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Gut Microbiota, Inflammation, and Behavioral Expression Following Social Defeat

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by

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Under the Direction of Kim Huhman, PhD

ABSTRACT

Social stress exacerbates symptoms of mood and anxiety disorders in humans. Here, we tested the hypothesis that social stress increases anxiety- and depression-like responses via changes in gut microbiota and inflammation. We used a social defeat model in Syrian hamsters to determine whether exposure to social stress alters the gut microbial community. We then tested whether alterations in the gut microbial community impacts susceptibility to social stress, and, if so, whether it might do so via immunological pathways. In Aim 1, the gut microbial community of hamsters was assessed by 16S mRNA Illumina sequencing after one and repeated agonistic encounters. Both dominant and subordinate hamsters exhibited alterations in the gut microbial community and reductions in species richness following social stress. LEfSE analysis revealed that some microbial taxa correlated with achieving dominant or subordinate status in a

future agonistic encounter. In Aim 2, hamsters were treated with either a probiotic for 2 weeks or an emulsifier for 12 weeks to test whether manipulating gut microbiota impacts behavioral susceptibility to social defeat. Probiotics are thought to promote a healthy microbial composition and emulsifiers have been shown to disrupt the gut microbial community. Probiotic treatment increased avoidance behavior and decreased social interaction following defeat. Probiotic treatment also altered the gut microbial community and serum cytokines following defeat. Emulsifier treatment had no effect on behavior. In Aim 3, neuroinflammation was assessed following social defeat. There was no increase in microglial activation in brain following defeat suggesting that exposure to mild social stress in hamsters does not induce robust neuroinflammation. As a positive control, we examined microglial activation following administration of lipopolysaccharide, a bacterial endotoxin, and were able to demonstrate a robust inflammatory response in hamster brain. Thus, the experiments in Aim 3 suggest that neuroinflammation is not necessary for behavioral responses to social stress in hamsters. Collectively, these data demonstrate that exposure to social stress can alter gut microbiota and that the microbiota can alter susceptibility to social stress. Future studies will be necessary to determine the mechanisms underlying this two-way relationship.

INDEX WORDS: Social stress, Social defeat, Syrian hamster, Gut microbiota, Probiotic, Microglia, Inflammation.

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by

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DEDICATION

To my family. My father, for his love of knowledge. My mother, for her curiosity and for instilling in me the importance of scientific pursuit. My sister, for being my confidant, my cheerleader, and for always brightening any day. And to my grandma, for her unconditional care and support for my goals.

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1 INTRODUCTION

1.1 Overview

Organisms adapt to threatening changes, or stressors, in the environment by producing a stress response. This response involves activation of systems, such as the hypothalamic pituitary adrenal (HPA) axis and the sympathetic nervous system, that produce physiological changes that allow individuals to respond appropriately to the environmental challenge. Thus, these stress responses can promote survival and fitness. If, however, the response is produced in the absence of a legitimate stressor, is not terminated appropriately, or becomes too prolonged, the effects can be detrimental to health or survival.

Social stress is arguably the most pervasive form of stress experienced by humans, occurring across the lifespan in social contexts such as school and work (Bjorkqvist et al., 2001). Social stress has been shown to cause, or to exacerbate the symptoms of, neuropsychiatric illnesses such as mood and anxiety disorders (Agid et al., 2000; Bjorkqvist et al., 2001; Lederbogen et al., 2011). These crippling disorders affect millions of people worldwide, but the currently-available treatment strategies are ineffective for many (Nestler et al., 2002; Trivedi et al., 2006). It is clear that we need a better and broader understanding of additional mechanisms whereby social stress might impact physiology and behavior so that we can develop new and better treatment options for patients suffering from these stress-related disorders.

We have over 100 trillion microorganisms, termed gut microbiota, living in our gastrointestinal tract (Eckburg et al., 2005). Recently, it has become clear that gut microbiota can influence the brain and behavior. Research suggests that perturbations of the gut microbial community, which can be caused by stress, for example (Lyte et al., 2011, Galley et al., 2014), can have a functionally relevant impact on the brain and, in turn, influence behavior (Holder et

al., 2019; Dinan et al., 2015; Parashar & Udayabanu, 2016; Bailey et al., 2011). Therefore, it is possible that gut microbiota influence susceptibility to stress-related neuropsychiatric disorders. We know that the "gut-brain" connection is partly mediated via immunological signals (Dinan et al., 2015) produced by gut microbiota (Ramakrishna, 2013; Chassaing et al., 2015). These immunological signals, particularly those that are pro-inflammatory, could explain, in part, the alterations to brain and behavior that often follow perturbations of the gut microbiota. It has also been proposed that the mechanism underlying mood disorders may be, at least in part, an inflammatory process (Tyring et al., 2006; Hodes et al., 2014; Zhang et al., 2016; Yirmiya et al., 2001), and that a lack of understanding about how inflammation contributes to mental health might be a factor in the limited therapeutic efficacy of currently-available treatments (Miller et al., 2009). This project will use a social stress model in Syrian hamsters to examine the role that the gut microbiota and inflammation play in social stress-induced, depressive- and anxiety-like changes in behavior. The purpose of this project is to test the overarching hypothesis that a pro-inflammatory state, driven in part by the dysbiosis of gut microbiota, exacerbates the behavioral response to social stress.

1.2 Studying the Effects of Stress: Social Stress and Social Defeat

Animal models are critical to understanding the neurobiological mechanisms that drive stress responding and the possible downstream neuropathology (Agid et al., 2000). Despite social stress being the primary form of stress experienced by humans (Brown & Prudo, 1981; Kessler, 1997; Bjorkqvist et al., 2001), the majority of animal studies investigating the effect of stress on brain and behavior have historically used nonsocial stressors such as cold water immersion, tail pinch, electric foot shock, immobilization, or physical restraint (Sutanto & Kloet, 1994). While these nonsocial stressors illicit a robust stress response, are highly controllable, and

are potentially useful in understanding the role stress plays in the development of neuropsychiatric disorders, these stressors are artificial and are not translatable to the common human stress experience (Kessler, 1997; Bjorkqvist et al., 2001). Because most of the stressors that humans and most other animals experience are social (Brown & Prudo, 1981; Kessler, 1997; Bjorkqvist et al., 2001), animal models of social stress are more ethologically relevant and have greater translational value (Chaouloff, 2013), particularly given that many animals respond to social stress in similar ways as do humans. For example, humans and many other animals show increased anhedonia, submissiveness, and social avoidance (Trew, 2011; Hammels et al., 2015; Nemeroff, 1998; Bjorkqvist et al., 2001; Agid et al., 2000; Gardner, 2001), along with changes in ingestive behavior, growth rate, metabolism, and sleep (Foster et al., 2006; Solomon et al., 2007; Kinn et al., 2008; Pulliam et al., 2010; Meerlo et al., 1996; Koolhaas et al., 2011; Meerlo et al., 1997; Chuang et al., 2010; Shively, 1998; Virgin & Sapolsky, 1997) in response to social stress.

The most common form of social stress used in animals is social defeat stress. Social defeat readily occurs as a result of an intraspecies agonistic encounter, characterized by a dominant animal displaying aggressive behaviors and a socially defeated, subordinate animal displaying submissive and defensive behaviors. Social defeat has been studied in a variety of species including lizards (Summers et al., 2003), zebra fish (Oliveira et al., 2016), *Drosophila* (Penn et al., 2010), crickets (Rillich et al., 2014), pigs (van der Staay et al., 2008), rats (Miczek, 1979), mice (Golden et al., 2011), hamsters (Huhman et al., 2003), and non-human primates (Fuchs & Flügge, 2002; Shively & Willard 2012; Sapolsky, 1990) and is thought to cause robust emotional and psychological stress as well as pronounced changes in physiology and behavior in the defeated animal (Hollis & Kabbaj, 2014; Huhman, 2006).

Laboratory animals and humans show remarkably similar responses to social defeat. Social defeat in humans, usually in the form of bullying or abuse, is also thought to cause emotional and psychological stress, and, behaviorally, both animals and humans show increased depression, anxiety, social withdrawal, and submission (Nemeroff, 1998; Agid et al., 2000; Bjorkqvist, 2001; Heim & Nemeroff; 2001; Gardner, 2001) following defeat. Additionally, because social defeat induces social stress in both humans and other animals, the behavioral and physiological changes following social defeat mimic symptoms often seen in patients diagnosed with mood and anxiety disorders, such as generalized depression (Blanchard et al., 1995; Kudryavtseva & Avgustinovich, 1998; Kampen et al., 2002), generalized anxiety, and post-traumatic stress disorder (Blanchard et al., 2001; Bremner, 2004). Importantly, many of the physiologically and behavioral consequences of social defeat in animal models can be reversed with the same treatments known to have antidepressant effects in humans (Fuchs et al., 2004; Meerlo et al., 1996; Berton et al., 1999). Due to the striking similarity between humans and animal models in their response to social defeat, the information gained from these studies has been critical to better understanding the neurobiological mechanisms that lead to social stress-related neuropsychiatric disorders. We will extend these findings by investigating whether two novel mechanisms, gut microbial and inflammation, drive susceptibility to social defeat in Syrian hamsters.

1.3 Syrian hamsters

Syrian hamsters provide a unique social defeat model because both males and females are highly territorial and do not require complex housing conditions to elicit conspecific aggression or reliable behavioral responses to defeat in the laboratory (Huhman et al., 2003; Solomon et al., 2007). This is in contrast to the majority of mammals wherein aggression directed towards or

between females is highly limited, or even absent, making it difficult if not impossible to study social defeat stress in both sexes. Conversely, male and female hamsters readily produce aggressive and territorial behavior when paired in either the home cage of one of the conspecifics or in a neutral arena, and this conflict rapidly results in the formation of a stable dominance relationship. After losing even a single agonistic encounter, subordinate hamsters abandon all territorial aggression and instead become highly submissive and socially avoidant, even when paired with a non-threatening stimulus animal (Potegal et al., 1993; Huhman et al., 2003; McCann & Huhman, 2012). This response has been termed conditioned defeat. A robust hormonal response accompanies conditioned defeat and is characterized by an increase in plasma adrenocorticotropin, cortisol, corticosterone, B-endorphin, and a decrease in plasma testosterone (Huhman et al., 1990, 1991). In contrast, winners do not show significant behavioral or hormonal changes following the agonistic encounter (Huhman, 2006).

Our lab has done extensive work to identify the neural circuitry that underlies the social defeat-induced change in behavior observed in losing hamsters. It is well known that the amygdala is necessary for processing and responding to emotional and fearful stimuli (Davis, 1992; Fanselow & Gale, 2003; McGaugh, 2004). In line with this, research from our lab demonstrated that synaptic transmission through, and protein synthesis in, the basolateral amygdala (BLA) is necessary for the acquisition of defeat-induced behavior (Jasnow & Huhman 2001; Markham et al., 2010). For example, microinjections of glutamate receptor antagonists (Jasnow et al., 2004) and gamma aminobutyric acid receptor agonists (Jasnow & Huhman, 2001), both of which blunt excitation, directly into the BLA and central amygdala block conditioned defeat. More recently, the medial prefrontal cortex (Markham et al., 2012), the bed nucleus of the stria terminalis (Markham et al., 2009), the medial amygdala (Markham &

Huhman, 2008), and the dorsal raphe nucleus (Cooper et al., 2008) have also been identified as important nodes for the acquisition and/or expression of defeat-induced behavioral responses.

Another strength of this social defeat model is that agonistic interactions in hamsters are highly ritualized so that they rarely result in physical injury; thus, it is possible to examine the behavioral, immunological, and physiological effects of social stress in the absence of physical injury or trauma and the concomitant inflammatory response resulting from such tissue damage. Further, unlike many models that require chronic social defeat stress to elicit behavioral and physiological changes, we observe many of the responses to defeat, such as elevated cortisol and social avoidance, after only a single defeat exposure in hamsters (Huhman et al., 1991; Huhman et al., 2003; McCann & Huhman, 2012). Thus, our model of social stress provides an excellent opportunity to study sex differences, to narrow temporally the time window within which behavioral and physiological responses to defeat occur, and to use a species wherein wounding is uncommon during brief social interactions.

1.4 Gut Microbiota and Social Stress

The trillions of gut microbiota that inhabit the gastrointestinal tract (Eckburg et al., 2005) share a mutually beneficial relationship with their host and are necessary for vital functions such as immunoregulation (Hrncir et al., 2008; Tlaskalova-Hogenova et al., 2004) and nutrient uptake and synthesis (Sommer & Backhed, 2013; Hill 1997). Various routes of communication, such as the vagus nerve, immune cell mediators, and neurotransmitter signaling, occur between the gastrointestinal tract and the brain (for a review, see Dinan & Cryan, 2012). Collectively, these routes of communication are termed the gut-brain axis. The existence of this axis suggests that the gut microbial community is able to communicate with and to impact physiological systems, such as the nervous and immune systems, and to influence behavior (Collins & Bercik, 2009;

Cryan, 2016). Therefore, gut microbiota may be an important mechanism to at least partly explain the abnormal behavioral phenotypes observed in many neuropsychiatric disorders (Sylvia & Demas, 2019). Evidence for this possibility comes from studies in germ-free mice that are born with no gut microbiota and raised in sterile conditions. These studies show that an absence of gut microbes causes changes in gene expression, an exaggerated HPA axis stress response, anxiogenesis, and deficits in cognitive functioning (Sudo et al., 2004; Neufeld et al., 2011; Clarke et al., 2013; Crumeyrolle-Arias et al., 2014; Desbonnet et al., 2014), suggesting that gut microbiota are necessary for normal neurological functioning.

Stress-induced alterations in this vibrant microbial community, often in the form of decreases in microbial species diversity, have been shown to cause inflammation, gastrointestinal distress, and changes to metabolism and behavior (Lyte et al., 2011). Notably, inflammation, gastrointestinal distress, and changes to metabolism and behavior are also symptoms of many mood and anxiety disorders (Kanuri et al., 2016; Kennedy et al., 2016; Qin et al., 2014), suggesting that stress-induced alterations in the gut microbial community could promote the development of these symptoms. Given that social stress has such important effects in humans, as described above, it is necessary to determine whether social stress could be impacting the brain and behavior, in part, via alterations to the gut microbial community. Thus, **Aim 1 will test whether social stress alters the gut microbial community in Syrian hamsters and whether the state of the gut microbial community can predict social behavior during an agonistic encounter.**

1.4.1 Probiotics

The term “probiotic” was first coined by Elie Metchnikoff after he found that a longer life span was linked to regular consumption of fermented milk in a group of Bulgarians (Metchnikoff et al., 2004). Today, probiotics are generally defined as supplements containing large quantities of gut-derived microbes that are thought to be beneficial for gastrointestinal health (Dinan et al., 2013). Recent research has focused on understanding the physiological and behavioral effects of these probiotic microbes. Two probiotic microbes, *Lactobacillus* and *Bifidobacterium*, are linked to lower plasma corticosterone and higher neurogenesis in mice following stress (Ait-Belgnaoui et al., 2014). These stress-protective effects may contribute to the decrease in anxiety- and depressive-like behavior observed in animals treated with probiotics (Bravo et al., 2011; O’Mahony et al., 2011).

In humans, probiotics improve gastrointestinal, immune, and cardiovascular health (Hungin et al., 2017; Khalesi et al., 2014). Recent evidence suggests that probiotics influence brain and behavior, as well. A meta-analysis on the effect of probiotics on anxiety- and depressive-like behavior suggests a general reduction in these behaviors after probiotic supplementation (Pirbaglou et al., 2016). Given this, it becomes important to investigate whether probiotic consumption can provide stress-protective effects in response to social stressors known to elicit anxiety- and depressive-like behavior. **Thus, Aim 2a will investigate whether probiotic treatment decreases susceptibility to social stress in Syrian hamsters.**

1.4.2 Emulsifiers

Emulsifiers are food additives used by the food industry to stabilize processed foods. As more processed foods enter our diet, our intake of emulsifiers increases. To date, only a few studies have been published looking at the effects of dietary emulsifiers on physiology. Among

those, Chassaing et al., (2015) was the first to demonstrate that chronic administration of emulsifiers to mice causes dysbiosis of the gut microbial community, characterized by a decrease in alpha diversity, and a disruption of the microbial-host relationship in the gut, characterized by the ability of bacteria and pathogens to penetrate the normally sterile mucous layer. Further, the emulsifier-induced dysregulation of the microbial community and its relationship with the gut results in low-grade level inflammation. This emulsifier-induced inflammation does not occur in germ free mice, suggesting that gut microbiota are necessary for the pro-inflammatory effect (Chassaing et al., 2015). Emulsifier treatment may also affect brain and behavior. After chronically consuming emulsifiers at a dose comparable to that of human consumption, mice show increased expression of agouti-related peptide and α-melanocyte stimulating hormone, two neuropeptides that can alter social and anxiety-related behaviors. Elevated expression of these neuropeptides positively correlated with alterations in social behavior, including an increase in some anxiety-like behaviors (Holder et al., 2019). Further research is necessary to extend these pioneering studies and to further investigate the effect of emulsifier treatment on brain and behavior. **Aim 2b will thus determine whether emulsifier treatment increases susceptibility to social stress in Syrian hamsters.**

1.5 Inflammation, Social Stress, and Neuropsychiatric Disorders

It has begun to be clear that inflammation can influence the brain and social behavior (Eisenberg et al., 2009, 2010; Moieni et al., 2015; Hodes et al., 2014; Wood et al., 2015; Menard et al., 2017). Through humoral and cellular pathways to the brain, the immune system alerts the central nervous system to stressors and other environmental changes (Maier & Watkins, 1998). Changes in inflammatory signaling in the brain is generally reversible, however chronic or abnormally robust signaling may exacerbate certain neuropsychiatric disorders, such as mood

and anxiety disorders (Dantzer et al., 2018). Diagnoses of many mood and anxiety disorders increase each year (Weinberger et al., 2018), and the current treatment options are ineffective for many patients (Zhang et al., 2016). In order to reach treatment-resistant patients, research investigating novel mechanisms, such as inflammation, that may drive or exacerbate these disorders may prove successful in helping treat these patients.

An exacerbated immune response has been observed in animals, including humans, that exhibit depressive- and anxiety-like symptoms. Previous studies in humans have described a positive correlation between pro-inflammatory signaling and increased anxiety and depressed mood (Irwin & Miller, 2007; Reichenberg, 2001). For example, post-mortem brains from depressed patients show increased pro-inflammatory gene expression in the pre-frontal cortex (Shelton et al., 2011), and many treatment-resistant patients show elevated levels of inflammatory markers in blood (Miller et al., 2009). In rodents, administering lipopolysaccharide (LPS), a widely used bacterial endotoxin that induces inflammation, also induces depressive-like behavior (Yirmiya et al., 2001; Frenois et al., 2007; Godbout et al. 2005, 2008; Dantzer et al., 2008), and the proinflammatory enzyme complex IkB kinase in the nucleus accumbens was found to be necessary and sufficient to induce anxiety-like behavior following social stress in mice (Christoffel et al. 2011). Relatedly, medications used in humans to treat inflammatory diseases also seem to cause some anti-depressant side effects (Tyring et al., 2006). Collectively, these findings support the hypothesis that inflammatory mechanisms induce depressive and anxiogenic symptoms.

It has been proposed that when an individual is faced with a socially stressful situation, inflammation is an adaptive response to prevent further wounding and to protect against bodily harm (Bluthe et al., 1992; Bluthe et al., 1994). One mechanism employed by the immune system

in these situations is to increase neuronal sensitivity to stress (Muscatell et al., 2016; Hariri et al., 2002) and other harmful stimuli (Eisenberger et al., 2009; Inagaki et al., 2012), and this change in neural sensitivity can impact current and future social behavior (Brachman et al., 2015). Following social defeat, many animals, including humans, show a pronounced increase in social avoidance and increased activity in brain regions necessary for this type of behavior, such as the amygdala (Muscatell et al., 2016, Sandi & Richter-Levin, 2009; Fekete et al., 2009; Bourne et al., 2013; Skórzewska et al., 2015). This marked increase in avoidant behavior could be beneficial, at first, by protecting the animal from further social stress, but promote the etiology of a mood or anxiety disorder if not terminated appropriately. Therefore, the link between inflammation and depressive and anxiogenic symptoms may, in part, derive from an inflammatory-induced increase in neural sensitivity to adverse social experiences (Eisenberger et al., 2017), which in turn could influence the behavioral response to a socially stressfully experience. It will be important to further investigate the mechanisms underlying the positive correlation between an increase in inflammatory markers and the development of neuropsychiatric disorders, as well as the role inflammatory mechanisms may play in the behavioral phenotypes associated with these disorders.

1.5.1 Microglia

The blood brain barrier blocks most immune cells from entering the brain (Lehmann et al., 2016), making it difficult for peripheral inflammatory cells to cause neuroinflammation unless the blood brain barrier is compromised. Certain central nervous system cells, however, have the ability to directly induce neuroinflammation. Microglia are one of the primary innate immune cells in the brain and, when activated, initiate a robust inflammatory signaling cascade (Kettenmann et al., 2011; Kim & Joh et al., 2006; Hanisch, 2002; Lehnardt, 2010; for a review,

see Gehrmann et al., 1995). Although microglia are necessary for proper neurological functioning, research in humans suggests that prolonged microglial activation increases susceptibility for many neuropsychiatric disorders, particularly those associated with a depressive state (Yirmiya et al., 2015; Wager-Smith et al., 2011; Nakagawa et al., 2014; Setiawan et al., 2015). Therefore, therapeutic drugs that inhibit microglia or return them to basal functioning may prove effective in treating diseases such as generalized depression and anxiety (Biber et al., 2016).

At rest, microglial cells modulate synapses for optimal neuronal communication, clear neuronal debris, and monitor for threats against homeostasis (Tremblay et al., 2011; Wu et al., 2015; Hanisch, 2002; Nimmerjahn et al., 2015; Kettenmann et al., 2011). When presented with a threat, microglia activate by increasing in number, changing morphology to a reactive profile (del Rio-Hortega, 1932; Hanisch & Kettenman 2007), and releasing cytokines, chemokines, prostaglandins, and reactive oxygen species (Lehmann et al., 2019; Ajmone-Cat et al., 2013; Hanisch, 2002; Kim & Joh et al., 2006; Hayes et al., 1987, 1988). Stress is one threat known to cause both proliferation and activation of microglia in stress-responsive brain regions such as the amygdala, prefrontal cortex, nucleus accumbens, and hippocampus (Tynan et al., 2010; Hinwood et al., 2012a, 2012b; Hanisch, 2002; Kreisel et al., 2014; Lehmann et al.,2016). Chronic social defeat has been shown to cause microglia activation and proliferation in many of these brain regions (Lehmann et al., 2016; Lehmann et al., 2018; Wohleb et al., 2011, 2014; Ramirez & Sheridan, 2016), resulting in neuroinflammation. Neuroinflammation caused by activated microglia is linked to physiological consequences in the brain such as increased phagocytosis and oxidative stress and to the behavioral consequences of social defeat (Lehmann et al., 2016, 2018, 2019). Further, research suggests microglia activation may be necessary for the behavioral

consequences of social defeat. For example, depleting microglia in mice before subjecting them to chronic social defeat eliminated the increase in anxiety-like behavior and abnormal social behavior normally observed following defeat (Lehmann et al., 2019). Interestingly, if microglia were allowed to repopulate after chronic social defeat, then the normal anxiogenic phenotype emerged despite no microglia being present during the social stressor (Lehmann et al., 2019). Although clearly valuable, this research has been conducted almost exclusively in male mice, where the social defeat protocol is chronic and where wounding is common. Notably, wounding of the defeated animal makes it extremely difficult to tease apart the effect of psychological stress versus physical injury on microglia activation. It will be important to extend these findings by analyzing microglia in response to social defeat in hamsters, where wounding is rare and where the hypothesis can be tested in both sexes. **Aim 3 will test the hypotheses that 1) social defeat in *male and female* hamsters increases the quantity and activation state of microglia in social stress-susceptible brain regions and 2) that the resulting neuroinflammation increases behavioral susceptibility to social defeat *in both sexes*.**

1.5.2 Cytokines

Cytokines are small signaling proteins released from leukocytes and other immune and non-immune cells (Barnes et al., 2009). These proteins impact physiological systems by mediating communication between the immune system and host tissue (Firestein et al., 2016). Cytokine signaling can promote a peripheral immune response to prevent infection and signal to the brain to alter behavior in response to an environmental threat. Depending on the nature of the environmental threat, cytokines can exert pro- and/or anti-inflammatory effects (Su et al., 2012). Cytokines can also profoundly affect brain and behavior by altering neurochemical signaling (Anisman & Merali, 2003; Camancho-Arroyo et al., 2009), neuroplasticity, and neuroendocrine

processes (Yirmiya & Goshen, 2011; Curfs et al., 1997). Although it has traditionally been believed that peripheral cytokines cannot pass the blood brain barrier, it is now recognized that stress and other insults can cause breakdown of this barrier allowing cytokines to penetrate the brain (Lochhead et al., 2017; Rodriguez-Arias et al., 2017; Lehmann et al., 2018). Peripheral cytokines are also actively transported into the brain via saturable transport molecules and can further affect brain function by activating epithelial cells lining the cerebral vasculature (Vitkovic et al., 2000; Banks, 2009; Konsman et al., 2004). As described above, microglia release cytokines to induce neuroinflammation. Therefore, a positive feedback mechanism exists between microglia and cytokines where peripheral cytokines stimulate microglia activation, and in turn, microglia release cytokines centrally. Interestingly, more recent research has shown that gut microbiota can stimulate cells in the gut mucous layer to release pro-inflammatory cytokines that are able to reach the brain via afferent vagus nerve fibers (Sternber, 1997). This suggests that changes to gut microbiota, caused by perturbations such as stress, can change inflammatory signaling to the brain and the neuroinflammatory profile (Eisenberger et al., 2017; Goehler et al., 1997; Dantzer et al., 2008; Maier & Watkins 1998; Dantzer et al., 2018).

Research over the past couple decades suggests that there is also a link between cytokine release and mood disorders, such as depression. In mice, cytokines have been shown to modulate depressive-like symptoms, such as anhedonia, and these symptoms are blocked by anti-depressant medication (Yirmiya et al., 2001). Depressive symptoms are also positively correlated with two widely studied pro-inflammatory cytokines, IL-6 and TNFα (Hannestad et al., 2011). IL-6 is the most consistently elevated pro-inflammatory cytokine in patients diagnosed with major depressive disorder and has been argued to be a predictive marker for depression (Hodes et al., 2006; Dowlati et al., 2010; Haapakoski et al., 2015). Interestingly, IL-6 levels in the

periphery positively correlate with susceptibility to social defeat stress (Hodes et al., 2014) and blocking IL-6 in socially defeated mice results in anti-depressant effects and normalizes the gut microbial community to that of no defeat controls (Zhang et al., 2017). Further, IL-6 may be necessary for a depressive-like phenotype. For example, IL-6 knockout mice fail to develop the depressive-like symptoms following constant darkness (Monje et al., 2011). Other proinflammatory cytokines, such as TNFα and IL-1, have also been shown to increase depressive-like behavior in mice, and blocking these cytokines or administering anti-depressant medication has been shown to reduce or eliminate the expression of depressive-like behaviors (Simen et al., 2006; Goshen et al., 2008). In addition to the cytokines previously mentioned, plasma levels of cytokines such as IL-1B, IL-2, IL-17, and IL-4 are elevated in response to social defeat in mice (Brachman et al., 2015). However, not all social defeat protocols result in elevated levels of cytokines. For example, neither an acute, repeated, or continuous social defeat protocol in Sprague Dawley rats was enough to elevate blood cytokine levels (Hueston et al., 2011). Although there is compelling evidence cytokines may be key mediators in the link between inflammation, social stress, and mood and anxiety disorders, additional data are necessary to confirm social stress-induced mechanisms by which inflammation may cause a depressive or anxiogenic phenotype. In addition, the majority of the previous research has been conducted solely in male subjects using a chronic social defeat protocol making it is unclear how cytokines may mediate the response to milder social stress in both males and females. **Aim 3 will investigate whether 1) social defeat in male and female Syrian hamsters is sufficient to induce a robust inflammatory response and if so, 2) whether an increase in pro-inflammatory cytokine signaling exacerbates the behavioral consequences of social defeat.**

1.6 Specific Aims Overview

1.6.1 Aim 1: Does social stress dysregulate gut microbiota?

A high co-morbidity exists between depression and gastrointestinal disorders, and many of the associated symptoms are thought to be caused or exacerbated by stress (Kanuri et al., 2016; Kennedy et al., 2016). However, little is known about the impact of social stress on gastrointestinal health. We will test the hypothesis that acute and repeated exposure to social stress in Syrian hamsters dysregulates gut microbiota. We will also test the hypothesis that the gut microbial community can predict future social behavior by analyzing the baseline microbial community of future dominant and subordinate hamsters.

1.6.2 Aim 2a: Can increasing "healthy" gut microbes prior to social stress reduce the behavioral response to social stress?

Probiotics can be used as tools to manipulate gut microbiota. Probiotics are thought to promote a healthy microbial composition and to reduce depressive-like stress responses (Mayer et al., 2015; Desbonnet et al., 2015; Aguilera et al., 2013). To test the hypothesis that manipulating gut microbiota prior to social stress impacts the behavioral response, the probiotic, Probiostick will be administered to Syrian hamsters prior to social defeat, and avoidance behavior will be tested 24hr later.

1.6.3 Aim 2b: Does disruption of the microbial-host relationship increase susceptibility to social stress?

Recently, new data have suggested that emulsifiers, commonly used food additives, also disrupt the microbial community, and in turn, the microbial-host relationship in the gut. These emulsifier-induced changes to the microbiota and the gut were shown to be necessary and sufficient for low-grade inflammation following emulsifier treatment in mice (Chassaing et al.,

2015). Because emulsifiers are consumed daily by most Americans, it is important to test further their impact on physiology and behavior. We will build on these novel findings by testing the hypothesis that chronic treatment with emulsifiers increases susceptibility to social stress in hamsters. Subjects will be given the commonly used emulsifiers, polysorbate 80 (P80) and carboxymethylcellulose (CMC), and avoidance behavior will be measured following social defeat.

1.6.4 Aim 3: Does social stress induce inflammation?

It is well known that stress can cause inflammation. However, it remains unknown whether social defeat in hamsters induces a pro-inflammatory state or whether pro-inflammatory signaling increases susceptibility to social defeat. We hypothesize that social defeat causes inflammation, as evidenced by increases in peripheral and central pro-inflammatory markers, in Syrian hamsters. To test this hypothesis, animals will be defeated, and blood and brain will be collected to measure central and peripheral inflammation. Microglia, key mediators of neuroinflammation, and pro-inflammatory cytokines that have previously been shown to be increased by stress, such as IL-6, will be measured.

2 ACUTE AND REPEATED EXPOSURE TO SOCIAL STRESS REDUCES GUT MICROBIOTA DIVERSITY IN SYRIAN HAMSTERS.

2.1 Introduction

Mood and anxiety disorders are strongly associated with somatic symptoms such as gastrointestinal distress (Bekhuis et al., 2014, Felice et al., 2015), and a high co-morbidity exists between stress-related neuropsychiatric symptoms and gastrointestinal disorders such as irritable bowel syndrome (Kanuri et al., 2016, Kennedy et al., 2012, Qin et al., 2014). One possibility is that stress impacts brain function and mental health via its effect on the gastrointestinal tract (for review see Dinan & Cryan, 2012, Dinan et al., 2015, Parashar & Udayabanu, 2016). Given that the available treatment strategies for a variety of stress-related neuropsychiatric disorders are inadequate for many, expanding our knowledge of a broader range of potential etiologic factors might lead to novel, more effective therapeutics (Culpepper et al., 2015, Haddad et al., 2015, Nestler et al., 2002).

The mammalian gastrointestinal tract houses over 100 trillion microorganisms (Eckburg et al., 2005), which are critical for vital functions such as processing and digestion of food, synthesis of vitamins, inhibition of pathogens, and immune system development and maturation (Ramakrishna, 2013). Thus, a stable and symbiotic relationship exists between these microorganisms, referred to as gut microbiota, and the host gastrointestinal system (Mayer et al., 2015). These microbiota are essential to homeostasis, and abrupt dysbiosis or absence of this vibrant community can compromise the physical and mental health of the host (Chang et al., 2008, Cryan, 2016, Luczynski et al., 2016, Turnbaugh et al., 2006). Recently, it has been demonstrated that there is bi-directional communication, referred to as the gut-brain axis, between the central nervous system and the gastrointestinal tract, and altering the gut microbiota

during early development or adulthood changes both stress-related behavior and responsivity of the stress axis (Desbonnet et al., 2014, Diaz et al., 2011, Collins et al., 2013, Sudo et al., 2004).

Many neuropsychiatric disorders and symptoms that are associated with gastrointestinal dysfunction are also caused or exacerbated by exposure to stress (Agid et al., 2000, Kessler, 1997, Saveanu & Nemeroff, 2012), and stress has been associated with significant alterations in the gut microbial community in mammals, including humans (Lyte et al., 2011). These stress-induced alterations are associated with consequences ranging from inflammation to increased anxiety-like behavior (Bailey & Coe, 1999, Bailey et al., 201l). Exposure to social stress, in particular, can cause or exacerbate disabling neuropsychiatric disorders, including depression and PTSD (Bjorkqvist et al., 2001, Qiao et al., 2016). Relatively little is known, however, about the direct impact of social stress on the gut microbial community and how these microbes, in turn, may affect behavior. Bailey et al. (2011) demonstrated that group-housed mice exposed to 6, 2 hr bouts of social disruption stress exhibited alterations of the gut microbial community characterized by a reduction in microbial diversity and richness. A similar response was observed in mice that were exposed to a more severe, 10-day social defeat procedure (Bharwani et al., 2016). There is even some evidence that a single, 2 hr exposure to a social defeat stressor in mice impacts gut microbiota (Galley et al., 2014), suggesting that even acute social stress might have effects on the gut.

The current study utilizes a well-characterized resident-intruder model in Syrian hamsters (Jasnow et al., 2001, Potegal et al., 1993) to investigate whether exposure to social stress affects the commensal gut microbiota and, in particular, whether it does so differently in individuals that "win" a social conflict (i.e., become dominant) versus those that "lose" (i.e., become subordinate). Syrian hamsters are ideal candidates for the study of social stress because when

weight- and age-matched conspecifics are paired, they readily produce aggressive and territorial behavior that rapidly results in the formation of a stable dominance relationship (Albers et al., 2002). This allows a direct comparison of commensal bacteria in dominants and subordinates. This comparison is not possible in mice because conspecifics generally do not fight; defeated mice are produced using a larger, more aggressive heterospecific (e.g., C57J/BL6 defeated by a CD-1 mouse). An additional benefit of using hamsters is that their agonistic behavior during brief encounters is highly ritualized and rarely results in any tissue damage, allowing us to focus on the psychological, as opposed to physical, aspects of social stress (Huhman & Jasnow, 2005). Furthermore, no studies have examined whether the baseline composition of the gut microbiota alters behavioral responses to social stress. Thus, we also measured whether the baseline gut microbiota composition can predict whether an animal becomes dominant or subordinate after a subsequent agonistic encounter.

2.2 Materials and Methods

2.2.1 Animals

Adult male Syrian hamsters (*Mesocricetus auratus*), weighing between 120 and 130 g, were obtained from Charles River Laboratory (Kingston, NY) at approximately 3 months of age. Hamsters were individually housed in polycarbonate cages (24x33x20cm) with corncob bedding, cotton nesting material, and a wire mesh top in a temperature controlled colony room under a 14:10hr light/dark cycle, which is standard to maintain reproductive gonadal status in hamsters. Food and water were available *ad libitum*. All hamsters were handled daily for 7 days to acclimate them to handling stress before the beginning of the experiment. Individual housing is not stressful for Syrian hamsters (Ross et al., 2017), and with the exception of the agonistic pairings described in Section 2.2, hamsters remained separated throughout the experiment. All

protocols and procedures were approved by the Georgia State University Institutional Animal Care and Use Committee prior to experimentation, and all methods align with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2.2 Behavioral procedures and fecal collection

Two days before testing occurred, hamsters were weighed and randomly assigned to one of three weight-matched treatment groups: Resident (n=9), Intruder (n=9), or Home Cage Control (n=5). Control animals experienced the same treatment and fecal collection protocol throughout the experiment, with the exception that they were never paired with another hamster, to control for all environmental variables experienced by the animals besides social stress. For fecal collection, animals were transferred to a clean cage and fecal samples (approximately 5 fecal boli) were collected from the bedding of each hamster's home cage. Collection from the home cage was done to avoid any additional stress to the animals. To help ensure that the samples were fresh, we collected fecal boli that were moist, on top of the bedding, and had no bedding stuck to them. These samples were collected in RNase-free microcentrifuge tubes and were immediately frozen and stored at -80° C until further processing. One day before testing began, all animals were transferred to a clean cage and fecal samples were collected 24hr later. Behavioral testing occurred over the next 5 days. All behavioral manipulations occurred during the first 3 hr of the dark phase of the daily light:dark cycle to control for circadian variation in microbiota and behavior and because this is when hamsters exhibit the majority of their agonistic behavior. Each day, all hamsters were moved into the behavioral suite 30 min prior to any manipulation to allow time to acclimate. Trials were run under dim red light and were recorded with a CCD camera for later scoring of behavior by observers blinded to the experimental condition. Home cage controls were not manipulated other than handling, transport to the testing

suite, and cage changes. For agonistic encounters, an intruder was placed in the home cage of a resident for 15 min on Day 1 and twice a day for 5 min on Days 2-5, and a clear plastic lid was placed over the resident's cage during each pairing to prevent escape. A dominance hierarchy was rapidly established during the first pairing, resulting in a winner (i.e., a dominant) who reliably attacked and defeated its losing partner (i.e., subordinate); the latter exhibited submissive and defensive behaviors such as upright defense, flee, and tail lift (Huhman et al., 1990). The scored behaviors were divided into four categories (i.e., social, aggression, submission, and nonsocial), as described in detail in Albers et al. (2002). Hamsters were transferred to a clean cage immediately after each agonistic interaction. Fecal samples were collected 24 hr after the first encounter from the animals' cages immediately before the animals were paired again on Day 2. Two additional pairings per day were conducted on Days 2-5, one at the start of the dark phase, as on Day 1, and the second 4 hr later. The same resident/intruder pairings were used throughout the experiment. On Day 5, after the final pairing, all hamsters were immediately transferred into clean cages, and fecal samples were again collected 24 hr later to assess the effect of repeated agonistic interaction on gut microbiota. All hamsters were carefully observed during each agonistic encounter for coprophagia and for any injury. No coprophagia or tissue damage occurred during these encounters.

2.2.3 Fecal microbiota composition analysis by 16S rRNA gene sequencing

Fecal 16S rRNA gene amplification and sequencing were done using Illumina MiSeq technology following the protocol of the Earth Microbiome Project with their modifications to the MO BIO PowerSoil DNA Isolation Kit procedure for extracting DNA (www.earthmicrobiome.org/emp-standard-protocols), as described previously (Caporaso et al.,

2012, Gilbert et al., 2010). In brief, bulk DNA was extracted from frozen feces using a PowerSoil-htp kit from MO BIO Laboratories (Carlsbad, California, USA) with mechanical disruption (bead-beating). The 16S rRNA genes, region V4, were PCR amplified from each sample using a composite forward primer and a reverse primer containing a unique 12-base barcode, designed using the Golay error-correcting scheme, which was used to tag PCR products from respective samples. We used the forward primer 515F 5'-*AATGATACGGCGACCACCGAGATCTACAC***TATGGTAATT*GT***GTGCCAGCMGCCGCGGTAA-3': the italicized sequence is the 5' Illumina adapter B, the bold sequence is the primer pad, the italicized and bold sequence is the primer linker and the underlined sequence is the conserved bacterial primer 515F. The reverse primer 806R used was 5'-*CAAGCAGAAGACGGCATACGAGAT* XXXXXXXXXXXX **AGTCAGTCAG *CC*** *GGACTACHVGGGTWTCTAAT*-3': the italicized sequence is the 3' reverse complement sequence of Illumina adapter, the 12 X sequence is the golay barcode, the bold sequence is the primer pad, the italicized and bold sequence is the primer linker and the underlined sequence is the conserved bacterial primer 806R. PCR reactions consisted of Hot Master PCR mix (Five Prime), 0.2 □M of each primer, 10-100 ng template, and reaction conditions were 3 min at 95°C, followed by 30 cycles of 45 s at 95°C, 60 s at 50°C, and 90 s at 72°C on a Biorad thermocycler. Two independent PCRs were performed for each sample, then combined and purified with Ampure magnetic purification beads (Agencourt), and products were visualized by gel electrophoresis. Products were then quantified (BIOTEK Fluorescence Spectrophotometer). A master DNA pool was generated from the purified products in equimolar ratios. The pooled products were sequenced using an Illumina MiSeq sequencer (paired-end reads, 2 x 250 bp) at Cornell University, Ithaca.

2.2.4 16S rRNA gene sequence analysis

The sequences were demultiplexed and quality filtered using the Quantitative Insights Into Microbial Ecology (QIIME, version 1.8.0) software package (Caporaso et al., 2010). Forward and reverse Illumina reads were joined using the fastq-join method (Aronesty, 2011, 2013). We used the QIIME default parameters for quality filtering as described in detail in Caporaso et al. (2010). Sequences were clustered using the UCLUST algorithm with a (Edgar, 2010) 97% homology threshold. Clusters were then classified taxonomically using the Greengenes reference database, Version 13.5 (McDonald et al., 2012). Clusters that did not match any Greengenes Operational taxonomic units (OTUs) were kept. A single representative sequence for each OTU was aligned and a phylogenetic tree was built using FastTree (Price et al., 2009).

The phylogenetic tree described above was used to assess beta and alpha diversity. Beta diversity measures the variation in microbiota composition between individual samples. Alpha diversity measures both the richness and evenness (or distribution) of unique microbial taxa within a sample (Mackos et al., 2017, Sekirov et al., 2010). Unweighted UniFrac distances between samples were computed, as done previously, to measure beta diversity (Lozupone et al., 2006, Lozupone & Knight, 2005) using rarefied OTU table count. Principal coordinates analysis (PCoA) plots were used to further assess and visualize beta diversity. Groups were compared for distinct clustering using PERMANOVA method using vegan R-package through QIIME. Kruskal-Wallis with Dunn's multiple comparisons test was used to compare the unweighted UniFrac distances within or between groups. The phylogeny-based metric, phylogenetic diversity whole tree (PD whole tree) measurements were determined with QIIME using an OTU table rarefied at various depths and the non phylogeny-based metric, Shannon measurements were

determined with QIIME using the alpha_diversity.py command line of the rarefied OTU table count. Area under the curve was calculated for each rarefaction curve and Kruskal-Wallis with Dunn's multiple comparison test was used to determine differences among groups. Due to technical limitations, not all samples could be amplified; therefore, we were unable to run repeated measures for the comparisons. Lastly, LEfSE (Linear Discriminate Analysis Effect Size) was used to investigate bacterial taxa that drive differences between groups by comparing the abundance of specific taxa between each experimental group (Segata et al., 2011), and Mann-Whitney U tests were performed where appropriate. All statistics were done in GraphPad Prism software, version 6.01 and IBM SPSS Statistics, version 22. Unprocessed sequencing data are deposited in the European Nucleotide Archive under accession numbers PRJEB25140.

2.3 Results

2.3.1 Behavioral responses to social stress

Each resident/intruder pair rapidly formed a dominant/subordinate relationship on Day 1, whereon either the resident or the intruder was defeated during the initial encounter. Dominant hamsters produced 211.1 ± 49.2 s of aggression and 1.2 ± 1.1 s of submission while their opponents (subordinates) produced 6.3 ± 3.1 s of aggression and 264.4 ± 49.5 s of submission during the initial 15min pairing. The dominance relationship within each pairing, with the exception of one, remained stable throughout the experiment. The pair in which the dominance hierarchy reversed after the first pairing was removed from the study and was not included in the analyses. While residence often confers dominance in resident-intruder models, pairing weight-matched animals tends to even those odds and has done so in our previous work. Nonetheless, in

this study, residents became the dominant animal more frequently (8 of 9 pairings) than expected for these weight-matched animals

2.3.2 Social stress alters gastrointestinal microbiota composition

Microbiota composition was analyzed by 16S rRNA Illumina sequencing of fecal DNA samples collected before any interaction ("baseline"), after the first ("acute") pairing, and after nine ("repeated") agonistic encounters. Microbiota composition was also analyzed from home cage control animals collected concurrently. 16S rRNA Illumina sequencing revealed that several samples had a low number of sequences and, therefore, were not included in the analyses. This included two samples from each group at baseline sampling, one home cage control at repeated sampling, and one subordinate at the acute sample. PERMANOVA analysis of the unweighted Unifrac distance (Lozupone & Knight, 2005) revealed that after acute and repeated pairing, microbiota composition of both dominant and subordinate hamsters was significantly altered when compared to their respective baseline values (Acute, dominant $p = 0.002$, subordinate $p = 0.004$; Repeated, dominant $p = 0.001$, subordinate $p = 0.001$) (Figure 1). It should be noted that before any social interaction there was no distinct clustering of the samples between home cage control and dominant animals ($p = 0.844$), between home cage control and subordinate animals ($p = 0.781$), nor between dominant and subordinate animals ($p = 0.695$) (Figure 2A). Further examination of the unweighted Unifrac distance revealed that, compared with home cage controls, both dominant and subordinate hamsters did not show a significant change in beta diversity after both an acute pairing (Figure 2B; dominant $p = 0.911$, subordinate $p = 0.414$) and repeated pairings (Figure 2C; dominant $p = 0.321$, subordinate $p = 0.086$) using the PERMANOVA test. This is likely due to the high variability between individuals. Further,

Kruskal-Wallis showed no differences in the unweighted Unifrac distance at baseline (Figure 2D) or after acute pairing (Figure 2E); however, after repeated pairings, there was a significant increase in the unweighted Unifrac distance between subordinate animals when compared with home cage controls (Figure 2F; $H(2,74) = 14.44$, $p < 0.001$, Dunn's multiple comparison test, $p < .05$) suggesting an increase in inter individual variation of the microbial composition in animals who lost. No differences in microbiota composition was observed between dominants versus subordinates after acute (Figure 2B; $p = 0.413$) or repeated stress (Figure 2C; $p = 0.820$).

The analysis of alpha diversity of the intestinal microbiota, reflecting the bacterial richness and evenness of the community, revealed a significant effect of stress using both phylogeny-based (PD whole tree) and non phylogeny-based (Shannon) measurements. Although home cage control animals demonstrated stable diversity over time (Figure 3A; $H(2, 9) = 1.414$, $p = 0.542$; Supplementary Figure 1; $H(2,9) = 0.695$, $p = .707$), post hoc analysis revealed that repeated stress reduced microbiota diversity in dominant animals compared with their baseline (Figure 3B; $H(2, 22) = 7.559$, $p = 0.023$, Dunn's multiple comparisons test, $p < .05$); Supplementary Figure 1; $H(2, 22) = 5.865$, $p = 0.053$, Dunn's multiple comparisons test, $p < .05$). The apparent reduction in microbiota diversity for subordinates following stress was not significant (Figure 3C; $H(2, 21) = 3.517$, $p = 0.17$; Supplementary Figure 1; $H(2, 21) = 3.88$, $p = 0.14$). Significance was not reached after an acute interaction (Figure 3D; $H(2, 19) = 0.120$, $p = 0.948$) when dominant, subordinate, and home cage control groups were compared; however, when these groups were compared following repeated interactions significance was reached (Figure 3E; $H(2, 19) = 8.117$, $p = 0.017$) with dominant hamsters harboring reduced microbiota diversity following repeated exposure to social stress compared with home cage controls (Figure 3E; Dunn's multiple comparisons test, $p < .05$). Subordinate animals showed a similar pattern

when compared to home cage controls, yet this observation was not significant (Figure 3E; Dunn's multiple comparison test, $p > .05$).

LEfSE analysis (Linear Discriminant Analysis (LDA) Effect Size) was then used to find differentially abundant taxa between groups following social interactions, in order to identify which bacterial taxa drove changes in the microbiota community. A LDA threshold of 2 was used to infer significance ($LDA > 2$, $p < 0.05$). Bacterial taxa were largely unchanged in home cage controls between the baseline measurement and the subsequent sampling (Figure 4A and B), indicating that the microbiota remained stable in controls over the duration of the experiment. However, within both dominant and subordinate groups, numerous taxa of the intestinal bacterial community were significantly altered compared with their baselines following an acute pairing (Figure 4C and E, Supplementary Figure 2A), with more changes observed following repeated pairing (Figure 4D and F, Supplementary Figure 2B and C). Further, when comparing home cage controls to dominant and subordinate animals after repeated pairing, significant differences were also observed (Figure 5A-B, Supplementary Figure 3).

Of note, microbiota from the order Lactobacillales and phyla Firmicutes were found to be significantly decreased, and microbiota from the phyla Bacteroidetes were found to be significantly increased following repeated social interactions in both dominant and subordinate animals (Figure 4D and F, Supplementary Figure 2B and C; $LDA > 2$, $p < 0.05$). The significant increase in phyla Bacteroidetes was also observed after the acute social interaction compared with baseline in both dominants and subordinates (Figure 4C and E, Supplementary Figure 2A; $LDA > 2$, $p < 0.05$). Differences between dominant and subordinate animals were also observed. Of particular interest, bacteria from the family Clostridiacea (Figure 4D, Supplementary Figure 2B; $LDA > 2$, $p < 0.05$) increased only in dominant animals, and bacteria from phyla Firmicutes

(Figure 4E, Supplementary Figure 2A; LDA > 2, p < 0.05) significantly reduced after acute defeat only in subordinate animals.

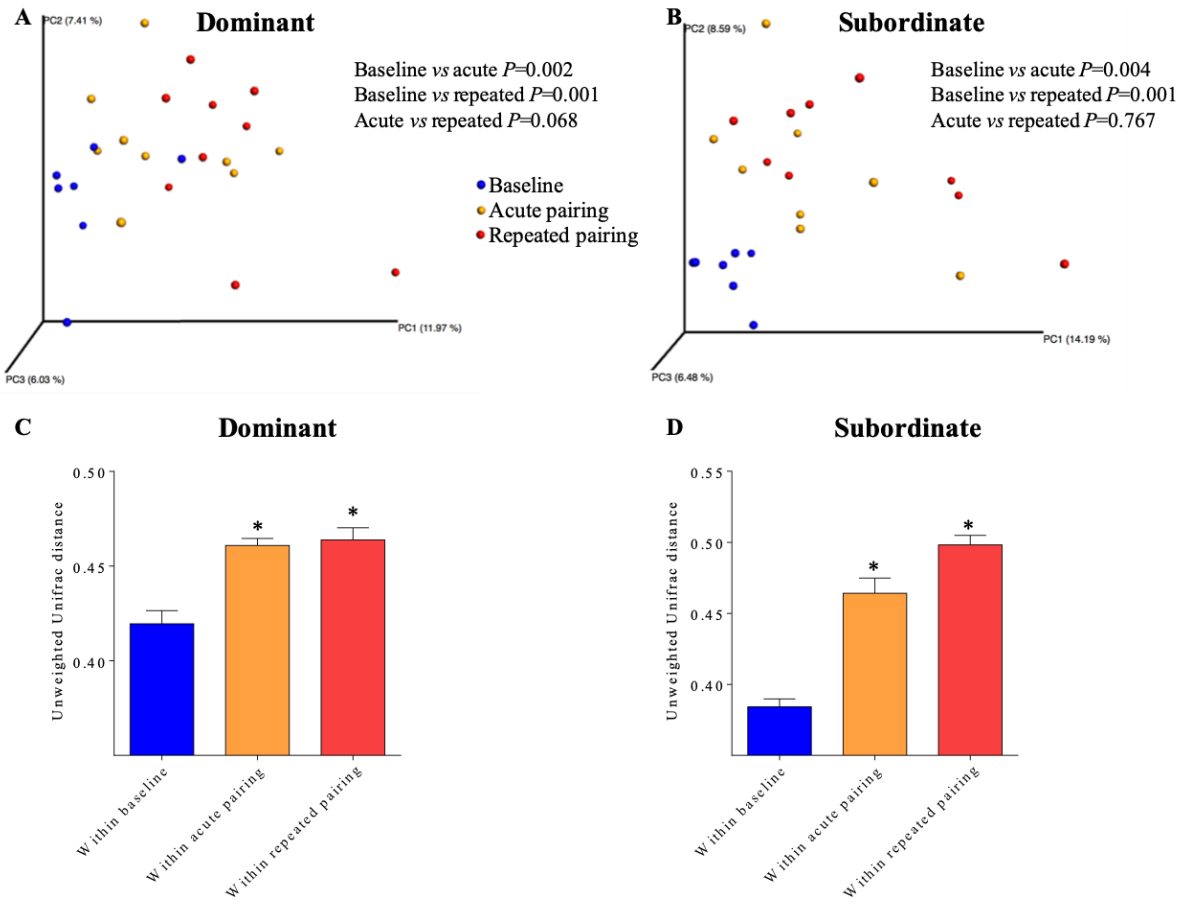

Figure 2.1 Social stress alters intestinal microbiota composition within dominant and subordinate hamsters compared to their baseline values.
Principal coordinate analysis (PCoA) of the unweighted UniFrac distance, illustrating changes in beta diversity, for dominant (**A**) and subordinate (**B**) hamsters comparing before social stress (baseline, blue dots), after one (acute pairing, orange dots), and repeated (repeated pairing, red dots) exposure to social stress. P values were determined using PERMANOVA analysis (**A-B**). No significant change in beta diversity occurred in home cage controls over the three collection time points (data not shown). Rarefied count: 5468 for **A**, 9288 for **B**. Panels C and D show the average (means ± SEM) unweighted Unifrac distance for dominant (**C**) and subordinate (**D**) hamsters, illustrating that within each social status there was a significant increase in the variation among samples at each time point compared with baseline. P values for C-D were determined using Kruskal-Wallis with Dunn's multiple comparison test. *denotes significantly greater than their respective baseline value (P<0.05).

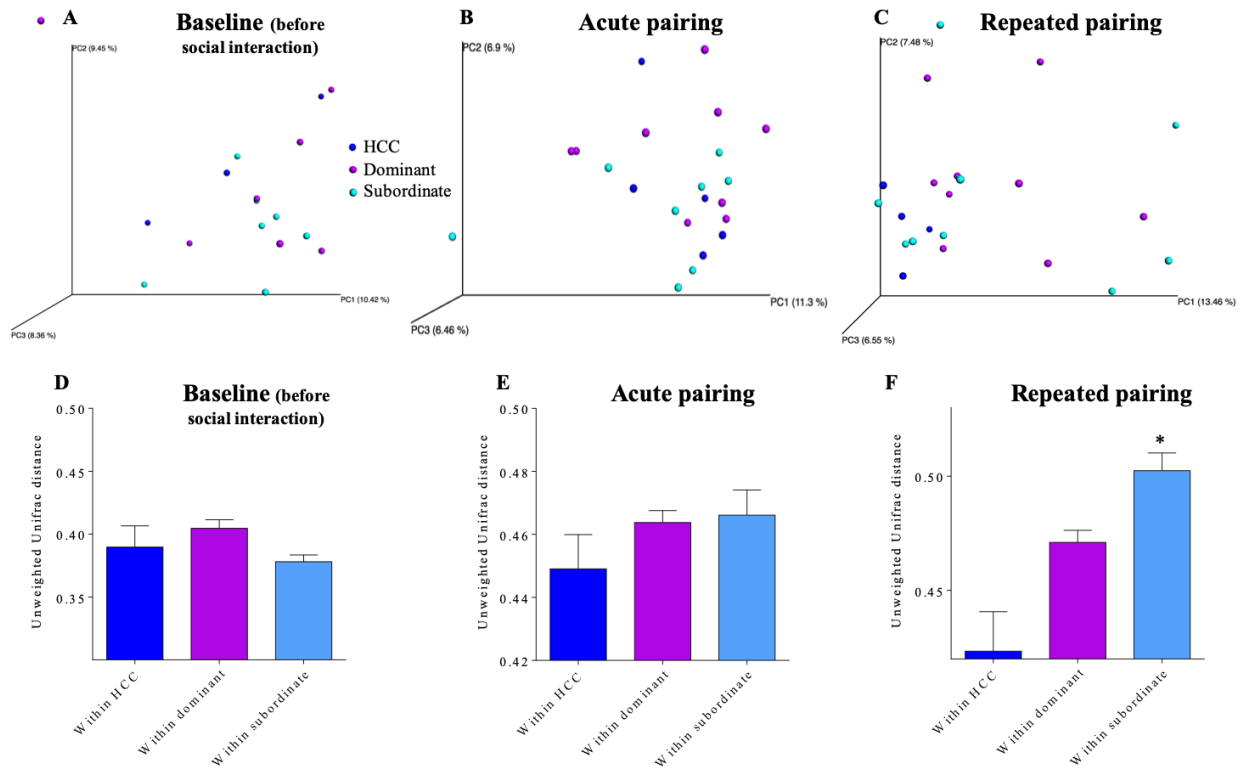

Figure 2.2 Social stress alters intestinal microbiota composition similarly in dominants and subordinates compared to home cage controls.
Principal coordinate analysis (PCoA) of the unweighted UniFrac distance at baseline (**A**), after acute pairing (**B**) and repeated pairing (**C**) for home cage control (blue dots), dominant (purple dots) and subordinate (light blue dots) hamsters. P values were determined using PERMANOVA analysis. Rarefied count: 5468 for **A**, 11242 for **B**, 9288 for **C**. Average (mean ± SEM) of the unweighted Unifrac distance within groups (control, dominant and subordinate) at baseline (**D**), after acute (**E**) or repeated pairing (**F**) illustrating that inter individual variation differed among groups after repeated pairing. P values in D-F were determined using Kruskal-Wallis with Dunn's multiple comparison test. *denotes significantly greater than home cage controls (HCC; $P<0.05$).

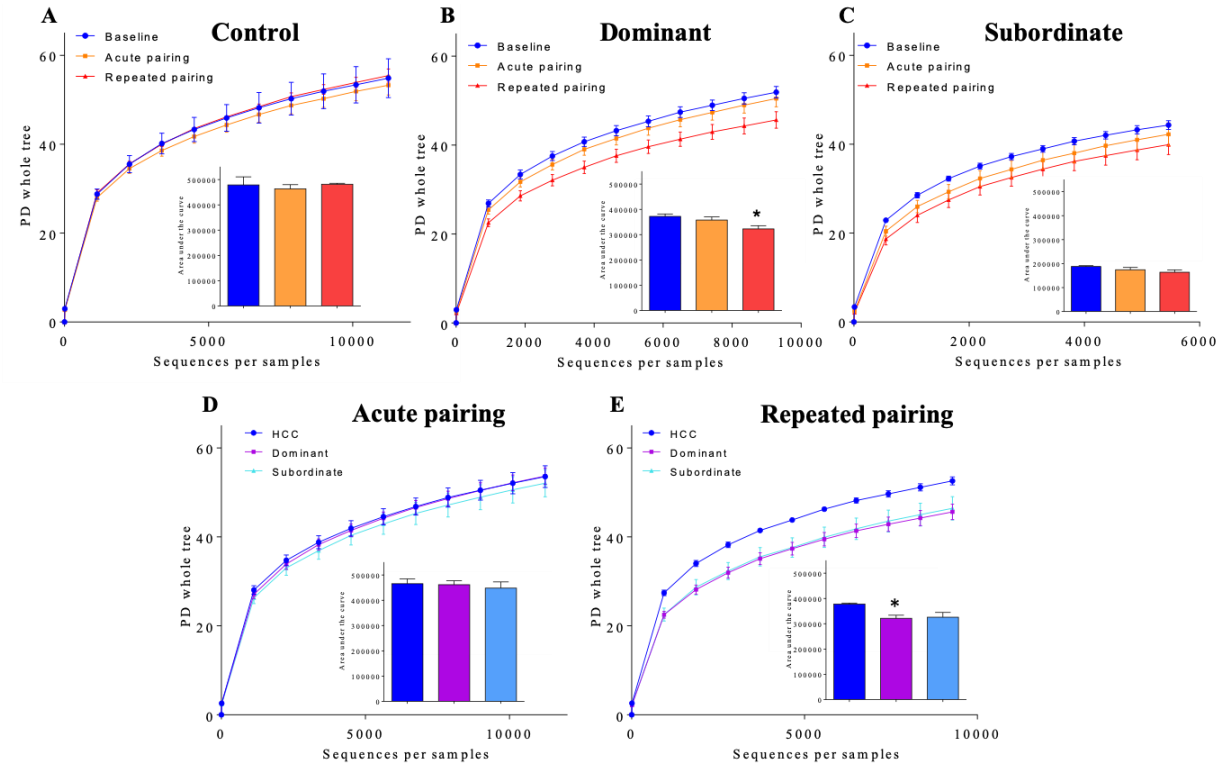

Figure 2.3 Social stress decreases intestinal microbiota diversity.
(**A-C**) Alpha diversity was determined by phylogenetic diversity whole tree measurement, as well as Shannon index (see Supplemental Figure 1) in control (**A**), dominant (**B**) and subordinate (**C**) hamsters before any social stress (baseline), after one (acute pairing) or repeated (repeated pairing) exposure to social stress. (**D-E**) PD whole tree and Shannon index measurements (see Supplemental Figure 1) were also used to compare alpha diversity across groups after one (acute pairing, **D**) or repeated (repeated pairing, **E**) exposure to social stress in home cage control (HCC), dominant and subordinate hamsters. Insets represent area under the curve for each group. Data are the means ± SEM; Comparisons done using Kruskal-Wallis with Dunn's multiple comparison test (Panel 3B and 3E). * denotes significantly reduced ($p<0.05$) in repeated pairing compared to baseline or home cage controls.

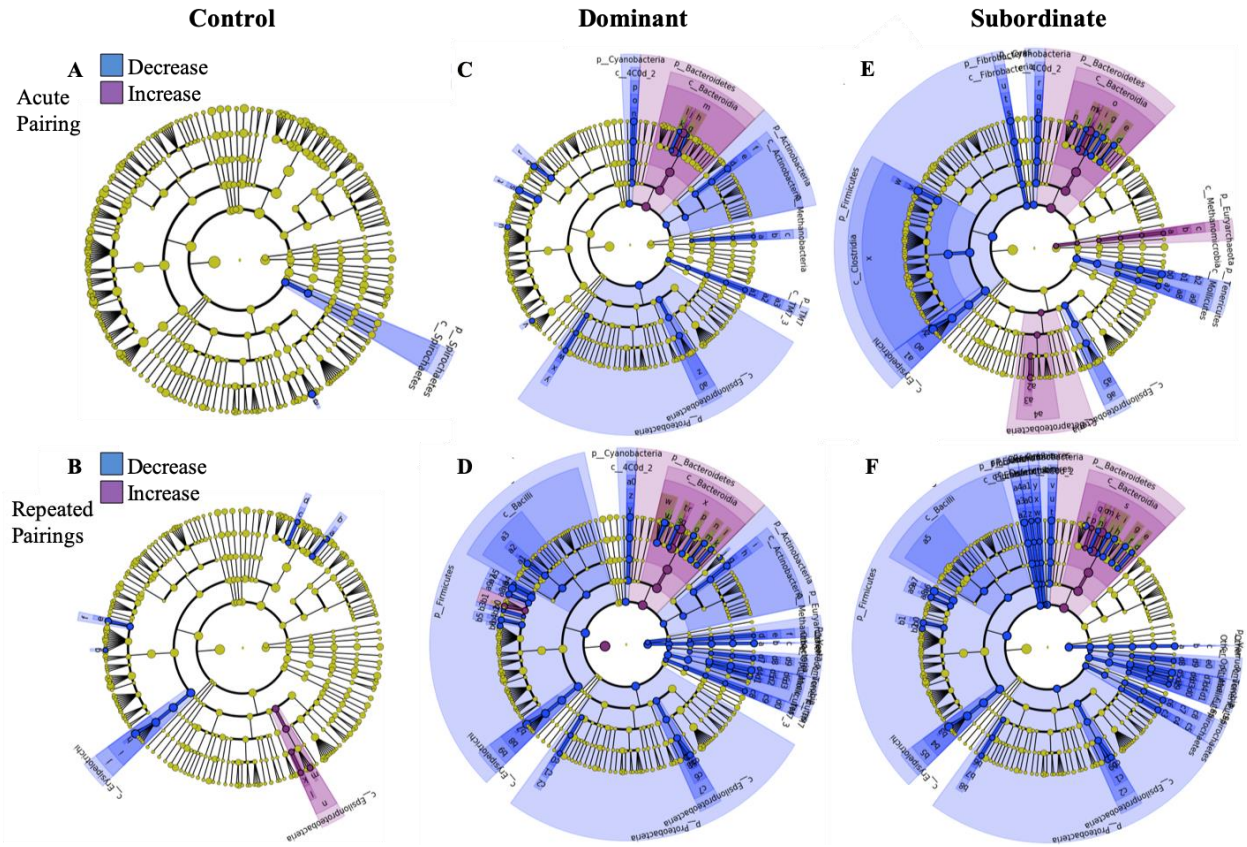

Figure 2.4 Identification of bacterial taxa altered following acute and repeated social stress compared to each group's baseline value.

Acute Social Stress (top row): LEfSE (Linear discriminant analysis Effect Size) was used to investigate bacterial taxa that drive differences between samples at baseline and after *acute* pairing in control (**A**), dominant (**C**) and subordinate (**E**) hamsters. Repeated Social Stress (bottom row): bacterial taxa that drive differences between samples at baseline and after *repeated* pairing in control (**B**), dominant (**D**) and subordinate (**F**) hamsters. Blue, taxa decreased after pairing compared with that group's baseline value; purple, taxa increased after pairing compared to baseline. Only taxa meeting an LDA significant threshold >2.0 are represented. Note: for a list of significantly altered taxa in panels where labels overlap (E, D, F) see Supplemental Figure 2.

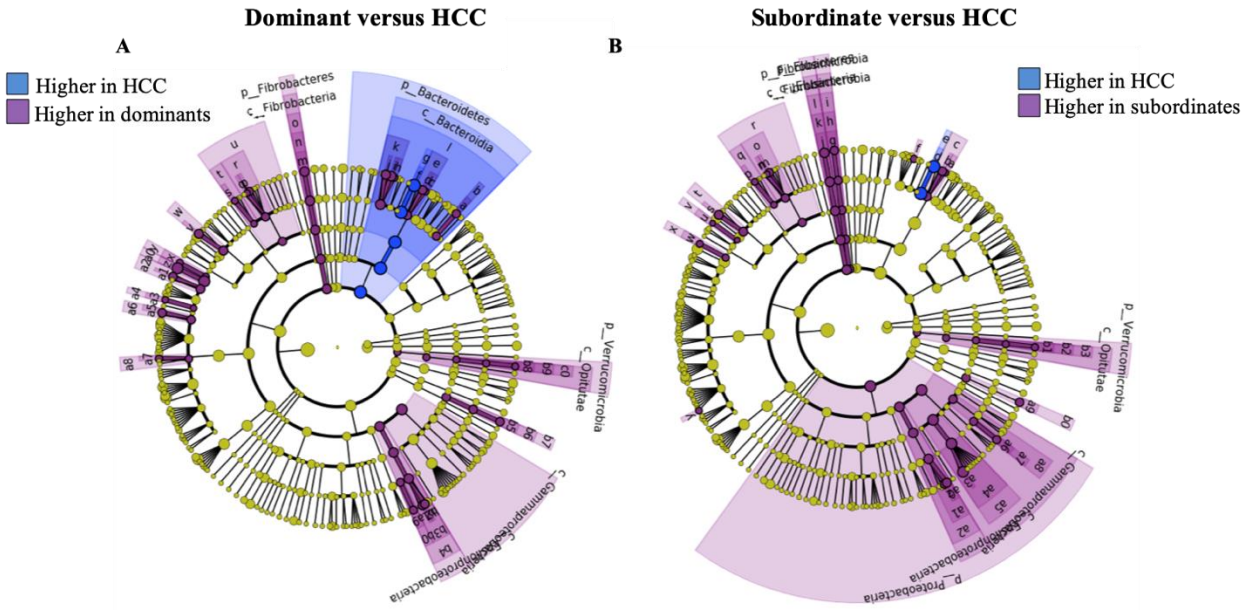

Figure 2.5 Identification of bacterial taxa altered by repeated social stress compared to home cage controls.
LEfSE (LDA Effect Size) was used to investigate bacterial taxa that drive differences between control and dominant hamsters (**A**) after repeated pairing and between control and subordinate hamsters after repeated pairing (**B**). Purple, taxa decreased compared to control; blue, taxa increased compared to control. Only taxa meeting an LDA significant threshold >2.0 are represented. Note: for a list of significantly altered taxa for both panels see Supplemental Figure 3.

2.3.3 Baseline microbiota composition can predict the outcome of a social conflict

We next investigated if the abundances of particular microbial taxa at baseline (i.e., before any social interaction) were different in animals that ultimately became dominant versus those that became subordinate. PERMANOVA analysis of the unweighted Unifrac distance revealed that there were no overall differences in microbiota community composition between future dominant or future subordinate hamsters before they were exposed to social conflict (Figure 2A; $p = 0.695$). However, LEfSE analysis revealed that the abundance of several individual bacterial taxa were significantly different at baseline in future dominant versus future subordinate hamsters (Figure 6A-F). Using a more stringent Mann-Whitney U test to compare individual OTUs, we observed significantly more Proteobacteria (Figure 6B; $U(12) = 4$, $p =$

0.007) and fewer Firmicutes (Figure 6F; $U(12) = 9$, $p = 0.053$) at baseline in future dominant animals compared with future subordinate animals. In addition, at the genus level, more *Prevotella* (Figure 6E; $U(12) = 8$, $p = 0.038$) was associated with future dominant status and more *Allobaculum* (Figure 6C; $U(12) = 6$, $p = 0.018$) with future subordinate status.

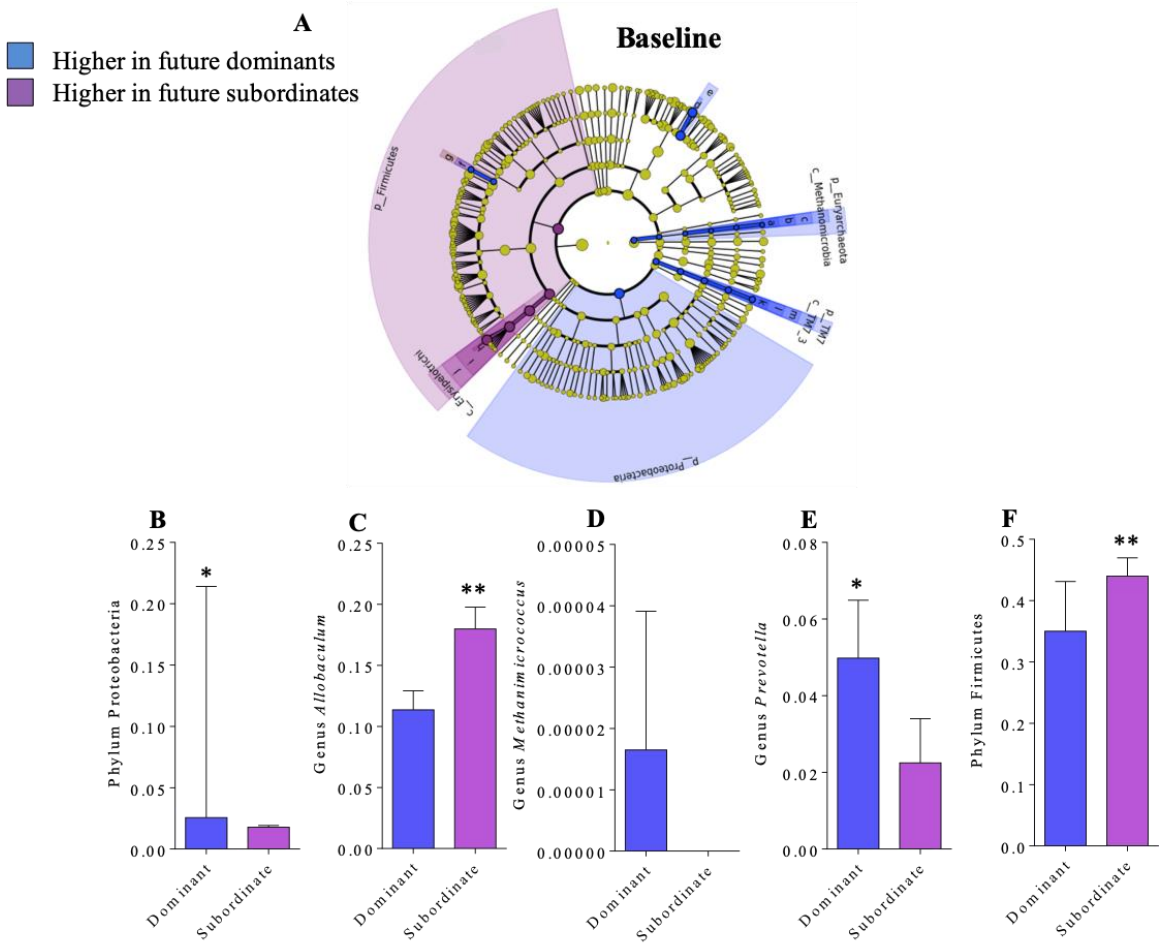

Figure 2.6 Identification of bacterial taxa that predict future dominant or subordinate status.

(**A**) LEfSE (LDA Effect Size) was used to investigate baseline bacterial taxa that predict dominant versus subordinate status upon pairing. Blue, future dominant-enriched taxa; purple, future subordinate-enriched taxa. Only taxa meeting an LDA significant threshold >2.0 are represented. Relative values of Phylum Proteobacteria (**B**), Genus *Allobaculum* (**C**), Genus *Methanimicrococcus* (**D**), Genus *Prevotella* (**E**), and Phylum Firmicutes (**F**) abundance at baseline in future dominant and subordinate hamsters. Data are the medians ± IQR. P values were determined using Mann-Whitney U test. * denotes significantly higher at baseline in future dominants; ** significantly higher at baseline in future subordinates ($P<0.05$).

2.4 Discussion

Using a well-characterized resident-intruder model in Syrian hamsters, we demonstrated that exposure to repeated and even to a single agonistic encounter causes alterations in gut microbiota in hamsters, whether they become dominant or subordinate. To our knowledge, this is the first time that the effects of social conflict on gut microbiota have been examined in hamsters, in animals that become dominant, and after both acute and repeated resident-intruder pairings in age- and weight-matched conspecifics. Intriguingly, we also found that certain microbes significantly differed in abundance between future dominants and subordinates, suggesting the possibility that baseline commensal gut bacteria in these animals could act as a predictor, or biomarker, of which animal would become dominant or subordinate during a subsequent social encounter. This exciting possibility would build on current evidence that gut microbiota can modulate social behavior and should be an area of future investigation.

PERMANOVA analysis revealed a significant shift from baseline values in the overall composition of the gut microbial community in hamsters after both one and repeated (e.g., nine) agonistic encounters. Interestingly, dominant and subordinate animals exhibited a similar increase in the inter individual variation of the microbial community after social conflict indicating that it is conflict, itself, that changes beta diversity independent of the outcome of the encounter; although it should be noted only subordinate animals reached significance when the unifrac distance was compared using Kruskal-Wallis. By contrast, home cage controls that experienced the same handling, transport, and cage changes, but no social interactions, exhibited little alteration in their gut microbes over the course of the study. Because we have previously observed a more pronounced hormonal response to social conflict in subordinates than in dominants (Huhman et al., 1990, 1991), we expected the microbial alterations in subordinate

hamsters to be exacerbated compared to that observed in dominants. At this point it is unclear if the observed changes to gut microbiota following social conflict have functional relevance or if the similar patterns of change observed in dominants and subordinates could possibly have different functional consequences for each group.

Shifts in microbial richness and evenness of the intestinal microbiota (i.e., a decrease in alpha diversity) was found in hamsters exposed to both acute and repeated agonistic encounters compared to their respective baseline values and to home cage controls following repeated encounters. Disruption to the richness and evenness of the intestinal microbiota can compromise the gastrointestinal epithelium, and such a compromise can be associated with bacterial translocation and a pro-inflammatory immune response (Chassaing et al., 2015, Maes, 2008). This phenomenon has been reported in a mouse model of social disruption and has also been linked to pathophysiology underlying major depressive disorder in humans (Bailey et al., 2011, Maes et al., 2008). We did not examine gastrointestinal status or immune responses in the current study, but we suggest that hamsters are ideal for testing these endpoints particularly because social stress can be examined in dominants and subordinates, in both males and females (Rosenhauer et al., 2017), and in the absence of social stress-induced tissue damage. Removing the confound of injury would thus allow an assessment of changes to the gastrointestinal epithelium and inflammation following exposure to a largely *psychological* stressor.

LEfSE analysis was used to identify specific microbial taxa driving changes to gut microbiota in dominants and subordinates. One of the taxa driving the altered composition in both dominants and subordinates following repeated fighting was the order Lactobacillales, which was significantly reduced following stress compared to baseline. One genus of this order, *Lactobacillus,* is often examined in the literature and a reduction in this genus is observed

following an acute and a prolonged social disruption stressor (Bailey et al., 2011, Galley et al., 2014). Although increases in certain members of lactobacilli have been associated with pathology, the majority of these bacteria are thought to be non-pathogenic or beneficial (Marin et al., 2017). Bailey et al., (2011), and Jones & Versalovic (2009) point out that many members of the genus *Lactobacillus* prevent the bacterial translocation that can trigger the production of pro-inflammatory cytokines. Certain members of lactobacilli are also thought to impact the behavior of their hosts (Dinan & Cryan, 2012). Supplementation with probiotic strains of lactobacilli in rodents reduces anxiety-like and depressive-like behavior and suppresses corticosterone release (Ait-Belgnaoui et al., 2014, Bravo et al., 2011). In humans, probiotics containing strains of lactobacilli reduce symptoms of anxiety in patients diagnosed with chronic fatigue syndrome and alleviate psychological distress in healthy human volunteers (Messaoudi et al., 2011, Rao et al., 2009). Given these findings, it is possible that the reduction within the order Lactobacillales observed after social stress is anxiogenic and impacts behavioral responses to subsequent stressors, however; this possibility requires further investigations into the particular strains driving the change in this order.

In addition to the potential effects discussed above, a reduction in certain strains of lactobacilli can also increase permeability of the gut epithelium to other bacteria, such as genus *Clostridium* (Bailey et al., 2011). *Clostridium* has been shown to increase in abundance following stress and is linked to gastrointestinal disease and inflammation (Aguilera et al., 2013, Brook, 2008, Libby & Bearman, 2009). *Clostridium* may act by producing propionic acid, which is thought to stimulate anxiety-like behavior, to further compromise an adaptive response to future stressors (Hanstock et al., 2004). Interestingly, both dominant hamsters in the current

study and mice subjected to a prolonged social disruption stressor (Bailey et al., 2011) exhibit increases in genus *Clostridium*.

Phyla Firmicutes and Bacteroidetes make up 90% of the bacteria in the gut of humans (Eckburg et al., 2005). Patients diagnosed with major depressive disorder and systemic lupus erythematosus consistently exhibit relatively lower Firmicutes and higher Bacteroidetes than do healthy controls (He et al., 2016, Jiang et al., 2015). Interestingly, LEfSe analysis revealed that Firmicutes were significantly lower and Bacteroidetes were significantly higher in both dominants and subordinates after repeated pairings compared to baseline. This finding extends the results of Bailey et al. (2011), who reported modest reductions in Firmicutes and increases in Bacteroidetes in mice following 6, 2hr bouts of social disruption stress. Further, the current study indicates that these shifts in microbial abundance are not specific only to hamsters that are being attacked, but are also observed in individuals on the winning end of a social conflict experience. Of course, we cannot rule out the possibility that the observed changes following social encounters may be due to social contact, itself, and not due to the agonistic behavior. The fact that we observed some differences in taxa changed between dominant and subordinate animals, however, suggests that the outcome of the agonistic encounters has at least some effect on gut microbiota.

The baseline abundance of certain microbial members has been previously associated with physiological and behavioral responses to subsequent stressors (Mika et al., 2016, Thompson et al., 2016). In this study, baseline abundance of some microbial species was differentially enriched in hamsters that would subsequently become dominant or subordinate in a social conflict situation. For example, bacteria of the phylum Proteobacteria and genus *Prevotella* were significantly higher in hamsters that went on to become dominant compared

with future subordinates. Interestingly, Thompson et al. (2016) found higher levels of Proteobacteria predicted greater REM sleep recovery following inescapable tail shock. Further, taxa from the genus *Prevotella* are thought to drive overall composition of the microbial community (Yatsunenko 2012) and reductions in these taxa has been associated with physiological consequences such as irritable bowel syndrome (Seksik 2003), eczema (Mah 2006), and rheumatoid arthritis (Vaahtovuo 2008). Therefore, increased levels of these taxa may be beneficial when presented with different physiological and psychological stressors. In contrast, phylum Firmicutes was significantly higher in hamsters that went on to lose their agonistic encounters compared with those that won. While this post-facto association would require replication to assess the notion that microbiota composition can predict an animal's likelihood of becoming dominant or subordinate following a resident-intruder interaction, it is in accord with recent data suggesting that gut microbiota can influence social behavior (Dinan et al., 2015, Parashar & Udayabanu, 2015). One possible explanation could be that microbiota composition influences, or is influenced by, hormones that control aggressive/subordinate behavior. These possibilities should be pursued in future studies.

In conclusion, this study used a social conflict model in Syrian hamsters to examine the effect of social conflict stress on commensal gut microbiota. We used this model in part because brief exposure to social conflict in this species causes pronounced and well-characterized responses in brain and behavior and because it is also possible to directly compare the responses of weight and age-matched hamsters that become both dominant and subordinate. Here, we demonstrated that 1) one agonistic encounter is sufficient to induce significant changes to gut microbiota, 2) the opportunity to engage in social interaction induces alterations to gut microbiota, although the particular microbial taxa that are altered are not completely overlapping

in dominants and subordinates, and 3) certain microbial taxa may predict the outcome of an agonistic encounter. Our study only examined the effect of agonistic encounters after one or nine pairings over the course of 5 days. Further studies should extend this timeline to assess chronic effects of social stress on the microbiome. Although the mechanistic link between gut bacteria and future social rank were not assessed in this study, our findings do raise a number of intriguing questions about how the gut might influence brain and behavior to shape responses to social stress. Future work should investigate the functional consequence of fecal transplant or manipulation of relevant bacteria in animals before exposure to social stress to further elucidate the role of gut microbiota in social conflict-related behavior.

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3 CAN INCREASING "HEALTHY" GUT MICORBES PRIOR TO SOCIAL STRESS REDUCE THE BEHAVIORAL RESPONSE TO SOCIAL STRESS?

3.1 Introduction

The human gastrointestinal tract houses a vastly abundant community of microorganisms and it has become increasingly clear that the state of this microbial community can impact a multitude of disease states (Eckburg et al., 2005; for a review, see Cryan et al., 2019; Scriven et al., 2018). It is well known that this community of microbes is necessary for general health and vital processes such as digestion (Sommer & Backhed, 2013; Hill 1997), gastrointestinal barrier protection, and immunoregulation (Hrncir et al., 2008; Tlaskalova-Hogenova et al., 2004), and it has recently become clear that this dynamic community can also impact the brain and behavior (Bercik & Collins, 2014; Cryan & O'Mahony, 2011). While it is well known that multiple, bidirectional routes of communication exist between the gastrointestinal tract and the brain (for a review, see Dinan & Cryan, 2012), including the vagus nerve, release of neurotransmitters, endocrine factors, and immune cell mediators, more research is necessary to understand the neurological and behavioral implications of this wide-spread communication. While recent research has begun to show that the gut microbiota may alter behavior in a number of contexts (Martin & Mayer, 2017), comparatively little is known as yet about whether this community alters social behavior in ethologically relevant models of social interaction in which we can observe behavioral responses that resemble the symptoms of neuropsychiatric disorders in humans. These data would illuminate whether the gut microbial community could be a potential target for the development of novel treatments for these neuropsychiatric disorders.

Social stress is the primary form of stress experienced by humans and is a major predictor for the onset of a variety of neuropsychiatric disorders (Brown & Prudo, 1981; Kessler, 1997;

Bjorkqvist et al., 2001), such as mood and anxiety disorders. Extensive research has explored how susceptibility to social stress may increase the likelihood of developing a mood or anxiety disorder (Agid & Lerer, 2000; Bjorkqvist et al., 2001; Lederbogen et al., 2011); however, there is limited research on the effect of gut microbiota on susceptibility to social stress. Research suggests the gut is responsive to stress, and stress-induced dysbiosis of the gut microbial community has been linked to negative health consequences such as breakdown of the gastrointestinal barrier and a heightened proinflammatory profile (Maltz et al., 2018; Lyte et al., 2011; Holder et al., 2019; Dinan et al., 2015; Parashar & Udayabanu, 2016; Chassaing et al., 2015). Our lab recently demonstrated that a single social defeat in Syrian hamsters is enough to cause dysbiosis of the gut microbial community and that these consequences are exacerbated following repeated bouts of social stress. Specific microbial taxa were also able to predict future dominant or subordinate status following an agonistic encounter (Partrick et al., 2018).

Therefore, to understand better how the state of the gut microbial community drives social behavior, we asked whether manipulating the gut microbial community with probiotics would alter susceptibility to social stress. Probiotics contain large quantities of gut-derived microbes that are thought to benefit the host (Dinan et al, 2013). Evidence for the therapeutic efficacy of probiotics continues to build with various microbial strains showing positive outcomes in animal models for diseases such as asthma (Fonseca et al., 2017), obesity (Li et al., 2016; Park et al., 2017), depression, and anxiety (Liu et al., 2016; Pirbaglou et al., 2016). Probiotic administration has been linked to lower plasma corticosterone and higher neurogenesis following water avoidance stress in mice (Ait-Belgnaoui et al., 2014) and is thought to decrease stress-induced anxiety- and depressive-like behavior (Bravo et al., 2011; O'Mahony et al., 2011). Further, probiotics are thought to promote an anti-inflammatory profile (Bermudez-Brito et al.,

2012; Laudanno et al., 2006; Singh et al., 2018). This claim is supported by data in humans and animal models suggesting that probiotics improve immune health (Hungin et al., 2017; Bharwani et al., 2017). To date, a limited number of studies have looked at the effect of probiotics on susceptibility to social stress. These studies have shown that probiotic supplementation increases social interaction with a conspecific (Maehata et al., 2018) and decreases anxiety-like behavior in the open field and light-dark box tests following chronic social defeat (Bharwani et al, 2017). Further, oral administration of the probiotic stain *Lactobacillus rhamnosus* eliminates the stress-induced immunoregulatory alterations observed following social defeat by preventing the activation of splenic dendritic cells and by promoting the proliferation of regulatory T-cells that produce the anti-inflammatory cytokine, interleukin-10 (IL-10) (Bharwani et al., 2017). Similarly, immunization of mice with the heat-killed probiotic strain *Mycobacterium vaccae* ameliorates the anxiety-like and fear-related behaviors normally observed in the elevated plus maze following 19 days of chronic subordinate colony housing, and this anxiolytic effect appears to be dependent on activation of regulatory T-cells. Intervention with *Mycobacterium vaccae* also causes an increase in the release of IL-10 (Reber et al., 2016). These studies suggest that probiotics have stress-protective effects on behavior and the immune system and that these effects could potentially decrease susceptibility to stress-related neuropsychiatric disorders. However, the current literature is somewhat limited in that the vast majority of studies have been conducted in a single species, mice, and, even among different mouse strains, the effect of probiotics on anxiety- and depressive-like behavior and immunoregulation can differ (Bharwani et al., 2017). Additionally, there is limited information on how probiotics impact the gut microbial community.

The current study uses a social defeat model in Syrian hamsters to test the hypothesis that probiotic (containing microbial strains *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) intervention regulates the gut microbial community and decreases susceptibility to social stress. Syrian hamsters provide an ideal model of social stress because when conspecifics are paired, they readily produce aggressive and territorial behavior that rapidly results in the formation of a stable dominance relationship (Albers et al., 2002). Agonistic behavior during these brief encounters is highly ritualized and rarely results in tissue damage, allowing us to focus on the psychological, as opposed to physical, aspects of social stress. Lack of injury also eliminates any potential confounding effect of physical injury on inflammation and the gut microbial community. We predict oral treatment with a probiotic containing *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 to prevent stress-induced dysbiosis of the gut microbial community and to decrease the usual defeat-induced effects on social avoidance and/or social interaction time. Additionally, we predict probiotic treatment to have an anti-inflammatory effect by upregulating circulating levels of anti-inflammatory cytokines and downregulating circulating levels of proinflammatory cytokines.

3.2 Methods

3.2.1 Animals

Adult male Syrian hamsters (*Mesocricetus auratus*), weighing between 120 and 130 g, were obtained from Charles River Laboratory (Kingston, NY) at approximately 3 months of age. Hamsters were individually housed in polycarbonate cages (24x33x20cm) with corncob bedding, cotton nesting material, and a wire mesh top in a temperature controlled colony room under a 14:10hr light/dark cycle, which is standard to maintain reproductive gonadal status in hamsters.

Food and water were available *ad libitum*. All hamsters were handled daily for 7 days to acclimate them to handling stress before the beginning of the experiment. Individual housing is not stressful for Syrian hamsters (Ross et al., 2017), and with the exception of the agonistic pairings described in Section 3.3, hamsters remained separated throughout the experiment. All protocols and procedures were approved by the Georgia State University Institutional Animal Care and Use Committee prior to experimentation, and all methods align with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

3.2.2 Probiotic intervention

Syrian hamsters were assigned to one of three treatment groups: placebo (n = 20), probiotic at a low dose of 10^9 colony forming units per day (n = 10) that is thought to be comparable to a dose normally consumed by humans, and probiotic high dose (10-fold higher than low dose) of 10^{10} colony forming units per day (n = 20). Hamsters were given either the commercial probiotic formulation Probio'stick (Lallemand Health Solutions Inc., Montreal, QC, Canada), containing freeze-dried lactic acid bacteria strains, *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 mixed with excipients (xylitol, maize derived matrodextrin, plum flavor, and malic acid) or the placebo formulation containing all excipients but no active bacterial strains. Placebo and probiotic solutions were prepared prior to administration by dissolving the formulation in distilled water (placebo: 6 g in 9 mL; probiotic low dose: 0.4 g in 6 mL; probiotic high dose: 4 g in 6 mL). Each hamster received a daily dose of 0.2 g. Hamsters received either the probiotic intervention or placebo for 14 days prior to the behavior experiment and throughout the experiment for a total treatment length of 21 days. Each day, hamsters were given 0.3 mL of the appropriate solution by syringe feeding (consuming the solution directly from an uncapped syringe held into their cage, as described in Tillmann & Wegener, 2018) at the

start of the active phase of the daily activity cycle. Due to the high likelihood of cross contamination between the probiotic intervention and placebo, extreme precaution was taken to ensure no cross contamination occurred. For instance, the probiotic and placebo solutions were prepared in separate lab spaces, lab coats were immediately disposed of after preparation, and different experimenters were used for administering either placebo or probiotic to hamsters.

3.2.3 Behavioral procedures

All behavioral manipulations were conducted during the dark phase of the daily light:dark cycle to control for circadian variation in behavior and because this is when hamsters are active and exhibit the majority of their agonistic behavior. Each day, all hamsters were moved into the behavior suite 30 min prior to any manipulation to allow time to acclimate. Behavior trials were run under dim red light and were recorded with a CCD camera.

For acute defeat training, hamsters were placed in the home cage of a novel same-sex aggressor (as described in Huhman et al., 2003) for 15 min at the start of the dark phase. For repeated defeat training, hamsters were placed in a novel, same-sex resident aggressor's home cage for 5 min twice a day for 4 days. The first pairing occurred at the start of the dark phase and the second occurred 4 hours later. A clear plastic lid was placed over the resident's cage during each pairing to prevent escape. The resident aggressor reliably attacked the experimental subject and the latter exhibited submissive and defensive behaviors such as upright defense, flee, and tail lift (for a detailed description of the behaviors scored see, Albers et al., 2002).

Social behavior testing (duration 5 min) took place approximately 24 h after acute and repeated defeat training, as described previously (McCann & Huhman, 2012). Hamsters were placed in a novel polycarbonate cage with a novel aggressor. These aggressors were confined to a small box on one side of the polycarbonate cage, allowing the subject to see, hear, and smell

the aggressor, but preventing any direct contact. Testing sessions were recorded in the same manner as the defeat training sessions and were later analyzed by observers blinded to condition to determine the time spent "far" (in the opposite half of the polycarbonate cage from the caged resident aggressor), which we define as social avoidance, as described previously (McCann et al., 2014; McCann & Huhman, 2012), and time spent in social interaction (defined as nose to caged aggressor on the near side). Additional behaviors were quantified including frequencies of flank marks (hamster rubs it's flank glands along the wall of the cage as a means of social communication (Song et al., 2014)), and several overt submissive behaviors including flees (hamster rapidly moves away from opponent often with tail lifted) and risk assessments (hamster stretches forward cautiously in a characteristic flat-back posture to investigate a potential threat (Blanchard et al., 2001; McCann & Huhman, 2012)). Social behavior comparisons were analyzed by Repeated Measures Two-way ANOVA with Tukey's post hoc analysis on GraphPad Prism 8.2.0 (GraphPad Software, La Jolla, CA). Differences in post hoc analyses were denoted as significant at $*p < 0.05$. All hamsters were carefully observed during each agonistic encounter for coprophagia and for any injury. No coprophagia or tissue damage occurred during training or testing.

3.2.4 Fecal collection and microbiota composition analysis by 16S gene sequencing

Fresh fecal samples were collected at three time points just before the beginning of the active (dark) phase of the daily light:dark cycle: 1) prior to the initial defeat (baseline samples), 2) 24 h after the acute defeat (acute defeat samples), and 3) 24 h after the final defeat (repeated defeat sample) to assess the microbial community before any stress, after one bout of social defeat, and after repeated bouts of social defeat. To avoid additional stress to the animal, hamsters were transferred into a clean cage and fecal samples were collected from the cage

approximately 1 h later. Samples were collected in RNase-free microcentrifuge tubes and were immediately frozen and stored at -80° C until further processing.

Fecal 16S rRNA gene amplification and sequencing were done using Illumina MiSeq technology following the protocol of the Earth Microbiome Project with their modifications to the MO BIO PowerSoil DNA Isolation Kit procedure for extracting DNA (www.earthmicrobiome.org/emp-standard-protocols). In brief, bulk DNA was extracted from frozen feces using a PowerSoil-htp kit from MO BIO Laboratories (Carlsbad, California, USA) with mechanical disruption (bead-beating). The 16S rRNA genes, region V4, were PCR amplified from each sample using a composite forward primer and a reverse primer containing a unique 12-base barcode, designed using the Golay error-correcting scheme, which was used to tag PCR products from respective samples. We used the forward primer 515F 5'-*AATGATACGGCGACCACCGAGATCTACAC***TATGGTAATT*GT***GTGCCAGCMGCCGCGGTAA-3': the italicized sequence is the 5' Illumina adapter B, the bold sequence is the primer pad, the italicized and bold sequence is the primer linker and the underlined sequence is the conserved bacterial primer 515F. The reverse primer 806R used was 5'-*CAAGCAGAAGACGGCATACGAGAT* XXXXXXXXXXXX **AGTCAGTCAG *CC*** *GGACTACHVGGGTWTCTAAT*-3': the italicized sequence is the 3' reverse complement sequence of Illumina adapter, the 12 X sequence is the golay barcode, the bold sequence is the primer pad, the italicized and bold sequence is the primer linker and the underlined sequence is the conserved bacterial primer 806R. PCR reactions consisted of Hot Master PCR mix (Five Prime), 0.2 □M of each primer, 10-100 ng template, and reaction conditions were 3 min at 95°C, followed by 30 cycles of 45 s at 95°C, 60 s at 50°C, and 90 s at 72°C on a Biorad thermocycler. Two independent PCRs were performed for each sample, then combined and purified with

Ampure magnetic purification beads (Agencourt), and products were visualized by gel electrophoresis. Products were then quantified (BIOTEK Fluorescence Spectrophotometer). A master DNA pool was generated from the purified products in equimolar ratios. The pooled products were sequenced using an Illumina MiSeq sequencer (paired-end reads, 2 x 250 bp).

3.2.5 16S gene sequencing analysis

The sequences were demultiplexed and quality filtered using the Quantitative Insights Into Microbial Ecology 2 (QIIME 2, version 3.5.5) software package (Bolyen et al., 2019). Forward and reverse Illumina reads were joined using the fastq-join method. We used the QIIME 2 default parameters for quality filtering as described in detail in Bolyen et al. (2019). Sequences were clustered using the UCLUST algorithm with a 97% homology threshold. Clusters were then classified taxonomically using the Greengenes reference database, Version 13.5. Clusters that did not match any Greengenes Operational taxonomic units (OTUs) were kept. A single representative sequence for each OTU was aligned and a phylogenetic tree was built using FastTree.

The phylogenetic tree described above was used to assess beta and alpha diversity. Beta diversity measures the variation in microbiota composition between individual samples. Alpha diversity metrics measure both the richness and/or evenness (or distribution) of unique microbial taxa within a sample (Mackos et al., 2017, Sekirov et al., 2010). Bray-Curtis, which accounts for taxa abundance, and Unweighted UniFrac, which accounts for phylogeny were used to compute distances between groups and measure beta diversity between groups using rarefied OTU table count. Different metrics analyze the data in slightly different ways. Therefore, it is important to analyze data using multiple metrics to gain more information, and to obtain a broader view of the data. Principal coordinates analysis (PCoA) plots were used to further assess and visualize beta

diversity. Groups were compared for distinct clustering using PERMANOVA method using vegan R-package through QIIME 2. The phylogeny-based metric, phylogenetic diversity whole tree (PD whole tree) measurements was determined with QIIME 2 using an OTU table rarefied at various depths and the non phylogeny-based metric, Observed OTUs was determined with QIIME 2 using the alpha_diversity.py command line of the rarefied OTU table count. Again, we used multiple metrics to analyze alpha diversity to gain more information from the data. Kruskal-Wallis with Dunn's multiple comparison test was used to determine differences in alpha diversity among groups. Lastly, LEfSE (Linear Discriminate Analysis Effect Size) was used to investigate bacterial taxa that drive differences between groups by comparing the abundance of specific taxa between each experimental group. All statistics were done in GraphPad Prism software, version 6.01 and IBM SPSS Statistics, version 22.

3.2.6 Multiplex assay procedure

24 h after the final defeat and immediately following behavior testing, hamsters were briefly anesthetized with isoflurane and euthanized by cervical dislocation. Trunk blood was collected and allowed to clot at room temperature for 2 h. After 2 h, blood was centrifuged for 20 min at 2000 x g to obtain serum, which was immediately frozen and stored at -80°C.

A Bio-Plex Pro™ Rat Cytokine 23-Plex Assay was conducted on hamster serum using a fluorescent bead-based instrument Bio-Plex 200 (Bio-Rad, Hercules, CA). Bio-Plex instruments were validated using a Bio-Plex Validation kit 24 h prior to conducting the assay, and instruments were calibrated immediately prior to performing the assay using a Bio-Plex Calibration Kit. The assay was conducted per the manufacturer's protocol using the recommended sample dilution (4x) and standard curve concentrations. All samples and standards were assayed in duplicates. This multi-plex technology uses "xMAP" based microspheres to

detect protein concentrations of target analytes. These microspheres are fluorescent-dyed and act as analyte identifiers. Bio-Plex uses flow cytometry techniques to quantify the analytes. Beads are transported single file through a cuvette by fluid flow. Dual lasers excite each bead causing the bead to fluoresce and the fluorescence is measured with avalanche photodiodes (bead identification) and a photomultiplier tube (reporter signal). A high-speed digital processor then identifies and quantifies each microsphere based on the bead-identifying fluorescent signal and the reporter signal. The concentration of each analyte is determined by Bio-Plex Manager, and the analyte concentrations are compared across groups by ANOVA with Tukey's post hoc comparisons or Kruskal-Wallis with Dunn's multiple comparisons test on GraphPad Prism 8.2.0 (GraphPad Software, La Jolla, CA). A rat assay was chosen because no equivalent multi-plex assay exists for hamster. Notably, several of the sequences for the analytes in the multi-plex assay share a 77%-90% homology with hamster sequences, suggesting a high likelihood of cross-reactivity of these analytes with the hamster protein.

3.3 Results

3.3.1 Probiotic intervention at a low dose increases susceptibility to social stress.

Following both acute and repeated defeat training, hamsters were tested for social avoidance and social interaction with a novel, caged opponent. Repeated Measures Two-way ANOVAs with Tukey's post hoc analyses were run to assess the effect of probiotic intervention (high and low dose) on social behavior. Analysis of social avoidance behavior revealed no interaction effect ($F(2, 44) = 0.5$, $p = 0.6$) or main effect of defeat ($F(1, 44) = 2.4$, $p = 0.1$) on avoidance behavior; however, there was a significant main effect of treatment ($F(2, 44) = 4.2$, $p = 0.02$). Tukey's pairwise comparisons indicated hamsters treated with a low dose of the

probiotic showed significantly more social avoidance following an acute defeat than did hamsters treated with a high dose of the probiotic ($p = 0.04$) or placebo-treated animals ($p = 0.02$) (Figure 3.1A). Following repeated defeats, no differences in avoidance behavior between treatment groups was observed; however, there was a trend for hamsters treated with the low dose of the probiotic to spend significantly more time avoiding a caged opponent compared to placebo-treated hamsters ($p = 0.055$) (Figure 3.1A). Analysis of social interaction revealed no interaction effect ($F(2, 44) = 1.5$, $p = 0.2$), yet there was a significant main effect of defeat ($F(1, 44) = 22.3$, $p < 0.0001$) and a main effect of probiotic treatment on social interaction ($F(2, 44) = 4.8$, $p = 0.01$). Tukey's post hoc analysis revealed that hamsters treated with a low dose of the probiotic spent significantly less time interacting socially with a caged opponent following an acute defeat compared to hamsters treated with a high dose of the probiotic ($p = 0.02$) or placebo ($p = 0.03$) (Figure 3.1B). Following repeated defeats, hamsters treated with a low dose of the probiotic exhibited less social interaction than did placebo-treated hamsters ($p = 0.03$) but did not differ from hamsters receiving the high dose of the probiotic ($p = 0.2$) (Figure 3.1B). Additional behaviors such as flank markings, risk assessments, and flees were rarely observed and thus were not compared across groups.

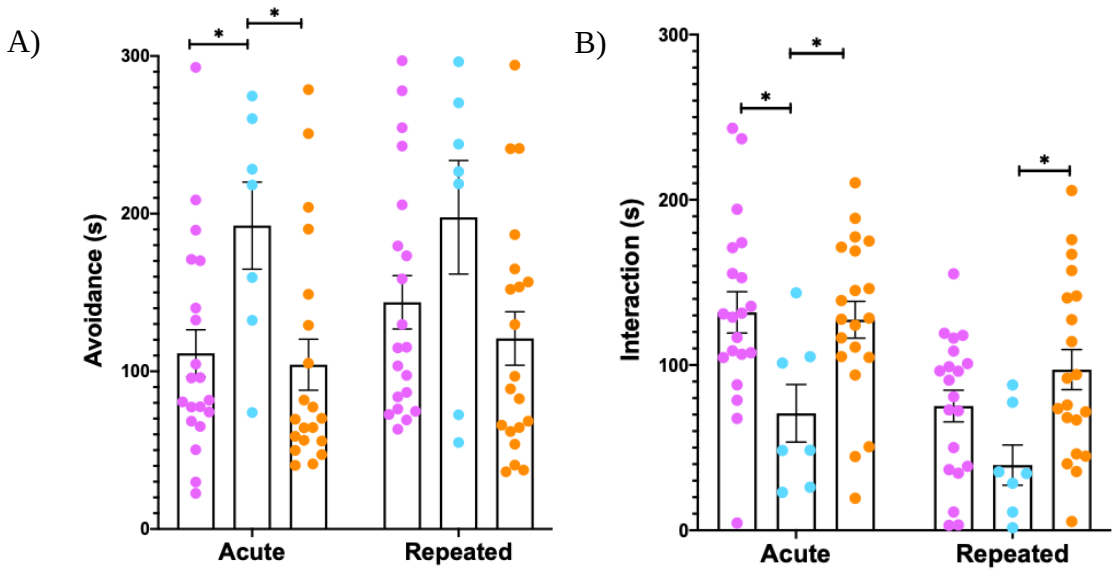
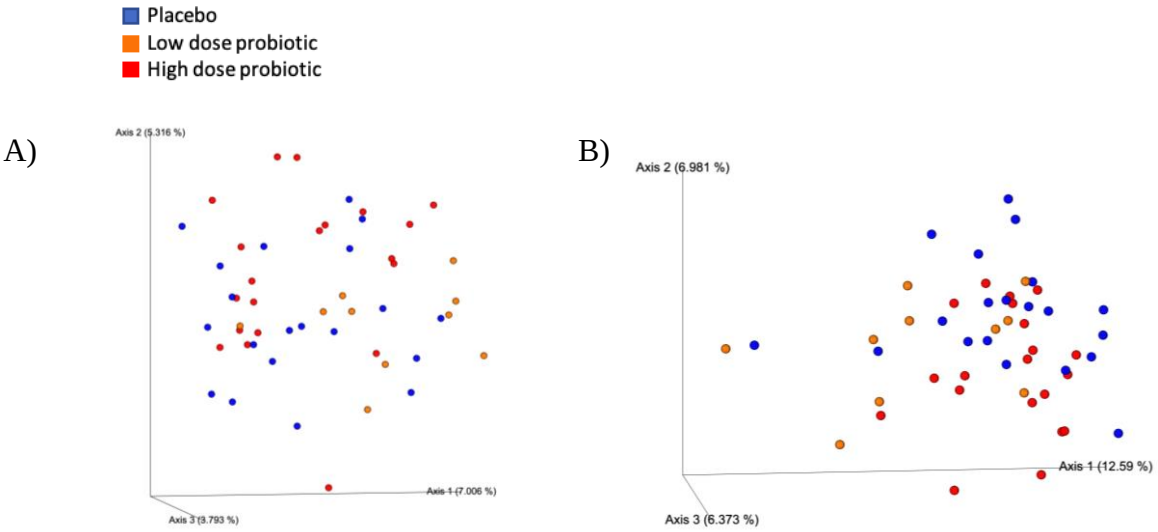


Figure 3.1 Probiotic intervention increases susceptibility to social stress.
Following the acute defeat, hamsters treated with a low dose of the probiotic (*n* = 7) (blue dots) avoided a novel opponent more (A) and interacted with the opponent less (B) than did hamsters treated with a high dose of the probiotic (*n* = 20) (purple dots) or the placebo (*n* = 20) (orange dots) (*p < 0.05). Behavioral data from 3 hamsters in the low dose probiotic group was unable to be scored and thus the number of animals in this group was reduced to 7 for these analyses. Following repeated defeats, there were no significant differences in avoidance behavior between treatment groups (A), yet low dose-treated hamsters (n = 7) (blue dots) interacted with a novel opponent significantly less than did placebo-treated hamsters (*n* = 20) (orange dots) (B; *p < 0.05).

3.3.2 Probiotic intervention alters gut microbiota composition.

Microbiota composition was analyzed by 16S rRNA Illumina sequencing of fecal DNA samples collected before defeat training (baseline), after the initial (acute) defeat, and after nine (repeated) bouts of social defeat. PERMANOVA analysis of the Bray-Curtis distance revealed that, at baseline, the microbial composition of hamsters given the low dose of the probiotic significantly differed from that of those given the high dose (p = 0.009) or placebo (p = 0.009), and a trend for a difference in microbial composition was observed following the acute defeat (low dose v high dose, p = 0.058; low dose v placebo, p = 0.054). After repeated defeats, the microbial composition of both probiotic groups differed from that of placebo (low dose v

placebo, p = 0.02; high dose v placebo, p = 0.02). Because different statistical models generate somewhat different outcomes, we also analyzed these data using the unweighted Unifrac distance between treatment groups. PERMANOVA analysis of the unweighted Unifrac distance revealed that the microbial composition of all treatment groups significantly differed from one another at baseline (low dose v high dose, p = 0.006; low dose v placebo, p = 0.006; high dose v placebo, p = 0.01). Following the acute defeat, the microbial composition of hamsters given the low dose of the probiotic differed significantly from that of hamsters administered the high dose (p = 0.05). After the acute defeat there was also a trend for the microbial composition of hamsters given either dose of the probiotic to significantly differ from that of placebo-treated hamsters (low dose v placebo, p = 0.057; high dose v placebo, p = 0.057). Following repeated defeats, the microbial composition of hamsters treated with a low dose of the probiotic differed from that of both hamsters treated with a high dose of the probiotic (p = 0.006) and placebo-treated hamsters (p = 0.04).



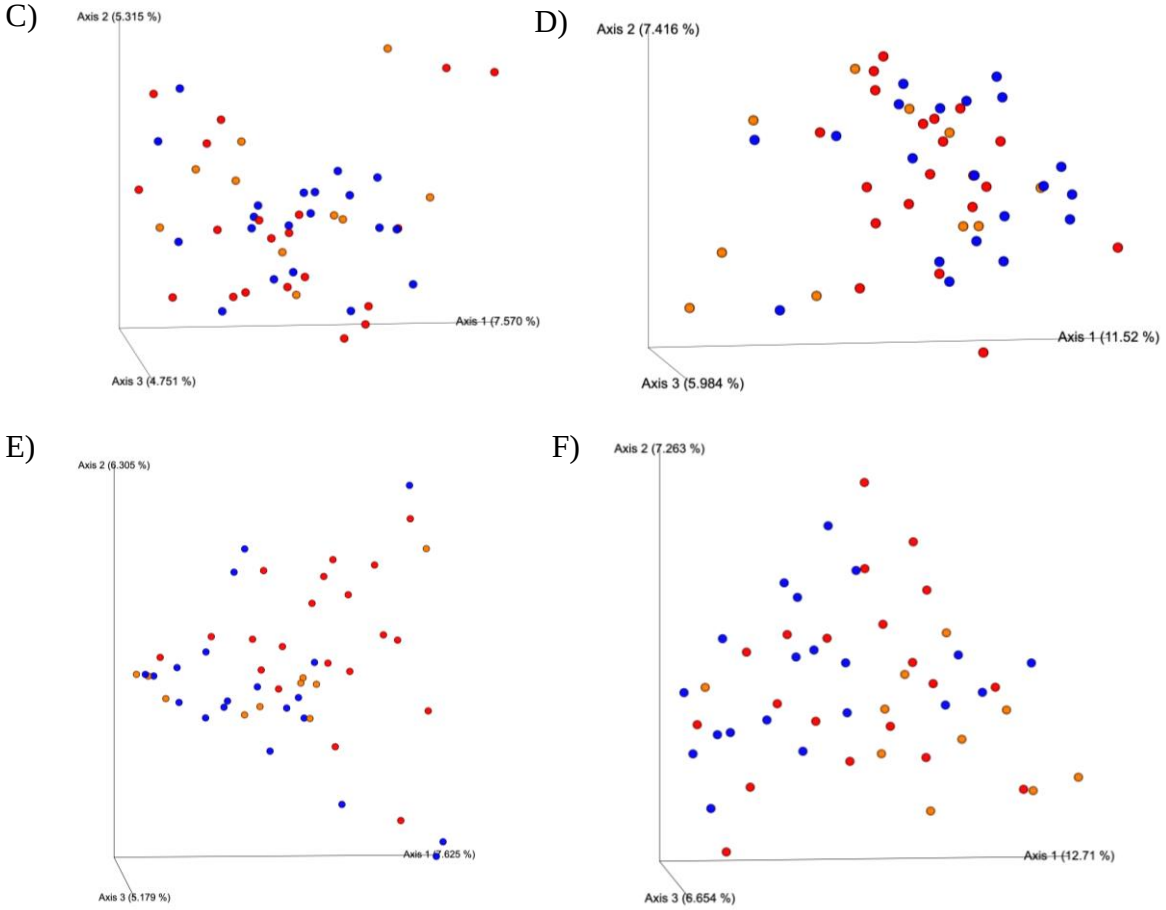


Figure 3.2 Probiotic intervention alters the gut microbial composition before and after social stress.

Principle coordinate analysis (PCoA) of the Bray-Curtis distance (A, C, E) and the unweighted unifrac distance (B, D, F) at baseline (A, B), after acute social stress (C, D), and after repeated social stress (E, F). Bray-Curtis revealed that the microbial composition of hamsters treated with a low dose of the probiotic (n = 10) (orange dots) differed from that of hamsters given placebo (n = 20) (blue dots) and the high dose of the probiotic (n = 20) (red dots) at baseline (A). After repeated bouts of social stress, the microbial composition of probiotic-treated hamsters differed from that of placebo-treated hamsters (E). Unweighted unifrac distance revealed that at baseline the microbial composition of all treatment groups differed from one another (B) and that, after acute social stress, the microbial composition of hamsters administered the low dose of the probiotic (n = 10) (orange dots) differed from that of hamsters administered the high dose (n = 20) (red dots) (D). Following repeated social stress, Unweighted unifrac revealed that the microbial composition of hamsters treated with a low dose of the probiotic (n = 10) (orange dots) was altered compared to placebo-treated hamsters (n = 20) (blue dots) and hamsters treated with a high dose of the probiotic (n = 20) (red dots) (F). P values were determined using PERMANOVA analysis and denoted as significant at P<0.05 (A-F).

The analysis of alpha diversity of the intestinal microbiota, reflecting the bacterial richness and evenness of the community, revealed a significant effect of treatment using both phylogeny-based (Faith Phylogenetic Diversity (PD) Whole Tree) (H = 14.54, p = 0.0007) and non-phylogeny-based (Observed Operational Taxonomic Units (OTUs)) (H = 10.33, p = 0.006) measurements. Dunn's multiple comparisons test revealed a significant decrease in alpha diversity for hamsters treated with a low dose of the probiotic compared to hamsters treated with a high dose of the probiotic (Faith PD Whole Tree, p = 0.001; Observed OTUs, p = 0.01) or with the placebo (Faith PD Whole Tree, p = 0.008; Observed OTUs, p = 0.02).

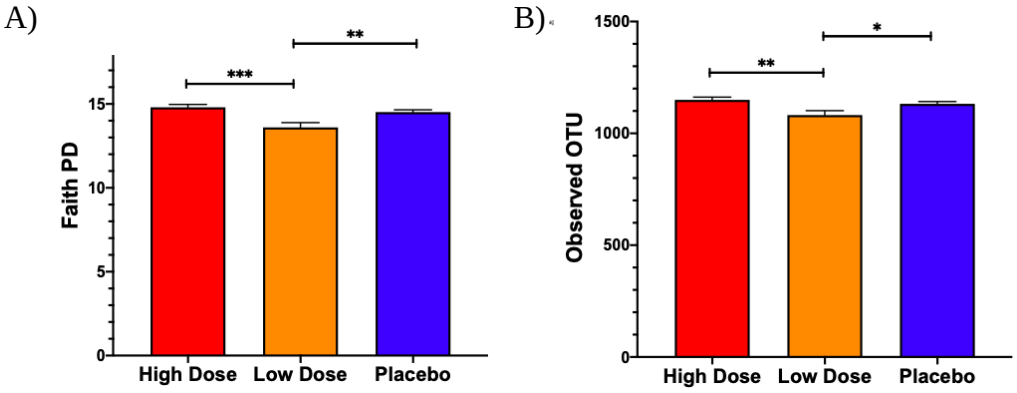


Figure 3.3 Probiotic administration at a low dose decreases intestinal microbiota diversity.

Alpha diversity was determined by Faith PD Whole Tree and Observed OTUs in hamsters treated with a high dose (red) or low dose (orange) of the probiotic and in placebo-treated hamsters (blue). Both Faith PD (A) and Observed OTUs (B) measurements indicated that hamsters given of low dose of the probiotic (orange) had lower diversity compared to hamsters given a high dose (red) or placebo (blue). * denotes $p < 0.05$; **denotes $p \leq 0.01$.

LEfSE analysis (Linear Discriminant Analysis (LDA) Effect Size) was used to identify bacterial taxa that were significantly altered by treatment or defeat training. An LDA threshold of 2 was used to infer significance (LDA > 2, $p < 0.05$). A relatively small number of taxa were significantly altered by probiotic treatment or defeat training. Notably, genus *Bifidobacterium* was significantly increased in hamsters treated with a low dose of the probiotic compared to all

other treatment groups following acute defeat (LDA > 2, $p < 0.05$). Following repeated defeats, genus *Prevotella* was significantly increased in hamsters treated with a high dose of the probiotic compared to their baseline and phyla Proteobacteria was significantly higher in placebo-treated animals compared to both probiotic treatment groups (LDA > 2, $p < 0.05$).

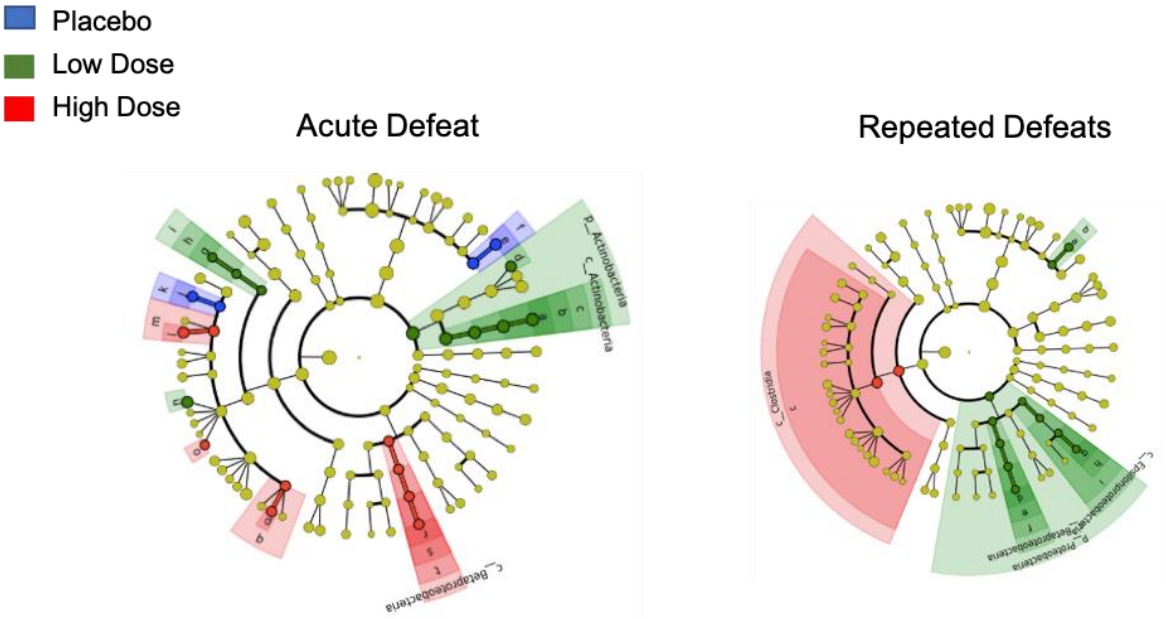


Figure 3.4 Identification of microbial taxa differentially altered across groups following acute or repeated social defeat.

LEfSE analysis was used to identify microbial taxa that differed in hamsters given either a low dose of the probiotic (green), high dose of the probiotic (red), or placebo (blue) after an acute defeat (A) or repeated defeats (B). Blue, taxa higher in placebo-treated hamster compared to both doses of the probiotic; green, taxa higher in hamsters treated with a low dose of the probiotic compared to the high dose or placebo; red, taxa higher in hamsters treated with a high dose of the probiotic compared to the low dose or placebo. Only taxa meeting an LDA significant threshold of > 2.0 are represented.

3.3.3 Probiotic intervention affects anti-inflammatory cytokine signaling.

A Bio-Plex Pro™ Rat Cytokine 23-Plex Assay was used to analyze circulating levels of cytokines. Only concentrations (pg/mL) of IL-7, IL-4, IL-10, GRO/KC, IL-5, and MIP-3α in hamster serum were detected in the majority of hamsters and were thus able to be reliably analyzed. Hamsters whose concentrations were out of range were given a value of 0 for analysis.

It should be noted that of the concentrations that were plotted on the standard curve, the majority were on the low end, which can increase variability and decrease reproducibility of results. No effect of treatment was found for IL-7, GRO/KC, and MIP-3α. One-way ANOVA revealed an effect of treatment for the anti-inflammatory cytokine IL-4 ($F(2, 48) = 12.12$, $p < 0.0001$) and Tukey's post hoc analysis found hamsters treated with both the low and high dose of the probiotic had elevated IL-4 compared to placebo-treated hamsters (high dose v placebo, $p = 0.0004$; low dose v placebo, $p = 0.0004$). The concentrations of IL-10 and IL-5 were not normally distributed, thus the nonparametric Kruskal-Wallis with Dunn's multiple comparisons test was used and revealed a significant effect of treatment for IL-10 ($H(3,51) = 19.29$, $p < 0.0001$) and IL-5 ($H(3,51) = 19.66$, $p < 0.0001$). Hamsters given either dose of the probiotic had higher concentrations of both the anti-inflammatory cytokine IL-10 and the anti-inflammatory chemokine IL-5 compared to hamsters treated with placebo (Dunn's multiple comparisons test; IL-10, high dose v placebo, $p = 0.002$, low dose v placebo, $p = 0.0021$; IL-5, high dose v placebo, $p = 0.003$, low dose v placebo, $p = 0.0002$).

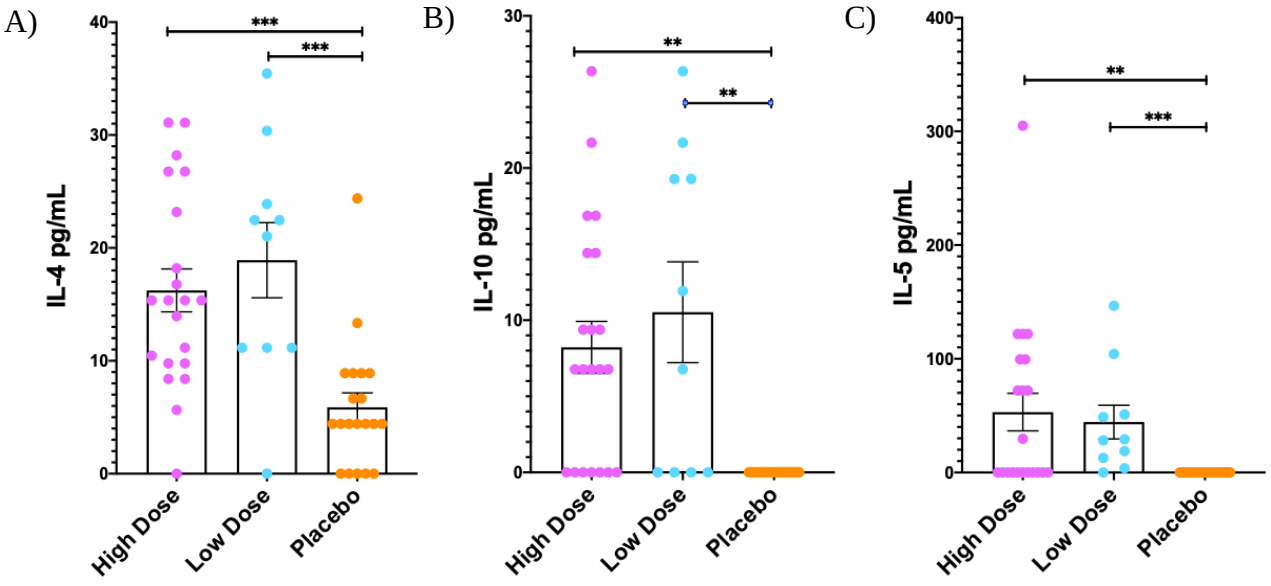


Figure 3.5 Probiotic intervention alters cytokine signaling following social stress.

Following repeated social defeat, circulating concentrations of IL-4 (A), IL-10 (B), and IL-5 (C) were altered by treatment. IL-4 (A), IL-5 (B), and IL-10 (C) concentrations (pg/mL) were significantly increased in hamsters treated with both doses of the probiotic (low dose, $n = 7$, blue dots) (high dose, $n = 20$, purple dots) compared with placebo ($n = 20$, orange dots) (**$p < 0.01$, ***$p < 0.001$).

3.4 Discussion

Our results demonstrate that a probiotic intervention at a dose that is equivalent to that used in humans can induce increases is social avoidance and decreases in social interaction, alterations in the gut microbial community, and modest changes in anti-inflammatory cytokine signaling in hamsters. We selected the commercially available probiotic Probio'stick containing the test organisms *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 because this probiotic has been reported to have reduce stress responses following exposure to water submersion or maternal separation (Ait-Belgnaoui et al., 2014, 2018; Gareau et al., 2007) and to decrease anxiety- and depressive-like behavior in humans and other animals (Messaoudi et al., 2011; Arseneault-Bréard et al., 2012). Thus, our behavioral findings were opposite of what was expected and contrast with previous literature (Dinan et al, 2013; Liu et al., 2016; Pirbaglou et al., 2016; Bravo et al., 2011; O'Mahony et al., 2011; Maehata et al., 2018; Bharwani et al, 2017; Reber et al., 2016; Warda et al., 2019). One possible explanation for this surprising finding may be that our behavioral endpoint is capturing something different than are the standard tests of anxiety-like behavior, such as the open field test or light-dark box test, that are commonly used in mice and other rodents (Bharwani et al., 2017, Bravo et al., 2011) or that the response in an ethologically relevant model is very different than that observed after exposure to a more artificial stressor. It may also be the case that the effects of probiotics are strain- or species-specific and do not necessarily translate across strains or species. This possibility is supported by evidence that different mouse strains exhibit strain-specific probiotic effects (Bharwani et al.,

2017, Bravo et al., 2011). Ultimately, it is unclear why our data differs from the majority of the previous literature indicating that probiotics are anxiolytic. However, a careful examination of differences in probiotic-induced changes to the gut microbial community and/or cytokine signaling may help elucidate why probiotic intervention at varying doses drives different responses in different models.

We measured the effect of probiotic intervention on the gut microbial composition. Beta diversity, which measures changes in the overall composition, was analyzed using both a weighted (Bray-Curtis distance) and an unweighted (unweighted Unifrac distance) metric. Bray-Curtis distance considers taxa abundance and unweighted Unifrac distance considers phylogeny of related taxa, thus each reveals a somewhat different assessment of the gut microbiota. Both the Bray-Curtis and unweighted Unifrac revealed that the microbial composition was altered in hamsters treated with a low dose of the probiotic compared to the high dose-treated and placebo-treated hamsters, suggesting that alterations to gut microbiota induced in hamsters receiving a low dose of the probiotic might drive the anxiogenic behavioral profile observed in this treatment group. It must be noted, however, that this alteration was observed at different time points when using the different metrics. That is, the Bray-Curtis indicated that at baseline there was a significant alteration in microbial composition following administration of the low dose and that there was a trend for this effect after the acute defeat. The unweighted Unifrac, which is the metric reported in most of the related studies that we have cited, indicated that, instead, the microbial composition of all treatment groups differed from one another at baseline and that there was a significant alteration in the microbial composition of hamsters administered the low dose of the probiotic after repeated social defeat. In either case, it is interesting that the microbial composition, like the behavior, seems to be altered differently by the same probiotic given at

different doses and that differences observed at baseline suggest that probiotic intervention in the absence of stress is sufficient to alter the microbial composition. Additionally, where we observed an altered microbial composition in hamsters treated with a low dose of the probiotic following repeated defeats using unweighted Unifrac, with Bray-Curtis we, instead, found that the microbial composition of both probiotic groups differed from that of placebo following repeated social stress. Thus, when accounting for taxa abundance, it appears the gut microbial composition responds differently to repeated social stress when a probiotic supplement is on board. These data verify that somewhat different results are obtained when metrics are used that take into account either abundance or phylogeny, and future work should more carefully take this into consideration.

We also assessed alpha diversity, a measure of microbial richness and abundance, using a phylogeny-based (Faith PD Whole Tree) and non-phylogeny-based (Observed OTUs) measurement. The measurements complimented one another, with both demonstrating a reduction in richness in hamsters given the low dose versus the high dose or the placebo. It is possible that a reduction in alpha diversity following social defeat in hamsters given the low dose of the probiotic drives, in part, the anxiogenic behavioral profile observed in this group. This is an interesting possibility that should be examined in future studies.

LEfSe analysis was used to identify microbial taxa that drive differences in beta and alpha diversity across groups. Although several microbial taxa were altered between treatment groups or within groups following defeat, there were fewer changes observed compared to our previous study that assessed the gut microbial community of hamsters following acute and repeated social defeat with no probiotic intervention (Partrick et al., 2018). One possibility is that the excipients (xylitol, maize-derived maltrodextrin, plum flavor, and malic acid) present in both

the placebo and probiotic solutions affected the gut to buffer somewhat the gut microbiota. Alternatively, it is also possible that these additives caused some level of gut dysbiosis that then masked the effect of our relatively mild social stressor. The latter possibility is certainly supported by the recent contention that maltodextrin is a stressor for the gut (Arnold & Chassaing, 2019). In the present study, genus *Bifidobacterium* was significantly higher in hamsters treated with a low dose of the probiotic after the acute defeat. *Bifidobacterium longum* is actively present in the probiotic formulation; thus, ingestion of the probiotic at a low dose may have allowed for heightened colonization of *Bifidobacterium* in the gut and/or increased proliferation of *Bifidobacterium* species. Further, phyla Proteobacteria was significantly higher in placebo-treated hamsters following repeated social stress and genus *Prevotella* was higher in hamsters given the high dose of the probiotic compared to their level at baseline. In our previous study, both Proteobacteria and *Prevotella* predicted dominance in an agonistic encounter (Partrick et al., 2018), and other research suggests these taxa may be beneficial or stress-protective for the host (Thompson et al., 2016; Warda et al., 2019). Therefore, the greater abundance of Proteobacteria or *Prevotella* in hamsters treated with the placebo or high dose probiotic may have reduced the behavioral response to social stress in these groups.

One mechanism whereby gut microbiota may drive differences in social behavior is by altering cytokine signaling, which is a well-characterized route of communication between the gut and brain (Dinan et al., 2015; Ramakrishna, 2013, Scriven et al., 2019). An increase in proinflammatory cytokines has been linked to exacerbated depressive- and anxiety-like behavior in animal models (Yirmiya et al., 2001; Hannestad et al., 2011; Hodes et al., 2014; Zhang et al., 2017; Monje et al., 2011; Simen et al., 2006; Goshen et al., 2008). Because the low dose probiotic produced changes to the gut microbial community and increased behavioral

susceptibility to social defeat, we investigated whether an increase in the expression of pro-inflammatory cytokines was correlated with these effects. Despite the high degree of homology of the protein sequences between rat and hamster cytokines, many of the cytokines targeted in the Luminex assay were out of detectable range. Thus, we were unable to detect several of the cytokines in which we had the most interest such as IL-6 and TNFα. This could indicate that there was a lack of cross-reactivity of some of these molecules, or, alternatively, this may simply indicate that many of the circulating cytokines were extremely low following the manipulations done in this study. Of the cytokines that were detectable (IL-7, IL-4, IL-10, GRO/KC, IL-5, MIP-3α) only three were found to be significantly altered by treatment. Although we observed an effect of probiotic dose on behavior and on the gut microbial community, this dose-dependency was not apparent when analyzing circulating cytokine signaling. Instead, both doses of the probiotic affected cytokine signaling similarly. Three anti-inflammatory cytokines, IL-4, IL-10 and IL-5, were significantly elevated in both probiotic (low and high dose) groups compared to placebo. Elevated serum concentrations of IL-10 have previously been observed following probiotic intervention with the probiotic strains *Lactobacillus rhamnosus* and *Mycobacterium vaccae* (Bharwani et al., 2017; Reber et al., 2016). Therefore, a therapeutic benefit of probiotic treatment may be to bias the immune system toward an anti-inflammatory profile following stress. Given the finding that we observed so few changes in cytokine concentrations, however, it could be the case that our model of social stress is too mild and/or that the current probiotic is ineffective in causing a robust increase in pro- or anti-inflammatory signaling and that it is not this signaling that underlies the ability of probiotics to alter behavior.

Collectively, the results of the current study demonstrate that the effect of probiotics on behavior and on gut microbial composition are probably species and dose dependent. This

highlights the importance of testing the effect of probiotics in multiple animal models and in various environmental contexts. Further, the dose-dependent differences in behavior and alterations to gut microbiota following probiotic treatment clearly illustrates that a higher probiotic dose does not necessarily predict a greater response. We have shown here that probiotic intervention can alter both behavioral responses to social stress and gut microbiota, but future work is necessary to establish whether changes to the gut microbiota are necessary for probiotic-induced behavioral alterations. Our data also suggest that cytokine signaling could be part of the mechanism whereby gut microbiota impacts behavior following probiotic treatment, thus future research should directly test this mechanism.

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4 DISRUPTION OF THE MICROBIAL-HOST RELATIONSHIP DOES NOT INCREASE SUSEPTIBILITY TO SOCIAL STRESS IN SYRIAN HAMSTERS.

4.1 Introduction

Our intestinal tracts house a diverse, complex, and abundant community of microbes, termed gut microbiota (Ley et al., 2008; Eckburg et al., 2005). Gut microbiota are critical for intestinal health, benefiting the intestinal tract by providing protection against foreign pathogens and mediating metabolism and immunoregulation (Hooper & Gordon, 2001; Hrncir et al., 2008; Tlaskalova-Hogenova et al., 2004). If the stability and diversity of the gut microbial community is compromised, this can compromise host health. Disturbances to the gut microbial community, particularly when they reflect a decrease in diversity, has been linked to gastrointestinal and inflammatory diseases (Podolskey, 2002; Targan & Karp, 2005; Lyte et al., 2011), such as irritable bowel syndrome, and to obesity-related diseases (Turnbaugh et al., 2006; Ley et al., 2006), such as metabolic syndrome. Further, a mucous membrane exists between the intestine and gut microbiota such that the gut microbiota do not come in direct contact with the intestinal epithelium. This membrane acts as a barrier that protects the intestine from bacterial encroachment, a side effect of microbial instability (Johanson et al., 2008). Consequently, it is sometimes the case that disturbances or perturbations to the gut microbial community cause breakdown of the mucosal barrier and this breakdown and the subsequent bacterial translocation has also been linked to gastrointestinal and inflammatory disease (Macpherson et al., 2006; Sartor, 1997; Targan & Karp, 2005; Robert et al., 2010; Chassaing et al., 2015).

A high co-morbidity exists between gastrointestinal disorders and neuropsychiatric disorders (Adams et al., 2011; Dinan et al., 2014). This maybe be, in part, because intestinal health and gut microbiota can impact brain and social behaviors, such as anxiety-like behavior

(Bravo et al., 2011; Buffington et al., 2016). Studies in germ-free mice or using antibiotics to significantly decrease gut microbe quantity and diversity reveal that the absence or lack of gut microbial abundance or stability alters anxiety-like behavior and other social behaviors in rodents (Clarke et al., 2013; Heijtz et al., 2011; Neufeld et al., 2011; Desbonnet et al., 2014; Leclercq et al., 2017). Future work examining other environmental disruptions to the gut microbial community and the subsequent impact on behavior are necessary to further understand the link between gut microbiota, intestinal health, and susceptibility to neuropsychiatric disorders.

Emulsifiers are detergent-like molecules commonly added to processed foods and drinks to stabilize lipids and to increase shelf-life (Lecomte et al., 2016). Recent evidence suggests these food additives may disrupt intestinal homeostasis. Specifically, *in vitro*, emulsifier compounds compromise the mucosal barrier between the gut microbiota and the intestinal tract, allowing potentially pathogenic bacteria to reach intestinal cells (Roberts et al., 2010). *In vivo*, treatment with emulsifiers in either food or water causes significant alterations to the gut microbial structure and bacterial encroachment onto the mucous membrane in mice, and these effects correlate with the onset of low-grade inflammation and metabolic syndrome (Chassaing et al., 2015). Further, experiments in germ free mice reveal that gut microbiota are necessary and sufficient for emulsifier-induced inflammation and metabolic syndrome (Chassaing et al., 2015). More recently, a mucosal stimulator of the human intestinal microbial ecosystem (M-SHIME) was used to replicate these results on human biology *in vitro* (Chassaing et al., 2017). Investigating the direct effect of emulsifiers on the human microbial community is currently not feasible due to various confounding variables and ethical concerns. Therefore, M-SHIME is an attractive alternative to *in vivo* studies because it is able to maintain the stable human microbial

community *in vitro*. M-SHIME mimicked the emulsifier-induced alterations to gut microbiota, the bacteria encroachment onto the mucous membrane, and the pro-inflammatory effect of emulsifier treatment previously observed in mice. Collectively, this work highly suggests that emulsifier consumption increases susceptibility to gastrointestinal, inflammatory, and metabolism-related diseases (Swidsinski et al., 2009); yet, it is still unknown whether emulsifiers could also promote symptoms commonly associated with neuropsychiatric disorders.

Two emulsifiers that are commonly in processed food and drinks produced for humans are Polysorbate 80 (P80) and Carboxymethycellulose (CMC). Polysorbate 80 has been investigated for its toxic and carcinogenic potential and is deemed safe by the US Food and Drug Administration at a concentration of up to 1% (Roberts et al., 2010; NTP Toxicology and Carcinogenesis Studies of Polysorbate 80 (CAS No. 9005-65-6) in F344/N Rats and B6C3F1 Mice (Feed Studies), 1992). Carboxymethylcellulose has not been extensively studied but is currently on the "generally regarded as safe" list at a concentration of up to 2% (Swidsinski et al., 2008, 2009). Chassaing et al., (2015, 2017) adminstered Polysorbate 80 (P80) and Carboxymethycellulose (CMC) at a 1% concentration and found that even at a tenth of the dose deemed safe for human consumption by the FDA, Polysorbate 80 produced pro-inflammatory and metabolic effects in mice (Chassaing et al., 2015).

To date, only one study has investigated the effect of emulsifiers on brain and behavior (Holder et al., 2019). After a 12 week treatment with either P80 or CMC administered in their drinking water, mice showed an increase in a subset of anxiety-like behaviors and alterations in other social behaviors, as well as changes in agouti-related peptide and alpha melanocyte stimulating hormone expression, two neuropeptides known to be involved in feeding and social and anxiety-related behavior. Although still in its infancy, this work suggests that chemicals

from our diet can influence our behavior and could thus contribute to the recent rise in mood and anxiety-related disorders (Holder et al., 2019). Because many mood and anxiety disorders are known to be caused or exacerbated by stress, future research is necessary to investigate the effect of emulsifiers on stress susceptibility. In particular, social stress is the most pervasive form of stress experienced by humans and is a major predictor for the onset of mood or anxiety disorders (Björkqvist, 2001; Lederbogen et al., 2011; Kessler, 1997). Therefore, in the current study we will test whether consumption of two commonly used emulsifiers, P80 and CMC, increases behavioral susceptibility to social stress. Polysorbate 80 and carboxymethylcellulose will be administered to Syrian hamsters in their drinking water for 12 weeks and their social avoidance and social interaction behavior will be measured following repeated social defeat. This study will improve our understanding of the impact of emulsifiers on our behavior and susceptibility to stress.

4.2 Materials and Methods

4.2.1 Animals

Adult male Syrian hamsters (*Mesocricetus auratus*), weighing between 120 and 130 g, were bred in-house. Hamsters were weaned on Day 24-26 and group-housed. At approximately 3 months of age, hamsters were individually housed in polycarbonate cages (24x33x20cm) with corncob bedding, cotton nesting material, and a wire mesh top in a temperature-controlled colony room under a 14:10hr light/dark cycle, which is standard to maintain reproductive gonadal status in hamsters. Food and water were available *ad libitum*. All hamsters were handled daily for 7 days to acclimate them to handling stress before the beginning of the experiment. Individual housing is not stressful for Syrian hamsters (Ross et al., 2017), and with the exception of the agonistic pairings described in Section 4.2.3, hamsters remained separated throughout the

behavioral experiment. All protocols and procedures were approved by the Georgia State University Institutional Animal Care and Use Committee prior to experimentation, and all methods align with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

4.2.2 Emulsifier treatment

Group-housed hamsters were randomly assigned to one of three water treatment groups: 1% Polysorbate-80 (P80) + water (n = 17), 1% Carboxymethylcellulose (CMC) + water (n = 16), or untreated water (n = 16). Appropriate treatments were administered to group housed cages for 10 weeks. Reverse-osmosis filtered Atlanta city water was given to the untreated water group and used to make emulsifier solutions. Water bottles were changed as needed, approximately every 5 days. After 10 weeks of treatment, hamsters were weighed and individually housed. After hamsters were individually housed, they remained on the same treatment (1% P80, 1% CMC, or untreated water) as when group housed. Water bottles were weighed periodically to compare drinking behavior across groups and cages were checked for normal urination. The emulsifier treatment period totaled 12 weeks. During this time, hamsters only had access to their assigned water treatments.

4.2.3 Behavioral procedures

Animals were weight-matched and assigned to either a social defeat or no defeat control group. This created six groups: Water/Defeat (n = 9), P80/Defeat (n = 9), CMC/Defeat (n = 9), Water/No Defeat (n = 6), P80/No Defeat (n = 8), CMC/No Defeat (n = 7). No defeat controls experienced the same protocol throughout the experiment, with the exception that they were never paired with another hamster, to control for all environmental variables experienced by the subjects besides social stress. All behavioral manipulations were conducted during the dark

phase of the daily light:dark cycle to control for circadian variation in behavior and because this is when hamsters exhibit the majority of their agonistic behavior. Each day, all hamsters were moved into the behavior suite 30 min prior to any manipulation to allow time to acclimate. Behavior trials were run under dim red light and were recorded with a CCD camera.

For defeat training, hamsters were placed in the home cage of a novel, same-sex resident aggressor (as described in Huhman et al., 2003) for 5 min twice a day for 4 days. Each day, the first pairing occurred at the start of the dark phase and the second occurred 4 hr later. A clear plastic lid was placed over the resident's cage during each pairing to prevent escape. The novel resident aggressor reliably attacked the experimental subject and the latter exhibited submissive and defensive behaviors such as upright defense, flee, and tail lift (for a detailed description of the behaviors scored see, Albers et al., 2002). For each encounter, subjects were paired with a novel resident aggressor.

Social behavior testing (duration 5 min) took place 24 h after defeat training, as described previously (McCann & Huhman, 2012). Both socially defeated hamsters and no defeat controls were placed in a novel polycarbonate cage with a novel aggressor. These aggressors were confined to a small box on one side of the polycarbonate cage, allowing the subject to see, hear, and smell the aggressor, but preventing any direct contact. Testing sessions were recorded in the same manner as the defeat training sessions and were later analyzed by observers blinded to condition to determine the time spent "far" (in the opposite half of the polycarbonate cage from the caged resident aggressor), which we define as social avoidance, as described previously (McCann et al., 2014; McCann & Huhman, 2012), and time spent in social interaction (defined as nose to caged aggressor on the near side). Additional behaviors were quantified including frequencies of flank marks (hamster rubs it's flank glands along the wall of the cage as a means

of social communication (Song et al., 2014)), and several overt submissive behaviors including flees (hamster rapidly moves away from opponent often with tail lifted) and risk assessments (hamster stretches forward cautiously in a characteristic flat-back posture to investigate a potential threat (Blanchard et al., 2001; McCann & Huhman, 2012)).

4.2.4 Tissue Collection

Immediately following social behavior testing, the fat-pad and the spleen were collected from all experimental animals and immediately weighed. Increased fat-pad mass is thought to be a marker for metabolic syndrome and an enlarged spleen as a marker of inflammation. Previous research has used these two markers to link emulsifiers to the onset of metabolic syndrome and a pro-inflammatory state in mice (Chassing et al., 2015). We collected both tissues to assess whether these physiological effects following emulsifier treatment in mice could be translated to hamsters.

4.2.5 Statistics

All statistical analysis was completed and visualized using GraphPad Prism 8.2.0 (GraphPad Software, La Jolla, CA). Fat-pad weight, spleen weight, drinking behavior, and social behavior comparisons across treatment groups were analyzed by ANOVA, with Tukey's post hoc analysis. Differences in post hoc analyses were denoted as significant at $*p < 0.05$. Social behavior comparisons between defeated and non-defeated animals were made using Student's *t*-test.

4.3 Results

4.3.1 Drinking behavior

Animals were weighed and singly housed two weeks prior to defeat training and individual drinking behavior was measured during this time by weighing individual bottles (g).

The weights of hamsters given P80 (164.5 g +/- 3.5 g), CMC (161 g +/- 6.1 g), or untreated water (158 g +/- 5.6 g) did not significantly differ, suggesting emulsifier treatment did not cause abnormal weight gain. ANOVA revealed a main effect of treatment on individual drinking behavior prior to defeat training (Figure 4.3; $F(2, 39) = 7.760$, $p = 0.0015$) and Tukey's post hoc analysis revealed that animals given CMC (1%) in their drinking water drank significantly less compared to animals given untreated water or water + P80 (1%) ($p = 0.01$ compared with water, $p = 0.002$ compared to P80). Notably, all animals consumed the fluids in sufficient amounts to support what appeared to be normal urination patterns.

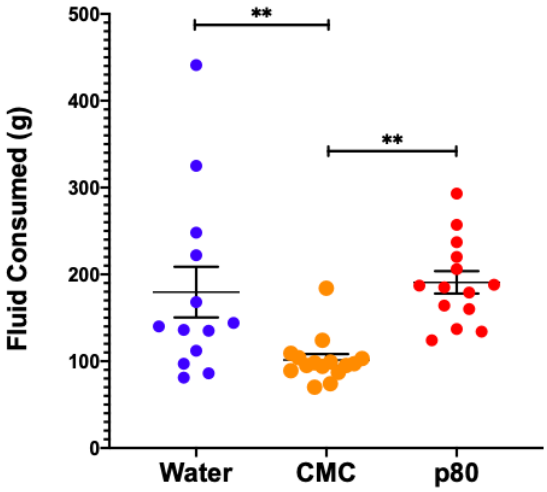

Figure 4.1 Individual drinking behavior.

Animals given CMC ($n = 16$) (orange dots) in their drinking water drank significantly less compared to animals given P80 ($n = 17$) (red dots) or untreated water ($n = 17$) (blue dots) (**$p < 0.002$).

4.3.2 Emulsifier treatment does not increase behavioral susceptibility to social stress.

Following 4 days of defeat training, hamsters were tested for social avoidance and social interaction with a novel, caged opponent. Due to a malfunction with the CDC camera, social behavior testing from 7 animals (2-3 animals per treatment group) was not recorded and was not analyzed. However, these animals were included in all other analyses. Defeated hamsters displayed significantly more avoidance (Figure 4.1A; $t(38) = 3.9$, $p = 0.0003$) and significantly

less social interaction (Figure 4.1B; $t(38) = 3.8$, p = 0.0004) compared to no defeat controls, regardless of treatment. ANOVAs were run to assess the effect of emulsifier treatment on social behavior separately for defeated and non defeated hamsters. When comparing treatment groups, for both defeated and non-defeated hamsters, ANOVA revealed no effect of emulsifier treatment on avoidance behavior (Figure 4.2A; $F(2, 19) = 0.66$, p = 0.5) (Figure 4.2B; $F(2, 15) = 0.54$, p = 0.6) or social interaction (Figure 4.2C; $F(2, 19) = 0.21$, p = 0.8) (Figure 4.2D; $F(2, 15) = 1.06$, p = 0.37). Tukey's post hoc analysis was used to compare treatment groups. For socially defeated hamsters, there were no differences between treatment groups for avoidance (Figure 4.2A; Water v CMC, p = 0.9; Water v P80, p = 0.5; P80 v CMC, p = 0.7) or social interaction (Figure 4.2C; Water v CMC, p = 1.0; Water v P80, p = 0.8; P80 v CMC, p = 0.9). Similarly, non-defeated hamsters showed no differences in behavior between treatment groups for avoidance (Figure 4.2B; Water v CMC, p = 0.9; Water v P80, p = 0.8; P80 v CMC, p = 0.6) or social interaction (Figure 4.2D; Water v CMC, p = 1.0; Water v P80, p = 0.5; P80 v CMC, p = 0.4). Additional behaviors such as flank markings, risk assessments, and flees were rare and thus the not compared across groups.

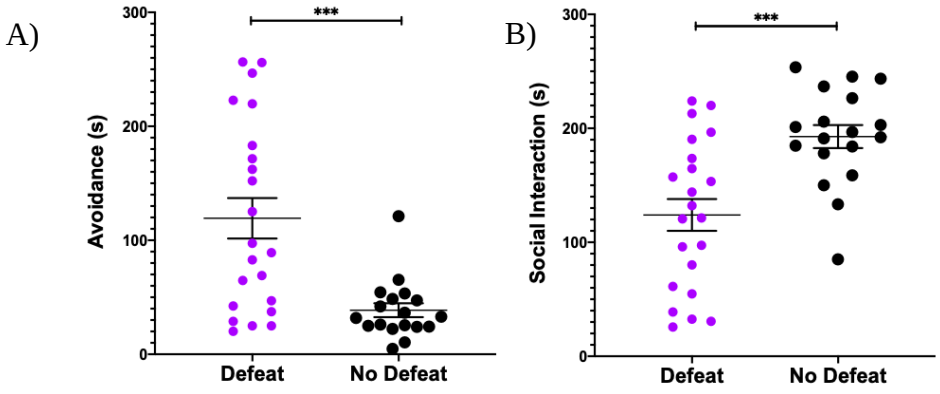

Figure 4.2 Defeated animals are more socially avoidant and less socially interactive compared to non-defeated animals. Defeated animals ($n = 23$) (purple dots) displayed significantly more A) social avoidance and significantly less B) social interaction compared to no-defeat controls ($n = 18$) (black dots) (***p < 0.0005).

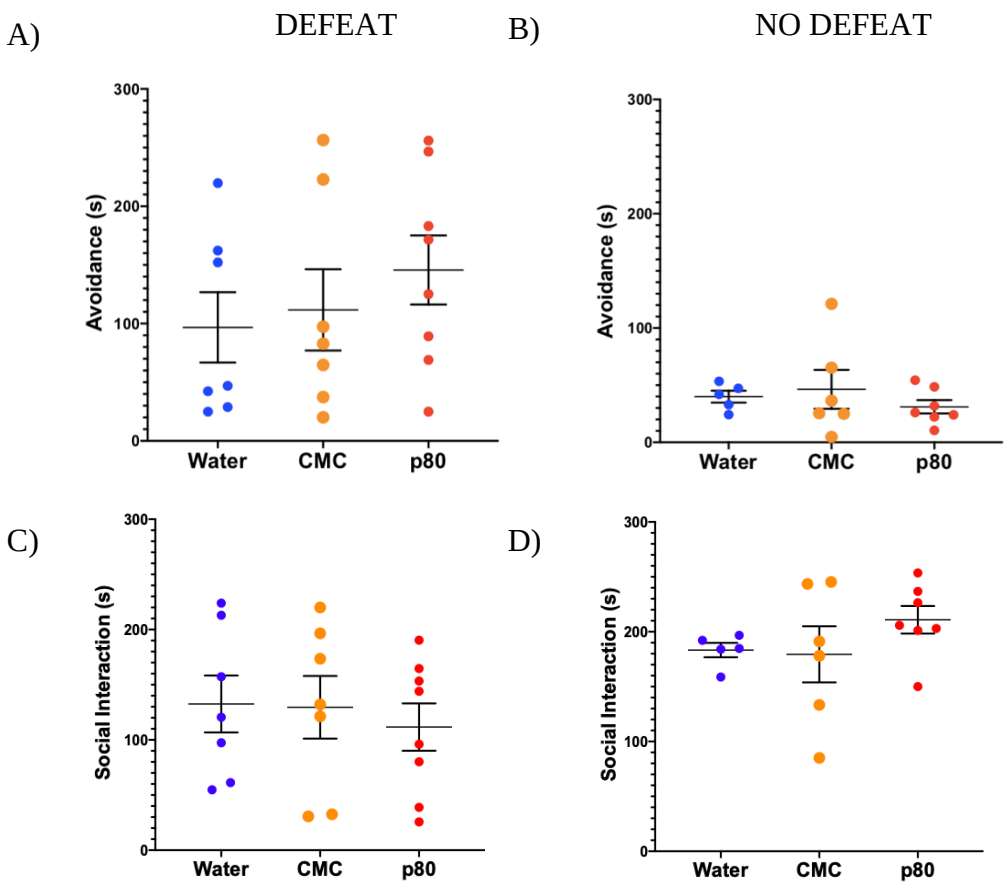

Figure 4.3 Emulsifier treatment does not affect behavioral susceptibility to social defeat. No significant differences were observed in avoidance (A, B) or social interaction (C, D) behavior in defeated animals (A, C) or in no defeat controls (B, D) after a 12 week treatment with either water (defeated, $n = 7$; no defeat $n = 5$) (blue dots), CMC (defeated, $n = 7$; no defeat $n = 6$) (orange dots), or P80 (defeated, $n = 8$; no defeat $n = 7$) (red dots).

4.3.3 Emulsifier treatment does not increase fat-pad or spleen weight.

Emulsifier treatment had no effect on either fat-pad or spleen weight in animals regardless of defeat status (Figure 4.4A; $F(2, 45) = 2.1$, $p = 0.14$) (Figure 4.4B; $F(2, 45) = 0.9$, $p = 0.4$). Defeated and non-defeated animals were collapsed because no defeat effect was observed for fat-pad weight or spleen weight. Further, Tukey's post hoc analysis revealed no differences between groups for either fat-pad (Figure 4.4A; Water v CMC, $p = 0.3$; Water v P80, $p = 0.9$; P80 v CMC, $p = 0.1$) or spleen weight (Figure 4.4B; Water v CMC, $p = 0.6$; Water v P80, $p = 0.4$; P80 v CMC, $p = 0.9$).

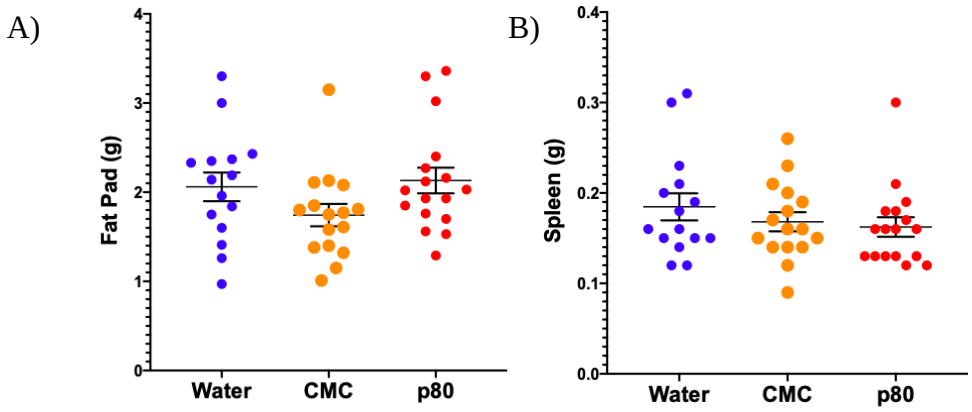

Figure 4.4 Fat-pad and spleen weight (g). Fat-pad (A) and spleen (B) weights were comparable across treatment groups (water (*n* = 15): blue dots; CMC (*n* = 16): orange dots; P80 (*n* = 17): red dots) in both defeated hamsters and no defeat controls.

4.4 Discussion

Processed foods are a staple of the Western diet, making the effect of emulsifiers on health a relevant and important area of research. Based on previous research in mice showing emulsifier treatment increased certain anxiety-like behaviors (Holder et al., 2019), we tested whether emulsifiers also increase stress susceptibility. We used a social defeat model in Syrian hamsters to determine whether emulsifier treatment exacerbated the behavioral response to social defeat. Specifically, we predicted that chronic emulsifier treatment would increase social avoidance and decrease social interaction following social defeat training, creating a heightened anxiety-like phenotype in the socially stressed animals. Our prediction was not supported. We observed no difference in anxiety-like behavior following defeat between hamsters that were administered emulsifier-treated water versus those that were given normal drinking. Emulsifier treatment also did not alter behavior in the absence of defeat, indicating that social defeat stress was not masking an underlying effect of emulsifiers on social approach or avoidance behavior. Although an increase in anxiety-like behavior has been previously observed in male mice, this increase was only observed in a subset of anxiety measures. For instance, male mice treated with

emulsifiers spent significantly less time in the center of open field than did water-treated controls, but no treatment effect was observed in another common test of anxiety-like behavior in mice, the light/dark box test. It is possible we did not observe a change in anxiety-like behavior because the only measure of anxiety-like behavior that we measured was social avoidance.

Neither defeated nor non-defeated hamsters exhibited the physiological changes that were previously observed in mice following emulsifier treatment (Chassaing et al., 2015; Holder et al., 2019). Namely, emulsifier treatment did not increase the weight of the fat-pad or spleen, an effect observed following emulsifier treatment in mice that was linked to the onset of metabolic syndrome and a pro-inflammatory state. Our results suggest that chronic treatment with emulsifiers has no effect on these factors in hamsters, suggesting that the potential impact of emulsifiers may be smaller than previously thought or may be species-specific. Alternatively, it could be that the dosage used in mice is too low to have an effect in hamsters or that hamsters drink significantly less than do mice.

Hamsters treated with CMC drank significantly less than did their conspecifics given P80 or untreated water. The CMC solution is more viscous compared to the P80 solution and untreated water; therefore, it is possible the animals were unable or less motivated to consume as much liquid due to the increased viscosity. It is also possible that less spillage occurred from the CMC water bottles due to the increased viscosity. Although it is unclear whether a significant decrease in water intake in the CMC group altered behavior in this study, future studies should identify whether differences in drinking behavior may impact study outcomes. Further, one possibility for why we did not observe a behavioral effect following emulsifier treatment may be due to the hamster's baseline drinking behavior. Although measuring individual drinking

behavior revealed hamsters were drinking sufficient amounts of liquid, hamsters are desert dwellers and have renal conservatory mechanisms that allow them to consume less water than most other small mammals. Therefore, it is possible that renal mechanisms or other mechanisms impacted drinking behavior in Syrian hamsters limit the effectiveness of solutions administered to these animals in their drinking water. Future studies choosing to treat hamsters via drinking water should consider this issue.

It is also possible that the emulsifier treatment had alternative effects, such as increasing susceptibility to gastrointestinal disease; however, this possibility was out of the scope of the current study. Instead, our results show that the previous physiological and behavioral changes observed in mice following emulsifier treatment are absent in Syrian hamsters and that emulsifier treatment does not increase susceptibility to social defeat stress. Previous work in our lab has shown that social stress causes dysbiosis of gut microbiota (Partrick et al., 2018). Future work should identify whether 1) emulsifier treatment causes dysbiosis of the gut microbial community and dysregulation of the microbial-host relationship in Syrian hamsters, and 2) emulsifier treatment exacerbates the dysbiosis of gut microbiota previously observed following social stress. This future direction will help explain whether no behavioral effect following emulsifier treatment was observed because emulsifier-induced dysregulation of the microbial-host relationship in mice is not translatable to hamsters or whether emulsifier consumption is simply not functionally relevant for social behavior in hamsters. To further investigate whether emulsifier treatment impacts stress susceptibility, the behavioral response to other non-social stressors, such as restraint stress, should be studied. Additionally, to better understand the impact emulsifiers may have on susceptibility to mood and anxiety disorders, tests measuring

depressive-like behaviors, such as the tail suspension test or sucrose preference, should be conducted following emulsifier treatment in multiple rodent species.

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5 INFLAMMATION IN SYRIAN HAMSTERS IN RESPONSE TO SOCIAL STRESS

5.1 Introduction

Animals, including humans, respond to environmental insults by producing an inflammatory response, characterized, in part, by peripheral and central infiltration of proinflammatory cytokines. Proinflammatory cytokines are released from sources such as macrophages, leukocytes, and microglia and can have a profound effect on physiology by mediating communication between the immune system and surrounding tissue (Barnes et al., 2009; Firestein et al., 2016). This increase in proinflammatory signaling can be adaptive by promoting a repertoire of responses including behaviors that decrease the likelihood of further insults. For example, inflammation-induced sickness behaviors cause organisms to become more non-social or even socially avoidant. Interestingly, there are many similarities between sickness behavior and the behavioral symptoms of mood and anxiety disorders. For example, sick and depressed patients both show increased anhedonia and social avoidance (Trew, 2011; Hammels et al., 2015; Nemeroff, 1998; Bjorkqvist et al., 2001; Agid et al., 2000; Gardner, 2001; Eisenberg et al., 2010; Kent et al., 1992), as well as changes in ingestive behavior and sleep (Shattuck & Muehenbein, 2015; Hart, 1988: Miller et al., 2005; Agargun & Somaz, 1997; Frost et al., 1982). These behavioral similarities have led some to propose that inflammatory mechanisms may underlie some of the behavioral symptoms associated with mood and anxiety disorders (Rainville & Hodes, 2019).

Mood and anxiety disorders affect a remarkable number of patients each year, and research investigating novel mechanisms that cause or exacerbate symptoms of these disorders is crucial to developing new, more effective therapeutics. Microglia and pro-inflammatory cytokines are two mediators of inflammation that appear to be key agents in the inflammatory

mechanisms that may, in part, cause or contribute to the symptoms of mood and anxiety disorders (Rainville & Hodes, 2019). Microglia are the primary immune cells in the brain (Hanisch, 2002). At rest, these cells maintain proper neurological function by regulating synapses and clearing neuronal waste (Walker et al., 2013). Further, microglia scan the local environment and become active and increase in number when presented with threatening signals (Hanisch & Kettenmann, 2007; Stence et al., 2011). Once activated, microglia are capable of inducing robust neuroinflammation via the release of pro-inflammatory cytokines and other inflammatory agents (Kreutzberg, 1996). Although this process is necessary for central nervous system health, prolonged activation of microglia and the associated proinflammatory cytokine release is maladaptive and is thought to promote the etiology of multiple pathologies, including depression (Prinz & Priller, 2014; Frick et al., 2013). Proinflammatory cytokines from the periphery are also thought to be pro-depressive; however, the exact mechanism of this effect remains unclear. Proinflammatory cytokines under normal conditions do not generally enter the brain; however, humoral pathways or a compromised blood brain barrier, which can happen after exposure to stress, may allow entry of proinflammatory cytokines (Menard et al., 2017; Tsyglakova et al., 2019). After reaching the brain, these signals can stimulate microglia and affect brain regions associated with affective states (Quan & Banks, 2007; Prinz et al., 2011). The purpose of the present study is to further understand whether exposure to mild social stress increases microglial activation and proinflammatory cytokine signaling

Social stress is a major predictor for the onset of mood or anxiety disorders, and both microglia and pro-inflammatory cytokines can impact responses to social stress. For example, microglia become activated following chronic social defeat, and depleting microglia in mice prior to chronic social defeat eliminates the increase in anxiety-like behavior normally observed

following defeat (Lehmann et al., 2019). Further, circulation of the well-studied, proinflammatory cytokine IL-6 positively correlates with susceptibility to social defeat stress and blocking IL-6 in socially defeated mice has anti-depressant effects (Hodes et al., 2014; Zhang et al., 2017). Therefore, microglial activation and IL-6 signaling may be necessary for the behavioral consequences of social stress. It is important to note, however, that research investigating the relationship between inflammation and social stress has been conducted almost exclusively in male mice using a relatively severe, chronic social defeat protocol in which tissue damage often occurs. The relationship between microglial activation or IL-6 signaling and mild social stress with no wounding or social stress in females remains unknown. Our social defeat model in Syrian hamsters allows for the study of mild social defeat in both sexes. Additionally, in this model, tissue damage is rare. Therefore, we can eliminate the confound of physical injury on inflammation. Both male and female hamsters readily produce territorial and aggressive behavior in the laboratory, and this social conflict quickly results in the formation of a stable dominance hierarchy. Even after a single social defeat, subordinate hamsters abandon all territorial aggression and become highly submissive and socially avoidant (Potegal et al., 1993; Huhman et al., 2003; McCann & Huhman, 2012). This behavioral response is termed conditioned defeat, and our lab has done extensive research to define the neural circuity underlying conditioned defeat. The prefrontal cortex (PFC) (Markham et al., 2012) and basolateral amygdala (BLA) (Jasnow & Huhman 2001; Markham et al., 2010) are two important nodes mediating the behavioral response to defeat. Interestingly, these brain regions are also known to be susceptible to neuroinflammation, characterized by an increase in microglia quantity and activation state, and both are implicated in mood disorders such as depression (Wohleb et al., 2011, 2014; Wrona, 2006; Maier & Watkins, 1998; Lehmann et al., 2016).

The current study will measure microglia proliferation and activation in the PFC and BLA, and IL-6 pro-inflammatory signaling in the periphery following mild social defeat. We hypothesize that social stress induces inflammation in both males and female hamsters. Specifically, we predict that there will be an increase in microglia proliferation and activation in the PFC and BLA, as well as increased circulating IL-6 following social defeat. Testing this hypothesis in both males and females will address the gap in knowledge of potential sex differences in the inflammatory response to social defeat. Further, we will examine whether there is a pro-inflammatory response following both acute and repeated bouts of defeat to test whether multiple socially stressful experiences are necessary to instigate a robust inflammatory response.

5.2 Materials and Methods

5.2.1 Animals

Adult male and female Syrian hamsters (*Mesocricetus auratus*), weighing between 120 and 130 g, were bred in-house from animals obtained from Charles Rivers Laboratories (Kingston, NY). Hamsters were weaned on Day 24-26 and group-housed with same-sex conspecifics. At approximately 3 months of age, hamsters were individually housed in polycarbonate cages (24x33x20cm) with corncob bedding, cotton nesting material, and a wire mesh top in a temperature-controlled colony room under a 14:10hr light/dark cycle as is common in this species to maintain gonadal patency. Food and water were available *ad libitum*. Individual housing is not stressful for Syrian hamsters (Ross et al., 2017), and with the exception of the agonistic pairings described in Section 5.2.2, hamsters remained separated throughout the behavioral experiment. All hamsters were handled daily for 7 days before the beginning of the experiment to acclimate them to handling stress. Phase of the estrous cycle was determined for all females by conducting daily vaginal swabs for the 8 days prior to defeat training. All

protocols and procedures were approved by the Georgia State University Institutional Animal Care and Use Committee prior to experimentation, and all methods align with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

5.2.2 Experiment 1: Behavioral procedures

Male and female hamsters were weight-matched and assigned to either a defeat (acute or repeated social defeat) group or a no defeat acute or repeated control group. Thus, experimental groups included: Acute Defeat (male n = 7, female n = 7), Acute No Defeat Control (male n = 2, female n = 2), Repeated Defeat (male n = 14, female n = 16), Repeated No Defeat Control (male n = 4, female n = 4). No defeat controls were not manipulated other than handling, transport to the testing suite, and cage changes. All behavioral manipulations were conducted during the dark phase of the daily light:dark cycle because this is when hamsters exhibit the majority of their agonistic behavior. Each day, all hamsters were moved into the behavior suite 30 min prior to any manipulation to allow time to acclimate. All testing was run under dim red light and was recorded with a CCD camera for later scoring by trained observers.

For acute defeat training, hamsters were placed in the home cage of a novel, same-sex aggressor (as described in Huhman et al., 2003) for 15 min at the start of the dark phase of the daily cycle, and tissue was collected 24 hr later. For repeated defeat training, hamsters were placed in the home cage of a novel, same-sex aggressor for 5 min, twice a day across 4 days, with one additional 5 min defeat on the 5^{th} day immediately before tissue collection. For repeated defeats, the first pairing occurred at the start of the dark phase and the second occurred 4 hr later. For each encounter, subjects were paired with a novel resident aggressor who reliably attacked the experimental subject, and the latter exhibited submissive and defensive behaviors such as upright defense, flee, and tail lift (Huhman et al., 1990).

5.2.3 Experiment 2: Thermochron iButton® implantation and recording and LPS injection

As a positive control (Experiment 2) to ensure that it was possible to measure inflammation in Syrian hamsters, we used Thermochron iButtons® (3.1g) (Dallas SemiConductor, Maxim, Sunnyvale, CA) were used to measure core body temperature following an immune challenge (see below). Thermochron iButtons® contain a thermometer, a clock, and a calendar encapsulated by stainless steel and have the ability to record body temperature at a specified date and time. The temperature range of the iButtons is 4-85°C with a resolution of ±0.0625°C. Thermochron iButtons® are reusable and do not require a telemetric recording device. Prior to implantation, iButtons were programmed to record temperature every 10 min and were coated with parrafin wax and vybar to waterproof them and to ensure that no tissue adhesion occurred after implantation. Before implantation, iButtons were sterilized overnight in 70% ethanol and then surgically implanted into the peritoneal cavity of hamsters. Anesthesia was induced with 5% isoflurane and hamsters were maintained at 3-5% isoflurane throughout surgery. Prior to surgery, the abdomen was shaved, and the skin was disinfected with three rounds of betadine + 70% ethanol. A single, 2 inch incision was made down the midline with a sterile scalpel blade, 1 inch above the tail. The iButton was then implanted into the peritoneal cavity and extreme precaution was made to ensure there was no disruption to any organs. The muscle wall was closed using sterile sutures and the skin closed with sterile wound clips. Forceps were used to ensure there were no openings in the muscle wall or skin. The wound was treated with betadine solution to prevent infection. Hamsters were returned to the housing facility for post-surgical monitoring and allowed to recover for 5-7 days prior to experimentation. After recovery, hamsters were injected intraperitoneally (i.p.) with Lipopolysaccharide (LPS)

(0.05mg/kg), a bacterial endotoxin known to induce inflammation and sacrificed 24 hr later. 24 hr before and after the injection, core body temperature was measured using the Thermochron iButtons®.

5.2.4 Tissue collection

For Experiment 1, tissue was collected from the acute defeat group and the respective no defeat control group 24 hr after the single, 15 min defeat. The repeated defeat group and their respective controls were sacrificed immediately after the ninth and final 5 min defeat. Notably, glial morphology and inflammatory gene expression do not appear to be dependent on the estrous cycle (Schwartz et al., 2012); therefore, we did not control for the estrous cycle when sacrificing female hamsters. For Experiment 2, male and female hamsters were sacrificed 24 hr after the LPS injection. Hamsters from both experiments were anesthetized with isoflurane and euthanized by cervical dislocation, and blood and brains were collected. After removal, brains were immediately postfixed in 5% acrolein and potassium phosphate buffer saline (KPBS) for 24 hr. Trunk blood was collected and allowed to clot at room temperature for 2 hr. After 2 hr, blood was centrifuged for 20min at 2000xg to obtain serum. Serum was collected and immediately frozen at -80°C. In Experiment 2, Thermochron iButtons® were removed from the peritoneal cavity.

5.2.5 Immunohistochemistry

After 24 hr in 5% acrolein and KPBS, brains were transferred to a 30% sucrose solution and stored at 4°C for one week. Brains were then sectioned coronally along the rostrocaudal axis on a cryostat at 30 µm and stored in cryoprotectant-antifreeze at -20°C until further processing. Sections were processed for ionized calcium binding adaptor molecule 1 (Iba-1) immunoreactivity. Iba-1 staining allows for both the quantitative and qualitative analysis of

microglia. In this study, we used a variation of an immunohistochemical procedure described previously (Murphy & Hoffman, 2001). In brief, free-floating sections in cryoprotectant were extensively rinsed in KPBS and incubated in rabbit anti-Iba-1 (WAKO Chemicals; 1:10 K) primary antibody solution containing KPBS and 1%Triton X-100 for 1 hr at room temperature followed by 48 hr at 4°C. Sections were then rinsed in KPBS and incubated in biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch; 1:600) for 1 hr followed by rinses in KPBS and an incubation in avidin-biotin peroxidase complex (ABC) (1:10, ABC Elite Kit; Vector Laboratories). After the ABC incubation, sections were rinsed in KPBS and sodium acetate (0.175 molar, pH 6.5), and exposed to a 3,3' -diaminobenzidine (DAB) solution containing nickel sulfate and 0.08% hydrogen peroxide in sodium acetate buffer. At this step, Iba-1 immunoreactivity was visualized as a purple/black reaction. After the DAB reaction, sections were rinsed in sodium acetate buffer followed by KPBS. To prepare sections for analysis, sections were mounted out of KPSB onto gelatin-subbed slides and allowed to air dry for 24-48 hr. Once dry, slides were quickly dipped in diH_2O, then dehydrated for 2 min each in EtOH 70%, 95%, and 100%. Immediately following the dehydration steps, slides were cleared for 2min in Cistrosolv and coverslipped using DPX.

5.2.6 Quantification and morphological analysis of microglia

Iba-1+ cells were analyzed in the prefrontal cortex (PFC) and basolateral amygdala (BLA), two key brain regions in the neural circuitry underlying conditioned defeat. A template was created for each region of interest (ROI) and photomicrographs were taken (2-3/animal) on StereoInvestigator software (MBF Biosciences, Williston, VT, USA). Manually, the total number of Iba-1+ cells were quantified by an observer blinded to condition. Iba-1+ cells were only counted if they were uniformly stained, within the plane of focus, and if the entire cell body

was visible. Further, because microglial morphology highly correlates with functional state (Karperien et al., 2013), Iba-1+ cells within the ROI were classified into four cell types (Type 1-4) with Type 1 microglia being the most activated and Type 4 microglia being as rest. Each type was characterized by cell shape and the configuration of processes. Type 1 was visualized as round/ameboid, Type 2 as stout with a large cell body and 1-2 thick/unramified processes, Type 3 as transitioning with thicker/longer processes, and Type 4 as fully ramified with small cell bodies and thinner/ramified processes, as previously described (Schwartz et al., 2012; Castillo-Ruiz et al., 2018) (Figure 1). Notably, perivascular macrophages also stain positive for Iba-1. However, previous research suggests these cells only account for approximately 4% of the Iba-1+ cell population within the brain and as such have negligible effects on results (Schwartz et al., 2012).

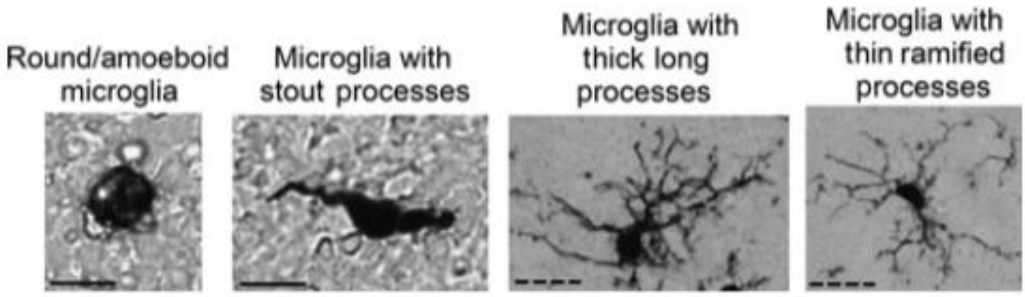

Figure 5.1 Classification of microglia morphology adapted from Schwartz et al., 2012. Each photo represents one of the four morphological classifications (Type 1-4, left to right) for microglial cells that are thought to correlate with differences in functional state.

5.2.7 Pro-inflammatory cytokine Interleukin 6 (IL-6) measurement

A mouse IL-6 ELISA kit was purchased from R&D Systems (Minneapolis, MN, USA) and a hamster IL-6 ELISA kit was purchased from Cusabio (Hubei, China). Both ELISA kits were used according to the manufacture's protocols to determine circulating levels of IL-6 protein in hamster serum.

5.2.8 Statistical analysis

All statistical analysis was completed and visualized using GraphPad Prism 8.2.0 (GraphPad Software, La Jolla, CA). A 2-Way ANOVA with Tukey's post hoc analysis was used to determine the effect of sex and defeat on microglia quantity. Where necessary, comparison of microglia quantity between groups was made using Student's *t*-test and microglial cell types (Type 1-4) were analyzed by Mutiple t-tests with Holm-Šidák correction. Differences between groups were denoted as significant at $*p < 0.05$.

Results

5.2.9 Social stress does not activate microglia in the prefrontal cortex or the basolateral amygdala.

Microglia quantity and morphology were analyzed in the prefrontal cortex (PFC) and basolateral amygdala (BLA) of socially-defeated male and female hamsters. Following repeated defeats, a 2-way ANOVA revealed no effect of defeat or sex on total microglia quantity in the PFC (Figure 5.2A; $F(1, 26) = 0.314$, $p = 0.58$) or the BLA (Figure 5.2B; $F(1, 25) = 0.742$, $p = 0.397$). No significant sex differences were found for measures of total microglia or microglia morphological type in either brain region; therefore, data was collapsed over sex for the remainder of analyses (data not shown). In the PFC, microglia were unable to be quantified due to poor staining for two hamsters in the acute, no defeat control group, reducing the *n* of that group to two for subsequent analyses. Following the acute defeat, there was no effect of defeat on microglia quantity in either the PFC (Figure 5.3A; $t(12) = 0.82$, $p = 0.43$) or the BLA (Figure 5.3B; $t(10) = 0.65$, $p = 0.5$). Multiple t-tests with Holm-Šidák method correction revealed no significant differences in any microglia morphological classification type between defeated and non-defeated animals in the PFC or BLA after both an acute (Figure 5.3C: Type 1, $t(12) = 0.01$,

p = 1.0; Type 2, *t*(12) = 0.7, p = 0.9; Type 3, *t*(12) = 0.7, p = 0.9; Type 4, *t*(12) = 0.2, p = 1.0) (Figure 5.3D: Type 1, *t*(10) = 1.5, p = 0.5; Type 2, *t*(10) = 0.6, p = 0.8; Type 3, *t*(10) = 0.3, p = 0.8; Type 4, *t*(10) = 1.2, p = 0.6), and repeated defeat (Figure 5.2C: Type 1, *t*(28) = 0.4, p = 0.9; Type 2, *t*(28) = 0.3, p = 0.9; Type 3, *t*(28) = 0.8, p = 0.8; Type 4, *t*(28) = 2.4, p = 0.1) (Figure 5.2D: Type 1, *t*(27) = 0.7, p = 0.9; Type 2, *t*(27) = 0.4, p = 0.9; Type 3, *t*(27) = 0.8, p = 0.9; Type 4, *t*(27) = 0.8, p = 0.9), suggesting microglia do not transition to an active state in response to defeat.

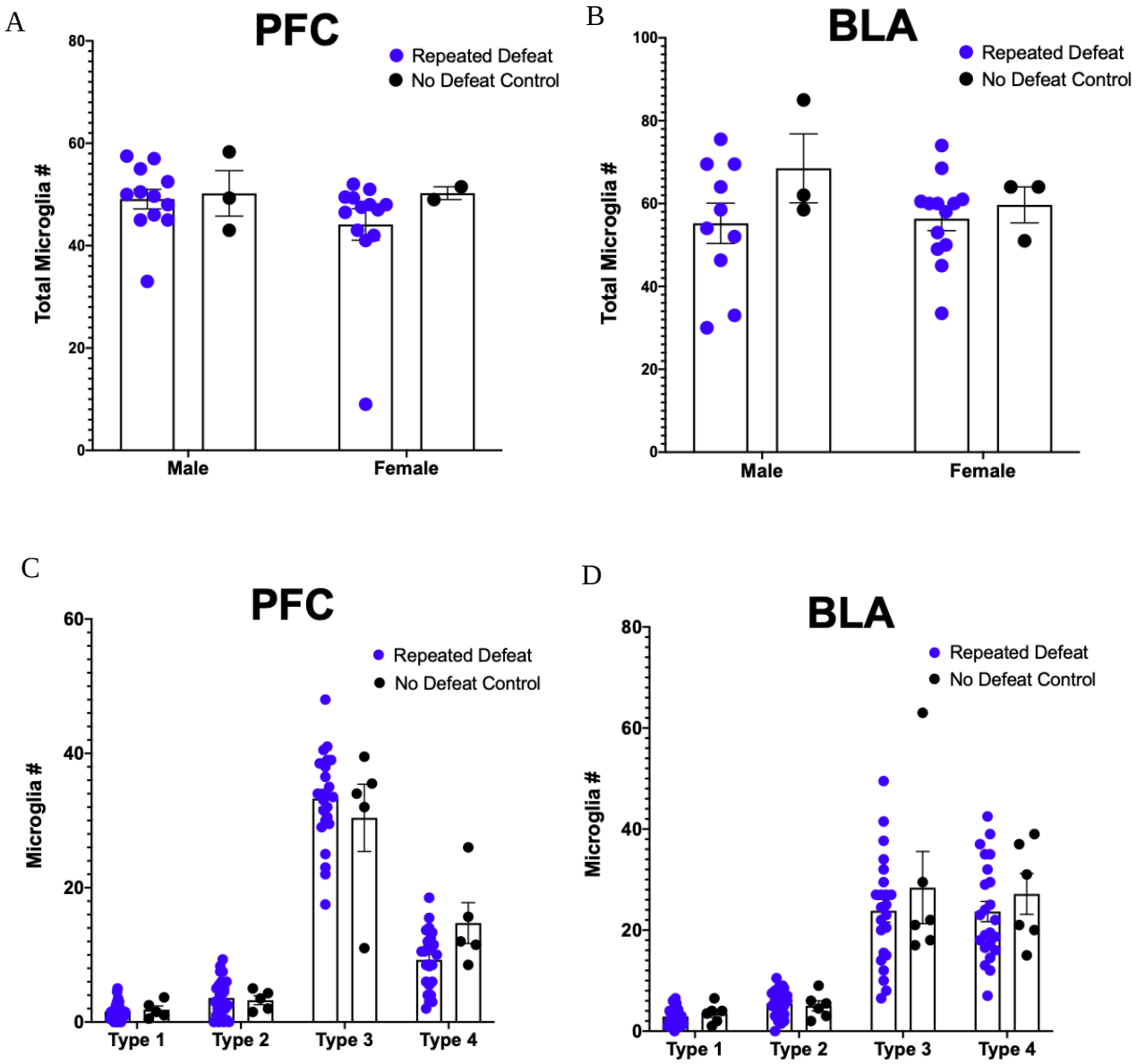

Figure 5.2 Repeated social stress does not affect microglia quantity or morphology in the prefrontal cortex or basolateral amygdala.

Following repeated social defeat, the number of microglia in the PFC (A) and BLA (B) was comparable across sex and between defeated hamsters (blue) and no defeat controls (black). Microglia (collapsed over sex) were categorized based on morphology and no differences were observed between defeated animals and no defeat controls for any morphological category (Type 1-4) in the PFC (C) or BLA (D).

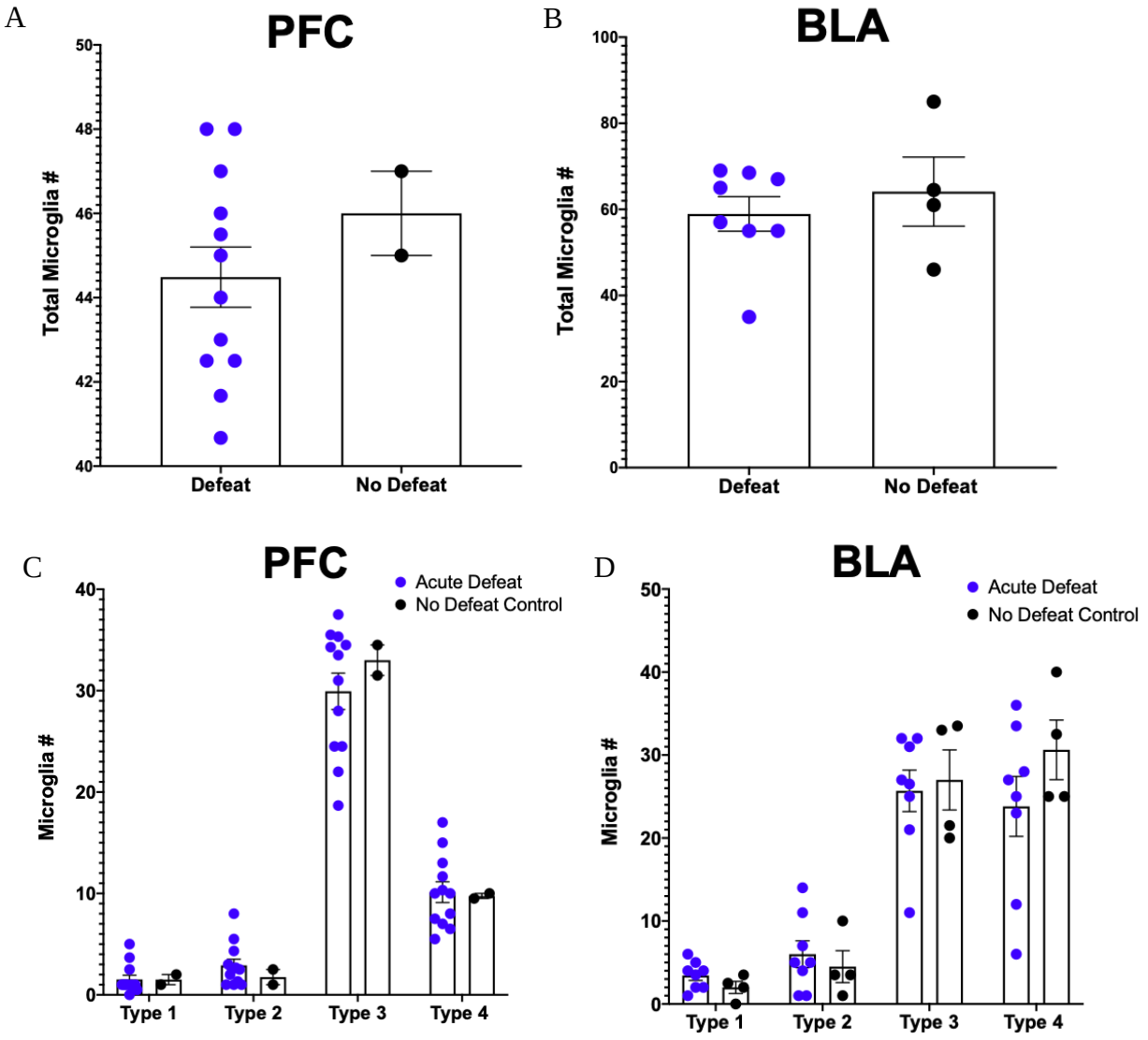

Figure 5.3 Acute social stress does not affect microglia quantity or morphology in the prefrontal cortex or basolateral amygdala.

Following an acute defeat, the number of microglia in the PFC (A) and BLA (B) (collapsed over sex) were comparable between defeated hamsters (blue) and no defeat controls (black). Microglia were categorized based on morphology and no differences were observed between defeated hamsters and no defeat controls for any morphological category (Type 1-4) in the PFC (C) or BLA (D).

5.2.10 IL-6 ELISA detection

The mouse and hamster IL-6 amino acid sequences were compared to determine the likelihood of cross-reactivity of the IL-6 antibody generated against mouse IL-6 with the hamster protein. The mouse IL-6 amino acid sequence showed > 90% homology with the hamster sequence. Therefore, we proceeded to test cross-reactivity of the mouse IL-6 ELISA kit from R&D Systems for detection of IL-6 protein in hamster serum. The assay was unsuccessful and failed to produce adequate detection (data not shown). Next, we used a hamster-specific IL-6 ELISA kit from Cusabio to detect IL-6 circulating protein levels in hamster serum. This kit also exhibited extremely low detection of IL-6.

5.2.11 LPS induces neuroinflammation.

By approximately 1 hr after injection, core body temperature was elevated in response to the injection of LPS. Body temperature peaked 11 hr post injection and remained elevated for 24 hr prior to tissue collection (Figure 5.4). Microglia quantity and morphology were analyzed in the prefrontal cortex (PFC) and basolateral amygdala (BLA) of hamsters 24 hr after the LPS injection and compared to that of controls. Again, there were no significant sex differences for measures of total microglia or microglia morphological type in either brain region; therefore, data was collapsed over sex for all analyses (data not shown). In the PFC, t-tests with Holm-Šidák correction revealed that animals treated with LPS had significantly more microglia in the Type 1, Type 2, and Type 3 morphological state compared to controls (Figure 5.5A; Type 1, $t(21) = 6.3$, $p < 0.0001$; Type 2, $t(21) = 7.8$, $p < 0.0001$; Type 3, $t(21) = 9.3$, $p < 0.0001$; Type 4, $t(21) = 0.7$, $p = 0.5$). Further, there were significantly more microglia present in the PFC of hamsters treated with LPS compared to controls (Figure 5.5A; $t(21) = 9.4$, $p < 0.0001$). These results suggest an increased proliferation of microglia and an increase in microglial activation in

the PFC following administration of LPS. In the BLA, there were significantly more microglia in the Type 2 morphological classification compared to controls, and control hamsters showed significantly more microglia in the Type 4 morphological classification than did hamsters treated with LPS (Figure 5.5B; Type 1, $t(24) = 2.2$, $p = 0.1$; Type 2, $t(24) = 3.6$, $p = 0.007$; Type 3, $t(24) = 0.5$, $p = 0.8$; Type 4, $t(24) = 2.9$, $p = 0.03$; Total, $t(24) = 0.4$, $p = 0.8$), suggesting that microglia move to a more activated state in response to an injection of LPS.

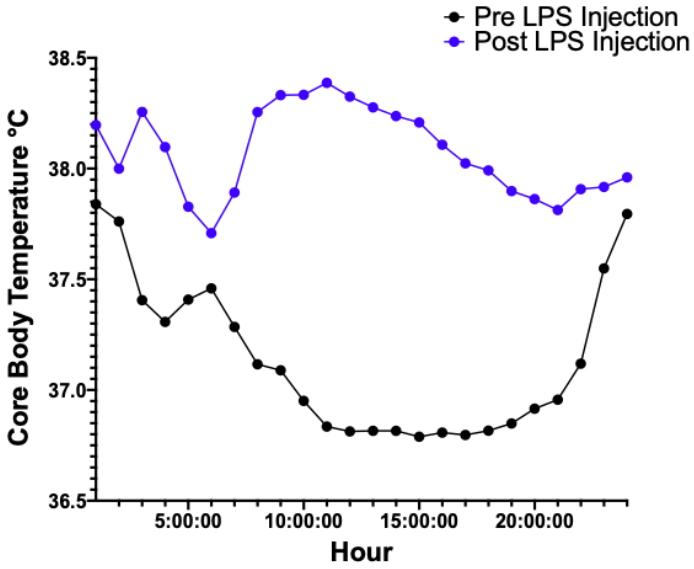

Figure 5.4 LPS increases core body temperature.

An i.p. injection of LPS (0.05mg/kg) increased core body temperature (blue line) compared to baseline (black line). Core body temperature remained elevated until animals were sacrificed 24 hr later.

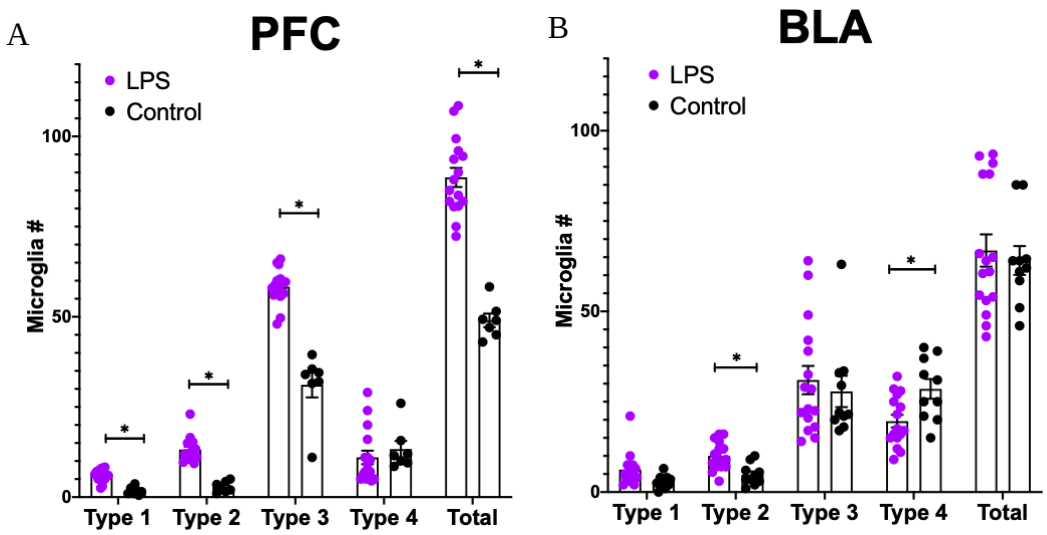

Figure 5.5 LPS activates microglia in the PFC and BLA.

Microglia were counted in the PFC (A) and BLA (B) and assigned to a morphological class (Type 1-4). An i.p. injection of LPS (0.05mg/kg) increased the total number of microglia in the PFC (A) as well as the total number of Type 1-3 microglia compared to controls (A, purple dots: LPS, black dots: control). In the BLA (B), hamsters injected with LPS had more Type 2 microglia compared to controls and control hamsters showed more Type 4 microglia compared to animals treated with LPS (B, purple dots: LPS, black dots: control).

5.3 Discussion

We predicted that both male and female hamsters would show increased inflammation, characterized by an increase in microglia quantity and activation state and increased circulating IL-6 protein, following mild social defeat stress. However, neither males nor females exhibited a significant inflammatory response after either acute or repeated social defeat. Inflammation was assessed by measuring microglia quantity and activation in brain tissue. We chose to focus on the PFC and BLA because these brain regions are necessary nodes in the circuity driving the behavioral response to social defeat, are susceptible to inflammation, and are implicated in mood and anxiety disorders (Wohleb et al., 2011, 2014; Wrona, 2006; Maier & Watkins, 1998; Lehmann et al., 2016). The number of microglia in the PFC or BLA did not increase and the residing microglia did not become more activated following either defeat protocol. This is in contrast to previous studies in mice showing microglia activation and proliferation in response to

social defeat in the PFC and amygdala (Wohleb et al., 2011, 2014; Lehmann et al.; 2016). These latter studies used a chronic social defeat protocol, which suggests that more severe or prolonged social stress may be necessary to produce a robust inflammatory response. Notably, there were no sex differences observed in the microglial response to defeat; however, further research is necessary to determine if a sex difference would become apparent if a significant increase in inflammation is observed. Unfortunately, the concentrations generated from the hamster and mouse IL-6 ELISA kits were below the level of detection, so we cannot draw conclusions about the peripheral inflammatory response following defeat. Despite a close homology of the IL-6 sequence between mouse and hamster, the mouse kit from R&D Systems does not seem to adequately detect IL-6 protein in hamster serum in this or previous studies (Zivcec et al., 2011), and the hamster kit from Cusabio appears to perform similarly. Hamsters offer a valuable model for immunological studies and immune-related disease states, but this value is under-utilized due to the lack of suitable assays to measure inflammation in this specie (Zivcec et al., 2011). There is an urgent need for the creation of hamster protein-specific immune assays in order to better utilize this animal model.

A recent study in mice using a relatively mild defeat protocol where subjects undergo an agonistic encounter for 5 min in the absence of injury followed by 24 hr of dyadic housing with the aggressor showed that 14 days of brief social defeat paired with dyadic dominant/subordinate housing does not cause changes in microglia morphology. However, LPS caused striking morphological changes such as an increase in roundness and soma size in the PFC and other brain regions. This research further demonstrates that the type of stressor and severity are important (Lehmann et al., 2016). Therefore, one hypothesis that can be drawn from our negative results is that mild social defeat in hamsters is not severe enough to induce robust inflammation,

given that hamsters are capable of producing an inflammatory response similar to that measured in mice. To test this hypothesis, male and female hamsters were given an injection of LPS, a bacterial endotoxin, at a dose known to cause potent inflammation, and brains were collected to assess microglia quantity and morphology in the PFC and BLA as in Experiment 1. In the PFC, LPS induced an increase in microglia quantity compared to controls. Animals treated with LPS also showed an increase in activated microglia, while the number of resting microglia remained comparable between groups. In the BLA, there was no significant difference in microglia quantity; however, there were more activated microglia in samples taken from hamsters treated with LPS and more resting microglia in samples from controls. Collectively, these data suggest LPS produces a robust neuroinflammatory response in multiple brain regions in hamsters that is similar to that observed in mice (Wohleb et al., 2011, 2014; Lehmann et al., 2016). Notably, the effect of LPS on microglia activation seems to be more pronounced in the PFC vs. the BLA, and future studies should investigate the downstream effects of microglia activation in the PFC in hamsters and other rodent models. No sex difference occurred in the microglial response to LPS suggesting males and females respond similarly to LPS. Ultimately, this experiment demonstrated that hamsters are capable of producing an inflammatory response similar to that of mice. Therefore, we conclude that mild social defeat, even if repeated, is not severe enough to produce a measurable increase in inflammation perhaps because of the absence of physical injury. However, we cannot rule out the possibility that a pro-inflammatory response would be captured if microglia were analyzed in other brain regions or if different pro-inflammatory cytokines such as TNF-alpha were analyzed in serum.

Still, this work has important implications moving forward for how inflammation may increase susceptibility to social stress-induced neuropsychiatric disorders. Our model of social

defeat is an ethologically-relevant stressor for hamsters, and it produces a robust behavioral change in the absence of inflammation. In contrast, social defeat in mice is somewhat artificial given that it is produced in inbred mouse strains that are subjected to defeat by a mouse of a different strain, which often results in physical injury. Additionally, prior research in mice shows that blocking microglia and proinflammatory cytokine signaling eliminates the behavioral response to defeat (Lehmann et al., 2019; Hodes et al., 2014; Zhang et al., 2017). Because we observe a pronounced behavioral change in response to social defeat in the absence of inflammation, our work suggests that the impact of inflammation on the behavioral response to social defeat may be smaller than previously thought or may be species-specific. Further, our lack of positive results questions the translational value that data collected in mice may have for humans given that it does not even necessarily translate among rodent species. To address this issue and to move forward with confidence in the translational value of previous and future work in this field, future studies in alternative models of social defeat must be tested.

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6 FINAL CONCLUSIONS

6.1 Summary of current findings

As described earlier, exposure to social stress can trigger or exacerbate a variety of neuropsychiatric disorders that present with symptoms of social avoidance. Despite a relatively large literature examining the mechanisms whereby social stress causes social avoidance, particularly in mice, there is still a lack of information about novel mechanisms that may increase susceptibility to social stress and thus, increase the likelihood of being diagnosed with a mood or anxiety disorder. If such new mechanisms can be identified, then these may lead to novel and more effective treatments for disorders that are characterized by these stress-related symptoms. The purpose of this project was to investigate the role of gut microbiota and inflammation in susceptibility to social stress. We sought to test the hypothesis that social stress increases anxiety- and depression-like responses via changes in gut microbiota and inflammation. We used a social defeat model in Syrian hamsters to test 1) whether social stress alters the gut microbial community, 2) whether manipulating the gut microbial community impacts anxiety-like behavior following social stress and 3) whether social defeat causes neuroinflammation in Syrian hamsters.

In Aim 1, we found that exposure to even a single social defeat leads to robust alterations in the gut microbial community, characterized by decreases in diversity and richness, in both dominant (i.e., winners) and subordinate (i.e., losers) hamsters. These changes to the gut microbiota became more pronounced with repeated bouts of social defeat, suggesting that ongoing social stress leads to increased consequences for the microbiome. These findings support our hypothesis that social stress induces changes to the gut microbial community. What we found even more potentially interesting was that certain microbial taxa might predict whether

an animal would become dominant or subordinate in a subsequent agonistic encounter. This exciting, preliminary finding suggests that the state of the gut microbial community might influence subsequent social behavior.

Therefore, Aim 2 investigated whether manipulating gut microbiota alters social behavior and anxiety-like responses following social stress. A probiotic was used to bias the microbial community to a "healthier", more diverse profile, and emulsifiers, commonly used food additives, were used to disrupt the gut microbial community, or produce gut dysbiosis. We predicted that the probiotic intervention would decrease, and that the emulsifier intervention would increase anxiety-like behavior following social defeat. Unexpectedly, probiotic treatment increased behavioral susceptibility to social defeat compared to controls, and chronic emulsifier treatment had no effect on social behavior in hamsters. Our findings contrast previously held assumptions that probiotics are anxiolytic and that emulsifiers increase anxiety-like behavior (Holder et al., 2019; Bravo et al., 2011; O'Mahony et al., 2011; Ait-Belgnaoui et al., 2014, 2018; Gareau et al., 2007; Messaoudi et al., 2011; Arseneault-Bréard et al., 2012). It is not entirely clear why our results differ from that of previous studies. It may be the case that the probiotic treatment altered the microbiota in a way that promoted anxiety-like behavior in hamsters. This hypothesis is supported by our data showing that hamsters given a biologically relevant dose of the probiotic demonstrated a reduction in gut microbial richness and diversity compared to controls. A reduction in gut microbial richness and diversity is thought to promote potentially harmful downstream effects such as inflammation, gastrointestinal distress and behavioral alterations (for a review, see Lyte et al., 2011). All of which are symptoms that are often observed in many stress-related neuropsychiatric disorders (Kanuri et al., 2016; Kennedy et al., 2016; Qin et al., 2014).

The lack of a behavioral effect of the emulsifier treatment could be due to several factors. The first is that it is possible that emulsifiers are, like the Food and Drug Administration maintains, relatively inert for most species. Thus, the modest anxiogenic-like effect that emulsifiers have been shown to have in mice may not generalize to other species. At the very least, this possibility underscores the importance of examining the effects of this, and similar, treatments in more than one species. Alternatively, the dose of emulsifier that we administered may have been too low to induce a behavioral change. Thus, it is possible that hamsters did not consume enough of the emulsifier during treatment to cause meaningful changes to gut microbiota and behavior. However, emulsifier-induced changes in gut microbiota was not tested here, and this limitation is discussed further in the next section. Additionally, it may be that we did not observe a change in anxiety-like behavior following emulsifier treatment because our behavioral endpoint is capturing something different than are the standard tests of anxiety-like behavior, such as the open field test or light-dark box test, that are commonly used in mice and other rodents. Unfortunately, we were unable to test whether emulsifiers would increase anxiety-like behavior in these tests because it is currently not possible to obtain reliable data from hamsters in the standard tests of anxiety-like behavior that are generated for mice and rats.

Aim 3 tested whether social defeat causes neuroinflammation in hamsters. We predicted that socially defeated hamsters would show an increase in microglial quantity and activation compared to no defeat controls. Our hypothesis was not supported. No increase in microglial activation in brain following defeat was observed. As a positive control, we examined microglial activation following administration of lipopolysaccharide, a bacterial endotoxin, and were able to demonstrate a robust inflammatory response in hamster brain with hamsters exhibiting increases in both microglial quantity and activation. Our model of social defeat is relatively mild in

comparison to the most common chronic social defeat model in mice, which is administered 24 hr a day for 10 days, and pronounced changes in inflammation have been observed in this chronic defeat model. Thus, it is possible that a more chronic form of social stress may be necessary to induce robust inflammation. However, a broader assessment of the response of multiple immune factors to social defeat is necessary to support this hypothesis and this will be further addressed in the next section. In any case, it is clear that the findings from these experiments demonstrate that neuroinflammation, as characterized by a change microglial morphology, is not necessary for behavioral responses to acute or repeated social stress in hamsters.

6.2 Limitations and future directions

Future work is necessary to understand further the putative role of gut microbiota and inflammation in susceptibility to social stress. In Aim 1, we observed social stress-induced dysbiosis of gut microbiota in both dominant and subordinate animals. Because dominant animals fail to show many of the hormonal and behavioral consequences of stress (Huhman et al., 1990, 1991), future studies should test the functional consequences of microbial dysbiosis for this group. An interesting possibility is that specific microbial changes to the gut microbial community that occurred in dominant animals is protective or that these changes can even drive their dominant behaviors. This possibility is consistent with that fact that we also obtained data suggesting that certain microbial taxa can predict the outcome of a social conflict. Replication of this finding is necessary to determine whether certain microbial taxa or a certain microbial profile can predict or drive the likelihood of becoming dominant or subordinate. Future intervention studies should be designed to test whether manipulating our so-called predictive microbial taxa is sufficient to cause changes in social behavior.

To test the hypothesis that social stress impacts the gut microbial community, we collected fecal matter from the hamsters' home cage before any social stress, 24 hr after one bout of social stress, and 24 hr after multiple bouts of social stress. A range of fecal collection protocols exist in the literature, and there is debate over which protocol best ensures the most valid identification of the microbial profile that is in direct response to the experimental manipulation. Here, we chose to collect fecal matter from the home cage to avoid any additional stress to the animal. We were unable to collect fecal matter directly from the colon due to the longitudinal nature of the experiment. It is possible, however, that the results would differ if a different fecal collection protocol was used, and this should be considered when interpreting the findings.

Our hypothesis that probiotic treatment would decrease susceptibility and that emulsifier treatment would increase susceptibility to social defeat was not supported in Aim 2. Instead, we found that probiotics increased social avoidance following defeat, while emulsifiers had no effect on behavior. Because no behavioral effect was observed following chronic emulsifier treatment, we did not assess either the gut microbial community or pro-inflammatory markers. This is a major limitation of the study. Testing the effect of emulsifiers on the gut microbial community and inflammation in Syrian hamsters will be an important future direction. If there are no or minimal changes to gut microbiota or proinflammatory markers, such as proinflammatory cytokines, these data would give more support for the lack of an emulsifier-induced increase in anxiety-like behavior observed in the present experiment and might suggest that ingestion of emulsifiers does not have adverse consequences in hamsters. However, if robust changes to gut microbiota and proinflammatory cytokine signaling are apparent following emulsifier treatment, this would suggest that our hypothesis that gut dysbiosis changes behavioral susceptibility to

social stress would not be supported. Alternatively, it may be that our measure of anxiety-like behavior, social avoidance, does not capture emulsifier-induced behavioral alterations or that only inbred mice are susceptible to these additives, and future experiments could be designed to test these possibilities.

A major limitation of Aim 2 was that we were unable to house the animals that received the probiotic with the animals that received the placebo treatment. Previous work in our lab and others using this probiotic revealed that cross contamination between probiotic-treated and placebo-treated animals is a major problem. Because of this, it is necessary that the probiotic and placebo solutions are made in separate lab spaces, and hamsters in either treatment group are kept separate during the experiment. Thus, it is possible that differences in housing conditions or the exposure to different experimenters could have impacted the anxiety-like behavior observed in hamsters given a biologically relevant (low) dose of the probiotic. However, both the high and low dose of the probiotic was made in the same lab space and administered by the same experimenter, and the behavioral effect was still observed when comparing hamsters given the high and low dose. Therefore, the likelihood of general housing conditions or the experimenter driving the behavioral effect in the hamsters treated with the low dose is unlikely.

In Aim 3, we tested the hypothesis that social defeat causes neuroinflammation by assessing microglial activation in hamster brains following social stress. We failed to detect an increase in neuroinflammation following acute or repeated bouts of social defeat. Although we set out to measure both neuroinflammation and peripheral inflammation, we were unable to measure serum cytokines successfully with the available mouse or hamster ELISA kits; therefore, the scope of this aim was limited to measuring neuroinflammation. It is possible that these commercial kits for measuring cytokines are failing to detect these signals or that they are

effective, and the circulating cytokines are simply very low in hamsters and more sensitive measures would be required. Microglial activation was measured in the prefrontal cortex and basolateral amygdala, two critical nodes in the neural circuit governing the behavioral response to social defeat (Jasnow & Huhman, 2001; Markham et al., 2010; Markham et al., 2012). It is possible that a proinflammatory response would have been detected if other brain regions in this circuit, such as the hippocampus, were examined. Microglial responses to social defeat have previously been observed in the hippocampus of mice (Wohleb et al., 2011, 2013, 2014; Lehmann et al. 2018). Therefore, future work should assess microglial responses in the hippocampus and other stress-responsive brain regions to better test how different models of social defeat affect neuroinflammation. Further, our experimental approach was limited to measuring microglial quantity and activation. Many other markers of neuroinflammation exist such as macrophage infiltration, oxidative stress, enrichment of pro-inflammatory cytokine mRNA, and enrichment of microglia mRNA that governs inflammatory pathways. Therefore, many options exist to gain a broader assessment of the relationship between neuroinflammation and mild social stress. In any case, however, the present findings do demonstrate that pronounced microglial activation in the medial prefrontal cortex and basolateral amygdala is not necessary for the behavioral changes that are observed in hamsters following mild social stress.

6.3 Conclusion

The present experiments demonstrate a two-way relationship whereby social stress alters gut microbiota and gut microbiota can alter susceptibility to social stress. Future studies are necessary to elucidate the mechanisms driving this relationship. We propose the exciting hypothesis that certain microbial taxa can drive future social behavior and mechanistic studies should be designed to test this hypothesis. Further, future research should expand on our work by

investigating a wider range of immune mechanisms whereby gut microbiota can influence behavioral susceptibility to social stress. Identifying these mechanisms is a critical next step to broaden the range of viable treatment options for those suffering from disorders characterized by social stress-related symptoms.

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