GASTROINTESTINAL FUNCTION AND MICROBIOTA DEVELOPMENT IN PRETERM INFANTS

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PROPOSITIONS

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1.

BIFIDOBACTERIA ARE WELL CAPABLE TO COLONISE AND TO BE METABOLICALLY ACTIVE IN THE PRE-TERM INFANT INTESTINAL TRACT. (THIS THESIS)

2.

AS LONG AS ANTIBIOTICS ARE A NECESSITY IN PRETERM INFANT CARE, DEVELOPMENT OF THEIR GUT MICROBIOTA REMAINS IMPEDED. (THIS THESIS)

3.

THE FULL POTENTIAL OF EXISTING -OMICS DATA SHOULD BE EXPLOITED BEFORE GENERATING NEW DATA.

4.

THE PERCEPTION THAT UNPAID OVERTIME, HIGH WORK-LOAD, LACK OF SUPPORT AND TEMPORAL CONTRACTS ARE NORMAL WORKING CONDITIONS, CONTRIBUTES TO THE HIGH RATES OF ANXIETY AND DEPRESSION IN ACADEMIA.
(LEVECQUE ET AL., RESEARCH POLICY, 2017).

5.

EXTENSIVE KNOWLEDGE ABOUT HUMAN NUTRITION AND GUT MICROBIOLOGY DOES NOT GUARANTEE HEALTHY DIETARY HABITS.

6.

WHILE BEING TYPICALLY PERCEIVED AS PETS FOR CHILDREN, RABBITS ARE BETTER SUITED FOR ADULT-ONLY HOUSEHOLDS.

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THESIS

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“CURIOUSER AND CURIOUSER!”

Lewis Caroll - Alice’s Adventures in Wonderland

“MOST MEN,

THEY’LL TELL YOU A STORY STRAIGHT THROUGH.

IT WON’T BE COMPLICATED,

BUT IT WON’T BE INTERESTING EITHER.”

Daniel Wallace - Big Fish

SUMMARY

Functioning of the gastrointestinal tract, and of the microbiota residing therein, is of significance for nutrient digestion and absorption, pathogen resistance and optimal immune performance. During early life, development of the gut microbiota coincides, and affects, development of the metabolic, cognitive and immune system. Proper establishment of the gut microbiota is therefore considered essential for healthy development. In early life, the intestinal microbiota is relatively unstable and responsive to perturbations. Factors that are recognised to influence neonatal microbiota development include gestational age, delivery mode, nutrition and antibiotic use. As such, gut microbiota establishment is likely to be impacted in preterm infants, since they have an immature gut and are commonly exposed to caesarean section delivery, specific feeding regimens and antibiotic treatment. Although gut microbiota development can be negatively impacted during early life, the developing microbiota also provides an opportunity to be targeted as means of therapeutic strategy to support healthy growth and development. In light of this, it is important to understand how the preterm infant gastrointestinal tract is functioning, which microbes colonise, what the microbes are doing and how microbiota establishment is affected. In this thesis, gastrointestinal function and microbiota development during the early life of preterm infants, and the impact of various host and environmental factors on this development, were studied.

The research described in this thesis was performed using material obtained during a single-centre, observational study including infants admitted to the neonatal unit born between 24-42 weeks gestation. A total of 238 infants (119 <32 weeks gestation, 119 >32 weeks gestation) were followed during the first six postnatal weeks, during which clinical factors were documented, and faeces and gastric aspirates were longitudinally collected for microbiota analysis. Microbiota development was not only approached compositionally via the application of qPCR and 16S rRNA gene amplicon sequencing, but also functionally via metaproteomics through LC-MS/MS, giving new insights into gut function and microbiota development in preterm infants.

Via a metaproteomics approach, gestational age-specific developmental patterns of the preterm infant gastrointestinal proteome were identified. Gestational and postnatal age were associated with quantity of specific markers for gut function and maturation, as well as with composition of the gut microbiota. The faecal proteome of very preterm infants indicated a gut environment dominated by *Bifidobacterium*, and with better digestive capacity, compared to extremely preterm infants. We showed that a *Bifidobacterium*-dominated community is associated with increased proteins involved in carbohydrate and energy metabolism, including those involved in the degradation of complex carbohydrates like human milk oligosaccharides. Regarding preterm infants, who commonly experience protein deficits and growth retardation, further exploration of the gut microbiota's metabolic traits is particularly relevant. The observed gestational age-specific developmental patterns were associated with the degree of exposure to perinatal antibiotics and respiratory support.

Studying the sole effect of gestational age on gut microbiota development remains challenging due to the cohesion between gestational age and the degree of special care.

Antibiotics are the most used therapeutics in neonatal intensive care units, prescribed for the prevention and treatment of infections and sepsis. It is important to understand the consequences of antibiotic treatment in neonates, as disturbances in microbiota development during this key developmental time window might affect early and later life health outcomes. We showed that two vancomycin dosages around time of removal of a central venous catheter presumably has no profound lasting effect on microbiota composition. However, postpartum amoxicillin/ceftazidime treatment impacts microbiota development, particularly by increasing the relative abundance of *Enterococcus* species, while decreasing *Bifidobacterium* abundance, during the first two postnatal weeks. In addition, more than five days of treatment seems to have a longer lasting effect on microbiota composition than less than three days of treatment as indicated by delayed (re)colonisation by *Bifidobacterium* species.

In addition to gestational age and perinatal antibiotics, we included various factors during data analysis to elucidate their impact on gut microbiota development in preterm infants. As well as factors acknowledged for their influence on microbiota development, such as delivery mode and feeding strategy, we identified a potential influence of gender, respiratory support and maternal preeclampsia. These findings could serve as incentive for the initiation of future studies to unravel the true influence of such factors on microbiota development.

The research described in this thesis contributes to current knowledge regarding gastrointestinal function and microbiota development during the early life of preterm infants and the factors associated with this development. This contribution could aid clinical practice and development of therapeutic strategies. In light of this, future work should consider 1) to implement functional analysis of the microbiota, 2) to study alternatives to antibiotics for the treatment and prevention of infections and 3) to elucidate the effect of microbiota targeting therapeutic strategies during preterm infant care.

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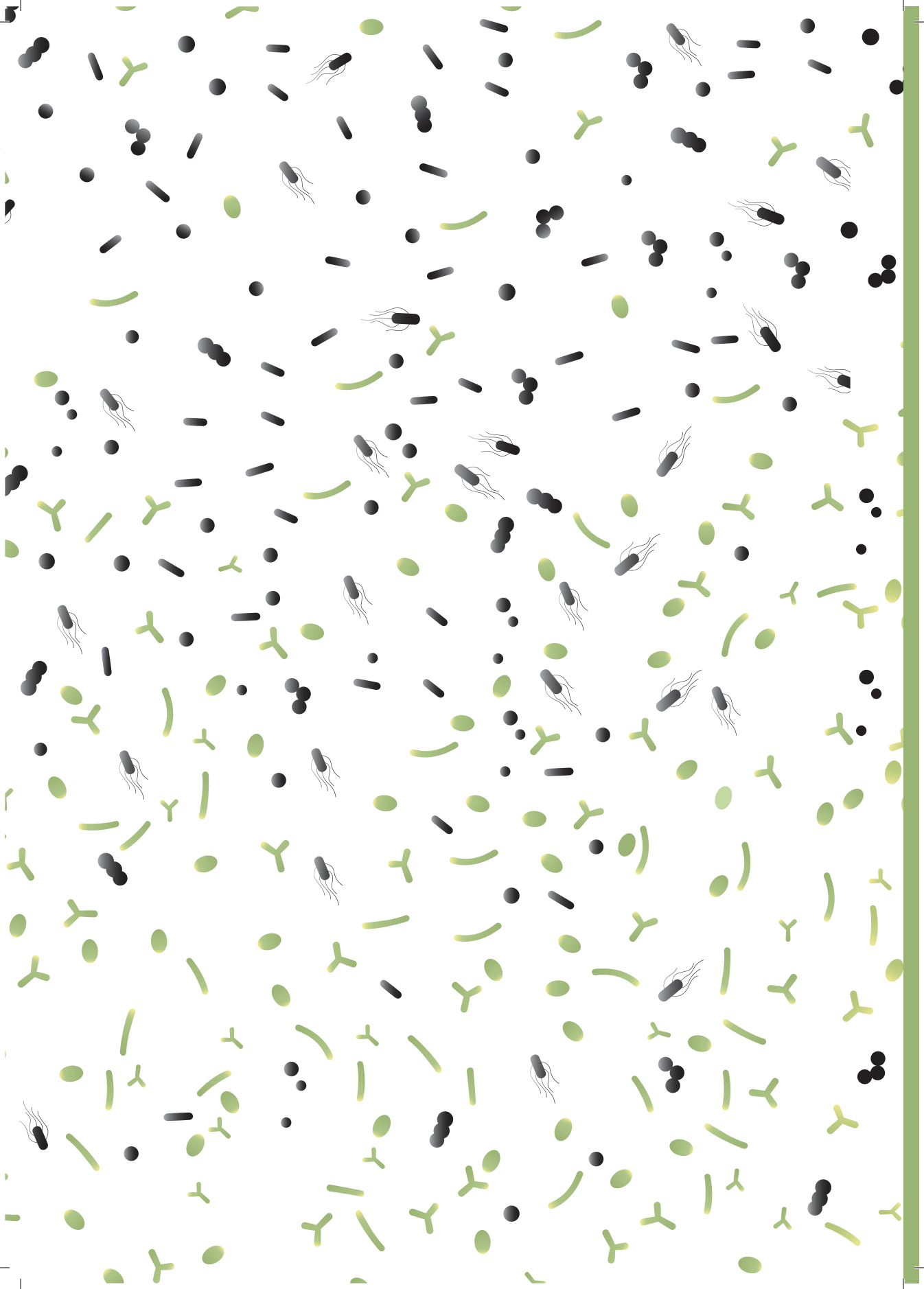


CHAPTER 1

GENERAL INTRODUCTION AND THESIS OUTLINE

PRETERM BIRTH

It is estimated that approximately fifteen million babies are born preterm every year and that complications associated with preterm birth are responsible for one million deaths worldwide[1]. In addition to mortality, preterm birth contributes to the development of early and later life health complications associated with organ immaturity[2]. Health complications are therefore related to gestational age, with babies born with lower gestational age generally needing more extensive care. Caring for the preterm neonate includes providing warmth, feeding support, breathing support, pain reduction and infection prevention[3]. Despite special care, preterm infants commonly suffer from complications during their early life which include respiratory distress syndrome, jaundice, infection, sepsis and necrotising enterocolitis (NEC). In addition, preterm birth is related to negative health outcomes in later life such as learning impairment, visual disorders, asthma and allergies, exerting a heavy burden on families, society and the health care system[2].

Preterm birth is defined as live birth before 37 complete weeks of pregnancy and is further categorised, based on gestational age, in extremely preterm (<28 weeks), very preterm (28 - <32 weeks) and moderate to late preterm (32 - <37 weeks) birth[4]. The incidence of preterm birth ranges from five percent in northern European countries to 18 percent in some African countries. Although preterm birth is generally higher in low-income countries, rates are high in some high-income counties, including the United States of America where one in eight babies are born preterm[1].

The pathway of labour consists of increased uterine contractility, cervical dilatation, and rupture of the chorioamniotic membranes. In the case of spontaneous preterm labour, one or more of these components is initiated too early[5]. Early initiation has been associated with vascular disorders, decidual senescence, uterine over-distension, cervical disease, stress, breakdown of maternal-foetal tolerance and infection[5]. Risk factors for preterm birth, related to abovementioned pathologies, are low or high maternal age, short pregnancy intervals, multiple pregnancies, infections like urinary tract infection, HIV, bacterial vaginosis and chorioamnionitis, low or high BMI, smoking, alcohol consumption, excessive physical work and bad psychological health[1].

FEEDING THE IMMATURE GASTROINTESTINAL TRACT

Development of the gastrointestinal tract (GIT) during gestation is generally subdivided in processes involved in cytodifferentiation, digestion, absorption and motility (Fig 1.1)[6,7]. Many structural and functional properties of the GIT develop within 24 weeks gestation. Anatomically, all parts of the GIT are developed within the first 12 weeks of gestation, while it takes up to 20 weeks for the villi and crypts to develop. In case of preterm birth, the infant particularly suffers from immaturity related to digestion and motility, since these are mainly developing during later stages of gestation (Fig 1.1). Digestive enzymes (e.g. lactase, sucrase, maltase, peptidase) can be detected from eight weeks gestation, but some enzymes are at that stage far below their full potential

concentration and activity. Lactase activity, important for the degradation of lactose from milk, starts maturing from 24 weeks onwards and reaches maximum activity at 40 weeks gestation. Maximum activity of lactase during gestation is estimated to be just 30% of the adult capacity, and its activity is particularly triggered by the first feeding after birth[6]. Sucking, swallowing, gastric emptying and intestinal motility develop during the third trimester and effective coordination of these processes is reached at term (Fig 1.1).

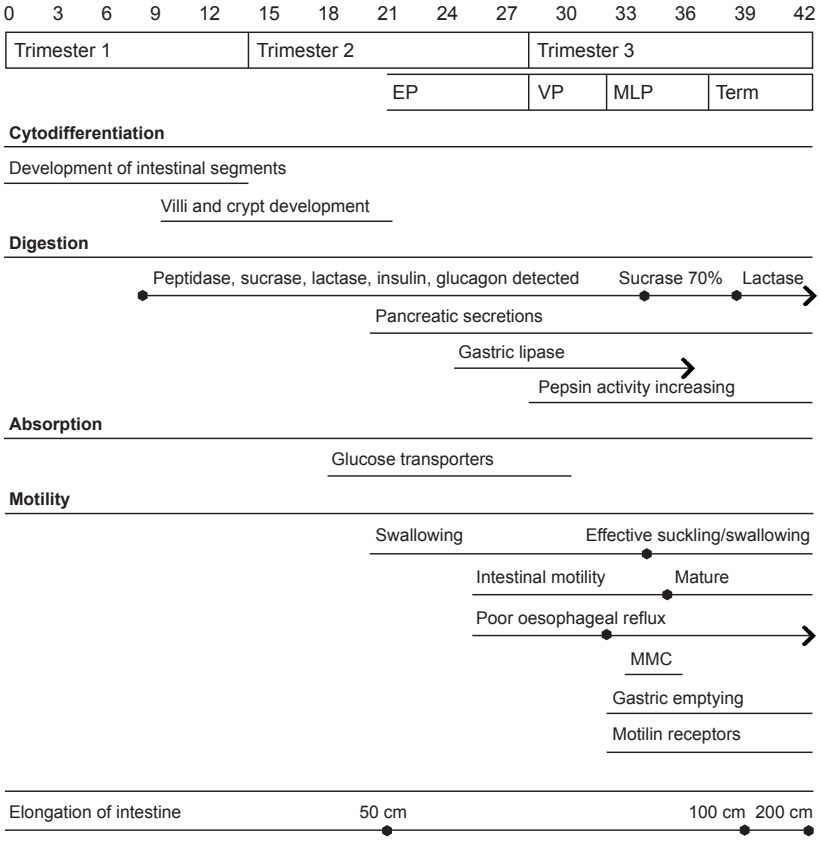


Figure 1.1 | **Timeline of structural and functional development of the gastrointestinal tract.** EP: extremely preterm, VP: very preterm, MLP: moderate to late preterm, MMC: migrating motor complex. Adjusted from Commare and Tappenden[6] and supplemented with information from Patole *et al*[7].

The decreased activity of digestive enzymes, in combination with immature motility functions and increased protein demands in preterm infants, raises a major challenge in meeting their nutritional needs[8]. Preterm infants, particularly those born before 32 weeks gestation, are prone to be intolerant to enteral feeding and most nutrients are provided intravenously via parenteral feeding for several weeks. However, the lack of enteral nutrients is not favourable and has been associated with a decrease in hormone activity, intestinal mucosa maturation, digestive enzyme activity, nutrient absorption and motility maturation and with an increase in gut permeability and bacterial translocation[8]. To stimulate functional maturation of the gastrointestinal tract of preterm infants, minimal enteral nutrition has been practiced widely in neonatal intensive care units[9]. During minimal enteral nutrition, small volumes (12 - 24 mL/kg/d) of breast milk

or formula are provided to the infant, without nutritive intend but aiming to reach full enteral feeding as quick as possible. Major determinants of withholding enteral feeding are NEC and feeding intolerance. Both, however, are not negatively affected by minimal enteral nutrition[10,11]. In addition to its function in nutrient digestion and absorption, the gastrointestinal tract is in close interaction with the endocrine, neural and immune system. Gastrointestinal immaturity may therefore impact processes distant from the gut. As mentioned before, preterm birth is associated with sepsis, NEC, learning impairment, asthma and allergies, all of which can be related to underdeveloped gut functioning and nutrient deficits during a critical developmental time window. Important to mention in this context is that the gastrointestinal tract is host to a complex community of microorganisms. Development of the gut microbiota coincides with maturation of the gastrointestinal tract and immune system and its establishment is stimulated by the dietary components in the gut lumen. Taking this all together, optimal feeding of the immature gastrointestinal tract is crucial for healthy growth and development of the infant.

HOST-MICROBE INTERACTIONS FOR HEALTH

The realisation that microorganisms are critical for human physiology has led to a new era in biology and medicine, expanding the number of studies aiming to find a relation between the human microbiota and health and disease. The human microbiota comprises bacteria, archaea, fungi and viruses that can be found on various body sites. Since bacteria achieve the highest cell density, most studies only consider the bacterial fraction of the microbiota, which also accounts throughout this thesis. It is estimated that the ratio of bacterial to human cells is around 1:1, depending on colon volume, blood volume, bacterial density of the colon and haematocrit[12]. The gastrointestinal tract is the most densely populated site of the human body[13], varying from 10^4-10^8 cells per ml luminal content in the small intestine to 10^{10}-10^{11} cells per gram faeces in the colon of adults[14-16].

The gut microbiota contributes to human physiology by its participation in several metabolic processes, its resistance to pathogen colonisation, its interaction with the immune system and its influence on organ development[17]. Germ-free mice, compared to specific-pathogen-free and wild-type mice, have different anatomical and physiological features including vitamin K and B deficiency, reduced liver size, reduced cardiac output and blood flow, smaller intestinal surface area, underdeveloped villi, high levels of mucin and longer transit time[18]. Although findings in mice cannot be directly extrapolated to humans, it demonstrates that host development and physiology are modulated by the microbiota. Regarding host metabolism, the gut microbiota has an important contribution via the breakdown of polysaccharides and polyphenols and via the synthesis of vitamins using enzymes that are not encoded in the human genome. In addition, the gut microbiota is involved in degradation of all sorts of dietary components and of some host-generated compounds, including bile acids and mucus[19]. Several studies have linked the gut microbiota to energy harvest capacity and adiposity of the host[20,21]. These metabolic features of the gut microbiota

deserve additional attention in relation to preterm infants, since these infants commonly suffer from nutrient deficits resulting in suboptimal growth outcomes. In addition to its metabolic function, the gastrointestinal tract, including its microbiota, plays a crucial role in host immune functioning. The mucosal immune system within the intestines provides tolerance to mutualistic microorganisms, is responsive to pathogens and acts a barrier against microbial translocation[17]. The role of the gut microbiota in providing colonisation resistance has been well demonstrated by the success rate of faecal microbiota transplantation as treatment for recurrent *Clostridium difficile* infection[22]. The contribution of the gastrointestinal tract and its microbiota to maturation and functioning of the immune system should be regarded in the case of preterm infants, who's immune compromised condition may have lifelong consequences. Thus far, an aberrant microbiota composition has been associated with the progression of numerous diseases and health conditions within and distant from the gastrointestinal tract, such as obesity, metabolic syndrome, several auto-immune diseases, irritable bowel syndrome and inflammatory bowel disease[23-26]. In most cases, however, it remains unclear whether an altered microbiota is a cause, a contribution or a consequence. Experiments in which specific microbes, a consortium of microbes or a complete microbiota is transplanted into healthy or diseased hosts are, so far, best in providing evidence for a causal relation between the gut microbiota and health outcomes [21,27-30].

Regarding early life, *Bifidobacterium* species are particularly abundant and regarded as beneficial for their positive influence on development and function of the metabolic and immune system and for their role in pathogen exclusion[31,32]. Bifidobacteria are gram-positive, anaerobic, non-motile, non-spore forming bacteria that can be branched shaped (bifid). Bifidobacteria produce lactic acid, which exhibits antimicrobial activity, as one of their main fermentation end products[31]. In addition, the ability of bifidobacteria to degrade glycan structures contained in human milk and mucus provides a competitive advantage during colonisation of the infant gut. Divergence in the diversity or abundance of *Bifidobacterium* species has been observed in several early and later life diseases and their beneficial properties are currently exploited as probiotics[33].

STUDYING THE GASTROINTESTINAL MICROBIOTA

For characterising the gut microbiota, most studies rely on the use of faecal samples as representative. Collection of faecal samples is relatively simple and non-invasive compared to alternatives such as biopsies and lavage collected via colonoscopy. It must be noted, however, that microbiota composition exhibits marked variation along different sites of the gastrointestinal tract and that findings vary with the use of different sample types[34-36].

For a long time, studying the gut microbiota consisted of cultivation, isolation and morphological and biochemical characterisation of microbial isolates. A major limitation of these conventional techniques is its selectivity towards readily cultivatable microbes, while the vast majority of microorganisms cannot be cultured[37]. Recent advances in technology and associated computational

methods allows for the assessment of the composition, function and activity of microorganisms in an ecosystem by studying their DNA (metagenomics), RNA (metatranscriptomics), proteins (metaproteomics) and metabolites (metabolomics). By combining these techniques, scientists attempt to answer the questions 'who are there?', 'what can they do?' and 'what are they doing?'. All technologies have their specific advantages and disadvantages related to information obtained, accuracy, costs and complexity.

In addition to metagenomics, in which the whole genomic content is sequenced, microbiota composition can be assessed by sequencing of specific genes such as the 16S rRNA gene and the 18S rRNA gene for prokaryotic and eukaryotic microorganisms respectively. Most studies, so far, applied sequencing of the 16S rRNA gene to determine the composition of the bacterial fraction of the gut microbiota, with the advantage that costs and complexity are relatively low, and the disadvantage that classification is up to genus level and no information about functionality is obtained. To understand the role of the gut microbiota in health and disease, it is of great importance to characterise its functional traits. In light of this, metaproteomics, in which the whole protein content of a sample is studied, is emerging[38]. This technique allows for the identification of expressed proteins and therefore provides insight in activity of the gut microbiota, thereby filling the functional gap created by sequencing of the 16S rRNA gene or the metagenome. However, identification of low abundant proteins remains challenging and the overall methodology is labour intensive compared to sequencing-based methods. Nevertheless, metaproteomics can provide a great amount of qualitative and quantitative data, giving insights in the host-microbiota relationship and its consequences to health and the development of complex diseases[39-42]. In 2009, Verberkmoes *et al.* reported the faecal metagenome and metaproteome of an adult twin pair[43]. They identified a common core proteome that was stable, but distinctive, for each individual. In addition, observed dissimilarities between the metagenome-based prediction of the proteome and the actual proteome highlight the added value of a metaproteomics approach.

INTESTINAL MICROBIOTA DEVELOPMENT

Foetal development is believed to occur in a sterile environment and colonisation of foetal membranes and amniotic fluid is particularly presented in the context of infections and preterm delivery. The concept of a sterile *in utero* environment has recently been challenged by studies showing bacterial signatures in the placenta, amniotic fluid, meconium and umbilical cord blood[44-46]. However, critical evaluation revealed that such studies generally lack good biological and technical controls and that they, when attempted, often failed to show viable bacteria[47]. Nevertheless, this field of research is emerging, and it may only be a matter of time before the answer to 'when do bacteria first colonise the human body?' is revealed.

It has been generally accepted that initial acquisition of the neonatal microbiota occurs during and after birth, a dynamic process during which microbial density and diversity are increasing and the community changes from being dominated

by aerotolerant to anaerobic bacteria[48]. The colonisation process is influenced by several host and environmental factors like gestational age, delivery mode, diet and antibiotic treatment. Delivery mode is a major determinant of which microbes are provided as first inoculum to the infant. The microbiota of vaginally delivered infants resembles that of the vagina and maternal faeces, while the microbiota of infants born through caesarean section resembles the skin and environmental microbiota[49,50]. In addition, delivery through caesarean section has been associated with delayed colonisation by specific taxa during the first six postnatal months[51]. During this time, the infant is solely exposed to milk feedings, either breastmilk or infant formula. The World Health Organisation recommends exclusive breastmilk feeding up till six months of age for the infant to benefit from the nutrients, immunoglobulin A and antimicrobial components contained in human breastmilk[52]. Exclusive breastmilk feeding selects for bacteria specialised in degradation of complex human milk oligosaccharides, particularly specific members of the *Bifidobacterium* genus[53]. This is shown by the observation that the microbiota of breastfed infants is typically dominated by *Bifidobacterium* species, while formula-fed infants harbour a more diverse microbiota. However, infant formulas have become more bifidogenic since the addition of prebiotics such as a mixture or short chain galactooligosaccharides (GOS) and long chain fructooligosaccharides (FOS)[54]. Solid food is generally introduced to an infant at around four to six months of age. This introduction exposes the gut microbiota to a larger array of non-digestible carbohydrates and other dietary components, thereby promoting bacterial species with the ability to utilise these substrates[55]. As such, transition towards a more diverse, adult-like microbiota is started and is achieved around three years of age[50,56]. The composition of the adult gut microbiota is generally stable and mainly comprises members of the Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia phyla[57]. Each individual harbours up to 1000 different bacterial species, with a host-specific composition and diversity that are influenced by lifestyle and dietary factors[56,58]. As humans age, the microbiota ages with them. Changes occur gradually over time, its rate and pattern being affected by age-associated alterations in lifestyle, diet, frailty and inflammation[59].

As mentioned before, the gut microbiota plays an essential role in human physiology and some consider the intestinal microbiota, containing its own specific functional traits, as an organ within an organ[60]. The human host and its microbiota must establish and maintain mutualistic symbiosis. Although the composition of the gut microbiota is individual-specific, it contains common compositional and functional signatures that, in healthy adults, are resilient and resistant to change[61]. Factors contributing to a resilient microbiota include its composition and diversity, as well as specific host-microbe and microbe-microbe interactions[61]. In early life, the intestinal microbiota can be considered simple, with low bacterial load, diversity and resilience and is therefore more responsive to perturbations. When circumstances are not allowing healthy microbiota development, like in the case of preterm birth, this may have serious consequences for health throughout life. However, the developing microbiota also provides a window of opportunity to manipulate the microbiota to our benefit.

INTESTINAL MICROBIOTA OF THE PRETERM INFANT

Preterm infants have an immature gut and are exposed to factors like extended hospitalisation, caesarean section, delayed enteral feeding and antibiotics. Therefore, establishment of their gut microbiota is likely to be impacted. Compared to term infants, preterm infants gut microbiota is less diverse and characterised by high levels of facultative anaerobic bacteria and delayed colonisation with obligate anaerobic bacteria like *Bifidobacterium*[62-64]. Immaturity of the gastrointestinal tract could affect the colonisation process and be an explanation for the observation that *Bifidobacterium* species colonise the preterm infant gut between 33-36 weeks postconceptional age, independent of postnatal age[65]. However, because of the strong association between gestational age and the extent and duration of care, it is practically impossible to examine the sole effect of gestational age on microbiota development.

Antibiotics are the most used therapeutics in neonatal intensive care units (NICUs), prescribed for the prevention and treatment of infections and sepsis[66]. Antibiotics, especially broad-spectrum antibiotics, such as vancomycin, penicillin's and cephalosporins, are commonly provided in a prophylactic and empirical manner. Early antibiotic treatment in preterm infants has been associated with perturbation of microbiota development by decreasing community diversity, delaying colonisation with *Bifidobacterium* and increasing the abundance of multi-drug resistant members of the *Klebsiella*, *Escherichia*, *Enterobacter* and *Enterococcus* genera[64,67,68]. Prolonged antibiotic treatment decreased microbiota diversity more profoundly than short antibiotic treatment[69,70]. In addition, maternal antibiotic use during the perinatal period has been related to aberrant gut microbiota establishment of the preterm infant and potentially affects the overall function of the developing bacterial community[67]. The early use of antibiotics and its associated disturbances of the gut microbiota have been associated with negative health outcomes including asthma, atopy and adiposity[71,72]. Adverse health outcomes, together with the risk of emerging multi-drug resistant strains, highlight the importance of limiting the unnecessary use of empiric antibiotics in the NICU[73,74].

Diet is acknowledged as major determinant for microbiota composition. During early life, nutritional needs are solely covered by milk feeding, preferably by breastfeeding. However, meeting the nutritional needs of preterm infants is challenging and requires the application of specific feeding strategies at individual level, including parenteral feeding, enteral feeding via a nasogastric tube, minimal enteral nutrition and supplementation of milk with fortifiers[75]. The influence of such feeding strategies on microbiota development are not well studied and outcomes are so far mainly presented in the context of growth rates, feeding intolerance, infections and NEC[10,76,77]. Up till date, studies have particularly focussed on the effect of various milk types, probiotics and prebiotics on the preterm infant gut microbiota. Mother's own milk seems most favourable compared to human donor milk and infant formula, promoting a more stable microbiota and colonisation with anaerobic bacteria like clostridia and bifidobacteria[78]. Like in term infants, a mixture of prebiotics GOS and FOS has bifidogenic capacities in preterm infants. However, whether probiotics affect

microbiota development remains to be elucidated since current findings are inconsistent[78]. In addition to the effect of diet on microbiota development, one should consider the role of the microbiota in processing these dietary inputs. Early differences in microbiota composition may affect the infants' food digestion capacity and subsequent energy harvest[21,79].

In addition to understanding how the gut microbiota of preterm infants develops and which factors influence this, it is of importance to comprehend the relation between the microbiota and health outcomes. NEC and infections are major contributors to mortality and morbidity among preterm infants. Several studies have shown abnormalities in gut microbiota composition and diversity before the onset of NEC and late onset sepsis[80]. However, a specific microbiota signature or causative bacterium has not been identified so far[81]. Associations have been made between NEC and the presence of members of the Proteobacteria phylum, specific *Clostridium* species and *Staphylococcus aureus*[77,80].Targeting the gut microbiota is considered as strategy to prevent the onset of infections and NEC in preterm infants. In light of this, several nutritional intervention strategies are being studied, of which human milk, probiotics and lactoferrin seem most promising[82].

To truly understand the relation between the gut microbiota and the developing preterm infant, one should not solely focus on microbiota composition but take microbiota activity and function into account. Multi-omics techniques are giving the first insights in functioning of the bacterial community in the preterm infant gut. Metagenomics has so far particularly shown activity of the preterm infant microbiota in view of antibiotic resistance and pathogenicity. Pressured by broad-spectrum antibiotics, the gut microbiota of preterm infants is likely to become dominated by potential pathogenic and antibiotic resistant species[62,83-85]. Analysing the metaproteome of preterm infant faeces revealed temporal changes in bacterial community structure alongside increasing activity towards the metabolism of complex carbohydrates[86,87]. In addition, this methodology has been applied to focus on the functionality of specific bacterial taxa contained in the preterm infant gut[88].The temporal increase in functional complexity of the preterm infant gut microbiota has also been shown by metabolomics, with metabolic complexity being related to weaning[89]. In addition, metabolomics has been applied to link specific metabolites to the onset of NEC and late-onset sepsis[90].

THE EIBER STUDY

'To investigate early life colonisation and establishment of the gut microbiota in extremely and very preterm infants' and 'to understand the effect of antibiotic treatment duration on gut microbiota development in preterm and term infants'. These two aims were the incentive for initiation of the EIBER study.The EIBER study is an observational, single-centre, non-intervention study conducted between 2011-2014 at Isala, Zwolle, The Netherlands. All preterm and term infants born between 24-42 weeks gestation that were admitted to the neonatal intensive care unit and the paediatric high care unit fulfilling the inclusion

criteria, but not the exclusion criteria, were eligible. In total, 119 preterm infants born between 24-32 weeks gestation and 119 preterm and term infants born between 32-42 weeks gestation were included. These infants were followed during the first six postnatal weeks, during which faeces and gastric aspirates were longitudinally collected for microbiota analysis. At all time, mothers were encouraged to provide human milk for tube feeding or to breastfeed when possible. The EIBER study provides the opportunity to get more insight in gastrointestinal function and microbiota development during the early life of preterm infants, and to link this development to various parameters including gestational age, nutritional factors and antibiotic treatment (Fig 1.2). Improved understanding of gastrointestinal functioning, which microbes colonise, what the microbes are doing and how microbiota establishment is affected in preterm infants may facilitate clinical practice and the development of effective strategies to prevent or treat diseases by targeting the gut microbiota.

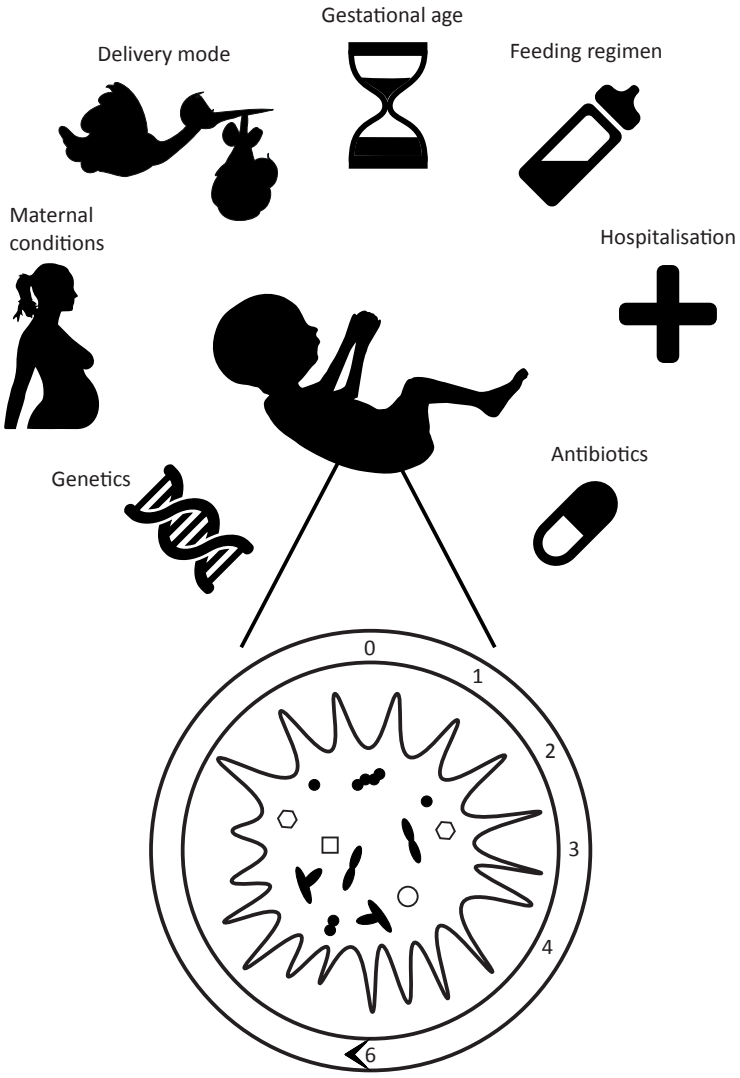


Figure 1.2 | **A schematic representation of the EIBER study.**

THESIS OUTLINE

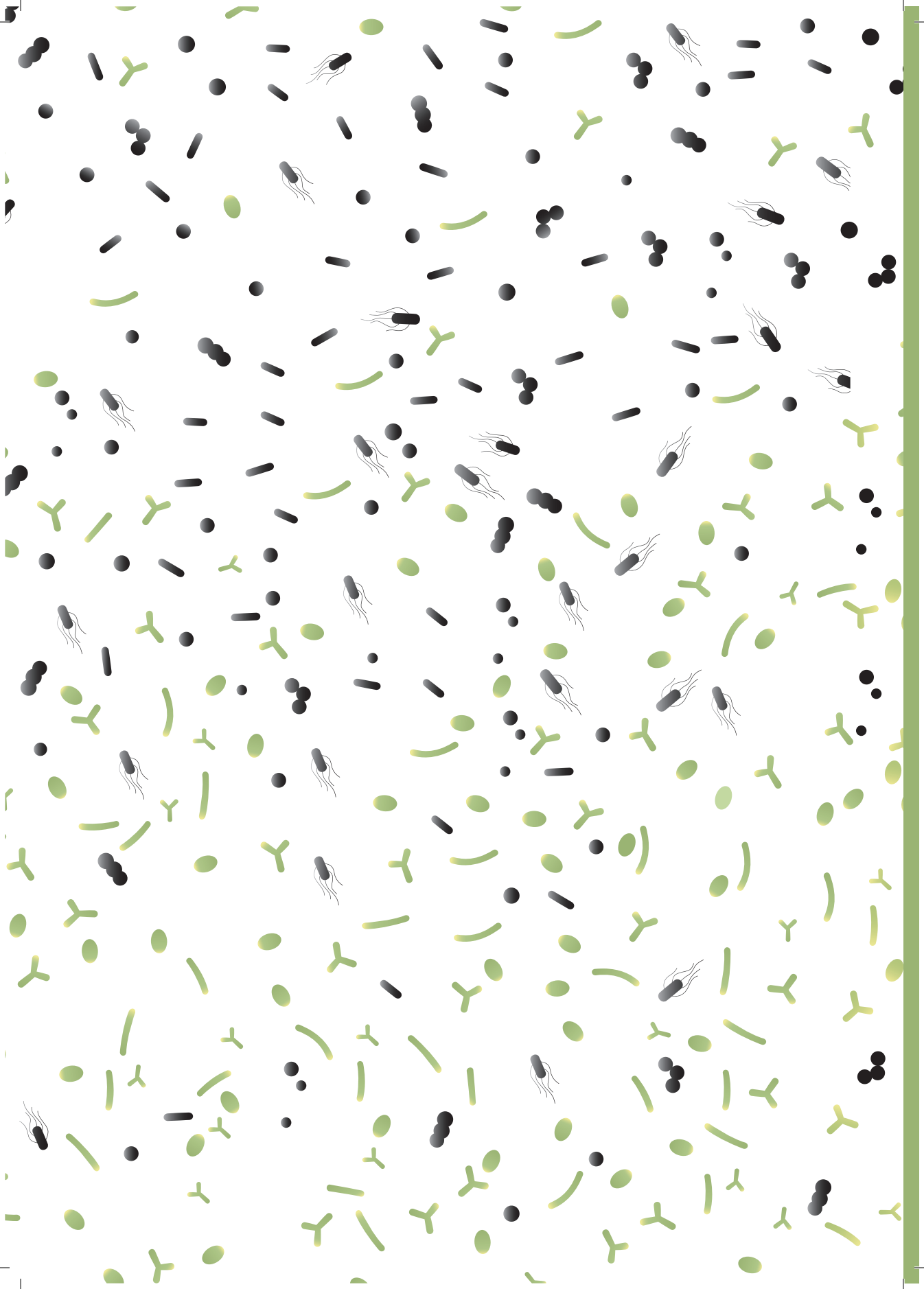
The perinatal period is regarded as window of opportunity for priming of lifelong metabolic, neurologic and immune functioning, with a profound role of the gut microbiota. Increasing the knowledge of gut microbiota development in preterm infants during this critical developmental period might aid clinical practice and improvement of early and later life health outcomes. The research described in this thesis aims to provide a better understanding of gastrointestinal function and microbiota development during the early life of preterm infants and to provide insight in which host and environmental factors play a key role in this development.

The first part of this thesis reports gastrointestinal function and microbiota development of extremely and very preterm infants. Current nutrition support strategies regularly fail to achieve *in utero* growth rates among preterm infants. In order to improve this, it is important to increase the understanding of how dietary inputs are processed by the immature and developing gastrointestinal system of preterm infants. **Chapter 2** describes the gastric and faecal metaproteome of ten infants as determined by LC-MS/MS. Here, were focussed on human- and bovine-derived proteins as an indication for gastrointestinal functioning and related this to bacterial protein-based microbiota composition. Regarding gut microbiota development in preterm infants, most studies so far focussed on microbiota composition, while knowledge about the functional signatures remains limited. In **chapter 3**, we concentrated on the bacterial fraction of the faecal proteome from the same ten infants and applied 16S rRNA gene amplicon sequencing as complementary approach to study functional and compositional development of the gut microbiota. We identified gestational age-specific developmental patterns of the preterm infant gastrointestinal tract and its microbiota and thereby increased the understanding of gastrointestinal function and maturation in relation to microbiota development. In addition, the observed gestational age-specific developmental patterns of the gut microbiota were associated with the degree of exposure to perinatal antibiotics and respiratory support.

The second part of this thesis aims to understand the effect of antibiotic treatment on early life gut microbiota development. The prophylactic and empirical use of antibiotics in the neonatal unit has led to decreased morbidity and mortality rates. However, it may also result in antibiotic overuse, antibiotic resistance and disturbed gut microbiota development. **Chapter 4** describes the effect of the prophylactic use of vancomycin during removal of a central venous catheter on gut microbiota development in a pilot of nineteen preterm infants born before 32 weeks gestation. Another pilot study, including fifteen infants born between 32 and 41 weeks gestation, is reported in **chapter 5**. Here we studied microbiota development during the early life of infants who varied in the duration of intravenous amoxicillin/ceftazidime treatment during the first postnatal week. As a continuation of this pilot study, **chapter 6** describes the effect of intravenous antibiotic treatment duration on intestinal microbiota development in 63 infants. We showed that elongation of postpartum antibiotic treatment with a few days potentially delays recovery of the microbiota with

weeks. Understanding the consequences of antibiotic treatment on microbiota development may support clinicians during the cost-benefit determination for antibiotic prescription (e.g. does the prophylactic use of antibiotics do more harm or good to the neonate?). In addition, we observed that bifidobacteria, despite being dominant, support a more rich and diverse community than other dominant bacterial taxa. In this way, bifidobacteria may contribute to development of a healthy and diverse ecosystem.

Chapter 7 provides a summary of the research described in this thesis and discusses its contribution to current knowledge in this field. In addition, it proposes future research directions to further increase and implement our knowledge about the preterm infant gut microbiota.



CHAPTER 2

CHARACTERISATION OF THE GASTRIC AND FAECAL PROTEOME TO UNRAVEL GASTROINTESTINAL FUNCTIONING AND MATURATION IN PRETERM INFANTS

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ABSTRACT

Despite the existence of various nutrition support strategies, preterm infants rarely meet *in utero* growth rates, and feeding the preterm infant remains challenging. In order to improve this, it is important to increase the understanding of how dietary inputs are processed by the immature and developing gastrointestinal system of preterm infants. We aimed to characterise the gastric and faecal proteome in order to obtain more insight in gastrointestinal function and maturation during the early life of preterm infants and to relate this to gut microbiota development. Therefore, gastric aspirates and faecal samples were collected from ten preterm infants during respectively the first two and first six postnatal weeks for metaproteomics through LC-MS/MS. In addition, gastric aspirates were collected daily during the first two postnatal weeks from 40 preterm infants for pH measurements and protease activity analysis. Gastric pH, pepsin and total protease activity showed high inter- and intra-individual variation. Median pH and pepsin activity were relatively stable over time, while median total protease activity increased during the second postnatal week. The gastric proteome was mainly affected by the ratio of human milk and formula feeding, with multiple bioactive components being significantly increased at times of human milk-predominated feeding. Composition of the faecal proteome was associated with gestational and postnatal age. Various enzymes, including lactase, sucrase-isomaltase and maltase-glucoamylase, were more abundant in very preterm infants than in extremely preterm infants, and were generally higher at higher postnatal age. Such proteins could therefore potentially serve as markers for gut maturation status. Quantity of various gut maturation markers could be associated with the abundance of specific bacterial taxa in faeces. It is important to combine gestational age-related digestive capacity, microbiota composition, and feeding type accordingly to further improve strategies for feeding the preterm infant. In light of this, specific host and microbial markers regarding gut function and maturation need to be identified. Insights in the gastric and faecal proteome, as described herein, might contribute to this.

INTRODUCTION

During early life, nutritional needs are completely covered by milk feeding, preferably by breastmilk feeding. The World Health Organisation recommends exclusive breastmilk feeding up till six months of age for the infant to benefit from the nutritional, immunomodulatory and antimicrobial components contained in human breastmilk[52]. Whether an infant is capable of meeting its nutritional needs by exclusive milk feeding is largely determined by the level of gastrointestinal (GI) maturity. Since some digestive enzymes and GI motility functions develop during later stages of gestation, preterm infants can experience feeding constraints involving abdominal distension, vomiting and gastric retention[8]. This raises a major challenge in meeting the nutritional needs of preterm infants and it therefore requires application of specific feeding strategies at individual level, including parenteral feeding, enteral feeding via a nasogastric tube, minimal enteral nutrition and supplementation of milk with fortifiers. Nevertheless, preterm infants rarely meet *in utero* growth rates and

feeding the preterm infant remains challenging[91]. Preterm infants generally have higher gastric pH than term infants, which may reduce activity of specific proteases[92]. However, proteolysis of the major milk proteins has been reported for preterm infants, in order from least to most resistant to degradation; β-casein, lactoferrin, serum albumin and α-lactalbumin[93]. A recent study showed that gastric protein digestion capacity is lower in preterm infants compared to term infants and that milk-derived proteases cannot compensate for this limitation[94]. The incomplete breakdown of proteins can be beneficial or harmful to the infant, depending on which specific protein remains intact (e.g. antimicrobials or allergens)[95]. In addition to the importance of gastrointestinal maturity for meeting nutritional needs, bacteria residing the human gastrointestinal tract play an essential role in metabolism of dietary components. The metabolic capacity of the gut microbiota is distinct, but complementary, to the activity of human gastrointestinal enzymes[96]. Variation in microbiota composition might differentially affect energy harvest and storage, and therefore weight gain, of the preterm infant[21,97,98]. In order to improve current feeding strategies, it is important to increase the understanding of how dietary inputs are processed by the immature and developing GI system of preterm infants. In addition, the relation between gut maturation and microbiota development needs to be further elucidated. In the study described herein, we characterised the gastric and faecal metaproteome of preterm infants by LC-MS/MS. In addition, the influence of multiple environmental factors on composition of the gastrointestinal proteome, and the relation between the proteome and gut microbiota development, were determined.

MATERIALS AND METHODS

Subjects and sample collection

This study was part of an observational, single-centre, non-intervention study involving (pre)term infants admitted to the hospital level III NICU or the level II neonatal ward of Isala in Zwolle, The Netherlands. The ethics board from METC Isala Zwolle concluded that this study does not fall under the scope of the Medical Research Involving Human Subjects Act (WMO). Informed consent was obtained from both parents of all individual participants included in the study. To get insight in digestive functioning of preterm infants, gastric aspirates and faecal samples were collected for metaproteomics (LC-MS/MS) and enzyme activity analysis. Aspirates of residual gastric content were collected daily during the first two postnatal weeks from 40 preterm infants. Faecal samples were collected right after birth and at postnatal weeks one, two, three, four and six from ten preterm infants. A sampling scheme can be found in table 2.1. All samples were stored temporally at -20°C until transfer to -80°C. Infant clinical characteristic can be found in table 2.2.

LC-MS/MS - protein extraction, in gel digestion procedures and data analysis

Gastric aspirates and faecal samples that were collected weekly from ten preterm infants during respectively the first two and six postnatal weeks were used (table 2.1). Proteins were mechanically extracted from faeces by repeated bead beating as described previously[99]. Gastric aspirates were defrosted on ice, centrifuged (3000rpm; 4°C) and the pellet was removed. pH of the supernatant

was determined, and the sample was centrifuged again to remove all debris (max rpm; 4°C). Faecal and gastric proteins were quantified using the Qubit® Protein Assay Kit a on a Qubit®2.0 fluorometer (LifeTechnologies, Carlsbad, CA, USA) and diluted in PBS to obtain a 3 µg/µl and 5 µg/µl concentration respectively. In gel digestion procedures and database construction were performed as previously described[99]. Obtained MS/MS spectra were analysed with MaxQuant 1.3.0.5[100] as previously described[99]. False discovery rates were set to 0.01 on peptide and protein level. Minimally two peptides were necessary for protein identification of which at least one is unique and at least one is unmodified. This led to the identification of 2315 protein groups, of which 867 were human and/or bovine derived. Each protein group was assigned to its corresponding KEGG Orthology (KO) identifier and annotated using the KEGG Brite Orthology and KEGG Brite Exosome databases[101]. 770 human- and bovine-derived protein groups were identified in gastric samples of which up to 40% and 70% could be functionally classified using respectively the KEGG Brite Orthology and Exosome databases. Classified proteins identified in gastric aspirates during the first two postnatal weeks were mainly assigned to CHO metabolism, transport and catabolism, signal transduction and lipid metabolism and were part of immune, digestive and endocrine functioning (Fig S2.1a, S2.1b). Exosomal proteins mainly derived from breast milk and hepatic cells (Fig S2.1c). 792 human- and bovine-derived protein groups were identified in meconium and faeces of which up to 70% and 30% could be functionally classified using respectively the KEGG Brite Orthology and Exosome databases. Classified proteins identified in meconium and faeces during the first six postnatal weeks contributed to signal transduction and transport and catabolism and were part of immune, digestive and endocrine functioning (Fig S2.1a, S2.1b). Exosomal proteins derived from breast milk, hepatic cells and other body fluids (saliva and urine) (Fig S2.1c). Intensity based absolute quantification (iBAQ) and label free quantification (LFQ) intensities were used for within and between sample comparisons respectively. When one protein could be classified into multiple functional categories, iBAQ intensity values were balanced between these categories for profile generation. Protein profiles based on iBAQ intensities were used for redundancy analysis using Canoco multivariate statistics software v5[102]. Here, a p-value of less than 0.05 was used as threshold for statistical significance. Further statistical analyses were performed in Perseus[103]. Here, LFQ intensities were 10Log transformed, samples were assigned to its designated study group, protein groups were filtered based on at least two valid values in at least one group, invalid values were replaced with 4, and a two-sample t-test was performed (Benjamini-Hochberg correction, S0=1). Bacterial-derived proteins contained in the faecal proteome were taxonomically classified and quantified as described previously[99]. To relate microbiota composition to human- and bovine-derived proteins, LFQ intensities of proteins identified in ≥50% of the samples (n=101 proteins) were correlated (Spearman correlation) with the abundance of the three most abundant bacterial genera: *Bifidobacterium*, *Klebsiella* and *Enterococcus*. A p-value of less than 0.05 was used as threshold for statistical significance.

Enzyme activity analysis

Gastric aspirates were collected daily during the first two postnatal weeks from 40 preterm infants and stored at -20°C until transfer to -80°C (Table 2.1). Samples were defrosted on ice, centrifuged (3000rpm; 4°C) and the cream layer was removed.The pH of the supernatant was determined, and the sample was centrifuged again to remove any remaining cream fraction (14000rpm; 4°C).Total protease and pepsin activity were determined using the green fluorescence EnzChek® Protease Assay Kit (Molecular Probes, Eugene, OR, USA) in duplicate and according to manufacturer's instructions. For determining total protease activity, 10mM TRIS buffer with pH 7.8 was used and the standard curve was generated using pancreatin from porcine pancreas (Sigma-Aldrich) to reach activities of 0, 0.78, 1.55, 3.10, 6.20, 12.40, 24.80 and 49.60 U/ml. For determining pepsin activity, 10mM HCl buffer with pH 2.2 was used and the standard curve was generated using pepsin from porcine gastric mucosa (Sigma-Aldrich) to reach activities of 0, 1.56, 3.13, 6.25, 12.50, 25, 50 and 100 U/ml.

Table 2.1 | **Sampling scheme.** Red: gastric aspirate, Black: faeces. Number next to circle indicates the number of available samples for that specific time point.

Infant	1	2	3	4	5	6	Wk 1	8	9	10	11	12	13	Wk 2	Wk 3	Wk 4	Wk 6
A				●●				●●					●	●	●	●	●
B	●							●●					●●		●		●
C				●●			●●						●	●	●	●	●
D						●●								●●	●	●	●
E		●●					●●						●	●	●	●	●
F			●●				●							●●	●	●	●
G	●	●					●●						●	●	●	●	●
H	●●				●		●							●●	●	●	●
I			●●					●●					●	●	●	●	●
J			●●			●	●						●	●	●	●	●
n=40	● 28	● 20	● 30	● 27	● 27	● 35	● 29	● 32	● 35	● 28	● 34	● 31	● 31	● 32			

Table 2.2 | **Infant characteristics.** A-J: individual infants, GA: gestational age, EP: extremely preterm, VP: very preterm, MLP: moderate- to late preterm, BW: birth weight, FI: food intolerance, FEF: full enteral feeding, AB1: first antibiotics course, AB2: second antibiotic course, Amx: amoxicillin, Ctz: ceftazidime, Va: vancomycin, Erm: erythromycin, mem: meropenem, Mtz: metronidazole. * Data was available for 24 infants: 9 EP and 15 VP infants. ** Percentage of human milk as enteral feeding at postnatal weeks 1, 2, 3, 4 and 6.

Infant	GA	BW (g)	Gender	Delivery mode	FI	Days until FEF	% Enteral feeding	% Human milk**	AB1	AB2
A	25+2	680	Female	Vaginal	Yes	10	39, 89, 100, 100, 100	91, 100, 100, 100, 98	Amx/Ctz/Va	Ctz/Va
B	26+4	670	Male	C-section	Yes	17	15, 49, 96, 98, 100	89, 96, 42, 22, 7	Amx/Ctz/Va	Mem/Va/Erm
C	26+6	630	Male	C-section	Yes	14	16, 63, 79, 100, 100	92, 100, 100, 100, 92	Amx/Ctz/Va	Ctz/Va/Mtz
D	27+0	1095	Male	C-section	No	10	43, 93, 22, 49, 100	96, 100, 100, 96, 100	Amx/Ctz/Erm	Ctz/Va
E	27+5	925	Male	C-section	Yes	16	6, 32, 98, 100, 100	51, 100, 100, 98, 89	Amx/Ctz	Ctz/Va
F	30+4	1260	Female	C-section	Yes	12	23, 79, 100, 100, 100	32, 90, 100, 100, 93	Amx/Ctz	-
G	30+4	1600	Female	C-section	No	8	50, 100, 100, 100, 100	4, 87, 100, 100, 66	-	-
H	30+4	1220	Female	C-section	No	12	25, 82, 100, 100, 100	58, 96, 100, 100, 100	Ctz/Va	-
I	30+6	1750	Male	Vaginal	No	8	51, 100, 100, 100, 100	91, 100, 100, 100, 100	Amx/Ctz	-
J	30+1	1675	Female	C-section	No	9	66, 100, 100, 100, 100	30, 95, 56, 9, 0	Amx/Ctz	-
	GA	**BW (g)**	**Female (%)**	**C-section (%)**	**FI (%)**	**Days until FEF***	**Enteral feeding (%)***	**Human milk (%)*, ****		
ALL (40)	28.7 ± 2.1	1177 ± 388	47.5	57.5	22.5	11	36, 84, 94, 92, 100	69, 93, 89, 86, 82		
EP (14)	26.3 ± 1.1	804 ± 150	42.9	50.0	35.7	13	26, 69, 85, 89, 100	69, 86, 82, 80, 76		
VP (23)	29.8 ± 1.1	1327 ± 287	52.2	56.5	17.4	10	42, 93, 100, 93, 100	69, 96, 93, 90, 86		
MLP (3)	33.7 ± 0.2	1768 ± 312	33.3	100.0	0.0	-	-	-		

RESULTS

Gastric pH and protease activity are highly variable during the first two postnatal weeks

Gastric pH, total protease and pepsin activity were determined in 40 preterm infants during the first two postnatal weeks and tested for associations with gestational age, postnatal age, delivery mode, gender, food intolerance, % enteral feeding and % human milk feeding. In general, gastric pH varied greatly between and within infants, with a mean difference of 4.2 ± 1.3 between the lowest and highest pH measured within infants over time. Median gastric pH fluctuated between 4.5 and 5.5 over time (Fig 2.1a) and was not related to gestational age or any other variable. Gastric pH was exceptionally high (>8.0) in six infants at day of birth, but their gastric pH did not vary from the other infants during the following six postnatal weeks. Total protease and pepsin activity showed high inter- and intra-individual variation. Nevertheless, median activity indicated that total protease activity was higher during the second than first postnatal week, while pepsin activity remained more stable with a minor increasing trend during the first five postnatal days and a gradual decrease thereafter (Fig 2.1b, 2,1c). Variation in pepsin activity significantly correlated with gastric pH (ρ=-0.404, p=5.9E-18), but pepsin- and total protease activity were not associated with gestational age, delivery mode, gender, food intolerance, % enteral feeding and % human milk feeding (data not shown). Pepsin could not be identified in the generated gastric proteome data. However, other proteases, like chymotrypsin-like elastase family members 2A, 3A and 3B, endo- and ceruloplasmin and trypsin, could be identified. Of these, trypsin (ρ=0.432, p=0.010) and chymotrypsin-like elastase family member 3A (ρ=0.475, p=0.004) correlated with total protease activity.

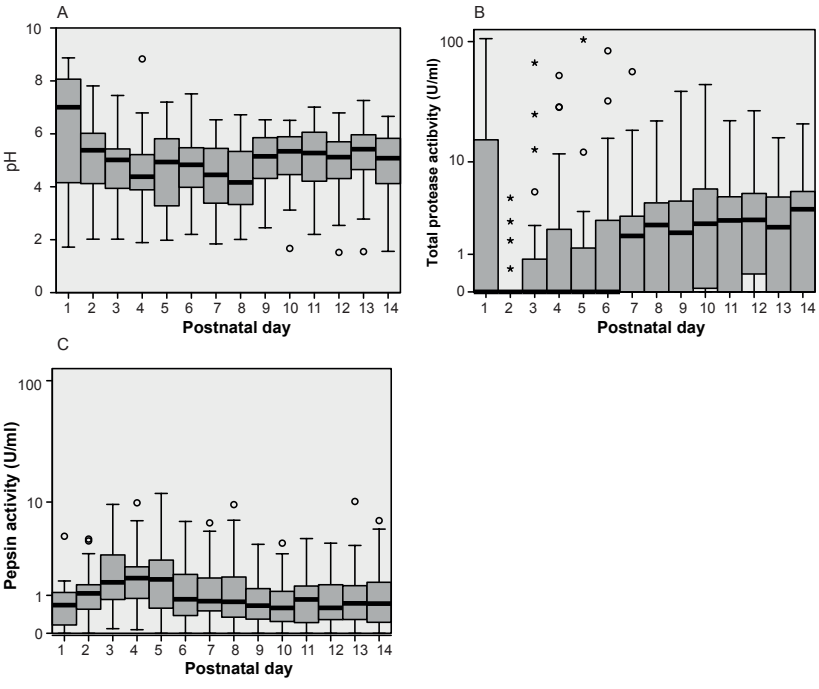


Figure 2.1 | **Dynamics of gastric pH (A), total protease activity (B) and pepsin activity (C) during the first two postnatal weeks.** Boxplots show the median, 25th and 75th percentiles, and minimal and maximal values with the exception of outliers (circles) and extremes (asterisks).

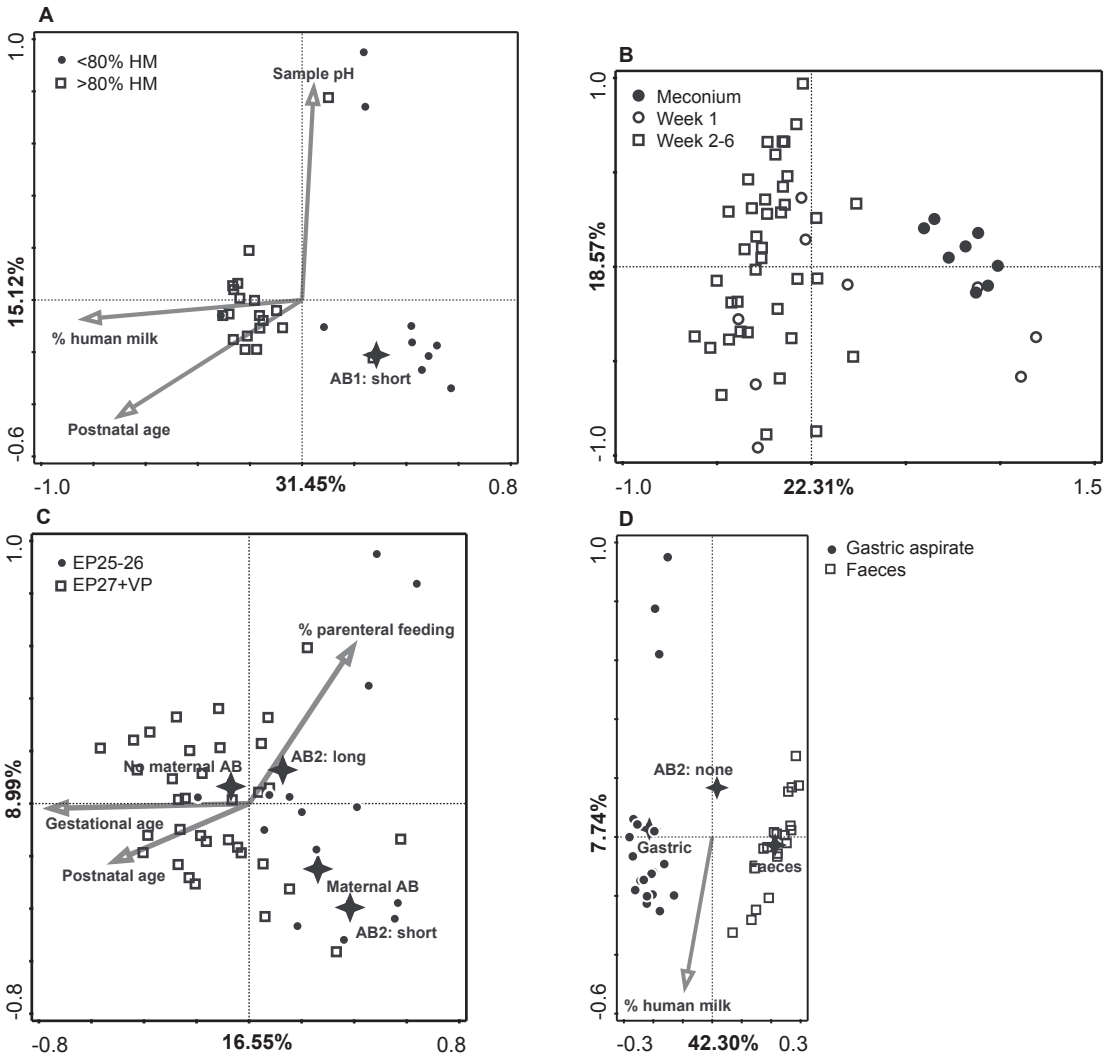
Human milk feeding is associated with an increase in digestive- and immune-related proteins

Throughout the first two postnatal weeks, milk-derived proteins, including lactotransferrin, alpha-lactalbumin and caseins, were particularly abundant in gastric samples (Table 2.3). Serum albumin, bile-salt activated lipase and several immunoglobulin structures were among the 25 most abundant proteins in gastric aspirates and are very likely to be milk-derived as well.The percentage of human milk in enteral feeding (22.7%, p=0.002) and sample pH (11.8%, p=0.002) were the main drivers for differences in the gastric proteome of preterm infants during the first two postnatal weeks (Fig 2.2a). Forty-five (out of 350) proteins were significantly different between gastric samples at time that enteral feedings contained more or less than 80% human milk (Fig 2.3a, Table S2.1). At the time when infants received less than 80% human milk, bovine-derived milk proteins were significantly increased. On the other hand, when enteral feeding consisted of more than 80% human milk, not the major human milk proteins, but proteins involved in digestive functioning (e.g. bile salt-activated lipase, mucin-4, α-amylase) and immune functioning (e.g. immunoglobulins, antigens) were significantly increased.The contribution of gestational age to composition of the gastric proteome was on the edge of significance (3.7%, p=0.056). Comparing the gastric proteome between extremely preterm (EP) and very preterm (VP) infants, however, mainly reflected their difference in the ratio of human milk to formula intake. Since the percentage of human milk as enteral feeding during the first two postnatal weeks was lower in VP infants, their gastric proteome contained significantly increased cow milk-derived whey proteins and caseins (Table S2.2).

Passage of milk-derived proteins through the GI-tract

The gastric proteome was very distinct from the faecal proteome, with sample type explaining 41.7% of the variation in proteome composition (p=0.002) (Fig 2.2d). Between gastric and faecal samples, 271 (out of 569) proteins were significantly different (Fig 2.3c, Table S2.3). In gastric aspirates, 137 proteins were more abundant of which milk-derived whey proteins and caseins, as well as bile-salt activated lipase and lysozyme C were among the most differentially abundant proteins. Although being significantly decreased, human milk-derived whey proteins serum albumin and lactotransferrin were still detected in high quantity in faecal samples of all preterm infants, while this was not observed for cow milk-derived proteins (Table 2.5). In addition, bile-salt activated lipase was still highly abundant in faeces of extremely preterm (EP) infants, but not in very preterm (VP) infants (Table 2.5). During the first postnatal week, bovine-derived whey proteins and caseins were abundant in gastric aspirates of very preterm infants receiving a lower proportion of human milk as enteral feeding (Table 2.5; infant F, G and J; 32%, 4%, 30% human milk). In faeces, various proteases and peptidases, including trypsin, chymotrypsin and several brush border enzymes were more abundant compared to gastric samples. A big variety of proteins were consistently identified in the gastric and faecal proteome, but did not differ in quantity, including prostasin, annexin A2, antithrombin-III, Ig alpha-1 chain C region, triosephosphate isomerase, ceruloplasmin, leucine-rich alpha-2-glycoprotein, galectin-3-binding protein, Ig lambda-2 chain C region, protein S100-A8, histone H4, alpha-1-antichymotrypsin, Ig kappa variable 3-11 and Ig kappa chain V-I region (data not shown).

Figure 2.2 | **Ordination analysis of the gastric and faecal proteome.**
A. Redundancy analysis of the gastric proteome during the first two postnatal weeks.
B. Principal component analysis of the meconium and faecal proteome during the first six postnatal weeks.
C. Redundancy analysis of the faecal proteome during postnatal weeks 1-6.
D. Redundancy analysis of the gastric and faecal proteome at postnatal weeks 1 and 2.
Arrows and stars indicate explanatory variables that are respectively continuous or a factor. AB: antibiotics, AB2: second antibiotic course, EP: extremely preterm, VP: very preterm, HM: human milk.


A
<80% HM
>80% HM
Sample pH
% human milk
AB1: short
Postnatal age
15.12%
31.45%
B
Meconium
Week 1
Week 2-6
18.57%
22.31%
C
EP25-26
EP27+VP
% parenteral feeding
No maternal AB
AB2: long
Gestational age
Postnatal age
Maternal AB
AB2: short
8.99%
16.55%
D
Gastric aspirate
Faeces
AB2: none
Gastric
Faeces
% human milk
7.74%
42.30%

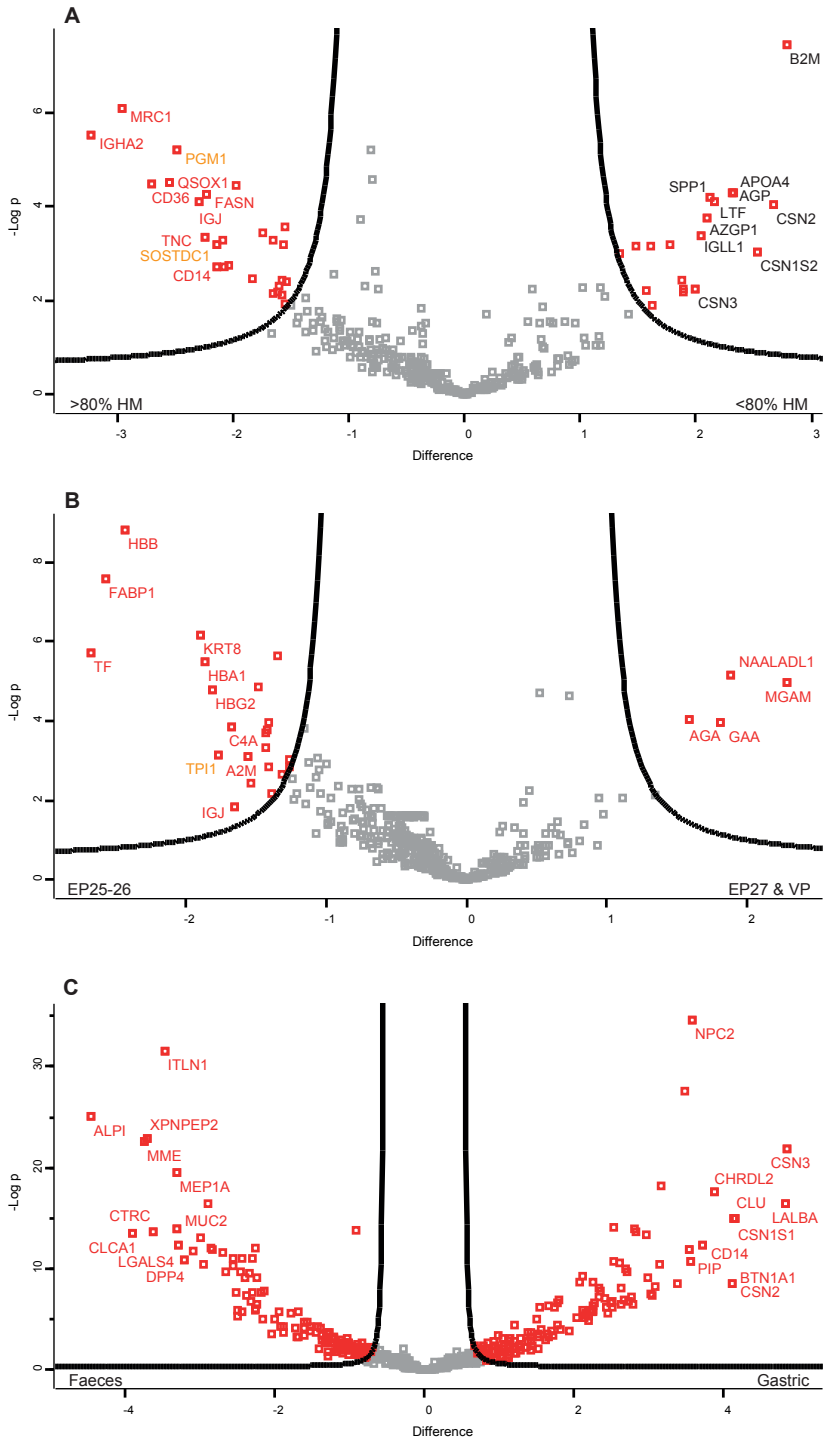


Figure 2.3 | **Volcano plots showing the difference within and between the gastric and faecal proteomes.** Label free quantification (LFQ) intensities were 10log transformed, samples were assigned to its designated study group, protein groups were filtered based on at least two valid values in at least one group, invalid values were replaced with 4, and a two-sample t-test with Benjamini-Hochberg correction and S0=1 was performed. p-values as indicated on the y-axis are -10log transformed. Text in red, orange and black indicate human-, humanbovine- and bovine-derived proteins respectively. **A.** Differentially abundant proteins in gastric aspirates at time that enteral feedings contained more or less than 80% human milk. HM: human milk. **B.** Differentially abundant proteins in faeces from preterm infants born at varying gestational age. EP25-26: extremely preterm born at 25 or 26 weeks gestation, EP27: extremely preterm born at 27 weeks gestation, VP: very preterm. **C.** Differentially abundant proteins between gastric and faecal samples at postnatal weeks 1 and 2.

The faecal proteome reflects gestational- and postnatal age

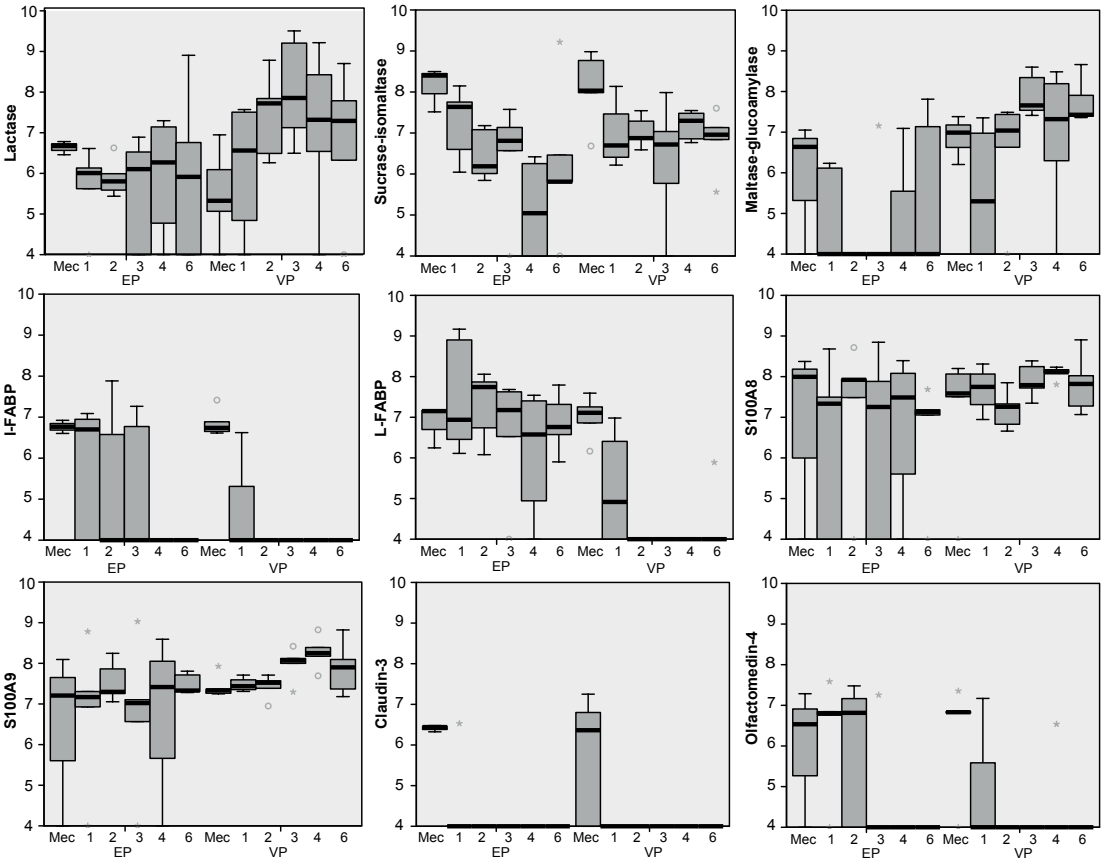
The proteome of meconium was distinct from that of faecal samples (Fig 2.2b). Differentiation was mainly associated with increased ras-related protein Rab-11A, claudin-3 and mucin-13 in meconium. Majority of the 25 most abundant proteins were shared between meconium and faecal samples of which alpha-1-antitrypsin, and immunoglobulin kappa variable 3-11 were particularly abundant (Table 2.4). Lactotransferrin and immunoglobulin alpha-1 chain C region were abundant in faeces, but not in meconium, and intestinal-type alkaline phosphatase was especially abundant during postnatal weeks 3-6 (Table 2.4). Gestational age (10.4%, p=0.002) and postnatal age (6.3%, p=0.002) were the main drivers for differences in the faecal proteome of preterm infants during the first six postnatal weeks (Fig 2.2c). Twenty-six (out of 447) proteins were significantly different between faecal samples from infants at 25-26 weeks gestation (EP25-26) compared to those born at 27 weeks (EP27) and 30 weeks (VP) gestation (Fig 2.3b, Table S2.4). Twenty-two proteins were increased in EP25-26 infants, of which serotransferrin, liver-type fatty acid binding protein and several haemoglobin subunits were most differentially abundant. Intestinal maltase-glucoamylase, n-acetylated-alpha-linked acidic dipeptidase, lysosomal alpha-glucosidase and aspartylglucosaminidase were more abundant in EP27 and VP infants. In addition to age, percentage parenteral feeding (4.6%, p=0.006) and antibiotic treatment-related factors (7.3%, p<0.05) contributed to the composition of the faecal proteome. Here, percentage parenteral feeding was positively associated with abundance of annexin A4 and A13, glutathione S-transferase A2 and dihydrolipoyl dehydrogenase (data not shown). The number of samples at time of high parenteral feeding was limited, which prohibited statistical analysis to determine differentially abundant proteins between samples collected at time of high or low parenteral feeding.

Assisting the quest towards markers for gut maturation and function

Several proteins have been suggested as potential marker for gut maturation, including digestive enzyme markers lactase, trehalase (TREH), fucosyltransferase (FUT), sucrase-isomaltase (SI), maltase-glucoamylase (MGAM), gut wall integrity and inflammation markers fatty acid binding protein (FABP), calprotectin, claudin-3, trefoil factors, S100A12 and intestinal stem cell marker olfactomedin-4. TREH, FUT, trefoil factors and S100A12 were not or only scarcely detected in the preterm infant faecal proteome. Human-derived lactase, sucrase-isomaltase, MGAM, FABP, calprotectin, claudin-3 and olfactomedin-4 were detected in the faecal proteome and their temporal patterns can be found in figure 2.4. Lactase showed an increasing pattern over time in very preterm infants, which was not observed in extremely preterm infants, resulting in higher lactase levels from the second postnatal week onwards in VP compared to EP infants. Sucrase-isomaltase levels were highest in meconium samples of preterm infants and while being stable from the first postnatal week onwards in VP infants, SI levels further decreased over time in EP infants. From the first postnatal week onwards, MGAM levels were higher in the faecal proteome of VP infants than of EP infants. In contrast, liver- and intestinal-type FABP were only observed during the first postnatal

week in VP infants, while also being observed at later postnatal age in EP infants, particularly liver-type FABP. Calprotectin, a heterodimer of S100A8/A9, was not detected as an entity, but S100A8 and S100A9 were separately identified. Both showed to be more consistently detected in VP infants than in EP infants. Claudin-3 and olfactomedin-4 were only abundant during early time points in both EP and VP infants.

Figure 2.4 | **Temporal dynamics of gastrointestinal integrity and maturation markers in the faecal proteome of extremely and very preterm infants.** The y-axis shows 10Log transformed label free quantification (LFQ) intensity. The x-axis shows the postnatal age in weeks for each gestational age group. Boxplots show the median, 25th and 75th percentiles, and minimal and maximal values with the exception of outliers (circles) and extremes (asterisks). Mec: meconium, EP: extremely preterm, VP: very preterm



Microbiota development coincides with GI maturation

To relate gastrointestinal function and maturation to the developing gut microbiota, relative abundances of the three most abundant bacterial genera contained in meconium and faeces were correlated to the amount of human- and bovine-derived proteins therein (Table 6). Various proteins, including I-FABP, serum albumin, and immunoglobulins negatively correlated with *Bifidobacterium*, of which the correlation with I-FABP was most profound. The quantity of antioxidant superoxide dismutase positively correlated with *Bifidobacterium* abundance, and negatively correlated with *Enterococcus*. While *Bifidobacterium* abundance also positively correlated with digestive enzymes maltase-glucoamylase and lactase, the abundance of respectively *Enterococcus* and *Klebsiella* correlated negatively with these enzymes. Aminopeptidase N, an enzyme involved in the degradation of peptides resulting from protein cleavage by gastric and pancreatic proteases, negatively correlated with *Enterococcus*, while being positively associated with *Klebsiella*. Regarding immune regulation, abundance of *Klebsiella* positively correlated with acid sphingomyelinase-like phosphodiesterase 3b, a protein acting as negative regulator of toll-like receptor signalling. Carcinoembryonic antigen-related cell adhesion molecules, a human glycoprotein belonging to the immunoglobulin superfamily, was also positively associated with abundance of *Klebsiella*, while negatively correlating with *Bifidobacterium* and *Enterococcus*.

First			Week 1			Week 2		
Protein	**Fraction**	**Host**	**Protein**	**Fraction**	**Host**	**Protein**	**Fraction**	**Host**
Serum albumin	0.2263	Human	Uncharacterized protein	0.1468	Bovin	Lactotransferrin	0.1533	Human
Uncharacterized protein	0.1791	Bovin	Lactotransferrin	0.1321	Human	Beta-casein	0.1461	Human
Haemoglobin subunit beta	0.0568	Human	Serum albumin	0.1167	Human	Serum albumin	0.1213	Human
Protein IGKV3-11	0.0446	Human	Beta-casein	0.0869	Human	Alpha-lactalbumin	0.1036	Human
Lactotransferrin	0.0410	Human	Alpha-lactalbumin	0.0756	Human	Kappa-casein	0.0684	Human
Haemoglobin subunit alpha	0.0353	Human	Kappa-casein	0.0380	Human	Polymeric immunoglobulin receptor	0.0431	Human
Alpha-lactalbumin	0.0302	Human	Protein IGKV3-11	0.0375	Human	Bile salt-activated lipase	0.0401	Human
Alpha-1-antitrypsin	0.0289	Human	Polymeric immunoglobulin receptor	0.0366	Human	Protein IGKV3-11	0.0390	Human
Beta-casein	0.0264	Human	Bile salt-activated lipase	0.0269	Human	Ig alpha-1 chain C region	0.0296	Human
Polymeric immunoglobulin receptor	0.0218	Human	Ig alpha-1 chain C region	0.0249	Human	Alpha-S1-casein	0.0215	Human
Ig alpha-1 chain C region	0.0202	Human	Alpha-lactalbumin	0.0193	Bovin	Ig lambda-3 chain C regions (Fragment)	0.0168	Human
Serotransferrin	0.0198	Human	Alpha-S1-casein	0.0158	Bovin	Clusterin	0.0118	Human
Ig lambda-3 chain C regions (Fragment)	0.0161	Human	Ig lambda-3 chain C regions (Fragment)	0.0124	Human	Uncharacterized protein	0.0116	Bovin
Alpha-lactalbumin	0.0136	Bovin	Clusterin	0.0120	Human	Lysozyme C	0.0113	Human
Haemoglobin subunit gamma-2	0.0126	Human	Alpha-S1-casein	0.0120	Human	Alpha-1-antichymotrypsin	0.0087	Human
Neutrophil defensin 3	0.0114	Human	Alpha-1-antichymotrypsin	0.0118	Human	Neutrophil defensin 3	0.0083	Human
Kappa-casein	0.0102	Human	Lysozyme C	0.0104	Human	Alpha-1-antitrypsin	0.0082	Human
Histone H4 (Fragment)	0.0091	Bovin	Alpha-1-antitrypsin	0.0099	Human	Histone H4 (Fragment)	0.0080	Bovin
Clusterin	0.0089	Human	Alpha-S2-casein	0.0078	Bovin	Immunoglobulin J chain (Fragment)	0.0077	Human
Haemoglobin subunit alpha	0.0083	Bovin	Immunoglobulin J chain (Fragment)	0.0071	Human	Monocyte differentiation antigen CD14	0.0065	Human
Alpha-1-antichymotrypsin	0.0078	Human	Haemoglobin subunit gamma-2	0.0067	Human	Histone H2A.J	0.0054	Bovin
Lysozyme C	0.0075	Human	Histone H4 (Fragment)	0.0055	Bovin	Haemoglobin subunit gamma-2	0.0049	Human
Immunoglobulin J chain (Fragment)	0.0072	Human	Kappa-casein	0.0054	Bovin	Lactadherin	0.0045	Human
Alpha-S1-casein	0.0069	Human	Protease serine 2 preproprotein	0.0051	Human	Haemoglobin subunit alpha	0.0040	Human
Histone H2A.J	0.0052	Bovin	Beta-casein	0.0050	Bovin	Butyrophilin subfamily 1 member A1	0.0036	Human

Table 2.3 | **Top 25 most abundant gastric proteins during the first two postnatal weeks.** Relative abundances are based on intensity based absolute quantification (iBAQ) intensities.

Table 2.4 | **Top 25 most abundant proteins in meconium and faeces during the first six postnatal weeks.** Relative abundances are based on iBAQ intensities.

Meconium			Week 1			Week 2		
Protein	**Fraction**	**Host**	**Protein**	**Fraction**	**Host**	**Protein**	**Fraction**	**Host**
Alpha-1-antitrypsin	0.2583	Human	Alpha-1-antitrypsin	0.1749	Human	Serum albumin	0.2089	Human
Protein IGKV3-11	0.1266	Human	Protein IGKV3-11	0.1080	Human	Ig alpha-1 chain C region	0.2060	Human
Transthyretin	0.0967	Human	Ig alpha-1 chain C region	0.0626	Human	Protein IGKV3-11	0.1204	Human
Aminopeptidase N	0.0533	Human	Lactotransferrin	0.0608	Human	Lactotransferrin	0.0813	Human
Serum albumin	0.0428	Human	Serum albumin	0.0508	Human	Alpha-1-antitrypsin	0.0740	Human
Alpha-1-antichymotrypsin	0.0281	Human	Ig lambda-3 chain C regions (Fragment)	0.0312	Human	Alpha-1-antichymotrypsin	0.0618	Human
Polyubiquitin-C (Fragment)	0.0213	Human	Histone H4 (Fragment)	0.0278	Bovin	Immunoglobulin J chain (Fragment)	0.0223	Human
Calcium-activated chloride channel regulator 1	0.0206	Human	Alpha-1-antichymotrypsin	0.0266	Human	Protein IGHV3-72	0.0204	Human
Ig lambda-3 chain C regions (Fragment)	0.0194	Human	Transthyretin	0.0261	Human	Ig lambda-3 chain C regions (Fragment)	0.0175	Human
Histone H4 (Fragment)	0.0171	Bovin	Aminopeptidase N	0.0162	Human	Ig alpha-2 chain C region	0.0138	Human
Ferritin light chain	0.0121	Human	Apolipoprotein D (Fragment)	0.0159	Human	Ig kappa chain V-III region WOL	0.0122	Human
Ig kappa chain V-III region WOL	0.0120	Human	Chymotrypsin-like elastase family member 3A	0.0156	Human	Alpha-2-macroglobulin	0.0091	Human
Mucin-13	0.0094	Human	Protein S100-A9	0.0154	Human	Trypsin-1	0.0079	Human
Histone H3	0.0086	Human	Ferritin heavy chain	0.0150	Human	Ig heavy chain V-I region EU	0.0073	Human
IgGFc-binding protein	0.0085	Human	Histone H2A.J	0.0139	Bovin	Transthyretin	0.0071	Human
Apolipoprotein D (Fragment)	0.0083	Human	Immunoglobulin J chain (Fragment)	0.0134	Human	Calcium-activated chloride channel regulator 1	0.0070	Human
Protein S100-A9	0.0083	Human	Ig kappa chain V-III region WOL	0.0132	Human	Ig lambda chain V-III region LOI	0.0070	Human
Protein IGHV3-72	0.0074	Human	Protein IGHV3-72	0.0128	Human	Protein IGKV2-28	0.0060	Human
Ferritin heavy chain	0.0074	Human	Calcium-activated chloride channel regulator 1	0.0113	Human	Polymeric immunoglobulin receptor	0.0056	Human
Chymotrypsin-like elastase family member 3A	0.0071	Human	Ferritin light chain	0.0110	Human	Protein IGLV1-47 (Fragment)	0.0052	Human
Dipeptidyl peptidase 4	0.0065	Human	Fatty acid-binding protein, liver	0.0106	Human	Serum amyloid P-component	0.0048	Human
Ig kappa chain V-IV region Len	0.0063	Human	Histone H3	0.0104	Human	Intestinal-type alkaline phosphatase	0.0043	Human
Protein IGKV1-17	0.0061	Human	Intestinal-type alkaline phosphatase	0.0102	Human	Protein IGKV1-17	0.0041	Human
Ig kappa chain V-I region EU	0.0061	Human	Polyubiquitin-C (Fragment)	0.0091	Human	IgGFc-binding protein	0.0039	Human
Protein S100-A8	0.0060	Human	Protein S100-A8	0.0086	Human	Ig heavy chain V-III region TRO	0.0039	Human

Week 3			Week 4			Week 6		
Protein	**Fraction**	**Host**	**Protein**	**Fraction**	**Host**	**Protein**	**Fraction**	**Host**
Alpha-1-antitrypsin	0.2809	Human	Alpha-1-antitrypsin	0.2115	Human	Alpha-1-antitrypsin	0.2216	Human
Intestinal-type alkaline phosphatase	0.0918	Human	Protein IGKV3-11	0.1316	Human	Protein IGKV3-11	0.1384	Human
Protein IGKV3-11	0.0871	Human	Intestinal-type alkaline phosphatase	0.1018	Human	Intestinal-type alkaline phosphatase	0.0922	Human
Ig lambda-3 chain C regions (Fragment)	0.0478	Human	Ig alpha-1 chain C region	0.0627	Human	Lactotransferrin	0.0788	Human
Ig alpha-1 chain C region	0.0431	Human	Ig lambda-3 chain C regions (Fragment)	0.0570	Human	Ig lambda-3 chain C regions (Fragment)	0.0579	Human
Alpha-1-antichymotrypsin	0.0418	Human	Alpha-1-antichymotrypsin	0.0547	Human	Ig alpha-1 chain C region	0.0455	Human
Serum albumin	0.0367	Human	Lactotransferrin	0.0460	Human	Aminopeptidase N	0.0310	Human
Uncharacterized protein	0.0360	Bovin	Immunoglobulin J chain (Fragment)	0.0247	Human	Serum albumin	0.0266	Human
Aminopeptidase N	0.0312	Human	Protein S100-A8	0.0181	Human	Chymotrypsin-like elastase family member 3A	0.0180	Human
Lactotransferrin	0.0223	Human	Aminopeptidase N	0.0180	Human	Uncharacterized protein	0.0149	Bovin
Protein S100-A8	0.0200	Human	Chymotrypsin-like elastase family member 3A	0.0174	Human	Protein IGHV3-72	0.0144	Human
Immunoglobulin J chain (Fragment)	0.0160	Human	Protein S100-A9	0.0149	Human	Alpha-1-antichymotrypsin	0.0137	Human
Chymotrypsin-like elastase family member 3A	0.0138	Human	IgGFc-binding protein	0.0135	Human	Chymotrypsin-C	0.0125	Human
IgGFc-binding protein	0.0136	Human	Protein IGHV3-72	0.0124	Human	Ferritin heavy chain	0.0117	Human
Protein S100-A9	0.0076	Human	Calcium-activated chloride channel regulator 1	0.0098	Human	Protease serine 2 preproprotein	0.0088	Human
Calcium-activated chloride channel regulator 1	0.0070	Human	Ig lambda chain V-III region LOI	0.0097	Human	IgGFc-binding protein	0.0086	Human
Protease serine 2 preproprotein	0.0066	Human	Ferritin heavy chain	0.0092	Human	Ig lambda chain V-III region LOI	0.0084	Human
Ferritin heavy chain	0.0064	Human	Ig heavy chain V-I region EU	0.0088	Human	Ig kappa chain V-III region WOL	0.0084	Human
Dipeptidyl peptidase 4	0.0064	Human	Chymotrypsin-C	0.0083	Human	Ig heavy chain V-I region EU	0.0075	Human
Apolipoprotein D (Fragment)	0.0063	Human	Carboxypeptidase A1	0.0082	Human	Protein IGKV1-17	0.0067	Human
Ig lambda chain V-III region LOI	0.0061	Human	Protease serine 2 preproprotein	0.0078	Human	Calcium-activated chloride channel regulator 1	0.0063	Human
Ig alpha-2 chain C region	0.0057	Human	Serum albumin	0.0075	Human	Apolipoprotein D (Fragment)	0.0061	Human
Protein IGHV3-72	0.0056	Human	Apolipoprotein D (Fragment)	0.0072	Human	Ferritin light chain	0.0060	Human
Deleted in malignant brain tumors 1 protein	0.0056	Human	Ig kappa chain V-III region WOL	0.0067	Human	Dipeptidyl peptidase 4	0.0059	Human
Ig kappa chain V-III region WOL	0.0054	Human	Protein IGKV1-17	0.0060	Human	Ig kappa chain V-I region EU	0.0058	Human

Table 2.5 | **Abundance of major milk-derived proteins in gastric aspirates and faeces during the first two postnatal weeks.** Log10 transformed LFQ values are shown. Colouring is based on abundance from most abundant (red) to medium abundant (orange) to least abundant (green). A-J: individual infants.

		Gastric					VP					Faeces					VP				
		EP										EP									
Host	Protein	A	B	C	D	E	F	G	H	I	J	A	B	C	D	E	F	G	H	I	J
Human	Kappa-casein	9.11	9.43	8.44	9.44	8.78	8.53	7.63	9.62	9.57	9.09	4.00	4.00	4.00	4.00	4.00	-	4.00	4.00	4.00	4.00
	Alpha-lactalbumin	9.72	9.60	9.32	9.79	9.38	9.35	9.31	9.46	9.20	9.30	6.75	4.00	4.00	7.83	4.00	-	4.00	4.00	4.00	4.00
	Alpha-S1-casein	8.41	9.07	7.83	8.12	8.58	8.65	9.21	8.74	8.90	7.95	4.00	4.00	4.00	7.42	4.00	-	4.00	4.00	4.00	4.00
	Beta-casein	9.63	9.07	8.78	9.76	8.16	9.47	9.18	9.54	9.67	8.93	6.35	4.00	4.00	6.55	4.00	-	4.00	4.00	4.00	4.00
	Bile salt-activated lipase	9.36	9.59	9.81	9.65	9.58	7.80	8.41	9.48	10.11	7.59	7.83	7.21	6.79	8.02	8.19	-	4.00	4.00	4.00	4.77
	Lysozyme C	7.77	8.74	7.97	8.66	8.43	8.66	9.22	8.79	9.18	8.71	4.00	4.00	7.54	7.54	7.14	-	4.00	4.00	7.84	4.00
	Serum albumin	10.42	10.39	10.38	10.13	10.38	10.69	10.24	10.17	10.41	10.40	10.05	10.27	9.70	9.26	9.88	-	8.40	9.24	7.13	7.35
	Lactotransferrin	10.59	10.55	10.64	10.60	10.55	9.75	9.66	10.60	10.66	9.73	10.18	7.91	6.57	9.57	7.25	-	9.77	9.36	10.41	9.70
Bovine	Alpha-lactalbumin	5.69	4.00	4.00	4.00	6.54	9.35	8.94	6.45	6.47	9.67	4.00	4.00	4.00	4.00	4.00	-	4.00	4.00	4.00	4.00
	Kappa-casein	6.07	4.00	4.00	4.00	5.49	8.93	8.71	5.81	7.48	9.42	4.00	4.00	4.00	4.00	4.00	-	4.00	4.00	4.00	4.00
	Alpha-S1-casein	5.16	4.00	4.00	4.00	5.05	9.25	7.34	5.73	6.67	10.13	6.41	5.92	4.00	4.00	4.00	-	4.00	7.11	4.00	4.00
	Beta-casein	4.00	4.00	4.00	4.00	4.00	8.92	8.37	4.00	4.00	9.27	4.00	4.00	4.00	4.00	4.00	-	4.00	4.00	4.00	4.00
	Alpha-S2-casein	4.00	4.00	4.00	4.00	6.34	9.38	8.13	4.00	4.00	9.76	4.00	4.00	4.00	4.00	4.00	-	4.00	7.09	7.55	4.00
	Lactotransferrin	4.00	4.00	4.00	4.00	4.00	7.28	7.57	4.00	4.00	7.32	4.00	4.00	4.00	4.00	4.00	-	4.00	4.00	4.00	4.00
Human	Kappa-casein	9.08	8.90	9.09	8.37	8.25	9.95	8.98	9.26	9.66	9.66	4.00	4.00	4.00	7.52	4.00	4.00	4.00	4.00	4.00	4.00
	Alpha-lactalbumin	9.40	9.66	9.52	9.74	9.67	8.96	9.28	9.51	9.65	8.26	4.00	4.00	4.00	8.45	4.00	4.00	4.00	4.00	4.00	4.00
	Alpha-S1-casein	8.29	8.18	8.31	7.80	9.29	9.10	9.04	9.23	8.43	8.32	4.00	4.00	4.00	8.69	4.00	4.00	4.00	4.00	4.00	4.00
	Beta-casein	9.88	4.00	9.39	9.52	9.31	4.00	9.66	10.31	9.37	4.00	4.00	4.00	4.00	7.87	4.00	4.00	4.00	4.00	4.00	4.00
	Bile salt-activated lipase	9.46	9.80	9.92	9.35	8.71	9.80	9.80	9.53	10.04	8.37	7.89	7.54	5.42	8.83	6.58	4.00	4.00	4.00	4.00	5.55
	Lysozyme C	7.41	8.20	8.45	8.90	8.97	7.92	9.33	8.89	8.68	7.92	4.00	4.00	7.26	7.63	7.49	4.00	7.00	4.00	4.00	4.00
	Serum albumin	10.38	10.40	10.32	10.53	10.05	10.36	10.25	10.17	10.44	10.31	10.31	9.87	9.29	10.27	9.45	8.52	7.77	8.32	6.88	5.97
	Lactotransferrin	10.71	10.57	10.67	10.35	10.18	10.47	10.30	10.54	10.78	10.48	9.93	10.05	9.29	9.98	9.88	10.14	9.50	10.39	9.21	10.21
Bovine	Alpha-lactalbumin	5.78	6.01	4.00	4.00	5.84	6.57	7.28	7.08	6.21	6.98	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
	Kappa-casein	4.00	4.00	4.00	4.00	7.19	4.00	7.20	6.12	5.62	6.38	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
	Alpha-S1-casein	6.09	5.28	4.00	4.00	5.22	7.47	5.97	5.74	6.39	7.42	4.00	4.00	4.00	6.18	4.00	4.00	4.00	7.03	4.00	4.00
	Beta-casein	6.17	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
	Alpha-S2-casein	4.00	4.00	4.00	4.00	4.00	6.51	6.58	7.35	4.00	6.32	4.00	4.00	7.63	4.00	4.00	4.00	4.00	4.00	7.59	4.00
	Lactotransferrin	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00

DISCUSSION

To get more insight in gastrointestinal function and maturation during the early life of preterm infants, the gastric and faecal proteome of ten preterm infants were determined by LC-MS/MS during respectively the first two and first six postnatal weeks. In addition, gastric pH, pepsin activity and total protease activity were determined in 40 preterm infants during the first two postnatal weeks.

Gastric pH, pepsin activity and total protease activity varied greatly between infants and within infants over time. Average gastric pH fluctuated between 4.5-5.5 during the first two postnatal weeks. A previous study among late preterm infants reported that gastric pH varied between 2-5 at two hours after feeding, with lowest pH being detected in the mid- and lower-stomach[104]. Extremely high gastric pH at day of birth, as observed in some infants, might be due to swallowing of alkaline amniotic fluid[105], and did not seem to affect pH during the following six weeks. Median pepsin activity was relatively stable during the first two postnatal weeks, and the observed variation between samples correlated with sample pH. While its activity could be measured, pepsin was not identified in the gastric proteome, which might be a result of the LC-MS/MS detection limit. Average gastric total protease activity increased during the second postnatal week. Since total protease activity was determined at pH 7.8, and major gastric protease pepsin is active between pH 1.5-4.5, active proteases measured in gastric aspirates were most likely milk-derived. Human milk contained proteases include trypsin, plasmin, pepsin and elastase[106]. Chymotrypsin-like elastase family member 3A and trypsin were identified in the gastric proteome as described herein, and correlated with total protease activity. This indicates that human milk derived proteases may aid protein digestion. A previous study, however, showed that human milk-derived proteases cannot compensate for the low gastric protein digestion capacity observed in preterm infants[94]. High pH, combined with low pepsin activity could therefore still affect gastric digestive capacity of preterm infants and thereby decrease their nutrient utilisation potential.

Composition of the gastric proteome was mainly driven by the percentage of human milk in enteral feedings. As expected, major cow milk-derived proteins were increased at times of more formula feeding. Human milk caseins and whey proteins, however, were not increased at times of more human milk feeding. Instead, various proteins with digestive and immune function were increased when feeding predominantly consisted of human milk. Among those proteins were various immunoglobulins, multiple antigens, bile salt-activated lipase, alpha-amylase and mucin-4. These proteins are present in human milk, but may not or to a lesser extent be available from infant formula[107,108]. More human milk feeding might therefore support digestive and immune function in preterm infants and positively influence health outcomes, like has been shown in term infants[109]. Compared to formula feeding, human milk feeding reduces the risk of NEC in preterm infants[110].

To obtain insights in digestive capacity and protein passage through the GI-tract, the gastric and faecal proteome were compared. The gastric and faecal proteome differed greatly from each other, driven by the high amount of milk-derived proteins in gastric aspirates and by the presence of various digestive enzymes in faecal samples. Lower levels of milk-derived caseins and whey proteins in faeces indicates that the preterm infant gastrointestinal tract is capable of degrading these proteins. However, the high amount of human milk-derived serum albumin and lactotransferrin remaining in faeces indicates that these proteins are to a certain extent resistant to proteolytic degradation, which has been previously reported[111]. The passage of lactotransferrin through the GI system might be beneficial to the infant, since it has been shown to possess antimicrobial and immune modulating properties and it has been suggested to protect against neonatal sepsis and NEC[112]. Alpha-1-antitrypsin and alpha-1-antichymotrypsin, which were among the most abundant proteins identified in the gastric and faecal proteome, limit the activity of proteolytic enzymes and have been shown to decrease degradation of lactotransferrin *in vitro*[113]. Various immunoglobulin structures, including Ig alpha-1 chain C region, which is part of immunoglobulin A (IgA), were also among the most abundant proteins identified in gastric aspirates and faeces. Secretory IgA levels are low or non-existent in newborn infants, and are acquired via human milk feeding. Since IgA represents the first line of defence for the neonate by preventing bacterial translocation, the passage of IgA through the GI system is considered beneficial[114].

Composition of the faecal proteome was mainly driven by gestational age and postnatal age. Although majority of the 25 most abundant proteins were shared between meconium and faeces, divergence was observed between them when taking into account all identified human- and bovine-derived proteins. Meconium particularly consists of material ingested *in utero*, including intestinal epithelial cells, mucus and amniotic fluid. Herein, this was reflected by increased ras-related protein Rab-11A, claudin-3 (both constituents of epithelial cells) and mucin-13, and by absence of milk-derived proteins lactotransferrin and IgA. Intestinal-type alkaline phosphatase (IAP), became part of the most abundant faecal proteins from the third postnatal week onwards. IAP is expressed by enterocytes and secreted into the mucosa and lumen, where it plays an important role in maintaining gut homeostasis[115]. Its deficiency has been linked to bacterial translocation in neonates[116]. The increase of this protein with age might indicate gut maturation. Considering gestational age, the faecal proteome of preterm infants born at 25-26 weeks differed from infants born at 27 or 30 weeks. Faeces of infants born at later gestation showed more glycolytic and proteolytic enzymes and less red blood cell-related proteins (serotransferrin, haemoglobin subunits) and liver-type fatty acid binding protein (FABP). Liver-type and intestinal-type FABP are respectively considered markers for hepatic and enterocyte damage, and the latter has been suggested as measure for the early diagnosis of NEC[117]. Both showed high levels in meconium of all infants, but only remained abundant at later time points in extremely preterm infants, particularly L-FABP. None of the infants included in our study developed NEC, hindering the validation of faecal

I-FABP as marker for its onset. Lactase, sucrase-isomaltase and maltase-glucoamylase are suggested enzyme markers for gut maturation[118], and were generally more abundant in very preterm compared to extremely preterm infants, particularly from the third postnatal week onwards. The identification of specific gestational age- and age-related proteins, including those mentioned above, might facilitate the quest towards markers for gastrointestinal function and maturation.

An important contributor to digestive capacity are the bacteria residing in the gut. Our previous work showed that a *Bifidobacterium*-dominated community, as observed in very preterm but not in extremely preterm infants, is associated with increased proteins involved in carbohydrate and energy metabolism, including those involved in the degradation of complex carbohydrates like human milk oligosaccharides[99]. Elucidating the sole effect of the gut microbiota on digestive functioning is challenging since the composition of the faecal proteome and microbiota were both related to gestational and postnatal age. The most abundant bacterial genera, *Bifidobacterium*, *Klebsiella* and *Enterococcus*, correlated significantly with various proteins, including some that are suggested as markers for GI maturation like lactase and maltase-glucoamylase. This demonstrates that gut maturation and microbiota development are coinciding. Whether these processes also affect each other could not be elucidated with the data described herein. Nevertheless, customising feeding regimen to microbiota composition is suggested as strategy to improve growth outcomes in preterm infants[98].

Our data provides insight in the gastric and faecal proteome of preterm infants and presents a link to its association with gut microbiota development. In addition, dynamics of gastric pH, pepsin and total protease activity are presented. Gastric pH and protease activity are highly variable during the immediate weeks after birth and are not associated with any of the clinical variables, including food intolerance. The gastric proteome is mainly affected by percentage human milk feeding. Human milk can be considered a source of proteins involved in digestive and immune functioning. Human milk-derived lactotransferrin and IgA are abundant in preterm infant faeces. Human milk and cow milk vary in composition, and their protein counterparts might possess different bioactivity. The faecal proteome reflects gestational and postnatal age. Various enzymes, including lactase, were more abundant in very preterm infants than in extremely preterm infants, an indication for gut maturation status. Gut maturation coincided with development of the gut microbiota. Deeper understanding of gastrointestinal maturation and functioning in preterm infants in relation to microbiota development, and including the processing of their feedings, might contribute to the improvement of current nutrition support strategies. In light of this, various strategies could be considered to improve growth and development of preterm infants, including pre-treatment of milk feeding by specific digestive enzymes and the addition of antimicrobials (e.g. immunoglobulins) and probiotics to formula feedings.

Supplementary data

Table S2.1 | **Differentially abundant proteins between gastric samples at time of more versus less than 80% human milk feeding.** A difference below zero indicates the protein is higher in abundance at times of >80% human milk.

Table S2.2 | **Differentially abundant proteins between gastric samples from extremely preterm versus very preterm infants.** A difference below zero indicates the protein is higher in abundance in EP infants.

Table S2.3 | **Differentially abundant proteins between gastric and faecal samples during postnatal weeks one and two.** A difference below zero indicates the protein is higher in abundance in faeces.

Table S2.4 | **Differentially abundant proteins between faecal samples from EP25-26 versus EP27 and VP infants during postnatal weeks one to six.** A difference below zero indicates the protein is higher in abundance in EP25-26 infants.

Figure S2.1 | **Gastric and faecal profiles as annotated to KEGG Brite Orthology level B (A), Organismal systems (B) and Exosome (C) databases.** Relative abundances are based on intensity based absolute quantification (iBAQ) intensities.

-Log p-value	Difference	Protein IDs	Protein names	KO	Host
5.50706	-3.23666	P01877	Ig alpha-2 chain C region	K06856	Human
6.091825	-2.96557	P22897	Macrophage mannose receptor 1	K06560	Human
4.470568	-2.70813	E7EU05	Platelet glycoprotein 4	K06259	Human
4.509782	-2.55503	O00391	Sulfhydryl oxidase 1	K10758	Human
5.208046	-2.4883	P36871	Phosphoglucomutase-1	K01835	HumanBovin
4.099251	-2.29561	D6RD17	Immunoglobulin J chain	K06856	Human
3.336462	-2.25081	P24821	Tenascin	K06252	Human
4.248159	-2.23497	P49327	Fatty acid synthase	K00665	Human
3.197202	-2.14536	Q2HJ20	Sclerostin domain-containing protein 1	-	HumanBovin
2.705126	-2.13861	P08571	Monocyte differentiation antigen CD14	K04391	Human
3.267333	-2.08735	E9PJK1	CD81 antigen	K06508	Human
2.714694	-2.0817	P02794	Ferritin heavy chain	K00522	Human
2.73336	-2.03373	E7ENC5	Mucin-4	K13908	Human
4.436551	-1.98034	P27105	Erythrocyte band 7 integral membrane protein	K17286	HumanBovin
2.465505	-1.83469	P19440	Gamma-glutamyltranspeptidase 1	K18592	Human
3.422953	-1.74731	X6R868	Bile salt-activated lipase	K12298	Human
3.265716	-1.65942	Q08431	Lactadherin	K17253	Human
2.141533	-1.65176	Q99541	Perilipin-2	K17284	Human
2.175327	-1.62367	P04745	Alpha-amylase 1	K01176	Human
2.295631	-1.60987	P63103	14-3-3 protein zeta/delta	K16197	HumanBovin
2.437494	-1.58276	Q16651	Prostasin	K08664	Human
2.101745	-1.57743	P07737	Profilin-1	K05759	Human
3.176606	-1.56065	P07996	Thrombospondin-1	K16857	HumanBovin
3.556275	-1.55312	A2VE41	EGF-containing fibulin-like extracellular matrix protein 1	K18262	HumanBovin
1.920706	-1.54922	P11021	78 kDa glucose-regulated protein	K09490	HumanBovin
2.397253	-1.5448	Q9P2E9	Ribosome-binding protein 1	K14000	Human
3.01088	1.336409	Q0P569	Nucleobindin-1	K20371	HumanBovin
3.155507	1.48516	F1N514	Uncharacterized protein	-	Bovin
2.222805	1.574554	G5E5H7	Uncharacterized protein	-	Bovin
3.154709	1.614098	Q0IIA2	Odorant-binding protein-like	-	Bovin
1.9113	1.627408	P81265	Polymeric immunoglobulin receptor	K13073	Bovin
3.174371	1.778531	V9GYE3	Apolipoprotein A-II	K08758	Human
2.425921	1.874571	P02662	Alpha-S1-casein	K17281	Bovin
2.167456	1.888943	P00711	Alpha-lactalbumin	K00704	Bovin
2.2379	1.894912	F1N726	Uncharacterized protein	K19899	Bovin
2.226647	1.998172	P02668	Kappa-casein	K17282	Bovin
3.372123	2.043895	F1MLW7	Uncharacterized protein	K06554	Bovin
3.753987	2.101781	Q3ZCH5	Zinc-alpha-2-glycoprotein	-	Bovin
4.187334	2.128034	F1MI46	Osteopontin	K06250	Bovin
4.098723	2.162103	P24627	Lactotransferrin	K17283	Bovin
4.291268	2.313393	Q32PJ2	Apolipoprotein A-IV	K08760	Bovin
4.293207	2.325902	Q5GN72	Alpha-1-acid glycoprotein	K17308	Bovin
3.032697	2.531097	P02663	Alpha-S2-casein	K17281	Bovin
4.043882	2.671918	P02666	Beta-casein	K17107	Bovin
7.439817	2.793574	P01888	Beta-2-microglobulin	K08055	Bovin

Table S2.1 | **Differentially abundant proteins between gastric samples at time of more versus less than 80% human milk feeding.** A difference below zero indicates the protein is higher in abundance at times of >80% human milk.

-Log p-value	Difference	Protein names	KO	Host
2.814517	-1.9169	Carboxypeptidase A1	K08779	Human
2.097275	-1.85968	Ig kappa chain V-II region TEW	K06856	Human
1.946705	-1.85762	Ig alpha-2 chain C region	K06856	Human
3.063118	-1.8449	L-lactate dehydrogenase A chain	K00016	Human
2.384905	-1.75755	Mucin-4	K13908	Human
2.521457	-1.71811	Gamma-glutamyltranspeptidase 1	K18592	Human
3.106728	-1.68606	Colipase	K14460	Human
2.216421	-1.59295	Chymotrypsin-like elastase family member 3A	K01345	Human
2.175924	1.616146	Polymeric immunoglobulin receptor	K13073	Bovin
2.603476	1.703413	Apolipoprotein A-IV	K08760	Bovin
4.016775	1.934035	Uncharacterized protein	-	Bovin
2.75029	2.060393	Kappa-casein	K17282	Bovin
3.317378	2.08754	Zymogen granule protein 16 homolog B	-	Human
2.40223	2.087646	Alpha-S2-casein	K17281	Bovin
3.801568	2.14862	Alpha-S1-casein	K17281	Bovin
3.428185	2.193123	Alpha-lactalbumin	K00704	Bovin

Table S2.2 | **Differentially abundant proteins between gastric samples from extremely preterm versus very preterm infants.** A difference below zero indicates the protein is higher in abundance in EP infants.

Table S2.3 | **Differentially abundant proteins between gastric and faecal samples during postnatal weeks one and two.** A difference below zero indicates the protein is higher in abundance in faeces.

-Log p-value	Difference	Protein names	KO	Host
25.09376	-4.46708	Intestinal-type alkaline phosphatase	K01077	Human
13.44494	-3.89651	Calcium-activated chloride channel regulator 1	K05027	Human
22.6324	-3.74009	Neprilysin	K01389	Human
22.82569	-3.69612	Xaa-Pro aminopeptidase 2	K14208	Human
13.62569	-3.62145	Chymotrypsin-C	K01311	Human
31.57058	-3.46235	Intelectin-1	K17527	Human
19.55465	-3.31728	Metalloendopeptidase	K01395	Human
13.90661	-3.30292	Mucin-2	K10955	Human
12.28733	-3.29774	Galectin-4	K10091	Human
10.81467	-3.20438	Dipeptidyl peptidase 4	K01278	Human
11.73002	-3.08569	IGLV1-47	K06856	Human
13.04377	-2.99248	Serpin B6	K13963	Human
10.38673	-2.95383	Zymogen granule membrane protein 16	-	Human
16.40163	-2.8924	Superoxide dismutase [Cu-Zn]	K04565	Human
12.11245	-2.85163	N-acetylglucosamine-6-sulfatase	K01137	Human
11.86395	-2.8343	Ig kappa chain V-IV region Len	K06856	Human
11.59421	-2.69903	IgGFc-binding protein	-	Human
9.660729	-2.65032	Serum amyloid P-component	-	Human
11.03776	-2.55858	Carboxypeptidase Q	K01302	Human
10.21915	-2.55196	Carcinoembryonic antigen-related cell adhesion molecule 5	K06499	Human
7.589604	-2.52503	Ig lambda chain V-IV region Hil	K06856	Human
5.363149	-2.49785	Trypsin-1	K01312	Human
5.824536	-2.49444	Chymotrypsin-like elastase family member 3A	K01345	Human
9.701286	-2.45268	Glutamate carboxypeptidase 2	K14592	Human
11.00804	-2.44731	Sucrase-isomaltase, intestinal	K01203	Human
5.769871	-2.43448	Ferritin light chain	K13625	Human
9.103516	-2.39713	Acid ceramidase	K12348	Human
7.389843	-2.3833	Acid sphingomyelinase-like phosphodiesterase 3b	K01128	Human
9.494332	-2.33281	Retinol-binding protein 2	K18271	Human
6.702665	-2.31486	Angiotensin-converting enzyme	K01283	Human
11.0202	-2.30367	N-sulphoglucosamine sulphohydrolase	K01565	Human
7.642232	-2.29781	Ectonucleotide pyrophosphatase/phosphodiesterase family member 7	K12354	Human
6.050263	-2.2701	Transthyretin	K20731	Human
5.879048	-2.26329	Annexin A4	K17093	Human
12.0344	-2.25556	Aminopeptidase N	K11140	Human
9.128276	-2.24332	N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase	K01444	Human
6.501842	-2.2401	Dipeptidase 1	K01273	Human
7.658119	-2.17146	Carcinoembryonic antigen-related cell adhesion molecule 1	K06499	Human
5.005836	-2.16617	Ig kappa chain V-III region WOL	K06856	Human
7.824743	-2.13384	Lactase-phlorizin hydrolase	K01229	Human
3.527874	-2.04826	Trypsin-2	K01312	Human
4.958143	-1.9949	Annexin A13	K17099	Human
5.739732	-1.93758	Aminoacylase-1	K14677	Human
4.210144	-1.9249	Fatty acid-binding protein, liver	K08750	Human
4.228546	-1.88101	Pancreatic secretory granule membrane major glycoprotein GP2	K19899	Human
3.734944	-1.87825	Ig heavy chain V-III region TRO	K06856	Human
5.63512	-1.79163	Neutral ceramidase	K12349	Human
4.128731	-1.73317	Protein IGHV3-72	K06856	Human
3.213611	-1.70679	Carboxypeptidase A1	K08779	Human
3.200024	-1.68626	Mucin-13	K17298	Human
5.693431	-1.68623	Nicastrin	K06171	Human
3.472017	-1.61476	Plasma protease C1 inhibitor	K04001	Human
4.150643	-1.61162	Apical endosomal glycoprotein	-	Human
4.752537	-1.6054	Sulfate transporter	K14701	Human
4.712509	-1.56313	Deoxyribonuclease-1	K11994	Human
3.895984	-1.53406	Glutathione reductase, mitochondrial	K00383	Human

3.7698	-1.53261	Cathepsin D	K01379	Human
4.227371	-1.52655	Dehydrogenase/reductase SDR family member 11	-	HumanBovin
3.639286	-1.44226	Cadherin-related family member 2	K16502	Human
4.259099	-1.4243	Long-chain-fatty-acid--CoA ligase 5	K01897	Human
2.049678	-1.40894	Ig kappa chain V-I region EU	K06856	Human
4.068925	-1.39418	Epidermal growth factor receptor kinase substrate 8-like protein 3	K17277	Human
2.898492	-1.36752	Villin-1	K05761	Human
3.43769	-1.36217	ADP/ATP translocase 2	K05863	HumanBovin
2.644483	-1.3307	Complement component C9	K04000	Human
3.158821	-1.31545	Carcinoembryonic antigen-related cell adhesion molecule 7	K06499	Human
3.295297	-1.31044	Microsomal triglyceride transfer protein large subunit	K14463	Human
2.742225	-1.29324	Ferritin heavy chain	K00522	Human
3.600475	-1.29293	Metalloendopeptidase	K08606	Human
1.393154	-1.2848	Ig lambda chain V-III region LOI	K06856	Human
3.646625	-1.2839	NHL repeat-containing protein 3	-	Human
1.738629	-1.28371	Ig kappa chain V-II region TEW	K06856	Human
3.64693	-1.27627	N-acetylated-alpha-linked acidic dipeptidase-like protein	K01301	Human
3.153011	-1.26608	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12	K04347	Human
2.607099	-1.24097	Protein S100	-	Human
3.09992	-1.22618	Voltage-dependent anion-selective channel protein 1	K05862	HumanBovin
3.188948	-1.20627	Desmoplakin	K10381	HumanBovin
3.14394	-1.20619	Kallikrein-1	K01325	Human
1.71956	-1.19981	Pancreatic triacylglycerol lipase	K14073	Human
1.788684	-1.16694	Carbonic anhydrase 1	K01672	Human
2.48068	-1.16546	Selenium-binding protein 1	K17285	Human
2.138065	-1.15095	Transthyretin	K20731	Bovin
2.304758	-1.14674	Olfactomedin-4	-	Human
2.022767	-1.14102	Eosinophil peroxidase	K10788	Human
2.969206	-1.11053	Guanine deaminase	K01487	Human
2.688112	-1.107	Nicotinate-nucleotide pyrophosphorylase [carboxylating]	K00767	Human
1.979262	-1.10597	Plasma serine protease inhibitor	K03913	Human
1.579184	-1.08231	Myeloperoxidase	K10789	Human
2.350154	-1.08009	Maltase-glucoamylase, intestinal	K12047	Human
2.612725	-1.06609	Small integral membrane protein 24	-	Human
2.723637	-1.06045	Ubiquitin-60S ribosomal protein L40	K04551	Human
1.896486	-1.05053	Azurocidin	-	Human
3.122508	-1.04098	Complement component C7	K03996	Human
2.622129	-1.02555	Hornerin	-	Human
2.035052	-1.01388	Alpha-2-macroglobulin	K03910	Human
2.690146	-1.01309	Sodium/glucose cotransporter 1	K14158	Human
1.449919	-1.01265	Lumican	K08122	Human
1.982676	-0.99947	Lysosomal alpha-glucosidase	K12316	Human
2.011267	-0.97261	Alpha-1-acid glycoprotein 1	K17308	Human
2.30346	-0.96567	Gamma-glutamyl hydrolase	K01307	Human
2.215805	-0.95261	Chloride anion exchanger	K14078	Human
2.217492	-0.93783	Fatty acid-binding protein, intestinal	K08751	Human
13.73433	-0.92269	Alpha-1-antitrypsin	K03984	Human
2.235524	-0.9176	Melanotransferrin	K06569	Human
2.197076	-0.91453	Cystatin-A	K13907	Human
1.529349	-0.90729	Cytosol aminopeptidase	K11142	Human
1.865	-0.90727	Ig kappa chain V-II region RPMI 6410	K06856	Human
1.617794	-0.89993	Apolipoprotein D	K03098	Human
2.551525	-0.89845	Filamin-C	K04437	HumanBovin
2.212369	-0.87568	Voltage-dependent anion-selective channel protein 2	K15040	HumanBovin
2.243479	-0.87185	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial	K09479	Human
1.849431	-0.86586	Glutathione S-transferase A2	K00799	Human
1.657315	-0.84414	Malate dehydrogenase, mitochondrial	K00026	HumanBovin
2.671186	-0.84303	Arylsulfatase A	K01134	Human
1.871733	-0.82325	Angiotensin-converting enzyme 2	K09708	Human
1.50818	-0.80705	Ig heavy chain V-I region EU	K06856	Human

2.268936	-0.80659	Tissue alpha-L-fucosidase	K01206	Human
2.254875	-0.80034	Complement C5	K03994	Human
2.250713	-0.79157	Unconventional myosin-Ia	K10356	Human
1.180558	-0.79005	Histone H2B	K11252	Bovin
1.50786	-0.78853	Chymotrypsin-like elastase family member 2A	K01346	Human
1.857129	-0.78816	Glia-derived nexin	K16643	Human
1.169543	-0.78798	Keratin, type I cytoskeletal 19	K07604	Human
1.838409	-0.78741	Dihydrolipoyl dehydrogenase, mitochondrial	K00382	Human
2.137576	-0.78591	Deleted in malignant brain tumors 1 protein	K13912	Human
1.69649	-0.78396	Sodium/potassium-transporting ATPase subunit alpha-1	K01539	HumanBovin
1.869727	-0.78048	Superoxide dismutase [Mn], mitochondrial	K04564	Human
1.8593	-0.77643	Cytochrome c	K08738	Human
1.301134	-0.77376	Peroxiredoxin-4	K03386	HumanBovin
1.863452	-0.76944	Thioredoxin-dependent peroxide reductase, mitochondrial	K20011	HumanBovin
1.857048	-0.75098	Ras-related protein Rab-11A	K07904	HumanBovin
1.822892	-0.74077	Aspartate aminotransferase, mitochondrial	K14455	Human
1.829629	-0.7145	Thyroxine-binding globulin	K20734	Human
2.218828	-0.6912	Xaa-Pro dipeptidase	K14213	HumanBovin
2.401273	0.669959	Phospholipase A2	K01047	Human
1.709281	0.712965	Calreticulin	K08057	HumanBovin
1.760937	0.74456	Actin, cytoplasmic 2	K05692	HumanBovin
1.717486	0.747565	Lactoperoxidase	K12550	Human
2.014561	0.774792	Tropomyosin alpha-4 chain	K10373	Human
1.698113	0.785788	Lipoprotein lipase	K01059	HumanBovin
2.411904	0.796159	Insulin-like growth factor-binding protein 1	K10138	Human
2.015633	0.820865	Ig alpha-2 chain C region (Fragment)	K06856	Human
0.964422	0.823346	Mucin-1	K06568	Human
1.060383	0.832489	Ig mu chain C region	K06856	Human
1.296064	0.836877	Beta-casein	K17107	Bovin
1.114008	0.843219	Erythrocyte band 7 integral membrane protein	K17286	HumanBovin
2.066449	0.848449	Kininogen-1	K03898	Human
2.664517	0.850805	Brain acid soluble protein 1	K17272	Human
1.304475	0.854267	Syntenin-1	K17254	Human
2.016271	0.854905	Pancreatic secretory granule membrane major glycoprotein GP2	K19899	Human
2.06375	0.861298	BPI fold-containing family B member 2	-	Human
1.192646	0.875628	Glyceraldehyde-3-phosphate dehydrogenase	K00134	Human
1.25496	0.886047	Vitronectin	K06251	Human
0.984169	0.888012	L-lactate dehydrogenase A chain	K00016	Human
1.071766	0.920803	Fibrinogen beta chain	K03904	Human
2.817484	0.922651	Plasminogen activator inhibitor 1 RNA-binding protein	K13199	HumanBovin
1.717927	0.935489	Programmed cell death 6-interacting protein	K12200	HumanBovin
1.14773	0.943545	Fibrinogen gamma chain	K03905	Human
2.398294	0.954307	Periplakin	K10386	Human
3.458847	0.981291	Lactotransferrin	K17283	Human
2.11518	0.991164	High mobility group nucleosome-binding domain-containing protein 4	K11302	Human
1.252575	1.032889	Hemoglobin subunit beta	K13823	Human
1.843898	1.033984	Uncharacterized protein	-	Bovin
1.412589	1.039781	Histone H1.5	K11275	Human
3.176515	1.050741	Histone H1x	K11275	Human
2.068581	1.054669	Cystatin-C	K13899	Human
2.053556	1.059834	Phosphatidylethanolamine-binding protein 1	-	HumanBovin
2.304287	1.060208	Pigment epithelium-derived factor	K19614	Human
0.956189	1.0802	Ig alpha-2 chain C region	K06856	Human
2.280811	1.116889	Uncharacterized protein	K19899	Bovin
1.453618	1.124051	Alpha-fetoprotein	K16144	Human
2.808067	1.126183	Beta-1,4-galactosyltransferase 1	K07966	Human
1.158861	1.133613	Immunoglobulin J chain	K06856	Human
2.419912	1.147362	40S ribosomal protein S14	K02955	Human
1.854921	1.148736	Alpha-amylase 1	K01176	Human
3.102516	1.148952	Coronin-1A	K13882	HumanBovin

2.208675	1.185629	Alpha-2-macroglobulin-like protein 1	K03910	Human
1.648172	1.196673	Hemoglobin subunit gamma-2	K13824	Human
4.401353	1.20076	Complement component C8 alpha chain	K03997	Human
2.045817	1.208372	Lithostathine-1-alpha	-	Human
2.874076	1.214295	Xanthine dehydrogenase/oxidase	K00106	Human
1.683957	1.223903	UTP--glucose-1-phosphate uridylyltransferase	K00963	HumanBovin
2.396141	1.273185	Keratin, type I cytoskeletal 13	K07604	Human
1.970598	1.279662	Alpha-S1-casein	K17281	Bovin
2.250845	1.281654	Cornulin	-	Human
3.048952	1.292423	Colipase	K14460	Human
2.169122	1.349201	14-3-3 protein zeta/delta	K16197	HumanBovin
2.222032	1.35753	Gamma-glutamyltranspeptidase 1	K18592	Human
2.845047	1.382222	Malate dehydrogenase, cytoplasmic	K00025	Human
3.731391	1.382299	40S ribosomal protein S18	K02964	HumanBovin
2.784779	1.418743	Angiotensinogen	K09821	Human
3.705013	1.423345	Polymeric immunoglobulin receptor	K13073	Bovin
3.254038	1.428878	Mucin-5AC	K13908	Human
2.006171	1.428903	Hemoglobin subunit alpha	K13822	Human
2.397428	1.440498	L-lactate dehydrogenase B chain	K00016	Human
5.031481	1.502334	Serum albumin	K16141	Human
3.541757	1.523183	Cornifin-A	-	Human
2.033081	1.526697	Carbonic anhydrase 6	K01672	Human
3.311638	1.527865	Sciellin	-	Human
6.18575	1.549819	Plastin-2	K17276	HumanBovin
3.975788	1.554291	Alpha-1B-glycoprotein	-	Human
2.919112	1.612798	78 kDa glucose-regulated protein	K09490	HumanBovin
3.555538	1.632684	Zinc-alpha-2-glycoprotein	-	Human
6.388124	1.656447	Myristoylated alanine-rich C-kinase substrate	K12561	HumanBovin
3.271841	1.667513	Histone H1.2	K11275	HumanBovin
3.77534	1.694085	Annexin A1	K17091	Human
3.46091	1.714334	Fatty acid synthase	K00665	Human
6.13638	1.733894	Non-histone chromosomal protein HMG-17	K11300	Human
4.412529	1.771607	CD81 antigen	K06508	Human
6.695169	1.781214	Intercellular adhesion molecule 1	K06490	Human
6.897817	1.800893	Protein AMBP		Human
3.922411	1.820034	Kappa-casein	K17282	Bovin
3.906188	1.946164	Serotransferrin	K14736	Human
5.127475	2.03566	Gelsolin	K05768	Human
8.605782	2.067162	Trefoil factor 2	-	Human
5.871166	2.107727	Mucin-5B	K13908	Human
9.247864	2.122904	Afamin	-	Human
5.094615	2.128814	CD59 glycoprotein	K04008	Human
5.420452	2.14729	Neutrophil defensin 3	K05230	Human
5.618481	2.147578	Gastric triacylglycerol lipase	K14452	Human
4.846356	2.190524	Fatty acid-binding protein, heart	K08752	Human
5.295207	2.204296	Fibrinogen alpha chain	K03903	Human
5.854271	2.215418	Mucin-4	K13908	Human
5.702605	2.242363	Alpha-lactalbumin	K00704	Bovin
6.369677	2.244624	Apolipoprotein A-I	K08757	Human
5.794502	2.247385	Alpha-enolase	K01689	Human
5.846826	2.256198	Beta-2-microglobulin	K08055	Human
8.63126	2.262198	Ribosome-binding protein 1	K14000	Human
6.642832	2.274194	Prosaposin	K12382	Human
8.133053	2.325265	Polymeric immunoglobulin receptor	K13073	Human
7.187705	2.334863	Complement factor H	K04004	Human
7.746131	2.345229	Phosphoglucomutase-1	K01835	HumanBovin
7.047976	2.422425	Perilipin-2	K17284	Human
6.230864	2.423571	Chitinase-3-like protein 1	K17523	Human
5.66476	2.454629	Complement C4-A	K03989	Human
6.606495	2.49818	Apolipoprotein E	K04524	Human

6.20343	2.505115	Thrombospondin-1	K16857	HumanBovin
6.797285	2.51676	Beta-2-glycoprotein 1	K17305	Human
10.74021	2.521944	Salivary acidic proline-rich phosphoprotein 1/2	K13910	Human
14.02157	2.53439	EGF-containing fibulin-like extracellular matrix protein 1	K18262	HumanBovin
10.58327	2.609303	Small proline-rich protein 3	-	Human
6.451511	2.621134	Platelet glycoprotein 4	K06259	Human
8.043074	2.626363	Haptoglobin	K16142	Human
6.888783	2.66327	BPI fold-containing family B member 1	-	Human
9.953033	2.692564	Neutrophil gelatinase-associated lipocalin	-	Human
9.65398	2.703362	Complement factor B	K01335	Human
6.699166	2.731617	Osteopontin	K06250	Human
7.142818	2.773215	Fructose-bisphosphate aldolase A	K01623	Human
6.417421	2.794992	Sulfhydryl oxidase 1	K10758	Human
13.95951	2.81531	Apolipoprotein A-IV	K08760	Human
13.71184	2.822483	Nucleobindin-1	K20371	HumanBovin
13.30885	2.958828	Alpha-2-HS-glycoprotein	-	Human
9.183569	2.985416	Macrophage mannose receptor 1	K06560	Human
9.143253	2.991422	Sclerostin domain-containing protein 1	-	HumanBovin
7.457353	3.023797	Lactadherin	K17253	Human
7.327101	3.048476	Tenascin	K06252	Human
8.299934	3.095148	Lysozyme C	K13915	Human
10.43425	3.151538	Vitamin D-binding protein	K12258	Human
18.25288	3.171959	Insulin-like growth factor-binding protein 2	K10138	Human
8.534266	3.382471	Bile salt-activated lipase	K12298	Human
27.49858	3.476053	Antileukoproteinase	-	Human
11.84738	3.539449	Prolactin-inducible protein	-	Human
10.75772	3.560811	Butyrophilin subfamily 1 member A1	K06712	Human
34.62338	3.588661	Epididymal secretory protein E1	K13443	Human
12.40428	3.721429	Monocyte differentiation antigen CD14	K04391	Human
17.56549	3.884707	Chordin-like protein 2	K17280	Human
8.589263	4.119535	Beta-casein	K17107	Human
14.95258	4.128901	Clusterin	K17252	Human
15.01235	4.145622	Alpha-S1-casein	K17281	Human
16.46909	4.822657	Alpha-lactalbumin	K00704	Human
21.8837	4.856932	Kappa-casein	K17282	Human

-Log p-value	Difference	Protein names	KO	Host
5.71821	-2.68333	Serotransferrin	K14736	Human
7.578675	-2.57419	Fatty acid-binding protein, liver	K08750	Human
8.810937	-2.43149	Haemoglobin subunit beta	K13823	Human
6.147903	-1.90143	Keratin, type II cytoskeletal 8	K07605	Human
5.490808	-1.86804	Hemoglobin subunit alpha	K13822	Human
4.767281	-1.80972	Hemoglobin subunit gamma-2	K13824	Human
3.128532	-1.76624	Triosephosphate isomerase	K01803	HumanBovin
3.862375	-1.6755	Complement C4-A	K03989	Human
1.847451	-1.6564	Immunoglobulin J chain	K06856	Human
3.094607	-1.55873	Alpha-2-macroglobulin	K03910	Human
2.435085	-1.54018	Glyceraldehyde-3-phosphate dehydrogenase	K00134	Human
4.839152	-1.48895	Alpha-fetoprotein	K16144	Human
3.706777	-1.43323	Alpha-enolase	K01689	Human
3.320974	-1.4272	Microsomal triglyceride transfer protein large subunit	K14463	Human
3.779303	-1.41779	Serum albumin	K16141	Human
2.84338	-1.41114	Actin	K05692	HumanBovin
3.969636	-1.4111	Guanine deaminase	K01487	Human
2.191973	-1.38689	Annexin A4	K17093	Human
5.646106	-1.34649	Complement C5	K03994	Human
2.655013	-1.31808	Fibrinogen gamma chain	K03905	Human
2.857688	-1.26023	Fibrinogen alpha chain	K03903	Human
3.048467	-1.25909	Malate dehydrogenase, mitochondrial	K00026	HumanBovin
4.03927	1.589653	N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase	K01444	Human
3.955492	1.81078	Lysosomal alpha-glucosidase	K12316	Human
5.169723	1.882697	N-acetylated-alpha-linked acidic dipeptidase-like protein	K01301	Human
4.953836	2.29195	Maltase-glucoamylase, intestinal	K12047	Human

Table S2.4 | **Differentially abundant proteins between faecal samples from EP25-26 versus EP27 and VP infants during postnatal weeks one to six.** A difference below zero indicates the protein is higher in abundance in EP25-26 infants.

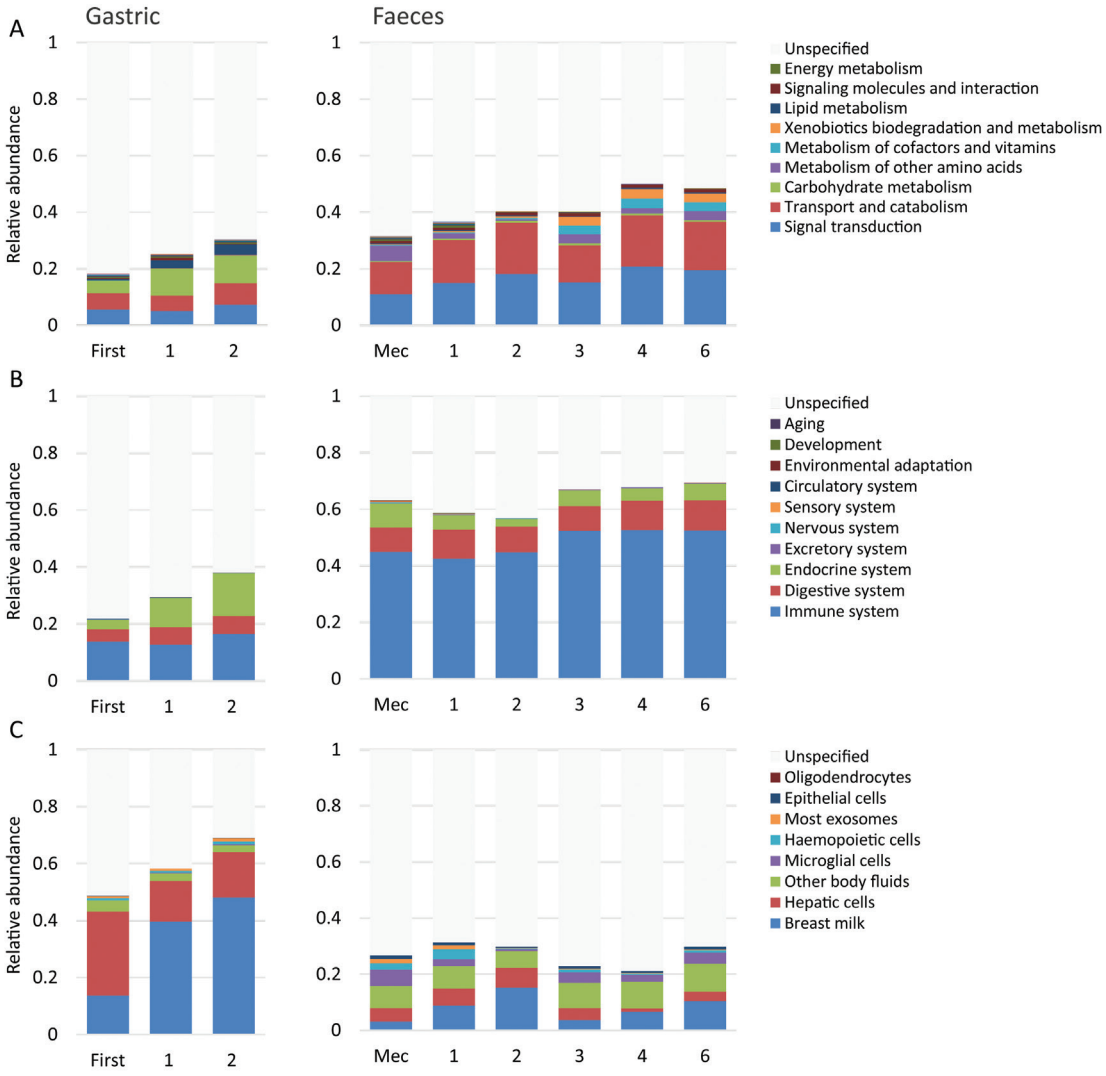
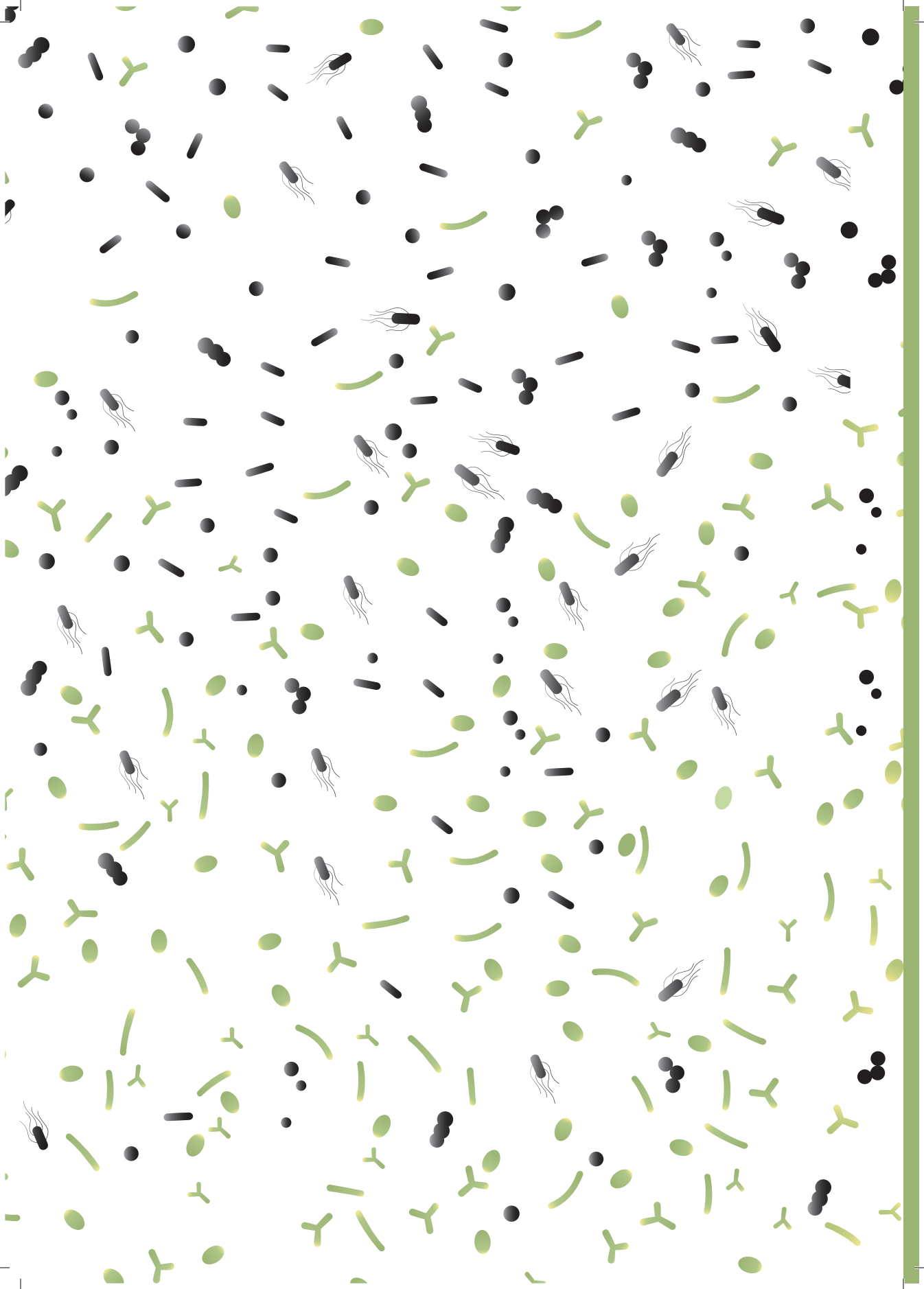


Figure S2.1 | **Gastric and faecal profiles as annotated to KEGG Brite Orthology level B (A), Organismal systems (B) and Exosome (C) databases.** Relative abundances are based on intensity based absolute quantification (iBAQ) intensities.



CHAPTER 3

METAPROTEOMICS REVEALS FUNCTIONAL DIFFERENCES IN INTESTINAL MICROBIOTA DEVELOPMENT OF PRETERM INFANTS

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ABSTRACT

Development of the gastrointestinal tract and immune system can be modulated by the gut microbiota. Establishment of the intestinal microbiota, in its turn, is affected by host and environmental factors. As such, development of the gut microbiota is greatly impacted in preterm infants, who have an immature gut and are exposed to factors like hospitalisation, caesarean section, antibiotics, and respiratory support. We analysed faecal microbiota composition and activity of ten preterm infants (gestational age 25-30 weeks; birthweight 630-1750 gram) during the first six postnatal weeks through metaproteomics (LC-MS/MS) and 16S rRNA gene sequencing. A gestational age-dependent microbial signature is observed, enabling microbiota-based differentiation between extremely preterm (25-27 weeks gestation) and very preterm (30 weeks gestation) infants. In very preterm infants, the intestinal microbiota developed towards a *Bifidobacterium*-dominated community, and was associated with high abundance of proteins involved in carbohydrate and energy metabolism. Extremely preterm infants remained predominantly colonised by facultative anaerobes and were associated with proteins involved in membrane transport and translation. Delayed colonisation by obligate anaerobes could be associated with antibiotic treatment and respiratory support. We speculate that gestational age and its associated intensity of care (e.g. antibiotics and respiratory support) affects intestinal microbiota composition and activity in preterm infants. As the gut microbiota plays a major role in development of the neonate, gestational age and its associated factors could set the state for early and later life health complications via interference with microbiota development.

INTRODUCTION

During birth and rapidly thereafter, microbes colonise the human gastrointestinal (GI) tract and eventually forms a stable, adult-like microbial population[55,56,119]. It is generally believed that the first colonisers are facultative anaerobes, primarily *Staphylococcus*, *Streptococcus*, *Enterococcus* and *Enterobacter*, who create an anaerobic environment to allow for colonisation by obligate anaerobes like *Bifidobacterium*, *Bacteroides* and *Clostridium*[48]. In early life, the intestinal microbiota is dynamic and its development is highly susceptible to host and environmental factors[120]. An abnormal pattern of microbial colonisation is characterised in preterm infants, with high levels of facultative anaerobes and delayed colonisation with obligate anaerobes like *Bifidobacterium*[121-123]. Furthermore, it has been shown that dominance of anaerobes occurs around postconceptional age 33-36 weeks, underlining the substantial influence of gestational age (GA) on microbiota development[65]. More frequent than term infants, preterm infants are exposed to caesarean section, hospitalisation, antibiotic treatment, delayed introduction of enteral feeding and formula feeding, contributing to a bacterial community rich in facultative anaerobes[124]. Respiratory support is a potential influencer of microbiota development, but is only occasionally mentioned in current studies. Administration of air or an air-oxygen mixture might interfere with microbiota development, particularly with colonisation of anaerobic bacteria. Altogether, the preterm infant is very likely to develop an altered intestinal microbiota, which can be associated

with adverse early and later life health outcomes. Host-microbe interactions influence GI-tract and immune system development[125], and disturbances in microbiota development have been related to development of disorders like NEC, infant colic, atopy, inflammatory bowel disease and obesity[126-129]. Despite increasing knowledge about microbiota composition in preterm infants, knowledge about the functional signatures of the intestinal microbiota remains limited. A metaproteomics case study of one preterm infant revealed that bacterial activity transits towards more complex metabolic functions in time[87]. The temporal increase in functional complexity has been confirmed by metabolomics in a bigger set of preterm infants, in which metabolic complexity was related to weaning[89]. The same group also showed increase in specific metabolites prior NEC diagnosis in preterm infants[130]. In the present study, 16S rRNA gene sequencing and metaproteomics are combined to study microbiota development during the first six postnatal weeks in preterm infants and to identify the factors associated with this development.

MATERIALS AND METHODS

Subjects and sample collection

This study was part of an observational, non-intervention study involving (pre) term infants admitted to the neonatal intensive care unit or the paediatric ward of Isala in Zwolle, The Netherlands. The ethics board from METC Isala Zwolle concluded that this study does not fall under the scope of the Medical Research Involving Human Subjects Act (WMO). Informed consent was obtained from both parents of all individual participants included in the study. Ten preterm infants were included for faecal microbiota characterisation. Five infants (infants A-E) were born extremely preterm (EP, 25-27 weeks gestation) and five (infants F-J) were born very preterm (VP, 30 weeks gestation). Infant clinical characteristics are shown in table 3.1. Meconium and faecal samples were collected during the first six postnatal weeks. For metaproteomics analysis, meconium and faecal samples collected at week one, two, three, four and six were used. For infant H, samples collected daily during the first two postnatal weeks were also included for metaproteomics analysis, resulting in a total of 64 samples for LC-MS/MS (Table S3.1). For 16S rRNA gene sequencing, meconium and faecal samples collected daily during the first two postnatal weeks, and collected at week three, four and six were used, resulting in 116 samples (Table S3.1). Samples were stored temporally at -20°C until transfer to -80°C.

Protein extraction

Proteins were extracted mechanically by repeated bead beating as described previously[131]. In short, approximately 0.125 g of meconium or faeces was resuspended in 375 µl PBS, mixed by vortexing and covered with gaseous nitrogen. Cells were lysed mechanically by five times bead beating with 0.1 mm zirconia/silica beads using the Precellys®24 instrument at 6.5 ms-1 for 45 s (Bertin Technologies, Montigny le Bretonneux, France). The mixture was centrifuged to remove beads (10.000 g; 4°C; 5min) and cell debris (14.000 g; 4°C; 8min). Proteins were quantified using the Qubit® Protein Assay Kit a on a Qubit®2.0 fluorometer (Life Technologies, Carlsbad, CA, USA).

In gel-digestion procedures

Protein extracts were diluted in PBS to obtain a 3 µg/µl concentration. 40 µl of each sample was mixed with 20 µl loading buffer and subsequently 50 µl was loaded on precast 10% acrylamide gels (PreciseTM Protein Gels, Thermo Scientific, Rockford, IL, USA) using the Mini-PROTEAN® Tetra Electrophoresis System (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer instructions. After short electrophoresis (20 mA; 10min) to allow for the complete sample to enter, the gels were stained with Coomassie Brilliant Blue. Proteins were reduced by incubating the SDS gels in 50mM dithiothreitol (60min; 60°C) while gently shaking. The gels were washed with water followed by protein alkylation by incubation in 100 mM iodoacetamide (60min; RT). The protein containing fraction of the gel was cut out with a clean scalpel, placed on parafilm, and further processed into 1mm2 pieces. In addition, a non-protein containing fraction was taken along as negative control. The gel pieces obtained were transferred to a 1.5 ml Eppendorf Protein LoBind tube and placed in 5 ng/µl trypsin solution to allow for in-gel digestion (overnight; RT). Protein digests were sonicated and centrifuged (14.000rpm; 5min). The pH of the obtained supernatant was adjusted to 2-4 with 10% trifluoroacetic acid. The peptide solutions were desalted and concentrated using in-house made C18 stage tip micro columns as described previously[132]. Sample volumes were reduced to 10 µl using a SpeedVac vacuum centrifuge at 35°C, and increased to 50 µl with 1 ml/l formic acid in water. Samples were analysed by nano-LC-LTQ-Orbitrap-MS as previously described[133].

Database construction

The obtained MS/MS spectra were searched against the public available Human Microbiome Project (HMP) reference genomes from the gastrointestinal tract, containing 457 bacterial genomes (2014, http://www.hmpdacc.org/HMRGD/). A smaller in-house database was constructed to be more representative to the study group and to decrease the chance of false matches. For this database, representative bacterial genera were selected based on the genera identified by using the HMP database or by 454 pyrosequencing. The proteomes of species within these genera were obtained from Uniprot (http://www.uniprot.org/proteomes/) and merged into one database together with the proteomes of human, cow, candida spp. and common contaminants (e.g. trypsin and keratin). This led to an in-house database containing 87 bacterial species, and a total size of 438537 sequences (Table S3.2).

Table 3.1 | **Infant characteristics.**

* Days to reach full enteral feeding (>140 ml/kg/day)

** Percentage of total feeding (enteral + parenteral)

*** Days until discharge

**** Respiratory support as mechanical ventilation and/or CPAP in days

Abbreviations: BW: birth weight, FI: food intolerance, #AB: number of antibiotic treatments, AB1: first course antibiotics, AB2: second course antibiotics, EF: enteral feeding, Amx: amoxicillin, Ctz: ceftazidime, Erm: erythromycin, Mem: meropenem, Mtz: metronidazole, Va: vancomycin

Infant	Gender	GA (week+day)	BW (gr)	Delivery mode	FI	#AB	AB1	AB2	Maternal AB	Days until FEF*	Human milk (% per week)**	Discharge ***	Respiratory support (days)****
A	Female	25+2	680	Vaginal	Yes	2	Amx/Ctz/Va	Ctz/Va	Yes	10	35, 89, 100, 100, 100	82	76
B	Male	26+4	670	C-section	Yes	2	Amx/Ctz/Va	Mem/Va/Erm	No	17	13, 47, 40, 21, 15	72	74
C	Male	26+6	630	C-section	Yes	2	Amx/Ctz/Va	Ctz/Va/Mtz	No	14	15, 63, 100, 100, 100	85	66
D	Male	27+0	1095	C-section	No	2	Amx/Ctz/Erm	Ctz/Va	Yes	10	41, 93, 22, 49, 100	61	58
E	Male	27+5	925	C-section	Yes	2	Amx/Ctz	Ctz/Va	No	16	3, 32, 98, 100, 100	64	57
F	Female	30+4	1260	C-section	Yes	1	Amx/Ctz	-	No	12	7, 71, 100, 100, 93	7	6
G	Female	30+4	1600	C-section	No	0	-	-	No	10	2, 87, 100, 100, 66	4	4
H	Female	30+4	1220	C-section	No	1	Ctz/Va	-	No	12	15, 79, 100, 100, 100	10	0
I	Male	30+6	1750	Vaginal	No	1	Amx/Ctz	-	No	8	45, 100, 100, 100, 100	8	2
J	Female	30+1	1675	C-section	No	1	Amx/Ctz	-	No	9	10, 95, 56, 9, 0	13	5

LC-MS/MS data analysis

The mass spectrometry data have been deposited to the ProteomeXchange Consortium[134] via the PRIDE partner repository with dataset identifier PXD005574. Obtained MS/MS spectra were analysed with MaxQuant 1.3.0.5[100] using the "Specific Trypsin/P" Digestion mode with maximally two missed cleavages, match between runs on in default settings, LFQ on in default settings, and default settings for the Andromeda search engine (first search 20 ppm peptide tolerance, main search 6 ppm tolerance, ITMSMS fragment match tolerance of 0.5 Da, Carbamidomethyl set as a fixed modification, while variable modifications were set for Protein N-terminal Acetylation and M oxidation which were completed by non-default settings for de-amidation of N and Q, the maximum number of modifications per peptide was 5)[135]. False discovery rates were set to 0.01 on peptide and protein level. Minimally two peptides were necessary for protein identification of which at least one is unique and at least one is unmodified. After filtering, 1641 protein groups could be identified of which 953 were bacterial derived. MaxQuant creates protein groups containing one or more proteins. 1021 protein groups with more than two proteins were created, meaning that those proteins cannot be discriminated based on the measured peptides. In case of ambiguous protein assembly, the protein with highest peptide count and highest number of unique peptides in its group was selected for further analysis. For each sample, intensity based absolute quantification (iBAQ) intensities were used for the generation of taxonomic and functional profiles[136]. For taxonomic classification, no further ranking than genus level was applied because of high protein sequence homology among species from the same genus. For functional classification, protein IDs were assigned to KEGG Orthology (KO) identifiers and functionally annotated using the KEGG Brite database on hierarchy level B. When one protein could be classified into multiple functional categories, iBAQ intensity values were balanced between these categories. Sample proteome, KO identifier, taxonomic and functional profiles and corresponding clinical data were imported in Canoco multivariate statistics software v5 for principal component analysis (PCA), redundancy analysis (RDA) and principal response curve analysis (PRC). Here, a p-value of less than 0.05 was used as threshold for statistical significance. Analysis were generally performed using Canoco's default settings. Specific settings are described in the figure captions.

DNA extraction

DNA was extracted from faeces by the repeated bead beating plus phenol/chloroform method as described previously[137]. DNA was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and by using a Qubit®2.0 fluorometer (Life Technologies, Carlsbad, CA, USA).

454 Pyrosequencing

Amplification of the V3-V5 regions of the 16S rRNA gene was performed using the *Bifidobacterium*-optimised 357F and 926Rb primers as described previously138. For each sample, the reverse primer included a unique barcode

sequence to allow for demultiplexing. PCR and 454 pyrosequencing (GS Junior, Roche) were performed by LifeSequencing S.L. (Valencia, Spain) as described previously[138]. Sequencing data is available in the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under study accession PRJEB18915.

Sequencing data analysis

Pyrosequencing data was analysed using the QIIME software package (v1.8)[139]. Fasta data was demultiplexed and filtered using default settings. Sequences were denoised using Acacia[140], followed by chimera removal using the Usearch algorithm[141]. UCLUST software[142] was used to pick de novo operational taxonomic units (OTUs) with 97% sequence similarity. A representative sequence from each OTU was picked and taxonomy assigned using the SILVA 111 reference database[143] clustered at 97% similarity and complying with the six taxonomic levels of Ribosomal Database Project (RDP) classifier. The obtained OTU table was filtered for OTUs with a total observation count of less than two and for OTUs that were present in less than two samples. This resulted in the identification of 2789 OTUs and the remaining of 975,238 sequences, representing 7332 ± 3022 reads per sample (mean+ SD). To compare the faecal microbial communities between and within infants, weighted unifrac distances were determined. The core microbiota was identified using the QIIME compute_core_microbiome.py script. OTUs present in at least 70% of the samples were considered to be part of the core microbiota. To study (dis)similarities in microbiota composition and relate changes in microbiota composition to clinical data, principal component analysis and redundancy analysis were performed using the Canoco multivariate statistics software v5. Specific settings are described in the figure captions.

16S rRNA gene sequence similarity

All 16S rRNA gene sequences from members of the *Enterobacter* (2515) and *Klebsiella* (1783) genus were downloaded from the SILVA SSU r126 RefNR database (www.arb-silva.de). *Enterobacter* sequences were blasted against *Klebsiella* sequences and vice versa and the average similarity of the hits was determined.

RESULTS

Microbiota development is highly variable during the first two postnatal weeks

Analyses of the faecal metaproteome revealed that the proportion of bacterial proteins was low (0.7-12.1%) till the second postnatal week in all preterm infants (Fig 3.1). Microbiota composition, as determined by 16S rRNA sequencing, showed high inter- and intra-individual variation during these first two postnatal weeks (Fig S3.1a, S3.1b). In all infants, the core microbiota consisted of *Enterococcus* and *Staphylococcus*, present in 73% and 97% of the samples respectively (Table 3.2). Other genera, including *Propionibacterium* and *Enterobacter*, were identified as highly abundant during the first two postnatal weeks, but their abundances were more sample specific (Fig S3.2).

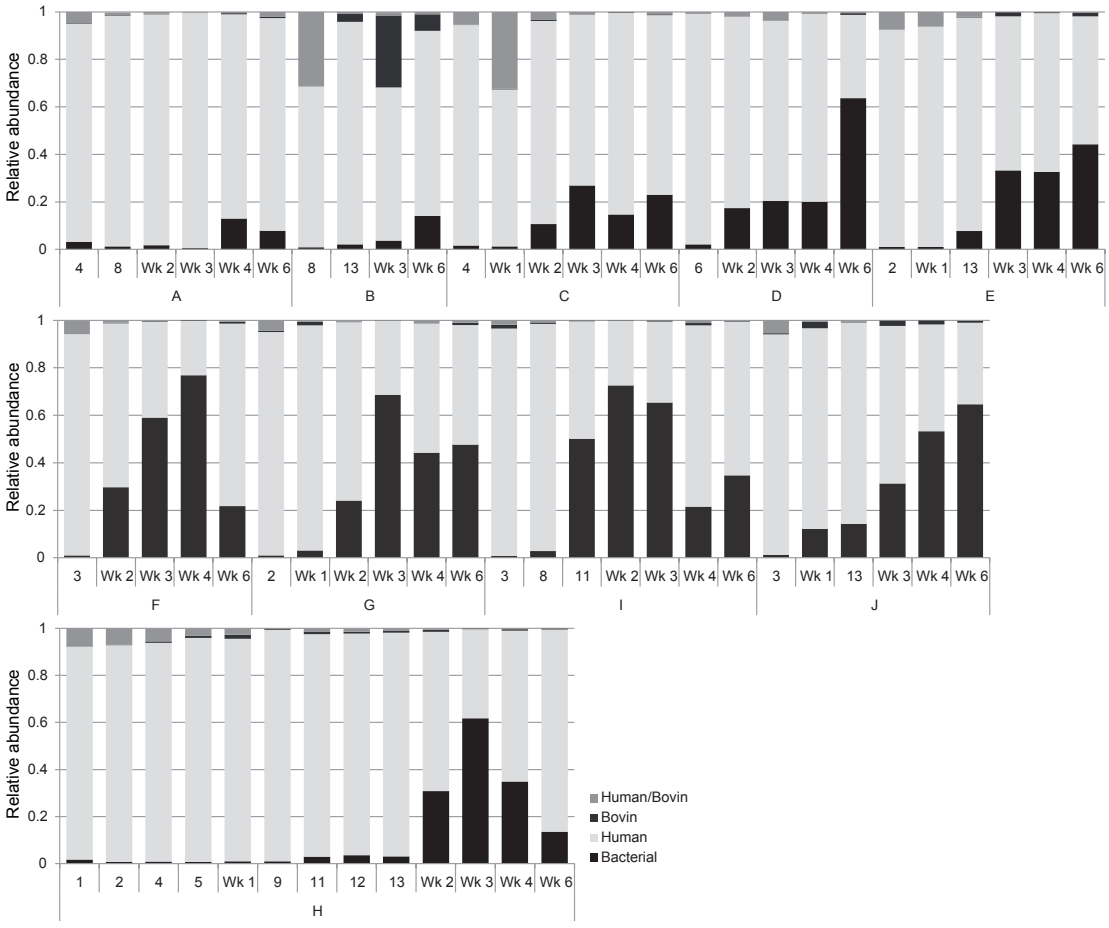


Figure 3.1 | **Distribution of bacterial-, human-, and bovine-derived faecal proteins.** Relative abundances were calculated using iBAQ intensities. Human and bovin-derived homologous proteins are indicated as Human/Bovin. Mec: meconium, A-J: individual infants

Table 3.2 | **Core fraction and relative abundance of the bacterial genera identified in faecal samples during the first two postnatal weeks.** Core microbiota and relative abundances of genera were identified using faecal samples from all preterm infants collected during the first two postnatal weeks.

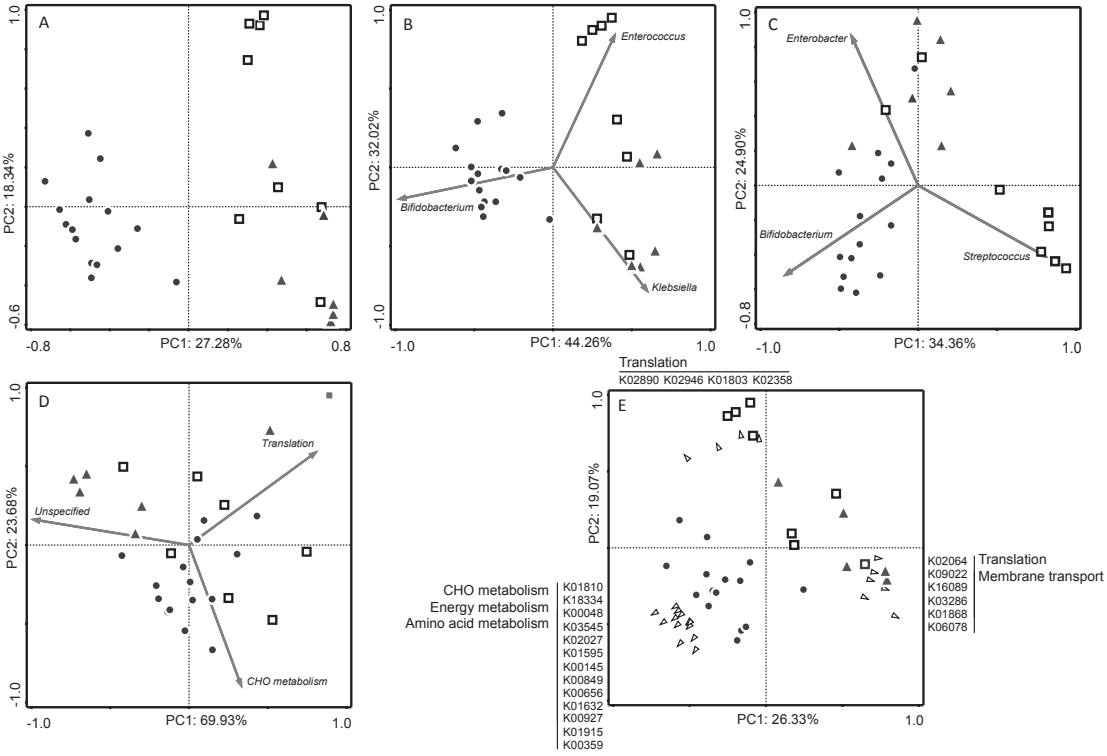
* Fraction of samples in which the genus is identified. Genera were considered to belong to the core microbiota when identified in at least 70% of the samples.

** Mean ± SD

Phylum	Genus	Core*	Rel. abundance**
Actinobacteria	*Bifidobacterium*	-	0.040 ± 0.137
	Propionibacterium	-	0.059 ± 0.128
Firmicutes	*Staphylococcus*	0.97	0.338 ± 0.330
	Enterococcus	0.73	0.146 ± 0.253
	Streptococcus	-	0.011 ± 0.036
	Veillonella	-	0.015 ± 0.046
	Clostridium	-	0.021 ± 0.084
	Lactobacillales; Other	-	0.005 ± 0.013
Proteobacteria	*Enterobacter*	-	0.058 ± 0.203
	Escherichia-Shigella	-	0.001 ± 0.003
	Enterobacteriaceae; Other	-	0.001 ± 0.002

Gestational age is predictive for microbial signatures during early microbiota development

From the third postnatal week onwards, the proportion of bacterial-derived proteins rapidly increased (Fig 3.1). However, this process was delayed in EP infants (infants A-E). Strikingly, the delay was most obvious in EP infants born at 25-26 weeks gestation (infants A-C). In addition, ordination analysis of the faecal bacterial proteome revealed a clear separation between samples from EP and VP infants, but also between samples from EP infants born at 25-26 or 27 weeks gestation (Fig 3.2a). For further analysis, the EP infants were therefore stratified as born extremely preterm at 25-26 or 27 weeks gestation (EP25-26; EP27). The GA-related separation of the faecal bacterial proteomes by ordination analysis could be explained by taxonomic differences (Fig 3.2b). VP infants were associated with increased abundance of *Bifidobacterium*-derived proteins while EP25-26 and EP27 infants were associated with increased abundance of *Enterococcus*- and *Klebsiella*-derived proteins respectively. These differences remained throughout postnatal weeks 3-6 (Fig S3.3a). Such separation of samples could also be observed based on 16S rRNA gene sequencing data, associated with the abundance of *Streptococcus*, *Enterobacter* and *Bifidobacterium* (Fig 3.2c). However, microbiota composition became more similar over time between EP25-26, EP27 and VP infants (Fig S3.3b). In general, EP infants were colonised with a higher proportion of aerobic and facultative anaerobic bacteria compared to VP infants (Fig S3.4). Bacterial protein based taxonomic classification revealed that the biggest proportion (66-90%) of identified proteins derived from *Klebsiella*, *Bifidobacterium* and *Enterococcus*, (Fig 3.3a). Based on 16S rRNA gene sequencing data, *Bifidobacterium*, *Enterobacter* and *Enterococcus*, comprised the most abundant genera (42-87%) (Fig 3.3b). Blasting revealed , that all SILVA derived 16S rRNA gene sequences from *Enterobacter* hit *Klebsiella* and vice versa, with an average 16S rRNA gene similarity of 97.7 ± 1.7% and 98.1 ± 1.5% (mean ± SD) respectively.


A
PC2: 18.34%
PC1: 27.28%
B
PC2: 32.02%
PC1: 44.26%
Enterococcus
Bifidobacterium
Klebsiella
C
PC2: 24.90%
PC1: 34.36%
Enterobacter
Bifidobacterium
Streptococcus
D
PC2: 23.68%
PC1: 69.93%
Translation
Unspecified
CHO metabolism
Translation
K02890 K02946 K01803 K02358
E
PC2: 19.07%
PC1: 26.33%
CHO metabolism
Energy metabolism
Amino acid metabolism
K01810
K18334
K00048
K03545
K02027
K01595
K00145
K00849
K00656
K01632
K00927
K01915
K00359
K02064
K09022
K16089
K03286
K01868
K06078
Translation
Membrane transport
Samples:
EP 25-26 EP 27 VP
Species:
Genera/Function
KO identifier

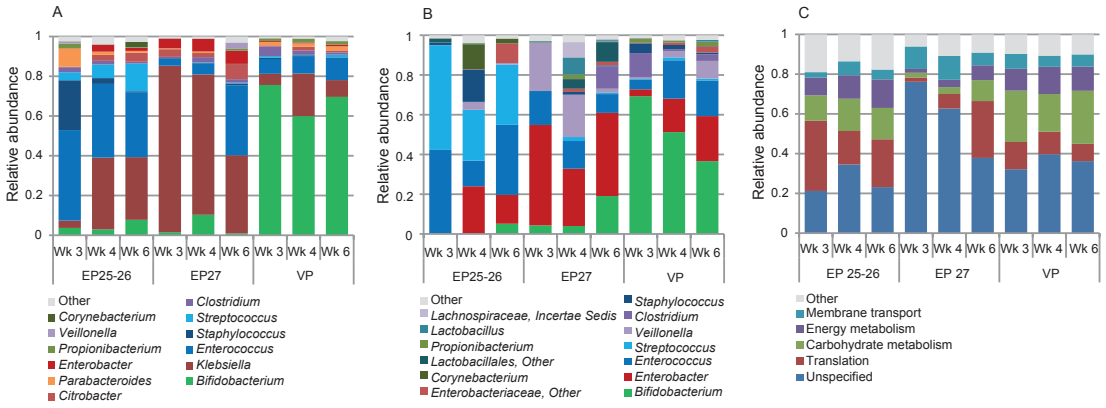

A
Relative abundance
Wk 3 Wk 4 Wk 6 Wk 3 Wk 4 Wk 6 Wk 3 Wk 4 Wk 6
EP25-26
EP27
VP
Other
Corynebacterium
Veillonella
Propionibacterium
Enterobacter
Parabacteroides
Citrobacter
Clostridium
Streptococcus
Staphylococcus
Enterococcus
Klebsiella
Bifidobacterium
B
Relative abundance
Wk 3 Wk 4 Wk 6 Wk 3 Wk 4 Wk 6 Wk 3 Wk 4 Wk 6
EP25-26
EP27
VP
Other
Lachnospiraceae, Incertae Sedis
Lactobacillus
Propionibacterium
Lactobacillales, Other
Corynebacterium
Enterobacteriaceae, Other
Staphylococcus
Clostridium
Veillonella
Streptococcus
Enterococcus
Enterobacter
Bifidobacterium
C
Relative abundance
Wk 3 Wk 4 Wk 6 Wk 3 Wk 4 Wk 6 Wk 3 Wk 4 Wk 6
EP 25-26
EP 27
VP
Other
Membrane transport
Energy metabolism
Carbohydrate metabolism
Translation
Unspecified

Figure 3.2 | **Principal component analysis of the faecal bacterial community using (A) proteome profiles, (B) protein-based taxonomic profiles, (C) 16S rRNA gene-based microbiota profiles, (D) protein-based KEGG Brite functional profiles and (E) protein-based KEGG Orthology identifier profiles.** A-D: Taxa and KEGG Brite level B categories fitting into the ordination space from 75-100% are shown. E: KEGG Orthology identifiers fitting into the ordination space from 50-100% are shown.

This means that these genera cannot be distinguished based on their 16S rRNA gene sequence, which could lead to misclassification and contributes to dissimilar findings when comparing sequencing data with metaproteomics data. Abundance of *Bifidobacterium*, *Enterobacter|Klebsiella*, *Enterococcus*, *Streptococcus* and *Clostridium* correlated significantly (Spearman correlation $p<0.01$) between the protein- and 16S-based approach (Table S3.3).

Divergence between bacterial activity in preterm infants of varying gestational age

Faecal bacterial proteins were matched to their corresponding KO ID and could be classified into 21 KEGG Brite functional categories. Proteins involved in translation, carbohydrate (CHO) metabolism, energy metabolism, membrane transport and unspecified processes were most abundant (64-93%) (Fig 3.3c). Ordination analysis using the KEGG Brite functional profiles revealed no clear functional differences related to gestational age (Fig 3.2d). Similar functional processes were covered by different bacterial genera (Fig S3.5). In VP infants, *Bifidobacterium* was the main genus involved in each functional process. In EP infants, metabolic processes CHO and energy metabolism were predominantly covered by *Enterococcus*. In EP27 infants, membrane transport proteins were mostly derived from *Klebsiella*, while in EP25-26 infants these derived from both *Klebsiella* and *Enterococcus*. Thus, different bacteria cover similar functional processes leading to comparable KEGG Brite functional profiles. Functional (dis)similarities between infants born at varying GA were further explored at protein level using KO ID-based profiles. Ordination analysis revealed a clear separation between EP and VP infants (Fig 3.2e). EP infants were associated with higher abundance of KO identifiers within functional categories translation and membrane transport. VP infants were associated with increased abundance of KO identifiers within CHO and energy metabolism. This could also be observed from the ten most abundant KO identifiers per GA group (Table 3.3). The ten most abundant proteins accounted for 63.5%, 77.6% and 58.8% of total proteins in EP25-26, EP27 and VP infants respectively. In VP infants, the top 10 KO identifiers mainly represented proteins involved in CHO and energy metabolism, while in EP infants they represented proteins involved in translation and membrane transport. Murein lipoprotein was particularly abundant in EP27 infants, but decreased over time resulting in profiles more similar to EP25-26 and VP infants (Fig S3.3c).

Figure 3.3 | **Protein-based (A) and 16S-based (B) taxonomic profiles and protein-based functional profiles (C) in EP25-26, EP27 and VP infants during postnatal weeks 3-6.** Per time point, average relative abundances for each gestational age group are shown. Relative abundances were calculated using iBAQ intensities and read counts for protein and 16S based profiles respectively. Mec: meconium.

Profiles per sample can be found in Fig S6.

GA group	KO ID	Protein	KEGG Brite Functional Category	Rel. abundance*
EP25-26	K01803	Triosephosphate isomerase	CHO metabolism, Energy metabolism	0.213
	K06078	Murein lipoprotein	x	0.162
	K02994	Small subunit ribosomal protein S8	Translation	0.063
	K02950	Small subunit ribosomal protein S12	Translation	0.053
	K02358	Elongation factor Tu	Translation, Transport and catabolism	0.034
	K00100	NADH-dependent butanol dehydrogenase A	CHO metabolism, Lipid metabolism, Xenobiotics biodegr. and metabolism	0.031
	K00134	Glyceraldehyde 3-phosphate dehydrogenase	CHO metabolism, Energy metabolism, Signal transduction	0.023
	K02040	Phosphate transport system substrate-binding protein	Membrane transport, Signal transduction	0.019
	K05878	Dihydroxyacetone kinase	Lipid metabolism	0.019
	K03286	OmpA-OmpF porin, OOP family	Membrane transport	0.018
EP27	K06078	Murein lipoprotein	x	0.537
	K01803	Triosephosphate isomerase	CHO metabolism, Energy metabolism	0.062
	K03286	OmpA-OmpF porin, OOP family	Membrane transport	0.053
	K03647	NrdI family protein	x	0.032
	K09475	Outer membrane pore protein C	Signal transduction	0.021
	K02986	Small subunit ribosomal protein S4	Translation	0.020
	K00134	Glyceraldehyde 3-phosphate dehydrogenase	CHO metabolism, Energy metabolism, Signal transduction	0.014
	K02950	Small subunit ribosomal protein S12	Translation	0.014
	K04744	LPS-assembly protein	Membrane transport	0.012
	K02871	Large subunit ribosomal protein L13	Translation	0.011
VP	K06078	Murein lipoprotein	x	0.132
	K00656	Formate C-acetyltransferase	CHO metabolism	0.128
	K01632	Xylulose-5-p/fructose-6-p phosphoketolase	Energy metabolism	0.072
	K01803	Triosephosphate isomerase	CHO metabolism, Energy metabolism	0.071
	K01810	Glucose-6-phosphate isomerase	CHO metabolism	0.042
	K00927	Phosphoglycerate kinase	CHO metabolism, Energy metabolism	0.038
	K00134	Glyceraldehyde 3-phosphate dehydrogenase	CHO metabolism, Energy metabolism, Signal transduction	0.031
	K01915	Glutamine synthetase	AA metabolism, CHO metabolism, Energy metabolism, Signal transduction	0.026
	K00359	Pyridine nucleotide-disulfide oxidoreductase	x	0.025
	K02906	Large subunit ribosomal protein L3	Translation	0.022

Table 3.3 | **Top 10 KEGG Orthology identifiers per gestational age group during postnatal weeks 3-6.** Per gestational age group, average relative abundances for each KEGG Orthology identifier were calculated based on iBAQ scores. Abbreviations: GA: gestational age, KO: KEGG Orthology, ID: identifier, CHO: carbohydrate, EP: extremely preterm, VP: very preterm.
* iBAQ scores were used to calculate mean relative abundance

A Bifidobacterium dominated community is associated with active metabolism towards human milk oligosaccharide degradation

VP infants showed to have a *Bifidobacterium*-dominated community, active in carbohydrate and energy metabolism. To metabolise complex CHO structures such as human milk oligosaccharides (HMOs), ABC transporters and glycolytic enzymes are required including galactosidases, fucosidases and sialidases. The glycolytic enzymes identified in our dataset were β-galactosidases derived from *Bifidobacterium*, *Enterobacter*, *Streptococcus* and *Clostridium*. *Bifidobacterium*-derived β-galactosidases were identified in all preterm infants, but were more abundant in VP than in EP infants (p=0.026) (Table S3.4). *Enterobacter*-, *Streptococcus*- and *Clostridium*-derived β-galactosidases could only be identified in very low abundance and just in a few samples. Similar accounted for ABC transporters for oligosaccharides; *Bifidobacterium*-derived ABC transporters were more abundant in VP than in EP infants (p=8.1E-06), while those *Klebsiella*- and *Eubacterium*-derived were rarely identified. ABC transporters for oligopeptides could only be identified in a few samples from both EP and VP infants.

Respiratory support and antibiotic treatment influences microbiota succession

The effect of clinical characteristics in association with microbiota composition and function were analysed by redundancy analysis. The GA-based separation of faecal microbiota composition and protein KO-ID profiles were mainly driven by the duration of respiratory support and antibiotic treatment-related factors (Fig 3.4). Respiratory support explained 24.1% and 4.9% of the variation in microbiota composition based on 16S and protein KO ID data respectively. Antibiotic treatment-related factors explained 25.6% and 44.6% of the variation respectively, and comprised the number and duration of treatment, and the administration of maternal antibiotics (Fig 3.4). Other factors, including mode of delivery, birth weight, feeding intolerance, proportion of human milk feeding and days until discharge did not have a significant influence. To provide more support for the association between microbiota composition and clinical factors, this analysis was repeated in all additional EP25-26 (n=14), EP27 (n=17) and VP30 (n=6) infants from the complete cohort, albeit solely based on 16S rRNA gene sequencing (Illumina MiSeq, data not shown). Ventilation remained the main factor associated with microbiota composition (4.7%, p=0.002). In addition, maternal antibiotics and duration of the first and second course of antibiotic treatment respectively explained 2.0%, 2.6% and 1.8% of the observed variation in microbiota composition ($p<0.05$).

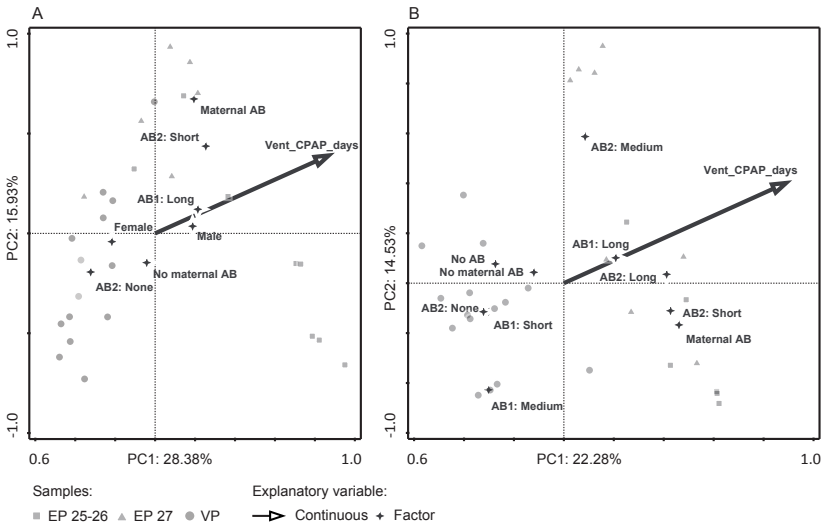


Figure 3.4 | **Redundancy analysis of the faecal bacterial community using (A) 16S rRNA gene-based microbiota profiles and (B) protein-based KEGG Orthology identifier profiles.** Clinical factors that significantly explain the variation are shown. AB: antibiotic, AB1: duration of first course of antibiotics, AB2: duration of second course of antibiotics, Vent_CPAP_days: duration of ventilation and/or CPAP in days.

DISCUSSION

The current study implements metaproteomics and 16S rRNA gene amplicon sequencing in a cohort of preterm infants to get insight into the establishment and activity of their intestinal microbiota. Metatranscriptomics and proteomics have only recently been applied to study GI- and microbiota function in preterm infants[87,144,145]. As such, a previous study showed that metaproteomics data is consistent with metagenomics and 16S rRNA gene analysis, and that bacterial activity transits towards more complex metabolic functions in time[87]. However, those findings were based on analysis of the faecal metaproteome of one preterm infant during the first three postnatal weeks. The current study adds up to this data by using a combination of metaproteomics and 16S rRNA gene sequencing to study microbiota development during the first six postnatal weeks in ten preterm infants of varying GA.

Analysis of the faecal proteome indicated low bacterial load till the second postnatal week in all preterm infants. Low bacterial load might have contributed to the observed variation in microbiota composition during the first two postnatal weeks. From the third postnatal week onwards, microbiota composition and function could be distinguished between infants of varying GA. Bacterial colonisation was delayed in EP25-26 and EP27 infants compared to VP infants, emphasising that microbial colonisation pattern is related to GA. Facultative anaerobes remained dominant throughout the first six postnatal weeks in EP infants. Colonisation with obligate anaerobes was delayed, which is consistent with previous findings[121-123]. In VP infants, obligate anaerobe and beneficial early life coloniser *Bifidobacterium* became predominant from the third postnatal week. This is in concordance with a previous study showing that dominance of obligate anaerobes occurs around postconceptional age 33-36 weeks in preterm infants[65]. The development towards a *Bifidobacterium*-dominant microbiota as observed in VP infants, but not in EP infants, is more

representative of microbiota development in term, vaginally born, breastfed infants, which is considered most beneficial during early life development.

The observed differences between proteome- and 16S-based taxonomy in EP infants could indicate that microbiota composition is not representative for metabolic activity of the microbiota. However, it should be noted that misclassification of bacterial genera could occur in case of high 16S rRNA gene similarity, which is the case for members of the *Enterobacter* and *Klebsiella* genera. Applying two complementary approaches, like metaproteomics and 16S rRNA gene amplicon sequencing, is therefore of great added value for data interpretation. In general, 16S rRNA gene amplicon sequencing confirmed the findings obtained by metaproteomics, showing clear taxonomic differences between infants born at varying GA, with high abundance of *Bifidobacterium* in VP infants.

In addition to taxonomic differences, our data shows clear differences in bacterial activity between GA groups. VP infants were associated with high abundance of proteins involved in CHO and energy metabolism, while proteins involved in membrane transport and translation were highly abundant in EP infants. Proteins related to HMO degradation were particularly abundant in VP infants, coinciding with the high abundance of *Bifidobacterium*. *Bifidobacterium* species are considered beneficial early life colonisers predominantly colonising the infant gut due to their ability to metabolise complex CHO structures, including HMOs. These findings indicate a well-established and metabolically active microbiota in VP infants, whereas in EP infants, the protein profile indicates a more active role on the generation and maintenance of biomass.

Our findings indicate that GA is associated with microbiota establishment and its activity in preterm infants. However, one should be aware of the factors that are associated with preterm birth and keep in mind that these factors can be greatly related to GA, including the extent and duration of intensive care. In this study, the observed differences in microbiota development between EP and VP infants were mainly driven by respiratory support and antibiotic treatment, which extent is negatively correlated to GA. One of the respiratory support strategies is continuous positive airway pressure (CPAP) in which pressure is combined with air/oxygen administration, thereby allowing air to reach the GI-tract[146]. This might create an oxygen-rich environment in the GI-tract, which could impede the passage and survival of obligate anaerobes, hence lead to establishment of an intestinal microbiota dominated by aerobes and facultative anaerobes. Indeed, our findings show dominance of aerobic and facultative anaerobic bacteria in extremely preterm infants. These infants all received respiratory support throughout the six weeks study period. These findings are supported by a previous study, showing that duration of respiratory support in preterm infants was associated with predominance of faecal aerobes/facultative anaerobes and with the onset of Staphylococcal late-onset sepsis[147]. In addition to longer respiratory support, the duration and number of antibiotic treatment showed to be significant drivers of the observed GA-dependent microbiota development. Ceftazidime and amoxicillin are broad spectrum β-lactam antibiotics, targeting

gram-negative and -positive bacteria. It has been shown that *Bifidobacterium* is sensitive to β-lactam antibiotics[148,149] and that treatment with amoxicillin can greatly influence the composition of *Bifidobacterium* species in infant intestinal microbiota[150]. Vancomycin particularly targets gram-positive bacteria, and has been shown to affect *Bifidobacterium*[148,149]. Prolonged and multiple antibiotic treatment in EP infants could therefore delay or prohibit establishment of a *Bifidobacterium*-dominated microbiota. Previous studies indeed show that antibiotic treatment greatly affects microbiota development in preterm infants[70,151,152]. Although we were able to replicate our findings considering the role of respiratory support and antibiotic treatment on microbiota development in the complete cohort, the effect appeared to be less apparent. This variation could be due to differences in the distribution of gestational age in the groups, with 50% (5/10) of infants being born at 30 weeks gestation in the current study and only 16% (6/37) in the complete cohort. Since factors such as respiratory support and antibiotic treatment strategies are associated with gestational age, further studies are needed to unravel the true contribution of these factors to microbiota development.

Overall, our findings indicate that gestational age is positively associated with abundance of *Bifidobacterium* and negatively associated with abundance of facultative anaerobic bacteria. Development of the intestinal microbiota most likely suffers from exposure to respiratory support and antibiotic treatment. High extent of exposure to these factors is common in EP infants, pressuring the bacterial community to become rich in facultative anaerobes and particularly active in translation and membrane transport. VP infants are to a lesser extent exposed to respiratory support and antibiotic treatment, allowing for development towards a more stable, metabolically active, *Bifidobacterium*-dominated microbiota. A microbial signature characterised by low abundance of *Bifidobacterium* and high abundance of facultative anaerobes has been associated with several negative health outcomes in early life, including NEC and late-onset sepsis[72,153]. In addition, disturbances in microbiota development have been related to development of disorders in later life. In light of this, our data indicates that gestational age and its associated intensity of care could greatly influence early and later life health of preterm infants by interfering with microbiota development.

Supplementary data

Table S3.1 | **Sampling scheme for metaproteomics and 16S rRNA sequencing.** Red dots: samples for metaproteomics, Black dots: samples for 16S rRNA gene sequencing, A-J: individual infants.

Table S3.2 | **Contents of the in-house generated protein database.**

Table S3.3 | **Correlation between 16S- and protein-based taxonomic classification.** P-values below 0.05 are considered significant (Spearman correlation with Monte Carlo permutation 10.000x).

Table S3.4: **Relative abundance of Bifidobacterium-derived beta-galactosidase and ABC transporters for oligosaccharides during postnatal weeks 3-6.** Relative abundances were calculated using iBAQ intensities.

Figure S3.1 | **Weighted unifrac distance between (A) and within (B) infants.** A: Unifrac distance was determined with samples collected from all infants at time points meconium, week 1 and week 2 to show variation between infants at these time points. B: Unifrac distance was determined with samples collected from each infant during the first two postnatal weeks to show variation within infants. Mec: meconium, A-J: individual infants

Figure S3.2 | **Microbiota composition profiles during the first two postnatal weeks.** Taxonomy is based on 16S rRNA gene sequencing. Taxonomic profiles are shown for each available time point (1-14) per infants (A-J).

Figure S3.3 | **Principal response curve analysis summarising the differences in protein-based taxonomic profiles (A), 16S-based taxonomic profiles (B) and protein-based KEGG Orthology identifier profiles between EP25-26, EP27 and VP infants throughout postnatal weeks 3-6.** Genera and proteins with a score lower than -0.85 or higher than 0.85 are shown on Resp1.

Figure S3.4 | **Proportion of aerobes/fac. anaerobes and anaerobes according to 16S rRNA gene sequencing (A) and metaproteomics (B) data.** Per time point, average relative abundance per gestational age group is shown.

Figure S3.5 | **Taxonomic profiles per functional category.** Taxonomic profiles were made for the most abundant KEGG Brite level B functional categories. Per time point, average relative abundances for each GA group are shown. Relative abundances were calculated using iBAQ intensities.

Figure S3.6 | **Protein-based (A) and 16S-based (B) taxonomic profiles and protein-based functional profiles (C) for all samples collected during postnatal weeks 3-6.**

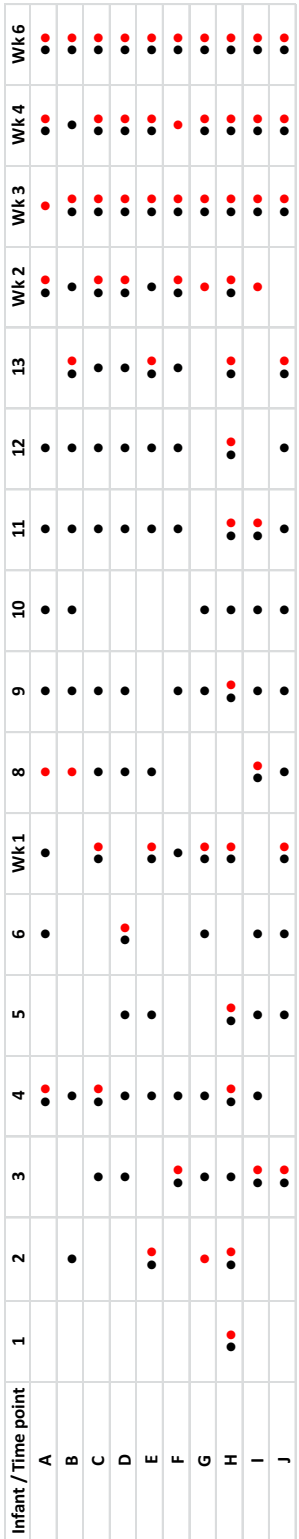


Table S3.1 | **Sampling scheme for metaproteomics and 16S rRNA sequencing.** Red dots: samples for metaproteomics, Black dots: samples for 16S rRNA gene sequencing, A-J: individual infants.

Kingdom	Species	Sequences	Taxonomy Uniprot ; reference proteome
Bacteria	Bifidobacterium	1723	206672 - Bifidobacterium longum (strain NCC 2705) ; Reference proteome
		1834	484020 - Bifidobacterium bifidum BGN4 ; Reference proteome
		1838	1254439 - Bifidobacterium thermophilum RBL67 ; Reference proteome
		1525	442563 - Bifidobacterium animalis subsp. lactis (strain AD011) ; Reference proteome
		2121	401473 - Bifidobacterium dentium (strain ATCC 27534 / DSM 20436 / JCM 1195 / Bd1) ; Reference proteome
		1629	367928 - Bifidobacterium adolescentis (strain ATCC 15703 / DSM 20083 / NCTC 11814 / E194a) ; Reference proteome
		1913	1385940 - Bifidobacterium breve JCM 7019
		1659	1263060 - Bifidobacterium pseudocatenulatum CAG:263
	Propionibacterium	2294	267747 - Propionibacterium acnes (strain KPA171202 / DSM 16379) ; reference proteome (Reference proteome)
	Xanthomonadales	4365	522373 - Stenotrophomonas maltophilia (strain K279a) ; Reference proteome
	Corynebacterium	2265	257309 - Corynebacterium diphtheriae (strain ATCC 700971 / NCTC 13129 / Biotype gravis) ; Reference proteome
		2508	548476 - Corynebacterium aurimucosum (strain ATCC 700975 / DSM 44827 / CN-1) ; Reference proteome
		2018	645127 - Corynebacterium kroppenstedtii (strain DSM 44385 / CCUG 35717) ; Reference proteome
	Bacteroides	3543	1263037 - Bacteroides caccae CAG:21 ; Reference proteome
		4782	226186 - Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482) ; Reference proteome
		4234	272559 - Bacteroides fragilis (strain ATCC 25285 / NCTC 9343) ; Reference proteome
		4465	357276 - Bacteroides dorei
	Lactobacillus	3038	220668 - Lactobacillus plantarum (strain ATCC BAA-793 / NCIMB 8826 / WCFS1); Reference proteome
		2688	321967 - Lactobacillus casei (strain ATCC 334) ; Reference proteome
		1865	557436 - Lactobacillus reuteri (strain DSM 20016) ; reference proteome
		2168	387344 - Lactobacillus brevis (strain ATCC 367 / JCM 1170) ; reference proteome
		1851	1069534 - Lactobacillus ruminis (strain ATCC 27782 / RF3) ; reference proteome
		1703	362948 - Lactobacillus salivarius (strain UCC118) ; Reference proteome
		1859	272621 - Lactobacillus acidophilus (strain ATCC 700396 / NCK56 / N2 / NCFM) ; Reference proteome
		2886	1088720 - Lactobacillus rhamnosus ATCC 8530
		2749	1446494 - Lactobacillus paracasei N1115

Table S3.2 | **Contents of the in-house generated protein database.**

	Staphylococcus	2889	93061 - Staphylococcus aureus (strain NCTC 8325) ; Reference proteome
		2463	176279 - Staphylococcus epidermidis (strain ATCC 35984 / Reference proteome62A) ; Reference proteome
	Enterococcus	3101	226185 - Enterococcus faecalis (strain ATCC 700802 / V583) ; Reference proteome
		2671	333849 - Enterococcus faecium DO ; Reference proteome
	Streptococcus	1946	862971 - Streptococcus anginosus C238
		1840	1069533 - Streptococcus infantarius (strain CJ18)
		2105	208435 - Streptococcus agalactiae serotype V (strain ATCC BAA-611 / 2603 V/R) ; Reference proteome
		2030	171101 - Streptococcus pneumoniae (strain ATCC BAA-255 / R6) ; Reference proteome
	Clostridium	2659	195102 - Clostridium perfringens (strain 13 / Type A); Reference proteome
		3752	272563 - Peptoclostridium difficile (strain 630) ; Reference proteome
	Enterobacter	5044	716541 - Enterobacter cloacae subsp. cloacae (strain ATCC 13047 / DSM 30054 / NBRC 13535 / NCDC 279-56) ; Reference proteome
	Escherichia	4305	83333 - Escherichia coli (strain K12) ; Reference proteome
	Shigella	3691	300267 - Shigella dysenteriae serotype 1 (strain Sd197)
	Klebsiella	4765	272620 - Klebsiella pneumoniae subsp. pneumoniae (strain ATCC 700721 / MGH 78578) ; Reference proteome
	Proteus	3606	529507 - Proteus mirabilis (strain HI4320) ; Reference proteome
	Veillonella	1843	479436 - Veillonella parvula (strain ATCC 10790 / DSM 2008 / JCM 12972 / Te3) ; Reference proteome
	Pseudomonas	5563	208964 - Pseudomonas aeruginosa (strain ATCC 15692 / PAO1 / 1C / PRS 101 / LMG 12228) ; Reference proteome
	Citrobacter	4991	290338 - Citrobacter koseri (strain ATCC BAA-895 / CDC 4225-83 / SGSC4696) ; Reference proteome
		4651	1333848 - Citrobacter freundii CFNIH1
	Bacillus	5219	226900 - Bacillus cereus (strain ATCC 14579 / DSM 31) ; Reference proteome
		5066	1367477 - Bacillus infantis NRRL B-14911 ; Reference proteome
	Paenibacillus	6210	324057 - Paenibacillus sp. (strain JDR-2) ; Reference proteome
	Faecalibacterium	2755	718252 - Faecalibacterium prausnitzii L2-6 ; Reference proteome
	Ruminococcus	2797	657313 - Ruminococcus torques L2-14 ; Reference proteome
		1811	657321 - Ruminococcus bromii L2-63
	Acinetobacter	3465	470 - Acinetobacter baumannii ; Reference proteome
		3598	871585 - Acinetobacter calcoaceticus (strain PHEA-2)
	Rhodanobacter	3773	666685 - Rhodanobacter denitrificans ; Reference proteome
	Eubacterium	4506	903814 - Eubacterium limosum (strain KIST612) ; Reference proteome

		3545	515619 - Eubacterium rectale (strain ATCC 33656 / VPI 0990) ; Reference proteome
		2309	1263078 - Eubacterium hallii CAG:12
	Anaerostipes	3141	1262699 - Anaerostipes sp. CAG:276 ; Reference proteome
	Roseburia	3629	718255 - Roseburia intestinalis XB6B4 ; Reference proteome
		2653	1263105 - Roseburia inulinivorans CAG:15 ; Reference proteome
		3351	585394 - Roseburia hominis (strain DSM 16839 / NCIMB 14029 / A2-183) ; Reference proteome
	Blautia	3087	1263061 - Blautia hydrogenotrophica CAG:147 ; Reference proteome
	Erysipelotrichaceae	3917	1262981 - Erysipelotrichaceae bacterium CAG:64 ; Reference proteome
	Fusobacterium	2046	190304 - Fusobacterium nucleatum subsp. nucleatum (strain ATCC 25586 / CIP 101130 / JCM 8532 / LMG 13131) ; Reference proteome
	Succinatimonas	1970	1262974 - Succinatimonas sp. CAG:777 ; Reference proteome
	Haemophilus	1707	71421 - Haemophilus influenzae (strain ATCC 51907 / DSM 11121 / KW20 / Rd) ; Reference proteome
		1694	233412 - Haemophilus ducreyi (strain 35000HP / ATCC 700724) ; Reference proteome
	Anaerococcus	1691	525919 - Anaerococcus prevotii (strain ATCC 9321 / DSM 20548 / JCM 6508 / PC1) ; Reference proteome
	Gardnerella	1365	525284 - Gardnerella vaginalis (strain ATCC 14019 / 317) ; Reference proteome
	Finegoldia	1631	334413 - Finegoldia magna (strain ATCC 29328) ; Reference proteome
	Rothia	1991	680646 - Rothia mucilaginosa (strain DY-18) ; Reference proteome
		2212	762948 - Rothia dentocariosa (strain ATCC 17931 / CDC X599 / XDIA) ; Reference proteome
	Parabacteriodes	3830	435591 - Parabacteroides distasonis (strain ATCC 8503 / DSM 20701 / NCTC 11152) ; Reference proteome
		2964	1263094 - Parabacteroides merdae CAG:48 ; Reference proteome
	Bradyrhizobium	8253	224911 - Bradyrhizobium diazoefficiens (strain JCM 10833 / IAM 13628 / NBRC 14792 / USDA 110); Reference proteome
	Achromobacter	6445	762376 - Achromobacter xylosoxidans (strain A8) ; Reference proteome
	Subdoligranulum	1341	1262970 - Subdoligranulum sp. CAG:314 ; Reference proteome
	Blastococcus	4793	1146883 - Blastococcus saxobsidens (strain DD2) ; Reference proteome
	Micrococcus	2207	465515 - Micrococcus luteus (strain ATCC 4698 / DSM 20030 / JCM 1464 / NBRC 3333 / NCIMB 9278 / NCTC 2665 / VKM Ac-2230) ; Reference proteome
	Dyella	4342	1379159 - Dyella jiangningensis ; Reference proteome
	Microbacterium	3671	979556 - Microbacterium testaceum (strain StLB037) ; Reference proteome
	Paracoccus	3363	1367847 - Paracoccus aminophilus JCM 7686 ; Reference proteome
		4403	318586 - Paracoccus denitrificans (strain Pd 1222) ; Reference proteome

	Burkholdia	4872	13373 - Burkholderia mallei
		6902	216591 - Burkholderia cenocepacia (strain ATCC BAA-245 / DSM 16553 / LMG 16656 / NCTC 13227 / J2315 / CF5610) ; Reference proteome
	Methylobacterium	6566	426117 - Methylobacterium sp. (strain 4-46) ; Reference proteome
	Bartonella	1466	283166 - Bartonella henselae (strain ATCC 49882 / Houston 1) ; Reference proteome
Fungi	Candida	9044	237561 - Candida albicans (strain SC5314 / ATCC MYA-2876) ; Reference proteome
		6226	294747 - Candida tropicalis (strain ATCC MYA-3404 / T1) ; Reference proteome
		5780	578454 - Candida parapsilosis (strain CDC 317 / ATCC MYA-4646) ; Reference proteome
		5209	284593 - Candida glabrata (strain ATCC 2001 / CBS 138 / JCM 3761 / NBRC 0622 / NRRL Y-65) ; Reference proteome
Animalia	Homo sapiens	67493	9606 - Homo sapiens ; Reference proteome
	Bos taurus	23898	9913 - Bos taurus ; Reference proteome

	Bifidobacterium	*Enterobacter/Klebsiella*	*Enterococcus*	*Streptococcus*	*Veillonella*	*Clostridium*	*Staphylococcus*
Spearman correlation	0.794*	0.739*	0.572*	0.686*	0.029	0.495*	0.226
p-value	7.537E-07	1.063E-05	0.002	7.809E-05	0.884	0.009	0.256
	Corynebacterium	*Propionibacterium*	*Lactobacillus*	*Escherichia*	*Finegoldia*	*Anaerococcus*	*Haemophilus*
Spearman correlation	0.206	0.114	0.052	-0.210	0.317	0.097	-0.099
p-value	0.304	0.573	0.798	0.293	0.107	0.629	0.622

* Significant correlation

Bifidobacterium derived beta-galactosidase

GA group	Mean	SD	25-26 vs 27	25-26 vs 30	27 vs 30
EP 25-26	1.38E-04	1.57E-04	0.258	0.029*	0.028*
EP 27	5.13E-05	9.70E-05			
VP	8.93E-03	1.40E-02			

Bifidobacterium derived ABC transporters for oligosaccharides

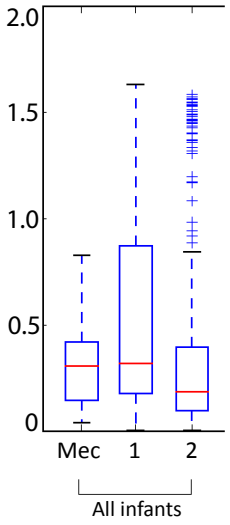
GA group	Mean	SD	25-26 vs 27	25-26 vs 30	27 vs 30
EP 25-26	2.60E-03	3.26E-03	0.171	0.00007**	0.00002**
EP 27	5.91E-04	8.85E-04			
VP	1.70E-02	1.01E-02			

Table S3.3 | **Correlation between 16S- and protein-based taxonomic classification.** P-values below 0.05 are considered significant (Spearman correlation with Monte Carlo permutation 10.000x).

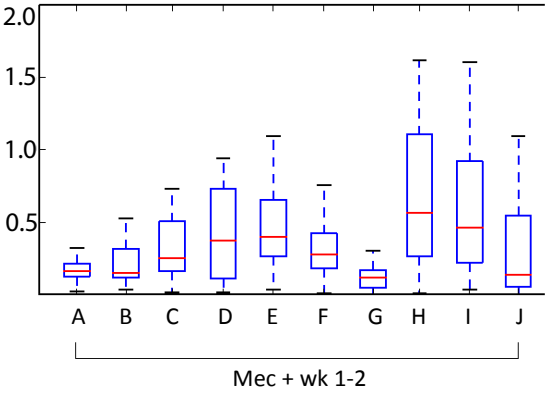
Table S3.4: **Relative abundance of Bifidobacterium-derived beta-galactosidase and ABC transporters for oligosaccharides during postnatal weeks 3-6.** Relative abundances were calculated using iBAQ intensities.
* Independent sample t-test p<0.05
** Independent sample t-test P<0.001

Figure S3.1 | **Weighted unifrac distance between (A) and within (B) infants.** A: Unifrac distance was determined with samples collected from all infants at time points meconium, week 1 and week 2 to show variation between infants at these time points. B: Unifrac distance was determined with samples collected from each infant during the first two postnatal weeks to show variation within infants. Mec: meconium, A-J: individual infants

A



B



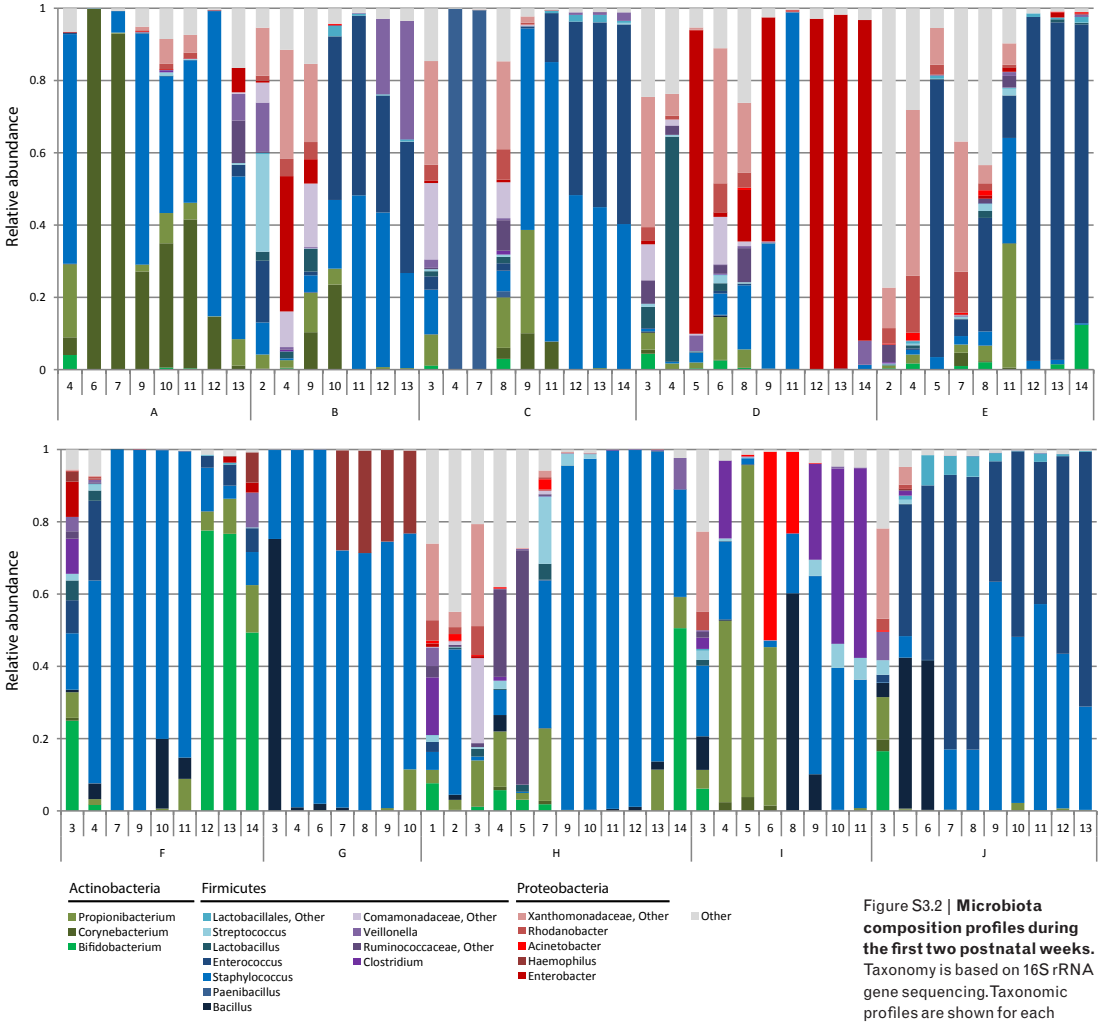
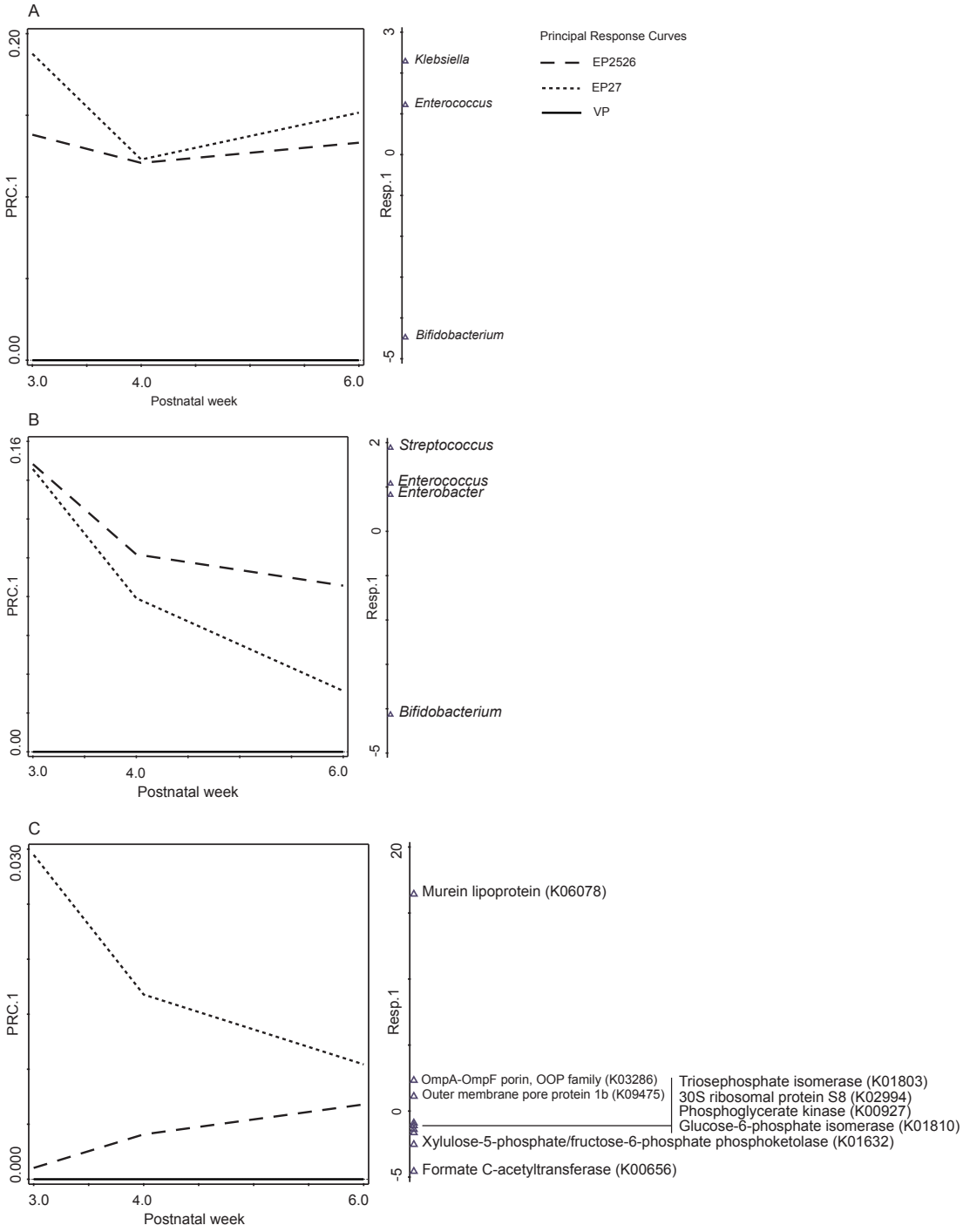


Figure S3.2 | **Microbiota composition profiles during the first two postnatal weeks.** Taxonomy is based on 16S rRNA gene sequencing. Taxonomic profiles are shown for each available time point (1-14) per infants (A-J).

Figure S3.3 | **Principal response curve analysis summarising the differences in protein-based taxonomic profiles (A), 16S-based taxonomic profiles (B) and protein-based KEGG Orthology identifier profiles between EP25-26, EP27 and VP infants throughout postnatal weeks 3-6.** Genera and proteins with a score lower than -0.85 or higher than 0.85 are shown on Resp1.


A
PRC.1
0.20
0.00
3.0
4.0
6.0
Postnatal week
Resp.1
3
0
-5
Klebsiella
Enterococcus
Bifidobacterium
Principal Response Curves
EP2526
EP27
VP
B
PRC.1
0.16
0.00
3.0
4.0
6.0
Postnatal week
Resp.1
2
0
-5
Streptococcus
Enterococcus
Enterobacter
Bifidobacterium
C
PRC.1
0.030
0.000
3.0
4.0
6.0
Postnatal week
Resp.1
20
0
-5
Murein lipoprotein (K06078)
OmpA-OmpF porin, OOP family (K03286)
Outer membrane pore protein 1b (K09475)
Triosephosphate isomerase (K01803)
30S ribosomal protein S8 (K02994)
Phosphoglycerate kinase (K00927)
Glucose-6-phosphate isomerase (K01810)
Xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (K01632)
Formate C-acetyltransferase (K00656)

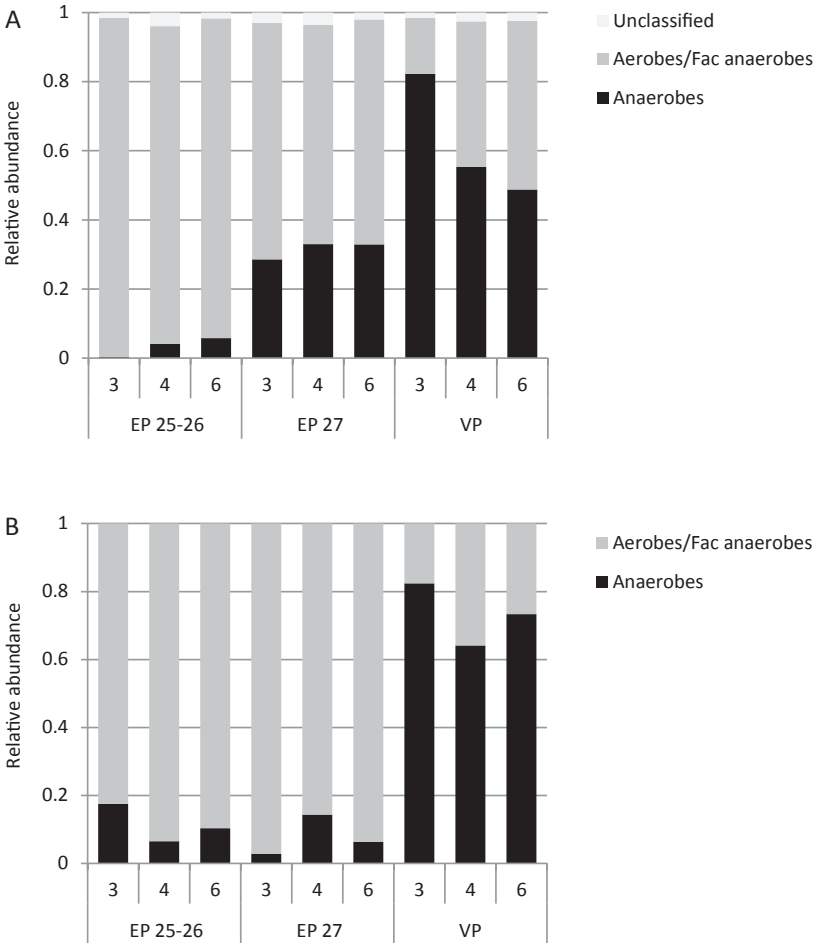


Figure S3.4 | **Proportion of aerobes/fac. anaerobes and anaerobes according to 16S rRNA gene sequencing (A) and metaproteomics (B) data.** Per time point, average relative abundance per gestational age group is shown.

Figure S3.5 | **Taxonomic profiles per functional category.** Taxonomic profiles were made for the most abundant KEGG Brite level B functional categories. Per time point, average relative abundances for each GA group are shown. Relative abundances were calculated using iBAQ intensities.

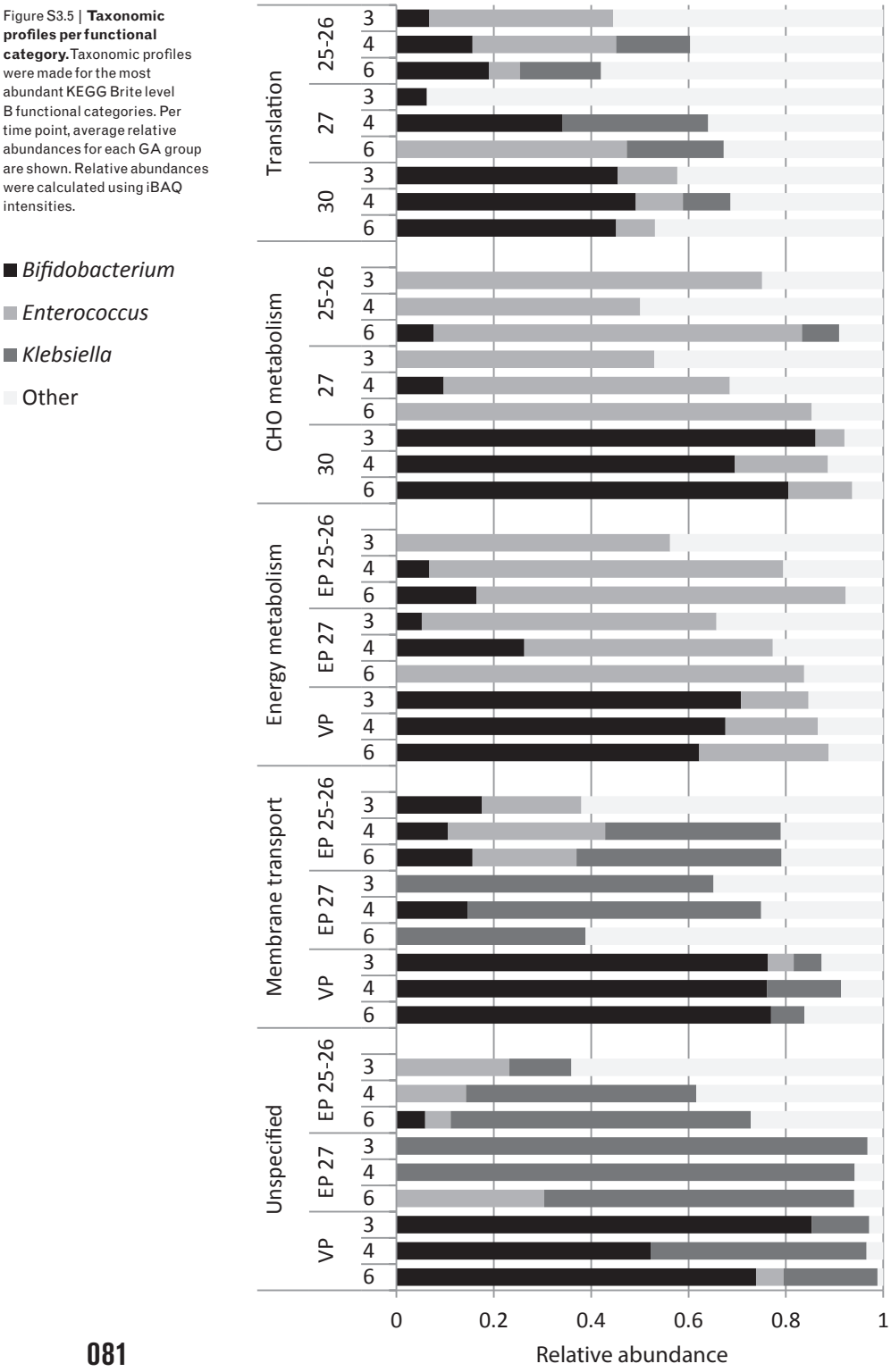
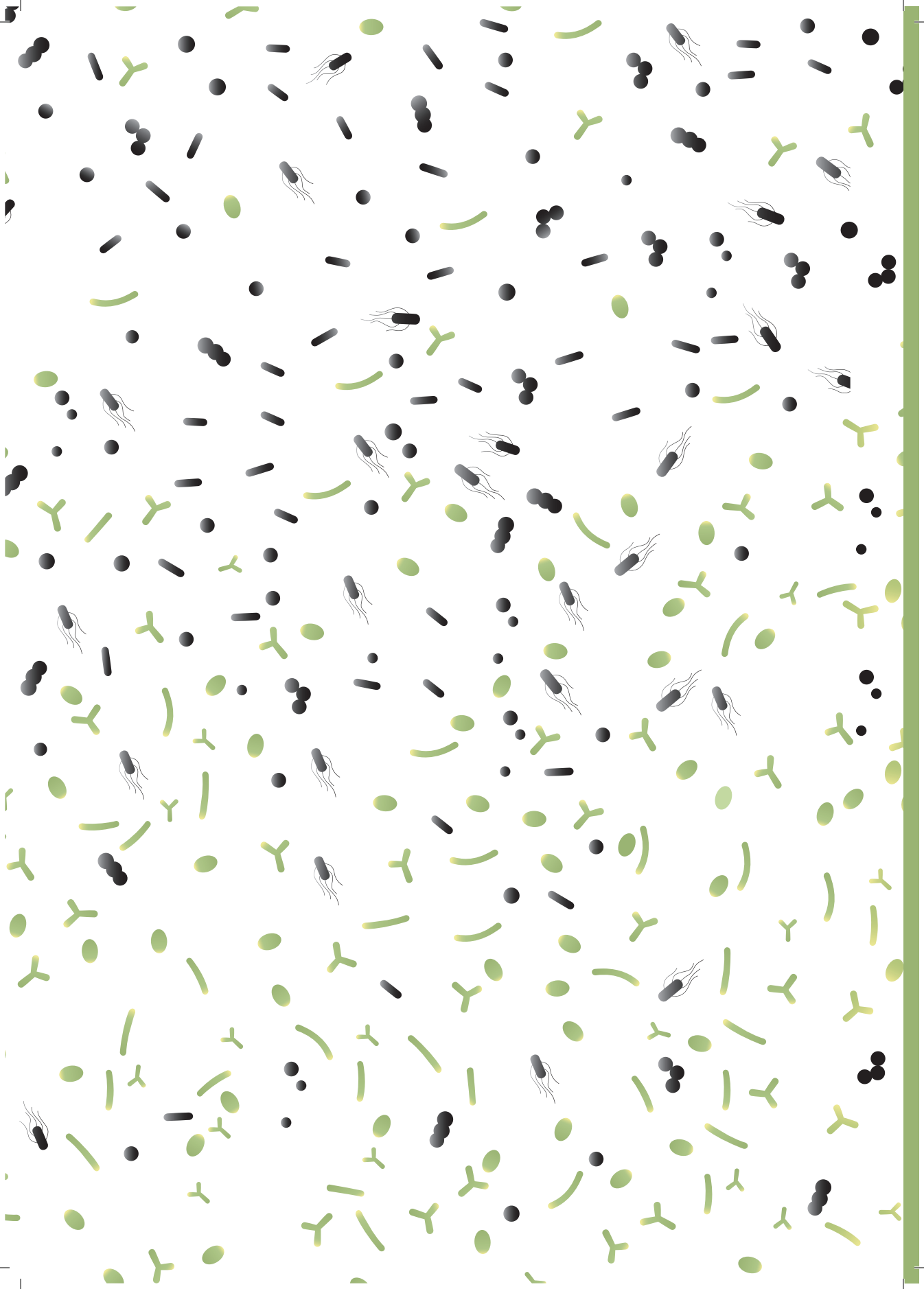


Figure S3.6 | **Protein-based (A) and 16S-based (B) taxonomic profiles and protein-based functional profiles (C) for all samples collected during postnatal weeks 3-6.**



CHAPTER 4

EFFECT OF VANCOMYCIN PROPHYLAXIS DURING REMOVAL OF A CENTRAL VENOUS CATHETER ON INTESTINAL MICROBIOTA COMPOSITION IN PRETERM INFANTS

ROMY D. ZWITTINK, ESTHER J. D' HAENS, CLARA BELZER, MARIEKE A.C. HEMELS, RICHARD A. VAN LINGEN, INGRID B. RENES, JAN KNOL AND DINY VAN ZOEREN-GROBBEN

ABSTRACT

Early life is a critical period for development of the intestinal microbiota, a process alongside metabolic and immune development, and its succession is highly susceptible to host and environmental factors. Two doses of vancomycin around time of removal of a central venous catheter reduces the incidence of central venous line removal associated sepsis, but might negatively affect development of the gut microbiota in preterm infants during a critical developmental time window. We studied microbiota development during the first six postnatal weeks in fourteen preterm infants receiving two vancomycin doses as CRAS-prophylaxis and compared their microbiota composition at postnatal week six to five preterm infants without prophylactic vancomycin treatment. The gut microbiota of preterm infants was less diverse and contained lower relative abundance of *Pseudomonas* and members of the *Comamonadaceae* family after vancomycin treatment than before treatment. In addition, decreased abundance of *Bifidobacterium* and increased abundance of *Staphylococcus* was observed in some infants after treatment. Throughout the first six postnatal weeks, the preterm infant gut microbiota became more stable and more similar between infants. At postnatal week six, treated and control infants did not differ in microbiota composition, richness and diversity, except for the abundance of *Veillonella*. Our findings indicate that two dosages of vancomycin as CRAS-prophylaxis might have short-term impact, but does not have profound lasting effects on the gut microbiota of preterm infants. Improved understanding of potential negative side effects of CRAS-prophylaxis, including its impact on microbiota development, is of clinical significance and could assist the cost-benefit determination of prophylactic antibiotic use.

INTRODUCTION

Central venous line removal associated sepsis (CRAS), defined as sepsis in the critical period 48 hours after removal of a central venous line, has an incidence of 11% in very low birthweight (VLBW, <1500 g) infants[154,155]. A randomised controlled trial showed the benefit of two prophylactic doses of antibiotics two hours before and 12 hours after removal of a central venous catheter (CVC) in reducing the incidence of CRAS[154]. It is important to consider advantages and disadvantages of CRAS-prophylaxis regarding disease burden, duration of hospital stay, antibiotic resistance and gut microbiota development. Disturbed development of the intestinal microbiota in premature infants and its potential lifelong consequences is a point of concern. Early life is a critical period for development of the intestinal microbiota, a process alongside metabolic and immune development, and its succession is highly susceptible to host and environmental factors[120,129,156]. Delivery mode, hospitalisation, timing of introduction of enteral feeding, formula feedings and antibiotics are examples of influencing factors. Premature infants are very likely to develop an altered intestinal microbiota[157,158]. Several observational studies showed an altered developmental pattern and composition of the gut microbiota in preterm infants and demonstrated the effect of short versus longer duration of antibiotics on microbiota development[69,70,159]. In the present pilot study, we evaluated microbiota development in preterm infants receiving two preventive doses of vancomycin. In addition, microbiota composition was compared with control

infants, who did not receive antibiotic prophylaxis, at the postnatal age of six weeks. Primary outcome was defined as the effect of two preventive doses of vancomycin around time of removal of a CVC on microbiota composition shortly after dosing and at postnatal week six. Secondary outcome was defined as the difference in microbiota composition at postnatal week six between infants who did and did not receive vancomycin prophylaxis.

MATERIALS AND METHODS

Subjects and sample collection

This pilot was part of an observational, non-interventional study performed from 2012 through 2013 in the level III NICU of Isala, Zwolle, The Netherlands. Included were all infants admitted to our unit with a gestational age less than 32 weeks and admitted before 96 hours of life. Written informed consent was obtained from both parents, and the study was approved by the Medical Ethical Committee of the hospital. Faecal samples were longitudinally collected during the first six postnatal weeks. Fourteen VLBW preterm infants with a CVC, but without episodes of (suspected) late-onset sepsis, all breastmilk fed were included. The infants only received antibiotics for suspected early onset sepsis composed of amoxicillin and ceftazidime during the first days of life. Prophylaxis consisted of two dosages of vancomycin (10 mg/kg) one hour before and 12 hours after removal of a CVC. Faecal samples were collected before (<5 days) and after prophylaxis (<7 days) and at the age of six weeks. During the study year, the protocol of antibiotic prophylaxis around removal of the central venous line was introduced. This provided the opportunity to include five control infants without prophylaxis and matched for gestational age and antibiotics, all receiving breastmilk and without a period of (suspected) late-onset sepsis. Additional clinical characteristics of all infants can be found in table 4.1. The timing of sampling, antibiotic use and prophylaxis of all infants are shown in figure 4.1.

Infant	GA	BW	Delivery mode	Days until FEF	PAB (days)
1	29	1090	C-section	8	5
2	28	1210	Vaginal	7	5
3	27	1090	Vaginal	9	6
4	29	1050	C-section	8	2
5	27	1130	C-section	11	2
6	27	1050	Vaginal	10	3
7	30	1420	C-section	10	2
8	26	1250	C-section	10	2
9	30	1310	Vaginal	10	2
10	28	1380	C-section	9	2
11	29	1400	Vaginal	9	3
12	29	1445	Vaginal	8	3
13	28	1230	C-section	11	3
14	30	1390	Vaginal	7	3
Mean (min-max)	**28.4 (26-30)**	**1246 (1050-1445)**		**9.1 (7-11)**	**3.1 (2-6)**
C1	27	1210	Vaginal	8	4
C2	27	1070	C-section	9	7
C3	31	2230	Vaginal	6	0
C4	27	1050	Vaginal	10	3
C5	27	1090	Vaginal	10	3
Mean (min-max)	**27.8 (27-31)**	**1596 (1050- 2230)**		**8.6 (6-10)**	**3.4 (0-7)**

Table 4.1 | **Infant characteristics.** Abbreviations: GA: gestational age, BW: birth weight, FEF: full enteral feeding, PAB: postpartum antibiotics, C-section: caesarean section

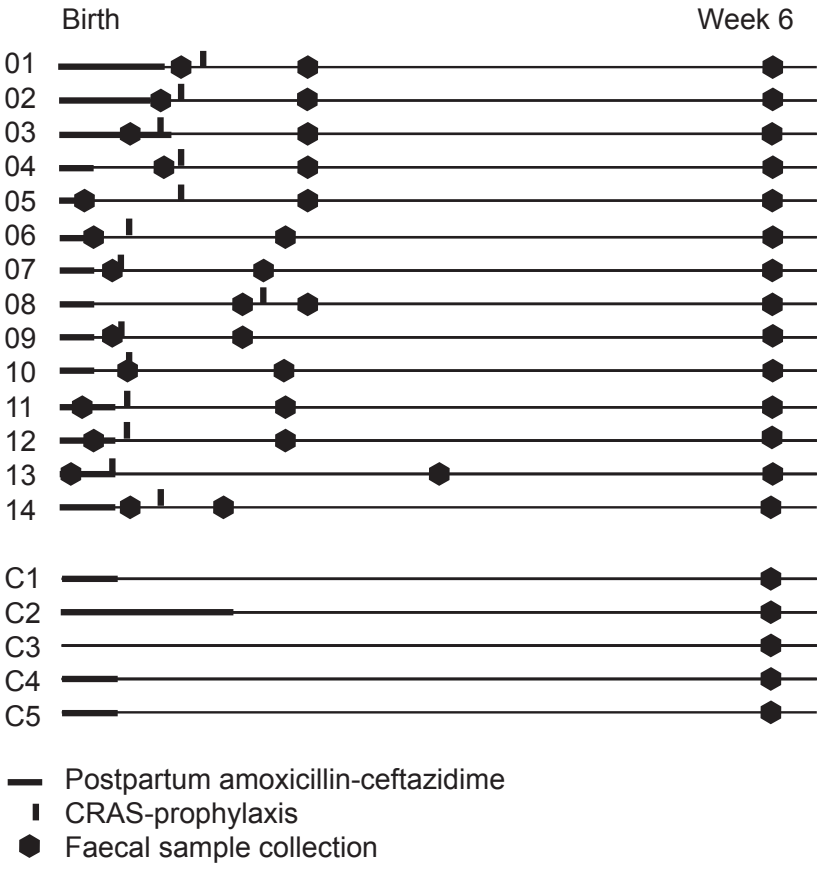


Figure 4.1 | **Timelines per infant regarding antibiotic treatment, faecal samples and CRAS-prophylaxis.**

16S rRNA gene amplicon sequencing

DNA extraction, library preparation and sequencing were performed by LifeSequencing S.L. (Valencia, Spain). DNA was extracted from 200 mg faeces using the QIAamp Fast DNA Stool Mini Kit (Qiagen), including enzymatic and chemical cell disruption by bead beating. DNA was purified and concentrated using the PowerMag DNA clean-up kit (MoBio) and 50 ng of DNA was amplified according to the Metagenomic Sequencing Library Illumina 15044223 B protocol (Illumina). For the first amplification step, primers were designed containing a universal linker sequence allowing amplicons for incorporation of indexes and sequencing primers by Nextera XT Index kit (Illumina) and 16S rRNA gene primers for region V3-V4 (Klindworth *et al*., 2013). Indexes were included during a second amplification step. Libraries were quantified using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermofisher) and pooled prior to sequencing on the MiSeq platform (Illumina, 300 bases paired-end). The size and quantity of the pool were respectively assessed on the Bioanalyzer 2100 (Agilent) and with the Library Quantification Kit for Illumina (Kapa Biosciences).

Data analysis

Read filtering, OTU-picking and taxonomic assignment were performed using the NG-Tax pipeline with the following settings: read length of 70, ratio OTU abundance of 2, classify ratio of 0.8, minimum percentage threshold of 0.5, identity level of 100%, error correction of 98.5, using the Silva_128_SSU Ref database[143,160]. For within infant (dependent) or between infants (independent) comparisons, the nonparametric Wilcoxon Signed Rank test and Kruskal-Wallis test, both with Monte Carlo permutation (10000x), were applied respectively. Before testing for differences in taxonomy, the OTU table was filtered for OTUs present in less than 25% of the samples. For analysing intra-individual and inter-individual differences in microbiota composition, the distance metric Unifrac was used. To relate microbiota composition to clinical data, redundancy analysis (RDA) was performed using Canoco multivariate statistics software v5. Factors were considered to have a significant influence when the false discovery rate corrected p-value was below 0.05.

Figure 4.2 | **Microbiota composition profiles.** The 15 most abundant taxa are shown, which were determined by calculating the average relative abundance of all samples. Relative abundance of all other identified taxa were summed under category 'other'.

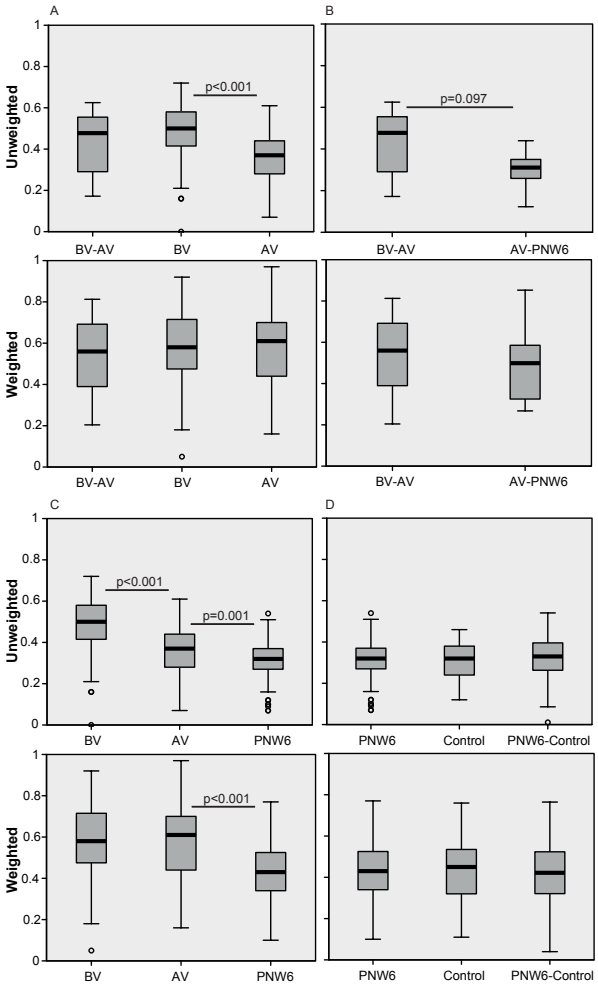


Figure 4.3 | **Unweighted and weighted unifrac distances.** BV-AV: within infants before and after treatment, BV: between infants before treatment, AV: between infants after treatment, AV-PNW6: within infants after treatment to postnatal week 6, PNW6: between treated infants at postnatal week 6, Control: between control infants at postnatal week 6, PNW6-Control: between treated and control infants. Boxplots show the median, 25th and 75th percentiles, and minimal and maximal values except for outliers (circles). For within infant or between infants comparisons, respectively the nonparametric Wilcoxon Signed Rank test and Kruskal-Wallis test with Monte Carlo permutation were applied.

RESULTS

Microbiota composition throughout the first six postnatal weeks

Gut microbiota composition was determined in fourteen infants before (BV) and shortly after (AV) two dosages of vancomycin, and at postnatal week six (PNW6) (Fig 4.2). *Staphylococcus*, *Enterococcus*, *Bifidobacterium*, *Enterobacteriaceae*, *Streptococcus*, *Peptoclostridium*, *Clostridium* and *Lactobacillus* were identified as abundant members, but their abundance, and the composition of the whole community, varied greatly between and within infants. Variation in microbiota composition within infants, as determined by comparing BV with AV samples, was of the same level as between infants before treatment and of higher level than between infants after treatment (Fig 4.3a). Microbiota composition became more stable over time, as indicated by a trend of higher unweighted unifrac distances between the BV and AV samples than between the AV and PNW6 samples (Fig 4.3b). In addition, microbiota composition became more similar between infants over time, as indicated by gradually decreasing unifrac distances (Fig 4.3c).

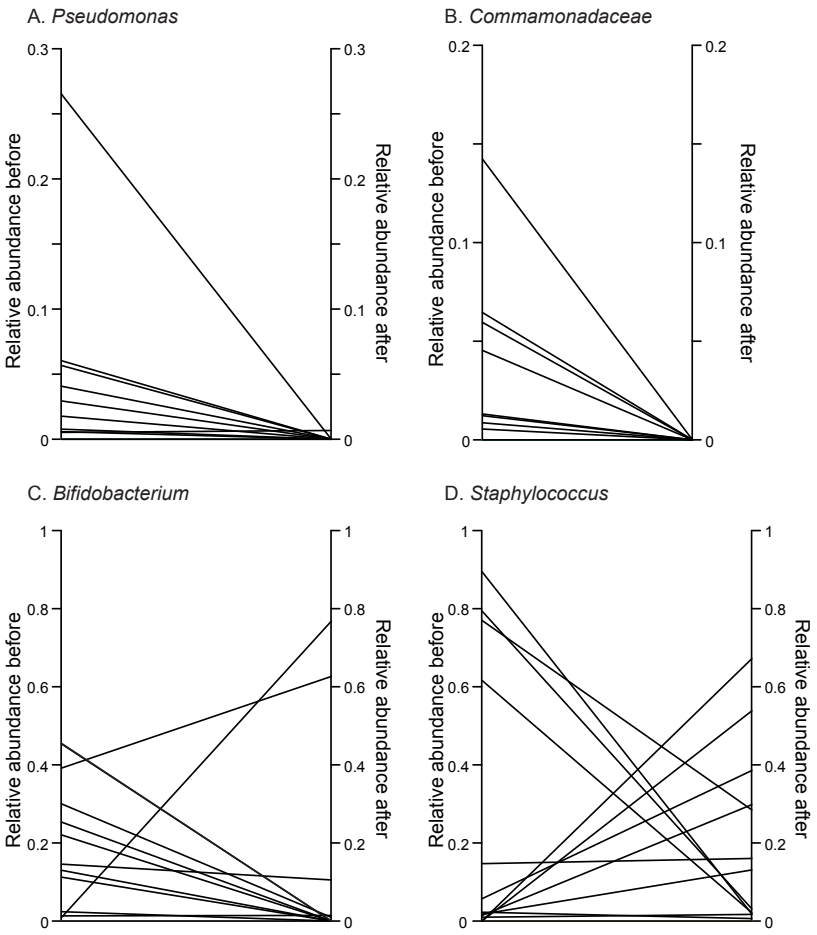


Figure 4.4 | **Taxa specific changes from before to after CRAS-prophylaxis.** Each line represents an infant.

Microbiota composition before and after CRAS-prophylaxis

To determine the effect of vancomycin prophylaxis, microbiota composition was compared between samples collected before and after prophylaxis. Members of the genus *Pseudomonas* and the family *Comamonadaceae* were significantly decreased after vancomycin prophylaxis (p=0.015, Fig 4.4a, 4.4b). Although not significantly different, relative abundance of *Bifidobacterium* decreased to non-detectible levels in six infants (43%) after vancomycin prophylaxis (Fig 4.4c). Strikingly, relative abundance of *Staphylococcus,* a target organism of vancomycin, increased in five infants (36%) after vancomycin prophylaxis (Fig 4.4d). Diversity of the gut microbiota was higher before than after treatment (p=0.035), and a trend towards decreased community richness could be observed (p=0.062, Fig 4.5a).

Microbiota composition at postnatal weeks six in infants with or without CRAS-prophylaxis

To elucidate whether CRAS-prophylaxis has long lasting effects, microbiota composition was determined at postnatal week six in preterm infants who did (PNW6) or did not (control) receive vancomycin treatment (Fig 4.2). Variation

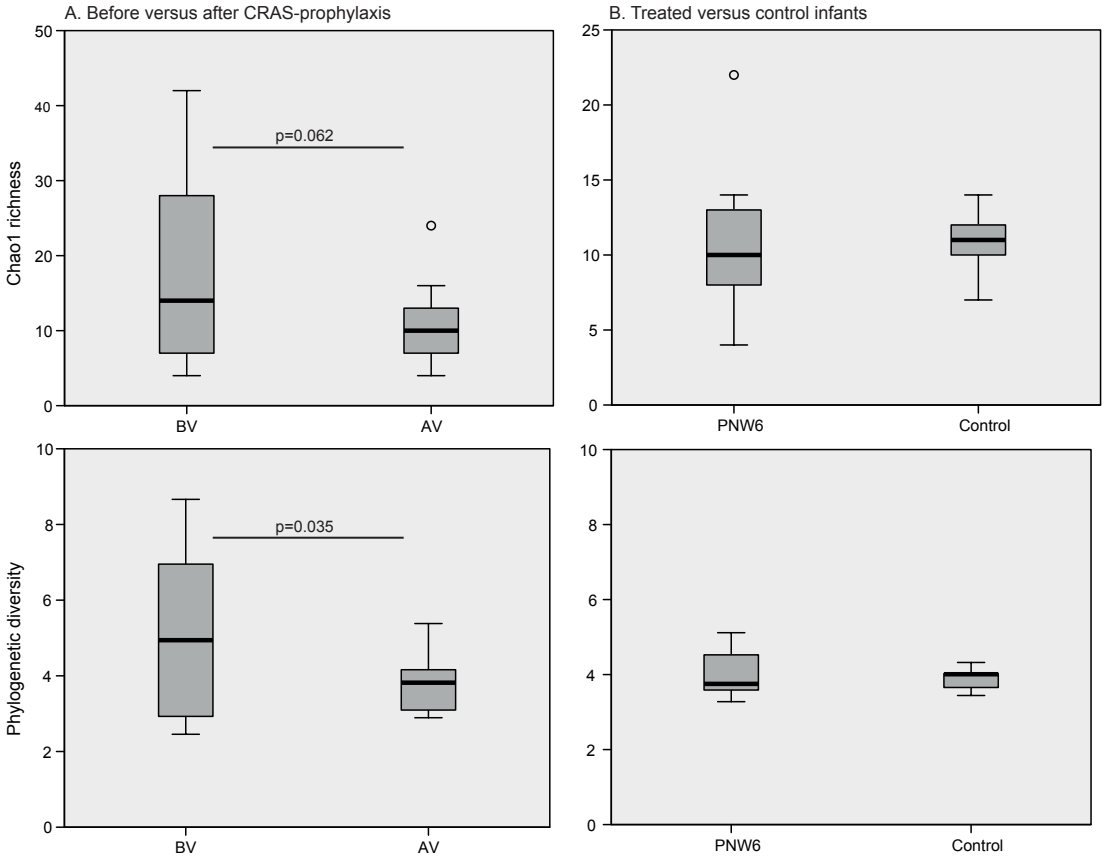


Figure 4.5 | **Community richness (choa1) and diversity (phylogenetic diversity) before and after CRAS-prophylaxis (A), and at postnatal week six in treated and control infants (B).** Boxplots show the median, 25th and 75th percentiles, and minimal and maximal values except for outliers (circles). For within infant or between infants comparisons, respectively the nonparametric Wilcoxon Signed Rank test and Kruskal-Wallis test with Monte Carlo permutation were applied.

in microbiota composition was similar between vancomycin treated infants, between control infants and between vancomycin treated and control infants (Fig 4.3d). In addition, no difference in community richness and diversity was observed (Fig 4.5b). Regarding taxonomy, relative abundance of *Veillonella* was lower in vancomycin treated infants (p=0.024), but there were no significant differences in the abundance of other bacterial taxa.

DISCUSSION

Two doses of vancomycin around time of removal of a central venous catheter reduces the incidence of CVC-associated sepsis[154,155]. It may, however, affect development of the gut microbiota in preterm infants during a critical developmental time window. We studied microbiota development during the first six postnatal weeks in 14 preterm infants receiving two vancomycin doses as CRAS-prophylaxis and compared their microbiota composition at postnatal week six to five preterm infants without prophylactic vancomycin treatment.

The gut microbiota of preterm infants was less diverse and contained lower relative abundance of *Pseudomonas* and members of the *Comamonadaceae* family after vancomycin treatment than before treatment. In addition, decreased

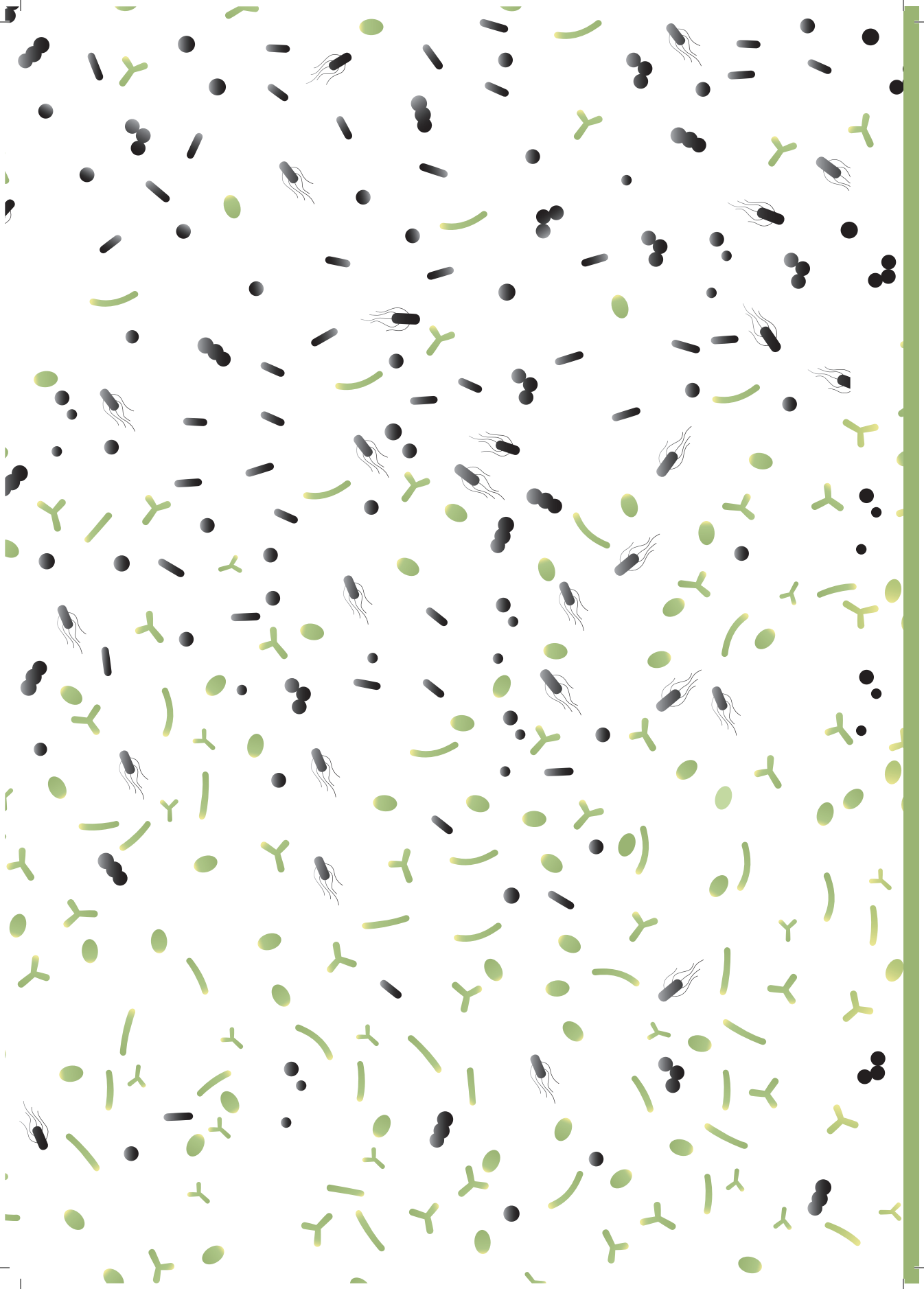
abundance of *Bifidobacterium* and increased abundance of *Staphylococcus* was observed in some infants after treatment. Since bifidobacteria are considered beneficial early life colonisers that are abundant during early life development of the gut microbiota, the observed decreased abundance of *Bifidobacterium* is a point of concern[161]. Bifidobacteria play an important role in milk digestion, limit the possibilities for pathogen colonisation and support development of the immune system[31,32]. We have previously associated postpartum antibiotic treatment with lower abundance of bifidobacteria during early development of the preterm infant gut microbiota[99,159]. Decreased abundance of *Bifidobacterium* species might therefore be a result of postpartum amoxicillin/ceftazidime treatment rather than of two vancomycin dosages. The lack of a control group during the first two postnatal weeks prohibits to determine the sole effect of CRAS-prophylaxis on microbiota composition. The increasing abundance of *Staphylococcus* is a striking observation, since vancomycin is the preferred antibiotic to prevent and treat staphylococcal infections. This observation could indicate resistance to vancomycin which has been previously documented for *S. capitis* strains in hospitalised neonates[162]. However, it must be noted that relative abundances were evaluated in this study, which is not a good indication for the absolute abundance of *Staphylococcus*, which may actually be stable or decreasing.

Throughout the first six postnatal weeks, the preterm infant gut microbiota became more stable and more similar between infants. At postnatal week six, treated and control infants did not differ in microbiota composition, richness and diversity, except for the abundance of *Veillonella*. This indicates that two dosages of vancomycin as CRAS-prophylaxis does not have profound lasting effects on the gut microbiota of preterm infants. A two-week vancomycin course for treatment of *Clostridium difficile* infection has shown to have individual-specific long-term impact on gut microbiota composition in adults[163]. In preterm neonates, the response to vancomycin treatment, in terms of shifts in microbiota composition, are individual-specific as well, but generally temporary[164]. It must be noted, however, that only 18 infants received vancomycin in that study, and that for 12 of these infants vancomycin was combined with cephalosporin[164].

Overall, our findings indicate that two dosages of vancomycin as CRAS-prophylaxis does not have profound lasting effects on the gut microbiota of preterm infants, which, together with decreased disease burden and hospital stay, favours its application. Small subject size, great inter-individual variation, and exposure to other antibiotics during the first postnatal week, however, could have prohibited the detection of consistent alterations in microbiota composition as a result of two vancomycin dosages. A randomised controlled trial with more inclusions might aid elucidating the sole effect of vancomycin prophylaxis on microbiota development in preterm infants. Improved understanding of potential negative side effects of CRAS-prophylaxis, including its impact on microbiota development, is of clinical significance and could assist the cost-benefit determination of prophylactic antibiotic use.

095

Effect of vancomycin prophylaxis during removal of a central venous catheter on intestinal microbiota composition in preterm infants



CHAPTER 5

ASSOCIATION BETWEEN DURATION OF INTRAVENOUS ANTIBIOTIC ADMINISTRATION AND EARLY LIFE MICROBIOTA DEVELOPMENT IN LATE PRETERM INFANTS

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ABSTRACT

Antibiotic treatment is common practice in the neonatal ward for prevention and treatment of sepsis, which is one of the leading causes of mortality and morbidity in preterm infants. Although the effect of antibiotic treatment on microbiota development is well recognised, little attention has been paid to treatment duration. We studied the effect of short and long intravenous antibiotic administration on intestinal microbiota development in preterm infants. Faecal samples from fifteen preterm infants (35±1 weeks gestation and 2871±260 gram birthweight) exposed to no, short (≤3 days) or long (≥5 days) treatment with amoxicillin/ceftazidime were collected during the first six postnatal weeks. Microbiota composition was determined through 16S rRNA gene amplicon sequencing and by qPCR. Short and long antibiotic treatment significantly lowered abundance of *Bifidobacterium* right after treatment (p=0.027) till postnatal week three (p=0.028). Long treatment caused *Bifidobacterium* abundance to remain decreased till postnatal week six (p=0.009). Antibiotic treatment was effective against members of the *Enterobacteriaceae* family, but allowed *Enterococcus* to thrive and remain dominant for up to two weeks after antibiotic treatment discontinuation. Community richness and diversity were not affected by antibiotic treatment, but were positively associated with postnatal age (p<0.023) and abundance of *Bifidobacterium* (p=0.003). Intravenous antibiotic administration during the first postnatal week greatly affects the infant's gastrointestinal microbiota. However, quick antibiotic treatment cessation allows for its recovery. Disturbances in microbiota development caused by short and more extensively by long antibiotic treatment, could affect healthy development of the infant via interference with maturation of the immune system and gastrointestinal tract.

INTRODUCTION

Over the last years, the intestinal microbiota has been well recognised as major contributor to human health and disease[165]. Despite its described importance, intestinal microbiota development is not completely understood as it is a highly dynamic process affected by multiple host and environmental factors of which gestational age, mode of delivery, diet and antibiotics are perceived as major influencing factors[120]. Previous studies showed that antibiotic treatment in early life can lead to short- and long-term alterations of the intestinal microbiota, which has been related to early and later life health outcomes such as asthma and adiposity[71,72,166]. Antibiotic treatment is common practice in the neonatal ward for prevention and treatment of sepsis, which is one of the leading causes of mortality and morbidity in preterm infants. Antibiotics, such as amoxicillin, ceftazidime, erythromycin and vancomycin are frequently used as they target a broad spectrum of pathogens. Intrauterine infections are a common cause of preterm birth, thus many preterm infants are born with suspicion of infection and are therefore treated with antibiotics from birth onwards. In preterm infants, group B streptococci and *Escherichia coli* are associated with onset of neonatal sepsis[167]. Since sepsis in preterm infants still has a high mortality and morbidity it is not possible to wait for test

results before starting antibiotic treatment. To reduce the antibiotic load in the neonatal ward it is common practice to evaluate the need for antibiotics after 48 hours and stop antibiotics if the infection is not proven. The applied antibiotic strategies in neonatology led to decreased mortality and morbidity rates. However, there is a risk of impeding gut microbiota development and increasing antibiotic resistance[168]. It has been shown that in infants intestinal microbiota composition and activity in early life is associated with gestational age and can be related to the degree of perinatal antibiotic administration[67,99]. In addition, it has been shown that preterm infants admitted to the neonatal intensive care unit (NICU) are particularly colonised by antibiotic resistant and virulent bacterial strains during early life, which was restored around two years of age[68]. Despite increased understanding of how antibiotic treatment affects preterm infant microbiota development, not much is known about the effect of duration of treatment. Previous studies showed that long antibiotic treatment (>5 days) in preterm infants results in lower diversity of the faecal microbiota than short treatment, but no clear differences in overall microbiota composition were observed[69,70]. This could be due to other factors that influence microbiota composition which were not accounted for during stratification of the infants, such as gestational age[70] or mode of delivery[69]. As gestational age and mode of delivery by itself influence early life microbiota development, the present study accounted for these in order to further understand the effect of antibiotic treatment duration on preterm infant microbiota development.

MATERIALS AND METHODS

Subjects and sample collection

This study was part of an observational, non-intervention study involving (pre) term infants admitted to the hospital level III NICU or the level II neonatal ward of Isala in Zwolle, The Netherlands. The ethics board from METC Isala Zwolle concluded that this study does not fall under the scope of the Medical Research Involving Human Subjects Act (WMO). Informed consent was obtained from both parents of all individual participants included in the study. For faecal microbiota profiling, fifteen late preterm infants (mean ± SD, 35.7 ± 0.9 weeks gestation, 2871 ± 261 grams birthweight) were longitudinally sampled during the first six postnatal weeks resulting in a total of 95 samples. Sampling days for each infant can be found in table 5.1. Infants received either no (control), short-term (ST, <3 days) or long-term (LT, >5 days) treatment with a combination of amoxicillin and ceftazidime during the first postnatal week. Infants started antibiotic treatment on clinical suspicion of a bacterial infection and upon negative or positive cultures antibiotic administration was respectively stopped (ST) after two to three days or continued (LT) up till a maximum of seven days. Of the LT infants, one was diagnosed with sepsis and three with pneumonia, and in all cases the causative pathogen was unknown. Meconium and faecal samples were collected at birth and at postnatal weeks one, two, three, four and six. Samples were stored temporally at -20°C until transfer to -80°C. Infant clinical characteristics can be found in table 5.1. All infants were born vaginally, only received enteral nutrition and did not have clinical signs of food intolerance.

Group	Infant	Gender	GA	BW (g)	AB (days)	Reason AB	Maternal AB	Days until FEF	Human milk (% per week)	Sampling days	Discharge	PREE	PROM	AM
Control	A	Female	35+2	2700	0	-	No	8	75, 98, 27, 0, 0	2, 7, 16, 22, 29, 39	9	No	Yes	No
Control	B	Male	35+5	3110	0	-	No	7	70, 100, 100, 100, 100	3, 5, 14, 21, 28, 42	5	No	No	No
Control	C	Male	34+2	2800	0	-	Yes	9	90, 100, 100, 100, 100	1, 6, 13, 21, 29, 41	12	No	No	Yes
Control	D	Male	35+1	3030	0	-	No	7	62, 100, 100, 100, 100	1, 6, 14, 22, 29, 43	7	No	No	No
Control	E	Male	35+1	2500	0	-	No	6	90, 100, 100, 100, 100	4, 6, 14, 21, 28, 43	11	Yes	No	No
Average ± SD			35.1 ± 0.5	2828 ± 221				7.4 ± 1.0			8.8 ± 2.6			
ST	F	Male	34+5	2385	3	Suspicion	Yes	7	30, 0, 0, 0, 0	3, 6, 7, 14, 22, 29, 44	10	No	Yes	No
ST	G	Male	35+2	3050	2.5	Suspicion	No	6	63, 100, 100, 100, 100	3, 6, 14, 21, 28, 42	6	No	No	No
ST	H	Male	35+2	2515	3	Suspicion	Yes	7	93, 100, 100, 100, 100	2, 6, 14, 21, 29, 43	10	No	No	Yes
ST	I	Male	37+0	2980	2	Suspicion	Yes	6	69, 89, 90, 86, 88	1, 4, 7, 14, 21, 28, 43	5	No	Yes	No
ST	J	Female	37+1	3130	2	Suspicion	No	7	80, 100, 100, 100, 100	2, 6, 13, 20, 29, 42	4	Yes	No	No
Average ± SD			35.8 ± 1.0	2812 ± 302				6.6 ± 0.5			7.0 ± 2.5			
LT	K	Male	36+6	2930	7	Pneumonia	No	6	74, 100, 100, 100, 47	3, 6, 10, 14, 21, 28, 42	8	No	Yes	Yes
LT	L	Female	35+3	2903	6	Sepsis	No	7	94, 100, 100, 100, 100	3, 6, 10 15, 21, 28, 42	15	No	No	Yes
LT	M	Male	34+5	2805	5	Susp. sepsis	Yes	6	22, 20, 19, 29, 29	4, 8, 14, 22, 28, 43	6	No	Yes	No
LT	N	Male	36+1	2830	7	Pneumonia	No	8	67, 97, 100, 100, 100	1, 5, 12, 20, 27, 41	14	No	Yes	No
LT	O	Male	37+1	3400	7	Pneumonia	No	7	7, 33, 25, 11, 10	4, 6, 11, 14, 21, 28, 42	8	Yes	No	No
Average ± SD			36.1 ± 0.9	2974 ± 218				6.8 ± 0.7			10.2 ± 3.6			

Table 5.1 | **Infant characteristics.**

Abbreviations: GA: gestational age, BW: birthweight, AB: antibiotics, FEF: full enteral feeding, PREE: preeclampsia, PROM: prolonged rupture of membranes, AM: antimycotics.

DNA extraction

DNA was extracted from faeces by the repeated bead beating plus phenol/chloroform method as described previously[137]. DNA was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and by using a Qubit®2.0 fluorometer (LifeTechnologies, Carlsbad, CA, USA) according to manufacturer's instructions.

454 pyrosequencing

Amplification of the V3-V5 regions of the 16S rRNA gene was performed using the Bifidobacterium optimised 357F and 926Rb primers as described previously[138]. For each sample, the reverse primer included a unique barcode sequence to allow for multiplexing. PCR and 454 pyrosequencing (GS Junior, Roche) were performed by LifeSequencing S.L. (Valencia, Spain) as described previously[138]. Sequencing data is available in the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under study accession PRJEB19937.

Sequencing data analysis

Pyrosequencing data was analysed using the QIIME software package (v1.8)[139] applying Acacia[140], USearch[141], UCLUST[142] and the SILVA 111 database[143] for denoising, chimera removal, OTU picking and taxonomic classification respectively. The obtained OTU table was filtered for OTUs with a number of sequences less than 0.005% of the total number of sequences[169]. To account for variation between samples total number of reads, rarefaction to 4085 reads per sample was applied. To identify bacterial taxa that were significantly different in abundance between control, ST and LT infants, the nonparametric Kruskal-Wallis test with Monte Carlo permutation (10000x) was applied. The Kruskal-Wallis test was done using absolute read counts for each taxonomic group and after the OTU table was filtered for OTUs present in less than 25% of the samples. To compare richness and diversity between samples, the Wilcoxon Signed Rank test, Mann-Whitney U test and Kruskal-Wallis test were applied for dependent, two groups of independent and more than two groups of independent samples respectively. To study (dis)similarities in microbiota composition and relate changes in microbiota composition to clinical data, principal component analysis (PCA) and redundancy analysis (RDA) were performed using the Canoco multivariate statistics software v5. For RDA analysis, factors were considered significant when the Bonferroni corrected p-value was below 0.05. Co-occurrence patterns were determined by Spearman correlation using the taxa that remained after the OTU table was filtered for OTUs present in less than 25% of the samples. Visualisation was done using the Gephi-0.9.1 platform (https://gephi.org/) and Adobe Illustrator CS6.

qPCR analysis

Real-time PCR amplification and detection were performed on a CFX384TM real-time PCR detection system (Bio-Rad). The reaction mixture was composed of 5 µl iQTM SYBR® Green Supermix, 0.2 µl forward and reverse primers (10 nmol), 1.6 µl nuclease-free water and 3 µl of DNA template (2 ng/µl). Primers used targeted total 16S[170], *Bifidobacterium*[171], *Enterococcus*[172] and *Enterobacteriaceae*[173]. The program for amplification of total 16S, *Bifidobacterium*

and *Enterococcus* was initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 20 seconds, annealing at 60°C for 20 seconds and elongation at 72°C for 50 seconds, followed by a melt-curve from 60°C to 95°C with 0.5°C steps. The program for amplification of *Enterobacteriaceae* was initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 55.8°C for 20 seconds and elongation at 72°C for 20 seconds, followed by a melt-curve from 60°C to 95°C with 0.5°C steps. Standard curves contained 10^1-10^9 16S rRNA copies/µl and were performed in triplicate. Data was analysed using CFX Manager™ software (Bio-Rad). Relative abundances of the taxa were determined by dividing taxa specific 16S rRNA gene copy number by total 16S rRNA gene copy number. qPCR and pyrosequencing data had a Spearman correlation of 0.758, 0.729 and 0.822 for *Bifidobacterium*, *Enterococcus* and *Enterobacteriaceae* respectively. To identify bacterial taxa that were significantly different in abundance between control, ST and LT infants, the nonparametric Kruskal-Wallis test with Monte Carlo permutation (10000x) was applied.

RESULTS

Antibiotic treatment delays colonisation by Bifidobacterium

Microbiota composition throughout the first six postnatal weeks was determined in fifteen infants with varying antibiotic treatment duration. Microbiota composition of control infants was characterised by a high abundance of *Bifidobacterium* throughout the first six postnatal weeks, with an average relative abundance of 45% in meconium increasing towards 73% at postnatal week six (Fig 5.1a). In three out of five infants, *Bifidobacterium* already covered more than 50% abundance in the meconium sample (Fig S5.1). These findings were confirmed by qPCR analysis (Fig 5.1b, S5.2). Despite common characteristics, control infants microbiota composition also contained individual specific profiles. Outstanding were: dominance of *Enterobacter* at postnatal weeks one and two in one infant (infant C), dominance of *Actinomyces* at postnatal weeks three and four in another infant (infant B), members of Proteobacteria were only identified in three infants, and in three infants *Enterococcus* was a dominant member in the meconium sample (Fig S5.1). Microbiota composition of ST and LT infants was not characterised by a particular microbiota profile that lasted throughout the first six postnatal weeks, but showed high variability between and within infants (Fig 5.1a, S5.1). To understand the effect of antibiotic treatment duration on intestinal microbiota development, microbiota composition was compared between control, ST and LT infants. ST and LT infants had a significantly lower abundance of *Bifidobacterium* right after antibiotic treatment ($p= 0.027, 0.027$) and at postnatal weeks one ($p=0.027, 0.021$), two ($p=0.016, 0.009$), and three ($p=0.028, 0.028$) compared to control infants. In LT infants, *Bifidobacterium* abundance also remained lower during postnatal weeks four ($p=0.086$) and six ($p=0.009$), whereas this could not be observed in ST infants. qPCR analysis confirmed the significantly lower quantity of *Bifidobacterium* right after a short ($p=0.033$) or long ($p=0.035$) antibiotic treatment compared to control infants. *Enterococcus* became a dominant member of the community in multiple ST and LT infants during the first postnatal week, which was not

observed in any of the control infants (Fig 5.1, S5.1, S5.2), however, except for postnatal week two, differences were not statistically significant. Total bacterial count, as determined by qPCR, was not significantly reduced by antibiotic treatment (Fig S5.3). However, in some ST and LT infants, lower total bacterial count at early time points suggests delayed colonisation due to antibiotic treatment. The differences in microbiota development over time were further explored via principal response curve and redundancy analysis. Temporal microbiota development was different between control, ST and LT infants (p=0.002) (Fig 5.2a). Short treatment allowed for development towards a microbiota composition more similar to control infants, characterised by high abundance of *Bifidobacterium* (Fig 5.2a, 5.2c). Abundances of *Bifidobacterium*, *Clostridium* and *Enterococcus* were different between control and antibiotic treated infants (Fig 5.2b). The abundance of *Bifidobacterium* and *Enterobacter* at postnatal week six explained the difference in temporal microbiota development between ST and LT infants (Fig 5.2b).

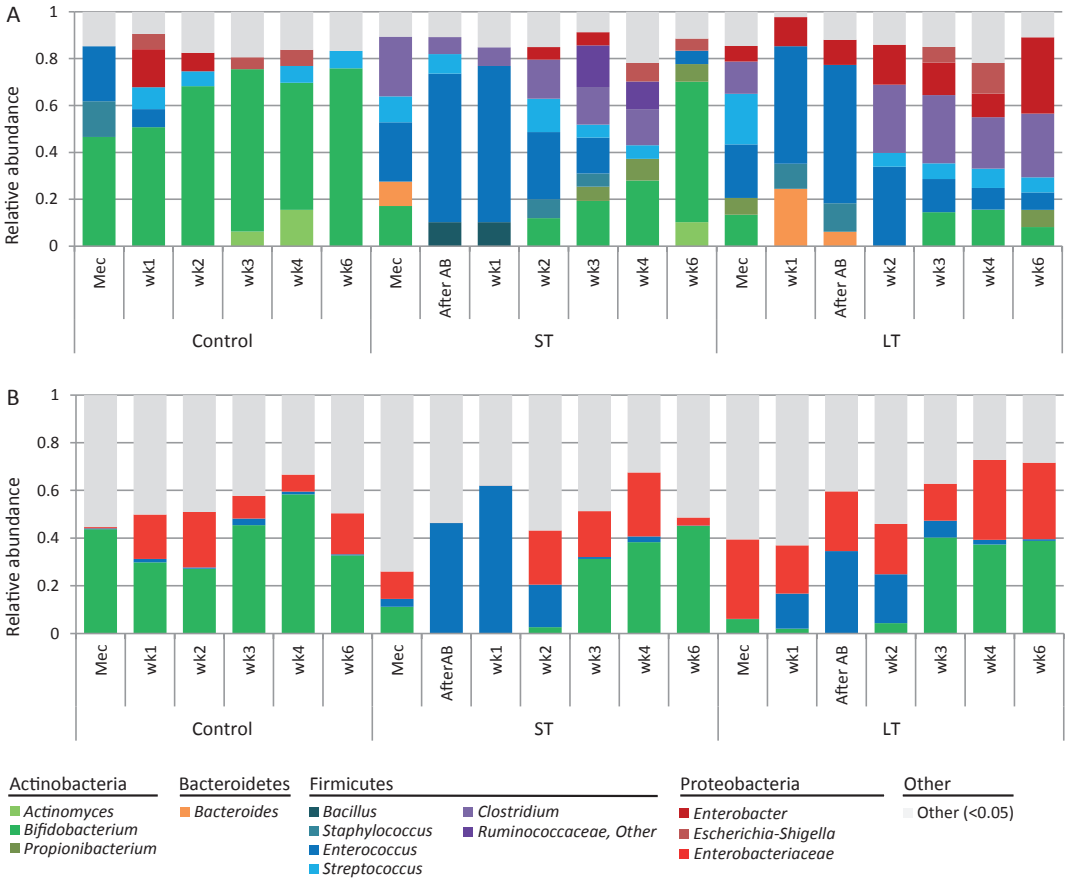


Figure 5.1 | **Microbiota composition profiles based on 16S rRNA-gene sequencing (A) and qPCR (B).** Per time point, averages of five infants are shown. For 16S rRNA gene sequencing data, genera with a relative abundance of more than 5% are shown.

Antibiotic treatment duration was the main factor significantly explaining the observed variation in microbiota composition between samples (p=0.002, Fig 5.2d). No, short and long antibiotic treatment explained 14.9%, 3.6% and 3.6% of the variation respectively. Other factors significantly explaining the variation were postnatal age (7.5%), gestational age (4.9%), preeclampsia (3.2%) and maternal antibiotics (2.9%) (Fig 5.2d). Gender, birth weight, proportion of human milk, days until full enteral feeding, days until discharge, indwelling catheters, pain medication, antimycotic use and prolonged rupture of membranes did not significantly affect microbiota composition.

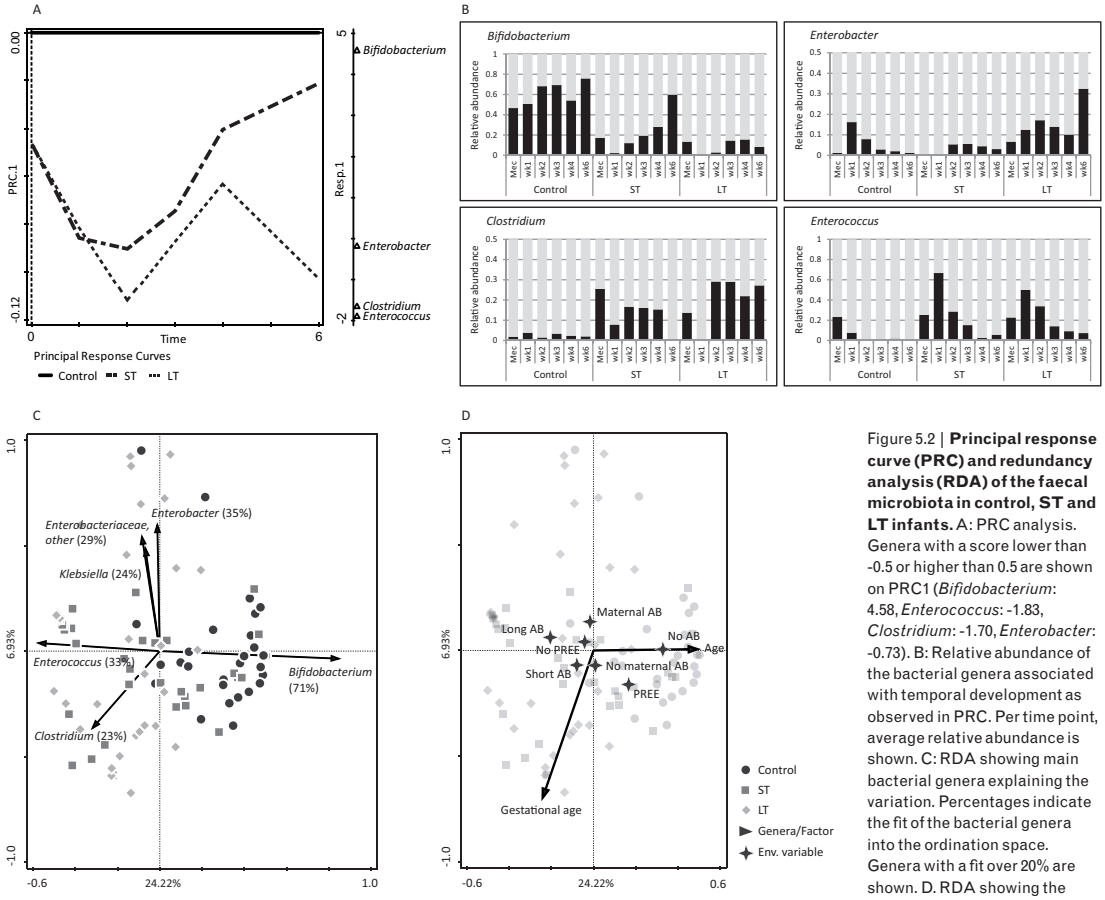


Figure 5.2 | **Principal response curve (PRC) and redundancy analysis (RDA) of the faecal microbiota in control, ST and LT infants.** A: PRC analysis. Genera with a score lower than -0.5 or higher than 0.5 are shown on PRC1 (*Bifidobacterium*: 4.58, *Enterococcus*: -1.83, *Clostridium*: -1.70, *Enterobacter*: -0.73). B: Relative abundance of the bacterial genera associated with temporal development as observed in PRC. Per time point, average relative abundance is shown. C: RDA showing main bacterial genera explaining the variation. Percentages indicate the fit of the bacterial genera into the ordination space. Genera with a fit over 20% are shown. D. RDA showing the clinical factors associated with microbiota composition. Clinical factors that significantly ($p<0.05$) explain the variation are shown. AB: Antibiotics, PREE: Preeclampsia.

Microiota community structure is associated with its dominating taxa

Short and long antibiotic treatment did not affect community richness and diversity (Fig 5.3a, S5.4). Instead, community richness and diversity were related to postnatal age, and depended on which taxa was dominant in the community (Fig 5.3b, 5.3c). It was observed that one of the major differences in microbiota composition between control, ST and LT infants was expressed by dominance of either *Bifidobacterium* or another genus like *Enterococcus*, *Enterobacter* or *Clostridium*. Dominance of *Bifidobacterium* in the bacterial community was related to significantly increased richness and diversity (Fig 5.3c). To increase the understanding of bacterial community structure dynamics in control, ST and LT infants, co-occurrence patterns based on Spearman correlation were visualised (Fig 5.4). In control infants, abundance of *Bifidobacterium* was negatively correlated to *Enterococcus*, *Veillonella*, *Clostridium*, *Escherichia-Shigella* and *Enterobacter*. In ST infants, *Enterococcus* was negatively correlated to *Bifidobacterium*, *Propionibacterium*, *Clostridium* and *Enterobacter*. In LT infants, *Enterococcus* was negatively correlated to *Clostridium*, *Serratia*, *Escherichia-Shigella*, *Enterobacter* and other *Enterobacteriaceae*.

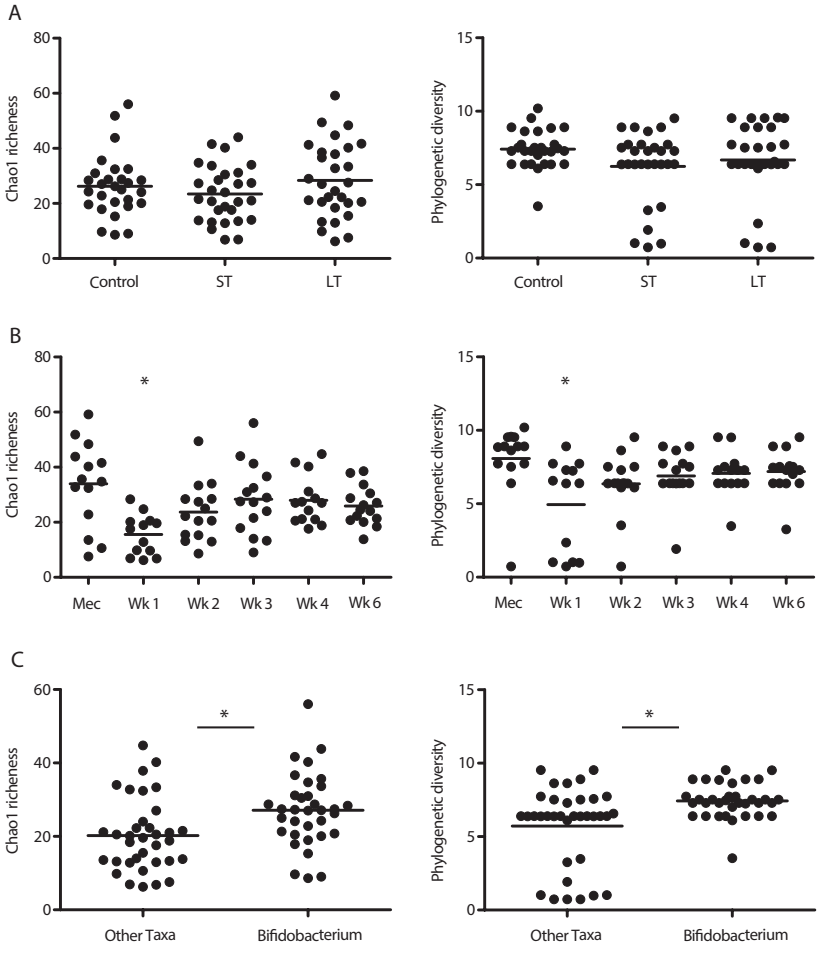


Figure 5.3 | **Bacterial community richness and diversity.** A: Samples stratified on antibiotic treatment duration. No significant difference observed. B: Samples stratified on sampling time point. Samples at postnatal week one were significantly lower compared to all other time points (* $p<0.05$; Mann-Whitney U test with Monte Carlo Permutation). C: Samples stratified on dominating taxa. * $p<0.01$ (Mann-Whitney U test with Monte Carlo Permutation)

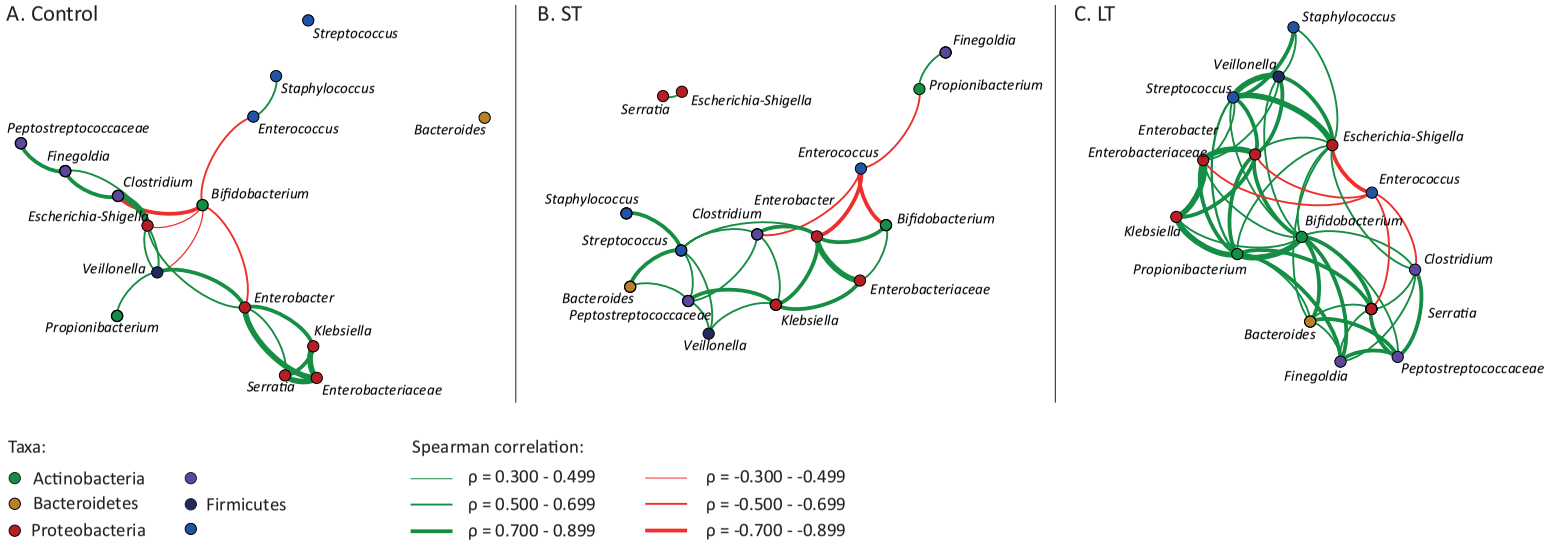


Figure 5.4 | **Co-occurrence patterns of the bacterial community in control, ST and LT infants.** Patterns are based on significant ($p<0.05$) Spearman correlations between genera.

DISCUSSION

Intravenous antibiotic administration for the prevention and treatment of infection and sepsis occurs frequently in preterm infants during the neonatal period. Therefore, studying the side effects of antibiotic treatment, including the effect on microbiota development, is of great relevance. The idea that short antibiotic use negatively affects clinical success and induces antibiotic resistance is gradually being replaced by the aim to avoid antibiotic overuse[174]. In this study, we focused on the effect of intravenous antibiotic treatment duration on intestinal microbiota development in preterm infants during the first six postnatal weeks. Our main findings are: 1) Both short and long treatment with amoxicillin/ceftazidime during the first postnatal week drastically disturbed normal colonisation pattern, 2) Short, but not long, antibiotic treatment allowed for recovery of *Bifidobacterium* levels within the first six postnatal weeks and 3) Community richness and diversity were not affected by antibiotic treatment, but were associated with postnatal age and with dominance of specific bacterial taxa leading to differences in microbial networks.

In the current study, 16S rRNA gene amplicon sequencing and qPCR analysis showed that infants faecal microbiota was dominated by *Bifidobacterium* throughout the first six postnatal weeks. *Bifidobacterium* species are considered beneficial early life colonisers, and are found in high abundance in term, vaginally delivered, breastfed infants[48]. Short and long treatment with a combination of amoxicillin and ceftazidime during the first postnatal week drastically disturbed normal colonisation pattern. Antibiotic treatment was effective against members of the *Enterobacteriaceae* family, but also negatively affected *Bifidobacterium* abundance and allowed *Enterococcus* to thrive. It must be noted that *Bifidobacterium* abundance was already lower in meconium samples of ST and LT infants compared to control infants, most likely a result of the relatively late (postnatal day 2-4) defecation of meconium samples by most preterm infants. *Enterococcus* remained dominant for up to two weeks after antibiotic treatment discontinuation. This might possess a health risk for the infants, as some *Enterococcus* species emerged from gut commensals to nosocomial pathogens via the acquisition of multi-drug resistance and other virulence determinants[175,176]. Short, but not long, antibiotic treatment allowed for recovery of *Bifidobacterium* levels within the first six postnatal weeks. Although the differences in average *Bifidobacterium* abundance between ST and LT infants was less apparent using qPCR instead of sequencing, it did show that *Bifidobacterium* levels recovered in 4/5 ST and only in 2/5 LT infants. In addition, both methods indicate that long antibiotic treatment results in increased abundance of members of the *Enterobacteriaceae* family at postnatal week six. Antibiotic treatment did not affect community richness and diversity. However, richness and diversity were affected by postnatal age and by dominance of specific bacterial taxa. Dominance of *Bifidobacterium* was negatively associated with abundance of other bacterial genera. It's dominance, however, allowed for higher community richness and diversity compared to dominance by other bacterial genera such as *Enterococcus*. We speculate that *Bifidobacterium* species control, but not outcompete, other bacterial species and that the microbial networks associated with *Bifidobacterium* species can

therefore play an important role in early life tolerance induction and immune system maturation. Microbiota profiles associated with antibiotic treatment could negatively influence immune system maturation via disturbance of the normal colonisation pattern. Indeed, previous studies showed that early life antibiotic exposure increased susceptibility to immune-related diseases such as asthma and allergy, and associated this with perturbations in microbial composition[129,177]. In addition to antibiotic treatment, our findings show that postnatal age, gestational age, preeclampsia and maternal antibiotics influenced microbiota composition. The latter two highlight the importance of maternal health status on infant microbiota development. Previous studies showed that microbes can be vertically transmitted, and that maternal health status, such as bodyweight and antibiotic use, affect infant microbiota development[178-180]. Maternal antibiotics could affect infant microbiota composition via prenatal exposure of the foetus to antibiotics, via alteration of the mothers microbiota and therefore the inoculum at birth, and via transfer of antibiotics through breastfeeding. In the study described herein, the use of perinatal antibiotics was unevenly distributed among the study groups. This, in addition to the relatively small sample size, hindered to unravel the true impact of maternal antibiotics on infant microbiota development. Preeclampsia is a condition characterised by high blood pressure and proteinuria, and is associated with maternal and neonatal morbidity and mortality, preterm birth and intrauterine growth restriction[181]. The aetiology of preeclampsia is unknown, but this disorder could be linked to genetic factors, obesity, abnormal formation of placental blood vessels and autoimmune disorders[181]. Our findings suggest that preeclampsia or its accompanying conditions are associated with infant microbiota composition. However, the relation between preeclampsia and infant microbiota development needs to be further elucidated as this study was not designed for studying this matter.

Overall, our findings show that intravenous antibiotic administration during the first postnatal week greatly affects infant gastrointestinal microbiota community structure. However, quick cessation of antibiotic treatment allows for recovery of the microbiota. Disturbances in microbiota development caused by short and more extensively by long antibiotic treatment, could affect healthy development of the infant via interference with maturation of the immune system and gastrointestinal tract. Clinicians should be aware of the disturbances that antibiotic treatment can cause and be strict in discontinuing antibiotic treatment as soon as possible to allow for a fast recovery of microbiota community structure.

Supplementary data

Figure S5.1 | **Microbiota composition profiles based on 16S rRNA gene sequencing in control (A), ST (B) and LT (C) infants.** Genera with a relative abundance of more than 5% are shown.

Figure S5.2 | **Microbiota composition profiles based on real-time qPCR data in control (A), ST (B) and LT (C) infants.**

Figure S5.3 | **Total bacterial count as determined by real-time qPCR.** No significant difference observed between gestational age groups at each time point as determined by the Kruskal-Wallis test with Monte Carlo Permutation.

Figure S5.4 | **Bacterial richness and diversity in control, ST and LT infants during the first six postnatal weeks.** No significant difference observed between gestational age groups at each time point as determined by the Kruskal-Wallis test with Monte Carlo Permutation.

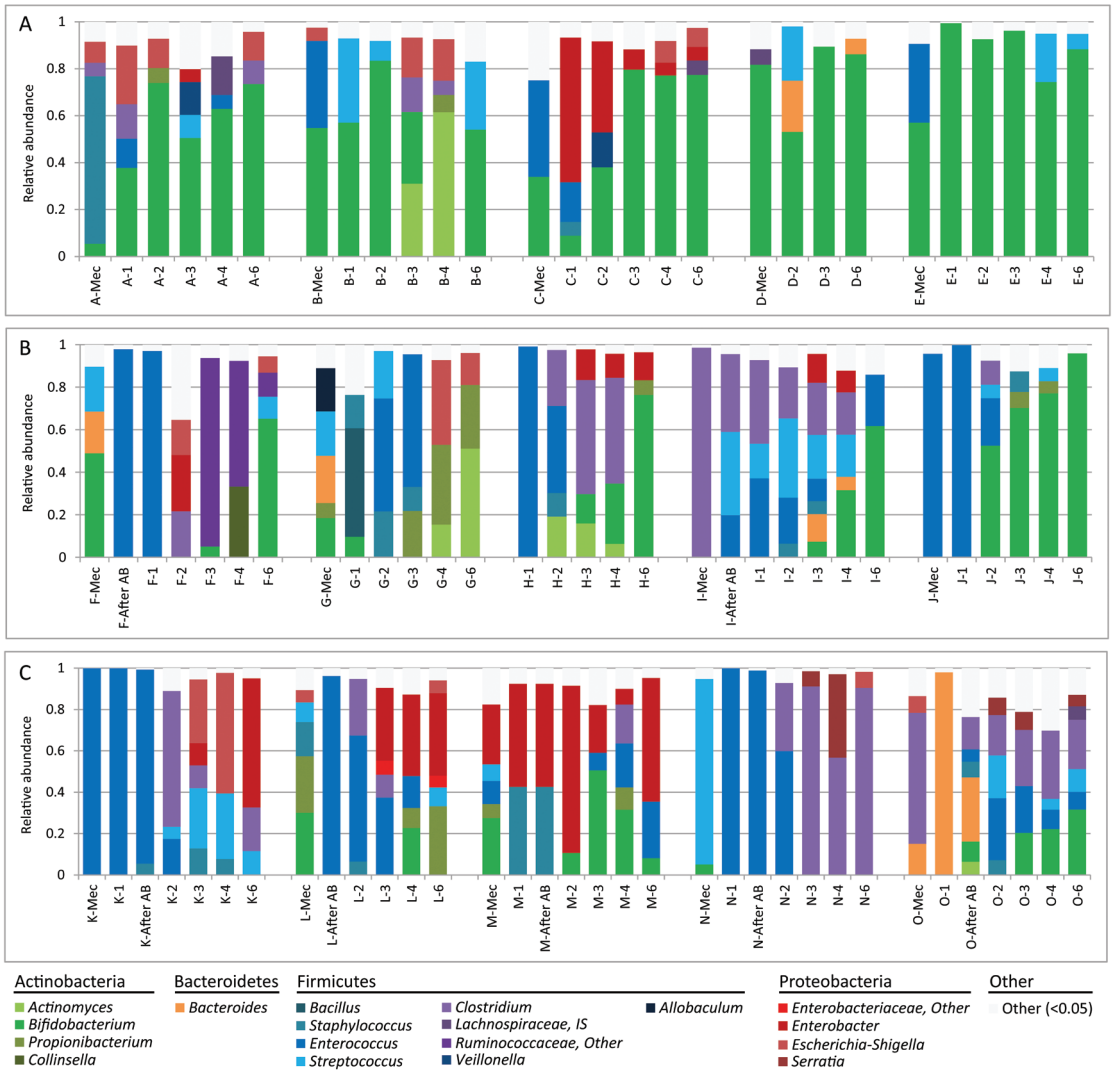


Figure S5.1 | **Microbiota composition profiles based on 16S rRNA gene sequencing in control (A), ST (B) and LT (C) infants.** Genera with a relative abundance of more than 5% are shown.

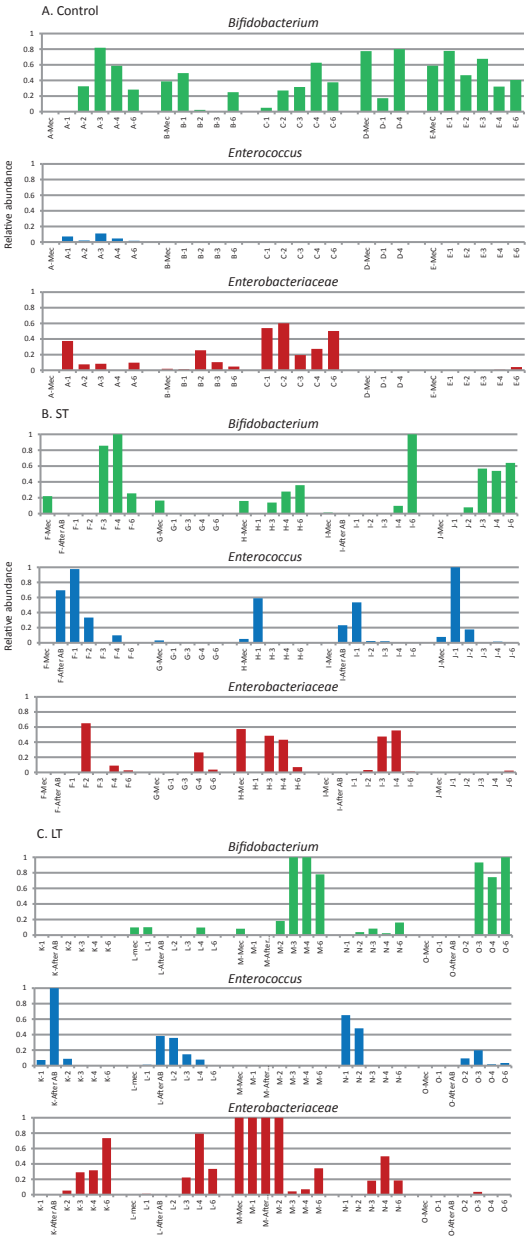


Figure S5.2 | **Microbiota composition profiles based on real-time qPCR data in control (A), ST (B) and LT (C) infants.**

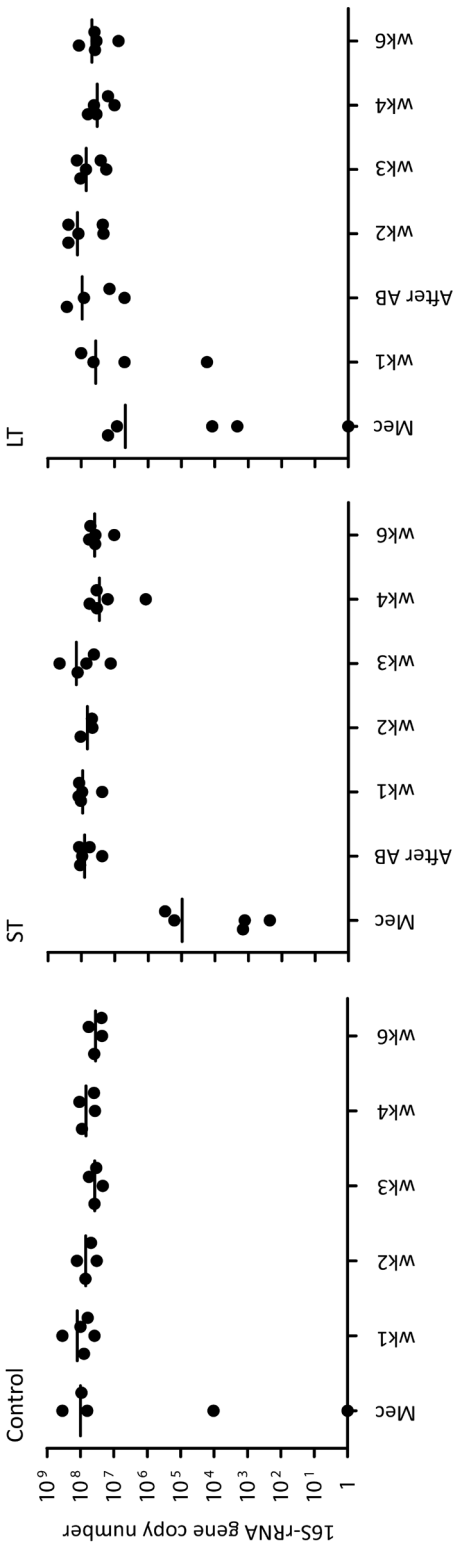


Figure S5.3 | **Total bacterial count as determined by real-time qPCR.** No significant difference observed between gestational age groups at each time point as determined by the Kruskal-Wallis test with Monte Carlo Permutation.

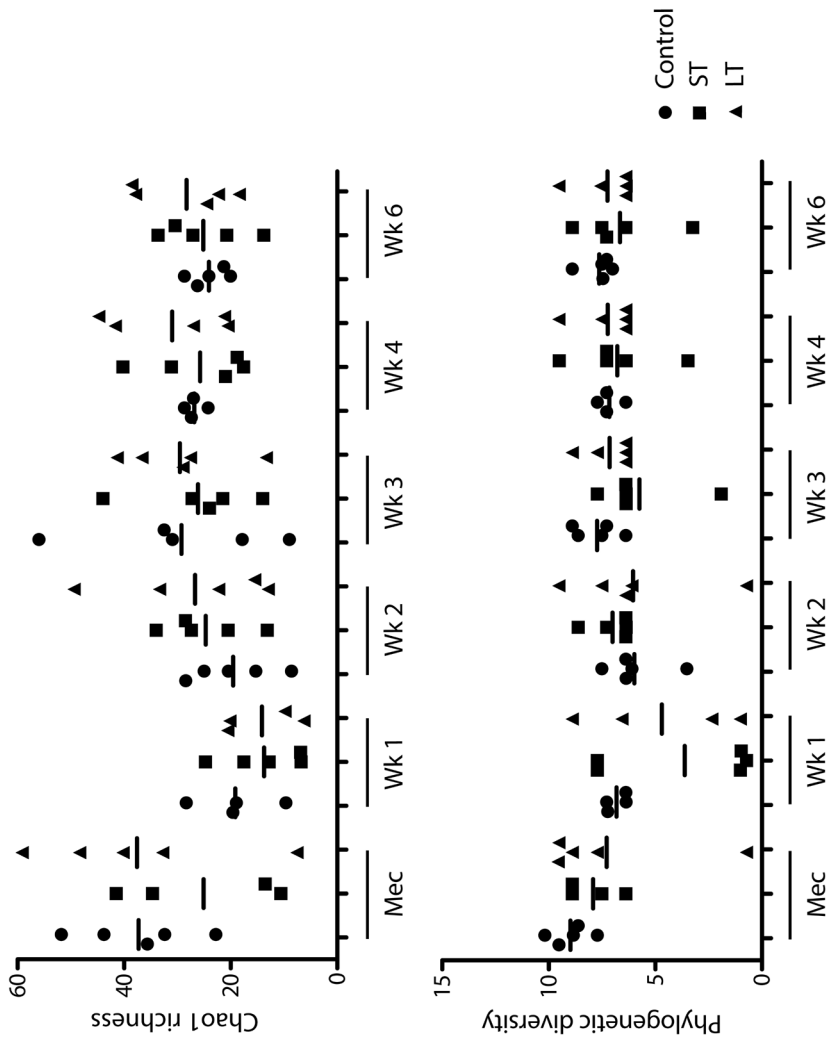
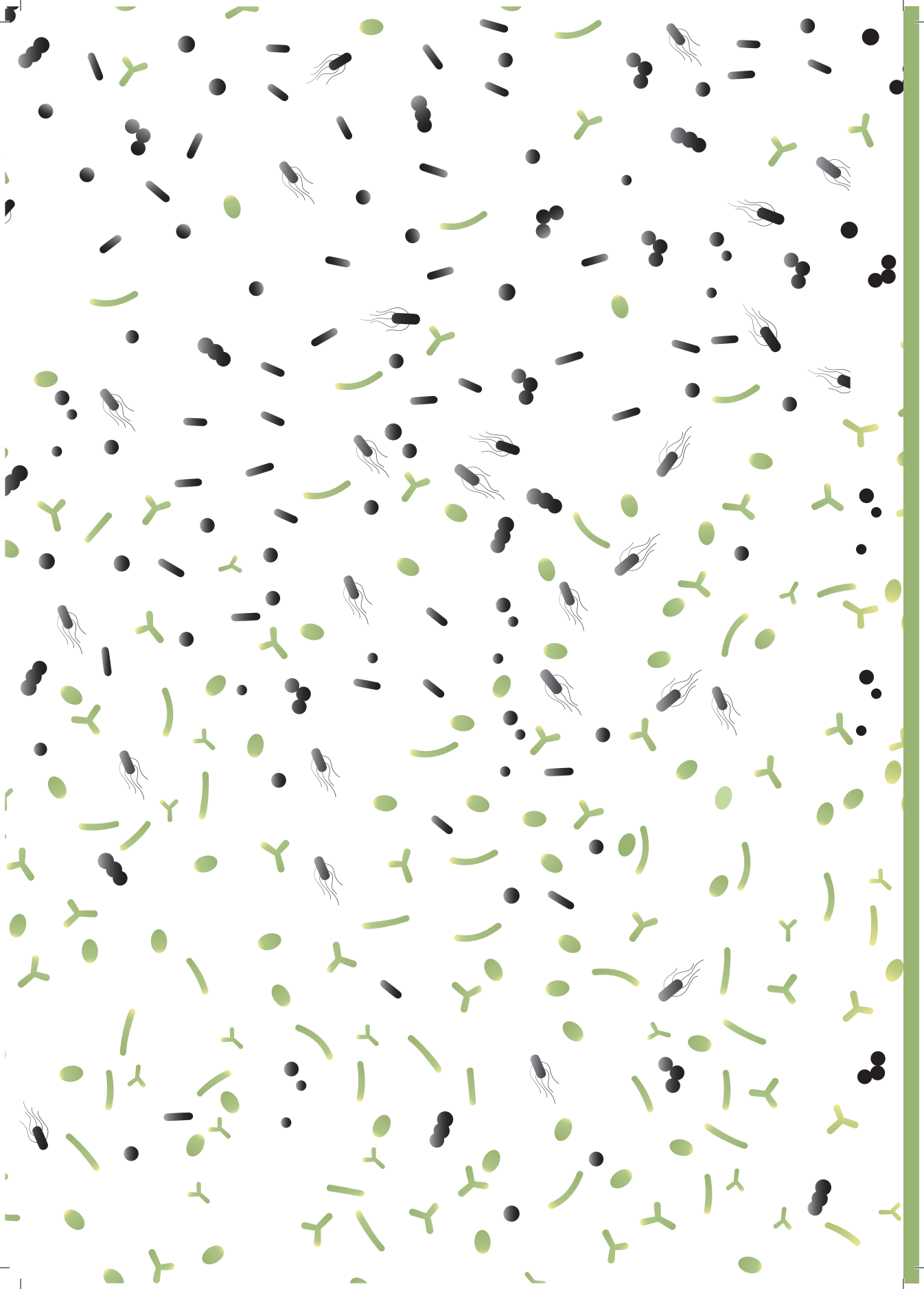


Figure S5.4 | **Bacterial richness and diversity in control, ST and LT infants during the first six postnatal weeks.** No significant difference observed between gestational age groups at each time point as determined by the Kruskal-Wallis test with Monte Carlo Permutation.



CHAPTER 6

INTRAVENOUS AMOXICILLIN/ CEFTAZIDIME TREATMENT EXERTS THRIVING OF *ENTEROCOCCUS* SPECIES IN PRETERM AND TERM INFANTS

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ABSTRACT

Most preterm infants receive broad spectrum antibiotics before infection is diagnosed. This strategy of prophylactic treatment has led to decreased mortality and morbidity among preterm neonates but may also lead to treatment of infants that not necessarily require antibiotics and increases the risk of antibiotic resistance and disturbed gut microbiota development. We studied the effect of intravenous amoxicillin/ceftazidime administration on intestinal microbiota development during the immediate postnatal life of preterm and term infants. Faecal samples from 63 infants were collected at postnatal weeks one, two, three, four and six (total of 263 samples). Infants received either no (control, n=28), short-term (<3 days, ST, n=22) or long-term (>5 days, LT, n=13) treatment with a combination of amoxicillin and ceftazidime during the first postnatal week. Microbiota composition was determined by amplicon sequencing of the 16S rRNA gene. Compared to control infants, ST and LT infants' microbiota contained a significantly higher abundance of *Enterococcus* during the first two postnatal weeks ($p<0.05$) at the expense of *Bifidobacterium* and *Streptococcus*. In addition, microbiota composition was less stable over time within ST and LT infants compared to control infants. Short and long antibiotic treatment both allowed for restoration of the intestinal microbiota within the first six postnatal weeks. However, *Enterococcus* and *Bifidobacterium* abundance were affected in fewer ST than LT infants. Overall, our findings show that intravenous administration of amoxicillin/ceftazidime affects intestinal microbiota composition, particularly by increasing the relative abundance of *Enterococcus* species during the first two postnatal weeks. Although being of short-term, the rise of antibiotic resistant enterococci at the expense of bifidobacteria and streptococci, including the potential effect of disturbed microbiota development on health outcomes in terms of metabolic and immune programming, should be considered as an aspect of the cost-benefit determination for antibiotic prescription.

INTRODUCTION

Neonatal infections are a major cause of mortality and morbidity, especially in preterm infants[182,183]. Since symptoms of infection are mostly nonspecific and infection can rapidly progress, most preterm infants are treated with broad-spectrum antibiotics before diagnosis. This, however, results in overtreatment and the risk of selection for resistant bacteria[184]. To reduce the use of antibiotics in a neonatal unit, it is recommended to evaluate the need for further antibiotic treatment after 36-48 hours and to stop antibiotics if infection is not proven. In addition to increasing the risk of antibiotic resistance, antibiotics might interfere with the development of the intestinal microbiota. During birth and thereafter, microbes rapidly colonise the human gastrointestinal (GI) tract, a process that is not yet completely understood as it is highly dynamic and influenced by multiple host and environmental factors[120]. An abnormal pattern of bacterial colonisation has been observed in preterm infants compared to term infants, which might be associated with greater exposure to factors like caesarean section, hospitalisation, formula feeding and antibiotic treatment[185,186]. Development of the gut microbiota coincides with, and influences, development

of the gastrointestinal tracts and immune system. Disturbances in early life microbiota development could therefore affect early and later life health outcomes[72,187]. Previous studies showed that the intestinal microbiota of preterm infants is affected by antibiotic treatment and characterised by high levels of facultative anaerobic bacteria and delayed colonisation with obligate anaerobes like *Bifidobacterium*[99,151,168]. Despite increasing knowledge about the effect of antibiotics on the intestinal microbiota, little research has focussed on treatment duration. In light of this, we previously performed a pilot study of 15 preterm infants exposed to no, short (<72 hours) or long (>5 days) antibiotic treatment during the first postnatal week[159]. We showed that short and long treatment affects the infant's gastrointestinal microbiota, however, short antibiotic treatment allows for its recovery within the first six postnatal weeks. In continuation of this pilot study, we aimed to verify these results in a bigger cohort. Therefore, we determined microbiota composition throughout the first six postnatal weeks in 63 infants in the present study. Herein, the primary outcome was defined as the effect of antibiotic treatment duration on microbiota composition. As secondary outcome, the effect of other parameters on microbiota composition were studied, including gestational age, delivery mode, maternal antibiotics, tolerance of enteral feeding, feeding type and respiratory support.

MATERIALS AND METHODS

Subjects and sample collection

This study was part of an observational, single centre, non-intervention study involving (pre)term infants admitted to the hospital level III NICU or the level II neonatal ward of Isala in Zwolle, The Netherlands. For the study described herein, all infants born between 32 and 42 weeks gestation, admitted to the level II neonatal ward and without major congenital malformation or malformations of the gastrointestinal tract, were eligible for inclusion. Informed consent was obtained from both parents of all individual participants included in the study. Antibiotic treatment was started on the suspicion of early-onset neonatal sepsis according to the hospital protocol and the judgement of the attending physician. After 48 hours, the need of antibiotic treatment was evaluated on the basis of clinical signs, blood culture and serial CRP. Faecal samples were collected from 63 infants at postnatal weeks one, two, three, four and six and stored at -20°C until transfer to -80°C. Infants received either no (control, n=28), short-term (<3 days, ST, n=22) or long-term (>5 days, LT, n=13) treatment with a combination of amoxicillin and ceftazidime during the first postnatal week. Their clinical characteristics can be found in table 6.1.

		Control	ST	LT
Infants	**n**	28	22	13
	Gestational age (weeks)	34.8 ± 1.4	34.2 ± 2.2	37.2 ± 3.1
	Birthweight (gram)	2309 ± 456	2406 ± 588	3111 ± 863
	Vaginal birth	13 (46.4%)	15 (68.2%)	6 (46.2%)
	Male	13 (46.4%)	13 (59.1%)	8 (61.5%)
	Preterm	24 (85.7%)	19 (86.4%)	7 (53.8%)
	Twin	11 (39.3%)	5 (22.7%)	2 (15.4%)
	AB treatment (days)	0	2.2 ± 0.5	7.5 ± 2.2
	CPAP	5 (17.9%)	10 (45.5%)	0 (0%)
	Food intolerant	0 (0%)	0 (0%)	1 (7.7%)
	TPN	2 (7.1%)	0 (0%)	1 (7.7%)
	Days until FEF	7.0 ± 1.0	7.2 ± 1.3	7.6 ± 0.9
	HM >50% throughout 6 PNW	19 (67.9%)	15 (68.2%)	7 (53.8%)
	% HM throughout 6 PNW	68 ± 27	71 ± 34	63 ± 32
	Cause of infection:			
	Proven sepsis			3 (23.1%)
	Clinical sepsis			6 (46.2%)
	Pneumonia			3 (23.1%)
	Meningitis			1 (7.7%)
	Causative pathogen:			
	Group B Streptococcus			4 (30.8%)
	Escherichia coli			1 (7.7%)
	Unknown			8 (61.5%)
Mothers	**Pre-eclampsia**	5 (17.9%)	3 (13.6%)	2 (15.4%)
	PROM	5 (17.9%)	5 (22.7%)	6 (46.2%)
	AB around birth	19 (67.9%)	15 (68.2%)	9 (69.2%)
	AB >48h after birth	2 (7.1%)	3 (13.6%)	4 (30.8%)

Table 6.1 | **Infant characteristics.** Abbreviations: AB: antibiotics, CPAP: continuous positive airway pressure, TPN: total parenteral nutrition, FEF: full enteral feeding, HM: human milk, PNW: postnatal week, PROM: prolonged rupture of membranes

16S rRNA gene amplicon sequencing

DNA extraction, library preparation and sequencing were performed by LifeSequencing S.L. (Valencia, Spain). DNA was extracted from 200 mg faeces using the QIAamp Fast DNA Stool Mini Kit (Qiagen), including enzymatic and chemical cell disruption by bead beating. DNA was purified and concentrated using the PowerMag DNA clean-up kit (MoBio) and 50 ng of DNA was amplified according to the Metagenomic Sequencing Library Illumina 15044223 B protocol (Illumina). For the first amplification step, primers were designed containing a universal linker sequence allowing amplicons for incorporation of indexes and sequencing primers by Nextera XT Index kit (Illumina) and 16S rRNA gene primers for region V3-V4[188]. Indexes were included during a second amplification step. Libraries were quantified using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermofisher) and pooled prior to sequencing on the MiSeq platform (Illumina, 300 bases paired-end). The size and quantity of the pool were respectively assessed on the Bioanalyzer 2100 (Agilent) and with the Library Quantification Kit for Illumina (Kapa Biosciences).

Data analysis

Read filtering, operational taxonomic unit (OTU)-picking and taxonomic assignment were performed using the NG-Tax pipeline with the following settings: read length of 70, ratio OTU abundance of 2, classify ratio of 0.8,

minimum percentage threshold of 0.5, identity level of 100%, error correction of 98.5, using the Silva_128_SSU Ref database[143,160]. For within infant (dependent) or between infants (independent) comparisons, the nonparametric Wilcoxon Signed Rank test and Kruskal-Wallis test, both with Monte Carlo permutation (10000x), were applied respectively. To correlate the abundance of taxa with each other, Spearman's rank correlation coefficient was determined. Before testing for differences in taxonomy, the OTU table was filtered for OTUs present in less than 25% of the samples. To relate microbiota composition to clinical data, redundancy analysis (RDA) was performed using Canoco multivariate statistics software v5. Clinical factors included in the analysis were gestational age, birthweight, delivery mode, singlet/twin/triplet, gender, postnatal age, maternal antibiotics around birth and at least 48 hours after birth, preeclampsia, prolonged rupture of membranes, ventilation, CPAP, days of total parenteral nutrition, days until full enteral feeding, food intolerance, proportion of parenteral/enteral nutrition per week, percentage human milk and formula feeding per week and average percentage of human milk feeding throughout the first six postnatal weeks and the duration of antibiotic treatment. Factors were considered to have a significant influence on microbiota composition when the false discovery rate corrected p-value was below 0.05.

RESULTS

Succession of the gut microbiota in infants receiving no, short or long antibiotic treatment

Faecal microbiota composition was determined during the first six postnatal weeks in moderate- to late preterm and term infants (32-42 weeks gestation) receiving either no (control), short-term (ST) or long-term (LT) antibiotic treatment during the first postnatal week. In control infants, the intestinal microbiota was characterised by high abundance of *Bifidobacterium*, *Streptococcus*, *Enterococcus*, *Staphylococcus*, *Escherichia-Shigella* and members of the *Enterobacteriaceae* family (Fig 6.1). During the first six postnatal weeks, average relative abundance of *Bifidobacterium* increased (18.9% to 41.1%; p=0.007), while *Staphylococcus* decreased (15.5% to 7.0%; p=0.0002). In addition, community richness and diversity gradually increased over time, being significantly higher at later time points compared to early time points (Fig 6.2a). Vaginal delivery and postnatal age explained most variation in microbiota composition between samples (7.7% and 5.5% respectively, Table 6.2). Delivery through primary and secondary caesarean section were associated with increased abundance of *Enterobacteriaceae* and *Enterococcus* respectively, while vaginal delivery was associated with increased *Bifidobacterium*, *Bacteroides* and *Escherichia-Shigella* (Fig 6.3a). In addition, days until full enteral feeding, amount of human milk feeding throughout the first six postnatal weeks, gender, continuous positive airway pressure, maternal antibiotics around birth and gestational age influenced microbiota composition (Table 6.2).

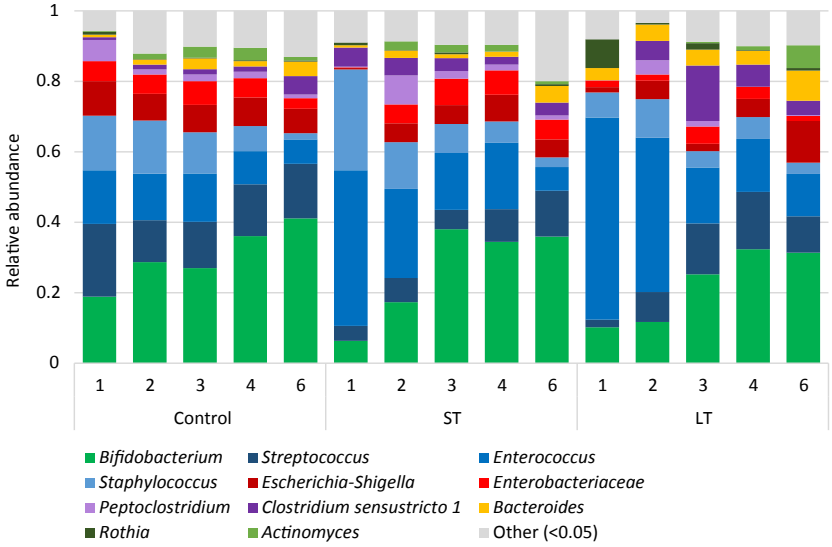
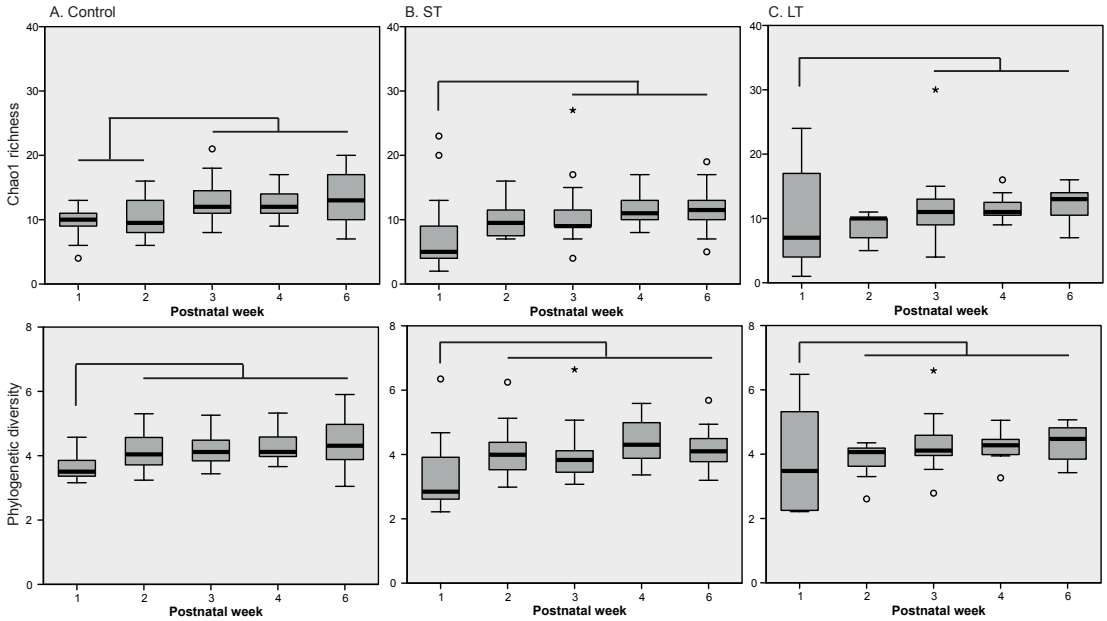


Figure 6.1 | **Microbiota composition profiles in control, ST and LT infants during the first six postnatal weeks.** Average relative abundances per time point are shown.

Figure 6.2 | **Community richness and diversity during the first six postnatal weeks in control (A), ST (B) and LT (C) infants.** Boxplots show the median, 25th and 75th percentiles, and minimal and maximal values with the exception of outliers (circles) and extremes (asterisks). Differences in richness and diversity between time points were determined using the Wilcoxon Signed Rank test with Monte Carlo permutation.



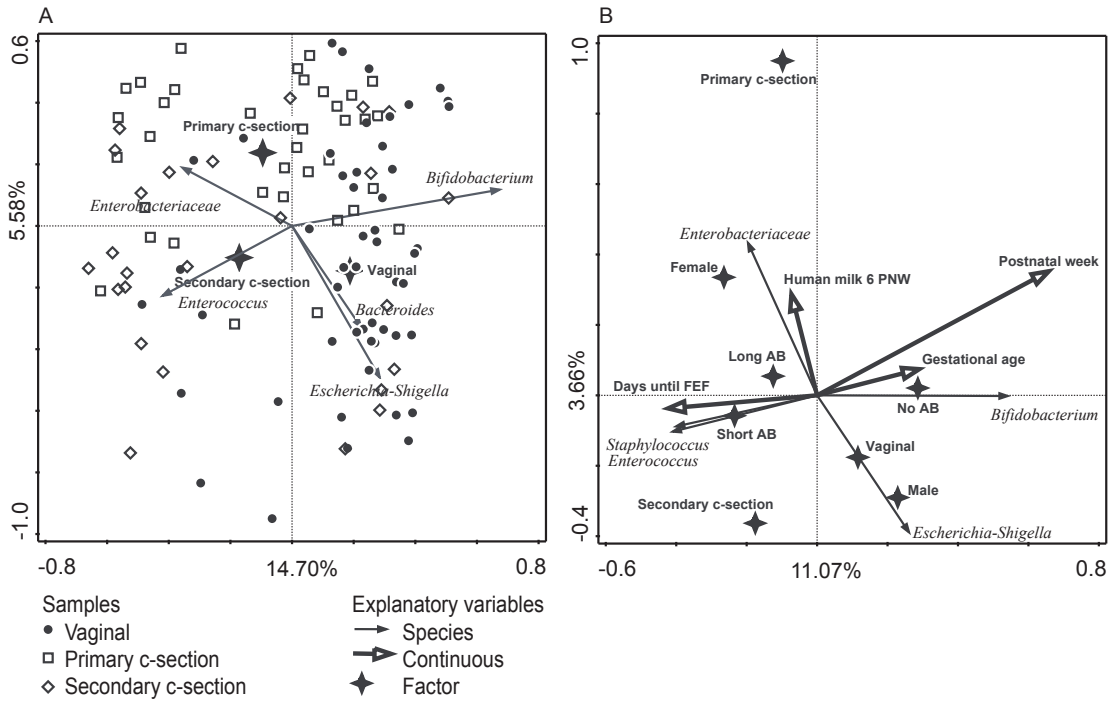


Figure 6.3 | **Redundancy analysis using microbiota composition profiles from (A) control infants and (B) all infants.** Species with a 15-100% fit into the ordination space and explanatory variables that significantly explain variation are shown. Abbreviations: C-section: caesarean section, AB: antibiotics, FEF: full enteral feeding, PNW: postnatal weeks.

Control			ST			LT			ALL		
Factor	Explains (%)	FDR p-value	Factor	Explains (%)	FDR p-value	Factor	Explains (%)	FDR p-value	Factor	Explains (%)	FDR p-value
Vaginal delivery	7.7	0.00433	Postnatal age	7.4	0.01533	Postnatal age	7.8	0.016	Postnatal age	5.5	0.006
Postnatal age	5.5	0.00433	PROM No	5.4	0.01533	Vaginal delivery	6.9	0.016	AB1 duration	2.5	0.006
Primary c-section	3.2	0.00433	PROM Yes	5.4	0.01533	TPN (days)	6.5	0.016	Female	2.9	0.006
Secundary c-section	3.2	0.00433	Male	4.9	0.01533				Male	2.9	0.006
Days until FEF	3.0	0.00433	Female	4.9	0.01533				Days until FEF	2.2	0.006
HM_6weeks (%)	2.9	0.00433							Primary c-section	2.0	0.006
Female	2.7	0.00433							GA (weeks)	1.5	0.006
Male	2.7	0.00433									
CPAP Yes	2.3	0.00743									
CPAP No	2.3	0.00743									
No Maternal AB	1.6	0.02022									
Maternal AB	1.6	0.02022									
GA (weeks)	1.6	0.02022									

Table 6.2 | **Factors explaining the variation in microbiota composition between samples as determined by redundancy analysis.** Factors significantly explaining the variation are shown.

Abbreviations: FDR: false discovery rate, C-section: caesarean section, FEF: full enteral feeding, HM: human milk, CPAP: continuous positive airway pressure, AB: antibiotics, GA: gestational age, PROM: prolonged rupture of membranes, TPN: total parenteral nutrition.

The intestinal microbiota of infants receiving short-term antibiotic treatment was characterised by a high abundance of *Bifidobacterium*, *Enterococcus*, *Staphylococcus*, *Streptococcus*, *Escherichia-Shigella*, *Clostridium* and members of the *Enterobacteriaceae* family (Fig 6.1). During the first six postnatal weeks, average relative abundance of *Bifidobacterium* (6.4% to 35.9%, p=0.001), *Streptococcus* (4.2% to 13.0%, p=0.001) and *Escherichia-Shigella* (0.4% to 5.1%, p=0.013) increased. In addition, there was a trend of decreasing *Enterococcus* (44.2% to 6.7%, p=0.077) and *Staphylococcus* (28.6% to 2.8%, p=0.053) throughout this time. Community richness and diversity showed a temporal increase, being significantly higher at later time points compared to early time points (Fig 6.2b). Postnatal age (7.4%), prolonged rupture of membranes (5.4%) and gender (4.9%) explained the variation in microbiota composition between samples (Table 6.2).

The intestinal microbiota of infants receiving long-term antibiotic treatment was characterised by a high abundance of *Bifidobacterium, Enterococcus, Clostridium, Staphylococcus, Escherichia-Shigella, Bacteroides* and members of the *Enterobacteriaceae* family (Fig 6.1). Over time, a trend of increasing *Bifidobacterium* (10.22% to 31.4%, p=0.077) and decreasing *Enterococcus* (57.3% to 12.0%, p=0.079) could be observed. Overall, community richness and diversity increased over time (Fig 6.2c). At the first postnatal week, however, richness and diversity varied greatly between infants. Postnatal age (7.8%), delivery mode (6.9%) and days of total parenteral nutrition (6.5%) explained the variation in microbiota composition between samples (Table 6.2).

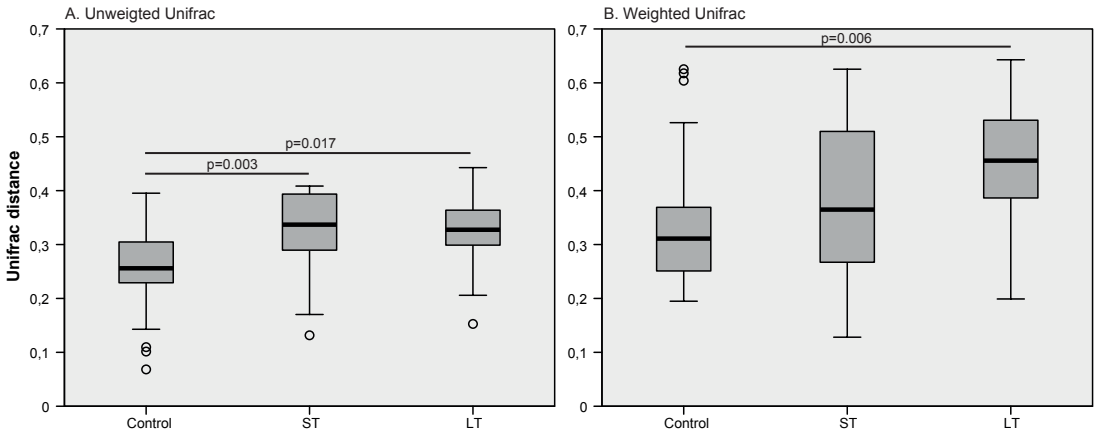
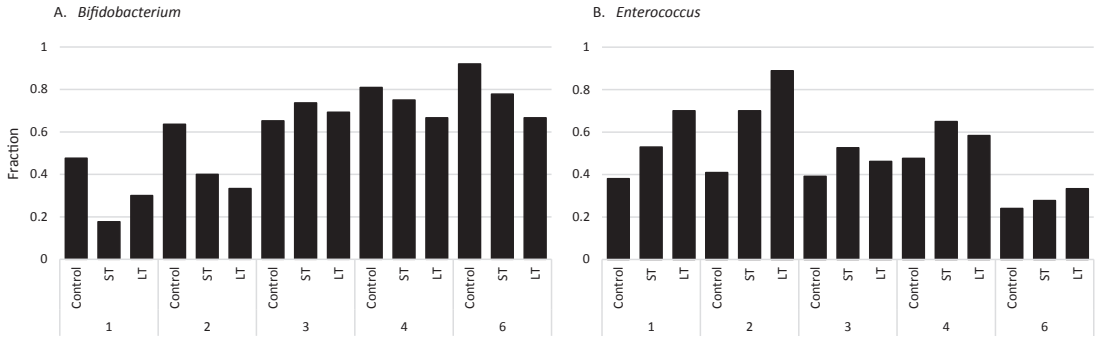


Figure 6.4 | **Unweighted (A) and weighted (B) unifrac distances within control, ST and LT infants.** Boxplots show the median, 25th and 75th percentiles, and minimal and maximal values with the exception of outliers (circles). Differences were determined using the Kruskal Wallis test with Monte Carlo permutation.



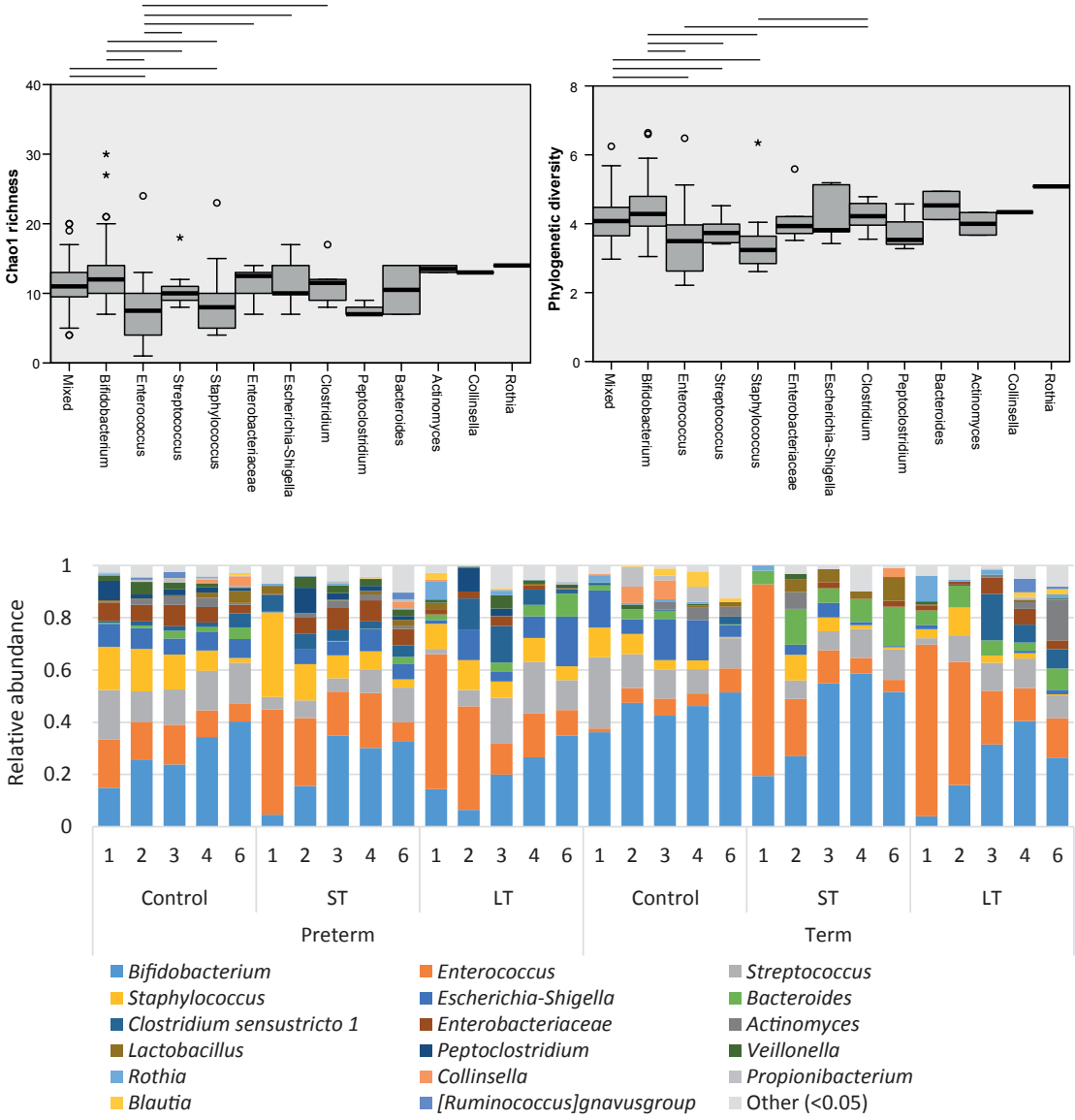
Antibiotic treatment exerts thriving of Enterococcus species

To understand the effect of antibiotic treatment duration on intestinal microbiota development, microbiota composition was compared between control, ST and LT infants. Compared to control infants, ST and LT infants' microbiota contained a significantly higher abundance of *Enterococcus* during the first two postnatal weeks ($p<0.05$). The abundance of *Enterococcus* negatively correlated with *Bifidobacterium* ($\rho=-0.260$, $p=1.3*10-5$) and *Streptococcus* ($\rho=-0.279$, $p=3*10-6$). In addition, microbiota composition was less stable over time within ST and LT infants compared to control infants as indicated by significantly higher unifrac distances (Fig 6.4). Microbiota composition did not significantly differ between ST and LT infants. However, *Enterococcus* became a dominant member of the community during the first two postnatal weeks in a higher percentage of LT than ST infants (Fig 6.5). In addition, *Bifidobacterium* was an abundant member of the community in a higher percentage of ST than LT infants at postnatal weeks four and six (Fig 6.5). Community richness and diversity were not consistently affected by antibiotic treatment. Instead, community richness and diversity related to which taxa dominated the community. In mixed communities, or communities dominated by *Bifidobacterium*, richness and diversity were higher than when the community was dominated by *Enterococcus*, *Streptococcus* or *Staphylococcus*, with the lowest richness and diversity being observed for *Enterococcus* (Fig 6.6).

Figure 6.5 | **Fraction of infants in which** Bifidobacterium **(A) or** Enterococcus **(B) was an abundant member of the bacterial community.** An abundant member was defined as representing an abundance of ≥10%.

Figure 6.6 | **Richness and diversity in samples with different dominating bacterial taxa.** Taxa were considered dominant in a sample when it was the most abundant taxon and at least 10% more abundant than the second most abundant taxon. When the difference between the two most abundant taxa was less than 10%, is was considered a mixed community. Boxplots show the median, 25th and 75th percentiles, and minimal and maximal values with the exception of outliers (circles) and extremes (asterisks).

Figure 6.7 | **Microbiota composition profiles during the first six postnatal weeks in preterm and term infants receiving no, short or long antibiotic treatment.** Average relative abundances per time point are shown.


Chao1 richness
Phylogenetic diversity
Mixed
Bifidobacterium
Enterococcus
Streptococcus
Staphylococcus
Enterobacteriaceae
Escherichia-Shigella
Clostridium
Peptoclostridium
Bacteroides
Actinomyces
Collinsella
Rothia
Relative abundance
Control
ST
LT
Preterm
Term
Bifidobacterium
Enterococcus
Streptococcus
Staphylococcus
Escherichia-Shigella
Bacteroides
Clostridium sensustricto 1
Enterobacteriaceae
Actinomyces
Lactobacillus
Peptoclostridium
Veillonella
Rothia
Collinsella
Propionibacterium
Blautia
[Ruminococcus]gnavusgroup
Other (<0.05)

Higher gestational and postnatal age, no antibiotic treatment and less days until full enteral feeding are associated with increased abundance of Bifidobacterium

The effect of clinical characteristics in association with microbiota composition were determined by redundancy analysis. Duration of antibiotic treatment explained 2.5% of the variation in microbiota composition between all samples (Table 6.2). In addition, postnatal age (5.5%), gender (2.9%), days until full enteral feeding (2.2%), delivery mode (2.0%) and gestational age (1.5%) were associated with differences in microbiota composition (Table 6.2). Boys were associated with increased abundance of *Escherichia-Shigella*, while girls were associated with increased abundance of other members of the *Enterobacteriaceae* family (Fig 6.3b). This difference was statistically significant at postnatal weeks two, three and four ($p<0.05$). Increased postnatal age, no antibiotic treatment, less days until full enteral feeding and higher gestational age were associated with increased abundance of *Bifidobacterium* (Fig 6.3b). Regarding gestational age, this study included infants born between 32-42 weeks gestation, thus comprised preterm and term infants. Comparing the intestinal microbiota between term and preterm infants showed that they respond similarly to antibiotic treatment (Fig 6.7). On average, abundance of *Bifidobacterium* was higher in term compared to preterm infants, however, this difference was not statistically significant.

DISCUSSION

Intravenous antibiotic administration for the prevention and treatment of infection and sepsis occurs frequently in the neonatal unit. Therefore, it is of great relevance to study the side effects of antibiotic treatment, including its effect on microbiota development. As a sequel to our pilot study[159], we studied the effect of intravenous antibiotic treatment duration on intestinal microbiota development in 63 (pre)term infants during the first six postnatal weeks.

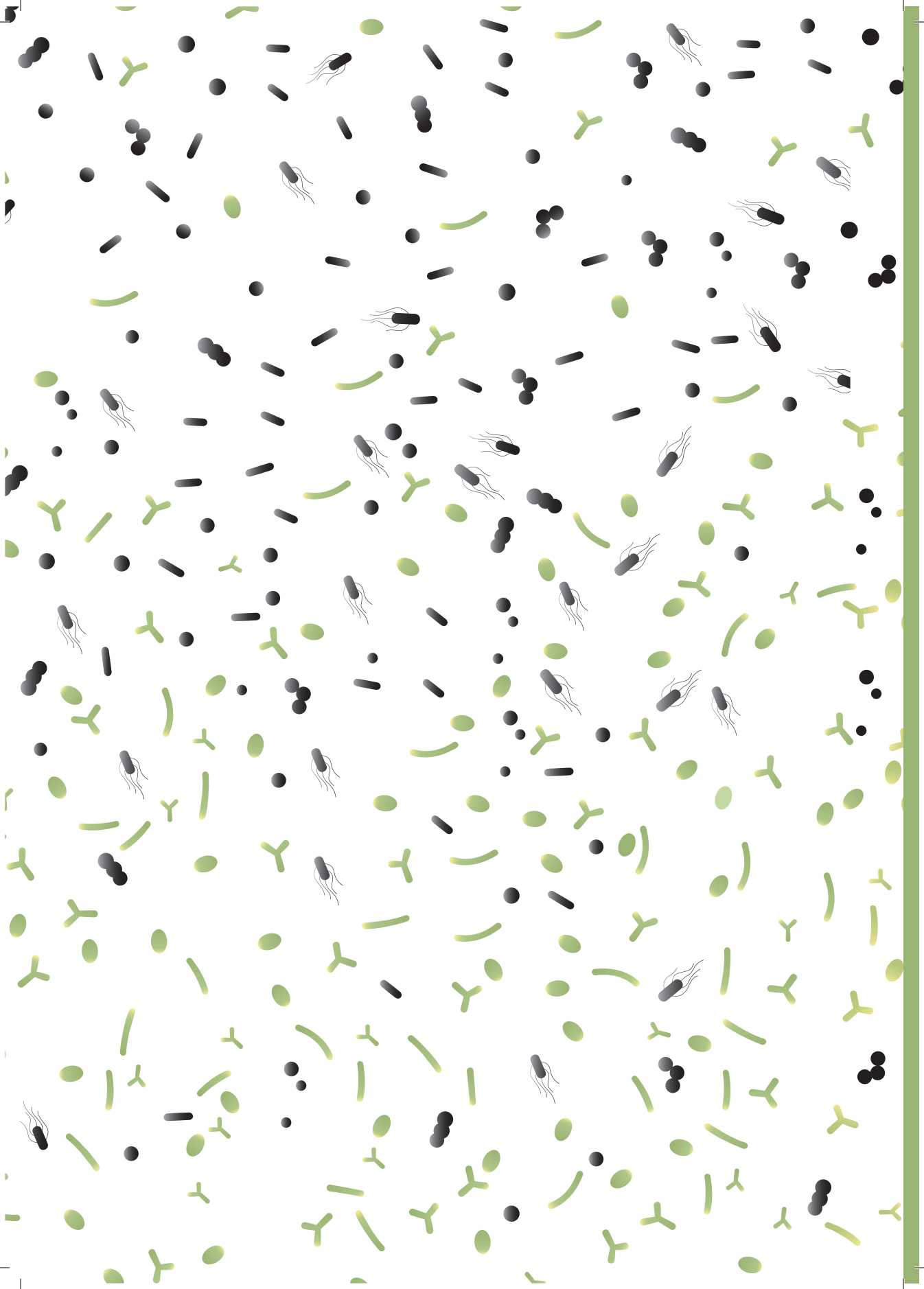
The genera *Bifidobacterium, Streptococcus, Enterococcus, Staphylococcus, Escherichia-Shigella* and members of the *Enterobacteriaceae* family made up the biggest proportion of the (pre)term infant faecal microbiota. Overall, relative abundances of *Enterococcus* and *Staphylococcus* were decreasing over the first six postnatal weeks, while abundances of *Streptococcus* and *Bifidobacterium* were increasing. Short and long antibiotic treatment with amoxicillin and ceftazidime affected microbiota composition by allowing *Enterococcus* to thrive during the first two postnatal weeks. *Enterococcus* thrived at the expense of *Bifidobacterium* and *Streptococcus*. These findings are in concordance with our previous study[159]. The increased abundance of *Enterococcus* at time of treatment could indicate antibiotic resistance as it is a target organism of amoxicillin. As some *Enterococcus* species emerged from gut commensals to nosocomial pathogens, this might possess a health risk for the infants[176]. *Enterococcus* species have been identified as causative organism in late-onset sepsis[189,190]. In contrast to the pilot study, no significant difference in microbiota composition could be observed between infants receiving short or long antibiotic treatment. However, it must be noted that *Enterococcus* became an abundant member of the community during the

first two postnatal weeks in a higher percentage of long than short treated infants. As well, *Bifidobacterium* did not become an abundant member of the community at postnatal weeks four and six in a higher percentage of long than short treated infants. This indicates that long antibiotic treatment has a more profound effect on microbiota development than short treatment, similar to what we have previously observed[159]. High inter-individual variation, in combination with the relatively small number of long treated infants, most certainly decreased statistical power. In addition, inclusion of all infants, instead of studying a carefully selected subset, did not allow to prevent possible bias by parameters like gestational age, delivery mode, gender, maternal antibiotics and feeding. For example, 46.2% of the long-treated infants were born at term, while only 13.6% of the short-treated infants were born at term.

Community richness and diversity were not consistently affected by antibiotic treatment. Instead, richness and diversity were increasing over time, and were related to which bacterial taxa was dominant. Richness and diversity were lower when the community was dominated by *Enterococcus*, *Streptococcus* or *Staphylococcus*, and higher when dominated by other taxa, including *Bifidobacterium*. As the abundance of *Bifidobacterium* was increasing over time, these two factors seem related and hinders to elucidate their sole effect on community richness and diversity.

In addition to antibiotic treatment duration, microbiota composition was associated with postnatal age, gender, days until full enteral feeding, delivery mode and gestational age. Increased gestational- and postnatal age and less days until full enteral feeding were associated with higher abundance of early life coloniser *Bifidobacterium*. A *Bifidobacterium*-dominated microbiota is more representative of microbiota development in term, vaginally born, breast-fed infants, which is considered most beneficial during early life development[161]. The beneficial effect of *Bifidobacterium* species is speculated to be due to its protection against pathogens and its immune modulating properties[32]. Since dominance by *Bifidobacterium*, compared to other bacterial taxa, allowed for higher community richness and diversity, we speculate that *Bifidobacterium* species control, but not outcompete, other bacterial species. Bifidobacteria could therefore play an important role in the development of a healthy and diverse ecosystem that promotes tolerance induction and immune system maturation. In addition, bifidobacteria are optimal milk degraders and are known for their role in the degradation of simple and complex sugars like human milk oligosaccharides[31]. Early differences in microbiota composition may affect the infants' food digestion capacity and subsequent energy harvest [21,79]. This is particularly relevant for infants born preterm with protein and energy deficits[98]. Regarding gender, boys' microbiota contained higher abundance of *Escherichia-Shigella,* while the microbiota of girls contained more members of the *Enterobacteriaceae* family that could not be classified to genus level. Several studies have shown that gut microbiota composition differs between adult males and females[191,192], but the gender-effect during early life is relatively unexplored[156].

Overall, our findings show that intravenous administration of amoxicillin and ceftazidime affects intestinal microbiota composition, particularly by increasing the relative abundance of *Enterococcus* species during the first two postnatal weeks. Short and long antibiotic treatment both allow for restoration of the intestinal microbiota within the first six postnatal weeks as characterised by increasing relative abundance of *Bifidobacterium* species. Long treatment, however, potentially has a more enduring effect on microbiota development than short treatment, but this needs to be further elucidated. Although being of short-term, the rise of antibiotic resistant enterococci at the expense of bifidobacteria and streptococci, including the potential effect of disturbed microbiota development on health outcomes, should be considered as an aspect of the cost-benefit determination for antibiotic prescription.



CHAPTER 7

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Preterm infants are prone to health complications during their early life including respiratory distress syndrome, jaundice, infection, sepsis and NEC. Health complications are mainly associated with organ immaturity and are therefore related to the infant's gestational age. Preterm infants are born with an immature gut and are therefore likely to experience feeding constraints, raising a major challenge in meeting their nutritional needs. In addition to its function in nutrient digestion and absorption, the gastrointestinal tract is in close interaction with the endocrine, neural and immune system. Gastrointestinal immaturity could therefore impact processes distant from the gut. The gastrointestinal tract is densely populated by microorganisms, the gut microbiota, playing a pivotal role in human physiology. Establishment of a healthy gut microbiota could benefit the preterm infant via its participation in metabolic processes, pathogen resistance and immune modulation. Development of the gut microbiota starts during birth and evolves further during life being affected by multiple host and environmental factors. Early life provides a window of opportunity since the gut microbiota is still developing and relatively responsive to interventions. Before the gut microbiota can be targeted as means of therapy in preterm infants, it is important to understand how their gastrointestinal tract is functioning, which microbes colonise, what the microbes are doing and how microbiota establishment is affected. The work in this thesis aimed to provide a better understanding of gastrointestinal function and microbiota development during the early life of preterm infants and to provide insight in which host and environmental factors play a key role in this development.

FUNCTIONING OF THE PRETERM GASTROINTESTINAL TRACT

When born preterm, the gastrointestinal tract may have immature motility functions and decreased digestive enzyme activity, with its degree of immaturity being associated with gestational age. Structural and functional maturation of the gastrointestinal tract are required for proper digestion and absorption of nutrients from milk feedings. Feeding constraints, reflected by abdominal distension, vomiting and gastric retention, are common, and preterm infants rarely meet *in utero* growth rates despite the existence of various nutrition support strategies[8,91]. Since sampling of various sites of the gastrointestinal tract is invasive and limited by ethical considerations, studying gastrointestinal development is challenging and often relies on animal models[193]. The application of metaproteomics analysis on faecal samples to study gut microbiota functioning in preterm infants (**chapter 3**) provided the opportunity to study host functioning since, in addition to bacterial proteins, many human- and bovine-derived proteins were identified. The inclusion of gastric aspirates from the same infants allowed to obtain insights in the gastric and faecal proteome as indication for gastrointestinal functioning and maturation, as described in **chapter 2**. The gastric proteome was mainly affected by percentage human milk feeding, while gestational age mainly affected the faecal proteome. The inability to functionally categorise a substantial proportion of identified proteins restricted comprehensive data interpretation. Nevertheless, our findings verified current knowledge considering the passage of proteins through the gastrointestinal tract and revealed gestational age-related developmental patterns for multiple

marker proteins. Although we were able to obtain first clues from the information contained in this complex dataset, we believe that more extensive analysis will provide further insights in gastrointestinal function and maturation in preterm infants. As Dallas *et al* stated, 'digestion is more complicated than the simple sum of the cleavage patterns of well-known proteases'[95]. Digestive capacity relies on the digestive environment (the combination of motility, secretory and regulatory functions), feeding strategy, and microbial colonisation. Bacteria residing the human gastrointestinal tract play an essential role in metabolism of dietary components, with their metabolic capacity being distinct, but complementary, to the activity of human enzymes[96]. In addition, variation in microbiota composition could differentially affect energy harvest and storage by the host[21,79]. Regarding preterm infants, who commonly experience protein deficits and growth retardation, further exploration of the gut microbiota's metabolic traits is particularly relevant[97,98]. Microbiota development coincides with gastrointestinal development, which was reflected by our observation that the quantity of gut maturation markers correlates with abundance of specific bacterial taxa (**chapter 2**). Overall, increased understanding of gastrointestinal function and maturation in relation to feeding regimen and microbiota development supports identification of diagnostic markers and aids improvement of nutritional and therapeutic strategies for preterm infants. In light of this, several nutritional strategies targeting the gut microbiota are being studied, of which human milk, probiotics and lactotransferrin seem most promising regarding infections and NEC[77,82]. Probiotics did so far not consistently improve growth outcomes in neonates[194,195], but they potentially reduce the number of days until full enteral feeding[196], which is of clinical relevance.

THE PRETERM INFANT GUT MICROBIOTA

The concept of a sterile *in utero* environment is being questioned since the observation of bacterial signatures in the placenta, amniotic fluid and meconium. Since meconium particularly consists of material ingested *in utero* it can serve as representative sample for studying the presence of an intra-uterine microbiota. Microbiota of meconium samples, as included in **chapter 3-5**, showed high compositional variability and a relatively high richness and diversity. It should be noted, however, that low biomass samples like meconium are prone to the introduction of PCR errors and could therefore result in overestimation of community richness and diversity, particularly when the number of PCR cycles is increased to obtain results[197]. Inclusion of proper negative controls during sampling and sample processing is therefore of foremost importance. In our attempt to study the meconium microbiota of preterm twins and triplets, around 85% of 59 samples seemed sterile as indicated by the inability to amplify the 16S rRNA gene by means of PCR and qPCR (data not shown). In 15% of the meconium samples, however, 104-106 copies of the 16S rRNA gene per 10ng DNA could be detected, and presence of bifidobacteria and members of the *Enterobacteriaceae* could be confirmed. Considering the analysis of meconium samples, is important to take the sampling day, high contamination risk, detection limit and bacterial viability into account. Based on these considerations, critical evaluation of current literature showed that

evidence for an *in utero* microbiome is weak[47]. Further studies are necessary to unambiguously confirm the existence of a viable intra-uterine microbiota and whether this affects postnatal development of the infant and its microbiota. Regardless of whether an infant is born sterile, majority of the microbiota residing the human body are acquired during and after birth. During the birthing process, the neonate is exposed to its mothers vaginal, faecal or skin microbiota, depending on delivery mode, thereby acquiring its first environmental-derived inoculum of microorganisms. After birth, the infant is exposed to a wide range of microorganisms that are residing in its direct environment, including parental skin and human milk feedings, which drives microbiota development further.

Bacterial load

In healthy neonates born at term, bacterial density reaches 1010 cells per gram faeces at the first postnatal week[156,198]. A study among very preterm infants showed that bacterial density reaches 10^9 cells per gram faeces at their tenth postnatal day[97]. In late preterm infants, quantification of total bacteria by qPCR showed the presence of 10^8 16S rRNA gene copies per 2ng faecal DNA from the first postnatal week onwards (**chapter 6**). Surprisingly, quantitative data of total bacteria in the gastrointestinal tract during early life is limited. Quantification has either focussed on specific bacterial taxa or total bacterial counts were solely used to transform absolute counts to relative abundances for specific taxa[62,137]. In addition, variation in methodology and in presentation of results hinders comparison between studies. The distribution of bacterial- and human-derived proteins, obtained by metaproteomics as described in **chapter 3**, indicates a low bacterial load in extremely and very preterm infants during the first two postnatal weeks, and a delayed colonisation up till the sixth postnatal week in extremely preterm infants. Equivalent results have previously been reported for one very preterm and four extremely preterm infants[86,87]. Low bacterial load could be an explanation for high variation in microbiota composition. It has recently been shown that bacterial load is a driver of microbiota composition[199]. In addition, it was stated that relative approaches, like sequencing of 16S rRNA gene amplicons, ignore the possibility that altered quantity of the microbiota by itself could be a marker of a disease-associated ecosystem and that relative profiling hampers linking microbiota composition to quantitative physiological parameters[199]. It must be noted, however, that quantitative and relative methods both have their limitations and that one should apply both complementary approaches to obtain full insight in microbiota composition and its temporal dynamics[200]. This understanding is currently resulting in the development of methodologies that integrate absolute and relative quantification[201-203].

Microbiota composition

Although the intestinal microbiota is dynamic during the first weeks of life, it is generally believed that the first colonisers are facultative anaerobic bacteria, particularly *Staphylococcus*, *Streptococcus*, *Enterococcus* and *Enterobacter*. These bacteria create an anaerobic environment to allow for colonisation by obligate anaerobes like *Bifidobacterium*, *Bacteroides* and *Clostridium* from the first postnatal week onwards. Previous studies have shown that preterm infants, compared to term infants, harbour a less diverse gut microbiota characterised

by high levels of facultative anaerobic bacteria and delayed colonisation with obligate anaerobic bacteria like *Bifidobacterium*[63,64,121]. The studies described in this thesis showed that *Staphylococcus*, *Enterococcus*, *Streptococcus*, *Enterobacter*, *Escherichia-Shigella*, *Enterobacteriaceae*, *Clostridium* and *Bifidobacterium* are abundant members of the bacterial community during the first six postnatal weeks in preterm infants (**chapters 3-6**). Community richness and diversity increased with increasing postnatal age in moderate to late preterm infants (**chapter 5 and 6**). In addition to postnatal age, richness and diversity were associated with the community's dominating taxa (**chapter 5 and 6**). When the community was dominated by one bacterial taxa, richness and diversity were generally lower, however, *Bifidobacterium* allowed for higher richness and diversity than *Enterococcus*, *Streptococcus* and *Staphylococcus*. Bifidobacteria may therefore play a significant role in the development of a healthy and diverse ecosystem. The correlation between postnatal age and abundance of these bacterial genera, however, hinders distinguishing their sole effect on community richness and diversity. Overall, abundance of facultative anaerobic bacteria and *Bifidobacterium* respectively decreases and increases with postnatal age, however, this process seems affected by gestational age and its associated factors. The observation that *Bifidobacterium* species colonise the preterm infant gut between 33-36 weeks postconceptional age, independent of postnatal age, indicates a substantial influence of gestational age on microbiota development[65,164]. When comparing microbiota establishment between extremely and very preterm infants, as described in **chapter 3**, we indeed observed that *Bifidobacterium* became an abundant member at the third postnatal week (33rd postconceptional week) in very preterm infants, while remaining low in extremely preterm infants up till the sixth postnatal week (31st-33rd postconceptional week). In another set of extremely and very preterm infants, as described in **chapter 4**, *Bifidobacterium* was an abundant member of the bacterial community at postnatal week six in 89% of the very preterm infants (35th-37th postconceptional week), while only in 50% of the extremely preterm infants (32nd-34th postconceptional week). Unfortunately, our dataset was limited to sampling up till the sixth postnatal week, disabling further elaboration about the existence of a postnatal/postconceptional age threshold for *Bifidobacterium* colonisation. In addition, colonisation of the gut by *Bifidobacterium* species does not solely depend on age. In moderate to late preterm infants, as described in **chapters 5 and 6**, *Bifidobacterium* was abundant at postnatal week six in 92-100%, 78-80% and 20-67% of infants receiving respectively no, short or long antibiotic treatment. The effect of various environmental factors on microbiota development is described later in this discussion. An overview of the relative abundance of *Bifidobacterium* at postnatal week six as observed throughout **chapters 3-6** is shown in figure 7.1. The incentive to explore the existence of an age threshold for colonisation by *Bifidobacterium* species, and to understand other factors impacting its colonisation derives from their beneficial effect on intestinal, metabolic and immune health. The gut microbiota of term, vaginally born, breastfed infants generally contains high abundance of *Bifidobacterium* species, in particular *B. breve* and *B. longum*. Elucidating *Bifidobacterium* colonisation patterns and a potential age threshold in preterm infants is important to advance therapeutic strategies targeting the microbiota.

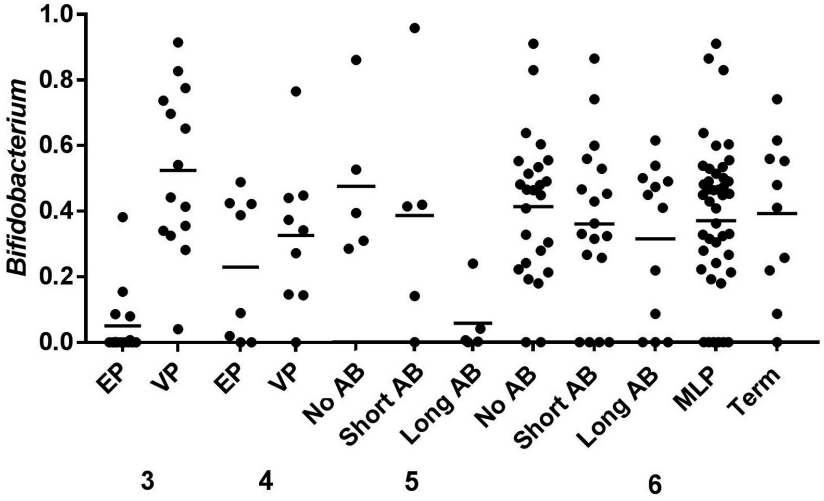


Figure 7.1 | **Relative abundance of *Bifidobacterium* at postnatal week six among all infants included in the studies described in chapters 3, 4, 5 and 6.** EP: extremely preterm, VP: very preterm, AB: antibiotics, MLP: moderate- to late preterm.

Microbiota activity

The number of studies aiming to unravel microbiota development in preterm infants is rising and, as such, knowledge about gut microbiota composition is increasing. However, despite the existence of various methodologies to study microbiota function based on DNA, RNA, proteins and metabolites, data regarding the functional signatures of the preterm infant intestinal microbiota remains limited. The application of metagenome sequencing allows for a functional prediction of the bacterial community based on gene content and has shown that the preterm infant gut microbiota is likely to become dominated by antibiotic resistant and pathogenic bacteria as pressured by broad-spectrum antibiotics[64,83-85]. Such findings are of clinical importance since antibiotic treatment is common in preterm infants due to their high susceptibility to infection and sepsis. In addition to antimicrobial resistance genes, stool metagenome data has been applied to determine host-microbe interactions as presented by metabolic- and immune-modulating capacities[204]. However, such functional traits of the preterm infant gut microbiota were not elaborated on in current literature. While the metagenome provides a static view of the community's functional capabilities, the metatranscriptome gives more dynamic insights by highlighting gene expression. However, metatranscriptomics has so far not been applied on preterm infant gastrointestinal samples. Some literature is available about the preterm infant faecal metaproteome, which may provide a better indication of the actual phenotype of the gut microbiota[86-88]. It must be noted, that these three studies were conducted within the same research group. Temporal development of the preterm infant gut microbiota composition was associated with increasing activity towards the degradation of complex carbohydrates[87]. However, this study only included one preterm infant who was longitudinally sampled up till the third postnatal week. The temporal development towards increasing carbohydrate degradation could be individual-specific as was shown in a study in which four preterm infants were included[86]. Here, the exploitation of carbohydrates, amino acids and lipids by the

gut microbiota showed large inter- and intra-individual variation, but could be related to the presence of specific bacterial taxa. Metaproteomics data of ten longitudinally sampled preterm infants, as described in **chapter 3**, revealed that a *Bifidobacterium*-dominated community is associated with increased proteins involved in carbohydrate and energy metabolism, including those involved in the degradation of complex carbohydrates like human milk oligosaccharides. Proteins involved in membrane transport and translation were abundant in communities dominated by *Enterococcus* or *Enterobacter*, confirming taxa-specific functional contributions to the bacterial community. Genomes of bifidobacteria contain gene cassettes devoted to the utilisation of various carbohydrates[205] and our metaproteomics data indicates that bifidobacteria are well capable to establish and to be metabolically active in the gut of very preterm infants. Early establishment and metabolic activity of bifidobacteria, and of the whole bacterial community, may be relevant considering the digestion capacity and potential energy harvest from milk feedings by preterm infants as mentioned earlier. It is worthy to further elucidate the metabolic capacities of the microbiota, and of its specific members, in relation to digestive functioning and growth outcomes in preterm infants[97,98]. Metabolomics studies showed that bifidobacteria are particularly abundant in healthy preterm infants, and are positively associated with raffinose, sucrose and acetic acid[206]. Specific metabolites involved in the 'C21-steroid hormone biosynthesis', 'linoleate metabolism' and 'leukotriene metabolism and prostaglandin formation from arachidonate' pathways are increased prior NEC diagnosis[130]. Like with metaproteomics, a metabolomics approach identified taxa-specific functional contributions. High abundance of *Enterococcus*, *Pseudomonas* and *Escherichia-Shigella*, as observed in antibiotic treated preterm infants, were associated with increased L-tyrosine and citric acid[207]. Complexity of the preterm infant faecal metabolome generally increases with age and can be associated with weaning[89]. Functional profiles are stable at 1-3 years of age and, at that time, cannot be associated with early life events including delivery mode, gestational age, antibiotic use and NEC/LOS[89]. It must be noted that all, except one, of these metabolome studies were conducted within the same research group, indicating that metaproteomics and metabolomics approaches are not yet widely applied to study gut microbiota functioning in preterm infants. To obtain more detailed insights in host-microbe interactions, functional analysis of the microbiota needs further implementation in current microbiota research.

FACTORS MAKING A DIFFERENCE

The findings presented in this thesis show that the faecal proteome and microbiota are associated with gestational age. The duration and extend of care for the preterm infant are strongly related to gestational age. Therefore, one should be aware that observed differences in the faecal proteome and microbiota between infants of varying gestational age might actually be attributable to gestational age-associated environmental factors rather than to gestational age itself. Early life microbiota development is influenced by factors like delivery mode, antibiotics and feeding type[120]. In preterm infants, various aspects of the NICU environment influence microbiota composition[208]. Multiple

variables were taken along during data analysis for the studies described in this thesis, among others gestational age, birthweight, gender, delivery mode, perinatal and postnatal antibiotics, feeding regimen, postnatal age and days until discharge. The gestational age-dependent microbiota signature in extremely and very preterm infants, as described in **chapter 3**, was associated with variation in exposure to antibiotics and with duration of respiratory support. Extremely preterm infants are to a greater extend exposed to antibiotics and respiratory support as compared to very preterm infants. The association between these factors, however, hinders to understand their sole effect on microbiota development. It is well recognised that antibiotics impact early life microbiota development[209]. In contrast, documentation regarding the effect of respiratory support on gut microbiota development is currently limited and deserves more attention[137,147]. While the EIBER study was not designed to further elucidate the effect of respiratory support strategies on microbiota development, it did aim to study the effect of antibiotics.

Postnatal antibiotics

Treatment with amoxicillin/ceftazidime during the first postnatal week altered the microbiota of late preterm infants to become dominated by enterococci at the expense of bifidobacteria (**chapter 5 and 6**). Despite inconsistencies, more than five days of treatment seems to have had a longer lasting effect on microbiota composition than less than three days of treatment. Elongation of antibiotic treatment with a few days potentially delays (re)colonisation by *Bifidobacterium* species with weeks. Two dosages of vancomycin during removal of a central venous catheter did not significantly alter microbiota composition and diversity (**chapter 4**). However, great inter- and intra-individual variation in microbiota composition in very preterm infants during the first postnatal weeks, and exposure to other antibiotics during the first postnatal week, could have prohibited the detection of consistent alterations in microbiota composition as a result of two vancomycin dosages. Considering the effect of antibiotics on intestinal microbiota development, antibiotic type, dose and duration should be taken into account. The by antibiotics affected microbiota, generally characterised by low abundance of *Bifidobacterium* and high abundance of facultative anaerobes including *Enterococcus* and *Enterobacter*, has previously been associated with onset of NEC and late-onset sepsis[72,153]. In addition, the early use of antibiotics and its associated disturbances of the gut microbiota have been associated to negative health outcomes in later life[73,74]. It must be noted, however, that applied antibiotic strategies decreased neonatal mortality and morbidity rates and that antibiotics are indispensable in preterm infant care. It is therefore of importance to further elucidate the cost-benefit balance of empiric antibiotic use in neonatology. As long as the use of broad-spectrum antibiotics is a necessity in caring for the preterm infant, development of their intestinal microbiota will most certainly remain impeded.

Perinatal antibiotics

In addition to postnatal antibiotic treatment, maternal antibiotics during the perinatal period were associated with microbiota composition of preterm infants (**chapter 3, 5 and 6**). Perinatal antibiotics are generally provided in

case of prolonged rupture of membranes, group B *Streptococcus* colonisation, caesarean section or chorioamnionitis[210]. Maternal antibiotics may affect infants' microbiota composition via prenatal exposure of the foetus to antibiotics, via alteration of the mothers' microbiota and therefore the inoculum at birth, and via transfer of antibiotics through breastfeeding. Previous studies showed that maternal antibiotic use during the perinatal period altered gut microbiota establishment of the preterm infant and potentially affected the functional capabilities of the developing bacterial community[67,211]. Another study, however, showed no profound difference in bacterial colonisation pattern of three days old infants born to mothers with or without intrapartum amoxicillin treatment, except for colonisation by *Clostridium*[212]. It is noteworthy, however, that *Bifidobacterium* species colonised 48% of infants born from non-exposed mothers, while this was observed in only 24% of infants born from antibiotic-exposed mothers[212]. This is in concordance with findings regarding postnatal antibiotic exposure, which generally delays colonisation by anaerobic bacteria. Like postnatal antibiotics, the use of perinatal antibiotics has been related to negative health outcomes of the infant in later life, but these antibiotics are also essential for preventing and curing maternal- and neonatal infections[213-216].

Feeding

Human milk is considered the best possible nutrition for all infants and the World Health Organisation therefore recommends exclusive breastfeeding during the first six postnatal months for the infant to benefit from the nutritive, immune modulating and antimicrobial components contained in human milk[52,217]. Human milk feeding is desirable regarding infant growth and for development of the digestive, immune and cognitive system. The ratio of human milk to infant formula in enteral feeding was the main driver of the composition of the gastric proteome, and also affected composition of the faecal proteome (**chapter 2**). More human milk feeding resulted in higher levels of digestive- and immune-related proteins that might be beneficial to preterm infants who are affected by immature gastrointestinal and immune functioning. Human milk and cow milk vary in composition, and their protein counterparts might possess different bioactivity. Human milk-derived, but not cow milk-derived, lactotransferrin as well as IgA, were abundant in preterm infant faeces. In addition to its influence on gastrointestinal and immune system maturation, human milk feeding is considered beneficial for development of the gut microbiota[120]. Human milk might in itself be a source of bacteria, but also contains complex oligosaccharides selectively stimulating bacteria that are specialised in the degradation of these compounds, like bifidobacteria. The percentage of human milk feeding, as well as other feeding parameters like food intolerance, ratio of enteral:parenteral feeding, days of total parenteral feeding and days until full enteral feeding were not identified as significant contributors to microbiota development in preterm infants (**chapter 3-5**). The effect of feeding regimen on microbiota development may have been eliminated or overshadowed by the detrimental effects caused by antibiotic administration in these infants. In moderate to late preterm infants who did not receive antibiotic treatment, days until full enteral feeding and percentage human milk feeding throughout the first six postnatal weeks were among the factors that significantly affected

microbiota composition (**chapter 6**). Delayed enteral feeding and less human milk feeding most certainly impedes colonisation by *Bifidobacterium* species. Delayed enteral feeding is generally not favourable and may delay maturation of the gastrointestinal tract and has been associated with increased permeability and bacterial translocation[8]. Furthermore, the combination of delayed enteral feeding and an immature immune system in preterm infants has been associated with negative health outcomes like NEC and sepsis. In addition to the effect of feeding regimen on microbiota development, one should consider the role of the gut microbiota in nutrient digestion and energy harvest[21,79]. Preterm infants require more energy and proteins for optimal development than term infants, and preterm infants rarely meet *in utero* growth rates despite application of various nutrition support strategies[91]. The gut microbiota should be considered as therapeutic target for optimising nutrient digestion and uptake and for improvement of growth outcomes in preterm infants[98].

Delivery mode

The birthing process provides the first major bacterial inoculum for an infant that, depending on delivery mode, contains bacteria representing the vaginal, faecal or skin microbiota. This initial inoculum is the initiator to a succession of events leading to the development of the infant's own microbiota. Delivery through caesarean section has been associated with the epidemic of autoimmune and allergic diseases, in which a disturbed gut microbiota may have been attributable[218]. In moderate to late preterm infants without antibiotic treatment, delivery mode was the main driver of microbiota composition during the first six postnatal weeks (**chapter 6**). The gut microbiota of vaginally delivered infants was compositionally distinct from infants delivered through primary or secondary caesarean section. Vaginal delivery was associated with increased abundance of *Bacteroides*, *Bifidobacterium* and *Escherichia-Shigella*, but microbiota composition still varied greatly between infants. In preterm infants receiving antibiotics, an effect of delivery mode was generally not observed (**chapter 3, 5 and 6**). Antibiotics greatly affect microbiota composition and possible influences of delivery mode may therefore not be detectable. The realisation that caesarean section negatively affects early life microbiota development and consequently may affect early and later life health outcomes, led to studying the application of vaginal microbiota transfer to caesarean section delivered neonates[219]. Vaginal microbiota transfer has shown effective and safe, its long-term effects, however, need to be elucidated. Nevertheless, vaginal microbiota transfer seems successful and its application should also be considered for preterm infants, particularly in those who do not receive antibiotics.

Other factors

In addition to antibiotics, feeding regimen and delivery mode, various other factors were considered for their potential effect on gastrointestinal function and microbiota development in preterm infants. Postnatal age influenced composition of the gastric and faecal proteome (**chapter 2**) and was consistently identified as factor influencing gut microbiota composition (**chapter 3-6**). With age, the gastrointestinal tract and immune system are maturing, which most

certainly affects bacterial community structure in the gut. In addition, an older infant has simply been exposed to the environment and various influencing factors longer and offered the microbiota more time to 'settle down'. Gender was identified as minor, but significant, contributor to microbiota composition (**chapter 3 and 6**). Currently, data considering a gender-effect during early life microbiota development is limited and our findings regarding its contribution were inconsistent and biased by other variables. A previous study among 108 infants showed that, during the first three postnatal months, the gut microbiota of girls contains more lactobacilli than the microbiota of boys[156]. Differences in microbiota composition related to gender have been identified in adults and is suggested to be driven by gender-specific functioning of the immune system[220]. In this context, it is relevant to note that preterm born girls generally 'perform better' relative to preterm born boys regarding health complications and mortality[221,222]. A better general health status most likely affects microbiota development. However, a gender-effect during early life gut microbiota development needs to be further elucidated. In **chapter 5**, preeclampsia was identified as factor contributing to microbiota composition, but our study design did not allow to further elucidate their relation. Preeclampsia is a maternal health complication characterised by high blood pressure, and is associated with preterm delivery[181]. One study describes a relation between preeclampsia and the gut microbiota, however, I consider their conclusions questionable[223].The aetiology of preeclampsia is unknown, but this disorder is linked to inflammation, which might have an infectious origin. Periodontitis is strongly associated with preeclampsia, and in addition to increased inflammatory status, bacterial translocation from the oral cavity to placental tissue has been suggested as underlying mechanism[224-226]. However, attempts to relate the presence of bacteria in the placental tissue of preeclamptic women have so far failed to identify consistent differences in their situation compared to woman without preeclampsia[227-229]. As for gender, the effect of preeclampsia on infant microbiota development needs to be further elucidated.

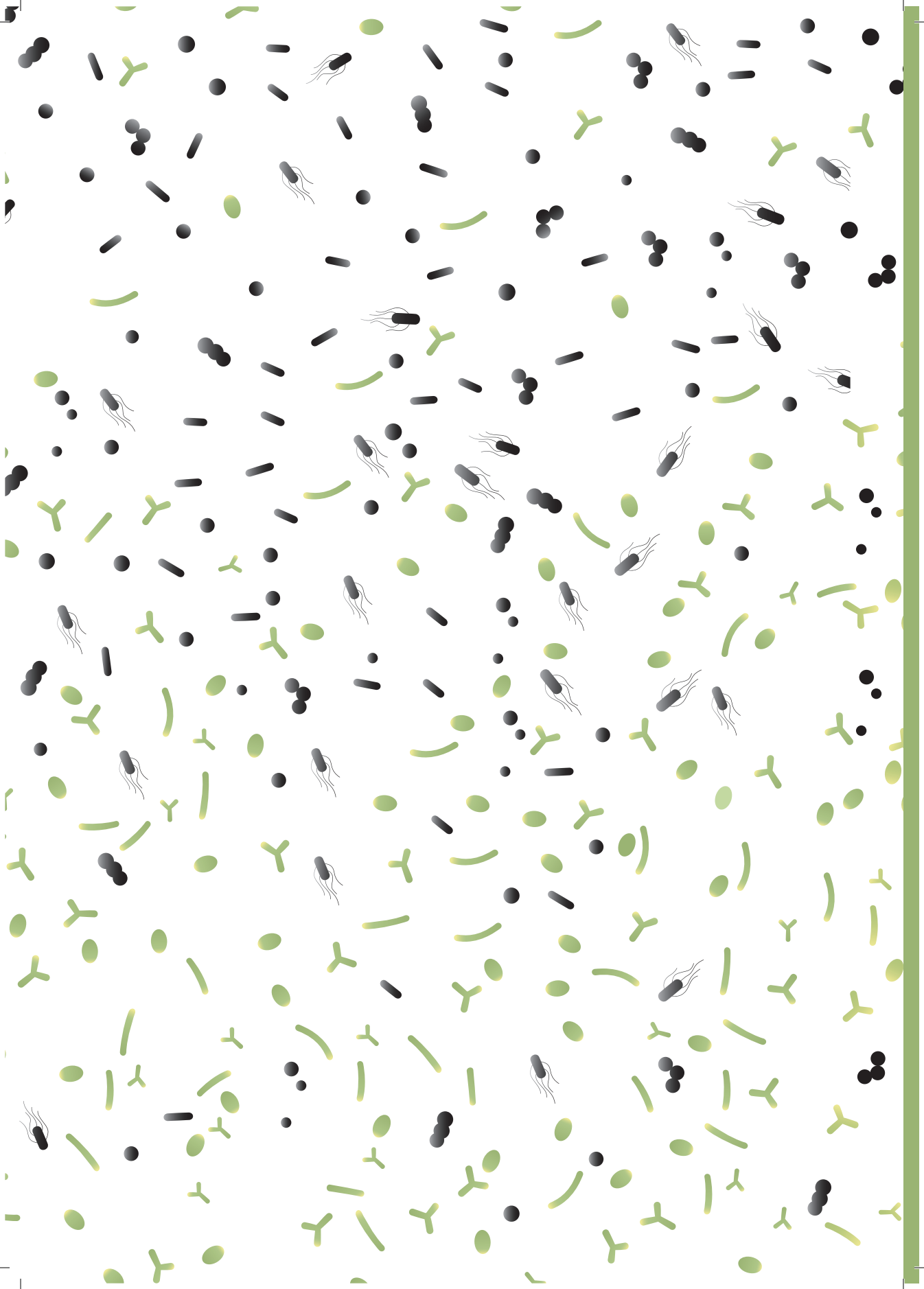
CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The research described in this thesis contributes to current knowledge regarding gastrointestinal function and microbiota development during the early life of preterm infants and the factors associated with this development. This contribution is not only relevant from microbial ecology perspective, but aids clinical practice and development of therapeutic strategies.The findings presented in this thesis confirmed previous knowledge, showing disturbed colonisation patterns in preterm infants, which is particularly associated with perinatal antibiotic treatment. However, it also revealed less expected associations, like the potential influence of respiratory support and maternal preeclampsia. Furthermore, we obtained new insights in gut microbiota functioning via the application of a metaproteomics approach.This approach also allowed for a sneak peek at human- and bovine-derived proteins contained in the gastric and faecal proteome of preterm infants, thereby providing insights in their gastrointestinal functioning from a different perspective. I hereby end the discussion of this thesis with a couple of concluding statements and the future perspectives related to this statement:

Human microbiota research is a composition-oriented field. To fully comprehend the host-microbiota relationship and its consequences to health and disease, functional analysis of the microbiota should be further implemented in current and future research. Depending on the research question; metatranscriptomics, metaproteomics, metabolomics, or a combination of these, can be applied to obtain functional insights. I acknowledge that implementation of such methodologies is costly and require expertise. This, however, stimulates extensive research collaborations and provides the opportunity to further advance the field.

Gut microbiota development in preterm infants remains impeded as long as antibiotics are a necessity during their care. To allow healthy development of the gut microbiota in preterm infants, antibiotics should be minimised and preferably not be provided at all. The latter, however, is currently impossible due to their role in infection prevention and treatment. Since antibiotic resistance became a major concern for health care and public health, the quest for alternatives is rapidly expanding. Alternatives under investigation include bacteriophages, bacteriocins and competitive exclusion by providing pre-, pro- or synbiotics, of which the latter brings me to the next statement.

Microbiota-targeting therapeutic strategies must be considered when caring for the preterm infant. Although not studied as part of this thesis, disturbed colonisation pattern during early life, as observed in preterm infants, has been related to a variety of negative health outcomes in early and later life. It is therefore of importance to prevent disturbances, or to early recover the microbiota whenever possible. Administration of probiotics or lactotransferrin, have shown effective for the prevention of infections and NEC. Although probiotics have so far failed to consistently improve neonatal growth outcomes, they potentially reduce days until full enteral feeding, which is relevant to elucidate further. Regarding probiotics, it is important to reach consensus about the used bacterial strains, their load and timing of administration. In this context, it is important to mention that human milk in itself is a source of bacteria, and that infant formula for term neonates is often supplemented with probiotic bacteria to better resemble human milk feeding. Since the safety and efficacy of probiotics for preterm infants are still inconsistent, routine administration of probiotics and addition of probiotics to preterm infant formula is not yet practiced routinely. In addition to microbiota-modulation via the oral route, results regarding vaginal microbiota transplantation to caesarean section born neonates are promising. When deemed safe and effective, also in preterm infants, vaginal microbiota transplantation is relatively simple and inexpensive to apply in daily clinical practice. Since disturbed microbiota is most certainly involved in negative outcomes like infections, NEC and allergies, which are common complications of preterm birth, microbiota targeting therapies have great potential for preterm infant care.



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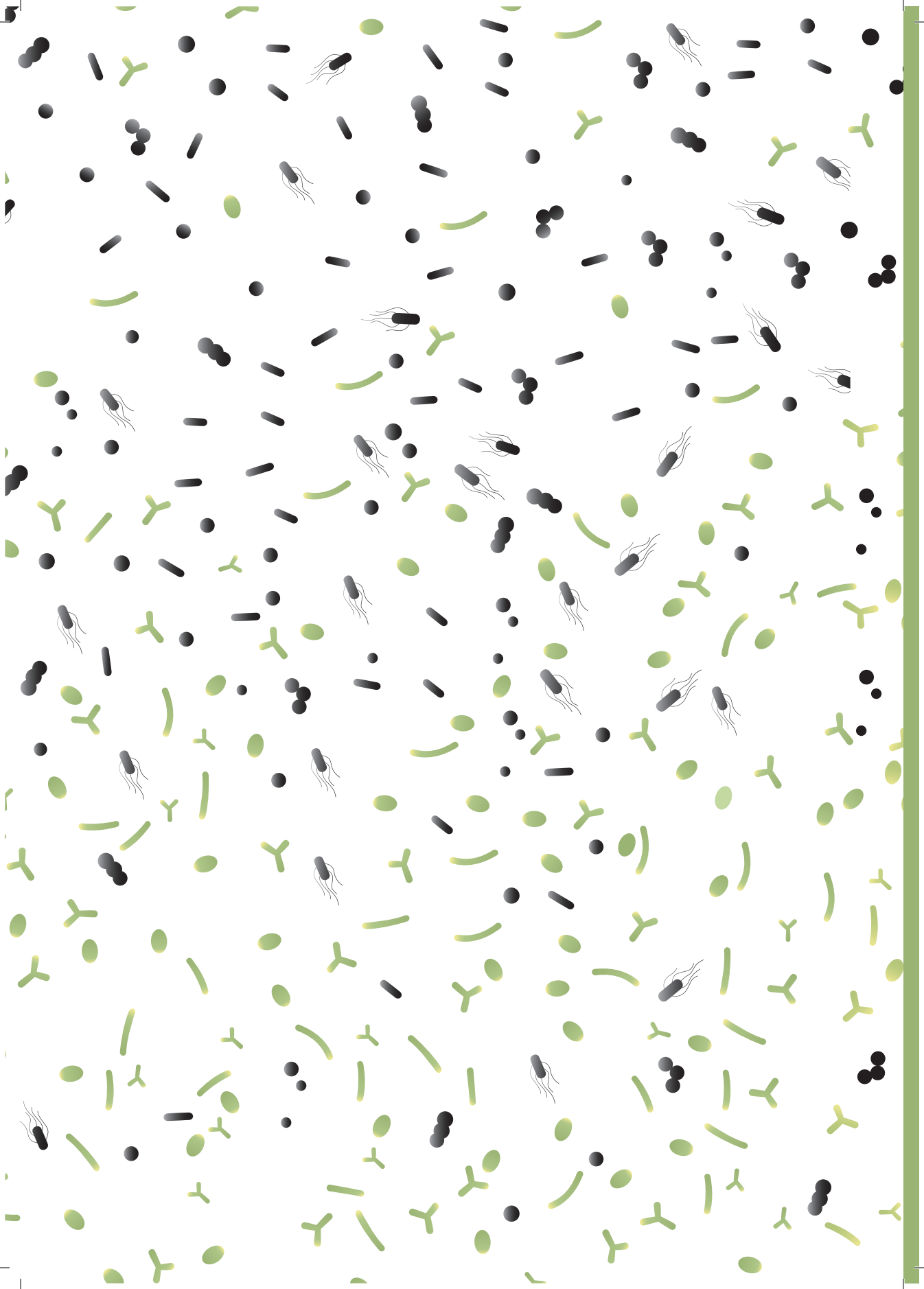

APPENDICES

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SAMENVATTING

Het maag-darmstelsel, en de bacteriën die het bevat, zijn essentieel voor de vertering en opname van voedingsstoffen, de weerstand tegen pathogenen en optimaal functioneren van het immuun systeem. In de vroege levensfase vindt de ontwikkeling van de darmbacteriën tegelijk plaats met de ontwikkeling van het metabole-, cognitieve- en immuunsysteem, en deze processen beïnvloeden elkaar. Goede ontwikkeling van de darmbacteriën, ofwel de darmmicrobiota, wordt daarom noodzakelijk geacht voor een gezond levensverloop. Tijdens de eerste paar jaar van een mensenleven is de samenstelling van de darmmicrobiota relatief onstabiel en extra vatbaar voor verstoringen. Zwangerschapsduur, wijze van bevallen, voeding en antibiotica gebruik zijn factoren welke de darmmicrobiota samenstelling van een baby sterk kunnen beïnvloeden. Te vroeg geboren baby's hebben een onderontwikkeld maag-darmstelsel, worden vaak geboren via een keizersnede, volgen een speciaal voedingsschema en worden regelmatig blootgesteld aan antibiotica. Zodoende, is een verstoorde ontwikkeling van de darmmicrobiota zeer aannemelijk bij prematuren. Ondanks dat de ontwikkeling van de darmmicrobiota verstoord kan worden tijdens de vroege levensfase, biedt de onstabiele en zich ontwikkelende darmmicrobiota ook de mogelijkheid als doelwit te dienen voor de ondersteuning van een gezonde groei en ontwikkeling. Vanuit dit oogpunt is het van belang de kennis te vergroten omtrent het functioneren van het maag-darmstelsel, de kolonisatie van de darm met bacteriën, wat deze doen, en welke factoren deze processen beïnvloeden. Voor dit proefschrift, werden de ontwikkeling van het maag-darm kanaal en de darmmicrobiota tijdens de vroege levensfase van premature baby's, en de factoren die hierop van invloed zijn, bestudeerd.

Voor de studies omschreven in dit proefschrift is gebruik gemaakt van monsters welke zijn verzameld tijdens observationeel onderzoek bij baby's welke zijn geboren tussen 24-42 weken zwangerschap en opgenomen op de neonatale intenstive care. Klinische informatie, fecale monsters en maagaspiraties werden gedurende de eerste zes weken na de geboorte verzameld voor het bestuderen van de microbiota. Naast het bepalen van de microbiota samenstelling door middel van qPCR en sequencen van 16S rRNA gen amplicons, is ook de activiteit en functie van de microbiota in kaart gebracht middels LC-MS/MS (metaproteomics). Dit geeft nieuwe inzichten betreffende het functioneren van het maag-darmstelsel en de darmbacteriën.

Door middel van metaproteomics hebben wij patronen in de ontwikkeling van het maag-darm proteoom kunnen identificeren welke specifiek zijn voor de zwangerschapsduur waarop het kindje is geboren. Zowel zwangerschapsduur als de leeftijd van de baby waren geassocieerd met eiwitten welke de rijping van het maag-darmstelsel en de microbiota aanduiden. De compositie van het fecale proteoom van ernstig premature baby's duidde meer bifidobacteriën en een betere spijsverteringscapaciteit aan, vergeleken met extreem prematuren. Wij hebben aangetoond dat wanneer de darmmicrobiota veel bifidobacteriën bevat, er meer enzymen aanwezig zijn welke een belangrijke rol spelen in koolhydraat- en energiemetabolisme, onder andere enzymen betrokken bij

de afbraak van complexe koolhydraten zoals humane melk oligosacchariden. Het gegeven dat prematuren vaak een eiwit tekort en groei achterstand hebben, maakt de metabole capaciteit van de darmmicrobiota extra relevant. De mate van vroeggeboorte hangt nauw samen met de mate van zorg. In deze studie waren de blootstelling aan antibiotica en duur van beademing significant geassocieerd met bovengenoemde zwangerschapsduur-specifieke patronen van de darmmicrobiota ontwikkeling. De samenhang tussen zwangerschapsduur en mate van zorg, maakt het bijna onmogelijk om enkel het effect van zwangerschapsduur op de microbiota te bepalen.

Premature baby's zijn extra vatbaar voor infecties, sepsis en necrotiserende enterocolitis . Antibiotica, ter voorkoming en behandeling hiervan, zijn de meest gebruikte medicatie op de neonatale intensive care. Gezien de rol van de darmbacteriën in gezondheid en ziekte, is het van belang de consequenties van antibiotica gebruik op microbiota ontwikkeling in kaart te brengen. Wij hebben aangetoond dat twee vancomycine toedieningen, rond de tijd van het verwijderen van een centraal veneuze katheter, vermoedelijk geen blijvend effect had op de samenstelling van de darmmicrobiota van prematuren. In tegendeel, postpartum amoxicilline/ceftazidim verstoorde de microbiota samenstelling aanzienlijk gedurende de eerste twee postnatale weken, ten voordele van *Enterococcus* soorten en ten nadele van *Bifidobacterium* soorten. Daarnaast lijkt het herstel van de microbiota met weken te zijn vertraagd in geval van een kuur van meer van vijf dagen, ten opzichte van een kuur van minder dan drie dagen.

Naast zwangerschapsduur en antibiotica gebruik, zijn er tijdens de studies beschreven in dit proefschrift meerdere factoren meegenomen in de bepaling, zodat hun effect op de microbiota ontwikkeling bepaald kon worden. Naast factoren welke al bekend zijn de microbiota te beïnvloeden, zoals wijze van geboorte en type voeding, hebben wij een mogelijk effect van geslacht, beademing en zwangerschapsvergiftiging aangetoond. Deze bevindingen kunnen aanleiding geven tot nader onderzoek betreffende het effect van deze factoren op de darmmicrobiota ontwikkeling.

Al met al draagt het onderzoek beschreven in dit proefschrift bij aan de huidige kennis omtrent de ontwikkeling van het maag-darm kanaal en de darmmicrobiota tijdens de vroege levensfase van premature baby's, en de factoren die hierop van invloed zijn. Onze bevindingen ondersteunen de klinische praktijk en de ontwikkeling van nieuwe therapieën. In het kader hiervan raden wij het volgende aan voor toekomstig onderzoek 1) implementatie van functionele analyse van de microbiota, 2) bestuderen van alternatieven voor antibiotica voor de behandeling en preventie van infecties, en 3) onderzoeken van de mogelijkheden van microbiota gerichte therapieën tijdens de zorg voor prematuren.

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You make me flourish!

“IK ZOU EEN WOORD WILLEN SPREKEN,

DAT WAAR EN VAN MIJ IS.

DAT DRAAGT WIE IK BEN, DAT HET HOUDT.

IK ZOU EEN WOORD WILLEN SPREKEN,

DAT RECHTOP STAAT ALS MENS DIE MIJ AANKIJKT EN ZEGT.

IK BEN JOUW ZUIVERSTE ZELF,

VREES NIET, VERSTA MIJ, IK BEN.”

Huub Oosterhuis - Ken je mij

ABOUT THE AUTHOR

Romy Daniëlle Zwittink was born on the 8th of February 1989 in The Hague, The Netherlands. After completing her high school education at Cals College in Nieuwegein in 2007, she continued her education at Wageningen University and obtained her BSc and MSc degree in Nutrition and Health in 2010 and 2012 respectively. She performed her MSc thesis research at the department of Cell Biology and Immunology on the effect of food-derived compounds on gut immunity by using Caco-2 cells and THP-1 macrophages as a model, under supervision of Dr Wasaporn Chanput and Professor Harry Wichers. For her internship, she worked at the R&D department of Mead Johnson Nutrition in Nijmegen to explore the potential of nutrition as therapeutic strategy for paediatric obesity via the interference with epigenetic programming, under supervision of Dr Marieke Schoemaker and Professor Harry Wichers. In May 2013, Romy started her scientific career as PhD student at the Molecular Ecology group at the Laboratory of Microbiology of Wageningen University. Her PhD project on gut microbiota development in preterm infants was done in collaboration with Nutricia Research and Isala, under supervision of Dr Clara Belzer and Professor Jan Knol. Since May 2017, she works as a postdoctoral researcher at the department of Medical Microbiology of the Leiden University Medical Center where she studies the human microbiota in health and disease.

LIST OF PUBLICATIONS

Romy D. Zwittink, Ingrid B. Renes, Diny van Zoeren-Grobben, Liesbeth J. Groot Jebbink, Sjef Boeren, Ruurd M. van Elburg, Richard A. van Lingen, Jan Knol and Clara Belzer. Characterisation of the gastric and faecal proteome to unravel gastrointestinal functioning and maturation in preterm infants. *In preparation.*

Esther J. d' Haens*, **Romy D. Zwittink***, Clara Belzer, Marieke A.C. Hemels, Richard A. van Lingen, Ingrid B. Renes, Jan Knol and Diny van Zoeren-Grobben. Effect of vancomycin prophylaxis during removal of a central venous catheter on intestinal microbiota composition in preterm infants. *In preparation.*

Romy D. Zwittink, Diny van Zoeren-Grobben, Ingrid B. Renes, Richard A. van Lingen, Obbe F. Norbruis, Rocio Martin, Liesbeth J. Groot Jebbink, Jan Knol and Clara Belzer. Intravenous amoxicillin/ceftazidime treatment exerts thriving of *Enterococcus* species in preterm and term infants. *Submitted.*

Romy D. Zwittink, Ingrid B. Renes, Richard A. van Lingen, Diny van Zoeren-Grobben, Prokopis Konstanti, Obbe F. Norbruis, Rocio Martin, Liesbeth J. Groot Jebbink, Jan Knol* and Clara Belzer*. (2018) Association between duration of intravenous antibiotic administration and early-life microbiota development in late-preterm infants. *European Journal of Clinical Microbiology & Infectious Diseases.*

Romy D. Zwittink, Diny van Zoeren-Grobben, Rocio Martin, Richard A. van Lingen, Liesbeth J. Groot Jebbink, Sjef Boeren, Ingrid B. Renes, Ruurd M. van Elburg, Clara Belzer* and Jan Knol*. (2017) Metaproteomics reveals functional differences in intestinal microbiota development of preterm infants. *Molecular and Cellular Proteomics.*

Shanna Bastiaan-Net, Wasaporn Chanput, Amelie Hertz, **Romy D. Zwittink**, Jurriaan J. Mes and Harry J. Wichers. (2013) Biochemical and functional characterization of recombinant fungal immunomodulatory proteins (rFIPs). *International Immunopharmacology.*

* Equal contribution

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities - Meetings

Symposium Intestinal Microbiology of Early life
(Wageningen, 2013)
15th Gut Day Symposium
(Groningen, 2013)
KNVM Fall Meeting
(Amsterdam, 2014)
16th Gut Day Symposium
(Amsterdam, 2014) – Poster presentation
KNVM/NVMM Scientific Spring Meeting
(Arnhem, 2015) – Oral presentation
ESPGHAN Annual Meeting
(Amsterdam, 2015) – Poster presentation
17th Gut Day Symposium
(Rotterdam, 2015) – Oral presentation – Best presentation award
KNVM/NVMM Scientific Spring Meeting
(Arnhem, 2016) – Oral presentation
INRA-Rowett Gut Microbiology Symposium
(Clermont-Ferrand, 2016) – Oral presentation
18th Gut Day Symposium
(Venlo, 2016) – Oral presentation
KNVM/NVMM Scientific Spring Meeting
(Arnhem, 2017) – Poster presentation
19th Gut Day Symposium
(Middelburg, 2017) – Poster presentation
KNVM/NVMM Scientific Spring Meeting
(Arnhem, 2018) – Oral presentation

Discipline specific activities - Courses

ARB/SILVA training
(Wageningen, 2014)
The intestinal microbiome and diet in human and animal health
(Wageningen, 2014)
Advanced Proteomics
(Wageningen, 2015)

General courses

Competence Assessment
(Wageningen, 2013)
VLAG PhD Week
(Baarlo, 2013)
Scientific Publishing
(Wageningen, 2015)
Career Perspectives
(Wageningen, 2016)
Programming in Python
(Wageningen, 2016)
BrainTraining
(Wageningen, 2017)

Optional activities

Preparation of PhD project proposal
PhD/postdoc meetings
Molecular Ecology group meetings
Early Life Microbiota team meetings
Microbiology PhD study trip to USA 2015
Wageningen Evolution and Ecology Seminars

COLOPHON

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