AGGRESSION AND THE GUT-BRAIN AXIS

By

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ABSTRACT

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Violence is a widespread public health and justice system problem with far-reaching consequences for victims, offenders, and their communities. Aggression, the cognitive and behavioral antecedent to violent action, is mainly understood in terms of the psychosocial risk factors that increase the likelihood of aggressive behavioral strategies. Neighborhood context is a principal risk factor for violent crime perpetration, but the mechanisms that mediate the effect of the environment on individual-level aggression behavior are poorly understood, especially the biological factors that may contribute to our understanding of violent behavior. In order to gain a better understanding of mechanisms that precipitate violence in specific geographic contexts, this dissertation explores the relationship between aggression behavior and the gut microbiome, a spatially determined physiological system that affects human health and behavior. Preclinical experiments elucidate the role of the gut microbiome in territorial, reactive aggression behavior in mice. Results show significant differences in gut microbiome composition across the spectrum of murine aggression behavior. Moreover, manipulation of the gut microbiome via administration of short-term antibiotics and sodium butyrate, a short-chain fatty acid byproduct of microbial fermentation, increases aggression behavior. The overall goal of this research is to use basic science findings in mice to better understand how environmental exposures could influence human health and behavior, thus revealing how community health affects individuals and supplying a potential target for future intervention.

In honor of my grandfathers, Władysław Kwiatkowski and Anthony Ambroziak, who collectively fought a war, crossed an ocean, and overcame abject poverty to give me this opportunity. Your sacrifices will never be forgotten.

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CHAPTER 1: INTRODUCTION

Crime is a persistent societal problem that threatens public safety and burdens the medical, public health, and criminal justice systems. In human populations, violence is a spatially specific phenomenon that is concentrated in impoverished neighborhoods alongside poor health and disadvantage (Bursik & Grasmick, 1993; LaVeist, Pollack, Thorpe, Fesahazion, & Gaskin, 2011; Sampson, 2012; Shaw & McKay, 1942). Indeed, geospatial context is one of the most important risk factors for perpetration of violence (Bingenheimer, Brennan, & Earls, 2005; Braga, Papachristos, & Hureau, 2010; Weisburd et al., 2016), directing criminological research efforts towards understanding the places where crime is committed and the motivations of the offender. However, while there is a long-standing empirical link between neighborhood context and crime perpetration, there is substantial variation among residents living in high crime areas. Importantly, most people living in high crime areas never behave violently despite exposure to the same psychosocial risk, indicating that there are individual-level factors that influence patterns of aggression behavior in humans.

This variation at the level of the individual necessitates a biopsychosocial approach to understanding violence, thereby incorporating biological mechanisms that can explain differential response to the known psychosocial risk factors that drive behavioral aggression. Emotional dysregulation is a critical, individual-level risk factor in the expression of aggression behavior (Sapolsky, 2017) that is also associated with exposure to early life adversity and structural disadvantage (Appleyard, Egeland, van Dulmen, & Alan Sroufe, 2005; Hay, Fortson, Hollist, Altheimer, & Schaible, 2006). Underlying this emotional dysregulation is dysfunction in physiological systems responsible for the processing of social behavior and associated emotional cues. Indeed, research suggests that aggression behavior manifests with a suite of canonical

psychological states, like impulsivity (e.g., Kwiatkowski, Robison, & Zeoli, 2018), elevating the brain as a logical target for further inquiry into the mechanisms of aggression behavior. Moreover, human imaging studies demonstrate that individuals exposed to disadvantage exhibit changes in the structure and function of the cortico-amygdala loop (Hackman, Farah, & Meaney, 2010), key systems in the regulation of socio-emotional behavior, and this is also observed among criminal offenders (Leutgeb et al., 2015). While the evidence overwhelmingly indicates an interdisciplinary approach to understanding aggression and violent behavior, there is still a pervasive separation between scientific disciplines over concerns of reductionism and biological determinism (Rafter, Posick, & Rocque, 2016). However, while the brain may act as a final common mediator of risk, it is the environment that shapes human physiology according to its needs (Sapolsky, 2017), underscoring the dependency of biological, psychological, and social factors in driving human aggression.

Background

Species-normative aggression behavior is a diverse and adaptive set of behaviors expressed in defense of self, offspring, or resources. Aggression behavior is generally offensive (i.e., territorial, reactive aggression) or defensive (i.e., threat). However, aggression behavior that occurs outside the contexts of competition and/or self-defense, is considered excessive, pathological aggression(Nelson & Trainor, 2007). Excessive aggression is associated with pathophysiological changes in the brain in regions that detect and regulate response to emotional stimuli. Though many regions have a known role in aggression behavior, the cortico-amygdala loop in among the most studied because of its role in processing emotional stimuli. The amygdala is one structure involved in the detection of emotional stimuli in the environment. The amygdala is an evolutionarily older, limbic structure (i.e., emotion center) responsible for the learning of emotion

associated with social behavior, and subsequently shaping behavior in response to emotional, or otherwise "motivationally salient," stimuli (Rosell & Siever, 2015). It is subdivided into nuclei (i.e., collections of cell bodies) that can be categorized as either gratification or aversion centers. With roles in fear and anger (i.e., negative emotional stimuli), the amygdaloid nuclei are overwhelmingly centers of aversion that guide social approach and avoidance behaviors (Haines, 2013).

Cues in the environment act as sensory input that activates the amygdala, directing attention to the stimulus and communicating its presence to other regions of the brain, like the cortex and hypothalamus (Davis & Whalen, 2001), thereby acting as a physiological alert system. Sufficient activation of the amygdala mobilizes innate defensive behaviors through its action on hypothalamic neurons that initiate the body's autonomic and stress responses (D. J. Anderson, 2012; Nelson & Trainor, 2007). Structures of the limbic system (i.e., emotional processing circuitry), including the amygdala, are well-connected throughout the brain to other aversion and gratification centers, which likely contributes to emotional stability (Haines, 2013). Emotional disturbances are thus related to atypical activation patterns in limbic structures. In particular, too much or too little activation in certain amygdaloid nuclei corresponds to states of hyperarousal and hypoarousal associated with reactive and instrumental aggression, respectively (Rosell & Siever, 2015). Though pathological amygdala activity "naturally" occurs in some cases (e.g., schizophrenia), these patterns are known to develop from exposure to psychosocial adversity (Sapolsky, 2017).

In contrast, the prefrontal cortex (PFC), the seat of executive function, is an essential structure in interpreting stimuli and integrating experience in novel contexts. While the amygdala is more of an automatic indicator of emotion, the frontal cortex, especially the PFC, is responsible

for processing feeling states (Gospic et al., 2011). In a healthy brain, the PFC receives sensory information about the environment and synthesizes emotional, experiential, and contextual knowledge to facilitate an appropriate response (Haines, 2013). Once sensory stimuli have been evaluated, the PFC's inhibitory projections clamp down on amygdala activity, conveying that the emotional stimuli or threats are benign or neutralized. When behavioral response is required, PFC projections to motor regions initiate conscious action. Like other regions in the brain, the PFC is subdivided into dorsolateral, ventrolateral, and orbitofrontal regions, all of which have a slightly varying role in the analysis of sensory information (Haines, 2013; Sapolsky, 2017).

Research shows that impairments in the amygdala-PFC circuit, the so-called cortico-amygdala loop, are associated with aggression behavior. Specifically, executive dysfunction results in the loss of inhibitory control over the amygdala, thereby disinhibiting the amygdala to initiate hard-wired, emergency behavioral responses or psychopathic disengagement, depending on which area of the PFC is disturbed (Sapolsky, 2017). As such, the pattern of activation in the PFC is more similar across different types of aggressive individuals with overall reductions in PFC activation and less functional coupling between the PFC and amygdala. In the absence of top-down control, pathophysiological changes in the amygdala occur, which can impair social behavior and facilitate aggression behavior. Again, natural deficits in the PFC can occur, but abnormal function in the PFC or its connectivity with the amygdala are associated with psychosocial adversity (Sapolsky, 2017).

Literature Review: Potential Connections between Aggression and the Gut-Brain Axis

Amygdala

In humans, instrumental aggression is associated with autonomic hypoarousal and hyporeactivity to emotional stimuli, creating both inattention to emotional stimuli and potential

impairments in fear conditioning (Rosell & Siever, 2015). As a result, individuals may develop "immunity" to distress, one's own or a victim's, which facilitates participation in harmful and potentially dangerous social behavior. For example, in a study investigating the underlying neurobiology of youth conduct problems, Lozier and colleagues (2014) found that youth with concomitant callous-unemotional traits showed significantly less activation of the right amygdala in response to fearful faces, a mediating effect that uniquely predicted proactive aggression. Activation of the right amygdala is associated with the identification of emotional distress and feelings of empathy, which normally inhibit aggression in healthy subjects. The absence of response in this region to emotional stimuli among youth with conduct problems and callous-unemotional traits suggests that they are unresponsive to the fearful emotions of their victims and therefore undeterred during aggressive action (Lozier, Cardinale, VanMeter, & Marsh, 2014). Relatedly, in another imaging study of adult inmates at a maximum security prison, self-reported criminal psychopaths (versus criminal non-psychopaths) showed significantly less activation in the amygdala and other limbic structures during an emotional memory task. Impaired emotional memory is consistent with the known inattention to emotional stimuli, which are likely not encoded if they are below notice. Thus, researchers concluded that deficits in emotional memory may result in impairments in fear conditioning that render individuals with psychopathic traits insensitive to their own distress (Kiehl et al., 2001). As a result, potential threats may not be incorporated into cognitive processes that guide behavior, thereby creating a destructive fearlessness that facilitates participation in proactive, instrumental aggression. Other imaging studies in clinical and healthy adult populations with psychopathic traits replicate significant reductions in amygdala activity during facial recognition tasks and even show hyporeactivity while considering harming others during moral decision-making (Dolan & Fullam, 2009; Glenn, Raine, & Schug, 2009). These

studies further advance the notion that offenders with psychopathic traits are emotionally undisturbed by the suffering and harming of others to obtain personal goals. Taken together, this work suggests that individuals with psychopathic traits characteristic of instrumental aggression demonstrate atypical neural activity associated with psychological disengagement that permits them to act without fear or remorse (Dolan & Fullam, 2009; Glenn et al., 2009; Shirtcliff et al., 2009).

In contrast, reactive aggression is associated with autonomic hyperarousal and hyperreactivity to emotional stimuli. For individuals who express this type of aggression, research suggests a hyperreactive activation pattern in the amygdala, indicating increased threat perception and attention to threat (Rosell & Siever, 2015). This hyperreactivity might override executive control to impulsively initiate defensive behaviors. For example, in their aforementioned study on youth and conduct problems, Lozier and colleagues (2014) found that youth without callous-unemotional traits demonstrated a hyperreactive activation pattern in the amygdala in response to fearful *and* neutral faces. This finding was associated with externalizing behavior, suggesting that these youth more readily perceive threat and react aggressively (Lozier et al., 2014). In other imaging studies of defensive reactivity in adults, researchers found that amygdala reactivity was associated with threat vigilance and subsequent recruitment of brain regions responsible for hard-wired, defensive behavioral action (Lueken et al., 2014; Mobbs et al., 2009; Mobbs et al., 2007). Critically, activity in the amygdala was shown to lessen only as these other areas activated and inhibited the amygdala, indicating that threat is neutralized by action in a defensive context. This becomes important when considering reactive aggression because the feeling of being under threat, real or perceived, is best ameliorated with defensive action, especially among those who might have executive impairments that limit non-aggressive coping strategies. Altogether, evidence

suggests that individuals who express reactive aggression demonstrate abnormal neural reactivity to environmental social cues that biases them to perceive threat and respond with defensive behaviors.

Relatedly, the gut microbiome has a potential role in the expression of deviance through its regulation of the physiological state of the amygdala. In a study of anxious behavior, Neufeld and colleagues (2011) found that germ-free (GF) mice (i.e., reared without microbes) demonstrated significantly less behavioral anxiety in exploration of open space, a task that is usually aversive to rodents. This behavioral phenotype was accompanied by significant reductions in central amygdalar mRNA expression of the N-methyl-D-aspartate (NMDA) receptor subunit NR2B, which has a known role in facilitating learning and brain plasticity (i.e., adaptive learning). Learning deficits associated with NMDA receptor dysfunction could impair fear conditioning and facilitate approach behavior (Neufeld, Kang, Bienenstock, & Foster, 2011). As described, insensitivity to normally distressing stimuli is a key factor in enabling aggressive behavior. The GF mice studied by Neufeld and colleagues demonstrate a behavioral phenotype reminiscent of this insensitivity as a result of pathophysiological changes in the same brain region known to facilitate aggressive behavior in humans. Consequently, these data raise the possibility that pathophysiological changes related to aggression may also be regulated, at least in part, by perturbations of the gut microbiome.

To more explicitly define the potential role of the gut microbiota in affective processing, Hoban and colleagues (2017) investigated the role of the microbiome in modulating amygdala activity and behavior in a classic fear conditioning paradigm. After conditioning, GF mice demonstrated significantly less freezing in response to a tone that signaled foot shock compared to conventional controls. Moreover, there was no evidence of retention during subsequent

extinction trials. This behavioral phenotype was associated with significant increases in the expression of immediate early genes (i.e., markers of neuronal activity), indicating a hyperresponsive amygdalar activation pattern in GF mice. Researchers concluded that amygdalar hyperreactivity reflected emotional dysregulation to a degree that impaired fear learning, leading to inappropriate behavioral responses to fear-inducing stimuli. Although amydalar hypo- rather than hyperreactivity is associated with impaired fear conditioning in humans, these findings still suggest some role for gut microbiome in the development of normative affective processing, especially emotional memory (Hoban et al., 2017). As such, these data raise the possibility that dysregulation in the amygdala related to aggression could be influenced by perturbations of the gut microbiome.

Preclinical findings point to a critical role for the gut microbiome in organizing and modulating neurophysiology, affective processing, and social behavior, but they come with substantial limitations because the GF condition is highly unnatural and never observed in humans. In an attempt to translate some of these findings to humans, researchers have begun exploring the relationship between the gut microbiome and affective processing in healthy adults. For example, Tillisch and colleagues (2013) investigated the role of the gut microbiota in modulating emotional attention to negative affect faces (i.e., fear, anger) in healthy adult women. Researchers conducted a double-blind, randomized, controlled trial of a fermented milk product with probiotic (FMPP) versus a nonfermented milk product or no intervention control. After four weeks of the intervention, researchers found that subjects receiving the FMPP demonstrated significantly less activity in emotional processing networks compared to the control conditions. Additionally, the FMPP group showed significant reductions in amygdala activation, in particular, compared to no intervention controls. Together, these findings suggest that the gut microbiota influence

responsiveness to emotional stimuli in healthy women (Tillisch et al., 2013), which may be indicative of a reduction in vigilance (Mayer, Knight, Mazmanian, Cryan, & Tillisch, 2014). In another study of healthy adults with mild risk for depression, Steenberger and colleagues (2015) conducted a placebo-controlled, randomized, pre- and post-intervention assessment of a probiotic treatment for cognitive reactivity to sad mood. After four weeks, researchers found significant reductions in self-reported aggression and rumination compared to baseline, but no change in the control group. This finding suggests a role for the microbiome of the gut in modulating cognitive reactivity during sad affective states (Steenbergen, Sellaro, van Hemert, Bosch, & Colzato, 2015). Though related to sad mood, a specific reduction in aggression associated with probiotic treatment is perhaps the most promising finding in support of a role for the gut microbiome influencing overall aggression. Consistent with previous findings, reductions in aggression and rumination may result from decreased neural reactivity to emotional stimuli, but that relationship is yet unclear. These data, however, do lend support to the importance of the gut microbiome in modulating sensitivity to emotional stimuli, which may extend to the emotional sensitization observed in reactive forms of aggression.

The Prefrontal Cortex

In general, pathological aggression results from uninhibited emotional responsiveness. In reactive forms of aggression, strong limbic activation in response to perceived threat may override executive control. In instrumental forms of aggression, impaired social learning and emotional disengagement may lead to a failure to recognize inappropriate social behavior. Both problems, however, reflect deficits in executive function. It is thus unsurprising that pathological aggression is broadly associated with deficits in the PFC. Accordingly, research shows deficits in PFC volume, activity, and functional coupling with the limbic system (Sapolsky, 2017). Despite some

inconsistencies in the literature due to methodological issues, imaging studies suggest that aggressive individuals show significant reductions in PFC volume (Y. Yang & Raine, 2009). For example, Leutgeb and colleagues (2015) found a significant reduction in prefrontal cortical gray matter (i.e., number of cell bodies) in the brains of violent criminals compared to controls. Among criminals, this finding was negatively correlated with psychopathic traits and associated with a significantly increased risk of criminal recidivism. Compared to controls, criminally violent subjects also demonstrated significant increases in gray matter volume in brain regions associated with motor elements of impulse control, which was positively correlated with psychopathic traits and recidivism risk (Leutgeb et al., 2015). This suggests that criminally violent individuals have impairments in self-regulation, but enhancements in motor output that facilitate violent behavior. In another imaging study of both reactive and instrumental forms of aggression, Bertsch and colleagues (2013) examined structural differences among (1) antisocial criminal offenders with borderline personality disorder (BPD), (2) antisocial criminal offenders with high psychopathic traits, and (3) healthy controls. As expected, both BPD-reactive and psychopathic-instrumental offenders exhibited significant overall reduction in gray matter volume in the PFC compared to controls. However, further investigation revealed that the source of this overall reduction varied between the two groups. Specifically, BPD-reactive offenders showed significant reductions in regions associated with impulse control while psychopathic-instrumental offenders showed significant reductions in regions associated with situational self-identification and moral reasoning. The authors therefore concluded that reactive forms of aggression are a product of emotional dysregulation whereas instrumental forms of aggression are related to in inability to place oneself in social situations and internalize social norms as well as impairments in moral judgment (Bertsch et al., 2013). In sum, this work shows significant neuroanatomical deficits in

the PFC associated with aggressive behavior, and evidence that suggests the PFC is differentially compromised in reactive versus instrumental forms of aggression.

In addition to reductions in PFC volume, evidence suggests that aggressive and deviant individuals exhibit significantly less functional coupling between the amygdala and PFC (Rosell & Siever, 2015; Sapolsky, 2017). In the absence of cortical control, behavior is guided by innate responses to emotional stimuli and/or deficient moral reasoning. Unfortunately, no single study attempts to characterize disruptions in functional connectivity between reactive and instrumental aggression, but independent investigations do provide some insight. In an imaging study of pathological reactive aggression, Coccaro and colleagues (2007) found that angry faces (versus other emotional expressions) elicited significant increases in amygdala activity, but a significantly diminished orbitofrontal (i.e., PFC subdivision) response among aggressive individuals. Additionally, researchers found that aggressive subjects failed to co-activate the amygdala and orbitofrontal cortex, which suggests less executive processing of emotional stimuli. Together, these findings suggest that reactive aggression is characterized by an attentional bias to negatively valenced emotional stimuli and emotional reactivity unmediated by executive control (Coccaro, McCloskey, Fitzgerald, & Phan, 2007). To examine instrumental forms of aggression associated with psychopathy, Contreras-Rodríguez and colleagues (2015) conducted a resting-state (i.e., no emotional provocation) functional connectivity imaging study of subjects with high levels of self-reported psychopathy and a history of a severe criminal offense versus healthy controls. Among the criminal offenders with psychopathy, researchers found significantly decreased PFC-amygdala coupling that was modulated by the severity of psychopathic traits. The authors suggest that a disconnect between emotional and cognitive neural systems leads to a failure to integrate emotional, experiential, and contextual information in social behavior. As a result, individuals

express inflexible behavioral patterns focused on goal attainment by any means necessary (Contreras-Rodríguez et al., 2015). Reference to social norms and moral reasoning (Bertsch et al., 2013) is a critical component of experiential and contextual information integration. Deficits in these areas could indeed decrease social adaptability and increase inflexible, aggressive behavioral patterns. Altogether, these studies indicate that neural deficits in executive function during affective state processing enable aggressive behavior.

Unfortunately, there is limited research on the relationship between the gut microbiome and the PFC. Current research is exploratory in nature and not related to the manifestation of any particular behavioral phenotype. Emerging evidence suggests, however, that the gut microbiome influences neuron myelination in the PFC and the maturation and function of glial cells (Cryan & Dinan, 2015; Erny et al., 2015; Heijtz et al., 2011; Hoban et al., 2016). Myelin is a fatty substance that coats neurons such that the conductivity, velocity, and overall efficiency of cell signaling increases. In its absence, communication between distal regions in the nervous system deteriorates and function is lost (e.g., multiple sclerosis) (Haines, 2013). To explore the role of the gut microbiota in the PFC, Hoban and colleagues (2016) conducted a genome-wide (RNA) sequencing of the PFC in GF, exGF, and conventionally colonized mice. Researchers found a significant upregulation in the expression of genes related to myelination in the PFC of male GF mice compared to conventional controls. Accordingly, male GF mice exhibited a significant, exaggerated increase in myelination in the PFC. This effect was rescued by colonization with a conventional microbiome post-weaning. Specifically, relevant gene expression and myelination returned to normal levels in the PFC of exGF mice. Taken together, these findings suggest that the gut microbiome regulates PFC myelination and plasticity throughout the life course (Hoban et al.,

2016). At this early stage, however, all that can be said is that the gut microbiota may influence neuronal signaling in the PFC, which may also have an effect on aggressive behavior.

Addressing Gaps

Though changes in the gut microbiome correspond to changes in brain systems related to aggression, little work has been done to directly explore the relationship between the gut microbiome and aggression. The work described in this thesis aims to directly examine the relationship between aggression and the gut microbiome.

Hypothesis and Specific Aims

Central Hypothesis:

Gut microbiome composition drives territorial, reactive aggression in mice

Aim 1: Examine the gut microbiome in murine territorial, reactive aggression behavior

- 1A | Development of a composite measure of aggression
- 1B | Characterization of aggressor gut microbiome biodiversity

Aim 2: Investigate potential causal role of the gut microbiome in aggression behavior in mice

- 2A | Effects of sodium butyrate on aggression behavior
- 2B | Effects of antibiotics on aggression behavior
- 2C | Effects of probiotics on aggression behavior
- 2D | Effects of gut microbiome recolonization from an aggressive donor

CHAPTER 2: MATERIALS AND METHODS

Preclinical Studies

Animals

All aggression experiments were performed with resident outbred, sexually experienced, retired breeder CD-1 male mice purchased from Charles River Laboratories (Wilmington, MA) at ≥ 16 weeks of age (**Fig 1**). Male inbred, sexually naïve C57BL/6J mice purchased from The Jackson Laboratory (Bar Harbor, ME) at 8 weeks of age were also used as novel intruders in the CD-1 home cage during behavioral trials. CD-1 mice were singly housed whereas C57BL/6J mice were housed in groups of 4-5 conspecifics. Mice were housed in a 12:12 hour light-dark cycle and provided ad libitum access to water and a standard laboratory diet. All experiments were approved by the Institutional Animal Care and Use Committee at Michigan State University (MSU) and conducted in accordance with guidelines from the Association for the Assessment and Accreditation of Laboratory Animal Care and National Institute of Health.

Behavioral Paradigms

Resident-Intruder Procedure

Aggression behavior in CD-1 male mice was evaluated using the resident-intruder (RI) task as previously described (Golden, Covington III, Berton, & Russo, 2011; Golden et al., 2016; Koolhaas et al., 2013; Olivier & Young, 2002). Male CD-1 retired breeder mice arrived at the MSU animal facility and were allowed to acclimate for 7-10 days to prevent a shift in the community of the gut microbiome during experimental procedures. Following this acclimation period, CD-1 mice were singly housed in 65 cm shoebox cages one week prior to screening to habituate and establish their territory. On each trial day, a novel male C57BL/6J intruder mouse was placed in the home cage of the CD-1 aggressor mouse and their social interaction was

observed for three minutes (180 s). The C57BL/6J intruder mice were rotated such that the experimental, resident mouse never interacted with the same intruder twice. This procedure was repeated over three consecutive days and measures of latency to the first attack, the number of attack bouts, and attack duration were collected each day via real-time experimenter evaluation.

An attack bout was defined as any successful aggressive contact unrelated to grooming or mounting. Aggressive contact included biting, pouncing, punching, boxing, grabbing, flipping, or pinning. An attack bout was considered to have ended either when the CD-1 (i) turned its back or, (ii) ceased activity for two or more seconds. An attack bout was considered unbroken if the CD-1 remained in active pursuit (e.g., chasing) of the C57BL/6J intruder. The number of attack bouts was counted on each trial day. The time from intruder introduction to the first attack was recorded and converted to seconds to provide a latency measure for each trial day. Animals that did not attack were assigned a latency value of 180, denoting that the entire trial period elapsed without attack. The length of each attack bout was also recorded and averaged across the entire trial to provide a measure of trial attack duration for each day. Altogether, nine indicator

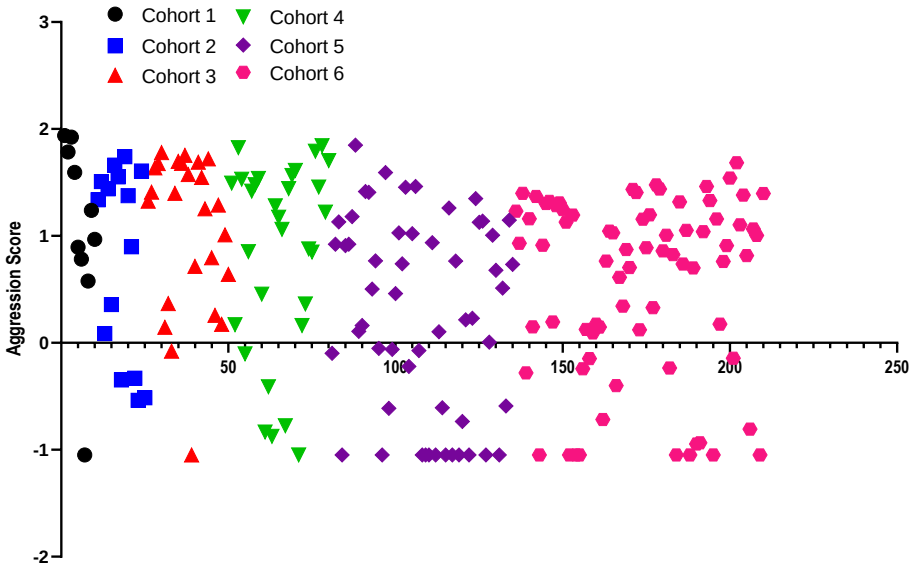

Figure 1 | Cohorts of CD-1 Mice. Schematic showing the distribution of aggression scores of sexually experienced CD-1 male mice who underwent aggression score using measures collected during the resident-intruder task.

variables were collected to characterize aggression behavior, henceforth referred to as latency, bouts, and duration for trial days 1-3.

Chronic Social Defeat Stress

Chronic social defeat stress (CSDS) was performed as previously described (Golden et al., 2011). Briefly, C57BL/6J mice were placed in the home cage of a CD-1 retired breeder mouse containing a perforated plexiglass divider bisecting the length of the cage. The experimental mice were allowed to physically interact with the CD-1 for 5-10 minutes. After the aggressive encounter, the experimental mice were placed on the other side of the divider allowing for sensory, but not physical, contact with the CD-1 for 24 hours. This procedure was repeated over 10 consecutive days with exposure to a new CD-1 aggressor every day. Behavioral testing began the day following the final day of stress.

Behavioral Tasks

Social Interaction

Social interaction (SI) testing was conducted as previously described (Berton et al., 2006; Krishnan et al., 2007). Under red light conditions, mice were placed in the center of a custom-made, opaque arena (25 in x 25 in x 15 in) containing an empty wire mesh cage (10 cm diameter) against one wall and allowed to freely explore for 150 seconds. The experimental mice were then removed from the arena and a novel CD-1 mouse was placed in the wire mesh cage. Experimental mice were reintroduced to the arena and allowed to free explore for another 150 seconds. The time spent in proximity (7.5 cm) of the wire mesh object was defined as the "interaction zone" time while the time spent in the two corners (9 x 9 cm square) farthest from the object was defined as "corner zone" time. The SI ratio was determined by calculating the

time spent in the interaction zone when the CD-1 was present divided by the time spent in the interaction zone when the CD-1 was absent.

Drug Administration

Sodium Butyrate Intraperitoneal Injections

Sodium butyrate (1.2 g/kg; dissolved in 0.9% saline) or a 0.9% saline solution was injected into the intraperitoneal cavity once daily at a total volume of 5 mL/kg. Behavioral testing was initiated two hours post injection. During trial days, a staggered drug administration strategy wherein small groups of animals were injected every 5 minutes was employed.

Antibiotic and Probiotic Treatment

Antibiotics and probiotics were administered to experimental animals via drinking water. Animals that were treated with antibiotics received a broad-spectrum cocktail of 1.0 g/L ampicillin and 0.5 g/L neomycin mixed in a UV-protected drinking bottle (Schepper et al., 2019). Animals that were treated with probiotics received a daily drinking solution of VSL#3 (L. Zhang et al., 2019), a commercially available probiotic supplement containing *Streptococcus thermophilus, Bifidobacterium breve, Bifidobacterium longum Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei, and Lactobacillus delbrueckii supsp. Bulgaricus.* Drinking volumes were noted to ensure consumption.

Molecular Techniques

Microbial DNA Extraction

DNA extraction for 16S ribosomal ribonucleic acid (rRNA) amplicon sequencing was conducted on fecal pellets collected during experimental rehousing or directly from the colon of CD-1 mice at the onset of transcardial perfusion (see *Immunohistochemistry* below). Fecal pellets were stored at -80º C until DNA extraction. DNA extraction was conducted using the

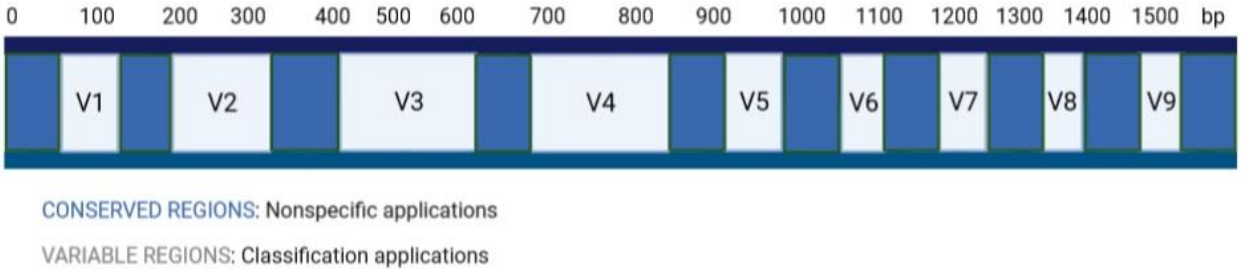

Figure 2 | 16S rRNA Gene. Schematic of the 16S rRNA gene depicting its conserved and highly variable regions.

DNeasy PowerSoil and PowerSoil Pro Kits (Qiagen, Hilden, Germany), a proprietary set of reagents designed to optimize DNA extraction from soil and fecal samples. To summarize, fecal pellets were lysed via chemical and mechanical homogenization, aided with the addition of 15 µl of lysozyme to the bead tube. This crude lysate was treated with the provided reagents to remove cellular debris and inhibitors that could impede the amplification process in downstream applications. The purified samples were then mixed with the provided DNA binding solution and passed through a silica membrane. Remaining contaminants were washed away and silica-bound DNA was eluted using 100 µl of a 10 mM Tris elution buffer. Sample DNA was quantified using Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) and normalized to a 1 ng/µl concentration. DNA samples and their normalized dilutions were stored at -20º C for downstream applications.

16S rRNA Amplicon Sequencing

To determine microbial community composition, 16S rRNA amplicon sequencing, a technique that leverages a common genetic marker to generate raw DNA sequences that identify microbiota, was conducted. The 16S rRNA gene is a standard genetic marker that codes for a component of the small ribosomal subunit (Hamady & Knight, 2009), a critical cellular component that facilitates protein production and thus a core cellular structure that is essential in sustaining life. The 16S rRNA gene is therefore a highly conserved gene, present in the DNA of

most organisms, yet still variable enough to distinguish between different taxa, or groups of organisms (Fox, Pechman, & Woese, 1977). Specifically, the 16S rRNA gene (**Fig 2**) consists of approximately 1,500 base pairs (bp) and contains both highly conserved and highly variable regions (Case et al., 2007). Importantly, mutations that have occurred within the highly variable regions have become fixed within lineages such that their DNA sequences are unique, allowing for the identification of microbiota across taxonomic levels to classify related organisms (Woese, 1987).

For the current study, normalized DNA samples (1 ng/µl) for both human and mouse subjects were submitted to the MSU Genomics Core (East Lansing, MI, USA) to prepare the 16S rRNA gene amplicon library and subsequent sequencing. The V4 hypervariable region of the 16S rRNA gene amplicon was amplified using region-specific, Illumina compatible, dual indexed primers [515 f (5'-GTG CCA GCM GCC GCG G-3') and 806 r (5'-TAC NVG GGT ATC TAA TCC-3')] as previously described (Kozich James, Westcott Sarah, Baxter Nielson, Highlander Sarah, & Schloss Patrick, 2013). Polymerase chain reaction (PCR) products were batch-normalized and pooled using Invitrogen SequalPrep DNA Normalization plates. The pool was cleaned up and concentrated using a QIAquick PCR Purification column followed by AMPureXP magnetic beads. The pool was quantified using a combination of Qubit dsDNA HS, Agilent 4200 TapeStation HS DNA1000 and Invitrogen Collibri Library Quantification qPCR assays. Pooled sequences were loaded on a MiSeq v2 standard flow cell and sequencing was carried out in a 2 x 250 bp paired-end format using a MiSeq v2, 500-cycle reagent cartridge. Custom sequencing with index primers complementary to the 515f/806r oligomers were added to the reagent cartridge to avoid primer sequencing. Filtering parameters were optimized for detecting low abundance phylogenetic diversity (Caporaso et al., 2012). Bases were called by the

Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.20.0.

Immunohistochemistry

Immunohistochemistry (IHC) was conducted on fixed, coronal tissue sections prepared from whole brain samples collected following transcardial perfusion, a tissue preservation procedure that utilizes an experimental animal's cardiovascular system for the systemic administration of a fixative solution. Specifically, CD-1 mice were transcardially perfused with cold PBS to evacuate the blood followed by 10% formalin to fix the tissue. Whole brain samples were extracted and post-fixed for 24 hours in 10% formalin. Samples were subsequently cryopreserved in 30% sucrose and sliced into 35μm sections. Tissue sections were incubated with anti-FosB (Cell Signaling FosB 5G4, 1:1000) primary antibody. A corresponding biotin-conjugated secondary antibody (Jackson Immunoresearch) was used, which was then visualized by 3,3′-diaminobenzidine staining (Vector Laboratories. Images of the prefrontal cortex, nucleus accumbens, amygdala, and ventromedial hypothalamus were taken with the Nikon Upright Eclipse N*i*_U M570e upright fluorescent microscope using a 20X objective. Positive nuclei were quantified by a blinded experimenter using Fiji software.

Analytic Strategy

Microbiome Data Analysis (performed with S. Kaszubinski)

To obtain meaningful sample characterizations from raw DNA sequences, a series of computationally-intensive data processing steps were conducted. First, the study team's collaborating data analyst pulled raw sequences through a standardized data processing pipeline to classify taxa present in the samples using the Quantitative Insights Into Microbial Ecology 2 (QIIME 2) program v2018.11 (Bolyen et al., 2019). Briefly, this entailed assembling paired-end

sequence reads from the raw fastq files provided by MSU Genomics Core, removing poor quality sequences, and taxonomic assignment, the details of which are outlined by the Human *Post Mortem* Microbiome team elsewhere (Kaszubinski, Pechal, Schmidt, et al., 2020). For classification, sequences were binned into operational taxonomic units (OTUs), or working groups of sequences with 99% similarity. From these OTUs, representative sequences were aligned to the SILVA small subunit database v132 (Quast et al., 2013). Non-bacterial sequences were removed from the dataset and final taxonomy tables were exported to CSV files to be used as input data for downstream analysis.

Next, the taxonomy tables were imported into R and RStudio for further data processing. In these final steps, a long-form dataset was created such that each OTU represented a row and every column was one of the decedents' five samples. The OTUs were labeled according to their taxonomic classification (down to the genus level), empty rows were removed, and the reformatted table was merged with combined study metadata using the *phyloseq* package for R and RStudio (McMurdie & Holmes, 2013). In an effort to standardize sample microbial communities, taxa with less than 0.01% of the mean library size, or the number of sequences, were dropped from the dataset. Sequence libraries were subsequently normalized via rarefaction, whereby sequence libraries were randomly subsampled to a specified minimum library size to prevent the effects of sample size bias on microbial community composition. Guided by rarefaction curves, mouse data were rarefied to standardize library size. Using normalized sequence libraries, measures of relative abundance and community diversity metrics were calculated to characterize the decedent's gut microbiome.

Statistics and Model Selection

Statistical analysis was conducted using a combination of R and RStudio version 4.0.5 (R Core Team, 2021; RStudio Team, 2020) and GraphPad PRISM software 9.0. Data distributions were examined with Shapiro-Wilks tests of normality and Bartlett's tests of homogeneity of variance to select the appropriate parametric or nonparametric tests.

CHAPTER 3: QUANTITATIVE STANDARDIZATION FOR STUDIES OF AGGRESSION AND SOCIAL DEFEAT

Note: Figures and text were previously published in (Kwiatkowski et al., 2021).

Author contributions:

Conceived and Designed Experiments: Kwiatkowski, Eagle, Goodwin, Golden, Robison

Performed Experiments: Kwiatkowski, Eagle, Ndlebe, Goodwin

Analyzed Data: Kwiatkowski, Akaeze, Bender

Contributed Materials or Analysis Tools: Moon

Introduction

Aggression is a common, adaptive animal behavior that broadly defines social conflict related to competition for resources or self-defense. However, aggression is an unobservable construct, or latent factor, that cannot be directly measured. Instead, aggression is defined by a unifying constellation of observable indicators that characterize an aggressive behavioral phenotype. One such behavioral phenotype in rodents is territorial, or reactive, aggression where a dominant male confronts and expels pubescent males from its marked territory (Miczek et al., 2004). Territorial aggression is typically studied with variations of the resident-intruder procedure, a multi-day (typically three-day) behavioral assay during which an intruder mouse is placed in the home-cage of a resident, and the subsequent social interaction behaviors are observed. The severity of the resident's aggressive behavior during resident-intruder testing is characterized by attack features, including latency to the first attack, the number of attack bouts, bout duration, attack consistency, attack site, level of tissue damage, bite number, and responsiveness to intruder submission behaviors (Golden et al., 2016; Golden, Jin, & Shaham, 2019; Miczek, de Boer, & Haller, 2013). However, individual variation of these measurements

between and even within experimental cohorts of mice makes a consistent overall determination of aggression behavior difficult. Currently, no one indicator exists that accurately encapsulates mouse aggression behavior for use in behavioral studies, leading to difficulty in comparing aggressive behavior between labs and decreased replicability in experimental set-ups that utilize aggressive behavior as a component.

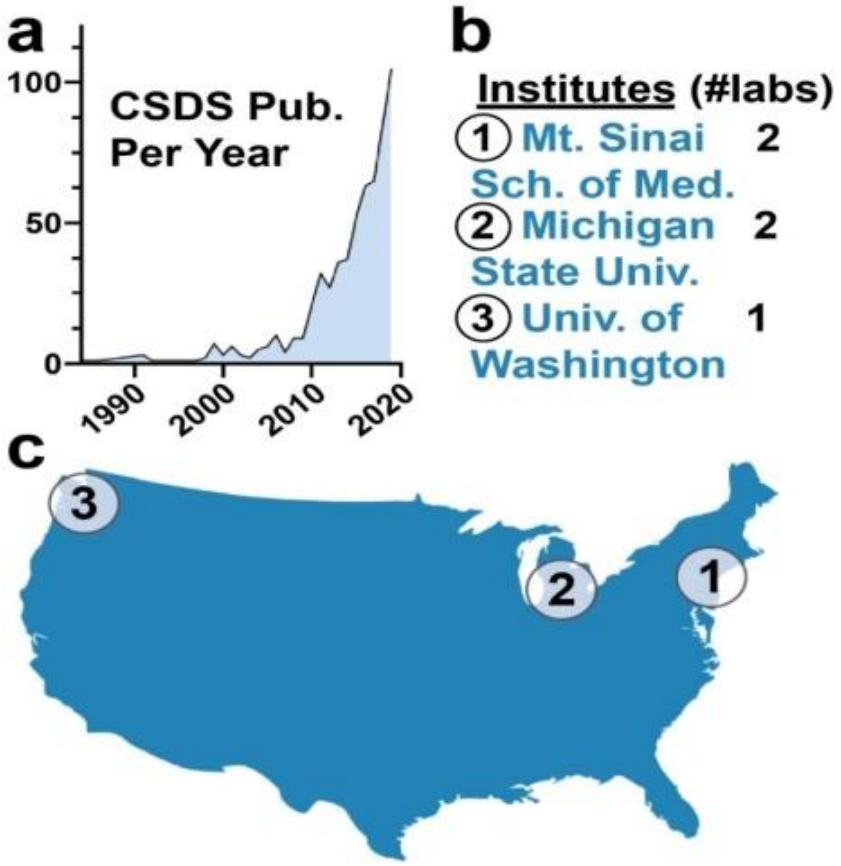

Figure 3 | Contributing Institutions. a) The number of studies using CSDS listed on NIH Pub Med each year since 1985. b) Data presented in this chapter are drawn from aggressor screenings at three different institutes located across the United States (c), performed over the course of ten years by five separate laboratories.

Mouse aggression is a key component of the chronic social defeat stress (CSDS) procedure (Golden et al., 2011; Krishnan et al., 2007; Kudryavtseva, Bakshtanovskaya, & Koryakina, 1991), a gold-standard model for the study of mood-related disorders in mice. Due to its etiological,

predictive, discriminative and face validity the CSDS procedure has grown enormously in popularity over the last decade (**Fig 3a**). In CSDS, inbred C57BL/6J male intruder mice are repeatedly subjected to bouts of social defeat by larger and more aggressive male outbred CD-1 resident mice, inducing enduring deficits in social interactions and other behavioral antecedents related to mood disorders like anhedonia and anxiety. However, the measurement of aggression exhibited by the resident CD-1 mouse is not standardized, introducing unnecessary variability into CSDS studies.

To evaluate aggression, the observed attack features may be used as dependent variables themselves, or to calculate a composite aggression score. A drawback to operationalizing aggression as any one of its observed indicators is that a single indicator may provide a limited view of behavior. This is especially true if the selected variable depends upon the escape behavior of the intruder mouse (e.g., attack duration), which can vary between intruders. Furthermore, unwanted variation due to measurement error contaminates any true score of aggression when only one indicator is used (Hamm & Hoffman, 2016). Alternatively, a composite aggression score can be generated by applying a rank or sum function to the observed indicators. This too is not optimal since, due to natural cohort-to-cohort variation, scores that depend on the group mean (e.g., z-score) will change when mice are added to analyses over time, or the same numerical score will reflect different behavior between mice in separate experimental cohorts. Furthermore, the validity of summed or aggregated indicators representing repeated measurements should also account for any expected change in behavior over the course of the behavioral assay (e.g., the "winner effect", wherein aggressive mice become more aggressive over time as they learn to rapidly and efficiently dominate intruders; Golden et al., 2017; Oyegbile & Marler, 2005), permitting appropriate weighting of the contribution of variables to the overall aggression score. To this end, we outline

a data-driven method to model aggression and systematically generate aggression scores that are comparable across experimental cohorts, at different repeated screenings, and between labs. This is a critical tool for social defeat experiments and direct studies of territorial reactive aggression, as these methods become more prominent and reproducibility more challenging.

Structural equation modeling (SEM) provides an ideal confirmatory framework for testing theoretical relations in data measured across multiple dimensions (e.g., latency to attack, bout number, and bout duration) of a latent factor (e.g., aggression). The assumption of these models is that shared variance among indicators is caused by a common relationship to the latent factor(s) under study, yielding a more stable representation of the latent construct, which in this case is aggression. Confirmatory factory analysis (CFA) is a particularly useful measurement tool for preclinical research because it utilizes foundational research, such as extensive behavioral research, to inform modeling by allowing researchers to specify the number and pattern of variable relationships (Jöreskog, 1969). Indeed, utilizing previous research to make a priori modeling decisions within this SEM framework is how CFA earns its "confirmatory" moniker.

Structural equation modeling, including CFA, compares a hypothesized covariance structure with an observed covariance matrix, and the results are judged based on multiple parameters reflecting the model's "goodness of fit". These indices demonstrate the extent to which the observed measures (i.e., attack characteristics) are intercorrelated because they are influenced by the same unobserved latent construct (i.e., aggression). Moreover, covariance is partitioned in the model such that common variance among indicators is leveraged to estimate latent factors while separately estimating the residual variance (e.g., measurement error) specific to individual indicators (Brown, 2015) that is unrelated to the latent factor. Thus, CFA in

aggression research can serve two purposes: 1) allow examination of theoretically-guided variable relationships that best represent the data; and 2) utilize these empirically validated relationships to systematically estimate reliable aggression scores for individual animals for direct comparison with scores from other cohorts or other laboratories.

The goal of the current study is to develop an empirically driven measurement model that explains variation in species-normative territorial, reactive aggression among male CD-1 retired breeder mice. To measure aggression, we selected latency to the first attack, attack bouts, and average attack duration because these observed indicators are the most common behavioral metrics used to quantify aggression without performing in-depth ethological analysis, making them suitable for high-throughput screening. We show that CFA generates an effective and consistent measurement model, the Mouse Aggression Detector (MAD), across four different laboratories in different institutes across the United States with CD-1 mice acquired from multiple vendors over a decade of experiments (**Fig 3b-c**). We then apply the model to additional, smaller cohorts of mice to further demonstrate the stability of aggression scores over repeated screening experiments and how aggressor selection predicts CSDS outcomes. Together, this approach facilitates statistically rigorous multivariate analyses of aggression to evaluate aggressor performance as well as consistent selection of aggressors for CSDS research. Use of MAD allows direct comparison of mouse aggression between labs and studies for the first time and will facilitate high-throughput screening for consistent and stable aggressors, improving the consistency and replicability of CSDS and related social stress studies.

Methods

Animals

All experiments involving male CD-1 and C57BL/6J mice were approved by the Institutional Animal Care and Use Committee at the University of Washington, Icahn School of Medicine at Mount Sinai, and Michigan State University and conducted in accordance with guidelines from the Association for the Assessment and Accreditation of Laboratory Animal Care and National Institute of Health. Mice were housed in a 12:12 hour light-dark cycle and provided ad libitum access to water and a standard laboratory diet.

Resident-Intruder Procedure

Aggression was evaluated using the resident-intruder procedure as previously described (Golden et al., 2011; Golden et al., 2016; Koolhaas et al., 2013; Olivier & Young, 2002). The procedure was repeated over three consecutive trial days and measures of latency to the first attack, the number of attack bouts, and attack duration were collected each day.

Chronic Social Defeat Stress

Chronic social defeat stress (CSDS) was conducted as previously described (Golden et al., 2011). Experimental C57BL/6J mice were subsequently evaluated for susceptibility to defeat in social interaction (SI), also as described (Eagle et al., 2020; Golden et al., 2011).

Aggregate Data

Five primary datasets were used in this study. The Robison and Mazei-Robison Labs at Michigan State University contributed aggression screening data from multiple cohorts for a combined total of 210 sexually experienced CD-1 male mice. Of these 210, there were some mice that underwent some form of experiment before or during the aggression screening and some that were experimentally naïve. Within this dataset, there are 131 experimentally naïve aggressors (Set

1) as well as 79 experimentally experienced mice that underwent some form of experimental manipulation prior to the initial aggression screening. The Russo Lab contributed data for 448 sexually experienced CD-1 male mice from aggression screenings conducted at the Icahn School of Medicine at Mount Sinai from other published work (Golden et al., 2016). All 448 animals were experimentally naive (Set 2) at the time of aggression screening. Together, the combined datasets include screening information for 579 experimentally naïve mice (Set 3) as well as a completely inclusive dataset of 658 experimentally naïve and experienced mice (Set 4) from the Robison, Mazei-Robison, and Russo Labs. Finally, the Golden Lab at the University of Washington contributed an independent dataset of 182 sexually experienced, experimentally naïve CD-1 male mice that underwent 10-minute aggression screenings using the resident-intruder procedure (Set 5) and observations were measured using the SimBA computer classification toolkit with an 'attack' predictive classifier generated as previously described (Nilsson et al., 2020). Set 5 remains independent of human scored animals in order to reduce scoring variation within datasets related to human versus computer assessment but tests the appropriateness of extending MAD's use to different experimental setups. Collectively, Sets 1-3, representing data solely for experimentally naïve animals, were used to develop measurement models of aggression to prevent variability in aggression related to an experimental manipulation (rather than natural variation in species-normative territorial aggression) from contaminating the model. Sets 4 and 5 were used to test applications for the MAD Model. Following these initial stages, additional cohorts of CD-1 mice were used to demonstrate the utility of the model.

In addition to needing a sufficiently large ($n > 200$), random sample as we have here, a key assumption of CFA is that the observed variables are normally distributed. This assumption is problematic in preclinical research where constraints of timed behavioral trials create censored

variables, which can affect their overall distribution. This issue had a pronounced effect on our latency variables, resulting in a bimodal distribution representing the difference between animals with any aggressive behavior and those that had none (and were assigned a latency of 180 or 600). Moreover, the variance of the raw latency variables was so large in comparison to the other variables that attempts to model them returned estimation errors. To account for these issues, we sought consensus between three experts in subdividing trial time into categories of initial attack times that characterize similarly aggressive animals. These groupings were used to reverse code the latency variables for days 1-3 into the categorical variables that were ultimately used in the CFA model. These three recoded variables along with the six variables for bouts and duration on trial days 1-3 were positively skewed, necessitating the use of the Maximum Likelihood-Robust (MLR) estimation with robust standard errors (Kline, 2015).

Data Analysis

We utilized the SEM framework to model attack behaviors (i.e., latency, bouts, duration) as manifest indicators representing theoretically related facets of an underlying aggression factor. To model aggression as a general trait, we developed two single-factor aggression models that included all nine indicator variables (i.e., latency, bouts, and duration on each of the three trial days) but differed in the groupings of the observed indicators. We established configural invariance for factor structure across the three trial days, demonstrating that we measured a qualitatively similar construct during each trial. We subsequently conducted a test of measurement invariance across trial days to test for potential changes in factor structure and the fidelity of construct measurement over time. However, we could not establish weak invariance across all trial days, the minimum threshold for invariant measurement and quantitative comparison of groups (Meredith, 1993; Putnick & Bornstein, 2016). In light of this, rather than specify separate but

correlated first-order factors for each day, we re-specified the model with all three trial day factors loading onto a common second-order factor.

Confirmatory factor analysis was conducted using the lavaan package (Rosseel, 2012) in RStudio version 3.6.2 (RStudio Team, 2020). Models were estimated using maximum likelihood estimation with robust standard errors. Confirmatory factor analysis subdivides variance such that common variance among indicators is leveraged to predict the value of observed variables as: $y = \Lambda_y \eta + \varepsilon$, where y is a *p*-variate vector of observed variables; Λ_y is a *p x m* matrix of factor loadings λ; η is an m-variate vector of factors, and ε is a *p*-variate vector of unique components (Brown, 2015). In turn, factor scores are generated with respect to model structure using a multivariate regression formula that utilizes factor loadings as regression coefficients (Thurstone, 1935).

To evaluate the CFA models, we examined several different indices reflecting goodness-of-fit between the hypothesized covariance structure and the observed data covariance matrix (Table 1). We explored fit indices, including the comparative fit index (CFI), Tucker Lewis index (TLI), root mean square error of approximation (RMSEA), and standardized root mean square residual (SRMR) (Bender & Raz, 2015). We evaluated our proposed measurement models using thresholds for good fit: RMSEA ≤ 0.06, CFI ≥ 0.95, TLI ≥ 0.95, and SRMR ≤ 0.08 (Hu & Bentler, 1999). To compare models, we performed Satorra-Bentler scaled chi-square difference tests (Satorra & Bentler, 2010). To evaluate the CFA models, we examined several different indices reflecting goodness-of-fit between the hypothesized covariance structure and the observed data covariance matrix. These indices also serve to assess the appropriateness of *a priori* modeling decisions. We first examined the X^2 index, a test of absolute fit between the true and predicted variance-covariance matrices, but given the stringency of this test, others have suggested it is an unrealistic metric in applied research (Brown, 2015). Therefore, we explored several alternative

fit indices, including the comparative fit index (CFI) and the Tucker Lewis index (TLI), both which compare model fit to the fit of the null model. We also examined the root mean square error of approximation (RMSEA), a measure of model misspecification, and the standardized root mean square residual (SRMR), a measure of explained variance (Bender & Raz, 2015). We evaluated our proposed measurement models using thresholds for good fit: RMSEA ≤ 0.06, CFI ≥ 0.95, TLI ≥ 0.95, and SRMR ≤ 0.08 (Hu & Bentler, 1999). To compare models, we performed Satorra-Bentler scaled chi-square difference tests (Satorra & Bentler, 2010). Once the final model was established (using control aggressor data from Set 3), we calculated aggression scores for smaller cohorts of mice using RStudio which inputs model parameters in a multivariate equation with novel data. To use the model in this way, materials are publicly available for download on GitHub (https://github.com/RobisonLab/MAD).

PRISM software 8.0 was utilized to compare aggression scores via Pearson correlation tests and analyze our observed indicators (i.e., latency, bouts, and duration) using repeated measures analysis of variance (ANOVA) tests with Tukey's multiple comparison tests for post-hoc analysis and their non-parametric equivalents. Though ANOVA is considered a robust test with minimal impact on the probability of a type I error (Harwell, Rubinstein, Hayes, & Olds, 1992; Lix, Keselman, & Keselman, 1996), skewed or kurtotic variable distributions affect the chances of false discovery. We therefore confirm our findings with Friedman tests given that our observed measures are not normally distributed, and we found that the results did not differ from those obtained with ANOVA.

Results

Attack behavior varies across trial days during the resident-intruder procedure

To determine potential changes in behavior during resident-intruder screenings (**Fig 4**), we independently examined changes in latency, bouts, and duration across trial days using experimentally naïve aggressor data generated at Michigan State University in 2018-2020 (Set 1; n = 131) and the Mount Sinai School of Medicine in 2010-2014 (Set 2; n = 448), as well as in a combined, experimentally naïve aggressor dataset (Set 3; n = 579). In accordance with previous research (Golden et al., 2017; Golden et al., 2016; Koolhaas et al., 2013), we show with repeated measures ANOVA that experimentally naïve, sexually experienced CD-1 male mice (Set 3) exhibit significant increases in aggression-related behavior across trial days (**Fig 5a-c**): reduced latency to the first attack and increased bout number and bout duration. We established a significant main

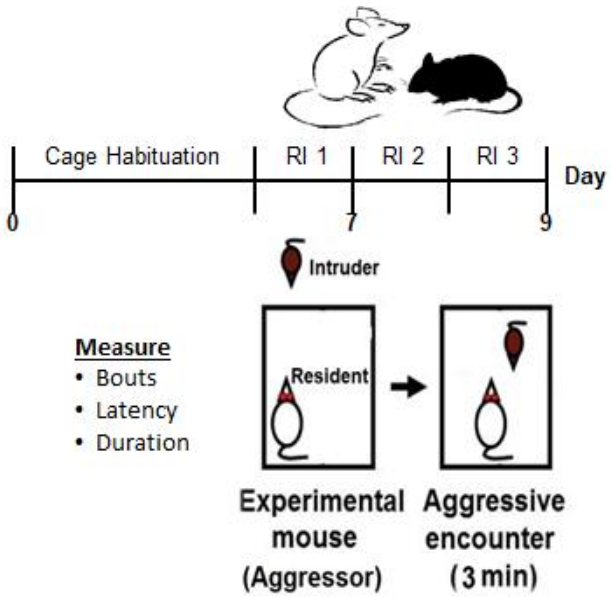

Figure 4 | The Resident-Intruder Procedure. The resident-intruder procedure is a 3-day behavioral assay that evaluates aggression during a timed social interaction. After habituation of retired breeder male CD-1 aggressors to the home cage, a different male C57BL/6J intruder is introduced for three minutes per day on each of three days. Attack features including bouts, latency, and duration are recorded for each resident mouse.

effect of trial day for bouts ($F(1.999, 1156) = 120.9$, $p < 0.0001$), latency ($F(1.940, 1121) = 248.5$, $p < 0.0001$), and duration ($F(1.958, 1132) = 36.12$, $p < 0.0001$). The number of bouts significantly increased only on day 2 versus day 1 ($\Delta M = 1.772$, $SE = 0.1398$, $p < 0.0001$) and day 3 versus day 1 ($\Delta M = 2.005$, $SE = 0.1418$, $p < 0.0001$). Latency significantly decreased across all trial days (day 2 v day 1 $\Delta M = 46.77$, $SE = 2.772$, $p < 0.0001$; day 3 v day 1 $\Delta M = 56.64$, $SE = 2.881$, $p < 0.0001$; days 2 v 3 $\Delta M = 9.874$, $SE = 2.474$, $p = 0.0002$). Duration significantly increased only on days 2 and 3 compared to day 1 (day 2 v day 1 $\Delta M = 1.210$, $SE = 0.1783$, $p < 0.0001$; day 3 v day 1 $\Delta M = 1.525$, $SE = 0.2020$, $p < 0.0001$). Taken together, these data suggest that behavior changes over trial days, underscoring the importance of developing an empirical model of aggression.

Next, we developed two single-factor CFA models to determine how to best calculate a composite measure of aggression. We compared a first-order model with freely estimated factor loadings (i.e., regression weights) for all nine indicators (**Fig 4a**) and a second-order model where indicators were grouped by trial day (**Fig 4b**). In the latter model, the estimated residual variance

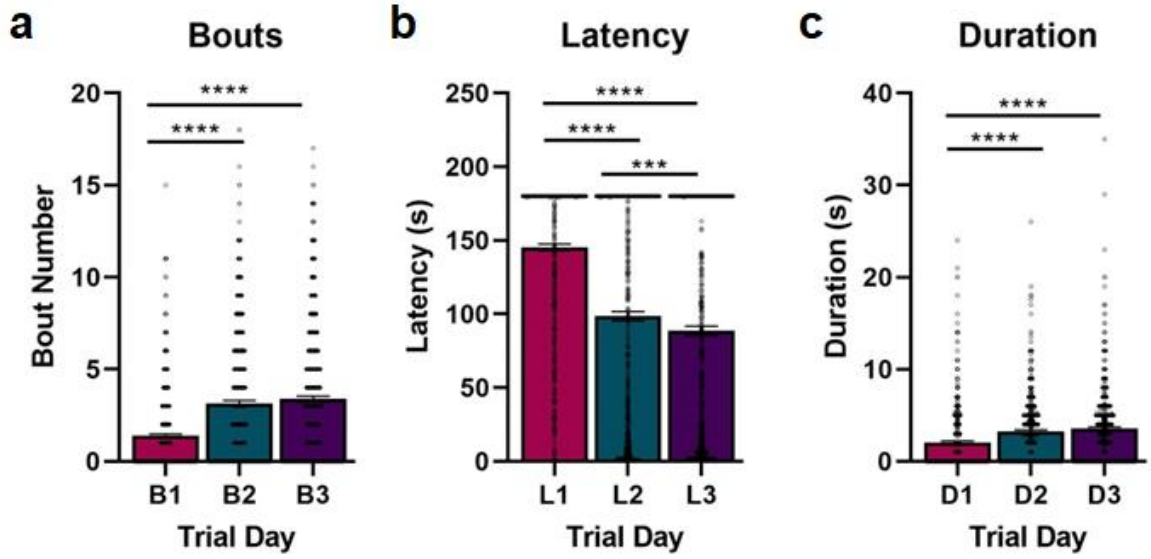

Figure 5 | Aggression Indicators. Among experimentally naïve aggressors (Set 3; n = 579), there was main effect of trial day for bouts ($p < 0.0001$), latency ($p < 0.0001$), and duration ($p < 0.0001$). e) The number of bouts increased on day 2 versus day 1 ($p < 0.0001$) and day 3 versus day 1 ($p < 0.0001$).

for latency on day 3 was negative, suggesting that all variability in this indicator was explained by the model. Therefore, residual variance for latency on day 3 was fixed to zero to improve estimation. For both models, the loadings for all indicators on their hypothesized factors were significant, suggesting that a unifying latent construct (i.e., aggression) is driving variation in all the observed variables.

As shown in **Table 1**, model fit was poor for Model 1 for Set 1 (RMSEA = 0.168; CFI = 0.721; TLI = 0.628; SRMR = 0.092), Set 2 (RMSEA = 0.161; CFI = 0.713; TLI = 0.617; SRMR = 0.078), and the combined Set 3 (RMSEA = 0.167; CFI = 0.712; TLI = 0.616; SRMR = 0.079). All reported fit indices improved dramatically in Model 2A versus Model 1. Specifically, model fit for Model 2A was good for Set 1 (RMSEA = 0.058; CFI = 0.970; TLI = 0.957; SRMR = 0.050), acceptable for Set 2 (RMSEA = 0.072; CFI = 0.947; TLI = 0.924; SRMR = 0.046), and acceptable for the combined dataset, Set 3 (RMSEA = 0.076; CFI = 0.945; TLI = 0.921; SRMR = 0.040). These data show Model 2A, in which the observable variables are grouped by trial day, has the most appropriate structure for aggression measurement. Taken together, this suggests that there is a credible latent structure in measuring aggression with the resident-intruder procedure and aggression is best represented as a composite of measures from all three trial days.

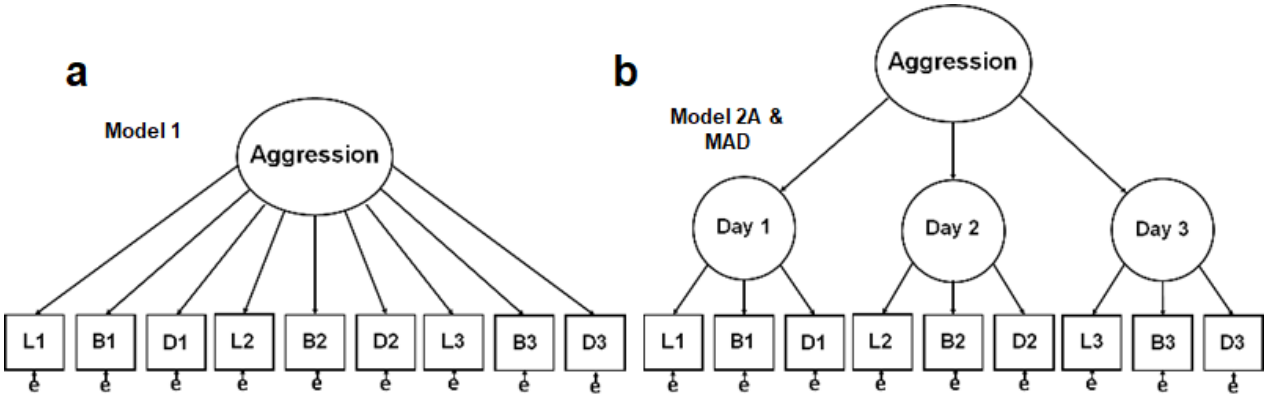

Figure 6 | Tested CFA Models. Bout, latency, and duration measurements generate 9 observed variables that can be structured in an (a) first-order or (b) second-order measurement model to calculate an aggression score.

To yield our final Mouse Aggression Detector (MAD) model, we further refined Model 2A based on model modification indices. This reveals additional sources of shared variance between the variables in the model that may improve model fit if consistent with theoretical considerations. For example, mice that have a shorter attack latency on the first trial day tend to also have a shorter attack latency on the second and third day. Acknowledging that behavior is correlated between trial days, we inspected the modification indices for relationships between trial days and mathematically accounted for those relationships in the model without changing its overall structure (**Fig 6b**). With this model, residual variance estimates for latency on days 2 and 3 were negative, suggesting not only that the model explains differences in these variables but that there was no residual covariance between them. Residual variance for latency on days 2 and 3 as well as their residual covariance were therefore fixed to zero. Model fit for MAD was excellent for Set 1 (RMSEA = 0; CFI = 1.000; TLI = 1.006; SRMR = 0.034), Set 2 (RMSEA = 0.045; CFI = 0.985; TLI = 0.971; SRMR = 0.035), and Set 3 (RMSEA = 0.042; CFI = 0.988; TLI = 0.976; SRMR = 0.028). We subsequently conducted Satorra-Bentler scaled chi-square difference tests to compare MAD to Model 1 across all three datasets (Satorra & Bentler, 2010). Results indicate that fit is significantly better for MAD for each comparison. In sum, we generated a model demonstrating excellent fit and significant factor loadings for three datasets. This demonstrates that our decision to include pre-selected variables for measurement (confirmatory factor analysis does allow for the *a priori* specification of variable relationship) are validated by the goodness-of-fit indices, highlighting the suitability of the MAD Model for measuring aggression behavior in the resident-intruder paradigm.

		X^2	*Df*	RMSEA (90% CI)	CFI	TLI	SRMR
Set 1							
	Model 1	127.342*	27	0.168 (0.142,0.196)	0.721	0.628	0.092
	Model 2A	35.862	25	0.058 (0.000,0.097)	0.970	0.957	0.050
	MAD	16.886	18	0 (0.000,0.074)	1.000	1.006	0.034
Set 2							
	Model 1	339.886*	27	0.161 (0.149,0.173)	0.713	0.617	0.078
	Model 2A	82.509*	25	0.072 (0.057,0.087)	0.947	0.924	0.046
	MAD	34.000*	18	0.045 (0.022,0.066)	0.985	0.971	0.035
Set 3							
	Model 1	463.704*	27	0.167 (0.156,0.178)	0.712	0.616	0.079
	Model 2A	107.877*	25	0.076 (0.062,0.089)	0.945	0.921	0.040
	MAD	36.026*	18	0.042 (0.021,0.061)	0.988	0.976	0.028

Table 1 | CFA Goodness-of Fit Indices show the statistics from multiple different indices that evaluate the goodness-of-fit of our measurement models for resident intruder data in Sets 1-3. We include the X^2 index, CFI, TLI, RMSEA, and SRMR. *Good fit: RMSEA ≤ 0.06, CFI ≥ 0.95, TLI ≥ 0.95, and SRMR ≤ 0.08*

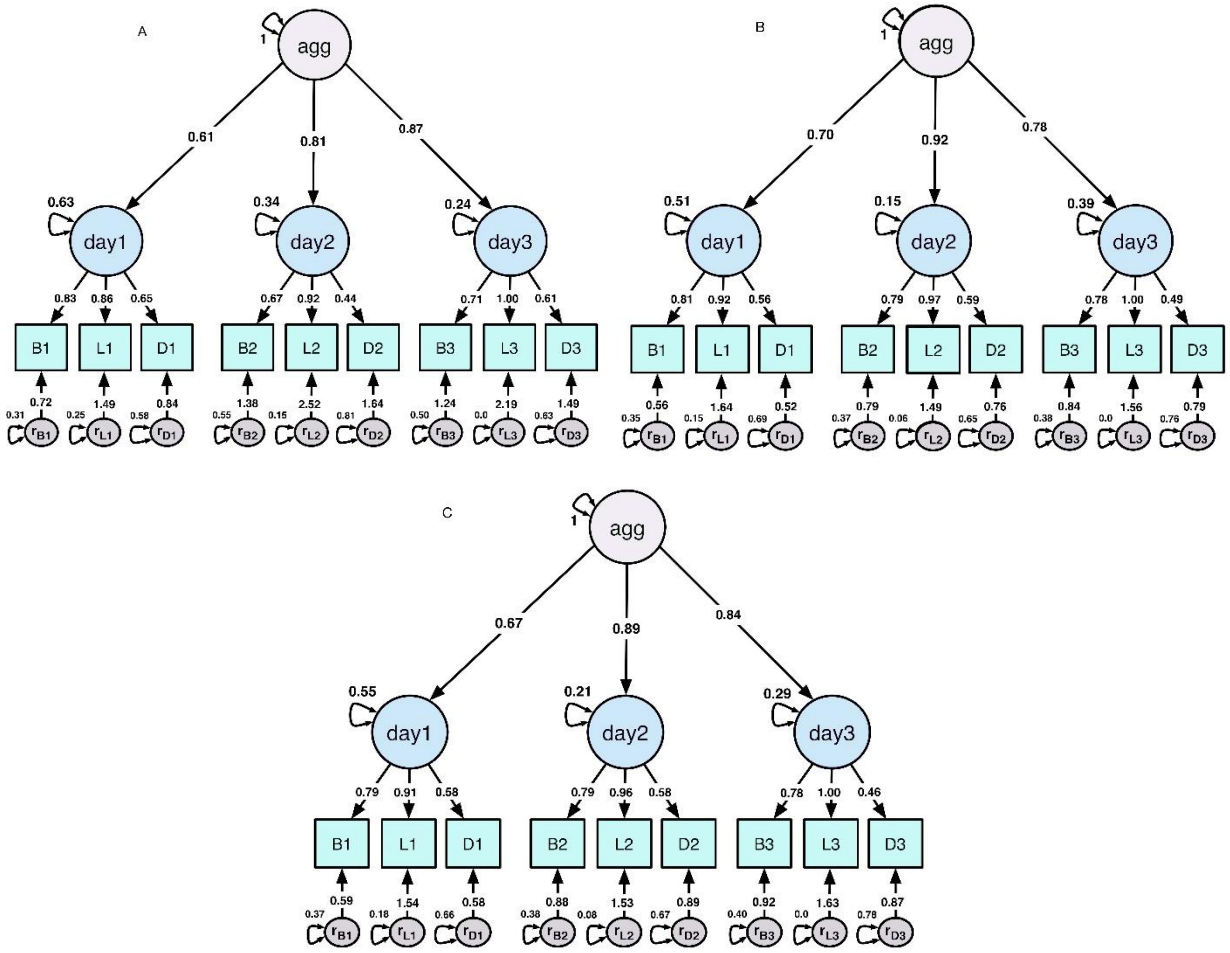

Figure 7 | Model Development. The final model (MAD) is a second-order measurement model wherein the observed variables are grouped by trial day before loading onto the higher-order factor, aggression. a-c) In each path diagram, circles represent latent (unobservable) factors, including an overall aggression score as well as a behavior score on days 1-3, while squares represent the observed indicators, bouts, latency, and duration, on days 1-3, and small circles with double-headed arrows represent indicator residuals and residual variance. Arrows containing factors loadings, or regression weights, are interpreted as regression coefficients, denoting the change in the indicated variable, latent or observed, for every one-unit change in the higher-order factor the arrow descends from. These values along with other model estimates are used in a multivariate formula to calculate aggression scores. MAD was developed 3 times using experimentally naïve aggressor data, thereby generating model estimates unique to (a) Set 1 (n = 131), (b) Set 2 (n = 448), and (c) Set 3 (n = 579).

Consistencies in model structure produce similar aggression scores when applied to novel data

Although the factor structure is the same, factor loadings differ between datasets (**Fig 7a-c**), thereby changing the regression coefficients used in the multivariate regression formula used to estimate aggression scores. In particular, the model estimates the same patterns of factor loadings in their contribution to generating an aggression score, where latency > bouts > duration. The model diverges, however, in the estimated factor loadings for trial days. For Set 1, factor loadings for day 3 > day 2 > day 1 in their contribution to the overall aggression score, while the factor loadings for Sets 2 and 3 followed the pattern of day 2 > day 3 > day 1 in their contribution to the aggression score. However, the 95% confidence intervals for these estimates were overlapping. To ensure that the model would be generalizable to novel datasets, we investigated the extent to which these differences change aggression scoring and if the scores remain comparable. Using the different model parameters calculated from Sets 1-3, we applied three iterations of MAD to Set 4, our combined dataset of 658 animals with and without (i.e., Set 3) experimental histories, yielding three different aggression scores for each animal to be used in subsequent analyses.

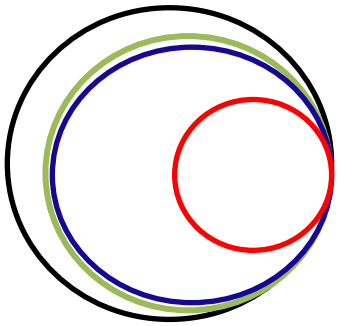

Figure 8 | Aggregate Data. Schematic representing model development datasets:
Set 1: Control Aggressors (n = 131)
Set 2: Control Aggressors (n = 448)
Set 3: Control Aggressors Combined (n = 579)
Set 4: All Aggressors Combined (n = 658)

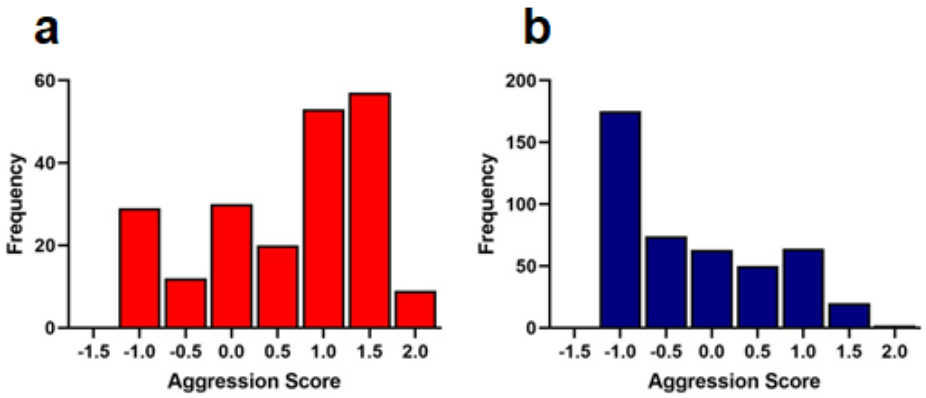

Figure 9 | MAD Aggression Score Distributions. Schematic showing the distributions of aggression scores calculated with Set 3 model estimates are depicted in separate histograms for (a) Set 1 and (b) Set 2 datasets.

To this end, we used lavaan's *predict* function to separately apply each of the three patterns of estimated factors loadings (i.e., for Set 1, Set 2, and Set 3) to the entire, aggregated sample (Set 4, n = 658) in order to generate three aggression scores for each animal (**Fig 8**). Aggression scores range from negative to positive with negative values denoting low aggression while positive values reflect high aggression (**Fig 9a-b**), and the level of aggression must be interpreted with respect to the range. Unsurprisingly, summary statistics for Set 4 represent moderately aggressive animals, or the average CD-1 behavior, but the numerical values differ between calculations made using parameters generated from MAD's application to Set 1 (M = -0.59, SD = 0.95, Mdn = -0.71, MIN = -1.73, MAX = 1.25), Set 2 (M = 0.31, SD = 1.07, Mdn = 0.16, MIN = -0.91, MAX = 2.52), and Set 3 (M = 0.06, SD = 0.94, Mdn = -0.05, MIN = -1.05, MAX = 1.94).

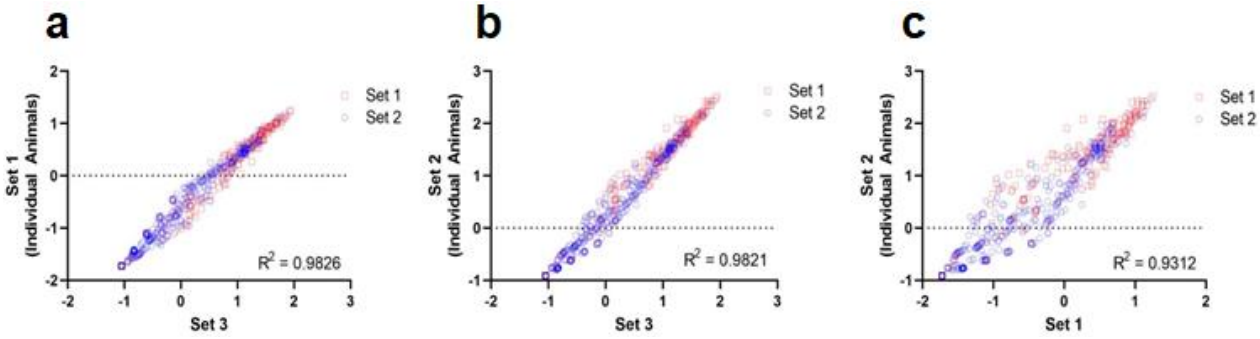

Figure 10 | MAD Model Iterations. Correlation analyses showed positive relationships between scores calculated using (a) Set 1 and Set 3 parameters ($p < 0.0001$); (b) Set 2 and Set 3 parameters ($p < 0.0001$); and (c) Set 1 and Set 2 parameters ($p < 0.0001$).

To assess the viability of applying MAD to different datasets, we fitted MAD to Set 4, (n = 658 aggressors from all conditions), and conducted a series of Pearson correlation tests between the three aggression scores generated for each animal. We found a significant, positive relationship between aggression scores calculated using Set 1 and Set 3 (**Fig 10a**; r = 0.9913, R^2 = 0.9826, p < 0.0001); using Set 2 and Set 3 (**Fig 10b**; r = 0.9910, R^2 = 0.9821, p < 0.0001); and using Sets 1 and 2, representing resident-intruder aggression screenings at two different institutions (**Fig 10c**; r = 0.9650, R^2 = 0.9312, p < 0.0001). Therefore, despite these differences in absolute values, MAD aggression scores consistently represent the spectrum of aggressive behavior. Taken together,

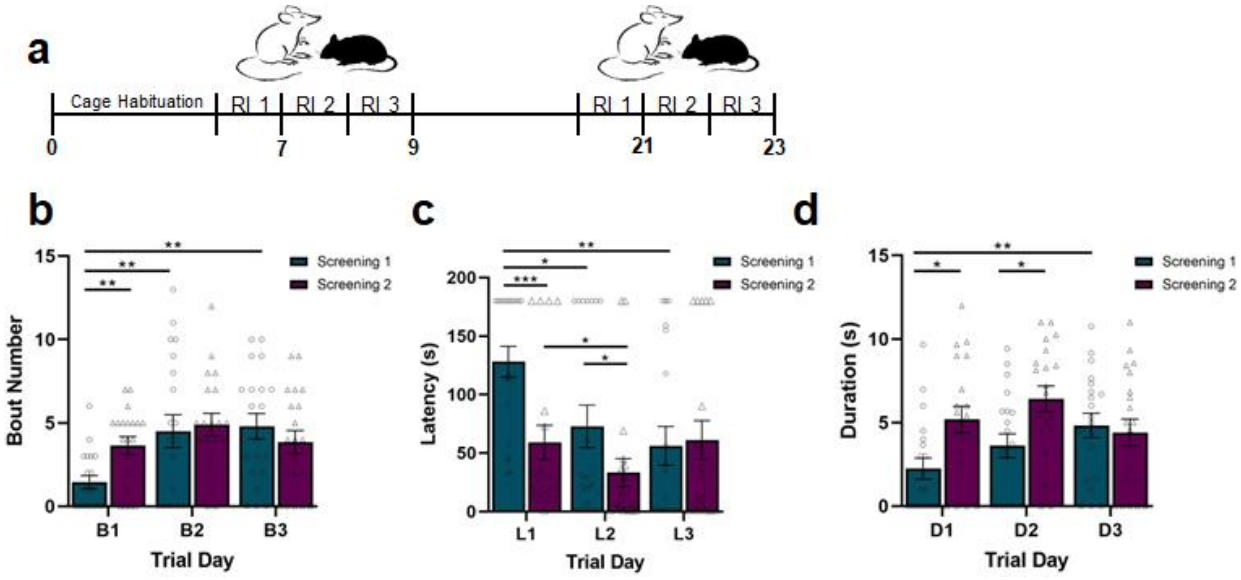

Figure 11 | MAD Aggression Scores Over Time. a) Schematic showing experimental timeline for a novel cohort (n = 20) measured for aggression via the resident-intruder procedure at 2 different time points. b) Across screenings, there was a main effect of trial day (p < 0.001) as well as an interaction effect between trial day and screening (p < 0.05). At T1, there was an increase in bouts on days 2 versus day 1 (p < 0.01) and 3 versus day 1 (p < 0.01). At T2, there were no differences in bout number between trial days. Between T1 and T2, we found an increase in the number of bouts on day 1 (p < 0.01), but not on days 2 or 3. c) For latency, there were main effects of both trial day (p < 0.01) and screening (p < 0.01) as well as an interaction effect between trial day and screening (p < 0.01). At T1, latency decreased versus day 1 on days 2 (p < 0.05) and 3 (p < 0.01) whereas only latency between days 1 and 2 at T2 (p < 0.05) decreased. Latency on days 1 (p < 0.001) and 2 (p < 0.05) decreased between T1 and T2.

these findings strongly suggest consistent factor-variable relationships and measurement model structure that accurately and consistently quantifies aggressive behavior.

Aggression scores are stable over time

To determine if aggression scoring is consistent over time, we repeated resident-intruder screening at two different time points in a novel cohort of n = 20 CD-1 mice, beginning 7 (T1) and 21 days (T2) after animal arrival (**Fig 11a**). There was a significant main effect of trial day ($F(1.862, 35.39) = 10.79$, $p = 0.0003$) as well as an interaction effect between trial day and screening time point ($F(1.957, 37.19) = 5.065$, $p = 0.0118$). At T1, there was an increase in bouts (**Fig 11b**) on days 2 and 3 compared to day 1 (day 2 v day 1 $\Delta M = 3.050$, $SE = 0.8223$, $p = 0.0045$; day 3 v day 1 $\Delta M = 3.350$, $SE = 0.7755$, $p = 0.0011$). At T2, there were no differences in bout number between trial days. Between T1 and T2, we found an increase in the number of bouts on day 1 ($\Delta M = 2.200$, $SE = 0.5161$, $p = 0.0013$), but not on days 2 or 3. For latency (**Fig 11c**), there were main effects of trial day ($F(1.693, 32.16) = 8.901$, $p = 0.0014$) and screening time ($F(1.00, 19.00) = 9.169$, $p = 0.0069$), as well as an interaction effect between trial day and screening time ($F(1.975, 37.52) = 7.497$, $p = 0.0019$). At T1, latency decreased on days 2 and 3 compared to day 1 (day 2 v day 1 $\Delta M = 55.20$, $SE = 17.76$, $p = 0.0173$; day 3 v day 1 $\Delta M = 71.85$, $SE = 16.70$, $p = 0.0012$), whereas only latency between days 1 and 2 at T2 decreased ($\Delta M = 26.00$, $SE = 9.586$, $p = 0.0409$). Latency on days 1 ($\Delta M = 68.80$, $SE = 15.22$, $p = 0.0007$) and 2 ($\Delta M = 39.60$, $SE = 14.25$, $p = 0.0355$) decreased between T1 and T2. At T1, we found an increase in duration (**Fig 11d**) between days 1 and 3 ($\Delta M = 2.586$, $SE = 0.7380$, $p = 0.0071$), but there were no other differences at T1 or T2. Between T1 and T2, there was an increase in duration on days 1 ($\Delta M = 2.941$, $SE = 0.9765$, $p = 0.0213$) and 2 ($\Delta M = 2.808$, $SE = 1.040$, $p = 0.0418$).

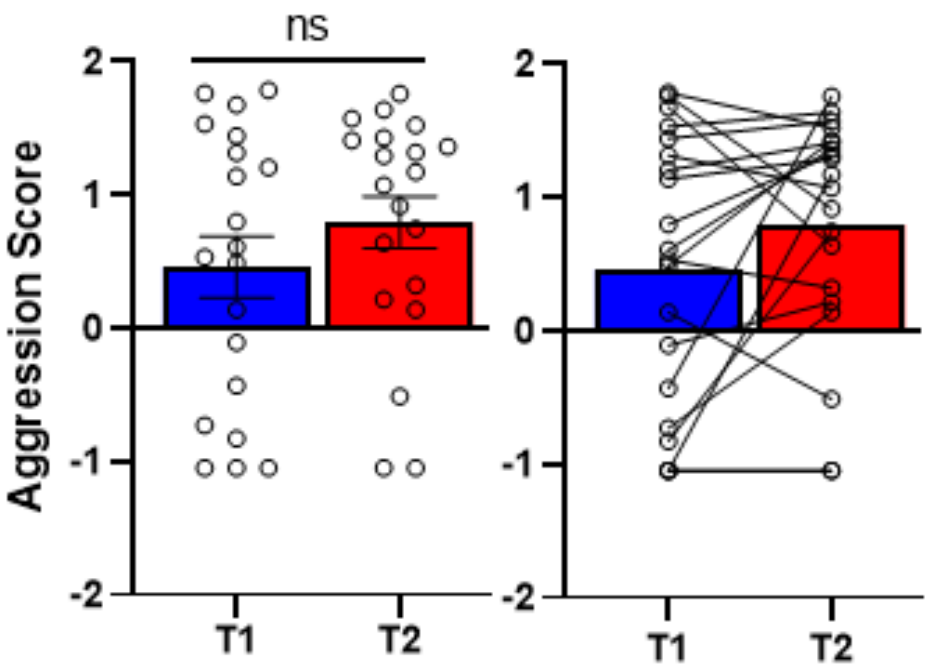

Figure 12 | MAD Aggression Score Stability. A paired t-test of aggression scores at Time 1 and Time 2 demonstrates no differences in aggression ($p > 0.05$). Among the top-most aggressive animals, however, the majority maintained aggression during the two screenings.

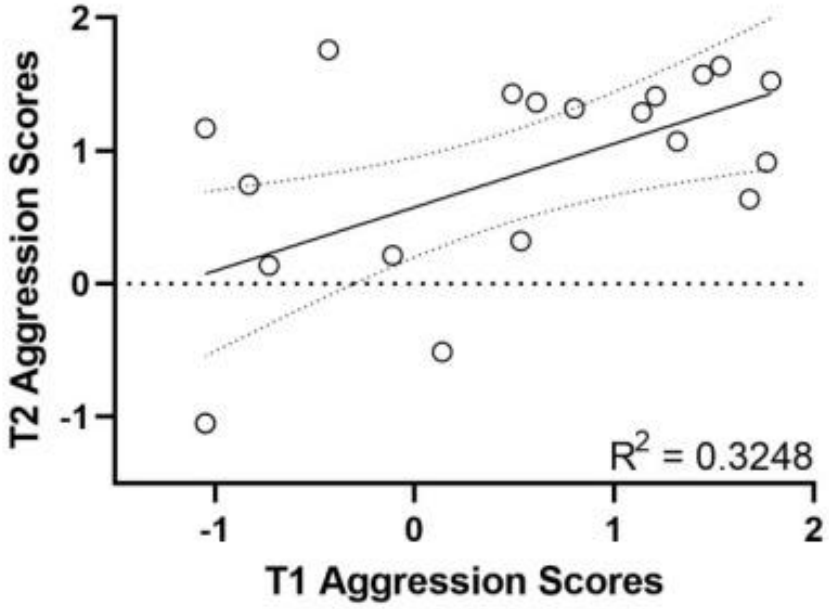

Figure 13 | MAD Correlation. Aggression scores are correlated for individual animals between T1 and T2 ($p < 0.01$). A simple regression analysis shows a relationship between aggression scores at T1 and T2 ($p < 0.01$).

Aggression scores were subsequently calculated by applying MAD to this novel dataset of n = 20 aggressors using parameters calculated with Set 3, our most inclusive dataset of experimentally naïve aggressors. We found that, though there were differences in the observed variables (**Fig 11b-d**), there was no difference in overall aggression over time (**Fig. 12**; $t(19) = 1.693$, $p = 0.1069$). However, we found that aggression scores at days 7 and 21 correlated (**Fig 13**; $r = 0.5699$, $p = 0.0044$) with aggression scores at T1 accounting for approximately 32% of variation in aggression scores at T2 ($R^2 = 0.3248$, $m = 0.4787$, $p = 0.0087$). Importantly, we showed that the majority of the most aggressive animals maintain their aggressive behavior between screenings

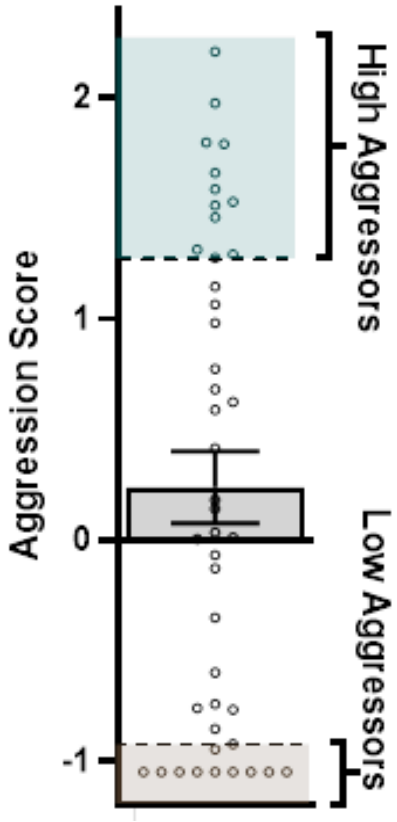

Figure 14 | MAD Model for CSDS Aggressor Selection. A novel cohort of aggressors (n = 42) was screened and those with the top 10 MAD scores (High Aggressors) and bottom 10 scores (Low Aggressors) were selected for subsequent CSDS.

(**Fig 12**), suggesting that aggressive animals behave consistently during repeated resident-intruder interactions, a typical circumstance for mice used as aggressors in multiple social defeat experiments. Taken together, these results indicate that our model provides a stable aggression score over time, making it well suited for initially selecting aggressors that will remain aggressive over multiple CSDS experiments.

MAD score predicts CSDS outcome

We next sought to determine whether a stratified aggressor exposure predicts susceptibility to CSDS-induced social interaction deficits. We screened 42 naïve CD1 aggressors and conducted CSDS using aggressors with high (n = 10) and low (n=10) aggression scores determined from the MAD model (**Fig 14**). Experimental juvenile C57BL/6J male mice were exposed to these two separate groups (n=19 High-expose, n=20 Low-exposed). Experimental mice were subsequently evaluated for susceptibility to social avoidance in the social interaction (SI) task. C57 mice exposed to high aggressors showed reduced interaction ratio (**Fig 15a**; t(37)=2.038, p=0.048) and increased corner time (**Fig 15b**; t(37)=2.539, p=0.014) compared to mice exposed to low

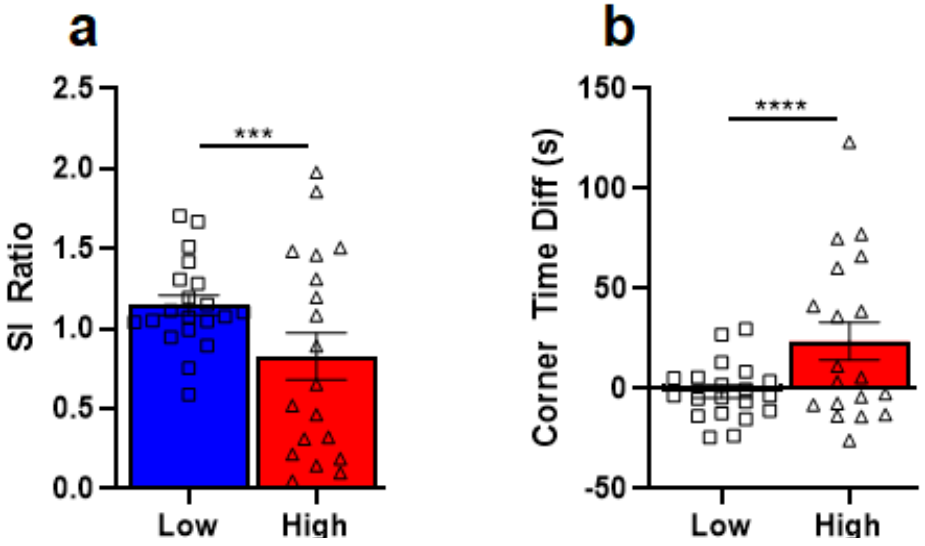

Figure 15 | CSDS Social Interaction. Adult male C57 mice exposed to CSDS with High versus Low Aggressors showed (a) reduced social interaction and (b) increased time in the corners.

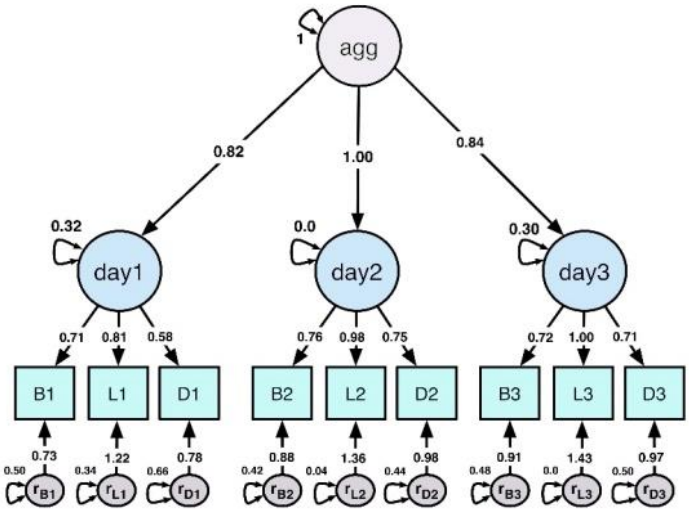

Figure 16 | MAD Model for SimBa Data. Schematic showing the path diagram for MAD model estimates generated using SimBA resident-intruder data from Set 5 (n = 182).

aggressors. Moreover, F test for inequality of variance showed that mice exposed to high aggressors had increased variance in SI (**Fig 15a**; F(18,19)=5.216, p=0.0008) compared to mice exposed to low aggressors, indicating a separation of susceptible from resilient animals.

MAD generated aggression scores generalize to automated aggression classification

To determine the extent to which the MAD model can be applied to resident-intruder data acquired from supervised machine-learning classification of encounter videos, we used MAD to generate aggression scores for 182 experimentally naïve, sexually experienced CD-1 male mice (Set 5) that were evaluated during 10-minute aggression trials using an attack classifier generated by SimBA, a supervised machine learning tool for social behavior classification (Nilsson et al., 2020). We observed the same consistent pattern of factor loadings for model estimates as seen in the primary analysis (**Fig 16**): latency > bouts > duration in their contribution to aggression score. Unlike the findings from the 3-minute screening data, there were significant differences between model estimates for all three variables on all three days. Moreover, model estimates for trial day factor loadings showed a pattern of day 2 > day 3 > day 1 in their contribution to the aggression

score. Model estimates for day 2 were different from days 1 and 3, but there was no difference between days 1 and 3. Together, these preliminary findings suggest that the behavioral characteristics defining aggression become more clearly distinguished from each other during a 10-minute trial compared to a 3-minute trial. However, 3-minute and 10-minute screenings both allow efficient definition of overall aggression in each mouse.

As shown in **Table 2**, however, metrics indicating MAD's goodness-of-fit for Set 5 decreased compared to the primary analyses, falling into the acceptable rather than good to excellent range. Though fit may be acceptable (RMSEA = 0.088; CFI = 0.963; TLI = 0.926; SRMR = 0.045), this finding suggests that there is additional variation in this dataset that is unaccounted

		X^2	*Df*	RMSEA (90% CI)	CFI	TLI	SRMR
MAD							
	Set 5	43.418*	18	0.088 (0.059,0.117)	0.963	0.926	0.045

Table 2 | CFA Goodness-of-Fit Indices for SimBa show statistics from multiple different indices, including the X^2 index, CFI, TLI, RMSEA, and SRMR, that evaluate the goodness-of-fit of our measurement models for resident intruder data in Set 5.

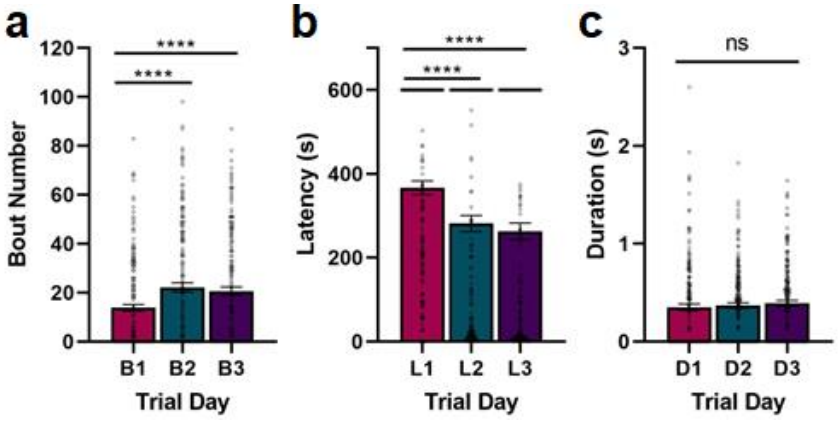

Figure 17 | SimBa Aggression Indicators. a-c) We found a main effect of trial day for bouts ($p < 0.0001$) and latency ($p < 0.0001$) but not duration ($p > 0.05$). a) Bouts increased day 2 versus day 1 ($p < 0.0001$) and day 3 versus day 1 ($p < 0.0001$). b) Latency decreased on day 2 versus day 1 ($p < 0.0001$) and day 3 versus day 1 ($p < 0.0001$). c) There were no differences in average bout duration across trial days.

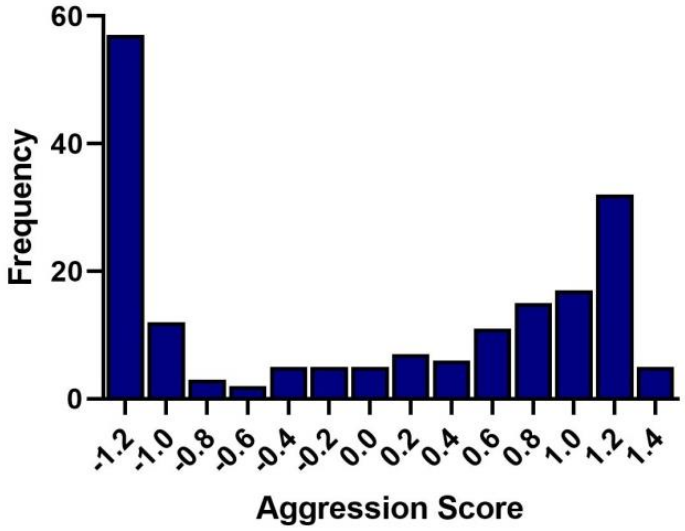

Figure 18 | MAD Aggression Score Distribution for SimBa Data. Histogram showing the distribution of aggression scores for Set 5 (n = 182).

for in the MAD model. Indeed, examination of the observed indicators revealed a slightly different pattern of aggression behavior across trial days. Using repeated measures ANOVA, we show that Set 5 mice demonstrate increases in only some aggression-related behaviors over time (**Fig 17a-c**). Specifically, we established a main effect of trial day for bouts ($F(1.930, 349.4) = 17.37$, $p < 0.0001$) and latency ($F(1.831, 331.4) = 28.97$, $p < 0.0001$) but not duration ($F(1.840, 333.1) = 0.8337$, $p = 0.4268$). Again, the number of bouts increased only on day 2 versus day 1 ($\Delta M = 8.313$, $SE = 1.607$, $p < 0.0001$) and day 3 versus day 1 ($\Delta M = 6.758$, $SE = 1.521$, $p < 0.0001$). Likewise, latency decreased only on days 2 and 3 compared to day 1 (day 2 v day 1 $\Delta M = 85.29$, $SE = 14.61$, $p < 0.0001$; day 3 v day 1 $\Delta M = 103.7$, $SE = 16.31$, $p < 0.0001$). Overall average attack duration among mice measured with SimBA is 0.37 seconds (versus 2.96 seconds among animals scored by human experimenters in Set 3) and the number of attack bouts is higher in Set 5, certainly because screening was over three times longer, but also likely a result of SimBA parsing attacks into many shorter, independent attack bouts. Taken together, this suggests that the attack classifier

used for this analysis measures aggressive behavior more granularly than human annotation, likely a function of unbiased annotation of every frame across all experimental videos. Importantly, these data show that MAD provides a useful mechanism for calculating aggression scores for high-throughput screening using automated supervised classification, as well as manual classification (**Fig 18**).

Three trial days allow for sufficient data collection for aggression scoring

To evaluate the number of trial days necessary for appropriately assessing aggression, we conducted 10 screening trials over 10 consecutive days. Results showed a significant main effect of trial day for latency (**Fig 19a**; $F(9) = 16.46$, $p < 0.0001$), bouts (**Fig 19b**; $F(9) = 7.711$, $p < 0.0001$), and duration (**Fig 19c**; $F(9) = 4.216$, $p < 0.001$). We found some differences in measured behaviors across individual days (**Tables 3-5**), but overall, we show that extended screenings do not provide substantively more information than three-day resident-intruder procedure. Moreover, we examined measurement invariance (a test of construct measurement fidelity) across trial days during model development. Overall, we could not establish metric invariance across all three trial days, precluding use of a three-factor model that grouped the observed indicators by time. We

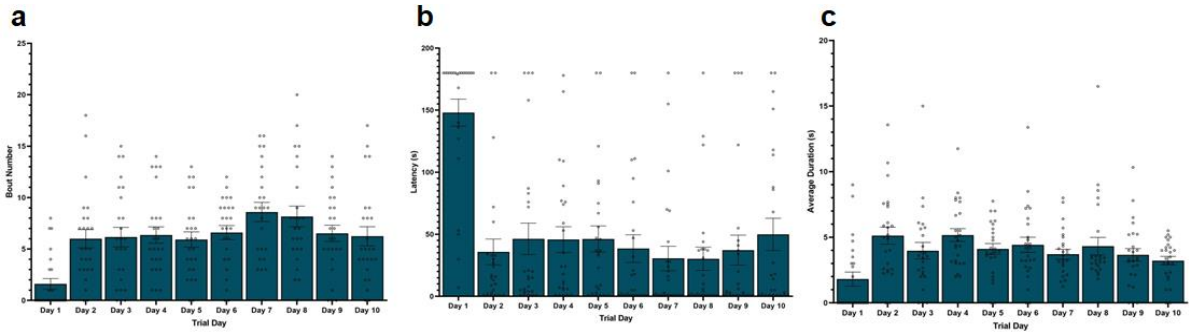

Figure 19 | 10-Day Aggression Indicators. a-c) The raw screening data for experimentally naïve, sexually experienced CD-1 male mice (n = 25) during a 10-day resident-intruder screening. a) Bout number for days 1-10. Day 1 is different than all other trial days ($p < 0.0001$). Bout number on day 5 is also different from that of day 7 ($p < 0.05$). b) Latency for days 1-10. Day 1 is different from all other trial days ($p < 0.0001$). There are no other differences between trial days. c) Duration for days 1-10. Day 1 is different ($p < 0.001$) from days 2-6, but not 7-10. Duration on day 4 is also different from duration on day 10 ($p < 0.05$).

further probed levels of measurement invariance between trial days. We could not establish measurement invariance between days 1 and 2, suggesting that the same construct is not being measured between these days. However, we established metric invariance between days 2 and 3, indicating that aggression behavior begins to stabilize on days 2 and 3. As such, the resident-intruder procedure is necessarily a multi-day experiment.

Table 3 | Tukey's Test 10-Day Bouts

Test details	Mean Diff.	SE of diff.	q	DF
Day 1 vs. Day 2	-4.4	0.9798	6.351	24
Day 1 vs. Day 3	-4.56	1.065	6.057	24
Day 1 vs. Day 4	-4.76	0.6935	9.707	24
Day 1 vs. Day 5	-4.32	0.6238	9.795	24
Day 1 vs. Day 6	-5	0.6856	10.31	24
Day 1 vs. Day 7	-7	0.7916	12.51	24
Day 1 vs. Day 8	-6.56	0.8681	10.69	24
Day 1 vs. Day 9	-4.92	0.7163	9.714	24
Day 1 vs. Day 10	-4.64	0.8581	7.647	24
Day 2 vs. Day 3	-0.16	1.072	0.2111	24
Day 2 vs. Day 4	-0.36	1.107	0.4598	24
Day 2 vs. Day 5	0.08	1.02	0.111	24
Day 2 vs. Day 6	-0.6	1.077	0.7878	24
Day 2 vs. Day 7	-2.6	1.115	3.298	24
Day 2 vs. Day 8	-2.16	1.27	2.406	24
Day 2 vs. Day 9	-0.52	1.214	0.6058	24
Day 2 vs. Day 10	-0.24	1.327	0.2558	24
Day 3 vs. Day 4	-0.2	1.125	0.2513	24
Day 3 vs. Day 5	0.24	1.032	0.329	24
Day 3 vs. Day 6	-0.44	0.7596	0.8192	24
Day 3 vs. Day 7	-2.44	1.181	2.923	24
Day 3 vs. Day 8	-2	1.023	2.765	24
Day 3 vs. Day 9	-0.36	1.258	0.4047	24
Day 3 vs. Day 10	-0.08	1.103	0.1026	24
Day 4 vs. Day 5	0.44	0.5918	1.051	24
Day 4 vs. Day 6	-0.24	0.8588	0.3952	24
Day 4 vs. Day 7	-2.24	0.8332	3.802	24
Day 4 vs. Day 8	-1.8	0.7257	3.508	24
Day 4 vs. Day 9	-0.16	0.9031	0.2506	24
Day 4 vs. Day 10	0.12	0.9171	0.185	24
Day 5 vs. Day 6	-0.68	0.7228	1.331	24

Table 3 (cont'd)

Day 5 vs. Day 7	**-2.68**	**0.7675**	**4.938**	**24**
Day 5 vs. Day 8	-2.24	0.9315	3.401	24
Day 5 vs. Day 9	-0.6	0.8622	0.9842	24
Day 5 vs. Day 10	-0.32	0.9861	0.4589	24
Day 6 vs. Day 7	-2	0.7746	3.651	24
Day 6 vs. Day 8	-1.56	0.8207	2.688	24
Day 6 vs. Day 9	0.08	0.8143	0.1389	24
Day 6 vs. Day 10	0.36	0.8998	0.5658	24
Day 7 vs. Day 8	0.44	0.9418	0.6607	24
Day 7 vs. Day 9	2.08	0.8122	3.622	24
Day 7 vs. Day 10	2.36	0.9914	3.366	24
Day 8 vs. Day 9	1.64	1.081	2.145	24
Day 8 vs. Day 10	1.92	0.8961	3.03	24
Day 9 vs. Day 10	0.28	0.799	0.4956	24

Table 4 | Tukey's Test 10-Day Latency

Test details	Mean Diff.	SE of diff.	q	DF
Day 1 vs. Day 2	112.2	11.92	13.32	24
Day 1 vs. Day 3	101.7	14.38	9.997	24
Day 1 vs. Day 4	102.3	11.65	12.42	24
Day 1 vs. Day 5	101.9	13.14	10.96	24
Day 1 vs. Day 6	109.5	12.47	12.42	24
Day 1 vs. Day 7	117.5	11.79	14.1	24
Day 1 vs. Day 8	117.8	12.82	12.99	24
Day 1 vs. Day 9	110.8	13.65	11.48	24
Day 1 vs. Day 10	98.08	14.96	9.273	24
Day 2 vs. Day 3	-10.52	12.74	1.168	24
Day 2 vs. Day 4	-9.88	14.51	0.9626	24
Day 2 vs. Day 5	-10.32	9.904	1.474	24
Day 2 vs. Day 6	-2.68	13.17	0.2877	24
Day 2 vs. Day 7	5.28	11.19	0.6672	24
Day 2 vs. Day 8	5.56	12.06	0.6518	24
Day 2 vs. Day 9	-1.4	14.16	0.1398	24
Day 2 vs. Day 10	-14.12	12.86	1.553	24
Day 3 vs. Day 4	0.64	16.33	0.05543	24
Day 3 vs. Day 5	0.2	12.35	0.02289	24
Day 3 vs. Day 6	7.84	12.46	0.8898	24
Day 3 vs. Day 7	15.8	12.41	1.801	24
Day 3 vs. Day 8	16.08	15.38	1.479	24
Day 3 vs. Day 9	9.12	17.72	0.7277	24
Day 3 vs. Day 10	-3.6	15.36	0.3315	24

Table 4 (cont'd)

Day 4 vs. Day 5	**-0.44**	**12.63**	**0.04927**	**24**
Day 4 vs. Day 6	7.2	11.36	0.8966	24
Day 4 vs. Day 7	15.16	10.16	2.109	24
Day 4 vs. Day 8	15.44	8.286	2.635	24
Day 4 vs. Day 9	8.48	14.08	0.852	24
Day 4 vs. Day 10	-4.24	13.96	0.4295	24
Day 5 vs. Day 6	7.64	11.36	0.9509	24
Day 5 vs. Day 7	15.6	8.697	2.537	24
Day 5 vs. Day 8	15.88	9.939	2.26	24
Day 5 vs. Day 9	8.92	13.21	0.9548	24
Day 5 vs. Day 10	-3.8	10.84	0.4956	24
Day 6 vs. Day 7	7.96	5.803	1.94	24
Day 6 vs. Day 8	8.24	7.304	1.595	24
Day 6 vs. Day 9	1.28	11.23	0.1612	24
Day 6 vs. Day 10	-11.44	11.93	1.356	24
Day 7 vs. Day 8	0.28	5.6	0.0707	24
Day 7 vs. Day 9	-6.68	9.897	0.9545	24
Day 7 vs. Day 10	-19.4	9.859	2.783	24
Day 8 vs. Day 9	-6.96	9.423	1.045	24
Day 8 vs. Day 10	-19.68	9.445	2.947	24
Day 9 vs. Day 10	-12.72	9.879	1.821	24

Table 5 | Tukey's Test 10-Day Duration

Test details	Mean Diff.	SE of diff.	q	DF
Day 1 vs. Day 2	-3.31	0.6991	6.696	24
Day 1 vs. Day 3	-2.162	0.6123	4.994	24
Day 1 vs. Day 4	-3.355	0.7985	5.942	24
Day 1 vs. Day 5	-2.296	0.5799	5.598	24
Day 1 vs. Day 6	-2.615	0.6958	5.316	24
Day 1 vs. Day 7	-1.895	0.6257	4.283	24
Day 1 vs. Day 8	-2.508	0.8929	3.973	24
Day 1 vs. Day 9	-1.844	0.6462	4.036	24
Day 1 vs. Day 10	-1.408	0.5393	3.691	24
Day 2 vs. Day 3	1.148	0.7715	2.104	24
Day 2 vs. Day 4	-0.0448	0.6766	0.09364	24
Day 2 vs. Day 5	1.014	0.7286	1.969	24
Day 2 vs. Day 6	0.6948	0.8195	1.199	24
Day 2 vs. Day 7	1.415	0.6301	3.175	24
Day 2 vs. Day 8	0.8016	0.9121	1.243	24
Day 2 vs. Day 9	1.466	0.8256	2.51	24
Day 2 vs. Day 10	1.902	0.6166	4.363	24

Table 5 (cont'd)

Day 3 vs. Day 4	**-1.193**	**0.733**	**2.301**	**24**
Day 3 vs. Day 5	-0.1336	0.5855	0.3227	24
Day 3 vs. Day 6	-0.4532	0.6203	1.033	24
Day 3 vs. Day 7	0.2668	0.7135	0.5288	24
Day 3 vs. Day 8	-0.3464	0.7574	0.6468	24
Day 3 vs. Day 9	0.3176	0.8387	0.5355	24
Day 3 vs. Day 10	0.7544	0.6527	1.635	24
Day 4 vs. Day 5	1.059	0.6681	2.242	24
Day 4 vs. Day 6	0.7396	0.6336	1.651	24
Day 4 vs. Day 7	1.46	0.4782	4.316	24
Day 4 vs. Day 8	0.8464	0.6257	1.913	24
Day 4 vs. Day 9	1.51	0.6797	3.143	24
Day 4 vs. Day 10	1.947	0.4966	5.545	24
Day 5 vs. Day 6	-0.3196	0.491	0.9205	24
Day 5 vs. Day 7	0.4004	0.4931	1.148	24
Day 5 vs. Day 8	-0.2128	0.7426	0.4053	24
Day 5 vs. Day 9	0.4512	0.5973	1.068	24
Day 5 vs. Day 10	0.888	0.4618	2.72	24
Day 6 vs. Day 7	0.72	0.6194	1.644	24
Day 6 vs. Day 8	0.1068	0.8561	0.1764	24
Day 6 vs. Day 9	0.7708	0.732	1.489	24
Day 6 vs. Day 10	1.208	0.5179	3.298	24
Day 7 vs. Day 8	-0.6132	0.7137	1.215	24
Day 7 vs. Day 9	0.0508	0.5242	0.137	24
Day 7 vs. Day 10	0.4876	0.4764	1.447	24
Day 8 vs. Day 9	0.664	0.6564	1.431	24
Day 8 vs. Day 10	1.101	0.6606	2.356	24
Day 9 vs. Day 10	0.4368	0.5677	1.088	24

Discussion

We present a data-driven method to generate a composite measure of aggression behavior for sexually experienced CD-1 male mice using confirmatory factor analysis. We showed the generalizability of MAD across labs and experimenters, and the stability of MAD in quantifying aggressor performance over time, thereby demonstrating the utility of our model as a critical tool for both CSDS and aggression research.

We also showed how MAD provides a useful measurement model for data collected using automated machine-learning based supervised classification. In short, any laboratory using the social defeat procedure can input aggressor screening data into MAD and use the resulting scores to select CD1 residents that are most aggressive and that will remain aggressive over multiple defeat experiments. These data can be collected manually or using automated approaches. Further, these scores can be reported and incorporated into the description of CSDS experiments, standardizing (or at least accounting for) CD-1 aggression levels across experiments and laboratories.

This approach allowed us to generate an internally consistent and generalizable model that can be used to study latent mouse aggression without the need for high-speed video monitoring, specialized hardware, or behavioral analysis software, and that is readily accessible to experimenters. However, our approach can be easily extended to computational neuroethological methods (Datta, Anderson, Branson, Perona, & Leifer, 2019) for the study of aggression-related behavior (Goodwin, Nilsson, & Golden, 2020), which generate datasets that contain many more observable measures and therefore require greater dimensionality reduction. As computational neuroethology becomes more common, our approach can further be used to standardize aggression scores between labs using manual and labs using automated approaches.

Currently, there is little standardization in the measurement of aggression within the resident-intruder procedure. Many studies rely on single variable measures to quantify offensive aggressive behavior. For example, average attack latency is widely used to evaluate aggressive behavior in both aggression (e.g., de Boer, van der Vegt, & Koolhaas, 2003) and CSDS research (Golden et al., 2011). Koolhaas and colleagues (2013) recommend summing offense behavior over three to four resident-intruder trials as a data reduction technique to score aggression across trial

days. In CSDS research, Golden and colleagues (2011) recommend selecting aggressors that attack on two consecutive days and have an attack latency of less than 60 seconds. In both cases, evaluating aggression requires the analysis of more than one aspect of aggressor behavior. The current study therefore builds on these approaches by offering a data-driven model to efficiently and systematically generate an aggression score that can be directly compared to those generated in other cohorts at different times, by different experimenters, and/or in different environments.

Confirmatory factor analysis utilizes variance and covariance to determine the structure of a measurement model, and mice are both sensitive to experimental conditions and do not often rigidly adhere to a pattern of behavior. Though there were no significant differences between the factor loadings for days 1-3, the model estimated a stronger factor loading for day 2. Looking at the raw data, we observed a small subset of animals that attacked only on day 2 and for an extended duration, likely driving this finding. As such, including cohorts of animals in aggregate data that may have been evaluated by different researchers or different screening methods (i.e., real-time versus video) certainly affects model structure, but building the model with data from multiple experimenters and institutes ensures its generalizability and thus its potential utility in all labs. We demonstrated here that aggression scores were highly correlated despite differential (but non-significant) numerical weighting of day 2 and 3 parameter estimates (**Fig 10c**). Thus, though variation in screening procedures likely produced differential parameter estimates that affect scoring, this only makes the model more amenable to different datasets and applications.

Another limitation of the current study is that the experimental animals were all male. In light of an ongoing effort to broaden our understanding of affective disorders, recent adaptations of the chronic social defeat stress model have been successfully applied to female mice (Newman et al., 2019; Warren, Mazei-Robison, Robison, & Iñiguez, 2020). Critically, this work has revealed

differences in attack behavior between intermale and rival female aggression (Newman et al., 2019). MAD does not account for any sex differences in aggression behavior and therefore cannot necessarily be applied to female aggressors. Though there may be some overlap in model structure, additional work is required to accurately characterize rival female aggression. It is likely that female aggression may be qualitatively different, both in measurement of behavior and the underlying circumstances that produce the aggression. While given similar conditions and behavioral measurements it can be presumed that the model may accurately predict aggression scores in both males and females, additional work would be necessary to test this hypothesis. Future work should apply SEM approaches to female aggressor data to develop an appropriate measurement model to aid the study of sex as a biological variable in aggression.

Conclusion

In the current study, we sought to develop a systematic, data-driven method of measuring aggression behavior in a preclinical model of territorial aggression. Structural equation modeling provides an ideal confirmatory approach that leverages foundational research to quantify aggression behavior. With this approach, we show that a multidimensional, multiday aggression screening provides a better characterization of aggression behavior. As such, utilizing this standard measurement approach, especially in tandem with other quantitative measurement tools (e.g., SimBA), facilitates reproducibility of research and collaboration across labs. Importantly, our model, MAD, can be used for high-throughput screening to streamline aggressor selection and reduce an element of variability in CSDS research.

CHAPTER 4: GUT MICROBIOME COMPOSITION IS A CRITICAL FACTOR IN AGGRESSION BEHAVIOR

Introduction

Emotional dysregulation is a critical risk factor in the expression of pathological aggression (Sapolsky, 2017), or aggression perpetrated outside the contexts of competition or self-defense. Underlying this emotional dysregulation is dysfunction in neural systems responsible for the processing of social behavior and associated emotional cues. Indeed, pathophysiological changes in the emotional processing centers of the brain and the cortical structures that regulate them are associated with aggression behavior. For example, human imaging studies show the prefrontal cortex (PFC), the seat of executive function and risk assessment, exerts less regulatory control over the amygdala, a nucleus for aggression and anxiety (Rosell & Siever, 2015). Aberrations in this system drive impairments in learning of social behavior as well as disruptions in processing of social stimuli and their integration with experiential and contextual information, permitting the expression of inappropriate social behaviors. Moreover, patterns of aggressive behavior may develop with the aid of the reward circuitry, which motivates future aggression following "success" of aggressive strategies (Golden et al., 2017). Overall, it is clear that changes in brain health influence the expression of aggressive and violent behavior.

In human populations, violence is also a spatially specific phenomenon that is often concentrated in impoverished neighborhoods alongside poor health and disadvantage (Bursik & Grasmick, 1993; LaVeist et al., 2011; Sampson, 2012). Neighborhood context is one of the most important risk factors for perpetration of violence (Bingenheimer et al., 2005; Braga et al., 2010), but most people living in so-called "crime hotspots" never behave violently despite exposure to the same psychosocial risk as violent residents. This variation at the level of the individual

necessitates a biopsychosocial approach to understanding aggression to gain a more comprehensive understanding of the risks that drive aggression behaviors. Indeed, human imaging studies demonstrate that individuals exposed to disadvantage and social disorder exhibit changes in the structure and function of the cortico-amygdala loop (Hackman et al., 2010), a key system in the regulation of socio-emotional behavior, and this is also observed among criminal offenders (Leutgeb et al., 2015). However, all existing data are correlational, and the causal links between environmental exposures or stressors and brain health that produce the individual variation observed in the expression of aggression behaviors remain unknown.

Though there is increasing interest in exploring the neurophysiological pathways that shape the cortico-amygdala loop and reward circuitry of the pathologically aggressive, little work has been done to uncover the role of the gut microbiome in modulating aggressive behavior. Thus, the current chapter explores how changes in the structure of the gut microbiome are related to aggression behavior in a preclinical model of territorial, reactive aggression.

Methods

Animals

Experiments were performed with outbred, sexually experienced, retired breeder male CD1 mice. Mice were singly housed in a 12:12 light-dark cycle with *ad libitum* access to food and water. To prevent shifts in gut microbiome composition during experimental procedures, mice were allowed to habituate to facility conditions for 10-14 days after arrival before beginning behavioral assessments. All experiments were approved by Institutional Animal Care and Use Committee at MSU.

Resident-Intruder Procedure

Aggression behavior was assessed via the resident-intruder procedure, wherein a novel intruder is introduced into a resident's cage and the social interaction is observed over three consecutive trial days. Animals were excluded from analysis if they 1) flooded their cages during any trial days or 2) exhibited significant stereotypies. Aggression scores were generated using the Mouse Aggression Detector (MAD) model described in Chapter 3. This model leverages features of attack behavior, including latency to the first attack, bout number, and bout duration, to calculate a composite aggression score.

Drug Administration

Cohorts receiving sodium butyrate (NaB) were given 1.2 g/kg NaB in 0.9% saline (or saline alone as vehicle control) via intraperitoneal injection. Cohorts receiving antibiotics or probiotics consumed treatment in their drinking water. Animals that were treated with antibiotics received a broad-spectrum cocktail of 1.0 g/L ampicillin and 0.5 g/L neomycin mixed in a UV-protected drinking bottle (Schepper et al., 2019). Animals that were treated with probiotics received a daily drinking solution of VSL#3 (L. Zhang et al., 2019).

Microbiome Recolonization

Microbiome recolonization was accomplished through a month-long hybrid strategy of oral gavage and donor bedding exposure. A donor cohort of ten sexually experienced CD-1 male mice was evaluated for aggression behavior using the resident-intruder procedure and MAD model scoring. The five most aggressive animals were selected as donors from which fecal pellets (week 1) and bedding (weeks 1-4) were collected. During the first week of recolonization following broad-spectrum antibiotic treatment, donor fecal pellets were combined in 5 mL PBS and homogenized by vortex. The mixture was subsequently centrifuged, and the supernatant was

administered via a stainless-steel gavage tool. This procedure was repeated for three total doses during the first week of recolonization. In parallel, donor bedding was collected, combined, and added to the cages of experimental mice (~60 g) once per week during cage change. Effects of this approach were confirmed via Qubit DNA quantification (using a fluorophore tag) at antibiotic and recolonization stages.

Sample Collection and Microbiome Data Processing (performed with S. Kaszubinski)

Whole brain and fecal pellet samples were collected during transcardial perfusion. Composition of the microbiome was assessed via 16S rRNA amplicon sequencing using the V4 region of DNA extracted from the fecal samples using the Qiagen PowerSoil and PowerSoil Pro kits. 16S rRNA amplicon DNA sequences were assembled, quality controlled, and aligned using QIIME2 as previously described (Kaszubinski, Pechal, Schmidt, et al., 2020). Further data processing and subsequent analyses were conducted using the *phyloseq* package for R and RStudio (McMurdie & Holmes, 2013; R Core Team, 2021; RStudio Team, 2020).

Permutational Analysis of Variance

Permutational analysis of variance (PERMANOVA) is a nonparametric test that analyzes group differences, similar to parametric analysis of variance (ANOVA) tests. Instead of means testing, however, PERMANOVA evaluates group differences based on user-specified distances that reflect similarities between sample clusters (M. J. Anderson, 2001, 2017). As such, PERMANOVA evaluates differences across centroids of clusters (e.g., experimental conditions). Here, dissimilarity matrices were calculated using Unifrac distances, a distance calculation that accounts for phylogenetic, or relatedness, among microbial community members.

Negative Binomial Mixed Models

Negative binomial mixed models (NBMM) were used to identify specific taxa associated with aggression behavior as previously described (Zhang et al., 2017; Zhang & Yi, 2020). This model was selected to account for features of microbiome data that are poorly dealt with by other analytic strategies. Specifically, microbiome data are count data with natural dependencies created by an inherent structure related to taxonomic levels (i.e., phylum, genus), phylogenetic relationships, gene function, etc. Moreover, the relative abundance of an OTU necessarily affects the abundance of all others in the sample. Together, these features violate the classic assumption of independence that most statistical tests require. In addition, sample OTU data are nested within an animal, incorporating unknown host factors that thus become random effects. The current study's model therefore includes OTUs as the modeled count variable, aggression score, a discrete variable, as the fixed effect, and sample ID, a mouse indicator, as the random effect.

Data Analysis

Data were analyzed using a combination of t-tests (Mann-Whitney U), correlation (Kendall's tau), ANOVA (Kruskal-Wallis), PERMANOVA, NBMM, and linear modeling in R and RStudio. Graphics were generated in both RStudio and PRISM 9.0.

Results

The mouse gut microbiome is associated with aggressive behavior

To explore aggression-related differences in gut microbiome composition, fecal 16S rRNA amplicon sequencing data was used to characterize the gut microbiome of CD-1 male mice scored for aggression using the resident-intruder procedure. After quality control and standardization, a final dataset comprised of 144 samples representing 1269 taxa was used in the analysis. Aggression indicators, including latency to the first attack, bout number, and bout duration across three trial

days, were used to calculate a composite aggression score using the MAD model. Aggression scores for experimentally naïve mice from five experimental cohorts were pooled to create a sufficiently large dataset to evaluate potential differences in microbial community structure.

Table 6 | Alpha Diversity Among Experimentally Naïve CD-1 Male Mice

Cohort	Chao1	Chao1 SD	Shannon	Shannon SD
1	106	22.7	3.49	0.38
2	114	22.2	3.34	0.21
3	189	53.2	4.05	0.32
4	243	42.0	4.26	0.32
6	317	37.0	4.14	0.22

Table 6 shows average alpha diversity values for the Chao1 and Shannon diversity indices for n = 144 experimentally naïve CD-1 male mice in cohort 1 (n = 10), cohort 2 (n = 15), cohort 3 (n = 16), cohort 4 (n = 30), and cohort 6 (n = 73).

In a first step to determine if there was a relationship between territorial, reactive aggression in CD-1 male mice and the composition of their gut microbiome, aggression-related differences in alpha diversity, an index of richness and evenness within gut microbiome samples, were explored. Using the experimentally naïve mice from five separate cohorts (n = 144), Chao1, a measure of richness, and Shannon diversity, a measure of richness and evenness, were calculated (**Table 6**). Kruskal-Wallis tests showed a strong cohort effect for both Chao1 ($H(4) = 106.76$, $p < 0.0001$) and Shannon diversity ($H(4) = 59.524$, $p < 0.0001$). Nemenyi *post hoc* tests revealed that effect of experimental cohort was robust (**Tables 7-8**), underscoring the effects of time on experimental outcomes. In an attempt to limit this effect, samples were subsequently subdivided by cohort and analyzed separately. However, no significant difference in alpha diversity by aggression score was found within cohort (**Table 9**), suggesting that the experimental habituation period with common exposures in the animal facility normalized gut communities.

To compare gut microbiome composition between samples collected from differentially aggressive CD-1 male mice, differences in beta diversity, a measure of between samples (dis)similarity, were explored. Given the strong cohort effect observed in the alpha diversity analyses, sample data were first aggregated to determine the effect of cohort membership on beta diversity. Unsurprisingly, PERMANOVA results showed a significant main effect of cohort on beta diversity (**Fig 20**; $F(4) = 15.362$, $R^2 = 0.307$, $p = 0.001$), indicating that groups of mice that are shipped together and arrive at our facility together have very similar gut microbiomes, as expected. Next, the relationship between aggression score and beta diversity was examined without accounting for cohort membership. Results revealed that there was no significant main effect of aggression ($F(1) = 1.559$, $R^2 = 0.011$, $p = 0.058$) on beta diversity. However, taking cohort membership into account, PERMANOVA results demonstrate a significant main effect of cohort

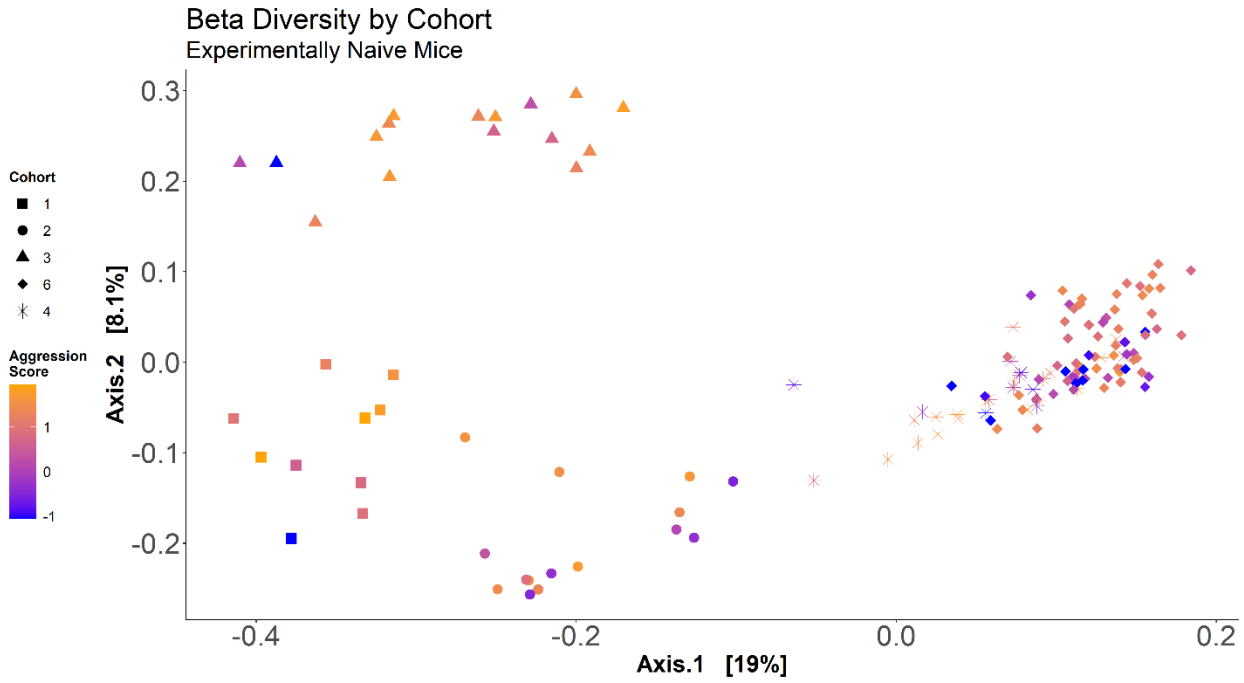

Figure 20 | PCoA Plot of Beta Diversity for Experimentally Naïve Mice. Principal coordinate analysis (PCoA) plot of mouse fecal microbiome beta diversity using Unifrac distances for all cohorts. Each point represents one mouse fecal sample while the color indicates aggression score associated with the mouse. Schematic depicts the clustering of samples by cohort. Permutational multivariate analysis of variance (PERMANOVA) of beta diversity detected significant differences ($p < 0.01$) among cohorts and for aggression scores.

(F(4) = 15.421, R^2 = 0.301, p = 0.001) and aggression score (F(1) = 2.223, R^2 = 0.011, p = 0.005) as well as an aggression score-cohort interaction (F(4) = 1.698, R^2 = 0.033, p = 0.008), suggesting that gut microbiome composition varies according to aggression behavior (**Fig 21**). Taken together, these data suggest that gut microbiome composition varies according to territorial aggression behavior in CD-1 male mice once experimental cohort membership is accounted for.

Table 7 | Nemenyi Post Hoc Tests Chao1 by Cohort

	1	2	3	4
2	0.9999			
3	0.6056	0.6107		
4	0.0188	0.0079	0.4084	
6	0.0000	0.0000	0.0000	0.0000

Table 7 shows p-values from Nemenyi post hoc tests for Chao1 diversity between 5 cohorts of CD-1 mice (n = 144).

Table 8 | Nemenyi Post Hoc Tests Shannon by Cohort

	1	2	3	4
2	0.9706			
3	0.0398	0.0011		
4	0.0000	0.0000	0.1780	
6	0.0005	0.0000	0.9156	0.2344

Table 8 shows p-values from Nemenyi post hoc tests for Shannon diversity between 5 cohorts of CD-1 mice (n = 144).

Table 9 | Correlation Between Alpha Diversity and Aggression Score

	Chao1			Shannon		
Cohort	z	tau	p	z	tau	p
1	1.16	0.29	0.24	0.98	0.24	0.33
2	-0.15	-0.03	0.88	-0.25	-0.05	0.80
3	1.35	0.25	0.18	1.35	0.25	0.18
4	0.20	0.03	0.84	0.02	0	0.99
6	0.42	0.03	0.67	-0.02	0	0.98

Table 9 shows the results of correlation tests with Kendall's tau, examining the relationship between Chao1 and Shannon diversity indices and aggression score for n = 144 experimentally naïve CD-1 male mice in cohort 1 (n = 10), cohort 2 (n = 15), cohort 3 (n = 16), cohort 4 (n = 30), and cohort 6 (n = 73).

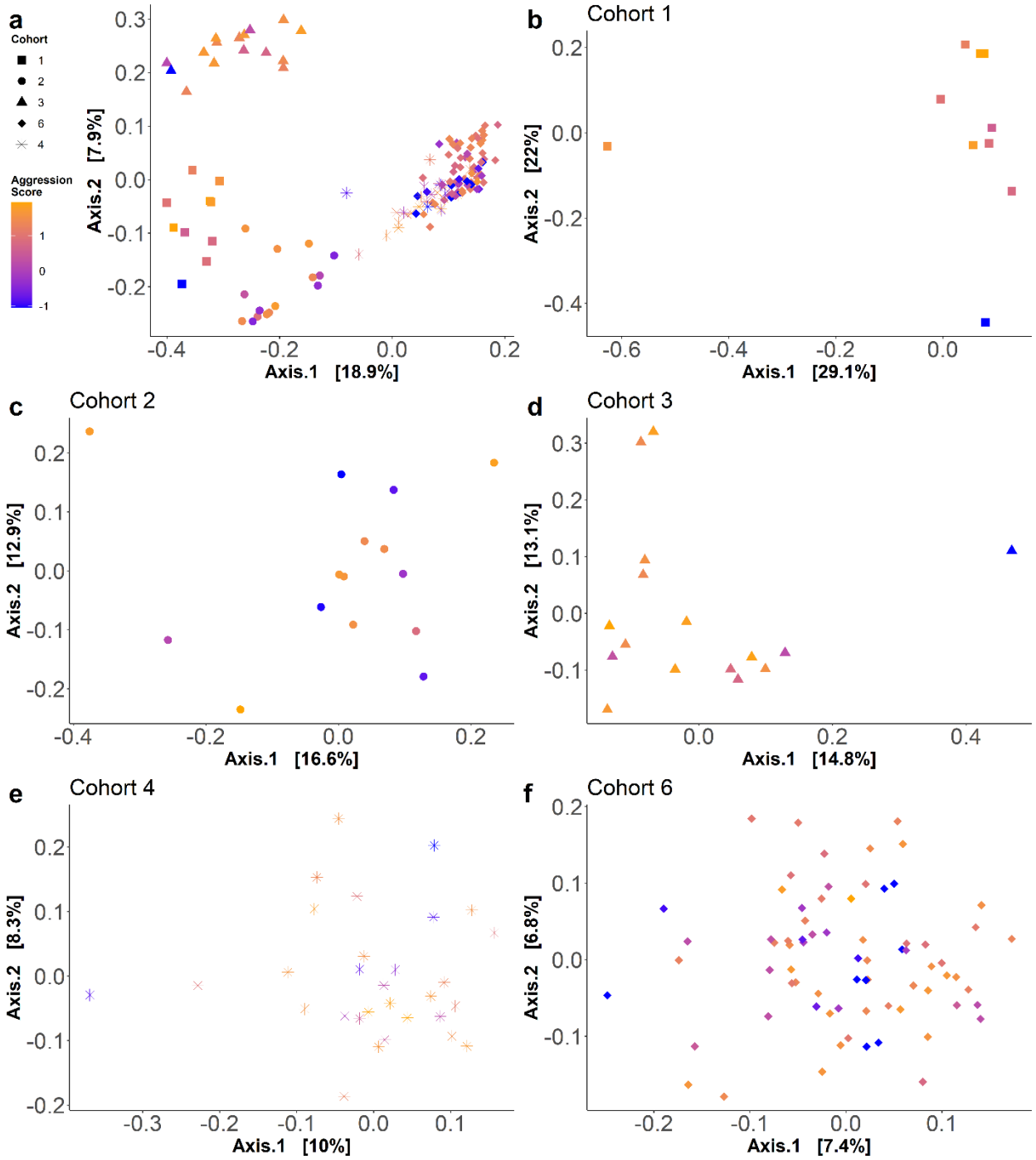

Figure 21 | PCoA Plots of Beta Diversity by Cohort. PCoA plots of mouse fecal microbiome beta diversity using Unifrac distances for a) all cohorts (1-4,6) b) cohort 1 c) cohort 2 d) cohort 3 e) cohort 4 and f) cohort 6. Each point represents one mouse fecal sample, while the color indicates aggression score associated with the mouse. PERMANOVA of beta diversity detected significant differences ($p < 0.01$) among cohort and for aggression scores.

Specific taxa drive aggression-related differences in beta diversity, suggesting short-chain fatty acids as potential mediators of the aggression-gut-brain link

To identify specific microbiota driving the relationship between the composition of the gut microbiome and aggression behavior, a negative binomial mixed model (NBMM) was used to model counts of microbial taxa with respect to aggression behavior. Here, data from the same group of experimentally naïve CD-1 male mice described in the previous section were used to detect specific taxa associated with aggression score. Specifically, OTU data, groupings of sequences with 99.99% similarity to describe bacterial abundance, were input as the model

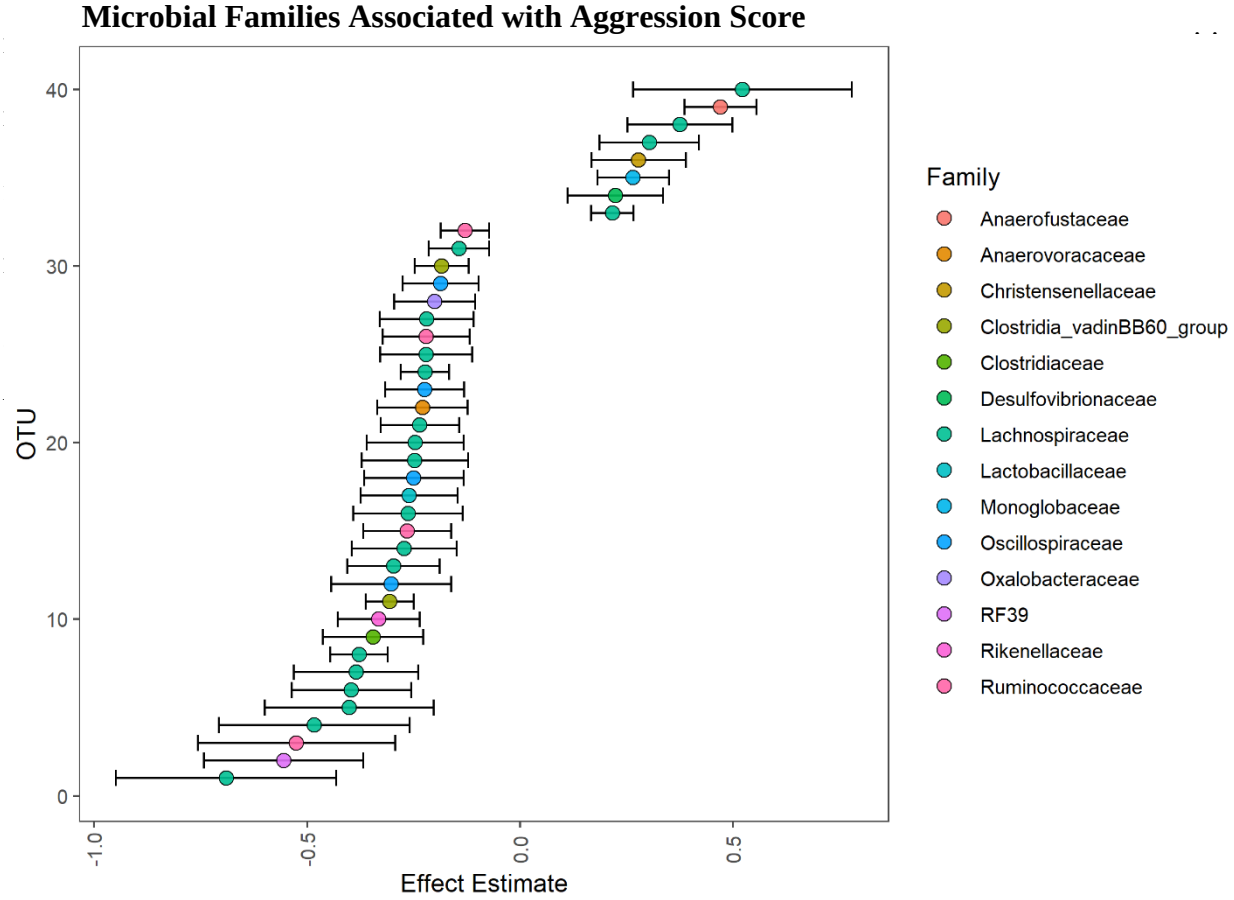

Figure 22 | Microbial Families Associated with Aggression Score. Significant effect estimates ($p<0.05$) for aggression score in modeling the distribution of microbial families among mouse fecal microbiomes. Each dot represents one operational taxonomic unit (OTU) or taxon, and the color of each dot corresponds to the microbial family. Standard error among the estimates is depicted by black bars. Negative estimate values indicate a negative association with aggression and taxon relative abundance, while positive estimate values indicate a positive association with aggression and taxon relative abundance.

Table 10 | Effect Estimates of Aggression Scores in Modeling Microbial Families

Phylum	Class	Order	Family	Genus	Estimate	Std.Error	p	p-adj
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Lachnospiraceae_NK4A136_group	-0.690	0.259	0.009	0.240
Firmicutes	Bacilli	RF39	RF39	RF39	-0.555	0.187	0.004	0.150
Firmicutes	Clostridia	Oscillospirales	Ruminococcaceae	Ruminococcus	-0.525	0.232	0.025	0.430
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Unassigned	-0.483	0.224	0.033	0.450
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Unassigned	-0.401	0.198	0.044	0.480
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Unassigned	-0.396	0.140	0.005	0.180
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Tyzzerella	-0.385	0.146	0.009	0.240
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Unassigned	-0.378	0.067	0.000	0.000
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Candidatus_Arthromitus	-0.345	0.118	0.004	0.150
Bacteroidota	Bacteroidia	Bacteroidales	Rikenellaceae	Alistipes	-0.332	0.096	0.001	0.047
Firmicutes	Clostridia	Clostridia_vadinBB60_group	Clostridia_vadinBB60_group	Clostridia_vadinBB60_group	-0.306	0.056	0.000	0.000
Firmicutes	Clostridia	Oscillospirales	Oscillospiraceae	Unassigned	-0.302	0.141	0.034	0.450
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	uncultured	-0.297	0.109	0.007	0.220
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Lachnoclostridium	-0.272	0.123	0.029	0.450
Firmicutes	Clostridia	Oscillospirales	Ruminococcaceae	uncultured	-0.265	0.103	0.011	0.240
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	A2	-0.263	0.128	0.042	0.480
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	-0.260	0.114	0.024	0.430
Firmicutes	Clostridia	Oscillospirales	Oscillospiraceae	Colidextribacter	-0.249	0.117	0.034	0.450
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	GCA-900066575	-0.247	0.125	0.049	0.490
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Unassigned	-0.246	0.114	0.033	0.450
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Unassigned	-0.235	0.092	0.011	0.240
Firmicutes	Clostridia	Peptostreptococcales-Tissierellales	Anaerovoracaceae	Family_XIII_UCG-001	-0.229	0.106	0.032	0.450
Firmicutes	Clostridia	Oscillospirales	Oscillospiraceae	Oscillibacter	-0.224	0.093	0.017	0.340
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Unassigned	-0.223	0.057	0.000	0.010
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Lachnoclostridium	-0.220	0.108	0.043	0.480
Firmicutes	Clostridia	Oscillospirales	Ruminococcaceae	[Eubacterium]_siraeum_group	-0.220	0.102	0.032	0.450
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Dorea	-0.219	0.110	0.047	0.480
Proteobacteria	Gammaproteobacteria	Burkholderiales	Oxalobacteraceae	Unassigned	-0.200	0.095	0.036	0.460
Firmicutes	Clostridia	Oscillospirales	Oscillospiraceae	Colidextribacter	-0.186	0.089	0.039	0.480
Firmicutes	Clostridia	Clostridia_vadinBB60_group	Clostridia_vadinBB60_group	Clostridia_vadinBB60_group	-0.184	0.063	0.004	0.150
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	A2	-0.143	0.071	0.046	0.480
Firmicutes	Clostridia	Oscillospirales	Ruminococcaceae	uncultured	-0.129	0.057	0.025	0.430
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Roseburia	0.217	0.050	0.000	0.003
Desulfobacterota	Desulfovibrionia	Desulfovibrionales	Desulfovibrionaceae	Bilophila	0.224	0.112	0.047	0.480
Firmicutes	Clostridia	Monoglobales	Monoglobaceae	Monoglobus	0.266	0.084	0.002	0.100
Firmicutes	Clostridia	Christensenellales	Christensenellaceae	uncultured	0.279	0.111	0.013	0.270
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Lachnospiraceae_NK4A136_group	0.304	0.117	0.010	0.240
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Roseburia	0.376	0.123	0.003	0.130
Firmicutes	Clostridia	Eubacteriales	Anaerofustaceae	Anaerofustis	0.471	0.084	0.000	0.000
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Unassigned	0.523	0.257	0.043	0.480

Table 10 lists the taxonomic classification of bacteria in the mouse fecal microbiome that have a significant relationship with aggression score ($p<0.05$). Effect estimates are presented with standard error, p-value, and a conservative, adjusted p-value.

Treatment with sodium butyrate increases aggressive behavior

To investigate the potential role of systemic butyrate in aggression, CD-1 male mice were treated with sodium butyrate (NaB) and evaluated for aggression behavior. Specifically, a novel cohort of 42 CD-1 male mice was screened for baseline aggression using the resident-intruder procedure over three consecutive trial days. Latency to the first attack, bout number, and bout duration were collected for aggression scoring using the MAD model. Aggression scores were used to classify experimental mice as having low, medium, or high baseline aggression. Within each categorical designation, mice were randomly selected for the treatment group with matched controls assigned to the control group, yielding an equivalent aggression mean and variance between the treatment ($M = 0.374$; $s^2 = 0.959$) and control ($M = 0.372$; $s^2 = 0.934$) conditions. Mice were subsequently treated with daily intraperitoneal NaB (1.2 g/kg in 0.9% saline) for seven days before initiating an aggression post-test with the resident-intruder procedure (**Fig 23**). As a preliminary measure to ensure any treatment effects were related to drug action and not an adverse drug effect, mouse weights were collected on the first and last day of drug treatment as a proxy measure for animal health (**Fig 24**). Analysis revealed no significant differences in weight in either condition between drug initiation and completion ($F(1, 40) = 0.6974$, $p = 0.409$).

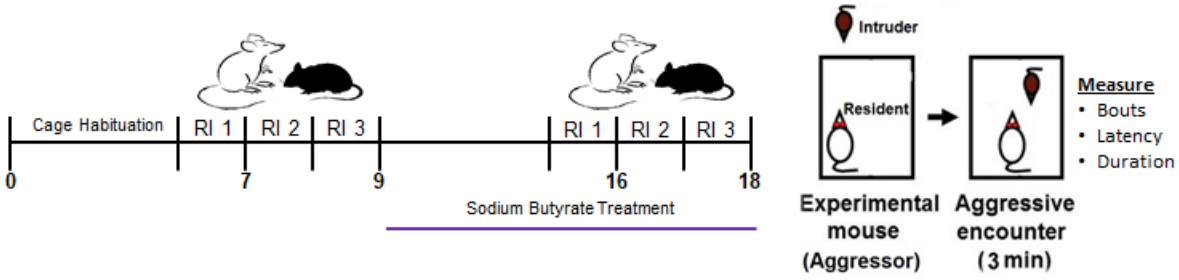

Figure 23 | Sodium Butyrate v Control Experimental Design. Schematic showing the experimental design for n = 42 CD-1 male mice that were evaluated for aggression and subsequently sorted into the NaB or control condition by aggression. The treatment group received NaB for 7 days prior to initiating post-test resident-intruder screening. Pre- and post-treatment aggression scores were compared.

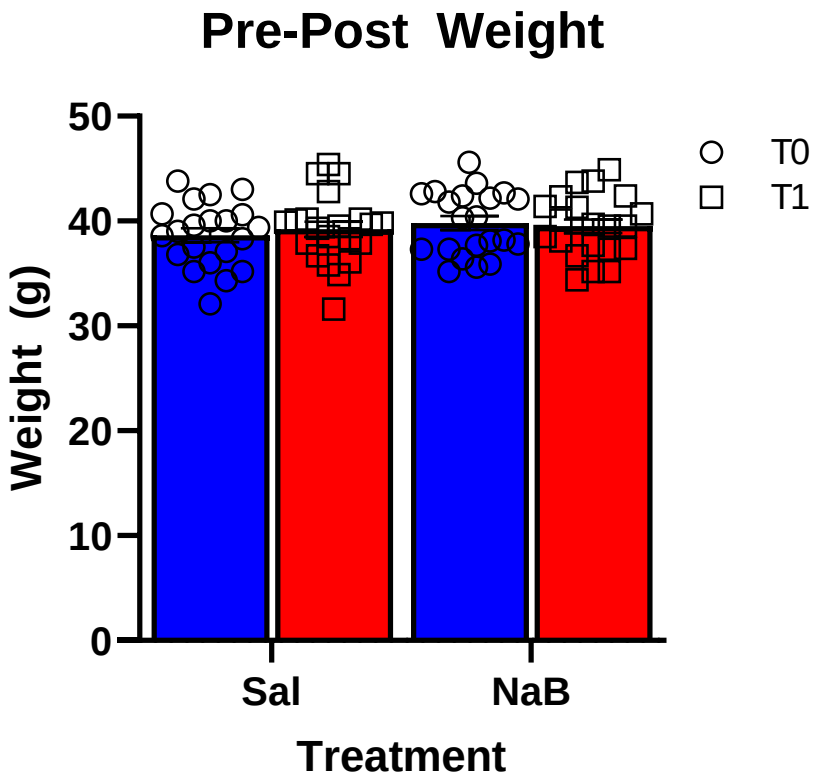

Figure 24 | Sodium Butyrate v Control Pre-Post Intervention Weights. Average weight in grams (g) at baseline (T0) and post-test (T1) resident-intruder screenings (n = 42). Error bars display SEM and symbols show individual data points.

To determine the effect of NaB on aggression behavior, a repeated measures ANOVA test was conducted. Results showed a main effect of time ($F(1, 40) = 5.981$, $p = 0.019$), but no main effect of treatment ($F(1, 40) = 0.5314$, $p = 0.470$). The treatment by time interaction term was also non-significant ($F(1,40) = 3.314$, $p = 0.0762$), suggesting that there was no difference in the change in aggression behavior between the treatment and control groups. However, post hoc analysis (with a Bonferroni correction) to identify which group(s) differed over time showed that only the NaB group (**Fig 25a-b**; $\Delta M = 0.4709$, $t(40) = 3.016$, $p = 0.0089$) but not the control group (**Fig 25a-b**; $\Delta M = 0.69$, $t(40) = 0.4420$, $p = 0.999$) significantly differed in aggression between baseline and the post-test. Overall, these findings indicate that another variable is introducing variation in the analysis.

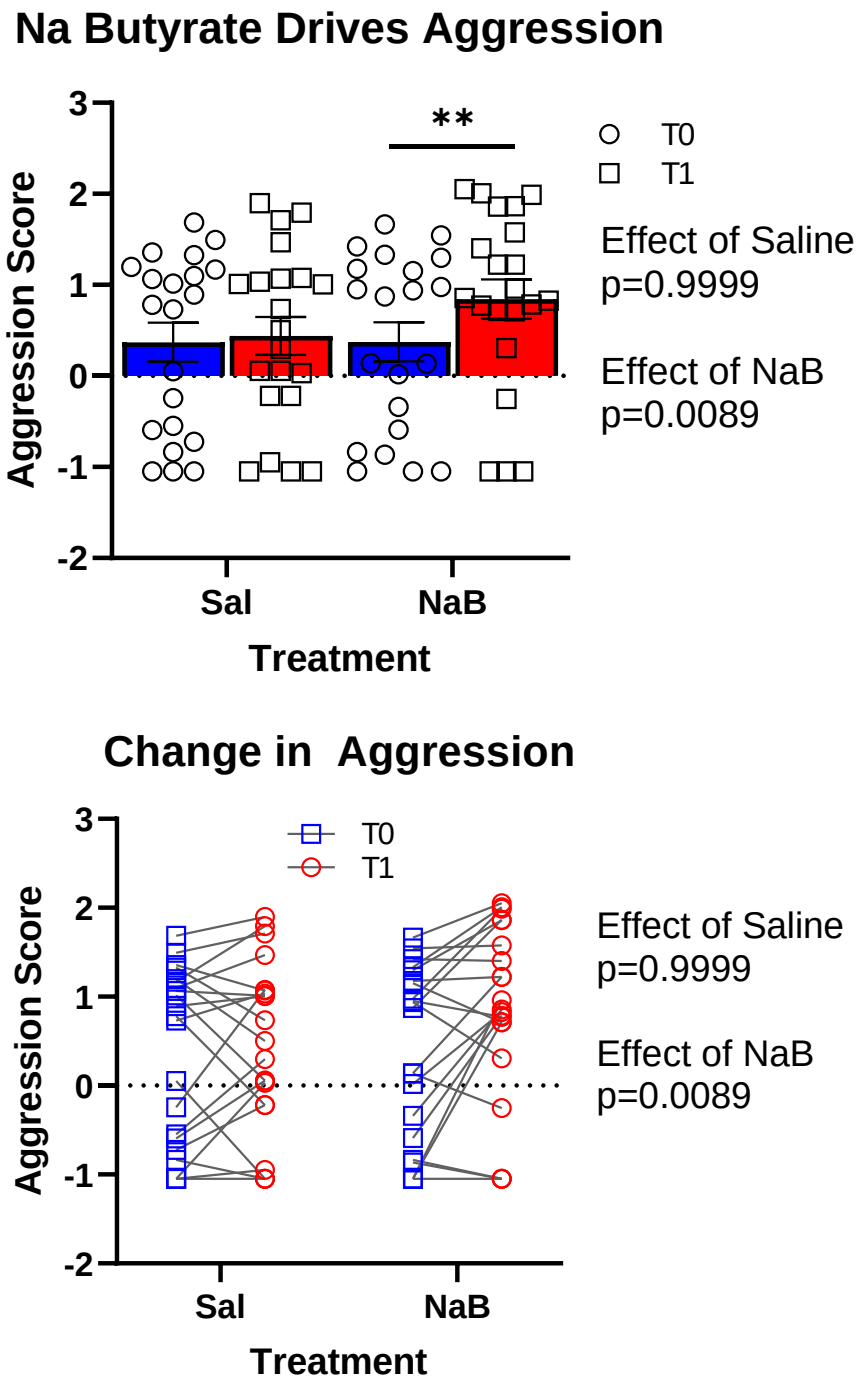

Figure 25 | Effect of Sodium Butyrate on Aggression. a-b) Post hoc tests for repeated measures ANOVA show differences in a) average aggression in the NaB ($p<0.05$) but not the saline group. A Bonferroni correction was used to correct p-values. Error bars display SEM and symbols show individual data points. b) Individuals in the NaB group showed greater change in aggression from baseline.

To determine the relationship between treatment and change in aggression behavior, a multivariate linear model was fitted to account for other factors that could drive aggression within experimental conditions. One potential confounding factor in a simpler analysis, like repeated measures ANOVA, is the inclusion of non-aggressors which has the effect of pulling down group averages. To account for this so-called attack status, the latency variable was queried across days 1-3 of the baseline resident-intruder screening for values of "180" such that a mouse was coded as a non-attacker if and only if there were no attack bouts during any of the three baseline resident-intruder trial days. With this, baseline aggression score was used to predict post-test aggression score, accounting for treatment condition and whether or not a CD-1 mouse displayed any aggression behavior at baseline. Results returned a collective significant effect of baseline aggression score, treatment, and attack status in predicting post-test aggression score ($F(3,38) = 33.71$, $p < 0.0001$, $R^2 = 0.7053$). There was a significant relationship between baseline aggression score and post-test aggression such that for every one unit increase in baseline aggression, there was a corresponding 0.50 increase in post-test aggression score ($t = 5.018$, $p < 0.0001$), accounting for all other factors. Likewise, there was an expected 0.40 increase in aggression in the NaB group ($t = 2.445$, $p = 0.019$), suggesting that treatment with NaB increased the likelihood of aggression behavior once baseline aggression score and attack status were accounted for. Unsurprisingly, there was an expected increase in post-test aggression score of 1.32 among animals that attacked during baseline resident-intruder trials ($t = 4.874$, $p < 0.0001$), distinguishing aggression phenotypes between mice that attack and those that do not and providing preliminary evidence that analyzing these two phenotypes together may confound simpler modeling approaches. Taken together, linear modeling shows that exposure to NaB, a

short-chain fatty acid and byproduct of gut microbiome fermentation, drives territorial aggression behavior in CD-1 male mice.

Short-term antibiotic treatment increases aggression behavior

In order to determine how gross changes in the gut microbiome would influence aggression behavior, a study of the effects of short-term, broad-spectrum antibiotics was conducted. In an experimental cohort of 60 CD-1 male mice, animals were randomly assigned to receive an antibiotic solution (n = 30) or standard drinking water (n = 30). Mice in the treatment group received a cocktail of broad-spectrum 1.0 g/L ampicillin and 0.5 g/L neomycin in their drinking water, beginning 48 hours prior to the initiation of resident-intruder screening and were maintained on antibiotics throughout the assessment. Animals then underwent aggression testing using the resident-intruder procedure and concomitant MAD model aggression scoring for a final sample of n = 28 antibiotic-treated and n = 26 control mice after removing animals that flooded cages during one of the trial days. Results of an unpaired t-test showed significantly increased aggression in the antibiotic group compared to controls (**Fig 26**; $t(52) = 2.060$, $p = 0.045$). Moreover, a parallel analysis of a small cohort of separate mice examined changes in weight over time as a proxy measure of the effects of antibiotics on health. Results showed no significant difference in weight between treatment (n = 4) and control (n = 4) groups over a period of 15 days (**Fig 27**; $F(14, 84) = 0.8006$, $p = 0.670$), indicating that the observed changes in behavior were not a product of an adverse drug effect. In sum, these data suggest that knock down of gut microbiome diversity drives territorial aggression behavior in CD-1 male mice.

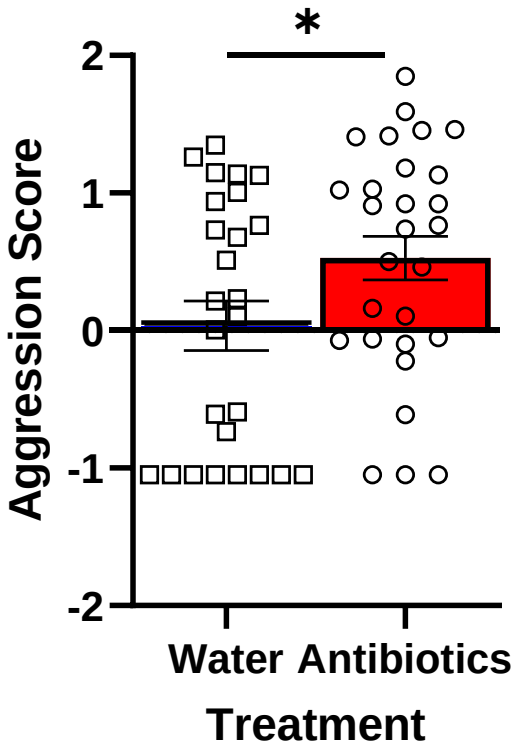

Figure 26 | Effects of Short-Term Antibiotics on Aggression. Results from an unpaired t-test of n = 54 CD-1 male mice show a significant increase ($p<0.05$) in the antibiotic group compared to controls. Error bars represent SEM while symbols show individual data points.

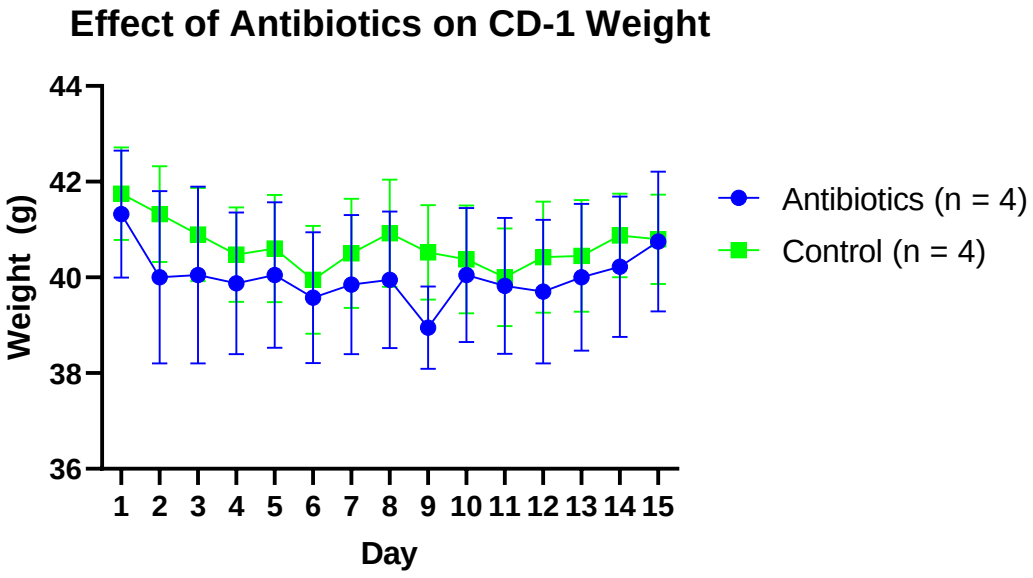

Figure 27 | Effects of Short-Term Antibiotics on Mouse Weight. Results from a repeated measures ANOVA of n = 8 CD-1 male mice showed no significant difference ($p>0.05$) in body weight (g) between antibiotic-treated and control mice. Error bars represent SEM.

To compare the structure of the gut microbiome community between antibiotic-treated and control CD-1 male mice, perimortem fecal samples were collected from the colon for downstream 16S rRNA amplicon sequencing. The final sample pool included 48 samples representing 1,020 taxa after normalization. Alpha and beta diversity measures were subsequently examined to describe community structure between groups. To assess alpha diversity, Chao1 diversity, a metric of bacteria richness, and Shannon diversity, a measure accounting for both richness and evenness, were calculated (**Table 11**). Results from Mann-Whitney U tests show a significant difference in Chao1 diversity between antibiotic-treated and control mice ($U = 0$, $p < 0.0001$). Likewise, there was a significant difference in Shannon diversity, a diversity between antibiotic-treated and control mice ($U = 1$, $p < 0.0001$). Indeed, such extreme values for the U-statistic indicate that every observation in the control condition is larger than the observations in the antibiotic-treated group, underscoring the marked decrease in diversity with antibiotics. Next, beta diversity, a sample (dis)similarity index, was examined using a PERMANOVA test. Findings indicate a strong effect of treatment condition on beta diversity ($F(1) = 30.091$, $R^2 = 0.436$, $p = 0.001$), delineating gut microbiome community structure between antibiotic-treated and control mice and highlighting the variability in the dysbiotic gut (**Fig 28**). Notably, there was no effect of aggression on beta diversity between conditions ($F(1) = 0.8626$, $R^2 = 0.01$, $p = 0.484$), but this is likely due to the spread of the antibiotic samples. Indeed, a test of beta dispersion, a measure of sample variance between conditions, showed significant differences between antibiotic and control mice ($F(1) = 87.892$, $p = 0.001$). Overall, these data show distinct microbial communities in the gut between antibiotic-treated and control mice.

Table 11 | Alpha Diversity Among Antibiotic-Treated and Control CD-1 Male Mice

Condition	Chao1	Chao1 SD	Shannon	Shannon SD
Control	388	53.7	4.28	0.314
Antibiotics	58.8	45.1	2.04	1.26

Table 11 shows average alpha diversity values for the Chao1 and Shannon diversity indices for n = 48 CD-1 male mice receiving antibiotics in their drinking water (n = 15) or controls (n = 26). Results from Mann-Whitney U test indicate significant differences ($p<0.0001$) in both alpha diversity metrics between treatment and control groups.

Sustained gut microbiome manipulation

In a follow-up analysis of sustained gut microbiome manipulation, the effects of long-term probiotics and antibiotics were explored in a novel cohort of 75 mice randomly assigned to receive a drinking solution of probiotics (n = 25), antibiotics (n = 25), or standard drinking water (n = 25) treatment. Mice assigned to the probiotic group received a commercially available VSL#3 probiotic supplement, beginning six weeks prior to the onset of resident-intruder testing. Mice in the antibiotic group were treated with a broad-spectrum antibiotic cocktail (1.0 g/L ampicillin, 0.5 g/L neomycin) for the two weeks leading up to aggression testing. Both drugs were maintained for the duration of the resident-intruder screening (**Fig 29**). Aggression testing was conducted for a period of 10 days (rather than three) using the resident-intruder procedure. Given the stability of attack behavior described in Chapter 3, aggression scores were calculated with indicator variables from the first three days of screening using the MAD model. Data were analyzed via one-way ANOVA to determine the effects of sustained treatment with probiotics and antibiotics on aggression behavior. Results indicate that there were no significant differences in aggression behavior between animals treated with probiotics or antibiotics compared to controls (**Fig 30**; $F(2, 72) = 1.456$, $p = 0.240$), suggesting that long-term manipulation of the gut microbiome is not associated with behavioral changes.

Beta Diversity of Mouse Fecal Microbiome for Antibiotic-Treated Mice

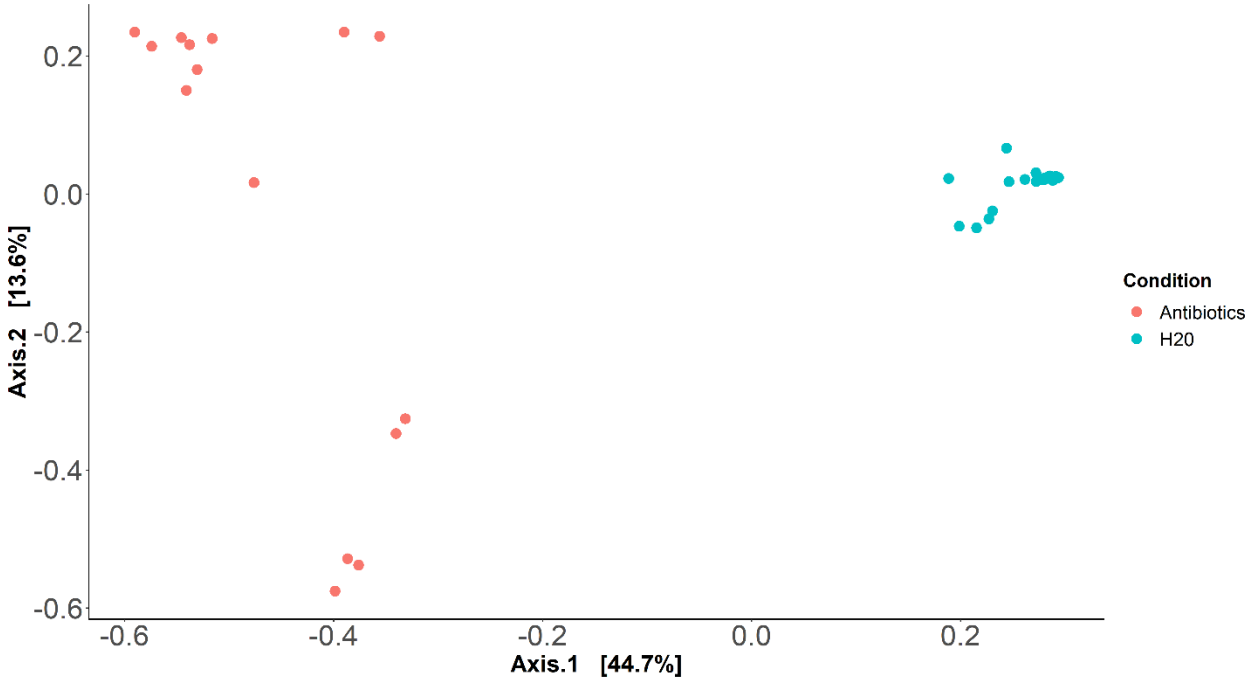

Figure 28 | PCoA Plot of Beta Diversity for Short-Term Antbiobiotic Treatment. PCoA plot of mouse fecal microbiome beta diversity using Unifrac distances. Each point represents one mouse fecal sample while the color indicates treatment condition. Schematic depicts the clustering of samples in the control condition and high variability in the antibiotic-treated group. PERMANOVA of beta diversity detected significant differences ($p<0.01$) between treatment conditions but not aggression score ($p>0.05$). Further analysis also showed a significant difference in beta dispersion ($p<0.01$).

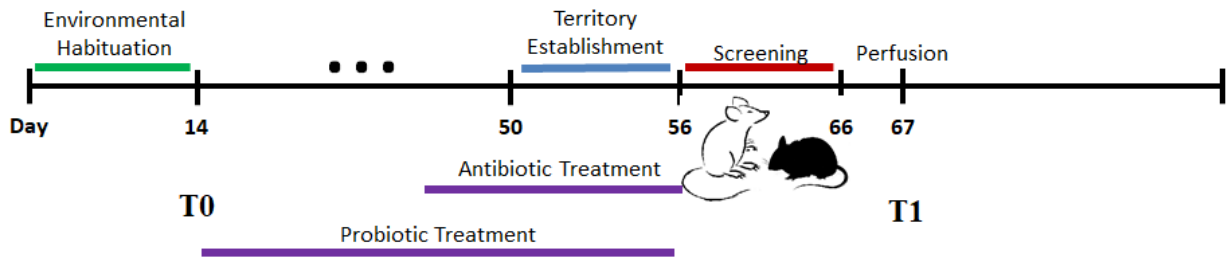

Figure 29 | Probiotic v Antibiotic v Control Experimental Design. Schematic showing the experimental design for n = 75 CD-1 male mice that received long-term treatment to alter the mouse fecal microbiome. Mice were evaluated for aggression using the resident-intruder procedure following treatment. Both baseline and post-test fecal samples were collected.

To investigate treatment-related changes in gut microbiome communities, both baseline and post-test fecal samples were collected for downstream analysis with 16S rRNA amplicon sequencing. The goal of this analysis was to examine changes in gut microbiome communities across groups over time, thereby ensuring fidelity of long-term treatments. Given the nature of drug effects, all the probiotic and control samples but only a subset of antibiotic samples were analyzed. As such, the final sample pool included 126 samples representing 924 taxa after library standardization. Communities were subsequently characterized using alpha and beta diversity measures (**Table 7; Fig 31**).

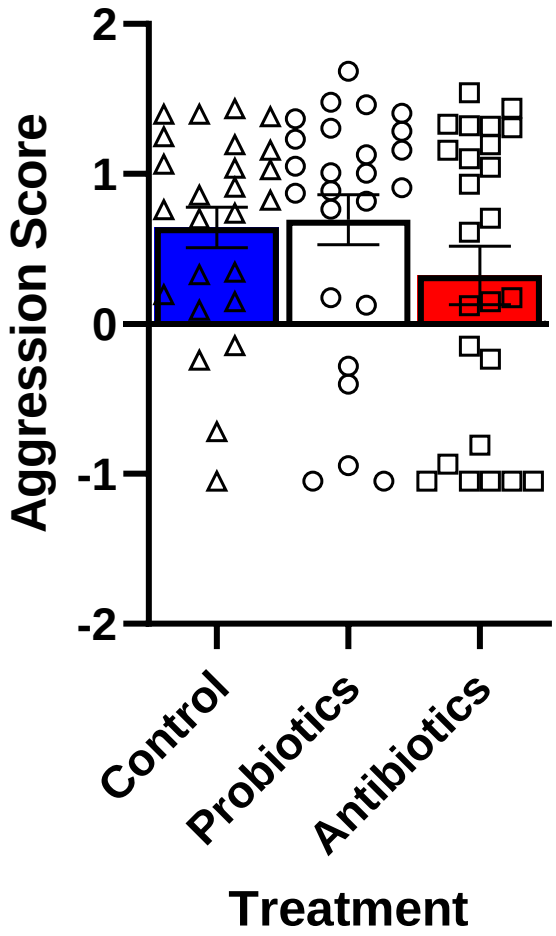

Figure 30 | Probiotic v Antibiotic v Control Treatment Effects. ANOVA results fail to show a significant difference in aggression behavior ($p>0.05$) between animals treated with probiotics versus antibiotics versus control. Error bars represent SEM while symbols depict individual data points.

Table 12 | Alpha Diversity by Treatment Group

	Chao1				Shannon			
	T0		T1		T0		T1	
Condition	M	SD	M	SD	M	SD	M	SD
Control	331	41.7	366	49.3	4.15	0.246	4.15	0.189
Probiotics	327	39.6	362	62.1	4.11	0.204	4.08	0.328
Antibiotics	332	30.2	106	189	4.20	0.205	1.98	1.60

Table 12 shows average alpha diversity values for the Chao1 and Shannon diversity indices at baseline (T0) and post-test (T1) for n = 53 CD-1 male mice that were treated with long-term probiotics (n = 24), antibiotics (n = 4), or control (n = 25). Kruskal-Wallis tests do not show significant differences between groups ($p>0.05$) at T1; though, low power in the antibiotic group is likely interfering with results.

Among control animals, there were significant changes in both alpha and beta diversity over time. Here, Shapiro-Wilk tests confirmed normality in the distributions of Chao1 and Shannon diversity metrics, permitting the use of parametric statistical tests. Thus, using paired t-tests to compare alpha diversity between baseline (T0) and post-test (T1), findings indicate a significant increase in Chao1 ($t(24) = -2.77$, $p = 0.011$) but not Shannon diversity ($t(24) = 0.0199$, $p = 0.984$), demonstrating a change in richness but not the distribution of taxa over time. Similarly, PERMANOVA testing demonstrated significant changes in beta diversity between baseline and post-test (**Fig 32;** $F(1) = 3.254$, $R^2 = 0.063$, $p = 0.001$), thereby describing distinct gut community structures between study timepoints. Overall, these findings suggest that time is a driving factor in gut microbiome composition among control animals, potentially confounding any aggression effects.

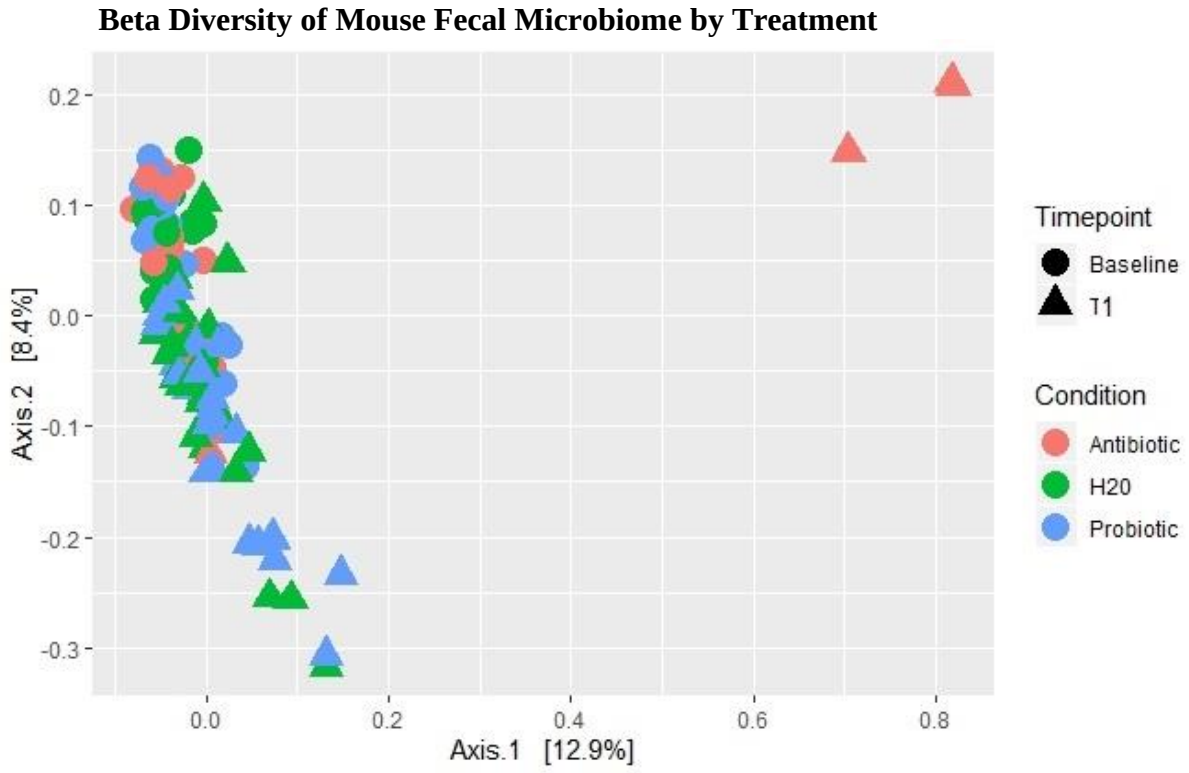

Figure 31 | PCoA Plot of Beta Diversity by Treatment. PCoA plot of mouse fecal microbiome beta diversity using Unifrac distances. Each point represents one mouse fecal sample while the color indicates sample condition. Symbol shape depicts sample collection timepoint at either baseline (T0) or post-test (T1) PERMANOVA of beta diversity detected significant differences ($p<0.01$) between conditions at T1. There were also significant

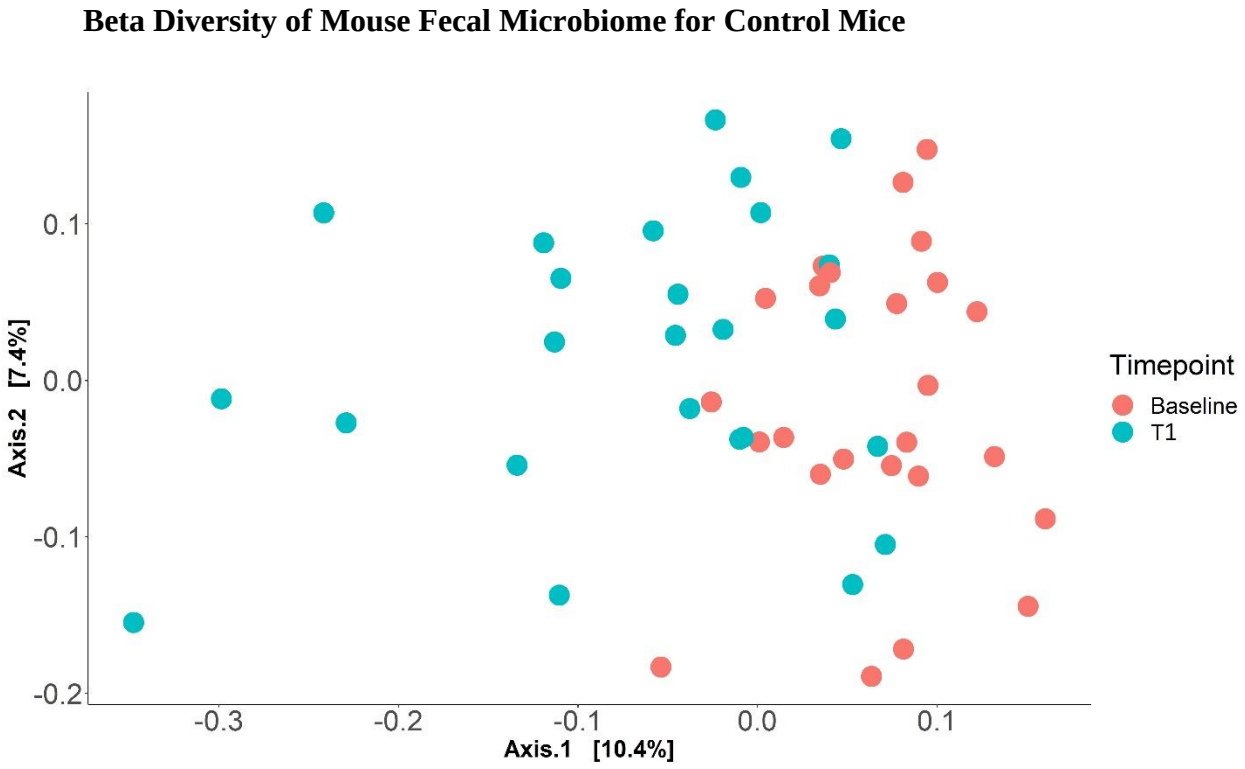

Figure 32 | PCoA Plot of Beta Diversity for Control Mice. PCoA plot of mouse fecal microbiome beta diversity using Unifrac distances. Each point represents one control mouse fecal sample while the color indicates sample timepoint. PERMANOVA of beta diversity detected significant differences ($p<0.01$) between timepoint.

Treatment fidelity was confirmed in both the probiotic and antibiotic groups by exploring changes in microbial community structure (i.e., alpha and beta diversity) secondary to sustained manipulation of the gut microbiome. Specifically, probiotics were expected to augment while antibiotics were expected to reduce diversity of the gut microbiome. As with controls, results of a paired t-test showed a significant increase in Chao1 ($t(23) = -2.43$, $p = 0.023$) but not Shannon diversity ($t(23) = 0.428$, $p = 0.673$) among probiotic-treated mice, suggesting that diversity but not distribution changed with probiotic treatment. Further analysis of beta diversity also demonstrated a significant difference between baseline and post-test sampling (**Fig 33**; $F(1) = 4.853$, $R^2 = 0.095$, $p = 0.001$), showcasing the efficacy of long-term probiotic treatment in shifting the mouse gut microbiome. Among antibiotic-treated mice, there were overall reductions in average Chao1 and Shannon diversity indices. However, formal testing with Kruskal-Wallis tests returned only a significant difference in Shannon ($H(1) = 5.280$, $p = 0.022$) but not Chao1 diversity ($H(1) = 2.482$, $p = 0.115$); though, it is likely these tests suffer from a lack of power. Likewise, PERMANOVA revealed significant differences in beta diversity between baseline and post-test samples collected from antibiotic-treated mice (**Fig 34**; $F(1) = 10.885$, $R^2 = 0.295$, $p = 0.001$). However, there was also a significant difference in beta dispersion (i.e., variance) between groups ($F(1) = 31.389$, $p = 0.001$), underscoring, again, the dissimilarity among the dysbiotic gut. Notably, there were some antibiotic-treated mice with high a very high number of reads in their sample sequence library but very low species diversity (**Fig 35**), suggesting a unique reconstitution of the gut microbiome with an antibiotic-resistant community. Taken together, these data confirm intended treatment effects and provide preliminary evidence that a non-diverse group of bacteria regrew in the antibiotic-treated animals over time.

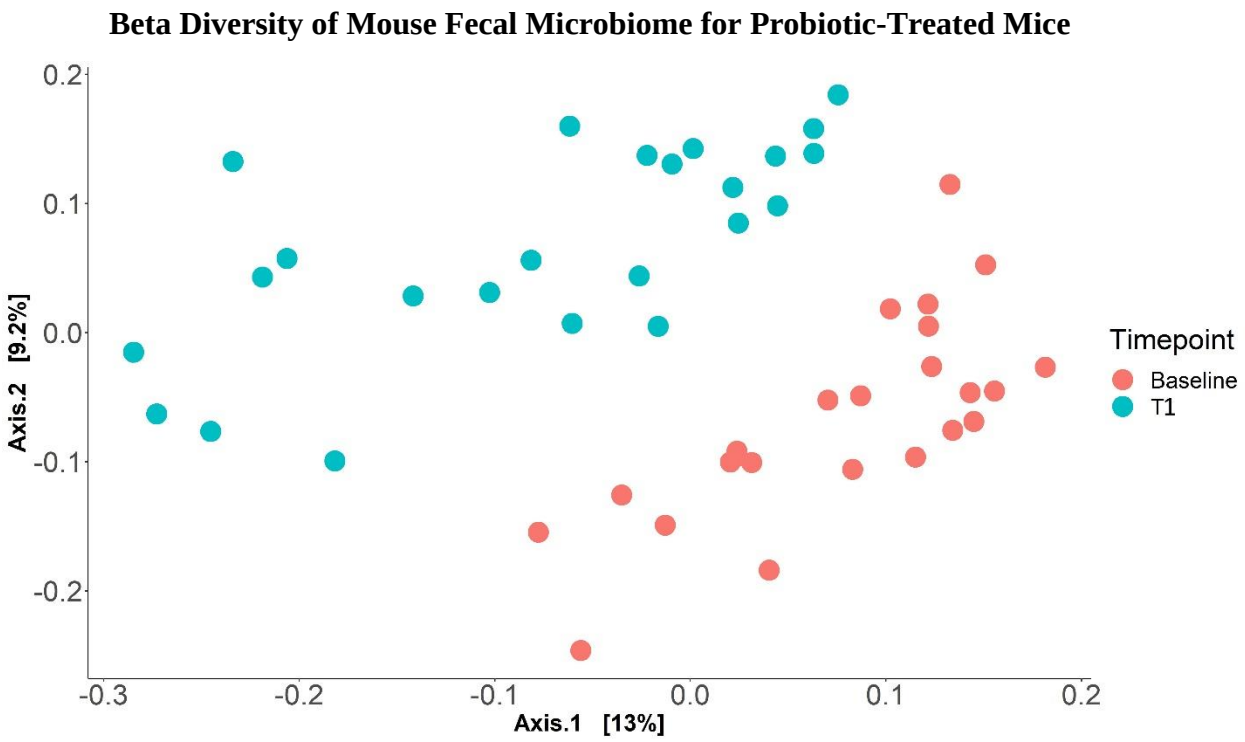

Figure 33 | PCoA Plot of Beta Diversity for Probiotic-Treated Mice. PCoA plot of mouse fecal microbiome beta diversity using Unifrac distances. Each point represents one probiotic-treated mouse fecal sample while the color indicates sample timepoint. PERMANOVA of beta diversity detected significant differences ($p<0.01$) between timepoint.

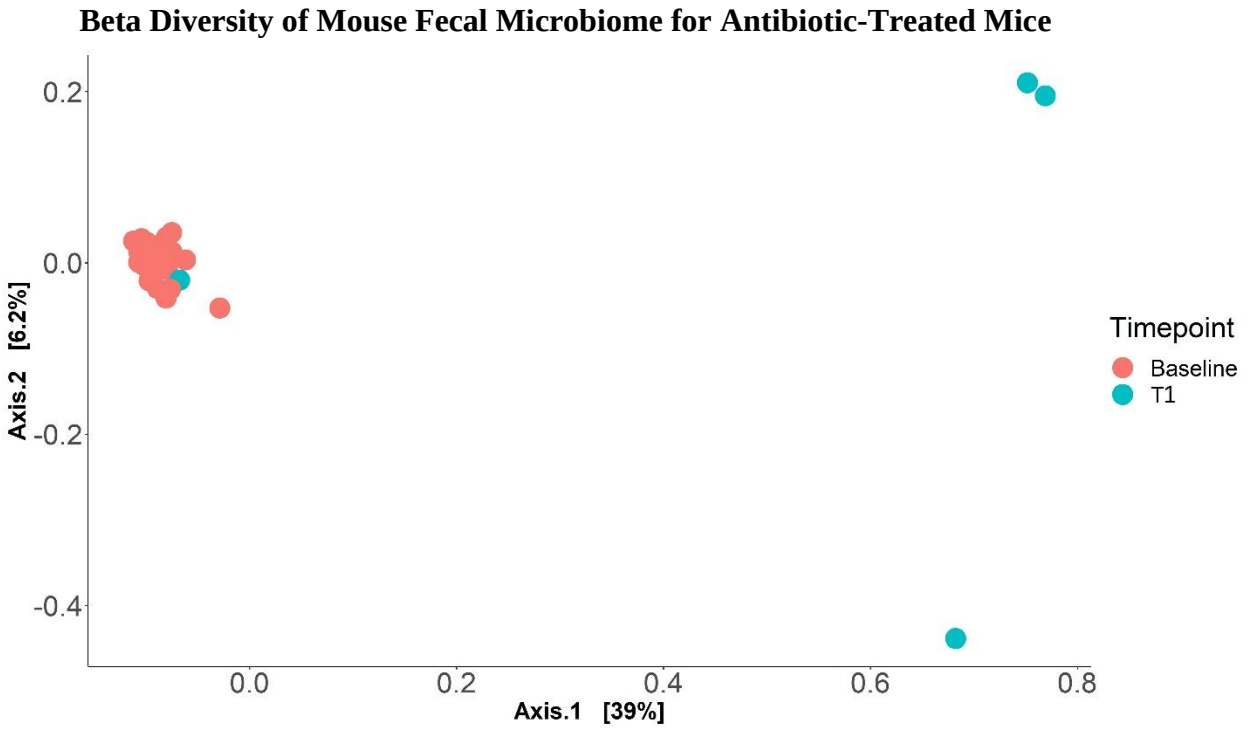

Figure 34 | PCoA Plot of Beta Diversity for Antibiotic-Treated Mice. PCoA plot of mouse fecal microbiome beta diversity using Unifrac distances. Points represents one antibiotic-treated mouse fecal sample and color shows sample timepoint. PERMANOVA of beta diversity detected significant differences ($p<0.01$) between timepoint.

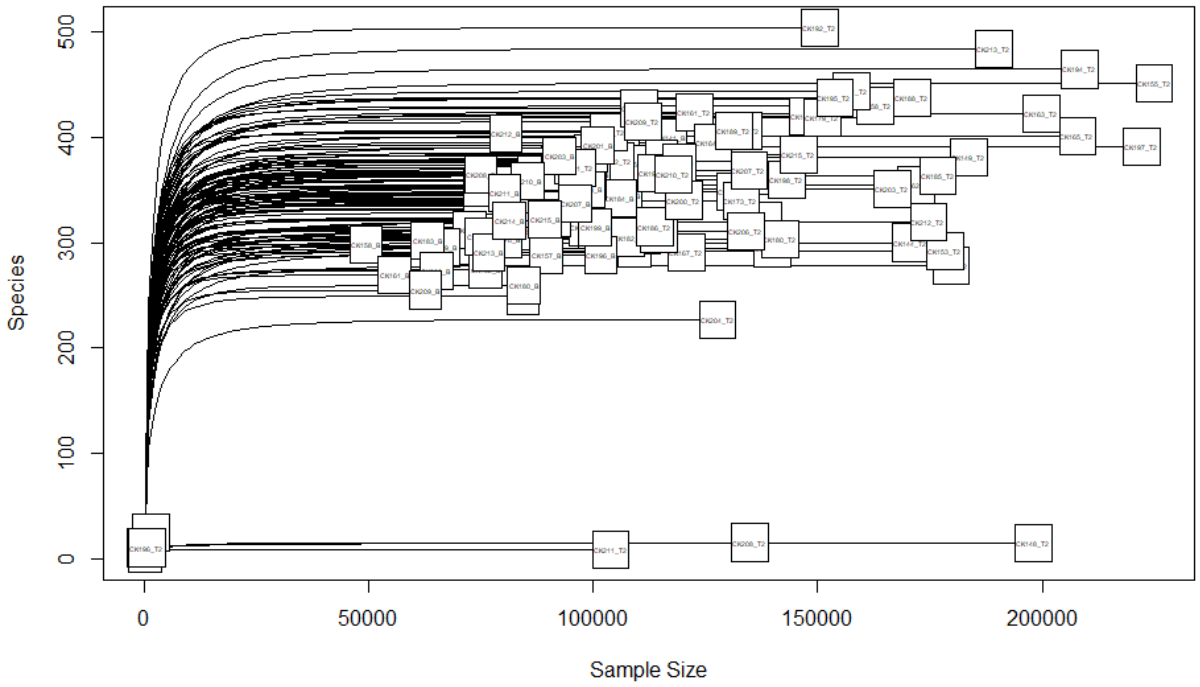

Figure 35 | Probiotic v Antibiotic v Control Rarefaction Curve. Rarefaction curve comparing the number of sequences in each sample sequence library (x-axis) and the number of bacteria (y-axis). A subset of antibiotic treated mice (bottom) demonstrated a high number of sequences but low overall diversity.

Pilot Study in Microbiome Transplantation

In an effort to determine the effects of transplantation with an aggressor donor microbiome, a pilot gut microbiome recolonization study was conducted (**Fig 36**). In this experiment, a cohort of 30 mice was screened for aggression at baseline, after a 48-hour treatment with broad-spectrum antibiotics (1.0 g/L ampicillin and 0.5 g/L neomycin) and following four weeks of recolonization with an aggressor donor microbiome. Within this cohort, 23 mice were randomly selected for treatment while the remaining seven served as controls. Following baseline resident-intruder screening (T0), experimental animals initiated a 48-hour regimen of a broad-spectrum antibiotic cocktail in their drinking water, after which aggression screening was repeated (T1). Following subsequent recolonization with an aggressor donor microbiome, a third and final resident-intruder screening was conducted (T2). Treatment efficacy was confirmed via Qubit DNA quantification (**Fig 37**).

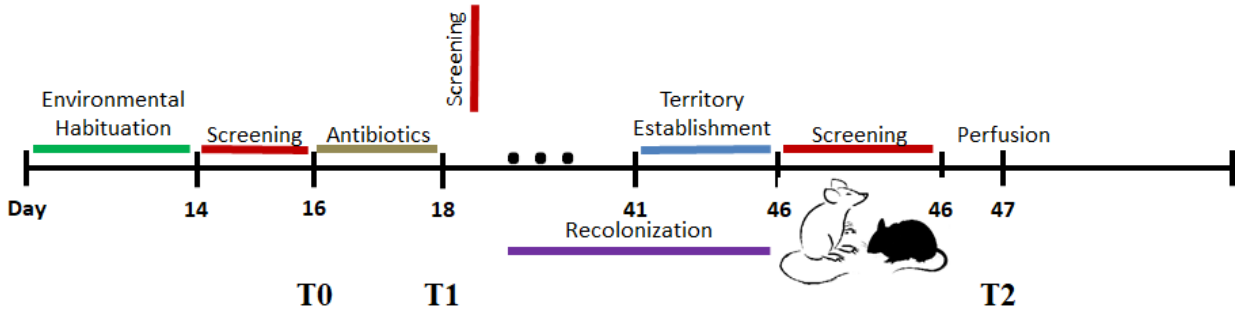

Figure 36 | Reconolonization Experimental Design. Schematic showing the experimental design for a pilot study of n = 30 CD-1 male mice that were evaluated for aggression at baseline (T0), following broad-spectrum antibiotic treatment (T1), and after recolonization (T2) with an aggressor donor microbiome. Fecal samples were collected at all three timepoints.

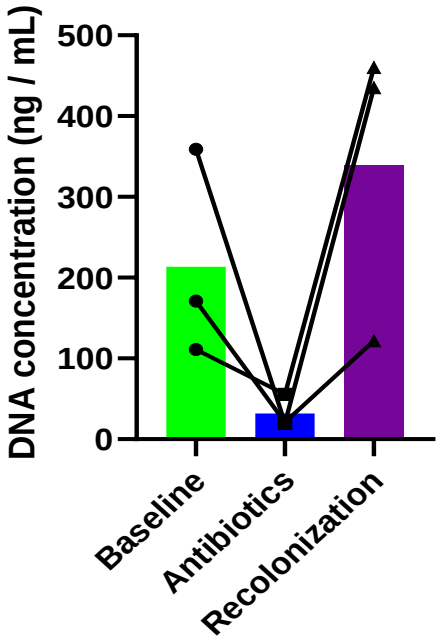

Figure 37 | Qubit DNA Concentrations. Schematic depicting Qubit DNA concentration (1 ng/mL) from a random sample of n = 3 mice undergoing gut microbiome elimination and recolonization. Samples were evaluated at baseline (T0), following broad-spectrum antibiotic treatment (T1), and after recolonization (T2). DNA concentrations were reduced at T1 and restored at T2.

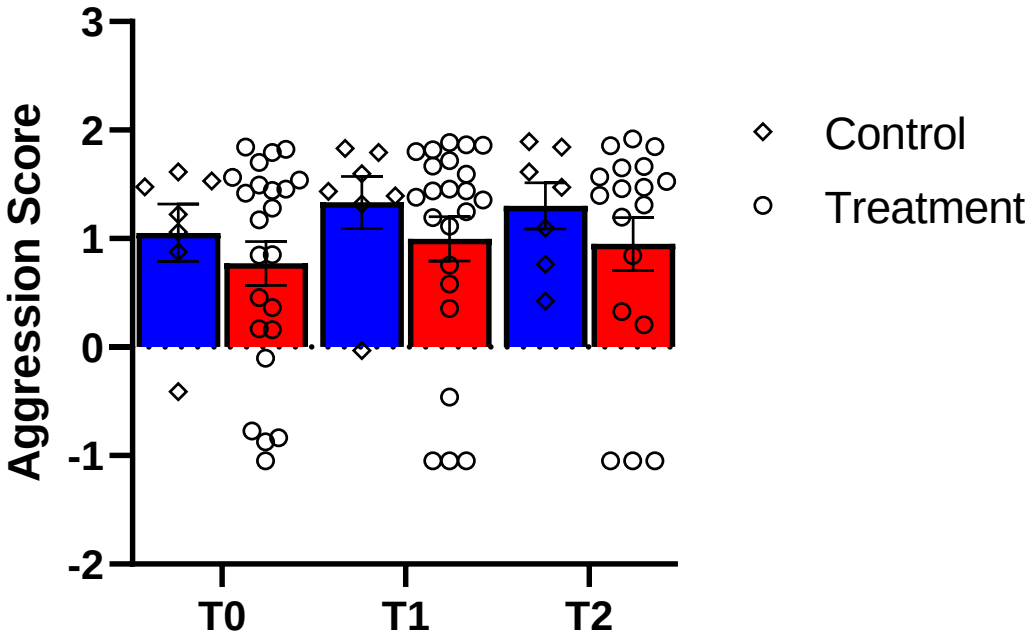

Figure 38 | Recolonization Treatment Effects. Results from a mixed effects ANOVA model show no significant differences in aggression score ($p>0.05$) in the treatment (n = 23) or control (n = 7) group over time. Aggression behavior did not change from baseline (T0), following broad-spectrum antibiotic treatment (T1), or after recolonization (T2) with an aggressor donor microbiome. Error bars represent SEM while bar color and symbol shape describe experimental condition.

Results indicate no overarching effect of antibiotic treatment or recolonization on aggression behavior (**Fig 38**). Though whole cohort analyses were hindered by unbalanced groups, a mixed effects ANOVA model was fitted, demonstrating a significant effect of time ($F(1.901, 48.47) = 4.176$, p 0.022) on aggression behavior but neither an effect of treatment ($F(1, 28)$, 0.7983, $p = 0.3792$) nor an interaction between treatment and time ($F(2, 51) = 0.2525$, $p = 0.7778$). Treatment conditions were subsequently separated and analyzed independently. Among controls, results from a repeated measures ANOVA show that there was no significant change in aggression over time ($F(1.880, 11.28) = 1.804$, $p = 0.210$). Likewise, repeated measure ANOVA with animals in the treatment group also showed no significant change in aggression over time ($F(1.484, 25.23) = 3.206$, $p = 0.070$). However, follow-up Tukey's post hoc test were used to explore the observed trend, which revealed a trend towards increased aggression between baseline and treatment with antibiotics ($\Delta M = 0.2568$, $p = 0.069$) but no effect of recolonization ($\Delta M = -0.1271$, $p = 0.1736$).

Neuronal activity and aggression scores

In order to identify regions of the brain driving changes in aggresion behavior among mice with differential gut microbiome composition, immunohistochemistry of ΔFosB, an immediate early gene correlate of long-term regional activation, was conducted in a cohort ($n = 25$) of experimentally naïve mice. Areas with a known role in aggression behavior in both mice and humans were explored, including prefrontal cortex (PFC), ventromedial hypothalamus (VMH), amygdala, and nucleus accumbens (NAc), as depicted in **Fig 39**. A series of correlation analyses were conducted to evaluate the extent to which the expression of ΔFosB in each of these regions is associated with mouse aggression score (**Fig 40**). Results showed no association between ΔFosB expression in the PFC infralimbic area ($r = -0.01$, $p = 0.95$), a region with a known role in emotional regulation (Halász, Tóth, Kalló, Liposits, & Haller, 2006), and aggression.

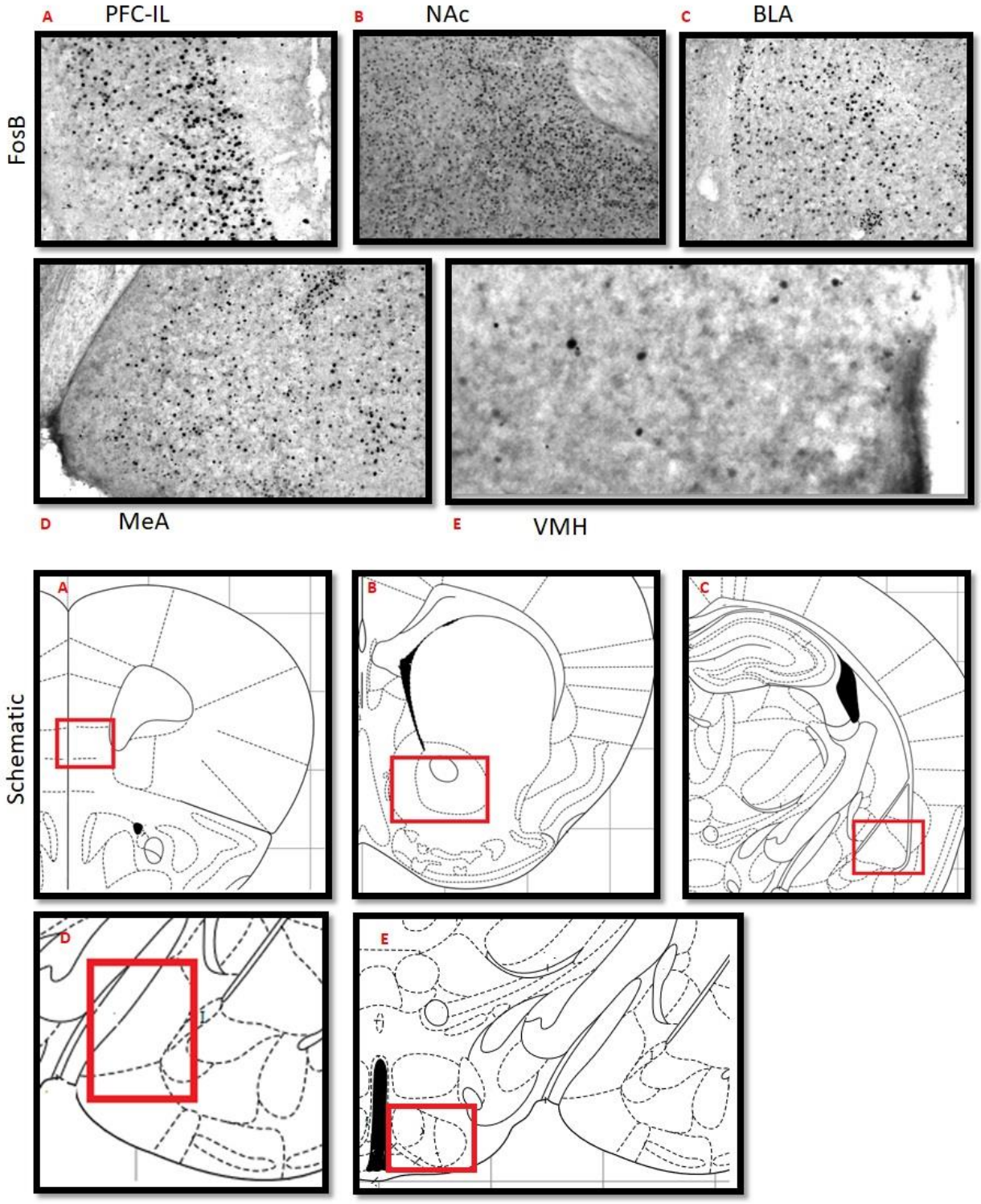

Figure 39 | ΔFosB Immunohistochemistry. a-e) Representative images of immunohistochemistry of ΔFosB in the a) PFC (n = 24), b) NAc Core (n = 24) and NAc Shell (n = 24), c) BLA (n = 21), d) MeA (n = 20), and e) VMH (n = 18).

Similarly, ΔFosB expression in the VMH, a region known for facilitating attack behavior (Falkner, Grosenick, Davidson, Deisseroth, & Lin, 2016), was not associated with aggression score (r = 0.11, p = 0.68). In the amygdala, neither the basolateral amygdala (BLA; r = 0.03, p = 0.91), with a role in learned defensive behaviors (Lingawi, Laurent, Westbrook, & Holmes, 2019), nor the medial amygdala (MeA; r = 0.07, p = 0.76), a region implicated in rivalry aggression (Jozsef Haller, 2018), demonstrated a relationship with aggression score. Likewise, expression of ΔFosB in both the NAc core (r = -0.32, p = 0.13) and NAc shell (r = -0.16, p = 0.46), regions involved in aggression reward (Golden et al., 2019), were not related to aggression. In sum, long-term regional

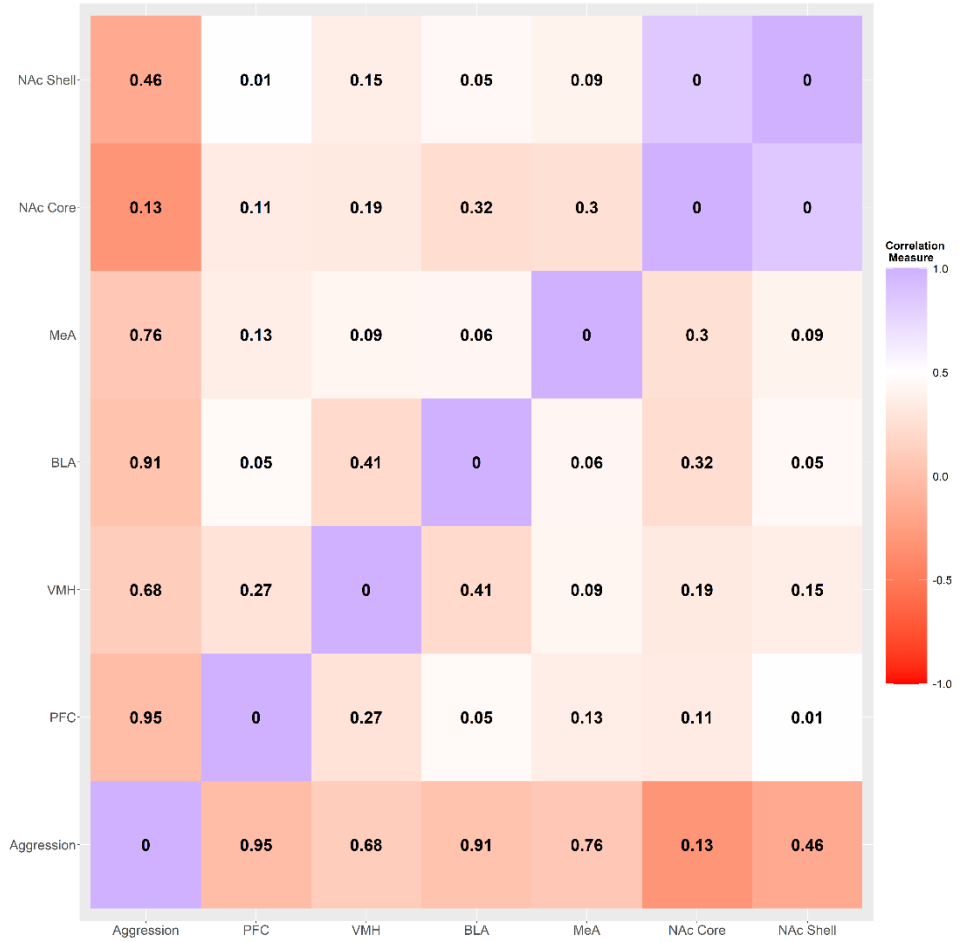

Figure 40 | ΔFosB Expression and Aggression Score. Correlogram showing results from a correlation analysis show no significant associations between expression of ΔFosB and aggression score (p>0.05) in the PFC (n = 24), VMH (n = 18), BLA (n = 21), MeA (n = 20), NAc Core (n = 24), or NAc Shell (n = 24). Color indicates the strength and direction of the correlation while the text shows the p-value of the test.

activation of aggression-related brain regions does not appear to have a relationship with territorial aggression in CD-1 male mice, showing the need for additional inquiry with a larger dataset and/or other methods of assessing brain region activity.

Discussion

These studies explore behavioral aggression and how it is related to the gut microbiome, a relationship that is currently not well understood. Initial experiments comparing gut microbiome composition across the spectrum of species-normative territorial, reactive aggression in experimentally naïve CD-1 male mice demonstrated a significant relationship between aggression behavior and microbiome beta diversity. Further analyses of bacterial abundance identified 40 OTUs that are both positively and negatively associated with aggression behavior. Among these groups, multiple genera from the *Lachnospiraceae*, a butyrate-producing family, were associated with aggression behavior. Indeed, we subsequently showed that exposure to systemic sodium butyrate (NaB) significantly increased aggression behavior in a novel cohort of mice. Overall, these data show strong support for the role of the gut microbiome in aggression behavior with preliminary evidence for NaB, a short-chain fatty acid produced by gut bacteria, as a potential mediator of the aggression-gut-brain link.

Emerging research shows that butyrate, a short-chain fatty acid critical in maintaining gut health, also plays a role in regulating neurophysiology and behavior. For example, Yamawaki and colleagues (2018) showed that chronic NaB administration rescued lipopolysaacharide (LPS)-induced depression behaviors and downregulated pro-inflammatory hippocampal microglia activation. This finding was associated with an increase in histone acetylation (Yamawaki et al., 2018), underscoring an epigenetic mechanism through which the gut microbiome can affect host behavior. Indeed, butyrate inhibits histone deacetylase (HDAC) activity to drive gene expression

(Tan et al., 2014), highlighting a potential mechanism through which differential expression of aggression behavior may manifest.

Further analysis into the aggression-related effects of changing gut microbiome diversity yielded mixed results. Specifically, the current study provides preliminary evidence that short-term (3 day) treatment with broad-spectrum antibiotics, an intervention designed to knock down microbial diversity, can increase aggression behavior. However, longer treatment (three weeks) with antibiotics did not show the same effect, possibly because antibiotic resistant bacteria recolonized the gut over this time, or because the mice homeostatically adapted to the altered gut microbiome, normalizing behavior over the course of weeks. On the other hand, treatment with probiotics, an intervention intended to promote gut health by augmenting the gut microbiome, had no significant effects on aggression behavior. Similarly, results showed no significant effect of recolonization with an aggressor donor microbiome, despite evident recolonization. In sum, short-term losses in microbial biodiversity are associated with increased aggression, but sustained treatments with longer potential for physiological adaptation had no effect on aggression behavior in these initial studies.

In a large sample of experimentally naïve CD-1 male mice, results failed to show any aggression-related differences in alpha diversity. This finding is unsurprising given that mice were specifically allotted time to habituate to the research facility. Michigan State University follows broader IACUC guidelines in animal care, which limits variation in animal care across universities in an effort to optimize animal welfare. Mice are therefore provided with standard chow, purified drinking water, stable housing with regular cage changes, and exposure to the same animal care staff when possible. As a result, environmental exposures are very controlled at any given cross section of time, resulting in very similar microbiome exposure for a given cohort of mice.

However, longitudinal variation can introduce a great deal of microbiome variation across cohorts separated by time.

For both alpha and beta diversity measures, results demonstrated a significant cohort effect on gut microbiome composition, showcasing the effects of time and cross-sectional exposures on microbial communities of the gut. Factors in the physical environment related to seasonal changes, building maintenance, staff changes, and colony composition may influence gut microbiome composition despite standardized approaches to animal care and the controlled conditions within an animal research facility. Indeed, 16S rRNA profiling data from fecal samples collected from control mice at weeks 1 and 10 show an increase in richness and a shift in beta diversity over time, suggesting that the gut microbiome community naturally varies in laboratory mice. This finding is in line with recent research that shows the microbiomes of laboratory mice are unstable and more responsive to environmental challenges compared to wild mice (Rosshart et al., 2019). As such, the described cohort effect has practical implications for experimental design, providing additional evidence that animal facility and laboratory conditions drive gut microbiome composition, an important physiological organizer that has the potential to affect behavioral outcomes over time.

The current study also demonstrated a significant interaction effect between cohort and aggression score, suggesting that the effect of cohort on gut microbiome composition is due, in part, to the differential distribution of aggression scores across experimental cohorts. This is unsurprising given that sample size varied between cohorts and that there is natural variation in the expression of species-normative territorial aggression in CD-1 male mice. In particular, previous research shows that approximately 70% of CD-1 males are aggressive to some degree while 30% are not (Golden et al., 2017). Therefore, cohorts with low sample sizes are not necessarily representative of all CD-1 male mice, in general. Thus, in addition to environmental

factors, there are cohort effects related to sample size and the physiology represented in smaller groups.

One limitation of the current work is the inclusion but lack of characterization of non-aggressive mice. Indeed, initial analyses of the effect of NaB on aggression behavior failed to demonstrate a significant treatment by time interaction effect, but *post hoc* analysis showed that only the NaB group significantly differed between baseline and the post-test, indicating the presence of a confounding factor in the analysis. One potential source of unaccounted variation is the behavior of non-aggressive animals, a phenotype which is poorly understood. The absence of attack behavior does not necessarily imply the lack of any behavior or the presence of a prosocial replacement behavior. Some animals engage in grooming or other dominance behaviors, which lends itself towards the development of aggressive behavior. Indeed, previous research describes a third, variably aggressive phenotype that emerges when specifically non-aggressors are repeatedly tested and begin to display aggression behavior (Golden et al., 2017). Currently, there are no tools to determine which non-aggressive mice are simply not aggressive versus which mice will develop this variably aggressive phenotype, potentially unbalancing otherwise homogenous groups. Future work should strive to better characterize this variably aggressive group to enable the use of more conventional statistical tests.

Summary

This chapter explored aggression-related differences in gut microbiome composition in a preclinical model of territorial, reactive aggression. Results showed significant differences in gut microbiome composition with specific, butyrate-producing taxa differing between aggressive and non-aggressive mice. Follow-up work demonstrated sodium butyrate drives aggression behavior, elevating short-chain fatty acids as a potential molecular mechanism in this relationship.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Summary

The work presented in this dissertation contributes to the field of neuroscience in several ways. Overall, the preclinical data describe a novel connection between the gut microbiome and territorial, reactive aggression behavior in mice that holds translational relevance for human public health. Together, these findings move toward a biopsychosocial model of aggression behavior, addressing an important gap in neuroscientific research. Specifically, current neurobiological research that uses animal models does not account for the broad array of social and environmental factors that shape the dysfunctional neural systems underlying aggression behavior in people. By extending my preclinical research to a system affected by environmental factors, this thesis describes the relationship between the gut microbiome and aggression behavior that could explain why context matters for human aggression behavior

To accomplish this research, the experiments described in Chapter 3 were conducted to develop a mathematical model for measuring aggression behavior in sexually experienced CD-1 male mice. Through collaboration with Drs. Sam Golden, Andrew Bender, and Hope Akaeze, a novel, quantitative aggression scoring model was developed using confirmatory factor analysis (CFA), a structural equation modeling (SEM) framework for measuring latent factors, like aggression. Using the MAD model, aggression scores were generated for 658 animals from five different laboratories at three different institutions across a decade of research that accurately represented CD-1 territorial aggression behavior, thereby standardizing aggression measurement and allowing for direct comparison of aggression behavior over time and between different laboratories. Moreover, aggression scores were stable over repeated measurements, which provides an avenue to improve aggressor selection for studies of chronic social defeat stress

(CSDS). Indeed, aggressor selection affected CSDS outcomes such that exposure to high performing, consistently aggressive CD-1 mice induced both resilient and stress-susceptible phenotypes in juvenile C57BL/6J mice, an effect that was not observed among similar mice exposed to low aggression CD-1s. Importantly, the MAD model allowed data aggregation for the remainder of the described preclinical work.

Experiments in Chapter 4 investigated the relationship between the gut microbiome and territorial, reactive aggression behavior in sexually experienced CD-1 male mice. Here, results from a large-scale analysis of experimentally naïve mice unveiled a previously unknown relationship between gut microbiome composition and aggression behavior. Negative binomial mixed models were subsequently used to identify which bacteria differed between aggressive and non-aggressive mice, returning multiple genera within the *Lachnospiracaeae* family, a taxon of butyrate-producing bacteria that were significantly associated with aggression. In a follow-up experiment examining the potential aggression-related effects of systemic exposure to sodium butyrate (NaB), a short-chain fatty acid (SCFA) regulating histone deacetylase (HDAC) activity, findings demonstrated a significant increase in aggression behavior in the NaB group compared to controls, supplying a molecular mechanism through which the microbiota of the gut could affect neurophysiological processes that drive aggression behavior. Further investigation of the effects of antibiotics on aggression behavior showed that short-term, but not long-term, ablation of the microbiome with antibiotics increased aggression behavior. Altogether, these data provide strong support for a causal role of the gut microbiome in territorial, reactive aggression behavior in CD-1 male mice.

Implications and Future Directions

Potential brain epigenetic effects of the gut microbiome

The structural and functional changes observed in the corticolimbic system of the brain underlying aggression behavior are in part due to changes in gene expression resulting from a normally adaptive process wherein unique environmental contexts confer preparedness for survival. To this end, epigenetics describes the mechanisms by which gene expression, but not the genes themselves, changes in response to the environment. One of the primary mechanisms regulating gene expression is histone modification. In a cell, histones are proteins around which DNA wraps, facilitating or prohibiting access to different sections, or amplicons, of DNA depending on their location within the chromatin superstructure. Histones have sites of molecular interaction that modify how the DNA is coiled, thereby exposing DNA so it can be transcribed and expressed. One modification is the addition of positively charged acetyl groups to lysine residues at the N-terminal tails of histones, leading to an open chromatin configuration permissive of gene transcription. Epigenetic regulators, like histone deacetylases (HDACs), remove these acetyl groups to reduce gene expression.

Experiments presented in Chapter 4 provide evidence of a causal relationship between gut microbiome composition and aggression behavior that is mediated by short-chain fatty acid signaling, underscoring the potential of the gut microbiome as a potential epigenetic regulator of aggression behavior. Though the gut microbiota exhibit multiple epigenetic mechanisms (Qin & Wade, 2018; Savidge, 2016; Tan et al., 2014), evidence suggests that short-chain fatty acids (SCFA), byproducts of gut bacteria fermentation of dietary fiber, have a regulatory role over HDAC activity. Specifically, butyrate inhibits HDAC activity (Choi & Friso, 2010), thereby disinhibiting gene expression, providing a direct molecular mechanism through which the gut

microbiome could exert its epigenetic effects. Thus, it is possible that butyrate produced in the gut can reach the brain via the circulatory system and inhibit histone deacetylase activity to alter gene expression. Such alterations in gene expression could lead to changes in activity of brain regions underlying aggression behavior, but further experiments are needed to determine whether this occurs and: 1) which genes are affected; 2) of those affected genes, which are causal of changes in neuronal function; and 3) in which brain regions does this occur to drive aggression.

Future Work

Additional research is needed to extend this dissertation work. The data presented here demonstrate a relationship between gut microbiome composition and aggression behavior in territorial, reactive CD-1 male mice. Moreover, results show behavioral data that support a causal effect of SCFA-mediated epigenetic changes in murine aggression behavior. However, these findings have yet to be connected to effects on aggression-related brain regions that ultimately drive aggression behavior. To this end, immunohistochemical research is needed, both bolstering sample sizes for further inquiry with ΔFosB and adding cFos to capture activity directly related to aggression behavior. To investigate epigenetic changes in histone acetylation related to HDAC activity, a pan-acetyl histone antibody needs to be used to quantify differences in histone acetylation related to gut microbiome composition. In addition, there is evidence that butyrate is a histone modifier in and of itself via lysine butyrylation (Chen et al., 2007), and further inquiry is needed to determine the actual epigenetic effects of butyrate, if any. As a complementary step, future research needs to move beyond 16S rRNA microbial profiling and utilize metabolomic approaches to assess systemic levels of butyrate and other microbial metabolites related to aggression behavior.

The effects of probiotics, antibiotics, and recolonization on behavior are widely discussed in the literature (Hsiao et al., 2013; Lach et al., 2020; Mörkl, Butler, Holl, Cryan, & Dinan, 2020), but results of resident-intruder screenings showed no effect of recolonization with an aggressor donor microbiome or long-term probiotics and antibiotics. This is perhaps a result of using a "dirty" model in that experiments were not conducted with germ-free mice, which is a standard model in preclinical gut microbiome research. Given that the resident-intruder screening is inherently social, with exposure of the would-be germ-free mice to intruder mice, future aggression research needs to be conducted with both germ-free residents and germ-free intruders, allowing complete control of the microbial contents of the mouse gut.

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