Sleep Duration and the Gut Microbiota in Infancy: an exploration of the determinants of sleep and the association between sleep and the gut microbiota at 3 months of age in a Canadian birth cohort

by

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Abstract

RATIONALE

Sleep duration is critical to growth, cognitive development, and overall health status in infancy. Strategies to ensure that national recommendations for sleep duration in infants are met require knowledge of perinatal factors that affect infant sleep. Parental socioeconomic status (income, education, and occupation) has been linked to shorter infant sleep duration. Study I attempts to explain which factors mediate this relationship. Recent research has shown that sleep and the gut microbiome are intimately linked € as little as a few days of sleep deprivation leads to overgrowth of the intestinal microbiome. The influence of short sleep on the gut microbiota in infancy may be exceedingly important, as the composition of the infant gut microbiota plays a significant role in the development of the immune system.

OBJECTIVES

Study I - To investigate the mechanistic pathways linking maternal education and infant sleep.

Study II € To investigate the relationship between sleep duration and gut microbiota composition at 3 months of age after adjustment for significant covariates.

METHODS

Study I - An observational study was conducted on 619 infants whose mothers were enrolled at the Edmonton site of the CHILD birth cohort. Infant sleep duration at 3 months was assessed using the Brief Infant Sleep Questionnaire (BISQ). Maternal education was collected via maternal report. Prenatal and postnatal depression scores were obtained from the 20-item Center for Epidemiologic Studies Depression Scale (CES-D). Birth records and maternal report were the source of covariate measures. Mediation analysis (PROCESS v3.0) was used to examine the indirect effects of maternal education on infant sleep duration mediated through prenatal depression and birth mode.

Study II - A sub-study was conducted on 437 infants for whom microbiome data was available whose mothers were enrolled at the Edmonton site of the CHILD birth cohort. Infant gut microbiota were profiled using 16S rRNA sequencing from faecal samples collected at 3 months of age. Nonparametric statistical testing and logarithmic regression modelling was used to examine the relationship between sleep and abundance of gut microbial taxa. MaAsLin was utilized to determine which microbial taxa were associated with shorter infant sleep while adjusting for covariates. Linear regression was conducted on arcsine square root transformed gut bacterial relative abundance.

RESULTS

Study I - At 3 months of age, infants sleep on average 14.1 hours. Sixty-one percent of infants met the National Sleep Foundation recommendation of •14 hours. Lower maternal education and p renatal depression were associated with significantly shorter infant sleep duration. Emergency cesarean section birth was associated with 1-hour shorter sleep duration at 3 months compared to vaginal birth [without intrapartum antibiotic prophylaxis] (95%CI: -1.51, -0.48). Thirty percent of the effect of lower maternal education on infant total sleep duration was mediated sequentially through prenatal depression and birth mode (Total Indirect Effects: -0.12, 95%CI: -0.22, -0.03, p<0.05).

Study II - Sleep duration (as a continuous measure) was negatively associated with the relative abundance of *Clostridium* (Spearman Rho: -0.10, p=0.03). Short sleepers (<14 hours per 24-hour period) were more likely to be colonized with *Enterococcus* (76.5% vs 65.2%, p=0.01; *Exact*) and *Clostridium* (80.6% vs 67.4%, p<0.01) and less likely to be colonized with *Erwinia* (5.9% vs 12.7%, p=0.02) than infants obtaining •14 hours of sleep. Additionally, arcsine square root transformed *Lachnospiraceae* was positively associated with continuous sleep duration in exclusively breastfed (,: -0.07, 95% CI: -0.12; -0.02;p=0.01) and in infants born vaginally without exposure to maternal antibiotics (,: -0.07, 95% Confidence Interval [CI]: -0.13; -0.01;p=0.02) following adjustment. *Erwinia* was the only bacterial taxa identified by MaAsLin to be associated with short sleep following adjustment for metadata (beta-coefficient: -0.00065, p=0.016).

CONCLUSIONS

Prenatal depression and birth mode sequentially mediate the effect of maternal education on infant sleep duration. Furthermore, the relative abundances and colonization frequency of biologically important gut bacteria for infant growth are significantly associated with infant sleep duration at three months of age. Additional counselling on infant sleep for mothers who experience prenatal depression, or an emergency caesarean section birth, may help to decrease the sleep duration gap between infants of mothers with and without postsecondary education.

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Preface

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Section 1.2.5 of Chapter 1 of this thesis consists of part of a literature review which has been submitted for publishing as ƒCircadian Rhythm, Sleep, and the Gut Microbiota,, to Sleep Medicine Reviews.

Chapter 3 of this thesis has been accepted and is …in press‡ as ƒPrenatal Depression and Birth Mode Sequentially Mediate Maternal Education†s Influence on Infant Sleep Duration,, at the journal Sleep Medicine.

Chapter 4 of this thesis will be submitted for publishing as ƒInfant sleep duration is associated with gut microbiota composition in the Canadian Healthy Infant Longitudinal Development (CHILD) study,, in future.

The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Canadian Healthy Infant Longitudinal Development (CHILD) Study, Study ID: Pro00002099, July 2009 (Appendix A).

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List of Abbreviations

BISQ	Brief Infant Sleep Questionnaire
CES-D	Centre of Epidemiological Studies-Depression scale
CHILD	Canadian Healthy Infant Longitudinal Development
CRP	C-reactive protein
CS	Caesarean section
HPA axis	Hypothalamic pituitary adrenal axis
IAP	Intrapartum antibiotic prophylaxis
IL	Interleukin
LPS	Lipopolysaccharide
MP	Muramyl peptide
NREM	Non-rapid eye movement (sleep)
OTU	Operational taxonomic unit
PG	Peptidoglycan
PGRP	Peptidoglycan recognition proteins
REM	Rapid eye movement (sleep)
SLEEP-E	Sleep, Learning, Education and hEalth Program € Edmonton
SES	Socioeconomic status
TNF-ˆ	Tumor necrosis factor alpha

Chapter 1: Introduction

1.1 Background

To be the first individual to explore fecal matter under a microscope would have been both exhilarating and terrifying. Interest in our relationship with the microbes that inhabit us began with the realization that these miniscule vectors were transmitting fatal illnesses from one individual to another (1). This evolved into a long-held fear of the mystifying microbial world, the development of antiseptics, and the widespread use of antibiotics. However, this path of inquiry inevitably led to the exploration of the relationship between our immune system and the microbes it protects against and, more importantly, tolerates. The very bugs we learned to despise are an undeniable part of us and are now considered as vital as most other internal organ. Leading hypotheses postulate that our own evolution is closely intertwined with our gut microbes, many of whom are as reliant on us as we are on them (2). The large intestine, an extreme environment in its own right, is equipped with a low pH and virtually zero oxygen. This microcosm, which ideally offers its tenants with the complex carbohydrates they require (3), provides the perfect, and often singular, home for our bacterial hitchhikers.

What is it that we gain from this relationship? It was discovered in 1989 that the incidence of hay fever is increased in adults with fewer older siblings compared to those with more older siblings (4). This led to the Hygiene Hypothesis: that exposure to increased richness and diversity of bacteria in infancy could promote immune tolerance and decreased atopy in later life (4). Since then, we have learned that not only exposure, but exposure to specific bacteria in infancy can have specific immunomodulatory effects which last into adulthood (5,6). These findings have revolutionized medicine and our understanding of the developmental origins of human health: systemic inflammation is an element of the most prevalent non-communicable diseases of the twenty-first century. Lack of exposure to bacteria in infancy can prime the immune system to be over-zealous, and the previous exposure, environment, diet, stress, and exercise which shape the composition of our gut bacteria are important keys in the etiology of multiple diseases. The careful investigation of the exposures which seed our gut microbiome in infancy

will one day contribute to the story of how we cured the most devastating of immune system-originating diseases (hopefully).

This thesis uses data from the Edmonton site of the Canadian Healthy Infant Longitudinal Development (CHILD) Study and examines sleep and the gut microbiota at 3 months of age. Sleep disturbances may impact the host - gut microbiota relationship in the same way as other physiological stresses (7).The purpose of this thesis is to: 1) explore the factors which contribute to infant sleep duration, with a special focus on sociodemographic factors and factors related to the microbiome and 2) investigate the association between sleep duration and the gut microbiota in infancy.

1.2 Literature Review

1.2.1 Importance of sleep in infancy

The National Sleep Foundation recommends 14-17 hours of sleep per day in 0-3 month old infants (8); however, according to a recent global meta-analysis undertaken by Galland et al, over 50% of infants are sleeping less than 14 hours per day by 3 months of age (9). Short sleep is significantly associated with increased risk of mortality, diabetes mellitus, hypertension, cardiovascular disease and coronary heart disease in adulthood (10) as well as increased BMI across the lifespan (11,12). In infancy, there is evidence that short sleep increases the future risk for obesity (13); however, short sleep may predispose the infant to any diseases known to be influenced by sleep duration in adulthood.

Sleep is integral for the overall health of an infant. Sleep plays an important role in the functioning of the immune system (14). Sleep loss or deprivation can lead to decreased barrier integrity (15), inflammation (16), and even enteric overgrowth (17). Sleep duration also increases transiently to support periods of rapid growth in infancy (18). Furthermore, in infancy and childhood, sleep duration is associated with the development of cognitive function (19). Understand the factors which contribute to sleep duration is the first step in the process of protecting and improving infant sleep.

1.2.2 Development of sleep in infancy

The infant circadian rhythm begins development in-utero and is not fully developed at birth (20,21). During the perinatal period, maternal rest-activity, heart rate, cortisol, melatonin and body temperature play a role in in the day-night rhythmicity of fetal heart rate (22). Infant sleep consists of 3 parts: quiet sleep (NREM), active sleep (REM), and indeterminate sleep. The proportion of sleep spent in active sleep is highest during periods of development of the central nervous system (23). Maturation of the infant circadian rhythm results in an increase in quiet sleep and a decrease in indeterminate and active sleep (23). REM sleep occupies approximately 55% of total sleep time during infancy, and declines to about 20-25% of sleep by five years of age (21). At birth, infants do not have a clear circadian rhythm and sleep patterns of day and night are not distinct (24). Instead, the full-term baby has a sleep-wake cycle of 2-4 hours which is thought to be predominantly entrained by feeding (22).

At birth, the ultradian and circadian rhythms are often disrupted by new environmental factors. In a study by McGraw et al. (25) when environmental factors (feeding, light) were optimally controlled, a clear circadian rhythm of body temperature in the infant was discernible at one week of age. By two weeks of age, the infant sleeps more during the night than during the day (20). At 3 months of age in the full-term infant, the infant undergoes a noticeable change from fetal sleep patterns to infantile sleep patterns (22).

Sleep duration, quality, and organization appear to be intrinsically linked. Infants who are €poor• sleepers tend to have a more chaotic sleep-wake cycle whereas €good• sleepers tend to have a more structured sleep-wake cycle (20). Inter-individual infant sleep variability decreases with increasing age (9). Increased requirements of sleep in infants are generally thought to reflect increased rates of neurosensory development and physical growth (18,23,24). Age plays a fundamental role in sleep duration and organization (24), with considerable sleep consolidation occurring between three and seven months of age (26). Infant sleep behaviour, independent of age, is driven by demographics, parental and infant factors, co-sleeping, feeding type, and environment (9,26, 30).

1.2.2.1 Sociodemographic factors

Short sleep is a sociodemographic problem in all age groups. In adults, short sleep is not only associated with, but also explains the relationship between low socioeconomic status (SES) and poor health (31). Due to the persistence of sleep problems which develop in early life (32), infancy is a critical period for influencing not only sleep, but health across the lifespan (33). Sleep improvements in infancy have the potential to positively influence both health and SES. James J. Heckman has written passionately about the complex web of exposures in childhood which contribute to the transgenerational transmission of economic disadvantage (34). Importantly, he identified that low parental SES exposes the infant to a number of factors which result in a lack of cognitive, emotional, and social skills which then predispose the child to future social and economic failure. Sleep is an important part of this puzzle， short sleep in infancy and childhood is associated with reduced cognitive development at 2 years of age (19), emotional and behavioural problems at 2-3 years of age (35), and inferior school performance (36). Additionally, compared to interventions implemented later in life, the rate of return to investment in human capital is exponentially larger for interventions which occur in preschool or sooner (34). Understanding how SES impacts sleep in infancy is an important first step in developing interventions to reduce the sleep gap between high and low SES infants.

SES is defined by the APA as a combination of an individual*ƒ*s income, education, and occupation (37). Previous studies have attempted to elucidate the socioeconomic determinants of infant sleep. In infants, familial material deprivation has been associated with reduced sleep quality (38), but the pathway between exposure and outcome is poorly understood. One such reason may be parental education level; however, studies of maternal education level*ƒ*s influence on infant sleep have found inconsistent results (38,39). Poor sleep habits in parents of reduced SES status may also explain differences in infant sleep duration (40). Sleep habits and disorders are moderately heritable, so children may genetically inherit their sleep patterns from their parents (41). Alternatively, circadian rhythm dysfunction can also be programmed in utero or during infancy due to maternal sleep problems and

subsequent melatonin levels (42). Sleep patterns of the mother strongly predict infant sleep in a unidirectional manner (40). Mothers who report better sleep tend to have infants with similar sleeping behaviour.

SES may influence infant sleep through maternal depression and maternal sleep quality during pregnancy (43, 45). Maternal prenatal depression and maternal sleep problems tend to co-exist in pregnancy (43). Reduced maternal melatonin associated with sleep problems in pregnancy can transmit sleep issues to the infant in utero (46,47). Maternal distress is also observed to mediate the association between lower household income and reduced maternal sleep quality (45). Finally, maternal prenatal and postnatal depression are independently associated with shorter infant sleep duration (39,48).

1.2.2.2 Gut microbiome related factors

Breastfeeding

The effect of breastfeeding on infant sleep appears to be age-dependent (40,49,50). At 12 weeks of age, infants who are breast fed have increased sleep duration and efficiency (49). These benefits may be due to increased tryptophan concentrations in breast milk preceding nighttime sleep (49). However, at 6-12 months of age, breastfeeding status does not appear to be associated with any significant effects on sleep (50,51).

Birth mode

The impact of birth mode on sleep quality and quantity has not been explored extensively. Caesarean section has been shown to influence time spent in wakefulness, as well as sleep organization on the first postnatal day; however, no effects were observed by the second postnatal day (52). Studies examining sleep duration in 3-month-old infants did not find a difference between vaginally and

caesarean born infants, but did not distinguish between scheduled versus emergency caesarean section type (53).

Antibiotics

Multiple studies have found a relationship between antibiotic administration and sleep. In clinical trials, up to 4 percent of people taking the antibiotic Levaquin® (levoflaxin) developed insomnia, which resolved upon discontinuation of the medication (54). When 200g of minocycline was administered to healthy men, slow wave sleep was decreased (55). However, when ampicillin was examined it did not appear to impact slow wave or REM sleep (55).

Hospitalization

Hospitalized infants may be at an increased risk of short sleep compared to non-hospitalized infants. In a study of behavioural state changes in response to noise and nursing interventions in 55 premature infants in NICU, 78% of infants changed their behavioural state in response to noise and nursing interventions (30). Most changes in behavioural state were from a regular or irregular sleep state to a fussy and crying state. Noise alone resulted in 43% of infants changing from the sleep state to the fussing and crying state.

Ethnicity

Children (0-12 years of age) in Asian countries tend to sleep an average of 1 hour less than their counterparts from Caucasian/ non-Asian countries (9). In the US, Black and Hispanic infants sleep less during the night and more during the day than white infants at 6 months of age, resulting in higher total sleep in white infants at 6 months, 1 and 2 years of age (39). Ethnic/racial differences in sleep duration may be due to co-sleeping, which is more common among African American and Hispanic ethnic groups (29). Co-sleeping infants have more awakenings per night although amount of nighttime spent awake is not different than solitary sleeping infants (29).

Probiotics

The impact of probiotic administration on sleep in a randomized controlled trial is reviewed in depth in Section 1.2.5.4.4.

1.2.3 Validity of common sleep measures in infants

There are four common methods for collecting infant sleep data: 1) polysomnography, 2) actigraphy, 3) parent report using a sleep log, and 4) parent report using the Brief Infant Sleep Questionnaire (BISQ). Polysomnography is considered the gold standard in sleep measurement and provides information on sleep organization through the collection of brain activity (electroencephalogram; EEG), eye movements (electrooculogram; EOG), muscle activity or skeletal muscle activation (electromyogram; EMG), heart rhythm (electrocardiogram; ECG), breathing functions, respiratory airflow, and peripheral pulse oximetry. This provides the clinician/researcher with adequate information to diagnose obstructive sleep apnea and to evaluate cardiorespiratory function in infants and children with chronic lung disease or neuromuscular disease (56). Polysomnography reliability in infant populations has been confirmed (57). However, polysomnography may be more difficult to conduct in infants and young children who may pull at the equipment and connecting cables during testing.

Actigraphy is the use of an actimetry sensor, usually worn on the wrist or waist, to measure and analyze activity in an individual. Algorithms are then applied to differentiate between sleep, wakefulness, and different levels of physical activity. In adult and pediatric populations, actigraphy has been validated against polysomnography and has been found to be no different when clinically significant thresholds are applied (58). Furthermore, actigraphy provides adequate measures of total sleep time when compared to polysomnography (overall mean difference from meta-analyses: 10.14 minutes in adults with insomnia and 14.54 minutes in adults being tested with home sleep apnea tests) (58). In pediatric populations, actigraphy is commonly noted to have poor specificity to detect wake after sleep onset (59). Subjective sleep reporting using a sleep log regularly overestimates the amount of total sleep time when compared to

actigraphy (58). The large demand on participant time to complete daily sleep logs lead to the creation of the BISQ. The BISQ is an 11-item questionnaire which requires the least amount of participant time compared to the 3 previously mentioned measurement methods. The test-retest correlations of the BISQ measurements are very high ($r > .82$) (60). Compared to actigraphy and sleep logs, sleep-onset time, nocturnal sleep duration, and night wakings from the BISQ are significantly correlated at an alpha level of 0.05 (60). Furthermore, the nocturnal sleep duration derived from the BISQ is statistically similar to that obtained using actigraphy (9.35±1.40 hours vs 9.48±0.82 hours; 43 children aged 6-29 months) (60).

1.2.4 The infant gut microbiota

The human gut microbiota is a multifaceted ecological network of bacteria, eukaryotes, Archaea, viruses, and phage which exist within the human gastrointestinal tract. This community of microbiota can protect the host against pathogenic microbes, stimulate and modulate the host immune system, produce essential metabolites, and nourish gastrointestinal cells (61). Microbial colonization and maturation of the gut takes place during the prenatal period until approximately 3 years of age and sets the stage for the adult microbiome (62). This development of the gut microbiota occurs during a critical time for immune system development, as evident in observed links between its composition and future immune system health (62, 65). The microflora hypothesis proposes that limited microbial exposure results in altered development of the infant gut microbiota due to an overly hygienic lifestyle (66). These alterations due to limited exposure are thought to impact the development of the immune system, and in turn impact the future risk of allergic disease. Maternal factors, delivery mode, breastfeeding status, antibiotic exposure, and home environment have been found to influence the composition of the infant gut microbiota (61,62,67, 69). The contribution of these exposures to the successive colonization of the infant gut has been reviewed in depth (61,64). In opposition to the sterile womb paradigm, genetically labelled bacteria administered orally to pregnant mice has been isolated from the meconium of the offspring (70). In 2012, Rautava et al. found that probiotic supplementation in mothers resulted in bacterial alterations of the

infant meconium and placenta (71). However, many studies provide evidence to refute the idea that microbial colonization of the placenta and womb occurs during healthy pregnancy (72,73).

In cases of vaginal birth, the infant*f*s first exposure to microbes occurs during contact with the microbiota of the vaginal tract and maternal faeces during birth (64,74). This initial inoculation begins the colonization of the infant gut microbiota. At first, the infant gut is an aerobic environment. The first to colonize the infant gastrointestinal tract, *Escherichia*, *Streptococcus*, and *Enterococcus* remove oxygen from the infant gut creating an anaerobic environment facilitating the shift to obligate anaerobes (64,75). Within a few days following birth, the vaginally-born infant*f*s intestinal microbiome resembles the maternal vaginal and fecal microbiota, distinguished by high relative abundance of *Lactobacillus*, *Prevotella*, and *Sneathia spp*. (61,74). Infants who are delivered via caesarean section instead tend to have a microbiome which somewhat resembles bacterial communities found on skin and within a hospital setting (61). Caesarean section born infants tend to have delayed and decreased colonization of *Bifidobacterium spp*. and *Bacteroides spp*., reduced diversity of the phylum Bacteroidetes, and increased colonization with *Staphylococcus, Corynebacterium, Propionibacterium spp*, and *Clostridium difficile* compared to vaginally born infants (61,74,76). Longitudinal studies have shown that gut microbiome changes associated with delivery by caesarean section can persist to 1 year of age and minor differences have been detected up to 7 years of age (61,77).

Breastfeeding, which functions as a source of both pre- and pro-biotics, results in an infant gut microbiome dominated by bifidobacterial species (64). Formula-fed infants, on the other hand, tend to have a more diverse microbial composition, with an increased abundance of *Escherichia coli, Clostridia,* and *Bacteroides* (61). The effects of birth mode and infant feeding exposures on the composition of the gut microbiome are additive and interactive. In vaginally born breastfed infants who have not been exposed to antibiotics, bifidobacteria comprises the largest group within the infant gut at 3 months of age (68). Alternatively, the increased abundance of Firmicutes at 1 year of age in infants delivered by emergency Caesarean section is ameliorated by exclusive breastfeeding for a minimum of 3 months (68).

The introduction of solid foods leads to increased diversity, increased abundance of *Bacteroides* and *Clostridium* and decreased abundance of *Lactobacillus* and *Bifidobacterium* (61).

Common medical interventions during birth and the immediate postnatal period (caesarean delivery, maternal intrapartum antibiotic prophylaxis (IAP), postnatal hospitalization, and postnatal infant antibiotic treatment) all lead to reduced abundance of bifidobacterial and members of the *Bacteroidaceae* family and increased abundance of *Clostridium* and *Enterbacteriaceae* (76). In 3 month old infants, maternal IAP results in differential gut microbiota community structure defined by reduced abundance of *Bacteroides* and *Parabacteroides* and increased abundance of *Enterococcus* and *Clostridium* (68). Once home from the hospital, the infant environment, including geography and presence of siblings and pets in the home, has been found to impact the infant microbiome (61,78). Geographical differences in infant gut microbiota composition only becomes apparent following weaning; consequently, geographic differences may be the result of diet variability (61). Alternatively, geographic differences in microbiota may also be driven by environmental exposure or genetic differences (61). The presence of older siblings in the home reduces *Peptostreptococcaceae* abundance and increases *Bifidobacterium* abundance (79). Early-life exposure to pets increases the abundance of *Oscillospira* and *Ruminococcus*, which have been negatively associated with childhood atopy and obesity (78).

Based on evidence accumulating from the 1980s, the composition and diversity of the gut microbiota during the critical window predisposes the infant to risk for several types of allergic disease (80). In mice, early antibiotic exposure has been shown to have lasting effects on immunity and metabolism, with earlier exposure resulting in significantly greater metabolic changes (6). Transplantation of the intestinal microbiota of antibiotic-exposed mice into a germ-free model results in a complete shift of metabolic and immune consequences to the new host. More recently, studies conducted in animal models and adult humans have provided evidence that obesity may be associated with the gut microbiota but some of this evidence is incongruent (81, 83). Comprehensive summaries of the literature on risk for overweight and allergic disease can be found in review papers by Bridgman et al. (80) and Koleva et al.

(82). As evident in associations between infant antibiotic treatment and childhood asthma or obesity (76,84), early life medical interventions that affect gut microbiota have long-term health sequela.

1.2.5 Sleep, circadian rhythm and the gut microbiome

Interest in the role of the gut microbiota in health and disease has allowed for rapid growth in this research area over the past 15 years. Multiple studies have highlighted the importance of colonization and succession of the gut during the critical window of infancy. During this period of infancy, the gut microbiome plays a vital role in the education of the immune system and development of cells in the gastrointestinal tract. The gut microbiota also plays a role in the regulation of the host metabolism throughout life. The infant gut microbiota composition has been associated with risk of obesity, asthma, and allergic disease. Understanding which factors play a role in the composition of the infant gut microbiota may lead to novel treatment options to prevent diseases which impact a significant portion of our population.

Globally, most infants at 3 months of age are not obtaining the recommended amount of sleep(9). Unfortunately, studies of sleep and the gut microbiome have been conducted in animal (85, 90) and adult (85,91, 93) populations, but not infants or children. Lack of sleep has the ability to activate pro-inflammatory pathways (14), which provides evidence that sleep may play a role in the health of the gut microbiome. The following sections aim to summarize the literature on the associations between sleep, circadian rhythm, and the gut microbiome.

1.2.5.1 Short chain fatty acids influence clock gene expression in vitro and in vivo

One of the most important cues humans receive daily are from microbial metabolites absorbed by the gastrointestinal tract, some of which have been associated with reduced risk for allergic and metabolic

disease (94). Some of the most important microbial metabolites - short-chain fatty acids acetate, propionate, and butyrate - change over the course of a day, with the highest concentrations in faecal samples occurring earlier and decreasing throughout the day (95). Indeed, gut microbial metabolites such as the short-chain fatty acids butyrate and acetate may influence clock gene expression. Leone et al. found that a lack of gut microbiota, and consequently a deficit of microbial metabolites, resulted in markedly impaired central and hepatic circadian clock gene expression (90), suggesting the possibility that gut microbiota play a role in propagating circadian rhythm at the molecular level. In vitro, Leone et al. found marked changes to clock gene Bmal1 and Per2 expression in hepatic cells of mice following the administration of sodium acetate and sodium butyrate (90). Depending on the light-dark or feeding cycle, Per2 expression was higher following acetate administration and lower following butyrate administration; Bmal1 expression was consistently higher following short-chain fatty acid treatment, especially for butyrate. In vivo in germ-free mice, 5 days of treatment with butyrate two hours after lights off (active period in mice) resulted in a significant increase in the Per2:Bmal1 mRNA ratio in hepatic cells (90). Additionally, the same treatment resulted in a non-significant increase in the Per2:Bmal1 mRNA ratio in medio-basal hypothalamic cells ($p=0.053$) (90). Clock genes such as Bmal1 and Per2 regulate circadian processes at the molecular level (96); their ratio is a marker of metabolic regulatory networks in the liver. An increase in the Per2:Bmal1 mRNA ratio has been associated with steatosis (97).

1.2.5.2 Sleep and the gut microbiome in animal and human adult studies

Almost 30% of adults obtained „6 hours of sleep per night in the United States in 2012, representing a 31% increase in the prevalence of short sleep duration since 1985 (98). Even more troubling, 23.9% of children aged 5-15 in Australia were found to sleep less than 9 hours per night, with short sleep duration strongly predicting obesity independent of sociodemographic variables, fruit and vegetable intake, and physical activity and inactivity (99). Short sleep in adulthood has been linked to increased risk of inflammatory-mediated diseases including obesity, diabetes mellitus, hypertension,

cardiovascular disease and coronary heart disease (10). Finally, short sleep reduces insulin sensitivity, increases ad libitum food intake, and leads to visceral white adipose tissue inflammation (100, 102).

Sleep fragmentation also promotes obesity, reduces leptin sensitivity (100), induces inflammatory cytokine expression and elevates serum corticosterone levels (103). Affecting up to 38% of some populations, obstructive sleep apnea is a leading cause of sleep fragmentation in adults (104).

Inflammation, sleep, and the gut microbiome are linked bi-directionally. Sleep loss increases inflammation, which must be mediated through the gut microbiome; inflammation and infection induce sleep; and sleep loss increases susceptibility to infection. As little as one night of total sleep loss is sufficient to raise circulating levels of the pro-inflammatory biomarkers, tumour necrosis factor alpha (TNF…) and C-reactive Protein (CRP); serum CRP levels rise progressively in response to 4 days of total sleep deprivation (105,106). Likewise, the interleukin (IL)-6 cytokine has been found to be elevated after one night of total sleep deprivation (107), while Shearer et al. reported elevated IL-6 levels only after the fourth day of total sleep loss (108). Greater 24-hour secretion of both IL-6 and TNF… is also observed in response to partial sleep deprivation over a 1 week period (4-6 hours of sleep per night) (109). Lengthier partial sleep restriction (10-11 days of 4 hours of sleep opportunity per night) results in progressively higher levels of CRP (106)and IL-6 (110), although greater mRNA expression for IL-6, and additional cytokines, IL-1† and IL-17, has been detected with only 5 nights of partial sleep deprivation (111). Increased cytokines in response to sleep loss, stress, or infection reciprocally induce sleep in the host (14).

Short sleep duration also results in a release of catecholamines and glucocorticoids; these hormones are known to stimulate mast cell degranulation, which leads to the breakdown of intestinal epithelial cell tight junctions (7,14). When the tight junctions are weakened, gut microbiota and their metabolites can infiltrate the circulation, resulting in systemic inflammation (7). In the absence of gut microbiota, Bailey et al found that environmental stress can no longer cause systemic inflammation in mice (112).

Circadian misalignment and sleep fragmentation are capable of inducing a breakdown of the intestinal epithelial barrier (88,113). Additionally, intestinal epithelial barrier integrity is influenced by oxidative stress, dietary components, the gut microbiota, and microbial metabolites (113, 115). When the tight junctions of the intestinal epithelium are disrupted, bacteria and their end-products are able to move through the ‡leaky-gut*f* into the circulatory system. Bacteria and their end products have been found to translocate to the mesenteric lymph nodes, liver, and the brain (17,116). Muramyl peptides (MPs), the building blocks of peptidoglycans (PGs), and lipopolysaccharide (LPS), are significant components of bacterial cell walls. Although PGs are associated with Gram-positive bacteria and LPS are associated with Gram-negative bacteria, some Gram-negative bacteria, such as *Enterobacter cloacae*, can upregulate peptidoglycan recognition proteins (PGRP) (116). Increased quantities and translocation of MPs and LPS induce fatigue in the host (117,118). This somnolence - specifically, enhanced NREM sleep - is at least partly mediated by TNF… and LPS activation of vagal afferents(119). LPS plays a significant role in the progression of obesity and metabolic dysfunction (120), and may partly explain the connection between sleep loss and metabolic disorder.

In addition to disrupting the epithelial barrier, extreme sleep deprivation disrupts the blood brain barrier (15). As a result, sleep deprivation upregulates PGRP expression in the brainstem and hypothalamus of rats (116). This upregulation of PGRP in the brain during sleep deprivation suggests that lack of sleep results in gram-positive microbial end-product intestinal translocation and the crossing of the blood brain barrier by these products. Furthermore, it suggests a potential role of PGRP in the homeostatic regulation of sleep.

For a comprehensive discussion on the interplay between sleep, microbial infection and bacterial translocation, please refer to Krueger and Opp (121).

1.2.5.3 Sleep fragmentation

Poroyko et al. found that the impact of sleep fragmentation on the development of metabolic syndrome may be mediated by altered gut microbiota in the mouse (88). Four weeks of chronic sleep fragmentation (awakening with horizontal bar sweeping cage every 2 minutes during light [7:00 am , 7:00 pm; inactive]) increased food intake and encouraged growth of *Lachnospiraceae* and *Ruminococcaceae* families , and decreased growth of the *Lactobacillaceae* family (88). Additionally, sleep fragmentation lowered the abundance of Bacteroidetes, Actinobacteria, and *Bifidobacteriaceae*. Various *Bifidobacterium* strains exert anti-obesity and anti-inflammatory effects in humans and mice (7), and *Bifidobacterium* (*infantis*) has been found to attenuate the exaggerated HPA stress response in germ free mice (122). Reductions in bifidobacteria due to sleep fragmentation could be especially detrimental in infant populations, as bifidobacteria dominate the gut of breastfed infants and protect against opportunistic pathogens (64). Dysbiosis characterized by increased abundance of *Prevotellaceae, Paraprevotellaceae* and *Lachnospiraceae*, has been reported in response to intermittent hypoxia, which is characteristic of obstructive sleep apnea (123). *Prevotellaceae* is positively associated with fiber intake and inversely associated with consumption of an animal-based diet, type 1 diabetes and Parkinson*ƒ*s disease (124, 126).

Germ-free recipients of microbiota from mice experiencing sleep fragmentation developed systemic and visceral white adipose tissue inflammation and altered insulin sensitivity, providing evidence that metabolic disturbances from sleep fragmentation are mediated by an altered gut microbiota (88). However, increased food intake during periods of sleep fragmentation may have confounded the effect of sleep fragmentation on the gut microbiota. Additionally, Wellman et al. found that mechanical stimulation using a sleep fragmentation chamber during either the light (inactive) or dark (active) period alone produced significant alterations in the gut microbiota (127). Stress due to this experimental design may be responsible for a proportion of the resultant gut dysbiosis, regardless of sleep disruption. However, mechanical activation during the light, but not dark, period resulted in suppression of

chemokines which regulate inflammation and maintain the immune mucosal barrier and increased inflammatory IL-33 in the cortex (127).

1.2.5.4 Sleep quality and duration

1.2.5.4.1 Observational studies of sleep quality

Subjective measures of sleep quality provide insight into important aspects of sleep such as sleep disturbances, latency, and efficiency which can occur independently of sleep duration. Observational studies of sleep quality and duration help to determine the consequences of chronic exposure to poor quality or abnormally long or short sleep which may not occur in response to acute exposures. Additionally, these studies may discover unanticipated confounding factors of the relationship between sleep and gut microbiota. However, these studies can face issues establishing temporality, direction of causation, and avoiding self-report bias. In a meta-analysis of sleep disturbance, sleep duration, and inflammation examining results from 72 studies (n > 50,000) conducted in 2016, self-reported sleep disturbance was associated with higher levels of CRP and IL-6 (128). Shorter self-reported sleep duration was associated with higher levels of CRP but not with IL-6 or TNF…, while the extreme of long sleep duration was associated with higher levels of CRP and IL-6 (128). Gut dysbiosis precedes and is necessary for stressor-induced systemic inflammation (112,129) and therefore is predicted to precede systemic inflammation subsequent to sleep disturbance or shortened sleep duration.

One of the most commonly used questionnaires to measure sleep disturbance in adults is the Pittsburgh Sleep Quality Index (PSQI), which has a high sensitivity and specificity (130). The PSQI is reported as a global sleep dysfunction score, which is a composite measure of seven areas: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medications, and daytime dysfunction. In a small group of breast cancer survivors (*n=12*), global sleep dysfunction was found to be associated with taxa abundance *of Paracoccus, Rikenellaceae*, and

Clostridium in fecal samples (92). Another observational study of 37 healthy older adult participants found that global sleep dysfunction was associated with lower proportions of the phyla Verrucomicrobia and Lentisphaerae (93). Interestingly, both Verrucomicrobia and Lentisphaerae have also been found to be reduced in individuals with post traumatic stress disorder (PTSD) (131), which provides additional evidence that a reduction in these two microbiota may contribute, or respond to, stress in the host. Furthermore, sleep dysfunction and stress are strongly intertwined, and either study may be confounded by sleep dysfunction or stress in the participants (132).

1.2.5.4.2 Experimental studies of sleep deprivation

Experimentally imposed short sleep duration (7 days of 20 hour per day forced activity in slowly rotating drums) in 12-week-old male Sprague-Dawley rats, fed ad libitum standard chow, resulted in reduced gut microbial species richness compared to baseline values; no species richness differences were observed with control rats (85). Principal coordinate analysis showed overlap in the gut microbial composition of sleep deprived and control rats; however, it was noted that one animal in the control group fell outside of the 95% confidence interval from the centroid of the control group. This raises questions over whether exposure or overall health was similar within control animals. Sleep deprived rats experienced increased TM7-3a relative abundance compared to baseline (85). TM7, the phyla containing TM7-3a, has been associated with inflammatory bowel disease, vaginosis, and periodontitis (133). Contrary to the results of sleep fragmentation studies in mice (88), sleep deprived rats experienced reduced age-related weight gain versus controls. This discrepancy was likely a function of a poor choice for the sleep deprivation intervention, which was activity-based and caused weight loss in study rats, whereas sleep restriction is generally associated with weight gain in humans (10,134). Sleep deprivation in humans is also associated with decreased physical activity (135).

While controlling activity level and dietary intake in humans, Benedict et al examined insulin sensitivity and gut microbiota of 9 normal-weight male subjects following two nights of normal sleep (sleep opportunity 22:30-07:00 h) and two nights of partial sleep deprivation (sleep opportunity 02:45-07:00 h) (91). Fasting and postprandial insulin sensitivity were reduced following partial sleep deprivation. Authors concluded that partial sleep deprivation resulted in an increased Firmicutes: Bacteroidetes ratio in gut microbiota and a higher abundance of the families *Coriobacteriaceae* ($p=0.049$) and *Eryspelotrichaceae* ($p=0.049$). Sleep loss also reduced the abundance of the Tenericutes phylum ($p=0.03$). No changes in microbial beta diversity, or fecal short-chain fatty acid concentrations were seen after sleep loss. Unlike other studies, false discovery rate for multiple comparisons were not employed; however, only the most abundant microbial taxa were examined, greatly reducing the number of comparisons conducted compared to other studies.

Conversely, in a study of 11 healthy male and female adult subjects, changes to gut microbial composition, as measured by beta diversity (weighted UniFrac), were not observed following 5 days of partial sleep deprivation (85), despite the fact that most subjects had experienced increases to BMI. However, this sample represented only half of subjects recruited due to the inability to adhere to sample timing criteria and recover DNA from samples. Six subjects underwent a second round of sleep deprivation as follows: baseline sleep for 2 days, 1st sleep restriction over 5 days, 1st recovery period for 5 days, 2nd sleep restriction over 5 days, 2nd recovery period for 2 days. Again, beta diversity in the pre-post sleep restriction periods and between study groups did not differ, nor did taxon abundance at the phyla or species level following false discovery rate correction. There were several aspects of the analysis of this study that were unclear, for example, whether data from six or 11 subjects were included in the analysis of individual taxa, or whether absolute (count) or relative abundance levels were assessed. Although the study followed a repeated measures design, repeated measure analysis methods were not pursued. This study is distinguished from that by Benedict et al. due to the entire protocol being conducted within the hospital and non-standardized dietary intake (91).

1.2.5.4.3 Complete sleep deprivation leads to enteric overgrowth and multi-organ infection

Everson and Toth elegantly explored the effects of complete sleep deprivation using a mechanical bar that pushed rats into water whenever they began to fall asleep as measured by electroencephalogram (17). To control for the stress of the mechanical bar set-up, yoked-controls were yoked to the experimental rats, allowing them to sleep whenever the experimental rat was active. Sham-control rats were not exposed to the mechanical bar set-up. Sleep deprivation led to significant overgrowth of intestinal microbiota. Based on culture methods, sleep-deprived rats had higher counts of aerobic bacteria, facultative anaerobic bacteria, *Enterobacter*, *Klebsiella pneumoniae*, and *E. coli* in either the ileum or cecum than sham-control rats. Compared to yoked rats, 72% vs 26% of sleep-deprived rats suffered from microbial invasion of their mesenteric lymph nodes by experimental day 20, with *Enterobacteriaceae* being the most common bacterial family cultured. *Enterobacteriaceae* is a microbial family which contains many opportunistic pathogens that produce substantial amounts of LPS. Lymph node infection was succeeded by polymicrobial infection of major organs. This study provides experimental evidence that sleep deprivation results in significantly altered gut microbial composition and overgrowth, and bacterial translocation independent of a stress pathway.

1.2.5.4.4 Probiotic interventions to improve sleep

Few studies have tested the effectiveness of manipulating gut microbiota to improve sleep. In a clinical trial of 32 medical students, the administration of the probiotic*, Lactobacillus gasseri* CP2305, was found to significantly improve sleep quality, as measured by the change in PSQI score (136). This improvement was more pronounced in male participants following probiotic use and may have been influenced by reduced sleep latency, which is the length of time required to fall asleep once in bed. Concomitantly, the relative abundance of 15 gut microbial species differed between the control and

probiotic groups, including reductions to *Bact. Vulgatus* and increases in *Dorea Longicatena* following probiotic use. Other psychobiotics have been shown to exhibit antidepressant and anxiolytic effects (137). These benefits may be due to the anti-inflammatory actions of some bacteria, or their capacity to reduce HPA-axis activity (137). Due to their interrelated nature, additional studies are required to determine if sleep improvements or anxiolytic effects predate the other in response to psychobiotics, or if both outcomes are a result of reduced systemic inflammation.

1.2.5.5 Summary

Sleep is an essential component of the human circadian cycle. Not unlike the rest of the human body, our gut microbiota fluctuates in response to circadian rhythm and feeding-fasting schedules. The ability to transmit metabolic outcomes due to sleep fragmentation and circadian disorganization through faecal transplant highlights the gut microbiota as an important target for therapeutic interventions. Alterations to the gut microbiota in response to jet lag in humans provide further evidence that the microbiome and circadian disruption are linked. How might this apply to infants, whose gut microbiota plays an integral role in the development of their immune system?

Observational studies of sleep deprivation are limited in their ability to take into account confounding factors such as diet, overweight, illness and physical activity. Importantly, the direction of the association can be unclear. What occurred first , was it gut dysbiosis or sleep impairment? Experimental designs are better able to impose temporality between shortened sleep and gut microbial composition, and control diet. Among two of three experimental studies conducted to date, partial sleep deprivation in adult humans and rats was followed by alterations to the relative abundance of bacterial taxa in the *Coriobacteriaceae* and *Erysipelotrichaceae* families, and the TM7 and Tenericutes phyla. In infants, *Coriobacteriaceae* are positively associated with reactive oxygen species generation (138), and may be associated with or the cause of increased inflammation in the gut. Sleep disruption can impact barrier integrity, leading to a disruption of the homeostatic relationship between the gut microbiome and the immune system. This disruption could predispose infants to metabolic and inflammatory disorders.

1.2.5.6 Application to an infant population

Based on the information presented above, it is plausible that sleep in the infant can influence the maturation and development of the gut microbiota. It is also possible that the gut microbiota and its metabolites in infancy can impact the development of the circadian rhythm and sleep in infancy. It is difficult to extrapolate data from adults and animals to infants, necessitating original research in the area of infant sleep and the gut microbiota. First, the nature of sleep and body rhythms in infants, and the composition of their gut microbiota, varies from that of adult humans and mice. Second, unlike in adults, the developing systems of sleep, circadian rhythm and gut microbiota are in flux. However, multiple bacterial taxa associated with sleep in adults and animals are of significance to infant health, including bifidobacteria, *Lachnospiraceae* and *Costridiaceae*. *Bifidobacteriaceae* are decreased during sleep fragmentation in mice (12), and make up the majority of the gut microbiota in breast fed infants (68). Infants with lower bifidobacterial counts at six and 12 months are more likely to be overweight at 7 years of age (139). Three-month-old infants with lower bifidobacterial counts were more likely to be overweight or obese at 10 years of age (140). *Lachnospira* have robust oscillations in the human gut and *Dorea*, of the *Lachnospiraceae* family, are decreased by a single instance of jet lag in adult humans (87). *Lachnospiraceae* is increased during sleep fragmentation (88) and in response to intermittent hypoxia (123), which may be the result of increased dietary intake in response to poor sleep quality. In infants, reduced relative abundance of *Lachnospiraceae* at 3 and 12 months of age is associated with higher risk of wheeze & atopy at 1 year of age (5), but higher *Lachnospiraceae* has been found to mediate the association between maternal and child overweight at 1 and 3 years of age (141). Clostridium taxa abundance is associated with global sleep dysfunction in breast cancer survivors (92). *Clostridium* is increased by common medical interventions during birth and the postnatal period (76) and is positively associated with stress and HPA-axis activation (112). If any of the microbial consequences of sleep loss or dysfunction in adults also occur in infants, they could have serious deleterious effects on future health. In fact, these microbial alterations could have a larger impact on health in the pediatric population than in

adults. However, the association between sleep and gut microbiota in the infant has not been studied. This thesis aims to describe the relationship between sleep duration and the composition of the gut microbiota in early infancy while controlling for the covariates which may confound this relationship. A secondary analysis (Chapter 3) focuses on the modifiable factors which tie SES to sleep duration in infancy.

1.3 Purpose Statement

The primary purpose of this work is to examine the associations between sleep duration and the composition of the gut microbiota in early infancy following adjustment for factors know to influence the infant gut microbiota. A secondary purpose of this work is to determine the influence of sociodemographic and gut microbiota-relevant factors on infant sleep duration at 3 months of age, with a special focus on the modifiable factors which link sleep duration in 3-month-old infants to their familial socioeconomic status.

1.4 Objectives

Primary Objective: To describe the association between sleep duration and the gut microbiota in infants at 3 months of age .

Secondary Objective: To examine whether in utero and early life exposures (breastfeeding status, birth mode, maternal depression, antibiotic exposure, colic, smoke exposure, siblings, ethnicity) explain differences in sleep duration in 3-month-old infants due to their familial socioeconomic status.

1.5 Hypotheses

Primary Hypothesis: Infants not meeting the National Sleep Foundation guidelines will have a gut microbiota composition lower in health promoting microbes (bifidobacteria) and higher in microbes

associated with sleep deprivation in adults and animals (*Coriobacteriaceae* and Enterobacter) than infants meeting the National Sleep Foundation guidelines.

Secondary Hypothesis: In utero and early life exposures (breastfeeding status, birth mode, maternal depression, antibiotic exposure, colic, smoke exposure, siblings, ethnicity) mediate the effect of familial socioeconomic status on sleep duration at 3-months of age.

Chapter 2: Materials and Methods

2.1 Study Population

The CHILD study is a general population-based birth cohort which has recruited 3624 pregnant women and 3542 infants. Pregnant mothers and their partners were recruited in their second or third trimester from Vancouver, Edmonton, Manitoba (Winnipeg, Morden, Winkler) and Toronto. For the purposes of this thesis, data from 843 mother/infant pairs from the Edmonton site were used in analysis due to their inclusion in the Sleep, Learning, Education, hEalth Project , Edmonton (SLEEP-E) sub-study and infant sleep data collection. Written informed consent was obtained from the mother at enrollment. Mothers of studied infants were enrolled during pregnancy between 2008 and 2012. Mothers and infants were required to meet strict inclusion/exclusion criteria (Table 2.2a).

Table 2.1a. Inclusion/Exclusion Criteria of the CHILD Study Cohort

Inclusion Criteria	• Pregnant women aged >18 years (>19 years in Vancouver). • Residential proximity (<50 Km) to participating delivery hospital. • Ability to read, write and speak English. • Willing to donate cord blood. • Planning to deliver at a designated recruitment • center participating hospital. • Infants born at or after 35 weeks.
Exclusion Criteria	• Children born with major congenital abnormalities or respiratory distress syndrome (RDS). • Expectation of moving away from a recruitment centre within 1 year of recruitment. • Children of multiple births.

	• Children resulting from in vitro fertilization. • Children who will not spend at least 80% of nights in the index home.

*Adapted from Subbarao et al. Thorax 2015

Participants of the CHILD study were recruited using fax (recruitment via obstetrics & gynaecology clinic staff), research assistants stationed in clinic waiting rooms, booths at trade shows, free and paid media and directly (when prospective participants called in) (142). Some minimal differences were noted between participants collected by different recruitment methods. Compared to participants recruited by fax, paternal participants recruited at tradeshows and in-clinic were less likely to be born in Canada; maternal participants recruited by tradeshows, in-clinic, and through paid media were less likely to be married or in a common law relationship; families recruited in-clinic were less likely to have a family income above $40,000; and participants recruited in-clinic were more likely to be above 24 weeks gestation while participants recruited through paid and free media were less likely to be above 24 weeks gestation (142). Compared to a general reference population of pregnant women identified through a multiple-physician obstetrical practice from the same city (Edmonton), women recruited to the Edmonton site of the CHILD study were more likely to have attended university, less likely to smoke, more likely to be Caucasian, Black, or First Nations, less likely to be East Indian, more likely to have a high income, and less likely to have high blood pressure or heart disease (142). Additionally, when the entire CHILD study sample was considered (recruited from Vancouver, Edmonton, Winnipeg, and Toronto, *n=3425*) 22.2% of mothers reported having asthma (143), compared to 9.2% of women aged 12 or older in Canada in 2014 (144).

2.2 Study Design

CHILD Study mothers completed questionnaires (www.canadianchildstudy.ca

/questionnaires.html) during pregnancy and when their child was 3 months of age (Table 2.3a). Questionnaires were utilized to collect information on family characteristics (eg. SES, siblings, and maternal mental health (Centre of Epidemiological Studies-Depression (CES-D) (145))), child characteristics (eg. Health, nutrition, medication) and environmental characteristics. The infant$f$s hospital birth chart was used to collection information on birth mode, medication administered to the mother during parturition, gestational age, date of birth, height and weight at birth. A home assessment was completed when the child was 3 months of age and included collection of the infant stool sample.

2.3 Sample Size Calculation

Two observational studies have examined the relationship between sleep changes and the gut microbiome in adults, however, a number of factors precluded these studies from use for the sample size calculation (92,93). The study by Paulsen et al. examined changes in gut microbiota composition associated with changes in cardiorespiratory fitness and psychosocial outcomes, including sleep (92). Unfortunately, this study could not be utilized due to reporting of the association between changes in sleep dysfunction and changes in the gut microbiota over time. Likewise, the study by Anderson et al. could not be used due the use of a sleep quality (PSQI) rather than duration measure and analysis conducted exclusively at the phyla level (93). As a result, experimental studies of sleep duration in humans were considered next.

The study by Benedict et al. (91) was an experimental study consisting of 2 consecutive days of sleep restriction (maximum sleep opportunity of 4 hours per night) in nine adult men (91). Although this study resulted in significant sleep deprivation, subjects were only sleep restricted for an acute period of time. Additionally, experimental conditions were optimally controlled in this study. As a result, this study was chosen as the comparative study for our sample size calculation. Benedict et al. found significant differences in the Firmicutes:Bacteroidetes ratio as well as the relative abundances of *Coriobacteriaceae*, *Erysipelotrichaceae* and Tenericutes (91). We chose *Coriobacteriaceae* for our calculation due to it$f$s

importance in the infant gut microbiome (138). We used the formula developed by Noether (146) to calculate the sample size for a two sided Mann-Whitney-U test. Based on the statistically significant p-value reported by Benedict et al. (91), we assumed the U value had to be „17. For our sample size calculation, we assumed a conservative p•=P(Y>X) of 0.21, based on a U value of 17 (the U value which corresponds to a p-value of 0.049). Assuming 80% power and a two-sided alpha of 5% a sample size of 31 infants (15.5 per group) will be required to detect a difference of 2.35% ($mu_1 = 7.26\%$; $mu_2 = 9.61\%$) in the mean relative abundance of *Coriobacteriaceae* (Appendix B). As data from 452 infants has been collected as part of the SLEEP-E sub study, there is a high probability that the proposed study will have adequate power to detect a difference in the relative abundance of *Coriobacteriaceae* across the in infants sleeping above and below the National Sleep Foundation guideline of 14 hours.

2.4 Study Variables

Table 2.4a. Characteristic used in Analyses and Time of Variable Extraction

Variable	Pregnancy		At Birth	Infant Age	
	18+ wks Gestation	36 wks Gestation		3 Months	6 Months
Maternal/ Family Characteristics					
Family Income	X				
Maternal Education	X				
Maternal age (at child*f*s birth)	X				
Maternal Prenatal Depression		X			
Maternal Postpartum Depression					X
Maternal IAP			X		
Siblings in the home	X				
Maternal Race/ Ethnicity	X				
Infant Characteristics					

Sex	X	
Birth Mode	X	
Gestational Age at Birth	X	
Weight (at delivery)	X	
Hospital Length of Stay	X	
Breastfeeding Status (3 month only)		X
Infant Direct Antibiotic Exposure		X
Colic/Acid Reflux Medication Exposure		X
Nighttime and Daytime Sleep Duration (From BISQ)		X
Infant Weight (3 month only)		X
Stool Sample (gut microbiota)		X

For the purposes of the study presented in Chapter 3, both income and maternal education level, or a composite measure of the two, were considered as potential markers of SES. A number of factors specific to our study contributed to our selection of maternal education over household income:

1. Education level is a robust predictor of potential earnings and may capture additional SES information such as parental SES in childhood (individuals who obtain a university degree are more likely to have parents with a university degree), and occupation (white collar vs blue collar). The binary categories utilized in this manuscript are €high school or some college• vs €has completed a university degree•. €Having a bachelor*f*s degree or higher• is a commonly utilized cut point and is associated with multiple measures of health (147).

2. In our sub-study, 7% (42/619) of respondents chose ‡prefer not to answer*f* when asked to report their household income, representing a sizeable amount of data which is missing not-at-random. Variables which are missing not-at-random are likely to increase the risk of bias whether the

missing data is included or excluded from analysis. Additionally, this precludes the missing values from imputation.

3. The majority of mothers in our study population are aged 30 to 39 (66%; 409/619) or 18 to 29 (30%; 185/619). Child care duties or being in school may transiently impact the income of parents in our study due to being in these age groups. Almost half of the infants in our study have at least one older sibling. As a result, one or both parents may have earned a reduced income in the past year due to child care duties. In Canada, mothers are guaranteed 12 months of paid maternity leave, and it is uncommon for mothers to return to work during this time.

Due to the reasons above, we believe that maternal education was the most suitable measure of SES for our study population.

CHILD participants from the Edmonton site completed additional sleep questionnaires and testing (i.e. in-home PSG, neurodevelopment). Subjects could verbally opt out of questionnaires completion and in clinic or at home testing at any point in the study. Data on covariates were obtained from hospital records (birth mode, gestational age at birth, hospital length of stay [HLOS], birth weight, antibiotic exposure, and intrapartum antibiotic prophylaxis [IAP]) or standardized questionnaires (maternal age, maternal race/ethnicity, infant sex, breastfeeding status, siblings and solid food intake).

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

Gastrointestinal issues. Infants were classified as having gastrointestinal issue before 3 months of age if they were taking a medication indicated for the treatment of colic/acid reflux or if colic/acid reflux was listed as the reason for taking a medication.

Direct infant antibiotic exposure. Infant antibiotic exposure before 3 months of age was collected from hospital chart at birth and parental report of medications administered from 0-3 months. Note that all

infants born via vaginal birth with IAP or caesarean section indirectly received antibiotic prophylaxis during parturition.

Maternal education. Maternal education level was collected from a standardized questionnaire. Mothers chose from: €1-high school or less•, €2-some university or college•, and €3-university degree obtained•. Categories 1 and 2 were then combined.

Infant sleep at 3 months of age. Infant total sleep duration was obtained from the parent self-reported Brief Infant Sleep Questionnaire (BISQ) administered at 3 months of age (60) (Appendix C. Parent self-report of infant day (7 am until 7 pm) and night (7 pm until 7 am) sleep duration in hours and minutes were combined to obtain infant total sleep duration per 24-hour period.

Depression symptoms. Depression symptoms were measured using the 20-item Center for Epidemiologic Studies Depression Scale (CES-D) (148) at 36 weeks of gestation and 6 months postpartum. Women self-reported how often they experienced various depressive cognitions, affect, and behaviors during the past week. Responses were given on a score ranging from 0 (None of the time; less than 1 day) to 3 (Most or all of the time; 5-7 days). Responses were summed, with higher scores indicating higher depressive symptoms (min=0, max=60). CES-D scores of 16 or greater represent significant risk for clinical depression (145).

Gut microbiota. Fecal samples were collected at (mean+/- SD) 4.2+/- 1.3 months of age via standard protocols during a planned home visit. Methods of sample collection, DNA extraction and amplification, 16S ribosomal RNA sequencing, and taxonomic classification have been described previously (78) (Appendix D).

2.5 Ethical Considerations

This study was conducted with respect for persons, concern for welfare, and justice. This study carries a very small probability and magnitude of risk to participants. Additionally, this study is a secondary analysis utilizing data from a previously established cohort study examining multiple research questions (www.canadianchildstudy.ca). This study contributes valuable information to the area of sleep-health research as well as our understanding of the infant gut microbiota, which plays a significant role in multiple leading causes of disease. Participant information utilized for the proposed study was be anonymized to protect the privacy and confidentiality of participants. Data was kept for a reasonable amount of time and then destroyed following accepted practices. Electronic information was encrypted, and password protected to prevent loss of information. Individuals were not unjustly excluded from participating in this research study due to ethnic, racial, or socioeconomic reasons. Furthermore, participation in this secondary analysis did not place participants in a position of increased vulnerability. This study only collected participant data which was necessary for the completion of the proposed study. The data from this study was not shared with a third party or utilized for research purposes other than those outlined in the consent form. The results of this study should not pose a threat to the safety of any vulnerable populations.

2.6 Integrated Knowledge Translation

Prior to study start, a small sample of parents were informally queried by me on their perceptions of the greatest threat to their infants*ƒ* sleep. This information was used to guide the emphasis on certain sleep measures in the study, as well as provide insight into potential confounding factors within the study.

2.6 Statistical Analysis

Study I , The Student*ƒ*s t-test and ANOVA test with Tukey post-hoc test were used to examine the association between infant total sleep duration and covariates. Univariate and multivariable linear

regression modelling was performed with total sleep duration as the outcome and maternal education as the exposure of interest. A final model was chosen using purposeful selection as described by Hosmer and Lemeshow (149). Mediation analysis was conducted using the Hayes PROCESS v3.0 macro for SPSS, version 23.0 (SPSS Inc) (150). A multiple mediation path model was evaluated to determine the indirect effects of sequential mediators: prenatal depression (mediator 1) and birth mode (mediator 2) in the path between maternal education and infant total sleep duration at 3 months of age. Bootstrapping (5000 bootstrap resamples) was used to generate to 95% CIs in mediation models. Sensitivity analyses were conducted to explore the potential confounding effect of postpartum depression on the multiple mediation model.

Study II - The Student*ʃ*s t-test and ANOVA test with Tukey post-hoc test were used to examine the association between infant total sleep duration and covariates. The association between sleep duration and bacterial relative abundance was examined using spearman correlation strati fied by birth mode and breastfeeding status. Univariate (crude) and multivariable logistic regression adjusted for birth mode, breastfeeding status at 3 months, maternal race, and maternal depression were used to examine the contribution of sleep to the prediction of % colonization. Univariate (crude) and multivariable linear regression adjusted for birth mode, breastfeeding status at 3 months, maternal race, and maternal depression were used to examine the contribution of sleep to the prediction of arcsine square root transformed bacterial relative abundance. Differentially abundant species according to infant sleep duration were identified using multivariate association with linear regression (MaAsLin) (151). Statistical tests were conducted in STATA unless otherwise specified.

Chapter 3: Prenatal Depression and Birth Mode Sequentially Mediate Maternal Education€s Influence on Infant Sleep Duration

Preamble: Chapter 3 presents the findings based on the secondary objective of this thesis. There is no lack of results showing that infants, children and adults of low SES suffer from shorter sleep duration than their higher SES counterparts. Infancy is an optimal period for the introduction of interventions to improve sleep across the lifespan， it is a time defined by amplified parental buy-in and increased return on investment. However, our lack of understanding of the factors which link SES to infant sleep leave us at a disadvantage. This study aims to explore these factors, providing researchers and clinicians with detailed information so that they can more accurately identify families who may benefit from additional counselling on infant sleep. Furthermore, this study aims to contribute to the current literature on factors which appear to directly impact infant sleep duration, thereby supporting future mechanistic studies on infant sleep. Pages 34-54 present a publication-ready manuscript on this topic.

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Drs. Kozyrskyj and Mandhane had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Matenchuk, Mandhane, Kozyrskyj.

Acquisition, analysis, or interpretation of data: Matenchuk, Tamana, Lou, Becker, Mandhane, Turvey, Subbarao, Sears, Kozyrskyj.

Drafting of the manuscript: Matenchuk, Kozyrskyj

Critical revision of the manuscript for important intellectual content: Matenchuk, Lou, Becker, Mandhane, Turvey, Subbarao, Moraes, Azad, Sears, Kozyrskyj, Tamana.

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Abstract

Rationale: Sleep duration is critical to growth, learning, and immune function development in infancy. Strategies to ensure that national recommendations for sleep duration in infants are met require knowledge of perinatal factors that affect infant sleep.

Objectives: To investigate the mechanistic pathways linking maternal education and infant sleep.

Methods: An observational study was conducted on 619 infants whose mothers were enrolled at the Edmonton site of the CHILD birth cohort. Infant sleep duration at 3 months was assessed using the Brief Infant Sleep Questionnaire. Maternal education was collected via maternal report. Prenatal and postnatal depression scores were obtained from the 20-item Center for Epidemiologic Studies Depression Scale (CES-D). Birth records and maternal report were the source of covariate measures. Mediation analysis (PROCESS v3.0) was used to examine the indirect effects of maternal education on infant sleep duration mediated through prenatal depression and birth mode.

Measurements and Main Results: At 3 months of age, infants slept on average 14.1 hours. Lower maternal education and prenatal depression were associated with significantly shorter infant sleep duration. Emergency cesarean section birth was associated with 1-hour shorter sleep duration at 3 months compared to vaginal birth [without intrapartum antibiotic prophylaxis] (†: -0.99 hours; 95% CI: -1.51, -0.48). Thirty percent of the effect of lower maternal education on infant total sleep duration was mediated sequentially through prenatal depression and birth mode (Total Indirect Effects: -0.12, 95%CI: -0.22, -0.03, $p<0.05$).

Conclusions: Prenatal depression and birth mode sequentially mediate the effect of maternal education on infant sleep duration.

Keywords: pediatric sleep, maternal education, prenatal depression, birth mode, emergency caesarean section

Abbreviations

CHILD - Canadian Healthy Infant Longitudinal Development; CS - Caesarean section; HPA，Hypothalamic-pituitary-adrenal; IAP - Intrapartum antibiotic prophylaxis; SES，Socioeconomic status

3.1 Introduction

Globally, over 50% of 3 month old infants obtain less than the recommended 14 hours of sleep per 24 hour period (8,9). Sleep plays a central role in growth, neurological development, learning and processing of memory, and optimal functioning of the immune system (21). In childhood, shorter sleep duration is associated with cognitive deficits (19), poor school performance and increased behavioral problems (36). Infants who sleep less are at risk for overweight (13) and those with frequent nocturnal awakenings are more likely to develop asthma (152). Sleep problems which arise in infancy and childhood tend to persist (32). In adults, short sleep duration has been linked to increased risk of mortality, diabetes mellitus, hypertension, obesity and coronary heart disease (4). Poor quality sleep in adulthood is identified as a pathway by which low socioeconomic status (SES) ‡gets under the skin*f* to cause disease (31) and conceivably, may be a missing link in the intergenerational transmission of SES inequalities in health (153). Hence, infant sleep is a logical target for government and public health agencies.

Be it short sleep in infants or sleep problems in toddlers, there is emerging evidence of the influence of family SES, including maternal educational attainment, on sleep in young children (38,39). When examining the various household factors that affect infant sleep, such as parental sleep (33,39,48), the upstream factor which is most likely candidate to mediate the relationship between SES and infant sleep duration is maternal prenatal depression (154). Mothers in distress have sleep problems during pregnancy (45), which can be ‡transmitted*f* to the fetus via the maternal suprachiasmatic nucleus or melatonin levels (46,47,155). Maternal depression also leads to elevated free cortisol levels during pregnancy (43), which in turn, appear to increase infant cortisol levels in response to stress (156,157). When cortisol levels are elevated, they preferentially bind to norepinephrine and glucocorticoid receptors and ultimately increase sleep EEG frequency, light sleep and frequent waking, and decrease short-wave sleep via stimulation of corticotropin releasing hormone (CRH) (158). Interestingly, maternal psychological health also plays a role in the birth process and birth outcomes (159, 161). If birth is stressful and/or leads to unexpected events such as cesarean delivery, infant sleep can be impacted through newborn exposure to hypothalamic

pituitary adrenal (HPA) axis hormones (162), reduced mother-infant bonding (163), or additional birth-associated medical interventions. There is a gap in the literature regarding the influence of the birth process on infant sleep duration beyond the second postnatal day (52).

While maternal education status has been linked to infant sleep duration (39) and maternal psychological health (44), the relatedness of these factors has not been studied nor has the birth process been taken into account. Reported associations between cesarean delivery and postpartum depression (164) may in fact be secondary to existing prenatal depression. Importantly, potential causes of childhood sleep duration are often examined after 1 year of age, excluding the first 6 months of life when critical development of the circadian rhythm, neurological function, and behavior takes place (21,155,165). We examined the association between maternal educational attainment and infant sleep duration at 3 months of age in the Canadian healthy Infant Longitudinal Development (CHILD) birth cohort. Second, we assessed whether prenatal depression and birth mode sequentially mediated the association between maternal education status and infant sleep duration. The CHILD cohort also provided a unique opportunity to test independence from putative confounding factors such as colic, often a suspected cause of sleep problems in infants (166) and antibiotic exposure, which has been found to induce transient insomnia (54) and decrease slow wave sleep (55).

3.2 Methods

Study Design

This study involved a subsample of 619 Canadian infants from the Edmonton site of the Canadian Healthy Infant Longitudinal Development (CHILD) birth cohort (http://www.childstudy.ca) (143). The Human Research Ethics board at the University of Alberta approved this study. Written informed consent was obtained from the mother at enrollment. Mothers of studied infants were enrolled during pregnancy between 2008 and 2012. Data on covariates were obtained from hospital records (birth mode, gestational

age at birth, birth weight, antibiotic exposure, and intrapartum antibiotic prophylaxis [IAP]) or standardized questionnaires (maternal age, maternal race/ethnicity, household income, infant sex, breastfeeding status, weight at 3 months, and solid food intake before 3 months). Infant colic status was determined from parent-reported infant medication questionnaires. Infants were classified as having colic before 3 months of age if they were taking a medication indicated for the treatment of colic or if colic was listed as the reason for taking a medication.

Birth mode. Birth mode was collected from maternal hospital records. Birth mode was categorized a vaginal birth without intrapartum antibiotic prophylaxis (IAP), vaginal birth with IAP, scheduled caesarean section (CS) and emergency CS.

Colic. Infant colic status was determined from parent-reported infant medication questionnaires. Infants were classified as having colic before 3 months of age if they were taking a medication indicated singularly for the treatment of colic or if colic was listed as the reason for taking a medication.

Breastfeeding status. Infant feeding status was collected from parental report at 3 months of age. Breastfeeding status was categorized as exclusive (breast milk only following hospital discharge from 0-3 months), partial (both breastmilk and formula consumed from 0-3 months), and formula (formula only from 0-3 months).

Household income. Household income was collected from maternal report at 18-36 weeks gestational age and was categorized as: 1) less than or equal to 39,999; 2) 40,000 to 79,999; 3) 80,000 to 99,999; 4) 100,000 or greater; 5) prefer not to answer.

Maternal race/ethnicity. Maternal race/ethnicity was collected from maternal report at 18-36 weeks gestational age. For the purposes of this study, maternal race was categorized as Caucasian, Asian (East Asian, South Asian and South East Asian) or other (Black, Hispanic, Middle Eastern and First Nations).

Infant sleep at 3 months of age. Infant total sleep duration was obtained from the parent self-reported BISQ (Appendix B) administered at 3 months of age (60). Parent self-report of infant day (7 am until 7 pm) and night (7 pm until 7 am) sleep duration in hours and minutes were combined to obtain infant total sleep duration per 24-hour period.

Maternal education. Maternal education level was collected from a standardized questionnaire. Mothers chose from: €1-high school or less•, €2-some university or college•, and €3-university degree obtained•. Categories 1 and 2 were then combined.

Depression symptoms. Depression symptoms were measured using the 20-item Center for Epidemiologic Studies Depression Scale (CES-D) (148) at 36 weeks of gestation and 6 months postpartum. Women self-reported how often they experienced various depressive cognitions, affect, and behaviors during the past week. Responses were given on a score ranging from 0 (None of the time; less than 1 day) to 3 (Most or all of the time; 5-7 days). Responses were summed, with higher scores indicating higher depressive symptoms (min=0, max=60). CES-D scores of 16 or greater represent significant risk for clinical depression (145).

Statistical analysis. The Student*f*s ŧtest and ANOVA test with Tukey post-hoc test were used to examine the association between infant total sleep duration and covariates. Univariate and multivariable linear regression modelling was performed with total sleep duration as the outcome and maternal education as the exposure of interest. A final model was chosen using purposeful selection as described by Hosmer and Lemeshow (149). Mediation analysis was conducted using the Hayes PROCESS v3.0 macro for SPSS, version 23.0 (SPSS Inc) (150). A multiple mediation path model was evaluated to determine the indirect effects of sequential mediators: prenatal depression (mediator 1) and birth mode (mediator 2) in the path between maternal education and infant total sleep duration at 3 months of age. Bootstrapping (5000 bootstrap resamples) was used to generate to 95% CIs in mediation models. Sensitivity analyses were conducted to explore the potential confounding effect of postpartum depression on the multiple mediation model.

3.3 Results

Table 1. Summary of maternal education level and infant total sleep duration at 3 months of age according to infant and maternal characteristics.

	Maternal Education			Total Sleep Duration (hours/24 hours)	
	No University Degree n = 284/619 (45.89 %)	University Degree n = 335/619 (54.11%)	p-value	Observations	Mean (SD) 14.17 (2.14)
Maternal education, No. (%)					
No University Degree	-	-	N.A.	284	13.94 (2.20)
University Degree	-	-		335	14.36 (2.08)
Infant Characteristics					
Gestational age, No. (%)					
Below 38 weeks	28 (9.96%)	44 (13.25%)	0.357	72	14.32 (2.25)
38 to 39 weeks	138 (49.11%)	148 (44.58%)		286	14.33 (2.27)
Over 40 weeks	115 (40.93%)	140 (42.17%)		255	13.93 (1.93)
Birth weight, No. (%)					
< 2500g	6 (2.12%)	14 (4.22%)	0.332	20	14.09 (2.23)
2500-3499g	147 (51.94%)	173 (52.11%)		320	14.19 (2.23)
3500-4499g	127 (44.88)	138 (41.57%)		265	14.14 (2.05)
> 4500g	3 (1.06%)	7 (2.11%)		10	13.95 (1.44)
Weight at 3 months, No. (%)					
< 5000g	6 (2.17%)	13 (3.93%)	0.302	19	14.26 (2.24)
5000-5999g	59 (21.38%)	73 (22.05%)		132	14.34 (2.26)
6000-7999g	185 (67.03%)	203 (61.33%)		388	14.16 (2.11)
>8000g	26 (9.42%)	42 (12.69%)		68	13.91 (2.10)
Gender, No. (%)					
Boy	150 (52.82%)	161 (48.06%)	0.259	311	14.25 (2.13)
Girl	134 (47.18%)	174 (51.94%)		308	14.09 (2.16)
Antibiotic exposure, No. (%)					
Yes	149 (53.41%)	161 (49.09%)	0.291	310	13.94 (2.16)
No	130 (46.59%)	167 (50.91%)		297	14.37 (2.10)
Birth mode, No. (%)					
Vaginal , IAP	144 (51.25%)	179 (53.92%)	0.177	323	14.39 (2.08)
Vaginal +IAP	56 (19.93%)	81 (24.40%)		137	13.98 (2.18)
Scheduled CS	36 (12.81%)	35 (10.54%)		71	14.36 (2.07)
Emergency CS	45 (16.01%)	37 (11.14%)		82	13.40 (2.20)
Breastfeeding status, No. (%)					
Exclusive	138 (48.59%)	204 (61.08%)	<0.001	342	14.26 (2.06)
Partial	79 (27.82%)	95 (28.44%)		174	14.09 (2.16)
Zero	67 (23.59%)	35 (10.48%)		102	14.00 (2.38)
Solids, No. (%)					
Yes	8 (2.92%)	7 (2.12%)	0.604	15	14.83 (1.54)
No	266 (97.08%)	323 (97.88%)		589	14.16 (2.16)
Colic, No. (%)					
Yes	43 (15.30%)	52 (15.66%)	0.911	95	13.89 (2.27)
No	238 (84.70%)	280 (84.34%)		518	14.22 (2.13)
Maternal Characteristics					
Maternal age, No. (%)					
18 to 29	125 (44.01%)	60 (17.91%)	<0.001	185	14.07 (2.08)

30 to 39	150 (52.82%)	259 (77.31%)		409	14.20 (2.17)
Over 40	9 (3.17%)	16 (4.78%)		25	14.32 (2.20)
Annual household income, No. (%)					
Less than 39,999	31 (10.95%)	7 (2.09%)	<0.001	38	13.86 (1.70)
40,000-79,999	83 (29.33%)	55 (16.42%)		138	14.01 (2.20)
80,000-99,9999	43 (15.19%)	49 (14.63%)		92	14.04 (2.34)
Greater than 100,000	101 (35.69%)	207 (67.79%)		308	14.41 (1.97)
Preferred not to answer	25 (8.83%)	17 (5.07%)		42	13.59 (2.76)
Maternal race, No. (%)					
White	215 (76.24%)	258 (77.01%)	<0.001	473	14.09 (2.09)
Asian	18 (6.38%)	50 (14.93%)		68	14.64 (2.46)
Other	49 (17.38%)	27 (8.06%)		76	14.21 (2.18)
Prenatal depression, No. (%)					
Yes	69 (27.27%)	41 (13.62%)	<0.001	110	13.65 (2.11)
No	184 (72.73%)	260 (86.38%)		444	14.23 (2.14)
Postnatal depression, No. (%)					
Yes	40 (17.62%)	30 (10.71%)	0.028	70	13.43 (2.54)
No	187 (82.38%)	250 (89.29%)		437	14.26 (2.03)
Maternal prenatal smoking, No. (%)					
No	256 (91.10%)	332 (99.40%)	<0.001	588	14.18 (2.14)
Yes	25 (8.90%)	2 (0.60%)		27	13.87 (1.94)
Siblings in the home, No. (%)					
No	124 (43.82%)	152 (45.65%)	0.685	270	13.70 (2.06)
Yes	159 (56.18%)	181 (54.35%)		340	14.54 (2.13)

Notes: -IAP: no intrapartum antibiotics; +IAP: with intrapartum antibiotics; CS: caesarean section.

In our population-based cohort of 619 mother-infant dyads, 54% of mothers had a university degree. Significant differences in maternal age, annual household income, maternal race, breastfeeding status, prenatal smoking, and pre- and post- natal depression were found between mothers with and without a university degree (See Table 1). Women with a university degree were older than those without a university degree. The majority of mothers with a university degree (67.79%) had an annual household income greater than $100,000. Annual household income greater than $100,000 was less prevalent when mothers did not have a university degree (35.69%). Two percent of mothers with a university degree and 10.95% of mothers without a university degree had a household income below $39,999. Mothers with a university degree were less likely to identify as a race other than white or Asian (8.1% vs 17.4%). In contrast, 6.4%of mothers without a university degree were Asian, compared to 14.9% of mothers with a university degree. Exclusive breastfeeding was higher at 3 months of age in mothers with a university degree (61.1% vs 48.6%).

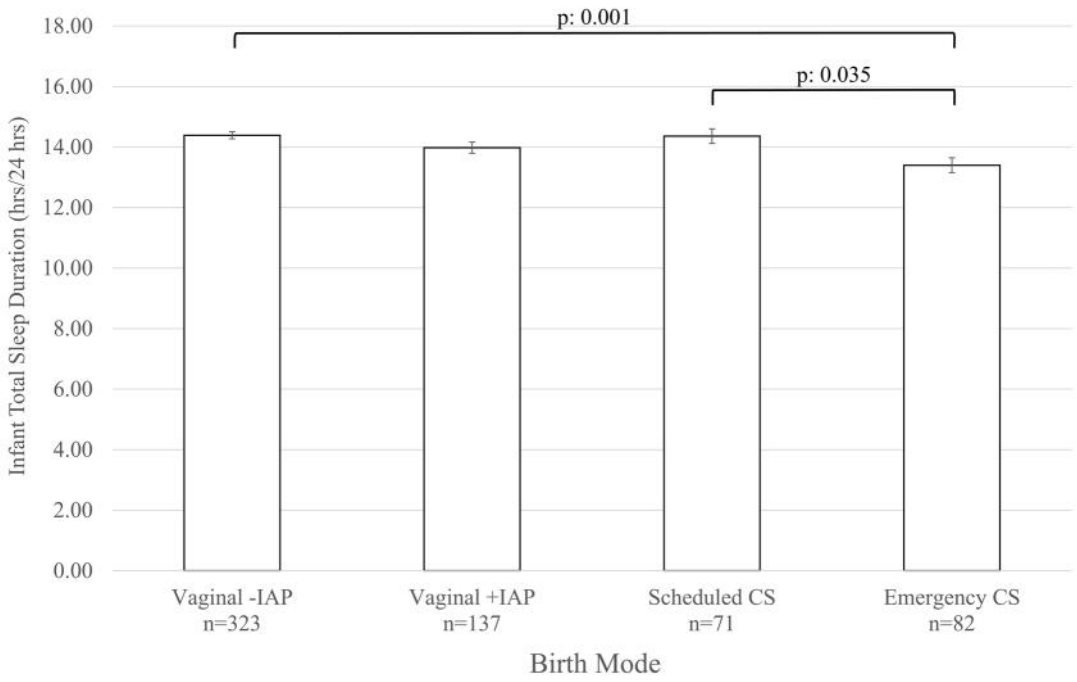

Figure 1. Infant total sleep duration at 3 months of age according to birth mode. Statistical significance determined by ANOVA with Tukey post hoc test. -IAP: no intrapartum antibiotics; +IAP: with intrapartum antibiotics; CS: caesarean section.

Mean infant total sleep duration at 3 months of age was 14.2 hours (Standard deviation [SD]: 2.14; Table 1). The National Sleep Foundation recommends 14-17 hours of sleep in this age group (8). Infants born to mothers without a university degree slept an average of 13.94 hours (SD: 2.20) compared to 14.36 hours (SD: 2.08) in infants born to mothers with university degrees. Both prenatal and postnatal depression were associated with significantly shorter infant sleep duration. Total sleep duration was significantly different according to birth mode. Tukey post-hoc test showed that infants born by emergency caesarian section (CS) slept significantly shorter than infants born vaginally without IAP or by scheduled CS (Figure 1).

Maternal education is associated with prenatal and postnatal depression.

Twenty-seven percent of mothers without a university degree had prenatal depression (CES-D score ˆ 16), while 13.6% of mothers with a university degree had prenatal depression. Similarly, 17.6% of mothers without a university degree had postnatal depression while only 10.7% of mothers with a university degree had postnatal depression. Women without a university degree had an almost 2 times higher relative risk of prenatal depression without postnatal depression (relative risk [RRR]: 1.91, 95% CI: 1.06, 3.43, p=0.03; figure E1 , Appendix E), 4.4 times higher relative risk of both prenatal and postnatal depression (RRR: 4.39, 95% CI: 1.82, 10.62, p<0.001), but no difference in relative risk of postnatal depression without prenatal depression (RRR: 0.95, 95% CI: 0.43, 2.08, p=0.89) compared to women with a university degree.

Maternal postsecondary education is positively associated with infant sleep duration.

Infants of mothers without a university degree had reduced sleep duration at 3 months of age (†: -0.42 hours, 95% Confidence Interval [CI]: -0.76, -0.08, p<0.01; Table 2) compared to mothers with a university degree. This association remained significant following adjustment for infant factors including gestational age at birth, gender, birth mode, breastfeeding status, solids, and colic (†: -0.42 hours, 95% CI: -0.78, -0.07, p<0.05; Model 2). However, the difference in sleep duration by maternal education status was not significant (†: -0.28 hours, 95% CI: -0.67, 0.11, p=NS; Model 3) when controlling for maternal characteristics including prenatal depression, maternal age, maternal race, siblings in the home, maternal prenatal smoking and all Model 2 variables.

Table 2. Crude and multivariable linear regression analyses predicting infant total sleep duration at 3 months of age.

	Crude		Model 1		Model 2		Model 3		Model 4	
	95% CI		95% CI		95% CI		95% CI		95% CI	
Coeff.	Lower, Upper	*Coeff.*	Lower, Upper	*Coeff.*	Lower, Upper	*Coeff.*	Lower, Upper	*Coeff.*	Lower, Upper

Maternal education ***(ref = university degree)***		**-0.42***	**-0.76, -0.08**	**-0.44****	**-0.78, -0.10**	**-0.42***	**-0.78, -0.07**	**-0.37**	**-0.75, 0.02**	**-0.29**	**-0.65, 0.06**
Infant Characteristics											
Block 1											
Gestational age at birth *(continuous)*		-0.12‰	-0.24, 0.01	-0.13‰	-0.26, 0.001	-0.15*	-0.28, -0.02	-0.03	-0.18, 0.12	-	-
Gender *(ref = female)*		0.16	-0.18, 0.50	0.12	-0.22, 0.46	0.09	-0.25, 0.44	0.16	-0.20, 0.52	-	-
Birth mode	Vag +IAP	-0.41‰	-0.83, -0.02	-0.49*	-0.92, -0.07	-0.50*	-0.93, -0.06	-0.48*	-0.93, -0.02	-0.35	-0.80, 0.09
(ref = vaginal - IAP)	Scheduled CS	-0.02	-0.57, 0.52	-0.11	-0.67, 0.45	-0.09	-0.66, 0.48	-0.22	-0.81, 0.37	-0.24	-0.81, 0.33
	Emergency CS	-0.99***	-1.51, -0.48	-0.94***	-1.45, -0.42	-0.98***	-1.51, -0.44	-0.68*	-1.26, -0.10	-0.70*	-1.25, -0.15
Block 2											
Breastfeeding status	Partial	-0.17	-0.56, 0.22	-	-	-0.20	-0.60, 0.21	-0.35‰	-0.77, 0.07	-	-
(ref = exclusive)	Zero	-0.27	-0.74, 0.21	-	-	-0.24	-0.74, 0.26	-0.30	-0.83, 0.23	-	-
Solids		0.68	-0.43, 1.78	-	-	0.89	-0.23, 2.00	0.96	-0.59, 0.43	-	-
Colic		-0.33	-0.81, 0.14	-	-	-0.40	-0.88, 0.08	-0.08	-0.59, 0.43	-	-
Maternal Characteristics											
Prenatal depression *(CES-D score)*		-0.03**	-0.06, -0.01	-	-	-	-	-0.03*	-0.06, 0.00	-0.03*	-0.05, -0.004
Maternal age	18-29	-0.14	-0.51, 0.24	-	-	-	-	-0.36‰	-0.78, 0.05	-	-
(ref = 30-39)	40+	0.12	-0.75, 0.99	-	-	-	-	-0.46	-1.41, 0.49	-	-
Maternal race	Asian	0.55*	0.01, 1.10	-	-	-	-	0.66*	0.06, 1.25	-	-
(ref = white)	Other	0.12	-0.40, 0.64	-	-	-	-	0.29	-0.28, 0.87	-	-
Siblings in the home *(ref = no)*		-0.85****	0.51, 1.18	-	-	-	-	0.79***	0.41, 1.17	0.79***	0.42, 1.16
Maternal prenatal smoking		-0.31	-1.14, 0.51	-	-	-	-	0.29	-0.66, 1.24	-	-

Notes: Model 1: maternal education, gestational age at birth, gender, and birth mode. Model 2: Model 1 with breastfeeding status, solids, and colic. Model 3: Model 2 with prenatal depression, maternal age, and maternal race. Model 4: maternal education, birth mode, prenatal depression, and siblings in the home [chosen by purposeful selection]. IAP: intrapartum antibiotics; CS: caesarean section. p<0.05*; p<0.01**; p<0.001***; p<0.0001****.

Emergency CS was associated with shorter sleep duration at 3 months of age compared to the reference group of infants born vaginally without IAP (Crude †: -0.99 hours, 95% CI: -1.51, -0.48, p<0.001). Each 1-point increase in mothers*ƒ* prenatal CES-D score was associated with a 0.03-hour decrease in infant sleep duration (Crude †: -0.03 hours, 95% CI: -0.06, -0.01, p<0.01). Infants of Asian mothers slept on average 0.59 hours more than infants of white mothers (Crude †: 0.59 hours, 95% CI: 0.05, 1.13, p<0.05).

Purposeful selection resulted in the inclusion of birth mode, prenatal depression (CES-D score) and siblings in the home in the regression model predicting infant sleep duration (Model 4). Maternal

education was included as an exposure of interest. Emergency CS (†: -0.70 hours, 95% CI: -1.25, -0.15, $p<0.05$; Model 4), prenatal depression (CES-D score) (†: -0.03 hours, 95% CI: -0.05, -0.004, $p<0.05$) and siblings in the home (†: 0.79 hours, 95% CI: 0.42, 1.16, $p<0.001$), significantly contributed to the prediction of infant sleep duration. Maternal education did not contribute to the model predicting infant sleep duration (†: -0.29 hours, 95% CI: -0.65, 0.06, p=NS) when adjusting for birth mode, prenatal depression and siblings in the home. Interactions between maternal education, prenatal depression, birth mode and siblings in the home did not significantly contribute to the model.

Prenatal depression and birth mode sequentially mediate the relationship between maternal education level and infant sleep duration.

Regression analysis was used to investigate the hypothesis that prenatal depression and birth mode sequentially mediate the effect of maternal education on infant total sleep duration (Figure 2). Lower maternal education was a significant predictor of prenatal depression (CES-D score) (†=2.68, SE=0.63, $p<0.0001$; Table E1 , Appendix E). Furthermore, prenatal depression (CES-D score) (†=0.01, SE=0.01, p=0.05), but not lower maternal education (†=0.15, SE=0.09, p=0.11) was a significant predictor of birth mode (classified as 1 = vaginal no IAP, 2 = vaginal IAP, 3 = scheduled CS, and 4 = emergency CS) when modelled concurrently. When evaluated in regression analysis together, prenatal depression (CES-D score) (†=-0.03, SE=0.01, p=0.04) and birth mode (†=-0.27, SE=0.08, $p<0.01$) but not maternal education (†=-0.27, SE=0.18, p=0.15) predicted infant total sleep duration.

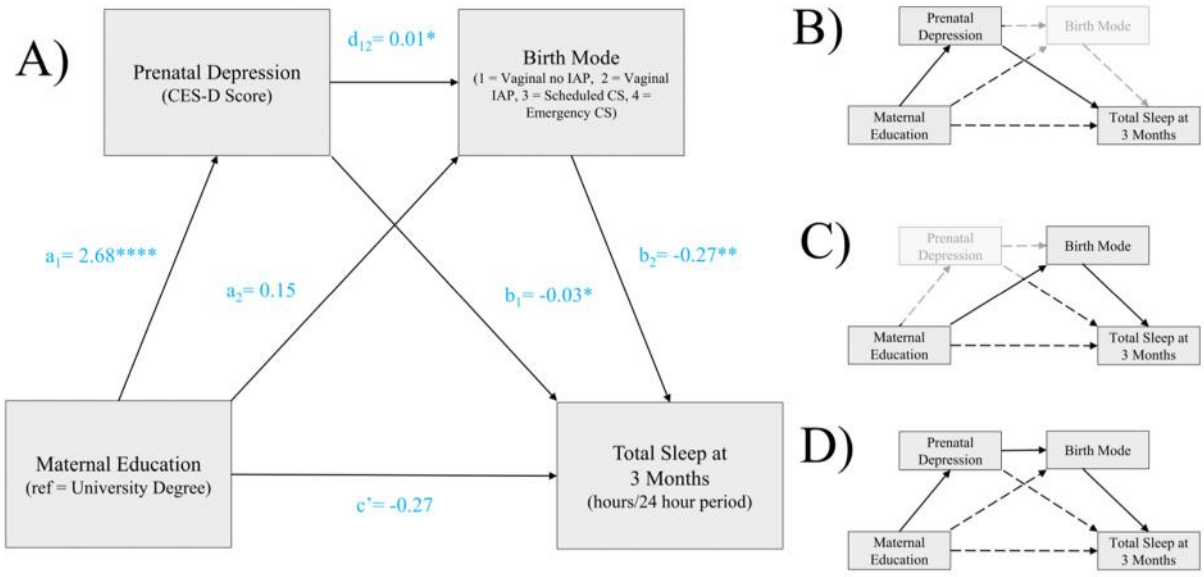

Figure 2. Sequential mediation model of associations between maternal education, prenatal depression, birth mode, and infant sleep duration. -IAP: no intrapartum antibiotics; +IAP: with intrapartum antibiotics; CS: caesarean section. p<0.1‰; p<0.05*; p<0.01**; p<0.001***; p<0.0001****.

Prenatal depression and birth mode sequentially mediate the relationship between maternal education and infant sleep duration. Following sequential mediation, the direct association of lower maternal education with infant total sleep duration (path c`) was no longer significant (Effect: -0.27, 95% CI: -0.63, 0.09, *p*=0.15; Table 3); however, the total indirect effects of lower maternal education on infant total sleep duration mediated sequentially through prenatal depression and birth mode were significant (Effect: -0.12, 95% CI: -0.22, -0.03, *p*<0.05). Combined, the direct and indirect effects of lower maternal education on infant sleep duration were significant (Effect: -0.38, 95% CI: -0.74, -0.03, *p*<0.05). Eighteen percent of the effect of lower maternal education on infant total sleep duration was mediated through prenatal depression alone (Effect: -0.07, 95% CI: -0.15, -0.01, *p*<0.05). The indirect effect of maternal education through birth mode alone was not significant (Effect: -0.04, 95% CI: -0.11, 0.01, *p*=NS). The

effect of lower maternal education on infant total sleep duration (2.3% of the total effect) was mediated sequentially through prenatal depression and birth mode directly (Effect: -0.01, 95% CI: -0.02, -0.0004, p<0.05). These associations were robust to sensitivity analyses for imputed missing values. Due to the high correlation between prenatal and postnatal CES-D scores, sequential mediation of the relationship between maternal education and infant sleep duration through prenatal and subsequently postnatal depression, as well as postnatal depression alone, was explored. Postnatal CES-D score was not found to mediate the relationship between maternal education and infant sleep duration with prenatal CES-D score or on its own.

Table 3. Breakdown of direct and indirect effects of maternal education on infant sleep duration at 3 months of age through prenatal depression (CES-D score) and birth mode.

	% Effect Explained	Effect	SE	p	95% CI
A) Total effect of maternal education (indirect + direct effects)	100%	-0.38*	0.18	0.04	-0.74, -0.03
B) Indirect effect 1 Maternal education → prenatal depression → sleep duration	17.7%	-0.07*	0.03	<0.05	-0.15, -0.01
C) Indirect effect 2 Maternal education → birth mode → sleep duration	10.5%	-0.04	0.03	NS	-0.11, 0.01
D) Indirect effect 3 Maternal education → prenatal depression → birth mode → sleep duration	2.3%	-0.01*	0.006	<0.05	-0.02, -0.0004
Total indirect effects (1 + 2 + 3)	30.4%	-0.12*	0.05	<0.05	-0.22, -0.03
Direct effect of maternal education	69.5%	-0.27	0.18	NS	-0.63, 0.09

Notes: p <0.05*.

3.4 Discussion

In our general population cohort of infants from an urban center in Canada, 38% of infants slept less than the recommended 14 hours per day; lower than global estimates of infant short sleep at 3 months of age (9). Infants born to mothers with a university degree slept an average of 0.42 hours longer than infants of mothers without a university degree. The association between maternal level of education and infant sleep duration persisted following adjustment for infant factors but diminished with additional adjustment for maternal characteristics, notably maternal prenatal depression (48). Further, we found that birth mode independently predicted infant sleep duration, with infants delivered by emergency caesarean sleeping approximately one hour less than infants born by vaginal birth. When combined, we found that maternal prenatal depression status and birth mode jointly mediated the association between maternal level of education and infant sleep duration. Previously, prenatal depression was found to be associated with shorter sleep duration in 1-2 year olds independent of household SES, and postnatal depressive symptoms in caregivers reported to influence the relationship between family demographics and sleep problems in toddlers (35,39). Our study is the first to suggest that prenatal depression has the capacity to mediate the relationship between maternal education level and infant sleep in the 3 months of age. Almost one-third of the indirect effect of maternal education was mediated through the joint action of prenatal depression and emergency caesarean.

The additional novelty of our study is the reduction in infant sleep three months after emergency caesarean delivery; this was not observed with scheduled caesarean or in vaginal deliveries with maternal antibiotic prophylaxis. Compared to vaginal delivery, both emergency and scheduled caesarean delivery have been shown to reduce active sleep in newborns on the first but not second postnatal day; however, an observed lack of diurnal rhythms in infant sleep/wakefulness with both surgical groups seems to persist (52). Netsi et al did not find an association between birth mode and sleep duration at age 3 months in a Brazilian cohort, in which many of the caesarean births would have been scheduled (53). One aspect of modern birth, the induction and augmentation of labour using synthetic oxytocin, is very common in

birth by emergency caesarean (167). In animal studies, synthetic oxytocin increases wakefulness (168), hypothesized to occur due to oxytocin*f*s influence on the HPA axis through an excitatory action on CRH (158,169,170). Upregulation of the CRH system has been implicated in the impairment of sleep quality in both human and animal studies (169).

Furthermore, emergency caesarean co-mediated with prenatal depression, the association between maternal SES and infant sleep. Little is known about the maternal physiological impact of emergency CS on the infant (171). Unexpected caesarean delivery can be a traumatic birth experience for the mother (172), interfering with parenting behaviours that promote self-soothing in the infant and longer sleep duration (173). Interestingly, both maternal depression during pregnancy and emergency caesarean birth have the capacity to disrupt development of the infant HPA axis and alter regulation of circadian rhythm (162,165). Smith et al. found that infants born by emergency but not scheduled caesarean, had higher levels of free cortisol in umbilical cord blood samples than vaginally born infants (162). Elevated cortisol levels can increase CRH, which are associated with reduced sleep quality (158). However, due to the development of the circadian clock genes and HPA axis during late gestation and early infancy, elevated cortisol at birth may have a lasting effect on the programming of these systems (165). Furthermore, infants born by emergency but not scheduled caesarean, have been found to have elevated C-reactive protein in the cord blood following birth (174). The administration of pro-inflammatory cytokines in animal studies promotes non-REM sleep, which is more common after sleep deprivation (175). Lastly, infants born by emergency caesarean are also more likely to exhibit gut microbial dysbiosis than infants born vaginally or by scheduled CS (68), compositional changes that may ultimately alter circadian rhythm and sleep patterns (176).

Our results also support the thesis that prenatal depression influences infant sleep through a fetal programming pathway (154). Infants born to mothers with prenatal depression slept on average 0.56 hours shorter than infants born to mothers without prenatal depression. The prenatal stress model, which is an approximate animal model of stress and depression in pregnancy, results in prolonged corticosterone

production after acute stress and reduced expression of glucocorticoids in the hippocampus in adult offspring (165). As a result, infants of mothers with prenatal depression may have an exaggerated stress-response which negatively impacts their sleep duration after birth. Prenatal depression is strongly linked to low SES (177); stressful life events during pregnancy and concern over finances have both been associated with frequent nocturnal awakening in toddlers (152). In our study, women without a university degree were much more likely to experience prenatal depression with or without postnatal depression but not postnatal depression without prenatal depression.

Strengths and Limitations

Our study has several strengths, including the ability to investigate birth mode in greater detail than previously examined in a birth cohort with a representative and large sample size. Also, the universal healthcare context of the Canadian populace provides an opportunity to study SES independent of accessibility to prenatal care healthcare (178). Limitations of this study include the unavailability of measures on maternal prenatal sleep, parenting behaviour and depressive symptoms in the postpartum period prior to 6 months.

3.5 Conclusions

Socioeconomic factors in early life have a strong influence on virtually all aspects of early human development (179). In our general population cohort from the CHILD study, infant sleep duration at 3 months of age was predicted by maternal education level, prenatal depression and birth mode. The maternal educational association with infant sleep was sequentially mediated by prenatal depression and birth mode. Our study provides evidence for a prenatal-birth pathway by which parental SES can impact infant sleep. Mothers who experience prenatal depression or emergency caesarean birth may benefit from advice on parenting style and infant stimulus control to increase infant sleep duration (173), so that these

problems do not persist in childhood. While we are at an early stage to discern the underlying biologic mechanisms, this study identifies prenatal depression and birth mode as targets for policy makers to improve infant sleep duration. Future work is required to determine if the impact of these exposures is mediated by oxytocin administration, cortisol level, maternal sleep, postpartum depression or parental behaviours.

3.6 Acknowledgements

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3.7 Appendix

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Chapter 4: Infant Sleep Duration is Associated with Gut Microbiota Composition in the Canadian Healthy Infant Longitudinal Development (CHILD) Study

Preamble: Chapter 4 presents the findings based on the primary objective of this thesis. Over the past twenty years, large strides have been made in determining which factors have a significant impact on the seeding and cultivation of the infant gut microbiome. Birth mode, antibiotic exposure, breastfeeding status, and exposure to siblings, pets and cleaning products in the home have been identified as major players in the shaping of the gut microbiome, and subsequent immune system development. Through their impact on the gut microbiome, these exposures have been linked to asthma, atopy, obesity and irritable bowel disease. In 1993, Everson and Toth discovered that complete sleep restriction in rats lead to death by an unlikely cause， enteric overgrowth. Since the publication of their intriguing study, researchers have determined that partial sleep restriction and circadian misalignment in humans and animals can induce changes in the gut microbiota composition as well. Furthermore, the resultant dysbiotic microbiota is capable of transmitting metabolic syndrome to germ-free recipients. This is the first study to explore the associations between sleep duration and the gut microbiota in infancy. Furthermore, this is the largest observational study on sleep and the gut microbiome to date. Pages 56-76 present a publication-ready manuscript.

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Drs. Kozyrskyj and Mandhane had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Abstract

Rationale: Sleep and the gut microbiome are intimately linked， as little as a few days of sleep deprivation leads to overgrowth of the intestinal microbiome. In infancy, both sleep and the gut microbiota play an integral role in the development of the immune system and subsequent health throughout life.

Objectives: The objective of this study was to investigate the impact of sleep duration on infant gut microbiota composition while controlling for factors known to influence the infant gut microbiota.

Methods: A sub-study was conducted on 437 infants whose mothers were enrolled at the Edmonton site of the CHILD birth cohort. Infant sleep duration at 3 months was assessed using the Brief Infant Sleep Questionnaire. Infant gut microbiota were profiled with 16S rRNA sequencing from faecal samples collected at 3 months of age. Birth records and maternal report were the source of covariate measures. Nonparametric statistical tests as well as logarithmic regression modelling was used to examine the relationship between sleep and the gut microbiota bacterial taxa. MaAsLin was utilized to determine which bacterial taxa were associated with shorter infant sleep while adjusting for the effect of metadata. Linear regression was conducted on arcsine square root transformed gut bacterial relative abundance.

Measurements and Main Results: Sixty-one percent of infants met the National Sleep Foundation recommendation of ˆ14 hours. Sleep duration (as a continuous measure) was negatively associated with the relative abundance of *Clostridium* (Spearman Rho: -0.10, p=0.03). Short sleepers (<14 hours per 24-hour period) were more likely to be colonized with *Enterococcus* (76.5% vs 65.2%, p=0.01; *Exact*) and *Clostridium* (80.6% vs 67.4%, p<0.01) and less likely to be colonized with *Erwinia* (5.9% vs 12.7%, p=0.02) than infants obtaining ˆ14 hours of sleep. Additionally, arcsine square root transformed *Lachnospiraceae* was positively associated with continuous sleep duration in exclusively breastfed (†: -0.07, 95% CI: -0.12; -0.02; p=0.01) and in infants born vaginally without exposure to maternal antibiotics (†:-0.07, 95% Confidence Interval [CI]: -0.13; -0.01;p=0.02) following adjustment. *Erwinia* was the only bacterial taxa identified by MaAsLin to be associated with short sleep following adjustment for metadata (beta-coefficient: -0.00065, p=0.016).

Conclusions: The relative abundances and colonization frequency of biologically important bacteria for infant growth are significantly associated with infant sleep duration at three months of age.

Keywords: pediatric sleep, infant gut microbiota, gut microbiome, birth mode,

Abbreviations

CHILD - Canadian Healthy Infant Longitudinal Development; CS - Caesarean section; HPA , Hypothalamic-pituitary-adrenal; IAP - Intrapartum antibiotic prophylaxis.

4.1 Introduction

The infant gut microbiota is a complex ecosystem which is colonized at birth and changes rapidly in response to the factors an infant is exposed to in the hours and weeks following birth (61). Each infant has a unique microbiome which is all her own, but can be linked back to her mode of birth, antibiotic exposure, food, and mom (61,76). The composition of the gut microbiota in infancy plays an important role in the education of the immune system. Increased incidence of hay fever in adults with fewer older siblings compared to those with more older siblings led to the Hygiene Hypothesis: that exposure to increased richness and diversity of bacteria in infancy could promote immune tolerance and decreased atopy in later life (4). Since the proposition of the Hygiene Hypothesis, the early life gut microbiome has been associated with future obesity (180), asthma (5), and even psychosocial development (181).

Both obesity (13) and asthma (152) in childhood have also been linked to sleep factors in infancy. Recently, an observational study found sleep duration and gut microbiota composition to be associated in older adults (93). Sleep, stress, immune function, and the gut microbiome exist in a carefully regulated cyclic relationship (14,121,182,183). In early life, the gut microbiota plays a significant role in the set-point of the hypothalamic-pituitary-adrenal (HPA) axis (182). Bacterial infection upregulates inflammatory cytokines, leading to increased NREM sleep (121). Conversely, sleep loss drastically increases the host*ƒ*s susceptibility to enteric infection via disruption of the epithelial barrier (17). As a result, homeostasis can be swiftly disrupted by a disturbance to just one of these factors. Recent studies have sought to determine what impact experimentally imposed bouts of short sleep have on the human gut microbiome; however, the results from these studies have been inconclusive and suffer from multiple methodological difficulties (85,91).

A recent global meta-analysis found that over 50% of 3 month old infants obtain less than the recommended 14 hours of sleep per 24 hour period (8,9). In comparison, 65%-74% of children aged 5-17 meet the National Sleep Foundation guidelines for their age group (99,184). Sleep plays a central role in growth, neurological development, and optimal functioning of the immune system (21). Furthermore, sleep is a mediator in the relationship between low socioeconomic status and poor health (31). Due to the

overlap of future health outcomes between sleep and gut microbiota in infancy, we sought to examine the relationship between sleep and the gut microbiota in infancy.

4.2 Materials and Methods

Study Design

This study involved a subsample of 437 infants from the Edmonton site of the Canadian Healthy Infant Longitudinal Development (CHILD) birth cohort (http://www.childstudy.ca) (143) (flow chart, Figure 1 , Appendix F). The Human Research Ethics board at the University of Alberta approved this study. Written informed consent was obtained from the mother at enrollment. Mothers of studied infants were enrolled during pregnancy between 2008 and 2012. Data on covariates were obtained from hospital records (birth mode, gestational age at birth, hospital length of stay [HLOS], birth weight, antibiotic exposure, and intrapartum antibiotic prophylaxis [IAP]) or standardized questionnaires (maternal age, maternal race/ethnicity, infant sex, breastfeeding status, siblings, solid food intake and direct infant exposure to antibiotics before 3 months). Infant gastrointestinal issue (colic or acid reflux only) was determined from parent-reported infant medication questionnaires. Infants were classified as having gastrointestinal issue before 3 months of age if they were taking a medication indicated for the treatment of colic/acid reflux or if colic/acid reflux was listed as the reason for taking a medication. Infant antibiotic exposure before 3 months of age included direct and indirect exposure. All infants born via vaginal birth with IAP or caesarean section (CS) indirectly received antibiotic prophylaxis during parturition.

Infant sleep at 3 months of age. Infant total sleep duration was obtained from the parent self-reported Brief Infant Sleep Questionnaire (BISQ) administered at 3 months of age (60). Parent self-report of infant day (7 am until 7 pm) and night (7 pm until 7 am) sleep duration in hours and minutes were combined to obtain infant total sleep duration per 24-hour period.

Depression symptoms. Depression symptoms were measured using the 20-item Center for Epidemiologic Studies Depression Scale (CES-D) (148) at 36 weeks of gestation and 6 months postpartum. Women self-reported how often they experienced various depressive cognitions, affect, and behaviors during the past

week. Responses were given on a score ranging from 0 (None of the time; less than 1 day) to 3 (Most or all of the time; 5-7 days). Responses were summed, with higher scores indicating higher depressive symptoms (min=0, max=60). CES-D scores of 16 or greater represent significant risk for clinical depression (145).

Gut microbiota. Fecal samples were collected at (mean+/- SD) 4.2+/- 1.3 months of age via standard protocols during a planned home visit. Methods of sample collection, DNA extraction and amplification, 16S ribosomal RNA sequencing, and taxonomic classification have been described previously (78) (Appendix D).

Statistical analysis. The Student*f*s ŧtest and ANOVA test with Tukey post-hoc test were used to examine the association between infant total sleep duration and covariates. The association between sleep duration and bacterial relative abundance was examined using spearman correlation stratified by birth mode and breastfeeding status. Univariate and multivariable logistic regression adjusted for birth mode, breastfeeding status at 3 months, maternal race, and maternal depression were used to examine the contribution of sleep to the prediction of % colonization. Univariate and multivariable linear regression adjusted for birth mode, breastfeeding status at 3 months, maternal race, and maternal depression were used to examine the contribution of sleep to the prediction of arcsine square root transformed bacterial relative abundance. Differentially abundant species according to infant sleep duration were identified using multivariate association with linear models (MaAsLin) (151). Statistical tests were conducted in STATA unless otherwise specified.

4.3 Results

In this population-based cohort of 437 three-month-old Canadian infants, 61.1% of infants met the National Sleep Foundation minimum recommended sleep duration of ˆ14 hours per 24 -hour period (See Table 1). Categorical infant sleep duration was similarly distributed according to infant gender, weight at delivery, hospital length of stay, direct antibiotic exposure before 3 months of age, gestational age at birth, birth mode, solids before 3 months, colic or acid reflux (gastrointestinal issues), breastfeeding status

at 3 months, maternal race/ethnicity, and maternal age. Infants whose mothers exhibited maternal prenatal depression were more likely to sleep less than 14 hours compared to infants whose mothers did not exhibit maternal prenatal depression (52.5% vs 36.6%; Exact *p-value: 0.01*). Infants with siblings were more likely to sleep more than 14 hours compared to infants who did not have siblings (69.5% vs 51.0%; Exact *p-value <0.001*). Average total sleep duration was 14.2 hours (SD: 2.2). Continuous sleep duration was not different according to infant gender, weight at delivery, gestational age at birth, solids before 3 months, colic or acid reflux, direct antibiotic exposure before 3 months, hospital length of stay, breastfeeding status at 3 months, maternal race/ethnicity, and maternal age (Table 1 , Appendix F). Continuous sleep duration was different according to birth mode (p<0.01; ANOVA), siblings (p<0.001; t-test), and maternal prenatal depression (p=0.01; t-test).

Table 1. Total sleep duration (categorical) according to infant and maternal characteristics.

		Infant Total Sleep Duration				
	Total	Sleep <14 hours		Sleep ^14 hours		
		N	(%)	*n*	(%)	*Exact P*
Characteristics		*170*	(38.9)	*267*	(61.1)	
Infant Gender						*0.08*
Girl	220	95	(43.2)	125	(56.8)	
Boy	217	75	(34.6)	142	(65.4)	
Weight at Delivery						*0.53*
Below 2500g	13	4	(30.8)	9	(69.2)	
2500 to 3499g	227	89	(39.2)	138	(60.8)	
3500 to 4499g	186	71	(38.2)	115	(61.8)	
Over 4500g	8	5	(62.5)	3	(37.5)	
Hospital Length of Stay						*1.00*
Less than 4 Days	350	134	(38.3)	216	(61.7)	
4+ Days	30	11	(36.7)	19	(63.3)	
Direct Antibiotic Exposure Before 3 Months						*0.09*
No	423	168	(39.7)	255	(60.3)	
Yes	14	2	(14.3)	12	(85.7)	
Gestational Age at Birth						*0.12*
Below 38 weeks	46	13	(28.3)	33	(71.7)	
38 to 39 weeks	205	76	(37.1)	129	(62.9)	
Over 40 weeks	183	80	(43.7)	103	(56.3)	
Birth Mode						*0.06*
Vaginal no IAP	233	78	(33.5)	155	(66.5)	
Vaginal with IAP	95	43	(45.3)	52	(54.7)	
Scheduled CS	48	19	(39.6)	29	(60.4)	
Emergency CS	56	28	(50.0)	28	(50.0)	

Solids Before 3 Months						*0.27*
No	417	164	(39.3)	253	(60.7)	
Yes	14	3	(21.4)	11	(78.6)	
Colic or Acid Reflux						*0.38*
No	311	117	(37.6)	194	(62.4)	
Yes	123	52	(42.3)	71	(57.7)	
Maternal Prenatal Depression						*0.01*
No	303	111	(36.6)	192	(63.4)	
Yes	80	42	(52.5)	38	(47.5)	
Breastfeeding Status at 3 Months						*0.30*
Exclusive	220	78	(35.5)	142	(64.5)	
Partial	128	52	(40.6)	76	(59.4)	
Zero	88	39	(44.3)	49	(55.7)	
Maternal Race/Ethnicity						*0.52*
White	340	136	(40.0)	204	(60.0)	
Asian	45	14	(31.1)	31	(68.9)	
Other	49	20	(40.8)	29	(59.2)	
Maternal Age						*0.23*
18 to 29	132	59	(44.7)	73	(55.3)	
30 to 39	285	105	(36.8)	180	(63.2)	
Over 40	20	6	(30.0)	14	(70.0)	
Siblings						*<0.001*
No	196	96	(49.0)	100	(51.0)	
Yes	239	73	(30.5)	166	(69.5)	

Notes: P-value calculated using Fischer*f*s Exact test. IAP-intrapartum antibiotic prophylaxis; CS-caesarean section.

Table 2. Median relative abundance in percent (IQR) of relevant bacterial taxa at the phyla, family, and genus level according to categorical sleep duration (<14 hours vs ˆ14 hours per 24 -hour period) at 3 months of age.

Bacterial Taxa	**Infant Total Sleep Duration**		
Phyla	<14 hours *n*=170	ˆ14 hours *n*=267	*P*
Family / Genus	Median Relative Abundance (%; IQR)	Median Relative Abundance (%; IQR)	
Actinobacteria	5.12 (0.84-16.88)	5.26 (1.46-14.83)	*0.75*
Bifidobacteriaceae	4.38 (0.48-16.24)	4.31 (1.15-14.01)	*0.74*
Bifidobacterium	4.38 (0.48-16.24)	4.31 (1.15-13.97)	*0.73*
Bacteroidetes	10.56 (0.07-60.17)	15.48 (0.08-57.98)	*0.82*
Bacteroides	8.26 (0.05-54.89)	7.82 (0.05-52.02)	*0.90*
Firmicutes	26.24 (11.59-47.78)	24.96 (11.27-47.41)	*0.52*
Enterococcaceae	0.03 (0.01-0.16)	0.02 (0.00-0.10)	*0.08*
Enterococcus	0.03 (0.01-0.15)	0.02 (0.00-0.10)	***0.05***
Streptococcaceae	0.67 (0.18-2.54)	0.54 (0.18-1.78)	*0.19*
Streptococcus	0.67 (0.18-2.54)	0.54 (0.18-1.78)	*0.19*

	Clostridiaceae	0.52 (0.06-2.37)	0.46 (0.05-2.53)	*0.52*
	Unclassified	0.22 (0.02-0.85)	0.18 (0.02-1.04)	*0.86*
	Clostridium	0.04 (0.01-0.74)	0.02 (0.00-0.40)	***0.02***
	Lachnospiraceae	4.54 (0.20-13.77)	4.91 (0.53-15.70)	*0.40*
	Unclassified	0.40 (0.01-4.98)	0.67 (0.02-4.98)	*0.43*
	Ruminococcus	0.10 (0.00-2.22)	0.12 (0.00-2.33)	*0.94*
	Ruminococcaceae	0.28 (0.01-1.63)	0.26 (0.01-1.96)	*0.40*
	Veillonellaceae	6.18 (1.35-16.62)	5.37 (1.02-16.28)	*0.33*
	Veillonella	5.49 (0.88-14.75)	3.16 (0.42-14.64)	*0.25*
Proteobacteria		16.60 (6.59-35.42)	16.58 (6.35-38.55)	*0.85*
	Enterobacteriaceae	13.62 (4.87-34.50)	15.13 (4.62-34.15)	*0.74*
	Unclassified	13.38 (4.78-33.80)	15.01 (4.57-34.01)	*0.74*

Notes: Significance calculated using Mann-Whitney U test.

Species richness, Chao index species richness, Shannon diversity, and Simpson diversity index were not different according to infant sleep duration (Figure 2， Appendix F). The relative abundance of *Enterococcus* was significantly higher in short sleepers when the full cohort was considered (Figure 1) as well as short sleepers born vaginally with IAP (Figure 3， Appendix F). *Clostridium* (Family *Clostridiaceae*) were significantly higher in short sleepers in the full cohort, as well as short sleepers who were formula fed. The relative abundance of *Lachnospiraceae* was significantly lower in short sleepers in exclusively breastfed infants, as well as short sleepers born vaginally without IAP. In infants born vaginally without IAP, relative abundances of Bacilli and Lactobacillales were significantly higher in short sleepers. In infants born by emergency CS, *Enterobacteriaceae* were significantly lower in short sleepers.

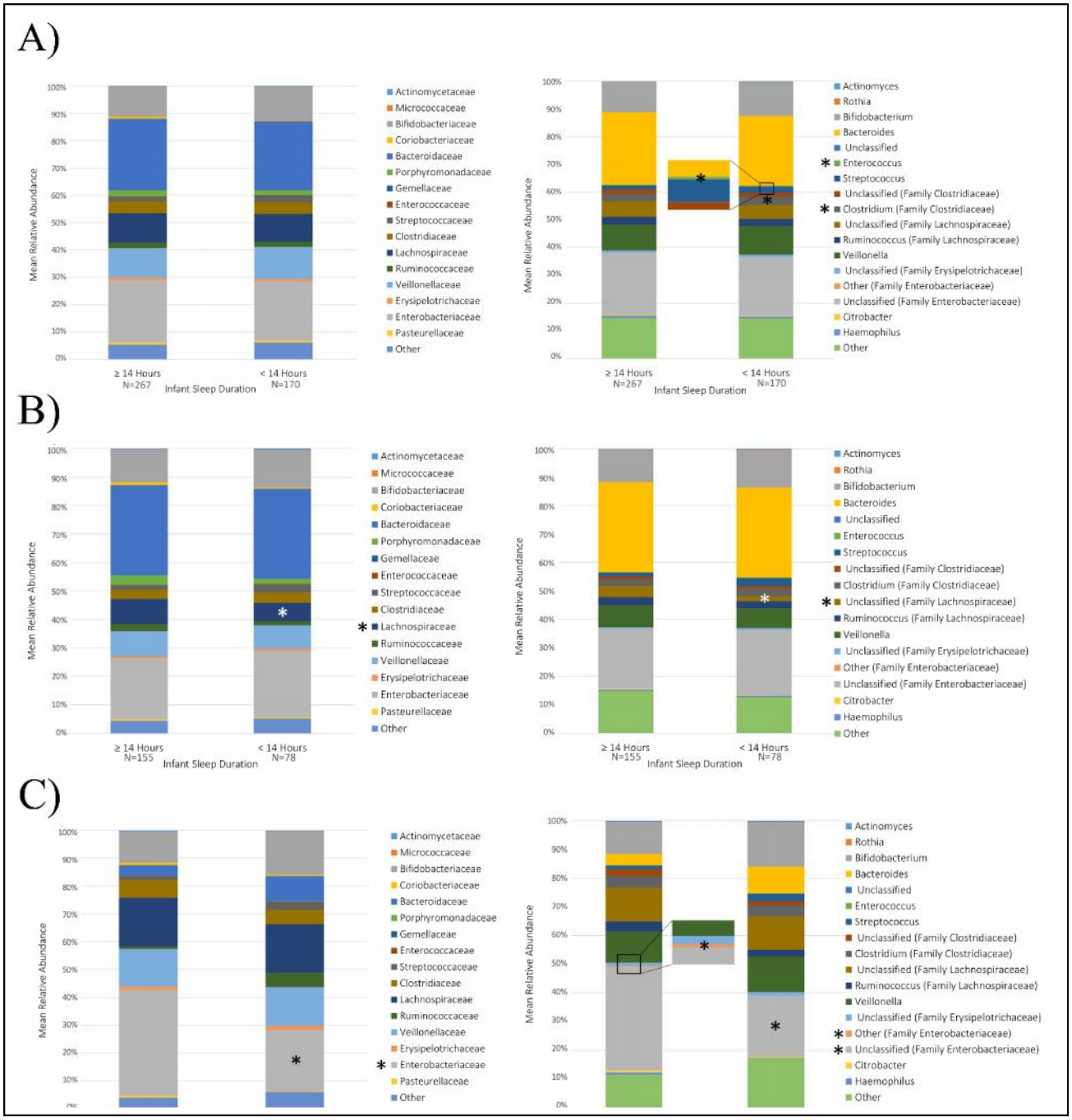

Figure 1. Stacked bar plots of bacterial mean relative abundance at the family and genus levels of A) all infants sleeping ˆ14 hours and < 14 hours; B) infants born vaginally without IAP sleeping ˆ vs < 14 hours; and C) infants born by emergency CS sleeping ˆ vs < 14 hours. Significance determined by Mann Whitney-U Test. *p-value<0.05. Restricted to bacterial taxa with colonization rate ˆ 50%.

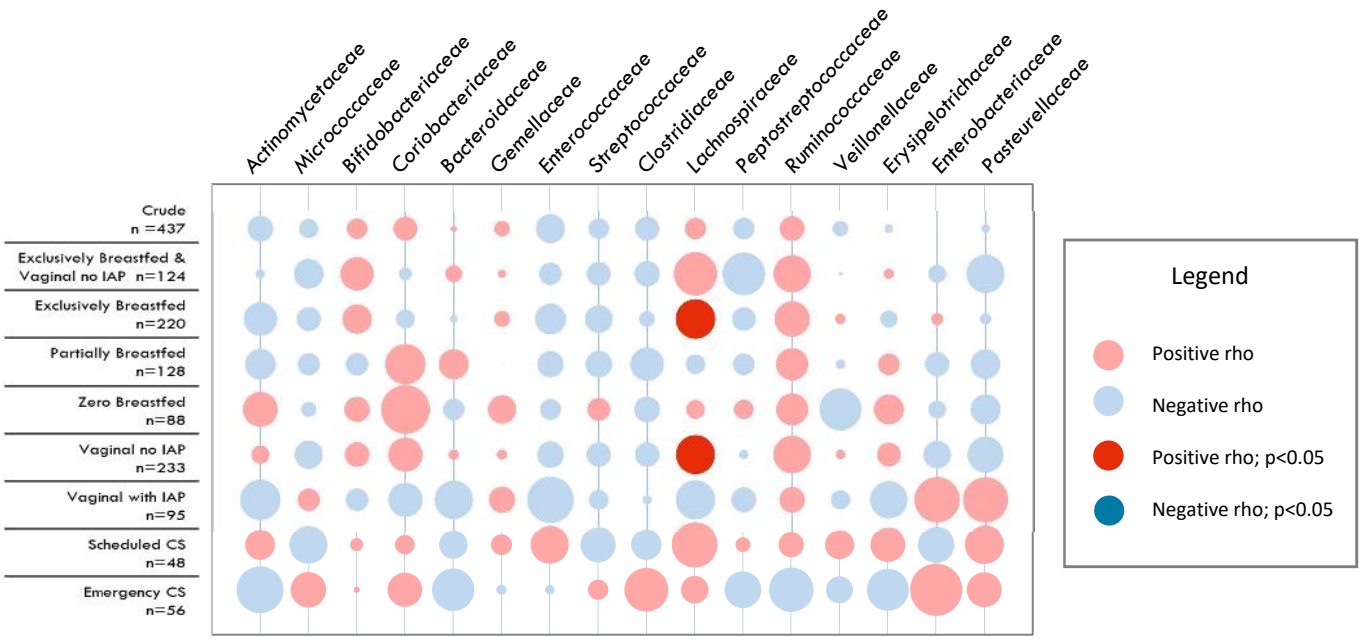

Figure 2. Spearman correlation at the family level between bacterial taxa relative abundance and continuous total sleep duration. Light blue: negative association; light red: positive association; dark blue: significant negative association ($p<0.05$); dark red: significant positive association ($p<0.05$).

At the family level, Actinomycetales (Rho: -0.144; p=0.033) and *Lachnospiraceae* (Rho: 0.136; p=0.044) were significantly associated with total sleep duration in exclusively breastfed infants according to Spearman Correlation of continuous sleep duration and microbial relative abundance (Figure 2). At the genus level, *Clostridium* (Family *Clostridiaceae*) was negatively associated with total sleep duration (Rho: -0.103; p=0.032; Figure 3). In exclusively breastfed infants born vaginally without IAP, no bacterial taxa were significantly associated with total sleep duration. No bacterial taxa were significantly associated with total sleep duration in partially breastfed, formula fed, or elective CS born infants. In infants born vaginally without IAP, *Lachnospiraceae* (Rho: 0.132; p=0.044) and *Oscillospira* (Rho: 0.137; p=0.036) were positively associated with total sleep duration, while *Clostridium* (family *Clostridiaceae*) (Rho: -0.135; p=0.040) was negatively associated total sleep duration. In infants born vaginally with IAP, Gammaproteobacteria (Rho: 0.207; p=0.044) and *Blautia* (Rho: -0.342; p=0.001) were significantly associated with total sleep duration. Genus *Other* (Family *Enterobacteriaceae*) (Rho: 0.338; p=0.011) was positively associated with total sleep duration in infants born by emergency CS. For Spearman Correlation results at the phyla, class and order level, see Figure 4 , Appendix F.

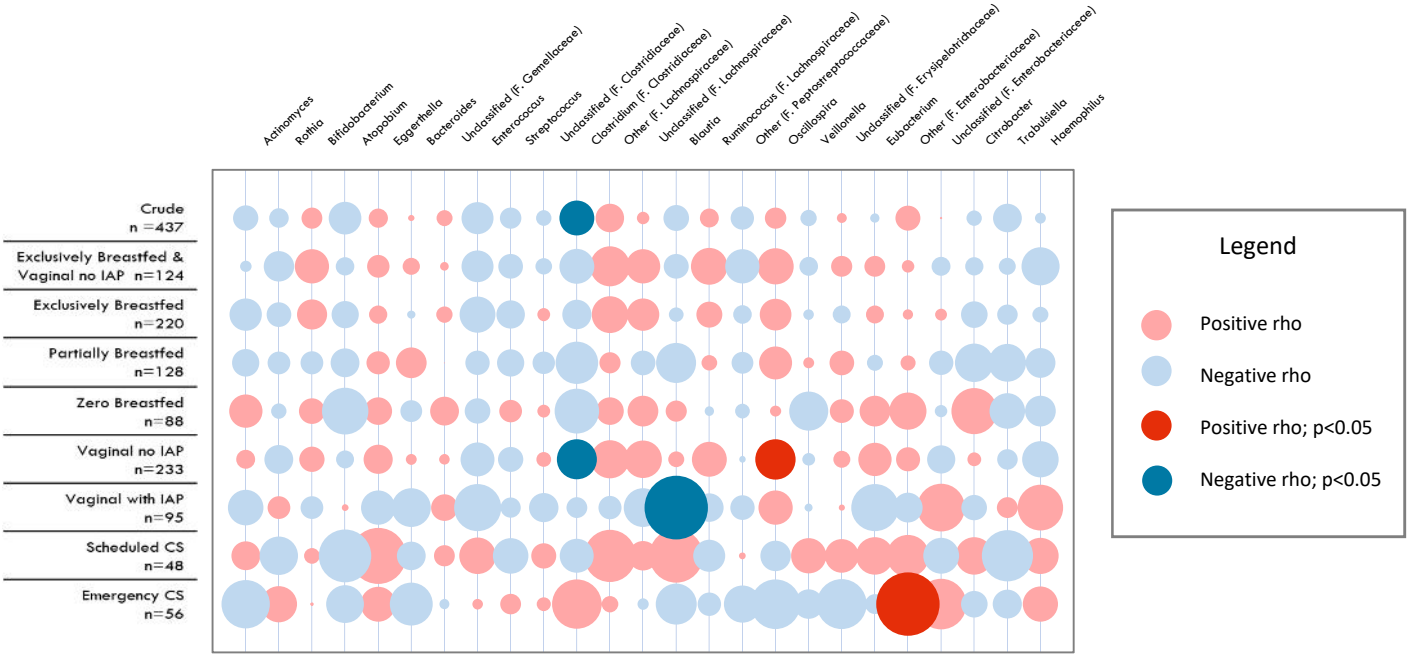

Figure 3. Spearman correlation at the genus level between bacterial taxa relative abundance and continuous total sleep duration. Light blue: negative association; light red: positive association; dark blue: significant negative association ($p<0.05$); dark red: significant positive association ($p<0.05$).

Bacterial taxa frequency (taxa present vs not present) was significantly different between infants sleeping ˆ vs <14 hours per 24 -hour period. Most importantly, odds of *Enterococcus* and *Clostridium* (family *Clostridiaceae*) being present were significantly higher while odds of *Erwinia* being present were significantly lower in short sleepers (Figure 4).

At the phyla level, categorical sleep duration (above vs below 14 hours per 24-hour period) significantly contributed to the prediction of phyla TM7 following adjustment for breastfeeding status, birth mode, and prenatal depression (OR for short sleepers: 0.53; Figure 5 , Appendix F) as well as adjustment for breastfeeding status, birth mode, and maternal race/ethnicity. TM7-3 was similarly associated with sleep duration. The order Actinomycetales was positively associated with short sleep when crude, adjusted for breastfeeding and birth mode, adjusted for breastfeeding, birth mode, and maternal race/ethnicity, but did not survive adjustment for breastfeeding status, birth mode, and prenatal depression.

At the family level, logistic regression determined that the odds of *Enterococcaceae* being present was higher in infants sleeping <14 hours compared to infants sleeping ˆ 14 hours (Figure 6 , Appendix F). This association survived adjustment for breastfeeding and birth mode, as well as breastfeeding and birth mode with maternal race, but did not survive adjustment when prenatal depression was included. Sensitivity analyses determined that hospital length of stay, direct infant antibiotic exposure, siblings, and age at stool sample collection did not confound the associations described above.

	Crude	Adjusted (Breastfeeding Status & Birth mode)	Adjusted (BF Status, Birth mode, & Depression)	Adjusted (BF Status, Birth mode, & Race/Ethnicity)
GENUS				
Actinomyces				
Varibaculum				
Rothia				
Bifidobacterium				
Atopobium				
Collinsella				
Eggerthella				
Bacteroides				
Parabacteroides				
Prevotella				
Unclassified (Family Rikenellaceae)				
Staphylococcus				
Unclassified (Family Gemellaceae)				
Other (Class Lactobacillales; Family Other)				
Granulicatella				
Other (Family Enterococcaceae)				
Enterococcus	O	O	O	O
Lactobacillus				
Streptococcus				
Other (C. Clostridiales; F. Other)				
Unclassified (C. Clostridiales; F. Unclassified)				
Other (Family Clostridiaceae)				
Unclassified (Family Clostridiaceae)			O	
Clostridium (Family Clostridiaceae)	OO	OO	OO	OO
Other (Family Lachnospiraceae)				
Unclassified (Family Lachnospiraceae)				
Blautia				
Clostridium (Family Lachnospiraceae)				
Coprococcus				
Dorea				
Epulopiscium				
Lachnospira				
Roseburia				
Ruminococcus (F. Lachnospiraceae)				
Other (Family Peptostreptococcaceae)				

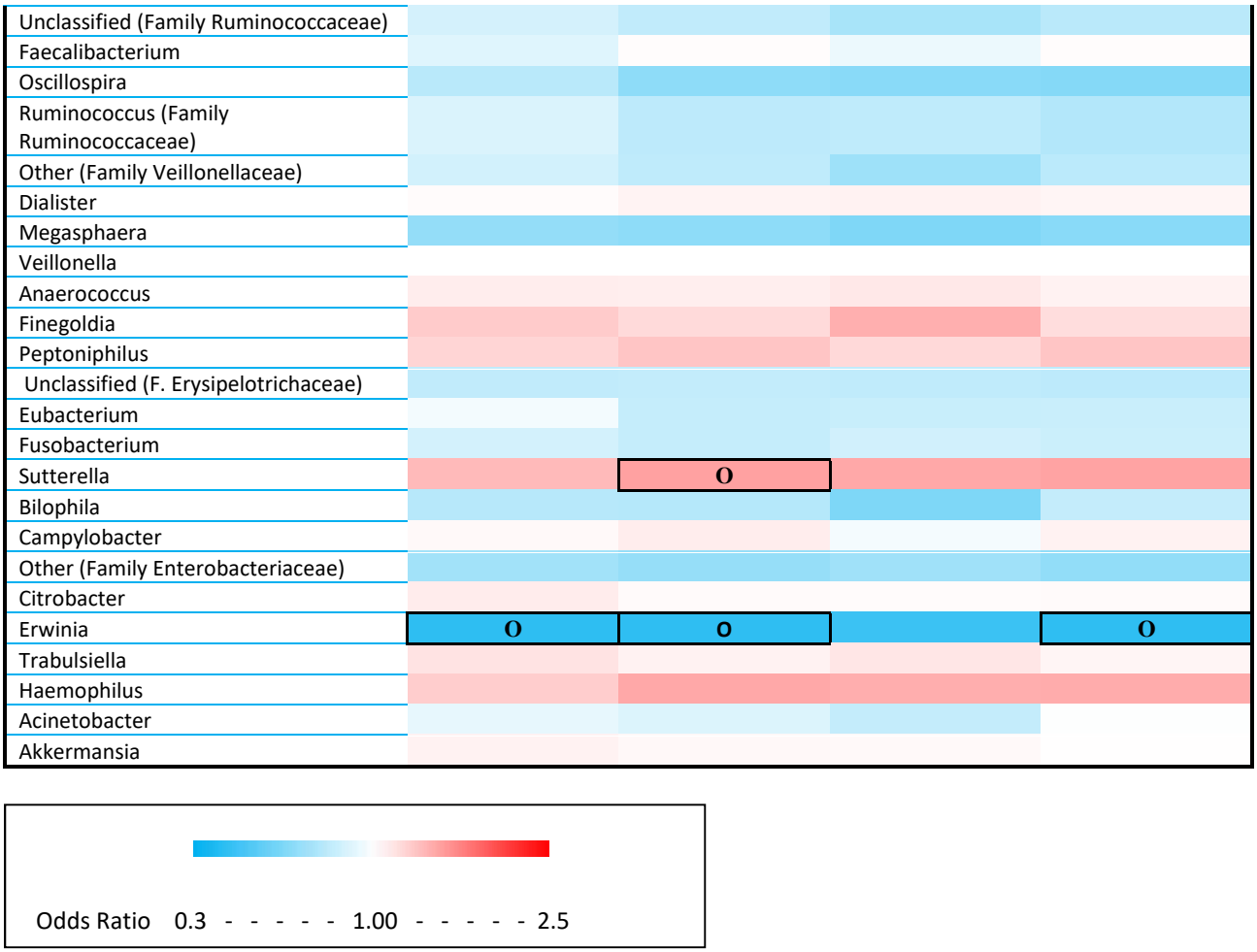

Figure 4. Heat map of odds ratios from logistic regression of bacterial taxa frequency. Odds Ratio = short sleepers (<14 hours) vs normal sleepers (ˆ14 h ours; *ref*). ‹ = statistical significance at an alpha level of 0.05; ‹ ‹ = statistical significance at an alpha level of 0.01.

The GLM-based model MaAsLin was used to determine which bacterial taxa were associated with categorical sleep after controlling for the effects of metadata. At an FDR level of 0.2, *Erwinia* was found to be negatively associated with short sleep while *Alcaligenaceae* was positively associated with short sleep (Table 3); however, *Alcaligenaceae* did not survive addition of prenatal depression to the model (Table 2 , Appendix F).

Table 3. MaAslin Results for total sleep ˆ 14 h ours (*ref*) vs < 14 hours with FDR alpha level 0.2.

Family	Infant Total Sleep Duration ˆ 14 h ours (*ref*) vs < 14 hours			
Genus	Coefficient	n not 0	*P value*	*Q value*
Enterobacteriaceae				

Erwinia	-0.00067	49	*0.013*	*0.085*
Alcaligenaceae	0.00079	124	*0.042*	*0.186*

Notes: Adjusted for metadata: Breast feeding status - exclusive, partial, or zero at 3 months; and birth mode， vaginal no IAP, vaginal with IAP, scheduled CS, or emergency CS.

GLM modelling was then conducted on arcsine square root transformed bacterial relative abundance to identify bacterial taxa which were not identified by MaAsLin due to covariate metadata which were stronger predictors of taxa relative abundance than short sleep. Using this method, *Streptococcaceae* and its genera *Streptococcus,* Bacilli, and Lactobacillales were found to be positively associated with being a short sleeper, independent of birth mode, breastfeeding, prenatal depression, hospital length of stay, siblings, and age at stool sample collection (Table 4 and Table 3-6， Appendix F). Collinsella and Erwinia were negatively associated with being a short sleeper, independent of the covariates mentioned above (Table 6， Appendix F). *Alcaligenaceae* and *Sutterella* were found to be confounded by prenatal depression while *Coriobacteriaceae* was found to be confounded by the presence of siblings.

Table 4. Multivariable linear regression predicting arcsine square root transformed relative abundance of bacterial taxa at the family level by categorical sleep duration (above vs below 14 hours) at the family level.

	Taxa Prevalence above 50%	**Crude**		**Adjusted (Breastfeeding status & Birth mode)**		**Adjusted (Breastfeeding status, Birth mode & Prenatal depression)**	
		n=435		n=424		n=371	
		Coef.	95% CI	Coef.	95% CI	Coef.	95% CI
FAMILY							
Actinomycetaceae	Œ	0.004	(-0.004, 0.013)	0.004	(-0.005, 0.012)	0.002	(-0.006, 0.009)
Micrococcaceae	Œ	0.004	(-0.001, 0.008)	0.004	(0.000, 0.009)	0.004	(-0.001, 0.009)
Bifidobacteriaceae	Œ	0.007	(-0.040, 0.054)	0.014	(-0.034, 0.062)	-0.001	(-0.052, 0.049)
Coriobacteriaceae	Œ	**-0.013***	**(-0.026, 0.000)**	**-0.014***	**(-0.026, -0.001)**	**-0.014***	**(-0.027, -0.001)**
Bacteroidaceae	Œ	-0.012	(-0.092, 0.069)	0.003	(-0.073, 0.079)	0.029	(-0.051, 0.110)
Porphyromonadaceae	Œ	-0.009	(-0.036, 0.019)	-0.009	(-0.036, 0.017)	-0.009	(-0.037, 0.019)
Prevotellaceae		0.001	(-0.010, 0.013)	0.001	(-0.011, 0.013)	0.002	(-0.009, 0.014)
Rikenellaceae		0.007	(-0.010, 0.024)	0.009	(-0.008, 0.026)	0.009	(-0.011, 0.028)
Staphylococcaceae		0.002	(-0.001, 0.005)	**0.003***	**(0.000, 0.007)**	0.003	(-0.001, 0.006)
Gemellaceae	Œ	0.000	(-0.003, 0.003)	0.000	(-0.003, 0.003)	0.000	(-0.003, 0.003)
Other (C. Lactobacillales)		-0.001	(-0.004, 0.003)	-0.002	(-0.005, 0.002)	-0.001	(-0.005, 0.003)
Carnobacteriaceae		0.000	(-0.001, 0.001)	0.000	(-0.001, 0.001)	0.000	(-0.001, 0.001)
Enterococcaceae	Œ	0.005	(-0.002, 0.012)	0.004	(-0.003, 0.012)	0.006	(-0.002, 0.013)

Lactobacillaceae		0.001	(-0.010, 0.012)	0.002	(-0.009, 0.014)	-0.005	(-0.014, 0.004)
Streptococcaceae	Œ	**0.019***	**(0.002, 0.036)**	**0.021***	**(0.004, 0.038)**	0.015	(-0.003, 0.034)
Other (C. Clostridiales)		0.000	(-0.002, 0.003)	0.000	(-0.003, 0.002)	0.000	(-0.003, 0.002)
Unclassified (C. Clostridiales)		0.009	(-0.004, 0.023)	0.008	(-0.006, 0.021)	0.007	(-0.009, 0.022)
Clostridiaceae	Œ	0.002	(-0.033, 0.037)	0.002	(-0.033, 0.037)	0.011	(-0.028, 0.049)
Lachnospiraceae	Œ	-0.015	(-0.059, 0.030)	-0.032	(-0.075, 0.010)	-0.040	(-0.085, 0.005)
Peptostreptococcaceae		0.000	(-0.003, 0.003)	0.000	(-0.004, 0.003)	0.000	(-0.004, 0.004)
Ruminococcaceae	Œ	-0.005	(-0.027, 0.017)	-0.011	(-0.032, 0.010)	-0.010	(-0.033, 0.013)
Veillonellaceae	Œ	0.019	(-0.021, 0.060)	0.012	(-0.028, 0.053)	0.006	(-0.039, 0.050)
Tissierellaceae		0.001	(-0.001, 0.004)	0.001	(-0.002, 0.004)	0.001	(-0.002, 0.004)
Erysipelotrichaceae	Œ	0.002	(-0.014, 0.018)	-0.001	(-0.017, 0.016)	-0.002	(-0.020, 0.016)
Fusobacteriaceae		-0.002	(-0.005, 0.001)	-0.002	(-0.005, 0.001)	-0.002	(-0.005, 0.001)
Alcaligenaceae		0.015	(0.000, 0.030)	**0.016***	**(0.001, 0.032)**	0.014	(-0.003, 0.031)
Desulfovibrionaceae		-0.005	(-0.013, 0.004)	-0.004	(-0.013, 0.004)	-0.006	(-0.015, 0.004)
Campylobacteraceae		-0.003	(-0.006, 0.000)	-0.003	(-0.006, 0.000)	-0.003	(-0.007, 0.000)
Enterobacteriaceae	Œ	-0.015	(-0.070, 0.040)	-0.010	(-0.064, 0.045)	-0.008	(-0.065, 0.049)
Pasteurellaceae	Œ	-0.010	(-0.025, 0.006)	-0.008	(-0.023, 0.007)	-0.012	(-0.029, 0.004)
Moraxellaceae		0.000	(-0.002, 0.002)	0.000	(-0.002, 0.002)	-0.001	(-0.002, 0.001)
Verrucomicrobiaceae		-0.006	(-0.040, 0.028)	-0.011	(-0.044, 0.023)	-0.008	(-0.045, 0.030)

Notes: Breastfeeding status (categorical): Exclusive (ref), partial, and formula fed; birth mode (categorical): vaginal without IAP (ref), vaginal with IAP, elective CS, emergency CS; prenatal depression(categorical): CESD score <16 (ref) vs „16. * p<0.05.

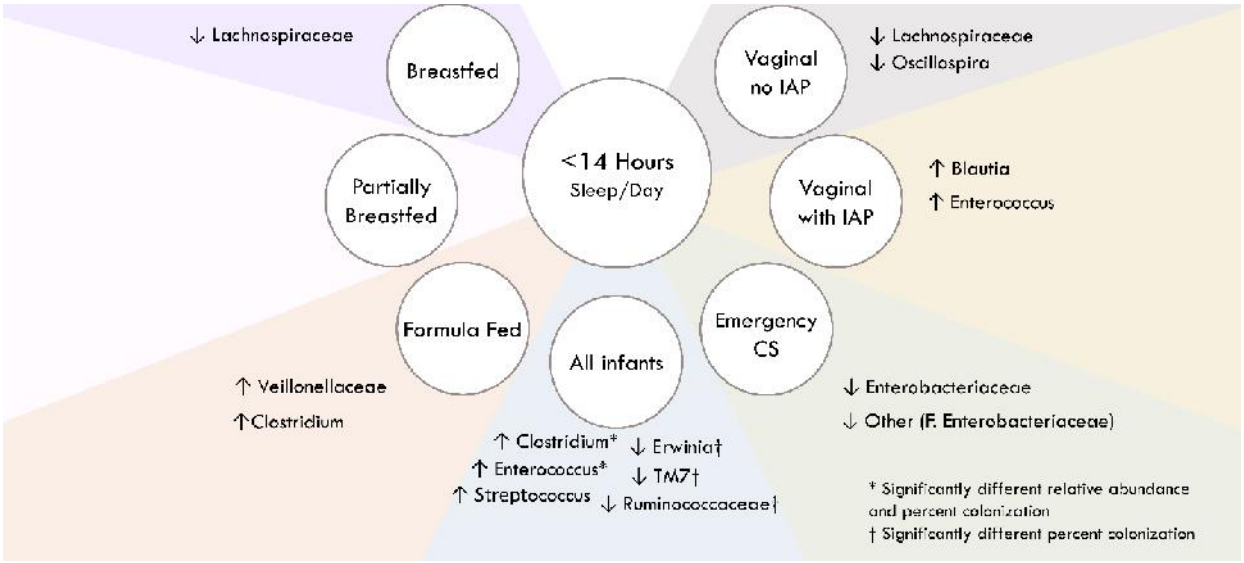

Figure 5. Summary of results at the family and genus level. * Significantly different relative abundance and percent colonization; ‰ significantly different percent colonization.

4.4 Discussion

In our general population birth cohort from Edmonton, Alberta, Canada, infant sleep duration at 3 months of age was associated with numerous differences in fecal microbiota composition. Compared with infants meeting National Sleep Foundation minimum recommended sleep duration (ˆ14 hours per 24 - hour period), short sleepers (<14 hours) had a reduced relative abundance of *Lachnospiraceae* (exclusively breastfed or vaginally born infants without IAP only). Previously, *Lachnospiraceae* has been found to play a role in the intergenerational transmission of weight status between mother and infant (141). We found that *Lachnospiraceae* was only associated with sleep duration in exclusively breastfed or vaginally born infants without IAP (no maternal antibiotic prophylaxis), which would both provide a route of transmission from mother to child. Of note, *Lachnospiraceae* was still significantly higher in infants who were born by caesarean section or formula fed. *Lachnospiraceae* has been shown to promote obesity, which is generally considered to be negative (180); however, infancy is a unique period requiring the careful orchestration of multiple biological systems including increased sleep duration (18) to facilitate normal growth. The direction of the association between *Lachnospiraceae* and sleep duration is currently unknown, and in fact both factors may increase concurrently in response to an unmeasured confounding factor which results in an increased rate of infant growth. Previous studies in animals have found experimentally imposed sleep fragmentation increases the relative abundance of *Lachnospiraceae* (88), which is in opposition to our findings. However, gut microbiota compositions enriched with *Lachnospiraceae* and *Enterobacteriaceae* are known to increase host inflammation (88,185). Due to the sleep-inducing role of inflammatory cytokines (14), *Lachnospiraceae* and *Enterobacteriaceae* effected inflammation may promote increased sleep duration. This highlights the importance of observational work and the potential important and drastic differences between acute experimental exposures and real life.

Short sleepers may have reduced gut microbiota age. *Streptococcaceae* and *Enterococacceae,* which we found to be increased in short sleepers, are associated with decreased infant gut microbiota age while *Lachnospiraceae* and *Ruminococcaceae* are associated with increased infant gut microbiota age (186).

However, *Enterobacteriaceae,* which were decreased in short sleepers born by emergency CS, are also negatively associated with infant gut microbiota age (186). This discrepancy may be due to the drastically different gut microbiota composition of infants born by emergency CS. Our findings survived adjustment for infant age at gut microbiota sample collection.

Erwinia relative abundance and percent colonization were found to be lower in short sleepers compared to those sleeping ˆ14 hours per 24 -hour period. Previously, this bacteria has been negatively associated with canine ownership in 3-6 month old infants enrolled in the VDAART cohort (187). *Erwinia* is a bacterium known for its pathogenic properties towards plants. There is the possibility that increased colonization with *Erwinia* may be a marker of increased exposure to plants (i.e. maternal intake of fresh fruits and vegetables or exposure to the outdoors). Some strains of *Erwinia carotovora,* a known plant pathogen*,* possess bacterial glutathione transferases, enzymes which can play a role in counteracting xenobiotic toxicity and abiotic stress (188). Glutathione is an antioxidant in plants, animals and some bacteria capable of preventing damage to important cellular components caused by reactive oxygen species. Glutathione peroxidase is a component of human breast milk, and is significantly associated with selenium concentrations (189). *Erwinia* may be increased in response to glutathione in the infants*f* diet. Furthermore, sleep deprivation results in a decrease of glutathione peroxidase in adults (190). This is due to increased production of reactive oxygen species, which leads to the depletion of glutathione. Similarly, glutathione may be utilized in sleep deprived or stressed infants, resulting in reduced glutathione availability for the *Erwinia* species to utilize.

Due to the cyclic nature of the relationship between sleep, stress, the immune system, and the gut microbiota, it is difficult to ascertain the direction of the associations described above. Additionally, an unmeasured confounder such as biological age or immune system activation may significantly contribute to the composition of the gut microbiome and concurrent infant sleep duration. However, this study provides considerable evidence that the infant gut microbiota may explain the positive relationship between sleep duration and weight gain in infancy.

Although many studies of the infant microbiota tend to design the analysis to identify factors which influence the microbiome in all infants, this study provides strong evidence that the relationship between factors and the microbiome can only be considered in reference to the previous factors which have shaped that specific infant*ƒ*s microbiome.

Strengths and Limitations

Our study has several strengths, including the application of high-thoroughput deep sequencing to profile gut microbiota in a birth cohort, with a representative and large sample size. We examined not only taxa median abundance but also taxa prevalence, which may give insight into the differences between the processes of microbial seeding and growth. We utilized taxon-specific arcsine square root transformed microbiota relative abundance modelling, allowing for a much deeper understand of the relationship between sleep and the infant gut microbiota. In this specific study, one important confounder may be the caloric amount, frequency and timing of infant feeding. There is the possibility that the associations we observed between the gut microbiota and sleep are influenced by a third, unmeasured factor.

4.5 Conclusions

Increased infant sleep duration at three months of age is associated with bacterial taxa which are associated with infant growth such as *Lachnospiraceae* and *Enterobacteriaceae*. We posit that bacterial composition may explain the association between infant sleep duration and weight gain in infancy.

4.6 Acknowledgements

The authors would like to thank the CHILD Study participants, without whom this research study would not be possible. The authors also thank the CHILD team, which includes interviewers, computer and laboratory technicians, clerical workers, research scientists, volunteers, managers, receptionists and nurses.

4.7 Appendix

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Chapter 5: Summary of Findings and Discussion

5.1 Prenatal depression and birth mode sequentially mediate the relationship between maternal education level and infant sleep duration

Infant sleep is one of the most common concerns of new parents (191). Infants of families with reduced SES are disproportionately impacted by infant sleep problems (38), which may serve as a conduit in the intergeneration transmission of SES. This study expands what is known about the modifiable factors which link SES to early infant sleep duration. Maternal education, a significant aspect of SES, and siblings in the home were significantly associated with longer infant sleep duration while maternal prenatal depression and emergency caesarean section birth were significantly associated with shorter infant sleep duration at 3 months of age. Furthermore, thirty percent of the association between maternal education and infant sleep duration was mediated by prenatal depression and birth mode. Our study supports previous research which has highlighted maternal antenatal depression as an important predictor of infant sleep duration (48). Maternal depression during pregnancy may impact the developing infant in a number of ways. Depression and sleep problems have high co-morbidity, and mothers with prenatal depression are known to have an increased risk of problems relating to sleep (43,45). As a result, the infant may be influenced in utero by maternal sleep dysregulation, circadian signalling and melatonin levels (46,47,155). Maternal depression during pregnancy is also strongly associated with postnatal depression (44). Maternal depression in the early postpartum period has been found to have a robust effect on infant sleep duration (192). The impact of maternal postpartum depression may be mostly due to the uni-directional relationship between maternal and infant sleep patterns (193). However, it is difficult to dissect which effects are due to prenatal versus postnatal depression (48). The impact of prenatal depression on birth outcomes is not well defined. Prenatal anxiety has been associated with increased risk of preterm birth and low birth weight (160), which suggests that prenatal depression may also increase the risk of adverse birth outcomes, including emergency caesarean section. We hypothesize that prenatal depression, and concurrent maternal sleep problems, may increase the risk of failure to progress during

labour, increasing the risk of emergency caesarean section. Some studies have found an association between caesarean section and postpartum depression while others have not (164), which may be due to potential confounding effects of prenatal depression.

The impact of emergency versus scheduled caesarean section has not been examined previously beyond the second postnatal day. Our study provides evidence that infants born by emergency caesarean section may represent a population at increased risk of short sleep. We can only speculate as to the cause of this association. A traumatic birth experience may have significant negative effects on the mother, impacting her mood, risk of depression, or sleep quality (164). As discussed previously, maternal sleep in the postpartum period has a significant effect on the development of sleep habits in the infant (193). Hurried anesthetic administration in response to medical emergencies during labour may also impact infant neuronal development (194,195). Many medications (i.e. antibiotics and oxytocin) are administered more frequently or in larger doses to women experiencing emergency caesarean section, whose impact on infant sleep has not been explored. The synthetic hormone oxytocin increases wakefulness (168), which may be due to oxytocin*'*s influence on the HPA axis (158,169,170). Antibiotics have been shown to not only influence sleep patterns (54,55), but also increase the risk of illnesses such as asthma and inflammatory bowel disease (IBD) when administered in infancy (84,196, 198). Despite the global use of these treatments, there is an urgent need for research exploring their long-term impacts on infant sleep.

For mom, emergency caesarean section is one of the most stressful modes of birth, resulting in infant exposure to significantly elevated free cortisol in labour during emergency caesarean birth (162). Stress and the circadian system are bidirectionally regulated through the HPA-axis, and both systems undergo critical development in the perinatal period (165). For example, in rats, the paraventricular nuclei, limbic system and hippocampus undergo significant neurogenesis in the period surrounding birth, while clock gene expression concurrently begins development (165). During this time, maternal stress signals (e.g., glucocorticoids, catecholamines, melatonin, and dopamine) reaching the embryo can lastingly change gene expression through the induction of epigenetic mechanisms (165). We believe that

maternal stress hormone production due to prenatal depression and emergency caesarean section birth may impact circadian rhythm through this pathway.

As a result, mothers who experience prenatal depression or emergency caesarean section birth may benefit from additional counselling on infant sleep. Furthermore, we provide supplementary evidence to support the global goal of reducing the rate of emergency caesarean section births (199).

5.2 Sleep duration is associated with the composition of the gut microbiota in infancy

We found that bacterial taxa associated with higher BMI (141) or adiposity (200) in toddlers, *Lachnospiraceae* and *Enterobacteriaceae*, were positively associated with sleep duration. Conversely, *Streptococcus*, *Enterococcus* and *Clostridium* were increased in infants who were short sleepers at 3 months of age. This work leads to interesting questions on how sleep and gut microbiota composition coordinate to result in growth at this critical time. Increased infant feeding frequency may result in increased carbohydrate utilizers in the gut such as *Lachnospiraceae*. Increased inflammation due to *Lachnospiraceae* and *Enterobacteriaceae* may directly influence sleep, resulting in increased sleep needs (128). Regardless of feeding type, sleep duration may provide caretakers with important information regarding growth trajectories of vulnerable infants. For instance, hospitalized infants who experience significant disruptions to their sleep may also have an altered gut microbiota composition, which may not be conducive to weight gain. Interventions to minimize disturbances to sleep or probiotics to increase energy utilization could serve as useful therapeutics in these special populations.

Poroyko et al. found that sleep fragmentation in mice led to an increase of *Lachnospiraceae* in mice, which at first glance appears to disagree with our findings (88). However, this discrepancy may be explained by the bi-directional relationship between sleep and the gut microbiota. Acute sleep fragmentation is associated with increased inflammation, which is a direct result of the consequent changes to the gut microbiota, including increased *Lachnospiraceae* (88). Increased inflammation over

time is both a result of sleep deprivation and a strong causal agent of fatigue and increased sleep needs (175). As a result, we hypothesize that increased inflammation due to *Lachnospiraceae* and *Enterobacteriaceae* may be the cause of increased sleep duration in infants meeting the National Sleep Foundation guidelines.

The clinical implication of *Clostridium* relative abundance, which we found to be higher and more prevalent in infants not meeting the National Sleep Foundation recommendations, is difficult to interpret. Historically, *Clostridioides difficile*, a pathogenic bacterium associated with life-threatening diarrhea, was classified as a member of the *Clostridium* genus. However, in the past decade, *C. difficile* has been reclassified as a member of the *Peptostreptococcaceae* family. As a result, previous studies have reported *Clostridium* as an early marker of topic dermatitis and atopy (201); however, these studies may or may not refer to the *Clostridium* genus containing *C. difficile*, or refer solely to *C. difficile*. *Clostridium* is a broad genus which contains species that are both pathogenic and commensal, making it difficult to draw conclusions on this finding. Future work is required to determine if *C. difficile* is elevated in short sleepers in early infancy, which may be a marker of atopy.

Chapter 6: Bias

Publication Bias. Due to the nature of scientific publishing, it is difficult to discern if negative results are underreported in the sleep-microbiome and infant microbiome areas of study. Articles reporting negative results such as Zhang et al. were identified and reviewed in Section 1.2 (85). Additionally, systematic reviews were consulted when available for topics covered in Chapter 1. A potential for post-publication bias should be considered, as animal and adult studies were reviewed exclusively in Section 1.2.5 due to a lack of relevant studies in infants. Finally, to minimize the risk of underreporting negative results in Sections 3 and 4, both negative and positive results are presented.

Selection Bias. To control selection bias, a strict set of inclusion and exclusion criteria were used to select participants (See Section 2.1). Participants were recruited from the general population using multiple recruitment methods. Mothers in the Edmonton site of the CHILD study were recruited at high and low volume maternity clinics, community locations (i.e. tradeshows, maternity stores), and through advertisements in local media (i.e. printed ads, radio) from August 2008 to April 2012 (142). Statistically significant differences in participant characteristics have been identified based on recruitment method (142). When compared to a reference population from an obstetric clinic in Edmonton, the CHILD study Edmonton cohort was found to be more educated and more likely to have a high household income (142). Furthermore, mothers from the Canada-wide CHILD cohort are more likely to have asthma or allergic disease than the general population (143). However, for a study of this magnitude and with such a large commitment from participants, optimal recruitment methods to decrease selection bias were employed. The results of the studies presented above may not be generalizable outside of Edmontonian families with higher than average educational attainment and household income. Furthermore, maternal asthma or allergic disease may increase the risk of asthma and allergic disease, microbial dysbiosis, and sleep disturbances in the infants in these studies.

Stool sample texture was an unforeseen source of selection bias in the CHILD study. In this study, infant stool samples were collected from diapers provided by the parents. Breastfeeding results in

runnier stools than formula feeding. This resulted in a positive selection bias toward formula and partially breast-fed infants because some samples from breastfed infants could not be analyzed after collection. As a result, breastfeeding prevalence at 3 months of age is underreported in Chapter 4. Human breastmilk also selectively encourages the growth of bifidobacteria, which is also likely to be underreported in this cohort. To control for this source of bias, analyses were conducted after stratification or adjustment for breastfeeding status at 3 months of age. The majority of gut microbiota studies conducted in early infancy are also likely to suffer from the inability to process runnier stools from breastfed infants in their cohorts as well.

Measurement Bias. Parent perspectives on the importance of infant sleep had the potential to introduce bias. Parental health literacy may be influenced by education level, income or cultural upbringing. Mothers with postsecondary education may also more accurately record infant sleep duration. However, previous studies have not found education level to be associated with increased accuracy of self-reported sleep (202). Infants who learn to self sooth have better reported quality of sleep (40,193). Parents who play an active role in infant settling may be more aware of their infant*f*s sleep patterns but also have a negative impact on their infant*f*s quality of sleep(193). Mothers who have a more complicated birth resulting in emergency CS may be more likely to dote on their infant and, as a result, disrupt the infant*f*s ability to self-sooth. However, this has not been confirmed by previous research. Infant self-soothing was not measured until after the 3-month time point, and therefore could not be included as a covariate.

The BISQ is a validated measure for the collection of infant sleep duration compared to actigraphy (60). Parent-reported number of night wakings was not utilized in this study, as this measure is reported to have reduced validity (60).

The use of the QIA DNA extraction kit may underreport the amount of *Bifidobacterium* in provided samples. Resultantly, the results presented are likely to underreport *Bifidobacterium*, one of the most significant bacterial taxa in the gut of the infant. A source of bias in gut microbiota studies highlighted by this work is the time of sample collection. The composition of the gut microbiota changes

over the course of the day. In infants, this effect is hypothesized to be reduced due to their increased frequency of feeding. Variability of sample collection time in this cohort is expected to reduce the risk of bias due to this factor.

Interpretation Bias. Protopathic bias is bias due to disregard for reverse causation. The results in Chapter 3 are at reduced risk of protopathic bias due to the temporality of the variables. Maternal education level precedes prenatal depression and birth mode, and all three variables precede the measurement of infant sleep at 3 months of age.

The results in Chapter 4 have been interpreted cautiously to avoid the risk of protopathic bias. There is evidence that the gut microbiota may impact sleep, and that sleep can impact the gut microbiota. As a result, statements on the direction of this relationship have been avoided.

Confounding Bias. Multiple sources of confounding bias have been identified and controlled in the preceding chapters. For Chapter 3 these sources include: breastfeeding status, infant gestational age and weight at birth, weight at 3 months of age, solids, colic, maternal race/ethnicity, maternal smoking and siblings. To control for these potential confounding factors, variables were modelled together and in a purposefully selected model, whereby confounding and effect modification were explored. Two sources of confounding bias which could not be controlled were the influence of maternal sleep and postpartum depression. These covariates could not be included because they were collected after 3 months postpartum. In Chapter 4, potential sources of confounding included: breastfeeding, birth mode, maternal race/ethnicity, prenatal depression, weight at delivery, hospital length of stay, antibiotic exposure, solids, colic and siblings. To control for these potential confounders, covariates were included in logarithmic and linear regression modelling.

Chapter 7: Future Directions, Knowledge Translation and Conclusions

7.1 Future Directions

The door has been opened to explore the relationship between sleep and the gut microbiota in the infant, and many questions remain unanswered. It is important to determine the direction of the relationship between these two factors. Interventions to improve sleep duration in infancy should also measure corresponding changes in the composition of the gut microbiota. Likewise, future probiotic studies in infants or lactating mothers should also take sleep duration into account to explore whether probiotics positively impact sleep in infancy like they do in adulthood. These questions could also be explored experimentally through animal models, although this may also present additional challenges. There are important differences between the gut microbiome of humans and murine animals. Certain important human infant exposures, such as emergency caesarean section, cannot be simulated in an animal model. Finally, current experimental models to restrict sleep duration in animals introduce significant bias such as stress and physical activity and would not be appropriate for use in infant animals.

The relationship between qualitative measures of sleep and circadian organization in infancy with gut microbiota have yet to be examined. Once future research has established the direction of the relationship, its role in the etiology of diseases such as obesity and asthma can be explored further. In animals, there is now sufficient evidence that glucometabolic dysfunction due to circadian misalignment can be transmitted from one animal to another through the gut microbiota. In pediatric populations, this causal pathway should also be explored.

A number of important confounding factors could not be scrutinized by this study. Maternal sleep is an important source of potential confounding not addressed in Chapter 3. Future work should examine if maternal sleep explains the associations between prenatal depression, birth mode and infant sleep. Stress hormones and the activation of the HPA-axis may also explain why emergency caesarean section

has such a large, negative impact on infant sleep duration. Studies examining cortisol at birth and infant sleep duration may help to elucidate this relationship.

Finally, this work highlights an important aspect in the reporting of infant gut microbiota research. The bearing of factors on the infant gut microbiota can only be considered in relation to previous influences on the gut microbiota. It is important to bear in mind that some infants may be more susceptible to dysbiotic influences and factors may lead to incongruent gut microbiota changes due to the seeding of the microbiome in early life.

End-of-Project Knowledge Translation. The knowledge translation goals of this project were to disseminate the findings of this study to relevant research groups as well as current and prospective parents. The main strategy for diffusion centered on preparing three manuscripts (one literature review and two original research papers) for submission to peer-reviewed journals for publication. Furthermore, the findings presented in this thesis were presented or submitted for presentation at the inFLAME 7th Annual Workshop, Women & Children*f*s Health Research Institute Research Day Conferences in 2017 and 2018, Canadian National Perinatal Research Meeting and AllerGen Research Conference, among others. A summary of findings will be sent to a select group of organizations which are known for providing health information to prospective parents such as Alberta Innovates, Today*f*s Parent, Parents Canada, Stollery Foundation of Edmonton, and the Women and Children*f*s Health Research Institute.

7.2 Conclusions

Early infancy is a critical period for the establishment of health across the lifespan. Infants of families with reduced SES are at increased risk of sleep problems, which may persist into childhood. We found prenatal depression and birth mode mediate the discrepancies in sleep duration between infants of mothers with and without postsecondary education. Consequently, increased counselling on infant sleep for mothers who experience prenatal depression or emergency caesarean section birth may have a

significant positive impact. The gut microbiota of infants is a strong candidate for the link between dysfunctional sleep, disrupted body rhythms and poor health. In 3-month old infants, we found that sleep duration is associated with the composition of the gut microbiota. Bacterial composition may explain the association between infant sleep duration and weight gain in infancy. However, further work is required to determine the direction of the relationship between the gut microbiota and sleep duration in infancy.

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Appendix A: CHILD Study University of Alberta Research Ethics Approval

Re-Approval Form

Date:	March 7, 2014
Principal Investigator:	Piushkumar Mandhane
Study ID:	Pro00002099
Study Title:	The Canadian Healthy Infant Longitudinal Development (CHILD) study
Approval Expiry Date:	March 13, 2015
Sponsor/Funding Agency:	Alberta Centre for Child, Family and Community Research CIHR - Canadian Institutes for Health Research
Sponsor/Funding Agency	AllerGen NCE Inc.

The Health Research Ethics Board - Biomedical Panel has reviewed the renewal request and file for this project and found it to be acceptable within the limitations of human experimentation.

The re-approval for the study as presented is valid for another year. It may be extended following completion of the annual renewal request. Beginning 45 days prior to expiration, you will receive notices that the study is about to expire. Once the study has expired you will have to resubmit. Any proposed changes to the study must be submitted to the HREB for approval prior to implementation.

All study-related documents should be retained so as to be available to the HREB on request. They should be kept for the duration of the project and for at least five years following study completion. In the case of clinical trials approved under Division 5 of the Food and Drug regulations of Health Canada, study records must be retained for 25 years.

Sincerely,

Dr. Glen J. Pearson, BSc, BScPhm, PharmD, FCSHP
Associate Chair, Health Research Ethics Board – Biomedical Panel

Note: This correspondence includes an electronic signature (validation and approval via an online system).

Appendix B

Sample size calculation:

n=sample 1

m=sample 2

N=n+m

c=m/N

m=n, therefore c=0.5

U=17

p•=U/$_{n1n2}$ =17/(9*9) = 0.21

$$N = \frac{(Z\alpha/2 + Z\beta)^2}{12c(1-c)(p" - 0.5)^2}$$

$$N = \frac{(1.96 + 0.84)^2}{12 * 0.5(1-0.5)(0.21-0.5)^2}$$

$$N = 31.1$$

n=15.6

m=15.6

Appendix C: The Brief Infant Sleep Questionnaire (BISQ)

Please mark only one (most appropriate) choice, when you respond to items with a few options.

Name of Responder: _____ Date: _____

Role of Responder: • Father • Mother • Grandparent • Other, Specify: _____

Name of the child: _____ Date of Birth: Month _____ Day: _____ Year: _____

Sex: • Male • Female Birth order of the child: • Oldest • Middle • Youngest

Sleeping arrangement:

• Infant crib in a separate room • Infant crib in parents' room

• In parents' bed • Infant crib in room with sibling

• Other, Specify: _____

In what position does your child sleep most of the time?

• On his/her belly • On his/her side • On his/her back

How much time does your child spend in sleep during the NIGHT (between 7 in the evening and 7 in the morning)? Hours: _____ Minutes: _____

How much time does your child spend in sleep during the DAY (between 7 in the morning and 7 in the evening)? Hours: _____ Minutes: _____

Average number of night wakings per night: _____

How much time during the night does your child spend in wakefulness (from 10 in the evening to 6 in the morning)? Hours: _____ Minutes: _____

How long does it take to put your baby to sleep in the evening? Hours: _____ Minutes: _____

How does your baby fall asleep?

• While feeding • Being rocked • Being held

• In bed alone • In bed near parent

When does your baby usually fall asleep for the night:

Hours: _____ Minutes: _____

Do you consider your child's sleep as a problem?

• A very serious problem • A small problem • Not a problem at all

Appendix D: Sample collection, DNA extraction and amplification

Adapted from Tun et al. (78): Methods of sample collection, DNA extraction and amplification, 16S rRNA sequencing and taxonomic classification have been previously described (68,78,203). Briefly, care givers were instructed to collect a sample (i.e. dirty diaper) prior to the 3-month home visit and refrigerate the sample immediately after collection. The sample was then collected by a research assistant and refrigerated during transport and stored at '80 °C until analysis. Genomic DNA was extracted from 80 to 200 mg of stool using the QIAamp DNA Stool Mini kit (Qiagen, Venlo, the Netherlands). The V4 hypervariable region of the bacterial 16S rRNA gene was amplified by PCR using universal bacterial primers: V4-515f: 5' AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT ATG GTA ATT GTG TGC CAG CMG CCG CGG TAA-3', V4-806r:5', CAA GCA GAA GAC GGC ATA CGA GAT XXXXXXXXXXXX AGT CAG TCA GCC GGA CTA CHV GGG TWT CTA AT-3'. For sample multiplexing, reverse primers were barcoded uniquely for each sample (barcoded sequence was denoted in the primer sequence by Xs). Each 25 "l PCR mixture contained 12.5 "l 2x Kapa2G Hotstart mix (Kapa Biosystems, Wilmington, MA), 0.6 "M of both forward and reverse primers and 2 "l genomic DNA (5 ng/"l). PCR amplification consisted of an initial denaturation step for 3 min at 94 °C, followed by 20 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 50 °C and an extension step for 30 s at 72 °C. PCR reactions for each sample were performed in triplicate with a negative control in each run. One hundred nanograms of pooled PCR product from each sample was concentrated using an Amicon Ultra-4 30K centrifugal filter (Millipore, Billerica, MA, USA), run on a 1.4% agarose gel, extracted and cleaned with the GENE-CLEAN Turbo Kit (MP Biomedicals Inc, Solon, OH, USA).

Pooled PCR amplicons were subjected to paired-end sequencing by Illumina Miseq platform. Using a QIIME pipeline (v1.6.0, qiime.org) (204), forward and reverse reads were assembled using PandaSeq for a final length of 144 bp (unassemblable sequences discarded), demultiplexed and filtered against the GREENGENES reference database (v13.8) (205) to remove all sequences with <60% similarity. Remaining sequences were clustered with Usearch61 at 97% sequence similarity against the GREENGENES database (closed picking algorithm), and taxonomic assignment was achieved using the RDP classifier (206) constrained by GREENGENES. After taxonomic assignment, operational taxonomic units (OTUs) representing bacterial origin were selected, and bacterial OTUs with colonization frequency below 10% were excluded from subsequence for downstream analyses. To avoid bias due to variation in sequencing depths among samples, data were rarefied to 13,000 sequences per sample.

Appendix E: Chapter 3 Supplementary Material

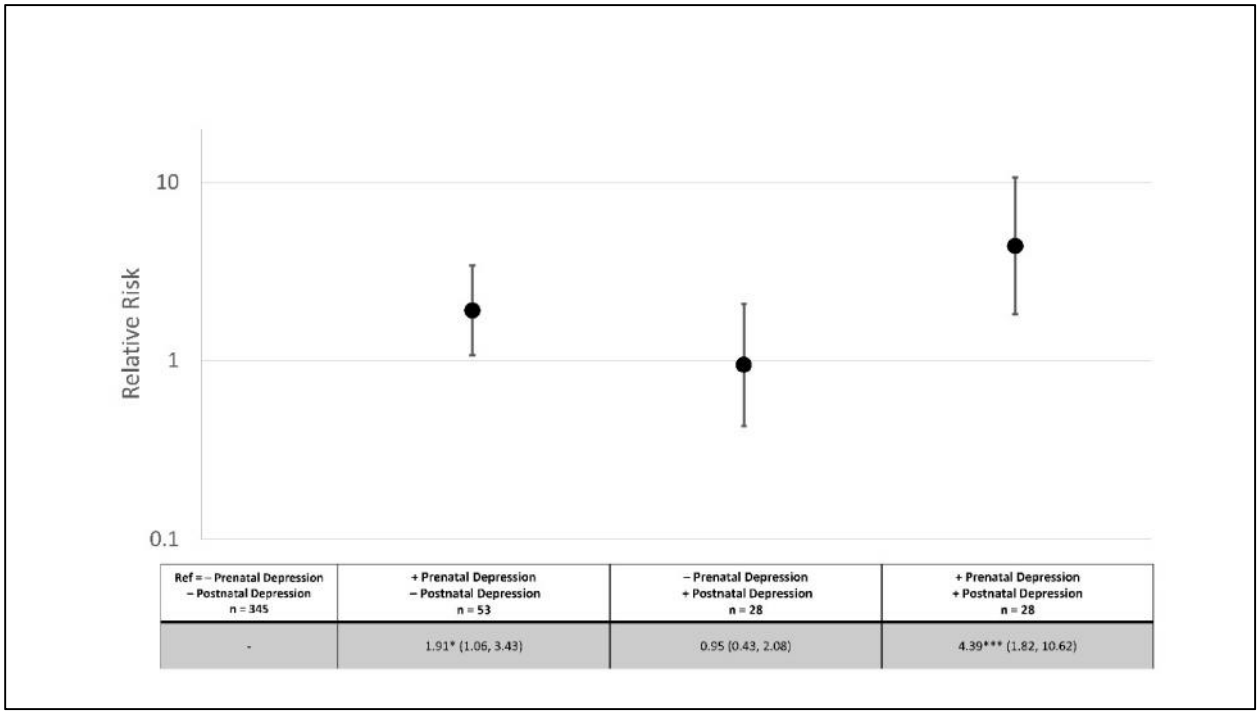


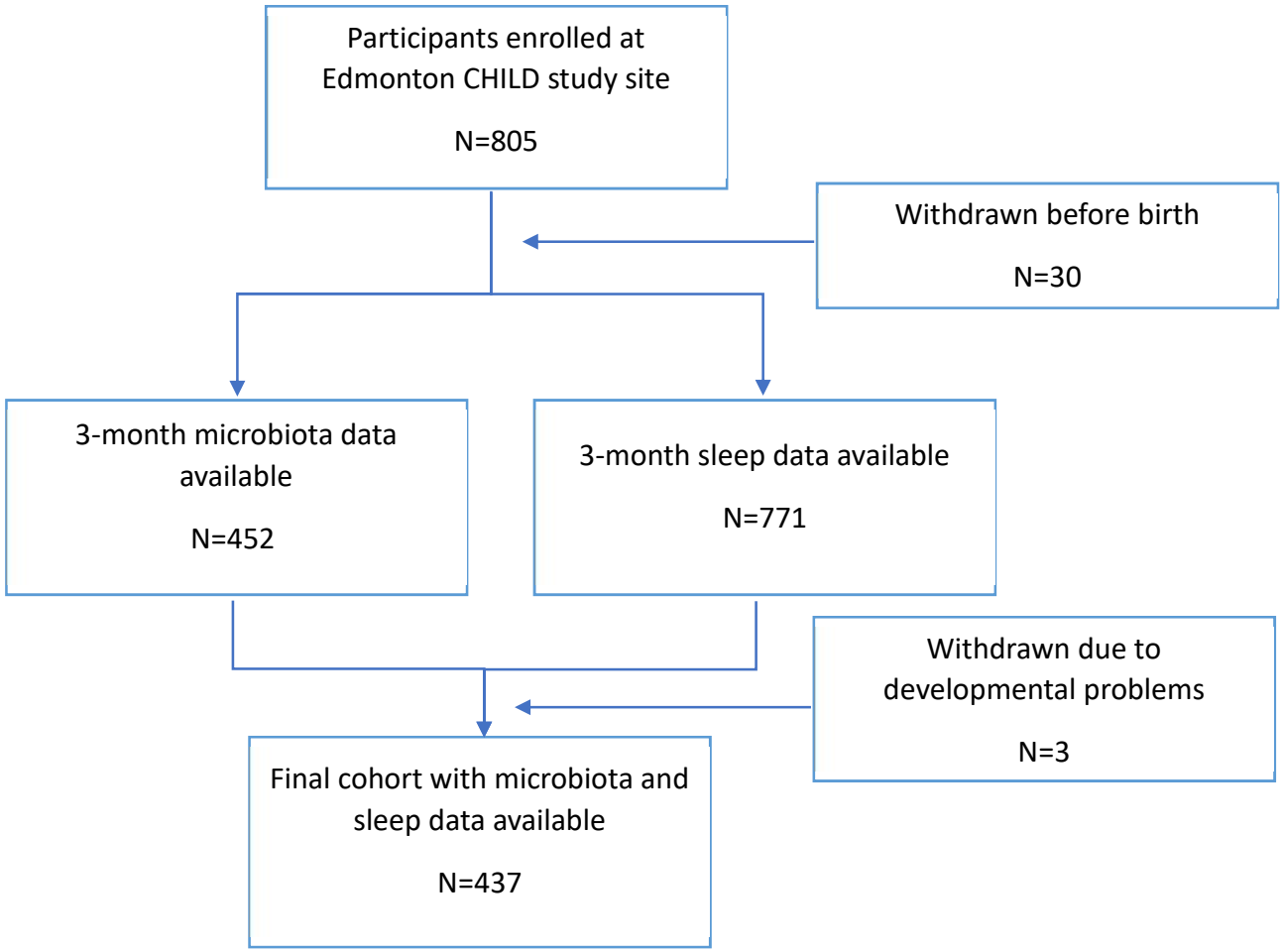
Figure E1. Relative risk of prenatal and postnatal depression associated with (lower) maternal education. Prenatal and postnatal depression classified as CES-D score ≥ 16.

Table E1. Linear regression results from sequential mediation of infant sleep duration due to maternal education level through prenatal depression and birth mode.

		Consequent									
		M_1 Prenatal depression (CES-D Score)				M_2 Birth mode				Y Total sleep duration (hours/24-hour period)	
Antecedent		Coeff.	SE	P		Coeff.	SE	P		Coeff.	SE	P
Maternal education	a_1	2.68	0.63	<0.0001	a_2	0.15	0.09	0.11	c'	-0.27	0.18	0.15
M_1 Prenatal depression (CES-D Score)		-	-	-	d_{21}	0.01	0.01	0.05	b_1	-0.03	0.01	0.04
M_2 Birth mode		-	-	-		-	-	-	b_2	-0.27	0.08	<0.01
Constant	i_{M_1}	9.28	0.47	<0.0001	i_{M_2}	1.78	0.06	<0.0001	i_Y	14.73	0.19	<0.0001
		$R^2 = 0.032$				$R^2 = 0.014$				$R^2 = 0.036$		
		$F(1,548) = 18.06, p<0.0001$				$F(2, 547) = 3.97, p = 0.02$				$F(3, 546) = 6.70, p = 0.0002$		

Notes: CES-D , Center for Epidemiologic Studies Depression Scale.

Appendix F: Chapter 4 Supplementary Material



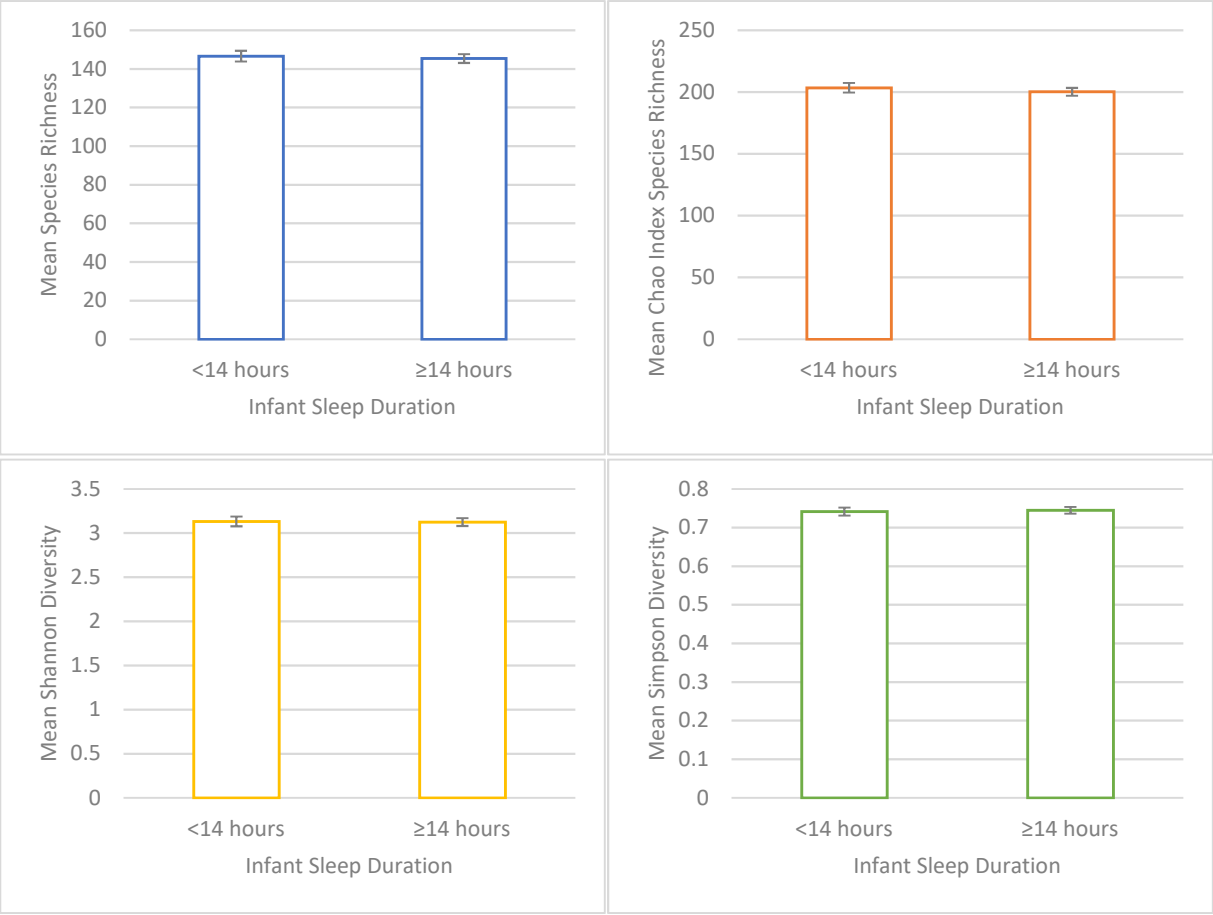
OS Figure 1. Flow chart of participant inclusion/exclusion.

OS Table 1. Total sleep duration (continuous) according to infant and maternal characteristics.

Infant Total Sleep Duration				
Characteristic	N	Mean	± SD	*P*
	437	14.2	± 2.2	
Infant Gender				*0.17*
Girl	220	14.0	± 2.2	
Boy	217	14.3	± 2.2	
Weight at Delivery				*0.98*
Below 2500g	13	14.2	± 1.8	
2500 to 3499g	227	14.1	± 2.3	
3500 to 4499g	186	14.2	± 2.1	
Over 4500g	8	13.9	± 1.5	
Gestational Age at Birth				*0.07*
Below 38 weeks	46	14.3	± 2.3	
38 to 39 weeks	205	14.4	± 2.3	
Over 40 weeks	183	13.9	± 2.0	
Birth Mode				*<0.01*
Vaginal no IAP	233	14.4	± 2.1	
Vaginal with IAP	95	13.9	± 2.3	
Scheduled CS	48	14.2	± 2.3	
Emergency CS	56	13.3	± 2.3	
Direct Antibiotic Exposure before 3 Months				*0.28*
No	423	14.1	± 2.2	
Yes	14	14.8	± 1.9	
Hospital Length of Stay				*0.98*
Less than 4 Days	350	14.3	± 2.1	
4+ Days	30	14.3	± 2.5	
Solids Before 3 Months				*0.53*
No	417	14.2	± 2.2	
Yes	14	14.5	± 1.8	
Colic or Acid Reflux				*0.32*
No	311	14.2	± 2.2	
Yes	123	14.0	± 2.2	
Maternal Prenatal Depression				*0.01*
No	303	14.3	± 2.2	
Yes	80	13.6	± 2.2	
Breastfeeding Status at 3 Months				*0.09*
Exclusive	220	14.4	± 2.0	
Partial	128	14.0	± 2.3	
Zero	88	13.9	± 2.4	
Maternal Race/Ethnicity				*0.23*
White	340	14.1	± 2.2	
Asian	45	14.7	± 2.4	
Other	49	14.1	± 2.2	
Maternal Age				*0.49*
18 to 29	132	14.0	± 2.1	
30 to 39	285	14.2	± 2.2	

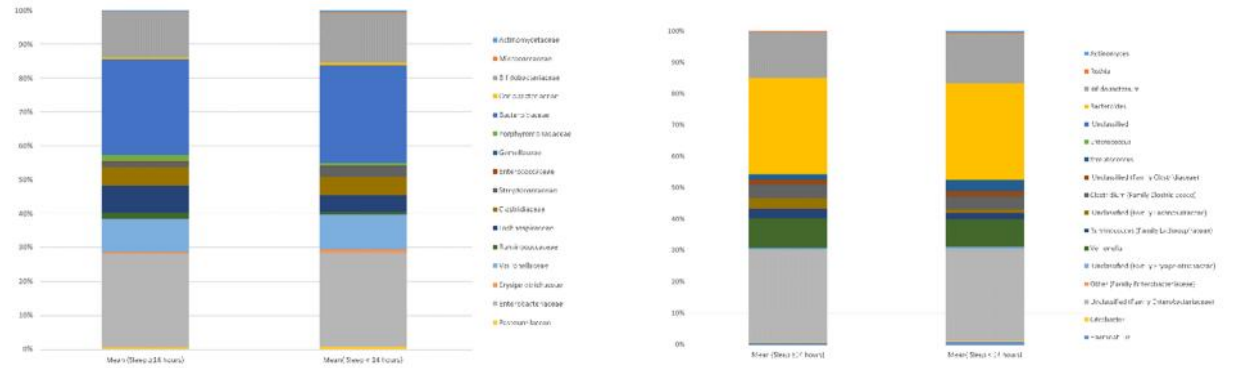
over 40	20	14.6	± 2.4	
Siblings				*<0.001*
No	196	13.7	± 2.1	
Yes	239	14.5	± 2.2	

Notes: P-value calculated using Student*f*s ŧ-test or ANOVA, where appropriate. IAP-intrapartum antibiotic prophylaxis; CS-caesarean section.



OS Figure 2. Species richness, Chao index species richness, Shannon diversity, and Simpson diversity index according to infant sleep duration (ˆ14 vs <14 hours per 24 -hour period). Significance obtained from Student*f*s ŧ-test.

D)



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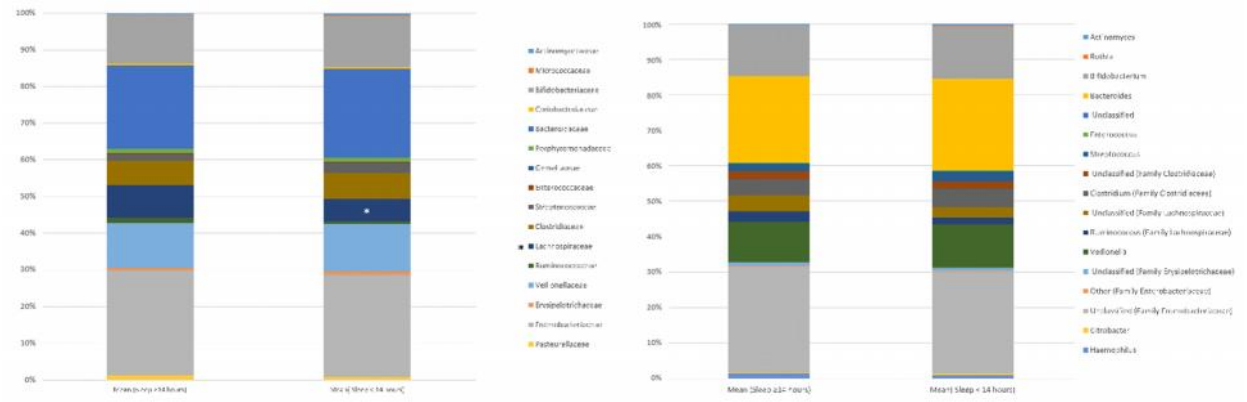

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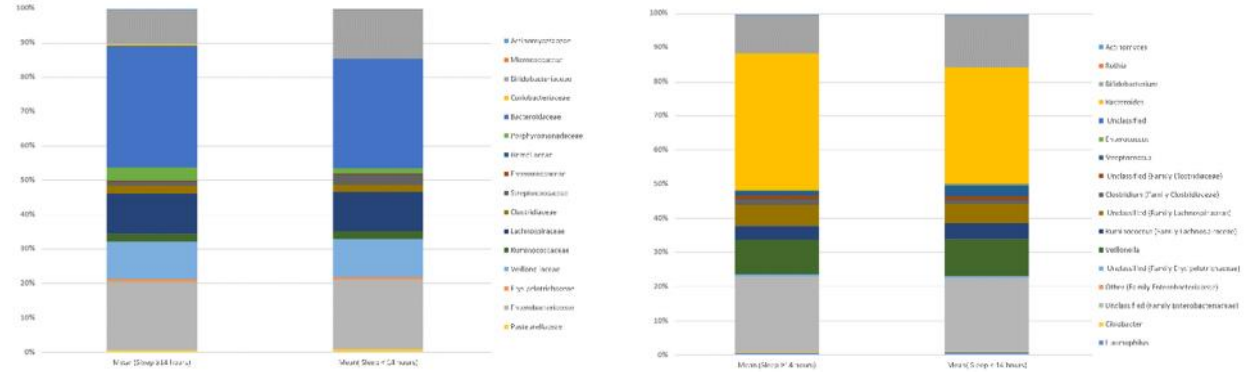
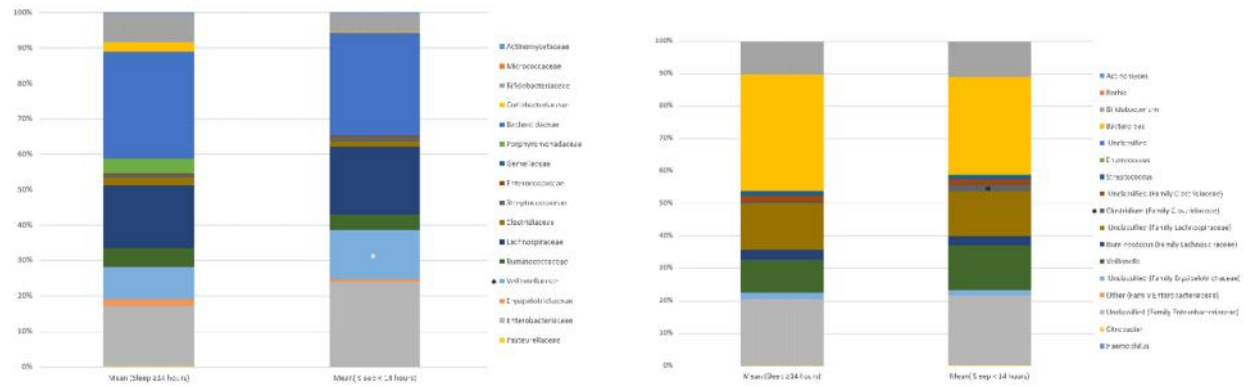
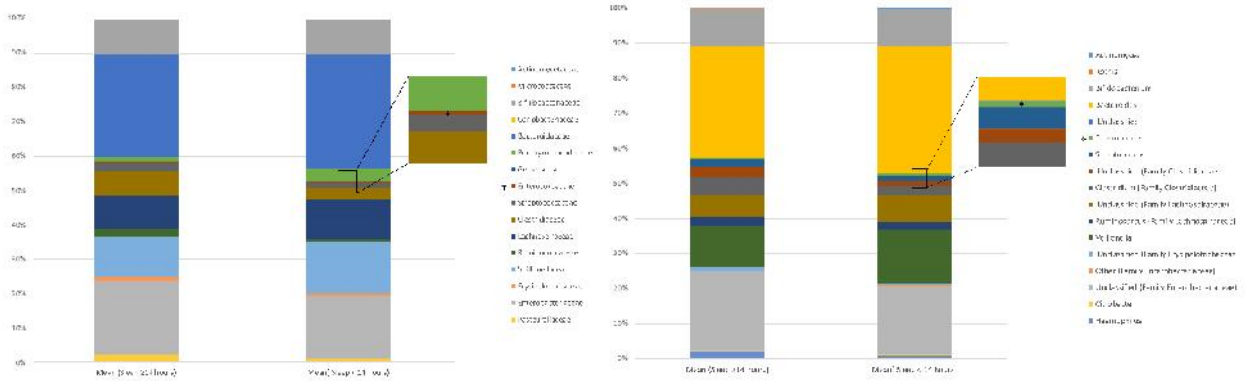


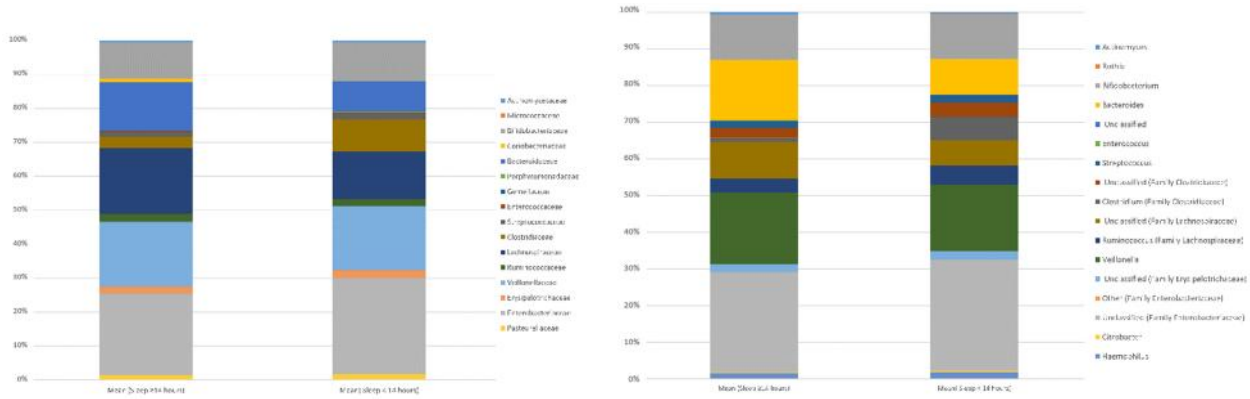
G)



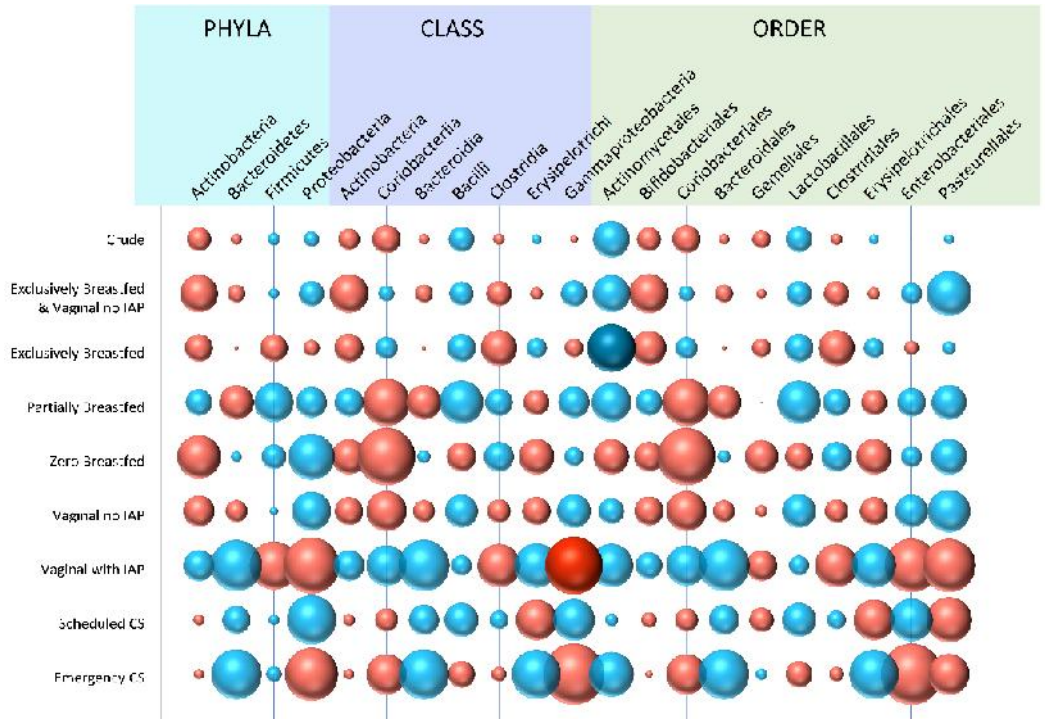
H)



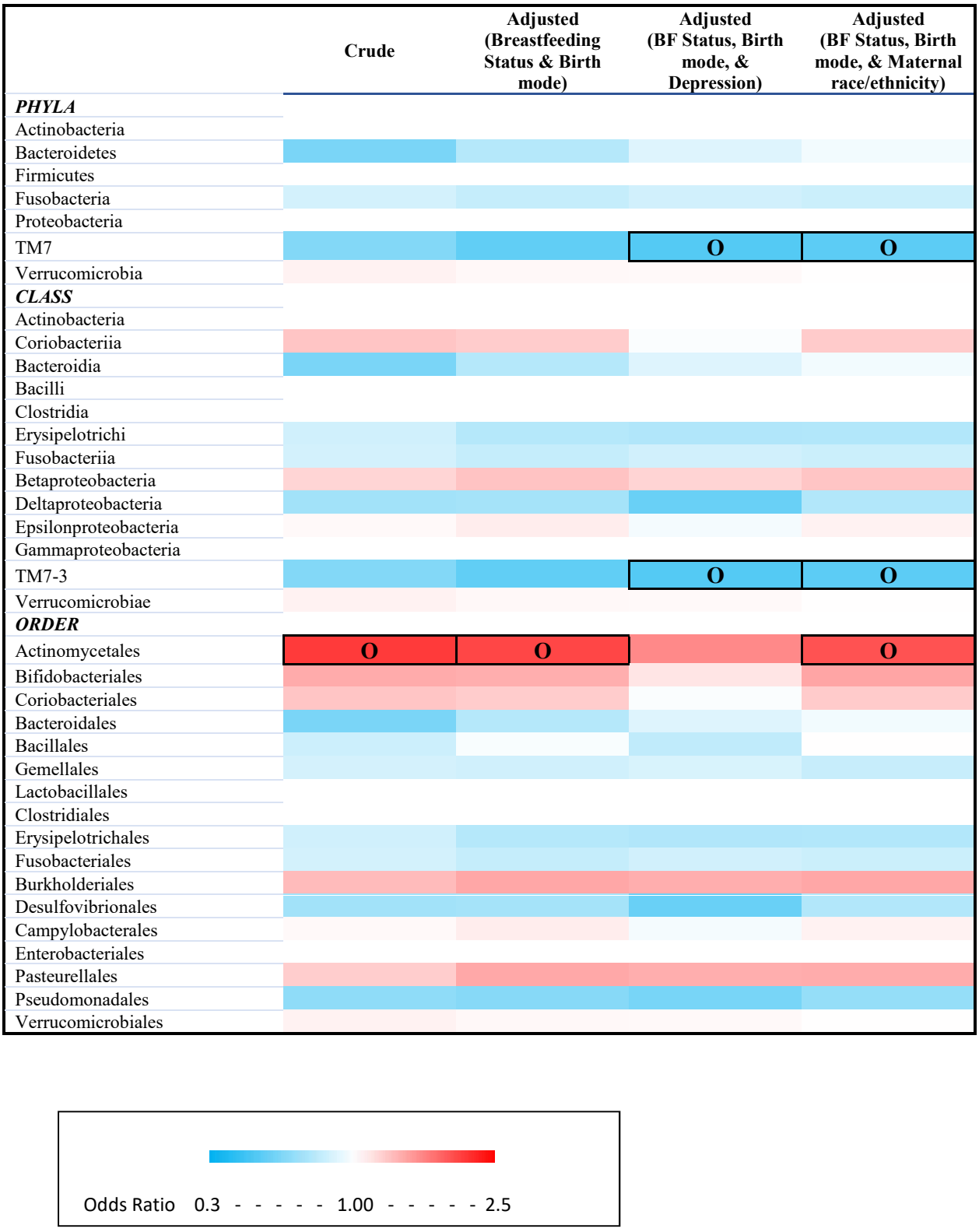
I)



OS Figure 3. Stacked bar plots of bacterial taxa at the family and genus levels of D) breastfed infants born vaginally without IAP sleeping ˆ14 hours and < 14 hours; E) breastfed infants sleeping ˆ vs < 14 hours; F) partially breastfed infants sleeping ˆ vs < 14 hours; G) formula fed infants sleeping ˆ vs < 14 hours; H) infants born vaginally with IAP sleeping ˆ vs < 14 hours; and I) infants born by elective CS sleeping ˆ vs < 14 hours. Significance determined by Mann Whitney -U Test. *p-value<0.05.

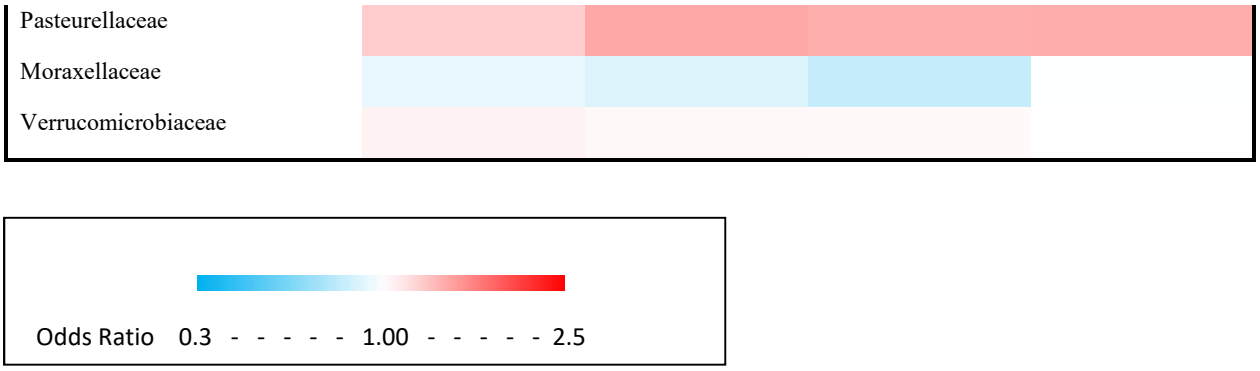


OS Figure 4. Spearman correlation between total sleep duration (hours) and relative abundance at the phyla, order, and class level. Bubble size indicates correlation magnitude. Light red = positive association; light blue = negative association; dark red = significant positive association (p-value < 0.05); dark blue = significant negative association (p-value < 0.05).



OS Figure 5. Heat map of odds ratios from logistic regression of bacterial taxa frequency at the phyla, class and order levels. Notes: Odds Ratio = short sleepers (<14 hours) vs normal sleepers (ˆ14 hours). ‹ = statistical significance at an alpha level of 0.05.

	Crude	Adjusted (Breastfeeding Status & Birth mode)	Adjusted (BF Status, Birth mode, & Depression)	Adjusted (BF Status, Birth mode, & Race/Ethnicity)
FAMILY				
Actinomycetaceae				
Micrococcaceae				
Bifidobacteriaceae				
Coriobacteriaceae				
Bacteroidaceae				
Porphyromonadaceae				
Prevotellaceae				
Rikenellaceae				
Staphylococcaceae				
Gemellaceae				
Other (Class Lactobacillales)				
Carnobacteriaceae				
Enterococcaceae	O	O		O
Lactobacillaceae				
Streptococcaceae				
Other (Class Clostridiales)				
Unclassified (Class Clostridiales)				
Clostridiaceae	O			
Lachnospiraceae				
Peptostreptococcaceae				
Ruminococcaceae		O	O	O
Veillonellaceae				
Tissierellaceae				
Erysipelotrichaceae				
Fusobacteriaceae				
Alcaligenaceae		O		O
Desulfovibrionaceae				
Campylobacteraceae				
Enterobacteriaceae				



OS Figure 6. Heat map of odds ratios from logistic regression of bacterial taxa frequency at the family level. Notes: Odds Ratio = short sleepers (<14 hours) vs normal sleepers (ˆ14 hours). ‹ = statistical significance at an alpha level of 0.05.

OS Table 2. MaAslin Results for total sleep ˆ 14 hours vs < 14 hours with FDR alpha level 0.2.

Family	Infant Total Sleep Duration ˆ 14 hours (*ref*) vs < 14 hours			
Genus	Coefficient	n not 0	*P value*	*Q value*
Enterobacteriaceae				
Erwinia	-0.00065	49	*0.016*	*0.128*

Notes: Adjusted for metadata: Breast feeding status - exclusive, partial, or zero at 3 months; birth mode , vaginal no IAP, vaginal with IAP, scheduled CS, or emergency CS; maternal prenatal depression; maternal race , white, Asian, or other.

OS Table 3. Multivariable linear regression predicting arcsine square root transformed relative abundance of bacterial taxa at the phyla level by categorical sleep duration (above vs below 14 hours) at the phyla level.

	Taxa Prevalence above 50%	Crude		Adjusted (Breastfeeding status & Birth mode)		Adjusted (Breastfeeding status, Birth mode & Prenatal depression)	
		n=435		n=424		n=371	
		Coef.	95% CI	Coef.	95% CI	Coef.	95% CI
PHYLA							
Actinobacteria	Œ	0.004	(-0.043, 0.052)	0.011	(-0.037, 0.059)	-0.004	(-0.055, 0.047)
Bacteroidetes	Œ	-0.007	(-0.091, 0.078)	0.007	(-0.071, 0.085)	0.033	(-0.049, 0.116)
Firmicutes	Œ	0.020	(-0.037, 0.077)	0.004	(-0.052, 0.059)	-0.008	(-0.067, 0.051)
Fusobacteria		-0.002	(-0.005, 0.001)	-0.002	(-0.005, 0.001)	-0.002	(-0.005, 0.001)
Proteobacteria	Œ	-0.008	(-0.062, 0.045)	-0.003	(-0.056, 0.050)	-0.005	(-0.060, 0.050)
TM7		-0.001	(-0.002, 0.001)	-0.001	(-0.003, 0.000)	-0.001	(-0.002, 0.001)
Verrucomicrobia		-0.006	(-0.040, 0.028)	-0.011	(-0.044, 0.023)	-0.008	(-0.045, 0.030)

Notes: Breastfeeding status (categorical): Exclusive (ref), partial, and formula fed; birth mode (categorical): vaginal without IAP (ref), vaginal with IAP, elective CS, emergency CS; prenatal depression(categorical): CESD score <16 (ref) vs „16. * p<0.05.

OS Table 4. Multivariable linear regression predicting arcsine square root transformed relative abundance of bacterial taxa at the class level by categorical sleep duration (above vs below 14 hours) at the class level.

	Taxa Prevalence above 50%	Crude		Adjusted (Breastfeeding status & Birth mode)		Adjusted (Breastfeeding status, Birth mode & Prenatal depression)	
		n=435		n=424		n=371	
		Coef.	95% CI	Coef.	95% CI	Coef.	95% CI
CLASS							
Actinobacteria	Œ	0.011	(-0.036, 0.058)	0.018	(-0.030, 0.065)	0.001	(-0.049, 0.051)
Coriobacteriia	Œ	**-0.013***	**(-0.026, 0.000)**	**-0.014***	**(-0.026, -0.001)**	**-0.014***	**(-0.027, -0.001)**
Bacteroidia	Œ	-0.007	(-0.091, 0.078)	0.007	(-0.071, 0.085)	0.033	(-0.049, 0.116)
Bacilli	Œ	**0.021***	**(0.001, 0.041)**	**0.024***	**(0.004, 0.044)**	0.014	(-0.006, 0.034)
Clostridia	Œ	0.005	(-0.052, 0.063)	-0.013	(-0.069, 0.043)	-0.015	(-0.075, 0.045)
Erysipelotrichi	Œ	0.002	(-0.014, 0.018)	-0.001	(-0.017, 0.016)	-0.002	(-0.020, 0.016)
Fusobacteriia		-0.002	(-0.005, 0.001)	-0.002	(-0.005, 0.001)	-0.002	(-0.005, 0.001)
Betaproteobacteria		0.012	(-0.004, 0.029)	0.014	(-0.003, 0.031)	0.011	(-0.008, 0.030)
Deltaproteobacteria		-0.005	(-0.013, 0.004)	-0.004	(-0.013, 0.004)	-0.006	(-0.015, 0.004)
Epsilonproteobacteria		-0.003	(-0.006, 0.000)	-0.003	(-0.006, 0.000)	-0.003	(-0.007, 0.000)
Gammaproteobacteria	Œ	-0.016	(-0.071, 0.039)	-0.010	(-0.064, 0.044)	-0.011	(-0.067, 0.046)
TM7-3		-0.001	(-0.002, 0.001)	-0.001	(-0.003, 0.000)	-0.001	(-0.002, 0.001)
Verrucomicrobiae		-0.006	(-0.040, 0.028)	-0.011	(-0.044, 0.023)	-0.008	(-0.045, 0.030)

Notes: Breastfeeding status (categorical): Exclusive (ref), partial, and formula fed; birth mode (categorical): vaginal without IAP (ref), vaginal with IAP, elective CS, emergency CS; prenatal depression(categorical): CESD score <16 (ref) vs „16. * p<0.05.

OS Table 5. Multivariable linear regression predicting arcsine square root transformed relative abundance of bacterial taxa at the order level by categorical sleep duration (above vs below 14 hours) at the order level.

	Taxa Prevalence above 50%	Crude		Adjusted (Breastfeeding status & Birth mode)		Adjusted (Breastfeeding status, Birth mode & Prenatal depression)	
		n=435		n=424		n=371	
		Coef.	95% CI	Coef.	95% CI	Coef.	95% CI
ORDER							
Actinomycetales	Œ	0.007	(-0.002, 0.016)	0.007	(-0.002, 0.016)	0.006	(-0.003, 0.014)
Bifidobacteriales	Œ	0.007	(-0.040, 0.054)	0.014	(-0.034, 0.062)	-0.001	(-0.052, 0.049)
Coriobacteriales	Œ	**-0.013***	**(-0.026, 0.000)**	**-0.014***	**(-0.026, -0.001)**	**-0.014***	**(-0.027, -0.001)**
Bacteroidales	Œ	-0.007	(-0.091, 0.078)	0.007	(-0.071, 0.085)	0.033	(-0.049, 0.116)
Bacillales		0.002	(-0.001, 0.005)	**0.003***	**(0.000, 0.006)**	0.003	(-0.001, 0.006)
Gemellales	Œ	0.000	(-0.003, 0.003)	0.000	(-0.003, 0.003)	0.000	(-0.003, 0.003)
Lactobacillales	Œ	**0.021***	**(0.001, 0.040)**	**0.023***	**(0.004, 0.043)**	0.014	(-0.006, 0.034)
Clostridiales	Œ	0.005	(-0.052, 0.063)	-0.013	(-0.069, 0.043)	-0.015	(-0.075, 0.045)
Erysipelotrichales	Œ	0.002	(-0.014, 0.018)	-0.001	(-0.017, 0.016)	-0.002	(-0.020, 0.016)
Fusobacteriales		-0.002	(-0.005, 0.001)	-0.002	(-0.005, 0.001)	-0.002	(-0.005, 0.001)
Burkholderiales		0.013	(-0.004, 0.030)	0.014	(-0.003, 0.031)	0.012	(-0.006, 0.031)
Desulfovibrionales		-0.005	(-0.013, 0.004)	-0.004	(-0.013, 0.004)	-0.006	(-0.015, 0.004)
Campylobacterales		-0.003	(-0.006, 0.000)	-0.003	(-0.006, 0.000)	-0.003	(-0.007, 0.000)
Enterobacteriales	Œ	-0.015	(-0.070, 0.040)	-0.010	(-0.064, 0.045)	-0.008	(-0.065, 0.049)
Pasteurellales	Œ	-0.010	(-0.025, 0.006)	-0.008	(-0.023, 0.007)	-0.012	(-0.029, 0.004)
Pseudomonadales		-0.001	(-0.003, 0.001)	-0.001	(-0.003, 0.001)	-0.001	(-0.003, 0.001)
Verrucomicrobiales		-0.006	(-0.040, 0.028)	-0.011	(-0.044, 0.023)	-0.008	(-0.045, 0.030)

Notes: Breastfeeding status (categorical): Exclusive (ref), partial, and formula fed; birth mode (categorical): vaginal without IAP (ref), vaginal with IAP, elective CS, emergency CS; prenatal depression(categorical): CESD score <16 (ref) vs „16. * p<0.05.

OS Table 6. Multivariable linear regression predicting arcsine square root transformed relative abundance of bacterial taxa at the genus level by categorical sleep duration (above vs below 14 hours) at the genus level.

	Taxa Prevalence above 50%	Crude		Adjusted (Breastfeeding status & Birth mode)		Adjusted (Breastfeeding status, Birth mode & Prenatal depression)	
		n=435		n=424		n=371	
		Coef.	95% CI	Coef.	95% CI	Coef.	95% CI
GENUS							
Actinomyces	Œ	0.004	(-0.003, 0.012)	0.004	(-0.005, 0.012)	0.002	(-0.005, 0.008)
Varibaculum		0.001	(0.000, 0.002)	0.001	(0.000, 0.002)	0.001	(0.000, 0.003)
Rothia	Œ	0.004	(-0.001, 0.008)	0.004	(0.000, 0.009)	0.004	(-0.001, 0.009)
Bifidobacterium	Œ	0.007	(-0.040, 0.054)	0.014	(-0.034, 0.062)	-0.001	(-0.052, 0.049)
Atopobium		0.002	(0.000, 0.005)	0.002	(0.000, 0.005)	0.001	(-0.001, 0.004)
Collinsella		**-0.013***	**(-0.026, 0.000)**	**-0.013***	**(-0.026, -0.001)**	-0.012	(-0.025, 0.000)
Eggerthella	Œ	-0.001	(-0.005, 0.003)	-0.002	(-0.006, 0.002)	-0.002	(-0.006, 0.002)
Bacteroides	Œ	-0.012	(-0.092, 0.069)	0.003	(-0.073, 0.079)	0.029	(-0.051, 0.110)
Parabacteroides		-0.007	(-0.035, 0.020)	-0.008	(-0.035, 0.018)	-0.008	(-0.036, 0.021)
Prevotella		0.001	(-0.010, 0.013)	0.001	(-0.011, 0.013)	0.002	(-0.009, 0.014)
Unclassified (F. Rikenellaceae)		0.007	(-0.010, 0.024)	0.009	(-0.008, 0.026)	0.009	(-0.011, 0.028)
Staphylococcus		0.002	(-0.001, 0.005)	**0.003***	**(0.000, 0.007)**	0.003	(-0.001, 0.006)
Unclassified (F. Gemellaceae)	Œ	0.000	(-0.003, 0.003)	0.000	(-0.003, 0.003)	0.000	(-0.003, 0.003)
Other (C. Lactobacillales; F. Other)		-0.001	(-0.004, 0.003)	-0.002	(-0.005, 0.002)	-0.001	(-0.005, 0.003)
Granulicatella		0.000	(-0.001, 0.001)	0.000	(-0.001, 0.001)	0.000	(-0.001, 0.001)
Other (F. Enterococcaceae)		0.000	(-0.001, 0.001)	0.000	(-0.001, 0.001)	0.000	(-0.001, 0.001)
Enterococcus	Œ	0.005	(-0.002, 0.012)	0.005	(-0.003, 0.012)	0.006	(-0.001, 0.013)
Lactobacillus		0.001	(-0.010, 0.012)	0.002	(-0.009, 0.014)	-0.005	(-0.014, 0.004)
Streptococcus	Œ	**0.019***	**(0.002, 0.036)**	**0.021***	**(0.004, 0.038)**	0.015	(-0.003, 0.034)
Other (C. Clostridiales; F. Other)		0.000	(-0.002, 0.003)	0.000	(-0.003, 0.002)	0.000	(-0.003, 0.002)
Unclassified (C. Clostridiales; F. Unclassified)		0.009	(-0.004, 0.023)	0.008	(-0.006, 0.021)	0.007	(-0.009, 0.022)
Other (F. Clostridiaceae)		0.000	(-0.002, 0.002)	0.000	(-0.002, 0.002)	0.000	(-0.002, 0.002)
Unclassified (F. Clostridiaceae)	Œ	-0.006	(-0.025, 0.014)	-0.008	(-0.028, 0.011)	-0.005	(-0.027, 0.016)
Clostridium (F. Clostridiaceae)	Œ	0.011	(-0.021, 0.043)	0.013	(-0.020, 0.045)	0.020	(-0.015, 0.055)
Other (F. Lachnospiraceae)	Œ	-0.004	(-0.014, 0.006)	-0.005	(-0.016, 0.005)	-0.008	(-0.019, 0.003)
Unclassified (F. Lachnospiraceae)	Œ	-0.008	(-0.046, 0.029)	-0.026	(-0.061, 0.010)	-0.022	(-0.059, 0.015)
Blautia		-0.005	(-0.020, 0.010)	-0.007	(-0.022, 0.008)	-0.009	(-0.026, 0.008)
Clostridium (F. Lachnospiraceae)		0.000	(-0.002, 0.002)	0.000	(-0.002, 0.002)	0.000	(-0.003, 0.002)
Coprococcus		0.001	(-0.002, 0.004)	0.001	(-0.002, 0.004)	0.000	(-0.003, 0.003)
Dorea		0.000	(-0.013, 0.013)	-0.004	(-0.017, 0.009)	-0.006	(-0.020, 0.008)
Epulopiscium		0.011	(-0.001, 0.024)	0.010	(-0.002, 0.023)	0.013	(-0.001, 0.027)
Lachnospira		0.006	(-0.007, 0.018)	0.005	(-0.008, 0.018)	0.004	(-0.011, 0.018)
Roseburia		0.003	(-0.002, 0.008)	0.002	(-0.003, 0.008)	0.002	(-0.004, 0.008)
Ruminococcus (F. Lachnospiraceae)	Œ	-0.004	(-0.031, 0.022)	-0.005	(-0.032, 0.022)	-0.014	(-0.042, 0.013)
Other (F. Peptostreptococcaceae)		0.000	(-0.003, 0.003)	-0.001	(-0.004, 0.003)	0.000	(-0.004, 0.004)

Unclassified (F. Ruminococcaceae)		-0.002	(-0.015, 0.011)	-0.004	(-0.017, 0.008)	-0.005	(-0.019, 0.009)
Faecalibacterium		-0.001	(-0.012, 0.011)	0.001	(-0.011, 0.012)	0.001	(-0.012, 0.014)
Oscillospira	Œ	-0.004	(-0.021, 0.013)	-0.010	(-0.026, 0.006)	-0.009	(-0.026, 0.009)
Ruminococcus (F. Ruminococcaceae)		0.001	(-0.004, 0.007)	0.000	(-0.006, 0.006)	0.000	(-0.006, 0.006)
Other (F. Veillonellaceae)		0.003	(-0.003, 0.008)	0.002	(-0.004, 0.008)	0.003	(-0.003, 0.009)
Dialister		-0.003	(-0.009, 0.003)	-0.002	(-0.009, 0.004)	-0.002	(-0.008, 0.005)
Megasphaera		-0.003	(-0.022, 0.016)	-0.004	(-0.023, 0.015)	-0.007	(-0.028, 0.015)
Veillonella	Œ	0.020	(-0.020, 0.061)	0.013	(-0.027, 0.054)	0.006	(-0.038, 0.050)
Anaerococcus		0.001	(-0.001, 0.002)	0.001	(-0.001, 0.002)	0.001	(-0.001, 0.002)
Finegoldia		0.001	(0.000, 0.002)	0.001	(0.000, 0.002)	0.001	(0.000, 0.002)
Peptoniphilus		0.000	(-0.001, 0.002)	0.000	(-0.001, 0.002)	0.000	(-0.002, 0.002)
Unclassified (F. Erysipelotrichaceae)	Œ	0.003	(-0.013, 0.019)	0.001	(-0.015, 0.017)	-0.001	(-0.018, 0.017)
Eubacterium		0.000	(-0.006, 0.006)	-0.002	(-0.008, 0.003)	-0.002	(-0.007, 0.004)
Fusobacterium		-0.002	(-0.005, 0.001)	-0.002	(-0.005, 0.001)	-0.002	(-0.005, 0.001)
Sutterella		0.015	(0.000, 0.030)	**0.016***	**(0.001, 0.032)**	0.014	(-0.003, 0.031)
Bilophila		-0.003	(-0.012, 0.005)	-0.003	(-0.012, 0.005)	-0.005	(-0.014, 0.005)
Campylobacter		-0.003	(-0.006, 0.000)	-0.003	(-0.006, 0.000)	-0.003	(-0.007, 0.000)
Other (F. Enterobacteriaceae)	Œ	-0.004	(-0.009, 0.001)	-0.004	(-0.009, 0.001)	-0.003	(-0.008, 0.001)
Unclassified (F. Enterobacteriaceae)	Œ	-0.014	(-0.068, 0.040)	-0.008	(-0.062, 0.046)	-0.007	(-0.063, 0.050)
Citrobacter	Œ	-0.003	(-0.011, 0.006)	-0.003	(-0.011, 0.005)	-0.003	(-0.012, 0.005)
Erwinia		**-0.001***	**(-0.002, 0.000)**	**-0.001***	**(-0.002, 0.000)**	**-0.001***	**(-0.001, 0.000)**
Trabulsiella		0.002	(-0.003, 0.008)	0.000	(-0.005, 0.006)	0.000	(-0.006, 0.006)
Haemophilus	Œ	-0.009	(-0.024, 0.006)	-0.008	(-0.022, 0.007)	-0.011	(-0.028, 0.005)
Acinetobacter		0.000	(-0.002, 0.002)	0.000	(-0.002, 0.002)	-0.001	(-0.002, 0.001)
Akkermansia		-0.006	(-0.040, 0.028)	-0.011	(-0.044, 0.023)	-0.008	(-0.045, 0.030)

Notes: Breastfeeding status (categorical): Exclusive (ref), partial, and formula fed; birth mode (categorical): vaginal without IAP (ref), vaginal with IAP, elective CS, emergency CS; prenatal depression(categorical): CESD score <16 (ref) vs „16. * p<0.05.