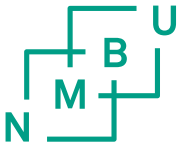
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Abstract

As the prevalence of infectious disease has decreased, the prevalence of allergic diseases has increased. The hygiene hypothesis points to a possible correlation between increased prevalence of allergic diseases, and modernization of homes, better hygiene, and smaller families. With the discovery of health benefits reaped from probiotic bacteria, several studies have been conducted to see if the intake of probiotica can have a preventive effect on the development of allergic diseases, and most of these studies have concluded that probiotica have a preventive effect on the development of allergic diseases. What is not known, however, is the mechanisms behind the preventive effect probiotica has on development of allergic diseases. One possible mechanism is thought to be that the intake of probiotica will cause alterations of the timeframe of when members of *Clostridium* clusters IV and XIVa colonize the infant gut, and start their production of the short chain fatty acid butyrate. As both the bacteria and the produced butyrate is known to have an effect on the immune system, the hypothesis is that a change in the normal colonization pattern can lead to the development of allergic diseases.

To test this hypothesis, fecal samples from the Pro-PACT study were treated with chloroform and heat to isolate, and then cultivate, endospores formed by members of *Clostridium* clusters IV and XIVa. Gas-liquid chromatography was used to detect the presence of butyrate in the cultivated samples. qPCRs specific for members of *Clostridium* clusters IV and XIVa, as well as Illumina sequencing were used to identify the bacteria present in the cultivated samples.

The results show that the main difference between the Biola and placebo groups is that there is a higher prevalence of members of *Clostridium* clusters IV and XIVa in the samples collected from the placebo group. The concentration of butyrate found in the samples are highly correlated with the amount of Clostridia in the samples. It was also discovered that the prevalence of Clostridia species is lower amongst children who have developed an allergic disease. However, this is true for both Biola and placebo groups, suggesting that the colonization of Clostridia is not a direct cause of development of allergic diseases, but rather that the differences between the bacterial composition in the Biola and placebo groups is a result of other bacteria who have colonized the infant gut instead of Clostridia, and thus is a more probable cause for the development of allergic diseases in children.

Sammendrag

Ettersom utbredelsen av infeksjonssykdommer har blitt mindre, har utbredelsen av allergiske sykdommer økt. Hygienehypotesen peker mot en mulig korrelasjon mellom økt utbredelse av allergiske sykdommer, og modernisering av hus, bedre hygiene, og mindre familier. Oppdagelsen av de helsemessige fordelene høstet fra probiotiske bakterier har ført til flere studier angående en eventuell preventiv effekt av probiotiske bakterier på utvikling av allergiske sykdommer, og flere av disse studiene har konkludert med at probiotika har en preventiv effekt på utvikling av allergiske sykdommer. Mekanismen bak den preventive effekten til probiotika på utviklingen av allergiske sykdommer er derimot fortsatt ukjent. En foreslått mekanisme er at inntak av probiotika vil forårsake endringer i tidsrammen for når medlemmer av *Clostridium* klynger IV og XIVa vil kolonisere fordøyelsessystemet hos spedbarn, og når disse bakteriene starter produksjon av den kort-kjedete fettsyren butyrat. Siden både bakteriene selv og butyrat har en kjent effekt på immunsystemet, så er teorien den at en endring av det normale koloniseringsmønsteret kan føre til utvikling av allergiske sykdommer.

For å teste denne hypotesen, ble fekale prøver samlet inn under Pro-PACT studien behandlet med kloroform og varme før kultivering for å isolere endosporer produsert av medlemmer fra *Clostridium* klynger IV og XIVa. Gass-væske-kromatografi ble brukt for å detektere butyrat i de kultiverte prøvene. I tillegg ble qPCRer spesifikke for medlemmer av *Clostridium* klynger IV og XIVa, og Illumina sekvensering brukt for å identifisere bakterier tilstede i de kultiverte prøvene.

Resultatene viser at hovedforskjellen mellom Biola og placebo gruppene var at det var en høyere tilstedeværelse av medlemmer av *Clostridium* klynger IV og XIVa i prøvene samlet inn fra placebo gruppen sammenlignet med Biola gruppen. Konsentrasjonen av butyrat var høyt korrelert til mengden Clostridia funnet i prøvene. Det ble også oppdaget at det var en lavere tilstedeværelse av Clostridia arter i prøver fra barn som hadde utviklet en allergisk sykdom, enn hos de som ikke hadde utviklet en allergisk sykdom. Siden denne forskjellen mellom allergiske og ikke-allergiske barn var synlig i både Biola og placebo gruppene, kan dette tyde på at koloniseringen av Clostridia ikke er en direkte årsak til utvikling av allergiske sykdommer, men heller at forskjellene i bakteriell sammensetning observert mellom Biola og placebo gruppene heller er et resultat av at andre bakterier som har kolonisert fordøyelsessystemet hos spedbarn istedenfor Clostridia. Det kan derfor virke som om disse andre, foreløpig ukjente bakteriene, har en mer direkte innvirkning på immunsystemet enn medlemmer av *Clostridium* klynger IV og XIVa.

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1 Introduction

1.1 Atopic disorders and probiotica

In the past, human life expectancy was very low. The low life expectancy was often caused by infectious diseases that we did not have the knowledge or technology to prevent or cure. At present, we have developed treatments for most infectious diseases and vaccines against others. Some infectious diseases have even become extinct.

Unfortunately, as a result of the decline of infectious diseases and increased living standards, we are faced with a new problem; autoimmune disorders, such as allergies, asthma, and atopic dermatitis (AD).

According to the American Academy of Allergy, Asthma & Immunology (AAAA)(1), allergy is a chronic condition where the sufferer has an unusually strong reaction to normally harmless allergens. The immune system views the allergens as harmful, and the white blood cells initiates production of immunoglobulin E (IgE). When IgE then attaches to mast cells, histamines are released, causing symptoms like itching, sneezing, runny or stuffy nose, and tearing eyes. The AAAA describes atopic dermatitis(2), or atopic eczema as a chronic or recurrent inflammatory skin disease, symptoms include red/brownish skin patches, itchiness, and dry caked or scaly skin. A person suffering from atopic dermatitis will normally begin to display symptoms during the first years of life. There is also a genetic tendency towards later development of other allergies, asthma, and/or allergic rhinitis.

When it comes to the amount of people suffering from allergies, the Asthma and Allergy foundation of America (Aafa) documents 60 million people in America(3), while Allergy UK says that the same numbers for Europe exceeds 150 million people, and that the number of people suffering from allergies increases by 5% each year(4). The prevalence of asthma has increased so much that it is almost considered an epidemic phenomenon, and the incident of atopic dermatitis has almost been tripled in most industrialized countries(5).

An explanation was proposed by David Strachan in 1989(6). During his study, he noticed that development of atopic dermatitis and hay fever were correlated with hygiene, household size and what position the child had within the family. In his article, he states that it is unlikely that the rise in allergic disease is the result of viral infections, as was believed at the time, but that infections during early childhood could actually prevent the development of allergic diseases. Modes of transmission of these early infections could for instance be older siblings, who brought these infections home with them from school. He also points out that improved living conditions, improved personal hygiene, and a decline in family size can be the cause of the increase in allergic diseases, because they limit a child's early exposure to infections that can help prevent allergic disease later in life.

A later article written by Wickens et.al.(7) looks at the different studies done in the first decade since the hygiene hypothesis was proposed. The conclusion of this article states that the hygiene hypothesis is a plausible explanation for the variations in development of allergy, based on factors such as time, geographical location, the socioeconomic status of the household, the size of the household, and an individual's position within the family.

1.1.1 Atopy and allergy

An atopic individual has a genetic disposition towards the development of allergic disorders. Atopic individuals may be symptom free, but can pass this genetic trait on to subsequent generations. An atopic disorder develops when the immune system marks harmless substances, such as food or pollen, as harmful, initiating a disproportionate response by the production of IgE. Among children, the most common atopic disorders are atopic dermatitis, allergic rhinitis, and asthma, caused by both genetic and environmental factors. Atopic dermatitis cause a chronic, recurrent inflammation of the skin, and is often a sign that the affected child will develop other types of allergy later in life(8).

1.1.2 Probiotics

In 2001 the World Health Organization (WHO) defined probiotics as live microorganisms that, when ingested, can modify the host's gut microflora in a beneficial way. Later on, several characteristics have been generally accepted as a definition of sorts when it comes to probiotic bacteria and their abilities; they are microorganisms that remain viable and stable after culturing, manipulation, and storage previous to ingestion, they are able to survive the disruptive environment in the stomach gastrointestinal tract, they are able to induce a response in the host upon entering the microbial ecosystem in the gut, and they are able to yield a functional or clinical benefit to the host when ingested(9). Two of the most used probiotic microorganisms are *Lactobacilli* and *Bifidobacterium*, both lactic acid bacteria common in the human intestine(10).

Even though the exact mechanism by which probiotics have a beneficial effect on human health is currently unknown, several different mechanisms has been proposed. Proposed mechanisms include improvement of the epithelial barrier, prevention of pathogen colonization, induction of host immune system, stabilization of the T helper cell type 1/ T helper cell type 2 (Th1/Th2) balance, and production of IgE, cytokines, and transformation of growth factor beta (TGF-β), amongst other(9).

There has been some concerns regarding the safety of probiotics use, as they are living microorganisms, but studies have shown that there is a very low incident rate of infection connected to the use of probiotics, and that all these infections occurred in patients with an underlying health issue. The US Food and Drug Administration (FDA) has approved *Bifidobacterium lactis* for commercial use in infant formulas, and its safety has been documented in infants from birth and in infants in vulnerable groups, such as preterm infants, malnourished infants, and infants with an HIV-infected mother. *Lactobacillus rhamnosus GG* (LGG) is also considered a safe probiotic appropriate for infants and older children.

As of late, when probiotics are used for therapeutic reasons, they are often paired with prebiotics in a symbiotic relationship. Prebiotics are non-digestible ingredients in food that can affect the host by selectively stimulating the growth of one or a few bacterial species already present in the gut. Specific prebiotics can then be selected to promote the growth of a particular probiotic, giving it an advantage when colonizing the gut(10).

1.2 Gut bacteria

There are approximately ten times more bacterial cells in the human body than human cells, and the majority of these are found in the gut(11). Most of the bacterial cells found in the gastrointestinal tract are commensal bacteria, and the absence of these bacteria can actually cause disease in humans. The gastrointestinal tract is the home of a complex community of microorganisms, and the commensal bacteria helps us with metabolizing and absorption of nutrients that are unavailable to the human cells, they can for instance produce hormones that we cannot synthesize on our own. In addition, the commensal gut microbiota prevents pathogens from establishing in the gut and cause disease by consuming the available nutrients, by producing substances that kill or inhibit the growth of pathogenic bacteria(12), or by actively fighting against the intruding bacteria(13). An even more interesting fact about commensal bacteria is that they can activate and develop the host's immune system; both mucosal and systemic innate and adaptive immune responses can be affected by the commensal microbiota.

Ivanov et.al.(13), has divided non-pathogenic bacteria into three groups. The first group are called probiotics, they are beneficial for gut health, but are not necessarily are part of the normal gut microbiota, and they can have an indirect effect on the beneficial microbiota present in the gut. Examples of probiotics include species from the *Bifidobacterium* and *Lactobacillus* genus. Their association with the host is transient, and they have an innocuous and/or immunostimulatory effect on host immune system, by mechanisms witch include induction of cytokines, activation of toll-like receptors (TLRs), production of lactic acid and short chain fatty acids (SCFAs), as well as suppressing pathobionts and pathogens. The second group is the autobionts, bacteria that are a part of the normal gut flora, and who has a direct effect on host immune cell homeostasis and function. Examples of autobionts include *Bacteroides fragilis,* members of *Clostridium* clusters IV and XIVa, and *Faecalibacterium prauznitsii*. Host association is permanent and symbiotic. Mechanisms involving the host immune system are mostly unknown, but it is likely that there are links to TLR2, metabolites, antigens, and interaction with intestinal epithelial cells (IECs). The third and last group is called pathobionts, and it contains bacteria such as *Helicobacter hepaticus, Clostridium difficile, Bilophila wadsworthia*, and species from the *Prevotella* and *Klebsiella* genus. These bacteria acts as pathogens in the absence of a healthy gut microbiota, but are do not exhibit pathogenic tendencies when the host is healthy. Host association is permanent and parasitic/infectious, with both innocuous and detrimental effects on host immune system, and mechanisms include invasiveness, spore formation and toxin production.

Both autobionts and pathobionts are permanent members of the gut microbiota, while the probiotics are transient; that is, they are introduced from the external environment, and they do not colonize the gut permanently. The transient bacteria have not coevolved with the gut as has the permanent bacteria, and therefore they cannot establish a mutualistic relationship with the gut. They try to survive in the gut, despite the defenses established by the permanent autobionts.

For a long time a lot was unknown about the gut microbiota, as most of these bacteria are obligate anaerobes, and therefore very difficult to culture, but new culture-independent techniques have revealed a lot about these bacteria. Most of the gut bacteria, about 98% of them, belongs to the phyla *Firmicutes* and *Bacteroidetes*. In these two phyla, three groups are more common than the rest; *Bacteroides*, *Clostridium* cluster IV (also known as the *Clostridium leptum* group), and

Clostridium cluster XIVa (also known as the *Clostridium coccoides* group). Species from *Clostridium* clusters IV and XIVa makes up 10-40% of the total gut microbiota, and given this high incidence of Clostridia in the gut, it is highly likely that they have a role in gut homeostasis, and that they can have an effect on the immune system.

1.2.1 The Clostridia class of bacteria

Clostridia is a large group of gram-positive, rod-shaped bacteria, who are obligate anaerobes capable of producing endospores. Clostridia is a very heterogeneous group, and is therefore divided into 19 clusters. Most of the pathogenic and toxin-producing Clostridia, such as *Clostridium perfringens, Clostridium difficile,* and *Clostridium tetani*, are in cluster I. Many of these bacteria can be found in the intestine, and while most are transient pathogens, others, such as *Clostridium difficile*, are pathobionts. Most of the Clostridia found in the gut are commensal bacteria, that does not produce toxins, and most of these bacteria belong to clusters IV and XIVa.

The currently available classification of Clostridia and its clusters is outdated, difficult to find, and in many cases, species from different genera have been placed in different clusters. For this thesis, the classification of *Clostridium* clusters IV and XIVa is based on the descriptions in the articles by Collins et.al.(14), Liu et.al.(15), and Lopetuso(12).

Cluster IV is described as a very heterogeneous group, with both mesophiles and thermophiles, and with a broad range of GC content in the chromosomal DNA. The cluster is also known as the *Clostridium leptum* group, and consists of species from the genera *Clostridium, Eubacterium, Ruminococcus*, and *Anaerofilum*.

Cluster XIV is the larger of the two clusters, containing more than 20 species, that have a generally high GC content, and the cluster is easily separated into two subclusters: XIVa and XIVb. Subcluster XIVa consists of a phenotypically heterogeneous group of microorganisms; there are even several non-spore forming cocci in this cluster. The cluster is also known as the *Clostridium coccoides – Eubacterium rectale* group, and consists of species from the genera *Clostridium, Eubacterium, Ruminococcus, Coprococcus, Dorea, Lachnospira, Roseburia,* and *Butyrivibrion*.

Because Clostridia are obligate anaerobes, they are unable to colonize the infant gut until there is an established anaerobic environment present. During the first days of life, aerobic bacteria from the external environment colonize the gut, and as time pass their metabolism consumes the available oxygen, leaving an anaerobic environment that is more suitable for the commensal permanent autobionts, including Clostridia. Clostridia as symbionts have developed a close relationship with intestinal cells, and will therefore occupy parts of the intestinal mucosa(12).

Most gram-positive anaerobic bacteria have the ability to produce short chain fatty acids, especially butyric acid, or butyrate, as a fermentation product. The article by Louis & Flint(16), has looked deeper into the different butyrate-producing bacteria, and lists the major butyrate-producer from the human intestine. This list contains several members of *Clostridium* cluster XIVa, one species from the XIV cluster, and three members of the IV cluster, among others. The numerically biggest group of butyrate-producers seems to be *Faecalibacterium prausnitzii* from the IV cluster, and *Eubacterium rectale* and *Roseburia* species from the XIVa cluster.

1.2.2 Butyrate production

The production of butyrate is a result of anaerobic fermentation, and most of the butyrate producing bacteria that inhabits the human gut are obligate anaerobes, that are generally difficult to culture(16). Anaerobic fermentation starts with the Embden-Meyerhof pathway, where a glucose molecule is phosphorylated, restructured, and split into two three-carbon compounds. During several steps, these three-carbon compounds are oxidized into two molecules of pyruvate(17).

After the Embden-Meyerhof pathway has produced pyruvate, a mixed acid fermentation occurs as follows(18):

Pyruvate →Acetyl-CoA→Acetoacetyl-CoA→Butyryl-CoA→Butyryl phosphate→Butyrate

According to Tortora et.al.(17), Clostridium bacteria can produce butyric acid, butanol, acetone, isopropyl alcohol, and CO_2 as end products of fermentation. The transformation of Acetoacetyl-CoA to butyryl-CoA is performed by butyryl-CoA dehydrogenase electron-transferring flavoprotein, or Bcd-Etf, whose genes are found in the genome of all butyrate producers found in the human gut(16).

Many butyrate producing bacteria use starch as a source for glucose molecules, and studies has shown that resistant starch(19), i.e. starch not degraded by amylases before it reaches the gut, tends to increase the amount of butyrate production in the gut.

A study that used in vitro gut models to examine the luminal and mucosal bacterial composition(20), showed that the luminal pH was kept at a consistent level, but that the mucosal pH was transient, responding to the changes in pH induced by accumulation of acids from fermentation. A decrease in pH will in addition select for the *Firmicutes* that produce these acids in the first place.

It is also thought that butyrate has an important role in the maintenance of gut health, both by being a major source of energy for the mucosal layer, but also as a regulating molecule, affecting gene expression, inflammation, differentiation, and apoptosis of host cells(16). In addition, butyrate producers may perform a selective degradation of prebiotics(20)

1.3 The effect of commensal bacteria on the immune system

The intestinal tract is lined with a protective mucosal layer, with an inner layer of lymphoid tissue(12). Under the lymphoid tissue, a single-cell layer of intestinal epithelial cells(IECs) creates a barrier between the mucosal layer and the microbiota, and the hosts own tissues and immune cells (13). The mucosal layer is also the home of Peyer's patches, where specialized immune cells internalize microorganisms and macromolecules, so that the macromolecules and antigens can be presented to T-lymphocytes. Many Clostridia are found close to these patches, possibly using this close contact as a way to affect the immune system. It is thought that the T-lymphocytes that express the antigens from the microorganisms internalized by the Peyer's patches adhere to receptors on the apical side of IECs, and that this activation of IECs, cause IEC-derived cytokines to be secreted on the basolateral side of the IEC layer, where they can influence the activity of the immune cells in the lamina propria (LP). This influence on e.g. dendritic cells will then have an effect on T-cell homeostasis(13).

There is a close connection between the commensal microbiota and the mucosal layer, and commensal bacteria from *Clostridium* clusters IV and XIVa occupy parts of the mucosa, and can affect the pH in the mucosal layer by production of organic acids as a result of fermentation. Under normal conditions in the gut, the microbiota can have an effect on the development and function of, among other, Immunoglobulin A (IgA) secreting plasma cells, Th17 cells, regulatory T cells(T_{reg}), invariant natural killer T(iNKT) cells, γδ cells, natural killer(NK) cells, macrophages, dendritic cells(DCs), and innate lymphoid cells(ILCs)(13).

Studies suggests that the close contact between Clostridia and the mucosal layer in the gut can lead to the activation of intraepithelial lymphocytes, IgA-producing cells, and dendritic cells, causing an increase in the amount of interleukin-6 and interleukin-7 (IL-6 and IL-7), and T_{reg}(12). It has also been shown that Clostridia have the gene for production of indoleamine 2,3-dioxygenase(IDO), a tryptophan-degrading enzyme that has been implicated in the induction of T_{reg}(13). This can cause naïve $CD4^+$ T cells to differentiate into antigen specific T_{reg} cells, thus giving an immune tolerance towards the commensal bacteria(12).

The active, antigen presenting dendritic cells can also initiate the differentiation of T helper cells that are normally divided into Th1 and Th2 types, and in infants, there is a higher amount of Th2 cells(21). The Th2 cells produce IL-4 and IL-10, which initiates the production of IgE, and IgE then use the FcεRI receptor to bind closely to mast cells(22). When an allergen then binds to the IgE present on the mast cell surface, there is a cross-linking between the receptors that causes the mast cells to produce and release chemical mediators. These chemical mediators may lead to the development of a "type 1 hypersensitivity reaction"(22).

There are also signs that short chain fatty acids produced by the commensal bacteria, in particular by Clostridia has an effect on the maintenance of the gut immune system homeostasis. The high level of butyrate production by Clostridia is thought to have an effect on the immune system and the development of childhood atopy although the exact mechanism is unknown. One theory says that if butyrate acts as an activating signal on GPRs, then this can activate a pathway to regulate the immune system and its inflammatory responses(13). Another theory includes the NF-κB pathway; butyrate can inhibit the NF-κB protein that causes transcription of several genes associated with immune responses. In addition to the inhibitory effect of butyrate on the NF-κB pathway, the production of IL-10 in T_{reg} cells, can cause a n intestinal and systemic anti-inflammatory effect(12).

The NF-κB pathway have a large effect on the innate immune system. Different receptors on the cell surface will activate the NF-κB pathway. In vertebrates, the pathway can be activated by e.g. Toll-like receptors (TLRs), tumor necrosis factor α (TNFα), and IL-1.

Several studies have also shown that a low level of Clostridia in the gut has been associated with childhood atopy (12, 13, 23), indicating that there is a connection between the colonization of Clostridia and their effect on the immune system.

1.4 Clinical studies

It has been proposed that specific commensal gut microbes are important in the prevention of atopic dermatitis, and that certain beneficial bacteria, or probiotics, can be used to prevent the development of atopic dermatitis and allergy. There are very many studies concerning this topic, three of which are presented here. These three studies have been chosen as they take slightly different approaches to the field of study, and they yield a lot of relevant information. The first study has a small cohort, but the children were also subjected to a follow-up study to see if the effect of probiotics were still present two years after the initial study was finished. The second study has a very large cohort, and they test the effect of four probiotic strains, as well as a prebiotic. The third study has a large cohort, and in addition to looking at the amount of children who develop allergies, they also have two in-depth substudies to look at the total fecal composition and the effect of probiotics vs. placebo in allergy development.

The first study is from Finland(24), where 159 mothers with a family history of atopic dermatitis, were randomly assigned probiotics or placebo during the last stages of pregnancy, and postnatally for six months. The children were examined for signs of atopic dermatitis and asthma at 3, 6, 12, 18, and 24 months of age. Skin-prick tests at 6, 12, and 24 months, as well as total and specific IgE (sIgE) assays in umbilical cord and at 3, 12, and 24 months was also performed. The children in the two groups all had a similar baseline. At two years of age, 35% of the children were diagnosed with atopic eczema, The results of this study shows that there have been a 50% reduction in atopic eczema frequency in children given probiotics vs. children given placebo, but that the total and specific IgE concentrations and skin-prick tests gave similar results between the groups. After two years, there was a follow-up study(25) to see if the preventive effect of probiotics was still in effect. From the original study, 107 children participated in the follow-up study. In the probiotic group, 14 of 53 children were diagnosed with atopic eczema. In the placebo group, 25 of 54 children were diagnosed with atopic eczema. The researchers concluded that the effect of probiotics were still effective at four years of age when it came to preventing atopic eczema.

A second study from Finland(26) had a much larger cohort, consisting of 1521 mothers of high-risk children (at least one parent had a diagnosed allergy), who were recruited for the study. A total of 925 infants were still in the study at the follow-up at two years of age, and IgE sensitization was measured in 916 of the children. This study also differs from the previously described Finnish study in that they investigate the effect of not one, but four probiotic strains, as well as prebiotic galacto-oligosaccharides. Mothers were given probiotics for 2-4 weeks before delivery, and the infants were given probiotics and prebiotics for six months. The children were examined for signs of allergic disease and asthma at 3, 6, 12, and 24 months of age. The children given probiotics and placebo had a similar baseline. The results in this study deviates from that of the previously described study; here it was shown that probiotics and prebiotics did not have a significant preventive effect on allergic diseases, but that the treatment reduced the prevalence of atopic dermatitis. The prevalence of eczema was reduced from 32% to 26%, and the prevalence of IgE-associated eczema was reduced from 18% to 12%. They also noted that previous studies with only one probiotic strain had a higher effect than this study, and that the probiotic treatment did not have an effect on the sensitization itself, but that it rather regulated the path from sensitization to clinical disease, thus increasing the amount of infants without symptoms of allergic disease.

The last study presented here is a study from Norway: Prevention of Atopy in Children in Trondheim, or the PACT study. According to NTNU(27) the main focus of the PACT study is to study how the intake of probiotics can affect three of the high risk factors of atopic diseases such as atopic dermatitis, asthma, and allergic airway disease, including allergic asthma and allergic rhinoconjunctivitis, in a randomized population of pregnant women and small children. Between 2000 and 2005, pregnant women in Trondheim were asked if they wanted to participate in the PACT study. When their children reached the age of two years, the women were to fill out a questionnaire, and the children were examined by a doctor for signs of atopic dermatitis. By the end of 2005, data had been collected for 4784 children. By using the questionnaire data, the prevalence of atopic dermatitis was found to be 16,5%(28). In addition to the main study, there were also two substudies concerning the development of atopic dermatitis in infants.

The aim of the IMPACT study was to look at how the gut microbiota composition affects development of the cytokine profile and the development of atopic disorders. From the pool of pregnant women who participated in the PACT study, the IMPACT study recruited 720 pregnant women for a more in depth study of the gut microbiota composition. A clinical examination of all the participating children at two years of age was performed, and the same clinical examination was offered to random, non-participating children at the ages of two and six years. The clinical examinations were performed to detect atopic dermatitis. In addition, skin-prick tests and specific IgE tests were performed, and blood and stool samples were collected. The article by Storrø et.al.(29), is dedicated to the analysis of the samples collected during the IMPACT study. The results show that low levels of *Escherichia coli* at three months and one year, higher levels of *Bifidobacterium longum* at one year, and lower levels of *Bacteroides fragilis* at two years were common in subjects with atopic sensitization, and that these patterns of colonization were associated with the development of specific IgE sensitization at two years of age. The researchers conclude that the results indicates that immune responses such as an elevation in sIgE is influenced by how the gut microbiota stimulates immunological maturation and the development of the intestinal mucosa.

1.4.1 The Pro-PACT study

The second substudy was called Probiotics in the Pact study, or Pro-PACT(30). In this study, as in the two studies from Finland, probiotics were given to women during pregnancy and three months after birth to see if there is any preventive effect on the development of atopic dermatitis. This study includes children both with and without a family history of atopy, and the participants in the study were randomly sorted into placebo and probiotic groups. Questionnaires were collected at baseline, six weeks, one and two years. Stool and blood (cord and venous) samples were collected at ten days, three months, one and two years of age. All children were examined at two years, and atopic sensitization was evaluated based on skin-prick tests and specific IgE levels. It was estimated that 40% of the children in the placebo group would develop atopic dermatitis, and that there would be a 40% reduction in the probiotic group. The results from this study shows that the preventive effect of probiotica is stronger for non-IgE-associated atopic dermatitis, and that there was no effect on IgE-associated AD, asthma, allergic rhinoconjunctivitis, or atopic sensitization. It was also noted that there often was a lower severity of atopic dermatitis among children in the probiotica group, and

that there were no significant prevention effect of probiotica among the children with a family history of atopy.

1.5 Real-time quantitative Polymerase Chain Reaction

Polymerase Chain Reaction, or PCR, is a method in molecular biology designed to use several thermal cycles to achieve an exponential increase of DNA in a sample. Most PCRs consists of four main steps; initialization, where the samples are heated to activate the polymerase used in the reactions; denaturation, where the samples are heated to break the hydrogen bonds between the bases, leaving two single strands of DNA; annealing, where the temperature is decreased, to allow a primer pair to anneal specifically to a desired position on the template DNA strands; and elongation, where the temperature is increased so the DNA polymerase can use the DNA template to synthesize new DNA strands complementary to the template strands. This temperature cycle is generally repeated between 20 and 40 times, depending on the DNA polymerase used in the reaction, the DNA concentration in the original sample, and the desired DNA concentration in the sample after the amplification process.

In real-time quantitative PCR, or RT qPCR, the DNA concentration is detected by a modified thermocycler (e.g., LightCycler) that measures the emission of light given by fluorescent probes attached to the double-stranded DNA (dsDNA). The result is can be read as the fractional PCR cycle number where the fluorescence level exceeds a set threshold value, called Ct value(31).

There are two main categories of fluorescent dyes used for RT qPCR. Specific fluorescent dyes, such as the TaqMan probe and dual hybridization probes, only emit a fluorescent signal after hybridization to a target sequence. The TaqMan probe has a reporter dye attached to the 3'-end, and a quencher dye attached to the 5'-end. When the dye then attaches to the target sequence, a Taq polymerase enzyme will cleave the probe at the 5'-end, thus separating the quencher and the reporter, allowing the reporter dye to emit a fluorescent signal(31). The dual hybridization probes consists of a set of two probes, where one probe carries a donor fluorophore at the 3'-end, while the other carries an acceptor fluorophore at the 5'-end. The probes will then hybridize head to tail on the target sequence during the annealing step, bringing the donor and the acceptor into close proximity. The donor fluorophore will then transfer energy to the acceptor fluorophore, allowing the acceptor to emit a fluorescent signal with a different wavelength than before the annealing(31).

Unspecific fluorescent dyes, such as the SYBR Green 1 and the EvaGreen probes, are reversible probes that binds to any dsDNA and emits a fluorescent signal, before detaching from the dsDNA. When SYBR Green 1 hybridize to dsDNA, it emits a fluorescent signal, which is detected at the end of each elongation step(31). The EvaGreen probe is a flexible spacer that separates to monomeric DNA-binding dyes with a looped conformation in the absence of DNA. Once DNA becomes available, the looped conformation shifts into a random conformation capable of binding DNA and emitting a fluorescent signal(32).

1.6 DNA sequencing

DNA sequencing is a collective term for several different methods used to find the correct sequence of bases in a strand of DNA. The first real method of DNA sequencing was Sanger sequencing, which is still in use. Modern methods termed Next Generation Sequencing (NGS) are capable of sequencing large amounts of material in a short amount of time. Two of the most common NGS methods are Illumina sequencing and Pyrosequencing. Recently a third generation of sequencing methods has been under development. Examples of third generation sequencing methods are Nanopore sequencing and Pacific Biosciences single-molecule real-time sequencing.

1.6.1 Sanger sequencing

Sanger sequencing is a DNA sequencing method developed in the 70s by F. Sanger and his group(33). The original Sanger sequencing used four parallel reactions, each containing all four 2'-deoxynucleotide triphosphates (dNTPs), and specific 2',3'-dideoxynucleotide triphosphates (ddNTPs). When a ddNTP is incorporated into the synthesizing DNA strand, synthesis is terminated. Because the amount of ddNTPs in each reaction solution in so small, the incorporation of ddNTPs happen rarely, resulting in a reaction solution with a mixture of DNA strands terminated at different points in synthesis(33).

To detect the different synthesized strands, radioactive phosphorus or sulfur isotopes is added to the reaction mix, where the isotope is incorporated into the synthesized strand via a precursor, normally a dNTP or the sequencing primer. The reaction solutions were then run through a polyacrylamide gel (PAG), which allows a very precise separation of the terminated strands based on size. Autoradiography was then used to detect the radioactive signals in the PAG(33).

The modern Sanger sequencing is a result of two breakthroughs by ABI and a team at Caltech. In 1986, the detection of fluorescent markers was developed, where four fluorescent markers were added to one reaction in one PAG tube. In 1990, the miniaturized variant of PAG, capillary gelelectrophoresis was introduced(33).

1.6.2 Pyrosequencing

Pyrosequencing is a sequencing-by-synthesis method based on the detection of released inorganic pyrophosphate (PPi) during the synthesis of DNA; this is possible thanks to the addition of various enzymes to the reaction solution(34).

The template DNA is fragmented and the sequencing primer hybridize to the single-stranded template. The four dNTPs are then added to the reaction solution in cycles, one at a time, as well as various enzymes(34). If the added dNTP is complimentary to the template strand, it will be added by the DNA polymerase, releasing PPi in an amount corresponding to the amount of dNTPs incorporated in the growing DNA strand. The PPi is then converted into ATP that cause the conversion of luciferin to oxyluciferin. Oxyluciferin is capable of emitting a detectable light directly proportional to the starting amount of PPi, and thus the number of dNTPs incorporated. A last enzyme will degrade the unincorporated dNTPs, before the next class of dNTPs are added to the reaction mix (34).

The combination of the light intensity emitted by oxyluciferin in the sample and the knowledge of which dNTP currently added to the reaction mix, makes it possible to determine the sequence of the template DNA.

The most used method at the moment is microfluid pyrosequencing produced by 454 Life Sciences. As stated in the article by Fakruddin et.al.(34), this method fragments the entire genomic DNA, before ligating the fragments to beads that can only hold one fragment at a time. After an amplification of these fragments, the beads are deposited in picolitre wells that only have place for one bead. The different enzymes and dNTPs are then flowed cyclically through the wells, and the generated light signal is detected by a camera.

1.6.3 Illumina sequencing

Illumina sequencing use dNTPs modified with a terminator that only allows one base at a time to be incorporated into the growing nucleotide strand. The terminator also contains a fluorescent label, making it possible to detect the incorporation of a new base. The attachment of the terminator is reversible to allow the incorporation of a new base to the growing chain(35).

The Illumina sequencing method starts with the fragmentation of the samples, and the addition of adapter molecules at both ends. These adapter molecules then attaches the fragmented DNA pieces to the surface of a flow cell. First, unlabeled nucleotides are added to the flow cell during "bridge amplification", thus initializing the creation of double-stranded bridges on the flow cell surface. Denaturation then leaves two individual single-stranded templates attached to the surface via the adapter molecules. During this "bridge amplification", several million double-stranded clusters are produced(36).

At the start of the first sequencing cycle, four labeled terminators are added to the flow cell, in addition to primers and DNA polymerase. As each base is added to the DNA template, a terminator causes the incorporation of new bases to stop. Because each of the labeled terminators emit a different fluorescent color, a detector will record the color emitted by the terminator. The terminator will then be cleaved from the dNTP already incorporated, and a new dNTP with an attached terminator is incorporated into the growing strand of nucleic acids. The Illumina software will then align the sequences based on a reference genome, thus giving the sequence of the sample(35).

A system of barcodes, or indexing primers, are used to allow sequencing of a large amount of samples at the same time. One reverse primer and one forward primer is added to each unique sample, before the samples are pooled into one sample that is sequenced. During the sequencing the primer sequence is also detected, and the Illumina software is then able to separate the pooled samples based on the individual set of forward and reverse primers added(37).

1.6.4 Nanopore sequencing

It was discovered that if a small voltage bias is directed through a membrane-bound nanopore, the current of ions going through the pore could be detected. It was then proposed that a change in the ion current could be detected if a strand of DNA or RNA was fed through the nanopore. The α-hemolysin pore was the first used for nanopore sequencing, as its internal diameter was just large enough for a single strand of nucleic acids to pass through. It was then hypothesized that if the ion current could show a characteristic change for each base, the changes in current could reflect the sequence of the nucleic acid strand(38).

The MspA porin is now used instead of α-hemolysin because it is much better at differentiating the four bases. The new development in nanopore technology also allows scientists to sequence not only

strands of nucleic acid, but also a wide variety of other substances, ranging from small molecules to posttranslationally modified proteins(39).

Nanopore sequencing begins with the incorporation of a nanopore into a lipid bilayer. When the sequencing sample binds to sites within the pore, the changes in ionic current is detected. Because the pores currently available for nanopore sequencing are not able to give a one-base/one-current output, the results are given as a set of bases instead; i.e. nanopore sequencing use a moving window that detects words, rather than individual letters.(39).

1.6.5 Pacific Biosciences (PacBio) single-molecule real-time sequencing

PacBio is a sequencing technology that reads a single molecule at a time, giving an output in real-time. It is a sequencing technology that can handle long reads without GC-bias or systematic errors, but it can suffer from modest throughput and low accuracy(40).

The technology use light detection, were a principle similar to light microscopes, only on a much smaller scale, makes it possible to detect very small samples amounts. A DNA template-polymerase complex is created, and immobilized above the light source, before dNTPs labeled with fluorophores are added. When one of the dNTPs are attached to the DNA template-polymerase complex, a spike in light intensity is detected. After the incorporation of the dNTP into the DNA template-polymerase complex, the phosphate chain is cleaved, releasing the attached fluorophore labeled dNTP, thus freeing the complex for the incorporation of a new dNTP. Several hundred sequencing reactions takes place in parallel, giving a high throughput of sequencing results(41).

1.7 Gas-liquid chromatography

In 1951, Martin and James published the first paper on gas-liquid chromatography, or GLC. The GLC method was based on the liquid-liquid partition chromatography method, published by Martin and Synge in 1941, where they noted that the mobile phase did not necessarily have to be a liquid(42). They had found it possible to detect ten unique acids during one full GLC run, where the identification of the acids were achieved by acid-base titration using sodium hydroxide, and phenol red as an indicator(43). In a paper from the end of 1952, the authors also comment on the dependence of better detection methods(43). They also mentioned that thermal conductivity detectors were already being used for the analysis of a variety of alcohols, esters, ketones and aromatic hydrocarbons, and could be adapted for gas-liquid chromatography.

GLC was developed as a way to separate the volatile components in a mixture. To achieve this, a small amount of the sample mixture is injected into a hot injector port where the components in the sample vaporize due to the high temperature, forming the gas phase. The separation of the components take place inside the column, where the carrier gas (helium is the most widely used carrier gas), or mobile phase, and the liquid, stationary phase carries the molecules through the column to a detector. When the molecules are detected, signals are sent to e.g. a computer, where the individual components are seen as peaks. The area under the peak gives is proportional to the injected amount of the different components(44).

1.8 Aim of thesis

A rise in the development of allergies and atopic dermatitis has caused an increase in studies on the topic of how the intake of probiotic bacteria can affect the development of allergies and AD in children. The clinical studies presented in this thesis examines the preventive effect that several different probiotics and prebiotics can have on the development of allergies and AD. The results of the clinical studies show that the intake of probiotics and prebiotics during the last few months of pregnancy, as well as the first few months of life, cause a reduced risk of developing allergies and AD, in particular in children without a family history of atopy. The results also showed that the intake of probiotica reduced the severity of AD in the children who were diagnosed with AD.

While the use of probiotics as preventive agents have shown promising results in several studies, the exact mechanism behind this preventive effect is currently unknown.

One of the proposed mechanisms is that butyrate-producing bacteria, such as members of *Clostridium* clusters IV and XIVa, can have an effect on the immune system when they colonize the infant gut. Members of *Clostridium* clusters IV and XIVa are obligate anaerobic bacteria that forms endospores and lives in close proximity to Peyer's patches, areas of the gut where microorganisms and macromolecules are internalized by specialized immune cells presenting them to T-lymphocytes. Members of these Clostridia clusters also produce butyrate as an end product of fermentation, and studies have shown that SCFAs can have an effect on the immune system. Thus, members of *Clostridium* clusters IV and XIVa can affect the immune system both by the presentation of antigens to immune cells, but also by butyrate causing activation of immunological pathways.

The hypothesis for this thesis is that the intake of probiotica can influence the colonization pattern of bacteria from *Clostridium* clusters IV and XIVa in the infant gut, as well as when they start to produce butyrate, and that this change in colonization pattern can cause the development of allergies and AD.

To determine when members of *Clostridium* clusters IV and XIVa colonize the infant gut, a combination of heat and chloroform was used to isolate the endospores formed by members of *Clostridium* clusters IV and XIVa in samples from mothers and their children who had participated in the Pro-PACT study. Gas-liquid chromatography was the method chosen for detection of butyrate in these samples. Specific qPCRs for members of *Clostridium* clusters IV and XIVa was then used to verify that the endospore isolation process had indeed isolated endospores formed by *Clostridium* clusters IV and XIVa. In addition, Illumina sequencing was then used to try to identify the main bacterial families present in the samples, particularly those belonging to *Clostridium* clusters IV and XIVa.

2 Materials and methods

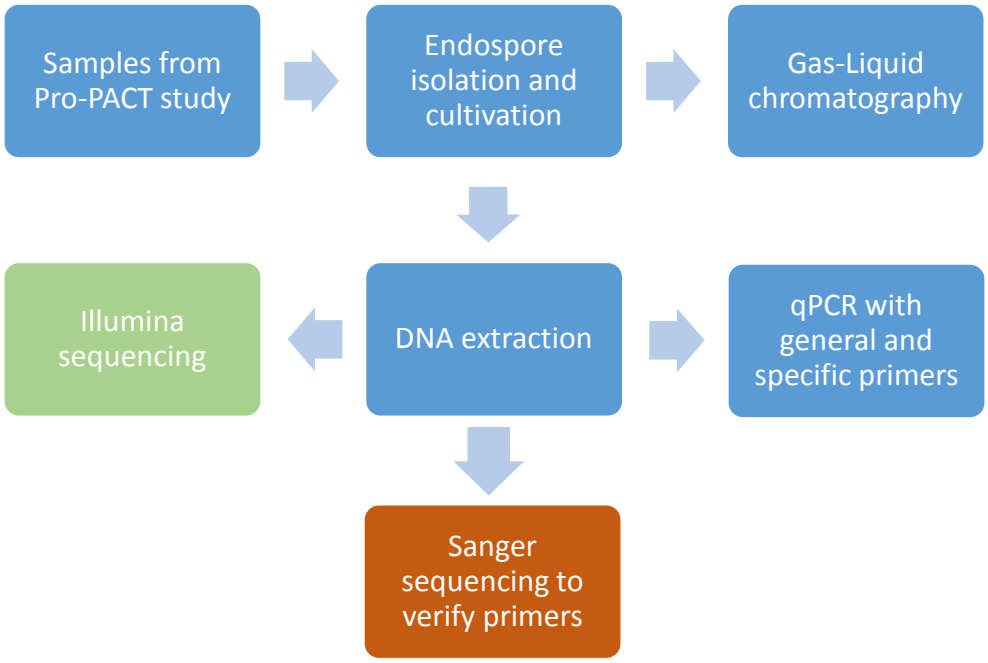


Figure 2.1: The figure shows the workflow of the thesis. Sanger sequencing (in orange) was performed by Krister Lundgård, and the results from the Illumina sequencing (in green) was analyzed with the help of Jane Ludvigsen.

2.1 Sample background

The Pro-PACT project was designed as a double-blind study to see the effect of probiotica on the development of atopic dermatitis. 415 pregnant women were recruited to the project, and randomly assigned to the Biola and placebo groups. Fecal samples were collected from mother at approximately 36 weeks of gestation (Mom1), from mother three months after birth (Mom2), and from the children at 10 days (10days), three months (3months), one (1year), and two years (2year) of age. A grand total of 1516 fecal samples were collected, and stored in Cary Blair media at -80°C. Data was collected for 1470 of the samples. The samples chosen for analysis in this thesis were chosen from the pool samples with all the necessary information available. To get the highest possible amount of data points, only samples from mother-child pairs that had contributed five or more fecal samples were chosen for analysis. The total amount of analyzed samples was 510, from a total of 85 mother-child pairs. For 83 of the mother-child pairs, there were six data points, from one pair there were seven data points, and from the last mother-child pair, five data points were available. Information about the samples used for this thesis is listed in Appendix A.

The primer pairs specific for *Clostridium* clusters IV and XIVa are based on the primer pairs used by Wise and Siragusa(45) in their article. The primer for *Clostridium* cluster IV is based on the *Clostridium leptum* subgroup, while the primer for *Clostridium* cluster XIVa is based on the *Clostridium coccoides – Eubacterium rectale* subgroup. To validate the primers, specific qPCRs were performed on a reference plate that contains a collection of reference bacterial strains found in the digestive system. The strains on the reference plate are listed in Appendix B.

2.2 Endospore isolation and cultivation

The samples were thawed on ice, vortexed for 10 seconds and then left to precipitate for 30 minutes. 250µl of the fecal samples was transferred to eppendorftubes (VWR, Norway), and chloroform (Merck, Norway) was added to a concentration of 6%. The samples were treated with chloroform at 270 rpm and 50°C for 60 minutes on a thermoshaker (TS-100, bioSan, Latvia). The samples were then centrifuged at 10000 rpm for 5 minutes. The supernatant was discarded and the pellet resuspended in 500µl PBS. The washing step was repeated once before 100µl of the washed sample was plated on TSA media, and 100µl added in 5ml anaerobic broth. Both plates and broth were cultivated anaerobic at 37°C and 100rpm for 48 hours. The optical density of the anaerobic broth containing the samples was measured by a McFarland reader (MF-unit DEN-1B, bioSan, Latvia) before and after incubation, and a 0,5 increase in optical density was set as the cutoff value for growth. All samples with a 0,5 or higher increase in optical density were marked as positive and stored at -20°C until further analyses. The endospore isolation is based on the methods described by Atarashi et.al.(46), and by Kelly et.al.(47) Recipes for anaerobic basal broth, TSA media, and PBS is listed in Appendix C.

2.3 DNA extraction

The samples marked as positive were centrifuged at 10000rpm for 10 minutes, and approximately 1ml of the supernatant was transferred to microtubes (2mL, VWR, Norway) containing 0,2g acid washed glass beads (<106µm, Sigma-Aldrich, Norway) and 250µl S.T.A.R. buffer (Stool Transport and Recovery buffer, Roche, Norway). The cells were then processed twice at 6500rpm for 20 seconds in a MagNaLyser (Roche, Norway), before the samples were centrifuged at 13000rpm for 5 minutes. The samples were then transferred to KingFisher 96 plates (Thermo Scientific, USA). A KingFisher Flex robot (Thermo Scientific, USA) was used for the proteinase treatment (program Proteinase LGC Mini), and the DNA extraction (program MagMiniLGC), using the chemicals provided by the MagMidi kit (LGC Genomics, Germany). The samples were then stored in 96 well plates until further analysis (PCR plates, Thermo Scientific, USA).

2.4 PCR amplification

Three qPCRs were performed on each sample, one using general 16S primers (PRK341F and PRK806R, Solis BioDyne, Estonia) and two with specific primers for clusters IV and XIVa. The following mastermix and final concentrations was used for all the qPCRs: 5X HOT FIREpol EvaGreen qPCR Mix Plus (N/A, Solis BioDyne, Estonia), Forward primer (0,2µl), and Reverse primer (0,2µl). The qPCRs were performed using a LightCycler 480(Roche, Norway) to read the fluorescence. The following thermo cycle protocol was used; 95°C for 15 minutes, (95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds)X40. The elongation step is the only variable point; for the general 16S qPCR with PRK primers the elongation temperature is 55°C, for the specific primer pairs for *Clostridium* clusters IV and XIVa, the elongation temperature is 60°C and 50°C, respectively.

2.5 DNA fragment size determination

A 1% agarose solution was prepared (agarose (Sigma-Aldrich, Norway) and 1% TAE buffer). Loading dye and gel red was mixed with a 2:2 ratio, before 2µl loading mix was mixed with 5µl DNA. The ladder was mixed with gel red with a 2:1 ratio. After the gel had set, 4µl of the ladder mix and 5µl of the DNA+loading mix, were placed in the wells. The gel was run with 75V and 400 mA for 35 minutes

2.6 Sequencing

2.6.1 Sanger sequencing

Sanger sequencing was performed on the reference plate to verify the bacterial strains. For the sequencing a BigDye Terminator v1.1 cycle sequencing kit (Life technologies, Thermo scientific, USA) was used, with CoverAll primers (Genetic Analysis, Norway). CoverAll primers are used because they cover almost the entire 16S gene. The sequencing was performed according to the manufacturer's instructions. Agencourt CleanSEQ (Beckman Coulter, Norway) was used to clean the samples before capillary gelelectrophoresis. The capillary gelelectrophoresis used was ABI 3130POP7BDv_1_1_E. The 16S sequences were identified using the Ribosomal Database Project, or RDP(48).

2.6.2 Illumina sequencing

All PCRs are performed as described under point 2.4 (PCR amplification). 1x AMPure XP beads (Beckman Coulter, Norway) were used to remove short DNA fragments from the amplified samples. Some of the samples were cleaned using the manual procedure provided by the producers of the AMPure beads, while most of the samples were cleaned using a BioMek 3000 robot (Beckman Coulter, Norway), following the same procedure as for manual clean up. After cleaning, a selection of the samples were checked on an agarose gel to make sure that the DNA fragments of the desired lengths were in the samples.

The next step is an indexing PCR where two individual primers are added to each sample, creating unique barcodes for each samples that are used to separate the samples during analysis of the sequencing output. A 1:100 dilution of the cleaned PCR-product was then made as preparation for an indexing PCR. To label the samples, forward primers 1-8 and reverse primers 7-19 were chosen and a new selection of samples were checked on an agarose gel after the indexing PCR to make sure that the PCR product were the right length.

The Taq-man probe (Solis BioDyne, Estonia) was then used for a quantification PCR, the results of which were used to normalize and pool the samples. The pooling of the samples was performed by the BioMek 3000, before a second cleaning of the samples using 0,8x AMPure XP beads. The pooled sample was then checked on an agarose gel to make certain that the correct fragments were present in the pooled sample.

The PerfeCta NGS Quantification Kit for Illumina sequencing platforms (Quanta BioSciences, USA) was then used to measure the DNA concentration in the pooled sample, and the measured concentration was then used to dilute the pooled sample to a 4nM concentration.

A PhiX control was then prepared according to the MiSeq producer's instructions. The PhiX control and the pooled sample was then diluted to an 8pM concentration, and loaded onto a version 3 Reagent cartridge and flow cell (Illumina, USA). The Reagent cartridge and flow cell were then loaded into the MiSeq (Illumina, USA). The results were analyzed using QIIME.

2.7 Gas-Liquid chromatography (GC)

Before endospore isolation and cultivation of the samples, 500µl of the original sample was transferred to an eppendorftube, and stored at -20°C until gas-chromatography analysis.

A Perkin Elmer AutoSystem Gas Chromatograph with an automatic sampler was used to analyze the samples. The column used was a Stabilwax-DA capped column/30m, 0,53mm ID, 0,25µm (Teknolab, Norway). Hydrogen was used as carrier gas. To prepare the stored samples for GC analysis, 400µl was centrifuged at 13000rpm for 10 minutes, before 300µl of the supernatant was transferred to centrifuge filters(0,2µm, 500µl, VWR, Norway), and centrifuged at 10000rpm for 5 minutes. The samples were then transferred to vials (Chromacol LTD 0,3FIV, VWR, Norway) and capped (caps alu, CI 11mm, VWR, Norway), before placement in the automatic sampler. The following program was used for oven temperature:

- Initial temperature: 70,0°C, hold for 3 minutes
- Ramp 1: 20,0°C/minute to 150,0°C, hold for 0,0 minutes
- Ramp 2: 8,0°C/minute to 170°C, hold for 0,0 minutes
- Ramp 3: 12,5°C/minute to 200°C, hold for 30,0 minutes

TotalChrom was used to initialize the GC-machine and to extract results.
The standards used for the standardcurve was 0,1M acetic acid, 0,1M propionic acid, 0,1M valeric acid, 0,1M isovaleric acid, 0,1M butyric acid, and 0,1M isobutyric acid (all standards acids purchased from Sigma Aldrich, Norway). The standards were mixed with a 1:1 relationship before used, and a minimum of four standard samples were included in each run to ensure that no contaminants caused irregular spikes in the baseline.
A filtered sample containing the anaerobic broth used for growth was also run through the GC, to identify the baseline of the broth without bacteria or short chain fatty acids present.

2.8 Data analysis

2.8.1 T-tests

Simple t-test were used for the statistical analysis of the data. A t-test is a statistical analysis that compare the difference between two groups, by looking at the ratio between the difference in the group means and the variability of groups. The result of a t-test is a p-value, or significance value. If the p-value is below 0,05, the difference between two groups is statistically significant, and if the value is above 0,05, the difference is not statistically significant.

2.8.2 Analysis of Illumina sequencing output

QIIME describes the main steps of analysis of Illumina sequencing outputs as following(49). The barcoded reads are assigned to the original sample during demultiplexing, and the samples are filtered by quality. After demultiplexing, the sequences are assigned an operational taxonomic unit, or OTU, and the sequences are then clustered based on the assigned OTU, giving an OTU table, listing how often each OTU appears in each sample. A threshold value for the amount of sequences in each sample is also chosen; here set at 2000, i.e. there has to be at least 2000 sequences in a sample for it to appear in the final OTU table. Diversity analysis are also performed on the sequences by the QIIME program, giving a number of different analysis results.

Among the different analysis results that are part of the QIIME output are information about the α-diversity in the samples shown as Rarefraction plots, and β-diversity in the samples shown as UniFrac plots. While α-diversity is information about the species diversity at one site, β-diversity is the species diversity between several sites. UniFrac plots is a presentation of the β-diversity in the samples, using the phylogenetic information from sequencing to compare the samples sequenced(50). Rarefraction plots are used to assess the species richness in the samples, based on the phylogenetic information from sequencing.

To look more closely at the phylogenetic relationship between the sequences in the samples, CLC Main Workbench version 7 was used to produce a phylogenetic tree.

2.8.3 Calculation of butyrate concentration

The output of a GC-run is the areal under a peak in the baseline. To calculate the molar concentration of butyrate in these samples, the average peak areal of all standards in a run was calculated. This average areal was used to make a standard-curve where the average peak areal was set as one. The formula of the trend line was used to correlate the butyrate peak areal in each sample of one run to the average peak areal for the standards for the same run.

After the peak areal in each sample had been correlated to the average peak areal in the standard, the concentration of butyrate in the standard mix was used to calculate the concentration of butyrate in each sample.

3 Results

3.1 Endospore isolation and cultivation

In the total pool of analyzed samples, 65 samples were collected from children at 10 days, three months, one year and two years of age, while 66 samples were collected from mothers during pregnancy, and 64 were collected from mothers three months after birth. From this total pool of analyzed samples, 221 of the samples were collected from the group given Biola, while 286 of the samples were collected from the group given placebo.

After endospore isolation and cultivation, 100 of the 510 samples were marked as positive after a 0,5 or higher increase in optical density had been measured as described in materials and methods. In the pool of samples with a sufficient increase in optical density after endospore isolation and cultivation, 40 of the samples were from the Biola group, and 60 of the samples were from the placebo group.

An overview of the increase in optical density in the individual samples, as well as average increase in optical density and standard deviation for the different age groups, is shown in Appendix D.

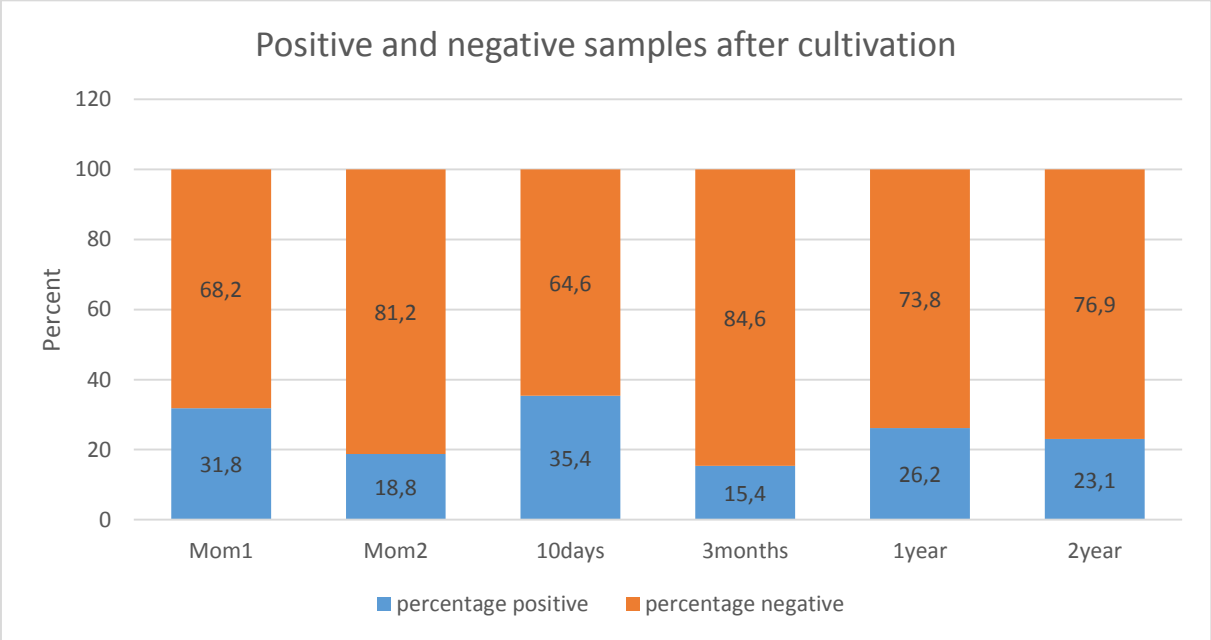


Figure 3.1: The figure shows an overview over what percentage of samples gave a positive result after endospore isolation and cultivation, divided by age groups.

Figure 3.1 shows the percentage of samples from each age group that reached a sufficient increase in optical density after endospore isolation and cultivation. The highest abundance of samples with a sufficient increase in optical density after cultivation were collected at 10 day old (35,4% of total amount of samples taken at 10 days), while the second largest amount of samples with a sufficient increase in optical density were collected from mothers during pregnancy (31,8%). In the samples taken from mothers three months (18,8%) after birth, there is a decline in the amount of positive samples. For the children, there was a decrease in abundance of positive samples at 3 months(15,4%) when compared to the amount of positive samples taken at 10 days, followed by an

increase in the amount of samples collected at one year of age(26,2%), before a new decrease in the amount of samples collected at two years of age (23,1%).

A randomly chosen selection of samples was also cultivated on TSA media after endospore isolation to assess the success rate of the treatment used for endospore isolation. After cultivation under the same conditions as the anaerobic broth, all the plates with TSA media showed growth. The colonies cultured on these plates varied greatly in morphology. Staining and wet mounds showed that there was a great diversity in bacterial morphology, as well as something reminiscent of fungal hyphae.

3.2 Specific qPCRs for *Clostridium* clusters IV and XIVa

The primer pairs specific for *Clostridium* clusters IV and XIVa were validated using the reference plate described in Materials and Methods. The validation yielded a number of positive reference strains for each of the primer pairs. One of the reference strains yielding positive amplification results for each primer pair was chosen as positive controls for the specific qPCRs on the samples marked as positive after cultivation. For cluster IV, *Clostridium leptum* was chosen as the positive control. For cluster XIVa, *Eubacterium rectale* was chosen as the positive control.

General qPCRs using 16S primer pairs were performed on all samples marked as positive after cultivation to make sure that there was enough available DNA for the specific qPCRs. All samples marked as positive after cultivation yielded a positive amplification result after the general qPCRs.

The results from the qPCRs with specific primer pairs for *Clostridium* clusters IV and XIVa are shown in Figure 3.2 and Figure 3.3, respectively. For both primer pairs, the highest abundance of samples with positive amplification results after qPCR is found in samples collected from mothers during pregnancy, with a decrease in abundance in samples collected from mothers after birth. For the children, there is a very small abundance of samples with a positive amplification result at 10 days, with an increase at three months and one year, before a slight decrease in abundance in samples collected at two years.

The primer pair specific for *Clostridium* cluster IV had the highest amount of samples not yielding any amplification results after qPCR. Common for both specific primer pairs is that the highest abundance of samples that does not yield amplification results is from the 10 day age group.

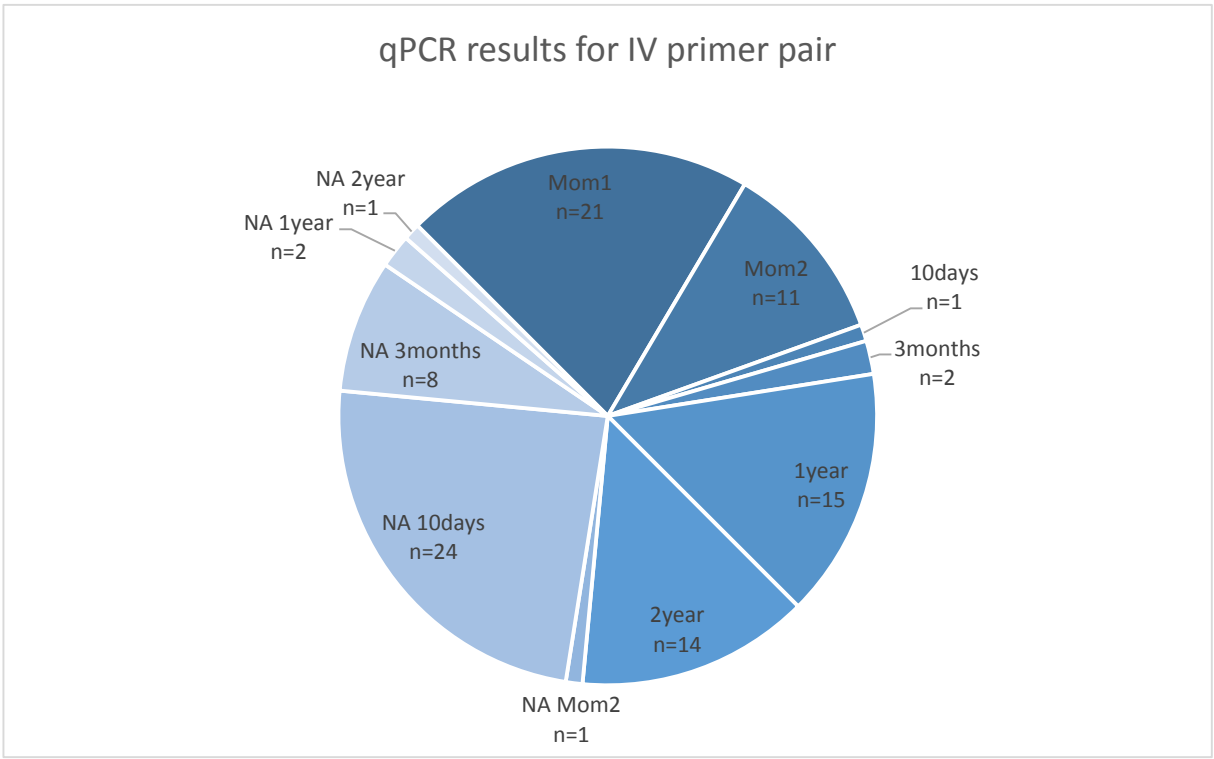


Figure 3.2: The figure shows an overview of the results of a qPCR with primers specific for Clostridium cluster IV. Samples not yielding amplification results are marked as NA, or No Amplification

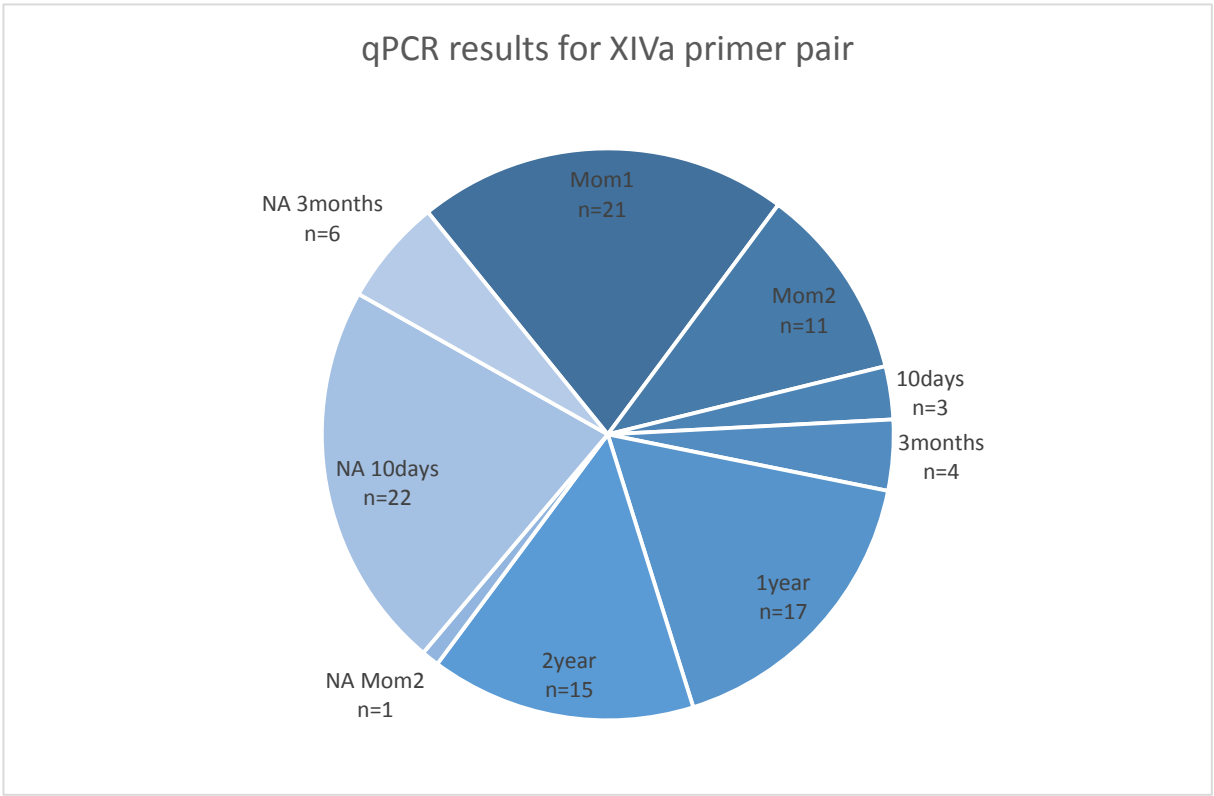


Figure 3.3: The figure shows an overview of the results of a qPCR with primers specific for Clostridium cluster XIVa. Samples not yielding amplification results are marked as NA, or No Amplification

3.3 Gas chromatography

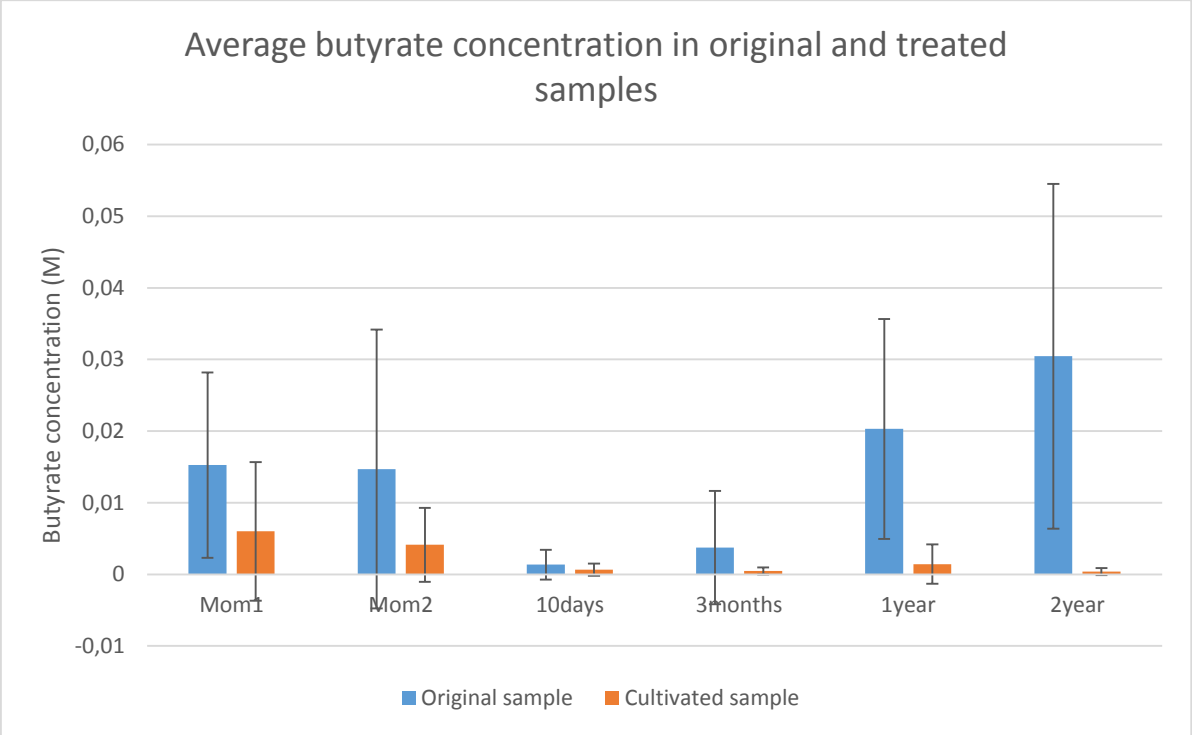


Figure 3.4: The figure shows the difference in average butyrate concentration in the original, untreated samples and in the same samples after endospore isolation and cultivation, as well as standard deviation in butyrate concentration.

All the samples marked as positive after endospore isolation and cultivation were analyzed by gas chromatography to find the butyrate concentration in the samples. The original, untreated versions of these samples were also analyzed by gas chromatography to see if there is a difference in butyrate concentration in the original fecal samples and the cultivated samples. When calculating the butyrate concentrations in the individual samples, the butyrate concentration in the standard used during the GC runs was calculated to be 0,01667M.

Figure 3.4 shows the average butyrate concentration in the original and the cultivated samples. The difference in concentration in the samples from mother are approximately the same both during pregnancy and after birth. In 10 day old infants, the butyrate concentration is almost the same in the original and the cultivated samples. At the other time points, the average butyrate concentration in the original fecal samples increases sharply throughout the first two years of life. The average butyrate concentration in the cultivated samples shows a slightly different pattern. In general, the average butyrate concentration is very low. In addition, the average butyrate concentration reaches a peak at one year of age, before a slight decrease in average butyrate concentration in the samples collected at two years of age.

T-tests confirms that the higher concentration of butyrate in the original samples versus the cultivated samples, is statistically significant. When looking at the butyrate concentrations for the different age groups in original samples versus cultivated samples, the p-values are the following; for mothers during pregnancy, the p-value is 0,02. For mothers three months after birth, the p-value is 0,03. At 10 days, the p-value is 0,17. At 3 months, the p-value is 0,28. At one year, the p-value is $6{,}8\text{x}10^{-5}$. At two years, the p-value is $2{,}6\text{x}10^{-4}$. When the t-test is expanded to include the difference in butyrate concentration in all original samples versus all cultivated samples, the p-value is $2{,}4\text{x}10^{-9}$.

3.4 Comparison of results from cultivation, qPCRs, and GC

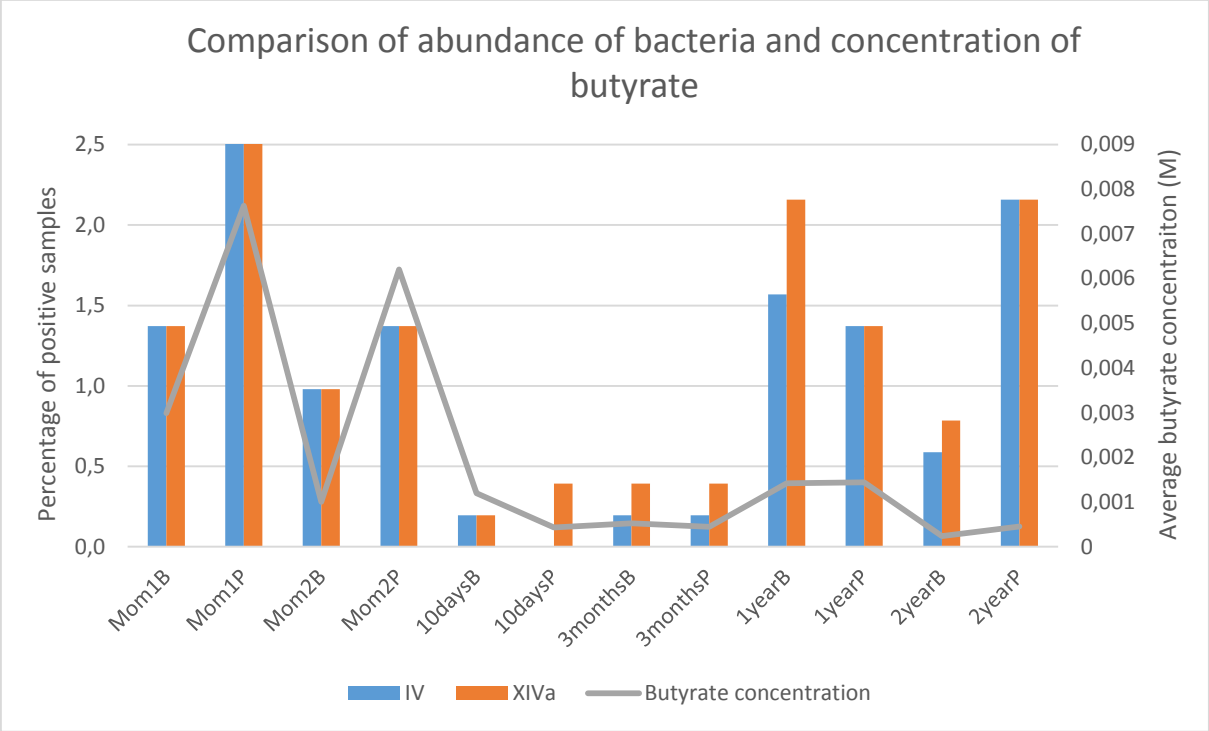


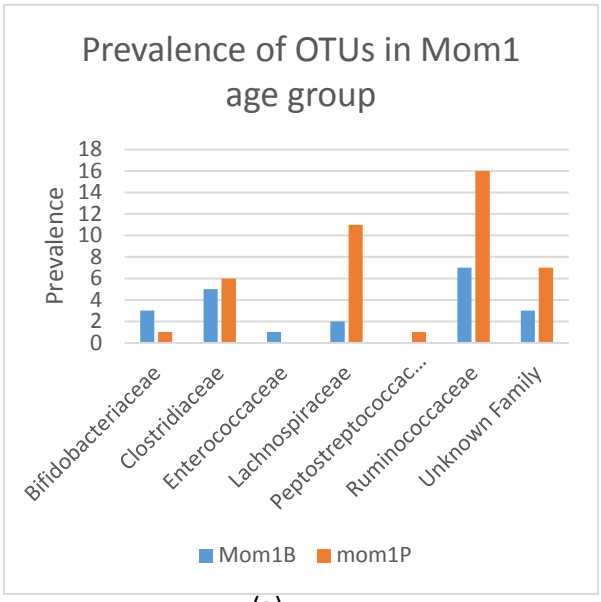
Figure 3.5: The figure compares the concentration of butyrate produced in cultivated samples, and the abundance of Clostridium cluster IV and XIVa bacteria found in samples belonging to Biola (B), and placebo (P) groups

Figure 3.5 shows the conneciton between butyrate produced in cultivated samples and the abundance of bacteria from clusters IV and XIVa after qPCRs. It also compares the butyrate concentration and bacterial abundance in samples from the Biola and the placebo group.

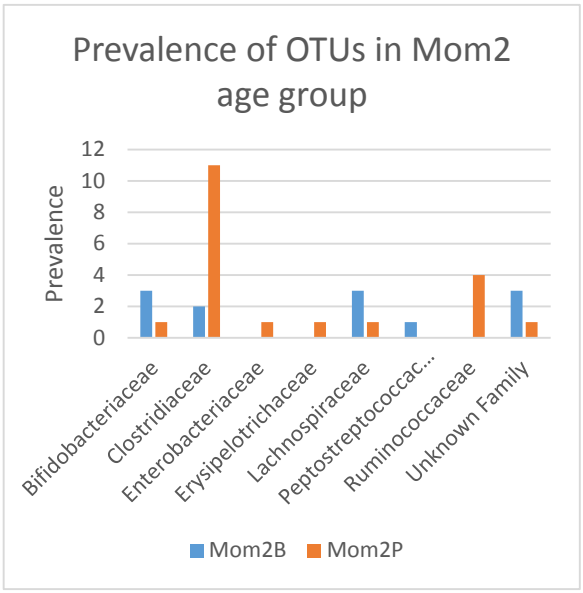
There is a higher prevalence of bacteria from *Clostridium* clusters IV and XIVa in samples collected from all age groups in the placebo group, with the exeption of samples taken at one year of age, where the highest abundance of *Clostridium* clusters IV and XIVa is found in the Biola group.

There seems to be a correlation between the average butyrate concentration and the abundance of *Clostridium* cluster IV and XIVa bacteria in the different age groups. T-tests confirms that there is a statistical significant correlation between the average butyrate concentration and bacteria abundance. A t-test on the difference in average butyrate concentration between samples with positive amplification results for qPCR with primers specific for *Clostridium* cluster IV gives a p-value of $5{,}35 \times 10^{-4}$. When comparing the difference in butyrate concentraion between samples with positive amplification results for qPCRs with primers specific for *Clostridium* cluster XIVa, the p-value is 0,001.

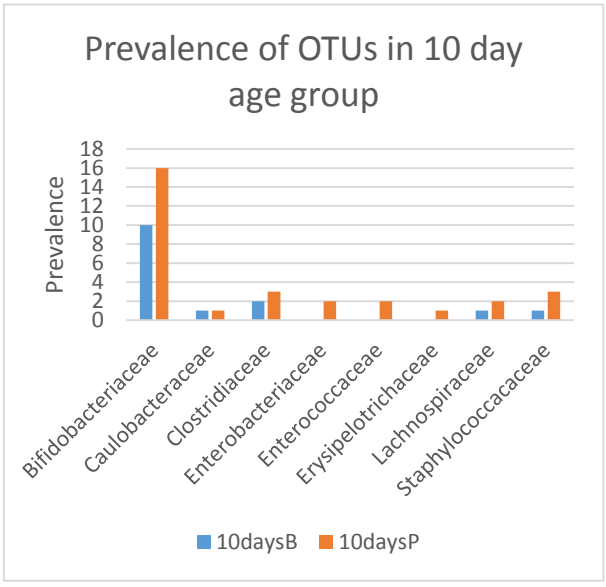
T-tests performed to see if there is a statistically significant correaltion between the butyrate concentration between the Biola and placebo groups yielded a p-value of 0,08.



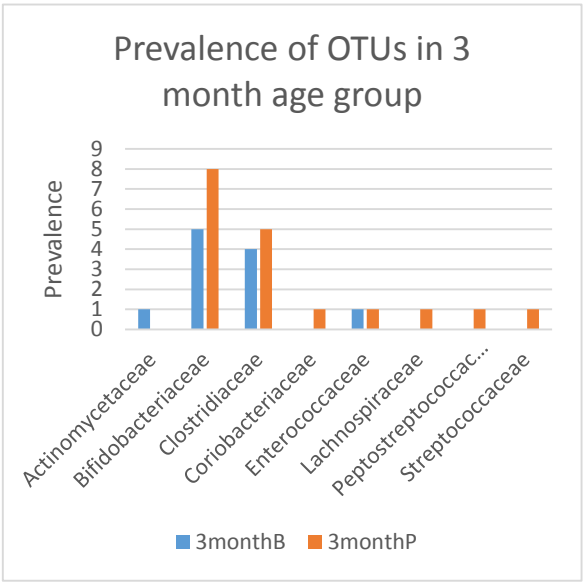
(a)



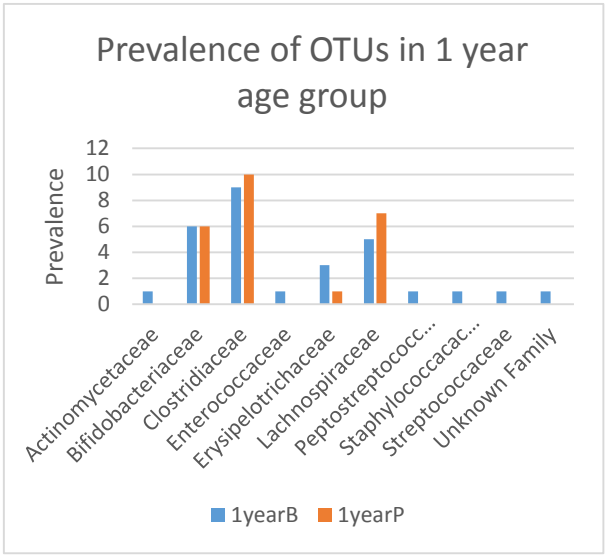
(b)



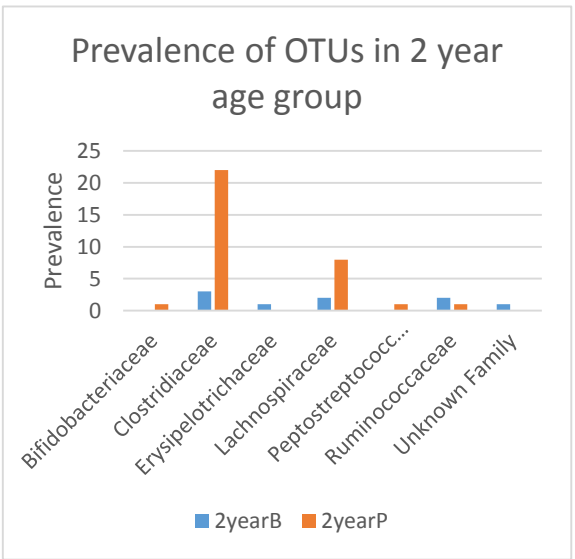
(c)



(d)



(e)



(f)

Figure 3.6: Figures a-f shows an overview of the most common OTUs present in the Biola (P) and placebo (P) groups divided by age group.

3.5 Illumina sequencing

The 100 samples marked as positive after cultivation was also subjected to Illumina sequencing. With a cut-of value of 2000 sequences, 88 of these 100 samples gave an output after sequencing. In total, 347 OTUs were found in the samples. When choosing the three most common OTUs from each individual sample, the number of OTUs was reduced to 63.

Figure 3.6a-f shows a presentation of prevalence data, while the amount of sequences found in the different OTUs are listed Appendix F.

Most of the 63 most common OTUs belong to phylum Firmicutes. The families belonging to the order Clostridiales include Clostridiaceae, Lachnospiraceae, Ruminococcaceae, and Peptostreptococcaceae. To identify which *Clostridium* clusters the families found during Illumina sequencing belongs to, articles by Collins et.al.(14) and Liu et.al.(15) have been used. In the Clostridiaceae family, most OTUs does not include a genus level, but those who do belongs to the genus *Clostridium*, and one sample belongs to the species *Clostridium perfringens*, that belongs to *Clostridium* cluster I. The genus *Clostridium* in itself is found in several clusters, including both clusters IV and XIVa. The Lachnospiraceae family contains the genera *Blautia, Coprococcus,* and *Lachnospira*, all of which have species belonging to *Clostridium* cluster XIVa. The Ruminococcaceae family contains the genera *Ruminococcus* and *Faecalibacterium*. The *Ruminococcus* genera contains species found in both *Clostridium* clusters IV and XIVa, while the *Faecalibacterium* genera contains species found in *Clostridium* cluster IV. The Peptostreptococcaceae family do not contain any information about species or genus level, and it can therefore not be assigned to any particular *Clostridium* clusters.

The family diversity is lowest at 10 days, and increases steadily throughout the first year, before the diversity decreases at two years, when the composition of bacterial families found in the samples more closely resembles that of the family composition in samples collected from mothers.

Some differences are visible when comparing the samples collected from mothers during pregnancy and three months after birth, shown in Figures 3.6a and 3.6b, respectively. The amount of Bifidobacterium, Lachnospira, and Ruminococcus have decreased in samples taken after birth, while the amount of Clostridia has increased, when compared to samples collected during pregnancy.

Figures 3.6c-f show the prevalence of OTUs in the samples collected at 10 days, three months, one and two years of age.

The Bifidobacteriaceae family is the most prevalent family at 10 days, but then decreases steadily, and is nearly absent in samples collected at two years. The OTUs Caulobacteriaceae, Coriobacteriaceae, Enterobacteriaceae, Enterococcaceae, Staphylococcaceae, Erysipelotrichaceae, Actinomycetaceae, and Streptococcaceae appear randomly in the different age group, and mostly with a low abundance.

The Clostridia family Peptostreptococcaceae seems to appear randomly in the Biola and placebo groups from three months of age, and always with a very low prevalence.

Clostridiaceae appears in the samples collected at 10 days. The prevalence of Clostridiaceae is higher in samples collected from the placebo group than in samples collected from the Biola group. The prevalence of Clostridiaceae in samples from the placebo group increases gradually throughout the

first two years of life. In the Biola group, however, there is a peak in the prevalence of Clostridiaceae in the samples collected at one year, with a sharp decrease in prevalence of Clostridiaceae in the samples collected at two years.

As with Clostridiaceae, Lachnospiraceae first appears in samples collected at 10 days, but the prevalence of Lachnospiraceae is lower than the prevalence of Clostridiaceae. At three months, the prevalence of Lachnospiraceae is very low in the placebo group, and completely absent in the Biola group. At one month, there is an increase in the prevalence of Lachnospiraceae in both group, and at two years, there is a decrease of Lachnospiraceae prevalence in the Biola group, as well as a slight increase in the placebo group.

Ruminococcaceae does not appear in samples collected from children until the age of two years. There are more Ruminococcaceae in the Biola group than the placebo group, but the overall abundance is very low.

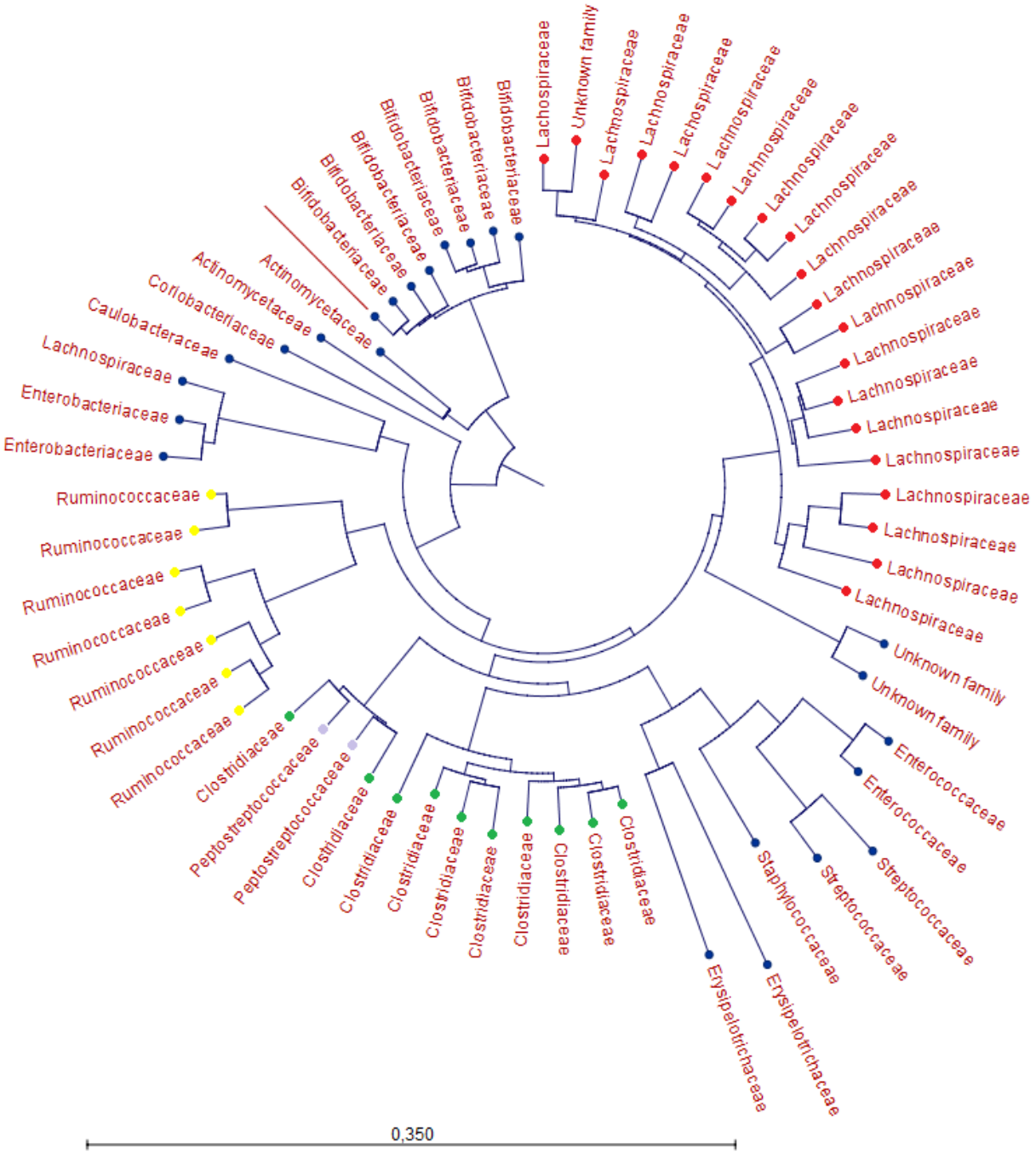


Figure 3.7: The figure shows a phylogenetic tree made with neighbor joining method over the 63 most common OTUs present in the samples. Red nodes show Lachnospiraceae, green nodes show Clostridiaceae, yellow nodes show Ruminococcaceae, light purple dots show Peptostreptococcaceae.

The phylogenetic tree in Figure 3.7 shows that the 63 most common OTUs found in the samples after Illumina sequencing are divided into three main clusters. The first cluster contains the Actinobacteria families Actinomycetaceae, Bifidobacteriaceae. The Actinobacteria family Coriobacteriaceae is clustered alone, but close to the rest of the Actinobacteria.

The second cluster contains the Gammaproteobacteria family Enterobacteriaceae, and the Alphaproteobacteria Coriobacteriaceae.

The third cluster contains a subcluster with the Bacilli class of Firmicutes, with the families Erysipelotrichaceae, Staphylococcaceae, Streptococcaceae, and Enterococcaceae. The Bacilli subcluster is located between two subclusters, one containing Lachnospiraceae (red nodes) and unknown families from the Clostridiales order. The other subcluster contains the Clostridiaceae (green nodes), Peptostreptococcaceae (light purple nodes), and Ruminococcaceae (yellow nodes) families.

In addition to the phylogenetic tree, UniFrac plots were generated based on the bacterial content of all samples after Illumina sequencing, showing how the samples clustered based on individual bacterial content.

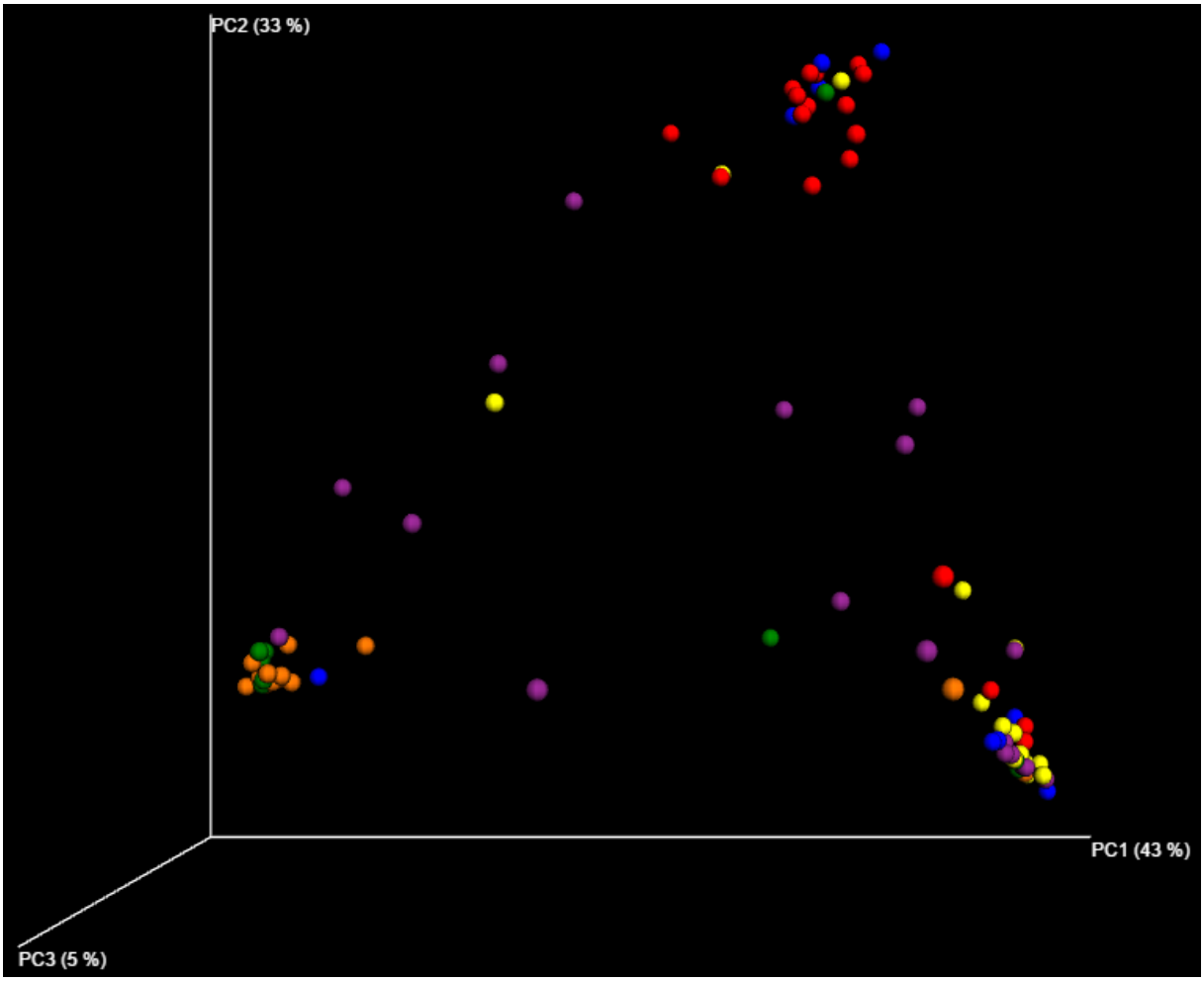


Figure 3.8: The UniFrac plot shows how the samples cluster based on age; mother during pregnancy (red), mothers three months after birth (blue), children at 10 days (orange), three months (green), one year (purple), and two years (yellow).

Figure 3.8 shows how the samples are clustered based on age. Most samples from children at 10 days (in orange) and three months (in green) of age are clustered together in the lower left corner of the plot. Samples from children at two years (in yellow) of age are clustered in the lower right corner of the plot, while the samples collected at one year (in purple) of age are more spread out, and most seems to be situated between the samples from 10 days and three months, and samples from two years. The last major clustering in the top right corner are mostly made up of samples from mothers during pregnancy (in red) and from mothers three months after birth (in blue).

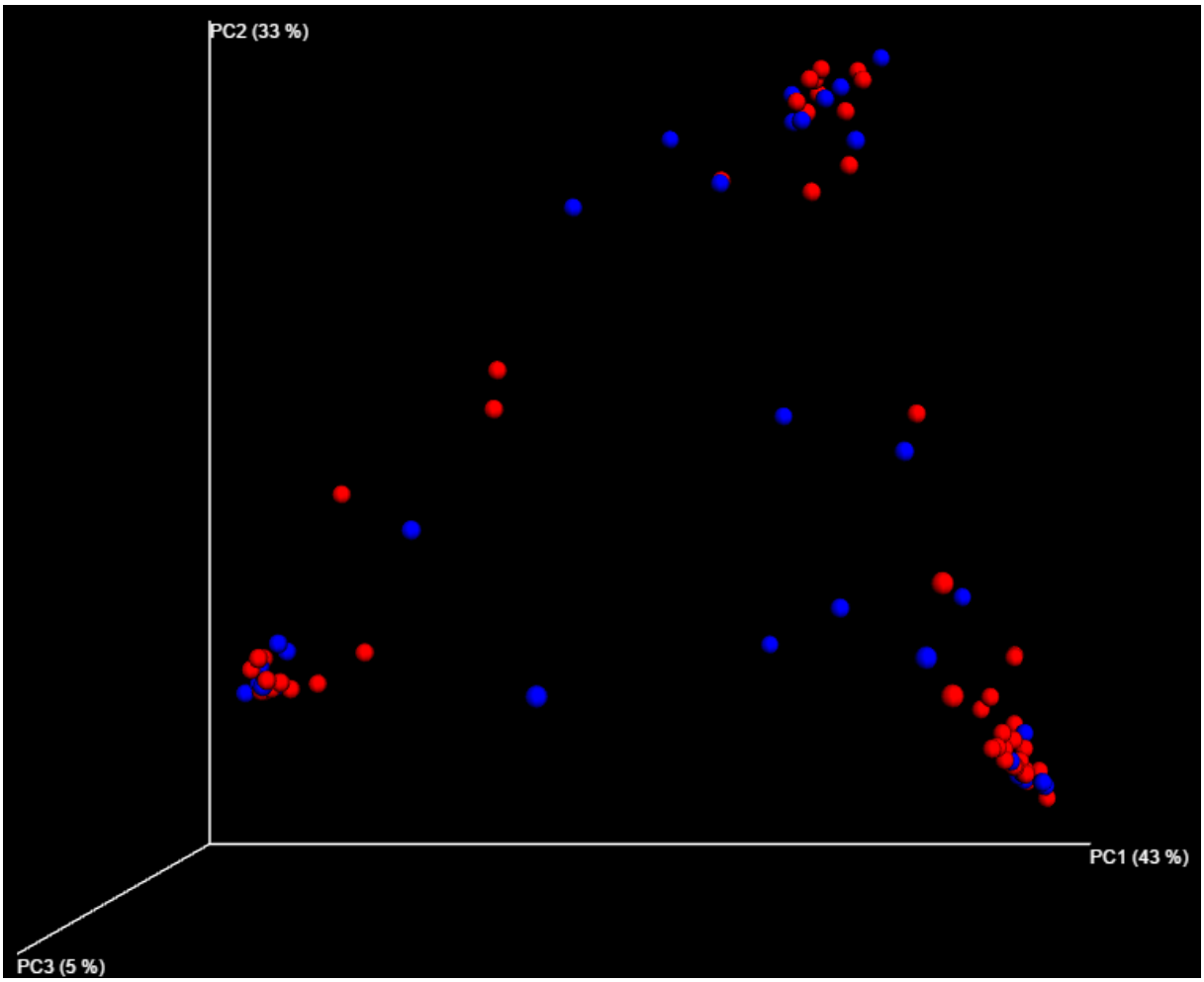


Figure 3.9: The UniFrac plot shows how the samples cluster based on intake of Biola (shown in blue) or placebo (shown in red).

The UniFrac plot in Figure 3.8 that shows the clustering based on the time point when the samples were taken shows a pretty clear clustering. This pattern of clustering is not as evident in Figure 3.9, which shows how the samples are clustered based on intake of Biola or placebo. The samples from the placebo group, shown in red, are more clustered than the samples from the Biola group, shown in blue. The cluster to the lower left corresponds to the clustering of samples taken at 10 days and three months. The cluster to the lower right corresponds to the clustering of samples taken at two years. The clustering to the top right corresponds to the clustering of samples taken form mothers during pregnancy and after birth. The samples from the Biola group are more spread out than the samples from the placebo group, and while it is represented in all the clusterings, there are more samples from the placebo group in the clusters.

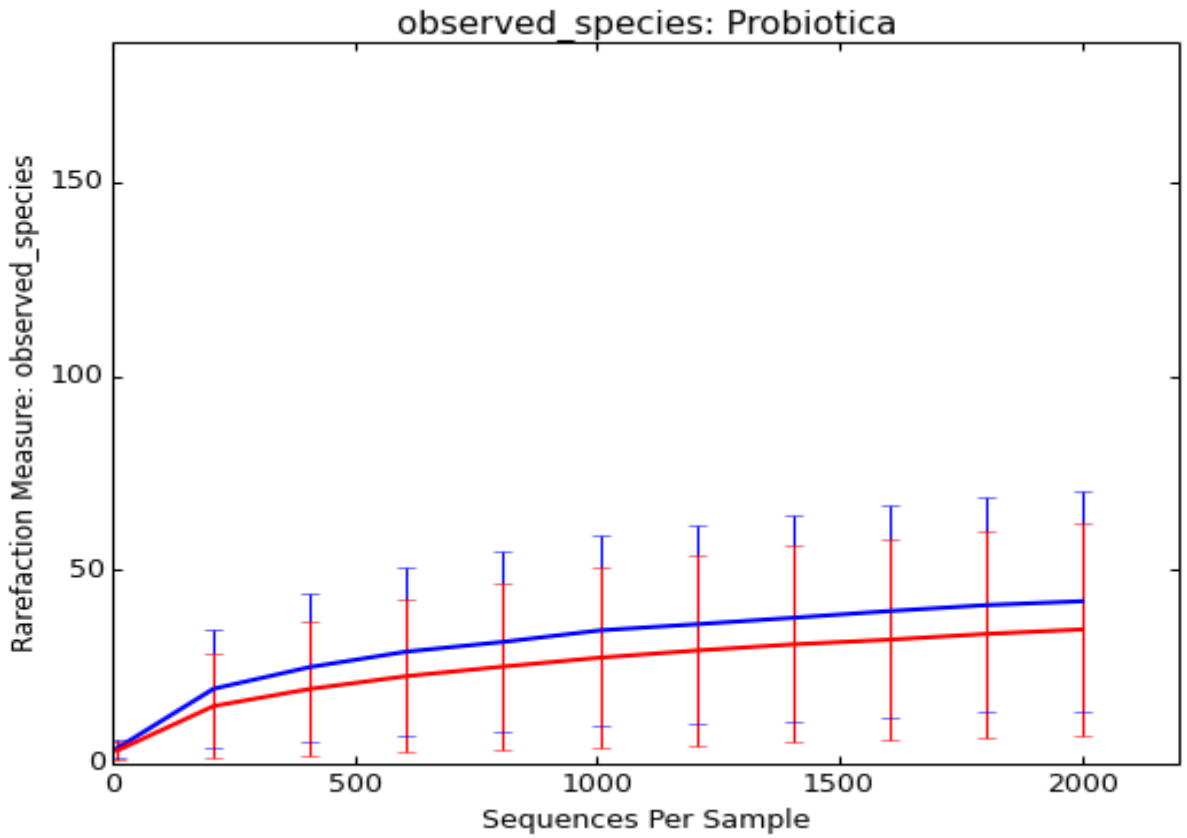
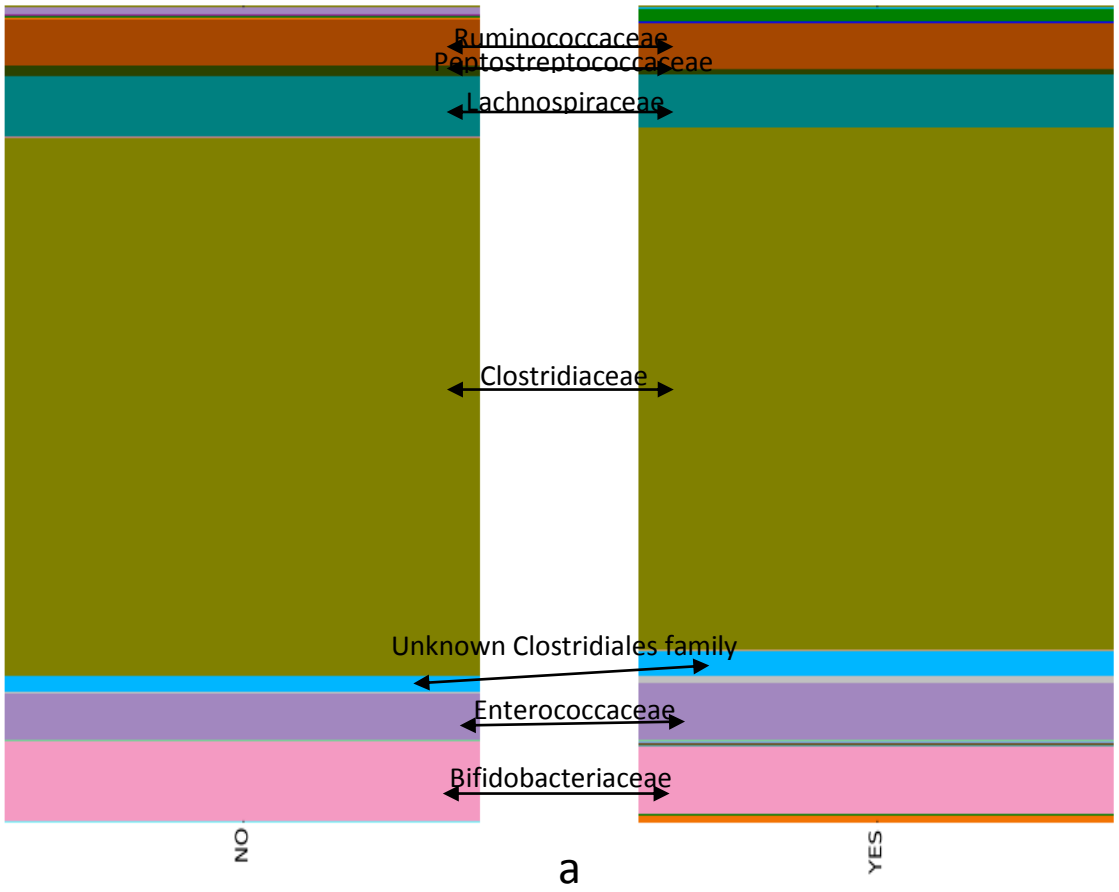


Figure 3.10: The figure shows a Rarefraction plot over the difference in observed bacterial species in the Biola and placebo groups, shown in blue and red, respectively.

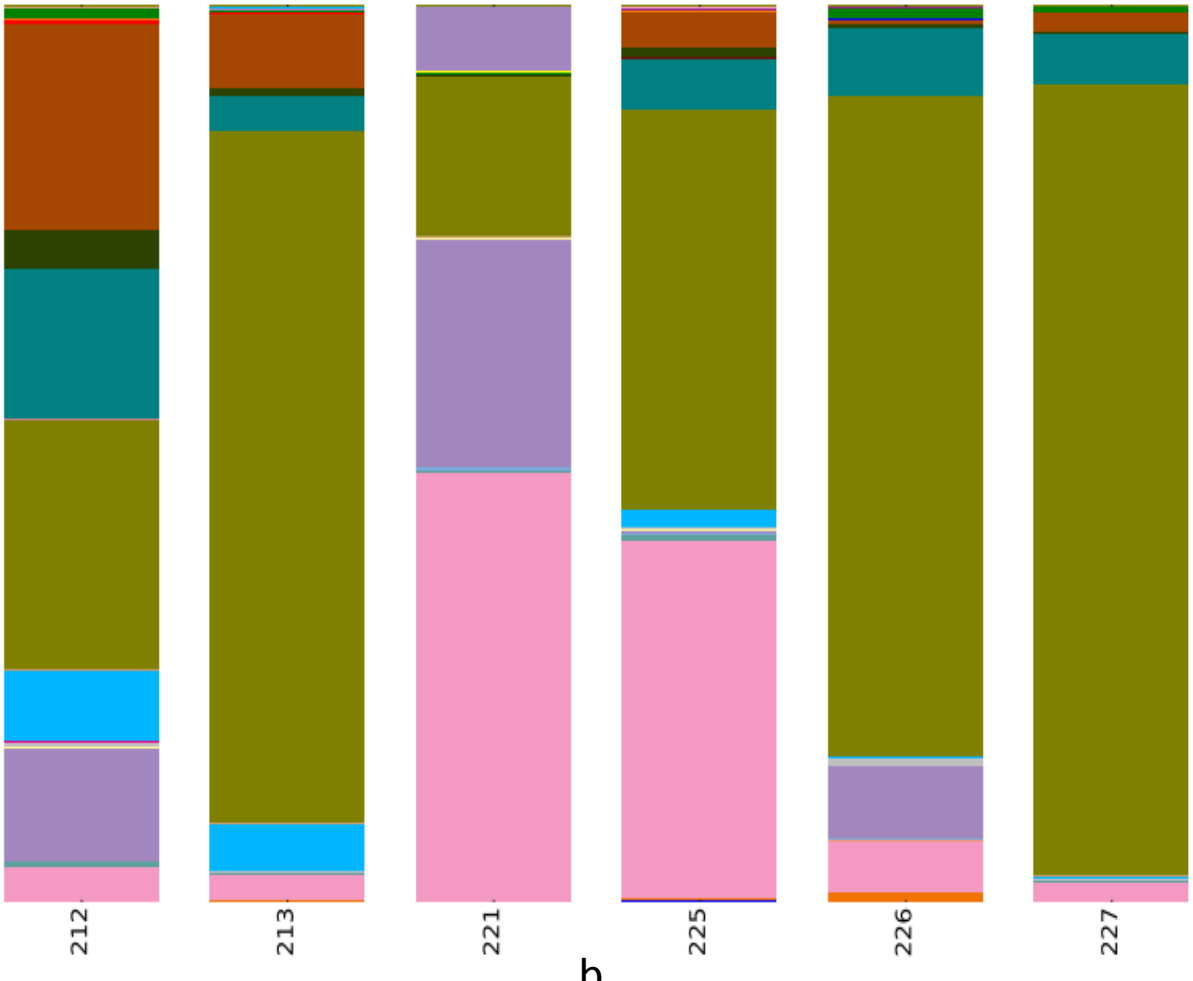
In addition to the UniFrac plots, the bacterial content in the samples has also been visualized as a Rarefraction plot, as shown in Figure 3.10. The figure shows that the amount of observed species is higher in samples collected from the Biola group.

A similar tendency can also be seen in the taxa plot in Figure 3.11a, that shows all OTUs present in the samples collected from the Biola and placebo samples. As seen in the bar graph over samples collected from the Biola group (marked as YES), there are several OTUs that are not present in the bar graph over samples collected from the placebo group (marked as NO). The Clostridiaceae family dominates in both groups, but there is a higher abundance of Clostridiaceae, Lachnospiraceae, Peptostreptococcaceae, and Bifidobacteriaceae in the placebo group than in the Biola group. on the other hand, the Ruminococcaceae family has a higher abundance in the Biola gorup than in the placebo group.

Figure 3.11b shows a similar bar graph as Figure 3.11a, but is divided based on the age group when the samples were collected. The amount of Bifidobacteriaceae is highest at 10 days (marked as 221), but the prevalence then decreases. Clostridiaceae is present at 10 days, and in the samples collected from the children, the amount of Clostridiaceae increases continuously during the first two years of life. The amount of the Ruminococcacea, Peptostreptococcaceae and Lachnospiraceae families is highest in samples collected from mothers during pregnancy (marked as 212), but they are present in all age groups, with the exception of samples collected at 10 days.



a



b

Figure 3.11: Figure A shows a taxonomic bar graph of the OTUs found in the samples based on intake of Biola or placebo. Figure B shows an overview over OTUs found in the samples based on the age group when the samples were collected (212=mother during pregnancy, 213=mother three months after birth, 221=10 days, 225=three months, 226=one year, 227=two years). The color-coding in Figure B is the same as in Figure B.

A similar colonization pattern as in Figure 3.11b is visible in the taxa plot in Figure 3.12. This taxa plot shows a complete overview of all OTUs found in all the samples that gave a postive result after Illumina sequencing.

The samples in the middle of the plot is dominated by the Bifidobacteriaceae family, and most of these samples were collected at 10 days and three months of age. There are a few samples dominated by pink at the right side of the taxa plot, and most of these samples are collected at one year of age.

During the first two years of life, there is a rather high abundance of species from the Clostridiaceae family. The Ruminococcaceae and Lachnospiraceae families start to appear in samples collected at three months of age.

The samples to the left of the taxa plot are samples collected from mothers during pregnancy and three months after birth, and shows a high abundance of the Ruminococcaceae and Lachnospiraceae species in most samples, while fewer samples are dominated by species from the Clostridiaceae family, as in samples collected from the children.

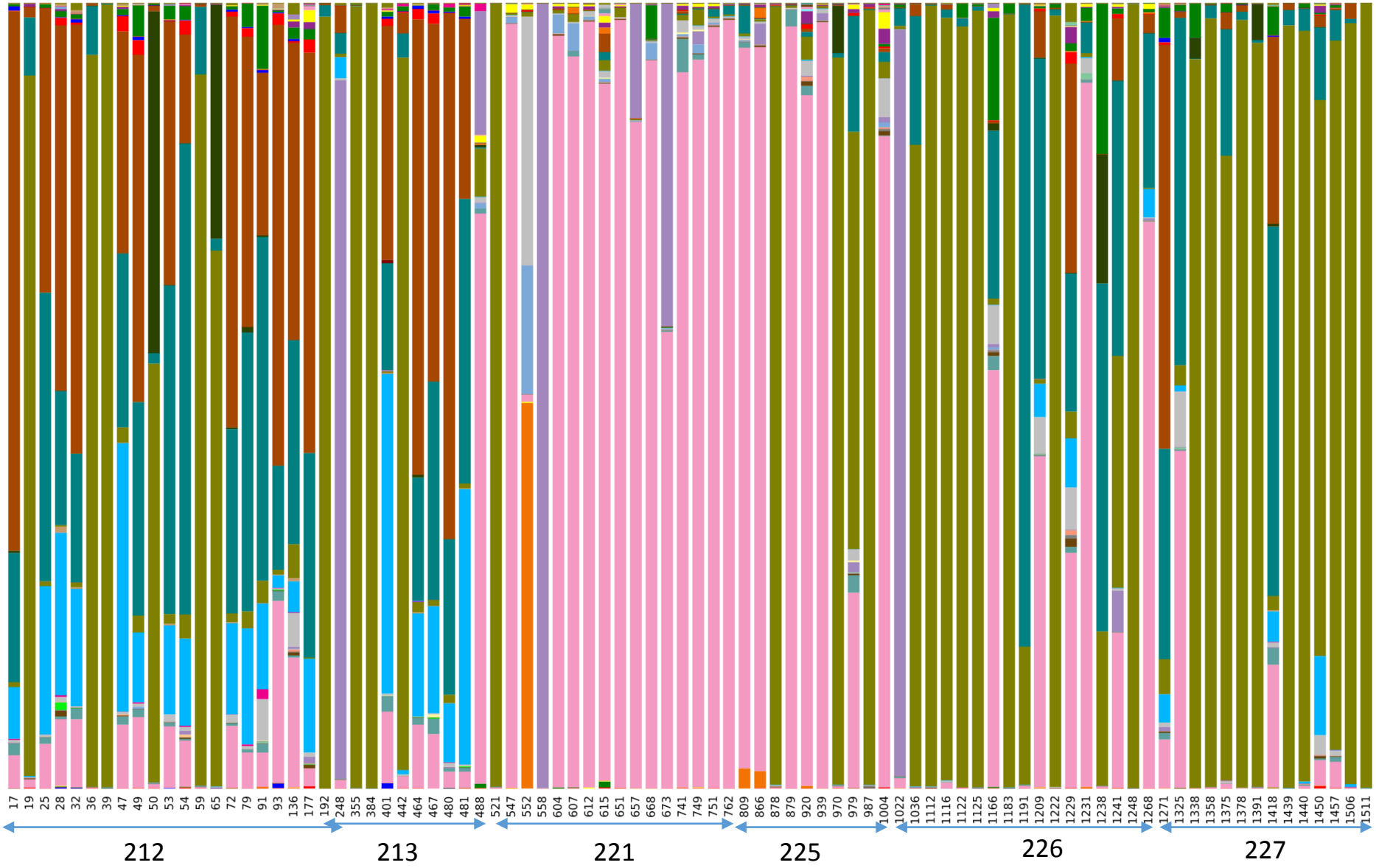


Figure 3.12: The figure shows an overview of all OTUs present in all samples that gave a positive result after Illumina sequencing. Both age codes and OTU color codes are the same as in Figure 3.11a.

3.6 Comparison of samples with AD and *Clostridium* clusters IV and XIVa

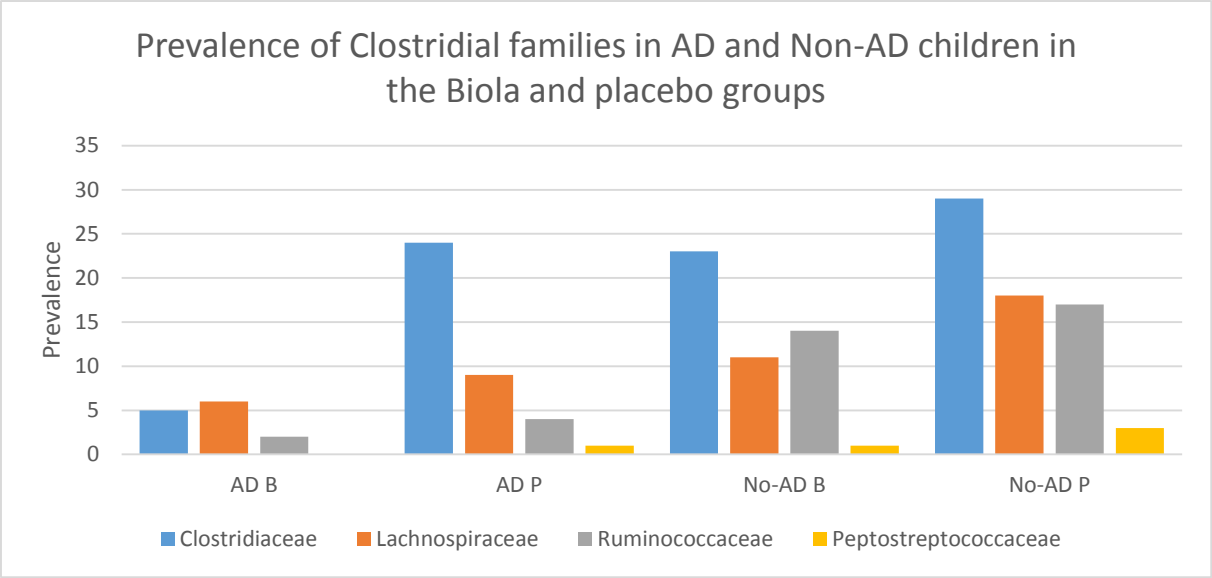


Figure 3.13: The figure show an overview of the prevalence of Clostridia families in children from the Biola and placebo groups who have or have not developed AD. AD B=AD positive children in the Biola group, AD P= AD positive children in the placebo group, NO-AD B= AD negative children in the Biola group, No-AD P=AD negative children in the placebo group.

In the 100 samples with a 0,5 or higher increase in optical density after endospore isolation and cultivation, 40 of the samples were collected from children in the Biola group, and 60 samples were collected from the placebo group. In the Biola group, 13 children developed AD, and 27 did not. For the placebo group, 21 children developed AD, while 39 did not.

Figure 3.13 shows the prevalence of the Clostridia families Clostridiaceae, Lachnospiraceae, Ruminococcaceae, and Peptostreptococcaceae in the samples marked as positive after cultivation. The samples have then been divided into AD-positive and AD-negative children in both the Biola and the placebo group. The figure shows that Clostridiaceae is generally the most abundant OTU, while, Peptostreptococcaceae is the OTU with the lowest abundance in all the groups.

As in the other figures where the results have been divided into Biola and placebo groups, there is a higher prevalence of members of *Clostridium* clusters IV and XIVa in the placebo group. As this figure in addition is divided based on the development of AD in the Biola and placebo groups, it is evident that the prevalence of members of *Clostridium* clusters IV and XIVa is lower in samples collected from children who have developed AD. This applies to samples collected from both the Biola and the placebo group.

4 Discussion and conclusion

4.1 Age related composition of gut microbiota

With the exception of a small amount of Erysipelotrichaceae in samples collected from mothers three months after birth, all OTUs found in the samples contain facultative or obligate anaerobes.

The samples collected from the children shows a higher prevalence of facultative anaerobic bacteria, and a lower prevalence of obligate anaerobes. As the children gets older, the prevalence of obligate anaerobic bacteria increases, suggesting that the gut microbiota in infants and young children change over time to become more similar to the gut microbiota found in adults. These results are similar to the results found by Adlerberth and Wold in 2008(51) and Palmer et.al. in 2007(11).

The highest amount of samples with a 0,5 increase in optical density was from the 10 day age group. After the qPCRs with specific primer pairs for members of *Clostridium* clusters IV and XIVa, the 10 day age group had the highest amount of samples without amplification results. Results from the Illumina sequencing showed that there was a very high prevalence of Bifidobacteriaceae found in the samples collected at 10 days. As there was a generally high increase in measured OD in the samples collected in this age group, it is likely that the Bifidobacteriaceae present in the untreated fecal samples have survived the chloroform and heat treatment implemented during endospore isolation. This suggests that members of the Bifidobacteriaceae family have some survival traits that are currently unknown, making it a topic for further studies, as there currently are no papers pertaining to this topic.

The samples collected at three months follow a similar pattern to the samples collected at 10 days. Of the samples that yielded a sufficient increase in optical density, only a few samples yielded amplification results after qPCRs specific for members of *Clostridium* clusters IV and XIVa. The results from the Illumina sequencing showed that the samples collected at three months of age also contained a high prevalence of Bifidobacteriaceae, though with a slightly lower prevalence when compared to the samples collected at 10 days.

Results from the qPCRs specific for members of *Clostridium* clusters IV and XIVa shows that there are amplification results in samples for all age groups. The low prevalence of Clostridia is found in samples collected at 10 days and three months is expected, as these are obligate anaerobic bacteria that have not yet colonized the gut. The prevalence of Clostridia then increased sharply in the samples collected at one year, with a slight drop in prevalence in samples collected at two years.

The results from the Illumina sequencing show a similar trend as the specific qPCRs. There is a very low prevalence of members of *Clostridium* clusters IV and XIVa at 10 days, with only a slight increase in prevalence in the samples collected at three months, and there is a much higher increase in prevalence in the samples collected at one year of age. In the samples collected at two years, however, there is an increase in the prevalence of Clostridia in the samples collected from children in the placebo group, while there is a sharp decrease in the prevalence of Clostridia in the samples collected from children in the Biola group. The number of sequences from the samples show a similar pattern as the prevalence of members of *Clostridium* clusters IV and XIVa.

Gas-liquid chromatography was performed on both untreated fecal samples and on the samples that had undergone endospore isolation and cultivation. Overall, the concentration of butyrate was higher in the untreated samples, and a t-test confirmed that the difference in average butyrate concentration in the untreated and the treated samples were statistically significant. This difference is most likely a result of a shorter period for butyrate production in the treated samples when compared to the untreated samples, as well as a lower amount of bacteria in general in the original, untreated fecal samples. In addition, non-spore-forming, butyrate producers such as *Roseburia intestinalis*, are present in the human gut, but they will not survive the treatment used to isolate endospores(52).

A selection of samples from each age group were cultivated on TSA media as well as in anaerobic broth after endospore isolation. In some of the cultures from samples collected at three months and one year, there were colonies that had a morphology slightly similar to fungi colonies. The samples with these types of colonies were stained and prepared as wet mounds. A microscopic examination showed that these samples contained large hyphal structures, which were tentatively identified as Actinomycetes species. Results from the Illumina sequencing supports this, as Actinomycetaceae were among the most common OTUs found in the samples collected in these age groups. A possible topic for further studies is how Actinomycetaceae survive or pass through the gut, as well as how members of the Actinomycetaceae family can affect the hosts' immune system.

4.2 Effect of Biola on gut microbiota composition

The mother-child pairs participating in the Pro-PACT study were randomly divided into a Biola group and a placebo group. Among the samples analyzed in this thesis, more samples belonged to the placebo group than to the Biola group. The relationship between the amount of samples belonging to the Biola and placebo groups in the pool of samples that had a 0,5 or higher increase in optical density after endospore isolation and cultivation was similar to the relationship between the amount of samples belonging to the Biola and placebo groups in the total pool of analyzed samples. This suggests that even though the intake of Biola causes changes to the composition of the gut microbiota, the changes are not drastic enough to cause any differences in how the gut microbiota responds to the treatment implemented during endospore isolation.

This suggests that the intake of Biola or placebo does not cause any alterations in the composition of the gut microbiota that promotes the survival rate or growth ability of bacteria in samples from one group over samples from the other group.

That being said, there are differences in the microbiota found in samples from the Biola and placebo groups. There are some differences in the prevalence of the OTUs found in the samples after Illumina sequencing. For instance, Actinomycetaceae are only found in samples collected from the Biola group, while Enterobacteriaceae and Coriobacteriaceae are only found in samples collected from the placebo group. All other OTUs found in the samples are found in both Biola and placebo groups, though not always present in samples collected from the same age group.

With the exception of the prevalence of Erysipelotrichaceae in the samples collected at one year, if an OTU is found in both Biola and placebo groups, it is found with a higher frequency in samples

collected from the placebo group. The amount of Bifidobacterium sequences found in the samples is higher in the placebo group in nearly all age groups. The samples collected at one year of age are the only samples where the total amount of Bifidobacterium sequences is higher in the Biola group than in the placebo group. These results are different from the results found by Kukkonen et.al.(26), who noted that Bifidobacterium appeared more frequent in samples collected from children who had been given probiotics.

There is no systematic pattern to the average butyrate concentration found in the Biola and placebo groups. A t-test was performed on the data, and yielded a high p-value, suggesting that the butyrate concentration in the samples were only dependent on the amount of bacteria from *Clostridium* clusters IV and XIVa, not on whether the samples had been collected from the Biola group or the placebo group.

4.3 Specific Clostridia and their relation to AD

Wise and Siragusa(45), Van den Abbeele et.al.(20), and Lopetuso et.al.(12) have all mentioned the butyrate producing abilities of members of *Clostridium* clusters IV and XIVa. When results from the qPCRs specific for members of *Clostridium* clusters IV and XIVa is compared to the average butyrate concentration found in the samples collected from the different age groups, there seems to be a correlation. A t-test performed on these results confirm that there is a statistically significant correlation between the average concentration of butyrate and the prevalence of *Clostridium* cluster IV and XIVa members in the different age groups.

In the Biola group, 28% of the children had developed AD, and all the children who had developed AD, had a close family member with atopy. In the placebo group, however, 39% of the children had developed AD, and AD seemed to develop independent of atopy in close family members. These results are similar to the ones found by Dotterud et.al.(30), where they noted that Biola did not seem to have a preventive effect in children with a family history of atopy.

A pattern seems to emerge after an examination of the prevalence of members of *Clostridium* clusters IV and XIVa in children from the Biola and placebo groups who had developed AD versus the children who had not developed AD. There is a higher prevalence of families from *Clostridium* clusters IV and XIVa in the samples collected from children who have not developed AD, than in samples collected from children who have developed AD. This decrease in prevalence of Clostridia is found in both the Biola and the placebo group.

4.4 Sources of error

The varying numbers of samples with a 0,5 or higher increase in optical density in each age group after endospore isolation and cultivation may be due to several factors. First of all, the amount of endospores in a given volume from a given fecal sample can vary greatly. Therefore, a more equal number of samples from each age group yielding a necessary increase in optical density after endospore isolation and cultivation could be achieved by analyzing duplicates of each sample. Secondly, it was observed that all the samples cultivated on TSA media showed growth, while the same sample cultured in anaerobic broth did not show a necessary increase in optical density, suggesting that there are non-spore-forming bacteria that survive treatment during endospore isolation, but are left in a weakened state, and are thus only able to grow on a media with a high nutrient content. This can be caused both by a higher tolerance towards chloroform and heat, but it can also be caused by the presence of fecal particles in the samples that can protect non-spore-forming bacteria against the chloroform and heat treatment implemented during endospore isolation.

The lack of duplicates analyzed may also be the reason why there is such a large difference in the number of samples yielding amplification results after the specific qPCRs, and those that did not yield an amplification result.

In the samples collected at 10 days, there was a very high prevalence of samples with a 0,5 or higher increase in optical density, and when the subsequent results from the qPCRs specific for members of *Clostridium* clusters IV and XIVa showed a sharp decrease in the prevalence of samples that were amplified, it was unclear why there was such a large deviation. The Illumina results then showed that there was a very high prevalence of Bifidobacteriaceae in the samples collected at 10 days. This can be a result of Bifidobacterium who have survived the endospore isolation, or it can be a result of DNA from dead cells. In several of the samples that were analyzed in this thesis, fecal particles were also present. These particles could possibly shield Bifidobacterium and other non-spore-forming bacteria from the endospore isolation, but the amount of such particles was very low in the samples collected at 10 days and three months.

The differences between the Bifidobacterium colonization found in this thesis and in the study performed by Kukkonen et.al. (4) can be due to several factors. The samples analyzed in this study were collected from children in Trondheim, where the Biola group received one probiotic bacteria, while the samples analyzed by Kukkonen et.al.(4) were from Finland, and had been given a mixture of four probiotic strains, as well as prebiotic galacto-oligosaccharides. There are possibly also differences in how the samples from this thesis and the Finnish study were analyzed. In addition, only a small pool of samples from the Pro-PACT study were analyzed for this thesis. Duplicates of each sample analyzed, as well as an increased pool of analyzed samples could possibly yield more similarities between the samples from the Finnish study and the samples from the Pro-PACT study.

4.5 Conclusions

As the decrease in prevalence of members of the *Clostridium* clusters IV and XIVa is found in both the Biola and the placebo group, the results suggest that bacteria from *Clostridium* cluster IV and XIVa and their production of butyrate is not a mechanism directly responsible for the development of AD in children.

As it was hypothesized that the members of *Clostridium* clusters IV and XIVa in themselves and the butyrate they produced, could activate immune system responses that could lead to the development of AD, the prevalence of these Clostridia families should have been higher in the Biola group than in the placebo group. Instead, the prevalence of these Clostridia is highest in the placebo group.

The samples collected at one and two years of age has a prevalence of members from *Clostridium* clusters IV and XIVa that is similar to what is found in the samples collected from the mothers, suggesting that the infant microflora becomes more similar to the microflora found in adults around this time.

Despite the fact that the prevalence of members of *Clostridium* clusters IV and XIVa found in samples collected at one and two years of age, is similar to the prevalence of these bacteria found in samples collected from the mothers(11, 51), the butyrate concentration is a lot lower in all the samples collected from children, suggesting that production of butyrate in as large quantities as found in adults, starts sometime after the children reaches two years of age.

Even though Clostridia and the butyrate they produce does not seem to be directly linked to the development of AD in children, there are still significant differences in the samples collected from the Biola group and the placebo group. These differences suggests that intake of Biola causes a change in the infant gut microbiota that cause other bacteria to colonize the gut, in place of members of *Clostridium* clusters IV and XIVa. The cause for these changes might be a more probable mechanism for how intake of probiotics gives a preventive effect on the development of AD.

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6 Appendixes

Appendix A: Overview of samples used in thesis

Form#	Biola	# Samples with growth	AD	Atopy fam.
20793	no	2	no	no
20251	no	3	no	yes
20301	no	1	no	yes
20309	yes	2	no	yes
20469	yes	3	no	yes
20787	yes	2	yes	yes
20850	no	1	no	yes
21116	no	1	no	yes
21251	no	1	yes	yes
21275	no	1	no	yes
21291	no	2	yes	no
21294	no	3	no	yes
21332	no	1	no	yes
21339	no	1	no	yes
21646	no	1	yes	no
21727	yes	2	no	no
21777	yes	1	no	yes
21822	no	2	no	no
21834	yes	1	yes	yes
21851	no	2	yes	yes
21908	no	1	yes	yes
21912	yes	1	no	no
21922	no	3	no	yes
21933	no	1	yes	yes
21947	no	3	no	yes
21968	yes	1	no	yes
22004	yes	1	no	no
22046	no	1	no	yes
22072	no	1	no	no
22083	yes	2	yes	yes
22095	yes	1	yes	yes
22148	yes	1	no	no
22212	no	2	yes	yes
22215	yes	1	no	yes
22242	no	2	no	no
22264	no	2	no	no
22270	no	1	no	yes

Form#	Biola	# Samples with growth	AD	Atopy fam.
22491	yes	2	yes	yes
22512	yes	1	yes	yes
22533	no	2	no	no
22546	yes	2	no	yes
22577	yes	1	no	no
22629	yes	1	yes	yes
22648	no	1	no	yes
22751	no	2	yes	yes
22778	yes	1	no	yes
22784	no	2	yes	yes
20318	yes	0	yes	yes
20308	no	0	yes	no
20933	yes	0	no	no
21117	no	0	yes	yes
22049	yes	0	yes	yes
21725	no	0	no	NA
21504	no	0	yes	yes
22640	no	0	no	yes
22200	no	0	no	no
22199	yes	0	yes	yes
22017	yes	0	no	yes
22034	yes	3	yes	yes
22714	no	2	yes	yes
22260	no	0	no	yes
22150	no	1	yes	yes
22135	no	1	yes	yes
22764	yes	1	no	yes
22694	no	0	no	yes
22623	no	1	yes	no
22596	yes	2	no	no
22085	no	1	no	yes
21395	no	4	no	yes
22673	yes	1	no	no
21942	yes	1	no	yes
22666	yes	0	no	yes
21825	no	0	no	no
22134	yes	0	no	no

Form#	Biola	# Samples with growth	AD	Atopy fam.
22303	yes	1	no	yes
22350	yes	1	no	yes
22419	no	2	yes	yes
22479	no	2	no	yes
22490	yes	1	no	yes

Form#	Biola	# Samples with growth	AD	Atopy fam.
22684	yes	0	yes	yes
22767	no	0	yes	yes
21853	yes	2	no	yes
22296	no	0	no	no
22341	yes	0	no	yes
22797	no	0	yes	no

Appendix B: Strain collection on reference plate after Sanger sequencing

	1	2	3	4	5	6
A	Roseburia sp	Bifidobacterium longum subsp	Bifidobacterium gallicum	Clostridium leptum	Eggerthella lenta	Faecalibacterium prausnitzii
B	No Sequence	Bifidobacterium longum bv. Infantis	Blautia coccoides	Clostridium perfringens	Enterobacter aerogenes	Finegoldia magna
C	Bacteroides cellulosilyticus	Bifidobacterium adolescentis	Burkholderia oklahomensis	Clostridium ramosum	Enterobacter hormaechei	NA
D	Bacteroides uncultured	Bifidobacterium angulatum	Butyrivibrio fibrisolvens	Enterococcus faecalis	Fusobacterium periodonticum	Fusobacterium periodonticum
E	Bacteroides intestinalis	NA	Campylobacter curvus	Corynebacterium aurimucosum	Enterococcus faecium	Gemella haemolysans
F	Bacteroides thetaiotaomicron	Bifidobacterium breve	Enterobacter sp	Desulfovibrio piger	Desulfovibrio piger	Aggregatibacter aphrophilus FEIL ID
G	Bacteroides uniformis	Bifidobacterium catenulatum	Clostridium XlVa uncultured	Dialister invisus	Eubacterium cylindroides	Helicobacter pylori
H	Bacteroides vulgatus	Bifidobacterium dentium	NA	Dorea uncultured	Eubacterium rectale	Klebsiella oxytoca

	7	8	9	10	11	12
A	Klebsiella pneumoniae	Lactobacillus paracasei	No Sequence	Proteus vulgaris	E.coli/Shigella	Streptococcus genomosp
B	Lactobacillus plantarum FEIL ID	Lactobacillus plantarum	Neisseria sp	Pseudomonas aeruginosa	Streptococcus pneumoniae gam	Streptococcus pneumoniae
C	Lactobacillus brevis	Lactobacillus reuteri	Oscillibacter valericigenes	Roseburia inulinivorans	Staphylococcus epidermidis	NA
D	Lactobacillus acidophilus	Lactobacillus salivarius	Parabacteroides johnsonii	Ruminococcus albus	Streptococcus luteciae	Streptococcus sanguinis
E	Lactobacillus amylolyticus	Lactococcus lactis subsp. lactis;	Parascardovia denticolens	Salmonella bongori	Streptococcus agalactiae	Veillonella dispar
F	Lactobacillus casei	NA	Prevotella marshii	Salmonella enterica subsp.	Streptococcus equinus	NA
G	Lactobacillus fermentum	Listeria monocytogenes	Prevotella oralis	Serratia marcescens	Escherichia coli O104:H4	Vibrio cholerae
H	Lactobacillus gasseri	Mobiluncus curtisii	Proteus mirabilis	Escherichia/Shigella dysenteriae	Streptococcus oralis	Negative control

Appendix C: Recipes

TSA blood agar:

- Tryptic soy broth(Merck, Norway): 30g/l
- Agar-agar(Merck, Norway): 14g/l
- Defibirnated horse blood(Thermo Scientific, Norway): 50 ml

Anaerobic basal broth:

- Anaerobic basal broth(Thermo Scientific, Norway): 35,4 g/l

PBS:

- Nacl(Thermo Scientific, Norway): 8,5g/l
- $Na_2HPO_4*2H_2O$(Thermo Scientific, Norway): 0,85g/l
- KH_2PO_4(Thermo Scientific, Norway): 0,25g/l

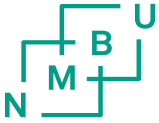
Appendix D: Increase in optical density after endospore isolation and cultivation

	Mom1	Mom2	10days	3months	1year	2year
	2,02	1,04	0,62	1,11	0,50	1,94
	0,54	0,83	0,67	0,90	4,23	0,76
	1,09	5,19	0,54	0,77	0,69	6,26
	1,39	3,62	0,68	0,59	0,52	4,59
	1,16	0,56	0,76	5,57	4,46	0,70
	0,71	5,40	1,11	1,82	0,79	3,35
	0,94	0,70	0,90	0,56	5,15	0,61
	0,59	0,64	0,85	0,70	4,86	5,53
	0,53	0,58	0,58	0,70	0,78	0,69
	0,51	0,57	0,97	6,41	0,53	0,53
	0,64	0,54	0,52		2,99	1,27
	0,50	0,62	0,58		1,68	0,71
	5,80	0,94	1,59		4,71	3,13
	1,77	3,68	0,65		1,28	5,19
	0,56		0,51		1,40	2,02
	0,54		0,93		4,36	
	0,64		0,84		2,67	
	3,35		0,54		2,00	
	0,50		6,63			
	0,53		0,70			
	3,08		0,76			
			0,53			
Average increase	**1,30**	**1,78**	**1,02**	**1,91**	**2,42**	**2,49**
Standard deviation	1,32	1,83	1,28	2,19	1,76	2,04

Appendix E: Number of sequences found in each OTU family

OTU	Mom 1 B	Mom 1 P	Mom 2 B	Mom 2 P	10day B	10day P	3month B	3month P	1year B	1yearP	2year B	2year P
Actinomycetaceae							38		982			
Bifidobacteriaceae	614	169	129	1446	7551	15824	4087	7262	3331	1880		328
Caulobacteraceae					28	2						
Clostridiaceae	3774	4466	1277	7869	1991	64	2952	3738	6396	9836	3084	16765
Coriobacteriaceae								44				
Enterobacteriaceae				318		1077						
Enterococcaceae	1773					2026	45	15	1909			
Erysipelotrichaceae				7		76			570	32	98	
Lachnospiraceae	534	2370	335	24	32	35		91	1760	2633	378	1298
Peptostreptococcaceae		870	600					134				91
Ruminococcaceae	1879	5106	2036	782					322		714	231
Staphylococcaceae					40	119			353			
Streptococcaceae							3		611			
Unknown Family	634	2491	944	130					123		201	

"B" stands for Biola group, "P" stands for placebo group.



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