Behavioural and biological effects of a Mediterranean-based diet in postpartum mice and prenatally stressed mice offspring

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Research Approval

The studies described in this thesis were all approved by the Animal Care Committee at the University of Ottawa (protocol number: HSe-3149), according to the guidelines of the Canadian Committee of Animal Care. In addition, before commencing the mouse experiments, I (and the co-authors where applicable) received training from the Animal Care and Veterinary Services and the Behavioural Core Facility at the University of Ottawa.

Abstract

Physiological changes in the perinatal period may increase the risk for postpartum mental disturbances. In the offspring, this risk for mental disorders may be compounded by adversities experienced by the mother during pregnancy. Alterations in inflammatory and neurotrophic factors, barrier function, and microbiota composition in the brain and/or intestinal environments have been suggested to underlie some of these mental disturbances in postpartum mothers and prenatally stressed offspring. The past years have seen a bloom of studies showing that dietary patterns based on the Mediterranean (Med) diet were associated with better mental health, reduced levels of systemic pro-inflammatory markers, and increased gut bacteria with health benefits in the intestinal tract. This thesis examined, in mice, whether a Med-based diet improved behaviours of postpartum dams and prenatally stressed offspring. Pro-inflammatory cytokines, brain-derived neurotrophic factor (BDNF), and tight junctions in brain and intestinal samples as well as gut microbiota metrics were also determined. Considering the sex biases in the outcomes of prenatal stress and the prevalence of depression and anxiety, sex differences in the effects of prenatal stress and the Med-based diet were investigated in the offspring. In Study 1, we examined behaviour and biological markers in brain and intestinal samples in postpartum dams fed a Control or a Med-based diet from preconception through early postpartum and showed that in addition to increasing self-care behaviour, the Med-based diet reduced pro-inflammatory cytokines and increased BDNF and claudin-5 in the hippocampus. Study 2 examined whether the Med-based diet limited socioemotional deficits and biological changes promoted by a prenatal stressor in neonatal offspring and showed that although it did not mitigate the reductions in vocalizations in stressed pups, the diet limited the cytokine increases and the BDNF decreases in the hippocampus and/or prefrontal cortex of these pups, particularly in females. Study 2 also looked at anxiety- and

depressive-like behaviours and biological markers in adult offspring subjected to the same manipulations as in the neonatal pups and showed that the Med-based diet limited the stress-induced increases in anxiety-like behaviours, hippocampal and colonic pro-inflammatory cytokines, and changes in gut microbiota composition, again particularly in females. Overall, this thesis demonstrates the potential of Med-based dietary patterns to support maternal and offspring's (particularly in female offspring) mental health, possibly by modulating inflammatory processes, neuroplasticity, and gut microbiota.

Contribution of Authors

This work was conducted by MaryAnn C. Udechukwu under the primary supervision of Dr. Marie-Claude Audet and co-supervision of Dr. Pierre Blier. The thesis was written by MaryAnn Chinonye Udechukwu with input from Dr. Marie-Claude Audet. Authors' contributions to the specific aspects of the thesis are as follows:

Dietary development (Chapter 3)

- The Control and Med-based diets used in this thesis were developed by MaryAnn C. Udechukwu, Dr. Krista Power, and Dr. Marie-Claude Audet, in collaboration with nutrition researchers at Research Diets Inc.

Manuscript #1 (Chapter 4)

- Study design: MaryAnn C. Udechukwu and Dr. Marie-Claude Audet
- Study execution: MaryAnn C. Udechukwu, Christophe Nadon, and Zoë Williams conducted the mouse experiment. MaryAnn C. Udechukwu performed the behavioural and RT-qPCR analyses. MaryAnn C. Udechukwu and J.K. Szyszkowicz conducted the microbiota sequencing analyses, and MaryAnn C. Udechukwu performed the bioinformatics analyses.
- Data interpretation and manuscript writing: MaryAnn C. Udechukwu and Dr. Marie-Claude Audet. The manuscript will be proof-read by all authors before its submission.

Manuscript #2 (Chapter 5)

- Study design: MaryAnn C. Udechukwu and Dr. Marie-Claude Audet
- Study execution: MaryAnn C. Udechukwu, Amanda Della Giustina, and Geneviève Lefebvre equally contributed to conducting the breeding, dietary, stressor, and behavioural manipulations. MaryAnn C. Udechukwu performed the USVs and RT-qPCR analyses.

MaryAnn C. Udechukwu and Ana Santos conducted the microbiota sequencing analyses, while MaryAnn C. Udechukwu performed the bioinformatics analyses.

- Data interpretation and manuscript writing: MaryAnn C. Udechukwu and Dr. Marie-Claude Audet. The manuscript will be proof-read by all authors before its submission.

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- Data interpretation and manuscript writing: MaryAnn Chinonye Udechukwu and Dr. Marie-Claude Audet. The manuscript will be proof-read by all authors before its submission.

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List of Abbreviations

BDNF: brain-derived neurotrophic factor

cDNA: complementary deoxyribonucleic acid

CRP: C-reactive protein

DASH: Dietary Approaches to Stop Hypertension

DNA: deoxyribonucleic acid

E: embryonic

GF: germ-free

HPA axis: hypothalamic pituitary adrenal axis

IL-1β: interleukin-1beta

IL-6: interleukin-6

KEGG: kyoto encyclopedia of genes and genomes

LPS: lipopolysaccharide

Med diet: Mediterranean diet

mRNA: messenger ribonucleic acid

NOD: nucleotide oligomerization domain

pCOA: principal coordinate analysis

PERMANOVA: permutational multivariate analysis of variance

PGN: peptidoglycan

PICRUSt: phylogenetic investigation of communities by reconstruction of unobserved states

PND: postnatal day

QIIME: quantitative insights into microbial ecology

rRNA: ribosomal ribonucleic acid

RT-qPCR: real time quantitative polymerase chain reaction

SCFAs: short-chain fatty acids

TNF-α: tumour necrosis factor- alpha

USVs: ultrasonic vocalizations

Chapter 1: General Introduction

Mental health disorders, such as depression and anxiety, contribute to global disease burden as leading causes of disability (Ferrari, 2022). In Canada, the prevalence of major depressive and generalized anxiety disorders doubled over the past decade, with young women accounting for the greater proportion of the diagnoses (Stephenson, 2023). Additionally, mental disorders enormously impact the Canadian economy, such that annual expenditures exceed $50 billion due to healthcare costs and losses from declined productivity and quality of life in affected individuals (Moroz et al., 2020). Without timely and effective interventions, the global prevalence of mental disorders is projected to increase further in the next 25 years (Wu et al., 2023). This underscores the importance of understanding the risk factors and biological mechanisms underlying mental disorders to facilitate the development of effective therapeutic and prophylactic interventions.

The postpartum period represents a time of increased vulnerability to mental disorders in the mother, partly attributed to the physiological changes occurring in the perinatal period (Rai et al., 2015). For instance, the maternal brain undergoes changes in neuroplasticity and immune functions during pregnancy and early postpartum (Barba-Müller et al., 2019; Duarte-Guterman et al., 2019), and the maternal gut microbiota is extensively remodeled towards an overall pro-inflammatory profile, leading to low-grade inflammation in the intestinal environment (Koren et al., 2012). While these physiological changes putatively support fetal development, parturition, and transition to motherhood (Barba-Müller et al., 2019; Koren et al., 2012; Mor et al., 2017), they could culminate in mental health disturbances at postpartum (Barba-Müller et al., 2019; Bränn et al., 2020), especially as they are consistent with the disordered physiological processes seen in stress-related mental health disorders. Approximately 10-15% of women suffer from postpartum

depression (Almond, 2009; Shorey et al., 2018), while about 8-12% experience severe anxiety within the first year of birth (Goodman et al., 2016; Miller et al., 2006). Beyond the detrimental effects on the mother's health and well-being, postpartum mental disturbances can compromise the quality of maternal care and interactions with the child, significantly impacting the child's postnatal development and health (Almond, 2009; Earls et al., 2010; Hoffman et al., 2017). The risk of developmental disturbances in the offspring may be compounded by adverse maternal experiences during pregnancy (referred to as prenatal stress), which have been extensively documented to disrupt fetal and postnatal development, predisposing the offspring to mental disorders across the lifespan (Van den Bergh et al., 2020; Weinstock, 2017). Prenatal stress can alter various aspects of brain development and function, particularly in regions involved in the stress response, such as the hippocampus and prefrontal cortex (Charil et al., 2010). For instance, prenatally stressed rodents displayed elevated markers of neuroinflammation, transcriptional and epigenetic changes in neurotrophic factors important in neuronal development and plasticity, and dysfunction in the blood-brain barrier (BBB) during neonatal and adult life (Badihian et al., 2020; Roshan-Milani et al., 2021; Zhao et al., 2022). Additionally, prenatal stress has been associated with perturbations in the establishment of the offspring's gut microbiota and intestinal inflammatory processes and barrier functions (Gur et al., 2017; Sun et al., 2021; Zhang et al., 2021). Importantly, the behavioural and biological effects of prenatal stress may depend on the sex of the offspring (Davis & Pfaff, 2014; Glover & Hill, 2012) and the timing of exposure (Charil et al., 2010; Herbison et al., 2017), thus highlighting the relevance of these factors in understanding and mitigating the impacts of prenatal stress in the offspring.

First-line treatments for depressive and anxiety disorders include selective serotonin reuptake inhibitors and selective norepinephrine reuptake inhibitors, which respectively inhibit

serotonin and norepinephrine reuptake and thereby improve their availability in the synaptic cleft (Goddard et al., 2010; Hillhouse & Porter, 2015). This treatment approach is based on the hypothesis that these disorders result from decreased levels of monoamine neurotransmitters (Delgado, 2000). Despite their widespread use, about 30-50% of patients do not respond to these treatments (Akil et al., 2018), and the therapeutic efficacy of these drugs is often impeded by side effects, including sleep disturbances, sexual dysfunction, increased suicidal risk as well as delayed onset of action and alleviation of symptoms in patients (Al-Harbi, 2012; David & Gourion, 2016; Ferguson, 2001; Hu et al., 2004). Importantly, pregnant and lactating mothers with mental health disturbances are often hesitant to take these medications due to safety concerns for their infants, thereby the limiting the use of these drugs (Battle et al., 2013). These challenges not only illustrate that processes other than monoamine deficiency contribute to mental disorders but, more crucially, emphasize the need to identify safe and sustainable therapeutic alternatives or adjuvant treatments for mental health promotion.

Emerging evidence indicates that diet may influence mental health, owing to the inherent capacity of some dietary components (e.g., omega-3 fatty acids, polyphenols, and fiber) to modulate various biological processes potentially involved in mental health and disease, including those related to the gut microbiota and inflammatory immune system (Marx et al., 2020). Particularly, dietary patterns based on the Mediterranean (Med) diet (regarded as gold-standards for optimal health) have been linked to improved mental health in healthy populations (Li et al., 2017; Ventriglio et al., 2021). The Med-based diet is traditionally characterized by high intakes of extra-virgin olive oil (main source of added fat), whole grains, fruits, vegetables, and legumes, moderate intakes of fish, dairy, nuts, and red wine (often with meals), and limited consumption of red meat (Trichopoulou et al., 2014). Notably, clinical dietary interventions based on the Med diet

led to significant improvements in depressive symptoms in individuals with major depression, particularly in female subjects (Firth et al., 2019). In relation to the perinatal period, women who adhered to Med-based dietary patterns during pregnancy had better mental health and well-being at this time (Casas et al., 2023; Flor-Alemany et al., 2022), and their children were less likely to develop depression and anxiety (House et al., 2018) or have cognitive and socioemotional deficits (Crovetto et al., 2023). Currently, there is limited information on whether Med-based diets can improve maternal mental health at postpartum. Likewise, no studies have examined if Med-based diets can mitigate the neuropsychiatric effects of prenatal stress in the offspring, and if these dietary impacts differ in females and males.

In this thesis, a Med-based diet was developed for mice according to human Med dietary patterns, and the palatability of the diet and its safety for breeding mice were established in a pilot study. The effects of the Med-based diet on the behaviours of postpartum dams and of prenatally stressed neonatal and adult offspring were examined. The biological processes associated with the behavioural effects of the Med-based diet were investigated by analyzing changes in the gene expression of inflammatory, tight junction, and neurotrophic factors in the brain and gut as well as changes in gut microbiota diversity, composition, and predicted metabolic capacity. Lastly, whether the effects of the prenatal stressor and Med-based diet differed in female and male offspring was also investigated. Findings from this thesis provide novel and fundamental information on the capacity of Med-based dietary patterns to promote positive mental health in mothers at postpartum and in offspring that experienced prenatal adversity. Also, the studies shed light on the potential biological processes underlying the behavioural impacts of the Med-based diet, an information that would be instrumental in future development of targeted and effective dietary guidelines and interventions for mitigating the risk for mental health disorders in

postpartum women and offspring exposed to prenatal insults. Lastly, by examining sex differences in the interactive effects of the prenatal stressor and Med-based diet in the offspring, this thesis provides crucial insights into the importance of sex considerations in dietary recommendations for promoting mental health in at-risk individuals.

This thesis consists of seven chapters, including the present introductory chapter. The second chapter elaborates on the influence of the perinatal period on key aspects of maternal physiology and their impacts on postpartum mental health. This chapter also reviews the impacts of maternal prenatal stress on offspring's mental health, discussing the potential underlying biological mechanisms, and the influence of the timing of the prenatal stressor and offspring's sex on the physiological and mental health outcomes. Lastly, the second chapter discusses the potential of diet, specifically based on Med dietary patterns, in promoting positive mental health in mothers at postpartum and in prenatally stressed offspring. The third chapter describes the development of the mouse Med-based diet and the pilot study conducted to establish its palatability and safety for breeding. Following this are the fourth, fifth, and sixth chapters, consisting of manuscripts detailing the studies conducted in the postpartum dams, the neonatal offspring, and the adult offspring, respectively. Finally, the seventh chapter provides further discussion on the overall thesis research findings, highlighting the contributions to knowledge and future research directions.

Chapter 2: Literature Review

2.1 Physiological changes in the perinatal period: links with postpartum mental health

Pregnancy and postpartum are unique times in a woman's life, accompanied by dramatic structural and functional changes in almost all maternal biological organs and systems to ensure a successful pregnancy and facilitate the transition to motherhood. Central to these changes is the progressive rise in reproductive hormones, specifically estrogens and progesterone, followed by a rapid decline immediately after delivery (Hendrick et al., 1998). These hormonal dynamics greatly modulate other physiological changes occurring during pregnancy and postpartum (Robinson & Klein, 2012). For instance, the perinatal period is associated with extensive remodelling of the brain (Barba-Müller et al., 2019; Duarte-Guterman et al., 2019). Studies have observed a reduction in brain size throughout pregnancy, which persists for six months postpartum before returning to a pre-pregnancy state (Oatridge et al., 2002). Pregnant women also exhibited decreased gray matter volume in several brain areas associated with social cognition, including the anterior and posterior midline, bilateral prefrontal cortex, and bilateral temporal cortex (Hoekzema et al., 2016; Kim et al., 2010). In early postpartum, region-specific changes in gray matter also occur, with increased volumes in the prefrontal cortex, parietal lobe, and midbrain areas (Kim et al., 2010; Nehls et al., 2024), and decreased volumes in the hippocampus, which subserves learning and memory functions and emotional behaviours (Hoekzema et al., 2016). Morphological changes such as increased number of apical dendritic spines in CA1 and CA3 regions of the hippocampus have also been reported in pregnant and early postpartum rodent dams (Kinsley et al., 2006; Pawluski & Galea, 2006), in addition to decreased neurogenesis, marked by reduced cell proliferation and survival in the CA1, CA3, and dentate gyrus of the hippocampus (Darnaudéry et al., 2007; Eid et al., 2019; Hillerer et al., 2014; Leuner et al., 2007; Pawluski & Galea, 2007). The decreases in

neurogenesis may partly result from changes in neurotrophins such as brain-derived neurotrophic factor (BDNF; important in neurogenesis and neuroplasticity), which progressively declines in the bloodstream during pregnancy (Christian et al., 2016; Lommatzsch et al., 2006).

Immunologically, decreased microglial numbers, density, and activity, along with changes in pro- and anti-inflammatory cytokine expression in the medial prefrontal cortex and hippocampus have been observed in pregnant and early postpartum rodents (Eid et al., 2019; Haim et al., 2017; Posillico & Schwarz, 2016). Parallel to these shifts in neuroimmune functions are coordinated and timed immune responses at the maternal-fetal interface (Mor et al., 2017). Contrary to the notion of constant immunosuppression in pregnancy, a pro-inflammatory immune activation, mediated by infiltration of innate and adaptive immune cells and release of pro-inflammatory cytokines and chemokines in the decidua, is necessary for the implantation of the blastocyst to uterine epithelium and subsequent placental emergence in the first trimester (Plaks et al., 2008; Zenclussen & Hämmerling, 2015). Following successful implantation and placentation, the maternal-fetoplacental immune system harmonizes into an anti-inflammatory state in the second trimester to promote immune tolerance to the rapidly growing fetus (Mor et al., 2017). Towards term, the immune system returns to a pro-inflammatory state in preparation for delivery, with pro-inflammatory cytokines mediating uterine contraction, fetal expulsion, and placental rejection (Mor et al., 2011). Studies have shown that intestinal factors such as the gut microbiota contribute to these peripheral immune fluctuations during the perinatal period. In addition to intestinal low-grade inflammation, marked by elevated levels of pro-inflammatory cytokines such as IL-6 and TNF-α, fecal bacterial richness is reduced, and beta diversity is increased due to compositional shifts involving increases in Proteobacteria (phylum composed of taxa containing the pro-inflammatory endotoxin lipopolysaccharide; LPS) and Actinobacteria (containing

pathogenic and symbiotic taxa) in the third trimester, persisting into early postpartum (Koren et al., 2012). Interestingly, transplanting the fecal microbiota of women in their third trimester into germ-free (GF) mice (raised in sterile environment and thus devoid of microbes) precipitated biomarkers of metabolic syndrome, including low-grade intestinal inflammation and insulin insensitivity (Koren et al., 2012), demonstrating that late pregnancy is associated with changes in the gut microbiota that may lead to pro-inflammatory fluctuations in the intestinal environment. It should be noted that although these compositional shifts in the gut microbiota during the perinatal period could lead to disease states in a non-pregnant condition, they are rather considered normal and vital to meeting the increased energy demands of pregnancy and postpartum (Koren et al., 2012).

The exact mechanisms underlying increased risk for postpartum mental disorders in the mother are not understood; however, evidence points to these changes in the maternal brain, immune system, neurotrophic activity, and gut microbiota normally occurring during the perinatal period. Women with postpartum depression had abnormalities in white matter in the left anterior limb of the internal capsule (Silver et al., 2018) and showed weaker activation of neural substrates underlying reward and motivation (e.g., nucleus accumbens) when presented with their own infant cry (Laurent & Ablow, 2012). Decreased serum BDNF levels were linked with more severe postpartum depressive symptoms and greater suicidal risk in postpartum women (Gao et al., 2016; Gazal et al., 2012; Pinheiro et al., 2012). In support of immune dysregulation in postpartum mental disorders, women with postpartum depression had higher serum IL-6 levels (Maes et al., 2000). Additionally, postpartum rats exhibiting depressive-like behaviour also had elevated prefrontal IL-6 expression (Posillico & Schwarz, 2016) and showed alterations in their gut microbiota, including

higher levels of Enterobacteriaceae, a Proteobacteria family (Liu et al., 2020; Tian et al., 2021; Zhao et al., 2022).

Although transient mood changes, often referred to as “baby blues”, are common in the first few days of birth, many mothers may experience more severe and persistent mood disturbances consistent with postpartum depression (O’Hara et al., 1991; O’Hara & Mc Cabe, 2013). Additionally, while it is natural for mothers to feel some apprehension about caring for their newborns, this feeling may escalate to significant anxiety disorders, which could compromise the health of the mother but also infant care (Fairbrother et al., 2016). These perturbations in maternal mental health could have serious implications for offspring development, and more so, if the mother experienced stress during pregnancy, which has been demonstrated to alter offspring’s development and increase the risk for mental disorders (Weinstock, 2008). Therefore, it is crucial to identify and implement strategies to help promote maternal mental health in both pregnancy and postpartum, for the mutual benefit of the mother and the offspring.

2.2 Prenatal stress as a risk factor for mental disorders in the offspring

The prenatal period is a critical time in development, during which the rapidly growing fetal organs and systems are highly sensitive and responsive to changes in the internal and external milieu (Davis & Narayan, 2020). This high degree of fetal sensitivity is aptly illustrated by the long-standing fetal origins of disease hypothesis, which posits that hostile maternal factors, such as stress, malnutrition, infection, alcohol, and drug use, among others, could permanently imprint on offspring’s physiological development and long-term health (Barker, 1998; Seckl & Holmes, 2007). Indeed, numerous preclinical and clinical evidence have supported this hypothesis by demonstrating links between prenatal insults and various physical and mental pathologies in the offspring (O’Donnell & Meaney, 2017; Padmanabhan et al., 2016). In humans, maternal prenatal

stress encompasses a broad spectrum of mild to severe stressors, such as daily life challenges, socioeconomic problems (e.g., poverty and food insecurity), pre-existing or pregnancy-related anxiety and depression, major life events (e.g., death of a loved one) as well as natural (e.g., the 1998 Quebec ice storm) or man-made (e.g., 9/11) traumatic incidents (Coussons-Read, 2013). These prenatal stressors have been linked with neuropsychiatric disorders in the offspring across the lifespan (Van den Bergh et al., 2020). For instance, maternal experience of high levels of perceived stress, anxiety, or depression during pregnancy were associated with emotional and behavioural problems or anxiety symptoms in infants and children (Davis et al., 2007; O'Connor et al., 2002; Van Den Bergh & Marcoen, 2004) and with depression in adolescents (Kingsbury et al., 2016). Likewise, adults whose mothers were exposed to stressful life events during pregnancy (e.g., the 1976 major earthquake in China) had higher depressive or anxiety symptoms and greater incidence of severe depression (Herbison et al., 2017; Watson et al., 1999). Notably, lessons from the 1944-1945 Dutch famine have clearly demonstrated the adverse lasting impacts of poor prenatal nutrition on the metabolic, cardiovascular, and neuropsychiatric health of the offspring (Roseboom et al., 2006).

While conducting research on prenatal stress and its impacts on the offspring is preferable in human subjects due to the relative ease of translation of the findings into public health and clinical interventions, ethical considerations surrounding such studies often restrict the acquisition of fundamental and critical information such as the biological mechanisms at play. Moreover, assessments of maternal prenatal stress typically rely on subjective self-reports, which can be inconsistent within and across studies, potentially biasing the results and their interpretations (Van den Bergh et al., 2020). Although this latter challenge can be addressed by examining direct prenatal exposure to natural uncontrollable events such as flood, earthquakes, and famine, these

events are infrequent and unpredictable, thus limiting the information that can be derived from such studies (Betts et al., 2015). In contrast, animal models, albeit not without limitations, do not only offer direct empirical evidence of behavioural abnormalities in offspring stressed *in utero* but also enable deeper exploration of the underlying molecular mechanisms at cellular and tissue levels. Furthermore, animal models allow for the control of important variables such as the timing, duration, and intensity of the prenatal stressor, providing valuable insights into the factors influencing prenatal stress outcomes in the offspring. Since most of the previous animal research were carried out in rodents (Weinstock, 2017), the studies presented in this thesis utilized mouse models to facilitate comparison of the results with existing literature.

2.2.1 Rodent models of prenatal stress

Animal studies of prenatal stress have endeavoured to recapitulate the diversity of stressors that can occur in human pregnancy as much as possible. Experimental prenatal stressors that have been used in rodents include noise, electric foot shocks, crowding, chronic variable stress, and physical restraint stress, applied at fixed or variable times during pregnancy. The prenatal noise stressor entails exposing the pregnant female to loud distressing sounds of 300-3000 hertz and 90-95 decibels (Barzegar et al., 2015; Jafari et al., 2017). These acoustic features are reminiscent of noise encountered in industrial sites and high traffic areas, which can constitute significant stress to a pregnant woman (Barzegar et al., 2015). The prenatal inescapable electric foot shock procedure mimics prenatal trauma and is performed by placing the pregnant female in a shock chamber and applying single electric foot shocks (0.5-3 milliamperes, 1-5 seconds) intermittently for up to 80 times daily during a given gestational period (Golub et al., 2016; Takahashi & Kalin, 1991; Velazquez-Moctezuma et al., 1993; Yang et al., 2006). Prenatal crowding is a less commonly used stressor, in which 3 to 5 pregnant females are housed together, mimicking the discomfort that

can arise from shared living environments in humans. This stressor is considered mild and usually paired with more potent stressors such as physical restraint (Hayashi et al., 1998; Murmu et al., 2006). The prenatal chronic variable stress paradigm, which models the unpredictability and randomness of daily life stressful challenges, involves subjecting the pregnant female to a combination of stressors, including physical restraint, odour, constant light, multiple cage changes in a day, overnight soiled bedding and food deprivation, cold, and noise (Subba et al., 2023). The pregnant female experiences one or two novel stressors every day during a specified trimester, thus precluding the possibility of habituation resulting from repeated exposure to a particular stressor (Mueller & Bale, 2006). Lastly, the physical restraint stressor is one of the common techniques used because it is painless and rarely inflicts bodily harm or enduring debilitation on the animal (Buynitsky & Mostofsky, 2009). As the name implies, the physical restraint stressor entails confining the animal to a fixed position for a given time. Typically, the rodent is placed, flat on the belly, in a restraining device (e.g., transparent cylindrical plastic bags or tubes) constructed in a way to restrict body movement while ensuring enough ventilation. In the context of pregnancy, this procedure is conducted once or several times daily, at specific times of the day, in a defined period of pregnancy, with the duration of each restraint session lasting for 30 minutes to a few hours, although variations in the frequency and duration of exposure exist (Weinstock, 2017). This type of prenatal stressor is thought to mimic uncontrollable psychological distress that can be experienced by a pregnant woman (Buynitsky & Mostofsky, 2009). It is note-worthy that although all these prenatal stressors effectively activate the hypothalamic-pituitary-adrenal (HPA) axis in pregnant dams (evidenced by a rapid increase in circulating corticosterone), they exhibit different strengths in their action (Barzegar et al., 2015; Golub et al., 2016; Mueller & Bale, 2006; Murmu et al., 2006). For instance, elevated serum corticosterone levels were more pronounced in pregnant

dams that underwent physical restraint stress compared to those subjected to crowding or chronic variable stress (Murmu et al., 2006). The physical restraint method was chosen for inducing prenatal stress in this thesis because of this demonstrated capacity to elicit robust stress responses from the maternal HPA axis and because preliminary findings from our lab and others showed that it increased anxiety- and depressive-like behaviours and brain pro-inflammatory cytokines (Diz-Chaves et al., 2012; Gur et al., 2019; Zhang et al., 2021), decreased brain BDNF (Jia et al., 2015; Liu et al., 2011), and altered the gut microbiota (Golubeva et al., 2015; Gur et al., 2017, 2019; Zhang et al., 2021) in the offspring, sex-specifically.

2.2.2 Evidence of anxiety- and depressive-like behaviours in postpartum dams and prenatally stressed rodent offspring

Unlike humans, it is impossible to ascertain whether a mouse or rat feels "anxious" or "depressed". However, under certain experimental conditions (e.g., stressor exposure) and using species-relevant tests, rodents can exhibit behaviours that are suggestive of some of the classical symptoms of human anxiety (e.g., fear) and depression (e.g., anhedonia and passive coping) (Weinstock, 2008, 2017). While rodent behaviours can be assessed at various ages during the lifespan, there are limited behavioural test options for neonatal pups. However, studies have utilized ultrasonic vocalizations (USVs) as a proxy for socioemotional phenotypes in young and pre-weaned pups. Rodent pups emit USVs progressively from birth until around the third postnatal week (Noirot, 1972). Although inaudible to humans, these USVs (also known as calls) serve as crucial means of communication between the pups and dams and effectively stimulate distinct maternal care behaviours, such as licking, nursing, nesting, and handling as well as discourage pup cannibalism (Hahn & Lavooy, 2005). In the test, pups are briefly separated from the dams, after which their calls are recorded from whole litters or from individual pups using an ultrasound

microphone in sound-attenuating chambers (Budylin et al., 2019). The acoustic file generated from the recording session is subsequently analyzed for the number, mean duration, peak frequency, and amplitude of the USVs. The call sonograms can also be classified into different sound types according to an existing database of rodent call shapes (Scattoni et al., 2008; Vogel et al., 2019), although the significance of these call types has yet to be entirely elucidated. Although the behavioural implications of USVs are not entirely understood, rodents emitting less or more USVs during the neonatal stage also exhibited more anxiety-like behaviours or less sociability in adulthood, respectively (Budylin et al., 2019; Ehrlich & Rainnie, 2015; Jones et al., 2010; Laloux et al., 2012). Additionally, it has been shown that subcutaneous or intraperitoneal administration of anxiolytics and anxiogenics to neonatal pups suppressed and increased their USVs emission, respectively, indicating that changes in USVs may reflect variations in anxiety-like states in the pups (Fish et al., 2000; Gardner, 1985). In the context of prenatal adversity, prenatal stressors altered USV emissions in neonatal pups, although the findings are inconsistent (likely due to differences in type and timing of the prenatal stressors as well as the rodent strain and age), as whereas some studies showed increased emission (Gulia et al., 2015; Harmon et al., 2009; Laloux et al., 2012; Williams et al., 1998; Zimmerberg & Blaskey, 1998), others reported fewer number of calls (Ehrlich & Rainnie, 2015; Jones et al., 2010; Takahashi et al., 1990).

Beyond the neonatal stage, adolescent and adult rodent offspring can be directly assessed for behaviours reminiscent of anxiety- and depressive-like states in different contexts (e.g., postpartum and prenatal stress). Tests assessing anxiety-like behaviours usually determine the presence of fear-related behaviours and are premised on two conflicting rodent behaviours, the innate desire to explore novel environments and the natural preference for dark and enclosed spaces where they deem safe and protected from predators (e.g., elevated plus maze, open field,

light-dark tests) or the fear of consuming novel unfamiliar foods (e.g., novelty-suppressed feeding tests) (Crawley & Bailey, 2008; Lezak et al., 2017). The tests consist of introducing animals to apparatus composed of open and bright areas that can be combined to enclosed, dark, or less illuminated areas for a short period of time (usually 5-10 minutes) and of determining the time spent, number of entries, and latency of entry into the different areas of the test apparatus as measures of anxiety-like behaviours (Lezak et al., 2017). Animals treated with anxiolytics (e.g., benzodiazepines) spend more time, make more entries, and take less time to enter the open and bright areas, which is considered reflective of lower levels of anxiety-like behaviours, therefore demonstrating the validity of these tests for probing anxiety-related phenotypes (Crawley & Goodwin, 1980; Merali et al., 2003; Pellow & File, 1986; Prut & Belzung, 2003). Using these tests, studies observed anxiety-like behaviours in postpartum dams (Maniam & Morris, 2010) and in adult rodent offspring exposed to the prenatal stressors described above (Golub et al., 2016; Salomon et al., 2011; Vallée et al., 1997; Wang et al., 2015; Zagron & Weinstock, 2006; Zhang et al., 2021; Zuena et al., 2008), although there are reports of no such behaviours in prenatally stressed offspring (Golubeva et al., 2015; Mueller & Bale, 2008), potentially owing to differences in the prenatal stressor and age of the offspring during the behavioural assessment.

Depressive-like behaviours are typically tested using the sucrose preference, forced swim, tail suspension, and splash tests. The sucrose preference test assesses anhedonia-like behaviors, characterized by the inability to derive pleasure in things that were once pleasurable and rewarding (Belovicova et al., 2017; Overstreet, 2012). The animal is first habituated to a sucrose solution in the first segment of the test and then presented with ordinary water and sucrose solution in a subsequent segment, during which their preference for the water or sucrose solution is determined. Reduced intake of the sucrose solution, denoting a decreased preference for a typically rewarding

stimulus, is considered suggestive of anhedonia-like behaviour (Overstreet, 2012). The Splash test evaluates grooming behaviour, based on the animal's motivation to engage in self-care behaviour and/or maintain personal hygiene, which is often impaired in depressed individuals (Kalueff et al., 2016; Smolinsky et al., 2009). In the test, the mouse is gently sprayed with a sucrose solution on both sides of the dorsal coat to elicit grooming, which is recorded with a video camera for up to 10 min. Time spent grooming, number of grooming sessions, and latency to first groom are determined as indices suggestive of depressive-like behaviours. Greater grooming time and frequency as well as lesser grooming latency indicate higher motivation to self-care, and hence less depressive-like behaviour (Smolinsky et al., 2009). The tail suspension and forced swim tests determine passive coping behaviour in the animal when subjected to an uncomfortable, stressful, and inescapable situation. Initially, the animal makes several attempts to escape the situation but eventually succumbs to prolonged periods of immobility after the attempts failed or in the absence of any rescue (Porsolt et al., 1977; Steru et al., 1985). At the end of the test period (usually 6 minutes), the total time spent immobile is calculated and used as a measure of passive coping response to stress, with a longer immobility time being suggestive of depressive-like behaviour. Classical antidepressants (such as fluoxetine) reversed depressive-like behaviours exhibited in these tests, indicating that the tests are valid for assessing such behavioural constructs in rodents (Borsini & Meli, 1988; Machado et al., 2012; Steru et al., 1985). Reduced sucrose intake, immobility time, and grooming has been reported in postpartum dams (Maniam & Morris, 2010; Posillico & Schwarz, 2016) or adult offspring subjected to the prenatal stressors above (Enayati et al., 2020; Isingrini et al., 2010; Mueller & Bale, 2008; Ślusarczyk et al., 2015; Szczesny et al., 2014; Zhang et al., 2021). Given that the sucrose solution used in the sucrose preference test may interact with the animal's gut microbiota (which is one of the biological variables in this thesis

studies) and that the forced swim test involves a high degree of stress to the animal, the tail suspension and splash tests were preferred for behavioural evaluations in the studies presented in this thesis.

2.3 Potential mechanisms underlying prenatal stress outcomes on offspring's mental health

The biological processes potentially involved in the behavioural and mental health effects of prenatal stress in the offspring are diverse and complex and can act independently or synergistically to produce robust and persistent impacts (Wu et al., 2024). This section discusses the processes that are most pertinent to the studies conducted in this thesis.

2.3.1 Dysfunction of the HPA axis

The primary mechanism proposed to mediate the negative impacts of prenatal stress on offspring's development and behaviour is excessive fetal exposure to glucocorticoids (cortisol in humans and corticosterone in rodents), which are part of the hormonal end-products of the HPA axis (Glover et al., 2010). Glucocorticoids play vital roles in the growth and maturation of fetal tissues and organs (Fowden et al., 2015), notably by stimulating surfactant production in the fetus lungs in late pregnancy, which is necessary for neonatal survival (Bolt et al., 2001). Synthetic glucocorticoids are clinically recommended for women at risk of preterm birth to accelerate fetal lung maturation, and their use remarkably reduces the incidence and severity of respiratory distress syndrome (a fatal preterm birth complication) and associated neonatal and infant mortality (Crowther et al., 2015; McGoldrick et al., 2020). Glucocorticoids promote cell proliferation, differentiation, and survival in the fetal brain (Carson et al., 2016; Moisiadis & Matthews, 2014). Additionally, glucocorticoids promote the differentiation of intestinal epithelial cells, expression of intestinal enzymes, and maturation of the immune and barrier functions of the intestinal

epithelium (Costalos et al., 2003; Nanthakumar et al., 2005; Quaroni et al., 1999; Van Beers et al., 1995). The availability of glucocorticoids for fetal development is ensured through deliberate and progressive increase in the maternal HPA axis activity throughout gestation (Brunton & Russell, 2011; Mastorakos & Ilias, 2003). In addition, increased levels of the human placental corticotropin-releasing hormone (CRH) during the last trimester stimulate cortisol production from the maternal and fetal HPA axis to meet the increased cortisol demands for late gestational organ maturation (Gangestad et al., 2012; Smith et al., 1998).

Under normal physiological conditions, only 10-20% of maternal glucocorticoids traverses the placenta into fetal circulation, due to the activity of a placental enzyme, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD-2), which converts cortisol into an inactive form, cortisone (Benediktsson et al., 1993). Additionally, circulating cortisol is bound to corticosteroid-binding globulin (CBG), limiting the amount of free and bioactive cortisol reaching the fetus (Siiteri et al., 1982). However, by further activating the maternal HPA axis, prenatal stress increases glucocorticoid transmission to the fetus beyond optimal and safe levels (Takahashi & Kalin, 1991; Wieczorek et al., 2019). Unlike the hypothalamic CRH, maternal cortisol surges due to stress stimulate placental CRH synthesis and release, which, in turn, increases cortisol secretion and ultimately sustains a positive feedback loop that promotes excessive fetal cortisol exposure (Sandman, 2018). Moreover, prenatal stress reduces the expression and activity of the placental 11β-HSD-2, thereby rendering the fetus unprotected from stress-induced elevations in maternal glucocorticoids (Appleton et al., 2013; Mairesse et al., 2007; O'Donnell et al., 2012; Peña et al., 2012; Welberg et al., 2005). Despite their importance in fetal growth, excessive levels of glucocorticoids can retard the developing fetal organs, including the brain (Harris & Seckl, 2011). The limbic areas, such as the hippocampus and prefrontal cortex, are particularly sensitive to the

detrimental effects of abnormally high glucocorticoid levels due to the vast concentration of glucocorticoid and mineralocorticoid receptors in these regions (Reul & De Kloet, 1985). Rodent and non-human primate offspring exposed to prenatal stress exhibited reduced neurogenesis, fewer number of dendritic spines and arborization, and synaptic loss in the hippocampus or frontal cortex Barros et al., 2006; Coe et al., 2003; Fujioka et al., 2006; Hayashi et al., 1998; Ishiwata et al., 2005; Kawamura et al., 2006; Lemaire et al., 2000; Valerie Lemaire et al., 2006). Excessive glucocorticoids stemming from maternal stress or dexamethasone administration could also lead to hyperactivity of the offspring's HPA axis, with potential behavioural implications. In line with this, prenatally stressed adult offspring displayed higher basal corticosterone secretion alongside increased anxiety- and depressive-like behaviours (Abe et al., 2007; De Vries et al., 2007; Levitt et al., 1996; Maccari et al., 1995; Uno et al., 1994; Weinstock et al., 1992). These offspring also showed greater corticosterone release when exposed to another stressor in adulthood (Takahashi & Kalin, 1991; Uno et al., 1994; Weinstock et al., 1992). Additionally, their stress-induced corticosterone secretion was protracted (Fride et al., 1986; Maccari et al., 1995; Morley-Fletcher et al., 2003; Uno et al., 1994), indicating delayed negative feedback inhibition of the HPA axis, likely due to reduced number of glucocorticoid and mineralocorticoid receptors in the hippocampus (Henry et al., 1994; Levitt et al., 1996; Szuran et al., 1994; Weinstock et al., 1992). These alterations in the offspring's HPA axis and associated anxiety-like behaviours were prevented by maternal adrenalectomy but reinstated by corticosterone administration to the adrenalectomized pregnant dams, pointing to a glucocorticoid-related mechanism in the developmental and behavioural effects of prenatal stress (Barbazanges et al., 1996; Salomon et al., 2011b; Zagron & Weinstock, 2006).

2.3.2 Dysfunction in the immune system

The role of the immune system in the development and pathophysiology of stress-related mental disorders has been established in human populations as well as in animal models. A consistent finding in individuals with major depressive and/or generalized anxiety disorders is the higher levels of pro-inflammatory factors such as C-reactive protein (CRP), tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β, and IL-6 in the blood, cerebrospinal fluid, or post-mortem brain (Enache et al., 2019; Osimo et al., 2020). Many individuals that responded to antidepressant treatment saw a reduction in their systemic levels of pro-inflammatory cytokines, although not always the case (Mosiołek et al., 2021), indicating that immune dysregulation may contribute to the pathophysiology of depression. In animal models, immunogenic substances (e.g., the bacterial endotoxin lipopolysaccharide; LPS) do not only elicit peripheral and brain inflammatory activation but also lead to behavioural disturbances, including increased anxiety- and depressive-like behaviours (Bay-Richter et al., 2011; Carabelli et al., 2020; Dang et al., 2018; Qiu et al., 2020; Sharma et al., 2018; Shi et al., 2017; Zhao et al., 2019). Compared to the HPA axis, fewer studies have investigated the role of the immune system in mediating the adverse behavioural outcomes of prenatal stress in the offspring. However, the interplay between glucocorticoids and inflammatory factors during the stress response (Zefferino et al., 2021) suggests that stress-induced activation of the maternal HPA axis could elicit changes in maternal immune system and, in turn, increase placental transmission of inflammatory markers into the fetal circulation, potentially impacting the developing organs and systems. In support of this, pregnant women that experienced greater psychological distress had higher serum levels of TNF-α and IL-6 as well as increased production of these cytokines from their lymphocytes upon stimulation (Coussons-Read et al., 2005, 2007, 2012). In mice, maternal chronic variable stress or physical restraint stress upregulated

the mRNA expression of IL-1β and/or IL-6 in the placenta and fetal brain (Bronson & Bale, 2014; Gur et al., 2017).

Although pro-inflammatory cytokines play crucial roles in fetal brain development, such as stimulating the expression of genes involved in the proliferation and differentiation of neural progenitor cells (Deverman & Patterson, 2009; Mousa & Bakhiet, 2013), excessive levels could lead to structural and functional alterations in the brain and increase the risk for neuropsychiatric disorders (Dammann & O'Shea, 2008; Smith et al., 2007). For instance, inflammation ensuing from maternal immune activation by polyriboinosinic–polyribocytidylic acid disrupted neurovascular unit formation in the fetal cortex, marked by a decrease in pericyte coverage of vascular endothelial cells (Zhao et al., 2022). This deficit in the neurovascular structure was associated with persistently increased BBB permeability and neuroinflammation, along with increased anxiety-like behaviours in the adult offspring (Zhao et al., 2022). Importantly, findings in mice and humans suggest that impaired BBB functions, promoted by reduced expression of the major tight junction claudin-5, may be involved in the pathogenesis of depression (Dion-Albert et al., 2022; Menard et al., 2017). Considering this, it is plausible that prenatal stress-induced increases in fetal inflammatory markers could disrupt the developing BBB among other potential developmental impacts, contributing to adverse neuropsychiatric consequences in the offspring. As further support that alterations in the immune development and functions may be involved in the behavioural effects of prenatal stress, adult mice and rats exposed to physical restraint stress during pregnancy exhibited increased anxiety- and depressive-like behaviours, in addition to increased basal and LPS-induced microglial activity and expression of TNF-α and IL-6 in the hippocampus and prefrontal cortex (Diz-Chaves et al., 2012, 2013; Gaviola et al., 2004; Gur et al., 2017; Ślusarczyk et al., 2015; Szczesny et al., 2014). Furthermore, prenatally stressed adult

offspring has been shown to have elevated pro-inflammatory cytokines and decreases in tight junction proteins in the colon, and these changes in the colonic environment may stem from perturbations in the gut microbiota (reviewed in the next section) (Sun et al., 2021). Collectively, the available evidence suggests that prenatal stress may lead to long-lasting disturbances in the establishment of brain and intestinal inflammatory immune processes and barrier functions, and that these changes could partly underlie the abnormal behaviours seen in the offspring.

2.3.3. Perturbations to gut microbiota development

The gut microbiota is increasingly recognized as a key player in stress-related mental disorders due to its influence on brain functions *via* neural, endocrine, immune, and metabolic pathways in the gut-brain axis (Cryan et al., 2019). The gut microbiota composition of individuals with major depression and generalized anxiety disorders differs from that of healthy individuals, with characteristic patterns of less *Bifidobacteria*, *Lactobacilli*, and *Faecalibacterium prausnitzii* (Aizawa et al., 2016; Jiang et al., 2018; Zheng et al., 2016). These bacteria are associated with mental health benefits partly due to their production of short-chain fatty acids (SCFAs), which are the main bacterial metabolites resulting from fiber fermentation that exert anti-inflammatory effects in the gut and brain as well as support intestinal barrier functions (Silva et al., 2020). Interestingly, GF mice or microbiota-depleted rats exhibited depressive- (anhedonia) and anxiety-like behaviours after receiving fecal microbiota transplants from major depressive individuals, pointing to a causal role for gut microbes in depression (Kelly et al., 2016; Zheng et al., 2016).

Microbial colonization of the offspring's gut primarily begins at birth, when the newborn encounters the maternal vaginal and/or skin microbial communities during vaginal and caesarean deliveries (Ferreti et al., 2018). A growing body of evidence demonstrates that prenatal stressors alter the maternal vaginal and gut microbial communities (the primary sources of microbes

colonizing the offspring's gut), thereby disrupting microbial colonization pattern of the offspring's gut (Aatsinki et al., 2020; Bailey et al., 2004; Golubeva et al., 2015; Gur et al., 2017, 2019; Hechler et al., 2019; Jašarević et al., 2015, 2017, 2018; Zhang et al., 2021; Zijlmans et al., 2015). While a causal link between disrupted gut microbial development and mental disorders in the offspring has yet to be established, prenatally stressed adult mice with different gut microbiota composition throughout their lifespan also displayed more anxiety- and depressive-like behaviours and reduced sociability (Gur et al., 2017, 2019; Zhang et al., 2021).

One way by which disturbances in the early-life establishment of the gut microbiota may impact mental health is through increasing pro-inflammatory activation in the gut-brain axis. Normally, the gut microbiota composition during the neonatal/infant stage is dominated by *Lactobacillus* and Proteobacteria, reflecting the maternal vaginal microbiota composition towards the third trimester and vertical transmission of these maternal microbes to the offspring's gut (Bergström et al., 2014; Jašarević et al., 2015). However, *Lactobacilli* is underrepresented and Proteobacteria is overrepresented in neonates and infants born to dams exposed to prenatal stress or to mothers that experienced significant distress during pregnancy (Golubeva et al., 2015; Gur et al., 2017; Zijlmans et al., 2015). Such gut microbiota patterns have been associated with increased intestinal inflammation and permeability, facilitating bacteria or LPS translocation to the intestinal epithelium or the bloodstream, activating the immune system and release of pro-inflammatory factors (Agus et al., 2016; Cani et al., 2009; Carvalho et al., 2012). Considering that peripheral inflammatory cytokines can cross the BBB and trigger immune response in the brain and the role of inflammation in the development of mental disorders, the possibility exists that stress-induced disturbances in microbial colonization of the offspring's gut could increase pro-inflammatory activation in the gut-brain axis during a critical period of brain maturation,

potentially influencing brain development and functions in a way that may increase susceptibility to mental disorders. Additionally, the findings that the gut microbiota drives postnatal maturation of intestinal innate and adaptive immune system suggest that abnormal microbial patterns in the postnatal period may alter the establishment and function of intestinal inflammatory processes (Gensollen et al., 2016; Hapfelmeier et al., 2010). Furthermore, there is evidence that the gut microbiota influences postnatal brain maturation, indicating another avenue by which altered gut microbial development may promote mental disease risk (Borre et al., 2014). For instance, GF mice exhibited higher protein levels of post-synaptic density-95 and synaptophysin in the striatum (Heijtz et al., 2011) and increased prefrontal expression of genes involved in myelination and myelin plasticity, leading to hypermyelination of PFC axons, which was reversed by post-weaning microbial colonization (Hoban et al., 2016). Altered fetal brain development has also been reported in GF mice, with findings of increased BBB permeability alongside reduced tight junction (occludin and claudin-5) expression (Braniste et al., 2014) and altered microglial gene expression (Thion et al., 2018). These alterations in the fetal GF mice brain are likely mediated by the maternal microbiome, as it influenced the expression of genes involved in thalamocortical axonogenesis (Vuong et al., 2020), suggesting that disturbances in the maternal microbiome due to prenatal stress may not only alter maternal-neonate microbial transmission but also brain development *in utero*, with potential impacts on offspring's mental health.

2.4 The timing of the prenatal stressor matters

Not every individual who experienced stress *in utero* will develop mental health disorders. One of the factors regulating this outcome is the time of fetal exposure to the prenatal stressor. While most biological organs and systems undergo their critical stages of development during the prenatal period (Barker, 2004), the timeline of development and hence the window of vulnerability

to prenatal insults differs across gestation (Rice & Barone, 2000; Semple et al., 2013). Brain development begins in the third week of the embryonic stage in humans and embryonic day (E)7 in mice (corresponding to early second trimester), marked by the folding of the neural plate to form the neural tube (E8.5 in mice), from which the brain and spinal cord differentiate (Chen et al., 2017; Semple et al., 2013). Subsequently, milestone events involving rapid cell proliferation, migration, and differentiation occur, forming the three primary brain vesicles, giving rise to the forebrain, mid brain, and hind brain at E9 (Chen et al., 2017). By E12.5, structures including the hippocampus and prefrontal cortex have established in the mouse brain and continue to mature for the rest of gestation and postnatally (Chen et al., 2017; Kolk & Rakic, 2022). Also, the neuroimmune system develops in the second trimester of mouse pregnancy (Holsapple et al., 2003), with microglia colonizing the fetal brain around E9.5 (Bilbo & Schwarz, 2012). Alongside neural plate formation, gastrulation occurs at E7.5 in mice, forming the endoderm, which folds into the primitive gut tube (Montgomery et al., 1999). At E8.5, the gut tube segments into the foregut, midgut, and hindgut, each giving rise to specific organs and structures (including liver, pancreas, and intestines), followed by maturation processes at E12.5, involving villi development, differentiation of gut layers, establishment of the enteric nervous system and vasculature (Chin et al., 2017). These developmental processes are critical to establishing the foundational elements of the brain and gut, and are thus highly sensitive to insults, such that any disruptions during this time, including altered expression and signaling of trophic factors, (e.g., cytokines and BDNF) regulating these processes, can have drastic effects on the establishment and function of the nervous system (Rice & Barone, 2000). Although the BBB is already present during embryogenesis with tight junctions detected in cerebral vessels and choroid plexus, it is not yet fully functional and thus more permeable, meaning that programming peripheral factors like

glucocorticoids and cytokines ensuing from maternal stress could easily access the fetal brain and influence its development (Daneman et al., 2010; Saunders et al., 2012). Also, the fetus may not be sufficiently protected from elevated levels of maternal glucocorticoids and cytokines elicited by stress in early pregnancy, given that placental barrier function is not fully established until around E10.5 (Woods et al., 2018) and the placental enzyme 11β-HSD2 may be absent or present in very low amounts in early gestation (Zheng et al., 2020; Zhu et al., 2018). Indeed, although most of the previous assessments of the effect of prenatal stress were done in the third trimester (reviewed in Weinstock, 2017), it appears that stress encountered in early gestation may produce the most influential impact in the offspring. For instance, stress during the first or second trimester, but not the third trimester, was associated with dysfunction in the HPA axis (marked by salivary cortisol) and elevated depressive and anxiety symptoms in adult men and adolescent girls (Herbison et al., 2017; Van Den Bergh et al., 2008). Maternal anxiety at 12-22 weeks but not 32-40 weeks was associated with anxiety disorder in 8-9 year olds (Van Den Bergh & Marcoen, 2004). Male mice only exhibited greater HPA axis responsivity to later-life stress and anhedonia if they were stressed in early pregnancy instead (Mueller & Bale, 2008). Moreover, women reported to be more sensitive and emotionally disturbed when they experienced stress in early pregnancy rather than a later time (Weinstock, 2017), suggesting the offspring may be more predisposed to early stressors during this time. Given that the development of the mouse hippocampus, prefrontal cortex, and colon (which are regions of interest in this thesis) commences in the second trimester of mouse pregnancy (Chen et al., 2017; Kolk & Rakic, 2022; Semple et al., 2013) and that offspring may be more susceptible to early prenatal stress, studies detailed in this thesis were conducted in the second trimester.

2.5 Sex differences in the physiological and behavioural effects of prenatal stress

It is unsurprising that prenatal stress differentially affects female and male offspring, considering the sexual dimorphism in development across the lifespan, including the brain (De Bellis et al., 2001; Premachandran et al., 2020). For example, compared to males, female mice had fewer numbers and more activated microglia in the CA1, CA3, and dentate gyrus of the hippocampus in neonatal and adolescent stages but their microglial numbers exceeded that of the males in adulthood (Schwarz et al., 2012). Moreover, the placental function is sex specific, such that placental 11β-HSD2 expression is generally lower in male placentas than the females (Stark et al., 2009), suggesting that the male fetus may be less protected from surges in maternal glucocorticoids due to stress. Additionally, in response to inflammation induced by maternal asthma, placental 11β-HSD2 activity is reduced in females, compromising fetal growth, in contrast to elevated enzymatic activity in the male placenta (Clifton, 2005). While most of the previous studies of the effects of prenatal stress (reviewed in Weinstock, 2017) were conducted in the male offspring, those that included males and females found sex differences in the physiological and behavioural outcomes assessed, although some reported no sex differences. In human studies, the incidence of major depression and anxiety was higher in males than females exposed to earthquake *in utero* (Herbison et al., 2017; Watson et al., 1999). In the animal literature, increased anxiety-like behaviours elicited by prenatal stress were mostly reported in the female offspring (Bowman et al., 2004; Richardson et al., 2006; Schulz et al., 2011; Van den Hove et al., 2014; Zagron & Weinstock, 2006) than the males (Zuena et al., 2008). Conversely, other studies reported increased anxiety-like behaviours in prenatally stressed offspring of both sexes (Fride & Weinstock, 1988; Palacios-García et al., 2015; Wang et al., 2015; Zhang et al., 2021; Zohar et al., 2015; Zohar & Weinstock, 2011). In contrast, males appear to be more sensitive to the physiological alterations

ensuing from prenatal stress. Altered HPA axis function and decreased neurogenesis have been mostly observed in prenatally stressed adult male offspring than their female counterparts (Mueller & Bale, 2008; Szuran et al., 1994; Van den Hove et al., 2014; Zuena et al., 2008). Also, stress-induced increases in mRNA expression of placental IL-1β and IL-6 (Bronson & Bale, 2014) and colonic TNF-a (Jašarević et al., 2018) were seen in only males. Sex differences were also shown to depend on the period of gestation when the stress occurred. For instance, males appeared to be predisposed to anxiety and depression if stressed early in pregnancy than later (Herbison et al., 2017; Mueller & Bale, 2008). This strong sex bias in the developmental and behavioural impacts of prenatal stressors highlights the crucial relevance of sex in studies examining prenatal stress and offspring's outcomes and, more importantly, in interventions aimed at mitigating the outcomes.

2.6 Diet may improve mental health in the mother and prenatally stressed offspring

Although still in the early stages, research demonstrates that diet may influence the behaviour and mental health of the mother and offspring. The findings that maternal consumption of diets high in saturated fat during pregnancy increased anxiety- and depressive-like behaviours in the adult offspring (Sullivan et al., 2015) does not only indicate a programming effect of diet on offspring's behaviour but also suggests that healthy dietary modifications could lead to positive behavioural outcomes in the offspring. It is well established that dietary nutrients, including protein, long-chain polyunsaturated fatty acids (notably, docosahexaenoic acid and arachidonic acid), iron, and iodine, are critical to early brain development and deficiency in these nutrients, especially during a period of increased demands, can lead to adverse physical and neurodevelopmental outcomes as well as behavioural abnormalities (Cusick & Georgieff, 2016). For instance, docosahexaenoic acid is necessary for neurodevelopmental milestones, including

neurogenesis, neuronal migration, synaptogenesis, and myelination in the prenatal and postnatal stages (Innis, 2008). Deficiency of docosahexaenoic acid in maternal and early postnatal diets led to deficits in neurogenesis as well as to social impairments and depressive-like behaviours in the adult offspring (Robertson et al., 2017), whereas maternal diets enriched with olive oil increased the neurotrophin BDNF (a key factor in neurogenesis) in the prefrontal cortex of adult rat offspring (Pase et al., 2015). Diets deficient in omega-3 fatty acids can lead to an imbalance in the omega-6/omega-3 ratio, which is associated with inflammation and chronic diseases such as cardiovascular diseases (Simopoulos, 2002). Additionally, the findings that dietary omega-3 fatty acids and polyphenols prevented or reduced inflammatory markers in intestinal inflammatory diseases or neurodegenerative models (Layé et al., 2018; Romier et al., 2009) demonstrates the capacity of dietary components to modulate pro-inflammatory activation, which is implicated in mental disorders in postpartum and prenatal stress contexts, as discussed in the previous sections.

Furthermore, diet is one of the major factors shaping the developmental trajectory of the offspring's microbiota (Bäckhed et al., 2015; Stewart et al., 2018). The maternal diet influences the composition of their vaginal and gut microbiota, which, as mentioned earlier, are the primary sources of microbes colonizing the offspring's gut after birth. This underscores not only the role of maternal prenatal diet in priming the initial microbial assembly in the offspring's gut but also suggests that diet can influence changes in the maternal gut microbiota in relation to postpartum mental health. Beyond the prenatal level, the infant's mode of feeding (breast milk versus formula) in the first 6 months profoundly modulates the gut microbial colonization (Stewart et al., 2018). While several species of *Lactobacilli* (e.g., *L. rhamnosus and L. johnsonii*) and *Bifidobacteria* (e.g., *B. breve and B. bifidum*) enrich the guts of breast-fed infants in the first 6 months, formula-fed infants exhibit a dominance of species found in the adult gut microbiota, including *Clostridium*

difficile, *Escherichia coli*, and *Citrobacter spp*. (Azad et al., 2013; Bäckhed et al., 2015; Harmsen et al., 2000; Stewart et al., 2018). These distinct gut microbial characteristics are due to the unique composition of the breast milk and formula. Besides being replete with nutrients that nourish, protect against infections, and ultimately promote infant growth, breast milk consists of several bioactive compounds that significantly contribute to establishing the gut microbes. Among these bioactive constituents are the human milk oligosaccharides (HMOs), which make up about 20% of the total carbohydrates and are the third most abundant solid component, after lactose and fat (Urashima et al., 2012). HMOs are insensitive to gastric pH and resist gastrointestinal digestion and absorption but are fermented by colonic bacteria to produce acetic acid (among other SCFAs), which reduces colonic pH and thus inhibits the growth of pathogenic bacteria. With varying HMOs degradation mechanisms and specificities, *Bifidobacterium* species are particularly known to utilize HMOs and contain genes encoding several HMOs-degrading enzymes (Asakuma et al., 2011; Egan et al., 2014; Garrido et al., 2011; Sakurama et al., 2013; Sela et al., 2008; Ward et al., 2006), which largely explains the high density of *Bifidobacteria* in the guts of breast-fed infants. Notably, the maternal diet influences breast milk's HMOs content, and thus indirectly contributes to shaping the offspring's gut microbial communities (Babakobi et al., 2020; Cortes-Macías et al., 2021; Hallam et al., 2014).

Weaning represents another milestone during the infant's gut microbiota development, and this is especially the case for breast-fed infants. This period is marked by the cessation of breast milk and subsequent introduction of solid foods, drives the maturation of the infant's gut microbiota, characterized by an increased bacterial richness and diversity and functional capacity (Bäckhed et al., 2015). During this time, the gut microbiota of breast-fed infants undergoes dramatic reductions in the once-dominant *Bifidobacterium* species and the appearance of species

that metabolize complex, indigestible plant polysaccharides (Bäckhed et al., 2015; Bergström et al., 2014). Such compositional and functional maturation in the gut microbiota are absent in undernourished infants, who rather exhibit persistent dominance of several Gram-negative pathogenic bacteria (Ghosh et al., 2014; Gupta et al., 2011; Mata et al., 1972; Monira et al., 2011; Subramanian et al., 2014; Smith et al., 2013). These dietary influences on the gut microbiota during early development provide an opportunity for dietary modifications to promote healthy gut microbial establishment, especially in prenatally stressed offspring who may be at risk for mental disorders arising from perturbations to the gut microbiota due to prenatal stress.

2.6.1 The Med-based diet as a dietary strategy for promoting mental health in postpartum mothers and prenatally stressed offspring

Originating in the Med basin, the Med diet is one of the popular dietary patterns that have received sustained interest in the scientific research community. Although civilization has led to an unfortunate departure from some of the cornerstone constituents of the Med diet over the years (Lăcătușu et al., 2019), the traditional Med diet is characterized by high consumption of olive oil (the main added fat), whole grains, legumes, fruits, and vegetables, moderate intakes of fish, dairy, nuts, and alcohol (commonly red wine taken with meals), and limited consumption of red meat and processed foods (Trichopoulou et al., 2014). The definition of the Med-based diet (and its health impacts) also integrates the cultural habits and lifestyle of the indigenous Med populations, which importantly promote communal interactions and social health (Trichopoulou et al., 2014). The health benefits of the Med diet, beyond basic nutrition, were first brought to public health limelight in the seminal Seven Countries Study by Ancel Keys and colleagues, in which individuals inhabiting the Crete Island of Greece were observed to have the lowest incidence of coronary heart disease and associated mortality, compared to counterparts in the United States and Northern

Europe (Keys et al., 1984). Inspired by Keys' findings, subsequent examinations of the Med dietary patterns have not only confirmed their protective or mitigating actions against cardiovascular adversities (Estruch et al., 2018), but also against several other pathologies, including stroke (Lakkur & Judd, 2015), cancer (Mentella et al., 2019), obesity (Meslier et al., 2020), type 2 diabetes (Salas-Salvadó et al., 2011), chronic inflammatory disease (Casas et al., 2014), and neurodegenerative diseases (Gardener & Caunca, 2018). Ultimately, these findings have contributed to the wide critical acclaim of the Med diet as a gold-standard diet for maintaining optimal health and lowering disease risk (Martinez-Gonzalez & Martin-Calvo, 2016).

Accumulating evidence points to the beneficial effects of the Med diet on mental health (Firth et al., 2019). In this regard, a seminal clinical intervention study found that dietary modifications based on the Med diet for 12 weeks significantly mitigated depressive symptoms in patients with major depression, compared to those that received only social support (Jacka et al., 2017), demonstrating that the Med-based diet may serve important adjuvant roles in the treatment depression. In context of maternal and offspring mental health, studies reported that women who mostly adhered to Med dietary patterns had fewer scores of perinatal anxiety and depression in women (Chatzi et al., 2011; Papadopoulou et al., 2023), and their children had lower risk for depression, anxiety, or socioemotional problems (Crovetto et al., 2023; House et al., 2018). How the Med-based diet may influence mental health is less known, but evidence suggests it could be through modulation of biological processes linked with mental disorders, including inflammation (Itsiopoulos et al., 2022), neurotrophic factors (Radd-Vagenas et al., 2018), and the gut microbiota (Bailey & Holscher, 2018). In support of this, observational studies (Hart et al., 2021) and randomized controlled trials (Koelman et al., 2022) in adults found that the Med-based diet was associated or reduced systemic markers of inflammation, including IL-6 and C-reactive protein

(CRP), in healthy and disease states (type 2 diabetes, arthritis, coronary heart disease), while no substantial effects on these inflammatory markers were observed for other dietary patterns, such as Dietary Adherence to Stop Hypertension (DASH) diet (Koelman et al., 2022). Higher adherence to Med-based diets was associated with lower odds for plasma BDNF reductions (Mohammadi et al., 2023), and a 2-year intervention with this diet supplemented with nuts increased plasma BDNF levels in individuals with depression (Sánchez-Villegas et al., 2011). Furthermore, Med-based diets increased the abundance of bacteria that produce short chain fatty acids (SCFAs) in pregnant women and in general adult populations (De Filippis et al., 2016; Garcia-Mantrana et al., 2018; Gutiérrez-Díaz et al., 2016; Miller et al., 2021; Mitsou et al., 2017), which have anti-inflammatory effects in the intestinal environment and support intestinal epithelial barrier function (Koh et al., 2016). It has been reported that these SCFAs mediated the improvements in intestinal barrier integrity seen in women that received a Med-based diet (Seethaler et al., 2022). Collectively, these lines of evidence suggest that Med-based diets could modulate changes in inflammatory processes, neurotrophic, and gut microbiota associated with the perinatal period in the mother and prenatal stress in the offspring, and potentially improve mental health.

2.7 Research questions, objectives, and hypotheses

As reviewed in the preceding sections, while biological changes during the perinatal period are necessary for fetal development, they can inadvertently increase maternal risk for postpartum mental disorders, potentially leading to developmental consequences for the offspring. These consequences can be exacerbated by maternal prenatal adversity, which is well-documented to disrupt the normal developmental trajectory of the offspring's organs and systems, predisposing to mental disorders across their lifespan in a sex-specific manner. However, growing evidence indicates that diet, particularly those based on Mediterranean (Med) dietary patterns, influences

mental health by modulating key biological factors such as inflammatory processes, tissue barrier functions, neurotrophic activity, and gut microbiota. Considering this evidence, this thesis aimed to address the following questions:

1. Can Med dietary patterns improve mental health outcomes in postpartum mothers and in offspring exposed to prenatal adversity?
2. Can Med dietary patterns mitigate alterations in immune, neurotrophic, and gut microbiota factors linked with mental health in postpartum mothers and prenatally stressed offspring?
3. Do the interactive effects of prenatal adversity and Med dietary patterns in prenatally stressed offspring produce different outcomes in females and males?

To address these questions, two mouse studies were conducted with the following objectives:

Objective 1 (Study 1; Chapter 3)

The first objective was to develop a Med-based diet for mice based on human dietary patterns as well as a matched Control diet and conduct a pilot study to confirm the diets were palatable and safe for breeding mice.

Objective 2 (Study 1; Chapter 4)

After validating the palatability and safety of the diets, the second objective was to assess whether the Med-based diet improved anxiety- and depressive-like behaviours in postpartum dams (in Objective 1), and whether the behavioural outcomes were related to changes in the gene expression of pro-inflammatory cytokines, BDNF, and tight junctions in the hippocampus and prefrontal cortex and/or colon (involved in physiological adaptations to the perinatal period and/or implicated in mental disorders) as well as changes in the fecal microbiota diversity, composition, and predicted function.

It was hypothesized that mice fed the Med-based diet would exhibit less anxiety- and depressive-like behaviours, lower mRNA expression of pro-inflammatory cytokines and higher mRNA expression of BDNF and tight junctions in the brain and/or gut regions assessed. Also, mice fed the Med-based diet were expected to have greater representation of fecal bacteria that produce SCFAs (e.g., *Akkermansia*) and less of those associated with inflammation (e.g., Proteobacteria).

Objective 3 (Study 2; Chapter 5)

The third objective was to determine whether the Med-based diet limited the impacts of a prenatal stressor in the offspring during early development by assessing changes in neonatal female and male pup's USVs as well as the gene expression of pro-inflammatory cytokines, BDNF, and tight junctions in their hippocampus and prefrontal cortex and their fecal microbiota diversity, composition, and predicted function.

It was hypothesized that the prenatal stressor would alter neonatal USVs and lead to sex-specific increases in pro-inflammatory cytokines and decreases in BDNF and tight junction markers in the brain as well as changes in the fecal microbiota, including increased Proteobacteria and reduced *Lactobacilli*. As well, it was expected that the Med-based diet would limit the biological impacts of the prenatal stressor in a sex-specific manner.

Objective 4 (Study 2; Chapter 6)

The fourth and last objective was to determine whether the Med-based diet limited the effects of the same prenatal stressor used in Objective 3 on anxiety- and depressive-like behaviours, gene expression of pro-inflammatory cytokines and tight junctions in the hippocampus, prefrontal cortex, and/or colon, and cecal microbiota diversity, composition, and predicted function in adult female and male offspring.

The prenatal stressor was hypothesized to elicit anxiety- and depressive-like behaviours, increase pro-inflammatory cytokines and decrease tight junction markers in the brain and colon, and lead to shifts in the cecal microbiota (e.g., increased Proteobacteria and reduced butyrate-producers). Similar to Objective 3, it was expected that the Med-based diet would limit these impacts of the prenatal stressor in a sex-specific manner.

Chapter 3: Development and validation of the experimental diets

3.1 Dietary development

The experimental diets used as part of the studies described in this thesis were developed in collaboration with scientists at Research Diets, Inc. (New Brunswick, NJ). The Control diet was developed using an Open Standard Diet (D12052701M, Research Diets Inc.) as a template. Extra casein was added to the Open Standard Diet to increase the total protein content to about 17% to meet mouse breeding requirements (Nutrition, 1995). Maltodextrin was also added to transform it from powder to pellets, which allowed for accurate measurements of food intake. The amount of cornstarch was reduced to adjust for the extra carbohydrates and calories from maltodextrin. Lastly, FD&C Yellow dye was removed from the Open Standard Diet since this ingredient provides no nutritional benefits and may interact with the gut microbiota (Zahran et al., 2024), which is one of the measures in the studies presented in this thesis. The Med-based diet was modeled after the 1999 Greek dietary guidelines, as outlined in Davis et al., 2015, which were founded on traditional Greek dietary patterns in the 1960's, a period when the Greek diet was linked to superior cardiovascular health, compared to the American and Northern European dietary patterns at that time (Keys et al., 1984). A mouse Med-based diet developed previously and commercially available at Research Diets, Inc. (Barrington et al., 2018) was used as a template to formulate the Med-based diet used in the present thesis. This template diet was extensively modified to increase its representation of some of the typical components (and their amounts) of Med dietary patterns in human populations. The modifications are described as follows:

3.1.1 Addition of legumes, fruits, vegetables, and walnuts:

First, legumes, fruits, vegetables, and nuts were included in the mouse Med-based diet, as they constitute the core components of the human Med diet (Davis et al., 2015). Chickpeas and

lentils were used as representatives of legumes and plant proteins in the diet (Naureen et al., 2022). Canned unsalted chickpeas were drained, rinsed, and blended into a puree. Dry red split lentils were cooked and blended to obtain a puree. The chickpea and lentil purees were then freeze-dried (Labconco Freeze-dryer), and the dry powders were analyzed for their macronutrient (carbohydrates, proteins, and fats) and fiber contents before incorporating them into the diet in equal proportions (Intertek, Saskatoon, Canada). Freeze-dried or air-dried fruits (apples, peach, strawberries, blueberries, and pomegranates) and vegetables (kale, spinach, broccoli, tomatoes, and carrots), freely supplied by FutureCeuticals, Inc. (Momence, Illinois, United States) along with their nutritional information, were added to the diet in equal amounts. Whole unsalted walnuts were ground and added to the diet. The choice of walnuts was based on previous findings that Med-based diets supplemented with nuts (predominantly walnuts, rich in omega-3 fatty acids) reduced the risk for myocardial infarction, stroke, and cardiovascular-related mortality (Estruch et al., 2018) and association of walnuts intake with lower risk for depression (Arab et al., 2019).

3.1.2 Addition of beta glucan, resveratrol, and maltodextrin:

As the human Med diet patterns are rich in fibers and polyphenols, additional sources of fiber and polyphenol were included in the mouse diet. Beta glucan was added to the diet, as this soluble fiber promoted anti-inflammatory gut bacteria, reduced colonic pro-inflammatory markers, and increased BDNF in the prefrontal cortex (Hu et al., 2022). Resveratrol, one of the major bioactive polyphenolic components in red wine (Fernández-Mar et al., 2012), was added to account for the intake of red wine with meals in human Med diet (Davis et al., 2015). Beta glucan powder (80%, 1/3, 1/6) and trans-resveratrol (50%) were purchased from PureBulk (Roseburg, Oregon, United States). Lastly, maltodextrin was added to pellet the diet.

3.1.3 Exclusion of sucrose, fructose, palm kernel oil, and dyes:

Sucrose, fructose, palm kernel oil, and FD&C red dye were removed from the original diet, as these ingredients are limited in human Med-based dietary patterns (Davis et al., 2015) or may interact with the gut microbiota (in the case of the dyes, as mentioned earlier).

3.1.4 Macronutrient modifications:

The amounts of casein, fish protein isolate, beef, wheatstarch, and olive oil in the original diet were adjusted to compensate for the additional proteins, carbohydrates, and fats from the new ingredients. Also, the overall protein content of the diet was increased to approximately 18% in accordance with the standards for breeding mice (Nutrition, 1995). The macronutrient caloric contribution of the mouse Med-based diet was kept within the range of values in the human Med diet, consisting of 15% protein, 37% fat, and 43% carbohydrates (Davis et al., 2015). The resulting modified Control and Med-based diets were isocaloric but had different macronutrient contribution to calories (Control diet: 22% protein, 18% fat, and 61% carbohydrates; Med-based diet: 17% protein, 39% fat, and 44% carbohydrates). The original and modified versions of the Control and Med-based diets are presented in Supplementary Table 1.

3.2 Pilot study with the Control and Med-based diets

Before using the newly developed Control and Med-based dietary formulas in the studies detailed in this thesis, it was imperative to confirm that the dietary modifications described above (especially the additional ingredients and modifications to macronutrient composition) did not change their palatability and/or lead to adverse breeding outcomes in mice. Food intake and body weights were measured daily as direct and indirect determinants of dietary palatability, respectively. Breeding outcomes were determined by measuring gestation length and litter characteristics (number of pups per litter and their body weight trajectory in the first postnatal week).

3.2.1 Animals

Naïve female (6-8 weeks) and male (7-9 weeks) C57BL/6N mice (Charles River Laboratories, St-Constant, Québec, Canada) were used as breeding stocks and housed individually in 19 cm x 29 cm x 13 cm polycarbonate N10 mouse cages (Ancare), enriched with a cotton nestlet, a cardboard house, and standard woodchip bedding. The housing room was maintained at a 12-h light-dark cycle (lights on 0700-1900 h), 21-23 °C, and 30-50% humidity. Free access to food and water was provided throughout the study. All experimental procedures were approved by the Animal Care Committee at the University of Ottawa (animal protocol number: HSe-3149), according to the guidelines of the Canadian Committee of Animal Care.

3.2.2 Experimental procedures

The experimental timeline is illustrated in Figure 1. Female and male mice were first fed the Control diet upon arrival and left to acclimate for one week before commencing the experiments. Following acclimation, a subset of females and males were randomly selected to remain on the Control diet (n = 8 females and 4 males), while the rest was switched to the Med-based diet (n = 9 females and 4 males). After two weeks of being fed their respective diets, females and males were paired for breeding using the Trio breeding scheme (Braden et al., 2017). Estrus was induced by placing nestlets and bedding from a male's housing cage into the cage of two individually housed females with which the male will be mated. Twenty-four hours later, the two females were introduced into the male's housing cage and left overnight for approximately 14 hours. Females were returned to their housing cages upon the detection of vaginal copulation plugs, designated E0.5. Two females in the Control diet group and one in the Med-based diet did not get pregnant after several rounds of breeding and were thus excluded from the study. Food intake and body weight of the females (Control: n = 6; Med: n = 8) were measured daily throughout

the study, and the palatability of the diets was determined using the data collected during the first two weeks of introducing the diets prior to pregnancy. Gestation length, litter size at birth, and pup weight from Postnatal Day (PND) 1 to 7 were recorded as indicators of pregnancy outcomes (Control: $n = 6$; Med: $n = 7$). All data were analyzed in GraphPad Prism version 10.1.0, except for food intake and body weight, which were analyzed in SPSS. Data for food intake and body weight of the dams as well as pup weight on PND 1 to 7 were analyzed using a two-way repeated measures analysis of variance (ANOVA), with Time (Day 1 to Day 14) and Diet (Control vs Med-based) as the within and between-group factors, respectively. Follow-up comparisons of the simple effects comprised *t* tests with a Bonferroni correction at an alpha level at $p < 0.05$. Data for gestation length and litter size were analyzed using a Mann-Whitney test (non-parametric), with Diet (Control vs Med-based) as the between-group factor. The alpha level was set to $p < 0.05$ for all analyses.

3.2.3 Results

Food intake was affected by Diet ($F_{(1, 6)} = 15.261$ $p = 0.008$), Time ($F_{(1, 6)} = 1.901$ $p = 0.043$), and Diet x Time interaction ($F_{(1, 6)} = 2.250$, $p = 0.014$), with follow-up comparisons for this interaction showing higher consumption of the Med-based diet on Day 11 ($p = 0.003$) and Day 14 ($p = 0.004$) compared to the Control diet (Fig.1). Body weight was also influenced by Time ($F_{(1, 6)} = 28.992$, $p = 0.002$) and Diet x Time interaction ($F_{(1, 6)} = 23.845$, $p = 0.003$), whose simple effects analyses revealed that mice fed the Med-based diet were heavier on Day 11 ($p = 0.019$), Day 12 ($p = 0.01$), Day 13 ($p = 0.011$), and Day 14 ($p = 0.003$) than their Control diet counterparts (Fig. 2). The weight gain in the Med-based diet dams is possibly due to the higher fat content of the Med-based diet (39%) compared to the Control diet (9%) (Supplementary Table 1). No group differences were observed for the gestation length, litter size at birth, and pup weight from birth

through PND 7 (p's > 0.05; Fig. 3). Overall, the findings demonstrated that the diets were well-eaten and did not negatively affect breeding (based on the pregnancy parameters assessed). After establishing that the diets are palatable and safe for breeding mice, subsequent studies were conducted in the postpartum dams and prenatally stressed offspring. These studies are presented in the next three chapters.

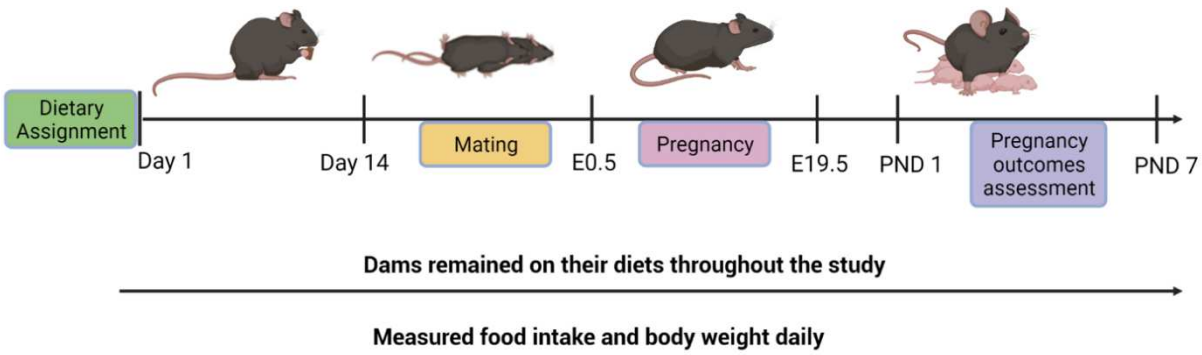

Figure. 1 Experimental design of the pilot study. Female mice were fed with the Control or Med-based diet and mated with males on the same diets after two weeks. Food intake and body weights of females were measured daily as direct and indirect indicators of dietary palatability, respectively. Gestation length, litter size at birth, and weight of the pups from birth until Postnatal Day (PND) 7 were determined as measures of pregnancy outcomes. The letter "E" stands for embryonic day. Figure created with BioRender.

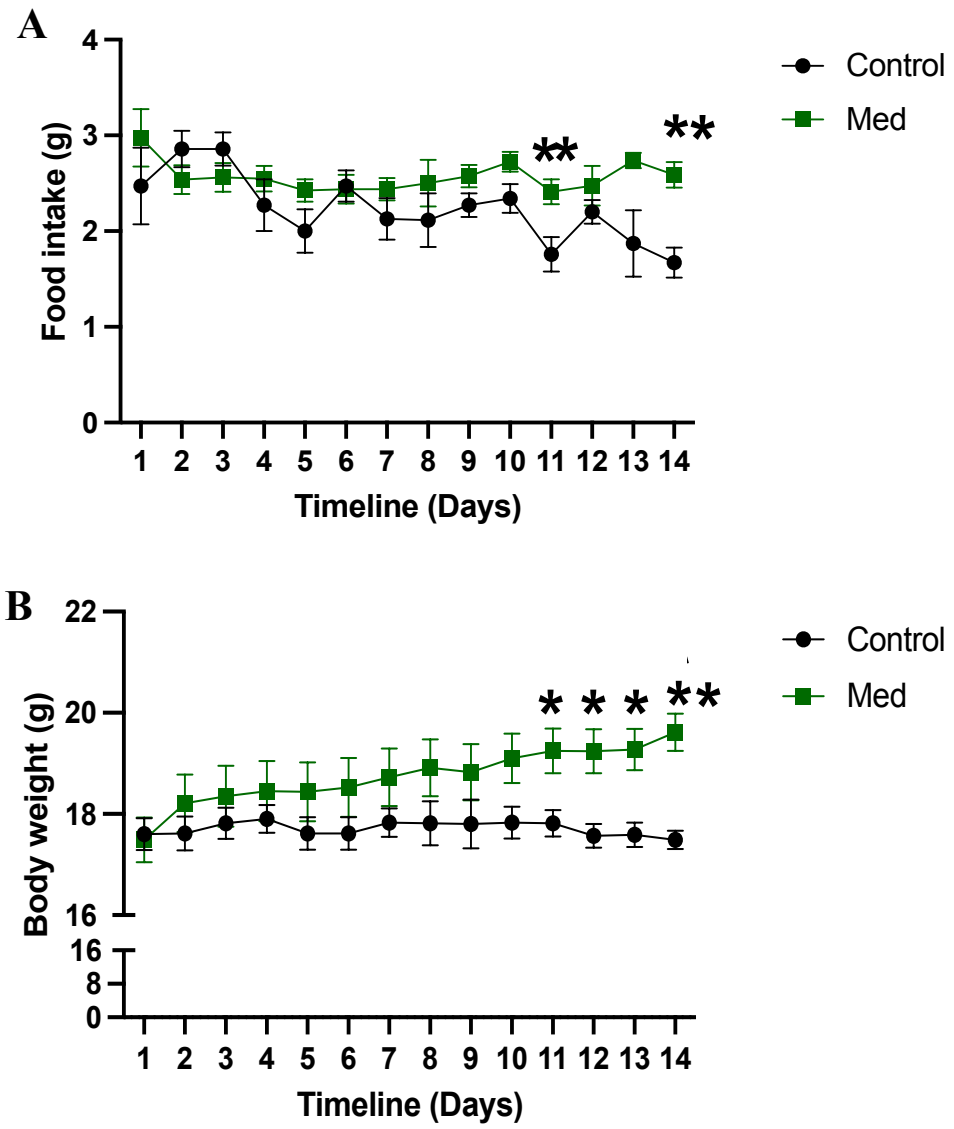

Figure 2. Food intake (A) and Body weight (B) of female mice fed the Control or Med-based diet. Data were analyzed using a two-way repeated measures analysis of variance, with Diet (Control vs Med) as the between-group factor and Time (Day 1 to Day 14) as the within-group factor. Data points for each timeline represent means ± S.E.M. Dams fed the Control diet (Control: $n = 7$); dams fed the Med-based diet (Med: $n = 8$). * $p < 0.05$ and ** $p < 0.01$ relative to Day 1 for the Control and Med-based diet groups.

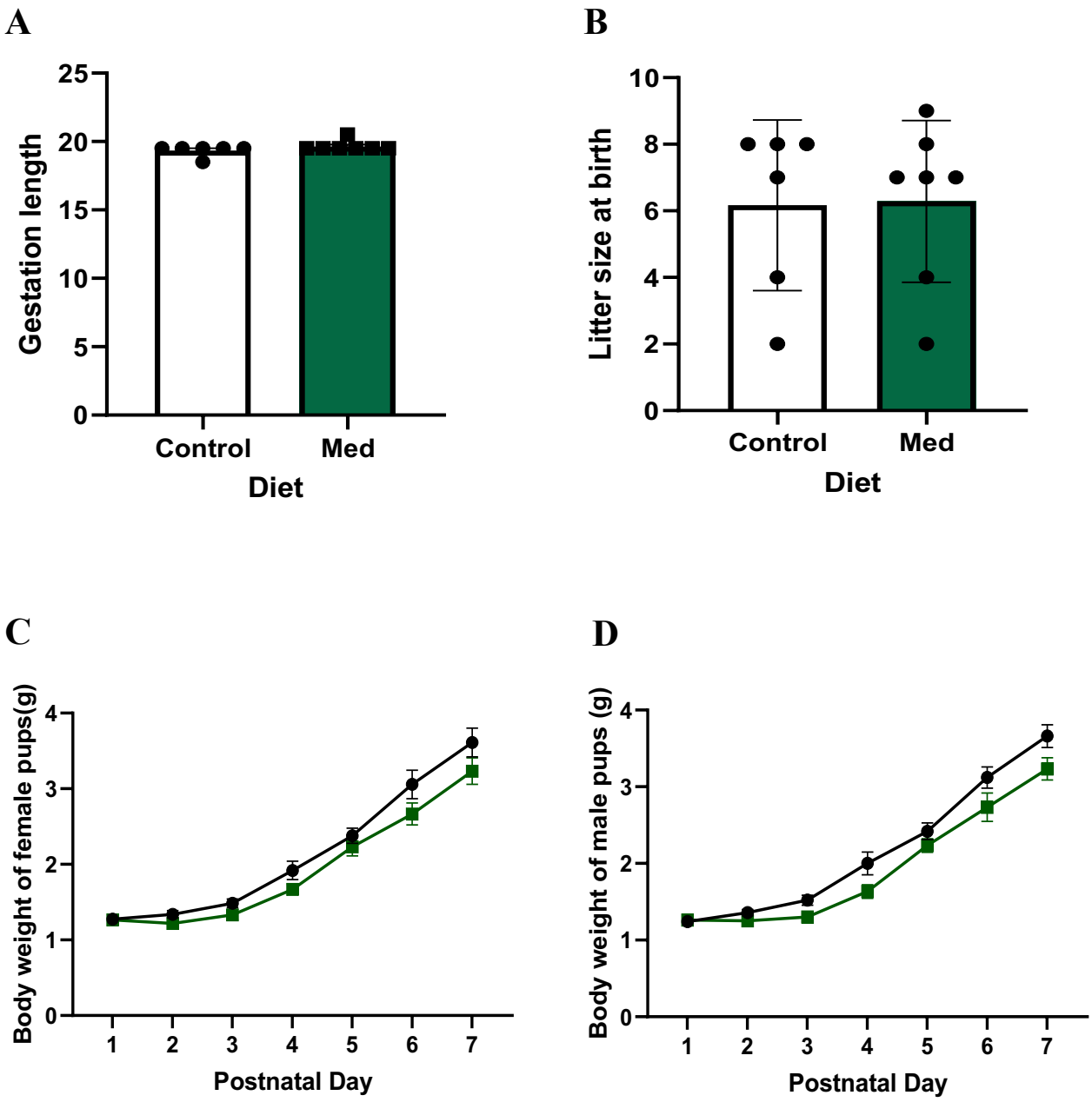

Figure 3. Gestation length (A), litter size at birth (B), and body weight of female (C) and male (D) pups born to dams fed the Control or Med-based diet. Data for gestation length and litter size at birth were analyzed using a one-way analysis of variance, while body weight data for each sex were analyzed using a two-way repeated measures analysis of variance. Bars and data points for each timeline represent means ± S.E.M. Control: $n = 6$; Med: $n = 7$.

Supplementary Table 1. Original and modified formula of the Control and Med-based diets

	Original Open Standard Control diet D12052701M	Modified Open Standard Control diet	Original Mediterranean-based diet	Modified Mediterranean-based diet
Ingredients	**gm**	**gm**	**gm**	**gm**
Casein	200	223	44	80
Fish Protein Isolate	0	0	27	18
Egg White	0	0	9	9
Beef, Cooked, Powdered, 5013	0	0	61.9	40
L-Cystine	3	3	3	3
Corn Starch	641	467.4	0	0
Maltodextrin	0	150	0	125
Wheat Starch	0	0	365	198.5
Chickpeas, Cooked, Dried	0	0	0	36
Lentils, Cooked, Dried	0	0	0	36
Sucrose	0	0	61	0
Fructose	0	0	19	0
Cellulose, BW200	75	75	40.7	14
Inulin	25	0	13.5	5
Beta-Glucans	0	0	0	5
Soybean Oil	70	70	0	0
Menhaden Oil (200 ppm tBHQ)	0	0	13.2	9
Palm Kernel oil	0	0	9.9	0
Butter, Anhydrous	0	0	7.4	5
Flaxseed Oil	0	0	6.1	6.5
Olive Oil	0	0	117.7	105
Walnuts, Dried, Powdered	0	0	0	20
t-BHQ	0	0.005	0	0.005
Mineral Mix S10026	10	10	10	10
Dicalcium Phosphate	13	13	13	13
Calcium Carbonate	5.5	5.5	5.5	5.5
Potassium Citrate, 1 H2O	16.5	16.5	16.5	16.5
Vitamin Mix V10001	10	10	10	10
Biotin (1%)	0	0	0.014	0.014
Choline Bitartrate	2	2	2	2
Fruit and Veggie Blend	0	0	0	100
Resveratrol (50% Trans Resveratrol)	0	0	0.045	0.045
FD&C Red Dye #5	0	0	0.05	0
FD&C Yellow Dye #40	0.05	0	0	0
Total	**1071.0549**	**1045.405**	**855.5113**	**872.064**
Macronutrient composition				
g				
Protein	177	197.0	115.7	156.4
Carbohydrate	570.3	552.9	406.7	402.1
Fat	72.4	72.7	172.3	157.8
Cholesterol	0	0.0	0.21	0.06
Total Fiber	93.8	75.0	50.8	55.0
Insoluble Fiber	75	75.0	40.7	37.8
Soluble Fiber	25	0.0	13.5	18.4
g%				
Protein	16.5	18.8	13.5	17.9
Carbohydrate	53.2	52.9	47.5	46.1
Fat	6.8	7.0	20.1	18.1
Cholesterol	0	0.0	0.024	0.007
Total Fiber	8.8	7.2	5.9	6.3
Insoluble Fiber	7.0	7.2	4.76	4.3
Soluble Fiber	2.33	0.0	1.58	2.1
kcal				
Protein	708	*788*	463	*626*
Carbohydrate	2281	*2212*	1627	*1608*
Fat	652	*654*	1551	*1420*
Total	***3641***	***3654***	***3641***	***3654***
kcal%				
Protein	19	*22*	12.71	*17*
Carbohydrate	63	*61*	44.69	*44*
Fat	18	*18*	42.6	*39*

Chapter 4: A Mediterranean-based diet limited postpartum changes in behaviour, neuroinflammation, and gut microbiota in mice

This chapter focuses on the first study investigating the Med-based diet's impact on postpartum behaviours and whether the behavioural effects are related to changes in brain and gut inflammatory, tight junction, and/or neurotrophic factors as well as the gut microbiota. The findings are detailed in the manuscript below.

A Mediterranean-based diet limited postpartum changes in behaviour, neuroinflammation, and gut microbiota in mice

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Abstract

Maternal dietary patterns during the perinatal period may influence postpartum mental health, potentially through their impact on the intestinal environment, with potential actions on brain processes. In this study, we investigated the effects of a Mediterranean (Med)-based diet on the behaviours of postpartum mice and examined whether these behaviours were related to brain and intestinal inflammatory, neurotrophic, tight junction, and microbiota metrics. Female C57BL/6N mice fed a Control or a Med-based diet before mating had their behaviour assessed on Postnatal Days (PNDs) 8 and 9 using the elevated plus maze, open field, Splash, and tail suspension tests, and their hippocampus, prefrontal cortex, colon, and feces collected on PND 10. Dams fed the Med-based diet engaged in more self-grooming behaviour in the Splash test and had a lower expression of interleukin (IL)-1β, IL-6, and occludin and a higher expression of BDNF and claudin-5 in the hippocampus. In contrast, they had more IL-6 and claudin-2 in the colon, alongside shifts in the fecal microbiota, including decreased *Akkermansia* and *Bilophila* and increased *Mucispirillum, Butyricicoccus, and Anaerovorax*. These findings indicate that Med-based dietary patterns could improve postpartum mental health, potentially by mitigating neuroinflammation and enhancing neuroplasticity and blood-brain barrier function.

Keywords: gut microbiota, inflammation, mediterranean diet, mental health, mouse model, neurotrophic factors, postpartum, tight junction

1. Introduction

The perinatal period is characterized by heightened vulnerability to mental health perturbations in the mother, with 10 to 15% of women suffering debilitating depression (Shorey et al., 2018) and 8 to 12% experiencing severe anxiety (Goodman et al., 2016) during early postpartum. Although the mechanism involved in the pathogenesis of postpartum depression and anxiety are not yet entirely elucidated, fluctuations in reproductive hormones during and after pregnancy have been suggested to play prominent roles in the onset of these mental disorders (Schiller et al., 2015). Beyond their putative direct influence on postpartum mental health, these perinatal hormonal changes may also drive extensive remodelling of the maternal brain structure and function during pregnancy and postpartum, which, although putatively supportive of fetal growth and in establishing maternal care behaviours, could also inadvertently increase the risk for postpartum mental health disorders (Barba-Müller et al., 2019; Paternina-Die et al., 2024). For instance, changes in neuroimmune functions in the early postpartum brain, marked by reduced microglial numbers, density, and activity, alongside altered cytokine expression in the medial prefrontal cortex and hippocampus, were reported in dams that also exhibited depressive-like behaviours (Eid et al., 2019; Haim et al., 2017; Posillico & Schwarz, 2016). Considering that alterations in neuroinflammatory processes can influence neurovascular functions (Gal et al., 2023) and that impairments in the blood-brain barrier (BBB) have been implicated in mental health disorders (Greene et al., 2020), changes in the maternal BBB reported during pregnancy (Cipolla, 2013) could also contribute to postpartum mental disorders. Moreover, circulating levels of brain-derived neurotrophic factor (BDNF), which is important in neurogenesis and synaptic plasticity (Kowiański et al., 2017) and known to bidirectionally interact with neuroimmune processes (Jin et al., 2019), are reduced during the perinatal period (Christian et al., 2016; Lommatzsch et al., 2006). Although information is lacking on how changes in brain BDNF may influence postpartum

mental health, lower serum BDNF levels, which may reflect central BDNF levels (Klein et al., 2011), were linked with depressive symptom severity and greater suicidal risk in postpartum women (Gao et al., 2016; Gazal et al., 2012; Pinheiro et al., 2012).

The gut microbiota has been implicated in stress-related mental disorders (Audet, 2019), and studies suggest that the pro-inflammatory shifts normally occurring in the maternal gut microbiota during the perinatal period may impact postpartum mental health. These pro-inflammatory changes involve increased levels of Proteobacteria (containing the endotoxin lipopolysaccharide; LPS) and reduced levels of *Faecalibacterium* (produces butyrate, a short chain fatty acid (SCFA) with anti-inflammatory effects (Louis & Flint, 2009)) (Koren et al., 2012). In relation to postpartum mental health, the fecal microbiota of women with postpartum depression was enriched *Enterobacteriaceae* (a Proteobacteria family) but depleted in *Faecalibacterium* and *Butyricicocus* (another butyrate producer) compared to postpartum women without this mental disorder (Zhou et al., 2020). Additionally, rodent dams exhibiting depressive-like behaviours in early postpartum showed higher levels of *Enterobacteriaceae* in their fecal microbiota (Liu et al., 2020; Tian et al., 2021; Yang et al., 2022; Zhao et al., 2022). Considering that these postpartum fecal microbial patterns have been linked with colonic inflammation and increased barrier permeability in non-pregnant and postpartum contexts (Agus et al., 2016; Bruce-Keller et al., 2015; Jang, et al., 2018), it is possible that changes in the gut microbiota during the perinatal period could increase pro-inflammatory signaling in the intestinal environment and influence inflammatory pathways in the gut-brain axis, potentially impacting maternal mental health (Audet, 2021). Additionally, the finding that perinatal probiotic administration reduced symptoms of postpartum depression and anxiety in women (Slykerman et al., 2017) and depressive-like behaviour in postpartum rats (Yang et al., 2022) illustrates not only the potential influence that gut microbes

may have on maternal mental health but also the prospects that modulating the gut microbiota during the perinatal period could improve postpartum mental health.

Accumulating evidence suggests that perinatal dietary patterns influence maternal mental health (Silva et al., 2019), suggesting that dietary modifications could serve as an avenue to promote optimal postpartum mental health. Particularly, a higher adherence to Mediterranean (Med) dietary patterns during pregnancy was associated with a lower risk of perinatal anxiety and depression in women (Chatzi et al., 2011; Papadopoulou et al., 2023), in addition to increasing the levels of gut bacteria that produce SCFAs (Miller et al., 2021). Also, adherence to Med-based diets have been shown to improve intestinal barrier integrity, and this effect was linked to the increased production of SCFAs (Seethaler et al., 2022). The mental health benefits of Med-based diets may be due to the capacity of some of their components to modulate inflammatory processes, neurotrophic factors, and the gut microbiota, which have all been implicated in mental health (Osimo et al., 2020; Reinhart et al., 2015; Zhang et al., 2023). For instance, omega-3 fatty acids and polyphenols reduced intestinal and brain inflammatory markers (Layé et al., 2018; Romier et al., 2009) and increased serum, hippocampal, or prefrontal BDNF levels (De Nicoló et al., 2013; Liu et al., 2023; Rathod et al., 2016) in rodents irrespective of sex. The mitigating actions of inulin fiber on high-fat induced anxiety- and depressive-like behaviours in postpartum mice were correlated with increases in fecal *Lactobacillus* (known to limit intestinal inflammation and promote barrier function (Dempsey & Corr, 2022)) and SCFAs (Liu et al., 2020).

In this study, we first examined whether a mouse diet designed based on human Med dietary patterns (Udechukwu & Audet, in preparation) administered throughout the perinatal period limited behaviours suggestive of anxiety- and depressive-like phenotypes in early postpartum dams. To investigate if the diet changed markers of neuroinflammation, neurotrophic

activity, and BBB permeability, known to be implicated in mental health and to fluctuate during the perinatal period (Haim et al., 2017; Johnson & Cipolla, 2015; Lommatzsch et al., 2006b), we assessed pro-inflammatory cytokines, tight junctions, and BDNF in the dorsal hippocampus and medial prefrontal cortex, which are brain regions involved in neural adaptations to pregnancy and postpartum (Duarte-Guterman et al., 2019; Haim et al., 2017; Posillico & Schwarz, 2016) and implicated in mental disorders (Gamo & Arnsten, 2011; MacQueen & Frodl, 2011). To determine whether the Med-based diet influenced intestinal factors, pro-inflammatory cytokines and tight junctions in the colon (exhibits immunological changes during the perinatal period (Koren et al., 2012) as well as fecal bacterial diversity, composition, and predicted metabolic capacity were assessed. Lastly, to determine if dams' behaviour at postpartum could be related to specific markers in the brain and intestinal environments, we established correlations between behavioural outcomes improved by the Med-based diet and the biological factors examined.

2. Materials and methods

2.1 Animals

Naïve female (6-8 weeks) and male (7-9 weeks) C57BL/6N mice (Charles River Laboratories, Montréal, QC, Canada) were housed singly in 19 cm x 29 cm x 13 cm polycarbonate N10 mouse cages (Ancare), enriched with a cotton nestlet, a cardboard house, and standard woodchip bedding. The housing room was set at a 12-h light-dark cycle (lights on 0700-1900 h), a temperature of 21-23 °C, and 30-50% humidity. Mice were provided with free access to water and food throughout the study. All experimental procedures were approved by the Animal Care Committee at the University of Ottawa (animal protocol number: HSe-3149), according to the guidelines of the Canadian Committee of Animal Care.

2.2 Summary of experimental procedures

The experimental procedures are illustrated in Figure 1. Upon their arrival and prior to commencing the experiments, all mice were acclimated to their new environment for one week and were fed a modified formula of an Open Standard Diet (D12052701M, Research Diets Inc.), referred to as the Control diet. At the end of acclimatization, a group of female and male mice were randomly selected to remain on the Control diet (n = 8 females and 4 males), while the rest was assigned to the Med-based diet (n = 9 females and 4 males). After two weeks on their respective diets, females and males fed the same diet were paired for breeding. Two females from the Control diet group and one from the Med-based diet group did not get pregnant and were therefore excluded from the study, reducing the sample size to 6 females in the Control diet group and 8 females in the Med-based diet group. After birth, which corresponded to embryonic day (E) 0.5, dams remained undisturbed with their litters. Anxiety- and depressive-like behaviours were assessed in the dams using standardized tests on postnatal days (PNDs) 8 and 9, which corresponded to 24 and 48 hours after being separated from their pups (which were euthanized on PND 7 as part of another study not reported here). Dams were euthanized on PND 10, approximately 24 hours after the last behavioural test, to collect their brains and colons for the analysis of pro-inflammatory, tight junction, and/or neurotrophic factors as well as their feces for microbiota assessments.

2.3 Experimental diets

The composition of the two experimental diets is presented in Supplementary Table 1. The details of the dietary development are available elsewhere (Udechukwu et al., in preparation). Briefly, an Open Standard Diet (D12052701M, Research Diets Inc.) was used as a base diet to formulate the Control diet. Extra casein was added to the base diet to increase the total protein content to approximately 17%, in accordance with the breeding requirements for mice (Nutrition,

1995). The Med-based diet was developed by modifying a previously reported mouse Med-based dietary formula (D12052702, Research Diets Inc.; Barrington et al., 2018). Freeze-dried chickpeas and lentils powders, processed in our laboratory and analyzed for their nutritional content (Intertek, Saskatoon, Canada), as well as freeze- or air-dried apples, peach, strawberries, blueberries, pomegranates, kale, spinach, broccoli, tomatoes, and carrots (generously supplied by FutureCeuticals, Inc., Momence, Illinois, United States), were added to account for legume as well as fruit and vegetable intake in the human Med-based diet (Naureen et al., 2022; Trichopoulou et al., 2014). Unsalted walnuts, also processed in our laboratory, were included based on findings that a Med-based diet supplemented with mixed nuts mainly comprising walnuts reduced the risk for myocardial infarction, stroke, and cardiovascular-related mortality (Estruch et al., 2018) and that walnuts consumption is associated with fewer depressive symptoms in adults (Arab et al., 2019). Finally, beta glucan powder and trans-resveratrol (PureBulk, Roseburg, Oregon, United States) were added to diversify the dietary fibers and account for the intake of red wine in the human Med diet, respectively (Trichopoulou et al., 2014). Finally, extra casein was added to the mouse Med-based diet to increase its protein content to approximately 18%, which is within the range required for mouse breeding (Nutrition, 1995). The resulting Control and Med-based diets were isocaloric, but the Med-based diet had different macronutrient (fat, protein, and carbohydrates) contributions to calories according to human dietary patterns (Davis et al., 2015).

2.4 Breeding procedures

Mice were bred using the Trio breeding scheme (Braden et al., 2017). Estrus was induced by placing nestlets and bedding from a male's housing cage into the cage of two singly housed females selected to be mated with this male. Twenty-four hours later, the two females were introduced into the male's housing cage and left overnight (approximately 14 hours) for breeding.

Females in which a vaginal copulation plug was detected, which was designated as E0.5, were monitored every three days to examine pregnancy status through body weight measurements. Pregnant mice were left undisturbed, except for body weight measurements, food, cage, and water bottle replacements, or occasional blood and fecal sample collection for another experiment beyond the scope of the present study.

2.5 Behavioural tests

2.5.1 Open field test

The open field test was used to assess the fear of open and bright spaces, indicative of anxiety-like behaviour (Lezak et al., 2017). The test apparatus consisted of an open arena made of white opaque acrylic plastic floor and walls, measuring 45 x 45 x 45 cm (Canus Plastics Incorporated). The test was conducted on PND 8 under room lighting of approximately 300 lux. After 1 hour of habituation to the testing room, dams were placed individually in the lower right corner of the arena, and their movements were recorded for 5 minutes using an overhead ceiling mounted camera. The test apparatus was cleaned between trials using a Quato 78 Plus solution (Swish Maintenance Limited). The number of entries and time spent in the center (15 x 15 cm) and corners (10 x 10 cm each) of the arena as well as the total distance travelled during the 5-minute test were later determined using a video tracking software (EthovisionXT, version 11.5, Noldus).

2.5.2 Splash test

The Splash test assessed the motivation to self-groom, a feature of depressive-like behaviour (Isingrini et al., 2010; Smolinsky et al., 2009). The test was conducted approximately 2 hours after the open field test under room lighting of approximately 530 lux. After 1 hour of habituation to the testing room, mice received two gentle spritzes of water on their lower back

and placed in an empty housing cage. Their grooming behaviour was videotaped for 10 minutes using a video camera in front of the cage. The videos were subsequently analyzed using the Behavioral Observation Research Interactive Software (BORIS) (Friard & Gamba, 2016) to determine the total time spent grooming, number of grooming sessions, and the latency to first groom.

2.5.3 Elevated plus maze test

The elevated plus maze test evaluated the fear of open, bright, and elevated spaces, characteristic of anxiety-like behaviour (Komada et al., 2008). The test apparatus consisted of a black acrylic plastic material, with one open arm (6-cm wide x 75-cm long) and one closed arm (6-cm wide x 75-cm long x 20-cm high) placed perpendicular to each other to form a cross. The maze was raised 74 cm above the floor. The test was performed on PND 9 under room lighting of approximately 100 lux. Following habituation to the testing room for 1 hour, mice were placed in the center of the maze facing the intersection of an open arm and a closed arm. Movements were tracked for 5 minutes using an overhead ceiling mounted camera and a video tracking software (EthoVisionXT, version 11.5, Noldus) to subsequently determine the number of entries and time spent in the open and closed arms. The maze was cleaned between trials using a Quato 78 Plus solution (Swish Maintenance Limited).

2.5.4 Tail suspension test

Approximately 2 hours after the elevated plus maze test, passive coping behaviours, suggestive of depressive-like behaviour, were assessed using the tail suspension test (Cryan et al., 2005). The test apparatus (Med Associates Inc) consisted of a tail suspension interface cabinet (DIG-735), a load cell amplifier hardware (ENV-505TS), and an associated user interface software (Tail Suspension SOF-821). After habituation for 1 hour under room lighting of approximately 100

lux, the mouse was hung upside down, with the tail anchored to an elevated aluminum bar with a small piece of surgical tape. The aluminum bar was connected to a strain gauge, which automatically detected movements. Mice were left suspended for 6 minutes, after which the time spent immobile (automatically calculated by the associated user interface software) was used as a measure of passive coping behaviour.

2.6 Euthanasia and sample collection

Mice were euthanized by rapid decapitation on PND10, approximately 15 hours after the last behavioural test. Whole brains were removed immediately from the skull, allowed to freeze on a sheet of Parafilm® M placed on dry ice and wrapped in the Parafilm® M sheet once frozen. In parallel, the gastrointestinal tract was rapidly extracted from the abdominal cavity and positioned on a nuclease-free frozen surface. Feces were collected from the rectum and placed into nuclease-free microcentrifuge tubes in dry ice. Whole colons were cut into small pieces, placed in nuclease-free cryotubes, and transferred in liquid nitrogen. All samples were stored at -80 ºC until further analyses. Frozen brains were subsequently dissected using the cryochamber of a ThermoFisher HM525 NX cryostat maintained at -20 ºC. Briefly, each whole brain was placed in a stainless-steel brain matrix (2.5 x 3.75 x 2.0 cm; slots spaced approximately 500 µm apart) positioned on an ice plate. Brains were sectioned coronally using razor blades guided by the matrix slots. Dorsal hippocampi and prefrontal cortices were dissected from the coronal sections following the Franklin and Paxinos mouse atlas (Franklin & Paxinos, 1997), placed into nuclease-free microcentrifuge tubes and stored at -80 ºC until subsequent analyses. The dorsal hippocampi or prefrontal cortices of one mouse from the Control diet group and two mice from the Med-based diet group were lost during the dissections, thereby reducing the sample size for analyses in these brain regions.

2.7 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Dorsal hippocampi, prefrontal cortices, and colons were homogenized using TRIzol, and their total RNA was extracted according to the manufacturer's instructions (Invitrogen, Burlington, Canada). RNA yield and purity of the samples were determined using a NanoDrop™ One Spectrophotometer (ThermoFisher Scientific). Samples with 260/80 and 260/230 ratios between 1.80 and 2.20 were included and thus the sample size and degrees of freedom associated with hippocampal, prefrontal, and colonic gene expression differ from other outcomes. Total RNA was reverse transcribed into complementary DNA (cDNA) using iScript™ Reverse Transcription Supermix and a T100 Thermal Cycler (Bio-Rad, Canada). Aliquots of cDNA were analyzed for the gene expression of tumor necrosis factor (TNF)-α, IL-1β, IL-6, BDNF, occludin, and claudin-2, 3, and 5, in duplicates of simultaneous quantitative polymerase chain reactions using the SsoAdvanced Universal SYBR® Green Supermix and a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Canada). Primers that amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Beta-Actin (Actb) were used as reference genes and their geometric mean was used to normalize the expression of the genes of interest. Fold changes for the mRNA expression for each gene of interest were calculated using the $2^{-\Delta\Delta CT}$ method, relative to the Control diet group (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). Primer sequences are provided in Supplementary Table 2.

2.8 Fecal microbiota analyses

2.8.1 16S ribosomal RNA (rRNA) gene sequencing

Fecal DNA was extracted using a Stool Nucleic Acid Isolation Kit following the manufacturer's instructions (Norgen Biotek Corp, Thorold, Canada). DNA concentration and purity of the samples were assessed using Quant-iT™ PicoGreen (Invitrogen). Two fecal samples

from the Control diet group and one from the Med-based diet group were excluded from subsequent analyses due to very low DNA concentrations. The 16S rRNA gene amplicons were prepared following the Illumina 16S library preparation procedures. Briefly, the V3 and V4 hypervariable regions of the 16S rRNA gene were amplified using the primers S-D-Bact-0341-b-S-17 (F: 5' TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG) and S-D-Bact-0785-a-A-21 (R: 5' GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C) (Klindworth et al., 2013). The resulting amplicons were tagged with Illumina nucleotide sequencing adapters and dual-index barcodes for Illumina MiSeq compatibility and sample identification, respectively. The samples were then pooled into a library, which was sequenced using a 600-cycle MiSeq Reagent Kit v3 and a MiSeq system, as directed by the manufacturer (Illumina, San Diego, CA, USA). The sequences were processed using QIIME 2 (Bolyen et al., 2019). Briefly, paired-end sequence reads that passed a median quality score of $Q \geq 30$ were denoised, filtered, and rarified using DADA2 (Callahan et al., 2016). All samples were above the retention threshold of 10,000 reads, except for one mouse from the Med-based diet group with 1556 reads, which was excluded from subsequent data processing. The relative abundance of bacteria at different taxonomic levels was calculated for each sample following the alignment of reads to taxa using the SILVA database (Quast et al., 2013). Data output from QIIME 2 was further analyzed using MicrobiomeAnalyst (Chong et al., 2020) to determine the Chao1 and Shannon alpha-diversity indices and the Bray-Curtis dissimilarity beta-diversity index. Beta-diversity was calculated using Bray-Curtis distance and visualized using Principal Coordinate Analysis (PCoA).

2.8.2 In silico prediction of metabolic activity

Bacterial metabolic activity was inferred from the 16S rRNA gene sequence data using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) Python package (Douglas et al., 2020). Briefly, the abundance of each Operational Taxonomic Unit was processed to predict the relative enrichment of the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs, KEGG pathways, and their associated BRITE functional hierarchies (Kanehisa et al., 2012). Based on *a priori* hypotheses that the Med-based diet would increase microbial short chain fatty acid production and reduce pro-inflammatory processes (Seethaler et al., 2022), KEGG pathways related to fatty acid biosynthesis, lipopolysaccharide biosynthesis, peptidoglycan biosynthesis, and NOD-like receptor signaling were examined.

2.9. Statistical analyses

Data analyses were performed using GraphPad Prism version 10.1.0. All data were tested for normality (Shapiro-Wilk test) and homogeneity of variances (Levene test) before comparisons using an unpaired *t*-test (parametric) or a Mann-Whitney test (non-parametric) at an alpha level set at $p < 0.05$, with Diet as the between-group factor. Multiple unpaired *t*-tests or Mann-Whitney tests comparing group differences in the relative family and genus abundance and the KEGG pathways were corrected for false discovery at a rate of 10%. Beta-diversity was determined using permutational multivariate analysis of variance (PERMANOVA) of Bray-Curtis distance matrices in MicrobiomeAnalyst (Chong et al., 2020). Pearson or Spearman correlation coefficients were used to determine relationships between the behavioural outcomes changed by the Med-based diet and biological factors determined in brain, colon, and fecal samples. The alpha level for correlations was set at $p < 0.01$, considering the high number of variables examined.

3. Results

3.1 The Med-based diet promoted grooming but did not change other behaviours suggestive of depressive- or anxiety-like phenotypes

Postpartum dams fed the Med-based diet spent more time grooming ($t_{12} = 4.522$, $p = 0.001$), engaged in more grooming sessions ($t_{12} = 4.045$, $p = 0.002$), and took less time to initiate the first grooming session ($U = 5$, $p = 0.013$) in the Splash test (Fig. 2). No differences between dams fed the Control diet and those fed the Med-based diet were apparent in the open field, elevated plus maze, and tail suspension tests (Supplementary Fig. 1).

3.2 The Med-based diet downregulated pro-inflammatory cytokines and upregulated neurotrophic and tight junction factors in the hippocampus only

Compared to the Control diet group, dams fed the Med-based diet exhibited a lower mRNA expression of IL-1β ($t_9 = 5.943$, $p = 0.001$) and IL-6 ($t_9 = 4.101$, $p = 0.003$) in the dorsal hippocampus, whereas that of TNF-α did not differ between the diet groups ($p > 0.05$; Fig. 3A-C). Dams fed the Med-based diet also had more BDNF ($t_{11} = 3.945$, $p = 0.003$) and claudin-5 ($U = 0$, $p = 0.004$) and less occludin ($t_{11} = 3.557$, $p = 0.006$) in the hippocampus, whereas claudin-3 remained unchanged ($p > 0.05$; Fig. 3D-G). No diet-mediated changes in pro-inflammatory cytokines, BDNF, or tight junctions were seen in the prefrontal cortex (p's > 0.05; Supplementary Fig. 2).

3.3 The Med-based diet increased colonic expression of IL-6 and claudin-2

As shown in Figure 4A-C, dams fed the Med-based diet had a higher mRNA expression of IL-6 in the colon than those fed the Control diet ($t_{11} = 2.508$, $p = 0.029$), while both groups of mice had comparable TNF-α and IL-1β (p's > 0.05). In terms of tight junctions, only claudin-2 differed between diet groups, being higher in dams fed the Med-based diet ($t_{11} = 2.763$, $p = 0.019$), whereas that of occludin, claudin-3 and 5 was unaffected by Diet (p's > 0.05; Fig. 4D-G).

3.4 Fecal microbiota

3.4.1 The Med-based diet changed the fecal beta diversity

There were no group differences in bacterial species richness and evenness, as measured by the Chao1 ($t_8 = 0.171$, $p = 0.869$) and Shannon ($t_8 = 1.198$, $p = 0.265$) diversity indices (Fig. 5A, B). In contrast, the bacterial communities in the Control and Med-based diet groups were dissimilar, as indicated by the Bray-Curtis beta diversity dissimilarity index ($t_{1,8} = 3.466$, $p = 0.007$; Fig. 5C).

3.4.2 The Med-based diet changed the relative abundance of specific fecal bacterial taxa

Nine phyla were detected in the fecal samples from each diet group (Supplementary Fig. 3), except for the phylum Cyanobacteria that was present in only the Control group and was thus excluded from the statistical analyses. Bacteroidota (48.31%) and Firmicutes (39.60%) were the most abundant phyla and were similar in relative abundance in the two diet groups (p's > 0.05) (Supplementary Fig. 3). Also, the relative abundance of Proteobacteria did not differ between the two diet groups ($p > 0.05$; Supplementary Fig. 3). In contrast, the Med-based diet decreased Verrucomicrobiota ($U = 0$, $p = 0.0095$) and Desulfobacterota ($t_8 = 5.328$, $p = 0.0007$) and increased Deferribacterota ($t_8 = 3.483$, $p = 0.0083$; Supplementary Fig. 3). Among the 54 families detected, 7 were present in only one sample and were thus excluded from subsequent statistical analyses. A series of unpaired *t*-tests or Mann-Whitney tests on the remaining 47 families revealed group differences in 9 families. However, only 6 families remained different between groups after correcting for multiple *t*-tests at a false discovery rate (FDR) of 10%. Mice fed the Med-based diet had less *Akkermansiaceae* ($U = 0$, $p = 0.0095$), *Desulfovibrionaceae* ($t_8 = 5.328$, $p = 0.0007$), and *Streptococcaceae* ($t_8 = 4.318$, $p = 0.0026$) but more *Deferribacteraceae* ($t_8 = 3.483$, $p = 0.0083$), *Butyricicoccaceae* ($t_8 = 4.285$, $p = 0.0027$), and *Anaerovoraceae* ($t_8 = 4.191$, $p = 0.003$)

(Supplementary Fig. 3). Among the 108 genera that were identified, 13 were present in only one sample and were thus discarded from the analyses. While group differences were observed in 16 genera after a series of unpaired *t*-tests or Mann-Whitney tests (Supplementary Fig. 4), only 9 genera passed as true discoveries following an FDR correction at 10%. As seen in Fig. 6, dams fed the Med-based diet had less *Akkermansia* ($U = 0$, $p = 0.0095$), *Bilophila* ($t_8 = 5.327$, $p = 0.0007$), *Lactococcus* ($t_8 = 4.364$, $p = 0.0024$), *Peptococcus* ($U = 2$, $p = 0.0238$), and *[Acetivibrio]_ethanolgignens_group* ($U = 3$, $p = 0.0333$), and more *Mucispirillum* ($t_8 = 3.483$, $p = 0.0083$), *Butyricicoccus* ($U = 0$, $p = 0.0095$), *Anaerovorax* ($t_8 = 4.348$, $p = 0.0025$), and *GCA-900066575* ($t_8 = 4.637$, $p = 0.0017$).

3.4.3 Inferred taxonomic metabolic activity

Taxonomic metabolic capacity for each fecal sample was predicted from the 16S data using PICRUSt2, which generated a catalog of KEGG orthologs and associated KEGG pathways. Among the 131 KEGG pathways identified, 3 were present in only one sample and were thus excluded. Unpaired *t*-tests or Mann-Whitney tests on the remaining 128 pathways showed differential enrichment of 19 pathways in the two diet groups (Supplementary Fig. 5), but only 7 pathways remained different between the groups after an FDR correction at 10%. Contrary to our *a priori* hypotheses that the Med-based diet would downregulate pathways associated with inflammation and upregulate pathways those involved in SCFAs production (Seethaler et al., 2022), the relative enrichment of lipopolysaccharide biosynthesis, peptidoglycan biosynthesis, NOD-like receptor signaling, and fatty acid biosynthesis pathways remained unchanged by Diet (p's > 0.05; Fig 7A-D). Although not part of our *a priori* hypotheses, the fecal microbiota of mice fed the Med-based diet (after a 10% FDR correction) was enriched in pathways related to histidine metabolism ($t_8 = 4.628$, $p = 0.0017$), nicotinate and nicotinamide metabolism ($t_8 = 8.400$, $p <$

0.0001), and RNA transport (t_8 = 4.834, p = 0.0013), while pathways related to lysine degradation (t_8 = 3.952, p = 0.0042), valine, leucine and isoleucine degradation (t_8 = 4.232, p = 0.0029), carotenoid biosynthesis (t_8 = 6.865, p = 0.0001), and steroid biosynthesis (U = 1, p = 0.0095) were less enriched in these mice (Supplementary Fig 6).

3.5 Improvements in total grooming time by the Med-based diet was linked to brain or fecal microbiota outcomes

Grooming time (the main behavioural indicator of motivation for self-care improved by the Med-based diet) correlated positively with hippocampal BDNF (r = 0.7725, p = 0.005) and claudin-5 (r_s = 0.7455, p = 0.008) and negatively with hippocampal occludin (r = -0.8188, p = 0.002) and IL-1β (r = -0.7026, p = 0.016) and *Peptococcus* (r_s = -0.750, p = 0.012) in all mice fed the Control and Med-based diets (Fig. 8). Also, grooming time tended to correlate positively with *Anaerovorax* (r = 0.717, p = 0.019) and negatively with hippocampal IL-1β (r_s = -0.7026, p = 0.016) and *Peptococcus* (r_s = -0.750, p = 0.012) in these mice and positively with *Lactococcus* (r = 0.8240, p = 0.044) in mice fed the Med-based diet only (Fig. 8).

4. Discussion

Pregnancy, ordinarily a rewarding experience, may also pose a risk for mental disorders in the mother, especially at postpartum. Although adherence to healthy dietary patterns such as those based on the Med diet have been shown to reduce maternal risk for postpartum depression (Papadopoulou et al., 2023), very little is known about the actions that these dietary patterns may have on biological processes known to fluctuate during the perinatal period, with potential impacts on mental health. In this study, we report that the consumption of a diet based on human Med dietary patterns from preconception through early postpartum in mice increased maternal motivation for self-care, suggestive of attenuated postpartum depressive-like behaviour. These

behavioural improvements were accompanied by a reduction of pro-inflammatory cytokines and an increase in BDNF and claudin-5 in the hippocampus. In contrast, the Med-based diet appeared to enhance pro-inflammatory activity and barrier permeability in the gut environment of the postpartum dams, as evidenced by increases in colonic IL-6 and claudin-2, together with shifts towards fecal bacterial patterns linked with intestinal inflammatory conditions. As revealed by the correlation analyses, enhanced motivation for self-care behaviours in the postpartum dams may be related to upregulation of BDNF and claudin-5 and downregulation of occludin in their hippocampus.

While dams fed the Control and Med-based diets had comparable levels of passive coping behaviour in the tail suspension test, those fed the Med-based diet were more motivated to self-groom after receiving water sprinkles in the Splash test. Similar to our findings, dietary supplementation with menhaden fish oil or omega-3 fatty acids, which were both present in our experimental mouse Med-based diet and are typically part of the human Med diet (Trichopoulou et al., 2014), attenuated depressive-like behaviours in rat models of postpartum depression (Arbabi et al., 2014; Aziz et al., 2020), suggesting that these ingredients may have contributed to the improvements in self-care behaviour in postpartum dams fed our Med-based diet. The absence of changes in anxiety-like behaviours in dams fed the Med-based diet is surprising, although Med-based dietary patterns have been more strongly associated with depression than anxiety in humans (Firth et al., 2019). Importantly, anxiety-like behaviours are generally attenuated in rodent dams in the few postnatal days or weeks compared to virgin females, as a behavioural adaptation that enhances maternal interaction and care for the altricial pups (Lonstein, 2007). While this is plausible, we cannot exclude the possibility that removing the litters from the dams' housing cage the day before testing elicited emotional distress in some of them, as previously reported (Lonstein,

2005), especially as the main indicators of anxiety-like behaviour (time in open field center and open arms of the elevated plus maze) were especially variable in our two groups of mice.

A growing body of evidence implicates immune dysregulation in postpartum mental health disturbances (Dye et al., 2021). Elevated plasma levels of pro-inflammatory cytokines, including IL-6 and TNF-α, were reported in women with postpartum depression (Achtyes et al., 2020; Bränn et al., 2020; Maes et al., 2000), and higher prefrontal expression of IL-6 in early postpartum rats was correlated to depressive-like behaviour relative to non-pregnant females (Posillico & Schwarz, 2016). In support for a role of IL-6 in postpartum depressive-like behaviour, an intra cisterna magna infusion of an IL-6 receptor antagonist on the day of birth prevented anhedonia-like behaviour in rat dams, in addition to decreasing prefrontal IL-6 as well as prefrontal and hippocampal BDNF (Gomez et al., 2019). In the current study, we observed that the Med-based diet reduced the mRNA expression of IL-1ß and IL-6 and increased that of BDNF in the hippocampus of postpartum dams, suggesting that the diet could limit aberrant pro-inflammatory activation in the brain that could otherwise predispose the mother to postpartum mental disorder alongside promoting neuroplasticity. Studies indicate that the neuroimmune system during the postpartum period establishes an anti-inflammatory state, with findings of decreased microglial activation in the CA1, CA3, and dentate gyrus of the hippocampus, while the mRNA expression of IL-1β is downregulated and that of IL-10 is upregulated in the medial prefrontal cortex (Haim et al., 2017; Posillico & Schwarz, 2016). An upregulation of peripheral and central levels of BDNF has also been reported during the postpartum period as adaptations to maternal care behaviour (Christian et al., 2016; Posillico & Schwarz, 2016), and reductions in maternal plasma BDNF levels during pregnancy and postpartum have been associated with poor mental health, including postpartum depression (Christian et al., 2016; Lommatzsch et al., 2006). Thus, our findings suggest

that the Med-based diet could reinforce the anti-inflammatory and neurotrophic brain status at postpartum by modulating hippocampal IL-1β, IL-6, and BDNF expression. This is consistent with previous findings that dietary supplementation with omega-3 fatty acids (bioactive components of the Med diet) increased hippocampal BDNF in mice (Venna et al., 2009; Wu et al., 2004) and serum BDNF levels in humans (Sohouli et al., 2023) as well as reduced pro-inflammatory cytokines in rodent brain (Layé et al., 2018). Surprisingly, the Med-based diet did not affect prefrontal expression of pro-inflammatory cytokines, BDNF, and tight junctions. Previous studies have reported that dietary supplementation with omega-3 fatty acids (present in the Med diet) increased both hippocampal and prefrontal BDNF levels, although in non-postpartum rats (Rathod et al., 2016). Considering this, the lack of effects of the Med-based diet in the PFC suggests the dietary impact in the postpartum brain may be region-specific.

The integrity of the BBB is indispensable to maintaining homeostasis in the brain, and structural and functional impairments in the BBB have been linked to neurological diseases and psychiatric disorders (Najjar et al., 2013). Lower expression of claudin-5, the most abundant BBB tight junction protein whose deficiency was shown to loosen the BBB and increase its permeability (Nitta et al., 2003), has been reported in postmortem hippocampi and nucleus accumbens of individuals with major depression and in mice exhibiting depressive-like behaviours (Dion-Albert et al., 2022; Greene et al., 2020; Menard et al., 2017). Here, we showed that the Med-based diet increased hippocampal claudin-5 expression, thus demonstrating the capacity of the diet to promote BBB structure and function. Also, available evidence demonstrates that tight junctions (claudin-5 and occludin) expression in the BBB is not altered during the perinatal period, as one of the cerebrovascular adaptations to pregnancy to protect the maternal brain from elevated peripheral vasoactive factors, including cytokines (Cipolla, 2013; Johnson & Cipolla, 2015). Thus,

the finding that the Med-based increased hippocampal claudin-5 expression suggests that the diet could reinforce this BBB protective mechanism, promoting postpartum brain functions and health. Surprisingly, the Med-based diet reduced occludin in the hippocampus of postpartum dams. Besides claudin-5, occludin is another important tight junction regulating BBB permeability and neuroinflammation (Sugiyama et al., 2023; Yuan et al., 2020). Thus, the functional implication of this downregulated occludin expression in the BBB is unclear and requires further elucidation.

Unexpectedly, dams fed the Med-based diet had increased IL-6 expression in their colon. As a pleiotropic cytokine, IL-6 has dual pro-inflammatory and anti-inflammatory physiological roles, depending on which receptor it is binding and the signaling pathway activated (Scheller et al., 2011). Whether the upregulated IL-6 expression in postpartum dams fed the Med-based diet resulted in pro- versus anti-inflammatory effects in the colon remains uncertain. Given that postpartum dams fed the Med-based diet also exhibited increased claudin-2 expression, it could be speculated that IL-6 exerted pro-inflammatory actions in the colon of these dams. Claudin-2 is a pore-forming tight junction regulating size-and-charge selective paracellular permeability of intestinal epithelial cells (Amasheh et al., 2002; Colegio et al., 2002). Overexpression of claudin-2 is frequently reported in ulcerative colitis and Crohn's disease and is strongly linked with intestinal barrier dysfunction observed in these inflammatory bowel diseases, in concert with downregulation of important tight junctions, including occludin and claudin-5 (Luettig et al., 2015; Prasad et al., 2005; Zeissig et al., 2007). In support for a role of claudin-2 in intestinal inflammation, transgenic overexpression of the tight junction exacerbated colitis in mice and was necessary and sufficient for IL-13-mediated increases in colonic permeability (Raju et al., 2020), effects that were both prevented by pharmacological inactivation of claudin-2 channel (Raju et al., 2020). Furthermore, intraperitoneal injection of IL-6 increased colonic permeability by

preferentially upregulating claudin-2 expression in a murine model of inflammatory bowel disease (Al-Sadi et al., 2014; Suzuki et al., 2011). Thus, our finding of co-upregulation of IL-6 and claudin-2 in dams fed the Med-based diet buttresses the conjecture that IL-6 could have elicited pro-inflammatory changes in the gut. Taken together, these findings hint that the Med-based diet promoted colonic pro-inflammatory activity, whose implication for postpartum mental health is not apparent at this time and needs to be further delineated.

The dominance of Bacteroidetes and Firmicutes in the fecal microbiota of the postpartum dams is consistent with previous reports (Weerasuriya et al., 2023). While the Med-based diet did not affect the number of fecal bacterial species and their relative proportion, the two diet groups markedly differed in terms of taxonomic composition. We expected the Med-based diet to promote the abundance of bacteria that produce SCFAs and reduce those with inflammatory potential, as is the case in studies in both pregnant and general adult populations (De Filippis et al., 2016; Miller et al., 2021; Mitsou et al., 2017; Seethaler et al., 2022). Strikingly, the genus *Akkermansia*, a known propionate producer with probiotic properties (Derrien et al., 2004; Lukovac et al., 2014), was depleted in postpartum dams fed the Med-based diet compared to those fed the Control diet. Fiber-rich diets are typically associated with increases in *Akkermansia* (Dao et al., 2016; Murga-Garrido et al., 2021; Rodriguez et al., 2020; Tagliamonte et al., 2021). The Med-based diet used in this study has a substantial fiber content (6.3 g% of the total dietary components) that is comparable to the Control diet (7.2 g%), and thus both diets would be expected to at least have similar levels of *Akkermansia*. However, the fat composition of the Med-based diet (18.1 g%; which is characteristic of human Med dietary patterns; Davis et al., 2015) was higher than that of the Control diet (7 g%). Based on reports that *Akkermansia* was depleted in mice fed a high-fat diet as well as in obese mice and humans (Everard et al., 2013; Rodriguez et al., 2020; Schneeberger et al., 2015),

it is possible that the higher fat content of our Med-based diet created unfavourable conditions for the growth of *Akkermansia*. Furthermore, diets containing flaxseed have been shown to reduce fecal and cecal abundance of *Akkermansia* in mice (Livingston et al., 2023; Määttänen et al., 2018; Power et al., 2016). Although the Med-based diet used in this study contains little amount of flaxseed oil (0.75 g%), this ingredient is completely absent in the Control diet, and thus we cannot exclude the possibility that it contributed to reducing *Akkermansia* in mice fed this diet.

In contrast to its effect on *Akkermansia*, we found that the Med-based diet favoured the presence of other bacteria that also produce SCFAs. In this regard, dams fed the Med-based diet exhibited a higher abundance of the butyrate-producing *Butyricicoccus* and Anaerovorax (Matthies et al., 2000) Devriese et al., 2017; Eeckhaut et al., 2013; Geirnaert et al., 2014), consistent with previous increases in *Butyricicoccus* reported following interventions with a Med-based diet in humans (Bourdeau-Julien et al., 2023; Choo et al., 2023; Zhu et al., 2020). Decreases in *Butyricicoccus pullicaecorum* have been frequently observed in inflammatory bowel diseases, and treatment with this bacterium reduced inflammation and intestinal barrier permeability in a mouse model of colitis (Devriese et al., 2017; Eeckhaut et al., 2013). Likewise, the abundance of *Anaerovorax* was negatively correlated with the severity of osteoarthritis, suggesting it may have a role in chronic inflammatory disease (Collins et al., 2021). In relation to postpartum mental health, *Butyricicoccus* was depleted in women with postpartum depression (Zhou et al., 2020), suggesting that the increased representation of this taxa in mice fed the Med-based diet could promote better mental health at postpartum.

Similar to *Akkermansia*, the overexpression of *Mucispirillum* in dams fed the Med-based diet could be partly attributed to its higher dietary fat composition, as high-fat diets stimulated the growth of this taxa (Kim et al., 2019; Ussar et al., 2015). The significance of *Mucispirillum* in host

health is less understood, as the existing literature has reported contradictory outcomes. Although its abundance has been mostly associated with intestinal inflammation in murine models of inflammatory bowel disease and chronic inflammatory conditions in humans (Herp et al., 2021), the species *Mucispirillum schaedleri* protected against salmonella-induced colonic inflammation and virulence in mice (Herp et al., 2019). Considering this evidence, the host implications of the increased abundance of *Mucispirillum* in dams fed the Med-based diet remains uncertain. Furthermore, the finding that the Med-based diet decreased the abundance of *Lactococcus and Peptococcus* (which have anti-inflammatory and probiotic properties (Luerce et al., 2014; Nishitani et al., 2009), but also decreased *Bilophila* (linked with inflammation and various disease conditions (Feng et al., 2017; Sen et al., 2017) suggests that the diet exerted complex immunomodulatory actions on the gut microbiota. Finally, although the Med-based diet led to distinct shifts in taxa known to produce short-chain fatty acids and/or have roles in intestinal inflammatory processes, we did not observe any changes in the relative enrichment of KEGG pathways involved in fatty acid biosynthesis, LPS or PGN biosynthesis, and NOD-like receptor signaling.

This study contributes to the growing evidence that perinatal dietary patterns may influence postpartum mental health by demonstrating improved self-care grooming behaviours, typically recognized as being suggestive of reduced depressive-like phenotypes (Smolinsky et al., 2009) in postpartum dams that fed a Med-based diet. The behavioural changes resulting from the Med-based diet were modest, potentially owing to the absence of prenatal challenge in our model. Although we did not link these behavioural improvements to any of the biological measures, our findings demonstrate that the Med-based diet reduced pro-inflammatory cytokines and increased neurotrophic factors and tight junctions in the brain, which are neurobiological changes generally

linked to positive mental health and could be relevant to maternal mental health at postpartum. Further studies are needed to disentangle the effects of the Med-based diet in the gut environment, particularly whether the effects are immunomodulatory or pathogenic in a postpartum context. In conclusion, our findings suggest that dietary modifications based on the Med-based diet may help support maternal brain and mental health at postpartum.

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6. Author Contributions: MCU and MCA designed the experiment. MCU, KAP, and MCA developed the experimental diets. MCU, CN, and ZW conducted the experiment. MCU performed the behavioural and molecular (RT-qPCR) analyses. MCU and JKS conducted the microbiota sequencing analyses. MCU performed the bioinformatics analyses. MCU and MCA interpreted the data and wrote the manuscript, which was edited by all authors.

7. Conflict of Interest Statement: All authors declare that the research work was conducted in the absence of any personal, professional, or financial relationships that could be construed as a conflict of interest.

8. List of Figures

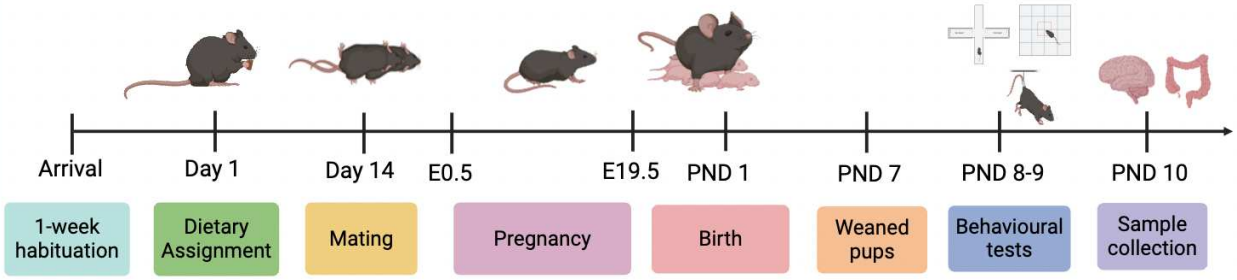

Figure 1. Experimental timeline. Female and male mice were fed a Control or a Med-based diet and mated after two weeks of being on their respective diets. After removing the litters from the dams' housing cages on postnatal day (PND) 7, anxiety- and depressive-like behaviours of the dams were assessed on PNDs 8 and 9, followed by their euthanasia and the collection of biological samples on PND 10. "E" refers to embryonic day. Figure created with BioRender.

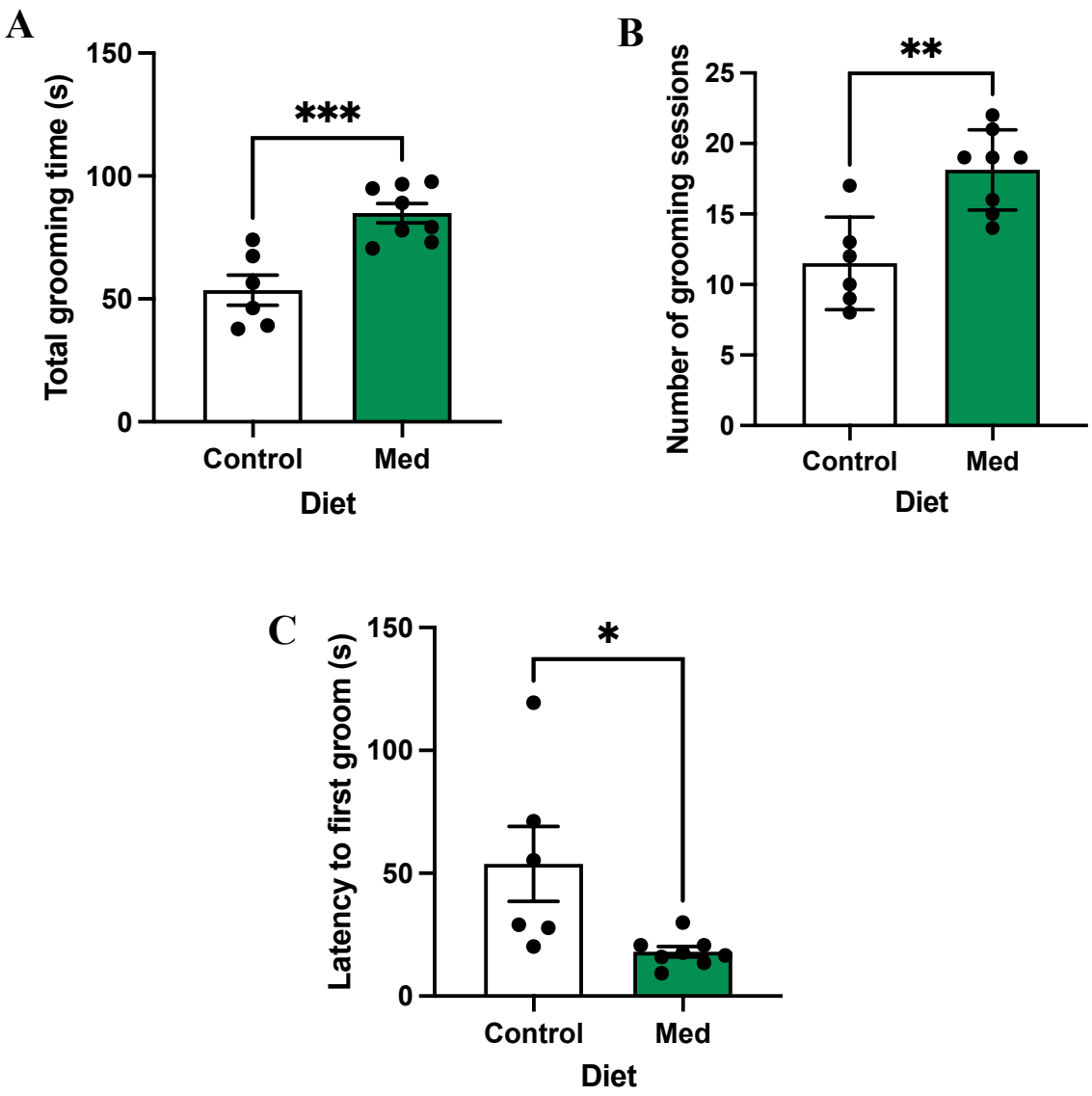

Figure 2. Perinatal access to a Med-based diet increased grooming metrics in the Splash test in postpartum dams. Dams fed the Med-based diet spent more time grooming (in seconds [s]) (A), engaged in more grooming sessions (B), and initiated the first grooming session more rapidly (in seconds [s]) (C) than dams fed the Control diet during the 10-minute test session. Data were analyzed using unpaired *t*-tests or Mann-Whitney test, with Diet (Control vs Med) as the between-group factor. Dots represent individual mice, while bars represent group means ± S.E.M. Dams fed the Control diet (Control: $n = 6$); dams fed the Med-based diet (Med: $n = 8$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ relative to dams fed the Control diet.

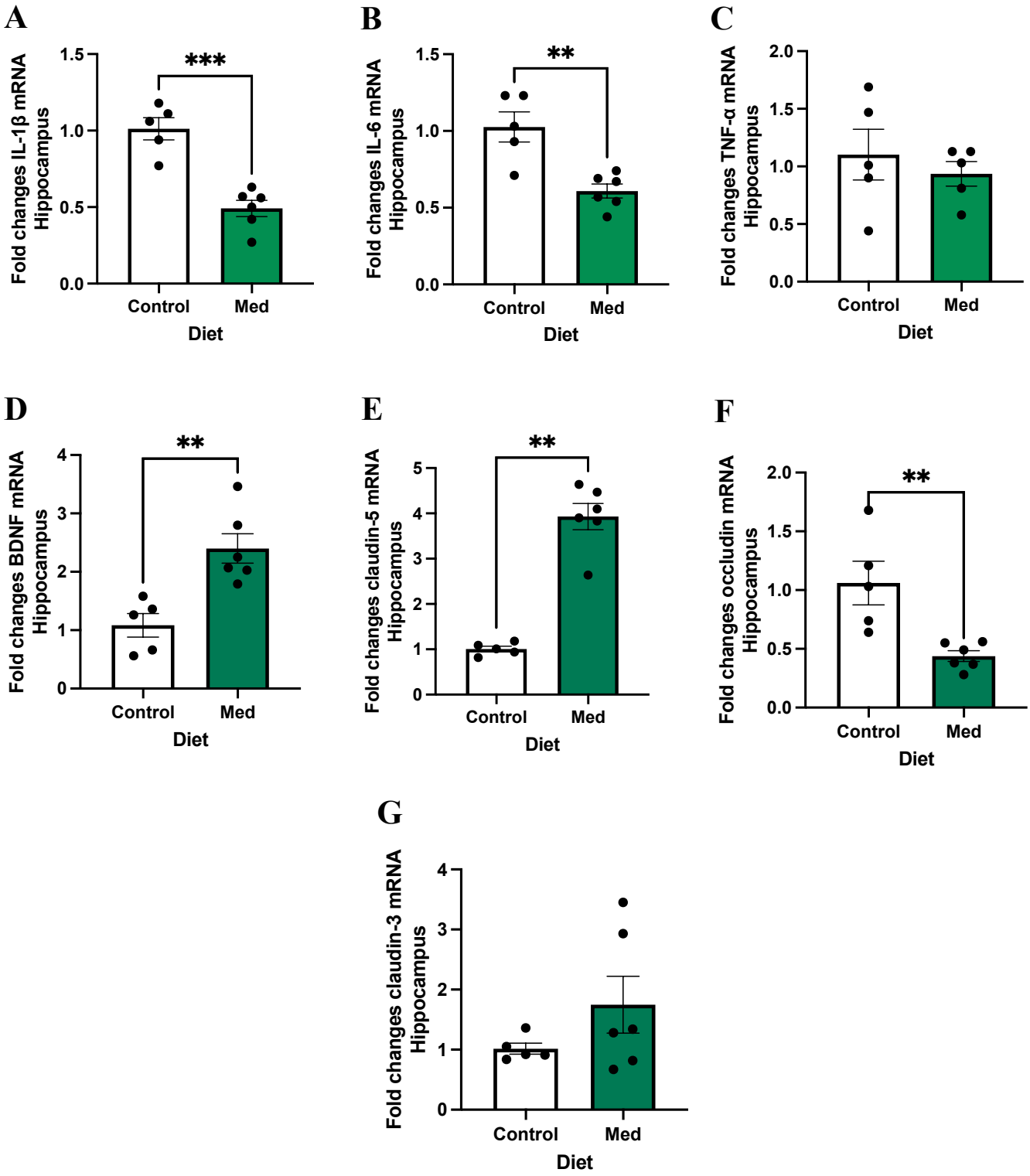

Figure 3. Perinatal access to a Med-based diet reduced fold changes in mRNA expression of pro-inflammatory cytokines and increased that of neurotrophic and tight junction factors in the hippocampus of postpartum dams. Dams fed the Med-based diet had less IL-1β (A), IL-6 (B), and occludin (F) and more BDNF (D) and claudin-5 (E) in the hippocampus than dams fed the Control diet. Data were analyzed using unpaired *t*-tests or Mann-Whitney test, with Diet (Control vs Med) as the between-group factor. Dots represent individual mice, while bars represent group means ± S.E.M. Dams fed the Control diet (Control: $n = 5$); dams fed the Med-based diet (Med: $n = 6$). ** $p < 0.01$ and *** $p < 0.001$ relative to dams fed the Control diet.

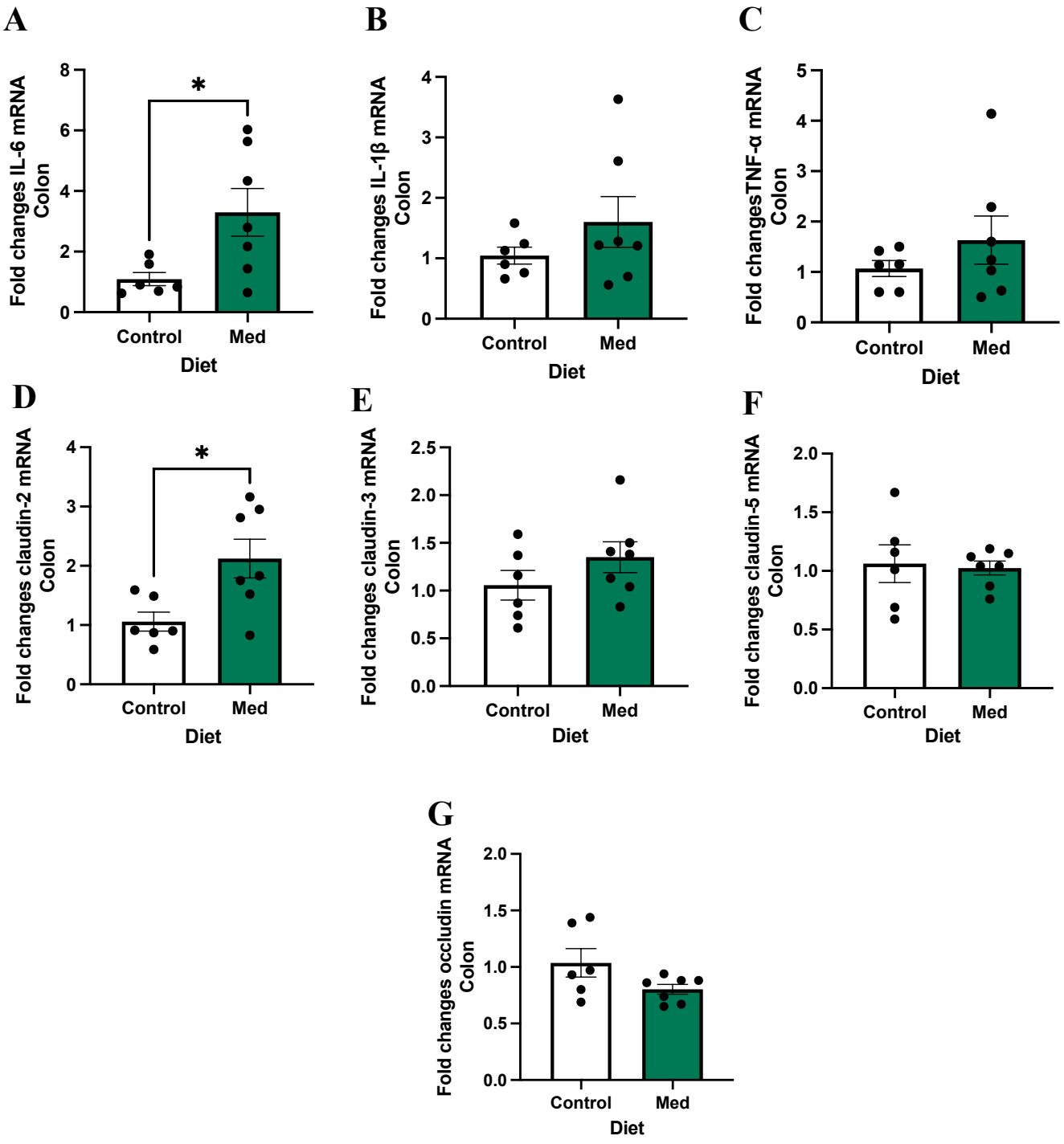

Figure. 4. Perinatal access to a Med-based diet increased fold changes in the gene expression of pro-inflammatory and tight junction factors in the colon of postpartum dams. Dams fed the Med-based diet had more IL-6 (A) and claudin-2 (D) in the colon than dams fed the Control diet. Data were analyzed using unpaired *t*-tests or Mann-Whitney test, with Diet (Control vs Med) as the between-group factor. Dots represent individual mice, while bars represent group means ± S.E.M. Dams fed the Control diet (Control: $n = 6$); dams fed the Med-based diet (Med: $n = 7$). * $p < 0.05$ relative to dams fed the Control diet.

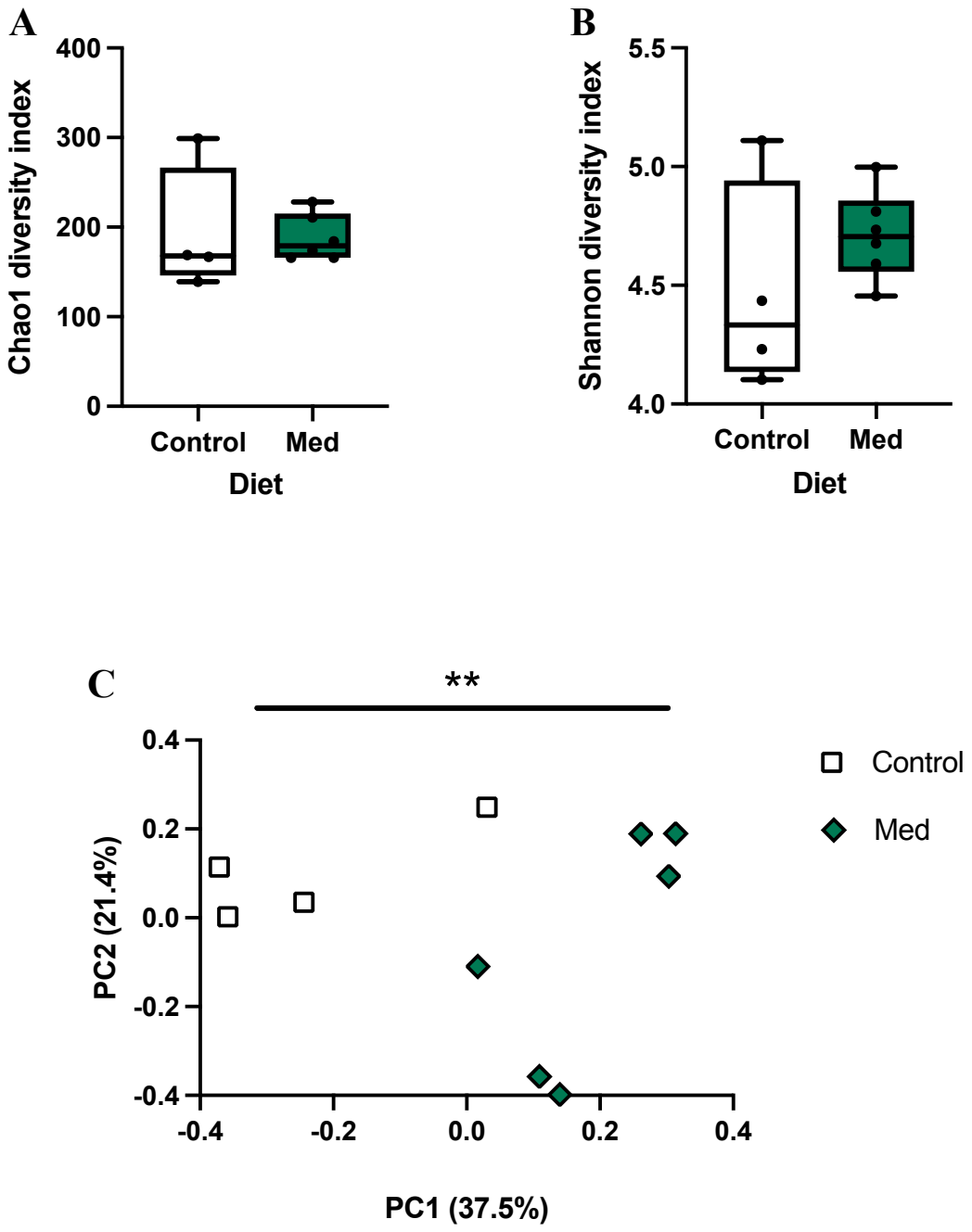

Figure 5. Perinatal access to a Med-based diet changed the Bray-Curtis beta diversity index of the fecal microbiota of postpartum dams. Fecal samples from dams fed the Med-based diet clustered separately from those fed the Control diet. Data for alpha diversity indices were analyzed using unpaired *t*-tests or Mann-Whitney test, with Diet (Control vs Med) as the between-group factor, while data for beta-diversity index was analyzed using permutational multivariate analysis of variance (PERMANOVA) of Bray-Curtis distance matrices in MicrobiomeAnalyst. Data points represent individual mice, while box plots in A and B represent Quartile 1 – media – Quartile 3. Dams fed the Control diet (Control: $n = 4$); dams fed the Med-based diet (Med: $n = 6$). ** $p < 0.01$ relative to dams fed the Control diet.

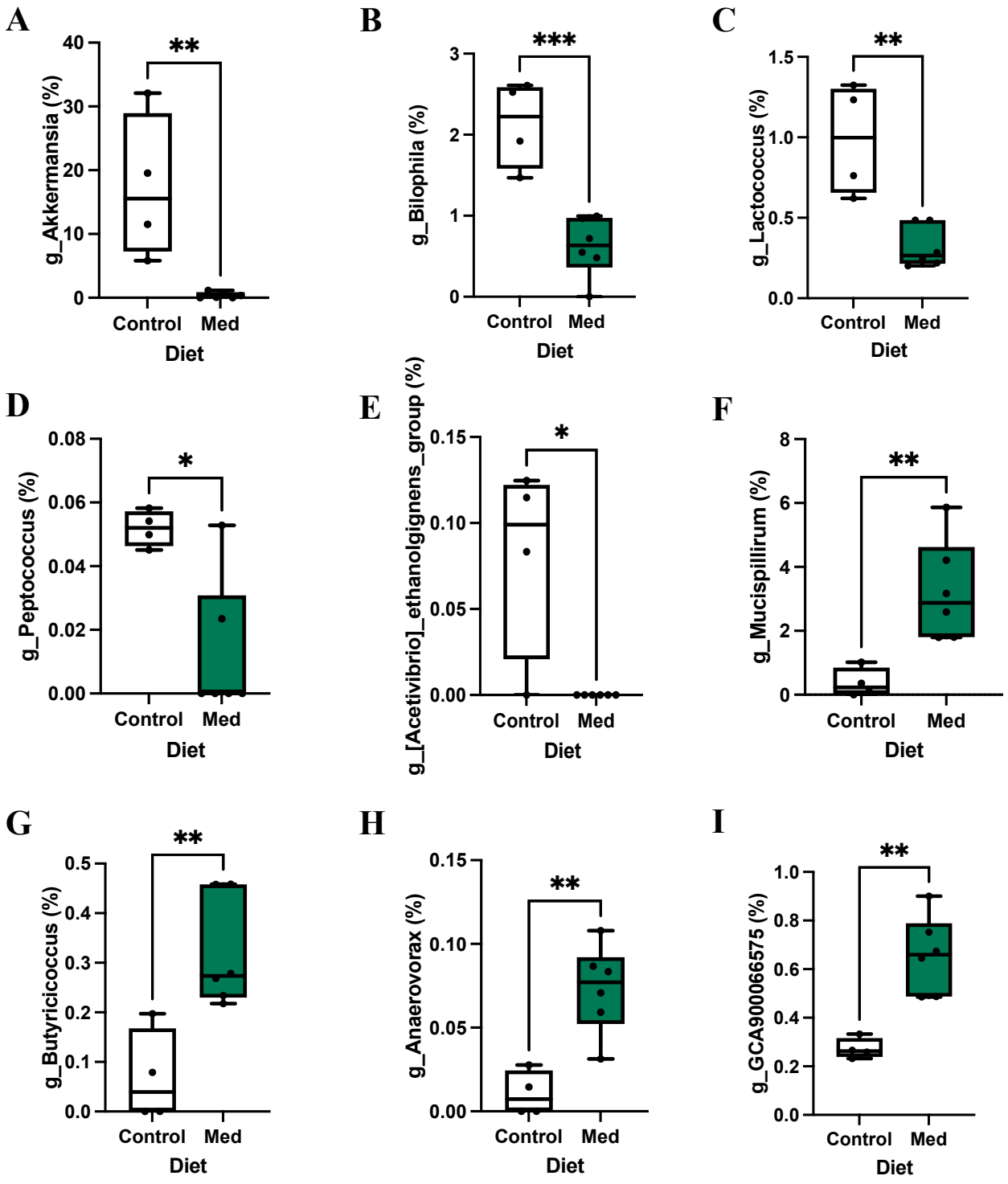

Figure 6. Perinatal access to a Med-based diet changed the relative abundance of genera in the fecal microbiota of postpartum dams. Dams fed the Med-based diet had less *Akkermansia* (A), *Bilophila* (B), *Lactococcus* (C), *Peptococcus* (D), and *Acetivibrio_ethanolignens_group* (E) and more *Mucispirillum* (F), *Butyricicoccus* (G), *Anaerovorax* (H), and *GCA900066575* (I) than dams fed the Control diet. Data were analyzed using unpaired *t*-tests or Mann-Whitney test, with Diet (Control vs Med) as the between-group factor. Dots represent individual mice, while box plots represent Quartile 1 – media – Quartile 3. Dams fed the Control diet (Control: $n = 4$); dams fed the Med-based diet (Med: $n = 6$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ relative to dams fed the Control diet.

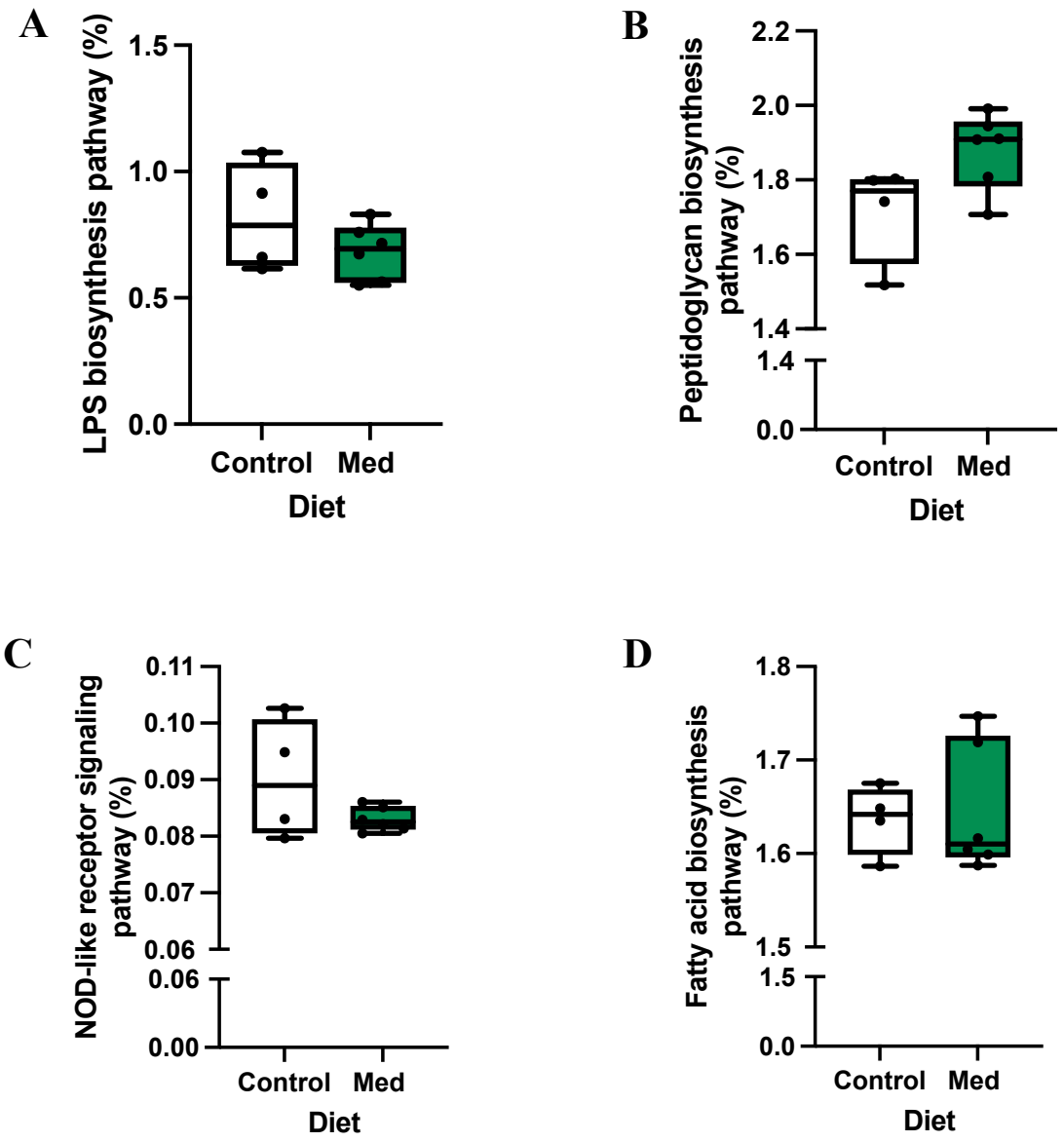

Figure 7. Perinatal access to a Med-based diet did not change the relative enrichment of KEGG pathways related to inflammation and fatty acid synthesis in the fecal microbiota of postpartum dams. Dams fed the Med-based diet had comparable enrichment of the LPS biosynthetic pathway (A), Peptidoglycan biosynthetic pathway (B), NOD-like receptor signaling pathway (C), and Fatty acid biosynthetic pathway (D) as dams fed the Control diet. Data were analyzed using unpaired *t*-tests or Mann-Whitney test, with Diet (Control vs Med) as the between-group factor. Dots represent individual mice, while box plots represent Quartile 1 – media – Quartile 3. Dams fed the Control diet (Control: $n = 4$); dams fed the Med-based diet (Med: $n = 6$).

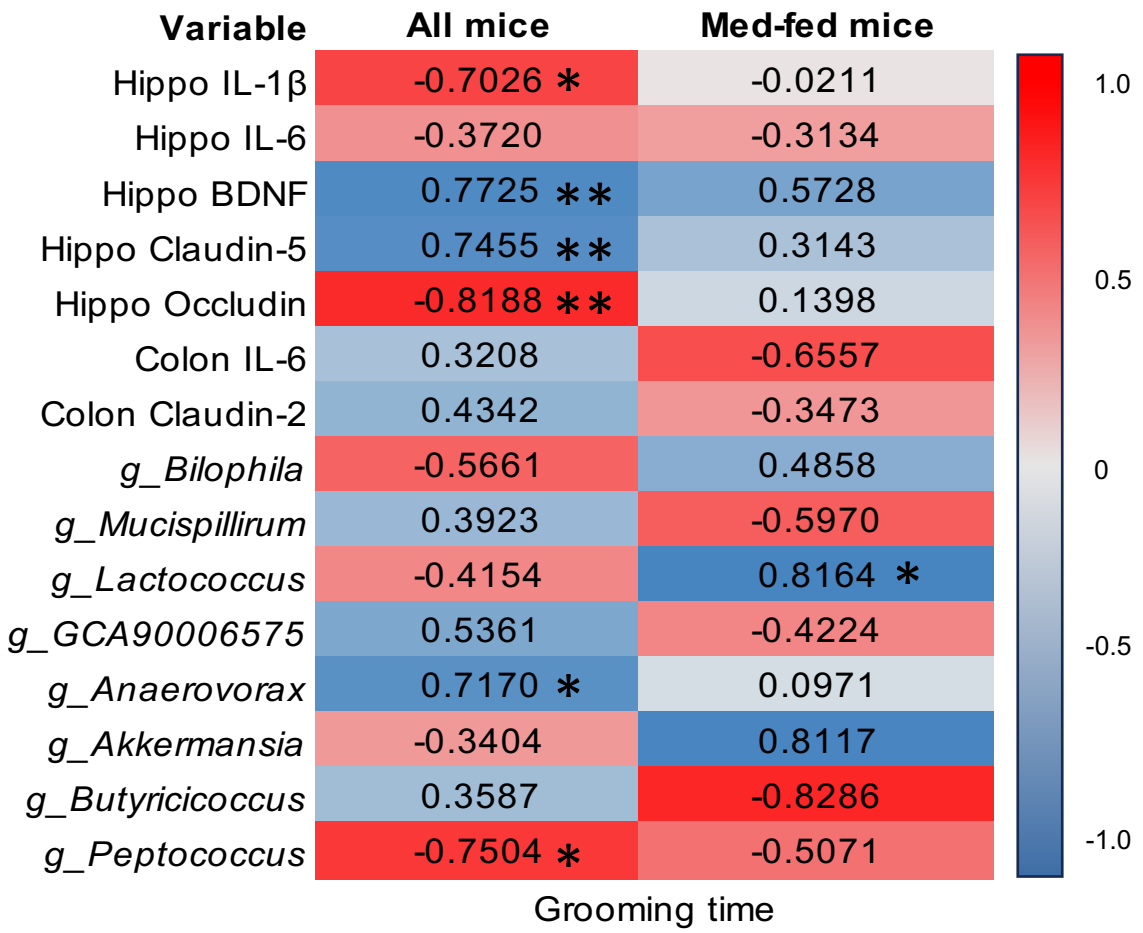

Figure 8. Changes in grooming behaviour correlated with neuroinflammatory, neurotrophic, tight junction, and fecal microbiota changes in the postpartum dams. Grooming time of all mice fed the Control and Med-based diet correlated positively with their brain-derived neurotrophic factor (BDNF) (p = 0.005) and claudin-5 (p = 0.008) and negatively with occludin (p = 0.002) in the hippocampus (Hippo) at a set alpha value of $p < 0.01$ for multiple variables assessed, while the same behavioural metric correlated negatively with interleukin (IL)-1β (p = 0.016), *Anaerovorax* (p = 0.019), and *Peptococcus* (p = 0.012) at $p < 0.05$. In dams fed the Med-based diet, grooming time positively correlated with *Lactococcus* (p = 0.044) at $p < 0.05$. Data were analyzed using Pearson (parametric) or Spearman (non-parametric) correlation coefficients test. Colour intensities indicate the strength of positive (blue) and negative (red) correlation coefficients. * $p < 0.05$ and ** $p < 0.01$

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10. Supplementary data

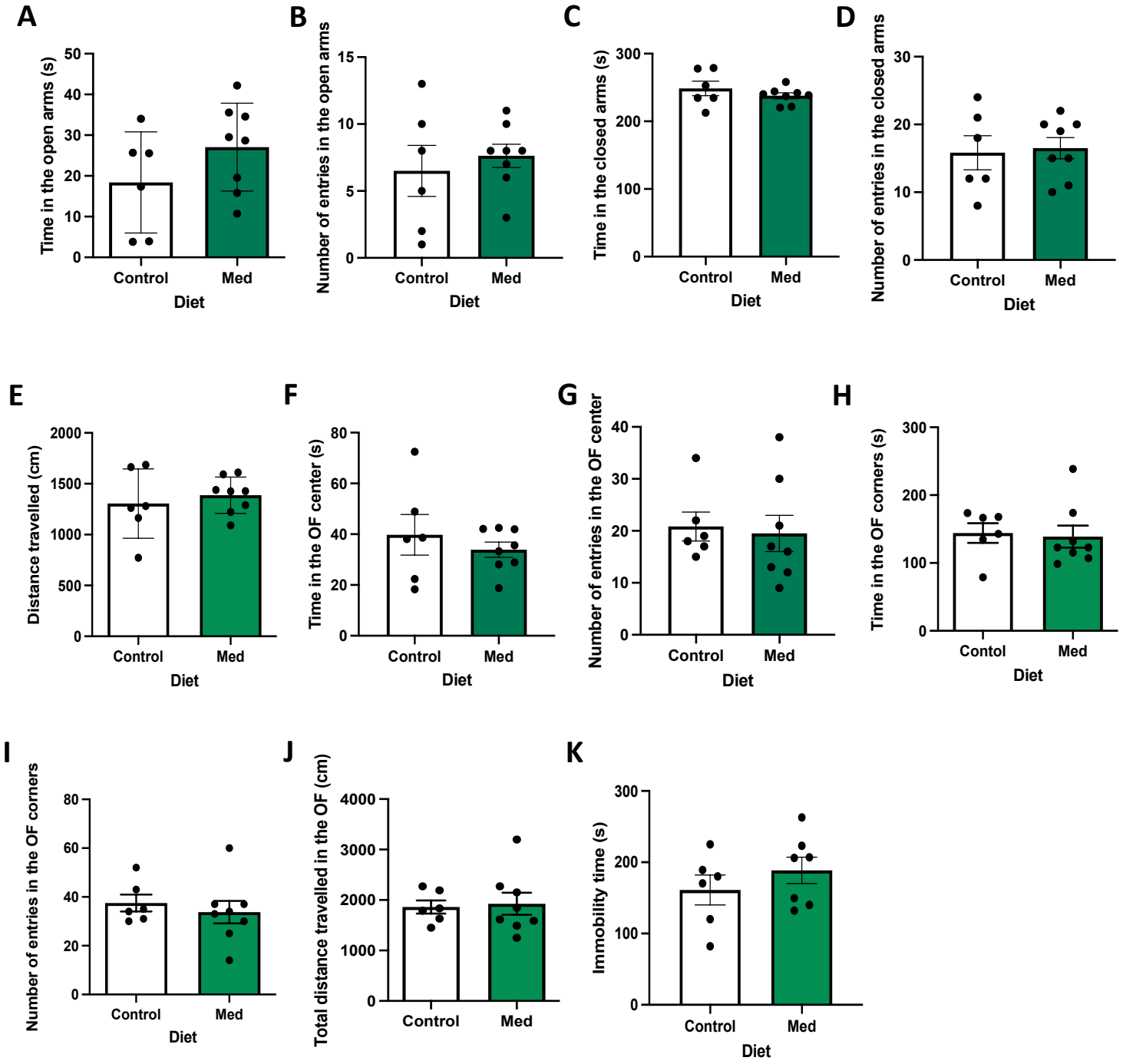

Figure 1. Perinatal access to a Med-based diet did not affect behaviours in the elevated plus maze (A-E), open field (F-J), and tail suspension tests (K) of postpartum dams. Data were analyzed using unpaired *t*-tests or Mann-Whitney test, with Diet (Control vs Med) as the between-group factor. Dots represent individual mice, while bars represent group means ± S.E.M. Dams fed the Control diet (Control: $n = 6$); dams fed the Med-based diet (Med: $n = 8$).

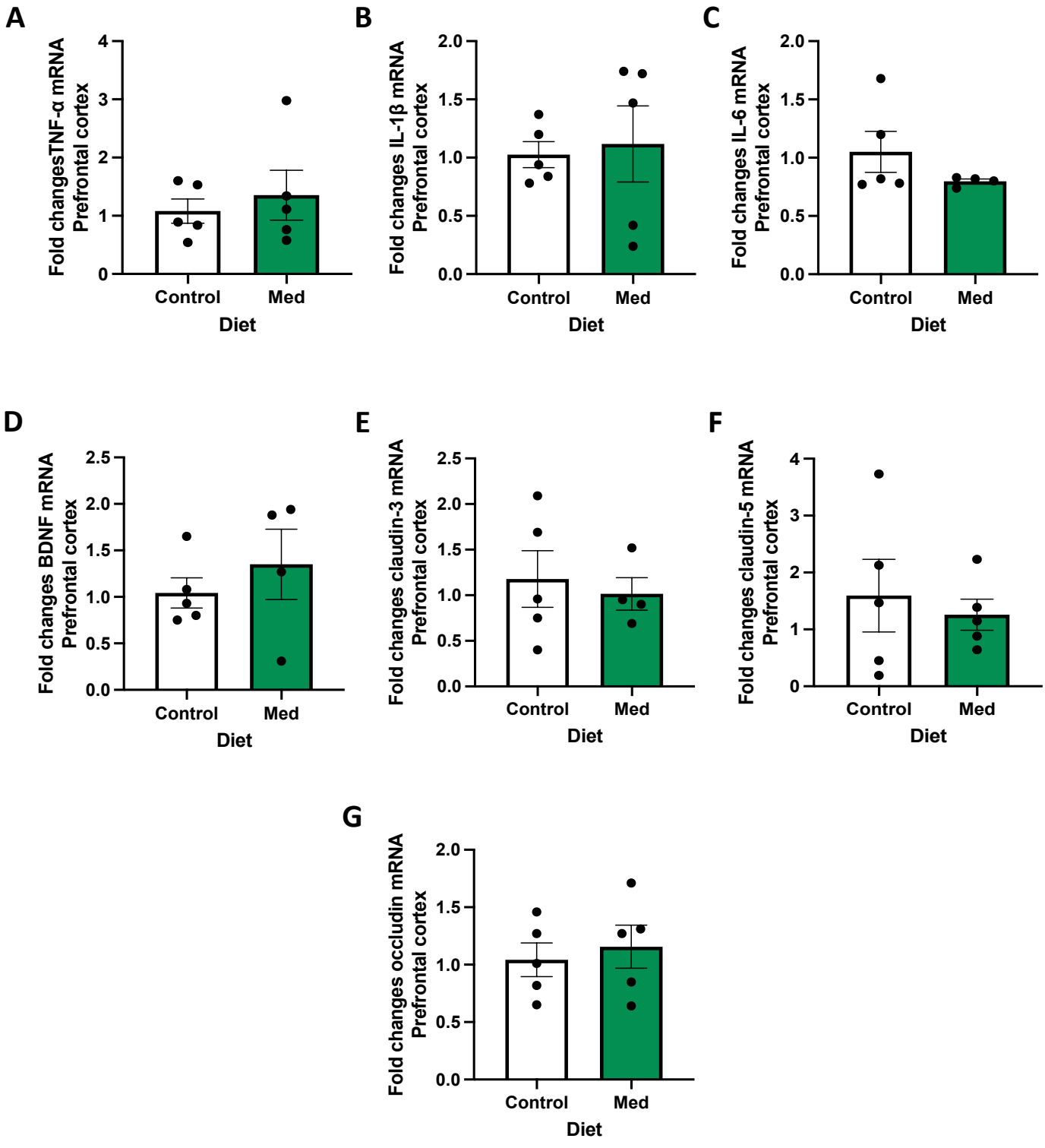

Figure 2. Perinatal access to a Med-based diet did not affect fold changes in the gene expression of pro-inflammatory (A-C), neurotrophic (D), and tight junction factors (E-G) in the prefrontal cortex of postpartum dams. Data were analyzed using unpaired *t*-tests or Mann-Whitney test, with Diet (Control vs Med) as the between-group factor. Dots represent individual mice, while bars represent group means ± S.E.M. Dams fed the Control diet (Control: $n = 5$); dams fed the Med-based diet (Med: $n = 4$-5).

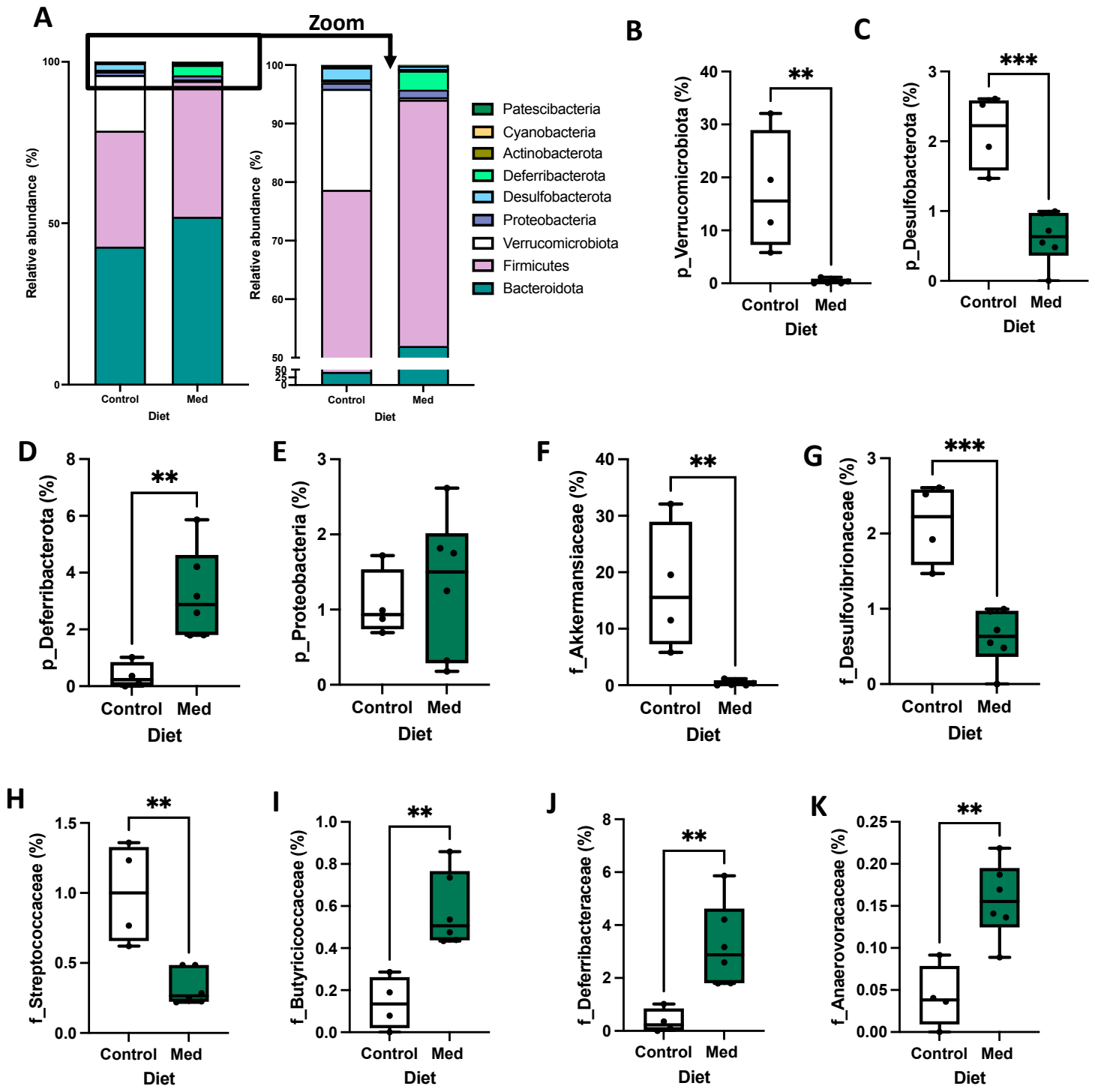

Figure 3. Perinatal access to a Med-based diet changed the relative abundance of phyla and families in the fecal microbiota of postpartum dams. Taxa bar plot of the phyla identified in the fecal microbiota of postpartum dams fed a Control diet and a Med-based diet (A). At the phylum level, dams fed the Med-based diet had less Verrucomicrobiota (B) and Desulfobacterota (C) but more Deferribacterota (D) than dams fed the Control diet. At the family level, dams fed the Med-based diet had less *Akkermansiaceae* (F), *Desulfovibrionaceae* (G), and *Streptococcaceae* (H) but had more *Deferribacteraceae* (I), *Butyricicoccaceae (J)*, and *Anaerovoraceae* (K) than those fed the Control diet. Data were analyzed using unpaired *t*-tests or Mann-Whitney test, with Diet (Control vs Med) as the between-group factor. Dots represent individual mice, while box plots represent Quartile 1 – media – Quartile 3. Dams fed the Control diet (Control: $n = 4$); dams fed the Med-based diet (Med: $n = 6$). ** $p < 0.01$ and *** $p < 0.001$ relative to dams fed the Control diet.

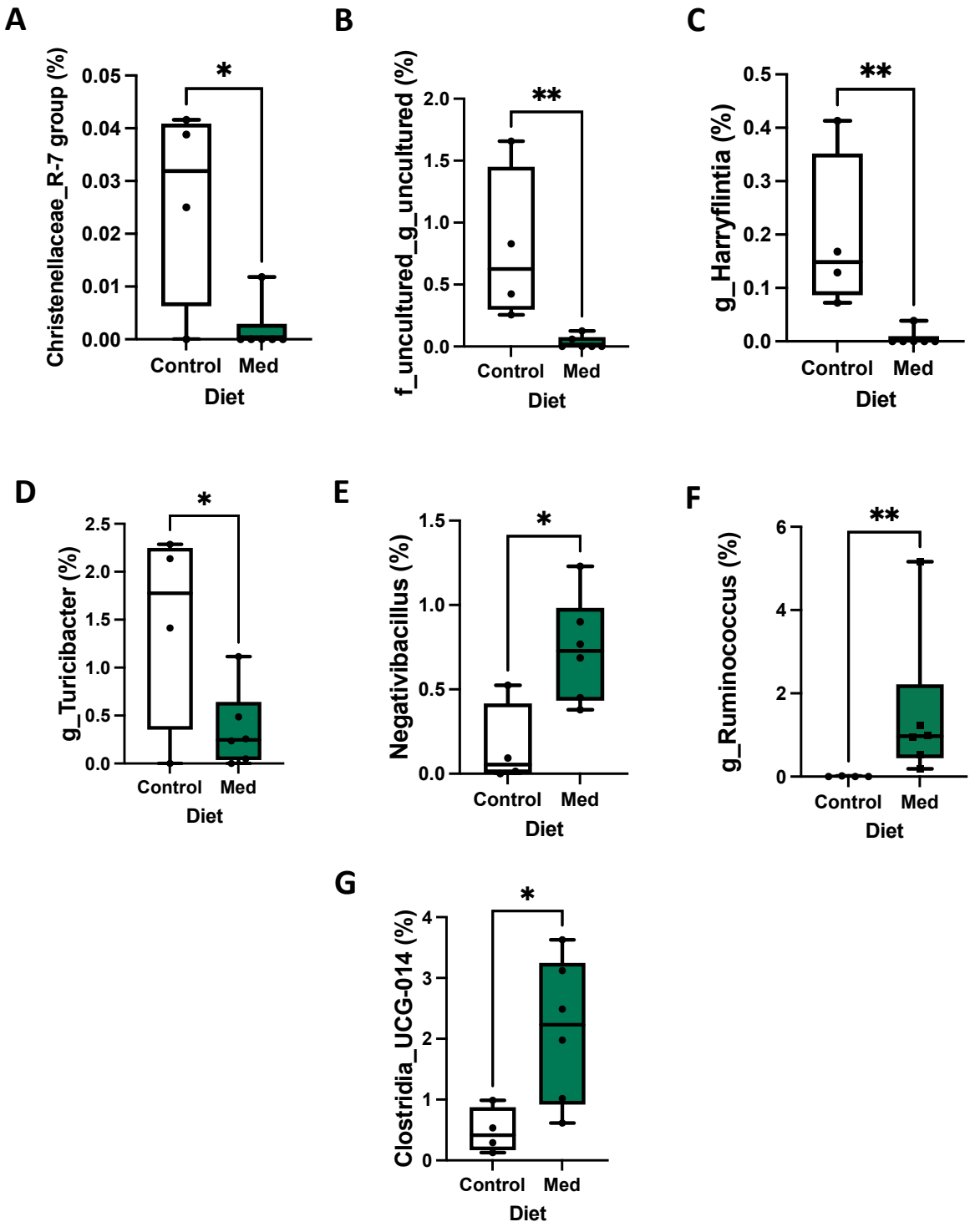

Figure 4. Fecal microbiota genera changed by a perinatal access to a Med-based diet in postpartum dams before 10% FDR corrections to multiple t-tests. Dams fed the Med-based diet had less *Christenellaceae_R-7 group* (A), uncultured genus (B), *Harryflintia* (C) and *Turicibacter* (D) but more *Negativibacillus* (E), *Ruminococcus,* and *Clostridia_UCG-014* (G) than dams fed the Control diet. Data were analyzed using unpaired *t*-tests or Mann-Whitney test, with Diet (Control vs Med) as the between-group factor. Dots represent individual mice, while box plots represent Quartile 1 – media – Quartile 3. Dams fed the Control diet (Control: $n = 4$); dams fed the Med-based diet (Med: $n = 6$). * $p < 0.05$ and ** $p < 0.01$ relative to dams fed the Control diet.

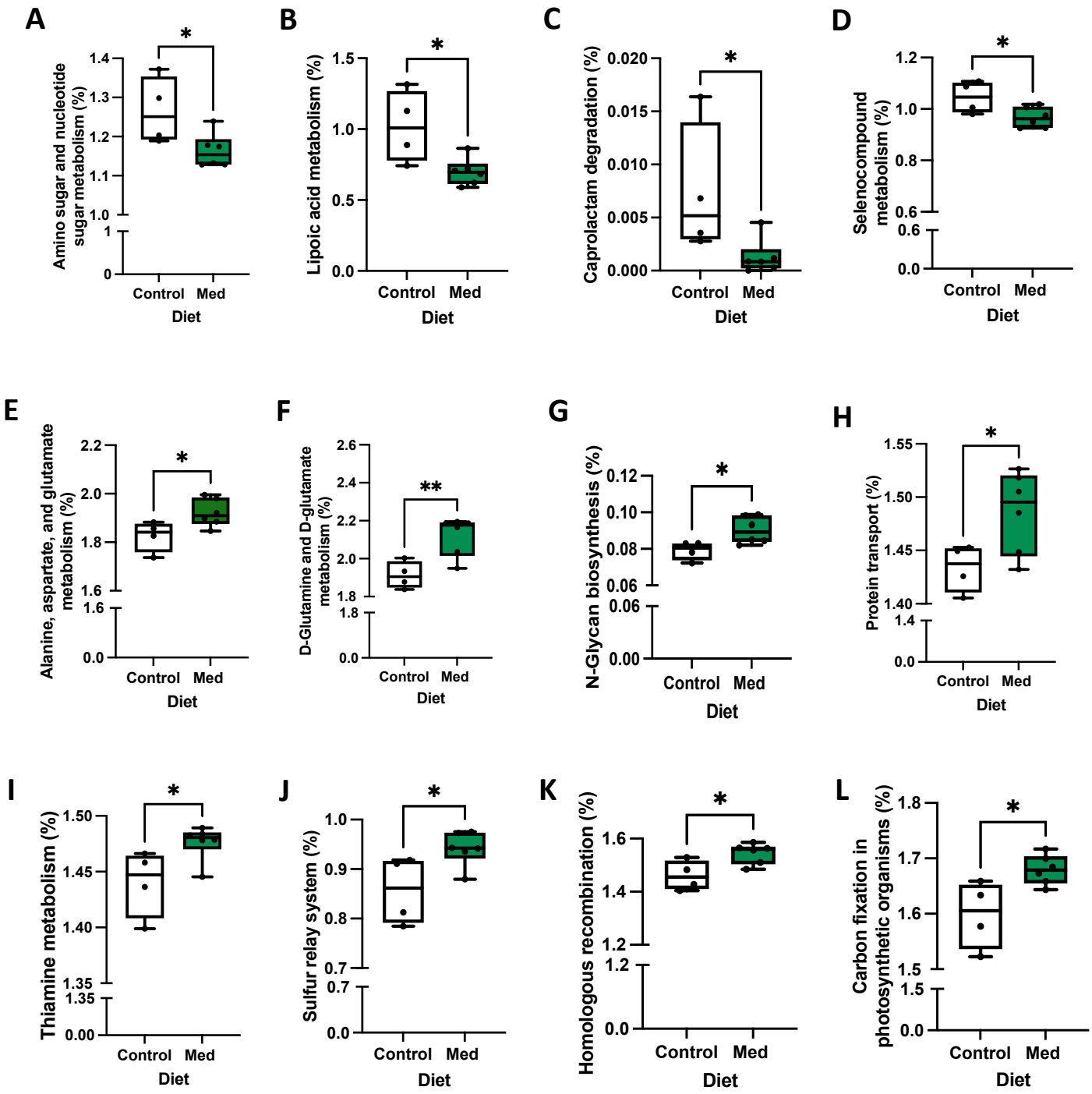

Figure 5. Fecal microbiota KEGG pathways changed by a perinatal access to a Med-based diet in postpartum dams before 10% FDR corrections to multiple t-tests. Dams fed the Med-based diet had less Amino sugar and nucleotide sugar metabolism (A), Lipoic acid metabolism (B), Caprolactam degradation (C) and Selenocompound metabolism (D) but more Alanine, aspartate, and glutamate metabolism (E), D-glutamine and D-glutamate metabolism (F), N-glycan biosynthesis (G), Protein transport (H), Thiamine metabolism (I), Sulfur relay system (J), Homologous recombination (K), and Carbon fixation in photosynthetic organisms (L) than dams fed the Control diet. Data were analyzed using unpaired *t*-tests or Mann-Whitney test, with Diet (Control vs Med) as the between-group factor. Dots represent individual mice, while box plots represent Quartile 1 – media – Quartile 3. Dams fed the Control diet (Control: $n = 4$); dams fed the Med-based diet (Med: $n = 6$). * $p < 0.05$ and ** $p < 0.01$ relative to dams fed the Control diet.

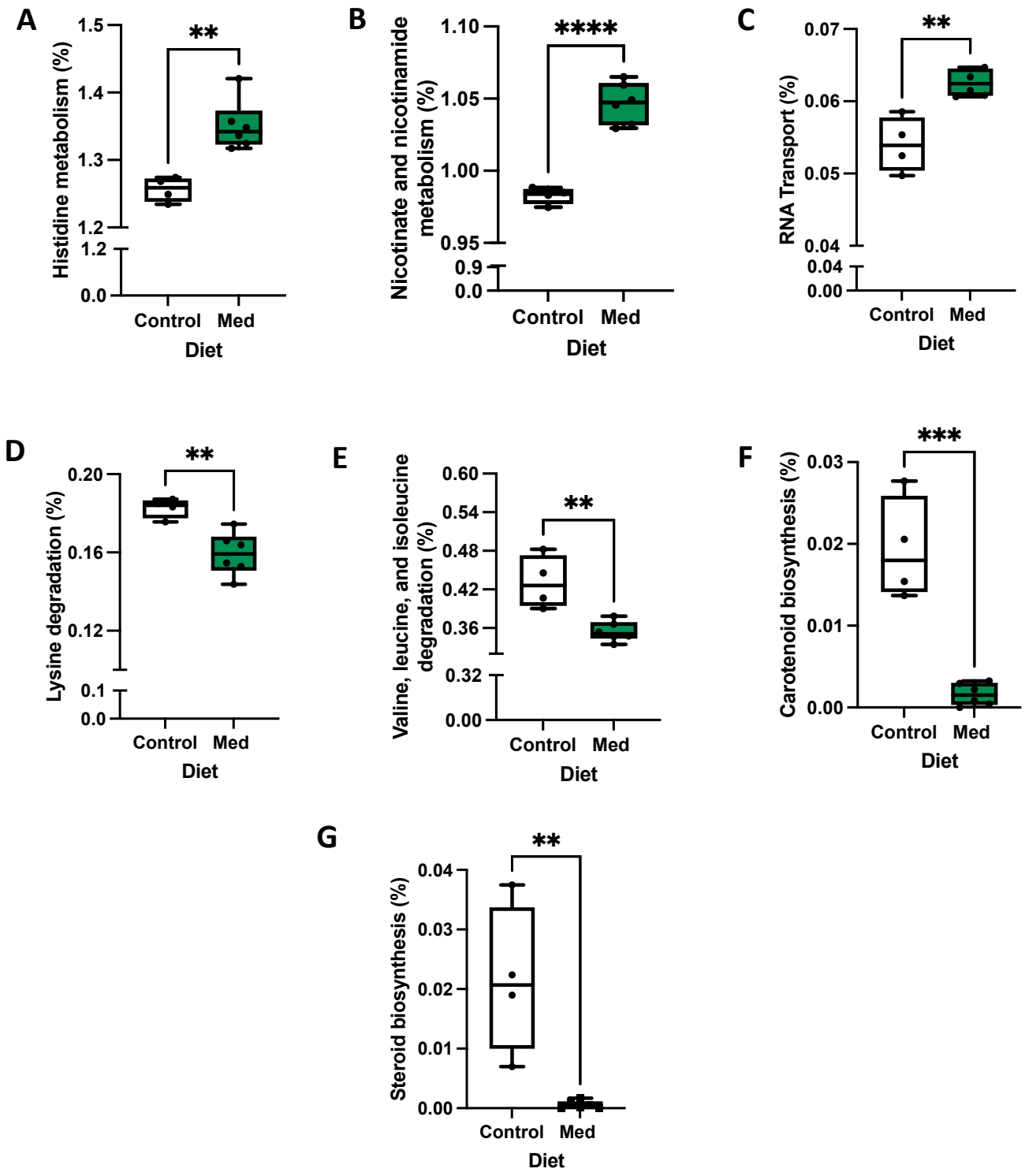

Figure 6. Fecal microbiota KEGG pathways changed by perinatal access to a Med-based diet in postpartum dams after 10% FDR corrections to multiple t-tests. Dams fed the Med-based diet had more Histidine metabolism (A), Nicotinate and nicotinamide metabolism (B), and RNA transport (C) and less Lysine degradation (D), Valine, leucine and isoleucine degradation (E), Carotenoid biosynthesis (F), and Steroid biosynthesis (G). Data were analyzed using unpaired *t*-tests or Mann-Whitney test, with Diet (Control vs Med) as the between-group factor. Dots represent individual mice, while box plots represent Quartile 1 – media – Quartile 3. Dams fed the Control diet (Control: $n = 4$); dams fed the Med-based diet (Med: $n = 6$). ** $p < 0.01$ and *** $p < 0.001$ relative to dams fed the Control diet.

Supplementary Table 1. Experimental Control and Med-based diet formula.

	Open Standard Control diet	Mediterranean-based diet
Ingredients	**Gm**	**gm**
Casein	223	80
Fish Protein Isolate	0	18
Egg White	0	9
Beef, Cooked, Powdered, 5013	0	40
L-Cystine	3	3
Corn Starch	467.4	0
Maltodextrin	150	125
Wheat Starch	0	198.5
Chickpeas, Cooked, Dried	0	36
Lentils, Cooked, Dried	0	36
Cellulose, BW200	75	14
Inulin	0	5
Beta-Glucans	0	5
Soybean Oil	70	0
Menhaden Oil (200 ppm tBHQ)	0	9
Butter, Anhydrous	0	5
Flaxseed Oil	0	6.5
Olive Oil	0	105
Walnuts, Dried, Powdered	0	20
t-BHQ	0.005	0.005
Mineral Mix S10026	10	10
Dicalcium Phosphate	13	13
Calcium Carbonate	5.5	5.5
Potassium Citrate, 1 H2O	16.5	16.5
Vitamin Mix V10001	10	10
Biotin (1%)	0	0.014
Choline Bitartrate	2	2
Fruit and Veggie Blend	0	100
Resveratrol (50% Trans Resveratrol)	0	0.045
Total	**1045.405**	**872.064**
Macronutrient composition		
g		
Protein	197.0	156.4
Carbohydrate	552.9	402.1
Fat	72.7	157.8
Cholesterol	0.0	0.06
Total Fiber	75.0	55.0
Insoluble Fiber	75.0	37.8
Soluble Fiber	0.0	18.4
g%		
Protein	18.8	17.9
Carbohydrate	52.9	46.1
Fat	7.0	18.1
Cholesterol	0.0	0.007
Total Fiber	7.2	6.3
Insoluble Fiber	7.2	4.3
Soluble Fiber	0.0	2.1
kcal		
Protein	*788*	*626*
Carbohydrate	*2212*	*1608*
Fat	*654*	*1420*
Total	***3654***	***3654***
kcal%		
Protein	*22*	*17*
Carbohydrate	*61*	*44*
Fat	*18*	*39*

Supplementary Table 2. Primer sequences used in RT-qPCR experiments.

Gene	Gene sequence/Assay ID
Mus GAPDH	Forward: 5'- GGT CGG TGT GAA CGG ATT TG -3'
	Reverse: 5'- TGC CGT GAG TGG AGT CAT ACT G -3'
Mus Actb	Forward: 5'- GAA CCC TAA GGC CAA CCG TG -3'
	Reverse: 5'- GGT ACG ACC AGA GGC ATA CAG G -3'
Mus TNF-α	Forward: 5'- CTC AGC CTC TTC TCA TTC CTG C -3'
	Reverse: 5'- GGC CAT AGA ACT GAT GAG AGG G -3'
Mus IL-1β	Forward: 5'- TGC CAC CTT TTG ACA GTG ATG -3'
	Reverse: 5'- GTG CTG CTG CGA GAT TTG AA -3'
Mus IL-6	Forward: 5'- ACG GCC TTC CCT ACT TCA CA -3'
	Reverse: 5'- TGC CAT TGC ACA ACT CTT TTC TC -3'
Mus BDNF	Forward: 5'- GTC TCC AGG ACA GCA AAG CCA C -3'
	Reverse: 5'- CCT TGT CCG TGG ACG TTT ACT TC -3'
Mus Occludin	Forward: 5'- ACC CGA AGA AAG ATG GAT CG -3'
	Reverse: 5'- CAT AGT CAG ATG GGG GTG GA -3'
Mus Claudin-2	Forward: 5'- GTC ATC GCC CAT CAG AAG AT -3'
	Reverse: 5'- CTG TTG GAC AGG GAA CCA GT -3'
Mus Claudin-3	Forward: 5'- GCA CAA AGA AAC CTC GCC CT -3'
	Reverse: 5'- CCC GTT TCA TGG TTT GCC TG -3'
Mus Claudin-5	Forward: 5'- GGC ACT CTT TGT TAC CTT GAC C -3'
	Reverse: 5'- CAG CTC GTA CTT CTG TGA CAC C -3'

Chapter 5: Sex-specific effects of a Mediterranean-based diet on behavioural, neuroinflammatory, and gut microbiota changes in prenatally stressed neonates

This chapter details the second study investigating whether the Med-based diet attenuated socioemotional deficits as well as brain inflammatory and neurotrophic factors and gut microbiota in prenatally stressed neonates. The sex-specific effects of the prenatal stressor and Med-based diet were also determined. The findings are presented in the manuscript below prepared for submission shortly.

Sex-specific effects of a Mediterranean-based diet on behavioural, neuroinflammatory, and gut microbiota changes in prenatally stressed mice neonates

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Abstract

Research indicates that prenatal stress alters the behaviour and physiology of the offspring during early development. Since maternal diet influences offspring's development, we examined whether dietary patterns based on the Mediterranean (Med) diet could modulate the effects of prenatal stress on behaviour, neuroinflammatory and neurotrophic factors, and gut microbiota in neonatal female and male mice. Female C57BL/6N mice were fed a Control diet or a Med-based diet (enriched with fruits, vegetables, legumes, fish and olive oils, and walnuts) and bred with males on respective diets after two weeks. In the second trimester, pregnant females were subjected to a physical restraint stressor or undisturbed. Ultrasonic vocalizations (USVs) of the pups were recorded on postnatal day (PND) 2. Brains were collected on PND 7 from one pup per sex in each litter to analyze the mRNA expression of tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, brain-derived neurotrophic factor (BDNF), and claudin-5 in the dorsal hippocampus and prefrontal cortex. Feces were also collected for the determination of microbiota diversity, composition, and inferred metabolic capacity. The results showed that prenatal stress decreased pup USVs regardless of diet. Conversely, the Med-based diet limited stress-induced increases in mRNA expression of TNF-α and IL-1β in the prefrontal cortex and decreases in that of BDNF in the hippocampus of females. No effect of prenatal stress was observed for claudin-5 expression in both brain regions and in the fecal microbiota. These findings suggest that early-life Med-based dietary patterns could alleviate detrimental impacts of prenatal stress on neuroinflammatory and neurotrophic processes in the developing neonatal brain, particularly in female offspring.

Keywords: prenatal stress, diet, behaviour, mental health, sex

1.0 Introduction

Maternal adversity during pregnancy may influence the early development of offspring's behaviour (Van den Bergh et al., 2020). Elevated maternal cortisol levels or psychological distress during pregnancy were associated with emotional and temperamental problems in infants (Davis et al., 2007; De Weerth et al., 2003; Hill et al., 2013). In rodents, alterations in ultrasonic vocalizations (USVs) emitted during brief isolation bouts from the dam have been reported in neonatal pups exposed to prenatal stress (Budylin et al., 2019; Jones et al., 2010; Laloux et al., 2012). Importantly, alterations in neonatal USVs due to prenatal stress have been linked to behavioural impairments later in life, with prenatally stressed neonatal rats that emitted fewer and shorter USVs being socially impaired in adulthood (Ehrlich & Rainnie, 2015), and those that called more frequently and for a longer time during the neonatal stage displaying more anxiety-like behaviours (Laloux et al., 2012).

Although the exact mechanisms underlying the behavioural effects of prenatal stress have yet to be entirely discovered, appreciable evidence shows that they may stem from alterations in the development of brain processes related to inflammation, homeostasis regulation, as well as neuronal growth and synaptic plasticity. Prenatal exposure to psychological or immunological stressors increased microglial activation and pro-inflammatory cytokines in the fetal and neonatal brain as well as decreased pericyte coupling to vascular endothelial cells and expression of brain-derived neurotrophic factor (BDNF) (Chen et al., 2020; Gur et al., 2017; Zhao et al., 2022; Fatima et al., 2019; Van Den Hove et al., 2006), leading to neuroinflammation, increased blood-brain barrier (BBB) permeability, and anxiety-like behaviours that persisted across the lifespan (Zhao et al., 2022). This is particularly important as increases in pro-inflammatory cytokines and reductions in tight junction proteins critical for BBB permeability were reported in the bloodstream and post-

mortem brains of individuals with major depression and anxiety disorders (Dion-Albert et al., 2022; Enache et al., 2019; Hou et al., 2017; Osimo et al., 2020), and reductions in BDNF are reported in depressive disorders (Reinhart et al., 2015). Prenatally stressed infants and neonatal mice also exhibited a higher abundance of Proteobacteria, a commensal phylum composed of Gram-negative bacteria that harbour the endotoxin lipopolysaccharide (LPS) as part of their outer membrane, and a lower abundance of *Lactobacilli* and *Bifidobacteria*, which have been associated with anti-inflammatory effects (Aatsinki et al., 2020; Bailey et al., 2004; Jašarević et al., 2015; Zijlmans et al., 2015). Given that the early establishment of the gut microbiota may influence postnatal brain development (Heijtz et al., 2011), such microbiota patterns during critical periods of brain maturation could disturb gut-brain inflammatory signaling, potentially affecting neuroimmune functions and behaviour. Supporting this, prenatally stressed mice with altered gut microbiota during the neonatal period also showed anxiety- and depressive-like behaviours and social impairments in adulthood (Gur et al., 2017, 2019; Zhang et al., 2021).

There is evidence that maternal diet during pregnancy may influence neonatal development and health. Dietary nutrients, such as long-chain polyunsaturated fatty acids, are indispensable to early brain development, affecting key developmental events, including neuronal and glial proliferation, synaptogenesis, and myelination (Prado & Dewey, 2014). Notably, children whose mothers adhered to dietary patterns based on the Mediterranean (Med) diet (abundant in whole grains, legumes, olive oil, fruits, vegetables, nuts, and seeds) during pregnancy were less likely to develop depression and anxiety (House et al., 2018) or have cognitive deficits and socioemotional problems (Crovetto et al., 2023). How dietary nutrients come to affect fetal development, with impact on neonatal and infant behaviour is uncertain, although it is well established that diet is an

important factor shaping postnatal microbial colonization of the offspring's gut (Stewart et al., 2018), which can influence brain development (Borre et al., 2014).

We previously developed a Med-based diet and demonstrated that its consumption from preconception to early postpartum reduced the mRNA expression of pro-inflammatory cytokines and increased that of BDNF and claudin-5 in the hippocampus of postpartum mouse dams (Udechukwu et al., 2024a; Udechukwu et al, in preparation). We also showed that this Med-based diet limited increases in anxiety-like behaviour and pro-inflammatory cytokines in the hippocampus and colon elicited by a prenatal physical restraint stressor in adult mice offspring (Udechukwu et al., 2024b). In this study, we investigated whether this Med-based diet would limit behavioural disturbances of neonatal mice exposed to the same intrauterine stress by assessing their USVs during brief isolation episodes from their mothers. To verify if the diet influenced biological changes in the neonates, we determined the mRNA expression of pro-inflammatory, tight junction, and neurotrophic factors in the dorsal hippocampus and prefrontal cortex, which are stress-sensitive brain regions (Charil et al., 2010), as well as fecal microbiota diversity, composition, and predicted metabolic capacity in prenatally stressed pups. Given the sex-specific impacts of prenatal adversity observed in our previous research (Udechukwu et al., 2024b) and that of others (Gur et al., 2017; Jašarević et al., 2017), we assessed these outcomes in female and male offspring separately.

2. Materials and methods

2.1 Animals

Naïve female (6-8 weeks) and male (7-9 weeks) C57BL/6N mice (Charles River Laboratories, Montréal, QC, Canada) were housed individually in 19 cm x 29 cm x 13 cm polycarbonate N10 mouse cages (Ancare), enriched with a cotton nestlet, a cardboard house, and

standard woodchip bedding. The housing room was maintained at a 12-h light-dark cycle (lights on 0700-1900 h), a temperature of 21-23 °C, and 30-50% humidity. Free access to food and water was provided throughout the study. All experimental procedures were approved by the Animal Care Committee at the University of Ottawa (animal protocol number: HSe-3149), according to the guidelines of the Canadian Committee of Animal Care.

2.2 Summary of the experimental procedures

Figure 1 illustrates the experimental timeline of the study procedures. Before starting the experiments, all mice were acclimated for one week, during which they were fed a modified formula of an Open Standard Diet (D12052701M, Research Diets Inc.), referred to as the Control diet. Following acclimation, a group of female and male mice were randomly selected to continue feeding on the Control diet (n = 12 females and 9 males), while the rest was assigned to the Med-based diet (n = 10 females and 7 males). After two weeks of receiving their respective diets, females and males fed the same diet were paired for breeding. After birth, which corresponded to Embryonic day (E) 0.5, dams remained undisturbed with their litters. During E7.5-12.5, a subset of pregnant females was exposed to a physical restraint stressor (Control: n = 6; Med: n = 5), while the rest remained undisturbed (Control: n = 6; Med: n = 5). On Postnatal Day (PND) 2, USVs were recorded from whole litters (4-10 pups) from each group. On PND 7, brains were collected from 1 female and 1 male pup from each litter to determine the mRNA expression of pro-inflammatory, tight junction, and neurotrophic factors, whereas feces were collected to assess microbiota diversity, composition, and *in silico* metabolic capacity.

2.3 Experimental diets

The Control and Med-based diets were developed as previously described (Udechukwu et al., in preparation). Briefly, an Open Standard Diet (D12052701M, Research Diets Inc.) was

modified to develop the Control diet by adding extra casein to increase the protein content to approximately 17% in line with mouse breeding requirements (Nutrition, 1995). The Med-based diet was developed by modifying an available mouse Med-based dietary formula (D12052702, Research Diets Inc.; Barrington et al., 2018). The main modifications included adding ingredients present in human Med dietary patterns, including olive oil, chickpeas, lentils, and walnuts (processed in our laboratory and analyzed for their nutritional content; Intertek, Saskatoon, Canada), fruits and vegetables (generously supplied by FutureCeuticals, Inc., Momence, Illinois, United States), as well as oat beta-glucan and trans-resveratrol (PureBulk, Roseburg, Oregon, United States). The experimental diets were isocaloric, but the Med-based diet had different macronutrient (fat, protein, and carbohydrates) contribution to calories according to human dietary patterns (Davis et al., 2015). The dietary formulas are provided in Supplementary Table 1.

2.4 Breeding procedures

Mice were bred following the Trio breeding scheme (Braden et al., 2017). Briefly, estrus was induced by placing nestlets and bedding from a male's housing cage into the cage of two individually housed females. Twenty-four hours later, these two females were introduced into the male's housing cage and left overnight for breeding, after which they returned to their housing cages. Females in which a vaginal copulation plug was detected, which was designated as E0.5, were monitored every three days to examine pregnancy status through body weight measurements. Pregnant dams were left undisturbed, except for body weight measurements, food, cage, and water bottle replacements, or occasional blood and fecal sample collection for another experiment beyond the scope of the present study.

2.5 Prenatal stressor procedures

The prenatal stressor was comprised of a validated physical restraint stressor paradigm in the second trimester (Buynitsky & Mostofsky, 2009). This stressor was chosen based on preliminary findings from our lab and others that it altered USVs (Laloux et al., 2012; Zimmerberg & Blaskey, 1998), increased pro-inflammatory cytokines (Diz-Chaves et al., 2012; Gur et al., 2019) and decreased BDNF (Jia et al., 2015; Liu et al., 2011) in the hippocampus and prefrontal cortex, and altered the gut microbiota (Golubeva et al., 2015; Gur et al., 2017, 2019; Zhang et al., 2021) in the offspring. The second trimester was selected as it corresponds to the period during which the mouse hippocampal and prefrontal cortical structures begin to develop (Kolk & Rakic, 2022; Semple et al., 2013) and as previous studies have shown that the offspring is more vulnerable to stress occurring in early trimesters (Charil et al., 2010; Herbison et al., 2017). The procedure was conducted by placing pregnant females in a triangular plastic bag with a small opening at the nose end to permit breathing and a tape at the tail end to restrict movement. The procedure was conducted for six consecutive days at 9:00, 12:00, and 15:00 h, with each session lasting 30 minutes, and during which breathing and proper limb positioning were constantly monitored (Gur et al., 2017).

2.6. Ultrasonic vocalizations

Ultrasonic vocalizations were recorded using UltraVox XT (Noldus, Wageningen, The Netherlands) equipped with a plug-and-play USB ultrasound microphone with a maximum sampling rate of 384 kHz, as previously reported (Budylin et al., 2019). The testing room was maintained at 21-23 °C (same as the mouse housing room) to prevent cold-induced vocalizations in the pups. Following habituation of the litter and the dam to the testing environment for 10 minutes under white light of approximately 100 lux, the dam was transferred to a fresh holding cage kept aside in the same testing room. The housing cage containing the litter was placed in a

45 x 45 x 45 cm sound-proof chamber made of white opaque acrylic plastic floor and walls (Canus Plastics Incorporated), with a microphone suspended 12 to 15 cm above the cage. USVs were recorded from whole litters for 5 minutes, after which the dam was returned to the housing cage and recordings were obtained for another 5 minutes. USV recordings were subsequently analyzed using the Ultravox XT software (Noldus, version 14) at a frequency above 40 kHz to allow for the detection of isolation calls from the pups (Hahn & Lavooy, 2005). Thereafter, the number of calls, mean duration of calls in milliseconds, and percentage of the different call types in the absence and presence of the dams were determined using a statistics file automatically generated from the software.

2.7 Euthanasia and sample collection

Pups were euthanized by rapid decapitation. Whole brains were immediately removed from the skull and allowed to freeze on a sheet of Parafilm® M placed on dry ice before their transfer to nuclease-free cryotubes in liquid nitrogen. The gastrointestinal tract was rapidly extracted from the abdominal cavity, placed on a nuclease-free frozen surface, and feces were collected from the rectum and placed into nuclease-free microcentrifuge tubes in dry ice. All samples were stored at -80 ºC until further analyses. Frozen brains were subsequently dissected using the cryochamber of a ThermoFisher HM525 NX cryostat maintained at -20 ºC to collect the dorsal hippocampus and the prefrontal cortex. Briefly, each whole brain was placed in a stainless-steel brain matrix (2.5 x 3.75 x 2.0 cm; slots spaced approximately 500 µm apart) positioned on an ice plate. Brains were sectioned coronally using razor blades guided by the matrix slots. Dorsal hippocampi and prefrontal cortices were dissected from the coronal sections following the Franklin and Paxinos mouse atlas (Franklin & Paxinos, 1997), placed into nuclease-free tubes, and stored at -80 ºC until further analyses.

2.8 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Dorsal hippocampi and prefrontal cortices were homogenized using TRIzol, and their total RNA was extracted according to the manufacturer's instructions (Invitrogen, Burlington, Canada). RNA concentration and purity were determined using a NanoDrop™ One Spectrophotometer (ThermoFisher Scientific). Samples with 260/80 and 260/230 ratios between 1.80 and 2.20 were included and thus the sample size and degrees of freedom associated with hippocampal and prefrontal gene expression differ from initial sample sizes. Total RNA was reverse transcribed into complementary DNA (cDNA) using iScript™ Reverse Transcription Supermix and a T100 Thermal Cycler (Bio-Rad, Canada). Aliquots of cDNA were analyzed for the gene expression of tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, BDNF, occludin, and claudin-5 in duplicates of simultaneous quantitative polymerase chain reactions using the SsoAdvanced Universal SYBR® Green Supermix and a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Canada). Primers that amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Beta-Actin (Actb) were used as reference genes and their geometric mean was used to normalize the expression of the genes of interest. Fold changes for the mRNA expression for each gene of interest were calculated for each sex (except for USV analyses where they were calculated for whole litters) using the $2^{-\Delta\Delta CT}$ method, relative to the group in the control conditions (pups born to non-stressed dams fed the Control diet (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). Primer sequences can be found in Supplementary Table 2.

2.9 Fecal microbiota analyses

2.9.1 16S ribosomal RNA (rRNA) gene sequencing

Fecal DNA was extracted using a Stool Nucleic Acid Isolation Kit, as instructed by the manufacturer (Norgen Biotek Corp, Thorold, Canada). DNA concentration and purity were

assessed using Quant-iT^{TM} PicoGreen (Invitrogen). Several fecal samples were excluded from subsequent analyses due to extremely low DNA concentrations, thereby reducing the sample size to 3 or 4 samples for some of the groups. The 16S rRNA gene amplicons were prepared following the Illumina 16S library preparation procedures. Briefly, the V3 and V4 hypervariable regions of the 16S rRNA gene were amplified using the primers S-D-Bact-0341-b-S-17 (F: 5' TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG) and S-D-Bact-0785-a-A-21 (R: 5' GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C) (Klindworth et al., 2013). The resulting amplicons were tagged with Illumina nucleotide sequencing adapters and dual-index barcodes for Illumina MiSeq compatibility and sample identification, respectively. All samples were then pooled into a library, which was sequenced using a 600-cycle MiSeq Reagent Kit v3 and a MiSeq system, following the manufacturer's directions (Illumina, San Diego, CA, USA). The resulting sequences were processed using QIIME 2 (Bolyen et al., 2019). Paired-end sequence reads that passed a median quality score of $Q \geq 30$ were denoised, filtered, and rarified using DADA2 (Callahan et al., 2016). All samples were above the retention threshold of 10,000 reads and were thus kept for subsequent data processing. Taxonomic assignment of sequence reads was performed using the SILVA database (Quast et al., 2013), after which the relative abundance of bacteria at the phylum, family, and genus taxonomic levels was calculated for each sample. The QIIME2 data output was further analyzed using MicrobiomeAnalyst (Chong et al., 2020) to determine the Chao1 and Shannon alpha-diversity indices and the Bray-Curtis dissimilarity beta-diversity index (calculated using Bray-Curtis distance and visualized using Principal Coordinate Analysis).

2.9.2 In silico prediction of bacterial metabolic capacity

Bacterial metabolic capacity was inferred from the 16S rRNA gene sequence data using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) Python package (Douglas et al., 2020). Briefly, the abundance of Operational Taxonomic Units was processed to obtain a catalog of Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs, KEGG pathways, and their associated BRITE functional hierarchies (Kanehisa et al., 2012), from which the relative enrichment of KEGG orthologs and pathways were calculated for each sample. Given our *a priori* hypothesis that the Med-based diet would limit the impacts of the prenatal stressor on inflammatory activity in the gut microbiota community (Madison & Bailey, 2024; Seethaler et al., 2022), we specifically examined changes in immune-related pathways, namely LPS biosynthesis and peptidoglycan (PGN) biosynthesis.

2.10. Statistical analyses

Statistical analyses were performed in SPSS version 29.0.2.0, and data were graphed in GraphPad Prism version 10.1.0. Data were first tested for normality (Shapiro-Wilk test) and homogeneity of variances (Levene test) and then analyzed using a series of two-way analyses of variance (ANOVAs), with Prenatal Stressor (non-stressed vs prenatally stressed) and Diet (Control vs Med-based) as the between-group factors. Brain and microbiota data were analyzed separately for each sex. Follow-up comparisons of the simple effects comprised *t* tests with a Bonferroni correction at an alpha level set at $p < 0.05$. Beta-diversity was determined using permutational multivariate analysis of variance (PERMANOVA) of Bray-Curtis distance matrices in MicrobiomeAnalyst (Chong et al., 2020). The alpha level was set at $p<0.05$ for all analyses.

3.0 Results

3.1. The Med-based diet did not limit reductions in ultrasonic vocalizations in stressed litters

As seen in Figure 2A, prenatally stressed litters emitted fewer USVs than non-stressed ones when their mothers were absent from the housing cages ($F_{(1,16)} = 4.643$, $p = 0.047$), regardless of whether they were fed the Control or the Med-based diets. No significant differences as a function of the Prenatal Stressor, Diet, or their interaction were observed in the number of USVs emitted when dams were returned to the housing cage, nor in the mean duration of USVs emitted in both the absence and presence of the dams (p's > 0.05; Fig. 2B-D).

3.2. The Med-based diet generally increased hippocampal pro-inflammatory cytokines in both sexes and limited stress-induced decreases in BDNF in females

In females, none of the pro-inflammatory cytokines in the hippocampus was affected by the Prenatal Stressor (p's > 0.05), but they all differed as a function of Diet or of its interaction with the Prenatal Stressor. As shown in Figure 3A, both non-stressed and prenatally stressed females fed the Med-based diet exhibited lower TNF-α expression compared to their counterparts fed the Control diet ($F_{(1,17)} = 18.823$, $p < 0.001$). In contrast, follow-up comparisons of the simple effects comprising the significant Prenatal Stressor and Diet interactions for IL-1β ($F_{(1,17)} = 7.279$, $p = 0.015$) and IL-6 ($F_{(1,17)} = 19.514$, $p < 0.001$) in females showed that the Med-based diet upregulated these cytokines only in pups born from non-stressed dams (p's < 0.001; Fig. 3B and 3C). Hippocampal BDNF expression in females was influenced by the Prenatal Stressor ($F_{(1,17)} = 21.547$, $p < 0.001$) and Diet ($F_{(1,17)} = 19.791$, $p < 0.001$), and although the Prenatal Stressor and Diet interaction did not reach statistical significance ($F_{(1,17)} = 3.267$, $p = 0.088$), follow-up comparisons were determined based on an *a priori* hypothesis that the Med-based diet would limit the impacts of the stressor on brain BDNF expression (Liu et al., 2011). Confirming this hypothesis, the prenatal stressor decreased hippocampal BDNF expression in females fed the Control diet ($p < 0.001$), but not in those fed the Med-based diet (Fig. 3D). No effect of the

manipulations was observed for claudin-5 hippocampal expression in females (*p's* > 0.05; Fig. 3E).

In males, TNF-α and IL-6 in the hippocampus were affected by the Prenatal Stressor (TNF-α: $F_{(1,16)} = 6.712$, $p = 0.020$; IL-6: $F_{(1,16)} = 21.230$, $p < 0.001$) and its interaction with Diet (TNF-α: $F_{(1,16)} = 7.596$, $p = 0.014$; IL-6: $F_{(1,16)} = 5.775$, $p = 0.029$), with IL-6 being also influenced by Diet ($F_{(1,16)} = 11.987$, $p = 0.003$). Follow-up comparisons revealed that TNF-α was higher in prenatally stressed males fed the Med-based diet relative to non-stressed ones ($p = 0.005$; Fig. 3A). In contrast, the stressor increased IL-6 in males fed the Control diet ($p < 0.001$), but not in those fed the Med-based diet (Fig. 3C). Lastly, none of the manipulations affected IL-1β, BDNF, and claudin-5 hippocampal expression in males (*p's* > 0.05; Fig 3B, 3D, and 3E).

3.3. The Med-based diet limited stress-induced increases in pro-inflammatory cytokines and promoted BDNF upregulation in the prefrontal cortex of females

In contrast to the hippocampus, TNF-α and IL-1β expression in the prefrontal cortex of females was affected by the Prenatal Stressor (TNF-α: $F_{(1,18)} = 15.517$, $p = 0.001$; IL-1β: $F_{(1,17)} = 6.435$, $p = 0.021$) and its interaction with Diet (TNF-α: $F_{(1,18)} = 7.215$, $p = 0.015$; IL-1β: $F_{(1,17)} = 6.527$, $p = 0.021$). Follow-up comparisons showed that increases in TNF-α ($p < 0.001$) and IL-1β ($p = 0.002$) prefrontal expression in prenatally females fed the Control diet were not apparent in those fed the Med-based diet (Fig. 4A and 4B). Prefrontal BDNF expression in females was only affected by Diet ($F_{(1,18)} = 13.617$, $p = 0.001$), with both non-stressed and prenatally stressed females fed the Med-based diet showing higher BDNF than those fed the Control diet (Fig. 4D). No group differences were observed for IL-6 and claudin-5 in females (*p's* > 0.05; Fig. 4C and 4E).

In males, TNF-α expression in the prefrontal cortex differed as a function of Diet ($F_{(1,16)}$ = 7.553, p = 0.014) and of its interaction with the Prenatal Stressor ($F_{(1,16)}$ = 4.577, p = 0.048). Follow-up comparisons showed that the Med-based diet upregulated this cytokine in the prefrontal cortex in non-stressed males only (p = 0.001; Fig. 4A). The other genes assessed in the prefrontal cortex of males were not influenced by the stressor and diet manipulations (p's > 0.05; Fig. 4B-E).

3.4 The Med-based diet, but not the prenatal stressor, affected fecal microbial diversity, composition, and predicted metabolic capacity

The Chao1 and Shannon alpha-diversity indices did not change due to the Prenatal Stressor, Diet, or their interaction in either sex (p's > 0.05; Fig. 5A and 5B). Similarly, the Bray-Curtis dissimilarity beta-diversity index did not show separate clustering of samples by the Prenatal Stressor or Diet in either sex (p's > 0.05; Fig. 5C-F). With respect to taxonomic composition, 9 phyla were detected in fecal samples, with Firmicutes (84%) being the most abundant, followed by Proteobacteria (13%) (Supplementary Fig. 1). Among the 9 phyla, 3 were present in only one sample in either of the groups and thus taxa from lower ranks belonging to these phyla were excluded from statistical analyses. At the family level, *Lactobacillaceae* (61%) and *Staphylococcaceae* (19%) comprised the major representatives of Firmicutes, while *Enterobacteriaceae* (10) was the most abundant family member of Proteobacteria. As shown in Figure 6, whereas *Lactobacillaceae* were not affected by any of the manipulations in either sex (p's > 0.05; Fig. 6A), the less abundant *Staphylococcaceae* were influenced by Diet in males only ($F_{(1,27)}$ = 10.403, p = 0.007), being less abundant in non-stressed and stressed males fed the Med-based diet compared to those fed the Control diet (Fig. 6B), and *Enterobacteriaceae* varied as a function of the significant Prenatal Stressor and Diet interactions in both females ($F_{(1,27)}$ = 6.516, p = 0.023) and males ($F_{(1,27)}$ = 4.667, p = 0.034), with follow-up comparisons showing that the

Med-based diet increased this family in prenatally stressed females (p = 0.007) and males (p = 0.026) but not in their non-stressed counterparts (Fig. 6C).

The *in silico* assessment of bacterial metabolic capacity resulted in a catalog of KEGG orthologs and associated KEGG pathways. Out of the 145 pathways identified, 8 were present in only one sample in either of the groups and thus were not included in subsequent analyses. Given our *a priori* hypothesis that the Med-based diet would limit the impacts of the prenatal stressor on inflammatory activity in the gut microbiota community (Madison & Bailey, 2024; Seethaler et al., 2022), we specifically examined changes in immune-related pathways, namely LPS biosynthesis and PGN biosynthesis. For the LPS biosynthesis pathway, follow-up comparisons of the significant Prenatal Stressor and Diet interaction ($F_{(1,27)}$ = 4.167, p = 0.048) in females showed that the Med-based diet enriched this pathway in those that were prenatally stressed only (p = 0.016) whereas in males, the diet increased this pathway in both non-stressed and prenatally stressed offspring ($F_{(1,27)}$ = 5.923, p = 0.030) (Fig. 7A). No changes due to the Prenatal Stressor, Diet, or their interaction were seen in the relative enrichment of the PGN biosynthesis pathway in either sex (p's > 0.05; Fig. 7B).

4.0 Discussion

Prenatal stress can alter offspring's behaviour and physiological development (Weinstock, 2017), and studies suggest that maternal diet can influence child mental health outcomes (House et al., 2018). In this study, we show that a prenatal stressor experienced during the second trimester of gestation led to deficits in socioemotional behaviour in neonatal offspring, alongside sex-specific changes in pro-inflammatory cytokine and BDNF expression in the hippocampus and prefrontal cortex. Importantly, we report, for the first time, that although they did not promote

major fecal microbiota shifts, dietary patterns based on the Med diet limited stress-induced changes in brain inflammatory and neurotrophic factors, particularly in neonatal females.

Previous findings regarding the impacts of prenatal stress on USVs emitted by neonatal pups when transiently separated from their mother are conflicting, perhaps due to differences in the type of prenatal stressor used and its timing during pregnancy, the age of pups at the time of assessment, and the rodent species examined. While some studies found increases in the number of USVs produced by prenatally stressed pups (Gulia et al., 2015; Harmon et al., 2009; Laloux et al., 2012; Williams et al., 1998; Zimmerberg & Blaskey, 1998), others reported fewer emissions (Ehrlich & Rainnie, 2015; Jones et al., 2010; Takahashi et al., 1990). Consistent with the latter studies, we found that exposure to a physical restraint stressor during the second trimester of gestation reduced the number of USVs emitted by pups when briefly isolated from the dams during the early neonatal period. In early postnatal life, rodent pups are heavily reliant on their mothers for growth and survival and USVs represent primary communication signals through which they can communicate their needs to their mothers and receive maternal care such as licking, nursing, and nesting (Hahn & Lavooy, 2005). Although we did not examine maternal behaviour when the dams were returned to the housing cage, previous reports showed that neonatal pups that emitted fewer number of USVs received less maternal retrieval and care (Abuaish et al., 2020) and exhibited fewer social interactions in adulthood (Budylin et al., 2019; Ehrlich & Rainnie, 2015; Jones et al., 2010), an outcome that we could not confirm, given that behaviours could not be evaluated at an advanced age. Unfortunately, our analyses failed to demonstrate an attenuating effect of the Med-based diet on the number of USVs emitted by prenatally stressed litters during the absence of the dam. Nonetheless, our results indicate that prenatal stress diminished the expression of important means of socioemotional communication during early development, with

potential consequences for maternal-neonate interactions and offspring mental health outcomes, as shown by others.

The prenatal stressor increased pro-inflammatory cytokines and reduced BDNF in the neonatal hippocampus and prefrontal cortex in a sex-specific manner. Similar increases in the hippocampal and prefrontal expression of pro-inflammatory cytokines and decreases in hippocampal BDNF have been reported in mice of both sexes stressed *in utero*, although these studies were conducted in fetal and adult stages (Diz-Chaves et al., 2012, 2013; Gur et al., 2019; Fatima et al., 2019; Liu et al., 2011; Van Den Hove et al., 2006). Unlike the observations for USVs, the Med-based diet counteracted these effects of prenatal stress in the neonatal brain, mostly in the females. Specifically, whereas the Med-based diet limited stress-induced hippocampal reductions in BDNF and prefrontal elevations of TNF-α and IL-1β in the females, it only limited increases in hippocampal IL-6, but not TNF-α, in the males. During early brain development, cytokines such as TNF-α, IL-1β, and IL-6 play critical roles in neural and glial proliferation and synaptic refinement (Deverman & Patterson, 2009), and BDNF promotes neuronal differentiation and survival as well as synaptic maturation (Park & Poo, 2012). However, excessive cytokine levels due to stress could alter neural circuitry and neurotransmission, potentially affecting behaviour (Dammann & O'Shea, 2008). Likewise, BDNF deficiencies due to genetic mutations were shown to impair axonal growth and dendritic arborization (An et al., 2008; Liao et al., 2015). Combined with observation that the Med-based diet decreased hippocampal TNF-a and increased prefrontal BDNF irrespective of stress, the finding that the diet attenuated pro-inflammatory cytokine elevations and BDNF reductions elicited by prenatal stress suggests that the diet could promote proper brain development. Treatment with eicosapentaenoic acid (Lynch et al., 2007) and various kinds of polyphenols (Tayab et al., 2022) prevented or reversed elevations of TNF-α, IL-1β, and

IL-6 in the hippocampus and prefrontal cortex associated with aging and stress in adult mice and rats of both sexes. As well, prenatal supplementation with docosahexaenoic acid prevented reductions in hippocampal BDNF in rats born to dams that experienced physical restraint stress during pregnancy (Liu et al., 2011). Considering that our Med-based diet contains fish oil, vegetables, and fruits, which are rich in omega-3 fatty acids and polyphenols, it is possible that these components contributed to mitigating stress-induced neuroinflammatory and BDNF changes in the neonates, with a potential to reduce abnormal pro-inflammatory signals and enhance BDNF availability for critical neurodevelopmental processes.

In contrast to females, the dietary and stressor manipulation elicited more subtle effects in males, suggesting that females could be more responsive to the neuroinflammatory and neurotrophic outcomes of prenatal adversity and, consequently, to the attenuating effect of a Med-based diet in this regard. These findings highlight the relevance of sex considerations when examining neurobiological outcomes of early-life stressors in rodent models and in future development of dietary interventions aimed at mitigating these outcomes. Unexpectedly, despite its anti-inflammatory effects in the context of prenatal stress, the Med-based diet increased the basal expression of IL-1β and IL-6 in the female hippocampus and that of TNF-α in the male prefrontal cortex. The functional significance of these findings is currently unclear and requires further investigation to determine if they have pro-inflammatory/pathological effects or contribute to promoting brain maturational processes influenced by cytokines during the neonatal stage.

Although no studies have examined the effects of prenatal stress on claudin-5 in the neonatal brain, findings in adult mice have consistently showed that adulthood stressors downregulate this tight junction in brain regions involved in the stress response and in mental health, and that the loss of claudin-5 increased BBB permeability (Dion-Albert et al., 2022;

Menard et al., 2017; Nitta et al., 2003). The lack of changes in claudin-5 in the neonatal brain of prenatally stressed pups of both sexes in this study suggests that the function of this tight junction with a major role in BBB permeability remained intact, although the absence of changes in BBB tight junctions does not necessarily translate to an intact BBB function, as previously reported (Zhao et al., 2022). Considering this, further examination of additional markers of BBB functions in prenatally stressed neonates is warranted.

The fecal microbiota of neonatal pups was predominantly composed of Firmicutes and Proteobacteria, which is typical of gut microbiota patterns in vaginally delivered neonates (Bäckhed et al., 2015). Contrary to previous studies showing decreased Lactobacillaceae/Lactobacillus and increased Proteobacteria/Enterobacteriaceae in maternal vaginal and neonatal offspring intestinal milieus (Jašarević et al., 2015, 2017, 2018; Weiss & Hamidi, 2023), we did not find changes in neonatal microbial diversity and composition due to prenatal stress in either sex. Interestingly, however, the Med-based diet increased *Enterobacteriaceae* in both stressed females and males, in addition to upregulating the LPS biosynthesis pathway, which is likely a metabolic consequence of increased *Enterobacteriaceae*, as this family contains Gram-negative bacteria with LPS as part of their outer membrane (Frirdich & Whitfield, 2005). These results are surprising and raise the question of whether the Med-based diet increased inflammation in the intestinal environment of these mice or maintained an otherwise "normal" pro-inflammatory status during the neonatal stage. Considering that *Enterobacteriaceae* are among the initial colonizers of the neonatal gut microbiota, priming the gut for subsequent bacterial colonization and driving early immune development (Karlsson et al., 2011), it is possible that the enhancing effect of the Med-based diet on *Enterobacteriaceae* may support normal developmental trajectory of the gut microbiota and the immune system.

Despite the important limitation related to the small sample size of some of the groups for microbiota and USVs assessments, resulting in a high variability of the data, findings from this study provide further support that prenatal stress alters offspring's behaviour and leads to sex-specific brain and intestinal changes in early postnatal life. Specifically, the data showed reduced neonatal USVs (an index of socioemotional behaviour), whereas pro-inflammatory cytokines are increased in the hippocampus of males and in the prefrontal cortex of females, and neurotrophic factor is decreased in the hippocampus of females. Importantly, we showed that maternal Med-based diet limited these neurodevelopmental effects of prenatal stress, mostly in females. Overall, these results suggest that Med-based dietary patterns could improve physiological outcomes in offspring that experienced prenatal adversity, notably in females, highlighting the potential benefits of perinatal dietary interventions for enhancing offspring's health outcomes.

5. Acknowledgements

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6. Author Contributions: MCU and MCA designed the experiment. MCU, ADG, and GL conducted the breeding, dietary, stressor, and behavioral manipulations included in the

experiment (equal contribution). MCU performed the USVs and molecular (RT-qPCR) analyses. MCU and AS conducted the microbiota sequencing analyses and MCU performed the bioinformatics analyses. MCU and MCA interpreted the data and wrote the manuscript, which was edited by all authors.

7. Conflict of Interest Statement: All authors declare that the research work was conducted in the absence of any personal, professional, or financial relationships that could be construed as a conflict of interest.

8. List of Figures

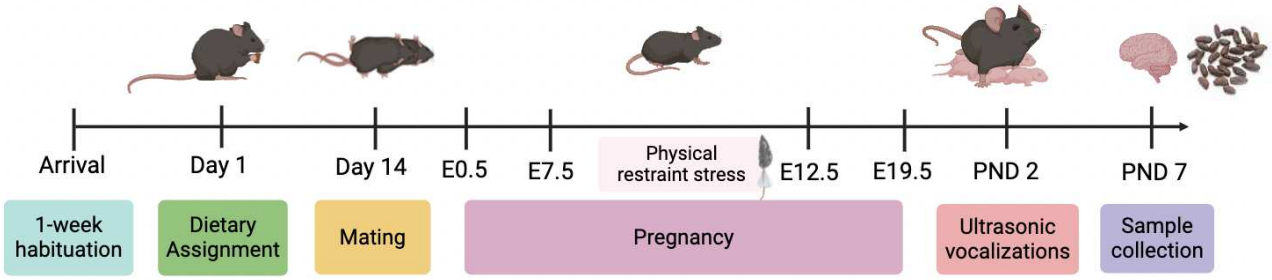

Figure 1. Experimental timeline. Female mice were fed a Control or a Med-based diet and mated with males fed the same diets after two weeks. Pregnant females in each diet group were exposed to a physical restraint stressor during the second trimester (E7.5-12.5) or left undisturbed. Ultrasonic vocalizations of whole litters were measured during a 5-minute isolation period from the dams and then for 5 minutes upon reintroduction of the dam to the housing cage on postnatal day (PND) 2. One female and one male pups from each litter was euthanized for the collection of biological samples on PND 7. "E" refers to embryonic day. Figure created with BioRender.

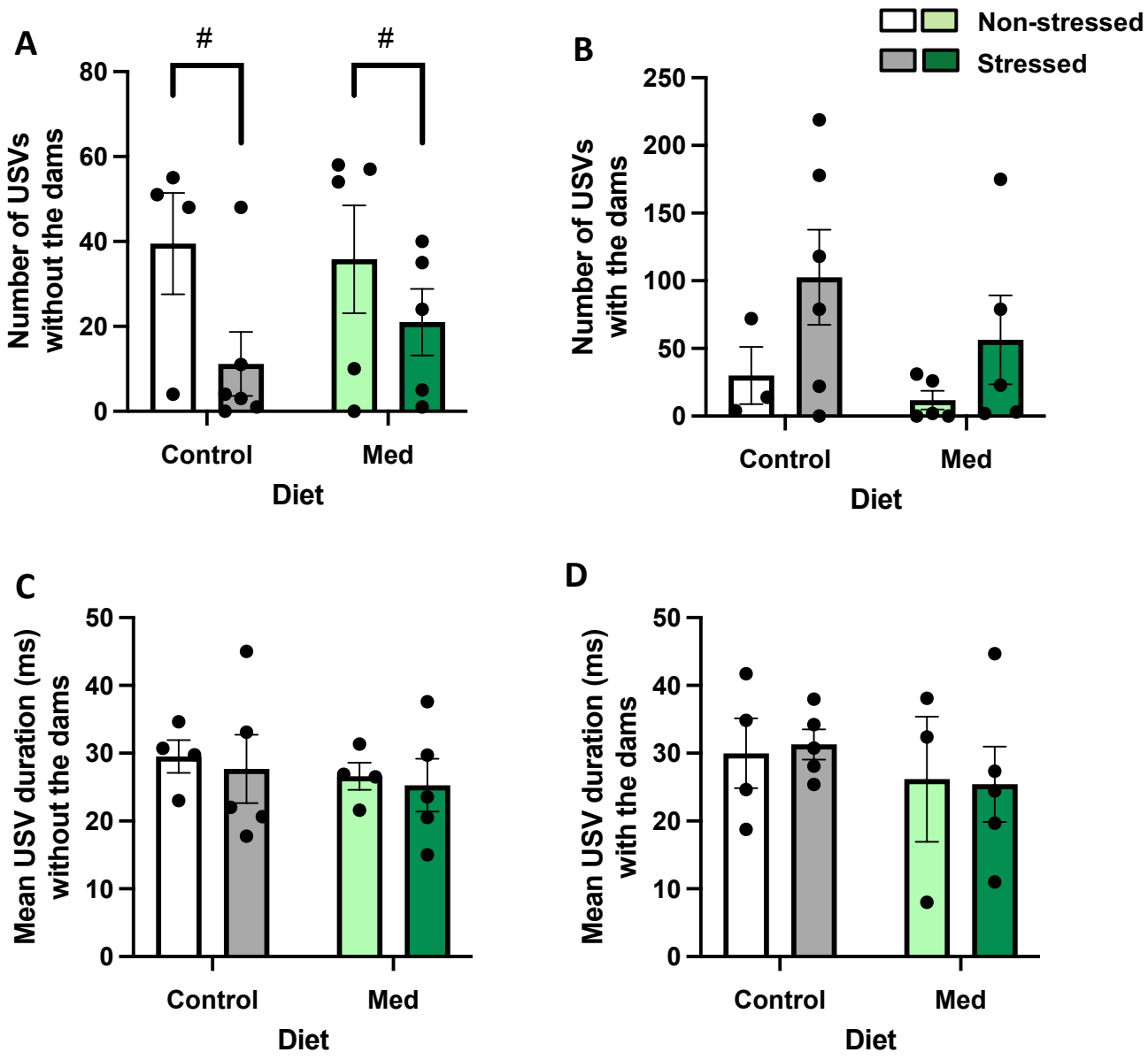

Figure 2. Access to the Med-based diet from preconception onwards did not limit the reductions in ultrasonic vocalisations (USVs) apparent in prenatally stressed litters upon separation from their mother on postnatal day 2. Whole litters born to dams stressed during the second trimester of pregnancy emitted less USVs in the absence of the dams, irrespective of the diet (A), but not upon reintroduction of the dams to the housing cage (B). Litters from the different stressor and diet groups emitted USVs of comparable duration in the absence (C) and presence (D) of the dams. Data was analyzed using 2-way analyses of variance, with Prenatal Stressor (non-stressed vs stressed) and Diet (Control vs Med) as the between-group factors, followed by Bonferroni-corrected multiple comparison *t* tests. Dots represent individual litters, while bars represent group means ± S.E.M. Litters fed the Control diet (non-stressed: n = 3-4, stressed: n = 5-6). Litters fed the Med-based diet (non-stressed: n = 3-5, stressed: n = 5). # $p < 0.05$ relative to litters born from non-stressed dams.

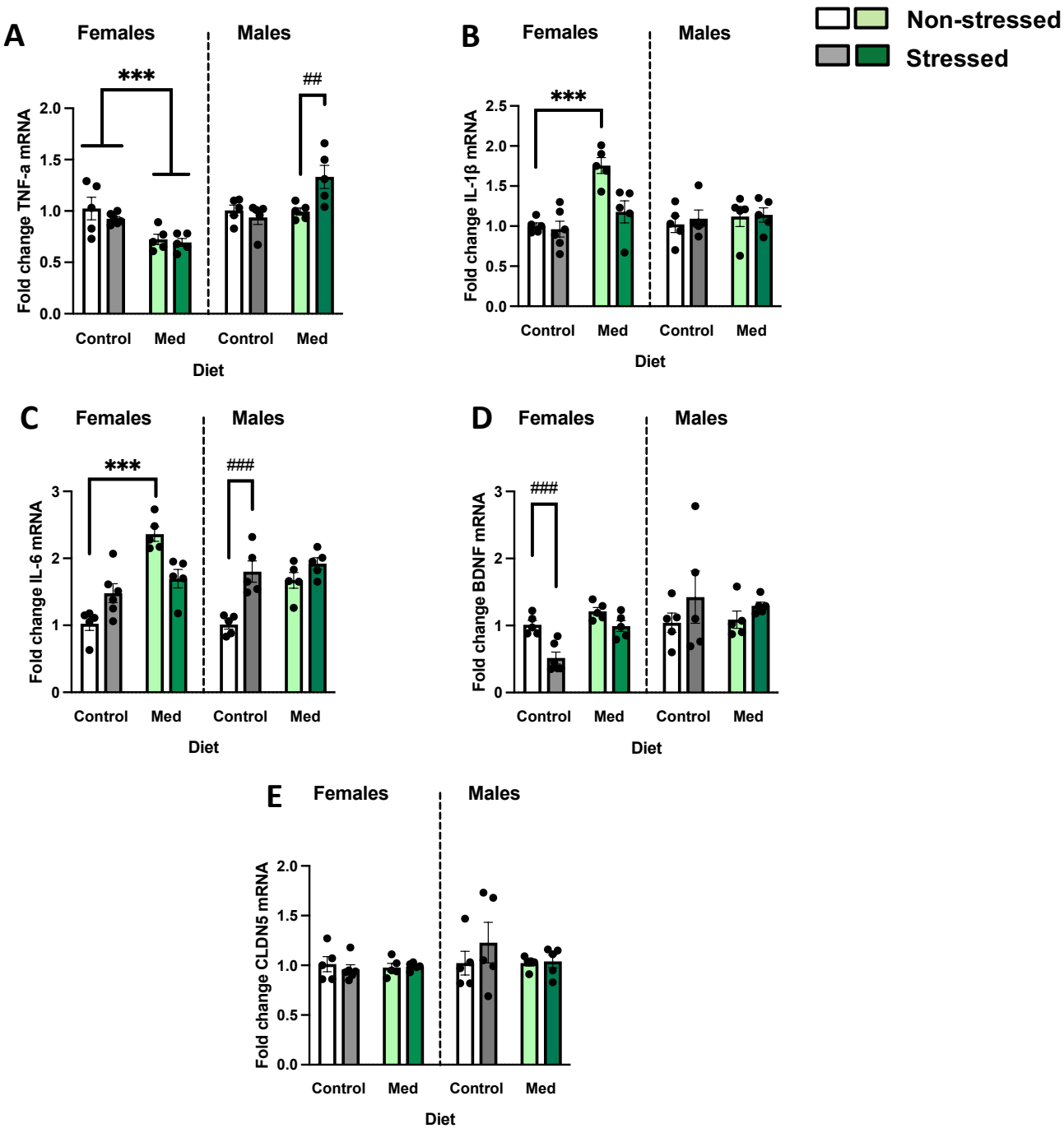

Figure 3. Hippocampal expression of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α (A), interleukin (IL)-1β (B), and IL-6 (C), the neurotrophin brain derived neurotrophic factor (BDNF; D), and the tight junction claudin-5 (E) on postnatal day 7 in female and male pups born to dams fed a Control or a Med-based diet and exposed to a physical restraint stressor or left undisturbed during the second trimester of pregnancy. Data for each sex were analyzed separately using a 2-way analysis of variance, with Prenatal Stressor (non-stressed vs stressed) and Diet (Control vs Med) as the between-group factors, followed by Bonferroni-corrected multiple comparison *t* tests. Dots represent individual mice, while bars represent group means ± S.E.M. Pups fed the Control diet (non-stressed females: $n = 5$, stressed females: $n = 6$; non-stressed males: $n = 5$, stressed males: $n = 5$). Pups fed the Med-based diet (non-stressed females: $n = 5$, stressed females: $n = 5$; non-stressed males: $n = 5$, stressed males: $n = 5$). ## $p < 0.01$ and ### relative to non-stressed pups and *** $p < 0.001$ relative to pups fed the Control diet.

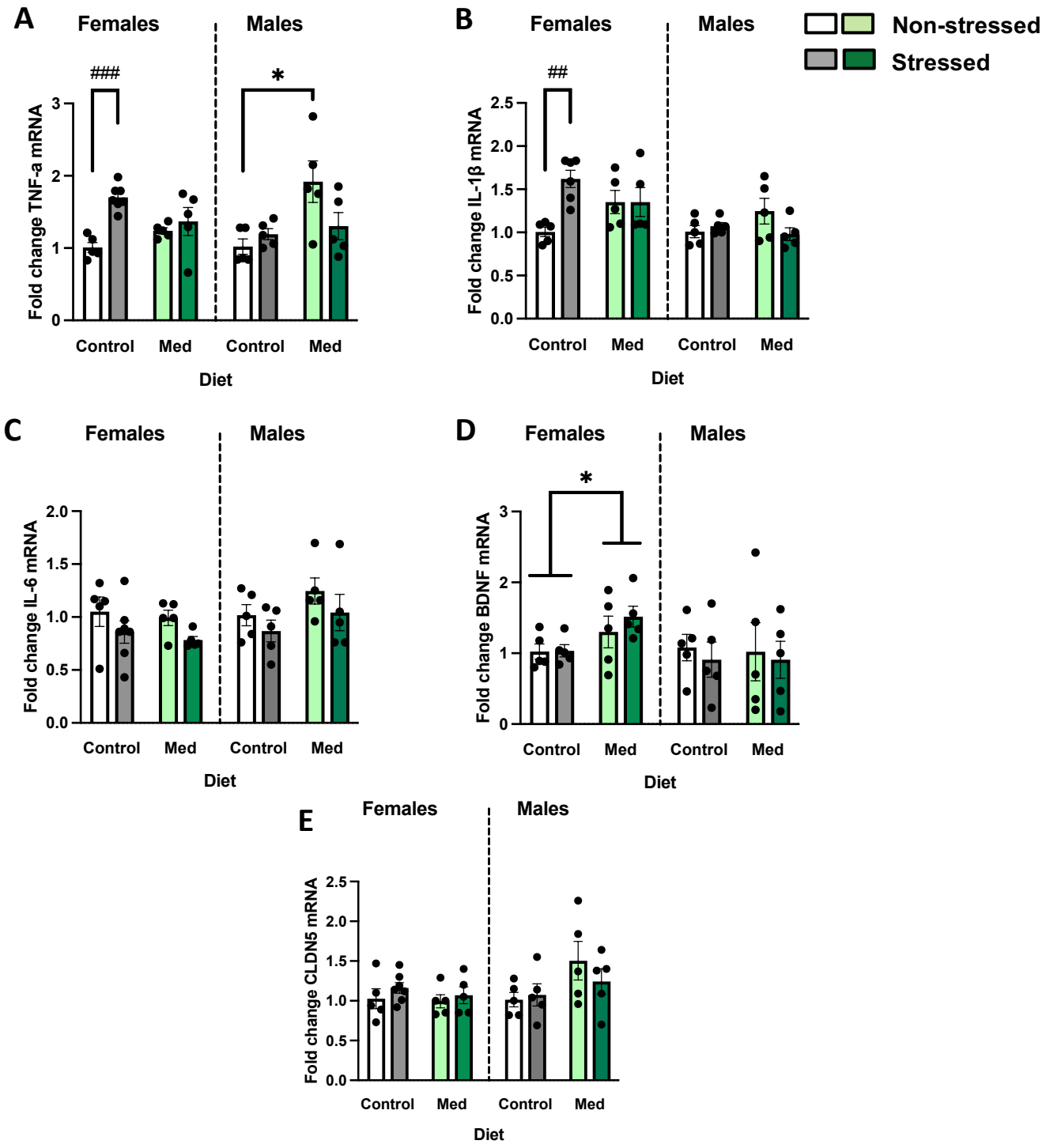

Figure 4. Prefrontal expression of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α (A), interleukin (IL)-1β (B), and IL-6 (C), the neurotrophin brain derived neurotrophic factor (BDNF; D), and the tight junction claudin-5 (E) on postnatal day 7 in female and male pups born to dams fed a Control or a Med-based diet and exposed to a physical restraint stressor or left undisturbed during the second trimester of pregnancy. Data for each sex were analyzed separately using a 2-way analysis of variance, with Prenatal Stressor (non-stressed vs stressed) and Diet (Control vs Med) as the between-group factors, followed by Bonferroni-corrected multiple comparison *t* tests. Dots represent individual mice, while bars represent group means ± S.E.M. Pups fed the Control diet (non-stressed females: $n = 5$, stressed females: $n = 6$; non-stressed males: $n = 5$, stressed males: $n = 5$). Pups fed the Med-based diet (non-stressed females: $n = 5$, stressed females: $n = 5$; non-stressed males: $n = 5$, stressed males: $n = 5$). ## $p < 0.01$ and ### $p < 0.001$ relative to non-stressed pups and * $p < 0.05$ relative to pups fed the Control diet.

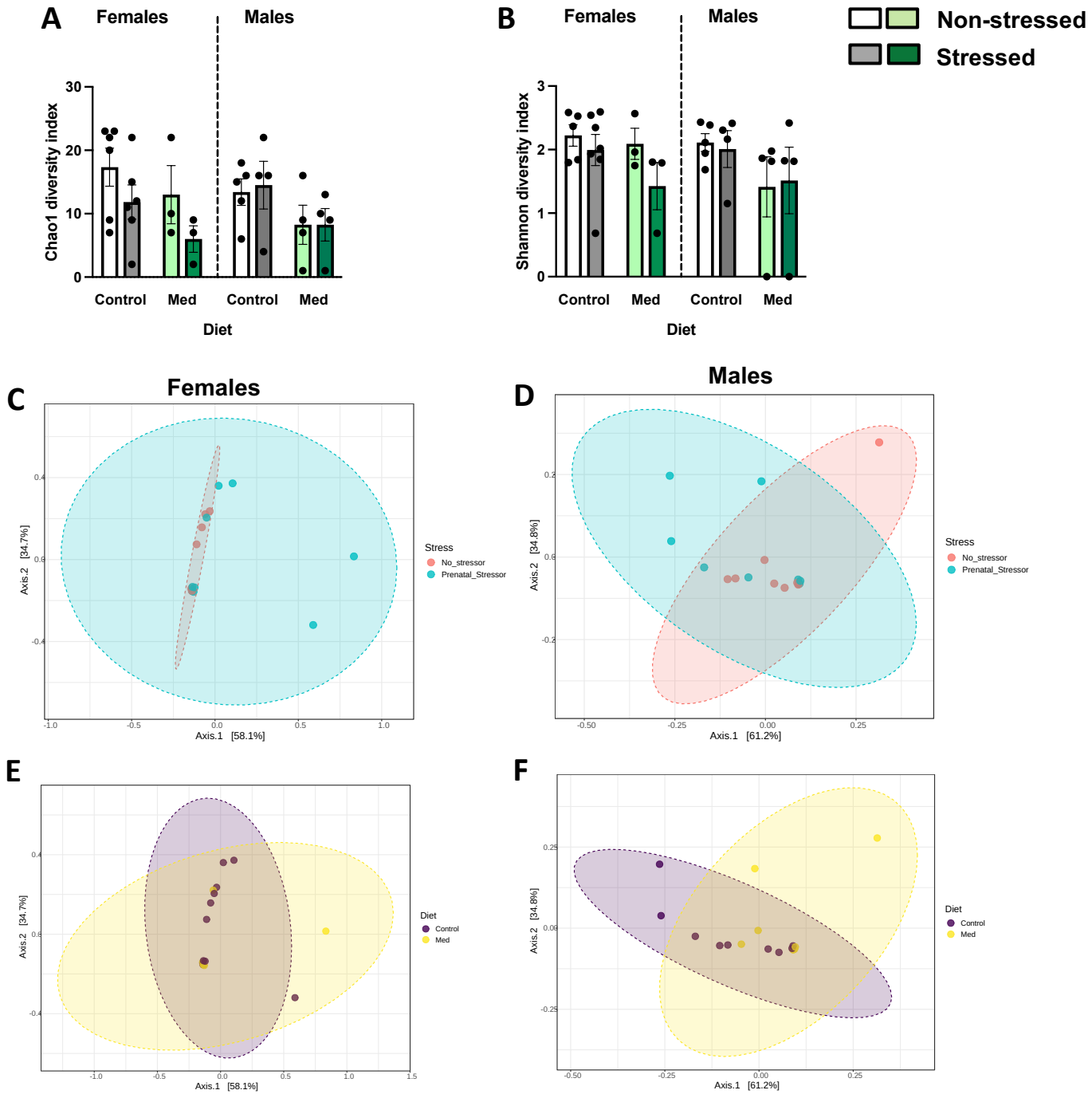

Figure 5. Chao1 (A) and Shannon (B) alpha diversity indices and Bray-Curtis dissimilarity indices as a function of Stress (C and D) and Diet (E and F) of fecal microbiota of female and male pups (postnatal day 7) born to dams fed a Control or a Med-based diet and exposed to a physical restraint stressor or left undisturbed during the second trimester of pregnancy. Chao1 and Shannon diversity indices for each sex were analyzed separately using a 2-way analysis of variance, with Prenatal Stressor (non-stressed vs stressed) and Diet (Control vs Med) as the between-group factors, followed by Bonferroni-corrected multiple comparison *t* tests. Data for beta-diversity index was analyzed using permutational multivariate analysis of variance (PERMANOVA) of Bray-Curtis distance matrices in MicrobiomeAnalyst. Dots represent individual mice, while bars represent group means ± S.E.M. Pups fed the Control diet (non-stressed females: $n = 6$, stressed females: $n = 6$-7; non-stressed males: $n = 5$, stressed males: $n = 4$). Pups fed the Med-based diet (non-stressed females: $n = 3$, stressed females: $n = 3$; non-stressed males: $n = 4$, stressed males: $n = 4$).

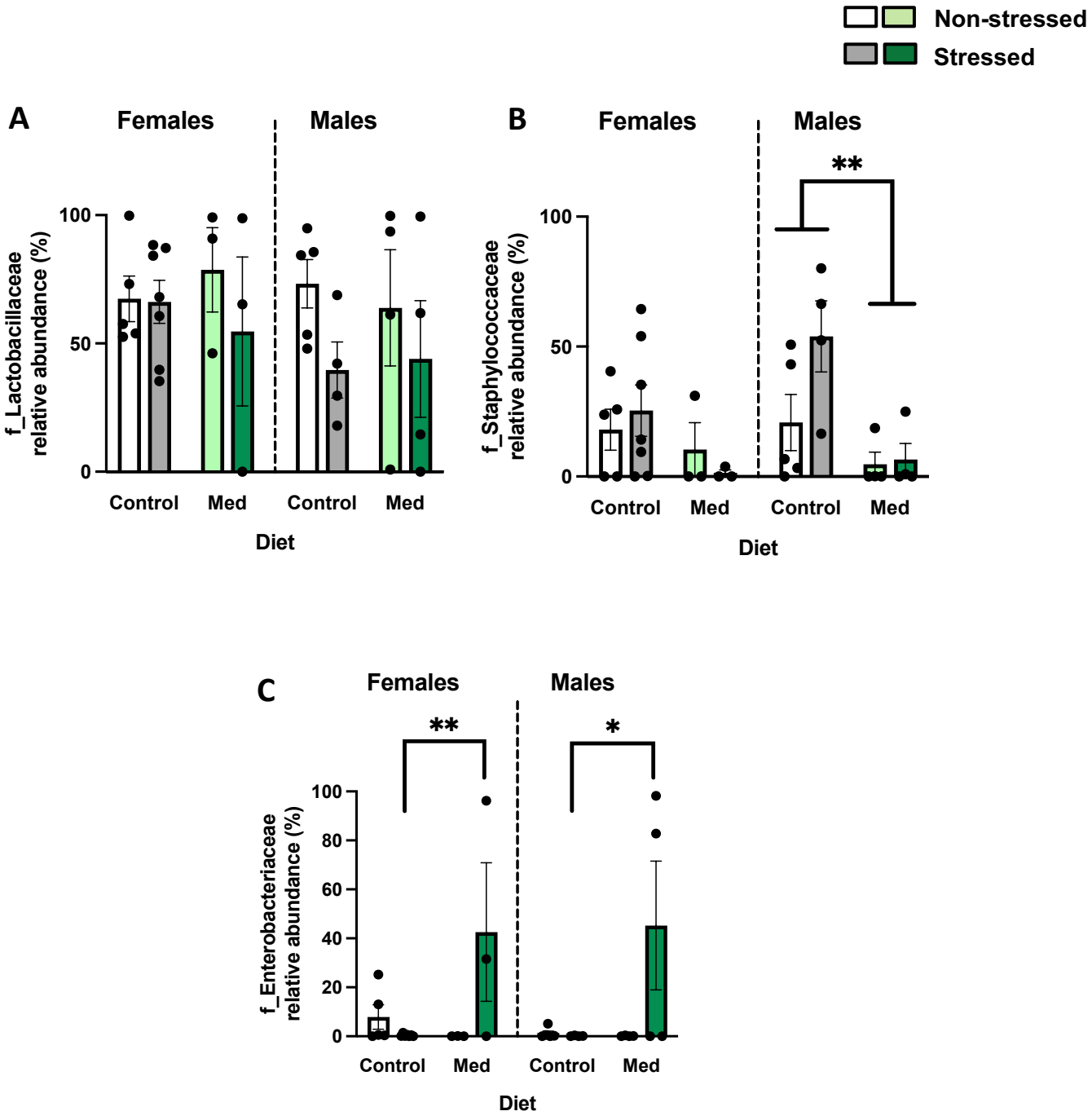

Figure 6. Relative abundance of families in the fecal microbiota of female and male pups (postnatal day 7) born to dams fed a Control or a Med-based diet and exposed to a physical restraint stressor or left undisturbed during the second trimester of pregnancy. Data for each sex were analyzed separately using a 2-way analysis of variance, with Prenatal Stressor (non-stressed vs stressed) and Diet (Control vs Med) as the between-group factors, followed by Bonferroni-corrected multiple comparison *t* tests. Dots represent individual mice, while bars represent group means ± S.E.M. Pups fed the Control diet (non-stressed females: $n = 5$, stressed females: $n = 7$; non-stressed males: $n = 5$, stressed males: $n = 4$). Pups fed the Med-based diet (non-stressed females: $n = 3$, stressed females: $n = 3$; non-stressed males: $n = 4$, stressed males: $n = 4$). * $p < 0.05$ and ** $p < 0.01$ relative to pups fed the Control diet.

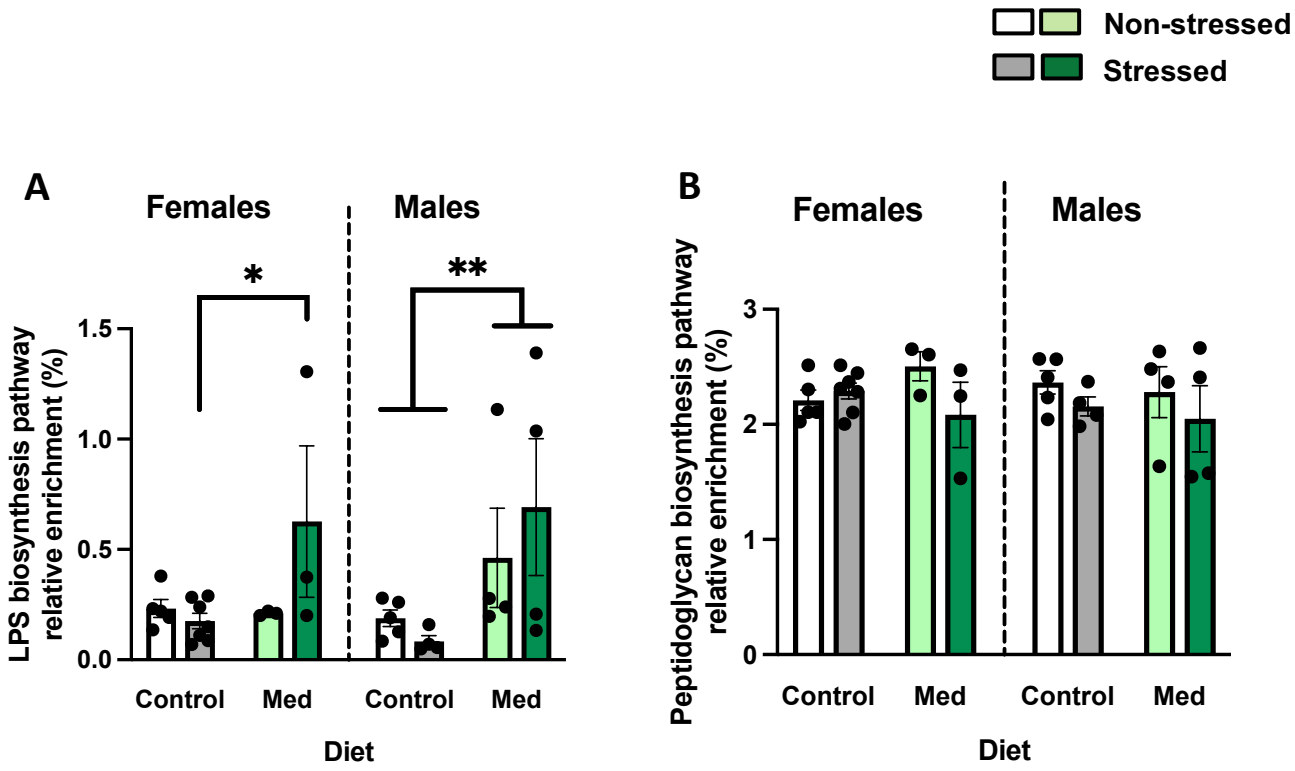

Figure 7. Relative enrichment of LPS (A) and Peptidoglycan (B) biosynthesis pathways in the fecal microbiota of female and male pups (postnatal day 7) born to dams fed a Control or a Med-based diet and exposed to a physical restraint stressor or left undisturbed during the second trimester of pregnancy. Data for each sex were analyzed separately using a 2-way analysis of variance, with Prenatal Stressor (non-stressed vs stressed) and Diet (Control vs Med) as the between-group factors, followed by Bonferroni-corrected multiple comparison *t* tests. Dots represent individual mice, while bars represent group means ± S.E.M. Pups fed the Control diet (non-stressed females: $n = 5$, stressed females: $n = 7$; non-stressed males: $n = 5$, stressed males: $n = 4$). Pups fed the Med-based diet (non-stressed females: $n = 3$, stressed females: $n = 3$; non-stressed males: $n = 4$, stressed males: $n = 4$). * $p < 0.05$ and ** $p < 0.01$ relative to pups fed the Control diet.

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10. Supplementary Data

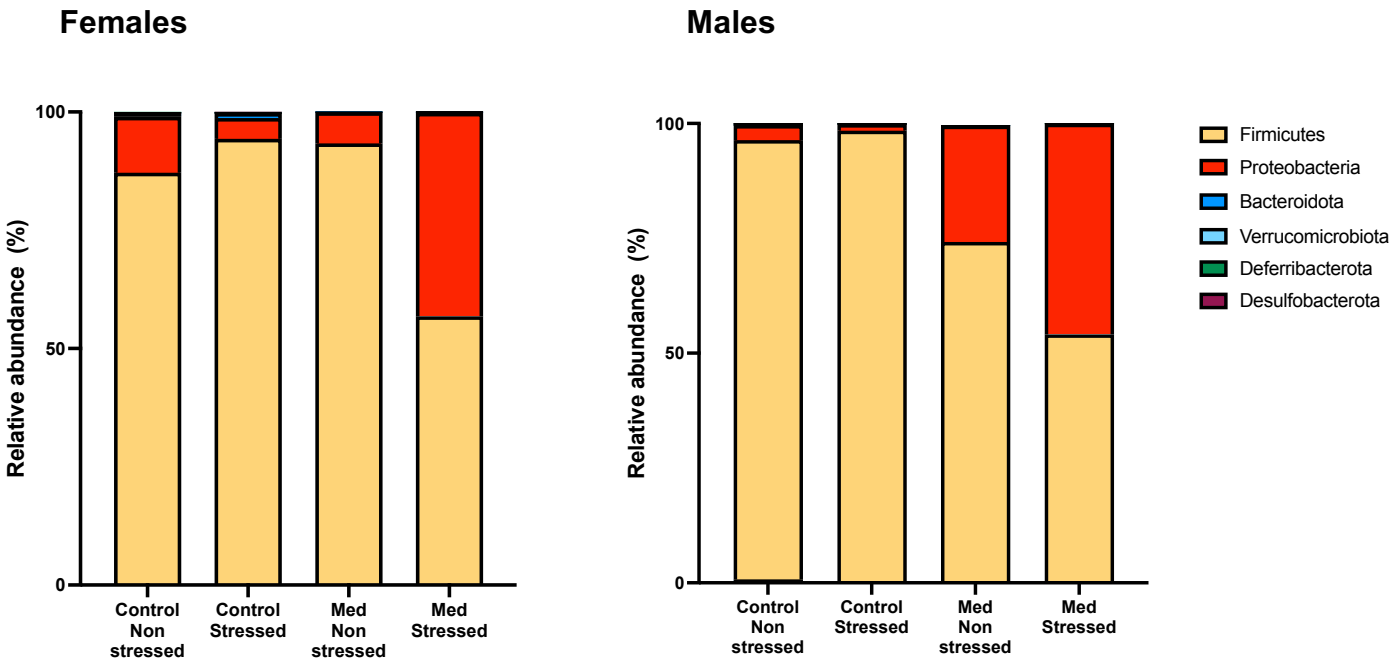

Figure 1. Phyla identified in the fecal microbiota of female and male pups (postnatal day 7) born to dams fed a Control or a Med-based diet and exposed to a physical restraint stressor or left undisturbed during the second trimester of pregnancy.

Supplementary Table 1. Experimental Control and Med-based diet formula

	Open Standard Control diet	Mediterranean-based diet
Ingredients	**gm**	**gm**
Casein	223	80
Fish Protein Isolate	0	18
Egg White	0	9
Beef, Cooked, Powdered, 5013	0	40
L-Cystine	3	3
Corn Starch	467.4	0
Maltodextrin	150	125
Wheat Starch	0	198.5
Chickpeas, Cooked, Dried	0	36
Lentils, Cooked, Dried	0	36
Cellulose, BW200	75	14
Inulin	0	5
Beta-Glucans	0	5
Soybean Oil	70	0
Menhaden Oil (200 ppm tBHQ)	0	9
Butter, Anhydrous	0	5
Flaxseed Oil	0	6.5
Olive Oil	0	105
Walnuts, Dried, Powdered	0	20
t-BHQ	0.005	0.005
Mineral Mix S10026	10	10
Dicalcium Phosphate	13	13
Calcium Carbonate	5.5	5.5
Potassium Citrate, 1 H2O	16.5	16.5
Vitamin Mix V10001	10	10
Biotin (1%)	0	0.014
Choline Bitartrate	2	2
Fruit and Veggie Blend	0	100
Resveratrol (50% Trans Resveratrol)	0	0.045
Total	**1045.405**	**872.064**
Macronutrient composition		
G		
Protein	197.0	156.4
Carbohydrate	552.9	402.1
Fat	72.7	157.8
Cholesterol	0.0	0.06
Total Fiber	75.0	55.0
Insoluble Fiber	75.0	37.8
Soluble Fiber	0.0	18.4
g%		
Protein	18.8	17.9
Carbohydrate	52.9	46.1
Fat	7.0	18.1
Cholesterol	0.0	0.007
Total Fiber	7.2	6.3
Insoluble Fiber	7.2	4.3
Soluble Fiber	0.0	2.1
Kcal		
Protein	*788*	*626*
Carbohydrate	*2212*	*1608*
Fat	*654*	*1420*
Total	***3654***	***3654***
kcal%		
Protein	*22*	*17*
Carbohydrate	*61*	*44*
Fat	*18*	*39*

Supplementary Table 2. Primer sequences used in RT-qPCR experiments.

Gene	Gene sequence/Assay ID
Mus GAPDH	Forward: 5'- GGT CGG TGT GAA CGG ATT TG -3'
	Reverse: 5'- TGC CGT GAG TGG AGT CAT ACT G -3'
Mus Actb	Forward: 5'- GAA CCC TAA GGC CAA CCG TG -3'
	Reverse: 5'- GGT ACG ACC AGA GGC ATA CAG G -3'
Mus TNF-α	Forward: 5'- CTC AGC CTC TTC TCA TTC CTG C -3'
	Reverse: 5'- GGC CAT AGA ACT GAT GAG AGG G -3'
Mus IL-1β	Forward: 5'- TGC CAC CTT TTG ACA GTG ATG -3'
	Reverse: 5'- GTG CTG CTG CGA GAT TTG AA -3'
Mus IL-6	Forward: 5'- ACG GCC TTC CCT ACT TCA CA -3'
	Reverse: 5'- TGC CAT TGC ACA ACT CTT TTC TC -3'
Mus BDNF	Forward: 5'- GTC TCC AGG ACA GCA AAG CCA C -3'
	Reverse: 5'- CCT TGT CCG TGG ACG TTT ACT TC -3'
Mus Claudin-5	Forward: 5'- GGC ACT CTT TGT TAC CTT GAC C -3'
	Reverse: 5'- CAG CTC GTA CTT CTG TGA CAC C -3'

Chapter 6: Sex-specific effects of a Mediterranean-based diet modulated on behavioural, inflammatory, and gut microbiota changes in prenatally stressed adult offspring

This chapter illustrates the third study investigating whether the Med-based diet limited the effects of the prenatal stressor (as in the previous chapter) on anxiety- and depressive-like behaviours, brain and gut inflammatory and neurotrophic factors, and gut microbiota in adult offspring. The sex-specific effects of the prenatal stressor and Med-based diet were also determined. The findings are detailed in a manuscript below prepared for submission shortly.

Sex-specific effects of a Mediterranean-based diet on behaviours, pro-inflammatory and tight junction markers, and gut microbiota in prenatally stressed adult mouse offspring

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Abstract

Maternal stress during pregnancy can predispose the offspring to mental health disorders. These neuropsychiatric effects could partly result from alterations in the development of brain inflammatory processes and in the postnatal establishment of the gut microbiota. Through their actions on these systems, early-life dietary patterns could potentially reduce the risk for neuropsychiatric disorders associated with prenatal stress. This study examined whether a Mediterranean (Med)-based diet from preconception onward attenuated behavioural, pro-inflammatory, and gut microbiota responses to a prenatal stressor in adult female and male mouse offspring. Pregnant C57BL/6N mice fed a Control diet or a Med-based diet experienced a physical restraint stressor during the second trimester or were left undisturbed. Female and male offspring had their anxiety- and depressive-like behaviours assessed on postnatal days (PNDs) 67 to 69, and different sections of their brains and intestines collected on PND 70. The Med-base diet limited anxiety-like behaviours, increases in the pro-inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β in the colon or hippocampus, and changes in the microbiota elicited by the prenatal stressor and increased tight junctions in the hippocampus in females only. These findings suggest that early-life adherence to Med-based dietary patterns could promote positive mental health in the context of prenatal adversity, potentially through the actions of the diet and its components on inflammatory and barrier function processes in the brain and intestinal environments, particularly in females.

Keywords: behaviour, gut microbiota, Mediterranean diet, mental health, mouse model, neuroinflammation, adult offspring, prenatal stress, sex differences

1.0 Introduction

Prenatal adversity can alter offspring's development, with impacts on health, including that in the mental domain (O'Donnell & Meaney, 2017; Seckl & Holmes, 2007). Generalized anxiety and major depression have been linked to stressful experiences experienced by the mother during pregnancy (Van den Bergh et al., 2020). In rodents, adult offspring born to dams stressed during pregnancy exhibited social impairments and phenotypes characteristic of depression and anxiety (Weinstock, 2008, 2017). Although the mechanisms underlying the effects of prenatal stress on offspring's mental health have yet to be entirely elucidated, it has been suggested that alterations in inflammatory processes could underlie some of the neuropsychiatric outcomes (Roshan-Milani et al., 2021). Plasma levels of C-reactive protein and pro-inflammatory cytokines were higher in adolescents or young adults whose mothers experienced psychological distress during pregnancy (Plant et al., 2016; Veru et al., 2015). In addition to increases in systemic and brain inflammation during the fetal stage (Chen et al., 2020; Gur et al., 2017; Ślusarczyk et al., 2015), prenatally stressed rodents had higher microglial activation and mRNA expression of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 in the hippocampus or prefrontal cortex, as well as sex-specific social impairments and increased anxiety- and depressive-like behaviours in adulthood (Diz-Chaves et al., 2012, 2013; Gur et al., 2017, 2019; Laviola et al., 2004; Ślusarczyk et al., 2015). Collectively, this suggests that prenatal adversity could alter neuroimmune development and potentially predispose the offspring to psychopathologies (Deverman & Patterson, 2009; Jiang et al., 2018), especially as pro-inflammatory activation has been reported in the post-mortem brains of individuals with major depression and generalized anxiety (Enache et al., 2019; Hou et al., 2017; Osimo et al., 2020).

Changes in neuroinflammatory processes due to intrauterine stress could also alter neurovascular development. Whether these changes resulted from alterations in neurovascular and/or neuroimmune development is uncertain, but fetal and neonatal offspring exposed to psychological or immunological stressors *in utero* exhibited decreased pericyte coverage of vascular endothelial cells alongside neuroinflammation (Gomez-Gonzalez & Escobar, 2006; Lu et al., 2023; Zhao et al., 2022). Additionally, the finding that the tight junction claudin-5 was decreased in the post-mortem hippocampi and nucleus accumbens of major depressive individuals (Dion-Albert et al., 2022; Greene et al., 2020; Menard et al., 2017) and that BBB impairments in prenatally stressed mice were linked to anxiety-like behaviours (Zhao et al., 2022) further support the view that increases in BBB permeability, in conjunction with neuroinflammation, could predispose or contribute to mental health disturbances.

It has been suggested that disturbances in neuroinflammatory and neurovascular processes could stem from alterations in the gut microbiota (Braniste et al., 2014). In line with the view that brain inflammatory changes elicited by prenatal adversity could result from disruptions in the developmental trajectory of the offspring's gut microbiota, with impact on mental health, prenatally stressed mice that exhibited compositional shifts in their gut microbiota both in early life and adulthood (including overrepresentation of taxa with pro-inflammatory potential such as Proteobacteria and decreases in taxa linked with anti-inflammatory outcomes such as *Lactobacilli* and *Bifidobacteria*) also had higher cortical IL-6 expression, less social interactions with conspecifics, and more depressive- and anxiety-like behaviours as adults (Chen et al., 2020; Gur et al., 2017, 2019; Zhang et al., 2021). Furthermore, non-stressed mice that received the fecal microbial transplant of those that were prenatally stressed exhibited increases in IL-1β and IL-6 and reduced occludin and claudin-3 in the colon (Sun et al., 2021). Such increases in intestinal

inflammation and downregulation of intestinal tight junctions could facilitate bacterial and/or LPS translocation to epithelium and the bloodstream, leading to release of pro-inflammatory molecules that could increase neuroinflammation by influencing inflammatory pathways in the gut brain axis, with potential impacts on mental disease risk (Audet, 2021).

Accumulating studies indicate that diet may influence mental health, potentially by modulating inflammatory processes and the gut microbiota (Marx et al., 2020). Dietary components such as omega-3 fatty acids and polyphenols have been shown to mitigate intestinal and brain inflammation (Layé et al., 2018; Romier et al., 2009), and dietary fibers stimulated the growth of bacteria that produce the anti-inflammatory short-chain fatty acids (Silva et al., 2020). Also, given that diet is critical in neurodevelopment, including neuronal and glial proliferation, synaptogenesis, and myelination (Prado & Dewey, 2014), and is one of the major contributors to postnatal development of the gut microbiota (Bergström et al., 2014), early-life dietary modifications could help modify the effects of prenatal stress in the brain as well as support healthy gut microbiota establishment, potentially improving mental health outcomes in the offspring. Mediterranean (Med) dietary patterns have been related to beneficial impacts on overall health, including mental health (Lassale et al., 2019). The diet is abundant in whole grains, legumes, olive oil, fruits, vegetables, nuts, and seeds, which comprise bioactive components such as omega-3 fatty acids, polyphenols, and fiber that have been linked to positive mental health (Trichopoulou et al., 2014). Notably, interventions based on the Med diet reduced symptom severity in major depressive individuals, highlighting the therapeutic prospects of this diet for depression (Jacka et al., 2017). In the context of pregnancy, children whose mothers adhered to dietary patterns based on the Mediterranean (Med) diet during pregnancy were less likely to develop depression and anxiety (House et al., 2018) or have cognitive deficits and socioemotional problems (Crovetto et

al., 2023), suggesting that the Med diet could improve mental health outcomes in the offspring. Additionally, the findings that adherence to Med diet promotes the abundance of gut bacteria that produce short-chain fatty acids (De Filippis et al., 2016; Miller et al., 2021; Mitsou et al., 2017), lower abundance of pro-inflammatory bacteria (Mitsou et al., 2017), and lower levels of circulating pro-inflammatory markers (Hart et al., 2021) provide support that the diet could mitigate the effects of prenatal stress on inflammatory processes and gut microbiota in the offspring.

Here, we investigated whether a diet based on human Med dietary patterns (described in Udechukwu et al, in preparation) mitigated the development of anxiety- and depressive-like behaviours in offspring exposed to a prenatal stressor. To examine whether the Med-based diet changed brain and intestinal inflammatory and BBB markers and the gut microbiota, we analyzed changes in the mRNA expression of pro-inflammatory and tight junction markers in the dorsal hippocampus, prefrontal cortex, and colon (based on reports that prenatal stress altered inflammatory processes or tissue barrier function in these brain and intestinal regions (Diz-Chaves et al., 2012; Sun et al., 2021; Zhao et al., 2022) and determined cecal microbiota diversity, composition, and predicted metabolic activity. Importantly, because of the sex differences in the outcomes of prenatal stress (Glover & Hill, 2012), in the pathophysiology and prevalence of mental disorders (Bangasser & Valentino, 2014; Kessler, 2003), and in antidepressant treatment response (Seifert et al., 2021), we assessed these outcomes in both female and male offspring. Ultimately, we hope that the findings would inform future development of targeted and effective dietary interventions for mental health promotion.

2. Materials and methods

2.1 Animals

Naïve female (6-8 weeks) and male (7-9 weeks) C57BL/6N mice (Charles River Laboratories, Montréal, QC, Canada) were housed singly in 19 cm x 29 cm x 13 cm polycarbonate N10 mouse cages (Ancare), enriched with a cotton nestlet, a cardboard house, and standard woodchip bedding. The housing room was maintained at a 12-h light-dark cycle (lights on 0700-1900 h), a temperature of 21-23 °C, and 30-50% humidity. Food and water were provided *ad libitum* throughout the study. All experimental procedures were approved by the Animal Care Committee at the University of Ottawa (animal protocol number: HSe-3149), according to the guidelines of the Canadian Committee of Animal Care.

2.2 Summary of experimental procedures

The experimental timeline is presented in Figure 1. Upon their arrival, all mice were fed a modified formula of an Open Standard Diet (D12052701M, Research Diets Inc.), which was considered the Control diet, and were left undisturbed for one week to acclimate to the housing environment. Following acclimation, female and male mice were randomly selected to remain on the Control diet (n = 10 females and 5 males), while the rest was assigned to the Med-based diet (n = 10 females and 5 males). After two weeks on their respective diets, females and males fed the same diet were paired for breeding. In the second trimester (Embryonic days [E] 7.5-12.5), pregnant females from each diet group were randomly assigned to a physical restraint stressor or were not manipulated (n = 5/group). Pups were removed at Postnatal day (PND) 7 for different assessments not included in this report. The remaining pups were weaned on PND 21 and housed in pairs of same-sex littermates until adulthood, with free access to food identical to that of their mothers and water. Behavioural were tested in 10 females and 10 males per group, with no more than 2 mice/sex from the same litter. Behavioural tests were conducted on PNDs 67 to 69, followed

by euthanasia and sample collection on PND 70, approximately 20-24 hours after the last behavioural test.

2.3 Experimental diets

The Control and Med-based diets were developed as previously described (Udechukwu et al., in preparation). Briefly, an Open Standard Diet (D12052701M, Research Diets Inc.) was modified to develop the Control diet by adding extra casein to increase the protein content to approximately 17% in line with mouse breeding requirements (Nutrition, 1995). The Med-based diet was developed by modifying an available mouse Med-based dietary formula (D12052702, Research Diets Inc.; Barrington et al., 2018). The main modifications included adding ingredients present in human Med dietary patterns, including olive oil, chickpeas, lentils, and walnuts (processed in our laboratory and analyzed for their nutritional content; Intertek, Saskatoon, Canada), fruits and vegetables (generously supplied by FutureCeuticals, Inc., Momence, Illinois, United States), as well as oat beta-glucan and trans-resveratrol (PureBulk, Roseburg, Oregon, United States). The experimental diets were isocaloric, but the Med-based diet had different macronutrient (fat, protein, and carbohydrates) contribution to calories according to human dietary patterns (Davis et al., 2015).The dietary formulas are provided in Supplementary Table 1.

2.4 Breeding procedures

Mice were bred following the Trio breeding scheme (Braden et al., 2017). Briefly, estrus was induced by placing nestlets and bedding from a male's housing cage into the cage of two individually housed females. Twenty-four hours later, these two females were introduced into the male's housing cage and left overnight for breeding, after which they returned to their housing cages. Females in which a vaginal copulation plug was detected, which was designated as E0.5, were monitored every three days to examine pregnancy status through body weight measurements.

Pregnant dams were left undisturbed, except for body weight measurements, food, cage, and water bottle replacements, or occasional blood and fecal sample collection for another experiment beyond the scope of the present study.

2.5 Prenatal stressor procedures

The prenatal stressor was comprised of a validated physical restraint stressor paradigm in the second trimester (Buynitsky & Mostofsky, 2009). This stressor was chosen based on preliminary findings from our lab and others that it altered USVs (Laloux et al., 2012; Zimmerberg & Blaskey, 1998), increased pro-inflammatory cytokines (Diz-Chaves et al., 2012; Gur et al., 2019) and decreased BDNF (Jia et al., 2015; J. Liu et al., 2011) in the hippocampus and prefrontal cortex, and altered the gut microbiota (Golubeva et al., 2015; Gur et al., 2017, 2019; Zhang et al., 2021) in the offspring. The second trimester was selected as it corresponds to the period during which the mouse hippocampal and prefrontal cortical structures begin to develop (Kolk & Rakic, 2022; Semple et al., 2013) and as previous studies have shown that the offspring is more vulnerable to stress occurring in early trimesters (Charil et al., 2010; Herbison et al., 2017). The procedure was conducted by placing pregnant females in a triangular plastic bag with a small opening at the nose end to permit breathing and a tape at the tail end to restrict movement. The procedure was conducted for six consecutive days at 9:00, 12:00, and 15:00 h, with each session lasting 30 minutes, and during which breathing and proper limb positioning were constantly monitored (Gur et al., 2017).

2.6 Behavioural tests

2.6.1 Behaviours suggestive of anxiety-like phenotypes

Behaviours indicative of anxiety-like states were tested using the elevated plus maze and open field tests, which both assess fear of open and bright spaces (Lezak et al., 2017). The open

field test was conducted on PND 67 in an apparatus consisting of an open arena made of white opaque acrylic plastic floor and walls, measuring 45 x 45 x 45 cm (Canus Plastics Incorporated). After habituation to the testing room for 1 hour under room lighting of approximately 300 lux, mice were placed individually in the top-right corner of the arena, and their movements were recorded using an overhead ceiling mounted camera and tracked for 5 minutes using the EthovisionXT video tracking software (Noldus, version 11.5). The number of entries and time spent in the center (15 x 15 cm) and corners (10 x 10 cm each) of the arena as well as the total distance travelled were used as measures of anxiety-like behaviour. The elevated plus maze test was done on PND 69 in a testing apparatus consisting of a black acrylic plastic material, with two perpendicularly crossed open (6-cm wide x 75-cm long) and closed (6-cm wide x 75-cm long x 20-cm high) arms raised 74 cm above the floor. Following habituation to the test room for 1 hour under room lighting of approximately 100 lux, mice were placed in the center of the maze facing the intersection of an open and closed arm. Movements were tracked for 5 minutes using an overhead ceiling mounted camera and a video tracking software (EthoVisionXT, Noldus, version 11.5). Anxiety-like behaviours were determined as the number of entries and time spent in the open and closed arms. The open field and the maze were cleaned between trials using a Quato 78 Plus solution (Swish Maintenance Limited).

2.6.2 Behaviours suggestive of depressive-like phenotypes

Depressive-like behaviours were determined using the tail suspension test, which assesses passive coping behaviours (Cryan et al., 2005). The test was conducted on PND 69 approximately 2 hours after the elevated plus maze test. The test apparatus (Med Associates Inc) consisted of a tail suspension interface cabinet (DIG-735), a load cell amplifier hardware (ENV-505TS), and an associated user interface software (Tail Suspension SOF-821). After habituation for 1 hour under

room lighting of approximately 100 lux, the mouse was hung upside down, with the tail anchored to an elevated aluminum bar with a small piece of surgical tape. The aluminum bar was connected to a strain gauge, which automatically detected movements. Mice were left suspended for 6 minutes, after which the time spent immobile (automatically calculated by the associated user interface software) was used as a measure of passive coping behaviour.

2.7 Euthanasia and sample collection

Mice were euthanized by rapid decapitation on PND70, approximately 15 hours after the last behavioural test. Whole brains were removed immediately from the skull, allowed to freeze on a sheet of Parafilm® M placed on dry ice and wrapped in the Parafilm® M sheet once frozen. In parallel, the gastrointestinal tract was rapidly extracted from the abdominal cavity and positioned on a nuclease-free frozen surface. Cecum contents and whole colons (cut into small pieces) were collected in nuclease-free cryotubes and flash-frozen in liquid nitrogen. All samples were stored at -80 ºC until further analyses. Frozen brains were later dissected using the cryochamber of a ThermoFisher HM525 NX cryostat maintained at -20 ºC. Briefly, each whole brain was placed in a stainless-steel brain matrix (2.5 x 3.75 x 2.0 cm; slots spaced approximately 500 µm apart) positioned on an ice plate. Brains were sectioned coronally using razor blades guided by the matrix slots. Dorsal hippocampi and prefrontal cortices were dissected from the coronal sections following the Franklin and Paxinos mouse atlas (Franklin & Paxinos, 1997), placed into nuclease-free microcentrifuge tubes and stored at -80 ºC until subsequent analyses.

2.8 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Dorsal hippocampi, prefrontal cortices, and colons were homogenized using TRIzol, and their total RNA was extracted according to the manufacturer's instructions (Invitrogen, Burlington, Canada). RNA concentration and purity of the samples were determined using a NanoDrop™ One

Spectrophotometer (ThermoFisher Scientific). Samples with 260/80 and 260/230 ratios between 1.80 and 2.20 were included and thus the sample size and degrees of freedom associated with hippocampal, prefrontal, and colonic gene expression differ from other outcomes. Total RNA was reverse transcribed into complementary DNA (cDNA) using iScript™ Reverse Transcription Supermix (Bio-Rad, Canada) and a T100 Thermal Cycler (Bio-Rad, Canada). Aliquots of cDNA were analyzed for the gene expression of TNF-α, IL-1β, IL-6, occludin, and claudin-2, 3, and 5 in duplicates of simultaneous quantitative polymerase chain reactions using the SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad, Canada) and CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Canada). Primers that amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Beta-Actin (Actb) were used as reference genes and their geometric mean was used to normalize the expression of the target genes. Fold changes for the mRNA expression for each target gene were calculated using the $2^{-\Delta\Delta CT}$ method, relative to the Control diet group (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). Primer sequences are provided in Supplementary Table 2.

2.9 Cecal microbiota analyses

2.9.1 16S ribosomal RNA (rRNA) gene sequencing

Cecal DNA was extracted using a Stool Nucleic Acid Isolation Kit, as instructed by the manufacturer (Norgen Biotek Corp, Thorold, Canada). DNA concentration and purity of the samples were assessed using Quant-iT™ PicoGreen (Invitrogen). The 16S rRNA gene amplicons were prepared following the Illumina 16S library preparation procedures. The V3 and V4 hypervariable regions of the 16S rRNA gene were amplified using the primers S-D-Bact-0341-b-S-17 (F: 5' TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG) and S-D-Bact-0785-a-A-21 (R: 5' GTC TCG TGG GCT CGG AGA TGT GTA TAA

GAG ACA GGA CTA CHV GGG TAT CTA ATC C) (Klindworth et al., 2013b). The resulting amplicons were tagged with Illumina nucleotide sequencing adapters and dual-index barcodes for Illumina MiSeq compatibility and sample identification, respectively. All samples were pooled into a library, which was sequenced on a MiSeq system using a 600-cycle MiSeq Reagent Kit v3, following the manufacturer's directions (Illumina, San Diego, CA, USA). The resulting sequence data was processed using QIIME 2 (Bolyen et al., 2019). Paired-end sequence reads that passed through a median quality score of $Q \geq 30$ were denoised, filtered, and rarified using DADA2 (Callahan et al., 2016). All samples were above the retention threshold of 10,000 reads. Alignment of reads to taxa was done using the SILVA database (Quast et al., 2013), after which relative abundances at the phylum, family, and genus taxonomic levels were calculated for each sample. The QIIME2 data output was further analyzed using MicrobiomeAnalyst (Chong et al., 2020) to determine the Chao1 and Shannon alpha diversity indices and the Bray-Curtis dissimilarity beta-diversity index, which was calculated using Bray-Curtis distance and visualized using Principal Coordinate Analysis.

2.9.2 In silico prediction of bacterial metabolic activity

Bacterial metabolic activity was inferred from the 16S rRNA gene sequence data using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) (Douglas et al., 2020). The abundance of Operational Taxonomic Units (OTUs) was processed to obtain a catalog of Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs and pathways (Kanehisa et al., 2012), from which the relative enrichment of KEGG orthologs and KEGG pathways were calculated for each sample. Based on *a priori* hypotheses that the Med-based diet would increase microbial short chain fatty acid production and reduce pro-inflammatory processes (Seethaler et al., 2022), KEGG pathways related to fatty acid biosynthesis,

lipopolysaccharide biosynthesis, peptidoglycan biosynthesis, and NOD-like receptor signaling were examined.

2.10. Statistical analyses

Statistical analyses were performed in SPSS version 29.0.2.0, and data were graphed in GraphPad Prism version 10.1.0. Data were first tested for normality (Shapiro-Wilk test) and homogeneity of variances (Levene test). Data for each sex were analyzed separately using a series of two-way ANOVA with Prenatal Stressor (non-stressed vs stressed) and Diet (Control vs Med-based) as the between-group factors. Follow-up comparisons of the simple effects comprised *t* tests with a Bonferroni correction. Correlation analyses were done using Pearson (parametric) or Spearman (non-parametric) correlation coefficients for each sex separately between the behavioural metrics changed by the prenatal stressor and the Med-based diet and the biological markers examined. The alpha level was set to $p<0.05$ for all analyses, except for correlations where the alpha level was set at $p<0.01$, considering the higher number of variables considered.

3.0 Results

3.1 The Med-based diet attenuated anxiety- and depressive-like behaviours in females only

As shown in Figure 2, the Med-based diet counteracted the increase in anxiety-like behaviours in the elevated-plus maze elicited by the prenatal stressor, decreased anxiety-like behaviours in the open field irrespective of prenatal experiences, and improved passive coping behaviours in prenatally stressed mice in the tail suspension test, exclusively in females. Specifically, although the Prenatal Stressor x Diet interaction for the time spent in the open arms of the elevated plus maze in females did not reach significance ($F_{(1,35)} = 3.155$, $p = 0.084$), follow-up comparisons of the simple effects comprising this interaction were conducted based on the a *priori* hypothesis that Diet would limit the behavioural impacts stemming from the Prenatal

Stressor. Analyses of the simple effects revealed that the reductions in the time spent in the open arms of the maze in prenatally stressed females fed the Control diet (p = 0.047) were not apparent in those fed the Med-based diet (Fig. 2A). In the open field test, both non-stressed and stressed females fed the Med-based diet spent more time in the center of the arena compared to their counterparts fed the Control diet ($F_{(1,34)}$ = 7.866, p = 0.008; Fig. 2B). Lastly, the time spent immobile in the tail suspension test in females varied as a function of the Prenatal Stressor × Diet interaction ($F_{(1,37)}$ = 5.487, p = 0.025), whereby follow-up comparisons confirmed that the Med-based diet decreased this behavioural measure in prenatally stressed mice only (p = 0.006; Fig 2C).

In addition to the main indicators of fear-related and passive coping behaviors, follow-up comparisons of the simple effects comprising the Prenatal Stressor x Diet interaction ($F_{(1,35)}$ = 4.278, p = 0.046) showed that non-stressed females fed the Med-based diet spent less time in the open field corners (p = 0.040), and that both non-stressed and stressed females fed this diet visited these corners less frequently ($F_{(1,36)}$ = 6.147, p = 0.018; Supplementary Fig. 1E and 1F). In contrast, in males the Med-based diet only decreased entries in the open field center in non-stressed offspring (p = 0.024; Prenatal Stressor x Diet: $F_{(1,34)}$ = 4.504, p = 0.041) and decreased the number of entries in their corners in both non-stressed and stressed offspring (Diet: $F_{(1,34)}$ = 4.938, p = 0.033; Supplementary Fig. 1C and 1F). Lastly, behavioural measures in the elevated plus maze and tail suspension test were not affected by the Prenatal Stressor, Diet, or their interaction (p > 0.05, Fig. 2A, 2C, and Supplementary Fig. 1A-B).

3.2 The Med-based diet limited stress-induced elevations of pro-inflammatory cytokines and promoted tight junction expression in the brain and colon, mostly in females

3.2.1 Hippocampus

The mRNA expression of TNF-α in the hippocampus varied as a function of the interaction between Prenatal Stressor and Diet in both females ($F_{(1,30)} = 5.864$, $p = 0.022$) and males ($F_{(1,24)} = 11.491$, $p = 0.002$) and was affected by Prenatal Stressor in females only ($F_{(1,30)} = 6.506$, $p = 0.016$). Follow-up comparisons revealed that the TNF-α increases in prenatally stressed females ($p = 0.001$) and males ($p = 0.007$) fed the Control diet were not apparent in those fed the Med-based diet (Fig. 3A). The other pro-inflammatory and tight junction markers were not affected by whether mice experienced stress *in utero* but differed as a function of Diet or its interaction with Prenatal Stressor in each sex. Specifically, the Med-based diet increased IL-1β ($p = 0.002$; interaction: $F_{(1,30)} = 6.714$, $p = 0.015$; Fig. 3B) and claudin-5 ($p = 0.026$; interaction: $F_{(1,31)} = 7.353$, $p = 0.011$; Fig. 3C) in non-stressed females and increased IL-6 in non-stressed males ($p = 0.018$; interaction: $F_{(1,24)} = 4.049$, $p = 0.056$; Fig. 3C). Lastly, the Med-based diet increased occludin in females ($F_{(1,30)} = 31.838$, $p < 0.001$), irrespective of Prenatal Stressor (Fig. 3E).

3.2.2 Prefrontal cortex

In contrast to the effects observed in the hippocampus, none of the pro-inflammatory cytokines or tight junctions assessed in females were affected by Prenatal Stressor, Diet, or their interaction (Fig. 4). Conversely, the prenatal stressor increased TNF-α ($F_{(1,31)} = 6.365$, $p = 0.017$) and occludin ($F_{(1,32)} = 6.604$, $p = 0.015$) in males, irrespective of their diets (Fig. 4A and 4E). As well, males fed the Med-based diet had higher IL-1β expression than those fed the Control diet ($F_{(1,31)} = 5.152$, $p = 0.030$), irrespective of whether they had been prenatally stressed (Fig. 4B). Lastly, IL-6 and claudin-5 prefrontal expression in males were not affected by any of the manipulations (p's > 0.05; Fig. 4C and 4D).

3.2.3 Colon

Similar to the hippocampus, most of the impact of the prenatal stressor or the Med-based diet in the colon were apparent in females. In this sex, TNF-α and IL-1b were affected by Prenatal Stressor (TNF-α: $F_{(1,36)} = 4.467$, $p = 0.042$; IL-1b: $F_{(1,36)} = 8.073$, $p = 0.007$), and follow-up comparisons of the Stress and Diet interactions (TNF-α: $F_{(1,36)} = 3.147$, $p = 0.085$; IL-1b: $F_{(1,36)} = 9.649$, $p = 0.004$) showed that the colonic TNF-α ($p = 0.009$; Fig. 5A) and IL-1β ($p < 0.001$; Fig. 5B) elevations in prenatally stressed females fed the Control diet were absent in those fed the Med-based diet. In contrast, follow-up comparisons of the Prenatal Stressor and Diet interactions showed that the Med-based diet increased IL-6 in the colon of non-stressed females ($p = 0.028$; interaction: $F_{(1,34)} = 5.242$, $p = 0.028$; Fig. 5C) and decreased claudin-3 in stressed females ($p = 0.028$; interaction: $F_{(1,34)} = 5.242$, $p = 0.028$; Fig. 5F). In males, only TNF-α, IL-1β, and occludin were affected by the manipulations. Whereas the Med-based diet increased TNF-α in the males irrespective of stress (Fig. 5A), follow-up comparisons of the Prenatal Stressor x Diet interactions showed a higher expression of IL-1β in stressed males ($p = 0.014$; $F_{(1,34)} = 5.242$, $p = 0.028$; Fig. 5B) and a lower expression of occludin in non-stressed males fed this diet ($p = 0.028$; interaction: $F_{(1,34)} = 5.242$, $p = 0.028$; Fig. 5D).

3.3 Cecal microbiota

3.3.1 Alpha- and beta-diversity

We first verified if the overall taxonomic composition of the cecal microbiota differed as a function of the Prenatal Stressor and Diet. The assessment of the Bray-Curtis beta diversity index revealed that whereas the apparent difference between non-stressed and prenatally stressed females failed to reach significance ($F_{(1,37)} = 1.8538$, $p = 0.079$; Fig. 6A), samples from prenatally stressed males clustered separately from the non-stressed ones ($F_{(1,34)} = 5.0245$, $p = 0.001$; Fig. 6B). Additionally, significant sample clustering according to Diet was observed in both females ($F_{(1,37)}$

= 6.561, $p = 0.001$; Fig. 6C) and males ($F_{(1,34)} = 5.6023$, $p = 0.001$; Fig. 6D). In terms of species richness, the Chao1 index varied as a function of the Prenatal Stressor x Diet interaction in both females ($F_{(1,37)} = 6.619$, $p = 0.014$) and males ($F_{(1,34)} = 6.619$, $p = 0.002$), in addition to being influenced by Diet in females ($F_{(1,37)} = 7.336$, $p = 0.010$). Follow-up comparisons revealed that the Chao1 index was higher in prenatally stressed females fed the Med-based compared to their non-stressed counterparts ($p = 0.035$), whereas in males, the stress-induced decrease in this index in mice fed the Control diet ($p < 0.001$) was not apparent in those fed the Med-based diet (Supplementary Fig. 2A). The Shannon index, which measures taxa richness and evenness, was higher in females fed the Med-based diet relative to their counterparts fed the Control diet ($F_{(1,37)} = 5.572$, $p = 0.024$), regardless of their prenatal stress experiences, but lower in non-stressed males specifically ($p < 0.001$; interaction $F_{(1,34)} = 11.065$, $p = 0.002$) (Supplementary Fig. 2B).

3.3.2 Taxonomic composition

The cecal bacterial communities of female and male offspring featured eight phyla, with Firmicutes (49%) and Bacteroidota (44%) being dominant (Supplementary Fig. 2C and 2D). In females, Firmicutes was higher in those fed the Med-based diet ($F_{(1,34)} = 44.182$, $p < 0.001$) regardless of stress (Supplementary Fig. 2C). Bacteroidota was influenced by Prenatal Stressor ($F_{(1,34)} = 5.857$, $p = 0.021$), Diet ($F_{(1,34)} = 23.971$, $p < 0.001$), and the Prenatal Stressor x Diet interaction ($F_{(1,34)} = 15.491$, $p < 0.001$), with follow-up comparisons showing lower Bacteroidota abundance in prenatally stressed mice fed the Control diet ($p < 0.001$), but not in those fed the Med-based diet (Supplementary Fig. 2C). In contrast, higher abundance of Proteobacteria was apparent in stressed females fed the Control diet ($p = 0.014$; $F_{(1,34)} = 10.123$, $p = 0.003$; Supplementary Fig. 2C), an outcome that was absent in those fed the Med-based diet. In males, the prenatal stressor increased Firmicutes ($F_{(1,33)} = 13.027$, $p = 0.001$) and decreased Bacteroidota

($F_{(1,33)} = 14.149$, $p = 0.001$) irrespective of diets (Supplementary Fig. 2D), whereas the Med-based diet decreased Proteobacteria ($F_{(1,33)} = 20.479$, $p < 0.0001$), irrespective of stress (Supplementary Fig. 2C). Lastly, the Med-based diet reduced the less abundant Verrucomicrobiota in both females ($F_{(1,34)} = 8.247$, $p = 0.007$) and males ($F_{(1,33)} = 7.349$, $p = 0.011$). The rest of the phyla in both sexes were unaffected by Prenatal Stressor, Diet, of their interaction (p's > 0.05).

At the genus level, the Med-based diet again appeared to limit disturbances elicited by the prenatal stressor almost exclusively in females. Decreases in *Bacteroides* ($p < 0.001$; interaction: $F_{(1,36)} = 18.016$, $p < 0.001$) and increases in *Lactococcus* ($p = 0.002$ interaction: $F_{(1,36)} = 6.231$, $p = 0.017$), *Harryflintia* ($p = 0.001$ interaction: $F_{(1,36)} = 5.781$, $p = 0.021$), and *Erysipelatoclostridium* ($p = 0.001$ interaction: $F_{(1,36)} = 5.635$, $p = 0.023$) in prenatally stressed females fed the Control diet were not apparent in those fed the Med-based diet (Fig. 7A-D). In contrast, prenatally stressed females fed the Med-based diet, but not those fed the Control diet, had lower levels of *Ruminococcaceae* ($p = 0.006$; interaction: $F_{(1,36)} = 18.016$, $p < 0.001$), relative to their non-stressed counterparts (Fig. 7E-F). In males, the decreases in *Bacteroides* ($F_{(1,33)} = 27.072$, $p < 0.001$) and the increases in *Anaeroplasma* ($F_{(1,33)} = 6.229$, $p = 0.018$) and *Turicibacter* ($F_{(1,33)} = 5.600$, $p = 0.024$) elicited by the prenatal stressor were apparent in both mice fed the Control and Med-based diets, (Fig. 7A, F, G). In contrast, the increases in *Harryflintia* ($F_{(1,33)} = 27.176$, $p < 0.001$) and *Parasutterella* ($F_{(1,33)} = 5.240$, $p = 0.016$) in stressed males fed the Control diet were not present in those fed the Med-based diet (Fig. 7D, J). Finally, *Roseburia* was increased by the prenatal stressor only in males fed the Med-based diet ($F_{(1,33)} = 7.800$, $p = 0.005$).

3.3.3 Inferred bacterial metabolic activity

In silico analyses of bacterial metabolic activity revealed differential enrichment of several KEGG pathways as a function of Prenatal Stressor, Diet, or their interactions. Based on the *a priori*

hypothesis that Prenatal Stressor and Diet would impact microbial inflammatory pathways (Madison & Bailey, 2024; Seethaler et al., 2022), we specifically examined changes in KEGG pathways related to LPS and PGN biosynthesis, as well as to NOD-like receptor signaling. In females, the LPS biosynthesis pathway was less enriched in the Med-based diet groups compared to the Control diet groups ($F_{(1,36)} = 18.137$, $p < 0.001$), regardless of their prenatal stressor experiences (Fig. 8A). Interestingly, the higher enrichment of the PGN biosynthesis pathway ($p = 0.003$; interaction: $F_{(1,36)} = 4.641$, $p = 0.038$) and the lower enrichment of NOD-like receptor signalling pathway ($p = 0.005$; interaction: $F_{(1,36)} = 4.638$, $p = 0.039$) in prenatally stressed females fed the Control diet were not seen in those fed the Med-based diet (Fig. 8B, C). In males, the prenatal stressor decreased the LPS biosynthesis pathway ($F_{(1,34)} = 17.077$, $p < 0.001$) but increased the PGN biosynthesis pathway ($F_{(1,34)} = 17.077$, $p < 0.001$), irrespective of Diet (Fig. 8A, B). Lastly, the NOD-like receptor signaling pathway was reduced by the prenatal stressor in mice fed the Med-based diet specifically ($p < 0.001$; interaction: $F_{(1,34)} = 4.967$, $p = 0.033$; Fig. 8C).

3.4. The time spent in the open arms of the elevated plus maze, reduced in prenatally stressed females and rescued by the Med-based diet, was linked to different biological metrics in females versus males

In females, the time spent in the open arms of the elevated plus maze showed a positive correlation with hippocampal claudin-5 levels and negative correlation with Bacteroides levels in stressed mice fed the Med-based diet (Fig. 9A). In males, this behavioural metric was negatively correlated with *Parasutterella* levels, irrespective of stress (Fig. 9B).

4. Discussion

It is well-established that maternal exposure to stress during fetal development can lead to mental health disturbances in the offspring (Van den Bergh et al., 2020). While the biological mechanisms underlying these outcomes have yet to be entirely defined, there is substantial evidence that they may involve alterations in brain inflammatory functions, potentially stemming from disturbances in early-life microbial establishment in the gut. Emerging research suggests that lifestyle factors, such as diet, can influence mental health (Marx et al., 2020). Here, we report novel findings that an early-life diet based on human Med dietary patterns limited anxiety-like behaviour, pro-inflammatory changes in the brain and gut environments, and alterations in the gut microbiota of adult mouse offspring exposed to prenatal stress. Importantly, these effects of the Med-based diet were mostly observed in females.

The prevalence of anxiety and depressive disorders is disproportionately higher in women than in men (Kessler, 2003; McLean et al., 2011). Rodent models of prenatal stress have also demonstrated that the female offspring is more susceptible than males in developing anxiety-like behaviours when exposed to prenatal stressors (Schulz et al., 2011; Verstraeten et al., 2019; Zagron & Weinstock, 2006). Consistent with this evidence, we found that a prenatal stressor experienced during the second trimester of pregnancy reduced the time spent in the open arms of the elevated plus maze, suggestive of increased anxiety-like behaviour, in females only. Importantly, this phenotype was not apparent in prenatally stressed females fed the Med-based diet, indicating that this diet could have imparted resilience against anxiety-like behaviours stemming from adverse prenatal experiences. Overall, we found that fear-related behaviours in the open field and passive coping behaviours in the tail suspension test were less pronounced in female offspring fed the Med-based diet, indicating that the Med-based diet mitigated anxiety- and depressive-like

behaviours in these mice. In humans, offspring whose mothers adhered to Med-based dietary patterns during pregnancy were less likely to display anxiety-related behaviours (House et al., 2018), supporting the view that the physiological effects of the Med-based diet throughout development could produce anxiolytic effects in the offspring. In line with the female-specific behavioural effects of the Med-based diet observed in this study, clinical interventions with Med-based diets reduced major depressive symptoms mostly in female subjects (Firth et al., 2019). Taken together, the present findings suggest that consuming dietary patterns based on the Med diet across the lifespan could reduce the risk of developing anxiety in offspring stressed during prenatal development while supporting overall mental health, at least in females.

To investigate if the prenatal stressor and dietary impacts on behaviour are related to changes in inflammatory processes and barrier permeability in the brain and gut environments, we analyzed the mRNA expression of pro-inflammatory cytokines and tight junction proteins in the hippocampus, prefrontal cortex, and colon. Consistent with previous observations of higher TNF-α and/or IL-1β in the hippocampus and colon of adult female and male mice stressed *in utero* (Diz-Chaves et al., 2012, 2013; Sun et al., 2021), TNF-α in the female hippocampus as well as TNF-a and IL-1β in the female colon were upregulated in offspring exposed to the prenatal stressor. Notably, the cytokine increases were not observed in mice fed the Med-based diet, suggesting that the diet counteracted the stress-induced elevations of pro-inflammatory markers in the female hippocampus and colon. In males, although the Med-based diet limited the stress-induced hippocampal TNF-α increases, it failed to inhibit the cytokine elevations in the prefrontal cortex, suggesting that the protective effect of the Med-based diet against prenatal stress-induced inflammation in males may be region-specific, perhaps due to sex differences in the immune system and the response to immune modulators (Audet, 2019; Gildawie et al., 2020; Klein &

Flanagan, 2016). Although we cannot point to the specific dietary components that may be responsible for the anti-inflammatory effects of our Med-based diet, the diet contains fish and olive oils, walnuts, fruits, and vegetables, which are abundant in omega-3 fatty acids or polyphenols that have anti-inflammatory and antioxidative properties (Hussain et al., 2016; Simopoulos, 2002). Thus, it is possible that some of these components could have individually and/or synergistically modulated the effects of the prenatal stressor on the development of brain and intestinal immune function in these offspring.

Interestingly, despite these anti-inflammatory effects of the Med-based diet in stressed mice, it was surprising to find that pro-inflammatory cytokine expression in the hippocampus and colon of non-stressed female and/or males were almost consistently increased, indicating that the effects of the Med-based diet could be contextual, in this case, based on non-stressed versus stressed conditions. Further studies would be important to elucidate the functional implications of these outcomes, particularly to determine whether they contribute to immunomodulation or promote pro-inflammatory/pathogenic phenotypes. Unexpectedly, while none of the tight junction markers assessed in the hippocampus and colon were affected by the prenatal stressor in either sex, occludin was upregulated in the prefrontal cortex of prenatally stressed male offspring, and the Med-diet did not rescue this effect. Although this finding contradicts the current understanding that stress downregulates occludin expression or promote barrier impairments in the brain (Welcome & Mastorakis, 2020), it is consistent with a previous report showing increased occludin in the prefrontal cortex of mouse offspring subjected to post-weaning isolation stress (Karailiev et al., 2021). As the males in the present study also exhibited an upregulated prefrontal TNF-α expression, we could speculate that the concurrent occludin elevation in this region is perhaps a protective mechanism against cytokine-induced BBB dysfunction. We also found that the Med-

based diet generally increased occludin and claudin-5 in the hippocampus of females, and this increase in claudin-5 correlated positively with their time spent in the open arms of the maze in the stressed mice fed this diet, although this correlation was not significant at our set *p* value of <0.01. Nonetheless, these tight junctions, particularly claudin-5, are indispensable to vascular endothelial function important in regulating the paracellular permeability of the BBB (Kadry et al., 2020), as claudin-5 deficiency in mice loosened and increased permeability of the BBB (Nitta et al., 2003), and loss of occludin was linked with degradation of the BBB in human brain tissue (Papadopoulos et al., 2001). Thus, by promoting the expression of these tight junctions, the Med-based diet demonstrates a capacity to preserve BBB structural integrity and function, at least in the hippocampus, thereby supporting optimal brain functions and health. Also, the fact that this was apparent only in females suggests that the diet may support BBB functions preferentially in females.

Since prenatal stress has been shown to disrupt the pattern of microbial assembly in the gut (Mepham et al., 2023), which can influence inflammatory immune development and function (Zheng et al., 2020) and contribute to mental health disturbances in the offspring (Nikolova et al., 2021), we examined different metrics in the cecal microbiota. While Chao1 was higher in prenatally stressed females fed the Med-based diet, this index was lower in prenatally stressed males fed the Control diet. These findings, albeit conflicting, illustrate sex-specific dietary influences on bacterial richness in the context of prenatal stress. Although the impact of diet or sex were not considered, previous studies reported increased (Zijlmans et al., 2015), reduced (Galley et al., 2021; Jahnke et al., 2021), or no change (Aatsinki et al., 2020) in alpha diversity in offspring whose mothers experienced stress during pregnancy, demonstrating varying effects of stress on bacterial richness, likely accounted for by the variations in prenatal stressor exposure and

offspring's diet and sex. Notably, our results showed that the cecal microbiota alterations resulting prenatal stress altered of the offspring, whereas females fed the Med-based diet were largely unperturbed, which is most likely attributed to their diet. Particularly, stress-induced decreases in Bacteroidota/*Bacteroides* and increases in *Lactococcus, Harryflintia, and Erysipelatoclostridium* in females were limited by the Med-based diet, while stressed females fed this diet only exhibited a decrease in bacteria related to *Ruminococcaceae.* In contrast, the Med-based diet did not counteract increases in *Anaeroplasma* and *Turicibacter* in the males, again demonstrating differential impacts of the diet in the female and male offspring. Given that treatment with *Bacteroides fragilis* prevented colonic inflammation (Mazmanian et al., 2008) and reversed anxiety-like behaviours induced by maternal immune activation (Hsiao et al., 2013), it is possible that the *Bacteroides* reductions in prenatally stressed females fed the Control diet could have contributed to their colonic pro-inflammatory markers and more pronounced anxiety-like behaviour. However, the lack of correlations between Bacteroides and colonic cytokines assessed renders this hypothesis implausible. Curiously, studies have demonstrated the anti-inflammatory properties of different species or strains of *Lactococcus* (Luerce et al., 2014; Nishitani et al., 2009) and *Anaeroplasma* (Beller et al., 2019, 2020) in *in vitro* and *in vivo* models of inflammatory bowel disease, yet prenatally stressed mice in this study that displayed higher levels of these bacteria also exhibited increased expression of colonic pro-inflammatory markers. In that same vein, some members of the *Ruminococcaceae* family are butyrate producers (Louis & Flint, 2017) and have been identified in lower levels in intestinal inflammatory conditions (Joossens et al., 2011; Morgan et al., 2012) as well as in generalized anxiety (Chen et al., 2019) and depression (Huang et al., 2018). The functional significance of the reduced abundance of *Ruminococcaceae* in prenatally stressed females fed the Med-based diet cannot be deduced, considering these mice did not exhibit

any altered pro-inflammatory or behavioural profiles and no other changes occurred in their cecal microbiota. Of particular interest was the impact of the prenatal stressor on Proteobacteria, given the pro-inflammatory and pathogenic nature of species in this phylum (Rizzatti et al., 2017). Consistent with previous findings in humans (Aatsinki et al., 2020; Jahnke et al., 2021; Zijlmans et al., 2015), prenatally stressed females exhibited higher levels of Proteobacteria, an effect that was counteracted by the Med-based diet, thus providing further basis for the anti-inflammatory effects of the Med-based diet in the intestinal milieu of the females. Also, *parasutterella* belonging to the Proteobacteria phylum was increased by stress in only the males fed the Control diet, again demonstrating that the diet discouraged the growth of pro-inflammatory bacteria in the gut.

As compositional changes in the gut microbiota do not certainly reflect changes in their metabolic functions, we employed an *in silico* predictive tool to gain insight into the functional changes associated with alterations in the cecal microbiota composition, resulting in a catalog of KEGG metabolic pathways in each sample. Contrary to our expectations, the prenatal stressor reduced the LPS biosynthesis pathway in males fed both diets, while no changes were observed in the females, despite the finding that females fed the Control diet displayed higher levels of Proteobacteria. In contrast, the PGN biosynthesis pathway was more enriched in all stressed groups of mice, again except females fed the Med-based diet. Considering that PGN can elicit pro-inflammatory responses (Laman et al., 2020; Royet et al., 2011), we speculate that the increased activity in its biosynthetic pathway may have contributed to the increased pro-inflammatory cytokines observed in these mice at the different sites examined, although no correlations were found between this pathway and the pro-inflammatory cytokines assessed. Finally, the prenatal stressor reduced the relative enrichment of NOD-like receptor signaling pathway in females fed the Control diet and in males fed the Med-based diet. Peptidoglycan is one of the potent activators

of NOD-like receptor signaling pathway (Franchi et al., 2009). Thus, the observation of simultaneous upregulation of PGN biosynthesis pathway and downregulation of NOD-like receptor signaling pathway in these stressed mice is rather puzzling and needs closer examinations.

This study provides further evidence that prenatal stress leads to sex-specific changes in the offspring's physiology and behaviour, and specifically showed that stress increased anxiety-like behaviours in females and led to microbiota alterations mostly in the males. Importantly, our findings demonstrate the capacity of dietary patterns based on the Med diet to minimize alterations in brain and intestinal inflammatory processes and gut microbiota due to prenatal stress, potentially improving mental health outcomes. However, considering that no major correlations were obtained between the behavioural, pro-inflammatory, and gut microbial outcomes of the Med-based dietary pattern, future studies are important to elucidate the specific molecular mechanisms underlying the dietary influence. Lastly, we showed that females had greater response to the beneficial impacts of the Med-based diet than males, highlighting the importance of sex considerations in future development of dietary interventions for mental health promotion.

5. Acknowledgements

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6. Author Contributions: MCU and MCA designed the experiment. MCU, ADG, and GL conducted the breeding, dietary, stressor, and behavioral manipulations included in the experiment (equal contribution). MCU performed the molecular (RT-qPCR) analyses. MCU and AS conducted the microbiota sequencing analyses and MCU performed the bioinformatics analyses. MCU and MCA interpreted the data and wrote the manuscript, which was edited by all authors.

7. Conflict of Interest Statement: All authors declare that the research work was conducted in the absence of any personal, professional, or financial relationships that could be construed as a conflict of interest.

8. List of Figures

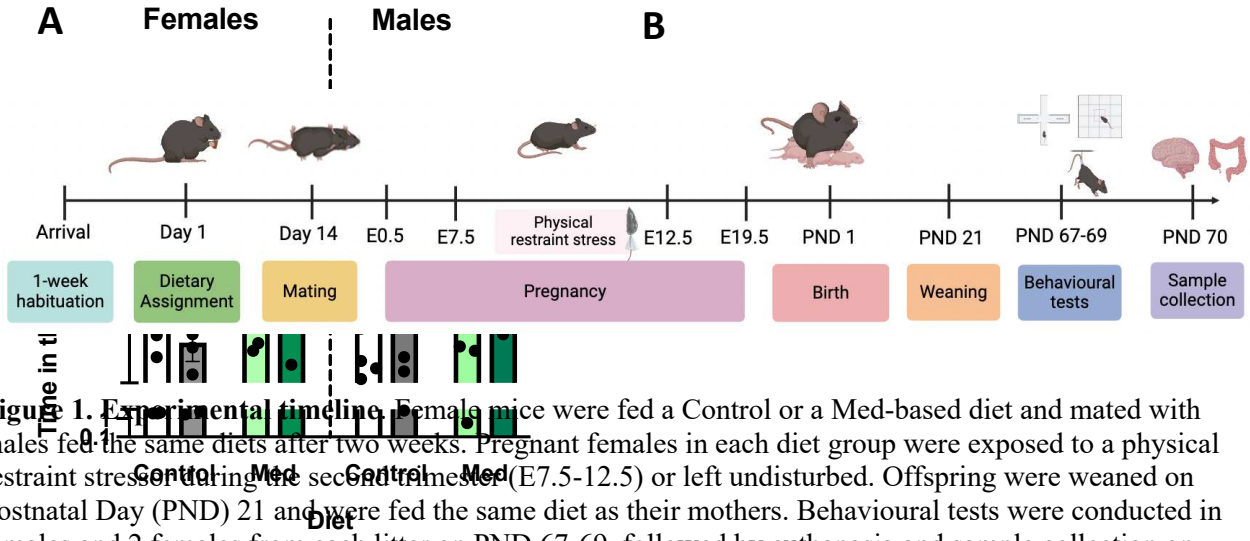
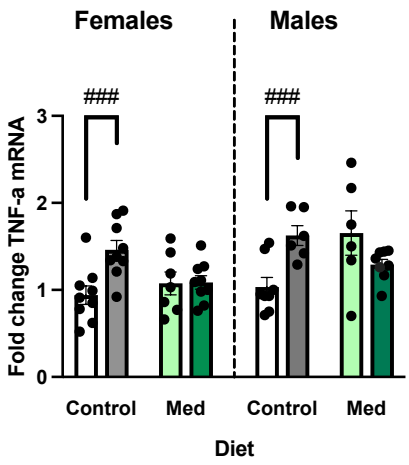
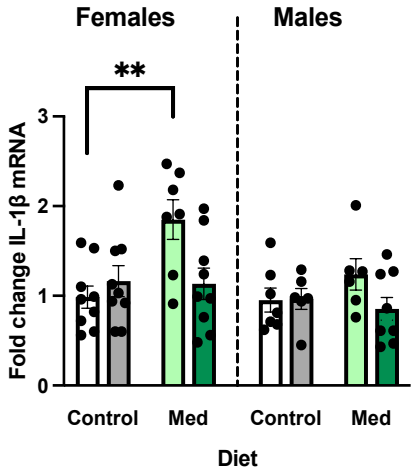
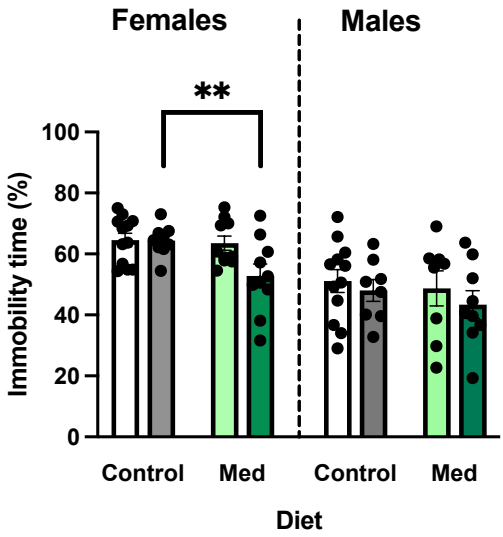


Figure 1. Experimental timeline. Female mice were fed a Control or a Med-based diet and mated with males fed the same diets after two weeks. Pregnant females in each diet group were exposed to a physical restraint stressor during the second trimester (E7.5-12.5) or left undisturbed. Offspring were weaned on Postnatal Day (PND) 21 and were fed the same diet as their mothers. Behavioural tests were conducted in 2 males and 2 females from each litter on PND 67-69, followed by euthanasia and sample collection on PND 70. "E" refers to embryonic day. Figure created with BioRender.

Figure 2. Time in the open arms of an elevated plus maze (A), center of an open field (B), and time spent immobile in the tail suspension test (C) in adult female and male adult offspring (postnatal day 70) born to dams fed a Control or a Med-based diet and exposed to a physical restraint stressor or left undisturbed during the second trimester of pregnancy. Data was analyzed using 2-way analyses of variance, with Prenatal Stressor (non-stressed vs stressed) and Diet (Control vs Med) as the between-group factors, followed by Bonferroni-corrected multiple comparison *t* tests. Dots represent individual mice, while bars represent group means ± S.E.M. Mice fed the Control diet (non-stressed females: $n = 12$, stressed females: $n = 7$-8; non-stressed males: $n = 5$, stressed males: $n = 5$). Litters fed the Med-based diet (non-stressed females: $n = 5$, stressed females: $n = 6$; non-stressed males: $n = 5$, stressed males: $n = 5$). # $p < 0.05$ relative to non-stressed mice and ** $p < 0.01$ relative to mice fed the Control diet







A

B

Non-stressed

Stressed

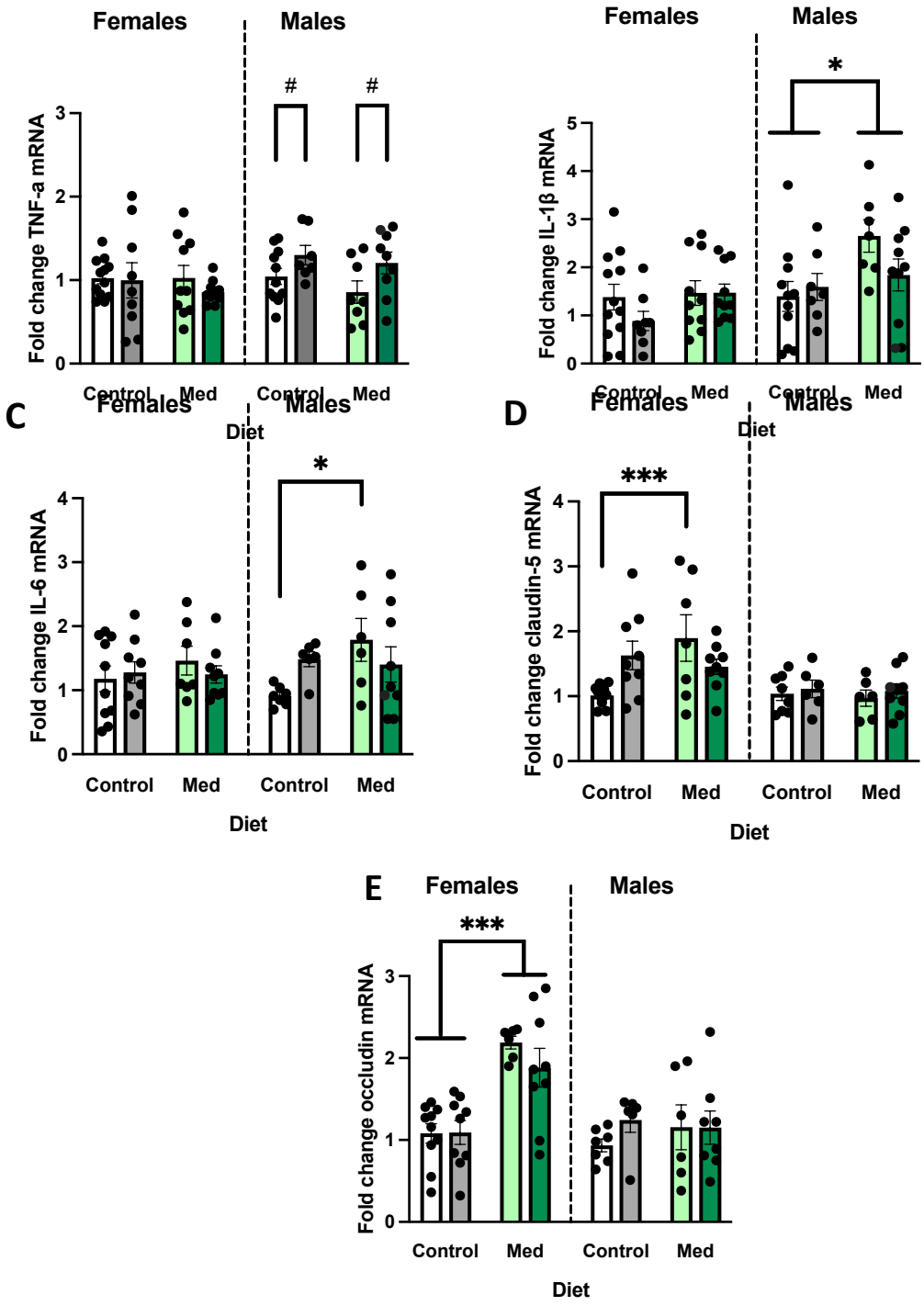
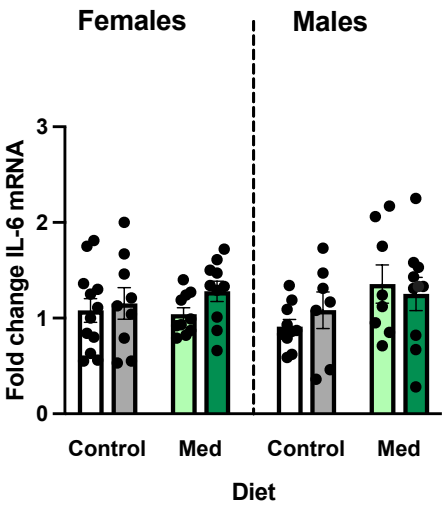


Figure 3. Hippocampal expression of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α (A), interleukin (IL)-1β (B), and IL-6 (C) and the tight junctions claudin-5 (D) and occludin (E) in adult female and male adult offspring (postnatal day 70) born to dams fed a Control or a Med-based diet and exposed to a physical restraint stressor or left undisturbed during the second trimester of pregnancy. Data for each sex were analyzed separately using a 2-way analysis of variance, with Prenatal Stressor (non-stressed vs stressed) and Diet (Control vs Med) as the between-group factors, followed by Bonferroni-corrected multiple comparison *t* tests. Dots represent individual mice, while bars represent group means ± S.E.M. Mice fed the Control diet (non-stressed females: $n = 10$, stressed females: $n = 9$; non-stressed males: $n = 7$-8, stressed males: $n = 6$). Mice fed the Med-based diet (non-stressed females: $n = 7$, stressed females: $n = 9$; non-stressed males: $n = 6$, stressed males: $n = 7$-9). ### $p < 0.001$ relative to non-stressed mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ relative to mice fed the Control diet.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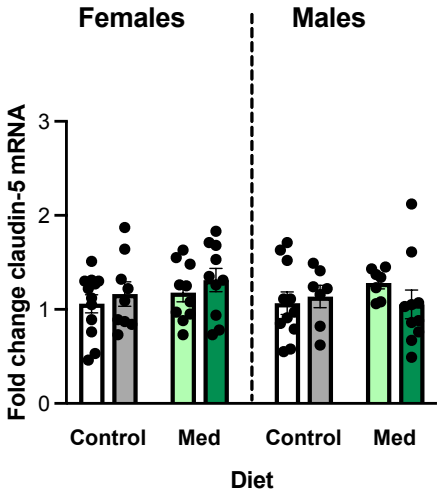
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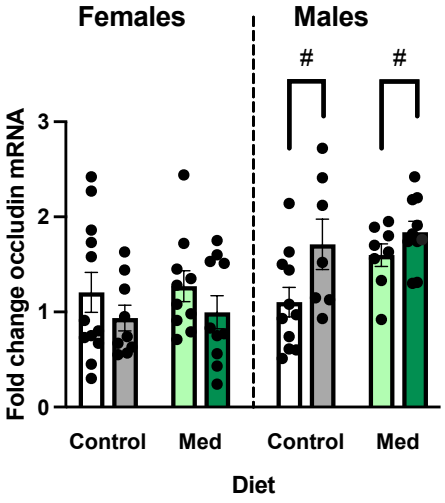
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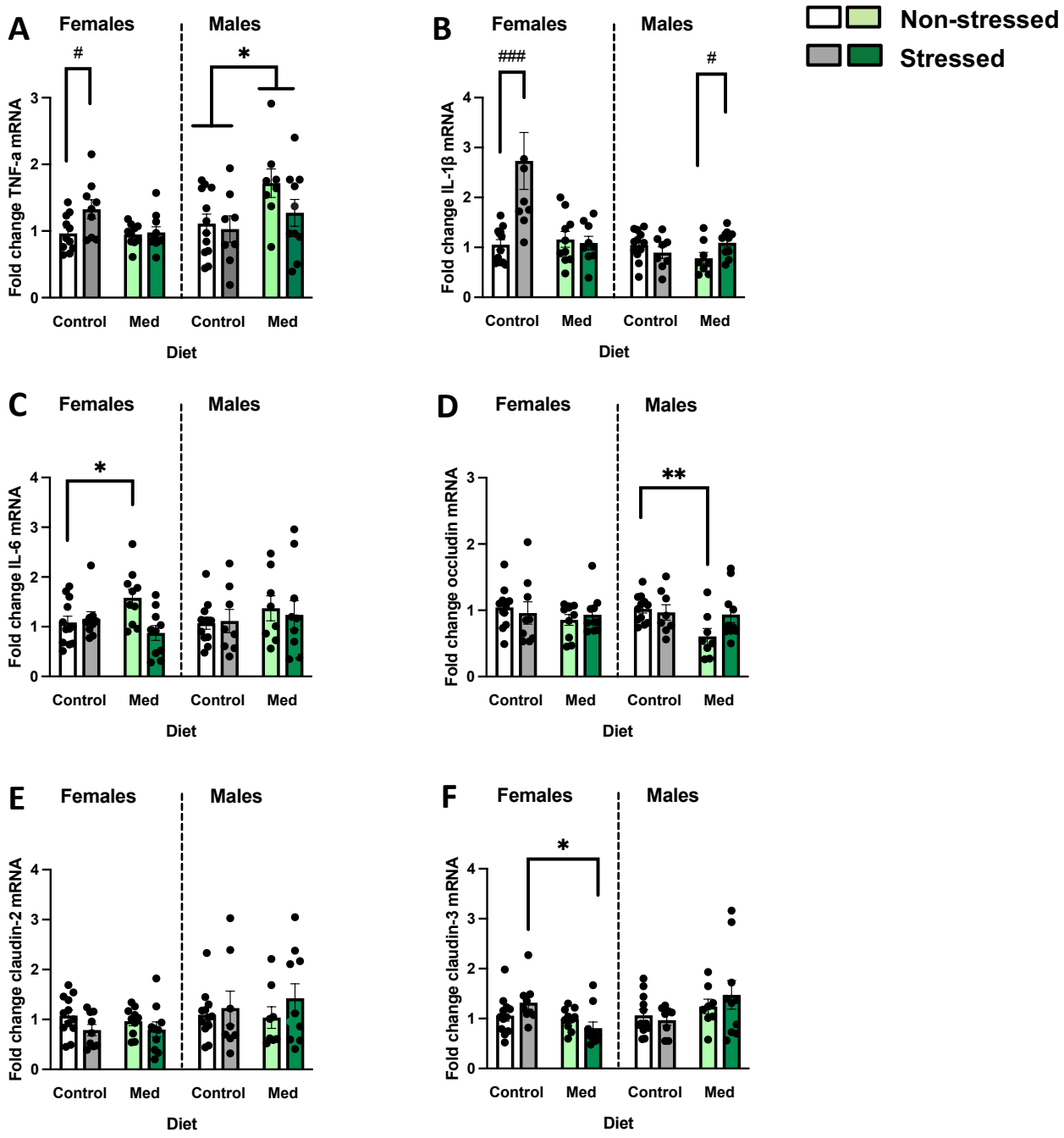

Figure 5. Colonic expression of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α (A), interleukin (IL)-1β (B), and IL-6 (C) and the tight junctions occludin (D), claudin-2 (E), and claudin-3 (F) in adult female and male offspring (postnatal day 70) born to dams fed a Control or a Med-based diet and exposed to a physical restraint stressor or left undisturbed during the second trimester of pregnancy. Data for each sex were analyzed separately using a 2-way analysis of variance, with Prenatal Stressor (non-stressed vs stressed) and Diet (Control vs Med) as the between-group factors, followed by Bonferroni-corrected multiple comparison *t* tests. Dots represent individual mice, while bars represent group means ± S.E.M. Mice fed the Control diet (non-stressed females: n = 11-12, stressed females: n = 9; non-stressed males: n = 12, stressed males: n = 8). Mice fed the Med-based diet (non-stressed females: n = 10, stressed females: n = 10; non-stressed males: n = 8, stressed males: n = 10). # $p < 0.05$ relative to non-stressed mice. * $p < 0.05$ and ** $p < 0.01$ relative to mice fed the Control diet.

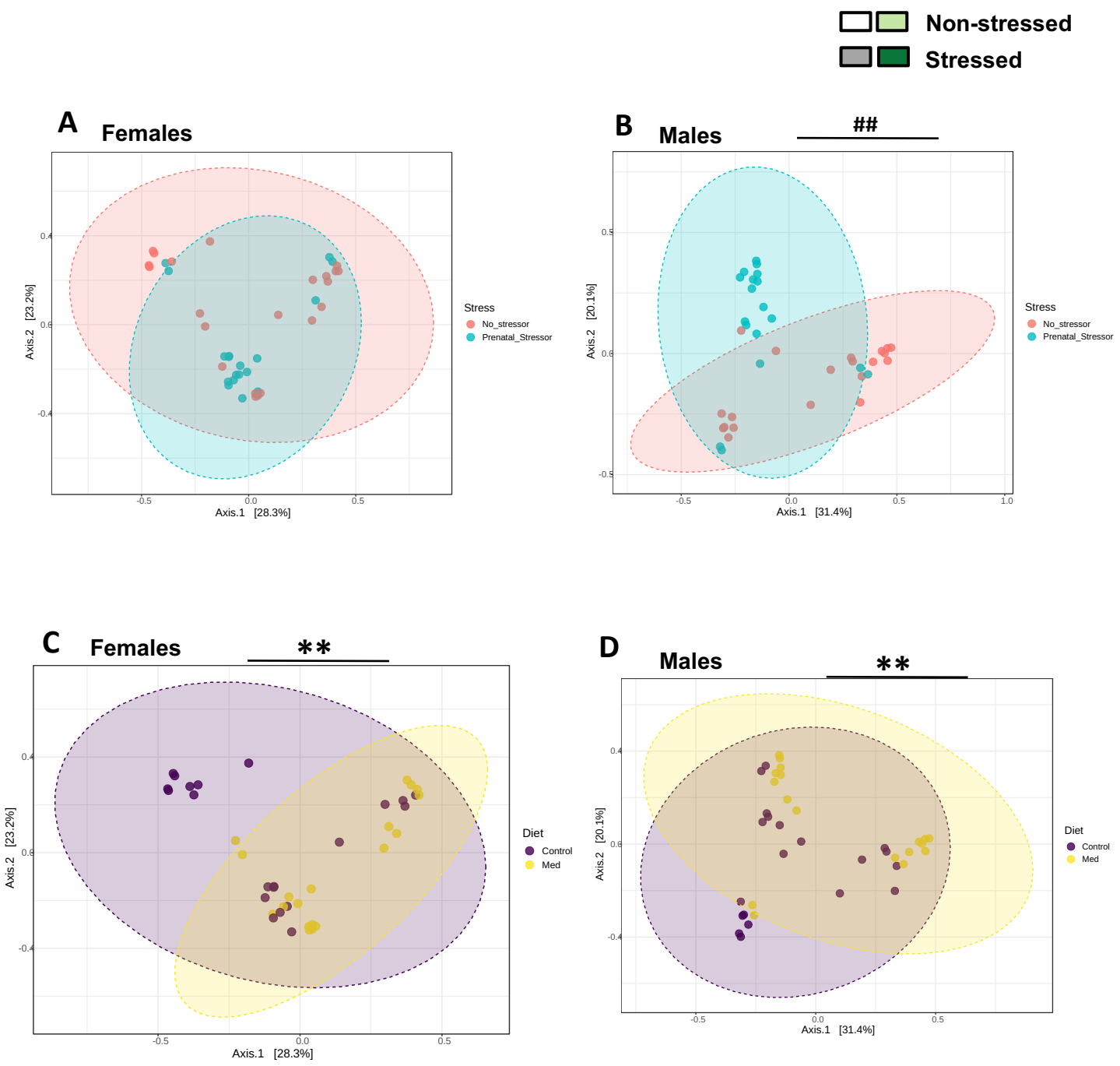

Figure 6. Bray-Curtis dissimilarity indices as a function of Stress (A and B) and Diet (C and D) of cecal microbiota of adult female and male offspring (postnatal day 70) born to dams fed a Control or a Med-based diet and exposed to a physical restraint stressor or left undisturbed during the second trimester of pregnancy. Data was analyzed using permutational multivariate analysis of variance (PERMANOVA) of Bray-Curtis distance matrices in MicrobiomeAnalyst. Dots represent individual mice. Mice fed the Control diet (non-stressed females: $n = 11$, stressed females: $n = 9$; non-stressed males: $n = 12$, stressed males: $n = 8$). Mice fed the Med-based diet (non-stressed females: $n = 10$, stressed females: $n = 10$; non-stressed males: $n = 8$, stressed males: $n = 10$). ## $p < 0.01$ relative to non-stressed mice and ** $p < 0.01$ relative to mice fed the Control diet.

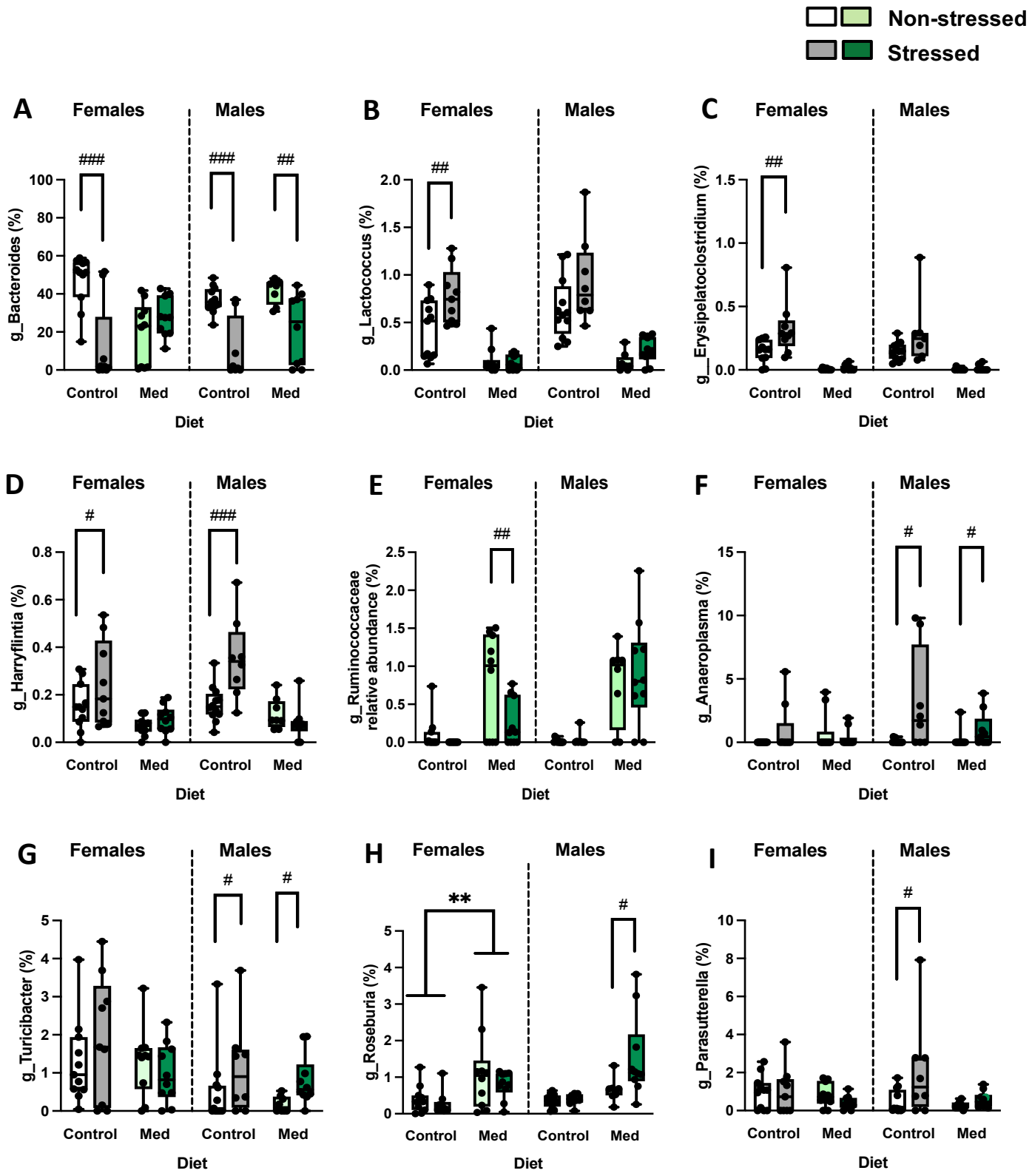

Figure 7. Relative abundance of genera in the cecal microbiota of adult female and male offspring (postnatal day 70) born to dams fed a Control or a Med-based diet and exposed to a physical restraint stressor or left undisturbed during the second trimester of pregnancy. Data for each sex were analyzed separately using a 2-way analysis of variance (ANOVA), with Stress (non-stressed vs stressed) and Diet (Control vs Med) as the between-group factors, followed by Bonferroni-corrected multiple comparison tests. Mice fed the Control diet (non-stressed females: $n = 11$, stressed females: $n = 9$; non-stressed males: $n = 12$, stressed males: $n = 8$). Mice fed the Med-based diet (non-stressed females: $n = 10$, stressed females: $n = 10$; non-stressed males: $n = 8$, stressed males: $n = 10$). # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ relative to non-stressed mice and ** $p < 0.01$ relative to mice fed the Control diet.

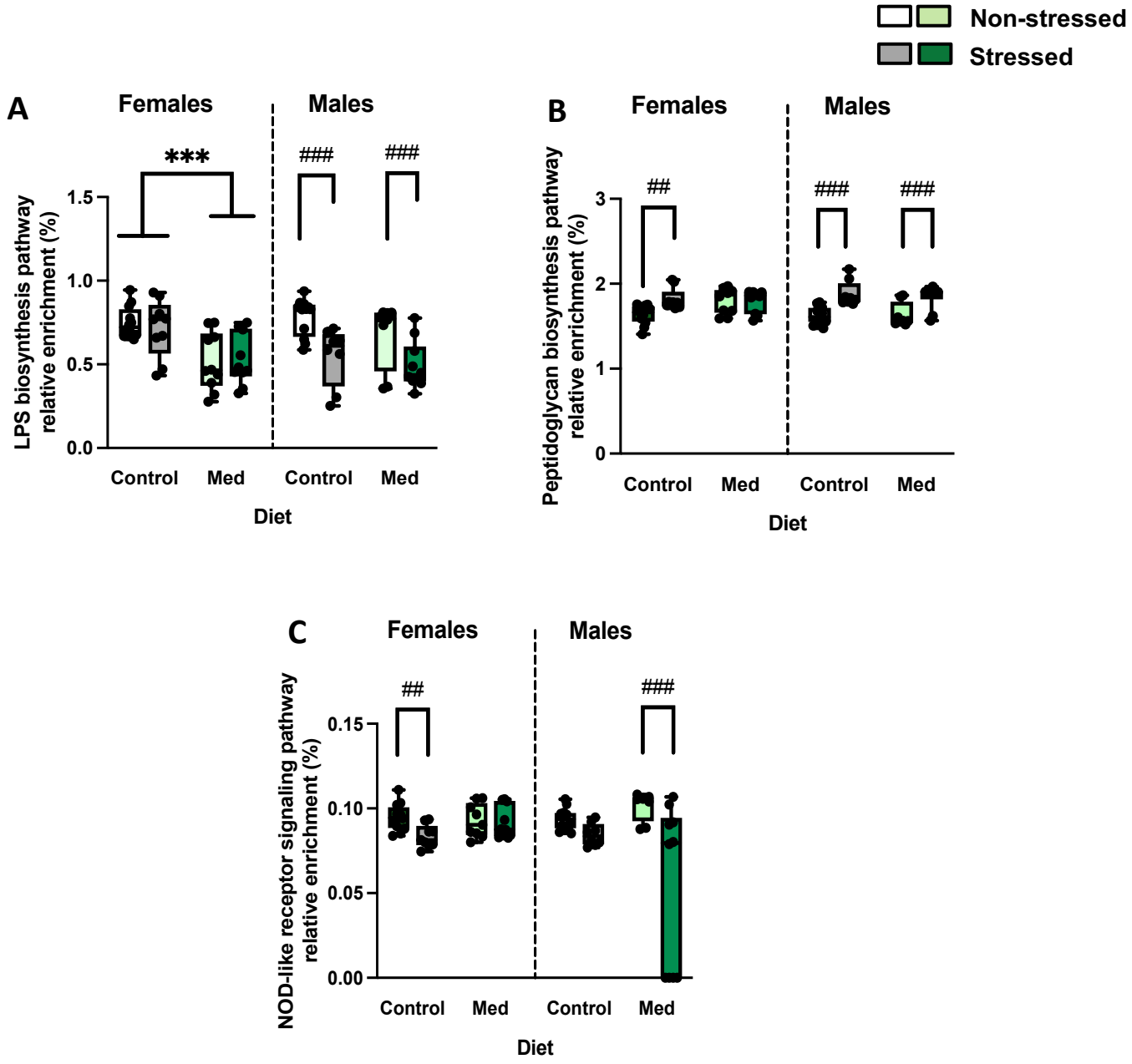

Figure 8. Relative enrichment of KEGG pathways in the cecal microbiota of adult female and male offspring (postnatal day 70) born to dams fed a Control or a Med-based diet and exposed to a physical restraint stressor or left undisturbed during the second trimester of pregnancy. Data for each sex were analyzed separately using a 2-way analysis of variance (ANOVA), with Stress (non-stressed vs stressed) and Diet (Control vs Med) as the between-group factors, followed by Bonferroni-corrected multiple comparison tests. Mice fed the Control diet (non-stressed females: $n = 11$, stressed females: $n = 9$; non-stressed males: $n = 12$, stressed males: $n = 8$). Mice fed the Med-based diet (non-stressed females: $n = 10$, stressed females: $n = 10$; non-stressed males: $n = 8$, stressed males: $n = 10$). ## $p < 0.01$ and ### $p < 0.001$ relative to non-stressed mice and *** $p < 0.001$ relative to mice fed the Control diet.

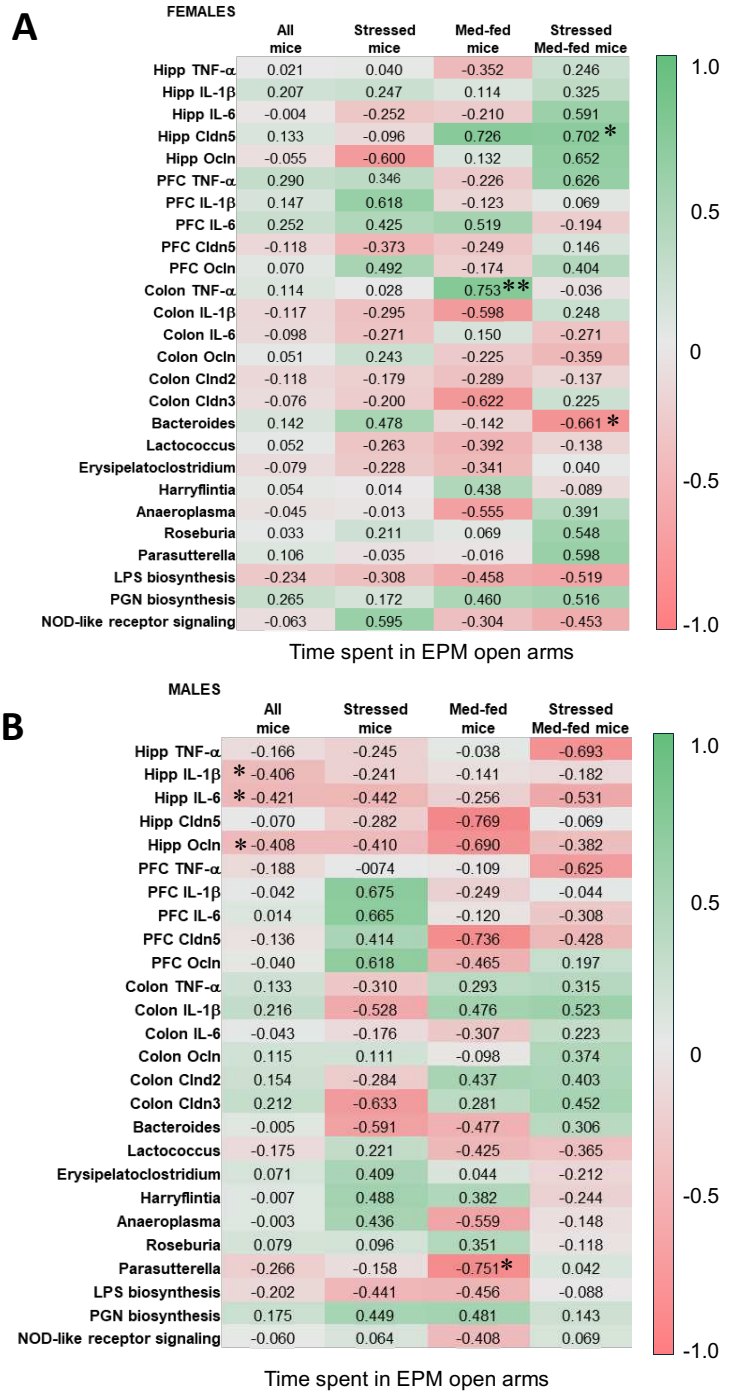

Figure 9. Correlations between the main behavioural parameter changed by the prenatal stressor and the Med-based diet and the different inflammatory, tight junction, and microbiota markers examined in the brain and intestinal environments of adult female (A) and male (B) offspring born to dams fed a Control or a Med-based diet and exposed to a physical restraint stressor or left undisturbed during the second trimester of pregnancy. (A) In females, the time spent in the open arms of the elevated plus maze (EPM) was positively correlated with the expression of tumor necrosis factor (TNF)-α in the colon of mice fed the Med-based diet (p = 0.010) and that of claudin-5 (Cldn5) in the hippocampus (HIPP) of prenatally stressed mice fed the Med diet (p = 0.035) but negatively correlated with the abundance of *Bacteroides* in the cecum contents of the same group (p = 0.038). (B) In males, the behavioural metric was negatively correlated with the expression of TNF-α (p = 0.029), interleukin (IL)-6 (p = 0.026), and occludin (p = 0.035) in the hippocampus of all mice and with the abundance of *Parasutterella* (p = 0.032) in mice fed the Med-based diet. Data for each sex were analyzed separately using Pearson and Spearman correlation coefficients tests. Colour intensities indicate the strength of positive (green) and negative (pink) correlation coefficients. * $p < 0.05$ and ** $p < 0.01$

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10. Supplementary Data

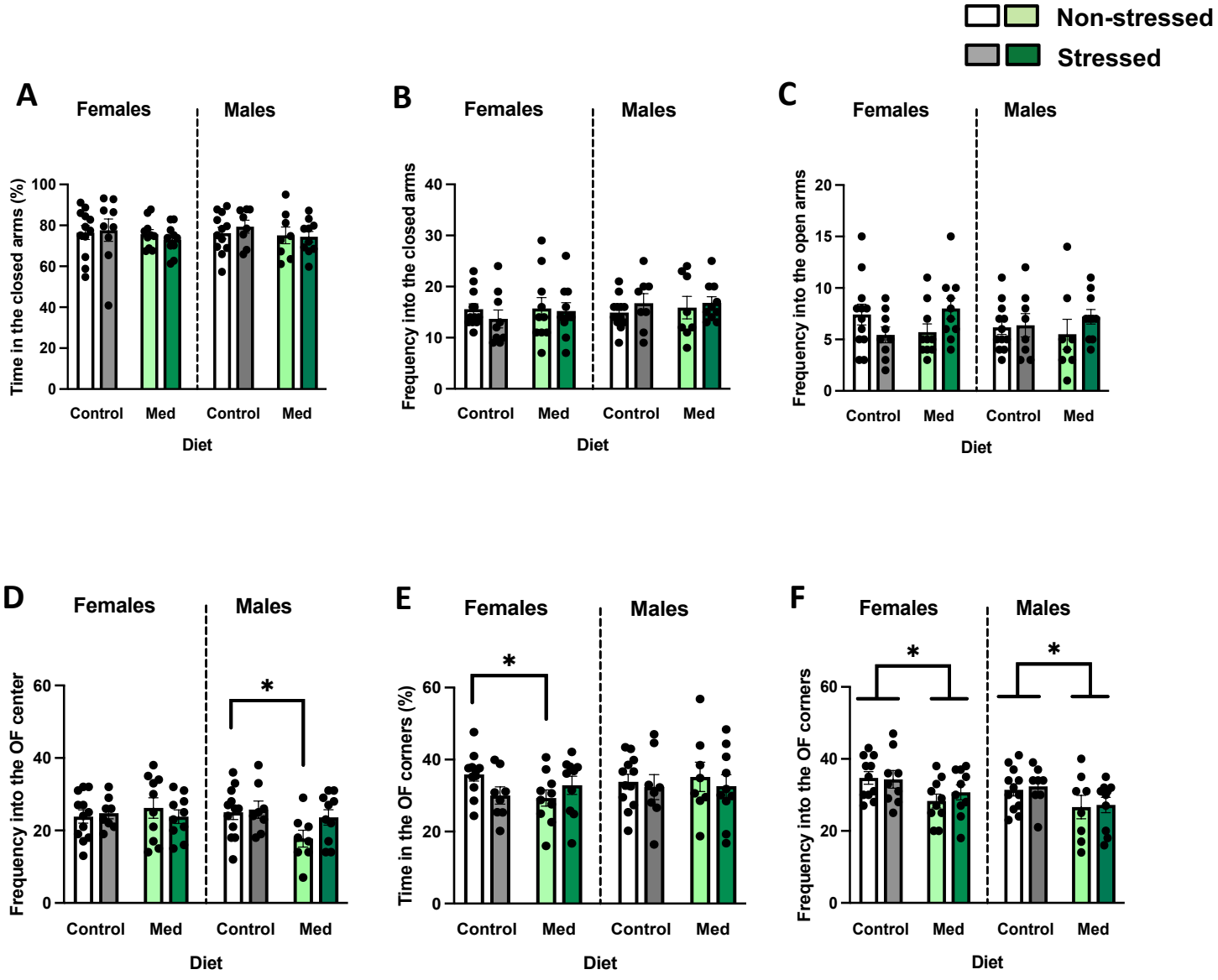

Figure 1. Behavioural measures in the elevated plus maze (A-C) and open field (D-F) of adult female and male offspring (postnatal day 70) born to dams fed a Control or a Med-based diet and exposed to a physical restraint stressor or left undisturbed during the second trimester of pregnancy. Data was analyzed using 2-way analyses of variance, with Prenatal Stressor (non-stressed vs stressed) and Diet (Control vs Med) as the between-group factors, followed by Bonferroni-corrected multiple comparison *t* tests. Dots represent individual mice, while bars represent group means ± S.E.M. Mice fed the Control diet (non-stressed females: $n = 12$, stressed females: $n = 7$-8; non-stressed males: $n = 5$, stressed males: $n = 5$). Mice fed the Med-based diet (non-stressed females: $n = 5$, stressed females: $n = 6$; non-stressed males: $n = 5$, stressed males: $n = 5$). * $p < 0.05$ relative to mice fed the Control diet

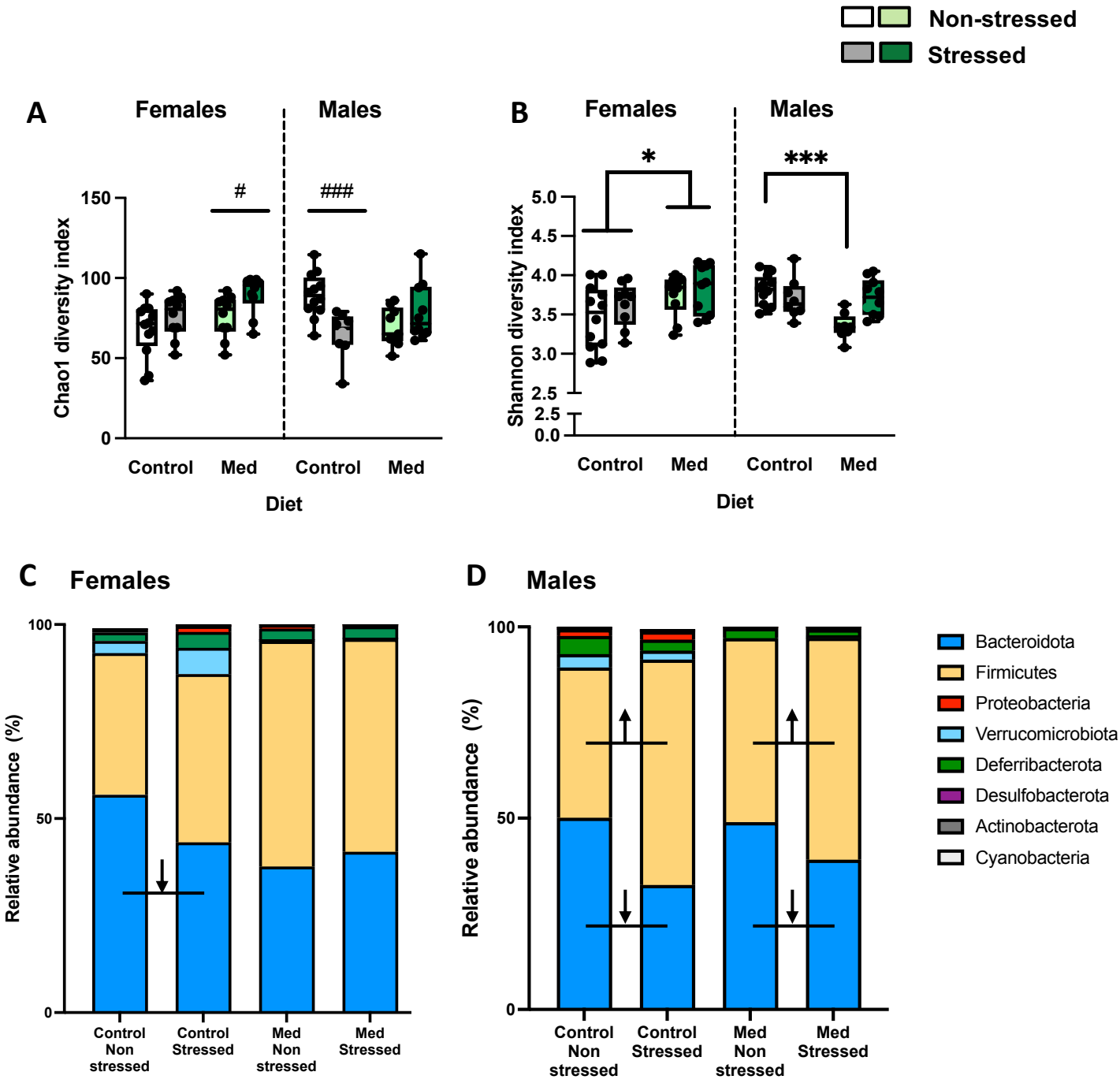

Figure 2. Chao1 (A) and Shannon (B) diversity indices and relative abundance of phyla (C and D) in the cecal microbiota of adult female and male offspring (postnatal day 70) born to dams fed a Control or a Med-based diet and exposed to a physical restraint stressor or left undisturbed during the second trimester of pregnancy. Data for each sex were analyzed using a 2-way analysis of variance (ANOVA), with Prenatal Stress (non-stressed vs stressed) and Diet (Control vs Med) as the between-group factors, followed by Bonferroni-corrected multiple comparison tests. Dots represent individual mouse, while box plots represent Quartile 1 – media – Quartile 3. Mice fed the Control diet (non-stressed females: $n = 11$, stressed females: $n = 9$; non-stressed males: $n = 12$, stressed males: $n = 8$). Mice fed the Med-based diet (non-stressed females: $n = 10$, stressed females: $n = 10$; non-stressed males: $n = 8$, stressed males: $n = 10$). # $p < 0.05$ and ### $p < 0.001$ relative to non-stressed mice and * $p < 0.05$ and *** $p < 0.001$ relative to mice fed the Control diet

Supplementary Table 1. Experimental Control and Med-based diet formula

	Open Standard Control diet	Mediterranean-based diet
Ingredients	**gm**	**gm**
Casein	223	80
Fish Protein Isolate	0	18
Egg White	0	9
Beef, Cooked, Powdered, 5013	0	40
L-Cystine	3	3
Corn Starch	467.4	0
Maltodextrin	150	125
Wheat Starch	0	198.5
Chickpeas, Cooked, Dried	0	36
Lentils, Cooked, Dried	0	36
Cellulose, BW200	75	14
Inulin	0	5
Beta-Glucans	0	5
Soybean Oil	70	0
Menhaden Oil (200 ppm tBHQ)	0	9
Butter, Anhydrous	0	5
Flaxseed Oil	0	6.5
Olive Oil	0	105
Walnuts, Dried, Powdered	0	20
t-BHQ	0.005	0.005
Mineral Mix S10026	10	10
Dicalcium Phosphate	13	13
Calcium Carbonate	5.5	5.5
Potassium Citrate, 1 H2O	16.5	16.5
Vitamin Mix V10001	10	10
Biotin (1%)	0	0.014
Choline Bitartrate	2	2
Fruit and Veggie Blend	0	100
Resveratrol (50% Trans Resveratrol)	0	0.045
Total	**1045.405**	**872.064**
Macronutrient composition		
g		
Protein	197.0	156.4
Carbohydrate	552.9	402.1
Fat	72.7	157.8
Cholesterol	0.0	0.06
Total Fiber	75.0	55.0
Insoluble Fiber	75.0	37.8
Soluble Fiber	0.0	18.4
g%		
Protein	18.8	17.9
Carbohydrate	52.9	46.1
Fat	7.0	18.1
Cholesterol	0.0	0.007
Total Fiber	7.2	6.3
Insoluble Fiber	7.2	4.3
Soluble Fiber	0.0	2.1
kcal		
Protein	*788*	*626*
Carbohydrate	*2212*	*1608*
Fat	*654*	*1420*
Total	***3654***	***3654***
kcal%		
Protein	*22*	*17*
Carbohydrate	*61*	*44*
Fat	*18*	*39*

Supplementary Table 2. Primer sequences used in RT-qPCR experiments.

Gene	Gene sequence/Assay ID
Mus GAPDH	Forward: 5'- GGT CGG TGT GAA CGG ATT TG -3'
	Reverse: 5'- TGC CGT GAG TGG AGT CAT ACT G -3'
Mus Actb	Forward: 5'- GAA CCC TAA GGC CAA CCG TG -3'
	Reverse: 5'- GGT ACG ACC AGA GGC ATA CAG G -3'
Mus TNF-α	Forward: 5'- CTC AGC CTC TTC TCA TTC CTG C -3'
	Reverse: 5'- GGC CAT AGA ACT GAT GAG AGG G -3'
Mus IL-1β	Forward: 5'- TGC CAC CTT TTG ACA GTG ATG -3'
	Reverse: 5'- GTG CTG CTG CGA GAT TTG AA -3'
Mus IL-6	Forward: 5'- ACG GCC TTC CCT ACT TCA CA -3'
	Reverse: 5'- TGC CAT TGC ACA ACT CTT TTC TC -3'
Mus Occludin	Forward: 5'- ACC CGA AGA AAG ATG GAT CG -3'
	Reverse: 5'- CAT AGT CAG ATG GGG GTG GA -3'
Mus Claudin-2	Forward: 5'- GTC ATC GCC CAT CAG AAG AT -3'
	Reverse: 5'- CTG TTG GAC AGG GAA CCA GT -3'
Mus Claudin-3	Forward: 5'- GCA CAA AGA AAC CTC GCC CT -3'
	Reverse: 5'- CCC GTT TCA TGG TTT GCC TG -3'
Mus Claudin-5	Forward: 5'- GGC ACT CTT TGT TAC CTT GAC C -3'
	Reverse: 5'- CAG CTC GTA CTT CTG TGA CAC C -3'

Chapter 7: General Discussion

This work was primarily undertaken in recognition of the need for holistic, sustainable, and safe approaches towards promoting mental health, beyond current pharmacological and psychological treatments, which have proven ineffective in reducing symptoms in a subset of individuals with mental health disorders. The approach explored in this thesis is diet, given the profound influence of dietary patterns on virtually all aspects of health and the relative ease for individuals to modify their dietary choices and habits. Using mouse models, this thesis investigated the capacity of a Med-based dietary pattern, critically acclaimed as a model diet for optimal health, to lower the risk for mental disorders in two vulnerable populations: postpartum mothers and offspring exposed to adversity during a critical period of development. The findings were envisioned to provide valuable information for the development of Med-based dietary guidelines and interventions for improving mental health in postpartum and prenatal stress contexts. The thesis also investigated whether the Med-based diet influenced some of the biological factors (inflammatory, tissue barrier, neurotrophic, and gut microbiota) implicated in mental health disorders in postpartum and prenatal stress contexts, thereby providing vital information for future development of targeted dietary guidelines and interventions. Also, a key information intended to be derived from this thesis was whether the effects of the Med-based diet in prenatally stressed offspring differed according to sex, given that female and male offspring do not have similar physiological and behavioural responses to prenatal stress (Sutherland & Brunwasser, 2018) and the sex bias in pathophysiology and prevalence of depression and anxiety as well as their treatment efficacy (Bangasser & Valentino, 2014; Kessler, 2003). The next section summarizes the key findings from the studies conducted in this thesis.

Summary of key findings

As a first step towards executing this study, a Med-based diet (as well as a matched Control diet) for mice were developed, mimicking the core characteristics of human Med dietary patterns as closely as possible (Chapter 3). Following the dietary development, a pilot study was conducted to confirm that the newly developed diets were palatable and safe for breeding mice, with the results demonstrating that the diets were well eaten and did not lead to adverse breeding outcomes (Chapter 3). Then, the effects of the Med-based diet on anxiety- and depressive-like behaviours of postpartum dams were determined, in addition to assessing changes in inflammatory markers, neurotrophic factors, and the gut microbiota in brain and intestinal regions involved in adaptation to pregnancy and postpartum (reviewed in Chapter 2). As detailed in Chapter 4, the Med-based diet increased grooming behaviours of the postpartum dams (indicative of motivation to self-care and characteristic of a reduced depressive-like state), which was accompanied by reduced expression of the pro-inflammatory cytokines IL-1ß and IL-6 and increased expression of the neurotrophin BDNF and the tight junction claudin-5 in the hippocampus. In contrast, the Med-based diet increased IL-6 and claudin-2 (potentially pro-inflammatory markers) in the colon, in addition to changing the levels of fecal genera that produce SCFAs (notably, *Akkermansia*, *Butyricicoccus*, and *Anaerovorax*) and those putatively involved in intestinal inflammation (*Mucispirillum*).

These effects of the Med-based diet in postpartum dams, particularly those in the brain, were considered promising and reinforced the motivation for a subsequent study investigating whether the diet rescued abnormal behaviours stemming from prenatal stress in neonatal and adult offspring and changes in inflammatory, tight junction, and/or neurotrophic factors (in stress-sensitive brain and intestinal regions) and the gut microbiota, while exploring sex differences in these behavioural and physiological outcomes. As illustrated in Chapter 5, although the Med-based

diet did not limit decreases in USVs emission in the prenatally stressed neonates, it counteracted stress-induced increases in pro-inflammatory cytokines and decreases in BDNF in the hippocampus and prefrontal cortex, predominantly in females. Similarly, in the adult offspring (Chapter 6), the Med-based diet limited anxiety-like behaviours elicited by prenatal stress in females and increases in pro-inflammatory cytokines in the hippocampus and colon, again mostly in females. Furthermore, in the females, the Med-based diet mitigated stress-induced alterations in cecal bacterial communities, notably increases in Proteobacteria, which is linked with colonic inflammation and impaired barrier function, as mentioned in the preceding chapters. Overall, these findings confirm most of the hypotheses in the present thesis that the Med-based diet can improve behavioural and biological outcomes in postpartum mothers and in prenatally stressed offspring. The findings also support the hypothesis that the behavioural and physiological effects of the prenatal stressor and the Med-based diet will vary by sex. The next subsections of this chapter offer an integrated discussion of these findings within a broader context of the literature, exploring their implications and highlighting areas for further research to enhance our current understanding.

7.1 Dietary guidelines based on the Med-based diet may be promising for enhancing mental health in postpartum women and prenatally stressed offspring

Women often experience mental health challenges in the first year of birth, stemming from the profound biological changes associated with pregnancy and postpartum, and compounded by individual and environmental factors, including changes in physical appearance, disruptions to normal daily life, and socioeconomic status. Disturbances in postpartum mental health are not only detrimental to maternal well-being but also pose significant stress on the family unit and social relationships. Importantly, disruptions in family dynamics owing to poor maternal mental health can create a hostile environment for infant care and development, potentially compromising the

offspring's emotional and psychological health across the lifespan. In addition to amplifying the risk for postpartum mental disturbances, adverse prenatal experiences in the mother can disrupt fetal development and further increase susceptibility to neuropsychiatric disorders in the offspring (Van den Bergh et al., 2020). While exposure to prenatal adversity may derail postnatal and lifelong development and health in the offspring, both prenatal and postnatal environmental factors can modify the developmental perturbations and risk for mental disorders associated with prenatal stress in the offspring. For instance, prenatal and postnatal environmental enrichments (which improve living conditions and enhance cognitive, sensory, and social stimulations) have been shown to prevent or reverse the behavioural and physiological impacts of prenatal stress in neonatal and adolescent rat offspring, including social deficits, increased anxiety-like behaviours, elevated cortical pro-inflammatory markers, and decreased spine density of hippocampal neurons in rats (Laviola et al., 2004; Li et al., 2012; Morley-Fletcher et al., 2003). This evidence suggests that improving environmental experiences for both the pregnant mother and the offspring could enhance maternal well-being and counteract the developmental abnormalities stemming from intrauterine stress, thus promoting healthier development trajectory.

While the Med-based diet in this study did not produce robust behavioural improvements in postpartum dams, these dams did exhibit grooming behaviours that suggested they had greater motivation for self-care. Disinterest in self-care and maintaining proper hygiene is one of the main symptoms of depression in postpartum women (O'Hara & Mc Cabe, 2013). Although rodents cannot fully replicate the complex repertoire of symptoms of postpartum depression in humans, which constitutes a potential limitation that could impede results translation to practice, the positive effect of the Med-based diet on grooming behaviours observed in this thesis suggests potential benefits of the diet for maternal self-care in humans. Similarly, the result in the adult

offspring indicates that the Med-based diet could limit the risk of developing anxiety in female offspring exposed to prenatal stress. These latter findings provide important novel and foundational evidence for future research on whether Med-based dietary patterns could mitigate behavioural disorders in adult human offspring exposed to prenatal adversity, which is a current knowledge gap in the literature. Also, an important area for such future research is to determine whether the Med-based diet could improve behavioural outcomes of prenatal stress in adolescents, given that anxiety and depressive disorders can emerge from this age (Solmi et al., 2021) and that prenatal stress also increases anxiety- and depressive-like behaviours in juvenile rodent offspring (Jia et al., 2015; Qulu et al., 2015; Schroeder et al., 2013).

7.2 How may the Med-based diet improve mental health of postpartum mothers and prenatally stressed offspring?

Although the studies conducted in this thesis were not designed to directly investigate the causal mechanisms between the behavioural and biological effects of the Med-based diet in the dams and prenatally stressed offspring, the results provide valuable insights into the biological processes potentially affected by the diet in the context of mental health related to postpartum and prenatal stress, thereby informing future mechanistic endeavours.

Influence on inflammatory, tight junction, and neurotrophic factors

Compelling evidence has demonstrated that the pathophysiology of mental disorders, including anxiety and depression, involves elevated systemic and central markers of inflammation (Enache et al., 2019; Osimo et al., 2020), reduced levels of neurotrophins such as BDNF (Ray et al., 2011), and impaired BBB functions marked by reduced levels of the tight junction claudin-5 (Dion-Albert et al., 2022; Greene et al., 2020). The present study demonstrates that the Med-based diet consistently reduced pro-inflammatory cytokine and enhanced BDNF and claudin-5

expression in the brain and/or intestinal environments of postpartum dams and prenatally stressed offspring. These findings further support the view that the Med-based diet can improve mental health in the mother and the offspring by mitigating neuroinflammation, promoting neuroplasticity, and supporting BBB functions. These biological markers influenced by the Med-based diet provide a foundation for future investigations into their direct involvement in the behavioural outcomes resulting from the Med-based diet. While the above impacts of the Med-based diet are promising, it is important to note unexpected and contradictory observations in dams and offspring fed this diet. Despite its anti-inflammatory effects in the brain of postpartum dams, the Med-based diet increased colonic expression of IL-6 (which exhibits both pro- and anti-inflammatory effects depending on receptor binding) and claudin-2 (a tight junction protein implicated in intestinal inflammation and often elevated in inflammatory bowel diseases). Although this may appear that the diet promoted pro-inflammatory activation in the colonic environment, these effects are likely adaptive in the postpartum context. As reviewed earlier, part of the immunological adaptations to pregnancy and postpartum include low-grade inflammation in the intestinal environment, partly promoted by pro-inflammatory shifts in gut microbiota composition (Koren et al., 2012). Additionally, the permeability of the intestinal barrier increases during the perinatal period, facilitating nutrient and ion transport to meet heightened nutrient and metabolic demands (Astbury et al., 2015). Although studies have not specifically examined changes in intestinal claudin-2 expression during pregnancy or postpartum, this tight junction is crucial for regulating paracellular permeability of intestinal epithelial cells to ions and sodium, and thus suggests that claudin-2 is upregulated in the perinatal period (Amasheh et al., 2002). Thus, the increased expression of IL-6 and claudin-2 in the dams fed the Med-based diet could reflect an adaptive response, and in which case, suggests that the diet supports essential physiological adaptations to the perinatal period. To

clarify these observations, future investigations could assess IL-6 and claudin-2 expression in the colons of non-pregnant dams to determine whether the Med-based diet also increased these markers at baseline or if its effects are specific to and "normal" in a postpartum context. Furthermore, analyzing the protein levels of this IL-6 and claudin-2, alongside other markers of intestinal inflammation and permeability in postpartum dams, could provide deeper insights into the inflammatory status in these mice. The opposite effects of the diet in the brain and gut can also be explained by the unique immune environment in these regions. Microglia constitutes the local immune cells in the brain with occasional exposure to bacterial factors. In contrast, the gut has diverse immune cells, including macrophages, dendritic cells, and natural killer cells with constant interaction with the bacterial populations. These differences in immune structure, composition, and interactions in the brain and gut can result in distinct effects of the Med-based diet, best suited to the unique immune environments in these regions.

Similar to its effects in postpartum dams, the Med-based diet increased pro-inflammatory cytokines in the brain and colon of non-stressed neonatal and adult offspring of both sexes, despite counteracting pro-inflammatory cytokine increases in the stressed mice. These observations also suggest that the Med-based diet might induce adaptive inflammatory responses, contingent on the physiological context. The increase in pro-inflammatory cytokines in non-stressed offspring indicates that the Med-based diet may contribute to priming the immune system. This could be particularly beneficial during critical developmental periods, where a certain level of immune activation is necessary for proper development and maturation of the brain and immune system and other physiological processes. In contrast, the Med-based diet's ability to counteract pro-inflammatory cytokine increases in stressed mice underscores its role in modulating excessive inflammatory responses elicited by adverse external factors (stress, in this case). This dual effect

implies that the Med-based diet can help maintain an optimal balance of immune activation, promoting resilience against stress-induced inflammatory damage while still supporting necessary immune functions. These findings have significant implications for dietary interventions aimed at promoting mental health. The adaptive immune impacts of the Med-based diet suggest that it could be a viable strategy to bolster immune function in individuals during key developmental stages or in those with compromised immune systems. On the other hand, its ability to modulate inflammation in stressed individuals points to potential therapeutic applications for managing stress-related disorders and enhancing overall stress resilience. In summary, the Med-based diet's influence on pro-inflammatory cytokines in both non-stressed and stressed offspring highlights its complex role in immune regulation.

Influence on the gut microbiota

One of the revelations in the past decades, facilitated by advances in genomic techniques, is the influence of the gut microbiota on the risk for depression and anxiety, with compelling findings of potentially causal roles of these microbes in these mental disorders (Kelly et al., 2016). Particularly, the gut microbiota has been suggested to contribute to the altered inflammatory immune mechanism suggested to play a role in depression, through its diverse influence on the gut-brain axis, a bidirectional communication network between the gut and brain through immune, neural, and endocrine pathways (Cryan et al., 2019). Specifically, disturbances in the gut microbiota composition (e.g., due to stress, poor diet, antibiotics) can trigger intestinal inflammation and impair the intestinal barrier, which can allow bacteria or their fragments such as the endotoxin LPS to access the circulation and activate immune response, leading to increased production of pro-inflammatory cytokines (Audet, 2021). These cytokines can cross the blood-brain barrier and contribute to neuroinflammation (Banks et al., 1995), disrupting the BBB,

impairing neuroplasticity, and altering neuroendocrine function, all of which can promote the development of mental disorders.

The Med-based diet has been shown to decrease pro-inflammatory bacteria and enhance the presence of SCFA-producing bacteria in the gut (Mitsou et al., 2017; Seethaler et al., 2022). It was therefore anticipated that this diet would yield similar benefits in both postpartum dams and prenatally stressed offspring. However, the results revealed unexpected outcomes, whereby *Akkermansia*, known for its probiotic and anti-inflammatory properties, decreased in the postpartum dams fed the Med-based diet, while *Mucispirillum*, whose role in host health remains unclear but has been linked to intestinal inflammatory conditions, was more enriched in dams fed this diet. During the perinatal period, there is typically a depletion of SCFA-producing bacteria in the gut and an overrepresentation of Proteobacteria containing pro-inflammatory LPS (Koren et al., 2012). The observed changes in gut microbiota composition in dams fed the Med-based diet may reflect adaptations to the postpartum environment. Interestingly, despite these shifts, metabolic profiling of the gut microbiota in the dams did not detect alterations in inflammatory pathways such as LPS biosynthesis, peptidoglycan biosynthesis, or the NOD-like receptor pathway, suggesting that the changes in microbial composition may not necessarily translate into functional changes at the host level. In prenatally stressed females, the Med-based diet effectively limited the increase in Proteobacteria (which is implicated in gut inflammation), and this result also coincided with the absence of elevated pro-inflammatory markers in the colonic environment of these mice, although no correlations were obtained for these outcomes. These findings demonstrate that the Med-based diet impacted the gut microbiota of the postpartum dams and offspring; however, whether these impacts influenced the behaviours of these mice remain unclear, especially given the lack of correlations between the microbiota behavioural changes in these mice.

7.3 Does sex matter in future development of Med-based dietary guidelines and interventions for mental health improvements in the context of prenatal adversity?

One of the novel contributions and strengths of this thesis is the demonstration that the Med-based diet's mitigating effects on behavioural and physiological alterations due to prenatal stress are predominantly observed in females during both neonatal and adulthood stages. This finding is particularly significant because previous studies on the dietary impact on mental and other health aspects often overlook the role of sex in biological outcomes. This oversight has led to a substantial gap in our understanding of how females and males might differentially benefit from specific dietary practices. The results of this thesis suggest that female and male offspring may require different prenatal/postnatal dietary modifications to mitigate the effects of prenatal adversity and promote optimal mental health outcomes. Consequently, dietary guidelines and interventions for mental health should not follow a one-size-fits-all approach, which is commonly seen in public health and nutrition practices. The findings emphasize the importance of considering sex as a critical variable in dietary research and practice. Moreover, exploring the underlying biological mechanism, such as hormonal influences, genetic factors, and differences in gut microbiota composition, will be crucial in understanding the sex-specific dietary impacts. By integrating sex-specific data into dietary guidelines, practitioners can develop more effective strategies that enhance mental health and overall well-being for both sexes. This thesis challenges the norm and underscores the necessity of including sex as a fundamental factor in dietary research, advocating for more nuanced and personalized dietary recommendations that account for the unique needs and responses of each sex.

7.4 Concluding remarks

This thesis provides novel information supporting the beneficial impact of Med-based diets for improving mental health in postpartum mothers and in offspring exposed to prenatal adversity, particularly in females. However, existing socioeconomic barriers could impede the successful implementation and effectiveness of dietary interventions for mental health promotion in these populations. Factors like poverty, income disparities, and the rising rate of food insecurity critically influence an individual's ability to adopt and maintain a healthy diet. When faced with limited access to food due to high costs or unavailability, individuals may prioritize food availability over quality, limiting opportunities for engagements in health-promoting dietary choices. This underscores the necessity of addressing socioeconomic determinants of health as part of any comprehensive strategy to improve mental health outcomes through dietary interventions. Tackling mental health issues requires a holistic approach that fundamentally addresses the influence of socioeconomic factors alongside existing pharmacological and behavioural interventions. Therefore, policies and interventions aimed at reducing poverty, closing income gaps, and ensuring food security are essential components of a broader strategy to enhance mental health. Despite these potential bottlenecks, diet, such as Med-based dietary patterns, remains a promising avenue for modifying mental disease risk, especially in the contexts of postpartum and prenatal adversity.

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