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# **Impact of early life intervention with four *Bifidobacterium* spp. on the infant faecal microbiota**

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## ABSTRACT

Colonization of the human intestinal tract is a complex process influenced by many factors such as mode of delivery and type of feeding. The succession of microbial species in the developing gut may be driven by the addition of bacteria to the infant's diet. However, the effect of bifidobacteria supplementation on the intestinal microbiota function and dynamic is not well studied.

In the current double blind, placebo-controlled study, 106 healthy infants were randomized to two formula groups, and studied in parallel to a breast-fed comparison group (n=9). Formula-fed infants either received a whey based infant formula (n=49) or the same formula containing 107 colony-forming units (CFU) of four *Bifidobacterium* spp. per gram powder (n=48). Faecal samples were collected monthly over a period of one year and at the age of two years. Breast milk samples were collected one month postnatally. High-throughput 16S rRNA amplicon sequencing by Illumina, high resolution metabolite analysis (UPLC-MS) and human milk oligosaccharide analysis were applied.

Bifidobacteria-supplemented formula altered the microbiota at early age and shifts in bacterial composition were primarily associated with a decreased occurrence of *Bacteroides* and *Blautia* spp. and with changes in lipids and unknown metabolites. Differences in bacterial and metabolite profiles between intervention and placebo groups disappeared over time, and no long-term colonization (24 months) of the supplemented bifidobacteria was detected.

Comparisons between infants fed by formula or breast showed that the differences between intervention and placebo groups were smaller and more temporal than those observed between breast- and formula-fed infants. The main hallmark of breastfeeding was a faecal ecosystem that was consistently less diverse throughout the first year of life, while at the age of two years, the initial feeding regime was not a significant determinant for the composition of the microbiota. Human milk analyses further revealed that the presence of the maternal fucosyltransferase-2 gene in breast milk can probably modulate the prevalence of one *Bifidobacterium* sp.

In conclusion, the study showed that the infant microbiome can be modulated by bifidobacteria-supplemented formula early in life, with no detectable long-term consequences for gut microbiota assembly or function.

## ZUSAMMENFASSUNG

Die Kolonisierung des menschlichen Darms ist ein komplexer Prozess, welcher durch unterschiedliche Faktoren, wie die Art der Geburt und die Art der Nahrung beeinflusst wird. Die Ansiedelung der Bakterien im gastrointestinalen Trakt kann möglicherweise bereits frühzeitig durch den Verzehr von zugesetzten Bakterien in der Nahrung beeinflusst werden. Dessen Einfluss auf die intestinale Dynamik und Funktion ist allerdings noch weitgehend ungeklärt.

In der vorliegenden doppelblinden, Plazebo kontrollierten Studie wurden 106 gesunde Neugeborene in zwei Gruppen randomisiert und parallel zu einer ausschließlich gestillten Kohorte analysiert (n=9). Die Plazebogruppe (n=49) erhielt eine Molkenbasierte Säuglingsmilch, die Interventionsgruppe dasselbe Produkt, jedoch mit  $10^7$  koloniebildenden Einheiten (KbE) der Bifidobakterienspezies pro Gramm Pulver (n=48). Stuhlproben wurden einmal monatlich bis zum ersten Lebensjahr, sowie ein weiteres Mal im Alter von zwei Jahren entnommen. Muttermilchproben wurden ein Monat nach der Geburt analysiert. 16S rRNA Illumina Sequenzierung sowie Analysen zur Bestimmung der Metabolitenprofile (UPLC-MS) und der Oligosaccharide (HMO) in der Muttermilch wurden vorgenommen.

Die Zugabe von Bifidobakterien zur Säuglingsmilch hat gezeigt, dass die bakterielle Zusammensetzung wenige Wochen nach der Geburt mit einer verminderten *Bacteroides* und *Blautia* spp. Konzentration einhergeht. Außerdem wurden Änderungen in der Menge von Lipiden und unbekanntem Metaboliten festgestellt. Die kompositionellen und funktionellen Unterschiede zwischen Interventions- und Plazebogruppe verschwanden mit zunehmendem Alter, wobei eine langfristige Kolonisierung (24 Monate) mit den zugefügten Bakterienstämmen ausgeschlossen wurde. Vergleiche zwischen gestillten und zugefütterten Kindern haben gezeigt, dass die beobachteten Diskrepanzen zwischen Interventions- und Plazebogruppe weitaus geringer und temporärer waren als jene zwischen Kindern, welche entweder Muttermilch oder Flaschennahrung erhielten. Die stärkste Ausprägung bei gestillten Kindern zeigte sich bei der geringen bakteriellen Diversität während des gesamten ersten Lebensjahres. Im Alter von zwei Jahren konnte die Mikrobiota nicht mehr auf Basis des ursprünglichen Fütterungsverhaltens unterschieden werden. Analysen zu maternalen Milcholigosacchariden deuteten auf einen Einfluss der mütterlichen

Präsenz des Fukosyltransferase-2 Gens auf die Bifidobakterienkonzentration bei den Neugeborenen hin.

Zusammenfassend lässt die Ernährung mit einer mit Bifidobakterien angereicherten Säuglingsmilch nur einen geringfügigen Einfluss auf die Kolonisierung des Darms zu. Zu den ausgeprägtesten Merkmalen der Interventionsgruppe zählten eine anfänglich geänderte Mikrobiota, sowie ein gänzlich unterschiedliches Metabolitenprofil.

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## INTRODUCTION

### 1. The human intestinal microbiota

#### 1.1. Composition and function

The complex community of microorganisms residing in or passing through the gastrointestinal tract is referred to as the intestinal microbiota and is composed of up to  $10^{11}$  cells per gram content in the large intestine (1, 2). The number of different species in the large intestine estimated by culturing and 16S rRNA sequencing methods is approximately 400 to 500 (3). However, extensive metagenomic analyses revealed that the number of different bacterial species is limited to approximately 160 per individual (4).

Healthy mature microbiota is dominated by four phyla: Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria (5). Approximately 60% of the healthy adult intestinal bacteria belong to the Bacteroidetes and Firmicutes phyla with highest abundance in the Firmicutes' *Clostridia* class (3, 6, 7). The duodenum and jejunum support lower numbers of commensal bacteria due to the low pH from stomach acid, pancreatic enzymes and motility patterns that hinder colonization (8). In contrast, the ileum harbors an increasing and diverse microbial population, only to be exceeded by the incontestably highest number of microorganisms in the colon, either found attached to the mucosa or in the contents (9).

Peyer's patches, usually intended to sample bacteria and to regulate the host's immune response in the small intestine, are lacking in the large intestine and as a result, the immune system can tolerate such high densities of bacterial communities (1).

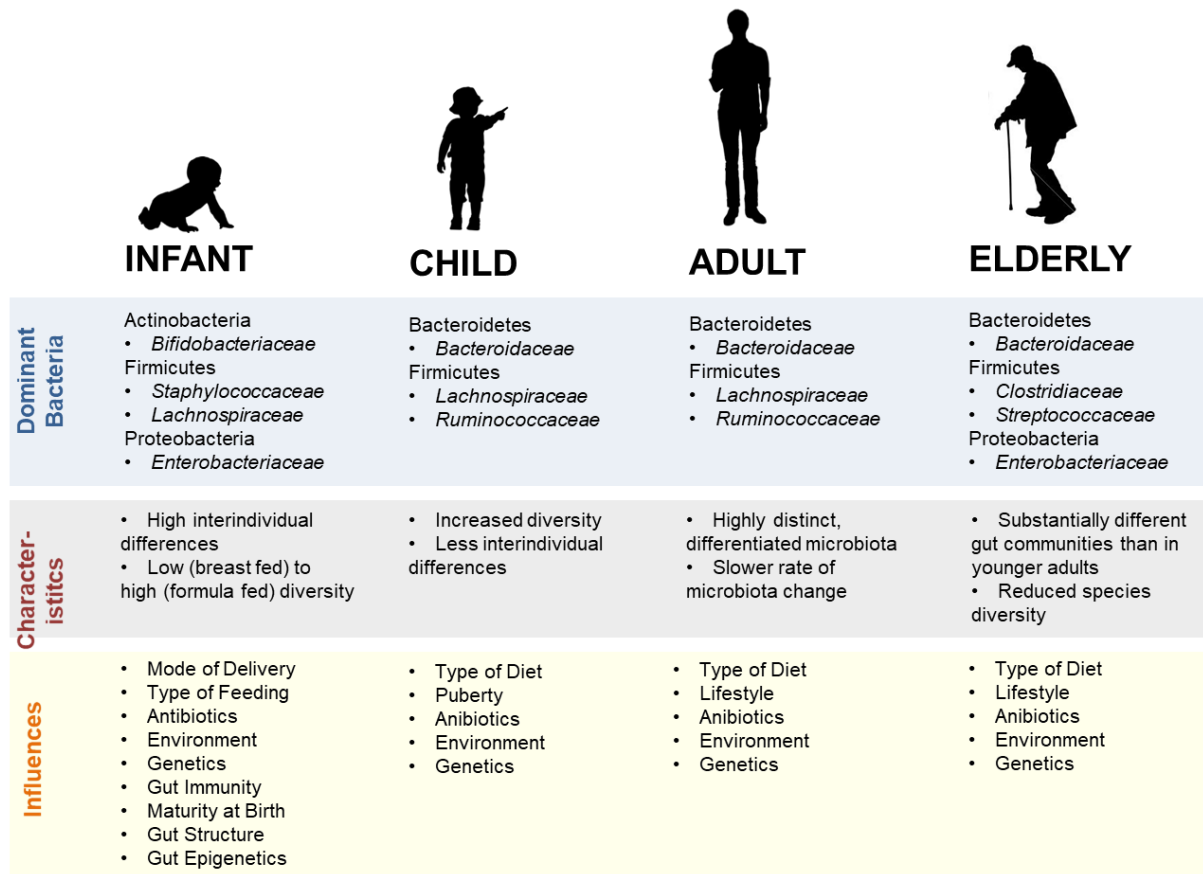
In a stable gastrointestinal ecosystem, all available habitats and niches are occupied by indigenous microorganisms, whereas transient species derived from food, water and other sources will normally not establish (i.e. colonize) and will pass through the tract (10).

The microbial ecosystem plays a crucial role in nutrient processing, regulation of host fat storage and protection against pathogens (11). Intestinal bacteria are indispensable for the normal function of the mammalian organism as they primarily but not exclusively extract energy and nutrients from food and protect the host from potentially pathogenic

microbes (12). Commensal bacteria supply energy to the host by the fermentation of non-digestible molecules, including cellulose, resistant starch and inulin in the colon (13).

The established host microbiota further acts as a protection against invading pathogens by the occupation of mucosal attachment sites, the secretion of bacteriocins and the competition for micronutrients (14). In addition, the mucosal and systemic immune systems are critically influenced by the microbial composition and changes may lead to immune-mediated diseases such as autoimmunity or allergies (15). Moreover, short-chain fatty acids (SCFAs), such as acetate, butyrate and propionate, are essential end products of bacterial fermentation and impose immunomodulatory effects on the host (16, 17). Other key roles of the intestinal microbiome include the modulation of intestinal permeability, and the mucus layer (18).

Besides multiple effects of environmental influences and host characteristics, the microbiota is strongly dependent on the host's age. The sterile gut of the unborn baby is challenged with a load of bacteria upon delivery, followed by high inter- and intra-individual differences, that is often characterized by a low bacterial richness and diversity (19). This continues then over the first 2-3 years of life, where the intestinal microbiota is characterized by a relatively low microbial diversity and stability, undergoing dynamic changes and thereby establishing highly adapted communities, which then represent a healthy microbiome in a state of homeostasis (20). Upon an imbalance in gut microbiota, a disturbed milieu can drive certain gastrointestinal diseases, such as necrotizing enterocolitis (NEC) in infants or inflammatory bowel diseases (IBD) in children and adults (21, 22). The elderly gut microbiota loses the high species richness from adult age and may degenerate into a state of dysbiosis, leading to chronic inflammation (inflamm-ageing) and reduced immune function (immunosenescence) (23). However, the alterations in microbiota in people from advanced age are not clear-cut due to the various physiological changes that elderly go through, such as modification in lifestyle and nutritional behavior. While different studies report conflicting outcomes, the negative effect on bacterial richness was observed in most of the studies (24).



**Figure 1: Changes in human microbiota composition throughout life.**

Depending on age, nutrition and health status, the microbial composition in the gut shifts considerably. While the unborn is not colonized at all, the baby has a balanced composition of all four dominant phyla. Later in life, Firmicutes and Bacteroidetes will become the predominating phyla. Elderly are characterized by a decreased bacterial richness (19, 24-28).

## 1.2. The infant colonization process

The colonization of the human intestinal tract is a complex process that is dependent on many factors such as gestational age at birth, maternal health and microbiota, antibiotic treatment, hospital hygiene, duration, place and mode of delivery, type of feeding and hospitalization after birth (29-33). It is widely accepted that the initial colonization of the *in utero* sterile gastrointestinal tract starts during birth when the newborn comes into contact with the maternal cervical and vaginal microbiota (34). Observations from a study on 5-10 minutes-old infants have shown that the infant microbiota assimilates that of the maternal cervix (35). The meconium, denoting the first stool of the baby after birth, was found to harbor enterococci and staphylococci genera, but also *Escherichia coli* and *Enterobacter* spp. (36). The source for the first

colonizers remains controversial, suggesting bacteria originating from the amniotic fluid and the placenta of the mother and the blood from the umbilical cord (37-40). However, analyses and corresponding results have to be handled with care as bacterial numbers found in these bodily parts remain low and may be resulting from contaminations during analysis.

The first bacteria that dominate in faecal samples of healthy infants are facultative anaerobes, such as staphylococci, enterobacteria (particularly *Klebsiella* spp. and *Escherichia coli*) and streptococci (19, 41). They remain predominant for the first two weeks of life and create a more reduced environment through the consumption of oxygen present in the intestine, which then allows the growth of obligate anaerobes, such as clostridia, bacteroides and bifidobacteria, with bifidobacteria becoming the most dominant ones (41-43).

However, there is a need for differentiation of microbial colonization between bottle- and breast-fed as studies have shown that the microbiota develops differently upon infant feeding type (44-48). The intestine of breast-fed infants is dominated by bifidobacteria due to the presence of human milk oligosaccharides (HMOs). Breast milk has a large percentage of indigestible oligosaccharides (up to 8% of total calories) with apparently no nutritional value for the newborn, but an indispensable function through the provision of substrates for the production of short-chain fatty acids, leading to the proliferation of health-promoting bacteria such as bifidobacteria and lactobacilli (49, 50). On the contrary, formula-fed infants show a more diverse flora that is shaped by bacteroides, enterobacteria, bifidobacteria and clostridia (45, 51).

Further succession of the microbiota is characterized by increasing total bacteria numbers, total anaerobes, bacterial diversity and community stability (52, 53). How the intestinal microbiota then further succeeds after these first weeks and months of initial colonization depends then on the bacteria's ability to compete for substrates and adhesion sites, their ability to produce metabolites, signal molecules and antimicrobial compounds and their capacity to interact with the intestinal tissue (25).

Start of complementary food intake is then the next major determinant for the development of bacterial species in the gut. Solid food may favor the colonization of clostridial species, as well as members of the *Lachnospiraceae* and *Ruminococcaceae* families (19).

However, weaning is considered to be the most critical and determining part of the complex colonization process, as the infant switches from a milk-based diet to a variety of food components (54).

Non-digestible carbohydrates are taken up by food and influence the microbiota by providing new substrates to specific bacteria thereby promoting their survival and growth (55). After weaning, *Clostridium coccooides*, *Bifidobacterium* spp. and *Bacteroides* spp. are the dominant bacterial populations to be found in infants and the faecal microbiota will be characterized by an increasingly homogenous composition (55, 56).

At 12 months, the infantile microbial composition of the large bowel of breast- and bottle-fed infants evolves toward a normal adult-like microbiota in number and composition together with a corresponding decrease in the number of facultative anaerobes (48). However, it remains to be determined to which extent internal (such as host genetics) or external (such as environment and diet) factors determine the timing, diversity and density of bacterial colonization of the infant gut (25). The shaping of the infant's microbiota profile will from then on be primarily determined by gastric acid secretion, gastrointestinal motility, innate immune response and dietary habit (57). From 18 to 36 months of age, the infant microbiome undergoes a final significant change that is characterized by a more stable microbial profile constituted primarily of Bacteroidetes and Firmicutes phyla (58). A host specific microbiota profile that remarkably decreased with regard to inter-individual differences will establish that is largely, but not exclusively shaped by the host's diet (59).

Once established, the microbiota contains trillions of microbes with a collective genome that outnumber the human genome up to one thousand times (60). Compared to the infant microbiota, the variability and dynamic of the adult microbial composition is remarkably decreased, so that it is characterized by a stable composition over time that may be subject to variations upon disease or ageing (3, 61, 62).

### 1.3. Crucial factors influencing the infant colonization process

#### 1.3.1. Preterm birth

Premature infants differ from term infants in kinds of exposures, particularly conditioned by the isolated environment in an incubator but also by a more or less extensive use of antibiotics after birth. Moreover, the immunological immaturity makes these infants particularly sensitive towards bacterial infections upon birth (25)

The colonization process of preterm infants with very low birth weight was shown to start with enterobacteria and streptococci, with both types of bacteria staying predominant for a longer time in the intestine than in full-terms (63). Also, enterococci were shown to dominate the preterm microbiota (64). Moreover, colonization with bifidobacteria was shown to be retarded and lower numbers than observed in full-term infants were detected (65).

A shift of the bacterial colonization in the intestine towards a lower species diversity and a predominance of *Enterobacteriaceae* such as *E. coli* and *Klebsiella pneumonia* or *Clostrida* spp. were shown to contribute to the most devastating intestinal disease in newborns – the necrotizing enterocolitis (66-68). However, it is still not known whether NEC is primarily initiated by a pathogenic bacterial invasion or a breakdown of the epithelial barrier followed by the invasion of pathogens (69). The disease primarily occurs in extremely low birth weight infants, with about 70% of cases occurring in babies born at less than 36 weeks of gestation and it often demands an urgent surgical intervention; postoperative survival rate is about 51% (69, 70). However, meta-analyses of numerous studies showed the positive effect of probiotics on the reduction of NEC and found a significant decline in the incidence of severe NEC and total mortality through the enteral supplementation of probiotics (71, 72).

Furthermore, not only NEC is a burden in prematurity. An increased intestinal permeability favors the translocation of potentially pathogenic bacteria to other organs, thereby inducing systemic infections in the preterm infant's body (73). In addition, antibiotic therapy, which is necessary to eliminate these pathogenic bacteria, sustains the vicious circle by preventing a normal microbiota development in the infant's gastrointestinal tract (74). One approach to compensate the deficiencies observed in the colonization process of preterms reported numerously, is also here the application of probiotics in order to tackle the problem of pathogenic bacteria overgrowth by the



blockage of free binding sites. Probiotics in premature neonates were also shown to significantly reduce regurgitation, mean daily crying time, but also gastric emptying, thereby mitigating symptoms of constipation (75, 76). However, the routine administration of probiotic bacteria to preterm infants is not yet fully recommended due to safety, right dosage and efficacy concerns (77).

### **1.3.2. Mode of delivery**

Approximately every third child is born by caesarean section in many developed countries. Numerous studies confirm a difference in early colonization patterns depending on the mode of delivery (78-82). During vaginal delivery, the infant's mouth comes into close contact with the maternal vaginal and faecal microbiota, which is an important source for the start of the newborn's intestinal colonization process. However, infants born by caesarean section lack this contact and are primarily influenced by the bacterial environment of the hospital and the mother's skin microbiota (82). The intestinal microbiota of infants born by cesarean section is characterized by a reduced number of *Bifidobacterium* spp. and *Bacteroides* spp. in the first days or even months of life (83-85). It was shown, that higher abundances of *Enterobacteriaceae*, veillonella and clostridia are found in faeces from infants born by caesarean section (80). Significant outcomes with regard to bacterial diversity were observed in some studies, with infants born by caesarean section having a reduced bacterial diversity when compared to normal births. However, after the initial difference in microbiota composition, bacterial habitats increasingly resemble between normal and caesarean born infants (84, 86). The microbiota serves as important stimuli for infant immune development, hence mode of delivery could affect the immune system due to different colonization processes. Reduced Th1-associated chemokines were found in infants born by caesarean section, likely caused by the reduced numbers of *Bacteroides* found in the same cohort, which were often associated with influences on the innate immune system (84, 87).

Results on the timely persistence of microbial differences between infants born by caesarean section and vaginal birth are still conflicting. For example, Grondlund et al suggested that the primary gut composition in infants born by caesarean section may be disturbed for up to six months with a delayed colonization and a rate of

*Bifidobacterium* and *Lactobacillus* species similar to that of normal deliveries more than one month later (88). Similarly, another report demonstrated that the microbiota is still retarded in its development at the age of six months, where vaginally born babies are dominated with *Bifidobacterium* and *Collinsella* compared to *Enterobacteriaceae* and *Streptococcus* sp. in caesarean sections (89). Differences in microbiota between the two delivery modes, however, can even persist up to seven years, as shown in children born normally with higher abundances in faecal *Clostridia* species than in children born by caesarean section (90).

Available epidemiological data further suggest a trend towards the development of allergic rhinitis and (hospitalization for) asthma for infants born by caesarean section when being compared to infants born by labor (91-93). However, these data have to be handled with care as there have been conflicting results about the long-term physiological effects caused by mode of delivery. To investigate the long-term effect of delivery modes, one has to carefully think of numerous confounding factors experienced by the subject during life. Several studies have pointed towards an effect in terms of incidences of IBD and have shown to be not significantly different upon different mode of delivery (94, 95). Also the immediate effect of delivery mode requires consideration of a delayed onset of lactation in mothers delivering via caesarean section as well as a lower volume of breast milk production, hence a lack of stimulation of the initial infant flora, which confounds the sole delivery effect on the microbiota (96).

### **1.3.3. Effect of feeding**

There are numerous studies showing that the faecal microbiota of breast-fed infants differs significantly from that of formula-fed infants (44, 45, 47, 48). While breast-fed infant faecal samples primarily harbor bifidobacteria and lactobacilli, formula-fed babies have an increased richness of species, with bacteroides, bifidobacteria, staphylococci, *Escherichia coli* and clostridia being the dominating bacterial communities (45, 79, 97, 98). The reason for this distinct colonization might be primarily caused by the virtual absence of highly complex human oligosaccharides (HMOs) in infant formula. Whereas, in contrast, human breast milk is an important source of HMOs that promotes growth of bifidobacteria in the gut (99). Further, high lactose, low casein and calcium phosphate and a low buffering capacity seem to favor the

colonization with bifidobacteria (100). Of the *Bifidobacterium* genus, *B. breve*, *B. infantis* and *B. longum* are the most frequently found species in breast-fed infants (101).

The retarded establishment of a normal gut microbiota in bottle-fed infants has been suggested to play an important role in postnatal maturation of the immune system and development of oral tolerance, probably leading to a higher susceptibility for allergic diseases in later life (102). However, the addition of probiotic bacteria to infant formula has shown that the pro-allergic effect of bottle-feeding can be mitigated, at least for some allergies (103). The tendency of breast-fed infants to gain less weight during infancy and therefore to be leaner than bottle-feds is, however, not due to nutritional deficits, but rather due to infant self-regulation of energy intake (104).

After weaning, it is still decisive whether the infant was breast- or bottle-fed before weaning, as bifidobacteria still dominate the intestinal microbiota of breast-fed babies and formula-fed infants show significantly higher proportions of *Bacteroides* sp. and *Clostridium coccooides* (55).

Recommendations for initial feeding practices, however, remain controversial. While the WHO (World Health Organization) encourages a sole breast-feeding from birth until six months of age, data from research have not uniquely concluded a recommendation for either of the two feeding practices. The consequence of the promotion of bifidobacteria growth upon breast-feeding is a reduced bacterial richness and diversity in the gut (79, 105). Reduced bacterial diversity has also been observed in fecal communities of preterm infants who develop necrotizing enterocolitis, compared with those who do not develop the disease (106). Further, reduced intestinal diversity has been linked to increased risk for the development of allergic diseases later in life (107). In contrast, an increased bacterial richness, calculated on the basis of microbial functional gene richness (high gene count vs. low gene count individuals) showed that high gene count adults have a greater repertoire of microbial metabolic functions, a functionally more robust microbiome, and a greater overall health (108). Consequently, and based on these and additional findings, it has been suggested that greater bacterial richness and diversity may be an indicator for health. The consequences of low bacterial richness and diversity during early life on adult health therefore need to be determined in long-term studies in order to make definite recommendations for initial feeding practices.

After initial liquid feeding, complementary food is required as breast milk or formula is no longer sufficient for nutritional reasons. According to the WHO, start of solid food is recommended after a six-month sole breast-feeding phase, while the European Society for Pediatric Gastroenterology Hepatology and Nutrition (ESPHGAN) generalizes this recommendation to a complementary feeding start between four and six months of age (109, 110). Numerous studies on the effect of an earlier start of complementary food, mostly smashed vegetables or fruits, have not led to the same conclusion, however, no adverse effects were observed later in life when initially introduced to solid food already at 4 months of life (111).

The ultimate impact of solid food introduction on the infant microbiota, however, seems to be rather low, when compared to the effect of cessation of breast-feeding. While infants that are being stopped from breast-feeding will be enriched with numbers of clostridia increasingly resembling the adult-like microbiota, the breast-fed microbiota is still dominated by bifidobacteria and lactobacilli after solid food introduction (112).

Nevertheless, there is strong evidence, that complementary foods drive numerous processes in the developing gut, including an increased abundance of Bacteroidetes, elevated faecal short-chain fatty acid levels, enrichment of genes associated with carbohydrate utilization, vitamin biosynthesis, and xenobiotic degradation, and a more stable community composition, all of which are characteristic of the adult microbiome (113). Moreover, a reduction in *Bifidobacterium* spp. abundance is often accompanied with introduction of solid food, however, depending on the type of species. For instance, *B. longum* and *B. breve* were shown to decrease upon complementary food uptake, while *B. adolescentis* and *B. catenulatum* increase (114).

The type of complementary food introduced also plays a crucial role in the development of the infant microbiome. For instance, an iron-fortified diet was shown to reduce relative abundances of bifidobacteria and lactobacilli in breast-fed infants, while a zinc-fortified diet lead to unchanged levels of these bacteria (115).

So the initial diet plays a substantial role in the development of the infant gut microbiota that is primarily determined by the choice between breast- and bottle-feeding and subsequently by the process of weaning followed by the introduction of complementary food.

#### 1.3.4. Antibiotic treatment

Particularly preterm infants are affected by an extensive use of antibiotics during the first days of life and as a consequence their colonization is strongly influenced by this condition (116). Similar microbial colonization delays as seen for infants born by caesarean section, namely a retarded growth of lactobacilli and bifidobacteria, occur in newborns that are exposed to antibiotic treatment (117). Bacterial diversity seems to be lower in antibiotic-treated infants coupled with a higher relative abundance of *Enterobacter* spp. (74).

However, the extent to which antibiotics may alter the infant microbiota remains a topic of current research and is likely to vary inter-individually. For example, a five-day course of ciprofloxacin altered the relative abundance of 30% of gut microbiota members. Most bacteria recovered to pre-treatment abundances after four weeks of antibiotic absence, while some bacterial communities were not able to fully recover after six months (118). Also, it was shown that even short-term antibiotic treatment might influence the colonization pattern of bifidobacteria, whose growth is perturbed up to 8 weeks after treatment with antibiotics, while Proteobacteria bloom during this phase (119). While changes in bacterial prevalence and abundance were observed, also alterations on the functional level are likely. Experiments in mice revealed that the majority of metabolites in murine faeces are altered qualitatively or quantitatively 24 hours after streptomycin treatment (120). In addition, more cases of necrotizing enterocolitis or death can be observed in infants that are treated with antibiotics during the first weeks of life compared to those not exposed to antibiotics (121, 122). To date, however, the number of antibiotic trials in early life is limited, so that only general conclusions on the extent of recovery to pretreatment levels are possible. From existing studies, recovery is dependent on a mix of factors, including the nature of the antibiotic, the duration of treatment and intrapersonal characteristics, such as host genetics, and probably microbiota composition (123). Moreover, the effect of early antibiotic administration on the adult gut microbiota is subject of current research. It was found that early life use of antibiotics changes *Bifidobacterium* and *Akkermansia* abundances in children (124). A suspected association between antibiotic use and development of IBD during childhood may emphasize the interrelationship between antibiotic administration and long-term microbiota alterations (125, 126). Hence, careful

consideration on the selection and duration of antibiotic treatment during infancy is important to minimize the risk for adverse long-term effects on the microbiota.

### 1.3.5. Other influences

The hygiene hypothesis has evolved increasingly during the past decades. It has been hypothesized that increased hygiene, reduced family size and subsequent reduced microbial exposure could explain the increases in global atopic disease prevalence (127). However, meta-analyses of studies from recent years have mitigated the hypothesis, showing that asthma prevalence in western countries have decreased during the last years, while occurrences in less clean countries such as from Latin America, have increased (128). Moreover, also other intestinal diseases such as IBD, which had initially been claimed to be linked to several hygiene-related factors, were shown not to be affected by an exaggerated cleanliness (129).

One further influence on the infant microbiota is the presence of a sibling, with infants having a sister or brother showing higher numbers of *Bifidobacterium* spp. than children without siblings (97). A recent study confirmed the bifidogenic effect of the presence of siblings, while single infants were more likely to develop a microbiota that is more *Escherichia*-dominant (130). The increased microbial exposure of siblings was further found to elevate gut bacterial richness and diversity and hence may substantiate the hygiene hypothesis (131).

It was further proposed that the presence of pets increases the bacterial richness and diversity in infants, while pets can also exhibit an under-representation of *Bifidobacteriaceae* and an over-representation of *Peptostreptococcaceae* (132). Among vaginally born infants, a major characteristic of the infant microbiota exposed to household pets included a significant decrease in *Streptococcus* abundance (133). In addition, *Oscillospira* and *Ruminococcus* species were increased, which both have been negatively associated with childhood atopy and obesity (133, 134).

The geographical location is also likely to affect the intestinal microbiota of infants. Investigations in infants born in Northern Europe have shown that they possess higher *Bifidobacterium* spp. and some *Clostridium* spp. and *Atopobium* spp. levels, while Southern infants have higher *Lactobacillus* and *Bacteroides* spp. abundances (135).

This difference in microbiota based on geographical background was also seen in adults, which substantiates the long-term power of initial colonization profiles (136). Major or minor influences on the developing intestinal microbiota may be driven for instance also by infant sex (137) and maternal gestational weight gain (138) but data are marginal and further investigations are required.

## **2. Probiotics and their use in early life**

### **2.1. Definition, role and selection criteria**

The WHO defines probiotics as live organisms which when administered in adequate amounts confer a health benefit on the host (139). The vast majority of probiotic bacteria are Gram-positive strains, mainly species of the *Lactobacillus* and *Bifidobacterium* genera, with *Lactobacillus rhamnosus* and *Bifidobacterium lactis* being the most popular ones. Moreover, *Lactococcus*, *Streptococcus* and *Enterococcus* species as well as some non-pathogenic *Escherichia coli* and certain yeast strains are qualified as probiotic (140). Probiotic bacteria possess a fermentive, obligatory, or facultative anaerobic character and are typically non-motile and of varying shapes (141).

Probiotics have been with us for as long as people have eaten fermented milks, however, their beneficial association to health has begun when Elie Metchnikoff studied the adverse effects of the gut microbiota on the host, suggesting that the ingestion of fermented milks can ameliorate this so called autointoxication (142). This hypothesis was stated in the beginning of the 20th century and Metchnikoff consequently proposed that lactic acid bacteria can replace or diminish the number of harmful bacteria in the gut and thus stabilize bowel health and prolong life (143). It took then several decades until the topic of beneficial microbes was resumed to reflect Metchnikoff's ideas. The term "probiotic" was used for the first time by Parker in 1974 to describe organisms and substances that contribute to intestinal microbial balance (142). In the last 20 years, scientific research in probiotics has advanced considerably, thereby selecting and typing potential probiotic cultures.

The roles of probiotic bacteria in dairy fermentations are diverse. First, probiotics assist in the preservation of milk by the generation of lactic acid and possibly antimicrobial

compounds (144). Second, probiotic bacteria in yoghurt and cheese are responsible for the production of flavor compounds such as acetaldehyde (144, 145). Apart from preservative and odorant properties, probiotics have been widely used in the prophylactic and therapeutic area. Main health benefits of probiotics include maintenance of healthy gut function, improved tolerance to antibiotics, prevalence of allergies in susceptible individuals, and overall reduced risk for different chronic diseases (146).

For the selection of bacteria with probiotic potential, specific selection criteria have been established. In a first step, a potential strain requires careful identification, including the bacterial origin, character, but also strain and genus safety properties, such as non-pathogenicity (147). An identification of the bacterial species or strain is inevitable for the linkage to essential information such as growth characteristics, genomic information and metabolic features (148).

Another selection criterion includes the safety aspect, which gives information about the innocuousness of the used strain. Regarding safety, there are three theoretical concerns, including the occurrence of disease (e.g. bacteremia), toxic or metabolic effects on the gastrointestinal tract and the transfer of antibiotic resistance in the intestinal microbiota (149).

Finally, also functional and physiological aspects, including viability and persistence in the gastrointestinal tract, immunomodulation and antagonistic and antimutagenic properties, should be assessed in order to qualify the selected species or strain as probiotic (150).

Tests on the selection of appropriate strains should be carried out *in vitro* and *in vivo* for the substantiation of the health effects in the target host (151). In addition, probiotic bacteria should be of human origin and should be able to resist technical processing (117).

An additional criterion for the selection of bacteria as probiotics is strain specificity. Some bacterial strains were shown to exert different health effects on the host. For example, different *Lactobacillus rhamnosus* strains have distinct properties when being applied in the treatment of rotavirus diarrhea, with one strain possessing the potential to reduce the duration of disease when being compared to another strain (152).



**Table 1: Most commonly used probiotic bacterial species in human trials.**

Lactobacilli and bifidobacteria are the most commonly used probiotic bacteria in food and pharmaceutical industry. Enterococcal and streptococcal species are occasionally used in food products. Their pathogenic potential upon ingestion cannot yet completely be excluded due to well known cases of bacteremia and other infections.

<b>Genus</b>	<b>Species</b>
<b><i>Lactobacillus</i></b>	<i>acidophilus</i>
	<i>bulgaricus</i>
	<i>casei</i>
	<i>fermentum gasseri</i>
	<i>gasseri</i>
	<i>helveticus</i>
	<i>johnsonii</i>
	<i>paracasei</i>
	<i>reuteri</i>
	<i>rhamnosus</i>
<b><i>Bifidobacterium</i></b>	<i>animalis</i>
	<i>bifidum</i>
	<i>breve</i>
	<i>infantis</i>
	<i>lactis</i>
	<i>longum</i>
<b><i>Enterococcus</i></b>	<i>faecium</i>
<b><i>Streptococcus</i></b>	<i>thermophilus</i>
<b><i>Escherichia</i></b>	<i>coli</i>

## **2.2. Effects of probiotics on diverse physiological functions**

In the recent decade, more than 1000 clinical trials were conducted in humans on the effect of probiotic bacteria on human health. Generally, probiotics are regarded as safe and positive outcomes from clinical trials outweigh negative implications or no change in health status through the use of orally administered probiotics (153).

Due to the modifying effect on the relatively simple infant microbiota, infants and children are a well researched target group for probiotics (154). However, the European Food Safety Authority (EFSA) has advised negatively about health claims

for probiotics to date (155), which emphasizes the need for studies on the clinical relevance of probiotic applications.

One proposed physiological effect of probiotics includes pathogen exclusion through competitive adhesion to stereo-specific receptors on the gut mucosal surface. This is dependent on the specificity of the bacteria and bacterial adhesins for the receptors as well as on the relative concentration of the competing bacteria, which in turn determines the effective dosage of a probiotic in a product (156). One of the most studied mucus-targeted bacterial adhesion is achieved by the mucus-binding-protein MUB of *Lactobacillus reuteri* (157). Furthermore, binding of other bacteria can be hindered through the establishment of an unfavorable environment for the competitors, e.g. by the production of bacteriocins (158).

Another major role of probiotics in the intestine is the enhancement of the epithelial barrier. The intact epithelial barrier is of utmost importance for the exclusion of bacterial and food antigens from the submucosa, which can result in an inflammatory response upon barrier disruption (159, 160). However, the mechanisms by which probiotics support epithelial barrier function are not yet fully understood. Recent studies have suggested bifidobacterial expression of genes involved in tight junction signaling (161) or promotion of mucus secretion thereby inhibiting pathogen translocation (162).

Moreover, specific probiotic bacteria were proven to have a protective effect on certain intestinal diseases. Particularly in patients with antibiotic-associated diarrhea or traveler diarrhea, successes were reported (163).

Further indications successfully prevented or treated by the use of single or combined probiotics particularly include IBS (irritable bowel syndrome) and NEC (see table 2 and 3 summarizing selected effects of probiotics in healthy and diseased groups of at least 20 individuals).

It is well known that changes in the microbiota pattern can change metabolite profiles in the intestinal ecosystem (164). Gut metabolites have diverse roles and their presence may drive beneficial downstream effects on immune pathways (17, 165). However, insufficient exposure to dietary metabolites was also proposed to deteriorate the host's health, leading to common western diet related diseases such as IBD (166). Upon a dietary uptake of probiotic bacteria, particularly SCFA levels were shown to be altered, mostly by increased concentrations (167, 168). One study revealed a normalization of IBS-induced disturbed energy metabolism, i.e. decreased glycogenesis and elevated lipid breakdown, through the administration of *L. paracasei*

(169). While probiotic consumption was moreover suggested to enhance specific metabolites and to further suppress the production of inflammatory cytokines, data in that field are scarce and the role of probiotics on metabolomics remains to be elucidated (170).

**Table 2: Effects of single probiotics used in human studies.**

Selection of clinical and non-clinical trials conducted within the last 20 years with at least 20 participants. Most clinical trials were performed in patients with acute and chronic diarrhea and constipation and in patients suffering from IBD. Preterm infants affected by necrotizing enterocolitis and eczema are also well studied trial groups. Only studies with significant outcomes are shown.

Probiotic	Condition	Form	Duration, Frequency	# Patients	Result	Reference
<i>B. animalis</i>	Healthy, active	Drink	4 months, 1x daily	465	Reduced risk of upper respiratory illness	(171)
<i>B. animalis</i>	Healthy, active infants	Formula	6 weeks, daily	172	Increased faecal <i>Bifidobacterium</i> counts	(172)
<i>B. bifidum</i>	Preterm delivery	Powder	Individually, 2x daily	36	Growth promotion	(173)
<i>B. bifidum</i>	IBS	Capsule	4 weeks, daily	122	Alleviation of symptoms	(174)
<i>B. breve</i>	Chemotherapy	Powder	6 weeks, 3x daily	42	Reduction in fever and antibiotic use	(175)
<i>B. lactis</i>	Constipation	Cheese	4 weeks, daily	30	Improvement of symptoms	(176)
<i>B. lactis</i>	IBS	Milk	4 weeks, 2x daily	34	Reduced symptomatology	(177)
<i>B. infantis</i>	Preterm delivery	Formula	5 weeks, daily	21	Increased faecal <i>Bifidobacterium</i> counts	(77)
<i>B. infantis</i>	IBS	Capsule	4 weeks, daily	362	Alleviation of symptoms	(178)
<i>B. longum</i>	Coeliac disease	Capsule	3 months, daily	33	Improvement of health status	(179)
<i>B. longum</i>	Healthy infants	Formula	Up to 4 months, daily	190	Resemblance to breast-fed microbiota	(180)

**Table 3: Effects of combined probiotics used in human studies.**

Selection of clinical and non-clinical trials conducted within the last 15 years with at least 20 participants. Most clinical trials were performed in patients with IBD and in preterm infants suffering from necrotizing enterocolitis and eczema. Only studies with significant outcomes are shown.

<b>Probiotic</b>	<b>Condition</b>	<b>Form</b>	<b>Duration, Frequency</b>	<b># Patients</b>	<b>Result</b>	<b>Reference</b>
<i>L. acidophilus, B. infantis</i>	Necrotizing enterocolitis	Capsule	14 days, daily	5351	Less NEC surgeries	(181)
<i>L. acidophilus, B. bifidum</i>	Cold	Capsule	3 months, 2x daily	80	Less fever, cough, rhinorrhea	(182)
<i>L. acidophilus, B. animalis</i>	Pregnancy	Yoghurt	9 weeks, daily	70	Lower hs-CRP levels	(183)
<i>L. acidophilus, B. animalis</i>	Cold, Influenza	Milk	6 months, 2x daily	326	Reduction in incidence and duration	(184)
<i>L. acidophilus, B. lactis</i>	Atopic dermatitis	Capsule	8 weeks, 2x daily	90	Improvement of symptoms	(185)
<i>L. acidophilus, B. longum</i>	Healthy women	Yoghurt	21 weeks, daily	29	Increase of HDL cholesterol	(186)
<i>L. acidophilus, L. delbrückii, B. bifidus</i>	Pouchitis	Capsule	9 months, 2-3x daily	43	Lower severity	(187)
<i>L. acidophilus, L. paracasei, B. animalis</i>	IBS	Capsule	6 months, 2x daily	131	No effect	(188)
<i>Lactobacilli, Bifidobacteria, Streptococci</i>	IBS	Capsule	4 weeks, 2x daily	49	Relieve of IBS symptoms	(189)
<i>Lactobacilli, Bifidobacteria, Propionibacteria</i>	IBS	Drink	5 months, daily	86	Alleviation	(190)
<i>Lactobacilli, Bifidobacteria, Streptococci</i>	Diabetes mellitus-2	Capsule	8 weeks, daily	54	Decrease in hs-CRP	(191)
<i>Lactobacilli, Bifidobacteria, Streptococci</i>	Healthy adults	Yoghurt	4 weeks, daily	58	Higher bifidobacteria counts	(192)
<i>Lactobacillus, Bifidobacteria</i>	Necrotizing enterocolitis	Powder	24 days (average), daily	611	Frequency reduction	(193)
<i>Lactococcus, Bifidobacteria</i>	Eczema	Powder	12 months	98	Preventive effect	(194)

### 2.3. Considerations for the administration of probiotics to infants

The incorporation of novel bacterial strains into food or therapeutic products requires a careful assessment of their safety. Continuously new bacterial strains are being discovered and incorporated in food products as probiotics, thereby claiming specific nutritional, functional or therapeutic characteristics (195). The absence of pathogenicity of any potential probiotic strain must be proven to show its safety. Safe doses of probiotics were shown to range from  $5 \times 10^5$  to  $1 \times 10^{12}$  colony forming units (CFUs), administered most often in form of yoghurt, milk and capsules (153).

The most common probiotic bacteria in food are lactic acid bacteria, such as *L. acidophilus*, *L. rhamnosus*, *L. casei*, *L. gasseri*, and *Bifidobacterium bifidum*. Species of the *Lactococcus* and *Lactobacillus* genera have been shown to be generally safe and are seldom isolated from infections (196-198). However, some strains of the *Streptococcus* and *Enterococcus* genera (e.g. *Streptococcus pyrogens*, *Streptococcus pneumoniae*, *Enterococcus faecium* etc.) can cause severe health-care associated infections. The use of *Enterococci*, primarily in southern European countries for cheese production, remains controversial. As a probiotic, enterococci are sold in form of probiotic fermented milk (Gaio, Denmark) and in other products even containing the infectious *E. faecium* strain, which can render a threat for individuals with poor health status due to the presence of virulence factors and acquired antibiotic resistance (198, 199). On the contrary, other strains such as *Streptococcus thermophilus*, are commensals of the human intestinal microbiome, and are considered to be safe for the use in yogurts and other products (200).

Depending on the form and country in which probiotics are administered and used, they are classified as dietary supplement, food, food component, or pharmaceutical. Each of these categories is subject to different regulations and burdens of proof in regard to the demonstration of health benefits and safety, which again differs between the countries (201). In Europe, probiotics are regulated under both common directives and national regulations (202).

Functional foods and dietary supplements are regarded as safe and therefore typically contain claims on their labelling about their benefits (203). These claims were harmonized on the European level by the European Union and its Regulation No 1924/2006 on nutrition and health claims made on foods. Nutrition claims state,

suggest or imply that a food has particular beneficial nutritional properties, such as that the product is low in salt (203). Article 13.1 claims are defined as “general function claims” and cover health claims describing or referring to the role of nutrient or other substance in growth, development and function of the body, or also to psychological and behavioral functions. Moreover, weight control by increase of satiety or slimming functions are covered by the article (204, 205).

Article 13.5 of the 1924/2006 EU regulation refers to any claim based on newly developed scientific evidence (205). Article 14 covers any claims for the reduction of disease risk and claims referring to children’s development and health (204). Any claims made under articles 13.1, 13.5 and 14 are then only authorized for use after a scientific assessment has been carried out by the Panel on Dietetic Products, Nutrition, and Allergies (NDA) of the EFSA (151, 202).

#### **2.4. Current knowledge about the effect of probiotics on infant physiology and development**

Probiotics are usually added to infant formula or they can be provided as droplets in a suspension or as capsules. The aim for the administration of either products is to assist in the development and maintenance of the gut microbiota in order to increase numbers of health-promoting bacteria, such as bifidobacteria or lactobacilli. Due to the high number of these bacteria in exclusively breast-fed bacteria, a supplementation with probiotics aims to mimic the breast-fed microbiota. However, the structural resemblance to breast milk oligosaccharides is difficult due to their high structural complexity. Increasingly, prebiotic mixtures in form of galacto-oligosaccharides and fructo-oligosaccharides are added to infant formula and were shown to exert effects on the newborn intestinal microbiota, in particular they increase bifidobacterial counts and lower the total number of pathogens when compared to infants fed with non-supplemented formula (99).

From 1994-2014, a total of 2817 published documents on probiotic research in pediatrics was counted, with highest activity in that field in the USA and Finland and the majority of trials in the field of necrotizing enterocolitis, allergies and diarrhea (206). The addition of probiotics was shown to affect numerous infant pathologies, such as rotavirus-associated diarrhea and gastroenteritis (207-209). One study revealed that

31% of infants from the placebo group developed diarrhea and 39% developing a rotavirus infection, while only 7% and 10%, respectively, from the intervention group developed the diseases (210, 211). The administration of bifidobacteria to the infant can also elevate faecal *Bifidobacterium* sp. concentrations and therefore lead to a more breast-fed adapted microbial structure than without the addition of the strains (180).

A substantial number of meta-analyses and systematic reviews of randomized controlled trials of a vast number of probiotic strains administered to preterm infants of different gestational ages and birth weights suggests a significant reduction in necrotizing enterocolitis, one of the major causes for death in premature babies (212, 213). Often, the preterm organism is also challenged by the use of antibiotics in order to prevent infections that could potentially endanger the baby. The use of probiotics, however, showed that the amount of antibiotics required, could be lowered significantly (214). There is also evidence for the reduction of atopic diseases in infants, with a reduction of up to 50% of affected infants when supplied with probiotic bacteria (215, 216). Particularly, it was demonstrated that atopic dermatitis can be reduced by the regular use of probiotics (217). Moreover, infantile colic, estimated to affect about 20% of newborns, may be palliated by the use of specific probiotic strains, such as *Lactobacillus reuteri* DSM 17938, however data have focused primarily on the administration to breast-fed infants instead of formula-feds (218). Further conditions that probiotics may benefit include gastrointestinal disorders such as acute gastroenteritis and IBD, which were shown to be ameliorated by the VSL#3 probiotic mix (a combination of 8 different bacteria strains) but here most data include analyses from children and adults (219). Other parameters, like faecal IgA and stool pH were shown to resemble more the breast-fed group when fed a *Bifidobacterium* strain (211). However, the number of studies that were not able to detect any major differences between placebo and intervention groups is also increasing – it was shown that the addition of probiotics does not exert any effect on diarrhea and febrile infections (211), sepsis (220, 221) , NEC (220), colic (222) and infant mortality (220, 221). Also effects on atopy were shown to be only marginal, particularly when measuring the long-term impact (223).

One of the major challenges in the measurement of a positive effect of probiotic feeding to infants lies in the persistence. While strains can be detected from faecal samples, providing evidence for their colonization potential, and some type of diseases might be



mitigated by their use, both - the long-term health effect and a long-term persistence of the strains in the gut - were not or only marginally measured to date (224, 225). Moreover, the selection of adequate strains for the prevention or treatment of different infantile pathologies requires more investigations in order to select a definite pool of bacteria appropriate for their intended use.

## **2.5. Genus *Bifidobacterium***

Currently, lactic acid bacteria are the most frequent bacteria to be used in industry for the production of probiotic food. Bifidobacteria were first described in 1900 by Tissier (226), who isolated V- or Y-shaped microorganisms from infantile faecal samples, suggesting that these bacteria were likely the reason for the lower incidence of infantile diarrhea in newborns (227, 228). In his subsequent work, he used these newly discovered bacteria to treat infantile diarrhea, thereby firstly executing an oral administration of live organisms for the treatment of a disease (226, 228). Searching for “bifidobacteria” on the Pubmed search tool (“*Bifidobacterium* clinical trial human”) gives a total of almost 600 trials that have been executed on humans by date. Most of the studies were done with *B. lactis* and *B. longum*.

*Bifidobacterium* species comprise a large group of bacteria, which are natural habitats of the human gastrointestinal tract, representing approximately 3%, of the adult faecal flora (229, 230). In contrast, the breast-fed infant microbiota harbors about 80-90% of *Bifidobacterium* spp. one week after birth (29).

Bifidobacteria belong to the phylum of Actinobacteria and the family of *Bifidobacteriaceae*. By date, the *Bifidobacteriaceae* family is comprised of nine genera with *Bifidobacterium*, *Gardnerella*, *Scardovia* and *Parascardovia* representing the dominant genera (231). The genus *Bifidobacterium* is grouped in a total of 41 species that have been identified by date, with *B. adolescentis* and *B. longum* being the principal ones of the human microbiota (232-234). While some other reports declared *B. catenulatum* as the most common taxon, one needs to consider the high similarity between *B. adolescentis* and *B. catenulatum*, which might have led to this confusion (101, 235).

Bifidobacteria are taxonomically not related to other lactic acid bacteria such as lactobacilli or lactococci, which are Firmicutes (230). Bifidobacteria are gram positive,

facultative anaerobic and rod-shaped. They are non-motile, non-sporulating, catalase-negative bacteria and possess a high GC content (55-67%). In general, these bacteria can be considered as safe, however, some species can cause peritonitis, pulmonary infections, urinary tract infections, endocarditis and they can favor the acidic environment for dental caries (230, 236).

The role of *Bifidobacterium* spp. in the large intestine is diverse and include the production of vitamins, mainly of the B group, as well as digestive enzymes and metabolic end products such as acetate and lactate to lower the pH for antibacterial effects (117, 237-239).



## **AIM OF THE WORK**

The diversity and composition of the infant gut microbiota is influenced by numerous factors, including initial feeding scheme. Although many commercially available formulas are supplemented with bacteria considered as probiotics, little is known about their ability to impact gut microbial composition and function. While preliminary studies reported on presumably positive effects of probiotic bacteria on specific infant and child diseases, such as necrotizing enterocolitis or inflammatory bowel diseases, data on the effect of bifidobacteria supplemented formula on the microbiota are marginal.

Hence, aim of this work was to address the question how bifidobacteria supplemented formula can shape the microbiota of healthy newborns in comparison to non-supplemented formula over the first year of life. Exclusively breast-fed infants were furthermore examined and compared to the formula groups in order to determine if a supplementation with bifidobacteria potentially shifts the microbiota towards a bifidobacteria enriched breast-fed microbial pattern. Of major interest was to subsequently determine the metabolite profiles of the different feeding groups and to correlate these analyses to the microbiota data – analyses were performed in collaboration with the Helmholtz Institute Munich. Results from metabolomics support the understanding for functional processes observed during the microbial succession of the infant gut. Furthermore, compositional investigations on samples of breast milk served to reveal the characteristic microbiota shaping in breast-fed infants.



## **METHODS**

### **1. Study setup**

#### **1.1. Subjects**

Pregnant women were recruited by gynecologists in Munich and surrounding areas and personally informed about all aspects of the study. Participation in the study trial was confirmed by a written informed consent of both parents prior enrollment. Women with antibiotic treatment 9 weeks prior delivery, maternal adiposity (BMI >30), anorexia (BMI <18,5), preterm delivery (gestational age <37 weeks), high risk pregnancy, chronic or mental diseases were excluded from the trial.

Infants with congenital malformations, gastrointestinal symptoms deviating from normal neonatal bowel behavior (such as NEC) or atopic predispositions were excluded from the study. Upon milk intolerance or dislike, infants were not considered for continuation. Exclusively breast-fed infants were kept in the trial as a control. A minimum number of 80 participants was aimed for the study.

#### **1.2. Study design**

The study was a double-blinded, parallel, randomized placebo-controlled trial. Parents were asked to provide blinded (supplemented or placebo) milk to their term infants (both sexes) in case of inability or non-willingness of breast feeding or after weaning. Mothers were not obliged to feed the provided formula at any time. Consequently, the milk was given to the babies either as a substitution to breast milk, in parallel to breast feeding or after weaning. Participants were

- breast-fed for the whole study period
- formula-fed for the whole study period
- breast-fed for a defined time (days to months), followed by the infant formula
- breast- and formula-fed in parallel

### 1.3. Sample collection

Faecal samples taken monthly over 12 months were collected by the parents preferably in the morning and collected in 50-mL polypropylene faecal collection tubes (Sarstedt, Germany). The tube was screwed halfway to allow oxygen to be taken up by a paper gas generating sachet (Oxoid, UK) which was put, together with the tube, into a sealed plastic pouch (W-Zip Plastic Pouches, Oxoid, UK). Oxygen was absorbed in the bag within 2,5 hours by the sachet, which reacted on contact with air by its ascorbic acid and activated carbon. This should have created ideal atmospheric conditions in particular for anaerobic bacteria. The bag with the sample was immediately put into the refrigerator and transported on ice to the laboratory within one day. The samples were stored as 100mg aliquots at -80°C until further analysis. At each visit, the amount and frequency of milk uptake (breast milk or infant formula) was recorded in questionnaires. Moreover, health status, antibiotic treatment, weight, size and the frequency and quantity of food intake were documented. Voluntarily, mothers could provide two breast milk samples, taken at different time points.

### 1.4. Study formulas

The experimental (supplemented) and placebo (non-supplemented) formula were standard whey based powder products provided by Töpfer GmbH (Dietmannsried, Germany). When prepared in accordance with the instructions they contained (per 100 ml): 281kJ, 67kcal, 1,7 g protein, 7,4 g carbohydrates, 3,5g fat, vitamins, minerals, choline, inositol, trace elements and dietary fibers. The experimental formula was supplemented with four types of *Bifidobacterium* species: *B. bifidum*, *B. breve*, *B. longum*, *B. longum* subsp. *infantis* (identified and typed by Cell Biotech Co, Ltd). Experimental formula was produced with a *Bifidobacterium* concentration of  $>1,0 \times 10^7$  cfu/g. All four species were added in same amounts to the product (25% each). Total viable cell numbers in the supplemented formula were monitored by the provider via cultivation on Bifidobacterium-selective agar and counts were confirmed to be  $10^7$  cfu/g throughout the study period. The supplemented and non-supplemented formula were almost identical in taste, smell and color and were blinded by the provider. Specimens

of formula were regularly checked for *Bifidobacterium* concentrations above  $3,0 \times 10^6$  cfu/g.

Moreover, according to the infant's appetite, a more satiable formula was provided as well, containing basically all constituents described above, but also glucose (0,03 g/ 100 ml) and maltose (0.1 g/ 100 ml). After the 6<sup>th</sup> month of life, follow-on milk with starch (1.1 g/ 100ml), was administered. The formula was stored at ambient temperature and provided to the parents in monthly intervals. Töpfer GmbH did not influence any steps of the work, including study design, contact with participants, sample collection and analysis, data management and interpretation of the results.

### **1.5. Randomization**

Randomization for feeding with supplemented or placebo formula was performed by an independent investigator using a manually generated randomization list with a block size of 8. Each block was evenly distributed for placebo and intervention and participants were successively added to the list. The formula was unlabeled and blinded. Only a label for indication of start-up or follow-on formula and a number for group differentiation were applied. Blinding of study formula was performed by the Töpfer GmbH.

### **1.6. Ethical approval and registration**

The study was approved by the Ethical Commission of the Faculty for Medicine of the Technical University of Munich (Germany). The study was registered at the German Register for Clinical Studies (DRKS) with the DRKS number DRKS00003660. All changes not in accordance with the initial study plan were approved by the Ethics Committee. All study participants were covered by a trial insurance covering all study associated harms.



## **2. Cultivation of bifidobacteria from faecal specimens**

Faecal samples were diluted in a 1:20 dilution with 40% Glycerol (v/v) and PBS. A dilution series was prepared ( $10^1 - 10^{-7}$ ) under anaerobic conditions (85% N<sub>2</sub>, 10% H<sub>2</sub>, 5% CO<sub>2</sub>) (Whitley H85 hypoxystation, Don Whitley Scientific Ltd, UK) and 10 µl aliquots of the dilutions were plated on Bifidobacterium Selective Medium (BSM, Sigma Aldrich) agar and incubated for 72 hours at 37°C. Cultural counts (CFUs) were determined after incubation and CFUs per gram faeces were calculated.

## **3. Illumina sequencing of 16S rRNA gene from infant faecal bacteria**

### **3.1. Bacterial DNA extraction**

Stool samples of defined weight were thawed on ice and re-suspended in 600 µl DNA Stabilization buffer (Stratec Molecular, Berlin, Germany) and 400 µl Phenol/Chloroform/Isoamylalcohol (25:24:1; Roth, Karlsruhe, Germany). Cells were mechanically lysed (3 x 6.5 m/ sec for 40 sec) with 500 mg 0.1 mm glass beads (Roth) using a bead-beater (MP Biomedicals) fitted with a cooling adaptor. After heat treatment (95°C, 8 min, 550 rpm) and centrifugation (16,000 rpm, 5 min, 4°C), 150 µl supernatant were incubated for 30 min with 15 µg RNase (0.1 µg/ml, Amresco, Ohio) at 37°C and 550 rpm.

Bacterial DNA was extracted using the NucleoSpin® gDNA Clean-up Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Briefly, 150 µl DNA solution were mixed with Binding Buffer and vortexed 5 sec. The solution was forced through a silica membrane of a NucleoSpin® gDNA Clean-up Column by 30 sec centrifugation at 11,000 x g. The membrane was washed twice with 700 µl Washing Buffer and 30 sec centrifugation, respectively. Residuals were removed by a 1 min centrifugation step at 11,000 x g. DNA was eluted by centrifugation with 50 µl Elution Buffer for 30 sec at 11,000 x g. DNA yield and quality (A<sub>260</sub>/A<sub>280</sub> ratio) were determined using a Nanodrop® system (ND-1000, LabTech International, Brampton, ON, Canada). If not processed immediately, samples were stored at -20°C.

### **3.2. 2-step tailed PCR with barcode adaptors**

A 2-step tailed PCR method was applied to amplify the V3/V4 region of the 16S rRNA genes and to tag samples with a unique barcode adaptor. Amplification was carried out with 24 ng of extracted DNA using bacteria-specific primers 341F and 785R (240). In a first step, duplicates of the following concentrations were prepared: 4 µl Phusion® HF Buffer (Thermo Fischer Scientific, USA), 0,4 µl dNTPs (20 µM, Bioline), 0.125 µl 341 forward HTS Primer (20 µM), 0.125 µl 785 reverse HTS Primer (20 µM), 0.1 µl Phusion® High-Fidelity DNA Polymerase Hotstart (Thermo Fischer Scientific, USA), 1.5 µl 100% DMSO (Thermo Fischer Scientific, USA) and 11.75 µl PCR water (Sigma Aldrich) and 2 µl of DNA (24 ng in total). The PCR was run in a thermocycler (Biometra, Germany) by a hot start at 98°C for 30 seconds, 15 amplification cycles (98°C 5 sec, 55°C 10 sec, 72°C 10 sec) and a final extension step at 72°C for 2 minutes. The first step was done in duplicates for each DNA sample. The second step of the PCR required a master mix consisting of 10 µl Phusion® HF Buffer, 1 µl dNTPs, 0.313 µl 341 F HTS Primer (20 µM), 0.313 µl 785 R HTS Primer (20 µM), 0.2 µl Phusion® High-Fidelity DNA Polymerase Hotstart, 1.5 µl 100% DMSO and 32.487 µl PCR water. To each mix a unique barcode sequence was added (2.5 µl, 20 µM) and 2 µl 16S rRNA were added and processed in the following PCR program: 98°C 30 sec, 10 cycles (98°C 5 sec, 55°C 10 sec, 72°C 10 sec) and 2 minutes at 72°C. Amplified products were visualized in a 2% agarose gel run at 120 V for 45 minutes.

### **3.3. Purification of 16S rRNA gene amplicon**

The 16S rRNA gene amplicon was purified from primer dimers and other contaminants using AMPure XP magnetic beads (Agencourt AMPure). 1.8 fold volumes of beads were incubated with the PCR product for 5 min at room temperature and then transferred to a magnetic rack for 3 min. The clear supernatant was removed carefully and each sample was washed twice with 70% ethanol. The samples were dried for 4 min at room temperature and were finally eluted with elution buffer. Samples were run on a 2% agarose gel at 120 V for 45 min. DNA concentrations were measured by Qubit Fluorometry.

### **3.4. 16S rRNA gene amplicon sequencing analysis of infant gut microbiota**

Data were analysed as described previously (241). Raw reads were processed using an in-house developed pipeline ([www.imngs.org](http://www.imngs.org)) based on the UPARSE approach (242). Sequences were demultiplexed, trimmed to the first base with a quality score <3 and then paired. Sequences with less than 380 and more than 420 nucleotides and paired reads with an expected error >3 were excluded from the analysis. Remaining reads were trimmed by ten nucleotides on each end to avoid GC bias and nonrandom base composition. The presence of chimeras was tested using UCHIME (243). Operational taxonomic units (OTUs) were clustered at 97% sequence similarity, and only those with a relative abundance >0.5% in at least one sample were kept. Taxonomies were assigned at 80% confidence level using the RDP classifier.

### **3.5. Statistical analysis for 16S rRNA gene amplicon sequencing data**

Processed reads clustered according to 97% sequence similarity were normalized to obtain an OTU table with relative sequence abundances for every subject at the defined time point. Phylogenetic trees were constructed by using fasttree (244) which is based on a Maximum Likelihood approach generated from an OTU sequence alignment made with MUSCLE (245). Generalized UniFrac distance metrics were calculated from the normalized OTU table and the phylogenetic tree using the GUniFrac package in R, version 1.0 (246). Based on the computed distance matrix a permutational multivariate analysis of variances was applied to construct non-metric multidimensional scaling (NMDS) plots with significance of variance for each group (R vegan function). Additionally, a pairwise group comparison was generated including p-value correction for multiple comparisons according to Benjamini-Hochberg method. Shannon effective counts and species richness were calculated in R from normalized OTU matrices using OTUs with a minimum relative abundance of 0.5%. To compare genera, class, family and phyla between formula- and breast-fed, caesarean and vaginal delivery, intervention and placebo fed, an ANOVA framework was used in R with a minimum cutoff of 0.5% relative abundance. Besides the required minimum abundance only OTUs with a prevalence of at least 30% are considered. Significance

( $p < 0.05$ ) was based on the Benjamini-Hochberg corrected  $p$  value of the non-parametric Wilcoxon Rank test. To provide pairwise comparisons a Post-hoc-test (Tukey test) was applied to significant group variances.

#### **4. Strain-specific analysis by PCR**

##### **4.1. Strain-specific primer design and validation**

The genomes of *Bifidobacterium bifidum* BF3, *Bifidobacterium breve* BR3, *Bifidobacterium longum* BG7 and *Bifidobacterium longum* subsp. *infantis* BT1 were sequenced by PacBio RS II sequencer at Cell Biotech Ltd, Korea. Strain specific primer design was kindly performed by University of Alberta, Edmonton, Canada (247). Briefly, unique genes were identified by comparison to annotated genomes in the JGI database and a putative primer was designed using Primer 3 software (248). After evaluation for hairpin and dimer formation by using Netprimer (Premier Biosoft International, Palo Alto, CA), each primer set was validated in silico by a BLAST search against NCBI database. Primers were also validated experimentally by qualitative PCR using DNA from type and additional available strains. Detection limit of the primers was determined by spiking faecal samples with known cell numbers of the strain ( $10^3$ - $10^8$ ) and PCR. Table 4 shows the 4 primer pairs used for analysis.

**Table 4: Primers used for strain-specific PCR.**

Sequences of primers for strain-specific analyses including melting temperature (T<sub>m</sub>) and product size (in nucleotides nt) are shown. Moreover, the genes targeted by the primer pairs are indicated.

Bifidobacterium bifidum CBT BF3				
	Sequence (5' -> 3')	T <sub>m</sub> [C]	Product size (nt)	Target gene
Forward	TCATCAGGGGGACACCGTTC	62	187	2629604485 hypothetical protein
Reverse	AGCGAGTAGAGTGGAGGGGG			

Bifidobacterium breve CBT BR3				
	Sequence (5' -> 3')	T <sub>m</sub> [C]	Product size (nt)	Target gene
Forward	CGATAACTCACGGCATTGTG	60	225	2629607000 oligosaccharide repeat unit polymerase
Reverse	CCATAATTGCTACCGCCAGT			

Bifidobacterium longum CBT BG7				
	Sequence (5' -> 3')	T <sub>m</sub> [C]	Product size (nt)	Target gene
Forward	GATTCGCTTCCCGATTCCCT	60	237	2629608866 type III restriction enzyme
Reverse	GTTCTTCAGCATCTCGCCCT			

Bifidobacterium longum subsp. infantis CBT BT1				
	Sequence (5' -> 3')	T <sub>m</sub> [C]	Product size (nt)	Target gene
Forward	GGTCACCACTGTGCCAATCA	60	94	2629609788 hypothetical protein
Reverse	CGATGCTCGCCAAAACCTCAC			

#### 4.2. Strain-specific qualitative PCR

Qualitative PCR was performed using a T-Gradient ThermoBlock instrument (Biometra, Germany). Each PCR was performed with 20- $\mu$ l volumes using 10  $\mu$ l ready-to-use MyTaq™ Mix (Bioline, Luckenwalde, Germany), 0,4  $\mu$ l forward primer (Sigma Aldrich) , 0.4  $\mu$ l reverse primer (Sigma Aldrich) - each at a concentration of 20  $\mu$ M - and 1  $\mu$ l genomic DNA (10 ng) filled up to 20  $\mu$ l with sterile water. PCR was performed with an initial 95°C denaturation step for 5 min followed by 30 cycles of amplification (15 sec at 95°C, 15 sec at 60°C, 10 sec at 72°C) and a final elongation at 72°C for 10 min. PCR products were run on a 2% agarose gel at 120 Volt and visualized under UV light.

## 5. Metabolomic analysis

### 5.1. UHPLC-Q-ToF-MS metabolite analysis of faecal methanol extracts

100 mg faecal aliquots were frozen after collection. Further analyses were performed at the Analytical BioGeoChemistry Research Unit of the Helmholtz Institute of Munich. Correlation to microbiota data was subsequently done in cooperation with the Technical University.

Approx. 100 mg faecal aliquots were centrifuged for 60 min (4°C, 12.000 rpm) and accrued faecal water was stored at -80°C. For metabolite extraction, 50 mg pellets were homogenized with 1 mL ice cold methanol (CHROMASOLV®, for HPLC, ≥99.9%, Sigma-Aldrich) and 500 mg ceramic beads using a TissueLyser II (Qiagen). The mixture was centrifuged (4°C, 10.000 rpm; 5 min) and the supernatant was used for measurement on a Waters ACQUITY UltraPerformance LC® system (Waters GmbH, Eschborn, Germany) coupled to a Bruker Daltonics (Bremen, Germany) maXis™ quadrupole time-of-flight mass spectrometer (Q-ToF-MS). Measurements were conducted on a VisionHT C18 HL 1,5 µm (150mm x 2.0mm) (W. R. Grace & Co, Columbia, USA) in randomized duplicates within ten batches in positive electrospray ionization mode.

Gradient separation with a total runtime of 15.5 minutes (flow rate: 0.4 mL/min, column temperature: 40°C, A: 5% ACN, 0.1 % formic acid, B: 100% ACN, 0.1% formic acid) was performed of 5 µL of each sample injected in partial loop with 99.5% A and 0.5% B as starting condition. After 1.12 minutes B was increased to 99.5% within 5.3 minutes and hold for 3.6 minutes, followed by a rapid decrease to 0.5% B in 0.5 minutes and hold for 5 minutes. For quality control and for the following normalization measurement, a QC (mixture of all samples) was injected after each 10 samples. For the calibration of the MS data a segment at the beginning of the chromatogram was added, whereas 1:4 diluted ESI-L Low Concentration Tuning Mix (G1969-85000, Agilent, Waldbronn, Germany) was injected. MS parameters were as follows: mass range: m/z 50 – 1000, dry gas: 8 L/min, dry temperature: 200°C, nebulizer gas: 2 bar.

## 5.2. Pre-processing of the positive ionization mode data

For positive ionization mode, data were processed using Genedata Expressionist® Refiner MS including filtering, calibration, alignment and peak clustering steps. Batch normalization was performed with Genedata Analyst™. For further analysis and metabolite/OTU correlation, the overall and monthly data matrices were filtered by mass defect above 0.9 and a 10% cutoff for zero presence values was applied. Masses were searched against the KEGG (Kyoto Encyclopedia of Genes and Genomes) (249), HMDB (Human Metabolome Database) (250, 251) and Lipid Maps (www.lipidmaps.org) databases as M+H, M+Na and M+K adducts using homo sapiens (hsa) as reference organism using the MassTRIX web server (252, 253) with a maximum error of 0.005 Dalton. The assignment of the mass signals by using the MassTRIX web server revealed 33 % of the total amount of metabolites listed in databases, whereas the rest of 67% remains unknown. In databases known metabolites were classified to compound classes using the MassTRIX assigned compound IDs of the HMDB and Lipid Maps database.

## 5.3. Statistical evaluation of metabolite data and correlation to OTUs

Unsupervised multivariate data analysis (principal component analysis) using SIMCA P-9.0 (Umetrics, Umeå, Sweden) was done to determine the influence of feeding and age on metabolite profiles. For metabolite/OTU correlations, both datasets were merged and evaluated through an orthogonal partial least squares discriminant analysis (OPLS-DA) using SIMCA-P 13.0.1 (Umetrics, Umeå, Sweden). In order to extrapolate the mass signals and correlated OTUs, the loadings of each time point of the OPLS-DA analysis were extracted. The most important mass signals and OTUs for each time point and group were then listed by rank from high to low importance.

Cross-validation analysis of variance (CV-ANOVA) for verification of robustness of each model and indicators (p-value to prove the significance of the different models), the goodness-of-fit  $R^2(Y)$ (cum) and the goodness-of prediction  $Q^2$ (cum) were reported and read as follows: Month 1:  $R^2(Y)=0.94$ ,  $Q^2=0.48$ ,  $p=6.72 \cdot 10^{-7}$ ; Month 7:  $R^2(Y)=0.53$ ,  $Q^2=0.38$ ;  $p=1.84 \cdot 10^{-8}$ ; Month 12:  $R^2(Y)=0.4$ ,  $Q^2=0.18$ ;  $p=0.0194$ . To

determine separation of the formula groups at month 12, an additional orthogonal component had been added to the model in order to confirm that no further separation among the Y-axis could be obtained.

#### **5.4. Short chain fatty acid analysis**

Methanol faecal extracts and solutions of each standard were derivatized according to the AMP+ Mass Spectrometry Kit (Caymen Chemicals, Hamburg, Germany) instructions. A total of 88  $\mu$ L derivatized solution per sample and standard was diluted with 352  $\mu$ L of a mixture of 99:1 solvent A:B. The following analysis of the SCFA derivatives was performed on a UHPLC-Q-ToF-MS in positive electrospray ionization mode. Gradient separation – with a total runtime of 22 min plus a 2 min pre-run – of 1  $\mu$ L each takes place on a Waters BEH C8 column (1.7  $\mu$ m, 2.1mmx150mm) with A as 5 mM ammoniumacetate and 0.1% acetic acid and B with 100% ACN. Gradient separation takes place as follows: Start with 99% A for 1 min; within 16 minutes from 99% A to 1% A, hold for further 2 min. Within 0.2 min % A was increased again to 99% A and on hold for 2.8 min. The flow rate was 0.3 mL/min and column temperature was 40°C. For calibration purpose a 1:4 diluted ESI-L Low Concentration Tuning Mix (Agilent, Waldbronn, Germany) was injected at the first 0.1 min of the analysis. MS parameters were as follows: Mass range: m/z 50 – 1200, capillary: 4500 V, end plate offset: -500 V, nebulizer gas: 2.0 bar, dry gas: 8 l L/min, dry heater: 200 °C.

SCFA were quantified through external calibration based on the extracted peak areas of each standard concentration via the calculated calibration function. Therefore, adequate solutions of varying concentrations of each standard were measured as well. Retention time (RT) was extracted using DataAnalysis Version 4.1 (Bruker Daltonics GmbH, Bremen, Germany). SCFA were evaluated and quantified using QuantAnalysis Version 2.1 (Bruker Daltonics GmbH, Bremen, Germany). Kruskal-Nemenyi significance test for the multiple comparisons of mean rank sums (package: 'PMCMR' version 4.1) (254) of each SCFA in the different groups was performed with R Studio Version 0.99.489.



## 6. HPLC-FL analysis of human milk oligosaccharides

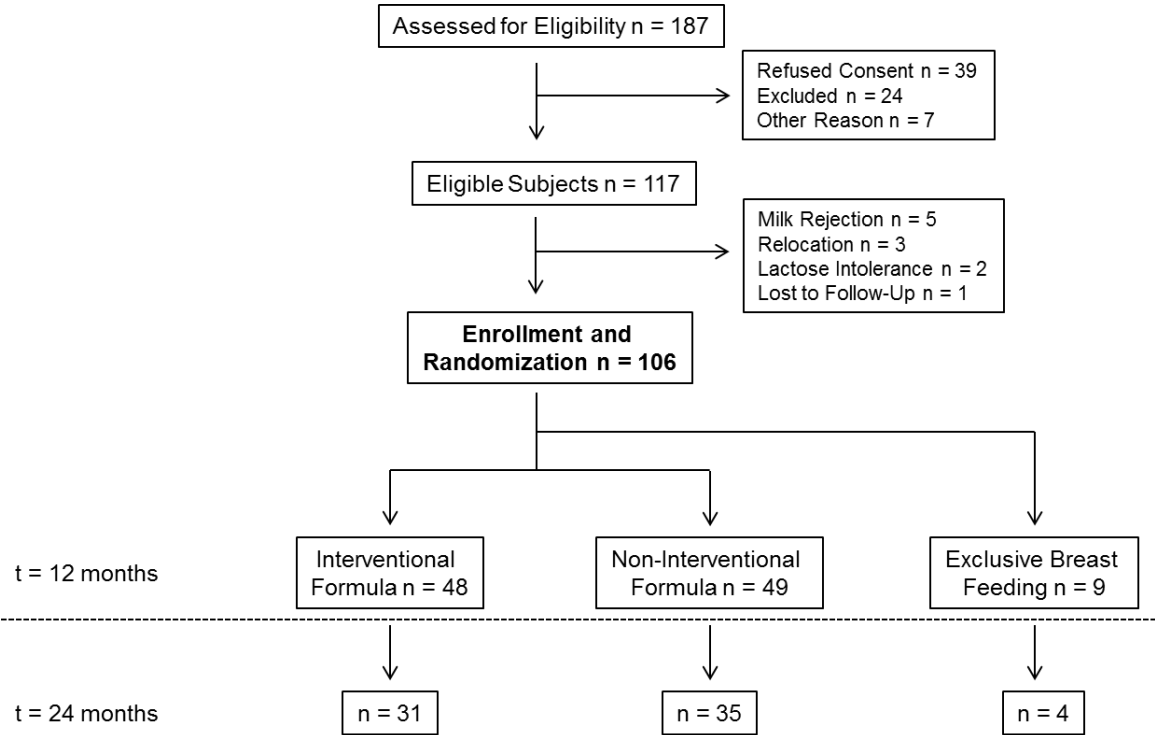
HMO analysis was performed at the University of California, San Diego, CA, USA, as previously described using high performance liquid chromatography after fluorescent derivatization (255). Raffinose was added to each milk sample as internal standard for absolute quantification. The total concentration of HMOs was calculated as the sum of the specific oligosaccharides detected. The following 16 HMOs were detected based on retention time comparison with commercial standard oligosaccharides and mass spectrometry analysis: 2'-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), 3'-sialyllactose (3'-SL), lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), lacto-N-fucopentaose (LNFP) I, LNFP II, and LNFP III, sialyl-LNT (LST) b and LSTc, difucosyl-LNT (DFLNT), disialyl-LNT (DSLNT), fucosyl-lacto-N-hexaose (FLNH), difucosyl-lacto-N-hexaose (DFLNH), fucosyl-disialyl-lacto-N-hexaose (FDSLNH) and disialyl-lacto-N-hexaose (DSLNH). Secretor status was defined by the presence of 2'FL or LNFP1.

# RESULTS

## 1. Study cohort

### 1.1. Population and baseline characteristics

A total of 187 neonates were assessed for eligibility, of which 70 were not considered for further continuation, mostly due to refusal on parental request or meeting exclusion criteria (Figure 2). Of 117 included infants, 11 (9.4%) dropped out during the study, primarily due to formula dislike (rate similar in all groups), relocation, lactose intolerance or as in one case, a motivational problem. A final number of 106 infants completed the 12-month follow-up, of which 57 were born by vaginal birth and 49 by caesarean section (however, the caesarean section rate cannot be seen as a random rate, as infants born by this mode were intentionally recruited for the trial). For the 24-month follow-up, faecal samples from 70 participants were collected. Baseline characteristics were similar in intervention, placebo and breast-fed groups (Table 5).



**Figure 2: Schematic diagram of participant enrolment and study progress.**

Participants were randomly assigned to receive either formula with ( $>1,0 \times 10^7$  cfu/g powder) (n=48) or without (n=49) bifidobacteria. Nine infants were breast-fed for the whole study period. Out of a total of 106 that completed the 12 month follow-up, 70 infants were followed up at two years of age.

**Table 5: Demographic and baseline characteristics of study participants.**

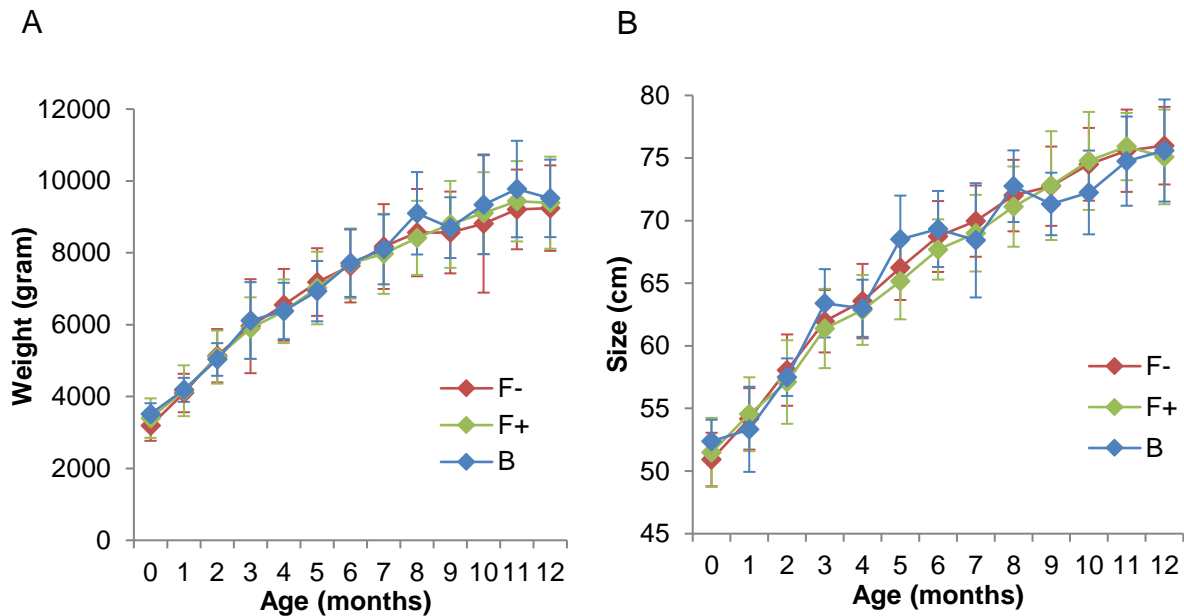
Infants were randomized into three groups based on intervention, placebo and breast-feeding. The table below lists general characteristics of the mother, delivery, feeding regimes and infantile disease patterns during the first year. The majority of infants was a nullipara and birth weight and size were similar between the groups. 78% of all infants from the intervention and placebo group were breast-fed for at least one month during the first year, while the remaining was solely formula-fed. Solid food was introduced on average after month 5. Probiotic intervention (n=3 for F+; n=6 for F-) in both formula groups was not exclusionary for study participation, but should have been prevented. No significant differences were detected between any of the groups. Values are mean (SD) or total numbers (%).

	<b>Intervention (n=48)</b>	<b>Placebo (n=49)</b>	<b>Breast Fed (n=9)</b>
<b>Maternal characteristics</b>			
BMI before pregnancy	23.6 (+/- 4.1)	22.4 (+/- 4.1)	23.3 (+/- 2.6)
Age at time of birth	31.8 (+/- 4.7)	32.4 (+/- 5)	33.2 (+/- 2.7)
Nullipara n (%)	35 (73)	41 (84)	7 (78)
<b>Birth characteristics</b>			
Gestational age (days)	279 (+/- 8)	278 (+/- 8)	281 (+/- 6)
Birth weight (g)	3399 (+/- 549)	3191 (+/- 424)	3515 (+/- 298)
Birth size (cm)	51 (+/- 3.2)	51 (+/- 2.1)	52 (+/- 1.7)
Vaginal Delivery n (%)	28 (58)	27 (55)	3 (33)
Caesarean section n (%)	20 (42)	22 (45)	6 (67)
Gender m/f	16 / 32	19 / 30	5 / 4
<b>Feeding characteristics</b>			
Breastfeeding during first year n (%)	37 (78)	38 (78)	9 (100)
<i>Mean duration (months)</i>	4.5 (+/- 3.6)	5 (+/- 3.6)	11.6 (+/- 1)
Exclusive formula feeding n (%)	11 (22)	11 (22)	0
Mean start of food intake (months)	5.2 (+/- 0.8)	5 (+/- 0.8)	5.7 (+/- 0.7)
Probiotic supplementation*	3 (6)	6 (12)	0
<b>Infantile Disease Pattern n (%)</b>			
Fever	33 (69)	24 (49)	4 (44)
Virus with diarrhoea	5 (10)	7 (14)	2(22)
Diarrhoea (non-viral)	8 (17)	8 (16)	2 (22)
Antibiotics	9 (19)	8 (16)	1 (11)
Drugs - except Antibiotics	28 (58)	17 (35)	1 (11)

\* Bigaia (*L. reuteri*) or Probiobact (8 *Lactobacillus* spp.)

## 1.2. Similar outcomes in growth and consumption measurements between the study cohorts

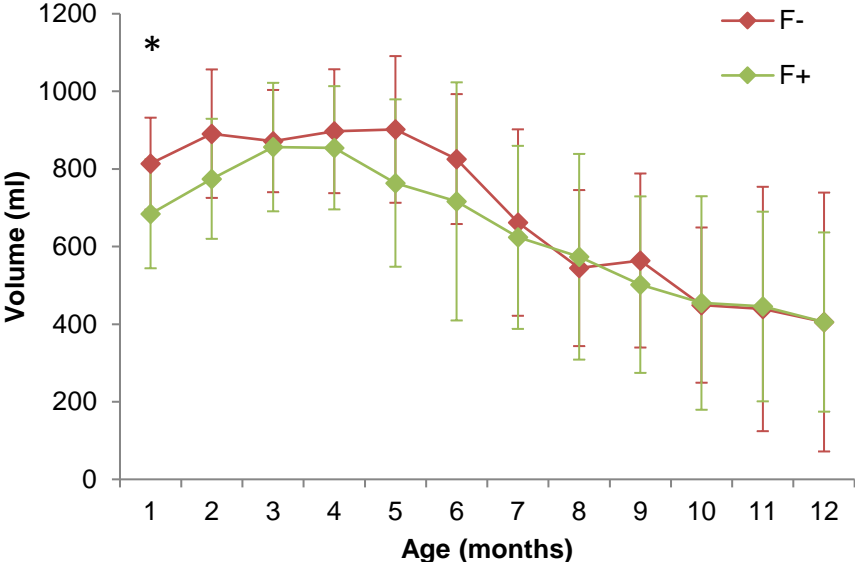
Body weight and body size were documented from birth until first year of life from all infants. Mean values and standard deviations are presented in figure 3. Differences in mean body weight and mean body size between the three main study populations, intervention (F+, n=48), placebo (F-, n=49) and breast-fed (B, n=9), were studied and found to be not significant at any time measured. The mean body weight at time of delivery was 3310 gram (considering all infants), expanding its range from 1920 to 4760 gram. Size at birth was on average 51 cm, ranging from 44 to 56 cm. At the age of one year, a mean body weight of 9326 gram (5600 – 12300 g) and a mean body size of 76 cm (66-84 cm) was measured.



**Figure 3: Mean gain in body weight and size in all breast (B), intervention (F+) and placebo (F-) groups.**

Monthly assessment of body weight (A) and size (B) revealed no significant difference between the different feeding groups from birth until first year of life.

The intervention group (n=48) was formula-fed for a mean duration of 9.1 (+/-3.1) months and received a mean volume of 393 ml/day (+/-233). The placebo group (n=49) was formula-fed for 9.3 (+/-3) months and consumed on average 406 ml/day (+/-216). The intervention cohort was introduced to solid food at the age of 5.2 months (+/-0,7), while the placebo group was confronted with complementary food at the age of 5 months (+/-0.8). Exclusively breast-fed infants were introduced to solid food at a mean age of 5.7 months (+/-0.6). Volume of daily formula uptake was comparable between the intervention and placebo groups (figure 4).



**Figure 4: Mean daily intake of intervention (F+) and placebo (F-) formula by exclusively formula-fed infants.**

Significantly more formula (n=11) was consumed by the F- group (n=11) within the first month compared to the F+ group (n=11). Volume of formula uptake reached its peak at month 3 in F+ and at month 5 in F- and declined continuously until the first year.

### 1.3. Probable effect of confounding factors on the faecal microbiota

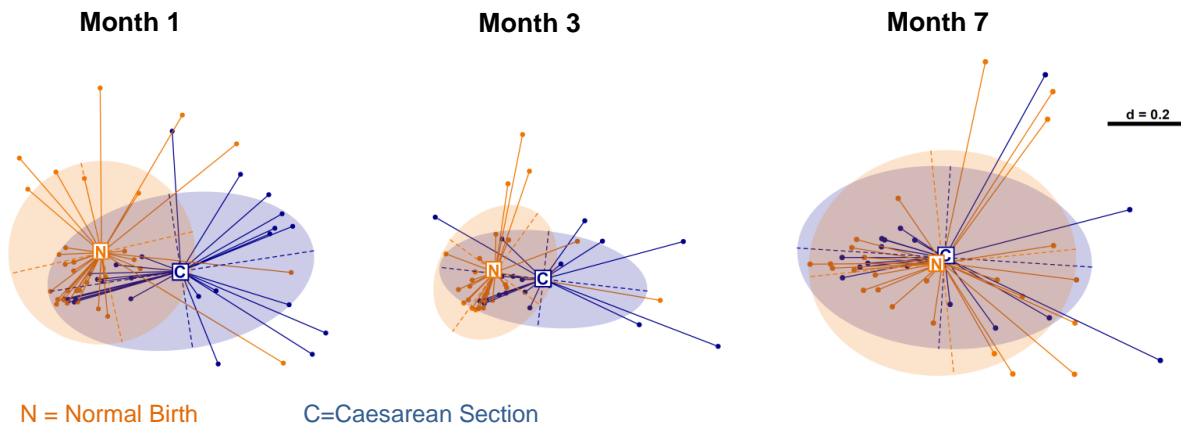
#### 1.3.1. Mode of delivery altered the microbiota temporarily

Reports on the effect of mode of delivery on microbiota development in early life have shown numerous times that the microbial succession is influenced by the type of delivery, while results on the extent and duration of the influence are conflicting (256). A recent study even revealed that no delivery effect can be detected from faecal samples of healthy newborns (257). While it was suggested, that infants born by normal birth are primarily influenced by the mother's cervical and faecal microflora, the intestinal microbiota of babies born by caesarean section seems to depend on the environment present during birth, such as the hospital and the skin from people coming into contact with the infant (258).

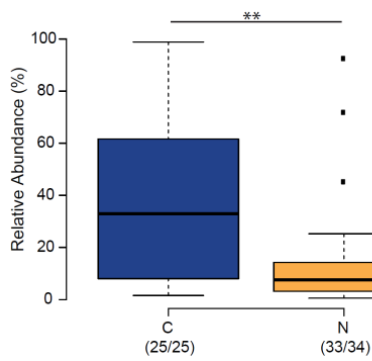
Here we analysed faecal samples from infants born by natural (N; orange) or caesarean (C; blue) birth at the age of 1 and 3 months, hereby only considering breast-fed infants in order to exclude effect of feeding. At month 1, 59 infants were analysed, and at month 3, 47 infants of that initial cohort were analysed. Moreover, at month 7, infants investigated at month 3 were compared (Figure 5).

The faecal microbiota differed significantly between infants born by caesarean section and normal birth at month 1 ( $p < 0.005$ ) and 3 ( $p < 0.005$ ) after birth. This effect, however, was not observed at month 7 ( $p = 0.91$ ), although a feeding effect cannot be excluded at month 7, as most of the infants had been weaned and solid food had been introduced at that time. A major driver for the distinct beta-diversity was the abundance of bacteria from the *Firmicutes* phylum, which were higher in caesarean deliveries.

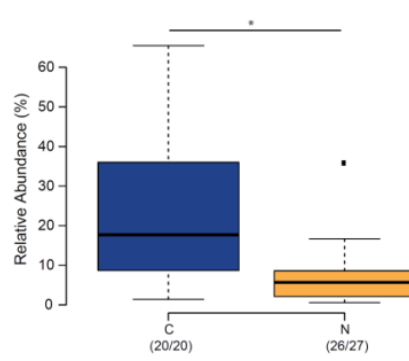
A



B



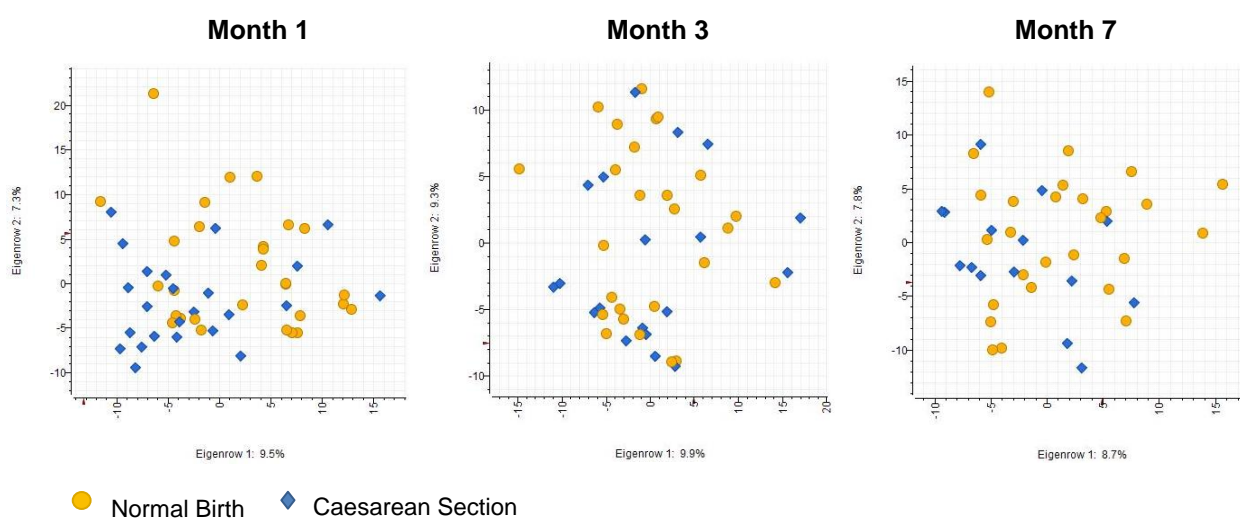
C



**Figure 5: Effect of mode of delivery on microbiota of 1-, 3- and 7 month old infants.**

The microbiota was significantly different between infants born by natural birth (N) and caesarean section at 1 and 3 months of age. At the age of 7 months, the effect diminished (A). The main difference that led to the distinct microbiota pattern was attributed to shifts in the Firmicutes abundances in caesarean section births, mainly represented by *Clostridium* spp. This effect was observed at month 1 (B) and persisted up to month 3 (C), but was not observed later on.

To further assess the influence of mode of delivery on metabolite profiles, unsupervised multivariate data analysis (principal component analysis) was performed using faecal extracts from exclusively breast-fed babies, either born by vaginal or caesarean section birth mode (figure 6). Also like with 16S data, months 1, 3 and 7 were analysed. In contrast to the differences observed for 16S rRNA data, UHPLC MS data revealed no significance at any time point measured.



**Figure 6: Principal component analysis of metabolites from infant faecal samples to determine the effect of mode of delivery at 1, 3 and 7 months of age.**

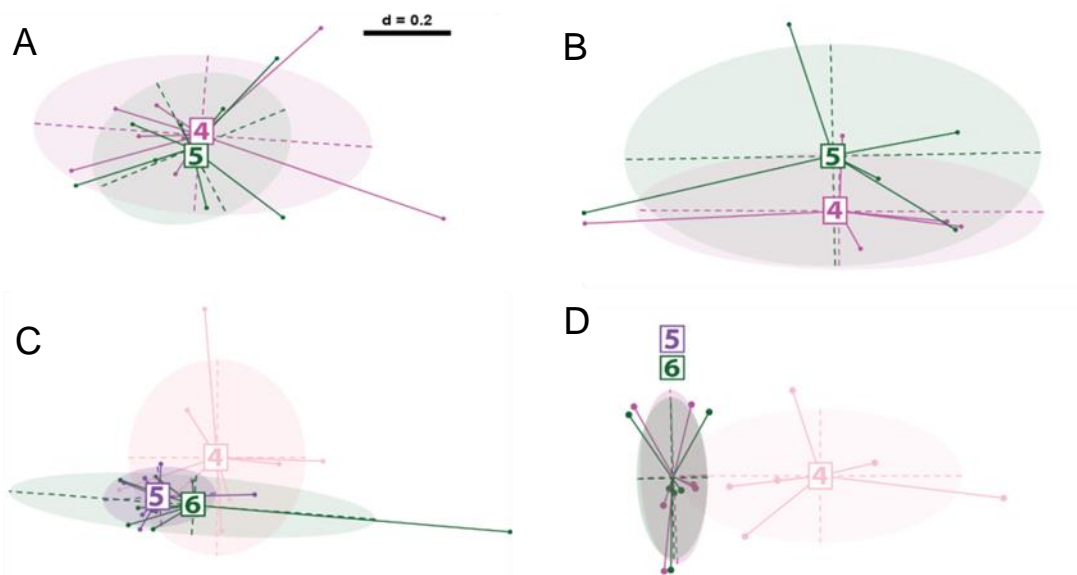
Overall metabolite profile of faecal samples taken 1, 3 and 7 months after delivery from 53, 45 and 42 infants, respectively. All infants of the analysis had been exclusively breast-fed at months 1 and 3. At month 7, the same infants as analysed at month 3, were used. Mode of delivery did not affect the overall metabolite profile at any of the measured time points.



### 1.3.2. Solid food did not alter the microbiota shortly after introduction

The introduction of complementary food is one of the major determinants for the development of the infant microbiota. There is strong evidence, that complementary foods drive numerous processes in the developing gut, including shifts in Bacteroidetes, and bifidobacteria abundances and elevated faecal SCFA levels (113, 114).

To investigate the influence of solid food introduction, infants that were breast-fed at least for the first six months were considered. First, it was analysed if the microbiota changed upon an introduction after month 4, thereby comparing month 4 to month 5. Second, it was investigated if a microbial change occurs between month 4, 5 and 6 upon introduction after month 5. Both analyses revealed that no significant change in microbiota *beta*-diversity occurred after introduction of complementary food (Figure 7).



**Figure 7: Introduction of solid food did not affect the *beta*-diversity of breast-fed infants.**

16S rRNA analysis of faeces from infants before and after introduction of solid food revealed no significant change of the overall microbiota composition. Independent of mode of delivery (A, C: normal birth; B, D: caesarean section), infants that were switched from exclusive breast-feeding to solid food parallel to breast-feeding after month 4 were not different in microbiota composition before food uptake (A:  $p=0.9$  and B:  $p=0.8$ ). Also, when food was introduced after month 5, no significance was detected in comparison to month 4 and 5 (C:  $p=1.0$  and D:  $p=0.9$ ).

### 1.3.3. Early life administration of antibiotics was not a major determinant for microbiota development

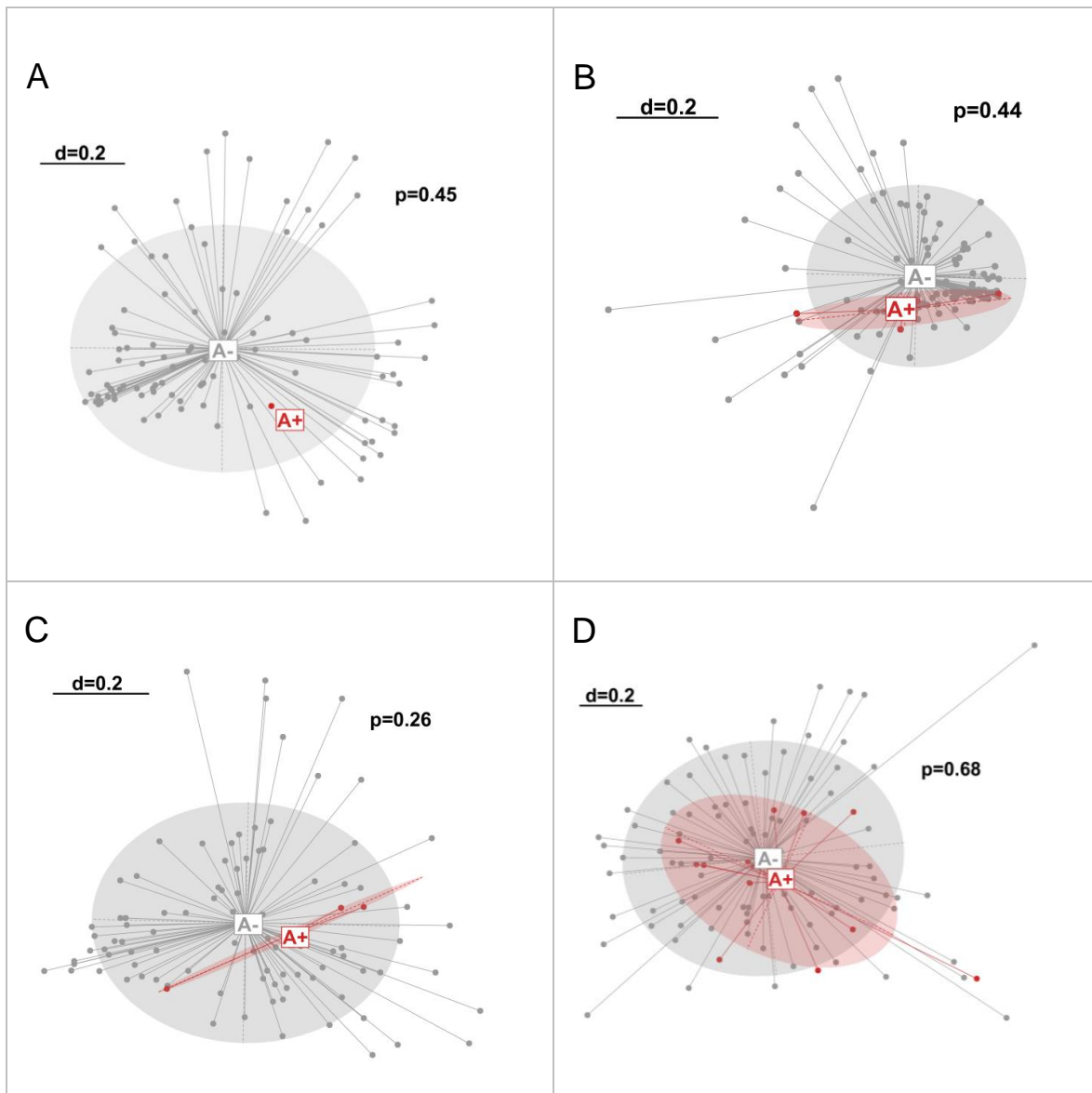
From birth until first year, 18 infants (17%) were subject to antibiotic treatment, including 8 infants (16%) from the placebo group, 9 infants (19%) from the intervention group and one infant (11%) from the exclusively breast-fed group (Table 6). Predominantly, amoxicillin was the antibiotic of choice (30% of all cases). Infants that were treated with antibiotics were compared to all infants not treated with antibiotics of the same age. In addition, each antibiotic-treated infant was compared to its feeding cohort (data not shown). In either case, antibiotic treatment did not significantly affect microbial communities (Figure 8).

**Table 6: Infants subject to antibiotic treatment within the first year of life.**

Summary of antibiotic treatments including type of antibiotic, administered age and duration, and the feeding applied at that time.

ID	Disease	Antibiotic	Age of Administration (months)	Duration of Administration (days)	Feeding at Time of Administration
91	Newborn Infection	Cefotaxim	1	7	B
63	Ophthalmitis	Penicillin	2	6	B
76	Rotaviral Infection	Amoxicillin	2	4	F-
29	Urinary Tract Infection	Amoxicillin	3	7	F-
77	Conjunctivitis	Floxal	3	5	B
67	Otitis Media	Unacid	4	7	F-
46	Bronchitis	Penicillin	5	7	F+
90	Otitis Media	Amoxicillin	7	10	B
98	Respiratory Infection	Roxihexal	8	3	F-
66	Conjunctivitis	Floxal	8	5	B
101	Tonsillitis	Cefpodoxim	9	5	F+
66	Bronchitis	Cefaclor	9	7	B
56	Otitis Media	Amoxicillin	10	7	F+
63	Bronchitis	Penicillin	10	5	F-
90	Otitis Media	Amoxicillin	11	10	F+
63	Conjunctivitis	Floxal	11	6	F-
67	Bronchitis	Penicillin	11	2	B
3	Bronchitis	Cefaclor	12	5	F+
44	Bronchitis	Amoxicillin	12	2	F+
72	Conjunctivitis	Floxal	12	2	F+
78	Otitis Media	Amoxicillin	12	7	F+
130	Cold	Cefaclor	12	10	F+
30	Cold	Cefaclor	12	6	F-

B: Breast-Fed; F+: Intervention Formula-Fed; F-: Placebo Formula-Fed



**Figure 8: The administration of antibiotics had no immediate effect on the overall faecal microbiota of 1 (A), 3 (B), 7 (C) and 12 (D) month old infants.**

Within the first month, one infant was subject to antibiotics (A) and showed no difference in beta-diversity to the other infants. The microbiota at month 3 (B) was not significantly altered within four infants that had previously had antibiotics. Same was observed at month 7 (C) and at month 12 (D). Also, no difference was observed when comparing antibiotics-treated infants explicitly to their corresponding feeding cohort.

Further possible confounding factors, like maternal age ( $p=0.89$ ), maternal antibiotic intake during the first seven months of pregnancy ( $p=0.31$ ), administration of supplemented probiotic bacteria to the infants (e.g. Probiobact, Bigaia) ( $p=0.7$ ), parental allergies ( $p=0.34$ ), nullipara ( $p=0.38$ ) and infant sex ( $p=0.11$ ) had no

significant effect on the development of the infant microbiota of breast-fed infants after birth (data not shown).

## **2. 16S rRNA gene amplicon analysis in the total study cohort**

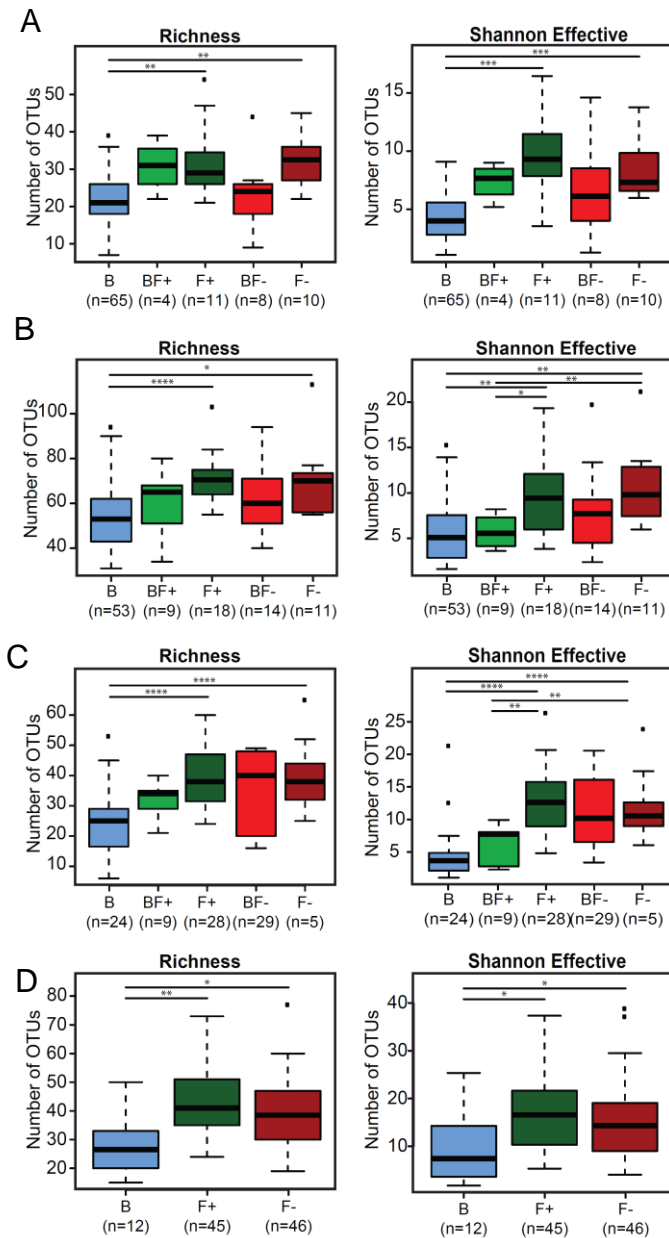
Based on the type of feeding, all infants (n=106) were grouped into five feeding groups: breast-fed (B), intervention (F+), placebo (F-) and mixed (B with F+ and B with F-) fed. All infants were assigned to one of the groups depending on feeding mode at the corresponding month. Exclusively fed infants were in the same group for the whole study period.

In order to process the vast number of samples collected during the study, sequence analysis was reduced to four time points – months 1, 3, 7 and 12. The time points were selected based on inclusion of a start (month 1) and end (month 12) point and two time points in between – month 3, intended to select more stable microbiota after birth, and month 7, intended to select a time point after introduction of solid food.

For 16S rRNA gene amplicon analysis, genomic DNA from infant faecal samples was extracted and sequenced by Illumina sequencing method. For *alpha*- and *beta*-diversity, an OTU table with normalized 97% sequence similarity clusters served as basis in order to obtain generalized UniFrac distance metrics. Permutational multivariate analysis of variances was applied to construct non-metric multidimensional scaling (NMDS) plots with a significance of variance for each group. Significance ( $p < 0.05$ ) was based on the Benjamini-Hochberg corrected p value of the non-parametric Wilcoxon Rank test. A post-hoc test for pairwise comparison was applied to determine significances between the different feeding groups.

## 2.1. Microbial *alpha*-diversity discriminated formula-fed infants significantly from breast-fed infants throughout the first year

The alpha-diversity describes the richness and evenness of the distribution of species within an ecosystem. Figure 9 represents the bacterial richness and Shannon effective counts for all five feeding groups from birth until 12 months.

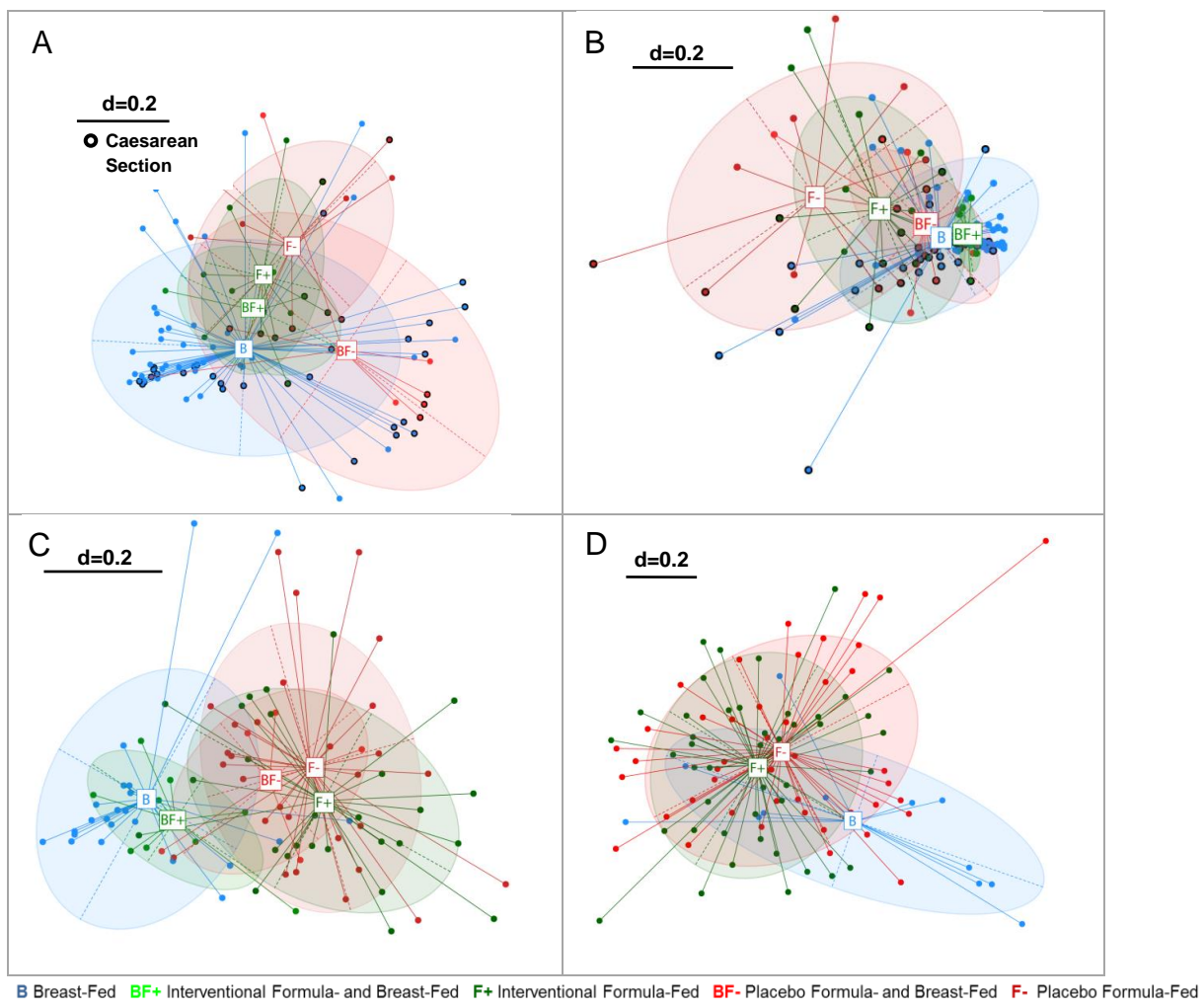


**Figure 9: Bacterial richness and Shannon effective counts from 1- (A), 3- (B), 7- (C) and 12- (D) month old infants fed with breast milk, (intervention/ placebo) formula or a mixture thereof.**

Bacterial richness and Shannon effective diversity boxplots display decreased numbers of molecular species in breast-fed infants when compared to the formula cohorts. While we observed no difference in the bacterial richness or Shannon diversity between intervention and placebo formula-fed microbiota, the intervention mixed fed microbiota resembled the breast-fed intestinal ecosystem, as observed at month 3 and 7. This, however, was probably due to lower formula intake in the intervention group. \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ , \*\*\*\* $p < 0.00005$

## 2.2. No major findings were observed in *beta*-diversity between intervention and placebo groups

In order to assess the effect of an intervention with bifidobacteria on the overall microbiota pattern, the *beta*-diversity was determined for all feeding groups throughout the first year. Generalized UniFrac distance metrics were calculated from normalized OTU tables and phylogenetic trees, presented by multidimensional scaling plots (Figure 10).



**Figure 10: Differences in the faecal microbial communities in 1- (A), 3- (B), 7- (C) and 12- (D) month old infants.**

NMDS plots of phylogenetic distances including all infants at the age of 1, 3, 7 and 12 months. At month 1, breast-fed infants (B, blue) developed a significantly different microbiota than both - exclusively (F-, red) and mixed (BF-, lightred) placebo groups, while both intervention groups (F+, green, BF+, lightgreen) show a microbiota that was more similar to the breast-fed microbiota. At months 3, 7 and 12, both exclusively formula-fed groups dissociated from breast-feds. At 7 months, the intervention mixed group still resembles the exclusively breast-fed cohort – an effect that was probably enhanced by the lower formula intake in that group.

The breast-fed microbiota differed significantly from the formula-fed microbiota with regard to *alpha*- and *beta*-diversity throughout the first year of life. A major characteristic was a lower bacterial richness and diversity in exclusively breast-fed infants (Figure 9 A-D). Notably, formula-feeding in parallel to breast-feeding shaped the microbiota in a way that was characterized by features from the breast- and formula-fed microbiota. For example, Shannon effective at month 1 was four counts for breast-feds, 7.4 counts for mixed feds and 9 counts for exclusively formula-fed infants.

When considering all five feeding groups, the biggest difference in *beta*-diversity was observed between breast and placebo groups ( $p=0.01$ ), and also between breast and breast/placebo groups in combination (parallel breast and formula feeding) ( $p=0.02$ ) at month 1 (Figure 10 A). No significance, however, was observed between breast and intervention groups ( $p=0.07$  between breast and intervention;  $p=0.41$  between breast and breast/intervention). No major difference was seen between intervention and placebo groups at any measured time point (Figure 10 A-D). At month 3 and 7, formula-fed infants developed a microbiota that was significantly different in composition from breast-fed infants (Figure 10 B and C). At month 7, the breast/intervention group seemed to resemble the breast-fed microbiota, but results were affected by higher breast milk consumption in this group (data not shown). At the age of one year, the breast-fed microbiota differed from the intervention and placebo fed microbiota ( $p=0.002$  for intervention and  $p=0.006$  for placebo) (Figure 10 D).

### 2.3. Breast-feeding promoted *Bifidobacteriaceae* growth

To further assess the cause for the distinct *alpha*- and *beta*-diversity observed between the five feeding groups, investigations on the faecal bacterial composition were done. Phylum and family abundances are shown for all groups in figures 11 and 12.

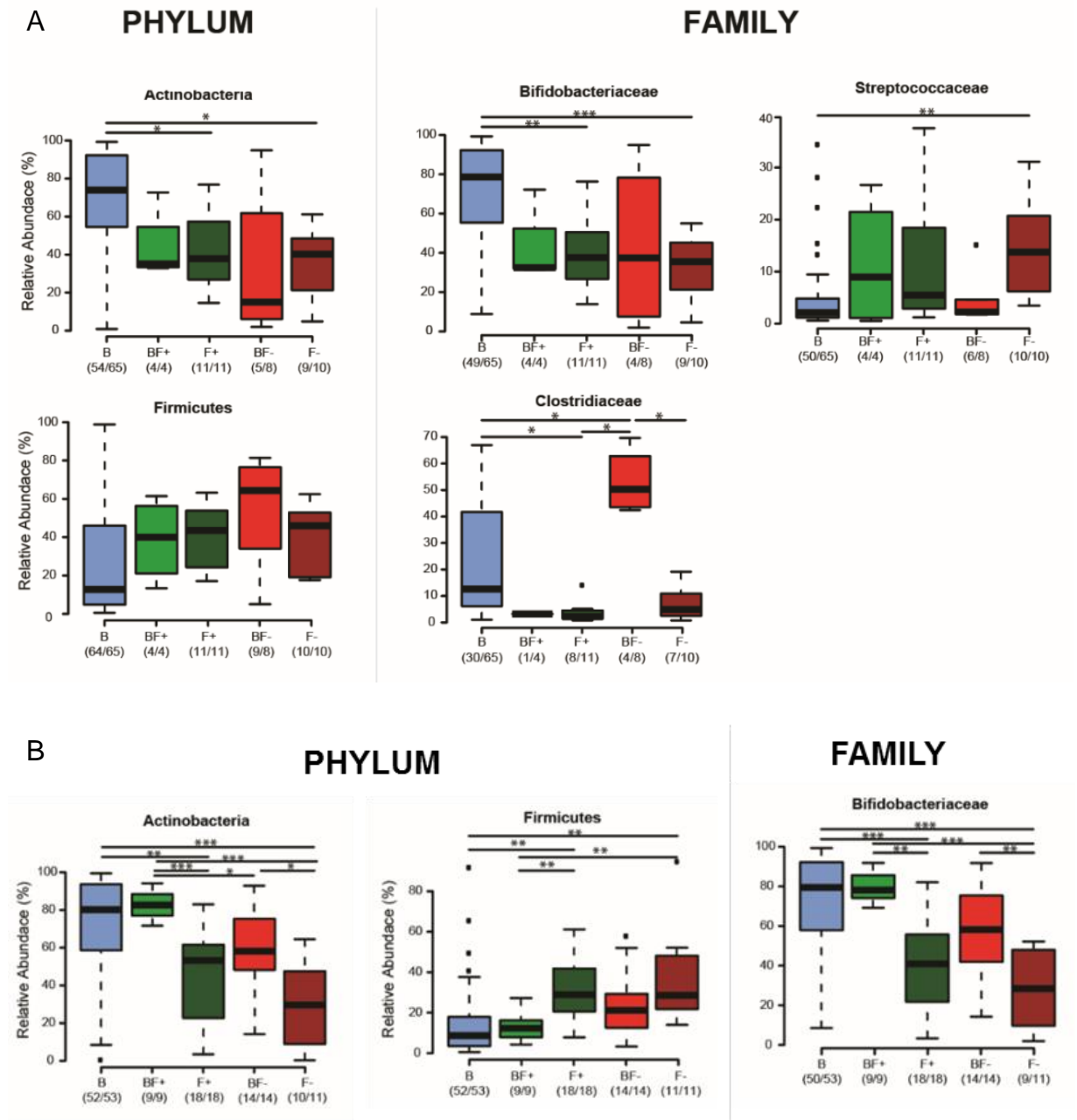
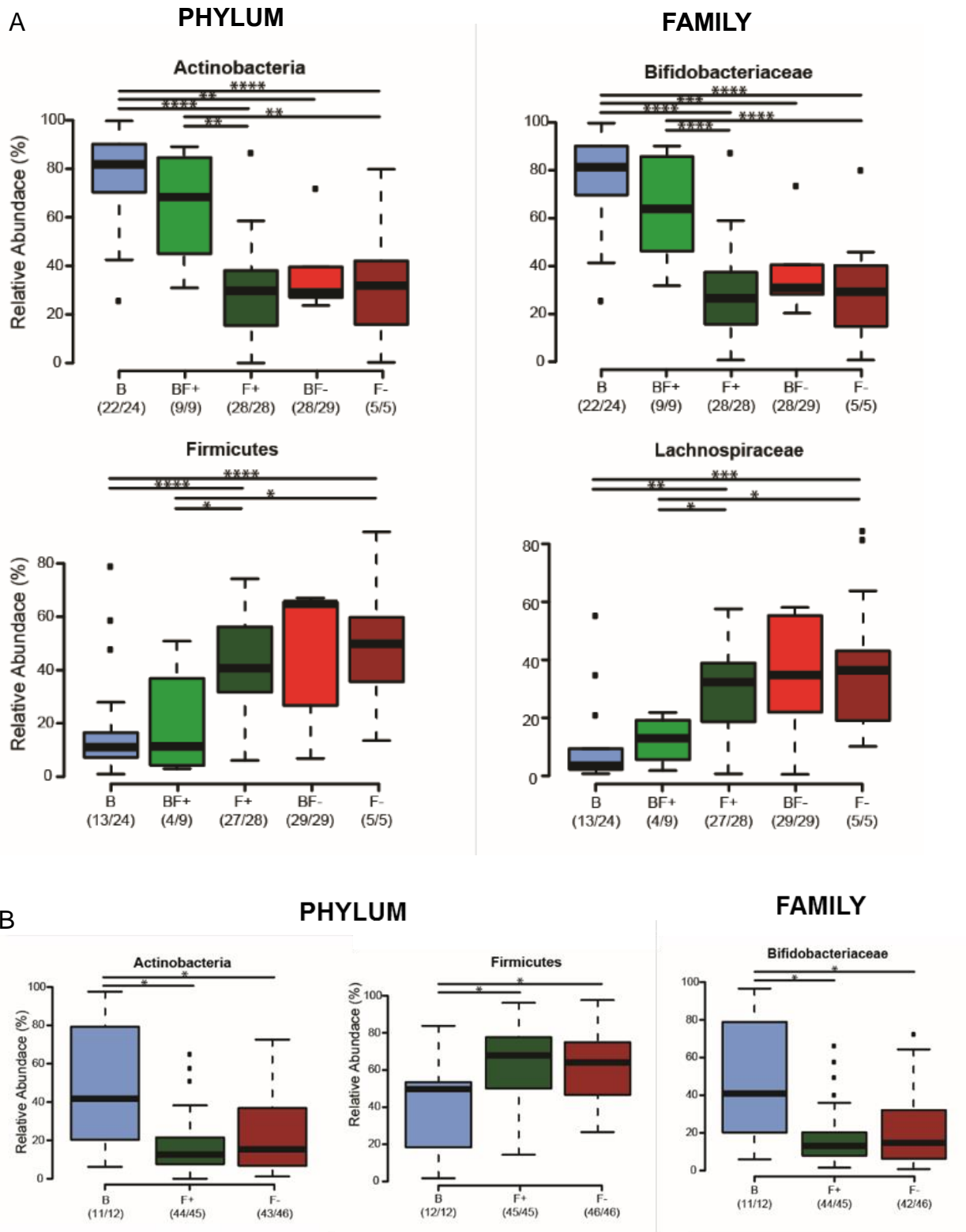


Figure 11: Most significant phyla and families in the total cohort at months 1 and 3.

Actinobacteria, mainly represented by *Bifidobacteriaceae*, dominated the microbiota of breast-fed infants at month 1 (A) and 3 (B), and were significantly higher than in formula-fed groups. Firmicutes were initially not higher in formula-fed infants, but increased with advanced age. *Streptococcaceae* was characteristic for formula feeding, particularly at month 1, while *Clostridiaceae* was enriched in the placebo group and breast-fed infants after delivery. \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ , \*\*\*\* $p < 0.00005$

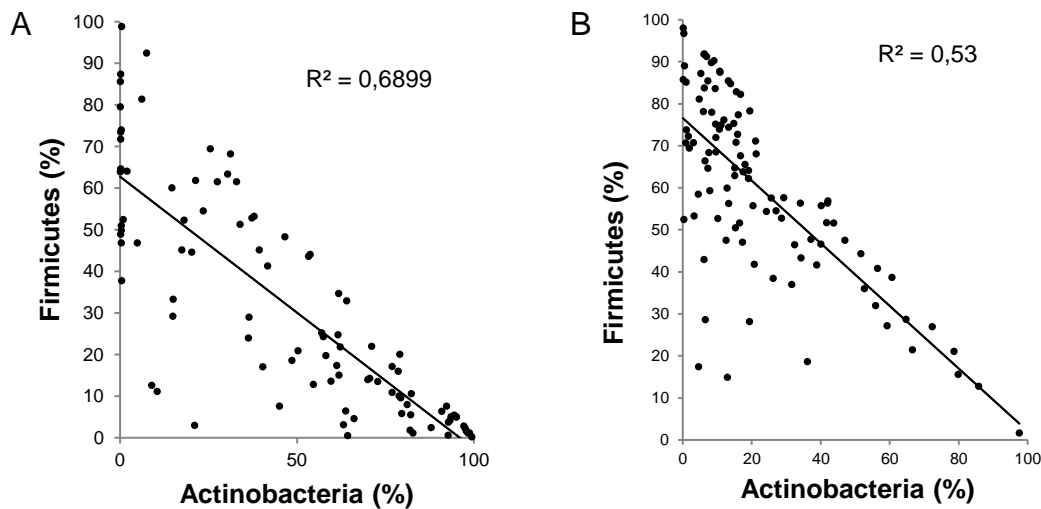




**Figure 12: Most significant phyla and families in the total cohort at months 7 and 12.**

Similarly, as observed during the initial months, Actinobacteria remained the most dominant phylum in the faecal microbiota of breast-fed infants at month 7 (A), while abundance was strongly reduced from then on, reaching only half of it at month 12 (B), but still significantly more than in formula-fed infants. Firmicutes were still higher in formula-

feds, with *Lachnospiraceae* becoming its most popular representative, particularly in placebo formula-fed infants.  
 \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ , \*\*\*\* $p < 0.00005$



**Figure 13: Correlation of Actinobacteria and Firmicutes at months 1 and 12.**

Actinobacteria dominated the breast-fed microbiota from birth until month 7, while the formula-fed microbiota was characterized by a weighted Actinobacteria/Firmicutes balance. The dominance of Actinobacteria consequently limited Firmicutes growth in breast-fed cohorts, while a reduction in Actinobacteria was accompanied by an increased Firmicutes abundance. At a later stage this increase was mainly due to higher amount of *Lachnospiraceae*.

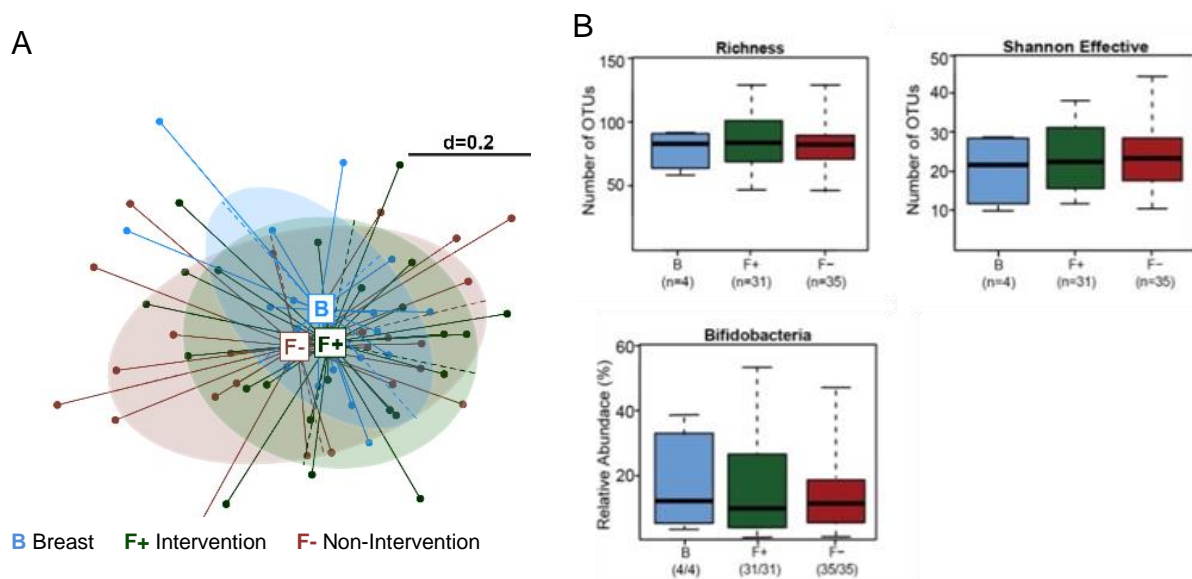
Members of the *Bifidobacteriaceae* family clearly dominated the microbiota of breast-fed infants (Figures 11 and 12). Their strong presence appeared until month 7 and was sharply reduced afterwards.

On the contrary, the formula-fed microbiota was not dominated by a single phylum, but was constituted of a rather mixed panel of representatives from the Actinobacterium and Firmicutes phyla. The placebo group was characterized by elevated *Clostridiaceae* and *Streptococcaceae* abundances during the first month, but levels evened out later on. At month 7, *Lachnospiraceae* increased remarkably in formula-feds, while levels still remained low in breast-fed infants.

The heterogeneous character in formula-fed infants persisted from birth until first year – a characteristic that was already seen by the higher *alpha*-diversity when compared to breast-fed infants.

## 2.4. There was no long-term effect of the initial feeding pattern on the microbiota at the age of two years

70 infants were analysed at the age of two years in order to determine if initial feeding habits affect microbial communities at a later stage. Infants were grouped into three groups, based on the duration of breast- or formula-feeding during the first 12 months of life. Infants that had been breast-fed for at least 8 months were classified as “B” (breast-fed, blue), while infants that had been fed with intervention (dark red) or placebo (green) formula for at least 8 months, are shown as “F+” and “F-“, respectively.



**Figure 14: Alpha- and beta-diversity of breast-fed, intervention and placebo formula-fed infants at the age of two years.**

(A) Meta NMDS plots of infants at the age of two years, grouped according to initial feeding regime. Infants that had been primarily (at least 8 months in the first year) breast- or formula-fed were grouped into one of the three groups. (B) Richness, Shannon Diversity and Bifidobacterium relative abundance were not different between breast- and intervention or placebo formula-fed infants at the age of two years.

No significances were detected between breast- and formula-fed children, and no difference was observed between intervention and placebo formula-fed children (Figure 14 A). Richness was found to comprise around 80 OTUs per infant with no differences among the groups (Figure 14 B). Also, Shannon effective counts and bifidobacteria abundance did not differ between the groups.

### **3. Microbiota and metabolite analyses in a selected sub-cohort**

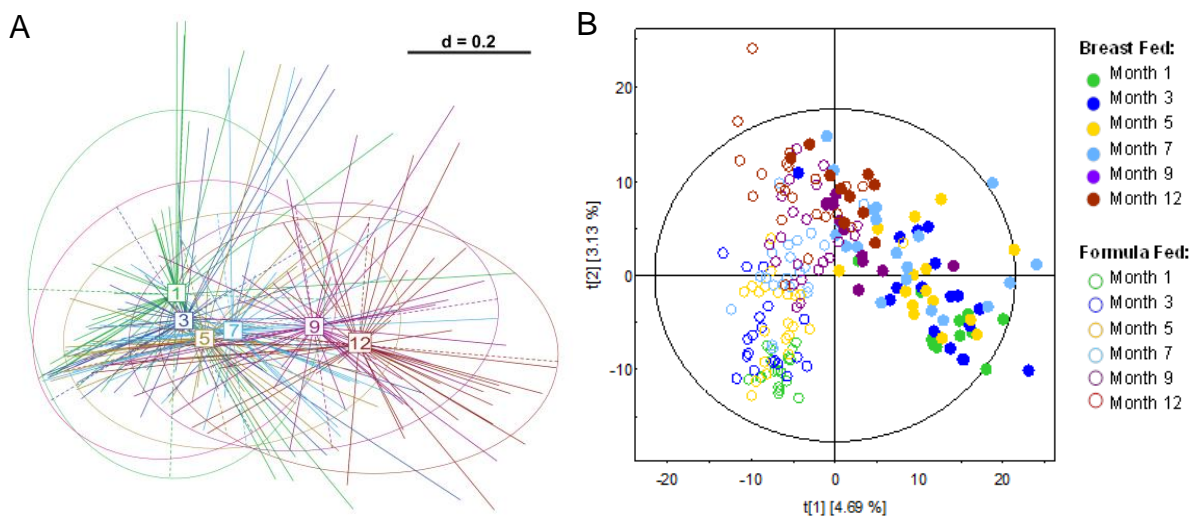
Real-time analysis of metabolites derived from gut microbiota is essential for understanding the metabolic functions of the gut microbiome and enables the characterization of metabolic footprints of mammalian hosts that correlate with the microbial community in the intestinal tract (259).

While the metabolomics field is increasingly becoming explored, there is still a tremendous number of unknown metabolites that require identification and functional assignment. In the pediatric field, a correlation was found between faecal microbiota dynamics and changes in metabolic pathways, with OTUs characterized by highly specialized metabolic functions that are triggered by milk nutrient digestion, microbial colonization, and host physiology (260).

In this study, microbiota data were correlated to UHPLC metabolite results in order to assess their inter-relationship during the infant phase. The majority of infants was breast-fed after birth, followed by formula-feeding at a mean age of five months. A sub-cohort of infants, however, was either exclusively breast-fed until month 7 (n=11) or even up to month 12 (n=9), or exclusively formula-fed (F+: n=11; F-: n=11) for 12 months. These cohorts were studied in more detail in order to address specific questions with regard to the effect of early life bifidobacteria intervention in comparison to placebo and breast-feeding.

### 3.1. The microbiota and metabolite profile continuously dissociated from the initial pattern

In a first step, the microbiota and metabolite dynamics from birth until one year of age was analysed from all infants of the sub-cohort. Six consecutive time points were selected and are presented in Figure 16 for the microbiota (A) and metabolites (B).



**Figure 15: OTU and metabolite dynamics from birth until first year of life.**

(A) MetaMDS plot of the microbiota of exclusively (breast and formula) fed infants at months 1, 3, 5, 7, 9 and 12. The microbiota developed over the first year by an increasing dissociation from the initial months. (B) The metabolite profile of exclusively breast- (filled circles) and formula- (empty circles) fed infants from birth until the first year shows that – similarly to the microbiota development – the metabolite profile changed with age, and resembled increasingly until the formula- and breast-fed groups merged at the age of 9 months.

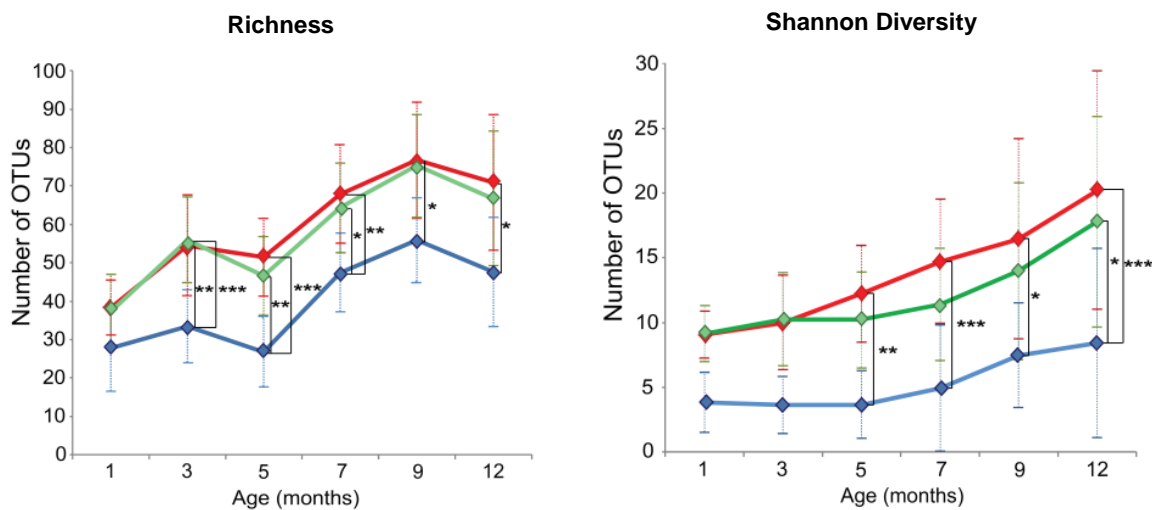
The overall faecal microbiota of infants fed either exclusively by formula or breast for the first year was characterized by a constant change, while with advanced age its composition continuously dissociated from the initial pattern (Figure 16 A). This was observed in all three feeding groups (data not shown). Microbiota change between two consecutive time points was small, but change from the initial (month 1 and 3) to later time points (month 9 and 12) was significant in breast- and formula-fed infants ( $p < 0.05$ ). Metabolite development was comparable to microbiota development, with a continuous compositional change from month 1 to 12 (Figure 16 B). Moreover, a convergence of initially different breast and formula groups was observed around month 9.

### 3.2. Bacterial richness and diversity increased constantly without significant differences between the exclusive formula groups

What drove microbiota change during the progression of its development was an alteration in bacterial richness and diversity, as observed in the total study cohort, where an increased alpha-diversity positively correlated with advanced age. Here, we compared the microbial richness and biodiversity for the three exclusively breast- and formula-fed groups. Assessments were done at month 1, 3, 5, 7, 9 and 12.

As observed in the total study cohort, we found that bacterial richness increased from month 1 to 3, particularly in the formula groups. From month 5 on richness further increased remarkably until the first year (Figure 17).

Shannon effective counts increased over time, but only marginally in the breast-fed group and more in the formula, particularly in the placebo group, which was significantly higher in OTU counts than the breast-fed group from month 5 to 12 (Figure 17).



**Figure 16: Bacterial richness and Shannon effective counts during the first year in exclusively breast-fed, intervention and placebo formula-fed infants.**

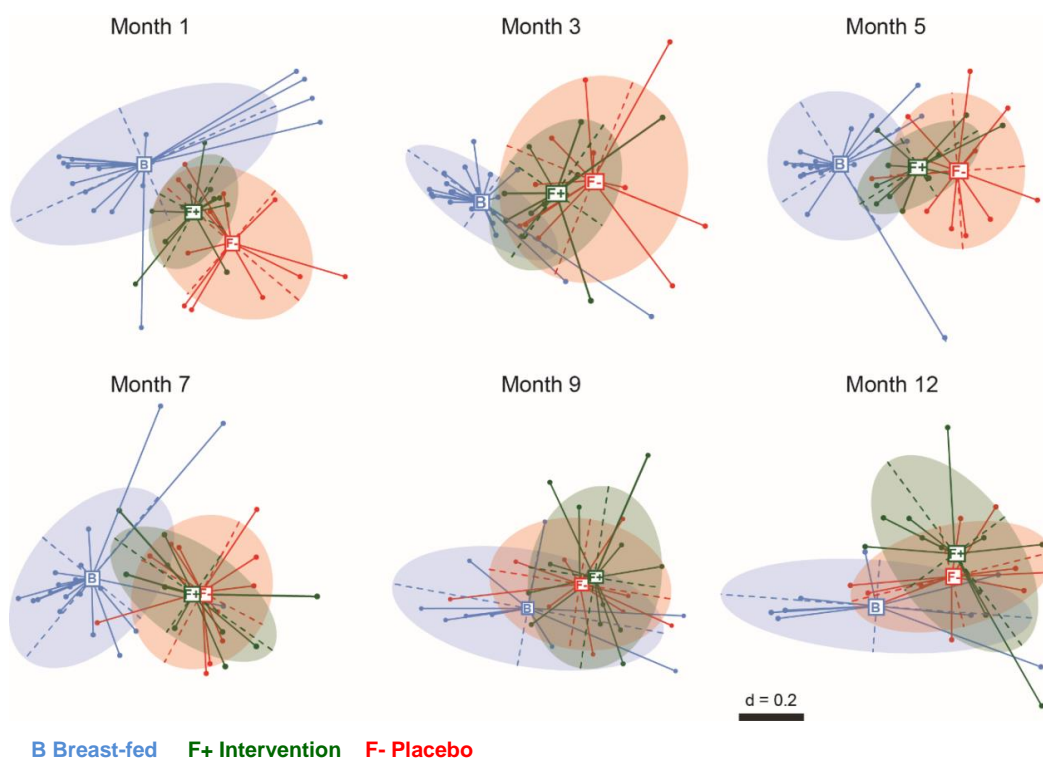
Bacterial richness increased constantly with advanced age and was significantly lower in the breast-fed group (blue) compared to both formula groups (A). The intervention group (green) was lower in richness than the placebo group (red), but not significantly. Shannon effective counts were lower in breast-fed infants than in placebo-fed infants from month 5 on, but not when compared to intervention-fed infants. Diversity also increased over time.

\* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ , \*\*\*\* $p < 0.00005$

### 3.3. The microbiota during the first year is significantly influenced by the type of feeding, but independent of supplementation

*Beta*-diversity analysis was assessed for all exclusively fed infants at months 1, 3, 5, 7, 9 and 12. NMDS plots were generated from phylogenetic trees and OTU tables and pairwise comparison was done by a post-hoc-test (Tukey test) applied to significant group variances.

We show that the intestinal ecosystem fed by breast milk develops significantly different compared to both formula groups from month 1 to 12 (months 1-9:  $p < 0.005$ , month 12:  $p < 0.05$ ) (Figure 18). No difference was observed between intervention and placebo groups at any time point.



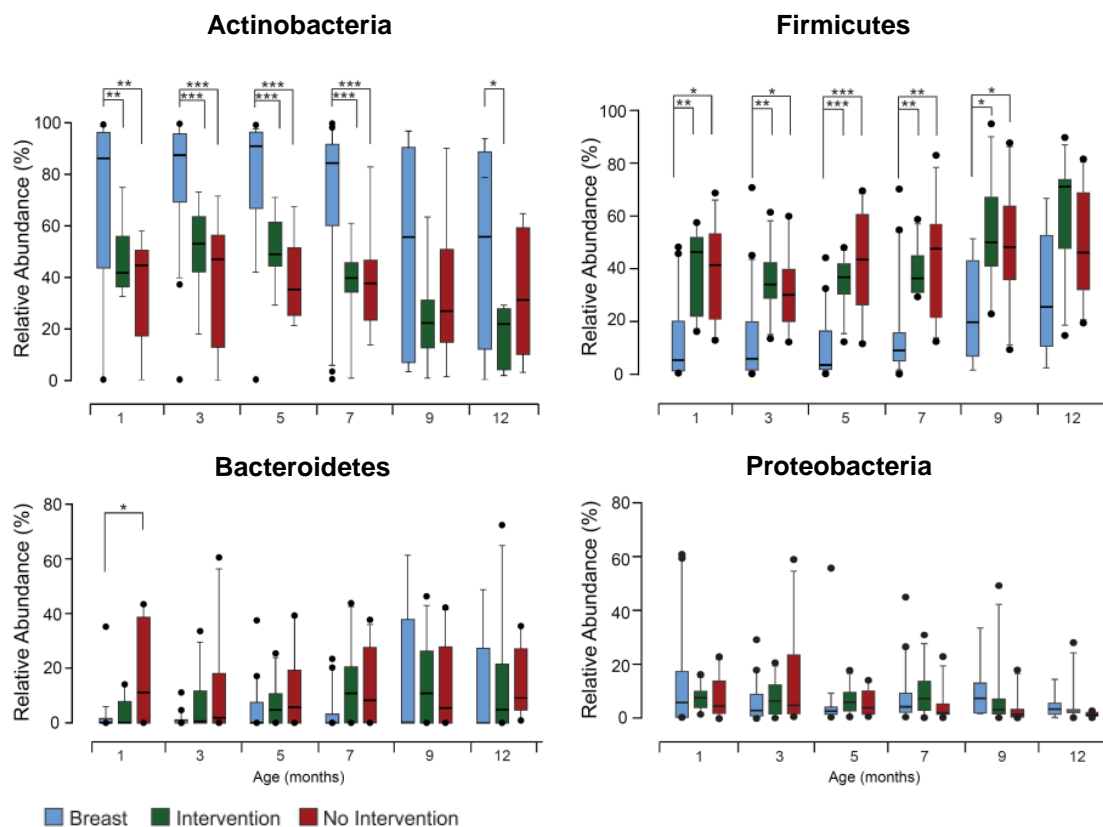
**Figure 17: An intervention with bifidobacteria did not influence the *beta*-diversity during the first year.**

Intervention (green) and placebo (red) groups did not differ in beta-diversity at any time point measured during the first year. The breast-fed control group (blue), however, was different from both formula groups from month 1 to 7, but not at a later time point. (months 1-9:  $p < 0.005$ ; month 12:  $p < 0.05$ ).

### 3.4. Comparison of Bacterial Abundances in the Three Feeding Groups

*Beta*-diversity of the three groups revealed that the microbiota observed in breast-fed infants differs remarkably from the microbiota observed in formula-fed infants. To investigate the drivers for this different colonization, phylum, family and OTU abundances were determined and compared between the groups at months 1, 3, 5, 7, 9 and 12.

#### 3.4.1. The formula-fed microbiota was characterized by a balanced distribution of Actinobacteria and Firmicutes



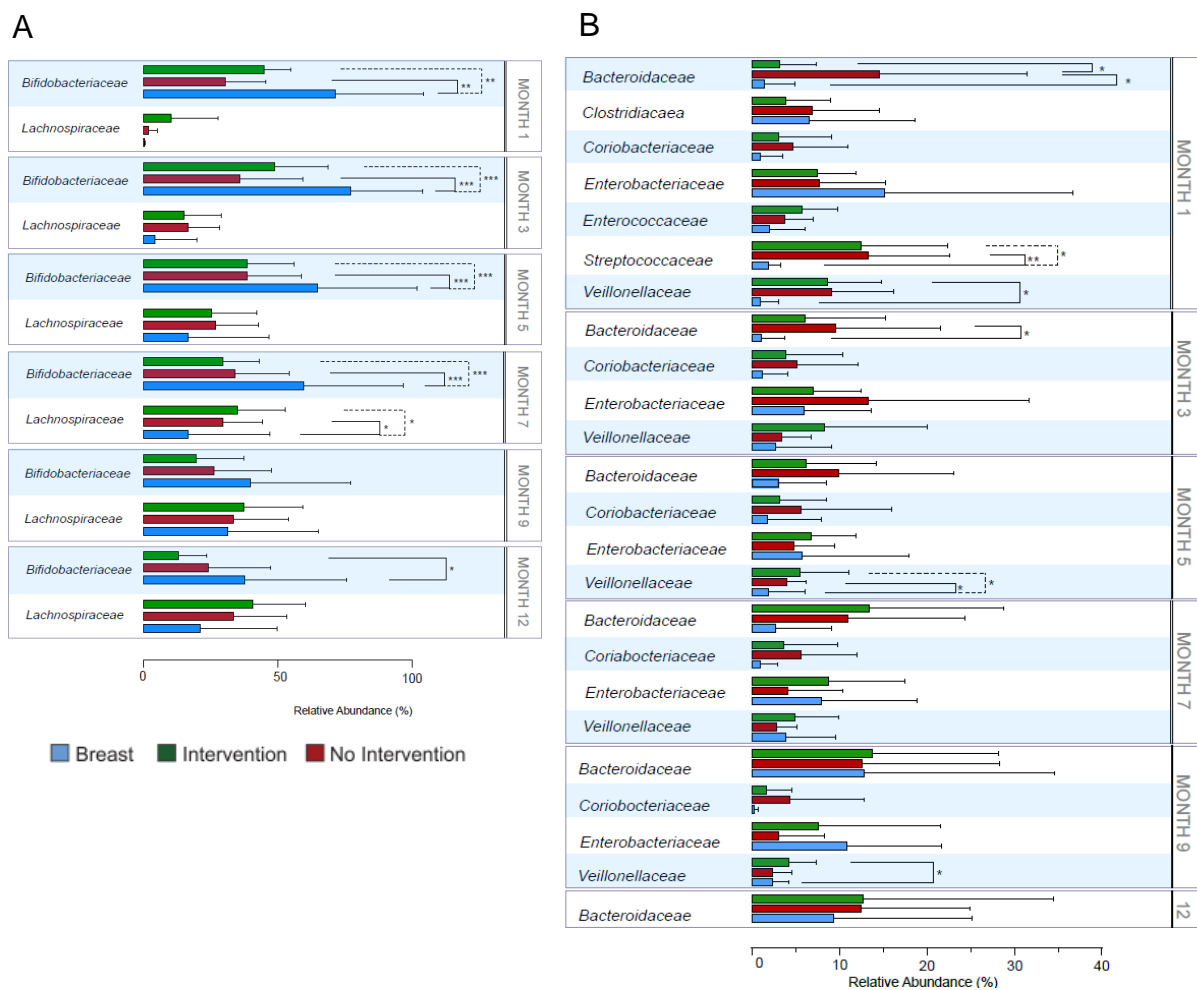
**Figure 18: Relative abundance of Actinobacteria, Firmicutes, Bacteroidetes and Proteobacteria in the three exclusive breast and formula study cohorts.**

Actinobacteria dominated the breast-fed faecal milieu and was reduced to half of its initial abundance at later months, being replaced primarily by members of the Firmicutes phylum. Formula-feeding was associated with a balanced Actinobacteria and Firmicutes abundance throughout the first year with no significant differences between intervention and placebo groups. Bacteroidetes levels were significantly higher in the placebo group at month 1, but evened out later on. Proteobacteria abundance was low in all groups with no differences among them. \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$



### 3.4.2. *Lachnospiraceae* were well established in formula-fed infants

While breast-feeding was associated with high Actinobacterium abundance, formula-feeding was characterized by an even distribution of Actinobacteria and Firmicutes. Both groups were low in Bacteroidetes and Proteobacterium levels. Representatives of the families with a minimum of 5% relative abundance in at least one of the three exclusive feeding groups are shown in figure 20.



**Figure 19: Most abundant bacterial families in exclusively breast-fed, intervention and placebo-fed infants from month 1 to 12.**

(A) *Bifidobacteriaceae* and *Lachnospiraceae* (right) were the most common families among the infants during the first year, with high, but constantly decreasing abundances of *Bifidobacteriaceae* in breast-feds and high and constantly increasing abundances of *Lachnospiraceae* in formula-feds. (B) Other families that were present throughout the first year with a minimum of 5% relative abundance included *Bacteroidaceae*, *Coriobacteriaceae*, *Enterobacteriaceae* and *Veillonellaceae*. *Clostridiaceae* and *Streptococcaceae*. \*p<0.05, \*\*p<0.005, \*\*\*p<0.0005

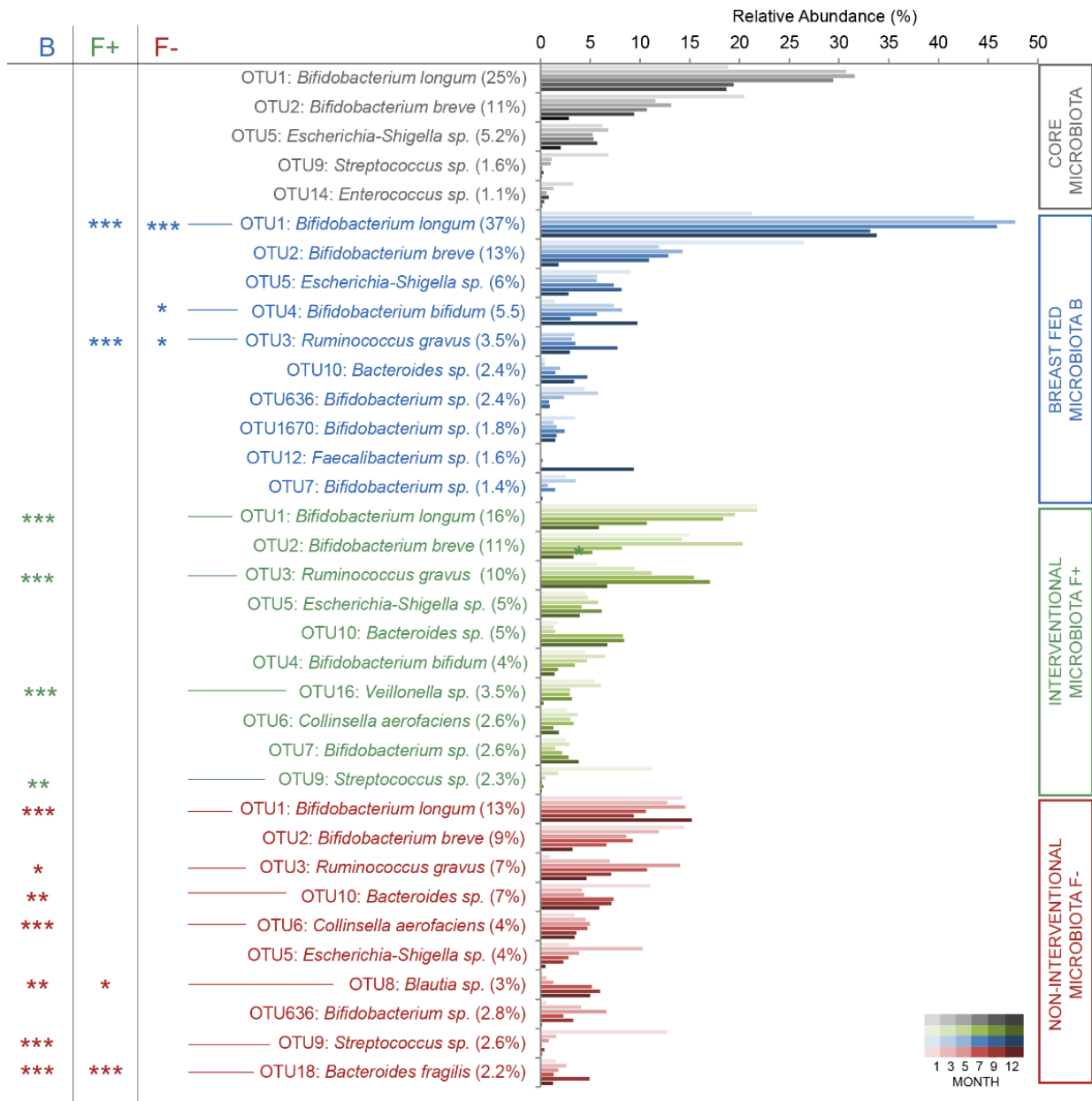
### 3.4.3. Decreased detection of *Bacteroides fragilis* and *Blautia sp.* was associated with bifidobacteria supplementation

From 16S rRNA gene amplicon sequencing data, each sequence was assigned on the genus level by comparison to sequence databases (RDP, Silva) and a more detailed identification tool (Eztaxon) to determine the corresponding species. An assignment of OTUs was based on >97% sequence identity. For each feeding group, the ten most abundant OTUs were selected and compared to the other groups. Also, a core microbiota was determined, which is composed of all OTUs found in any infant during the first year.

Comparison of the three feeding groups revealed differences in the hierarchy and abundance of OTUs (Figure 21). Placebo formula-feeding was associated with a significantly higher relative abundance of *Bacteroides fragilis* (OTU18; 2.2 +/- 1.3%;  $p < 0.005$ ) and one *Blautia sp.* (OTU8; 3.0 +/- 2.4%;  $p < 0.005$ ) over the first year, while these species were low in bifidobacteria-supplemented (0.04 +/- 0%; 1.4 +/- 1.3%) and breast-fed infants (0.3 +/- 0.7%; 1.0 +/- 1.4%). One *Bifidobacterium longum* (OTU1) was highly characteristic of faecal microbiota in breast-fed infants compared to formula-fed infants throughout the first year (37% +/-9.8% vs. 15.5% +/-4.8%;  $p < 0.0005$ ), while one *Streptococcus sp.* (OTU9) was specific for both formula groups at month 1 (11.9 +/-8.9% vs. 1.1 +/- 1.3%;  $p < 0.0005$ ) and *Ruminococcus gnavus* (OTU3) was higher in formula-fed than in breast-fed infants throughout the first year (9.1 +/-4.5% vs. 3.5 +/-1.8%,  $p < 0.05$ ).

Analysis of the 10 most abundant OTUs further revealed that 4 respectively 3 OTUs of the intervention and placebo group belong to the genus *Bifidobacterium*. Sequence comparison using the EzTaxon database showed that OTU1, OTU2 and OTU4 have a 100% sequence identity with *B. longum* subsp. *infantis*, *B. breve* and *B. bifidum*, respectively.

Moreover, a “core microbiota” was defined, which included all species that were present in each of the 106 infants at least once during the first year. These species were two *Bifidobacterium* spp. and one species each of the *Escherichia-Shigella*, *Streptococcus* and *Enterococcus* genera.



**Figure 20: Core microbiota and 10 most abundant OTUs in breast-fed, intervention and placebo formula-fed infants during the first year.**

The core microbiota is composed of five species, that all share the feature of being present in any of the 106 infants during the first year of life at one of the time points measured. OTU1 (*B. longum*) was the most dominant OTU in all feeding groups. The placebo group had higher *Blautia sp.* and *Bacteroides fragilis* than breast- and intervention-feds. Percentages in brackets indicate mean relative abundance of the specific OTU within the first year. Asterisks show significances of OTUs to the corresponding feeding cohort; \* $<0,05$ , \*\* $<0,005$ , \*\*\* $<0,0005$

#### **3.4.4. The breast-fed microbiota was characterized by a remarkably low number of OTUs**

A constant increase in total OTUs was observed in all three feeding groups from birth until first year, with the breast-fed group possessing lowest numbers of total OTUs at all time points measured (Table 7). Similarly, OTUs per infant were lowest in breast-feds at all time points, with 28 OTUs at month 1 and 48 OTUs at month 12, while in the intervention group this was 37 and 67, and in the placebo group it was 38 and 71 at months 1 and 12, respectively. A remarkable increase in OTU numbers was observed from month 1 to 3, with a mean increase of +230% in all feeding groups. In formula-fed infants, a mean of 8 OTUs was found in every formula-fed infant in the first year, while in breast-fed infants only a mean of 4 OTUs were present in every infant.

The breast-fed microbiota was characterized by a low richness, as seen when calculating the abundance of the five most abundant OTUs. At month 3, for example, the five most abundant OTUs made up 80% of the total breast-fed microbiota, while in intervention and placebo groups, this was only 52% and 42%, respectively. When considering the ten most abundant OTUs, the breast-fed microbiota was made up of even 92%, and intervention fed infants by 66% and placebo fed infants by 55%.

The number of OTUs that was present in both formula groups, but not in the breast-fed group, was a lot higher at any time point (except at month 3) than the OTUs shared between one of the formula groups and the breast-fed group, hence more identical OTUs are present in the two formula groups than in one formula and breast group. Moreover, each of the groups harbored its specific OTUs that were not found in either of the other two feeding groups. For example, OTU1670 (*Bifidobacterium* sp.) was found at month 1 solely in three infants from the breast-fed group at a mean relative abundance of 3.46%. *Bacteroides massiliensis* was detected at month 9 in four infants of the intervention group with a mean relative abundance of 1.85%, but was absent in the other feeding groups. An unknown genus from the *Lachnospiraceae* family and *Bacteroides fragilis* were detected in 5 infants at month 9, respectively 12, solely in the placebo group.

**Table 7: OTU characteristics of intervention-, placebo-, and breast-fed infants.**

Richness and prevalence characteristics of OTUs are summarized below for the three exclusive feeding cohorts B (breast-fed), F+ (intervention fed) and F- (placebo fed).

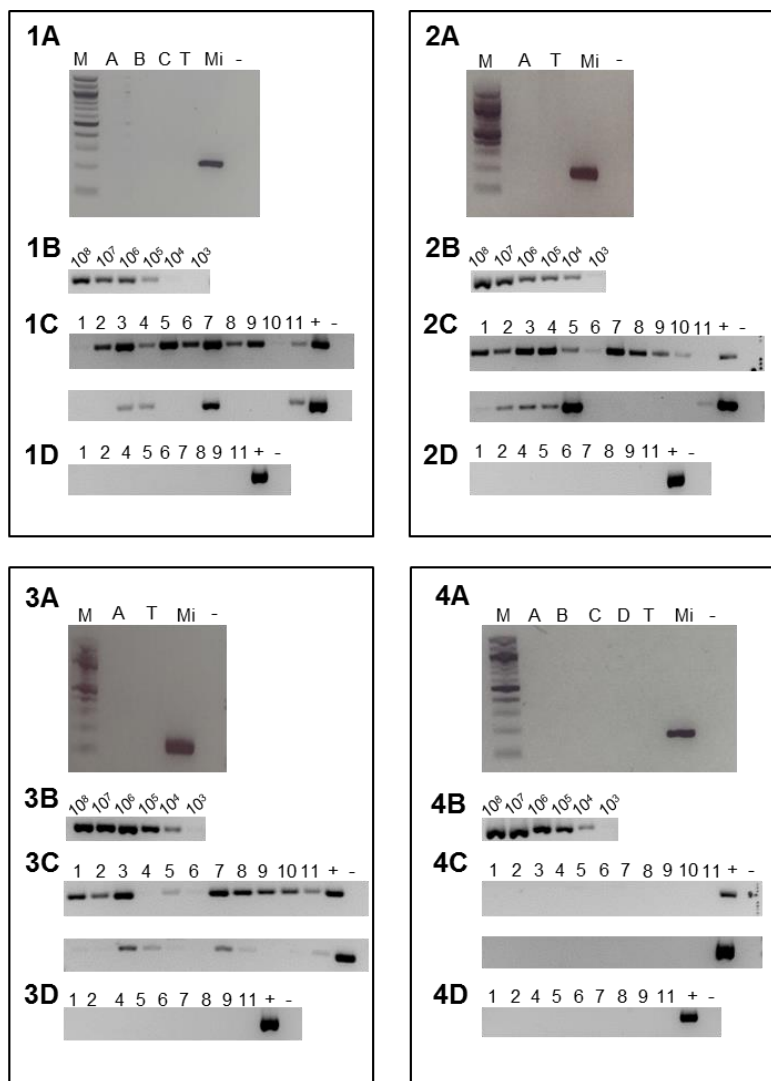
		MONTHS AFTER DELIVERY																	
		1			3			5			7			9			12		
		B	F+	F-	B	F+	F-	B	F+	F-	B	F+	F-	B	F+	F-	B	F+	F-
RICHNESS and DIVERSITY	Number of Infants	20	11	11	20	11	11	20	11	11	20	11	11	12	11	11	9	11	11
	Number of Analysed Samples	19	11	11	20	11	11	19	11	11	20	11	11	9	11	10	8	11	10
	Total Number of OTUs	171			198			194			222			226			232		
	Detected OTUs	63	66	67	139	154	156	119	150	141	183	186	186	154	195	197	144	195	186
	OTUs present in every infant (100% Abundance)	2	7	5	3	10	8	1	7	6	4	7	9	7	10	10	6	9	10
	Mean OTUs/Infant (Richness)	28	37	38	34	56	54	27	47	51	47	64	68	56	75	77	48	67	71
	Relative Abundance of 5 Predominant OTUs	65%	59%	56%	80%	52%	42%	79%	61%	49%	75%	54%	43%	64%	47%	36%	56%	34%	37%
Relative Abundance of 10 Predominant OTUs	80%	79%	72%	92%	66%	55%	88%	74%	65%	85%	79%	60%	79%	64%	55%	68%	53%	51%	
PREVALENCE	Number of Common OTUs																		
	Between B and F+	3			10			0			4			4			6		
	Most Commonly (Mean Prevalence B/ F+, in %)	OTU320: <i>Streptococcus</i> sp. (0.09/0.8)			OTU318: <i>Bifidobacterium</i> sp. (0.73/0.35)			-			OTU21: Unknown <i>Clostridiaceae</i> (2,44/0.73)			<i>Bacteroides ovatus</i> (1.39/0.39)			<i>Bacteroides ovatus</i> (0.35/0.15)		
	Between B and F-	2			14			6			11			8			6		
	Most Commonly (Mean Prevalence B/ F-, in %)	OTU 396: <i>Bifidobacterium</i> sp. (3/0.06)			OTU27: <i>Bacteroides</i> sp. (1.27/0.48)			OTU25: <i>Veillonella</i> sp. (0.93/0.94)			OTU1670: <i>Bifidobacterium</i> sp. (2,41/1,4)			<i>Bacteroides fragilis</i> (3.1/4.9)			OTU69: Unknown <i>Lachnospiraceae</i> (0.24/1.62)		
	Between F+ and F-	12			3			22			21			25			23		
	Most Commonly (Mean Prevalence F+/ F-, in %)	OTU 33: Unknown <i>Lachnospiraceae</i> (1.9/0.7)			<i>Actinomyces radingae</i> (0.85/0.1)			<i>Flavonifractor plautii</i> (1.87/0.87)			OTU22: Unknown <i>Lachnospiraceae</i> (0,82/2.07)			OTU6: <i>Collinsella aerofaciens</i> (1.28/3.59)			OTU6: <i>Collinsella aerofaciens</i> (2.0/3.42)		
	OTUs Only Found in the Corresponding Feeding Group	22	9	14	31	11	14	17	15	21	19	15	19	12	25	24	16	27	24
	Most commonly in B																		
	Highest Mean Prevalence (%/ Infants above 0.5%)	OTU1670: <i>Bifidobacterium</i> sp. (3.46/3)			OTU66: <i>Hungatella</i> sp. (0.61/4)			OTU1670: <i>Bifidobacterium</i> sp. (1.62/2)			OTU13: Unknown <i>Clostridiaceae</i> (0.94/4)			OTU1841: Unknown <i>Bacteroidaceae</i> (1.0/1)			OTU396: <i>Bifidobacterium</i> sp. (2.65/3)		
	Highest Number of Infants with Prevalence >0.5 (No.)	OTU 55: <i>Haemophilus</i> sp. (6)			OTU66: <i>Hungatella</i> sp. (4)			OTU1653: Unknown <i>Bifidobacterium</i> (6)			OTU9: <i>Streptococcus</i> sp. (5)			OTU1653: Unknown <i>Bifidobacteriaceae</i> (1)			OTU396: <i>Bifidobacterium</i> sp. (3)		
	Most commonly in F+																		
	Highest Mean Prevalence (%/ Infants above 0.5%)	OTU28: <i>Megamonas</i> sp. (1.33/1)			OTU396: <i>Bifidobacterium</i> sp. (2.52/1)			OTU28: <i>Megamonas</i> sp. (1.84/1)			OTU28: <i>Megamonas</i> sp. (1.66/1)			OTU46: <i>Bacteroides massiliensis</i> (1.85/4)			OTU38: Unknown <i>Lachnospiraceae</i> (1.164)		
	Highest Number of Infants with Prevalence >0.5 (No.)	OTU898: Unknown <i>Enterobacteriaceae</i> (1)			OTU38: Unknown <i>Lachnospiraceae</i> (2)			OTU302: <i>Enterococcus</i> sp. (2)			Enterobacteriaceae sp. (21)			OTU27: <i>Bacteroides</i> sp. (5)			OTU38: Unknown <i>Lachnospiraceae</i> (4)		
Most commonly in F-																			
Highest Mean Prevalence (%/ Infants above 0.5%)	OTU67: Unknown <i>Coriobacteriaceae</i> sp. (0.81/1)			OTU72: <i>Barnesiella intestinihominis</i> (0.57/1)			<i>Bacteroides fragilis</i> (1.77/4)			<i>Eubacterium eligens</i> (0.52/2)			OTU38: Unknown <i>Lachnospiraceae</i> (1.69/5)			<i>Bacteroides fragilis</i> (1.25/5)			
Highest Number of Infants with Prevalence >0.5 (No.)	OTU84: <i>Lactobacillus</i> sp. (2)			OTU1458: Unknown <i>Bifidobacteriaceae</i> (1)			<i>Bacteroides fragilis</i> (4)			<i>Clostridium aldense</i> (2)			OTU38: Unknown <i>Lachnospiraceae</i> (5)			<i>Bacteroides fragilis</i> (5)			

#### **4. Exogenous bifidobacteria did not colonize the infant gut**

The use of strain-specific primers allowed to specifically target the added formula strains. Primers designed to amplify a region of only one bacterial strain require some analysis effort, a task that was delegated to the group of Jens Walter from the Microbes and Gastrointestinal Health Unit of the University of Alberta (Edmonton).

After primer design, the four *Bifidobacterium* strains from formula were first analysed against type strains and all other strains of the same species available at the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ, Germany). After successful testing, faecal samples from exclusively intervention formula-fed infants at months 4, 12 and 24 were examined for strain presence.

Results show that the designed primers targeted only the formula strains when compared to all other strains from the same species that were available from the DSMZ (Figure 28). Further, the detection limit was assessed by testing the primers on samples with a pre-determined DNA concentration. Finally, primers were tested on infant faecal DNA samples from 11 intervention formula-fed infants at month 4 and 12 and also at month 24. We also tested the primers on samples from the placebo group and amplification was present, but only to a marginal extent (data not shown).



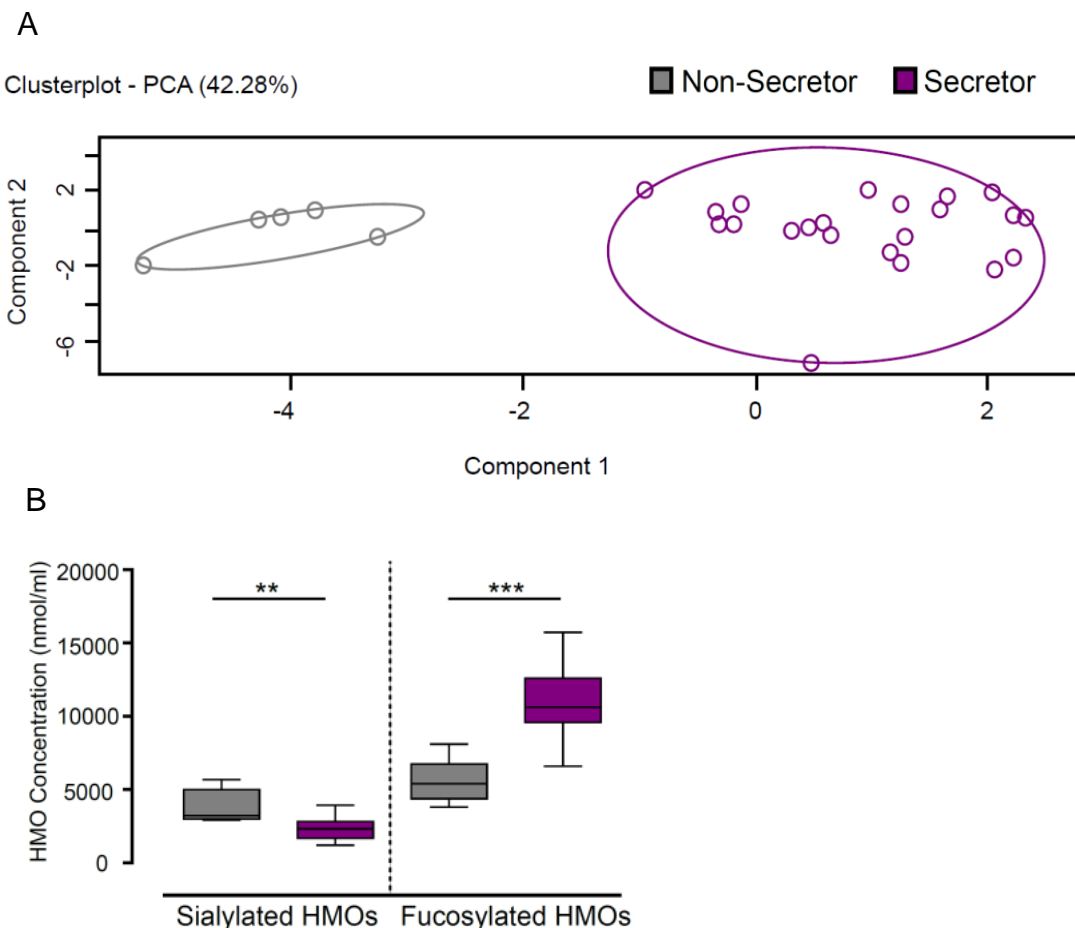
**Figure 21: Strain-specific analysis for the detection of formula-added *Bifidobacterium* spp. in infant faeces.**

Strain-specific primers were used to target *B. bifidum* BF3 (1A-D), *B. breve* BR3 (2A-D), *B. longum* BG7 (3A-D) and *B. longum* subsp. *infantis* BT1 (4A-D). Primers were tested for specificity against available strains and type strain of the corresponding species (A, B, C, D=other strains; T=type strain; Mi=Milk strain; “-“=negative control) (1-4A). Detection limits were measured using defined amounts of DNA added to the PCR reaction (1-4B). Infants aged 4, 12 (1-4C) and 24 months (1-4D) that were exclusively intervention formula-fed during the first year were tested with the strain-specific primers (1-11=infant code).

*B. bifidum* BF3, *B. breve* BR3 and *B. longum* BG7 were detected in almost all eleven infants at month 4 (BF3: 82%, BR3: 91%, BG7: 82%), but in less than half of them at month 12 (BF3: 36%, BR3: 45%, BG7: 45%). At month 24, no infant was positive for any of the strains. *B. longum* subsp. *infantis* BT1 was not detected at any time point measured.

## 5. Presence of fucosylated human milk oligosaccharides correlated with the occurrence of bifidobacteria

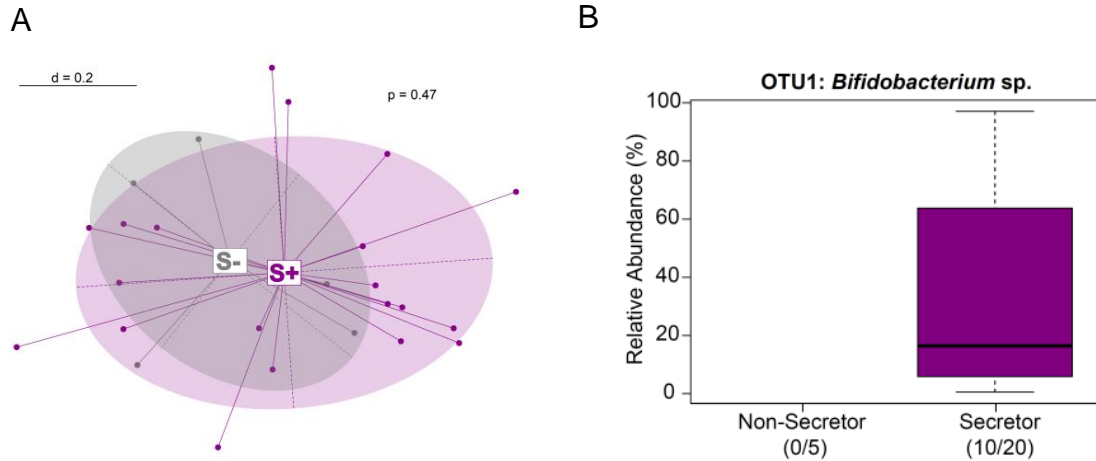
Maternal breast milk from 27 mothers was analysed for differences in composition applying liquid chromatography and compared to outcomes from 16S sequencing data from corresponding infants. Analysis was performed by the lab from Lars Bode (University of California, San Diego). Human milk oligosaccharides were determined qualitatively and quantitatively and maternal secretor status, which is an indicator for the presence (secretor) or absence (non-secretor) of the intact fucosyltransferase-2 (FUT-2) allele, was analysed.



**Figure 22: The maternal secretor status determined the composition of the breast milk.**

(A) Principal component analysis of maternal breast milk samples with a separation of secretor (purple) from non-secretor (grey) mothers ( $p < 0.05$ ). (B) Depending on the maternal secretor status, the composition of the human milk oligosaccharides (HMOs) differed significantly. While sialylated HMOs were higher in non-secretor maternal breast milk ( $p < 0.005$ ), fucosylated HMOs were higher in secretor mothers ( $p < 0.0005$ ).





**Figure 23: Effect of maternal secretor status on the *beta*-diversity and on the abundance of OTU1 (*Bifidobacterium longum*) at month 1.**

(A) The different composition of the maternal breast milk as a consequence of the presence or absence of the intact FUT-2 gene (S+: secretor; S-: non-secretor) did not affect the overall microbiota composition of the corresponding infants at month 1 ( $p=0.47$ ). (B) Prevalence of *Bifidobacterium longum* was affected by the maternal secretor status as shown by the absence of the species in infants from non-secretor mothers and a 50% presence in infants from secretor mothers.

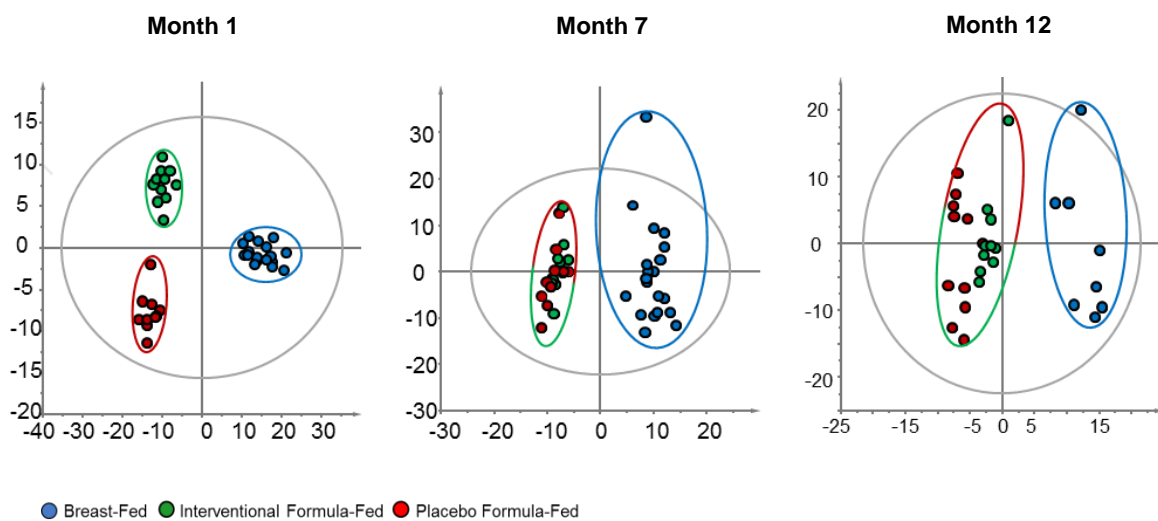
HMO analysis revealed that the concentration of sialylated and fucosylated oligosaccharides depends on the mother's secretor status (Figure 29). The presence of an intact FUT-2 allele, and hence a secretor-status, led to a lower sialylated, but higher fucosylated HMO concentration. This in turn did not affect the infant's faecal beta-diversity ( $p=0.47$ ), but the presence of OTU1, *Bifidobacterium longum* (Figure 30). This species was present in 50% of all infants from secretor mothers, while no infant from a non-secretor mother harbored this species one month after birth.

## **6. Metabolomic Analysis and Correlation to Microbiota Data**

The analysis of gut microbiota-derived metabolites was applied to investigate potential metabolic shifts in dependence on microbiota dynamics. Analyses were done in cooperation with the Helmholtz Institute of Munich, where trained staff received fecal aliquots and further processed them for LC-MS metabolite analysis. Correlation to OTU data, which were formerly obtained from us, was done by a statistician from the institute. While the major part of the metabolite analysis and the generation of the figures shown hereafter were done by staff from the Helmholtz Institute, it is important to show these data in the context of microbiota analysis in order to gain insights into functional changes within the intestinal ecosystem, notably of SCFAs, the major fermentive end products of gut bacteria.

### **6.1. Faecal metabolites discriminated interventional and placebo formula-fed infants at early age**

Orthogonal partial least squares discriminant analysis (OPLS-DA) was performed to determine differences in metabolite profiles between the exclusively breast milk, intervention and placebo groups. We observed that faecal metabolites and OTUs discriminate intervention and placebo formula-fed infants at the age of 1 month (Figure 22). At months 7 and 12, metabolites were different between breast-fed and formula-fed infants, but not between intervention and placebo groups.



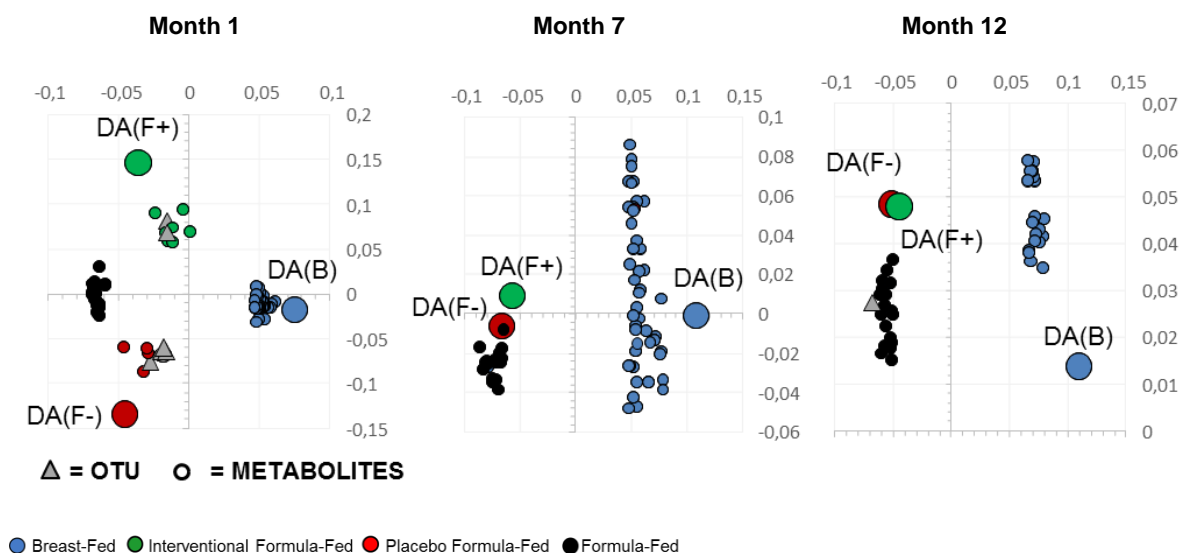
**Figure 24: OPLS-DA scores plot of Main Discriminating Features of Breast-Fed, Intervention and Placebo Formula-Fed Infants at the Age of 1, 7 and 12 Months.**

Metabolite profiles of exclusively breast-fed (blue) and exclusively intervention (green) and placebo (red) formula-fed infants are significantly different at month 1. At months 7 and 12, formula-fed infants differ from breast-fed infants, but no separation was observed between intervention and placebo fed infants.

## 6.2. Metabolites and not OTUs were the major determinants for the discrimination between intervention and placebo groups at month 1

Metabolites and OTUs were correlated in order to assess if both or only one of them contributes to the separation of the groups at month 1. Both datasets were therefore merged and evaluated through an orthogonal partial least squares discriminant analysis (OPLS-DA).

The correlation showed that 6 OTUs were involved in the feeding-specific shaping of the faecal ecosystem at month 1 and therefore contributed to the separation of the feeding groups (Figure 23). At month 7 and 12, no OTU or metabolite specific profile was observed though. Differences between breast and formula groups were maintained until the end of intervention at one year. The metabolites and microbial species involved in the discrimination of the groups can be seen in the following tables (Tables 8-10).



**Figure 25: OTU/Metabolite Correlations of Exclusively Breast-Fed, Intervention and Placebo Formula-Fed Infants at the Age of 1, 7 and 12 Months.**

At month 1, four OTUs clustered with metabolites from the placebo group (red) and two OTUs clustered with metabolites from the intervention group (green), while no OTUs correlated with breast-fed-specific metabolites (blue). At month 7, no correlations were observed for any feeding group. At month 12, one OTU correlated with metabolites from both formula-fed groups.

ID	Feed	Order	m/z or taxonomy	Lipid Maps classification	HMBD classification
Cluster_4611	B	1	552,3366878		
Cluster_1816	B	2	621,7141547		
Cluster_5216	B	3	621,2105299		
Cluster_0644	B	4	256,0821987		
Cluster_5825	B	5	694,2352887		
Cluster_3214	B	6	376,2631225		Benzopyrans
Cluster_5970	B	7	713,2216027		
Cluster_5136	B	8	613,1922285		
Cluster_2992	B	9	341,293116		
Cluster_2002	B	10	721,2122736		
Cluster_3467	F	1	407,245529		
Cluster_3230	F	2	377,2296393		Lipids
Cluster_0971	F	3	386,7162076		
Cluster_3644	F	4	427,3609756		Lipids
Cluster_3593	F	5	421,2189653		
Cluster_3745	F-	1	440,2844395		
OTU_10	F-	2	<i>Bacteroides</i> sp,		
Cluster_3789	F-	3	445,3706539	Sterol Lipids [ST]	Prenol Lipids
Cluster_4023	F-	4	472,4172805		
Cluster_5412	F-	5	644,4008303		
OTU_18	F-	6	<i>Bacteroides fragilis</i>		
OTU_96	F-	7	<i>Odoribacter</i> sp,		
Cluster_5175	F-	8	616,3489547		
OTU_68	F-	9	<i>Alistipes</i> sp.		
Cluster_6345	F-	10	764,4230286		
Cluster_0385	F+	1	510,3339711		
Cluster_4506	F+	2	537,386372		
OTU_4	F+	3	<i>Bifidobacterium</i> sp.		
Cluster_6749	F+	4	813,5680134	Glycerophospholipids [GP]	
Cluster_1515	F+	5	520,762328		
OTU_142	F+	6	<i>Lactococcus</i> sp,		
Cluster_3067	F+	7	353,2506538		
Cluster_2260	F+	8	144,0825079		
Cluster_3573	F+	9	417,3345252		

B=Breast-fed; F=Formula-Fed; F+=Intervention Formula-Fed; F-= Placebo Formula-Fed

**Table 8: Feeding cohort specific metabolites and correlation to OTUs of month 1 in breast-fed (B) and formula-fed (F) infants differentiating in intervention (F+) and placebo (F-) fed infants ranked from high to low importance (order) by significance on OPLS-DA loadings plot.**

While breast-feeding was distinguished from the other groups primarily by the dominance of 10 metabolites and no OTU, the differentiation of both formula groups was achieved through a specific OTU/metabolite profile. As found in the OTU analyses from sequencing, *Bacteroides fragilis* was a discriminating factor for the placebo group. m/z = mass-to-charge ratio of positive electrospray ionization. Molecular formula and classification assigned using the MassTRIX webserver with an error of 0.05 Da, assignment by KEGG, LipidMaps and HMDB.

ID	Feed	Order	m/z or taxonomy	Lipid Maps classification	HMBD classification
Cluster_4141	B	2	491,330764		
Cluster_4188	B	3	497,1767158		
Cluster_3864	B	4	453,2158723		
Cluster_4611	B	5	552,3366878		
Cluster_3868	B	6	453,2406183		
Cluster_3207	B	7	375,2921489	Sterol Lipids [ST]	Steroids and Steroid Derivatives
Cluster_3088	B	8	357,2827519		Fatty Acids and Conjugates
Cluster_4029	B	9	473,2536532	Sterol Lipids [ST]	Steroids and Steroid Derivatives
Cluster_3946	B	10	462,3440454	Sphingolipids [SP]	Sphingolipids
Cluster_3580	F	1	418,3461543		
Cluster_3425	F	2	401,3093941	Sterol Lipids [ST]	Prenol Lipids
Cluster_3573	F	3	417,3345252		
Cluster_0971	F	4	386,7162076		
Cluster_3493	F	5	410,320276		
Cluster_3683	F	6	431,3553964	Sterol Lipids [ST]	Prenol Lipids
Cluster_3644	F	7	427,3609756		Lipids
Cluster_3230	F	8	377,2296393		Lipids
Cluster_4577	F	9	547,4244787		
Cluster_3803	F	10	447,3479744		

B=Breast-fed; F=Formula-Fed

**Table 9: Feeding cohort specific metabolites and correlation to OTUs of month 7 in breast-fed (B) and formula-fed (F) infants differentiating in intervention (F+) and placebo (F-) fed infants ranked from high to low importance (order) by significance on OPLS-DA loadings plot.**

Due to the similarity of the metabolite and OTU profile between intervention and placebo groups, only breast-fed and formula-fed groups were compared at month 7. The top 10 most characteristic factors of groups B and F were metabolites, with most of the identified ones belonging to the sterol lipid class. m/z = mass-to-charge ratio of positive electrospray ionization. Molecular formula and classification assigned using the MassTRIX webserver with an error of 0.05 Da, assignment by KEGG, LipidMaps and HMBD.

ID	Feed	Order	m/z or taxonomy	Lipid Maps classification	HMBD classification
Cluster_2524	B	1	261,1469315	primary amine	Amino Acids and Derivatives
Cluster_4871	B	2	583,2605075		
Cluster_4197	B	3	498,0621014		
Cluster_2250	B	4	118,0879665		
Cluster_1342	B	5	466,1913538	Polyketides [PK]	
Cluster_2753	B	6	301,1126443		
Cluster_2352	B	7	206,0829788		
Cluster_3088	B	8	357,2827519		Fatty Acids and Conjugates
Cluster_4611	B	9	552,3366878		
Cluster_3207	B	10	375,2921489	Sterol Lipids [ST]	Steroids and Steroid Derivatives
OTU_29	F	1	Flavonifractor sp,		
Cluster_3642	F	2	427,3608033		Lipids
Cluster_3613	F	3	423,3295783		Fatty Acid Esters
Cluster_3666	F	4	430,331642	Fatty Acyls [FA]	
Cluster_3781	F	5	445,371389	Sterol Lipids [ST]	Prenol Lipids
Cluster_3783	F	6	445,3707973	Sterol Lipids [ST]	Prenol Lipids
Cluster_3689	F	7	431,3536514	Sterol Lipids [ST]	Prenol Lipids
Cluster_3493	F	8	410,320276		
Cluster_3914	F	9	458,3999067	Sterol Lipids [ST]	
Cluster_3683	F	10	431,3553964	Sterol Lipids [ST]	Prenol Lipids

B=Breast-fed; F=Formula-Fed;

**Table 10: Feeding cohort specific metabolites and correlation to OTUs of month 12 in breast-fed (B) and formula-fed (F) infants differentiating in intervention (F+) and placebo (F-) fed infants ranked from high to low importance (order) by significance on OPLS-DA loadings plot.**

At month 12, OTU29 (*Flavonifractor* sp.), belonging to the Clostridium class of the Firmicutes phylum, was characteristic for formula-feeding. Similarly to month 7, most identified metabolites were sterol lipids. m/z = mass-to-charge ratio of positive electrospray ionization. Molecular formula and classification assigned using the MassTRIX webserver with an error of 0.05 Da, assignment by KEGG, LipidMaps and HMBD.

### 6.3. Short chain fatty acid (SCFA) profiles were similar between intervention and placebo groups

We further assessed SCFA profiles in faeces from the exclusively breast- and formula-fed infants using UHPLC-Q-ToF mass spectrometry.

Our results showed that breast-feeding led to lower proportions of propionate, butyrate, valerate and isovalerate (Figure 24). In addition, no differences in the SCFA profiles were detected between the intervention and placebo groups. Pyruvic and lactic acid were detected at significantly higher concentrations in breast-fed infants. Independent of feeding, the concentrations of propionic, butyric, isovaleric and valeric acid increased over time.

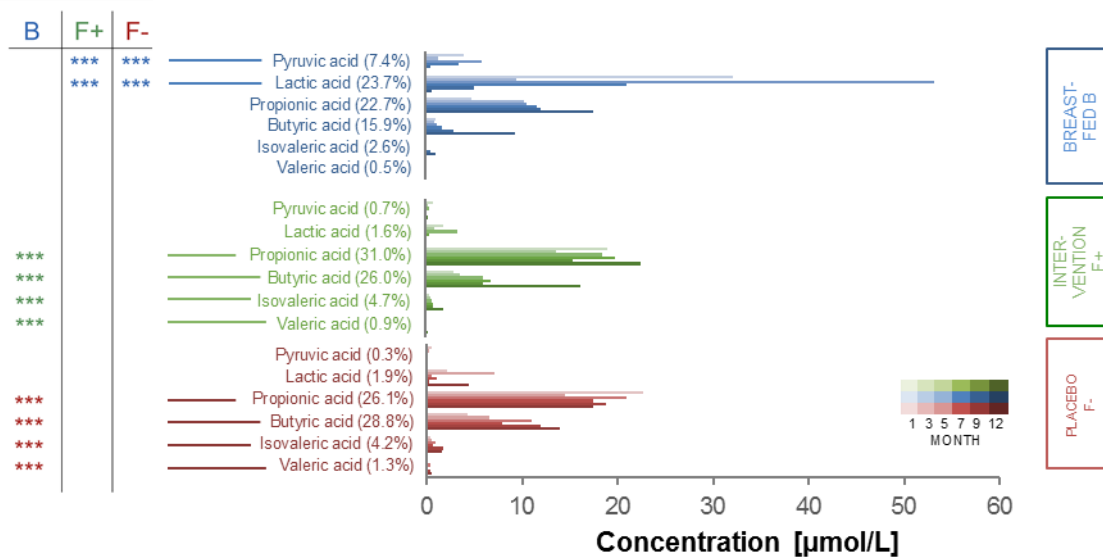


Figure 26: SCFA profiles in the different feeding groups.

Faecal concentrations of SCFA in breast- (blue), intervention (green) and placebo (red) formula-fed infants at the age of 1, 3, 5, 7, 9 and 12 months as indicated by colour code from light to dark. Similar concentrations were observed in intervention and placebo fed infants, with no significances between these groups. Formula-feeding compared to breast-feeding, however, resulted in a strongly different SCFA profile as shown by high concentrations of pyruvic acid and lactic acid in breast-fed infants and high concentrations of propionic and butyric acids in formula-fed infants. Asterisks show significances of OTUs or SCFA to the corresponding feeding cohort; \* $<0,05$ , \*\* $<0,005$ , \*\*\* $<0,0005$

## DISCUSSION

Infant colonization is a highly dynamic, rapidly changing and complex process that still remains an unpredictable phenomenon onto which numerous internal and external factors have an influence (97). The *in utero* sterile gut of the unborn will be challenged by a massive number of bacteria upon delivery and initial feeding regimes were shown to possess one of the most dominating powers to affect bacterial habitation (44). Due to the proven bifidogenic effects on the infant microbiota, it is well accepted, that breast-feeding should be done during the first months (261). To compensate the lack of potential to produce enough amounts of breast milk, the goal of assimilating commercial, whey based formula to breast milk, was set by providers in order to offer a substitution to their breast-fed counterparts. The complexity of maternal milk composition has led to new ideas in formula research, such as the addition of pre- and probiotics. While prebiotics were reported to stimulate growth and/or activity of a limited number of bacterial species already resident in the colon, probiotics in the form of bifidobacteria or lactobacilli can colonize the gut upon ingestion through infant formula (76).

However, although many commercially available infant formulas are supplemented with such bacteria, little is known about their ability to impact gut microbial community composition and function. The superiority of bacterially supplemented formula over sterile formula has been shown in numerous studies, particularly when providing evidence for the mitigating and preventive effect in several pathologies, such as necrotizing enterocolitis (262) – one of the major causes for deaths in preterm births, but also in less serious diseases like infantile colic and atopic dermatitis (263). Regrettably, there is still a huge gap of knowledge in determining effects of bacterial supplementation on a healthy infant ecosystem.

This study aimed to address the question of how the supplementation of four different *Bifidobacterium* spp. affect microbial communities in the faecal ecosystem, demonstrating that an intervention with bifidobacteria modulates microbiota composition and metabolite profiles during the very first months of life. Some of the induced changes, such as decreased relative abundance of *Bacteroides fragilis*, have been linked to benefits in infants, warranting the exploration of probiotics for disease prevention.



## 1. Development of the infant microbiota

The colonization of the infant gut starts already during birth and is a highly dynamic process up to the age of 2-3 years, when the intestinal microbiota resembles the adult-like composition (19). The initial feeding scheme, also including cessation of breast-feeding and introduction of a complementary feeding, is among the major drivers in shaping microbial community structures (114). Moreover, early life microbiota development is characterized by high inter- and intra-individual differences, which continuously decrease with advanced age and finally merge over time to a more comparable microbiota around the age of 18 months and older (54). Here, in agreement with published data, we show that the microbiota is in a dynamic process from birth on, dissociating progressively from its initial composition. The most significant change in microbiota composition occurred from month 7 to month 9. This was observed in exclusively breast- and formula-fed infants, both groups not going through the phase of cessation of breast-feeding at this time. The change is therefore likely to be caused by the introduction of solid food, which occurred at a mean age of around 5 months. In general, the early microbiota was dominated by Actinobacteria and Firmicutes, whereas results have to be handled with care when differentiating between the feeding groups. For example, at month 1, breast-fed infants were colonized by more than two thirds with Actinobacteria, more precisely, with family members of *Bifidobacteriaceae*, while formula-fed or mixed-fed (formula- and breast-fed) infants were evenly colonized by Actinobacteria and Firmicutes. Abundances of Bacteroidetes and Proteobacteria were rather low in all groups. This Actinobacteria dominance in breast-fed infants continued until month 3 and 7, with abundances around 80% of total bacteria. The Actinobacteria/Firmicutes balance in formula- and mixed fed infants decreased at month 3, where a slight shift to higher *Bifidobacteriaceae* levels was observed, particularly in the intervention mixed-fed group. Firmicutes decreased to levels of 10-25% in formula-fed infants at month 3, while they recovered similarly to Actinobacteria to 35-40%. At month 12, Actinobacteria decreased sharply, particularly in the formula-fed cohorts (breast-fed: 40%, formula-fed: 10%) and the microbiota of all feeding groups was then dominated by Firmicutes (breast-fed: 45%, formula-fed: 70%). In consistence with another study, we found that there is a dominance of Actinobacteria and/or Firmicutes during the initial months of life, while Bacteroidetes and Proteobacteria remained rather low (132).

Further, we observed ranges from a highly stable to a dramatically instable and constantly changing microbiota in breast-fed infants during the first year of life, while formula-feeding influenced the development of the faecal microbiota rather moderately. The stability of microbial composition in breast-fed infants was primarily caused by a strong dominance of bifidobacteria that resided over the first twelve months, while other breast-fed infants were characterized by a mixed and permanently changing microbial pattern. In comparison, exclusively formula-fed infants were characterized by a constant change in microbiota, without any major deviations from the preceding composition. This circumstance is likely to be a result of the stable nutritional behaviour in this group, which does not account for breast-fed infants, which are subject to differing maternal breast milk compositions due to e.g. alternating nutrition of the mother affecting the breast milk.

Effects of complementary food introduction were observed to be not immediate and are likely to require some adaptation time by the intestinal microbiota. We compared solely breast-fed infants to the same infants after introduction of solid food (time between the measurements was one month) and found no change in beta-diversity and no alterations in bacterial abundances. Backhed et al also described the comparatively minor influence of complementary foods on the microbiota composition. Primary influencers on the contrary were found in cessation of breast-feeding and initial feeding behaviour (112). Similarly, Fallani et al disclosed a marginal difference between infants before and after solid food introduction, while still the initial feeding regime determined the microbial composition for a longer time. This marginal influence included e.g. a promotion of *Bifidobacterium* spp., *Clostridium coccooides* and *Bacteroides* in the baby's intestinal ecosystem (55). Hence our data emphasize the prolonged duration that is required to change a microbiota that is dominated by initial factors, such as breast- or formula-feeding or delivery mode, to a microbiota that increasingly resembles the complex adult-like composition.

## **2. Analysis of confounding factors affecting the colonization process**

Confounding factors, such as delivery mode and antibiotic treatment, that have been reported to play a crucial role during the infant colonization process were studied here as well. While maternal and gestational age, infant sex, parental allergy history, nullipara and presence of pets had no ultimate influence on microbiota development

(measured in 1- and 3-month old breast-fed infants, n=44), delivery mode affected its composition at the age of 1 and 3 months after birth. This difference was characterized by higher Firmicutes, more exact *Clostridium* spp., abundance in caesarean section births, an effect that was not present at the age of 7 months anymore. One recent study also revealed that *Clostridium* spp., particularly *Clostridium perfringens* was significantly higher in infants born by caesarean section than in vaginally delivered infants. Also, other skin-related bacteria were found to be higher in caesarean section infants, as observed in another study, where species from the *Staphylococcus*, *Corynebacterium* and *Propionibacterium* genera were more common (78). These species, however, were not increased in our study cohort. While *Clostridium* spp. abundance was also described to be dependent not only on the delivery mode, but also on the initial feeding method, this influence can be excluded for our study, as infants studied were solely fed by breast milk (55).

Furthermore, we investigated the effect of antibiotic administration on the developing microbiota. Those infants receiving antibiotics during the months preceding the measured time points (e.g. when an infant was treated with antibiotics at month 6, a faecal sample from month 7 was analysed), were compared to infants not receiving antibiotics. In our study, we found that there is no change in the alpha- and beta-diversity as well as in bacterial phylum or family abundances. This accounted for antibiotic provision to the infant during early stages in life, but also during advanced age of about one year. From literature, conflicting results were presented. For example, it was shown that antibiotics administered to infants during the perinatal phase do not influence the post-weaning microbiota (55). The majority of studies, however, revealed an effect of antibiotics on the developing microbiota, as shown for example by Palmer et al who found a drastic reduction in total bacterial density and alterations in population composition in antibiotic treated babies and also by Dardas et al who similarly registered a shift towards a reduced bacterial diversity in antibiotic treated infants (97, 264). One report, however, emphasized the need for differentiation, showing that there is a strong dependence on the type of antibiotic administered. For example, cefotaxime significantly reduced bacterial richness while vancomycin and gentamycin, both very commonly used for infants, did not exert uniform effects on the richness (116). However, our results have to be handled with caution, as the time between antibiotic treatment and collection of the faecal sample was more than 2 months for some of the infants. However, for this study it was important to determine if an effect of antibiotics

can be observed even after several weeks or months in order to find out if the collected sample was altered through the antibiotic treatment. We therefore concluded that none of the samples, analysed at any of the provided time points (months 1, 3, 7 and 12), were influenced by antibiotic exposure.

In conclusion, our results show that delivery mode influenced the gut microbiota during early age characterized by a promotion of clostridia, with no detectable long-term effects on the faecal composition. While some studies also presented an influence of the presence of older siblings (131) or pets (265) and infant gender (266) on the colonization process, we could not detect any significances. The influence of parental allergy history, maternal and gestational age on the infantile microbiota is only poorly studied while from our study, no influence was detected.

### **3. The supplementation of *Bifidobacterium* spp. during infancy**

Numerous studies have been conducted during the past years with outcomes that do not yet support the clear idea of beneficial effects of probiotic bacteria such as bifidobacterial or lactobacilli. A recent study revealed that their use does not affect rates of necrotizing enterocolitis, sepsis and mortality in preterm deliveries (267). Another study disclosed effects of probiotics on lower *Clostridium histolyticum* abundances, while the remaining microbiota was only marginally affected (76). In order to further investigate minor differences observed in the *beta*-diversity between the different groups, we studied Operational Taxonomic Units (OTUs), which are sequence reads clustered by relatedness to provide a phylogenetic description of the microbial community (74). In our study, OTUs were clustered based on a minimum sequence similarity of 97%.

Our data suggest that bifidobacteria-supplemented formula alters the microbiota through shifts in bacterial composition, primarily a decreased occurrence of one *Bacteroides fragilis* (OTU 18) and one *Blautia* sp. (OTU 8) was observed. *B. fragilis* was described to contribute to the development and maturation of the infant immune system (87), and due to its potential to encode virulent metalloproteases, a pathogenic character is assumed for that species (268). One recent study revealed that *Bacteroides* spp. are less abundant in Russian infants, but are dominant in Finnish and Estonian infant cohorts, who are known to be more often affected from early onset of

autoimmune diseases, suggesting that increased *Bacteroides* abundance in the latter two populations might contribute to immunological pathologies (269).

To date, there is only a small number of confirming studies measuring *B. fragilis* abundance in infants, thereby evaluating the influence of exogenous or indigenous factors on its colonization potential. Fallini et al described a strong dependence of *Bacteroides* abundance in infants on geographical location of birth and living, pointing towards a strong environmental influence on the richness of that genus (55). Another study focused on *B. fragilis* abundance during infancy on childhood weight development, including 909 one-month old infants, thereby revealing that infants with higher numbers of *B. fragilis*, have a higher risk for the development of an elevated BMI later in life (270). Moreover, it was disclosed, that *B. fragilis* and *Blautia coccooides* were absent in several infants at the age of less than 6 months, while both species were found to be present later in life in the same infants (62).

In our study, we observed the presence of *B. fragilis* in four infants from the placebo group (36%), but in no infant from the intervention and breast-fed groups. The abundance of *B. fragilis* during the first year was significantly higher in the placebo group than in either of the other groups ( $p < 0.0005$ ). Due to the low number of infants in any of the groups, results have to be handled carefully, but could give a hint towards the assumption of a feeding-specific microbiota development. This is partially in consistency with another study that found lower *B. fragilis* rates in breast-fed than in formula-fed infants (97). Whether or not *B. fragilis* colonization in the placebo group can be traced back solely to the feeding regime in that group, remains to be determined by further investigations. A conflict for this theory can be retrieved from literature, where *B. fragilis* colonization is dependent on multiple factors as the species favors not only a specific feeding regime of the host, but also seems to colonize infants born by caesarean section less frequently (266). Also Gronlund et al suggested a correlation between mode of delivery and *B. fragilis* abundances, with infants born by vaginal mode being colonized with more than double rates of *B. fragilis* (88). This factor is likely to have influenced our results as well, as the four infants with *B. fragilis* presence were born by vaginal birth as well, however, all infants were in the placebo group, while no infant from the intervention group born by normal birth was colonized by the species. It is therefore likely, that both – mode of delivery and feeding regime affected the

growth of *B. fragilis*, with infants born by vaginal delivery and being fed the placebo milk possessing highest rates.

As mentioned before, one *Blautia* sp. (OTU8) was also significantly more abundant in the placebo group throughout the first year compared to breast-fed and intervention formula-fed infants. *Blautia* species are butyrate-producing bacteria, which could be an explanation for the high abundance of butyric acids in the placebo group, with rates more than 10% higher than in the intervention group and almost double rates than in the breast-fed cohort. *Blautia* sp. were recently described to be strongly reduced in patients with Crohn's Disease, supporting the understanding that the species plays an important role in the physiology of the healthy gut (271). However, detailed reports for the importance and role of the species in the developing infant gut are lacking, requiring more investigations.

Besides OTU analyses, the *alpha*- and *beta*-diversity was not affected by the type of formula. A trend, however, was observed in the mixed fed group that received intervention milk in parallel to breast milk. This trend was characterized by a *beta*-diversity that assimilated the one of breast-fed infants through month 1 and 7. Due to a lower formula intake by the intervention group than by the placebo group, the effect is possibly not only due to the bifidobacteria intervention.

#### **4. Strain-specific analysis of *Bifidobacterium* spp. in faecal samples**

In order to determine the potential of exogenous *Bifidobacterium* spp. to colonize the infant gut, strain-specific primers were designed to target formula strains in faecal samples. 4 months after birth, three (*B. bifidum*, *B. breve*, *B. longum*) of the four strains were detected in more than 90% of all exclusively intervention formula-fed infants. When testing in the same infant cohort at month 12, a strong reduction of the presence of added strains in faecal samples was observed. Less than 50% of the infants harbored at least one of the strains. Also, the concentration of exogenous *Bifidobacterium* spp. was clearly lower as observed by band thickness on the agarose gel while using same DNA concentrations. *B. longum* subsp. *infantis*, however, was not detected at months 4 or 12 in any infant. At the age of two years none of the formula strains was detected in any exclusively intervention formula-fed infant.

The reduction in the presence of added strains in faecal samples from month 4 to 12 can be due to different reasons. It is likely, that this is due to a decrease in daily milk consumption with increasing age. While daily formula uptake was approximately 800 ml at month 4, this volume was about half at month 12. The absence of any strain at the age of two years and after a non-provision of intervention formula for one year supports this theory. It is likely, that there is a minimum required dose of bacteria that needs to be ingested in order to be detectable with strain-specific primers. We have shown that the detection limit of the assay is  $10^4$  to  $10^5$  cells, which is comparable to other reports (272). Also, a replacement of formula strains by the high load of diverse bacteria introduced by solid food is probable.

The competition for the colonization of specific niches in the developing gut is high and an inhabitation by more age-specific bacteria is likely (273). The absence of *B. longum* subsp. *infantis* at any age measured opened many questions. Detection limit for *B. longum* subsp. *infantis* was found to be  $10^4$  and it is therefore likely that the strain was not able to colonize the infant gut. Despite the fact that the strain-specific primers were developed with specific software and tested against positive and negative controls, still some uncertainty remains if the strain could not be detected from faecal samples. Maybe the strain was not able to grow under provided conditions or the colonies picked from the plates did not include *B. longum* subsp. *infantis*.

From literature, there is a substantial number of studies, reporting the successful detection of specific strains from faecal samples. For example, Elli et al found strains provided by yoghurt in faecal samples from healthy adults, while Pagnini et al found that levels of added bacterial strains are increased after administration (274, 275). However, data for long-term persistence in the gut are missing and reports often lack a placebo comparison group when determining strain-specificity of a selected method (276). Our analysis included faecal samples from placebo fed infants, serving as an additional negative control. We detected marginal amplifications, but we were certain that a mix-up error can be excluded. However, results from the intervention group were assumed correct as the difference to the placebo group was remarkable. The difficulty in the detection of strains is moreover hampered by the massive bacterial load found in the gut and consequently in faecal samples of the developing infant. As bifidobacteria are among the most dominant bacterial genera during the first year, a

vast number of different species and thus strains is present in the intestinal microbiota, aggravating the detection of a selected strain.

From our results, we can conclude that bifidobacteria strains added to formula and ingested through its regular uptake, can be detected from faecal samples, while no long-term persistence seems to occur. The choice for an adequate detection method has to be considered carefully though, including adequate validation procedures, such as inclusion of a negative control from the placebo group.

### **5. Maternal secretor status influenced the selection of *Bifidobacterium* spp.**

It is well accepted that cell numbers of bifidobacteria increase through breast feeding, thanks to the utilization of human milk oligosaccharides (HMOs) (277). HMOs are a family of structurally diverse unconjugated glycans that are highly abundant in and unique to human milk (278). Numerous roles have been assigned to HMOs, such as prevention of pathogen attachment to infant mucosal surfaces and HMO-mediated *B. infantis* dominance, that potentially keeps harmful bacteria in check as they compete for limited nutrient supply (279). In our study, breast-feeding was associated with higher relative abundances of OTU1 (100% sequence identity to *Bifidobacterium longum*), OTU3 (100% to *Ruminococcus gravus*) and OTU4 (100% to *Bifidobacterium bifidum*) compared to formula-fed infants. OTU1 was found to be by far the most dominant species among breast-fed infants during the first year (mean relative abundance of 37%), suggesting that OTU1 *B. longum* was one of the main HMO-utilizers.

Analyses of the maternal breast milk by HPLC-FL revealed, that 22 mothers were “secretors”, while 5 mothers were “non-secretors”. “Non-secretor” mothers are characterized by inactivating variants of fucosyltransferase-2 (FUT-2) on both alleles. The FUT-2 plays a crucial role in the mediation of the inclusion of fucose in sugar moieties of glycoproteins and glycolipids and associations between “non-secretors” and Norovirus resistance, but also Crohn’s disease were found (280). Here in our study, we found that the prevalence of OTU1 was dependent on the maternal secretor status, as 50% of infants from “secretor” mothers were positive for OTU1, while no infant from “non-secretor” mothers (n=5) was positive. These results are consistent with another study reporting that relative abundances of bifidobacteria correlate with



the maternal secretor status (281). The authors suggested that an inactive allele of the maternal FUT-2 gene (in “non-secretor” mothers) leads to a delayed and decreased colonization by bifidobacteria. Moreover, adults that harbor an inactive FUT-2 gene were found to possess lower levels of bifidobacteria in their own gut, which could mean that when inheriting the inactive allele to their offspring, these infants have lower levels of bifidobacterial due to inheritance reasons as well (280). Also, as shown by a study on 71 healthy adults, the microbiota between secretors and non-secretors differs significantly, mostly characterized by a lower species richness in non-secretors. As the maternal microbiota is forwarded at least partially during normal birth to the infant, this could be a reason for the altered microbiota between infants from secretor and non-secretor mothers (282).

Further studies in that field are necessary, as there are only few reports on the breast milk’s composition and its effect on the baby’s microbiota. Recent reports have focused primarily on the breast milk’s microbiota, suggesting staphylococci and streptococci as one of the major bacteria found in maternal milk (283). Research in this field, however, has consequently mitigated the effects of other compositional ingredients and more focus should be laid on these.

## **6. Metabolomics and SFCA analyses**

Metabolomics, the study of metabolites in a given sample, allows the direct monitoring of the end products of bacterial metabolism (284). It can be carried out on a local basis, thereby analysing the total metabolite profile in a sample, or in a targeted way for particular groups of metabolites, such as SCFAs (285). SCFAs, primarily of acetic, butyric and propionic acids, are produced by the metabolic activity of the gut microbiota. The acids were reported to possess anti-inflammatory effects and the potential to inhibit growth of specific cancer cell lines (286, 287).

While the number of reports on metabolomics has increased significantly within recent years, most of them have focused on the adult and not on the developing infant. Moreover, the metabolomics approach is applied to any bodily fluid, such as blood plasma or urine or to different compartmentalizations, decreasing the number of data even lower for those in faecal samples. From the marginal amount of reports present, one investigated effects of initial feeding regime on the faecal metabolome in primates,

revealing that faecal metabolomic differences between breast- and formula-fed persisted from month 6 to month 12 of age. In particular, formula-fed animals showed increased amino acids such as asparagine in stools (288). However, recently, analysis on SCFAs – the major metabolic products – has emerged to one of the most extensively studied areas in the metabolomics field, with numerous studies suggesting effects of probiotic administration on faecal SCFA profiles. One study on infants with the potential to develop eczema revealed that SCFA levels can be increased through probiotics and therefore decreases the risk for eczema occurrence, as the disease is typically characterized by its low SCFA levels (289).

In our study, we determined metabolites from faeces by UHPLC mass spectrometry. In order to pinpoint singular hallmarks of the intervention, metabolite and microbiota data were further combined to search for differences at month 1, 7 and 12. We found that metabolite profiles were clearly distinct at neonatal age between breast- and formula-fed infants, and they converged over time to reach profiles that were very different from the initial time point. This deviation from neonatal profiles was mirrored by overtime shifts in the microbiota and is in consistence with published data (54). Most interestingly, it was found that faecal metabolites and OTUs discriminate intervention and placebo formula-fed infants at the age of 1 month. These correlations revealed a relation between F+ specific metabolites and two molecular species (OTU 4, *Bifidobacterium bifidum*; OTU142, *Lactococcus* sp.), while F- specific metabolites showed a correlation with 4 species (*Bacteroides* spp.: OTU10, OTU18; *Odoribacter* sp.: OTU96; *Alistipes* sp. OTU68).

Of the top 50 ranked metabolites at month 1, none correlated with any OTU, hence encouraging the assumption that the metabolites are a major driver for the discrimination of breast- from formula-fed groups. At months 7 and 12, metabolites were different between breast-fed and formula-fed infants, but not anymore between intervention and placebo groups. One recent study evaluated the use of probiotics in preterm infants and their impact on the microbiome and metabolome, concluding that metabolite profiles are different between probiotic and control groups, which strengthens our results on the discrimination of breast- and formula-fed infants over time (290). Another study disclosed that upon probiotic milk consumption, significant changes in microbiota-encoded enzymes involved in diverse metabolic pathways occur, while the microbiota remained mostly unchanged (291). Although little insight is

available for non-targeted metabolomics of healthy breast-fed and formula-fed infants, Wang et al. identified 15-methylhexadecanoic acid, galactitol and maltose as discriminating metabolites for breast-feeding and beta-alanine, dodecanoic acid, glycolic acid, decanoic acid and tyramine for formula-feeding (292). However, we identified dodecanoic acid to be associated with breast-fed infants at month 1, clearly demonstrating the need to perform additional validation studies.

Moreover, one metabolite assigned as a glycerophospholipid was increased in the breast-fed group at all measured time points. In contrast, one sterol lipid-like metabolite was dominant in faeces from formula-fed infants in the first 7 months. Database comparison and MS/MS experiments of those two important metabolites, however, did not provide any identity information. It still remains one of the main challenges of metabolomics to assign particular spectra to specific compounds due to the immense amount of different and yet unknown metabolites (293). Nevertheless, it should be mentioned that the faecal metabolome is a complex matrix, containing human and microbial metabolites (294). The classification and identification of the “unknown” metabolites are challenging, for which several databases are used (294, 295) in order to gain a first overview of the metabolite composition. Nevertheless, the vast majority (80 %) of them still remains unknown and a conclusion related to health or disease susceptibility is not possible at this stage.

We further assessed short-chain fatty acid (SCFA) profiles in faeces from the exclusively breast- and formula-fed infants. SCFAs are a major product of gut microbiome fermentation, with acetic, propionic and butyric acid being the principal metabolites produced in the human gut (296). The roles and characteristics of the single SCFAs are diverse. Propionic acid, for example, was shown to positively regulate weight gain in obese humans, while butyric acids were found enriched in cases where a low-fat diet was applied (297, 298). Reports from previous studies suggested that acetic and lactic acid are the dominant SCFAs in faeces from breast-fed infants, while formula-fed infants have higher concentrations of propionic and butyric acid (299).

Similarly, we showed that breast-feeding leads to lower proportions of propionate, butyrate, valerate and isovalerate. As *Clostridiales* are primary butyrate producers and we observed that *Clostridiaceae* were increased in the placebo group at month 1, this would justify the high butyrate levels in the placebo group. In addition, we observed

that differences in the SCFA profiles were not detectable between the intervention and placebo groups. Pyruvic and lactic acid were observed at significantly higher concentrations in breast-fed infants. As bifidobacteria were highest in the breast-fed group, it seems likely that lactic acid, a fermentation product of carbohydrates converted by bifidobacteria, is highly present in the breast-fed group (300). Although the general understanding is, that many bacteria can rapidly metabolize simple carbohydrates to lactic acid, acetic acid or propionic acid (300), it seems that the mix of *Bifidobacterium* strains used in this study did not affect the SCFA levels. Independent of feeding, the concentrations of propionic, butyric, isovaleric and valeric acid increased over time.



## CONCLUSION

The definition of a healthy infant microbiome remains controversial and inconsistencies in published data have not yet provided adequate evidence for it. Adding bifidobacteria to infant formula has been a major innovation in order to feed the developing baby with presumable health-promoting bacteria that would, in the best case, settle in and confer a benefit to the host. A recent advance in the use of next generation sequencing methods has propagated this research topic, thereby providing new insights in the microbial pattern. While traditionally, studies included cell culture and animal based models, the number of trials using sequencing techniques, mostly by the 16S ribosomal DNA and total DNA, on human faecal samples has increased recently (301). Furthermore, “metagenomic” studies, involving the sequencing of all the genetic material recovered from environmental samples, have evolved to one of the most promising tools in the microbiological field (302). While the number of trials using solid identification tools increases, the results of them remain conflicting, providing no clear conclusion for the optimum nutrition to be provided to babies. While the WHO gives clear recommendations to breast feed a baby at least for a duration of six months, ideally up to the age of two years ([www.who.int/nutrition/topics/exclusive\\_breastfeeding/en/](http://www.who.int/nutrition/topics/exclusive_breastfeeding/en/)), it was also suggested that the reduced bacterial richness and diversity in breast-fed infants is likely to be linked to numerous pathologies (106, 107). However, the partly conflicting results in that field highlight that the exact role of initial feeding practices and later pathologies remains to be elucidated.

In conclusion, this placebo-controlled intervention study clearly shows that bifidobacteria-supplemented formula modulates the infant metabolome at very early stages in life, with no detectable long-term consequences for gut microbiota assembly or function. Minor differences were detected in the intervention group with regard to specific OTUs. For example, one *Bacteroides fragilis* and one *Blautia* sp. were found at similar levels between intervention and breast-fed infants, but were found to be more prevalent in placebo infants. One additional hallmark of this study is the observation that bifidobacteria from formula were washed-out from the infant gut over time, failing to persistently colonize beyond intervention, despite the high prevalence of bifidobacteria in all feeding groups even after two years of age. The colonization with supplemented bacteria is therefore assumed to be only temporary and not on a long-

term basis. This might only be possible to achieve by the continuous administration of supplemented milk or yoghurt, also with advanced age.

Besides existing studies, this study has provided new insights in the healthy developing microbiome and metabolome, pointing towards a minor, but not underestimating effect of bifidobacterial supplementation when provided during early life. Key areas for future research hence include the selection of appropriate probiotic species and strains, timing and duration of treatment, and cross-interactions with other factors, such as delivery mode and cessation of parallel breast-feeding.

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## Abbreviations

B	Breast-fed
BF+	Breast- and intervention formula-fed in parallel
BF-	Breast- and placebo formula-fed in parallel
BSM	Bifidobacterium Selective Medium
CFU	Colony-forming unit
EFSA	European Food Safety Authority
F+	Formula with Bifidobacteria
F-	Formula without Bifidobacteria (Placebo)
FL	Fluorescence
FUT-2	Fucosyltransferase-2
IBD	Inflammatory Bowel Diseases
IBS	Irritable Bowel Syndrome
IL	Interleukin
LC	Liquid Chromatography
MS	Mass Spectrometry
NEC	Necrotizing Enterocolitis
NMDS	Non-Metric Multidimensional Scaling
OTU	Operational Taxonomic Unit
UHPLC	Ultra-High Performance Liquid Chromatography
WHO	World Health Organization



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## Curriculum Vitae

### PERSONAL DETAILS

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### WORK EXPERIENCE

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- 12/ 2011 – 03/ 2016 Technical University of Munich, Chair of Nutrition and Immunology  
Prof. Dirk Haller  
**Freising, Germany (GER)**  
*“Impact of Early Life Intervention with four Bifidobacterium spp. on Infant Faecal Microbiota”*
- 07/ 2010 – 01/ 2011 Australian Institute of Marine Science (Master’s Internship), Genetics Unit  
Dr. David Bourne  
**Townsville, Australia (AUS)**  
*“Development of a Quantitative Real-Time PCR Assay for the Detection of Vibrio corallilyticus targeting the Metalloprotease Gene VcpA”*
- 07/ 2008 – 02/ 2009 Medical University of Vienna, Institute of Cancer Research (Bachelor’s Internship), Hepatocellular Carcinoma Unit  
Prof. Bettina Grasl-Kraupp  
**Vienna, Austria (AUT)**  
*“Role of Peroxidized Fatty Acids in the Development of Hepatocellular Carcinoma in Rats”*

### EDUCATION

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- 09/ 2011 Master of Science in Engineering (MSc), passed with Distinction
- 2009 – 2011 Master Program at the University of Applied Sciences  
Medical and Pharmaceutical Biotechnology  
**Krems, Austria (AUT)**
- 06/ 2009 Bachelor of Science in Engineering (BSc)
- 2006-2009 Bachelor Program, University of Applied Sciences  
Medical and Pharmaceutical Biotechnology  
**Krems, Austria (AUT)**

## HONORS

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Stipend for excellent academic performance from the University of Applied Sciences, Krems (AUT)

Announced as "Top 5" Master Student at the University of Applied Sciences, Krems (AUT)

Master of Science with Distinction

## **Publications and Presentations**

### **Peer-reviewed original manuscripts in course of the doctoral thesis**

Hemmerling J., Heller K., Hörmannspurger G., **Bazanella M.**, Clavel T., Kollias G., Haller D.

Fetal exposure to maternal inflammation does not affect postnatal development of genetically-driven ileitis and colitis.

**PLoS One.** 2014 May 21;9(5):e98237. doi: 10.1371/journal.pone.0098237

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**Bazanella M.**, Maier T.V., Clavel T., Lagkouvardos I., Lucio M., Maldonado-Gómez M.X., Autran C., Walter J., Bode L, Schmitt-Kopplin P., Haller D.

Randomized controlled trial on the impact of early life intervention with bifidobacteria on the healthy infant fecal microbiota and metabolome

**Am J Clin Nutr.** 2017 (Accepted).

### **Oral Presentations**

**Bazanella M.**, Maier T.V., Clavel T. Lagkouvardos I., Schmitt-Kopplin P., Haller D.: Impact of Early Life Intervention with Bifidobacterium sp. on the Infant Fecal Microbiota. 53. Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung (DGE): Der Mensch ist, was er isst. Hochschule Fulda, Germany. 02-04 Mar 2016.

### **Poster Presentations**

**Bazanella M.**, Maier T.V., Clavel T. Lagkouvardos I., Schmitt-Kopplin P., Haller D.: Impact of Early Life Intervention with Four Bifidobacterium spp. on Infant Fecal Microbiota. The 8th International Yakult Symposium: Probiotics, a proactive approach to health. Dbb Forum, Berlin, Germany. 23-24 Apr 2015.

**Bazanella M.**, Maier T.V., Clavel T. Lagkouvardos I., Schmitt-Kopplin P., Haller D.: Impact of Early Life Intervention with Four Bifidobacterium spp. on Infant Fecal

Microbiota. 8<sup>th</sup> Seeon Conference and Science Camp: From Sequencing to Function.  
Seeon, Germany. 10-12 July 2015.