Cesarean Section and other Birth Interventions: impact on *Clostridioides difficile (C. difficile)* colonization in the infant gut microbiota in the Canadian Healthy Infant Longitudinal Development (CHILD) birth cohort

by

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Abstract

Background Medical interventions during childbirth are increasing, with cesarean section (CS) delivery exceeding recommended rates by 13-15 % in Canada. CS has been associated with gut dysbiosis in early life. Infants who bypass this beneficial maternal bacterial inoculation during vaginal birth have been found to be commonly colonized by opportunistic bacteria such as *C. difficile*, but factors leading to colonization remain unknown. This study aimed to determine the impact of medical interventions during birth on the colonization of *C. difficile* in infants at 3 months, 1 year and longitudinal persistent colonization throughout the first year of life.

Methods This was a prospective cohort study utilizing data on 1477 mother-infant pairs at 3 months, 1836 mother-infant pairs at 1 year and 1226 mother-infant pairs longitudinally from the Canadian Healthy Infant Longitudinal Development (CHILD) population-based birth cohort. Medical interventions (i.e. cesarean delivery, anesthetics and oxytocin-like drugs to stimulate labor such as oxytocin, carbetocin, prostaglandins), and maternal and infant covariates were collected from hospital charts or maternal questionnaires. *C. difficile* was detected in infant fecal samples collected at 3-4 months and 1 year of age using quantitative polymerase chain reaction and classified as present/absent. Logistic regression models were run to determine whether medical interventions and mode of delivery were associated with *C. difficile* colonization, adjusted for covariates.

Results Almost one-third of infants were colonized with *C. difficile* at 3 months of age which extended to almost fifty percent at 1 year of age with a fifteen percent persistence colonization rate at 3 months and 1 year of age. Overall, mode of delivery effects were most prominent at 3 months of age; *C. difficile* rates were 28%, 31%, 41% and 38% in infants born vaginally with no maternal intrapartum antibiotic prophylaxis (IAP), vaginally with IAP, emergency CS with IAP and elective CS with IAP, respectively. In unadjusted analysis, the risk of colonization with *C. difficile* was significantly increased in infants born by emergency CS and elective CS compared to vaginal birth with no IAP (OR 1.76, 95% CI: 1.27-2.44 p=0.001 and OR 1.55, 95% CI: 1.06-2.26 p=0.024, respectively). Following adjustment for maternal gravida status, birthweight, anaesthetic and oxytocin use during delivery, hospital length-of-stay, maternal

ethnicity and age, prenatal depression, postnatal smoking and breastfeeding and other covariates, the association remained significant for infants born by emergency CS compared to vaginal birth with no IAP (aOR 1.91, 95% CI: 1.27-2.88 p=0.002). Oxytocin-like drugs and anesthetics were used in 47% and 77% of all births, respectively. After stratification for these drugs, among mothers who received both anesthetics and oxytocin-like drugs during delivery, this increased risk of *C. difficile* in infants born by emergency CS was further amplified (aOR 2.29, 95% CI: 1.21-2.83 p=0.004). Mode of delivery effects persisted longitudinally to 1 year of age among first born infants and exclusively formula fed at 3 months; similarly, mode of delivery effects at 3 months were most evident in first born infants. Oxytocin and anesthetics appeared to have little effect on *C. difficile* colonization in early life; however, the 'first born effect' questions other medical interventions during birth that could disrupt the natural assembly and balance of the infant gut microbiota at birth.

Conclusions Emergency cesarean delivery was significantly associated with *C. difficile* colonization during infancy and this did not appear to be related to oxytocin-like drugs or anesthetics during delivery.

Preface

This is an original work done by Cara McLean. This thesis follows the traditional thesis format as outlined by the Faculty of Graduate Studies and Research at the University of Alberta.

Chapter 1 comrpises of a literature review on the history of birth, medical interventions during birth, the infant gut microbiota, *C. difficile* colonization in early life and potential confounding variables. As well as gaps in the literature, study objectives, hypotheses, and sample size calculation.

Chapter 2 consists of the materials and methods section with all the relevant variables associated with mode of delivery, medical interventions and *C. difficile* included in this study. As well as study design, statistical analysis and qPCR methods.

Chapter 3 includes the results section for this thesis. Chapter 4 includes the discussion, summary and interpretations of findings, strengths and limitations, clinical importance, bias and confounding and implications for future studies and conclusion

Dedication

An ode to women, written by a woman, for women

Dedicated to my family, friends and my dogs for making this journey happen

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CHAPTER 1

1.1 Birth Practices

1.1.1 History of birth

Indigenous to human nature, childbirth is an inexplicably natural event that has evolved tremendously over the last millions of years. Evolutionary constrains imply childbirth has become distressfully daunting when hominins evolved to walk upright, coinciding with narrow pelvises and bigger heads, which coined the term "obstetrical dilemma" (1). Challenging this notion, evidence of obstetric competence proves that the pelvis is extremely variable in size between individuals extending to large variations between populations potentially leading to an explanation of varying cesarean section (CS) rates around the globe. (2). This idea has shifted the focus from "obstetric dilemma" which is still believed today to "obstetric adaptation", whereby as oppose to high maternal and neonatal risk in childbirth due to the morphology of the human pelvis, actually may have deep rooted explanations in cultural, ecological succession and more recently socio-economical and historical factors amplified by biomedical models and clinician interventions (3).

1.1.2 Rising cesarean section rates

CS delivery rates have largely surpassed recommended rates of 10-15% set by the World Health Organization (WHO), whereby a rate above this limit deems little advantage to maternal or infant mortality rates (4, 5). CS rates have reached 32.3% in North America, with Canadian rates fairing close to this national average of 28%, which is 10% higher compared to 1990 (3) and well above the average global rate of 21.1% (6). Albeit efforts to promote vaginal births and evidence to suggest it is the safest for the infant, CS rates have continued to climb with great variation across the country.

The largest component of CS delivery in Canada is contributed to repeat CS followed by elective CS and CS after failed induction (7). Rising CS rates are driven mainly by non-medically indicated CS as well as medically indicated (8). Changes in maternal characteristics have accounted for much of the increase in primary CS rates, most notably increases in age, parity and weight gain as well as changes in obstetric practice (8). Decreasing use of forceps and increasing use of CS for breech presentation, labor induction, epidural anesthesia and obstetrician delivery have accounted for changes in obstetric practice in Canada (8). Unravelling maternal characteristics from obstetric practice proves challenging. Maternal characteristics often dictate mode of delivery, such as increasing maternal age and parity being a risk factor for CS delivery, induction and anesthesia (9).

With repeat CS rates being disproportionately high, prevention of primary CS delivery is of considerable interest. Indications for primary CS most commonly include labor dystocia, fetal distress and fetal malpresentation (10). First time mothers often undergo more medical interventions during delivery leading to more CS deliveries. Therefore, understanding the role of medical interventions such as oxytocin and anesthesia use during delivery may provide an important insight into the reduction of primary CS rates. Medical interventions such as synthetic oxytocin and anesthesia during delivery have undoubtedly paralleled rising CS rates but these factors may not provide the entire picture to these stubborn growing CS rates. In 2018, several Cochrane reviews were performed which indicated that induction of labour at term or beyond term as well as epidural use did not result in increased CS delivery or increased perinatal deaths; but may result in more instrumental deliveries (11, 12). In contrast, oxytocin use during labour to accelerate slow labour may not have favourable outcomes on CS deliveries (13).

Labor dystocia, defined as an abnormal progression of labor, is one of the highest contributing factors to primary CS deliveries along with failed induction of labour (10). Accordingly, the American College of Obstetricians and Gynecologists recently set forth guidelines for an updated definition of labor dystocia and failed induction of labor (10). This guideline was formed on the basis that labour progresses at a rate slower than previously thought and this may reduce unnecessary interventions during delivery. In addition, these guidelines may reduce primary CS and subsequently repeat CS. These recently updated guidelines have shown promising results. With the introduction of the new guidelines allowing a prolonged second stage of labour; primary CS rates have shown to decline (14, 15). However, a reduction in CS after failed induction was not successful among women with epidural anesthesia (16).

1.1.3 Reducing Cesarean Section rates

Halting the CS epidemic has largely been unsuccessful to date, with rates continuing to rise. Reducing CS delivery has proven to be a multifactorial process which undoubtedly has garnered much concern. Limitations of CS studies have failed to differentiate between elective and emergency CS indications (17), with primary CS more commonly resulting from emergency CS indications. Underlying etiology for indication of CS may partially explain these rising rates along with maternal and infant mortality and morbidity (17). In a recent three-part *Lancet* series; global rising CS rates, short and long-term consequences of CS and interventions to reduce unnecessary CS rates were addressed (6, 17, 18).

This series highlighted growing concern over rising CS rates, while immediate benefits of surgical interventions may not outweigh short and long-term risks. CS delivery increases maternal mortality and morbidity compared to vaginal births (17). As well as negative health effects extending to infants and children. This series also suggested addressing non-clinical

interventions may be more beneficial than clinical interventions in reducing CS rates (18). Medical indication for CS is a small percentage of these rising rates (6). Therefore, introducing more labour support, collaborative midwife-obstetrician model of care, and psychoeducational training for mothers to fully engage a multifactorial approach is of focus (18). These interventions stem from the updated World Health Organization recommendations on non-clinical interventions to reduce unnecessary CS rates (19).

Physiological alterations have been found in infants undergoing CS that may due to differences in exposures at birth (17). Growing concern around bacterial alterations at birth and during infancy from CS delivery have recently been elucidated. The infant gut microbiota has allowed another angle of revaluation into the short and long-term effects of CS delivery which will be the focus of this study. Indeed, CS delivery has been associated with a higher risk of immunological and metabolic disorders such as allergies and obesity which will be discussed in the following section (1.1.3).

1.1.4 Long-term effects of CS delivery and medical interventions

Interventions such as oxytocin and anesthesia during delivery may not increase the risk of CS delivery; however, they may have short and long-term effects. Controversy has ensued for over a decade regarding long-term effects of CS delivery which is inherently linked to these medical interventions during delivery. Recent emerging evidence suggests that medical interventions may carry short-term risks to children (20). A recent population based study analyzed 491,590 healthy low risk women who gave birth at 37-41 weeks (20). Follow-up in children was analyzed at 28 days and 5 years of age, with findings suggestive of increased likelihood of jaundice in infants who underwent an instrumental birth following induction or augmentation compared to a spontaneous vaginal birth. Alarmingly, there was increased risk of

feeding problems within 28 days in infants who underwent births with induction or augmentation in instrumental vaginal births and emergency CS compared to spontaneous vaginal birth. Medical interventions have limited evidence of long-term implications in children. However, CS delivery has consistently, yet controversially, been associated with long-term health outcomes in children.

Several systematic and meta-analyses have shown CS predisposes children to allergic disease (21, 22) and obesity (23-25); however, these reviews have not considered potential confounding factors such as maternal BMI, breastfeeding, maternal smoking and several others. After adjustment for maternal body mass index, Kuhle et al. found a 29% increased odds of obesity in children who were delivered by CS (26). More recently, a bias adjusted meta-analysis found a small but insignificant increased risk of obesity and overweight combined in early childhood, adolescence and adulthood; however, could not make strong conclusions due to residual confounding and publication bias (27).

In the most up to date comprehensive systematic review and meta-analysis covering several child outcomes in which included randomized controlled trials and prospective cohort studies in high income countries found CS to be associated with asthma up to 12 years of age, childhood overweight and obesity up to 5 years of age. The authors did not find an association with childhood wheeze, allergies, dermatitis or atopy but found a decrease risk of inflammatory bowel disease (28). Most of these findings were from observational studies including studies adjusting for several important confounders. However, heterogeneity is high and several residual confounding factors remain. One limitation is the failure to disentangle elective vs emergency CS and the association with their outcomes (28). Differences have been found in childhood asthma risk between elective and emergency CS (29, 30).

Similarly, accounting for differences between elective and emergency CS, a large US prospective study (22,068 individuals aged 9-14 and 20-28) adjusted for major confounding factors, found a 15% increased risk of obesity delivered by CS, which was stronger for women with no indication for CS (elective CS) with a 30% higher risk (31). There was a decreased likelihood of becoming obese in infants born vaginally after CS delivery compared to repeat CS delivery. Interestingly, within family analysis showed 64% higher risk of obesity in offspring if they were delivered by CS compared to a sibling born vaginally (31). Additionally, the most recent population based study reported a 17% higher risk of asthma in infants undergoing emergency CS compared to spontaneous vaginal birth, with a smaller risk of 7% associated with elective CS (20). A higher risk of metabolic outcomes were also found for elective and emergency CS delivery. A higher risk was found for emergency CS delivery compared to spontaneous vaginal delivery; however, maternal obesity was not adjusted for.

1.1.5 Summary

Mechanisms explaining this association between CS delivery and allergies and obesity are limited. However, accumulating evidence suggests this relationship may be partially mediated through the gut microbiota (32, 33). Evidence for medical interventions such as oxytocin and anesthesia are limited in scope and little research has been done regarding maternal satisfaction during birth, postnatal side effects to the mother and infant and complications resulting from these drugs. With these interventions often accompanying CS delivery, further insight is required to evaluate potential amplifying effects of CS delivery with these interventions, specifically on the infant gut microbiota.

1.2 Infant Gut Microbiota

1.2.1 Infant gut microbiota in early life

Essential and advantageous to the adaptive succession of the infant and equally as important as mother-infant bonding is the maternal-infant microbial transfer at birth. Although it is generally assumed neonates are rapidly colonized with maternal microbes at birth, there is recent evidence to suggest there is bacterial presence in the placenta and intrauterine environment (34). Therefore, there is pressing need to understand perinatal and birth related factors affecting fetal microbial seeding and subsequently infant gut microbial colonization.

Studies of the infant gut microbiota have recently transformed the way we think about birth. Microbial stimulation is integral to the success of the infant both in the short term and long-term health. The gut microbiota is an essential component in the development of the immune system and function. Most notably it acts as a barrier to pathogenic bacteria, with the most critical window of development being the first 100 days of life (35). Normally, infants are inoculated by the vaginal bacteria and maternal gut microbiota, which is the primary acquisition of beneficial microbes at birth. Vaginal transmission is a crucial factor in establishing subsequent microbial succession. Infants born vaginally harbour skin, nasal and gut microbiota very much closely resembling their mother's vaginal microbiota (36).

Vaginally born infants are characterized by an abundance of *Bacteroides, Bifidobacterium, Lactobacillus and Clostridium* in their gut microbiota (37). The vaginal microbiota changes over the course of gestation suggesting a preparation mechanism for the transfer of microbes to the infant. Characterized by decreasing diversity and species richness with a enrichment of *Lactobacillus, Clostridiales, Bacteroidales and Acintomycetales* throughout pregnancy (38). Likewise, the maternal gut microbiota undergoes several changes over the course of pregnancy with Proteobacteria and Actinobacteria increasing and paralleling a reduced

diversity (39). This coincides with hormonal fluctuations, immunological and metabolic changes which benefit the growth of the fetus and postnatal demands (39).

Accordingly, the magnitude of maternal transmission has been altered due to the medicalization of birth practices. Nonindigenous microbes are passed on with little known consequences. Although an undeniably lifesaving procedure, CS delivery has profoundly altered the initial maternal-infant route of inoculation at birth. A systematic review of seven studies found infants born by means of CS commonly have delayed microbial colonization and reduced diversity. This specifically reduces beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* and an increase in the phylum Firmicutes. Of particular importance are microbial changes seen up to 3 months yet disappearing by 6 months (37). These changes are particularly concerning in infants born by CS as these microbial differences may render infants more susceptible to pathogenic bacteria. Accordingly, CS has been associated with a myriad of diseases in the short-term and long-term and may be evidence of a poor immune system.

Commensal bacterial succession is crucial during early infancy. Facultative oxygen tolerant anaerobic bacteria dominate during the first days of life. Proteobacteria and Actinobacteria later establishes into an adult like state with strict anaerobic bacteria taking precedence such as Fimicutes and Bacteroidetes (40). Stemming from the hygiene hypothesis, whereby children raised in poor, large families or farm environments with animals seem to be protected against allergies. It is postulated that microbial stimulation in life is necessary for immune regulation (40).

1.2.2 Maternal microbial deprivation in early life by cesarean section

Modifications during this critical window of development may render infants more susceptible to inflammatory disease such allergic disease, asthma, obesity and autoimmune

disease (41). Consequently, reduced stimulation by microbes in early life and CS delivered infants results in delayed microbial colonization leading to altered homeostatic balance between Th1-Th2 immunity (42). The immune system is under the crucial influence from microbial colonization. Experimental evidence suggests there is a certain postnatal period in which T cells (Treg) and natural killer cells are educated by microbes in which enables long-term tolerance (43). Thus, a reduction in *Bacteroidetes*, being the most notable depletion in regards to CS at 3 months, has a role in the reduction of Th1 immunity (43). Vaginally born infants are characterized by a Th1 dominant immune response which is evident up to 2 years (42). CS delivery, which is characterized by a delay in microbial stimulation along with reduced beneficial bacteria, provides the optimal environment for potentially pathogenic bacteria such as *Clostridioides difficile* (*C. difficile*).

1.2.3 Consequences of medical interventions on the infant gut microbial colonization

i) Delayed breastfeeding and skin to skin initiation and duration with CS delivery

CS delivery has been associated with reduced initiation of breastfeeding and duration compared to a vaginal birth (44). Similarly, skin to skin is largely delayed after CS delivery while the mother recovers. In the meantime, the infant may receive a bath, encounter hospital surfaces and will be handled by many healthcare professionals (45). CS delivery has also been associated with disrupted microbial composition of the breast milk and colostrum (46, 47). However, the origin of microbes in breastmilk has yet to be determined and could result from the oral microbiota. Therefore, efforts to increase skin to skin regardless of breastfeeding may be beneficial, and even integral to stimulate initiation of breastfeeding.

ii) Indirect/direct effects of oxytocin and anesthetics on the infant gut microbiota

It is not a coincidence that endogenous oxytocin increases gut motility (48), especially in labouring women. Fecal-oral transmission is a natural occurrence during delivery and this transmission is essential to the infant but is limited in the hospital setting. Similar to its effects on the contractility of uterine lining, this effect has also been seen on gastric motility in women (48). Indeed, the gut carries oxytocin receptors which could be influenced by synthetic oxytocin given during labour (49). Oxytocin receptors can be found in the breast, epithelial wall and uterine muscle (48). A downregulation by 300-fold in the myometrium lining has been shown in women with oxytocin-induced labours (50). During pregnancy, the oxytocin receptor expression increases as a preparation mechanism for increased surge of endogenous oxytocin during labour (51). Therefore, a saturation and desensitization of oxytocin receptors by synthetic oxytocin during labour could have detrimental effects on other areas of the body, specifically the gut microbiota in infants which remains to be elucidated.

In conjunction with oxytocin passing through the placental barrier, medication such as fentanyl during labour also has the capacity to pass through into the fetal circulatory system (52). Most concerning to the infant gut microbiota, is the accumulating evidence for oxytocin and anesthetics to reduce the initiation and duration of breastfeeding. Intrapartum exposure to high doses of fentanyl and synthetic oxytocin were a significant predictor in whether the infant suckled. A stepwise decreasing fashion followed with the amount of fentanyl in the proportion of infants suckling while skin-to-skin in vaginal births (52). Meaning, higher doses of fentanyl and oxytocin had more negative effects on the infant suckling.

Likewise, epidural analgesia and oxytocin may negatively influence endogenous oxytocin together which may interfere with milk let down (53). Endogenous oxytocin stimulates the release of prolactin, which in the presence of synthetic oxytocin may desensitize this

relationship by altering the breast receptors and weakening the milk let down. Consequently, neonates exposed to synthetic oxytocin may undergo altered behavior which negatively influences breastfeeding (54). Accordingly, the most recent review synthesized evidence from 1978 to 2015 and found 50% of the breastfeeding measures studied were associated with less optimal breastfeeding outcomes if synthetic oxytocin was given, while 23% found no findings and 26% found mixed findings (55). However, other smaller studies have found no evidence of shorter duration or initiation with synthetic oxytocin (56). Large variations have been found between parity, breastfeeding intentions, birth setting, and obstetrical risk (55). Thus, no conclusive evidence remains between anesthetics, oxytocin and breastfeeding as it is likely time and dose dependent. Additionally, it is hard to disentangle the effects of oxytocin and anesthetics separately as they often are used simultaneously.

Thus, in light of CS delivered infants initially having a delayed microbial colonization; oxytocin and anesthetics may further amplify these effects. Breastfeeding is known to be the most crucial factor in restoring the gut microbiota after birth. Any delay in breastfeeding initiation or duration could disrupt in the infant gut microbial colonization allowing potentially pathogenic bacteria to colonize, such as *Clostridioides difficile (C. difficile)*.

1.3 *Clostridioides Difficile (C. difficile)* Colonization in Early Infancy

1.3.1 *Clostridioides difficile* colonization patterns

C. difficile was first discovered in 1935 as a gram-positive anaerobic spore forming bacillus, which presents naturally in the infant gut microbiota with no symptoms (57). Over the last ten years, *C. difficile* infection (CDI) has increased in the pediatric population (58). Although, infants under the age of 2 are most often asymptomatic carriers, it is inconclusive

whether this early life carriage affects later colonization. *C. difficile* is predominantly hospital acquired, as its spores can survive and resist chemical agents and antibiotics. However, recently community acquisition has also become an important source. Colonization rates vary geographically and are likely age dependent with 25-30% of neonates (< 1 month) found to be colonized, 10-25% of infants aged 1 month to 1 year and 5-10% of children over the age of 1 to be colonized (59). With this, an early acquisition phase and a late acquisition phase has been reported on (60).

Consequently, *C. difficile* likely colonizes in the absence of suppressive anaerobic microbes. Comparing studies from 1980 until recently, *C. difficile* was more commonly acquired during the first 6 months of life in 1980 (61). More recent studies suggest the colonization remains high until 1 year of age (62). Explanations being delayed complex microbial structure. *C. difficile* largely gets excluded when the diversity increases especially with the introduction of foods rendering the microbiota more adult like. To understand the above phenomenon, 55 samples were taken from one infant born by CS from 5.5 months to 17 months undergoing the weaning process before the beginning of the study (63). A gradual increase in diversity was seen throughout the study coinciding with increase in fecal pH. Within days of breast milk cessation drastic shifts in the microbiota were observed. A rapid increase in Bacteroidetes and Firmicutes which followed likely suppressed *C. difficile* in which it never returned after 1 year (59). Nontoxigenic strains were more common in early life which transitioned into toxigenic strains. Also, well as several strains were acquired throughout the study owing evidence for community acquisition (63).

Early cross-sectional evidence comparing differences in geography and confirming evidence of the hygiene hypothesis indicated high colonization rates of *C. difficile* in 1 year old

Swedish infants (34%) compared to Estonian children (4%). Lactobacilli was notably higher in Estonian children (64). Industrialization was deemed as a possible cause due to Estonia largely adhering to traditionally diets and lactic acid fermented foods whereas Sweden had adopted a more westernized lifestyle. Although antibiotics were not able to be analyzed in this study, more recent evidence suggest similar findings when comparing private school children to children who grew up in slums in Brazil (65).

Accordingly, Brazilian children aged 5-11 who were living in slums harboured lower colonization rates (43% vs 100%) rates and lower *C. difficile* counts (1.69 vs 8.85 CFU/g x 10^3) compared to children attending private school (65). Although these differences are strong and indicative of persistence colonization into childhood, several other factors likely play a role. Brazil has extremely high CS rates and in this study 80% of the private school children compared to 17% of slum children were born by CS delivery. Additionally, private school children had increased BMI scores and prematurity likely additionally affecting the gut microbiota and *C. difficile* colonization. However, these findings do reinforce socioeconomic status as a proxy for increased sanitation and altered microbial composition potentially leading to increased pathogenic bacteria.

1.3.2 Maternal transmission of C. difficile at birth

Considering recent evidence suggesting saline enemas during the active first stage of labor may reduce the colonization of *C. difficile* in infants at one week of age (66), the following section will explore the possible role of maternal transmission of *C. difficile* at birth.

i) Vaginal transmission at birth

Infant *C. difficile* colonization arises commonly with its first opportunity at birth and during the first few weeks of life. This could be due to direct contact from the birth canal or oral-fecal transmission or indirectly through hospital acquisition. Evidence confirming maternal transmission to infants is limited. Although using 16s rRNA, the vaginal canal has been found to harbour the Clostridium genus (38). However, few studies have confirmed *C. difficile* species colonization in the vaginal canal pre-or post-delivery. In one small-scale study, 22% of women were colonized with *C. difficile* which extended to 89% of their babies testing positive for *C. difficile* within 4 days. This is in comparison to 56% of infants testing positive in mothers who were not colonized (67). These results have not been able to be replicated, likely due to failure of culture enrichment methods.

To investigate the source of inoculation in the hospital setting, Al-jumail et al., collected fecal samples from nurses, samples from the environment in the unit, and vaginal swabs from the mother. It was found that the environment was deemed to be the culprit as the nurses fecal sample and mothers' vaginal swabs failed to return positive samples while 16 infants tested positive for *C. difficile* (68). Seeing as vaginal delivery poses a lower risk for colonization rates and counts of *C. difficile* compared to cesarean section, it is highly unlikely that direct maternal-infant transmission poses as a strong risk factor for infant colonization.

ii) Fecal- oral transmission at birth

In opposition to high colonization rates of *C. difficile* during infancy, adults carry a low rate of asymptomatic *C. difficile* in the gut microbiota. Colonization may present even lower in pregnant women due to their young and healthy status (69). Leading to rare reports of mothers passing *C. difficile* to their infant through fecal oral transmission. Asymptomatic colonization

rates in pregnant women have been proven to be quite low in one Chinese cohort (3.5%), although likely to vary based on geography (70). A study of 40 Japanese neonates confirmed fecal-oral transmission in one singular mother-infant dyad case, determined by PCR ribotyping, pulsed-field gel electrophoresis and toxin gene type (71). Similarly, an epidemic strain was found in both a mother and infant pair, with evidence to suggest that the infant played a role in recurrent infection in the mother (72). Although it is impossible to determine if the mother previously had *C. difficile*, the researchers were unable to find positive samples of that epidemic strain in the neonatal unit where the baby resided for 2 days.

With this evidence, it is presumable that *C. difficile* is predominantly environmentally acquired, as rectal swabs nor fecal samples rarely confirm the presence of *C. difficile* in the maternal gut microbiota (61). Thus, evidence for enemas reducing *C. difficile* is unlikely and may be partly due to study bias, such as co-intervention bias whereby the hospital room in which women undergoing enemas during labour was thoroughly decontaminated compared to control group where this decontamination did not occur. Additionally, due to the unpredictable nature of birth, contamination bias likely has a role as the control group may have accidentally received an enema and vice versa.

iii) Protective role of vaginal microbiota in the colonization of C. difficile

Normally, the gastrointestinal tract of newborns is rapidly inoculated at birth from the birth canal predominantly colonized with *Lactobacillus species, Staphylococcus, Streptococcus, Enterococcus, and Clostridiu*m (37). The vaginal microbiota of pregnant women is characterized by lower diversity and richness than non-pregnant women (73). It endures several changes over the course of pregnancy (39) and labour (74). Vaginal bacteria have not proved to

be long-time colonizers in the infant gut. Discrepancies between bacteria have been noted in the vaginal microbiota based on bioethnicity (74). Hence, these pioneer bacteria likely initially educate the immune system, favor the succession of other subsequent beneficial bacteria which persist throughout adulthood (75). Thus, the fact that meconium does not represent vaginal colonization as an important source of inoculation may be ignoring that certain species are abundant during labour. This is likely a preparation mechanism for maternal-infant transmission and many studies are not able to study species specific effects.

The vaginal canal also harbours a lower pH, which may be neutralized during the breaking of waters. Inhibitory effects with lactobacillus has been shown, proving another beneficial factor in maternal vaginal transmission to infant (76). But, common practice in North America to prevent transmission of G*roup B Streptococcus* (GBS) through the vaginal canal in neonates is maternal intrapartum antibiotic prophylaxis (IAP) in labor to reduce neonatal sepsis. Administered in up to 10-30% (77) of women during labor for GBS positivity and all women undergoing CS which renders this rate higher, these practices are concerning due to the increase antibiotics use in general and specifically during birth which likely renders beneficial vaginal microbiota transmission even lower.

iv) Consequences of maternal IAP during labour on the infant gut microbiota

Routine use of maternal IAP during labour is not common practice in all countries. Despite successful efforts to reduce GBS transmission, GBS burden remains high in early infancy (78). Several countries like Canada, United States and several European countries including Spain, France, Belgium and Germany use culture-based approaches for testing maternal GBS colonization (78). Others have adopted risk based approaches such as the UK,

Netherlands and Norway which is in opposition to the Centers for Disease Control and Prevention guidelines. This opposition disagrees with universal rectovaginal screening during pregnancy (78). Although universal screening has been effective, early onset sepsis still differs between both practices with US rates being notably higher than Norway (.98 per and 0.54 per 1000 infants) using culture-confirmed early onset sepsis (79). Returning to practices in Norway, it is of importance to add they have a low elective CS rates along with no routine administration of IAP to women undergoing this CS delivery (80). Universal screening methods have not proved effective. In recent light these practices may prove to have consequences on the maternal and infant gut microbiota.

Consequences of routine use of IAP during labor on the infant gut microbiota have not been well documented, especially in regards to *C. difficile.* In the first study to analyze the effects of IAP on the infant gut microbiota, 50 mother-infant dyad pairs were studied, half of whom received antibiotic treatment for GBS. Stool samples were taken at 3 days in infants, where it was found that the *Clostridium* genus was depleted in the antibiotic exposed group (81). More recently, Azad et al., found infants exposed to maternal IAP had an abundance of the *Clostridium* genus (82). *Clostridium* was associated with vaginal births and emergency CS but not elective CS at 3 month. Similarly, richness was decreased in IAP exposed infants born vaginally and through emergency CS with an overall pattern of increased Proteobacteria with maternal IAP exposure was noted (82). Given the importance of the first bacterial inoculum at birth, maternal IAP could be the first exposure leading to an altered infant gut microbiota. Due to maternal transmission being reduced, altered or transmission of maternal IAP into breastmilk this could have detrimental effects on pathogenic bacteria, with cumulative exposures in infants undergoing CS delivery, specifically emergency CS.

1.3.3 Role of mode of delivery and the colonization of *Clostridioides difficile*

i) Colonization from birth to 3 month

Evidence for maternal vaginal or fecal-oral transmission is low in the transmission of *C. difficile*. These findings are also confirmed using meconium samples in neonates. In one Japanese cohort, colonization in neonates was also substantially low and *C. difficile* was only found in one neonate out of 151 within 48 hrs (83). Similarly, in another Japanese cohort zero neonates were colonized within 14 days (84). However, in an Indian cohort infants born by CS harboured relatively large amounts whereas they could not be detected in infants born vaginally at day 7 (85). In a Polish study, maternal vaginal swabs were taken with a follow up in infant samples in meconium. They noted colonization rates of 30% in vaginally born, exclusively breastfed infants (86). No mothers were colonized with Clostridia in the vaginal microbiota. But, Lactobacilli in the mother's vaginal microbiota was associated with significantly decreased counts of Clostridia in the newborn meconium. However, the authors concluded with confusing results for *C. difficile*. The authors indicated no comparison between meconium of *C. difficile* colonized infants in whom were born to lactobacilli colonized mothers *versus* no lactobacilli mothers. It seems Lactobacilli may not have played a role in *C. difficile* counts in meconium (86). Additionally, limited maternal variables were collected for, as maternal IAP, infant antibiotics, hospital length of stay were not reported on. These differences in *C. difficile* are likely due to geographical differences and timing of sampling as it appears *C. difficile* colonizes towards the end of the first week and most dominantly in the first month.

At 1 month of age, earlier studies using culture methods have not found differences between infants born vaginally, instrumental vaginally or CS delivery in *C. difficile* colonization

rates. (87). Although a small sample size was studied (n=59), along with probable inability to detect low levels of the organism, may have understated the role of birth mode. In two recent larger studies utilizing real time PCR from the same cohort of 1000 infants in the Netherlands, both found stark differences between vaginally home birth infants, vaginally hospital born infants, artificial delivery and CS infants with *C. difficile* colonization rates of 19.1%, 26%, 34% and 42%, respectively (32, 88). In addition to differing colonization rates, counts of *C. difficile* were 100-fold higher in CS infants compared to vaginally home born infants even after adjustment (88). Furthermore, *Clostridium perfringens*, which can result in co-infection with *C. difficile* in adults, has been found to be higher in CS delivered infants (89). Similarly, the *Clostridium* genus, although very heterogeneous, seems to be elevated at 3 week and 2 months in infants born by CS delivery (90).

ii) Colonization from 3-6 months

At 3 months of age, in a small subset of the Canadian Healthy Infant Longitudinal Development (CHILD) cohort, no differences were found in the prevalence of *C. difficile* between elective CS, emergency CS and vaginal birth (91). However, diversity was higher in emergency CS while elective CS had lower diversity and richness than vaginal birth. This may be due to reduced beneficial bacteria from labour onset. Presumably, these differences in diversity and richness among mode of delivery would alter the colonization of *C. difficile* in a larger sample (91). Similarly, in the same cohort with a subsample of 47 infants, *C. difficile* was less likely in infants born by CS (92). However, a larger sample size is needed to fully understand this relationship. In analyzing infants born vaginally with varying maternal vaginal microbiota, *C. difficile* counts were found to be significantly higher in infants born to mothers who had a lack of Lactobacilli in the vaginal microbiota compared to neonates of mothers who

did. Although it was a repeat sample, is not a valid comparison as *C. difficile* counts in infants at 3 months of age did not vary by the status of Lactobacillus in the vaginal canal (86). Infant samples were taken at 3 months of age with an overall colonization rate of 36%. It should be noted that colonization rates were not reported between the mothers with lactobacilli and without (86).

iii) Colonization from 6 months to 1 year

There is a paucity of literature of longitudinal studies on the impact of mode of delivery and *C. difficile* in later infancy. Differences in microbial composition show little differentiation between mode of delivery at 1 year. However, long-term effects of emergency CS have been shown with increased diversity at 1 year of age. Notably, *Bacteroidaceae* was persistently lower at 1 year of age if the infant was not exclusively breastfed (93). A depletion in *Bacteroidetes* has been shown to promote the colonization of *C. difficile* in adults. This is associated with increased abundance of *Ruminococcus gnavus* which produce trypsin-dependent antimicrobial substances which are not active against *C. difficile* (94). These findings have also been confirmed in infants in the first year of life, where biodiversity was not altered but *Ruminococcus gnavus* and *Klebsiella pneumoniae* were significantly associated with the presence of *C. difficile* (95). Meanwhile, *Bifidobacterium longum* was associated with the absence of *C. difficile*. This, in turn, may lead to a mucosal immunity role of Bifidobacteria which has been shown in many studies to be depleted in CS delivery and in the presence of *C. difficile* (95).

Notably, infants born by CS have been shown to acquire *C. difficile* ribotype 014 more often than vaginal born infants. *C. difficile* ribotype 014 is predominantly acquired beyond 2 months as it is common in the environment (62). This ribotype also shown to be a long-term (>6 months) colonizer, and is a toxin producer with the capacity to cause disease in adults (62, 63).

Thus, understanding colonization patterns in early infancy and later infancy is needed to better understand colonization rates, which most likely are not accurately represented when one sample is taken. Additionally, long-term effects of mode of delivery on potentially pathogenic bacteria is not fully understood.

1.3.4 Protective role of breastfeeding on the colonization of Clostridioides difficile

Breastfeeding may exert the most protective effects in the exclusion of *C. difficile*. Consistently, breastfeeding has been associated with lower colonization rates in infants compared to formula fed infants. In a study of 170 infants during the first year of life, formula fed infants were found to have four times the colonization rates of *C. difficile* than breastfed infants. With an intermediate effect for mixed feeding using counterimmunoelectropheresis (CIE) methods (96). Similarly, using culture methods, 343 infants from a Swedish cohort found breast fed infants to harbour significantly less C. *difficile* than formula fed infants at 6 weeks (21% vs 47%) and 6 months (21% vs 47%) (97).

More recently using realtime PCR, Penders et al., confirmed these findings in 1000 infants at 1 month of age as *C. difficile* colonization rates were more than two times lower in breastfed infants than formula fed infants (14% vs 30%). *C. difficile* counts were also significantly lower in breast fed infants (98). A small cohort of 8 vaginally born infants were studied. There were 4 breast fed (one mixed fed) and 4 formula fed infants and 136 samples were taken in the first 12 weeks of life with high sampling frequency. *C. difficile* emerged in the second to third month of life in all the formula-fed infants, and only one breastfed infant after the introduction of complementary formula using (99).

These protective effects are likely due to immunomodulatory and antibacterial properties of breastmilk which contains human milk oligosaccharides (HMO's) capable of neutralizing *C. difficile* toxins (100), as well as high amounts of IgA are found in breastmilk. High fecal IgA has been found to be inversely associated with *C. difficile* colonization (92). Breastmilk has also been associated with low fecal pH of the infant, likely from *Lactobacillus* and *Bifidobacterium* producing lactic acid and acidic fermentation of HMO's (101). HMO's select for very specific microbes allowing low diversity and richness which may play a role of exclusion of *C. difficile*. Interestingly, and more recently in the past century there has been an increase in fecal pH from 5.0 to 6.5 from 1926 to 2017. This has coincided with diminished *Bifidobacterium* in breastfed infants in westernized countries (101). Most importantly, a depletion in *Bifidobacterium* was the only bacteria associated with this shift despite acid production by other bacteria. Additionally, *Bifidobacterium* is notably depleted in CS infants which may be another insight into gut dysbiosis and subsequent rise in *C. difficile* colonization induced by westernized living.

1.3.5 Colonization of Clostridioides difficile as a risk factor for later life disease

It is not clear whether early *C. difficile* colonization affects CDI in children. Asymptomatic colonization may be associated with the progression to CDI. This is especially so if the strain is toxigenic, in which it is common for infants to harbour toxigenic strains (102). Similarly, infants do carry strains that cause infection in adults, therefore could be transmitting *C. difficile* to adults. On the other hand, asymptomatic colonization with possible non-toxigenic strains may be protective. This may be partly due to immune responses and production of antibodies which likely could prevent CDI or could integrate itself as part of the commensal bacteria (102).

Long-term evidence for *C. difficile* colonization in early life posing as a risk factor for later disease in limited. Allergic disease in later childhood has been found to be associated with early colonization of *C. difficile* as three prospective studies have shown this. Symptomatic infants aged 1-12 months colonized with *C. difficile* had an increased risk of developing at least one allergic disease at the age of 2 (aOR:5.60 95% CI: 1.52 to 20.74)(103). Food allergy prevalence was higher in the *C. difficile* positive group; however, an inadequate sample size limited the results as there were no infants with food allergy in the *C. difficile* negative group. In a larger cohort, a 54% increase in allergic sensitization up to the age of 6 or 7 years of age was found in infants colonized with *C. difficile* at 1 month (aOR:1.54;95% CI:1.02 to 2.31)(104). Similarly, in the same cohort an association between food sensitization and *C. difficile* was only found when stratified by parental history of allergies (32). Furthermore, mediation analysis confirmed a relationship between *C. difficile* as an intermediary step between mode of delivery and place and atopic outcomes (32). These two studies were limited to only one sampling point at 1 month, which may have biased the results due to the common occurrence of colonization of *C. difficile* up to 12 months. With limited evidence to date, it is uncertain how individual species affect the allergic disease. *C. difficile* may not directly influence allergic disease, but it may be an biomarker of an aberrant gut environment predisposing infants to later immune and metabolic disease.

1.3.6 Microbial compositional changes in relation to Clostridioides difficile infection (CDI)

Several bacterial taxa have shown to be depleted prior to *C. difficile* infection, similarly the presence of *C. difficile* has been associated with significant modifications in gut microbiota of infants in early life (95). Normally, the gut microbiota functions as a strong inhibitory agent

for harmful bacteria through the release of antimicrobial compounds, competitive exclusion and immune responses to prevent *C. difficile* infection (CDI) (105). In adults, CDI has shown to be associated with lower diversity and richness, with notable differences between CDI positive patients undergoing antibiotic treatment vs CDI negative patients. Taxa that were over represented in CDI negative patients who underwent antibiotic treatment, indicative of protective species, mainly belonged to the class Clostridia including *Ruminococcus, Subdoligranulum, Oscilibater, Roseburia,* in which the authors suggested a "niche exclusion" as a colonization resistant mechanism. Where similar members of microbiota compete for nutrients; therefore, depleting others by competition (105). These effects are also seen in infants, as *Ruminococcus* is more common in breastfed infants, and is thought to inhibit the growth of Clostridia and likely *C. difficile* as colonization rates in breastfed infants are significantly lower than formula fed infants (59).

1.3.7 Mechanisms to colonization: summary on the role of the infant gut microbiota

Microbial alterations by CS delivery and possibly medical interventions provides the ideal environment for the colonization of *C. difficile*. Recently reviewed trends have emerged regarding CS delivery, with striking evidence for consequences on the infant gut microbiota. Montoya-Williams et al., reviewed and found strong trends in infants born by CS delivery having notable reductions in overall diversity, especially in elective CS, with fewer health promoting bacteria and an increase in potentially pathogenic bacteria (36). It is not a coincidence opportunistic bacteria such as *C. difficile* colonizes in the absence of beneficial bacteria as the literature has shown consistent results with increased colonization with formula feeding, antibiotics and hospital environmental. All of which have detrimental effects on the diversity, richness and composition of the infant gut microbiota. CS delivery mimics these effects, showing

consistent alterations in the infant gut microbiota which likely allows the establishment of opportunistic bacteria.

Additionally, if one were to consider the cumulative effects of birth with antibiotics, oxytocin and anesthetics. These interventions may reduce and delay protective effects of breastfeeding, the most notable factor in the exclusion of *C. difficile*. Therefore, it is an additional cost to the microbiota as breastfeeding has been shown to mitigate these effects on the gut microbiota of CS delivered infants in later life (91). Thus, the literature has provided sufficient evidence to suggest *C. difficile* is more abundant where beneficial bacteria is absent. How birth method, specifically differences between elective and emergency CS and medical interventions play a role in this relationship is lacking with little known consequences for later life. Figure 1 illustrates possible mechanisms to colonization through mode of delivery and medical/birth interventions.

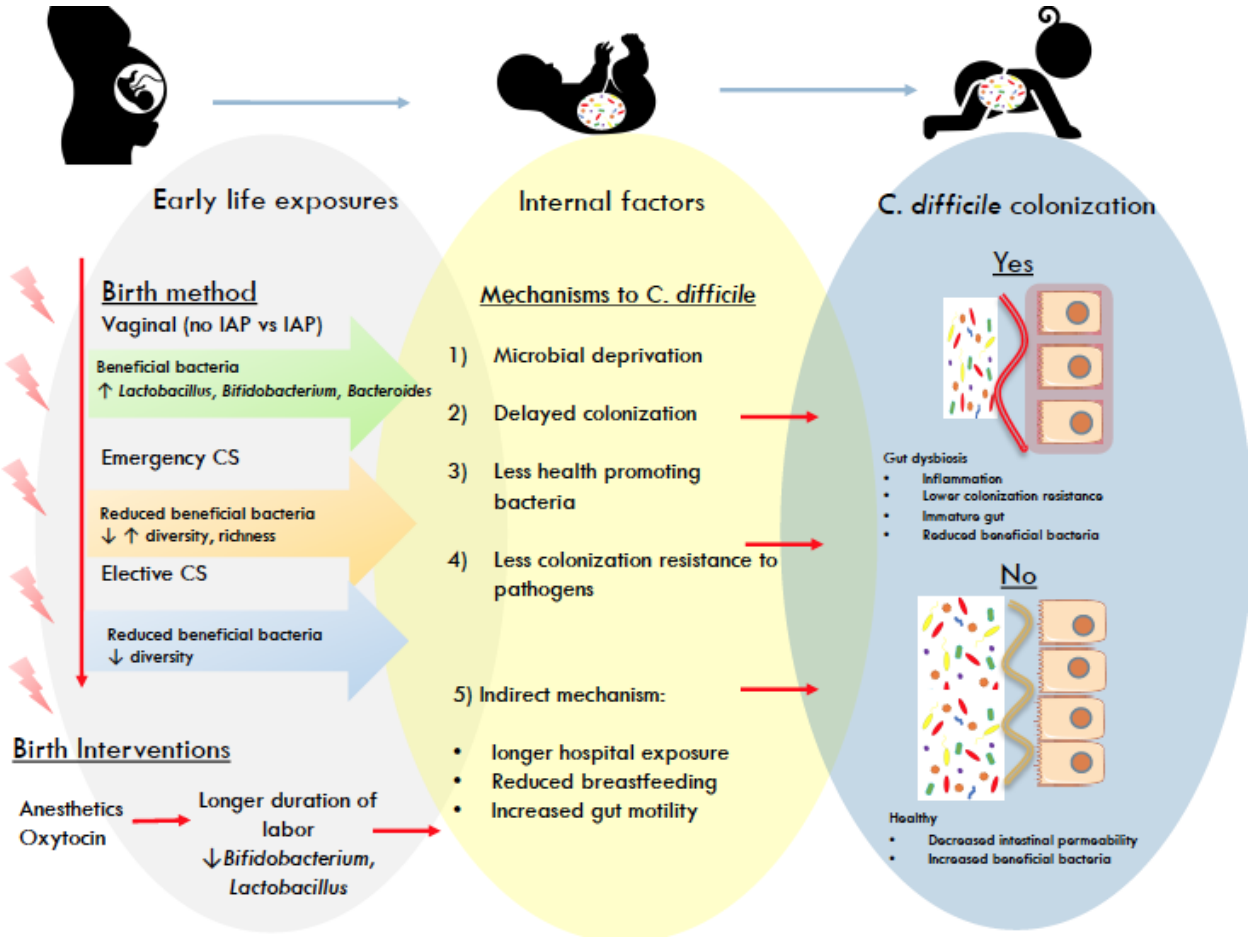


Figure 1. Conceptual framework with possible mechanisms to *C. difficile* colonization through mode of delivery

1.4 Potential Covariates affecting birth mode, medical interventions at birth and the infant gut microbiota

i) Prenatal characteristics

Gravida

Gravida, defined as the number of pregnancies the women has had including the present pregnancy. It is often an indication of cumulative medical interventions as first time mothers often undergo more medical intervention. Longer duration of labour and more complications are common which may dictate mode of delivery. This is known as 'first born effect', whereby more

medical interventions at birth often leads to more severe effects on the infant gut microbiota, which has been shown to increase opportunistic such as Clostridia and *Enterobacteriaceae* at 1 year of age with reduced amounts of anaerobic bacteria (40).

Pre-pregnancy BMI

Obesity is on the rise, with maternal obesity notably being predictor of childhood obesity. Moreover, maternal obesity has been associated with an increased risk of CS delivery (106) which essentially poses as another risk factor for childhood overweight as maternal obesity has been associated with gut dysbiosis as well as CS delivery. The mechanism by which obesity translates into the offspring is largely unknown, but it may however be mediated through changes in the maternal microbiome as it is given to the newborn during delivery or after (107). Higher pre-pregnancy BMI was associated with an increased *Bacteroides, Clostridium and Staphylococcus*, while excess gestational weight gain was associated with an increased *Bacteroides* and decreased *Bifidobacterium* in third trimester (108). Most recently, evidence for obesogenic microbial transmission was highlighted as infants born to an overweight or obese mothers were more likely to be overweight into childhood with potential for mediation through the microbiota (enrichment of *Lachnospiraceae.*) which was differentially impacted by birth mode (33). Similarly, the risk of childhood overweight was exacerbated by overweight mothers who gave birth vaginally and by CS delivery, with the latter at a much greater risk of childhood overweight (106). Additionally, breast milk from overweight mothers has been shown to be less diverse than normal weight mothers, as well as pregnant women undergoing elective CS had an altered composition compared to a vaginal delivery, suggesting there is a protective factor associated with labour (109)

Maternal age and ethnicity

Maternal age is a significant risk factor for CS delivery, as women are more commonly delaying childbirth leading to more women over the age of 35 giving birth increasingly by means of CS delivery (110). Complications during pregnancy and labour such as gestational diabetes and hypertension are more common with increasing age (111). Similarly, prolonged labour and hemorrhaging are also more common among older women (111).

Recently, in two Canadian prospective cohorts, differences in the gut microbiota were found between infants of mothers of white Caucasian *versus* South Asian ethnicity at 1 year of age. Specifically, South Asian mothers harboured more *Bifidobacterium, Lactococcus, Streptococcus* and *Enterococcus* in which are lactic producing bacteria (112). Infants born to white Caucasian mothers were found to have higher abundance of the order *Clostridiales,* in which the authors suspected dietary differences may be explaining differences; however, was not solely reliant on diet.

Pre-and postnatal depression

The microbiota- gut-brain-axis is generating considerable interest and pre-and postnatal depression is a risk factor for morbidity of the mother and child (113). The complex interplay of hormones during pregnancy and subsequently labour and the postnatal period are orchestrated by the Hypothalamic-Pituitary-Adrenal-Axis (HPA axis) which is the maternal stress system, as well as the immune system and metabolism (113). As the microbiota is integral linked to the immune system, emerging evidence suggest the microbiota may play a strong role in the gut-brain axis (113), whereby alterations in the gut microbiota may induce improper depressive responses and behaviors which could dictate labour and birth interventions. Indeed, women

requesting elective CS have shown to have higher antepartum depression and anxiety and mode of deliverymay affect post-partum depression (114), although inconclusive (115); however, post-natal depression is significantly associated with breastfeeding status. Women suffering from postpartum depression are more likely to discontinue breastfeeding, with early introduction to foods which ultimately leads to poor mother-infant bonding (116).

Maternal smoking

Maternal smoking is a significant risk factor for low birth weight which subsequently leads to child overweight with mechanisms largely remaining unknown (117). Accumulating evidence has recently suggested the term 'smoking-induced' gut dysbiosis, reviewed by Savin et al., 2018 (118), which highlights alterations in the gut microbiota of adults and mice; however, little evidence remains in infants. Gosalbes et al., found the meconium of infants born to mothers who smoked during the entire pregnancy had elevated *Enterobacteriaceae* (119) while Levin et al., found lingering effects at 6 months of age with elevated *Staphylococcus* and *Bacteroides* in infants (120). Similarly, consistent findings in adults and mice were reduced bacterial diversity, and an increase in *Bacteroides, Prevotella, Enterobacteria,* and *Clostridium* which seemed to favor cigarette exposure (118). Likewise, viable bacterial cells ranging from soil microorganisms to human bacteria have been found in cigarettes which are naturally present during the curing and fermentation process (107), however it is of concern as *C. difficile* has also been found in cigarettes with uncertainty whether these spores can survive the high temperature of smoking process and able to colonize the gut. Furthermore, third hand smoke has been recently discovered whereby smokers obtain remnants of the tobacco on their hands which passes to the extremely absorbent skin of the infant in which likely acts as a contaminant just like

second hand smoke, and now additionally could potentially transfer *C. difficile.* Lastly, household tobacco smoke during pregnancy was shown to increase the diversity, richness and evenness in neonates at 1 month of age (120).

There is also an overrepresentation of obesity and smoking among women of lower socioeconomic status (121). Among women who quit smoking during pregnancy, breastfeeding rates were lower in obese compared to normal weight women (122). Interestingly, Shenassa et al., (123) found small for gestational age infants of heavy smokers exposed to breastmilk containing tobacco metabolites gained more weight than non-exposed infants, leading to a possible mechanism through the infant gut microbiome.

ii) Postnatal characteristics

Hospitalization

As *C. difficile* infection largely results from nosocomial sources, hospitalization duration is an important factor in the acquisition. It has been shown that everyday an infant remained in the hospital, the prevalence of *C difficile* increased 13% per day compared to infants without hospitalization (88). Colonization rates has also been shown to be higher in NICU environments at 61% with the nontoxigenic strain being identical from infant to infant, confirming the acquisition was hospital acquired (124).

Gender

Discrepancies in findings in the infant gut microbiota between genders have been found and may require further stratification by ethnicity to see differences (125). Likewise, several studies have found no differences in the infant gut microbiota between genders; however, the

CHILD study cohort further restricted to their analyses to caucasian infants and found *Bacteroide*s to be depleted in males at 3 months compared to females (125). Currently, a gender-related phenomenon has been reported on, with male fetal gender as a significant risk factor for adverse pregnancy outcomes, likely dependent on parity status. Studies have found male gender specific effects on CS delivery rates with 25-47% higher risk in women undergoing spontaneous labour, while a 83-251% higher risk in women undergoing induced labour (126). Explanations likely owing to higher birthweight, head circumference and different fetal placental response to induction (126). These findings suggest that differences in the gut microbiota between male and female infants may be partly explained by mode of delivery and birth interventions such as induction and potentially anesthetics as it often accompanies induction.

Perinatal antibiotic use

Antibiotic use during infancy is a common factor associated with *C. difficile* colonization in infants as it is characterized by a depletion in the microbial barrier function resulting in loss of protection against pathogenic bacteria. A delayed maturation of the microbiota has been shown in children exposed to antibiotics (127). Antibiotics in infancy was associated with a decrease in *Clostridiales* and *Ruminococcus* from 3-9 months of life (127). In the NICU setting, prior antibiotic use has shown to decrease the risk of *C. difficile* colonization; however, following the cessation of antibiotics renders the gut microbiota to a vulnerable state allowing colonization (59). It is not common for infants to receive antibiotics in the presence of *C. difficile* and is rarely tested for under the age of 2. Nonetheless, gut diversity measures have been shown to be decreased with the use of antibiotics and it has been reported that recovery may not be attainable leading to long-term effects.

Prematurity

Penders et al, 2006 found that premature infants were more commonly colonized with *C difficile* than term infants (64% vs 23%), although limited to 11 infants the significance after adjustment did not remain due to low number of infants. Most recently, in 43 late preterm infant's intestinal colonization was delayed compared to full term infants recruited from a probiotic RCT. Clostridium species increased over time, with *C. difficile* only detectable at 6 months of age in full term infants (however early term shows there was detection to a lesser amount) (128). In contrast, in very low birth weight premature babies, *C. difficile* was more abundant within 72hrs than at 1 month and was not found in healthy term infants (129). Interestingly, preterm infants who were formula fed harboured D beta lactamase genes, where *C. difficile* strains harboring this gene were at higher abundance in formula fed infants compared to *C. difficile* lacking this gene (130).

Postnatal smoke exposure

In recent light of bacteria which thrive in a smoke loving environments, postnatal smoke exposure is important to adjust for as it could be a direct or indirect exposure. Two cohort studies have eluded to postnatal smoke as a significant risk factor for alterations in the gut microbiota, leading to a disruption in the normal commensal bacterial barrier which is a known cause for colonization of *C. difficile.* In a US cohort, neonates at 1 month of age exposed to household smoke had increased *Ruminococcus* and *Akkermansia* (120), similarly in a Canadian cohort with a larger sample size found 3-4 months old infants exposed to postnatal or pre-and postnatal smoke had increase *Ruminococcus* and *Lachnospiraceae* (131). Likewise, if the mother was a

smoker postnatally, infants at 6 months of age has increased *Bacteroides* and *Staphylococcus* (120).

Introduction to foods

The introduction of food is characterized by an increase in diversity which renders the infant gut microbiota more complex and adult like which has been show to exclude *C. difficile* (63). Therefore, depending on the timing of introduction of foods, it is likely that earlier introduction could be a sign of cessation of breastfeeding which may increase the risk of *C. difficile* or earlier introduction could increase the diversity in which excluding the colonization of *C. difficile.*

Pets and Siblings

Evidence for zoonotic transmission from animal to humans is low, however *C. difficile* is found in the soil, water and food stuff which pets may bring into the home and could be possible route for environmental acquisition. Accordingly, the risk of *C. difficile* colonization in children under 2 years of age has been shown to be greater in homes that have a pet dog, as carriages rates in household pets are common (11-40%) and can be asymptomatic and could be a potential source of acquisition for infants (132). As well as exposure to pets has been associated with an abundance of Clostridium species as well as *Peptosteptococcaceae*, in which *C. difficile* belongs (133). Stemming from the hygiene hypothesis, where growing up in larger families or on farms is protective against allergies, it is likely siblings would have an impact on the infant gut microbiota. Indeed, it has been reported that first born infants are commonly colonized with

Clostridia and Enterobacteria which differs with the amount of siblings as Bifidobacteria, *Bacteroides* and *Lactobacillu*s are more predominant in the presence of siblings (133).

1.5 Summary

There is no doubt medicalization of birth has altered the course of the symbiotic relationship between humans and microbes. Most importantly, these changes have drastically influenced the infants first maternal inoculate at birth which is essential to the ecological succession and subsequent health outcomes in infants.

1.6 Gaps in Literature

To date, there is a paucity of research regarding *C. difficile* colonization in infants between modes of delivery, notably differences between elective CS and emergency CS, and medical interventions. Many studies lack multiple sampling points which is required for accurate colonization rates as there is evidence of an early acquisition and a later acquisition. Discrepancies between colonization rates have been found between sampling points, with low and high colonization rates in meconium (0%-30%), varying colonization rates at 1-3 months of age (30-50%) which remain high until 1 year of age; although, evidence is limited at this time point. Colonization rates have shown to swiftly decline between 1-2 years of age. These differences are based on geographical locations, sampling techniques and timing, maternal and infant characteristics, limited number of infants included, lack of longitudinal measures and differential *C. difficile* outcomes.

Currently, studies on the colonization patterns of *C. difficile* exist in regards to mode of delivery (32, 62, 83, 85, 88, 91); however, most are restricted to small sample sizes and aren't

able to adjust for important pre and postnatal factors as well as birth factors which limits their findings. Most importantly, differentiation between elective CS and emergency CS has not been reported on. One cohort study out of the Netherlands of substantial size (n=1000) has reported on mode of delivery and *C. difficile* colonization at 1 month with evidence to suggest differences do exist between CS delivery and vaginal delivery and these differences have long-term effects; however, their analysis is based on single study point which requires additional follow-up (32, 88). The only longitudinal study followed 42 infants up until the age of 1 with several sample points throughout the first year, with evidence to suggest CS delivered infants carry a toxigenic strain that is a persistent long-term that is acquired from the community, and what is also concerning is the ability for this ribotype strain to cause disease in adults (62). Additionally, medical interventions at birth are a completely understudied area in regards to the infant gut microbiota, with studies to suggest these practices interfere with the natural progression of birth and the most protective factor for the infant gut microbiota, breastfeeding. Any insight to repair this relationship is needed. Therefore, there is a pressing need to understand *C. difficile* colonization patterns in relation to mode of delivery and medical interventions at birth over the first year of life, as well as persistent colonization utilizing a larger sample size, and more specifically following Canadian children as this has not been reported on in a large-scale study.

1.7 Research Question, Hypothesis and Objectives

i) Research question: Is *Clostridioides difficile* differentially impacted by mode of delivery and is this relationship further amplified by medical interventions during delivery?

ii) Hypothesis

This thesis aimed to test whether *C. difficile* colonization will be differentially impacted by mode of delivery and additionally medical interventions during labour. The hypothesis for this study is that infants born by means of CS delivery will have higher colonization rates compared to infants born vaginally. Additionally, the use of maternal IAP during labour will increase the risk between vaginal deliveries. Secondly, another hypothesis is that medical interventions may further this increased colonization risk either directly or indirectly through the use of oxytocin and anesthetics during labour.

iii) Objectives

Determine the association between:

1) *C. difficile* colonization at 3 months and 1 year in relation to mode of delivery
2) *C. difficile* persistent colonization at 3 months and 1 year in relation to mode of delivery
3) *C. difficile* colonization and mode of delivery stratified by medical interventions (oxytocin like drugs and anesthetics)

1.8 Sample Size Calculation

Differences have been found in *C. difficile* counts and colonization rates between C-section delivered infants and vaginally delivered infants. One study found colonization rates to be 43.4% in CS delivered infants compared to 27.2% in vaginally born infants. This sample size calculation was done comparing CS vs vaginally born infants using colonization percentage rates. Assuming 80% power and a two-sided alpha or .05, a sample size of 133 infants in each group is required to detect a difference of .162 in the colonization rates of *C. difficile.* The

CHILD cohort has over 2000 *C. difficile* samples with ~ 50% *C. difficile* colonization rate; therefore, the proposed study should have adequate power.

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

$$n = (1.96 + 0.84)^2 * \frac{0.272(1-0.272) + 0.434(1-0.434)}{(0.272-0.434)^2}$$

$$n = 133$$

CHAPTER 2: METHODS AND MATERIALS

2.1 Study design

This proposed study is a prospective cohort study examining the colonization patterns of *Clostridioides difficile* among infants born to different modes of delivery and medical interventions during labour (oxytocin-like drugs and anesthetics) using a subset of infants from the Canadian Healthy Longitudinal Development (CHILD) (www.childstudy.ca) national population-based general birth cohort whose mothers were enrolled during the between November 2009 and February 2012. CHILD recruited 3624 pregnant women who gave birth to 3542 eligible infants, of which 3455 were followed prospectively with clinical assessments done at birth, 3 months, and 1, 3 and 5 year visits. Strict inclusion/exclusion criteria were performed to ensure a healthy status for the pregnant mother and infant for this cohort. Table 1 provides information regarding the inclusion/exclusion criteria performed for the CHILD cohort. Participation rate in the CHILD cohort is 92% at 1 year of age (134). This study will involve infants from Edmonton, Vancouver and Winnipeg sites who have stool samples analyzed for *Clostridioides difficile* available at three months and one year with complete data on mode of

delivery and maternal intrapartum antibiotic prophylaxis (IAP) collected from hospital records or maternal questionnaires. This data will be used to assign each infant to one of four exposure groups: Vaginal birth with IAP (Intrapartum antibiotic prophylaxis during labour), vaginal without IAP, elective C-section, IAP and emergency C-section, IAP. Additionally, medical interventions (anaesthetics and oxytocin-like drugs to stimulate labour consisting of oxytocin, carbetocin and prostaglandins) will be analyzed using stratification to additionally analyze their effects on *C. difficile* while comparing mode of delivery within each stratum of medical intervention.

Table 1. Eligibility Criteria for the CHILD study

Inclusion Criteria	Exclusion Criteria
● Pregnant women that were 18 years of age or older ● Were able to read and speak English ● Planned on giving birth at one of the recruitment centres affiliated hospitals in Edmonton, Vancouver, Winnipeg ● Had a valid number and address ● Were willing to give informed consent ● Infants born at 35 weeks gestational age or greater	● Pregnant women who were planning on moving away for their recruitment centre within a 1 ● infants of multiple births ● infants resulting from in vitro fertilization ● Were born with major congenital abnormalities ● Did not spend at least 80% of nights in the participants home

2.2 Exposures

This study has the outcome variable of *C. difficile* as a binary variable (colonized: yes or no). As *C. difficile* was collected longitudinally at 3 months and 1 year, persistent colonization will also be analyzed as a binary variable using a smaller subset of the same infants who have samples at 3 months and 1 year. This study has an exposure variable of 1) mode of delivery: Vaginal birth with IAP (Intrapartum antibiotic prophylaxis during labour), vaginal without IAP, elective CS, with IAP and emergency CS, with IAP and 2) and medical interventions (oxytocin-

like drugs and anesthetics) which will be categorized as no oxytocin/anesthetics, oxytocin only, anesthetics only and both oxytocin/anesthetics. It is standard practice for all infants undergoing CS to receive IAP during delivery. Mode of delivery, maternal antibiotic prophylaxis and medical interventions were retrieved from hospital records. Oxytocin-like drugs were defined as drugs given which stimulated contractions as oxytocin, prostaglandins and duratocin (carbetocin). Anesthetics were defined as epidural, spinal, general anesthetics, gas (nitrous oxide), and local anesthetic. Stratifications will be done by important covariates and by medical interventions. Covariates collected from questionnaires data and hospital records will be used for maternal and infant characteristics which will be included in statistical models as potential confounding variables. Please see appendix A and section 2.6 for detailed list of covariates and exposures.

2.3 qPCR amplification and extraction

Fecal samples (fresh or frozen) were collected at 3 months at the home visit and or brought to the clinic at 1 year. All fecal samples were stored and transported at -80°C. Quantitative polymerase chain reaction for targeted analysis of *C. difficile* was followed and has been previously described (88). Universal 16 S primers and probes from Nadkarni *et al.,* (135), were used to quantify to total bacterial charge of the samples. Primers and probe from Penders *et al*., (98) were used for amplification of *C. difficile* 16S region. Primers and probe efficiency was determined by Standard curve procedure by making five 1:10 serial dilutions of *C. difficile* ATCC 9689D-5 genomic DNA starting at 1ng/uL. For each plate a non-template control was used. An efficiency between 90 and 110 % and an R^2 equal or higher than 0.9 for the primers and probes combination were used as quality control parameters for each run. Please see Table F1 in

Appendix F for a summary of current methods for *C. difficile* quantification, extraction and toxin detection.

2.4 Statistical Analysis

C. difficile colonization at 3 months, 1 year of age and persistent (3 months and 1 year age) will be analyzed using binary variable colonization (yes or no). Crude unadjusted associations will be tested using Pearson Chi-square test and fisher exact tests to compile a descriptive table of all maternal and infant demographics associated with the *C. difficile*. Crude unadjusted associations will be tested using Pearson Chi-square test and fisher exact tests to compile a descriptive table of all maternal and infant demographics associated with mode of delivery and medical interventions (no oxytocin or anesthetics, oxytocin only, anesthetics only, both oxytocin and anesthetics). Simple and multiple logistic regression will be used to detect differences between mode of delivery and *C. difficile* colonization (yes or no), after adjusting for potential confounding variables. To assess for potential confounders and effect modification all covariates (medical interventions, birthweight, infant sex, gravida, gender, maternal race, maternal age, maternal smoking and postnatal exposure, infant antibiotic exposure, maternal pre-pregnancy weight, prenatal and postnatal stress/depression, breastfeeding status, furry pets, siblings, introduction to foods, hospital length of stay, geography) will be adjusted for separately. Stratification will be performed within vaginal birth with IAP, vaginal birth without IAP, emergency CS and elective CS as well as for medical interventions and covariates deemed important to affect *C. difficile*. Variables that change the regression coefficients of other variables by more than 10-15% will remain in the final model or if they have a p value below

.05. The final model will provide adjusted odd ratios and 95% CI for *C. difficile* colonization. A p value of .05 is considered significant. All analysis will be conducted using STATA version 13.

2.6 Definition of potential covariates

Several covariates were analyzed in this study including: birthweight, gender, gravidity, child medication (antibiotic use), gestational age, breastfeeding at 3 months and 6 months, introduction to foods, maternal race, geography, maternal prenatal smoking, postnatal smoke exposure, maternal prenatal depression, maternal postnatal depression (6 mo), maternal prenatal stress, maternal age, maternal postnatal stress (36 weeks), maternal pre-pregnancy BMI, pets in the home, hospital length of stay, siblings and age at infant stool sample collection. All variables were created from CHILD questionnaires.

Birthweight (in grams) was collected from hospital records as a continuous variables which was then categorized into a 4 category variable due to the non-linear relationship with *C. difficile.* Gender was determined through hospital records. Gestational age was collected from hospital records, and was categorized as 37-38 weeks and 39-41 weeks at 3 months but at 1 year was left as a continuous variable due to linear relationship with *C. difficile*. Gravida was defined as total number of pregnancies, including present pregnancy, and was collected from hospital records. Hospital length of stay was recorded in hospital records, where a categorical variable was made into ≤24hrs, 2-3 days, ≥4 days. Birth chart questionnaires were formulated from hospital records for CHILD cohort use.

Breastfeeding status at time of stool collection was defined as exclusively breastfed, partially breastfed (breastfed and formula fed), and zero breastfeeding (formula fed) and was collected by maternal questionnaires at 3 and 6 months during home visits. Introduction of foods

was reported in months and collated into a variable whereby it as indicated if solid food as introduced before 3 months or after 3 months of age. Infant age at stool collection was determined at the home visit at 3 months where the infant age was recorded in weeks, this variable was left as continuous.

Maternal prenatal smoking and postnatal smoke exposure was defined if the mother smoked during pregnancy which was determined from recruitment questionnaires, and postnatal smoke exposure was defined as anyone in the home smoked after the delivery of the infant based on a home 3-month questionnaire. Siblings were defined as if there were any living children. Pets in the home was defined as any furry pet living in the home before the 3-month home visit determined from home environment questionnaires. Geography was defined as which study site they resided in (Vancouver BC, Edmonton, Ab, Winnipeg, MB, Canada).

Maternal pre-pregnancy BMI was determined based on BMI scored before the pregnancy using cut offs for underweight, normal weight, overweight and obese. Maternal race was defined as white, Asian and other. Maternal age at the time of enrollment was a continuous variable and was left continuous as it was linear with *C. difficile.* Maternal prenatal and postnatal depression was determined using the 20-item center for Epidemiological Studies Depression, where mothers self-reported various behaviors, which scores were given and collated into a sum. Maternal prenatal and postnatal stress was determined through a 10-item version of the Perceived Stress Scale, in which mothers reported various behaviors and were given a mean score using a cut off of 17. Maternal depression and stress were utilized as a binary outcome (Y/N).

CHAPTER 3: RESULTS

3.1 CHILD cohort overall population

The overall CHILD cohort did not differ than the three subsamples chosen for this study; 3months, 1 year and persistent colonization (Table 2).

Table 2. Proportion of mode of deliveryand other covariates at three time points: 3 months, 1 year and persistence colonization compared to the entire CHILD cohort

	C. difficile 3-month subsample % (N=1477)	*C. difficile* 1 year subsample % (N=1836)	*C. difficile* persistent subsample %. (N=1226)	CHILD cohort (three sites) % N=2373
Mode of delivery				
Cesarean section	23	24	23	24
Vaginal	77	76	77	76
Breastfeeding				
Yes	83	86	83	84.8
No	17	14	17	15.2
Gravida				
Primigravida	37	37	38	35.8
Multigravida	63	63	62	64.2
Pets				
Yes	54	55	54	45.2
No	46	45	46	54.8
Postnatal Smoke exposure				
Yes	15	15	14	15.7
No	85	85	86	84.3
Gender				
Male	53	52	53	52.3
Female	47	48	47	47.7

3.2 Study population (3 months of age)

i) 3 month characteristics of mother-infant pairs

In this subsample of 1477 infants at 3 months, 462 (31.2%) were colonized with *C. difficile.* Table 3. describes the characteristics of the mother-infant pairs according to colonization present or absent of *C. difficile* at 3 months of age. Samples were taken at a mean of 3.62 months (range 1.3-9.86). Overall, vaginal delivery with no IAP (54.7%) was most common route of delivery followed by vaginal delivery with IAP (22.8%), emergency CS (13%) and elective CS (9%). According to *C. difficile* colonization, there were significant differences

between mode of delivery (p=.002), with *C. difficile* colonization rates consisting of vaginal, no IAP (27%), vaginal, IAP (31%), emergency CS (40.6%) and elective CS (37.5%). Several other factors were significantly associated with the colonization of *C. difficile* at 3 months with respect to medical interventions (no oxytocin or anesthetics, oxytocin only, anesthetics only, both oxytocin and anesthetics)(p=.032), birthweight (p=.018), breastfeeding status at 3 months (p<.0001), introduction to foods (p=.005), maternal race (p<.0001), geography (p<.0001), maternal prenatal smoking (p=.043), postnatal smoke exposure (p<0001), maternal prenatal depression (p=.002), maternal pre-pregnancy BMI (p=.001), pets in the home (p=.001), hospital length of stay (p<.0001), and siblings (p=.029).

Table 4 describes frequency percentages in relation to mode of delivery and medical interventions using row percentages. Regarding mode of delivery, significant differences were found between gestational age (p=<.0001), with vaginal, no IAP delivery and elective CS birth being more common among early term births. Overall 54% of infants were exclusively breastfed at 3 months, notably exclusive breastfeeding was less common among elective CS and emergency CS. Moreover, gender (p=.004), prenatal depression (p=.647), gravida (p=<.0001), siblings (<.0001), geography (p=.001) and hospital length of stay p<.0001) differed between mode of delivery.

Among medical interventions at birth, oxytocin-like drugs and anesthetics were commonly used in 47% and 77% of all births, respectively. Infant born by emergency CS were more likely to receive both medical interventions at birth, whereas elective CS most commonly received just anesthetics which is shown in Table 4. Women undergoing vaginal birth with IAP were also more likely to receive both medical interventions compared to vaginal, no IAP. Several other factors differed between medical interventions such as gestation age (p=.01), maternal pre-

pregnancy BMI (p<.0001), breastfeeding at 3 months (p=.001), gender (p=.040), gravida (p<.0001), siblings (p<.0001), geography (p<.0001), and hospital length of stay (p<.0001). Most notably, exclusive breastfeeding rates were decreased with the use of both medical interventions, exclusive breastfeeding rates were 47.5% with both medical interventions compared to no medical interventions at 64% exclusive breastfeeding rates (data not shown). Likewise, mixed feeding rates were 31.5% with the use of both medical interventions compared to 22% in no medical interventions, which mirrored formula feeding rates at 21% and 14%, respectively (data not shown). Row percentages of frequency distribution between exposures and covariates are shown in Table 4.

Several sensitivity analyses were performed to analyze whether potential major microbiota disrupting factors affected this subsample such as homebirths, prematurity and age at sample collection. Although homebirths and prematurity was an exclusion criteria for this cohort, several infants were still included. However, sensitivity analysed showed that homebirths and prematurity (<36 weeks) did not affect the results when they were included or excluded from this study which was assessed by calculating whether the odds ratio differed by 10% with infants included vs excluded. Therefore, infants born premature remained in the study; however, it was decided that homebirths were to be removed due to the nature of maternal characteristic in which several factors lead to the decision of a homebirth. Women who undergo homebirths may not be representable to the general population as they may follow alternative diet patterns and lifestyles. Additionally, medical intervention rates during a homebirth are extremely low compared to hospital births, and missing information was high for home births. Finally, there was great variation between sampling points, as the sample point for 3 months varied from 1.3-9.8 months of age. There is evidence that after the introduction of foods around 6 months of age, the infant

gut microbiota increases in complexity and becomes more adult like, whereby *C. difficile* often gets outcompeted; however, a sensitivity analyses concluded infants with sample points above 6 months of age included in the study did not differ than if they were excluded. Please refer to Appendix D; table D1-D3 for full sensitivity analyses.

Table 3. Descriptive characteristics associated with *C. difficile* colonization at 3 months and 1 year.

		C. difficile colonization at 3 months N= 1477 Overall: 31% colonized			*C. difficile* colonization at 1year N=1836 Overall: 46% colonized		
Row percentages		**No N (%)**	**Yes N (%)**	**P value (x^2 exact)**	**No N (%)**	**Yes N (%)**	**P value (x^2 exact)**
Mode of delivery	N=1477				N=1836		
	Vaginal, No IAP	583(73%)	225(27%)	.002	553(57%)	418(43%)	.024
	Vaginal, IAP	230(69%)	106(31%)		205(49%)	210(51%)	
	Emergency CS, IAP	117(59%)	80(41%)		130(10%)	134(51%)	
	Elective CS, IAP	85(62.5%)	51(37.5%)		98(53%)	88(47%)	
Medical interventions	N=1473				N=1818		
	No oxytocin/anesthetics	174(74%)	60(26%)	.033	167(60%)	111(40%)	.008
	Oxytocin only	74(76%)	24(24%)		73(60%)	46(39%)	
	Anesthetics only	373(69%)	171(31%)		367(53%)	319(47%)	
	Both oxytocin and anesthetics	390(65%)	207(35%)		366(50%)	370(50%)	
Birthweight	N=1463				1836		
	1590-3160 g	266(72%)	101(28%)	.016	223(49%)	233(51%)	.009
	3160-3460g	249(69%)	116(31%)		230(51%)	225(49%)	
	3460-3783g	260(72%)	105(28%)		265(58%)	188(42)	
	3783-5103	228(63%)	138(37%)		257(56%)	198(44%)	
Gravida	N=1477				N=1836		
	Primigravida	360(66%)	182(33%)	.162	329(48%)	351(51%)	.001
	Multigravida	655(70%)	280(29%)		658(56%)	501(43%)	
Child medication 3mo	N=1477				N=1838		
	No	983(69%)	448(31%)	1.0	948(54%)	827(46%)	.305
	Yes	32(70%)	14(30%)		38(60%)	25(40%)	
Gestational age	N=1476				N=1833		
	39-42 weeks	789(69%)	348(31%)	.911	740(55%)	605(45%)	.141
	37-38 weeks	230(69%)	101(31%)		207(50%)	199(50%)	
	34-36 weeks	39(67%)	19(33%)		38(46%)	44(54%)	
Breastfeeding (3 months)	N=1470				N=1831		
	Exclusive	610(77%)	186(23%)	<.0001	606(55%)	507(45%)	.355
	Partial	265(64%)	149(35%)		232(51%)	225(49%)	
	Formula Fed	133(50%)	127(50%)		142(55%)	115(45%)	
Breastfeeding (6 months)					N=1811		
	Exclusive	-	-		175(53%)	153(47%)	.646
	Partial	-	-		569(53%)	506(47%)	
	Formula	-	-		227(56%)	182(44%)	
Introduction to foods	N=1477				N=1836		
	≤3 months	29(51%)	28(49%)	.005	41(68%)	19(32%)	.025
	>3 months	986(69%)	434(31%)		945(53%)	831(47%)	
Maternal race	N=1461				N=1817		
	White	738(67%)	363(33%)	<.0001	762(55%)	627(45%)	<.0001
	Asian	158(82%)	35(18%)		95(40%)	140(60%)	
	Other	109(66%)	58(34%)		118(61%)	75(39%)	
Geography	N=1477				N=1229		
	Vancouver	351(77%)	103(23%)	<.0001	208(52%)	191(47%)	.575
	Edmonton	232(58%)	168(42%)		159(56%)	125(44%)	
	Winnipeg	432(69%)	191(30%)		299(55%)	247(45%)	
Maternal prenatal smoking	N=1441				N=1792		
	No	954(69%)	419(31%)	.043	918(53%)	800(47%)	.154
	Yes	39(58%)	29(42%)		46(62%)	28(38%)	

Postnatal smoke exposure	N=1454				N=1758		
	No	870(71%)	360(28%)	<.0001	795(54%)	700(47%)	.640
	Yes	130(58%)	94(41%)		144(55%)	119(45%)	
Maternal prenatal depression	N=1453				N=1806		
	No	780(71%)	318(28%)	.002	743(53%)	640(46%)	1.00
	Yes	221(62%)	134(38%)		227(54%)	196(46%)	
Maternal Postnatal depression(6mo)					N=1601		
	No	-	-		740(54%)	637(46%)	.098
	Yes	-	-		107(47%)	117(52%)	
Maternal prenatal stress (36 weeks)	N=1370				N=1707		
	No	498(69%)	217(30%)	.448	505(54%)	430(46%)	.733
	Yes	443(67%)	212(32%)		410(53%)	362(47%)	
Maternal postnatal stress (6 mo)					N=1603		
	No	-	-		491(54%)	420(46%)	.364
	Yes	-	-		357(52%)	335(48%)	
Maternal pre-pregnancy BMI	N=1406				N=1804		
	Underweight	27(82%)	6(18%)	.001	24(43%)	33(57%)	.212
	Normal weight	588(73%)	230(27%)		568(55%)	471(45%)	
	Overweight	218(68%)	103(32%)		223(53%)	206(47%)	
	Obese	137(58%)	97(42%)		156(56%)	123(44%)	
Pets in the home 0-3 mo	N=1455				N=1753		
	No	571(72%)	218(27%)	.001	497(52%)	460(48%)	.179
	Yes	427(65%)	235(34%)		441(55%)	357(45%)	
Baby Gender	N=1477				N=1840		
	Male	524(67%)	261(33%)	.092	552(58%)	402(42%)	<.0001
	Female	491(71%)	201(29%)		434(49%)	448(51%)	
Hospital Length of Stay	N=1408				N=1836		
	24 hr or less day	285(75.5)	92(24%)	<.0001	266(57%)	194(42%)	.044
	2-3 day	587(67%)	285(33%)		576(53%)	504(47%)	
	4 or more days	92(58%)	57(42%)		144(48%)	152(51%)	
Siblings	N=1480				N=1833		
	No	493(66%)	253(34%)	.033	450(47%)	492(52%)	<.0001
	Yes	521(71%)	209(29%)		536(60%)	359(40%)	
Maternal age	N=1477				N=1836		
		Mean (SD)	Mean (SD)	<.0001	Mean (SD)	Mean (SD)	.164
		31.94(4.6)	30.69(4.83)		31.68(4.5)	31.97(4.5)	

ii) 3-month crude analyses

Overall, 31.26% of infants were colonized with *C. difficile* at 3 months, when stratified to include only infants that were vaginally delivered without antibiotics, exclusively breastfed and no medical interventions (oxytocin or anesthetics), this colonization rate was significantly

reduced to 19.5% (p=.006) (Figure 2). In crude analyses, mode of delivery was significantly associated with *C. difficile* colonization (p=.002, Table 3). *C. difficile* colonization rates between mode of delivery are shown in Figure 3. Similarly, the magnitude of this effect was shown using simple logistic regression with emergency CS and elective CS (OR:1.76 95% CI: 1.27-2.44 p=.001, OR:1.55 95% CI:1.06 p=.024) having higher odds of *C. difficile* colonization at 3 months of age compared to vaginal, no IAP as shown in Figure 4 and Table 5. Infants born vaginally with the use of IAP did not significantly predict the odds of *C. difficile* colonization compared to infants without the use of IAP (Figure 4).

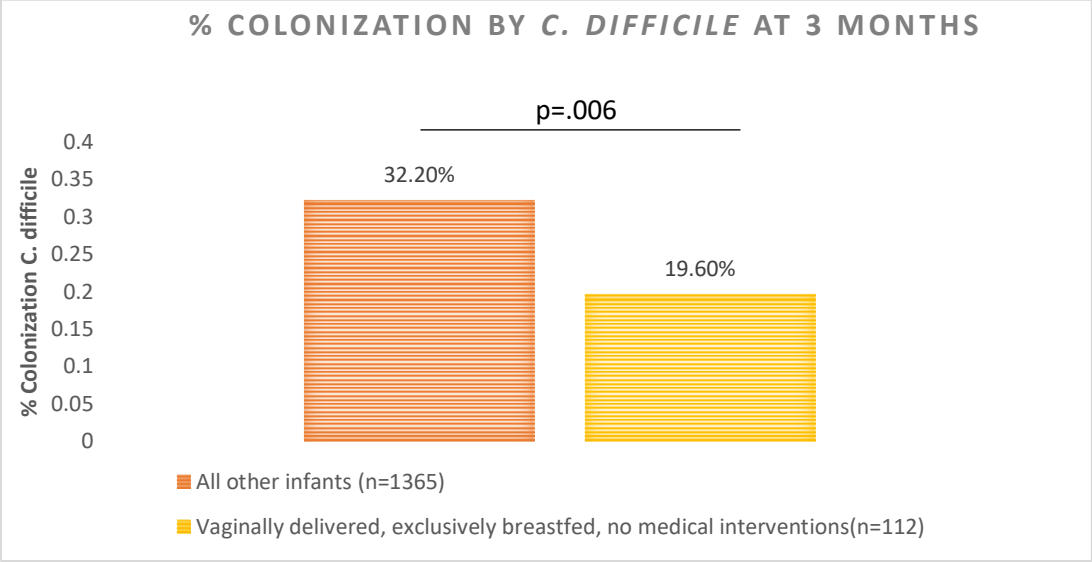


Figure 2. Colonization rates between *C. difficile* at 3 months in all other infants compared to *C. difficile* colonization between infants who were vaginally delivered, exclusively breastfed and received no medical interventions at birth (oxytocin and anesthetics)

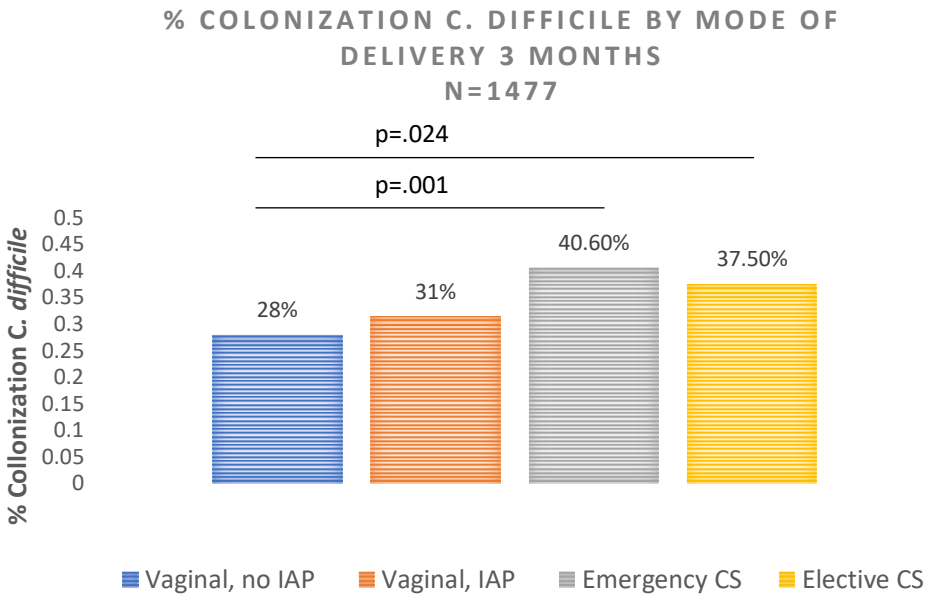


Figure 3. Colonization rates between mode of delivery and *C. difficile* at 3 months of age

iii) 3-month adjusted analyses

Logistic regression was performed to predict the odds of *C. difficile* colonization as a binary outcome (present/absent) in relation to the exposure of interest: infants born vaginally without IAP (reference), vaginally with IAP, emergency CS and elective CS. Simple and multiple logistic regression was performed to compile an adjusted model using a purposeful selection. Variables were initially selected at $p<.20$ using fishers exact test in crude analyses. Confounding factors were assessed after each model and were kept in the model if the regression coefficient changed by >10%. Interaction terms were analyzed in the final model and were included at $p\leq.05$. The adjusted model is shown in Table 5 for the 3 month analyses. In model 1, adjustment was made for medical interventions (no oxytocin or anesthetics, oxytocin only, anesthetics only and both oxytocin and anesthetics), in which the odds of colonization with *C. difficile* was significantly increased with infants born by emergency CS and elective CS

compared to infants born vaginally with no IAP (reference) (aOR:1.60 95% CI:1.14-2.24 p=.006; aOR:1.50 95% CI:1.0-2.24 p=.047).

In model 2, after adjustment for prenatal characteristics (gravida, birthweight, gender, maternal race, maternal smoking, maternal BMI, mothers age and maternal prenatal depression), infants born by emergency CS and elective CS had increased odds of *C. difficile* colonization which remained significant compared to infants born vaginally with no IAP (aOR:1.94 95% CI:1.36-2.78 p=<.0001; aOR:1.91 95% CI:1.26-2.92 p=.002), while infants born vaginally with IAP was not significant compared to the reference. Of note, to understand why the odds of *C. difficile* became significant in infants born by elective CS in this model, individual adjustment was performed where maternal age was a variable that was considerably masking this effect in crude analyses (Appendix B-Table B1).

In model 3, after adjustment for postnatal characteristics (breastfeeding status at 3 months, postnatal smoke exposure, introduction to foods, pets, hospital length of stay, siblings, geography and age at sample collection), infants born by emergency CS remained significant in the model (aOR:1.72 95% CI:1.16-2.54 p=.006) compared to infants born vaginally with no IAP. Infants born by elective CS had a higher odds of *C. difficile* colonization; however, it did not remain significant in the model compared to vaginal with no IAP (Table 5). In the final model, after adjustment for medical interventions, maternal race, mothers age, prenatal depression breastfeeding at 3 months, postnatal smoke exposure, pets, hospital length of stay, siblings, geography and age at sample collection, infants born by emergency CS remained the only mode of delivery in which the odds for *C. difficile* colonization remained significant compared with infants born vaginally with no IAP (OR: 1.91 95% CI:1.27-2.88, p=.002).

To understand where elective CS lost significance, individual adjustment was performed (Figure 4) and to a fuller extent in Appendix B Table B1. After adjustment for breastfeeding, the association between elective CS and *C. difficile* colonization was lost (Figure 4). In this final model, *C. difficile* colonization was significantly less common in infants whose mother was of the Asian race and whose mother was of older age. *C. difficile* colonization was significantly increased if the infant was partially breastfed or formula fed, was older at stool collection and was born in Edmonton, Alberta, Canada (Table 5).

A sensitivity analyses was performed with infants born vaginally with IAP as the reference in an adjusted analysis (Appendix D- Table D7). As all CS deliveries receive IAP, this analysis was to ensure the greater risk found between infants born by emergency CS and *C. difficile* colonization was independent of maternal IAP. Infants born by emergency CS was compared to infants born vaginally with IAP, and the increased risk remained borderline significant (aOR: 1.51 95% CI: .98-3.32; p=.060; Appendix D- Table D7).

Table 4. Percentage distribution of descriptive characteristics in relation to mode of delivery and medical interventions at birth (3 months)											
Column percentages		Vaginal, NO IAP	Vaginal, IAP	Emergency CS	Elective CS		No oxytocin/anesthetics	Oxytocin only	Anesthetics only	Both oxytocin and anesthetics	
		N (%)	N (%)	N (%)	N (%)	P value(x^2)	N (%)	N (%)	N (%)		P value(x^2)
Mode of delivery											
	Vaginal, no IAP	-	-	-	-		185(79%)	77(79%)	260(48%)	288(48%)	**.0001**
	Vaginal, IAP	-	-	-	-		49(30%)	21(21%)	101(19%)	165(28%)	
	Emergency CS	-	-	-	-		0	0	63(12%)	130(22%)	
	Elective CS	-	-	-	-		0	0	120(22%)	16(3%)	
Gestational age											
	Term (39-42 weeks)	639(79%)	239(71%)	142(72%)	67(49%)	**.0001**	190(81%)	77(79%)	378(70%)	439(74%)	**.040**
	Early Term (37-38)	150(20%)	66(20%)	43(21%)	63(46%)		37(16%)	19(19%)	141(26%)	133(23%)	
	Premature (<36 weeks)	10(1%)	30(9%)	12(6%)	6(4%)		7(3%)	2(2%)	24(4%)	25(4%)	
Maternal Pre-pregnancy BMI											
	Underweight	20(3%)	6(2%)	6(3%)	2(2%)	**.004**	2(.9%)	5(5%)	10(2%)	16(3%)	**.0001**
	Normal weight	485(63%)	175(55%)	96(51%)	62(48%)		149(67%)	54(55%)	312(60%)	302(54%)	
	Overweight	164(21%)	78(24%)	44(23%)	35(27%)		58(26%)	28(27%)	108(21%)	127(23%)	
	Obese	103(13%)	58(18%)	44(23%)	29(23%)		14(6%)	12(13%)	91(18%)	117(21%)	
Maternal Race											
	White	616(77%)	248(75%)	139(71%)	98(73%)	.473	183(80%)	78(80%)	411(76%)	427(72%)	.083
	Asian	96(12%)	44(13%)	31(16%)	22(16%)		27(12%)	9(9%)	75(14%)	82(14%)	
	Other	86(11%)	41(12%)	27(14%)	14(10%)		19(8%)	11(11%)	52(10%)	84(14%)	
Breastfeeding 3 mo											
	Exclusive	445(55%)	192(58%)	104(53%)	55(40%)	**.038**	149(64%)	55(57%)	307(57%)	283(48%)	**.001**
	Partial	217(27%)	88(26%)	56(28%)	53(39%)		51(22%)	27(28%)	146(27%)	188(32%)	
	Formula Fed	141(18%)	54(16%)	37(19%)	28(21%)		32(14%)	15(16%)	89(17%)	124(21%)	
Gender											
	Male	411(51%)	170(51%)	127(65%)	77(57%)	**.004**	109(47%)	47(48%)	309(57%)	318(53%)	**.045**
	Female	397(49%)	166(49%)	70(36%)	59(43%)		125(53%)	51(52%)	235(43%)	279(47%)	
Prenatal depression											
	Yes	610(78%)	248(75%)	141(73%)	99(74%)	.654	171(75%)	76(78%)	413(77%)	440(74%)	.612
	No	185(23%)	83(25%)	53(27%)	34(26%)		57(25%)	21(22%)	122(23%)	153(26%)	
Gravida											
	Primigravida	273(34%)	148(44%)	98(50%)	23(17%)	**.0001**	61(26%)	34(35%)	160(29%)	285(48%)	**.0001**
	Multigravida	535(66%)	190(56%)	99(50%)	113(83%)		173(74%)	64(65%)	384(71%)	312(52%)	
Siblings											
	No	363(45%)	198(59%)	151(77%)	34(25%)	**.0001**	73(31%)	39(40%)	222(41%)	408(68%)	**.0001**
	Yes	445(55%)	137(41%)	46(23%)	102(75%)		161(69%)	59(60%)	321(59%)	389(32%)	
Geography											
	Vancouver	214(27%)	114(34%)	74(38%)	52(38%)	**.001**	54(23%)	21(21%)	175(32%)	203(34%)	**.0001**
	Edmonton	220(27%)	86(26%)	50(27%)	44(33%)		32(14%)	13(13%)	167(31%)	188(32%)	
	Winnipeg	374(46%)	136(41%)	73(37%)	40(29%)		148(63%)	64(65%)	202(37%)	206(35%)	
Hospital length of stay											
	24hrs or less	290(38%)	84(22.5%)	0(0%)	3(2%)	**.0001**	116(54%)	39(43%)	117(22%)	105(18%)	**.0001**
	2-3 days	433(57%)	211(65%)	129(68%)	99(73%)		92(43%)	45(50%)	344(65%)	390(67%)	
	4 days or more	35(4.6%)	29(9%)	61(32%)	34(34%)		6(3%)	6(7%)	65(12%)	81(14%)	

Pets											
	No	445(56%)	170(51%)	101(51%)	73(55%)	.360	139(60%)	53(7%)	289(56%)	308(52%)	.200
	Yes	346(43%)	162(49%)	95(49%)	60(45%)		91(40%)	41(6%)	247(44%)	281(48%)	
Maternal Smoking											
	No	750(54%)	318(23%)	183(13%)	126(9%)	.284	220(97%)	90(94%)	504(95%)	557(95%)	.411
	Yes	43(63%)	9(13%)	10(14%)	6(9%)		6(3%)	6(6%)	27(5%)	29(5%)	

Table 5. Crude simple and multiple logistic regression with birth, prenatal and postnatal characteristics predicting *C. difficile* colonization at 3 months of age

	Crude (unadjusted)			**Model 1 (adjusted for birth characteristics)**			**Model 2 (adjusted for prenatal characteristics)**			**Model 3 (adjusted for postnatal characteristics)**			**Model 4 (Final model)**		
Outcome: Clostridioides difficile 3 months (Y/N)	Main exposure of interest: Birth method	Crude Univariate OR	P value	Main exposure of interest: Birth method	Adjusted OR	P value	Main exposure of interest: Birth method	Adjusted OR	P value	Main exposure of interest: Birth method	Adjusted OR	P value	Main exposure of interest: Birth method	Adjusted OR	P value
Birth method:	N=1477			N=1477			N=1356			N=1351			N=1351		
	Vaginal no IAP	(reference)		Vaginal no IAP	-		Vaginal no IAP	-		Vaginal no IAP	-		Vaginal no IAP	-	
	Vaginal, IAP	1.19(.89-1.55)	.239	Vaginal, IAP	1.13(.85-1.50))	.565	Vaginal, IAP	1.22(.90-1.65)	.184	Vaginal, IAP	1.22(.90-1.66)	.185	Vaginal, IAP	1.26(.92-1.73)	.148
	Emergency CS, IAP	1.76(1.27-2.44)	**.001**	Emergency CS, IAP	1.60(1.14-2.24)	**.006**	Emergency CS, IAP	1.94(1.36-2.78)	**<.0001**	Emergency CS, IAP	1.72(1.16-2.54)	**.006**	Emergency CS, IAP	1.91(1.27-2.88)	**.002**
	Elective CS, IAP	1.55(1.06-2.26)	**.024**	Elective CS, IAP	1.50(1.0-2.24)	**.047**	Elective CS, IAP	1.91(1.26-2.92)	**.002**	Elective CS, IAP	1.37(.87-2.14)	.166	Elective CS, IAP	1.51(.86-2.25)	.095
Block 1: Birth characteristics															
	Medical inteventions: None(ref) Oxytocin and anesthetics Oxytocin only Anesthetics only Both	.94(.54-1.62) 1.32(.93-1.87) 1.54(1.10-2.17)	.826 .112 **.012**	Medical interventions: None(ref) Oxytocin and anesthetics Oxytocin only Anesthetics only Both	.93(.52-1.54) 1.12(.79-1.64) 1.34(.96-1.944)	.824 .517 .095								Confounding	.65(.35-1.24) .93(.61-1.42) .97(.64-1.49)	.193 .749 .926
Block 2: Pre-natal Characteristics															
	Gravida(ref-primigravida)	.83(.66-1.04)	.124				Gravida(ref-primigravida)	.89(.69-1.15)	.391						
	Birthweight(grams) 1590-3160(Ref) 3160-3460 3460-3783 3783-5103	1.20(.87-1.64) 1.05(.763-1.45) 1.57(1.15-2.15)	.259 .752 **.004**				Birthweight (grams) 1590-3160(ref) 3160-3460 3460-3783 3783-5103	1.18(.83-1.66) 1.07(.75-1.52) 1.45(1.02-2.05)	.341 .712 .034						
	Gender(ref-male)	.82(.66-1.02)	.079				Gender(ref-male)	.86(.67-1.09)	.221						
	Maternal race (ref-white) Asian Other	.44(.32-.68) 1.10(.75-1.47)	**<.001** .566				Maternal race (ref-white) Asian Other	.53(.35-.81) .93(.63-1.36)	**.003** .738					.49(.35-.83) .88(.56-1.28)	**.001** .563
	Maternal smoking (Y/N)	1.69(1.03-2.7)	.036				Maternal smoking (Y/N)	1.14(.66-1.99)	.631					-	

	Maternal BMI (ref-normal weight) Underweight Overweight obese	.56(.22-1.37) 1.2(.90-1.57) 1.81(1.40-2.5)	.218 .181 **<.001**				Maternal BMI (ref-normal weight) Underweight Overweight obese	.539(.21-1.37) 1.13(.84-1.51) 1.40(1.02-1.94)	.193 .413 .037					-	
	Mothers age(continuous)	.94(.92-.96)	**<.001**				Mothers age(continuous)	.94(.92-.981)	**<.001**					.96(.936-.99)	**.033**
	Maternal Pre-natal depression (Y/N)	1.58(1.23-.201)	**.002**				Maternal prenatal depression (Y/N)	1.41(1.06-1.85)	**.015**					1.31(.97-1.73)	.065
Block 3: Post-natal Characteristics															
	Breastfeeding 3 mo (ref-exclusive) Partial Formula fed	1.85(1.42-2.4) 3.17(2.36-4.2)	**<.001** **<.001**							Breastfeeding 3 mo (ref-exclusive) Partial Formula fed	1.77(1.35-2.4) 2.82(2.25-4.30)	**<.001** **<.001**		1.87(1.4-2.49) 2.86(2.04-4.01)	**<.0001** **<.0001**
	Postnatal smoke exposure	1.77(1.32-2.33)	**<.001**							Postnatal smoke exposure	1.33(1.03-1.99)	**.079**	Confounding	1.32(1.00-2.05)	.109
	Intro to foods (ref-<= 3 mo)	.45(.26-.75)	**.004**							Intro to foods (ref-<= 3 mo)	.78(.43-1.40)	.504		-	
	pets	1.38(1.11-1.72)	**.003**							Pets(Y/N)	1.19(.95-1.52)	.161	Confounding	1.06(.84-1.40)	.655
	Hospital length of stay (continuous) 24hr or less(eef) 2-3 day 4 days or more	1.50(1.14-1.98) 2.26(1.52-3.35)	**.003** **<.0001**							Hospital length of stay 24hr or less 2-3 day 4 days or more	.95(.692-1.68) 1.03(.63-1.68)	.776 .302	Confounding	.95(.68-1.32) 1.12(.67-1.84)	.778 .660
	Siblings(Y/N)	.78(.614-.949)	**.027**							Siblings(Y/N)	.75(.57-.97)	.041		.77(.52-.95)	.087
	Age at stool collection(continuous)	1.38(1.27-1.55)	**<.001**							Age at stool collection(continuous)	1.45(1.28-1.64)	**<.001**		1.45 (1.22-1.62)	**<.0001**
	Geography Vancouver(ref) Edmonton Winnipeg	2.46(1.83-3.31) 1.5091.14-1.98)	**<.0001** **.004**							Geography Vancouver(ref) Edmonton Winnipeg	1.78(1.25-2.53) 1.52(1.11-2.08)	**.001** **.008**		1.62(1.12-2.34) 1.23(.87-1.74)	**.016** .290
	Child medication (Y/N)	.95(.50-1.81)	.900							Child medication	.99(.50-1.96)	.992		.867(.42-1.76)	.694

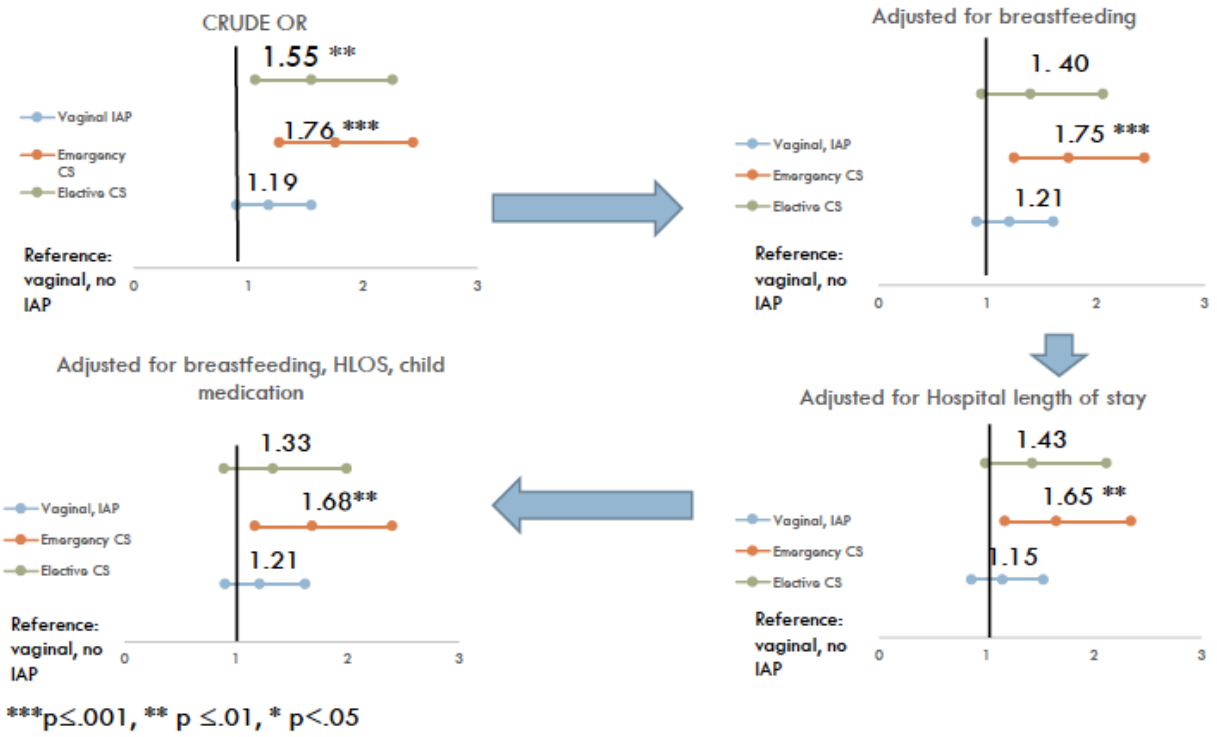


Figure 4: Individual adjustment for important covariates between mode of delivery and *C. difficile* colonization at 3 months of age. Results from simple and multiple logistic regression.

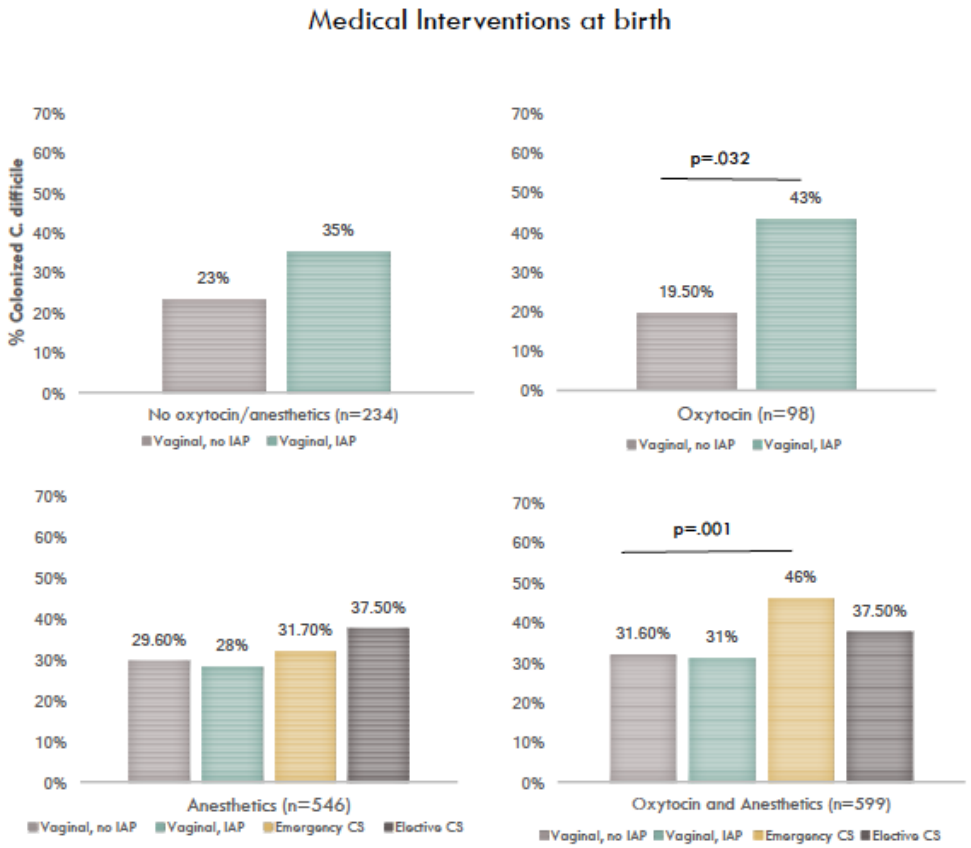
iv) Unadjusted stratified results at 3 months of age

Several unadjusted stratifications were performed at 3 months by important covariates to further understand the effect of mode of delivery on *C. difficile* colonization (Appendix C table. C1). Stratification by medical interventions, provided evidence for oxytocin alone and both oxytocin and anesthetics to differentially affect *C. difficile* colonization between birth modes. Among women who received oxytocin during labour, *C. difficile* colonization rates for vaginal, IAP compared to vaginal, no IAP significantly differed, 19.5% vs 43% (p=.032), respectively (Figure 5.A). Similarly, infants born by emergency CS had higher *C. difficile* colonization rates (46%) compared to infants born vaginally with no IAP (31.6%), among mothers who received both oxytocin and anesthetics combined (p=.001) (Figure 5. A). To examine whether *C. difficile* colonization between medical interventions were significantly different within each birth mode

of delivery, stratification by mode of delivery was performed to compared this relationship (Figure 5. B). The *C. difficile* colonization of 46% in infants born by emergency CS with the use of both oxytocin and anesthetics combined was higher than infants born by emergency CS with the use of anesthetics only (32%; p=.058; Figure 5. B). Similarly, among infants born vaginally with IAP, the rate of *C. difficile* colonization was significantly higher when the mother received oxytocin and anesthetics combined compared to no oxytocin or anesthetics (31% vs 23%, p=.050; Figure 5. B). However, interaction between these covariates were not significant in global unadjusted or adjusted models (Appendix E- Table E1).

Breastfeeding, being the most important determinant of the infant gut microbiota in early life, was stratified into infants who were exclusively breastfed, partially breastfed and formula fed infants whereby *C. difficile* colonization between mode of delivery was compared within each stratification. Most notably, among exclusively breastfed infants at 3 months of age, no significance was found between mode of delivery and *C. difficile* colonization (Appendix F-Figure F1). Among partially breastfed infants, infants born by emergency CS had significantly higher colonization rates compared to vaginal, no IAP birth (p=.001). Likewise, infants born by emergency CS had the highest colonization rates among formula fed infants, compared to infants born vaginally with no IAP (p=.036) (Appendix F-Figure F1). An individual interaction term between emergency CS and partial breastfeeding was significant (p=.050) in adjusted and unadjusted stratified analyses; however, did not remain in a global unadjusted or adjusted test (p=.082 and p=.263, respectively; Appendix E-Table E1). No other interaction terms were significant.

A)


Medical Interventions at birth
% Colonized C. difficile
70%
60%
50%
40%
30%
20%
10%
0%
23%
35%
No oxytocin/anesthetics (n=234)
Vaginal, no IAP
Vaginal, IAP
p=.032
19.50%
43%
Oxytocin (n=98)
29.60%
28%
31.70%
37.50%
Anesthetics (n=546)
Emergency CS
Elective CS
p=.001
31.60%
31%
46%
37.50%
Oxytocin and Anesthetics (n=599)

B)

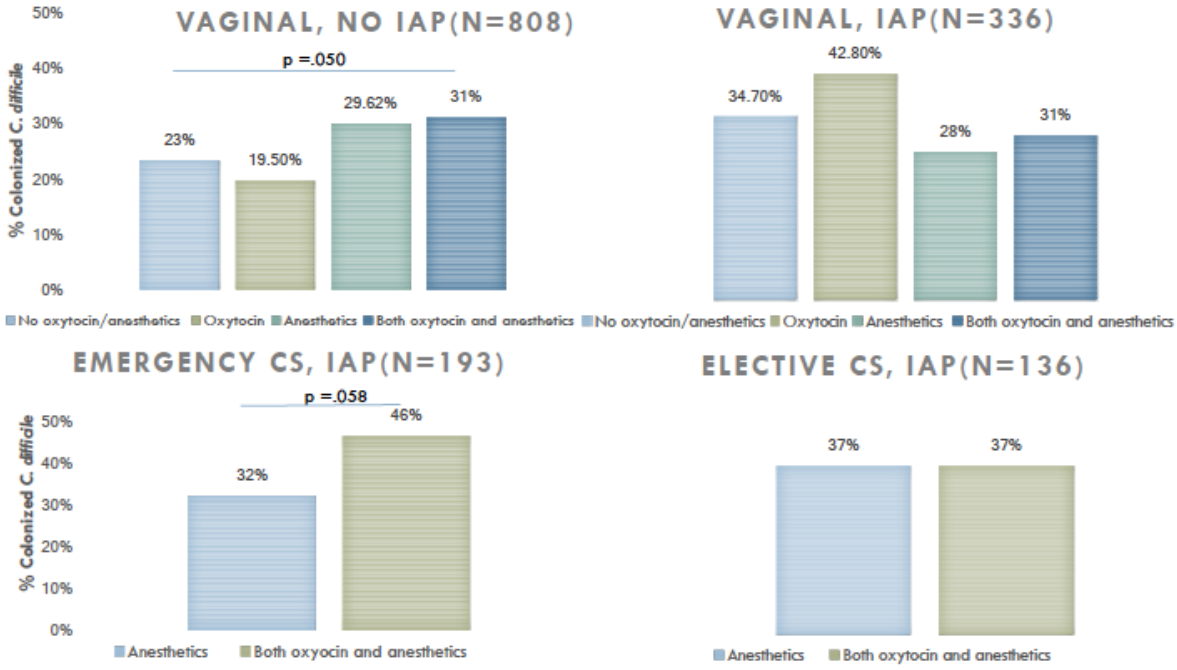

VAGINAL, NO IAP(N=808)
% Colonized C. difficile
50%
40%
30%
20%
10%
0%
p =.050
23%
19.50%
29.62%
31%
No oxytocin/anesthetics
Oxytocin
Anesthetics
Both oxytocin and anesthetics
VAGINAL, IAP(N=336)
34.70%
42.80%
28%
31%
EMERGENCY CS, IAP(N=193)
p =.058
32%
46%
Anesthetics
Both oxyocin and anesthetics
ELECTIVE CS, IAP(N=136)
37%
37%

Figure 5. A) *C. difficile* colonization rates between mode of delivery stratifies by medical interventions during birth at 3 months of age B) *C. difficile* colonization rates within medical interventions stratified by mode of delivery at 3 months of age

Although gravida status was not significant in our final model, it is an important factor when considering mode of delivery as the first born often undergoes more medical interventions than their sibling counterparts. Therefore, stratification was performed for gravida status, differences between mode of delivery on the colonization of *C. difficile* were only evident among primigravida infants, in which gravida is defined by the number of pregnancies the women has had. Among primigravida mothers, significantly higher colonization rates in infants delivered by emergency CS (47%) and elective CS (56%) compared to vaginal, no IAP was found (28%) (p=.006 and p=.001, respectively; Appendix Figure F2). Unadjusted interaction terms were not significant; however, after adjustment, this interaction term became borderline significant in a global test (p=.056; Appendix E- Table E1). However, making strong conclusions regarding infants born by elective CS in primigravida status may be limited due to small sample size (n=23).

After stratifying for several other covariates, significant differences were seen in colonization rates of *C. difficile* and birth methods. Among infants with no siblings, higher colonization rates were seen in emergency CS (44%) and elective CS (47%) compared to vaginal no, IAP (29%) (p=.036 and p=.001; Appendix F-Figure F3). No significant interactions existed using a global test between these groups in unadjusted or adjusted analysis. Significant differences were found among homes where pets were present, higher *C. difficile* colonization rates were seen in elective CS (43%) and emergency CS (52%) compared to vaginal, no IAP

(30%) (p=.049 and p=.0001, respectively; Appendix F-Figure F4). This interaction term was not significant in unadjusted or adjusted analyses using a global test (Appendix E- Table E1).

v) Adjusted stratified results at 3 months of age

Stratifications were performed in the adjusted analyses if interaction terms were significant or borderline significant using a global test (Appendix E-Table E1). Although interaction was not significant between mode of delivery and breastfeeding and mode of delivery and medical interventions on the colonization of *C. difficile*, we performed stratifications due to exposure status of medical interventions and clinical relevance with breastfeeding. After stratification by medical interventions, it appeared that these interventions were not playing a strong role; although the effects of emergency CS was amplified when the mother received oxytocin and anesthetics combined during labour after adjustment (Figure 6). No other relationships were found and this interaction was not significant (p=.387). After stratification for breastfeeding status at 3 months, exclusive breastfeeding did slightly attenuate the effects of emergency CS on *C. difficile* colonization while partial breastfeeding amplified the effects of emergency CS on *C. difficile* colonization after adjustment (Appendix F-Figure F5). This relationship was the opposite for elective CS whereby the effects were amplified in exclusive breastfeeding and attenuated with partial breastfeeding and formula feeding after adjustment (Appendix F-Figure F5). As previously stated, gravida itself was not a significant predictor of *C. difficile* and did not make it in the final model; however, stratification was performed due to differences in first born infants showing stronger mode of delivery effects in previous studies. After adjustment, effects of mode of delivery were stronger among primigravida mothers, with infants born by emergency CS and elective CS to be significantly associated with an increased

odds of *C. difficile* colonization at 3 months (p=.003 and p=.024; Appendix F-Figure F6). This adjusted interaction term almost reached statistical significance using a global test (p=.056; Appendix E- Table E1). Of note, hospital length of stay was masking this borderline interaction effect in unadjusted analyses, in which it was more common for first born infants to be hospitalized longer.

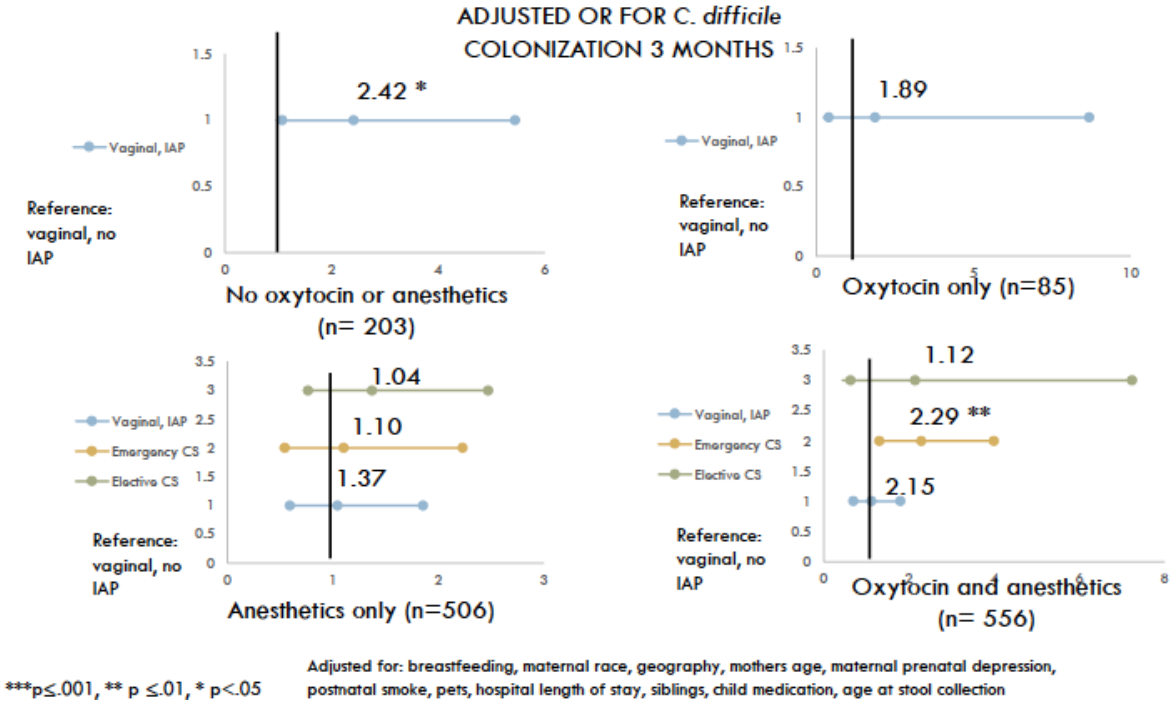


Figure 6. Results from adjusted multiple logistic regression when stratifying within stratums of medical interventions while comparing mode of delivery effects on *C. difficile* colonization. Global interaction term was not significant p=.341.

3.3 Study population (1 year of age)

i) 1 year characteristics of mother-infant pairs

In this subsample of 1836 infants, 850 (46%) of infants were colonized with *C. difficile* at 1 year of age. Table 3 describes the mother-infant characteristics associated with *C. difficile* colonization at 1 year. Similar characteristics were seen at 3 months and 1 year between overall

birth methods, as the same infants were followed; however, due to sampling timing and stool consistencies, 1 year samples were more easily obtained, rendering the sample size larger at 1 year. Also of note, some 3 month samples were missing where 1 year samples were obtained. Stool samples were collected at a mean of 12.43 days (range 9.32-24.21). Overall vaginal births with no IAP were most common (53%) followed by vaginal IAP births (22.6%), emergency CS (14%) and elective CS (10%). According to *C. difficile* colonization, colonization rates differed significantly between mode of delivery (p=.024; Table 3). Infants born by emergency CS had the highest rates of *C. difficile* colonization (51%), which was the same for infants born vaginally with IAP (51%) followed by elective CS (47%), and vaginal, no IAP having the lowest colonization rates (43%) (Figure. 8).

Several other covariates were significantly associated with the colonization of *C. difficile* with respect to medical interventions at birth (p=.006), gravida (p<.001), introduction to foods (p=.025), maternal race (p<.0001), gender (p<.0001), and siblings (p<.0001) (Table 3). Table 6 shows frequency distribution between covariates and mode of delivery and medical interventions at birth in the 1 year sample. Similar characteristics were seen between medical interventions, mode of delivery and covariates at 3 months compared to 1 year. Overall, breastfeeding rates at 6 months were 18% for exclusive breastfeeding, 59% for partial feeding and 23% for formula feeding. Sensitivity analyses were also performed to ensure premature, homebirths and age at stool sample collection did not affect the results. Results showed these potential confounding factor did not affect the results; however, for the same reason noted at 3 months, homebirths were excluded from the analysis due to missing information and several maternal characteristics associated with the decision to opt for a homebirth which do not represent the general population. Please see Appendix D- Table D4-D6 for sensitivity analyses.

i) 1 year crude analyses

Overall, 850 (46%) of infants were colonized at 1 year of age, in which stratification by vaginally delivery, exclusively breastfed at 3 months or 6 months, with no medical interventions did not affect colonization rates at 1 year of age (Figure 7). In crude analyses, mode of delivery was significantly associated with *C. difficile* colonization (p=.024; Table 2) with higher colonization rates found in infants born by emergency CS (51%), vaginal delivery with IAP (51%), elective CS (47%) and lastly, vaginal delivery without IAP (43%). In simple logistic regression, infants born by emergency CS had 36% higher odds of *C. difficile* colonization compared to infants born vaginally with no IAP (OR: 1.36; 95% CI:1.03-1.78; p=.026). Infants born vaginally with IAP also had a higher odds of *C. difficile* colonization compared to vaginal without IAP (OR: 1.35; 95% CI:1.07-1.70; p=.010; Table 7).

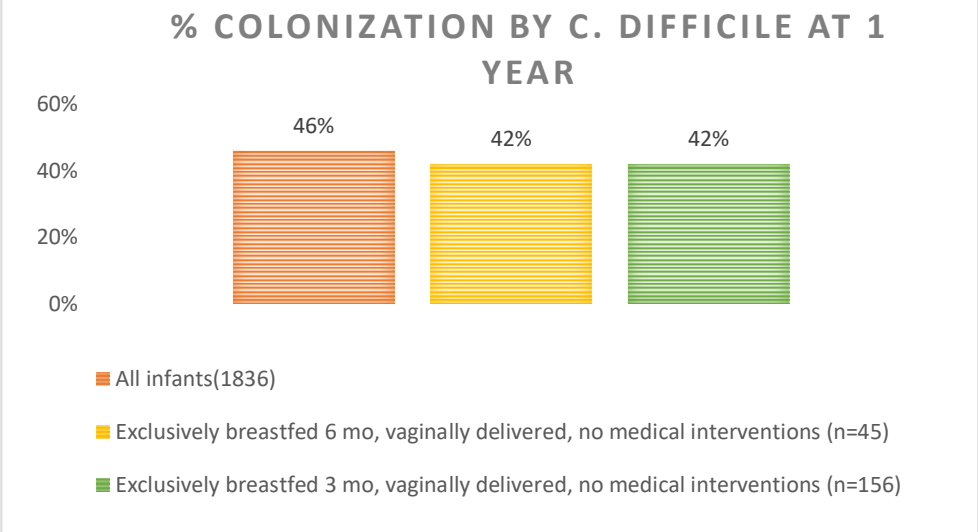


Figure 7. Colonization rates between *C. difficile* at 1 year in all infants compared to C. difficile colonization between infants who were vaginally delivered, exclusively breastfed at 3 months or 6 months and received no medical interventions

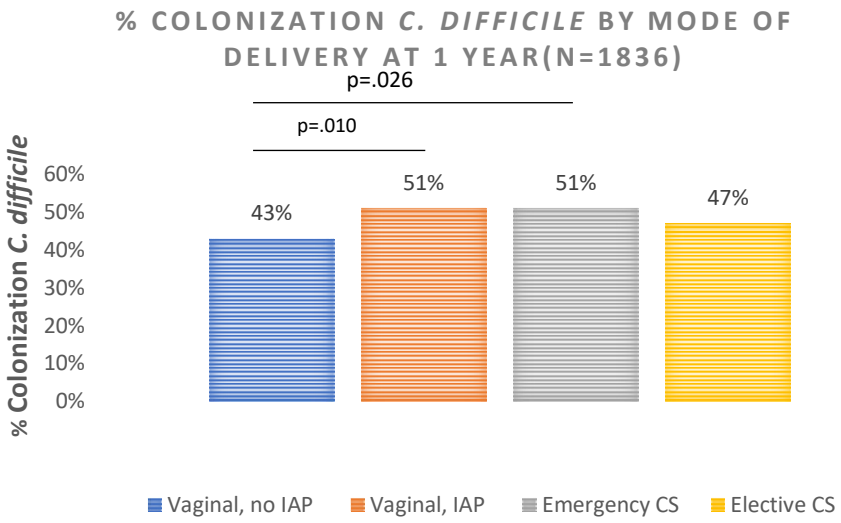


Figure 8. Colonization rates between mode of delivery and *C. difficile* at 1 year of age

ii) 1 year adjusted analyses

Logistic regression was performed to predict the odds of *C. difficile* colonization as binary outcome (present/absent) in relation to the exposure of interest: infants born vaginally without IAP (reference), vaginally with IAP, emergency CS and elective CS. Simple and multiple logistic regression was performed to compile an adjusted model using a purposeful selection. Variables were initially selected at p<.20 using fishers exact test in crude analyses. Adjusted models were performed with birth characteristics, prenatal characteristics and postnatal characteristics (Table 7). Variables that were significant in these models at p<.05 were carried to the final model. Confounding effects were analyzed after each model, whereby a change in the regression coefficient by >10% deemed the variable a confounding factor and was kept in the model, all confounding variables are noted in Table 7. Interaction terms were analyzed in the final model and were kept in the final model if they were significant at p<.05 using a global test.

After adjustment for medical interventions (no oxytocin or anesthetics, oxytocin only, anesthetics only and both oxytocin and anesthetics) in model 1, the only mode of delivery that

remained significantly associated with the odds of *C. difficile* colonization were infants born vaginally with IAP (OR:1.28; 95% CI: 1.01-1.62 p=.039; Table 7). In model 2, adjustment was made for prenatal characteristics (gravida, birthweight, gender, gestational age, maternal race and mothers age), where infants born vaginally with IAP remained a significant predictor in the odds of *C. difficile* colonization (OR:1.27; 95% CI: .97-1.68 p=.052; Table 7). Infants born by emergency CS or elective CS were not significant in this model. In model 3, after adjustment for postnatal characteristics (breastfeeding at 6 months, introduction to foods, pets, hospital length of stay, siblings, postnatal depression and age at sampling collection) infants born by elective CS became a significant predictor of the odds of *C. difficile* colonization (OR:1.57; 95% CI: 1.07-2.31 p=.029; Table 7).

To understand which variable was masking the effect of elective CS in previous models, individual adjustment was performed (Appendix B- table B1) and it was noted that after adjusted for age at stool collection at 1 year, infants born by elective CS became significantly associated with *C. difficile* colonization in model 3. Finally, in model 4, the final model, adjustment was made for all variables in the previous three models if the variable was significant at $p<.05$ as well as for confounding variables. Thus, the final model was adjusted for medical interventions, gravida, gender, maternal race, mothers age, introduction to foods, pets, hospital length of stay, siblings, postnatal depression at 6 months and age at sample collection. Mode of delivery was not a significant predictor of the odds of *C. difficile* colonization in the final adjusted model. Interaction terms were not significant between any variables and the exposures in the final model. Anesthetics use during labour in the final model was associated with an increased risk of *C. difficile* colonization by 39% independent of tested covariates compared to no use of oxytocin or anesthetics during, although it did not reach statistical significance (p=.066; Table 7).

Significance for other medical interventions were not found. Furthermore, the significance of both anesthetics and oxytocin during labour in the crude analysis and model 1 was taken away by sibling and gravida status as the use of both these drugs were more common during the first delivery.

To understand where each mode of delivery lost significance in relation to the odds of *C. difficile* colonization, individual adjustment was performed (Figure 10) and to a fuller extent Appendix B-Table B1. The significance for infants born by emergency CS was individually taken away by medical interventions at birth, siblings, geography and hospital length of stay. No individual factor alone took away the significance for infants born vaginally with IAP. After adjustment for hospital length of stay, breastfeeding at 6 months, siblings and postnatal depression the significance was lost for infants born vaginally with IAP (Appendix B-Table B1). In this final model, *C. difficile* colonization at 1 year of age was significantly more common in females, infants born from mothers of the Asian race and infants who were introduced to foods above 3 months. Additionally, *C. difficile* colonization was significantly less common in infants whose homes had a furry pet and a sibling (Table 7).

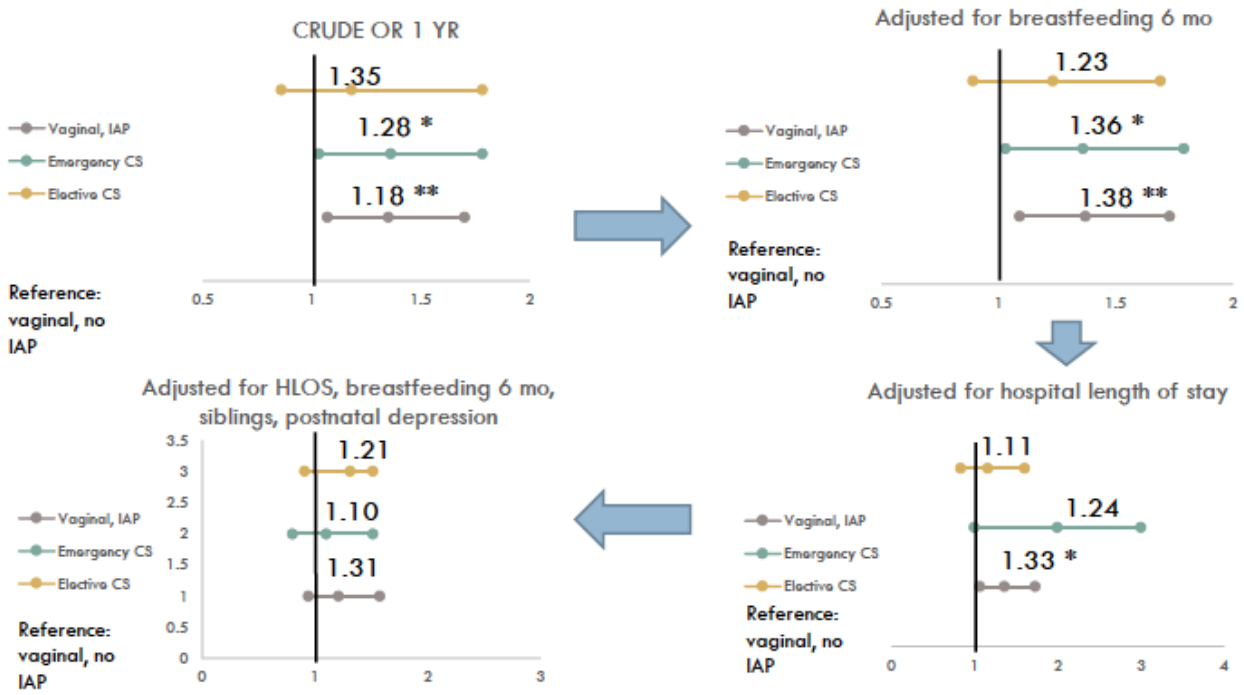
Table 6. Percentage distribution of descriptive characteristics in relation to mode of delivery and medical interventions at birth (1 year)											
Column percentages		**Vaginal, NO IAP Overall: 53%**	**Vaginal, IAP Overally:23 %**	**Emergency CS, ISP Overall:14%**	**Elective CS, IAP Overall:10%**		**No oxytocin/anesthetics Overall: 15%**	**Oxytocin only Overall: 6.5%**	**Anesthetics only Overall: 37%**	**Both oxytocin and anesthetics Overall: 40%**	
		N (%)	N (%)	N (%)	N (%)	P value(x^2)	N (%)	N (%)	N (%)		P value(x^2)
Mode of delivery											
	Vaginal, no IAP	-	-	-	-		228(82%)	92(77%)	325(47%)	320(43%)	**.0001**
	Vaginal, IAP	-	-	-	-		51(18%)	27(23%)	117(17%)	218(30%)	
	Emergency CS	-	-	-	-		0	0	78(12%)	180(25%)	
	Elective CS	-	-	-	-		0	0	167(24%)	18(2%)	
Gestational age											
	Term (39-42 weeks)	779(79%)	295(71%)	187(71%)	94(51%)	**.0001**	225(81%)	92(78%)	461(67%)	550(41%)	**.001**
	Early Term (37-38)	188(19%)	83(20%)	54(20%)	81(44%)		47(17%)	22(19%)	185(27%)	152(37%)	
	Premature (≤36 weeks)	14(1%)	35(8%)	23(9%)	10(5%)		6(2%)	4(3%)	38(6%)	33(4%)	
Maternal Pre-pregnancy BMI											
	Underweight	29(3%)	14(3%)	8(3%)	6(3%)	**.001**	6(2%)	7(6%)	22(3%)	21(3%)	**.0001**
	Normal weight	589(62%)	228(56%)	128(49%)	94(52%)		181(66%)	59(49%)	399(59%)	393(54%)	
	Overweight	222(23%)	96(24%)	64(25%)	47(26%)		70(25%)	37(31%)	154(23%)	162(22%)	
	Obese	115(12%)	69(17%)	60(21%)	35(19%)		19(7%)	17(14%)	98(15%)	145(20%)	
Maternal Race											
	White	757(79%)	309(75%)	182(69%)	141(76%)	**.023**	216(79%)	90(76%)	527(78%)	539(74%)	.091
	Asian	110(12%)	51(12%)	46(17%)	28(15%)		33(12%)	10(8%)	90(13%)	102(14%)	
	Other	90(9%)	52(13%)	35(13%)	16(9%)		23(8%)	19(16%)	63(9%)	89(12%)	
Breastfeeding 6 mo											
	Exclusive	159(17%)	92(22%)	48(18%)	29(16%)	.092	67(24%)	27(23%)	121(18%)	109(15%)	**.002**
	Partial	593(61%)	231(56%)	150(57%)	102(56%)		163(59%)	70(59%)	407(60%)	426(59%)	
	Formula Fed	209(22%)	87(21%)	63(24%)	50(27%)		47(17%)	21(18%)	145(22%)	192(26%)	
Gender											
	Male	499(51%)	208(50%)	152(57%)	95(51%)	.253	141(51%)	63(51%)	362(52%)	380(52%)	.937
	Female	472(49%)	207(50%)	112(42%)	91(49%)		138(49%)	58(49%)	324(47%)	356(48%)	
Prenatal depression											
	Yes	746(78%)	312(76%)	184(71%)	141(77%)	.161	212(78%)	87(73%)	527(78%)	546(75%)	.465
	No	210(22%)	97(23%)	74(29%)	42(23%)		59(22%)	31(26%)	147(22%)	179(25%)	
Gravida											
	Primigravida	328 (34%)	188(45%)	128(48%)	35(19%)	**.0001**	69(25%)	38(32%)	212(31%)	357(49%)	**.0001**
	Multigravida	643(66%)	226(55%)	136(52%)	151(81%)		211(75%)	83(68%)	476(69%)	378(51%)	
Siblings											
	No	432(45%)	254(62%)	202(77%)	50(27%)	**.0001**	83(30%)	44(37%)	293(43%)	511 (70%)	**.0001**
	Yes	538(55%)	159(38%)	62(23%)	136(73%)		195(70%)	75(63%)	391(57%)	223(30%)	
Geography											
	Vancouver	185(28%)	104(38%)	66(39%)	44(39%)	**.004**	50(26%)	19(23%)	147(33%)	182(37%)	**.0001**
	Edmonton	160(24%)	55(20%)	39(23%)	30(27%)		24(12%)	7(8%)	114(26%)	138(28%)	
	Winnipeg	324(48%)	118(43%)	66(39%)	38(34%)		122(62%)	57(69%)	182(41%)	177(36%)	
Hospital length of stay											
	24hrs or less	357(37%)	98(23%)	1(.4%)	4(2%)	**.0001**	144(52%)	46(39%)	145(21%)	121(17%)	**.0001**
	2-3 days	514(53%)	269(65%)	160(61%)	137(74%)		106(38%)	59(48%)	432(63%)	478(65%)	
	4 days or more	100(10%)	48(12%)	103(39%)	45(24%)		20(28%)	16(13%)	111(16%)	137(19%)	
Pets											

	No	514(55%)	208(53%)	132(52%)	101(57%)	.717	158(57%)	71(63%)	352(54%)	366(52%)	.119
	Yes	417(45%)	183(47%)	121(48%)	77(43%)		116(43%)	44(37%)	297(45%)	336(48%)	
Maternal Smoking											
	No	907(52%)	393(23%)	246(14%)	176(10%)	.582	265(99%)	108(91%)	636(95%)	696(96%)	**.007**
	Yes	44(59%)	14(19%)	11(15%)	5(7%)		4(1%)	11(9%)	33(5%)	26(4%)	

Table 7. Simple and multiple logistic regression models with birth, prenatal and postnatal characteristics predicting the odds of *C. difficile* colonization at 1 year of age.

	Crude (unadjusted)			**Model 1 (adjusted for birth characteristics)**			**Model 2 (adjusted for prenatal characteristics)**			**Model 3 (adjusted for postnatal characteristics)**			**Model 4 (Final model)**		
Outcome: Clostridioides difficile 1 year (Y/N)	Main exposure of interest: Birth method	Crude OR	P value (x^2)	Main exposure of interest: Birth method	Adjusted OR	P value (x^2)	Main exposure of interest: Birth method	Adjusted OR	P value (x^2)	Main exposure of interest: Birth method	Adjusted OR	P value (x^2)	Main exposure of interest: Birth method	Adjusted OR	P value (x^2)
Birth method:	N=1836			N=1818			N=1770			N=1487			N=1464		
	Vaginal no IAP	(reference)		Vaginal no IAP	-		Vaginal no IAP	-		Vaginal no IAP	-		Vaginal no IAP	-	
	Vaginal, IAP	1.35(1.07-1.70)	**.010**	Vaginal, IAP	1.28(1.01-1.62)	**.039**	Vaginal, IAP	1.27(.99-1.61)	**.052**	Vaginal, IAP	1.27(.97-1.66)	.073	Vaginal, IAP	1.26(.92-1.62)	.096
	Emergency CS, IAP	1.36(1.03-1.78)	**.026**	Emergency CS, IAP	1.24(.92-1.65)	.142	Emergency CS, IAP	1.26(.94-1.68)	.114	Emergency CS, IAP	1.08(.78-1.49)	.272	Emergency CS, IAP	1.05(.73-1.46)	.764
	Elective CS, IAP	1.18(.86-1.78)	.283	Elective CS, IAP	1.10(.79-1.55)	.552	Elective CS, IAP	1.12(.80-1.57)	.490	Elective CS, IAP	1.57(1.07-2.31)	**.019**	Elective CS, IAP	1.28(.83-1.89)	.239
Block 1: Birth characteristics															
	Medical interventions: No oxytocin/anesthetics Oxytocin only Anesthetics only Both oxytocin/anesthetics	 .96(.624-1.49) 1.32(.99-1.75) 1.53(1.16-2.03)	 .880 **.052** **.003**	Medical interventions: No oxytocin/anesthetics (ref) Oxytocin only Anesthetics only No oxytocin/anesthetics	.93(.60-1.45) 1.24(.92-1.6) 1.39(1.04-1.87)	.771 **.142** **.026**								Confounding	.89(.55-1.53) 1.38(.98-1.96) 1.27(.90-1.80)	.760 .066 .164
Block 2: Pre-natal Characteristics															
	Gravida (ref-primigravida)	.71(.58-.845)	**.001**				Gravida (ref-primigravida)	.71(.57-.87)	**.001**					.95(.67-1.29)	.776
	Birthweight (grams) 1590-3150(ref) 3150-3455 3455-3778 3778-5103	.99(.99-.99)	**.003**				Birthweight (grams) 1590-3150(ref) 3150-3455 3455-3778 3778-5103	 1.05(.79-1.39) .82(.60-1.08) .94(.69-1.27)	 .724 .186 .685						
	Gender(ref-male)	1.41(1.13-1.63)	**<.001**				Gender (ref-male)	1.44(1.14-1.67)	**<.001**					1.53(1.24-1.90)	**<.0001**
	Gestational age	.912(.84-.98)	**.013**				Gestational age	.95(.85-1.006)	.253						
	Maternal race (ref-white) Asian	1.79(1.40-2.44)	<.001 .105				Maternal race (ref-white) Asian	1.66(1.23-2.2)	**.001** .080					1.62(1.16-2.26)	**.004** .757

	Other	.77(.587-106)					Other	.75(.54-1.01)						.94(.68-1.35)	
	Maternal smoking (Y/N)	.69(.43-1.12)	.142				Maternal smoking (Y/N)	.85(.51-1.39)	.543					-	
	Mothers age(continuous)	1.01(.99-1.03)	.194				Mothers age(continuous)	1.01(.94-1.03)	.134				Confounding	1.07(.99-1.04)	.156
Block 3: Post-natal Characteristics															
	Breastfeeding 6 mo (ref-exclusive) Partial Formula fed	 1.01(.79-1.29) .917(.68-1.22)	 .915 .898							Breastfeeding 6 mo (ref-exclusive) Partial Formula fed	.95(.72-1.26) .85(.60-1.18)	.773 .335			
	Intro to foods (ref-<= 3 mo)	1.89(1.06-3.21)	**.023**							Intro to foods (ref-<= 3 mo)	2.29(1.33-4.75)	**.004**		2.29(1.20-4.36)	**.011**
	Pets	.87(.73-1.05)	.164							Pets (Y/N)	.76(.61-.95)	**.012**		.78(.63-.99)	**.034**
	Hospital length of stay 24hrs or less(ref) 2-3 days 4 days or more	 1.19(.95-1.48) 1.43(1.07-1.93)	.118 **.015**							Hospital length of stay 24hrs or less(ref) 2-3 days 4 days or more	1.10(.847-1.43) 1.41(.98-2.02)	.472 .062	Confounding	 1.05(.95-1.16) 1.31(.90-1.03)	.723
	Siblings(Y/N)	.61(.49-.711)	**<.001**							Siblings(Y/N)	.63(.51-.79)	**<.001**		.67(.49-.96)	**.023**
	Postnatal depression (6 mo)	1.28(.96-1.70)	.084							Post-natal depression (6mo)	1.30(.99-1.84)	.084	Confounding	1.27(.93-1.77)	.133
	Age at sampling 1 yr	.97(.91-1.03)	.353							Age at sampling 1 yr	.955(.89-1.02)	.360	Confounding	.97(.90-1.03)	.418



***p≤.001. ** p ≤.01. * p<.05

Figure 9. Individual and multiple adjustment for important covariates between mode of delivery and *C. difficile* colonization at 1 year of age. Results from simple and multiple logistic regression.

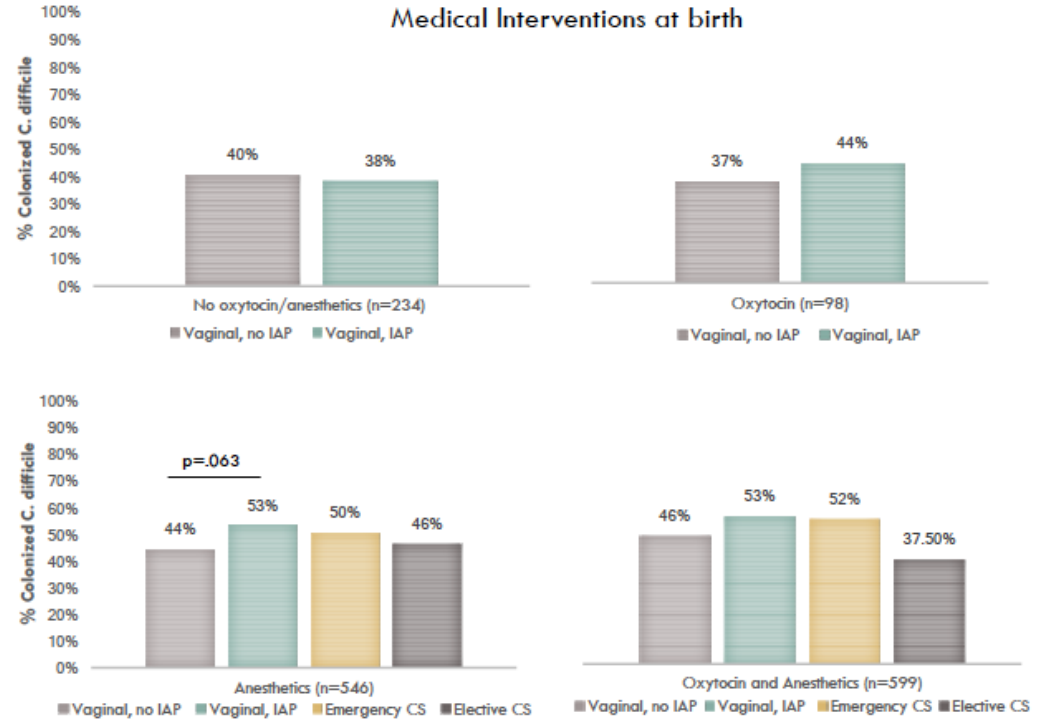
iii) Unadjusted stratified results at 1 year of age

Several unadjusted stratifications were performed at 1 year by important covariates to further understand the effect of mode of delivery on *C. difficile* colonization (Appendix C- Table C1 and Appendix F). After stratification by medical interventions (Figure 10. A), higher *C. difficile* colonization rates were found between infants born vaginally with IAP compared to infants born vaginally without the use of IAP, among women who received anesthetic (53% vs 44%); however, this did not reach statistical significance (p=.063). No other relationships were found after stratifying by medical interventions. Additionally, no significant global interaction terms were found between this relationship in unadjusted or adjusted analysis (Appendix E-Table E1). To understand the differences between medical interventions within each mode of delivery

on *C. difficile* colonization, stratification was performed by mode of delivery (Figure 10.B). Among infants born vaginally with IAP, higher *C. difficile* colonization rates were found when mothers received both oxytocin and anesthetics combined in labour compared to no oxytocin or anesthetic use (53% vs 38%, p=.053). No other differences were found between medical interventions when stratified by birth method. Again, no significant global interaction terms were found between this relationship in unadjusted or adjusted analysis using a global test (Appendix E-Table E1).

After stratification for breastfeeding status at 6 months of age (Appendix F-Figure F7), no differences between mode of delivery existed in exclusively breastfed infants or formula fed infants. Among infants who were partially breastfed at 6 months, higher *C. difficile* colonization rates were found in infants born vaginally with IAP compared to infants born vaginally without IAP (p=.014). After stratifications by other covariates, higher *C. difficile* colonization rates were found in infants born vaginally with IAP compared to infants born vaginally with no IAP, among infants who were multigravida (p=.024; Appendix F-Figure F8) and among infants where no pets resided in the home (p=.039; Appendix F-Figure F10). Similarly, infants born by emergency CS had higher *C. difficile* colonization rates among infants who were male (p=.052; Appendix F-Figure F9) and among infants where no pet resided in the home (p=.026; Appendix F-Figure F10) compared to the reference group. Finally, infants born by elective CS had higher colonization rates among infants who were male (p=.016; Appendix F-Figure F9). No differences were seen between any mode of delivery among infants who were female. No significant interactions were found for any mentioned covariates in unadjusted or adjusted stratification using a global test. Subsequent unadjusted stratifications were performed in Appendix E-Table E1.

A)


Medical Interventions at birth
% Colonized C. difficile
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
40%
38%
No oxytocin/anesthetics (n=234)
Vaginal, no IAP
Vaginal, IAP
37%
44%
Oxytocin (n=98)
Vaginal, no IAP
Vaginal, IAP
p=.063
44%
53%
50%
46%
Anesthetics (n=546)
Vaginal, no IAP
Vaginal, IAP
Emergency CS
Elective CS
46%
53%
52%
37.50%
Oxytocin and Anesthetics (n=599)
Vaginal, no IAP
Vaginal, IAP
Emergency CS
Elective CS

B)

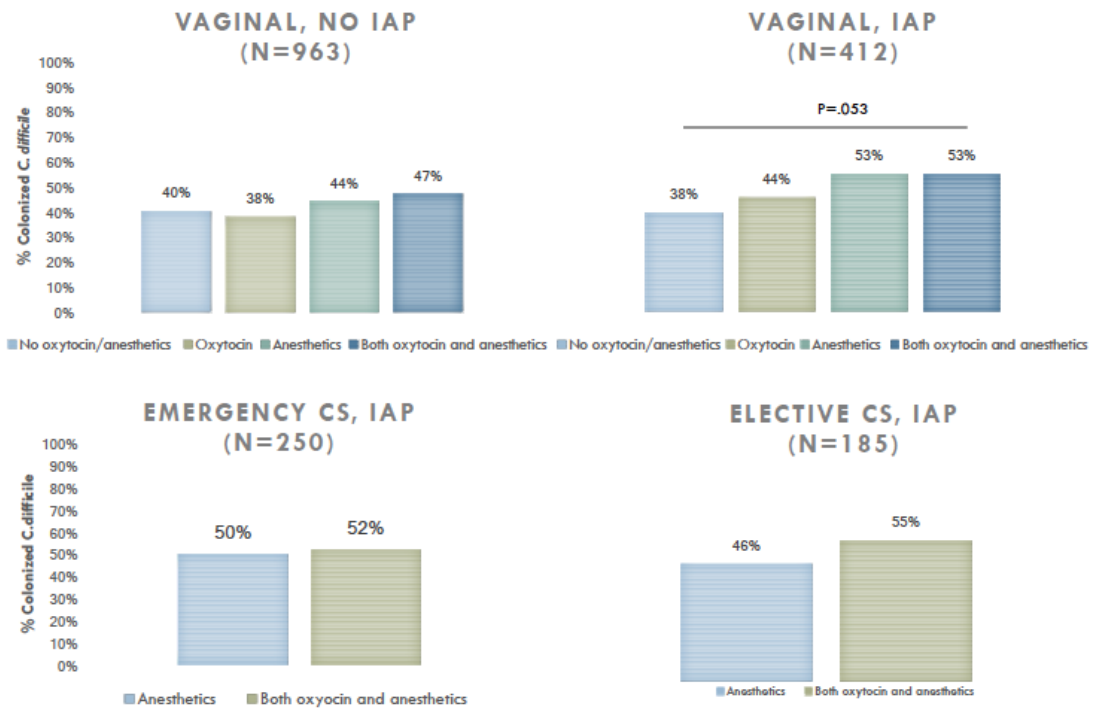

VAGINAL, NO IAP
(N=963)
% Colonized C. difficile
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
40%
38%
44%
47%
No oxytocin/anesthetics
Oxytocin
Anesthetics
Both oxytocin and anesthetics
VAGINAL, IAP
(N=412)
P=.053
38%
44%
53%
53%
No oxytocin/anesthetics
Oxytocin
Anesthetics
Both oxytocin and anesthetics
EMERGENCY CS, IAP
(N=250)
% Colonized C.difficile
50%
52%
Anesthetics
Both oxyocin and anesthetics
ELECTIVE CS, IAP
(N=185)
46%
55%
Anesthetics
Both oxytocin and anesthetics

Figure 10. A) *C. difficile* colonization rates between mode of delivery stratifies by medical interventions during birth at 1 year of age B) *C. difficile* colonization rates between medical interventions stratified by mode of delivery at 1 year of age
at 1 year of age

iv) Adjusted stratified results at 1 year of age

No significant interaction was found between any variable and mode of delivery at 1 year of age; however, anesthetic use in the final model was borderline predicting *C. difficile* colonization and thus stratification was performed within medical interventions. Among women who received anesthetics during labour, *C. difficile* was further amplified in infants born vaginally with IAP; however, this interaction was not significant (p=.741). No other effects were found.

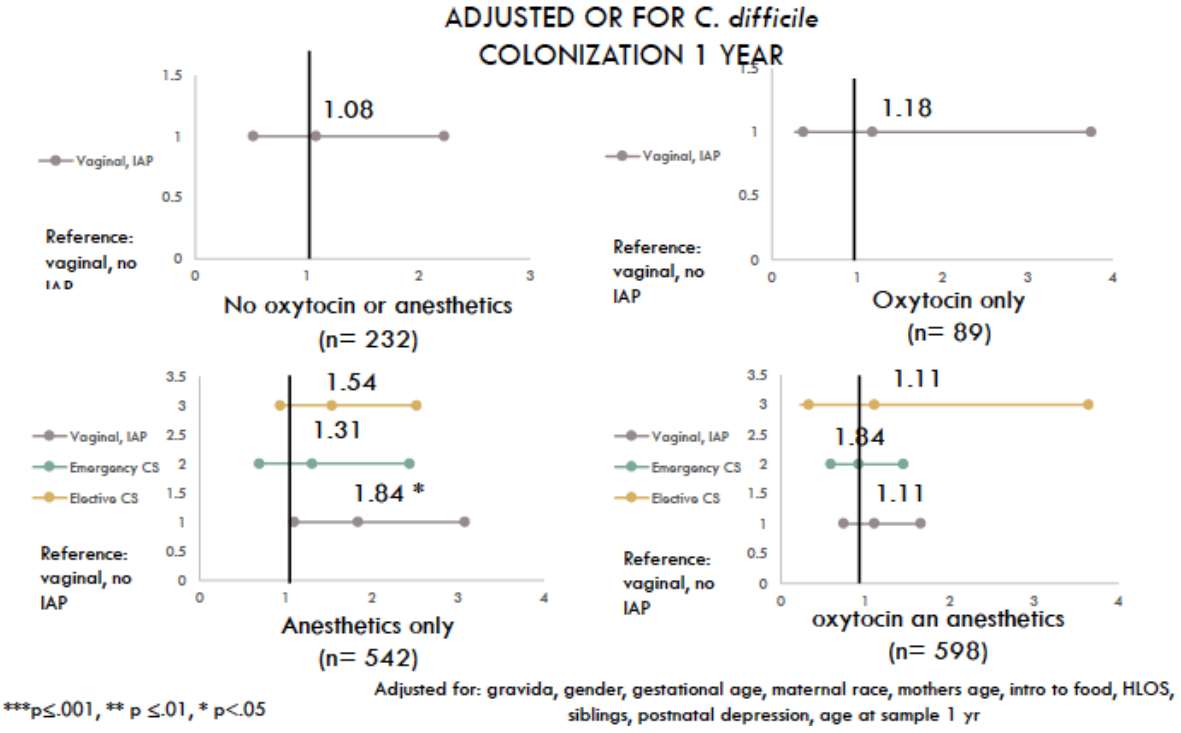


Figure 11. Results from adjusted multiple logistic regression when stratifying within stratums of medical interventions status while comparing mode of delivery effects on *C. difficile* colonization at 1 year. Global interaction term was not significant p=.741

3.4 Study population (persistence colonization)

i) Persistence colonization characteristics of mother-infant pair

In this subsample of 1226 infants, 190 (15%) were colonized with *C. difficile* at 3 months and 1 year (persistence colonization). Table 8 describes the mother-infant characteristics associated with *C. difficile* persistent colonization. Overall, 55% were delivered vaginally with no IAP, 23% were delivered vaginally with IAP, 14% were delivered by emergency CS and 9% were delivered by elective CS. According to *C. difficile* persistent colonization, colonization rates differed significantly between mode of delivery (p=.004; Table 8). Infants born by emergency CS had the highest rates of colonization (23%), followed by infants born by elective CS (18%), vaginal, IAP (17%) and vaginal, no IAP having the lowest colonization rates (12%). Several other factors were significantly associated with the persistent colonization of *C. difficile* with respect to gravida (p=.001), birthweight (p=.018), breastfeeding status at 3 months (p<.0001) and at 6 months (p<.0001), siblings (p=<.0001), maternal postnatal depression (p=.050), hospital length of stay (p<.0001), geography (p=.017).

Table 8. Descriptive characteristics associated with *C. difficile* colonization, 3 months and 1 year (persistent colonization). Row percentages, chi square exact

		C. difficile colonization at 3 months & 1 year (persistent colonization) N= 1226 Overall: 15% colonized		
		No	**Yes**	**P value (x^2 exact)**
Mode of delivery	N=1226			
	Vaginal, No IAP	585 (88%)	83(12%)	.004
	Vaginal, IAP	229(83%)	48(17%)	
	Emergency CS, IAP	131(77%)	39(23%)	
	Elective CS, IAP	91(82%)	20(18%)	
Gender	N=1226			
	Male	549(85%)	100(15%)	.937
	Female	487(85%)	90(15%)	
Gravidity	N=1226			
	Primigravida	371(80%)	94(20%)	.001
	Multigravida	666(87%)	97(13%)	
Medical interventions	N= 1171			
	No oxytocin/anesthetics	152(88%)	20(11%)	.104
	Oxytocin	55(90%)	6(10%)	

	Anesthetics	374(85%)	67(15%)	
	Both oxytocin/ anesthetics	406(82%)	91(18%)	
Breastfeeding (3 months)	N=1223			
	Exclusive	605(89%)	76(11%)	<.0001
	Partial	273(80%)	66(20%)	
	Formula Fed	155(76%)	48(24%)	
Breastfeeding (6 months)	N=1212			
	Exclusive	195(91%)	19(9%)	<.0001
	Partial	589(85%)	99(15%)	
	Formula	241(77%)	71(23%)	
Introduction to foods	N=1226			
	<=3 months	34(85%)	6(15%)	1.0
	>3 months	1003(84%)	185(16%)	
Siblings	N=1225			
	No	507(80%)	129(20%)	<.0001
	Yes	528(89%)	61(10%)	
Pets in the home 0-3 mo	N=1208			
	No	558(85%)	92(14%)	.176
	Yes	464(82%)	96(17%)	
Maternal pre-pregnancy BMI	N=1204			
	Underweight	25(92%)	2(7%)	.063
	Normal weight	617(86%)	95(13%)	
	Overweight	224(81%)	51(18%)	
	Obese	156(81%)	36(18%)	
Gestational age	N=1174			
	39-41 weeks	766(84%)	136(15%)	.398
	37-38 weeks	230(83%)	44(16%)	
	<36 weeks	40(84%)	11(22%)	
Maternal Race	N=1215			
	White	794(84%)	148(15%)	.622
	Asian	135(87%)	20(13%)	
	Other	100(83%)	20(16.6%)	
Child medication 3mo	N=1226			
	No	1005(84%)	185(15%)	1.0
	Yes	32(84%)	6(16%)	
Postnatal smoke exposure	N=1213			
	No	884(85%)	153(15%)	.057
	Yes	140(79%)	36(20%)	
Maternal prenatal smoking	N=1200			
	No	977(84%)	177(15%)	1.00
	Yes	41(85%)	7(14%)	
Maternal Pre-natal depression	N=1205			
	No	800(85%)	133(14%)	.085
	Yes	223(81%)	51(18%)	
Maternal Post-natal depression(6mo)	N=1076			
	No	794(85%)	134(14%)	.050
	Yes	118(79%)	31(21%)	
Hospital length of stay	N=1226			
	24 hrs or less	293(91%)	25(8%)	.0001
	2-3 days	592(82%)	126(17%)	
	>4 days	152(79%)	39(20%)	
Geography	N=1225			
	Vancouver	348(88%)	49(12%)	.017
	Edmonton	226(79%)	58(20.5%)	
	Winnipeg	461(85%)	83(15%)	

i) Persistent colonization crude analyses

Overall, 15% of infants were colonized with *C. difficile* at 3 months and 1 year (persistence), when stratified to include only infants that were vaginally delivered, exclusively breastfed at 3 months and no medical interventions (antibiotics, oxytocin or anesthetics), this colonization rate was reduced to 8.5% (p=.063) (Figure 12). In crude analyses, mode of delivery was significantly associated with persistent *C. difficile* colonization (p=.004, Table 10). Colonization rates between mode of delivery are shown in Figure 13. Similarly, the magnitude of this effect was shown in simple logistic regression with infants born by emergency CS and vaginal, IAP (OR:2.09 95% CI: 1.37-3.20 p=.001, OR:1.47 95% CI:1.0-2.17 p=.048) having higher odds of persistent *C. difficile* colonization compared to vaginal, no IAP as shown in Figure 14; Table 10.

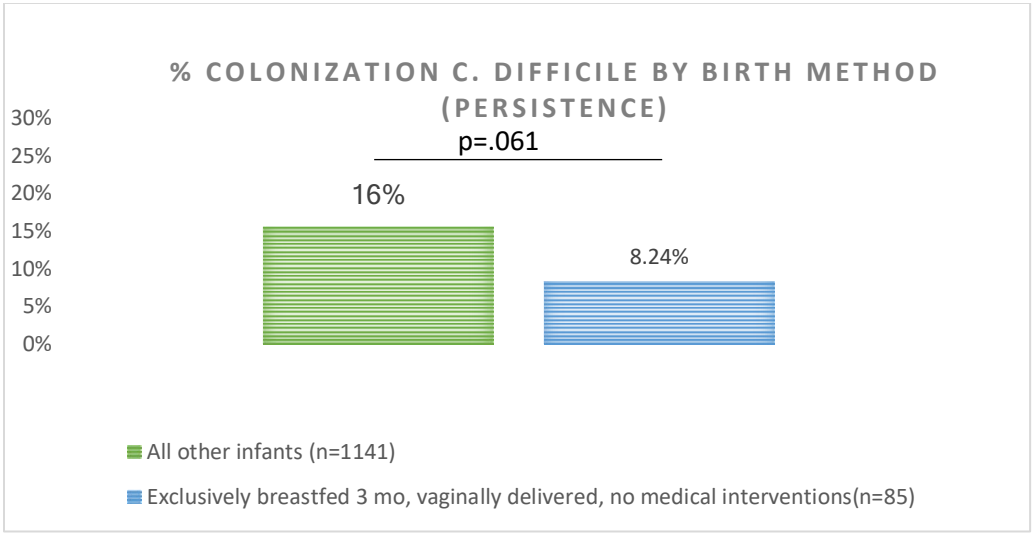


Figure 12. Colonization rates between persistent *C. difficile* in all infants compared to C. difficile colonization between infants who were vaginally delivered, exclusively breastfed at 3 months or 6 months and received no medical interventions

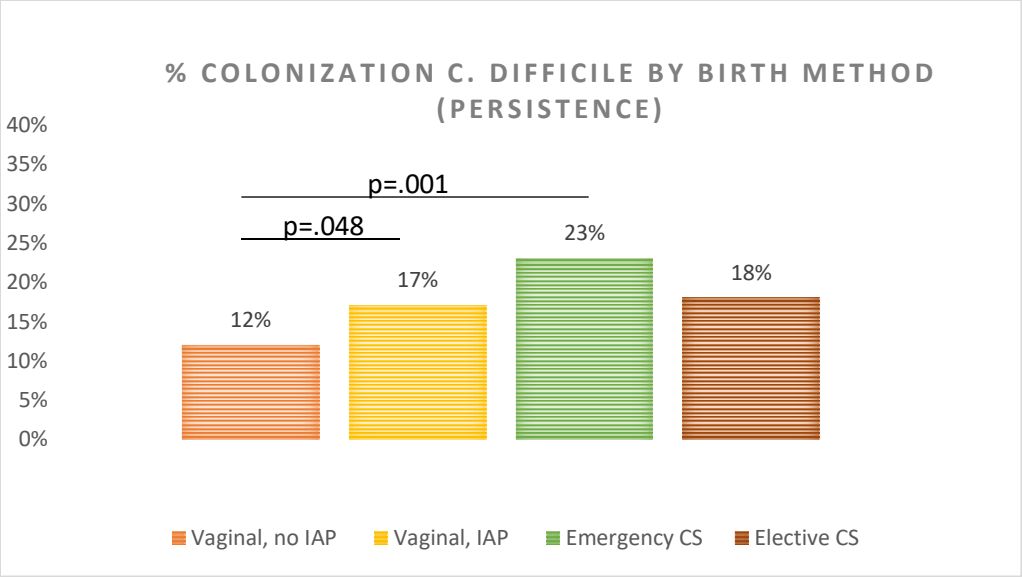


Figure 13. Colonization rates between mode of delivery and persistent *C. difficile* colonization

ii) Persistent C. difficile colonization adjusted analyses

Logistic regression was performed to predict the odds of persistent *C. difficile* colonization as binary outcome (present/absent) in relation to the exposure of interest: infants born vaginally without IAP (reference), vaginally with IAP, emergency CS and elective CS. Simple and multiple logistic regression were performed to compile an adjusted model using a purposeful selection. Variables were initially selected at $p<.20$ using fishers exact test. Adjusted models were performed with birth characteristics, prenatal characteristics and postnatal characteristics (Table 10). Variables that were significant in these models at $p<.05$ were carried to the final model. Confounding effects were analyzed after each model, whereby a change in the regression coefficient by >10% deemed the variable a confounding factor and was kept in the model, all confounding variables are noted in Table 10. Interaction terms were analyzed in the final model and were kept in the final model if they were significant at $p<.05$. Table 10 explains the final model for simple and multiple logistic regression.

After adjustment for medical interventions in model 1, infants born by emergency CS remained significantly associated with increased odds of persistent *C. difficile* colonization (OR:1.92 95% CI:1.22-3.0 p=.004), whereas infants born vaginally with IAP lost significance after adjustment. In model 2, after adjustment for gravida, maternal pre-pregnancy BMI and maternal prenatal depression, infants born by emergency CS remained significant (OR:1.86 95% CI:1.16-2.8 p=.007), as well as infant born by elective CS became significantly associated with increased odds of persistent *C. difficile* colonization (OR:1.82 95% CI:1.04-3.14 p=.033). Elective CS became significant when gravida and maternal prenatal depression were added separately to the model, conferring evidence that these variables may be masking this association. In model 3, after adjustment for postnatal characteristics such as breastfeeding at 3 months and 6 months, postnatal smoke exposure, hospital length of stay and siblings, all significance for mode of delivery were taken away. Similarly, in the final model, after adjustment for medical interventions, gravida, maternal prenatal depression, breastfeeding at 3 months and 6 months, pets, hospital length of stay, and siblings, no significance remained for mode of delivery predicting the odds of persistent colonization of *C. difficile* (Table 10).

To understand where mode of delivery lost significance in the model, individual adjustment was performed in Figure 14 and Appendix B-Table B1. After adjustment for breastfeeding at 3 months, significance in the crude odds ratio did not change which was similar for adjustment for breastfeeding at 6 months of age. After adjusting for breastfeeding at 3 months and hospital length of stay, infants born by elective CS lost significance. Finally, after adjustment for breastfeeding at 3 months, hospital length of stay and siblings, mode of delivery did not remain significantly associated with the odds of persistent *C difficile* colonization (Figure 14). In this model, persistent *C. difficile* colonization was more common among infants who were partially

breastfed at 3 months and formula fed at 6 months, and in infants whose hospital length of stay was 2 days or more. Persistent *C. difficile* colonization was less common in infants with a sibling (Table 10).

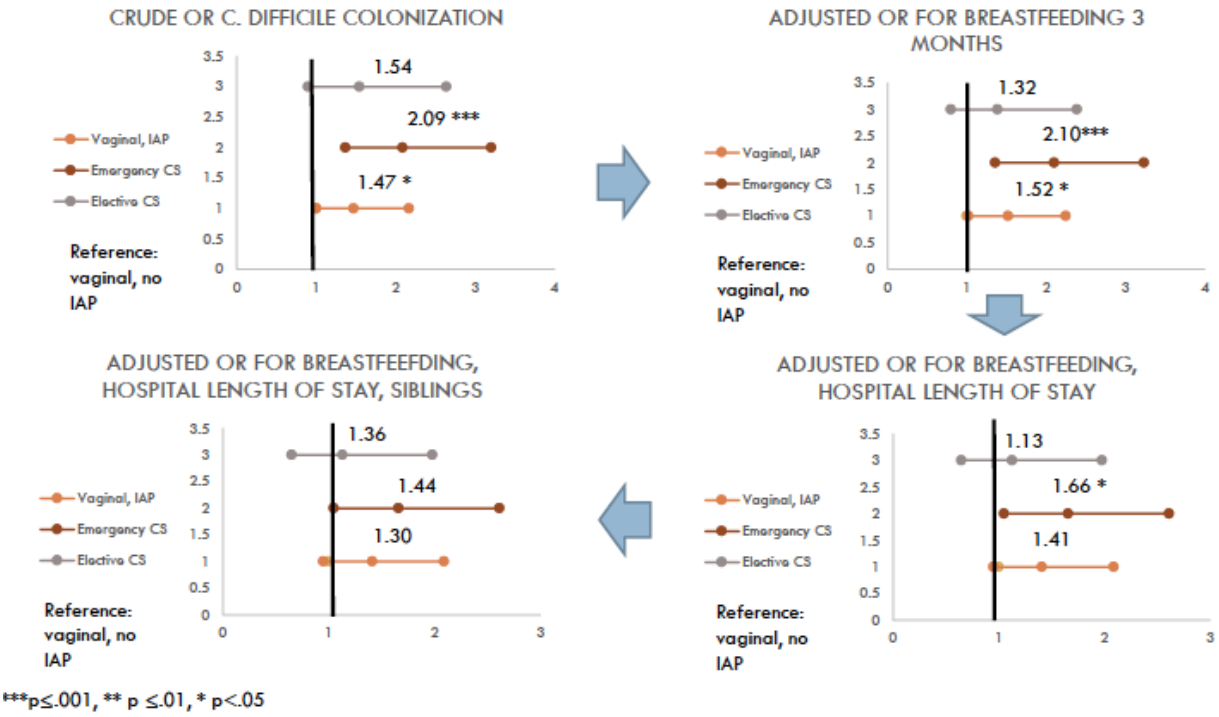


Figure 14. Individual and multiple adjustment for important covariates between mode of delivery and persistent *C. difficile* colonization. Results from simple and multiple logistic regression.

i) Persistent C. difficile colonization unadjusted stratified analyses

Several unadjusted stratifications were performed to further understand differences between mode of delivery and persistent *C. difficile* colonization. After stratification for medical interventions during labour, among women who received anesthetics and oxytocin, infants born by emergency CS had significantly higher persistent *C. difficile* colonization rates compared to vaginal, no IAP (25% vs 15%, p=.036; Figure 15.A). Further stratification by mode of delivery to compare differences between medical interventions proved no significance (Figure 15.B). No significant interaction was found in unadjusted or adjusted stratification between this relationship

using a global test. After stratification for breastfeeding status at 3 months, among partially breastfed infants, persistent *C. difficile* colonization was higher in infants born by emergency CS (30% vs 14%; p=.012; Appendix F-Figure F11). Similarly, among formula fed infants at 3 months, infants born by emergency CS had the highest persistent colonization rates compared to vaginal, no IAP (56% vs 14%; p=.0001; Appendix F-Figure F11). Significant interaction was found between breastfeeding at 3 months and mode of delivery in unadjusted stratification (p=.043); however lost significance in adjusted stratification (p=.057). However, effects seen in emergency CS may be due to limited number of infants (n=27) who were formula fed.

After stratification for breastfeeding status at 6 months, among infants who were formula fed, higher persistent colonization was found in infants born by emergency CS (50%) and vaginal IAP (26%) compared vaginal, no IAP (15%; p=.0001 and p=.057, respectively; Appendix F-Figure F12). Unadjusted interaction terms were significant between this relationship; however, after adjustment it did not remain significant (Appendix E- Table E1). After stratification for gravida status, among infants whose mothers were primigravida, higher persistent *C. difficile* colonization rates were found in infants born by emergency CS (p=.001; Appendix F-Figure F13). Interaction between this relationship was not significant in unadjusted analysis (p=.068); however, became borderline significant in adjusted analysis using a global test (p=.059). Finally, after stratification for sibling status, infants born by emergency CS and elective CS had significantly higher persistent *C. difficile* colonization compared to infants born vaginally with no IAP, among infants with no siblings (p=.001 and p=.025, respectively; Appendix F-Figure F14). Significant unadjusted or adjusted interactions were not present with these covariates using a global test (Appendix E-Table E1).

ii) Persistent C. difficile colonization adjusted stratified analyses

Stratifications were performed in the adjusted analyses if interaction terms were significant or borderline significant using a global test (Appendix E-Table E1). In this persistence model, borderline significant interactions were found between breastfeeding at 3 months and mode of delivery and gravida status and birth method, both in relation to *C. difficile* persistence colonization. After stratification by breastfeeding status at 3 months, infants born by emergency CS had significantly higher odds of persistent *C. difficile* colonization among infants who were formula fed in an adjusted model compared to infants born vaginally with no IAP (aOR: 4.59 95% CI: 1.58-13.35 p=.005; Appendix F-Figure F15), whereas no other mode of delivery effects were found among exclusive or partially breastfed infants. Similar to the 3-month model, interaction was found between gravida status and mode of delivery and the colonization of persistence *C. difficile* colonization. Among infants born to primigravida mothers, infants born by emergency CS had significantly higher odds of persistent *C. difficile* colonization in an adjusted model compared to infants born vaginally with no IAP (Appendix F-Figure F16), whereas these effects were not found between any mode of delivery among multigravida status. After stratification for medical interventions, in an adjusted model, no significance was found between mode of delivery within stratums of medical interventions (Figure 16) and the interaction term was not significant (p=.996). An odds ratio with the stratum of oxytocin only was not performed due to small sample size (Figure 16).

Table 9. Percentage distribution of descriptive characteristics in relation to mode of delivery and medical interventions at birth (persistence colonization)											
Column percentages		Vaginal, NO IAP	Vaginal, IAP	Emergency CS	Elective CS		No oxytocin/anesthetics	Oxytocin only	Anesthetics only	Both oxytocin and anesthetics	
		N (%)	N (%)	N (%)	N (%)	P value(x^2)	N (%)	N (%)	N (%)		P value(x^2)
Mode of delivery											
	Vaginal, no IAP	-	-	-	-		133(77%)	48(79%)	218(49%)	227(46%)	.0001
	Vaginal, IAP	-	-	-	-		39(23%)	13(21%)	75(17%)	142(29%)	
	Emergency CS	-	-	-	-		0	0	52(12%)	113(23%)	
	Elective CS	-	-	-	-		0	0	96(22%)	15(3%)	
Gestational age											
	Term (39-42 weeks)	530(79%)	194(21%)	124(14%)	53(6%)	.0001	140(81%)	46(5%)	301(35%)	370(43%)	.050
	Early Term (37-38)	130(19%)	56(20%)	35(12%)	53(19%)		27(15%)	14(5%)	118(44.7%)	105(40%)	
	Premature (≤ 36 weeks)	8(1%)	26(9%)	11(6%)	5(4%)		5(3%)	1(2%)	21(5%)	22(4%)	
Maternal Pre-pregnancy BMI											
	Underweight	15(2%)	5(2%)	5(3%)	2(2%)	.007	2(1%)	3(5%)	8(2%)	12(2%)	.0001
	Normal weight	419(64%)	153(57%)	85(51%)	55(50%)		116(68%)	33(54%)	265(61%)	268(55%)	
	Overweight	144(22%)	64(24%)	37(22%)	30(27%)		45(26%)	18(30%)	89(20%)	107(22%)	
	Obese	81(12%)	48(18%)	40(24%)	23(21%)		7(4%)	7(11%)	72(17%)	101(21%)	
Maternal Race											
	White	523(79%)	208(75%)	126(74%)	84(76%)	.515	128(76%)	46(75%)	346(79%)	371(75%)	.500
	Asian	77(12%)	36(13%)	24(14%)	18(16%)		26(15%)	7(11%)	57(13%)	64(13%)	
	Other	58(9%)	32(12%)	20(12%)	9(8%)		15(9%)	8(13%)	35(8%)	58(12%)	
Breastfeeding 6 mo											
	Exclusive	101(15%)	65(24%)	32(19%)	16(15%)	,067	44(26%)	12(20%)	80(18%)	70(14%)	.002
	Partial	391(59%)	146(53%)	93(55%)	58(54%)		92(53%)	42(69%)	247(57%)	278(56%)	
	Formula Fed	169(26%)	65(24%)	44(26%)	34(32%)		36(21%)	7(11%)	106(24%)	146(29%)	
Gender											
	Male	341(51%)	138(50%)	111(65%)	59(53%)	.006	81(13%)	28(47%)	245(46%)	264(56%)	.189
	Female	327(49%)	139(50%)	59(35%)	52(47%)		91(17%)	88(53%)	196(54%)	233(47%)	
Prenatal depression											
	No	514(78%)	210(77%)	122(73%)	85(77%)	.546	133(80%)	48(28%)	340(79%)	376(77%)	.776
	Yes	142(22%)	62(28%)	45(27%)	25(23%)		34(20%)	12(72%)	92(21%)	115(23%)	
Gravida											
	Primigravida	232(34%)	129(45%)	84(49%)	19(17%)	.0001	49(28%)	23(38%)	134(30%)	247(50%)	.0001
	Multigravida	436(65%)	148(53%)	86(51%)	92(83%)		123(72%)	38(62%)	307(70%)	250(30%)	
Siblings											
	No	306(46%)	171(62%)	131(77%)	28(25%)	.0001	58(49%)	25(41%)	186(42%)	350(70%)	.0001
	Yes	362(54%)	105(38%)	39(23%)	83(75%)		114(66%)	36(59%)	254(58%)	147(30%)	
Geography											
	Vancouver	184(28%)	104(37%)	65(38%)	44(40%)	.003	49(28%)	18(30%)	146(33%)	181(37%)	.0001
	Edmonton	160(24%)	55(20%)	39(23%)	30(27%)		24(14%)	7(11%)	114(26%)	138(28%)	
	Winnipeg	323(48%)	118(43%)	66(39%)	37(33%)		99(58%)	36(59%)	181(41%)	177(36%)	
Hospital length of stay											
	24hrs or less	246(37%)	69(25%)	0	3(3%)	.0001	85(49%)	25(41%)	97(22%)	88(18%)	.0001
	2-3 days	350(52%)	176(64%)	109(64%)	82(74%)		66(38%)	30(49%)	278(63%)	320(64%)	
	4 days or more	72(11%)	32(12%)	61(36%)	26(23%)		21(12%)	6(10%)	66(15%)	89(18%)	
Pets											

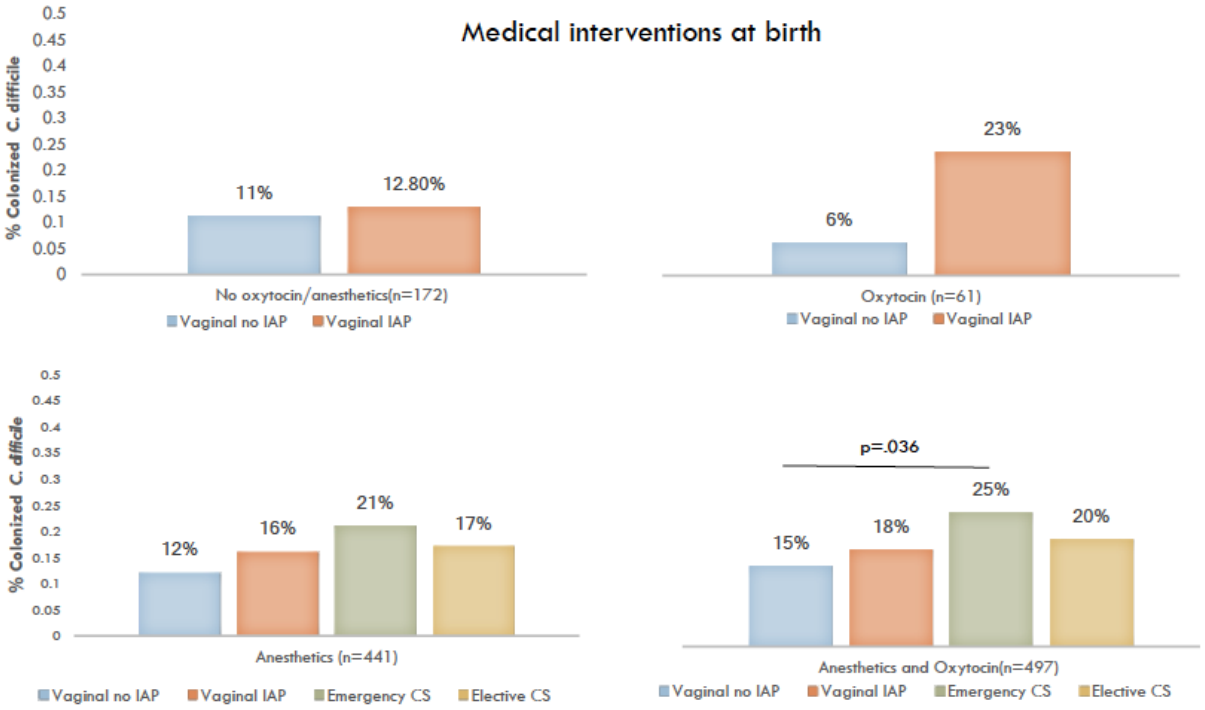
	No	358(55%)	141(52%)	86(51%)	63(57%)	.623	95(56%)	31(52%)	233(53%)	251(51%)	.744
	Yes	298(45%)	132(48%)	83(49%)	47(43%)		75(44%)	29(48%)	203(47%)	239(48%)	
Maternal Smoking											
	No	621(54%)	265(23%)	159(14%)	107(9%)	.141	164(98%)	57(97%)	410 (95%)	470(96%)	.380
	Yes	33(69%)	6(12.5%)	7(15%)	2(4%)		3(2%)	2(3%)	21(5%)	19(4%)	

Table 10. Simple and multiple logistic regression with birth, prenatal and postnatal characteristics predicting *C. difficile* colonization at 3 months and 1 year of age (persistence colonization)

	Crude (unadjusted)			**Model 1 (adjusted for birth characteristics)**			**Model 2 (adjusted for prenatal characteristics)**			**Model 3 (adjusted for postnatal characteristics)**			**Model 4 (Final model)**		
Outcome: *Clostridioides difficile* persistence (Y/N)	Main exposure of interest: Birth method	Crude OR	P value	Main exposure of interest: Birth method	Adjusted OR	P value	Main exposure of interest: Birth method	Adjusted OR	P value	Main exposure of interest: Birth method	Adjusted OR	P value	Main exposure of interest: Birth method	Adjusted OR	P value
Birth method:	N=1226			N=1171			N=1185			N=1197			N=1129		
	Vaginal no IAP	(reference)		Vaginal no IAP	(reference)		Vaginal no IAP	(reference)		Vaginal no IAP	(reference)		Vaginal no IAP	(reference)	
	Vaginal, IAP	1.47(1.0-2.17)	**.048**	Vaginal, IAP	1.3(.91-2.0)	.132	Vaginal, IAP	1.39(.92-2.09)	.108	Vaginal, IAP	1.24(.81-1.8)	.301	Vaginal, IAP	1.26(.82-1.94)	.281
	Emergency CS, IAP	2.09(1.37-3.20)	**.001**	Emergency CS, IAP	1.92(1.22-3.0)	**.004**	Emergency CS, IAP	1.86(1.16-2.87)	**.007**	Emergency CS, IAP	1.32(.82-2.12)	.241	Emergency CS, IAP	1.36(.82-2.23)	.230
	Elective CS, IAP	1.55(.89-2.61)	.109	Elective CS, IAP	1.51(.83-2.68)	.153	Elective CS, IAP	1.82(1.04-3.14)	**.033**	Elective CS, IAP	1.41(.78-2.5)	.241	Elective CS, IAP	1.38(.74-2.5)	.303
Block 1: Birth characteristics															
	Medicalization: No oxytocin/anesthetics (ref) Oxytocin only Anesthetics only Both oxytocin/anesthetics	 .829(.316-2.17) 1.35(.79-2.31) 1.72(1.02-2.89)	 .703 .261 **.040**	Medicalization: No oxytocin/anesthetics (ref) Oxytocin only Anesthetics only Both oxytocin/anesthetics	 .83(.317-2.18) 1.15(.66-2.02) 1.42(.83-2.44)	 .709 .609 .193							Confounding	 .53(1.16-1.68) 1.04(.56-1.90) .90(.50-1.90)	 .286 .896 .733
Block 2: Pre-natal Characteristics															
	Gravida(ref-primigravida)	.574(.42-.78)	**<.0001**				Gravida (ref-primigravida)	.55(.39-.77)	**.001**					1.10(.70-1.70)	.672
	Maternal BMI (ref-normal weight) Underweight Overweight obese	 .519(.12-2.29) 1.47(1.01-2.14) 1.49(.982-2.28)	 .378 **.040** .060				Maternal BMI (ref-normal weight) Underweight Overweight obese	 2.16(.49-9.44) 3.21(.72-14.24) 2.92(.65-13.1)	 .303 **.046** .181				-		

	Maternal Pre-natal depression (Y/N)	1.37(.96-1.96)	**.079**				Maternal prenatal depression (Y/N)	1.43(.99-2.06)	.054					1.08(.72-1.61)	.682
Block 3: Post-natal Characteristics															
	Breastfeeding 3 mo (ref-exclusive) Partial Formula fed	1.92(1.34-2.76) 2.52(1.68-3.76)								Breastfeeding 3 mo (ref-exclusive) Partial Formula fed	1.53(.84-2.10) 1.60(.81-3.14)	**.046** .171	Confounding	1.62(1.05-2.51) 1.55(.78-3.12)	**.029** .229
	Breastfeeding 6 mo (ref-exclusive) Partial Formula fed	1.72(1.02-2.89) 3.06(1.78-5.25)	**.039** **<.0001**							Breastfeeding 6 mo (ref-exclusive) Partial Formula fed	1.56(.87-2.78) 2.03(.93-4.39)	.171 .072	Confounding	1.65(.89-3.06) 2.27(1.0-5.0)	.107 **.045**
	Postnatal smoke exposure	1.48(.9-2.22)	.055							Postnatal smoke exposure	1.19(.80-1.89)	.408	-		
	pets	1.25(.91-1.71)	.153							Pets(Y/N)	1.06(.75-1.45)	.725	Confounding	1.06(.75-1.45)	.729
	Hospital length of stay 24 hrs or less(Ref) 2-3 days 4 days or more	2.39(1.53-3.74) 2.89(1.69-4.92)	**<.0001** **<.0001**							Hospital length of stay 24 hrs or less(Ref) 2-3 days 4 days or more	1.80(1.12-2.93) 1.99(1.1-3.5)	**.016** **.022**	Hospital length of stay 24 hrs or less(Ref) 2-3 days 4 days or more	1.77(1.06-2.95) 2.05(1.09-3.84)	**.028** **.024**
	Siblings(Y/N)									Siblings(Y/N)	.53(.35-.78)	**.001**	Siblings(Y/N)	.44(.27-.73)	**.002**

A)



B)

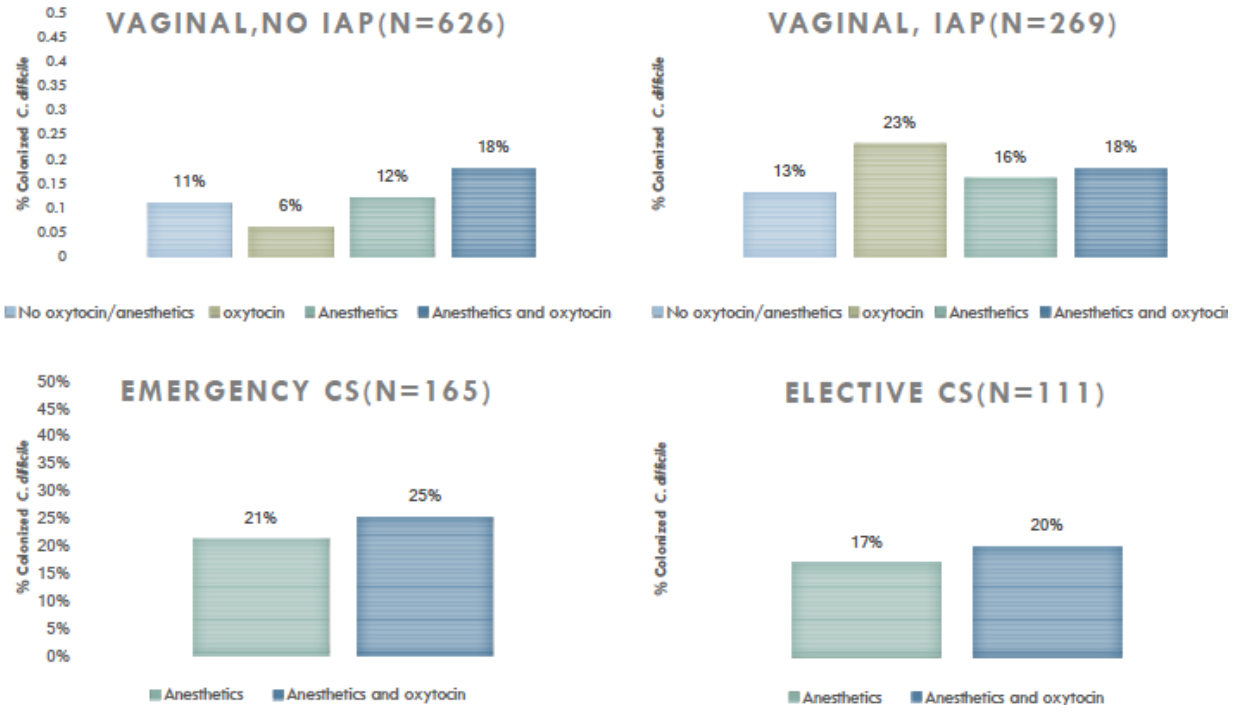
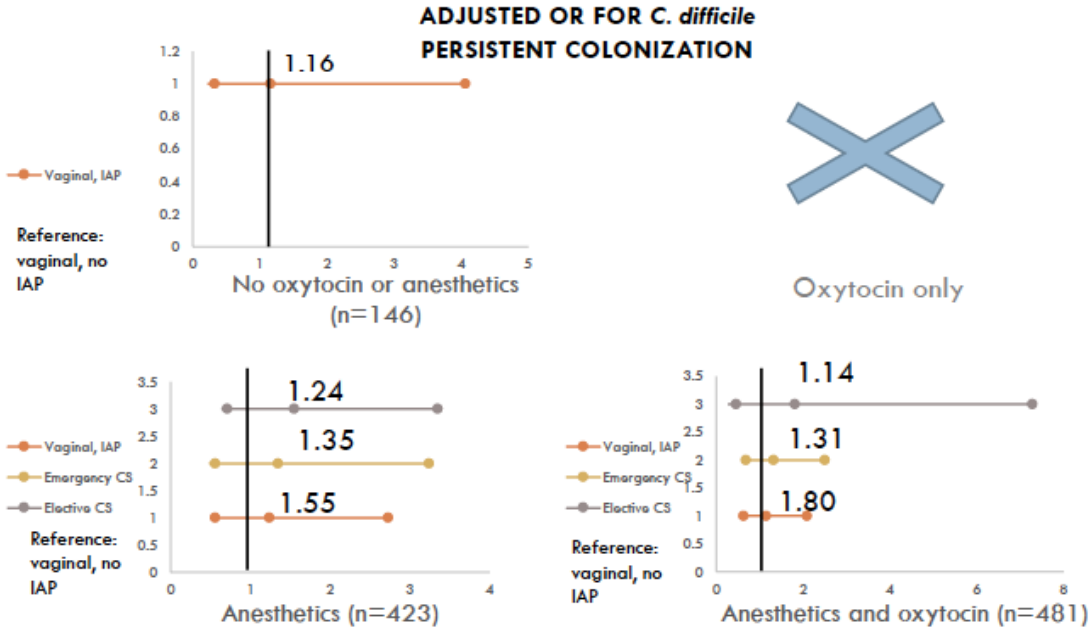


Figure 15. A) *C. difficile* colonization rates between mode of delivery stratified by medical interventions during birth at <u>3 months of age and 1 year (persistence)</u> B) *C. difficile*

colonization rates within medical interventions stratified by mode of delivery at 3 months of age and 1 year (persistence)



Adjusted for medical interventions, prenatal depression, breastfeeding 3 & 6 months, siblings, pets, hospital length of stay

***p≤.001, ** p ≤.01, * p<.05

Figure 16. Results from adjusted multiple logistic regression when stratifying within stratums of medical interventions status while comparing mode of delivery effects on persistent *C. difficile* colonization. Global interaction term was not significant p=.996

CHAPTER 4: DISCUSSION AND CONCLUSIONS

4.1 Summary and interpretations of findings

4.1.1 General overview

This large prospective study performed in healthy, full term infants at 3 months, 1 year and persistent colonization over the first year of life elucidates and confirms the susceptibility and vulnerability of the gut microbiota of infants undergoing birth interventions. Overall, mode of delivery effects were most prominent at 3 months of age. Infants who were born by CS delivery, specifically emergency CS delivery, had significantly increased odds of *C. difficile* colonization independent of medical interventions, prenatal and postnatal characteristics. Emergency CS effects persisted, defined as the colonization of *C. difficile* at 3 months and 1

year, among first born infants. This suggests other interventions during birth or postnatal factors may play a role regardless of oxytocin and anesthetics during delivery.

Almost one third of infants were colonized with *C. difficile* at 3 months which increased to almost half of infants at one year of age with a persistent colonization rate of 15% at both time points which is consistent with previous studies in healthy infants (86, 88, 136). Higher *C. difficile* colonization rates have been found between infants born by CS delivery (32, 85, 88) compared to vaginal deliveries with lowest rates found in infants delivered at home (32). Novel evidence from this study elucidates and disentangles differences between infants born by emergency CS and elective CS and the risk for *C. difficile*, a biomarker for an aberrant gut environment and later allergic disease (32).

4.1.2 C. difficile colonization is differentially impacted by mode of delivery at 3 months of age

It is evident *C. difficile* is more common in the absence or disruption of maternally acquired microbes such as CS delivery, formula feeding, and antibiotic use (59). Disturbances in the fecal microbiota of infants have been shown in vaginal births exposed to hospital environment compared to home deliveries (32, 137), CS deliveries (36, 82), with and without exposure to antibiotics (82) and breastfed *versus* formula fed (91, 138). These common exposures affect the assembly of pioneer bacteria and subsequently raises threats of *C. difficile* colonization. Any threat to the microbiota during the critical window of development may pose short and long-term consequences to the infant (33). Mode of delivery shapes the bacterial populations at birth. Vaginally born infants closely resemble the vaginal microbiota of their mothers while CS delivered infants commonly harbour skin bacteria (42, 139).

Evidence for differences between the gut microbiota of infants born by emergency CS and elective CS are limited which is a major gap of knowledge in this field. Nevertheless, limited evidence suggests disruptions are more severe and long lasting in infants born by emergency CS. A depletion of *Bacteroides* and an increase in the phylum Proteobacteria and genus *Clostridium* in early life were noted in infants born by emergency CS. Persistent depletion of *Bacteroides* was found in infants born by emergency CS especially among non-breastfed infants at 1 year of age (82). Moreover, bacterial richness was increased in infants born by emergency CS at 1 year of age, suggesting long-term influences of hospital interventions (82).

Similarly, Chu et al., found the initiation of labour may have the greatest effect on the origin of the infant gut microbiota. Similarities were seen for vaginally born infants compared to infants born by CS delivery only with the onset of labour. Vaginally acquired microbes and maternal skin microbiota dominated in infants born with the onset of labour. In contrast, infants born by elective CS without the onset of labour harboured predominantly maternal skin microbiota (140). These findings are of particular importance as it highlights and follows up with a previous study, where infants born by elective CS were more similar to vaginal deliveries (139); however, how labour played a role was not explored in this study.

Infants born by elective CS completely lack the onset labour and potential microbial inoculation through the breaking of membranes compared to vaginal births and emergency CS delivery. Findings from this study suggest the limited magnitude of maternal inoculation in infants born by emergency CS may not play a substantial role in the exclusion of *C. difficile*. Furthermore, these findings bring to light to further perinatal, in utero and stress related factors in the mother affecting the maternal and infant gut microbiota.

Given recent evidence for possible intrauterine bacterial colonization (34), mothers undergoing emergency CS may have predisposing conditions in which render them more susceptible to an emergency CS delivery. Likewise, these conditions could alter the bacterial colonization in utero if it does exist. Underlying aetiologies for CS delivery such as hypertension, gestational diabetes and medical indication for emergency CS could be playing a role and were not controlled for in this study. Mothers who undergo emergency CS most likely intended to have a vaginal birth. Therefore, mothers may have been Group B *Streptoccocus* positive and cumulative exposures of IAP during labour and subsequently emergency CS delivery and postnatally may have been given. The CHILD cohort has previously reported 43% of mothers undergoing emergency CS delivery received antibiotics during the first 3 months in a sub sample in which correlated with higher *Clostridium* species in their infants (82). Additionally, transmission of antibiotics from breastmilk to the infant has been reported in which resulted in perturbations in the infant gut microbiota (141).

Differences between Infants born by emergency CS and elective CS could lie in the hypothalamic-pituitary-adrenal (HPA) axis. Infants who undergo elective CS display a lower stress response compared to vaginal deliveries (142). Adrenaline, noradrenaline and cortisol in umbilical cord blood are also lower in infants born by CS delivery, likely influencing systolic blood pressure which is lower infants born by elective and emergency CS delivery compared to vaginal deliveries (143). Consequently, infants born by CS delivery lack necessary 'vaginal squeeze' which prepares the infants lung development through physical force which is widely different in infants born by CS delivery (143). Again, differences between elective and emergency CS remain to be understudied in this area.

Elective and emergency CS have shown discrepancies between risk of allergies and obesity (29-31). Authors have recently suggested the risk of emergency CS and obesity to be independent of the vaginal microbiota (144). Infants born by emergency CS are technically inoculated with the onset of labour which renders them similar to vaginal births in this regard. These authors found no relationship between elective CS and childhood obesity, which suggests confounding by indication for CS delivery. However, this inoculum for infants born by emergency CS may not be comparable to vaginal delivery. Maternal fecal-oral transmission likely plays a stronger role than vaginal microbiota. These vaginal bacteria, such as *Lactobacillus,* have not been found to colonize long-term and are transient in nature (145). Thus, it is likely the vaginal microbiota cannot reside in the conditions in the gut microbiota long-term. Indication for emergency CS has not been explored in relation to the infant gut microbiota, and may likely to a consequence of maternal factors. However, all evidence does not support the conclusion that elective CS renders no risk for childhood overweight or obesity and in fact the opposite has been shown (29-31).

4.1.3 Exclusive breastfeeding did not modify the effects of emergency CS on C. difficile at 3 months of age

C. difficile colonization rates and counts have shown to be consistently lower in exclusively breastfed infants (88). Likewise, this study demonstrated partial breastfeeding and formula feeding to be independent predictors of *C. difficile* colonization at 3 months. Several components in breastmilk have shown to exclude *C. difficile* colonization. Human milk oligosaccharides (HMO's) can neutralize *C. difficile* toxins (100). Breastmilk contains

immunomodulatory properties, antibacterial substances and high amounts of IgA in which has shown to be inversely associated with *C. difficile* colonization (146).

Exclusive breastfeeding has potential modifying effects against microbiota assaults such as CS delivery (82). The fact that emergency CS effects on *C. difficile* were independent of breastfeeding demonstrates the importance of microbial assembly and establishment at birth. Exclusive breastfeeding did slightly attenuate the effects of *C. difficile* in infants born by emergency CS compared to infants born by elective CS. Although, differences overall were not significant between stratums of breastfeeding. Moreover, disruptions from emergency CS delivery may not successfully be recovered solely by maternal microbes from breastfeeding or from the vaginal microbiota.

Differences in the breastmilk and colostrum microbiota have been found to differ between mode of delivery (46, 47) which may also account for some of these observed changes. Breastfeeding is often more difficult to initiate after CS delivery, is of shorter duration and may affect the infants suckling initiation. These effects of oxytocin and anesthetics during delivery may further amplify these difficulties (11, 52, 55). Similarly, these interventions could potentially disrupt the breastmilk microbiota. Moreover, synthetic oxytocin and fentanyl have the capability to disrupt milk let down. Desensitization of oxytocin receptors has been found in the presence of synthetic oxytocin which often coincides with fentanyl through epidural use (52). With endogenous oxytocin stimulating prolactin and subsequently milk let down, this relationship could be affected by synthetic oxytocin. Therefore, the quality and the quantity of breastmilk could be diminished in infants born by CS delivery (52).

4.1.4 Emergency CS effects were stronger among first born infants

Primigravida women are more likely to receive a range of medical interventions at birth (147), very few of which have been studied in relation to the infant gut microbiota. At 3 months of age and persistent colonization to 1 year of age, the relationship between emergency CS and *C. difficile* was most evident among first born infants. This suggest that the 'first born' effect is a factor in *C. difficile* colonization. Persistent *C. difficile* colonization in CS delivered infants has been shown. Toxigenic strains have been shown to colonize after 2 months in which persists long-term (>6 months) and are environmentally acquired in CS infants (62). Infants born by CS delivery display a lower strict/facultative anaerobe ratio at 12 months (62). This could possibly be explaining persistent colonization in infants born by emergency CS delivery among women who were primigravida. Lastly, the gut microbiota of infants without siblings, and similarly primigravida women, resemble those of CS delivered infants (145). First born infants tend to harbour less *Bifidobacterium* than infants with siblings (145). Thus, the combination of CS delivery and the first born effect likely renders the gut microbiota less complex and mature leading to the inability to exclude *C. difficile* (145).

4.1.5 Effects of oxytocin-like drugs and anesthetics on C. difficile colonization

We did not find strong evidence for oxytocin or anesthetic use during labour to impact the colonization of *C. difficile* independent of mode of delivery or breastfeeding at 3 months of age. This could be due to lack of timing, dosage and indication for these interventions. Synthetic oxytocin may imprint on infant health through epigenetic changes (143), disruption in gut motility, alteration in endogenous oxytocin receptor sensitivity, and potentially the infant gut microbiota (48). At 1 year of age, anesthetics during delivery borderline predicted *C. difficile*

colonization independent of tested covariates. This suggests potential long-term consequences of interventions during birth; however, strong evidence remains to be elucidated in humans.

Animal models have suggested inhaled anesthetic agents, such as isoflurane during the neonatal period can disrupt the gut microbiota of juvenile male rats (148). Alterations were seen at the phylum level with increased Proteobacteria and Firmicutes in rats who were exposed to isoflurane compared to controls in which Bacteroidetes was more abundant. At the family level *Lachnospiraeae, Bacillaeae* and *Burkholderiaceae* were enriched in exposed rats (148). Similarly, morphine can significantly suppress intestinal mucus and in turn disrupt the epithelium through shifts in *Pseudomonas aeruginosa*, a common intestinal pathogen (149). Epidemiological findings suggest anesthetic agents may render host immunity susceptible to *Pseudomonas aeruginosa* and *C. difficile* infection which may have underlying commonality in neurodevelopmental disorders (150). These effects require further large scale follow up during the neonatal period in human studies to fully understand this relationship.

4.1.6 Mode of delivery effects did not extend to 1 year of age

Overall, fewer birth mode associated effects were seen at 1 year of age; nevertheless, infants born vaginally with IAP did show a slight but insignificant increased risk for *C. difficile* colonization. This suggests a 'cesarean effect' may not play a strong role long term with *C. difficile*, and could be due to dosage of maternal IAP given during labour. It was shown in this study, mothers undergoing vaginal delivery with IAP were somewhat just as susceptible to medical interventions in labour than mothers undergoing emergency CS. Oxytocin and anesthetics could be a proxy for longer duration of labour and multiple courses of antibiotics. Indeed, the Society of Obstetrics and Gynecology (SOGC) mandates women to receive

antibiotics if their membranes have been ruptured for more than 18 hours to reduce early onset neonatal sepsis (151).

It is not uncommon for mothers undergoing vaginal deliveries in hospitals to endure several interventions before and during delivery. Recent evidence suggests maternal vaginal and fecal microbiota differ by place of delivery- hospital *versus* home with most profound differences found at birth (137). Maternal vaginal microbiota alpha diversity was significantly altered in hospital deliveries and remained so throughout the first month of life. Similarly, these findings extended to alterations in the infant gut microbiota in hospital deliveries (137). Hospital interventions such as vaginal exams and washes, instrumentation, separation of mother and baby, delayed breastfeeding and baby baths (137) are common and could likely disturb the assembly of pioneer bacteria and could be one explanation for this finding at 1 year of age.

Additionally, *C. difficile* colonization may be a sign of reduced colonization resistance. Colonization resistance is defined as the suppression of facultative bacteria and potentially pathogenic bacteria (*C. difficile*) with increased complexity of strict anaerobes (145). By one year of age the infant gut microbiota becomes more complex and mature. This often outcompetes *C. difficile,* especially with the introduction of foods, cessation of breastfeeding and postnatal microbial exposures. In this study, more infants were colonized at one year age compared to 3 months of age. Possible explanations being delayed gut maturity. It has been hypothesized that infants were more commonly colonized in early life in the 1980's whereas recently *C. difficile* has been persisting into later life. Possibly due to a lower strict/facultative anaerobe ratio (40), which may be a biomarker for modernized sanitary living. Other groups have suggested high colonization rates around 7-9 months coincide with the weaning period (136). As exclusive breastfeeding is the strongest excluder of *C. difficile* colonization, this transition period may

render the infant gut microbiota more susceptible to invaders. This period also may be a time that infants naturally acquire *C. difficile* and could be a part of a normal gut microbial composition at this stage.

4.1.7 Independent predictors of C. difficile colonization at 1 year of age

Several factors were predicting *C. difficile* at 1 year of age, independent of mode of delivery, medical interventions and other predictor variables. Independent predictors were female gender and later introduction of foods (>3 months) as risk factors for increased odds of *C. difficile* colonization. Females had significantly higher *C. difficile* colonization rates compared to males. Although, after stratification by gender to compare between mode of delivery, males were profoundly influenced by mode of delivery whereas this relationship was not found in female infants. Pets and siblings were associated with decreased odds of *C. difficile* colonization at 1 year of age.

Gender has rarely been studied regarding the infant gut microbiota and subsequently *C. difficile* colonization. Reviews have suggested infant gender plays little role on the colonization of *C. difficile* during the first year of life (61). However, Martin et al., reported at birth boys harbour higher bacterial counts in their gut microbiota. At birth and during the first few days of life, infant girls were more commonly colonized by *Lactobacillus ruminis*, *Lactobacillu gasseri and Lactobacillus reuteri* (133). Caucasian male infants at 3 months of age have shown to be depleted of *Bacteroides* compared to female infants (125). How these changes extend to 1 year of age are unknown. Considering male infants may be more likely to be born by CS delivery due to higher birthweight, this could be explaining these differences between mode of delivery at 1

year of age in males and *C. difficile* colonization. Although, the reasoning behind higher colonization rates overall in females remains to be undetermined.

Zoonotic transmission from animals to humans in unclear, *C. difficile* colonization has been found to be higher in infants with dogs in the home (132). *Peptostreptococcaceae*, in which *C. difficile* belongs, appears to be elevated in infants whose homes have a pet (133). Prenatal pet ownership has shown to have favourable outcomes on the infant gut microbiota, with reductions in allergic disease (152). In this study, stronger differences were found between mode of delivery in homes that had pets, with higher *C. difficile* colonization in infants born vaginally with IAP and by emergency CS. Whereas differences between mode of delivery were not found in homes with a pet. Thus, exposure to pets may subject infants to an abundant array of microbes during early life that may be protective against *C. difficile* at 1 year of age.

The introduction of food renders the infant gut microbiota more diverse and complex, allowing resemblance to an adult gut microbiota which has been show to exclude *C. difficile* (63). On one hand, early introduction of foods could be a sign of cessation of breastfeeding allowing an increased risk of *C. difficile.* On the other hand, earlier introduction of food could increase the diversity in which excluding the colonization of *C. difficile.* This study demonstrated early introduction of foods as a protective factor for *C. difficile*. A small number of infants received foods before 3 month; therefore, this finding remains inconclusive.

4.2 Strengths of this study

This study was the first to emphasize and disentangle differences between infants born by emergency CS and elective CS and the colonization of *C. difficile* which has not been previously reported on. This study also had the advantage of studying the effects of intrapartum maternal

antibiotic prophylaxis, which are becoming increasingly more common during delivery. Although *C. difficile* has been reported on in relation to different birth modes, these studies were limited to single sample points and smaller sample size in which they were not able to comment on various modes of delivery. Oxytocin and anesthetics, which is inherently linked to rising CS rates, have never been reported on in relation to the infant gut microbiota. This study did not find these interventions to strongly influence the colonization of *C. difficile*; nevertheless, they may influence whether infants undergo emergency CS. They may also be a risk factor for exclusive breastfeeding difficulties and subsequently an indirect factor influencing colonization; however, this indirect factor was not the focus of this study. With a large sample size performed at multiple time points, multiple adjustments were possible which allowed deeper insights into variables that were confounding and effect modifiers.

Temporality is a strength, as birth and medical intervention exposure variables occurred first followed by stool sample collection at a mean of 3.6 months of age and 12.43 months of age. Although stool sample collection had a wide range (age in months: 1.3-9.8, and 9.32-24.21, respectively), sensitivity analyses was performed to analyze whether stool samples after 6 months or after 16 months altered the results of the study; in which we concluded it did not. *C. difficile* is most commonly acquired in early life, where it has been shown that after the introduction of foods at 6 months, colonization rates diminish; however, in our adjusted analysis, older age at stool sample collection was a risk factor for the colonization of *C. difficile* independent of mode of delivery and tested covariates. In which it may suggest that environmental acquisition in later infancy is a more important source of *C. difficile* in this cohort of healthy infants.

Longitudinal measures were not analyzed in the 3 month and 1 year sample analysis due to the crossectional nature in which it was performed, this ensured maximization of the sample size to accurately report on colonization rates at this time point. Persistent colonization was performed to investigate longitudinal analysis, in which the same infants were followed from birth to 1 year with stool sample collection at 3 months and 1 year. Little research has been done on the longitudinal nature of *C. difficile* large cohorts in relation to birth, especially factors affecting persistent colonization in healthy infants.

Although one species was analyzed in this study, it is commonly noted as a biomarker for an aberrant and dysbiotic gut in infants, in which beneficial bacteria have been reduced in its presence. Allergic disease in later life has also been associated with *C. difficile* colonization in infancy; therefore, species specific effects provide valuable insight into microbiome research. With the rise in pediatric CDI and populations previously at low risk becoming more at risk for infection, it is essential to understand early life factors that may predispose infants to early colonization to potentially mitigate later infection.

4.3 Limitations of this study

This study also presents some limitations. We cannot report on the transient nature or stable colonization, as it is limited to two time points, and multiple strains could have been acquired. Moreover, non-toxigenic strains and toxigenic strains could not be deciphered due to the nature of PCR methods. With evidence of infants born by emergency CS to be at a higher risk of *C. difficile* colonization, meconium samples would have been indicative of a baseline aberrant gut profile which would have been useful for microbial succession leading to 3 months and is a limitation of this study.

Dosage of oxytocin and anesthetics have previously been reported on, and potentially could have missed possible relationship between dose, indication and at which stage of labour these drugs were given and *C. difficile* colonization. Third stage of labour, when the placenta is delivered has been noted as a possible susceptible time of influence on the neonatal gut due to accumulation of oxytocin in breastmilk. Similarly, IAP dosage was not reported on, and it is not uncommon for multiple doses to be given to women delivering vaginally, and through emergency CS delivery.

As the area of homebirths is accumulating evidence, it is hypothesized that greater effects would have been noted if this reference was used. Lastly, we cannot report on asymptomatic nature of infants due to the lack of diarrhea information for infants and maternal microbiota samples were not available for mothers. *C. difficile* may be transmitted from mother to infants; however, asymptomatic rates in pregnant women is low and transmission is likely to be low based on current evidence but this is a limitation.

4.4 Bias and Confounding

4.4.1 Selection Bias

Selection bias occurs when the sample obtained is not representative of the population. This is minimized in the CHILD cohort as multiple sample sites were used to obtain participants, additionally participants were recruited through multiple different means such as clinics, booths at tradeshows, fax and by phone calls. Participants in the CHILD study were mainly from urban residencies, were mostly educated, had higher allergy rates than the general population and lacked large information about aboriginal communities which may limit the generalizability of

this study to its entirety. Of note, the primary outcome for the CHILD study was asthma; although this was not advertised and unlikely to affect recruitment.

The CHILD cohort has low rates of loss to follow up, with 92% retention rate to date. Missing data for some infants may cause attrition bias leading to poor adjustment for some variables; however, comparisons were made to the overall cohort and differences were not found (Table 1) and missing variables data was low in this study. Due to the watery consistency of breastfed babies, stool from these infants were difficult to collect, which may skew our results towards formula fed infants which are known to be associated with altered gut profiles and higher C. *difficile* colonization rates and counts and is a source of selection bias. However, this is an unlikely issue due to the large sample size in this study as well adjustment was made for breastfeeding status.

4.4.2 Measurement Bias

Measurement bias occurs as a result of systematic error in the measurement or classification of an outcome or exposure. Due to the nature of an observational cohort study, this is minimized, as the lab technician will be blinded to the results of mode of delivery and medical interventions such oxytocin and anesthetics, reducing the chances of measurement bias. Errors in measuring exposure variables is limited due to the retrieval of birth methods, medical interventions and maternal IAP from hospital charts. However, bias may occur when hospital charts are used due to the nature of the medical system which may reduce validity and reliability as exposures could be missed or misclassified. Medical interventions such as oxytocin and anesthetics could be subject to misclassification bias, but it likely determined to be non-differential misclassification in which study participants are equally likely to be exposed to this

bias. In this study, missing rates (n=3) were low for oxytocin or anesthetics. Additionally, home births were excluded for this reason as well, as accurate reporting of birthing events may have been low.

Measurement bias may arise by using questionnaires, which may produce inaccurate results due to miscommunication between the interviewer and participant. Language barriers were addressed in the inclusion/exclusion criteria where participants unable to read or write in English were excluded. Ascertainment of exposure is deemed to be low in exposures chosen for this study, but variables such as maternal smoking status, breastfeeding status (i.e formula use), certain socioeconomic status indicators and maternal age may be under-reported or misreported, especially in interviewer based questionnaires due to social pressures which could lead to non-response or inaccuracy. Questionnaire bias was reduced in this cohort and efforts to reduce recall bias were made by giving validated questionnaires to all participants in the same fashion at several time points in order to minimize this potential bias. Questionnaires were collected at multiple time points to observe changes in response.

Finally, inherent bias from real time qPCR methods may be present but this is likely reduced by removing false positives which were positive but quantifiable amounts were not attainable due to levels being below a level of detection. Additionally, *C. difficile* genomes that exceeded total bacteria were marked as errors (inhibitory effects). Although PCR is efficient and very sensitive it may not be able to detect species below a certain threshold; however, this did not seem to be the case in this study. Specific primers and probes were used to quantify *C. difficile* were 16S region and one lab technician ran the qPCR in the CHILD cohort, reducing measurement bias. Pre-amplification steps are important with *C. difficile* PCR based methods due to *C. difficile* spores which carry genomic DNA; however, cannot be accessed unless broken

with culture methods. Culture methods were not used in this study, therefore some infants who harboured spores may have been deemed not colonized; however, this is likely to be non-differential misclassification and will not result in measurement bias.

4.4.3 Confounding bias

As with all observational studies, confounding bias presents if there is an additional variable explaining the result between the exposure and outcome which can only be examined through data analysis. An established priori hypothesis will be effective to examine the relationship between *C. difficile*, mode of delivery and medical interventions. Extensive collection of perinatal covariates occurred in the CHILD study. Given this is a secondary study, some covariates may not be available which could lead to potential problems with confounding bias, such as maternal diet, maternal microbiome (stool and vaginal), maternal and infant probiotic use, maternal infection status and maternal antibiotics. Please see covariates collected and unattainable or not collected in Appendix A, Table A1. Although based on current literature, covariates seem to be adequately collected for in this study. CS delivery could be associated with maternal infections; therefore *C. difficile* may be a result of these factors in infants which we cannot rule out as an explanation. Additionally, infants undergoing medical interventions and CS delivery are more susceptible to hospital stays, antibiotic use, formula supplementation and antibiotics use; however, this was mitigated by adjusting for hospital length of stay, antibiotic use and breastfeeding status. Any factors that were related to the exposure and outcome of interest were adjusted for using multiple logistic regression in the analysis, similarly, fisher exact tests were performed to identify any variables that were significantly different in distribution between the exposures of interest (Table 4,6,9). Furthermore, stratified analysis was performed

for mode of delivery and medical interventions to further control for confounding effects, collinearity and to understand effect modification (interaction).

Within infants born by emergency CS, almost 10% of women did not go into labour, which was deemed as an emergency CS; although, indication was not included in this study. This may bias the results as there could have been complications with the mother which could predispose the infant to further possible infections and subsequent in utero gut dysbiosis. This may lead to increased risk of *C. difficile* colonization. We cannot exclude confounding by indication for the reasons why women underwent CS delivery, received IAP during delivery or anesthetics or oxytocin. As we found a lesser risk in elective CS compared to emergency CS in this study, and with the absence of labour likely to inhibit the passage of microbes with on the onset of labour, a sensitivity analyses was performed to exclude infants born by emergency CS without labour and no differences were found in the outcome (Appendix D- Table D8).

Differing birth practices along with obstetric guidelines during recruitment period could have changed over time; however, this is unlikely during such a short recruitment period from 2009-2012. However, site specific effects may occur with different practices more common at different hospitals between Vancouver, Edmonton and Winnipeg. As this study site effect was found in this study, differing practices could have been evident such as the use of water tubs at the women's hospital in Vancouver which mimics home births as *C. difficile* colonization was less likely at the Vancouver site, this practice could be playing a role, or maternal race, such as maternal Asian race which has been associated with microbiota differences, could be explaining this as well.

4.5 Clinical Importance

Any disturbance to the natural physiological process of birth has the opportunity to negatively influence infant health outcomes. Women opt for CS delivery for multiple reasons including cultural, societal pressures, psychology fear and trauma which undoubtedly necessitates options of CS delivery. Health education to promote the natural physiological process of birth should be encouraged, while supportive labour partners and medical staff should be trained in natural birth. Minimization of fear and stress while increasing a safe environment for women should be top priority, while incorporating trained midwifes into medical practice.

Medical interventions which have been widely accepted and perceived as superior have been associated with short and long-term health consequences. As we have come full circle to understanding the integral role of maternally acquired pioneer bacteria through vaginal, fecal oral transmission and breastmilk, it is imperative now more than ever to preserve inherited indigenous maternal bacteria. With fecal transplants ironically superior than antibiotics in the treatment of CDI, the sterility of the modern day birth places have become a considerable area of research. Fecal-oral transmission is often prevented and limited due to the sterile nature of interventions in hospital settings during birth.

With *C. difficile* commonly colonizing in the absence of beneficial bacteria, modern birthing practices have been scrutinized. Considering rises in CS rates worldwide, anyway to mitigate these rates have become a priority as they have far surpassed recommended rates. With no evidence to suggest benefits to maternal and infant outcomes without medical indication. Findings from this study suggests infants born by emergency CS, although independent of oxytocin and anesthetics, allows the colonization of potentially pathogenic bacteria (*C. difficile)*. Moreover, emergency CS effects were particularly evident among first born infants. First born infants are known to undergo more medical interventions and disruptions during labour which

elucidates the need for more supportive labour environments and personnel for first time mothers. Education on the risk of CS delivery, supportive breastfeeding relationships, skin to skin and harmful household exposures that may further disrupt the infant gut microbiota postnatally should be emphasized.

4.6 Implications for future research

Future studies should prioritize ways to reduce CS delivery, specifically primary CS rates which more often lead to emergency CS and subsequently repeat CS deliveries. Any way to preserve inherited maternal microbial transmission is crucial, while establishing more nurturing and supportive hospital environments that encourage breastfeeding, skin to skin and education for mothers.

4.7 Conclusion

CS delivery has undoubtedly altered and extinguished the course of the first microbial inoculation at birth which subsequently poses great risk to the developing infant. Balanced indigenous maternal transmission during birth are integral for immune development and homeostasis. Disruptions to the natural physiological process of birth delays and inhibits the assembly of pioneer bacteria which consequently allows pathogenic bacteria to colonize with little known ramifications or mitigations for these disturbances.

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Appendices

Appendix A

Table A1- Covariates collected/ unattainable	
Potential Confounding Variables	
Covariates (collected)	*Covariates (not collected or unattainable)*
• Birthweight • Infant sex • Gravida • Oxytocin/anesthetics • Hospital length of stay • Maternal Education • Maternal Race • Maternal age • Maternal smoking • Postnatal smoking • Infant antibiotic exposure • Maternal pre-pregnancy BMI • Breastfeeding status • Furry pets and siblings • Introduction of solid foods • Pre-postnatal stress • Pre-postnatal depression • Geography • Age at stool collection	• Maternal microbiome (vaginal microbiome, stool) • Breast milk composition • Maternal diet • probiotics
Variables	Definition/Source
Exposures: Mode of delivery **i) Vaginal, no IAP** **ii) Vaginal, IAP** **iii) Emergency CS, IAP** **iv) Elective CS, IAP**	Source: birth questionnaires **i) Vaginal, NO IAP** - Vaginal unassisted - Vaginal forceps assisted - Vaginal vacuum extracted - Assisted breech - Maternal IAP: NO **ii) Vaginal, IAP** - Vaginal unassisted - Vaginal forceps assisted - Vaginal vacuum extracted - Assisted breech - Maternal IAP: YES

	iii) Emergency CS - Caesarean (during labour) - Emergency Caesarean section (during labour) - Emergency Caesarean section (without labour) - Maternal IAP: YES **iv) Elective CS** - Elective Caesarean section - Maternal IAP: YES

Questionnaire examples

A2) Mode of delivery

12. **Method of Delivery:** CBIRTHCDQ12

[1] Vaginal Unassisted
[2] Vaginal Forceps assisted
[3] Vaginal Vacuum Extracted
[4] Assisted Breech (normal delivery often with forceps)
[5] Breech Extraction (rare/emergency when baby remains in uterus)
[6] Elective Caesarean section
[7] Caesarean section (during labour)

12. [8] Emergency Caesarean section (during labour)
[9] Emergency Caesarean section (without labour)
[10] Not recorded
[11] Other

Specify (AlphaNumeric - Length: 120) CBIRTHCDQ12a

A3) Anesthetics

9. **Did the mother have any anesthetics during delivery?** CBIRTHCDQ9

9. [1] Yes
[0] No
[9] Not recorded

9.1 **If yes, indicate type:**

[1]/[0] Epidural CBIRTHCDQ9_1a
[1]/[0] Spinal CBIRTHCDQ9_1b
[1]/[0] General Anesthetic CBIRTHCDQ9_1c
[1]/[0] Gas (nitrous oxide) CBIRTHCDQ9_1d
[1]/[0] Local Anesthetic CBIRTHCDQ9_1e
[1]/[0] Other CBIRTHCDQ9_1f

Specify (AlphaNumeric - Length: 120) CBIRTHCDQ9_1g

A4) Oxytocin-like drugs

11. **Did the mother have any Oxytocin-like or related drugs which stimulate contractions (e.g., Oxytocin, Duratocin, Prostaglandins)?** CBIRTHCDQ11

[1] Yes
[0] No

11.1 **If yes, indicate reason:**

[1]/[0] For induction CBIRTHCDQ11_1a
[1]/[0] For augmentation of labour CBIRTHCDQ11_1b

Appendix B- INDIVIDUAL AND COMBINED ADJUSTMENTS

Table B1- Individual and combines adjustments with covariates and mode of deliveryat 3 months, 1 year and persistent colonization

	Exposure= birth method, outcome= C.*difficile* =(Y/N)	3 months OR(CI) N=1477	P value (x^2)	1 year OR(CI) N=1836	P value (x^2)	Persistent colonization OR(CI) N=1226	P value (x^2)
CRUDE OR							
Mode of deliverywith IAP							
	Vaginal, No IAP	**(reference)**		**(reference)**		**(reference)**	
	Vaginal, IAP	1.18(.89-1.55)	.239	1.35(1.07-1.70)	.010	1.35(1.07-1.70)	.058
	Emergency CS, IAP	1.76(1.27-2.44)	.001	1.36(1.03-1.78)	.026	1.36(1.03-1.78)	.001
	Elective CS, IAP	1.55(1.06-2.26)	.024	1.18(.86-1.78)	.285	1.18(.86-1.78)	.119
ADJUSTED FOR							
Child gender							
	Vaginal, IAP	1.13 (.239)	.372	1.35(1.07-1.70)	.011	1.45(.98-2.13)	.011
	Emergency CS, IAP	1.65(1.25-2.39)	.003	1.39(1.06-1.83)	.017	2.08(1.36-3.20)	.001
	Elective CS, IAP	1.55(1.05-2.47)	.034	1.18(.86-1.62)	.287	1.53(.89-2.61)	
Medical interventions (oxytocin & anesthetics)							
	Vaginal, IAP	1.13(.85-1.50))	.565	1.27(1.01-1.61)	.041	1.33(.89-1.98)	.157
	Emergency CS, IAP	1.60(1.18-2.24)	.006	1.21(.91-1.62)	.178	1.88(1.20-2.94)	.005
	Elective CS, IAP	1.50(1.03-2.32)	.047	1.12(.80-1.57)	.504	1.50(.85-2.65)	.161
Gestational age							
	Vaginal, IAP	1.22(.91-1.62)	.171	1.31(1.03-1.66)	.025	1.41(.95-2.09)	.080
	Emergency CS, IAP	1.81(1.30-2.53)	.000	1.35(1.02-1.80)	.034	2.04(1.33-3.12)	.001
	Elective CS, IAP	1.51(1.01-2.24)	.042	1.15(.83-1.60)	.295	1.5(.86-2.59)	.145
Breastfeeding 3 mos							
	Vaginal, IAP	1.21(.91-1.61)	.170	1.37(1.09-1.73)	.006	1.50(1.01-2.22)	.041
	Emergency CS, IAP	1.75(1.26-2.44)	.001	1.36(1.04-1.79)	.025	2.08(1.35-3.19)	.001
	Elective CS, IAP	1.40(.95-2.07)	.085	1.16(.85-1.60)	.338	1.37(.80-2.37)	.247
Breastfeeding 6 mos							
				1.37 (1.09-1.73)	.007	1.53(.103-2.27)	.031
				1.36 (1.03-1.79)	.027	2.05(1.33-3.16)	.001
				1.23 (.89-1.699)	.193	1.51(.88-2.60)	.133
Introduction to foods							
	Vaginal, IAP	1.18(.89-1.56)	.231	1.35(1.07-1.70)	.010	1.4(.98-2.13)	.058
	Emergency CS, IAP	1.76(1.27-2.44)	.001	1.37(1.04-1.80)	.023	2.07(1.35-3.17)	.001

	Elective CS, IAP	1.54(1.05-2.26)	.025	1.18(.86-1.62)	.293	1.53(.89-2.6)	.119
Pets in the home (Y/N)							
	Vaginal, IAP	1.13(.85-1.50)	.374	1.37(1.08	.008	1.36(.92-2.02)	.116
	Emergency CS, IAP	1.70(1.22-2.35)	.001	1.35(1.02-1.79)	.032	1.96(1.28-3.01)	.002
	Elective CS, IAP	1.43(.97-2.11)	.067	1.25(.912-1.73)	.161	1.52(.89-2.60)	.123
Siblings							
	Vaginal, IAP	1.15(.87-1.5)	.322	1.26(1.0-1.60)	.046	1.30(.88-1.92)	.186
	Emergency CS, IAP	1.65(1.18-2.30)	.003	1.17(.89-1.56)	.249	1.67(1.08-2.59)	.020
	Elective CS, IAP	1.62(1.10-2.37)	.013	1.29(.94-1.78)	.108	1.82(1.0-3.15)	.031
Ethnicity (white=reference, Asian, other)							
	Vaginal, IAP	1.22(.92-1.61)	.160	1.37(1.08-1.72)	.008	1.49(1.01-2.21)	.041
	Emergency CS, IAP	1.85(1.33-2.56)	.0001	1.32(1.00-1.74)	.045	2.13(1.39-3.27)	.0001
	Elective CS, IAP	1.61(1.09-2.38)	.015	1.17(.85-1.61)	.323	1.58(.92-2.71)	.092
Geography (Vancouver= reference, Edmonton, Winnipeg)							
	Vaginal, IAP	1.25(.94-1.65)	.120	1.41(1.06-1.87)	.017	1.54(1.05-2.28)	.028
	Emergency CS, IAP	1.91(1.37-2.66)	.0001	1.31(.93-1.84)	.112	2.18(1.42-3.35)	.0001
	Elective CS, IAP	1.61(1.09-2.38)	.015	1.36(.91-2.04)	.130	1.58(.92-2.72)	.093
Maternal prenatal smoking(Y/N)							
	Vaginal, IAP	1.22(.92-1.62)	.157	1.32(1.05-1.67)	.017	1.52(1.02-2.25)	.036
	Emergency CS, IAP	1.82(1.31-2.52)	.0001	1.37(1.04-1.81)	.023	2.16(1.40-3.33)	.0001
	Elective CS, IAP	1.50(1.02-2.22)	.038	1.19(.86-1.64)	.280	1.63(.95-2.81)	.073
Postnatal smoke exposure(Y/N)							
	Vaginal, IAP	1.16(.87-1.53)	.297	1.37(1.08-1.73)	.009	1.40(.95-2.07)	.084
	Emergency CS, IAP	1.71(1.23-2.38)	.001	1.34(1.01-1.77)	.037	1.98(1.29-3.30)	.002
	Elective CS, IAP	1.40(.95-2.07)	.085	1.25(.90-1.72)	.168	1.52(.89-2.61)	.122
Maternal pre-pregnancy BMI(underweight=reference, normal weight, overweight, obese)							
	Vaginal, IAP	1.14(.85-1.52)	.363	1.36(1.08-1.72)	.009	1.42(.96-2.12)	.077
	Emergency CS, IAP	1.72(1.23-2.41)	.001	1.36(1.03-1.79)	.028	1.96(1.27-3.04)	.002
	Elective CS, IAP	1.54(1.0-2.28)	.031	1.19(.274)	.274	1.51(.88-2.59)	.134

Mothers age							
	Vaginal, IAP	1.21(.91-1.60)	.171	1.35(1.07-1.70)	.010	1.48(1.01-2.19)	.044
	Emergency CS, IAP	1,97(1.41-2.74)	<.0001	1.34(1.02-1.76)	.034	2.20(1.43-3.38)	<.0001
	Elective CS, IAP	1.87(1.26-.277)	.002	1.15(.84-1.58)	.374	1.67(.97-2.88)	.063
Hospital length of stay (categorical)							
	Vaginal, IAP	1.13(.85-1.51)	.380	1.33(1.05-1.67)	.016	1.34(.91-1.99)	.133
	Emergency CS, IAP	1.51(1.06-2.15)	.022	1.24(.93-1.66)	.143	1.59(1.01-2.48)	.041
	Elective CS, IAP	1.31(.87-1.9)	.184	1.11(.80-1.42)	.520	1.21(.70-2.09)	.487
Prenatal depression							
	Vaginal, IAP	1.20(.90-1.58)	.199	1.35(1.07-1.70)	.011	1.51(1.02-2.24)	.037
	Emergency CS, IAP	1.77(1.28-2.46)	.001	1.39(1.05-1.83)	.018	2.12(1.37-3.2)	.001
	Elective CS, IAP	1.51(1.03-2.46)	.034	1.23(.89-1.69)	.195	1.62(.94-2.78)	.079
Postnatal depression							
	Vaginal, IAP	1.22(.90-1.65)	.197	1.29(1.0-1.66)	.042	1.35(.88-2.06)	.161
	Emergency CS, IAP	2.05(1.45-2.89)	.0001	1.35(1.01-1.80)	.040	2.12(1.35-3.31)	.001
	Elective CS, IAP	1.58(1.02-2.43)	.037	1.27(.90-1.79)	.171	1.67(.934-3.0)	.083
Child medication							
	Vaginal, IAP	1.18(.89-1.55)	.237	1.35(1.07-1.70)	.010		
	Emergency CS, IAP	1.76(1.27-2.44)	.001	1.36(1.03-1.78)	.026		
	Elective CS, IAP	1.55(1.06-2.27)	.023	1.18(.86-1.62)	.285		
Age at stool collection 3 mo							
	Vaginal, IAP	1.20(.91-1.59)	.192				
	Emergency CS, IAP	1.79(1.29-2.48)	<.0001				
	Elective CS, IAP	1.51(1.02-2.22)	.036				
Age at stool collection 1 yesr							
	Vaginal, IAP			1.43(1.13-1.83)	.003		
	Emergency CS, IAP			1.35(1.02-1.79)	.035		
	Elective CS, IAP			1.42(1.02-1.98)	.037		
Combined Adjusted OR							
3-MONTH MODEL							
Adjusted for Hospital length of stay + breastfeeding 3 mo							
	Vaginal, IAP	1.19(.89-1.60)	.222				
	Emergency CS, IAP	1.57(1.09-2.25)	.015				

	Elective CS, IAP	1.24(.82-1.87)	.289				
Adjusted for Hospital length of stay + breastfeeding + siblings							
	Vaginal, IAP	11.19(.89-1.60)	.229				
	Emergency CS, IAP	1.54(1.06-2.23)	.020				
	Elective CS, IAP	1.27(.84-1.93)	.250				
Adjusted for Hospital length of stay + breastfeeding + siblings + geography							
	Vaginal, IAP	1.28(.95-1.72)	.102				
	Emergency CS, IAP	1.81(1.24-2.65)	.002				
	Elective CS, IAP	1.50(.98-2.65)	.060				
Adjusted for Hospital length of stay + breastfeeding + siblings + geography + age at sample collection							
	Vaginal, IAP	1.27(.94-1.72)	.114				
	Emergency CS, IAP	1.77(1.20-2.600	.003				
	Elective CS, IAP	1.52(.98-2.34)	.059				
Adjusted for Hospital length of stay + breastfeeding + siblings + geography + age at sample collection + pets							
	Vaginal, IAP	1.23(.91-1.67)	.175				
	Emergency CS, IAP	1.73(1.17-2.55)	.006				
	Elective CS, IAP	1.39(.89-2.17)	.147				
1 YEAR MODEL							
Adjusted for hospital length of stay + breastfeeding 6 mo							
	Vaginal, IAP			1.34(1.06-1.70)	.013		
	Emergency CS, IAP			1.24(.92-1.66)	.149		
	Elective CS, IAP			1.15(.83-1.60)	.392		

Adjusted for hospital length of stay + breastfeeding 6 mo + siblings							
	Vaginal, IAP			1.27(1.0-1.62)	.042		
	Emergency CS, IAP			1.11(.83-1.50)	.459		
	Elective CS, IAP			1.29(.92-1.81)	.129		
Adjusted for hospital length of stay + breastfeeding 6 mo + siblings + postnatal depression							
	Vaginal, IAP			1.20(.93-1.55)	.149		
	Emergency CS, IAP			1.10(.80-1.51)	.534		
	Elective CS, IAP			1.31(.91-1.68)	.139		
PERSISTENCE MODEL							
Adjusted for hospital length of stay + breastfeeding 6 mo							
	Vaginal, IAP					1.41(.95-2.10)	.085
	Emergency CS, IAP					1.60(1.02-2.53)	.041
	Elective CS, IAP					1.22(.70-2.13)	.474
Adjusted for hospital length of stay + siblings							
	Vaginal, IAP					1.24(.83-1.84)	.280
	Emergency CS, IAP					1.38(.87-2.17)	.162
	Elective CS, IAP					1.48(.84-2.60)	.169

APPENDIX C- STRATIFICATIONS

Table C1. Unadjusted stratification by covariates with mode of delivery as the exposure and *C. Difficile* colonization at 3 months and 1 year as the outcome

		3-month colonization (Y/N) N= 1477 OR(CI)	**P value**		**1 year colonization (Y/N) N= 1836 OR(CI)**	**P value**
Crude birth method	**N=1477**			**N=1836**		
	Vaginal, No IAP	(reference)		Vaginal, No IAP	(reference)	
	Vaginal, IAP	1.18(.89-1.55)	.239	Vaginal, IAP	1.35(1.07-1.70))	.010
	Emergency CS, IAP	1.76(1.27-2.44)	.001	Emergency CS, IAP	1.36(1.03-1.78)	.026
	Elective CS, IAP	1.55(1.06-2.26)	.024	Elective CS, IAP	1.18(.86-1.78))	.285
UNADJUSTED STRATIFIED						
Breastfeeding (3 mo)				**Breastfeeding (6 mo)**		
Exclusive	**N= 799**			**N=328**		
	Vaginal, No IAP	-		Vaginal, No IAP	-	
	Vaginal, IAP	1.33(.90-1.97)	.148	Vaginal, IAP	1.19(.71-2.00)	.497
	Emergency CS, IAP	1.12(.67-1.87)	.655	Emergency CS, IAP	1.41(.74-2.7)	.291
	Elective CS, IAP	1.67(.90-3.1)	.100	Elective CS, IAP	1.39(.63-1.08)	.408
Partial	**N=414**			**N=1075**		
	Vaginal, No IAP	-		Vaginal, No IAP	-	
	Vaginal, IAP	1.43(.85-2.41)	.175	Vaginal, IAP	1.47(1.08-1.98)	.013
	Emergency CS, IAP	2.96(1.62-5.1)	.001*	Emergency CS, IAP	1.23(.85-1.76)	.257
	Elective CS, IAP	1.56(.84-2.92)	.157	Elective	1.35(.88-2.05)	.162
Formula Fed	**N=261**			**N=408**		
	Vaginal, No IAP			Vaginal, No IAP	-	
	Vaginal, IAP	.748(.396-1.41)	.370	Vaginal, IAP	1.35(.81-2.23)	.260
	Emergency CS, IAP	2.26(1.05-4.86)	.036	Emergency CS, IAP	1.69(.95-2.95)	.068

	Elective CS, IAP	.943(.418-2.12)	.888	Elective CS, IAP	.96(.51-1.81)	.911
Gravida						
Primigravida	**N=543**			**N=680**		
	Vaginal, No IAP	-		Vaginal, No IAP		
	Vaginal, IAP	1.19(.77-1.83)	.432	Vaginal, IAP	1.20(.84-1.72)	.311
	Emergency CS, IAP	2.26(1.40-3.6)	.001	Emergency CS, IAP	1.43(.95-2.16)	.084
	Elective CS, IAP	1.31(.85-2.03)	.006*	Elective CS, IAP	1.62(.79-3.30)	.181
Multigravida	**N=938**			**N=1159**		
	Vaginal, No IAP			Vaginal, No IAP		
	Vaginal, IAP	1.16(.81-1.67)	.394	Vaginal, IAP	1.41(1.04-1.91)	.024
	Emergency CS, IAP	1.35(.86-2.14)	.188	Emergency CS, IAP	1.19(.82-1.73)	.352
	Elective CS, IAP	1.31(.85-2.03)	.215	Elective CS, IAP	1.17(.81-1.67)	.388
Gender						
Male	**N=788**			N=903		
	Vaginal, No IAP			Vaginal, No IAP		
	Vaginal, IAP	1.01(.69-1.49)	.938	Vaginal, IAP	1.36(.98-1.88)	.062
	Emergency CS, IAP	1.48(.98-2.24)	.058	Emergency CS, IAP	1.56(1.08-2.25)	.016
	Elective CS, IAP	1.34(.80-2.2)	.256	Elective CS, IAP	1.54(.052)	.052
Female	**N=693**					
	Vaginal, No IAP	-		Vaginal, No IAP		
	Vaginal, IAP	1.39(.93-2.08)	.101	Vaginal, IAP	1.34(.96-1.86)	.080
	Emergency CS, IAP	2.16(1.27-3.6)	.004	Emergency CS, IAP	1.21(.80-1.83)	.357
	Elective CS, IAP	1.82(1.02-3.23)	.041	Elective CS, IAP	.90(.575-1.13)	.653
Postnatal Smoke exposure						
Yes	**N=225**			**N=226**		
	Vaginal, No IAP	-		Vaginal, No IAP	-	
	Vaginal, IAP	1.99(1.02-3.87)	.041	Vaginal, IAP	1.16(.63-2.14)	.628
	Emergency CS, IAP	1.92(.86-4.25)	.106	Emergency CS, IAP	1.94(.95-3.95)	.065
	Elective CS, IAP	1.92(.813-4.54)	.136	Elective CS, IAP	1.01(.43-2.36)	.979

No	**N=1233**			**N=1496**		
	Vaginal, No IAP	-		Vaginal, No IAP		
	Vaginal, IAP	1.03(.755-1.40)	.849	Vaginal, IAP	1.41(1.09-1.82)	.008
	Emergency CS, IAP	1.68(1.17-2.40)	.004	Emergency CS, IAP	1.25(.92-1.69)	.147
	Elective CS, IAP	1.31(.84-2.03)	.226	Elective CS, IAP	1.29(.918-1.83)	.139
Siblings						
Yes	**N=731**			**N=895**		
	Vaginal, No IAP	-		Vaginal, No IAP	-	
	Vaginal, IAP	1.14(.75-1.74)	.529	Vaginal, IAP	1.16(.406)	.406
	Emergency CS, IAP	1.06(.54-1.09)	.851	Emergency CS, IAP	1.00(.588-1.73)	.971
	Elective CS, IAP	1.41(.89-2.23)	.139	Elective CS, IAP	1.30(.88-1.90)	.175
No	**N=749**			**N=942**		
	Vaginal, No IAP	-		Vaginal, No IAP	-	
	Vaginal, IAP	1.18(.81-1.72)	.371	Vaginal, IAP	1.35(.99-1.85)	.053
	Emergency CS, IAP	1.94(1.31-2.88)	.001	Emergency CS, IAP	1.26(.90-1.76)	.171
	Elective CS, IAP	2.17(1.06-4.41)	.032	Elective CS, IAP	1.24(.68-2.2)	.472
Pets						
Yes	**N=663**			**N=798**		
	Vaginal, No IAP	-		Vaginal, No IAP	-	
	Vaginal, IAP	1.21(.81-1.80)	.341	Vaginal, IAP	1.34(.94-1.9)	.099
	Emergency CS, IAP	2.34(1.47-3.72)	<.001*	Emergency CS, IAP	1.16(.77-1.75)	.454
	Elective CS, IAP	1.75(1.0-3.0)	.049	Elective CS, IAP	1.01(.62-1.66)	.947
No	**N=792**			**N=957**		
	Vaginal, No IAP	-		Vaginal, No IAP	-	
	Vaginal, IAP	1.07(.72-1.59)	.716	Vaginal, IAP	1.40(1.01-1.93)	.039
	Emergency CS, IAP	1.23(.76-1.97)	.388	Emergency CS, IAP	1.54(1.05-2.2)	.026
	Elective CS, IAP	1.19(.69-2.06)	.512	Elective CS, IAP	1.48(.96-2.27)	.072
HLOS						
1 day or less	**N=366**			**N=460**		
	Vaginal, No IAP	ref		Vaginal, No IAP	-	
	Vaginal, IAP	1.09(.63-1.91-1.9)	.742	Vaginal, IAP	1.32(1.03-1.812)	.222
	Emergency CS, IAP	-		Emergency CS, IAP	-	

	Elective CS, IAP	-		Elective CS, IAP	4.38(.45-42.5)	.202
2-3 days	**N=875**			N=1083		
	Vaginal, No IAP	ref		Vaginal, No IAP	-	
	Vaginal, IAP	1.31(.96-2.07)	.126	Vaginal, IAP	1.30(.84-1.06)	.080
	Emergency CS, IAP	1.46 (.97-2.21)	.067	Emergency CS, IAP	1.17(.82-1.67)	.382
	Elective CS, IAP	1.25(.78-1.99)	.337	Elective CS, IAP	1.07(.73-1.56)	.706
4 days or more	**N=159**					
	Vaginal, No IAP	-		Vaginal, No IAP	-	
	Vaginal, IAP	.39(.12-1.20)	.102	Vaginal, IAP	1.50(.75-3.0)	.244
	Emergency CS, IAP	1.45(.62-3.36)	.386	Emergency CS, IAP	1.45(.83-2.52)	.184
	Elective CS, IAP	1.5(.57-3.89)	.405	Elective CS, IAP	1.12(.55-2.27)	.747
Medical interventions:						
No oxytocin/anesthetics	**N=234**			**N=279**		
	Vaginal, No IAP	ref		Vaginal, No IAP	-	
	Vaginal, IAP	1.75(.88-3.4)	.105	Vaginal, IAP	.87(.46-1.64)	.683
	Emergency CS, IAP	-		Emergency CS, IAP	-	
	Elective CS, IAP	-		Elective CS, IAP	-	
Oxytocin only	**N=98**			**N=119**		
	Vaginal, No IAP	-		Vaginal, No IAP	-	
	Vaginal, IAP	3.1(1.10-8.70)	.032	Vaginal, IAP	1.36(.57-3.25)	.483
	Emergency CS, IAP	-		Emergency CS, IAP	-	
	Elective CS, IAP	-		Elective CS, IAP	-	
Anesthetics only	**N=546**			**N=688**		
	Vaginal, No IAP	-		Vaginal, No IAP	-	
	Vaginal, IAP	.93(.61-2.00)	.783	Vaginal, IAP	1.49(.98-2.28)	.063
	Emergency CS, IAP	1.10(.61-2.00)	.741	Emergency CS, IAP	1.30(.79-2.14)	.292
	Elective CS, IAP	1.42(.90-2.24)	.127	Elective CS, IAP	1.11(.76-1.62)	.565
Both oxytocin and anesthetics	**N= 599**			**N= 599**		
	Vaginal, No IAP	-		Vaginal, No IAP	-	
	Vaginal, IAP	.96(.64-1.46)	.879	Vaginal, IAP	1.28(.912-1.81)	.149
	Emergency CS, IAP	1.85(1.21-2.83)	.004	Emergency CS, IAP	1.23(.85-1.78)	.251
	Elective CS, IAP	1.29(.45-3.68)	.623	Elective CS, IAP	1.41(.545-3.6)	.475
Maternal race						

White race	**N=1104**			**N=1391**		
	Vaginal, No IAP	-		Vaginal, No IAP	-	
	Vaginal, IAP	1.10(.80-1.51)	.554	Vaginal, IAP	1.44(1.11-1.88)	.006
	Emergency CS, IAP	2.01(1.38-2.93)	.0001	Emergency CS, IAP	1.40(1.01-1.94)	.041
	Elective CS, IAP	1.82(1.17-2.81)	.007	Elective CS, IAP	1.16(.81-1.67)	.405
Asian	N=193			N=235		
	Vaginal, No IAP	-		Vaginal, No IAP	-	
	Vaginal, IAP	1.95(.80-4.74)	.139	Vaginal, IAP	.77(.39-1.51)	.464
	Emergency CS, IAP	1.12(.37-3.42)	.834	Emergency CS, IAP	1.07(.53-2.17)	.836
	Elective CS, IAP	1.72(.54-5.42)	.353	Elective CS, IAP	1.73(.70-4.27)	.234
Other race	N=168			N=195		
	Vaginal, No IAP	-		Vaginal, No IAP		
	Vaginal, IAP	1.54(.71-3.34)	.266	Vaginal, IAP	1.65(.82-3.30)	.153
	Emergency CS, IAP	1.74(.72-4.23)	.216	Emergency CS, IAP	1.19(.53-2.65)	.667
	Elective CS, IAP	.59(.15-2.31)	.454	Elective CS, IAP	.59(.17-1.9)	.401
***Individual Interaction terms present:<.20**						

APPENDIX C-STRATIFICATIONS

Table C2. Unadjusted stratification by covariates with mode of delivery as the exposure and persistent *C. difficile* colonization as the outcome

	Exposure= birth method, outcome= C.*difficile* =(Y/N)	Persistent colonization N= 1226 OR(CI)	P value (x^2)
Mode of delivery	**N=1226**		
	Vaginal, No IAP	(reference)	
	Vaginal, IAP	1.45(.98-2.13)	.058
	Emergency CS, IAP	2.07(1.35-3.16)	.001
	Elective CS, IAP	1.53(89-2.61)	.119
UNADJUSTED STRATIFIED			
Breastfeeding 3 mo			
Exclusive	**N= 682**		
	Vaginal, No IAP	(reference)	
	Vaginal, IAP	1.29(.742-2.25)	.362
	Emergency CS, IAP	.90(.42-1.94)	.804
	Elective CS, IAP	1.08(.40-2.91)	.869
Partial	**N=339**		
	Vaginal, No IAP	-	
	Vaginal, IAP	1.58(.76-3.27)	.213
	Emergency CS, IAP	2.57(1.22-5.38)	.012*
	Elective CS, IAP	2.05(.942-4.48)	.070
Formula Fed	**N=204**		
	Vaginal, No IAP	-	
	Vaginal, IAP	1.85(.81-4.24)	.143
	Emergency CS, IAP	6.18(2.50-15.289)	.0001*
	Elective CS, IAP	.87(.232-3.27)	.841
Breastfeeding 6mo			
Exclusive	**N= 214**		
	Vaginal, No IAP	-	
	Vaginal, IAP	1.18(.39-3.57)	.767
	Emergency CS, IAP	1.20(.29-4.83)	.795
	Elective CS, IAP	1.66(.31-8.6)	.546
Partial	**N=688**		
	Vaginal, No IAP	-	
	Vaginal, IAP	1.41(.83-2.37)	.199
	Emergency CS, IAP	1.10(.57-2.13)	.759
	Elective CS, IAP	1.59(.77-3.28)	.203
Formula Fed	**N=313**		
	Vaginal, No IAP	-	
	Vaginal, IAP	1.96(.98-3.92)	.057
	Emergency CS, IAP	5.53(2.7-11.4)	.0001*
	Elective CS, IAP	1.43(.56-3.6)	.446

Gravida			
Primigravida	**N=465**		
	Vaginal, No IAP	-	
	Vaginal, IAP	1.29(.73-2.28)	.377
	Emergency CS, IAP	2.98(1.67-5.30)	.0001*
	Elective CS, IAP	2.63(.93-7.32)	.068
Multigravida	**N=763**		
	Vaginal, No IAP	=	
	Vaginal, IAP	1.51(.89-2.57)	.122
	Emergency CS, IAP	1.03(.504-2.14)	.917
	Elective CS, IAP	1.41(.74-2.69)	.287
Gender			
Male	**N=651**		
	Vaginal, No IAP	-	
	Vaginal, IAP	1.37(.79-2.38)	.252
	Emergency CS, IAP	2.02(1.16-3.49)	.012
	Elective CS, IAP	1.41(.66-3.0)	.361
Female	**N=577**		
	Vaginal, No IAP	-	
	Vaginal, IAP	1.52(.88-2.63)	.125
	Emergency CS, IAP	2.17(1.09-4.29)	.026
	Elective CS, IAP	1.66(.77-3.56)	.193
Postnatal Smoke exposure			
Yes	**N=176**		
	Vaginal, No IAP	-	
	Vaginal, IAP	1.93(.79-4.67)	.145
	Emergency CS, IAP	1.94(.69-5.42)	.206
	Elective CS, IAP	1.31(.33-5.23)	.696
No	**N=1037**		
	Vaginal, No IAP	-	
	Vaginal, IAP	1.30(.84-2.01)	.228
	Emergency CS, IAP	1.98(1.24-3.18)	.004
	Elective CS, IAP	1.56(.87-2.8)	.130
Siblings			
Yes	**N=731**		
	Vaginal, No IAP	1.20(.601-2.41)	.598
	Vaginal, IAP	.77(.227-2.65)	.690
	Emergency CS, IAP	1.42(.69-2.94)	.335
	Elective CS, IAP		
No	**N=638**		
	Vaginal, No IAP	-	
	Vaginal, IAP	1.39(.86-2.24)	.173
	Emergency CS, IAP	1.99(1.22-3.25)	.006
	Elective CS, IAP	2.49(1.06-5.83)	.035
Pets			
Yes	**N=663**		

	Vaginal, No IAP	-	
	Vaginal, IAP	1.50(.87-2.60)	.143
	Emergency CS, IAP	1.91(1.03-3.52)	.037
	Elective CS, IAP	2.21(1.05-4.61)	.034
No	**N=792**		
	Vaginal, No IAP	-	
	Vaginal, IAP	1.24(.70-2.17)	.448
	Emergency CS, IAP	2.03(1.11-3.69)	.021
Hospital length of stay			
24hrs or less	**N=316**		
	Vaginal, No IAP	-	
	Vaginal, IAP	1.05(.40-2.74)	.906
	Emergency CS, IAP	-	
	Elective CS, IAP	-	
2-3 days			
	Vaginal, No IAP	-	
	Vaginal, IAP	1.54(.97-2.45)	.064
	Emergency CS, IAP	1.50(.87-2.59)	.142
	Elective CS, IAP	.96(.48-1.89)	.915
4 days or more			
	Vaginal, No IAP	-	
	Vaginal, IAP	.79(.23-2.70)	.710
	Emergency CS, IAP	1.97(.83-4.65)	.121
	Elective CS, IAP	2.46(.86-7.05)	.093
Geography			
Vancouver			
	Vaginal, No IAP	-	
	Vaginal, IAP	1.52(.72-3.24)	.269
	Emergency CS, IAP	1.78(.77-4.13)	.175
	Elective CS, IAP	2.18(.87-5.44)	.094
Edmonton			
	Vaginal, No IAP	-	
	Vaginal, IAP	1.93(.91-4.07)	.083
	Emergency CS, IAP	3.17(1.44-6.95)	.004
	Elective CS, IAP	1.41(9.52-3.82)	.492
Winnipeg			
	Vaginal, No IAP	-	
	Vaginal, IAP	1.36(.76-2.43)	.293
	Emergency CS, IAP	1.96(1.1-3.81)	.043
	Elective CS, IAP	1.29(.50-3.28)	.587
Medical interventions			
No oxytocin/anesthetics	**N=172**		
	Vaginal, No IAP	-	
	Vaginal, IAP	1.15(.39-3.41)	.792
	Emergency CS, IAP	-	
	Elective CS, IAP	-	

Oxytocin only	**N=61**		
	Vaginal, No IAP		
	Vaginal, IAP	4.5(.78-24.65)	.090
	Emergency CS, IAP	-	
	Elective CS, IAP	-	
Anesthetics only	**N=442**		
	Vaginal, No IAP	-	
	Vaginal, IAP	1.32(.63-2.77)	.452
	Emergency CS, IAP	1.89(.87=4.13)	.106
	Elective CS, IAP	1.52(.78-4.13)	.213
Both oxytocin and anesthetics	**N= 498**		
	Vaginal, No IAP		
	Vaginal, IAP	1.23(.70-2.15)	.456
	Emergency CS, IAP	1.81(1.03-3.17)	.036
	Elective CS, IAP	1.37(.36-5.13)	.632
***Individual interaction term present p<.05**			

APPENDIX D- SENSITIVITY ANALYSES						
Table D1. Sensitivity analyse with homebirths in the model vs no homebirths in the model 3 months						
Exposure= birth method, outcome= C.*difficile* 3 month=(Y/N)	**Sensitivity analyses with home included (n=1518)**	**3 months**	**P value**	**Sensitivity analyses w/ home excluded (n=1481)**	**3 months**	P value
CRUDE OR						
Mode of delivery with IAP						
	Vaginal, No IAP	(reference)		Vaginal, No IAP	(reference)	
	Vaginal, IAP	1.18(.89-1.55)	.229	Vaginal, IAP	1.18(.89-1.55)	.239
	Emergency CS, IAP	1.83(1.32-2.52)	**<.0001**	Emergency CS, IAP	1.76(1.27-2.44)	**.001**
	Elective CS, IAP	1.60(1.10-2.34)	**.014**	Elective CS, IAP	1.55(1.06-2.26)	**.024**
Adjusted for breastfeeding						
	Vaginal, No IAP	Ref		Vaginal, No IAP	ref	
	Vaginal, IAP	1.21(.918-1.61)	.171	Vaginal, IAP	1.21(.91-1.61)	.170
	Emergency CS, IAP	1.80(1.29-2.51)	**<.0001**	Emergency CS, IAP	1.75(.001)	**.001**
	Elective CS, IAP	1.44(.975-2.12)	**.066**	Elective CS, IAP	1.40(.95-2.07)	.085
Adjusted for child medication						
	Vaginal, No IAP	ref		Vaginal, No IAP	Ref	
	Vaginal, IAP	1.18(.89-1.55)	.228	Vaginal, IAP	1.18(.89-1.55)	.237
	Emergency CS, IAP	1.83(1.32-2.53)	**<.0001**	Emergency CS, IAP	1.76(1.27-2.44)	**.001**
	Elective CS, IAP	1.60(1.10-2.35)	**.014**	Elective CS, IAP	1.55(1.06-2.27)	**.023**
Adjusted for Oxytocin/Anesthetics						
	Vaginal, No IAP	ref		Vaginal, No IAP		
	Vaginal, IAP	1.06(.80-1.42)	.653	Vaginal, IAP	1.13(.85-1.50)	.372
	Emergency CS, IAP	1.61(1.15-2.27)	**.005**	Emergency CS, IAP	1.60(1.14-2.24)	**.006**
	Elective CS, IAP	1.55(1.03-2.32)	**.035**	Elective CS, IAP	1.49(1.02-1.26)	**.037**
Adjusted for Gravida						
	Vaginal, No IAP	ref		Vaginal, No IAP	Ref	
	Vaginal, IAP	1.16(.88-1.53)	.287	Vaginal, IAP	1.16(.88-1.53)	.290
	Emergency CS, IAP	1.78(1.28-2.46)	**<.0001**	Emergency CS, IAP	1.72(1.24-2.38)	**.001**
	Elective CS, IAP	1.65(1.12-2.41)	**.010**	Elective CS, IAP	1.59(1.08-2.33)	**.017**

Table D2. Sensitivity analyses with premature births (<37 weeks) in the model vs not in the model 3 months						
Exposure= birth method, outcome= C.*difficile* 3 month=(Y/N)	**Sensitivity analyses with premature babies excluded (n=1416)**	**3 months**	**P value**	**Sensitivity analyses w/ premature babies included (n=1481)**	**3 months**	P value
CRUDE OR						
Mode of deliverywith IAP						
	Vaginal, No IAP	(reference)		Vaginal, No IAP	(reference)	
	Vaginal, IAP	1.21(.91-1.61)	.185	Vaginal, IAP	1.18(.89-1.55)	.239
	Emergency CS, IAP	1.81(1.30-2.52)	**<.0001**	Emergency CS, IAP	1.76(1.27-2.44)	**.001**
	Elective CS, IAP	1.47(.99-2.17)	**.052**	Elective CS, IAP	1.55(1.06-2.26)	**.024**
Adjusted for breastfeeding						
	Vaginal, No IAP	Ref		Vaginal, No IAP	ref	
	Vaginal, IAP	1.25(.93-1.67)	.133	Vaginal, IAP	1.21(.91-1.61)	.170
	Emergency CS, IAP	1.80(1.28-2.51)	**.001**	Emergency CS, IAP	1.75(.001)	**.001**
	Elective CS, IAP	1.33(.89-1.98)	**.158**	Elective CS, IAP	1.40(.95-2.07)	.085
Adjusted for child medication						
	Vaginal, No IAP	ref		Vaginal, No IAP	Ref	
	Vaginal, IAP	1.21(.91-1.61)	.183	Vaginal, IAP	1.18(.89-1.55)	.237
	Emergency CS, IAP	1.81(1.30-2.52)	**<.0001**	Emergency CS, IAP	1.76(1.27-2.44)	**.001**
	Elective CS, IAP	1.47(1.0-2.18)	**.049**	Elective CS, IAP	1.55(1.06-2.27)	**.023**
Adjusted for Oxytocin/Anesthetics						
	Vaginal, No IAP	ref		Vaginal, No IAP		
	Vaginal, IAP	1.16(.87-1.55)	.302	Vaginal, IAP	1.13(.85-1.50)	.372
	Emergency CS, IAP	1.63(1.15-2.3)	**.005**	Emergency CS, IAP	1.60(1.14-2.24)	**.006**
	Elective CS, IAP	1.40(.92-2.12)	**.108**	Elective CS, IAP	1.49(1.02-1.26)	**.037**
Adjusted for Gravida						
	Vaginal, No IAP	ref		Vaginal, No IAP	Ref	
	Vaginal, IAP	1.19(.88-1.53)	.230	Vaginal, IAP	1.16(.88-1.53)	**.290**
	Emergency CS, IAP	1.75(1.25-2.44)	**.001**	Emergency CS, IAP	1.72(1.24-2.38)	**.001**
	Elective CS, IAP	1.51(1.02-2.24)	**.010**	Elective CS, IAP	1.59(1.08-2.33)	**.017**

Table D3. Sensitivity analyses with age at stool collection (>6 month excluded vs > 6 months included 3 months						
Exposure= birth method, outcome= C.*difficile* 3 month=(Y/N)	**Sensitivity analyses with >6 months included (n=1481)**	**3 months**	**P value**	**Sensitivity analyses w/ >6 months excluded (n=1409)**	**3 months**	**P value**
CRUDE OR						
Mode of deliverywith IAP						
	Vaginal, No IAP	(reference)		Vaginal, No IAP	(reference)	
	Vaginal, IAP	1.18(.89-1.55)	.239	Vaginal, IAP	1.14(.86-1.53)	.340
	Emergency CS, IAP	1.76(1.27-2.44)	**.001**	Emergency CS, IAP	1.77(1.26-2.47)	**.001**
	Elective CS, IAP	1.55(1.06-2.26)	**.024**	Elective CS, IAP	1.50(1.01-2.22)	**.044**
Adjusted for breastfeeding 3 mo						
	Vaginal, No IAP	ref		Vaginal, No IAP	ref	
	Vaginal, IAP	1.21(.91-1.61)	.170	Vaginal, IAP	1.19 (.89-1.6.227)	.227
	Emergency CS, IAP	1.75(.001)	**.001**	Emergency CS, IAP	1.78(1.26 2.5)	**.001**
	Elective CS, IAP	1.40(.95-2.07)	.085	Elective CS, IAP	1.37(.91- 2.0)	.121
Adjusted for child medication						
	Vaginal, No IAP	Ref		Vaginal, No IAP	ref	
	Vaginal, IAP	1.18(.89-1.55)	.237	Vaginal, IAP	1.15(.86-1.53)	.336
	Emergency CS, IAP	1.76(1.27-2.44)	**.001**	Emergency CS, IAP	1.77(1.27-2.47)	**.001**
	Elective CS, IAP	1.55(1.06-2.27)	**.023**	Elective CS, IAP	1.50(1.01-2.23)	**.041**
Adjusted for Oxytocin/Anesthetics						
	Vaginal, No IAP	ref		Vaginal, No IAP	ref	
	Vaginal, IAP	1.13(.85-1.50)	.372	Vaginal, IAP	1.09(.81-1.45)	**.551**
	Emergency CS, IAP	1.60(1.14-2.24)	**.006**	Emergency CS, IAP	1.56(1.10-2.20)	**.011**
	Elective CS, IAP	1.49(1.02-1.26)	**.037**	Elective CS, IAP	1.47(.97-2.24)	.069
Adjusted for Gravida						
	Vaginal, No IAP	Ref		Vaginal, No IAP	ref	
	Vaginal, IAP	1.16(.88-1.53)	.290	Vaginal, IAP	1.12(.84-1.50)	.424
	Emergency CS, IAP	1.72(1.24-2.38)	**.001**	Emergency CS, IAP	1.71(1.22-2.39)	**.002**
	Elective CS, IAP	1.59(1.08-2.33)	**.017**	Elective CS, IAP	1.56(1.05-2.32)	**.028**

Table D4. Sensitivity analyses with home births included excluded 1 YEAR						
Exposure= birth method, outcome= C.*difficile* 1 YEAR (Y/N)	**Sensitivity analyses with home included (n=1888)**	**3 months**	**P value**	**Sensitivity analyses w/ home excluded (n=1840)**	**3 months**	**P value**
CRUDE OR						
Mode of deliverywith IAP						
	Vaginal, No IAP	(reference)		Vaginal, No IAP	(reference)	
	Vaginal, IAP	1.38(1.09-1.72)	**.005**	Vaginal, IAP	1.35(1.07-1.70)	**.010**
	Emergency CS, IAP	1.39(1.06-1.83)	**.015**	Emergency CS, IAP	1.36(1.03-1.78)	**.026**
	Elective CS, IAP	1.21(.88-1.65)	.233	Elective CS, IAP	1.18(.86-1.62)	.285
Adjusted for breastfeeding 6 mo						
	Vaginal, No IAP	ref		Vaginal, No IAP	ref	
	Vaginal, IAP	1..40(1.11-1.76)	**.004**	Vaginal, IAP	1.37(1.09-1.73)	**007**
	Emergency CS, IAP	1.40(1.06-1.84)	**.015**	Emergency CS, IAP	1.36(1.03-1.79)	**.027**
	Elective CS, IAP	1.25(.91-1.73)	.154	Elective CS, IAP	1.23(.89-1.69)	.193
Adjusted for child medication						
	Vaginal, No IAP	ref		Vaginal, No IAP	ref	
	Vaginal, IAP	1.38(1.09-1.73)	**.005**	Vaginal, IAP	1.35(1.07-1.70)	**.010**
	Emergency CS, IAP	1.39(1.06-1.82)	**.017**	Emergency CS, IAP	1.35(1.03-1.78)	**.029**
	Elective CS, IAP	1.21(.88-1.66)	.228	Elective CS, IAP	1.18(.86-1.62)	.281
Adjusted for Oxytocin/Anesthetics						
	Vaginal, No IAP	ref		Vaginal, No IAP	ref	
	Vaginal, IAP	1.27(1.0-1.6)	**.046**	Vaginal, IAP	1.27(1.01-1.61)	**.041**
	Emergency CS, IAP	1.23(.92-1.65)	.145	Emergency CS, IAP	1.21(.91-1.62)	**.178**
	Elective CS, IAP	1.09(.78-1.53)	.593	Elective CS, IAP	1.12(.61-1.47)	.504
Adjusted for siblings						
	Vaginal, No IAP	ref		Vaginal, No IAP	ref	
	Vaginal, IAP	1.28(1.02-1.62)	**.033**	Vaginal, IAP	1.26(1.0-1.60)	**.046**
	Emergency CS, IAP	1.19(.90-1.58)	.203	Emergency CS, IAP	1.17(.89-1.56)	.249
	Elective CS, IAP	1.32(.96-1.81)	**.083**	Elective CS, IAP	1.29(.94-1.78)	.108

Table D5. Sensitivity analyses with infants born <37 weeks included and excluded 1 YEAR						
Exposure= birth method, outcome= C.*difficile* 1 YEAR (Y/N)	**Sensitivity analyses with <37 weeks excluded (n=1757)**	**3 months**	**P value**	**Sensitivity analyses with <37 weeks included (n=1840)**	**3 months**	**P value**
CRUDE OR						
Mode of deliverywith IAP						
	Vaginal, No IAP	(reference)		Vaginal, No IAP	(reference)	
	Vaginal, IAP	1.31(1.03-1.67)	**.023**	Vaginal, IAP	1.35(1.07-1.70)	**.010**
	Emergency CS, IAP	1.36(1.02-1.81)	**.031**	Emergency CS, IAP	1.36(1.03-1.78)	**.026**
	Elective CS, IAP	1.21(.88-1.67)	.236	Elective CS, IAP	1.18(.86-1.62)	.285
Adjusted for breastfeeding 6 mo						
	Vaginal, No IAP	ref		Vaginal, No IAP	ref	
	Vaginal, IAP	1.33(1.04-1.69)	**.019**	Vaginal, IAP	1.37(1.09-1.73)	**007**
	Emergency CS, IAP	1.36(1.02-1.81)	**.033**	Emergency CS, IAP	1.36(1.03-1.79)	**.027**
	Elective CS, IAP	1.27(.91-1.76)	.148	Elective CS, IAP	1.23(.89-1.69)	.193
Adjusted for child medication						
	Vaginal, No IAP	ref		Vaginal, No IAP	ref	
	Vaginal, IAP	1.31(1.03-1.67)	**.024**	Vaginal, IAP	1.35(1.07-1.70)	**.010**
	Emergency CS, IAP	1.35(1.02-1.80)	**.035**	Emergency CS, IAP	1.35(1.03-1.78)	**.029**
	Elective CS, IAP	1.21(.88-1.67)	.234	Elective CS, IAP	1.18(.86-1.62)	.281
Adjusted for Oxytocin/Anesthetics						
	Vaginal, No IAP	ref		Vaginal, No IAP	ref	
	Vaginal, IAP	1.25(.98-1.59)	**.071**	Vaginal, IAP	1.27(1.01-1.61)	**.041**
	Emergency CS, IAP	1.21(.90-1.64)	.197	Emergency CS, IAP	1.21(.91-1.62)	**.178**
	Elective CS, IAP	1.14(.81-1.62)	.428	Elective CS, IAP	1.12(.61-1.47)	.504
Adjusted for siblings						
	Vaginal, No IAP	ref		Vaginal, No IAP	ref	
	Vaginal, IAP	1.23(.97-1.57)	.083	Vaginal, IAP	1.26(1.0-1.60)	**.046**
	Emergency CS, IAP	1.16(.86-1.56)	.307	Emergency CS, IAP	1.17(.89-1.56)	.249
	Elective CS, IAP	1.32(.51-.76)	.092	Elective CS, IAP	1.29(.94-1.78)	.108

Table D6. Sensitivity analyses with age at stool collection>16months included vs excluded 1 YEAR						
Exposure= birth method, outcome= C.*difficile* 1 YEAR (Y/N)	**Sensitivity analyses with >16 months excluded (n=1796)**	**3 months**	**P value**	**Sensitivity analyses with >16 months included (n=1836)**	**3 months**	**P value**
CRUDE OR						
Mode of deliverywith IAP						
	Vaginal, No IAP	(reference)		Vaginal, No IAP	(reference)	
	Vaginal, IAP	1.32(1.05-1.67)	**.017**	Vaginal, IAP	1.35(1.07-1.70)	**.010**
	Emergency CS, IAP	1.32(1.0-1.74)	**.047**	Emergency CS, IAP	1.36(1.03-1.78)	**.026**
	Elective CS, IAP	1.23(.89-1.69)	.198	Elective CS, IAP	1.18(.86-1.62)	.285
Adjusted for breastfeeding 6 mo						
	Vaginal, No IAP	ref		Vaginal, No IAP	ref	
	Vaginal, IAP	1.35(1.04-1.71)	**.012**	Vaginal, IAP	1.37(1.09-1.73)	**007**
	Emergency CS, IAP	1.32(1.02-1.74)	**.049**	Emergency CS, IAP	1.36(1.03-1.79)	**.027**
	Elective CS, IAP	1.28(.93-1.77)	.148	Elective CS, IAP	1.23(.89-1.69)	.193
Adjusted for child medication						
	Vaginal, No IAP	ref		Vaginal, No IAP	ref	
	Vaginal, IAP	1.32(1.05-1.67)	**.017**	Vaginal, IAP	1.35(1.07-1.70)	**.010**
	Emergency CS, IAP	1.31(.99-1.73)	**.051**	Emergency CS, IAP	1.35(1.03-1.78)	**.029**
	Elective CS, IAP	1.21(.88-1.69)	.202	Elective CS, IAP	1.18(.86-1.62)	.281
Adjusted for Oxytocin/Anesthetics						
	Vaginal, No IAP	ref		Vaginal, No IAP	ref	
	Vaginal, IAP	1.25(.99-1.59)	**.058**	Vaginal, IAP	1.27(1.01-1.61)	**.041**
	Emergency CS, IAP	1.21(.90-1.62)	.201	Emergency CS, IAP	1.21(.91-1.62)	**.178**
	Elective CS, IAP	1.14(.81-1.62)	.400	Elective CS, IAP	1.12(.61-1.47)	.504
Adjusted for siblings						
	Vaginal, No IAP	ref		Vaginal, No IAP	ref	
	Vaginal, IAP	1.25(.97-1.57)	.065	Vaginal, IAP	1.26(1.0-1.60)	**.046**
	Emergency CS, IAP	1.15(.86-1.56)	.328	Emergency CS, IAP	1.17(.89-1.56)	.249
	Elective CS, IAP	1.34(.52-.77)	.070	Elective CS, IAP	1.29(.94-1.78)	.108

Table. D7 Crude simple and multiple logistic regression with birth, prenatal and postnatal characteristics predicting *C. difficile* colonization at 3 months of age- sensitivity analyses with vaginal IAP as reference

	Crude (unadjusted)			Model 1 (adjusted for birth characteristics)			Model 2 (adjusted for prenatal characteristics)			Model 3 (adjusted for postnatal characteristics)			Model 4 (Final model)		
Outcome: Clostridium difficile 3 months (Y/N)	Main exposure of interest: Birth method	Crude Univariate OR	P value	Main exposure of interest: Birth method	Adjusted OR	P value	Main exposure of interest: Birth method	Adjusted OR	P value	Main exposure of interest: Birth method	Adjusted OR	P value	Main exposure of interest: Birth method	Adjusted OR	P value
Birth method:	N=1477			N=1477			N=1356			N=1351			N=1351		
	Vaginal, IAP	(reference)		Vaginal, IAP	(reference)		Vaginal, IAP	(reference)		Vaginal, IAP	(reference)		Vaginal, IAP	(reference)	
	Vaginal, no IAP	.837(.63-1.10)	.209	Vaginal, no IAP	.87(.65-1.15)	.332	Vaginal, no IAP	.80(.59-1.08)	.156	Vaginal, no IAP	.831(.59-1.10)	.185	Vaginal, no IAP	.78(.57-1.07)	.138
	Emergency CS, IAP	1.48(1.27-2.44)	**.035**	Emergency CS, IAP	1.44(.99-2.1)	**.051**	Emergency CS, IAP	1.62(1.09-2.42)	**.017**	Emergency CS, IAP	1.40(.92-2.12)	.109	Emergency CS, IAP	1.51(.98-2.32)	.060
	Elective CS, IAP	1.30(.85-1.97)	.214	Elective CS, IAP	1.34(.86-2.09)	.183	Elective CS, IAP	1.51(.95-2.41)	**.**078	Elective CS, IAP	1.11(.69-1.79)	.655	Elective CS, IAP	1.19(.71-1.99)	.503
Adjustment				Adjusted for: medical interventions			Adjusted for: gravida, birthweight, gender, maternal race, maternal smoking, maternal BMI, mothers age, maternal prenatal depression			Adjusted for: breastfeeding 3 mo, child medication, postnatal smoke exposure, intro to foods, pets, hospital length of stay, siblings, geography, age at stool collection			Adjusted for: medical interventions, maternal race, geography, mothers age, maternal prenatal depression, breastfeeding 3 mo, postnatal smoke, pets, HLOS, siblings, child medication, age at stool collection		

Table. D8 Crude simple and multiple logistic regression with birth, prenatal and postnatal characteristics predicting *C. difficile* colonization at 3 months of age- sensitivity analyses with emergency CS (NO LABOUR) removed

	Crude (unadjusted)			Model 1 (adjusted for birth characteristics)			Model 2 (adjusted for prenatal characteristics)			Model 3 (adjusted for postnatal characteristics)			Model 4 (Final model)		
Outcome: Clostridium difficile 3 months (Y/N)	Main exposure of interest: Birth method	Crude Univariate OR	P value	Main exposure of interest: Birth method	Adjusted OR	P value	Main exposure of interest: Birth method	Adjusted OR	P value	Main exposure of interest: Birth method	Adjusted OR	P value	Main exposure of interest: Birth method	Adjusted OR	P value
Birth method:	N=1459			N=1477			N=1339			N=1351			N=1351		
	Vaginal, no IAP	(reference)		Vaginal, no IAP	(reference)		Vaginal, no IAP	(reference)		Vaginal, no IAP	(reference)		Vaginal, no IAP	(reference)	
	Vaginal, IAP	1.19(.90-1.57)	.209	Vaginal, IAP	1.15(.86-1.52)	.327	Vaginal, IAP	1.22(.90-1.65)	.195	Vaginal, IAP	1.20(.88-1.63)	.234	Vaginal, IAP	1.26(.92-1.73)	.138
	Emergency CS, IAP	1.74(1.24-2.43)	**.001**	Emergency CS, IAP	1.62(1.14-2.30)	**.007**	Emergency CS, IAP	1.90(1.32-2.75)	**.001**	Emergency CS, IAP	1.60(1.08-2.39)	**.019**	Emergency CS, IAP	1.86(1.21-2.84)	**.004**
	Elective CS, IAP	1.55(1.06-2.27)	**.023**	Elective CS, IAP	1.53(1.02-2.30)	**.038**	Elective CS, IAP	1.87(1.23-2.86)	**.003**	Elective CS, IAP	1.30(.83-2.03)	.240	Elective CS, IAP	1.49(.92-2.43)	.103
Adjustment				Adjusted for: medical interventions			Adjusted for: gravida, birthweight, gender, maternal race, maternal smoking, maternal BMI, mothers age, maternal prenatal depression			Adjusted for: breastfeeding 3 mo, child medication, postnatal smoke exposure, intro to foods, pets, hospital length of stay, siblings, geography, age at stool collection			Adjusted for: medical interventions, maternal race, geography, mothers age, maternal prenatal depression, breastfeeding 3 mo, postnatal smoke, pets, HLOS, siblings, child medication, age at stool collection		

APPENDIX E

Table E1- Interaction terms between mode of delivery and covariates in the final model at 3 months, 1 year and persistent colonization with *C. difficile* colonization (global test for interaction)		
3 months		
Variables	**Unadjusted (p value)**	**Adjusted (p value)**
Breastfeeding (3 months)	.082	.263
Medical interventions (oxytocin and anesthetics)	.132	.387
Maternal Race	.355	.341
Prenatal depression	.543	.219
Postnatal smoke exposure	.322	.160
Pets in the home	.269	.752
Hospital length of stay	.304	.585
Siblings	.379	.515
Geography	.934	.962
Gravida	.123	.056
1 year		
Medical interventions (oxytocin and anesthetics)	.866	.741
Gravida	.579	.817
Gender	.329	.524
Maternal race	.255	.773
Introduction to foods	.850	.585
Pets in the home	.568	.366
Postnatal depression	.581	.804
Breastfeeding 6 mo	.823	.639
Persistence Colonization		
Medical interventions (oxytocin and anesthetics)	.855	.996
Breastfeeding 3 months	.043	.057
Breastfeeding 6 months	.049	.091
Gravida	.068	.059
Prenatal depression	.371	.128
Pets in the home	.569	.334
Hospital length of stay	.424	.382
Siblings	.440	.628

APPENDIX F

3 months-stratified unadjusted analyses

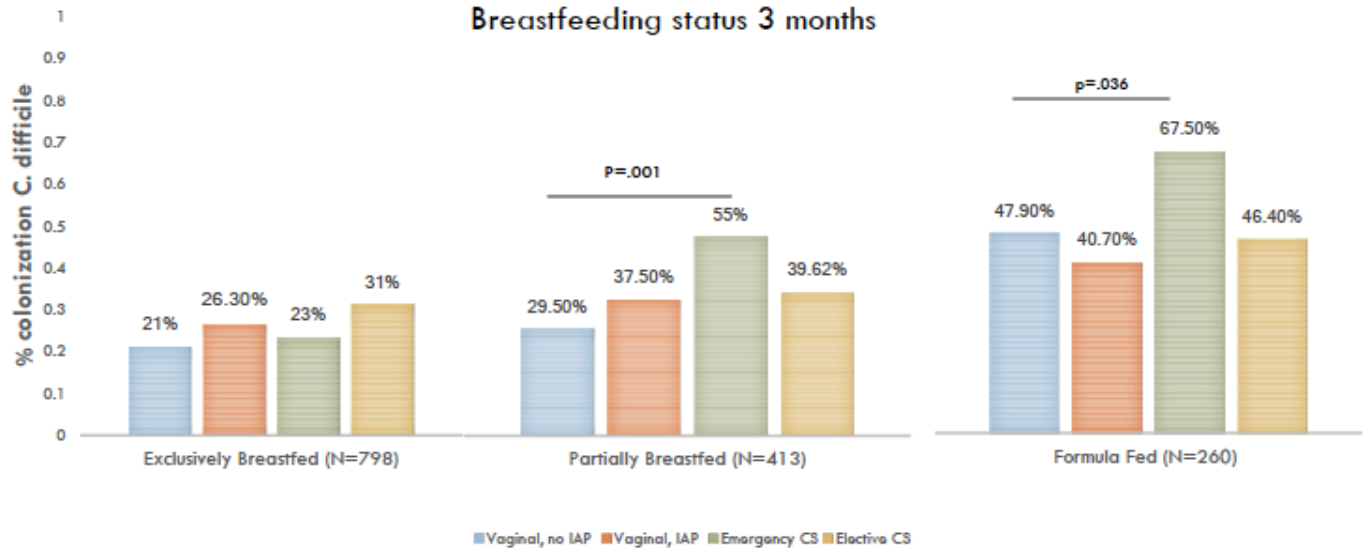


Figure F1. *C. difficile* colonization rates between mode of delivery stratified by breastfeeding status at 3 months of age

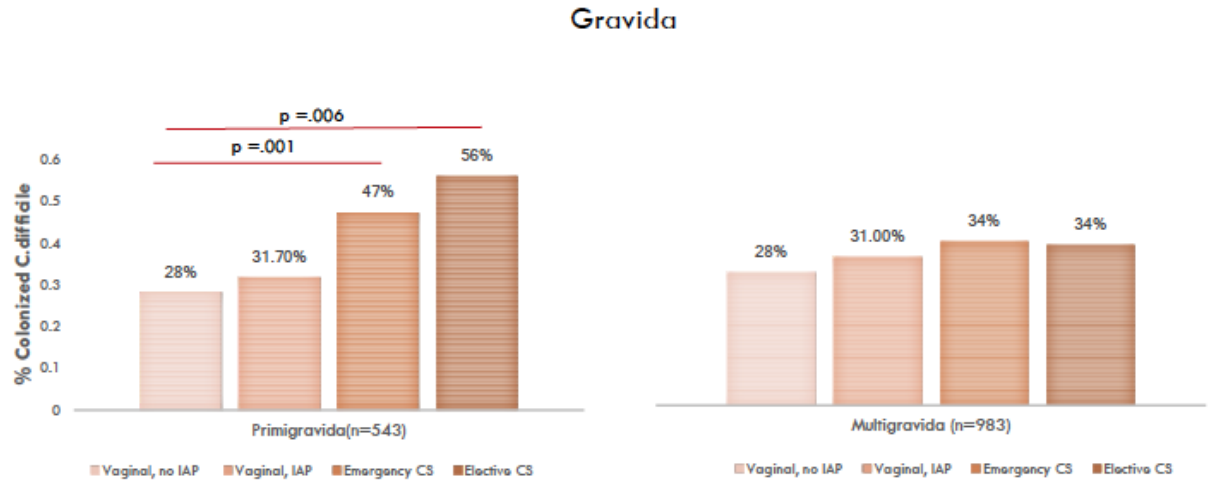


Figure F2. *C. difficile* colonization rates between mode of delivery stratified by gravida status at 3 months

Figure F3. *C. difficile* colonization rates between mode of delivery stratified by siblings present or absent at 3 months

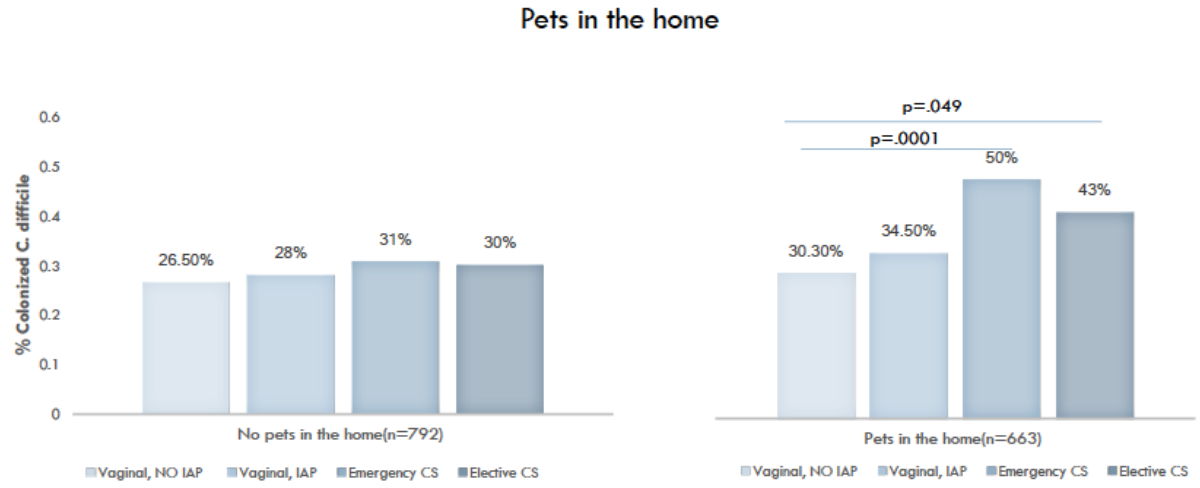
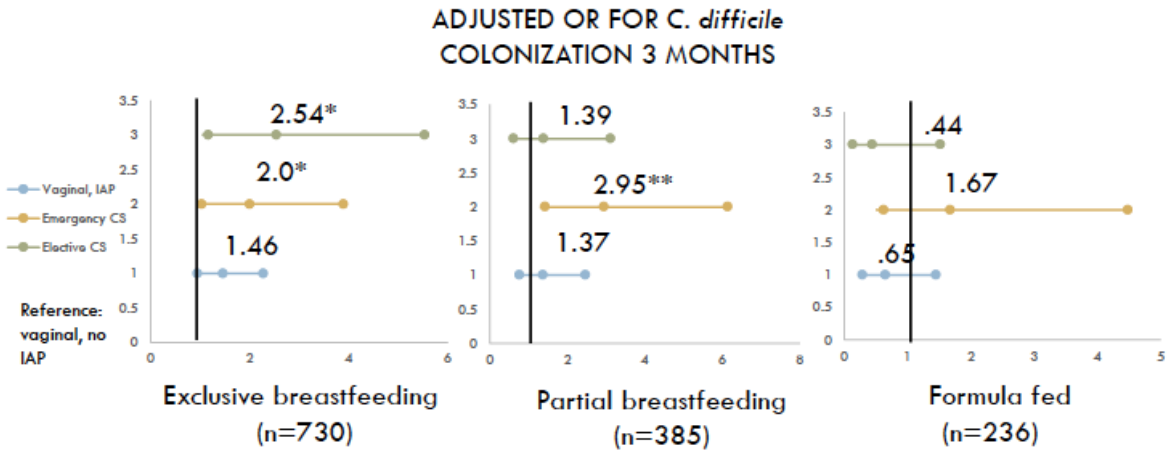


Figure F4. *C. difficile* colonization rates between mode of delivery stratified by pets present or absent in the home at 3 months

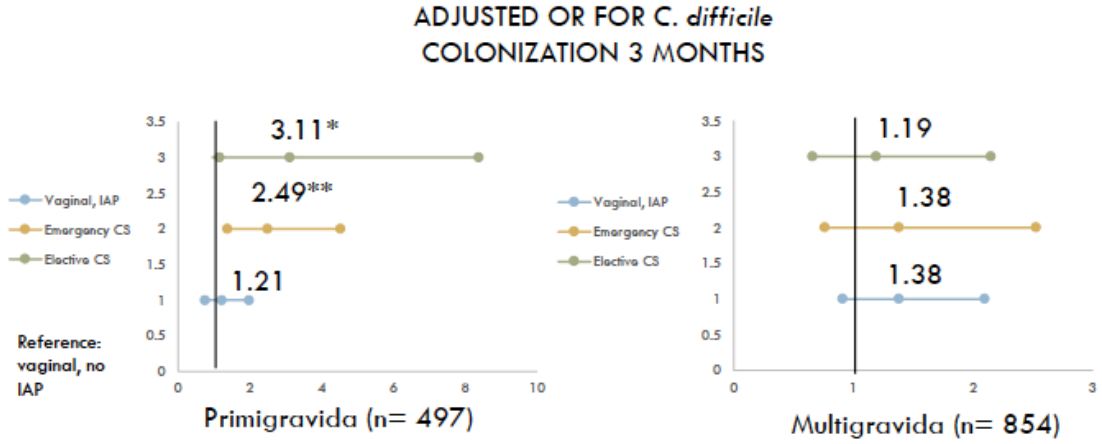
3-months stratified adjusted analyses



Adjusted for: anesthetics, oxytocin, maternal race, geography, mothers age, maternal prenatal depression, postnatal smoke, pets, hospital length of stay, siblings, child medication, age at stool collection

***p≤.001, ** p ≤.01, * p<.05

Figure F5. Results from adjusted multiple logistic regression when stratifying within stratums of breastfeeding while comparing mode of delivery effects with *C. difficile.* Global interaction term was not significant p=.263



Adjusted for: anesthetics, oxytocin, breastfeeding, maternal race, geography, mothers age, maternal prenatal depression, postnatal smoke, pets, hospital length of stay, siblings, child medication, age at stool collection

***p≤.001, ** p ≤.01, * p<.05

Figure F6. Results from adjusted multiple logistic regression when stratifying within stratums of gravida status while comparing mode of deliveryeffects on *C. difficile* colonization. Global interaction term was borderline significant p=.057

1 year- stratified unadjusted analyses

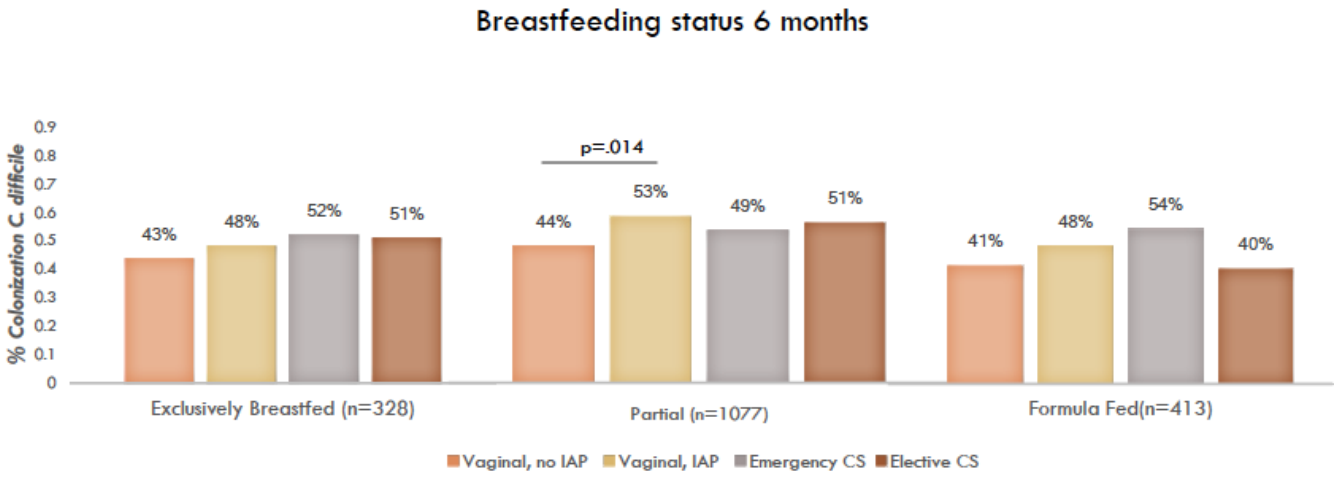


Figure F7. *C. difficile* colonization rates between mode of delivery stratified by breastfeeding status at 1 year of age

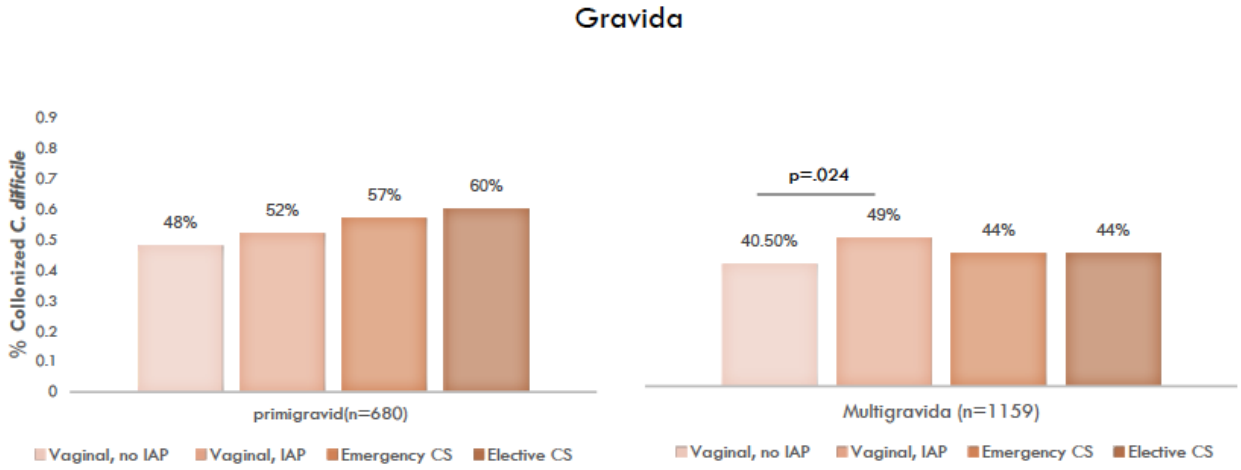


Figure F8. *C. difficile* colonization rates between mode of delivery stratified by gravida status at 1 year of age

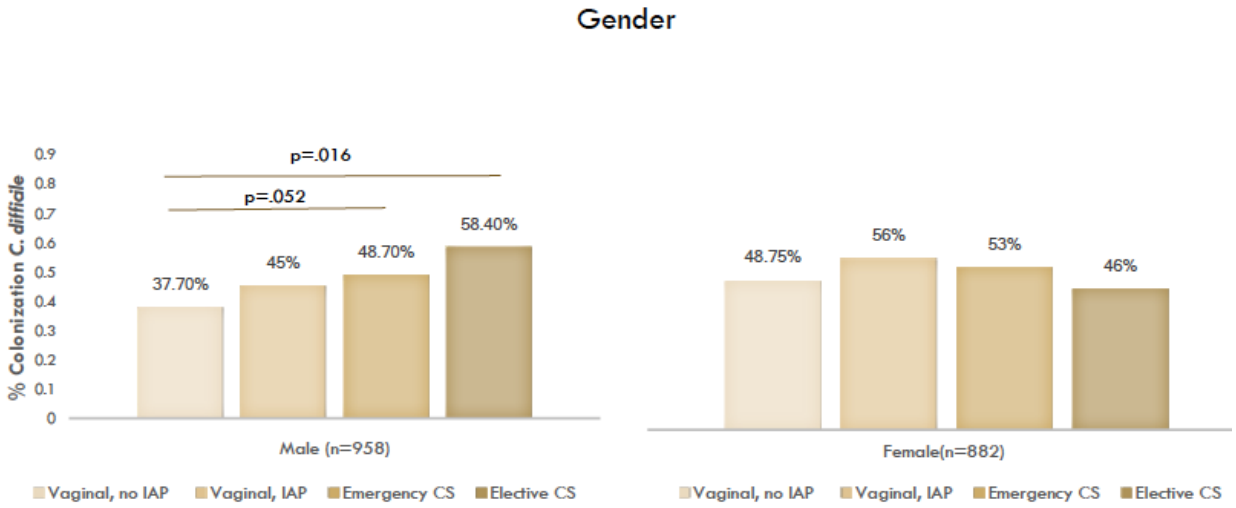


Figure F9. *C. difficile* colonization rates between mode of delivery stratified by infant gender at 1 year of age

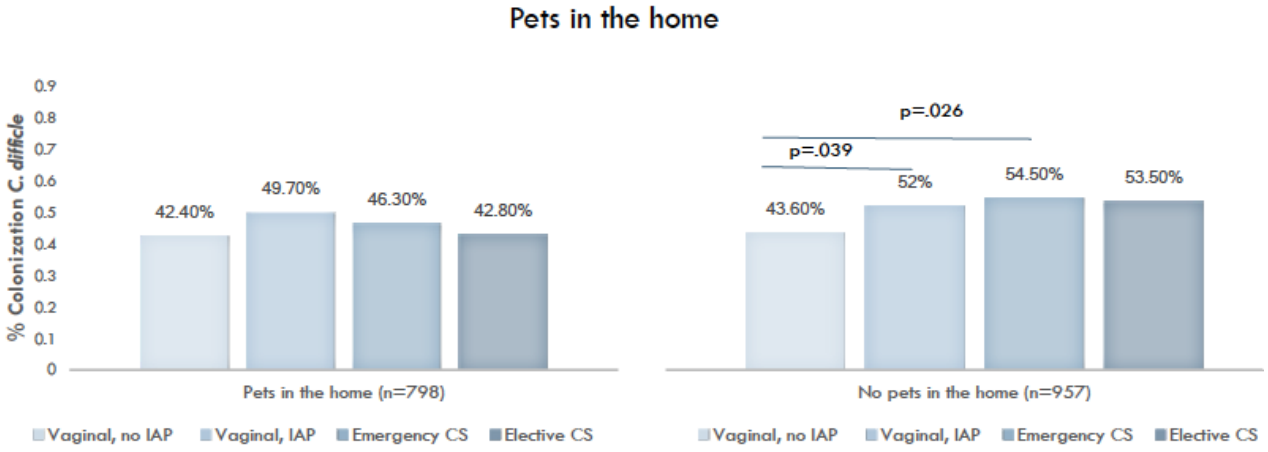


Figure F 10. *C. difficile* colonization rates between mode of delivery stratified by gravida status at 1 year of age

Persistent colonization- unadjusted stratified analyses

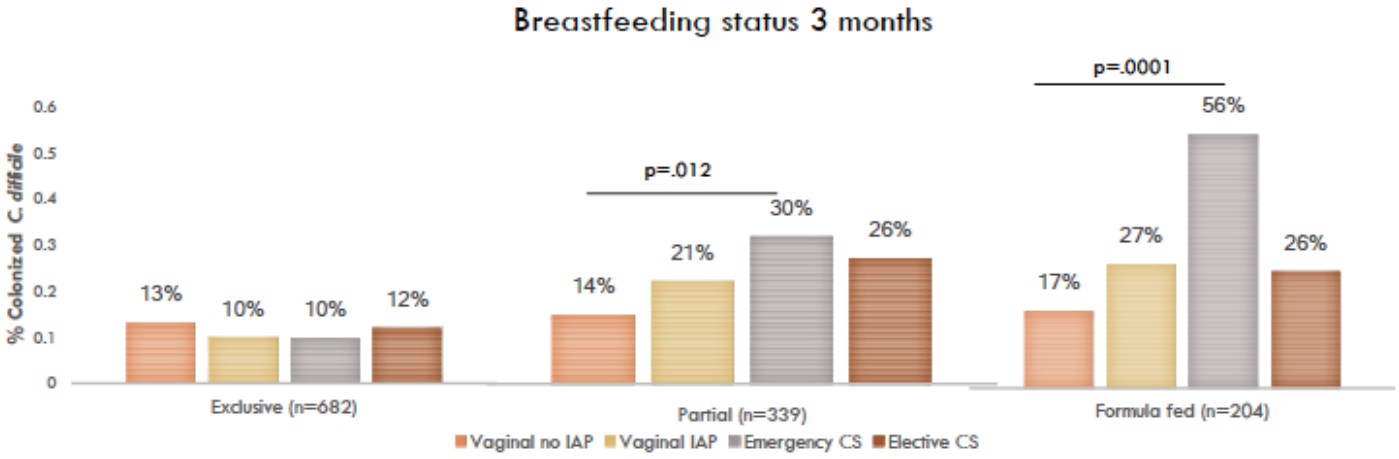


Figure F11. Persistent *C. difficile* colonization rates between mode of delivery stratified by breastfeeding status at 3 months of age

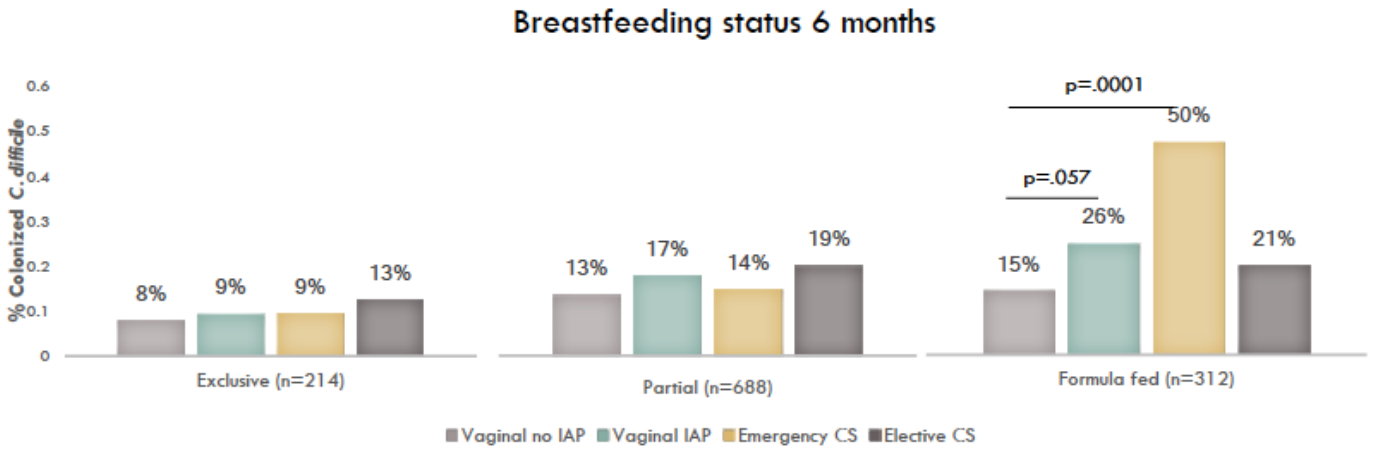


Figure F12. Persistent *C. difficile* colonization rates between mode of delivery stratified by breastfeeding status at 6 months of age

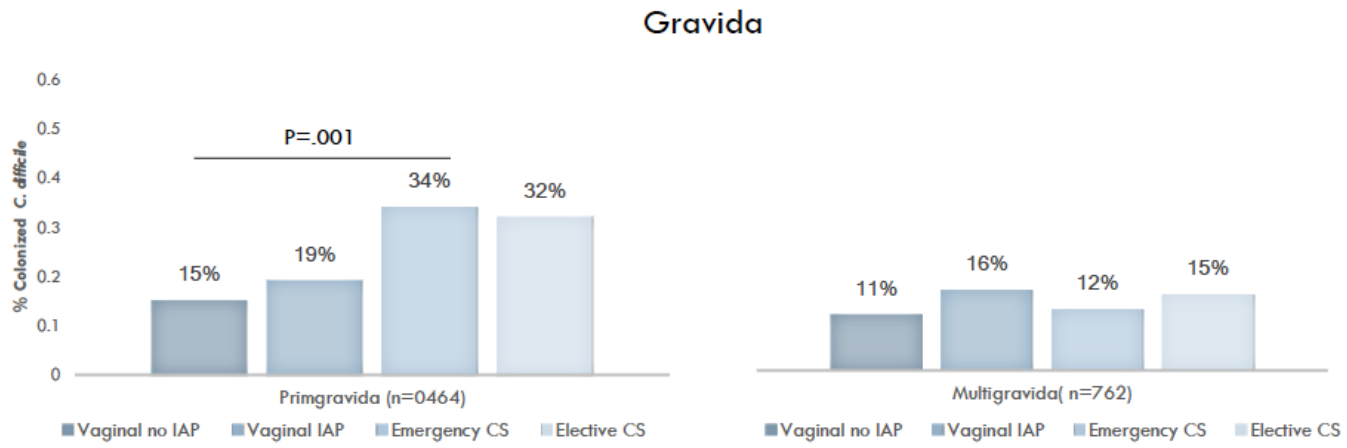


Figure F13. Persistent *C. difficile* colonization rates between mode of delivery stratified by gravida status

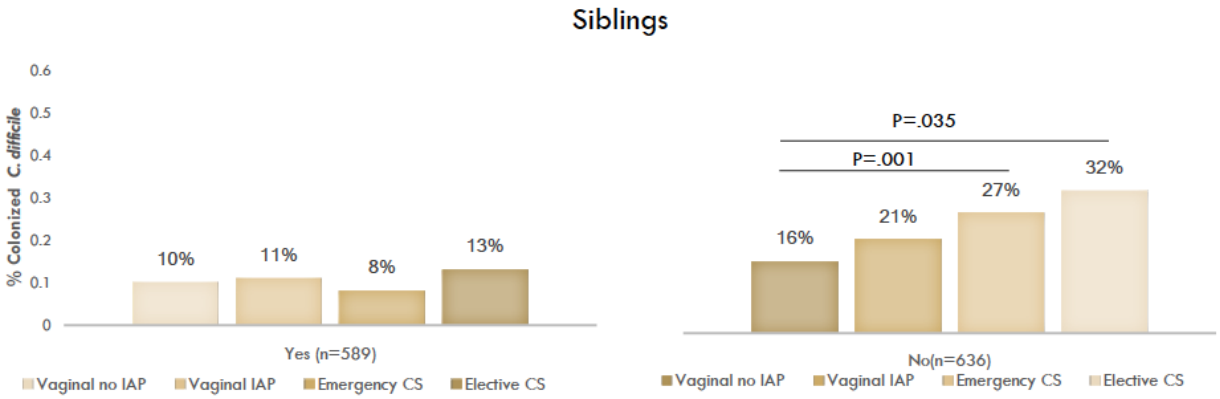
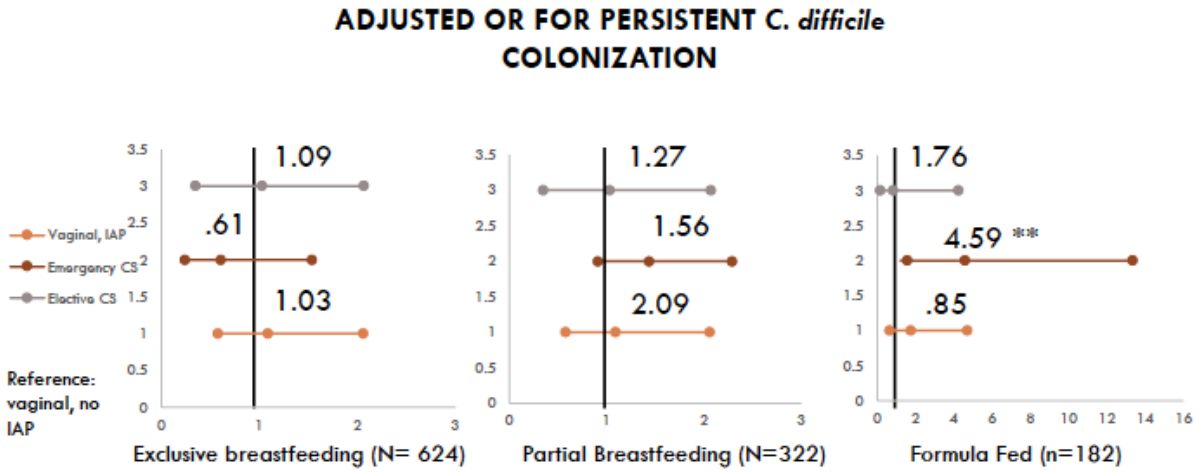


Figure F14. Persistent *C. difficile* colonization rates between mode of delivery stratified by sibling status

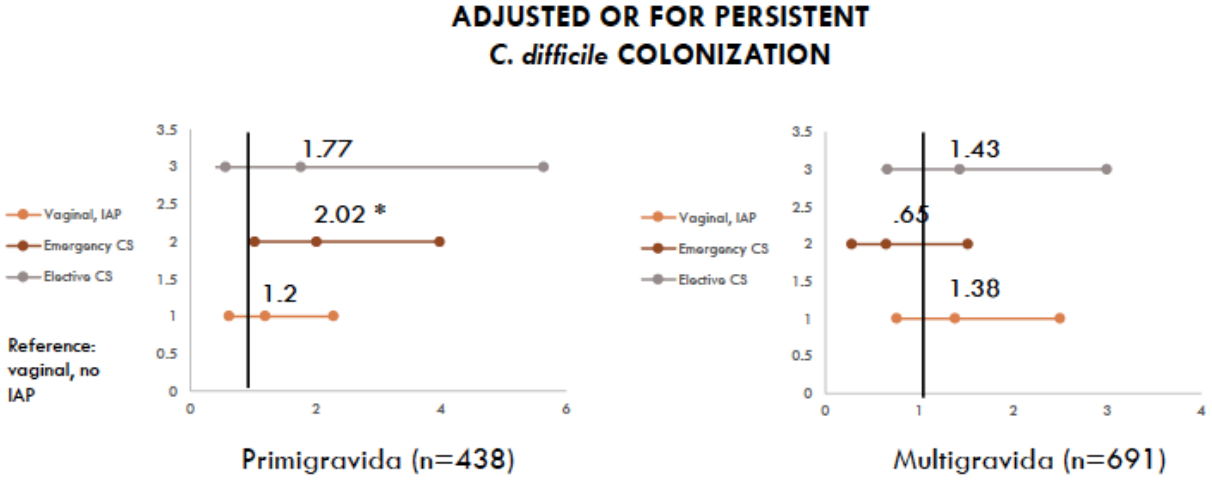
Persistent colonization- Adjusted stratified analyses



Adjusted for medical interventions, gravida, prenatal depression, breastfeeding 6 months, siblings, pets, hospital length of stay

***p≤.001. ** p ≤.01. * p<.05

Figure F15. Results from adjusted multiple logistic regression when stratifying within stratums of breastfeeding while comparing mode of delivery effects on *C. difficile* colonization. Global interaction term was borderline significant p=.057



Adjusted for medical interventions, prenatal depression, breastfeeding 3 & 6 months, siblings, pets, hospital length of stay

***p≤.001, ** p ≤.01, * p<.05

APPENDIX G- Table with current *C. difficile* methods and colonization in relation to mode of delivery

Main Findings		Found 10 times higher count of c difficile in infants whose mothers has no lactobacilli in their vaginal flora comparing to newborns from lactobacilli-colonized mothers at 3 months	C diff found in one vag baby (1/151). Differences in the ratio of facultative vs. obligatory anaerobes and also in the relative proportions of different bacteria between VG and CS infants
Methods	**EIA test for C difficile bacteria Yes / No If Yes, name of assay**	no	no
	Characterization of C difficile strains Yes / No If Yes, method and/or citation of method	no	no
	Molecular testing for C difficile Yes / No If Yes, method and/or citation of method	no	Bacterial counts were quantified by using a highly sensitive culture- independent reverse-transcription-quantitative-PCR (RT-qPCR) . Methods cited: (Matsuda et al., 2009)
	Culture for C difficile Yes / No If Yes, media and/or citation of method	bacterial swabs were taken from the posterior vaginal fornix, anaerobic bacteria were incubated for 4-5days in anaerobic consition at 37c in GENbox anaer. C difficile was cultured on Clostridium Difficile Agar.	no
	EIA test for C difficile toxin Yes / No If Yes, name of assay	no	no
Subject Characteristics	**Population tested (Inclusion/exclusion criteria)**	inclusoin criteria: healthy newborns at term, no congenital defects, no signs of infection until 3 days after delivery . Exclusion criteria: children born from ART pregnancies, matern-complicated pregnancies (diabetes, thyroid disorders, autoimmue diseases) and with clinical signs of infection both at mother and child until 3 days after delivery.	Inclusion: breastfed infants only, healthy infant and mothers with no indication of disease or intrauterine infection.
	Population tested (Time points/Age of infants (Number of infants	n= 79 maternal-neonatal pairs , sample at birth and 3 months . C difficile colonization: 29/79 (36%)	n= 151 healthy full-term infants [134 vaginally-born (VG); 17 elective esarean-
	Population tested (geogpraphic location, time period of study)	poland	Japan
		Gabriel et al., 2017. The influence of matenal vaginal flora on the intestinal colonization in newborns and 3-month-old infants	Nagpal et al.,2016. Sensitive Quantitative Analysis of the Meconium Bacterial Microbiota in Healthy Term Infants Born Vaginally or by Cesarean Section

C diff was found in a higher frequency and high counts in children living in satisfactory living conditions (private school children) compared to slum children . Persistence of c diff	elective cesarean delivery had the lowest richness and diversity compare to vaginal delivery .no strong differences by mode of delivery in the prevalence of C. difficile. Formula fed infants had an overrepresentation of C. diff. c-secton infants had a decrease in escerichia-shigella and bacteroides compared to vaginal	In the c-section infants Clostridium difficile was recovered in relatively large numbers; however, they could not be detected in the vag infants. The presence of this species in the CB infants may be an indication of the aberrant gut microbial composition in these infants,. The percentage of the C.	birth place and mode were strongly associated with coloniation by c diff, colonization rates for vaginally home-born, vaginally hospital borh and c-section were 19.1%, 27.2%, 43.4%. Inc hildren with a positive family history of atopy, vaginal home delivery decreased the off for
no		no	no
no	no	no	no
real time PCR	quantitative PCR.method cited: Penders et al., 2006.	PCR amplification was performed using 16S rRNA universal primers, 16S rDNA cloning and sequencing	real time PCR. Methods cited: (Belanger et al., 2004)
no	no	no	no
no	no	no	no
Inclusion: absence of diarhea for a minimum of 30 days, and delivery of a stool sample for use in gut microbiota analysis. Exclusion criteria: recent use of antibiotics and clinical evidence of serious illness. Children whose caregivers were unable to report the amount of time since using antibiotics were excluded	Exclusion criteria: infants of multiple births, Pregnant women who were planning on moving away for their recruitment centre within a 1 year, infants resulting from in vitro fertilization, were born with major congenital abnormalities, did not spend at least 80% of nights in the participants home	Inclusion criteria: healthy full term delivered and exclusively breastfed infants, no antibiotic use,	Exclusion: newborns with congenital abnormalities, premature infants (<37 weeks), children who received antibiotics in the first month of life
n=100 children in slums, n=30 private school children , ages 5-11. c diff Brazil	n= 24, sample collected at 4 months. C diff colonization: emergency c-section (~70%) vs vaginal (~ 55%), Winnipeg, Manitoba,Canada, 2009-2011	n=24 infants , sample at day 7, 11% c-section infants colonized with c.diff India	n= 952, 1 month samples, vaginally home-born, vaginally hospital born Netherlands, 2000-2002
Mello et al., 2016. Gut microbiota differences in children from distinct socioecomic levels living in the same urban area in Brazil	Azad et al., 2013. Gut microbiota of healthy Canadian infants: profiles by mode of delivery and infant diet at 4 months	Pandey et al., 2012. Comparative analysis of fecal microflora of healthy full-term Indian infants born with different methods of delivery (vaginal vs cesarean): Acinetobacter sp. prevalence in vaginally born infants	Van Nimwegen et al., 2011. Mode and place of delivery, gastrointestinal mirobiota and their influence on asthma and atopy

Neither atopic eczema nor food-specific IgE by 18 months of age were associated with time of acquisition of any particular bacterial group. Cesarean section delayed colonization by *Escherichia coli* and *Bacteroides* and *Bifidobacterium* species, giving way to, for example, *Clostridium* species. Lack of older siblings was associated with earlier	Infants born to c-section were more often colonized by c difficile, infant formula fed were more likely to be colonized with c diff compared with breastfed infants. Hospitalization(increased ~13% per dayof hospitalization compared to non-hospitalization) and prematurity(64% for prematurity vs 23% for term) were associated with higher counts of c diff	The rates of C. difficile positivity by at least one culture method were 13.54% and 37.63% in neonates in the enema and control groups, respectively (P<0.001). . difficile toxin positivity was detected in 22.92% and 53.76% of cases in the enema and control groups, respectively (P<0.001). ** Questionable findings and interpretation**
no	no	no
no	no	no
no	real time PCR Taq-man assays	no
Clostridium species were defined as straight gram-positive or gram-labile rods isolated from alcohol-treated samples (which kills vegetative cells bu leaves spores intact), cultivated on brucella blood	no	Three culture methods were used for *C. difficile*: alcohol shock followed by inoculation supplemented with 7% horse blood on to blood agar; direct culture on selective cycloserine-cefoxitin fructose agar (Oxoid, Basingstoke, UK); and enrichment culture in cycloserine-cefoxitin fructose broth supplemented with
no	no	C. difficile was confirmed biochemically in colonies morphologically suspected as being C. difficile. Direct detection of Toxin A and Toxin B from stool samples by enzyme-linked immunosorbent assay (ELISA) was performed using the RIDASCREEN C. difficile Toxin A/B ELISA (C0801) (Clinilab, Cairo, Egypt), according to the manufacturer's instructions.
inclusion criteria: a normal singleton pregnancy. Exclusion: language difficulty, moving house soon, prematurity (<37)	-exclusion: premature infants, infants who received antimicrobial agents, infants with insufficient stool sample, infants whose faeces were not collected between 3 and 6 weeks of age, infants with missing questionnaires	Aymptomatic others with uncomplicated vaginal delivery and their neonates without diarrhoae were included. Women eligible for vaginal delivery with a singleton, term baby were recruited into this study. The inclusion criteria were: asymptomatic mother with no diarrhoea, maternal age 20–40 years, and maternal body mass index 20–35 kg/m2. Maternal exclusion criteria were: preterm rupture of membranes, complicated or instrumental delivery, CS and prematurity. Neonatal exclusion criteria were: administration of medication, neonatal intensive care unit admission, any surgical condition and diarrhoea.
Infants were recruited perinatally in Göteborg (n = 116), London (n = 108), and Rome (n =	n=1032 infants , vaginally home-born, vaginally hospital born, artificial delivery and c-section	n= 189. sample collection: one week. C. difficile was detected in 13.54% and 37.63% of stool cultures from the enema group and the control group,
Goteborg, london, rome	Netherlands, -2000-2002	Egypt
Alderberth et al., 2007.Gut microbiota and development of atopic eczema in 3 European birth cohorts	Penders et al.,2006. Factors influencing the composition of the intestinal microbiota in early infancy	Nada et al., Does saine enema during the first stage of labour reduce the incidence of Clostridium difficile colonization in neonates? A randomized controlled trial

no difference was found between c. diff colonization and mode of deliveryor gestational age.	chance of acquiring a c. diff strain was highest in the 1st week of life. At 12 months of age acquiring new strains dropped sharply. No more than 1/3 of strains were toxin negative.36% were long-term colonizers, toxin producing strains reached hgiher population counts than toxin-negative ones in 1-week old. C-section children tended to carry more strains of ribotype 014(toxin producing) more often than vaginally delivered children (46% vs 17%), no diff between
no	no
no	*C. difficile* isolates were ribotyped on the basis of profiles obtained after PCR amplification of the 16S-23S rRNA intergenic spacer region .method as cited:
The identity of C. difficile isolates was confirmed by amplification of the 16S rRNA gene using C. difficile specific primers (PG48 and B) PCR reactions were used for the detection of C. difficile toxin genes A and B (TcdA andTcdB) and to detect genes encoding the enzymatic (cdtA) and binding (cdtB) components of the binary toxin as described by (Terhes et al..,2004) Mutation detection in gyrA and gyrB genes was carried out using PCR as reported by Dridi et al. 2002.	realtime PCR
The specimens were first treated by absolute ethanol (v/v) for 1 h before inoculation into Clostridium difficile moxalactam-norfloxacin agar plates (CDMN, Oxoid, England) which was supplemented with 7% (v/v) defibrinated horse blood. Culture plates were incubated for 48 hours at 37°C under anaerobic condition, and	Culture supernatants from all isolates were used to detect toxin B production by a direct cell cytotoxicity neutralization assay
PCR reactions were used for the detection of C. difficile toxin genes A and B (TcdA andTcdB) and to detect genes encoding the enzymatic (cdtA) and binding (cdtB) components of the binary toxin as described by Terhes et al. [14]. Mutation detection in gyrA and gyrB genes was carried out using PCR as reported by Dridi et al. [15].	- All *C. difficile* isolates were investigated for carriage of toxin genes by using PCR for the detection of genes for toxin A (*tcdA*) and multiplex real-time PCR for the detection of genes for toxin B (*tcdB*) and binary toxin (*cdtA/B*)
	Inclusion: infants with positive stool culture of c.diff - all infants delivered by c-section -first 20 C. difficile-positive children in the cohort, all caesarean section-delivered C. difficile-positive infants born during 2000 to 2003 (n 12), and for each of these caesarean section-delivered infants, the next born C. difficile-positive vaginally de- livered infant (n 12)
A total of 37/287 (12.9%) of C. difficile isolates were recovered from infants aged ≤1 year, of these 20/37 (54.1%) were toxigenic strains.	42 children
Jordan, 2015	Sweden,1998-2003
Aby-Kahder et al., 2017. Epidemiological features of Clostridium difficile Colonizing the Intetsine of Jordanian Infants	Adlerberth et al., 2014. Toxin-producing Clostridium difficile strains as long-term gut colonizers in healthy infants.

community-acquired C. difficile accounted for 66.7 % (66/ 99) of cases. Community-acquired C. difficile was also the main mode of acquisition whatever the strain, toxigenic (14/21, 66.7 %) or not (52/78, 66.7 %). Infants are widely colonized with non-toxigenic strain, but adult toxigenic strains circulate with no symptoms in infants too.. the carriage rate of C. difficile was 35 % for infants under 1 year of age, and 28 % for infants between 1 and 2 years

no

The diversity of the strains was analysed by PCR- ribotyping and multilocus sequence typing (MLST)

PCR

Faecal samples were tested for the presence of C. difficile using toxigenic culture, considered as a reference method, on fresh stools. The selective culture and C. difficile identification were performed as previously described (Rousseau et al., 2010). Bacteria obtained after 48 h anaerobic

Toxin-encoding genes were screened by PCR following the procedure described by Lemée et al. (2004b) for tcdA and tcdB, and by Stubbs et al. (2000) for cdtA and cdtB (binary toxin)

294 infants, 99 infants tested positive for c diff (33.7%). 21 of the c diff carriers were colonized with a toxigenic strain (7.1%). The distribution of infants according to age was as follows: 62 newborns aged 0–1 month, 189 infants aged 1 month–1 year, and 43 infants aged 1–2 years. Colonization prevalence was 25.8 % (16/62, with 3 toxigenic strains) for newborns, 37.6% (71/189, with 13 toxigenic strains) for infants aged 1 month–1 year, and 27.9 % (12/43, with 5 toxigenic strains) for infants aged 1–2 years old.. The colonization rate reached a maximum of 72.2% (13/18) for infants aged between 7 and 9 months. The proportion of toxigenic strains

Paris, France , 2008-2009

Rousseau et al., 2011. Prevalence and diversity of Clostridium difficile strains in infants

cesearean delivery and hospital following birth was associated with higher colonisation rates of both toxigenic and non-toxigenic strains. Exclusively breastfed infants were less colonized often colonized with non-toxigenic strains compared to formula fed infants. Colonisation of non-toxigenix strains increased the risk of develiping eczema and sensitization to food allergens. Therefore, Toxin A and B were not responsible for the increased risk. Recurrent wheeze was positively associated with toxin-positive c difficile

no

yes method as cited: (Belanger et al., 2003)

realtime PCR

no

real time fluorescence-based multiplex PCR cytotoxicity assay
-smart cycler

957 infants

- 1 month old fecal samples

- 200 infants (20.9%) colonized with non toxigenic strain

- 36 (.4%) colonized with toxigenic strains

exclusion: premature infants, infants who received antimicrobial agents, infants with insufficient stool sample, infants whose faeces were not collected between 3 and 6

Netherlands
- prospective cohort study
- 2000-2002

Penders et al., 2008. Toxigenic and non-toxigenic Clostridium difficile: determinants of intestinal colonisation and role in childhood atopic manifestations