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Colonization of *Bifidobacterium* in the Human Infant Gut

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Abstract

The early bacterial flora and bacterial colonization of a human infant's gut have been studied broadly over the years due to it being the key to the development of a healthy gut. Several of these studies have shown a bacterial association between infants and their mothers, where mode of delivery and breastfeeding are the sources of transmission. *Bifidobacterium* is commonly found in the gut of both infants and adults. However, the time of colonization is still uncertain. This thesis main aim was to investigate the presence of *Bifidobacterium* in mother-infant pairs, where the hypothesis was that the mother's milk is the reason for selection and colonization of different *Bifidobacterium* species.

The samples included in this study were received from the Prevent Atopic Dermatitis and Allergies (PreventADALL cohort). The samples were collected from 147 mother-infant pairs, where 99 infants were born by vaginal delivery and the remaining 48 from caesarean section (c-section). In total, 536 samples collected from four different sample categories were analysed. These four categories included skin swabs from newborns (taken <10 minutes after birth), meconium, and stool from 3-month-old infants and their mothers.

The bacterial taxonomic composition in each sample category was determined using 16S rRNA sequencing and sequencing based on *Bifidobacterium* specific primers (*clpC*). To study the overlap of *Bifidobacterium* species in the mother-infant pairs, operational taxonomic units (OTU) and amplicon sequence variants (ASVs) profiles were studied by processing them with different pipelines in RStudio.

Bifidobacterium was present in all sample categories, except in the meconium of c-section delivered infants. The species *Bifidobacterium longum* was the most abundant, and the highest mother-infant association was seen between mothers and 3-month-olds, indicating a selection of *B. longum* sometime after birth, presumably due to breastfeeding.

In conclusion, the data collected support the claim that *Bifidobacterium* selection is not affected by mode of delivery, but by consumption of breastmilk. The lack of information about which infant was breastfed and the number of sequences not corresponding to a *Bifidobacterium* species, makes it impossible to draw a full conclusion. Therefore, further research of *Bifidobacterium* specific primers should be conducted, and more information about the mother-infant pairs should be collected, to better understand the transmission and colonization time of this bacterium.

Sammendrag

Bakteriefloraen og koloniseringen av et menneske-spedbarns tarm har vært et yndet forskningsobjekt gjennom årene på bakgrunn av sin essensielle rolle i å skape et sunt og friskt tarmmiljø. Flere av disse studiene viser en klar bakteriell sammenheng mellom mors tarmflora og spedbarnets, der fødselsmetode og amming har blitt studert som overføringskilde for bakteriene. Bifidobakterier finnes som regel i tarmen til både spedbarn og voksne, imidlertid er det fortsatt en del usikkerhet rundt når koloniseringstidspunktet finner sted. Hovedmålet med denne masteroppgaven var å se på tilstedeværelsen av bifidobakterier i mor-barn par, hvor hypotesen var at morsmelk er grunnen for selektering og kolonisering av ulike arter av bifidobakterier.

Studien er bygget på prøver som ble mottatt fra kohortstudien Prevent Atopic Dermatitis and Allergies (PreventADALL). Prøvene ble samlet inn fra 147 mor-barn par, der 99 barn ble født vaginalt, og de resterende 48 ved keisersnitt. Totalt ble 536 prøver, fra fire prøvekategorier, analysert. Disse fire kategoriene var: hudprøver fra nyfødte, tatt <10 minutter etter fødsel, mekonium (barnebek), og avføring fra både 3 måneder gamle barn og deres mødre.

Den taksonomiske bakteriesammensetningen i prøvekategoriene ble studert ved 16S rRNA-sekvensering og sekvensering basert på bifidobakterium-spesifikke primere, *clpC*. For å studere overlappingen av arter av bifidobakterier i parene mellom mor og spedbarn ble operative taksonomiske enheter (OTU) og amplikon sekvens variant (ASV) profiler studert ved å behandle dem med ulike pipelines i RStudio.

Med unntak av mekonium hos spedbarn født ved keisersnitt, var bifidobakterier til stede i alle prøvekategorier. Arten *Bifidobacterium longum* var den mest utbredte, og den høyeste mor-barn assosiasjonen ble sett mellom mødre og 3 måneder gamle spedbarn. Dette indikerer at koloniseringen av bifidobakterier ikke skjer ved fødsel, men senere i spedbarns-fasen.

Studien konkluderer med at det er mange faktorer som tilsier at *B. longum* selekteringen i barn er påvirket av amming, og ikke fødselsmetode. Det er visse begrensninger rundt det å dra en endelig konklusjon, ettersom det mangler informasjon om hvilke barn som er ammet, og antallet ASV-er som ikke svarer til en art av bifidobakterier er høy. Ytterligere forskning rundt bifidobakterium-spesifikke primere, og en grundigere innhenting av informasjon rundt mor-barn parene anbefales for å få en dypere innsikt i overføringen av denne bakterielle gruppen.

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Abbreviations

ASV	Amplicon Sequence Variant
BLAST	Basic Local Alignment Search Tool
BSM-Broth	Bifidus Selective Medium Broth
bp	Base pair
clpC	Caseinolytic protease C
C-section	Caesarean section
Cq value	Quantification cycle value
DADA2	Divisive Amplicon Denoising Algorithm 2
ddNTP	Dideoxynucleotide Triphosphates
dNTP	Deoxynucleotide Triphosphates
DNA	Deoxyribonucleic Acid
dsDNA	Double Stranded DNA
ESwab	Elution Swab
GI	Gastrointestinal
HMO	Human Milk Oligosaccharide
NCBI	The National Center for Biotechnology Information
NGS	Next Generation Sequencing
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PreventADALL	Preventing Atopic Dermatitis and Allergies in children
QIIME	Quantitative Insights Into Microbial Ecology
qRT-PCR	Quantitative Real Time PCR
rRNA	Ribosomal Ribonucleic Acid
ssDNA	Single stranded DNA

1. Introduction

1.1 The Human Gut Microbiota

The human gastrointestinal (GI) tract harbours a microbial community of great richness and complexity. An assemblage of co-existing microbes present in an environment can be referred to as a microbial community (Callieri et al., 2019). Two terms often used when describing microbial communities are microbiota and microbiome. The first is defined as the different microorganisms in a specified habitat, while the latter is the set of genomes contained in the microbiota of that environment. The microbiota found in the gut of humans include members from all three domains of life: *Archaea*, *Bacteria*, and *Eukarya*. They are often harmless, and many live in a healthy symbiosis with their host (Milani et al., 2017; Thursby & Juge, 2017). They contribute to a normal immune function, protect us against pathogens, and provide essential services, including the production of metabolites and vitamins needed for a normal gut function (Browne et al., 2017).

Four dominant bacterial phyla colonize the human gut: Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria (Eckburg, 2005; Lozupone et al., 2012). According to Thursby and Juge (2017), 93.5% of bacteria found in the gut belong to these four phyla. The core microbiota in adults largely consists off the first two, while the two latter are found in most adults but in a lower scale. However, the gut microbiota varies between human individuals depending on factors such as age, diet, and health (Milani et al., 2017). For example, the gut microbiota found in infants varies from adults and goes through a transformation from sterile to adult-like microbiota from birth to around 2.5 to 3 years of age (Milani et al., 2017; Yatsunenko et al., 2012).

1.1.1 Development of an infant gut microbiota

There are two different hypotheses concerning the moment of microbial colonization of an infant's gut. The first says the placenta barrier keeps the infant sterile until birth, making delivery mode and gestational age the first factors (Browne et al., 2017; Rehbinder et al., 2018). The sterile womb was an accepted dogma for decades because of numerous studies supporting it (Fanaro et al., 2007; Pham et al., 2016). Recent studies have challenged the hypothesis of a sterile *in utero* environment by implicating that a fetus is exposed to bacteria already in the

uterus, via the placenta, and through the umbilical cord, and amniotic fluid (Avershina et al., 2016; Digiulio et al., 2010).

During the infant gut microbiota development, both diversity and composition change rapidly due to different factors (figure 1.1) (Matamoros et al., 2013). Samples taken from one individual over a given interval showed more resemblance than samples taken from different individuals. This suggests an individual variation with a stable environment of microbes (Lozupone et al., 2012).

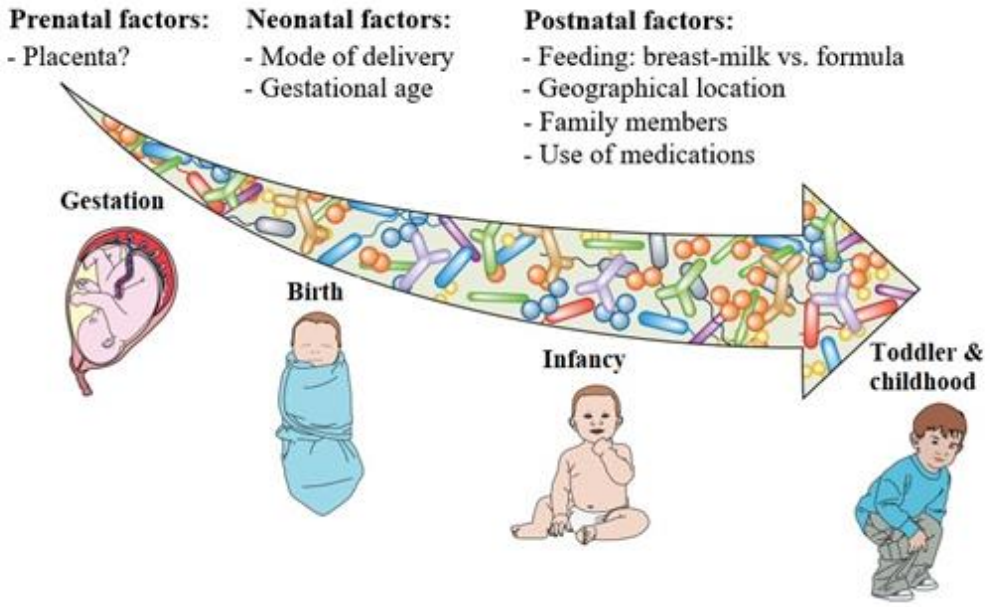

Figure 1.1: Schematic representation of prenatal, neonatal, and postnatal factors influencing the gut microbiota development. A representation of factors that contribute to the development of gut microbiota in infants. Figure modified from Milani et al. (2017).

If the hypothesis of a sterile *in utero* environment is correct, the first factor influencing an infant's gut is the delivery mode, i.e., vaginal birth or caesarean section (c-section). An infant born vaginally is in direct contact with the mother's vaginal and perineal area, leading to direct transmission of bacteria such as *Lactobacillus*, *Prevotella*, and *Bacteroides* (Gregory et al., 2015; Milani et al., 2017). The vaginal tract contains epithelial cells with glycogen, leading to an acidic environment (pH ~4.5) as a result of the production of lactic acids as a biproduct during glycogen metabolism (Caillouette et al., 1997; King & Brucker, 2011). In contrast, children born by c-section are exposed to bacteria from the skin and the environment, e.g., *Firmicutes* and *Proteobacteria.* These infants also show a reduced complexity of some bacteria, and colonization of *Bacteroides* is rare (Milani et al., 2017).

Premature birth is a second neonatal factor that affects the microbiota in infants. An infant born prematurely will have a low birth weight and is thereby more vulnerable to serious health challenges, leading to the use of antibiotics and other medical treatments (Milani et al., 2017). Antibiotics harm the gut's natural microbiota and can lead to an immature gut resulting in an infant with many immune, respiratory, and neurological difficulties (Angelakis et al., 2013; Lozupone et al., 2012).

An important postnatal factor affecting gut microbial development in infants is the form of feeding: breastmilk or formula. A difference in microbiota composition in breastfed and formula-fed new-borns has been documented, especially in the number of *Bifidobacterium* species (Solís et al., 2010). Breastmilk contains a mixture of prebiotics and antimicrobial agents favouring the development of milk-oriented microbiota (Lawson et al., 2020; Martín et al., 2003). It also contains human milk oligosaccharides (HMO), known to multiply the growth and function of microbes beneficial to the gut. Members of the Bifidobacteriaceae family are highly represented in breastfed infants, especially *Bifidobacterium longum* (Avershina et al., 2016). Contrary, formula-fed newborns are exposed to various carbohydrates and bacteria that affect the intestinal flora by making it more diverse. Domination of *Staphylococci, Bacteroides, Clostrida, Enterococci,* and *Enterobacteria* is documented in these infants (Milani et al., 2017). Davis et al. (2016) observed that switching from human milk to cow milk had a strong influence on the microbiota. Only five days after breast-feeding ceased, *Bifidobacterium* and *Lactobacillus* decreased, and an increase in *Bacteroides* and *Ruminococcus* was documented. Due to the differences in, and the influence of feeding method, as well as breastfeeding being the traditional biological standard for most mammals, breastfed infants' microbiota can be considered the standard for a healthy gut flora (Solís et al., 2010).

Family lifestyle and geographical location are both examples of environmental factors affecting an infant. The presence of older siblings appears to be associated with increased intestinal microbial diversity and richness during early childhood. A study on gut microbiota in adopted infants showed a substantial overall similarity in housemates in a shared home (Tavalire et al., 2021). Geographical location also influences the microbiota as a result of the differences in diet and lifestyle ranging from country to country (Milani et al., 2017). A study by Fallani et al. (2010) showed that Northern Europe had a higher number of *Bifidobacterium* than other parts

of Europe. In this part of Europe, breastfeeding is more common and may be the reason for the higher number of *Bifidobacterium* (Fallani et al., 2010).

1.2 *Bifidobacterium* and its role in the gut

The first mention of *Bifidobacterium* in a scientific paper is dated back to the early 1900s (Tissier, 1900). The discovery was made by the French paediatrician Henry Tissier while working with stools from breast-fed infants. *Bifidobacterium* is now known to be a large group of Gram-positive, non-motile, non-sporulating, anaerobic bacteria belonging to the phylum *Actinobacteria* (Butta et al., 2017). Over 30 species of the genus are recognized and isolated from the GI tract, vagina, mouth, and faeces of mammals, including humans, and their infants. Strains have also been isolated from other environments such as fermented milk and sewage (Masco et al., 2004), which may both be a result of the aforementioned mammals.

Bifidobacterium is characterized as a probiotic bacterium. Probiotics are living organisms that provide beneficial properties when consumed by either stimulating the growth or the activity of other bacteria in the colon (Picard et al., 2005). Already in the early 1900s, *Bifidobacterium* was suggested as a supplement for patients with diarrhea (Tissier, 1900). In a study conducted by Valdés-Varela et al. (2016), two species of *Bifidobacterium* (*B. longum* and *Bifidobacterium breve)* showed a high reduction of *Clostridium difficile* toxicity. *C. difficile* is an opportunistic bacterium, which may cause mild diarrhea or life-threatening conditions like hypoalbuminemia (Heinlen & Ballard, 2010; Leffler & Lamont, 2015). Over the years, *Bifidobacterium* has proven to be efficient against more than just diarrhea. *Bifidobacterium* can lower the pH in the colon as they produce acetic and lactic acids. This ability protects humans against colonization of pathogens and helps to restore a healthy gut of patients with intestinal infections and colonic transit disorders (Mavroudi, 2012; Picard et al., 2005). It has also been shown to have a direct effect on other organisms by producing bacteriocins with an antimicrobial effect (Cheikhyoussef et al., 2008), adhering to epithelial cells and thereby blocking adherence to pathogens (Collado et al., 2007), and secretion of factors that interfere with the invasion of host epithelial cells (Ingrassia et al., 2005).

1.2.1 Infants dependency of *Bifidobacterium*

Breast milk is considered the optimal nutrition for infants (Lessen & Kavanagh, 2015). It contains proteins, carbohydrates, and viable bacteria that protect the infant against infection and

contribute to the development of normal gut flora. As mentioned, HMOs are highly abundant in breastmilk. This glycan is, behind lactose and lipids, the third most abundant solid component in milk (Triantis et al., 2018). HMOs are also resistant to digestion in the gut, leading to many studies investigating how infants can utilize a glycan that they cannot digest. The answer seems to lay with *Bifidobacterium* and their utilization of milk oligosaccharide via the fermentative pathway using metabolomic and proteomic approaches (Sela et al., 2008). The knowledge and study of the transmission and colonization of this bacteria is therefore essential to understand the development of a healthy infant gut. Several previous studies have shown that breast-fed infants have higher *Bifidobacterium* levels than formula-fed infants (Bode, 2012; Hauck et al., 2011; Tissier, 1900).

1.2.2 Cultivation approaches

Cultivation is a well-known approach when studying microorganisms (Hitchens & Leikind, 1939). This is an approach where the organisms reproduce themselves in a predetermined media under controlled conditions, making the determination and study of an organism possible. The media used provides a replicable environment favourable for the organism of interest, including the correct pH, growth factors, and carbon and energy source. A pure culture is created by letting one colony grow separately on an agar plate, resulting in plates consisting exclusively of this bacterium. Agar is a polysaccharide extracted from different red algae and can create different solidifications of specific nutrients (e.g., Tryptic Soy Agar, Mannitol Salt Agar, Blood agar, or specific agars for different bacteria) (Williams & Phillips, 2000). Approaches to grow *Bifidobacterium* exploits the anaerobic properties of the bacteria, and the use of peptone and meat extract as sources of carbon, nitrogen, and minerals is favourable. There are some mediums that favour *Bifidobacterium*, such as Bifidus Selective Medium Broth (BSM-broth), blood- and liver agar, and *Bifidobacterium* agar plates.

1.3 Microbiota study based on nucleic acid approaches

Cultivation methods have several limitations; it is time-consuming, there are high risks of contaminations, they are often selective to specific microorganisms, and there are some unculturable bacteria (Bodor et al., 2020). The use of different nucleic acid approaches has been studied, and the development of various techniques has simplified the work for microbiologists and other scientists worldwide.

1.3.1 Polymerase Chain Reactions

Polymerase Chain Reactions (PCR) is a method often used to study diversity in complex microbial communities by amplifying biomarkers (Ochman et al., 1988). The method makes detection of a Deoxyribonucleic Acid (DNA) fragment possible by amplifying the fragment of interest by introducing it to a mix of reagents, including specially designed primers for the fragment, DNA polymerase, and deoxyribose nucleotide triphosphates (dNTPs). Then, the mixture of the reagents and DNA is transferred to a PCR machine which follows a thermal cycle containing three main steps:

(1) Denaturation: the two DNA strands are separated at a high temperature, 95-96°C.

(2) Annealing: the primers attach to the end of the DNA strand after the temperature lowers, 50-56°C.

(3) Elongation/extension: the temperature is raised to 72°C, making it possible for the polymerase to extend the 3' hydroxyl (OH^-) end of the DNA template by adding complementary dNTPs.

The three steps described above are repeated a given number of times, during which the DNA is doubled in each cycle (Garibyan & Avashia, 2013).

PCR can be separated into two main techniques, qualitative PCR, and quantitative PCR, where both follow the same forementioned steps. Qualitative, or conventional PCR, can make millions of copies of a specific DNA sample, making endpoint detection and further DNA analysis possible. A possible endpoint analysis is gel electrophoresis, which shows the presence or absence of the sequence of interest and indicates the size of the amplicon.

Quantitative PCR, also known as quantitative real-time-PCR, is a method used to detect and quantify gene expression in real-time. Real-time detection is possible due to the combination of amplification and detection. These measurements are done by using different fluorescent dyes that bind either specifically (e.g., TaqMan® probes) or un-specifically (e.g., SYBR® Green I or EvaGreen®) (Brankatschk et al., 2012). An increase in the fluorescence is proportional to the increase of the PCR product, where the reagents are the limitation. The number of cycles necessary for reaching the fluorescent threshold value is defined as the quantification cycle (Cq) value. A high concentration of DNA results in a low Cq value, whilst a low concentration result in a high Cq value.

1.3.2 Genetic markers for microbial studies

The use of the 16S rRNA gene

Molecular methods based on the 16S rRNA gene have revolutionized the scientific community regarding taxonomic determination (Dubnau et al., 1965; Fox et al., 1977). The 16S ribosomal RNA (rRNA) gene has been used extensively when dealing with the classification of bacteria and archaea down to a low taxonomic order. The gene is highly conserved, present in all prokaryotes, and classification is possible due to its 9 variable regions (V1-V9). The variation is, however, not big enough to be able to classify down to species level. Within some taxa the interspecies similarity can be as high as 99.9% (García-López et al., 2014), leading to the search of other molecular markers usable for studying bacteria down to species level.

Detection of *Bifidobacterium*

Within the *Bifidobacterium*-taxa, the interspecies similarity of the 16S rRNA sequence has a mean of 95% (ranges from 87.7-99.5%) (Ventura et al., 2006), making it difficult to investigate the different species of *Bifidobacterium* found in a sample. Thus, classification of *Bifidobacterium* down to (sub)species level has been a challenge, and several molecular markers have been studied. This includes *recA, atpD, dnaK,* and *groEL,* but these markers are not ideal because of the lack of databases available.

In 2005, Ventura et al. (2005) proposed the *clpC* gene as a genetic marker for *Bifidobacterium*. The *clpC* gene is a housekeeping gene that encodes the ATPases Associated with diverse cellular Activities (AAA+) superfamily protein *ClpC* (Kojetin et al., 2009). This protein belongs to the functional chaperone category, functioning in stress tolerance when exposed to heat. The study described in Ventura et al. (2005) included the partial sequence of seven genes, *clpC, dnaB, dnaG, dnaJ1, purF, rpoC,* and *xfp*. However, phylogenetic positioning showed that *clpC* had a general agreement with 16S rRNA sequences, but it possessed a higher power of discrimination.

1.3.3 Sanger sequencing

Sanger sequencing is a first-generation sequencing technique using dNTPs and visualization with electrophoresis (Sanger et al., 1977). The process involves amplification of DNA, and heat is used to denature double-stranded DNA (dsDNA) to single-stranded DNA (ssDNA). A primer is added on the 5' side of the DNA. The primed DNA is then dispersed into four reaction vessels. Next, DNA polymerase is added to the four tubes, together with four deoxynucleotide

triphosphates (dNTPs). One out of four specially modified dideoxynucleotide triphosphates (ddNTPs) is added to the reaction vessels. ddNTPs are analogue to the monomer dNTPs, but they differ in a OH-group in the 3'-position on the dNTP. The polymerase attaches the dNTPs to the template strand at the primer until a ddNTP is base paired. Once this is base paired, the sequence is terminated as a result of the lacking hydroxyl group. The chain termination leads to formation of DNA fragments across the reaction vessels (Valencia et al., 2013).

To sequence DNA, polyacrylamide gel electrophoresis is used. The negative charge in DNA leads to migration of the DNA from a negative to a positive pole. The smaller DNA lengths will travel further through the gel than the longer ones, as the same force is applied to a smaller mass. It is vital to use polyacrylamide gel instead of agarose gel because of its high resolving power, and it can separate DNA strands that differ in length by one base pair. The sequence is then read from the bottom, and the process gives us the complementary sequence of DNA. In 1986 an automated Sanger sequencing appeared, including automation of gel electrophoresis, detection of fluorescent DNA band patters, and analysis of bands (Smith et al., 1986).

1.3.4 Illumina dye sequencing

Illumina dye sequencing is a next-generation sequencing (NGS) technique, also referred to as second-generation sequencing, based on reversible dye-terminators that enable the identification of single bases as they are introduced into DNA strands (Illumina, 2021). The NGS made its arrival 15 years ago and has been revolutionary due to the possibility of sequencing DNA and RNA faster and less expensively than previously used sequencing methods.

The NGS revolves around three basic steps. First, there is a sample preparation of extracted DNA, where adapters are ligated to the DNA. The adapters contain sequencing binding sites and a complementary sequence that makes hybridization to the flow cell possible. Next, heat is added to denature the double-stranded DNA (dsDNA), and the single-stranded DNA (ssDNA) attaches to the flow cell because of the adapters. DNA polymerase synthesizes the complementary strand of the ssDNA, and the original strand is washed away. The complementary strand attaches to another oligonucleotide on the flow cell and is synthesized. This process is called bridge amplification because of the design's similarity to a bridge. Denaturation makes two ssDNA that are both attached to the flow cell. The flow cell has hundreds of thousands of oligonucleotides loaded on to it, so the bridge amplification is

repeated multiple times, resulting in the amplification of the DNA fragment into millions of ssDNA copies in a process called cluster generation. The reverse strand is cleaved off the flow cell, and sequencing begins by binding a primer to the oligosaccharide, and sequencing by synthesis is performed with specific fluorescently labelled nucleotides. Finally, the fluorescent signal is read by a machine (Illumina, 2021).

1.4 Taxonomy assignment

1.4.1 The National Center for Biotechnology Information

The need for bioinformatic tools and databases has grown in parallel with the increasing use of sequencing. The National Center for Biotechnology Information (NCBI) is an online library for biotechnology databases necessary for bioinformatics tools and analysis (NCBI, 1988). Over the past decade, the library has been filled with DNA sequencing databases and algorithms, characterizations and information about genes and proteins. In addition, numerous articles and books are available for scientists worldwide. The NCBI library contains the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990), a program that provides a statistical significance of taxonomic matches. This is a database often used when analysing sequencing retrieved from Sanger sequencing (see section 1.3.3).

1.4.2 Operational taxonomic units

The most common method of taxonomy assignment is to construct an operational taxonomic unit (OTU) table. This is a clustering method that clusters based on similar sequences. The similarity is given a specific threshold value, often 97 %. One disadvantage of this technique is that many bacteria species have variations on a nucleotide level, which leads to more than one bacterium species specific to one OTU. Therefore, this approach is often triangulated with 16S rRNA sequencing, where detection on a species level is unnecessary. The processing of the sequences can be conducted by different RStudio pipelines, such as QIIME (Quantitative Insight Into Microbial Ecology). The QIIME-pipeline is used to process the FastQ files by removing primers, demultiplexing, filtering by quality, and creating an OTU table. Assigning taxonomy to the OTU table is then performed by a database, e.g., the SILVA database (Pruesse et al., 2007). The SILVA database is an up-to-date quality-controlled database including sequencing for both the small and large rRNA subunits (Quast et al., 2012).

1.4.3 Amplicon sequence variants

The OTU method is, as previously mentioned, limited with a 97 % threshold value, leading to the rise of alternative methods. The use of amplicon sequence variants (ASVs) is another approach that, in contrast to the use of OTUs, gives a separation down to the nucleotide level. A pipeline that is compatible with ASVs is DADA2 (Divisive Amplicon Denoising Algorithm 2) (Callahan et al., 2016). This software R-package involves functions to perform every processing step from demultiplexed FastQ files to a finished amplicon sequence variant profile. The resulting ASV profile can provide information about how many times the exact ASV was observed in the different samples (Callahan et al., 2015), and taxonomy is assigned by running it through a database. A suitable database for ASVs is the Kraken database, which provides genus-level taxonomy assignment to shorter DNA sequences (Wood & Salzberg, 2014).

1.5 PreventADALL

The current master thesis is a part of Preventing Atopic Dermatitis and Allergies in Children (PreventADALL) (OUS, 2021). This is an ongoing, population-based study focusing on the increase of allergic and immune-related diseases in children. The study aims to identify factors contributing to such diseases, investigate measures to prevent allergies in infants, and translate the research into public health interventions. One of the latest discoveries conducted by PreventADALL was that skin emollient and introduced complementary feeding did not influence the development of atopic dermatitis by the age 12 months (Skjerven et al., 2020).

The current thesis uses data from the PreventADALL cohort. The cohort includes biological samples (i.e., stool- and skin samples) and detailed questioning sheets collected from 2397 mother-child pairs from Oslo, Østfold, and Stockholm. The recruitment period for the mothers was between December 2014 and October 2016, while the children were enrolled after birth. The mothers delivered their first and only stool sample in week 18 of their pregnancy, and the infants delivered stool samples in months 0, 3, 6, 12, 24, and 36. In addition to stool samples, a skin swab from the elbow-hook of the infants was taken within 10 minutes after birth. Included in the current thesis are stool from months 0 and 3, the skin swab, and stool from the corresponding mother.

1.6 Aim of thesis

Bifidobacterium is identified as one of the earliest colonizers in an infant's gut (Ruiz et al., 2017; Turroni et al., 2019). A potential route of colonization seems to be linked to a mother-infant transmission through breastfeeding (Lawson et al., 2020; Matamoros et al., 2013; Underwood et al., 2015). However, the exact timing of colonization remains unsettled. The hypothesis addressed in the current thesis is that *Bifidobacterium* is colonized in the human infant gut through a selection by mother's milk.

The main aim of this thesis was to investigate mother to infant association of *Bifidobacterium* using material from PreventADALL. To address the main objective, the following sub-goals were included:

- Examine the *Bifidobacterium* association of mother-infant pairs.
- Examine the difference in microbiota of infants with different modes of delivery.
- Cultivate samples to investigate the presence of living Bifidobacterial cells in meconium.

The main aim and sub-goals were addressed by extracting *clpC*- and 16S rRNA genes from bacteria in fecal- and skin samples from mother-infant pairs and sequencing them on an Illumina MiSeq.

2. Material and methods

2.1 Outline of experimental design

The current thesis is part of an ongoing mother-infant study of the human gut microbiota. The experiments conducted during this master period and beforehand by another student are shown in figure 2.1.

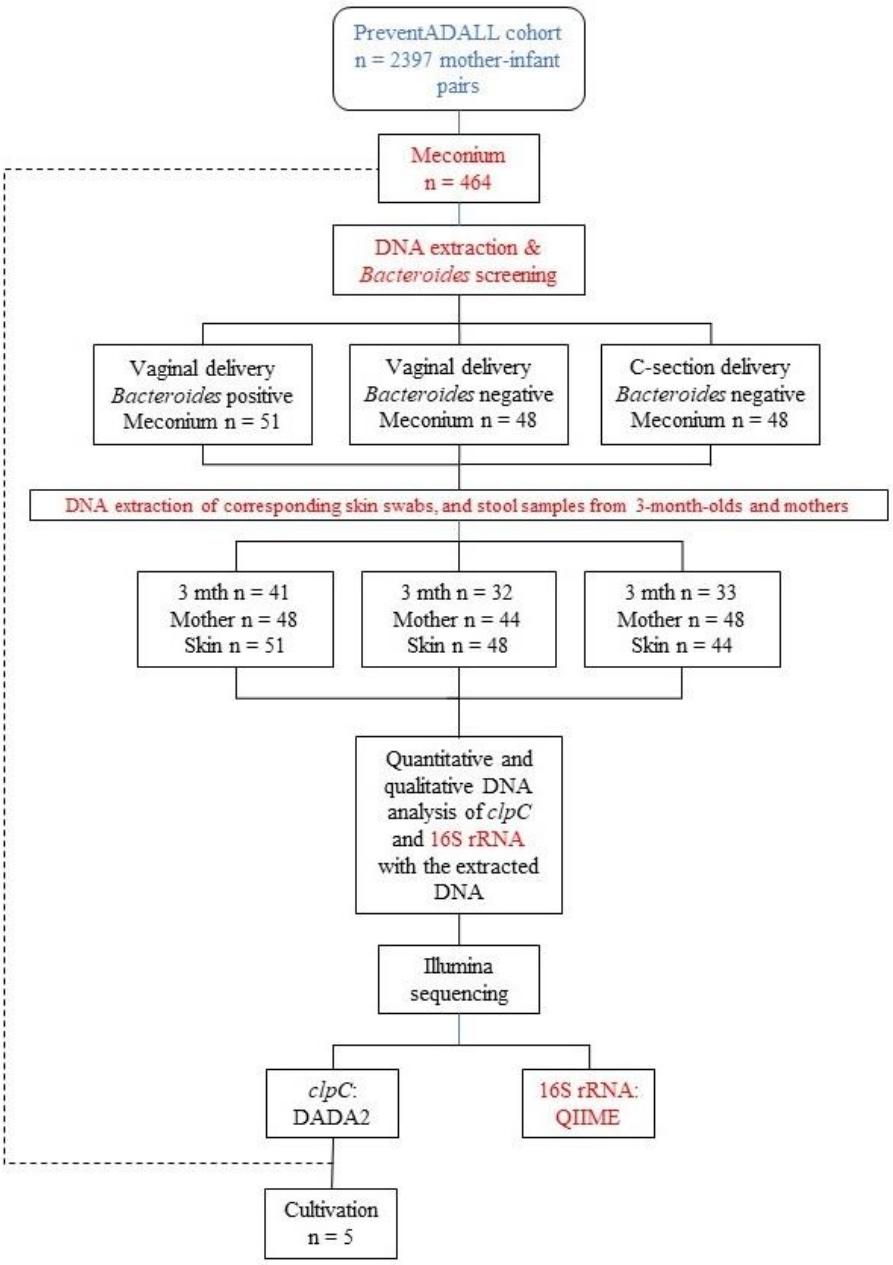

Figure 2.1: Workflow of this thesis. Collection of the samples were done by the PreventADALL cohort. DNA extraction and screening for *Bacteroides* was performed, sorting the meconium into three groups: "Positive for *Bacteroides* vaginal delivery", "Negative for *Bacteroides* C-section" and "Negative for *Bacteroides* vaginal delivery". Skin swabs and stool samples from 3-month-olds and mothers, corresponding to the meconium, were included and DNA extraction was performed. Samples were prepared for Illumina sequencing with both *Bifidobacterium*-selective primers, *clpC*, and 16S rRNA-primers. Post-processing included the use of the DADA2- and QIIME-pipeline on the output from the *clpC*- and 16S rRNA-sequences, respectively. Cultivation of five meconium samples positive for *Bifidobacterium* was done to investigate the presence of living bacteria. The steps marked in red were performed by Regina Sørensen and Morten Nilsen.

2.2 Sample description and preparation

2.2.1 Sampling and storage

The samples used in this thesis were collected from 147 mother-infant pairs, where 99 infants were born by vaginal delivery and the remaining 48 by c-section. This include meconium (an infant's first stool) collected within 72 hours after birth, elution swabs (E-Swabs)/skin samples from the elbow hook of newborns, stool samples from when the child was 3 months old, and stool samples from the mothers when they were 18 weeks pregnant (table 2.1). All the stool samples used for DNA analysis were stored in a DNA stabilizing buffer (1:10) (Nordic Biolabs, Sweden) to avoid DNA degradation. The E-Swabs were stored in 1 mL Amies transport media. After delivery to the Norwegian University of Life Sciences, the samples were stored at -80°C until the DNA analysis started. The nutrients for the 3-month-olds are listed in table A.1, appendix A.

Table 2.1. Number of samples included in this study derived from skin, meconium, and stools from 3-month-old infants and their mother stratified by vaginal and c-section delivery.

Skin	Meconium	3-month-olds	Mothers
Vaginal delivery			
99	98	73	92
C-section delivery			
44	48	33	40

2.2.2 Initial handling

The stool and skin samples were thawed on ice and vortexed to homogenize the solution. Next, 1-1.2 mL of the stool samples were transferred to Eppendorf tubes and pulse centrifuged at 12 000 rpm to remove big particles. The initial handling and DNA extraction for the skin samples were carried out employing the protocols described in Rehbinder et al. (2018). To harvest the bacterial cells from these samples, 1 mL of the centrifuged stool samples were transferred to new tubes and centrifuged at 13 000 rpm for 15 minutes.

Further, the cell pellet from the skin samples were resuspended and homogenized in Stool Transport and Recovery (S.T.A.R) buffer (Roche, USA), which can inactivate infectious organisms, limit degradation of nucleic acids and increase binding of nucleic acids to magnetic particles (Espy et al., 2006). The stool was processed in DNA shield buffer.

2.3 DNA extraction and quality control

2.3.1 Isolation of DNA from stool- and skin samples

DNA extraction was performed with a combination of mechanical and chemical cell lysis. Mechanical lysis ensures the lysis of most bacterial cells and was achieved by mixing the sample in FastPrep tubes containing 3 different glass beads: 0.2 g acid-washed beads <106 µm (Sigma-Aldrich, USA), 0.2 g acid-washed beads 425-600 µm (Sigma-Aldrich, USA), and 2 x 2.5-3.5 mm acid-washed beads (Sigma-Aldrich, USA). The tubes were processed twice at 1800 rpm for 40 sec on a FastPrep 96 (MP 20 Biomedicals, USA). Further, the lysed bacterial cells were centrifuged at 13 000 rpm for 5 min at 4˚C. Contrarily, to avoid stiffening of the S.T.A.R. buffer, the skin samples were centrifuged at 25˚C.

Most of the samples were extracted automatically by a KingFisher Flex robot (Thermo Fischer Scientific, USA) with a MagMidi LGC extraction kit (LGC Biosearch Technologies, UK). Magnetic particles, in combination with positively charged salt, extract DNA by utilizing its negative charge. Finally, nuclease-free water (VWR, USA) was added to elute the DNA.

2.3.2 DNA extraction following cultivation

DNA extraction was performed on bacterial colonies grown on Bifidus Selective Medium (BSM) (Sigma-Aldrich, Germany). A description of the media is provided in appendix B. The colonies were mixed with 200 µl STAR buffer and the extraction was performed as described in the section above (section 2.3.1). Another method used for extraction was boiling the colonies in 25 µl 1x TE-buffer at 99°C for 10 minutes.

2.3.3 Gel electrophoresis

To investigate the presence of DNA, all PCR products were checked for successful DNA amplification on a 1.5 % agarose gel, before and after purification. The gel was prepared by dissolving agarose (Sigma Aldrich, Germany) in 1x tris-acetate EDTA (TAE) buffer. PeqGreen (Peqlab, Germany) was added to the agarose mixture before casting. This is a DNA/RNA dye that insert itself between the base pairs in the double helix. In addition, 6x loading dye (Thermo Fischer Scientific, USA) was added to the purified PCR products for visual tracking of the DNA migration.

Determination of the amplicon size is possible due to an electric current and the migration abilities of the negative DNA fragments to a positive electrode. The electrophoresis ran at 80 V with 400 mA for 35 minutes. A 100 bp DNA ladder (Solis Biodyne, Estonia) was added to the first well of each gel as a size marker. The gel was visualized by The Molecular Imager® Gel Doc™ XR Imaging system with Quantity One 1-D (BioRad, USA).

2.3.4 Qubit dsDNA High Sensitivity Assay

To quantify the amount of genomic DNA in the samples and verify the DNA extraction, a Qubit fluorometer (Life Technologies, Waltham, MA) was used. The quantification was done with the double-stranded DNA high sensitivity assay kit (Life Technologies, USA). Assay tubes were prepared with 2 µl DNA samples and 198 µl working solution, containing Quant-iT™ reagent in a volume of 1:200 in Quant-iT™ buffer.

2.4 Polymerase chain reaction

To identify the presence of the *clpC*-gene, polymerase chain reaction was performed on eight different 96 plates, including 536 samples from 147 mother-infant pairs divided into vaginally delivered and c-section delivered infants. In addition, positive and negative extraction controls, and PCR controls were added to each plate. The primers used in this study are listed in table 2.2. Details of the 16S rRNA analysis are available in a previous study (Sørensen, 2020).

Table 2.2. Overview of the primers used in this study. F stands for "Forward primer" and R for "Reverse primer".

Primer name	Target	Primer sequence (5'-3')	Reference
***clpC*-F**	*Bifidobacterium* (position 2457-3018)	GAGTACCGCAAGTACATCGAG	(Ventura et al., 2006)
***clpC*-R**	*Bifidobacterium* (position 2457-3018)	CATCCTCATCGTCGAACAGGAAC	
PRK341F	V3-V4 region of the 16S rRNA gene	CCTACGGGRBGCASCAG	(Yu et al., 2005)
PRK806R	V3-V4 region of the 16S rRNA gene	GGACTACYVGGGTATCTAAT	
Mangala F-1	16S rRNA – total bacteria	TCCTACGGGAGGCAGCAG	Genetic analysis
16SUR	16S rRNA – total bacteria	TCCTACGGGAGGCAGT	Genetic analysis

2.4.1 Quantitative PCR

Quantitative PCR, or qPCR, was used to quantify the number of bacteria from the extracted samples based on the *clpC* gene. With a total volume of 20 µl, each reaction was added to a Lightcycler 480 multiwell plate 96 (Roche, Germany). The wells contained 1x HOT FIREPol®EvaGreen® qPCR supermix* (Solis Biodyne, Germany), 0.2 µM of primers targeting *clpC* (positions 2457–3018) (according to table 2.2), nuclease-free water, and 2 µl template DNA. A sample of *B. bifidum* 38 ng/µl (DSM 20456) (DSMZ, Germany) was diluted 10-fold, leading to a final concentration of 3.8 ng/µl. 2 µl (~7.6 ng) of this sample was added as a positive control. In addition, a negative control, nuclease-free water, was also included. Initial denaturation was set to 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 30 sec, an annealing step for 30 sec at 51 °C, elongation at 72°C for 45 sec, and final elongation at 72 °C for 7 min before cooling at 10 °C ∞. All reactions were performed on a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad).

2.4.2 Qualitative PCR

Each reaction, with a total volume of 25 µl, contained 1x HOT FIREPol® Blend Master Mix Ready to Load (Solis Biodyne, Germany), 0.2 µM primers (according to table 2.2), nuclease-free water, and 2 µl template DNA. In addition, *B. bifidum* DSM 20456 (diluted, ~7.6 ng), and nuclease-free water were added as positive and negative control. The mixture was applied to a 96 PCR Plate (VWR, Germany). Initial denaturation was set to 95 °C for 15 min, followed by 33 cycles, 30 cycles for the mother samples, of denaturation at 95 °C for 30 sec, an annealing step for 30 sec at 51 °C, elongation at 72°C for 45 sec, and a final elongation at 72 °C for 7 min before cooling at 10 °C ∞. The qualitative PCR was performed on a 2720 Thermal Cycler and checked on 1.5% agarose gel (described in section 2.3.3).

2.4.3 CoverAll® PCR

DNA extracted from cultivation was prepared for Sanger sequencing by PCR with CoverAll® primers (table 2.2). The reaction mix was as described in section 2.4.2, the only difference being the primers, which targets a larger portion of the 16S rRNA gene. Initial denaturation was set to 95 °C for 15 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, an annealing step for 30 sec at 55 °C, elongation at 72°C for 1 min and 20 sec, and a finally a cooling at 10°C.

2.4.4 Purification of the PCR products

To obtain pure PCR products (originating from the work described in section 2.4.2 and 2.4.3), a purification step was performed. Most of the PCR products were purified automatically, following the protocol for AMPure purification on Biomek 3000 (Beckman Coulter, USA). To bind and isolate PCR products, 0.1 % SeraMag Speed Beads (Sigma Aldrich, Germany) were used. The concentration of the Sera-Mag Speed Beads was decided by looking at the amplicon size. The ratio used between the beads and PCR products was 1:1. To ensure removal of inhibitors, primer dimers, salt, and larger DNA fragments, the amplicons were cleaned twice with 80 % ethanol. Nuclease-free water was used to elute the cleaned PCR products. To validate the purification, some of the samples were checked on 1.5 % agarose gel (as described in 2.3.3). Some of the PCR products (n=100) were purified manually, following the same protocol as the Biomek 3000.

2.5 DNA sequencing

2.5.1 Sanger sequencing

Sanger sequencing was performed on two different occasions; to identify DNA extracted from 20 colonies from cultivation of meconium samples, and to identify DNA extracted from liquid BSM broth after 0 and 48 hours (the media and solutions used in cultivation are described in appendix B). 5 µl of extracted DNA was mixed with 5 µl of forward primer. The extracted DNA was processed with CoverAll-primers (see table 2.2). The mixtures were sent to GATC BioTech (Oslo) for sequencing. Sequences retrieved from the Sanger sequencing were identified from the NCBI database using the Nucleotide BLAST.

2.5.2 Illumina sequencing

Index PCR

Index PCR involved an extension of the fragments with Illumina-specific adapters using modified Illumina-indexed *clpC*- and 16S rRNA-primers (performed in Sørensen (2020), primers listed in appendix C, table C.1). A total of 16 forward and 36 reverse index primers (listed in appendix C, table C.2) were designed for the *clpC* sequencing, making it possible to map all modified samples back to the original sample. Each reaction, with a total volume of 25 µl, contained 1x FIREPol® Master Mix Ready to Load (Solis Biodyne, Germany), nuclease-free water, 0.2 µM unique primer sets, and 2 µl extracted DNA. The unique primer sets were added using an Eppendorf epMotion 5070. The thermal conditions were as followed; 95 °C for

5 min, followed by 10 cycles of 95 °C for 30 sec, 51 °C for 1 min, and 72 °C for 45 sec, with a final elongation step at 72 °C for 7 min before cooling at 10 °C ∞. Further, the PCR products were normalized and pooled together as described in the next sections.

Quantification and Normalization

Quantitative DNA measurements were performed using a Cambrex-FLX800 CSE robot (Thermo Fischer Scientific, USA). A Qubit Working solution was prepared for all samples, and a standard curve was made using the fluorescence data from the lowest to highest fluorescence value by adding Qubit reagent. Further, DNA concentration was calculated based on the standard curve. Normalization and pooling were done on Biomek 3000. To prevent dilution of the pooled library, samples without sufficient DNA were not included further. This selection process consisted of a combination of Cq values and bands' visibility on the gel electrophoresis of the indexed PCR products. The samples from the categories within vaginally delivered infants and their mothers selected for sequencing included 14.14% (n=14) of the skin swab samples, 37.76% (n=37) of the meconium samples, 68.49% (n=50) of the stool samples from 3 months of age, and 85.87% (n=79) of the mother's stool samples. From the c-section delivery, the samples included were 11.36% (n=5) of the skin swabs samples, 56.25% (n=27) of the meconium samples, 84.85% (n=28) of the stool samples from 3 months of age, and 83.33% (n=40) of the mother's stool samples. After normalization, a purification step was performed using a 1:2 ratio of the library and AMPure beads (full description in section 2.4.4).

Quantification of Illumina library

To measure the amount of DNA present in the library, quantification using KAPA Library Quantification Kit for Illumina Platforms (Roche, Germany) was performed. 20 µl were added to a Lightcycler plate, with 2 µl of DNA, and 18 µl of the following master mix; PCR mix containing 2x KAPA SYBR FAST qPCR Master Mix and 10x Primer premix, and nuclease-free water. The kit included 6 DNA standards where 2 µl of each were added to different wells. In addition to the standards, 2 µl of 10^{-4} to 10^{-7} dilutions of the library and negative control were added to appropriate wells. The qPCR was performed with the following cycling protocol: denaturation for 5 min at 95 °C, annealing for 30 sec at 95 °C, extension for 45 sec at 60 °C, in addition, a melting curve analysis from 65-95 °C.

Denaturation of DNA and combination of amplicon library and PhiX

For Illumina MiSeq sequencing, the Miseq V3 kit (Illumina, USA) was used. The pooled library was diluted to 4 nM in nuclease-free water. The dilution was mixed with 0.2 M NaOH and diluted a second time to 6 pM in hybridization buffer, HT1. A 6 pM PhiX control was combined with the diluted library, giving a final concentration of 15% PhiX. The combined mixture was incubated at 96°C for 2 minutes, mixed and placed on ice until it was loaded on Illumina Miseq Sequencing Platform by the lab personnel.

2.5.3 QIIME for 16S rRNA sequences

The QIIME-pipeline was used to process the files by removing primers, demultiplexing, and filtering by quality. Contaminants were removed using the R-package "decontam". In addition, a contaminant, *Burkholderiales-Paraburkholderiales*, found throughout many of the samples, including the negative control, was removed. The resulting OTU-table was run through the Silva database to assign a bacterial taxonomy with a similarity of 97%, and samples with less than 5000 sequences were not investigated further.

2.5.4 DADA2 for *clpC* sequences

DADA2 was used for phylogenetic analysis of the *Bifidobacterium* sequences retrieved from Illumina sequencing. The pipeline was run through RStudio, version 4.0.3 (appendix D.1). First, demultiplexing was performed to separate sequences with unique indexes to make it possible to map samples back to the original sample. After demultiplexing, sequences were filtered based on quality scores, dereplicated, base incorporation errors were learned and applied, chimeras removed, and finally, forward and reverse reads were merged. The resulting ASVs were annotated taxonomy using the Kraken standard database.

2.6 Statistical analysis

2.6.1 Wilcoxon Signed-Rank Test

The Wilcoxon Signed-Rank Test (Wilcoxon, 1945) was performed to investigate the *Bifidobacterium* association between the mother and the infant at age 0 and 3 months. This is a non-parametric test used to compare two related samples with no normal distribution, and the null hypothesis is that there is no difference. The closer the p-value is to 1, the higher the association.

2.6.2 Chi-square (X^2) test

A Pearson's chi-square test with Yates ' continuity correction (Pearson, 1900) was performed to investigate if there was a significant difference between the association of *Bifidobacterium* across the different modes of delivery. The test works from a null hypothesis where no difference is true. All ASVs of the sequenced samples were merged to the highest taxonomic level detected, and a binarizing assigning presence of a species of *Bifidobacterium* to 1 and no sequences present to 0, was performed.

2.7 Cultivation strategies for *Bifidobacterium*

The overview of the cultivation experiment is illustrated in figure 2.2.

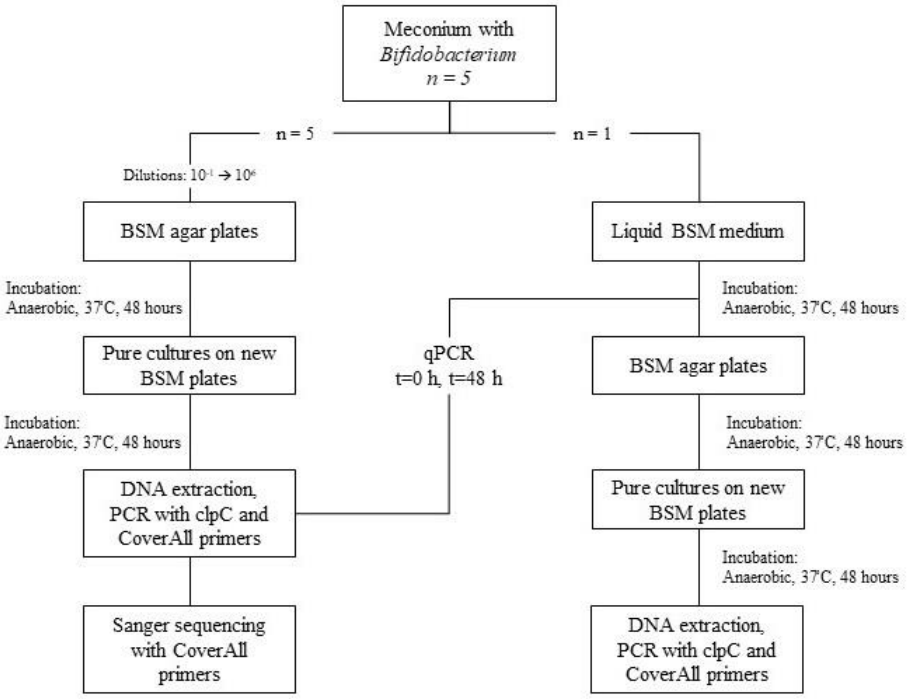

Figure 2.2. Workflow of the cultivation experiment. Investigation of living *Bifidobacterium* cells was done by using Bifidus Selective Medium (BSM).

Investigation of the presence of living cells of *Bifidobacterium* in meconium was performed by cultivating five meconium samples with positive hits for *Bifidobacterium* after sequencing. The samples were diluted 10-, 100-, 1 000-, 10 000-, 100 000, and 1 000 000-fold in Milli-Q water, streaked on BSM plates, and incubated anaerobically in chambers at 37°C for 48 hours. 2.5 L AnaeroGen (Oxoid, USA) were added to the chambers to create anaerobic conditions. After incubation, pure cultures were streaked on new BSM plates and a second anaerobic incubation at 37°C for 48 hours was performed. A lab strain of *B. infantis* (DMS 20088) was used as a positive control. The approach and ingredients for the BSM are described in Appendix B. An additional incubation step was added for one of the meconium samples by mixing it directly into liquid BSM broth before streaking it on BSM agar plates. qPCR with *clpC*-primers was performed on the samples to investigate the amount of *Bifidobacterium* before and after incubation.

3. Results

3.1 Qualitative analysis by qPCR

To get an overview of the 16S rRNA- and *clpC* gene quantity in the different sample categories, a qPCR analysis was performed. This section represents the bacterial load within the sample categories skin, meconium, and stool from 3-month-olds and mother, but not the difference between delivery modes.

3.1.1 Determination of bacteria load based on the 16S rRNA gene

The Cq values from the qPCR targeting the 16S rRNA gene showed a high variation between the different sample categories, ranging from 21 to 40. The lowest Cq values, and therefore the highest bacterial load, were observed in the mothers and 3-month-olds, with an average Cq of 23 and 29, respectively. The samples with the highest Cq values and the lowest bacteria load were observed on the skin (average 35) and meconium samples (average 35). More details about Cq values, and the number of N/A-samples are presented in appendix E, table E.1.

3.1.2 Determination of bacteria load based on the *clpC* gene

The Cq values of the *clpC* gene for all sample categories varied, but overall, the values were high. The lowest Cq values were observed in samples from mother and 3-month-olds, with an average of approximately 34 for both. The skin and meconium samples both showed an average Cq value of around 37. In addition to high Cq values, there was also an increased number of samples (n=141) that did not give a Cq value. This indicates that there is an insufficient quantity of DNA from this gene in the samples, or no presence of *Bifidobacterium*. More details about Cq values, and the number of N/A-samples are presented in appendix E, table E.1.

3.2 Analysis of the 16S rRNA- and *clpC*-sequencing data

The sequencing data was divided into two main groups for the data analysis: vaginally- and c-section delivered infants. In addition, each main group was divided into four sample categories: skin, meconium, and stool from 3-month-olds and mothers. The division was done to make comparison between the sample categories within the different mode of deliveries possible.

3.2.1 Taxonomic composition from mother and infants based on the 16S rRNA gene

The QIIME-pipeline was used to process the sequences retrieved from Illumina sequencing with 16S rRNA primers. Based on the files retrieved from the QIIME-pipeline, the taxonomic composition within each group could be investigated (figure 3.1). From the vaginally delivered group, the samples selected for sequencing included 90 (90.91%) of the skin swab samples, 96 (97.96%) of the meconium samples, 53 (72.60%) of the stool samples from 3 months of age, and 89 (96.74%) of the mother's stool samples. From the c-section delivered infants, the samples included 100% of the skin swabs samples, meconium samples and mother's stool samples, and 20 (60.61%) of the stool samples from 3 months of age.

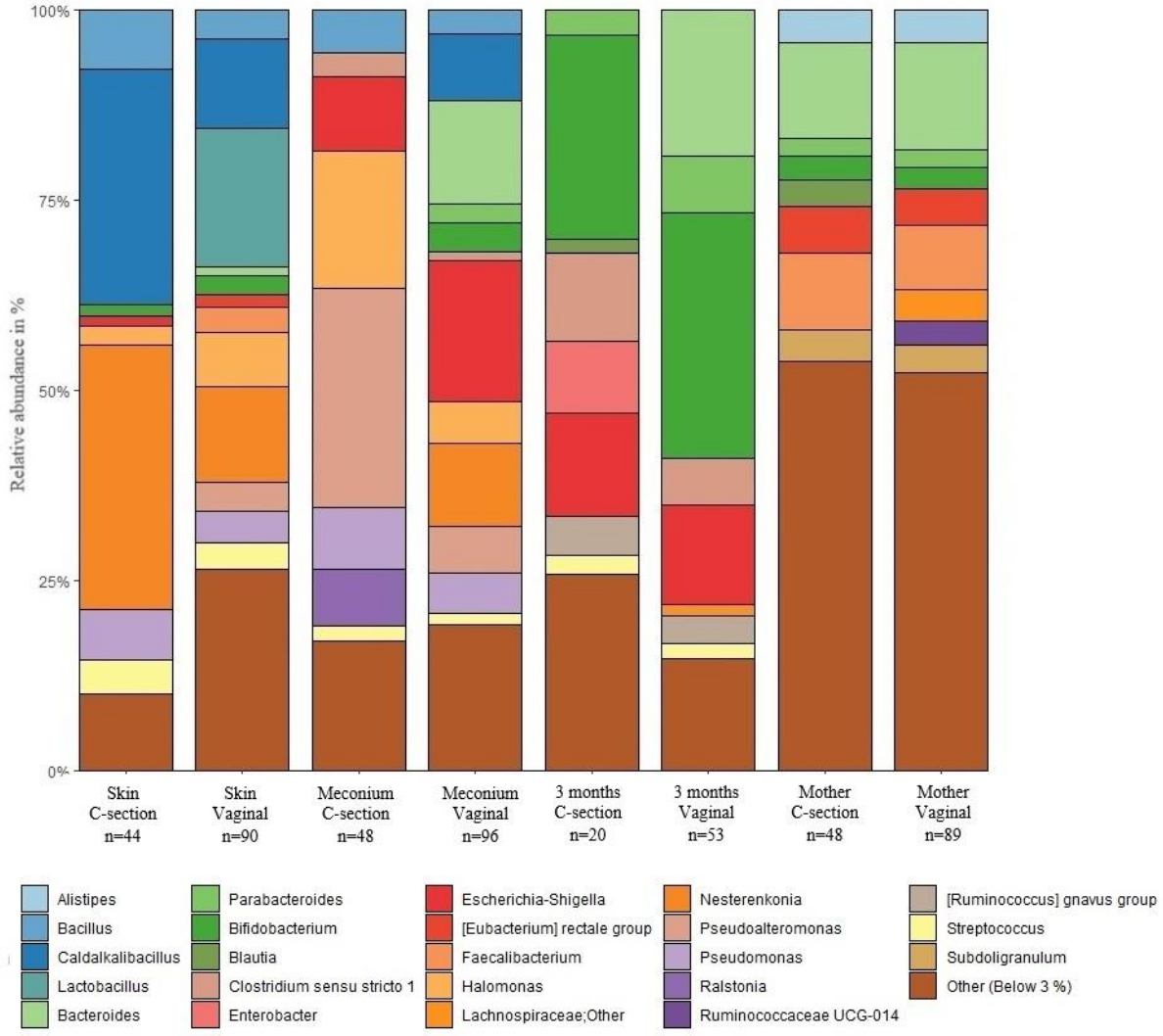

Figure 3.1 Taxonomic composition on the lowest possible taxonomic level based on 16S rRNA sequencing. The average relative bacteria abundance (y-axis) within the different sample categories (x-axis) were obtained from data used in Sørensen (2020). The different bacteria species are presented with different colours. The total number of sequenced samples is represented as n. The "Other (Below 3%)"-group represents 27 bacteria-classifications with a relative abundance of under 3% for all the sample categories.

Composition of *Bifidobacterium* in the different sample categories

The taxonomic composition varied within each of the sample categories (figure 3.1). The highest abundance of *Bifidobacterium* was found in the stool of 3-month-olds, with 32.18% in the vaginally delivered infants and 26.87% in the c-section delivered children (figure 3.1). The skin and meconium samples had a low quantity of *Bifidobacterium,* with 2.45% and 3.78% in vaginally delivered infants and 1.51% and 0% in c-section delivered infants, respectively (figure 3.1). The relative abundance of *Bifidobacterium* was approximately 3% in mothers, regardless of how they gave birth.

Composition of other species

The elution swabs from skin of vaginally delivered infants had *Lactobacillus* (18.02%) and *Nesterenkonia* (34.73%) as the most abundant species (figure 3.1). Meconium samples from these infants had other dominant bacteria: *Escherichia-Shigella* (18.53%) and *Bacteroides* (13.51%). The bacteria found on the skin and in meconium from c-section delivered infants are most likely contaminations. The skin samples had *Caldalkalibacillus* (30.91%) as the most dominant species, while with the meconium samples, *Pseudoalteromonas* (28.73%) and *Halomonas* (18%), was the most abundant (figure 3.1). The two mother categories had the highest amount of diversity, with over 50% of the bacteria belonging to the "Other (Below 3%)" group (figure 3.1). This group represents all bacteria with a relative abundance under 3% (appendix F).

3.2.2 *Bifidobacterium* composition based on *clpC* sequencing

The sample selection for the sequencing run with the *clpC* gene was performed by investigating the visibility of bands from the gel electrophoresis after index PCR. The sequences retrieved from the Illumina sequencing of *clpC,* resulted in 125 444 sequences belonging to 1039 unique ASVs. Out of the 1039 ASVs, only 128 (12%) were allocated to a Bifidobacterial species, where 55 092 (44%) of the sequences belonged to these ASVs. The Bifidobacterial ASVs were identified as 10 different *Bifidobacterium* groups: *Bifidobacterium, Bifidobacterium adolescentis, Bifidobacterium animalis, Bifidobacterium bifidum, B. breve, Bifidobacterium catenulatum, B. longum, B. longum* subsp. *infantis, B. longum* subsp. *longum,* and *Bifidobacterium pseudocatenulatum*.

Bifidobacterium, B. animalis, B. catenulatum, B. longum subsp. *longum* and *B. pseudocatenulatum* had a relative abundance of below 1% for each sample category and were therefore merged into one group "Other (Below 1%)".

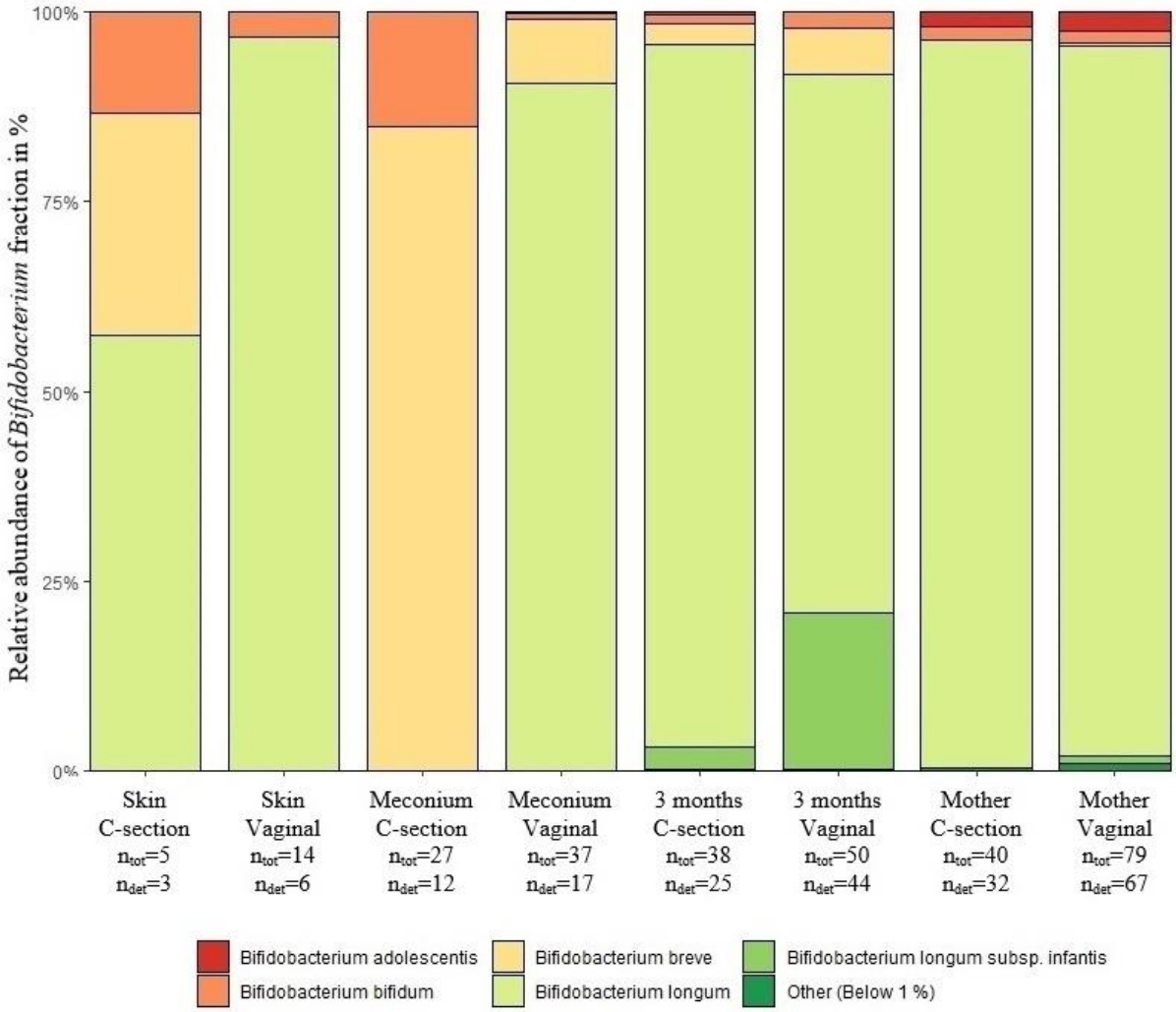

Figure 3.2 *Bifidobacterium* species composition based on *clpC* sequence data. The total number of sequenced samples is represented as n_{tot} under the bars, and the number of these samples with presence of *Bifidobacterium* is represented as n_{det}. The relative *Bifidobacterium* abundance (y-axis) is based on the accumulated number of *Bifidobacterium* reads within each sample category. The different species of *Bifidobacterium* is presented with different colours. The "Other (Below 1%)"-category represents species of *Bifidobacterium* with a relative abundance of under 1% for all the sample categories.

Composition of *Bifidobacterium* in vaginally delivered infants

B. longum was the most abundant species (over 90%) for all sample categories within the vaginally delivered group (figure 3.2) (appendix D.2, RStudio-pipeline). It was found in 36% of the sequenced skin samples (n_{det}=5, n_{tot}=14), 41% of the meconium samples (n_{det}=15, n_{tot}=37), 84% (n_{det}=42, n_{tot}=50) of the 3-month-olds' stool and 80% (n_{det}=64, n_{tot}=79) of the

mothers (appendix G, table G.1). The second most abundant species of *Bifidobacterium* on the skin of newborns was *B. bifidum* (figure 3.2), found in 14% (n_{det} =2, n_{tot}=14) of the samples with a relative abundance of 3.27%. In meconium and 3-month-olds, *B. breve* was the second most abundant species (figure 3.2). It was present in 5% of the meconium samples (n_{det}=2, n_{tot}=37), and 8% (n_{det}=4, n_{tot}=50) of the 3-month-olds, and amounted approximately 7% of the *Bifidobacterium* content for both categories. *B. adolescentis* and *B. bifidum* was also observed in small amounts in the meconium (figure 3.2). In mother's stool, *B. adolescentis* and *B. bifidum* were the second and third most abundant *Bifidobacterium* (figure 3.2), found in 22% (n_{det}=17, n_{tot} = 79) and 15% (n_{det} 12, n_{tot} = 79) of all samples, respectively, and both amounted to ~2% of the composition.

Composition of *Bifidobacterium* in c-section delivered infants

B. longum was the most abundant species for three sample categories: skin, 3-month-olds, and mothers (figure 3.2). It was observed in 40% (n_{det}=2, n_{tot} = 5), 85% (n_{det}=24, n_{tot} = 28) and 78% (n_{det}=31, n_{tot} = 40) of the samples, respectively, and amounted 57% of the composition on the skin, and over 94% for the two latter (appendix G, table G.2). On the skin, *B. bifidum* and *B. breve* was the second and third most abundant bacteria, found in 40% and 20% (n=1) of all samples, and amounted 13.36% and 29.18% of the composition, respectively. In the stool of 3-month-olds, *B. breve* was the second most abundant species of *Bifidobacterium* (figure 3.2), accounting for 2.73% of the composition and was found in 7% (n_{det}=2, n_{tot} = 28) of the samples. In mother's stool, *B. adolescentis* and *B. bifidum* were the second and third most abundant *Bifidobacterium,* found in 10% (n_{det} 4, n_{tot} = 40) and 8% (n_{det}=4, n_{tot} = 40) of all samples, respectively, and both amounted to ~2% of the composition. In the meconium samples, *B. breve* and *B. bifidum* was the only two species observed (figure 3.2). *B. breve* was found in 4% (n_{det}=1, n_{tot} = 27) of the samples, and amounted to 85% of the composition, while *B. bifidum* was observed in 40% (n_{det}=11, n_{tot} = 27) of the samples and amounted to 15% of the composition.

3.2.3 Other species detected from the *clpC* sequencing run

In total, 911 of the 1039 (88%) ASVs were identified as a different species than *Bifidobacterium*. After removing all bacteria with a relative abundance of below 3% and merging the same bacterial groups together, 16 taxa remained. The largest group was the unclassified bacteria, with 41% of the sequences belonging to this group (n=52 047,

n_{tot}=125 444). It was found in 51% of the samples sequenced (n=143, n_{tot}=280), and amounted to a relative abundance of over 50% for all categories. For the 3-month-olds, the unclassified group had an abundance of over 94% regardless of the delivery mode. In addition, vaginally delivered infants had 5.2% *Bacteroides uniformis*, while c-section born infants had no classified bacteria with an abundance over 3%. The skin samples from vaginally born infants showed high *Alistipes shahii* (15.2%) quantities, while the c-section infants had *Streptomyces griseorubiginosus* (10.7%) as the most abundant classified bacteria. In meconium, *Burkholderia cepacia complex* was the bacteria with the highest abundance for both the vaginally- and c-section delivered infants (49.4% and 19.7%, respectively). The mothers showed the highest variations of different bacteria species, including *A. shahii, Pseudomonas* and *B. uniformis*.

3.3 Presence of *Bifidobacterium* in mother-infant pairs.

In total, 147 mother-infant pairs were analysed, where 98 were considered complete pairs. Complete pairs means that information about all sample categories is available, and these were studied further. Of the 98 pairs, 65 belonged to the vaginally delivered group, and 33 belonged to the c-section delivered group. The association of *B. bifidum, B. breve,* and *B. longum* between mothers and infants was investigated by looking at the meconium, and the stool of 3-month-olds, and mothers. Due to the low levels of bacteria on the skin, and for the bacteria *B. adolescentis, B. longum* subsp. *infantis*, and the "other below 3%"-group, these were excluded from further analysis. The presence of the association of *B. bifidum, B. breve,* and *B. longum* is presented in figure 3.3A-F (appendix D.3, RStudio-pipeline).

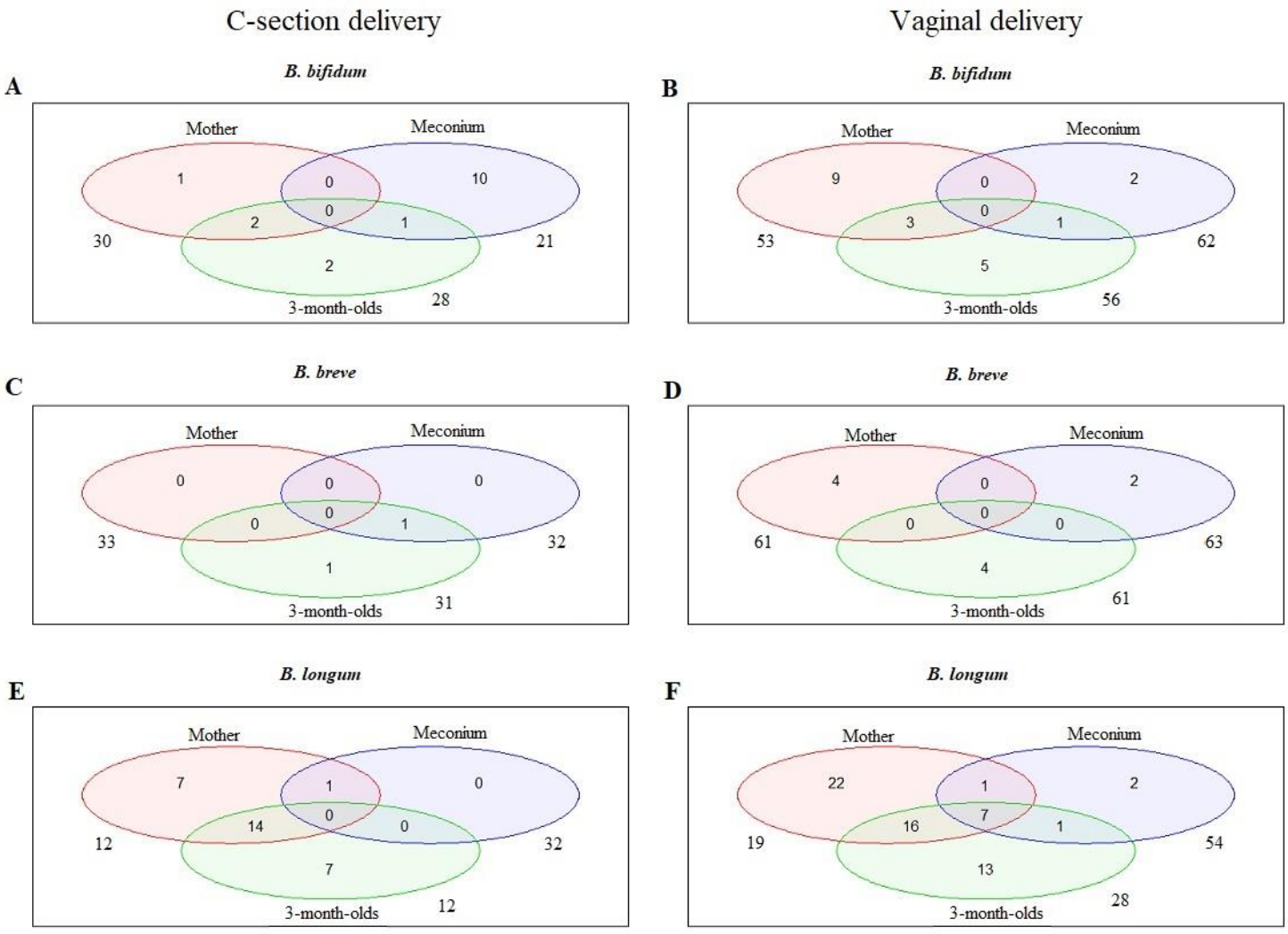

Figure 3.3 Association between mother-child pairs based on *B. breve, B. bifidum* and *B. longum*. To investigate the association, the ASVs for each sample were binarized (presence of a *Bifidobacterium* ASV was set to 1, no presence was set to 0). The association was checked with the species *B. bifidum* (A, B), *B. breve* (C, D) and *B. longum* (E, F). The c-section delivered group is represented in A, C and E, while the vaginally delivered group in B, D and F. The number on the side of the diagrams represent the number of samples with no ASVs representing the *Bifidobacterium* species of interest.

3.3.1 *Bifidobacterium* association within the same delivery mode

A Wilcoxon Signed-Rank Test based on the binarized numbers of ASVs was performed to investigate if there was a significant difference or association between the sample categories within the same delivery mode (table 3.1). Due to the low quantity and lack of association observed within *B. breve* and *B. bifidum* (figure 3.3A-D), the test was performed on *B. longum* exclusively. In the vaginally delivered group, the highest significant difference was observed between meconium and mothers ($p<0.0001$). The c-section delivered infants showed an equally significant difference ($p<0.0001$) for the meconium and mother, and meconium and 3-month-olds categories. Similarity was highest between 3-month-olds and mothers, regardless of delivery mode (table 3.1).

Table 3.1 Result of the Wilcoxon Signed-Rank test within different sample categories. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0001$

Categories	p-value	Significance
Vaginal delivery		
Meconium/mother	$4.60x10^{-8}$	****
Meconium/3-month-olds	$6.21x10^{-5}$	****
3-month-olds/mother	0.03	*
C-section delivery		
Meconium/mother	$2.98x10^{-6}$	****
Meconium/3-month-olds	$8.55x10^{-6}$	****
3-month-olds/mother	0.64	

3.3.2 *B. longum* association between the delivery modes

Chi-squared tests based on the binarized number of ASVs between the delivery modes within meconium and 3-month-olds show that there were no significant differences (table 3.2) between the mother-infant associated based on delivery mode.

Table 3.2 Result of the Chi-square tests with Yates correction based on presence and absence of *B. longum* between different infant categories and their corresponding mothers across the delivery modes.

Categories	X^2	p-value
Meconium	1.28	0.27
3-month-olds	0.37	0.54

3.4 Cultivation and identification of colonies

3.4.1 Identification of colonies

In order to investigate the growth of living *Bifidobacterium*, a 48-hours anaerobic cultivation at 37°C on Bifidus selective medium was performed. Meconium from vaginally delivered infants was streaked directly on agar plates and in a liquid BSM medium. The cultivation on agar plates resulted in pink, round colonies, visually identical to the positive control (*B. longum* subs. *infantis*). The DNA extracted from 20 colonies growing on the BSM agar plates and from the liquid BSM medium from t=0 and t=48 hours were processed with CoverAll-primers. The sequences retrieved from the Sanger sequencing were identified using nucleotide BLAST search, where all samples had a 99% identity match to an *Enterococcus faecalis* strain.

3.4.2 Quantification of *Bifidobacterium* in liquid medium over time

qPCR was performed to investigate bacterial growth in liquid BSM over time (t=0, t=48 hours). DNA was extracted and diluted 10-, 100-, and 1000-fold, and processed with both 16S rRNA- and *clpC* primers. Both genes showed a clear decrease of Cq values from t=0 and t=48 hours. The 16S rRNA gene had a decrease in Cq values from 25.46 to 19.54, and the *clpC* gene from 37.26 to 31.06 (table H.1, appendix H). The starting Cq values are lower for the 16S rRNA compared to the *clpC* products, thus there is a high growth of other bacteria than *Bifidobacterium.*

4. Discussion

4.1 Presence of *Bifidobacterium* in mothers and their infants

4.1.1 *Bifidobacterium* species identified in the mother-infant pairs

The natural development of *Bifidobacterium* in an infant's gut is a topic that lacks knowledge. Breastmilk, and especially HMOs found in breastmilk, seems to be the source of the selection for the bacterium in the gut (Lawson et al., 2020; Matamoros et al., 2013; Underwood et al., 2015). In this study, the highest probability of detecting *B. longum* in a mother-infant pair was observed when comparing 3-month-olds and their mothers, regardless of delivery modes (figure 3.3E-F). This indicates that there is a form of selection of the same *Bifidobacterium* species in infants as found in their mothers. Many of the infants has received breastmilk, so a possible source of this selection is breastmilk (appendix A, table A.1). Unfortunately, the information about which infants were breastfed or not is lacking from the database used in this study, and it is therefore not possible to compare breastfed and not breastfed infants.

There was no evidence for an association between mothers and infants when investigating *B. bifidum* and *B. breve.* This result differs from other studies where these species of *Bifidobacterium* have been observed as dominant bacteria in an infant's gut (O'Callaghan & Van Sinderen, 2016; Turroni et al., 2019). The reason for this result may lie with differences in the environmental conditions for the infants investigated in this study compared to the infants of previous studies. These conditions include duration of breastfeeding, antimicrobial use, and breastmilk composition (Lewis & Mills, 2017).

4.1.2 Composition of *Bifidobacterium*

The Illumina sequencing targeting the *clpC* gene showed high abundance of *B. longum* in all sample categories, except meconium from c-section delivered infants. The other species of *Bifidobacterium* were not abundant in any of the categories. *Bifidobacterium* is a known abundant bacterium in a human's gut. According to Turroni et al. (2019), *B. bifidum, B. breve* and *B. longum* are typically found in infants, and *B. adolescentis* and *B. catenulatum* in adults. The explanation of the low diversity of *Bifidobacterium* species in the samples may lie with the number of "unclassified bacteria" (see section 3.2.3). The c-section- and vaginally delivered infants showed variations in the meconium samples, with *B. longum* only observed in vaginally delivered infants. This can be an indication that a vaginal birth leads to a quicker *B. longum*

colonization. At 3-months of age, however, there are evidence of a convergence toward a *B. longum* colonization for both delivery modes (figure 3.3E-F). The variations in microbiota composition documented in vaginally- and c-section delivered infants have shown a gradual decrease, where breastfeeding is the most probable source (Milani et al., 2017). There could be other reasons for the significant increase of *Bifidobacterium* at 3-months. For example, the early colonizers' (e.g., Enterobacteriaceae) ability to deplete the gut for oxygen makes the environment in the gut suitable for facultative anaerobic bacteria (Matamoros et al., 2013).

4.2 Differences in bacterial composition based on delivery mode

4.2.1 Birth canal as the first exposure site of transmission to the skin of newborns

The results from the Illumina sequencing run based on the 16S rRNA gene showed differences in the composition on the skin of infants born different ways. Infants from both delivery modes showed low levels of *Bifidobacterium.* The infants' skin of vaginally delivered infants was dominated by *Lactobacillus* and *Nesterenkonia*, while c-section delivered infants had *Caldalkalibacillus* as the dominant species (figure 3.1). *Nesterenkonia* is a bacterium that seems to thrive in alkalic environments (pH 7-12) (Zhang et al., 2015). Therefore, this bacterium is unlikely to be transmitted from the mother's vaginal tract to the infants unless a dysbiosis of the vaginal tract has led to a more alkalic environment. A better explanation of these findings may be that these are contaminants found either in the water or in the Taq polymerases used during PCR (Hughes et al., 1994; Iulia et al.; Maiwald et al., 1994). *Caldalkalibacillus* found on c-section delivered infants also thrives in alkaline environments, and there is no report of this bacterium being found in the incision site or on mothers' skin. Thus, a valid explanation could be the bacterium's ability to form endospores, either by being a contamination found in the PCR-water, at the hospital, or via the doctors.

Working from the hypothesis of a sterile *in utero* environment, an infant's first exposure to microbes occurs during birth, either through the vaginal tract or a surgical incision in the mother's abdomen. Vaginally delivered infants are expected to show higher similarities to their mother's vaginal tract, and c-section delivered infants to the environment. *Lactobacillus* is a well-known bacterium that dominates the vaginal tract of healthy women (Cribby et al., 2008; Redondo-Lopez et al., 1990; Walker et al., 2017), due to its ability to form biofilms and hence persist in the harsh environment the vaginal tract create (Salas-Jara et al., 2016). Therefore, there is a possibility that these bacteria were transmitted vertically from mother to infant.

4.2.2 Mode of delivery affects the meconium taxonomic composition

The mode of delivery has proven to be an essential factor that strongly influences gut microbiota development (Mitchell et al., 2020). The vaginally delivered infants are in direct contact with the mothers' perineal area, hence contacting faeces and vaginal fluids, resulting in an oral transmission of microbes (Moore & Townsend, 2019). C-section infants, however, does not get the same transmission, resulting in variation of which microbes is transmitted during birth. In this study, the 16S rRNA sequencing identified *Bifidobacterium* in a low scale in the meconium of vaginally born infants, and not detected in meconium of c-section delivered infants. This indicates that vaginally born infants may have an earlier colonization of the bacterium than c-section infants.

One of the most abundant bacteria found in meconium of vaginally delivered infants was *Bacteroides,* which is a bacterium that is seemingly directly transmitted from mother to infant through vaginal delivery (Ferretti et al., 2018; Karlsson et al., 2011; Sørensen, 2020). In c-section delivered infants the results pointed towards amplification and sequencing of contaminants. *Pseudoalteromonas* and *Halomonas* were the dominating bacteria found in these infants. *Pseudoalteromonas* is a marine bacterium known to produce extracellular agents (Isnansetyo & Kamei, 2003), while *Halomonas* is an aerobic or facultatively anaerobic bacteria known to grow in saline environments (Ventosa et al., 1996). *Pseudoalteromonas* and *Halomonas* are bacteria not documented on mothers' skin or at the c-section site.

4.3 Investigation of living cells in meconium samples

4.3.1 Cultivation on agar plates indicates presence of living cells

Sequencing techniques do not discriminate between living and dead cells; therefore, a cultivation experiment was performed to investigate living bacteria. The five samples chosen for cultivation had a *Bifidobacterium* abundance of ~4% and were selected exclusively from the vaginally delivered infants. Meconium samples from c-section delivered infants were not cultivated because of the lack of positive *Bifidobacterium* samples after sequencing. The BSM-medium chosen for the current thesis gave a growth of a positive control: *B. longum* subs. *infantis* strain. The morphology of the colonies from meconium were the same as the positive control, with pink and round colonies forming on all plates. However, the bacterium identified from the colonies through Sanger sequencing was *Enterococcus faecalis*. The broth used for the agar plates specifies that *Enterococcus* will not grow due to the presence of a

specific salt. This may indicate a resistance encountered by the *Enterococcus faecalis* (Heo et al., 2019).

4.3.2 Investigation of bacterial growth using a combination of qPCR and cultivation

Quantitative PCR was performed to investigate the presence of bacteria in a liquid medium over time, hours 0 and 48. The Cq values decreased for both the 16S rRNA- and *clpC* gene after a 48-hour incubation in *Bifidobacterium's* favourable environment. The decrease of Cq values indicates growth of bacteria, supporting the claim of living bacteria present in meconium (section 4.3.1). The increase in products amplified with *clpC* primers may indicate growth of *Bifidobacterium,* but further investigation is needed as other bacteria can be amplified. The use of another medium such as a Wilkins Chalgren anaerobic agar with the antibiotic's mupirocin and norfloxacin added, could be an option. The mupirocin selects most anaerobic bacteria; however, several anaerobic bacteria are known to be resistant to mupirocin (Moy et al., 1990; Vlková et al., 2015). In Vlková et al. (2015), norfloxacin was reported to inhibit the growth of most anaerobic bacteria, excluding *Bifidobacterium*.

4.4 Technical considerations, difficulties, and strengths

4.4.1 DNA extraction of meconium and skin of newborns with low DNA yield

Quantitative PCR indicates that both the infant meconium and skin samples contain less bacterial DNA than their mothers. There is a general tendency of there being low DNA quantities in these kinds of samples, and the results from this thesis might be because of that. Another possibility is that the extraction method is inefficient, especially for meconium. Meconium DNA extraction is affected by its unique texture, not allowing a full dissolvement, and a complete representation of the bacterial DNA present may be false. In addition, meconium consists of a high concentration of PCR inhibitors. These inhibitors are not yet identified, but studies suggest that meconium includes bile salts and acids, which are known PCR inhibitors (Stinson et al., 2018). When processing meconium and skin samples with low DNA yield, qualitative PCR cycles were increased. This increase leads to a high possibility of contaminants being amplified in addition to the bacteria of interest. Lastly, preservation buffers and freezing condition have been identified as possible sources of variations in microbial composition (Antosca et al., 2020).

4.4.2 Cross-reaction of the *clpC* primers

The *clpC* gene is universal in most bacteria and has been used for distinguishing closely related species of *Bifidobacterium* (Ventura et al., 2005). However, in this study, the Kraken database assigned 56% of the sequences to another species than *Bifidobacterium.* The group with the highest abundance was "unclassified bacteria", indicating a lack of genomes collected for the reference database. A solution to this problem may be to use a different database, such as the newly established HumGut (Hiseni et al., 2020). This is a genome collection of gut microbes aiming to be a universal reference database for all human gut microbiota studies. According to Hiseni et al. (2020), the HumGut collection outperforms the Kraken database.

The *clpC* gene is commonly used in studies concerning *Bifidobacterium*. The gene is a single-copy gene in *Bifidobacterium* and allows better taxonomic assignments of bifidobacterial taxa compared to the 16S rRNA gene, which is limited to ~97% clustering. The result from this thesis indicates that the primers do not give a high enough exclusion of the bacterium of interest. A possible solution to this problem is the use of other primers. Junick and Blaut (2012) used the *groEL* as the target gene. In this case the limitation of this study was the lack of complete *groEL* sequences for the different *Bifidobacterium* species. A full investigation of the sequences of this gene for all *Bifidobacterium* could be interesting for further studies of the transmission and colonization of the bacteria to the infant's gut.

4.4.3 Selection of positive *Bifidobacterium* samples

Even though there was a limitation of the *clpC* primers, the result was still sufficient for investigating the main objective of the thesis. A challenge with this thesis was that most skin and meconium samples were not positive for the *clpC* gene. Prior to any Illumina sequencing run, there is a selection process to avoid too much dilution of the library and under-clustering of the flow-cell. Thus, many samples from the *clpC* PCR products were excluded from sequencing due to the high Cq values and no band visual on the gel electrophoresis after index PCR. Some of the samples excluded may still be *clpC* positive, which can lead to an inconclusive representation of the *Bifidobacterium* amount in the sample categories.

5. Conclusion and further perspectives

In conclusion, this thesis supports the claim of *Bifidobacterium* selection sometime after birth. The study showed that the highest probability of detecting *B. longum* in a mother-infant pair is observed when the infant is 3 months old, regardless of the delivery mode. This indicates a selection of the same species of *Bifidobacterium* in an infant as found in the mothers. A difference in this study, compared to previous studies, is that *B. longum* seems to be the only species with a mother-infant association. A similar association between both *B. bifidum* and *B. breve* was not detected. The reason for this may be a difference in the environmental conditions for the infants, or technical difficulties with the *clpC* primers.

Further perspectives of this study could be to include more complete mother-infant pairs with the inclusion of breastmilk-samples from each mother to study the effects of breastmilk versus formula on colonization of *Bifidobacterium*. Furthermore, get access to more information about specific children and mothers. The addition of placenta samples would also be interesting to investigate whether the uterus is sterile or not. Lastly, a study of genetic markers other than *clpC* should be considered.

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Appendix

Appendix A: Nutritional distribution of the 3-month-olds

The diet of the 3-month-olds is listed in table A.1. Due to privacy policy, the information about exactly which infant has received which nutrition is not known.

Table A.1. The nutritional distribution of the 3-month-olds.

	C-section	**Vaginal delivery**
Breastfeeding and formula	8	11
Breastfeeding	10	42
Formula	3	5
Breastmilk by breastfeeding and bottle	8	6
Missing	4	9

Appendix B: Cultivation medium

In this study, Bifidus Selective Medium Broth (Sigma-Aldrich, USA) and BSM-supplement were used. This mixture is suitable for isolation, identification, and enumeration of *Bifidobacterium.* Violet/brown colonies grow within 24-48 hours.

For 1 L BSM-medium, agar-plates:

42.5 g BSM-broth and 15 g agarose-powder was dissolved in 1 L MQ-water. The medium was then sterilized by autoclaving at 121 ºC for 15 minutes and cooled to 55 ºC. Parallel to the autoclaving, the BSM-supplement was prepared. 0.116 g was suspended in 3.5 ml MQ-water. When the medium hit 55 ºC, the supplement was added. The broth was then poured into 50 petri-dishes. After the stiffening of the broth, homogenized meconium samples were diluted 10^{-1}-10^{-6} and streaked on the BSM agar-plates.

For 1 L BSM-medium, liquid:

42.5 g BSM-broth and 10 g agarose-powder was dissolved in 1 L MQ-water. The medium was then sterilized by autoclaving at 121 ºC for 15 minutes and cooled to 55 ºC. Parallel to the autoclaving, the BSM-supplement was prepared. 0.116 g was suspended in 3.5 ml MQ-water. When the medium hit 55 ºC, the supplement was added. The broth was then poured into autoclaved test tubes, and meconium were added directly to the liquid medium.

Appendix C: Index primers

Table C.1: The index-primers used for library preparation for sequencing targeting 16S rRNA.

Primer name	Primer sequence
16S forward primers	
F1 16S	AatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctagtcaaCCTACGGGR BGCASCAG
F2 16S	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctagttccCCTACGGGR BGCASCAG
F3 16S	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctatgtcaCCTACGGGR BGCASCAG
F4 16S	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctccgtccCCTACGGGR BGCASCAG
F5 16S	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtagagCCTACGGGR BGCASCAG
F6 16S	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtccgcCCTACGGGR BGCASCAG
F7 16S	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtgaaaCCTACGGGR BGCASCAG
F8 16S	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtggccCCTACGGGR BGCASCAG
F9 16S	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtttcgCCTACGGGRB GCASCAG
F10 16S	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctcgtacgCCTACGGGR BGCASCAG
F11 16S	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgagtggCCTACGGGR BGCASCAG
F12 16S	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctggtagcCCTACGGGR BGCASCAG
F13 16S	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctactgatCCTACGGGR BGCASCAG
F14 16S	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctatgagcCCTACGGGR BGCASCAG
F15 16S	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctattcctCCTACGGGRB GCASCAG
F16 16S	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctcaaaagCCTACGGGR BGCASCAG
16S reverse primers	
R1 16S	caagcagaagacggcatacgagatCGTGATgtgactggagttcagacgtgtgctcttccgatctGGACTA CYVGGGTATCTAAT
R2 16S	caagcagaagacggcatacgagatACATCGgtgactggagttcagacgtgtgctcttccgatctGGACTA CYVGGGTATCTAAT
R3 16S	caagcagaagacggcatacgagatGCCTAAgtgactggagttcagacgtgtgctcttccgatctGGACTA CYVGGGTATCTAAT
R4 16S	caagcagaagacggcatacgagatTGGTCAgtgactggagttcagacgtgtgctcttccgatctGGACTA CYVGGGTATCTAAT

R5 16S	caagcagaagacggcatacgagatCACTCTgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
R6 16S	caagcagaagacggcatacgagatATTGGCgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
R7 16S	caagcagaagacggcatacgagatGATCTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
R8 16S	caagcagaagacggcatacgagatTCAAGTgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
R9 16S	caagcagaagacggcatacgagatCTGATCgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
R10 16S	caagcagaagacggcatacgagatAAGCTAgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
R11 16S	caagcagaagacggcatacgagatGTAGCCgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
R12 16S	caagcagaagacggcatacgagatTACAAGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
R13 16S	caagcagaagacggcatacgagatTTGACTgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
R14 16S	caagcagaagacggcatacgagatGGAACTgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
R15 16S	caagcagaagacggcatacgagatTGACATgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT

Table C.2: The index-primers used for library preparation for sequencing targeting *clpC*.

Primer name	**Primer sequence**
	***clpC* forward primers**
F1 *ClpC*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctagtcaaGAGTACCGCAAGTACATCGAG
F2 *ClpC*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctagttccGAGTACCGCAAGTACATCGAG
F3 *ClpC*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctatgtcaGAGTACCGCAAGTACATCGAG
F4 *ClpC*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctccgtccGAGTACCGCAAGTACATCGAG
F5 *ClpC*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtagagGAGTACCGCAAGTACATCGAG
F6 *ClpC*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtccgcGAGTACCGCAAGTACATCGAG
F7 *ClpC*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtgaaaGAGTACCGCAAGTACATCGAG
F8 *ClpC*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtggccGAGTACCGCAAGTACATCGAG
F9 *ClpC*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtttcgGAGTACCGCAAGTACATCGAG

F10 *ClpC*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctcgtacgGAGTACCGCA AGTACATCGAG
F11 *ClpC*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgagtggGAGTACCGCA AGTACATCGAG
F12 *ClpC*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctggtagcGAGTACCGCA AGTACATCGAG
F13 *ClpC*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctactgatGAGTACCGCA AGTACATCGAG
F14 *ClpC*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctatgagcGAGTACCGCA AGTACATCGAG
F15 *ClpC*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctattcctGAGTACCGCAA GTACATCGAG
F16 *ClpC*	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctcaaaagGAGTACCGCA AGTACATCGAG
clpC reverse primers	
R1 *ClpC*	caagcagaagacggcatacgagatCGTGATgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R2 *ClpC*	caagcagaagacggcatacgagatACATCGgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R3 *ClpC*	caagcagaagacggcatacgagatGCCTAAgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R4 *ClpC*	caagcagaagacggcatacgagatTGGTCAgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R5 *ClpC*	caagcagaagacggcatacgagatCACTCTgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R6 *ClpC*	caagcagaagacggcatacgagatATTGGCgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R7 *ClpC*	caagcagaagacggcatacgagatGATCTGgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R8 *ClpC*	caagcagaagacggcatacgagatTCAAGTgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R9 *ClpC*	caagcagaagacggcatacgagatCTGATCgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R10 *ClpC*	caagcagaagacggcatacgagatAAGCTAgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R11 *ClpC*	caagcagaagacggcatacgagatGTAGCCgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R12 *ClpC*	caagcagaagacggcatacgagatTACAAGgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R13 *ClpC*	caagcagaagacggcatacgagatTTGACTgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R14 *ClpC*	caagcagaagacggcatacgagatGGAACTgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R15 *ClpC*	caagcagaagacggcatacgagatTGACATgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R16 *ClpC*	caagcagaagacggcatacgagatGGACGGgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R17 *ClpC*	caagcagaagacggcatacgagatCTCTACgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC

R18 *ClpC*	caagcagaagacggcatacgagatGCGGACgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R19 *ClpC*	caagcagaagacggcatacgagatTTTCACgtgactggagttcagacgtgtgctcttccgatctCATCCTCA TCGTCGAACAGGAAC
R20 *ClpC*	caagcagaagacggcatacgagatGGCCACgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R21 *ClpC*	caagcagaagacggcatacgagatCGAAACgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R22 *ClpC*	caagcagaagacggcatacgagatCGTACGgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R23 *ClpC*	caagcagaagacggcatacgagatCCACTCgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R24 *ClpC*	caagcagaagacggcatacgagatGCTACCgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R25 *ClpC*	caagcagaagacggcatacgagatATCAGTgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R26 *ClpC*	caagcagaagacggcatacgagatGCTCATgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R27 *ClpC*	caagcagaagacggcatacgagatAGGAATgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R28 *ClpC*	caagcagaagacggcatacgagatCTTTTGgtgactggagttcagacgtgtgctcttccgatctCATCCTCA TCGTCGAACAGGAAC
R29 *ClpC*	caagcagaagacggcatacgagatTAGTTGgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R30 *ClpC*	caagcagaagacggcatacgagatCCGGTGgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R31 *ClpC*	caagcagaagacggcatacgagatATCGTGgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R32 *ClpC*	caagcagaagacggcatacgagatTGAGTGgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R33 *ClpC*	caagcagaagacggcatacgagatCGCCTGgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R34 *ClpC*	caagcagaagacggcatacgagatGCCATGgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R35 *ClpC*	caagcagaagacggcatacgagatAAAATGgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC
R36 *ClpC*	caagcagaagacggcatacgagatTGTTGGgtgactggagttcagacgtgtgctcttccgatctCATCCTC ATCGTCGAACAGGAAC

Appendix D: RStudio - pipelines

D.1: Dada 2-pipeline

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

#Locating the files
path <- "R lab/DADA2/fastq/"
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)

#Looking at the Quality scores from sequencing
plotQualityProfile(fnFs[1:1])
plotQualityProfile(fnRs[1:2])

#Filtering the files
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(240,160),
                    maxN=0, maxEE=c(2,2), truncQ=2, rm.phix=TRUE,
                    compress=TRUE, verbose=TRUE)
```

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

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


#Learning the errors
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)

#Applying the errors
dadaFs <- dada(derepFs, err=errF, multithread = TRUE)
dadaRs <- dada(derepRs, err=errF, multithread = TRUE)

dadaFs[[1]]

#Merging of the sequences
mergers <- mergePairs(dadaFs, derepFs, dadaRs, derepRs, verbose = TRUE)
head(mergers[[1]])

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

dim(seqtab.nochim)
sum(seqtab.nochim)/(sum(seqtab))

#Converting the colums into a .fasta file
uniquesToFasta(getUniques(seqtab.nochim), fout = "R lab/DADA2/kraken/uniqueSeqs_fasta/",
               ids=paste0("Seq", seq(length(getUniques(seqtab.nochim)))))

#Creating a new table
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")
```

```
#Taking the taxonomy into the dada.table
dada_table <- read.delim("kraken/dada_table.txt", row.names=NULL)

#Reading results from Kraken
library(tidyverse)
report.file <- "kraken/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

#Combine the Taxonomy output from kraken to our sequence table
combined.df <- cbind.data.frame(dada_table, table.id$Tax.ID)

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

D.2: Pipeline for bar-plot

```
library(readxl)
library(tidyverse)
library(microseq)

Bifido_full <- read_excel("Relativ_prosent_all_groups.xlsx")

Bifido_full_s <- stack(Bifido_full[,-1])
view(Bifido_full_s)
list1 <- rep(Bifido_full$Variabel)
Bifido_Stacked <- cbind(list1, Bifido_full_s)
as.data.frame(Bifido_Stacked)

colnames(Bifido_Stacked) <- c('Category','Prosent','Bacteria')
Bifido_Stacked$Category <- factor(Bifido_Stacked$Category,
                                  levels = c("Skin_CS", "Skin_V", "Meconium_CS",
                                             "Meconium_V", "3_months_CS", "3_months_V",
                                             "Mother_CS", "Mother_V"))
view(Bifido_Stacked)

#Libraries needed for bar chart
library(ggplot2)


ggplot(Bifido_Stacked) +
  geom_col(aes(x = Category, y = Prosent, fill = Bacteria), width = 0.8, colour = "Black") +
  theme(axis.text.x = element_text(angle = 90)) +
  labs(x=" ", y="Relative abundance in %", title="ClpC sequencing data") +
  scale_fill_brewer(palette="RdYlGn") +
  scale_y_continuous(labels = function(x) paste0(x*1, "%"), expand = c(0,0)) +
  scale_x_discrete(expand = c(0,0)) +
  theme_classic() + theme(legend.position="bottom")
```

D.3: Pipeline for VennDiagrams

```
install.packages("VennDiagram")
library('ggplot2')
library('limma')

#Bifidobacterium bifidum
til_cs_bifidum <- read_excel("til_cs_bifidum.xlsx")
til_vag_bifidum <- read_excel("til_vag_bifidum.xlsx")

cs_1 <- vennCounts(til_cs_bifidum)
vag_1 <- vennCounts(til_vag_bifidum)

#Bifidobacterium breve
til_cs_breve <- read_excel("til_cs_breve.xlsx")
til_vag_breve <- read_excel("til_vag_breve.xlsx")

cs_2 <- vennCounts(til_cs_breve)
vag_2 <- vennCounts(til_vag_breve)

#Bifidobacterium longum
til_cs_longum <- read_excel("til_cs_longum.xlsx")
til_vag_longum <- read_excel("til_vag_longum.xlsx")

cs_3 <- vennCounts(til_cs_longum)
vag_3 <- vennCounts(til_vag_longum)
```

```
attach(mtcars)
par(mfrow=c(3,2))
vennDiagram(cs_1,
            circle.col = c("red", "blue", "green3"),
            counts.col = 'black', main = "Bifidobacterium bifidum")
vennDiagram(vag_1,
            circle.col = c("red", "blue", "green3"),
            counts.col = 'black', main = "Bifidobacterium bifidum")
vennDiagram(cs_2,
            circle.col = c("red", "blue", "green3"),
            counts.col = 'black', main = "Bifidobacterium breve")
vennDiagram(vag_2,
            circle.col = c("red", "blue", "green3"),
            counts.col = 'black', main = "Bifidobacterium breve")
vennDiagram(cs_3,
            circle.col = c("red", "blue", "green3"),
            counts.col = 'black', main = "Bifidobacterium longum")
vennDiagram(vag_3,
            circle.col = c("red", "blue", "green3"),
            counts.col = 'black', main = "Bifidobacterium longum")
```

Appendix E: Quantitative PCR

Table E.1: Cq-values, number of N/A samples and baseline result after qPCR targeting 16s rRNA and *clpC*.

16S rRNA

Sample category	Cq-min	Cq-max	Average Cq	Number of N/A	Baseline (RFU)
Skin (mixed)	29.76	39.96	37.36	22	700
Skin (positive *Bacteroides*)	26.33	39.79	34.95	0	1000
Meconium c-section (negative *Bacteroides*)	32.05	39.95	36.14	8	700
Meconium vaginal (mixed)	31.11	37.24	34.83	0	1000
Meconium vaginal (positive *Bacteroides*)	22.73	37.75	31.71	0	1200
3 months (mixed)	21.15	38.70	29.73	0	1100
Mother (mixed)	19.55	31.57	23.92	0	1100
Mother (positive *Bacteroides*)	17.88	29.28	23.08	0	1600
clpC					
Skin (mixed)	31.04	39.94	37.62	12	95
Skin (positive *Bacteroides*)	35.88	39.55	37.74	12	800
Meconium c-section (negative *Bacteroides*)	37.69	39.68	38.72	41	75
Meconium vaginal (mixed)	33.03	39.91	36.95	28	70
Meconium vaginal (positive *Bacteroides*)	36.44	39.77	38.05	26	300
3 months (mixed)	25.49	39.70	34.08	10	450
Mother (mixed)	31.37	39.57	35.39	9	380
Mother (positive *Bacteroides*)	29.91	39.76	33.39	3	1680

Appendix F: “Other below 3%”-group from the 16S rRNA sequencing

D_0__Archaea;D_1__Euryarchaeota;D_2__Methanobacteria;D_3__Methanobacteriales;D_4__Methanobacteriaceae;D_5__Methanobrevibacter

D_0__Bacteria;D_1__Acidobacteria;D_2__Subgroup 2;D_3__uncultured Acidobacteria bacterium;D_4__uncultured Acidobacteria bacterium

D_0__Bacteria;D_1__Actinobacteria;D_2__Acidimicrobiia;D_3__Acidimicrobiales;D_4__uncultured;D_5__uncultured bacterium

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Actinomycetales;D_4__Actinomycetaceae;D_5__Actinobaculum

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Actinomycetales;D_4__Actinomycetaceae;D_5__Actinomyces

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Actinomycetales;D_4__Actinomycetaceae;D_5__Actinotignum

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Actinomycetales;D_4__Actinomycetaceae;D_5__Mobiluncus

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Actinomycetales;D_4__Actinomycetaceae;D_5__Varibaculum

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Actinomycetales;D_4__Actinomycetaceae;D_5__uncultured

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Bifidobacteriales;D_4__Bifidobacteriaceae;D_5__Scardovia

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Corynebacteriales;D_4__Corynebacteriaceae;D_5__Corynebacterium 1

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Corynebacteriales;D_4__Corynebacteriaceae;D_5__Lawsonella

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Corynebacteriales;D_4__Dietziaceae;D_5__Dietzia

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Corynebacteriales;D_4__Mycobacteriaceae;D_5__Mycobacterium

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Corynebacteriales;D_4__Nocardiaceae;D_5__Rhodococcus

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Micrococcales;D_4__Bogoriellaceae;D_5__Georgenia

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Micrococcales;D_4__Cellulomonadaceae;D_5__Actinotalea

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Micrococcales;D_4__Intrasporangiaceae;D_5__Janibacter

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Micrococcales;D_4__Intrasporangiaceae;D_5__Ornithinicoccus

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Micrococcales;D_4__Microbacteriaceae;D_5__Frigoribacterium

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Micrococcales;D_4__Microbacteriaceae;D_5__Leifsonia

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Micrococcales;D_4__Micrococcaceae;D_5__Arthrobacter

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Micrococcales;D_4__Micrococcaceae;D_5__Rothia

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Micrococcales;D_4__Sanguibacteraceae;D_5__Sanguibacter

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Propionibacteriales;D_4__Nocardioidaceae;D_5__Nocardioides

D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Propionibacteriales;D_4__Propionibacteriaceae;D_5__Propionibacterium

D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Coriobacteriaceae;D_5__Atopobium

D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Coriobacteriaceae;D_5__Collinsella

D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Coriobacteriaceae;D_5__Eggerthella

D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Coriobacteriaceae;D_5__Enterorhabdus

D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Coriobacteriaceae;D_5__Olsenella

D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Coriobacteriaceae;D_5__Senegalimassilia

D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Coriobacteriaceae;D_5__uncultured

D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Coriobacteriaceae;Other

D_0__Bacteria;D_1__Actinobacteria;D_2__Nitriliruptoria;D_3__Nitriliruptorales;D_4__Nitriliruptoraceae;Other

D_0__Bacteria;D_1__Actinobacteria;D_2__Rubrobacteria;D_3__Rubrobacterales;D_4__Rubrobacteriaceae;D_5__Rubrobacter

D_0__Bacteria;D_1__Actinobacteria;D_2__Thermoleophilia;D_3__Gaiellales;D_4__Gaiellaceae;D_5__Gaiella

D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidetes vadinHA17;D_3__uncultured bacterium;D_4__uncultured bacterium;D_5__uncultured bacterium

D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Bacteroidales S24-7 group;D_5__uncultured bacterium

D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Porphyromonadaceae;D_5__Barnesiella

D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Porphyromonadaceae;D_5__Butyricimonas

D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Porphyromonadaceae;D_5__Coprobacter

D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Porphyromonadaceae;D_5__Macellibacteroides

D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Porphyromonadaceae;D_5__Odoribacter

D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Porphyromonadaceae;D_5__Porphyromonas

D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Paraprevotella

D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotella

D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotella 2

D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotella 6

D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotellaceae NK3B31 group

D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;Other

D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Rikenellaceae;D_5__Rikenellaceae RC9 gut group

D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__uncultured;Other

D_0__Bacteria;D_1__Bacteroidetes;D_2__Cytophagia;D_3__Cytophagales;D_4__Cytophagaceae;D_5__Hymenobacter

D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Capnocytophaga

D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Chryseobacterium

D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Cloacibacterium

D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Epilithonimonas

D_0__Bacteria;D_1__Bacteroidetes;D_2__Flavobacteriia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__uncultured

D_0__Bacteria;D_1__Bacteroidetes;D_2__Sphingobacteriia;D_3__Sphingobacteriales;D_4__Chitinophagaceae;D_5__Heliimonas

D_0__Bacteria;D_1__Bacteroidetes;D_2__Sphingobacteriia;D_3__Sphingobacteriales;D_4__Chitinophagaceae;D_5__Sediminibacterium

D_0__Bacteria;D_1__Bacteroidetes;D_2__Sphingobacteriia;D_3__Sphingobacteriales;D_4__Chitinophagaceae;D_5__uncultured

D_0__Bacteria;D_1__Chloroflexi;D_2__KD4-96;D_3__uncultured Chloroflexi bacterium;D_4__uncultured Chloroflexi bacterium;D_5__uncultured Chloroflexi bacterium

D_0__Bacteria;D_1__Cyanobacteria;D_2__Chloroplast;D_3__Gerbera hybrid cultivar;D_4__Gerbera hybrid cultivar;D_5__Gerbera hybrid cultivar

D_0__Bacteria;D_1__Cyanobacteria;D_2__Cyanobacteria;D_3__SubsectionII;D_4__FamilyII;D_5__Chroococcidiopsis

D_0__Bacteria;D_1__Cyanobacteria;D_2__Melainabacteria;D_3__Gastranaerophilales;D_4__uncultured bacterium;D_5__uncultured bacterium

D_0__Bacteria;D_1__Cyanobacteria;D_2__Melainabacteria;D_3__Gastranaerophilales;Other;Other

D_0__Bacteria;D_1__Cyanobacteria;D_2__Melainabacteria;D_3__Obscuribacterales;D_4__uncultured bacterium;D_5__uncultured bacterium

D_0__Bacteria;D_1__Deinococcus-Thermus;D_2__Deinococci;D_3__Thermales;D_4__Thermaceae;D_5__Meiothermus

D_0__Bacteria;D_1__Deinococcus-Thermus;D_2__Deinococci;D_3__Thermales;D_4__Thermaceae;D_5__Thermus

D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales;D_4__Alicyclobacillaceae;D_5__Effusibacillus

D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales;D_4__Bacillaceae;D_5__Aeribacillus

D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales;D_4__Bacillaceae;D_5__Anoxybacillus

D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales;D_4__Bacillaceae;D_5__Geobacillus

D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales;D_4__Bacillaceae;D_5__Oceanobacillus

D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales;D_4__Family X;D_5__Thermicanus

D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales;D_4__Paenibacillaceae;D_5__Paenibacillus

D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales;D_4__Planococcaceae;D_5__Planomicrobium

D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Aerococcaceae;D_5__Abiotrophia

D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Carnobacteriaceae;D_5__Alkalibacterium

D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Carnobacteriaceae;D_5__Dolosigranulum

D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Enterococcaceae;D_5__Vagococcus

D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Leuconostocaceae;D_5__Weissella

D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Lactococcus

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Christensenellaceae;D_5__uncultured

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Christensenellaceae;Other

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Clostridiaceae 1;D_5__Proteiniclasticum

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Clostridiales vadinBB6na group;D_5__uncultured bacterium

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Defluviitaleaceae;D_5__Defluviitaleaceae UCG-na11

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Eubacteriaceae;D_5__Anaerofustis

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Eubacteriaceae;D_5__Eubacterium

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XI;D_5__Anaerococcus

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XI;D_5__Ezakiella

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XI;D_5__Finegoldia

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XI;D_5__Helcococcus

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XI;D_5__Peptoniphilus

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;D_5__Family XIII AD3na11 group

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;D_5__Family XIII UCG-nana1

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;D_5__[Eubacterium] brachy group

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;D_5__[Eubacterium] nodatum group

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;D_5__uncultured

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;Other

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Anaerosporobacter

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Coprococcus 1

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Coprococcus 2

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Eisenbergiella

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Howardella

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Hungatella

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Johnsonella

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae FCSna2na group

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NC2nana4 group

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae ND3nana7 group

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NK4B4 group

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae UCG-nana1

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae UCG-nana3

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae UCG-nana4

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae UCG-na1na

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Marvinbryantia

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Moryella

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Oribacterium

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Sellimonas

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Tyzzerella

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Tyzzerella 3

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Tyzzerella 4

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Eubacterium] ruminantium group

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Eubacterium] ventriosum group

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Eubacterium] xylanophilum group

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Ruminococcus] gauvreauii group

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Ruminococcus] torques group

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptococcaceae;D_5__Peptococcus

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptococcaceae;D_5__uncultured

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptostreptococcaceae;D_5__Filifactor

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptostreptococcaceae;D_5__Intestinibacter

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptostreptococcaceae;D_5__Paeniclostridium

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptostreptococcaceae;D_5__Peptoclostridium

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptostreptococcaceae;D_5__Peptostreptococcus

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptostreptococcaceae;D_5__[Eubacterium] yurii group

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Anaerofilum

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Anaerotruncus

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Butyricicoccus

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Candidatus Soleaferrea

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Hydrogenoanaerobacterium

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Oscillibacter

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Oscillospira

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 1

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 5

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 6

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 9

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae NK4A214 group

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-nana3

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-nana4

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-nana5

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-nana9

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-na1na

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-na11

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-na13

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__uncultured

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;Other

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Thermoanaerobacterales;D_4__Thermoanaerobacteraceae;D_5__Gelria

D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Catenibacterium

D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Catenisphaera

D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Coprobacillus

D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Erysipelatoclostridium

D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Erysipelotrichaceae UCG-nana3

D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Erysipelotrichaceae UCG-nana4

D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Erysipelotrichaceae UCG-nana7

D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Holdemanella

D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Holdemania

D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Turicibacter

D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__[Clostridium] innocuum group

D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__uncultured

D_0__Bacteria;D_1__Firmicutes;D_2__Negativicutes;D_3__Selenomonadales;D_4__Acidaminococcaceae;D_5__Acidaminococcus

D_0__Bacteria;D_1__Firmicutes;D_2__Negativicutes;D_3__Selenomonadales;D_4__Acidaminococcaceae;D_5__Phascolarctobacterium

D_0__Bacteria;D_1__Firmicutes;D_2__Negativicutes;D_3__Selenomonadales;D_4__Veillonellaceae;D_5__Allisonella

D_0__Bacteria;D_1__Firmicutes;D_2__Negativicutes;D_3__Selenomonadales;D_4__Veillonellaceae;D_5__Megamonas

D_0__Bacteria;D_1__Firmicutes;D_2__Negativicutes;D_3__Selenomonadales;D_4__Veillonellaceae;D_5__Megasphaera

D_0__Bacteria;D_1__Firmicutes;D_2__Negativicutes;D_3__Selenomonadales;D_4__Veillonellaceae;D_5__Mitsuokella

D_0__Bacteria;D_1__Firmicutes;D_2__Negativicutes;D_3__Selenomonadales;D_4__Veillonellaceae;D_5__Negativicoccus

D_0__Bacteria;D_1__Fusobacteria;D_2__Fusobacteriia;D_3__Fusobacteriales;D_4__Fusobacteriaceae;D_5__Fusobacterium

D_0__Bacteria;D_1__Fusobacteria;D_2__Fusobacteriia;D_3__Fusobacteriales;D_4__Leptotrichiaceae;D_5__Leptotrichia

D_0__Bacteria;D_1__Lentisphaerae;D_2__Lentisphaeria;D_3__Victivallales;D_4__Victivallaceae;D_5__Victivallis

D_0__Bacteria;D_1__Lentisphaerae;D_2__Lentisphaeria;D_3__Victivallales;D_4__vadinBE97;D_5__uncultured bacterium

D_0__Bacteria;D_1__Planctomycetes;D_2__Planctomycetacia;D_3__Planctomycetales;D_4__Planctomycetaceae;D_5__Gemmata

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Caulobacterales;D_4__Hyphomonadaceae;Other

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__Beijerinckiaceae;D_5__uncultured

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__Bradyrhizobiaceae;D_5__Bosea

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__Bradyrhizobiaceae;D_5__Bradyrhizobium

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__Brucellaceae;Other

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__Methylobacteriaceae;D_5__Methylobacterium

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__Phyllobacteriaceae;D_5__Mesorhizobium

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__Phyllobacteriaceae;D_5__Phyllobacterium

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__Rhizobiales Incertae Sedis;D_5__Phreatobacter

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodospirillales;D_4__Rhodospirillaceae;D_5__uncultured

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rickettsiales;D_4__Mitochondria;D_5__Betula platyphylla

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Sphingomonadales;D_4__Sphingomonadaceae;D_5__Novosphingobium

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Sphingomonadales;D_4__Sphingomonadaceae;D_5__Sphingobium

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Sphingomonadales;D_4__Sphingomonadaceae;D_5__Sphingomonas

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Sphingomonadales;D_4__Sphingomonadaceae;D_5__Sphingopyxis

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Alcaligenaceae;D_5__Achromobacter

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Alcaligenaceae;D_5__Parasutterella

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Alcaligenaceae;D_5__Verticia

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Burkholderiaceae;D_5__Cupriavidus

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Burkholderiaceae;D_5__Lautropia

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Burkholderiaceae;D_5__Polynucleobacter

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Comamonadaceae;D_5__Acidovorax

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Comamonadaceae;D_5__Comamonas

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Comamonadaceae;D_5__Hydrogenophaga

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Comamonadaceae;D_5__Limnohabitans

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Comamonadaceae;D_5__Variovorax

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Comamonadaceae;D_5__uncultured

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Comamonadaceae;Other

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Oxalobacteraceae;D_5__Duganella

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Oxalobacteraceae;D_5__Oxalicibacterium

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Oxalobacteraceae;D_5__Oxalobacter

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Hydrogenophilales;D_4__Hydrogenophilaceae;D_5__Hydrogenophilus

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Hydrogenophilales;D_4__Hydrogenophilaceae;D_5__Tepidiphilus

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Methylophilales;D_4__Methylophilaceae;D_5__Methylotenera

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Neisseriales;D_4__Neisseriaceae;D_5__Alysiella

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Neisseriales;D_4__Neisseriaceae;D_5__Neisseria

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Neisseriales;D_4__Neisseriaceae;D_5__uncultured

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Rhodocyclales;D_4__Rhodocyclaceae;D_5__Azospira

D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Rhodocyclales;D_4__Rhodocyclaceae;D_5__Dechlorobacter

D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Bdellovibrionales;D_4__Bacteriovoracaceae;D_5__Bacteriovorax

D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Desulfovibrionales;D_4__Desulfovibrionaceae;D_5__Desulfovibrio

D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Myxococcales;D_4__P3OB-42;D_5__uncultured bacterium

D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Myxococcales;D_4__Polyangiaceae;D_5__Sorangium

D_0__Bacteria;D_1__Proteobacteria;D_2__Epsilonproteobacteria;D_3__Campylobacterales;D_4__Campylobacteraceae;D_5__Campylobacter

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Alteromonadaceae;D_5__Marinobacter

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Idiomarinaceae;D_5__Idiomarina

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Shewanellaceae;D_5__Shewanella

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Cellvibrionales;D_4__Cellvibrionaceae;D_5__Cellvibrio

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Chromatiales;D_4__Chromatiaceae;D_5__Rheinheimera

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Citrobacter

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Proteus

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;Other

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Methylococcales;D_4__Methylococcaceae;D_5__Methyloglobulus

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pasteurellales;D_4__Pasteurellaceae;D_5__Haemophilus

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pasteurellales;D_4__Pasteurellaceae;D_5__uncultured

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Moraxellaceae;D_5__Enhydrobacter

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Moraxellaceae;D_5__Moraxella

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Xanthomonadales;D_4__Xanthomonadaceae;D_5__Dokdonella

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Xanthomonadales;D_4__Xanthomonadaceae;D_5__Luteimonas

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Xanthomonadales;D_4__Xanthomonadaceae;D_5__Lysobacter

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Xanthomonadales;D_4__Xanthomonadaceae;D_5__Stenotrophomonas

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Xanthomonadales;D_4__Xanthomonadaceae;D_5__Thermomonas

D_0__Bacteria;D_1__SR1 (Absconditabacteria);D_2__uncultured bacterium;D_3__uncultured bacterium;D_4__uncultured bacterium;D_5__uncultured bacterium

D_0__Bacteria;D_1__Saccharibacteria;D_2__uncultured bacterium;D_3__uncultured bacterium;D_4__uncultured bacterium;D_5__uncultured bacterium

D_0__Bacteria;D_1__Spirochaetae;D_2__Spirochaetes;D_3__Spirochaetales;D_4__Spirochaetaceae;D_5__Treponema 2

D_0__Bacteria;D_1__Synergistetes;D_2__Synergistia;D_3__Synergistales;D_4__Synergistaceae;D_5__Cloacibacillus

D_0__Bacteria;D_1__Synergistetes;D_2__Synergistia;D_3__Synergistales;D_4__Synergistaceae;D_5__Jonquetella

D_0__Bacteria;D_1__Tenericutes;D_2__Mollicutes;D_3__Mollicutes RF9;D_4__uncultured bacterium;D_5__uncultured bacterium

D_0__Bacteria;D_1__Tenericutes;D_2__Mollicutes;D_3__Mollicutes RF9;D_4__uncultured rumen bacterium;D_5__uncultured rumen bacterium

D_0__Bacteria;D_1__Tenericutes;D_2__Mollicutes;D_3__Mollicutes RF9;Other;Other

D_0__Bacteria;D_1__Tenericutes;D_2__Mollicutes;D_3__Mycoplasmatales;D_4__Mycoplasmataceae;D_5__Mycoplasma

D_0__Bacteria;D_1__Tenericutes;D_2__Mollicutes;D_3__Mycoplasmatales;D_4__Mycoplasmataceae;D_5__Ureaplasma

D_0__Bacteria;D_1__Tenericutes;D_2__Mollicutes;D_3__NB1-n;Other;Other

D_0__Bacteria;D_1__Verrucomicrobia;D_2__Opitutae;D_3__Opitutae vadinHA64;D_4__uncultured bacterium;D_5__uncultured bacterium

D_0__Bacteria;D_1__Verrucomicrobia;D_2__Verrucomicrobiae;D_3__Verrucomicrobiales;D_4__Verrucomicrobiaceae;D_5__Akkermansia

Appendix G: Presence of *Bifidobacterium* in samples sequenced

Table G.1. Observed *Bifidobacterium* species on the newborns skin, meconium, and stool of 3-month-olds and mothers in the vaginally delivered group. For each species, the ASV quantity of total is based on the presence of ASVs of that species within each category. The numbers are based on the number of samples sequenced with the species present.

	Skin samples Total = 14		**Meconium Total = 37**		**3-month-olds Total = 50**		**Mothers Total = 79**	
Species	Presence in samples	ASV quantity of total %	Presence in samples	ASV quantity of total	Presence in samples	ASV quantity of total %	Presence in samples	ASV quantity of total %
Bifdobacterium	0	0	0	0	1	0.01	3	0.12
B. adolescentis	0	0	1	0.19	2	0.04	17	2.57
B. animalis	0	0	0	0	0	0	6	0.68
B. bifidum	2	3.27	3	0.79	9	2.16	12	1.63
B. breve	0	0	2	8.39	4	5.96	4	0.30
B. catenulatum	0	0	0	0	0	0	0	0
B. dentium	0	0	0	0	3	0.11	0	0
B. longum	5	96.73	15	90.60	35	71.05	63	93.58
B. longum* subsp. *infantis	0	0	0	0	7	20.67	1	1.02
B. longum* subsp. *longum	0	0	0	0	0	0	2	0.07
B. pseudocatenulatum	0	0	1	0.03	0	0	1	0.03

Table G.2. Observed *Bifidobacterium* species on the newborns skin, meconium, and stool of 3-month-olds and mothers in the c-section delivered group. For each species, the ASV quantity of total is based on the presence of ASVs of that species within each category. The numbers are based on the number of samples sequenced with the species present.

	Skin samples Total = 5		**Meconium Total = 27**		**3-month-olds Total = 28**		**Mothers Total = 40**	
Species	Presence in samples	ASV quantity of total %	Presence in samples	ASV quantity of total %	Presence in samples	ASV quantity of total %	Presence in samples	ASV quantity of total %
Bifdobacterium	0	0	0	0	0	0	0	0
B. adolescentis	0	0	0	0	1	0.40	4	1.86
B. animalis	0	0	0	0	1	0.03	1	0.19
B. bifidum	2	13.36	11	15.08	5	1.16	3	1.86
B. breve	1	29.18	1	84.92	2	2.73	0	0
B. catenulatum	0	0	0	0	0	0	1	0.16
B. dentium	0	0	0	0	2	0.13	0	0
B. longum	2	57.46	0	0	20	92.66	31	95.92
B. longum* subsp. *infantis	0	0	0	0	4	2.99	0	0
B. longum* subsp. *longum	0	0	0	0	0	0	0	0
B. pseudocatenulatum	0	0	0	0	0	0	0	0

Appendix H: Cultivation in liquid BSM medium

Table H.1 The Cq values of DNA extracted at t=0 and t=48 hours of products processed with 16S rRNA primers and *clpC* primers.

Sampling point	No dilution	10^{-1}	10^{-2}	10^{-3}	Baseline (RFU)
16S rRNA					
t=0 h	25.46	29.66	33.29	37.33	970
t=48 h	19.54	23.27	26.79	30.15	847
clpC					
t=0 h	37.26	N/A	N/A	N/A	202
t=48 h	31.06	36.14	39.41	38.22	235

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