Understanding the role of *Clostridioides difficile* and Vitamin D supplementation in shaping the gut microbiome of Canadian infants

by

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Abstract

Introduction

The gut microbiome is shaped during infancy and has an important role in the development of both the immune and metabolic systems. Many early life exposures and events contribute to the composition of this complex intestinal environment, notably early infant diet and breastfeeding. *Clostridioides difficile* colonization occurs in up to half of infants under the age of 3 months and is strongly predicted by formula feeding. Although this microbe does not appear to pose any immediate risks for infants (colonization is largely asymptomatic), its presence has been associated with susceptibility to chronic disease later in childhood, perhaps by promoting changes in the gut microbiome including increased opportunity for colonization with other pathogenic bacteria. Infant feeding (breast and formula) has an important role in shaping gut microbiome and predicting *C. difficile* colonization, yet little is known about the relationship between Vitamin D and the gut microbiome of young infants. Meanwhile, Vitamin D supplementation is recommended by pediatric health professionals in Canada for all breastfed infants, and many maternal multivitamins/prenatal vitamins contain Vitamin D which could influence the Vitamin D intake of infants during pregnancy and lactation. Vitamin D has also been associated with changes to the microbiome and *C. difficile* abundance in infancy, thus meriting further investigation.

Objectives

This thesis aims to describe the differences in gut microbiome according to *C. difficile* colonization status and breastfeeding status (Study 1) and determine if maternal and infant intake

of an important micronutrient, Vitamin D, is associated with *C. difficile* colonization in breastfed infants at 3 months of age (Study 2).

Methods

Study 1 (presented in Chapter 2) is a descriptive analysis conducted on 1562 infants whose mothers were enrolled at the Edmonton, Vancouver or Winnipeg Canadian Healthy Infant Longitudinal Development Study sites. Study 2 (presented in Chapter 3) was a cross-sectional analysis conducted on 1157 mother/infant pairs. All participants had complete data describing breastfeeding status at 3 months and each provided a stool sample which was profiled using 16S rRNA sequencing and targeted detection of *C. difficile* with real time qPCR. Fecal metabolites and sIgA were also detected using NMR and an Immundiagnostik ELISA kit. Parametric and non-parametric tests, where appropriate, were used to describe the relationship between *C. difficile* colonization, Vitamin D supplement intake, metabolites, sIgA and gut microbiome diversity. Multivariate linear regression (MaAslin) was used to determine associations between *C. difficile* and the gut microbiota composition (Study 1) as well as Vitamin D supplementation and the gut microbiota composition (Study 2). PERMANOVA was used to determine the significance of global diversity changes (beta diversity) with *C. difficile* colonization. Logistic regression analyses were performed to determine the association between Vitamin D supplementation and *C. difficile* colonization, while adjusting for relevant covariates.

Results

C. difficile colonization was differentially associated with the gut microbiome in breastfed versus formula fed infants, with more notable differences among exclusively breastfed

infants and very few among exclusively formula fed infants. These differences included increased alpha diversity, significant between sample heterogeneity (beta-diversity), decreased fecal sIgA, increased abundance of Firmicutes, decreased Bifidobacteriaceae and increased concentrations of SCFA's in those colonized with *C. difficile* relative to non-carriers. Neither maternal (pre or post-natal) nor infant Vitamin D supplementation was associated with *C. difficile* colonization at 3 months of age, although there was a trend towards lower odds of colonization with the use of infant Vitamin D drops in exclusively breastfed infants, following adjustment for relevant covariates (OR: 0.86, 95% CI: 0.51-1.46).

Conclusion

The findings of Study 1 provide evidence of important changes in the infant gut microbiome that are associated with *C. difficile* colonization, particularly in exclusively breastfed infants and that there were important differences between all feeding groups. Upon exploring a possible predictor of *C. difficile* colonization in exclusively breastfed infants (Study 2), we found no significant association between Vitamin D supplementation and *C. difficile* carriage. Thus, current guidelines surrounding Vitamin D supplementation do not appear to influence *C. difficile* colonization.

Preface

This thesis is an original work by Kelsea Michelle Drall. The thesis was written in accordance to the guidelines set by the Faculty of Graduate Studies and Research at the University of Alberta.

This thesis is comprised of 4 separate sections/chapters:

Chapter 1 consists of a thorough literature review on Vitamin D, breastfeeding, infant gut microbiota and asymptomatic *Clostridioides difficile* colonization including animal models studies, as well as clinical studies in adults, infants and children. The review is followed by an outline of the overall purpose, objectives, hypotheses, and sample size calculation for the study.

Chapter 2 presents the first research study and study findings. This descriptive study explores differences in the gut microbiome according to *Clostridioides difficile* colonization and breastfeeding status of 3 month old infants.

The results of the second study are presented in Chapter 3. The impact of Vitamin D supplements (both maternal and infant) on *C. difficile* colonization and the infant gut microbiota was examined for 3-month old infants enrolled in the Canadian Healthy Infant Longitudinal Development Study.

General discussion and conclusions based on these works are presented in the final chapter of this thesis (Chapter 4). This chapter also covers limitations, strengths, bias assessment and a summary of the significance of these findings for families and in clinical practice.

Dedication

I dedicate this thesis to my mother: although I may blame you for my allergies and dysbiotic gut microbiome, I would not be where I am today without your support and love. And to the extra-ordinary families who participated in the CHILD Study: for without them, I would have no thesis.

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My sincerest gratitude goes out to the families and staff of the CHILD (Canadian Healthy Infant Longitudinal Development) study for the opportunity to work with such an amazing cohort and such valuable data. My interest in research began in second year of undergraduate studies, when I volunteered at the Edmonton CHILD site, so a special thanks to their team for sparking an interest that has led me to this point in my research career.

Last but not least, I would like to thank my family and friends who have been so generous, caring and supportive. You inspire me and I am grateful your support.

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List of Abbreviations

BMI	Body Mass Index
CHILD	Canadian Healthy Infant Longitudinal Development Study
CPS	Canadian Pediatric Society
CDI	*Clostridium/Clostridioides difficile* Infection
CD	*Clostridioides difficile* Colonization
HMO	Human Milk Oligosaccharide
IL	Interleukin
IDT	Integrated DNA Technologies
IBD	Irritable Bowel Disease
IU	International Units
KO	Knock Out
LPS	Lipopolysaccharide
OTU	Operational Taxonomic Unit
MaAslin	Multivariate Association with Linear Models
NMR	Nuclear Magnetic Resonance Spectroscopy
PERMANOVA	Permutational Multivariate Analysis of Variance
PCoA	Principle Coordinate Analysis
PCR	Polymerase Chain Reaction
SCFA	Short Chain Fatty Acid
sIgA	Secretory Immunoglobulin A
TLR	Toll-like Receptors
VDR	Vitamin D Receptor
VDD	Vitamin D Deficiency
UVB	Ultraviolet Light B
UC	Ulcerative Colitis
25(OH)D	25 Hydroxy-Vitamin D (Calcifediol)
$1,25(OH)_2D_3$	Calcitriol

Chapter 1

1. Introduction

1.1 Literature Review

1.1.1 The development of the gut microbiome in infancy

Each and every one of us are inhabited by a complex community of microorganisms, which are often collectively referred to as our microbiome. The majority of bacteria, also called microbiota, can be found in the large intestine, where they have evolved alongside humans to assist us with many biological functions including immune development, barrier to toxins and pathogens and food digestion (1,2). The gut microbiome is shaped during infancy by numerous factors which contribute to the heterogeneity and individuality of our adult microbiota: however, the natural progression follows a similar discourse.

Microbial colonization begins at birth: vaginally born infants are first exposed to their mothers fecal and vaginal bacteria (ie. Lactobacilli and Escherichia) whereas infants born via caesarean section are exposed to microbes commonly found on the skin (ie. Staphylococcus) (3–5). The newborn intestinal environment is quite different from the adult gut as it is oxygenated and contains aerobic bacteria, such as Enterobacteria, that can utilize the oxygen and promote colonization by obligate anaerobic species (5,6). Over time, strict anaerobes such as Bifidobacteria, Bacteroides spp. and members of the Firmicutes phylum begin to colonize, although the microbiota profile remains distinct based on mode of delivery. Notably, those infants born via caesarean section have a higher alpha diversity, a lower abundance of Bacteroides spp. and a higher abundance of Clostridium spp. and these differences persist through infancy (7,8) The infant gut microbiota continue to shift dynamically and increase in diversity based on a variety of exposures until around 3 years of age, where it will then stabilize

and persist throughout adulthood (6). Other influential exposures, besides mode of delivery, that contribute the infant gut microbiota development include antibiotic exposure, infant age, geographic location, pets, siblings, and of course, infant diet (4,9,10). Our infant gut microbiota are important for future health, as they play a role in gut homeostasis by creating and contributing to the intestinal functional and physical barrier defense (11). Commensal gut microbiota produce short chain fatty acids and other signalling molecules that stimulate the production of mucus, anti-inflammatory cytokines, antimicrobial peptides and sIgA which all act as a functional barrier to external pathogens and antigens (12). These molecules in return, further control the composition of the gut microbiota by ensure a tolerogenic response. The physical barrier of tightly adhering intestinal epithelial cells serves as a physical line of defense preventing microbe invasion and subsequent dysbiosis (11).

1.1.2 Breastfeeding and the infant gut microbiome

Another major driver of gut microbiota composition is infant feeding mode (ie. breast or formula feeding). As the nutrient and biomolecular components of infant formula and breastmilk differ considerably, infants who are exclusively breastfed have a microbiome that is undoubtedly distinct from those who are exclusively formula fed (5,10,13). Breastmilk human milk oligosaccharides (HMOs) have a unique and complex structure and they selectively promote gut colonization with beneficial microbiota, notably *Bifidobacteria* spp (14). These bacteria dominate the microbiome of breastfed infants making the diversity of microbes in these babies' lower relative to formula fed infants (15). Although some brands of formula attempt to incorporate prebiotics (ie. galacto- and fructo-oligosaccharides) as equivalents to HMOs, these have not yet proven to have the same beneficial effect on the gut microbiota composition and are

not widely available (16). Furthermore, breastmilk contains other active molecules such as secretory immunoglobulin A (sIgA) and lactoferrin which contribute to the microbiome composition by binding and inhibiting infiltration by potentially pathogenic gut microbes and priming the immune system (17,18). Furthermore, differences in the infant gut microbiota and diet influences the metabolites they produce, including the short chain fatty acids (SCFA) acetate, butyrate and propionate (19,20). The most abundant SCFA is acetate, which can be produced by most gut microbiota, notably Bifidobacteria, making it more abundant in breastfed infants (21). Butyrate contributes to a very small proportion of all SCFA's produced in early infancy and is utilized by the epithelial cells of the large intestine (22). Butyrate producers included Lachnospiraceae and Ruminoccocaceae, which are characteristic of a more mature gut and more commonly found in formula fed infants or after weaning (23). Propionate is also absorbed and transported to the liver by the portal vein. Propionate is produced by microbes of the Bacteroidetes and Firmicutes phyla. This coincides with the fact that propionate and butyrate are more abundant in formula fed infants, where the gut microbiota are more distinguished (23,24). It is thought that a combination of breastmilk microbiota, macronutrients, micronutrients, biomolecules and their effect on gut microbiota and metabolites contribute to beneficial outcomes in breastfed children including reduced risk for obesity and allergic disease (25–27). Incidentally, formula fed infants tend to be at higher risk of developing these chronic diseases in later childhood and are more often colonized with potentially harmful microbes, including *Clostridioides difficile* (28–30).

1.1.3 Clostridioides difficile colonization in infants

Clostridioides (previously *Clostridium*) *difficile* colonization is characterized by the positive detection of *C. difficile* in a fecal sample. The presence of *C. difficile* in infants is largely asymptomatic but is the main cause of antibiotic-associated diarrhea and colitis in adults (31,32). This gram-positive spore forming obligate anaerobe achieves it pathogenicity through production of toxins A and B (tcdA and tcdB) which contribute to the infectious symptoms experienced with *Clostridioides difficile infection* (CDI). These may range from mild diarrhea to fatal colitis and are dependent on other risk factors including age, length of hospital stay and antibiotic or proton pump inhibitor use (33). The prevalence of *C. difficile* colonization in infants is typically between 10-50% (compared to 3-5% in adults), with less than 20% of cases being toxigenic (34,35). There are a number of hypotheses as to why *C. difficile* colonization in infants is asymptomatic: toxin receptors are absent at a young age, infants are predominantly colonized with non-toxigenic strains and breastmilk factors, including HMOs and secretory IgA, may neutralize *C. difficile* toxins (35–38). In infants, colonization status is strongly predicted by formula feeding whereas breastfeeding is associated with lower relative abundances and prevalence of *C. difficile* (15,39). Other factors that may contribute to colonization status are season of birth, length of hospital stay and age at stool sample collection (34).

Although *C. difficile* colonization in infants may not be accompanied by diarrheal illness, it has been associated with later asthma, food allergy, antibiotic resistance and microbial dysbiosis (29,30,40). The relationship between *C. difficile* and these childhood diseases, including the effect on other gut microbiota, requires further examination in infants. In healthy adults, the resident commensal gut microbiota provide defense and protection against *C. difficile* colonization, also known as colonization resistance, by creating a selective niche that hinders *C.*

difficile proliferation (ie. low availability of nutrients, less favourable pH, less access to gut mucosal epithelial cells) (41). The infant gut microbiota is still developing until nearly 2 year of age and thus may not sustain the same protection against colonization (6). Of the limited findings to date, we see that bifidobacteria are inversely correlated and ruminoccoci are positively correlated with *C. difficile* colonization in infancy, which differs from the expected succession during development (42). Altered gut homeostasis and gut microbiota development can also result when micronutrient sources are low, including Vitamin D. Talsness et al. (2017) found that maternal Vitamin D supplementation in pregnancy was associated with lower abundance of *C. difficile* colonization in a group of 1 month old infants (43).

1.1.4 The importance of Vitamin D and breastfeeding in pediatric health

Infancy is a time of nutritional demand where breastmilk, formula and Vitamin D supplements or drops serve as the only source of Vitamin D for an infant. Despite the relatively low Vitamin D content of breastmilk, bioavailability may be greater than from other sources and breastfeeding has been associated with a number of positive health outcomes including reduced risk for overweight, obesity and allergic diseases (44–46). There are also recommendations around supplementation for optimal nutrition (for both the infant and the mother) during breastfeeding; however, here we will focus specifically on Vitamin D (16,18,47–49). Vitamin D is the most commonly used supplement among infants in Canada (50). This is because it is shown to be a vital nutrient in many biological processes including calcium absorption and bone mineralization for infant growth, immune system development and protection against infection (51).

1.1.5 Current breastfeeding and supplementation guidelines in Canada

In an effort to prevent infant Vitamin D deficiency, the Canadian Paediatric Society recommends 400 IU of Vitamin D_3 for all breastfed infants (52). Thus, it is no surprise that three-quarters of Canadian breastfed infants are supplemented with a liquid preparation of Vitamin D_3 (50). In the Canadian Healthy Infant Longitudinal Development (CHILD) Study, nearly 75% of exclusively breastfed infants received a Vitamin D_3 supplement during the first 3 months of life, compared to 25% use among exclusively formula fed infants as formula is already supplemented. Furthermore, the Government of Canada outlines supplement recommendations for mothers during pregnancy (53). Supplement use is recommended during pregnancy to prevent maternal deficiency, but also because Vitamin D has been shown to cross the placenta and influence circulating levels in the fetus (54). For this reason, Vitamin D deficient mothers who are subsequently supplemented with Vitamin D give birth to infants with higher umbilical cord serum 25(OH)D levels (55). During the postnatal period, the prevalence of breastfeeding in Canada is 90% (2012) with one-quarter of mothers continuing exclusive breastfeeding for greater than 6 months (53). Although maternal breastmilk is considered the best form of early life nutrition for infants, the bioavailability of Vitamin D in breastmilk is low, with less than 20% of maternal Vitamin D being transferred to the infant via breastfeeding (49,56). Maternal supplementation can increase the concentrations of Vitamin D in breastmilk and increase circulating concentrations in both the mother and the breastfed infant (57).

Vitamin D dosing and deficiency measures are based on the amount needed for bone mineralization and calcium absorption but specific levels and dosing of Vitamin D for other diseases such as inflammatory bowel disease and allergic diseases have yet to be confirmed. This includes the potentially beneficial or adverse effects on intestinal homeostasis and the gut

microbiota. Multiple meta-analyses, have shown that the odds being Vitamin D deficient are around 2 times greater in patients with either Crohn's disease and ulcerative colitis (UC), compared to healthy controls (58,59). The evidence surrounding allergic diseases, such as allergic rhinitis, asthma and atopic dermatitis, is less strong but still suggests that lower serum Vitamin D is correlated with higher prevalence of these conditions (60–62). As shown, Vitamin D deficiency seems to be associated with various health outcomes at the population level, making a mechanistic and a physiological look at its role in disease desirable.

1.1.5 Vitamin D: Sources, metabolism and physiological response

Vitamin D is a fat-soluble vitamin with many molecular forms. Its precursor (Vitamin D_3) is derived primarily from the metabolism of 7-dehydrocholesterol in epidermal cells that have been exposed to ultra-violet (UVB) light (**Figure 1.1**) (63). Other dietary sources of the vitamin include dietary supplements, milk, fortified foods and fish oils (64). In general, there are two forms of Vitamin D in supplements and food: Vitamin D_2 and Vitamin D_3. Breastmilk and most supplements contain Vitamin D_3 (65); however, evidence shows that both perform the same biological roles and are generally considered equivalent in potency and efficacy (66).

Vitamin D insufficiency is determined by measuring serum calcifediol (25(OH)D) and is classified as <50 nmol/L, while deficiency is even lower at <30 nmol/L (66) . These serum amounts are set based on optimal calcium absorption and circulating parathyroid hormone levels for health. Dietary intake of Vitamin D from supplements and food is correlated with an increase in serum 25(OH)D, however the relationship is not linear (66). Particularly, those with higher serum 25(OH)D typically require a larger dose or intake of Vitamin D to increase their circulating levels, compared to those with lower (<50 nmol/L) serum 25(OH)D.

In order for Vitamin D to elicit a physiological response, it must be metabolized into its active form, calcitriol (1,25$(OH)_2D_3$), and then bind with the nuclear Vitamin D receptor (VDR)(67). The conversion of preVitamin D_3 to 1,25$(OH)_2D_3$ begins in the liver. The hepatic enzyme, 25-hydrolase (CYP2R1), catalyzes the conversion of preVitamin D_3 to calcifediol (25(OH)D) which is then metabolized by 1,α-hydroxylase (CYP27B1) to make 1,25$(OH)_2D_3$ in either the kidney or extra-renal cells, especially those that possess VDRs (Figure 1.1).

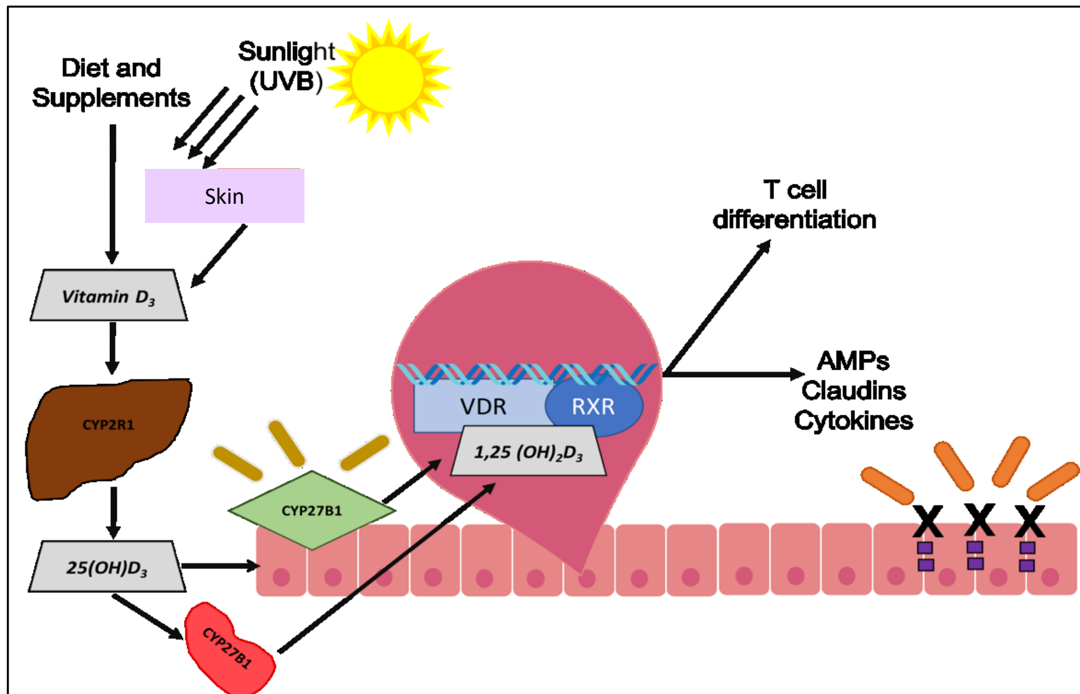

Figure 1.1: The acquisition and metabolism of Vitamin D for activation of Vitamin D receptors. VDRs can then act as transcription factors for a variety of molecular pathways that change the intestinal environment and gut homeostasis. Microbes can also play a role in the expression of enzymes involved in Vitamin D metabolism (ie. CYP27B1).

Cells where VDR is found include T cells, B cells, dendritic cells and importantly VDR is expressed in intestinal enterocytes (68). Active $1,25(OH)_2D_3$ ultimately binds VDR, which then acts as a transcription factor and initiates signaling pathways involved in both adaptive and innate immunity, inflammatory responses, secretion of antimicrobial peptides and production of tight-junction proteins (51). VDR is highly expressed in the proximal colon and acts as a transcription factor responsible for approximately 3% of the human genome (over 1000 genes) (69,70). These include genes for production of defensins, cathelicidin, claudins, TLR2, zonulin occludens, and NOD2. Importantly, these proteins help with the maintenance of the host intestinal physical and functional barrier and promote tolerance to gut microbiota. Any disruption in the physical gut epithelial cell barrier and resident microbiota may increase the chance for invasion and colonization by pathogens, including *C. difficile.*

1.1.6 Current evidence regarding the gut microbiome and Vitamin D

A recent systematic review summarizes all the up-to-date information regarding Vitamin D and the gut microbiota in animal, cell and human (*in vivo*) studies (71). Of these studies, few are in adults and even fewer in infants. Luthold and colleagues (2017) performed a cross-sectional analysis of a group of 150 healthy adults and determined that Vitamin D levels were not associated with levels of inflammatory markers including IFN-γ, TNF-α, IL-6 and IL-10 (72). Those that were placed into the highest tertile of Vitamin D intake (7.56-39.87 μg/day) had an increased abundance of *Prevotella*, but decreased *Haemophilus* and *Veillonella* compared to the lowest tertile of intake (1.66-4.95 μg/day). *Bifidobacteria* and *Coprococcus* abundances decreased as serum 25(OH)D increased.

Another cohort took biopsies from along the length of the GI tract and stool samples from 15 healthy adult volunteers (73). Samples were taken both before and after supplementation with Vitamin D_3. Each participant received 980 IU/kg body weight of Vitamin D_3 per week. After 4 weeks, the dose was reduced to 490 IU/kg body weight per week. Following 8 weeks of supplementation, the authors observed an increase in microbial richness in the gastric antrum and duodenum in those supplemented with the higher dose of Vitamin D. The most significant compositional changes were observed in the upper GI tract, namely increased abundance of Proteobacteria with Vitamin D supplementation. Interestingly, there were no significant changes to the microbial composition in patients stool samples.

Sordillo et al. (2017) looked at gut microbiota diversity and composition in stool samples of infants enrolled in the Vitamin D Antenatal Asthma Reduction Trial (VDAART) (10). Mothers of these infants received either 4000 IU Vitamin D and prenatal vitamins or 400 IU Vitamin D and prenatal vitamins during the first trimester of pregnancy. There were no observed differences between the diversity of microbiota in the two exposure groups; however, an increased abundance of Lachnospiraceae and *Lachnobacterium* and decreased abundance of *Lactococcus* were observed in infants with higher cord blood Vitamin D ($\beta = 0.014$, $p=0.004$). A second study examined the effects of maternal Vitamin D supplementation, maternal serum Vitamin D and infant Vitamin D supplements in shaping gut microbiota (43). Interestingly, infant direct supplementation (ie. via liquid drops) did not alter the gut microbial composition; however, indirect Vitamin D exposure, specifically maternal prenatal multivitamin supplementation, was correlated with an increased abundance of *Bacteroides fragilis* and decreased abundances *Bifidobacterium* spp. and *C. difficile*. Accordingly, human adults who are deemed to have an insufficient serum Vitamin D level ($<$30ng/mL 25(OH)D) have 2 times greater odds of acquiring

Clostridium difficile infections at hospitalization, compared to patients who are Vitamin D sufficient (≥50 nmol/L serum 25(OH)D) (74).

Studies done in animals typically use VDR knock out (KO) mice. Microbial "dysbiosis" has been characterized by an increase in the relative abundance of Proteobacteria, *Bacteroides spp.* and *Clostridium spp* (75,76). Knowing that maternal Vitamin D levels can influence the Vitamin D status of their offspring, Villa and colleagues (2017) fed C57BL/6J mothers a diet of either 5000 IU or 25 IU Vitamin D_3/kg body weight. *Bacteroides* and *Prevotella* enterotypes were examined and the offspring of mice on the high Vitamin D_3 diet had more *Bacteroides* spp. in the proximal colon, compared to those born to un-supplemented mothers (77). The authors claim that the observed *Bacteroide*s levels were independent of maternal fecal *Bacteroides* spp. as these, in addition to maternal diet, did not differ in stool samples collected from the mothers during pregnancy.

1.1.7 Gap in literature

To date, there have been no studies that have examined the gut microbiome in response to *C. difficile* colonization in infants. Additionally, only one study of direct infant Vitamin D supplementation has been conducted in infants (43). Meanwhile, preliminary studies (as mentioned above) have demonstrated that breastfeeding and Vitamin D may play a role in *C. difficile* colonization and gut microbiota composition. This thesis work aims to further examine these phenomena in infants at 3 months of age, as this is a critical period for gut microbiome development.

1.2 Thesis Overview

1.2.1 Objectives and Hypotheses

The primary aim of this thesis is to understand how *Clostridioides difficile* and Vitamin D supplementation are associated with the developing infant gut microbiome. This aim was investigated in two separate research studies:

The primary objective of the first study, presented in Chapter 2, was to examine ecological changes in the infant gut microbiome following colonization with *C. difficile* at 3-4 months of age. A secondary objective was to classify any changes according to breastfeeding status (exclusively breastfed versus mixed fed versus exclusively formula fed) to differentiate patterns among groups with distinct microbiomes. Accordingly, we hypothesized that the microbiomes of infants colonized with *C. difficile* would be distinct from those who were not and that these changes to the microbial environment would also be different between breastfed, partially breastfed and formula fed infants.

The primary objective of the second study, presented in Chapter 3, was to determine the association between maternal prenatal and postnatal and infant postnatal Vitamin D supplementation and colonization with *C. difficile* at 3-4 months of age. The secondary objective was to determine if Vitamin D supplementation was associated with the infant gut microbiota composition. Based on the findings from the first study and our literature review, we hypothesized that exclusively breastfed infants receiving Vitamin D drops or breastfed by a mother taking Vitamin D supplements would be less likely to be colonized with *C. difficile* and have a different gut microbiota composition compared to infant not receiving Vitamin D supplements.

1.2.2 Sample size calculations

To determine the effective sample size for Study 1 (presented in Chapter 2), a statistically significant difference in the mean microbial alpha diversity index was used. In another SyMBIOTA study from the CHILD cohort, Azad et al. (2015) found differences in infant gut microbiota alpha diversity with delivery mode and intrapartum antibiotic (IAP) exposure (3). A mean Shannon diversity index of 3.16 (SD = 0.44) was observed for infants who were delivered via emergency cesarean section with IAP. The reference group (vaginal birth, no IAP) had a mean Shannon diversity of 2.72 (SD = 0.5). After performing a sample size calculation, assuming 80% power and a two-sided alpha of 5%, it was determined that 21 infants per variable group will be required for the proposed study (**Figure 1.2**). With our sample numbers in study 1 (n = 1562), we will have sufficient power to detect meaningful differences in gut microbiota indices and other microbiome compositional changes.

$$n = 2\left[\left(\frac{Z\alpha}{2} + Z\beta\right) * \frac{SD}{Mean1 - Mean2}\right]^2$$

$$n = 2\left[(1.96 + 0.84) * \frac{0.5}{3.16 - 2.72}\right]^2 = 21$$

Figure 1.2: Sample size calculation for Study 1

We calculated our sample size for study 2 (presented in Chapter 3) referencing a study examining Vitamin D supplementation and *C. difficile* colonization in infants (43). Talsness et al. found a significant decrease in *C. difficile* counts with maternal multivitamin supplement intake of greater than or equal to 10 μg (400 IU) Vitamin D per day in the prenatal period compared to those who had no Vitamin D supplements during pregnancy. The proportion of mothers who were not taking a supplement was 36.68% (84/229) and the proportion of mother who were taking greater than or equal to 10 μg/day was 48.03% (110/229). After performing a sample size calculation, assuming 80% power and a two-sided alpha of 5%, it was determined that 294 infants per variable group will be required for the proposed study (**Figure 1.3**). With our sample numbers in this study (n = 1157), we should have sufficient power to detect meaningful differences in *C. difficile* colonization with Vitamin D intake.

$$n = (\mathrm{Z}\alpha/2 + \mathrm{Z}\beta)^2 * \frac{\mathrm{p1}(1-\mathrm{p1}) + \mathrm{p2}(1-\mathrm{p2})}{(p1-p2)^2}$$

$$n = (1.96 + 0.84)^2 * \frac{0.3668(1-0.3668) + 0.4803(1-0.4803)}{(0.3668-0.4803)^2}$$

$$n = 293.2$$

Figure 1.3: Sample size calculation for Study 2

1.2.3 The Canadian Healthy Infant Longitudinal Development (CHILD) Study and the Synergy in Microbiota Research (SyMBIOTA) Program

The CHILD Study is a prospective population-based birth cohort that recruited mothers during their third trimester of pregnancy at four Canadian sites including Vancouver, Edmonton, Winnipeg and Toronto between 2009 and 2012. A more detailed outline of the general cohort inclusion and exclusion criteria can be found in Appendix A (**Figure S1.3**) or www.childstudy.ca. Recruitment has ended but the study is still ongoing, and the study investigators have collected information and continue to collect information from enrolled families at various time points as the children grow. Some of the information includes questionnaire data (demographics, health, home life, etc) and clinical data (medical records, urine, blood and fecal samples, etc) environmental exposures (research assistant assessment of home environment). The main objective of the CHILD Study was to determine the developmental, environmental and genetic determinants of later allergy and asthma in childhood. Specific CHILD Study investigators have taken on individual projects to answer secondary research questions relevant to the overarching aims set by CHILD.

Relevant to this thesis, the Synergy in Microbiota (SyMBIOTA) Research program, led by Dr. Anita Kozyrskyj and Dr. James Scott, is a sub-study of the CHILD study to investigate associations between early life exposures and the infant gut microbiome and how these compositional changes may contribute to or protect against future childhood allergic and metabolic disease. This sub-study was funded by the Canadian Institutes of Health Research (CIHR) and AllerGen NCE. which enabled the team to sequence microbiota profiles and perform metabolite analysis on fecal samples collected from infants at the Vancouver, Edmonton and Winnipeg study sites at birth, 3 months and 1 year of age.

1.2.4 Ethical considerations

The conducted studies align with all ethics requirements for research on human subjects: respect for persons, concern for patient welfare and justice. Informed consent was received from all mothers enrolled in the CHILD study. All personal identifying information remained confidential during analysis and will remain confidential during publication of study results. Furthermore, the risks associated with participating in the CHILD Study were low and these secondary analyses did not introduce any additional risks. During analysis, all study data were saved on a protected server to prevent loss, alteration of and sharing of valuable study data. The study data will be kept for a specified period of time and then destroyed (according to standard practices). Justice has been achieved by enrolling a diverse cohort and by providing equal opportunity for participation within Canada. Finally, the studies only retrieved participant data that were necessary to achieve the stated research objectives. Finally, the Human Research Ethics Boards at the University of Manitoba, University of Alberta and University of British Columbia have approved the CHILD and SyMBIOTA studies.

1.2.5 Study variables

All study data was obtained from the CHILD and SyMBIOTA study database. Covariate data were collected from 2 sources: 1) hospital records including information on birth mode, antibiotic exposure, and intrapartum antibiotic prophylaxis (IAP) or 2) standardized questionnaires for information regarding maternal age, maternal ethnicity, infant sex, breastfeeding status, age at stool collection and solid food intake.

Birth mode. Birth mode was collected from hospital records. Birth mode was categorized as vaginal birth without IAP, vaginal birth with IAP, scheduled cesarean section and emergency cesarean section.

Direct infant antibiotic exposure. Infant antibiotic exposure before 3 months of age was collected by parental report of medications administered from 0-3 months.

Maternal education. Each mother's highest level of education was collected from a standardized questionnaire. Mothers chose from: 1) less than high school, 2) some high school, 3) high school diploma, 4) some college, 5) college complete, 6) some university, 7) university complete 8) Master's degree, or 6) PhD degree. These categories were combined to create a three-category variable: 1) high school or less, 2) some university or college, and 3) degree complete.

Maternal ethnicity. At enrolment, mothers were asked to report their ethnicity in a standardized demographics questionnaire. Based on the distribution of difference ethnicities in the CHILD study, a 3-category variable was created to ensure ample sample size for analysis: 1) White, 2) Asian, and 3) Other ethnicities.

Breastfeeding status and solid foods. In the 3-month infant nutrition questionnaire, mothers were asked to report their infants' diet, including breastfeeding, formula feeding and use of solid foods. A 3-category variable was created for infant breastfeeding status at the time of stool sample collection and questionnaire administration: 1) exclusively breastfed, 2) partially (or mixed) breastfed and 3) exclusively formula fed. The time point at which solid foods were introduced was reported and included as a continuous variable in relevant analyses.

Gut microbiota measures. Fecal samples were collected at 3-4 months of age via standard protocols during a planned home visit. Methods of sample collection, DNA extraction and amplification, 16S ribosomal RNA sequencing, and taxonomic classification have been described previously (78).

1.2.6 Personal interest

I first joined the CHILD study investigative team as a second-year undergraduate science student, unfamiliar with epidemiologic and clinical research. I was interested in working with the team as I am one of many Canadians who has allergies and was interested in learning more about the origins of related diseases. The welcoming community of staff and students at the Edmonton study site encouraged me to pursue research further. After 4 months with the team, a seed was planted and my curiosity, thirst for knowledge and drive have all helped me grow into the researcher I am today. I approached Dr. Kozyrskyj, wanting to work on a microbiome project using CHILD data, returning back to the roots of my research journey which has made this entire experience very special.

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Chapter 2

2. Characterization of the gut microbiome among infant carriers of Clostridioides difficile at 3 months of age

2.1 Abstract

Background. C. difficile colonization is common during infancy, particularly in formula fed infants, and although it is largely asymptomatic, its presence has been associated with allergic and metabolic diseases in later childhood. *C. difficile* may be associated with changes in the intestinal environment, including altered gut microbial diversity, changes to microbiota composition and differentially abundant bacterial metabolites and proteins that facilitate or result from *C. difficile* expansion.

Objectives. The primary objective of this study was to describe the gut microbiome of infants at 3 months of age according to *C. difficile* colonization status and subsequently by feeding mode.

Methods. A descriptive study was conducted on 1562 infants enrolled in the CHILD birth cohort at the Edmonton, Winnipeg and Vancouver study sites. Mothers reported on breastfeeding status at 3-month in standardized questionnaires. Infant gut microbiota were profiled with 16S rRNA sequencing and targeted qPCR detection for *C. difficile* from fecal samples collected at 3 months of age. Fecal metabolites and sIgA were detected using NMR spectroscopy and ELISA, respectively. Population characteristics were described using fishers' exact tests. Mann-Whitney U-tests and students' t-tests were used to describe Chao1 and Shannon richness and diversity indices, as well as fecal metabolite and sIgA concentrations according to colonization status within each feeding group. Multivariate linear regression (MaAslin) was performed to determine the association between *C. difficile* colonization and gut microbiota composition, stratified by feeding mode.

Results. C. difficile colonization was differentially associated with microbiome changes in breastfed versus formula fed infants, with more notable differences in the exclusively breastfed group and very few among exclusively formula fed infants. In exclusively breastfed infants, these changes included increased alpha diversity, significant between sample heterogeneity (beta-diversity), decreased fecal sIgA, a higher abundance of Firmicutes and SCFA's and lower relative abundance of Bifidobacteriaceae in those colonized with *C. difficile* relative to non-carriers. Partially breastfed infants also had higher Firmicutes and alpha diversity when colonized with *C. difficile*, but no differences in the relative abundance of Bifidobacteriaceae, beta-diversity or sIgA levels were noted. Finally, partially breastfed and exclusively formula fed infants colonized with *C. difficile* did have lower relative abundances of Staphyloccus spp. (partially breastfed) and bacteria belonging to the Gemellaceae, and Streptoccaceae families (exclusively formula fed), compared to non-carriers of the same feeding group.

Conclusions. C. difficile colonization appears to be differentially associated with altered gut microbiome development in breastfed versus formula fed infants and these are characteristic of a composition that has previously been associated with childhood atopy and obesity.

2.2 Introduction

*Clostridioides (*formerly *Clostridium) difficile* is a pathogenic bacterium that is present in the intestine of nearly 40% of infants at one month of age, and 30% of infants between the ages of 1 and 6 month (1). Acquisition of *C. difficile* during infancy has been attributed to a number of environmental exposures, including hospitalization, formula feeding, household pets, season of birth and age (1–5). Adults carrying *C. difficile* are at risk for toxigenic, infectious and sometimes fatal conditions, such as CDI (*Clostridioides difficile* infection) (6). On the contrary, infants who are colonized with this microbe are typically asymptomatic during the first year of life but more likely to develop allergic and metabolic diseases later in childhood, including asthma and obesity (7–9). Despite the lack of immediate risks related to carriage of *C. difficile* in infants, this gram-negative spore forming bacterium is capable of inducing gut inflammation and disrupting the intestinal epithelial barrier (10,11). As a result, these less than desirable influences on the intestinal environment may be associated with changes in succession and abundance of commensal gut microbiota and overall microbial ecology.

Infancy is a critical period for establishment of the gut microbial ecosystem and immune system priming in order to confer protection and reduce the risk of negative health outcomes associated with dysbiosis. *C. difficile* is associated with the presence of non-commensals and pathogenic bacteria, although this phenomenon has received little attention in infants. One study reported changes in gut microbiota composition in a small group of infants (n=53) (12). They found that *Ruminococcus gnavus* and *Klebsiella pneumoniae* species were more prevalent in infants colonized with *C. difficile*, while non-carriers were more frequently colonized by *Bifidobacterium longum*. Consequently, infants colonized with *C. difficile* may manifest distinct and persistent changes in their gut ecology, including changes in metabolites, secretory proteins

and resident microbiota: we have explored these differences in a cohort of 1562 Canadian infants from the Canadian Healthy Infant Longitudinal Development (CHILD) Study. This descriptive study presents findings on the differences in gut microbiome according to colonization status (*C. difficile* present yes/no), including measures of diversity, composition, fecal metabolites and sIgA of the infant gut at 3-months of age. We also explored these differences in exclusively breastfed, partially breastfed and exclusively formula fed infants to examine the microbial community and *C. difficile* colonization in infants with distinct diets.

2.3 Methods

2.3.1 Study Design and Population

This study includes a sub-set of 1562 families enrolled in the Canadian Healthy Infant Longitudinal Development (CHILD) general population birth cohort. Mothers were recruited during the third trimester of pregnancy between January 2009 and December 2012 from the Vancouver, Edmonton and Winnipeg study sites (inclusion and exclusion criteria outlined at www.childstudy.ca). All infants included in this subsample provided a fecal sample at 3-months of age, which was sequenced and analyzed using qPCR with targeted detection of *Clostridioides difficile*. Mothers provided informed consent upon enrollment and the Human Research Ethics Boards at the University of Manitoba, University of Alberta, and University of British Columbia have approved this study.

2.3.2 qPCR for Clostridioides difficile detection

Total bacterial charge of each infant fecal sample was determined using universal 16S primers and probes (13). A targeted 16S primer was used for amplification and quantification of

C. difficile and followed the methods set by Penders et al. (14). To summarize, a standard curve was created and employed to determine the efficiency of the *C. difficile* primers and probes by performing five 1:10 serial dilutions of *C. difficile* ATCC 9689D-5 genomic DNA starting at 1ng/uL. qPCR cycling condition were as follows: initial denaturation for 2 mins at 95.0˚C, 40 cycles of denaturation for 5 seconds at 95˚C and annealing/extension/reading for 20 seconds at 60˚C. Oligonucleotides were manufactured by IDT (Integrated DNA Technologies Inc, Coralville, IA, USA). All reactions were performed on the MiniOpticonTM Real-Time PCR System (Bio-Rad, Hercules, CA, USA). Infants were classified as colonized (*C. difficile* present in fecal sample, yes/no) prior to subsequent analysis.

2.3.3 Fecal Microbiome Analysis

Fecal samples were collected from infants during home-visits conducted at ~3-months of age by a research assistant or parents according to an approved protocol (Appendix A, **Figure S1.1**). Samples were aliquoted stored at -80˚C until analyzed. DNA extraction and amplification of bacterial V4 hypervariable region of the bacterial 16S rRNA gene was followed by sequencing and taxonomic classification and was conducted as previously described (15). To summarize, microbiota DNA was extracted from the frozen stool samples (80 to 200 mg) using the QIAamp DNA Stool Mini kit according to the manufacturer protocol (Qiagen Inc, Valencia CA). Next, the bacterial 16S rRNA genes were amplified at the hypervariable V4 region using PCR with appropriate primers. PCR products were combined for sequencing, performed using the Illumina MiSeq platform (San Diego, CA). Resultant sequences were taxonomically classified and matched at >97% similarity against the GREENGENES reference database in QIIME and filtered/excluded if <60% similarity. Finally, microbiota data were rarefied to 13,000

sequences per sample. At this time, microbiota diversity within samples (alpha diversity) was calculated using standardized estimators of OTU richness and/or evenness): Chao1 and Shannon diversity indices.

i. SCFA and fecal metabolites: A sub-set of infant samples were analysed for fecal metabolites (N=501) using nuclear magnetic resonance spectroscopy (NMR). Homogenization and centrifugation were used as necessary for sample cleaning. After extraction, fecal water was buffered and transferred to an NMR tube for spectral analysis. The ^{1}H-NMR spectra and metabolic profiles of infant faecal samples were obtained using a Varian 500 MHz Inova spectrometer. Acquisition of the spectra was completed 25˚C using the first transient of the Varian tnnoesy pulse sequence and an 80-gauss bandwidth. Spectra were collected with 128 transient and 8 steady-state scans, using a 4 second acquisition time (48,000 complex points) and a 1 second recycle delay. ^{1}H-NMR spectra were processed and profiled using a Chenomx NMR Suite Professional software package version 8.1 (Chenomx Inc., Edmonton, AB)(16). Metabolites were quantified as μmol/gram feces.

ii. Fecal secretory IgA: A sub-sample of infant samples were analyzed for fecal detection of sIgA (N=736) at 3 months of age using enzyme-linked immunosorbent assay (ELISA, Bethyl Laboratories Human IgA kit, TX, USA). Samples were run in duplicate, as previously described (17) and quantified as the average milligram of sIgA per gram total sample protein.

2.3.4 Covariates Data

Potential covariates that may correlate with early colonization with *C. difficile* colonization status in infancy were identified in the literature, including breastfeeding status, mode of delivery, antibiotics, household pets, season of birth, maternal BMI and hospitalization.

Breastfeeding status was determined through administration of questionnaires to mothers at 3 months. Mothers reported exclusive, partial or no breastfeeding (exclusive formula) at 3-months including if there had been an introduction of solid foods. Mode of delivery, gender and season of birth were assessed from hospital records, while antibiotic exposure and household pet ownership were ascertained from questionnaires given to mothers at 3-months postpartum.

2.3.5 Statistical Analysis

All statistical analysis was conducted using Stata (version 13) and R-based packages (phyloseq, ggplot2, vegan), and the Galaxy platform (MaAslin) between September 2018 and December 2018. The impact of environmental factors on *C. difficile* colonization were described using fisher's exact tests. Mann-Whitney U-tests and students t-tests, where appropriate, were used to compare alpha diversity indices, fecal metabolites and fecal sIgA according to colonization status. Differences in microbiota community structure (beta diversity) were tested using Bray-Curtis distances and permutational analysis of variance (PERMANOVA). Differences in taxon abundance according to *C. difficile* colonization status were determined using the multivariate association with linear models method developed by the Huttenhower lab (MaAslin) (18). Statistical significance was defined as a p or q-value <0.05, after FDR correction for multiple comparisons.

2.4 Results

2.4.1 Prevalence of C. difficile colonization and participant characteristics

At a mean age 3.56 months (SD: 1.00), 1562 infants provided a useable fecal sample for qPCR analysis. The prevalence of *C. difficile* colonization among all infants was 30.9%

(n=482), which aligns with previously reported rates (1). These colonization rates significantly differed between feeding groups: 22.6% for exclusively breastfed infants, 36.0% for partially breastfed infants and 49.6% for formula fed infants ($p < 0.001$, **Figure 2.1**). *C. difficile* colonization was not associated with post-birth hospitalization (p=0.507), infant antibiotic use (p=0.751), or gender (p=0.089) (**Table 2.1**). On the contrary, *C. difficile* was unevenly distributed in our study sample with regards to birth mode (p=0.001), maternal pre-pregnancy BMI ($p<0.001$), season of birth (p=0.037) and household pet ownership (p=0.003) (Table 2.1). *C. difficile* colonization was more common in infants born to overweight (31.5%) and obese (41.9%) mothers compared to those born to normal weight mothers (27.7%). Furthermore, 34.6% of infants in families with furry pets were colonized with *C. difficile* compared to 27.6% in pet-less homes. Interestingly, approximately 40% of infants born via caesarean section (both emergency and elective) were colonized with *C. difficile* compared to ~30% of vaginal births (both no IAP and IAP) (Table 2.1). There were also more infants colonized with *C. difficile* born in the fall and winter months (32.8%) compared to the spring and summer (28.7%). All subsequent analyses were stratified by feeding mode as it is a strong and consistent predictor *C. difficile* status, and a variable with sufficient sample size for analysis (all group > 50 infants).

2.4.2 Gut microbiome according to colonization status at 3 months of age

Microbiota alpha diversity was measured using Chao1 and Shannon indices. T-tests were used due to the normal distribution of the alpha diversity indices in this study (**Figure S2.1**). The mean Shannon index was 3.00 (95% CI: 2.96-3.05) for infants who lacked *C. difficile* and 3.23 (95% CI: 3.17-3.30) for infants colonized with *C. difficile.* Similarly, the mean Chao1 index was greater in colonized infants (206.58, 95% CI: 201.72 – 211.43) compared to infants without *C.*

difficile (191.11, 95% CI: 187.99-194.22). Thus, colonized infants had a greater number of species in their sample relative to other infants, and this difference was statistically significant (**Figure S2.2**, p<0.001). These effects persisted in exclusively breastfed and partially breastfed infants after stratifying for feeding mode, but no difference in alpha diversity was observed for exclusively formula fed infants (**Figure 2.2**).

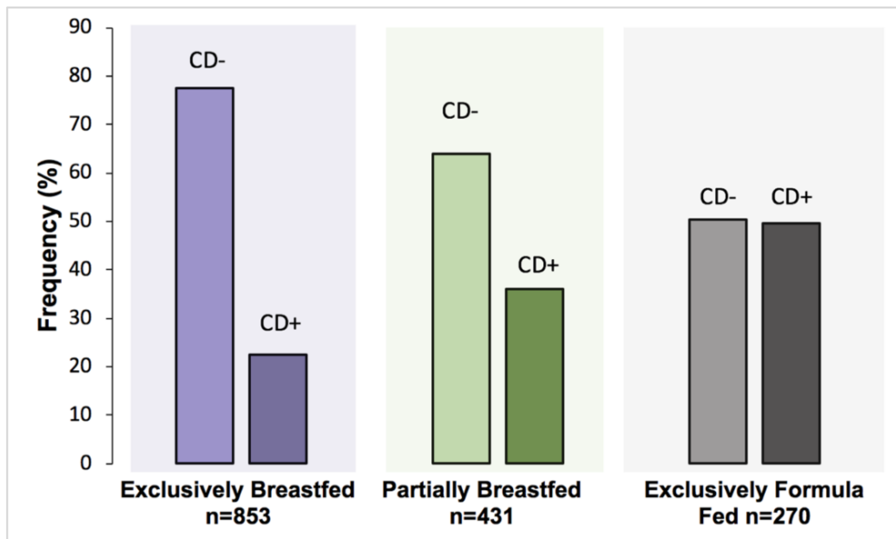

Figure 2.1: Infant colonization status according to feeding mode. Colonization rates differ among feeding groups, 22.63% of exclusively breastfed infants, 35.96% of partially breastfed infants and 49.63% of formula fed infants (Fishers' exact p<0.001). Colonized with *C. difficile* = CD+, non-carrier = CD-.

Table 2.1: Population characteristics and environmental exposures by *Clostridioides difficile* colonization status at 3 months. Fisher's exact p-values calculated using Stata (version 13).

	C. difficile **colonization at 3 months (n=482/1562, 30.8%)**		
N (row %)	No	Yes	p-value
Birth mode (n=1521)			
Vaginal no IAP	610 (72.79)	228 (27.21)	
Vaginal IAP	239 (69.28)	106 (30.72)	**0.001**
Elective CS	86 (61.87)	53 (38.13)	
Emergency CS	119 (59.80)	80 (40.20)	
Feeding mode (n=1554)			
Exclusively breastfed	660 (77.37)	193 (22.63)	
Partially breastfed	276 (64.04)	155 (35.96)	**<0.001**
Exclusively formula fed	136 (50.37)	134 (49.63)	
Hospitalization since birth (n=1533)			
No	994 (69.51)	436 (30.49)	0.507
Yes	68 (66.02)	35 (33.98)	
Infant antibiotics (n=1562)			
No	1048 (69.22)	466 (30.78)	0.751
Yes	32 (66.67)	16 (33.33)	
Furry pets in the home (n=1535)			
No	605 (72.37)	231 (27.63)	**0.003**
Yes	457 (65.38)	242 (34.62)	
Mom's pre-pregnancy weight (n=1484)			
Underweight	28 (82.35)	6 (17.65)	
Normal weight	627 (72.32)	240 (27.68)	**<0.001**
Overweight	231 (68.55	106 (31.45)	
Obese	143 (58.13)	103 (41.87)	
Season of birth (n=1561)			
October to March (Fall/Winter)	560 (71.61)	260 (33.38)	**0.037**
April to September (Spring/Summer)	519 (66.62)	222 (28.39)	
Infant gender (n=1561)			
Male	560 (67.23)	273 (32.77)	0.089
Female	519 (71.29)	209 (28.71)	

Changes in beta diversity were also apparent and determined using permutational multivariate analysis of variance (PERMANOVA) with the vegan and phyloseq packages in R (19,20). Bray-Curtis dissimilarity matrices were created from microbiota relative abundance data and compared before and after stratifying by feeding mode to examine community level differences between infant samples. We found significant inter-sample variability (beta-diversity) between infant samples with *C. difficile* and infant samples without *C. difficile* ($p=0.01$). Exclusively breastfed infants also demonstrated significant inter-sample variability ($p=0.01$) but no significant differences in beta-diversity were found for partially breastfed and exclusively formula fed infants (**Figure S2.3**).

Next, we sought to determine which microbiota explained these differences in diversity that were observed between *C. difficile* carriers and non-carriers. We performed multivariate association with linear models, MaAslin, on arc-sine square root transformed data, which revealed changes in relative abundance of key microbiota. In all infants, at the family level we saw that Bifidobacteriaeceae were decreased and Peptostreptococcaceae, Clostridiaceae, Ruminococcaceae and Lachnospiraceae were increased in abundance when comparing infants colonized with *C. difficile* with non-carriers. These family and also genus level differences were then examined following stratification for feeding mode at 3-months of age. Only taxa that were differentially abundant according to *C. difficile* colonization status were reported in **Figure 2.3** (FDR corrected $q<0.05$). Bacteria of the genus *Bifidobacterium* (family Bifidobacteriaceae) were decreased in exclusively breastfed infants colonized with *C. difficile* compared to non-carriers of the same diet ($q=0.02$, Figure 2.3, **Table S2.1**).

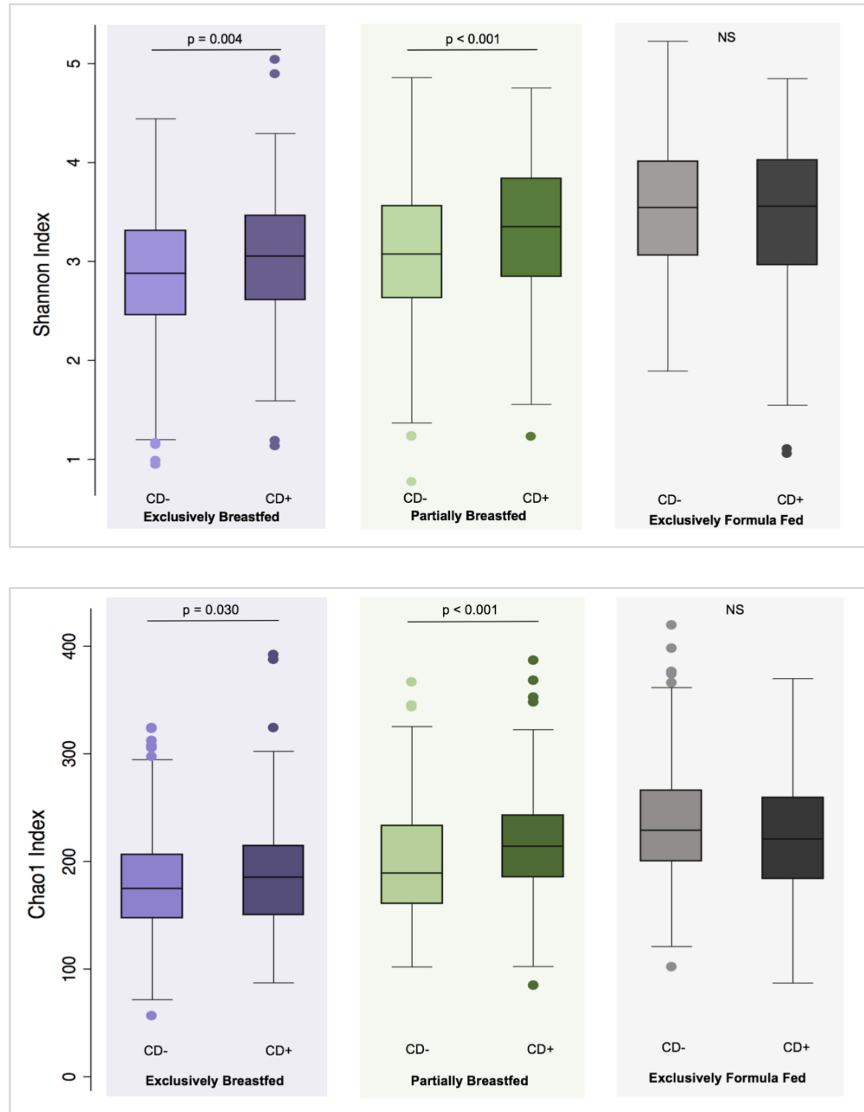

Figure 2.2: Infant microbial alpha-diversity indices, Chao1 index of species richness and Shannon diversity index, according to infant colonization and feeding mode. Higher α-diversity observed for infants colonized with *C. difficile* (CD+) and breastfed (either exclusively or partially) compared to non-carriers (CD-) on the same diet. Students' t-test p-values reported.

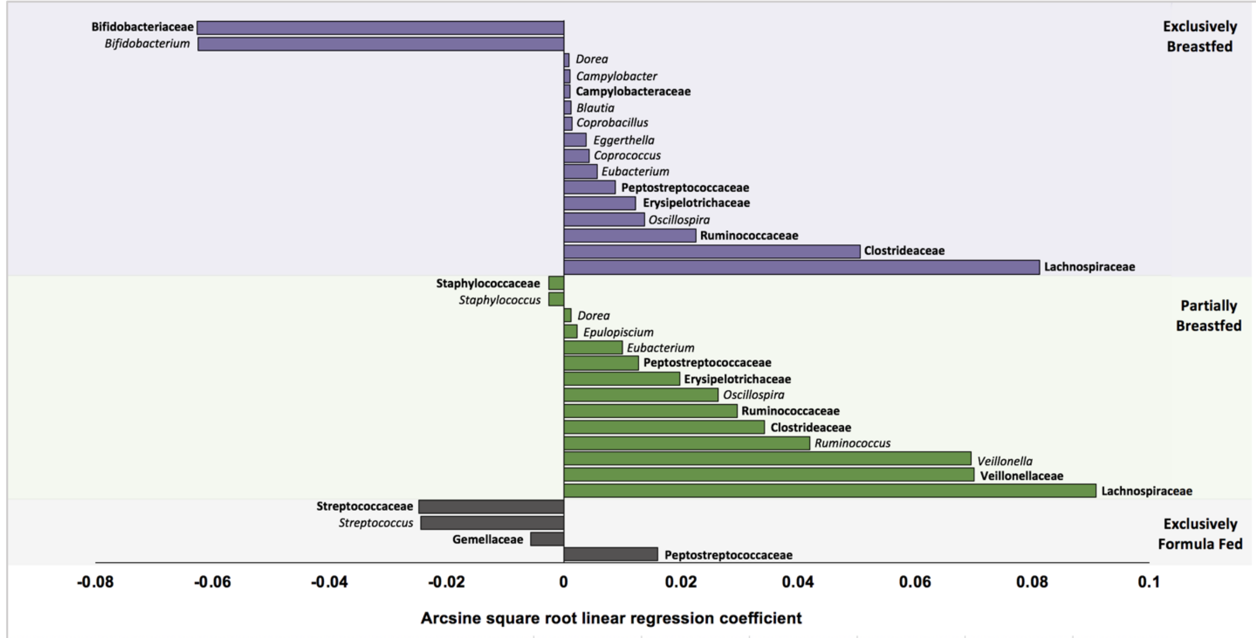

Figure 2.3: Multivariate linear regression results for family level taxa that are differentially associated with *C. difficile* colonization at 3 months (x-axis = coefficient, arc sine square root transformation, reference = no *C. difficile* in 3-month sample). Data shown only for taxa with FDR corrected q-value < 0.05. Coefficient > 0 means significantly higher abundance with *C. difficile* colonization, coeff. <0 means significantly decreased in abundance in the presence of *C. difficile*.

Futhermore, in exclusively breastfed infants, *C. difficile* was associated with higher abundances of Lachnospiraceae including *Blautia* (q = 0.003), *Copocococcus* (q < 0.001) and *Dorea* spp. (q = 0.01) but also *Campylobacter* (family Campylobacteraceae, q = 0.006), *Eggerthella* (family Coriobacteriaceae, q = 0.008), *Oscillospira* (family Ruminococcaceae, q <0.001) and *Eubacterium* (family Erysipelotrichaceae, q = 0.002) spp. The relative magnitude of these changes to the gut microbiota are reported in Figure 2.3 (arc-sine square root transformed coefficients on the x-axis).

Partially breastfed infants colonized with *C. difficile* exhibited compositional differences similar to those who were exclusively breastfed at the family level, notably a greater abundance of Lachnospiraceae ($q < 0.001$), Ruminococcaceae ($q = 0.006$) and Erysipelotrichaceae ($q < 0.001$). Associations that were unique to partially breastfed infants colonized with *C. difficile* compared to non-carriers include a higher abundance of *Veillonella* (family Veillonellaceae, $q = 0.002$), *Ruminococcus* (family Lachnospiraceae, $q < 0.001$), *Epulopiscium* (family Lachnospiraceae) and species of the Clostridiaceae family ($q < 0.001$). There was also a lower abundance of bacteria of the genus *Staphylococcus* (family Staphylococcaceae, $q < 0.001$) in partially breastfed infants positive for *C. difficile*, compared to non-carriers.

Finally, exclusively formula fed infants colonized with *C. difficile* had substantially less compositional changes, relative to breastfed (exclusive and partial) infants. The only family of microbes that was significantly increased in exclusively formula fed *C. difficile* carriers versus non-carriers was Peptostreptococcaceae ($q < 0.001$), which is the family to which *C. difficile* belongs. Evidently, exclusively breastfed and partially breastfed *C. difficile* carriers also exhibited significantly higher abundances of Peptostreptococcaceae compared to non-carriers ($q < 0.001$). Other significant associations in exclusively formula fed infants include a lower abundance of *Steptococcus* (family Streptococcaceae, $q =0.005$) and bacteria of the genus Gemellaceae ($q = 0.001$) (Figure 2.3).

To further understand how these compositional changes may affect the intestinal environment, we measured and analyzed concentrations of relevant infant fecal metabolites. Using Mann-Whitney U-tests, we explored metabolites that are physiologically relevant to *C. difficile*, infant health and most gut microbiota, including short chain fatty acids (SCFA: acetate, butyrate and propionate), as well as glutamate, p-cresol and succinate. Overall, acetate was the

most abundant fecal metabolite (median: 66.19µmol/g, IQR: 47.94 - 91.38), and p-cresol was the least abundant (median: 0.00 µmol/g, IQR: 0.00 – 0.075). Exclusively breastfed infants colonized with *C. difficile* had higher concentrations of all three SCFA's ($p < 0.05$) compared to non-carriers of the same diet group (**Figure 2.4**). Contrastingly, there were no statistically significant differences in fecal SCFA concentrations between *C. difficile* positive and *C. difficile* negative infants in the exclusively formula fed group (Figure 2.4).

Concentrations of glutamate, which have shown to be essential for establishment and colonization of *C. difficile in vivo* (21)*,* were not differentially associated with *C. difficile* colonization status in any of the infant feeding groups, although its median concentration was greater in exclusively formula fed infants (exclusively formula fed, median: 7.61 µmol/g, IQR: 5.08 - 13.25 | partially breastfed, median: 6.88 µmol/g, IQR: 3.52-10.81) | exclusively breastfed, median: 3.98 µmol/g, IQR: 1.64 - 8.02). Similarly, *C. difficile* may utilize succinate for expansion and colonization (22). However, succinate concentrations only differed according to *C. difficile* colonization status among exclusively breastfed infants (lower in the CD+ infants, median of 5.73 µmol/g versus 10.25 µmol/g, Figure 2.4).

Finally, p-cresol, an end product of *C. difficile* metabolism (23), was greater in all infants colonized with *C. difficile* ($p < 0.05$) regardless of their diet, although the overall detectable concentration of this metabolite was very small (data not shown).

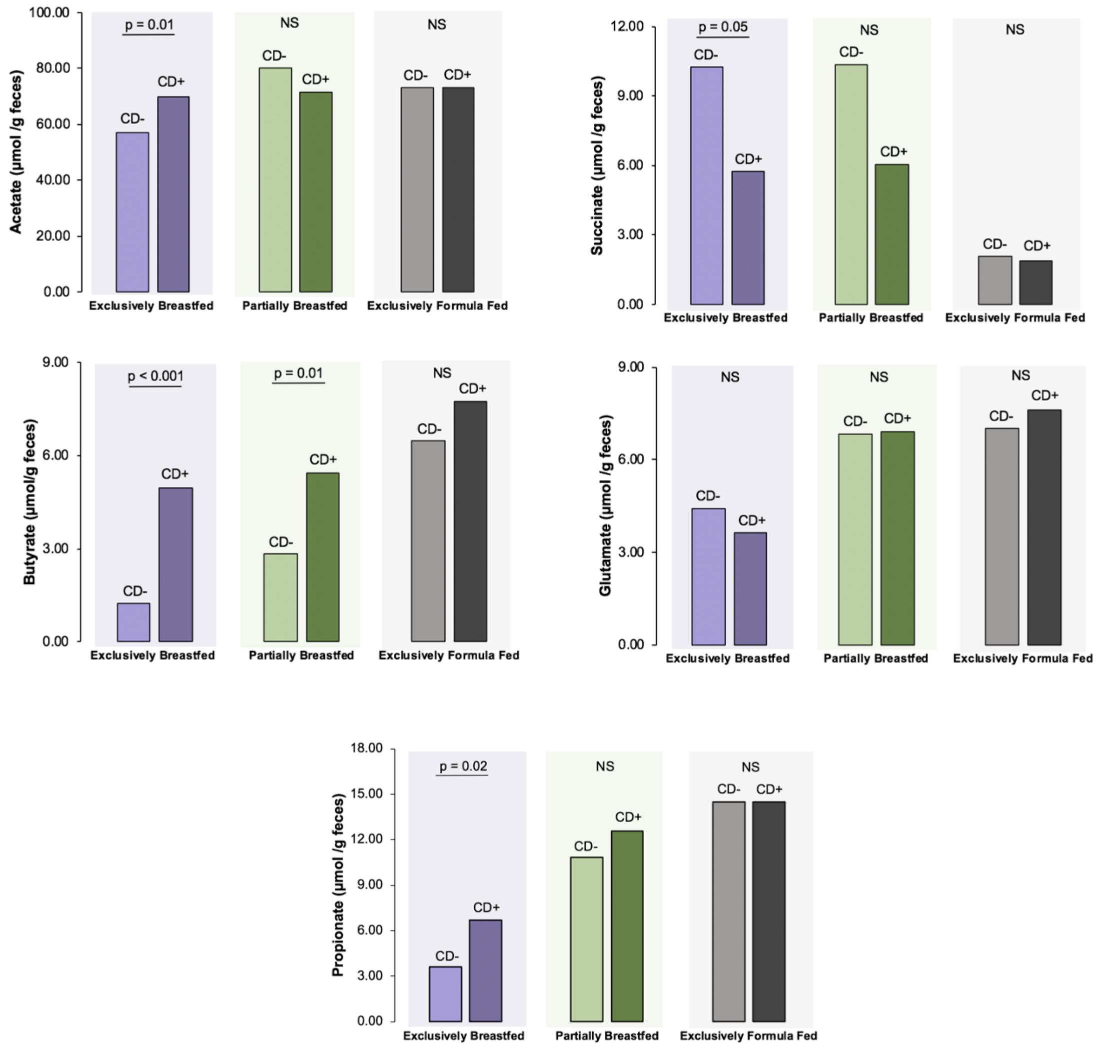

Figure 2.4: Mann-Whitney U-test of fecal metabolites associated with *C. difficile* colonization, stratified by infant feeding mode. Concentrations of fecal metabolites of differentially associated with *C. difficile* colonization and infant diet. (CD+ = colonized with *C. difficile*, CD- = non-carriers).

In addition to examining fecal metabolites, we also tested the collected infant stool sample for secretory IgA, a marker of intestinal homeostasis and mucosal immunity (24). Secretory sIgA concentrations were right-skewed, thus data were treated with non-parametric

Mann-Whitney U-tests for statistical analysis (and plotted as log transformed values in **Figure 2.5**). Among all study infants, those colonized with *C. difficile* had a lower concentration of fecal sIgA (median: 5.71 mg/g versus 3.78 mg/g, p = 0.001). Exclusively breastfed infants were the only group that had significantly lower sIgA concentrations in the presence of *C. difficile* following stratification by feeding mode at 3-months compared to non-carriers of the same diet (Figure 2.5). Overall, the concentration of sIgA was lowest in exclusively formula fed infants colonized with *C. difficile* (median: 2.37, IQR: 1.48-4.20) and highest in exclusively breastfed infants who were not colonized (median: 8.39, IQR: 4.87-12.60).

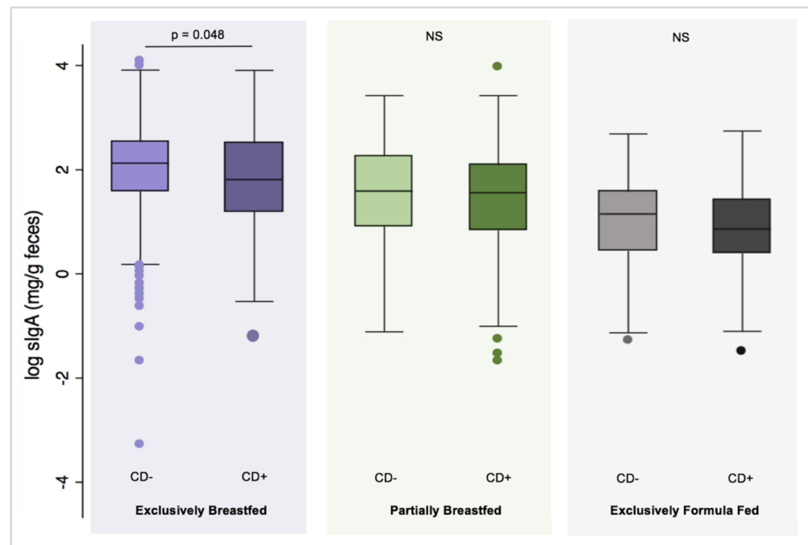

Figure 2.5: Mann-Whitney U-test of log transformed fecal sIgA (mg/g) between infants colonized with *C. difficile* (CD+) and infants lacking *C. difficile* (CD-) at 3 months of age, according to infant diet. Exclusively breastfed CD+ infants had lower fecal sIgA compared to CD- infants of the same diet.

2.5 Discussion

Our results suggest a distinction between the gut microbiome of infants colonized with *Clostridioides difficile* and those who are not, which fills a gap in our understanding of how *C. difficile* colonization in infancy may be associated with the development of the microbial ecosystem during infancy. Our report is novel, in that we are the first to describe the relationship between *C. difficile* and the infant gut microbiome according to breastfeeding status.

In our study, one third of infants were *C. difficile* carriers at 3 months of age, a prevalence that agrees with the previously reported findings (1,25,26). It is well known that infant diet (ie. breastfeeding) is a strong predictor of colonization status, with a greater proportion of formula fed infants being positive for *C. difficile*. Also, infant diet contributes to profound changes in other gut microbiome factors (14,27). Interestingly, the most noticeable microbiome differences with *C. difficile* colonization observed in this study were among exclusively breastfed infants. There were also many associations among partially breastfed infants, but these were less abundant and consistent than in the exclusively breastfed group. This may be due to the fact that partially and exclusively formula fed infants typically have a more heterogeneous gut microbiota, compared to exclusively breastfed infants, thus colonization with *C. difficile* creates more obvious changes in the overall community structure amongst infants who typically have a less diverse microbiome composition, as in exclusively breastfed infants (28).

Breastfed infants with *C. difficile* had increased diversity and richness compared to uncolonized infants (Figure 2.2). Exclusive breastfeeding is generally associated with a low microbial alpha diversity, compared to other methods of feeding, due to the dominant nature of *Bifidobacterium spp*. in these infants (15,29). Bifidobacteria are gram-positive anaerobic bacteria

thrive on human-milk oligosaccharides and are often used in probiotics due to their numerous reported health benefits (30). One of these benefits is that the presence of these commensals anaerobes has been linked to suppression in *C. difficile* acquisition (12,30,31). In accordance, our multivariate linear regression models reveal that *Bifidobacterium spp*. are decreased in exclusively breastfed infants colonized with *C. difficile* compared to those who are not colonized. Thus, Bifidobacteria no longer dominate the gut of exclusively breastfed infants when *C. difficile* is present. Other changes in composition follow *C. difficile* colonization, including an increase in microbial alpha-diversity, changes in beta-diversity and increased abundances of bacteria or the Firmicutes and Proteobacteria phyla in exclusively breastfed infants. Rousseau et al. (2011) did not find any differences in biodiversity with *C. difficile* colonization (12): however, their cohort of infants were not separated according to feeding mode and was relatively small (n=53), which may account for the fact that they did not detect significant differences in diversity with *C. difficile* colonization.

Furthermore, our multivariate linear regression models found significant associations between *C. difficile* colonization and microbiota composition including greater abundance of members of the Lachnospiraceae and Ruminococcaceae families among breastfed infants (both exclusive and partially breastfed) which agrees with the finding of Rousseau et al. (2011) (12). Lachnospiraceae, including *Coprococcus*, *Blautia, Dorea, Ruminococcus*, *Eubacterium* spp. feed on dietary sugars to produce the short chain fatty acid butyrate and propionate using different metabolic pathways (32–34), and these metabolites are also more concentrated in the fecal samples of breastfed *C. difficile* carriers. Meanwhile, acetate is formed as a product of carbohydrate metabolism by most intestinal microbiota (33). Although butyrate and other short chain fatty acids are usually thought of as positive for gut and overall health (32), increased

abundance of Lachnospiraceae microbes has been linked to later childhood obesity (35). The reason behind this association requires further investigation, but may be attributed to an higher concentration of SCFA's, relative to the energy requirements and absorption capability of the colon, when Firmicutes microbiota outnumber Bacteroidetes (36,37). One of the changes in microbiota abundance that is consistent among all infants (regardless of feeding mode) is the increase in Peptostreptococcaceae with *C. difficile* colonization. This is expected as *C. difficile* is a member of the Peptostreptococcaceae family.

Of the few compositional changes noted in exclusively formula fed infants, we did observe an association between *C. difficile* colonization and decreased relative abundance of Streptoccocaceae and Gemellaceae, two families that have been linked to dysbiosis (38). On the contrary, *Streptococcus* spp. have also been linked to reduced adiposity at 6 months of age (39). As this observation was only noted in exclusively formula fed infants, these microbes may be associated with formula feeding but may be out-competed by *C. difficile* for nutrients like amino acids and sugars, thus lowering their abundance in its presence.

It has been noted that high concentrations of succinate and glutamate in the intestine may create an ideal environment for *C. difficile* expansion (22). *C. difficile* can ferment succinate into butyrate and can therefore thrive well under these conditions. Accordingly, in our infant fecal samples that were positive for *C. difficile*, we observe decreased concentrations of succinate and increased concentrations of butyrate, which may result from *C. difficile* fermentation of succinate (33). Succinate is not easily or rapidly absorbed by the colonic cells, thus when diet is controlled for, a lower concentration of succinate may be due to the microbial composition and cross-feeding of *C. difficile* with Bacteroidetes and members of the Negativicutes branch of Firmicutes (ie. *Veillonella* spp.) which are increased in partially breastfed *C. difficile* carriers (Figure 2.3)

(40,41). Furthermore, *C. difficile* possesses the ability to utilize glutamate for expansion; however, glutamate levels were not differentially associated with feeding and colonization status. Although this amino acid is the essential for *C. difficile* pathogenesis and resulting inflammation, it may not be essential for asymptomatic colonization in infants.

C. difficile can also degrade other amino acids into 4-methylphenol, otherwise known as p-cresol (23). We observed that among all infants, regardless of their diet, there was a greater concentration of p-cresol in the fecal samples of those infants who were colonized with *C. difficile*. The concentration of this metabolite was extremely low in our study, and like with glutamate, we can assume that it might be more concentrated in pathogenic cases of *C. difficile* infection which is often not the case in infancy.

Finally, exclusively breastfed infants who were colonized with *C. difficile* had lower fecal concentrations of secretory IgA compared to non-carriers (Figure 2.5). This was not the case for partially breastfed and exclusively formula fed infants. Previously, we have reported an inverse association between *C. difficile* colonization and sIgA in a smaller subset of infants (n = 47) and these findings have been confirmed in our larger population where we were able to stratify by breastfeeding status (17). Although no general consensus has been achieved in the literature, this decrease in sIgA may correspond to an increase in relative abundance of bacteria which are bound by sIgA, such as bacteria of the Erysipelotrichaceae family, Campylobacteraceae family and *C. difficile* itself (42–44). This binding of sIgA and *C. difficile* may also inhibit the toxigenic effects of *C. difficile* in infants (43). Maternal sources of sIgA in breastmilk, which represent a large portion of breastfed infant sIgA at 3 months, may also be depleted due to some maternal factor, such as depression (45) thus predisposing the infant to colonization by *C. difficile* and

other pathogens. Bile acids also play a role in the pathogenesis and toxigenic properties of *C. difficile* (46), but we were unable to measure these in our study.

Other study limitations include our inability to measure the strains and toxigenic properties of *C. difficile*. Should our study findings continue to align with previous findings, we might expect a prevalence of toxigenic strains to be less than 10% among those with *C. difficile* positive samples (31,47). Also, fecal metabolites are only representative of final products of metabolism and unabsorbed molecules. We were unable to measure mucosal and circulating (*in vivo)* metabolite concentrations which may have given us a more accurate representation of the relationships between microbiota and their fermentation products. Furthermore, fatty acids are volatile, and thus the absolute concentrations reported may not be representative of fecal concentrations: however, the amount lost in these samples is not expected to differ drastically between samples as they were all collected according to a standard protocol (Appendix A, Figure S1.1). We are also unable to determine the direction of the observed associations: whether *C. difficile* is causing the observed changes, or if the observed microbiome changes increase infant susceptibility to *C. difficile* colonization. This could be improved by measuring the *C. difficile* colonization status of infants longitudinally (at more than one time point) to see if *C. difficile* colonization is transient or persistent and whether the microbiome changes precede or follow colonization. Nevertheless, our study is of large sample size (n = 1562), enabling us to be the first to describe differences in the microbiome that are associated with *C. difficile* colonization, according to infant diet during a point during the critical of infant gut microbiome development (ie. 3 months of age).

2.6 Conclusions

The most noticeable alterations in microbial ecology, among all measured parameters, were observed in exclusively breastfed and partially breastfed infants who were colonized with *C. difficile,* although there were also distinct differences between these breastfed infant (exclusive versus partial). This may be due to the fact that exclusively formula fed infants typically have a more heterogeneous gut microbiota, thus colonization with *C. difficile* is not associated with as obvious differences in the microbial community compared to exclusively breastfed infants who are typically dominated by Bifidobacteria spp. prior to weaning. *C. difficile* colonization in linked to poor health outcomes including obesity and allergy in infancy, and these changes in gut microbiome that are associated with its presence may partially explain this relationship. The exact reason behind *C. difficile* colonization in exclusively breastfed infants remains to be determined and is important for understanding early life influences on later infant health. Further research in partial breastfed or mixed fed infants is essential as these infants appeared to have unique differences in their microbiome according to *C. difficile* colonization status. Finally, the direction of the association remains unknown, although we were able to describe important differences with *C. difficile* colonization according to breastfeeding status.

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Chapter 3

3. Vitamin D supplementation in pregnancy and lactation in relation to gut microbiota and *Clostridioides difficile* colonization in infancy

3.1 Abstract

Background. Vitamin D supplements are recommended for all breastfed infants in Canada, but the impact on the gut microbiota during this critical period remains largely unknown. Supplementation with this micronutrient has been shown to alter the gut microbiota composition and decrease the presence of *C. difficile* in infants.

Objectives. The primary objective of this study was to investigate the association between maternal and infant Vitamin D supplementation and *C. difficile* colonization at 3 months of age, particularly among exclusively breastfed infants. The secondary objective of this study was to investigate the association between maternal and infant Vitamin D supplementation and the infant gut microbiota composition in these same infants.

Methods. A cross -sectional study was conducted on 1157 mother infant pairs enrolled in the CHILD birth cohort at the Edmonton, Winnipeg and Vancouver study sites. Maternal and infant Vitamin D supplement intake and breastfeeding status at 3 month were self-reported in standardized questionnaires. Infant gut microbiota were profiled with 16S rRNA sequencing and targeted qPCR detection for *C. difficile* from fecal samples collected at 3 months of age. Population characteristics were described using fishers' exact tests. Logistic regression analyses were used to assess the association between Vitamin D supplementation and *C. difficile* colonization. Multivariate linear regression (MaAslin) was performed to determine the association between Vitamin D supplementation and gut microbiota composition.

Results. At 3-months of age, 64.65% of all infants received a Vitamin D supplement, while 74.81% of exclusively breastfed infants received a supplement. Maternal supplementation patterns did not differ based on whether or not they were exclusively breastfeeding. *C. difficile* colonization status was not associated with maternal pre or postnatal Vitamin D supplementation after adjusting for breastfeeding status. Furthermore, infant Vitamin D supplementation was not associated with *C. difficile* colonization, although there was a trend towards lower odds of colonization with this microbe in infants receiving Vitamin D compared to those with no direct Vitamin D supplementation among exclusively breastfed infants (aOR: 0.84, 95% CI: 0.42 - 1.67). Finally, both maternal and infant supplementation were associated with decreased relative abundance of *Bilophila* spp., but only maternal pre and postnatal supplementation was associated with lower relative abundance of Lachnospiraceae and increased abundance of *Haemophilus* spp.

Conclusions. Vitamin D supplementation in infancy and lactation is not associated with *C. difficile* colonization but it is associated with other important compositional changes to the infant gut microbiota which may impact later childhood health.

3.2 Introduction

Beginning at birth, there are key medical, lifestyle and environmental factors which contribute to the establishment of the complex communities of our gut microbiota, notably mode of delivery and infant feeding modality (1–3). The impact of infant feeding, either breast, mixed or formula, on the infant gut microbiome has been researched extensively (4,5). More recently, attention is shifting beyond breastfeeding alone, examining the role of specific micronutrients in the early infant diet (6–8). In Canada, a liquid preparation of 400 IU of Vitamin D_3 is recommended for all breastfed infants according to guidelines set by the Canadian Pediatric Society in 2010, making them the most frequently-used micronutrient supplement during early infancy (9). Vitamin D is also present in infant formula so that the recommended 400 IU per day is achieved regardless of feeding mode.

Until recently, Vitamin D intake and supplementation have been largely ignored in infant microbiome studies, yet this vitamin has many physiological roles that could impact microbial composition. These include the production of antimicrobial peptides, tight junction proteins that maintain intestinal epithelial barriers, and stimulation of intestinal immune cells resulting in the production of anti-inflammatory cytokines (10). All these factors contribute to overall gut homeostasis which is important for the exclusion of opportunistic pathogens and maintenance of commensal gut microbiota (11). A recent systematic review summarizes *in vivo* studies that studying Vitamin D, Vitamin D receptor and gut microbiota composition, however, only two studies have been conducted in infants (12,13).

Maternal supplementation during pregnancy with Vitamin D containing multivitamins ($>$ 10 μg/day) has previously been associated with a decrease in abundance of *C. difficile* among breastfed infants at 1 month of age, but not linked to maternal serum circulating 25(OH)D levels

or direct infant supplementation via drops (14). Additionally, lowered *C. difficile* abundance was accompanied by an increased abundance of *Bifidobacterium longum,* a probiotic bacteria, in infants whose mothers were taking vitamins containing Vitamin D in pregnancy, following adjustment for place of birth, mode of delivery, siblings, sex, feeding mode and maternal vaginitis (14). These findings suggest that maternal and infant supplementation, as sources of Vitamin D, may be associated with the gut microbiota composition and *C. difficile* prevalence. *C. difficile* colonization occurs in nearly 30% of infants between the ages of 1-6 months, is largely asymptomatic, but may predispose infants to later allergic and metabolic diseases in childhood (15–17). As noted in Chapter 2, *C. difficile* colonization is associated with changes to the natural discourse of microbiome development, particularly in exclusively breastfed infants. If Vitamin D is in fact associated with *C. difficile* colonization and gut microbiota composition, exclusively breastfed infants are at greater risk of unfavourable compositional changes because they require supplements to achieve similar Vitamin D levels as formula fed infants who receive a standard dose of 400 IU/day through formula (18). *C. difficile* is of particular interest as it Vitamin D deficiency has also been shown to be correlated with *Clostridium difficile* infection (CDI) in adults (19–22). Furthermore, there is evidence to suggest that supplementation during infant development is important for preventing diseases associated with low Vitamin D, including susceptibility to infection and colitis (23). These conditions have also been associated with imbalances in gut microbiota, suggesting a potential connection between Vitamin D and the gut microbiome that merits investigation in infants (11,12,24).

The primary objective of this study was to determine the association between Vitamin D supplementation during the first 3 months of life and *C. difficile* colonization following maternal and infant supplement use in a large cohort of infants from the CHILD Study. The secondary

objective of this study was to determine the association between Vitamin D supplementation and the composition of the gut microbiota.

3.3 Methods

3.3.1 Study Design and Population

This observational study includes a subset of 1157 families enrolled in the CHILD general population birth cohort. Mothers were recruited during their third trimester of pregnancy between January 2009 and December 2012 from either the Vancouver, Edmonton or Winnipeg sites (inclusion/exclusion criteria outlined at www.childstudy.ca). All infants in this subsample provided a fecal sample at 3-4 months of age, which was analyzed for targeted detection of *C. difficile* and 16S rRNA gene analysis for other gut microbiota. In addition to the stool samples, mothers in this study completed a nutrition questionnaire for their child at 3 months postpartum describing breastfeeding status and Vitamin D supplement intake. Mothers provided informed consent upon enrollment and the Human Research Ethics Boards at the University of Manitoba, University of Alberta, and University of British Columbia have approved this study.

3.3.2 Microbiota outcome variables: qPCR for C. difficile and fecal microbiome analysis

Please refer to sections 2.3.2 and 2.3.3 in Chapter 2 for a detailed description of all microbiome sequencing and qPCR methods (excluding SCFA/metabolite and sIgA sections which are not relevant to this Chapter). *C. difficile* outcomes were modelled as a binary outcome (present, yes or no) and all other microbiota were modelled as continuous arc-sine square root transformed relative abundances.

3.3.3 Vitamin D exposure variables

Maternal Vitamin D and multivitamin supplement use was collected in a supplement/vitamin questionnaire at recruitment (around 27 weeks gestation) and 3 months postpartum, whereas child supplement intake was recorded in a child nutrition questionnaire at 3 months of age. Mothers were asked to report whether they used a prenatal vitamin, a multivitamin or a Vitamin D supplement, including the dose and frequency of supplementation. The infant questionnaire required mothers to describe whether they gave their infant any supplements, including Vitamin D drops. This questionnaire did not ask about dosing or frequency of dosing. Maternal food frequency questionnaires reported daily milk consumption during pregnancy, which was used as an estimate (proxy) for maternal dietary Vitamin D intake (80-100 IU in one cup of fortified milk or plant-based milk alternative) as previous reports have classified milk as the major dietary source of Vitamin D during pregnancy (25,26). Additionally, 2 cups of milk or fortified milk alternative daily (500mL) were, at the time of the study, recommended for pregnant and lactating women by Health Canada (27). Maternal and infant intake variables were created as follows:

Maternal prenatal Vitamin D intake. Mothers were asked to report their vitamin and supplement intake in a standardized questionnaire (**Figure S3.1**). A 3-category exposure variable was created from the relevant questionnaire information: 1) First group: One supplement ***or*** no supplements containing Vitamin D, 2) Second group: Two supplements containing Vitamin D and 3) Third group: Three or more supplements containing Vitamin D.

Postnatal Vitamin D intake. Mothers were asked to report their vitamin and supplement intake in a standardized questionnaire (**Figure S3.2**). A 4-category exposure variable was created from the relevant questionnaire information: 1) First level: Low (ie. one maternal source, no infant

direct) ***or*** no Vitamin D from supplements, 2) Second level: Two maternal supplements with Vitamin D ***or*** infant direct D, 3) Third level: Infant direct ***and*** one maternal supplement, and 4) Fourth level: Two or more maternal supplements ***and*** infant direct Vitamin D.

Maternal perinatal intake based on the dosing information. The current Canadian guidelines for mothers surrounding Vitamin D supplementation suggest 400-600 IU per day during pregnancy and breastfeeding (recommended dietary allowance, RDA, per day) (28). We created binary variables with a cut-off at 400 IU per day based on the information available from the maternal supplement questionnaire (Appendix C: Figure S3.1 and Figure S3.2). Reference category was less than 400 IU per day and the second category represented greater than or equal to 400 IU per day in either the prenatal, postnatal period or at both time points. A final 4 category variable was created with the guidelines in mind: 1) No maternal Vitamin D or less than 400 IU per day, 2) Prenatal only maternal Vitamin D supplementation ≥400 IU/day, 3) Postnatal only maternal Vitamin D supplementation ≥400 IU/day and 4) Prenatal and Postnatal supplementation ≥400 IU/day.

Maternal milk consumption. Mothers reported their milk intake in a food frequency questionnaire (FFQ) administered during the prenatal period. We assumed similar intake in the postnatal period and used this variable to account for dietary sources of Vitamin D that could have confounded our ability to detect a relationship between Vitamin D supplementation and *C. difficile* colonization. They were asked to report their daily milk/fortified substitute beverage consumption (1 cup), milk/fortified substitute use on cereal (1/2 cup) and milk/fortified substitute in tea/coffee, etc. (1 Tbsp). A 3-category variable was created from this information: 1) 1 or fewer cups/day, 2) 2 cups/day, 3) 3 or more cups/day.

3.3.4 Other Covariate Data

Additional covariate data for this study include the season of birth, maternal pre-pregnancy age and BMI, infant age at stool sample collection, hospital length of stay at birth, mode of delivery, infant sex, feeding mode, antibiotics use, household income, ethnicity, maternal depressive symptoms, pets in the home and study centre. This information was collected from relevant CHILD study questionnaires or from medical charts. A dichotomous variable was created to describe the season of birth as sunlight and UVB exposure are environmental sources of Vitamin D that could have confounded our ability to detect a relationship between Vitamin D supplementation and *C. difficile* colonization: 1) infants born in low UVB season (born October through March, when negligible amounts of Vitamin D are produce subcutaneously) and 2) infants born in high UVB season (born April through September) (29). Mode of delivery was collected from hospital records and was categorized as vaginal birth without IAP, vaginal birth with IAP, scheduled cesarean section and emergency cesarean section. Infant antibiotic exposure before 3 months of age was collected by parental report of medications administered from 0-3 months. In the 3-month infant nutrition questionnaire, mothers were asked to report their infants' diet, including breastfeeding, formula feeding and use of solid foods. A 3-category variable was created for infant breastfeeding status at the time of stool sample collection and questionnaire administration: 1) exclusively breastfed 2) partially breastfed and 3) exclusively formula fed. The time point at which solid foods were introduced was included as a continuous variable in relevant analyses.

3.3.5 Statistical analysis

All analyses and variable creation were completed using Stata (version 13.0) statistical software and the online Galaxy platform (version 1.0.1). Relationships between Vitamin D supplementation and *C. difficile* colonization were explored using fishers' exact tests, where appropriate. Logistic regression analyses were performed to determine the association between Vitamin D supplement use and *C. difficile* colonization (binary: present or absent). Models were built according to the purposeful selection of covariates method with an initial significance value of $p \leq 0.2$ (univariate selection) (30). Variables were removed from the multivariable model if $p \leq 0.05$ and no interaction, confounding or biological/clinical significance was present. Analyses were also stratified by breastfeeding status at 3 months due to the strong association between breastfeeding and infant direct Vitamin D supplementation. Other measures of microbiota composition were compared using Multivariate Association with Linear Models (MaAslin) among exclusively breastfed infants (31). Microbiota relative abundances were arcsine square root transformed, then run through the multivariate model in Galaxy with covariate data, subjected to FDR correction and reported if $q \leq 0.05$. Figures and tables were created using Microsoft Excel (version 16.21.1) and Stata.

3.4 Results

3.4.1 Study participant characteristics

Among the 1157 infants for which there was available data on Vitamin D intake and *C. difficile* colonization, the average age at stool sample collection was 3.52 months (SD: 0.97 months). Of these infants, 64.65% received a Vitamin D supplement in the form of infant Vitamin D drops and 29.73% were colonized with *C. difficile* (**Table 3.1**). As expected, a higher

proportion (74.81%) of exclusively breastfed babies received Vitamin D supplements and only 22.10% were colonized with *C. difficile.* Interestingly, some of the partially (67.83%) and exclusively formula fed infants (25.64%) also received Vitamin D drops, but supplementation was less frequent in these groups ($p<0.001$, Table 1). The study site that had the lowest prevalence of infant direct Vitamin D supplementation was Winnipeg (43.43% overall, 53.41% in exclusively breastfed infants, EBF), compared to Edmonton (78.62% overall, 91.11% EBF) and Vancouver (82.59% overall, 88.71 EBF) ($p<0.001$). In addition to breastfeeding status, covariates differed between infants colonized with *C. difficile*: these include maternal age, maternal pre-pregnancy BMI, birth mode, maternal ethnicity, maternal pre-pregnancy milk consumption, household pets and infant Vitamin D supplementation. Of those infants who were colonized with *C. difficile*, their mothers were usually younger, of a higher BMI, and less frequently of Asian ethnicity (Table 3.1). Furthermore, a smaller proportion of infants colonized with *C. difficile* were born to mothers who consumed at least 3 cups of milk a day. Colonized infants were also more frequently living in homes with furry pets, in Edmonton and less likely to receive Vitamin D drops.

Table 3.1: Study participant characteristics according to Vitamin D supplementation and Clostridioides difficile colonization in all infants *P* value calculated using fisher's exact tests in Stata (version 13.0).

	Maternal supplement intake ≥400 IU				*Infant supplement intake ≥400 IU*		*C. difficile colonization*	
Covariates	**Prenatal n=108/1126 (9.59%)**	**Postnatal n=85/1126 (7.55%)**	**Both n=758/1126 (67.32%)**	**p-value**	**Yes n=409/1157 (64.65%)**	**p-value**	**Yes n=344/1157 (29.73%)**	**p-value**
Age at stool sample collection, months (Mean, SD)	3.52 (0.98)	3.42 (0.96)	3.53 (0.98)	0.787	3.58 (0.98)	**0.002**	3.76 (1.18)	**<0.001**
Maternal age, years (Mean, SD)	31.43 (4.79)	31.68 (4.40)	32.13 (4.54)	**<0.001**	32.30 (4.60)	**<0.001**	31.03 (4.87)	**<0.001**
Maternal pre-pregancy BMI (Mean, SD)	25.44 (6.24)	24.55 (5.49)	24.56 (5.15)	**0.02**	24.36 (5.05)	**<0.001**	25.82 (6.35)	**<0.001**
Hospital length of stay, days (Mean, SD)	4.83 (15.59)	10.56 (27.43)	6.03 (18.84)	0.139	4.56 (14.69)	**<0.001**	5.98 (18.13)	0.905
Birth mode, n (row %)								
Vaginal no IAP	55 (8.94)	49 (7.97)	406 (66.02)		393 (62.09)		167 (26.38)	
Vaginal IAP	22 (8.76)	19 (7.57)	173 (68.92)	0.349	168 (64.62)	0.060	72 (27.69)	**0.001**
CS Elective	12 (13.64)	5 (5.68)	57 (64.77)		63 (71.59)		31 (35.23)	
CS Emergency	17 (11.33)	8 (5.33)	111 (74.00)		111 (72.08)		65 (42.21)	
Baby Gender, n (row %)								
Female	43 (8.35)	40 (7.77)	357 (69.32)	0.436	338 (63.65)	0.537	147 (27.68)	0.175
Male	65 (10.64)	45 (7.36)	401 (65.63)		410 (65.50)		197 (31.47)	
Feeding mode, n (row %)								
Exclusively breastfed	59 (9.39)	52 (8.28)	423 (67.36)		484 (74.81)		143 (22.10)	
Partially breastfed	29 (9.42)	15 (4.87)	217 (70.45)	0.354	213 (67.83)	**<0.001**	110 (35.03)	**<0.001**
Exclusively formula fed	20 (10.58)	18 (9.52)	118 (62.43)		50 (25.64)		91 (46.67)	
Solid foods at 3 months, n (row %)								
No	160 (15.09)	82 (7.74)	718 (67.74)	0.617	708 (65.19)	0.120	318 (29.28)	0.065
Yes	1 (4.55)	0 (0.00)	17 (77.27)		11 (47.83)		11 (47.83)	
Infant antibiotics, n (row %)								
No	103 (9.41)	82 (7.49)	741 (67.67)	0.335	725 (64.44)	0.456	333 (29.60)	0.560
Yes	5 (16.13)	3 (9.68)	17 (54.84)		23 (71.88)		11 (34.38)	
Household income, n (row %)								
≤ $39999	10 (11.49)	12 (13.79)	48 (55.17)		44 (46.81)		30 (31.91)	
$40,000 to $79,999	32 (10.88)	19 (6.46)	185 (62.93)		178 (59.93)		84 (28.28)	
$80,000 to $99,999	19 (11.88)	9 (5.62)	112 (70.00)	**0.027**	107 (66.46)	**<0.001**	46 (28.57)	0.940
≥ $100,000	33 (7.08)	38 (8.15)	333 (71.46)		341 (72.86)		136 (29.06)	
Preferred not to answer	14 (13.33)	7 (6.67)	69 (65.71)		58 (55.24)		33 (31.43)	

Covariates	***Maternal supplement intake ≥400 IU/day***				***Infant supplement intake ≥400 IU/day***		***C. difficile colonization***	
	Prenatal n=108/1126 (9.59%)	**Postnatal n=85/1126 (7.55%)**	**Both n=758/1126 (67.32%)**	**p-value**	**Yes n=409/1157 (64.65%)**	**p-value**	**Yes n=344/1157 (29.73%)**	**p-value**
<u>Maternal ethnicity, n (row %)</u>								
Caucasian	81 (9.60)	68 (8.06)	567 (67.18)		550 (64.03)		270 (31.43)	
Asian	14 (8.43)	7 (4.22)	127 (76.51)	**0.008**	121 (72.02)	0.075	31 (18.45)	**0.002**
Other	13 (11.30)	10 (8.70)	63 (54.78)		73 (60.33)		39 (32.23)	
<u>Maternal Depression, n (row %)</u>								
None	63 (8.33)	60 (7.94)	530 (70.11)		513 (66.88)		217 (28.29)	
Prenatal	16 (10.67)	10 (6.67)	91 (60.67)	0.068	94 (60.26)	0.110	58 (37.18)	0.155
Postnatal	11 (10.48)	7 (6.67)	69 (65.71)		72 (62.61)		32 (27.83)	
Both	18 (15.65)	8 (6.96)	68 (59.13)		67 (57.26)		37 (31.62)	
<u>Season of birth, n (row %)</u>								
October through March (low UV)	62 (10.56)	51 (8.69)	392 (66.78)	0.151	345 (62.61)	0.176	170 (28.05)	0.198
April through September (high UV)	46 (8.53)	34 (6.31)	366 (67.90)		403 (66.50)		174 (31.58)	
<u>Maternal prenatal milk consumption, n (row %)</u>								
1 or less cups a day	4 (5.19)	5 (6.49)	59 (76.62)		54 (68.35)		27 (34.18)	
2 cups a day	32 (9.33)	27 (7.87)	232 (67.64)	0.654	216 (62.25)	0.421	120 (34.58)	**0.014**
3 or cups a day	69 (10.62)	43 (6.62)	435 (66.92)		430 (65.85)		171 (26.19)	
<u>Pets in the home, n (row %)</u>								
No	58 (9.56)	47 (7.74)	411 (67.71)	0.960	409 (65.97)	0.354	165 (26.61)	**0.014**
Yes	49 (9.51)	38 (7.38)	345 (66.99)		337 (63.35)		177 (33.27)	
<u>Study centre, n (row%)</u>								
Edmonton	26 (9.70)	19 (7.09)	182 (67.01)		217 (78.62)		115 (41.67)	
Vancouver	31 (8.29)	25 (6.68)	276 (73.80)	**0.022**	313 (82.59)	**<0.001**	87 (22.96)	**<0.001**
Winnipeg	51 (10.54)	41 (8.47)	300 (67.32)		218 (43.43)		142 (28.29)	
<u>Maternal intake, n (row%)</u>								
None or below recommendation					87 (49.71)		50 (28.57)	
Prenatal					76 (70.37)	**<0.001**	32 (29.63)	0.996
Postnatal					51 (60.00)		25 (29.41)	
Both					517 (68.21)		225 (29.68)	
<u>Infant intake, n (row%)</u>								
No	32 (8.10)	34 (8.61)	241 (61.01)	**<0.001**			137 (33.50)	**0.043**
Yes	76 (10.40)	51 (6.98)	517 (70.73)				207 (27.67)	
<u>*C. difficile* colonization, n (row%)</u>								
No	76 (9.57)	60 (7.56)	533 (67.13)	0.996	541 (66.54)	**0.043**		
Yes	32 (9.64)	25 (7.53)	225 (67.77)		207 (60.17)			

Table 3.2: Study participant characteristics according to Vitamin D supplementation and Clostridioides difficile colonization in exclusively breastfed infants. *P* value calculated using fisher's exact tests in Stata (version 13.0).

	Maternal supplement intake ≥400 IU/day				*Infant supplement intake ≥400 IU/day*		*C. difficile colonization*	
Covariates	**Prenatal n=59/628 (9.39%)**	**Postnatal n=52/628 (8.28%))**	**Both n=423/628 (67.36%)**	**p-value**	**Yes n=484/647 (74.81%)**	**p-value**	**Yes n=143/647 (22.10%)**	**p-value**
Age at stool sample collection, months (Mean, SD)	3.55 (0.87)	3.33 (0.82)	3.53 (0.97)	0.494	3.60 (0.97)	**<0.001**	3.73 (0.93)	**0.003**
Maternal age, years (Mean, SD)	31.90 (4.90)	32.43 (3.65)	32.35 (4.39)	0.074	32.45 (4.50)	**<0.001**	31.12 (4.62)	**0.005**
Maternal pre-pregancy BMI (Mean, SD)	24.36 (5.20)	24.59 (5.72)	23.67 (4.22)	0.433	23.75 (4.52)	0.203	24.77 (5.36)	**0.009**
Hospital length of stay, days (Mean, SD)	6.84 (20.90)	9.94 (26.33)	6.72 (20.81)	0.465	4.63 (15.37)	**<0.001**	6.34 (19.85)	0.919
Birtmode, n (row %)								
Vaginal no IAP	32 (9.17)	29 (8.31)	234 (67.05)	0.501	263 (73.06)	0.054	67 (18.61)	0.083
Vaginal IAP	12 (7.84)	15 (9.80)	102 (66.67)		114 (71.25)		43 (26.88)	
CS Elective	5 (13.89)	2 (5.56)	21 (58.33)		32 (88.89)		11 (30.56)	
CS Emergency	9 (11.11)	5 (6.17)	61 (75.31)		67 (81.71)		20 (24.39)	
Baby Gender, n (row %)								
Female	23 (7.96)	23 (7.96)	204 (70.59)	0.424	222 (74.00)	0.717	65 (21.67)	0.849
Male	36 (10.62)	29 (8.55)	219 (64.60)		262 (75.50)		78 (22.48)	
Solid foods at 3 months, n (row %)								
No	54 (8.90)	52 (8.57)	411 (67.71)	n/a	469 (75.28)	n/a	136 (21.83)	n/a
Yes	0 (0.00)	0 (0.00)	0 (0.00)		0 (0.00)		0 (0.00)	
Infant antibiotics, n (row %)								
No	56 (9.18)	50 (8.20)	412 (67.54)	0.556	468 (74.52)	0.430	140 (22.29)	0.779
Yes	3 (16.67)	2 (11.11)	11 (61.11)		16 (84.21)		3 (15.79)	
Household income, n (row %)								
≤ $39999	5 (12.82)	4 (10.26)	25 (64.10)		30 (68.18)		9 (20.45)	
$40,000 to $79,999	20 (11.36)	11 (6.25)	110 (62.50)		120 (67.80)		37 (20.90)	
$80,000 to $99,999	9 (10.11)	8 (8.99)	61 (68.54)	0.684	72 (80.00)	**0.001**	18 (20.00)	0.992
≥ $100,000	21 (7.75)	26 (9.59)	190 (70.11)		222 (81.32)		60 (21.98)	
Preferred not to answer	4 (9.30)	3 (6.98)	29 (67.44)		25 (58.14)		8 (18.60)	
Maternal ethnicity, n (row %)								
Caucasian	44 (9.09)	44 (9.09)	326 (67.36)		371 (74.95)		113 (22.83)	
Asian	7 (7.87)	3 (3.37)	67 (75.28)	0.157	67 (74.44)	0.987	12 (13.33)	0.067
Other	8 (14.55)	5 (9.09)	30 (54.55)		44 (75.86)		16 (27.59)	

Covariates	Maternal Intake ≥ 400 IU/day: Prenatal n=59/628 (9.39%)	Postnatal n=52/628 (8.28%))	Both n=423/628 (67.36%)	p-value	Infant intake ≥400 IU/day: Yes n=484/647 (74.81%)	p-value	C. difficile colonization: Yes n=143/647 (22.10%)	p-value
Maternal Depression, n (row%)								
None	39 (8.61)	36 (7.95)	314 (69.32)	0.577	351 (76.30)	0.455	97 (21.09)	0.390
Prenatal	8 (10.96)	7 (9.59)	41 (56.16)		53 (68.83)		23 (29.87)	
Postnatal	6 (12.00)	5 (10.00)	34 (68.00)		39 (71.91)		12 (21.82)	
Both	6 (11.54)	4 (7.69)	34 (65.38)		40 (74.07)		11 (20.37)	
Season of birth, n (row %)								
October through March (low UV)	31 (9.72)	27 (8.46)	217 (68.03)	0.866	231 (72.87)	0.278	72 (21.82)	0.925
April through September (high UV)	28 (9.06)	25 (8.09)	206 (66.67)		253 (76.67)		71 (22.40)	
Maternal prenatal milk consumption, n (row %)								
1 or less cups a day	1 (2.38)	1 (2.38)	34 (80.95)		35 (81.40)		15 (34.88)	
2 cups a day	18 (9.78)	16 (8.70)	127 (69.02)	0.417	142 (75.94)	0.640	47 (25.13)	**0.025**
3 or cups a day	39 (10.51)	30 (8.09)	245 (66.04)		278 (74.53)		70 (18.77)	
Pets in the home, n (row %)								
No	37 (10.45)	26 (7.34)	244 (68.93)	0.335	274 (75.69)	0.714	63 (17.40)	**0.002**
Yes	22 (8.06)	26 (9.52)	179 (65.57)		210 (74.20)		79 (27.92)	
Study centre, n (row%)								
Edmonton	13 (10.08)	12 (9.30)	90 (69.77)		123 (91.11)		49 (36.30)	
Vancouver	20 (8.16)	17 (6.94)	178 (72.65)	0.105	220 (88.71)	**<0.001**	36 (14.52)	**<0.001**
Winnipeg	26 (10.24)	23 (9.06)	155 (61.02)		141 (53.41)		58 (21.97)	
Maternal intake, n (row%)								
None or below recommendation					53 (56.38)	**<0.001**	19 (20.21)	0.664
Prenatal					50 (84.75)		12 (20.34)	
Postnatal					37 (71.15)		8 (15.38)	
Both					332 (78.49)		97 (22.93)	
Infant intake, n (row%)								
No	9 (5.77)	15 (9.62)	91 (58.33)	**<0.001**			39 (23.93)	0.514
Yes	50 (10.59)	37 (7.84)	332 (70.34)				104 (21.49)	
***C. diffcile* colonization, n (row%)**								
No	47 (9.55)	44 (8.94)	326 (66.26)	0.664	380 (75.40)	0.514		
Yes	12 (8.82)	8 (5.88)	97 (71.32)		104 (72.73)			

After stratifying by feeding status, the covariates that continued to differ among colonized and not colonized and exclusively breastfed infants were maternal and infant age, maternal BMI, maternal milk consumption, pets in the home and city of birth. The difference in frequency of Vitamin D drops use was no longer statistically significant among colonized and not colonized infants (23.93% no, versus 21.49% yes, p=0.514) (**Table 3.2**). Fifteen percent of infants were born to mothers who did not report taking supplements with Vitamin D or took a dose <400IU/day during pregnancy and lactation, compared to 67% whose mothers reported taking Vitamin D supplements (≥400 IU/day) in both the prenatal and postnatal period, and these proportions stayed the same following restriction of the study population to only exclusively breastfed infants.

3.4.2 Maternal and infant Vitamin D supplement intake is not associated with infant C. difficile colonization status

Simple and multiple variable logistic regression models were run for all infants to determine the crude and adjusted odds ratios for *C. difficile* colonization with Vitamin D supplementation. In the crude models, infant direct supplementation (OR: 0.75, 95% CI: 0.58 – 0.99, **Figure 3.1**), as well as ≥ 2 maternal postnatal supplements coupled with infant direct supplementation (OR:0.67, 95% CI: 0.45 – 0.98, Figure 3.1), were associated with lower odds of *C. difficile* colonization compare to those who did not take supplements. These associations were lost upon adjustment and stratification for feeding mode (**Figures 3.2 and Figure 3.3**). There were no associations in the crude or adjusted models with maternal Vitamin D supplementation meeting the RDA guidelines (≥400 IU/day) or with supplementation ≥400IU/d in the prenatal period.

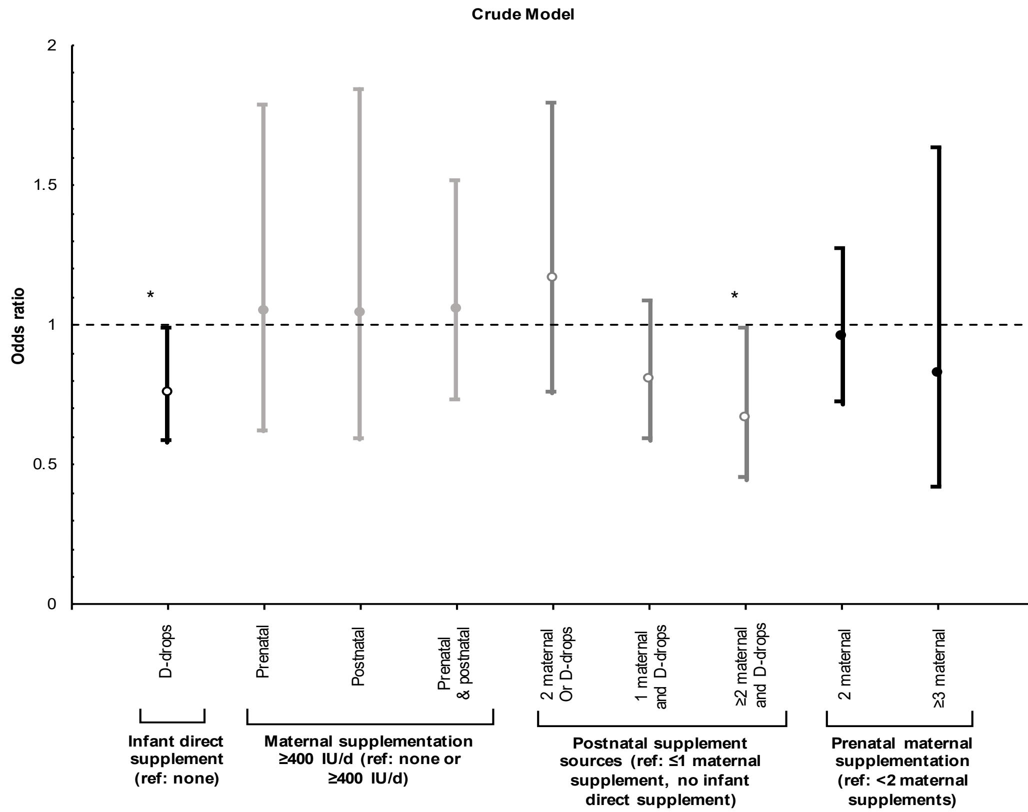

Figure 3.1: Forest plot of crude odds ratios for *C. difficile* colonization according to maternal or infant Vitamin D supplementation in the perinatal period. OR's calculated using logistic regression in Stata (version 13.0) (*p<0.05).

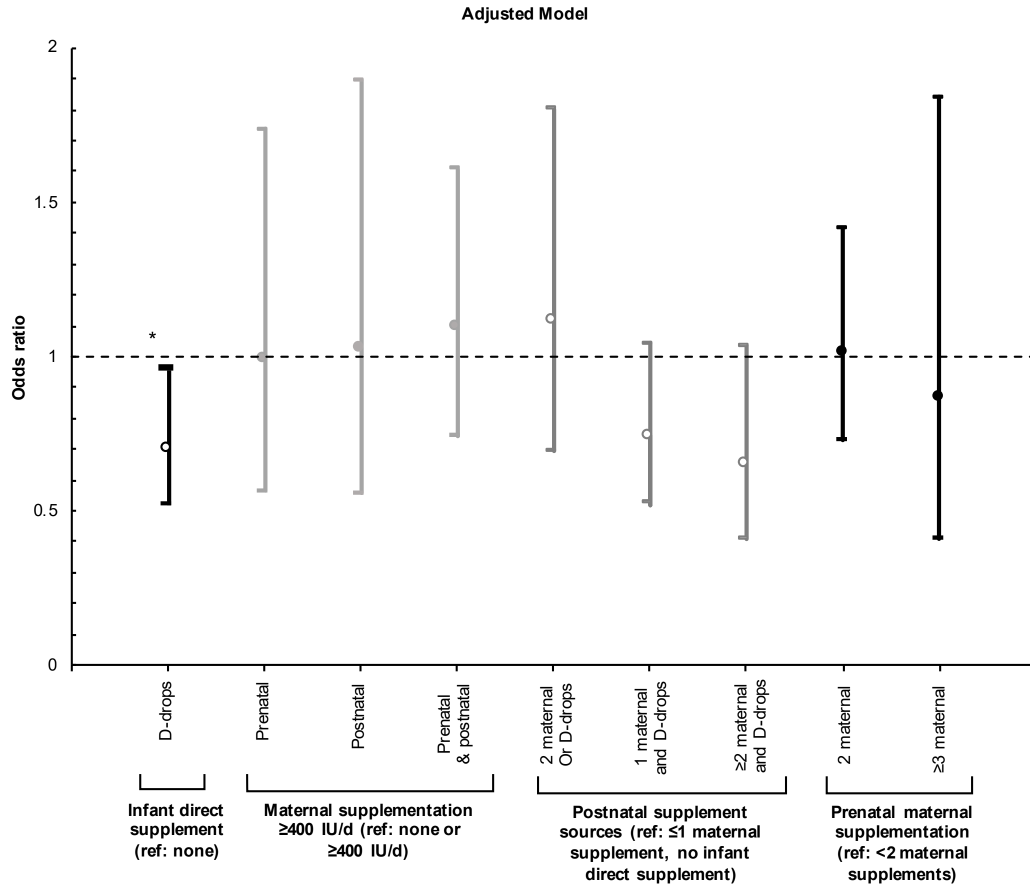

Figure 3.2: Forest plot of adjusted odds ratios for *C. difficile* colonization according to maternal or infant Vitamin D supplementation in the perinatal period. OR's calculated using logistic regression (*p<0.05). Adjusted for mode of delivery, maternal milk consumption during pregnancy, household pets, age at stool sample collection, study centre and other supplement categories.

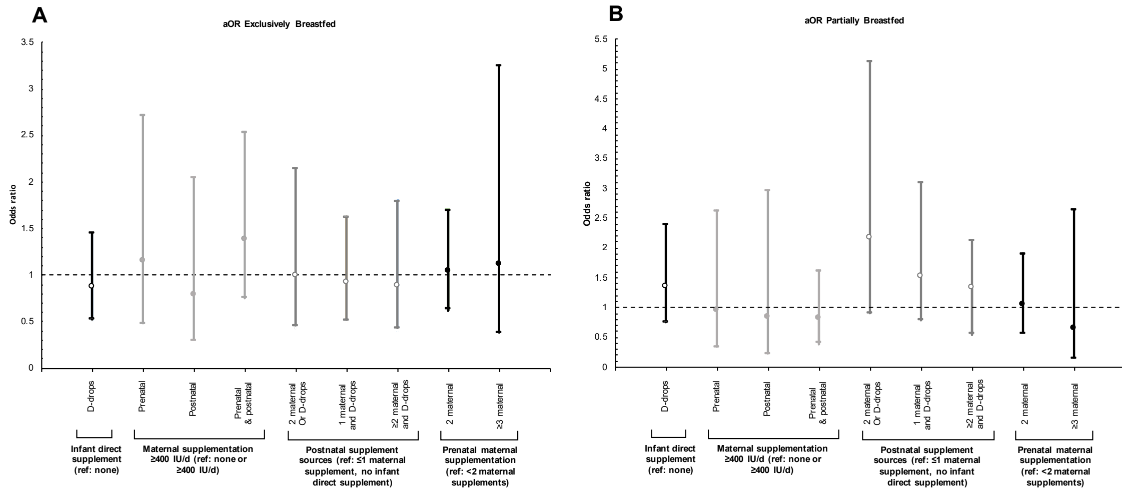

Figure 3.3: Forest plot adjusted odds ratios for *C. difficile* colonization according to maternal or infant Vitamin D supplementation in the perinatal period, before and after stratification by feeding mode. **A:** Exclusively breastfed infants, **B:** Partially breastfed infants. Exclusively formula fed infants (excluded non-direct postnatal, ie. maternal sources during lactation from this model, presented in **Figure S3.3**). Adjusted for birth mode, maternal milk consumption during pregnancy, household pets, age at stool sample collection, study centre and other supplement categories. OR's calculated using logistic regression in Stata (version 13.0).

In our stratified models, associations between infant Vitamin D drops, maternal Vitamin D supplementation and *C. difficile* status were tested using logistic regression analyses (**Table 3.3**). Crude odds ratios were not significant at alpha = 0.05 but trended towards lower likelihood of *C. difficile* colonization with infant Vitamin D drops (OR: 0.87, 95% CI: 0.57 - 1.32, p = 0.517) and maternal postnatal Vitamin D supplementation (≥400 IU) during breastfeeding (OR: 0.72, 95% CI: 0.29 - 1.78, p = 0.473). Covariates that were statistically significant at $p<0.20$ were added individually to the crude model to test for confounding, however; the OR's for the association between maternal and infant Vitamin D supplementation did not reach statistical significance after individual variable adjustments (Table 3.3). The final logistic model was

created based on purposeful selection method and included birth mode, maternal milk consumption, household pets, study centre and age at sample collection. There were no significant interaction terms (effect modification) discovered between variables. In the final model, maternal and infant Vitamin D intake (compared to intake <400 IU/day) did not prove to be significant predictors of *C. difficile* colonization (Table 3.3), but the trend towards decreased odds of *C. difficile* colonization with infant direct supplementation compared to no supplementation remained (aOR: 0.86, 95% CI: 0.51-1.46, p=0.585). Interestingly, maternal milk consumption greater than or equal to 3 cups per day, compared to 1 or less cups per day, was associated with lower odds (aOR: 0.40, 95% CI: 0.19-0.82, p=0.012 **Table S3.1**) of *C. difficile* colonization in exclusively breastfed infants.

3.4.3 Vitamin D supplementation is associated with other compositional changes in the infant gut microbiota in exclusively breastfed infants

Next, we sought to determine changes in arcsine-square root transformed microbiota relative abundances with Vitamin D intake using a multivariate linear model. All analyses were adjusted for birth mode, breastfeeding and all other gut microbiota. Although many microbiota strongly correlated with feeding and birth mode, those of the genus *Megamonas* (Veillonellaceae family) were of significantly lower abundance when the infant was supplemented with Vitamin D (q = 0.01), while *Peptostreptococcus* (Peptostreptococcaceae family) and *Eubacterium* (Eubacteriaceae family) were of lower abundance in supplemented infants compare to those that were not but statistical significance was lost upon correction for multiple comparisons (**Table S3.4**).

Table 3.3: Logistic regression analyses of Vitamin D supplementation and *C. difficile* colonization in exclusively breastfed infants (purposeful method of covariate selection). Calculated using Stata (version 13.0). None significant at $p<0.05$.

Ref: *C. difficile* absent	OR (95% CI) infant direct supplementation and *C. difficile* colonization	OR (95% CI) maternal prenatal only supplementation (≥400 IU/d) and *C. difficile* colonization	OR (95% CI) maternal postnatal only supplementation (≥400 IU/d) and *C. difficile* colonization	OR (95% CI) maternal pre and postnatal supplementation (≥400 IU/d) and *C. difficile* colonization
Crude	0.87 (0.57 - 1.32)	1.01 (0.45 - 2.26)	0.72 (0.29 - 1.78)	1.17 (0.68 - 2.04)
Adjusted for child age at sample col.	0.79 (0.52 - 1.21)	1.02 (0.45 - 2.29)	0.77 (0.31 - 1.91)	1.19 (0.68 - 2.07)
Adjusted for maternal age	0.95 (0.62 - 1.46)	1.06 (0.47 - 2.38)	0.78 (0.31 - 1.94)	1.27 (0.73 - 2.22)
Adjusted for pre-pregnancy maternal BMI	1.00 (0.64 - 1.54)	1.02 (0.43 - 2.38)	0.67 (0.26 - 1.77)	1.33 (0.74 - 2.37)
Adjusted for birth mode	0.84 (0.55 - 1.28)	1.00 (0.44 - 2.26)	0.72 (0.29 - 1.79)	1.14 (0.65 - 1.99)
Adjusted for maternal ethnicity	0.92 (0.60 - 1.41)	1.00 (0.44 - 2.26)	0.69 (0.28 - 1.72)	1.21 (0.69 - 2.11)
Adjusted for season of birth	0.87 (0.57 - 1.33)	1.01 (0.45 - 2.28)	0.72 (0.29 - 1.78)	1.18 (0.68 - 2.04)
Adjusted for maternal milk consumption	1.00 (0.63 - 1.57)	0.99 (0.42 - 2.30)	0.85 (0.33 - 2.16)	1.19 (0.67 - 2.13)
Adjusted for pets in the home	0.89 (0.58 - 1.37)	1.15 (0.50 - 2.61)	0.75 (0.30 - 1.88)	1.30 (0.74 - 2.29)
Adjusted for study centre	0.81 (0.51 - 1.30)	0.96 (0.42 - 2.18)	0.66 (0.26 - 1.67)	1.17 (0.66 - 2.05)
Adjusted for maternal Vitamin D supplement intake	0.91 (0.57 - 1.43)	N/A	N/A	N/A
Adjusted for infant Vitamin D supplement intake	N/A	1.03 (0.46 - 2.35)	0.73 (0.29 - 1.80)	1.19 (0.68 - 2.10)
Adjusted for all the above	1.00 (0.57 - 1.76)	1.14 (0.43 - 3.01)	0.94 (0.33 - 2.68)	1.68 (0.85 - 3.33)
Final purposeful model (includes: birth mode, milk consumption, pets, study centre and child age at stool sample collection)	0.86 (0.51 - 1.46)	1.15 (0.46 - 2.87)	0.95 (0.36 - 2.54)	1.43 (0.75 - 2.70)

Data were then stratified by breastfeeding status and were tested for compositional differences within exclusively breastfed infants according to supplementation status. These infants exhibited a lower relative abundance of Proteobacteria with infant Vitamin D drops, including those of the genus *Bilophila* (**Table 3.4**). Furthermore, exclusively breastfed infants born to mothers who were meeting or exceeding ≥400IU/d for Vitamin D supplementation during pregnancy and lactation had a lower abundance of bacteria belonging to the Lachnospiraceae family and a higher abundance of microbes belonging to the Pasteurellaceae (ie. Haemophilus spp.) at 3-months of age compare to infants nursed by mothers taking < 400IU/d. (Table 3.4).

Table 3.4: Multivariate linear regression (MaAslin) predicting arc-sine square root transformed relative abundances of microbiota in exclusively breastfed infants according to postnatal maternal and infant Vitamin D supplementation practices. Analyses adjusted for birth mode and all other gut microbiota (q-values FDR corrected, presented if q≤0.05).

Maternal Pre and Postnatal Vitamin D Supplementation (ref: none or <400IU/day)	Coefficient	N	Non zero N	p-value	q-value
Proteobacteria|Deltaproteobacteria|Desulfovibrionales|Desulfovibrionaceae	-0.0037	647	96	0.0010	0.013
Bilophila	-0.0038	647	95	0.0007	0.009
Firmicutes|Clostridia|Clostridiales|Lachnospiraceae	-0.0207	647	602	0.0016	0.018
Lachnospiraceae|Other	-0.0010	647	229	0.0015	0.018
Proteobacteria|Gammaproteobacteria|Pasteurellales|Pasteurellaceae	0.0057	647	522	0.0020	0.025
Haemophilus	0.0058	647	520	0.0018	0.021
Postnatal Infant Vitamin D Supplementation (ref: none)					
Proteobacteria|Deltaproteobacteria|Desulfovibrionales|Desulfovibrionaceae *Bilophila*	-0.0038	647	95	0.0007	0.009

3.5 Discussion

This study is the first to report on Vitamin D supplementation in Canadian mothers and infants in association with *C. difficile* colonization and infant gut microbiota composition. This is a key area of interest due to the discussion surrounding the health benefits of Vitamin D supplementation among Canadians and breastfed infants in biological systems but without much consideration of the potential effects on gut homeostasis and microbiota composition. We found that neither infant nor maternal Vitamin D intake (during pregnancy and/or lactation) were associated with *C. difficile* colonization in infants at 3 months of age, although results trended towards decreased likelihood of colonization when supplemented. The KOALA birth cohort from the Netherlands, who collected stool samples from infants at 1 month of age, found a significant decrease in the counts of *C. difficile* among infants born to mothers taking ≥400 IU (10 μg/d) of Vitamin D during pregnancy via multivitamins compared to no supplements after adjustment for relevant covariates (14). This study was conducted in the early 2000's when supplementation recommendations in the Netherlands were beginning to be introduced (32). Consequently, the proportion of mothers who did not take a Vitamin D containing supplement during pregnancy in the KOALA cohort was relatively large (as opposed to our proportion in CHILD), serving as a more powerful reference group for detecting differences in *C. difficile* colonization in the regression analyses. Similar to findings in other studies, the lack of association between direct infant Vitamin D supplementation and *C. difficile* colonization may be due to a true lack of relationship (ie. Vitamin D does not impact *C. difficile*) or due to a low prevalence of infants not receiving supplements among exclusively breastfed infants.

Other microbiota were found to be associated with reported Vitamin D supplementation, including a lower relative abundance of *Bilophila* (phylum Proteobacteria, maternal pre-post and

infant supplementation) and Lachnospiraceae (phylum Firmicutes, maternal pre-post supplementation) and increased abundance of *Haemophilus* spp. (phylum Proteobacteria, maternal pre-post supplementation). Previous work in mice and adults has found that similar associations, suggesting that Vitamin D may consistently alter the composition of certain gut microbiota, despite heterogeneity in study population and Vitamin D doses (12). Others have found that *Haemophilus* spp. are enriched in healthy infants whereas Lachnospiraceae are enriched in infants with diagnosed eczema (33). Furthermore, *Bilophila* spp. have been linked to inflammation and colitis in mice (34,35). Thus, the association between Vitamin D supplementation and the composition of the gut microbiota of exclusively breastfed infants could contribute to some of the noticeable health benefits related to Vitamin D in later childhood, beyond the expected benefit of supplementation for bone mineralization. Further exploration of how the microbiota may mediate the relationship between Vitamin D, colitis and allergic outcomes is warranted.

In this study, we did not have access to maternal or infant serum concentrations of Vitamin D: however, we do not believe that this greatly impacts the study results deficiency cut-offs are not necessarily relevant to microbiota measures. Additionally, the recent studies suggest that the measuring serum Vitamin D concentrations may not provide any additional benefit for understanding infant growth and immune development, and that RDA's provide sufficient information (36–38). Due to the non-linear relationship between serum measurements and supplement/dietary intake (mentioned in the introduction) (39), it may be helpful to have both measures in future work, as a more wholesome and robust measure of Vitamin D status.

Interestingly, we found that maternal milk consumption greater than or equal to 3 cups per day was associated with decreased odds of *C. difficile* colonization in exclusively breastfed

infants, even after adjustment for covariates (aOR: 0.40, 95% CI: 0.19-0.82, Table OS1). This suggests that a combination of dietary factors, rather than just a specific nutrient (ie, Vitamin D) may be associated with *C. difficile* colonization. There is evidence that maternal dietary patterns (40–43) and other lifestyle factors (ie. cleaning product use) (44) influence the development of the infant gut microbiota and later childhood health, thus future studies should include measures of both specific nutrients and larger overarching dietary patterns and their association with the gut microbiota in infancy. Unfortunately, we were not able to measure maternal milk consumption in the postnatal period, which limited our ability to accurately adjust for postnatal dietary sources of Vitamin D. Also, maternal report of infant Vitamin D supplementation in Winnipeg was very low (43.43% of all infants, 53.41% of exclusively breastfed infants). This is problematic due to under-reporting, or lack of adherence to the supplementation guidelines in Winnipeg, but we were unable to determine the exact reason behind this low prevalence of supplementation. A sensitivity analysis was conducted to ensure that our results were not affected by the low report at the Winnipeg site, and no differences were noted for the relationship between supplementation and *C. difficile* colonization when the infants and mothers from the Winnipeg study site were removed from the analysis (data not shown).

Another major limitation of this study is that we rely on self-report in questionnaires that were not designed to provide a robust measure of maternal Vitamin D intake or supplementation. Our study would have benefited from a more specific and validated nutrition/supplement questionnaire, such as the supplement intake questionnaire used in the Alberta Pregnancy Outcomes and Nutrition (APrON) study, that was pilot-tested to ensure efficient and detailed collection of vitamin and mineral data (45).

We did not see any relationship between Vitamin D or *C. difficile* and infant sex; however, we did see some important differences in our measure with regards to ethnicity. Notably, Asians were less frequently colonized with *C. difficile* while Asian and Caucasian mothers had a higher prevalence of supplementation with Vitamin D (≥400IU/day) in both the pre and postnatal periods relative to those of other ethnicities. Unfortunately, each of the ethnic groups in the "other" group did not have sufficient sample size to discern which ethnicities were supplementing less frequently. This is an important limitation as those with darker skin pigmentation have been shown to supplement less frequent and produce less subcutaneous Vitamin D, making these groups at greater risk of low Vitamin D (39). Residual confounding could remain and limit the generalizability of our study results to these groups who are of interest.

Finally, these study findings are encouraging for nursing mothers and nutritionists, in that the current recommendations surrounding Vitamin D supplementation appear to support the development of a healthy gut microbiota as supplementation was associated with lower abundance of pro-inflammatory microbes (ie. *Bilophila*) and these exclusively breastfed infants were characterized by other microbial markers of a healthy gut (ie. higher Haemophilus and lower Lachnospiraceae) at 3-months of age. Additionally, Vitamin D supplementation may not influence *C. difficile* colonization and other predictors should be explored in future; however, it is essential to confirm our negative finding to fully understand the relationship between Vitamin D, *C. difficile* colonization and the gut microbiota composition in infancy.

3.6 Conclusion

Vitamin D supplementation and *C. difficile* carriage were not associated in this cohort of infants. Future studies with Vitamin D supplements may be beneficial to confirm this negative finding and determine other predictors of *C. difficile* colonization in exclusively breastfed infants. Vitamin D supplementation does, however, shape the composition of the gut microbiota of exclusively breastfed infants during a critical period, including lowered Lachnospiraceae and Proteobacteria. Ultimately, maternal and infant Vitamin D supplementation are associated with a microbiota composition that may be protective against inflammation and allergic disease, suggesting that current guidelines may foster healthy intestinal environment and contribute to infant health beyond bone mineralization and should thus continue to be reinforced while nursing.

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Chapter 4

4. Key findings and general conclusions

4.1 Thesis summary

This research was conducted to understand the role of breastfeeding, *Clostridioides difficile* colonization and vitamin D supplementation in shaping the gut microbiome of Canadian infants. Two separate but related studies were conducted and are presented in chapters 2 and 3. Chapter 2 characterized the gut microbiome of infants at 3 months of age, according to *Clostridioides difficile* colonization status and infant diet (ie. breastfeeding versus formula). Chapter 3 examined the association between maternal and infant vitamin D supplementation and *Clostridioides difficile* colonization at 3 months of age. A secondary objective of chapter 3 was to examine the association between maternal and infant vitamin D supplementation and the infant gut microbiota composition at 3-months of age. The major findings, which represent important steps towards understanding factors that shape the infant gut microbiome, will be summarize in this chapter (chapter 4) followed by a discussion of the strengths and limitations of these studies (including an assessment of bias). The final objective of this section is to discussion the implications and potential links with future research and policy making to advance research in the area of perinatal nutritional health and developmental origins of health and disease.

Key finding #1. *Clostridioides difficile* colonization differentially impacts the gut microbiome of breastfed infants versus formula fed infants, with more significant associations and differences in gut microbiome development among exclusively breastfed infants.

Up to half of infants under the age of 1 year may be colonized with *C. difficile* without any obvious or immediate risks (colonization is largely asymptomatic). The presence and

abundance of this pathogenic microbe has been linked to susceptibility to chronic disease later in childhood, and we hypothesized that this may because *C. difficile* promotes changes in the microbial ecosystem of infants, including increased opportunity for colonization with other pathogenic bacteria and altered mucosal immunity. Formula feeding is a strong predictor of *C. difficile* colonization, thus we sought to explore microbiome changes with *C. difficile* carriage in infants with three distinct diets: exclusively breastfed infants, partially breastfed infants and exclusively formula fed infants. We found a higher prevalence of *C. difficile* in exclusively formula fed infants but were unable to determine the direction of this association. The most notable changes in gut microbiota composition, fecal metabolites and fecal secretory IgA were among exclusively breastfed infants colonized with *C. difficile* at 3 months of age (with less but some important shifts in partially breastfed babies). Exclusively breastfed infants colonized with *C. difficile* had increased microbial alpha diversity and significant inter-sample variability (beta diversity) compare to non-carriers, coupled by an increase in the relative abundance of bacteria from the Lachnospiraceae, Clostridiaceae, Ruminococcaceae, Erysipelotrichaceae, Coriobacteriaceae and Campylobacteraceae families.

Furthermore, these changes in bacteria were accompanied by changes in the concentrations of fecal metabolites, including a higher concentration of fecal short chain fatty acids (acetate, butyrate and propionate). The exclusively breastfed infants' gut microbiome is typically quite homogeneous, dominated by *Bifidobacterium* spp., and sIgA, which were also lower when *C. difficile* was present versus absent. We can appreciate that the typically and relatively uniform intestinal environment of an exclusively breastfed infant may be more susceptible to change in the presence of *C. difficile* relative to the more heterogeneous gut of infants receiving formula. Furthermore, these changes have potentially adverse consequences on

later infant metabolic and immune health. Further research is required to understand what causes *C. difficile* colonization in exclusively breastfed infants in order minimize colonization and associated microbiome shifts during development.

Key finding #2. Maternal and infant vitamin D supplement intake was not associated with *Clostridioides difficile* colonization exclusively breastfed infants.

Vitamin D supplements, in the form of liquid drops, are recommended for all breastfed infants in Canada (400 IU per day) (1). Vitamin D has received substantial attention in recent years, particularly in research surrounding optimal dosing during pregnancy and lactation to prevent adverse health outcomes in mothers and infant (2). Previous works have shown that *C. difficile* is less abundant in infants whose mothers took a vitamin D supplement in pregnancy and an increase in bacteria from the Clostridiales order when vitamin D intake is low (3–5). Thus, we thought it reasonable to hypothesize that a lack of vitamin D supplementation (or low amounts of this micronutrient) in pregnancy and lactation could be associated with *C. difficile* colonization in exclusively breastfed infants. In doing so, we aimed to answer the question that remained at the end of our previous study (chapter 2 study, key finding #1). When we explored this research question, we did not find any significant associations between maternal prenatal, postnatal, infant direct or cumulative vitamin D supplementation (mother and infant) and *C. difficile* colonization at 3 months of age. This negative finding is important to replicate in future research with more detailed, robust and controlled measures of vitamin D supplementation.

Key finding #3. Maternal and infant vitamin D supplementation were associated with compositional changes in the infant gut microbiota of exclusively breastfed infants.

Vitamin D serves as a transcription factor for molecules involved in many biological processes of the gut and the intestinal functional and physical barriers, including immune responses, intestinal cell to cell adhesion (ie. tight junction proteins) and antimicrobial peptides (6,7). Although we were not able to find any significant association between vitamin D supplementation and *C. difficile* colonization, this does not mean that vitamin D is not associated with other important shifts in the gut microbiota composition. A systematic review of all *in vivo* studies examining vitamin D and gut microbiota composition concluded that, despite large heterogeneity between studies, vitamin D supplementation and deficiency were associated with shifts in the gut microbiota composition that could have important implications for childhood health (8). Specifically, we found that *Bilophila* spp. and bacteria belonging to the Lachnospiraceae family were decreased while *Haemophilus* spp. were increased in the 3-month fecal samples of exclusively breastfed infants whose mothers took pre and postnatal vitamin D supplements compared to those who did not supplement, or who took less than 400 IU per day. *Bilophila* spp. were also decreased in the group of infant who were directly supplemented with vitamin D compared to infants who did not receive these drops. Bilophila has been shown to stimulate pro-inflammatory immune pathways and is observed in colitis (9,10). Changes to Bilophila abundance with vitamin D supplementation may contribute to the protective effects of vitamin D in colitis (11).

Others have found that *Haemophilus* spp. are enriched in healthy infants whereas Lachnospiraceae are enriched in infants with diagnosed eczema (12). This could explain, in part, the apparent associations between vitamin D supplementation in mothers and infants and the development of eczema later in childhood (13,14). These findings are important to replicate in future research with more detailed and controlled measures of vitamin D intake and perhaps a

higher versus standard dose randomized controlled trial. A germ-free mouse model could be used to discern whether these effects are due to an association between vitamin D and the immune system, or rather a vitamin D-microbiota-inflammatory connection. Furthermore, more frequent measures of gut microbiota longitudinally could provide important insight into the effect of specific micronutrients, such as vitamin D, on the gut microbiota composition.

4.2 Strengths and limitations

This thesis work has several strengths and limitations, which will be mentioned briefly in this section and discussed further in the next section on bias and confounding (Section 4.3). A significant strength of this work, although cross-sectional, is the longitudinal, collaborative, detailed and representative nature of the data gathered in the CHILD cohort. For this thesis, we had both a sufficient sample size and an abundance of covariate data to adjust for many important confounders in multi-variable and multivariate regression analyses. We were also able to stratify by feeding mode, with sufficient N in each feeding group to make important detections. Although we cannot report on causal relationships, longitudinal collection of data and fecal samples allows us to be sure that any prenatal and early life exposures came prior to microbiome analyses (outcome), thus ensuring study temporality for these specific cases.

Vitamin D supplementation, the exposure of interest in the second study (presented in chapter 3), and some of the study covariates, including breastfeeding status, were collected from questionnaires. Although these questionnaires were standardized, they rely on accurate and complete reporting by mothers. More objective measures of breastfeeding and vitamin D supplementation would have increased the reliability and validity of our study findings.

Participants recruited for CHILD were mainly residing in urban city centres, including Vancouver, Edmonton and Winnipeg, thus our results may not be generalizable to Canadian living in rural communities. Nevertheless, more than 80% of Canadians live in urban areas or very close to urban centres (15). Furthermore, more than 25% of CHILD infants had a parent of non-white ethnicity, but we were only able to classify the cohort into 3 ethnic groups (Caucasian, Asian and Other). This general "other" ethnic category presents a limitation that may have impacted our vitamin D results, as those with darker pigmented skin are known to have lower vitamin D levels (16) but also limits the generalizability of our study findings to specific ethnic groups beyond Asians and Caucasians.

Another strength of this study is the high-throughput gene sequencing that was used to profile the microbiota in our 3-month infant fecal samples. This allowed deep-level phylogenetic identification of microbial communities. When compared to sequencing platforms that were available at the time of our microbiome analyses, the Illumina MiSeq platform out-competed others: lower error rates, highest read lengths per run, reasonable cost per run and high alignment coverage of reads (17). Additionally, use of the V4 region helped reduce the loss of sensitivity and specificity for taxonomic classification, relative to hypervariable regions V1-V3 (18). Using these methods, we were unable to detect microbiota changes at the species level; however, little is known about the importance of gut microbiota at the species level for health outcomes, except with certain key bacteria such as *C. difficile* (19).

The use of both real time qPCR, ELISA and NMR methods makes our findings more robust and extensive as we were able to detect *C. difficile,* metabolites and sIgA in our infant 3-month fecal samples. Each of these methods and assays do have inherent limitations. Notably, qPCR detection of *C. difficile* was not accompanied with culture and pre-amplification steps

which are necessary for detection of toxins or spores (20,21). Additionally, prior to analysis, all infants with *C. difficile* counts below the level of detection were re-classified as non-colonized and those where *C. difficile* counts exceeded total bacterial counts were excluded in an effort to reduce the number of false positives. The ELISA kit which was used for detection of fecal sIgA states that it is able to detect both free sIgA and sIgA that was bound to bacteria, although there may still be some uncertainty surrounding these claims. Finally, NMR spectroscopy may have lower sensitivity and specificity, compared to mass spectrometry. However, it is commonly used for targeted quantification and identification of metabolites in cohort studies as it requires less sample for processing due to the less complex pre-processing steps and has high reproducibility (22). We selected NMR over MS in our study due to limited valuable sample quantity from our fecal samples for metabolomic analysis.

Overall, this research contributes to the literature surrounding breastfeeding, vitamin D supplementation and *C. difficile* colonization in infancy in novel and significant ways.

4.3 Bias and confounding

The observational nature of any epidemiological research study comes with inherent and unavoidable sources of bias. Although the CHILD study is a cohort design, and all data were collected prospectively, the main objective of this cohort was to study early life environmental and health exposures that are associated with allergies and asthma in childhood (23). Seeing as our sub-study objectives differ from the main objective of the cohort, an assessment of the internal validity, or rather degree to which the study findings are true, was conducted by considering the three main sources of bias.

4.3.1 Selection Bias

Selection bias was controlled for in the initial recruitment period, as per the CHILD study protocols. Inclusion and exclusion criteria were specified *a priori* (Appendix A, Figure S1.3) and participants were recruited from the general Canadian population using multiple methods to control selection bias. Recruitment methods included faxing study endorsements to maternal healthcare providers to be administered during a prenatal visit, research assistant recruitment directly in clinic, tradeshow booths, free and paid and media endorsements, word of mouth and other advertisements (24). Attrition rates are also very low in CHILD, with nearly 92% of initially enrolled mother infant pairs remaining in the study when infants reached 1 year of age (15). We did exclude all mothers/infants from the Toronto study site, as stool samples were not available from this study site but this is unlikely to contribute to selection bias as all study participants were recruited using the same methods and information.

As *C. difficile* colonization in infants is largely asymptomatic and was determined after enrollment, it is not likely that infants would have been selected for our secondary analysis differentially according to the outcomes of interest (*C. difficile* and microbiome measures). Contrastingly, exclusively breastfed infants did provide stool samples that were of a more liquid consistency, making it challenging to collect a large or adequate sample weight for analysis. Therefore, some breastfed infants may be unintentionally and systematically excluded from this study due to the lack of viable stool sample for microbiome and *C. difficile* measurements. Furthermore, the PCR and metabolite (ie. SCFA) analyses could not be performed at the same time as the microbial16S rRNA gene sequencing. These analyses were thus performed with secondary aliquots of the fecal sample that may have had a smaller quantity of viable sample (or no sample) available for analysis, particularly among breastfed infants. In terms of vitamin D,

this cohort is a high-income cohort thus those families of low income, who perhaps could not afford vitamin D supplements, may have been unintentionally excluded making our reference groups (ie. no or low vitamin D) under-powered or under-represented.

4.3.2 Measurement bias

Measurement bias arises when we have inconsistencies in measurements between groups of interest for the exposures and outcomes of interest (25). This type of bias is most common in intervention studies, where it is important to blind the assessor to the intervention while they are assessing the outcome or blind the study participants to the intervention. In our prospective cohort study, the vitamin D intake and breastfeeding exposure variables were created from data gathered from study participants self-report using a nutrition/supplementation questionnaire. Also, the way the questionnaires were worded did not allow for us to classify mothers according to dose, after a level of 400 IU vitamin D (Appendix C, Figure S3.1, S3.2). Our study would have benefited from a more specific and validated nutrition/supplement questionnaire, such as the supplement intake questionnaire used in the Alberta Pregnancy Outcomes and Nutrition (APrON) study, that was pilot-tested to ensure efficient and detailed collection of vitamin and mineral dosing information (26). This would have included a section for mothers to report the exact dose of Vitamin D that they were taking, or the specific brand of multivitamin/prenatal vitamin. As vitamin D supplementation is recommended for all breastfed infants, maternal perspectives on the importance of reporting vitamin D drop use for their baby was not a likely source of bias. In contrast, we did not have a measure of compliance for infant direct supplementation, and this made the assumption that if mothers reported supplementing their infant, it was daily at 400 IU in accordance with the current guidelines. Future questionnaires

and studies should also ask mothers about the frequency and dose of Vitamin D supplementation in their infant.

Standardized operating procedures were used for stool sample collection and processing (23). However, in any microbiome study there are multiple steps: sample collection, DNA extraction, gene amplification and sequencing and finally taxonomic classification which is limited by the database entries available. All of these factors increase the opportunity for measurement bias. The SyMBIOTA research team has attempted to reduce this bias as much as possible by having the DNA extraction, amplification and sequencing steps all performed by the same research technician for every 3-month fecal sample. Furthermore, infant samples were not all collected at the same time point but this potential source of measurement and confounding bias was recorded and adjusted for in regression analyses (see confounding bias section). Finally, we may have lost some valuable information by categorizing many of our measures (particularly our vitamin D doses). This was done as a way to answer important research questions despite the lack of a robust measure of vitamin D supplementation in our study (see confounding bias section).

4.3.3 Confounding Bias

When determining the best estimate of an association between an exposure and an outcome in an epidemiologic study, there is always the possibility of confounding. Confounding variables are not in the causal pathway of interest but are independently associated with both the exposure and the outcome of interest (25). The first study, presented in chapter 2, was a descriptive study, and not meant to answer a hypothesis driven question, but rather to characterize and describe any differences in the gut microbiome of infants associated with *C.*

difficile colonization. As there was not specific exposure or outcome, confounding factors were not considered. In the second study, presented in chapter 3, many factors were associated with both vitamin D supplementation (the exposure) and *C. difficile* colonization (the outcome) or microbiota composition. The CHILD study collected data on many covariates, from both questionnaires, biological measures and medical records, which were assessed for confounding and adjusted for in regression analyses when evaluating the relationship between vitamin D supplementation and *C. difficile* as necessary. Some of the measured covariates included mode of delivery, maternal milk consumption, household pets, study centre and age at sample collection. Socioeconomic status (ie. household income) had an apparent association with vitamin D supplementation, which is to be expected as vitamins and supplements are not free or covered under health insurance in Canada. Furthermore, although more highly educated women will have knowledge regarding the health benefits of this nutrient (27), we did not find any association between education or income and *C. difficile* colonization and therefore we did not consider these covariates as a source of confounding bias in our regression analyses (Chapter 3). In another study involving Canadian children, *C. difficile* infection was independent of a lower or higher socioeconomic status (28).

Unfortunately, since the proposed study is a secondary analysis, we did not have access to data for all potential confounders for our research questions and residual confounding may exist. Covariates that were not collected or unavailable include maternal vaginal and fecal microbiota measures, maternal sunlight exposure, maternal diet, breastmilk samples, cord blood samples, infant and maternal serum 25(OH)D levels. There are other important maternal nutritional factors, such as fiber and iron intake, that may have been associated with Vitamin D supplementation and microbiota composition or *C. difficile* colonization but unfortunately, we

were not able to account for these in this project. Additionally, our questionnaires were not specific enough to determine exact doses of vitamin D from supplements and food during pregnancy and the postpartum period. Best efforts were taken to control for these unavailable measures, such as testing for confounding based on the season in which the baby was born (proxy for maternal and infant sunlight exposure and subcutaneous vitamin D production) and maternal milk consumption (proxy for maternal dietary intake of vitamin D). Future work in this area should consider including and collecting the measures mentioned above to ensure the study findings are free from confounding bias.

4.4 Significance and clinical relevance

The development of the infant gut microbiome can impact future health and disease susceptibility in childhood. To date, the majority of the focus has been placed on breastfeeding and mode of delivery as early life events that shape this complex ecosystem. The findings of this thesis are novel in that they explore other factors that are associated with breastfeeding but may shape the infant gut microbiome in a unique and independent way, including vitamin D supplementation and *Clostridioides difficile* colonization. Significant shifts in the microbiome of breastfed infant colonized with *C. difficile* versus breastfed infants who were not colonized were observed and these differences may have long term health consequences. In future, we need to explore how we can minimize *C. difficile* colonization in breastfed infants to avoid undesirable shifts in microbiome development that may predispose an infant to obesity and allergy in later life.

Upon exploring a potential predictor of *C. difficile* colonization in breastfed infants, vitamin D, we found no association. This is reassuring to families and health care providers in

that barriers to vitamin D supplementation, including financial barriers, will not likely increase the likelihood of *C. difficile* colonization in these infants. Therefore, the current recommendations surrounding supplementation in pregnancy and lactation are likely to be safe. Additionally, maternal and infant vitamin D supplementation was associated with a microbiota composition that may be protective against inflammation and allergic disease, suggesting that current guidelines may foster a healthy intestinal environment and contribute to infant health beyond bone mineralization.

On the contrary, we found that maternal milk consumption in the pregnancy was associated with decreased risk of *C. difficile* colonization. In light of the updated Canadian Food Guide, which has a reduced emphasis on consumption dairy products, and an increased emphasis on a plant based diet (29), further research needs to be done to understand the potentially protective relationship between milk consumption and *C. difficile* colonization to inform these guidelines as necessary. Furthermore, we did find that vitamin D supplementation influenced gut microbiota other than *C. difficile,* thus adding to the body of literature surrounding vitamin D and health.

4.5 Concluding remarks

This thesis examined the association between *C. difficile* colonization, vitamin D supplementation and the infant gut microbiome development in infancy. In stratifying by distinct feeding groups, we were able to tease apart the effects of these unique exposures that are associated with breastfeeding and determined that breastfed infants are more susceptible to the changes in gut microbiota composition with *C. difficile* colonization compared to formula fed infants. Vitamin D supplementation (both maternal and infant) was also associated with

differences in the gut microbiota composition that may be protective against allergic disease and inflammation, but not with *C. difficile* colonization. Our observational findings generate new research questions and contribute to our understanding of modifiable population-level exposures that can play a role shaping the infant gut microbiome and later childhood health.

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APPENDIX A: Supplementary Material for Chapter 1

Figure S1.1: 3-Month Stool Sample Collection Protocol and Form

CHILD Study ID

Event Name: 3MONTHC

Sample Full Name: Child's Stool

Sample Short Name: STOOL

Sample ID:

Research Staff

Sample Collected: ◯ Yes ◯ No

Sample Collection Date and Time: Y M D T : (24 hr clock)

Weight: mg

Notes (e.g. if not collected or protocol deviation):

Instructions

Collection:

1. Label a stool specimen jar with the mother's name, CHILD study ID, and HealthDiary Specimen label.
2. The day before the visit, ask the mother to save the first poopy diaper and place in fridge in bag provided, writing the time of collection on the label.
3. Wearing nitrile gloves, take the poopy diaper into the bathroom, and collect stool to fill up to one half of the pre-labeled specimen jar.
4. Discard diaper as per the mother's instructions.
5. Ensure cap is screwed on tightly.
6. Place in cooler for transport to the Study centre.
7. On this form, record the time and date the child pooped.

Processing:

1. Freeze at -80 degrees Celsius.
2. Enter volume and collection information in HealthDiary.

CHILD Study Information

Canadian Healthy Infant Longitudinal Development Study

Toronto: 416-586-4800 X2977 Winnipeg: 204-789-3978 Edmonton: 780-407-8084 Vancouver: 604-875-2345 X5370

Coordinating Study Centre 905-522-1155 X35228

Page 1 of 1 www.CanadianChildStudy.ca © HealthDiary Inc F37SPECIMEN v.2008M12D15

Figure S1.2: Sub-samples of the Canadian Healthy Infant Longitudinal Development

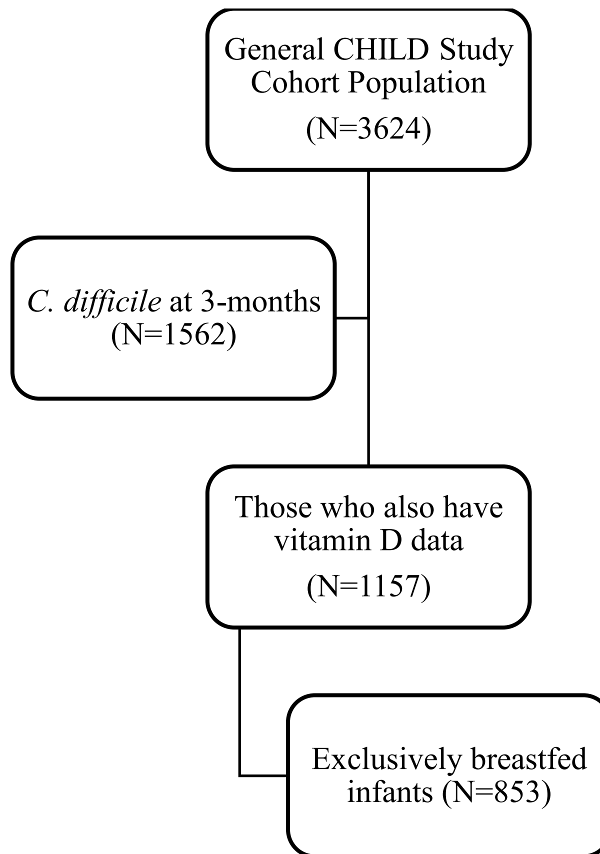


Figure S1.3: Inclusion and exclusion criteria for the CHILD Study

CHILD Study Enrolment Criteria (www.canadianchildstudy.ca/methods)	
Inclusion	**Exclusion**
[1] pregnant women aged 18 years and older (19 in Vancouver) [2] residence in reasonable proximity to the delivery hospital [3] able to read, write, and speak English [4] willing to provide informed consent [5] willing to consent to cord blood collection for the study [6] planning to give birth at a designated recruitment centre participating hospital [7] infants born at or after 35 weeks [8] able to provide name, address and telephone numbers of two alternate contact individuals	[1] children born with major congenital abnormalities or respiratory distress syndrome (RDS) [2] expectation of moving away from a recruitment area within 1 year [3] children of multiple births [4] children resulting from in vitro fertilization [5] children who will not spend at least 80% of nights in the index home [6] children born before 35 weeks gestation

APPENDIX B: Supplementary Material for Chapter 2

Figure S2.1: Chao1 and Shannon alpha diversity indices are normally distributed in our sub-sample of 1562 infants.

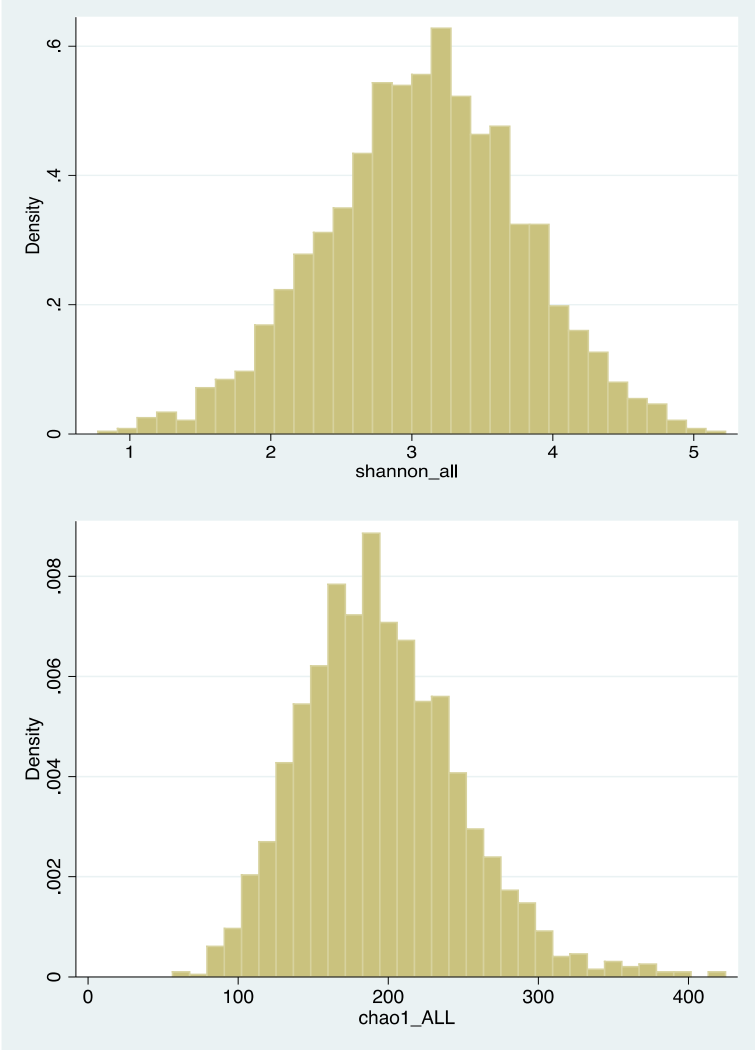


Table S2.1 Multivariate linear regression (MaAslin) predicting arc-sine square root transformed relative abundances of microbiota in infants according to *C. difficile* colonization status in exclusively breastfed infants (q-values FDR corrected).

Colonized with *C. difficile* (ref: not colonized)	*coeff*	*N*	*Non-zero N*	*p-value*	*q-value*
Firmicutes|Clostridia|Clostridiales|Peptostreptococcaceae	0.009	853	203	0.000	0.000
Firmicutes|Clostridia|Clostridiales|Lachnospiraceae	0.081	853	800	0.000	0.000
Coprococcus	0.004	853	90	0.000	0.000
Dorea	0.001	853	152	0.002	0.011
Blautia	0.001	853	227	0.000	0.004
Lachnospira	0.008	853	78	0.001	0.008
Firmicutes|Clostridia|Clostridiales|Ruminococcaceae	0.023	853	540	0.000	0.000
Oscillospira	0.014	853	357	0.000	0.000
Firmicutes|Erysipelotrichi|Erysipelotrichales|Erysipelotrichaceae	0.012	853	467	0.001	0.005
Coprobacillus	0.001	853	25	0.000	0.002
Eubacterium	0.006	853	171	0.000	0.002
Proteobacteria|Epsilonproteobacteria|Campylobacterales|Campylobacteraceae	0.001	853	164	0.001	0.006
Campylobacter	0.001	853	164	0.001	0.006
Actinobacteria|Coriobacteriia|Coriobacteriales|Coriobacteriaceae *Eggerthella*	0.004	853	354	0.001	0.008
Firmicutes|Clostridia|Clostridiales|Clostridiaceae	0.051	853	742	0.002	0.010
Actinobacteria|Actinobacteria|Bifidobacteriales|Bifidobacteriaceae	-0.063	853	832	0.004	0.021
Bifidobacterium	-0.063	853	831	0.004	0.021

Figure S2.2: Infant microbial alpha-diversity indices, Chao1 index of species richness and Shannon diversity index, according to infant colonization. Higher α-diversity observed for infants colonized with *C. difficile* compared to non-carriers. Measured in all infants before stratification by feeding mode.

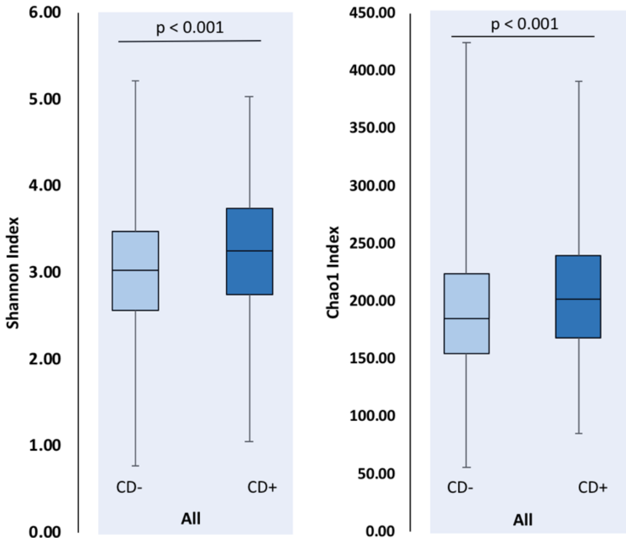
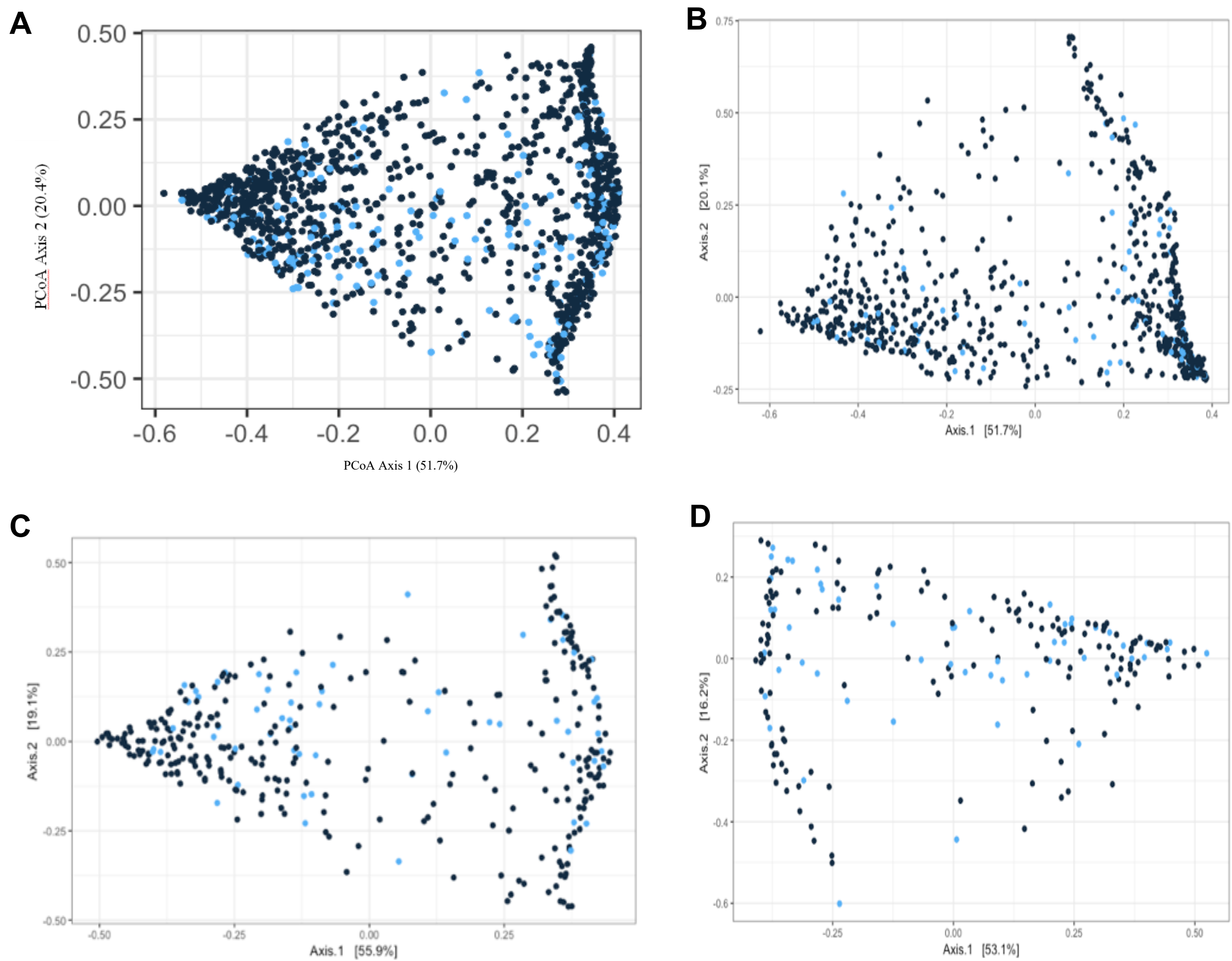


Figure S2.3: Principle coordinate analysis of *C. difficile* colonization and gut microbiota (beta-diversity), stratified by breastfeeding status based on Bray-Curtis distances calculated for each infant sample in R using the vegan package. Each dot represents an infant, light blue are those colonized with *C. difficile*, dark blue are non-carriers. A: All infants, PERMANOVA p=0.01, B: Exclusively breastfed infants, PERMANOVA p=0.01, C: Partially breastfed infants, PERMANOVA p>0.05, D: Exclusively formula fed infants p>0.05.



APPENDIX C: Supplementary Material for Chapter 3

Figure S3.1: Prenatal Maternal Supplementation Questionnaire (relevant questions only)

Mother's Vitamins and Supplements ID Y 2 0 M D

PART B: VITAMINS AND SUPPLEMENTS TAKEN SINCE YOU KNEW YOU WERE PREGNANT.

MULTIVITAMINS

2. How often did you take multivitamins (not Materna or other pregnancy-specific multivitamins)?

◯ Never ◯ < 1 per month ◯ 1-3 times per month ◯ 1-3 times per week ◯ 4-6 times per week ◯ Every day

2a. Did your multivitamin contain iron?

◯ Yes ◯ No ◯ Not applicable

2.1 How often did you take Materna or other pregnancy-specific multivitamins?

◯ Never ◯ < 1 per month ◯ 1-3 times per month ◯ 1-3 times per week ◯ 4-6 times per week ◯ Every day

2.7 How often did you take vitamin D?

◯ Never ◯ < 1 per month ◯ 1-3 times per month ◯ 1-3 times per week ◯ 4-6 times per week ◯ Every day

2.7a If you took a vitamin D supplement, how much vitamin D did you take in one day?

◯ ≤ 124 IU ◯ 125-249 IU ◯ 250-399 IU ◯ ≥ 400 IU ◯ Don't know

Figure S3.2: 3 Month Maternal Supplementation Questionnaire and Infant Nutrition Questionnaire (relevant questions only)

Mother Vitamins/Supplements 3 Mth

ID | Y 2 0 | M | D

The following questions ask about the vitamins and supplements that you took DURING THE LAST TRIMESTER of your pregnancy.

MULTIVITAMINS

1. How often did you take Materna or other pregnancy-specific multivitamins during this time period?
   - ○ Never, go to Q2
   - ○ 1-3 times per month
   - ○ 4-6 times per week
   - ○ < 1 per month
   - ○ 1-3 times per week
   - ○ Every day

   1.1 Brand name of this multivitamin: ________

2. How often did you take multivitamins during this time period (do not include pregnancy-specific multivitamins)?
   - ○ Never, go to Q3
   - ○ 1-3 times per month
   - ○ 4-6 times per week
   - ○ < 1 per month
   - ○ 1-3 times per week
   - ○ Every day

   2.1 Did this multivitamin contain iron? ○ Yes ○ No ○ N/A

   2.2 Brand name of the multivitamin: ________

8. How often did you take vitamin D during this time period?
   - ○ Never, go to Q9
   - ○ 1-3 times per month
   - ○ 4-6 times per week
   - ○ < 1 per month
   - ○ 1-3 times per week
   - ○ Every day

   8.1 If you took a vitamin D supplement, how much vitamin D did you take in one day?
   ○ ≤ 125 IU ○ 125-249 IU ○ 250-399 IU ○ ≥ 400 IU ○ Don't know

Child Nutrition 3 Months

ID | Y 2 0 | M | D

1. Did you breastfeed your child for any duration (more than a few days) since birth? ○ Yes ○ No, go to Q5

   1.1 If Yes, are you currently breastfeeding your child (whether or not feedings are supplemented)? ○ Yes ○ No, go to Q4

4. If you are not currently breastfeeding, how old was your child when you stopped breastfeeding?
   If you are currently breastfeeding your child, enter 88. If you never breastfed your child, enter 00.
   __ weeks old

5. Are you currently giving your child any infant formula? ○ Yes ○ No, go to Q6

   5.1 How old was your child when you started giving him/her any type of infant formula?
   __ weeks old

7. Are you giving your child any vitamins or supplements? ○ Yes ○ No, go to Q8

   7.1 If Yes, which are given? (Check all that apply)
   - ☐ D-drops or D-Vi-Sol (Vitamin D)
   - ☐ Tri-Vi-Sol (Vitamins A, C, D)
   - ☐ Poly-Vi-Sol (Vitamins A, C, D, B1, B2, B3, B6)
   - ☐ Poly-Vi-Sol with iron
   - ☐ Fer-In-Sol (iron)
   - ☐ Other, specify below:

   If Other, specify: ________

Figure S3.3: Forest plot adjusted odds ratios for *C. difficile* colonization according to maternal or infant vitamin D supplementation in the perinatal period. Exclusively formula fed infants OR's calculated using logistic regression in Stata (version 13.0).

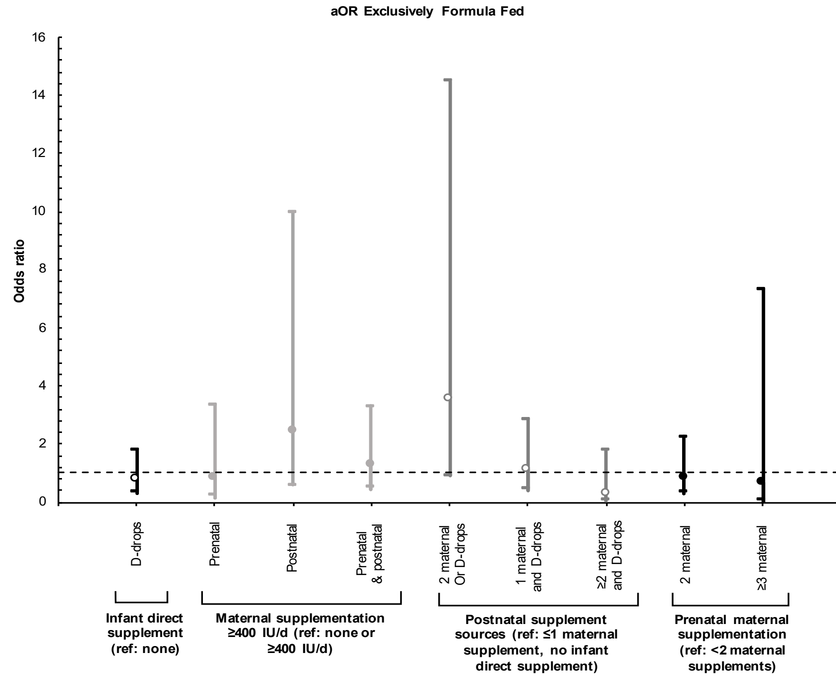


Table S3.1: Final purposeful regression model for predicting *C. difficile* colonization in exclusively breastfed infants.

	OR for *C. difficile* colonization	**p-value**	**95% Conf. Interval**
Ref: no *C. difficile* colonization			
Infant direct supplementation (ref: none)	0.86	0.585	0.51 -1.46
Maternal prenatal only supplementation ≥400 IU/day (ref: <400 IU or none)	1.15	0.759	0.46 - 2.87
Maternal postnatal only supplementation ≥400 IU/day (ref: <400 IU or none)	0.95	0.923	0.36 - 2.54
Maternal pre and postnatal supplementation ≥400 IU/day (ref: <400 IU or none)	1.43	0.274	0.75 - 2.70
Vaginal IAP (ref: vaginal no IAP)	**1.78**	**0.022**	1.09 - 2.92
CS-Elective (ref: vaginal no IAP)	**2.53**	**0.031**	1.09 - 5.86
CS-Emergency (ref: vaginal no IAP)	1.78	0.070	0.95 - 3.31
2 cups milk per day (ref: ≤1 cup per day)	0.56	0.136	0.26 - 1.20
≥3 cups milk per day (ref: ≤1 cup per day)	**0.40**	**0.012**	0.19 - 0.82
Age at stool sample collection (continuous)	1.25	0.051	1.00 - 1.55
Furry pets (ref: no furry pets)	**1.81**	**0.006**	1.19 - 2.75
Vancouver (ref: Edmonton)	**0.32**	**0.000**	0.18 - 0.57
Winnipeg (ref: Edmonton)	0.60	0.096	0.33 - 1.09

Table S3.2: Multivariate linear regression (MaAslin) predicting arc-sine square root transformed relative abundances of microbiota in infants according to postnatal maternal and infant vitamin D supplementation practices. Analyses adjusted for birthmode, feeding mode and all other gut microbiota (q-values FDR corrected).

Any Postnatal Infant Vitamin D Supplementation	*coeff*	*N*	*Non zero N*	*p*	*q*
Firmicutes|Clostridia|Clostridiales|Veillonellaceae| *Megamonas*	-0.0048866	1157	45	0.0022	0.012
Firmicutes|Clostridia|Clostridiales|Peptostreptococcaceae| *Peptostreptococcus*	-0.0016505	1157	54	0.038	0.173
Any Pre and Postnatal Maternal Vitamin D Supplementation					
Firmicutes|Clostridia|Clostridiales|Eubacteriaceae| *Eubacterium*	-0.0004473	1157	38	0.028	0.127