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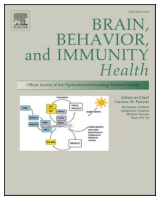

Chronic variable stress leads to sex specific gut microbiome alterations in mice

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A B S T R A C T

Stress has been implicated in the incidence and severity of psychiatric and gastrointestinal disorders. The immune system is capable of modulating the activity and composition of the gut following stress and vice versa. In this study we sought to examine the sequential relationship between immune signaling and microbiome composition occurring in male and female mice over time using a variable stress paradigm. Tissue was collected prior to, during, and after the stress paradigm from the same mice. Cytokines from plasma and brain were quantified using a multiplexed cytokine assay. Fecal samples were collected at the same timepoints and 16S rRNA amplicon sequencing was performed to determine the relative abundance of microbiota residing in the guts of stressed and control mice. We found sex differences in the response of the gut microbiota to stress following 28 days of chronic variable stress but not 6 days of sub-chronic variable stress. Immune activation was quantified in the nucleus accumbens immediately following Sub-chronic variable when alterations of gut composition had not yet occurred. In both sexes, 28 days of stress induced significant changes in the proportion of *Erysipelotrichaceae* and *Lactobacillaceae*, but in opposite directions for male and female mice. Alterations to the gut microbiome in both sexes were associated with changes in cytokines related to eosinophilic immune activity. Our use of an animal stress model reveals the immune mechanisms that may underly changes in gut microbiome composition during and after stress. This study reveals potential drug targets and microbiota of interest for the intervention of stress related conditions.

1. Introduction

The stress response is an important biological mechanism that encourages organisms to avoid danger, and/or noxious stimuli. When stress becomes chronic, it can become pathological and is associated with immune system modulation as well as psychological disorder incidence and severity (Glaser et al., 1987; Kiecolt-Glaser et al., 1996; Brady and Sinha, 2005; McEwen, 2017). Recently, the gut microbiome has been identified as contributing to the stress response, and it is also heavily impacted by psychiatric and gastrointestinal disorders (Kelly et al., 2015; Foster et al., 2017; Maes et al., 2019; Dinan and Cryan, 2017). The immune system has a modulatory role in the gut, is also activated during stress, and is implicated in the etiology of psychiatric disorders (Fung et al., 2017; Kiecolt-Glaser et al., 1996). It is currently thought that there is a microbiota-gut-brain axis that has yet to be fully identified and forms a network that is relevant to a wide range of stress related conditions (Kentner et al., 2019; Cryan et al., 2019). Understanding the mechanisms that underlie gut alterations due to stress and immune activity could provide insight into potential therapeutics and drug targets for gastrointestinal and psychiatric conditions.

Changes in gut microbial composition due to psychological stress occur through vagal nerve signaling, glucocorticoid regulation, enteric neurotransmission, and immune activation (Fig. 1A, B, 1C) (Kinsey et al., 2007; Furness, 2012; Breit et al., 2018; Fülling et al., 2019; Xu et al., 2020; Muller et al., 2020; Müller et al., 2022). Alterations that occur in the gut microbiome can be compensatory in an effort to relieve the conditions that caused it, or deleterious, where alterations cause harm to the host organism (Zhang et al., 2015). Stress also causes the loosening of the intestinal barrier that surrounds the gut, which has been deemed the "leaky gut theory" (Maes et al., 2008, 2019). When the barrier surrounding the gut loosens, it makes it more likely for the passage of bioactive elements into and out of the gut. The leaky gut also makes it more likely that circulating immune molecules, such as cytokines and chemokines, will enter the gut (Fig. 1D) (Keita and Söderholm, 2010; Camilleri, 2019). The leaky gut in combination with alterations to gut microbiome composition result in altered release bioactive materials such as short-chain fatty acids, cytokines, and lipopolysaccharides (LPS) into the laminal space (Fig. 1E) (Schirmer et al., 2016; Piero Portincasa et al., 2022; Marcello Candelli et al., 2021). When the gut is leakier, these biologically active elements can get into the circulatory system and affect various parts of the body. Because the gut has resident immune cells that regulate microbial composition in the gut, activation of

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these cells by cytokines entering the barrier will change immune cell activity, and therefore change composition of the gut microbiome (Kamada and Núñez, 2014). When the gut microbial composition is changed, the immune elements that the microbes of the gut release will also change, triggering further modulation of immune activity (Fig. 1F). Modulation of immune activity around the body will lead to altered activity in the ongoing repair process for stress in the brain (Fig. 1G). By understanding stress-induced alterations of gut microbial populations and parallel changes immune signaling molecules, we can begin to understand how the immune system and gut interact due to stress in a sex specific way.

Historically, animal models used in neuroscience over-represented male subjects or have not been designed to properly detect sex differences (Beery and Zucker, 2011; Rechlin et al., 2022). Therefore sex-specific mechanisms, including those that underlie stress, have been missing from the literature. Sex specific immune activity in the brain is linked to sex differences found in depression and anxiety (Kentner et al., 2010; Mandakh Bekhbat and Neigh, 2018). Microbial profiles in the gut of both humans and rodents are individualistic and sex specific (Yong Sung Kim et al., 2020). The hypothalamic–pituitary–adrenal (HPA) axis is activated during stress and shows sex differences that start during development (Leonidas Panagiotakopoulos and Neigh, 2014). Transplantation of gut microbiota between sexes can confer some of the sex specific immune properties of the host into a recipient of the other sex (Markle et al., 2013). Because the gut microbiome is related to psychiatric conditions, immune activation, and stress, uncovering the effects of stress on both gut microbes and the immune system may indicate potential targets or intervention strategies for stress and gut dysbiosis. Doing so in a sex specific way will allow for these potential treatments to be more generalizable to a wider clinical population that suffers from stress-related conditions.

Stress is ubiquitous in mammals and therefore can be robustly generated and studied. In mouse models, the chronic variable stress model is a well-established paradigm to induce stress-related behaviors, immune activation, and elevated circulating corticosterone (LaPlant et al., 2009; Hodes et al., 2015; Johnson et al., 2021). Subchronic (6 days) variable stress causes behavioral changes compared to controls in female mice but not male mice. When variable stress becomes chronic (28 days), both males and females display altered behavior, including decreased motivation for food reward, decreased time grooming, and increased passive coping responses (Hodes et al., 2015; Johnson et al., 2021). To our knowledge, there have been no studies to date on the effects of subchronic or chronic variable stress on alterations in the composition of the gut microbiome in either sex. To determine how stress impacts the gut microbiome in both sexes as stress shifts from an acute to chronic condition, we examined alterations that occur in male and female mice over the course of 28 days compared to unstressed control mice. Samples of blood and feces were collected before stress, after 6 days, and after 28 days of variable stress. We hypothesized that the stress group would suffer from dysbiosis and that the controls would not, with females showing signs of dysbiosis after 6 days of stress and males showing signs after 28 days of stress. Next generation sequencing was performed on fecal samples and a multiplexed cytokine assay was used to determine the relationship between immune activity and microbiome alterations. These targets were chosen in order to understand the co-occurring actions of cytokines and gut microbiome changes due to stress.

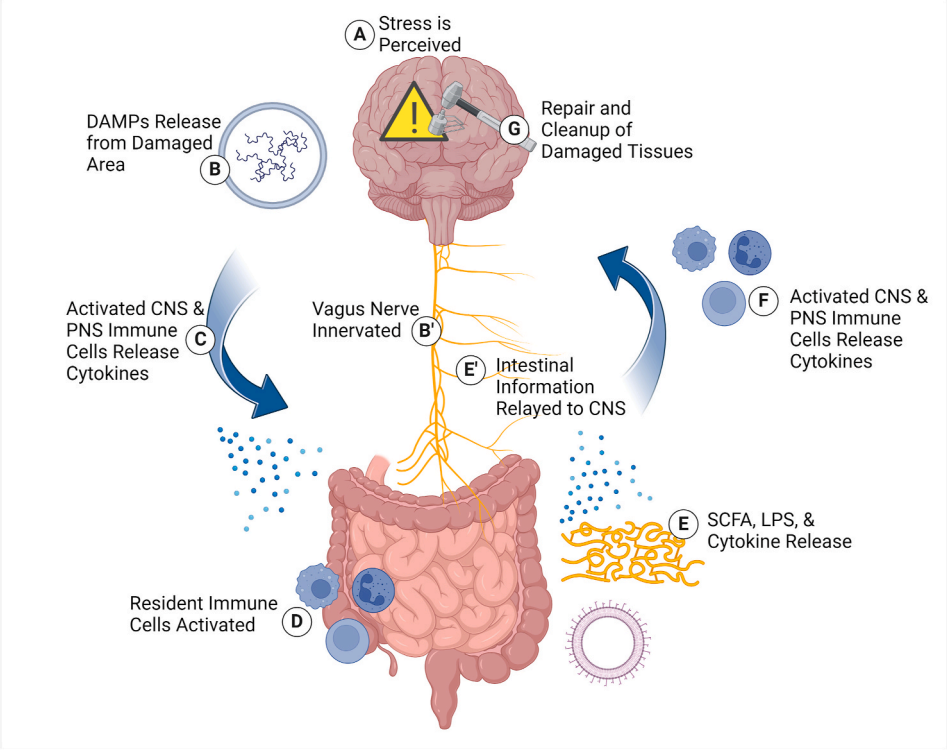

Fig. 1. Conceptual overview of stress perception translating into immune activity and compositional gut change. The stress response occurs after a psychological stress is perceived in the organism (A). Upon stress, damage-associated molecular patterns are generated in the brain (B). At the same time that this is occurring, vagal signaling is activated going from the brain to the gut (B′). DAMPs generated in the brain migrate into the bloodstream and around the body, activating pattern recognition receptors on immune cells causing them to release immune signaling molecules (C). These immune signaling molecules travel through the bloodstream and activate receptors on resident immune cells in and around the gut (D). These activated resident immune cells release their own cytokines and alter the composition of gut microbiota thus altering the release of short-chain fatty acids and LPS from the microbiota (E). At the same time, the gut is signaling about altered activity in the gut to the brain via the vagus nerve (E′). These alterations lead to further activation of immune cells around the body (F). This altered immune and microbe profile effect the ongoing immune effort of repair and cleanup in the brain that was triggered by stress perception in the brain (G).

2. Materials and methods

2.1. Animals

All procedures were performed in accordance with the Institutional Animal Care and Use Committee guidelines of Virginia Tech, Protocol #s: 19–181 and 19–202.

2.1.1. Cohort 1

C57BL/6J male and female mice (Jackson Laboratory) were 8 weeks old at the start of the study. All animals were pair housed with paper bedding and maintained on a 12-h light/dark cycle with ad libitum access to food and water. Feces were collected from one mouse per cage to avoid bias associated with coprophagy (the ingestion of feces). For the stress group, we used an n = 12 (6 males and 6 females); for the control group, an n = 12 was used (6 males and 6 females). Fecal samples were collected at three timepoints resulting in 72 microbiome samples total.

2.1.2. Cohort 2

C57BL/6J male and female mice (Jackson Laboratory) at 8 weeks of age were used for the second cohort. For the stress group, we used an n = 20 (10 males and 10 females); for the control group, an n = 20 was used (10 males and 10 females). Variable stress was conducted over the course of 6 days. Animals were sacrificed immediately following the final stressor. All procedures were performed in accordance with the Institutional Animal Care and Use Committee guidelines of Virginia Tech.

2.2. Variable stress

A variable stress model previously described (LaPlant et al., 2009; Hodes et al., 2015) was used to induce stress in male and female mice over the course of 28 days. This paradigm consists of three stressors given once per day: a 2 s foot shock of 0.45 mA administered every 36 s over the course of an hour, tail suspension for 1 h, or restraint in a 50 mL conical restraint tube placed in their home cage for 1 h. These stressors were repeated in the same order over the course of 28 days for the stress group of animals.

2.3. Tissue/sample collection

2.3.1. Cohort 1

Blood was collected from the same individuals by submandibular bleed 4 days prior to stress, 24 h after 6 days of stress and 24 h after 28 days of stress. Blood samples were centrifuged (Eppendorf 5424R) at 4 °C, 1500RCF for 15 min to isolate plasma and was aliquoted for cytokine quantification. Fecal samples were collected from one mouse per cage to avoid redundancy due to coprophagia. Fecal samples were collected immediately prior to blood collection. Left NAc (3.83, 5.677, 4.434), right NAc (3.83, 5.677, 6.648), PFC (3.83, 4.502, 5.601), left hippocampus (7.485, 1.989, 3.873), and right hippocampus (7.485, 1.989, 7.433), were micropunched and immediately frozen for analysis (Lein et al., 2007). All samples were stored at −80 °C following collection to preserve until the time of processing/analysis.

2.3.2. Cohort 2

All mice in cohort 2 were immediately sacrificed following 6 days of variable stress. At the time of sacrifice, blood was collected and brains were removed. The brains were immediately sliced in a brain matrix and punched for PFC and Nucleus Accumbens in the same areas as described for cohort 1. Blood samples were centrifuged (Eppendorf 5424R) at 4 °C, 1500RCF for 15 min to isolate plasma and was aliquoted for cytokine quantification. All samples were stored at −80 °C following collection to preserve until the time of processing/analysis.

2.4. Microbiome sequencing and analysis

DNA isolation was performed with Quick-DNA™ Fecal/Soil isolation kit (Zymo Research). The 16S rRNA V4 region gene was amplified by PCR and sequenced utilizing the MiSeq platform as previously described (Kumar et al., 2014). Sequencing data was denoised and dereplicated using the DADA2 package in RStudio (Callahan et al., 2016). Forward reads were truncated at 240bp and reverse reads were truncated at 160bp. Samples below a quality score of 4 were truncated and the maximum expected errors allowed was 5. Taxonomy was assigned to sequences using the open-source RNA SILVA file repository (Quast et al., 2012). ASV tables were generated with the count and taxonomic data and transformed into a phyloseq object using the phyloseq package (McMurdie and Holmes, 2013). Relative abundance of taxa was plotted using the phyloseq package. The phyloseq object was converted to a DESeq object and the DESeq function was run with a wald test and a local fit type (Love et al., 2014). Volcano Plots and histograms plotted with ggplot2 using DESeq data. Correlations calculated and plotted using corrplot v0.92 (Wei and Simko, 2021).

2.5. Plasma and brain cytokine assay

Inflammatory immune activity was measured in male and female mice at various timepoints using a MILLIPLEX® MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (MCYTMAG-70K-PX32, EMD Millipore) which quantifies 32 cytokines concurrently. Blood samples were centrifuged and plasma was collected via submandibular bleed and stored at −80 °C. Brain tissue was isolated using micropunch, flash-frozen in isopentane and stored at −80 °C until analysis. The tissue was homogenized in 0.25 ml of lysis buffer (100 mM PIPES, pH 7.0, 500 mM NaCl, 2 mM EDTA, 0.1% sodium azide, 0.2% Triton X-100, 5 μg/ml aprotinin, 0.1 μg/ml pepstatin A, and 0.5 μg/ml antipain). The homogenate was centrifuged at 2500 RPM for 30 min at 4 °C. The supernatant was removed, and the amount of protein in each sample was measured in duplicate by detergent compatible (DC) protein assay (BioRad, 5000111). Following protein quantification on a microplate reader (EMAX) all samples were diluted to a concentration of 2 mg/ml of protein in lysis buffer. At the time of analysis, the plasma samples were vortexed and centrifuged immediately prior to use. Samples were measured in a Luminex MAGPIX and then quantified in the MILLIPLEX® Analyst 5.1 software. The cytokines/chemokines included in the quantification were: Eotaxin, G-CSF, GM-CSF, IFN-y, IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(P40), IL-12(P70), IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1a, MIP-1b, MIP-2, TANTES, TNFa, and VEGF. Significant results are reported graphically in the figures, all cytokine data collected can be found in the supplementary tables.

2.6. Data analysis

Next-generation sequencing and analysis are described above. Cytokine and microbial family statistics were analyzed in GraphPad Prism (Version 9.1.0, GraphPad, La Jolla California USA). Normal distributions in the data were validated using 2-way ANOVA or mixed effects analysis. Tukeys or Šidák's multiple comparison test was performed where appropriate to determine significant differences between groups. All figures are represented as mean ± SEM. Statistical significance was determined by an alpha value below 0.05.

3. Results

The gut microbiome of animals and humans is sex specific (Yong Sung Kim et al., 2020). Some studies suggest that microbiome changes can occur within 24 h of a dietary change (David et al., 2014; Sonnenburg and Bäckhed, 2016; Leeming et al., 2019). At 6 days of variable stress, female mice exhibit significant behavioral changes that are not

seen in males (Hodes et al., 2015; Williams et al., 2020; Johnson et al., 2021). Therefore, we tested whether a 6 day sub-chronic course of variable stress (Fig. 2A) was sufficient to induce microbiome alterations in males or females. The sub-chronic variable stress model did not produce substantial alterations in gut microbial composition following 6 days of stress as shown through principal components analysis (PCA) (Fig. 2B). Although there was no observation of changes to gut microbes following 6 days of stress, male and female mice separated distinctly in the first principal component (44% of variance) (Fig. 2B). There was no clear distinction on either principal component for the effect of stress after 6 days. An unbiased clustering of the top 25 variable microbes confirmed the distinct separation of the microbes in the guts of the mice by sex, but not stress (Fig. 2C). This demonstrates that stress had little to no effect on gut microbial composition following 6 days of stress, however sex differences were clear and present at this timepoint. Following this conclusion, we sought to understand whether gut dysbiosis or immune signaling in the brain occurs first following six days of variable stress. To test this, we generated a separate cohort of male and female mice so that immune activation in the brain could be quantified immediately following the stressor on the sixth day of subchronic variable stress. Cytokines in the nucleus accumbens of stressed male and female mice differed significantly from controls. 2-way ANOVA revealed that there was significant variation between control and stress groups in levels of interleukin-4 ($F(1, 36) = 18.53$ $P = 0.0001$), Tukey's multiple comparisons revealed the stressed group had significantly lower IL-4 in males (p-adjusted = 0.0232) and females (p-adjusted = 0.0201). There was significant variation between control and stress groups in levels of interleukin-9 ($F(1, 36) = 26.28$ $P < 0.0001$), Tukey's multiple comparisons revealed the stressed group had significantly lower IL-9 in males (p-adjusted = 0.0035) and females (p-adjusted = 0.0064). Another cytokine with significant variation in concentration between control and stress was interleukin-13 ($F(1, 36) = 6.638$ $P = 0.0142$), Tukey's multiple comparisons revealed the stressed group had significantly higher IL-13 in females (p-adjusted = 0.0191) but not in males (p-adjusted = 0.9456). MCP1 showed significant variation in concentration between control and stress ($F(1, 34) = 7.920$ $P = 0.0081$), Tukey's multiple comparisons revealed the stressed group had significantly higher MCP1 in females (p-adjusted = 0.0078) but not in males (p-adjusted = 0.9520). Mip1a showed significant differences in concentration between control and stress ($F(1, 36) = 4.740$ $P = 0.0361$), Tukey's multiple comparisons revealed the stressed group had significantly higher Mip1a in females (p-adjusted = 0.0430) but not in males (p-adjusted = 0.9884). VEGF showed significant variation in concentration between control and stress ($F(1, 36) = 5.797$ $P = 0.0213$), Tukeys multiple comparisons revealed the stressed group had significantly higher VEGF in females (p-adjusted = 0.0204) but not in males (p-adjusted = 0.9865) (Fig. 2D). Together these data show that there is significant activation of the immune system in the nucleus accumbens before significant changes to gut composition occur. Females show more significant activation of immune signaling molecules in the NAc compared to males immediately following 6 days of stress. This is consistent with trends seen in microglia morphology and stress phenotypes in females who have undergone 6 days of variable stress (Hodes et al., 2015; Tsyglakova et al., 2021). These data suggest that immune signaling in the brain is occurring immediately following stress and at a time where gut microbiome composition changes have not yet been observed.

Next, we examined if a chronic variable stress paradigm would cause compositional alterations to gut microbial communities within mice over time. Chronic variable stress was carried out in cohort 1 for a total of 28 days (Fig. 3A). Chronic variable stress produced substantial alterations in the gut microbe composition of male and female mice as revealed by PCA (Fig. 3B). In the PCA stress separated the baseline timepoint from the 28 day timepoint on the second principal component (9% of variance). Consistent with the PCA from the subchronic variable stress timepoint (Fig. 2B), male and female mice distinctly separated across the first principal component (35% of variance) (Fig. 3B). An unbiased clustering of the top 25 variable microbes confirmed the sex specific effects seen in the PCA from all timepoints (Fig. 3C). This shows that gut microbial composition is altered following 28 days of variable stress and that sex differences in the gut microbiome persist through stress and time.

Because the sex of the mouse accounted for the largest percentage of variance at both timepoints, and there were no discernible compositional alterations after 6 days of stress, we segregated data by sex at 28 days to examine sex specific gut microbe alterations. We found that between the baseline and the 28 day timepoints of stress that males (Fig. 4A) had 28 microbes significantly altered across 5 phyla (Firmicutes, Bacteroidota, Proteobacteria, Cyanobacteria, and Actinobacteria) and 10 families (*Lachnospiraceae*, *Muribaculaceae*, *Rikenellaceae*, *Lactobacillaceae*, *Bifidobactericeae*, *Bacteroidaceae*, *Erysipelotrichaceae*, *Prevotellaceae*, Unknown Proteobacteria, Unknown Cyanobacteria) (Fig. 4B

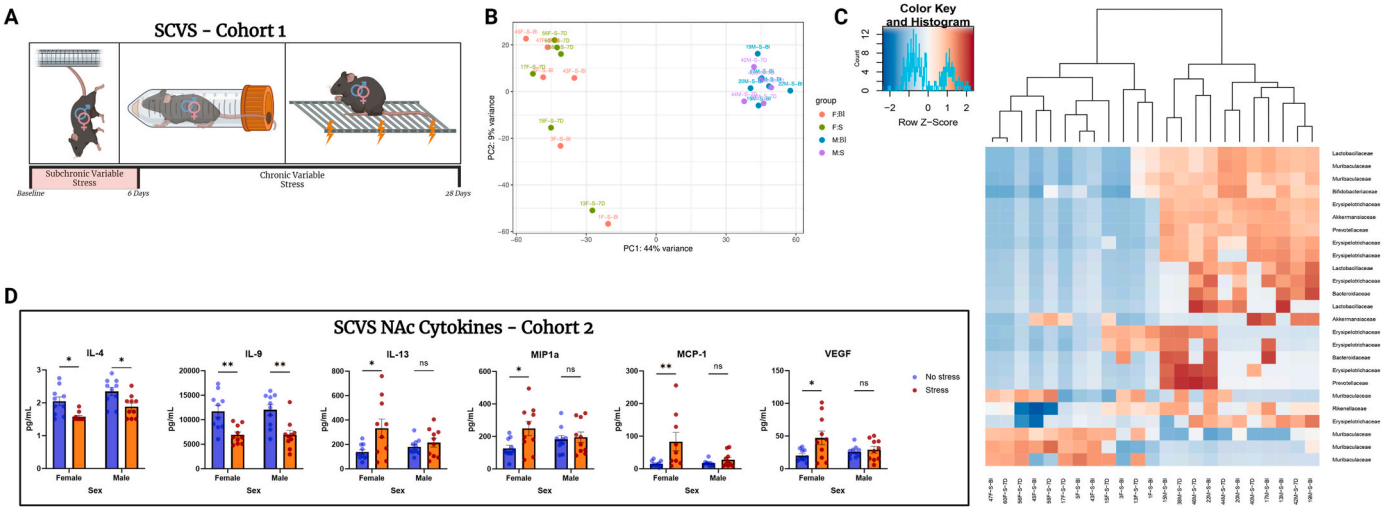

Fig. 2. Gut microbiome shows sex differences 6 days into stress. Conceptual framework of variable stress paradigm (A). Principal components analysis of all mice gut microbiomes after 6 days of variable stress (B). Phylogenetic clustering of gut microbiome after 6 days of stress using the top 25 variable microbes between baseline and 6 days (C). Levels of IL-4, IL-9, IL-13, MIP1a, MCP-1, and VEGF in the nucleus accumbens of male and female cohort 2 mice who underwent 6 days of variable stress (D). All bar graphs are represented as mean ± SEM. A (*; $p < 0.05$) or (**; $p < 0.01$) represents statistical significance between groups, a (#; $p < 0.05$) or (##; $p < 0.01$) denotes a significant interaction effect between groups, (ns) denotes no significance between groups.

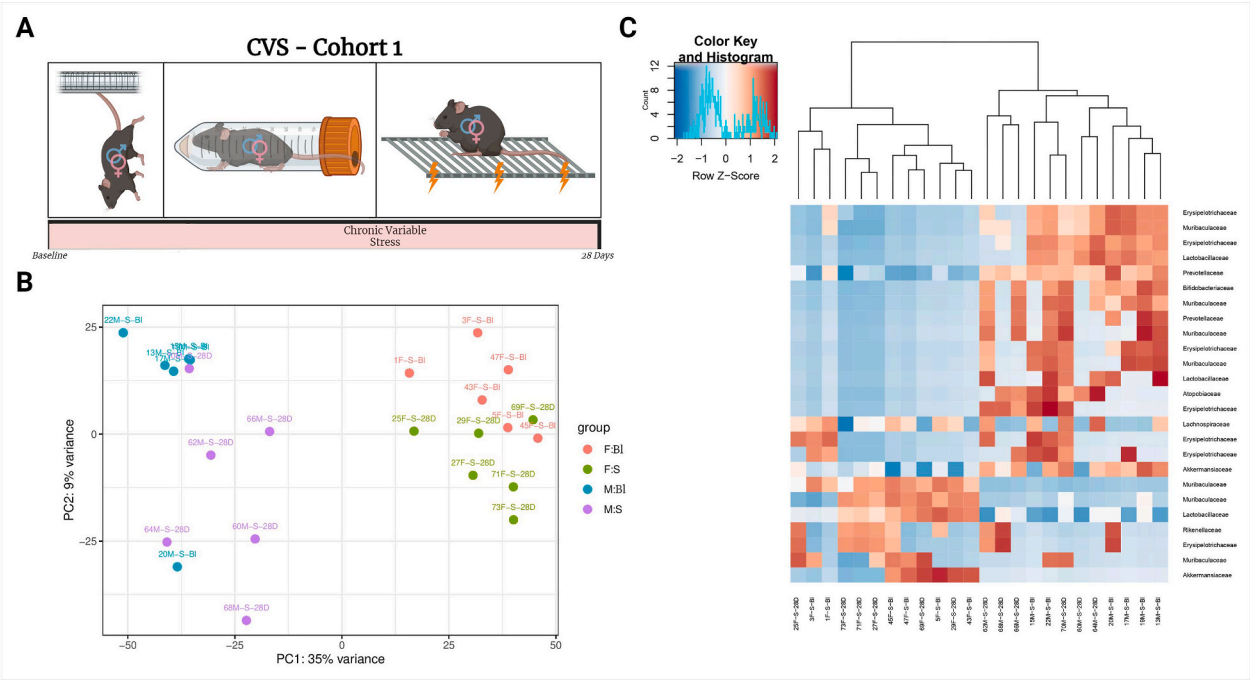

Fig. 3. Gut microbiome altered after 28 days of stress in male and female mice. Conceptual framework of variable stress paradigm (A). Principal components analysis of all mice gut microbiomes after 28 days of variable stress (B). Phylogenetic clustering of gut microbiome after 28 days of stress using the top 25 variable microbes between baseline and 6 days (C). All bar graphs are represented as mean ± SEM. A (*; $p < 0.05$) or (**; $p < 0.01$) represents statistical significance between groups, a (#; $p < 0.05$) or (##; $p < 0.01$) denotes a significant interaction effect between groups, (ns) denotes no significance between groups.

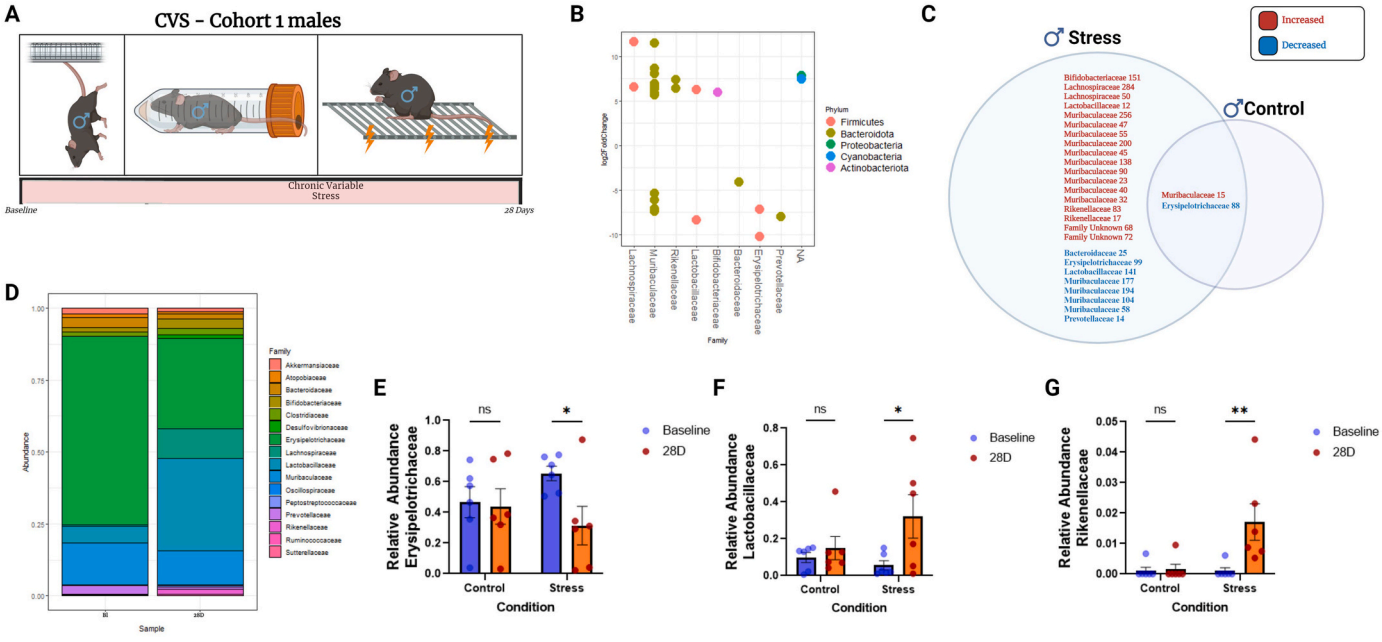

Fig. 4. Microbiome alterations in male mice following 28 days of stress. Conceptual framework of variable stress paradigm (A). Log fold change of significantly altered microbiota (p-adjusted <0.001) with phylum and family level distinction (B). Venn diagram comparing the microbes that were significantly altered from baseline to 28 days in stressed and control male mice, red represents a significant increase over time and blue represents a significant decrease over time, microbes in the middle section were significantly altered in both stressed and control mice (C). Relative abundance of gut microbes in stressed animals at baseline and at 28 days of stress (D). Relative abundance of gut microbes at the family level at baseline and after 28 days in stressed or control conditions (E–G). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and C). In addition to changes of specific microbes over time, there were also alterations to gut composition at the family level. Although the two-way ANOVA did not reveal significant variation between stress and control changes over time ($F(1, 10) = 3.573$ $P = 0.0880$). Šidák's multiple comparisons test revealed that the male 28-day stress group had a significant reduction in *Erysipelotrichaceae* over 28 days (p-adjusted = 0.0308) whereas control mice did not have a significant change in the relative abundance of this family over time (p-adjusted =

0.9664) (Fig. 4D and E). *Lactobacillaceae* did not show significant variation between stress and control over time (F (1, 10) = 2.525 P = 0.1432). However, Šidák's multiple comparisons test revealed that the stress group had a significant increase in the abundance *Lactobacillaceae* over time (p-adjusted = 0.0382) whereas controls saw no significant alterations over time (p-adjusted = 0.8421) (Fig. 4D and F). *Rikenellaceae* however did display significant variation between stress and control groups over time (F (1, 10) = 5.975 P = 0.0346). Šidák's multiple comparisons test showed that the stress group had a significant increase in relative abundance of *Rikenellaceae* (p-adjusted = 0.0040) that was not found in controls (p-adjusted = 0.9933) (Fig. 4D and G).

Analysis of alterations in the gut microbiome due to stress was also performed in females, both at the compositional family level and at the level of specific microbes (Fig. 5A). There were 10 significantly altered microbes from females that spanned across a single phylum (Firmicutes) and 3 families (*Erysipelotrichaceae*, *Lachnospiraceae*, *Lactobacillaceae*) (Fig. 5B and C). A two-way ANOVA did not show significant variation between stress and control changes over time (F (1, 10) = 2.661 P = 0.1339). Šidák's multiple comparisons test however showed that the relative abundance of *Erysipelotrichaceae* increased over time in stressed females (p-adjusted = 0.0374) but not controls (p-adjusted = 0.8664) (Fig. 5D and E). These alterations are directly in opposition to relative abundance *Erysipelotrichaceae* in the male stress group over time. There was no significant variation between groups over time (F (1, 10) = 0.1246 P = 0.7314) however Šidák's multiple comparisons test showed a significant decrease in *Lactobacillaceae* for the female stress group (p-adjusted = 0.0138) and control group (p-adjusted = 0.0322) (Fig. 5D and F). Once again this is opposite for what was measured in males. For *Sutterellaceae* There was once again no significant variation between groups over time F (1, 10) = 2.111 P = 0.1769), Šidák's multiple comparisons test revealed a significant decrease for the female stress group (p-adjusted = 0.0336) but not control group (p-adjusted = 0.6859) (Fig. 5D and G).

Stress has been linked to proinflammatory immune activation, and when chronic, a dysregulation of systemic immune activity (Morey et al., 2015). Microbial communities in the gut also interact closely with the immune system (Wu and Wu, 2012). As a result, cytokines in the plasma of males and females were quantified in order to observe the relationship between circulating immune elements, stress, and the gut. In male mice a mixed effects analysis revealed an interaction effect with eotaxin (F (2, 44) = 6.234 P = 0.0041), where control males had an increase in eotaxin over time and stressed males had an initial increase in eotaxin followed by a decrease by day 28 (Fig. 6A). Four microbes of the *Muribaculaceae* family correlated moderately to strongly to eotaxin in male mice (Fig. 6B). Microbes with a negative correlation to eotaxin were significantly increased in stressed mice, and those with a positive correlation were significantly decreased in stressed mice (Fig. 6B and C). In female mice stress led to a significant vairiation in IL-5 between control and stress (F (1, 11) = 4.858 P = 0.0497), Šidák's multiple comparisons test showed that there is a significant difference in IL-5 at the 28 day timepoint (P = 0.03980). There was also a significant interaction effect (F (2, 21) = 6.450 P = 0.0066) where stressed females experienced a significant increase in IL-5 between day 6 and day 28 as opposed to control females which experienced a consistent decrease in IL-5 over time (Fig. 6D). Microbes that were negatively correlated with IL-5 belonged to the *Erysipelotrichaceae* family and a microbe that was negatively associated with IL-5 belonged to the *Lactobacillaceae* family (Fig. 6E). The three microbes belonging to the *Erysipelotrichaceae* family were significantly increased by day 28 and the microbe belonging to the *Lactobacillaceae* family was significantly decreased by day 28 (Fig. 6F).

4. Discussion

Psychological stress is capable of activating the immune system and altering the composition of gut microbiota (Xu et al., 2020). Stress, alterations to gut microbe composition, and chronic immune activation have all been implicated in the onset of psychiatric disorders (Brady and Sinha, 2005; McEwen, 2017). The purpose of our study was to elucidate

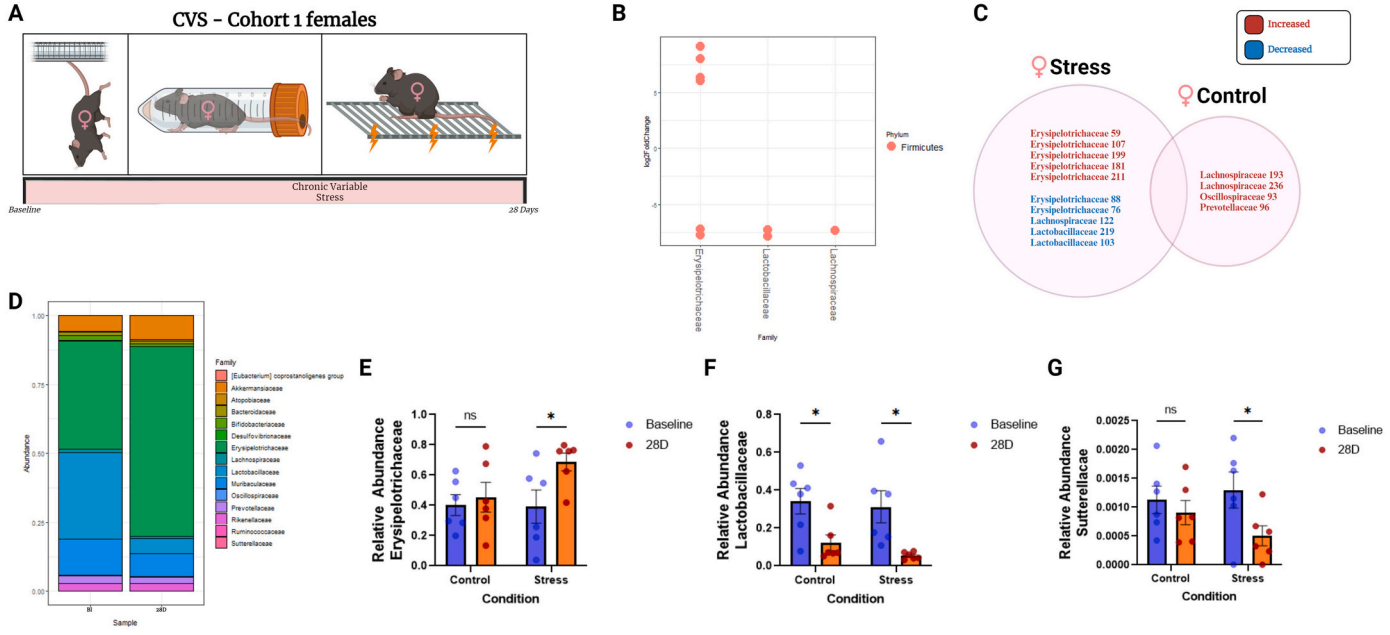

Fig. 5. Microbiome alterations in female mice following 28 days of stress. Conceptual framework of variable stress paradigm (A). Log fold change of significantly altered microbiota (p-adjusted <0.001) with phylum and family level distinction (B). Venn diagram comparing the microbes that were significantly altered from baseline to 28 days in stressed and control female mice, red represents a significant increase over time and blue represents a significant decrease over time, microbes in the middle section were significantly altered in both stressed and control mice (C). Relative abundance of gut microbes in stressed animals at baseline and at 28 days of stress (D). Relative abundance of gut microbes at the family level at baseline and after 28 days in stressed or control conditions (E–G). All bar graphs are represented as mean ± SEM. A (*; $p < 0.05$) or (**; $p < 0.01$) represents statistical significance between groups, a (#; $p < 0.05$) or (##; $p < 0.01$) denotes a significant interaction effect between groups, (ns) denotes no significance between groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

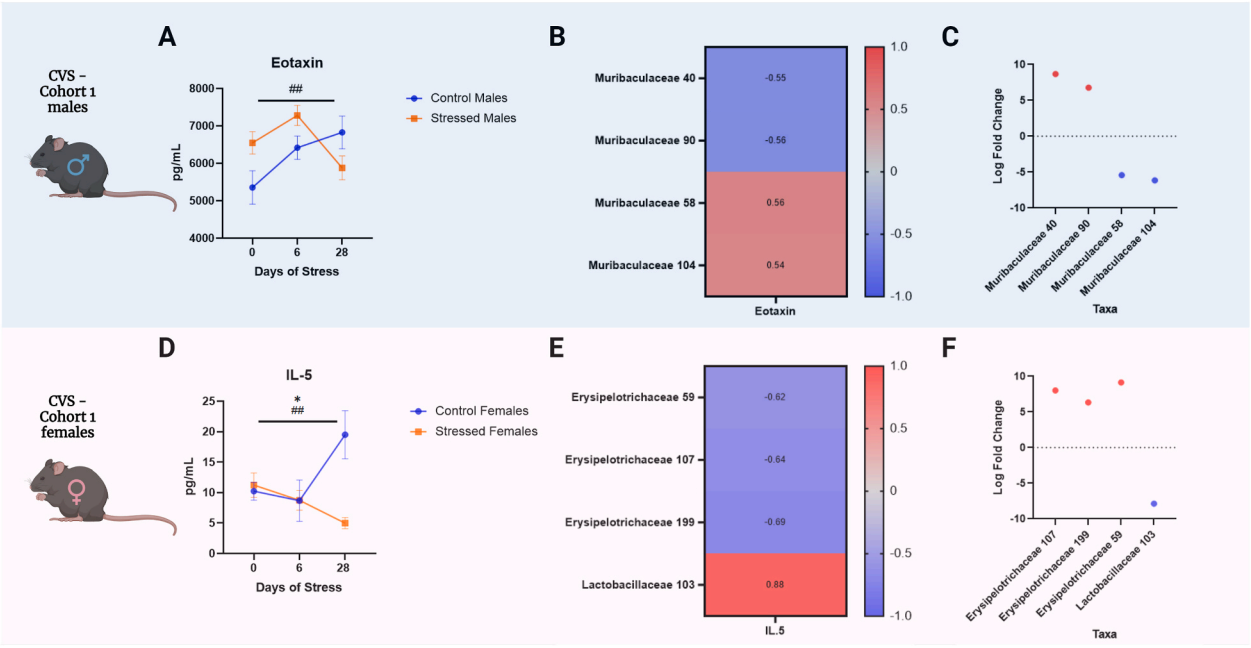

Fig. 6. Circulating cytokines correlate with significantly altered gut microbes after 28 days of variable stress. Levels of circulating eotaxin in control and stress males at baseline, 6 days, and 28 days of stress or control conditions (A). Significantly altered microbes that correlates with eotaxin in males (Pearson $r < -0.5$; $r > 0.5$) (B). Log fold change of significantly altered microbes that correlate with eotaxin in males (C). Levels of circulating IL-5 in control and stress females at baseline, 6 days, and 28 days of stress or control conditions (D). Significantly altered microbes that correlates with IL-5 in females (Pearson $r < -0.5$; $r > 0.5$) (E). Log fold change of significantly altered microbes that correlate with IL-5 in females (F). All line graphs are represented as mean ± SEM. A (*; $p < 0.05$) or (**; $p < 0.01$) represents statistical significance between groups, a (#; $p < 0.05$) or (##; $p < 0.01$) denotes a significant interaction effect between groups, (ns) denotes no significance between groups.

some of the changes that occur in circulating cytokines and alterations to gut microbes in C57BL/6J mice across the timespan of a 28 day variable stress paradigm. Variable stress over the course of 6 days did not result in composition level alterations to the gut microbiome in males or females. Variable stress is capable of generating immune activity in the brain after 6 days, at this timepoint we did not measure changes to gut microbe composition. After 28 days of stress, there were significant alterations to the composition of the gut microbiome of male and female mice versus controls. The gut microbiomes of male and female mice were distinct from each both at baseline and following stress. Both male and female mice had alterations in the level of cytokines that promote eosinophilic immune activity that occurred after 28 days of variable stress. Microbes of the family *Erysipelotrichaceae* and *Lactobacillaceae* were significantly impacted by stress but interacted with sex resulting in opposite regulation in stressed males vs. stressed females. Significantly altered microbes of the *Muribaculaceae* family correlated with eotaxin in males in females, members of the *Erysipelotrichaceae* and *Lactobacillaceae* families correlated with IL-5. Together, these data suggest that the gut composition of microbes change due to stress in a sex dependent way and coincide with alterations to eosinophilic activity. This highlights the importance of sex as a biological variable (SABV) in gut microbiome research as the exclusion of either sex may result in ineffective or even dangerous interpretations of preclinical results, eventually leading to poor clinical outcomes.

4.1. Implications of gut compositional changes

Erysipelotrichaceae is a family of gram-positive bacteria that was first classified in 2004 (Verbarg et al., 2004). Our study reveals that the relative abundance of *Erysipelotrichaceae* was significantly decreased in males and increased in females after stress. Eosinophil related immune signaling molecules were also altered following chronic variable stress. Previous studies have demonstrated that an unnamed microbe of the *Erysipelotrichaceae* family has a high affinity for immunoglobulin A binding (Palm et al., 2014; Wu et al., 2021). Interestingly, immunoglobulin A is a powerful trigger for granulocyte release from eosinophils (Abu-Ghazaleh et al., 1989). It is possible that these changes to the relative abundance of *Erysipelotrichaceae* occur in order for the host organisms to modulate the level of eosinophilic activity and granule release occurring in the gut during chronic variable stress. This would help to explain why there is such a high degree of variability between individuals when it comes to compositional gut microbe changes during immune active states. More experiments on the causal relationship between *Erysipelotrichaceae* changes and gut microbe composition would be necessary to confirm if this link exists. Every individual will have different baseline levels of both *Erysipelotrichaceae*, and gut eosinophils so individualistic changes may be required to bring the level of cytotoxic granulocyte release and activity to a state that promotes health in the host organism. More work needs to be done on altering eosinophilic activity during stress in order to understand the directionality and mechanisms that underly this potential relationship.

Lactobacillaceae is a family of gram-positive bacteria that live in the guts of humans and other animals. This family is associated with positive health outcomes to the host through immune modulation as well as transportation of bioactive metals like copper and zinc (Huynh and Zastrow, 2023). Our data show that *Lactobacillaceae* was significantly altered in both male and female mice following 28 days of stress. There was a decrease in the relative abundance of this family in both the control and stress conditions for the female mice, and an increase in the relative abundance of just stressed males. Many studies have identified *Lactobacillaceae* or members of it as health promoting due to its probiotic and anti-inflammatory properties (Walter, 2008; Hill et al., 2014; Oscarsson et al., 2021). This raises the question of why males and females in our study had opposing trends in relative abundance of this family. It may be that *Lactobacillaceae* is beneficial sex specifically. This is supported by the fact that a microbe in the *Lactobacillaceae* family correlated with IL-5 in the families but that all of the microbes related to eotaxin in the males were of the *Muribaculaceae* family. Non-obese male

mice have a greater relative abundance of *Lactobacillaceae* at baseline compared to female mice (Leonid Yurkovetskiy et al., 2013). More work would have to be done on the use of a wide range of members of the *Lactobacillaceae* family on males and females to parse out their exact relationship to gut health, or if that positive effect is sex dependent. It is also interesting that the control females experienced a significant decrease in relative abundance of *Lactobacillaceae*. This is likely not due to female hormonal fluctuations as gut composition is not significantly altered across the estrous cycle (Wallace et al., 2018). More work would have to be done to understand why *Lactobacillaceae* is variable at baseline for females but that this trend is not seen in males.

Very little is known about the role of *Rikenellaceae* in the gut of mice or humans. Relative abundance of the family *Rikenellaceae* in our study was increased in stressed male mice, but not in females. There is some evidence in the literature to suggest that *Rikenellaceae* is associated with negative health outcomes. A high-fat diet in mice is associated with an increase in abundance of *Rikenellaceae* (Kim et al., 2012). A high-fat diet in mice is also associated with increased levels of LPS-containing microbes in the gut (Cani et al., 2007). Furthermore, this change in abundance of *Rikenellaceae* may increase the levels of LPS that get released from the gut, thus promoting further proinflammatory activity around the body (Marcello Candelli et al., 2021). This inflammatory activity would be increased if the gut was leaky, which would allow for more LPS to cross the intestinal barrier into the bloodstream. In humans the greater relative abundance of *Rikenellaceae* was associated with lower levels of visceral fat (Tavella et al., 2021). This means that caution should be taken in the interpretation of mouse microbial results as they may not be perfectly generalizable to humans. This could be due to the highly individualistic nature of gut microbes, or it could be due to differences in host species. Further studies looking at the specific function of these microbes in the gut would help to elucidate why results vary between species of host organism.

Sutterellaceae is a family of gram-reactive-negative bacteria that was first classified in 2010 (Masami Morotomi et al., 2011). Very little has been published on the association between stress and abundance of *Sutterellaceae* in the gut. In adult humans with depression there is an increase in the abundance of the *Sutterellaceae* family (Barandouzi et al., 2020). In young adults the opposite is true where depressed patients had decreased abundance of *Sutterellaceae* (Chen et al., 2023). It is unclear whether these opposing results occur due to individualistic differences in sample groups, if it was due to the age of participants in the study, or due to specificity of microbes within the *Sutterellaceae* family. Our data showed that in female mice the relative abundance of *Sutterellaceae* decreased after 28 days of stress. More work needs to be done on manipulating levels of gut *Sutterellaceae* in mice and humans to see what members of this family promote positive or negative health effects in the host species.

4.2. Immunity and gut microbiome alterations

Eosinophils play a major role in gut health and homeostasis. Eosinophils are granulocytes that originate from the bone marrow and can release cytotoxic chemicals to combat parasites and allergens, it can also release cytokines to help coordinate an immune response (A. Straumann and Simon, 2004). The majority of eosinophils created in the bone marrow migrate to the gut in order to maintain mucosal barrier immunity as well as microbial homeostasis in the gut (Alexandre Loktionov, 2019). Certain alterations of bacteria in the gut have been associated with immune activity in the host organism. A mixed granulocyte allergy model increased the relative abundance of the family *Erysipelotrichaceae* in the guts of male C57BL/6J mice (Gu et al., 2022). Eosinophil levels are increased in the guts of patients and mice with irritable bowel disease (S. Al-Haddad and Riddell, 2005). During psychological stress eosinophils release corticotrophin releasing hormone (CRH), a hormone capable of activating proinflammatory immune machinery as well as activating the HPA axis (Zheng et al., 2009; Kanamori et al., 2022). Together these data suggest that eosinophils may be implicated in gut alterations that occur due to stress. Whether eosinophilic activity drives microbiome changes or whether microbiome changes precede eosinophilic signaling is unknown. More studies need to be done to understand the directionality of this relationship as well as to what degree eosinophils are able to modify gut microbe compositions.

4.3. Limitations

There are several limitations to 16S rRNA amplicon microbiome sequencing and how it taxonomically classifies gut microbes. The first notable exception is the lack of genus and species level classifications that exist in current taxonomic databases. This makes it so that although analysis of gut composition can be specific to the level of a single microbe, it is not abundantly clear what that microbe is. This creates an issue where we can understand gut alterations taxonomically down to the family level but lose substantial taxonomic clarity at the genus and species levels, which is necessary for understanding the specific gut function of individual microbial species. This is apparent in the male microbiome data where members of the same family correlate in opposing directions with certain cytokines. The other notable limitation is the threshold for taxonomic classification based on sequencing. Classification occurs when a sequence meets a threshold (based on calculated experimental error rates) that matches with a sequence of a known taxonomy. This makes it so that a single sequence may be classified as several microbes whose similarity is within this threshold of error. The transient nature of cytokine based molecular signaling also presents a limitation to these results. Cytokines are constantly secreted and exist within the body in order to maintain homeostatic immunity. Given the challenges associated with real time monitoring of circulating cytokines, the best we can do is gather a snapshot of current immune modulatory activities. In this study we identified cytokine signaling consistent with eosinophilic immune activity. This suggests that at 28 days of stress eosinophils are being modulated in order to bring the body to homeostasis. However more studies will need to be done to understand the complex dynamics of cytokine activity over time.

5. Conclusion

Chronic stress is associated with proinflammatory immune activity and changes to the composition of the gut microbiome (Fung et al., 2017; Madison and Kiecolt-Glaser, 2019). The gut microbiome composition of male and female mice does not show substantial alterations after 6 days of variable stress but does show them after 28 days. Immune activation was measured in the nucleus accumbens of another cohort of mice after 6 days of stress, a timepoint in which gut dysbiosis at the composition level is not yet seen. Alterations to the male microbiome have greater diversity and magnitude compared to the alterations that occur in stressed females. Both males and females show significant alterations to the families *Erysipelotrichaceae* and *Lactobacillaceae*, however these alterations are in opposing directions. Males had significant alterations in the family *Rikenellaceae* and females had significant alterations to the family *Sutterellaceae*. Levels of circulating eotaxin in males correlated with the *Muribaculaceae* family, circulating levels of IL-5 were associated with members of the *Erysipelotrichaceae* family and the *Lactobacillaceae* family. Significantly altered cytokines in males and females suggest that eosinophils are associated with changes that occur in the gut due to chronic variable stress in mice. Further study of *Erysipelotrichaceae* and *Lactobacillaceae* as well as eosinophilic activity in the gut may yield important information about potential targets for the treatment of psychiatric and gastrointestinal disorders.

CRediT authorship contribution statement

Dawson R. Kropp: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization,

Writing – original draft, Writing – review & editing, Software, Supervision, Validation. **Jennifer R. Rainville:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Supervision, Writing – original draft. **Matthew E. Glover:** Data curation, Formal analysis, Resources. **Mariya Tsyglakova:** Investigation, Methodology. **Rupabali Samanta:** Investigation. **Tamer R. Hage:** Investigation. **Audrey E. Carlson:** Investigation. **Sarah M. Clinton:** Conceptualization, Project administration, Resources, Supervision, Writing – original draft, Funding acquisition. **Georgia E. Hodes:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – original draft, Project administration, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors report no financial or potential conflicts of interest.

This manuscript has not previously been published, nor is it under consideration in any other journals.

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Appendix A. Supplementary data

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