The Impact of Maternal Distress on Child Neurodevelopment in a Sex-Specific Manner: Examining the Mediating Role of the Infant Gut Microbiome

by

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Abstract

Introduction

Depression has taken over as the leading cause of disability worldwide. In Alberta, 7% of mothers reported prenatal depressive symptoms and more than 25% of women worldwide reported mental health-related concerns after childbirth. The DOHaD hypothesis aims to emphasize that both the prenatal and postnatal periods encompass sensitive windows of developmental plasticity in which exposures during the early life could impact the development of health and disease throughout the lifespan. Substantial evidence has demonstrated the negative consequences of maternal distress on offspring brain development with recent literature demonstrating potential sex differences. In a newly emerging field, maternal stress additionally appears to impact the developing gut microbiome and gut-brain axis; however, stress-microbiome pathways have not been fully explored in humans. The purpose of this research was to demonstrate the impact of maternal distress on child neurodevelopment in a sex-specific manner while exploring whether breastfeeding and the infant's gut microbiome sequentially mediated this association.

Methods

This study consisted of 646 healthy term infants from the Canadian Healthy Infant Longitudinal Development (CHILD) birth cohort. Maternal distress was measured using the Centre of Epidemiological Studies Depression Scale (CES-D) and Perceived Stress Scale (PSS) administered at several time points through pregnancy. Child neurodevelopment was assessed using the Bayley Scales of Infant Development (BSID-III) administered at one and two years of age. Exclusive breastfeeding duration was reported during infancy with infant fecal samples collected from a home assessment at four months of age. Microbiota from fecal samples were sequenced using 16S sequencing with *C. difficile* analyzed using qPCR with the appropriate

primers. Microbial metabolites were identified using NMR sequencing. Covariates were measured from study questionnaires or hospital birth records. A DAG approach was used to select the minimal adjustment set from potential covariates. Firstly, we tested multivariable linear regression models with child sex interactions to test whether the impact of maternal distress at different time points through pregnancy had sex-specific impacts on child neurodevelopment scores. Secondly, we built adjusted structural equation models to test sequential mediations of exclusive breastfeeding duration and infant gut microbial mediators on this association.

Results

In our study, approximately 16% of mothers experienced clinically significant depressive symptoms and 31% experienced higher than average perceived stress during the perinatal period. We found six significant interactions demonstrating that maternal distress had a time-and-sex-specific impact on child language and motor scores. Moreover, children's cognitive and social-emotional scores did not demonstrate this sex-specific effect and were significantly reduced among both sexes following exposure to persistent distress. Exploring the gut-brain axis, we found several sex-specific mediation pathways. We found that 43% of healthy infants were colonized with *C. difficile* at four month of age and that the impact of prenatal depressive symptoms on child neurodevelopment scores was sequentially mediated by breastfeeding duration and *C. difficile* abundance. Specifically, depressive symptoms decreased exclusive breastfeeding duration which increased *C. difficile* abundance during infancy and lowered two-year cognitive and language scores among boys. We found a significant intervention in this pathway, in which increasing exclusive breastfeeding duration can reduce infant *C. difficile* abundance and increase boys' cognitive, language, and motor scores. The positive impact of

exclusive breastfeeding duration on neurodevelopment scores was further mediated by increasing acetate abundance during infancy, specifically in girls.

Conclusion

Alongside the DOHaD hypothesis, the prenatal and postnatal periods represent sensitive windows in which stressors may impact a child's developmental trajectory. We demonstrated that maternal distress had a time-and-sex specific impact on child neurodevelopment scores. Furthermore, colonization with *C. difficile* during infancy may not be as benign as the current literature supports as we revealed that *C. difficile* abundance during infancy had both direct and indirect effects of lowering neurodevelopment scores in childhood. Interestingly, we also found pathways demonstrating a significant intervention on this association in which supporting women to breastfeed for a longer duration could reduce the abundance of *C. difficile* and increase the abundance of microbial SCFA acetate during infancy which was associated with increased two-year neurodevelopmental scores. Future research should explore whether supplementation with acetate during infancy could mitigate the effects of shorter breastfeeding duration on neurodevelopment scores. This research emphasizes the significance of supporting women's mental health through pregnancy and promoting exclusive breastfeeding duration as we revealed novel pathways indicating maternal distress symptoms may impact the development of the gut-brain axis among healthy children.

Keywords: gut microbiome, maternal distress, child neurodevelopment, sex-specificity, early life, infancy, microbial metabolites, *C. difficile*, acetate

Preface

This thesis is original work by Carmen Tessier. The research project in which this thesis is a part of received ethics approval from the University of Alberta Research Ethics Board (# Pro00103296).

This thesis was written in a traditional thesis format following the Faculty of Graduate Research and Studies formatting guidelines at the University of Alberta.

No part of this thesis has been previously published.

This thesis comprises of four-chapter sections:

CHAPTER 1: INTRODUCTION
Chapter 1 consists of a literature review followed by the project aims and hypotheses.

CHAPTER 2: METHODS AND MATERIALS
Chapter 2 consists of the methods, sample size calculation, and statistical analysis plan.

CHAPTER 3: RESULTS
Chapter 3 reveals the sample characteristics and the results of both the primary and secondary aims of the study.

CHAPTER 4: DISCUSSION AND CONCLUSION
Chapter 4 discusses the summary and interpretation of results, the strengths and limitations, clinical relevance, implications for future research, bias assessment, and the final conclusions.

Dedication

"Cogito, ergo sum"
— René Descartes

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Firstly, I would like to thank my mentor and supervisor, Dr. Anita Kozyrskyj, for accepting me as your student and for your dedicated time and guidance through my Master's program. My early research career has taught me the importance of mentorship and having such a supportive, understanding, and brilliant research supervisor has shaped my research career. Looking back at when I started my Master's program, I owe the researcher that I have become today to your continued guidance and I am eternally thankful. Thank you for your undoubted trust in my abilities and I feel honoured to have had the opportunity to work under your supervision.

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List of Abbreviations

Abbreviated term	Abbreviation phrase
ANOVA	Analysis of Variance
BDNF	Brain-Derived Neurotrophic Factor
BSID-III	Bayley Scales of Infant Development Third Edition
BMI	Body Mass Index
CI	Confidence Interval
C-section	Caesarean Section
CESD	Center for Epidemiological Studies Depression Scale
CHILD	Canadian Healthy Infant Longitudinal Development Study
DAG	Directed Acyclic Graph
DOHaD	Developmental Origins of Health and Disease
GF	Germ-Free (referring to animal models with no exposure to microbiota)
IAP	Intrapartum Antibiotic Prophylaxis
IgA	Immunoglobulin A (sIgA refers to secretory IgA)
PFC	Prefrontal Cortex
PSS	Perceived Stress Scale
SCFA	Short Chain Fatty Acid
SEM	Structural Equation Model
SES	Socioeconomic Status

CHAPTER 1: INTRODUCTION

Chapter 1 introduces the associations between maternal distress through pregnancy and its impacts on child neurodevelopment while also exploring breastfeeding, the gut microbiome, sex-specificity, and how the covariates in this study are associated with both the exposure and outcome. This chapter also summarizes gaps in the literature and how this study can fill those gaps.

CHAPTER 1: INTRODUCTION

1.1 Introduction

Stress is ubiquitous to all species; however, it can be complex to define. Stress may be defined as exposure to an unpredicted change in environment, an innate physiological response, a perceived psychosocial construct- or if one were to ask a physicist- it may be described as a force that acts against resistance. Hans Selye coined the term *stress* in 1936 to describe the phenomenon he witnessed in which patients that were exposed to various types of stressors had the same triad of symptoms: an enlarged adrenal cortex, atrophy of the thymus, and gastrointestinal ulcers (Selye, 1936). Stress was thus defined as the "non-specific response of the body to any demand made upon it" (Selye, 1936). The triad of symptoms as a result of chronic stress is noteworthy as they demonstrate that stress can have a systemic effect on the body by altering stress hormone release, the immune system, and gastrointestinal function.

A newly emerging field referred to as the gut-brain axis explores the bidirectional relationship between the central nervous system and the enteric nervous system and plays a vital role in overall health. The gut microbiome consists of microbiota and their gene products that reside in the gastrointestinal tract. Colonization of the gut microbiome begins after birth, with infancy presenting as a sensitive period for the co-development of the infant's brain and gut microbiome. In humans, meta-analytic evidence from 14 studies linked maternal postpartum depression to lower cognitive scores in infants on the Bayley's neurodevelopmental scale (Y. Liu et al., 2017). Increasing evidence from animal models has demonstrated the negative effects of prenatal stress on offspring brain development (Charil et al., 2010) with the prenatal and postnatal periods described as critical windows for the impact of stress on the gut-brain axis (Cowan et al., 2020). However, stress-microbiome pathways have not been fully explored in humans.

Gut microbiota produce many active metabolites that are absorbed and have the potential to access the brain; hence, changes in the infant gut microbiota after stress that alter the metabolite composition have the capacity to influence the developing brain (Ganci et al., 2019). The purpose of this study is to examine the impact of maternal distress on child neurodevelopment by exploring the mediating role of infant gut microbiota and metabolites using the Canadian Healthy Infant Longitudinal Development (CHILD) birth cohort.

1.2. Part 1 of the Triad: Stress

The first symptom of Selye's triad after exposure to chronic stress was an increase in size of the adrenal cortex. This enlargement has also been discovered among patients with major depression (Nemeroff et al., 1992). One of the main functions of the adrenal cortex is to release hormones, such stress hormones, including glucocorticoids. The stress response includes the activation of two physiological pathways. First, the body will rapidly activate the sympathetic nervous system (the "fight or flight" response) to increase energy availability in the body as an evolutionary adaptation to either fight or flee from stressors such as predators. Secondly, activation of the hypothalamic-pituitary-adrenal (HPA) axis, which releases glucocorticoids, will be sustained until the stress subsides. Unfortunately, if the stressor persists, a state of chronic stress may be induced altering hormone balance within the body which has been associated with major depressive symptoms (Zunszain et al., 2011). Maternal distress refers to experiencing stress and/or depressive symptoms during the pregnancy period. Mothers may experience distress for a variety of reasons, including as a response to pregnancy, pregnancy-related concerns, or life events. Experiencing distress in either the prenatal, postnatal or persistently through pregnancy could affect the developmental trajectory of the offspring through prenatal fetal mechanisms and postnatal mother-infant interactions.

1.2.1 Prenatal Distress

Evidence from natural disasters and famines have demonstrated the extent to which prenatal stress can alter the developmental trajectory of the fetus with cognitive impairments remaining through childhood (Laplante et al., 2008; Painter et al., 2005). Spelt was the first to demonstrate that the human fetus could learn from its environment *in vivo* (Spelt, 1948). The fetal programming hypothesis expands on the notion that the fetus is learning from its environment and suggests that early exposures in utero "set the stage" for what to expect from the postnatal environment (Barker, 1990). The literature demonstrates that both prenatal stress and depression lead to alterations in circulating maternal stress hormones which can pass through the placenta and induce alterations in gene expression through epigenetic mechanisms (Charil et al., 2010; E. P. Davis et al., 2007a; Shao et al., 2020; Stroud et al., 2019). This mechanism could explain literature demonstrating that exposure to prenatal stress in utero increased offspring HPA axis hormone release in rhesus monkeys (A. S. Clarke et al., 1994). The mothers exposed to the highest stress treatment group had offspring that displayed the greatest alteration in stress

hormones indicating the level of exposure to stress can increase responsivity to stressors later in life. In this example, the fetal programming hypothesis would suggest that after exposure to increased stress hormones in utero, the fetus would learn that the environment was stressful and adapt mechanisms, such as a heightened stress response, to cope with a predicted high-stress postnatal environment. Exploring brain regions impacted by stress, researchers have demonstrated that prenatal stress results in an increase in the offspring's amygdala with this brain region being associated with fear, anxiety, and stress response mechanisms within the body (Jones et al., 2019). An increased amygdala size would allow for the offspring to initiate a quicker stress response. However, increased amygdala size has been associated with a decrease in other brain regions such as the pre-frontal cortex (PFC), as well as increased anxiety-like behaviour (M. Davis, 1992; McEwen et al., 2016). In addition to the amygdala and PFC, prenatal stress appears to impact areas within the limbic system such as the hippocampus as well as sensory regions, which demonstrates a potential impact on emotion, cognition, and motor function (Bock et al., 2015; Lemaire et al., 2000; Patin et al., 2004). These alterations are long-lasting with prenatal depression increasing late childhood thinning of the frontal lobe region which mediated children's externalizing behaviour at 6-9 years of age (Sandman et al., 2015). In Alberta, 7% of mothers experienced prenatal depression and 20% reported prenatal anxiety during their third trimester of pregnancy (Bayrampour et al., 2015). Maternal distress symptoms may alter circulating maternal stress hormones which pass through the placenta and have the potential to impact stress reactivity, structural areas of the brain involved in cognition and emotion, as well as behavioural changes among children.

1.2.2 Postnatal Distress

In addition to the prenatal period, experiencing postnatal distress may alter maternal behaviour. Building from the prenatal programming hypothesis, the Developmental Origins of Health and Disease (DOHaD) approach emphasizes that both the prenatal and postnatal environments influence the developmental trajectory of children (Barker, 2007). Attachment theory focuses on the postnatal interactions between the mother and child in the first few years of life. According to the theory, primary caregivers that are supportive and responsive to their infants' needs allow the infant to develop a sense of security (Ainsworth, 1979). A disruption in the formation of this mother-infant attachment was proposed to result in long-term cognitive, social, and emotional difficulties in the infant (Bowlby, 1969). Animal research has

demonstrated that postnatal stress-induced maternal HPA axis reprogramming leads to increased maternal depression-like symptoms and deficits in maternal care (Brummelte & Galea, 2010; Murgatroyd et al., 2015). The impact of the stress response on the PFC and amygdala have been a target of both animal and human research, with studies demonstrating links from these brain regions to maternal responsiveness, mood, anxiety, and maternal care behaviours (Afonso et al., 2007; Febo et al., 2010; Murgatroyd et al., 2015; Numan et al., 2010). In humans, brain imaging methods demonstrated that postnatal depressive symptoms were associated with decreased maternal brain region activation when presented with the sound of their infant's crying as well as a lower response to positive photos of their infant's faces (Barrett et al., 2012; Laurent & Ablow, 2012). This demonstrates depressive symptoms may reduce activation in maternal behaviour brain networks in mothers with potential subsequent impacts on mother-infant attachment. A systemic review revealed that mother-infant interactions impacted cognitive, language, motor, and social development in infants at twelve months of age (Adriana Cicuto Ferreira Rocha et al., 2020). With more than a quarter of women worldwide experiencing mental health problems after giving birth (Shorey et al., 2018), maternal postnatal distress may pose a risk to mother-child attachment and child neurodevelopment scores.

Experiencing distress in either the prenatal, postnatal or persistently through pregnancy could impact the developmental trajectory of the offspring through prenatal fetal mechanisms and postnatal mother-infant interactions.

1.3 Part 2 of the Triad: Breastfeeding

The second of Selye's triad was the atrophy of the thymus which demonstrated the impact of stress on the immune system. In infants, breastfeeding is essential to initiate the newborns' immune system by providing passive immunity from bioactive molecules, such as immunoglobulin A (IgA) (Koenig et al., 2005). Exploring the impact of stress, both prenatal and postnatal maternal distress have been associated with decreased breastfeeding duration (Bascom & Napolitano, 2016; Wallenborn et al., 2018).

Breastfeeding may facilitate mother-infant bonding (Fergusson & Woodward, 1999) as well as provide the developing infant with various bioactive molecules to promote brain development, gut microbial composition, and the development of their immune system (Ballard & Morrow, 2013; Gibbs et al., 2018). Within CHILD, we found that breastfeeding was associated with infant microbial SCFA abundance (Bridgman et al., 2017a). Breastmilk contains

sugars that select for beneficial bacteria in the infant such as Bifidobacterium species which make SCFA such as acetate, propionate, and butyrate (Oozeer et al., 2013). Thus, a decreased breastfeeding duration could alter the initial gut microbiota and metabolite composition among infants which may impact their developing brain.

In addition to altering the infant's gut microbiome, psychological distress during pregnancy was associated with decreasing the immunological benefits of breastmilk by reducing IgA concentration (Moirasgenti et al., 2019). Initially provided to the newborn through maternal breastmilk, IgA plays a role in infant microbial tolerance and gut barrier maintenance through its ability to bind both commensal microbiota as well as elicit immune response pathways against microbial pathogens (Bunker & Bendelac, 2018). An off-balanced gut microbiome characterized by pathogen overgrowth, referred to as gut dysbiosis, has been associated with altered intestinal barriers, increased permeability, and increased inflammation, and is a suggested mechanism in the pathway from the altered gut microbiota to altered brain function (Rogers et al., 2016). Recently, IgA has been associated with not only gut barrier maintenance but plays an additional role in defending the brain barrier as well (Fitzpatrick et al., 2020). Therefore, breastmilk composition may play a role in the development of both the gut and brain barriers during infancy in addition to preventing gut dysbiosis and inflammation.

Infancy presents itself as a sensitive period in which disruptions to early colonization, such as decreased breastfeeding duration after exposure to maternal distress, may affect immune tolerance mechanisms to commensal microbiota, impair both gut and brain barrier formation, and impact the developing gut-brain axis.

1.4 Part 3 of the Triad: The Gut Microbiome

After exposure to chronic stress, Selye reported gut symptom pathologies as the final part of the triad. Stress in early life can alter the HPA axis with evidence emerging of crosstalk between the HPA axis and commensal microbiota (Frankiensztajn et al., 2020). In a human study, prenatal stress was associated with infant colonization with fewer beneficial microbiota (Zijlmans et al., 2015). Moreover, after exposure to maternal prenatal stress, male mice displayed increased stress hormone release, neuroinflammation, altered gut microbial composition, and decreased social behaviour (Gur et al., 2019). Therefore, altered HPA axis response after exposure to stress may impact the development of the gut-brain axis in infants. Germ-free mice, which are mice with an absent microbiome, were found to have an exaggerated stress response as

well as a reduced brain-derived neurotrophic factor (BDNF) (Sudo et al., 2004). Reduced BDNF has been implicated with several neurological disorders, such as schizophrenia and autism as well as individuals living with major depressive disorder (Jiang et al., 2015; Skogstrand et al., 2019). Interestingly, post-colonization of these stress-exposed GF mice only reversed some of these alterations and only if it was administered during early developmental stages (Sudo et al., 2004). In summary, altered microbiota and their metabolites after exposure to stress have the capacity to impact the developing brain, and interventions such as re-colonization and supplementation during an early critical window may restore some of these negative effects.

The first thousand days of life represent the greatest window of opportunity for influences and modulation of the microbiome (Robertson et al., 2019). The active and dynamic development of the gut microbiome coincides with key neurodevelopmental processes such as neurogenesis, cell differentiation, myelination, synaptogenesis, and microglial activation which provides a clear window of opportunity in which disruptions in gut colonization could affect brain development (Borre et al., 2014). The infant's gut microbiota and their metabolites may be impacted by maternal distress which may affect neurodevelopmental outcomes in childhood.

1.4.1 The Brain in the Absence of a Microbiome

Studies with germ-free (GF) animals demonstrate the extent of pathology with the lack of an early life commensal gut microbiome. Concerning brain development, GF animals display increased blood-brain barrier (BBB) permeability (Braniste et al., 2014), hypermyelination in the prefrontal cortex (PFC) (Hoban et al., 2016), alterations in cytokines (Morgane S. Thion et al., 2018), neurotransmitters (noradrenaline, dopamine, serotonin, serotonin receptors (G. Clarke et al., 2013; Heijtz et al., 2011a)), and tryptophan concentrations (G. Clarke et al., 2013), altered microglial cell size, number, and maturity (Castillo-Ruiz et al., 2018; Erny et al., 2015; Morgane Sonia Thion et al., 2018a), reduced brain-derived neurotrophic factor (BDNF) (Bercik et al., 2011; Erny et al., 2015), and exaggerated stress hormone signalling (ACTH, corticosterone, glucocorticoid receptor) (G. Clarke et al., 2013; Neufeld et al., 2011; Sudo et al., 2004). Recolonization of GF mice with microbiota or their SCFA metabolites can only restore some of these effects (Braniste et al., 2014; G. Clarke et al., 2013; Sudo et al., 2004). The absence of microbiota and their metabolites display a detrimental and time-sensitive effect on brain development with a potential to disrupt immune functioning, emotional and stress responses, motor activities, fear and anxiety-like behaviours, and overall cognitive functioning.

1.4.2 Colonization Resistance*: C. difficile*

Exploring specific bacteria, approximately 30-60% of infants are colonized with *Clostridioides difficile* (*C. difficile*) with prevalence on the rise (Kubota et al., 2016a; Zilberberg et al., 2008). Although *C. difficile* is a known pathogen in adults; interestingly, infants appear asymptomatic (Jangi & Lamont, 2010). Within the CHILD cohort, prenatal depressive symptoms were associated with reduced immunoglobulin A (IgA) and reduced IgA is a risk factor for *C. difficile* colonization (Meghan B. Azad et al., 2012a; L. J. Kang et al., 2018). Exploring breastfeeding, the sugars found in breastmilk can select for beneficial bacteria (Musilova et al., 2014) which may serve as a function of colonization resistance. Colonization resistance is the ability of microbes to prevent other, potentially pathogenic microbes, from colonizing. *Clostridioides difficile* is acquired from the environment and a deficiency of breastfeeding, and thus lack of beneficial bacteria present within the infant's gut, may increase the likelihood for *C. difficile* to colonize. Recently within CHILD, multilevel mediation pathways revealed that *C. difficile* colonization in infancy was associated with altered microbial composition, altered microbial metabolites, as well as colonization being identified in pathways towards development of negative childhood outcomes such as atopy and obesity (Vu et al., 2021). Colonization with *C. difficile* altered each of the microbial metabolite levels more than any of the other microbiota present (Vu et al., 2021). Whether prenatal depressive symptoms increasing *C. difficile* colonization and altering SCFA metabolites has an impact on child neurodevelopment requires further attention.

1.4.3 Short-Chain Fatty Acids (SCFA)

The microbiome metabolites include short-chain fatty acids (SCFA) such as butyrate, propionate, and acetate, all of which can cross the blood-brain barrier and influence the central nervous system (Cryan et al., 2019). The primary source of SCFA comes from microbial fermentation of dietary fibres, as well as the breakdown of proteins by microbiota (Cryan et al., 2019). Researchers found that SCFA (acetic acid, butyric acid, and propionic acid) were decreased in mice exposed to chronic stress (Maltz et al., 2018). Examining neurodevelopment, researchers found that re-colonization with SCFA resulted in restoring brain microglia homeostasis in GF mice (Erny et al., 2015). However, the researchers supplemented with propionate, acetate, and butyrate together, making it no longer possible to differentiate between SCFA. Microbial SCFA can also act as substrates for astrocytogenesis (development of

astrocytes) and maintenance (Wyss et al., 2011). Astrocytes are necessary in providing structure and support for the blood-brain barrier (Abbott et al., 2006) and thus altering SCFA metabolite concentrations during infancy may impact the developing brain barrier. Animal model researchers found that chronic psychosocial stress disrupted gut microbiota composition with significant behavioural deficits; however, supplementation with butyrate, propionate and acetate ameliorated intestinal permeability and had antidepressant and anxiolytic effects (van de Wouw et al., 2018a). Supplementation with SCFA after stressful events may pose as a likely intervention.

1.4.4 Tryptophan

In addition to SCFA, the gut microbiome is also associated with amino acid metabolites. The gut microbiome makes 90% of the serotonin in the body through the tryptophan pathway and tryptophan metabolites are involved in the bidirectional communication between the brain and the gut (Agus et al., 2018). Germ-free mice with an absent microbiota that were exposed to stress had altered tryptophan concentrations and displayed anxiety-like behaviour (G. Clarke et al., 2013). Therefore, exposure to stress has the ability to alter tryptophan concentrations and affect limbic system areas of the brain that are responsible for emotional regulation. Relating the serotonergic systems to brain microglia, microbial metabolites derived from the tryptophan pathway have been implicated in microglial control in the central nervous system (Dodd et al., 2017). Therefore, both neuroimmune homeostasis and the serotonergic pathway may be regulated by microbial tryptophan metabolites and could be impacted by depression and stress.

1.5 Sex Differences

Researchers have noted sex-specific differences in HPA axis response as well as neurodevelopment scores. In preterm infants, male sex was related to lower cognitive, language, and motor scores on the BSID-III (Velikos et al., 2015). In animals, male GF mice had increased serum tryptophan and decreased hippocampal serotonin when compared to female offspring (G. Clarke et al., 2013). The hippocampal serotonergic system is involved in stress, anxiety, and depression; therefore, maternal distress could affect this system in a sex-specific manner. Reviews demonstrate sex differences in HPA responses; thus, the impact of maternal distress on the development of the offspring's HPA axis response may be sex-specific and with the sexes impacted differentially (Goel et al., 2014; Panagiotakopoulos & Neigh, 2014). Moreover, gut microbiome disruption after antibiotic treatment pre-and-postnatally was associated with sex-

specific alterations in microglia that lasted into adulthood (Morgane Sonia Thion et al., 2018a). The male animals were affected to a greater extent by prenatal exposures while the impact of female exposure may not surface until later in life. This literature demonstrates that the timing of perturbation on the microbiome impacted microglia in a time-and-sex-specific manner. Taken together, there is evidence of sex-specific stress responses, males demonstrating lower neurodevelopmental scores on the BSID-III, and germ-free males displaying increased detriments after exposure to prenatal stress and microbiome disruptions.

Figure 1 summarizes the co-development of the gut microbiome alongside the brain with infancy characterized as a sensitive period for immune system priming. Immune system priming occurs during colonization of the gut microbiome during infancy in which immune cells tolerize commensal microbiota to prevent increased inflammatory responses later in life (Al Nabhani & Eberl, 2020). Exposures such as maternal distress and breastfeeding may impact gut colonization and subsequently impact the gut-brain axis.

Figure 1. Early life co-development of the gut-brain axis and immune priming stages.

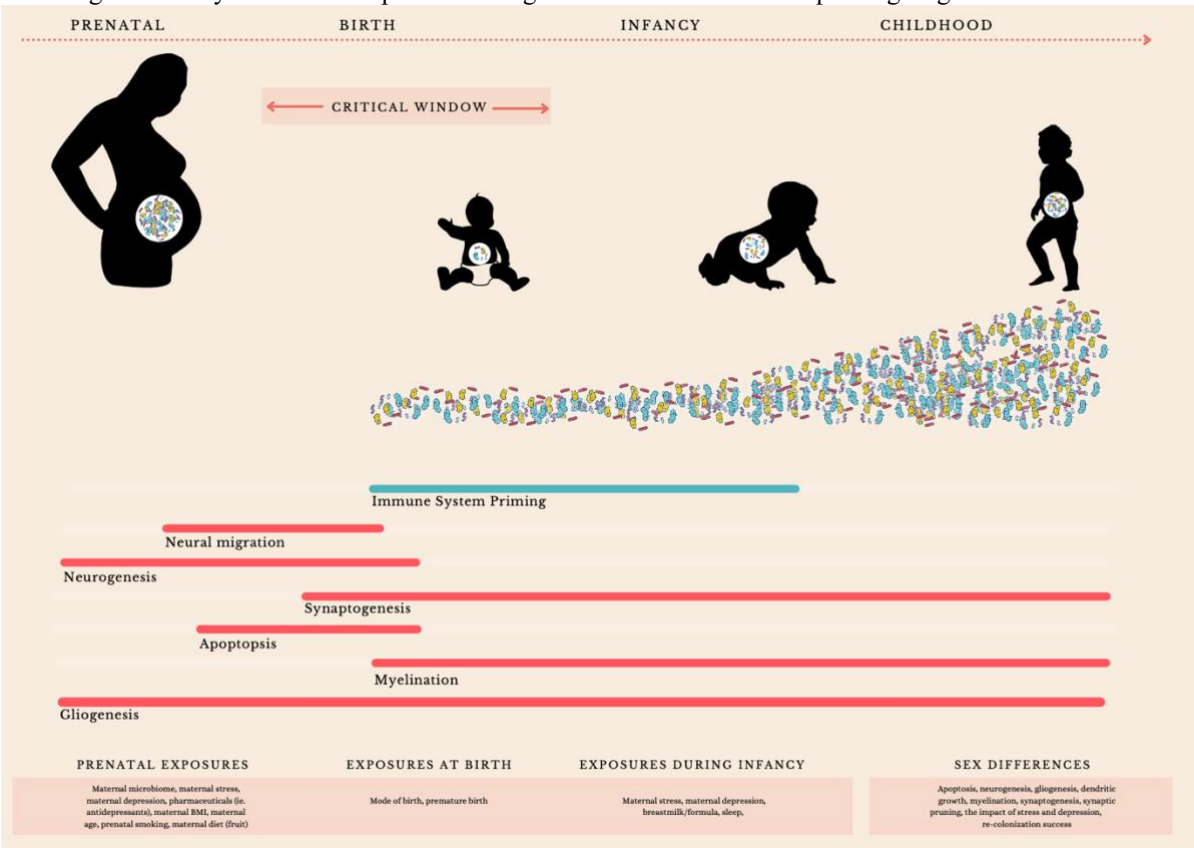


Figure 1. Conceptual figure demonstrating the co-development of the brain alongside colonization of the gut microbiome and immune system priming time-points. The figure presents a critical window between the prenatal period and postnatal infancy in which the brain may be increasingly susceptible to environmental stressors. Exposures that may impact the developing brain are noted and explained in further

detail (**1.6 Covariates** section). Neurophysiological sex differences in the development of the brain (McCarthy et al., 2017) are summarized as well as sex-specific differences on the impact of stressors and gut microbiome recolonization success (Erny et al., 2015; Morgane Sonia Thion et al., 2018b).

1.6 Covariates

1.6.1 Maternal Education (SES): In a longitudinal prospective cohort study, participants of lower SES experienced a higher prevalence of depression (Huurre et al., 2007). Maternal education is associated with both cognitive and language scores of the BSID-III (Ko et al., 2013) with a multisystem model demonstrating that maternal education increases child academic outcomes through pathways such as cognition (Harding et al., 2015).

1.6.2 Maternal Age: The prevalence of distress is significantly higher in both older and younger mothers (Muraca & Joseph, 2014; Reis, 1988). In a large Australian cohort, multivariate analysis demonstrated that a maternal age between 30-34 compared to 25-29 was associated with sex-specific behavioural outcomes such as increased anxiety, stress, and depression symptoms in daughters later in adulthood (Tearne et al., 2016). Dutch population-based cohort found a negative linear relationship between parental age and attention problems (younger parents were associated with more attention problems), and maternal age-positive and linearly related to child IQ and educational achievement (Veldkamp et al., 2020). However, this association was partly confounded by socioeconomic status (SES). Older maternal age has also been associated with an increased risk of pregnancy and delivery complications, which could impact the developing fetus and infant (Seoud et al., 2002).

1.6.3 Pre-Pregnancy Weight: Compared to pre-pregnancy normal weight, women that were underweight and obese experienced higher rates of postpartum depression (Lacoursiere et al., 2010). Moreover, increased pre-pregnancy BMI scores were associated with both higher prenatal and anxiety scores (Holton et al., 2019). Exploring child development, compared to normal weight, all other BMI weight was associated with lower mental development index in children at 2 years of age, meaning both underweight and overweight can affect children's neurodevelopment (Hinkle et al., 2012). Meta-analysis of pre-pregnancy weight found compared to normal weight, overweight or obesity was associated with lower neurodevelopment scores, increased risk for ADHD, ASD, developmental delay, and emotional and behavioural problems

(Sanchez et al., 2018). In summary, a variation from a normal pre-pregnancy BMI was associated with perinatal distress symptoms as well as lower neurodevelopment scores in children.

1.6.4 Prenatal Smoking: In a systematic review of fifteen articles, prenatal smoke exposure was associated with negative neurodevelopmental outcomes in children (Chen et al., 2013). In a multiple mediation analysis, maternal psychological symptoms were a mediating variable in the association from prenatal smoking on postnatal parenting stress in infancy, meaning increased post-partum psychological distress in mothers (Lynch et al., 2011). In a systematic review, there appears to be a bidirectional relationship between smoking and symptoms of depression and anxiety (Fluharty et al., 2017). Factors associated with smoking cessation during pregnancy include higher SES and education, planned breastfeeding, no depression, and low stress during pregnancy (Riaz et al., 2018). Therefore, there is a bidirectional association with smoking and maternal distress with distress severity affecting a mother's decision to cease smoking during pregnancy, with this association also being confounded by SES.

1.6.5 Maternal Diet: Fruit Intake: A meta-analysis found an inverse relationship between depressive symptoms and fruit consumption (X. Liu et al., 2016). Maternal prenatal fruit intake was previously associated with infant cognition at one year of age within the CHILD birth cohort (Bolduc et al., 2016).

1.6.6 Infant Sleep Duration: The CHILD cohort reported that infants with shorter sleep duration had lower cognitive and language scores at two years of age (Smithson et al., 2018). Moreover, the CHILD cohort also discovered pathways from prenatal depressive symptoms on infant sleep duration (Matenchuk et al., 2019).

1.6.7 Antidepressants: Antidepressants are pharmaceutical treatments that aim to reduce depressive symptoms. Infants exposed to mothers taking antidepressants scored significantly lower on motor, social-emotional, and behaviour subscales of the BSID-III (Hanley et al., 2013).

1.6.8 Gestational Age: A meta-analysis found that maternal depressive symptoms during pregnancy significantly increased the risk of preterm birth and remained significant with correction for bias (Grote et al., 2010). Moreover, healthy preterm infants had

significantly lower cognitive and language scores on the BSID-III compared to term infants (Ionio et al., 2016).

1.6.9 Birth mode: The CHILD cohort reported that prenatal depression was a significant predictor of birth mode in a pathway commencing from maternal education (Matenchuk et al., 2019). An Australian cohort study found that caesarean section (c-section) born children had a significantly lower cognitive performance on several measures in children at eight and nine years of age (Polidano et al., 2017). However, other factors such as maternal health and breastfeeding accounted for part of the cognitive gap.

1.6 Summary of Gaps

Researchers have explored the association between maternal distress on child neurodevelopment; however, most studies have used higher-risk infants such as preterm newborns. Preterm birth results in a delay in the colonization of the newborn's gut microbiome compared to term infants (Henderickx et al., 2019). Further research is needed to explore these impacts in healthy term newborns and the role of the microbiome on this association.

Moreover, there is a gap in research exploring the timing of the distress and its impact on neurodevelopmental outcomes. Reviews have demonstrated that there is ample evidence on the impacts of prenatal stress and postnatal depression on child neurodevelopment, with research on the impacts of prenatal depression and postnatal stress less common (Ahun et al., 2021; Brand & Brennan, 2009; Y. Liu et al., 2017). Additionally, studies on prenatal and postnatal distress have not explored distress trajectories in which researchers can further identify a persistent distress group that may further impact neurodevelopment.

Regarding sex-specificity, contrary to their hypothesis, a longitudinal cohort study found that maternal distress symptoms were associated with higher scores on child developmental outcomes with no significant sex differences. However, mothers that remained in the study (only 68% of the original sample) were older, married, more educated, and non-minority compared to mothers that did not remain in the study, indicating potential loss to follow-up bias (DiPietro et al., 2006). Further research with more representative sample is required to further explore maternal distress during pregnancy on child neurodevelopment in a sex-specific manner.

Examining the infant gut microbiome, there is an overall lack of research on the impacts of colonization of *C. difficile* in infancy and its potential impact on the developing gut-brain axis. Research on SCFA and neurodevelopment vary, with propionate injections during development

being used as a model to induce autistic-like behaviour as well as contrary associations that it has neuroprotective effects on the blood-brain barrier (Hoyles et al., 2018; MacFabe et al., 2007). These inconsistencies in whether SCFA act as neuroprotective or as mechanisms in pathology warrant further investigation. Moreover, researchers commonly supplement with mixed solutions of SCFA, which makes it no longer possible to differentiate between them.

Finally, stress-microbiome pathways have not been fully explored in humans. A substantial amount of microbiota and metabolite research uses animal models; however, the microbiota and metabolism of animal models vary from the human microbiome, as reportingly only 15% of gut bacterial lineages are shared between humans and mice (Ley et al., 2005). This will be the first study to explore the mediating role of the gut microbiota and metabolome during infancy on child neurodevelopment after exposure to maternal distress trajectories among healthy term children.

1.7 Research Aims

Early child development presents itself with key developmental windows in which exposures to distress and alterations in the gut microbiome can have lasting effects on the developing brain. Animal models with GF mice showed altered brain regions and neuroimmune defects with re-colonization of the gut microbiota at early critical windows having the potential to restore negative outcomes (Erny et al., 2015). Indications of sex differences are demonstrated in lower BSID scores (Romeo et al., 2012), as well germ-free animal models demonstrated sex-specific differences in brain microglial gene expression (Morgane Sonia Thion et al., 2018a) and serotonergic systems after exposure to stress (G. Clarke et al., 2013). Within CHILD, we previously reported that prenatal depressive symptoms decreased breastfeeding duration (Rodriguez, under review), that breastfeeding impacts infant SCFA abundance (Bridgman et al., 2017a), and animals supplemented with SCFA after exposure to stress alleviated specific stress-induced outcomes (van de Wouw et al., 2018b).

The purpose of this research is to explore the impact of maternal distress on child neurodevelopment while examining the sequential mediating role of both exclusive breastfeeding duration and the infant gut microbiome on this association.

1. The primary aim of this study is to determine the impact of maternal pre and postnatal distress trajectories (stress and depression) on child neurodevelopment at one and two years of age in a sex-specific manner using the CHILD cohort study.

2. A secondary objective is to determine the sequential mediating role of exclusive breastfeeding duration and the infant gut microbiome and metabolome (microbiota enterotype clusters, SCFA, *C. difficile*, and tryptophan) on this association.

We hypothesize that maternal distress will negatively impact child neurodevelopment, with males affected to a greater extent. Moreover, we hypothesize that maternal distress will impact the length of exclusive breastfeeding duration, impacting the gut microbiome. Regarding the role of the gut microbiome, we hypothesize that *C. difficile* colonization would have a negative effect on neurodevelopment, while butyrate and acetate would have a positive effect on neurodevelopment, with an exploratory approach to propionate, formate, and tryptophan. Overall, we hypothesize that exclusive breastfeeding duration and the infant gut microbiome will sequentially mediate the association from maternal distress on child neurodevelopmental scores.

CHAPTER 2: METHODS AND MATERIALS

Chapter 2 defines the study design and population, the variables used in this study, the sample size calculation, and the statistical analyses that were performed for both the primary and second aims of the study.

CHAPTER 2: METHODS AND MATERIALS

2.1 Study Design

This is a quantitative study using data collected from Canadian Healthy Infant Longitudinal Development (CHILD), a national prospective longitudinal birth cohort study that follows families across Canada from pregnancy to early childhood.

2.2 Study Population

This study was comprised of a sub-sample with 646 mother-child pairs that were enrolled into the CHILD birth cohort recruited from Edmonton. These infants were selected based on maternal distress trajectories and 1-year cognitive score data availability (Figure 2). Written informed consent for mothers and infants were obtained from the mothers, as well as consenting fathers when available, at enrollment. Participants were enrolled in their second or third trimester of pregnancy between 2008 and 2012. Subbarao et al. (2015) discuss the inclusion and exclusion criteria for the cohort (Subbarao et al., 2015a).

Figure 2. Study sample flow chart.

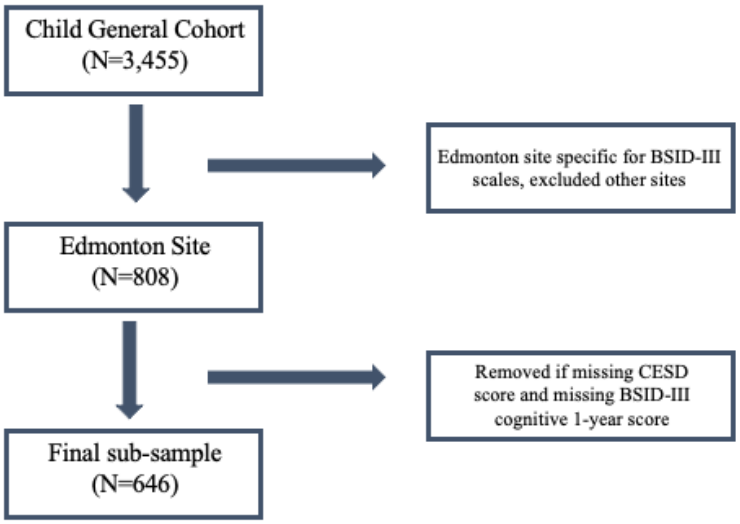


Figure 2. Study population sub-sample flow chart describing the participants in this study. The Edmonton recruitment site is the only CHILD site with BSID-III scores. The final sample contains participants with both exposure and outcome variables, specifically CESD trajectory scores and BSID-III cognitive 1-year scores.

2.3 Exposures (Maternal Distress)

2.3.1 Maternal Depression

Maternal depression symptoms were measured using the 20-item Center for Epidemiologic Studies Depression Scale (CES-D). The CES-D is a brief self-report scale to

measure depressive symptoms experienced in the past week. The scale ranges from 0 (rarely or none) to 3 (most or almost all of the time). The scores range from 0 to 60, with higher scores indicating greater depressive symptoms. A cut-off score of 16 or greater identifies individuals at risk for clinical depression (Brenner & Penzenik, 2018). Substantial construct validity, as well as concurrent validity by clinical and self-report criteria, have been demonstrated (Radloff, 1977).

2.3.2 Maternal Stress

Maternal perceived stress was measured using the 10-item Perceived Stress Scale (PSS). The PSS is a validated instrument and is widely used as a psychological measurement for participant's appraisal of the degree of stressful situations in their lives (S. Cohen et al., 1983). The PSS is a five-point Likert scale, from 0 (never) to 4 (very often) on participants' perceived stress during the last month. The scores are then summed, with higher scores indicating higher perceived stress. The PSS score shows high reliability and is correlated with life events, depressive symptoms, and social anxiety (S. Cohen et al., 1983). Since the PSS does not have established criteria for a cut-off, the mean value across all six waves (12.96) was used as the cut-off for a high level of perceived stress (Chow et al., 2019). This mean was similar to the mean in the general US population among women (Sheldon Cohen & Williamson, 1988).

2.3.3 Primary Aim Exposure: Maternal Distress Trajectories

Mothers reported perceived distress (stress and depressive symptoms) at several time points through the pregnancy and postpartum period (recruitment, 36 weeks of gestation and then 6, 12, 18, 24 months of infant age). From these timepoints, previous CHILD researchers identified five mutually exclusive distress trajectories (L. J. Kang et al., 2020). The trajectories are named Prenatal, Postnatal, Persistent, Never (moderately low) and Never (low). This study combined the two Never (moderately low) and Never (low) as both scores are below the cut-off values for clinical significance to increase the sample size in the reference group, creating a four-level variable. Persistent trajectory consists of mothers with high symptoms throughout the pre-and-postnatal period. Prenatal trajectory consists of high symptoms primarily in the prenatal pregnancy period, while postnatal trajectory consists of high symptoms primarily in the postnatal period after delivery.

2.3.4 Secondary Aim Exposure: Prenatal Distress

Although the maternal distress trajectories provide information regarding distress experienced at various times through the perinatal period, the structural equation modelling

(SEM) method to test for sequential mediations does not allow factorial exposures. Thus, with CHILD researchers identifying associations between prenatal distress decreasing exclusive breastfeeding duration (Rodriguez et al. Under Review), we tested the prenatal (36w) stress and depression scores in the mediation models. Only the prenatal (36w) distress time point was tested in the mediation models as the postnatal timepoint (six months) does not precede the fecal samples (four months).

2.4 Outcomes (Neurodevelopment)

Infant neurodevelopment was measured using the Bayley Scales of Infant Development (BSID-III) at one and two years of age. The BSID-III has excellent validity and reliability (Viezel et al., 2014). The BSID-III is comprised of five domains and four were available for this study: Cognitive, language, motor, and social-emotional. The cognitive scale assesses sensorimotor development, exploration, object relatedness, memory, concept formation, and aspects of cognitive processing. The language scale includes both a receptive and expressive communication scaled score and includes vocabulary development, social referencing, verbal comprehension, and preverbal communication such as babbling and turn taking. The motor scale includes a fine and gross motor scores and includes motor planning, reaching, object manipulation, locomotion, coordination, and balance. The social-emotional scale assesses social and emotional milestones such as self-regulation, communicating needs, engaging others, using emotional signals to solve problems, and establishing relationships.

The BSID-III was improved from the BSID-II for early childhood assessment from birth through three years of age (Albers & Grieve, 2007). A primary caregiver was present in an examination room while a trained research assistant conducted testing during a time of day when the child was fully awake and not drowsy. The research assistant was assessed on their administration of the BSID-III annually or semi-annually by a registered educational psychologist to ensure reliability. The cognitive, language and motor scales were administered by trained research assistants while the social-emotional scale was parent-reported. Raw scores were converted to composite scores, and the age-standardized population mean for the composite score was 100 (standard deviation of 15). A higher score on the BSID-III is an indication of better abilities.

2.5 Mediators

We aimed to explore the mediating role of exclusive breastfeeding duration and the gut microbiome. Exploring the gut microbiome, the metabolites and microbiota selected as potential mediators in this study were *C. difficile* abundance, microbiota enterotype clusters, tryptophan concentration, and the abundance of propionate, acetate, butyrate, and formate. The structural equation modelling (SEM) method to test sequential mediations assumes linear outcomes; therefore, the mediators in this study were either rank or continuous variables.

2.5.1 Exclusive Breastfeeding Duration

Mothers reported exclusive breastfeeding duration at the one year to follow-up (range 0-9 months). This variable was re-coded to have a maximum of four months to precede the fecal sample in the mediation analyses (N=629; Mean=2.5; SD=1.7; Range: 0-4 months). Mothers also reported breastfeeding status (exclusively breastfed, partial breastfed, and formula-fed) at four months of age during a home visit which was used for sample characteristics purposes.

2.5.2 Gut Microbiota

Infant gut microbiota was assessed by 16SrRNA gene sequencing from fecal samples collected during a 4-month visit at a mean age of 4.17 months (SD=1.2). *Clostridioides difficile* (*C. difficile)* was specifically sequenced using qPCR with the appropriate primers. Fecal samples were fresh or refrigerated for a short period and collected during standard home visits. Samples were transported and stored at -80°C until analysis. Microbial samples were clustered into three mutually exclusive enterotypes using partitioning around medoids (PAM) clustering algorithm which has been previously described (Tamana et al., 2021). Cluster 1 is a Proteobacteria and Firmicutes-dominant cluster, cluster 2 is Firmicutes-dominant cluster, and cluster 3 is a Bacteroidetes dominant cluster. To test the clusters in the mediation models, we created a rank variable. The enterotype clusters were ranked according to *Lachnospiraceae* family colonization as there was a significant ANOVA ($F=29.61$, $p<0.0001$) with all three clusters being significantly different from each other (Firmicutes-Proteobacteria $p=0.009$, Firmicutes-Bacteroidetes $p<0.001$, Bacteroidetes-Proteobacteria $p<0.001$) to fulfill the linear assumption for the rank variable required for mediation analysis. The rank variable is in the following ascending order: Cluster 2 (Firmicutes), cluster 1 (Proteobacteria), then cluster 3 (Bacteroidetes).

2.5.3 Metabolome

Metabolome profiling to determine short-chain fatty acid metabolites and amino acid derivatives were analyzed and quantified using NMR spectroscopy sequencing from the same four-month fecal samples. NMR sequencing has been successfully applied to metabolite measurement in fecal samples (Matysik et al., 2016). For this study, the metabolites selected to include were propionate, acetate, butyrate, formate, and tryptophan. Tryptophan was analyzed as absolute concentration (μmol/g). Acetate, butyrate, propionate, and butyrate were measured as relative abundance (in which total SCFA was the sum of acetate, propionate, butyrate, formate, and valerate for this equation). All metabolites (tryptophan and SCFA) were z-score standardized.

2.54 Techniques

Bacterial DNA was extracted from fecal samples. It was analyzed using the QIAamp DNA Stool Mini Kit, followed by 16S gene sequencing to identify individual microbes. Specifically, for *Clostridioides difficile*, additional qPCR analysis was completed. Peaks observed from NMR sequencing identified microbial metabolites. NMR spectroscopy allows for simultaneous measurement of a wide range of metabolites (Matysik et al., 2016).

2.6 Definition of Candidate Covariates

2.6.1 Maternal Education (SES): Mothers reported their education on the standardized questionnaire at recruitment and was analyzed in the multivariable models as a continuous variable of years of maternal education achieved. For the sample characteristics, maternal education was categorized as (1) was defined as high school or less, (2) as university or college, and (3) a university degree obtained.

2.6.2 Maternal Age: Mothers reported their age on the standardized questionnaire at recruitment. Maternal age was analyzed as a continuous variable in the multivariable models and was categorized into three levels for the sample characteristics: 18-29, 30-39, and over 40.

2.6.3 Pre-Pregnancy Weight: Maternal pre-pregnancy weight was based on BMI scores that were calculated using weight in kilograms divided by height in meters squared, using height and self-reported pre-pregnancy weight or estimated from measured weight at 1 year postpartum. Pre-pregnancy BMI scores were analyzed as a continuous variable in the multivariable models and were categorized for the sample characteristics: defined as normal weight (BMI, 18.5-24.9) and overweight (BMI, ≥25.0).

2.6.4 Prenatal Smoking: Prenatal smoking was measured from the recruitment questionnaire, defined as smoked during pregnancy.

2.6.5 Maternal Diet: Fruit Intake: Maternal diet was measured using a food frequency questionnaire by a nutritional epidemiologist trained and certified in dietary data entry at the Fred Hutchinson Cancer Research Center modified to reflect Canadian multi-ethnic food choices. This 175-item self-administered test was completed at study enrolment. The questions consisted of average frequency and portion sizes of food consumed since becoming pregnant. The total fruit intake ("5-a-day" method) was the sum of "servings of fruit, not including juices" plus "servings of juice" per day.

2.6.6 Infant Sleep Duration: Parents reported the amount of infant sleep duration using the 13-item Brief Infant Sleep Questionnaire (BISQ) (Sadeh, 2004). Total sleep was calculated by summing the number of times parents reported infant sleeping during the day and night.

2.6.7 Antidepressants: Maternal antidepressant use was determined prenatal and postnatally from the maternal medication questionnaire. The antidepressant variable combined pre-and/or postnatal antidepressant use.

2.6.8 Gestational Age: The CHILD study is primarily term infants, with the few born below 37 weeks defined as premature.

2.6.9 Birth mode: Birth mode was defined with a four-level categorical variable: vaginal birth with no IAP, vaginal birth with IAP, emergency caesarean delivery, and elective caesarean delivery.

2.6.10 DAG: A Directed Acyclic Graph (DAG) is a visual representation with unidirectional arrows to conceptualize the minimal adjustment set of covariates that were identified from a literature review. According to the DAG model, maternal years of education, prepregnancy BMI, and maternal age were identified as confounds to achieve a total direct effect from maternal distress to child neurodevelopment.

Figure 3. Directed Acyclic Graph (DAG).

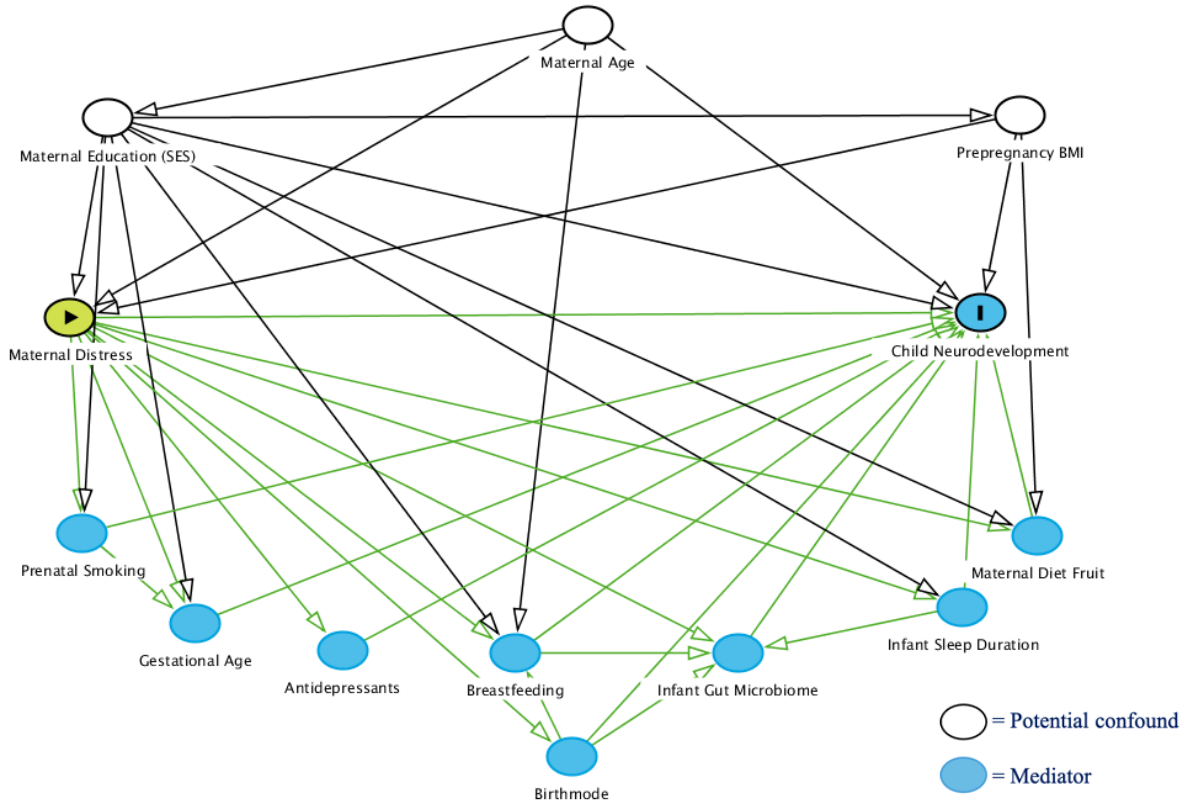


Figure 3. Directed Acyclic Graph (DAG) visualized variables that are associated with both the exposure and the outcome (confounding variables). A DAG allows researchers to differentiate between a potential confounding variable (variables in white- which may need to be adjusted for in the statistical analysis) and a mediating variable (variables in blue- adjusting for a mediating variable can bias the estimates).

2.7 Sample Size Calculation

To complete the sample size calculation, we aimed to use previous CHILD studies as they would represent similar sample characteristics. We combined two studies to calculate the sample size required to explore the association of maternal distress on child neurodevelopment. We used a CHILD study exploring the impact of maternal depression on infant sleep duration as well as a second CHILD study exploring the impact of infant sleep duration on child BSID-III scores (Matenchuk et al., 2019; Smithson et al., 2018). The researchers reported that pre and postnatal depression were associated with shorter infant total sleep duration and that infants with a shorter total sleep duration score an estimated 4.66 (95%CI: -7.95, -1.37) lower cognitive scores on the BSID-III at two years of age. Taking these two CHILD cohort studies together, infants exposed to maternal depression may score a 4.66 clinical difference on the BSID-III, compared to non-exposed infants. The confidence interval (3.29) and sample size (n=157) from

Smithson et al. (2018) were used to estimate the standard deviation to calculate the required sample size.

$N= (1.96xSD/confidence\ limit)^2 \rightarrow 157= (1.96 \times SD/3.29)^2$
SD=21.03
To determine the sample size with a 2-sides *a* of 0.05 and β of 0.20 (power=80%), the Power Index (PI) will be: 1.96+0.84= 2.80
$N= 2(PI \times SD/clinical\ difference)^2 \rightarrow N= 2(2.80 \times 21.03/4.66)^2$
N=319
The minimum required number of participants is approximately *319*.

2.8 Statistical Analyses

Exploring sample characteristics, the percentage distribution of study sample characteristics across depression and stress trajectories was analyzed using Fishers exact test or chi2 tests for categorical covariates and ANOVA for continuous covariates. Sample characteristics and their associations with 1- and 2-year neurodevelopmental scales were analyzed by t-test or ANOVA (categorical variables) and linear regressions (continuous variables). Statistical significance was defined as a p-value<0.05.

2.8.1 Primary Aim Statistical Analyses

Linear multivariable regressions were used to test the associations of stress and depression trajectories on neurodevelopmental scores. Collinearity was found between depression and stress trajectories; therefore, they were analyzed in separate models. Each neurodevelopmental outcome was tested separately in a multivariable model. A directed acyclic graph (DAG) model-building approach was used to refine the selection of the adjusting variables for the multivariable linear regressions. Covariates identified from the DAG were then tested through the hypothesis model approach for each separate model, meaning each model had its own unique minimal adjustment set. The covariates remained in the adjustment set if there were a greater than 10% change in estimate with the inclusion of that covariate (Supplement Table S1). Linear multivariable regressions were used to test the associations of stress and depression trajectories on neurodevelopmental scores. Collinearity was found between depression and stress trajectories; therefore, they were analyzed in separate models. To test sex-specificity, each model was tested for sex interactions with significant interactions remaining in the models. Stratified multivariable models were also analyzed to explore both sexes. Statistical analyses were completed using STATA version13. The final multivariable models were bootstrapped with 1000

resample repetitions to compute a re-sampled distribution due to low sample sizes in the persistent distress groups.

2.8.2 Secondary Aim Statistical Analyses

Mediation analyses were completed using structural equation modelling (SEM) in STATA version 16. Structural equation models in STATA assume linear associations; therefore, all mediators and outcomes in the model were in their linear form. The exposures were prenatal stress and depression scores from questionnaires administered at 36w gestation. The first mediator, exclusive breastfeeding duration, was maternally reported and re-coded to range from zero to four months of infant age. The second mediators, the gut microbiome, included *C. difficile*, enterotype clusters rank, tryptophan concentration, and the relative abundance of propionate, acetate, butyrate, and formate, which were all analyzed from fecal samples taken after home assessments at four months of age. The outcomes consisted of the BSID-III neurodevelopment scales administered at one and two years of child age. To narrow down on the metabolites that were tested within SEM models, first, we tested whether there were significant crude regression associations between prenatal stress or depression scores on the microbial mediators as well as significant crude associations between the microbial mediators on neurodevelopment outcomes. Only potential microbial mediators with significant associations both from maternal prenatal distress and on neurodevelopmental outcomes were further tested within the SEM sequential mediation models.

Temporality is an assumption within mediation analyses; therefore, the exposure must precede the first mediator, the first mediator must precede the second mediator, and the exposure and both mediators must precede the outcome. Figure 4 displays the variables in the mediation models and their temporal associations. Sex-specific models were additionally tested. All sequential mediations were bootstrapped with 1000 resampled repetitions to compute a sample distribution. All mediations were adjusted for the minimal adjustment set described in the DAG which included maternal years of education, maternal age, and maternal pre-pregnancy BMI scores. Statistical analyses were completed using STATA version16.

Figure 4. Data collection timeline demonstrating temporality for the variables tested in the sequential mediation models.

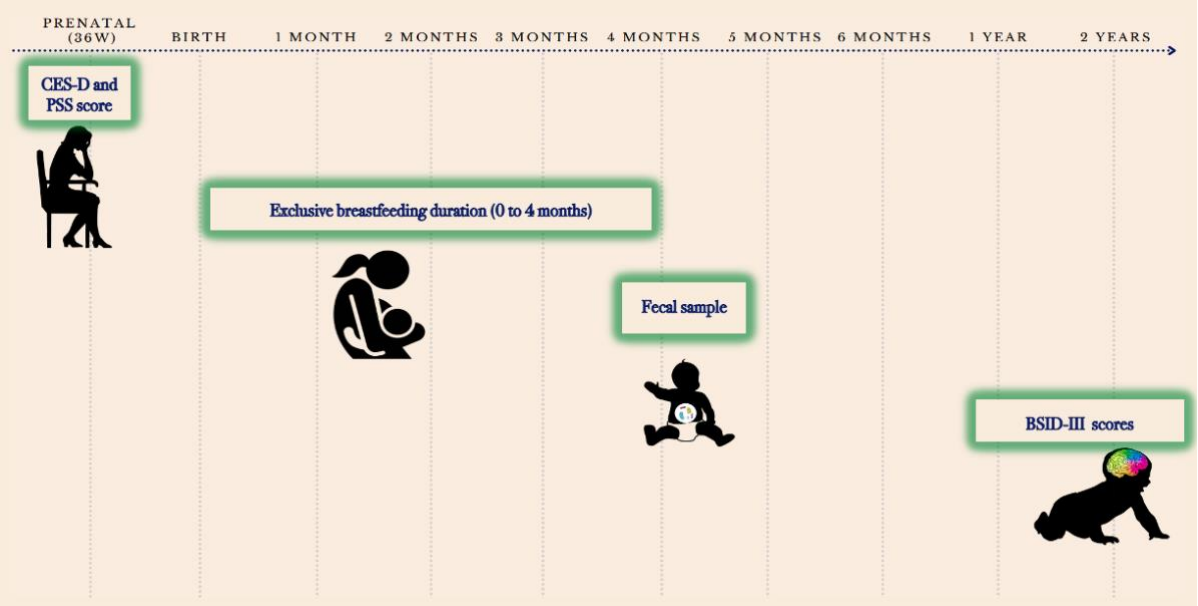


Figure 4. Data collection timeline for the variables chosen for the sequential mediation analyses. Prenatal stress and depression scores were collected at 36 weeks gestational age, exclusive breastfeeding duration ranged from zero to four months (Mean 2.5; SD=1.7), fecal samples were collected at four months (Mean: 4.17; SD= 1.2), and BSID-III scores were assessed at one and two years of age.

CHAPTER 3: RESULTS

Chapter 3 firstly provides the child sub-sample characteristics of the study population. Secondly, this chapter explores the results of the primary aim of the study exploring the impact of maternal distress trajectories on child neurodevelopment in a sex-specific manner. Finally, this chapter explores the second aim of this study which is to explore mediation analyses that provide insight on the impact of maternal distress and exclusive breastfeeding duration on the early development of the gut-brain axis in children.

CHAPTER 3: RESULTS

3.1 Sub-Sample Characteristics

3.1.1 Maternal Characteristics

Exploring maternal distress (Table 1), 6.9% of the mothers experienced clinically significant depression during the prenatal pregnancy period and 6.8% experienced depressive symptoms during the postnatal period. Additionally, 1.9% experienced persistent depressive symptoms pre-and-postnatally, with a total of 15.6% of mothers experienced clinically significant depression at some point during pregnancy. Regarding stress, 4.5% of mothers experienced prenatal stress, 20.6% experienced postnatal stress, and 6.2% experienced persistent stress pre-and-postnatally. In total, 31.6% of mothers experienced perceived stress at some point during pregnancy. Approximately half of the mothers in the sample had a family income above $100,000 (53%), had a university degree (54.4%), and had a normal pre-pregnancy weight (55.8%). The most frequent maternal age was between 30-39 (66.1%) and mothers ate approximately 3 servings of fruit a day during the prenatal period. Approximately 7% of the mothers took antidepressants pre and/or postnatally and only 4% reported smoking during the prenatal period.

3.1.2 Infant Characteristics

Regarding infant characteristics, half of the sample were boys (50.6%), with infants primarily vaginally born (75.9%). Only 5.6% of the infant in this sample were born prematurely as the CHILD cohort is predominantly term infants. More than half of the infants were exclusively breastfed (57%) at four months of age with infants sleeping just over 14 hours a day.

Regarding the infant gut microbiome, the average age of fecal samples was taken when the infants were just over 4 months old (M=4.17) (Table 2). The most abundant SCFA was acetate followed by propionate, butyrate, formate. Moreover, 43.2% of the infants were colonized with *C. difficile*. Microbiota composition at four months of age was characterized as 40% dominated with the Proteobacteria cluster, 35% dominated by the Firmicutes cluster, and 25% dominated by the Bacteroides cluster.

Table 1. Frequency characteristics for categorical variables in the study sample of infants with both maternal distress and neurodevelopmental data at 1 year of age (*n*=646)

Maternal characteristics	Total N	n (%)	**Infant characteristics**	Total N	n (%)
CESD	646		**Child Sex**	646	
Never		545 (84.4)	Boys		327 (50.6)
Prenatal		45 (6.9)	Girls		319 (49.4)
Postnatal		44 (6.8)	**Breastfeeding 3 months**	646	
Persistent		12 (1.9)	None		101 (15.6)
PSS	646		Partial		171 (26.5)
Never		444 (68.7)	Exclusive		371 (57.4)
Prenatal		29 (4.5)	**Birthmode**	642	
Postnatal		133 (20.6)	Vaginal no IAP		339 (52.8)
Persistent		40 (6.2)	Vaginal IAP		148 (23.0)
Family income	585		CS-Elective		71 (11.1)
Less than 39,999		36 (6.2)	CS-Emergency		84 (13.1)
40,000 to 79,999		145 (24.8)	**Gestational Age**		
80,000 to 99,999		94 (16.0)	37 weeks+	646	610 (94.4)
Exceeds 100,000		310 (53.0)	Less than 37 weeks		36 (5.6)
Maternal Education	621		***C. difficile* colonization 4 months**	370	
Some/finished high school		48 (7.7)	Yes		160 (43.2)
Some university/college		235 (37.8)	No		210 (56.8)
University degree		338 (54.4)	**Enterotype Clusters 4 Months**	404	
Maternal Age	646		Cluster 1: Proteobacteria^{+}		163 (40.3)
18 to 29		193 (28.9)	Cluster 2: Firmicutes		142 (35.2)
30 to 39		427 (66.1)	Cluster 3: Bacteroides		99 (24.5)
Over 40		26 (4.0)			
Prenatal smoking	624				
Yes		26 (4.2)			
No		598 (95.8)			
Pre-pregnancy weight	625				
Overweight		276 (44.2)			
Normal weight		349 (55.8)			
Antidepressants	646				
Never		599 (92.7)			
Pre and/or Postnatal Use		47 (7.3)			

Note: CESD= Center for Epidemiological Studies Depression Scale; PSS= Perceived Stress Scale; CS=caesarean section; $^{+}$= Proteobacteria and Firmicutes-dominant cluster.

Table 2. Descriptive characteristics for continuous variables in the study sample of infants with both maternal distress and neurodevelopmental data at 1 year of age (*n*=646).

Continuous variables	Total N	Mean (SD)	Min	Max
BSID-III cognitive 1 year	646	110.14 (10.29)	75	145
Missing, n (%)	0 (0)			
BSID-III language 1 year	646	108.22 (12.25)	65	147
Missing, n (%)	0 (0)			
BSID-III motor 1 year	646	103.49 (14.57)	64	154
missing, n (%)	0 (0)			
BSID-III social-emotional 1 year	619	102.65 (13.88)	60	145
Missing, n (%)	27 (4)			
BSID-III cognitive 2 year	541	105.67 (14.30)	70	145
Missing, n (%)	105 (16)			
BSID-III language 2 year	542	100.15 (12.00)	70	145
Missing, n (%)	104 (16)			
BSID-III motor 2 year	541	98.89 (9.50)	68	135
Missing, n (%)	105(16)			
BSID-III social-emotional 2 year	533	108.70 (15.68)	67	127
Missing, n (%)	113 (18)			
Maternal pregnancy fruit intake	610	3.22 (2.19)	0.14	19.67
Missing, n (%)	36 (6)			
Propionate abundance^{+}	168	0.12 (0.09)	0.004	0.45
Missing, n (%)	478 (74)			
Acetate abundance^{+}	168	0.78 (0.12)	0.23	0.99
Missing, n (%)	478 (74)			
Butyrate abundance^{+}	168	0.07 (0.07)	0.001	0.51
Missing, n (%)	478 (74)			
Formate abundance^{+}	139	0.03 (0.04)	0.0	0.21
Missing, n (%)	507 (79)			
Tryptophan concentration^{+} (μmol/g)	163	0.24 (0.20)	0.04	1.32
Missing, n (%)	483 (75)			
Infant sleep duration	575	14.23 (2.09)	7.0	20.0
Missing, n (%)	71 (11)			
Age at 4-month microbiota sample	404	4.17 (1.22)	1.33	9.87
Missing, n (%)	242 (64)			

Note: BSID-III= Bayley Scales of Infant Development; SD=standard deviation

$^{+}$Relative abundance of SCFA and tryptophan concentration are presented in their non-standardized format but were z-score standardized for analyses.

3.1.3 Potential Covariates Across Distress Trajectories

Exploring the potential covariates across distress trajectories, family income, maternal education, prenatal smoking, pre-pregnancy weight, antidepressant use, gestational age, and infant sleep duration varied across both the stress and depression trajectories (Supplementary Table S2, S3, S4, and S5). Additionally, birth mode and the colonization of *C. difficile* varied across depression trajectories but were evenly distributed across stress trajectories.

3.1.4 Potential Covariates Across Neurodevelopmental Scores

Exploring covariates across the neurodevelopmental outcomes, maternal education was associated with neurodevelopment means across cognitive, language, and social-emotional scores at both 1 and 2 years of age with a greater effect size at 2 years of age (Supplementary Table S6 and S7). weight was associated with neurodevelopment scores on the one-year cognitive and motor scores. Breastfeeding status was associated with language scores at both 1 and 2 years of age. Birth mode was marginally associated with cognitive and language scores at 2 years of age. Infant sex was associated with language at 1 year of age as well as cognitive, language, motor, and social-emotional scores at 2 years of age. Infant sleep duration was associated with cognitive scores at 1 year of age and both motor and social-emotional scores at 2 years of age. Maternal prenatal fruit intake was associated with language and social-emotional scores only at 1 year of age. Antidepressant use was associated with motor scores at 1 year of age and language scores at 2 years of age. Gestational age was associated with language and motor scores at 1 year of age as well as cognitive, language, and motor scores at 2 years of age.

There were many associations between the infant gut microbiome at 4 months of age and neurodevelopment scores. Firstly, *C. difficile* was associated with both cognitive and language scores at 2 years of age. Exploring neurodevelopment scores at 1 year of age, tryptophan was associated with motor scores and formate was associated with both motor and cognitive scores. Exploring scores at 2 years of age, propionate, acetate, and butyrate were associated with cognitive scores, propionate, butyrate, formate, and tryptophan were associated with language scores, and butyrate and tryptophan were associated with motor scores.

3.2 Primary Aim

The primary aim of this study was to explore the impact of maternal distress (stress and depression) trajectories on neurodevelopmental scores at 1 and 2 years of age in a sex-specific manner.

3.2.1 Maternal Distress on Neurodevelopment

Multivariable linear regressions (Table 3 and Table 4**)** were built to explore the impact of maternal distress on neurodevelopment. Due to collinearity between stress and depression trajectories, separate models were built for each exposure variable. Each model has a unique set of covariates that were chosen based on the DAG model (which consisted of maternal years of

education, maternal age, and maternal pre-pregnancy BMI scores) paired with a covariate 10% change in estimate assessment assess if the covariates identified in the DAG are true confounds in each model (refer to Figure 3 in Chapter 2: Methods).

3.2.1.1 Maternal Depression on Neurodevelopment

Children exposed to prenatal depression had significantly lower social-emotional scores at two years of age (Coef: -6.32; 95%CI: -11.99, -0.065; p=0.029) adjusted for covariates. Persistent depression significantly lowered children's cognitive scores at 2 years of age (Coef: -6.76; Bootstrap 95%CI: -10.37, -3.15; p<0.001), adjusted for covariates. No significant findings were found for postpartum depression on neurodevelopment outcomes.

Table 3. Univariate and multiple variable linear regression analyses of maternal depression trajectories on BSID-III scores at 1 and 2 years of age.

Multivariable Linear Regression Analyses						
Cognitive Scores 1 Year of Age						
	Crude Estimate (95%CI)	**p-value**	**Adjusting for Sex** Estimate (95%CI)	**p-value**	**Fully Adjusted Model** Estimate (Bootstrap 95%CI)	**p-value**
CESD						
Never*	-		-		-	
Prenatal	-1.69 (-4.84, 1.40)	0.281	-1.74 (-4.84, 1.35)	0.269	-1.32 (-5.29, 2.75)	0.469
Postnatal	0.96 (-2.18, 4.13)	0.553	0.89 (-2.27, 4.05)	0.580	0.29 (-2.88, 3.46)	0.862
Persistent	7.87 (1.99, 13.74)*	0.009	7.66 (1.76, 13.55)*	0.011	3.87 (-3.01, 10.75)	0.294
Child Sex						
Girls*	-		-		-	
Boys	-0.85 (-2.44, 0.74)	0.295	-0.71 (-2.29, 0.88)	0.380	-0.87 (-2.56, 0.83)	0.316
Maternal Education						
Years of Education	0.22 (-0.08, 0.52)	0.145	-	-	0.24 (-0.06, 0.54)	0.117
Maternal Age						
Age in Years	0.06 (-0.12, 0.24)	0.500	-	-	-	-
Prepregnancy Weight						
Maternal BMI	0.13 (-0.01, 0.27)*	0.074	-	-	0.14 (-0.008, 0.29)*	0.065
Language Scores 1 Year of Age						
	Crude Estimate (95%CI)	**p-value**	**Adjusting for Sex** Estimate (95%CI)	**p-value**	**Fully Adjusted Model** Estimate (Bootstrap 95%CI)	**p-value**
CESD						
Never*	-		-		-	
Prenatal	-2.69 (-6.42, 1.04)	0.153	-2.94 (-6.58, 0.70)	0.114	-3.09 (-8.48, 1.50)	0.170
Postnatal	0.92 (-2.85, 4.69)	0.633	0.56 (-3.16, 4.28)	0.766	-3.15 (-7.84, 1.59)	0.188
Persistent	-1.79 (-8.82, 5.22)	0.615	-2.87(-9.81, 4.07)	0.417	-5.19 (-15.07, 4.69)	0.347
Child Sex						
Girls*	-		-		-	
Boys	-3.89 (-5.76, -2.03)*	<0.001	-3.98 (-5.85, -2.11)*	<0.001	-4.62 (-6.74, -2.51)*	<0.001
Maternal Education						
Years of Education	0.16 (-0.20, 0.52)	0.378	-	-	0.04 (-0.32, 0.41)	0.785

Maternal Age						
Age in Years	-0.01 (-0.23, 0.21)	0.923	-	-	-	-
Prepregnancy Weight						
Maternal BMI	0.13 (-0.04, 0.30)	0.121	-	-	0.14 (-0.04, 0.31)	0.128
Sex-specific Interaction						
Prenatal#Girls*	-	-	-	-	-	-
Prenatal#Boys	-	-	-	-	0.86 (-7.61, 9.33)	0.842
Postnatal#Girls*	-	-	-	-	-	-
Postnatal#Boys	-	-	-	-	9.16 (1.99, 16.34)*	0.012
Persistent#Girls*	-	-	-	-	-	-
Persistent#Boys	-	-	-	-	3.99 (-11.48, 19.25)	0.620
Motor Scores 1 Year of Age						
	Crude Estimate (95%CI)	**p-value**	**Adjusting for Sex** Estimate (95%CI)	**p-value**	**Fully Adjusted Model** Estimate (Bootstrap 95%CI)	**p-value**
CESD						
Never*	-	-	-	-	-	-
Prenatal	-2.87 (7.33, 1.83)	0.213	-2.96 (-7.49, 1.57)	0.200	-4.48 (-9.19, 0.22)*	0.062
Postnatal	1.22 (-3.43, 5.82)	0.605	1.10 (-3.52, 5.72)	0.641	0.99 (-4.08, 6.05)	0.703
Persistent	4.48 (-4.15, 13.08)	0.307	4.12 (-4.51, 12.75)	0.349	-3.04 (-8.45, 2.37)	0.271
Child Sex						
Girls*	-	-	-	-	-	-
Boys	-1.40 (-3.72, 0.92)	0.235		0.258	-1.18 (-3.60, 1.22)	0.335
Maternal Education						
Years of Education	-0.15 (-0.59, 2.79)	0.485	-	-	-0.23 (-0.65, 0.19)	0.284
Maternal Age						
Age in Years	-0.03 (-0.19, 0.24)	0.849	-	-	-	-
Prepregnancy Weight						
Maternal BMI	0.26 (0.05, 0.46)*	0.014	-	-	0.28 (0.07, 0.49)*	0.006
Social-Emotional Scores 1 Year of Age						
	Crude Estimate (95%CI)	**p-value**	**Adjusting for Sex** Estimate (95%CI)	**p-value**	**Fully Adjusted Model** Estimate (Bootstrap 95%CI)	**p-value**

CESD						
Never*	-		-		-	
Prenatal	-2.65 (-6.97, 1.68)	0.223	-2.63 (-6.91, 1.65)	0.228	-2.03 (-6.49, 2.44)	0.373
Postnatal	-1.11 (-5.59, 3.35)	0.623	-1.09 (-5.56, 3.38)	0.632	-1.78 (-6.61, 3.07)	0.473
Persistent	-5.27 (-13.57, 3.04)	0.213	-5.14 (-13.48, 3.19)	0.226	-8.43 (-17.54, 0.68)*	0.070
Child Sex						
Girls*	-		-		-	
Boys	0.53 (-1.65, 2.72)	0.632	0.36 (-1.83, 2.55)	0.749	0.71 (-1.60, 3.06)	0.558
Maternal Education						
Years of Education	0.48 (0.07, 0.89)*	0.021	-	-	0.52 (0.12, 0.91)*	0.010
Maternal Age						
Age in Years	0.04 (-0.21, 0.29)	0.768	-	-	-	-
Prepregnancy Weight						
Maternal BMI	0.13 (-0.06, 0.33)	0.181	-	-	0.17 (-0.03, 0.37)*	0.098
Cognitive Scores 2 Years of Age						
	Crude Estimate (95%CI)	**p-value**	**Adjusting for Sex** Estimate (95%CI)	**p-value**	**Fully Adjusted Model** Estimate (Bootstrap 95%CI)	**p-value**
CESD						
Never*	-		-		-	
Prenatal	-1.57 (-7.31, 3.28)	0.555	-2.01 (-7.17, 3.14)	0.443	-2.94 (-8.44, 2.57)	0.212
Postnatal	-1.86 (-6.47, 2.90)	0.438	-2.52 (-7.15, 2.12)	0.287	-2.11 (-6.81, 2.60)	0.381
Persistent	-7.96 (-17.91, 2.13)	0.120	-9.46 (-19.38, 0.46)*	0.062	-6.76 (-10.37, -3.15)*	<0.001
Child sex						
Girls*	-		-		-	
Boys	-4.96 (-7.35, -2.58)*	<0.001	-5.22 (-7.61, -2.83)*	<0.001	-5.51 (-7.96, -3.05)*	<0.001
Maternal education						
Years of education	-0.79 (0.34, 1.23)*	0.001	-	-	0.74 (0.28, 1.22)*	0.002
Maternal age						
Age in years	-0.14 (-0.43, 0.14)	0.333	-	-	-	-
Prepregnancy weight						
Maternal BMI	-0.02 (-0.23, 0.20)	0.881	-	-	0.07 (-0.13, 0.27)	0.483
Language Scores 2 Years of Age						

	Crude Estimate (95%CI)	**p-value**	**Adjusting for Sex** Estimate (95%CI)	**p-value**	**Fully Adjusted Model** Estimate (Bootstrap 95%CI)	**p-value**
CESD						
Never*	-		-		-	
Prenatal	0.40 (-4.26, 4.59)	0.857	-0.14 (-4.24, 4.06)	0.946	0.02 (-4.86, 4.90)	0.993
Postnatal	-1.54 (-5.42, 2.41)	0.440	-2.35 (-6.13, 1.42)	0.222	-1.63 (-5.80, 2.53)	0.443
Persistent	-10.19 (-18.54, 1.77)*	0.017	-12.04 (-20.13, -3.95)*	0.004	-6.83 (-13.75, 0.08)*	0.053
Child sex						
Girls*	-		-		-	
Boys	-6.23 (-8.18, -4.28)*	<0.001	-6.49 (-8.44, -4.54)*	<0.001	-6.44 (-8.51, -4.38)*	<0.001
Maternal Education						
Years of Education	0.72 (0.35, 1.10)*	<0.001	-	-	0.83 (0.48, 1.19)*	<0.001
Maternal age						
Age in Years	-0.14 (-0.38, 0.10)	0.257	-	-	-0.25 (-0.48, -0.02)*	0.032
Prepregnancy Weight						
Maternal BMI	-0.06 (-0.24, 0.12)	0.534	-	-	0.02 (-0.12, 0.21)	0.790
Sex-specific interaction						
Prenatal#Girls*	-	-	-	-	-	-
Prenatal#Boys	-	-	-	-	-9.87 (-19.66, -0.08)*	0.048
Postnatal#Girls*	-	-	-	-	-	-
Postnatal#Boys	-	-	-	-	-2.49 (-11.03, 6.05)	0.568
Persistent#Girls*	-	-	-	-	-	-
Persistent#Boys	-	-	-	-	10.38 (3.93, 16.83)*	0.002
Motor Scores 2 Years of Age						
	Crude Estimate (95%CI)	**p-value**	**Adjusting for Sex** Estimate (95%CI)	**p-value**	**Fully Adjusted Model** Estimate (Bootstrap 95%CI)	**p-value**
CESD						
Never*	-		-		-	
Prenatal	-0.94 (-4.90, 2.14)	0.605	-1.07 (-4.53, 2.38)	0.541	-1.81 (-6.34, 2.72)	0.434
Postnatal	-0.79 (-3.91, 2.33)	0.620	-1.03 (-4.13, 2.08)	0.517	-1.46 (-4.36, 1.44)	0.325
Persistent	-0.92 (-7.59, 5.75)	0.787	-1.46, -8.11, 5.19)	0.666	1.14 (-11.69, 13.97)	0.862
Child sex						

Girls*	-		-		-	
Boys	-1.83 (-3.42, -0.24)*	0.024	-1.90 (-3.51, -0.30)*	0.020	-2.23 (-3.97, -0.50)*	0.012
Maternal Education						
Years of Education	0.19 (-0.11, 0.49)	0.231	-	-	0.23 (-0.12, 0.52)	0.227
Maternal Age						
Age in Years	-0.07 (-0.26, 0.12)	0.458	-	-	-0.13 (-0.33, 0.08)	0.175
Prepregnancy Weight						
Maternal BMI	-0.05 (-0.19, 0.09)	0.520	-	-	-0.04 (-0.18, 0.10)	0.835
Sex-specific Interaction						
Prenatal#Girls*	-	-	-	-	-	-
Prenatal#Boys	-	-	-	-	-11.45 (-19.18, -3.71)*	0.004
Postnatal#Girls*	-	-	-	-	-	-
Postnatal#Boys	-	-	-	-	-2.52 (-8.33, 3.22)	0.390
Persistent#Girls*	-	-	-	-	-	-
Persistent#Boys	-	-	-	-	31.52 (27.09, 35.95)*	<0.001
Social-Emotional Scores 2 Years of Age						
	Crude Estimate (95%CI)	**p-value**	**Adjusting for Sex** Estimate (95%CI)	**p-value**	**Fully Adjusted Model** Estimate (Bootstrap 95%CI)	**p-value**
CESD						
Never*	-		-		-	
Prenatal	-6.64 (-12.60, -0.88)*	0.024	-6.97 (-12.71, -1.23)*	0.017	-6.32 (-11.99, -0.65)*	0.029
Postnatal	-1.02 (-6.20, 4.13)	0.699	-1.48 (-6.62, 3.67)	0.574	-1.06 (-6.30, 4.17)	0.692
Persistent	-14.31(-25.98, -2.67)*	0.016	-15.13 (-26.73, -3.52)*	0.011	-9.96 (-22.42, 2.50)	0.117
Child sex						
Girls*	-		-		-	-
Boys	-2.95 (-5.60, -0.29)*	0.029	-3.30 (-5.95, -0.66)*	0.014	-3.11 (-5.89, -0.32	0.029
Maternal Education						
Age in Years	0.97 (0.47, 1.46)*	<0.001	-	-	0.88 (0.38, 1.39)*	<0.001
Maternal Age						
Continuous Age	-0.07 (-0.38, 0.24)	0.667	-	-	-	-

Prepregnancy Weight					
Maternal BMI	0.01 (-0.22, 0.25)	0.922	-	0.08 (-0.16, 0.32)	0.539
			-		

All models tested for infant sex interactions. Only significant interactions are shown (p<0.10) and if interactions were not significant the interaction term was dropped from the full model. Fully adjusted models are multivariable linear regressions with covariates that were both identified in the DAG model and that had greater than 10% estimate change. Due to the smaller sample sizes in the depression trajectories, all the fully adjusted models were bootstrapped (reps=1000). Model diagnostics are presented in supplementary (Table S8).
Note: CESD= Center for Epidemiological Studies Depression Scale; CI=confidence interval, Estimate=Beta Coefficient
*=reference
*=p-value<0.05
*=p-value between 0.05 and 0.10

3.2.1.2 Maternal Stress on Neurodevelopment

Exposure to persistent stress during pregnancy significantly lowered children's social-emotional scores at 1 year of age (-5.64; Bootstrap 95%CI: -9.52, -1.76; p=0.004), with this finding remaining at 2 years of age (Coef: -5.40; Bootstrap 95%CI: -10.72, -0.08; p=0.047), adjusting for covariates. No significant findings emerged from prenatal or postnatal stress on child neurodevelopmental outcomes.

Table 4. Univariate and multiple variable linear regression analyses of maternal stress trajectories on BSID-III scores at 1 and 2 years of age.

Multivariable Linear Regression Analyses

Cognitive Scores 1 Year of Age						
	Crude Estimate (95%CI)	**p-value**	**Adjusting for Sex** Estimate (95%CI)	**p-value**	**Fully Adjusted Model** Estimate (Bootstrap 95%CI	**p-value**
PSS						
Never*	-		-		-	
Prenatal	-2.13 (-5.97, 1.74)	0.278	-2.11 (-5.98, 1.75)	0.283	-0.57 (-4.80, 3.55)	0.787
Postnatal	-2.03 (-4.02, -0.05)*	0.043	-2.05 (-4.044, -0.06)*	0.043	-2.07 (-4.22, 0.08)*	0.060
Persistent	2.53 (-0.69, 5.95)	0.130	2.38 (-0.93, 5.69)	0.158	0.94 (-2.75, 4.63)	0.619
Child sex						
Girls*	-		-		-	
Boys	-0.85 (-2.44, 0.74)	0.295	-0.68 (-2.27, 0.91)	0.402	-0.80 (-2.48, 0.89)	0.355
Maternal Education						
Years of Education	0.22 (-0.08, 0.52)	0.145	-	-	0.23 (-0.06, 0.53)	0.119
Maternal Age						
Age in Years	0.06 (-0.12, 0.24)	0.500	-	-	-	-
Prepregnancy Weight						
Maternal BMI	0.13 (-0.01, 0.27)*	0.074	-	-	0.14 (-0.008, 0.29)*	0.068
Language Scores 1 Year of Age						
	Crude Estimate (95%CI)	**p-value**	**Adjusting for Sex** Estimate (95%CI)	**p-value**	**Fully Adjusted Model** Estimate (Bootstrap 95%CI	**p-value**
PSS						
Never*	-		-		-	
Prenatal	0.83 (-3.79, 5.45)	0.724	0.97 (-3.58, 5.52)	0.676	2.14 (-2.28, 6.56)	0.343
Postnatal	-0.82 (-3.20, 1.56)	0.496	-0.82 (-3.16, 1.53)	0.495	-0.67 (-3.21, 1.88)	0.608
Persistent	-1.52(-5.47, 2.48)	0.447	-2.42 (-6.32, 1.47)	0.223	-2.89 (-7.78, 1.99)	0.246
Child sex						
Girls*	-		-		-	
Boys	-3.89 (-5.76, -2.03)*	<0.001	-4.02 (-5.90, -2.15)*	<0.001	-3.93 (-5.83, -2.04)*	<0.001
Maternal Education						
Years of Education	0.16 (-0.20, 0.52)	0.378	-	-	0.06 (-0.30, 0.31)	0.702
Maternal Age						
Continuous Age	-0.01 (-0.23, 0.21)	0.923	-	-	-	-
Prepregnancy Weight						

Maternal BMI	0.13 (-0.04, 0.30)	0.121	-	-	0.14 (-0.03, 0.31)	0.112
Sex-specific Interaction						
Prenatal#Girls*	-	-	-	-	-	-
Prenatal#Boys	-	-	-	-	1.76 (-7.08, 10.59)	0.696
Postnatal#Girls*	-	-	-	-	-	-
Postnatal#Boys	-	-	-	-	4.91 (0.24, 9.57)*	0.039
Persistent#Girls*	-	-	-	-	-	-
Persistent#Boys	-	-	-	-	4.04 (-7.01, 15.09)	0.473
Motor Scores 1 Year of Age						
	Crude Estimate (95%CI)	**p-value**	**Adjusting for Sex** Estimate (95%CI)	**p-value**	**Fully Adjusted Model** Estimate (Bootstrap 95%CI)	**p-value**
PSS						
Never*	-				-	
Prenatal	-2.67(-8.36, 2.97)	0.355	-2.62 (-8.28, 3.04)	0.363	-2.11 (-7.63, 3.41)	0.454
Postnatal	-0.19 (-3.15, 2.70)	0.894	-0.19 (-3.11, 2.72)	0.896	0.45 (-2.41, 3.31)	0.757
Persistent	1.24 (-3.40, 6.36)	0.613	0.94 (-3.90, 5.79)	0.702	-1.87 (-7.93, 4.18)	0.544
Child Sex						
Girls*	-		-		-	
Boys	-1.40 (-3.72, 0.92)	0.235	-1.33 (-3.66, 1.01)	0.265	-1.15 (-3.54, 1.25)	0.349
Maternal Education						
Years of Education	-0.15 (-0.59, 2.79)	0.485	-	-	-0.21 (-063, 1.25)	0.345
Maternal Age						
Continuous Age	-0.03 (-0.19, 0.24)	0.849	-	-	-	-
Prepregnancy Weight						
Maternal BMI	0.26 (0.05, 0.46)*	0.014	-	-	0.27 (0.08, 0.47)*	0.006
Social-Emotional Scores 1 Year of Age						
	Crude Estimate (95%CI)	**p-value**	**Adjusting for Sex** Estimate (95%CI)	**p-value**	**Fully Adjusted Model** Estimate (Bootstrap 95%CI)	**p-value**
PSS						
Never*	-		-		-	
Prenatal	-0.13 (-5.34, 5.09)	0.961	-0.14 (-5.36, 5.08)	0.958	0.48 (-6.90, 7.86)	0.899
Postnatal	-1.73 (-4.52, 1.05)	0.222	-1.73 (-4.51, 1.06)	0.224	-1.32 (-4.29, 1.65)	0.383
Persistent	-4.05 (-8.71, 0.62)*	0.087	-3.95 (-8.58, 0.69)*	0.095	-5.64 (-9.52, -1.76)*	0.004
Child sex						
Girls*	-		-		-	
Boys	0.53 (-1.65, 2.72)	0.632	0.33 (-1.87, 2.53)	0.769	0.66 (-1.55, 2.87)	0.559

Maternal Education						
Years of Education	0.48 (0.07, 0.89)*	0.021	-	-	0.51 (0.10, 0.92)*	0.013
Maternal Age						
Continuous Age	0.04 (-0.21, 0.29)	0.768	-	-	-	-
Prepregnancy Weight						
Maternal BMI	0.13 (-0.06, 0.33)	0.181	-	-	0.17 (-0.03, 0.37)*	0.088
Cognitive Scores 2 Years of Age						
	Crude Estimate (95%CI)	**p-value**	**Adjusting for Sex** Estimate (95%CI)	**p-value**	**Fully Adjusted Model** Estimate (Bootstrap 95%CI)	**p-value**
PSS						
Never*	-		-		-	
Prenatal	-0.93 (-7.2, 5.43)	0.774	-0.98 (-7.23, 5.26)	0.757	-0.52 (-7.58, 6.54)	0.884
Postnatal	0.06 (-3.03, 3.15)	0.971	-0.002 (-3.05, 3.05)	0.999	0.41 (-2.66, 3.48)	0.794
Persistent	-0.18 (-5.31, 4.95)	0.946	-1.35 (-6.43, 3.74)	0.604	1.56 (-4.02, 7.13)	0.584
Child sex						
Girls*	-		-		-	
Boys	-4.96 (-7.35, -2.58)*	<0.001	-5.03 (-7.44, -2.63)*	<0.001	-5.09 (-7.48, -2.69)*	<0.001
Maternal Education						
Years of Education	-0.79 (0.34, 1.23)*	0.001	-	-	0.95 (0.45, 1.46)*	<0.001
Maternal Age						
Continuous Age	-0.14 (-0.43, 0.14)	0.333	-	-	-0.34 (-0.65, -0.03)*	0.030
Prepregnancy Weight						
Maternal BMI	-0.02 (-0.23, 0.20)	0.881	-	-	0.07 (-0.14, 0.29)	0.504
Language Scores 2 Years of Age						
	Crude Estimate (95%CI)	**p-value**	**Adjusting for Sex** Estimate (95%CI)	**p-value**	**Fully Adjusted Model** Estimate (Bootstrap 95%CI)	**p-value**
PSS						
Never*	-		-		-	
Prenatal	0.25 (-5.00, 5.59)	0.926	0.19 (-4.92, 5.29)	0.943	-0.21 (-7.58, 7.16)	0.955
Postnatal	-0.673(-3.26, 1.90)	0.581	-0.79 (-3.28, 1.70)	0.533	-0.49 (-3.15, 2.17)	0.720
Persistent	-1.36(-5.97, 2.73)	0.534	-2.83 (-6.99, 1.33)	0.182	-0.04 (-3.83, 3.76)	0.995
Child sex						
Girls*	-		-		-	
Boys	-6.23 (-8.18, -4.28)*	<0.001	-6.37 (-8.34, 4.41)*	<0.001	-6.31 (-8.30, -4.31)*	<0.001

Maternal Education						
Years of Education	0.72 (0.35, 1.10)*	<0.001	-	-	0.86 (0.50, 1.21)*	<0.001
Maternal Age						
Continuous Age	-0.14 (-0.38, 0.10)	0.257	-	-	-0.27 (-0.51, -0.03)*	0.025
Prepregnancy Weight						
Maternal BMI	-0.06 (-0.24, 0.12)	0.534	-	-	0.01 (-0.14, 0.17)	0.856
Motor Scores 2 Years of Age						
	Crude Estimate (95%CI)	**p-value**	**Adjusting for Sex** Estimate (95%CI)	**p-value**	**Fully Adjusted Model** Estimate (Bootstrap 95%CI)	**p-value**
PSS						
Never*	-		-		-	
Prenatal	-0.38 (-4.57, 3.81)	0.858	-0.40 (-4.57, 3.77)	0.850	-0.47 (-6.51, 5.57)	0.879
Postnatal	0.49 (-1.56, 2.53)	0.638	0.47 (-1.57, 2.50)	0.651	0.72 (-1.28, 2.72)	0.483
Persistent	1.29 (-2.12, 4.66)	0.463	0.85 (-2.54, 4.25)	0.622	1.38 (-2.18, 4.89)	0.451
Child sex						
Girls*	-		-		-	
Boys	-1.83 (-3.42, -0.24)*	0.024	-1.79 (-3.39, -0.18)*	0.029	-2.08 (-3.72, -0.44)*	0.013
Maternal Education						
Years of Education	0.19 (-0.11, 0.49)	0.231	-	-	0.23(-0.08, 0.55)	0.147
Maternal Age						
Continuous Age	-0.07 (-0.26, 0.12)	0.458	-	-	-0.12 (-0.33, 0.08)	0.244
Prepregnancy Weight						
Maternal BMI	-0.05 (-0.19, 0.09)	0.520	-	-	-0.05 (-0.20, 0.09)	0.479
Social-Emotional Scores 2 Years of Age						
	Crude Estimate (95%CI)	**p-value**	**Adjusting for Sex** Estimate (95%CI)	**p-value**	**Fully Adjusted Model** Estimate (Bootstrap 95%CI)	**p-value**
PSS						
Never*	-		-		-	
Prenatal	-2.53 (-9.40, 4.32)	0.470	-2.55 (-9.39, 4.28)	0.463	-3.86 (-12.63, 4.90)	0.388
Postnatal	-2.48 (-5.86, 0.89)	0.150	-2.54 (-5.90, -.83)	0.139	-1.79 (-5.04, 1.47)	0.283
Persistent	-7.33 (-13.15, -1.53)*	0.013	-8.11 (-13.93, -2.30)*	0.006	-5.40 (-10.72, -0.08)*	0.047
Child sex						
Girls*	-		-		-	
Boys	-2.95 (-5.60, -0.29)*	0.029	-3.33 (-5.98, -0.68)*	0.014	-3.05 (-5.78, -0.32)*	0.029

Maternal Education						
Years of Education	0.97 (0.47, 1.46)	<0.001	-	-	0.90 (0.41, 1.39)*	<0.001
Maternal Age						
Continuous Age	-0.07 (-0.38, 0.24)	0.667	-	-	-	-
Prepregnancy Weight						
Maternal BMI	0.01 (-0.22, 0.25)	0.922	-	-	0.07 (-0.17, 0.31)	0.578

All models tested for infant sex interactions. Only significant interactions are shown ($p<0.10$) and if interactions were not significant the interaction term was dropped from the full model. Fully adjusted models are multivariable linear regressions with covariates that were both identified in the DAG model and that had greater than 10% estimate change. Due to the smaller sample sizes in the stress trajectories, all the fully adjusted models were bootstrapped (reps=1000). Model diagnostics are presented in supplementary (Supplementary Table S8).
PSS: Perceived Stress Scale; CI=confidence interval, Estimate=Beta Coefficient
*=reference
*=p-value<0.05
*=p-value between 0.05 and 0.10

3.2.1.3 Impact of Covariates Independent of Maternal Distress

Each model had a unique set of maternal education, maternal age, and maternal prepregnancy BMI as covariates. Exploring the impact of the covariates on neurodevelopmental outcomes within the multivariable models and independently of maternal distress trajectories, maternal years of education was associated with increased social-emotional (at 1 and 2 years of age), cognitive (2 years of age), and language (2 years of age) scores (Tables 3 and 4). Exploring maternal prepregnancy BMI, increased BMI was associated with motor scores (at 2 years of age). Maternal age was associated with lower language (at 2 years of age) scores independently of maternal distress. Independent of stress but not depression trajectories, maternal age was also associated with lower cognitive scores (at 2 years of age).

3.2.1.4 Impact of Child Sex on Neurodevelopmental Scores, Independent of Maternal Distress

Exploring child sex, at one year of age boys had significantly lower language scores compared to girls, independent of maternal distress trajectories, maternal years of education, maternal age, and maternal prepregnancy weight (Tables 3 and 4). At 2 years of age, boys had lower scores on all of the neurodevelopment subscales (cognitive, language, motor, and social-emotional), independent of maternal distress trajectories, maternal years of education, maternal age, and maternal years of education.

3.3.2 Impact of Maternal Distress on Neurodevelopment Scores in a Sex-Specific Manner

Within the multivariable models, each model was tested whether exposure to distress trajectories varied by child sex (Tables 3 and 4). We found six significant interactions within four of the multivariable models (Figure 5). Sex-specific stratifications are presented in the supplementary (Figures S1 and S2).

3.3.2.1 Maternal Distress and Child Sex Interactions

Prenatal depression differentially affected the sexes, with a significant interaction in which boys exposed to prenatal depression had an estimated -9.87 lower language scores (Bootstrap 95%CI: -19.36, - 0.38; p=0.041) and -11.44 lower motor scores (Bootstrap 95%CI: -18.72, -4.17; p=0.002) at two years of age compared to girls exposed to prenatal depression, adjusted for covariates.

Boys exhibited higher scores compared to girls after exposure to postnatal and persistent depression and postnatal stress. Boys had higher language scores at one year of age after exposure to both postnatal stress (Coef: 4.91; Bootstrap 95%CI: -0.28, 10.09; p=0.064) and postnatal depression (Coef: 9.16; Bootstrap 95%CI: 2.35, 15.97; p=0.008) compared to girls exposed to postnatal maternal stress or depression. After exposure to persistent depression, boys had significantly higher two-year language scores (Coef: 10.38; Bootstrap 95%CI: 3.71, 17.05; p=0.002) and two-year motor scores (Coef: 31.52; 95%CI:26.96, 36.08; p<0.001) than girls' exposure to maternal persistent depression; however, there were few boys within the persistent depression group (N=3).

Figure 5. Predictive Margins Graphs of the Interactions of Maternal Distress Trajectories and Child Sex on Neurodevelopment Scores.

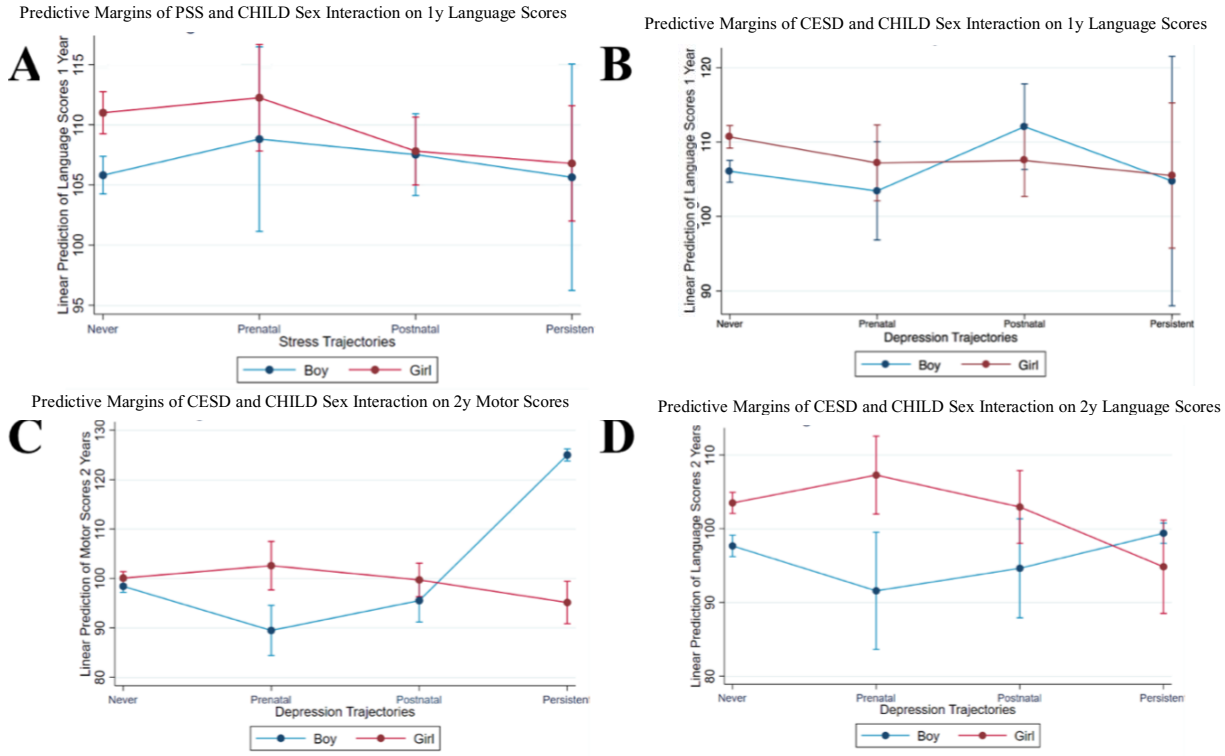


Figure 5. Interactions of maternal stress or maternal depression trajectories and child sex on neurodevelopment scales. Interactions were bootstrapped with 1000 repetitions. Interaction estimate coefficients are presented with 95% confidence intervals. All models have the same adjustment set as their corresponding multivariable model shown in Tables 3 and 4. We identified six significant interactions within four multivariable models. **A**= Interaction between postnatal stress and boys' language scores at 1 year of age. **B**=Interaction between postnatal depression and boys' language scores at 1 year of age. **C**= Interaction between prenatal depression and boys' motor scores at 2 years of age. **D**=Interaction between prenatal depression and boys' language scores at 2 years of age.

3.3 Secondary Aim

The second aim of this study is to explore the mediating role of exclusive breastfeeding duration and the gut microbiome at 4 months of age on the associations between maternal prenatal distress on neurodevelopment scores. Mediations were adjusted for years of education, maternal age, and maternal prepregnancy BMI scores which were previously identified in the DAG. Table S9 provides sensitivity analyses adjusting for the age of the stool sample. Supplementary tables S10 and S11 provide sub-sample characteristics for the *C. difficile* (n=370) and metabolite (n=168) sub-samples as well as the full CHILD cohort characteristics (N=3472). Only microbial mediators with significant associations with maternal prenatal distress and neurodevelopmental outcomes were further tested in structural equation modelling. While many microbial metabolites were associated with neurodevelopment scores (summarized in section

3.3.4), we only found significant associations among prenatal depression scores on acetate abundance and *C. difficile* abundance. There were no associations from prenatal stress on potential microbial mediators. Table S12 demonstrates the summary of associations among prenatal depression, acetate and *C. difficile* abundance, and neurodevelopment outcomes that were further tested in sequential mediation models. The percent effect explained by the mediation models is demonstrated in the supplementary (Supplementary Table S13).

3.3.1 Sequential Mediation Models

We discovered five significant mediation paths with neurodevelopment scores at two years of age (Figure 6). There were no significant indirect effects with pathways starting from prenatal depressive symptom scores. Significant pathways were found from exclusive breastfeeding duration on child neurodevelopment scores mediated by acetate and *C. difficile* abundance.

Adjusted indirect effect pathways with acetate abundance as a mediator

We found two significant indirect paths with acetate abundance in which 35.9% of the association from exclusive breastfeeding duration on child cognitive scores at two years of age was explained by increasing acetate abundance, adjusted for maternal pre-pregnancy BMI scores, maternal years of education, and maternal age. Similarly, 10.5% of the association from exclusive breastfeeding duration on child language scores at two years of age was explained by increasing acetate abundance, adjusted for covariates.

Adjusted indirect effect pathways with C. difficile abundance as a mediator

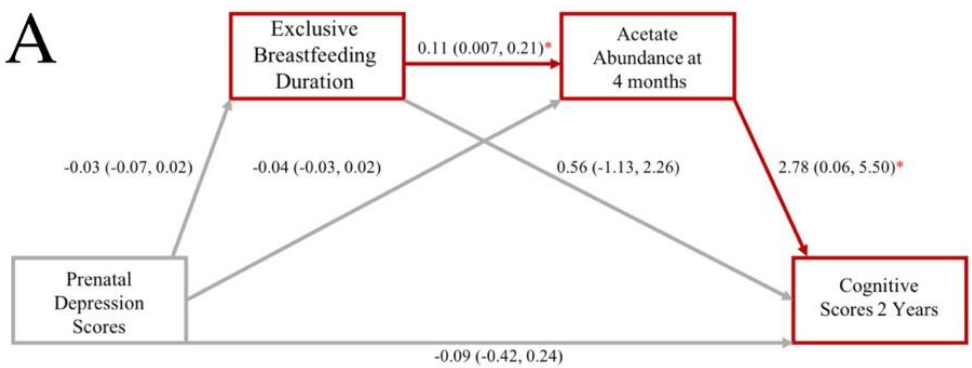
We found three paths in which *C. difficile* significantly mediated the associations from exclusive breastfeeding duration on neurodevelopment scores: *C. difficile* explained 18.1% of the effect on cognitive scores, 6.5% of the effect on language scores, and 18.5% of the effect on child motor scores, adjusted for covariates.

Adjusted direct effects

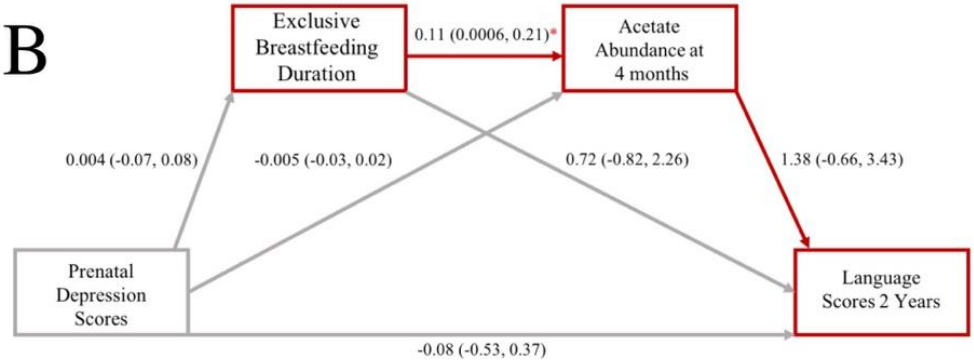
Exploring direct effects, exclusive breastfeeding duration significantly increased acetate abundance in both the cognitive (Coef: 0.11; Bootstrap 95%CI: 0.007, 0.21; p=0.037) and language (Coef: 0.11; Bootstrap 95%CI: 0.0006, 0.21; p=0.49) mediation models, adjusted for covariates. Exclusive breastfeeding duration also significantly decreased *C. difficile* abundance in the cognitive, language, and motor models, adjusted for covariates. Acetate abundance at four

months of age significantly increased child cognitive scores at 2 years of age (Coef: 2.78; Bootstrap 95%CI: 0.06, 5.50; p=0.045), while abundance of *C. difficile* significantly decreased child two-year cognitive scores (Coef: -0.52; Bootstrap 95%CI: -0.98, -0.05; p=0.030). Exclusive breastfeeding duration significantly increased child cognitive (Coef: -0.29; Bootstrap 95%CI: -0.51, -0.06; p=0.012), language (Coef: -0.29; Bootstrap 95%CI: -0.51, -0.08; p=0.008) and motor (Coef: -0.29; Bootstrap 95%CI: -0.51, -0.06; p=0.015) scores within the *C. difficile* mediation models.

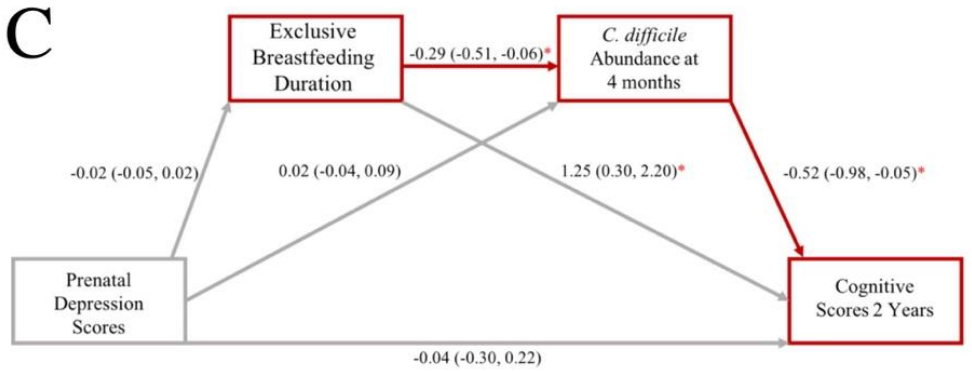
Figure 6. Indirect effects from exclusive breastfeeding duration on neurodevelopment outcomes through the mediating paths of acetate and *C. difficile* abundance.



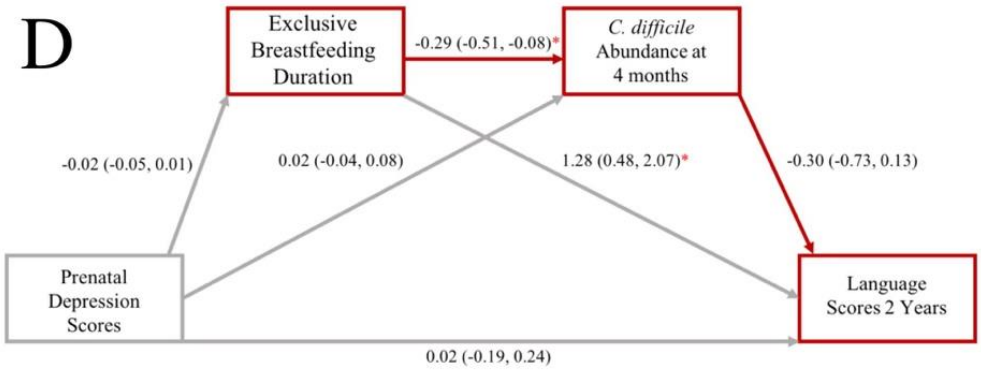
Indirect Coef: 0.31; 95%CI: 0.02, 0.61; p=0.037



Indirect Coef: 0.15; 95%CI: 0.0008, 0.30; p=0.049



Indirect Coef: 0.15; 95%CI: 0.03, 0.26; p=0.012



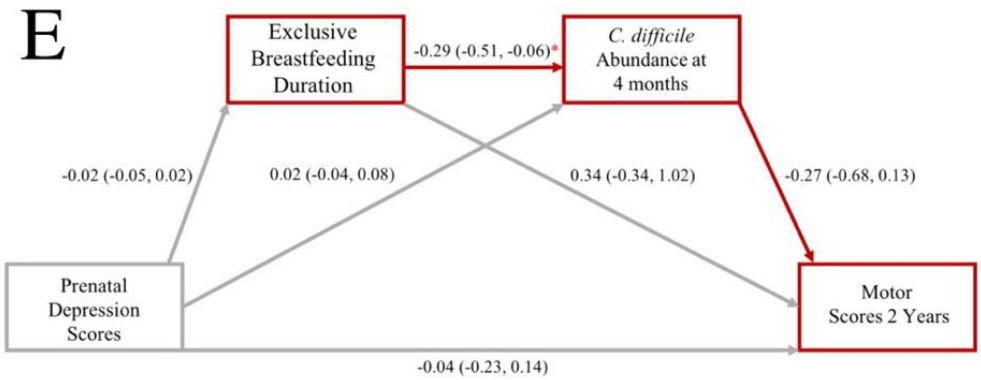


Figure 6. Adjusted mediation models demonstrating the five mediation paths discovered. These models represent the full sample sizes available for each mediation. Each mediation model is bootstrapped with 1000 repetitions and adjusted for maternal years of education, maternal BMI, and maternal age. For the indirect pathways, highlighted in red indicates a statistically significant indirect path ($p<0.05$), and grey represents no significant indirect effects.

→ = indirect effect p-value<0.05

*=direct effect p-value<0.05

*=direct effect p-value 0.05>p<0.10

Direct effect coefficients with bootstrap 95% confidence intervals are presented in the models. Indirect coefficients and bootstrap 95% confidence intervals are presented underneath significant pathways. Model fit indices are presented in supplementary Table S14. **A**= Significant indirect effect demonstrating that exclusive breastfeeding duration increases child cognitive scores at 2 years of age by increasing acetate abundance at 4 months. **B**= Significant indirect effect demonstrating that exclusive breastfeeding duration increases child language scores at 2 years of age by increasing acetate abundance at 4 months. **C**= Significant indirect effect demonstrating that exclusive breastfeeding duration increases child cognitive scores at 2 years of age by decreasing *C. difficile* abundance at 4 months. **D**= Significant indirect effect demonstrating that exclusive breastfeeding duration increases child language scores at 2 years of age by decreasing *C. difficile* abundance at 4 months. **E**= Significant indirect effect demonstrating that exclusive

breastfeeding duration increases child motor scores at 2 years of age by decreasing *C. difficile* abundance at 4 months.

3.3.2 Sex-specific Sequential Mediation Models

To test whether the sequential mediation models were sex-specific, we stratified the fixe significant models by child sex and found eight significant indirect paths with neurodevelopment scores at two years of age (Figure 7), adjusted for covariates. Supplementary Table S13 summarizes the percent of the effect that is explained through the mediation for the marginal and significant paths.

Adjusted indirect effect pathways with acetate abundance as a mediator: Sex-specific

Exploring acetate, there were no significant indirect effects in boys on cognitive or language models. In girls, acetate abundance explained 44.9% of the effect of exclusive breastfeeding duration significantly increasing cognitive scores as well as a marginal indirect effect of acetate mediating this association on language scores. Additionally, a second marginal indirect effect emerged in which prenatal depression marginally lowered acetate abundance by decreasing exclusive breastfeeding duration. In summary, we found that the indirect paths of acetate abundance as a mediator and increased neurodevelopment scores are sex-specific and were only found among girls.

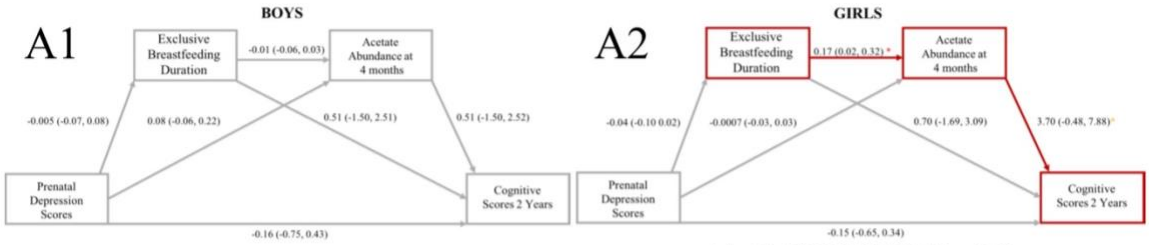
Adjusted indirect effect pathways with C. difficile abundance as a mediator: Sex-specific

In the *C. difficile* models, there were no indirect effects in girls on cognitive, language, or motor scales (Figure 7). In boys, we found two significant sequential mediations in which prenatal depressive symptoms decreased exclusive breastfeeding duration which increased infant *C. difficile* abundance and decreased boys cognitive and language scores. The sequential mediation from exclusive breastfeeding duration on infant *C. difficile* abundance explained 18.6% of the impact of prenatal depressive symptoms on cognitive scores and 19.4% on language scores. Moreover, we found three additional mediation paths in which exclusive breastfeeding duration significantly increased boys' cognitive, language, and motor scores by decreasing *C. difficile* abundance. These effects mediated through infant *C. difficile* explained 17.7% of the effect of breastfeeding duration increasing cognitive scores, 5.1% of the effect of increasing language scores, and 57.6% of the effect of increasing motor scores at two years of age. In summary, we found significant indirect pathways from prenatal depressive symptoms on

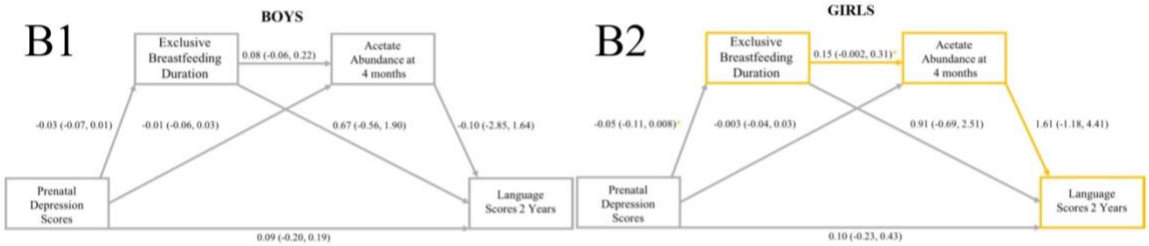
neurodevelopment scores through the sequential pathway of exclusive breastfeeding duration and *C. difficile* abundance emerge within the sex-specific models and are exclusively found among boys.

Figure 7. Sex-specific mediation pathways from prenatal depressive symptoms on neurodevelopment outcomes through the mediating paths of acetate and *C. difficile* abundance.

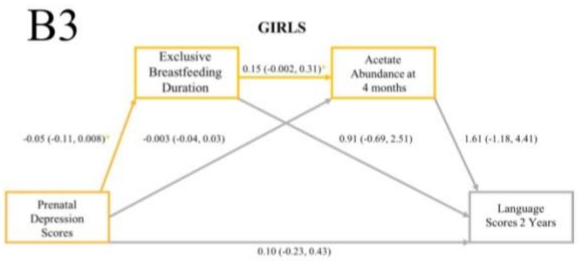
MALES **FEMALES**



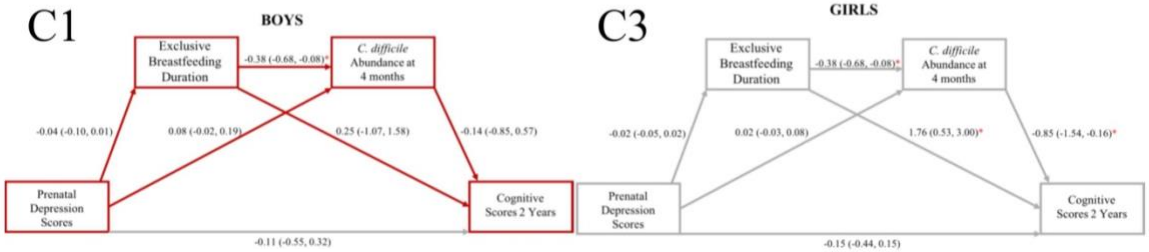
Indirect Coef: 0.63; 95%CI: 0.06, 1.19; p=0.030



Indirect Coef: 0.25; 95%CI: -0.002, 0.50; p=0.052



Indirect Coef: -0.008; 95%CI: -0.017, 0.001; p=0.089



Indirect Coef: -0.03; 95%CI: -0.05, -0.002; p=0.031

MALES **FEMALES**

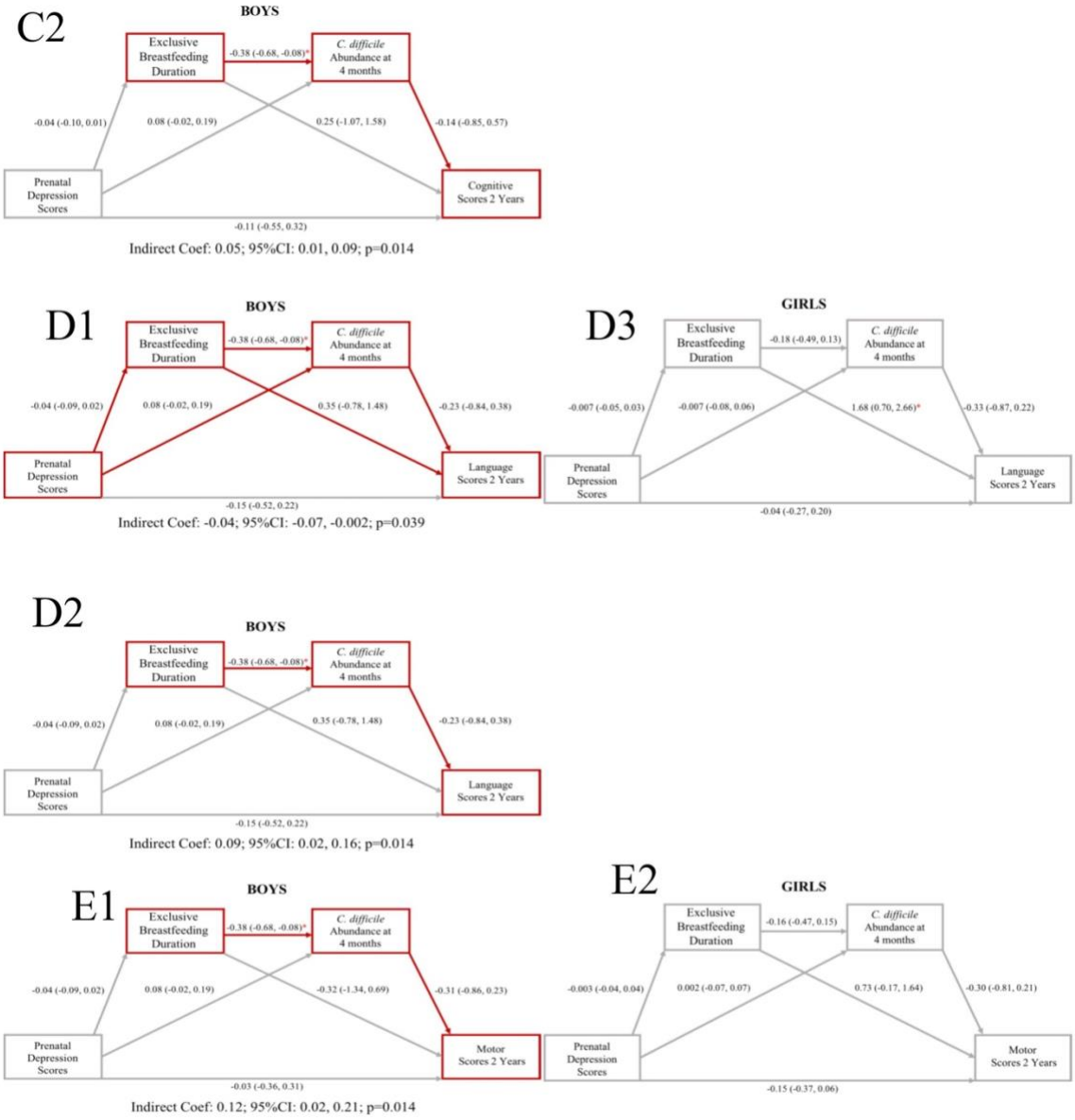


Figure 7. Adjusted sex-specific mediation models. The five mediation paths were further stratified by sex. Each mediation model is bootstrapped with 1000 repetitions and adjusted for maternal years of education, maternal BMI, and maternal age. For the indirect pathways, highlighted in yellow represents a marginally significant indirect path ($0.05>p<0.10$), highlighted in red indicates a statistically significant indirect path ($p<0.05$), and grey represents no significant indirect effects.

→ = indirect effect p-value<0.05

→ = indirect effect p-value $0.05>p<0.10$

*=direct effect p-value<0.05

*=direct effect p-value $0.05>p<0.10$

Direct effect coefficients with bootstrap 95% confidence intervals are presented in the models. Indirect coefficients and bootstrap 95% confidence intervals are presented underneath significant pathways. Model fit indices are presented in supplementary Table S14. **A**= Models with acetate on cognitive scores. *A1*=No indirect effects in boys. *A2*= Significant indirect effect demonstrating that exclusive breastfeeding duration increases girls' cognitive scores at 2 years of age by increasing acetate abundance at 4 months. **B**= Models with acetate on language scores. *B1*=No indirect effects in boys. *B2*= Marginally significant indirect effect demonstrating that exclusive breastfeeding duration increases girls' scores at 2 years of age by increasing acetate abundance at 4 months. *B3*=Marginally significant indirect effect demonstrating that prenatal depressive symptoms decreased acetate abundance by decreasing exclusive breastfeeding duration. **C**= Model with *C. difficile* and cognitive scores. *C1*= Significant indirect effect demonstrating that prenatal depressive symptoms decreased boys cognitive scores at 2 years of age by decreasing exclusive breastfeeding duration and increasing *C. difficile* abundance. *C2*= Exclusive breastfeeding duration increased boys' cognitive scores at 2 years of age by decreasing *C. difficile* abundance at 4 months. *C3*= No indirect effects in girls. **D**= Model with *C. difficile* and language scores. *D1*= Significant indirect effect demonstrating that prenatal depressive symptoms decreased boys' language scores at 2 years of age by decreasing exclusive breastfeeding duration and increasing *C. difficile* abundance. *D2*= Exclusive breastfeeding duration increases boys' language scores at 2 years of age by decreasing *C. difficile* abundance at 4 months. *D3*= No indirect effects in girls. **E**= Model with *C. difficile* and motor scores. *E1*=Exclusive breastfeeding duration increased boys' motor scores at 2 years of age by decreasing *C. difficile* abundance at 4 months. *E2*= No indirect effects in girls.

Do variables within the mediation models vary by sex?

We tested whether prenatal depressive symptoms, breastfeeding duration, and abundance of acetate or *C. difficile* were different between the sexes. The abundance of acetate (t=1.23, p=0.2181) and *C. difficile* (t=1.12, p=0.261) did not differ between the sexes. Girls (Mean=2.58 months) were breastfed slightly longer than boys (Mean months=2.35) (t= -1.67, p=0.0945) but were exposed to mothers with higher prenatal depression scores (t=2.46, p=0.0143). Figure 8 demonstrates the association between maternal prenatal depressive symptoms on exclusive breastfeeding duration by child sex. We further explored this association within regression models stratified by sex and found that prenatal depressive symptoms significantly decreased exclusive breastfeeding duration in both sexes (Boys: Coef: -0.034; 95%CI: -0.06, -0.004, p=0.028 and Girls: Coef: -0.036; 95%CI: -0.06, -0.01; p=0.003). However, after adjusting for covariates (maternal education, pre-pregnancy BMI, and maternal age), this association only remained significant among girls (Coef: -0.028; 95%CI: -0.05, -0.002, p=0.034).

Figure 8. The impact of maternal prenatal depressive symptoms on exclusive breastfeeding duration stratified by sex.

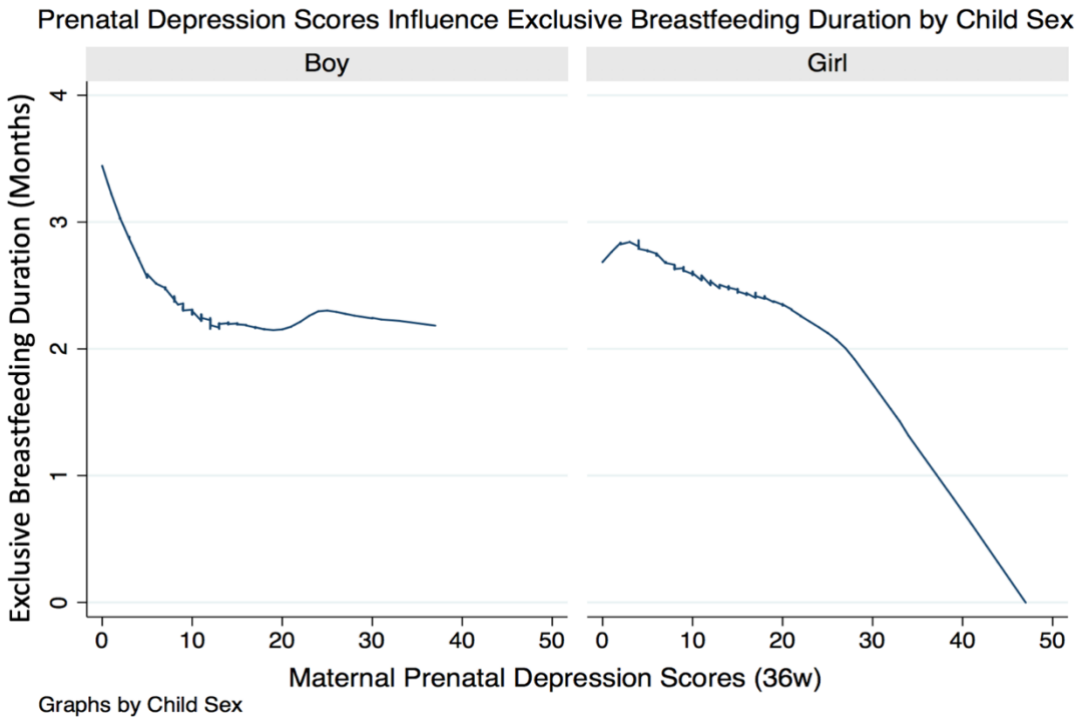


Figure 8. Lowess (locally weighted scatterplot smoothing) plot demonstrating the impact of maternal prenatal depressive symptoms CES-D scores on exclusive breastfeeding duration (Range: 0-4 months) stratified by child sex.

3.3.3 Direct and Indirect Effects from Covariates

Sequential models were adjusted for maternal years of education, pre-pregnancy BMI scores, and maternal age. We found two sex-specific indirect effects from pre-pregnancy BMI scores increasing *C. difficile* abundance in boys through the indirect path of increasing maternal prenatal depression scores and decreasing exclusive breastfeeding duration within the two-year language (Coef: 0.03; Bootstrap 95%CI: -0.003. 0.06; p=0.079) and motor (Coef: 0.03; Bootstrap 95%CI: -0.003. 0.06; p=0.079) mediation models.

Table S15 summarizes the marginal and significant direct effects among the mediation models with regards to covariates. In each of the mediation models, maternal education had a direct effect of significantly increasing prenatal depression scores among girls.

Within the *C. difficile* models, pre-pregnancy BMI had a direct effect of increasing prenatal depression scores as well as decreasing cognitive, language, and motor scores with sex stratification revealing this finding were only significant among girls. Among boys, pre-

pregnancy BMI had a direct effect of decreasing exclusive breastfeeding duration within the language and motor models.

3.3.4 Gut Microbiome and Neurodevelopment Associations

Although only *C. difficile* and acetate abundance were further tested in the mediation models due to their significant association with prenatal depression, there were many associations of the metabolites on neurodevelopment scales. Table 5 summarizes the marginal and significant crude regression associations between the enterotype clusters and metabolites that were not mediators from prenatal depression but were significantly associated with neurodevelopment scores. Tryptophan concentration had a positive effect on one-year motor scores and a negative effect on two-year motor and language scores. Formate abundance was positively associated with cognitive scores at one year of age and language scores at two years of age. Contrary to our hypothesis, butyrate abundance had a negative impact on cognitive and motor scores at two years of age. We further explored this association and found that this negative association was only significant among infants that were colonized with *C. difficile* (Cognitive Coef: -6.63; 95%CI: -10.60, -2.65; p=0.001; Motor Coef: -2.63; 95%CI: -5.63, 0.37; p=0.085) and no association from butyrate abundance on either cognitive or motor scores at two years was found in infants that were not colonized with *C. difficile* (Cognitive Coef: 0.27; 95%CI: -3.57, 4.11; p=0.890; Motor Coef: 0.92; 95%CI: -3.58, 1.74; p=0.494). Propionate had a negative impact on cognitive and language scores at two years of age. The microbial ranked clusters based on *Lachnospiraceae* family abundance had a negative impact on cognitive scores at one year of age.

Table 5. Summary of the crude linear regression associations from the infant gut microbiome on neurodevelopment scores.

Crude Associations of Gut Microbiome on Neurodevelopment Scores Summary			
Potential Mediator (M)	BSID-III Scale (Y)	Crude M→Y Estimate (95%CI)	p-value
Tryptophan concentration			
	Motor 1 year	2.72 (0.20, 5.13)*	0.028
	Language 2 year	-2.39 (-4.27, -0.51)*	0.012
	Motor 2 year	-2.79 (-3.89, -0.49)*	0.012
Formate abundance			
	Cognitive 1 year	1.48 (-0.27, 3.22)*	0.097
	Language 2 year	1.99 (0.12, 3.87)*	0.038
Butyrate abundance			
	Cognitive 2 year	-2.79 (-5.45, -0.16)*	0.038
	Motor 2 year	-1.67 (-3.51, 0.16)*	0.073
Propionate abundance			
	Cognitive 2 year	-3.31 (-5.62, -1.00)*	0.005
	Language 2 year	-2.71 (-4.49, -0.92)*	0.003
Enterotype clusters rank at 4 months			
	Cognitive 1 year	-1.22 (-2.56, 0.13)*	0.077

Table 5. Summary of the M→Y significant crude associations between significant potential mediators and neurodevelopment scores. Only potential mediators with significant X→M associations (acetate abundance and *C. difficile* abundance) were further tested in structural equation models for indirect effects.
*=p-value<0.05
*=p-value between 0.05 and 0.10

Exploring sex-specificity, we found that tryptophan concentration during infancy had a positive impact on boys' motor scores at one year and a negative impact on girls' motor scores at two years of age (Supplementary Table S16). Increased formate abundance increased boys' cognitive scores at two years, while the enterotype cluster rank based on *Lachnospiraceae* family abundance marginally decreased girls' cognitive scores at 1 year. Increased butyrate abundance decreased girls cognitive and motor scores at two years of age, while propionate abundance was associated with lower cognitive scores in both sexes at two years and decreased two-year language scores specifically in boys. Within the stratification, the association from formate increasing two-year language scores was no longer significant which could be due to reduced statistical power after the stratification.

Microbial metabolites, C. difficile, and neurodevelopment correlations

Supplementary Table S17 summarizes the correlations among neurodevelopment scores at both one and two years of age and gut microbiome variables. Acetate abundance was positively correlated with butyrate abundance and cognitive and language scores at two years while also being negatively associated with tryptophan concentration. Butyrate was negatively

correlated with formate and cognitive scores at two years. Formate was positively correlated with language scores at two years and negatively correlated with propionate abundance and the enterotype cluster rank variable. Propionate abundance was positively correlated with tryptophan concentration and negatively correlated with both cognitive and language scores at two years of age. Tryptophan was positively correlated with motor scores at one year but negatively correlated with motor scores at two years, as well as negatively correlated with language scores at two years. The abundance of *C. difficile* was positively correlated with the enterotype cluster rank, as well as cognitive, language, and motor scores at two years of age. There were many positive correlations among neurodevelopmental scores at both one and two years of age.

CHAPTER 4: DISCUSSION AND CONCLUSION

Chapter 4 provides a summary and interpretation of the research findings. This chapter also explores both the strength and limitations of this study, the significance and clinical relevance of this study, implications for future research, and an overall bias assessment of the study cohort. This chapter will also provide key concluding remarks about the overall findings.

CHAPTER 4: DISCUSSION AND CONCLUSION

4.1 Summary and Interpretation of Findings

4.1.1 Primary Aim

The primary aim of this study was to explore the impact of maternal distress trajectories on child neurodevelopment scores in a sex-specific manner. We hypothesized that maternal distress would negatively impact child neurodevelopment with males to a greater extent. To test this hypothesis, we used multivariable models and tested sex-specific interactions which revealed several significant interactions demonstrating that this association is both time-and-sex specific.

4.1.1.1 Sex-specific Impact of Maternal Distress Trajectories

A mismatch environment

We found that after exposure to maternal depression only in the prenatal period, that boys had lower language and motor scores compared to girls. In contrast, when exposed to only postnatal distress (stress or depression), boys had higher language scores than girls. These findings agree with the large (N=8354) Avon Longitudinal Study of Parents and Children (ALSPAC) cohort of mother-child pairs that found a three-way interaction in which prenatal depression moderated the association between postnatal depression and child emotional symptoms in a sex-specific manner (Braithwaite et al., 2020). Specifically, the impact of postnatal depression on girls was stronger if they were exposed to lower prenatal depression. This is demonstrated in our results by boys having higher scores than girls after both experiencing high postnatal depression but low prenatal depression. The mismatch hypothesis theorizes that adaptations from fetal exposures may be advantageous as a form of developmental plasticity but can be disadvantageous when the postnatal environment does not match the level of distress experienced in utero (Daskalakis et al., 2013). These observations are further validated by epigenetic studies in which postnatal depression was associated with increased stress hormone gene methylation and anxious-depressive symptoms among daughters that were exposed to postnatal but not prenatal depression (J. Hill et al., 2017). Meaning, HPA axis methylation mediated the mismatch between prenatal and postnatal maternal depression on emotional symptoms specifically in females. Moreover, our findings alongside several reviews demonstrate that boys may be at greater vulnerability to prenatal stressors while girls may be affected to a

greater extent by postnatal stressors (Ahun et al., 2021; Eriksson et al., 2010; Sandman et al., 2013; Sutherland & Brunwasser, 2018; Weinstock, 2007). Maternal distress in the prenatal period may predict vulnerability to postnatal distress differentially between the sexes.

Sex-specific motor and language development

Our findings demonstrated sex-specific differences in language and motor scores dependent on the timing of distress. There are small but reliable sex differences in both verbal and spatial learning between the sexes in which females have greater language, verbal, and non-verbal communication in the first three years of life while male development appears to favour spatial regions (Adani & Cepanec, 2019; Eaton & Enns, 1986; Johnson & Moore, 2020). Examining motor development in rats, researchers have demonstrated prenatal stress can impair motor reflexes (Patin et al., 2004) as well as stress having a sex-specific impact on motor exploratory behaviour (Pallarés et al., 2007). The sex-specific differences in language and spatial development may explain differences in scores with the timing of stressors having differential impacts. Interestingly, germ-free mice showed altered gene expression pathways in neuronal circuits related to motor control (Heijtz et al., 2011b). In section **4.1.2** we will discuss sex-specific microbial mediation pathways involving language and motor scores.

A matched environment

Furthermore, after exposure to persistent depression, boys had the highest language and motor scores. This demonstrates a match between the prenatal and postnatal environment in which adaptations to distress in utero successfully anticipated postnatal distress conditions. Our findings are consistent once again with the large ALSPAC cohort which found that after exposure to persistent maternal depression, boys had the highest outcome scores (Braithwaite et al., 2020). However, our persistent depression group consisted of fewer participants with sex stratification further reducing the sample; thus, these findings should be replicated in a larger sample for further reliability.

Non-sex-specific impacts of maternal distress

This match effect of persistent depression predicting higher neurodevelopment scores was sex-specific to the language and motor scales. In all children, persistent depression lowered two-year cognitive scores as well as persistent stress lowering both one and two-year social-

emotional scores. This demonstrates that maternal distress may impact the development of children's cognitive and emotional processing independent of child sex. Cognitive development contributes to the development of self, alongside a secure attachment style, with consequences impacting social skills and emotional regulation. The social-emotional scale assesses self-regulation and the infant's ability to communicate its needs. Prenatal distress has been implicated in preferentially impacting brain areas related to cognition and emotional regulation such as the pre-frontal cortex and amygdala, as well as studies demonstrating prenatal stress predicting infants' stress regulation and temperament (Braithwaite et al., 2016; E. P. Davis et al., 2007b, 2011; Jones et al., 2019; King & Laplante, 2005; Tollenaar et al., 2011). Moreover, maternal postpartum depression has been associated with lower maternal reports of infant bonding, insecure attachment, higher infant emotional sensitivity, and increased risk for depressive symptoms in adolescence (Murray et al., 2006; Nonnenmacher et al., 2016). Therefore, prenatal distress may lead to brain alterations associated with increased infant stress reactivity and emotional sensitivity which is further impacted by postnatal distress being associated with additional difficulties in mother-infant bonding.

Exploring mother-infant attachment, researchers reported that attachment moderated the negative impact of amniotic cortisol levels on cognitive scores on the BSID-II (Bergman et al., 2010). Meaning, the negative association from prenatal cortisol levels on children's cognitive scores was only significant among infants that had an insecure attachment style. Attachment is described as the "lasting psychological connectedness between human beings" (Bowlby, 1969) and emerges from synchrony between the newborn and mother providing support and responsiveness to the infant's needs. Interestingly, we found that persistent stress was associated with lower one-year social-emotional scores which preceded the impact of persistent distress on lower two-year cognitive and social-emotional scores. Evidence has emerged that insecure attachment can impact child cognitive development (Ding et al., 2014; Mikulincer et al., 2003; O'Connor & McCartney, 2007) with researchers theorizing a secure attachment enables infants to explore the world leading to increased stimulation and cognitive processing. Lower social-emotional scores at one-year following persistent distress may predict the co-development of two-year social-emotional and cognitive scores. Interventions on improving attachment styles during infancy may prevent lower social-emotional and cognitive scores among children exposed to persistent distress.

Figure 9. Conceptual figure summarizing the mismatch theory and our primary aim findings.

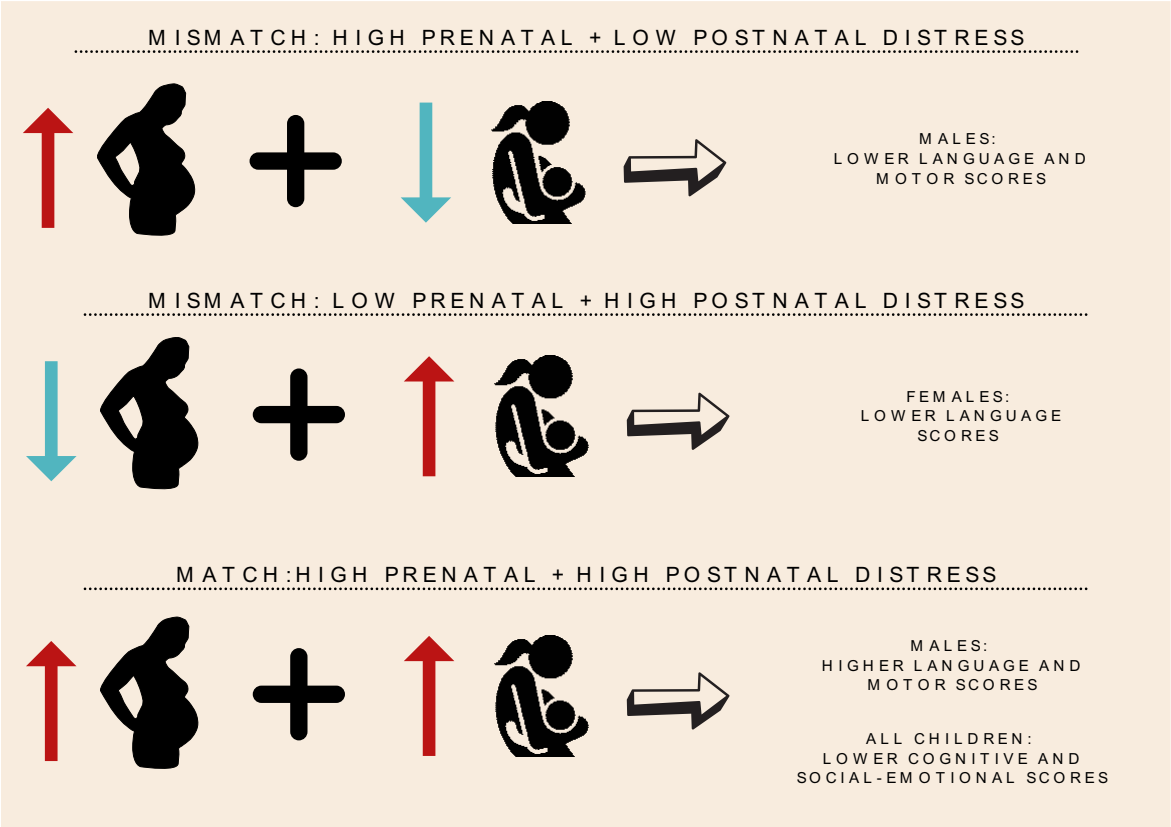


Figure 9. Conceptual figure summarizing the mismatch theory and our primary aim findings. Our findings demonstrate that males may be at greater vulnerability to prenatal stressors while females may be more vulnerable to postnatal stressors, with persistent distress having sex-specific impacts and detriments to both sexes.

4.1.2 Secondary Aim

The secondary aim of this study was to explore whether the gut microbiome mediated the impact of maternal distress on child neurodevelopment. We found two sex-specific mediation paths in which the impact of prenatal depressive symptoms on neurodevelopment scores was sequentially mediated by exclusive breastfeeding duration and *C. difficile* abundance.

4.1.2.1 Mediations with *C. difficile* Abundance

Sequential mediations from prenatal depression: C. difficile

We found two significant sequential mediation pathways demonstrating associations from prenatal depressive symptoms decreasing exclusive breastfeeding duration which increased *C. difficile* abundance during infancy and decreased two-year cognitive and language scores among boys. Thus, the negative impact of *C. difficile* abundance on boys' neurodevelopment scores is explained by the pathway from prenatal depressive symptoms to breastfeeding duration. Breastfeeding provides the infant with immunoglobulin A (IgA) which protects the intestinal epithelial barrier from pathogens as well as plays a critical role in tolerance mechanisms to commensal microbiota (Corthésy, 2013). Within CHILD, reduced IgA increased the likelihood of *C. difficile* colonization (Meghan B. Azad et al., 2012b). Moreover, researchers have found negative correlations between breast milk sIgA levels and mothers' postpartum negative mood states (anxiety, depression, anger-hostility, fatigue) (Kawano & Emori, 2015). This finding in addition to our pathways demonstrates that maternal mood may decrease infant sIgA concentrations in two ways: by decreasing sIgA within breastmilk and by decreasing breastfeeding duration, with both contributing to the sequential pathways.

With scarce research on *C. difficile* in infancy, we can only hypothesize potential mechanisms in which *C. difficile* could impact the brain via breastfeeding pathways. In addition to stimulating the infants' immune system via microbial tolerance, this vital immunoglobulin can defend the brain barrier and downregulate inflammatory processes (Fitzpatrick et al., 2020). While IgA can defend against barriers, *C. difficile* can potentially disrupt them, as an intestinal epithelial cell culture model demonstrated that *C. difficile* was found to disrupt tight junction proteins (Hecht et al., 1988; Nusrat et al., 2001). Disruption of tight junction proteins leads to increased intestinal permeability known as "leaky gut" in which substances, such as bacteria and toxins, pass through the leaky barrier and increase inflammatory mechanisms. However, it is unknown whether *C. difficile* can cause similar epithelial barrier disruption in infants. Figure 10 summarizes our working hypothesis theorizing that colonization with *C. difficile* disrupts the microbial composition affecting both the intestinal epithelial and barriers, leading to systemic inflammation. The inflammatory response may affect higher-order processes such as cognitive and language development.

As discussed in **4.1.1,** males may be more vulnerable to prenatal stressors which could explain this pathway from prenatal depressive symptoms only emerging in boys. Moreover, literature has demonstrated that male brain development may be more vulnerable to microbial perturbations than females (G. Clarke et al., 2013; Jaggar et al., 2020; Morgane Sonia Thion et al., 2018a). Exploring inflammatory processes, increased inflammation has been found to alter microglia in a sex-specific manner with males affected to a greater extent (Gómez-González & Escobar, 2010; Hui et al., 2018; Lebovitz et al., 2018; Morgane S. Thion et al., 2018; Morgane Sonia Thion et al., 2018a). The pro-inflammatory state hypothesized by increased *C. difficile* abundance and potential disruption to barriers may preferentially impact male neuroimmune cells to a greater extent than females, resulting in sex-specific detriments to neurodevelopment scores.

To summarize, we found two novel pathways in which prenatal depressive symptoms decreased exclusive breastfeeding duration which increased *C. difficile* abundance and lowered cognitive and language scores among boys.

Figure 10. Potential mechanism demonstrating the impact of *C. difficile* on gut barrier integrity and systemic inflammation in the developing infant.

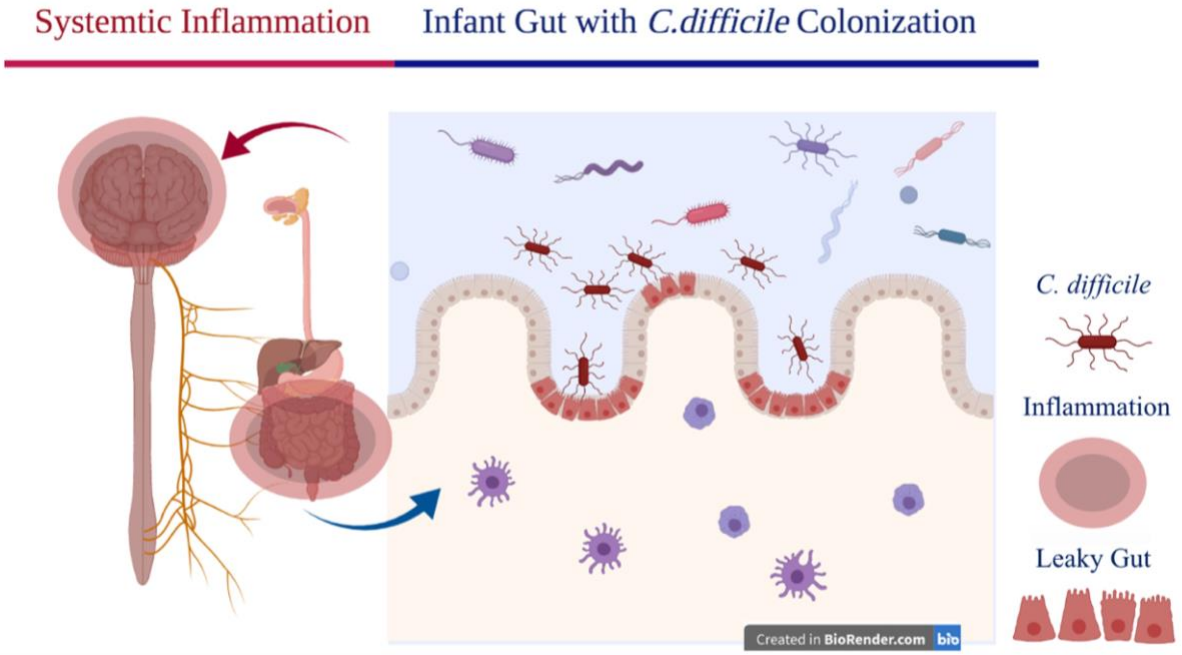


Figure 10. Potential mechanism demonstrating the colonization of *C. difficile* may impact gut barrier integrity, increasing pro-inflammatory immune cells, and leading to systemic inflammation in the developing infant.

In girls, we found a direct association of *C. difficile* lowering two-year cognitive scores. Independently from the breastfeeding pathway, *C. difficile* can uniquely produce para-cresol (p-

cresol) which increases its virulence to cause adult *C. difficile* infection (CDI). The production of p-cresol alters the microbial composition and the viability of surroundings bacteria resulting in a competitive advantage for *C. difficile* colonization in the adult gut (Passmore et al., 2018). Increased urinary and fecal p-cresol have been implicated as a potential biomarker for autism spectrum disorder (Altieri et al., 2011; De Angelis et al., 2013; Gabriele et al., 2014; D. W. Kang et al., 2018; Pascucci et al., 2020; Sharon et al., 2019). This metabolite has also been associated with pro-inflammatory activity once it has been absorbed into the bloodstream (Gryp et al., 2017). With only 18 infants in our study with detectable levels of p-cresol, we were not able to associate its concentration with neurodevelopmental outcomes. Further research is needed to explore whether this is a possible mechanism among infants and if it could explain the direct mechanisms of *C. difficile* abundance impacting neurodevelopment scores.

Mediations from exclusive breastfeeding duration: C. difficile

We found three significant paths in which exclusive breastfeeding duration decreased infant *C. difficile* abundance and increased cognitive, language, and motor scores among boys. The ability of IgA to bind to pathogens (Bunker & Bendelac, 2018) and for breastmilk to promote beneficial bacteria during infancy (Ho et al., 2018) may play a vital role in decreasing *C. difficile* abundance during infancy and promoting healthy brain development. This is a fascinating finding as the path itself demonstrates a significant opportunity for intervention. Interventions to prevent the colonization of *C. difficile* in infancy should focus on promoting exclusive breastfeeding duration.

4.1.2.2 Mediations with Acetate Abundance

Mediations from exclusive breastfeeding duration: acetate

We found that acetate abundance mediated the positive association from exclusive breastfeeding duration on two-year cognitive scores with a trend towards increasing two-year language scores. In the gut, acetate can stimulate the formation of gut barrier tight junctions (Feng et al., 2018). In the brain, acetate can cross the blood brain barrier (BBB) and can reduce inflammation by regulating astrocytes to promote the integrity of the BBB as well as its ability to regulate neuroimmune inflammatory microglial cells (Soliman et al., 2012, 2013) (Figure 10). Astrocytes are involved in the structure of the BBB while microglia are the main immune defense in the brain (Abbott et al., 2006; Li & Barres, 2018). Therefore, acetate has been found

to promote both the gut and brain barriers while also reducing inflammation. Exclusive breastfeeding duration can increase acetate abundance by selecting beneficial bacteria that produce SCFA, such as Bifidobacterium (which are enriched in breastfed infants) which can metabolize sugars found in breastmilk to produce acetate (M. B. Azad et al., 2016; Bridgman et al., 2017b; Fukuda et al., 2011).

Exploring sex-specificity, this mediation pathway was only found among girls. There were no differences in acetate abundance across child sex. As the main immune defense in the brain, microglia are altered in size, number, and maturity in GF mice which demonstrates the importance of microbial colonization on the development of microglial cells (Castillo-Ruiz et al., 2018; Erny et al., 2015; Morgane Sonia Thion et al., 2018a). Further exploring sex differences in GF mice, the absence of microbiota appears to have a time and sex-specific impact on microglia as prenatal GF environments impacted male embryonic microglia while females demonstrated microglial impairment in adulthood (Morgane Sonia Thion et al., 2018a). This suggests that gut microbiome perturbations have a greater impact on male microglia during gestation while perturbations to microglia appear postnatally in females. Exploring our acetate findings and acetate's ability to regulate microglia, perhaps the pathway from exclusive breastfeeding duration increasing acetate abundance and increasing neurodevelopmental scores only being found among girls could be explained by female microglia appearing to be affected to a greater extent postnatally which may increase their response to interventions such as increased breastfeeding duration and acetate abundance. In summary, our working hypothesis theorizes that breastfeeding duration increases acetate abundance which has an anti-inflammatory response on neuroimmune cells. Thus, acetate has a beneficial effect on both gut and brain barrier maintenance which may improve cognitive and language scores in childhood.

Figure 11. Potential mechanism of the beneficial effect of acetate on the gut and brain barriers.

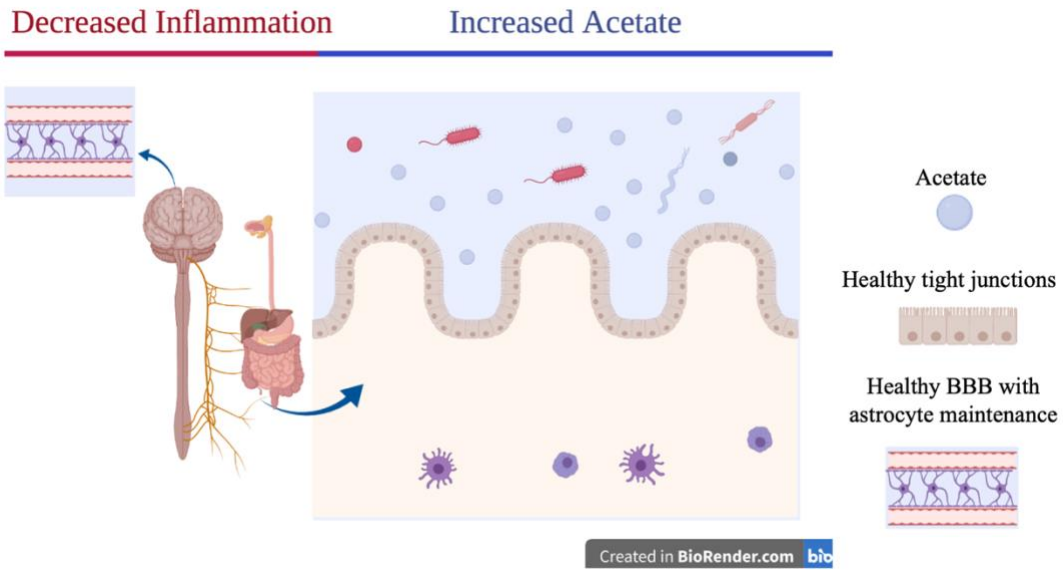


Figure 11. Potential mechanism demonstrating that increased abundance of acetate is associated with healthy tight junctions within the gut while also supporting astrocyte maintenance in the blood-brain barrier.

4.1.2.3 Role of the Microbiota and Metabolome on Neurodevelopment

We found several metabolites that were crudely associated with neurodevelopment scores that were not mediators from prenatal distress. Firstly, against our hypothesis, we found that butyrate abundance was negatively associated with cognitive and motor scores at two years of age. Regarding sex-specificity, this association was only significant in girls. We further investigated this association and found that butyrate decreasing cognitive and motor scores was only significant in children that were colonized by *C. difficile.* This is not too surprising, as *C. difficile* produces butyrate (Karlsson et al., 2000) and CHILD researchers demonstrated that the presence of *C. difficile* within the infant's gut alters butyrate, propionate, formate, and acetate concentrations (Vu et al., 2021). Among girls, propionate also contributed to lower two-year cognitive scores. Within the mediation pathways, we saw a direct effect of *C. difficile* lowering girls' two-year cognitive scores and whether propionate and butyrate are involved in this direct effect require further exploration. There is ample research on the protective effects of butyrate on several aspects of brain development within animal models and cell lines; however, animal research does not take into account infant *C. difficile* colonization. In the presence of *C. difficile,*

adding butyric acid was found to enhance toxin production (Karlsson et al., 2000). Nevertheless, it is still not clear whether *C. difficile* toxins affect the infant intestinal epithelium in the same manner as adults since infants appear to be asymptomatic even with the presence of toxigenic strains (Kubota et al., 2016b). Regardless, further research is required on the mechanisms of *C. difficile* colonization during infancy and interventions that explore infant supplementation with SCFA should proceed with caution and test for *C. difficile* colonization prior to supplementation.

Secondly, we found that increased tryptophan concentration significantly increased one-year motor scores in boys but significantly decreased two-year language and motor scores among girls. Tryptophan is an amino acid and acts as a precursor for many molecules such as neurotransmitters, hormones, and vitamins (Heine et al., 1995). Tryptophan has the potential to contribute to depressive and anxiety symptoms, infant sleep, cognitive and social function, and cardiovascular health (Friedman, 2018). Our findings suggest that tryptophan had a sex-and-time-specific impact on neurodevelopment outcomes by increasing boys' scores at one year but decreasing girls' scores at two years. With tryptophan being involved in many metabolite pathways within the body, further research is necessary to tease apart these time and sex specific impacts of tryptophan on the developing brain.

4.1.3 Associations from Covariates

Within the mediation models, we found that maternal pre-pregnancy BMI scores marginally increased *C. difficile* abundance by sequentially increasing prenatal depression scores and decreasing exclusive breastfeeding duration, specifically in boys. This once again highlights the importance of the prenatal period among males. Within CHILD, maternal pre-pregnancy BMI scores were associated with differences in breastmilk composition which impacted human milk microbiota (Moossavi et al., 2019). Altered breastmilk composition and milk microbiota could impact the initial seeding of the newborn gut, resulting in altered microbial colonization during infancy and an increased risk for *C. difficile* colonization. Moreover, increased pre-pregnancy BMI scores have been associated with higher prenatal depression and anxiety scores demonstrating potential comorbidities among maternal pre-pregnancy weight and mood disorders (Holton et al., 2019). The literature demonstrates that the association between pregnancy weight and maternal distress could be bidirectional; however, in our models, we found a greater model fit with pre-pregnancy BMI impacting prenatal depression scores rather

than the reversed association. Women presenting with increased pre-pregnancy weight should be screened for comorbidities such as stress, depression, and/or anxiety to improve maternal mental health and weight during pregnancy as well as decrease risk factors associated with infant *C. difficile* colonization.

Within the multivariable models, we found that maternal years of education increased two-year cognitive, two-year language, and one-and-two year social-emotional scores independent of maternal stress or depression trajectories. These findings are consistent with findings demonstrating that maternal education increases neurodevelopment scores on the BSID-III (Ko et al., 2013). Within the mediation models, we found that maternal education had a significant and sex-specific direct effect of decreasing prenatal depressive symptoms among mothers that gave birth to daughters within each sex-specific mediation model. There were no sex differences among maternal education levels; however, mothers that gave birth to daughters experienced higher prenatal depressive symptoms which may explain the direct effects from maternal education level on prenatal depressive symptoms emerging in the sex-specific models.

4.2 Study Strengths

The prospective CHILD cohort has many strengths. Firstly, the CHILD cohort study has a high retention rate with 92% of participants remaining at the one-year follow-up (Subbarao et al., 2015b). Secondly, the cohort has extensive documentation on covariates which allows us to characterize our sample and adjust for confounders in the multivariable analyses. The multivariable regressions allowed us to control for significant covariates and strengthens our associations. Moreover, the DAG model-building approach allowed us to differentiate between covariates and mediators which reduced potential bias in our estimates. The prospective nature of the cohort and data collection allowed us to fulfill the temporal requirements required to pursue structural equation modelling to test the mediating effect of the infant gut microbiome on the association of maternal distress on child neurodevelopment. The clear temporal relationship among variables in this study provides further strength to our analysis and is one of the Bradford Hill criteria for causality (A. B. Hill, 1965). Although we can comment on the temporality of associations in the study, we cannot comment fully on causality due to potentially unobserved sources of bias. Using the structural equation modelling method to test the sequential mediations allowed us to not only adjust for covariates but also explore pathways from covariates within the

model, such as maternal BMI, which provided further insight amongst these associations and timepoints for potential intervention. Finally, exploring microbial metabolites offers a mechanistic approach to how the gut microbiome in infancy may impact childhood neurodevelopment scores.

4.3 Study Limitations

Maternal depression and stress were tested in separate multivariable regression models due to collinearity, thus these findings may be better interpreted as the co-occurrence of stress and depression and not independent of one another. Moreover, 2.9% of women that did not experience clinically significant depression or higher perceived stress during any pregnancy time point had taken antidepressants either during the prenatal or postnatal period. These women may have experienced pre-pregnancy distress and were prescribed medications and reported lower distress during pregnancy revealing successful intervention and improvement of their symptoms. History of mental illness may increase susceptibility to perinatal distress; however, researchers found that perinatal depression, rather than pre-pregnancy history of depression, was associated with infant cortisol reactivity which further demonstrates that the timing of distress has differential outcomes on the offspring (Brennan et al., 2008). Although the CHILD cohort aims to represent Canadian families, some inclusion criteria such as the ability to read, write, and speak English may have led to the CHILD study being primarily white, urban, and of moderate to higher income (Subbarao et al., 2015b). Thus, these findings may not represent lower-income families. Due to the BSID-III scores only being administered at the Edmonton CHILD site, we had lower sample sizes than anticipated in the maternal distress trajectories, specifically the persistent trajectory. Additionally, the mediation samples were further reduced due to the availability of stool samples. The acetate mediation models' goodness of fit was moderate, which is very likely due to the smaller metabolite sample size that was available. With the purpose of this study exploring sex-specific differences, stratifying with a smaller sample size reduced our statistical power as well as limits generalizability. To support the smaller sample size, we proceeded with bootstrapping replications to both our multivariable and mediation models to aid with our sample distributions (Bollen & Stine, 1990; Shrout & Bolger, 2002).

4.4 Sources of Bias

Understanding bias within a study is a critical method to assess validity. Identifying biases allows the researcher to counter and control for the bias and minimize its impact. One bias is referred to as attrition bias/withdrawal bias in which there is unequal participant loss during the study, especially as a longitudinal study design. To account for this bias, there must be rigorous maintenance to contact subjects and avoid withdrawal and loss of follow-ups. As mentioned, the CHILD cohort has a high retention rate (Subbarao et al., 2015b) minimizing withdrawal bias. Subsequently, three main biases can arise within research studies including selection bias, measurement bias, and confounding bias.

4.4.1 Selection Bias

Selection bias, also known as susceptibility bias, occurs during recruitment and arises when there are baseline differences in susceptibility of the outcome before the exposure (Hartman et al., 2002). Selection bias can occur when the study sample does not represent the target population. The CHILD cohort has specific inclusion and exclusion criteria that were used to recruit participants across the four study sites with multiple methods to control for selection bias (Subbarao et al., 2015b). The inclusion had several criteria including the ability to read, write, and speak English, which could explain the CHILD cohort being predominantly white, urban, and from a higher socioeconomic status. Moreover, due to the difficulty to extract stool samples from infants that were breastfed, there is a potential source of selection bias among the microbiome sub-samples which could limit generalizability.

4.4.2 Measurement Bias

Measurement bias occurs when an inconsistent measurement of variables occurs between groups or data collected, which can under/over-estimate the association (Hartman et al., 2002). Measurement bias can arise from differences in data collection between clinics or among exposure groups. This study only used data from the Edmonton site, minimizing measurement bias arising from different study sites. Moreover, there are proper protocols in place for each clinic to ensure data and specimens are stored and collected as similarly as possible to avoid any variation due to clinic collection. Measurement bias can also arise from differences in outcome measurement according to the exposure. The prospective nature of the CHILD cohort design minimizes this type of bias. Therefore, the trained research assistants administering the

neurodevelopment scales (cognitive, language, and motor) would be blinded to the maternal distress trajectory membership. The research assistants were also assessed bi or semi-annually by registered education psychologists ensuring that the administration of the BSID-III was consistent across all study participants. Moreover, the neurodevelopment scales were administered at a time of day that the children were not sleepy to ensure accurate measurement and reliability. The questionnaires used in this study have also been validated to ensure accurate measurement of the variables of interest.

Although the questionnaires within the CHILD cohort used have been validated, many covariates were collected from standardized questionnaires completed by the mothers (i.e. maternal distress, SES, breastfeeding, the social-emotional scale). A limitation of standardized questions is that they rely on accurate maternal reporting in which bias can arise due to challenges with recall as well as social desirability bias. Moreover, the data depends on completion. For example, in this study, we used maternal years of education as our SES variable due to fewer missing variables compared to family income. A more accurate measure of SES or increased completion of the family income questions would add validity to our measures, although maternal years of education have been found to have stronger associations on child outcomes compared to neighbourhood income as a measure of SES (Luo et al., 2006). Self-report bias can also lead to underreporting, specifically in depressive symptoms (Hunt et al., 2003). Due to the depression and stress trajectories relying on self-report data and the documented literature on underreported depression (Cox et al., 2016), mothers may have experienced higher symptoms than were reported.

4.4.3 Confounding Bias

Further exploring bias, mothers were allocated into maternal distress groups based on their distress levels rather than random allocation meaning there were nonrandom assignments to study groups with the potential for confounding bias. Confounding bias occurs when factors that are associated with both the exposure and the outcome are present and cannot be randomly allocated, which can also under/over-estimate the association (Hartman et al., 2002). To minimize confounding bias, we identified potential confounds through a literature review and created a DAG (refer to Chapter 2) to identify our minimal adjustment set for this study to adjust for confounds that could not be randomly allocated across our exposures. Although the DAG is a

robust approach to model adjustment, other potential unmeasured confounds could contribute to the association from maternal distress to child neurodevelopment (i.e. mother-infant attachment, maternal coping and help-seeking, social support). Age of stool sample varied among the sample and efforts to control for this potential confound included conducting a sensitivity analysis in which we tested for the confounding effect of age at stool sample collection by comparing the indirect effects of the mediation models with the indirect effects after adjusting for age at stool sample (refer to Supplementary).

4.5 Study Significance and Clinical Relevance

4.5.1 Study Significance

This novel study revealed that *C. difficile* abundance during infancy had both a direct and indirect effect on lowering healthy newborns' neurodevelopmental scores in childhood. This study warrants future research on whether testing for *C. difficile* during infancy may be a biomarker of gut dysbiosis with implications on brain development. Moreover, we demonstrated pathways in which microbial SCFA abundance in infancy, such as acetate, may improve executive function among children which may lead to microbial SCFA as possible interventions for neurodevelopmental delay. Supplementation with microbial metabolites after exposure to early life stress may mitigate the effects of stress on the developing brain.

4.5.2 Clinical Relevance

Although the rates of initiation of breastfeeding in Canada have increased to 90% in 2015, only 35% of mothers breastfeed until the recommended six months (Statistics Canada, 2021). Within our mediation models, we found a significant intervention in which increasing exclusive breastfeeding duration can increase child neurodevelopmental scores by decreasing the colonization of a pathobiont and increasing microbial acetate abundance. Moreover, we revealed that prenatal depressive symptoms were associated with decreased exclusive breastfeeding duration demonstrating that proposed interventions to target breastfeeding duration should begin during the prenatal period.

We found many associations among covariates and their impact on both maternal distress and child neurodevelopment. We propose a multidisciplinary approach to interventions aimed at supporting women through pregnancy with continued support in the postnatal period. Such

interventions should focus on maternal mental health, breastfeeding, and BMI scores with particular attention to women of lower socioeconomic status or with fewer years of education achieved as maternal education directly impacted maternal depressive scores and child neurodevelopment. Cognitive-behavioral therapy among mothers that experienced maternal distress mitigated the impact of distress on child development (Stein et al., 2018) and may be a likely intervention to support women's mental health through pregnancy.

4.6 Implications for Future Research

Future research is vital to explore mechanistic pathways to explain the pathogenesis of *C. difficile* abundance during infancy and its consequences on the developing brain. Moreover, future research with a larger sample is needed to determine the impact of persistent distress during pregnancy on term infant's neurodevelopment. To further explore the potential activational effects from exposure to early life distress, researchers should explore whether the timing of maternal distress impacts child development with sex-specific reactivity to stressors potentially emerging during adolescence and the role of the early gut-brain axis on this association. Future research should explore whether supplementation with acetate during infancy could mitigate the effects of shorter breastfeeding duration on neurodevelopment scores.

4.7 Conclusion

This study aimed to explore the impact of maternal distress trajectories on child neurodevelopment in a sex-specific manner and whether breastfeeding and the infant gut microbiome were mediators in this association among a sample of healthy term infants from the CHILD birth cohort.

4.7.1 Key finding #1

"Stress, in addition to being itself, was also the cause of itself, and the result of itself"
— Hippocrates

#1- We found that maternal perinatal distress had a timing and sex-specific impact on child neurodevelopment. Our findings align with the mismatch hypothesis in which early life stressors can induce physiological changes within the developmental trajectory of children and can have either adaptive or consequential impacts on development depending on whether the pre-and-post stressor environment is matched or mismatched. The adaptive mechanisms of a matched

environment were sex-specific and distinct to motor and language scores, as persistent distress significantly lowered cognitive and social-emotional scores among all children compared to no maternal distress.

Figure 12. Conceptual figure demonstrating the time and sex-specific impacts of maternal distress on child neurodevelopment.

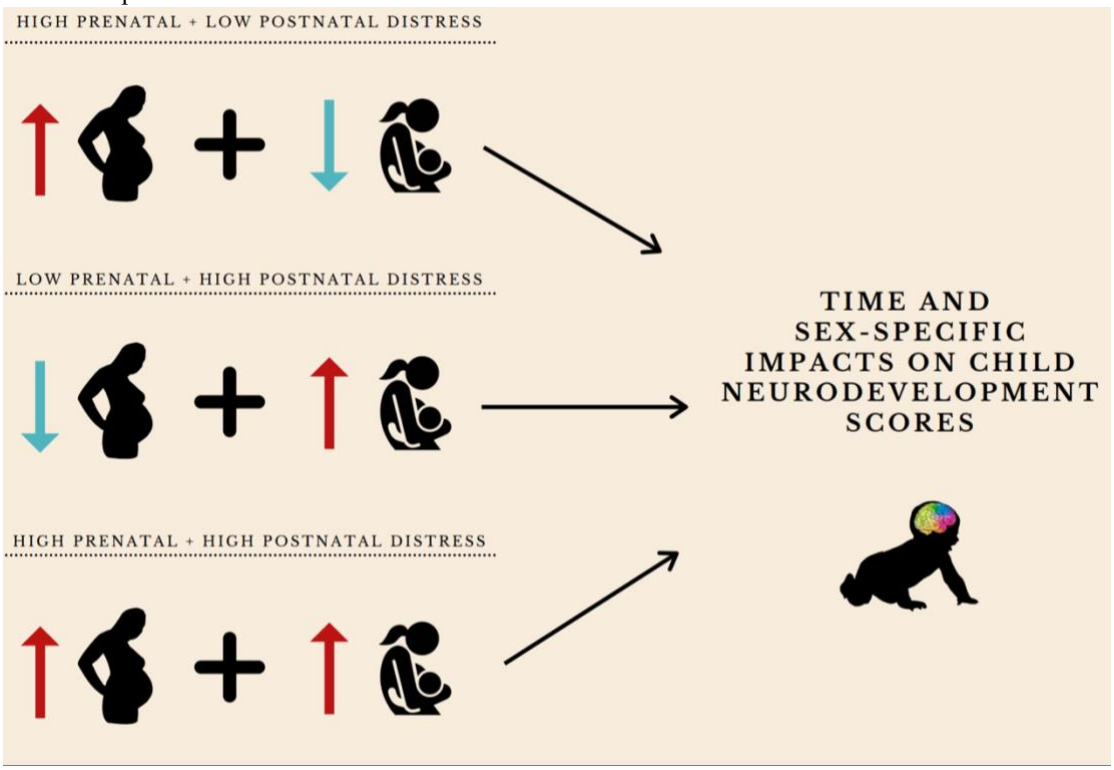


Figure 12. Key finding #1 conceptual figure demonstrating the time and sex-specific impacts of maternal distress on child neurodevelopment. We found that the timing of maternal distress through pregnancy impacted several neurodevelopment scores in a sex-specific manner.

4.7.2 Key finding #2

"All disease begins in the gut."
— Hippocrates

#2- This is the first study to explore the impact of infant *C. difficile* on brain development. Among boys, we found that exclusive breastfeeding duration and *C. difficile* abundance were significant sequential mediators on the impact of prenatal depressive symptoms on cognitive and language scores at two years of age. In girls, we found that *C. difficile* abundance directly lowered two-year cognitive scores. We also found that increasing exclusive breastfeeding

duration during infancy can be a significant intervention on the consequential impact of infant *C. difficile* abundance on child neurodevelopment.

Figure 13. Conceptual figure demonstrating the significant sequential mediation pathway through *C. difficile* abundance.

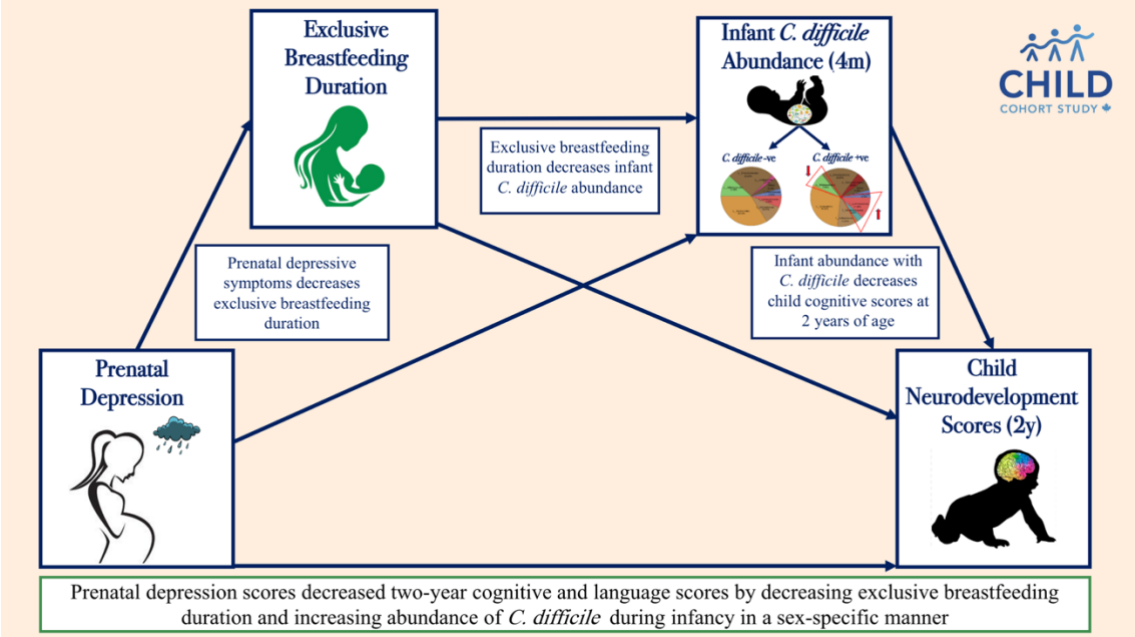


Figure 13. Key finding #2 conceptual figure demonstrating the sequential mediation pathway in which the impact of prenatal depressive symptoms can impact child two-year cognitive scores by decreasing exclusive breastfeeding duration and increasing the abundance *C. difficile* during infancy in a sex-specific manner.

Note: The pie charts within the "Infant *C. difficile* Abundance" square demonstrate the impact of *C. difficile* presence altering the infant gut microbiota composition demonstrated by previous CHILD researchers (Pie chart figure by Khanh Vu (Vu et al., 2021)).

4.7.3 Concluding Statement

Seyle coined the term stress in 1936 as the "non-specific response of the body to any demand made upon it" and linked the stress response to immune and gastrointestinal function (Selye, 1936). Colonization with *C. difficile* was discovered in 1935 among breastfed infants just days after birth (Hall, 1935). Spelt, in 1948, was the first to discover that the human fetus could learn from its environment *in utero*, revealing that exposures during the prenatal period could impact the developing brain (Spelt, 1948). More than have of a century later, this study discovered that maternal distress has a time-and-sex-specific impact on child neurodevelopment

and revealed novel pathways in which the prenatal depression can increase *C. difficile* abundance and influence the developing brain. Interestingly, we also found pathways demonstrating a significant intervention on this association in which supporting women to breastfeed for a longer duration could increase neurodevelopment scores by reducing the abundance of *C. difficile* and increasing the abundance of microbial SCFA acetate in infancy. Future research should explore whether supplementation with acetate during infancy could mitigate the effects of shorter breastfeeding duration on neurodevelopment scores. Alongside the DOHaD hypothesis, the prenatal and postnatal periods represent sensitive windows in which stressors may impact a child's developmental trajectory and colonization with *C. difficile* during infancy may not be as benign as the current literature supports. This research emphasizes the significance of supporting women's mental health through pregnancy with findings revealing pathways from maternal distress symptoms to the development of the gut-brain axis in children.

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APPENDIX

Supplement Table S1. Example of the multivariable linear regression model building process demonstrating the decision to include covariates identified from the DAG in the model.

Cognitive Scores at 1-year Model					
Maternal Depression Trajectory Model (CESD)	Estimate CESD (95%CI)	Covariate	Estimate Covariate (95%CI)	10% change in CESD Estimate?	Decision
Crude CESD					
Never* Prenatal Postnatal Persistent	- -1.69 (-4.84, 1.40) 0.96 (-2.18, 4.13) 7.87 (1.99, 13.74)				
Adjusted for Sex					
Never* Prenatal Postnatal Persistent	- -1.74 (-4.84, 1.35) 0.89 (-2.27, 4.05) 7.66 (1.76, 13.55)	Girls* Boys	- -0.71 (-2.29, 0.88)	2.9% 7.3% 2.6%	Clinically relevant
Adjusted for Maternal Education					
Never* Prenatal Postnatal Persistent	- -1.50 (-4.75, 1.74) 0.57 (-2.71, 3.85) 9.76 (3.29, 16.24)	Maternal education (years)	0.24 (-0.07, 0.54)	11.2% 40.6% 24.0%	Yes
Adjusted for Maternal Age					
Never* Prenatal Postnatal Persistent	- -1.63 (-4.73, 1.47) 0.98 (-2.18, 4.13) 7.84 (1.97, 13.72)	Maternal age (years)	0.06 (-0.13, 0.24)	3.6% 2.0% 0.4%	No
Adjusted for Pre-pregnancy weight					
Never* Prenatal Postnatal Persistent	- -1.25 (-4.64, 2.13) 0.72 (-2.43, 3.87) 2.92 (-3.50, 9.34)	Pre-pregnancy BMI	0.13 (-0.01, 0.27)	26.0% 25.0% 62.8%	Yes

Note: CESD= Center for Epidemiological Studies Depression Scale; CI= Confidence interval.
Example of the model building process in which each potential confound (maternal years of education, maternal age, and maternal pre-pregnancy BMI) were tested for a 10% change in estimate. Only confounds that had greater than a 10% change were included in the multivariable models. Child sex was included in the models to test for interactions.

Supplement Table S2. Percentage distribution of potential categorical covariates across depressive trajectories (n=646).

Categorical variables	Never depressed (84.4% overall)	Prenatal depression (6.9% overall)	Postnatal depression (6.8% overall)	Persistent depression (1.9% overall)	Row total	p-value
	N(%)	N(%)	N(%)	N(%)	N(%)	
Child sex						0.183
Boys	284 (52)	21 (47)	19 (43)	3 (25)	327 (50.6)	
Girls	261 (48)	24 (53)	25 (57)	9 (75)	319 (49.4)	
Family income						<0.001
Less than 39,999	26 (5)	5 (14)	3 (8)	2 (20)	36 (6)	
40,000 to 79,999	113 (23)	13 (35)	14 (37)	6 (60)	145 (25)	
80,000 to 99,999	76 (15)	9 (24)	8 (21)	1 (10)	94 (16)	
Exceeds 100,000	286 (57)	10 (27)	13 (34)	1 (10)	310 (53)	
Maternal education						<0.001
Some/finished high school	33 (6)	9 (21)	2 (5)	4 (40)	48 (8)	
Some university/college	186 (35)	20 (48)	24 (58)	5 (50)	235 (38)	
University degree	309 (58)	13 (31)	15 (37)	1 (10)	338 (54)	
Maternal age						0.380
18 to 29	155 (28)	19 (42)	15 (34)	4 (33)	193 (29)	
30 to 39	366 (67)	24 (53)	29 (66)	8 (67)	427 (66)	
Over 40	24 (4)	2 (4)	0 (0)	0 (0)	26 (4)	
Prenatal smoking						0.012
Yes	18 (3)	6 (14)	1 (3)	1 (10)	26 (4)	
No	513 (97)	36 (86)	40 (97)	9 (90)	598 (96)	
Breastfeeding 3 months						0.188
None	79 (15)	8 (18)	10 (23)	4 (33)	101 (16)	
Partial	142 (26)	15 (34)	10 (23	4 (33)	171 (27)	
Exclusive	322 (59)	21 (48)	24 (54)	4 (33)	371 (57)	
Birth mode						0.015
Vaginal no IAP	299 (55)	16 (35)	21 (49)	3 (25)	339 (52.8)	
Vaginal IAP	119 (22)	17 (37)	7 (16)	5 (42)	148 (23.0)	
CS-Elective	54 (10)	8 (17)	6 (14)	3 (25)	71 (11.1)	
CS-Emergency	69 (13)	5 (11)	9 (21)	1 (8)	84 (13.1)	
Pre-pregnancy weight						0.057
Overweight	227 (43)	21 (53)	21 (100)	7 (100)	276 (44)	
Normal weight	304 (57)	19 (47)	0 (0)	0 (0)	349 (56)	
Antidepressants						<0.001
Never	520 (96)	30 (65)	39 (89)	10 (83)	599 (92.7)	
Pre and/or Postnatal Use	24 (4)	16 (35)	5 (11)	2 (17)	47 (7.3)	

Gestational Age						0.068
37 weeks+	521 (95)	40 (90)	40 (91)	12 (100)	613 (95)	
Less than 37 weeks	26 (5)	6 (10)	4 (9)	0 (0)	36 (5)	
***C. difficile* colonization 4 months**						0.083
Yes	131 (41)	13 (68)	13 (52)	3 (50)	160 (43)	
No	189 (59)	6 (32)	12 (48)	3 (50)	210 (57)	
Enterotype Clusters 4 Months						0.863
Cluster 1: Proteobacteria[+]	143 (41)	9 (41)	10 (38)	1 (16)	99 (24.5)	
Cluster 2: Firmicutes	121 (35	7 (32)	10 (39)	4 (67)	163 (40.3)	
Cluster 3: Bacteroides	86 (25)	6 (27)	6 (23)	1 (16)	142 (35.2)	

Statistical analysis completed using Fishers exact test

Supplement Table S3. Percentage distribution of potential categorical covariates across stress trajectories (n=646)

Categorical variables	Never stressed (68.7% overall)	Prenatal stress (4.5% overall)	Postnatal stress (20.6% overall)	Persistent stress (6.2% overall)	Row total	p-value
	N(%)	N(%)	N(%)	N(%)	N(%)	
Child sex						0.059
Boys	230 (52)	16 (55)	69 (52)	12 (30)	327 (51)	
Girls	214 (48)	13 (45)	64 (48)	28 (70)	319 (49)	
Family income						<0.001[1]
Less than 39,999	15 (4)	3 (12)	13 (10)	5 (16)	36 (6)	
40,000 to 79,999	82 (20)	9 (36)	38 (31)	16 (52)	145 (25)	
80,000 to 99,999	62 (15)	4 (16)	22 (18)	6 (19)	94 (16)	
Exceeds 100,000	246 (61)	9 (36)	51 (41)	4 (13)	310 (53)	
Maternal education						<0.001
Some/finished high school	22 (5)	4 (15)	10 (8)	12 (33)	48 (8)	
Some university/college	153 (36)	14 (52)	51 (39)	17 (47)	235 (38)	
University degree	253 (59)	9 (33)	69 (53)	7 (19)	338 (54)	
Maternal age						0.360
18 to 29	126 (28)	14 (48)	38 (29)	15(37)	193 (29)	
30 to 39	298 (67)	15 (52)	90 (68)	24 (60)	427 (66)	
Over 40	20 (5)	0 (0)	5 (4)	1(3)	26 (4)	
Prenatal smoking						0.008
Yes	13 (3)	3 (11)	5 (4)	5 (14)	26 (4)	
No	419 (97)	24 (89)	123 (96)	32 (86)	598 (96)	
Breastfeeding 3 months						0.082
None	64 (15)	6 (20)	24 (18)	7 (18)	101 (16)	
Partial	112 (25)	10 (35)	32 (24)	17 (44)	171 (27)	
Exclusive	267 (60)	13 (45)	76 (58)	15 (38)	371 (57)	
Birth mode						0.371
Vaginal no IAP	244 (55)	12 (41)	67 (52)	16 (39)	339 (52.8)	
Vaginal IAP	95 (21)	8 (28)	32 (25)	13 (32)	148 (23.0)	
CS-Elective	43 (10)	4 (14)	18 (14)	6 (15)	71 (11.1)	
CS-Emergency	60 (14)	5 (17)	13 (10)	6 (15)	84 (13.1)	
Pre-pregnancy weight						0.020[1]
Overweight	184 (42)	13 (46)	55 (44)	24 (67)	276 (44)	
Normal weight	251 (58)	15 (54)	71 (56)	12 (33)	349 (56)	
Antidepressants						<0.001
Never	423(95)	23 (79)	120 (90)	33 (80)	599 (92.7)	
Pre and/or Postnatal Use	20 (5)	6 (21)	8 (10)	8 (20)	47 (7.3)	
Gestational Age						0.003
37 weeks+	42 (96)	24 (83)	128 (96)	35 (85)	613 (95)	
Less than 37 weeks	20 (4)	5 (17)	5 (4)	6 (15)	36 (5)	

***C. difficile* colonization 4 months**						0.568
Yes	109 (42)	9 (60)	33 (43)	9 (47)	160 (43)	
No	151 (58)	6 (40)	43 (57)	10 (53)	210 (57)	
Enterotype Clusters 4 Months						0.778
Cluster 1: Proteobacteria[+]	111 (39)	7 (39)	36 (43)	9 (45)	99 (24.5)	
Cluster 2: Firmicutes	98 (35)	9 (50)	28 (34)	7 (35)	163 (40.3)	
Cluster 3: Bacteroides	74 (26)	2 (11)	19 (23)	4 (20)	142 (35.2)	

Statistical analysis completed using Fishers exact test

[1]=Statistical analysis completed using Chi2 test

Supplement Table S4. Mean comparisons of continuous covariates across depressive trajectories (N=646)

Continuous variables	Never depressed (84.4% overall)	Prenatal depression (6.9% overall)	Postnatal depression (6.8% overall)	Persistent depression (1.9% overall)	p-value
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	
BSID-III cognitive 1 year	110.05 (10.04)	108.33 (12.06)	111.02 (9.92)	117.92 (13.22)	0.0345
BSID-III language 1 year	108.18 (13.14)	105.69(13.13)	109.30 (10.77)	106.58 (11.69)	0.5512
BSID-III motor 1 year	103.18 (15.55)	100.62(15.28)	104.57 (16.06)	107.83 (15.15)	0.4513
BSID-III social-emotional 1 year	102.99 (13.88)	100.35(12.41)	101.88 (15.47)	97.73 (13.30)	0.3904
BSID-III cognitive 2 year	106.07 (14.39)	104.00(13.80)	104.23 (14.80)	98.13 (5.94)	0.3484
BSID-III language 2 year	100.40 (11.83)	100.57(13.01)	98.90 (13.22)	90.25 (9.45)	0.1062
BSID-III motor 2 year	99.05 (9.47)	97.67(10.50)	98.26 (8.77)	98.13 (11.89)	0.8390
BSID-III social-emotional 2 year	109.32 (15.66)	102.59(14.74)	108.29 (15.65)	95.00 (11.55)	0.0144
Maternal pregnancy fruit intake	3.21 (2.13)	2.86 (2.09)	3.66 (2.80)	3.56 (2.60)	0.3940
Propionate abundance	0.02 (1.04)	-0.35 (0.74)	-0.03 (0.77)	0.40 (1.09)	0.5714
Acetate abundance	0.01 (0.97)	0.23 (0.64)	-0.0007 (0.85)	-1.57 (2.65)	0.6474
Butyrate abundance	-0.03 (0.92)	-0.03 (0.70)	-0.07 (0.83)	1.72 (3.70)	0.9208
Formate abundance	-0.05 (0.99)	0.27 (0.93)	0.27 (1.17)	0.16 (0.48)	0.4982
Tryptophan concentration	-0.02 (0.97)	0.09 (1.63)	-0.03 (0.64)	0.71 (0.64)	0.1752
Infant sleep duration	14.34(2.07)	13.55(1.99)	13.54(2.35)	13.61(1.87)	0.0168

Note: SD= standard deviation; CS=caesarean section

Statistical analysis of means completed using ANOVA or Kruskal-Wallis

Supplement Table S5. Mean comparisons of continuous covariates across stress trajectories (N=646)

Continuous variables	Never stressed (68.7% overall)	Prenatal stress (4.5% overall)	Postnatal stress (20.6% overall)	Persistent stress (6.2% overall)	p-value
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	
BSID-III cognitive 1 year	110.49(10.10)	108.38(9.84)	108.46(10.54)	113.13(11.08)	0.0399
BSID-III language 1 year	108.20(13.25)	109.28(11.41)	107.62(12.60)	106.95(12.18)	0.8617
BSID-III motor 1 year	103.19(15.97)	100.72(13.14)	103.20(14.33)	104.90(16.66)	0.7510
BSID-III social-emotional 1 year	103.24(13.63)	103.10(16.98)	101.50(13.98)	99.19(13.62)	0.2685
BSID-III cognitive 2 year	105.70(14.24)	104.86(15.11)	105.84(14.48)	105.16(14.67)	0.9887
BSID-III language 2 year	100.37(11.50)	100.67(16.54)	99.69(12.90)	96.75(11.80)	0.8596
BSID-III motor 2 year	98.76(9.45)	98.38(12.25)	99.25(9.45)	99.66(8.49)	0.9212
BSID-III social-emotional 2 year	109.70(15.92)	107.14(18.34)	107.19(14.76)	102.24(12.07)	0.0541
Maternal pregnancy fruit intake	3.20 (2.10)	2.65 (2.08)	3.35 (2.40)	3.47 (2.53)	0.4421
Propionate abundance	0.05 (1.04)	-0.26 (0.52)	-0.09 (0.98)	0.001 (0.93)	0.8129
Acetate abundance	-0.02 (0.96)	0.09 (0.67)	0.09 (0.98)	-0.19 (1.61)	0.8978
Butyrate abundance	-0.02 (0.86)	0.17 (0.74)	-0.02 (1.05)	0.13 (2.008)	0.3698
Formate abundance	-0.03 (1.02)	0.05 (0.94)	-0.003 (0.96)	0.26 (1.09)	0.8378
Tryptophan concentration	-0.02 (0.97)	0.68 (2.25)	-0.05 (0.87)	0.01 (0.68)	0.8600
Infant sleep duration	14.36(2.07)	14.14(2.55)	14.04(2.09)	13.36(1.80)	0.0464

Note: SD= standard deviation; CS=caesarean section
Statistical analysis of means completed using ANOVA or Kruskal-Wallis

Supplement Table S6. Covariates and their associations with infant 1-year BSID-III neurodevelopment scales (N=646).

Covariates and their associations with infant 1-year BSID-III neurodevelopment scales (N=646).									
		Cognitive Score		Language Score		Motor Score		Social-Emotional Score	
Variable	**N(%)**	**Mean (*SD*)**	***p*-valu**	**Mean (*SD*)**	***p*-value**	**Mean (*SD*)**	***p*-value**	**Mean (*SD*)**	***p*-value**
Categorical factors - Mean (SD)[a]									
Infant sex			0.243		<0.001		0.177		0.689
Boys	327 (51)	109.7(10.7)		106.3(11.9)		102.8(13.3)		102.9(14.3)	
Girls	319 (49)	110.6(9.9)		110.2(12.3)		104.4(14.9)		102.4(13.5)	
Family income			0.176		0.189		0.713		0.651
Less than 39,999	36 (6)	106.8(11.2)		103.9(13.6)		101.1(12.2)		102.2(17.3)	
40,000 to 79,999	145 (25)	109.8(10.3)		108.3(14.5)		103.9(15.3)		102.4(14.1)	
80,000 to 99,999	94 (16)	110.9(10.6)		108.7(12.3)		104.3(15.2)		103.9(15.1)	
Exceeds 100,000	310 (53)	110.4(9.8)		108.6(12.0)		103.1(13.1)		101.8(12.7)	
Maternal education			0.023		0.079		0.117		0.068
Some/finished high school	48 (8)	106.3(10.7)		104.3(11.3)		102.3(12.9)		97.9(13.6)	
Some university/college	235 (38)	110.7(10.9)		108.5(13.4)		105.2(13.4)		102.2(13.0)	
University degree	338 (54)	110.4(9.8)		108.5(11.7)		102.4(13.3)		103.1(14.2)	
Maternal age			0.140		0.872		0.275		0.496
18 to 29	193 (29)	109.1(9.7)		107.8(12.9)		103.4(13.8)		103.4(14.9)	
30 to 39	427 (66)	110.7(10.5)		108.4(11.9)		103.5(14.5)		102.2(13.3)	
Over 40	26 (4)	108.3(10.6)		108.1(11.5)		107.9(10.9)		104.6(15.6)	
Prenatal smoking			0.917		0.097		0.162		0.854
Yes	26 (4)	110.4(9.9)		112.2(12.8)		107.3(16.0)		101.9(10.1)	
No	598 (96)	110.2(10.3)		103.4(14.07)		103.4 (14.1)		102.5(13.9)	

***C. difficile* colonization 4m**			0.118		0.711		0.981		0.952
Yes	160 (43)	109.3(10.9)		108.9(11.6)		104.1(14.6)		102.5(13.2)	
No	210 (57)	110.9(9.7)		108.3(12.9)		104.0(14.9)		102.6(13.3)	
Breastfeeding 3 months			0.854		0.062		0.374		0.261
None	101 (16)	109.7(10.9)		107.4(12.5)		102.6(14.6)		100.8(14.6)	
Partial	171 (27)	109.9(11.1)		106.6(13.3)		102.2(17.2)		102.3(13.2)	
Exclusive	371 (57)	110.3(9.7)		109.2 (11.6)		104.1(14.1)		103.3(13.9)	
Birth mode			0.1981		0.8477		0.1069		0.4578
Vaginal no IAP	339 (52.8)	110.8(10.4)		108.6(12.5)		103(16.2)		103.5(14.0)	
Vaginal IAP	148 (23.0)	109.8(10.7)		107.9(11.5)		102.9(14.3		101.4(13.5)	
CS-Elective	71 (11.1)	108.2(8.5)		107.5(13.2)		101.1(12.9)		102.2(14.7)	
CS-Emergency	84 (13.1)	109.4(10.4)		107.9(11.5)		106.7(11.7)		102.0(13.4)	
Pre-pregnancy weight			0.088		0.134		0.016		0.141
Overweight	276 (44)	110.9(10.1)		109.0(12.2)		104.9(14.5)		103.9(13.7)	
Normal weight	349 (56)	109.5(10.1)		107.6(12.1)		102.0(15.1)		101.9(14.1)	
Antidepressants			0.4166		0.4216		0.0470		0.4794
Never	599 (93)	110.2 (10.2)		108.3 (12.2)		103.6(15.1)		102.5 (14.0)	
Pre and/or Postnatal Use	47 (7)	108.9 (11.9)		106.8 (12.2)		99.1 (12.3)		104.0 (12.5)	
Gestational Age			0.6696		0.0480		0.0082		0.1991
37 weeks+	613 (95)	110.2 (10.4)		108.4 (12.3)		103.7 (15.1)		102.8 (13.9)	
Less than 37 weeks	36 (5)	109.4 (9.2)		104.3 (10.1)		96.9 (11.8)		99.7 (13.2)	
Enterotype Clusters 4 Months			0.1645		0.8279		0.0553		0.8129
Cluster 1: Proteobacteria^{+}	163 (40.3)	109.9 (10.5)		108.9(13.0)		102.1(18.9)		103.1(13.1)	
Cluster 2: Firmicutes	142 (35.2)	111.8(10.6)		109.4(12.0)		106.7(14.6)		102.1(14.0)	
Cluster 3: Bacteroides	99 (24.5)	109.5(10.6)		108.4(12.1)		104.1(14.2)		102.1(12.2)	

Continuous factors – or β (95% CI)[b]									
Infant sleep duration	575 (89)	-0.39(-0.79, 0.0009)	0.051	0.02(-0.45, 0.49)	0.926	-0.49(-1.09, 0.09)	0.102	0.43(-0.09, 0.96)	0.107
Maternal prenatal fruit intake	610 (94)	0.24 (-0.13, 0.61)	0.204	0.41 (-0.03, 0.86)	0.071	0.44 (-0.12, 0.99)	0.116	0.72 (0.20, 1.23)	0.007
Propionate	168 (26)	-0.08 (-1.84, 1.68)	0.929	0.05 (-1.78, 1.89)	0.955	-0.32 (-2.72, 2.08)	0.792	-2.12 (-2.27, 1.95)	0.838
Acetate	168 (26)	-0.98 (-2.74, 0.77)	0.272	0.009 (-1.83, 1.84)	0.992	-1.07 (-3.46, 1.33)	0.379	0.02 (-2.03, 2.08)	0.985
Butyrate	168 (26)	0.63 (-1.13, 2.39)	0.480	-0.27 (-2.10, 1.57)	0.771	1.21 (-1.18, 3.60)	0.319	0.57 (-1.47, 2.62)	0.581
Formate	139 (23)	1.48 (-0.27, 3.22)	0.097	0.14 (-1.70, 1.98)	0.878	1.63 (-0.76, 4.01)	0.180	-0.09 (-2.14, 1.95)	0.926
Tryptophan	163 (25)	0.70 (-1.11, 2.51)	0.445	1.17 (-0.70, 3.04)	0.217	2.72 (0.30, 5.13)	0.028	0.27 (-1.82, 2.36)	0.800

Note: BSID-III=Bayley Scales of Infant Development Third Edition; SD=standard deviation; *β=Coefficient*

[a]Analyzed by t-test or one-way analysis of variance.

[b]Analyzed by linear regression

The total number of observations (N) represents those participants with maternal distress data and cognitive data collected at the 1-year study visit

Supplement Table S7. Covariates and their associations with infant 2-year BSID-III neurodevelopment scales (N=646).

Covariates and their associations with infant 2-year BSID-III neurodevelopment scales (N=646).

Variable	N(%)	Cognitive Score		Language Score		Motor Score		Social Emotional Score	
		Mean (*SD*)	*p*-valu	Mean (*SD*)	*p*-value	Mean (*SD*)	*p*-value	Mean (*SD*)	*p*-value
Categorical factors - Mean (SD)[a]									
Infant sex			0.0001		<0.0001		0.029		0.022
Boys	327 (51)	103.4(13.5)		97.2(11.8)		98.0(9.5)		107.2(16.1)	
Girls	319 (49)	108.2(14.8)		103.4(11.4)		99.8(9.5)		110.3(15.1)	
Family income			0.574		0.083		0.501		0.104
Less than 39,999	36 (6)	102.0(18.7)		95.2(13.5)		98.0(13.1)		102.2(17.7)	
40,000 to 79,999	145 (25)	105.1(14.6)		99.7(13.1)		99.1(10.2)		108.2(14.9)	
80,000 to 99,999	94 (16)	106.1(12.6)		101.9(10.8)		100.4(9.9)		109.1(16.1)	
Exceeds 100,000	310 (53)	105.9(13.9)		100.7(13.4)		98.6(8.9)		110.1(15.7)	
Maternal education			0.0001		0.0002		0.208		0.0007
Some/finished high school	48 (8)	99.2(12.4)		94.9(10.5)		96.5(8.9)		101.0(13.8)	
Some university/college	235 (38)	103.5(13.3)		98.6(11.9)		98.7(10.0)		107.2(16.3)	
University degree	338 (54)	107.6(14.5)		101.9(11.9)		99.4(9.3)		110.6(15.2)	
Maternal age			0.254		0.126		0.572		0.922
18 to 29	193 (29)	107.2(15.2)		101.6(11.4)		99.6(9.6)		109.1(15.1)	
30 to 39	427 (66)	104.9(13.9)		99.4(12.2)		98.6(9.6)		108.6(15.9)	
Over 40	26 (4)	107.1(15.1)		102.3(12.6)		99.1(8.5)		108.2(14.8)	
Prenatal smoking			0.142		0.359		0.667		0.272
Yes	26 (4)	100.8 (13.2)		97.6(9.6)		98.0(8.0)		104.7(16.8)	
No	598 (96)	105.6(14.3)		100.2(12.1)		98.9(9.7)		108.8(15.6)	

***C. difficile* colonization 4m**			0.033		0.045		0.101	0.939
Yes	160 (43)	104.2(13.1)		98.9(11.4)		97.8(9.8)	108.0(16.5)	
No	210 (57)	107.6(14.5)		101.6(11.9)		99.6(9.6)	108.2(15.0)	
Breastfeeding 3 months			0.310		0.002		0.353	0.115
None	101 (16)	102.9(10.6)		98.0(10.4)		97.7(8.2)	107.2(17.8)	
Partial	171 (27)	104.4(13.3)		98.0(11.1)		98.6(9.5)	106.9(15.9)	
Exclusive	371 (57)	107.0(15.4)		101.7(12.6)		99.3(9.8)	109.9(14.9)	
Birth mode			0.0595		0.0896		0.2034	0.7610
Vaginal no IAP	339 (53)	107.0(15.1)		101.1(11.1)		99.6(8.9)	109.0(16.3)	
Vaginal IAP	148 (23)	104.4(12.4)		100.2(12.7)		98.2(9.1)	108.2(14.2)	
CS-Elective	71 (11)	101.9(14.2)		96.8(13.2)		97.1(10.9)	107.0(16.2)	
CS-Emergency	84 (13)	106.1(12.2)		99.4(12.9)		99.1(10.5)	109.7(15.3)	
Pre-pregnancy weight			0.200		0.146		0.249	0.746
Overweight	276 (44)	104.8(13.9)		99.4(11.9)		98.3(10.0)	108.5(15.5)	
Normal weight	349 (56)	106.4(14.7)		100.9(11.9)		99.3(9.0)	108.9(15.9)	
Antidepressants			0.6983		0.0210		0.5095	0.4866
Never	599 (93)	105.8 (14.5)		100.5 (11.8)		99.0 (9.3)	108.8(15.6)	
Pre and/or Postnatal Use	47 (7)	104.9(13.4)		95.8(13.0)		97.9 (11.5)	107.0(16.9)	
Gestational Age								
37 weeks+	613 (95)	106.1 (14.2)	0.0087	100.5 (11.7)	0.0165	99.2 (9.4)	0.0177	108.9 (15.7)	0.1671
Less than 37 weeks	36 (5)	99.2 (14.5)		95.2 (15.3)		95.0 (9.9)		104.8 (14.0)	
Enterotype Clusters 4 Months			0.8114		0.5419		0.2783	0.7481
Cluster 1: Proteobacteria^{+}	163 (40)	105.2(13.5)		101.2(11.1)		99.4(9.9)	107.7(15.6)	
Cluster 2: Firmicutes	142 (35)	106.3(15.2)		100.3(12.9)		98.4(9.3)	108.7(16.3)	
Cluster 3: Bacteroides	99 (25)	105.7(13.3)		99.4(10.7)		97.3(9.4)	109.2(15.6)	

Continuous factors – or β (95% CI)[b]									
Infant sleep duration	575 (89)	0.32(-0.29, 0.93)	0.304	0.32(-0.19, 0.83)	0.220	0.59(0.19, 0.99)	0.004	0.76(0.11, 1.41)	0.020
Maternal prenatal fruit intake	610 (94)	-0.46 (-1.08, 0.16)	0.143	0.37 (-0.15, 0.89)	0.162	0.15 (-0.27, 0.56)	0.479	0.55 (-0.13, 1.23)	0.115
Propionate	168 (26)	-3.31 (-5.62, -1.00)	0.005	-2.71 (-4.49, -0.92)	0.003	0.43 (-1.22, 2.08)	0.609	-1.21 (-3.88, 1.46)	0.371
Acetate	168 (26)	3.89 (1.48, 6.30)	0.002	2.46 (0.59, 4.33)	0.010	0.40 (-1.34, 2.13)	0.652	0.70 (-2.12, 3.52)	0.624
Butyrate	168 (26)	-2.80 (-5.43, -0.16)	0.038	-1.62 (-3.66, 0.42)	0.118	-1.67 (-3.51, 0.16)	0.073	-0.20 (-.32, 2.82)	0.898
Formate	139 (23)	1.91 (-0.56, 4.37)	0.128	1.99 (0.11, 3.88)	0.038	0.12 (-1.60, 1.85)	0.889	1.14 (-164, 3.94	0.417
Tryptophan	163 (25)	-1.18 (-3.68, 1.31)	0.350	-2.39 (-4.27, -0.51)	0.013	-2.19 (-3.89, -0.49)	0.012	-0.69 (-3.40, 2.03)	0.618

Note: BSID-III=Bayley Scales of Infant Third Edition; SD=standard deviation; *β=Coefficient*

[a]Analyzed by t-test or one-way analysis of variance.

[b]Analyzed by linear regression

Total number of observations (N) represents those participants with maternal distress data and cognitive data collected at the 1-year study visit

Table S8. Model diagnostics for both the fully adjusted CESD and PSS multivariable models.

Multivariable Linear Regression Model Diagnostics			
	Adjusted R^2	**Variation Explained by th Model (%)**	**Levene's Robust Test o Homogeneity**
Cognitive Scores 1 Year of Age			
CESD Model	0.0140	1.4%	p=0.449
PSS Model	0.0185	1.9%	p=0.763
Language Scores 1 Year of Age			
CESD Model	0.0425	4.3%	p=0.947
PSS Model	0.0399	4.0%	p=0.968
Motor Scores 1 Year of Age			
CESD Model	0.0193	1.9%	p=0.0660
PSS Model	0.0153	1.5%	p=0.793
Social-Emotional Scores 1 Year of Age			
CESD Model	0.0215	2.2%	p=0.379
PSS Model	0.0244	2.4%	p=0.706
Cognitive Scores 2 Years of Age			
CESD Model	0.0617	6.2%	p=0.226
PSS Model	0.0666	6.7%	p=0.966
Language Scores 2 Years of Ag			
CESD Model	0.1218	12.2%	p=0.551
PSS Model	0.1081	10.8%	**p=0.035**
Motor Scores 2 Years of Age			
CESD Model	0.0577	5.8%	p=0.752
PSS Model	0.0209	2.1%	p=0.360
Social-Emotional Scores 2 Years of Age			
CESD Model	0.0471	4.7%	p=0.572
PSS Model	0.0439	4.4%	p=0.147

Note: CESD= Center for Epidemiological Studies Depression Scale; PSS= Perceived Stress Scale. Diagnostics are for the full adjusted models presented in tables 3 and 4. The adjusted R^2 demonstrates the variance explained by the model, adjusted for the number of variables that are included. Levene's test of homogeneity tests the linear regression assumption of equality of variance (a significant p-value means there is significant inequality of variance). Indications of poor model fit are in **bold** (Levene's test $p<0.05$). The only model indicating poor fit is the PSS two-year language model in which we did not find any statistical impacts of maternal stress trajectories on two-year language scores.

Figure S1. Multivariable Linear Regression Models Examining the Impact of Maternal Depression Trajectories on Neurodevelopment Scores Stratified by Child Sex

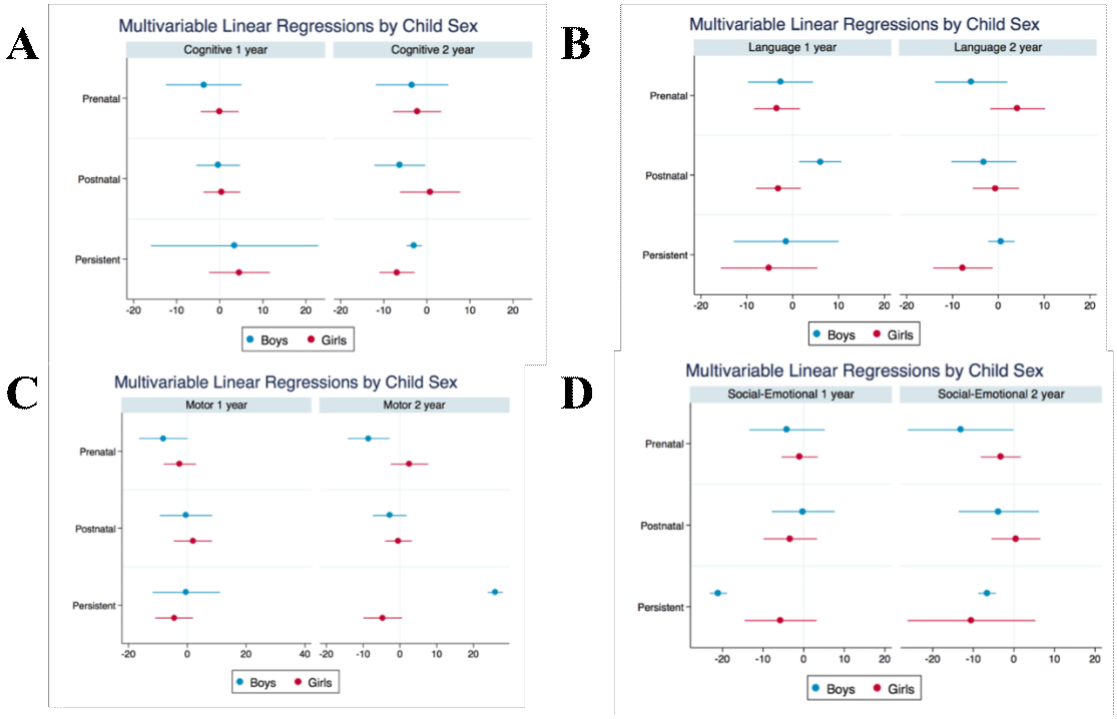

Figure S1. Multivariable regressions of the impact of maternal depression trajectories on neurodevelopment scales at 1 and 2 years of age, stratified by sex. The reference group is the never experiencing maternal distress category. These models are bootstrapped (reps 1000) and contain the same covariates as **Table __**, with the covariates not shown in the figures to focus the stratification of the impact of depression trajectories by child sex. **A**= Multivariable models of depression trajectories on cognitive scores at 1 and 2 years of age, by child sex. Both boys (Coef: -2.93; Bootstrap 95%CI: -4.71, -1.15; p=0.001) and girls (Coef: -6.94; Bootstrap 95%CI: -10.80, -3.08; p=0.001) exposed to persistent depression had lower cognitive scores at 2 years of age. Postnatal depression significantly lowered boys two-year cognitive scores (Coef: -6.30; 95%CI: -12.14, -0.45; p=0.035). **B**= Multivariable models of depression trajectories on language scores at 1 and 2 years of age, by child sex. Postnatal depression increased boy's language scores at one year of age (Coef: 6.01; Bootstrap 95%CI: 1.36, 10.67; p=0.011). Persistent lowered girls' two-year language scores (Coef: -7.70; Bootstrap 95%CI: -14.15, -1.25; p=0.019). **C**= Multivariable models of depression trajectories on motor scores at 1 and 2 years of age, by child sex. Prenatal depression decreased boy two-year motor scores (Coef: -8.47; Bootstrap 95%CI: -14.42, -2.42; p=0.005). Persistent depression increased boys two-year motor scores (Coef: 25.93, Bootstrap 95%CI: 23.87, 27.99; p=<0.001). Persistent depression marginally decreased girls two-year motor scores (Coef: -4.70; Bootstrap 95%CI: =10.02, 0.62; p=0.083). **D**= Multivariable models of depression trajectories on social-emotional scores at 1 and 2 years of age, by child sex. Prenatal depression lowered boys two-year social-emotional scores (Coef: -13.11; Bootstrap 95%CI: -25.52, -0.69; p=0.039). Persistent depression lowered boys one-year (Coef: -20.99; Bootstrap 95%CI: -23.14, -18.83; p<0.001) and two-year (Coef: -6.62; Bootstrap 95%CI: -8.78. -4.45; p<0.001) social-emotional scores.

Figure S2. Multivariable Linear Regression Models Examining the Impact of Maternal Stress Trajectories on Neurodevelopment Scores Stratified by Child Sex.

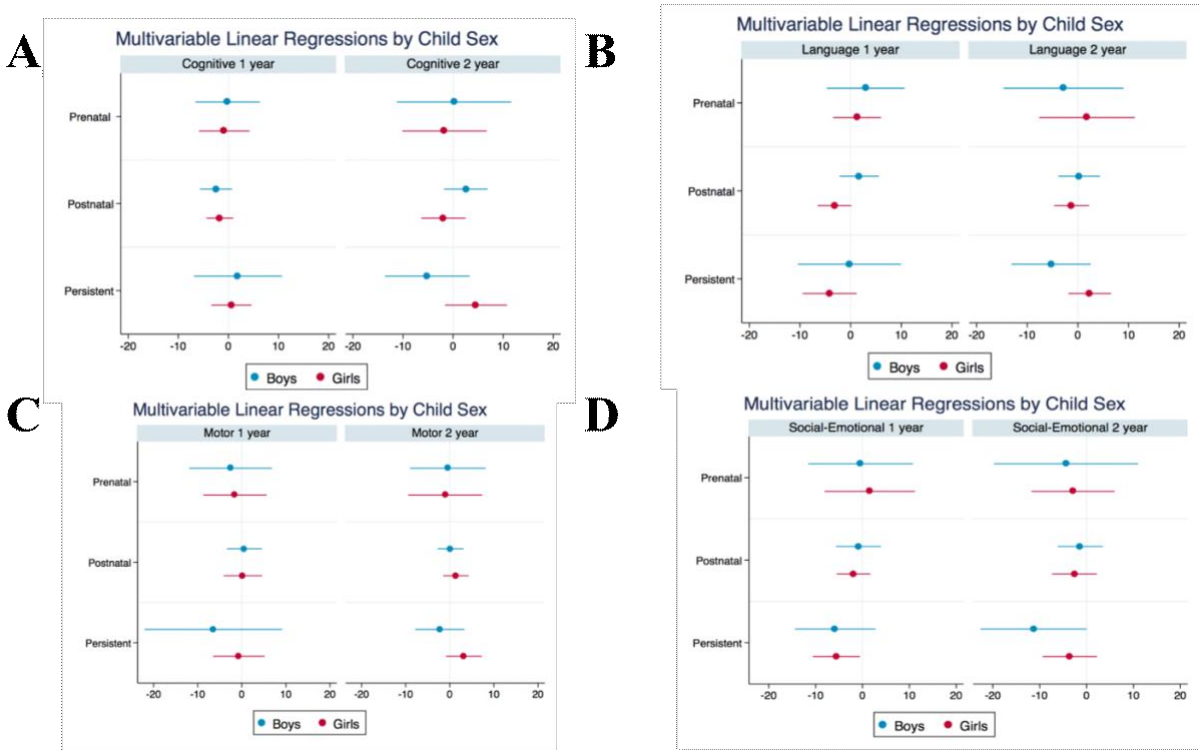

Figure 2. Multivariable regressions of the impact of maternal stress trajectories on neurodevelopment scales at 1 and 2 years of age, stratified by sex. These models are bootstrapped (reps 1000) and contain the same covariates as **Table __**, with the covariates not shown in the figures to focus the stratification of the impact of depression trajectories by child sex. The reference group is children with mothers that did not experience perinatal stress. There were no significant associations among maternal stress trajectories and neurodevelopment scores when stratified by child sex, with the exception of a few marginal trends. **A**= Multivariable models of stress trajectories on cognitive scores at 1 and 2 years of age, by child sex. **B**= Multivariable models of stress trajectories on language scores at 1 and 2 years of age, by child sex. Postnatal stress marginally decreased girl's language scores at one-year (Coef: -3.17; Bootstrap 95%CI: -6.47, 0.13; p=0.059). **C**= Multivariable models of stress trajectories on motor scores at 1 and 2 years of age, by child sex. **D**= Multivariable models of stress trajectories on social-emotional scores at 1 and 2 years of age, by child sex. Persistent stress significantly decreased girls social-emotional scores at one-year of age (Coef: -5.57, Bootstrap 95%CI: -10.38, -0.75; p=0.023), with this finding marginal in boys at two years of age (Coef: -11.31, Bootstrap 95%CI: -22.86, 0.23; p=0.055).

Table S9. Sensitivity analyses for the mediation indirect effects after addition of adjustment for age of stool sample.

Mediator	BSID-III Scale	Indirect pathway	Indirect effects of fully adjusted model Coef (Bootstrap 95%CI)	Indirect effects of fully adjusted model adding adjustment of age of stool sample Coef (Bootstrap 95%CI)
C. difficile abundance				
	Cognitive 2 year	Exclusive breastfeeding duration → *C. difficile* abundance → cognitive scores 2 years	0.14 (0.03, 0.26)	0.16 (0.05, 0.27)
	Language 2 year	Exclusive breastfeeding duration → *C. difficile* abundance → language scores 2 years	0.09 (0.02, 0.15)	0.09 (0.03, 0.16)
	Motor 2 year	Exclusive breastfeeding duration → *C. difficile* abundance → motor scores 2 years	0.08 (0.02, 0.14)	0.08 (0.02, 0.14)
Acetate abundance				
	Cognitive 2 year	Exclusive breastfeeding duration → acetate abundance → cognitive scores 2 years	0.31 (0.02, 0.61)	0.34 (0.04, 0.63)
	Language 2 year	Exclusive breastfeeding duration → acetate abundance → language scores 2 years	0.15 (0.0008, 0.30)	0.15 (-0.001, 0.29)

Note: BSID-III= Bayley Scale of Infant Development Third Edition; CI=Confidence Interval
Sensitivity analyses exploring whether age of stool sample collection (Mean=4.2, SD=1.2) affected the sequential mediation analyses. The indirect path from prenatal CESD scores on neurodevelopment scales were not significant in the full models; therefore, the significant indirect paths from breastfeeding to neurodevelopment scales were tested. The fully adjusted models (not sex-specific) were tested and were adjusted for maternal education (SES), maternal age, and maternal BMI scores. Sensitivity analyses consist of the full sample mediation models and not the sex-specific mediation models.

Table S10. Frequency characteristics for variables in the study sample of infants with *C. difficile* colonization data (*n*=370)

	Full CHILD Cohort (N=3472)	Study sub-sampl (n=646)	*C. difficile* sul sample (n=37(	Metabolite sub-sample (n=168)
Categorical variables	n (%)	n(%)	n (%)	n (%)
CESD	N=3321	N=646	N=370	N=156
Never	2846 (86)	545 (84)	320 (87)	130 (83)
Prenatal	178 (5)	45 (7)	19 (5)	15 (10)
Postnatal	222 (7)	44 (7)	25 (7)	9 (6)
Persistent	75 (2)	12 (2)	6 (2)	2 (1)
PSS	N=3321	N=646	N=370	N=156
Never	2164 (65)	444 (69)	260 (70)	106 (68)
Prenatal	148 (5)	29 (5)	15 (4)	6 (4)
Postnatal	766 (23)	133 (21)	76 (21)	36 (23)
Persistent	243 (7)	40 (6)	19 (5)	8 (5)
Child Sex	N=2534	N=646	N=370	N=168
Boys	1347 (53)	327 (51)	189 (51)	91 (54)
Girls	1187 (47)	319 (49)	181 (49)	77 (46)
Family income	N=2894	N=585	N=340	N=152
Less than 39,999	263 (9)	36 (6)	16 (5)	4 (3)
40,000 to 79,999	686 (24)	145 (25)	88 (24)	41 (27)
80,000 to 99,999	424 (15)	94 (16)	56 (15.1)	23 (15)
Exceeds 100,000	1521 (53)	310 (53)	180 (49)	84 (55)
Maternal Education	N=3196	N=621	N=329	N=163
Some/finished high school	185 (9)	48 (8)	24 (7.3)	15 (9.2)
Some university/college	919 (39)	235 (38)	145 (44.1)	69 (42.3)
University degree	1992 (62)	338 (54)	190 (57.8)	79 (48.5)
Maternal Age	N=3388	N=646	N=359	N=168
18 to 29	846 (25)	193 (29)	107 (28.9)	50 (29.8)
30 to 39	2297 (68)	427 (66)	245 (66.2)	115 (68.5)
Over 40	245 (7)	26 (4)	18 (4.9)	3 (1.8)
Prenatal smoking	N=3297	N=624	N=362	N=163
Yes	127 (4)	26 (4)	35 (9.7)	19 (11.7)
No	3062 (93)	598 (96)	327 (90.3)	144 (88.3)
Breastfeeding 3 months	N=3154	N=646	N=370	N=168
None	447 (14)	101 (16)	70 (19)	45 (27)
Partial	921 (26)	171 (27)	116 (31)	55 (33)
Exclusive	1886 (59)	371 (57)	184 (50)	68 (41)
Birth mode	N=3209	N=642	N=365	N=153
Vaginal no IAP	1696 (53)	339 (53)	206 (56)	80 (52)
Vaginal IAP	699 (22)	148 (23)	74 (20)	39 (26)
CS-Elective	347 (11)	71 (11)	38 (10)	18 (12)
CS-Emergency	467 (15)	84 (13)	47 (13)	16 (11)
Pre-pregnancy weight	N=2302	N=625	N=332	N=162
Overweight	916 (40)	276 (44)	198 (51.0)	84 (51.8)
Normal weight	1386 (57)	349 (56)	163(49.0)	78 (48.2)
Antidepressants	N=3472	N=646	N=368	N=167
Never	3309 (95)	599 (93)	366 (91.3)	155 (92.8)
Pre and/or Postnatal Use	163 (5)	47 (7)	32 (8.7)	12 (7.2)
***C. difficile* colonization 4 months**	N=1564	N=370	N=370	N=150

Yes	484 (31.0)	160 (43)	160 (43.2)	80 (53.3)
No	1080 (69.0)	210 (57)	210 (56.8)	70 (46.7)
Gestational Age	N=3222	N=646	N=160	N=156
37 weeks+	3086 (96)	610 (94)	152 (95.0)	146 (93.6)
Less than 37 weeks	136 (4)	36 (6)	8 (5.0)	10 (6.4)
Enterotype Clusters 4 Months	N=414*	N=404	N=367	N=84
Cluster 1: Proteobacteria^{+}	170 (41.0)	163 (40)	158 (43.0)	36 (42.9)
Cluster 2: Firmicutes	144 (42.5)	142 (35)	120 (32.7)	27 (32.1)
Cluster 3: Bacteroides	100 (24.2)	99 (24)	89 (24.3)	21 (25.0)
	Full CHILD Cohort (N=3472)	Study sub-sample (n=646)	*C. difficile* sul sample (n=37(	Metabolite sub-sample (n=168)
Continuous variables	Mean(SD)	Mean(SD)	Mean (SD)	Mean(SD
Maternal pregnancy fruit intake	N= 3052	N=610	N=354	N=156
	3.27 (2.12)	3.22 (2.19)	3.40 (2.45)	3.38 (2.18)
Infant sleep duration	677*	575	363	167
	14.12 (2.14)	14.23 (2.09)	14.2 (2.09)	14.3 (2.30)

Note: BSID-III= Bayley Scales of Infant Development; CESD= Center for Epidemiological Studies Depression Scale; CS=caesarean section; PSS= Perceived Stress Scale; SD=standard deviation
*=Infant sleep was only collected at the Edmonton CHILD site and enterotype cluster analysis was only completed on infants with microbiota data and BSID-III scores which is also exclusive to the Edmonton CHILD site.

Table S11. Mediation sub-sample comparisons of neurodevelopment scores.

BSID-III Scores	Study Sample (N=646) Mean (SD)	*C. difficile* sub-sample (N=370) Mean (SD)	Metabolite sub-sample (N=170) Mean (SD)
BSID-III cognitive 1 year	110.1 (10.3)	110.2 (10.2)	110.0 (10.0)
BSID-III language 1 year	108.2 (12.3)	108.6 (12.3)	108.1 (12.9)
BSID-III motor 1 year	103.5 (14.6)	103.6 (16.2)	101.4 (15.9)
BSID-III social-emotional 1 year	102.7 (13.9)	102.5 (13.2)	100.9 (13.4)
BSID-III cognitive 2 year	105.7 (14.3)	106.0 (13.9)	107.4 (15.4)
BSID-III language 2 year	100.2 (12.0)	100.4 (11.8)	100.5 (12.2)
BSID-III motor 2 year	98.9 (9.5)	98.8 (9.8)	99.2 (9.6)
BSID-III social-emotional 2 year	108.7 (15.7)	108.1 (15.7)	109.8 (15.2)

Mean comparisons of neurodevelopmental scores between the *C. difficile* sub-sample and the metabolite sub-sample.

Table S12. Summary of potential mediators that were further tested in SEM models.

Model with Prenatal Depression Exposure (X)				
Potential Microbial Mediator (M)	BSID-III Scale (Y)	X→Y Estimate (95%CI)	X→M Estimate (95%CI)	M→Y Estimate (95%CI)
C. difficile abundance(N=370)			1.43 (0.18, 2.69) p=0.025*	
	Cognitive 2 year	-2.74 (-7.43, 1.95) p=0.251		-0.54 (-1.03, -0.05) p=0.031*
	Language 2 year	-1.65 (-5.57, 2.26) p=0.407		-0.44 (-0.86, -0.03) p=0.034*
	Motor 2 year	-0.85 (-3.95, 2.25) p=0.589		-0.36 (-0.71, -0.02) p=0.037*
Acetate abundance (N=168)			-0.10 (0.01, 0.19) p=0.030*	
	Cognitive 2 year	-0.11 (-0.29, 0.06) p=0.211		3.89 (1.48, 6.29) p=0.002*
	Language 2 year	-0.08 (-0.23, 0.06) p=0.260		2.46 (0.59, 4.33) p=0.010*

*=p-value<0.05

Summary table of the potential gut microbiome mediators that had significant prenatal depression → potential microbial mediators (X→M) and potential microbial mediators to neurodevelopmental outcome (M→Y) associations. This is a summary of the two microbial mediators that had significant (p<0.10) X→M and M→Y associations that were further tested in the SEM models for indirect effects. There were no associations from prenatal stress on potential microbial mediators.

Table S13. Percent effect explained for the mediation models.

Mediation Model	Indirect Effect Coefficient	Total Effect Coefficient (Indirect +Direct effects)	Percent Effect Explained
Full models			
Acetate models			
A	0.3144	0.8765	35.9%
B	0.1472	1.3993	10.5%
C. difficile models			
C	0.1486	0.8194	18.1%
D	0.0881	1.3631	6.5%
E	0.0782	0.4222	18.5%
Sex-specific models			
Acetate models			
A2	0.6252	1.3929	44.9%
B2	0.2487	1.1615	-
B3	-0.0081	-0.0111	-
C. difficile models			
C1	-0.0251	-0.1346	18.6%
C2	0.0541	0.3052	17.7%
D1	-0.0364	-0.1870	19.4%
D2	0.0886	1.7383	5.1%
E	0.1180	-0.2049	57.6%

Percent of the indirect effects refers to how much of the direct association from the exposure to the outcome can be explained through the indirect effect. The percent explained by the indirect effect is calculated by dividing the indirect effect coefficient by the total effect coefficient. Absolute values were taken to produce the percent effect. The indirect effects within the acetate and two-year language score models (B2 and B3) were marginal and percent effects were only calculated for significant indirect effects.

Table S14. Structural equation model fit.

Mediation Model	$\chi 2$ (p-value)	RMSEA (90% CI)	pclose	AIC	CFI	SRMR	CD
Full models							
Acetate models							
A	**9.537 (0.049)**	**0.112** (0.007, 0.205)	0.111	4287	**0.674**	0.043	0.107
B	**9.861 (0.043)**	**0.115** (0.019, 0.209)	0.099	4186	**0.689**	0.045	0.129
C. difficile models							
C	2.115 (0.347)	0.015 (0.0, 0.125)	0.552	10690	0.997	0.019	0.105
D	2.046 (0.360)	0.009 (0.0, 0.124)	0.563	10553	0.999	0.018	0.116
E	2.115 (0.347)	0.015 (0.0, 0.125)	0.552	10534	0.995	0.019	0.089
Sex-specific models							
Acetate models							
A	10.629 (0.224)	**0.077** (0.0, 0.186)	-	4304	**0.618**	0.044	0.163
B	10.995 (0.202)	**0.083** (0.0, 0.190)	-	4194	**0.603**	0.046	0.163
C. difficile models							
C	4.107 (0.392)	0.014 (0.0, 0.134)	-	10671	0.997	0.023	0.169
D	3.987 (0.408)	0.0 (0.0, 0.132)	-	10529	1.0	0.023	0.174
E	4.107 (0.392)	0.014 (0.0, 0.134)	-	10520	0.996	0.023	0.150

AIC, Akaike information criterion; CD, coefficient of determination; CFI, comparative fix index; RSMEA, root mean square error of approximation; SRMR, standardized root mean residuals. No pclose is available for multiple groups when stratifying models. Indications of **lower model fit are in bold**: $\chi 2$ p-value <0.05, CFI <0.9, RMSEA > 0.05, and SRMR>0.08, pclose<0.05. Poor model fit within the acetate mediations (models A and B) can potentially be attributed to small sample size.

Table S15. Summary of marginal and significant direct effects among mediation models from covariates.

Mediation Model	Child Sex	Direct effect Estimate (95%CI)	p-value
Full models			
Acetate models			
A			
Maternal education → Prenatal depression scores	-	-0.63 (-1.09, -0.19)	0.005
B			
Maternal education → Prenatal depression scores	-	-0.63 (-1.07, -0.18)	0.006
C. difficile models			
C			
Maternal education → Prenatal depression scores	-	-0.41 (-0.69, -0.14)	0.003
Pre-pregnancy BMI → Prenatal depression scores	-	0.16 (0.03, 0.29)	0.015
D			
Maternal education → Prenatal depression scores	-	-0.41 (-0.66, -0.15)	0.002
Pre-pregnancy BMI → Prenatal depression scores	-	0.16 (0.03, 0.28)	0.015
Maternal education → Language scores	-	0.74 (0.27, 1.20)	0.002
E			
Maternal education → Prenatal depression scores	-	-0.41 (-0.67, -0.15)	0.002
Pre-pregnancy BMI → Prenatal depression scores	-	0.16 (0.02, 0.30)	0.021
Sex-specific models			
Acetate sex-specific models			
A			
Maternal education → Prenatal depression scores	Girls	-0.98 (-1.73, -0.22)	0.012
B			
Maternal education → Prenatal depression scores	Girls	-0.95 (-1.71, -0.19)	0.014
C. difficile sex-specific models			

C			
Maternal education → Prenatal depression scores	Girls	-0.62 (-1.08, -0.16)	0.009
Pre-pregnancy BMI → Prenatal depression scores	Girls	0.27 (0.06, 0.48)	0.013
Pre-pregnancy BMI → Cognitive scores	Girls	-0.85 (-1.54, -0.16)	0.016
Maternal age→ Cognitive scores	Girls	-0.71 (-1.20, -0.22)	0.004
D			
Maternal education → Prenatal depression scores	Girls	-0.61 (-1.07, -0.14)	0.011
Pre-pregnancy BMI → Prenatal depression scores	Girls	0.26 (0.05, 0.47)	0.016
Pre-pregnancy BMI → Language scores	Girls	0.42 (0.14, 0.72)	0.004
Maternal education → Language scores	Girls	0.70 (0.04, 0.135)	0.037
Maternal age → Language scores	Girls	-0.41 (-0.80, -0.03)	0.036
Pre-pregnancy BMI → Exclusive breastfeeding duration	Boys	-0.06 (-0.11, -0.009)	0.022
E			
Maternal education →Prenatal depression scores	Girls	-0.62 (-1.08, -0.16)	0.009
Pre-pregnancy BMI → Prenatal depression scores	Girls	0.27 (0.06, 0.48)	0.013
Pre-pregnancy BMI → Motor scores	Girls	0.26 (0.09, 0.64)	0.009
Pre-pregnancy BMI → Exclusive breastfeeding duration	Boys	-0.06 (-0.11, -0.009)	0.022

Table S16. Summary of the crude linear regression associations from the infant gut microbiome on neurodevelopmental scores stratified by child sex.

Crude Associations of the Gut Microbiome on Neurodevelopment Scores by Child Sex					
Potential Mediator (M)	BSID-III Scale (Y)	**Boys** Estimate (95%CI)	**p-value**	**Girls** Estimate (95%CI)	**p-value**
Tryptophan concentration					
	Motor 1 year	4.93 (2.19, 7.68)*	<0.001	0.65 (-3.40, 4.70)	0.749
	Language 2 year	-0.82 (-3.46, 1.81)	0.536	-3.81 (-6.24, -1.37)*	0.003
	Motor 2 year	-1.26 (-3.75, 1.23)	0.316	-3.10 (-5.44, -0.77)*	0.010
Formate abundance					
	Cognitive 1 year	3.49 (0.37, 6.61)*	0.029	0.21 (-1.89, 2.33)	0.840
	Language 2 year	1.62 (-1.81, 5.05)	0.349	1.38 (-0.78, 3.54)	0.207
Butyrate abundance					
	Cognitive 2 year	-1.03 (-4.27, 2.20)	0.526	-5.57 (-10.40, -0.75)*	0.024
	Motor 2 year	-0.72 (-3.08, 1.64)	0.544	-3.17 (-6.38, 0.04)*	0.053
Propionate abundance					
	Cognitive 2 year	-2.61 (-5.57, 0.35)*	0.083	-3.92 (-7.75, -0.09)*	0.045
	Language 2 year	-2.25 (-4.54, 0.04)*	0.054	-2.28 (-5.05, 0.49)	0.105
Enterotype clusters rank at 4 months					
	Cognitive 1 year	-0.75 (-2.75, 1.26)	0.465	-1.68 (-3.49, 0.12)*	0.068

Summary of gut microbiome four-month crude associations on neurodevelopment stratified by child sex.
*=p-value<0.05
*=p-value between 0.05 and 0.10

Table S17. Correlations among neurodevelopmental scores and gut microbiome variables.

	Acetate	Butyrate	Formate	Propionate	Tryptophan	*C. difficile*	Clusters 4m	Cognition 1y	Language 1y	Motor 1y	Social Emotion 1y	Cognition 2y	Language 2y	Motor 2y	Social Emotion 2y
Acetate	-														
Butyrate	0.593*	-													
Formate	-0.0200	-0.154*	-												
Propionate	-0.732*	0.002	-0.270*	-											
Tryptophan	-0.245*	0.172*	-0.095	0.220*	-										
C. difficile	-0.102	0.093	-0.178	0.106	0.035	-									
Clusters 4m	0.002	0.126	-0.222*	-0.004	-0.099	0.120*	-								
Cognitive 1y	-0.085	0.055	0.128	-0.007	0.060	-0.080	-0.088	-							
Language 1y	0.001	-0.023	0.012	0.004	0.097	0.020	-0.033	0.415*	-						
Motor 1y	-0.068	0.077	0.104	-0.021	0.173*	-0.041	-0.071	0.415*	0.442*	-					
Social-emotional 1y	0.002	0.044	-0.007	-0.016	0.021	0.010	-0.0002	0.144*	0.108*	0.076	-				
Cognitive 2y	0.261*	-0.175*	0.129	-0.234*	-0.234*	-0.121*	-0.020	0.139*	0.166*	0.097*	0.021	-			
Language 2y	0.216*	-0.133	0.176*	-0.247*	-0.247*	-0.119*	-0.024	0.174*	0.299*	0.128*	0.091*	0.697*	-		
Motor 2y	0.038	-0.151	0.012	0.044	-0.044	-0.117*	-0.032	0.024	0.044	0.056	0.024	0.514*	0.478*	-	
Social-emotional 2y	0.042	-0.011	0.070	-0.077	-0.076	0.045	0.009	0.119*	0.106*	0.031	0.408*	0.183*	0.323*	0.153*	-

*= $p<0.05$

Correlation matrix amongst neurodevelopmental scores at one and two years of age and gut microbiome mediators (SCFA abundance, tryptophan concentration*, C. difficile* abundance, and enterotype cluster ranks).

Table S18. Study population characteristics for participants with Bayley Scales of Infant Development (BSID-III) data at 1 year (N=646) compared to 2 years (N=541), missing data for 105 (16.3%).

Categorical variables	BSID-III data present at 1 year (N=646) n(%)	BSID-III data present at 2 years (n=541) n(%)
CESD		
Never	545 (84.4)	466 (85.7)
Prenatal	45 (6.9)	31 (5.7)
Postnatal	44 (6.8)	39 (7.2)
Persistent	12 (1.9)	8 (1.5)
PSS		
Never	444 (68.7)	383 (70.4)
Prenatal	29 (4.5)	21 (3.86)
Postnatal	133 (20.6)	107 (19.7)
Persistent	40 (6.2)	33 (6.1)
Child Sex		
Boys	327 (50.6)	282 (51.8)
Girls	319 (49.4)	262 (48.2)
Family income		
Less than 39,999	36 (6.2)	26 (5.3)
40,000 to 79,999	145 (24.8)	122 (24.7)
80,000 to 99,999	94 (16.0)	82 (16.6)
Exceeds 100,000	310 (53.0)	165 (53.5)
Maternal Education		
Some/finished high school	48 (7.7)	36 (6.9)
Some university/college	235 (37.8)	195 (37.4)
University degree	338 (54.4)	291 (55.8)
Maternal Age		
18 to 29	193 (28.9)	151 (27.8)
30 to 39	427 (66.1)	372 (36.4)
Over 40	26 (4.0)	21 (3.9)
Prenatal smoking		
Yes	26 (4.2)	52 (9.9)
No	598 (95.8)	474 (90.1)
Breastfeeding 3 months		
None	101 (15.6)	78 (14.4)
Partial	171 (26.5)	140 (27.5)
Exclusive	371 (57.4)	315 (58.1)
Birth mode		
Vaginal no IAP	339 (52.8)	280 (51.2)
Vaginal IAP	148 (23.0)	130 (24.1)
CS-Elective	71 (11.1)	58 (10.8)
CS-Emergency	84 (13.1)	71 (13.2)
Pre-pregnancy weight		
Overweight	276 (44.2)	236 (43.9)
Normal weight	349 (55.8)	301 (56.1)
Antidepressants		
Never	599 (92.7)	504 (93.0)
Pre and/or Postnatal Use	47 (7.3)	38 (7.0)

***C. difficile* colonization 4 months**		
Yes	160 (43.2)	145 (45.7)
No	210 (56.8)	172 (54.3)
Gestational Age		
37 weeks+	610 (94.4)	513 (94.3)
Less than 37 weeks	36 (5.6)	31 (5.7)
Continuous variables	Mean(SD)	Mean(SD)
Maternal pregnancy fruit intake	3.22(2.2)	3.16 (2.0)
Infant sleep duration	14.23 (2.1)	14.19 (2.1)

Comparison of sample characteristics between children with neurodevelopment scores at one-year of age compared to children that had scores at two-years of age.