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Vertical Transmission of *Bacteroides* in Mother-Child Pairs in Relation to Vaginal Delivery

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Abbreviations

ASV	Amplicon Sequence Variants
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
C-section	Caesarean section
DADA	Divisive Amplicon Denoising Algorithm
DNA	Deoxyribonucleic Acid
E-swab	Elution swab
HMO	Human Milk Oligosaccharide
HRM	High Resolution Melting
NGS	Next Generation Sequencing
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
rpoB	RNA Polymerase β-subunit
qRT-PCR	Quantitative Real-Time PCR
QIIME	Quantitative Insights Into Microbial Ecology

Abstract

Early colonization of beneficial bacteria in the infant gut is key for the development of healthy gut microbiota, immune system and for health later in life. Microbes are shared between mothers and their children. There is an ongoing debate about whether the first transmission occurs inside the uterus or after rupture of the amniotic membrane. Therefore, there still lacks information regarding the time of transmission and mechanisms for transfer. Previous studies have identified an association of *Bacteroides* in mother-child pairs. Therefore, this study aimed to identify vertical transmission of *Bacteroides* species from mother to child.

Overlap of *Bacteroides* in 144 mother-child pairs in relation with mode of delivery were investigated. Ninety-five mother-child pairs were related to vaginal delivery, and 48 mother-child pairs were related to caesarean section delivery. Samples from mother's stool, meconium, skin from newborns, and feces from 3 months old infants were analyzed to determine bacterial overlap. Material included in this study were received from the PreventADALL cohort. Bacterial composition in samples was studied by Illumina sequencing using the 16S rRNA gene and the *rpoB* gene. Amplicon sequence variant (ASV) profiles were used to study overlap of *Bacteroides* species in mothers and children. Indication of live bacterial cells in meconium was identified by cultivation.

Bacteroides was present in all sample categories and represented 26.4% of the composition in meconium from vaginal delivered infants. Most samples with presence of *Bacteroides* were related to vaginal delivered infants. *B. vulgatus* was the predominant species in most samples. Several species of *Bacteroides* were present in mother-child pairs, and *B. vulgatus* and *B. dorei* had a significant association ($p<0.05$) between presence in mother-child pairs and vaginal delivery. *B. vulgatus* discovered in infants could be directly linked to mother's stool, which indicates mother's gut as an important reservoir for mother-child transmission.

In conclusion, the results support that *B. vulgatus* is vertically transmitted from mother to child by vaginal delivery and persists in the infant gut up until 3 months of age. These findings indicate a stable colonization of *B. vulgatus* in the infant gut. Further research is essential to investigate if *B. vulgatus* is stable in the infant gut for a longer period, and the effect of mode of delivery on infant gut composition.

Sammendrag

Sammenhengen mellom menneskets tarmmikrobiota og helse og sykdom har blitt avdekket de siste årene. Tidlig kolonisering av gunstige bakterier i nyfødtes tarm er essensielt for utviklingen av en sunn tarmflora, immunsystemet og for helse senere i livet. Mikrober overføres fra mor til barn. En debatt pågår om hvorvidt den første overføringen skjer i livmoren eller når fostervannsmembranen blir brutt. Tiden for første overføring og mekanismene bak overføringen mangler derfor fremdeles. Tidligere studier har identifisert en assosiasjon av *Bacteroides* i mor-barn par. Målet til denne studien er derfor å identifisere en vertikal overføring av arter av *Bacteroides* fra mor til barn.

Overlappen av *Bacteroides* 144 mor-barn par i sammenheng med fødselsmetode ble undersøkt. 95 av mor-barn parene var i forbindelse med vaginal fødsel, og 48 mor-barn i forbindelse med keisersnitt. Prøver fra mors avføring, mekonium (barnebek), nyfødts hud og avføring fra 3 måneder gamle barn ble analysert for å studere bakteriell overlapp. Materialet i denne studien er en del av PreventADALL kohorten. Sammensetningen av bakterier i de ulike prøvene ble studert ved Illumina sekvensering, ved bruk av 16S rRNA genet og *rpoB* genet. Amplikon sekvensvarianter (ASV) profiler ble brukt til å studere sammensetningen av *Bacteroides* i mødre og barn. Indikasjon på levende bakterier i mekonium ble undersøkt ved kultivering.

Bacteroides var til stede i alle prøvekategorier, og representerte 26.4% av bakteriesammensetningen i mekonium fra nyfødte som ble født vaginalt. De fleste prøvene med *Bacteroides* til stede var i sammenheng med vaginal fødsel. *B. vulgatus* var den dominerende arten i de fleste prøvene. Flere arter av *Bacteroides* var til stede i mor-barn par, og *B. vulgatus* og *B. dorei* viste en signifikant assosiasjon ($p<0.05$) mellom tilstedeværelse i mor-barn par og vaginal fødsel. *B. vulgatus* som ble funnet i prøver fra barn kunne bli linket direkte til mors avføring, som indikerte at mors tarm er en viktig kilde for overføring fra mor til barn.

Resultatene i denne studien støtter opp om at *B. vulgatus* blir vertikalt overført fra mor til barn ved vaginal fødsel, og vedvarer i tarmen opptil 3 måneders alder. Funnene indikerer en stabil kolonisering av *B. vulgatus* i nyfødtes tarm. Videre studier er nødvendige for å undersøke om *B. vulgatus* forholder seg stabil i spedbarnets tarm over en lengre periode, og hvilken effekt fødselsmetode har på sammensetningen av spedbarns tarm.

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1.0 Introduction

1.1 Human microbiota

1.1.1 Diversity, stability, and function of the human gut microbiota

Microorganisms can be found on both external and internal surfaces of the human body, such as the skin, saliva, and in the gastrointestinal tract (GI-tract) (Sender, Fuchs, & Milo, 2016). The majority of microbes in the human body are located in the GI-tract, where the colon harbors the majority of the microbes (Sender et al., 2016). The human GI-tract is inhabited by a complex and diverse population of microorganisms called the gut microbiota (Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012; Thursby & Juge, 2017). The gut microbiota is made up of organisms from Bacteria, Archaea and Eukarya, and forms a mutually beneficial partnership with the host (Thursby & Juge, 2017). Additionally, the majority of species in the gut microbiota belong to Bacteria (Thursby & Juge, 2017). The majority of gut bacteria are strict anaerobes, but facultative anaerobes and aerobic bacteria are also found in smaller magnitudes (Sekirov, Russell, Antunes, & Finlay, 2010). The ratio of bacterial cells to human cells in the average human body was believed to be 10:1, but recent studies have shown that this ratio is closer to 1:1(Sender et al., 2016). The number of bacterial genes, however, differs vastly from the number of human genes. There are approximately 300 to 500 bacterial species in the human gut, which comprises nearly 2 million genes (Quigley, 2013), though the exact number of both species and genes have yet to be defined.

Studies have shown that healthy adults share a core gut microbiota, meaning they share most of the same bacterial species (Lozupone et al., 2012). The majority of these bacteria belong to a few phyla, where Bacteroidetes and Firmicutes are the most common dominating phyla (Lozupone et al., 2012; Robles Alonso & Guarner, 2013). However, a considerable variation in bacterial composition can be found among individuals when studying the human gut microbiota on lower taxonomical levels (strain or species) (Robles Alonso & Guarner, 2013). Strain-level diversity between individuals suggests a distinctive pattern of bacterial composition unique to each individual (Robles Alonso & Guarner, 2013). The bacterial composition is relatively undisturbed through life unless an imbalance is provoked by external factors such as dietary changes, antibiotic usage or pathogens (Levy, Kolodziejczyk, Thaiss, & Elinav, 2017). Unbeneficial alterations in function and composition of the gut microbiota is commonly referred to as dysbiosis, easily explained as an unhealthy gut flora (Levy et al.,

2017). Unless the gut microbiota can revert to its healthy state and the dysbiosis continues, the host has a higher chance of developing inflammation and disease than with a healthy gut flora (W. A. Walker, 2017).

Throughout the GI tract, the epithelium is covered by a mucus layer, which varies in thickness depending on the location, creating a boundary between the gut lumen and the host tissue (Donaldson, Lee, & Mazmanian, 2016). The small intestine harbors a single layer of mucus attached to the epithelium, whereas the colon harbors two layers (Donaldson et al., 2016). The outer layer in the colon is loose and continuously sloughed off into the fecal stream, whereas the inner layer is dense and firmly attached to the epithelium (Donaldson et al., 2016). The inner layer is essentially sterile, while the outer layer is colonized by mucin-degrading bacteria (Donaldson et al., 2016).

The composition of bacteria alongside the GI tract differs, because of the different chemical, nutritional and immunological gradients along the gut (Thursby & Juge, 2017). The small intestine has higher levels of oxygen and antimicrobial peptides than the colon and is more acidic (Donaldson et al., 2016). Therefore, the bacteria which reside in the small intestine are mainly fast-growing aerobic or facultative anaerobic bacteria, e.g. Lactobacillaceae and Enterobacteriaceae (Donaldson et al., 2016). The bacteria residing in the small intestine compete with the host for nutrients and combined with the exposure to acids and antimicrobials, the bacterial growth in the small intestine is limited to smaller densities than in the colon (Thursby & Juge, 2017). The concentration of antimicrobials in the colon is lower and the transit time of digesta slower, resulting in a higher density and diversity of bacteria (Donaldson et al., 2016). Bacteria that reside in the mucus layer in the intestines are most likely to participate in immunologic reactions, whereas bacteria that reside in the lumen are more likely to participate in metabolism (Quigley, 2013).

Important functions of the gut microbiota include their role in metabolism, as colonic bacteria can break down non-digestible carbohydrates such as cellulose, resistant starch, and xylans, which are not metabolized during the transit through the small intestine (Donaldson et al., 2016; Thursby & Juge, 2017). These carbohydrates are fermented by colonic bacteria and provide energy for microbial growth, but also leads to the generation of metabolites such as short-chain fatty acids (SCFA) (Tremaroli & Bäckhed, 2012). The SCFAs mainly produced by microbial degradation of carbohydrates are acetate, propionate, and butyrate, which have a

large effect on gut health (Tremaroli & Bäckhed, 2012). Butyrate is an energy substrate for colonic epithelium, while acetate and propionate are energy substrates for peripheral tissue (Donaldson et al., 2016).

While the gut microbiota plays a large role in human metabolism, it also has a large effect on the immune system (Quigley, 2013). By colonizing the gut, the bacteria stimulate the production of the antibody immunoglobulin A, the differentiation of T-helper cells and the development of T-regulatory cells (Cerf-Bensussan & Gaboriau-Routhiau, 2010). However, for the gut bacteria to be accepted by the immune system as a friend rather than a foe, the colonization needs to begin early in the development of the immune system (Cerf-Bensussan & Gaboriau-Routhiau, 2010).

1.1.2 Transmission of bacteria from mother to infant

Bacterial transmission from mother to child is collectively agreed on. However, the time of transmission is not yet fully understood. The common belief is that the human fetus is developed in a sterile environment and is therefore sterile before birth, referred to as the "sterile womb" hypothesis (Rehbinder et al., 2018). However, several studies challenge the "sterile womb" hypothesis. Studies have reported detection of bacterial DNA in both meconium and amniotic fluid, and suggest that the fetus is exposed to microbial DNA prior to birth (Stinson, Boyce, Payne, & Keelan, 2019). The latest findings also suggest that the correct time for the first colonization may be after the rupture of the amniotic membrane during birth, and as the infant move through the birth canal they receive their first inoculation of microbes via the vaginal flora and by swallowing amniotic fluid (Moore & Townsend, 2019). Despite these different findings, several studies agree that infants develop an initial microbiome from birth and that this development continues during their first years of life (Eck et al., 2020; Rutayisire, Huang, Liu, & Tao, 2016). As mentioned previously, the "sterile womb" hypothesis is a debated topic, where both sides propose different mechanisms for transfer.

During pregnancy, the mother's immune and hormonal function undergoes changes, which drives the intestinal and vaginal microbiota to undergo changes in community structure (Blaser & Dominguez-Bello, 2016). The vaginal microbiota is characterized by low diversity and stability through pregnancy, with *Lactobacillus* dominating the microbiota (R. W.

Walker, Clemente, Peter, & Loos, 2017). This dominance may inhibit infections which could lead to the fetus being exposed to pathogenic microbes, but it can also ensure the colonization of beneficial microbes during delivery (R. W. Walker et al., 2017). The gut microbiota during pregnancy is characterized by a higher diversity and richness than the vaginal microbiota (R. W. Walker et al., 2017). Especially butyrate-producing bacteria increase in representation in the gut, as they increase the production of T-regulatory cells which may reduce the chances of maternal rejection of the fetus (Blaser & Dominguez-Bello, 2016).

The physical changes the women go through during pregnancy may induce translocation of bacteria to the intrauterine environment (R. W. Walker et al., 2017). Studies have proposed mechanisms for the transfer of bacteria to the sterile womb, via either the amniotic fluid or the placental barrier, by vaginal ascension where the bacteria travel up to the uterus, or by hematogenous translocation through the cord blood (R. W. Walker et al., 2017). These mechanisms could potentially mean that bacteria are transmitted from mother to child before birth, which could suggest that the womb is not sterile. However, the placenta and amniotic fluid contain low amounts of bacteria, which makes analysis of these samples difficult (Perez-Muñoz, Arrieta, Ramer-Tait, & Walter, 2017). Contamination is an ongoing problem when analyzing samples with a low biomass, where low amounts of bacteria are related to a high amount of contamination (Perez-Muñoz et al., 2017). Additionally, another problem the non-sterile hypothesis face is the viability of the bacteria found in the samples, meaning whether the bacteria is alive or not.

Other studies indicate that the placenta and amniotic fluid is sterile, which support the “sterile womb” hypothesis. Hence, the transmission of bacteria from mother to infant may happen during or after birth. Strain-level analysis of microbes has provided evidence for vertical transmission of bacteria from mother to child (Wang et al., 2020). Possible sources of bacterial transmission have been identified as breast milk and vaginal-, gut- and oral microbiota (Ferretti et al., 2018). The mother’s gut microbiota dominates the transmission, followed by the vagina, oral cavity, and lastly the skin, and the contribution from the mother’s gut is also the most persistent over time compared to the other sources (Wang et al., 2020).

Bacteria that are transmitted vertically from mother to child have a larger chance of forming stable colonization in the infant’s gut over time when compared to the other sources of maternal transmission (Korpela et al., 2018). Especially the genus *Bacteroides* has been

observed in the infant's gut after 1 year and are indicated to be transmitted by vaginal delivery (Korpela et al., 2018). However, delivery by caesarean section (C-section) has been shown to disrupt the vertical transmission since the microbiota in the mother's gut and vagina are unavailable to the infant with this birth method (Wang et al., 2020). Additionally, the majority of C-section delivered infants do not experience a transmission of *Bacteroides* from their respective mothers but rather a transmission from their skin microbiota (Wang et al., 2020).

1.1.3 Establishment of the infant gut microbiota

The mode of delivery has been shown to have a large influence on the early colonization of the infant gut (Munyaka, Khafipour, & Ghia, 2014; Rutayisire et al., 2016). During and immediately after birth, the infant's GI tract is colonized by microbes from both the mother and the surrounding environment (Munyaka et al., 2014). Vaginal delivered children are exposed to maternal fecal and vaginal microbiota, while children delivered by C-section are not (Milani et al., 2017; Yassour et al., 2018). Therefore the gut flora in vaginal delivered children resembles the composition of bacteria found in the mothers gut and vagina (Francino, 2018). However, children delivered by C-section have been shown to be colonized by bacteria found on skin (Francino, 2018). Additionally, the bacterial diversity and richness of the gut in C-section delivered infants are significantly lower than in vaginal delivered infants (Mitchell et al., 2020). Especially the prevalence of colonization by the obligate anaerobic *Bacteroides* species has been shown to be lower in children delivered by C-section (Mitchell et al., 2020). Long-term studies of infant gut composition (up to 2 years) have shown an association of low diversity and delayed colonization of *Bacteroides* with C-section (Jakobsson et al., 2014).

Mode of delivery is not the only factor affecting the development of the infant's gut microbiota. Particularly diet affects the phases the gut microbiota undergoes during its establishment (Derrien, Alvarez, & de Vos, 2019). During the first 3-14 months of life, the infant's gut microbiota is in its developmental phase, where the diet consists of either breastmilk or formula (Derrien et al., 2019). During this stage, the diversity in the gut is still relatively low but dominated by the species *Bifidobacterium*, as the selection in the gut is driven towards bacteria that are able to degrade human milk oligosaccharides (HMO's) (Derrien et al., 2019*)*. As more complex carbohydrates are introduced with solid foods, both the bacterial load and diversity increase and is dominated by bacteria belonging to phyla such as Bacteroidetes and Firmicutes (Derrien et al., 2019). When the diet consists of only solid

foods, around 31-46 months of age, the majority of the development of the gut bacteria is finished, and the composition is in its stable phase with a predominance of Firmicutes (Derrien et al., 2019).

1.1.4 Human vaginal microbiota

During an infant's travel through the birth canal, they are exposed to the mother's vaginal microbiota. The healthy vaginal microbiota is composed of predominantly Lactobacilli, but other bacteria, such as *Bacteroides*, *Bifidobacterium* and *Enterococcus* are also found (Mendling, 2016). Lactobacilli, with their antibacterial properties and immunologic factors, create a defense system against dysbiosis and infections in the vagina, which is responsible for ensuring a healthy pregnancy and delivery (Mendling, 2016).

1.1.5 Human skin microbiota

The impact of bacterial colonization of an infant is not limited to the gut and vagina only. The skin offers an ecological niche to bacteria, yeast, and mold, and is divided into three regions: dry, moist, and sebaceous areas (Cundell, 2018). The dry sites on the skin are the most diverse, where one can find bacteria from phyla such as Actinobacteria, Proteobacteria, Firmicutes and Bacteroidetes (Grice & Segre, 2011). Mode of delivery influences the infant's skin microbiota, where vaginal delivery leads to colonization of vaginal bacteria such as *Lactobacillus* and *Prevotella*, whereas C-section leads to colonization of maternal skin bacteria such as *Staphylococcus* and *Corynebacterium* spp. (Cundell, 2018).

1.1.6 Genus Bacteroides

One of the dominating phyla in the human gut is Bacteroidetes. Belonging to this phylum is species of *Bacteroides*, which amount to ~25% of the gut bacteria (Wexler, 2007). *Bacteroides* are bile-resistant, non-spore-forming gram-negative bacteria with a rod shape (Wexler, 2007). While they are obligate anaerobes they are also described as aerotolerant, meaning they will not use oxygen for ATP production but can survive with oxygen present (Bacic & Smith, 2008). The relationship between the host and *Bacteroides* can be described as mutualistic, where *Bacteroides* produce SCFA's and promote the development of the immune system in the host, while the host provide the bacteria with nutrients and a safe environment (Wexler, 2007). *Bacteroides* can adapt to the nutritional conditions in the host's gut by using the available dietary and host-derived glycans (Rios-Covian, Salazar,

Gueimonde, & de los Reyes-Gavilan, 2017). Bacteria from the phylum Bacteroidetes mainly use polysaccharides and peptides for growth, and Bacteroidetes have been suggested as the main producer of the SCFA propionate, utilizing the succinate pathway to produce propionate from hexose sugars (Reichardt et al., 2014). Another system *Bacteroides* utilize to adapt to the host's gut is a modulation of their surface polysaccharides, where they "flip" their promoters for expression on and off to evade the host's immune system (Wexler, 2007).

In order to thrive in the human gut, *Bacteroides* is dependent on stable colonization. Some bacteria harbors genes which benefit them in the colonization in an infant's gut (Lee et al., 2013). Especially *B. fragilis* has been described to have a unique conserved class of polysaccharide utilization loci, called commensal colonization factors (*ccf*) which are upregulated during colonization at the colonic surface (Lee et al., 2013). This locus, discovered by Lee et al. allows *B. fragilis* to penetrate the mucus layer and mediates species-specific colonization of *B. fragilis* in the crypt channels of the colon (Lee et al., 2013). The study proposed that the CCF-system formed by the *ccf* genes allowed for species of *Bacteroides* to utilize specific glycan structures in the colonic crypts much more efficiently than other gut bacteria (Lee et al., 2013). The *ccf* genes encode a specific subset of polysaccharide utilization loci which promotes stable and resilient colonization, as crypt-associated bacteria create reservoirs that can repopulate the gut in case of disruption by disease or antibiotic treatment (Lee et al., 2013).

Intestinal colonization influences the infant's immune system and plays an important role in the maturation of the intestinal mucus layer and the gut-associated lymphoid tissue (GALT) (Francino, 2018). Species of *Bacteroides* play different parts in developing the infant's immune system by colonizing its gut. Mice studies have shown that colonization by *B. fragilis* is correlated with the development of thymic lymphocytes which further develops into T cells, which is involved in the maturation of the infant's immune system (Ennamorati et al., 2020; Pronovost & Hsiao, 2019). *B. ovatus* have been described as responsible for the majority of the production of IgA by inducing B cells to secrete the antibody (Hand, 2020).

The participation in the development of the immune system is not the only contribution of *Bacteroides* to the immunologic functions of the host. Several species of *Bacteroides* produce antimicrobial peptides and anti-inflammatory components. *B. fragilis* produce zwitterionic polysaccharides (ZPS), a unique carbohydrate that has both positively and negatively charged

functionalities (Zhang, Overkleeft, van der Marel, & Codée, 2017). These polysaccharides can activate $CD4^+$ T cells which can prevent inflammatory responses in the gut (Mazmanian, Round, & Kasper, 2008). *B. thetaiotaomicron* has been shown to stimulate the production of Ang4, an antibiotic Paneth cell protein, which can kill pathogenic organisms (Wexler, 2007). Severeal *Bacteroides* species excrete antimicrobial toxins which antagonizes closely related species and strains, with *B. fragilis* as the main producer (Shumaker, Laclare McEneany, Coyne, Silver, & Comstock, 2019). While this toxin secretion could be beneficial for bacterial competition, it may also be a threat to the health of the host (Shumaker et al., 2019).

While *Bacteroides* contribute beneficially regarding the host's health, their large genome bank may pose a threat. *Bacteroides* can evade the host's immune system by modulating their surface polysaccharides and destroying the gut tissue, which can lead to bacteremia (Wexler, 2007). Especially *B. fragilis* is described as an opportunistic pathogen. *B. fragilis* can be divided into two groups, enterotoxigenic and nontoxigenic, which produces toxins and does not produce toxins respectively (Sun et al., 2019). Infection by enterotoxigenic *B. fragilis* can cause a loss of tight junctions between epithelial cells, generating a barrier dysfunction (Sun et al., 2019). Antibiotic treatment of *Bacteroides* may be difficult, as many of the species are resistant to several antibiotics (Wexler, 2007). While the potential threat of *Bacteroides'* virulence factors is present, the benefits often shadow the disadvantages.

1.2 Analysis of taxonomic composition

Taxonomic classification in microbiology used to be based on culture-dependent techniques, where phylogenetic traits such as morphology, antimicrobial resistance, and enzyme activity were used to identify microbial diversity. However, culture-based methods are both time- and resource-intensive, as requirements such as atmosphere, nutrients, temperature, and incubation time must be fulfilled for bacteria to grow. When studying bacteria from diverse environments, it may be difficult to fulfill the requirements for all bacteria, which in turn will lead to a limited overview of the bacterial diversity. In the present, culture-dependent techniques are still in practice, but analysis of taxonomic composition has moved towards molecular methods. Molecular methods do not face the same challenges as culture-based methods, as these methods assess DNA-sequences and have the potential to recover most of the bacterial diversity in the environment by targeting specific genetic markers. A molecular

approach to examine bacterial diversity is the 16S rRNA gene, which is the most important tool today.

1.2.1 16S rRNA-sequencing

A common gene used in taxonomic classification of community compositions in prokaryotes is the 16S ribosomal RNA gene (16S rRNA). Sequencing this gene is a culture-independent technique often used in microbial studies. The gene is present in all prokaryotes and is highly conserved, with 9 hypervariable regions (V1-V9). These regions can be used to distinguish bacteria on a higher taxonomical level by using targeted primers, while the whole gene can be used to identify bacteria on lower taxonomical levels (Robles Alonso & Guarner, 2013). While 16S rRNA-sequencing is an efficient and low-cost analysis, it does not allow for separation of closely related species because of the low sequence divergence (Ko et al., 2007).

1.2.2 Genetic markers for *Bacteroides*

Diversity studies use genetic markers to separate closely related species to the highest taxonomical level. The gene *rpoB,* which encodes the ß-subunit of bacterial RNA polymerase, has been suggested as genetic marker in phylogenetic studies (Ogier, Pagès, Galan, Barret, & Gaudriault, 2019). The gene has been used in phylogenetic studies to separate similar strains of the genus *Bacteroides* because of its greater resolution and genetic variation when compared to the V3-V4 regions of 16S rRNA (Ko et al., 2007). False positives occur at a much lower rate in *rpoB*-based sequencing (Ogier et al., 2019). The divergence in sequences in *rpoB* between species of *Bacteroides* makes it a formidable marker for differentiating and identifying closely related species (Ko et al., 2007).

1.2.3 Bioinformatic tools for taxonomic assignment based on DNA sequence data

Assigning taxonomy to DNA sequences is a crucial step in any DNA analysis. Bioinformatics has become a vital tool for taxonomic assignment using reference databases to transform sequences into bacterial names (Sierra et al., 2020).

1.2.3.1 Databases for taxonomic assignment

To assign taxonomy to sequences retrieved from approaches such as Illumina and Sanger, several databases can be used. Two main technical components of a taxonomic analysis are the reference database and the binning approach used, either aligning reads against reference sequences or sorting into *k*-mers (Balvočiūtė & Huson, 2017). Examples of reference databases that assign taxonomy based on 16S rRNA sequences are Greengenes, RDP (Ribosomal Database Project), and SILVA (Balvočiūtė & Huson, 2017). HumGut is a newly established reference database with the intent to be used as a universal reference for all human gut microbiota studies (Hiseni, Rudi, Wilson, Hegge, & Snipen, 2021).

BLAST (Basic Local Alignment Search Tool, NCBI) is a bioinformatic search tool that finds similar regions between sequences and calculates the statistical significance (Wood & Salzberg, 2014). Kraken is another system for assigning taxonomy and is often used to assign taxonomy to short sequences. This system assigns sequences into weighted *k*-mers, a nucleotide sequence with length *k*, by the last common ancestor (LCA) (Wood & Salzberg, 2014). The Kraken system provides precision and sensitivity on genus-level similar to the fastest BLAST-program (Wood & Salzberg, 2014). Databases are created for different content and purposes, which should be considered when selecting a database for assigning taxonomy.

1.2.3.2 Amplicon sequence variants

Amplicon sequence variants (ASV) is an approach that can separate sequences down to nucleotide level by targeting genetic markers (Callahan, McMurdie, & Holmes, 2017). ASV determines which sequences were read and how often, and gives a broad understanding of community diversity both within and between the samples (Callahan et al., 2017). Other popular approaches such as operational taxonomic unit (OTU) clustering uses either a reference database or creates clusters based on observed sequences (Callahan et al., 2017). While these approaches are easy and reliable to a certain degree, ASV provides a more precise sequence variation compared to OTU clustering (Callahan et al., 2017). In order to obtain the ASV profiles, bioinformatic tools such as the DADA2 pipeline could be used. DADA2 is a reimplantation of the Divise Amplicon Denoising Algorithm (DADA), an algorithm used to correct amplicon errors when constructing OTU's (Callahan et al., 2016). The DADA2 pipeline was developed for Illumina sequencing amplicons to detect variations

in microbial communities and obtaining fewer incorrect sequences, while also possibly be a better approach for detecting strain-level variation (Callahan et al., 2016).

1.3 Polymerase-chain reactions

Polymerase-chain reaction (PCR) is a simple enzymatic assay used for the amplification of specific DNA-fragments. It was first discovered in 1990 and is still a widespread assay often used when studying microbial communities (Garibyan & Avashia, 2013). Each assay requires template DNA, primers, nucleotides, and DNA polymerase (Garibyan & Avashia, 2013). PCR requires three steps with different temperatures and the steps are repeated in a given number of cycles. The first step is denaturation, where the reaction mix is heated to a temperature where double-stranded DNA melts and the strands separate (Garibyan & Avashia, 2013). Annealing, the second step, is where the temperature is lowered to a point where primers can bind to the desired DNA-fragments, followed by the final step, extension, where DNA polymerase extends the sequence (Garibyan & Avashia, 2013). By repeating these three steps, the amount of DNA grows exponentially (Garibyan & Avashia, 2013). PCR can be used to indicate the presence of the desired DNA or indicate the amount of DNA present, by using qualitative or quantitative PCR respectively. These differences should be taken into amount when deciding the appropriate method for a study.

1.3.1 Qualitative PCR

Qualitative PCR is used to indicate whether the target DNA is present. Qualitative PCR includes end-point analysis in order to confirm the presence of the target DNA. One of these analyses is agarose gel electrophoresis, where the amplified product can be stained using dyes such as ethidium bromide or peq green (Garibyan & Avashia, 2013). Gel electrophoresis separates the PCR products based on size and charge, where negatively charged DNA will travel through the gel and give an indication of the size of the fragment.

1.3.2 Quantitative PCR

Quantitative real-time PCR (qRT-PCR) is a PCR assay that indicates the amount of a specific gene or DNA-fragment in the sample. Two methods are commonly used to detect and quantify the amount of the desired product – non-specific fluorescent dyes and sequence-specific probes (Garibyan & Avashia, 2013). Real-time assays allow the detection and

quantification to be measures with each cycle, while the targeted DNA-fragment is being synthesized (Garibyan & Avashia, 2013). The concentration of DNA can be calculated using the Cq-value the assay provides, which is the number of cycles needed to reach the fluorescent threshold (Kubista et al., 2006). A low Cq-value indicate a high amount of DNA, while a high value indicates a low amount. A standard curve based on serial dilutions of a standard containing known amounts of the target sequence can be used to calculate the concentration of target DNA in samples (Kubista et al., 2006). Additionally, to compare the fragments, a melt curve analysis could be included.

1.4 Sequencing techniques

1.4.1 First-generation sequencing

The major breakthrough in DNA sequencing technology came in 1997 when Frederick Sanger and colleagues developed a chain-termination technique which was named Sanger-sequencing (Heather & Chain, 2016). This technique is based on using deoxynucleotide triphosphates (dNTPs) which are monomers of DNA strands and specific for each DNA base (Heather & Chain, 2016). Dideoxynucleotide triphosphates (ddNTPs), which lack the 3'hydroxyl group and cannot form bonds with the 5'phosphate on the next dNTP, are added to the mix (Heather & Chain, 2016). By mixing labeled ddNTPs into an extension reaction with dNTPs, the ddNTPs will be incorporated randomly as the strand extends (Heather & Chain, 2016). The reaction produces DNA fragments with different lengths, which can be separated by gel electrophoresis and thereby identified (Heather & Chain, 2016).

1.4.2 Second-generation/Next-generation sequencing

Second-generation sequencing, commonly referred to as next-generation sequencing (NGS), allows for analysis of larger sequences (Fadrosh et al., 2014). A popular NGS technique is Illumina sequencing. The workflow for Illumina sequencing requires three steps: library preparation, sequencing and data analysis (Illumina, 2021). First, the DNA must be fragmented and mixed with a unique set of Illumina index primers that attach to each end of the fragment. (Illumina, 2021). Index primers contain an adapter and a barcode, where the adapters attach to each side of the fragment and allow the oligonucleotides to be hybridized on the flow cell (Illumina, 2021). The unique barcode sequences make it possible for the fragments to be mapped back to the original sample, allowing several samples to sequenced

together on the flow cell (Illumina, 2021). Second, when the library is loaded onto the flow cell and sequencing occurs, the DNA fragments are amplified into millions of single-stranded DNA, generating clusters (Illumina, 2021). Moreover, nucleotides bind to complementary template DNA in a process called sequencing by synthesis, where each nucleotide contains a fluorescent tag and a reversible terminator (Illumina, 2021). These tags indicate which nucleotide has been added, and the reversible terminator blocks the incorporation of a new base until a fluorescent signal from the tag has been emitted (Illumina, 2021). The forward reads are read first and then washed away, and the process repeats for the reverse strands resulting in paired-end sequencing (Illumina, 2021). Thirdly, after sequencing, the nucleotides are identified, and the data can be further analyzed by bioinformatic tools such as the DADA2 pipeline (Callahan et al., 2016). Next-generation sequencing such as Illumina allows for high-throughput sequencing of larger DNA-fragments and leads to efficient analysis of DNA.

1.5 Culturing techniques

While molecular methods provide a larger overview of microbial diversity in an environment, there are limitations to these methods which culture-dependent techniques can solve. One of these limitations is the viability of bacteria, where molecular methods provide no information on whether the bacteria is alive. As mentioned earlier, low biomass provides difficulties for molecular methods as the prevalence of contamination is high (Perez-Muñoz et al., 2017) Popular cultivation approaches are solid and liquid mediums with specific nutrients designed to facilitate the growth of the bacteria of interest. However, most of the human gut microbiota is deemed “unculturable” (Browne et al., 2016). Molecular methods may provide information about genetic traits and mechanisms which can be used to create mediums and techniques allowing for the study of “unculturable” bacteria.

Cultivation of *Bacteroides* requires several considerations. As mentioned previously, *Bacteroides* is obligate anaerobes, but are also one of the most aerotolerant anaerobic bacteria (Bacic & Smith, 2008). Therefore, the cultivation of *Bacteroides* requires anaerobic conditions but they will tolerate certain amounts of oxygen. Hydrolysis of esculin have been proposed as a formidable phylogenetic marker, as *Bacteroides* will form a black/brown complex on solid mediums (Livingston, Kominos, & Yee, 1978). The addition of bile to mediums ensures inhibition of non-bile resistant bacteria, and the addition of hemin and

vitamin K stimulates growth of *Bacteroides* (Bacic & Smith, 2008). Fulfillment of these conditions may result in the successful cultivation of *Bacteroides*.

1.6 PreventADALL cohort

"Preventing Atopical Dermatitis and Allergies in children", the PreventADALL study is an ongoing multinational population-based birth-cohort study highlighting the upwards trend of allergic and immune-related diseases in the Western world. The PreventADALL study is a population-based, randomized clinical trial aiming to deter if early life factors can influence the development of allergies and diseases such as asthma, obesity, and diabetes. Recently, a study aimed to determine whether regular skin emollients or early complementary feeding could prevent atopic dermatitis by age 12 months and food allergy by 36 months respectively (Skjerven et al., 2020). Unfortunately, the study showed that there was no effect on the development of these ailments (Skjerven et al., 2020). In the PreventADALL study, data from 2397 mother-child pairs were collected, with pregnant women recruited between December 2014 to October 2016, and the infants recruited from April 2015 to 2017. The data included detailed questionnaires and biological samples (stool and elution swabs from skin). The samples from mothers were collected at 18 weeks into pregnancy, while the samples from infants were collected at age 0 (meconium), 3, 6, 12, 24, and 36 months. There are follow-up studies planned for the children at age 4 (Lødrup Carlsen et al., 2018). Analyzing samples from children at different ages allows for an investigation of the establishment and development of the infant gut microbiota.

A previous study (Sørensen, 2020) screened 465 meconium samples from the PreventADALL cohort for *Bacteroides* by amplifying the *rpoB*-gene. Of the 465 samples, 51 meconium samples had an amplification of the gene, where 48 of these could be matched to their corresponding mothers, resulting in these samples being analyzed further on. All the meconium samples that had a positive hit were infants born vaginally, therefore, in this study, we included the same number of infants that did not have amplification of *rpoB* born by C-section, or by vaginal delivery.

1.7 Aim of thesis

Bacteroides are one of the first colonizers of the human gut and is thought to be transferred from mother to child (Gregory, LaPlante, Shan, Kumar, & Gregas, 2015). A potential route of colonization is the transmission from mother to child during or after birth, although it is uncertain exactly when *Bacteroides* are transferred (Yassour et al., 2018). Even though the time of transmission is yet to be identified, it is hypothesized that *Bacteroides* are vertically transferred from mother to child during vaginal delivery.

The main goal of this thesis is therefore to identify the mother-child association of *Bacteroides* species using material from the PreventADALL study. To achieve this goal, we had the following subgoals:

- Investigate the presence of *Bacteroides* in samples from mother's stool, meconium, feces from 3 months old infants, and infant's skin using the *rpoB* gene as a positive marker for *Bacteroides.*
- Investigate the bacterial composition in samples from mother's stool, meconium, feces from 3 months old infants, and infant's skin based on 16S rRNA sequencing.
- Determine the presence of live *Bacteroides* in meconium samples with amplification of *rpoB* by cultivation.

2.0 Materials and methods

2.0.1 Work progress

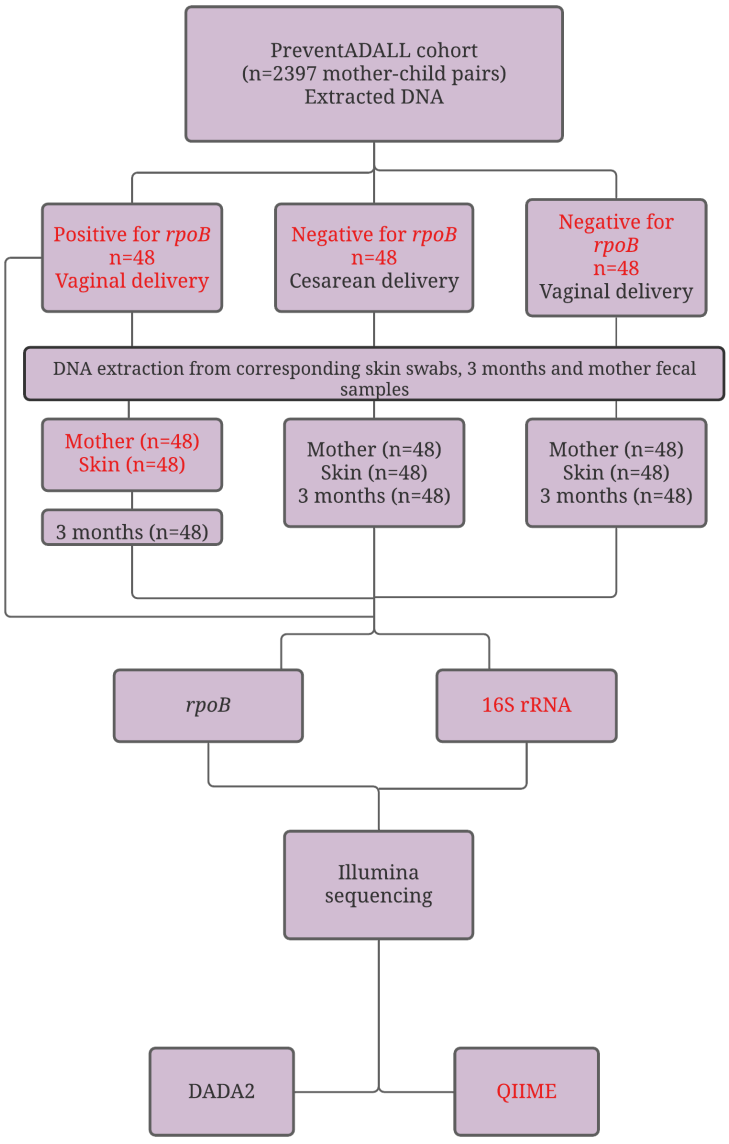

Figure 2.1. Workflow overview of the sequencing part of this master thesis. Samples were collected by the PreventADALL cohort and meconium samples were screened for *rpoB* by MSc Regina Sørensen and PhD Morten Nilsen. Meconium samples from vaginal delivered children with positive hits for *rpoB* and their corresponding samples from mother's stool and elution swabs were sequenced using 16S rRNA as a marker. Everything marked in red was performed by Sørensen and Nilsen. This thesis builds on their work by including samples from mother's stool, elution swabs and feces from 3 months old children from the groups "negative for *rpoB* – cesarean delivery" and "negative for *rpoB* – vaginal delivery". These samples were screened for *rpoB* and positive hits were sequenced using *rpoB* specific primers and sequenced using Illumina.

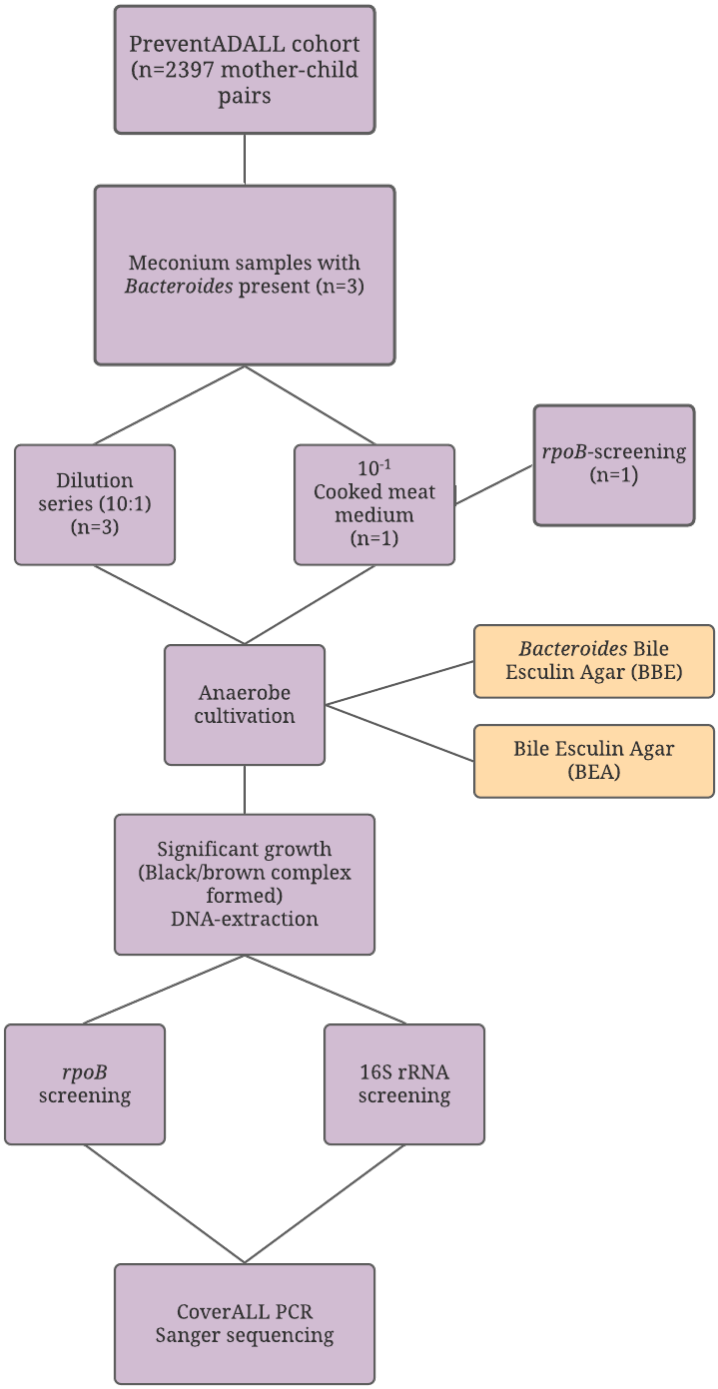

Figure 2.2. Workflow describing the cultivation process. Meconium samples with a high abundance of *Bacteroides* present, especially *Bacteroides vulgatus,* were selected. One sample was screened for *rpoB* before incubation on *Bacteroides* specific mediums. A dilution series, 10^{-1} to 10^{-6}, was made for each sample and the series were incubated on two *Bacteroides* specific mediums. Colonies with promising morphological qualities such as esculin hydrolyzation were selected for Sanger sequencing.

2.1 Sample description

The samples used in this project were retrieved from the PreventADALL cohort (see the introduction for details) and DNA was extracted beforehand. The samples include elution swabs (E-Swabs >10 minutes after birth) from newborn's skin, feces from 3 months old children, and feces from mother at 18 weeks into their pregnancy (Figure 2.1). Samples used in cultivation were meconium collected immediately after being passed by the infant (Figure 2.2). The extracted DNA was stored at -20 ºC between usage, and meconium used in cultivation was stored at -80ºC without buffer until they were used.

2.2 DNA extraction and quality analysis

2.2.1 Mechanical lysis

Samples were thawed on ice and homogenized with nuclease-free water before they were mixed with 3 different sizes of glass beads. To a 200 µl sample, 0.2 g acid-washed glass beads (Sigma-Aldrich, <106 µm), 0.2 g acid-washed glass beads (Sigma-Aldrich, 425-600 µm) and 2 2.5-3.5 mm acid-washed beads (Sigma-Alridch) were added. Samples were processed twice on a FastPrep 96 (MP Biomedicals) for 40 seconds at 1800 rpm and centrifuged at 13000 rpm for 5 minutes. Genomic DNA was extracted from the lysed samples and stored on -20 ºC until further use.

2.2.2 DNA extraction

DNA was extracted from the lysed samples using a MagMidi LGC Kit (LGC Biosearch Technologies, UK), where magnetic particles in combination with salt were used to extract DNA. Negatively charged DNA were absorbed by the magnetic particles because of a cation bridge formed between the magnetic particles and positively charged salt (Tian, Hühmer, & Landers, 2000). The DNA/particle complex was washed to remove impurities and DNA was eluted from the particles using nuclease-free water (VWR, USA). Eluted samples were stored at -20 ºC until further use.

2.2.3 Qubit dsDNA High Sensitivity Assay

DNA extraction was verified using Qubit, where DNA concentrations were measured using the Qubit dsDNA High Sensitivity Assay. A Working Solution was made by diluting Quant-iT™ (Invitrogen, Thermo Fischer Scientific) reagent 1:200 in Quant-iT™ buffer (Invitrogen, Thermo Fischer Scientific). The samples were prepared and measured using the

manufacturer's recommendation. Qubit was also used to quantify the DNA concentration, using the same protocol.

2.2.4 Agarose gel electrophoresis

PCR products were run on a 1.5 % Agarose gel (Invitrogen, Thermo Fischer Scientific) at 80 V for 30 minutes (Bio-Rad, USA) to separate the negatively charged DNA-fragments based on their size, using a 100 bp ladder (New England BioLabs, USA) as a reference. The fragments were visualized using a Gel Doc XR (Bio-Rad, USA).

2.3 Polymerase Chain Reactions

Polymerase chain reactions, both qualitative and quantitative, were used to identify the presence of *Bacteroides* in samples from mother and child. The primers used in the described reactions are listed in Table 2.1.

Table 2.1. Overview of the primers used in quantitative and qualitative PCR. F stands for Forward primer and R for reverse primer for all primers used.

Target area	**Primer name (F/R)**	**Amplicon length (bp)**	**Primer sequence F/R (5'-3')**	**Annealing temperature**	**Reference**
***rpoB* (RNA polymerase ß-subunit)**	BF BR	358	CACTTGAGCAAYCGTCGTRT CCTTCAGGAGTYTCAATNGG	55 ºC	(Ko et al., 2007)
V3-V4 region of the 16S rRNA gene	PRK 341F PRK 806R	466	CCTACGGGRBGCASCAG GGACTACYVGGGTATCTAAT	55 ºC	(Yu, Lee, Kim, & Hwang, 2005)
16S rRNA gene	CoverALL® F CoverALL® R	1200	TCCTACGGGAGGCAGCAG CGGTTACCTTGTTACGACTT	55 ºC	

2.3.1 Qualitative PCR

Each reaction contained a mix of 1xHotFirePol® Blend Master Mix Ready to Load (Solis Biodyne, Estonia), primer sets of 0.2 µM *rpoB* Forward and Reverse primers (Table 2.1), 2 µl template DNA, and nuclease-free water (VWR, USA) to a total volume of 25 µl. Amplification of the *rpoB* gene was achieved using a thermal cycler (Applied Biosystems, USA) with the following program: 95 °C for 15 minutes, 30 cycles of 95°C for 30 seconds, 55 °C for 30 seconds and 72 °C for 45 seconds, finished by 72 °C for 7 minutes and 10 °C until further use.

2.3.2 Quantitative PCR

Quantitative measurement of bacteria was performed for both 16S rRNA and *rpoB* using quantitative PCR. Primers targeting the V3-V4 regions of the 16S rRNA genes (Table 2.1) were used for 16S rRNA, and primers targeting the *rpoB* gene (Table 2.1) were used for *rpoB*. Amplification and quantification of the samples were done on a LightCycler480 (BioRad, USA), and each reaction contained 1x HOTFirePol® EvaGreen qPCR supermix (Solis Biodyne, Estonia), primer sets of 0.2 µM, 2 µl template DNA, and nuclease-free water (VWR, USA). The final volume was 20 µl. The PCR programs for both 16S rRNA and *rpoB* were 95 °C for 15 minutes, followed by 40 cycles of 95 °C for 30 seconds, 55 °C for 45 seconds and 72 °C for 30 seconds. The fluorescence was measured, and a High Resolution Melting (HRM) analysis was included.

2.3.3 CoverAll® PCR

DNA fragments from the cultivation process were prepared for Sanger sequencing using PCR with CoverAll® primers (Table 2.1) which targets the 16S rRNA gene. Each reaction contained a mix of 5x HOTFirePol® DNA Polymerase Ready to Load (Solis Biodyne, Estonia), 10 µM of each CoverAll® primer, 2 µl template DNA, and nuclease-free water (VWR, USA) to a total volume of 25 µl. The samples were amplified on a thermal cycler (Applied Biosystems, USA) with the following program: 95 °C for 15 minutes, followed by 30 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 1 minute and 20 seconds, and lastly 10 °C until further use.

2.3.4 High Resolution Melting (HRM) Analysis

In order to separate PCR products based on their melting point, an HRM analysis was added to the qRT-PCR program on the LightCycler 480 II. This analysis made it possible to separate false positives (e.g. primer dimers) from the PCR products, since primer dimers showed a lower melting temperature (Kuperman et al.) than the longer target sequence (Kubista et al., 2006). The changes in temperature ranged from 60-95 °C. Real-time data and melt curve analysis were analyzed with BioRad CFX Maestro 1.0, version 4.0.2325.0418 (BioRad 2017, USA)

2.3.5 PCR product clean-up

To remove enzymes, nucleotides, primers and buffers from the amplified products, a clean-up was performed using Sera-Mag beads (0.1%), where a 2x volume of beads was added to 10 µl of DNA sample. The ratio of beads was determined due to the size of the DNA-fragments. The products were washed with freshly made 80% ethanol to ensure removal of inhibitors and eluted in nuclease-free water (VWR, USA).

2.4 Illumina and Sanger sequencing

Next-generation sequencing in the form of Illumina Mi-Seq was used to determine the presence and amount of *Bacteroides* in samples from mother and child. Samples with no positive hits for *rpoB* were excluded and were not sequenced. Sanger sequencing, a first-generation sequencing technique, was used to determine if growing colonies from cultivation were identified as species of *Bacteroides*.

2.4.1 Indexing fragments for Illumina sequencing

Each DNA sequence can be mapped back to its original sample using unique primer combinations, made possible by *rpoB*-specific index primers. A combination of 24 reverse and 15 forward index primers (1 µM) were made (Appendix A, Table A1), and used to attach *rpoB*-fragments. The reaction mix for each index PCR contained 1xFIREPol® Master Mix Ready to Load (Solis BioDyne, Estonia), unique primer sets (0.2 µM) for each sample, 5 µl template DNA and nuclease-free water (VWR, USA) to a total volume of 25 µl. The indexed PCR samples were amplified in a thermal cycler with the following program: 95 °C for 5 minutes, followed by 10 cycles of 95 °C for 30 seconds, 55 °C for 1 minute and 72 °C for 45

seconds, and 72 °C for 7 minutes before storage at 10 °C. The indexed PCR products were normalized and pooled together according to their DNA concentration to create an amplicon library. Clean-up of the pooled library was performed using the ratio of 0.1 % Sera-Mag beads (2:1), and AMPure's protocol for PCR clean-ups.

2.4.2 Quantification and normalization

Qualitative DNA measurements were performed using the Cambrex FLX 800 CSE robot. A Working Solution (the same as described above) was prepared for all samples. The standard curve made from the measurements from Cambrex was used to estimate DNA concentrations in samples, based on fluorescent values from lowest to highest. The samples were normalized according to the highest DNA concentration, and the Biomek 3000 robot was used for normalization and pooling of DNA samples to one library.

2.4.3 Illumina sequencing set up

The pooled library was diluted to 4 nM, using a KAPA library quantification kit (Kapa Biosystems, Roche Diagnostics). In order to reach a concentration of 4 nM, the library was diluted with Tris-HCl. The dilution ratio was determined using qPCR and Qubit to create a standard curve and calculate the library concentration, which was 55 nM. 5 µl of the 4 nM library was mixed with 5 µl 0.2 M NaOH in an Eppendorf tube, and a PhiX control was made in another tube, with the same volumes and concentrations. The tubes were incubated at room temperature for 5 minutes, and 990 µl were then added to each tube. This resulted in a 20 pM concentration in both tubes, which were diluted to 6 pM with a total volume of 500 µl. The amplicon and PhiX control were combined, using 90 µl PhiX and 510 µl diluted amplicon library. The combined sample was denatured and incubated for 2 minutes on 96 °C, mixed and then placed on ice until it was loaded on Illumina MiSeq by lab personnel.

2.4.4 Sanger sequencing

The DNA extracted from the cultivation was prepared for Sanger sequencing by performing a CoverALL® PCR for the 16S rRNA gene. The amplified products were run on a 1.5 % agarose gel (Invitrogen, Thermo Fischer Scientific) and visualized on Gel Red XR (Bio-Rad, USA) as described earlier. The PCR-products were cleaned using AMpure's protocol and a 1:1 ratio of DNA and 0.1 % Sera-Mag beads. The cleaned products were prepared for Sanger sequencing using only CoverALL® forward primer (Table 2.1).

2.5 Data processing from Illumina and Sanger sequencing

2.5.1 DADA2 for *rpoB* sequences

For phylogenetic analysis of the *rpoB* sequences retrieved from Illumina sequencing, the DADA2 pipeline was used. The pipeline was run in R-studio version 4.0.3, and the background for the pipeline is described in the introduction. The data files provided by Illumina sequencing were demultiplexed by PhD Morten Nilsen, which provided FASTQ-files of *rpoB* sequences without barcodes. These files were processed through the DADA2-pipeline, where several steps were used to create amplicon sequence variants (ASV). These steps include trimming and filtering by quality, where the forward reads were trimmed at position 240 and reverse reads at position 220. Dereplication was performed, where data was condensed by collapsing together all reads which encoded the same sequence. Sequence variants that were not random noise were kept by the function denoising, and errors formed during PCR amplification, chimeras, were removed. Furthermore, a merging of paired forward and reverse reads was performed, merging the reads if they overlapped exactly. The full script for the pipeline can be found in Appendix B. The output from DADA2 was amplicon sequence variant (ASV) profiles of *rpoB* sequencing, which provided information about how many times an ASV was observed in the different samples (Callahan et al., 2016). The profiles were run through the Kraken2 HumGut database by PhD Morten Nilsen, to assign bacterial taxonomy to the different ASV. The file obtained from Kraken was used to assign the sequencing results with their corresponding sample ID's.

2.5.2 QIIME for 16S rRNA sequences

The QIIME pipeline was used to assign taxonomy to 16S rRNA sequences from samples from mothers' stool, meconium, and elution swabs (see the introduction for details). 16S rRNA sequences retrieved from Illumina sequencing were processed before using the QIIME-pipeline. The processing included removal of primers, demultiplexing, and filtering by quality (Sørensen, 2020). Demultiplexing was used to separate sequences with unique indexes in order to map samples back to their origins. An OTU (Operation Taxonomic Unit) table was made with a 97% or higher sequence similarity (Rapin, Pattaroni, Marsland, & Harris, 2017). The SILVA-database was used to assign bacterial taxonomy (Sørensen, 2020). The bacterial

diversity was determined using the QIIME pipeline, and the resulting data were visualized in bar charts.

2.5.3 BLAST for Sanger sequencing

Bacterial species from growing colonies were determined by Sanger sequencing and data processing of the retrieved results. FASTA-files of forward reads were retrieved from Sanger sequencing, and the sequences were identified using Nucleotide BLAST (NCBI).

2.6 Cultivating strategies for Bacteroides

To investigate whether live *Bacteroides* species were found in meconium, several cultivation strategies were used. Strains of *B.ovatus* and *B.vulgatus* were used as positive controls for *Bacteroides* and were used in a pilot study in order to determine whether *Bacteroides* could be cultivated on the chosen mediums. Plates were incubated anaerobically at 37 °C in chambers with 3.5 L AnaeroGen (Oxoid, Thermo Scientific). Colonies growing on mediums were prepared for Sanger sequencing using CoverAll® PCR (16S rRNA gene), or *rpoB*-specific PCR, as well as qRT-PCR. Samples with positive hits for *Bacteroides* were sequenced using Sanger sequencing. The retrieved sequences were identified using Nucleotide BLAST (NCBI). The media and solutions used in cultivation are described in appendix C.

2.6.1 Bile esculin agar (± amikacin)

Meconium samples were diluted from 10^{-1} to 10^{-6} and spread on plates with bile esculin agar BEA (Oxoid, Thermo Scientific), and plates with bile esculin agar with amikacin BBE (VWR, USA). The plates were incubated and monitored for 2-5 days, and cultures growing on the media were selected based on whether a black/brown complex was formed, indicating a hydrolyzation of esculin. One sample was screened for live *Bacteroides* by adding 100 µl of its 10^{-1} dilution to cooked meat medium (Oxoid, Thermo Scientific) before incubation, and performing a PCR before and after incubation. As many colonies from each dilution as possible were picked and placed on a 96-well PCR plate with TE-buffer. The samples were heated up to 99 °C for 10 minutes in order for cell lysis. PCR using *rpob* specific primers (Table 2.1) was used to screen for possible colonies of *Bacteroides*, which were sequenced using Sanger sequencing.

2.7 Statistical approaches and analysis

2.7.1 Handling of 16S rRNA and *rpoB* sequence data

Based on result from 16S rRNA sequencing the relative bacterial composition in all sample categories were studied. Based on *rpoB* sequencing the composition of *Bacteroides* in each sample category were studied, and the abundance of each species of *Bacteroides* was calculated for all sample categories. The abundance of *Bacteroides* species in meconium in connection with sampling time was calculated, as well as the standard error.

2.7.2 Fischer's exact test

The Fischer's exact test was performed to analyze the association between mode of delivery and presence of *Bacteroides* species in mother-child pairs. Before the tests were performed the two vaginal groups were merged, and mother-child pairs were assigned 1 if the *Bacteroides* species was present in both, and 0 if not. The test was performed for the binarized data with a 95% confidence interval ($p < 0.05$).

2.7.3 T-test

Two-tailed t-tests were performed in Microsoft Excel to detect any differences in the content of *Bacteroides* in meconium at the different sampling times with a confidence interval at 95% ($p < 0.05$). The tests were performed with the assumption that there was an unequal variance between the sampling time points.

3.0 Results

3.1 Analysis of 16S rRNA sequencing data to determine overall microbiota composition

3.1.1 Bacterial composition in mother's stool, meconium, infant's skin, and 3 months old children's stool

Samples from meconium, infant's skin, and feces from 3 months old infants, and their corresponding mother's stool were divided into three groups based on delivery mode and the potential presence of *Bacteroides*. This was determined by amplification of the *rpoB* gene, where a positive hit indicated the potential presence of *Bacteroides*. The result was two groups where the infant was delivered vaginally and one group where the infant was delivered by C-section. Therefore, samples from vaginal delivered infants and their mothers with a potential presence of *Bacteroides* will henceforth be referred to as Vaginal A. Samples from vaginal delivered infants and their mothers without a potential presence of *Bacteroides* will henceforth be referred to as Vaginal B. Samples from infants delivered by C-section and their mothers without a potential presence of *Bacteroides* will be referred to as C-section.

Using the QIIME pipeline, sequences of the 16S rRNA gene retrieved from Illumina sequencing were analyzed. For details regarding the QIIME pipeline, see the introduction and materials and methods. The OTU-table yielded from the QIIME pipeline contained information about bacterial abundance from kingdom to genus level.
The bacterial composition based on 16S rRNA data of each sample category from Vaginal A, Vaginal B, and C-section are presented in Figure 3.1.

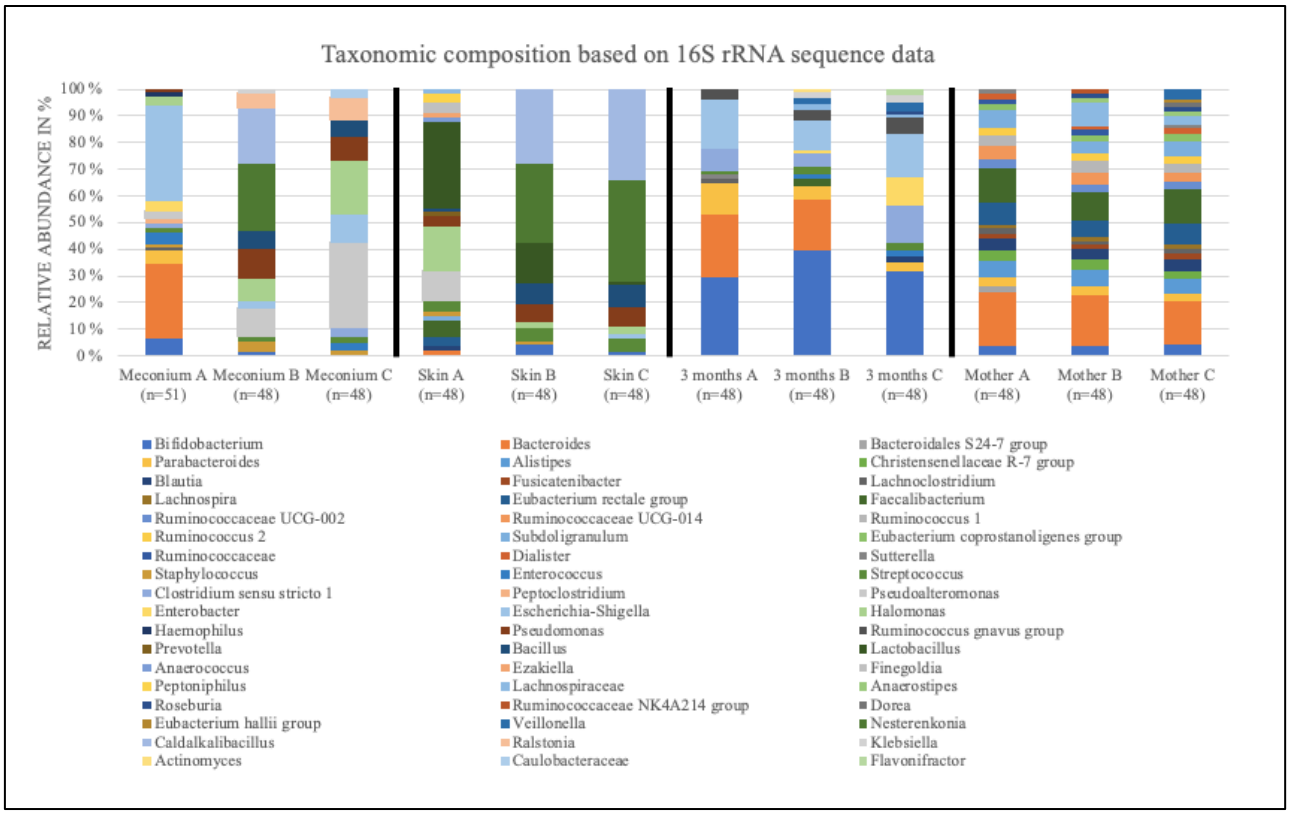

Figure 3.1. Composition of bacterial genus based on 16S rRNA sequence data. Based on 16S rRNA analysis, the average taxonomic composition (genus-level) within the sample categories of the three groups (X-axis) were obtained. The average relative abundance of bacterial genus within each sample category was used for the content of bacterial genus (Y-axis) with a limit of > 1%. The colors indicating the different genus is described at the bottom of the figure. Sample categories marked with A belongs to Vaginal A, categories marked with B belongs to Vaginal B, and categories marked with C belongs to infants delivered by C-section.

The bacterial composition in mothers was similar (Figure 3.1). Mother's stool was dominated by *Bacteroides* (13.4%) and *Faecalibacterium* (8.5%) in samples from mothers who delivered vaginally with potential presence of *Bacteroides* (Vaginal A). Mother's stool from mothers who delivered vaginally without potential presence of *Bacteroides* was also dominated by *Bacteroides* (14.7%) and *Faecalibacterium* (8.4%) (Vaginal B). Lastly, the stool samples from mothers who delivered by C-section were dominated by *Bacteroides* (12.7%) and *Faecalibacterium* (10.2%) as well.

The bacterial composition in the other sample categories varied. The predominant genus in meconium samples from vaginal delivered infants (Vaginal A) was *Escherichia-Shigella* (34%) and *Bacteroides* (26.4%). However, meconium samples from vaginal delivered infants,

without potential presence of *Bacteroides*, was dominated by *Nesterenkonia* (22.6%) and *Caldalkalibacillus* (18.4%) (Vaginal B). Meconium from infants delivered by C-section was dominated by *Pseudoalteromonas* (28.7%) and *Halomonas* (18.4%). Fecal samples from vaginally delivered 3 months old infants with potential presence of *Bacteroides* were dominated by *Bifidobacterium* (26%) and *Bacteroides* (21%) (Vaginal A). Samples from the other vaginal delivered 3 months old infants were dominated by *Bifidobacterium* (37%) and *Bacteroides* (17%) (Vaginal B). However, fecal samples from 3 months old infants delivered by C-section were dominated by *Bifidobacterium* (26.9%) and *Escherichia-Shigella* (13.5%). The bacterial composition in skin samples from vaginal delivered infants with potential presence of *Bacteroides* were dominated by *Lactobacillus* (24.7%) and *Halomonas* (13%) (Vaginal A). The skin composition in the other two groups were dominated by *Nesterenkonia* and *Caldalkalibacillus*. On the skin of vaginal delivered infants, *Nesterenkonia* amounted to 23.4% of the composition and *Caldalkalibacillus* amounted to 22.2% (Vaginal B), while on the skin of infant's delivered by C-section, *Nesterenkonia* amounted to 34.7% and *Caldalkalibacillus* 30.9%.

3.2 Analysis of *rpoB* sequencing data to determine *Bacteroides* composition

3.2.1 Composition of *Bacteroides* in sample categories

Using the DADA2 pipeline, sequences of the *rpoB* gene retrieved from Illumina sequencing were analyzed. For details regarding the DADA2 pipeline, see the introduction and materials and methods. The final output from the DADA2 pipeline was an amplicon sequence variant profile containing 1122 unique ASV's. 91% of all fragments in the amplicon sequence profile belonged to species of *Bacteroides*. In total 22.19% (n=249) ASV's were identified as different species of *Bacteroides.* From these 249 ASV's, the distribution of different *Bacteroides* species was: *B. thetaiotamicron* (12.85%), *B. fragilis* (11.65%), *B. intestinalis* (8.84%), *B. salanitronis* (8.84%), *B. vulgatus* (8.43%), *Bacteroides sp.* (8.03%), *B. dorei* (6.02%), *B. cellulositycus* (5.22%), *B. helcogenes* (5.22%), *B. ovatus* (5.22%), *Bacteroides (unspecified)* (5.22%), *B. caccae* (4.02%), *B. uniformis* (4.02%), *Parabacteroides* (3.21%), *B. caecimuris* (2.81%) and *B. heparinolyticus* (0.40%). In total through this study, 16 different species of *Bacteroides* were identified, where 14 were analyzed further (Figure 3.2).

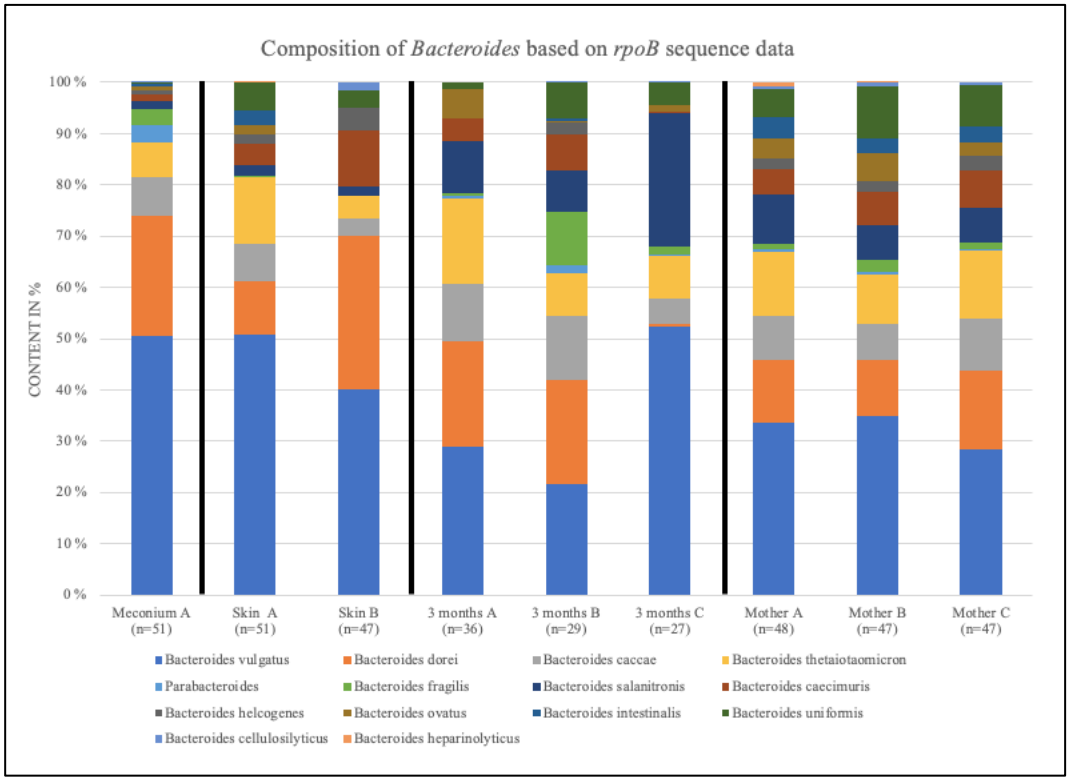

Figure 3.2. Composition of *Bacteroides* species based on *rpoB* sequence data. The relative bacterial abundance is based on the average amount of each *Bacteroides* species in each sample category. The bacterial content in % is presented on the Y-axis, and the sample categories on the X-axis. Sample categories **Mother A**, **Meconium A** and **Skin A** are retrieved from a data set from a previous study (Sørensen, 2020). The colors indicating the different species of *Bacteroides* are presented at the bottom of the figure. Sample categories marked with **A** belongs to **Vaginal A**, categories marked with **B** belongs to **Vaginal B**, and categories marked with **C** belongs to infants delivered by **C-section**.

Composition of *Bacteroides* in samples related to vaginal delivery with potential presence of *Bacteroides* (Vaginal A)

B. vulgatus was the most abundant species of *Bacteroides* in three sample categories from vaginal delivered infants with potential presence of *Bacteroides* (Figure 3.2) (Vaginal A). Both skin (50.6%) and meconium (50.5%) were the sample categories with the highest abundance of *B. vulgatus* of all *Bacteroides* ASV's identified in the sample categories. Additionally, *B. vulgatus* was found in 96% (n=49, of total n=51) of meconium samples within the group (Appendix D, Table D1), and 88% (n=45, of total n=51) of the skin samples (Appendix D, Table D1). Further, *B. vulgatus* was present in all samples from mother's stool (n=48) (Appendix D, Table D1) and represented 33.5 % of the bacterial content in mother's stool with potential presence of *Bacteroides* (Figure 3.2) (Vaginal A). In feces from vaginal delivered 3 months old infants, with potential presence of *Bacteroides*, the most abundant species of *Bacteroides* was *B. vulgatus*, representing 29% of the composition (Figure 3.2) (Vaginal A). Additionally, *B. vulgatus* was found in 44.6% of the samples from 3 months old infants (n=16, of total n=36) (Appendix D, Table D1).

The second most abundant species of *Bacteroides* in samples from vaginal delivered 3 months old infants was *B. dorei*, amounting to 20.5% of the composition of *Bacteroides* (Figure 3.2), and was found in 30.6% of the samples (n=11, of total n=36) (Appendix D, Table D1) (Vaginal A). *B. dorei* was the second most abundant species in meconium, amounting to 23.6% of the composition of *Bacteroides* (Figure 3.2), and was found in 88.2% of the meconium samples (n=45, of total n=51) (Appendix D, Table D1) (Vaginal A). *B. thetaiotaomicron* was the second most dominating species of *Bacteroides* in stool from mothers who delivered vaginally with potential presence of *Bacteroides*, amounting to 12.6% of the composition (Figure 3.2), and was found in 95.8% of the samples (n=46, of total n=48) (Appendix D, Table D1) (Vaginal A). *B. thetaiotaomicron* was also the second most abundant species of *Bacteroides* in samples from skin from vaginal delivered infants with potential presence of *Bacteroides*, amounting to 12.8% of the composition (Figure 3.2). *B. thetaiotaomicron* was found in 52.9% of elution swabs from skin (n=27, of total n=51) (Appendix D, Table D1) (Vaginal A). All *Bacteroides* species were present in the different sample categories, except *B. helcogenes*, *B. heparinolyticus* and *B. cellulositycus*. *B. helcogenes* and *B. cellulositycus* were not present in fecal samples from 3 months old infants, while *B. heparinolyticus* was not present in meconium or on infant's skin (Vaginal A).

Composition of *Bacteroides* in samples related to vaginal delivery without potential presence of *Bacteroides*

From elution swabs from skin of vaginal delivered infants without potential presence of *Bacteroides*, only 1 sample (of total n=47) was sequenced. The results presented hereafter are therefore based on 1 skin sample. The most abundant species of *Bacteroides* on infant's skin was *B. vulgatus* which amounted to 50.6% of the bacterial content on skin (Figure 3.2) and was found in 2.12% of the samples (n=1, of total n=47) (Appendix D, Table D2) (Vaginal B). *B. vulgatus* was the dominating species in the other two sample categories in this group. In mother's stool *B. vulgatus* was found in all samples (n=47) and amounted to 34.8% of the composition (Figure 3.2) (Appendix D, Table D2) (Vaginal B). In feces from vaginal delivered 3 months old infants *B. vulgatus* represented 29.7% of the composition (Figure 3.2) and was found in 46.7% of the samples (n=14, of total n=30).

The second most abundant species of *Bacteroides* in the sample categories was *B. dorei*. *B. dorei* was present in 2.12 % of the skin samples (n=1, of total n=47) (Appendix D, Table D2) and amounted to 30% of the bacterial content on skin (Figure 3.2). In stool from mother's who delivered vaginally, without potential presence of *Bacteroides*, *B. dorei* was found in 97.9% of the samples (n=46, of total n=47) (Appendix D, Table D2) and amounted to 11 % of the composition (Figure 3.2) (Vaginal B). In feces from vaginal delivered infants *B. dorei* represented 20.3 % of the composition (Figure 3.2) and was found in 43.3% of the samples (n=13, of total n=30) (Appendix D, Table D2) (Vaginal B). The third most abundant species in feces from vaginal delivered 3 months old infants was *B. caccae*, found in 40% of the samples (n=12, of total n=30) (Appendix D, Table D2), and amounting to 14.7% of the bacterial content (Figure 3.5) (Vaginal B). The third most abundant species in mother's stool was *B. uniformis* (10.1%) (Figure 3.2) which was found in 97.8% of the samples (n=46, of total n=47) (Appendix D, Table D2) (Vaginal B). All species were present in all sample categories except *B. heparinolyticus, B. intestinalis* and *B. ovatus*. *B. heparinolyticus* was not present on skin or in feces from 3 months old infants (Vaginal B). *B. intestinalis* was not present on infant's skin (Vaginal B).

Composition of *Bacteroides* in samples related to C-section delivery without potential presence of *Bacteroides*

B. vulgatus was the most abundant species in both sample categories from C-section delivery (Figure 3.2). In stool samples from mothers who delivered by C-section, *B. vulgatus* amounted to 28.5% of the composition (Figure 3.2) and was found in 91.5% of the samples (n=43, of total n=47) (Appendix D, Table D3). In feces from C-section delivered 3 months old infants, *B. vulgatus* represented 52.4% of the composition (Figure 3.2) and was found in 11% of the samples (n=3, of total n=27) (Appendix D, Table D3).

In stool from mothers who delivered by C-section the second most abundant species of *Bacteroides* was *B. dorei* (Figure 3.2). *B. dorei* was present in 91.5% of stool samples from mothers who delivered by C-section (n=43, of total n=47) (Appendix D, Table D3) and represented 15.4% of the composition (Figure 3.2). In feces from C-section delivered infants, the second most abundant species were *B. salanitronis* which amounted to 26.2% of the composition (Figure 3.2) and was found in 18.5% of the samples (n=5, of total n=27) (Appendix D, Table D3). The third most abundant species in feces from C-section delivered infants was *B. thetaiotaomicron* which represented 8.3% of the composition (Figure 3.2) and was found in 11% of the samples (n=3, of total n=27) (Appendix D, Table D3).
All species of *Bacteroides* were found in both mothers and 3 months old infants, except *B. heparinolyticus* and *B. intestinalis. B. heparinolyticus* was not found in either mother's stool or feces from 3 months old infants, and *B. intestinalis* was not found in feces from 3 months old infants.

3.2.2 Presence of *Bacteroides* species in mother-child pairs

In this study, from the 144 mother-child pairs available, 138 complete mother-child pairs were analyzed. Complete pairs mean information about meconium, E-swabs (skin), feces from 3 months old infants, and mother's stool are available from Vaginal A, Vaginal B, and C-section delivery. First, mother's stool, meconium and skin were analyzed, and feces from 3 months old infants were excluded from this analysis. From samples related to vaginal delivery with potential presence of *Bacteroides*, 48 complete mother-child pairs were analyzed. From samples related to vaginal delivery without potential presence of *Bacteroides*, 47 complete mother-child pairs were analyzed, and lastly 43 complete mother-child pairs from C-section

delivery. Another analysis was performed using mother's stool and feces from 3 months old infants from Vaginal A (n=36), Vaginal B (n=30), and C-section delivery (n=27).

Mother's stool, meconium, and skin

The presence of *Bacteroides* species in each group was studied (Figure 3.3, Appendix E, Figure E1 and E2). Sequences within each sample category were binarized, either representing the presence or absence of the given *Bacteroides* species. Further, the presence or absence were calculated within each sample category in all groups indicated in their respective figure legends (Figure 3.3, Appendix E, Figure E1 and E2). Complete mother-child pairs, indicating the presence of the given *Bacteroides* species in all sample categories is shown as the category "All".

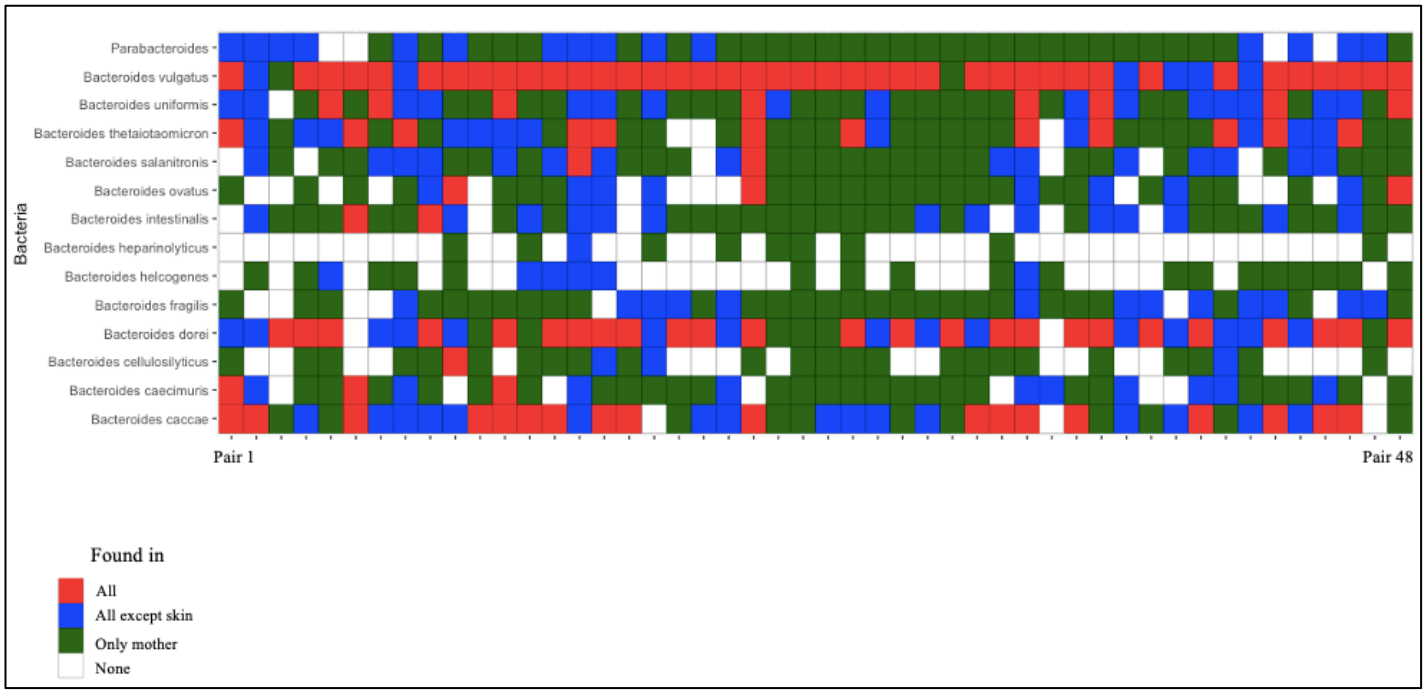

Figure 3.3. Presence of *Bacteroides* species in 48 mother-child pairs related to vaginal delivery with potential presence of *Bacteroides* (Vaginal A). The figure illustrates the presence of different *Bacteroides* species found in mother's stool, meconium and on newborn's skin. Complete mother-child pairs are presented on the X-axis and the different *Bacteroides* species are presented on the Y-axis. The colors coding for the different categories are given on the legend at the bottom left of the figure.

The species dominating within the 48 complete mother-child pairs from samples related to vaginal delivery and potential presence of *Bacteroides* was *B. vulgatus* (Vaginal A), as shown in Figure 3.3. The second and third most dominating species were *B. dorei* and *B. caccae*. The species found in the least mother-child pairs were *B. heparinolyticus* and *B. helcogenes*. The

presence of *Bacteroides* species were varying in the complete mother-child pairs, and most *Bacteroides* found only in one category was in mother's stool. In samples related to vaginal delivery without potential presence of *Bacteroides* (Vaginal B), there was only one of the 47 mother-child pairs where *Bacteroides* species were found in other samples than mother's stool (Appendix E, Figure E1) (Vaginal B). In samples from C-section delivery, species of *Bacteroides* were only found in samples from mother's stool in the 43 mother-child pairs (Appendix E, Figure E2).

Based on *rpoB* sequencing data the presence of *Bacteroides* species in mother's stool, meconium, and skin swabs from vaginal delivered infants were analyzed to determine an association of *Bacteroides* with mother or mother-child association (Vaginal A). Five species of *Bacteroides* were selected for study: *B. vulgatus, B. dorei, B. fragilis, B. uniformis* and *B. caecimuris*. The results presented as Venn diagrams are found in Figure 3.4A-E. *B. vulgatus* was most abundant of the *Bacteroides* species, and was found in all mother's stool samples, 45 meconium samples (of total n=48) and 39 of the skin swabs samples (of total n=48) (Figure 3.4A). In total, *B. vulgatus* was found in all three sample categories in 39 mother-child pairs (of total n=48) and was associated with mother-child pairs (Figure 3.4A-E). *B. fragilis* was not found in skin samples from infants and was only found in 20 meconium samples (of total n=48) (Figure 3.4C). Additionally, *B. fragilis* was found in 41 samples from mother's stool (of total n=48) and showed a stronger association with mother (Figure 3.4C).

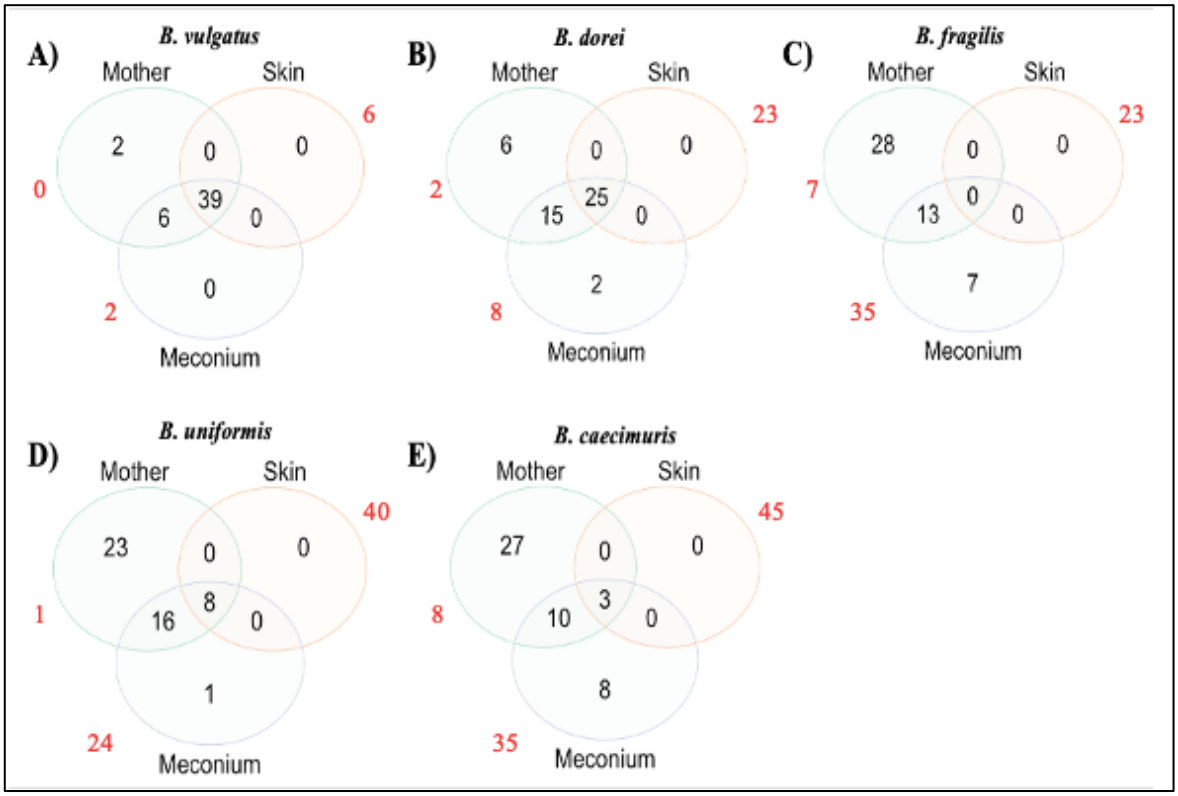

Figure 3.4A-E. Presence of *Bacteroides* in samples from mother's stool, meconium, and infant's skin. The presence of *Bacteroides* species in sample categories from vaginal delivery with an amplification of *rpoB* was binarized, representing either presence or absence of the given species. Mother-child pairs were analyzed together to determine the number of shared species in the pairs, and the number of shared species between sample categories, presented as Venn diagrams. **A)** *B. vulgatus* **B)** *B. dorei* **C)** *B. fragilis* **D)** *B. uniformis* and **E)** *B. caecimuris*. The number of pairs where species of *Bacteroides* were either not present in mother's stool, meconium or elution swabs from infant's skin are marked with numbers in red next to the sample category.

Fischer's exact test was performed to test the association between mother and child, based on mode of delivery and presence of *Bacteroides* in mother-child pairs. The test was performed using information from mother's stool samples, meconium, and samples from infant's skin. The two vaginal groups were merged, and the association between mode of delivery and presence of *Bacteroides* was tested on three species of *Bacteroides*: *B. vulgatus, B.dorei* and *B. caecimuris*. Results from Fischer's exact test indicated a significant association ($p < 0.05$) between *B. vulgatus* and vaginal delivery ($p = 1.0e\text{-}5$) and a significant association between *B. dorei* and vaginal delivery ($p = 1.0e\text{-}4$). There was not a significant association between *B. caecimuris* and vaginal delivery ($p = 0.552$). The results from these tests indicate that *B. vulgatus* and *B. dorei* are associated to vaginal delivery, but not *B. caecimuris*. All p-values from Fischer's exact test are presented in Appendix F, Table F1.

Mother's stool and feces from 3 months old infants

The presence of *Bacteroides* species in all groups were studied (Figure 3.5 and 3.6, Appendix E, Figure E3). The sequences were binarized, indicating presence or absence of the given Bacteroides species. Further, the presence or absence were calculated within each sample category in all groups indicated in their respective figure legends (Figure 3.5, 3.6, Appendix E, Figure E3). Mother-child pairs where *Bacteroides* was present in both mother and child is shown as the category "Mother and 3 months".

The species of *Bacteroides* which was the most abundant in both mother's stool and feces from 3 months old vaginal delivered infants was *B. vulgatus*, followed by *B. dorei* and *B. thetaiotaomicron*, as shown in Figure 3.5 (Vaginal A). *B. vulgatus* was found in both mother and 3 months old infant in 16 pairs (of total n=36), while *B. dorei* was found in 11 pairs (of total n=36) and *B. thetaiotaomicron* in 10 pairs (of total n=36) (Vaginal A). Three species were only found in feces from 3 months old infants and not in mother's stool: *B. ovatus* (n=1, of total n=36), *B. dore* (n=1, of total n=36), and *B. caecimuris* (n=1, of total n=36). *B. heparinolyticus* was found only in 6 of mother's stool samples. *B. helcogenes, B. heparinolyticus* and *B. cellulositycus* was not found in feces from 3 months old infants.

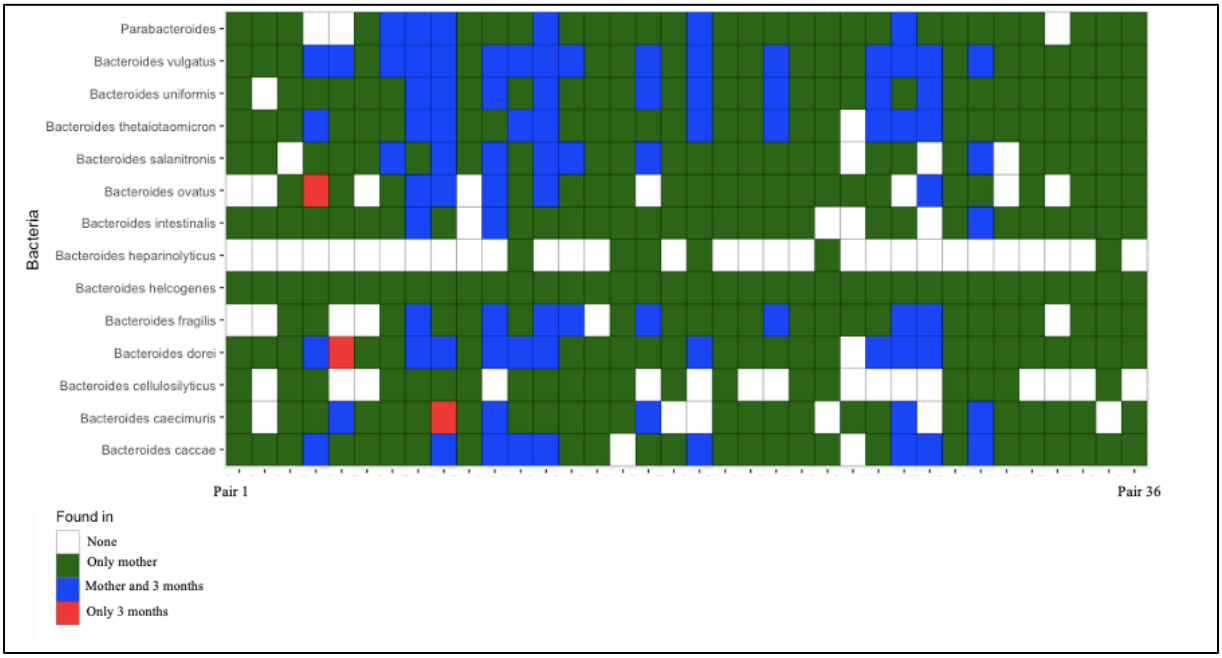

Figure 3.5. Presence of *Bacteroides* species in 36 mother-child pairs from samples related to vaginal delivery with potential presence of *Bacteroides* (Vaginal A). The figure illustrates the presence of different *Bacteroides* species found in mother's stool and feces from 3 months old infants. Mother-child pairs with data available from both mother's stool and feces from 3 months old infants are presented on the X-axis and the different *Bacteroides* species are presented on the Y-axis. The colors coding for the different categories are given on the legend at the bottom left of the figure.

The species of *Bacteroides* found most often in samples related to vaginal delivery without potential presence of *Bacteroides* (n=30) was *B. vulgatus*, followed by *B. thetaiotaomicron, B. dorei* and *B. uniformis* (Figure 3.6) (Vaginal B). *B. vulgatus* was found in samples from both mother's stool and feces from 3 months old infants in 14 mother-child pairs (of total n=30) (Figure 3.6). *B. thetaiotaomicron, B. dorei* and *B. uniformis* were found in 12 pairs (of total n=30). All bacteria were found in mother's stool and feces from 3 months old infants, except for *B. heparinolyticus* which was found in only in one sample from mother's stool. *B. helcogenes, B. ovatus* and *B. cellulosilyticus* were found in only one mother-child pairs.

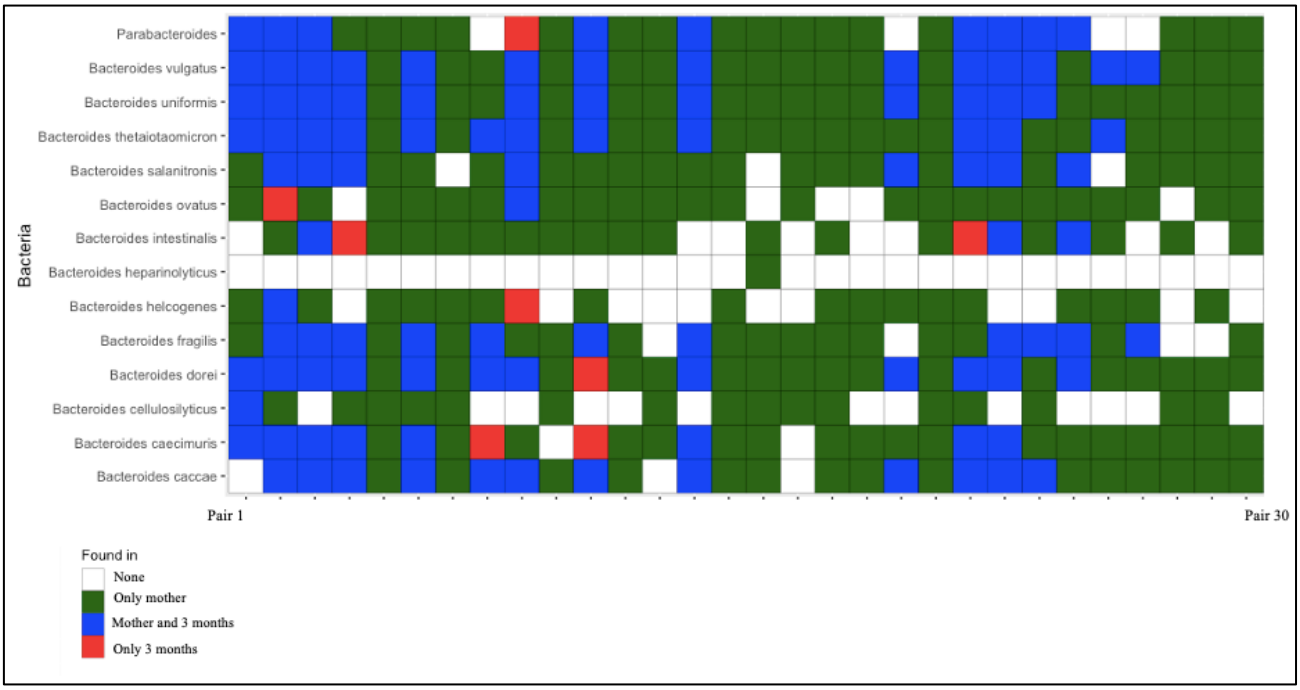

Figure 3.6. Presence of *Bacteroides* species in 30 mother-child pairs from samples related to vaginal delivery without potential presence of *Bacteroides* (Vaginal B). The figure illustrates the presence of different *Bacteroides* species found in mother's stool and feces from 3 months old infants. Mother-child pairs with data available from both mother's stool and feces from 3 months old infants are presented on the X-axis and the different *Bacteroides* species are presented on the Y-axis. The colors coding for the different categories are given on the legend at the bottom left of the figure.

The most abundant species of *Bacteroides* in samples related to C-section delivery were *B. dorei, B. caecimuris* and *B. salanitronis* which were found in both mother's stool and feces from 3 months old infants in 5 mother-child pairs (of total n=27) (Appendix E, Figure E3). *B. fragilis, B. uniformis, and B. caccae* were found in 4 mother-child pairs (of total n=27) (Appendix E, Figure E3). *B.vulgatus* was found in 3 mother-child pairs (of total n=27). (Appendix E, Figure E3). Three species of *Bacteroides* were found in only feces from 3 months old infants: *B. cellulositycus, B. helcogenes,* and *B. heparinolyticus* (Appendix E,

Figure E3). *B. heparinolyticus* was found in only feces from 3 months old infants in two infants.

Based on *rpoB* sequencing data the presence of *Bacteroides* species in mother's stool and feces from 3 months old vaginal delivered infants (Vaginal A and Vaginal B), and C-section delivered infants were analyzed to determine an association of *Bacteroides* with mother or mother-child association. Three species of *Bacteroides* were selected for analysis: *B. vulgatus, B. dorei* and *B. uniformis*. The most abundant species of *Bacteroides* shared between mother and 3 months old infants was *B. vulgatus* (Figure 3.7A), which was shared between 16 vaginal delivery pairs with potential presence of *Bacteroides* (of total n=36) (Vaginal A) and 14 vaginal delivery pairs without potential presence of *Bacteroides* (of total n=30) (Figure 3.7A) (Vaginal B). *B. vulgatus* was present in 3 mother-child pairs from C-section delivery (of total n=27) (Figure 3.7A). *B. dorei* was present in both mother's stool and vaginal delivered 3 months old infants in 10 mother-child pairs from Vaginal A (of total n=36), and 12 pairs from Vaginal B (of total n=30) (Figure 3.7B). *B. dorei* was not present in mother's stool in one mother-child pair from Vaginal A (of total n=30) (Figure 3.7B). In C-section delivered 3 months old infants and mother's stool, *B. dorei* was present in 5 mother-child pairs (of total n=27) (Figure 3.7B). In C-section delivered 3 months old infants, *B. uniformis* was present in 4 mother-child pairs (of total n=27) (Figure 3.7C). *B. uniformis* was present in 9 mother-child pairs from Vaginal A (of total n=36) and was not present in 1 sample from mother's stool (of total n=36) (Figure 3.7C). *B. uniformis* was present in 12 mother-child pairs from Vaginal B (of total n=27) (Figure 3.7C).

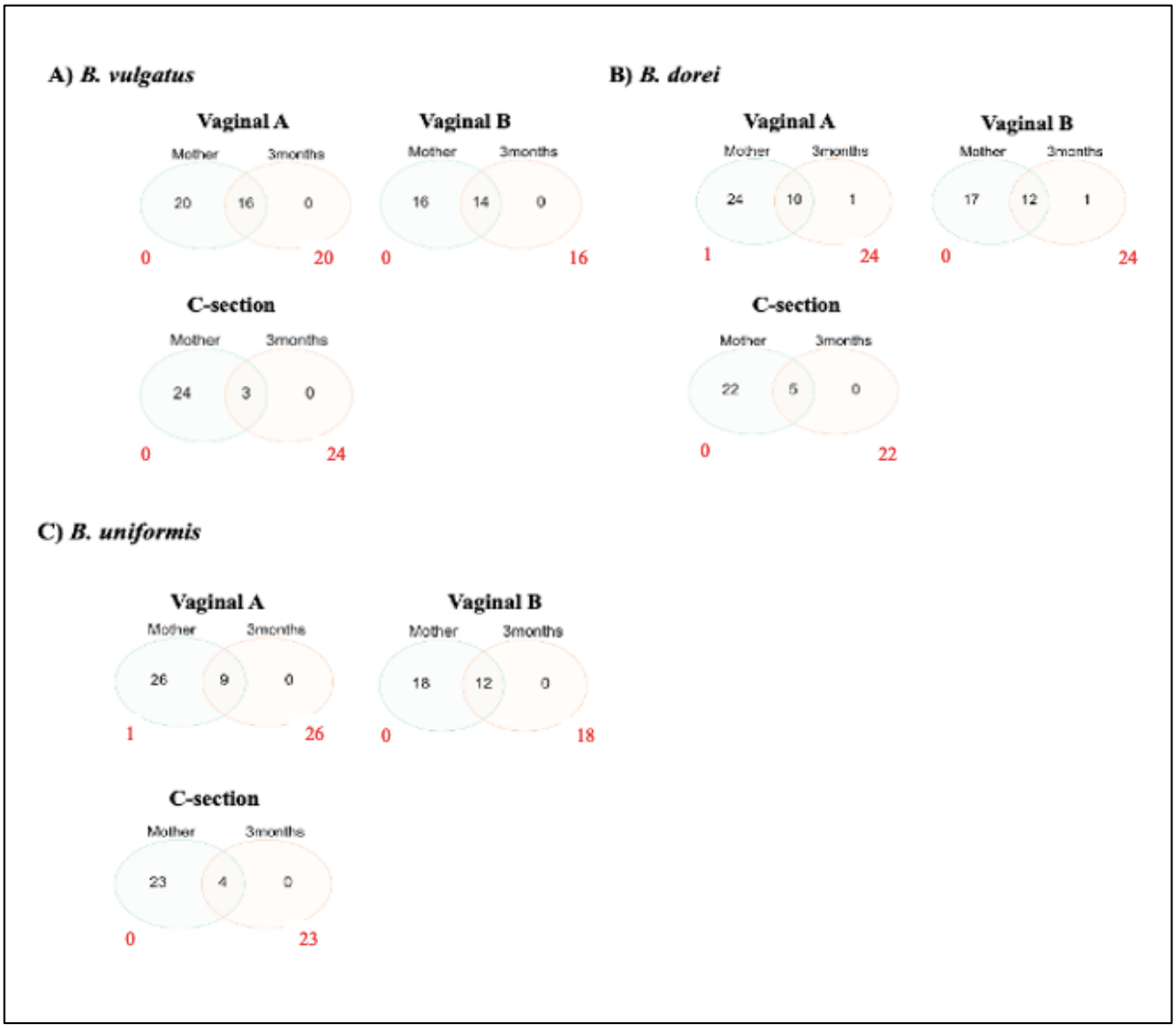

Figure 3.7A-C. Presence of *Bacteroides* in samples from mother's stool and feces from 3 months old infants. The presence of *Bacteroides* species in sample categories from all delivery groups were binarized, representing either presence or absence of the given species. Mother-child pairs were analyzed together to determine the number of shared species in the pairs, presented as Venn diagrams. **A)** *B. vulgatus* **B)** *B. dorei* and **C)** *B. uniformis*. The number of pairs where species of *Bacteroides* were either not present in mother's stool or feces from 3 months old infants are marked with red numbers next to the sample category.

Fischer's exact test was performed to test the association between mother and child, based on mode of delivery and presence of *Bacteroides* in mother-child pairs. The test was performed using information from mother's stool samples and fecal samples from 3 months old infants. The two vaginal groups were merged, and the association between mode of delivery and presence of *Bacteroides* was tested on 14 species of *Bacteroides.* Results from Fischer's exact test indicated a significant association ($p < 0.05$) between *B. vulgatus* and vaginal delivery ($p = 2.3e\text{-}3$) and a significant association between *B. dorei* and vaginal delivery ($p = 0.01$). The results from these tests indicate that *B. vulgatus* and *B. dorei* are associated between mode of

delivery and presence of *Bacteroides.* There was no significant association between mode of delivery and the remaining species of *Bacteroides*. All p-value results from Fischer's exact tests are presented in Appendix F, Table F1.

3.3 Presence of Bacteroides in meconium in connection with collection time

The collection time for meconium was registered for most samples. Of all meconium samples included in the main study, the collection time was registered for 65.73% (n=305, of total n=464) of the samples. Therefore, the results presented hereafter are based on meconium samples with registered collection time. Most meconium samples were registered within 24 hours after birth (70.8%, n=216). Further, 21.3% (n=65) were collected 24-48 hours after birth, and 7.8% (n=24) were collected later than 48 hours after birth. All meconium samples with registered sampling time points were from vaginal delivered infants with potential presence of *Bacteroides* (Vaginal A). The collection time for meconium samples from Vaginal A (n=51), was registered for 74.5% (n=38) of all samples. Of the *Bacteroides*-positive meconium samples 28.9% (n=11) were collected within 24 hours, 52.6% (n=20) were collected between 24 and 48 hours, and 18.4% (n=7) were collected after 48 hours. The abundance of *Bacteroides* species were studied in connection to meconium collection time (Figure 3.8).

Bacteroides species which amounted to more than 5% of the composition in meconium at the different sampling times are presented in Figure 3.8. *B. vulgatus* was the predominant species at all sampling time points, with the highest abundance within 24-48 hours (Figure 3.8). *B. dorei* was the second most abundant species in all sampling time points, with the highest abundance in samples collected within 24 hours. The percentages of *Bacteroides* species at each sample time are presented in Appendix G, Table G1. Two-tailed t-tests were performed to detect any differences in the content of *Bacteroides* in meconium at the different sampling times with a confidence interval at 95% ($p < 0.05$). The results from two-tailed t-test indicated no difference in the content of *Bacteroides* at different sampling points, except for *B. cellulositycus* ($p = 0.019$) when comparing the sampling points between 24-48 hours and over 48 hours (Appendix G, Table G2). Additionally, the content of *B. cellulositycus* did not amount to more than 5% of the composition in meconium at any of the sampling time points and is therefore not presented in Figure 3.8. All results from two-tailed t-test are presented in Appendix G, Table G2.

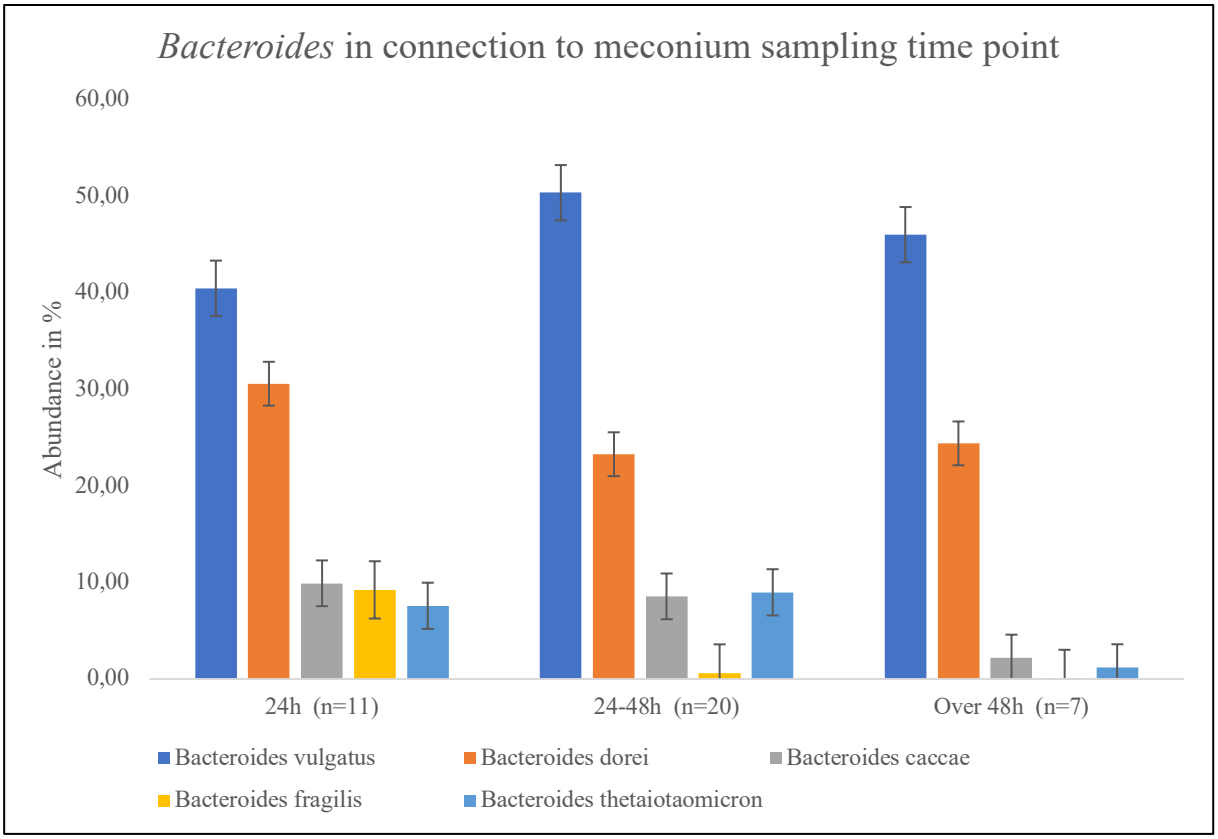

Figure 3.8. Composition of *Bacteroides* in connection with meconium sampling time points. The numbers are based on average values of *Bacteroides* in each sampling time point. Standard error of the data is shown in each bar. The different registered sampling time points for meconium are shown on the X-axis, and the abundance of *Bacteroides* species in % is shown on the Y-axis. The colors indicating the different species of *Bacteroides* are given in the figure legend.

The species of *Bacteroides* which amounted to more than 5% of the composition in meconium were studied in connection with their presence in corresponding skin samples (Appendix G, Figure G1, G2 and G3). *B. vulgatus* was in total found in 84.2 % of the skin samples (n=32, of total n=38) corresponding with meconium samples from all collection sampling time points. Moreover, *B. vulgatus* was most abundant on skin of infants when the meconium collection time was between 24-48 hours (n=19, of total n=20) (Figure G2). *B. dorei* was the second most abundant species on skin and was in total found in 60.5% of the skin samples (n=23, of total n=38). Additionally, *B. dorei* was also most abundant on skin of infants when the meconium collection time was between 24-48 hours (n=15, of total n=20) (Figure G2). The abundance of *Bacteroides* species in the different sampling time points connected with the presence of *Bacteroides* species are presented in Appendix G, Figure G1, G2 and G3).

3.4 Cultivating and isolating *Bacteroides*

Based on *rpoB* sequencing data, 3 meconium samples with an amplification of *rpoB* were selected for cultivation to determine if live species of *B. vulgatus* were found in meconium. The samples were selected due to their high abundance of *Bacteroides* and were the samples with the highest percentage of *B. vulgatus* of the meconium samples with an amplification of *rpoB*. The abundance of *B. vulgatus* in each sample based on *rpoB* sequencing data in percent (%) is listed in Table 3.1. Selective media was used to cultivate *Bacteroides*: Bile Esculin agar (BEA) and *Bacteroides* Bile Esculin agar (BBE). 24 colonies from sample 1 were sequenced, and 75 colonies from sample 3 (Appendix H, Table H1). Sample 1 showed no growth on either medium after 5 days of incubation. Two species from growing colonies were identified as species of *Bacteroides*: *B. ovatus* and *B. dorei* (Table 3.1, Appendix G, Table G1). One species were identified as *Klebsiella* (Table 3.1, Appendix G, Table G1). The species were identified with BLAST, and the query coverage was >97% for all bacterial species. However, *B. vulgatus* was not identified in either of the samples (Table 3.1).

Table 3.1. Based on *rpoB* sequencing data, the percentage of *B. vulgatus, B. ovatus* and *B. dorei* was calculated in 3 meconium samples and selected for cultivation of *B. vulgatus*. After cultivation of 3 meconium samples on BBE and BEA mediums, 2 species of *Bacteroides* and one species of *Klebsiella* was cultivated. NA indicates no growth.

Meconium samples	**% Abundance of *B. vulgatus***	**% Abundance of *B. ovatus***	**% Abundance of *B. dorei***	**Bacteria cultivated**
Sample 1	99.81	0.00	0.00	NA
Sample 2	92.80	0.02	1.67	*B. ovatus* *B. dorei*
Sample 3	79.04	6.45	0.04	*Klebsiella*

4.0 Discussion

4.1 Transmission of *Bacteroides* from mother to child

4.1.1 Birth canal as infants first bacterial exposure site

An infant's first exposure to microbes is during its descent through the birth canal, where they are exposed to their mother's vaginal and fecal microbes (Moore & Townsend, 2019). Women's vaginal microbiota is dominated by *Lactobacillus*, therefore the skin microbiota of vaginal delivered children are often dominated by *Lactobacillus* (Moore & Townsend, 2019). Based on 16S rRNA sequencing data, the dominating group on newborn's skin varied between vaginal delivered infants and C-section delivered infants in this study. Vaginal delivered newborn's skin with potential presence of *Bacteroides* was dominated by *Lactobacillus* (Vaginal A), while in the other vaginal group and C-section delivered infants, *Lactobacillus* was present in lower abundances. Vaginal delivered newborn's skin (Vaginal B) and C-section delivered newborn's skin were dominated by *Nesterenkonia* and *Caldalkalibacillus*. Vaginal delivered infants are expected to have a greater similarity to their mother's vaginal microbiota than infants delivered by C-section (Blaser & Dominguez-Bello, 2016). The origin of *Lactobacillus* found on newborn's skin in this study is most likely from their mother's vaginal microbiota. However, as the vaginal microbiota in mothers was not investigated in this study, this is not confirmed. Additionally, the dominating presence of *Nesterenkonia* and *Caldalkalibacillus* on newborn's skin in this study is most likely from contamination, and the data retrieved from these samples may not be an accurate representation of the actual skin microbiota in C-section delivered infants. Strains of these bacterial species have been observed as a contaminant in several Taq polymerases used in PCR-reactions (Iulia, Bianca, O., & Octavian, 2013). The number of cycles for 16S rRNA in this study was 30, which could result in more contaminations.

4.1.2 Differences in bacterial exposure due to mode of delivery

Mother's gut is the largest bacterial reservoir for potential transmission, and colonization of microbiota from mother's gut is more persistent over time compared to maternal vaginal-, skin-, and oral microbiota (Bäckhed et al., 2015). Therefore, the lack of exposure to maternal fecal microbiota may delay or disrupt the colonization of gut bacteria associated with a healthy gut flora, such as *Bacteroides*. Children delivered by C-section have been reported to have a lower diversity in gut flora, which have been linked to a larger chance of developing immune mediated diseases (Jakobsson et al., 2014).

The gut microbiota of children delivered by C-section resembles maternal skin microbiota and has shown to harbor less *Bacteroides* than children delivered vaginally (Eck et al., 2020; Wang et al., 2020). However, in this study, mother's skin microbiota was not studied and similarity between mother's skin and C-section delivered infant's gut microbiota could not be examined. In this study, vaginal delivered infant's where *Bacteroides* was present on skin, and in meconium and feces from 3 months old infants, represented the majority. In infants delivered by C-section, *Bacteroides* species were not found on either skin or in meconium, which strengthens the assumption that exposure to maternal vaginal and fecal microbiota is necessary for transmission of *Bacteroides*. In this study, results from statistical analyzes indicated a significant association between presence of *Bacteroides* species such as *B. vulgatus*, and vaginal delivery.

In C-section delivery, only mother's stool and feces from 3 months old infants showed a presence of *Bacteroides*. Additionally, in this study, the lack of *Bacteroides* species in meconium and skin from C-section delivered infants further supports the assumption that *Bacteroides* is not transmitted from mother to child by C-section delivery.

Although *Bacteroides* species were present in feces from C-section delivered 3 months old infants, there is a possibility of other factors than the maternal gut microbiota being the source of *Bacteroides*. Mother's vaginal, skin, and oral microbiota have been identified as the origin of strains found in infant's gut (Ferretti et al., 2018). Studies have shown that one of the earliest transmission routes of microbiota, other than from mother to child, is exposure to surfaces in hospitals such as stethoscopes and pacifiers (Moore & Townsend, 2019). Stools from mothers who delivered by C-section had a high abundance of *Bacteroides*, indicating the potential for transmission from mother to child. Interestingly, studies have reported a difference in the development of gut microbiota between elective and emergency C-sections (Hoang, Levy, & Vandenplas, 2021). The rupture of the amniotic sac was defined as one of the key differences, which was more common in emergency C-sections. The rupture of the amniotic sac resulted in higher richness and diversity in the gut microbiota in emergency C-section delivered infants compared to elective (Hoang et al., 2021). However, the study did not indicate whether rupture of the amniotic sac influenced the colonization of *Bacteroides*. Moreover, the delayed colonization by *Bacteroides* in C-section delivered infants and the subsequent lower diversity in gut microbiota resulted in higher abundance of opportunistic pathogens related to healthcare environment (Shao et al., 2019). In this study, rupture of the

amniotic sac was not studied, therefore the potential difference in gut microbiota composition in C-section delivered infants could not be examined and should be studied further.

4.1.3 Vertical transmission of *B. vulgatus* from mother to child

The significant association between *B. vulgatus* and vaginal delivery indicates vertical transmission of the species from mother to child. Several studies have indicated a vertical transmission of *B. vulgatus* from mother to child (Ferretti et al., 2018; Li et al., 2020; Yassour et al., 2018). Additionally, *B. vulgatus* have been shown to persist from birth up until 4 months of age (Ferretti et al., 2018). A newly created database, HumGut, indicates that *Bacteroides* species are the most prevalent genomes in the human gut, of which *B. vulgatus* lies at the top (Hiseni et al., 2021). In this study, the high abundance of *B. vulgatus* in all sample categories from vaginal delivered infants indicates vertical transmission of *B. vulgatus* from mother to child based on *rpoB* sequencing data.

4.2 Presence of Bacteroides in mothers and their children

Some species of *Bacteroides* were abundant in mother-child pairs, particularly in samples from vaginal delivered infants and their mothers (Vaginal A). Especially *B. vulgatus*, *B. dorei* and *B. caccae* were most often observed in both mother's stool and all sample categories from their respective children in this group. In vaginal delivered infants, without potential presence of *Bacteroides, Bacteroides* was mainly found in only mother's stool, except for one mother-child pair where species of *Bacteroides* was found on newborn's skin (Vaginal B).

B. vulgatus and *B. dorei* have been linked to mother-child association in several studies (Bäckhed et al., 2015; Ferretti et al., 2018; Yassour et al., 2018). However, some vaginal delivered infants have shown to harbor a less diverse gut microbiota more similar to infants delivered by C-section, which cannot be explained by technical variation or variables (Wang et al., 2020). This may be a possible explanation for the absence of *Bacteroides* in meconium and skin from vaginal delivered infants without potential presence of *Bacteroides.* Proper fecal microbiota seeding from mother's stool during vaginal delivery have been shown to be significant in establishing growth of beneficial bacteria in the infant gut (Reyman et al., 2019). Additionally, bacteria associated with mother's gut, such as *Faecalibacterium*, were only present in feces from vaginal delivered 3 months old infants in this study. The absence of

Faecalibacterium in C-section delivered 3 months old infants further highlights the importance of proper fecal microbiota seeding from mother's stool for beneficial gut colonization in infants (Reyman et al., 2019).

Fecal samples from 3 months old infants showed a presence of *Bacteroides* in samples from all delivery groups, with the highest abundance in vaginal delivery. Therefore, *Bacteroides* may have colonized the infant gut after birth, but the source and time of transmission is uncertain. However, both *B. vulgatus* and *B. dorei* showed a positive association between presence of *Bacteroides* in mother's stool and fecal samples from 3 months old infants, and vaginal delivery. This could indicate a mother-child transmission by vaginal delivery, where *B. vulgatus* and *B. dorei* were able to colonize the infant gut, as both species were found in meconium.

The relative abundance of *Bacteroides* in meconium from vaginal delivered infants (Vaginal A), was shown to decrease over time, except for *B. vulgatus* and *B. thetaiotaomicron*. The presence of these two species increased from two sampling time points, within 24 hours and between 24-48 hours, and the relative abundance of *B. vulgatus* and *B. thetaiotaomicron* decreased when the sampling time point was over 48 hours after birth. *B. dorei*, as the second most abundant species of *Bacteroides* in meconium, decreased from the 1st sampling point to the 3rd. The lower abundance between sampling time points could indicate colonization of bacteria who may compete with *Bacteroides* species for nutrients. A study performed by Yassour et al. (2018) reported a colonization of the infant gut by mother's non-dominating strains. These non-dominating strains harbor a starch utilizing system enabling them to utilize starch and colonize the gut, which the dominating strains do not have (Yassour et al., 2018). Secondary strain transmission of *B. dorei* from mothers have been observed more often than their dominant strain (Yassour et al., 2018). However, the study did not investigate bacterial traits or presence of non-dominating strains over a longer period. Therefore, inheritance of mother's non-dominating strains such as *B. dorei* cannot be described.

4.2.1 Indication of live bacteria in meconium

Results from cultivation indicates presence of live bacteria in meconium. In this study, analysis of 16S rRNA sequencing data indicated presence of genus *Escherichia-Shigella* and *Bacteroides*. Analysis of *rpoB* sequencing data indicated presence of *Bacteroides* species such

as *B. vulgatus*, *B. dorei*, and *B. thetaiotaomicron* in meconium. Furthermore, there was indication of bacterial growth, as *B. ovatus*, *B. dorei* and *Klebsiella* were identified from growing colonies. The stool samples from corresponding mothers had a high abundance of *Bacteroides*, particularly *B. vulgatus* and *B. dorei*. Therefore, the presence of *B. dorei* in both mother's stool and meconium indicates a vertical transmission of *B. dorei* from mother to child. *B. ovatus* was present in both mother's stool and meconium, indicating a vertical transmission from mother to child, but had a lower abundance than *B. dorei*. However, a study performed by Li et al. (2020) indicated that species which are vertically transmitted from mother to child are not always the species with a high abundance in mothers. Since *B. vulgatus* was present in all meconium samples but did not grow on either medium used in this study, a conclusion regarding living cells of *B. vulgatus* in meconium could not be drawn. *B. vulgatus* may have been outcompeted by other bacterial species during the cultivation process, or the mediums used may not have been optimal for growth of *B. vulgatus*. However, this was not confirmed, and should be investigated further. A study done by Lee et al., (2013) described the presence of a conserved class of polysaccharide utilization loci encoding a starch utilization system in *B. fragilis* (*ccf*) and proposed that this system may be present in other species of *Bacteroides.* A potential presence of this loci in *B. dorei* or *B. ovatus* could explain why these species were able to grow on the mediums used in this study despite being much lower in abundance than *B. vulgatus*. BBE-mediums used in this study contains starch in the form of papaic digest of soybean meal (Appendix C), which the potential starch utilization system in *Bacteroides* species could have utilized. However, presence of this loci was not examined in this study and would need to be investigated further.

4.3 Methodological considerations

4.3.1 Extraction of DNA from samples with low biomass

Samples from meconium, skin, and feces from 3 months old infants contain less bacterial DNA than samples from mother's stool. The low biomass in these samples, along with other factors, may influence the quality of DNA extracted. Freezing conditions and preservation mediums have been identified as possible sources of variation in microbial composition in stool samples (Antosca et al., 2020). Meconium could contain PCR inhibitors such as urea, glycolipids, and bile salts and acids (Stinson, Keelan, & Payne, 2018). Additionally, meconium could be difficult to dissolve due to its varying texture. Extracting a representative

amount of DNA from poorly dissolved samples adds further barriers for efficient DNA extraction (Stinson et al., 2018). In this study, a combination of glass beads was used for mechanical lysis in DNA extraction (see material and methods for more information). A combination of beads is more efficient than using only one type, and leads to a higher diversity and less bias when cells are mechanically lysed (Bakken & Frostegard, 2006). However, the chance of aggravating the DNA is higher (Bakken & Frostegard, 2006). Low amounts of DNA in samples are often combined with a high amount of contamination and could potentially detect more false positives (Kuperman et al., 2020). Therefore, inclusion of controls is important to reduce the risk of false positives. In this study, negative controls were evaluated after PCR reactions and after each step in sequencing library preparations. Negative controls with positive hits for *rpoB* were included in sequencing to determine possible sources of contamination. Analysis of sequencing data determined the contamination in negative controls did not affect the results in this study. Based on negative controls in 16S rRNA sequencing, potential contaminants were removed from the 16S rRNA data set.

4.3.2 Sequence analysis of the 16S rRNA gene

The 16S rRNA gene has been a pillar in bacterial analysis of microbial composition for decades (Johnson et al., 2019). Sequencing data from 16S rRNA analysis provides information about bacterial diversity between and within samples on genus level. 16S rRNA sequencing of samples from mothers and their children made it possible to determine bacterial overlap of *Lactobacillus* from mother to child. Additionally, 16S rRNA sequencing revealed the dominance of *Bacteroides* in mother's stool, and therefore identified mother's gut as a large reservoir for vertical transmission of *Bacteroides* from mother to child. However, on lower taxonomic levels, species such as *Bacteroides* are not separated due to taxonomic similarity between closely related species (Ravi et al., 2018). Therefore, 16S rRNA sequence analysis was not suited for determining bacterial overlap between mother and child on species level. Additionally, 16S rRNA sequencing could not determine the dominant species of *Bacteroides* in mother's stool, or in samples from infants. Although 16S rRNA sequencing analysis provides information about bacterial diversity, its limitation should be considered when investigating diversity at lower taxonomic levels. Furthermore, the low amount of DNA in samples with low biomass, such as meconium, required a higher number of cycles than mother's stool in this study. Higher number of cycles in PCR leads to a higher sequencing

error rate, which should be taken into consideration for analysis of the data generated (Sze & Schloss, 2019).

4.3.3 Sequence analysis of the *rpoB* gene

The *rpoB* gene is a universal gene found in most bacteria, and has been proposed as a tool for separating closely related species and strains in taxonomic analysis of microbial composition (Adékambi, Drancourt, & Raoult, 2009). Comparison of partial *rpoB* sequences have allowed for distinguishing closely related species of *Bacteroides* (Ko et al., 2007). In this study, *rpoB* based sequence analysis allowed for determining the presence of different species of *Bacteroides* in all samples. Additionally, the Kraken HumGut database also assigned other species to *rpoB*. However, the information regarding *Bacteroides* was sufficient for making conclusions and further assumptions for this study. The initial screening performed in a previous study (Sørensen, 2020) combined with negative hits for *rpoB* after qualitative PCR, allowed for the assigning of samples as *rpoB* negative and therefore negative for *Bacteroides* in this study. However, analysis of samples which were assigned as *Bacteroides* negative indicated a presence of *Bacteroides*. Therefore, assigning samples as *Bacteroides* negative based on *rpoB* screening may lead to loss of valuable data if they are not investigated due to being assigned as *Bacteroides* negative.

Additionally, the *rpoB* sequencing data the information regarding presence of *Bacteroides* in mother's stool, meconium, and skin swabs from infant's (Vaginal A) stem from a sequencing data set from a previous study (Sørensen, 2020), which should be taken into consideration. A stronger conclusion could be drawn if samples from mother's stool, meconium, and skin swabs from vaginal delivered infants (Vaginal A) were analyzed together with the samples in this study. Combining two different data sets could potentially generate errors or false observations. In addition, potential errors during sample preparation, library preparation, and sequencing in the study done by Sørensen (2020) are unknown and should be taken into consideration.

4.3.4 Cultivation of *Bacteroides* species

Members of the genus *Bacteroides* are obligate anaerobes and will not divide in the presence of oxygen (Bacic & Smith, 2008). However, species of *Bacteroides* are one of the most aerotolerant anaerobic species and have been reported to slowly divide in nanomolar

concentrations of oxygen (Baughn & Malamy, 2004). Therefore, the anaerobic cultivating techniques used in this study was sufficient for the growth of two species of *Bacteroides*. Esculin hydrolysis have been used as a phylogenetic marker for *Bacteroides*, where black/brown complexed formed provides evidence for identification of *Bacteroides* (Livingston et al., 1978). However, *Klebsiella* also formed a black/brown complex. Therefore, inclusion of other phylogenetic markers for *Bacteroides* should be considered. Species of *Bacteroides* require nutritional features such as hemin and vitamin K for optimal cultivation (Varel & Bryant, 1974). Although the anaerobic and nutritional conditions for growth of *Bacteroides* were presumably met in this study, *B. vulgatus* was not able to grow on the provided mediums. Hence, genetic markers in *B. vulgatus* could be investigated to determine whether this species demand other conditions or supplements. Indication of living cells of *B. vulgatus* in meconium would further strengthen the assumption of vertical transmission related to vaginal delivery.

4.3.5 Strength and weaknesses of this study

There are several strengths to this study. The sample size is a considerable strength, as it made it possible to investigate the *Bacteroides* association between 95 mother-child pairs related to vaginal delivery, and 47 mother-child pairs related to C-section delivery. Information regarding mode of delivery made it possible to determine the effect of delivery method had on vertical transmission of *Bacteroides*. Additionally, as the meconium samples with presence of *Bacteroides* were all from vaginal delivered infants, the hypothesis of vertical transmission of bacteria through the birth canal could be strengthened. Cultivation of live species of *Bacteroides* from meconium samples from vaginal delivered infants further strengthened the assumption of association between transmission of *Bacteroides* from mother to child and vaginal delivery.

When it comes to the weaknesses of this study, conclusions regarding differences in bacterial composition in meconium between vaginal delivered infants and C-section delivered infants on species level could not be drawn. Additionally, no conclusion could be drawn on the differences in bacterial composition on species level on newborn's skin. Mother's vaginal microbiota were not studied, which could have provided more information about vertical transmitted bacteria. Furthermore, the rupture of the amniotic sac was not studied, which could have explained differences in the bacterial composition in C-section delivered infant's gut.

5.0 Conclusion and further perspectives

Several species of *Bacteroides* were mother child associated, and these findings corroborate with previous studies. Our results indicated the significant association between transmission of *B. vulgatus* from mother to child and vaginal delivery. Interestingly, the infant's gut appears to be favorable to colonization of *B. vulgatus*, as it was one of the most abundant in meconium and feces from vaginal delivered infants. Genetic markers for functional abilities in *B. vulgatus* should be investigated to further attempt cultivation of *B. vulgatus* from meconium. Future studies should investigate whether the colonization of *B. vulgatus* persists longer than 3 months after birth. Additionally, the differences in gut composition between vaginal delivered and C-section delivered infants should be investigated over a longer period.

More research could be done by including samples from mother's vaginal fluids to investigate the vaginal microbiota composition. Additionally, information regarding breastfeeding could be included to study the effect of breastmilk versus formula on infant's gut composition, and composition of *Bacteroides*. Furthermore, the functional genes of *Bacteroides* could be investigated to provide information regarding the importance of *Bacteroides* in the human gut. Regarding C-section delivered infants, information about rupture of the amniotic sac could be included to study the effect on gut composition in C-section delivered infants.

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Appendix A: Illumina index-primers

Table A1. Primer sequences for the index-primers used for library preparations for sequencing *rpoB*

Primer name	Primer sequence '5 → 3'	Target gene	Reference
F1 *rpoB*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccg atctagtcaaCACTTGAGCAAYCGTCGTRT	*rpoB*	(Ko et al., 2007)
F2 *rpoB*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccg atctagttccCACTTGAGCAAYCGTCGTRT	*rpoB*	(Ko et al., 2007)
F3 *rpoB*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccg atctatgtcaCACTTGAGCAAYCGTCGTRT	*rpoB*	(Ko et al., 2007)
F4 *rpoB*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccg atctccgtccCACTTGAGCAAYCGTCGTRT	*rpoB*	(Ko et al., 2007)
F5 *rpoB*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccg atctgtagagCACTTGAGCAAYCGTCGTRT	*rpoB*	(Ko et al., 2007)
F6 *rpoB*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccg atctgtccgcCACTTGAGCAAYCGTCGTRT	*rpoB*	(Ko et al., 2007)
F7 *rpoB*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccg atctgtgaaaCACTTGAGCAAYCGTCGTRT	*rpoB*	(Ko et al., 2007)
F8 *rpoB*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccg atctgtggccCACTTGAGCAAYCGTCGTRT	*rpoB*	(Ko et al., 2007)
F9 *rpoB*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccg atctgtttcgCACTTGAGCAAYCGTCGTRT	*rpoB*	(Ko et al., 2007)
F10 *rpoB*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccg atctcgtacgCACTTGAGCAAYCGTCGTRT	*rpoB*	(Ko et al., 2007)
F12 *rpoB*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccg atctggtagcCACTTGAGCAAYCGTCGTRT	*rpoB*	(Ko et al., 2007)
F13 *rpoB*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccg atctactgatCACTTGAGCAAYCGTCGTRT	*rpoB*	(Ko et al., 2007)
F14 *rpoB*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccg atctatgagcCACTTGAGCAAYCGTCGTRT	*rpoB*	(Ko et al., 2007)
F15 *rpoB*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccg atctattcctCACTTGAGCAAYCGTCGTRT	*rpoB*	(Ko et al., 2007)
F16 *rpoB*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccg atctcaaaagCACTTGAGCAAYCGTCGTRT	*rpoB*	(Ko et al., 2007)
R1 *rpoB*	caagcagaagacggcatacgagatCGTGATgtgactggagttcagacg tgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R2 *rpoB*	caagcagaagacggcatacgagatACATCGgtgactggagttcagacg tgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R3 *rpoB*	caagcagaagacggcatacgagatGCCTAAgtgactggagttcagacg tgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R4 *rpoB*	caagcagaagacggcatacgagatTGGTCAgtgactggagttcagacg tgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)

R5 *rpoB*	caagcagaagacggcatacgagatCACTCTgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R6 *rpoB*	caagcagaagacggcatacgagatATTGGCgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R7 *rpoB*	caagcagaagacggcatacgagatGATCTGgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R8 *rpoB*	caagcagaagacggcatacgagatTCAAGTgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R9 *rpoB*	caagcagaagacggcatacgagatCTGATCgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R10 *rpoB*	caagcagaagacggcatacgagatAAGCTAgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R11 *rpoB*	caagcagaagacggcatacgagatGTAGCCgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R12 *rpoB*	caagcagaagacggcatacgagatTACAAGgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R13 *rpoB*	caagcagaagacggcatacgagatTTGACTgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R14 *rpoB*	caagcagaagacggcatacgagatTTGACTgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R15 *rpoB*	caagcagaagacggcatacgagatGGAACTgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R16 *rpoB*	caagcagaagacggcatacgagatTGACATgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R17 *rpoB*	caagcagaagacggcatacgagatGGACGGgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R18 *rpoB*	caagcagaagacggcatacgagatCTCTACgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R19 *rpoB*	caagcagaagacggcatacgagatGCGGACgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R20 *rpoB*	caagcagaagacggcatacgagatTTTCACgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R21 *rpoB*	caagcagaagacggcatacgagatGGCCACgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R22 *rpoB*	caagcagaagacggcatacgagatCGAAACgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R23 *rpoB*	caagcagaagacggcatacgagatCGTACGgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)
R24 *rpoB*	caagcagaagacggcatacgagatCCACTCgtgactggagttcagacgtgtgctcttccgatctCCTTCAGGAGTYTCAATNGG	*rpoB*	(Ko et al., 2007)

Appendix B: R-script

Copy of R-script used in the DADA2-pipeline to generate the ASV profile.

```
library(dada2)
library(ShortRead)
library(ggplot2)
library(phyloseq)
library(tidyverse)

path <- "masterproject/fastq/Data/"
fnFs <- sort(list.files(path, pattern = "_R1.fastq", full.names = TRUE))
fnRs <- sort(list.files(path, pattern = "_R2.fastq", full.names = TRUE))
sample.names <- sapply(strsplit(basename(fnFs), "_"), `[`, 1)

plotQualityProfile(fnFs[1:2])
plotQualityProfile(fnRs[1:2])

filtFs <- file.path(path, "filtered", paste0(sample.names, "_F_filt.fastq.gz"))
filtRs <- file.path(path, "filtered", paste0(sample.names, "_R_filt.fastq.gz"))
names(filtFs) <- sample.names
names(filtRs) <- sample.names

filt_path <- file.path(path, "filtered")
if(!file_test("-d", filt_path)) dir.create(filt_path)
filtFs <- file.path(filt_path, paste0(sample.names, "_F_filt.fastq.gz"))
filtRs <- file.path(filt_path, paste0(sample.names, "_R_filt.fastq.gz"))


for(i in seq_along(fnFs)) {
  fastqPairedFilter(c(fnFs[i], fnRs[i]), c(filtFs[i], filtRs[i]),
                    truncLen=c(240,220),
                    maxN=0, maxEE=c(2,2), truncQ=2, rm.phix=TRUE,
                    compress=TRUE, verbose=TRUE)
}

derepFs <- derepFastq(filtFs, verbose = TRUE)
derepRs <- derepFastq(filtRs, verbose = TRUE)

names(derepFs) <- sample.names
names(derepRs) <- sample.names

dadaFs.lrn <- dada(derepFs, err=NULL, selfConsist = TRUE, multithread = TRUE)
errF <- dadaFs.lrn[[1]]$err_out
dadaRs.lrn <- dada(derepRs, err=NULL, selfConsist = TRUE, multithread = TRUE)
errR <- dadaRs.lrn[[1]]$err_out

plotErrors(dadaFs.lrn[[1]], nominalQ=TRUE)

dadaFs <- dada(derepFs, err=errF, multithread = TRUE)
dadaRs <- dada(derepRs, err=errF, multithread = TRUE)

dadaFs[[1]]

mergers <- mergePairs(dadaFs, derepFs, dadaRs, derepRs, verbose = TRUE)
head(mergers[[1]])

seqtab <- makeSequenceTable(mergers[names(mergers) != "Mock"])
dim(seqtab)
table(nchar(getSequences(seqtab)))

seqtab.nochim <- removeBimeraDenovo(seqtab, verbose = TRUE)
dim(seqtab.nochim)
sum(seqtab.nochim)/(sum(seqtab))

uniquesToFasta(getUniques(seqtab.nochim), fout = "masterproject/fastq/UniqueSeqs.fasta", ids=paste0("Seq",
seq(length(getUniques(seqtab.nochim)))))
```

```
library(phyloseq)
otab <- otu_table(seqtab.nochim, taxa_are_rows=FALSE)
colnames(otab) <- paste0("Seq", seq(ncol(otab)))
otab = t(otab)
write.table(otab, "dada_table.txt",quote=FALSE,sep="\t")


dada_table <- read.delim("dada_table.txt", row.names=NULL)

library(tidyverse)
report.file <- "masterproject/reads.txt"
read_delim(report.file, delim = "\t",
           col_names = c("Class/Unclass", "Seq.Nr",
                         "Tax.ID", "ReadLength", "LCA"),
           trim_ws = T) %>%
  select(Seq.Nr, Tax.ID) -> table.id

combined.df <- cbind.data.frame(dada_table, table.id$Tax.ID)

write.table(combined.df, "combined_table.txt",quote=FALSE,sep="\t")
```

Appendix C: Media and solutions for cultivation

The media used in this study is described below. Dilution series (10^{-1}-10^{-6}) were made from homogenized meconium samples without buffer and added to petri dishes containing bile esculin agar (BEA) (Oxoid) and *Bacteroides* bile esculin agar with amikacin (BBE) (VWR, USA). BEA plates and BBE plates were incubated anaerobically at 37ºC for 2-5 days. In addition, one 10^{-1} dilution of sample 3 was added to cooked meat medium (Oxoid) and incubated anaerobically at 37ºC for 2-5 days. *Bacteroides* are reported to forming black/brown complexes on mediums with esculin present (Bacic & Smith, 2008).

Bile Esculin Agar (BEA)

For 1 L BEA medium:

Agar 15.0 g

Esculin 1 g

Ferric citrate 0.5 g

Meat extract 3 g

Meat peptone 5 g

Ox-bile 40 g

Bacteroides bile esculin agar with amikacin (BBE)*:

For 1 L BBE medium:

Pancreatic Digest of Casein 14.5 g

Papaic Digest of Soybean Meal 5.0

Sodium Chloride 5.0

Esculin 1.0

Ferric Ammonium Citrate 0.5

Oxgall 15.0

Hemin 0.01

Amikacin 0.075

Vitamin K1 0.01

Agar 14.0

Growth Factors 1.8

* Premade from manufactory

Cooked meat medium*:

For 1 L cooked meat medium:

Heart muscle 454.0 g

Peptone 10.0 g

‘Lab-Lemco’ powder 10.0 g

Sodium chloride 5.0

Glucose 2.0

***** Premade from manufactory

Appendix D: Presence of Bacteroides in different sample categories

Table D1. The observed species of *Bacteroides* in mother's stool, meconium, newborn's skin, and feces from 3 months old infant related to vaginal delivery with potential presence of *Bacteroides* (Vaginal A). For each species, the percentage is based on the presence of ASV in the sample categories. The percentage presence of ASV within each sample category is shown on the right side of the observed species amount, the numbers are based on the average amount of ASV within the sample category. The % ASV quantity for each Bacteroides species is visualized in Figure 3.2.

Vaginal A	**Mother's stool (n=48) ***		**Meconium (n=51) ***		**Skin (n=51) ***		**Infant's feces 3 months (n=36)**	
Species	% Presence in sample category	% ASV quantity of total	% Presence in sample category	% ASV quantity of total	% Presence in sample category	% ASV quantity of total	% Presence in sample category	% ASV quantity of total
B. caccae	95.83	8.61	74.51	7.31	50.98	7.24	27.78	11.24
B. caecimuris	81.25	4.97	33.33	1.24	23.53	4.36	22.22	4.45
B. cellulosilyticus	60.42	0.61	13.73	0.02	7.84	0.10	0.00	0.00
B. dorei	93.75	12.31	88.24	23.61	58.82	10.66	30.56	20.48
B. fragilis	85.42	1.12	29.41	3.09	5.88	0.14	22.22	0.52
B. helcogenes	50.00	2.32	19.61	0.95	3.92	1.84	0.00	0.00
B. heparinolyticus	14.58	0.75	0.00	0.00	5.88	0.00	0.00	0.00
B. intestinalis	87.50	4.36	35.29	0.53	11.76	2.95	8.33	0.03
B. ovatus	70.83	3.71	27.45	0.60	13.73	1.69	16.67	5.66
B. salanitronis	85.42	9.48	39.22	1.54	29.41	2.11	19.44	10.08
B. thetaiotaomicron	95.83	12.62	56.86	6.77	52.94	12.90	27.78	16.61
B. uniformis	97.92	5.28	45.10	0.33	31.37	5.29	25.00	1.40
B. vulgatus	100	33.50	96.08	50.46	88.24	50.67	44.44	29.01
Parabacteroides	NA	0.35	NA	3.53	NA	0.4	16.67	0.53

*Data retrieved from Sørensen (2020)

Table D2. The observed species of *Bacteroides* in mother's stool, newborn's skin, and feces from 3 months old infant related to vaginal delivery without potential presence of *Bacteroides* (Vaginal B). For each species, the percentage is based on the presence of ASV in the sample categories. The percentage presence of ASV within each sample category is shown on the right side of the observed species amount, the numbers are based on the average amount of ASV within the sample category. The % ASV quantity for each Bacteroides species is visualized in Figure 3.2.

Vaginal B	**Mother's stool (n=47)**		**Skin (n=47)**		**Infant's feces 3 months (n=30)**	
Species	% Presence in sample category	% ASV quantity of total	% Presence in sample category	% ASV quantity of total	% Presence in sample category	% ASV quantity of total
B. caccae	93.62	6.83	2.12	3.40	40.00	12.46
B. caecimuris	89.36	6.58	2.12	11.04	33.33	7.04
B. cellulosilyticus	59.57	0.82	2.12	1.67	3.33	0.00
B. dorei	97.87	11.13	2.12	29.96	43.33	20.32
B. fragilis	87.23	2.33	2.12	0.01	36.67	10.47
B. helcogenes	61.70	1.88	2.12	4.25	6.67	2.17
B. heparinolyticus	2.13	0.00	0.00	0.00	0.00	0.00
B. intestinalis	70.21	2.83	0.00	0.00	16.67	0.42
B. ovatus	80.85	5.55	0.00	0.00	6.67	0.46
B. salanitronis	93.62	6.82	2.12	1.73	26.67	8.05
B. thetaiotaomicron	100.00	9.86	2.12	4.46	40.00	8.34
B. uniformis	97.87	10.15	2.12	3.39	40.00	7.01
B. vulgatus	100.00	34.81	2.12	40.09	46.67	21.69
Parabacteroides	82.98	0.41	2.12	0.02	33.33	1.55

Table D3. The observed species of *Bacteroides* in mother's stool, and feces from 3 months old infant related to C-section delivery. For each species, the percentage is based on the presence of ASV in the sample categories. The percentage presence of ASV within each sample category is shown on the right side of the observed species amount, the numbers are based on the average amount of ASV within the sample category. The % ASV quantity for each Bacteroides species is visualized in Figure 3.2.

C-section	**Mother's stool (n=47)**		**Infant's feces 3 months (n=27)**	
Species	% Presence in sample category	% ASV quantity of total	% Presence in sample category	% ASV quantity of total
B. caccae	89.36	9.98	14.81	4.88
B. caecimuris	87.23	7.43	11.11	0.05
B. cellulosilyticus	51.06	0.53	3.70	0.10
B. dorei	91.49	15.42	11.11	0.48
B. fragilis	85.11	1.45	14.81	1.42
B. helcogenes	38.30	2.85	7.41	0.06
B. heparinolyticus	0.00	0.00	0.00	0.00
B. intestinalis	70.21	3.10	0.00	0.00
B. ovatus	74.47	2.59	3.70	1.38
B. salanitronis	76.60	6.68	18.52	26.24
B. thetaiotaomicron	91.49	13.24	11.11	8.30
B. uniformis	91.49	8.00	14.81	4.32
B. vulgatus	91.49	28.45	11.11	52.41
Parabacteroides	74.47	0.28	7.41	0.36

Appendix E: Illustration of mother-child association of Bacteroides

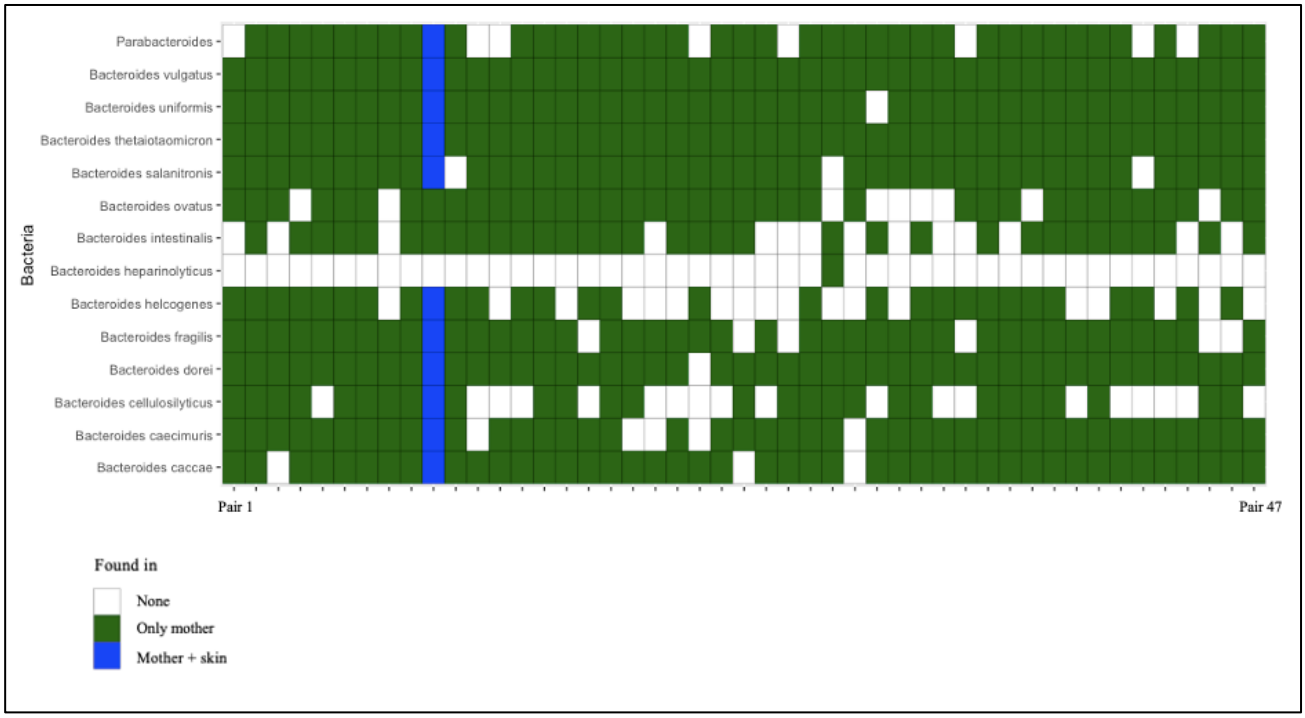


Figure E1. Presence of *Bacteroides* species in 47 mother-child pairs related to vaginal delivery without potential presence of *Bacteroides*. The figure illustrates the presence of different *Bacteroides* species found in mother's stool and on newborn's skin. Complete mother-child pairs are presented on the X-axis and the different *Bacteroides* species are presented on the Y-axis. The colors coding for the different categories are given on the legend at the bottom left of the figure.

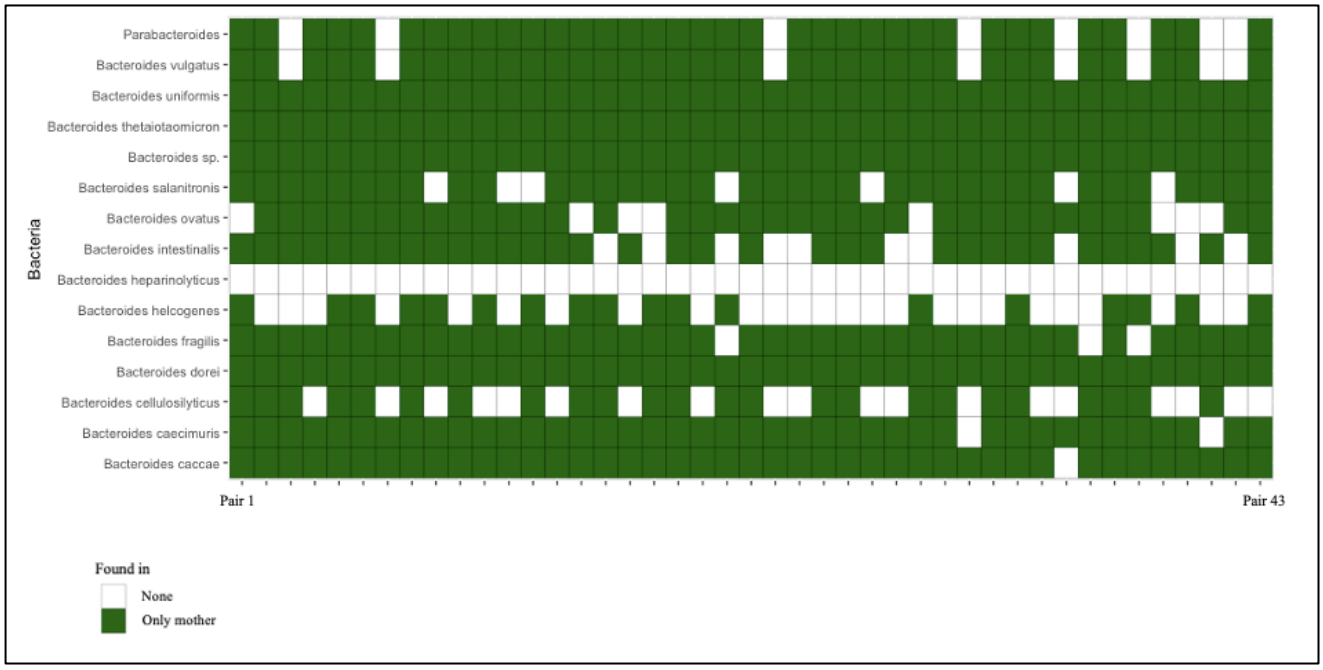


Figure E2. Presence of *Bacteroides* species in 43 mother-child pairs related to C-section delivery.

The figure illustrates the presence of different *Bacteroides* species found in mother's stool and on newborn's skin. Complete mother-child pairs are presented on the X-axis and the different *Bacteroides* species are presented on the Y-axis. The colors coding for the different categories are given on the legend at the bottom left of the figure.

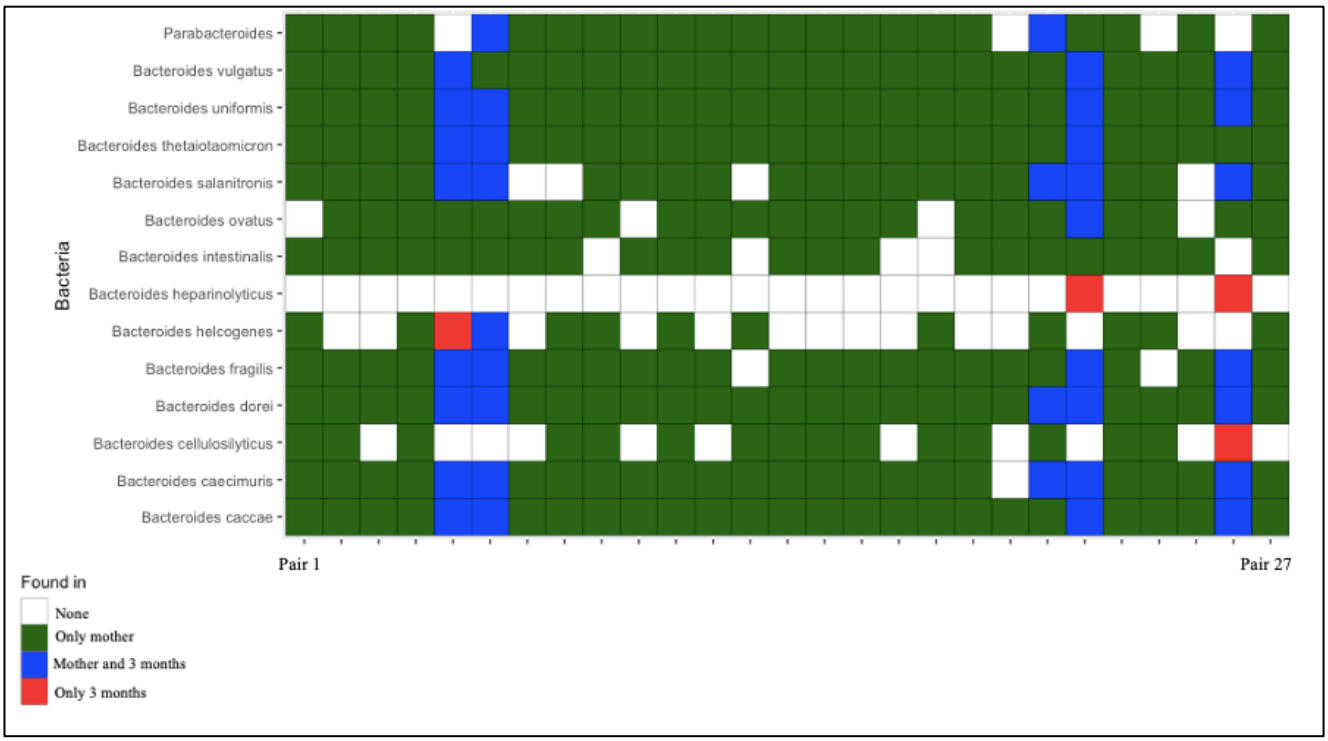


Figure E3. Presence of *Bacteroides* species in samples from mother's stool and feces from 3 months old infants from C-section delivery. The figure illustrates the presence of different *Bacteroides* species found in mother's stool and in feces from corresponding 3 months old infants. Mother-child pairs are presented on the X-axis and the different *Bacteroides* species are presented on the Y-axis. The colors coding for the different categories are given on the legend at the bottom left of the figure.

Appendix F: P-values indicating mother-child association of Bacteroides

Table F1. Results from Fischer's exact test.

Fischer's exact test p-value		
Species	Presence of *Bacteroides* – mode of delivery (Mother's stool – meconium + skin)	Presence of *Bacteroides* – mode of delivery (Mother's stool – 3 months)
Parabacteroides *	2.8E-3	0.215
Bacteroides vulgatus **	1.0E-5	2.3E-3
Bacteroides uniformis	0.0573	0.286
Bacteroides thetaiotaomicron *	0.018	0.253
Bacteroides salanitronis	1.00	1.00
Bacteroides ovatus	0.552	0.669
Bacteroides intestinalis	1.00	0.099
Bacteroides heparinolyticus	1.00	0.205
Bacteroides helcogenes	1.00	1.00
Bacteroides fragilis	1.00	0.192
Bacteroides dorei ***	1.0E-4	0.010
Bacteroides cellulosilyticus	1.00	1.00
Bacteroides caecimuris	0.552	0.055
Bacteroides caccae *	8.0E-4	0.122

*Significant p-value (<0.05) by Fischer's exact test (vaginal delivery – presence of *Bacteroides*)
**Significant p-value (<0.05) by Fischer's exact test (vaginal delivery – presence of *Bacteroides*)

Appendix G: Meconium collection time and abundance of *Bacteroides*

Table G1. Relative abundance of *Bacteroides* in meconium at different sampling time points. Values are calculated based on the total average of species of *Bacteroides* at each sampling time point.

Species	% Within 24 hours	% Within 24-48 hours	% Over 48 hours
Bacteroides vulgatus	40.47	50.37	46.02
Bacteroides dorei	30.59	23.29	24.41
Bacteroides caccae	9.92	8.56	2.23
Bacteroides fragilis	9.24	0.63	0.07
Bacteroides thetaiotaomicron	7.59	8.98	1.21
Bacteroides ovatus	1.57	0.38	0.00
Parabacteroides	0.39	0.76	20.01
Bacteroides intestinalis	0.18	0.18	2.55
Bacteroides salanitronis	0.02	2.99	0.01
Bacteroides caecimuris	0.02	1.99	1.43
Bacteroides uniformis	0.01	0.43	0.56
Bacteroides cellulosilyticus	0.01	0.01	0.10
Bacteroides helcogenes	0.01	1.43	1.40

Table G2. Results from two-tailed t-test. p-values indicate differences in abundance of *Bacteroides* at two sampling time points.

Species	Within 24 hours and 24-48 hours	24-48 hours and over 48 hours	Within 24 hours and over 48 hours
Parabacteroides	0.374	0.096	0.215
Bacteroides vulgatus	0.587	0.682	0.946
Bacteroides uniformis	0.287	0.942	0.174
Bacteroides thetaiotaomicron	0.764	0.163	0.511
Bacteroides salanitronis	0.216	0.323	0.217
Bacteroides ovatus	0.282	0.327	0.384
Bacteroides intestinalis	0.945	0.124	0.258
Bacteroides heparinolyticus [a]	-	-	-
Bacteroides helcogenes	0.467	0.923	0.220
Bacteroides fragilis	0.198	0.406	0.425
Bacteroides dorei	0.78	0.873	0.751
Bacteroides cellulosilyticus	0.538	0.019 *	0.109
Bacteroides caecimuris	0.271	0.739	0.220
Bacteroides caccae	0.99	0.355	0.388

[a] Average and standard deviation was 0, test could not therefore not be performed

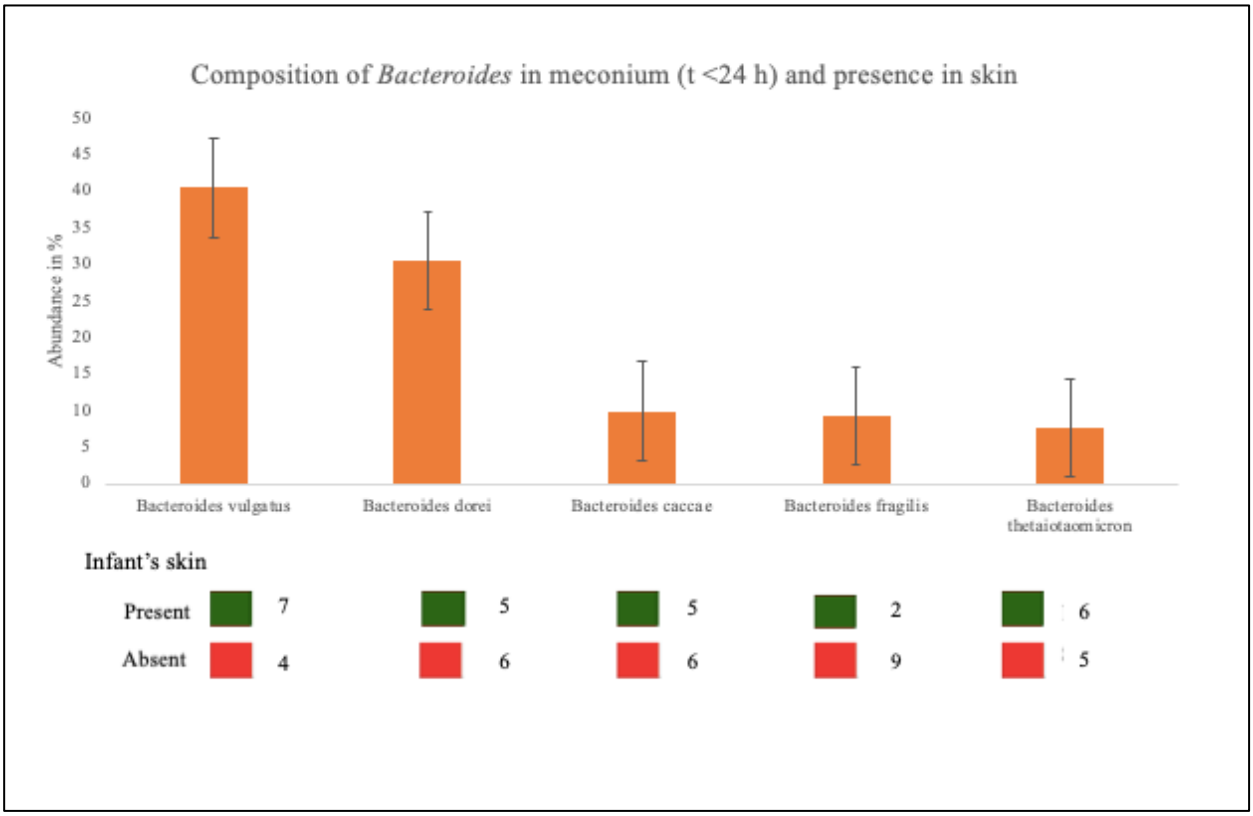


Figure G1. Composition of *Bacteroides* in connection with meconium sampling time points and presence on infant skin. The numbers are based on average values of *Bacteroides* in sampling time point 'within 24 hours'. Standard error of the data is shown in each bar. The different *Bacteroides* species are shown on the X-axis, and the abundance of *Bacteroides* species in % is shown on the Y-axis.

The amount of skin samples with the given species present or absent is shown in the figure legend.

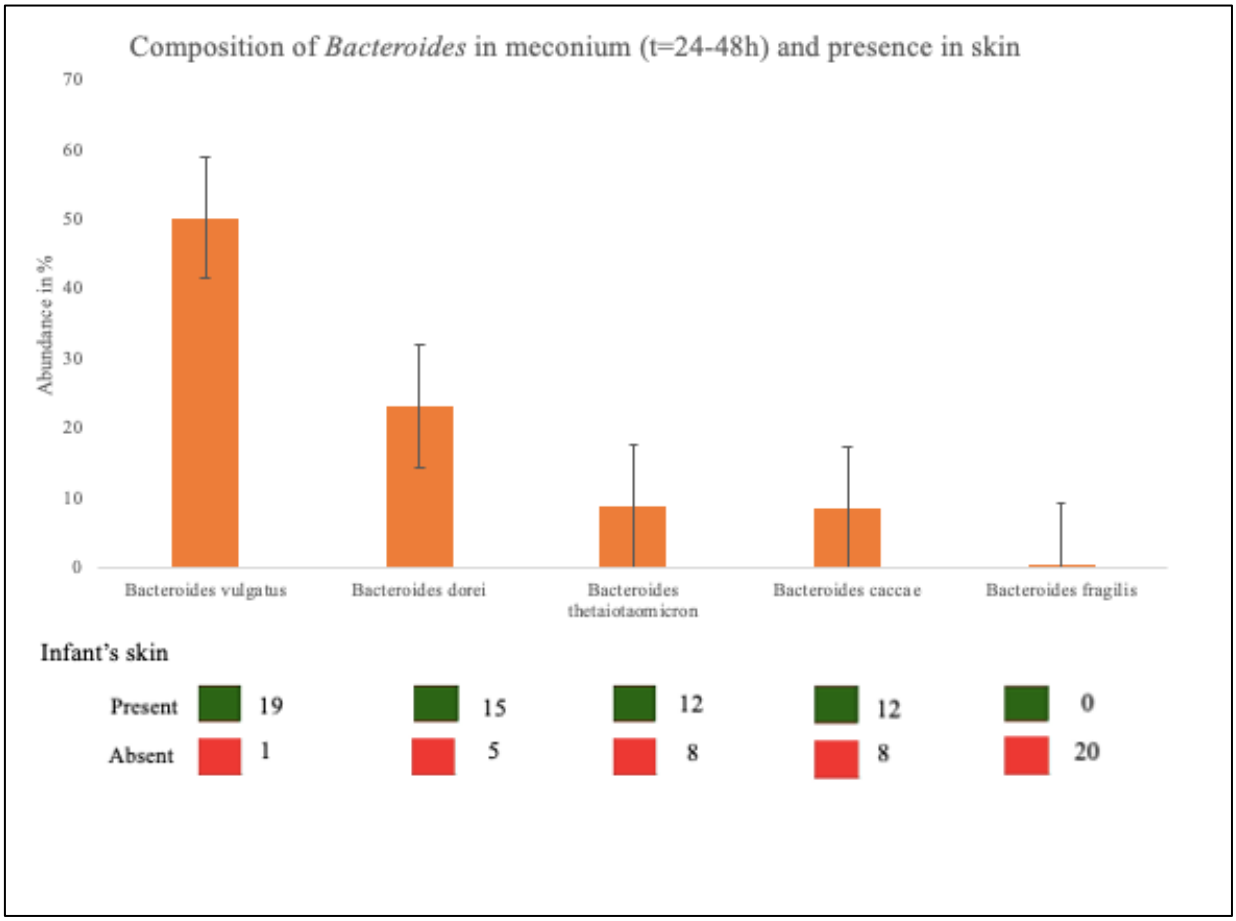


Figure G2. Composition of *Bacteroides* in connection with meconium sampling time points and presence on infant skin. The numbers are based on average values of *Bacteroides* in sampling time point 'between 24 hours and 48 hours'. Standard error of the data is shown in each bar. The different *Bacteroides* species are shown on the X-axis, and the abundance of *Bacteroides* species in % is shown on the Y-axis. The amount of skin samples with the given species present or absent is shown in the figure legend.

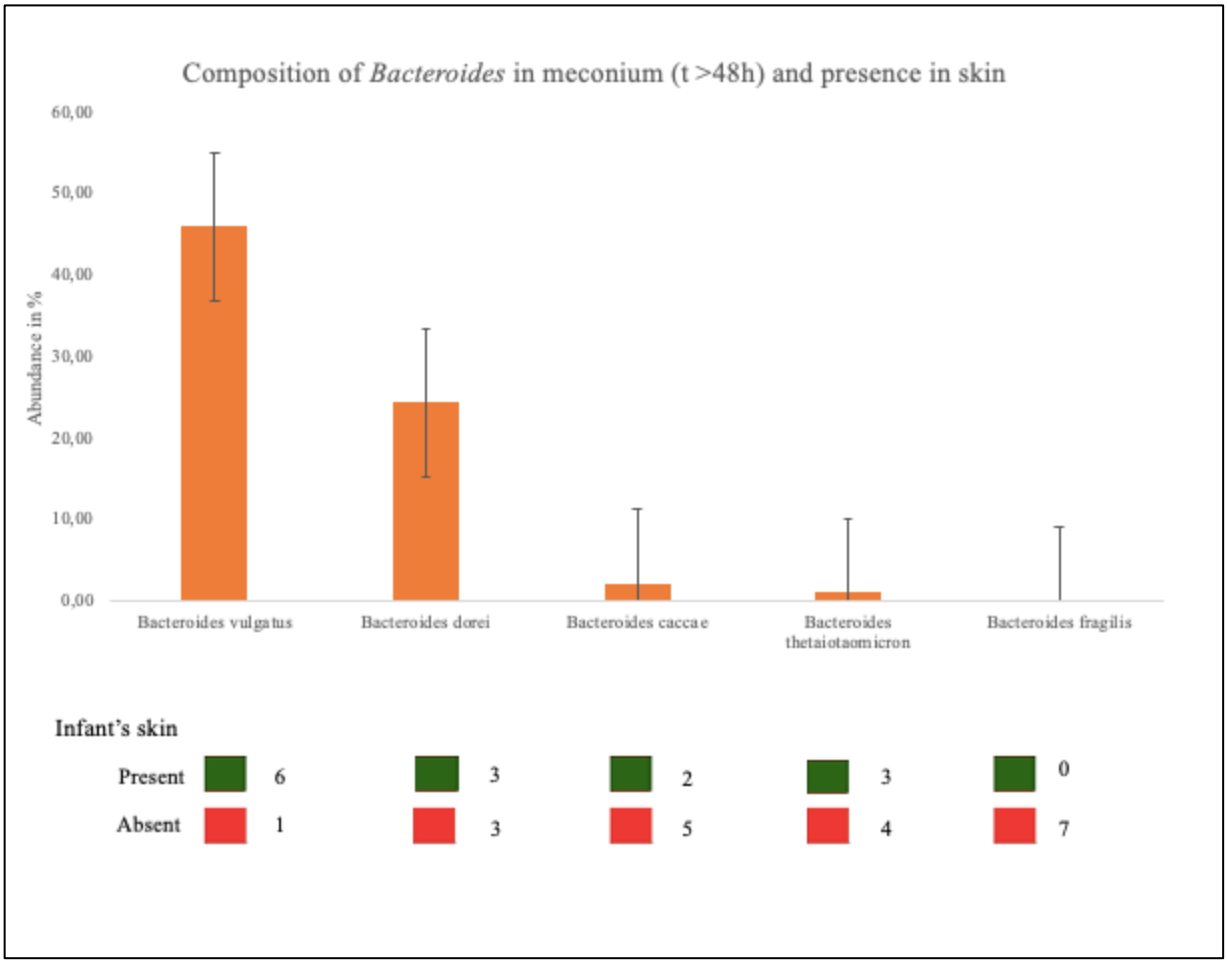
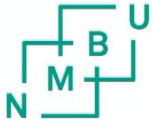


Figure G3. Composition of *Bacteroides* in connection with meconium sampling time points and presence on infant skin. The numbers are based on average values of *Bacteroides* in sampling time point 'over 48 hours'. Standard error of the data is shown in each bar. The different *Bacteroides* species are shown on the X-axis, and the abundance of *Bacteroides* species in % is shown on the Y-axis. The amount of skin samples with the given species present or absent is shown in the figure legend.

Appendix H: Selective media for cultivating *Bacteroides*

Table H1. Results after cultivation. 3 meconium samples chosen based on their 16S rRNA sequencing data where *B. vulgatus* was the dominating Bacteroides in each sample. The + and – signs indicate positive or negative hit/growth. NA indicates not attempted.

Meconium sample	16S sequencing data *B. vulgatus* present	BBE-medium - growth	BEA-medium - growth	Cooked meat medium - growth	rpoB positive hits after incubation	Pure cultured colonies sequenced	Result after Sanger sequencing
Sample 1	+	-	-	NA	-	0	*NA*
Sample 2	+	+	+	NA	+	24	*Bacteroides dorei* *Bacteroides ovatus*
Sample 3	+	+	+	+	+	75	*Klebsiella*



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