Impact of Pre-eclampsia and Emergency Cesarean Section without Labour on *Bifidobacterium* Levels in Infant Gut Microbiota

by

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Abstract

Background:

Pre-eclampsia affects 8-10% of all pregnancies, resulting in significant maternal-fetal morbidity, including fetal distress, abnormal fetal heart rate, and placental abruption. These conditions also increase the risk of emergency cesarean delivery for pre-eclamptic women[1] before a trial of labour. Furthermore, pre-eclamptic women face a higher likelihood of experiencing premature rupture of membranes (PROM), which has negative consequences for both mothers and infants. Recent findings have indicated that there are distinct gut microbiota changes in groups with pre-eclampsia compared to healthy control groups, thus confirming the potential link between dysbiosis of the gut microbiota and the occurrence of pre-eclampsia. However, there is lack of knowledge regarding gut microbiota alterations in infants born to pre-eclamptic mothers. Since critical gut microbiota and immune system interactions can be affected by microbial dysbiosis resulting from maternal health conditions, cesarean birth, and other birth events, the current study aimed to investigate the association between pre-eclampsia and the levels of key microbiota, *Bifidobacterium*, in the infant gut at 3 months of age. Additionally, this study aimed to explore whether this association is independent of emergency cesarean delivery without labour and premature rupture of membranes (PROM), as well as to determine if these factors mediate the observed association.

Methods:

This was a prospective cohort study utilizing data on 1429 mother-infant pairs, longitudinally from the Canadian Healthy Infant Longitudinal Development (CHILD) population-based birth cohort.

[1] In this study, the term 'women' is utilized to refer to pregnant persons.

Data on delivery mode, pre-eclampsia, PROM, breastfeeding status, maternal pre-pregnancy BMI and other maternal and infant covariates were collected from hospital records or standard maternal questionnaires. Infant fecal samples, collected at 3-4 months of age, were profiled by qPCR for levels of total *Bifidobacterium*. Fisher's exact tests was employed to assess crude unadjusted associations between maternal and infant characteristics with pre-eclampsia, emergency cesarean section without labour, and *Bifidobacterium* levels. To enhance data normality, *Bifidobacterium* values underwent Box-Cox transformation before logistic regression analysis. Simple and multiple logistic regressions (using STATA version 17) were utilized to identify the impact of pre-eclampsia on *Bifidobacterium* levels in the infant gut microbiome, while considering adjustments for potential confounding variables as suggested by the Directed Acyclic Graph (DAG). Additionally, mediation analyses were conducted using the Hayes PROCESS v3.5 macro in SPSS (version 26, SPSS Inc., Chicago, IL, USA) to examine whether emergency cesarean delivery without labour and premature rupture of membranes (PROM) are in the pathway between pre-eclampsia and *Bifidobacterium* levels in infant.

Results:

In our study, we observed a significant inverse association between pre-eclampsia and *Bifidobacterium* levels in the gut microbiota of infants at 3 months of age in the crude logistic analysis (βc: -1.48, 95% CI: -2.9, -0.05, $p < 0.05$). However, after adjusting for potential confounding variables, this association lost its significance (βa: 0.31, 95% CI: -1.28, 1.90, $p > 0.05$). Furthermore, our investigation into the mediation process using simple and sequential analyses did not fully support the hypothesis that emergency cesarean delivery without labour and premature rupture of membranes (PROM) act as mediators between pre-eclampsia and *Bifidobacterium* levels in the infant gut microbiota. However, when examining the role of birth

mode as a mediator, our mediation analyses revealed a significant inverse association between cesarean section (elective and emergency CS with and without labour) and *Bifidobacterium* levels in the infant gut microbiota at 3 months of age. Additionally, we found similar results in the second mediation model, indicating that rupture of membranes has an inverse impact on *Bifidobacterium* levels in the infant gut.

Conclusion:

To summarize, our findings indicate that infants born to mothers with pre-eclampsia had decreased levels of *Bifidobacterium* in their gut at 3 months of age. However, it is important to note that the statistical significance of this association was not maintained after adjusting for potential confounding variables due to the limited sample size. Our study also revealed that cesarean section including elective and emergency CS with and without labour, had an inverse influence on *Bifidobacterium* levels, aligning with previous research findings. Furthermore, we observed an inverse association between rupture of membranes and *Bifidobacterium* levels, which warrants further investigation.

Preface

This is an original work done by Maryam Paraktoon. The thesis adhered to the guidelines established by the Faculty of Graduate Studies and Research at the University of Alberta.

Chapter 1: comprises a literature review that focuses on understanding pre-eclampsia, its impact on both mothers and infants, the connection between pre-eclampsia and the human gut microbiota, and the importance of early-life gut microbiota development. Additionally, it addresses the gaps in the existing literature, presents the study objectives, hypotheses, and provides details on the sample size calculation.

Chapter 2: comprises the materials and methods section, which defines all the potentially relevant variables associated with pre-eclampsia and *Bifidobacterium* levels in the infant gut microbiota at 3 months of age. Additionally, it covers the study design, statistical analysis, mediation analysis and qPCR methods.

Chapter 3: presents the findings of the analyses. In this chapter, the findings are presented through descriptive tables, simple and multivariable logistic regression tables, as well as simple and sequential mediation analysis models.

Chapter 4: covers the discussion and interpretations of the findings, along with the exploration of strengths and limitations, clinical importance, bias and confounding, and implications for future studies, ultimately leading to the conclusion.

Dedication

I would like to dedicate my thesis to my mother, who serves as my moral role model, constantly encouraging and supporting me in every step of life. Mom, I love you for your endless love and all the support you have provided throughout my life.

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I would like to express my heartfelt gratitude to my supervisor, Dr. Anita Kozyrskyj, who has been an incredible mentor and source of inspiration throughout my academic journey. As a newcomer to Canada, her endless support, kindness, and expertise have had a profound impact not only on my academic pursuits but also on my personal growth. Without her guidance, patience, and belief in my abilities, this thesis would not have been possible. Thank you so much for being an extraordinary supervisor.

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as potential mediators. Beta-coefficient with 95% confidence interval of each path exhibits and significant differences ($p < 0.05$) are indicated in red.

CHAPTER 1

1. Introduction

1.1 Literature Review

1.1.1 Hypertensive Disorders of Pregnancy

Hypertensive disorder of pregnancy (HDP) is one of the most common pregnancy complications, creating a death triad with hemorrhaging and infection, and refers to a group of conditions defined by high blood pressure (1). The incidence of hypertensive disorders of pregnancy varies from 4 to 25%, and it makes a significant contribution to maternal and perinatal mortality(2,3). The American College of Obstetricians and Gynecologists (ACOG) characterises hypertension in pregnancy as systolic blood pressure higher than or equal to 140 mmHg and/or diastolic blood pressure over or equal to 90 mmHg on two different occasions at least 6 hours apart after twenty weeks of gestation for pregnancy induced hypertension or before 20 weeks of gestation for chronic hypertension (4,5). The American Congress of Obstetricians and Gynecologists guidelines on hypertensive disorders complicating pregnancy published in 2013 divided HDP into four classifications: gestational hypertension, pre-eclampsia/eclampsia, chronic hypertension, and chronic hypertension superimposed pre-eclampsia/eclampsia (3). Furthermore, the World Health Organization (WHO) indicated that hypertensive disorders of pregnancy are responsible approximately 14.0% of global maternal mortality (6). Hypertensive disorders of pregnancy were responsible for 25.7% of maternal deaths in Latin American and Caribbean nations; 9.1% of maternal mortality in Asian and African countries, and around 16% in Sub-Saharan African countries. Hypertension in pregnancy is a global public health hazard in both developed and

developing countries. However, the risk of a developing-country woman dying from the complications of hypertensive disorders of pregnancy is roughly 300 times greater than that of a developed-country woman (5,7,8).

1.1.2 The Classification and Definition of the Hypertensive Disorders of Pregnancy

The American College of Obstetrics and Gynecology (ACOG) divided pregnancy hypertension into four categories: chronic hypertension, gestational hypertension, pre-eclampsia, and chronic hypertension with superimposed pre-eclampsia (9).

Definition:

"Chronic hypertension (cHTN)" is characterized as having an elevated systolic blood pressure (SBP) of 140 mmHg and/or diastolic blood pressure (DBP) of 90 mmHg prior to pregnancy or detected before 20 weeks of gestation (9).

"Gestational hypertension (GH)" is persistent hypertension that emerges at or after 20 weeks gestation in the absence of any maternal or fetal pre-eclampsia symptoms; and hypertension normalises by 12 weeks postpartum (10). The earlier the gestational hypertension is detected, the more severe the hypertension is and the more likely the woman would develop pre-eclampsia or have a poor pregnancy outcome. Additionally, severe hypertension (170/110 mmHg) is linked with a greater likelihood of serious pregnancy outcomes (11,12).

"Pre-eclampsia" is characterized by the new onset of hypertension along with one or more of the following new-onset conditions at or after 20 weeks of gestation; however, all signs will resolve by 12 weeks postpartum (10,13).

1. Proteinuria (> 0.3 g/24 hours).

2. Maternal organ dysfunctions including:

a) Liver involvement in the absence of any basic disease (elevated transaminases, e.g., ALT or AST > 40 IU/l with or without serious persistent right upper quadrant or epigastric abdominal pain which cannot be recognised as other diseases and is treatment resistant)

b) Progressive kidney impairment (creatinine level greater than 1.0 mg/dl, other renal disorders excluded)

c) Stroke and other neurological dysfunction (including clonus, eclampsia, visual field disorder, severe headaches except for primary headache etc.)

d) Haematological consequences (thrombocytopenia because of HDP - platelet count less than 150,000/L, DIC, hemolysis)

3. Uteroplacental impairment (fetal growth restriction, stillbirth)

"Chronic hypertension with superimposed pre-eclampsia" takes place while women who have chronic high blood pressure prior to pregnancy develop worse high blood pressure, protein in the urine, or other health complications of pregnancy (12).

1.1.3 Pre-eclampsia (PE) and its Classification

Pre-eclampsia, a human-pregnancy-specific disease, is the most common medical complication of pregnancy, with an increasing global incidence (14). Pre-eclampsia occurs in approximately 2-8% of all pregnancies and is associated with significant maternal mortality and morbidity and affects about 50,000 deaths worldwide yearly (15). Thus, decreasing maternal mortality and morbidity has become one of the most important goals of the World Health Organization (WHO) in recent years. The International Society for the Study of Hypertension in Pregnancy (ISSHP) revised its

guideline in 2014 and provided an update indicating that pre-eclampsia as the new onset of hypertension (systolic BP≥140 mm Hg and/or diastolic BP ≥ 90 mm Hg conducted at least 4-6 hours apart) at or after the 20 weeks of gestation combined with evidence of maternal organ dysfunction, which includes new-onset proteinuria >300mg/day or other evidence of renal impairment, haematological consequences such as thrombocytopenia, liver dysfunction, or neurological complications including visual disorders, and evidence of uteroplacental impairment such as fetal growth restriction (16,17). This definition has been reviewed and confirmed by both the American College of Obstetricians and Gynecologists (ACOG) and the United States Preventive Services Task Force (USPSTF) (18). In recent years, it has become evident that pre-eclampsia could be classified into several separate categories. In addition to the differences between pre-eclampsia, gestational hypertension, and pre-eclampsia superimposed on chronic hypertension, pre-eclampsia can be classified as either with or without severe symptoms, based on blood pressure, clinical observations, and the level of proteinuria (13). Mild pre-eclampsia is described as blood pressure (BP) of 140/90 mm Hg or above, with proteinuria ranging from 0.3 to 3 g/day. Severe pre-eclampsia, on the other hand, includes blood pressure greater than 160/110 mmHg, proteinuria of 3 to 5 g/day, platelet count less than 100,000/L, liver enzymes elevated to twice normal concentrations, persistent right upper quadrant pain stating severe liver involvement, serum creatinine higher than 1.1mg/dL or double than the normal serum creatinine, pulmonary edema, and visual dysfunction (19). Furthermore, one unique combination of severe characteristics is known as HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets). HELLP syndrome is related to a higher perinatal risk and accounts for 15.1% of individuals diagnosed with severe pre-eclampsia (20). This classification as a subtype of pre-eclampsia aids in the diagnosis of HELLP syndrome and distinguishes it from thrombotic thrombocytopenic purpura by lactate

assessment (20). Another key factor in pre-eclampsia diagnosis is the distinction between early and late onset (before or after 34 weeks of gestation) (19). Based on the data acquired from the two groups, it is considered that this may certainly identify two separate aetiologies of pre-eclampsia. So, the early-onset produces a more serious form, and the late-onset typically presents mild or moderate disease (21). From clinical point of view, early-onset pre-eclampsia presents a significant additional maternal risk since maternal mortality is approximately 20-fold higher at less than 32 weeks of gestation than when pre-eclampsia emerges at term (22). Additionally, evidence suggests that early-onset pre-eclampsia may be a distinct disorder. The pathophysiology of early-onset pre-eclampsia differs from that of late-onset disease in terms of neutrophil function and cytokine levels (19,21). Furthermore, early-onset pre-eclampsia is associated with a higher likelihood of recurrence in subsequent pregnancies, as well as an increased risk of later cardiovascular disease and mortality (23). In a pregnancy complicated with pre-eclampsia, giving birth before 37 weeks raises the risk of death from cardiovascular disease by 7.1 to 8.1-fold. Furthermore, early-onset pre-eclampsia is associated with increased fetal growth restriction as well as pathological evidence of placental malperfusion. This is not the case with late-onset pre-eclampsia, which is likewise less likely to predict cardiovascular disease in later life (24,25).

1.1.4 Pathophysiology of Pre-eclampsia

The understanding of pre-eclampsia pathophysiology is still evolving. Pre-eclampsia is a complicated and diverse syndrome with several probable mechanisms leading to disease progression. It is a systemic disease characterized by endothelial dysfunction, excessive immune system activity and responses, as well as multiorgan failure, all of which impact the health of both the mother and the fetus (26). The main concept in pre-eclampsia pathophysiology is Redman's two-stage model, which he introduced in 1991. The first stage is known as the placental stage and

is caused by impaired placentation produced by an insufficient trophoblastic invasion of the maternal spiral arteries, resulting in decreased placental perfusion. The second stage is characterized as the clinical/maternal stage and is thought to be a result of stage one in which the release of several biological components from the ischemic placenta causes endothelial dysfunction and the development of acute maternal syndrome with systemic multiorgan failure (27). Almost 30 years after the two-stage PE model was established, there is substantial proof of many factors linking both disease stages. They include angiogenic and antiangiogenic factors (vascular endothelial growth factor VEGF, placental growth factor PlGF, soluble FMS-like tyrosine kinase 1 sFlt-1, soluble endoglin sEng), hypoxia-induced factor 1α (HIF-1α), endothelin-1, syncytiotrophoblast microparticles (STBM), angiotensin II 1 receptor autoantibodies (AT1-AA), oxidative stress, and endoplasmic reticulum (ER) stress with unfolded protein response (26). Redman and Staff published an upgrade to the two-stage PE model in 2014 and 2019, respectively. The most significant observation is that there are at least two or maybe more distinct routes leading to stage one meaning it is not just poor placentation with insufficient modification of the maternal spiral arteries, but also intraplacental malperfusion due to mechanical restrictions causing syncytiotrophoblast (STB) stress (28,29). The first pathway represents an "extrinsic cause" of placental failure that develops early in pregnancy and leads to early-onset PE, which is frequently accompanied by fetal growth restriction. The second pathway represents the condition in which the placenta becomes too large for the uterine capacity late in pregnancy and the terminal villi become compacted, restricting intervillous perfusion. This is considered as an "intrinsic cause" of placental impairment and is associated with late-onset PE with normal fetal growth. Based on research studies, abnormalities in placental function might lead to the release of inflammatory components, resulting in increased vascular resistance and subsequent maternal blood pressure. In

other words, excessive immune activity, along with elevated levels of proinflammatory cytokines and antiangiogenic factors, could suggest the role of inflammation in the pathogenesis of pre-eclampsia to some extent. These changes occur both within the intrauterine environment and the maternal endothelium. (30,31). Another enhancement to the two-stage PE model is the incorporation of data showing maternal variables may influence disease progression at both phases. Maternal characteristics such as genetic predisposition, immunological variables, and chronic diseases can all influence placentation, placental growth, and function of placenta as well as maternal susceptibility and response to substances released by placental tissue, thereby increasing the onset of clinical symptoms in the mother (29). In addition to all of the possible causes of pre-eclampsia discussed above, recent evidence suggests that the human body has co-evolved with a set of microbiota that can alter the mother's metabolism. Any changes in the structure and composition of maternal microbiota (known as dysbiosis) are associated with inflammation, hypertension, and may also play a role in the development of pre-eclampsia (32).

1.1.5 Risk Factors of Pre-eclampsia

According to the research studies several important factors have been identified that place pregnant woman at the greater risk of developing pre-eclampsia; however, the clear and specific etiology of PE remains unknown due to the complexity and heterogeneity of PE (33). Based on the 2019 National Institute for Health and Care Excellence (NICE) guidelines, a woman is at high risk of pre-eclampsia if she has a history of hypertensive disease during a previous pregnancy or if she has a maternal disease such as chronic kidney disease, autoimmune diseases, diabetes, or chronic hypertension. Overall, women who are nulliparous, young maternal age (< 18) and age ≥ 40 years old, have a body mass index (BMI) of 35 kg/m or greater, a family history of pre-eclampsia, a multifoetal pregnancy, or a pregnancy interval of more than 10 years are at moderate risk. Pre-

eclampsia occurs in 40 to 50% of multiparous women with a diagnosis of pre-eclampsia who have previously experienced pre-eclampsia during their previous pregnancy (34). If pre-eclampsia required delivery at or before 32 weeks' gestation during a previous pregnancy, the probability for recurrence becomes over 40% (35). Interestingly, large-scale epidemiological studies and detailed clinical studies have supported nulliparity as an important risk factor for pre-eclampsia which nulliparous black women are twice as likely to experience pre-eclampsia as white women as they are also more likely to experience hypertension independently of pregnancy (36). Excessive gestational weight gain and high pre-pregnancy body mass index (BMI) were found to be important risk factors for PE in a recent systematic review and meta-analysis study by Ulhaq et al. (2021). Obesity and overweight were both linked to an increased risk of pre-eclampsia (37). Also, pregnant women who gained weight below the standard during pregnancy were protected against pre-eclampsia, whereas pregnant women who gained weight above the standard had a nearly doubled risk. Another possible risk factor for pre-eclampsia is pre-existing diabetes mellitus; prevalence rate varies from 9% to 66% among women with pre-existing diabetic nephropathy (38). Environmental factors may also play a role in the onset of pre-eclampsia. For example, the high prevalence of pre-eclampsia in many developing countries indicates that a poor diet may be a risk factor in this regard. Calcium, zinc, vitamins C and E, and n-3 essential fatty acid deficiencies have been proposed as relevant dietary factors, therefore routine antenatal care should include recommendations for a balanced daily diet during pregnancy (39). Because of the protective effects that nutritional supplements, dietary and lifestyle interventions might have against pre-eclampsia. In terms of lifestyle interventions, it has been revealed that rates of pre-eclampsia might be lower in those pregnant women who had higher vegetable and plant-based diet and vitamin D supplementation may also provide some benefit in decreasing pre-eclampsia development (34).

Despite the fact that supplementation is frequently advised in clinical practise, strong randomised controlled trial (RCT) data is still needed to demonstrate its efficacy. Other than factors mentioned above, there are additional factors that significantly increase the risk of pre-eclampsia, including endothelial dysfunction, oxidative stress, disturbed placental implantation, an excessive inflammatory response to pregnancy (33), polycystic ovarian syndrome, sleep disordered breathing, various infections such as periodontal disease, urinary tract infections, and helicobacter pylori which have been reported to play a role in the development of PE. In addition, in vitro fertilization (IVF) with the use of oocyte donation carries a greater risk of developing pre-eclampsia in such cases than IVF without oocyte donation or normal conception (34). The etiology of pre-eclampsia within pregnancies involving donor oocytes remains in the process of clarification. However, it is plausible to consider immunologically mediated mechanisms as potential contributors, given the indication of immunological elements in the pathogenesis of pre-eclampsia. In egg donation, the embryo genetically aligns with that of the donor rather than the recipient. This genetic disparity between the embryo and the maternal host as the recipient might trigger immune reactions during pregnancy that increases the risk of pre-eclampsia. Additionally, egg donation disrupts the reorganization of fetal HLA-C (a ligament of the immunoglobulin-type receptor of natural killer cells) by the maternal immune system, as it is entirely allogeneic. This disruption can impair the blood flow to the placenta, which consequently contributes to the onset of pre-eclampsia through placental dysfunction (31,40).

1.1.6 Adverse Maternal Consequences Associated with Pre-eclampsia

Maternal morbidity and mortality rates in pre-eclamptic women have risen in recent years. HELLP syndrome, eclampsia, cerebrovascular bleeding, and retinal detachment are all short-term maternal complications. According to the previous research studies, pre-eclampsia is becoming identified

for its long-term impacts, which can appear up to 15 years after giving birth. Numerous studies have found that pre-eclampsia increases the risk of chronic hypertension, ischemic heart disease, stroke, development of end-stage kidney disease (ESKD), and death from cardiovascular events (13). Over 700,000 women were studied in a Danish Registry-Based Cohort Study with a median follow-up of 14.6 years have shown that severe pre-eclampsia is associated with a 6-fold rise in hypertension, a 1.7-fold increase in ischemic heart disorder, a 1.9-fold increase in thromboembolism, and a 4-fold increase in type 2 diabetes (41). The few studies that have evaluated the consequences of PE at different gestational ages imply that early-onset PE is more severe than late-onset PE. When PE began before 28 weeks gestation, maternal mortality rates were 24 times higher than when it began at term. Irgens et al discovered that, compared to women with non-pre-eclamptic term deliveries, the risk of long-term cardiovascular disease was about 2-fold for women with late-onset PE, but this risk increased to over 8-fold for women with PE before 37 weeks gestation. When compared to late-onset PE, early-onset PE was associated with a higher risk of complications (reoccurring PE and reoccurring early-onset PE, small for gestational age infants, preterm birth, stillbirth, caesarean section, and placental abruption) and a lower risk of large for gestational age in subsequent pregnancies (42).

1.1.7 Adverse Infant Consequences Associated with Pre-eclampsia

Notably, pre-eclampsia not only influences mother's health, but also her offspring (13). As previously documented pre-eclampsia pathophysiology is complex and not entirely understood, however, it is known to involve abnormal placentation, systemic inflammation, and oxidative stress. Abnormal placentation takes place when the spiral arteries are unable to change properly, leading to a higher resistance in the flow of blood to the placenta and a decrease in the placenta's blood supply. This can cause a lack of oxygen and blood to the developing fetus, resulting in poor

outcomes such as intrauterine growth restriction, a high percentage of premature birth, low amniotic fluid levels, the separation of the placenta from the uterus, distress in the fetus, and stillbirth. (34,43). The frequency of fetal problems varies based on the onset of pre-eclampsia, and the early onset of pre-eclampsia has been linked to significantly greater rates of poor fetal outcomes, including IUGR, oligohydramnios, and fetal death (44). Infants of women with pre-eclampsia often have neonatal comorbidities such as respiratory distress syndrome and intraventricular hemorrhage. These babies are more at the risk of neurodevelopmental and behavioral problems and cardiovascular disease as they grow up (45). Furthermore, women with pre-eclampsia are at a 2.27-times greater risk of experiencing premature rupture of membranes (PROM) compared to those with normal pregnancies. PROM is a significant concern for obstetricians and can lead to maternal and neonatal morbidity that may be partially caused by pre-eclampsia. Pre-eclampsia and premature rupture of membranes are also known to increase the risk of lower neonatal birth weight and length. Other complications associated with PROM that can affect the health of the fetus include infection, fetal distress, chorioamnionitis, trauma, intraventricular hemorrhage, and respiratory distress syndrome which may increase the need for a cesarean delivery to protect the health of both the mother and the baby. (46–48).

1.1.8 Management of Pre-eclampsia

Although our understanding of the pathophysiology of pre-eclampsia has improved in recent years, an accurate diagnostic tool or biomarkers for early screening and diagnosis of pre-eclampsia are still lacking. Pre-eclampsia is a significant cause of maternal and fetal morbidity and mortality and has adverse effects on mothers after pregnancy. Early detection, monitoring, and management are crucial, as clinical data suggest (49). The management of pre-eclampsia depends on both the severity of the condition and the gestational age at diagnosis. If pre-eclampsia is discovered after

36–37 weeks of pregnancy, there is no advantage in continuing the pregnancy, regardless of its severity. However, if severe pre-eclampsia is diagnosed before 24 weeks of pregnancy, termination of pregnancy is recommended due to the high risk of maternal complications and poor neonatal prognosis (50). Expectant management is advised for mild pre-eclampsia, but immediate delivery is necessary in case of severe pre-eclampsia or when certain signs are present such as uncontrolled severe hypertension, acute pulmonary edema, thrombocytopenia, abruption placenta, fetal distress, or eclampsia. The goal of expectant management of pre-eclampsia is to reduce the risk of maternal and neonatal complications by administering anti-hypertensive drugs such as nifedipine, methyldopa, labetalol, alone or in combination and anti-convulsant such as magnesium sulfate or phenytoin to manage hypertension and seizure. According to gestational age, Betamethasone, a corticosteroid, can also be administered to enhance infant pulmonary maturation, and reducing the risk of neonatal mortality (51,52). To conclude, while the aim of all clinical interventions for pre-eclampsia is to prevent maternal morbidity and mortality, seizures, and fetal injury, it should be noted that there is currently no known cure for pre-eclampsia, and the most effective treatment is delivery of the baby and placenta(51).

1.1.9 Pre-eclampsia and Emergency Cesarean Section

Many investigations have been carried out to determine the most effective delivery method and timing for pre-eclampsia. International guidelines suggest that delivery should be considered in cases where pre-eclampsia occurs at week 37 of gestation (53). Typically, the mode of delivery is determined based on the clinical condition of both the mother and the fetus. When the fetus is nearing the anticipated delivery date, vaginal delivery is usually preferred. A study called the Hypertension and Pre-eclampsia Intervention Trial at Term investigated the effectiveness of induction of labour versus expectant monitoring in women with pre-eclampsia between weeks 36

to 41 of gestation. The study found that induction of labour (IOL) resulted in better outcomes for mothers than expectant monitoring. Inducing labour is a medical intervention aimed at stimulating the initiation of labour artificially before the onset of spontaneous labour and the most frequent and efficient pharmacological approaches for labour induction involve the use of prostaglandins (in oral or vaginal form) and oxytocin (administered intravenously) (54). Pre-eclampsia is one of the indications for IOL, and numerous guidelines consistently recommend that women who develop pre-eclampsia at or after 37 weeks of gestation be offered IOL. However, varying recommendations exist regarding the timing of IOL for women with pre-term pre-eclampsia (i.e., before 37 weeks), particularly in cases of mild to moderate hypertension. While some guidelines suggest delaying induction of labour for women with mild to moderate pre-eclampsia until 37 weeks, others recommend inducing labour from 34 weeks. Notably, only two guidelines emphasize the importance of considering women's preferences and needs in the decision-making process around the timing of induction of labour for hypertensive disorders (55). While vaginal delivery might be safe for women with pre-eclampsia, research indicates that cesarean sections are more common among pre-eclamptic women compared to those without the condition (56,57). According to a study conducted by Pacher et al., elective cesarean section was the most frequently used delivery method for women with pre-eclampsia. The decision to perform an elective cesarean section was primarily based on maternal health conditions or a previous cesarean section due to pre-eclampsia. In addition, 12.3% of pre-eclamptic women had an emergency cesarean section because of a sudden threat to the life of the mother or fetus, such as acute fetal distress, abnormal fetal heart rate, malpresentation during labour, or unsuccessful induction of labour (56). Roberts et al. conducted a study that investigated the reasons for using different delivery methods among pregnant women. The study revealed that in cases of severe pre-eclampsia, emergency cesarean

section without labour was performed in 30% of cases, especially when fetal distress or placental abruption was present (58,59). Among their study cohorts, other indications for performing emergency cesarean section without labour included twin pregnancies, antepartum hemorrhage, and premature pre-labor rupture of membranes (59).

1.1.10 Human Gut Microbiota

The human gastrointestinal (GI) tract is one of the most fundamental connections in the human body between the host, antigens, and the environment. In a typical lifetime, around 60 tonnes of nutrients travel through the human GI tract, accompanied by a plethora of microorganisms from the environment, creating a significant danger to gut integrity (60). The term 'gut microbiota' is used to indicate the combination of bacteria, archaea, and eukarya that colonize the GI tract and have accompanied and evolved with the host for thousands of years to develop a beneficial interaction with the host (61). The number of microorganisms in the GI tract has been estimated to be over 10^{14} and *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Proteobacteria* are the four main bacterial phyla in the human gut canal. Thursby and Juge (2017) estimate that these four phyla account for 93.5% of microorganisms identified in the intestine (62). It is worth noting that the gut microbiota composition might be different among human individuals depending on several factors such as age, diet, genetic, and the host's health condition. For instance, infant gut microbiota differs from adult gut microbiota and undergoes dynamic changes from sterile to adult-like bacteria from birth to approximately 2.5 to 3 years of age (63). Furthermore, gut microbiota provides numerous benefits, including enhancing gut integrity or modifying the intestinal epithelium, gathering energy, protecting the host against infections and diseases, and regulating host immunity. However, these systems may be interrupted because of a change in microbial composition, which is called dysbiosis (62).

1.1.11 The Development of Gut Microbiota in Early Infancy

It is generally accepted that the development of the microbiota begins at birth, but some studies have found evidence of microbes in placental tissue, intrauterine environment, and other womb tissues, which challenges this belief (62). The process of birth, specifically the rupture of the amniotic membranes surrounding the embryo and separating it from the uterus, marks the beginning of the establishment of the neonate's microbiota. In other words, when the membranes rupture and the newborn passes through the birth canal, it becomes exposed to the colonized surfaces of the mother's body and the environment, initiating the establishment of its own unique microbiota (64). Therefore, the colonization of the neonate by bacteria is an important aspect of a normal birth and it is important to understand the various factors that influence the presence and colonization of microbes in the fetus and newborn's gut during the perinatal period and at birth. It's been reported that before the age of 3 years, microbial communities are rapidly evolving into adult-like communities during the first hours and days after birth. An extensive study on almost 600 infants found that the mode of delivery had the strongest influence on the composition of the infant gut microbiota (65). Infants born vaginally are exposed to the maternal vaginal canal and fecal microbiota during birth which leads to the colonization of the infant gut by microbes such as *Lactobacillus* and *Prevotella*, *Bacteroides*, *Bifidobacterium* while infants born via caesarean section do not come into direct contact with maternal microbes and are more likely to be colonized by microbes from the hospital environment, maternal skin, or hospital staff leading to a different microbial composition (63). The composition of bacteria in the gut can be affected by the maternal diet, maternal health and pregnancy condition. During pregnancy, these factors can impact the mother's gut bacteria, which can then impact the infant both in utero and after birth by altering the infant's gut bacteria composition (66). It is also important to note that exposure to antibiotic therapy

and its effects on the human microbiome can start in utero and continue throughout crucial growth and development stages. A study by Tanaka et al. in 2009 showed that changes in the gut bacteria of infants whose mothers were treated with antibiotics were similar to the changes seen in infants treated with antibiotics, highlighting the impact of maternal medications on infant health (67). For example, in one previous study, it was observed that prenatal antibiotics in mice reduce the diversity and structure of the microbiota in offspring (68). Infant feeding is also a significant factor that determines the early colonization of bacteria and, as a result, affects the composition of the neonatal gut microbiome and gastrointestinal function. The difference in gut microbial composition between breastfed and formula-fed infants is well-established, with higher levels of *Bifidobacterium* being found in infants who are breastfed (66). Other important factors that influence the development of the infant gut microbiome include the gestational age of the infant, the mother's body mass index (BMI), the ethnicity of the mother, whether the mother smoked during pregnancy, whether the mother experienced pre/postnatal depression, and the mother's level of education. All of these factors can contribute to the programming of the infant's development, and early changes to the gut microbiota of infants have been linked to numerous inflammatory, immune-mediated, allergic, and metabolic disorders in later life (66,69).

1.1.12 Role of Cesarean Section on Infant Gut Microbiota Composition

During pregnancy, women have the option of delivering their babies through natural vaginal delivery or through a surgical procedure known as cesarean section (CS). CS is a worldwide method of ending pregnancy that involves making an incision in the abdominal and uterine walls to deliver either live or deceased fetuses (70). It has become increasingly common in many countries and is often used to save lives. Studies have shown that the rate of CS in developed countries ranges from 10-25%, while in developing countries it can vary from 8-36%. Currently,

it is estimated that around 20 million CS deliveries take place annually worldwide, making it the most common type of abdominal surgery among adults (70). CS can be further classified into elective and emergency procedures, depending on whether the operation is planned in advance or needs to be done urgently. The most common indications for emergency CS are dystocia, oligohydramnios, hypertension, pre-eclampsia, placental abruption, fetal distress, and prolonged rupture of membranes (71). In addition to the types and medical indications of CS, the mode of delivery through cesarean section is considered as one of the most important factors shaping the early composition of an infant's gut microbiota (72). Several studies using various techniques have found that the gut microbiota of infants born via caesarean section is different from that of vaginally born infants. These infants often have a gut microbiota dominated by *Proteobacteria* and *Firmicutes* in the early days after birth, with *Actinobacteria* appearing later. The gut microbiota of infants born via caesarean section is also less diverse and less likely to be colonized by bacteria such as *Bifidobacterium* and *Bacteroides*, which are usually passed on to the infant during birth and associated with the fermentation of breast milk oligosaccharides in the gut. Similarly, a recent study by Chen et al. revealed that CS with or without labour inversely influence the absolute quantities of *Bifidobacterium* in infants (73). Instead, they are more frequently colonized by certain types of *Clostridium* (63). These differences between vaginally and C-section delivered infants gradually diminish. However, C-section-delivered infants remain more diverse than vaginally born infants for up to a year after birth (66). Also, short-chain fatty acids (SCFAs) are created when gut bacteria break down certain types of carbohydrates that cannot be digested by the body. The main types of SCFAs are acetate, propionate, and butyrate (74). These substances play important roles in regulating various functions, such as the immune system and metabolism. Research studies have shown that infants born via C-section have a greater likelihood of

developing metabolic disorders later in life compared to those born vaginally and this difference in risk may be linked to variations in the levels of SCFAs present in the bodies of these two groups of infants (75).

1.1.13 Role of Breastfeeding on Infant Gut Microbiota

Human milk, which is typically the first source of nutrition for newborns, is an important link between mothers and their infants. Its composition is complex and dynamic, and it is vastly different from formula-based products in terms of nutritional value and the presence of certain growth factors and enzymes (76). Bioactive compounds found in human milk, such as human milk oligosaccharides, are beneficial for infants as they not only aid in development but also strengthen the immune system, provide protection against allergies, and may even offer protection from certain health conditions like coeliac disease, obesity, type-2 diabetes, and diarrhea (77–79). The World Health Organization recommends that infants should be breastfed for at least the first six months of life, after which solid foods should be introduced (66). While a single component of breast milk does not significantly impact the gut microbiome of infants, there is evidence that human milk oligosaccharides (HMOs) play a crucial role by promoting the growth of beneficial bacteria such as *Bifidobacteria* and *Bacteroides* (66,80). Additionally, HMOs modulate the health of infants through their prebiotic effects, modulation of innate immune responses and intestinal cell responses, and anti-inflammatory effects (66). Furthermore, it has been shown that human milk contains certain proteins and stimulation factors that enhance the growth of beneficial bacteria in the infant's gut, which help in breaking down complex oligosaccharides present in human milk (80). Many researchers have attempted to study the impact of feeding habits on the gut microbial diversity of infants. Cong et al. (2016) investigated the relationship between feeding types and the infant gut microbiome and found that breast milk feeding is associated with a higher diversity of

the infant's gut microbiome compared to non-breast milk feeding (81). Furthermore, it has been reported that *Lactobacillus* and *Bifidobacterium* species, specifically *B. breve*, *B. longum*, *B. dentium*, *B. infantis*, and *B. pseudocatenulatum*, are the most prevalent *Actinobacteria* in breastfed infants. Breastfed and vaginally delivered term infants also have lower levels of *C. difficile* and *E. coli* and higher levels of *Bifidobacterium* spp., which are beneficial for infant health (66,82). On the other hand, formula-fed infants are exposed to different carbohydrates, bacteria, and (micro) nutrients, leading to different microbial colonization patterns in the gut (63). *Clostridium* and *Streptococcus* species, *Bacillus subtilis*, *Escherichia coli*, and *Enterococcus* were found to be significantly higher in formula-fed infants than those in breastfed infants (83). As a result of these microbiota differences, the levels of short-chain fatty acids (SCFAs) in the stools of breastfed and formula-fed infants are also different, with propionate and butyrate being present at higher levels in the formula-fed group (84). Additionally, it seems that infants fed with formula milk achieve an early divergence towards an adult-like microbiota composition (63).

1.1.14 Human Gut microbiota and Its Association with Pre-eclampsia

The human gut microbiota, which is a complex and massive community of microorganism, plays a key role in host metabolism and immunity (85). Recently, studies have demonstrated that gut microbiome dysbiosis can cause intestinal barrier disruption and bacterial translocation, resulting in a state of persistent systemic inflammation and disease pathogenesis such as obesity, type 2 diabetes, atherosclerosis, non-alcoholic fatty liver disease, hypertension, and chronic kidney diseases (86,87). Dysbiosis of the gut microbiome is an important point in blood pressure (BP) regulation and it can lead to the development of proteinuria, which can cause kidney failure disease (88–90). Recent findings suggest a disrupted gut microbiota composition in patients with pre-eclampsia (PE) in late pregnancy, but there is no causative analysis, and little is known about the

gut microbiota composition and relationship in patients with PE (91,92). A few other studies have been conducted which connected microbial-associated molecular patterns to inflammation and cardiometabolic disorder (93). Kell and Kenny (2016) believed that microbial infection is the root cause of PE, and bacterial products like LPS (also defined as endotoxin) are well recognised to be strongly inflammagenic and may induce immune reaction that irritates inflammation response (94). However, only several studies have investigated the link between gut microbiota dysbiosis and PE. A research study demonstrated that dysbiosis of the gut microbiome is an important point in blood pressure (BP) regulation and can contribute to the development of proteinuria, which may result in kidney disease (93). Wang et al (2020) reported that the gut microbiota of PE patients in T3 was significantly different from that of the control group with the decreased relative abundances of *Firmicutes* and the increased relative abundances of *Bacteroidetes*, *Proteobacteria*, and *Enterobacteriaceae* (33). A recent study from East China described those alterations in the abundance of bacteria of certain genera such as increased abundance of *Blautia* and *Ruminococcus* and decreased abundance of *Bifidobacterium* were associated with PE development to some degree (95). Furthermore, a recent systematic review published in 2021 investigated the gut microbiota changes in PE groups versus healthy groups as controls. They observed substantial dysbiosis of the gut microbiota in PE mothers, confirming the possibility of a link between dysbiosis of the gut microbiota and the occurrence of pre-eclampsia; however, there is a lack of knowledge on how pre-eclampsia, a maternal condition, impacts the gut microbiota of infants during pregnancy. Alpha- and beta-diversity, showed an alteration of the gut microbiota in PE patients. *Fusobacterium* and *Ruminococcus* were also enhanced in PE, whereas *Lachnospira*, *Akkermansia*, *Faecalibacterium*, *Bifidobacterium*, and *Alistipes* were diminished (96). Additionally, it has been reported that LPS biosynthesis pathways were significantly overrepresented in the microbiota of

pre-eclamptic mothers. *Bacteroidetes* have been identified as the primary contributors to LPS biosynthesis. LPS is being used experimentally to stimulate a condition like PE in several animal models studies. LPS injection in rats caused PE-like signs including hypertension, proteinuria, maternal complications, and maternal inflammatory reactions, both systemically and locally at the placenta, according to (Liu et al. 2017). In pre-eclampsia, LPS can result inflammation via the Toll-like receptor 4 (TLR4) signalling pathway. Proinflammatory cytokines TNF- and IL-6 improved in PE patients' circulation and placental trophoblast cells, while anti-inflammatory cytokines IL-10 and IL-4 lessened (93).

1.1.15 Gap in Literature

Several studies have been conducted in recent years to investigate the critical role of human gut microbiota on immune system and host health since birth. Maternal and environmental factors, as well as vertical microbial transfer from the mother, and factors relating to the infant, all play an important role in altering the gut microbiota, particularly members of the genus *Bifidobacterium* (66,98). Reduced levels of *Bifidobacterium* have been observed in various perinatal circumstances, including premature infants, infants delivered by C-section with and without labour, or after antibiotic treatment. The reduction of *Bifidobacterium* is linked to the development of metabolic, immune, and neurodevelopmental diseases in later life (98). It has been noted that cesarean section can reduce the presence of *Bifidobacterium*. This can be either an emergency or elective procedure clinically. Emergency CS is defined as a surgical delivery which takes place in an urgent condition, before or when labour is established. The most frequent indications for an emergency CS are fetal distress, failure to labour progress, when labour was deemed to be arrested in the first or second stage, and pre-eclampsia with the goal of saving mother and child’s lives. However, elective cesarean delivery is mainly considered as a planned procedure or maybe performed immediately

after the onset of labour in anyone due to a planned section (99–101). Chen et al. (2021) recently, found the influence of birth mode on the abundance of *Bifidobacterium* in infant gut microbiota at 3 months of age with the finding that maternal IAP exposure during vaginal birth significantly decreased the absolute and relative abundance of *Bifidobacterium* compared to vaginal birth with no IAP. Furthermore, absolute quantities of *Bifidobacterium* were inversely affected by CS birth, with or without labour. In terms of clinical categories of CS, they found statistically significant reductions in *Bifidobacterium* quantity between emergency CS delivery vs vaginal birth no IAP. The value of *Bifidobacterium* was also lower in emergency CS delivery vs elective CS delivery; however, it was not statistically significant. Additionally, when CS delivery was re-categorized into CS with labour and without labour *Bifidobacterium* differences were no longer evident so that there were not statistically significant differences between CS without labour compared to elective CS birth and CS with labour to emergency CS delivery (73). Based on their findings cesarean section influenced *Bifidobacterium*; however, reduction of *Bifidobacterium* was not due to the labour in emergency CS group when it was categorized based on the onset of labour. Since the reason behind of the *Bifidobacterium* levels in emergency CS birth has not been investigated completely in their study, further explorations are needed to understand why infants delivered by emergency CS without labour have lower *Bifidobacterium* to fill the missing link in the literature.

1.2 Thesis Overview

1.2.1 Hypothesis, Objective and Research Questions

As noted, *Bifidobacterium* could be negatively affected by cesarean section with and without labour. The onset of labour is the main difference between elective and emergency CS, but there was not a big difference within the re-categorized CS groups. Therefore, we hypothesize there are other factors which may play a key role than labour that are not explored yet. To be more specific,

emergency CS could be happened before the onset of labour due to some indications such as fetal distress or maternal complications such as pre-eclampsia. More importantly, there have been reported that pre-eclampsia influences mothers gut microbiota with the reduction of *Bifidobacterium* (95) which may be transferred to their fetal because of the vertical transmission of maternal gut microbiome to the infant and through premature rupture of membranes. Altogether, maternal health conditions like pre-eclampsia could have an effect on the levels of *Bifidobacterium* in the emergency CS without labour groups which have to be further tested in order to fill the gaps in the literature since it has not been investigated so far. Therefore, our study aims to determine the effect of pre-eclampsia and birth mode on the levels of *Bifidobacterium* in the infant gut to test the following objectives:

a) To determine the association between pre-eclampsia and *Bifidobacterium* levels in infant gut microbiota
b) To determine if this association is independent of emergency cesarean section without labour and premature rupture of membranes (PROM)
c) To determine if this association is mediated by emergency cesarean section without labour and PROM

1.2.2 The Canadian Healthy Infant Longitudinal Development (CHILD) Study

This study was a prospective cohort study that aimed to investigate the effect of pre-eclampsia and emergency cesarean birth without labour on the levels of *Bifidobacterium* in the gut microbiota of infants. The study used a subset of 1429 term infants from the Canadian Healthy Longitudinal Development (CHILD) national population-based general birth cohort. The CHILD study recruited 3624 pregnant women who gave birth to 3542 eligible infants, of which 3455 were followed prospectively with clinical assessments conducted at birth, 3 months, and 1-, 3- and 5-

year visits and were enrolled between November 2009 and February 2012 from either Vancouver, Edmonton, or Winnipeg sites. Strict inclusion and exclusion criteria were used to ensure that the pregnant mothers and infants were healthy for this cohort (Table 2.1). All infants in this study provided fecal samples at 3-4 months of age, which were analyzed for *Bifidobacterium* levels using qPCR analysis. The mothers participated in the study during their third trimester of pregnancy and completed a breast-feeding status questionnaire. Data regarding delivery mode, pre-eclampsia, labour duration, maternal body-mass-index (BMI), and intrapartum antibiotic prophylaxis (IAP) were obtained from hospital records. In this study, the mode of delivery was classified into five groups: vaginal birth without IAP (as reference group), vaginal birth with IAP, elective cesarean section, emergency cesarean section with labour, and emergency cesarean section without labour.

1.2.3 Study Variables

In this study potential variables were collected from the CHILD cohort database. Hospital records provide data regarding mode of delivery, maternal intrapartum antibiotic prophylaxis (IAP), labour duration, premature rupture of membranes, pre-eclampsia, and maternal BMI. Additionally, information regarding maternal age, maternal ethnicity, maternal education, prenatal smoking, infant sex, gestational age, breastfeeding status, pre/postnatal depression obtains from standardized questionnaires.

1.2.4 Potential Covariates Affecting Pre-eclampsia and Infant Gut Microbiota

Maternal BMI

Obesity is a significant global public health issue, causing more than 2 million avoidable deaths each year. This inflammatory condition is generally identified as having a body mass index (BMI) of 30 kg/m2 or higher (102). While obesity is more widespread in developed countries, its occurrence is rising in developing nations as well. Recent evidence suggests that obesity increases

the risk of all forms of pre-eclampsia, including severe and mild pre-eclampsia occurring in both early and late gestation, particularly in overweight and obese women (103). The exact mechanisms involved are not yet fully understood; however, excessive weight gain during pregnancy or pre-existing obesity and overweight conditions may be associated with hyperinsulinism, insulin resistance, oxidative stress, maternal systemic inflammation, and altered vascular function. These mechanisms may lead to endothelial dysfunction, hypertension, proteinuria, multi-organ damage, and high maternal morbidity and mortality (104). Also, there have been recent reports of vascular inflammation occurring in both pre-eclamptic and obese women. This suggests that if the vasculature of obese women is inflamed, they may have a higher risk of developing pre-eclampsia during pregnancy and are subjected to additional pregnancy complications (105). During pregnancy, obesity increases the risk of metabolic disorders in offspring. Extensive research involving humans and animals has revealed that the gut microbiota plays a crucial role in the development of obesity and related diseases. Maternal intestinal microbial community's composition and diversity are also affected by obesity during pregnancy (106). Gut microbial changes have been linked to maternal weight, BMI, and gestational weight gain, with significant variations observed between the first and third trimesters. Evidence suggests that obese and overweight mothers and those who gain excessive weight during pregnancy have lower levels of *Bifidobacterium* spp. compared to healthy mothers. Additionally, overweight pregnant women have lower levels of *Bifidobacterium* spp. and *Bacteroides* spp., but higher levels of *Staphylococcus* and *Escherichia coli* spp. compared to normal weight mothers (107,108). Although it was widely believed that the establishment and colonization of the infant gut microbiota occurred after birth, recent studies have revealed the presence of microbiota in various prenatal sources, such as the placenta, amniotic fluid, umbilical cord blood, and meconium. This

finding strongly suggests that the microbiome of offspring is likely to be transmitted from the mother before birth (109–111). Consequently, maternal obesity during pregnancy, which is associated with gut microbiota dysbiosis, could potentially impact microbiota transmission from mother to offspring and lead to metabolic disturbances in offspring (106).

Birth Mode

Pre-eclamptic women have the option of delivering their infant through either vaginal delivery or cesarean section. However, according to most of the literature, the decision regarding the preferred method of delivery is strongly linked to factors such as the gestational age, the health conditions of the mother and fetus, as well as the severity of the pre-eclampsia. While vaginal delivery is a safe option in the absence of any threat to maternal or fetal health, the incidence of cesarean section, including both elective and emergency procedures, remains relatively high (56). When considering the influence birth mode on the infant gut microbiota colonization, it is important to note that the birth mode has a significant impact, which may persist for several months, potentially affecting normal physiological processes and disease development. Research has shown that there are notable distinctions in the colonization of gut microbiota between infants delivered via cesarean section and those born vaginally, with a less developed microbiota in CS group (66). Cesarean section newborns have been found to have lower levels of *Bifidobacteria* and *Bacteroides* in their gut microbiota due to the absence of passage through the vaginal canal, which is characteristic of infants born vaginally (66). Furthermore, other factors that could contribute to the gut microbiota of a newborn delivered via C-section include the use of intrapartum antibiotics, maternal obesity, feeding practices, gestational age of fetus, and absence of labour. Intrapartum antibiotics are antibiotics given during labour and delivery. They frequently used to treat pre-existing infections or as prophylaxis for premature rupture of membranes, Group B *Streptococcus*

(GBS), cesarean section, or chorioamnionitis. A number of studies have shown that administering intrapartum antibiotics may be linked to changes in microbial diversity, as well as declines in the relative abundance of bacteria believed to be beneficial to health, such as *Bifidobacterium* and *Lactobacillus* in CS delivered infants (112,113).

Maternal Age and Ethnicity

Maternal age is a significant risk factor in the development of pre-eclampsia as well as CS delivery which could potentially cause gut microbiota dysbiosis (114,115). Research studies demonstrate that pregnant women aged 35 and above are more susceptible to develop pre-eclampsia than younger women. The underlying causes of this phenomenon are not fully understood, but it is believed to be linked to the physiological alterations associated with aging, such as reduced placental blood flow and vascular function (115). Moreover, the risk of pre-eclampsia is higher in women of black and South Asian ethnicity compared to white women, and it is believed that both genetic and environmental factors may contribute to this ethnic disparity (116). In two prospective Canadian cohorts, it was investigated that there are differences in the gut microbiota of infants born to mothers of white Caucasian ethnicity compared to those of South Asian ethnicity at the age of one year. The South Asian infants had higher levels of certain lactic acid bacteria (LAB) such as *Bifidobacterium*, *Lactococcus*, *Streptococcus*, and *Enterococcus*, while the white Caucasian infants had a greater abundance of genera within the *Clostridiales* order(117).

Maternal Smoking

Smoking while pregnant is a significant risk factor for preterm delivery and low birth weight in infants. Infants born to smokers have a higher chance of experiencing sudden infant death syndrome and respiratory problems, and they are more likely to develop obesity, cardiovascular disease in the future. Recent research indicates that changes in gut microbe balance may contribute

to obesity and cardiovascular disease (118). Exposure to environmental smoke before and after birth may lead to increased gut bacterial richness in infants, particularly in the *Firmicutes* phylum, at 3 months of age, which is linked to a higher risk of obesity and overweight at 1-3 years of age. Moreover, maternal smoking could elevate the abundance of *Bacteroides* and *Staphylococcus* in the gut microbiome of the infant by six months of age, while decreasing levels of *Actinobacteria*. Exposure to environmental smoke in the early stages of life may also raise the levels of *Ruminococcus* and *Akkermansia* in the infant's gut microbiota (119). The link between smoking and pregnancy-induced hypertension (PIH) is still unclear, and there are inconsistencies in research findings. However, understanding this relationship is crucial since smoking is a common habit among a significant portion of the population in Europe, and it is also the primary preventable cause of various pregnancy-related complications (120). Although some studies indicate that smoking during pregnancy reduces the risk of PIH, other studies have demonstrated opposing results, and the underlying mechanisms explaining the positive or negative effects of smoking on PIH risk are not yet fully determined. A recent cohort study found that smoking during the first trimester increases the risk of pregnancy-induced hypertension (PIH) and pre-eclampsia (PE) compared to women who never smoked. The study also revealed that women who quit smoking before pregnancy had a lower likelihood of PIH and PE, while those who stopped smoking during pregnancy had a much higher risk of PIH and PE. These findings align with previous research indicating that smoking induces inflammation, oxidative stress, and reduced placental blood flow, all of which can contribute to PIH and PE (121).

Premature Rupture of Membranes

While the spontaneous rupture of membranes (ROM) during labour and delivery is normal, it is considered abnormal when it happens before the onset of labour and uterine contractions, known

as preterm rupture of membranes (PROM) (46). PROM can occur at any gestational age and is categorized as either term PROM or preterm PROM (PPROM) depending on whether it occurs after or before the 37th week of gestation (122). PROM presents potential risks to both the mother and infant, including mortality and morbidity. Neonatal complications associated with PROM include fetal distress, prematurity, intraventricular hemorrhage, respiratory distress syndrome, and intrauterine infection. In addition, maternal complications commonly linked to PROM are endomyometritis, bacteremia, and postpartum hemorrhage (46,122). A recent population-based cohort study investigated the relationship between gestational hypertension, pre-eclampsia, and premature rupture of membranes (PROM). Their findings revealed that the incidence of PROM was significantly higher in women with gestational hypertension and pre-eclampsia, with respective rates of 17.7% and 8.9%, as opposed to 5.9% in the normotensive group. In addition, the study indicated that the odds of PROM were 4.21 times higher in women with gestational hypertension and women with pre-eclampsia had 2.27 times higher odds than those with normotension. Although the exact mechanism linking gestational hypertension and pre-eclampsia to PROM remains unclear, current understanding suggests that inflammation may trigger a host response that leads to the production of prostaglandins by the fetal membranes. These prostaglandins may cause uterine irritability and collagen degradation within the membranes, ultimately resulting in weakened fetal membranes that are more susceptible to rupture (48). The rupture of the amniotic membranes that enclose the fetus and separate it from the uterine cavity is the first exposure of the neonate to the birth canal and environment. This event marks the beginning of microbiota establishment and transmission from the mother to the baby. The newborn's microbiota colonization starts after exposure to maternal microbiota via the rupture of membranes and passage through the birth canal (64). In cases of premature rupture of membranes, intrapartum

antibiotics are commonly prescribed to reduce the risk of infection (123). However, these antibiotics have been shown to alter the developing infant microbiome up to 6 months after exposure. This alteration is characterized by changes in microbial counts, leading to a decrease in commensal bacteria such as *Bifidobacterium* and a persistence of potentially pathogenic bacteria. It should be noted that these changes might be further influenced by feeding method (124).

Maternal Education

The socioeconomic status (SES) is frequently employed in health research to differentiate and categorize individuals based on their economic and social circumstances. It comprises of education, occupation, and income, which are the primary variables used to evaluate SES (125). Pre-eclampsia is a complex condition that is also influenced by environmental and socioeconomic factors. A recent research study has demonstrated a significant association between education, pre-eclampsia, and its severity, as measured by the total SES score. Women with low education and low income are more likely to develop pre-eclampsia, and the condition tends to be more severe in individuals from a low socioeconomic background (125). Therefore, low maternal education is recognized as a significant risk factor for pre-eclampsia. There are many factors that could potentially explain this correlation, and lifestyle elements such as access to healthcare, exercise habits, and diet are among the most important. According to findings from a prospective cohort study, women with lower levels of education were more likely to have maternal overweight and obesity(126), which subsequently increases their susceptibility to pre-eclampsia due to a higher body mass index (BMI). Moreover, low socioeconomic status, including low education and low income were associated to prenatal depression and higher levels of depressive symptoms during late pregnancy and postpartum (127,128). Mothers who experience postnatal depression are less likely to begin breastfeeding and tend to stop breastfeeding earlier than non-depressed

mothers(129). Studies have reported that prenatal depression and a more severe form of postnatal depression are also linked to a decreased probability of exclusive breastfeeding and shorter duration of breastfeeding (130). Regarding infant gut microbiota composition, it's worth considering that maternal education may have a significant impact, particularly in cases of obesity and disrupted breastfeeding patterns linked to maternal depression. Maternal obesity and feeding status have a known impact on early gut microbiota composition, making maternal education a potentially critical component to consider in research and interventions aimed at improving and investigating early-life gut health.

1.2.5 Sample Size Calculation

To determine the effective sample size for our research, we collected data from a previous CHILD cohort study from SyMBIOTA group conducted by Chen et al. (2021) which demonstrated reduction levels of *Bifidobacterium* in emergency CS without labour delivered compared to 3 months in both crude model (cβ: -1.609, multiple R^2 = 0.01266, $p < 0.05$ for all infants) and adjusted model (aβ: -1.602, adjusted R^2 = 0.01298, $p < 0.05$ for all infants) compare to the reference group (73). To calculate sample size following equations related to the R^2 were used (131). Assuming 80% power and an α level of 0.05, a sample size of 618 infants for the crude model and 602 infants for the adjusted model are required.

$$n = 3 + \frac{(z_{\left(1-\frac{\alpha}{2}\right)} + z_{1-\beta})^2}{(\widetilde{\rho^*_{yx}} - h^*)^2} \quad \text{(Eq. 1)}$$

$$\widetilde{\rho^*_{yx}} = \frac{1}{2}\ln\frac{1+\widetilde{\rho_{yx}}}{1-\widetilde{\rho_{yx}}} \quad \text{(Eq. 2)}$$

$$\widetilde{\rho_{yx}} = \sqrt{R^2} \quad \text{(Eq. 3)}$$

Sample size based on the Crude model: $n = 3 + \frac{(1.96+0.84)^2}{(0.112995-0)^2} = 617.04 \approx 618$

$\widetilde{\rho_{yx}^{*}} = \frac{1}{2}\ln\frac{1+\widetilde{\rho_{yx}}}{1-\widetilde{\rho_{yx}}} = \frac{1}{2}\ln\frac{1+0.112517}{1-0.112517} = 0.112995$

$\widetilde{\rho_{yx}} = \sqrt{R^2} = \sqrt{0.01266} = 0.112517$

Sample size based on the Adjusted model: $n = 3 + \frac{(1.96+0.84)^2}{(0.114427-0)^2} = 601.77 \approx 602$

$\widetilde{\rho_{yx}^{*}} = \frac{1}{2}\ln\frac{1+\widetilde{\rho_{yx}}}{1-\widetilde{\rho_{yx}}} = \frac{1}{2}\ln\frac{1+0.11393}{1-0.11393} = 0.114427$

$\widetilde{\rho_{yx}} = \sqrt{R^2} = \sqrt{0.01298} = 0.11393$

CHAPTER 2

2. Methods and Materials

2.1 Study Design

This prospective cohort study aimed to investigate the impact of pre-eclampsia and emergency cesarean births without labour on *Bifidobacterium* levels in infant gut microbiota. Our study used a subsample of 1429 infants from the Canadian Healthy Infant Longitudinal Development (CHILD) study, which is the largest longitudinal, population-based birth cohort study in Canada, and is considered one of the most informative studies of its kind globally. (www.childstudy.ca). The CHILD study recruited 3624 pregnant women who gave birth to 3542 eligible infants. Of

these, 3455 were followed up prospectively with clinical assessments conducted at birth, 3 months, and 1, 3, and 5-year visits. The study employed strict inclusion and exclusion criteria to ensure that pregnant mothers and infants were healthy for this cohort (Table 2.1). In the current study, mothers of the studied infants were recruited in their second or third trimester of pregnancy. Infants in this study were from the Edmonton, Vancouver, and Winnipeg sites and provided fecal samples when they were 3-4 months old. Fecal samples were analyzed using 16s rRNA sequencing and qPCR analysis to detect total *Bifidobacterium*. Hospital records provided data on delivery mode, pre-eclampsia, maternal body-mass-index (BMI), intrapartum antibiotic prophylaxis (IAP), and premature rupture of membranes (PROM). Data on maternal and infant characteristics, such as maternal ethnicity, age, education, prenatal smoking, pre/postnatal depression, infant sex, breastfeeding status, and gestational age, were obtained from standard questionnaires completed by mothers. In this study birth mode is classified into five groups: vaginal birth without IAP (as the reference group), vaginal birth with IAP, elective cesarean section, emergency cesarean section with labour, and emergency cesarean section without labour. Furthermore, a directed acyclic graph (DAG) was created using the daggity.net program to demonstrate the potential relationship between pre-eclampsia and *Bifidobacterium* levels in the gut of infants at 3 months old. This program enables the identification of the minimum necessary covariates for adjustment. The DAG selected several possible confounding factors, including maternal education, premature rupture of membranes (PROM), prenatal smoking, and emergency cesarean section without labour (Figure 1). In the multivariable regression models, these variables will be adjusted if they have a p-value below 0.05 or if they change the regression coefficients of other variables by more than 10-15%.

Table 2.1 Eligibility Criteria for the CHILD study.

Inclusion Criteria	Exclusion Criteria
▪ Pregnant women that were 18 years of age or older ▪ Were able to read and speak English ▪ Had a valid number and address ▪ Planned on giving birth at one of the recruitment centres affiliated hospitals in Edmonton, Vancouver, Winnipeg ▪ Were willing to give informed consent ▪ Infants born at 35 weeks gestational age or greater	▪ Children born with major congenital abnormalities or respiratory distress syndrome (RDS) ▪ Expectation of moving away from a recruitment area within 1 year ▪ Children of multiple births ▪ Children resulting from in vitro fertilization ▪ Children who will not spend at least 80% of nights in the index home ▪ Children born before 35 weeks gestation

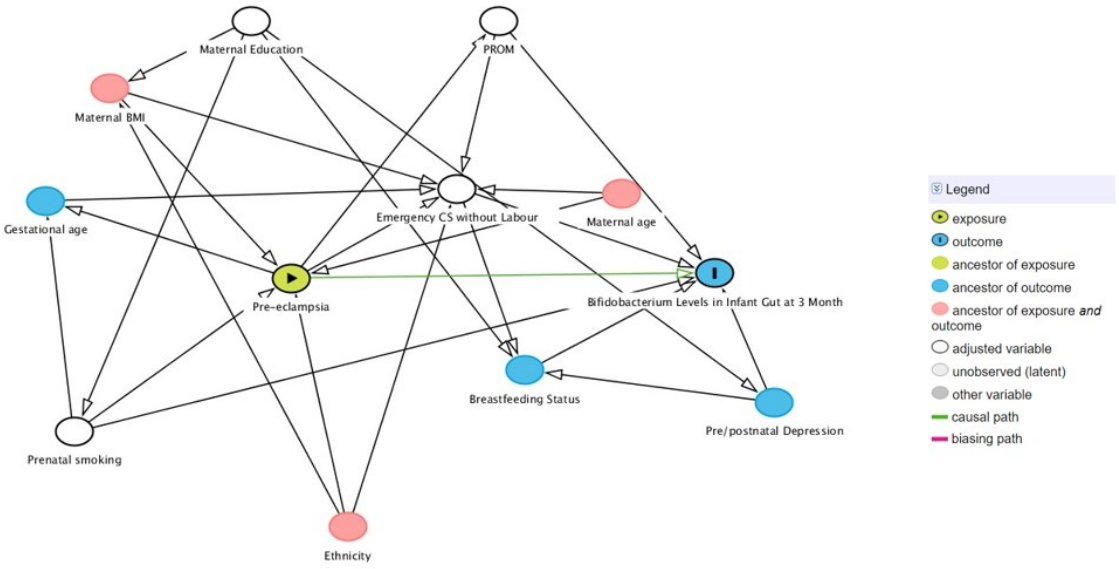


Figure 2.1. DAG exploring the association between pr-eclampsia and *Bifidobacterium* levels in infant gut microbiota

2.2 Fecal Sample Collection, DNA Extraction and qPCR Amplification

Samples of fecal matter weighing between 5 to 10 grams were taken from infant diapers during home-visits conducted when the infants were around 3 to 4 months old. These collections were done by a research assistant or the parents, following an approved protocol. The samples were refrigerated after collection, divided into smaller portions, and stored at a temperature of -80 degrees Celsius until analysis. Genomic DNA was extracted from the frozen stool samples, ranging from 80 to 200 milligrams, using the QIAamp DNA Stool Mini kit, following the instructions provided by the manufacturer, Qiagen, which is based in Venlo, the Netherlands. The DNA extracted from infant fecal samples underwent a quantitative PCR test, following a published protocol. To reduce variations in inhibitory effects caused by varying concentrations of genomic template DNA, all DNA samples were normalized by diluting them to 1 ng/µL. A multiplex assay was then created by adding 1X QuantiNova Multiplex PCR Kit, 0.4 µM of each primer, 0.25 µM

of each probe, and 1 µL (1 ng/µL) of sample DNA to reach a final volume of 20 µL. The relative abundance of total *Bifidobacteria* was determined by computing the percentage of their respective gene copies per gram of stool, relative to the total number of bacterial gene copies per gram of stool measured using qPCR. To assess the level of agreement, we compared the relative abundance of *Bifidobacterium* with the relative abundance of 16S. When determining the qPCR-based abundance, we observed that almost every DNA product amplified from the targeted 16S rDNA regions resulted in a larger denominator and lower abundance values compared to the 16S relative abundance. Despite certain variations between the two methods, there was a significant correlation between qPCR-based relative abundance and 16S-based relative abundance. We found that in the lower range of abundance values, qPCR-based relative abundance was smaller than 16S-based relative abundance, whereas in the higher range of abundance, qPCR-based relative abundance was greater. In this context, the values of gene-copies/g stool are referred to as absolute quantities to distinguish them from the calculated relative abundance values.

2.3 Statistical Analysis

In this study all analyses performed using STATA version 17. *Bifidobacterium* levels was categorized as a binary variable based on the median value. This categorization allowed for the division of samples into high and low *Bifidobacterium* groups. Samples with values equal to or higher than the median were considered as the high *Bifidobacterium* group, and those with values lower than the median were categorized as the low *Bifidobacterium* group. To test crude unadjusted associations of maternal and infant characteristics with pre-eclampsia, emergency cesarean section without labour and *Bifidobacterium*, Fisher's exact tests was used, and a descriptive table was provided. Linear and logistic regressions were employed to identify the impact of pre-eclampsia on *Bifidobacterium* levels in infant gut microbiome while considering the

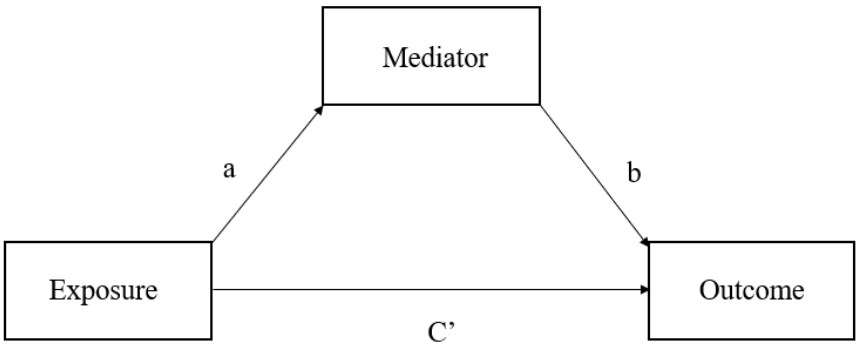
adjustment for potential confounding variables. Values of *Bifidobacterium* levels were Box-Cox transformed before linear and logistic regressions to improve the normality of data. To evaluate potential confounding factors, all potential covariates suggested by the Directed Acyclic Graph (DAG), such as maternal education, premature rupture of membranes (PROM), prenatal smoking, and emergency cesarean section without labour, individually adjusted for. Variables that result in a change of more than 10-15% in the regression coefficients of other variables will be retained in the final model, as well as those with a p-value below 0.05. The final model will provide crude and adjusted beta coefficients and 95% confidence intervals (CI) for *Bifidobacterium* levels. A p-value of 0.05 is considered significant.

2.4 Testing the Potential Mediating Roles of Birth Mode and Premature Rupture of Membranes

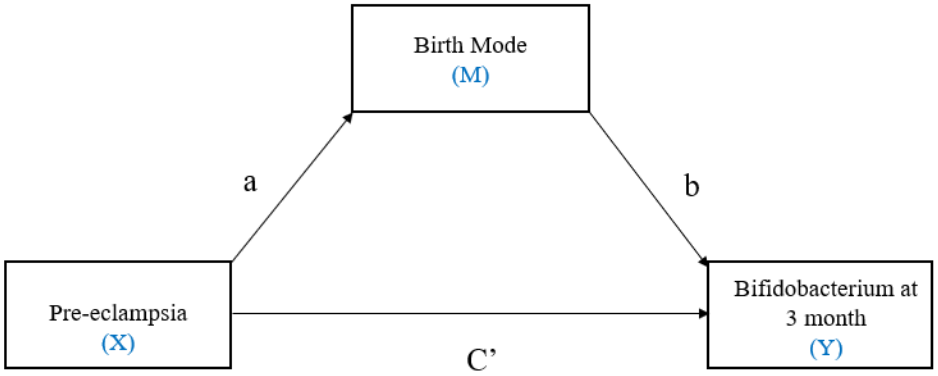
A mediation model establishes a connection between a potential cause (X) and an assumed effect (Y) by means of a mediator variable (M). The mediator variable can encompass various aspects, such as psychological states, cognitive processes, affective responses, or any other conceivable "mechanism" through which X exerts its influence on Y. In our study, we considered emergency cesarean section without labour and premature rupture of membranes (PROM) as potential mediators. From a biological standpoint, pre-eclampsia has the potential to impact the types of birth mode, which, in turn, may affect the infant gut microbiota. Therefore, it is anticipated that pre-eclampsia indirectly influences *Bifidobacterium* levels in infant gut microbiota through its impact on mode of delivery. This reasoning also applies to PROM, as women with pre-eclampsia are more susceptible to experiencing PROM, which subsequently affects the infant gut microbiome. Thus, PROM is identified as a mediator in our study. Figure 2.2A, illustrates the schematic diagrams of the simple mediation models. The analysis was carried out using the Hayes

PROCESS v3.5 macro in SPSS (version 26, SPSS Inc., Chicago, IL, USA) (132). To overcome the limitations of Hayes PROCESS v3.5 macro package in the SPSS software, we treated the mediator variables as categorical, ensuring they had a minimum of three categories, rather than using them as binary variables. Considering the various childbirth possibilities for women with pre-eclampsia, such as vaginal birth, elective cesarean section, and emergency cesarean section with or without labour, we modified the first mediation model by considering three rank-order variables: vaginal birth without IAP, vaginal birth with IAP, and a combined category including elective cesarean section, emergency cesarean section with labour, and emergency cesarean section without labour (Figure 2.2B). In the second model, the duration of rupture of membranes (ROM) was utilized as a mediator. The zero value for ROM represented the absence of membrane rupture, which was grouped together as a single variable. Non-zero values, indicating the duration of membrane rupture, were log-transformed to ensure normalization, and categorized into four distinct groups based on percentiles (Figure 2.2C). This allowed for the incorporation of categorical variables in the model. Moreover, due to the influence of birth mode on breastfeeding status, which in turn may impact the infant gut microbiota, a sequential mediation analysis was conducted. In this model, the first mediator is birth mode, following the structure of the initial simple mediation model. The second mediator is breastfeeding comprising three rank-order variables: exclusive breastfeeding (exclusive-BF), partial breastfeeding (partial-BF), and no breastfeeding (non-BF) (Figure 3A, B). The process of bootstrapping was employed in mediation analysis to create 95% confidence intervals, using 10000 bootstrap resamples.

A.


Mediator
a
b
Exposure
Outcome
C'

B.


Birth Mode
(M)
a
b
Pre-eclampsia
(X)
Bifidobacterium at
3 month
(Y)
C'

C.

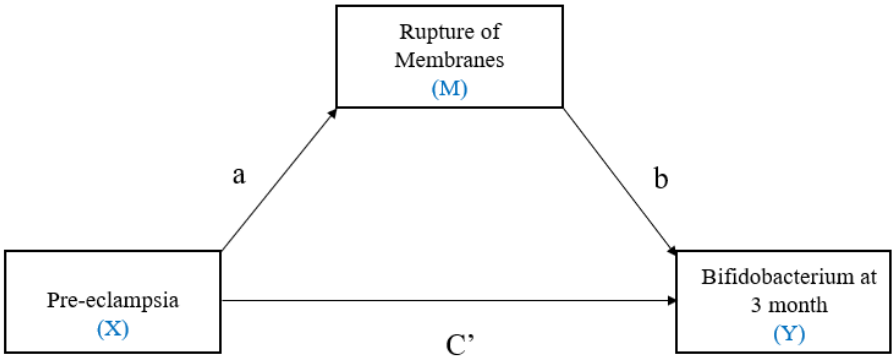
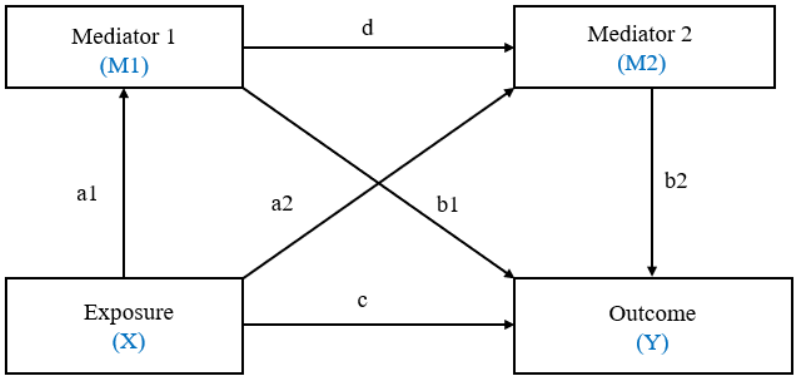


Figure 2.2. **A)** A schematic diagram of the simple mediation model. **B)** A schematic diagram of the simple mediation model by birth mode as a potential mediator. **C)** A schematic diagram of the simple mediation model by ROM as a potential mediator. Exposure (X) and mediator (M) are categorical variables and outcome(Y) is considered as continuous form.

A.



B.

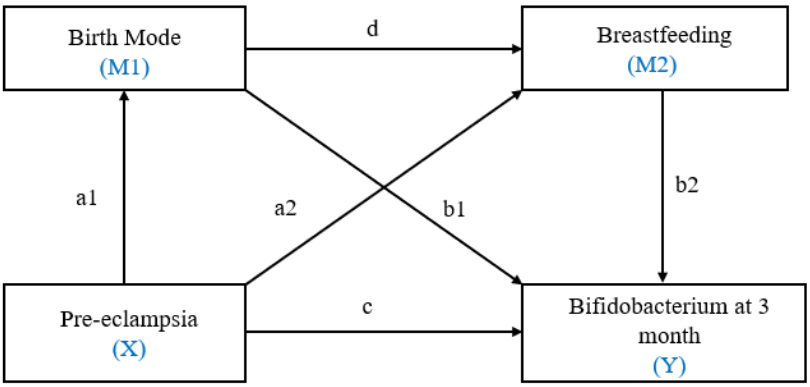


Figure 2.3. **A)** A schematic diagram of the sequential mediation model. **B)** Causal diagram of the association between pre-eclampsia(X) and *Bifidobacterium* levels at 3-month (Y) mediating by birth mode (M1) and breastfeeding status (M2).

2.5 Definition of Potential Covariates

Our study analyzed several potential covariates, including mode of delivery, maternal pre-pregnancy BMI, maternal education, maternal age, breastfeeding status, gestational age, premature rupture of membranes (PROM), maternal ethnicity, maternal prenatal and postnatal depression, and maternal smoking. All these variables were obtained from the CHILD dataset, which gathered the data from hospital records and standard questionnaires. Mode of delivery, medication use such as antibiotics, and maternal complications including pre-eclampsia were obtained from hospital records. Birth chart questionnaires were formulated from hospital records for CHILD cohort use to gather information about these variables. According to the guideline from the Society of Obstetricians and Gynaecologists of Canada (SOGC), pre-eclampsia defined as gestational

hypertension with new-onset proteinuria or the presence of one or more adverse conditions, such as maternal end-organ complications or signs of uteroplacental dysfunction which aligns with other established guidelines. According to its recent update in 2022, the SOGC is now suggesting the inclusion of angiogenic markers, if they are accessible, as they indicate central placental dysfunction, a key factor in the development of pre-eclampsia. This inclusion enhances the accuracy of diagnosis, better identifying women and infants at risk of unfavorable outcomes(133). However, it is important to highlight that we used the clinical definition of pre-eclampsia in this study, as documented in the hospital charts of pregnant mothers. Therefore, we defined pre-eclampsia as the new onset of hypertension and proteinuria, with no consideration given to biomarkers assessment for the diagnosis of pre-eclampsia. This definition aligns with earlier guidelines, given that the CHILD cohort participants were enrolled during the period from 2009 to 2012. Mode of delivery was collected from hospital records and classified into five categories in our study: vaginal birth-no IAP, vaginal birth-IAP, elective cesarean section, emergency cesarean section with labour, emergency cesarean section without labour. Maternal pre-pregnancy BMI was determined based on BMI scored before the pregnancy calculated as pre-pregnancy weight in kilograms divided by the square of height in meters and categorized into underweight (<18.5 Kg/m^2), normal weight (18.5-24.9 Kg/m^2), overweight (25.0-29.9Kg/m^2), and obese (>30.0 Kg/m^2). Maternal education ascertained from questionnaires and categorized as follows: high school or less, some post secondary or university degree, and postgraduate degree. Breastfeeding status was defined as exclusively breastfed, partially breastfed (breastfed and formula fed), and only formula fed and was collected by maternal questionnaires at 3 months of age. Mothers age classified into three groups: 18 to 29, 30 to 39, and Over 40 based on the questionnaires. Hospital records provided data on the PROM, and it was defined as the rupture of membranes before the

onset of labour at or after 37 weeks of gestation which considered as binary variables (Y/N) in this study. Gestational age also was obtained through hospital records and classified to 39-42 weeks, 37-38 weeks, and 34-36 weeks. Maternal smoking was defined if the mother had prenatal smoking which was determined from recruitment questionnaires (Y/N). Maternal prenatal and postnatal depression was determined using the 20-item center for Epidemiological Studies Depression and provided by questionnaires (Y/N). Maternal ethnicity was defined as Caucasian, first nation, and other according to the questionnaires completed by mothers.

CHAPTER 3

3. Results

3.1 Overall Population Characteristics

Table 3.1 describes the results of maternal and infant characteristics in the subsample of CHILD study cohort (N=1429). As indicated by the table, 53.3% of the infants were male and 46.7% were female. The infants were mostly exclusively breastfed (54.8%) and were born with a gestational age of around 39-42 week (74.4%). Among this cohort, vaginal birth without IAP was the most common mode of birth (53.4%), followed by vaginal birth with IAP (22.6%), emergency CS without labour (10.9%), elective CS (10%), and emergency CS with labour (3%). Mothers who were obese or overweight comprised 17.1% and 22% of the population, respectively, while mothers who were normal weight constituted 60.9% of the population. A small proportion of mothers (3.3%) had pre-eclampsia while 96.7% of them were not pre-eclamptic. Premature rupture of membranes took place in 14.1% of the population while most of the mothers in this cohort (85.9%) did not have premature rupture of membranes. Furthermore, the majority of mothers were

Caucasian (76.6%), educated to some post secondary, university degree (72.6%), were aged between 30 and 39 (65.5%), did not smoke prenatally (92.6%), and did not experience prenatal or postnatal depression (76.0% and 80.4%, respectively).

Table 3.1 Maternal and infant characteristics in subsample of CHILD study cohort (N=1429)	
Characteristics	N (%)
Infant Sex	
Male	756 (53.3%)
Female	663 (46.7%)
Breastfeeding Status	
Exclusive	779 (54.8%)
Partial	393 (27.7%)
Formula Fed	249 (17.5%)
Gestational Age	
39-42 weeks	1041(74.4%)
37-38 weeks	307 (21.9%)
34-36 weeks	52 (3.7%)
Mode of Delivery	
Vaginal-no IAP	411 (53.4%)
Vaginal-IAP	174 (22.6%)
Elective CS	77 (10.0%)
Emergency CS with Labour	23 (3.0%)
Emergency CS without Labour	84 (10.9%)
Pre-eclampsia	
No	1346 (96.7%)
Yes	46 (3.3%)
PROM	
No	644 (85.9%)
Yes	105 (14.1%)
Maternal Pre-Pregnancy BMI	
Normal Weight	811 (60.9%)
Overweight	293 (22.0%)
Obese	228(17.1%)
Maternal Education	
High School or Less	140 (10.1%)
Some Post Secondary, University Degree	1002 (72.6%)
Postgraduate Degree	239 (17.3%)
Maternal Age	
18 to 29	420 (29.4%)
30 to 39	935 (65.5%)

Over 40	73 (5.1%)
Maternal Ethnicity	
Caucasian	1085 (76.6%)
First Nation	92 (6.5%)
Asian	171 (12.1%)
Other	69 (4.8%)
Maternal Prenatal Depression	
No	1041(76.0%)
Yes	329 (24.0%)
Maternal Postnatal Depression	
No	1102 (80.4%)
Yes	268 (19.6%)
Prenatal Smoking	
No	1257 (92.6%)
Yes	100 (7.4%)

IAP, intrapartum antibiotic prophylaxis; CS, Cesarean Section; PROM, Premature Rupture of Membranes; BMI, Body Mass Index

3.2 Study Population and Pre-eclampsia, Birth mode, and *Bifidobacterium*

Table 3.2 shows descriptive characteristics of mothers and infants in relation to pre-eclampsia (Y/N), emergency CS without labour vs vaginal no IAP and *Bifidobacterium* levels (High/Low) at 3 months. In relation to pre-eclampsia, there were some factors significantly associated with pre-eclampsia, including gestational age ($p=0.015$), PROM ($p<0.001$), maternal pre-pregnancy BMI ($p<0.001$), and maternal age ($p=0.023$). However, no significant differences were detected in breastfeeding status at 3 months ($p=0.123$), maternal antibiotic at birth ($p=0.224$), maternal ethnicity ($p=0.869$), maternal prenatal depression ($p=1.0$), maternal postnatal depression ($p=0.706$), and maternal smoking ($p=0.571$) according to pre-eclampsia. Regarding emergency CS without labour vs vaginal no IAP, significant differences found between PROM groups ($p<0.001$). Comparing emergency CS without labour vs vaginal birth no IAP group, all the mothers who had premature rupture of membranes underwent emergency CS without labour. Maternal pre-pregnancy BMI ($p=0.006$) and gestational age ($p=0.02$) were other factors which demonstrated significant differences with emergency cs without labour vs vaginal birth no IAP. Mothers who

had emergency CS without labour were much more likely to be overweight or obese. According to the levels of *Bifidobacterium* at the 3 months of age, significant differences were observed in terms of breastfeeding status at 3 months (p=0.004), and low *Bifidobacterium* levels were more common in infants who were formula fed. Significant differences were also detected among other factors such as mode of delivery (p=0.013), PROM (p=0.005), and maternal prenatal depression (p=0.021).

Table 3.2 Percentage distribution of descriptive characteristics in relation to pre-eclampsia, Emergency CS without labour vs Vaginal no IAP and *Bifidobacterium* levels at 3 months

		Pre-eclampsia		p-value*	Emergency CS without labour vs Vaginal no IAP		p-value*	*Bifidobacterium* levels at 3 Months**		p-value*
Covariates N (%)		No	Yes		Emergency CS without labour	Vaginal no IAP		High	Low	
Breastfeeding Status at 3 Month N=1385										
	Exclusive	738(97.2%)	21(2.8%)	0.123	40(16.1%)	208(83.9%)	0.67	375(49.4%)	384(50.6%)	**0.004**
	Partial	364(95.0%)	19(5.0%)		26(17.1%)	126(82.9%)		211(55.1%)	172(44.9%)	
	Formula Fed	237(97.5%)	6(2.5%)		17(20.2%)	67(79.8%)		101(41.6%)	142(58.4%)	
Gestational Age N=1364										
	39-42 weeks	987(97.4%)	26(2.6%)	**0.015**	59(15.6%)	319(84.4%)	**0.02**	514(50.7%)	499(49.3%)	0.31
	37-38 weeks	282(94.0%)	18(6.0%)		20(19.4%)	83(80.6%)		143(47.7%)	157(52.3%)	
	34-36 weeks	49(96.1%)	2(3.9%)		4(57.1%)	3(42.9%)		21(41.2%)	30(58.8%)	
Mode of Delivery N=753										
	Vaginal-no IAP	396(97.8 %)	9(2.2%)	0.053	-	-	-	223(55.1%)	182(44.9%)	**0.013**
	Vaginal-IAP	159(95.2%)	8(4.8%)		-	-		68(40.7%)	99(59.3%)	
	Elective CS	73(96.0 %)	3(4.0%)		-	-		38(50.0%)	38(50.0%)	
	Emergency CS with labour	19(86.4%)	3(13.6%)		-	-		9(40.9%)	13(59.1%)	
	Emergency CS without labour	80(96.4%)	3(3.6%)		-	-		35(42.2%)	48(57.8%)	

PROM N= 735										
	No	606(72.5%)	25(27.5%)	**<0.001**	14(3.4%)	396(96.6%)	**<0.001**	325(51.5%)	306(48.5%)	**0.005**
	Yes	103(99.0%)	1(1.0%)		66(100.0%)	0		39(37.5%)	65(62.5%)	
Maternal Pre-Pregnancy BMI N= 1301										
	Normal weight	783(98.5%)	12(1.5%)	**<0.001**	41(14.2%)	248(85.8%)	**0.006**	378(47.5%)	417(52.5%)	0.096
	Overweight	273(95.5%)	13(4.5%)		14(14.9%)	80(85.1%)		157(54.9%)	129(45.1%)	
	Obese	202(91.8 %)	18(8.2%)		24(30.0%)	56(70.0%)		112(50.9%)	108(49.1%)	
Maternal Education N= 1346										
	High school or less	133(97.8%)	3(2.2%)	0.636	7(19.4%)	29(80.6%)	0.631	67(49.3%)	69(50.7%)	0.768
	Some post secondary, University degree	940(96.3%)	36(3.7%)		60(16.9%)	295(83.1%)		479(49.1%)	497(50.9%)	
	Postgraduate degree	228(97.4 %)	6(2.6 %)		11(13.1%)	73(86.9%)		121(51.7%)	113(48.3%)	
Maternal Age N=1392										
	18 to 29	401(98.0%)	8(2.0%)	**0.023**	20(14.7%)	116(85.3%)	0.222	212(51.8%)	197(48.2%)	0.590
	30 to 39	878(96.5%)	32(3.5%)		61(18.8%)	263(81.2%)		444(48.8%)	466(51.2%)	
	Over 40	67(91.8%)	6(8.2%)		2(7.1%)	26(92.9%)		37(50.7%)	36(49.3%)	
Maternal Ethnicity N=1381										
	Caucasian	1021(96.7%)	35(3.3%)	0.869	60(16.0%)	316(84.0%)	0.467	532(50.4%)	524(49.6%)	0.588
	First Nation	87(96.7 %)	3(3.3%)		7(22.6%)	24(77.4%)		41(45.6%)	49(54.4%)	
	Other	63(95.5%)	3(4.5%)		3(18.8%)	13(81.2%)		33(50.0%)	33(50.0%)	
Maternal Prenatal Depression	No	978(96.5%)	35(3.5%)	1.0	57(15.3%)	316(84.7%)	0.108	484(47.8%)	529(52.2%)	**0.021**
	Yes	311(96.6%)	11(3.4%)		24(22.2%)	84(77.8%)		178(55.3%)	144(44.7%)	

N=1335										
Maternal Postnatal Depression N=1335										
	No	1037(96.6%)	36(3.4%)	0.706	70(17.3%)	335(82.7%)	0.619	539(50.2%)	534(49.8%)	0.370
	Yes	252(96.2%)	10(3.8%)		11(14.5%)	65(85.5%)		123(47.0%)	139(53.0%)	
Prenatal Smoking N=1322	No	1183(96.6%)	41(3.4%)	0.571	72(16.1%)	374(83.9%)	0.072	605(49.4%)	619(50.6%)	0.917
	Yes	94(95.9%)	4(4.1%)		9(28.1%)	23(71.9%)		49(50.0%)	49(50.0%)	

*Fisher's exact test.
** *Bifidobacterium* was categorized as a binary variable based on the mean value (High/Low).
IAP, Intrapartum Antibiotic Prophylaxis; CS, Cesarean Section; PROM, Premature Rupture of Membranes; BMI, Body Mass Index. Significant p-values are shown in bold.

3.3 Association Between Pre-eclampsia and *Bifidobacterium* Levels in the Infant Gut Microbiota at 3 Months

To investigate the relationship between pre-eclampsia and *Bifidobacterium* levels in the infant gut microbiota at 3 months, we initially conducted a linear regression analysis using the absolute quantity of the *Bifidobacterium* genus. However, the univariable linear regression did not reveal a statistically significant association (Table S3.1). As a result, the *Bifidobacterium* values were transformed into a binary variable instead of being continuous. This alteration allows us to conduct both crude and adjusted logistic regression analyses. The crude model revealed a significant inverse association between pre-eclampsia and *Bifidobacterium* levels in the infant gut microbiota at 3 months (βc: -1.48, 95% CI: -2.9, -0.05, $p < 0.05$). This finding indicates that infants born to pre-eclamptic mothers have lower *Bifidobacterium* levels compared to the control group. To address potential confounding variables and adhere to the 10-15% change estimate rule, we adjusted the model by considering different potential confounding variables including maternal education, premature rupture of membranes (PROM), prenatal smoking, and emergency cesarean section (CS) without labour. Among these variables, only PROM and emergency CS without labour passed the rule and were retained in the final model. Then, to test whether the association between pre-eclampsia and *Bifidobacterium* levels in infant gut at 3 months is independent of emergency CS without labour and PROM, adjusted logistic regression analyses were conducted. However, after adjusting the model for emergency CS without labour as a potential confounding factor, a statistically significant association was not found. Additionally, the statistically significant association was not evident when adjusting the model for PROM (Table 3.3).

Table 3.3 Associations between pre-eclampsia and *Bifidobacterium* levels in infant gut at 3 months, by crude and adjusted logistic regression analyses.

		Beta Coefficient (95% CI)	*P* value
Pre-eclampsia	Crude Model	-1.48 (-2.90, -0.05)	**0.04**
	Adjusted Model	0.31 (-1.28, 1.90)	0.70
	Adjusted for Emergency CS without Labour	-0.27 (-1.77, 1.23)	0.72
	Adjusted for PROM	-0.86 (-2.32, 0.59)	0.24

Analysis is conducted by logistic regression. The reference group is normotensive women. Model is adjusted for PROM and emergency CS without labour. Significant p values are <0.05.

3.4 Simple Mediation Analysis by Birth Mode as a Potential Mediator

A mediation analysis was conducted to examine the hypothesis of whether birth mode acts as a mediator in the relationship between pre-eclampsia and *Bifidobacterium* levels in the infant gut microbiota at 3 months. Regarding the first path (a), there was no statistically significant association between pre-eclampsia (X) and birth mode (M), with a β-coefficient of 0.31 (95% CI -0.01, 0.63; P = 0.06). However, we did find a significant inverse association between birth mode (M) and *Bifidobacterium* levels in the infant gut microbiota (Y) in the second path (b), with a β-coefficient of -0.43 (95% CI -0.74, -0.11; P < 0.05). This suggests that infants born via cesarean section have lower *Bifidobacterium* levels compared to those delivered vaginally. The indirect effect, which represents the relationship from pre-eclampsia (X) to birth mode (M) as a mediator and then to *Bifidobacterium* levels in the infant (Y) as the dependent variable (a*b), did not yield statistically significant results. However, the value was close to be a significant which could be due to sample size. (a*b: -0.13; bootstrap 95% CI -0.34, 0.01). Furthermore, the direct effect in

path (c') between X and Y did not show a significant association (c': βc: -0.38; 95% CI -1.82, 1.04; P = 0.59) (Figure 3.1).

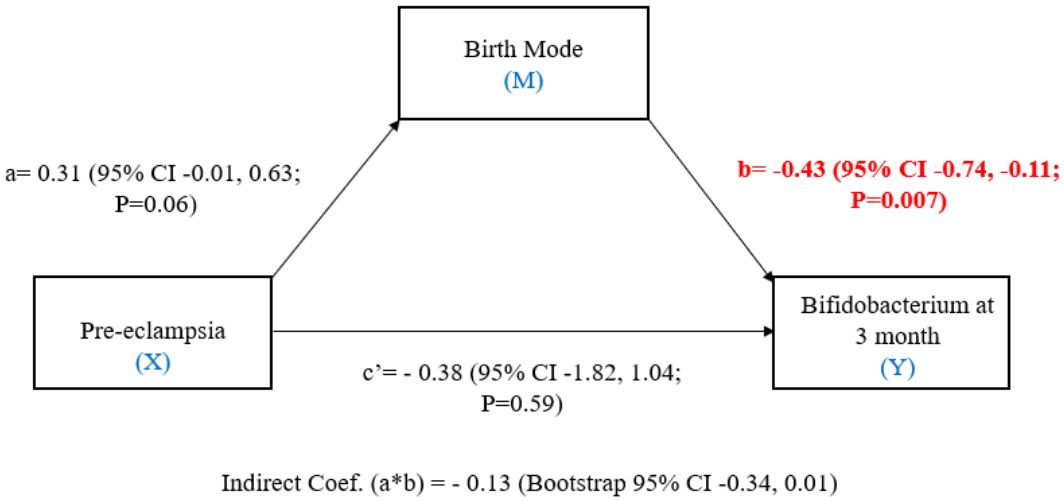


Figure 3.1 Simple mediation diagram of the association between pre-eclampsia and *Bifidobacterium* levels in infant gut microbiota at 3 month, and birth mode as a potential mediator. Beta-coefficient with 95% confidence interval of each path exhibits and significant differences ($p < 0.05$) are indicated in red.

3.5 Simple Mediation Analysis by Rupture of Membranes (ROM) as a Potential Mediator

The second mediation analysis aimed to investigate whether rupture of membranes (ROM) plays a mediating role in the relationship between pre-eclampsia and *Bifidobacterium* levels in the infant gut microbiota at 3 months. Only the second path (b) in the model demonstrated a significant inverse association between ROM (M) and *Bifidobacterium* levels in the infant gut microbiota (Y) with a β-coefficient of -0.28 (95% CI -0.48, -0.06; $P < 0.05$). This suggests that the rupture of

membranes is linked to lower levels of *Bifidobacterium* in the infant gut microbiota. As the occurrence of rupture of membranes increases, the levels of *Bifidobacterium* in the infant gut microbiota tend to decrease. However, the other paths and the indirect effect did not exhibit statistically significant associations, thereby failing to provide support for the mediating role of ROM in the relationship between pre-eclampsia and *Bifidobacterium* levels in the infant gut microbiota (Figure 3.2).

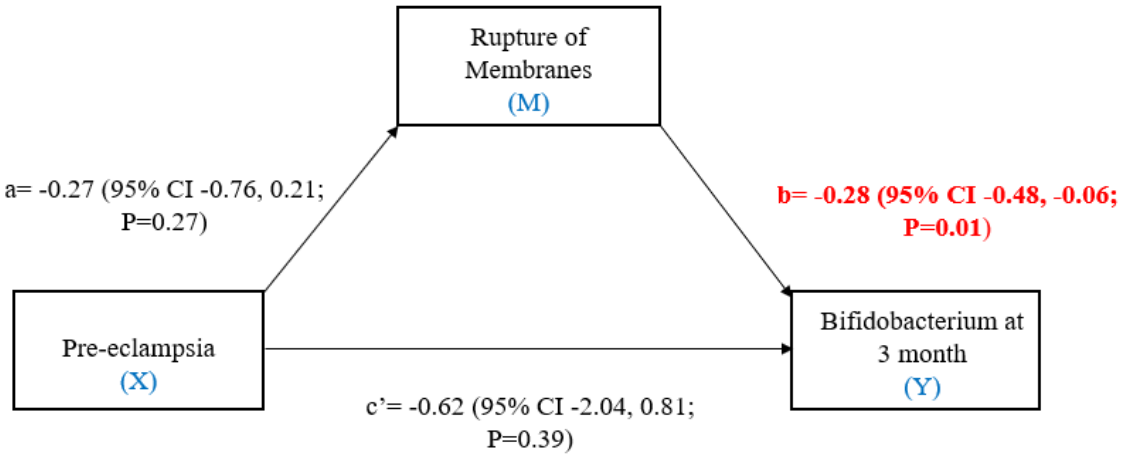


Figure 3.2 Simple mediation diagram of the association between pre-eclampsia and *Bifidobacterium* levels in infant gut microbiota at 3 months, and ROM as a potential mediator. Beta-coefficient with 95% confidence interval of each path exhibits and significant differences ($p < 0.05$) are indicated in red.

3.6 Sequential Mediation Analysis by Birth Mode and Breastfeeding Status

Since different modes of delivery can influence breastfeeding status, we employed a sequential mediation model with two potential mediators to explore whether the association between pre-

eclampsia and *Bifidobacterium* levels in infants could be mediated by birth mode and breastfeeding status. However, as shown in Figure 3.3, we only found a statistically significant association in the path (b1) between birth mode (M1) and *Bifidobacterium* levels in infants (Y) (βc: -0.42; 95% CI -0.74, -0.11; P=0.009) which is consistent with the results of the first mediation model (Figure 3.1). Overall, the indirect effect of pre-eclampsia on *Bifidobacterium* levels through birth mode and breastfeeding (a1*d*b2) did not exhibit any significant association in this model (Figure 3.3).

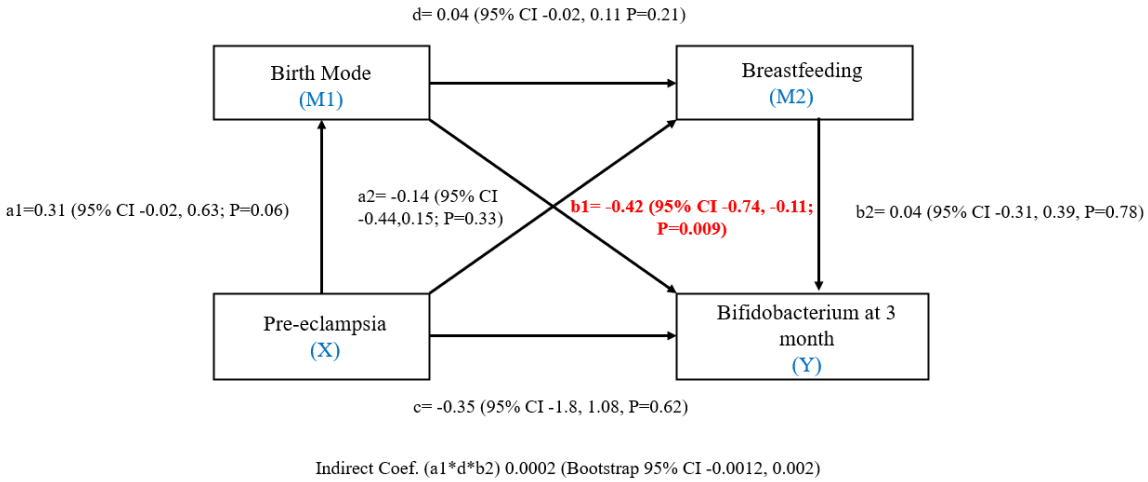


Figure 3.3 Sequential mediation analysis on the association between pre-eclampsia and *Bifidobacterium* levels in infant gut microbiota at 3 months, by birth mode and breastfeeding status as potential mediators. Beta-coefficient with 95% confidence interval of each path exhibits and significant differences ($p < 0.05$) are indicated in red.

CHAPTER 4

4. Discussion and Conclusions

4.1 Summary and Interpretations of Findings

In this prospective cohort study comprising 1429 healthy term infants, we examined the association between pre-eclampsia and the levels of *Bifidobacterium* in the gut microbiota of infants at 3 months of age. Additionally, we aimed to determine whether this association is independent of emergency cesarean section without labour and premature rupture of membranes (PROM) or if this association is mediated by birth mode and PROM. Overall, we discovered a significant inverse association between pre-eclampsia and *Bifidobacterium* levels in infant gut microbiota at 3 months of age (βc: -1.48, 95% CI: -2.9, -0.05, $p < 0.05$). However, this significant finding was not observed when we adjusted our model for potential confounding variables. In the next step, mediation analyses did not fully provide support for the hypothesis that birth mode and premature rupture of membranes (PROM) serve as pathways linking pre-eclampsia to *Bifidobacterium* levels in the gut microbiota of infants. To our knowledge this study represents the first investigation into the effects of pre-eclampsia on infant *Bifidobacterium* levels, a topic that has not been explored in existing literature. While there have been studies on gut microbiota dysbiosis in pre-eclamptic mothers, which suggest that these mothers exhibit variations in their gut microbiota compared to the normotensive group, particularly in crucial species like *Bifidobacterium*, which is found to be lower in such cases(95).

The human body can be considered a complex "super organism" made up of cells and a variety of symbiotic microorganisms. These microorganisms, particularly bacteria, outnumber our own cells and possess a significantly larger number of genes. Different parts of the body, such as the intestinal tract, skin, respiratory tract, and reproductive tract, harbor specific colonies of bacteria

that interact with their surrounding environment. Among these, the intestinal tract is the most intricate colonization environment, hosting the largest bacterial population in the human body. It is estimated that approximately 150 grams of microorganisms inhabit the intestines, forming what is known as the intestinal flora (62). This flora comprises numerous species of microbes that establish themselves from birth and are influenced by factors such as the mode of birth, infant feeding, lifestyle, medication, and the genetic makeup of the host. These microbial populations interact with each other and constantly exchange information with the host cells, playing crucial roles in digestion, maintaining and strengthening the intestinal barrier, immune defense, regulation of the nervous system, nutrition, and metabolism. Any disturbances in the gut microbiota can contribute to the development of various common metabolic disorders, including obesity, type 2 diabetes, hypertension, gestational mellitus, etc.(62,134).

Pre-eclampsia (PE) is a complex disorder that occurs specifically during pregnancy, involving multiple systems and presenting with hypertension and proteinuria after the 20th week of gestation. It represents a major global concern, contributing to significant maternal and perinatal morbidity and mortality, with an incidence of 3-8% in pregnancies worldwide. The underlying causes of PE include inadequate remodeling of spiral arteries, oxidative stress, dysfunction in the maternal vascular endothelium, and an exaggerated inflammatory response to pregnancy(135). However, the precise mechanisms responsible for PE development remain poorly understood. There is limited knowledge regarding the relationship between the gut microbiota and PE. Some studies have discovered the potential role of gut microbiome dysbiosis, which disrupts the integrity of the intestinal barrier and leads to the translocation of bacteria. This process triggers persistent systemic inflammation, ultimately contributing to the pathogenesis and progression of hypertension and PE(88,93). According to the findings of Kell et al., they propose that microbial infection serves as

the underlying cause of pre-eclampsia (PE). Bacterial products, particularly lipopolysaccharides (LPS) or endotoxins, are widely recognized for their potent ability to stimulate inflammation through the Toll-like receptor 4 (TLR4) signalling pathway. These substances are known to possess highly inflammatory properties and can trigger an innate immune response, thereby exacerbating the inflammatory process even further (94). In a study conducted by Liu et al. in 2017, it was observed that the injection of lipopolysaccharides (LPS) in rats resulted in the manifestation of signs resembling pre-eclampsia (PE). These signs included hypertension, proteinuria, maternal complications, as well as systemic and localized maternal inflammatory responses occurring at the placenta(97). In addition to these discoveries, a recent study conducted in East China identified notable changes in the abundance of specific bacterial genera, which were linked to the development of pre-eclampsia (PE). This study revealed an increased presence of *Blautia* and *Ruminococcus*, accompanied by a decreased level of *Bifidobacterium*(95). Notably, another research study demonstrated that hypertensive rats had a lower abundance of SCFA-producing bacteria in their gut microbiota compared to normotensive rats indicating an association between the levels of short-chain fatty acids (SCFAs) such as acetic acid, propionic acid, and butyric acid produced by the gut microbiota and hypertension because of their crucial role in influencing vascular tone(136,137). Interestingly, administering probiotics has shown promising results in reducing blood pressure, highlighting the significant role of the gut microbiota in regulating blood pressure(138). The genus *Bifidobacterium*, commonly employed in probiotics, stands out as it produces substantial quantities of SCFAs, which serve to inhibit pathogen accumulation by lowering intestinal pH (139,140). Moreover, the findings from the study conducted in East China revealed a strong negative correlation between a diminished presence of *Bifidobacterium* bacteria and key factors associated with hypertension and pre-eclampsia,

including systolic blood pressure (SBP), diastolic blood pressure (DBP), hyperlipidemia, and the level of aspartate aminotransferase. These findings underscore the potential contribution of *Bifidobacterium* and its associated SCFA production to the development of pre-eclampsia(95). Additionally, the study also identified a positive correlation between the increased abundance of bacteria belonging to the genera *Blautia* and *Ruminococcus* with obesity and dyslipidemia, both known risk factors for PE(95). Furthermore, Wang et al. (2020) investigated the gut microbiota composition of PE patients in the third trimester (T3) and found significant differences compared to the control group. Specifically, there were decreased levels of *Firmicutes* and increased levels of *Bacteroidetes*, *Proteobacteria*, and *Enterobacteriaceae* within the gut microbiota of PE patients(33).

In contrast to the existing literature that focuses on alterations in the maternal gut microbiota caused by pre-eclampsia, our study aimed to investigate the impact of pre-eclampsia on the gut microbiota of infants. The results of our analysis using the crude model indicate that infants born to pre-eclamptic mothers had lower levels of *Bifidobacterium* at 3 months of age compared to the normotensive group. However, this difference did not reach statistical significance in the adjusted model, which may be attributed to our small sample size (only 3.3% of our population had pre-eclampsia). Nevertheless, from a physiological standpoint, we believe that there is a possibility of early gut microbiota dysbiosis in infants born to pre-eclamptic mothers. This is because it is well-known that the vertical transmission of maternal microbes is a major pathway for establishing the initial gut microbiome in infants. In line with this, a study conducted on an Italian cohort of mother-infant pairs revealed that 50.7% of the microbial species present in the infant gut on the day of delivery originated from the mother's gut, vagina, oral cavity, or skin, and this proportion remained relatively stable over the following 4 months(141). Consequently, infants may inherit some aspects

of their mothers' gut microbiota, so it could be possible that infants born to pre-eclamptic mothers exhibit lower levels of *Bifidobacterium* due to the reduced abundance of this species in the maternal gut microbiota, which aligns with our statistical findings as well (Table 3.3).

Pre-eclamptic women face a higher risk of undergoing emergency cesarean section due to various medical reasons such as fetal distress and abnormal fetal heart rate. It is widely acknowledged that the mode of delivery plays a significant role in the initial colonization of the gut microbiota, with notable differences observed between infants delivered via cesarean section and those born vaginally(142). Cesarean section delivery disrupts the natural pattern of vertical transmission of the mother-to-infant microbiota. During vaginal delivery, the infant's gut is seeded with microorganisms from the mother's vaginal community, which is not available to infants born via cesarean section(143). For infants delivered by cesarean section, the first major microbial exposures originate from the maternal skin and the surrounding environment, with dominant genera such as *Staphylococcus*, *Corynebacterium*, and *Propionibacterium*, as opposed to the birth canal for vaginally born infants(143). Jakobsson et al. and Penders et al. have reported a reduced transmission of maternal *Bifidobacterium* and *Bacteroides* to infants born via cesarean section(144,145). As a subsequent impact, it is plausible that infants born to pre-eclamptic women may exhibit lower levels of *Bifidobacterium* in their gut due to the lack of exposure to maternal vaginal canal microbiota during a cesarean section, particularly in cases of elective CS and emergency CS without labour. These explanations align with the results of our mediation analyses, which are discussed in detail.

We conducted two simple mediation analyses and one sequential mediation analysis to examine whether the impact of pre-eclampsia on infant *Bifidobacterium* levels is mediated by birth mode and PROM (premature rupture of membranes) as potential mediator variables (Figure 3.1-3).

Contrary to our expectations, the indirect effects in these models did not yield statistically significant results, although they were very close. However, we obtained important findings in each of the mediation models. Firstly, in the simple mediation model involving birth mode as the mediator, we observed a significant inverse association in path "b", which represents the relationship between the mediator (birth mode) and the outcome (*Bifidobacterium* levels in infants) (Figure 3.1). In this analysis, we categorized birth mode into three ranks based on the transmission of microbiota to the infant: vaginal birth without IAP, vaginal birth with IAP, and the combination of three types of cesarean sections (elective CS + emergency CS with labour + emergency CS without labour). This analysis revealed that cesarean section delivery resulted in lower *Bifidobacterium* levels in infants compared to those delivered vaginally. These findings align with the results of Chen et al., who demonstrated an inverse relationship between *Bifidobacterium* quantities and cesarean section births, with and without labour(73). Additionally, we found in this model the rates of IAP administration increased from vaginal birth to cesarean section in our model, which contributes to alterations in the infant gut microbiota and reduction of vertical transmission maternal microbes to infant, consistent with other studies(73,146). Secondly, the sequential mediation analysis involving birth mode and breastfeeding status demonstrated a statistically significant association in path "b1," further supporting the inverse impact of cesarean section on infants' *Bifidobacterium* levels (Figure 3.3). Thirdly, we obtained an interesting result when examining the role of rupture of membranes (ROM). It is worth noting that due to the limited number of cases with premature rupture of membranes (PROM) in our database, we included the ROM variable, which encompasses PROM as well, to increase the sample size. Similarly, path "b" revealed a significant inverse association between ROM and *Bifidobacterium* levels in infants. This suggests that as the occurrence of rupture of membranes increases the levels of

Bifidobacterium in the gut microbiota of infants born to pre-eclamptic mothers tend to decrease (Figure 3.2). To the best of our knowledge, there is limited evidence regarding the association between rupture of membranes (ROM) and early gut microbiota composition. However, our findings align with previous literature and are supported by biological considerations. It is recognized that the initial exposure of the neonate to the birth canal and the surrounding environment occurs through the rupture of the amniotic membranes(64). This critical event marks the beginning of microbiota establishment and transmission from the mother to the baby. Nevertheless, further analyses are required to understand how *Bifidobacterium* levels in infants could be reduced through ROM, particularly in the context of pre-eclampsia.

4.2 Strengths of This Study

This thesis work possesses several strengths and limitations. One notable strength is its originality, as it represents the first investigation into the impact of pre-eclampsia and emergency CS without labour on *Bifidobacterium* levels in the infant gut microbiota. This unique aspect adds novelty to the thesis, as no prior reports on this topic have been published. Furthermore, this thesis used data gathered from participants at three different sites such as Edmonton, Winnipeg, and Vancouver of the CHILD longitudinal cohort. This birth cohort is well-characterized and provides a representative sample of the Canadian general population. The prospective longitudinal study design enables the collection of information over time, allowing for the measurement of changes in the outcome variable as time progresses. Temporal sequencing is another strength considered in this study. The exposure variables, such as pre-eclampsia and birth mode, occurred prior to the collection of stool samples. This temporal relationship enhances the validity of the findings. Moreover, the study obtained data on pre-eclampsia, birth mode, intra-partum antibiotics prophylaxis (IAP), duration of rupture of membranes, breastfeeding status, and other variables

from hospital records or CHILD questionnaires. These sources undergo an internal validity test, standardization, and validation before the study's commencement, ensuring the reliability of the collected data. Finally, the inclusion of statistical mediation analyses that align with our biological hypothesis represents another strength of this research work. These analyses provide support for the hypothesis from a biological perspective.

4.3 Limitations of This Study

Like any observational study, our research has certain limitations that should be considered. Firstly, a remarkable limitation is the small sample size, which significantly impacts the power of our study. In this study, we were unable to include women who had experienced premature delivery in order to increase the sample size of pre-eclamptic women, as prematurity was among the exclusion criteria in the CHILD cohort study. Despite our efforts to employ a multiple regression model to address potential confounding variables suggested by the Directed Acyclic Graph (DAG), the limited number of pre-eclamptic women in our sample prevented us from detecting significant differences in the adjusted models. Therefore, future studies with larger sample sizes and the inclusion of a wider range of possible confounders are necessary to further explore the association between the exposure and outcome variables. The second limitation relates to the lack of sufficient information regarding labour induction among our participants, particularly among those who were diagnosed with pre-eclampsia. It is well-established that labour induction is performed in some cases of pre-eclampsia to deliver the baby and placenta. However, we believe that the duration and specific methods of labour induction, such as the use of oxytocin, may influence early gut microbiota alterations. Unfortunately, we were unable to consider this potential factor in our thesis due to the lack of available data. Furthermore, in this study, we did not assess women with pre-eclampsia who were younger than 18 years old or became pregnant through in

vitro fertilization (IVF) due to the limitations within the CHILD study database, as these groups are excluded from the CHILD cohort. Younger age and IVF are both risk factors for developing pre-eclampsia, and including young preeclamptic women or those who underwent IVF in the study would offer greater insights. Also, due to the limited information on pre-eclamptic mothers in this study, we were unable to categorize them based on the timing of pre-eclampsia development, distinguishing between early onset (< 34 weeks) and late onset pre-eclampsia (≥ 34 weeks). These factors which mentioned have the potential to influence the findings and provide more comprehensive insights. Another limitation of our study is that the majority of participants were recruited from urban areas, which may restrict the generalizability and external validity of our findings. This geographical bias limits our ability to draw conclusions that are applicable to more diverse populations or those residing in rural areas. Therefore, caution should be exercised when extrapolating our results to broader populations. Lastly. it is important to note that we only assessed total *Bifidobacterium* and measuring specific species of *Bifidobacterium* might provide more information for us.

4.4 Bias and Confounding

The inherent and unavoidable presence of bias is a characteristic of any epidemiological research study conducted in an observational manner. Despite the fact that the CHILD study was designed as a cohort study, and all data were collected using rigorous protocols to minimize bias, it is still important to consider the possibility of some sources of bias.

4.4.1 Selection Bias

Selection bias arises when the groups being studied lack comparability, and the obtained sample does not accurately represent the overall population. In the CHILD cohort, efforts were made to minimize this bias by utilizing multiple sample sites and employing diverse recruitment methods,

such as clinics, tradeshows, fax, and phone calls. Also, the CHILD cohort has low rates of loss to follow up, with 92% retention rate which enhances the study's integrity and facilitates the reliable tracking of participants over time. Furthermore, specific inclusion and exclusion criteria were predetermined, resulting in a highly homogeneous participant pool. However, it is worth noting that specific inclusions and exclusion criteria, combined with the predominantly urban and well-educated White Caucasian background of the CHILD study participants, could have introduced some selection bias. This limits the generalizability of our findings to other population groups, as the entire general population may not have been adequately represented.

4.4.2 Measurement Bias

Measurement bias occurs when there is a systematic error in measuring or categorizing an outcome or exposure. This type of bias is commonly observed in intervention studies, where it is crucial to ensure that the assessor remains unaware of the intervention while assessing the outcome, or to blind the study participants to the intervention. To minimize measurement bias in this study, information on the mode of delivery and pre-eclampsia condition within the respective groups was gathered using hospital records, validated CHILD questionnaires, and structured interviews conducted by trained interviewers at specific time points. These data collection methods were implemented consistently across all study sites to ensure uniformity and reduce the potential for measurement bias. Although it is possible for inherent biases to arise from sample collection, DNA extraction, gene amplification/sequencing, and qPCR methods, these potential biases are likely minimized due to strict and standardized CHILD protocol. Moreover, these procedures are consistently carried out by the same research specialist for every 3-month stool sample, further reducing the likelihood of measurement bias. Furthermore, in order to accommodate the limitations of the Hayes PROCESS v3.5 macro package in the SPSS software for conducting

mediation analysis, we categorized the birth mode mediator variable as a categorical variable with a minimum of three categories. Instead of utilizing only one or two categories, we included vaginal birth without IAP, vaginal birth with IAP, and a combination of all cesarean section (CS) types as the mediator variable. Although the original hypothesis focused on emergency CS without labour as a potential mediator, we had to consider the various childbirth possibilities for women with pre-eclampsia by including at least three variables and combining the different CS types that occur for pre-eclamptic women. This categorization aligns with biological considerations due to the diversity of childbirth experiences in this population. While the act of combining all CS types into a categorical variable for birth mode in the mediation analysis could introduce measurement bias, we aimed to mitigate this bias by distinguishing between different CS types, such as elective cesarean sections and emergency cesarean sections with and without labour. This approach allows us to investigate the effect of emergency CS without labour on the variables of interest in our mediation analysis. However, it would be preferable to explore alternative statistical approaches that facilitate the specific inclusion of emergency CS without labour as a mediator variable to examine its effect.

4.4.3 Confounding bias

Confounding bias arises when a third factor distorts the relationship between an exposure and an outcome. In simpler terms, confounding variables are associated with both the exposure and the outcome but not on the causal pathway. It is crucial to account for confounding during data analysis when seeking to obtain the most accurate estimation of the association between an exposure and an outcome in an epidemiological study. This ensures that any potential confounding factors are appropriately controlled for, leading to more reliable findings. In our study, we aimed to minimize confounding bias by identifying and adjusting for potential confounding variables associated with

the exposure and outcome of interest. To accomplish this, we utilized a Directed Acyclic Graph (DAG) and performed multiple logistic regression to adjust our models accordingly. Despite our efforts to adjust for potential covariates suggested by the DAG, it is possible that there may still be some degree of confounding bias present in our study. This is indicated by the lack of statistically significant results in the adjusted logistic regression model. Therefore, it is plausible that there may be additional unidentified confounders that were not controlled for in our study.

4.5 Clinical Importance

The microbiome undergoes rapid colonization in early life and is highly influenced by environmental factors that impact its composition and function. Any disruption in their balance, known as dysbiosis, can have long-lasting effects on health and increase the risk of various diseases during infancy and later stages of life. Factors such as mode of delivery, antibiotic use, and maternal health conditions like pre-eclampsia can negatively affect the early gut microbiota, particularly the levels of *Bifidobacterium*. Alterations in *Bifidobacterium* levels have been associated with conditions like obesity, allergies, and other immune and metabolic diseases in infancy and childhood. Therefore, it is crucial to be mindful of the possibility of reduced levels of *Bifidobacterium* in infants born to pre-eclamptic mothers or delivered through cesarean section (CS).

While there is no known cure for pre-eclampsia, it is possible to reduce some risk factors in susceptible pregnant women. Maternal obesity and high BMI not only negatively impact *Bifidobacterium* levels in the infant gut microbiota but are also major risk factors for developing pre-eclampsia. A recent review research study has shown that high maternal pre-gestational body mass index is associated with an altered infant microbiome from the early days of life until two years of age, with *Bifidobacterium* being one of the most affected genera and linked to a higher

risk of overweight and obesity in infants(98). As a result, healthcare professionals should educate pregnant women during prenatal visits, encouraging them to maintain a healthy diet and engage in physical activity to maintain a normal BMI and reduce the risk of pre-eclampsia, thus preventing early disruptions in the gut microbiota. Furthermore, high maternal BMI can also influence the composition and diversity of the breast milk microbiota, negatively impacting *Bifidobacterium* levels(98).

In cases where pre-eclamptic women undergo cesarean section, it is important to promote exclusive breastfeeding as it can help compensate for the reduction in *Bifidobacterium* levels caused by the CS. Human milk oligosaccharides (HMOs) have been found to play a crucial role in promoting the growth of beneficial bacteria like *Bifidobacterium* and *Bacteroides*(80). HMOs have prebiotic effects, modulate innate immune and intestinal cell responses, and possess anti-inflammatory properties, all of which contribute to the overall health of infants(66,80). Therefore, maintaining a normal maternal BMI, healthy diet and encouraging breastfeeding can be beneficial for early gut microbiota development in infants born to pre-eclamptic mothers.

4.6 Future Research Consideration

Further research is required with larger sample sizes and the inclusion of additional confounding variables thoroughly investigate the impact of pre-eclampsia and birth mode on *Bifidobacterium* levels in the gut microbiota of infants at three months of age. Additionally, future studies should investigate the role of human milk oligosaccharides (HMOs) in promoting the growth of beneficial bacteria, including *Bifidobacterium*, in infants born to mothers with pre-eclampsia. Comparative studies comparing infants born to pre-eclamptic mothers with those born to mothers with a normal BMI and uncomplicated pregnancies could provide valuable insights into the specific effects of pre-eclampsia and maternal obesity on the gut microbiota, *Bifidobacterium* levels, and disease

outcomes. Finally, conducting a case-control study involving pregnant women with gestational hypertension as the control group and pre-eclamptic patients as cases could be a suitable study design for future research.

4.7 Conclusions

In conclusion, infants born to pre-eclamptic mothers exhibited lower levels of *Bifidobacterium* in their gut at 3 months of age. The results of multiple logistic regression suggested that emergency CS without labour and PROM were confounding/mediating factors. There is a possibility that cesarean section (CS), including elective and emergency, with and without labour, might mediate the association between pre-eclampsia and Bifidobacterium in infant gut at 3 months.

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Appendix: Supplementary Material for Chapter 3

Table S3.1 Associations between pre-eclampsia and the absolute quantity of genus *Bifidobacterium* in infant gut at 3 months, by univariable linear regression analysis.

		Beta Coefficient (95% CI)	*P* value
Pre-eclampsia	Crude Model	-3.71 (-11.16, 3.75)	0.33

Analysis is conducted by linear regression. To improve the normality, the absolute quantity of genus Bifidobacterium is Box–Cox transformed. The reference group is normotensive women. Significant p value is <0.05.