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Investigating the Association Between Prenatal Stress and Neurodevelopment: The Contribution of the Gut Microbiota

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Investigating the Association Between Prenatal Stress and Neurodevelopment: The Contribution of the Gut Microbiota

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Abstract

Prenatal stress is common among pregnant individuals and has been associated with adverse neurodevelopmental outcomes in children. Emerging evidence has independently linked children's gut microorganisms (i.e., gut microbiota) to prenatal stress and neurodevelopment. For this reason, the current study wanted to determine if the child's gut microbiota mediates the relationship between prenatal stress and Full-Scale Intelligence Quotient (FSIQ) in children aged 3-4. Prenatal stress was operationalized for 248 women using maternal salivary cortisol samples and mood questionnaires to generate a psychological distress score at each trimester. Children collected fecal samples and completed a standardized neurocognitive assessment to evaluate gut microbiota features and FSIQ, respectively. Results indicate that prenatal stress was associated with the child's gut alpha diversity (i.e., different bacteria types), bacteria relative abundance, and selective metabolic pathways in a trimester-dependent manner. Also, we found that the child's gut microbiota features are not associated with FSIQ. Even though prenatal stress was associated with the child's gut microbiota, the study findings indicate that child's gut microbiota does not mediate the relationship between prenatal stress and FSIQ in children aged 3-4. Despite previous studies providing strong evidence linking gut microbiota to neurodevelopment, the current study does not provide direct or indirect support for this relationship.

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I. Introduction

Pregnant individuals make significant life adjustments in their careers, finances, and social life, which can be very stressful (La Marca-Ghaemmaghami & Ehlert, 2015). Approximately 30% of pregnant individuals worldwide report moderate stress levels during pregnancy (Bleker et al., 2019). Excessive prenatal stress is associated with preterm birth, low birth weight, and adverse neurodevelopmental outcomes (Dunkel Schetter & Tanner, 2012). Emerging evidence suggests that prenatal stress influences many biological systems in both the mother and baby. Recent evidence indicates that this includes bacterial colonization of the gastrointestinal tract of infants (Zijlmans et al., 2015). Gut microbiota (e.g., gut bacteria) can produce metabolites, including amino acids and short-chain fatty acids (SCFAs), which the brain can sense through various physiological routes, including the vagus nerve and innervation of the gastrointestinal tract, immune system, and various metabolic pathways. Importantly, the gut microbiota can communicate and be sensed by the brain during critical windows of neurodevelopment (Cryan et al., 2019). Recent studies have linked the infant gut microbiota with cognitive development and brain functional connectivity (Carlson et al., 2018; Gao et al., 2019). Yet, it is currently unclear whether prenatal stress has long-term implications on children's gut microbiota composition and how these compositional changes affect neurodevelopment. For this reason, my thesis aimed to examine the relationship between prenatal stress, gut microbiota, and Full-Scale Intelligence Quotient (FSIQ) in children aged 3-4.

Stress Physiology During Pregnancy

Stress can be defined as the relationship between an individual and their environment which they evaluate to be demanding or surpassing resources for coping (Lazarus & Folkman, 1984). Stress can lead to imbalances in the body and stem from physical, emotional, and

immunological factors. The hypothalamic-pituitary-adrenal (HPA) axis is a central neuroendocrine stress response system within the human body (Dickens & Pawluski, 2018). Activation of the HPA axis results in the secretion of cortisol, a steroid hormone responsible for increasing gluconeogenesis and protein and fat metabolism (Frankiensztajn et al., 2020). The HPA axis plays an integral role in maintaining the body's homeostasis, it is always active and regulates glucocorticoid production (Bellavance & Rivest, 2014). The HPA axis consists of a hormone cascade beginning in the brain and extending to the adrenal gland. The cascade begins at the paraventricular nucleus of the hypothalamus, which releases corticotropin-releasing hormone (CRH). CRH stimulates the anterior pituitary gland to release adrenocorticotropic hormone (ACTH) into systemic circulation. ACTH will stimulate the adrenal glands to release cortisol. The hypothalamus and hippocampus detect high cortisol levels in systemic circulation and cease to release CRH in negative feedback control (Frankiensztajn et al., 2020).

During gestation, cortisol has additional functions aside from maintaining the homeostasis of the mother's body, and that is to prepare the infant for life outside the uterine environment. Cortisol aids in the maturation of thermoregulation, lung development, brain development, and glucose metabolism (Dickens & Pawluski, 2018; Matthews, 2001). The placental structure secretes placental CRH, stimulating the maternal pituitary gland, which leads to an increase in the overall abundance of cortisol in systemic circulation during gestation via positive feedback (Lindsay & Nieman, 2005). To counteract this positive feedback mechanism, the levels of corticosteroid-binding globulin (CBG) increase to capture free circulating cortisol in the maternal bloodstream. However, CBG has a higher affinity for progesterone, another steroid hormone secreted by the placenta (Dickens & Pawluski, 2018). In turn, the placenta will release an enzyme called 11β-hydroxysteroid hydrogenase type 2 (11β-HSD2), which converts

glucocorticoids into their inactive form cortisone to protect the developing fetus from excess maternal cortisol exposure (Edwards et al., 1993; La Marca-Ghaemmaghami & Ehlert, 2015). This is not to suggest that 11β-HSD2 inactivates all maternal cortisol. Particular concentrations of cortisol need to cross the placenta and reach the fetus to stimulate organ development. For instance, cortisol stimulates surfactant production, a compound composed of proteins and phospholipids that helps lower the surface tension within the alveolus, the air-filled sacs of the lungs where gas exchange occurs. The production of surfactants is vital as it prevents lung collapse when breathing upon birth (Bolt et al., 2001). However, excessive maternal cortisol exposure has also been studied for its negative effects on neurodevelopment (Lenniger et al., 2020).

Prenatal Stress Affects Neurodevelopment

Contemporary studies highlight that prenatal stress is associated with adverse neurodevelopmental outcomes (Van den Bergh et al., 2020). These negative outcomes include lower developmental scores in gross motor movements and adaptive and social behaviour (Lin, Xu, et al., 2017). Other outcomes include poorer cognitive development and academic achievement (Li et al., 2013; Schechter et al., 2017; Tarabulsy et al., 2014). Previous studies investigating the relationship between prenatal stress and neurodevelopment have focused predominantly on maternal and infant HPA axes because the fetal brain has cortisol receptors present within the limbic system, the brain network that is associated with emotion, cognition, and behaviours (Catani et al., 2013; Matthews, 2001). As such, much research on prenatal stress has focused on understanding the implications of maternal cortisol exposure on neurodevelopment. For example, animal studies have highlighted that maternal cortisol exposure can influence neurogenesis, synaptogenesis, myelination, and the development of both axons and

dendrites in brain areas where cortisol receptors are present like the amygdala, prefrontal cortex, and hippocampus (Charil et al., 2010; Davis et al., 2016).

Human-based studies have also provided evidence that maternal cortisol exposure has timing effects on child cognitive performance. Children exposed to higher maternal cortisol concentrations in the third trimester displayed increased cognitive performance at 6-9 years of age (Davis et al., 2016). Additionally, an inverse relationship has been found between fetal cortisol exposure in the second trimester and infant cognitive development at 17 months of age (Bergman et al., 2010). Furthermore, infants exposed to elevated maternal cortisol levels in early pregnancy had lower development scores at the age of 1. In comparison, children exposed to elevated maternal cortisol levels in late pregnancy had higher development scores at the same age (Davis & Sandman, 2010). Moreover, maternal cortisol exposure has sex-specific associations with child neurodevelopmental outcomes. Female children exposed to elevated maternal cortisol levels during pregnancy exhibited increased internalizing symptoms compared to male children (Graham et al., 2019).

However, other physiological mechanisms in addition to maternal cortisol can explain the relationship between prenatal stress and neurodevelopment. For example, prenatal stress can affect maternal immunity, lead to lifestyle changes (e.g., sleep, physical activity, eating), and is associated with fetal epigenetic modifications, all of which can affect neurodevelopment (Beijers et al., 2014). Particularly important to our discussion is that prenatal stress is associated with alterations of the infant gut microbiota, which is associated with neurodevelopment (Warner, 2019; Zijlmans et al., 2015). To understand how gut bacteria can influence neurodevelopment, there must be an in-depth discussion of the microbiota-gut-brain axis.

The Microbiota-Gut-Brain Axis

The gut microbiome refers to the total genetic makeup present within humans' gastrointestinal (GI) tract. The GI tract harbours bacteria, fungi, viruses, and protozoa (Corrigan et al., 2018). Collectively, bacteria, archaea, and eukarya present in the gut are called gut microbiota (Thursby & Juge, 2017). There are approximately 100 trillion microorganisms present within the GI tract (Valdes et al., 2018). This astounding number has led researchers to question the role of the gut microbiome in human health. Experimental findings show that gut bacteria aid in metabolism, have a role in immune response and communicate bidirectionally with the central nervous system (CNS) **(Figure 1)** (van de Wouw et al., 2017; Yoon et al., 2014; Zhang et al., 2015). Gut microbiota can produce metabolites including amino acids, neurotransmitters, and SCFAs. The bacterial-derived metabolites are a primary focus of health research due to their ability to interact with the host physiology via autonomic nervous stimulation and through endocrine, immune, and metabolic pathways (Cryan et al., 2019). For example, stimulation of the vagus nerve (a cranial nerve connected to the GI tract) is altered in response to the type of metabolites produced by gut microbiota, and the CNS senses this stimulation (Averina & Danilenko, 2017). From a health perspective, alterations in the composition of the gut microbiota are associated with various human diseases (e.g., obesity, irritable bowel syndrome, dementia, asthma), psychiatric disorders, cognition, and social behaviour (Cryan et al., 2019; Foster & McVey Neufeld, 2013; Zhang et al., 2015). Regarding neurodevelopment, the argument is that alterations to the gut microbiota composition will lead to different signals being produced and received by the CNS, leading to specific neurodevelopmental changes in early childhood when neurons are forming and refining connections with one another (Cryan et al., 2019; de Weerth, 2017; Dinan & Cryan, 2017).

Changes in neurodevelopment can impact memory, motivation, mood, and stress reactivity (Schlotz & Phillips, 2009). To understand how bacteria could influence neurodevelopment, we must first discuss how and when bacteria colonize the infant's gut.

Bacterial colonization of the gut begins during birth and has short- and long-term health implications (Francavilla et al., 2018). Children born via cesarean section (c-section) have their GI tract predominantly colonized by bacterial strains native to the maternal skin. In contrast, children born vaginally have their GI tract predominantly colonized by bacterial strains native to the maternal vagina (Arboleya et al., 2018; Eshraghi et al., 2018). In addition, children born via c-section have delayed colonization of keystone species (e.g., *Bacteroides*) compared to vaginally born children. Keystone species are associated with mucosal barrier function, which reduces the growth of pathogenic strains like *Clostridium difficile* (Codagnone et al., 2019; Mueller et al., 2015). In addition, bacterial colonization of the infant's GI tract coincides with critical windows for immune and CNS development (Houghteling & Walker, 2015; Warner, 2019). As a result, the maternal microbiome matters, and its composition and function are influenced by diet, level of stress/anxiety, lifestyle, genes, and overall health. However, feeding practices (e.g., formula-feeding vs. breastfeeding), number of people within the household, proximity to animals, and antibiotic use are factors that also alter the GI tract composition after birth (de Weerth, 2017). However, the mode of delivery does not have a significant influence on the child's gut microbiota composition past 6 months of age (Ríos-Covian et al., 2021). A child's gut microbiota composition changes significantly during the first two years of life and stabilizes around age 3 when it begins to resemble adult composition (Arrieta et al., 2014). Therefore, there is a transfer of maternal bacteria to the neonate during birth, whether it be vaginal or skin

bacteria (Arboleya et al., 2018). Yet, this understanding alone does not explain how bacteria within the gut can affect neurodevelopment or the CNS in general.

Germ-free (GF) models provide a proof of principle for the connection between gut microbiota and the CNS. Mice devoid of bacteria display a wide array of alterations in the CNS. For example, GF mice display increased permeability of the blood-brain barrier (BBB) associated with decreased expression of tight junction proteins (Braniste et al., 2014). They also show increased myelination of the prefrontal cortex, a brain region implicated in neuropsychiatric disorders (e.g., depression, autism) (Hoban et al., 2016). Other changes include decreased maturation of microglia, the cells in the CNS responsible for immune response (Erny et al., 2015). To further add, GF mice display distinct alterations within the hippocampus, including decreased neurogenesis and dendritic connections, and sex-specific changes in brain-derived neurotrophic factor (BDNF) (Clarke et al., 2013; Luczynski et al., 2016; Ogbonnaya et al., 2015). GF mice also display alterations in levels of neurotransmitters (e.g., serotonin), amino acids, and SCFAs, and all of these molecules influence neurophysiology (Cryan et al., 2019). For instance, particular gut bacteria can produce intermediate molecules (e.g., tryptophan) for serotonin production and regulate its release; importantly, serotonin is implicated in neuropsychiatric disorders (Agus et al., 2018; Hata et al., 2017).

Human-based studies are less numerous in microbiome research; however, they further support the relationship between gut microbiota and the CNS. For example, supplementation of *Bifidobacterium longum* 1714 improves hippocampus-dependent visuospatial performance in adult males subjected to acute stress (Allen et al., 2016). Likewise, supplementation of *Lactobacillus plantarum* P8 is associated with reduced stress and anxiety and improved emotional and verbal memory among moderately stressed adults (Lew et al., 2019). To further

add, the consumption of fermented milk with a probiotic is associated with alterations in brain connectivity (e.g., somatosensory network, prefrontal cortex). These changes in connectivity were associated with changes in emotional attention tasks among healthy female adults (Tillisch et al., 2013). Therefore, both the GF model and human studies illustrate a link between gut microbiota and the CNS.

To summarize, children are first colonized with bacteria at birth and this initial colonization event matters because it takes place during critical windows of both the immune and CNS development (Arboleya et al., 2018). Additionally, the initial colonization event sets the stage for the programming of the adult microbiota composition (Houghteling & Walker, 2015). Bacterial-derived metabolites are "sensed" by the CNS and are associated with changes in mood, cognition, social behaviour, and various human diseases (Cryan et al., 2019).

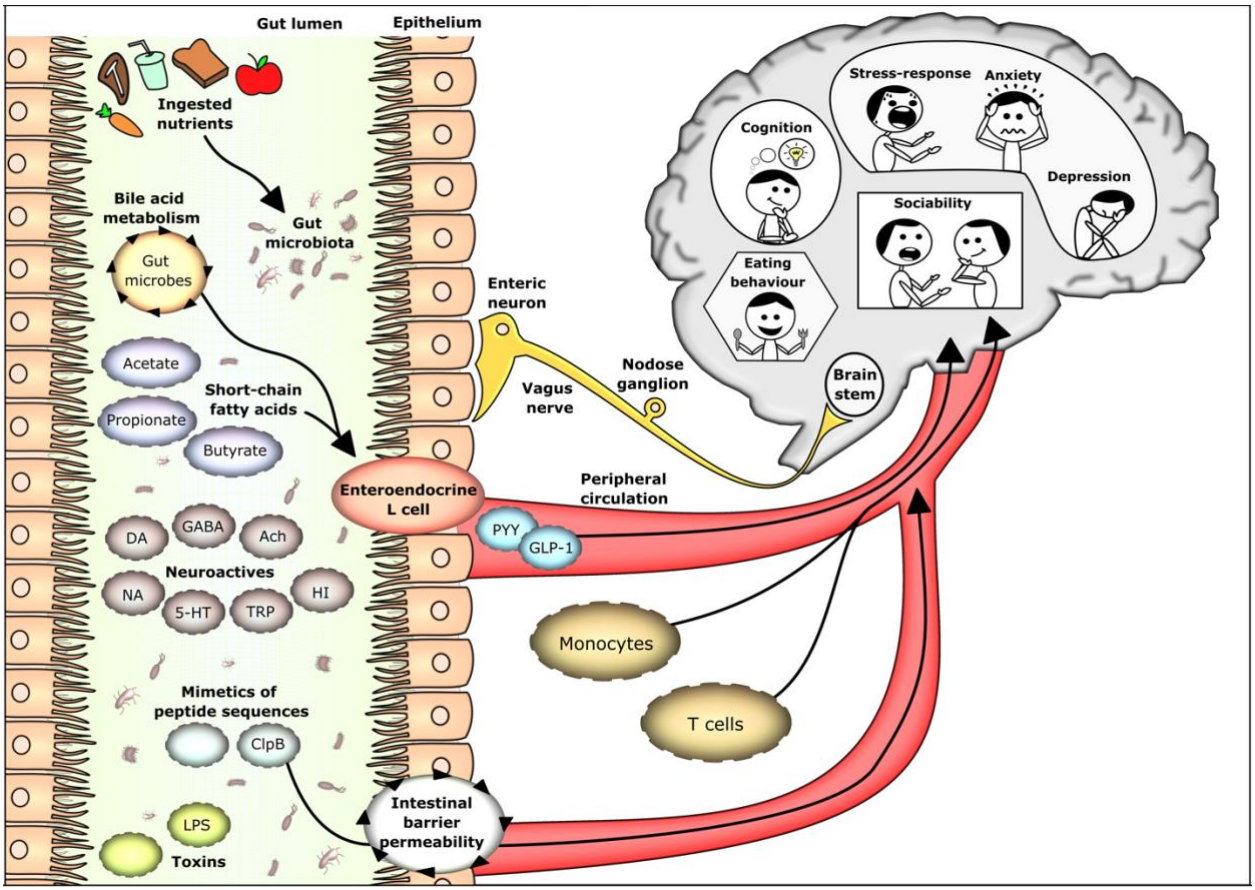

Figure 1. Diagram illustrating bidirectional communication between gut and brain. Provided by Marcel van de Wouw adapted from "Microbiota-Gut-Brain Axis: Modulator of Host Metabolism and Appetite" by Marcel van de Wouw and colleagues, 2017, *The Journal of Nutrition*, 147(5), 727–745.

Prenatal Stress in Relation to Infant Gut Microbiota

The general principle is that bacteria, whether skin or vaginal, gets transferred to the neonate during the birthing process. As such, there is the potential to pass on imbalances present in the maternal microbiota, which implicates the infant's health (Amabebe & Anumba, 2018; Jašarević et al., 2018; Jašarević, Howerton, et al., 2015). For example, vaginal microbiota predicts components of the infant's microbiota (Jašarević et al., 2021). Keystone species found within the vagina during pregnancy, like *Lactobacillus spp.,* are the first bacterial genera to colonize infants' gut and establish a commensal relationship with the host (Jašarević, Rodgers, et al., 2015).

Relevant to our topic, is the fact that prenatal stress can affect the composition of the vaginal microbiota. For example, prenatal stress results in the upregulation of the maternal HPA axis leading to cortisol secretion, which disrupts vaginal glycogen production needed to mature the vaginal epithelium and maintain *Lactobacillus spp.* The disruption to glycogen production decreases *Lactobacillus spp.* and lactic acid production needed to maintain the vagina at a low pH to prevent the growth of pathogenic bacterial strains, and this is associated with the alteration of the vaginal microbiome (Amabebe & Anumba, 2018; Jašarević et al., 2018). To be more specific, prenatal stress has been associated with the loss of commensal bacteria present in the vaginal microbiome (Jašarević, Howerton, et al., 2015). This is important because the birth canal is the site of vertical transmission of bacteria from mother to infant in vaginal birth (Arboleya et al., 2018; Jašarević, Howerton, et al., 2015). Both animal and human studies provide support for this claim.

For instance, dams subjected to chronic prenatal stress had a reduced abundance of vaginal *Lactobacillus,* which was associated with decreased transmission and with the type of

amino acids present in the brain tissue of pups (Jašarević, Howerton, et al., 2015). Also, other sex-specific alterations were observed when comparing the abundance of *Odoribacter*, *Desulfovibrio*, *Flexispira*, *Mucispirillium*, *Lachnospiraceae*, and *Clostridiales* (Jašarević et al., 2017). Similarly, women who experienced significant prenatal stress had offspring colonized with a greater abundance of bacteria commonly associated with adverse health outcomes (*Escherichia*, *Serratia*, and *Enterobacter*) and had a lower number of bacterial strains associated with positive health outcomes (*Lactobacillus*, *Aerococcus*, *Lactococcus*, *Bifidobacteria*) (Zijlmans et al., 2015). To further add, mothers with high prenatal stress and cortisol levels had children with increased *Proteobacteria* (phylum) present within their fecal sample at 2.5 months of age. The *Proteobacteria* phylum contains genera that have been linked to host inflammation (Aatsinki et al., 2020). Lastly, it has also been found that greater pregnancy-related anxiety is associated with reduced alpha diversity and overall abundance of *Enterococcaceae* in newborn children (Hu et al., 2019).

Collectively, these research findings are crucial because they demonstrate that prenatal stress is associated with the type and abundance of bacterial colonization. Evidence suggests that compositional changes of the gut microbiota post-birth endure long enough to affect neurodevelopment (Mueller et al., 2015; Salminen et al., 2004). Considering that previous research has indicated that prenatal stress has long-term effects on neurodevelopment, it is necessary to further investigate the relationship between prenatal stress and gut microbiota in preschool children. Since (1) the composition of the gut microbiota stabilizes in the early preschool years, and (2) gut microbiota is associated with neurodevelopment (Arrieta et al., 2014; Carlson et al., 2018; Rothenberg et al., 2021).

The Relationship Between Gut Microbiota and Child Cognitive Outcomes

Recent studies have demonstrated a link between child gut microbiota composition and cognitive outcomes in early childhood. For example, children who have a high abundance of *Clostridiales* scored lower in communication and personal and social skills at the age of 3. In comparison, children with reduced fine motor skills had a higher abundance of *Bacteroides* at the same age (Sordillo et al., 2019). Similarly, children who have greater bacterial diversity (i.e., alpha diversity) had an overall lower composite score (a score similar to IQ) at the age of 2 on The Mullen Scales of Early Learning cognitive assessment test. Specifically in the subset of expressive language and visual perception (Carlson et al., 2018). In addition, there is a positive association between the abundance of *Faecalibacterium, Sutterella,* and *Clostridium* cluster XIAa and scores on the Mental Development Index and Psychomotor Developmental Index in children aged 3 (Rothenberg et al., 2021). Altogether, previous research findings provide support for the link between gut microbiota and neurodevelopment.

The Current Study

Accordingly, at this point, it may seem that there are two main conclusions based on previous work: (1) Prenatal stress is associated with adverse neurodevelopmental outcomes and (2) the microbiota-gut-brain axis affects neurodevelopment. However, a gap exists in the literature as there are no current studies, to my knowledge, that examine the relationship between prenatal stress, gut microbiota, and typical neurodevelopment. Therefore, I have addressed this gap by examining the association of prenatal stress on the composition and function of the gut microbiota at preschool age and how the gut microbiota, in turn, is associated with child FSIQ scores. FSIQ is a global measure that is used to access intellectual functioning. Therefore, it is an appropriate measure to access neurodevelopment.

Previous studies have focused on understanding the relationship between these variables in children with neurodevelopmental disorders. This is another component of novelty in my work, as I will be doing research in the context of typical development. An advantage of looking at the relationship between all variables in the context of typical development is that it will elucidate the relative contribution of gut microbiota in neurodevelopment. The current study sought to understand how prenatal stress relates to cognitive outcomes in childhood and investigate the contribution of the gut microbiota in this relationship.

Aims and Hypotheses

Aim 1: Determine if features of the child's gut microbiota and metabolic profile are different depending on the amount of prenatal stress exposure.

First Hypothesis: Children born to mothers who experienced a greater amount of prenatal stress will have an altered gut microbiota composition and function compared to children born to mothers who experienced less prenatal stress.

Aim 2: Determine if children's FSIQ and primary indices scores (verbal comprehension, visual spatial, working memory) are different depending on the amount of prenatal stress exposure.

Second Hypothesis: Children born to mothers who experienced greater prenatal stress will have lower FSIQ and primary indices scores compared to children born to mothers who experienced less prenatal stress.

Aim 3: Determine if children's FSIQ and primary indices scores (verbal comprehension, visual spatial, working memory) are different depending on features of the child's gut microbiota (alpha diversity, taxa abundance) and metabolite profile.

Third Hypothesis: Children with a higher FSIQ and primary indices scores will have an altered gut microbiota composition and function compared to children with a lower FSIQ and primary indices scores.

II. Methods

Overview of Study

During each trimester of pregnancy, participants completed several mental health questionnaires (i.e., POMS-15, EPDS, SCL-90-R) and collected a salivary sample (i.e., cortisol levels). After pregnancy when children were aged 3-4 years, they completed a neurocognitive assessment (WPPSI-IV^{CDN}) and collected a stool sample (gut microbiota, metabolomics). A parent or legal guardian of the participating child additionally completed a dietary and home environment questionnaire (food frequency and HOME-SF questionnaire, respectively). Child diet and home environment are relevant covariates for the gut microbiota and child cognitive outcomes (Leeming et al., 2019; Tong et al., 2007) **(Figure 2) (Supplementary Table S1).**

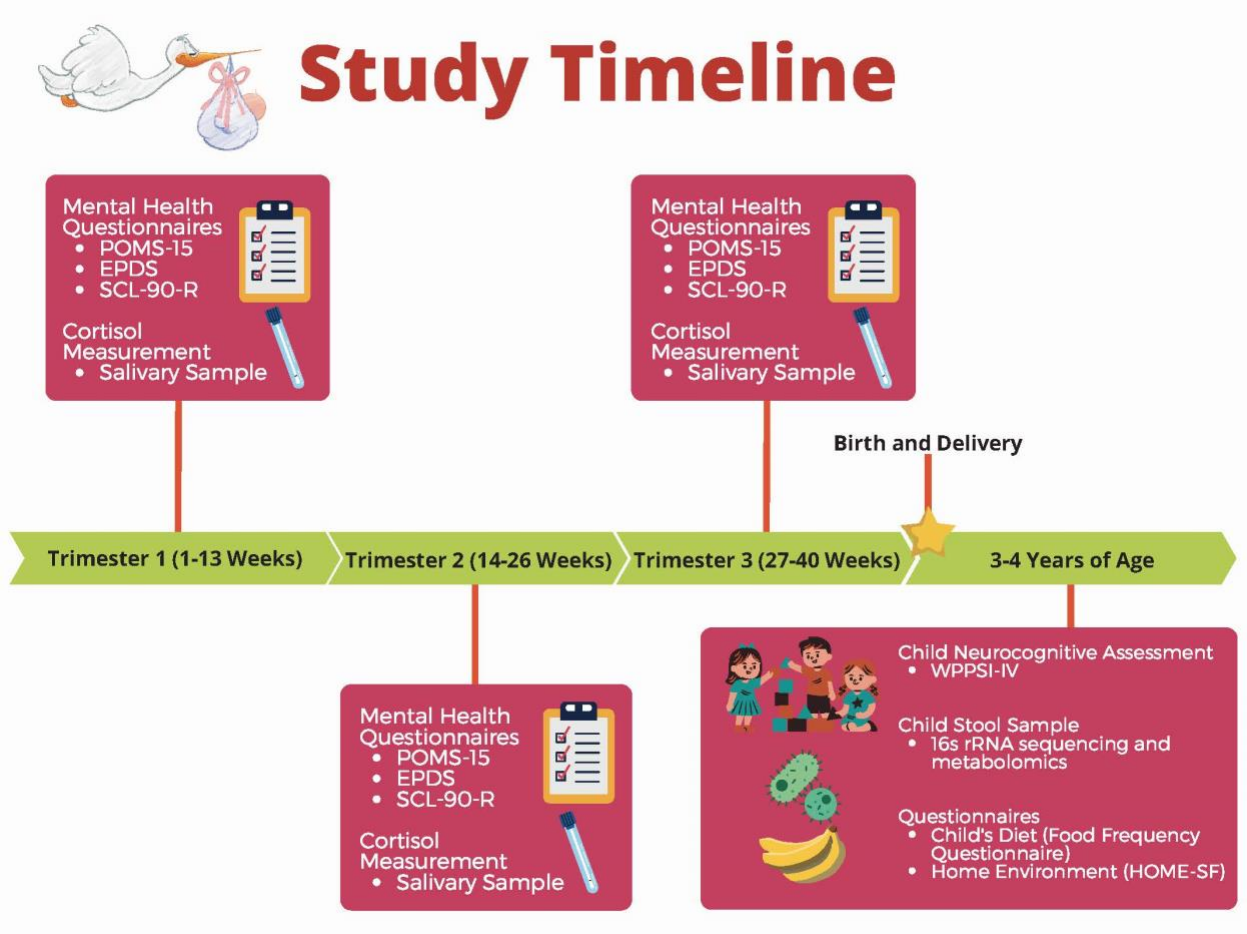

Figure 2. Figure illustrating assessment timeline.

Participant Recruitment

Participants for the current study were drawn from the Alberta Pregnancy Outcomes and Nutrition (APrON) study (Kaplan et al., 2014). At the time of recruitment, women had a gestational age of < 22 weeks. Participants were excluded if they could not read or speak English. Participants were also excluded depending on particular health behaviours and/or obstetric factors, which included: a) administration of exogenous steroids for management of preterm labour, b) administration of corticosteroids (e.g., for management of asthma), c) maternal smoking, alcohol consumption, or recreational drug use (or history of these during current pregnancy), d) multiple birth pregnancy, and e) genetic fetal abnormalities. A parent or legal guardian of participating children provided informed written consent before sample and data collection. Children were excluded if they were diagnosed with neurocognitive deficits (including suspected diagnosis of autism) or if they had recent antibiotic exposure (2 weeks before the fecal sample collection).

Maternal Mood Questionnaires

Participants completed 3 separate mood questionnaires. One questionnaire was the profile of mood states-15 (POMS-15) which provides a multidimensional measure of mood. Items on POMS-15 were rated on a 5-point Likert scale from “not at all” to “extremely” the scale assesses 5 mood dimensions: anger, sadness, anxiety, depression, fatigue, and vigour/positive affect. The total negative mood score was obtained by subtracting the vigour subscale from the sum of other subscales (Giesbrecht et al., 2015). Participants were also asked to complete items from the demands scale which relate to perceived external stress (Fliege et al., 2005). The POMS-15 is a valid and reliable instrument for detecting within-person changes in mood (Cranford et al., 2016; Giesbrecht et al., 2015; Giesbrecht et al., 2013).

In addition, participants completed the *Edinburgh Postnatal Depression Scale* (EPDS) and the *Symptom Checklist-90-R* (SCL-90-R) questionnaires during each trimester. The EPDS is a 10-item self-report questionnaire (Levis et al., 2020). It is a valid instrument for detecting depression during pregnancy and post-partum (Rubertsson et al., 2011). The instrument has a sensitivity of 77% and a specificity 94% for detecting depression among pregnant individuals using a cut-off score of ≥13 (Rubertsson et al., 2011). The SCL-90-R is a multidimensional self-report questionnaire used to access psychological symptoms, this instrument has 9 symptom scales (Derogatis, 2021). For this study, I used the anxiety subscale which consists of 10-items. The SCL-90-R is an instrument that is both reliable and valid to access psychological distress (Lin, Zhou, et al., 2017).

Maternal Salivary Cortisol Collection

Participants collected their saliva at 4-time points on 2 consecutive weekdays in early (<22 weeks gestation) and late pregnancy (32 weeks gestation). The 4 time points included: immediately upon waking, 30 min post waking, mid-morning, and 9 PM. In total, participants provided 16 saliva samples. Participants were asked to refrain from food or beverages (except water) 30 minutes before saliva collection. Each participant received a personal digital assistant (PDA) that would provide reminders to collect saliva samples throughout the day and recorded the time of sample collection - considering that cortisol displays day-to-day variation, adherence to the study protocol was critical (Dahlgren et al., 2009). To ensure this, data was reviewed using two criteria: (1) the early morning sample was collected within 15 minutes from when the PDA provided a reminder alarm and (2) the other collection samples were collected within 30 minutes from the reminder alarm. At the time of saliva collection, participants were asked to complete the POMS-15 questionnaire. Participants were to collect saliva samples by placing a synthetic swab

underneath their tongue. Samples were stored at −20°C and were assayed in duplicate for cortisol using 510k cleared high-sensitive enzyme immunoassay (Salimetrics, LLC, State College, PA). Repeated salivary cortisol samples were used to calculate the Area-Under-Curve with respect to ground (AUC_G) (i.e., total cortisol secretion throughout the day) which uses the trapezoid method as described by Pruessner and colleagues (2003) (Pruessner et al., 2003).

Cumulative Stress Score

The inspiration behind the cumulative stress score came from the work of Zijlmans and colleagues (2015) who created a maternal prenatal stress score using 5 mood questionnaires (e.g., Prenatal Daily Hassle, State-trait Anxiety Inventory) and 5 separate cortisol concentrations (e.g., AUCg). Likewise, a cumulative stress score was created which included a combination of both subjective measures from the POMS-15 questionnaire (e.g., anxiety, depressed, external demands) and objective measures which included maternal salivary cortisol. However, unlike Zijlman's and colleagues (2015), who used a median cut-off score to generate a prenatal stress score, the cumulative stress score was created using a quartile approach. The benefit of this approach is it allows naturally continuous variables (e.g., AUCg) to remain continuous when generating the cumulative stress score which in turn reduces the loss of information from the sample (Bakhshi et al., 2012). Quartiles for each stress index were assigned a value from 1- 4. For example, if the raw score for anxiety was in the first quartile the value assigned was 1 (1 = first quartile, 2 = second quartile, 3 = third quartile, 4 = fourth quartile). Next, the assigned scores for each stress index were added and averaged to produce a cumulative stress score ranging from 1- 4. This approach avoids the unnecessary categorization of participants into groups (e.g., high prenatal stress versus low prenatal stress).

Maternal Psychological Distress

A psychological distress score was generated for each participant at each trimester by (1) converting the raw scores of the EPDS and SCL-90-R into z-scores and then (2) generating an average score. There are two primary reasons why taking an average score is justified in this case. First, the two constructs are theoretically related in practice and are highly comorbid. Secondly, in our sample, they were strongly correlated ($r = .63$, $p < .001$). Psychological distress encompasses non-specific symptoms of stress, anxiety, and depression and has been found to impact 21.1% of pregnant North American women (Obrochta et al., 2020; Viertiö et al., 2021).

Dietary Assessment

Parents completed a 100-item semi-quantitative Food Frequency Questionnaire (FFQ) for their child at 3-years of age. It is important to note that the diet of children is relatively stable at the ages of 3-4 (Gasser et al., 2017). The FFQ lists food items that are recommended by Canada's Food Guide and parents indicate how often their child consumes certain food items (Morrison et al., 2009). The options include 1–3 times per week; 4–6 times per week; 1 time per day; 2–3 times per day; 4–6 times per day; and >6 times per day. Parents can also write down additional food items not listed on the FFQ in the open-ended section. The FFQ was used to generate a diet quality index score (DQI) which was constructed by calculating the ratio between consumption versus recommended intake for the following 6 food components: (1) Vegetables and Fruit, (2) Grain Products, (3) Milk and Alternatives, (4) Meat and Alternatives, (5) Candy/Snacks, and (6) Sugar-Sweetened Beverages. The ratio for each component was then summed to give a total score ranging from 0 to 6, where higher values indicate greater adherence to the recommended values from Canada's Food Guide. The DQI is an index that has been

previously used to assess diet quality among Canadian preschool children in Alberta, who had a mean DQI value of 3.7 (Jarman et al., 2020).

Home Environment

To access factors of the home environment the Home Observation and Measurement of the Environment – Short Form (HOME-SF) was used. The HOME-SF has been extensively used in child development research (Mott, 2004). The parent or legal guardian completed the HOME-SF questionnaire, which was used to assess cognitive stimulation within the home environment which affects child development (Mott, 2004).

Child Stool Sample Collection

Stool samples were collected from 3–4-year-old children throughout 2014-2018. Parents collected child stool samples at home in a sterile 50-ml plastic conical collection tube with a plastic applicator. Stool samples were kept in the home freezer (-20°C) for up to 24 h before being transported back to the study lab in a cooler surrounded by freezer packs. At the lab, stool samples were stored in a -80°C freezer. DNA from fecal samples were extracted using FastDNA® Spin Kit for Feces (MP Biomedicals, Santa Ana, California, US) following the manufacture's instruction.

16S rRNA amplicon sequencing

The abundance of gut bacteria was measured using 16S rRNA gene amplicon sequencing which was performed using the MiSeq platform at the Centre for Health Genomics and Informatics (University of Calgary, Calgary, Canada). PCR amplification of the V3 and V4 region of the 16S rRNA gene was performed using manufacturer recommended primers, followed by a quality check of sequences (Ewels et al., 2016; Martin, 2011). For this analysis both alpha diversity (observed OTUs, Faith phylogenetic diversity, Shannon diversity, Chao1 diversity) and gut bacterial taxa

were used; taxa were centred log-ratio transformed (Gloor et al., 2017). The 4 different alpha diversity measures selected for the analyses are frequently used in gut microbiome research to determine two components of a particular biological community which include species richness and evenness of the distribution (Wang et al., 2022; Willis, 2019). Observed OTUs measures the number of different species found within the sample, Chao1 measures species richness, Faith PD measures the phylogenetic distance between OTUs, and Shannon measures relative abundance and evenness of taxa present within the sample (Hagerty et al., 2020).

16s sequencing data were transformed into relative pathway abundance data using PICRUST2 (Douglas et al., 2020). Significant metabolites related to maternal stress indices as determined by the MetaboAnalyst analysis were used to select a priori pathways from the PICRUST2 output.

Metabolomics

To quantify metabolites, faecal metabolomic analysis was performed at the Calgary Metabolomics Research Facility. Faecal samples (100-200 mg) were diluted 5 times (w/v) into 50% methanol/water solution. First, diluted samples were homogenized using Tissue Lyser II (QIAGEN) and were then incubated on ice for 30 min for full extraction. Next, samples were centrifuged (approximately 13,000 rpm) and 500 µl of supernatant was collected for metabolomic analysis. Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo-Fisher) and Vanquish™ UHPLC System (Thermo-Fisher) were used to perform metabolomics runs. Chromatographic separation was done on a Syncronis HILIC UHPLC column (2.1mm x 100mm x 1.7um, Thermo-Fisher) using a binary solvent system at a flow rate of 600uL/min. Analysis of metabolite data was done by El-MAVEN software package. Identification of metabolites was done by matching observed m/z signals (+/-10ppm) and

chromatographic retention times to those observed from commercial metabolite standards (Sigma-Aldrich). Automated feature detection function in EL-MAVEN with a minimum signal intensity threshold of 50,000 signal intensity was used to generate raw untargeted data.

To quantify SCFAs (Bihan D, 2019), an extraction process was done (1:2 ratio wet sample weight (mg) to extraction solvent (μL)) from fecal samples with ice-cold extraction solvent (50% water/acetonitrile, v/v). SCFAs were then spiked with stable isotope-labeled internal standards (IS) (acetic acid-1,2-13C2, 4 mM, final concentration; propionic acid-13C3, 1 mM; butyric acid-1,2-13C2, 1 mM; isobutyric acid-d7, 250 μM and valeric acid-d9, 500 μM), homogenized at 30 Hz for 3 min with a tissue lyser (Qiagen), derivatized with N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and aniline. The final step was to conduct liquid chromatography mass/tandem mass spectrometry (LC-MS/MS).

Stool Metabolome Analysis

The web-based statistical software MetaboAnalyst was used to analyze children's metabolome (Pang et al., 2021). MetaboAnalyst can perform comprehensive data normalization prior to statistical analysis from a concentration table of metabolites (McGill, 2022). Untargeted metabolomics covering 142 metabolites was performed. Specifically, a pathway analysis was performed to determine (1) concentration differences in child metabolites based on maternal stress exposure variables at each trimester and (2) what pathways significant metabolites are found in (i.e., significant metabolic pathways related to the maternal stress exposure variable at each trimester).

Neurodevelopmental Assessments

Children aged 3 and 4 were administered *The Wechsler Preschool and Primary Scale of Intelligence – 4th (Canadian)* (WPPSI-IV^{CDN}). The WPPSI-IV^{CDN} is used to assess FSIQ in

children aged 2 to 7. FSIQ in children aged 3 includes assessments in 3 domains of intelligence: verbal comprehension (e.g., receptive vocabulary, information, picture naming), visual spatial (e.g., block design, object assembly), and working memory (e.g., picture memory, zoo locations) (Wechsler, 2012). FSIQ in children aged 4 includes assessments in 5 domains of intelligence: verbal comprehension (e.g., information, similarities, vocabulary, comprehension), visual spatial (e.g., block design, object assembly), fluid reasoning (e.g., matrix reasoning, picture concepts), working memory (e.g., picture memory, zoo locations), and processing speed (e.g., bug search, cancellation, animal coding) (Wechsler, 2012). Raw scores for each subset are scaled, and the scaled values are added together. The scaled scores make up the composite score for domains comprising FSIQ. The population mean for FSIQ is 100 with a standard deviation of 15. For the current analysis, FSIQ scores and primary indices of FSIQ (verbal comprehension, visual spatial, working memory) were used for children aged 3 and 4 **(Supplementary Table S2).** The WPPSI-IV^{CDN} is a reliable and valid instrument for assessing intellectual functioning in children aged 2.5-7.7 (Wechsler, 2012).

Data Analytic Plan

Aim 1: I used SPSS software version 27 (IBM Corp) to perform all statistical analyses. First, I used Pearson correlations to evaluate associations between the (1) cumulative stress score and alpha diversity measures and (2) maternal cortisol concentration and psychological distress at each trimester with alpha diversity measures. Next, I evaluated associations between maternal cortisol and psychological distress at each trimester with microbial taxa abundance, using Spearman rank correlations and 95% confidence intervals (95% CI). I used the Benjamini-Hochberg method to correct P-values for false discovery rate, where a $q<0.15$ is considered significant (Storey & Tibshirani, 2003). Next, I analyzed the relationship between maternal

cortisol concentration and psychological distress at each trimester with the child's stool metabolomic pathways using MetaboAnalyst. Lastly, I used relative pathway abundance data (acquired using PICRUST2) to verify if metabolomic pathways were differentially expressed in the gut microbiota.

Aim 2: I used a multivariable linear regression to determine if children's FSIQ and primary indices scores are different depending on maternal cortisol concentration and psychological distress at each trimester. In this model, the primary predictor variable is maternal cortisol concentration and psychological distress at each trimester, which are all quantitative variables. The model will include 5 covariates. Dichotomous covariates include child sex (male/female) and breastfed (yes/no). Quantitative covariates include cognitive support in the home environment, socioeconomic status (SES) (income and education will be used), and diet quality index. The outcome variable(s) are FSIQ score and score on primary indices of FSIQ.

Aim 3: I used Pearson correlations to evaluate the associations between features of the child's gut microbiota (i.e., alpha diversity and taxa abundance) with FSIQ and primary indices scores. The Benjamini-Hochberg method was used to correct P-values for false discovery rate, where a $q<0.15$ is considered significant (Storey & Tibshirani, 2003). I analyzed the relationship between FSIQ and primary indices scores with the child's metabolome functioning using MetaboAnalyst.

III. Results

Population Characteristics

The final analysis included 248 children who completed a stool sample. Mothers were primarily white (86.3%), married (86.7), university-educated (55.2%), with an annual income of $100, 000 or greater (60.5%), and with an average age of 32.4 years (SD 4.1) **(Table 1).** Children had an average age of 3.8 years (SD 0.4) at the time of stool collection and 52.4% and 47.6% were biologically male and female, respectively **(Table 2).**

Table 1. Maternal sociodemographic characteristics for the study sample (n = 248)

	Mean (SD)	Range
Maternal Age (years)	32.4 (4.1)	20-44
	n	%
Marital Status		
Single	5	2.0
Married	215	86.7
Cohabiting	27	10.9
Separated	1	0.4
Education		
Postgraduate Degree	51	20.6
University Degree	137	55.2
Completed Trade/Technical Degree	46	18.5
Completed High School Diploma	14	5.6
Annual Income		
$100,000+	150	60.5
$70,000-$99,999	57	23.0
$40,000-$69,999	27	10.9
$20,000-39,999	8	3.2
Less than $20,000	5	2.0
Missing	1	0.4
Ethnicity		
White (Caucasian)	214	86.3
Black	1	0.4
Hispanic/Latinx	6	2.4
Chinese	10	4.0
Filipino	4	1.6
Japanese	3	1.2
Southeast Asian	7	2.8
Arab	1	0.4
Other	2	0.8

Table 2. Child characteristics for the study sample (n = 248)

	Mean (SD)	Range
Child Age (Years)	3.8 (0.4)	3-4
Diet Quality Score	3.7 (0.7)	1.9-5.7
Sum of Cognitive Support Items	9.6 (1.2)	5-11
	n	%
Infant Sex		
Male	130	52.4
Female	118	47.6
Delivery Mode		
Vaginal	187	75.4
C-section	60	24.2
Missing	1	0.4
Breastfeeding Status (3 months exclusive)		
Yes	119	48.0
No	128	51.6
Missing	1	.4
Antibiotic Exposure		
Yes	150	39.5
No	98	60.5

Aim 1: Determine if features of the child's gut microbiota at 3-4 years of age are different depending on the amount of prenatal stress exposure

The Cumulative Stress Score was not Associated with Alpha Diversity

The cumulative stress was not associated with the child's gut alpha diversity **(Figure 3).**

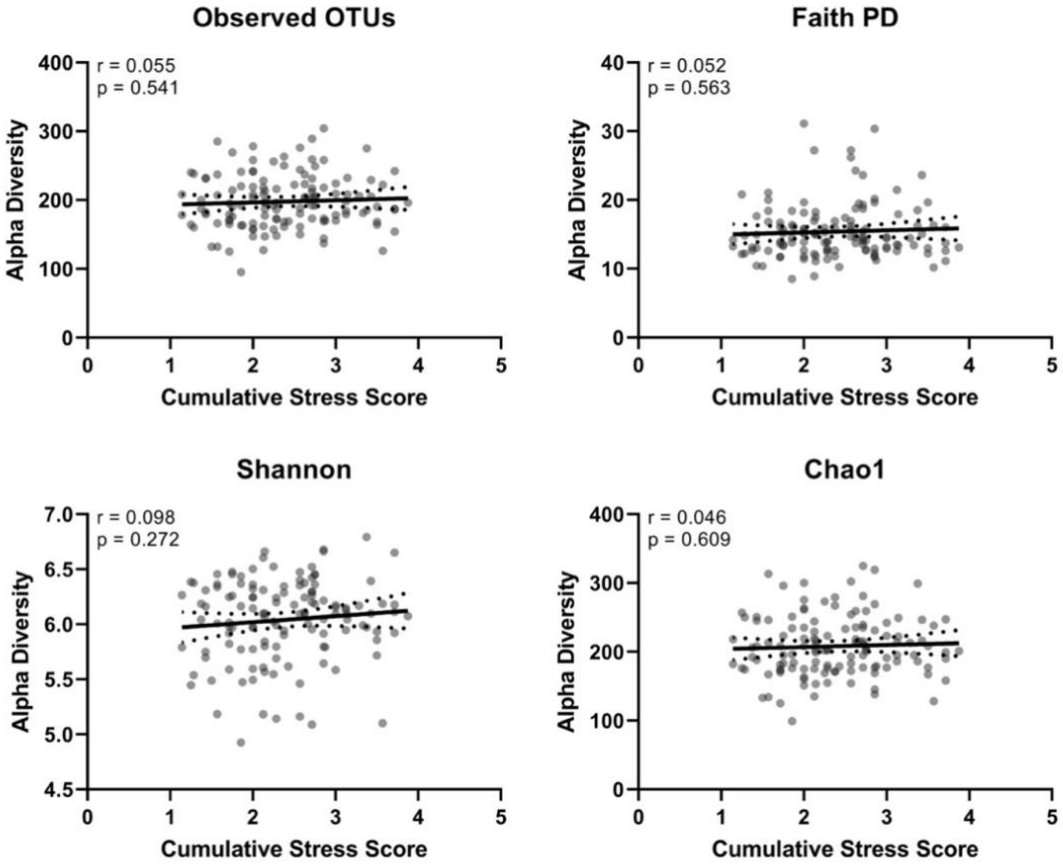

Figure 3. Correlation plots between cumulative stress score and alpha diversity measures (*n* = 127).

The Mode of Delivery was not Associated with Alpha Diversity

The mode of delivery was not associated with the child's gut alpha diversity **(Figure 4),** and therefore it was not considered as a covariate in analyses.

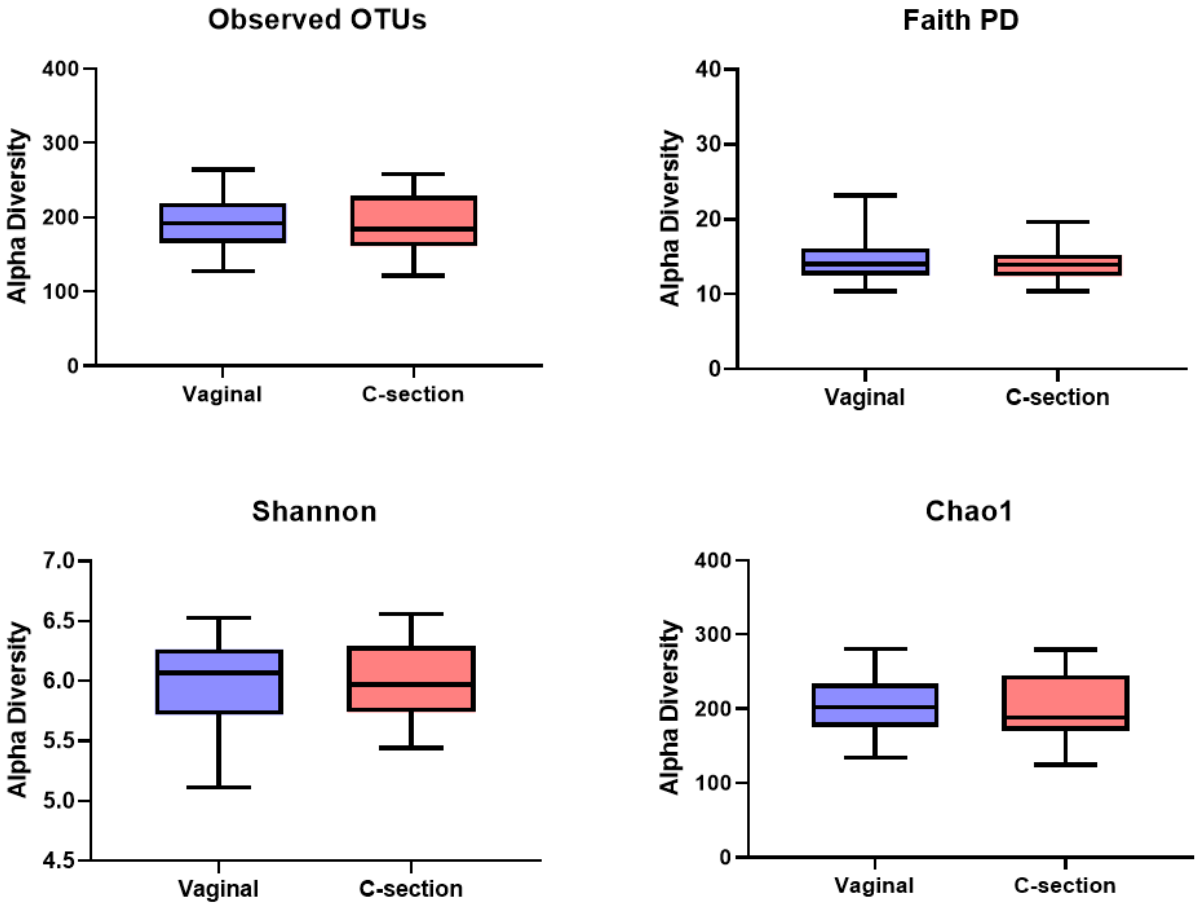

Figure 4. Boxplots examining the relationship between mode of delivery and alpha diversity measures (*n* = 248).

Preschool Gut Microbiota Alpha Diversity was Associated with Prenatal Cortisol and Psychological Distress in a Trimester-Dependent Manner

There was a significant positive correlation between total maternal cortisol concentration in Trimester 1 and Faith PD alpha diversity of the child's fecal microbiota, (r = .32, p = .036). In contrast, there was a significant negative correlation between total maternal cortisol concentration in Trimester 2 and Shannon alpha diversity, (r = -.27, p = .008). Comparatively, the maternal psychological distress score in Trimester 2 was positively associated with Observed OTUs, Shannon, and Chao1 alpha diversity (r = .23, p = .009; r = .12, p = .037; r = .24, p = .006, respectively) (**Figure 5**).

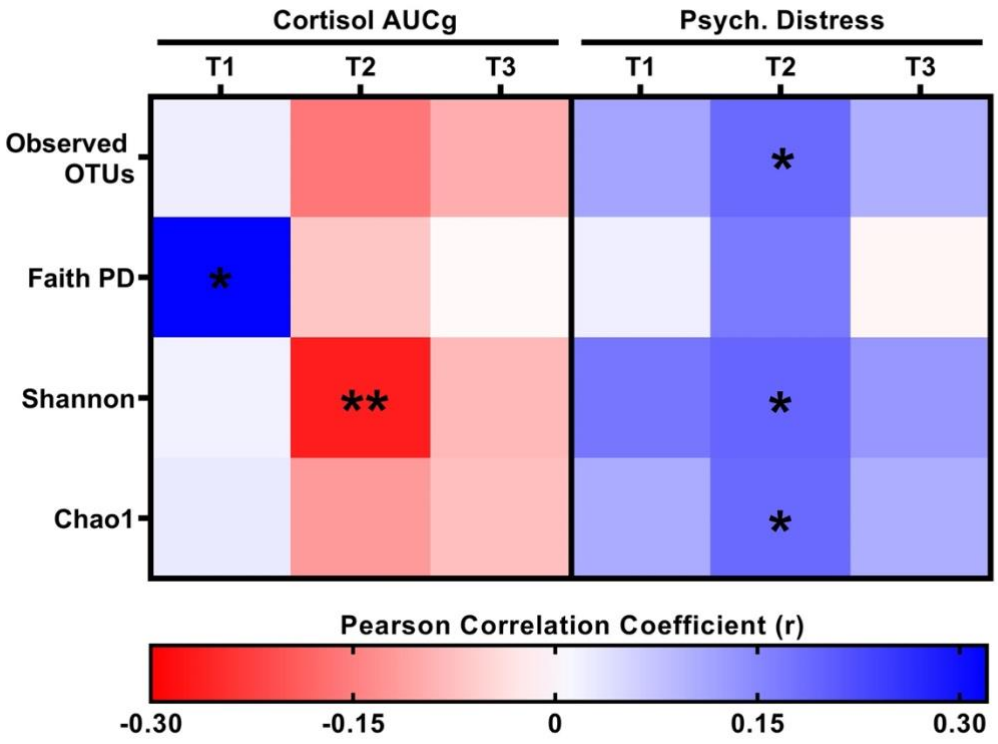

Figure 5. Correlation heatmap examining the association between maternal cortisol concentration and psychological distress in each trimester with child alpha diversity measures. All associations were FDR corrected at a cut-off of q <.15. AUCg = Area under the curve with respect to ground, Psych. Distress = Psychological distress, and T = trimester. (n = 44 for Cortisol AUCg-T1, n = 97 for Cortisol AUCg-T2, n = 127 for Cortisol AUCg-T3, n = 83 for Psychological Distress-T1, n = 127 for Psychological Distress-T2, and n = 127 for Psychological Distress-T3) *< .05, ** < .01.

Total Maternal Cortisol Concentration in Trimester 2 was Associated with Bacterial Taxa Abundance

Ten bacterial taxa were significantly correlated with total maternal cortisol concentration in Trimester 2 (**Figure 6**) **(Supplementary Table S3).** Maternal psychological distress was not significantly associated with bacterial taxa abundance.

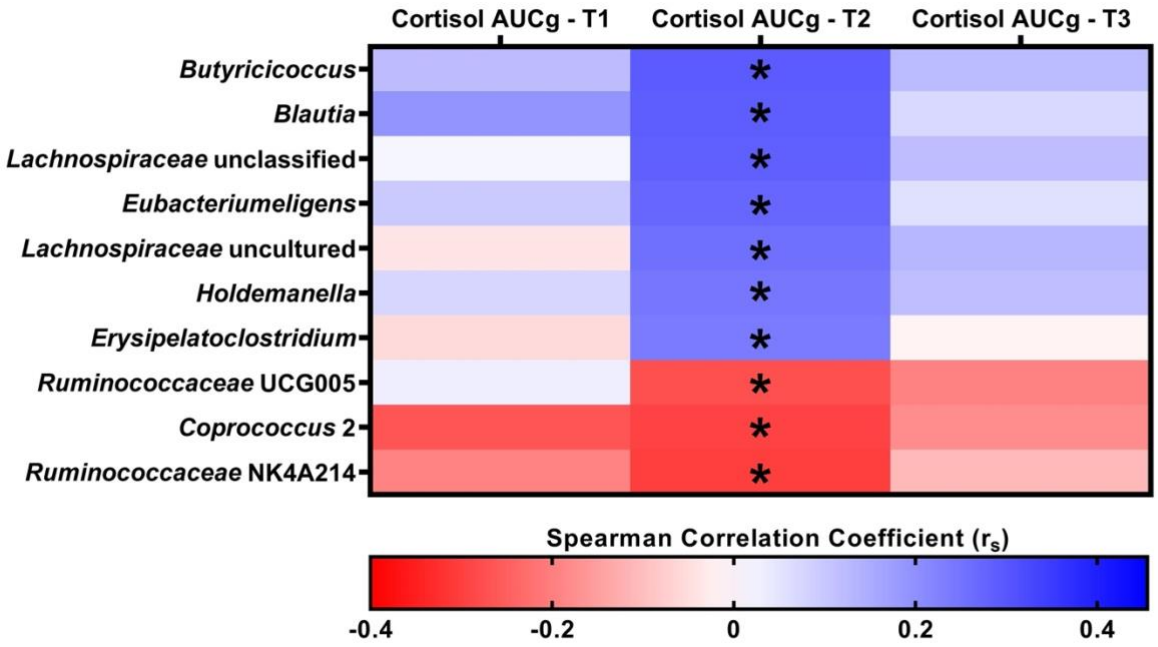

Figure 6. Correlation heatmap examining the association between maternal cortisol concentration at each trimester and child's gut microbial taxa abundance. All associations were FDR corrected at a cut-off of q<0.15. AUCg = Area under the curve with respect to ground and T = trimester. (*n* = 44 for Cortisol AUCg-T1, *n* = 97 for Cortisol AUCg-T2, *n* = 127 for Cortisol AUCg-T3) *< .05.

Total Maternal Cortisol Concentration was Associated with the Child Metabolome in a Trimester-Dependent Manner

Twelve fecal metabolic pathways were significantly associated with total maternal cortisol concentration in Trimester 1, and three child fecal metabolic pathways were significantly associated with total maternal cortisol concentration in Trimester 3 **(Figure 7) (Supplementary Table S5-S7).** In addition, eight distinct metabolites were significantly associated with total maternal cortisol concentration in Trimester 1. Succinate was significantly associated with total maternal cortisol concentration in Trimester 2, (r = .22, p = .034). Lastly, succinate and 2-oxobutanoate (r = .25, p = .007; r = .21, p = .021, respectively) were significantly associated with total maternal cortisol concentration in Trimester 3 **(Figure 8) (Supplementary Table S4).** Maternal psychological distress was not significantly associated with the child's stool metabolome.

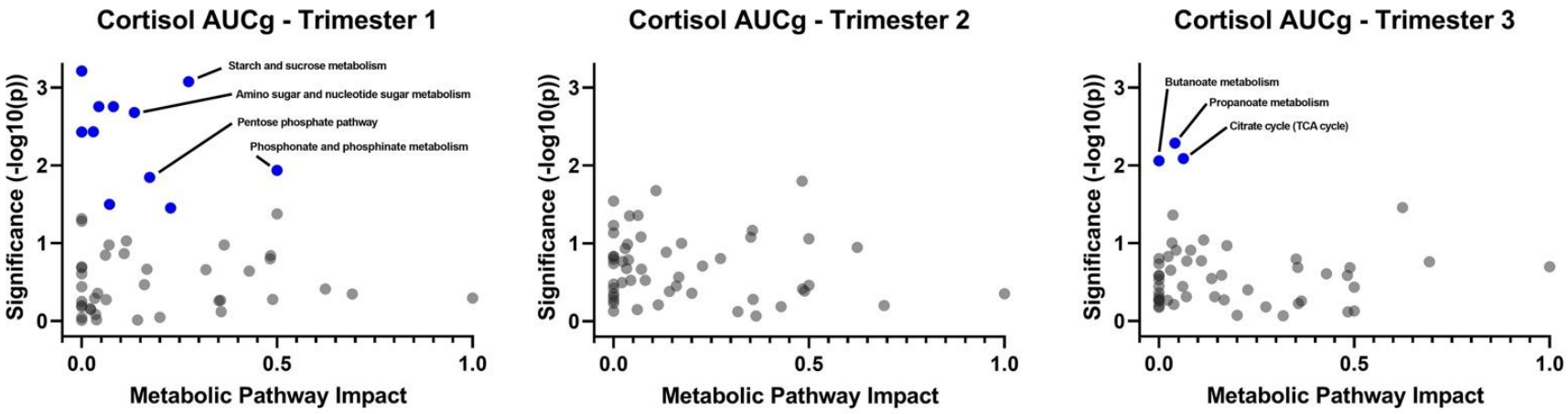

Figure 7. Plots examining the relationship between total maternal cortisol concentration at each trimester and child metabolic pathways. Blue dots indicate a statistically significant relationship between total maternal cortisol concentration and metabolic pathways in each trimester. All associations were FDR corrected at a cut-off of q<0.15. AUCg = Area under the curve with respect to ground. (*n* = 44 for Cortisol AUCg-T1, *n* = 97 for Cortisol AUCg-T2, *n* = 127 for Cortisol AUCg-T3).

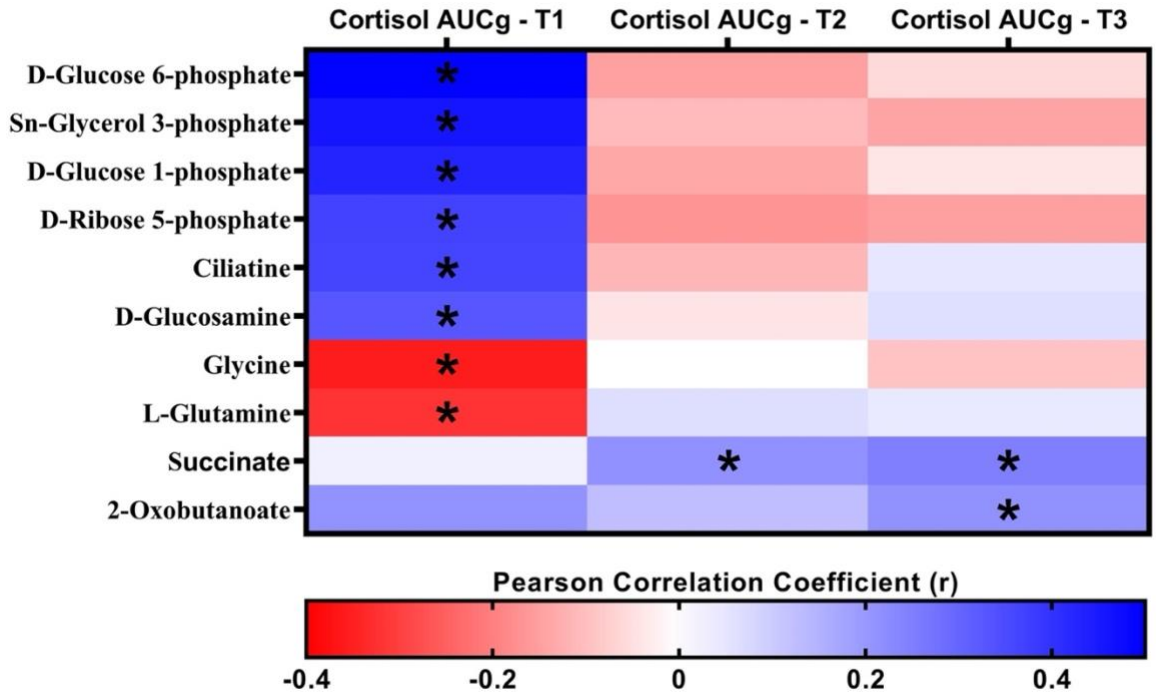

Figure 8. Correlation heatmap examining the relationship between total maternal cortisol concentration in each trimester and child stool metabolite concentration. AUCg = Area under the curve with respect to ground and T = trimester. (*n* = 44 for Cortisol AUCg-T1, *n* = 127 for Cortisol AUCg-T2, and n = 127 for Cortisol AUCg-T2.) *< .05.

Total Maternal Cortisol Concentration was Associated with Gut Microbial Glucose-1-Metabolic Pathway and Reductive TCA cycle II in a Trimester-Dependent manner

There was a significant positive association between total maternal cortisol concentration in trimester 1 and the Glucose-1- Metabolic Pathway, (r_s = .387, p = .009). Additionally, there was a significant negative association between total maternal cortisol in trimester 3 and the Reductive TCA cycle II, (r_s = -.190, p = .039) **(Figure 9).** Both Glucose-1- Metabolic Pathway and Reductive TCA cycle II are pathways expressed by the gut microbiota.

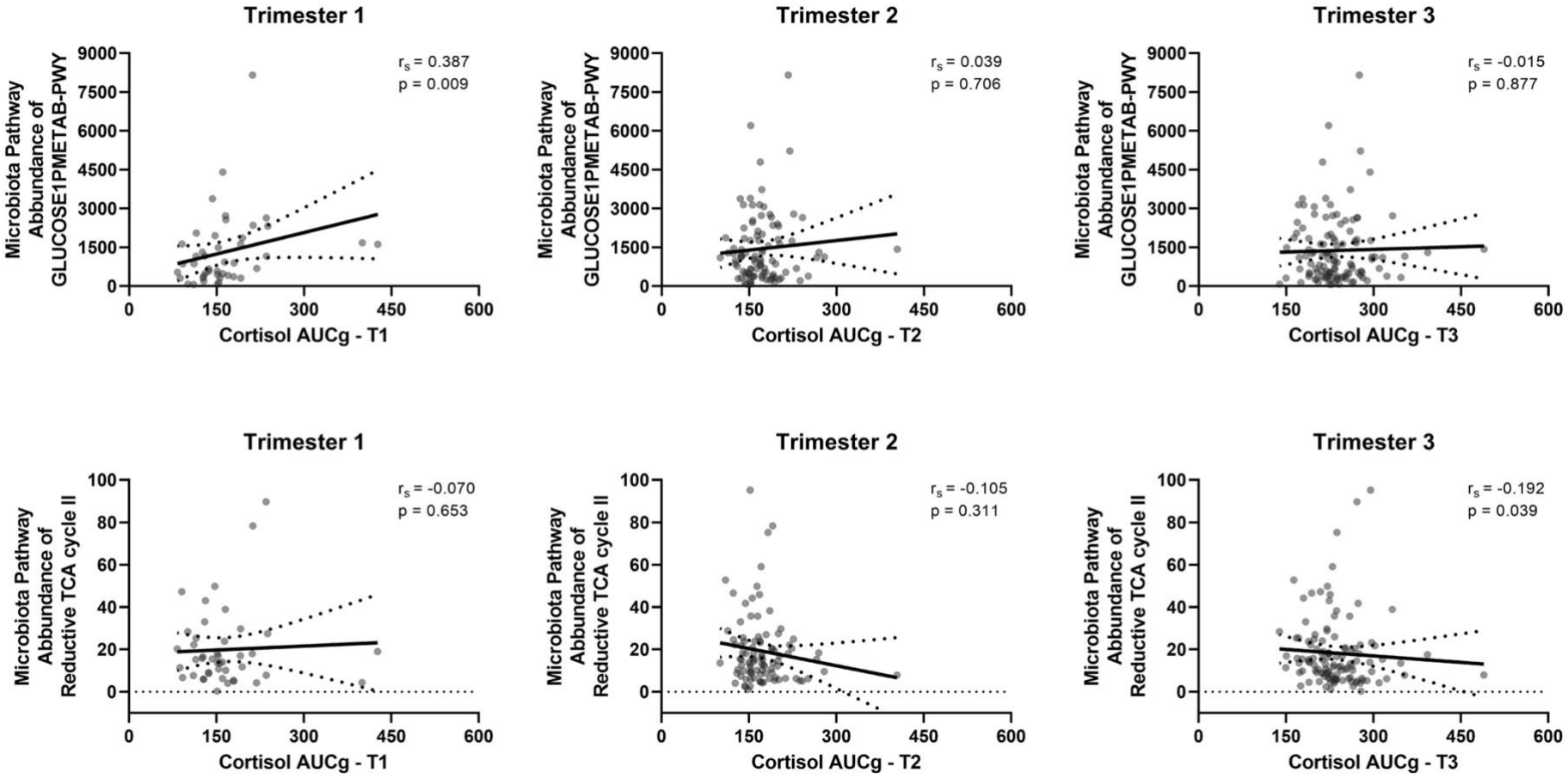

Figure 9. Plots examining the relationship between total maternal cortisol concentration at each trimester and child metabolic pathways from gut microbiota. All associations were FDR corrected at a cut-off of q<0.15. (*n* = 44 for Cortisol AUCg-T1, *n* = 97 for Cortisol AUCg-T2, *n* = 127 for Cortisol AUCg-T3).

Aim 2: Determine if children's FSIQ and primary indices scores are different depending on the amount of prenatal stress exposure

Prenatal Stress is not Significantly Associated with FSIQ Score in Preschool Children when Accounting for Relevant Covariates

There was a significant negative correlation between maternal psychological distress in trimester 3 and child FSIQ score, (r = -.167, p = .019). Maternal psychological distress in Trimester 3, child sex, SES, cognitive support, DQI, and breastfed status were used in a stepwise multiple regression analysis to predict child FSIQ score. The correlation between FSIQ and maternal psychological distress in Trimester 3 and SES was statistically significant (**Table 3**). The final linear regression model contained 6 predictor variables and was not statistically significant, $F(6,147) = 1.638$, p =.141, and accounted for approximately 2.4% of the variance of FSIQ score (R^2= .063, adjusted R^2= .024). SES accounted for .03% of the variance in FSIQ score, which was the only predictor that remained significant in the final model **(Table 4).** Excluding maternal psychological distress in trimester 3, all other stress indices (maternal cortisol and psychological distress) were not associated with child FSIQ or primary indices scores, at any time point during pregnancy.

Table 3. Correlation analysis between psychological distress in trimester 3 and child FSIQ, with relevant *covariates* (*n* =154)

Variables	**2**	**3**	**4**	**5**	**6**	**7**
1. Full Scale Composite	-.167*	-.033	.212*	.046	.055	.034
2. Psychological Distress T3	--	.006	-.225	.008	-.167	-.073
3. Child Sex		--	.065	.066	-.087	-.003
4. SES			--	.143	.202	.066
5. Cognitive Support				--	.040	.199
6. Diet Quality Score					--	.130
7. Breastfed (3 Months Exclusive)						--

Note. The correlation between Full Scale Composite with Psychological Distress T3 and SES was statistically significant, * $p < .05$.

Table 4. Regression results of psychological distress in trimester 3 predicting child FSIQ score (*n* =154)

Model	b	SE-b	β
Constant	101.115	9.309	
Psychological Distress T3	-2.288	1.501	-0.126
Child Sex	-1.058	1.841	-0.046
SES*	3.348	1.527	0.185
Cognitive Support	0.220	0.800	0.022
Diet Quality Score	-0.170	1.440	-0.010
Breastfed (3 Months Exclusive)	0.246	1.853	0.011

Note. The dependent variable was Full Scale Composite, * $p < .05$.

Aim 3: Determine if children's FSIQ and primary indices scores are different depending on features of the child's gut microbiota

Verbal Composite Score was Negatively Associated with Shannon Alpha Diversity

There was a significant negative association between Shannon alpha diversity and the verbal composite score, (r = -.14, p = .032) **(Figure 10).** Associations with other measures of alpha diversity were not significant.

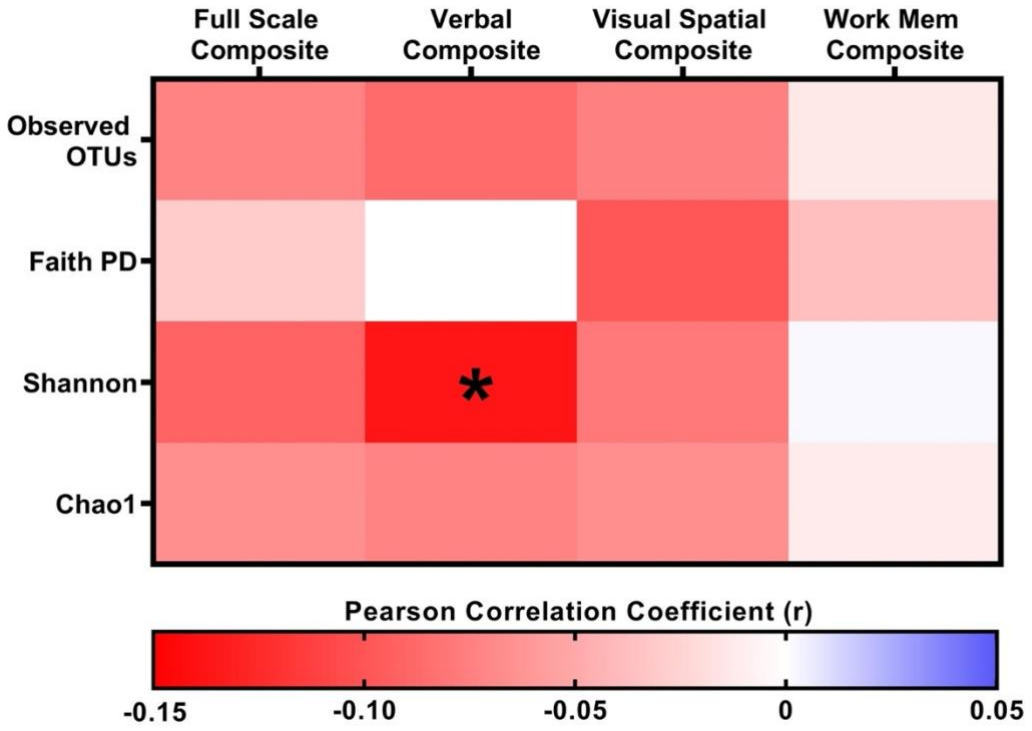

Figure 10. Correlation heatmap examining the relationship between alpha diversity and FSIQ and primary indices scores. All associations were FDR corrected at a cut-off of q<0.15. (n = 241 for Full Scale Composite, n = 243 for Verbal Composite, n = 244 for Visual Spatial Composite, n = 244 for Working Memory Composite) *< .05.

FSIQ and Primary Indices Scores were not Significantly Associated with Child Bacterial Taxa Abundance and Metabolome at Preschool Age

FSIQ and primary indices scores were not significantly associated with bacterial taxa abundance or child metabolome **(Figure 11).**

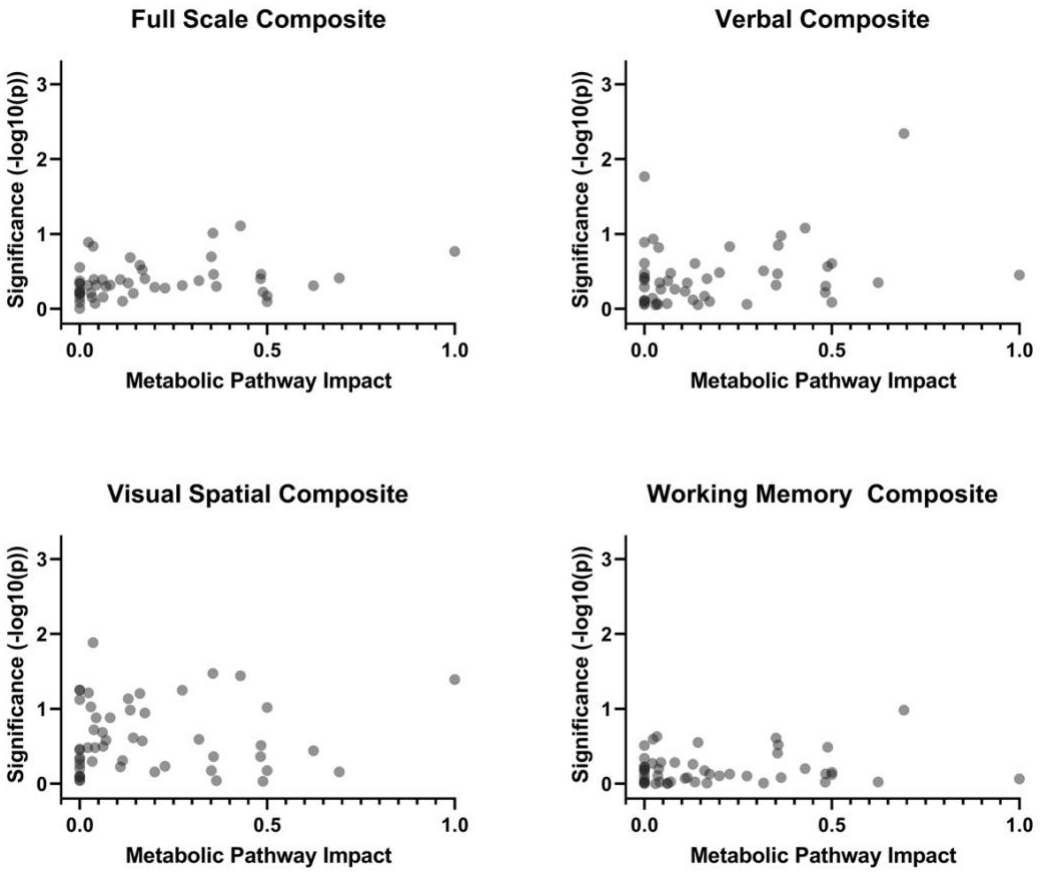

Figure 11. Plot examining the relationship between FSIQ and primary indices scores with child metabolic pathways. (*n* = 241 for Full Scale Composite, *n* = 243 for Verbal Composite, *n* = 244 for Visual Spatial Composite, *n* = 244 for Working Memory Composite).

IV. Discussion

The present study investigated if the gut microbiota contributes to the relationship between prenatal stress and child FSIQ at the age of 3-4. Gut microbiota may mediate the relationship, considering that prenatal stress is associated with features of the child's gut microbiota. However, study findings do not provide evidence of mediation because features of the child's gut microbiota that we examined were not associated with child FSIQ scores at the age of 3-4.

For Aim 1, the objective was to determine if features of the child's gut microbiota were different depending on the amount of prenatal stress exposure. The cumulative stress score was not associated with the child's gut alpha diversity. The cumulative stress score was composed of total maternal cortisol concentration and the POMS-15 questionnaire (i.e., anxiety, depressed, external demands) in each trimester. Total maternal cortisol concentration and scales of the POMS-15 were for the most part associated with children's gut alpha diversity in opposite directions throughout pregnancy. As a result, significant relationships between maternal stress indices and child alpha diversity measures were masked by the cumulative stress score.

For this reason, we decided to investigate alternative approaches for conceptualizing prenatal stress. A major hurdle in stress research is determining what measurements to use, as stress can be experienced at multiple levels – social, psychological, and physiological. As such, there is no standardized way to conceptualize stress (Epel et al., 2018). In addition, the association between self-reported stress (e.g., mood questionnaires) and biological markers like cortisol is weak (Dalile et al., 2022). In line with this literature, in our own data set, total maternal cortisol concentration and psychological distress were not associated **(Supplementary Figure S1)**, indicating a weak association between self-reported stress and biological markers of

stress. To add another level of complexity, there are trimester-specific effects, meaning that the timing of a particular exposure matters as it seems to affect the fetus differently. This indicates that specific exposures need to be presented at a particular time point in pregnancy to impact the developmental trajectory of the fetus (i.e., developmental windows) (Moisiadis & Matthews, 2014). Therefore, creating a cumulative stress score for stress experienced during pregnancy may be inappropriate because it neglects trimester-specific effects that stress exposures (e.g., maternal cortisol) can have on the developing fetus. Considering that the initial results indicate that there was no association between the cumulative stress score and alpha diversity, we decided to operationalize prenatal stress using subjective (psychological distress) and objective (maternal cortisol) measures separately for each trimester. Moreover, the mode of delivery was not associated with the child's gut alpha diversity. This was excepted as there is evidence to suggest that the effects of the mode of delivery on an infant's gut microbiota composition disappear around 6 months of age (Ríos-Covian et al., 2021). For both of these reasons, the birth mode was not controlled for when analyzing gut microbiota composition and functioning in preschool children.

Using this new approach, we found that total maternal cortisol concentration in trimesters 1 and 2 were associated with Faith PD and Shannon alpha diversity, respectively. In addition, it was found that the maternal psychological distress score in trimester 2 was positively associated with Observed OTUs, Shannon, and Chao1 alpha diversity. It is important to mention that (1) Observed OTUs and Chao1 measure the number of different species, independent of the richness, (2) Faith PD measures bacterial phylogeny, (3) and Shannon measures different species with relative high abundance. Based on this information, the signal between maternal cortisol in trimester 1 and Faith PD alpha diversity was driven by taxa that are phylogenetically very

different (i.e., species that are genetically very different from one another) and the signal between maternal cortisol in trimester 2 and Shannon alpha diversity was driven by bacterial taxa that are highly abundant in children's gut microbiota. Moreover, the signal between maternal psychological distress in trimester 2 was driven by different types of bacterial species that were highly abundant in children's gut microbiota.

Considering that total maternal cortisol concentration in trimester 1 was positively associated with Faith PD alpha diversity and not other indices, it suggests that greater maternal cortisol exposure in early pregnancy was associated with a gut microbiota composed of bacterial taxa that are not similar in children aged 3-4, but not with the amount (i.e., richness) and distribution (i.e., evenness) of the bacteria found within the gut. Furthermore, considering that total maternal cortisol in trimester 2 was negatively associated with Shannon alpha diversity and not other indices, it suggests that greater maternal cortisol exposure in mid-pregnancy was associated with reduced bacterial abundance in children aged 3-4 and not with bacterial relatedness (i.e., phylogeny) and distribution (i.e., evenness). Lastly, considering that maternal psychological distress in trimester 2 was positively associated with observed OTUs, Shannon, and Chao1 alpha diversity and not Faith PD alpha diversity, it suggests that greater maternal psychological distress in mid-pregnancy was associated with an increased number of different bacterial species and amount (i.e., richness) in children aged 3-4, and not with bacterial relatedness (i.e., phylogeny). Overall the first set of findings indicates trimester-specific effects of maternal cortisol exposure and psychological distress on the composition of the child's gut microbiota. Specifically, study results suggest that greater maternal cortisol exposure in early and mid-pregnancy was associated with increased bacterial diversity and a reduction in bacterial

abundance, respectively, and greater maternal psychological distress in mid-pregnancy is associated with an increased number of different bacterial species and amount.

Additionally, total maternal cortisol concentration in trimester 2 was significantly associated with ten bacterial taxa. Similarly, previous studies have also found that children born to mothers who reported high stress and elevated cortisol concentrations had a lower relative abundance of lactic acid bacteria (e.g., *Lactobacillus, Lactoccus, Aerococcus*) and a higher abundance of proteobacterial groups (Zijlmans et al., 2015). Animal studies indicate that prenatal stress is associated with a decreased relative abundance of *Lactobacillus*, and an increased abundance of *Oscillibacter*, *Anaerotruncus*, and *Peptococcus* (Golubeva et al., 2015). Therefore, both total maternal cortisol concentration and maternal psychological distress were associated with bacterial diversity (i.e., alpha diversity) in a trimester-specific manner. In addition, total maternal cortisol concentration was associated with bacterial taxa abundance in a trimester-specific manner.

The next step of the analysis was to examine the relationship between the maternal stress indices and the child's fecal metabolome using pathway analysis. We found that total maternal cortisol concentration in trimesters 1 and 3 were associated with 12 and 3 metabolic pathways, respectively. In addition, total maternal cortisol concentration in trimester 1 was associated with eight distinct metabolites, trimester 2 with one metabolite, and trimester 3 with two metabolites. The pathways and metabolites that were significantly associated with total maternal cortisol concentration from the pathway analysis (using MetaboAnalyst) were related to two pathways from the PICRUST2 analysis. PICRUST2 data was used to investigate whether the metabolomic pathways were also differentially expressed in the gut microbiota. These two pathways were

Glucose-1- Metabolic Pathway and Reductive TCA cycle II, which were significantly associated with total maternal cortisol levels in trimesters 1 and 3, respectively.

The Glucose-1-Metabolic Pathway and its metabolite D-glucose-6-phosphate are both central in gluconeogenesis. This is noteworthy because during times of stress the HPA axis is activated and results in the release of cortisol which increases gluconeogenesis which is the generation of glucose to supply the body with energy to deal with the current stressor (Seal & Turner, 2021). D-glucose-6-phosphate metabolite is associated with the HPA axis, which might indicate that HPA axis functioning is altered. To support this claim, the gut microbiota has also been associated with HPA axis development and functioning in other research (Misiak et al., 2020). The activation of the HPA axis is an adaptive response to acute stress, with the intention of preparing the body to deal with a momentary stressor (e.g., removing oneself from a dangerous environment). Considering that total maternal cortisol concentration was associated with Glucose-1-Metabolic Pathway it suggests that increased maternal cortisol exposure programs the fetus and later the child to deal with stress possibly by influencing HPA axis activity through the gut microbiota, which can be adaptive.

Furthermore, total maternal cortisol concentration in trimesters 2 and 3 were significantly associated with succinate, a metabolite that is part of the Reductive TCA cycle II (Martínez-Reyes & Chandel, 2020). The TCA cycle is the second stage of cellular respiration and its main objective is to break down organic molecules like glucose (Britannica, 2013). Bacteria within the gut can produce succinate as a by-product of the fermentation of dietary fibers. In turn, succinate is used as a substrate for intestinal gluconeogenesis, which is connected to HPA axis functioning (De Vadder et al., 2016). Importantly, intestinal gluconeogenesis is associated with signaling of the brain via gastrointestinal nerves (de Vadder & Mithieux, 2018). Therefore, the current study

findings may illustrate the programming effects of maternal cortisol exposure on the infant gut microbiota composition which relates to alterations in metabolic functioning. Altogether, results from Aim 1 indicate that the total maternal cortisol concentration during pregnancy relates to both the child's gut microbiota composition and fecal metabolome in a trimester-specific manner.

For Aim 2, the objective was to determine if children's FSIQ and primary indices scores are different depending on the amount of prenatal stress exposure. There was a significant negative association between maternal psychological distress in trimester 3 and FSIQ. However, prenatal stress was no longer associated with children's FSIQ after accounting for SES. This is in line with previous work showing that there is no association between prenatal stress and child IQ at the age of 6 when controlling for both maternal IQ and SES (Cortes Hidalgo et al., 2020). Maternal IQ was not directly accounted for in the current model, however, the SES variable used encompassed maternal education and income, and level of education is positively associated with IQ (Feinkohl et al., 2021). Previous research findings have elucidated the significant contribution of postnatal factors like SES on child cognitive performance (e.g., IQ, academic achievement). Children born into lower SES households tend to have (1) reduced access to nutritious food and quality education (e.g., textbooks, toys, home enrichment), (2) experience psychosocial pressures (e.g., overcrowding, interpersonal violence, disruptive relationship with caregivers), and are (3) less likely to be breastfed, all such factors are negatively associated with neurodevelopment (Bush et al., 2020). In our sample, children were predominantly born to mothers with high SES. As most children within our study sample reside in Calgary, Alberta a high SES status is considered normative and would be representative of the community sample (CalgaryEconomicDevelopment, 2021). Therefore, our study results suggest that SES can act as

a protective factor because it prevented maternal psychological distress in trimester 3 from having a significant negative effect on child FSIQ, and also illustrates that even small variations in SES can have an effect on children's cognitive outcomes.

For Aim 3, the objective was to determine if children's FSIQ and primary indices scores correlated with the gut microbiota at preschool age. The verbal composite score was negatively associated with Shannon alpha diversity, indicating that this relationship was driven by bacteria with relative high abundance in children's gut microbiota. As such, this finding indicates that higher verbal composite scores were associated with reduced bacterial diversity. This result is in line with the previous work showing that higher alpha diversity at the age of 2 was associated with lower visual reception and expressive language scores on the Mullen Scales of Early Learning (Carlson et al., 2018). Similarly, a significant negative association between verbal IQ (VIQ) of the WPPSI-III and Shannon, Faith PD, and observed OTUs had been found in children age 3 (Streit et al., 2021). The specific finding between VIQ and Shannon alpha diversity matches our current study findings. Taxa abundances were not significantly associated with children's FSIQ and primary indices scores at the age of 3-4. These results are in line with a recent longitudinal study that demonstrates that both infant and child fecal samples are not significantly associated with executive function in middle childhood (Eckermann et al., 2022). Based on this information, it would be reasonable to conclude that there is no significant association between taxa abundance and cognitive outcomes (e.g., FSIQ score) past early childhood (0-2 years of age). This can partly be explained by the fact that the effects of prenatal stress might change over time. In early development, the effects of prenatal stress on the gut microbiota may be more pronounced due to the fact that birth mode and breastfeeding are significant factors contributing to the development of the gut microbiota from 5-31 weeks post-

birth. Therefore, mothers are contributing greatly to their children's gut microbiota in early life. For example, prenatal stress is associated with altering the composition of the vaginal microbiome which can be passed to the neonate through vaginal birth. However, after the cessation of breastfeeding, diet becomes the most significant factor influencing gut microbiota composition (Galazzo et al., 2020). As such, the influence of other external factors like diet is influencing development to a greater extent in later life as opposed to prenatal stress. Finally, FSIQ and primary indices scores were not associated with the child metabolome. Considering that the effects of the gut microbiota are predominantly mediated by the metabolites they produce it can reasonably be concluded that the gut microbiota is not associated with children's FSIQ and primary indices scores at the age of 3-4.

Strengths and Limitations

The current study had several strengths. First, the study used repeated samples for cortisol and psychological measures at each trimester to operationalize prenatal stress. The advantage of having repeated samples throughout pregnancy is it allows us to examine trimester-specific effects of stress on an outcome of interest which in this case was child gut microbiota features and FSIQ scores. In addition, by analyzing the effects of maternal cortisol and psychological distress separately, the study acknowledged the complexity of stress research. Stress is multifaceted and can be experienced on many levels: social, psychological, and physiological (Epel et al., 2018). In other words, a single measure of stress at a single time point would not accurately characterize prenatal stress. Second, when analyzing the relationship between prenatal stress and FSIQ, well-known confounding variables were taken into consideration in the multivariate analysis. For example, the breastfeeding status, cognitive support in the home environment, biological sex, diet, and SES (Ronfani et al., 2015; Strøm et

al., 2019). Third, the study examined both gut microbiota features and their functioning by analyzing the child's fecal metabolome in relation to prenatal stress and FSIQ. The effects of gut bacteria are frequently mediated through the metabolites they produce, meaning that analyzing these metabolites is a crucial component for understanding microbiota-gut-brain axis communication.

It is also equally as important to acknowledge the limitations of the current study. One limitation is the lack of repeated sampling for the child stool samples (i.e., cross-sectional design) which makes it challenging to ascertain the stability of the gut microbiota features that we are analyzing (Bäckhed et al., 2015; de Meij et al., 2016; de Muinck & Trosvik, 2018). Another limitation would be the generalizability of the study results, considering that 86.3% of participants were white and 60.5% had an annual income over $100,000, there is a possibility that study results may not apply to individuals who identify as non-white or of lower SES. Furthermore, the *n* numbers for maternal stress variables are not the same throughout all three trimesters. This is a result of mothers joining the study at different time points (trimester 1 or 2) and missing data. As a result, analyses with trimester 1 data may be underpowered compared to trimesters 2 and 3. However, all studies that employ longitudinal data collection are faced with this challenge. In this case, a longitudinal approach was necessary as it provides a means to see patterns of specific exposures during pregnancy and how they relate to child outcomes (e.g., gut microbiota composition, FSIQ).

Future Directions

Given the results, a few pressing questions remain for future exploration. First, considering that total maternal cortisol concentration was associated with children’s bacterial diversity, abundance, and metabolome but not FSIQ, the gut microbiota is possibly mediating

another type of health-related outcome not examined in this study like the child's HPA axis. Prenatal stress is associated with an altered gut microbiota composition in infants and the early life gut microbiota has been linked to the development of the HPA axis (Frankiensztajn et al., 2020; Zijlmans et al., 2015). The results of this thesis show that total maternal cortisol concentration was associated with metabolic pathways related to gluconeogenesis (Glucose-1-Metabolic Pathway, Reductive TCA cycle II), which in turn has been linked to the HPA axis (Seal & Turner, 2021). As such, excessive prenatal maternal cortisol exposure is associated with an altered gut microbiota composition in infants such that bacteria present have altered metabolic functioning which is sensed by the brain influencing HPA development and functioning. An indicator of altered HPA functioning would be glucose metabolism because the end product of the HPA axis is cortisol which increases gluconeogenesis. Therefore, a follow-up study should investigate the relationship between prenatal stress and HPA axis development and the mediating role of the gut microbiota in this relationship.

Additionally, one way to validate if total maternal cortisol concentration and psychological distress are associated with the child's gut microbiota composition and metabolome would be to perform a longitudinal intervention study. For instance, does providing additional mental health support and resources during pregnancy affect the specific gut microbiota composition and function features identified in this study in children at 3-4 years of age.

Conclusion

To summarize, findings from the current study indicate that total maternal cortisol concentration was associated with alpha diversity, bacterial abundance, and metabolome in children aged 3-4 years in a trimester-specific manner. In addition, maternal psychological

distress was associated with alpha diversity in a trimester-specific manner. However, prenatal stress and gut microbiota features were not associated with children's FSIQ scores at the age of 3-4. Even though previous studies have linked gut microbiota to neurodevelopment, the current study findings do not support the gut microbiota mediating the relationship between prenatal stress and FSIQ. The gut microbiota may influence neurodevelopment seeing that prenatal stress was associated with children's gut microbiota composition and functioning. The long-lasting effects of prenatal stress on children's gut microbiota require further exploration to understand the overall implication of such alterations on child health outcomes. Overall, the current study findings should make us optimistic that prenatal stress was not significantly associated with adverse cognitive outcomes in preschool children when accounting for relevant covariates (e.g., SES). Considering that postnatal factors seem to moderate the relationship between prenatal stress and cognitive outcomes in preschool children, families and caregivers can positively impact their children's development postbirth and not feel that their children are burdened by everyday normative stress during pregnancy.

VI. References

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Supplemental Information

Supplementary Table S1.
Subjective and Objective Measures of Prenatal Stress

	n	Mean (SD)	Range
Psychological Distress			
Trimester 1	83	-.3 (.7)	-1.3 – 2.6
Trimester 2	127	-.2 (.8)	-1.2 – 3.4
Trimester 3	127	-.1 (.8)	-1.2 – 3.4
CortAUCg			
Trimester 1	44	164.8 (68.0)	82.7 – 426.6
Trimester 2	97	173.8 (42.2)	100.6 – 404.2
Trimester 3	127	236.3 (50.0)	138.7 – 489.7
Cumulative Stress Score	127	2.4 (.7)	1.1 – 3.9

Supplementary Table S2.
Child Neurocognitive Assessments (*N* = 248)

	Mean (SD)	Range	n	Missing	%
Full Scale	103.8 (12.4)	70-144	241	7	2.8
Verbal Composite	106.2 (13.6)	54-138	243	5	2.0
Visual Spatial Composite	103.2 (14.5)	65-145	244	4	1.6
Working Memory Composite	104.8 (13.3)	65-146	245	3	1.2

Supplementary Table S3.

Spearman Correlations Between Total Maternal Cortisol Concentration at Each Trimester and Child Bacterial Taxa Abundance

		Bacterial Taxa									
		Butyricicoccus	*Blautia*	*Lachnospiraceae* unclassified	*Eubacterium eligens* group	*Lachnospiraceae* uncultured	*Holdemanella*	*Erysipelatoclostridium*	*RuminococcaceaeUCG005*	*Coprococcus2*	*Ruminococcaceae NK4A214group*
Cortisol AUCg - T1	Correlation Coefficient	0.119	0.190	0.015	0.094	-0.266	0.072	-0.058	0.029	-0.268	-0.196
	Sig. (2-tailed)	0.441	0.217	0.921	0.546	0.081	0.640	0.709	0.850	0.078	0.202
Cortisol AUCg - T2	Correlation Coefficient	0.292**	0.286**	0.283**	0.271**	0.255*	0.243*	0.235*	-0.275**	-0.298**	-0.303**
	Sig. (2-tailed)	0.004	0.005	0.005	0.007	0.012	0.016	0.021	0.006	0.003	0.003
Cortisol AUCg - T3	Correlation Coefficient	0.120	0.067	0.116	0.054	0.127	0.113	-0.019	-0.197*	-0.137	-0.078
	Sig. (2-tailed)	0.197	0.471	0.210	0.559	0.170	0.222	0.838	0.032	0.138	0.399

Note. All associations were FDR corrected at a cut-off of q<0.15. (*n* = 44 for Cortisol AUCg-T1, *n* = 97 for Cortisol AUCg-T2, *n* = 127 for Cortisol AUCg-T3) *< .05.

Supplementary Table S4.

Pearson Correlations Between Total Maternal Cortisol Concentration at Each Trimester and Child Metabolite Concentration

		Bacterial Taxa									
		D-Glucose 6-phosphate	Sn-Glycerol 3-phosphate	D-Glucose 1-phosphate	D-Ribose 5-phosphate	Ciliatine	D-Glucosamine	Glycine	L-Glutamine	Succinate	2-Oxobutanoate
Cortisol AUCg - T1	Correlation Coefficient	0.496**	0.458**	0.428**	0.370*	0.363*	0.328*	-0.357*	-0.320*	0.028	0.210
	Sig. (2-tailed)	0.001	0.002	0.004	0.014	0.016	0.029	0.017	0.034	0.856	0.171
Cortisol AUCg - T2	Correlation Coefficient	-0.148	-0.107	-0.137	-0.169	-0.116	-0.042	0.000	0.064	0.215*	0.125
	Sig. (2-tailed)	0.148	0.182	0.182	0.097	0.258	0.680	1.000	0.534	0.034	0.222
Cortisol AUCg - T3	Correlation Coefficient	-0.058	-0.143	-0.040	-0.150	0.047	0.062	-0.095	0.042	.248**	.212*
	Sig. (2-tailed)	0.533	0.123	0.670	0.104	0.615	0.506	0.307	0.655	0.007	0.021

Note. All associations were FDR corrected at a cut-off of q<0.15. (*n* = 44 for Cortisol AUCg-T1, *n* = 97 for Cortisol AUCg-T2, *n* = 127 for Cortisol AUCg-T3) *< .05.

Supplementary Table S5.

Pathway Results for Total Maternal Cortisol Concentration Trimester 1, N = 44 (MetaboAnalyst)

Pathways	Total Compounds	Hits	Raw p	-log10(p)	Holm adjust	FDR	Impact
Neomycin, kanamycin and gentamicin biosynthesis	2	D-Glucose 6-phosphate	0.000611	3.2139	0.031163	0.01427	0
Inositol phosphate metabolism	30	D-Glucose 6-phosphate	0.000611	3.2139	0.031163	0.01427	0
Starch and sucrose metabolism	18	D-Glucose 1-phosphate D-Glucose 6-phosphate	0.0008394	3.076	0.041132	0.01427	0.2734
Glycerophospholipid metabolism	36	sn-Glycerol 3-phosphate	0.0017566	2.7553	0.084318	0.01773	0.0809
Glycerolipid metabolism	16	sn-Glycerol 3-phosphate	0.0017566	2.7553	0.084318	0.01773	0.0436
Amino sugar and nucleotide sugar metabolism	37	N-Acetyl-D-glucosamine D-Glucose 1-phosphate alpha-D-Glucose D-Glucosamine	0.0020861	2.6807	0.095962	0.01773	0.1347
Glycolysis / Gluconeogenesis	26	D-Glucose 1-phosphate alpha-D-Glucose Acetate	0.0036884	2.4332	0.16598	0.02379	0.0299
Pentose and glucuronate interconversions	18	D-Glucose 1-phosphate	0.0037316	2.4281	0.16598	0.02379	0

Phosphonate and phosphinate metabolism	6	2-Aminoethylphosphonate CMP-2-aminoethylphosphonate	0.011575	1.9365	0.49771	0.06559	0.5
Pentose phosphate pathway	22	D-Ribose 5-phosphate D-Ribose	0.014298	1.8447	0.6005	0.07292	0.1735
Galactose metabolism	27	Raffinose alpha-D-Glucose Glucose 1-phosphate D-Sorbitol	0.03168	1.4992	1	0.14688	0.0711
Glyoxylate and dicarboxylate metabolism	32	Glycolate L-Serine Glycine L-Glutamate Acetate L-Glutamine	0.035254	1.4528	1	0.14983	0.2275
D-Glutamine and D-glutamate metabolism	6	3	0.04192	1.3776	1	0.16446	0.5
Nitrogen metabolism	6	2	0.048426	1.3149	1	0.17641	0
Porphyrin and chlorophyll metabolism	30	2	0.052208	1.2823	1	0.17751	0
Terpenoid backbone biosynthesis	18	1	0.09353	1.0291	1	0.29813	0.1143

Cysteine and methionine metabolism	33	5	0.10513	0.97829	1	0.29902	0.3647
Purine metabolism	65	13	0.10554	0.9766	1	0.29902	0.0697
Glutathione metabolism	28	3	0.13648	0.86492	1	0.35121	0.1084
Pyruvate metabolism	22	2	0.14275	0.84542	1	0.35121	0.0607
Histidine metabolism	16	6	0.14462	0.83978	1	0.35121	0.4836
Arginine biosynthesis	14	8	0.15839	0.80028	1	0.36717	0.4822
Sphingolipid metabolism	21	1	0.20052	0.69783	1	0.42965	0
Lysine degradation	25	2	0.20801	0.68192	1	0.42965	0
Aminoacyl-tRNA biosynthesis	48	17	0.21698	0.66359	1	0.42965	0.1667
Tyrosine metabolism	42	5	0.21904	0.65948	1	0.42965	0.3177
Taurine and hypotaurine metabolism	8	2	0.22808	0.64192	1	0.43081	0.4286
Ubiquinone and other terpenoid-quinone biosynthesis	9	2	0.24733	0.60672	1	0.45049	0
beta-Alanine metabolism	21	6	0.34062	0.46773	1	0.59902	0.1605
Biosynthesis of unsaturated fatty acids	36	1	0.36184	0.44149	1	0.61512	0
Alanine, aspartate and glutamate metabolism	28	8	0.38782	0.41137	1	0.63802	0.6234

Propanoate metabolism	23	3	0.4407	0.35585	1	0.69454	0.0406
Caffeine metabolism	10	1	0.44941	0.34736	1	0.69454	0.6923
Phenylalanine, tyrosine and tryptophan biosynthesis	4	2	0.5064	0.29551	1	0.71691	1
Fructose and mannose metabolism	20	2	0.51075	0.2918	1	0.71691	0.0331
Glycine, serine and threonine metabolism	33	7	0.52952	0.27611	1	0.71691	0.489
Citrate cycle (TCA cycle)	20	2	0.53077	0.27509	1	0.71691	0.0625
Pyrimidine metabolism	39	11	0.54244	0.26565	1	0.71691	0.3551
Arginine and proline metabolism	38	7	0.54957	0.25997	1	0.71691	0.3511
Valine, leucine and isoleucine biosynthesis	8	6	0.56229	0.25004	1	0.71691	0
Nicotinate and nicotinamide metabolism	15	2	0.63013	0.20057	1	0.78381	0
Selenocompound metabolism	20	1	0.66165	0.17937	1	0.80343	0
Valine, leucine and isoleucine degradation	40	4	0.69139	0.16028	1	0.82002	0.0217
Primary bile acid biosynthesis	46	3	0.71442	0.14605	1	0.82808	0.0232

Phenylalanine metabolism	10	3	0.75959	0.11942	1	0.86087	0.3571
Pantothenate and CoA biosynthesis	19	5	0.83005	0.0809	1	0.92027	0.0357
Biotin metabolism	10	2	0.89845	0.04651	1	0.96152	0.2
Synthesis and degradation of ketone bodies	5	1	0.90496	0.04337	1	0.96152	0
Steroid hormone biosynthesis	85	2	0.96916	0.01361	1	0.97853	0.0376
Tryptophan metabolism	41	1	0.97471	0.01113	1	0.97853	0.1431
Butanoate metabolism	15	4	0.97853	0.00943	1	0.97853	0

Supplementary Table S6.

Pathway Results for Total Maternal Cortisol Concentration Trimester 2, N = 97 (MetaboAnalyst)

Pathways	Total Compounds	Hits	Raw p	-log10(p)	Holm adjust	FDR	Impact
Arginine biosynthesis	14	8	0.01584	1.8004	0.80761	0.396	0.4822
Glutathione metabolism	28	3	0.02108	1.6762	1	0.396	0.1084
Butanoate metabolism	15	4	0.02866	1.5427	1	0.396	0
Citrate cycle (TCA cycle)	20	2	0.04377	1.3588	1	0.396	0.0625
Propanoate metabolism	23	3	0.04431	1.3535	1	0.396	0.0406
Porphyrin and chlorophyll metabolism	30	2	0.05867	1.2316	1	0.396	0
Pyrimidine metabolism	39	11	0.06798	1.1677	1	0.396	0.3551
Nitrogen metabolism	6	2	0.07327	1.1351	1	0.396	0
Purine metabolism	65	13	0.08299	1.081	1	0.396	0.0697
Arginine and proline metabolism	38	7	0.08354	1.0781	1	0.396	0.3511
D-Glutamine and D-glutamate metabolism	6	3	0.08719	1.0595	1	0.396	0.5
Pentose phosphate pathway	22	2	0.09992	1.0004	1	0.396	0.1735
Pantothenate and CoA biosynthesis	19	5	0.10287	0.98769	1	0.396	0.0357
Alanine, aspartate and glutamate metabolism	28	8	0.11246	0.94899	1	0.396	0.6234
Glycolysis / Gluconeogenesis	26	3	0.11651	0.93365	1	0.396	0.0299
Amino sugar and nucleotide sugar metabolism	37	4	0.12982	0.88665	1	0.396	0.1347
Neomycin, kanamycin and gentamicin biosynthesis	2	1	0.14778	0.83037	1	0.396	0
Inositol phosphate metabolism	30	1	0.14778	0.83037	1	0.396	0

Starch and sucrose metabolism	18	2	0.15631	0.80602	1	0.396	0.2734
Nicotinate and nicotinamide metabolism	15	2	0.16069	0.794	1	0.396	0
Steroid hormone biosynthesis	85	2	0.16359	0.78623	1	0.396	0.0376
Primary bile acid biosynthesis	46	3	0.17081	0.76748	1	0.396	0.0232
Pentose and glucuronate interconversions	18	1	0.18199	0.73994	1	0.4036	0
Glyoxylate and dicarboxylate metabolism	32	6	0.19623	0.70724	1	0.417	0.2275
Fructose and mannose metabolism	20	2	0.21112	0.67548	1	0.4221	0.0331
Galactose metabolism	27	4	0.21517	0.66722	1	0.4221	0.0711
Aminoacyl-tRNA biosynthesis	48	17	0.27145	0.56631	1	0.5127	0.1667
Glycerophospholipid metabolism	36	1	0.29865	0.52484	1	0.5252	0.0809
Glycerolipid metabolism	16	1	0.29865	0.52484	1	0.5252	0.0436
Valine, leucine and isoleucine degradation	40	4	0.31873	0.49657	1	0.5419	0.0217
Valine, leucine and isoleucine biosynthesis	8	6	0.33471	0.47534	1	0.5449	0
Phosphonate and phosphinate metabolism	6	2	0.34618	0.4607	1	0.5449	0.5
beta-Alanine metabolism	21	6	0.3526	0.45272	1	0.5449	0.1605
Ubiquinone and other terpenoid-quinone biosynthesis	9	2	0.37713	0.42351	1	0.5606	0
Histidine metabolism	16	6	0.3847	0.41488	1	0.5606	0.4836

Glycine, serine and threonine metabolism	33	7	0.40945	0.3878	1	0.5682	0.489
Tryptophan metabolism	41	1	0.41369	0.38333	1	0.5682	0.1431
Biotin metabolism	10	2	0.43809	0.35844	1	0.5682	0.2
Phenylalanine, tyrosine and tryptophan biosynthesis	4	2	0.44414	0.35248	1	0.5682	1
Selenocompound metabolism	20	1	0.44563	0.35103	1	0.5682	0
Sphingolipid metabolism	21	1	0.48385	0.31529	1	0.6019	0
Phenylalanine metabolism	10	3	0.52438	0.28036	1	0.6368	0.3571
Lysine degradation	25	2	0.54003	0.26758	1	0.6405	0
Synthesis and degradation of ketone bodies	5	1	0.58984	0.22927	1	0.6837	0
Terpenoid backbone biosynthesis	18	1	0.61629	0.21021	1	0.6985	0.1143
Caffeine metabolism	10	1	0.63084	0.20008	1	0.6994	0.6923
Taurine and hypotaurine metabolism	8	2	0.65197	0.18577	1	0.7075	0.4286
Pyruvate metabolism	22	2	0.71209	0.14747	1	0.7566	0.0607
Biosynthesis of unsaturated fatty acids	36	1	0.74267	0.1292	1	0.7713	0
Tyrosine metabolism	42	5	0.75613	0.1214	1	0.7713	0.3177
Cysteine and methionine metabolism	33	5	0.85467	0.0682	1	0.8547	0.3647

Supplementary Table S7.

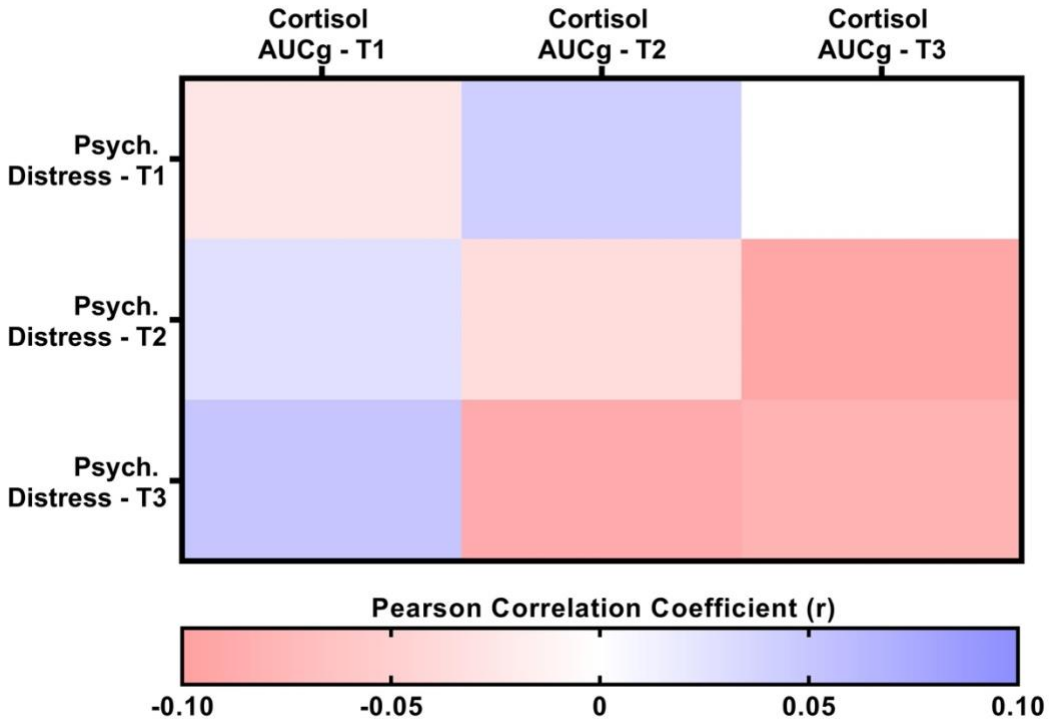
Pathway Results for Total Maternal Cortisol Concentration Trimester 3, N = 127 (MetaboAnalyst)

Pathways	Total Compounds	Hits	Raw p	-log10(p)	Holm adjust	FDR	Impact
Propanoate metabolism	23	Succinate 2-Oxobutanoate Propanoate	0.005175	2.2861	0.26391	0.1484	0.0406
Citrate cycle (TCA cycle)	20	Succinate Fumarate	0.008155	2.0886	0.40776	0.1484	0.0625
Butanoate metabolism	15	(R)-3-Hydroxybutanoate L-Glutamate Butanoic acid Succinate	0.008731	2.0589	0.42782	0.1484	0
Alanine, aspartate and glutamate metabolism	28	28	0.034931	1.4568	1	0.4429	0.6234
Pantothenate and CoA biosynthesis	19	19	0.04342	1.3623	1	0.4429	0.0357
Terpenoid backbone biosynthesis	18	18	0.091022	1.0409	1	0.5165	0.1143
Fructose and mannose metabolism	20	20	0.099314	1.003	1	0.5165	0.0331
Pentose phosphate pathway	22	22	0.10751	0.96853	1	0.5165	0.1735
Glycerophospholipid metabolism	36	36	0.12288	0.91051	1	0.5165	0.0809
Glycerolipid metabolism	16	16	0.12288	0.91051	1	0.5165	0.0436
Primary bile acid biosynthesis	46	46	0.14862	0.82792	1	0.5165	0.0232

Synthesis and degradation of ketone bodies	5	5	0.15829	0.80054	1	0.5165	0
Arginine and proline metabolism	38	38	0.15955	0.7971	1	0.5165	0.3511
Glutathione metabolism	28	28	0.16822	0.77412	1	0.5165	0.1084
Galactose metabolism	27	27	0.17043	0.76844	1	0.5165	0.0711
Caffeine metabolism	10	10	0.17341	0.76094	1	0.5165	0.6923
Nicotinate and nicotinamide metabolism	15	15	0.18384	0.73557	1	0.5165	0
Phenylalanine, tyrosine and tryptophan biosynthesis	4	4	0.20052	0.69784	1	0.5165	1
Pyrimidine metabolism	39	39	0.20486	0.68853	1	0.5165	0.3551
Glycine, serine and threonine metabolism	33	33	0.20702	0.68398	1	0.5165	0.489
Glycolysis / Gluconeogenesis	26	26	0.22235	0.65297	1	0.5165	0.0299
Taurine and hypotaurine metabolism	8	8	0.2477	0.60608	1	0.5165	0.4286
beta-Alanine metabolism	21	21	0.25701	0.59005	1	0.5165	0.1605
Porphyrin and chlorophyll metabolism	30	30	0.25874	0.58714	1	0.5165	0
Arginine biosynthesis	14	14	0.25886	0.58694	1	0.5165	0.4822
Selenocompound metabolism	20	20	0.2633	0.57954	1	0.5165	0
Amino sugar and nucleotide	37	37	0.28466	0.54568	1	0.5365	0.1347

sugar metabolism							
Valine, leucine and isoleucine biosynthesis	8	8	0.29455	0.53084	1	0.5365	0
Lysine degradation	25	25	0.35794	0.44619	1	0.6064	0
Pyruvate metabolism	22	22	0.36147	0.44192	1	0.6064	0.0607
D-Glutamine and D-glutamate metabolism	6	6	0.3686	0.43344	1	0.6064	0.5
Glyoxylate and dicarboxylate metabolism	32	32	0.39834	0.39975	1	0.6349	0.2275
Nitrogen metabolism	6	6	0.43441	0.3621	1	0.6714	0
Sphingolipid metabolism	21	21	0.48712	0.31236	1	0.6839	0
Purine metabolism	65	65	0.48727	0.31223	1	0.6839	0.0697
Tryptophan metabolism	41	41	0.48836	0.31126	1	0.6839	0.1431
Neomycin, kanamycin and gentamicin biosynthesis	2	2	0.5335	0.27287	1	0.6839	0
Inositol phosphate metabolism	30	30	0.5335	0.27287	1	0.6839	0
Aminoacyl-tRNA biosynthesis	48	48	0.53493	0.27171	1	0.6839	0.1667
Valine, leucine and isoleucine degradation	40	40	0.54172	0.26622	1	0.6839	0.0217
Cysteine and methionine metabolism	33	33	0.5523	0.25782	1	0.6839	0.3647

Ubiquinone and other terpenoid-quinone biosynthesis	9	9	0.56324	0.24931	1	0.6839	0
Phenylalanine metabolism	10	10	0.59526	0.22529	1	0.706	0.3571
Steroid hormone biosynthesis	85	85	0.61361	0.2121	1	0.7112	0.0376
Biosynthesis of unsaturated fatty acids	36	36	0.64467	0.19067	1	0.7268	0
Starch and sucrose metabolism	18	18	0.65908	0.18106	1	0.7268	0.2734
Pentose and glucuronate interconversions	18	18	0.66978	0.17407	1	0.7268	0
Phosphonate and phosphinate metabolism	6	6	0.74248	0.12931	1	0.7889	0.5
Histidine metabolism	16	16	0.76357	0.11715	1	0.7947	0.4836
Biotin metabolism	10	10	0.84484	0.07323	1	0.8551	0.2
Tyrosine metabolism	42	42	0.8551	0.06798	1	0.8551	0.3177



Supplementary Figure S1. Correlation heatmap examining the association between total maternal cortisol concentration and psychological distress in each trimester, T= Trimester. (*n* = 144 for Cort-T1, *n* = 279 for Cort-T2, *n* = 318 for Cort-T3, *n* = 282 for Psych. Distress-T1, *n* = 363 for Psych. Distress-T2, *n* = 349 for Psych. Distress-T3) *< .05.