Jannie G.E. Henderickx

# The immature gut

MICROBES AND NUTRITION ORCHESTRATE MATURATION OF THE PRETERM GASTROINTESTINAL TRACT

## Propositions

1. Prematurity is a chronic condition.
   (this thesis)

2. Human milk mitigates immaturity of the gut.
   (this thesis)

3. Evidence-based practices should rely on clinical, rather than statistical, significance.
   (Sharma et al., 2021)

4. The normalization of insecurities would eradicate the imposter syndrome in academia.

5. The appointed women's quota will amount to little more than window dressing.

6. More so than physically, weightlifting makes you strong mentally.

Propositions belonging to the thesis, entitled:

**"The immature gut: microbes and nutrition orchestrate maturation of the preterm gastrointestinal tract"**

**Jannie G.E. Henderickx**

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## Microbes and nutrition orchestrate maturation of the preterm gastrointestinal tract

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# The immature gut

## Microbes and nutrition orchestrate maturation of the preterm gastrointestinal tract

**Jannie G.E. Henderickx**

**Thesis**

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*Let everything happen to you*
*Beauty and terror*
*Just keep going*
*No feeling is final*

*Rainer Maria Rilke*

# SUMMARY

Preterm birth interrupts the natural, intrauterine growth path of infants that occurs during the third trimester. As a result of preterm birth, there is a discrepancy between the maturation status of the gastrointestinal tract and the process of microbial colonization in early life. Yet, the concordant maturation of the gastrointestinal tract and the microbiome is pivotal for growth and health of the preterm infant. Neonatal support in the early life of preterm infants offers the opportunity to orchestrate the maturation of the immature gastrointestinal tract and the colonizing microbes. Despite continuous improvements in preterm infant care, optimal feeding is challenging due to the infant's immaturity.

The intestinal bacteria and fungi are pivotal in health and disease. In preterm infants, variation in the gut microbiota (in this thesis the bacterial community) and mycobiota (the fungal community of the microbiota) is introduced due to a unique set of environmental conditions. Within this thesis, we hypothesized that prematurity of the gut microbiota may be an inconspicuous challenge in nutritional neonatal care. Moreover, we expected the intestinal fungi to be affected by clinical variables in early life. A better understanding of the development of the gastrointestinal tract and the microbes in preterm infants is key for optimal nutritional support. The work in this thesis therefore studied the maturation of the gastrointestinal tract and of the intestinal microbes in preterm infants with their implications for infant growth, development and health.

The infants studied in this thesis participated previously in a single-center, observational study in which they were admitted to the neonatal intensive care unit or the pediatric ward of the Isala Women and Children's Hospital (Zwolle, The Netherlands). The preterm and full-term infants were born between 24 and 42 weeks of gestation and were followed in the first six postnatal weeks, during which clinical variables and feces were collected weekly. Gastric aspirates were additionally collected daily in preterm infants during the first two postnatal weeks. Within this thesis, infants were selected from this cohort based on gestational age and antibiotic use of the mother and the infant. The infant's gastrointestinal and microbial functionality was assessed by enzyme activity analyses of host proteins and by metaproteomics with LC-MS/MS analyses. The mycobiota's composition was characterized by sequencing the internal transcribed spacer region 2 (ITS2).

Functional analyses on the host and the microbiota were performed to improve the understanding of gastrointestinal maturation during the early life of preterm infants. We identified host and microbial marker proteins for digestion and barrier defense, which were indicative for gastrointestinal maturation in the first six postnatal weeks. The combination of enzyme activity analyses and metaproteomics showed that preterm infants were capable to digest human milk, albeit to a lesser extent than full-term infants. Moreover, gastrointestinal barrier proteins were compromised in preterm infants compared to full-term infants in the first six postnatal weeks. The maturation status of the infant was additionally found to have implications on the microbiome. Despite the immature status, human milk offers a protective function as shown by multiple bioactive proteins detected in the preterm gastrointestinal tract.

Within this thesis we also present a clinical study design for the "From Mum to Bum" study, in which we aim to investigate the effect of preterm birth on the microbiota's functionality and its relation to anthropometric outcomes. This pilot study will be an observational, single-center study performed at the neonatal intensive care unit at Isala Women and Children's Hospital. A cohort of preterm and full-term mother–infant pairs will be followed during the first six postnatal weeks with follow-up at three- and six-months postnatal age. Compositional and functional methods will be combined to analyze multiple samples along the length of the gastrointestinal tract. As such, we follow digestion of human milk from the breast of the mother throughout the gastrointestinal tract of the infant, or "From Mum to Bum".

Besides the preterm intestinal bacteria, the work in this thesis also describes preterm intestinal fungi. Like bacteria, the colonization and development of the mycobiota in the preterm intestine was hypothesized to be affected by clinical variables. We detected fungi and other eukaryotic kingdoms in the intestinal tract of preterm and full-term infants in the first six postnatal weeks. The gut mycobiota composition and development was influenced by gestational and postnatal age patterns, individuality and mode of delivery. Our data support the hypothesis of vertical transmission of fungi and underpin the role of the mode of delivery in the development of the mycobiota in preterm infants.

The research described within this thesis contributes to current knowledge of the preterm gastrointestinal maturation and its intestinal microbes during the early life of preterm infants, as well as the clinical influences on their development. The intestinal barrier proves to be an important environment where microbes interact with the intestinal epithelium and the immune system to drive growth, development and health of the preterm infant. In light of its clinical relevance, future research should consider the functionality of the preterm microbiota in human milk digestion coupled to anthropometric outcomes as well as the interkingdom interactions in the (preterm) infant intestine. Based on the research described in this thesis, the microbiome and nutrition hold promising applications for preterm infant care that help to orchestrate maturation of the gastrointestinal tract. Microbiota modulation offers hope for future improvements in preterm infant care that pave the way for systemic and lifelong effects. Before nutritional therapies targeting the microbiome can be implemented in preterm infant care, the mechanisms by which microbes are involved in preterm infant health need to be thoroughly assessed. As such, the preterm infant gut microbiome remains a research priority.

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# CHAPTER 1

## General introduction and thesis outline

## Preterm birth and its complications

One out of ten infants are born prematurely each year, which amounts to a total of approximately fifteen million infants per year on a global scale[1]. In addition to burdening the health of the infant and causing psychological stress for parents, premature birth is associated with rising healthcare costs and a major socioeconomic impact[2,3]. Preterm birth is defined by the World Health Organization as babies born alive before 37 weeks of pregnancy, and these births are subdivided according to gestational age in extremely preterm (< 28 weeks); very preterm (28–32 weeks); and moderate to late preterm (32–36 weeks)[1]. Preterm births can either be spontaneous or iatrogenic[4]. Spontaneous preterm birth occurs due to early induction of one of the labor processes, including increased uterine contractility, cervical dilatation and rupture of the chorioamniotic membranes[4]. These processes can be triggered spontaneously or by inflammatory processes at the mother-child interface[5]. Contrastingly, iatrogenic preterm birth is medically indicated due to complications during pregnancy and includes medical labor induction or caesarean delivery without undergoing labor. While genetics are most likely involved in spontaneous preterm birth, other risk factors include previous pregnancies, a multifetal pregnancy, malnutrition, infections, stress and chronic conditions of the mother[1,4]. Interestingly, preliminary results suggest that the recent incidence of preterm birth has declined with the implementation of policies related to the COVID-19 pandemic[6–8]. The proposed underlying mechanisms for this decline are a decrease in the incidence of infections and in maternal stress[6,8].

Prematurity and associated complications are responsible for one third of the deaths in children under the age of five years[1,9]. Over the past decades, advances in technology have increased chances of surviving preterm birth and have decreased mortality rates, although these are dependent on the countries' income[1,9]. Clinicians have made major efforts to support the infants optimally, but care is often a double-edged sword in which advantages of preventing infections must be weighed against contraindications. Strategies to support the infant include nutritional support, respiratory support and medication (including antibiotics and antimycotics)[10]. Fact is, the younger the gestational age at which an infant is born, the higher the chances are for short- and long-term complications[11]. Short-term complications associated with prematurity are increased risk of developing neonatal respiratory conditions, necrotizing enterocolitis (NEC), sepsis, neurological conditions and feeding difficulties[12]. NEC and sepsis are among the most prevalent nosocomial infections and are associated with high mortality rates among preterm infants in early life. Long-term consequences include asthma, impaired cognitive development and psychosocial problems[13–16].

## Meeting the nutritional needs of preterm infants

Preterm birth interrupts the natural, intrauterine growth of infants that occurs during the third trimester. While all anatomical parts of the gastrointestinal tract are developed within the first 12 weeks of gestation, structural and functional properties only develop within 24 weeks of gestation[17]. Consequently, digestive enzymes are being produced upon preterm birth, albeit below their full potential concentration and activity[18]. The activity of lactase—important for

lactose degradation from milk—increases progressively from 24 weeks onwards and reaches maximum activity at term age[17]. Furthermore, the processes of sucking, swallowing and gastric emptying have not been developed completely at preterm birth and their effective coordination is only reached at term age[17]. As such, the preterm gastrointestinal tract is structurally and functionally not fully developed to process human milk feedings as it would at a term age. Maturity of the gastrointestinal tract is needed to digest and absorb nutrients from milk feedings efficiently. Therefore, nutritional support is a crucial part of neonatal care that supports the infant in accordance with its gastrointestinal maturation status.

Nutritional strategies are needed to support the growth and (organ) development of the preterm infant optimally, in which tissue growth and body composition of a fetus of the same postmenstrual age is approximated[19]. The rapid growth that infants normally undergo in the third trimester can hardly be reached by the preterm infant due to an abrupt change in environment from womb to "world"[20]. The preterm infant is suddenly exposed to an environment outside the maternal uterus that requires high energy expenditure for thermal and metabolic homeostasis[20]. Particularly high amounts of protein are required to develop new tissues[21]. Consequently, fat-free mass accretion is pursued in nutritional support. Preterm infants develop a lack of fat-free mass accretion at term equivalent age, subsequently negatively affecting neurodevelopment[22]. Fat-free mass accretion is stimulated by human milk proteins that are an important source of amino acids. Yet, the importance of nutritional strategies reaches further than growth and developmental outcomes alone. The gastrointestinal tract and immune system mature simultaneously in early life. A complex community of microorganisms in the gastrointestinal tract—the gut microbiota—interacts with both processes and orchestrates further intestinal and immunological development of the infant[11]. Additionally, human milk stimulates the maturation of these three elements: the gut microbiota, the gastrointestinal tract and the immune system[21,23]. First, human milk establishes and shapes the gut microbiota with its pre- and probiotic components[24,25]. Second, human milk feeding stimulates the structural and functional development of the gastrointestinal tract and maintains the intestinal barrier[17,24]. The intestinal barrier is compromised upon preterm birth and makes infants prone to NEC and sepsis[26–31]. Lastly, human milk contains many immunoglobulins and protective components with which it supports the naturally immature immune system of neonates[32]. Even more so than full-term infants, preterm infants have an immature immune system that supposedly contributes to the risk of NEC[31–33]. In summary, the support of the infant's fat-free mass accretion together with the stimulation of the gut microbiota, the gastrointestinal tract and the immune system should be important aspects of nutritional neonatal care.

Immediately after birth, nutritional care is initiated with parenteral feeding in which amino acids, lipids and other macro- and micronutrients are administered intravenously[34,35]. While parenteral feeding improves growth in the first postnatal weeks, it is also associated with metabolic and infectious complications[36]. In contrary, enteral feeding stimulates the development of the gastrointestinal tract—including endocrine and metabolic processes—and reduces local and systemic inflammation[34,37]. Therefore, the aim is to transition to full enteral feeding as soon as possible and to use parenteral feeding only complementary[34]. In practice, however, full enteral

feeding is often delayed due to concerns for increased risk of NEC. As such, the nutritional recommendation is to feed parenterally in the early phase while transitioning into minimal enteral feeding of (donated) human milk and human milk fortifiers[34,35,37,38]. Minimal enteral nutrition is practiced widely in neonatal intensive care units (NICUs), in which small volumes of human milk, which is the golden standard for enteral feeding, or formula are provided and are gradually increased (15-30 mL/kg/day) to continuous enteral feeding[37–39]. Besides the aforementioned bioactive components, the major nutritious constituents of human milk are lactose and lipids[40]. When mother's production is insufficient, pasteurized donor milk can be used as alternative and may potentially be personalized in the future with mother's own milk as inoculum[41–44]. Additionally, fortification of human milk with micro- and macronutrients deriving from bovine milk is necessary to provide sufficient nutrients to the infant and to improve growth in weight, length and head circumference[37–39,45].

In practice, nutritional management proves to be a major challenge in preterm infants. Recommended full enteral feeding is not always feasible in critically ill infants or infants born before 32 weeks of gestation who are prone to be intolerant to feeding, which is characterized by abdominal distension, emesis and diarrhea[46,47]. Aspirates of gastric residuals generally act as putative indicator for feeding intolerance and are used to monitor the infant's status and guide feeding advancements as such[48–51]. Despite these efforts, more than half of the hospitalized preterm infants are being discharged with ongoing severe postnatal growth impairment[52]. With a central role for nutrition, optimal maturation of the preterm infant is stimulated with nutritional care.

## The intestinal microbiota and mycobiota and their interactions with the host

Technological advances have made it possible to progress our understanding on the prevalence and diversity of microorganisms associated with the human body. Various human body sites—including skin, vagina and gastrointestinal tract—harbor a complex community of microorganisms[53,54]. Microbial diversity depends on body site, complexity and aggregate functions of the community and is correlated to health status, diet and hygiene[53]. Of those body sites, and along the length of the gastrointestinal tract, the large intestine is most densely populated with bacteria, fungi, archaea and viruses[53,55]. Collectively, the assembly of these living microorganisms comprise the gut microbiota[55]. Within this thesis, however, we will refer to the "microbiota" and "mycobiota" to indicate the assembly of microorganisms solely belonging to the kingdom "Bacteria" and "Fungi", respectively. The terms "microbiome" and "mycobiome" will be used to indicate this assembly of microorganisms and their "theater of activity" comprising of microbial structures, molecules, mobile genetic elements and relic DNA embedded in the environmental conditions of the habitat[55]. While bacteria reach high cell densities of approximately $10^{11}$ cells per gram of feces in the adult large intestine (collectively called the "microbiota"), fungi are present in substantially lower numbers[56–58]. Fungi are estimated to comprise 0.1% of the microorganisms in the large intestine and consist of $10^5$-$10^6$ cells per gram of feces (collectively called the "mycobiota"), although these numbers may be an underestimation[56,59,60]. While smaller in cell

counts, fungal cells are 10-fold longer and 100-fold larger in volume than bacterial cells. Hence, the fungal biomass and the metabolites they produce cannot be compared with the microbiota by solely considering cell counts[61].

Both bacteria and fungi have indispensable functions that influence many processes in the human body and thereby are pivotal for health[53,62–64]. The microbiome and mycobiome are therefore increasingly appreciated as a fundamental and necessary component of human physiology[53,59]. The microbial gene pool in the intestine consists of five million unique genes[65]. Despite interpersonal differences in microbial community composition, there is a high level of functional redundancy between microbial community members[66].



## The gut microbiome's functions

The microbiome interacts with the physiology and the maturation of the gastrointestinal tract. First, the gut microbiota's metabolic functions are distinct, yet complementary to human enzymes. Certain intestinal bacteria are involved in the biosynthesis of vitamins B9, B12 and K and of amino acids[67]. Others aid in absorption of ions such as calcium, iron and magnesium[53]. Second, the gut microbiota degrades undigested carbohydrates such as those in human milk. While infants can digest lactose from human milk, other complex carbohydrates remain undigested and pass to the colon where members of the gut microbiota consume the so-called "human milk oligosaccharides" (HMOs). More specifically, *Bifidobacterium* spp. and *Bacteroides* spp. have genes encoding for glycosyl hydrolases, enzymes involved in HMO degradation[68–70]. One of the intermediate metabolites of HMO degradation is lactate and, thus, is dominant in early life. HMO degradation by the microbiota yields short-chain fatty acids (SCFAs) including butyrate, acetate and propionate[53], and thereby contributes to intestinal homeostasis and microbiota development[70]. SCFAs act as nutrient for the microbiota itself[71,72], and are important for gastrointestinal maturation by upregulating tight junction proteins that maintain the intestinal barrier[71,73]. Butyrate is the main energy source of epithelial cells and specifically stimulates epithelial cell differentiation while inhibiting epithelial cell proliferation[53]. Yet, the levels of butyrate and the abundance of butyrate-producing bacteria are low in infant's feces and succeed with increasing age[74,75]. Given their predominance in early life, acetate and lactate may thus be more relevant for maturation of the infant intestine[74,75]. The importance of the microbiome in intestinal epithelial development is epitomized in germ-free mice that have a smaller intestinal surface area, decreased epithelial cell turnover and underdeveloped villi and crypts compared to mice colonized with intestinal bacteria[76]. However, not only the presence, but also the composition of the microbiota may affect intestinal epithelial development[77].

The microbiome also develops and regulates the immune system. The simultaneous development of the microbiota and the immune system in early life is critical for intestinal homeostasis, as it may prevent inappropriate inflammatory responses toward commensal bacteria[53,67]. Thereby, disturbances in these processes can have long-lasting health consequences[67,78]. Interactions between the microbiota and the immune system occur at the gut-associated lymphoid tissue (GALT), which is part of the mucosal immune system and is the primary site of interaction

between the microbiome, the immune system and antigens[67]. Pathogen Recognition Receptors (PRRs) or G-protein coupled receptors can recognize Microbial Associated Molecular Patterns (MAMPs). Also, Microfold (M) cells facilitate direct interaction of bacteria in the lumen with lymphoid cells by transporting bacteria across the epithelial cell layer. The intestinal mucus lining forms a niche for bacteria with mucin-degrading capacities, including *Bifidobacterium* spp., *Bacteroides* spp., *Ruminococcus gnavus* and *Akkermansia muciniphila*[79]. Niche occupation of the mucosal surface establishes a physical barrier for potential pathogens, but also leads to the production of compounds forming a chemical barrier[53,67]. Moreover, attachment and invasion of pathogens is inhibited by competing for nutrient availability along with production of antimicrobials. These mechanisms contribute to so-called "colonization resistance", defined as the ability of commensal bacteria to prevent expansion of potential pathogens[80].

## The gut mycobiome's functions

There are many similarities and interactions between the microbiota and the mycobiota as they share a niche environment. Although it is still unclear if fungi are residents of the intestine or rather transients, it is likely that fungi can exert bioactive functions[58]. Like bacteria, intestinal fungi are involved in gastrointestinal physiology and maturation. First, fungi exert metabolic functions, although these are not studied extensively in the infant intestine. One example is the production of SCFAs, of which particularly butyrate is produced[58,81,82]. Moreover, some fungi may be related to carbohydrate digestion as the fecal abundance of *Candida* spp. has been positively correlated to a carbohydrate-rich diet[57,58,61]. These metabolites may then be fermented by intestinal bacteria into several by-products[57]. Besides degrading carbohydrates directly, *Saccharomyces boulardii* has been reported to stimulate the activity of brush border enzymes—amongst others sucrase, lactase and maltase—and nutrient transport[81]. Additionally, some fungal species may promote growth of lactic acid bacteria by secreting amino acids[83]. In that way, fungi might stimulate lactose degradation indirectly and play an important role in human milk degradation. Future studies are needed to elucidate the ability of fungi to degrade human milk components directly and the possible products this would generate, as well as the effects of these products on host processes.

The mycobiome additionally affects the host immune system functions[58,61,63,84]. Upon stimulation by fungi in the intestine, structural development of lymph nodes is initiated and lymphocyte homing to the intestine is induced[85]. Furthermore, fungi can modulate immune responses reaching further than the gastrointestinal tract, as fungal dysbiosis in the intestine has been shown to affect immune responses in distal organs[86–89]. Such immune responses are mediated through some of their immunomodulating metabolites. The most studied immunomodulators include β-glucans and mannans that both derive from the fungal cell wall, as well as prostaglandin E2[90–95]. Although understudied, the commensal fungi may provide local and systemic protection. For example, commensal intestinal fungal species can functionally replace intestinal bacteria by reducing susceptibility to intestinal injury and extra-intestinal infection[96]. These benefits are mediated by the fungal cell wall mannans that confer protection against mucosal tissue injury and upregulate the responsiveness of circulating immune cells[96]. Additionally, commensal

*Candida albicans* protects against systemic invasive infection by extracellular pathogens through the stimulation of systemic Th17 immune responses[89]. Other reported protective actions by the probiotic yeast *S. boulardii* include pathogen binding and the production of antimicrobial peptides, although many of the mechanistic details underlying these observations are still missing[81].



## Studying the intestinal microbiota and mycobiota

The intestinal microbiota and mycobiota are mostly studied by using fecal samples as proxy for the intestinal content. Collection of feces is non-invasive compared to other used methods such as biopsies and endoscopic procedures[97]. However, microbiota, and possibly mycobiota, composition varies along the intestinal tract, as well as across the biostructure of a stool sample and with the consistency of the stool[97–102].

Conventionally, the gut microbiota has been studied by culturing and characterizing bacterial isolates. The major limitation of these techniques is the selectivity toward the readily cultivatable bacteria[103], which has been termed "the great plate count anomaly"[104]. Yet, new high-throughput techniques have revived microbial cultivation[103,105–109]. Nowadays, the microbiota composition is most often characterized by a culture-independent method that sequences the highly variable regions of the conserved ribosomal RNA (rRNA) 16S subunit with amplicon sequencing. Due to its variability, the 16S rRNA subunit allows to classify identified bacteria up to species level[110]. Amplicon sequencing comprises extraction of DNA from complex, biological samples containing a mixture of microorganisms. Additionally, many technological and computational advances now allow for culture-independent, high-throughput "-omic" methods. These methods include the use of DNA (metagenomics), RNA (metatranscriptomics), proteins (metaproteomics) and metabolites (metabolomics). With the advances in "-omic" techniques the questions "Who are there?", "What can they do?" and "What are they doing?" have become the mantra of the gut microbiologist. In fact, it is easier than ever to study composition, functionality and activity of microorganisms in any given ecosystem although interpretation of biological implications can still be challenging.

In contrast to prokaryotes, no consensus has been reached in biomarkers for studying eukaryotic fungal communities[59,111–113]. The internal transcribed spacer (ITS) region has been proposed as "universal barcode marker for fungi"[114]. The ITS regions of fungal ribosomal DNA (rDNA) consists of two regions, termed "ITS1" and "ITS2", that allow for classification at species level[112,115]. The ITS2 region has been recommended because of its better taxonomic resolution and low primer bias compared to ITS1[112,116,117]. Other biomarkers for the fungal community include the small (18S) and large (28S) rRNA subunit[114]. Although being more conserved, its variability is too low for classification at species level[114]. Besides the ongoing debate on the genomic target regions and the optimal primer sets, the fungal databases present another challenge[59,111,112,118]. While bacterial 16S rRNA databases are well-characterized, the fungal reference sequence database is still more limited[59]. Moreover, within the fungal databases, many of the fungal sequences are annotated as "uncultured"[59]. Lastly, sexual (teleomorph) and asexual (anamorph) forms of a fungus are often classified into a different taxon[59].

## Intestinal microbiota development in preterm infants

In early life, the gut microbiota of a healthy full-term, vaginally delivered and exclusively breastfed infant is considered the golden standard for an optimal microbiota development[119]. The mode of delivery is important for which bacteria first colonize the infant intestine[25,120–123]. Vaginally born infants typically acquire bacteria resembling the vaginal microbiota of their mother, whereas caesarean (C-)section infants acquire bacteria resembling the skin microbiota and show reduced diversity[121–123]. Generally, the intestine of infants is colonized with facultative anaerobic bacteria during and shortly after birth due to the presence of low amounts of oxygen in this environment[124]. These facultative anaerobic bacteria belong to the genera *Enterobacter*, *Enterococcus*, *Staphylococcus* and *Streptococcus*[125]. As facultative anaerobic bacteria consume the residual oxygen in the infant intestine, the resulting lowered redox potential allows obligate anaerobic bacteria to thrive[124]. *Bifidobacterium* spp., *Bacteroides* spp. and *Clostridium* spp. proliferate and become the predominant genera, of which particularly *Bifidobacterium* and *Bacteroides* species have a selective advantage in early life in light of HMO degradation[25,68–70,126–128]. Breastfed infants are for this reason typically characterized by a microbiota dominated by bifidobacteria, while formula-fed infants have a more diverse microbiota[25]. The degradation of HMOs by *Bifidobacterium* spp. and *Bacteroides* spp. generates beneficial nutritious components for epithelial cells and intestinal bacteria, and is therefore considered a beneficial process supporting gastrointestinal health in early life[68–70].

Microbial colonization of the neonate's intestine is already impacted before birth by maternal factors, such as the microbiota composition of the mother, and prenatal or intrapartum antibiotic administration[129]. From the moment after birth, the gut microbiota composition develops by selective pressures, including gestational age, antibiotic treatment and diet (Fig. 1.1)[130–132]. Of those variables, gestational age influences the gut microbiota development strongly[133,134]. Preterm infants typically display delayed microbial colonization of obligate anaerobes and limited microbial diversity compared to full-term infants[135–137]. At the same time, potential pathogenic and facultative anaerobic bacteria are increased[125,138–140]. The genera *Enterobacter*, *Enterococcus*, *Escherichia*, *Klebsiella* and *Prevotella* are predominantly present in preterm infants and less in full-term infants[131,141–143]. Contrastingly, *Bifidobacterium* spp. levels are generally less abundant in preterm infants compared to full-term infants[127,144]. Other selective pressures relevant in preterm infants are the NICU environment, antibiotic treatment of the infant and respiratory support (Fig. 1.1**)**. The hospital environment may act as reservoir for microbes, selected by lavish antibiotic use, that subsequently colonize the infant intestine[11,145]. Moreover, antibiotic exposure and respiratory support have been shown to affect microbiota composition and functionality in preterm infants considerably by delaying colonization with beneficial, obligate anaerobic bacteria like *Bifidobacterium* spp.[137,144,146]. As a consequence, shifts in obligate and facultative anaerobic bacteria may have an impact on HMO degradation in the preterm infant intestine[11,137]. In turn, this may affect acetate and lactic acid production that is important to reduce intestinal pH and inhibit growth of pathogens[147].

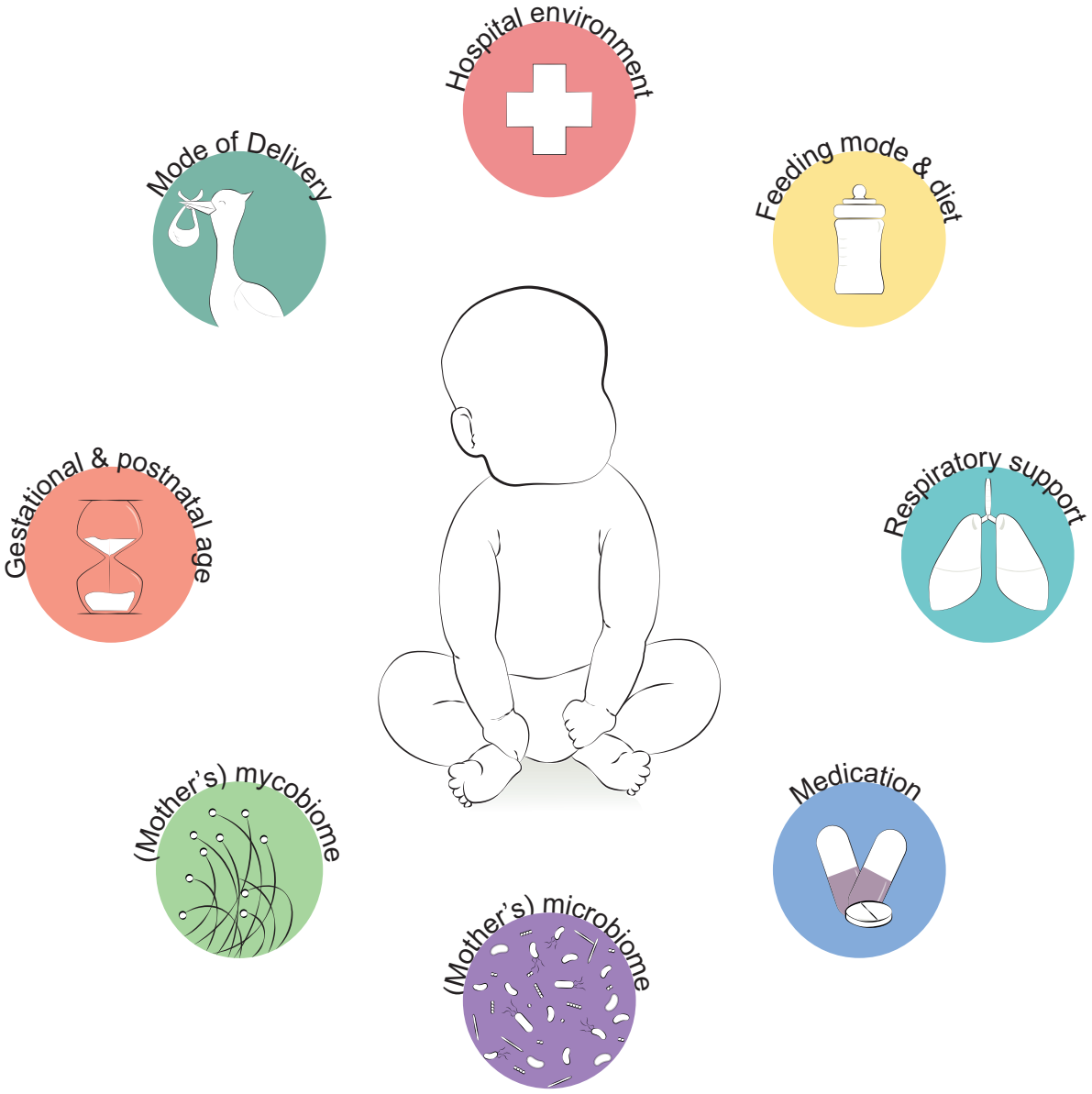


**Figure 1.1** Environmental influences on the microbiome and mycobiome of (preterm) infants.

The disrupted microbiota development in preterm infants is a risk factor for development of NEC and sepsis[148–150]. As mentioned before, other risk factors include a decreased intestinal barrier and a naïve immune system, which is common in preterm infants[30,151,152]. Although the etiology of these nosocomial infections is multifactorial, the microbiota plays an indispensable role in the development of both diseases[31,152,153]. While no causative microorganism has been identified, low microbial diversity and predominance of a single genus of the Proteobacteria phylum are frequently observed in preterm infants with NEC[31,148,153]. In sepsis, Gram-negative bacteria including *Escherichia coli, Klebsiella* spp. and *Pseudomonas* spp., and Gram-positive bacteria including *Enterococcus* spp., *Streptococcus* spp. and coagulative-negative Staphylococci (CoNS) are frequently identified[151]. Moreover, a delay in colonization with obligate anaerobic bacteria may be a predisposing factor[151]. However, some bacteria may have protective effects as indicated by their decreased abundance in

preterm infants with NEC or sepsis. More specifically, the abundance of phyla Actinobacteria, Bacteroidetes and Firmicutes are generally decreased in NEC cases, while low bifidobacterial abundance has been observed in preterm infants with sepsis[154]. Furthermore, these taxa are more abundant in healthy preterm infants[154]. Yet, the mechanisms of the involvement of microbes in pathogenesis or protection of NEC and sepsis are still largely unknown.

## Intestinal mycobiota development in preterm infants

Normally, the intestine of a vaginally delivered infant is colonized by *Candida* spp. through vertical transmission from mother to infant[54,155,156]. Although the fungal genus *Candida* is a commensal of the vaginal mycobiota, *Candida* species are also frequently identified in vaginal infections[155]. Other fungal species observed in infants include *Aspergillus*, *Cladosporium*, *Cryptococcus*, *Debaryomyces*, *Elmerina*, *Eurotiomycetes*, *Leptosphaerulina*, *Malassezia*, *Nectriaceae*, *Penicillium*, *Saccharomyces*, *Stereum*, *Tremellomycetes* and *Trichosporon*[54,58,155–158]. In contrast to healthy infants, the preterm infant gut mycobiota is often dominated by a single species, typically a yeast such as *Candida* spp.[157,158]. More specifically, *C. albicans* and *C. parapsilosis* are highly prevalent and persistent in preterm infants[157]. Other fungi identified in stools of extremely low birth weight and preterm infants include those of the Saccharomycetales order and species of the *Cladosporium* and *Cryptococcus* genus[59,136].

Similar to the microbiota, environmental variables drive mycobiota development and include gestational and postnatal age, mode of delivery, hospital environment, antibiotic exposure and diet (Fig. 1.1)[54,155,158–161]. The mother's mycobiota and the infant's bacterial microbiota additionally influence the mycobiota composition and diversity[155]. At birth, the mode of delivery determines vertical transfer of fungi from mother to infant. This is well studied for *Candida* spp. in particular, but has also been described for the Saccharomycetales order and species of the *Cladosporium* and *Cryptococcus* genus[156,162]. Furthermore, the mycobiota is affected by the hospital environment, which may hold a reservoir of fungi that could colonize the intestine of hospitalized (preterm) infants[161]. After birth, fungal diversity generally increases with gestational and postnatal age[157]. Diet continues to shape the mycobiota with increasing postnatal age. Early in life, human milk may be a source of viable fungi for the infant gut mycobiota[161,163–167]. Moreover, HMOs within human milk inhibit hyphal morphogenesis as well as fungal interaction with premature intestinal epithelial cells and thereby shape the mycobiota[168]. After weaning, the food-associated *Saccharomyces cerevisiae* becomes most abundant in infants[157]. As mentioned before, it is unclear if this food-associated yeast becomes a resident of the intestine or rather is a transient.

Fungal colonization in early life and its potential health effects is a relatively new field of research[54,58]. Important to note is that fungi can be dimorphic[59]. For example, *Candida* spp. have the ability to grow in unicellular budding yeast forms and in filamentous hyphal forms. Immune cells may recognize these two morphologies differently, which therefore can have implications for the fungi's pathogenicity and their role in immuneprogramming[169–174]. Many fungi are commensal and some have been shown to confer health benefits[58,62,155,175]. For example, administration of the probiotic strain *S. boulardii* has been shown to be effective in ameliorating gastrointestinal diseases[176,177]. In contrast to health benefits, the mycobiota can, however, also act as a reservoir for opportunistic

pathogens in immunocompromised hosts such as preterm infants[161]. Preterm infants often experience overgrowth of an opportunistic pathogenic fungus after antibiotic treatment, which typically results in invasive systemic candidiasis[178–181]. Morbidity and associated mortality rates of invasive systemic candidiasis in preterm infants are as high as 10% and 30%, respectively[182–184]. Invasive candidiasis is most often caused by *C. albicans*, which might transition from commensal to opportunistic pathogen in response to perturbations in the microbiota and/or weakening of the immune system[185–188]. Another suggested mechanism is the primary invasion and subsequent infection of physiological barriers after they have been breached, for example by NEC[181,186–188]. Factors that might trigger the transition include long-term or repeated use of broad-spectrum antibiotics, use of central venous catheters, parenteral nutrition and a naïve immune system[180,182,185]. Indeed, antibiotics may promote overgrowth by *Candida* spp. through induction of genetic changes leading to increased fitness of *C. albicans* in the intestine[189].

## Interkingdom interactions in the intestine

Different types of microorganisms in the intestinal tract have co-evolved with their host into a complex ecosystem. Sharing the same niche environment allows for intimate crosstalk between bacteria, fungi, archaea and viruses[190]. Such interactions—referred to as "interkingdom" (used in this thesis), "transkingdom" or "multibiome" interactions—have been observed in the intestine. Interkingdom interactions start in early life, in which bacteria and fungi correlate inversely in the infant intestine[191]. Causal implications have been established in mice for intestinal fungi with microbial ecology and with host immune functionality[192]. Described interactions between bacteria and fungi are mutualistic, commensalistic and competitive[61].

Most mutualistic interactions have been observed in the oral and vaginal environments between *C. albicans* and various *Streptococcus* species in mixed biofilms[61,193–195]. Bacteria and fungi co-occur in mixed biofilms providing extra protection against host immune responses compared to monoculture biofilms[195–199]. On the one hand, bacteria are protected by a fungal polysaccharide matrix and become more resistant to antibacterial compounds[195–197,200]. On the other hand, fungi benefit from the extracellular matrix of bacteria that enhances virulence factors for fungal filamentation[201], even though inhibition by bacteria is also reported[202,203]. Indeed, emerging evidence suggests that mixed biofilms decrease antimicrobial efficacy *in vitro*[198]. Another mutualistic, interkingdom interaction is cross-feeding. The yeast *S. cerevisiae* has been shown to enable growth of lactic acid bacteria *in vitro*. In nitrogen-rich environments, *S. cerevisiae* adapts its metabolism by secreting metabolites, of which especially amino acids[83]. Subsequently, *Lactiplantibacillus plantarum* and *Lactococcus lactis* benefit from these amino acids and produce galactose and glucose that are consumed by the yeast[83].

One of the described commensal interactions is the ability of *C. albicans* to promote growth and proliferation of bacteria, such as *Clostridium difficile*[61]. The yeast consumes oxygen by mitochondrial activity and thereby creates a favorable anaerobic environment for obligate anaerobic bacteria like *C. difficile*[204,205]. Other commensal interactions have been described in which Enterobacteriaceae positively affect growth of yeasts in the mouse's intestine[206].

Competitive interactions between bacteria and fungi are mainly described from the bacterial perspective. The negative effect of bacteria on fungi is most evident in fungal overgrowth after antibiotic treatment. Both in murine models and in humans, antibiotic treatment has been shown to support *Candida* spp. colonization and fungal overgrowth[185,207,208]. Intestinal bacteria have a diverse repertoire of molecules capable of disturbing fungal growth and differentiation[61]. Bacteria can reduce virulence factors of *C. albicans* by affecting their filamentation, adherence and biofilm formation[209–212]. Reduction of virulence factors of *C. albicans* may in turn lead to reduced protection of filamentous bacteria in mixed biofilms[61]. Future research should shed light on deleterious effects of fungi on bacteria, as these are currently understudied. As mentioned before, bacteria outnumber fungi substantially in the intestine. For this reason, future studies additionally need to account for the absolute abundance of bacteria and fungi in these interkingdom interactions.

## Research aim and thesis outline

Preterm birth interrupts the natural, intrauterine growth of infants that occurs during the third trimester. As a result of preterm birth, there is a discrepancy between the maturation status of the gastrointestinal tract and the process of microbial colonization in early life. Yet, the concordant maturation of the gastrointestinal tract and the microbiome is pivotal for growth and health of the preterm infant. Neonatal support in the early life of preterm infants offers the opportunity to orchestrate the maturation of the immature gastrointestinal tract and the colonizing microbes. A better understanding of the development of the gastrointestinal tract and the microbes in preterm infants is key for optimal nutritional support. The work in this thesis therefore aimed to study the maturation of the gastrointestinal tract and of the intestinal microbes in preterm infants with their implications for infant growth, development and health. To this end, we provide new understanding on how the intestinal maturation status of the infant and the microbiota's functionality are affected by preterm birth. Moreover, we present a novel clinical study design toward a targeted approach to investigate the role of the preterm microbiome in human milk digestion. We additionally describe the composition of intestinal fungi in preterm infants and the effect of clinical variables on them.

This thesis comprises of six chapters, including a literature review, research papers and a study protocol.

**Chapter 2** reviews the interaction between prematurity and nutrition in the concordant maturation of gut microbiota, gastrointestinal tract and immune system in early life of preterm infants. This chapter describes the preterm gut microbiota composition and unique environmental conditions contributing to this, and the interaction between human milk and the gut microbiota, the gastrointestinal tract and the immune system.

**Chapter 3** discusses the functionality of the preterm gastrointestinal tract and of the microbiota therein. A functional gastrointestinal tract and microbiome are essential for growth and development of preterm infants. As such, the aim of this study was to improve the understanding

of gastrointestinal functionality and maturation during the early life of preterm infants by means of gastrointestinal enzyme activity assays and metaproteomics. This study was part of the EIBER study; an observational, single-center, non-intervention study conducted between 2011-2014 at Isala Women and Children's Hospital (Zwolle, The Netherlands). The main objective of the EIBER study was to investigate early life colonization and establishment of the gut microbiota in extremely and vey preterm infants as well as to understand the effect of antibiotic treatment duration on gut microbiota development in preterm and full-term infants.

**Chapter 4** describes the study protocol for the clinical study "From Mum to Bum". The "From Mum to Bum" study is an observational, single-center, non-intervention study, with the main objective to investigate how the intestinal microbiota of preterm and full-term infants differ in their ability to extract energy and nutrients from oligosaccharides and proteins in human milk. As a follow-up of the EIBER study, the "From Mum to Bum" study started in 2020 at the Isala Women and Children's Hospital (Zwolle, The Netherlands) and in the area of Zwolle.

**Chapter 5** elaborates on the fungal colonization in early life of antibiotic-treated preterm infants. Like bacteria, the colonization and development of intestinal fungi in preterm infants was hypothesized to be affected by interactions with clinical variables. We therefore aimed to characterize the composition and diversity of the preterm infant mycobiota and the effect of clinical variables on it in the first six postnatal weeks. This study was part of the EIBER study.

**Chapter 6** summarizes the research findings generated within this thesis and discusses its main findings in a broader ecological and clinical context. Furthermore, this chapter identifies challenges and future research directions that could complement future neonatal care.

# CHAPTER 2

## The preterm gut microbiota: an inconspicuous challenge in nutritional neonatal care

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## Abstract

The nutritional requirements of preterm infants are unique and challenging to meet in neonatal care, yet crucial for their growth, development and health. Normally, the gut microbiota has distinct metabolic capacities, making their role in metabolism of dietary components indispensable. In preterm infants, variation in microbiota composition is introduced while facing a unique set of environmental conditions. However, the effect of such variation on the microbiota's metabolic capacity and on the preterm infant's growth and development remains unresolved. In this review, we will provide a holistic overview on the development of the preterm gut microbiota and the unique environmental conditions contributing to this, in addition to maturation of the gastrointestinal tract and the immune system in preterm infants. The role of prematurity, as well as the role of human milk, in the developmental processes is emphasized. Current research stresses the early life gut microbiota as cornerstone for simultaneous development of the gastrointestinal tract and the immune system. Besides that, literature provides clues that prematurity affects growth and development. As such, this review is concluded with our hypothesis that prematurity of the gut microbiota may be an inconspicuous clinical challenge in achieving optimal feeding besides traditional challenges, such as preterm human milk composition, high nutritional requirements and immaturity of the gastrointestinal tract and the immune system. A better understanding of the metabolic capacity of the gut microbiota and its impact on intestinal and immune maturation in preterm infants could complement current feeding regimens in future neonatal care and thereby facilitate growth, development and health in preterm infants.

## Background information

Preterm infants, born before 37 weeks of gestation, are increasingly affected both by prematurity and by complications associated with decreasing gestational age. Complications of prematurity include impaired maturation of the gut microbiota, gastrointestinal tract and immune system (Fig. 2.1). Yet, simultaneous maturation of the gut microbiota, gastrointestinal tract and immune system in early life orchestrates further infant growth—that is, weight gain—and organ development. As they are playing a cornerstone role in infant growth and development, impaired maturation of the gut microbiota, gastrointestinal tract and immune system could have serious health consequences. Preterm infants with extremely low birth weight are susceptible to infections, which in turn is associated with poor neurocognitive functioning [213,214]. Therefore, preterm infants would benefit from weight gain, implicating growth can be considered as health indicator[77,215]. Strict feeding regimens are needed in the neonatal period to stimulate maturation processes, growth and organ development.



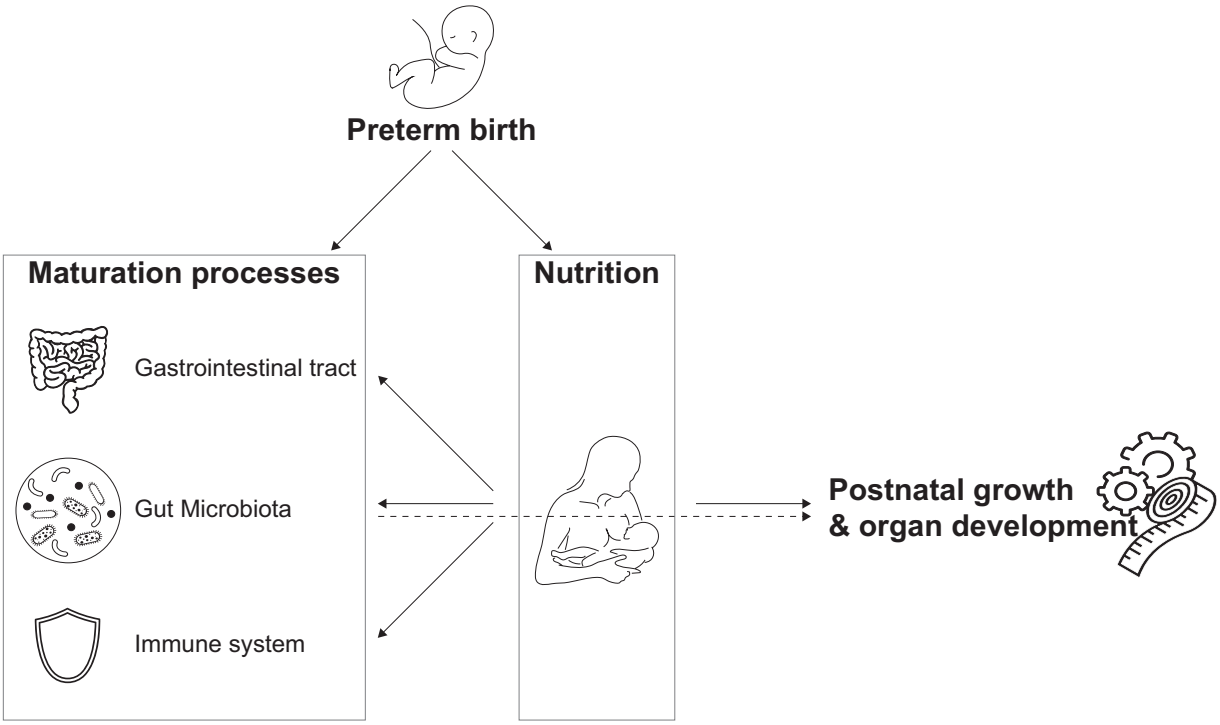


**Figure 2.1** Preterm birth influences human milk composition and affects maturation processes. Human milk stimulates maturation of the gastrointestinal tract, gut microbiota and immune system, which, together with its dietary components, promotes postnatal growth and organ development. While preterm birth influences human milk composition and affects maturation processes, it remains unknown to what extent the preterm gut microbiota is involved in human milk digestion and how it contributes to postnatal growth and organ development.[1]

1 Icons were retrieved from The Noun Project. All retrieved icons are licensed as public domain or creative commons (CC BY). Icons were designed by: Cristiano Zoucas (Measuring tape), Design Science (Immune System), Gregor Cresnar (Gears), Jannie Henderickx (baby), Julia Amadeo (Gastrointestinal tract), Julie McMurry (breastfeeding) and Maxim Kulikov (Gut microbiota).

Despite continuous improvements in preterm infant care, optimal feeding for individual infants is challenging. One of the challenges is the differential composition of human milk associated with preterm delivery[216] (Fig. 2.1). Besides that, the specific nutritional needs of preterm infants are challenging to meet[46]. Another difficulty to achieve optimal feeding regimens is underdevelopment of the gastrointestinal tract that hinders motility and nutrient absorption, factors that might lead to abdominal distension, vomiting and gastric retention[46]. Lastly, underdevelopment of the immune system could trigger exacerbated inflammatory responses to antigens, such as those from undigested food or bacterial compounds, which could contribute to the development of necrotizing enterocolitis (NEC)[46]. As a consequence of these challenges, more than half of the hospitalized preterm infants are being discharged with ongoing severe postnatal growth impairment[52].

While meeting nutritional needs is challenging partly due to underdevelopment of the gastrointestinal tract and immune system, there is a gap in knowledge on the involvement of the gut microbiota in meeting nutritional requirements of preterm infants (Fig. 2.1). The gut microbiota has distinct metabolic capacities, making their role in metabolism of dietary components indispensable to the host. In preterm infants, variation in gut microbiota composition is introduced due to a unique set of environmental conditions, including the hospital environment of the neonatal intensive care unit (NICU) and its associated common clinical practices and feeding regimens. This variation in microbiota composition could interfere directly and indirectly with energy harvest and storage and thereby with weight gain of the preterm infant[52,215,217].

In this review we hypothesize that variation in gut microbiota composition could have serious consequences on growth and development in preterm infants by differential digestion and absorption of human milk. We will support this hypothesis by describing the preterm gut microbiota composition and unique environmental conditions contributing to this; and by describing the interaction between human milk and the gut microbiota, gastrointestinal tract and immune system.

## A unique set of conditions shapes the gut microbiota of preterm infants

In early life, the gut microbiota of a full-term, vaginally delivered and exclusively breastfed infant is considered the golden standard for a healthy infant microbiota[119]. Generally, the intestine of these infants is colonized with facultative anaerobic bacteria during and shortly after birth due to the presence of low amounts of oxygen in this environment[124]. These facultative anaerobic bacteria belong to genera *Enterobacter*, *Enterococcus*, *Staphylococcus* and *Streptococcus*[125]. As facultative anaerobic bacteria thrive on residual oxygen in the infant intestine, the resulting lowered redox potential allows obligate anaerobic bacteria to grow[124]. *Bifidobacterium*, *Bacteroides* and *Clostridium* proliferate and become the predominant genera associated with early life[128]. Further gut microbiota development is driven by host and environmental factors, such as antibiotic treatment, delivery mode, diet and gestational age[130]. Gestational age is among the strongest influencers of gut microbiota development[133,218]. In comparison to full-term infants, the gut

microbiota of preterm infants is characterized by delayed colonization and by limited microbial diversity[135]. In addition, levels of commensal, obligate anaerobic bacteria are generally decreased, while levels of potential pathogenic and facultative anaerobic bacteria are increased[125,138–140] (Fig. 2.2). Comparison of the gut microbiota composition of preterm and full-term infants showed that *Enterobacter*, *Enterococcus*, *Escherichia* and *Klebsiella* were predominantly present in preterm infants and not so much in full-term infants[141,142].

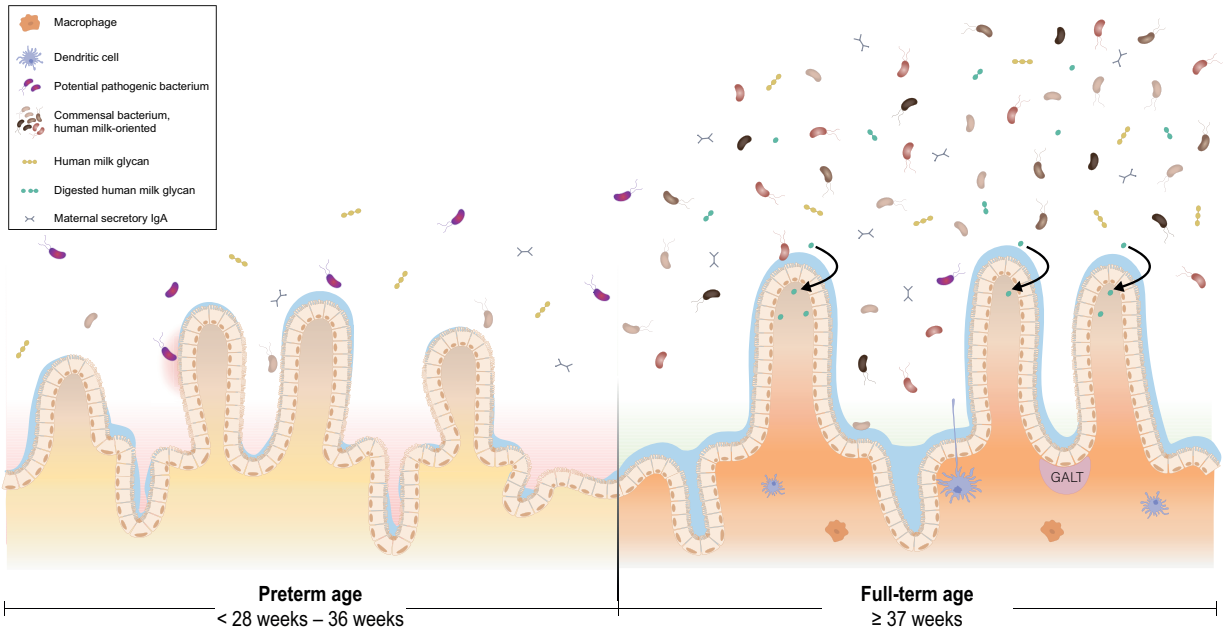


**Figure 2.2** The preterm and full-term situation of the intestine in early life. In the intestine of infants, maturation of the gut microbiota, gastrointestinal tract and immune system occur at the same time. In the preterm situation, the gut microbiota is low in abundance and in diversity due to the unique set of environmental conditions the infant is exposed to. In the full-term situation, the gut microbiota is higher in abundance and diversity and more oriented toward human milk digestion. The gastrointestinal tract is more mature in full-term infants compared to preterm infants with regard to enzyme production and activity, nutrient absorption and intestinal motility. Lastly, the preterm situation is characterized by a pro-inflammatory state partly due to a discrepancy in crosstalk between the gut microbiota and immune system, while in the full-term situation there is oral tolerance.

Not only gestational age shapes gut microbiota composition of preterm infants, but an additional unique set of environmental conditions, including the hospital environment, common clinical practices in neonatal care and feeding regimens further contributes to abnormal gut microbiota development[52,219].

## The hospital environment converges differences in microbiota composition of preterm infants

Environmental conditions are acknowledged for having great influence on bacterial colonization of the intestine[130]. Most preterm infants are exposed to a restricted hospital environment during the first postnatal weeks of life, in which the length of hospital stay is strongly associated with gestational age and bodyweight at birth[220,221]. Not surprisingly, inter-

individual differences in microbiota composition of hospitalized very low birth weight (VLBW) infants become smaller with increasing stay[141,222]. More specifically, the microbiota of hospitalized VLBW infants converges toward a core microbiota mainly composed of bacterial families *Enterobacteriaceae* (genera *Klebsiella* and *Escherichia* in particular) and *Enterococcaceae*[222,223]. The NICU-associated core microbiota is very different from healthy full-term infants, which is commonly composed of *Bifidobacterium*, *Bacteroides* and *Clostridium* species in early life[128]. In addition to decreased differences in microbiota composition between infants within one care unit, variations in infant microbiota composition and succession between different hospitals have been observed, further supporting the influence of the hospital environment on the microbiota composition[224]. A NICU-specific microbiota composition might be explained by the hospital environment acting as reservoir for microbes, selected by lavish antibiotic use, that subsequently colonize the infant intestine[145]. Another explanation for a NICU-specific microbiota is transmission of bacteria between patients within one care unit and between patients and caregivers[225–227]. Knowledge on the role of the hospital environment on the gut microbiota composition is particularly relevant in preventing colonization with potential pathogenic bacteria, such as *Enterobacter* species that cause outbreaks of nosocomial infections within NICUs[226]. Among prevalent nosocomial infections are NEC and sepsis, these are both infections in which the gut microbiota has been implicated[148–150].

### Caesarean delivery enriches the gut microbiota for skin and environmental microbes

The mode of delivery is considered the first and foremost determinant that affects early life microbiota composition[121,228]. The maternal fecal and vaginal microbiota serve as inoculum for the infant's gastrointestinal tract during passage through the birth canal[229]. As such, the gut microbiota of vaginally delivered infants resembles the maternal fecal and vaginal microbiota, with a dominance of genera *Lactobacillus*, *Prevotella* and *Sneathia*[121,123]. In contrast, the microbiota of infants born by caesarean (C)-section is dominated by common skin and environmental microbes, including the genera *Staphylococcus*, *Propionibacterium* and *Corynebacterium*[121,123]. Changes in microbial diversity and colonization with specific taxa have been associated with C-section during the first three postnatal months[120]. Although microbiota composition of infants born by natural birth or C-section gradually becomes similar, differences in abundance and diversity of specific bacterial taxa can remain apparent until 12–24 months of age[122,230].

More frequently than full-term infants, preterm infants are born by C-section[137], thereby contributing significantly to perturbations of their gut microbiota. These perturbations may have health consequences on both short term and long term[231]. On short term, perturbations of the gut microbiota, as a result of caesarean delivery, may affect developing mucosal and systemic immune functions[232,233]. Together with limited diversity and pathogen dominance, this makes preterm infants prone to nosocomial infections, such as NEC and sepsis[148,234,235]. Long-term consequences, like asthma, allergies and obesity, are a result of a discrepancy between the simultaneously developing gut microbiota and immune system. Commensal bacteria are responsible for stimulating development of the immune system and for educating the immune system which

antigens it should respond to or should tolerate[236]. Normally, immune responses toward orally administered antigens, including commensal bacteria, are not triggered, a phenomenon known as "oral tolerance". Abnormal microbiota development in preterm infants could have long-lasting changes in the way the immune system was programmed, resulting in a "skewed" tolerance that plays a role in diseases, such as asthma, allergies and obesity[32]. Indeed, these diseases have been related to the changes in microbiota composition upon caesarean delivery[32].

In attempt to alleviate changes in microbiota composition associated with C-section, pioneer pilot studies transferred vaginal bacteria from mothers to full-term, caesarean-delivered infants[237]. This vaginal microbial transfer, or "vaginal seeding", partially restored the infant's intestinal, oral and skin microbiota to become more similar to the microbiota of vaginally delivered infants[237]. Albeit of great potential to beneficially alter the gut microbiota, vaginal seeding has not yet been performed in preterm infants. There is a great need to further assess the ratio between the benefit and risk of vaginal seeding in infants[238]. At the moment, there is a negative advice for extending this practice, because not enough evidence currently exists about the proposed long-term benefits outweighing the costs and potential risks[238].



### Antibiotic treatment perturbs gut microbiota development

Antibiotic treatment is one of the most common practices in NICUs for preventing and treating infections and sepsis[144]. Prenatal and perinatal antibiotic treatment of the mother has been associated with abnormal gut microbiota establishment in the preterm infant[239,240]. In addition, broad-spectrum antibiotics, such as amoxicillin, ceftazidime, erythromycin and vancomycin are often administered from birth onwards[144]. While antibiotics decrease mortality and morbidity rates on the one hand, they disrupt gut microbiota development on the other hand[241]. Such disruptions are characterized by: (1) decreased bacterial diversity[242,243]; (2) delayed *Bifidobacterium* spp. colonization[144]; and (3) increased presence of antibiotic resistance genes or abundance of multidrug resistant members of *Klebsiella*, *Escherichia*, *Enterobacter* and/or *Enterococcus* genera[122,144,239,242–245]. Not only administration of antibiotics, but also the duration of the treatment has an effect on the gut microbiota[144,242,243]. For example, microbial diversity decreases significantly with increasing duration of antibiotic treatment in preterm infants[242,243]. In addition, the time to recover from low *Bifidobacterium* spp. abundance prolongs in preterm infants receiving long antibiotic treatment (≥ 5 days) compared to preterm infants receiving short treatment (≤ 3 days)[144]. The influence of antibiotics is sustained for at least two months after termination of treatment[246].

The disturbance of the gut microbiota development by antibiotic administration may influence crosstalk with the immune system. As such, sustained alterations in gut microbiota composition could have long-lasting consequences for health. In fact, pre- and postnatal antibiotic use increases the risk of disease later in life, such as asthma and other allergic diseases[247–251]. Also, other regularly prescribed medication in neonatal healthcare, like gastric acid suppressive medication, has been associated with allergic disease in early childhood, possibly by causing intestinal dysbiosis[252].

## Respiratory support shifts the ratio of facultative to obligate anaerobic bacteria in the intestine

Respiratory support has recently been shown to drive differences in microbiota development between extremely and very preterm infants[137]. Prolonged duration of respiratory support in preterm infants was associated with predominance of fecal aerobic and facultative anaerobic bacteria[151]. The presence of aerobic and facultative anaerobic bacteria suggests that respiratory support in the form of positive airway pressure may introduce oxygen in the otherwise anoxic gastrointestinal tract[137,139,151]. As a result of an immature gastrointestinal tract, oxygenation of the gastrointestinal tract could also occur through a permeable intestinal epithelium[151]. This oxygenation could impede passage and survival of obligate anaerobic bacteria, allowing aerobic and facultative anaerobic bacteria to thrive[137].

With a shift in the ratio of facultative to obligate anaerobic bacteria, defense against pathogenic bacteria may be impaired. The most relevant nosocomial infectious agents for preterm infants are among facultative anaerobic bacteria[253]. Obligate anaerobic bacteria prevent bacterial translocation by strengthening the intestinal mucosal barrier, adhering to the intestinal mucosa and impeding pathogen invasion[254]. As such, absence or reduction of obligate anaerobic bacteria in the intestine increases the risk of facultative anaerobic bacteria crossing the intestinal barrier[254]. Another effect that accompanies a shift in the ratio of facultative to obligate anaerobic bacteria, is that metabolism may become aerobic in specific niches of the intestine[255]. Overall, this could result in aerobic degradation of human milk or infant formula instead of anaerobic fermentation, which presumably affects production of energy, nutrients and bioactive compounds[255].

## Glycosylated compounds in human milk are affected by preterm delivery

Human milk is the preferred source of nutrition for preterm infants because of its immunological and nutritional benefits. Besides that, mother's own milk contains prebiotic and probiotic components and thereby has the ability to shape the infant's microbiota[34,256]. In absence of mother's milk, preterm infants receive pasteurized donor human milk as alternative[257]. Recently, also pasteurized donor human milk has been shown to shape the microbiota by favoring a gut microbiota composition more similar to breastfed infants compared to formula-fed infants[257]. Yet, more research is needed to investigate the impact of pasteurized donor human milk on the preterm infant's gut microbiota composition and its potential biological implications.

In mother's own milk, human milk oligosaccharides (HMOs) are prebiotic components belonging to a group of glycosylated compounds in human milk called "glycans". They comprise a collection of structurally complex sugars that display an array of α-linkages and β-linkages[40,258]. Particularly *Bifidobacterium* species, but also some *Bacteroides* species, have genes encoding for enzymes required for HMO digestion[68,69]. The milk of mothers who deliver preterm is much more variable in HMO composition and percentage of fucosylated HMOs compared to mothers delivering at term[259]. Bacteria thriving on selective HMOs will be affected by this higher variation in fucosylated HMOs, which is supported by findings showing that colonization by *Bifidobacterium breve* in the preterm infant's intestine was influenced by HMO fucosylation[260].

In addition, fucosylated HMOs prevent intestinal bacterial adhesion to epithelial surfaces and can have an impact on the gut microbiota composition as such[259,261].

Digestion of HMOs results in production of short-chain fatty acids (SCFAs) that not only serve as energy source for the infant, but also lower luminal pH that subsequently inhibits potential pathogens from colonizing[78,262]. Like HMOs, SCFAs are thus involved in managing gut microbiota composition. In preterm infants it has been shown that the total fecal SCFA concentrations increased with gestational or postnatal age, regardless of diet[263,264]. However, it remains unknown if lower fecal SCFA concentrations in preterm infants is due to lower bacterial production, due to higher uptake by epithelial cells or both[263].



Besides prebiotic components, human milk has its own (probiotic) microbiota that is mainly composed of bacteria associated with the skin and the intestine, like the genera *Bifidobacterium*, *Staphylococcus*, *Streptococcus* and *Pseudomonas*[265–267]. Many other bacterial genera, such as *Bacteroides*, *Lactobacillus* and *Ruminococcus* have been reported in human milk[166,265,268,269]. Methodologic differences in human milk collection, DNA extraction, amplification, sequencing and bioinformatics may have contributed to the discrepancy in reported human milk microbiota composition[267]. So far, only few studies have investigated the effect of preterm birth on the human milk microbiota, while many more studies have investigated the effect of preterm birth on nutrient composition of human milk[270]. The bacterial composition of preterm vs. full-term human milk has been reported to be comparable[269,271]. The colostrum of mothers who delivered preterm contained the genera *Staphylococcus*, *Streptococcus* and *Lactobacillus*, while in more mature milk of the same mothers the genera *Enterococcus* and *Enterobacter* were additionally found[271]. Besides changes in composition, bacteria are less abundant in preterm human milk[271]. The enteromammary pathway involves translocation of bacteria by intestinal monocytes from the intestine to mesenteric lymph nodes and mammary glands, and occurs solely in the last weeks before term delivery[272,273]. In preterm birth, this pathway is not functional or less active, which results in a reduced absolute abundance of bacteria in human milk. In addition, mothers who deliver preterm may already receive antibiotics during delivery, which could impact bacterial counts in the mammary glands[274]. Still, more research is needed to assess the impact of preterm birth on the human milk-associated microbiota composition and absolute abundance of bacteria.

## Prematurity and diet interact with maturation of the immune system

While at term birth both the innate and adaptive immune system are not fully functional, they are competent to handle infections and to respond to immunization[78]. Together with microbiota development, the immune system matures in an age-dependent manner from a Th2-biased immune response toward a balanced Th1/Th2 immune response[78]. The complete process of immune system maturation and its interaction with the gut microbiota is beyond the scope of this review but is described extensively for the first 1000 days of life by Wopereis et al. (2014)[67]. In short, the gut-associated lymphoid tissue (GALT) is the primary site where the immune system interacts with environmental antigens and commensal bacteria[67] (Fig. 2.2). These commensal

bacteria and their products interact with the host via, for example, Pathogen Recognition Receptors (PRRs) that specifically recognize Microbial Associated Molecular Patterns (MAMPs) or by signaling through G-protein-coupled receptors, such as GPR43[67].

Breastfeeding plays a crucial role in immune system development[24]. Besides nutrients, it continuously provides immunological components that promote immune system development[24,275]. Among them are secretory immunoglobulin A (SIgA); leukocytes—primarily macrophages and neutrophils—that actively engulf microbial pathogens by phagocytosis; and lymphocytes[24,67,275]. In addition to these components, HMOs interact with the immune system by modulating cytokine production of lymphocytes, subsequently influencing the balance between Th1 and Th2 cells[68]. It also reduces selectin-mediated cell-cell interactions and decreases leukocyte rolling on activated endothelial cells[68]. This could lead to reduced mucosal leukocyte infiltration and activation[68]. Human milk additionally contains non-specific factors that have antimicrobial and antipathogenic effects. These non-specific factors include enzymes and proteins that inhibit growth of many bacterial species by disrupting the proteoglycan layer; and lactotransferrin, which limits bacterial growth by removing essential iron[24]. Other components contribute to passive protection in the gastrointestinal tract by preventing adherence of pathogens to the mucosa[24]. A meta-analysis investigating the health benefits of breastfeeding has shown a lower risk of gastrointestinal infection and other diseases in breastfed infants[24].

Preterm birth has major consequences on immune system development. One consequence of preterm birth is a change in the immunological composition of human milk. For example, milk of mothers who delivered before 32 weeks of gestation contained more SIgA in comparison to mothers who delivered term[276]. Higher levels of SIgA in preterm human milk offer greater protection against infections, implicating compensation for immaturity of the immune system of preterm infants[276]. In addition to changes in immunological human milk composition, immaturity of the immune system is more pronounced in preterm infants compared to full-term infants. According to Melville and Moss (2013) this immaturity is characterized by: "a smaller pool of monocytes and neutrophils, impaired ability of these cells to kill pathogens and lower production of cytokines which limits T cell activation and reduces the ability to fight bacteria and detect viruses in cells, compared to full-term infants"[33]. The immune system of preterm infants also plays a role in NEC, a disease characterized by an exacerbated inflammatory response of the intestines[30,78]. In full-term infants, the response of the innate immune system is biased toward a Th2 phenotype and against Th1-cell-polarizing cytokines[32]. This bias allows for microbial homing and colonization, but also makes the infant susceptible to opportunistic pathogens shortly after birth[32]. After multiple pathogenic encounters, a time- and age-dependent shift takes place from Th2 toward a balanced Th1/Th2 response[32]. A state of disrupted gut microbiota composition in preterm infants promotes a strong Th1 bias, pushing the immune system to be pro-inflammatory under the influence of IL-12 and IFN-γ secretion, supposedly contributing to NEC[32] (Fig 2.2). Another mechanism contributing to gastrointestinal inflammation is disruption of the liver-bile acid-microbiota axis upon alterations in gut microbiota composition[277].

## Prematurity and diet interact with maturation of the gastrointestinal tract

Structural and functional maturation of the gastrointestinal tract are required for efficient digestion and absorption of nutrients from milk feedings. Development of the gastrointestinal tract during gestation is generally subdivided in processes involved in cytodifferentation, digestion, absorption and motility[17,278]. Anatomically, all parts of the gastrointestinal tract are developed within the first 12 weeks of gestation, while it takes up to 20 weeks for the villi and crypts to develop[17]. Many structural and functional properties of the gastrointestinal tract develop within 24 weeks of gestation. Digestive enzymes (e.g., lactase, sucrase, maltase and peptidase) can be detected from 8 weeks of gestation, but some enzymes are far below their full potential concentration and activity at that stage[18]. Lactase activity, important for the degradation of lactose from milk, increases progressively from 24 weeks onwards and reaches maximum activity at 40 weeks of gestation[17]. Sucking, swallowing, gastric emptying and intestinal motility develop during the third trimester and effective coordination of these processes is reached at term. Although not yet reaching its full potential, the gastrointestinal tract of infants born at term is ready to receive and process milk feedings. Further maturation of gastrointestinal tract functioning is stimulated by milk feeding itself. This particularly accounts for lactase activity, which rapidly increases from the first milk feeding onwards[17].



In case of preterm birth, the infant particularly suffers from immaturity related to digestion and motility, since these develop during the third trimester (Fig. 2.2). The combination of decreased activity of digestive enzymes, immature motility functions, limited absorptive capacity and increased protein demands in preterm infants, raises a major challenge in meeting their nutritional needs[46]. Preterm infants, particularly those born before 32 weeks of gestation, are prone to be intolerant to enteral feeding and therefore nutrients are provided intravenously via parenteral feeding for the first 2–4 weeks. Withholding enteral feeding is not favorable and has been associated with reduced gastrointestinal function and structural integrity. These include a decrease in hormone activity, intestinal mucosa maturation, digestive enzyme activity, nutrient absorption and motility maturation; and an increase in intestinal permeability and bacterial translocation[46,279,280]. To stimulate functional maturation of the gastrointestinal tract of preterm infants, minimal enteral nutrition has been practiced widely in NICUs[281]. During minimal enteral nutrition, small volumes of human milk or formula are given to the infant without nutritive intent, but with the aim to prevent mucosal atrophy and to stimulate intestinal motility in order to reach full enteral feeding as quick as possible. Human milk in particular can aid in intestinal maturation, as HMOs in human milk directly affect intestinal epithelial cells and modulate their gene expression, leading to changes in cell surface glycans and other cell responses[68]. Furthermore, the presence of dietary components in the intestinal lumen is essential for establishing and shaping of the gut microbiota. In turn, bacteria residing in 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[282]. In addition, the gut microbiota is involved in the degradation of some host-generated compounds, including bile acids and mucus[283]. Besides its role in digestion, the gut microbiota plays an essential role in structural development of the gastrointestinal tract.

Germ-free mice, among others, have smaller intestinal surface area, decreased epithelial cell turnover and underdeveloped villi and crypts compared to specific pathogen-free and wild-type mice[76]. The essential role of gut microbiota in structural development of the gastrointestinal tract has been further supported in a study with preterm infant's gut microbiota, showing that gut microbiota, body weight and intestinal epithelial development are closely related[77]. Microbiota transplantation from preterm infants with normal weight gain to germ-free mice increased villus height, crypt depth, cell proliferation and numbers of goblet and Paneth cells when compared to mice inoculated with microbiota from preterm infants with poor weight gain. In addition, tight junctions were enhanced in germ-free mice colonized with microbiota from normal-weight-gain infants[77]. Although findings in mice cannot be extrapolated to humans directly, it demonstrates that structural development of the gastrointestinal tract is affected by the microbiota. Hence, abnormal microbial colonization of the intestine in preterm infants affects the gastrointestinal tract in terms of the intestinal barrier and nutrient absorption.

## The preterm gut microbiota challenges nutritional neonatal care

As described throughout this review, prematurity and nutrition affect maturation of the gut microbiota, gastrointestinal tract and immune system. These processes are rather intertwined, and consequences of prematurity affect the infant on a systemic level in terms of growth and development.

Preterm infants require adequate feeding and subsequent digestion and absorption of nutrients. However, caretakers have to overcome nutritional challenges in feeding preterm infants to reach optimal growth and development. The first challenge is the high nutritional requirement of preterm infants in particular for protein[39,51]. Even though protein content is higher in preterm human milk, it still is not sufficient to meet the preterm infant's high nutrient requirements[39,40,216]. Therefore, fortification of preterm human milk with proteins, minerals and vitamins is needed to achieve adequate growth and development[39,284].

Another challenge that caretakers need to overcome in preterm infant feeding is the immature gastrointestinal tract. As a result of ongoing gastrointestinal development, carbohydrate, protein and lipid digestion does not occur to the full extent in preterm infants[51] (Fig. 2.2). In case of carbohydrate digestion, most importantly, lactase activity is low in preterm infants; its activity increases from 24 to 40 weeks of gestation[51]. Being built on a basic lactose core, low lactase activity could affect HMO digestion[40,68]. Also, mechanisms for protein digestion are underdeveloped in preterm infants. While activity of most milk-derived proteases is not affected by gestational age, limited gastric acid secretion and low enterokinase activity impedes protein hydrolysis[51,285,286]. Consequently, preterm infants digest proteins to a lesser extent than full-term infants[287,288]. Lastly, lipid digestion in VLBW infants is affected by lower duodenal concentrations of bile acids that are critical for efficient fat digestion and absorption[51]. Lower duodenal concentrations of bile acids are a result of lower synthesis and ileal reabsorption of bile[51]. After digestion of carbohydrates, proteins and lipids, subsequent nutrient absorption could additionally be lower. The intestine and thus the absorptive surface is still elongating in the third trimester[17]. In addition, hampered motility could lead to retention of undigested

content in the intestinal lumen for a considerable longer time period, which may initiate an inflammation cascade[17].

Practical hurdles with regard to nutrient requirements and gastrointestinal prematurity are relatively conspicuous. However, we hypothesize that prematurity of the gut microbiota may be an additional inconspicuous challenge in preterm nutritional care (Fig. 2.2). In a healthy state, the gut microbiota contributes to growth and development in two ways. First, the gut microbiota has a distinct, yet complementary, metabolic capacity to human gastrointestinal enzymes. As a result of bacterial digestion, otherwise unavailable energy and nutrients are provided to the host[289]. Second, the gut microbiota is involved in host body weight management[217,290–292]. The gut microbiota manages body weight by being involved in production of metabolites and in the harvest, storage and expenditure of energy from food components by affecting the intrinsic metabolic machinery of host cells[289,293]. The most convincing involvement of gut microbiota in body weight management is the induction of an impaired growth phenotype upon microbiota transplantation from undernourished children to germ-free mice[292]. While germ-free mice receiving microbiota from undernourished children showed growth impairment, their littermates receiving microbiota from healthy children showed a healthy phenotype[292]. Moreover, the impaired growth phenotype could subsequently be ameliorated by introducing two invasive bacterial species, *Ruminococcus gnavus* and *Clostridium symbiosum*[292].



While several studies suggest the involvement of the gut microbiota in body weight and growth management in adults and children, little is known about this role in preterm infants[294]. Literature on this topic is scarce and thereby represents a major gap in this field of research. Given that preterm birth impedes "normal" gut microbiota development, the role of the preterm gut microbiota in altered digestion of milk feedings and in intestinal maturation—and thereby affecting postnatal growth and development—becomes increasingly likely. Even though research is scarce and mechanisms remain unknown, some studies in preterm infants suggest an association between the gut microbiota, growth and development in early life[215]. Grier et al. (2017) identified microbiota phases in preterm infants that were each characterized by distinct metabolic functions[52]. Significant associations were found between nutrition, microbiota phase and preterm infant growth[52]. Also, Arboleya et al. (2017) associated specific bacterial families and genera with weight gain[215]. Especially *Enterobacteriaceae* and *Streptococcus* levels at two days of age and *Bacteroides*-group levels at 10 days of age were associated with weight gain at one month of age[215]. In addition to that, some bacterial genera—including *Staphylococcus* and *Enterococcus*—were negatively associated with weight gain, while *Weissella* spp. were positively associated with weight gain in preterm infants[215]. These genera, or specific species or strains within these genera, may affect infant food digestion capacity and subsequent energy harvest[217,289,291]. Possible mechanisms of these taxa could be differential abundance of genes involved in metabolism of carbohydrates, proteins and/or lipids[52]. In fact, differences have been reported in microbial proteins involved in metabolic activity between preterm infants of varying gestational and postnatal age[137,295]. Most likely, microbial effects on infant growth are strain-specific, each having distinct genes encoding for proteins involved in metabolism[255,296]. Besides specific taxa, also microbial diversity appears to play a role in achieving digestive tolerance and weight gain[125].

Based on these clues in current research, it becomes increasingly likely that prematurity of the gut microbiota may be an additional clinical challenge in achieving optimal feeding. The preterm gut microbiota may have a differential metabolic capacity compared to full-term infants due to variation in the abundance of genes that are involved in metabolism of carbohydrates, proteins and/or lipids. By having a differential food digestion capacity and energy harvest, the preterm gut microbiota could be involved in preterm infant weight gain and development as such. We expect that the variation in gut microbiota of preterm infants will be mainly emphasized in digestion of glycosylated carbohydrates (HMOs) and proteins (glycoproteins) from human milk, since intestinal bacteria have genes encoding for enzymes that digest these components[69]. However, we should not exclude the possibility of changes in the type of bioactive compounds, or in the activity of these compounds, considering that human milk contains many bioactive compounds and the gut microbiota is involved in their production[228]. Changes in bioactivity of degraded compounds could subsequently influence the antimicrobial properties or crosstalk with the intestinal epithelium and immune system that manage inflammatory responses. However, to date, it remains undiscovered to what extent HMO and glycoprotein digestion takes place in the preterm intestine and how the intact or digested compounds contribute to the nutritional value and the health benefits for preterm infants.

## Conclusion

The preterm infant is predisposed to health complications, both on short and long term, due to underdevelopment of the gut microbiota, gastrointestinal tract and immune system. Specifically, the gut microbiota of preterm infants is shaped by a unique set of environmental conditions, which we hypothesized as inconspicuous clinical challenge in nutritional neonatal care. Current research provides clues that prematurity affects infant growth and development. Exploration of the metabolic capacity of the preterm gut microbiota, with HMO-degrading *Bifidobacterium* spp. and *Bacteroides* spp. in particular, would contribute to a better understanding of production of energy and metabolites that become available to the preterm infant and impact intestinal maturation and overall host metabolism. This knowledge could complement current nutritional neonatal care and benefit infant growth, development and health in the future. As such, the preterm infant gut microbiota remains a research priority, in which a reference for a healthy, preterm microbiota composition and its interactions with the gastrointestinal tract and immune system need to be incorporated to thoroughly understand mechanisms by which the gut microbiota is involved in preterm infant growth, development and health.

# CHAPTER 3

## Maturation of the preterm gastrointestinal tract can be defined by host and microbial markers for digestion and barrier defense

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## Abstract

Functionality of the gastrointestinal tract is essential for growth and development of newborns. Preterm infants have an immature gastrointestinal tract, which is a major challenge in neonatal care. This study aims to improve the understanding of gastrointestinal functionality and maturation during the early life of preterm infants by means of gastrointestinal enzyme activity assays and metaproteomics. In this single-center, observational study, preterm infants born between 24 and 33 weeks ($n$ = 40) and full-term infants born between 37 and 41 weeks ($n$ = 3), who were admitted to Isala Women and Children's Hospital (Zwolle, The Netherlands), were studied. Enzyme activity analyses identified active proteases in gastric aspirates of preterm infants. Metaproteomics revealed human milk, digestive and immunological proteins in gastric aspirates of preterm infants and in feces of preterm and full-term infants. The fecal proteome of preterm infants was deprived of gastrointestinal barrier-related proteins during the first six postnatal weeks compared to full-term infants. In preterm infants, bacterial oxidative stress proteins were increased compared to full-term infants and higher birth weight correlated to higher relative abundance of bifidobacterial proteins in the third until sixth postnatal weeks. Our findings indicate that gastrointestinal and beneficial microbial proteins involved in gastrointestinal maturity are associated with gestational and postnatal age.

## Introduction

Preterm birth interrupts the natural, intrauterine development of the gastrointestinal tract, immune system and microbiota that occurs during the third trimester[11,20]. The gastrointestinal tract continues to develop after birth with the environment deviating from mother's womb. Early exposure to a deviating environment affects the infant and maturation processes on a systemic level.

Strict feeding regimens are implemented to orchestrate optimal maturation of the gastrointestinal tract, which is crucial for infant growth, development and health. Human milk is the first choice for both full-term and preterm infants as it has nutritious, immunomodulatory and microbial benefits[297]. Whether nutritional components of human milk can be absorbed and digested largely depends on gastrointestinal maturity of the infant[17]. Some digestive enzymes and gastrointestinal motility functions develop during later stages of gestation, leading to suboptimal functioning upon preterm birth[17,298]. This affects proteolysis of major milk proteins in preterm infants for example, even though it still occurs[288,299]. Incomplete breakdown of proteins can be beneficial or harmful, depending on which proteins remain intact[284]. Additionally, underdeveloped gastrointestinal motility may lead to nutrient retention that could initiate a sequence of events with translocation of microbes or their toxic products as a consequence[46].



Gastrointestinal maturity additionally plays an important role in gastrointestinal barrier functioning, which is crucial for maintaining gastrointestinal homeostasis and infant health[300]. Preterm infants have a leaky gut in the first weeks of life as the intestinal barrier develops[27–29]. Along with gestational age, multiple factors affect intestinal permeability in preterm infants, including infection, inflammation, feeding type and antibiotic exposure[301–303].

Host-microbe interactions occur at the intestinal barrier and impact physiological development of the gastrointestinal tract, the immune system and human milk digestion[11,78]. Preterm infants are particularly susceptible to sepsis and necrotizing enterocolitis (NEC) due to immaturity of the intestinal epithelial barrier, the immune system as well as their microbiota development[29,46,304].

Metaproteomics offers great potential to functionally characterize organisms and is increasingly used to supplement compositional profiling of the human microbiome[295,305–308]. This study aims to improve the understanding of gastrointestinal functionality and maturation during the early life of preterm infants by means of gastrointestinal enzyme activity assays and metaproteomics. Here, we add new enzyme activity analyses to previously acquired metaproteomics data[137]. Moreover, gastric aspirates and full-term infants were newly added to the metaproteomics analyses, which focus on human proteins and their implications for interactions with previous findings on the microbiome[137].

## Materials and methods

### Ethics declaration

The board of the Medical Ethics Committee of Isala Women and Children's Hospital (Zwolle, The Netherlands) 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.

### Study description

The study was part of the EIBER study; a single-center, observational study involving full-term and preterm infants admitted to the neonatal intensive care unit or the pediatric ward of Isala Women and Children's Hospital in Zwolle, The Netherlands. Mothers were encouraged to breastfeed at all times. If needed, infants were supplemented or fed with preterm formula (Nutrilon Nenatal Start, Nutricia, The Netherlands).

As part of the EIBER study, gastric aspirates were obtained on a daily basis during the first 14 days of life in all preterm infants having a nasogastric tube on clinical grounds (Fig. 3.1, gestational age of 24–33 weeks, $n$ = 40 infants). Enteral feeding was started as soon as possible with gradual increments but was supplemented with parenteral nutrition if necessary. Samples of this part were used to perform pH measurements and enzyme activity analyses.

Another part of the EIBER study included gastric aspirate and fecal sample collection immediately after birth and at postnatal weeks one, two, three, four and six. This part included both preterm and full-term infants that were selected based on gestational age (Fig. 3.1). Out of the forty preterm infants, ten preterm infants were selected based on a gestational age < 32 weeks, whereas three full-term infants were selected based on a gestational age between 37 and 41 weeks. The amount of collected gastric aspirate was roughly 1 mL; the minimal amount of collected feces was one scoop. Samples of this part were used to perform metaproteomics and were used previously for 16S rRNA gene amplicon sequencing as well[137].

### Subjects and sample selection

#### *pH and enzyme activity analyses*

From the ten preterm infants ($n$ = 100 samples), as well as thirty additional preterm infants ($n$ = 325–334 samples), aspirates of residual gastric content were collected daily during the first two postnatal weeks (Fig. 3.1). The thirty additional preterm infants were selected if gastric aspirates of minimally eight timepoints were available. At collection, samples were frozen at −20 °C and stored at −80 °C.

#### *Metaproteomics*

Ten preterm infants and three full-term infants from the EIBER study were selected based on gestational age (Fig. 3.1, Table S3.1)[137]. From all these infants, fecal samples were collected

right after birth and at postnatal weeks one, two, three, four and six (*n* = 81). Sixty-four samples derived from preterm infants and 17 from full-term infants. On the same timepoints, gastric aspirates were collected from preterm infants in the first two postnatal weeks (*n* = 35). The metaproteomes of these infants have been generated and described previously, with the main objective to characterize the bacterial part of the preterm infant's fecal metaproteomes[137].

### Enzyme activity analysis

Gastric aspirate samples were thawed on ice, centrifuged (3000 rpm; 4 °C) and the cream layer was removed[309]. pH of the supernatant was determined (*n* = 425 samples) and samples were centrifuged to remove any remaining cream fraction (14,000 rpm; 4 °C)[309]. Total protease (*n* = 433 samples) and pepsin activity (*n* = 434 samples) were determined using the green fluorescence EnzChek Protease Assay Kit (Molecular Probes, Eugene, OR, USA) in duplicate according to manufacturer's instructions. For determining total protease activity, 10mM TRIS buffer (pH 7.8) was used and the standard curve was generated using pancreatin from porcine pancreas (Sigma-Aldrich, Saint Louis, MO, USA)[309]. 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)[309].



### Sample processing for metaproteomic analysis

Extraction of proteins from feces was performed mechanically by repeated bead beating as described previously (*n* = 81 samples)[137,310]. Gastric aspirates were thawed on ice, centrifuged (3000 rpm; 4 °C) and pellet was removed (*n* = 35 samples). pH of the supernatant was determined, and samples were centrifuged to remove debris (max rpm; 4 °C). Fecal and gastric proteins were quantified using Qubit Protein Assay Kit on a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA) and diluted in PBS to obtain a 3 µg/µl and 5 µg/µl concentration, respectively. In gel digestion procedures, database construction, analysis of MS/MS spectra and protein grouping with MaxQuant 1.3.0.5[311] were performed as previously described[137]. In the current study, no additional mapping of initial search results was performed to functionally classify proteins.

### Metaproteomic database construction

The metaproteomic database has been constructed by Zwittink et al.[137]. The in-house database was accommodated to the study group in order to decrease the chance of false identification. Bacterial genera were selected based on their identification in infants by the Human Microbiome Project reference genomes and by 454 pyrosequencing[137]. Selected bacterial genera were retrieved from UniProt and their proteomes were merged with proteomes of human, cow, *Candida* spp. and common contaminants[137]. In total, 87 bacterial species and 438,537 sequences were captured in the in-house database (contents of the in-house generated protein database are presented in Zwittink et al.[137]). Taxonomic classification of MS/MS spectra was performed until genus level as there was high protein sequence homology among species of the same genus[137].

## Data transformation

Eight fecal samples of preterm infants were not collected on correct timepoints and were excluded, resulting in 73 fecal samples ($n$ = 56 preterm samples; $n$ = 17 full-term samples) and 108 samples in total for further analyses. In Perseus version 1.6.2.1[312], Label-Free Quantification (LFQ) intensities were $\log_{10}$-transformed. Resulting NaN values were replaced by the value 4.0, which was lower than minimally observed, and further processed as described before[137]. These pre-processed LFQ intensities were used for analyses. Intensity Based Absolute Quantification (iBAQ) intensities were used to determine relative abundances of iBAQ intensities (riBAQ) like described previously[313].

Growth velocity was calculated based on clinical data according to the exponential model as described by Patel et al.[314]:

$$[1000 \times \ln(W_n/W_1)] / (D_n - D_1)$$

where W is the weight in grams, D is day; 1 indicates the beginning of the time interval and n is the end of the time interval, in days[314]. In all cases, birth weight was selected as $W_1$.

## Statistical analysis

All analyses were performed in R version 3.6.1[315]. In all cases, (adjusted) *P*-values below 0.05 were considered statistically significant.

Redundancy analysis (RDA) was performed with the *vegan* package version 2.5-6[316]. Missing values of explanatory variables were omitted and LFQ intensities were mean centered. Forward and reverse automatic stepwise model selection for constrained ordination was performed using *ordistep* from the *vegan* package. For robustness, *ordiR2step* was also performed. Both methods corresponded in selection of significant terms. Resulting *P*-values were adjusted with *p.adjust* from the *Stats* package[315] using FDR correction.

Volcano plots were generated in Perseus version 1.6.2.1[312]. First, distribution of LFQ values was visually inspected per sample for normality. A two-sided Student's t-test on LFQ intensities of human- and bovine-derived proteins between two groups was performed with an s0 of 1 and a permutation-based FDR with 250 permutations and an FDR of 0.01.

Temporal dynamic plots were generated per protein of interest with the *ggplot2* package version 3.3.0[317]. Default non-parametric LOESS regression was performed with a 95% confidence interval to generate a smooth fitted line. Individual data points were additionally plotted.

Spearman correlations of clinical data to bifidobacterial proteins were performed on riBAQ intensities using the *ggscatter* function of the *ggpubr* package version 0.3.0[318]. Birth weight was not significantly higher in very preterm infants compared to extremely preterm infants based on a one-sided Mann-Whitney U test ($P$ > 0.99) and were therefore grouped for correlation

analyses. Mean riBAQ intensities were calculated per gestational age category and postnatal week. Individual data points as well as a regression line with a 95% confidence interval were plotted. For bacterial oxidative stress proteins, mean riBAQ intensities were calculated per gestational age category and postnatal week. Of each sample, the sum iBAQ intensities of listed oxidative stress proteins were divided by the sum iBAQ intensities of all bacterial proteins (Table S3.6). Comparison between gestational age groups in specific postnatal weeks was performed with Dunn's test.

## Data availability

The mass spectrometry data have been deposited to the ProteomeXchange Consortium[319] via the PRIDE partner repository with dataset identifier PXD005574.

# Results

## Metaproteomic characterization

740,343 MS/MS spectra were recorded of which 89,294 were identified. After omitting samples (Fig. 3.1), metaproteomics generated 11,885 unique peptides and 3181 protein groups in 108 samples. 2317 protein groups remained after protein filtering. Of the identified protein groups, 886 proteins were human- and/or bovine-derived and 1431 proteins were bacterial-derived. On average, 1409 ± 456.6 unique peptides and 206.3 ± 66.5 protein groups were detected in a sample. 199.3 ± 62.8 and 243.8 ± 74.6 protein groups were detected on average in preterm and full-term infants, respectively.

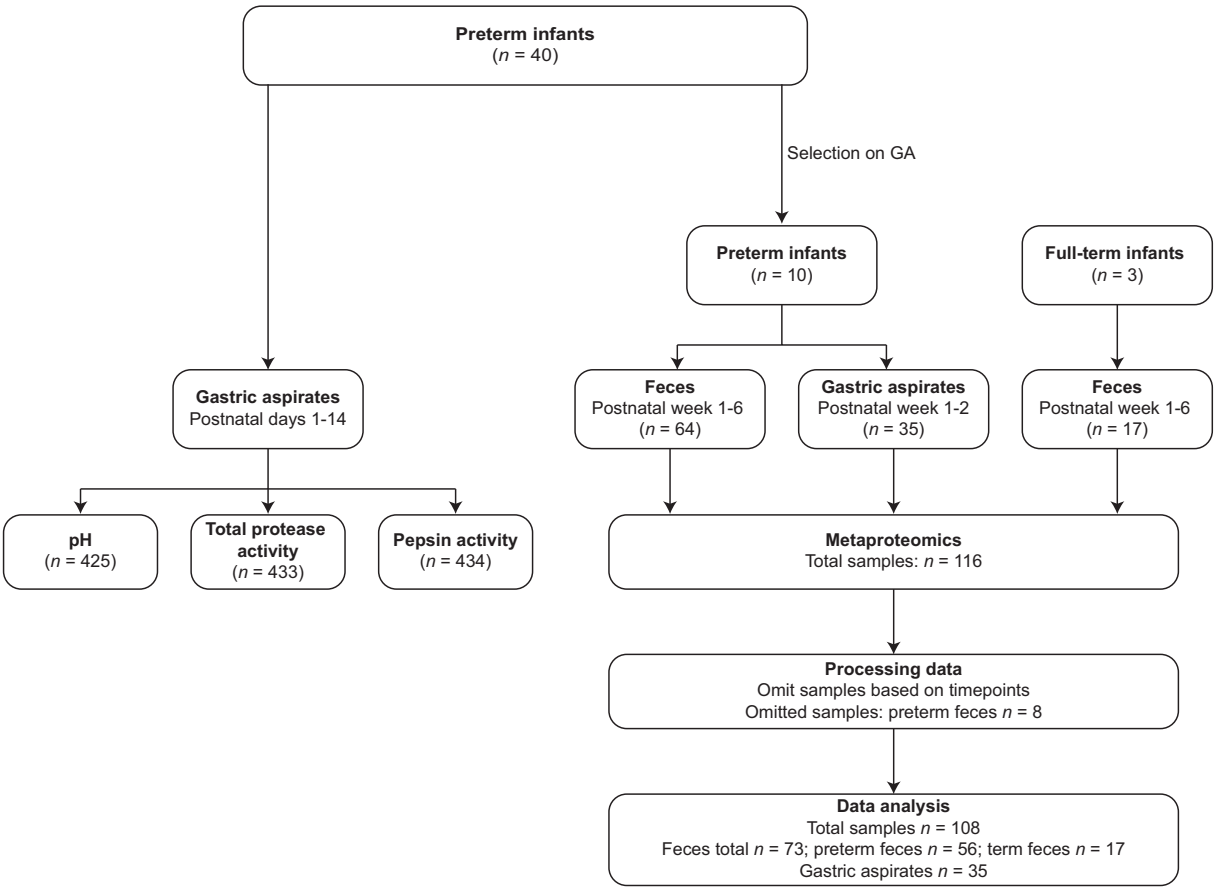


**Figure 3.1** Overview and workflow of this study. Preterm and full-term infants were part the EIBER study. Of forty preterm infants, gastric aspirates were collected during postnatal days 1–14 (left). From ten out of forty preterm infants, feces were additionally collected in postnatal week 1–6 with the exception of week 5; gastric aspirates of the first two postnatal weeks were included for metaproteomics if they were collected on similar timepoints as the fecal samples. Three full-term infants were included as healthy reference. Feces of those infants were collected in postnatal week 1–6 with the exception of week 5. GA: gestational age.

## Gastric proteases and peptidases are present and active in the preterm gastric proteome

Forty preterm infants were part of this study. Of all these infants, gastric aspirates were collected during postnatal days 1–14 (Fig. 3.1). These samples were used for pH measurements, total protease activity and pepsin activity. At some timepoints, samples were unavailable or insufficient in volume to conduct all measurements, resulting in 425 samples for pH measurement, 433 for total protease activity assays and 434 for pepsin activity assays (Fig. 3.1).

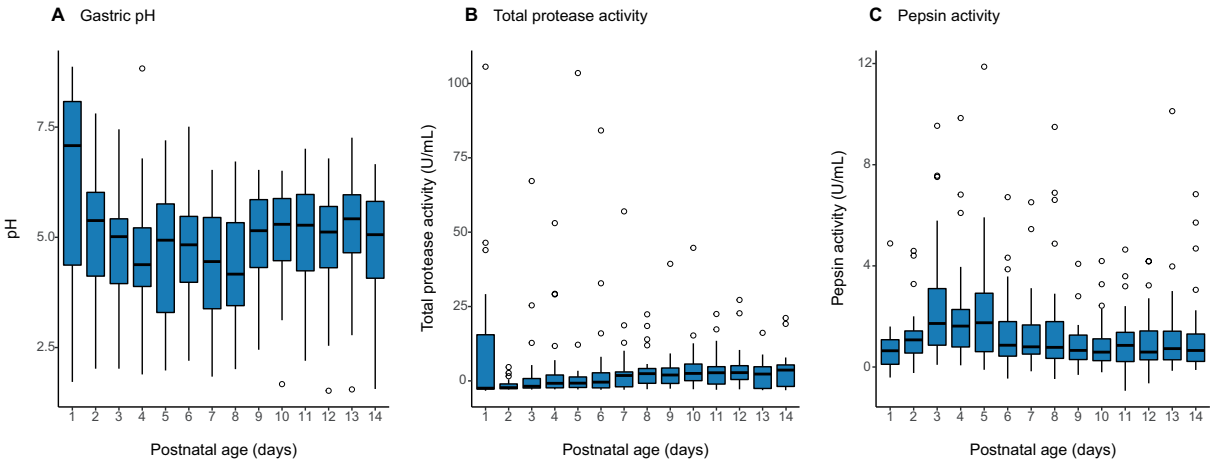


**Figure 3.2** Gastric pH and enzyme activity during the first two postnatal weeks of preterm infants. Dynamics of (A) gastric pH, (B) total protease activity and (C) pepsin activity. Boxplots show the median, 25th and 75th percentiles, and minimal and maximal values with the exception of outliers (circles, lower or higher than 1.5 * inter-quartile range).

Median gastric pH of preterm infants fluctuated between 4.5 and 5.5 over time (Fig. 3.2A). In ten out of forty infants, the gastric pH was exceptionally high (> 8.0 pH) at day of birth but mean gastric pH did not differ significantly from the other infants in the days thereafter ($P$ > 0.99). Intra-individual differences were high, with a mean difference of 4.2 ± 1.3 (SD) between the lowest and highest pH measured during the first two weeks of life. Total protease and pepsin activity showed high variation between and within infants. While median total protease activity was higher in the second than the first postnatal week, pepsin activity remained relatively stable (Fig. 3.2B and C). Being a pH-dependent enzyme, pepsin activity decreased with higher gastric pH and was not affected by postnatal age ($\rho = -0.32$, $P = 1.3 \times 10^{-11}$). Interestingly, pepsin was not detected in the gastric proteome by means of LC-MS/MS. However, other proteases, like trypsin and chymotrypsin-like elastase family members 2A, 3A and 3B could be identified.

## Human and microbial proteins across the gastrointestinal tract

Ten out of the forty preterm infants were selected for metaproteomics based on gestational age (Fig. 3.1, Table S3.1)[137]. Additionally, three full-term infants from the EIBER study were included as reference. From all these infants, fecal samples ($n$ = 81) were collected right after birth and at postnatal weeks one, two, three, four and six. Sixty-four fecal samples derived from

the ten preterm infants and 17 from the three full-term infants. Gastric aspirates collected from the ten preterm infants during the first two postnatal weeks were included for metaproteomics if they were collected on similar timepoints as the fecal samples ($n$ = 35). Eight fecal samples from preterm infants were collected in between the intended timepoints and were therefore omitted before data analysis, leaving a total of 56 fecal samples of preterm infants (Fig. 3.1).

### *Specific human milk proteins resist degradation in the preterm gastrointestinal tract*

In addition to the presence of proteases, milk-derived proteins were present in gastric aspirates and feces of preterm infants throughout the first two postnatal weeks. These included bile-salt activated lipase, lactotransferrin, caseins, alpha-lactalbumin and serum albumin (Table S3.2). In the gastric aspirates of extremely preterm infants in the first two postnatal weeks, more than 30.0% of identified human milk proteins consisted of casein fragments. In feces, only 0.07% and 0.2% of identified human milk proteins were casein fragments in week one and two, respectively. In contrast to extremely preterm infants, the relative abundance of casein fragments in gastric aspirates of very preterm infants was higher with 48.1% and 47.5% in week one and two respectively. No casein fragments were detected in feces. Human milk-derived lactotransferrin and serum albumin were also detected in fecal samples of all preterm and full-term infants, while no bovine-derived proteins were observed (Fig. 3.3 and Fig. S3.1).

### *Birth weight positively correlates to bifidobacterial protein abundance in preterm infants from the third postnatal week onwards*

In full-term infant's feces, relative abundance of bacterial proteins gradually increased from 18% to 34% over the first six weeks, while the abundance of host- and dietary-derived proteins decreased. The ratio bacterial to eukaryote proteins developed more stochastically in preterm infants (Fig. S3.2). The bacterial proteins' abundance in extremely preterm infants was 6% and remained significantly lower than that of full-term infants up till the end of the six weeks ($P$ = 0.04). For very preterm infants, bacterial protein abundance increased to 31%, reaching similar levels as that of full-term infants in week six. Moreover, extremely preterm infants were characterized by a low mean relative abundance of bifidobacterial-derived proteins fluctuating between 6% and 10% throughout the six-week period (Fig. S3.3). In contrast, bifidobacterial-derived proteins reached proportions as high as 41% and 52% in the sixth week of very preterm and full-term infants, respectively.

Weight parameters were correlated to relative abundance of bifidobacterial-derived proteins in preterm infant's feces. For full-term infants, no data on weight gain was available. For preterm infants, higher birth weight was significantly correlated to higher proportions of bifidobacterial proteins from week three onwards ($\rho > 0.75$ and $P < 0.05$) (Fig. S3.4). In contrast, higher *Bifidobacterium* spp. relative abundance did not significantly correlate to growth velocity during the first six postnatal weeks ($P \geq 0.23$) (Fig. S3.5).

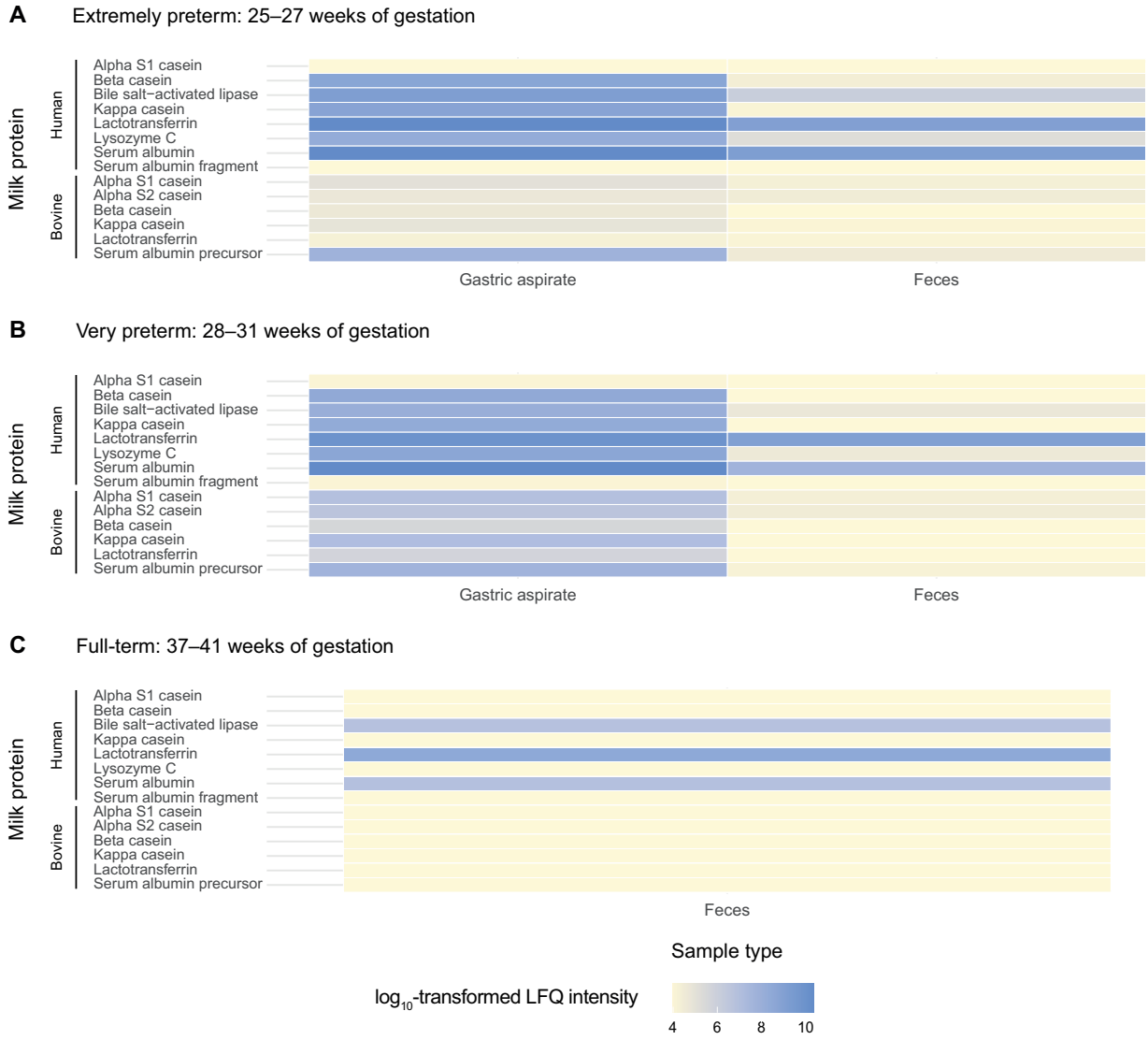


**Figure 3.3** Normalized abundance of milk-derived proteins of human and bovine source in gastric aspirates and feces during the first two postnatal weeks. Milk-derived proteins in gastric aspirates and feces of (A) extremely preterm, (B) very preterm and (C) full-term infants. $Log_{10}$-transformed LFQ values are shown. Coloring is based on abundance from least abundant (yellow) to most abundant (blue).

### *Gastrointestinal barrier-related proteins are less abundant in preterm infant's feces*

Based on redundancy analysis, gestational age was identified as significant driver for differences in the fecal proteome of preterm and full-term infants during the first six postnatal weeks (Fig. 3.4, Table S3.3). Thirteen (out of 804) human-derived proteins' abundances were significantly lower in preterm infants' feces compared to full-term infants' feces during the first six postnatal weeks (Fig. 3.5, Table S3.4). These proteins included gastrointestinal barrier and innate mucosal immune proteins mucin-5AC (MUC5AC), trefoil factor 2 (TFF2), trefoil factor 3 (TFF3) and neutrophil defensin 3 (DEFA3) and proteins involved in lipid metabolism. As such, these proteins were further analyzed longitudinally by temporal dynamic patterns. MUC5AC showed a 2.7-fold change in full-term infants compared to preterm infants (Fig. 3.5) and was detected

in low levels of preterm infants during the first two postnatal weeks (Fig. 3.6A). From the third week onwards, MUC5AC intensity decreased further in preterm infants while it increased in full-term infants. From the third postnatal week onwards, MUC5AC levels were significantly higher in full-term infants compared to that of preterm infants ($P < 0.05$). A similar pattern was observed for TFF2 (Fig. 3.6B) with a 3.6-fold change difference between full-term infants and preterm infants (Fig. 3.5). In contrast to full-term infants, TFF3 was not detected in preterm infants during the first six weeks of life (Fig. 3.6C). TFF3 had a 2.5-fold change in full-term infants compared to preterm infants (Fig. 3.5). DEFA3 was significantly higher in full-term infants compared to preterm infants during the whole period six-week period ($P < 0.05$) (Fig. 6D) and reached 4.5-fold change difference in full-term infants compared to preterm infants (Fig. 3.5).

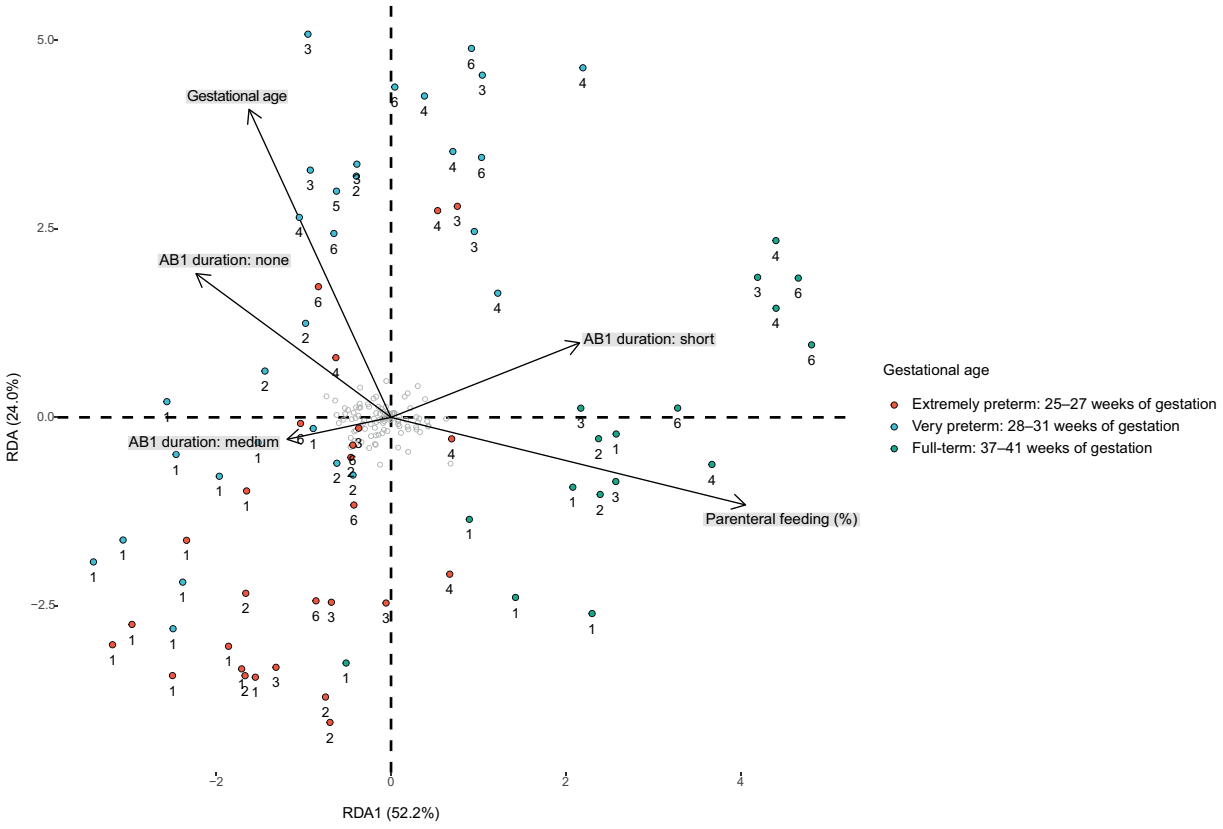


**Figure 3.4** Redundancy analysis on the fecal proteome of preterm and full-term infants during the first six postnatal weeks. Clinical variables shown by arrows significantly explain variation in the proteome as selected by forward and reverse automatic stepwise model selection. Colored points indicate infant samples of one timepoint, numbers indicate the corresponding postnatal week and grey points indicate centroids of identified proteins. AB1: first antibiotic treatment including no treatment and treatment of short (< 3 days) or medium (3–5 days) duration.

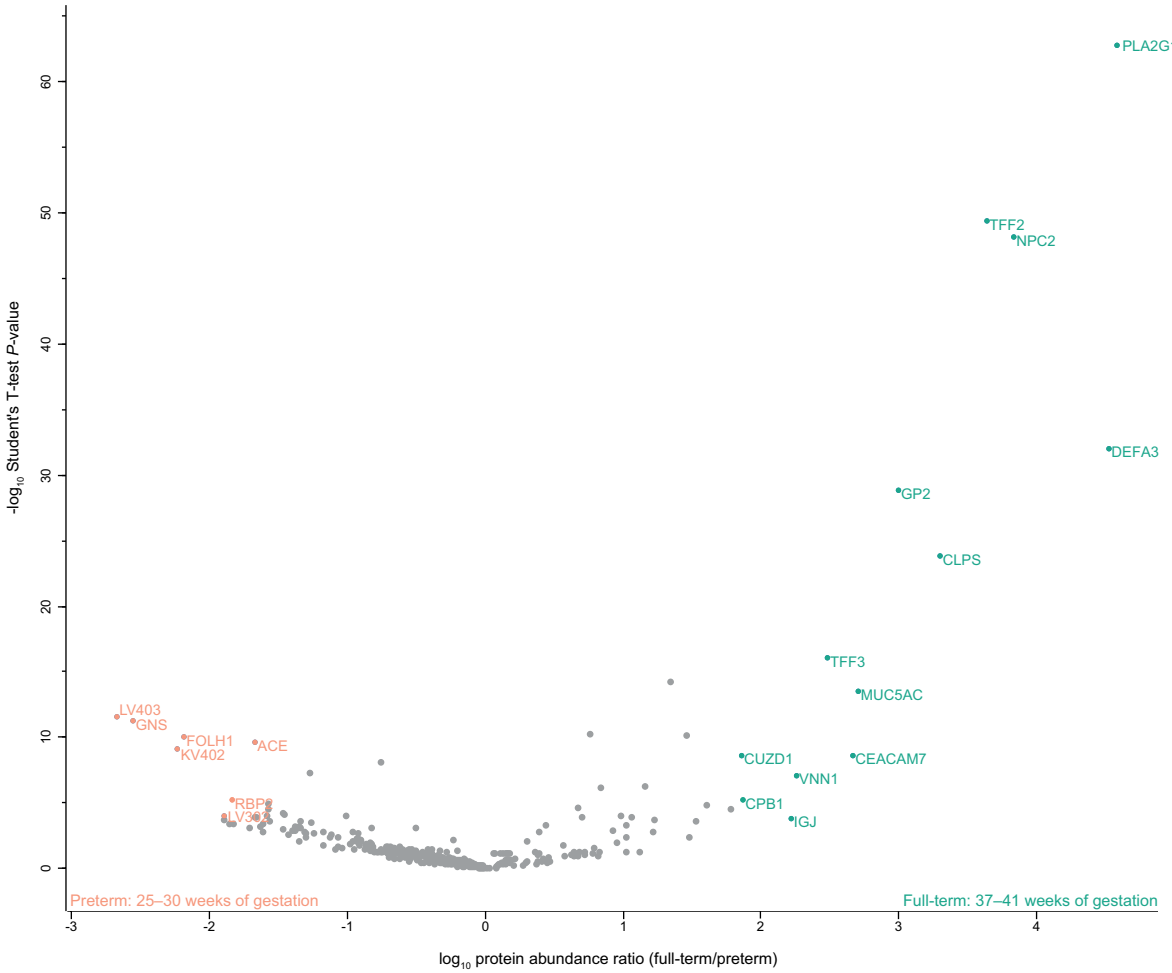


**Figure 3.5** Volcano plot showing differences within the fecal proteome of preterm and full-term infants. LFQ intensities of fecal samples from preterm ($n$ = 56) and full-term ($n$ = 17) infants were used to generate the volcano plot in Perseus version 1.6.2.1[312]. LFQ intensities were $\log_{10}$-transformed, samples were assigned to its designated study group and compared with a Student's two-sample t-test with permutation-based FDR correction. *P*-values as indicated on the y-axis are -$\log_{10}$-transformed. The differentially expressed proteins are marked by gene names. Upregulated proteins in preterm infants include: LV403: Ig lambda chain V-IV region Hil; GNS: Glucosamine (N-acetyl)-6-sulfatase; FOLH1: Glutamate carboxypeptidase 2; ACE: Angiotensin-converting enzyme; KV402: Ig kappa chain V-IV region Len; RBP2: Retinol-binding protein 2 (fragment); LV302: Ig lambda chain V-II region LOI. Upregulated proteins in full-term infants include PLA2G1B: Phospholipase A2; TFF2: Trefoil factor 2; NPC2: Epididymal secretory protein E1 (fragment); DEFA3: Neutrophil defensin 3; GP2: Pancreatic secretory granule membrane major glycoprotein GP2 (fragment); CLPS: Colipase; TFF3: Trefoil factor 3; MUC5AC: Mucin-5AC; CUZD1: CUB and zona pellucida-like domain-containing protein 1; CEACAM7: Carcinoembryonic antigen-related cell adhesion molecule 7; VNN1: Pantetheinase; CPB1: Carboxypeptidase B; IGJ: Immunoglobulin J chain (fragment).

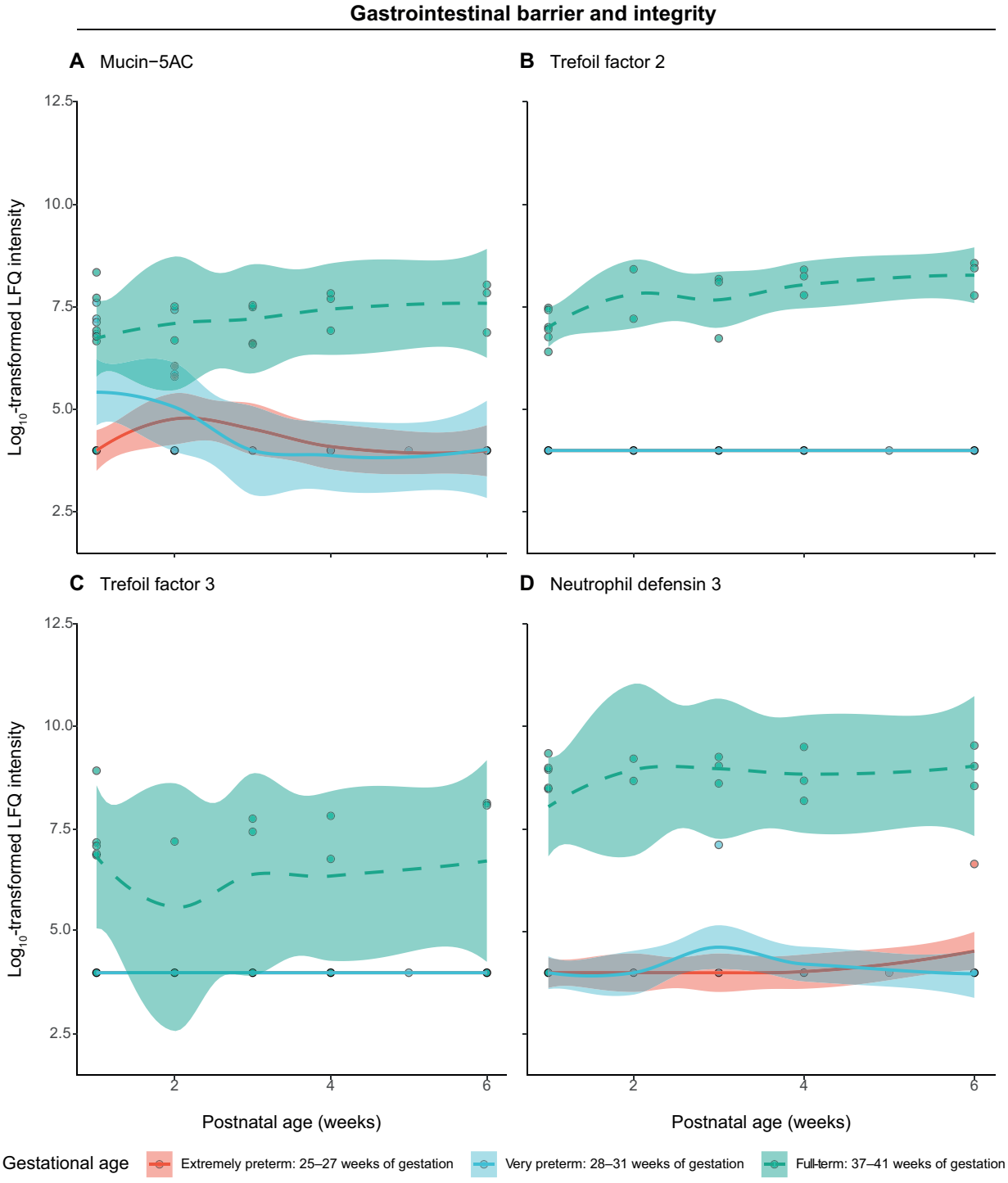


**Figure 3.6** Temporal dynamics of gastrointestinal barrier and integrity maturation markers in the fecal proteome of preterm and full-term infants. (A) Mucin-5AC, (B) trefoil factor 2, (C) trefoil factor 3 and (D) neutrophil defensin 3. The y-axis shows $log_{10}$-transformed LFQ intensity. The x-axis shows the postnatal age in weeks for each gestational age group.

### *Preterm infants express higher levels of microbial oxidative stress proteins compared to full-term infants*

Our previous work indicated that delayed colonization by beneficial, obligate anaerobes was associated with respiratory support strategies[137]. Samples with low levels of bifidobacterial proteins showed relatively high levels of proteins derived from opportunistic pathogens including *Enterococcus* spp., *Escherichia* spp. and *Klebsiella* spp. (Fig. S3.3). Being facultative anaerobic bacteria, expression of oxidative stress proteins may provide a competitive advantage. These genera expressed oxidative stress proteins at different levels in the gestational age groups. Extremely preterm infants, characterized by a low mean relative abundance of bifidobacterial-derived proteins, had significantly higher levels of oxidative stress proteins compared to full-term infants in the second till fourth postnatal week ($P \leq 0.01$) (Fig. S3.6).



### *Human digestive and immunological proteins are consistently present in the preterm infant's gastrointestinal tract*

Many bioactive proteins were consistently identified in preterm infants. For example, immunoglobulin structures and other innate immune proteins were attributes with highest contribution to variation in the gastric proteome by principal component analysis (Fig. S3.7) as well as by comparison of fecal protein abundances in preterm and full-term infants (Fig. 3.5, Table S3.4). Immunoglobulin structures that were significantly more abundant in preterm infants included Ig lambda chain V-III region LOI, Ig kappa chain V–IV region Len and Ig lambda chain V–IV region Hil (−1.9-, −2.2- and −2.7-fold change, respectively, between preterm and full-term infants). Additionally, human catabolic enzymes were more abundant in preterm infants (Fig. 3.5, Table S3.4) including angiotensin-converting enzyme, glutamate carboxypeptidase 2 and N-acetylglucosamine-6-sulfatase (−1.7-, −2.2- and −2.6-fold change, respectively, between preterm and full-term infants). Apart from significant differences, a big variety of human-derived proteins involved in digestion and immune responses were consistently identified in both gastric and fecal proteomes of preterm infants (Table S3.5).

## Discussion

Our findings show that preterm infants express enzymes for human milk protein degradation, albeit to a lesser extent than full-term infants. Digestion likely starts in the gastric environment with proteases derived from mother and/or infant. Moreover, the gastrointestinal barrier of preterm infants is impaired during the first six postnatal week together with less milk-degrading microbes and more bacterial oxidative stress proteins. Although digestive enzymes and gastrointestinal permeability are known to be suboptimal in preterm infants, our findings address these issues for the first time by combining the proteomic profiles of infant gastric aspirates and feces. Other metaproteomic studies in preterm infants have addressed functionality of the microbiota[295,307]. We have added a host and developmental perspective to this by monitoring in the first six postnatal weeks.

Similar to findings by Omari et al., we found average gastric pH fluctuated during the first two postnatal weeks[320]. Extremely high gastric pH at day of birth, as observed in some infants in our study, might be due to swallowing of alkaline amniotic fluid[321]. Median pepsin activity was relatively stable, yet very low. High pH combined with low pepsin activity could affect gastric digestive capacity of preterm infants and thereby decrease their nutrient utilization potential[284,288]. Although enzyme activity analyses showed pepsin was active in the stomach, we could not identify pepsin in the gastric proteome by means of LC-MS/MS. Yet, our results suggest preterm infants are equipped to degrade human milk proteins, as we identified proteases, peptidases and various other digestive enzymes in the gastric and fecal proteome. The activity of these enzymes depends on the maturation status of the infant and may thereby introduce variation in the protein groups identified in each infant, as was shown by our high protein identity variety across samples. The identified enzymes could also be derived from human milk[322,323]. A previous study, however, showed that human milk-derived proteases cannot compensate for the low gastric protein digestion capacity observed in preterm infants[288]. In agreement with our findings, other studies have shown lower proteolytic capacity of gastric enzymes for human milk proteins in preterm infants compared to full-term infants[288]. Milk peptides, including caseins, can survive gastrointestinal digestion, which we could confirm in our study in a quantitative and longitudinal manner[324]. As milk peptides are an important source of peptides and amino acids for rapidly growing infants, the impaired degradation and/or absorption of human milk proteins in preterm infants could have serious consequences on energy acquisition and subsequent growth in early life[325]. However, providing infants with protein hydrolysates did not improve growth and weight gain[326]. The microbiota composition in preterm infants may further influence metabolic activities as shown by other metaproteomic studies in preterm infants that identified microbiota-associated metabolic shifts[295,307]. In our previous work, we similarly have shown that a *Bifidobacterium*-dominated community, as observed in very preterm but not in extremely preterm infants, is associated with increased bacterial proteins involved in carbohydrate and energy metabolism[137]. Here, we have described a correlation between bifidobacterial proteins and birth weight in preterm infants. Infants with higher birth weight are less likely to encounter complications and are more likely to have better early neonatal circumstances compared to low-birth-weight infants. Less and shorter antibiotic treatments as well as less respiratory support

might allow beneficial obligate anaerobes, such as bifidobacteria, to thrive. Delayed colonization of such bacteria was observed with more and longer antibiotic treatments as well as with increased duration of respiratory support[137,144,327]. As a consequence of a higher birth weight, maturation of the gastrointestinal tract as well as bifidobacterial abundance would be promoted, further supporting human milk utilization capacity of the microbiota and weight gain. Potential associations between microbiome and weight gain should be further investigated in a larger cohort of preterm infants.

In addition to digestion, we have shown impaired levels of gastrointestinal barrier-related proteins in preterm infants over the first six postnatal weeks. While MUC5AC is a gel-forming glycoprotein lining gastric and respiratory tract epithelia, trefoil factors are mucin-associated peptides involved in protection and repair of the gastrointestinal mucosa by being involved in restitution and stimulation of immunocyte migration[328–330]. Interaction of TFF2 or TFF3 with MUC5AC has not been reported so far, but is likely due to proven interactions between trefoil factor 1 and MUC5AC as well as homology within a conserved trefoil domain[331]. Therefore, our findings could indicate a less thick and stable mucus layer in the gastrointestinal tract of preterm infants that could subsequently impair the intestinal barrier as described previously[27]. Other metaproteomic studies in preterm infants identified proteins related to intestinal mucosal barrier development and protection, including MUC5AC and trefoil factors[295,307]. Our results showed an inverse association of gastrointestinal barrier-related proteins with bacterial oxidative stress proteins of facultative anaerobes. In our previous work, delayed colonization by beneficial, obligate anaerobes was associated with respiratory support strategies including ventilation and continuous positive airway pressure (CPAP)[137]. Hence, we hypothesized that respiratory support might introduce oxygen into the lumen. Subsequently, the aerobic environment could decrease abundance of beneficial, obligate anaerobic microbes such as *Bifidobacterium* spp., that produce short-chain fatty acids involved in the production of anti-inflammatory cytokines and stimulation of the intestinal barrier function[71,73,303,332]. This in turn might sustain an aerobic environment in which facultative anaerobes, such as *Enterococcus* spp. and *Klebsiella* spp., benefit from oxygen while restraining oxidative stress[333]. While *Bifidobacterium* spp. may protect against intestinal barrier dysfunction, products of *Enterococcus* spp. could compromise the intestinal epithelial barrier[334,335]. Our previous work on the same cohort indicated a delay in bacterial colonization in extremely preterm infants compared to infants born at later gestational ages, as well as decreased abundance of *Bifidobacterium*-derived proteins, suggesting that this gestational age category is particularly prone to an impaired intestinal barrier and a leaky gut[137].

Digestive and immune proteins were consistently identified in the gastric and fecal proteome. Proteins related to innate immune responses, including immunoglobulins and antibacterial proteins, have previously been identified in preterm infants[295,307]. It remains unknown whether these proteins are active and whether they are produced by the preterm infant itself or derived from human milk, even though many proteins have been detected in human milk and preterm infants are immunocompromised[33,322,323,336,337]. Some bioactive proteins are more evident to derive from human milk, such as secretory immunoglobulin A[338]. Other bioactive proteins that we identified in feces include casein fragments, lactotransferrin and serum albumin, as described

previously[339]. By surviving gastrointestinal digestion, these components could confer functional properties that could protect against neonatal sepsis and NEC although certainty of evidence in these cases is low[340,341]. Human milk, thus, acts at the intestinal barrier interface where it supports functional development of the gastrointestinal tract, shapes the microbiome and positively influences health outcomes[24,26].

While our findings indicate impaired digestion and gastrointestinal barrier defense in preterm infants, we acknowledge the relatively small number of particularly full-term infants described in this study. As the main objective of this pilot study was to elucidate metaproteomes of preterm infants, few full-term infants were recruited. This should be taken into account when interpreting the data. Moreover, metaproteomics of the human intestine has its challenges[306]. Future studies should focus on increasing depth and coverage of the microbiome, sample preparation throughput and multiplexity of MS measurements. Moreover, technical barriers for bioinformatic data processing require additional efforts[305,306].

## Conclusion

Our findings indicate that gastrointestinal and beneficial microbial proteins involved in gastrointestinal maturity are associated with gestational and postnatal age. While digestive enzymes and gastrointestinal permeability are known to be suboptimal in preterm infants, this is the first study measuring both human and microbial proteins in stomach and feces during the first six postnatal weeks. The intestinal barrier proves to be an important environment where gastrointestinal epithelium, immune system and microbiome interact to drive growth, development and health of the preterm infant. More insights might lead to the design of optimized nutrition support strategies based on the characteristics of the preterm infant and its intestinal maturation status.

## Supplementary information

**Maturation of the preterm gastrointestinal tract can be defined by host and microbial markers for digestion and barrier defense**

**Figure S3.1** Cumulative relative abundance of casein fragments in feces of preterm and full-term infants during the first six postnatal weeks.

**Figure S3.2** Distribution of proteins derived from human, bovine, human or bovine and bacterial source per postnatal week.

**Figure S3.3** Distribution of proteins derived from bacterial genera *Bifidobacterium*, *Enterococcus*, *Klebsiella* and other genera per postnatal week.

**Figure S3.4** Spearman correlations between preterm infant's relative abundance of *Bifidobacterium*-derived proteins in postnatal weeks 1–6 and birth weight.

**Figure S3.5** Spearman correlations between preterm infant's relative abundance of *Bifidobacterium*-derived proteins in postnatal weeks 1–6 and growth velocity.

**Figure S3.6** Relative abundance of bacterial oxidative stress proteins per postnatal week.

**Figure S3.7** Principal component analysis on the gastric proteome of preterm infants during the first two postnatal weeks.

**Table S3.1** Infant characteristics of two subsets of the EIBER cohort.

**Table S3.2** Differentially abundant human- and bovine-derived proteins between gastric and fecal samples of preterm infants during postnatal weeks one and two.

**Table S3.3** Tables of RDA data.

**Table S3.4** Differentially abundant human- and bovine-derived proteins in feces during the first six postnatal weeks between gestational age groups preterm (25–31 weeks of gestation) and full-term (37–41 weeks of gestation).

**Table S3.5** Human- and bovine-derived proteins identified in more than 50% of the gastric and fecal proteomes of preterm infants (25–31 weeks of gestation).

**Table S3.6** Bacterial oxidative stress proteins from opportunistic pathogens including *Enterococcus* spp., *Escherichia* spp. and *Klebsiella* spp.

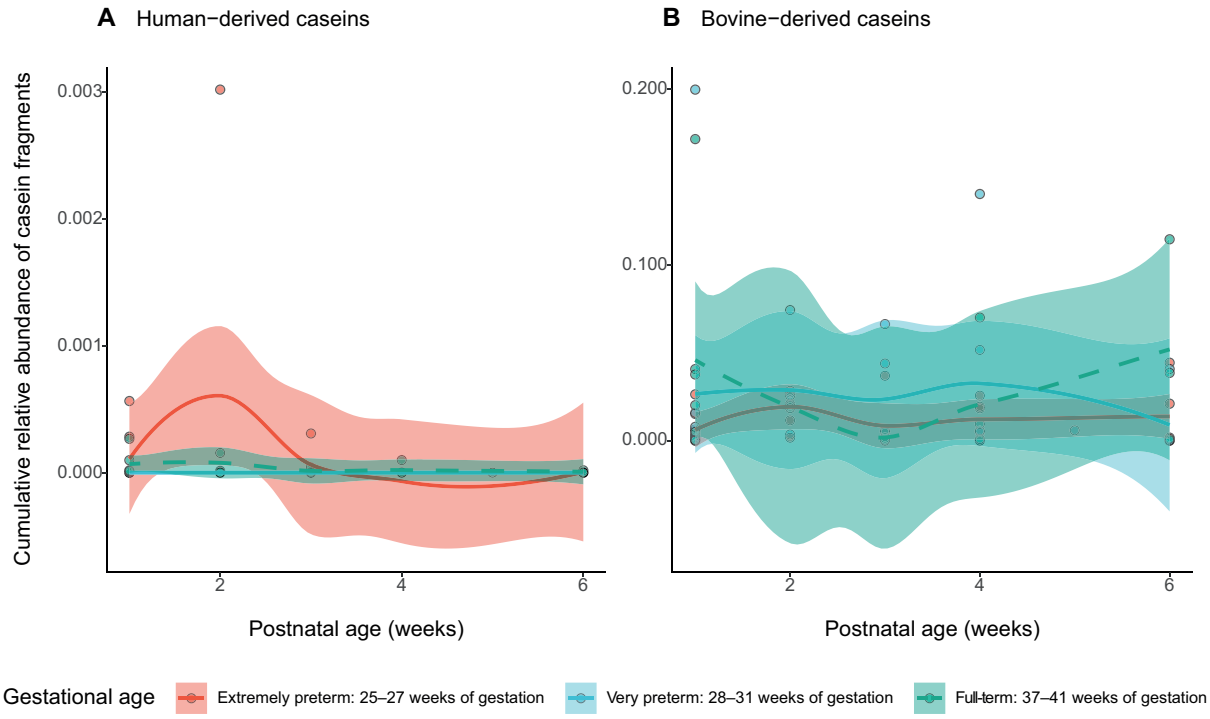


**Figure S3.1** Cumulative relative abundance of casein fragments in feces of preterm and full-term infants during the first six postnatal weeks. (A) Human-derived and (B) bovine-derived alpha-, beta- and kappa-casein fragments in feces of preterm and full-term infants during the first six postnatal weeks. riBAQ was used to calculate relative abundances and were calculated with respect to all human-derived or all bovine-derived proteins. Non-parametric LOESS regression with a 95% confidence interval was used to generate a smooth fitted line per gestational age group.

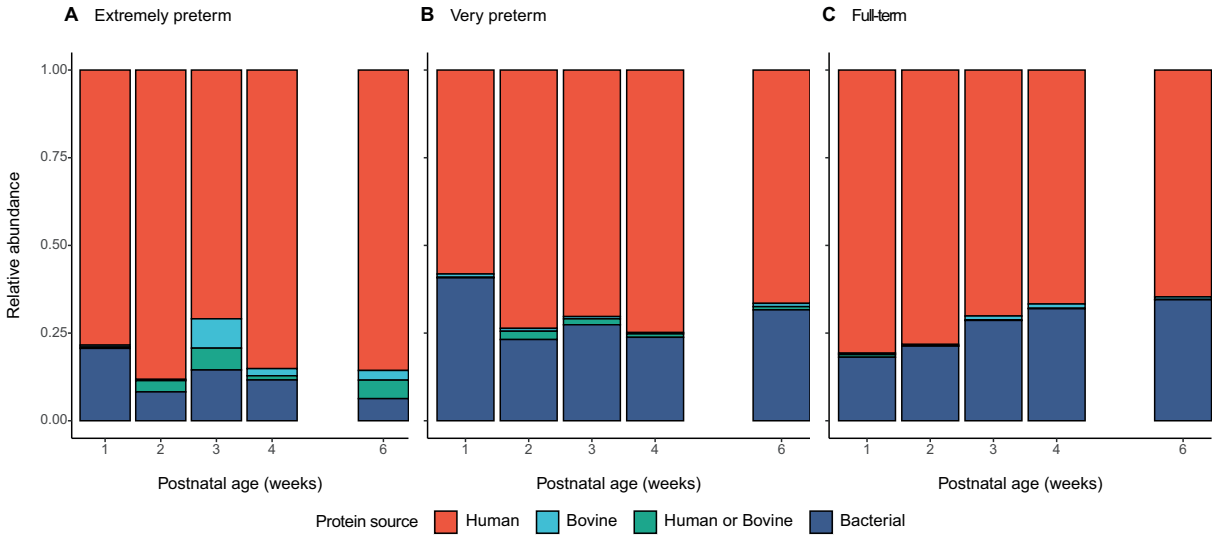


**Figure S3.2** Distribution of proteins derived from human, bovine, human or bovine and bacterial source per postnatal week. Distribution of proteins identified in feces of (A) extremely preterm, (B) very preterm and (C) full-term infants. riBAQ was applied by dividing by the sum of all proteins.

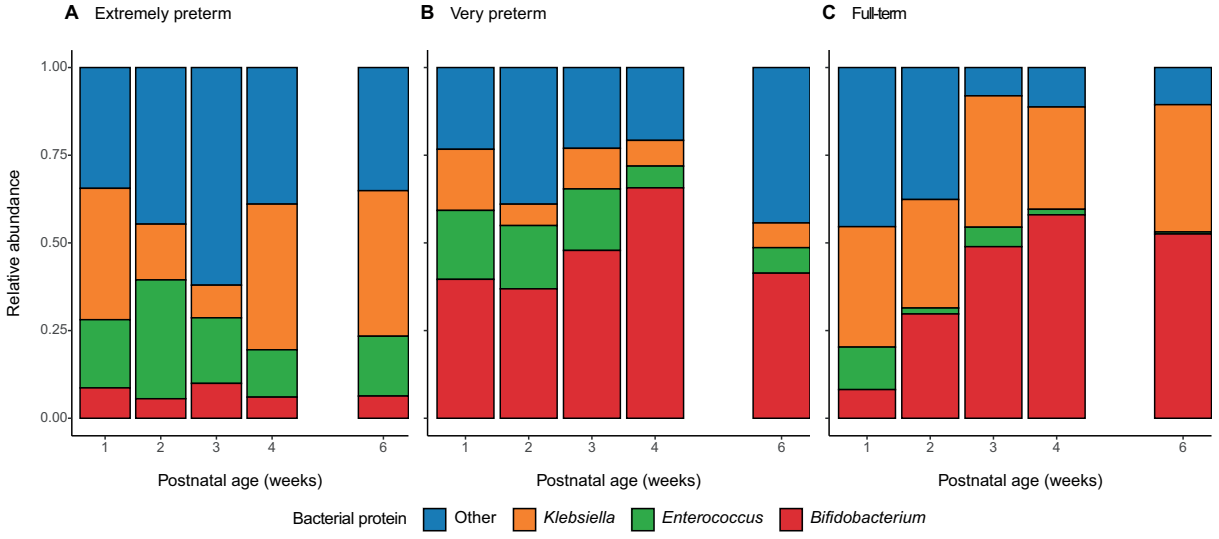


**Figure S3.3** Distribution of proteins derived from bacterial genera *Bifidobacterium*, *Enterococcus*, *Klebsiella* and other genera per postnatal week. Distribution of proteins identified in feces of (A) extremely preterm, (B) very preterm and (C) full-term infants. riBAQ was applied by dividing by the sum of all bacterial proteins.

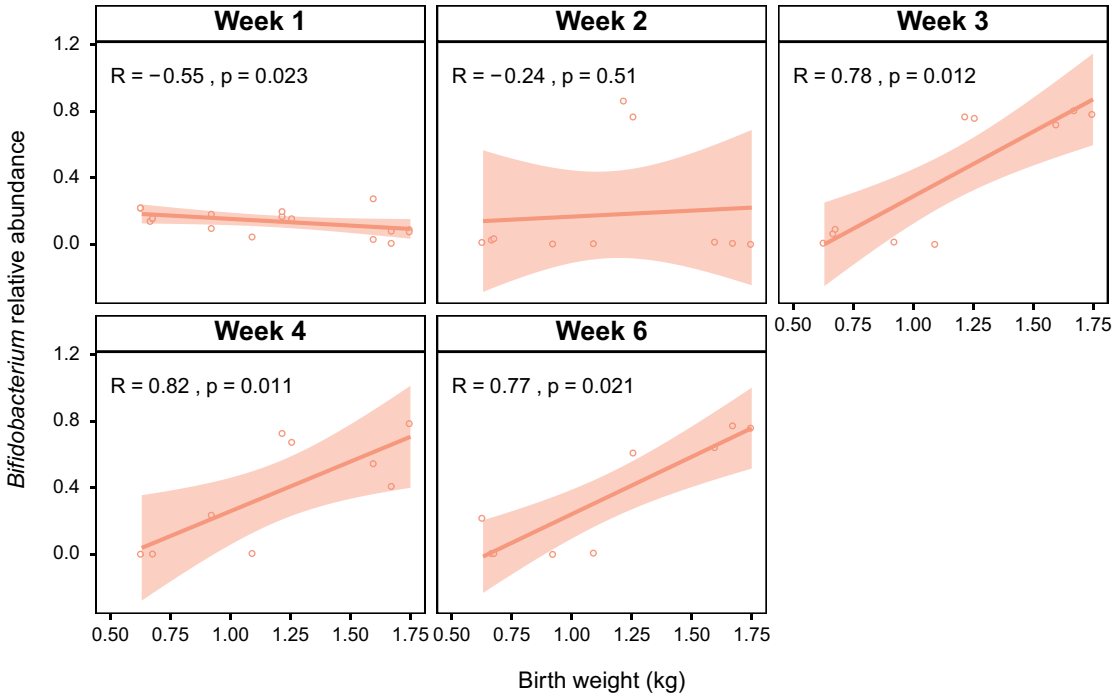


**Figure S3.4** Spearman correlations between preterm infant's relative abundance of *Bifidobacterium*-derived proteins in postnatal weeks 1–6 and birth weight. *Bifidobacterium*-derived proteins as displayed on the y-axis were calculated using riBAQ with respect to all bacterial-derived proteins. *Bifidobacterium* relative abundance is displayed in the panel's corresponding postnatal week. Birth weight in kilograms is displayed on the x-axis.

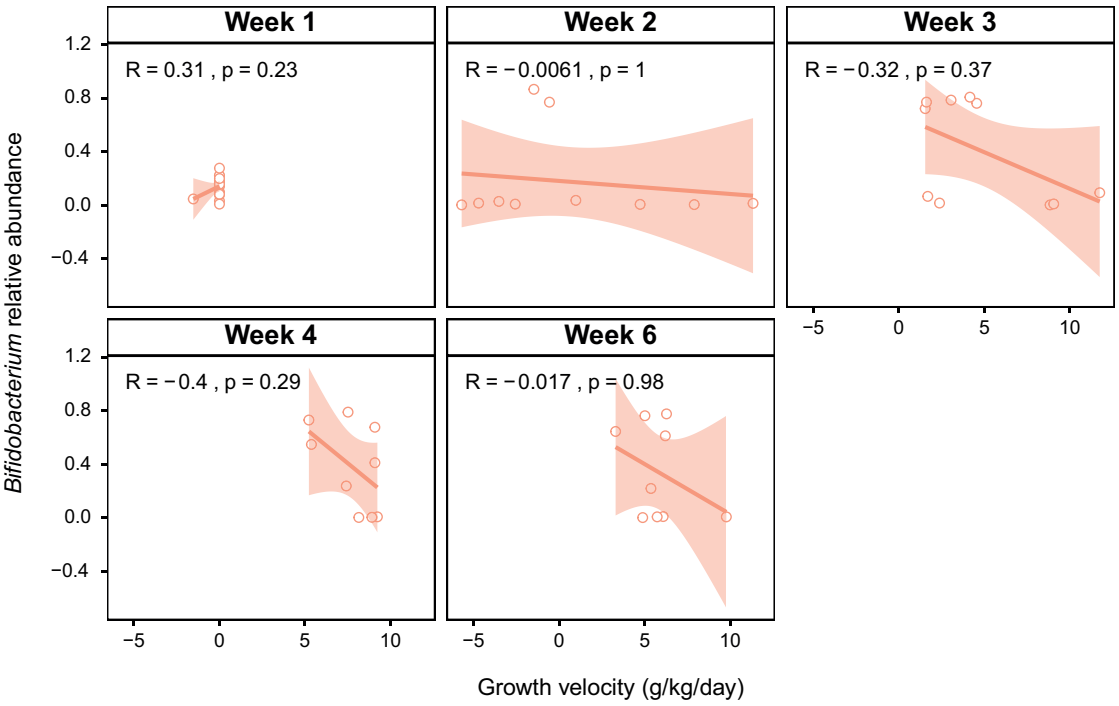


**Figure S3.5** Spearman correlations between preterm infant's relative abundance of *Bifidobacterium*-derived proteins in postnatal weeks 1–6 and growth velocity. *Bifidobacterium*-derived proteins as displayed on the y-axis were calculated using riBAQ with respect to all bacterial-derived proteins. *Bifidobacterium* relative abundance is displayed in the panel's corresponding postnatal week. Growth velocity in g/kg/day is displayed on the x-axis.

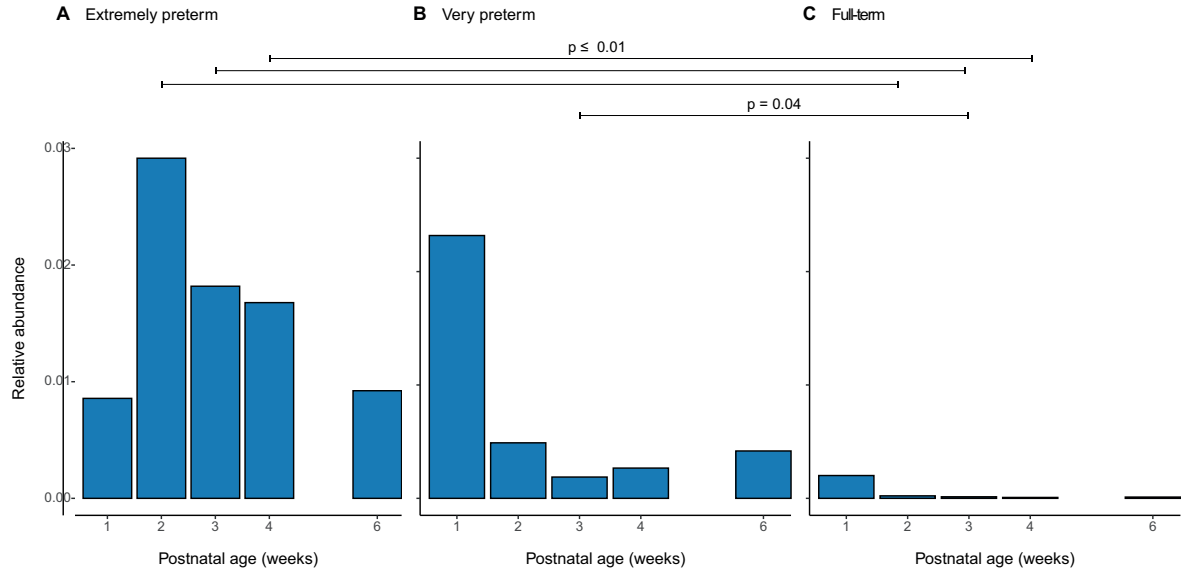


**Figure S3.6** Relative abundance of bacterial oxidative stress proteins per postnatal week. Bacterial oxidative stress proteins from opportunistic pathogens including *Enterococcus* spp., *Escherichia* spp. and *Klebsiella* spp. identified in feces of (A) extremely preterm, (B) very preterm and (C) full-term infants (Table S3.6). riBAQ was applied by dividing by the sum of all bacterial proteins.

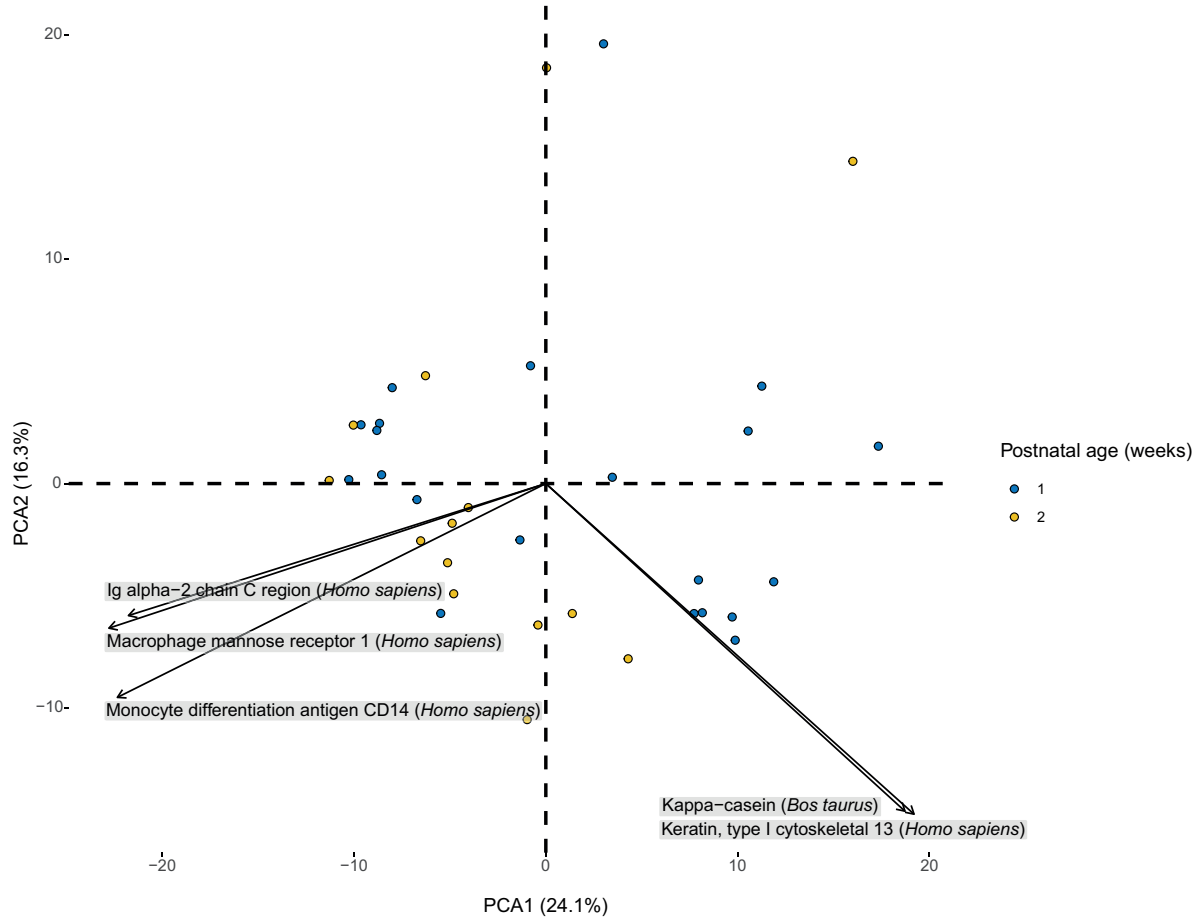


**Figure S3.7** Principal component analysis on the gastric proteome of preterm infants during the first two postnatal weeks. Arrows display proteins explaining most variation on the first two principal components. Colored points indicate infant samples of one timepoint.

**Table S3.1** Infant characteristics of two subsets of the EIBER cohort.

Table S3.1A

| | Gestational age (mean ± SD) | Birth weight (kg ± SD) | Female (%) | C-section (%) | Food Intolerance (%)[a] | Enteral feeding (%)[b, c] | Human milk (%)[b, c, d] |
|---|---|---|---|---|---|---|---|
| ALL ($n$ = 40) | 28.5 ± 2.4 | 1.18 ± 0.39 | 47.5 | 57.5 | 22.5 | 39, 88 | 72, 95 |
| EP ($n$ = 14) | 25.9 ± 1.2 | 0.80 ± 0.16 | 42.9 | 50.0 | 35.7 | 28, 77 | 74, 91 |
| VP ($n$ = 23) | 29.5 ± 1.0 | 1.33 ± 0.29 | 52.2 | 56.5 | 17.4 | 47, 95 | 71, 97 |
| MLP ($n$ = 3) | 33.0 ± 0.0 | 1.77 ± 0.38 | 33.3 | 100.0 | 0.0 | - | - |

Table S3.1B

| | Gestational age (mean ± SD) | Birth weight (kg ± SD) | Weight gain (kg ± SD) | Female (%) | C-section (%) | Food Intolerance (%)[a] | Enteral feeding (%)[e] | Human milk (%)[d, e] |
|---|---|---|---|---|---|---|---|---|
| EP ($n$ = 5) | 26.2 ± 0.8 | 0.80 ± 0.18 | 0.74 ± 0.16 | 20.0 | 80.0 | 80.0 | 22, 66, 79, 87, 100 | 20, 66, 68, 86, 77 |
| VP ($n$ = 5) | 30.0 ± 0.0 | 1.52 ± 0.22 | 0.71 ± 0.15 | 80.0 | 80.0 | 20.0 | 42, 89, 100, 100, 100 | 18, 84, 91, 82, 65 |
| FT ($n$ = 3) | 38.7 ± 2.1 | 3.22 ± 0.68 | - | 33.3 | 66.7 | 0.0 | 100,100,100,100,100 | 50, 98, 69, 67,67 |

Infant characteristics of two subsets of the EIBER cohort: (A) Forty preterm infants of whom pH, protease and pepsin activity were analyzed daily during the first two postnatal weeks; and (B) Ten preterm infants of whom gastric proteome was analyzed weekly in the first two weeks and fecal proteome was analyzed weekly during the first six postnatal weeks, as well as three full-term infants of whom fecal proteome was analyzed weekly during the first six postnatal weeks. EP: extremely preterm, VP: very preterm, MLP: moderate to late preterm, FT: full-term. [a] Defined as infants not bearing an increase in amount of food as assessed by (increasing) retention, vomiting or diarrhea or the appearance of clinical symptoms including tense and extended abdomen, diminished bowel movements or bloody stool. [b] Data was available for 35 infants; 14 EP and 21 VP infants. For one out of three MLP infants, data was available and was therefore not included in analyses. [c] Percentage at postnatal weeks 1 and 2 respectively. [d] Percentage of human milk as enteral feeding. [e] Percentage at postnatal weeks 1, 2, 3, 4 and 6, respectively.

**Table S3.2** Differentially abundant human- and bovine-derived proteins between gastric and fecal samples of preterm infants during postnatal weeks one and two.
Table S3.2A

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >sp\|P00709\|LALBA_HUMAN Alpha-lactalbumin OS=Homo sapiens GN=LALBA PE=1 SV=1; >tr\|F8VWU1\|F8VWU1_HUMAN Alpha-lactalbumin OS=Homo sapiens GN=LALBA PE=3 SV=1 | LALBA | Alpha-lactalbumin | 21.44 | 0.00 | -4.50 | -3.47 |
| >sp\|P07498\|CASK_HUMAN Kappa-casein OS=Homo sapiens GN=CSN3 PE=1 SV=3 | CSN3 | Kappa-casein | 25.18 | 0.00 | -4.37 | -3.51 |
| >sp\|P05814\|CASB_HUMAN Beta-casein OS=Homo sapiens GN=CSN2 PE=1 SV=4 | CSN2 | Beta-casein | 17.14 | 0.00 | -4.21 | -3.13 |
| >sp\|P10909\|CLUS_HUMAN Clusterin OS=Homo sapiens GN=CLU PE=1 SV=1; >tr\|E7ERK6\|E7ERK6_HUMAN Clusterin beta chain (Fragment) OS=Homo sapiens GN=CLU PE=4 SV=1 | CLU | Clusterin; Clusterin beta chain | 22.13 | 0.00 | -3.95 | -3.15 |
| >sp\|P47710\|CASA1_HUMAN Alpha-S1-casein OS=Homo sapiens GN=CSN1S1 PE=1 SV=1; >tr\|E9PDQ1\|E9PDQ1_HUMAN Alpha-S1-casein OS=Homo sapiens GN=CSN1S1 PE=1 SV=1 | CSN1S1 | Alpha-S1-casein; Casoxin-D | 16.86 | 0.00 | -3.88 | -2.93 |
| >sp\|P03973\|SLPI_HUMAN Antileukoproteinase OS=Homo sapiens GN=SLPI PE=1 SV=2 | SLPI | Antileukoproteinase | 23.68 | 0.00 | -3.54 | -2.93 |
| >sp\|P12273\|PIP_HUMAN Prolactin-inducible protein OS=Homo sapiens GN=PIP PE=1 SV=1 | PIP | Prolactin-inducible protein | 17.37 | 0.00 | -3.54 | -2.75 |
| >sp\|Q13410\|BT1A1_HUMAN Butyrophilin subfamily 1 member A1 OS=Homo sapiens GN=BTN1A1 PE=1 SV=3; >tr\|Q4VAN1\|Q4VAN1_HUMAN BTN1A1 protein OS=Homo sapiens GN=BTN1A1 PE=2 SV=1 | BTN1A1 | Butyrophilin subfamily 1 member A1 | 12.27 | 0.00 | -3.16 | -2.35 |
| >sp\|Q6WN34\|CRDL2_HUMAN Chordin-like protein 2 OS=Homo sapiens GN=CHRDL2 PE=1 SV=1; >tr\|A0A087WZH6\|A0A087WZH6_HUMAN Chordin-like protein 2 OS=Homo sapiens GN=CHRDL2 PE=4 SV=1 | CHRDL2; CHL2 | Chordin-like protein 2 | 14.36 | 0.00 | -3.13 | -2.41 |
| >tr\|G3V2V8\|G3V2V8_HUMAN Epididymal secretory protein E1 (Fragment) OS=Homo sapiens GN=NPC2 PE=1 SV=1; >tr\|J3KMY5\|J3KMY5_HUMAN Epididymal secretory protein E1 OS=Homo sapiens GN=NPC2 PE=1 SV=1 | NPC2 | Epididymal secretory protein E1 | 16.92 | 0.00 | -3.08 | -2.45 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >sp\|P06727\|APOA4_HUMAN Apolipoprotein A-IV OS=Homo sapiens GN=APOA4 PE=1 SV=3 | APOA4 | Apolipoprotein A-IV | 25.56 | 0.00 | -3.05 | -2.62 |
| >sp\|P02765\|FETUA_HUMAN Alpha-2-HS-glycoprotein OS=Homo sapiens GN=AHSG PE=1 SV=1; >tr\|C9JV77\|C9JV77_HUMAN Alpha-2-HS-glycoprotein OS=Homo sapiens GN=AHSG PE=1 SV=1 | AHSG | Alpha-2-HS-glycoprotein; Alpha-2-HS-glycoprotein chain A | 18.59 | 0.00 | -3.03 | -2.46 |
| >sp\|P18065\|IBP2_HUMAN Insulin-like growth factor-binding protein 2 OS=Homo sapiens GN=IGFBP2 PE=1 SV=2; >tr\|C9JMY1\|C9JMY1_HUMAN Insulin-like growth factor-binding protein 2 OS=Homo sapiens GN=IGFBP2 PE=4 SV=1 | IGFBP2 | Insulin-like growth factor-binding protein 2 | 19.27 | 0.00 | -2.92 | -2.41 |
| >sp\|P02774\|VTDB_HUMAN Vitamin D-binding protein OS=Homo sapiens GN=GC PE=1 SV=1; >tr\|D6RBJ7\|D6RBJ7_HUMAN Vitamin D-binding protein OS=Homo sapiens GN=GC PE=1 SV=1 | GC | Vitamin D-binding protein | 14.83 | 0.00 | -2.91 | -2.29 |
| >sp\|P61626\|LYSC_HUMAN Lysozyme C OS=Homo sapiens GN=LYZ PE=1 SV=1; >tr\|F8VV32\|F8VV32_HUMAN Lysozyme C OS=Homo sapiens GN=LYZ PE=1 SV=1 | LYZ | Lysozyme C | 12.84 | 0.00 | -2.90 | -2.22 |
| >tr\|B4E1Z4\|B4E1Z4_HUMAN Uncharacterized protein OS=Homo sapiens PE=2 SV=1; >sp\|P00751\|CFAB_HUMAN Complement factor B OS=Homo sapiens GN=CFB PE=1 SV=2 | CFB | Complement factor B; Complement factor B Ba fragment | 16.48 | 0.00 | -2.84 | -2.29 |
| >sp\|P19013\|K2C4_HUMAN Keratin, type II cytoskeletal 4 OS=Homo sapiens GN=KRT4 PE=1 SV=4; >sp\|P19013\|K2C4_HUMAN Keratin, type II cytoskeletal 4 OS=Homo sapiens GN=KRT4 PE=1 SV=4 | KRT4 | Keratin, type II cytoskeletal 4 | 9.50 | 0.00 | -2.79 | -2.04 |
| >sp\|Q08431\|MFGM_HUMAN Lactadherin OS=Homo sapiens GN=MFGE8 PE=1 SV=2; >tr\|F5GZN3\|F5GZN3_HUMAN Lactadherin short form OS=Homo sapiens GN=MFGE8 PE=4 SV=1 | MFGE8 | Lactadherin; Lactadherin short form | 9.03 | 0.00 | -2.78 | -2.01 |
| >sp\|P02749\|APOH_HUMAN Beta-2-glycoprotein 1 OS=Homo sapiens GN=APOH PE=1 SV=3; >tr\|J3QLI0\|J3QLI0_HUMAN Beta-2-glycoprotein 1 (Fragment) OS=Homo sapiens GN=APOH PE=4 SV=1 | APOH | Beta-2-glycoprotein 1 | 11.56 | 0.00 | -2.77 | -2.10 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >sp\|P24821\|TENA_HUMAN Tenascin OS=Homo sapiens GN=TNC PE=1 SV=3;>tr\|J3QSU6\|J3QSU6_HUMAN Tenascin OS=Homo sapiens GN=TNC PE=1 SV=1; >tr\|E9PC84\|E9PC84_HUMAN Tenascin OS=Homo sapiens GN=TNC PE=1 SV=1 | TNC | Tenascin | 8.39 | 0.00 | -2.77 | -1.98 |
| >sp\|P01833\|PIGR_HUMAN Polymeric immunoglobulin receptor OS=Homo sapiens GN=PIGR PE=1 SV=4 | PIGR | Polymeric immunoglobulin receptor; Secretory component | 11.71 | 0.00 | -2.75 | -2.10 |
| >sp\|Q8TDL5\|BPIB1_HUMAN BPI fold-containing family B member 1 OS=Homo sapiens GN=BPIFB1 PE=1 SV=1 | BPIFB1 | BPI fold-containing family B member 1 | 9.28 | 0.00 | -2.73 | -1.99 |
| >sp\|Q0P569\|NUCB1_BOVIN Nucleobindin-1 OS=Bos taurus GN=NUCB1 PE=2 SV=1; >sp\|Q02818\|NUCB1_HUMAN Nucleobindin-1 OS=Homo sapiens GN=NUCB1 PE=1 SV=4 | NUCB1 | Nucleobindin-1 | 16.26 | 0.00 | -2.70 | -2.19 |
| >tr\|J3KPS3\|J3KPS3_HUMAN Fructose-bisphosphate aldolase OS=Homo sapiens GN=ALDOA PE=1 SV=1; >sp\|P04075\|ALDOA_HUMAN Fructose-bisphosphate aldolase A OS=Homo sapiens GN=ALDOA PE=1 SV=2 | ALDOA | Fructose-bisphosphate aldolase A | 10.93 | 0.00 | -2.67 | -2.02 |
| >sp\|P00738\|HPT_HUMAN Haptoglobin OS=Homo sapiens GN=HP PE=1 SV=1; >tr\|J3QLC9\|J3QLC9_HUMAN Haptoglobin (Fragment) OS=Homo sapiens GN=HP PE=1 SV=1 | HP | Haptoglobin | 12.97 | 0.00 | -2.65 | -2.08 |
| >sp\|P08603\|CFAH_HUMAN Complement factor H OS=Homo sapiens GN=CFH PE=1 SV=4 | CFH | Complement factor H | 12.92 | 0.00 | -2.58 | -2.03 |
| >sp\|P02649\|APOE_HUMAN Apolipoprotein E OS=Homo sapiens GN=APOE PE=1 SV=1; >tr\|E9PEV4\|E9PEV4_HUMAN Apolipoprotein E (Fragment) OS=Homo sapiens GN=APOE PE=1 SV=1 | APOE | Apolipoprotein E | 10.85 | 0.00 | -2.54 | -1.95 |
| >tr\|X6R8F3\|X6R8F3_HUMAN Neutrophil gelatinase-associated lipocalin OS=Homo sapiens GN=LCN2 PE=1 SV=1; >sp\|P80188\|NGAL_HUMAN Neutrophil gelatinase-associated lipocalin OS=Homo sapiens GN=LCN2 PE=1 SV=2 | LCN2 | Neutrophil gelatinase-associated lipocalin | 12.70 | 0.00 | -2.52 | -1.99 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >sp\|P02671\|FIBA_HUMAN Fibrinogen alpha chain OS=Homo sapiens GN=FGA PE=1 SV=2; >tr\|A0A087WUA0\|A0A087WUA0_HUMAN Fibrinogen alpha chain OS=Homo sapiens GN=FGA PE=1 SV=1 | FGA | Fibrinogen alpha chain | 10.63 | 0.00 | -2.50 | -1.92 |
| >tr\|Q2HJ20\|Q2HJ20_BOVIN Sclerostin domain containing 1 OS=Bos taurus GN=SOSTDC1 PE=2 SV=1; >sp\|Q6X4U4\|SOSD1_HUMAN Sclerostin domain-containing protein 1 OS=Homo sapiens GN=SOSTDC1 PE=1 SV=2 | SOSTDC1 | Sclerostin domain-containing protein 1 | 8.31 | 0.00 | -2.47 | -1.82 |
| >sp\|Q9UBC9\|SPRR3_HUMAN Small proline-rich protein 3 OS=Homo sapiens GN=SPRR3 PE=1 SV=2; >tr\|B1AN48\|B1AN48_HUMAN Small proline-rich protein 3 (Fragment) OS=Homo sapiens GN=SPRR3 PE=4 SV=3 | SPRR3 | Small proline-rich protein 3 | 12.02 | 0.00 | -2.40 | -1.90 |
| >tr\|A0A087WYF5\|A0A087WYF5_HUMAN Salivary acidic proline-rich phosphoprotein 1/2 (Fragment) OS=Homo sapiens GN=PRH1 PE=4 SV=1; >tr\|A0A087WYT0\|A0A087WYT0_HUMAN Salivary acidic proline-rich phosphoprotein 1/2 OS=Homo sapiens GN=PRH1 PE=4 SV=1 | PRH1 | Salivary acidic proline-rich phosphoprotein 1/2 | 11.99 | 0.00 | -2.40 | -1.89 |
| >sp\|Q9HC84\|MUC5B_HUMAN Mucin-5B OS=Homo sapiens GN=MUC5B PE=1 SV=3 | MUC5B | Mucin-5B | 8.77 | 0.00 | -2.32 | -1.75 |
| >tr\|A2VE41\|A2VE41_BOVIN EGF-containing fibulin-like extracellular matrix protein 1 OS=Bos taurus GN=EFEMP1 PE=2 SV=1; >sp\|Q12805\|FBLN3_HUMAN EGF-containing fibulin-like extracellular matrix protein 1 OS=Homo sapiens GN=EFEMP1 PE=1 SV=2 | EFEMP1 | EGF-containing fibulin-like extracellular matrix protein 1 | 12.16 | 0.00 | -2.28 | -1.82 |
| >sp\|P02647\|APOA1_HUMAN Apolipoprotein A-I OS=Homo sapiens GN=APOA1 PE=1 SV=1; >tr\|F8W696\|F8W696_HUMAN Truncated apolipoprotein A-I OS=Homo sapiens GN=APOA1 PE=1 SV=1 | APOA1 | Apolipoprotein A-I; Proapolipoprotein A-I | 9.93 | 0.00 | -2.27 | -1.76 |
| >sp\|P08571\|CD14_HUMAN Monocyte differentiation antigen CD14 OS=Homo sapiens GN=CD14 PE=1 SV=2; >tr\|D6RFL4\|D6RFL4_HUMAN Monocyte differentiation antigen CD14, urinary form (Fragment) OS=Homo sapiens GN=CD14 PE=4 SV=1 | CD14 | Monocyte differentiation antigen CD14; Monocyte differentiation antigen CD14, urinary form | 5.71 | 0.00 | -2.25 | -1.58 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >tr\|X6R868\|X6R868_HUMAN Bile salt-activated lipase OS=Homo sapiens GN=CEL PE=3 SV=1; >sp\|P19835\|CEL_HUMAN Bile salt-activated lipase OS=Homo sapiens GN=CEL PE=1 SV=3 | CEL | Bile salt-activated lipase | 6.05 | 0.00 | -2.21 | -1.57 |
| >sp\|P0C0L4\|CO4A_HUMAN Complement C4-A OS=Homo sapiens GN=C4A PE=1 SV=2; >sp\|P0C0L5\|CO4B_HUMAN Complement C4-B OS=Homo sapiens GN=C4B PE=1 SV=2 | C4A; C4B | Complement C4-A; Complement C4 beta chain | 7.39 | 0.00 | -2.20 | -1.63 |
| >sp\|P07098\|LIPG_HUMAN Gastric triacylglycerol lipase OS=Homo sapiens GN=LIPF PE=1 SV=1 | LIPF | Gastric triacylglycerol lipase | 7.59 | 0.00 | -2.18 | -1.63 |
| >sp\|P59666\|DEF3_HUMAN Neutrophil defensin 3 OS=Homo sapiens GN=DEFA3 PE=1 SV=1; >sp\|P59665\|DEF1_HUMAN Neutrophil defensin 1 OS=Homo sapiens GN=DEFA1 PE=1 SV=1 | DEFA3; DEFA1 | Neutrophil defensin 3;HP 3-56; Neutrophil defensin 1;HP 1-56 | 6.74 | 0.00 | -2.17 | -1.59 |
| >sp\|Q03403\|TFF2_HUMAN Trefoil factor 2 OS=Homo sapiens GN=TFF2 PE=1 SV=2 | TFF2 | Trefoil factor 2 | 11.48 | 0.00 | -2.11 | -1.70 |
| >sp\|O00391\|QSOX1_HUMAN Sulfhydryl oxidase 1 OS=Homo sapiens GN=QSOX1 PE=1 SV=3 | QSOX1 | Sulfhydryl oxidase 1 | 5.57 | 0.00 | -2.10 | -1.50 |
| >tr\|A0A087WXL8\|A0A087WXL8_HUMAN Ig gamma-3 chain C region OS=Homo sapiens GN=IGHG3 PE=4 SV=1; >tr\|A0A075B6N8\|A0A075B6N8_HUMAN Ig gamma-3 chain C region (Fragment) OS=Homo sapiens GN=IGHG3 PE=4 SV=1 | IGHG3 | Ig gamma-3 chain C region | 5.72 | 0.00 | -2.03 | -1.47 |
| >sp\|P43652\|AFAM_HUMAN Afamin OS=Homo sapiens GN=AFM PE=1 SV=1 | AFM | Afamin | 9.85 | 0.00 | -2.02 | -1.60 |
| >sp\|P10451\|OSTP_HUMAN Osteopontin OS=Homo sapiens GN=SPP1 PE=1 SV=1; >tr\|D6R9C5\|D6R9C5_HUMAN Osteopontin (Fragment) OS=Homo sapiens GN=SPP1 PE=1 SV=1 | SPP1 | Osteopontin | 5.57 | 0.00 | -1.98 | -1.44 |
| >sp\|Q9P2E9\|RRBP1_HUMAN Ribosome-binding protein 1 OS=Homo sapiens GN=RRBP1 PE=1 SV=4; >tr\|A0A087WVV2\|A0A087WVV2_HUMAN Ribosome-binding protein 1 OS=Homo sapiens GN=RRBP1 PE=1 SV=1 | RRBP1 | Ribosome-binding protein 1 | 8.94 | 0.00 | -1.98 | -1.55 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >tr\|C9JIZ6\|C9JIZ6_HUMAN Prosaposin OS=Homo sapiens GN=PSAP PE=1 SV=2; >tr\|B1AVU8\|B1AVU8_HUMAN Saposin-D OS=Homo sapiens GN=PSAP PE=1 SV=1 | PSAP | Prosaposin; Saposin-D | 7.78 | 0.00 | -1.97 | -1.51 |
| >sp\|P22897\|MRC1_HUMAN Macrophage mannose receptor 1 OS=Homo sapiens GN=MRC1 PE=1 SV=1 | MRC1 | Macrophage mannose receptor 1 | 5.69 | 0.00 | -1.93 | -1.41 |
| >sp\|P13646\|K1C13_HUMAN Keratin, type I cytoskeletal 13 OS=Homo sapiens GN=KRT13 PE=1 SV=4; >tr\|K7ERE3\|K7ERE3_HUMAN Keratin, type I cytoskeletal 13 OS=Homo sapiens GN=KRT13 PE=1 SV=1 | KRT13 | Keratin, type I cytoskeletal 13 | 5.54 | 0.00 | -1.93 | -1.40 |
| >tr\|S4R371\|S4R371_HUMAN Fatty acid-binding protein, heart (Fragment) OS=Homo sapiens GN=FABP3 PE=1 SV=1; >sp\|P05413\|FABPH_HUMAN Fatty acid-binding protein, heart OS=Homo sapiens GN=FABP3 PE=1 SV=4 | FABP3 | Fatty acid-binding protein, heart | 4.91 | 0.00 | -1.86 | -1.34 |
| >sp\|P16403\|H12_HUMAN Histone H1.2 OS=Homo sapiens GN=HIST1H1C PE=1 SV=2; >sp\|A7MAZ5\|H13_BOVIN Histone H1.3 OS=Bos taurus GN=HIST1H1D PE=1 SV=1 | HIST1H1C; HIST1H1D | Histone H1.2 | 5.86 | 0.00 | -1.86 | -1.38 |
| >sp\|P05362\|ICAM1_HUMAN Intercellular adhesion molecule 1 OS=Homo sapiens GN=ICAM1 PE=1 SV=2; >tr\|E7ESS4\|E7ESS4_HUMAN Intercellular adhesion molecule 1 OS=Homo sapiens GN=ICAM1 PE=1 SV=1 | ICAM1 | Intercellular adhesion molecule 1 | 9.14 | 0.00 | -1.84 | -1.47 |
| >tr\|E7EU05\|E7EU05_HUMAN Platelet glycoprotein 4 (Fragment) OS=Homo sapiens GN=CD36 PE=4 SV=2; >sp\|P16671\|CD36_HUMAN Platelet glycoprotein 4 OS=Homo sapiens GN=CD36 PE=1 SV=2 | CD36 | Platelet glycoprotein 4 | 4.65 | 0.00 | -1.83 | -1.31 |
| >sp\|P02787\|TRFE_HUMAN Serotransferrin OS=Homo sapiens GN=TF PE=1 SV=3 | TF | Serotransferrin | 6.68 | 0.00 | -1.83 | -1.40 |
| >sp\|P36222\|CH3L1_HUMAN Chitinase-3-like protein 1 OS=Homo sapiens GN=CHI3L1 PE=1 SV=2; >tr\|H0Y3U8\|H0Y3U8_HUMAN Chitinase-3-like protein 1 (Fragment) OS=Homo sapiens GN=CHI3L1 PE=3 SV=1 | CHI3L1 | Chitinase-3-like protein 1 | 5.53 | 0.00 | -1.83 | -1.35 |
| >sp\|P35321\|SPR1A_HUMAN Cornifin-A OS=Homo sapiens GN=SPRR1A PE=1 SV=2 | SPRR1A | Cornifin-A | 6.18 | 0.00 | -1.76 | -1.34 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >sp\|P02538\|K2C6A_HUMAN Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3 | KRT6A | Keratin, type II cytoskeletal 6A | 4.11 | 0.00 | -1.75 | -1.24 |
| >sp\|Q99541\|PLIN2_HUMAN Perilipin-2 OS=Homo sapiens GN=PLIN2 PE=1 SV=2 | PLIN2 | Perilipin-2 | 5.13 | 0.00 | -1.71 | -1.27 |
| >sp\|P06733\|ENOA_HUMAN Alpha-enolase OS=Homo sapiens GN=ENO1 PE=1 SV=2 | ENO1 | Alpha-enolase | 5.58 | 0.00 | -1.68 | -1.27 |
| >sp\|P07996\|TSP1_HUMAN Thrombospondin-1 OS=Homo sapiens GN=THBS1 PE=1 SV=2; >sp\|Q28178\|TSP1_BOVIN Thrombospondin-1 OS=Bos taurus GN=THBS1 PE=2 SV=2 | THBS1 | Thrombospondin-1 | 4.01 | 0.00 | -1.61 | -1.16 |
| >tr\|H0YL18\|H0YL18_HUMAN Beta-2-microglobulin form pI 5.3 OS=Homo sapiens GN=B2M PE=1 SV=1; >sp\|P61769\|B2MG_HUMAN Beta-2-microglobulin OS=Homo sapiens GN=B2M PE=1 SV=1 | B2M | Beta-2-microglobulin; Beta-2-microglobulin form pI 5.3 | 4.75 | 0.00 | -1.60 | -1.19 |
| >sp\|P06396\|GELS_HUMAN Gelsolin OS=Homo sapiens GN=GSN PE=1 SV=1;>tr\|Q5T0H9\|Q5T0H9_HUMAN Gelsolin OS=Homo sapiens GN=GSN PE=1 SV=1; >tr\|Q5T0H8\|Q5T0H8_HUMAN Gelsolin OS=Homo sapiens GN=GSN PE=1 SV=1 | GSN | Gelsolin | 4.23 | 0.00 | -1.59 | -1.16 |
| >sp\|P36871\|PGM1_HUMAN Phosphoglucomutase-1 OS=Homo sapiens GN=PGM1 PE=1 SV=3; >sp\|Q08DP0\|PGM1_BOVIN Phosphoglucomutase-1 OS=Bos taurus GN=PGM1 PE=2 SV=1 | PGM1 | Phosphoglucomutase-1 | 5.13 | 0.00 | -1.51 | -1.16 |
| >sp\|P16401\|H15_HUMAN Histone H1.5 OS=Homo sapiens GN=HIST1H1B PE=1 SV=3 | HIST1H1B | Histone H1.5 | 3.47 | 0.00 | -1.50 | -1.07 |
| >sp\|P04217\|A1BG_HUMAN Alpha-1B-glycoprotein OS=Homo sapiens GN=A1BG PE=1 SV=4; >tr\|M0R009\|M0R009_HUMAN Alpha-1B-glycoprotein (Fragment) OS=Homo sapiens GN=A1BG PE=4 SV=3 | A1BG | Alpha-1B-glycoprotein | 4.58 | 0.00 | -1.49 | -1.13 |
| >sp\|P05204\|HMGN2_HUMAN Non-histone chromosomal protein HMG-17 OS=Homo sapiens GN=HMGN2 PE=1 SV=3 | HMGN2 | Non-histone chromosomal protein HMG-17 | 6.23 | 0.00 | -1.49 | -1.17 |

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|---|---|---|---|---|---|---|
| >sp\|P02771\|FETA_HUMAN Alpha-fetoprotein OS=Homo sapiens GN=AFP PE=1 SV=1; >tr\|J3KMX3\|J3KMX3_HUMAN Alpha-fetoprotein OS=Homo sapiens GN=AFP PE=1 SV=1 | AFP | Alpha-fetoprotein | 3.14 | 0.00 | -1.47 | -1.04 |
| >sp\|O95171\|SCEL_HUMAN Sciellin OS=Homo sapiens GN=SCEL PE=1 SV=2 | SCEL | Sciellin | 4.36 | 0.00 | -1.46 | -1.10 |
| >tr\|E7ENC5\|E7ENC5_HUMAN Mucin-4 beta chain OS=Homo sapiens GN=MUC4 PE=4 SV=1; >tr\|E9PDY6\|E9PDY6_HUMAN Mucin-4 beta chain OS=Homo sapiens GN=MUC4 PE=4 SV=1 | MUC4 | Mucin-4 beta chain | 4.36 | 0.00 | -1.45 | -1.09 |
| >tr\|A0A087WZH7\|A0A087WZH7_HUMAN Myristoylated alanine-rich C-kinase substrate OS=Homo sapiens GN=MARCKS PE=1 SV=1; >sp\|P29966\|MARCS_HUMAN Myristoylated alanine-rich C-kinase substrate OS=Homo sapiens GN=MARCKS PE=1 SV=4 | MARCKS | Myristoylated alanine-rich C-kinase substrate | 6.14 | 0.00 | -1.44 | -1.14 |
| >tr\|E9PNW4\|E9PNW4_HUMAN CD59 glycoprotein OS=Homo sapiens GN=CD59 PE=1 SV=1; >tr\|E9PR17\|E9PR17_HUMAN CD59 glycoprotein OS=Homo sapiens GN=CD59 PE=1 SV=1 | CD59 | CD59 glycoprotein | 3.99 | 0.00 | -1.41 | -1.05 |
| >sp\|P02768\|ALBU_HUMAN Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2; >tr\|B7WNR0\|B7WNR0_HUMAN Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=1 | ALB | Serum albumin | 8.71 | 0.00 | -1.40 | -1.17 |
| >sp\|P69892\|HBG2_HUMAN Hemoglobin subunit gamma-2 OS=Homo sapiens GN=HBG2 PE=1 SV=2; >tr\|E9PBW4\|E9PBW4_HUMAN Hemoglobin subunit gamma-2 OS=Homo sapiens GN=HBG2 PE=1 SV=1 | HBG2 | Hemoglobin subunit gamma-2 | 3.28 | 0.00 | -1.37 | -1.00 |
| >sp\|P25311\|ZA2G_HUMAN Zinc-alpha-2-glycoprotein OS=Homo sapiens GN=AZGP1 PE=1 SV=2; >tr\|C9JEV0\|C9JEV0_HUMAN Zinc-alpha-2-glycoprotein OS=Homo sapiens GN=AZGP1 PE=3 SV=1 | AZGP1 | Zinc-alpha-2-glycoprotein | 4.23 | 0.00 | -1.37 | -1.04 |
| >tr\|A0A087X2C0\|A0A087X2C0_HUMAN Ig mu chain C region OS=Homo sapiens GN=IGHM PE=1 SV=1; >tr\|A0A075B6N9\|A0A075B6N9_HUMAN Ig mu chain C region (Fragment) OS=Homo sapiens GN=IGHM PE=1 SV=2 | IGHM | Ig mu chain C region | 2.76 | 0.00 | -1.36 | -0.96 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >tr\|E9PJK1\|E9PJK1_HUMAN Tetraspanin OS=Homo sapiens GN=CD81 PE=1 SV=1; >tr\|E9PRJ8\|E9PRJ8_HUMAN Tetraspanin (Fragment) OS=Homo sapiens GN=CD81 PE=1 SV=1 | CD81 | CD81 antigen | 4.36 | 0.00 | -1.36 | -1.04 |
| >tr\|F8W148\|F8W148_HUMAN Carbonic anhydrase 6 (Fragment) OS=Homo sapiens GN=CA6 PE=4 SV=1; >sp\|P23280\|CAH6_HUMAN Carbonic anhydrase 6 OS=Homo sapiens GN=CA6 PE=1 SV=3 | CA6 | Carbonic anhydrase 6 | 2.52 | 0.00 | -1.32 | -0.92 |
| >sp\|P49327\|FAS_HUMAN Fatty acid synthase OS=Homo sapiens GN=FASN PE=1 SV=3 | FASN | Fatty acid synthase | 3.25 | 0.00 | -1.29 | -0.95 |
| >sp\|P11021\|GRP78_HUMAN 78 kDa glucose-regulated protein OS=Homo sapiens GN=HSPA5 PE=1 SV=2; >sp\|Q0VCX2\|GRP78_BOVIN 78 kDa glucose-regulated protein OS=Bos taurus GN=HSPA5 PE=2 SV=1 | HSPA5 | 78 kDa glucose-regulated protein | 3.10 | 0.00 | -1.29 | -0.94 |
| >sp\|Q92522\|H1X_HUMAN Histone H1x OS=Homo sapiens GN=H1FX PE=1 SV=1 | H1FX | Histone H1x | 5.42 | 0.00 | -1.28 | -1.02 |
| >tr\|A0A096LPK4\|A0A096LPK4_HUMAN Mucin-5AC OS=Homo sapiens GN=MUC5AC PE=4 SV=1; >sp\|P98088\|MUC5A_HUMAN Mucin-5AC (Fragments) OS=Homo sapiens GN=MUC5AC PE=1 SV=3 | MUC5AC | Mucin-5AC | 2.99 | 0.00 | -1.27 | -0.93 |
| >sp\|P02788\|TRFL_HUMAN Lactotransferrin OS=Homo sapiens GN=LTF PE=1 SV=6; >tr\|E7ER44\|E7ER44_HUMAN Lactotransferrin OS=Homo sapiens GN=LTF PE=1 SV=1 | LTF | Lactotransferrin | 5.28 | 0.00 | -1.26 | -1.01 |
| >sp\|P00747\|PLMN_HUMAN Plasminogen OS=Homo sapiens GN=PLG PE=1 SV=2 | PLG | Plasminogen | 3.94 | 0.00 | -1.23 | -0.95 |
| >sp\|P62263\|RS14_HUMAN 40S ribosomal protein S14 OS=Homo sapiens GN=RPS14 PE=1 SV=3; >tr\|H0YB22\|H0YB22_HUMAN 40S ribosomal protein S14 (Fragment) OS=Homo sapiens GN=RPS14 PE=1 SV=1 | RPS14 | 40S ribosomal protein S14 | 3.60 | 0.00 | -1.22 | -0.93 |
| >tr\|D6RD17\|D6RD17_HUMAN Immunoglobulin J chain (Fragment) OS=Homo sapiens GN=IGJ PE=1 SV=3; >sp\|P01591\|IGJ_HUMAN Immunoglobulin J chain OS=Homo sapiens GN=IGJ PE=1 SV=4 | IGJ | Immunoglobulin J chain | 1.63 | 0.01 | -1.20 | -0.79 |

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|---|---|---|---|---|---|---|
| >tr\|K7EKI8\|K7EKI8_HUMAN Periplakin OS=Homo sapiens GN=PPL PE=1 SV=1; >sp\|O60437\|PEPL_HUMAN Periplakin OS=Homo sapiens GN=PPL PE=1 SV=4 | PPL | Periplakin | 3.87 | 0.00 | -1.19 | -0.92 |
| >sp\|P13796\|PLSL_HUMAN Plastin-2 OS=Homo sapiens GN=LCP1 PE=1 SV=6; >tr\|F1MYX5\|F1MYX5_BOVIN Uncharacterized protein OS=Bos taurus GN=LCP1 PE=4 SV=1 | LCP1 | Plastin-2 | 5.05 | 0.00 | -1.19 | -0.96 |
| >sp\|P68871\|HBB_HUMAN Hemoglobin subunit beta OS=Homo sapiens GN=HBB PE=1 SV=2; >tr\|F8W6P5\|F8W6P5_HUMAN LVV-hemorphin-7 (Fragment) OS=Homo sapiens GN=HBB PE=1 SV=1 | HBB | Hemoglobin subunit beta; LVV-hemorphin-7 | 2.13 | 0.00 | -1.19 | -0.83 |
| >sp\|P02675\|FIBB_HUMAN Fibrinogen beta chain OS=Homo sapiens GN=FGB PE=1 SV=2; >tr\|D6REL8\|D6REL8_HUMAN Fibrinogen beta chain OS=Homo sapiens GN=FGB PE=1 SV=1 | FGB | Fibrinogen beta chain | 2.29 | 0.00 | -1.19 | -0.84 |
| >sp\|Q9UBG3\|CRNN_HUMAN Cornulin OS=Homo sapiens GN=CRNN PE=1 SV=1 | CRNN | Cornulin | 2.54 | 0.00 | -1.16 | -0.85 |
| >sp\|P31146\|COR1A_HUMAN Coronin-1A OS=Homo sapiens GN=CORO1A PE=1 SV=4; >sp\|Q92176\|COR1A_BOVIN Coronin-1A OS=Bos taurus GN=CORO1A PE=1 SV=3 | CORO1A | Coronin-1A | 4.48 | 0.00 | -1.16 | -0.92 |
| >sp\|Q3T0R1\|RS18_BOVIN 40S ribosomal protein S18 OS=Bos taurus GN=RPS18 PE=2 SV=3; >sp\|P62269\|RS18_HUMAN 40S ribosomal protein S18 OS=Homo sapiens GN=RPS18 PE=1 SV=3 | RPS18 | 40S ribosomal protein S18 | 4.28 | 0.00 | -1.16 | -0.91 |
| >sp\|Q8N4F0\|BPIB2_HUMAN BPI fold-containing family B member 2 OS=Homo sapiens GN=BPIFB2 PE=1 SV=2 | BPIFB2 | BPI fold-containing family B member 2 | 3.56 | 0.00 | -1.15 | -0.89 |
| >sp\|P02751\|FINC_HUMAN Fibronectin OS=Homo sapiens GN=FN1 PE=1 SV=4; >tr\|H0Y7Z1\|H0Y7Z1_HUMAN Ugl-Y3 (Fragment) OS=Homo sapiens GN=FN1 PE=1 SV=1 | FN1 | Fibronectin; Ugl-Y3 | 2.80 | 0.00 | -1.15 | -0.85 |
| >sp\|P80723\|BASP1_HUMAN Brain acid soluble protein 1 OS=Homo sapiens GN=BASP1 PE=1 SV=2 | BASP1 | Brain acid soluble protein 1 | 4.81 | 0.00 | -1.14 | -0.92 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >sp\|P31025\|LCN1_HUMAN Lipocalin-1 OS=Homo sapiens GN=LCN1 PE=1 SV=1 | LCN1 | Lipocalin-1 | 2.31 | 0.01 | -1.08 | -0.79 |
| >sp\|P02760\|AMBP_HUMAN Protein AMBP OS=Homo sapiens GN=AMBP PE=1 SV=1; >tr\|S4R471\|S4R471_HUMAN Protein AMBP (Fragment) OS=Homo sapiens GN=AMBP PE=4 SV=1 | AMBP | Protein AMBP | 2.60 | 0.01 | -1.05 | -0.79 |
| >sp\|P04083\|ANXA1_HUMAN Annexin A1 OS=Homo sapiens GN=ANXA1 PE=1 SV=2; >tr\|Q5T3N1\|Q5T3N1_HUMAN Annexin (Fragment) OS=Homo sapiens GN=ANXA1 PE=1 SV=1 | ANXA1 | Annexin A1; Annexin | 2.62 | 0.01 | -1.05 | -0.79 |
| >tr\|A1L5B7\|A1L5B7_BOVIN SERPINE1 mRNA binding protein 1 OS=Bos taurus GN=SERBP1 PE=2 SV=1; >sp\|Q8NC51\|PAIRB_HUMAN Plasminogen activator inhibitor 1 RNA-binding protein OS=Homo sapiens GN=SERBP1 PE=1 SV=2 | SERBP1 | Plasminogen activator inhibitor 1 RNA-binding protein | 4.31 | 0.00 | -1.04 | -0.84 |
| >sp\|P07357\|CO8A_HUMAN Complement component C8 alpha chain OS=Homo sapiens GN=C8A PE=1 SV=2 | C8A | Complement component C8 alpha chain | 4.34 | 0.01 | -0.96 | -0.79 |

Table S3.2B

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >sp\|P51148\|RAB5C_HUMAN Ras-related protein Rab-5C OS=Homo sapiens GN=RAB5C PE=1 SV=2; >sp\|Q58DS9\|RAB5C_BOVIN Ras-related protein Rab-5C OS=Bos taurus GN=RAB5C PE=2 SV=1 | RAB5C | Ras-related protein Rab-5C | 4.79 | 0.01 | 0.94 | 0.78 |
| >sp\|Q96JP2\|MY15B_HUMAN Unconventional myosin-XVB OS=Homo sapiens GN=MYO15B PE=1 SV=2 | MYO15B | Unconventional myosin-XVB | 3.88 | 0.01 | 0.94 | 0.77 |
| >tr\|F5H3C5\|F5H3C5_HUMAN Superoxide dismutase [Mn], mitochondrial (Fragment) OS=Homo sapiens GN=SOD2 PE=1 SV=1; >tr\|F5H4R2\|F5H4R2_HUMAN Superoxide dismutase [Mn], mitochondrial (Fragment) OS=Homo sapiens GN=SOD2 PE=1 SV=1 | SOD2 | Superoxide dismutase [Mn], mitochondrial | 3.83 | 0.01 | 0.95 | 0.77 |
| >sp\|Q9UHR4\|BI2L1_HUMAN Brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 1 OS=Homo sapiens GN=BAIAP2L1 PE=1 SV=2 | BAIAP2L1 | Brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 1 | 4.33 | 0.01 | 0.96 | 0.79 |
| >sp\|P01009\|A1AT_HUMAN Alpha-1-antitrypsin OS=Homo sapiens GN=SERPINA1 PE=1 SV=3 | SERPINA1 | Alpha-1-antitrypsin | 15.45 | 0.00 | 0.96 | 0.89 |
| >sp\|O14745\|NHRF1_HUMAN Na(+)/H(+) exchange regulatory cofactor NHE-RF1 OS=Homo sapiens GN=SLC9A3R1 PE=1 SV=4; >tr\|J3QRP6\|J3QRP6_HUMAN Na(+)/H(+) exchange regulatory cofactor NHE-RF1 (Fragment) OS=Homo sapiens GN=SLC9A3R1 PE=1 SV=1 | SLC9A3R1 | Na(+)/H(+) exchange regulatory cofactor NHE-RF1 | 3.87 | 0.01 | 0.97 | 0.79 |
| >sp\|P12277\|KCRB_HUMAN Creatine kinase B-type OS=Homo sapiens GN=CKB PE=1 SV=1; >sp\|Q5EA61\|KCRB_BOVIN Creatine kinase B-type OS=Bos taurus GN=CKB PE=1 SV=1 | CKB | Creatine kinase B-type | 2.97 | 0.01 | 0.98 | 0.76 |
| >sp\|P35705\|PRDX3_BOVIN Thioredoxin-dependent peroxide reductase, mitochondrial OS=Bos taurus GN=PRDX3 PE=1 SV=2; >sp\|P30048\|PRDX3_HUMAN Thioredoxin-dependent peroxide reductase, mitochondrial OS=Homo sapiens GN=PRDX3 PE=1 SV=3 | PRDX3 | Thioredoxin-dependent peroxide reductase, mitochondrial | 3.89 | 0.01 | 0.98 | 0.79 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >sp\|P02763\|A1AG1_HUMAN Alpha-1-acid glycoprotein 1 OS=Homo sapiens GN=ORM1 PE=1 SV=1 | ORM1 | Alpha-1-acid glycoprotein 1 | 2.68 | 0.01 | 1.01 | 0.77 |
| >tr\|F8VPF3\|F8VPF3_HUMAN Myosin light polypeptide 6 (Fragment) OS=Homo sapiens GN=MYL6 PE=4 SV=1; >tr\|F8W1R7\|F8W1R7_HUMAN Myosin light polypeptide 6 OS=Homo sapiens GN=MYL6 PE=4 SV=1 | MYL6 | Myosin light polypeptide 6 | 3.43 | 0.01 | 1.01 | 0.80 |
| >tr\|A0A075B6R9\|A0A075B6R9_HUMAN Protein IGKV2D-24 (Fragment) OS=Homo sapiens GN=IGKV2D-24 PE=4 SV=1 | IGKV2D-24 | Protein IGKV2D-24 | 3.41 | 0.01 | 1.02 | 0.80 |
| >sp\|P40926\|MDHM_HUMAN Malate dehydrogenase, mitochondrial OS=Homo sapiens GN=MDH2 PE=1 SV=3; >tr\|G3XAL0\|G3XAL0_HUMAN Malate dehydrogenase OS=Homo sapiens GN=MDH2 PE=1 SV=1 | MDH2 | Malate dehydrogenase, mitochondrial; Malate dehydrogenase | 3.22 | 0.01 | 1.02 | 0.79 |
| >tr\|H7C012\|H7C012_HUMAN Phospholipase B1, membrane-associated (Fragment) OS=Homo sapiens GN=PLB1 PE=4 SV=1; >tr\|H7BYX7\|H7BYX7_HUMAN Phospholipase B1, membrane-associated (Fragment) OS=Homo sapiens GN=PLB1 PE=4 SV=1 | PLB1 | Phospholipase B1, membrane-associated | 4.37 | 0.00 | 1.02 | 0.83 |
| >sp\|Q8NDH3\|PEPL1_HUMAN Probable aminopeptidase NPEPL1 OS=Homo sapiens GN=NPEPL1 PE=1 SV=3; >tr\|H0YEP3\|H0YEP3_HUMAN Probable aminopeptidase NPEPL1 (Fragment) OS=Homo sapiens GN=NPEPL1 PE=4 SV=1 | NPEPL1 | Probable aminopeptidase NPEPL1 | 4.98 | 0.00 | 1.02 | 0.84 |
| >sp\|P29622\|KAIN_HUMAN Kallistatin OS=Homo sapiens GN=SERPINA4 PE=1 SV=3 | SERPINA4 | Kallistatin | 3.91 | 0.01 | 1.02 | 0.82 |
| >sp\|Q8N5I2\|ARRD1_HUMAN Arrestin domain-containing protein 1 OS=Homo sapiens GN=ARRDC1 PE=2 SV=1; >tr\|Q5T370\|Q5T370_HUMAN Arrestin domain-containing protein 1 (Fragment) OS=Homo sapiens GN=ARRDC1 PE=4 SV=1 | ARRDC1 | Arrestin domain-containing protein 1 | 3.79 | 0.01 | 1.02 | 0.81 |
| >tr\|A0A075B6S3\|A0A075B6S3_HUMAN Protein IGKV2-30 (Fragment) OS=Homo sapiens GN=IGKV2-30 PE=4 SV=1; >sp\|P06310\|KV206_HUMAN Ig kappa chain V-II region RPMI 6410 OS=Homo sapiens PE=4 SV=1 | IGKV2-30 | Ig kappa chain V-II region RPMI 6410 | 3.41 | 0.01 | 1.04 | 0.81 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >sp\|Q9BV40\|VAMP8_HUMAN Vesicle-associated membrane protein 8 OS=Homo sapiens GN=VAMP8 PE=1 SV=1; >tr\|B8ZZT4\|B8ZZT4_HUMAN Vesicle-associated membrane protein 8 OS=Homo sapiens GN=VAMP8 PE=1 SV=1 | VAMP8 | Vesicle-associated membrane protein 8 | 4.97 | 0.00 | 1.04 | 0.86 |
| >sp\|P15289\|ARSA_HUMAN Arylsulfatase A OS=Homo sapiens GN=ARSA PE=1 SV=3 | ARSA | Arylsulfatase A | 6.15 | 0.00 | 1.04 | 0.88 |
| >tr\|E9PBP6\|E9PBP6_HUMAN Microsomal triglyceride transfer protein large subunit OS=Homo sapiens GN=MTTP PE=1 SV=1; >sp\|P55157\|MTP_HUMAN Microsomal triglyceride transfer protein large subunit OS=Homo sapiens GN=MTTP PE=1 SV=1 | MTTP | Microsomal triglyceride transfer protein large subunit | 3.78 | 0.00 | 1.04 | 0.83 |
| >sp\|P04066\|FUCO_HUMAN Tissue alpha-L-fucosidase OS=Homo sapiens GN=FUCA1 PE=1 SV=4 | FUCA1 | Tissue alpha-L-fucosidase | 4.99 | 0.00 | 1.05 | 0.86 |
| >sp\|P40879\|S26A3_HUMAN Chloride anion exchanger OS=Homo sapiens GN=SLC26A3 PE=1 SV=1 | SLC26A3 | Chloride anion exchanger | 4.33 | 0.00 | 1.06 | 0.85 |
| >sp\|Q12929\|EPS8_HUMAN Epidermal growth factor receptor kinase substrate 8 OS=Homo sapiens GN=EPS8 PE=1 SV=1 | EPS8 | Epidermal growth factor receptor kinase substrate 8 | 3.65 | 0.00 | 1.06 | 0.84 |
| >sp\|Q5E9I6\|ARF3_BOVIN ADP-ribosylation factor 3 OS=Bos taurus GN=ARF3 PE=2 SV=3; >sp\|P84080\|ARF1_BOVIN ADP-ribosylation factor 1 OS=Bos taurus GN=ARF1 PE=1 SV=2 | ARF3; ARF1 | ADP-ribosylation factor 1;ADP-ribosylation factor 3 | 4.43 | 0.00 | 1.07 | 0.86 |
| >tr\|K7EIG7\|K7EIG7_HUMAN Unconventional myosin-Id OS=Homo sapiens GN=MYO1D PE=1 SV=1; >sp\|O94832\|MYO1D_HUMAN Unconventional myosin-Id OS=Homo sapiens GN=MYO1D PE=1 SV=2 | MYO1D | Unconventional myosin-Id | 4.87 | 0.00 | 1.08 | 0.88 |
| >sp\|Q6PIF6\|MYO7B_HUMAN Unconventional myosin-VIIb OS=Homo sapiens GN=MYO7B PE=1 SV=2 | MYO7B | Unconventional myosin-VIIb | 4.43 | 0.00 | 1.08 | 0.87 |
| >sp\|Q9Y2T3\|GUAD_HUMAN Guanine deaminase OS=Homo sapiens GN=GDA PE=1 SV=1; >tr\|Q5SZC6\|Q5SZC6_HUMAN Guanine deaminase OS=Homo sapiens GN=GDA PE=1 SV=1 | GDA | Guanine deaminase | 5.19 | 0.00 | 1.08 | 0.89 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >sp\|P01040\|CYTA_HUMAN Cystatin-A OS=Homo sapiens GN=CSTA PE=1 SV=1; >tr\|C9J0E4\|C9J0E4_HUMAN Cystatin-A OS=Homo sapiens GN=CSTA PE=1 SV=1 | CSTA | Cystatin-A; Cystatin-A, N-terminally processed | 3.41 | 0.00 | 1.09 | 0.85 |
| >tr\|X6RBG4\|X6RBG4_HUMAN Uromodulin OS=Homo sapiens GN=UMOD PE=4 SV=1; >sp\|P07911\|UROM_HUMAN Uromodulin OS=Homo sapiens GN=UMOD PE=1 SV=1 | UMOD | Uromodulin; Uromodulin, secreted form | 4.39 | 0.00 | 1.10 | 0.88 |
| >sp\|Q9ULC5\|ACSL5_HUMAN Long-chain-fatty-acid-CoA ligase 5 OS=Homo sapiens GN=ACSL5 PE=1 SV=1 | ACSL5 | Long-chain-fatty-acid-CoA ligase 5 | 4.96 | 0.00 | 1.10 | 0.89 |
| >sp\|P21589\|5NTD_HUMAN 5-nucleotidase OS=Homo sapiens GN=NT5E PE=1 SV=1; >tr\|Q96B60\|Q96B60_HUMAN 5-nucleotidase OS=Homo sapiens GN=NT5E PE=2 SV=1 | NT5E | 5-nucleotidase | 4.33 | 0.00 | 1.11 | 0.88 |
| >sp\|P12429\|ANXA3_HUMAN Annexin A3 OS=Homo sapiens GN=ANXA3 PE=1 SV=3; >tr\|D6RFG5\|D6RFG5_HUMAN Annexin (Fragment) OS=Homo sapiens GN=ANXA3 PE=1 SV=1 | ANXA3 | Annexin A3; Annexin | 2.92 | 0.00 | 1.12 | 0.84 |
| >tr\|E9PEX6\|E9PEX6_HUMAN Dihydrolipoyl dehydrogenase, mitochondrial OS=Homo sapiens GN=DLD PE=1 SV=1; >sp\|P09622\|DLDH_HUMAN Dihydrolipoyl dehydrogenase, mitochondrial OS=Homo sapiens GN=DLD PE=1 SV=2 | DLD | Dihydrolipoyl dehydrogenase, mitochondrial | 4.89 | 0.00 | 1.16 | 0.93 |
| >sp\|P62833\|RAP1A_BOVIN Ras-related protein Rap-1A OS=Bos taurus GN=RAP1A PE=1 SV=1; >sp\|P61223\|RAP1B_BOVIN Ras-related protein Rap-1b OS=Bos taurus GN=RAP1B PE=2 SV=1 | RAP1A; RAP1B | Ras-related protein Rap-1A; Ras-related protein Rap-1b | 3.58 | 0.00 | 1.18 | 0.90 |
| >sp\|P08582\|TRFM_HUMAN Melanotransferrin OS=Homo sapiens GN=MFI2 PE=1 SV=2 | MFI2 | Melanotransferrin | 4.89 | 0.00 | 1.18 | 0.94 |
| >sp\|Q07075\|AMPE_HUMAN Glutamyl aminopeptidase OS=Homo sapiens GN=ENPEP PE=1 SV=3 | ENPEP | Glutamyl aminopeptidase | 4.85 | 0.00 | 1.20 | 0.96 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >sp\|Q14315\|FLNC_HUMAN Filamin-C OS=Homo sapiens GN=FLNC PE=1 SV=3; >tr\|E1BE25\|E1BE25_BOVIN Uncharacterized protein OS=Bos taurus GN=FLNC PE=4 SV=1 | FLNC | Filamin-C | 6.46 | 0.00 | 1.20 | 0.99 |
| >sp\|Q9UBC5\|MYO1A_HUMAN Unconventional myosin-Ia OS=Homo sapiens GN=MYO1A PE=1 SV=1; >tr\|G3V342\|G3V342_HUMAN Unconventional myosin-Ia OS=Homo sapiens GN=MYO1A PE=4 SV=1 | MYO1A | Unconventional myosin-Ia | 6.18 | 0.00 | 1.20 | 0.99 |
| >sp\|Q9H6S3\|ES8L2_HUMAN Epidermal growth factor receptor kinase substrate 8-like protein 2 OS=Homo sapiens GN=EPS8L2 PE=1 SV=2 | EPS8L2 | Epidermal growth factor receptor kinase substrate 8-like protein 2 | 4.93 | 0.00 | 1.21 | 0.96 |
| >sp\|Q8SQH5\|ADT2_BOVIN ADP/ATP translocase 2 OS=Bos taurus GN=SLC25A5 PE=2 SV=3; >sp\|P05141\|ADT2_HUMAN ADP/ATP translocase 2 OS=Homo sapiens GN=SLC25A5 PE=1 SV=7 | SLC25A5 | ADP/ATP translocase 2; ADP/ATP translocase 2, N-terminally processed | 5.21 | 0.00 | 1.21 | 0.98 |
| >sp\|P48052\|CBPA2_HUMAN Carboxypeptidase A2 OS=Homo sapiens GN=CPA2 PE=1 SV=3 | CPA2 | Carboxypeptidase A2 | 2.71 | 0.00 | 1.25 | 0.90 |
| >sp\|P45879\|VDAC1_BOVIN Voltage-dependent anion-selective channel protein 1 OS=Bos taurus GN=VDAC1 PE=1 SV=3; >tr\|F1MIN1\|F1MIN1_BOVIN Voltage-dependent anion-selective channel protein 1 OS=Bos taurus GN=VDAC1 PE=4 SV=2 | VDAC1 | Voltage-dependent anion-selective channel protein 1 | 5.49 | 0.00 | 1.26 | 1.01 |
| >tr\|A0A087WTM7\|A0A087WTM7_HUMAN Apolipoprotein B-100 OS=Homo sapiens GN=APOB PE=1 SV=1; >sp\|P04114\|APOB_HUMAN Apolipoprotein B-100 OS=Homo sapiens GN=APOB PE=1 SV=2 | APOB | Apolipoprotein B-100 | 3.33 | 0.00 | 1.28 | 0.95 |
| >sp\|P51884\|LUM_HUMAN Lumican OS=Homo sapiens GN=LUM PE=1 SV=2 | LUM | Lumican | 2.81 | 0.00 | 1.30 | 0.93 |
| >sp\|P11678\|PERE_HUMAN Eosinophil peroxidase OS=Homo sapiens GN=EPX PE=1 SV=2 | EPX | Eosinophil peroxidase | 4.21 | 0.00 | 1.30 | 1.00 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >sp\|P29992\|GNA11_HUMAN Guanine nucleotide-binding protein subunit alpha-11 OS=Homo sapiens GN=GNA11 PE=1 SV=2; >sp\|P38409\|GNA11_BOVIN Guanine nucleotide-binding protein subunit alpha-11 OS=Bos taurus GN=GNA11 PE=2 SV=2 | GNA11 | Guanine nucleotide-binding protein subunit alpha-11 | 5.47 | 0.00 | 1.31 | 1.04 |
| >sp\|P05543\|THBG_HUMAN Thyroxine-binding globulin OS=Homo sapiens GN=SERPINA7 PE=1 SV=2 | SERPINA7 | Thyroxine-binding globulin | 5.47 | 0.00 | 1.33 | 1.06 |
| >tr\|C9JFW8\|C9JFW8_HUMAN N-acetylated-alpha-linked acidic dipeptidase-like protein OS=Homo sapiens GN=NAALADL1 PE=4 SV=1; >tr\|C9JX16\|C9JX16_HUMAN N-acetylated-alpha-linked acidic dipeptidase-like protein OS=Homo sapiens GN=NAALADL1 PE=4 SV=2 | NAALADL1 | N-acetylated-alpha-linked acidic dipeptidase-like protein | 6.85 | 0.00 | 1.34 | 1.09 |
| >sp\|P28838\|AMPL_HUMAN Cytosol aminopeptidase OS=Homo sapiens GN=LAP3 PE=1 SV=3 | LAP3 | Cytosol aminopeptidase | 4.63 | 0.00 | 1.34 | 1.04 |
| >sp\|P05154\|IPSP_HUMAN Plasma serine protease inhibitor OS=Homo sapiens GN=SERPINA5 PE=1 SV=3; >tr\|G3V5Q9\|G3V5Q9_HUMAN Plasma serine protease inhibitor (Fragment) OS=Homo sapiens GN=SERPINA5 PE=3 SV=1 | SERPINA5 | Plasma serine protease inhibitor | 3.93 | 0.00 | 1.34 | 1.01 |
| >sp\|Q07837\|SLC31_HUMAN Neutral and basic amino acid transport protein rBAT OS=Homo sapiens GN=SLC3A1 PE=1 SV=2; >tr\|A0A087X0R9\|A0A087X0R9_HUMAN Neutral and basic amino acid transport protein rBAT OS=Homo sapiens GN=SLC3A1 PE=4 SV=1 | SLC3A1 | Neutral and basic amino acid transport protein rBAT | 4.97 | 0.00 | 1.35 | 1.05 |
| >tr\|A0A087WW89\|A0A087WW89_HUMAN Protein IGHV3-72 OS=Homo sapiens GN=IGHV3-72 PE=4 SV=1 | IGHV3-72 | Protein IGHV3-72 | 4.37 | 0.00 | 1.35 | 1.03 |
| >sp\|P07093\|GDN_HUMAN Glia-derived nexin OS=Homo sapiens GN=SERPINE2 PE=1 SV=1 | SERPINE2 | Glia-derived nexin | 5.20 | 0.00 | 1.39 | 1.09 |
| >sp\|P10643\|CO7_HUMAN Complement component C7 OS=Homo sapiens GN=C7 PE=1 SV=2 | C7 | Complement component C7 | 6.34 | 0.00 | 1.40 | 1.12 |
| >tr\|K7EMV3\|K7EMV3_HUMAN Histone H3 OS=Homo sapiens GN=H3F3B PE=1 SV=1; >tr\|B4DEB1\|B4DEB1_HUMAN Histone H3 OS=Homo sapiens GN=H3F3A PE=1 SV=1 | H3F3B; H3F3A | Histone H3 | 3.91 | 0.00 | 1.41 | 1.05 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >sp\|Q9HBB8\|CDHR5_HUMAN Cadherin-related family member 5 OS=Homo sapiens GN=CDHR5 PE=1 SV=3 | CDHR5 | Cadherin-related family member 5 | 4.95 | 0.00 | 1.43 | 1.10 |
| >sp\|Q9BYF1\|ACE2_HUMAN Angiotensin-converting enzyme 2 OS=Homo sapiens GN=ACE2 PE=1 SV=2 | ACE2 | Angiotensin-converting enzyme 2 | 5.57 | 0.00 | 1.43 | 1.12 |
| >sp\|P09210\|GSTA2_HUMAN Glutathione S-transferase A2 OS=Homo sapiens GN=GSTA2 PE=1 SV=4; >sp\|P08263\|GSTA1_HUMAN Glutathione S-transferase A1 OS=Homo sapiens GN=GSTA1 PE=1 SV=3 | GSTA2; GSTA1 | Glutathione S-transferase A2; Glutathione S-transferase A1 | 5.51 | 0.00 | 1.44 | 1.13 |
| >tr\|H3BMH2\|H3BMH2_HUMAN Ras-related protein Rab-11A (Fragment) OS=Homo sapiens GN=RAB11A PE=3 SV=1; >tr\|H3BSC1\|H3BSC1_HUMAN Ras-related protein Rab-11A OS=Homo sapiens GN=RAB11A PE=1 SV=1 | RAB11A | Ras-related protein Rab-11A | 6.17 | 0.00 | 1.44 | 1.15 |
| >tr\|E7ER45\|E7ER45_HUMAN Maltase-glucoamylase, intestinal OS=Homo sapiens GN=MGAM PE=3 SV=2; >sp\|O43451\|MGA_HUMAN Maltase-glucoamylase, intestinal OS=Homo sapiens GN=MGAM PE=1 SV=5 | MGAM | Maltase-glucoamylase, intestinal | 5.81 | 0.00 | 1.46 | 1.14 |
| >sp\|Q14002\|CEAM7_HUMAN Carcinoembryonic antigen-related cell adhesion molecule 7 OS=Homo sapiens GN=CEACAM7 PE=1 SV=1 | CEACAM7 | Carcinoembryonic antigen-related cell adhesion molecule 7 | 5.49 | 0.00 | 1.46 | 1.14 |
| >sp\|Q6UX06\|OLFM4_HUMAN Olfactomedin-4 OS=Homo sapiens GN=OLFM4 PE=1 SV=1 | OLFM4 | Olfactomedin-4 | 5.76 | 0.00 | 1.48 | 1.16 |
| >tr\|R4GN98\|R4GN98_HUMAN Protein S100 (Fragment) OS=Homo sapiens GN=S100A6 PE=1 SV=1; >sp\|P06703\|S10A6_HUMAN Protein S100-A6 OS=Homo sapiens GN=S100A6 PE=1 SV=1 | S100A6 | Protein S100; Protein S100-A6 | 5.41 | 0.00 | 1.48 | 1.15 |
| >sp\|Q92820\|GGH_HUMAN Gamma-glutamyl hydrolase OS=Homo sapiens GN=GGH PE=1 SV=2 | GGH | Gamma-glutamyl hydrolase | 5.58 | 0.00 | 1.49 | 1.16 |
| >sp\|P62871\|GBB1_BOVIN Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 OS=Bos taurus GN=GNB1 PE=1 SV=3; >sp\|P62873\|GBB1_HUMAN Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 OS=Homo sapiens GN=GNB1 PE=1 SV=3 | GNB1 | Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 | 5.58 | 0.00 | 1.49 | 1.16 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >sp\|P13866\|SC5A1_HUMAN Sodium/glucose cotransporter 1 OS=Homo sapiens GN=SLC5A1 PE=1 SV=1 | SLC5A1 | Sodium/glucose cotransporter 1 | 6.89 | 0.00 | 1.49 | 1.20 |
| >sp\|P12104\|FABPI_HUMAN Fatty acid-binding protein, intestinal OS=Homo sapiens GN=FABP2 PE=1 SV=2 | FABP2 | Fatty acid-binding protein, intestinal | 6.82 | 0.00 | 1.50 | 1.20 |
| >sp\|P00915\|CAH1_HUMAN Carbonic anhydrase 1 OS=Homo sapiens GN=CA1 PE=1 SV=2; >tr\|E5RHP7\|E5RHP7_HUMAN Carbonic anhydrase 1 (Fragment) OS=Homo sapiens GN=CA1 PE=1 SV=1 | CA1 | Carbonic anhydrase 1 | 3.33 | 0.00 | 1.51 | 1.07 |
| >tr\|F5H265\|F5H265_HUMAN Polyubiquitin-C (Fragment) OS=Homo sapiens GN=UBC PE=4 SV=1; >tr\|J3QS39\|J3QS39_HUMAN Ubiquitin (Fragment) OS=Homo sapiens GN=UBB PE=4 SV=1 | UBC; UBB | Ubiquitin-60S ribosomal protein L40 | 5.93 | 0.00 | 1.57 | 1.22 |
| >sp\|Q13228\|SBP1_HUMAN Selenium-binding protein 1 OS=Homo sapiens GN=SELENBP1 PE=1 SV=2 | SELENBP1 | Selenium-binding protein 1 | 4.84 | 0.00 | 1.58 | 1.19 |
| >sp\|Q5JS37\|NHLC3_HUMAN NHL repeat-containing protein 3 OS=Homo sapiens GN=NHLRC3 PE=2 SV=1; >tr\|C9J973\|C9J973_HUMAN NHL repeat-containing protein 3 OS=Homo sapiens GN=NHLRC3 PE=4 SV=1 | NHLRC3 | NHL repeat-containing protein 3 | 7.60 | 0.00 | 1.58 | 1.27 |
| >sp\|P01762\|HV301_HUMAN Ig heavy chain V-III region TRO OS=Homo sapiens PE=1 SV=1 | | Ig heavy chain V-III region TRO | 4.05 | 0.00 | 1.62 | 1.17 |
| >sp\|P07339\|CATD_HUMAN Cathepsin D OS=Homo sapiens GN=CTSD PE=1 SV=1; >tr\|F8W787\|F8W787_HUMAN Cathepsin D light chain (Fragment) OS=Homo sapiens GN=CTSD PE=3 SV=1 | CTSD | Cathepsin D; Cathepsin D light chain | 7.08 | 0.00 | 1.65 | 1.30 |
| >sp\|P05155\|IC1_HUMAN Plasma protease C1 inhibitor OS=Homo sapiens GN=SERPING1 PE=1 SV=2; >tr\|E9PGN7\|E9PGN7_HUMAN Plasma protease C1 inhibitor OS=Homo sapiens GN=SERPING1 PE=3 SV=1 | SERPING1 | Plasma protease C1 inhibitor | 5.00 | 0.00 | 1.66 | 1.23 |
| >sp\|P02748\|CO9_HUMAN Complement component C9 OS=Homo sapiens GN=C9 PE=1 SV=2 | C9 | Complement component C9 | 5.26 | 0.00 | 1.70 | 1.27 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >tr\|A0A087X1V9\|A0A087X1V9_HUMAN Protein IGKV2-28 OS=Homo sapiens GN=IGKV2-28 PE=4 SV=1; >sp\|P01617\|KV204_HUMAN Ig kappa chain V-II region TEW OS=Homo sapiens PE=1 SV=1 | IGKV2D-28 | Ig kappa chain V-II region TEW | 4.00 | 0.00 | 1.75 | 1.23 |
| >sp\|O75264\|SIM24_HUMAN Small integral membrane protein 24 OS=Homo sapiens GN=SMIM24 PE=2 SV=2; >tr\|K7EKM7\|K7EKM7_HUMAN Small integral membrane protein 24 OS=Homo sapiens GN=SMIM24 PE=4 SV=1 | SMIM24 | Small integral membrane protein 24 | 7.45 | 0.00 | 1.76 | 1.38 |
| >sp\|Q6UWP2\|DHR11_HUMAN Dehydrogenase/reductase SDR family member 11 OS=Homo sapiens GN=DHRS11 PE=1 SV=1; >sp\|Q3ZBV9\|DHR11_BOVIN Dehydrogenase/reductase SDR family member 11 OS=Bos taurus GN=DHRS11 PE=2 SV=1 | DHRS11 | Dehydrogenase/ reductase SDR family member 11 | 8.43 | 0.00 | 1.79 | 1.42 |
| >sp\|Q9UBI6\|GBG12_HUMAN Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12 OS=Homo sapiens GN=GNG12 PE=1 SV=3 | GNG12 | Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12 | 8.50 | 0.00 | 1.82 | 1.44 |
| >sp\|P01598\|KV106_HUMAN Ig kappa chain V-I region EU OS=Homo sapiens PE=1 SV=1; >tr\|A0A075B6S8\|A0A075B6S8_HUMAN Ig kappa chain V-I region HK102 (Fragment) OS=Homo sapiens GN=IGKV1-5 PE=4 SV=1 | IGKV1-5 | Ig kappa chain V-I region EU | 5.06 | 0.00 | 1.85 | 1.34 |
| >sp\|P55259\|GP2_HUMAN Pancreatic secretory granule membrane major glycoprotein GP2 OS=Homo sapiens GN=GP2 PE=2 SV=3 | GP2 | Pancreatic secretory granule membrane major glycoprotein GP2 | 6.52 | 0.00 | 1.87 | 1.41 |
| >sp\|P15085\|CBPA1_HUMAN Carboxypeptidase A1 OS=Homo sapiens GN=CPA1 PE=1 SV=2; >tr\|C9JUF9\|C9JUF9_HUMAN Carboxypeptidase A1 OS=Homo sapiens GN=CPA1 PE=4 SV=1 | CPA1 | Carboxypeptidase A1 | 5.85 | 0.00 | 1.88 | 1.39 |
| >sp\|Q92542\|NICA_HUMAN Nicastrin OS=Homo sapiens GN=NCSTN PE=1 SV=2; >tr\|H0Y6T7\|H0Y6T7_HUMAN Nicastrin (Fragment) OS=Homo sapiens GN=NCSTN PE=1 SV=1 | NCSTN | Nicastrin | 10.39 | 0.00 | 1.89 | 1.53 |
| >sp\|P09327\|VILI_HUMAN Villin-1 OS=Homo sapiens GN=VIL1 PE=1 SV=4 | VIL1 | Villin-1 | 8.25 | 0.00 | 1.93 | 1.50 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >sp\|P50443\|S26A2_HUMAN Sulfate transporter OS=Homo sapiens GN=SLC26A2 PE=1 SV=2 | SLC26A2 | Sulfate transporter | 11.21 | 0.00 | 2.01 | 1.62 |
| >sp\|Q6UXC1\|AEGP_HUMAN Apical endosomal glycoprotein OS=Homo sapiens GN=MAMDC4 PE=1 SV=2 | MAMDC4 | Apical endosomal glycoprotein | 9.30 | 0.00 | 2.02 | 1.58 |
| >sp\|P00390\|GSHR_HUMAN Glutathione reductase, mitochondrial OS=Homo sapiens GN=GSR PE=1 SV=2 | GSR | Glutathione reductase, mitochondrial | 10.27 | 0.00 | 2.02 | 1.61 |
| >sp\|P02794\|FRIH_HUMAN Ferritin heavy chain OS=Homo sapiens GN=FTH1 PE=1 SV=2;<br>>tr\|G3V192\|G3V192_HUMAN Ferritin OS=Homo sapiens GN=FTH1 PE=1 SV=1 | FTH1 | Ferritin heavy chain; Ferritin | 6.43 | 0.00 | 2.08 | 1.52 |
| >tr\|A6XMV8\|A6XMV8_HUMAN Protease serine 2 preproprotein OS=Homo sapiens GN=PRSS3P2 PE=2 SV=1;<br>>tr\|A0A096LNX4\|A0A096LNX4_HUMAN Putative trypsin-6 OS=Homo sapiens GN=PRSS3P2 PE=4 SV=1 | PRSS2 | Trypsin-2 | 5.94 | 0.00 | 2.08 | 1.50 |
| >sp\|P09848\|LPH_HUMAN Lactase-phlorizin hydrolase OS=Homo sapiens GN=LCT PE=1 SV=3;<br>>tr\|H0Y4E4\|H0Y4E4_HUMAN Lactase (Fragment) OS=Homo sapiens GN=LCT PE=3 SV=1 | LCT | Lactase-phlorizin hydrolase;<br>Lactase | 13.66 | 0.00 | 2.09 | 1.73 |
| >sp\|Q9BYE9\|CDHR2_HUMAN Cadherin-related family member 2 OS=Homo sapiens GN=CDHR2 PE=1 SV=2 | CDHR2 | Cadherin-related family member 2 | 10.00 | 0.00 | 2.10 | 1.65 |
| >sp\|P07148\|FABPL_HUMAN Fatty acid-binding protein, liver OS=Homo sapiens GN=FABP1 PE=1 SV=1;<br>>tr\|A8MW49\|A8MW49_HUMAN Fatty acid-binding protein, liver OS=Homo sapiens GN=FABP1 PE=1 SV=1 | FABP1 | Fatty acid-binding protein, liver | 8.51 | 0.00 | 2.10 | 1.61 |
| >sp\|Q8TE67\|ES8L3_HUMAN Epidermal growth factor receptor kinase substrate 8-like protein 3 OS=Homo sapiens GN=EPS8L3 PE=1 SV=2 | EPS8L3 | Epidermal growth factor receptor kinase substrate 8-like protein 3 | 9.61 | 0.00 | 2.10 | 1.64 |
| >sp\|Q9NR71\|ASAH2_HUMAN Neutral ceramidase OS=Homo sapiens GN=ASAH2 PE=1 SV=2;<br>>tr\|E9PBM9\|E9PBM9_HUMAN Neutral ceramidase soluble form OS=Homo sapiens GN=ASAH2 PE=4 SV=1 | ASAH2;<br>ASAH2C | Neutral ceramidase;<br>Neutral ceramidase soluble form | 13.00 | 0.00 | 2.11 | 1.73 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >sp\|Q03154\|ACY1_HUMAN Aminoacylase-1 OS=Homo sapiens GN=ACY1 PE=1 SV=1; >tr\|C9JMV9\|C9JMV9_HUMAN Protein ABHD14A-ACY1 OS=Homo sapiens GN=ABHD14A-ACY1 PE=4 SV=1 | ACY1; ABHD14A-ACY1 | Aminoacylase-1 | 11.79 | 0.00 | 2.13 | 1.72 |
| >sp\|P24855\|DNAS1_HUMAN Deoxyribonuclease-1 OS=Homo sapiens GN=DNASE1 PE=1 SV=1 | DNASE1 | Deoxyribonuclease-1 | 10.81 | 0.00 | 2.18 | 1.73 |
| >sp\|P13688\|CEAM1_HUMAN Carcinoembryonic antigen-related cell adhesion molecule 1 OS=Homo sapiens GN=CEACAM1 PE=1 SV=2 | CEACAM1 | Carcinoembryonic antigen-related cell adhesion molecule 1 | 13.38 | 0.00 | 2.25 | 1.83 |
| >sp\|P06731\|CEAM5_HUMAN Carcinoembryonic antigen-related cell adhesion molecule 5 OS=Homo sapiens GN=CEACAM5 PE=1 SV=3; >tr\|A0A024R0K5\|A0A024R0K5_HUMAN Carcinoembryonic antigen-related cell adhesion molecule 5 OS=Homo sapiens GN=CEACAM5 PE=4 SV=1 | CEACAM5 | Carcinoembryonic antigen-related cell adhesion molecule 5 | 12.77 | 0.00 | 2.33 | 1.87 |
| >sp\|P01717\|LV403_HUMAN Ig lambda chain V-IV region Hil OS=Homo sapiens PE=1 SV=1 | | Ig lambda chain V-IV region Hil | 9.82 | 0.00 | 2.34 | 1.79 |
| >sp\|Q6UWV6\|ENPP7_HUMAN Ectonucleotide pyrophosphatase/ phosphodiesterase family member 7 OS=Homo sapiens GN=ENPP7 PE=1 SV=3 | ENPP7 | Ectonucleotide pyrophosphatase/ phosphodiesterase family member 7 | 12.99 | 0.00 | 2.35 | 1.89 |
| >sp\|P20933\|ASPG_HUMAN N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase OS=Homo sapiens GN=AGA PE=1 SV=2; >tr\|H0Y9C7\|H0Y9C7_HUMAN N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase (Fragment) OS=Homo sapiens GN=AGA PE=1 SV=3 | AGA | N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase | 19.10 | 0.00 | 2.40 | 2.04 |
| >sp\|Q9H3R2\|MUC13_HUMAN Mucin-13 OS=Homo sapiens GN=MUC13 PE=1 SV=3 | MUC13 | Mucin-13 | 7.86 | 0.00 | 2.41 | 1.76 |
| >sp\|P51688\|SPHM_HUMAN N-sulphoglucosamine sulphohydrolase OS=Homo sapiens GN=SGSH PE=1 SV=1; >tr\|I3NI22\|I3NI22_HUMAN N-sulphoglucosamine sulphohydrolase (Fragment) OS=Homo sapiens GN=SGSH PE=4 SV=1 | SGSH | N-sulphoglucosamine sulphohydrolase | 18.92 | 0.00 | 2.44 | 2.07 |
| >sp\|P27216\|ANX13_HUMAN Annexin A13 OS=Homo sapiens GN=ANXA13 PE=1 SV=3 | ANXA13 | Annexin A13 | 11.22 | 0.00 | 2.46 | 1.91 |

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|---|---|---|---|---|---|---|
| >sp\|Q92485\|ASM3B_HUMAN Acid sphingomyelinase-like phosphodiesterase 3b OS=Homo sapiens GN=SMPDL3B PE=2 SV=2; >tr\|F8VWW8\|F8VWW8_HUMAN Acid sphingomyelinase-like phosphodiesterase 3b OS=Homo sapiens GN=SMPDL3B PE=4 SV=1 | SMPDL3B | Acid sphingomyelinase-like phosphodiesterase 3b | 13.81 | 0.00 | 2.47 | 1.98 |
| >sp\|P01623\|KV305_HUMAN Ig kappa chain V-III region WOL OS=Homo sapiens PE=1 SV=1; >sp\|P18135\|KV312_HUMAN Ig kappa chain V-III region HAH OS=Homo sapiens PE=2 SV=1 | | Ig kappa chain V-III region WOL; Ig kappa chain V-III region HAH | 8.23 | 0.00 | 2.48 | 1.82 |
| >tr\|D6RB89\|D6RB89_HUMAN Retinol-binding protein 2 (Fragment) OS=Homo sapiens GN=RBP2 PE=3 SV=1; >sp\|P50120\|RET2_HUMAN Retinol-binding protein 2 OS=Homo sapiens GN=RBP2 PE=1 SV=3 | RBP2 | Retinol-binding protein 2 | 15.34 | 0.00 | 2.54 | 2.06 |
| >tr\|A0A087WW55\|A0A087WW55_HUMAN Trypsin-1 OS=Homo sapiens GN=PRSS1 PE=4 SV=1; >tr\|E7EQ64\|E7EQ64_HUMAN Trypsin-1 OS=Homo sapiens GN=PRSS1 PE=3 SV=1 | PRSS1 | Trypsin-1 | 9.02 | 0.00 | 2.59 | 1.91 |
| >tr\|E7EMM4\|E7EMM4_HUMAN Acid ceramidase OS=Homo sapiens GN=ASAH1 PE=1 SV=1; >sp\|Q13510\|ASAH1_HUMAN Acid ceramidase OS=Homo sapiens GN=ASAH1 PE=1 SV=5 | ASAH1 | Acid ceramidase | 16.42 | 0.00 | 2.61 | 2.13 |
| >tr\|A0A087WXI2\|A0A087WXI2_HUMAN IgGFc-binding protein OS=Homo sapiens GN=FCGBP PE=4 SV=1; >sp\|Q9Y6R7\|FCGBP_HUMAN IgGFc-binding protein OS=Homo sapiens GN=FCGBP PE=1 SV=3 | FCGBP | IgGFc-binding protein | 15.33 | 0.00 | 2.64 | 2.13 |
| >sp\|P09093\|CEL3A_HUMAN Chymotrypsin-like elastase family member 3A OS=Homo sapiens GN=CELA3A PE=1 SV=3 | CELA3A | Chymotrypsin-like elastase family member 3A | 9.85 | 0.00 | 2.64 | 1.97 |
| >tr\|A0A087WYY1\|A0A087WYY1_HUMAN Carboxypeptidase Q OS=Homo sapiens GN=CPQ PE=4 SV=1; >sp\|Q9Y646\|CBPQ_HUMAN Carboxypeptidase Q OS=Homo sapiens GN=CPQ PE=1 SV=1 | CPQ | Carboxypeptidase Q | 19.09 | 0.00 | 2.68 | 2.24 |

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|---|---|---|---|---|---|---|
| >sp\|P16444\|DPEP1_HUMAN Dipeptidase 1 OS=Homo sapiens GN=DPEP1 PE=1 SV=3 | DPEP1 | Dipeptidase 1 | 14.24 | 0.00 | 2.69 | 2.14 |
| >sp\|P12821\|ACE_HUMAN Angiotensin-converting enzyme OS=Homo sapiens GN=ACE PE=1 SV=1 | ACE | Angiotensin-converting enzyme | 12.59 | 0.00 | 2.70 | 2.09 |
| >sp\|Q04609\|FOLH1_HUMAN Glutamate carboxypeptidase 2 OS=Homo sapiens GN=FOLH1 PE=1 SV=1; >sp\|Q9HBA9\|FOH1B_HUMAN Putative N-acetylated-alpha-linked acidic dipeptidase OS=Homo sapiens GN=FOLH1B PE=2 SV=1 | FOLH1; FOLH1B | Glutamate carboxypeptidase 2; Putative N-acetylated-alpha-linked acidic dipeptidase | 19.76 | 0.00 | 2.75 | 2.30 |
| >sp\|P15144\|AMPN_HUMAN Aminopeptidase N OS=Homo sapiens GN=ANPEP PE=1 SV=4 | ANPEP | Aminopeptidase N | 17.91 | 0.00 | 2.78 | 2.28 |
| >sp\|P02743\|SAMP_HUMAN Serum amyloid P-component OS=Homo sapiens GN=APCS PE=1 SV=2 | APCS | Serum amyloid P-component | 17.26 | 0.00 | 2.82 | 2.30 |
| >sp\|O60844\|ZG16_HUMAN Zymogen granule membrane protein 16 OS=Homo sapiens GN=ZG16 PE=1 SV=2 | ZG16 | Zymogen granule membrane protein 16 | 16.02 | 0.00 | 2.83 | 2.27 |
| >sp\|P09525\|ANXA4_HUMAN Annexin A4 OS=Homo sapiens GN=ANXA4 PE=1 SV=4; >tr\|Q6P452\|Q6P452_HUMAN Annexin OS=Homo sapiens GN=ANXA4 PE=1 SV=1 | ANXA4 | Annexin A4; Annexin | 14.19 | 0.00 | 2.85 | 2.23 |
| >sp\|P02766\|TTHY_HUMAN Transthyretin OS=Homo sapiens GN=TTR PE=1 SV=1; >tr\|A0A087WT59\|A0A087WT59_HUMAN Transthyretin OS=Homo sapiens GN=TTR PE=4 SV=1 | TTR | Transthyretin | 11.52 | 0.00 | 2.91 | 2.18 |
| >sp\|P14410\|SUIS_HUMAN Sucrase-isomaltase, intestinal OS=Homo sapiens GN=SI PE=1 SV=6 | SI | Sucrase-isomaltase, intestinal | 19.66 | 0.00 | 2.93 | 2.42 |
| >sp\|P02792\|FRIL_HUMAN Ferritin light chain OS=Homo sapiens GN=FTL PE=1 SV=2; >tr\|A0A087X1B9\|A0A087X1B9_HUMAN Ferritin light chain OS=Homo sapiens GN=FTL PE=1 SV=1 | FTL | Ferritin light chain | 13.89 | 0.00 | 2.94 | 2.28 |

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|---|---|---|---|---|---|---|
| >sp\|P01625\|KV402_HUMAN Ig kappa chain V-IV region Len OS=Homo sapiens PE=1 SV=2; >sp\|P06314\|KV404_HUMAN Ig kappa chain V-IV region B17 OS=Homo sapiens PE=2 SV=1 | | Ig kappa chain V-IV region Len; Ig kappa chain V-IV region B17 | 17.79 | 0.00 | 2.94 | 2.38 |
| >tr\|H7C3P4\|H7C3P4_HUMAN Glucosamine (N-acetyl)-6-sulfatase (Sanfilippo disease IIID), isoform CRA_b OS=Homo sapiens GN=GNS PE=1 SV=1; >tr\|F6S8M0\|F6S8M0_HUMAN N-acetylglucosamine-6-sulfatase OS=Homo sapiens GN=GNS PE=1 SV=1 | GNS | N-acetylglucosamine-6-sulfatase | 23.97 | 0.00 | 2.96 | 2.52 |
| >sp\|P00441\|SODC_HUMAN Superoxide dismutase [Cu-Zn] OS=Homo sapiens GN=SOD1 PE=1 SV=2; >tr\|H7BYH4\|H7BYH4_HUMAN Superoxide dismutase [Cu-Zn] OS=Homo sapiens GN=SOD1 PE=1 SV=1 | SOD1 | Superoxide dismutase [Cu-Zn] | 26.28 | 0.00 | 2.99 | 2.58 |
| >tr\|A0A075B6I8\|A0A075B6I8_HUMAN Protein IGLV1-47 (Fragment) OS=Homo sapiens GN=IGLV1-47 PE=4 SV=1 | IGLV1-47 | Protein IGLV1-47 | 17.13 | 0.00 | 3.02 | 2.42 |
| >sp\|P35237\|SPB6_HUMAN Serpin B6 OS=Homo sapiens GN=SERPINB6 PE=1 SV=3; >tr\|A0A024QZX5\|A0A024QZX5_HUMAN Serpin B6 OS=Homo sapiens GN=SERPINB6 PE=1 SV=1 | SERPINB6 | Serpin B6 | 20.12 | 0.00 | 3.04 | 2.51 |
| >sp\|P56470\|LEG4_HUMAN Galectin-4 OS=Homo sapiens GN=LGALS4 PE=1 SV=1; >tr\|M0QZ93\|M0QZ93_HUMAN Galectin (Fragment) OS=Homo sapiens GN=LGALS4 PE=4 SV=1 | LGALS4 | Galectin-4; Galectin | 20.51 | 0.00 | 3.37 | 2.74 |
| >sp\|P27487\|DPP4_HUMAN Dipeptidyl peptidase 4 OS=Homo sapiens GN=DPP4 PE=1 SV=2 | DPP4 | Dipeptidyl peptidase 4 | 16.53 | 0.00 | 3.40 | 2.64 |
| >sp\|Q8WWA0\|ITLN1_HUMAN Intelectin-1 OS=Homo sapiens GN=ITLN1 PE=1 SV=1 | ITLN1 | Intelectin-1 | 53.14 | 0.00 | 3.45 | 3.25 |
| >sp\|Q02817\|MUC2_HUMAN Mucin-2 OS=Homo sapiens GN=MUC2 PE=1 SV=2 | MUC2 | Mucin-2 | 27.59 | 0.00 | 3.55 | 3.02 |

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|---|---|---|---|---|---|---|
| >tr\|B7ZL91\|B7ZL91_HUMAN Metalloendopeptidase OS=Homo sapiens GN=MEP1A PE=2 SV=1; >sp\|Q16819\|MEP1A_HUMAN Meprin A subunit alpha OS=Homo sapiens GN=MEP1A PE=1 SV=2 | MEP1A | Metalloendopeptidase; Meprin A subunit alpha | 35.26 | 0.00 | 3.56 | 3.16 |
| >sp\|Q99895\|CTRC_HUMAN Chymotrypsin-C OS=Homo sapiens GN=CTRC PE=1 SV=2 | CTRC | Chymotrypsin-C | 26.23 | 0.00 | 3.74 | 3.12 |
| >sp\|O43895\|XPP2_HUMAN Xaa-Pro aminopeptidase 2 OS=Homo sapiens GN=XPNPEP2 PE=1 SV=3 | XPNPEP2 | Xaa-Pro aminopeptidase 2 | 33.83 | 0.00 | 3.79 | 3.31 |
| >sp\|P08473\|NEP_HUMAN Neprilysin OS=Homo sapiens GN=MME PE=1 SV=2 | MME | Neprilysin | 39.78 | 0.00 | 3.99 | 3.56 |
| >sp\|A8K7I4\|CLCA1_HUMAN Calcium-activated chloride channel regulator 1 OS=Homo sapiens GN=CLCA1 PE=1 SV=3 | CLCA1 | Calcium-activated chloride channel regulator 1 | 21.08 | 0.00 | 4.02 | 3.16 |
| >sp\|P09923\|PPBI_HUMAN Intestinal-type alkaline phosphatase OS=Homo sapiens GN=ALPI PE=1 SV=2 | ALPI | Intestinal-type alkaline phosphatase | 44.98 | 0.00 | 4.50 | 4.06 |

Proteins derived from human or bovine source that were more abundant in (A) gastric aspirates or (B) feces. A Student's T-test difference below zero indicates the protein is higher in abundance in gastric aspirates compared to feces. *P*-values are -$\log_{10}$-transformed. In case protein groups consisted of multiple proteins, the two proteins with highest protein existence (PE) value were selected.

**Table S3.3** Tables of RDA data.

Table S3.3A

| | Df | AIC | F | Pr(>F) |
|---|---|---|---|---|
| Gestational age | 1 | 388.1 | 2.648 | 0.0075** |
| Percentage parenteral feeding | 1 | 386 | 4.092 | 0.0075** |
| Duration first antibiotic treatment | 3 | 387.2 | 1.506 | 0.0500* |

Table S3.3B

| | R2.adj | Df | AIC | F | Pr(>F) |
|---|---|---|---|---|---|
| Percentage parenteral feeding | 0.04668 | 1 | 386.3 | 4.5256 | 0.002** |
| All variables | 0.07647 | | | | |

Table S3.3C

| | RDA1 | RDA2 |
|---|---|---|
| Eigenvalue | 15.5138 | 7.137 |
| Proportion explained | 0.5215 | 0.24 |
| Cumulative proportion | 0.5215 | 0.761 |

(A) ANOVA table, (B) $R_2$-adjusted table and (C) accumulated constrained eigenvalues. Scaling 2 for species and site scores. Species are scaled proportional to eigenvalues. Sites are unscaled: weighted dispersion equal on all dimensions. General scaling constant of scores: 10.99205. $^{**}P \leq 0.01$, $^{*}P \leq 0.05$. *P*-values are adjusted with False Discovery Rate (FDR).

**Table S3.4** Differentially abundant human- and bovine-derived proteins in feces during the first six postnatal weeks between gestational age groups preterm (25–31 weeks of gestation) and full-term (37–41 weeks of gestation).

Table S3.4A

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >sp\|P01717\|LV403_HUMAN Ig lambda chain V-IV region Hil OS=Homo sapiens PE=1 SV=1 | | Ig lambda chain V-IV region Hil | 11.57 | 0.00 | -2.67 | -2.03 |
| >tr\|H7C3P4\|H7C3P4_HUMAN Glucosamine (N-acetyl)-6-sulfatase (Sanfilippo disease IIID), isoform CRA_b OS=Homo sapiens GN=GNS PE=1 SV=1; >tr\|F6S8M0\|F6S8M0_HUMAN N-acetylglucosamine-6-sulfatase OS=Homo sapiens GN=GNS PE=1 SV=1 | GNS | N-acetylglucosamine-6-sulfatase | 11.23 | 0.00 | -2.55 | -1.95 |
| >sp\|P01625\|KV402_HUMAN Ig kappa chain V-IV region Len OS=Homo sapiens PE=1 SV=2; >sp\|P06314\|KV404_HUMAN Ig kappa chain V-IV region B17 OS=Homo sapiens PE=2 SV=1 | | Ig kappa chain V-IV region Len; Ig kappa chain V-IV region B17 | 9.17 | 0.00 | -2.23 | -1.70 |
| >sp\|Q04609\|FOLH1_HUMAN Glutamate carboxypeptidase 2 OS=Homo sapiens GN=FOLH1 PE=1 SV=1; >sp\|Q9HBA9\|FOH1B_HUMAN Putative N-acetylated-alpha-linked acidic dipeptidase OS=Homo sapiens GN=FOLH1B PE=2 SV=1 | FOLH1; FOLH1B | Glutamate carboxypeptidase 2; Putative N-acetylated-alpha-linked acidic dipeptidase | 10.03 | 0.00 | -2.18 | -1.69 |
| >sp\|P80748\|LV302_HUMAN Ig lambda chain V-III region LOI OS=Homo sapiens PE=1 SV=1 | | Ig lambda chain V-III region LOI | 4.00 | 0.01 | -1.90 | -1.30 |
| >tr\|D6RB89\|D6RB89_HUMAN Retinol-binding protein 2 (Fragment) OS=Homo sapiens GN=RBP2 PE=3 SV=1; >sp\|P50120\|RET2_HUMAN Retinol-binding protein 2 OS=Homo sapiens GN=RBP2 PE=1 SV=3 | RBP2 | Retinol-binding protein 2 | 5.25 | 0.01 | -1.83 | -1.34 |
| >sp\|P12821\|ACE_HUMAN Angiotensin-converting enzyme OS=Homo sapiens GN=ACE PE=1 SV=1 | ACE | Angiotensin-converting enzyme; Angiotensin-converting enzyme, soluble form | 9.68 | 0.01 | -1.67 | -1.36 |

Table S3.4B

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >tr\|I3L4Y3\|I3L4Y3_HUMAN Pancreatic secretory granule membrane major glycoprotein GP2 (Fragment) OS=Homo sapiens GN=GP2 PE=4 SV=1; >tr\|I3L486\|I3L486_HUMAN Pancreatic secretory granule membrane major glycoprotein GP2 (Fragment) OS=Homo sapiens GN=GP2 PE=1 SV=1 | GP2 | Pancreatic secretory granule membrane major glycoprotein GP2 (Fragment) | 28.84 | 0.00 | 3.00 | 2.59 |
| >tr\|A0A096LPK4\|A0A096LPK4_HUMAN Mucin-5AC OS=Homo sapiens GN=MUC5AC PE=4 SV=1; >sp\|P98088\|MUC5A_HUMAN Mucin-5AC (Fragments) OS=Homo sapiens GN=MUC5AC PE=1 SV=3 | MUC5AC | Mucin-5AC | 13.55 | 0.00 | 2.71 | 2.11 |
| >sp\|Q86UP6\|CUZD1_HUMAN CUB and zona pellucida-like domain-containing protein 1 OS=Homo sapiens GN=CUZD1 PE=2 SV=1 | CUZD1 | CUB and zona pellucida-like domain-containing protein 1 | 8.59 | 0.00 | 1.86 | 1.46 |
| >sp\|P15086\|CBPB1_HUMAN Carboxypeptidase B OS=Homo sapiens GN=CPB1 PE=1 SV=4 | CPB1 | Carboxypeptidase B | 5.25 | 0.01 | 1.87 | 1.36 |
| >tr\|D6RD17\|D6RD17_HUMAN Immunoglobulin J chain (Fragment) OS=Homo sapiens GN=IGJ PE=1 SV=3; >sp\|P01591\|IGJ_HUMAN Immunoglobulin J chain OS=Homo sapiens GN=IGJ PE=1 SV=4 | IGJ | Immunoglobulin J chain | 3.80 | 0.00 | 2.22 | 1.42 |
| >sp\|O95497\|VNN1_HUMAN Pantetheinase OS=Homo sapiens GN=VNN1 PE=1 SV=2 | VNN1 | Pantetheinase | 7.07 | 0.00 | 2.25 | 1.64 |
| >sp\|Q07654\|TFF3_HUMAN Trefoil factor 3 OS=Homo sapiens GN=TFF3 PE=1 SV=1; >tr\|X6R3S7\|X6R3S7_HUMAN Trefoil factor 3 OS=Homo sapiens GN=TFF3 PE=4 SV=2 | TFF3 | Trefoil factor 3 | 16.04 | 0.00 | 2.48 | 2.02 |
| >sp\|Q14002\|CEAM7_HUMAN Carcinoembryonic antigen-related cell adhesion molecule 7 OS=Homo sapiens GN=CEACAM7 PE=1 SV=1 | CEACAM7 | Carcinoembryonic antigen-related cell adhesion molecule 7 | 8.60 | 0.00 | 2.66 | 1.92 |
| >sp\|P04118\|COL_HUMAN Colipase OS=Homo sapiens GN=CLPS PE=1 SV=2; >tr\|A0A087WZW1\|A0A087WZW1_HUMAN Colipase OS=Homo sapiens GN=CLPS PE=4 SV=1 | CLPS | Colipase | 23.83 | 0.00 | 3.30 | 2.72 |

| Fasta header | Gene name | Protein name | -Log Student's T-test *P*-value | Student's T-test q-value | Student's T-test Difference | Student's T-test Test statistic |
|---|---|---|---|---|---|---|
| >sp\|Q03403\|TFF2_HUMAN Trefoil factor 2 OS=Homo sapiens GN=TFF2 PE=1 SV=2 | TFF2 | Trefoil factor 2 | 49.36 | 0.00 | 3.64 | 3.33 |
| >tr\|G3V2V8\|G3V2V8_HUMAN Epididymal secretory protein E1 (Fragment) OS=Homo sapiens GN=NPC2 PE=1 SV=1; >tr\|J3KMY5\|J3KMY5_HUMAN Epididymal secretory protein E1 OS=Homo sapiens GN=NPC2 PE=1 SV=1 | NPC2 | Epididymal secretory protein E1 | 48.11 | 0.00 | 3.83 | 3.48 |
| >sp\|P59666\|DEF3_HUMAN Neutrophil defensin 3 OS=Homo sapiens GN=DEFA3 PE=1 SV=1; >sp\|P59665\|DEF1_HUMAN Neutrophil defensin 1 OS=Homo sapiens GN=DEFA1 PE=1 SV=1 | DEFA3; DEFA1 | Neutrophil defensin 3; HP 3-56; Neutrophil defensin 1; HP 1-56 | 31.98 | 0.00 | 4.52 | 3.73 |
| >tr\|F8W062\|F8W062_HUMAN Phospholipase A2 OS=Homo sapiens GN=PLA2G1B PE=3 SV=1; >sp\|P04054\|PA21B_HUMAN Phospholipase A2 OS=Homo sapiens GN=PLA2G1B PE=1 SV=3 | PLA2G1B | Phospholipase A2 | 62.78 | 0.00 | 4.58 | 4.26 |

Fecal proteins derived from human or bovine source that were more abundant in (A) preterm infants or (B) full-term infants. A Student's T-test difference below zero indicates the protein is higher in abundance in preterm infants compared to full-term infants. *P*-values are -$\log_{10}$-transformed. In case protein groups consisted of multiple proteins, the two proteins with highest protein existence (PE) value were selected.

**Table S3.5** Human- and bovine-derived proteins identified in more than 50% of the gastric and fecal proteomes of preterm infants (25–31 weeks of gestation).

| | **Fasta header** |
|---|---|
| 1 | >tr\|A0A075B6L0\|A0A075B6L0_HUMAN Ig lambda-3 chain C regions (Fragment) OS=Homo sapiens GN=IGLC3 PE=4 SV=2;<br>>tr\|A0A075B6K9\|A0A075B6K9_HUMAN Ig lambda-2 chain C regions (Fragment) OS=Homo sapiens GN=IGLC2 PE=4 SV=1 |
| 2 | >tr\|A0A087WW89\|A0A087WW89_HUMAN Protein IGHV3-72 OS=Homo sapiens GN=IGHV3-72 PE=4 SV=1 |
| 3 | >tr\|A0A087WXI2\|A0A087WXI2_HUMAN IgGFc-binding protein OS=Homo sapiens GN=FCGBP PE=4 SV=1;<br>>sp\|Q9Y6R7\|FCGBP_HUMAN IgGFc-binding protein OS=Homo sapiens GN=FCGBP PE=1 SV=3 |
| 4 | >tr\|A0A087WZW8\|A0A087WZW8_HUMAN Protein IGKV3-11 OS=Homo sapiens GN=IGKV3-11 PE=4 SV=1 |
| 5 | >tr\|A0A087X0N5\|A0A087X0N5_HUMAN Protein IGKV1-17 OS=Homo sapiens GN=IGKV1-17 PE=4 SV=1;<br>>sp\|P80362\|KV125_HUMAN Ig kappa chain V-I region WAT OS=Homo sapiens PE=1 SV=1 |
| 6 | >tr\|A0A087X2C0\|A0A087X2C0_HUMAN Ig mu chain C region OS=Homo sapiens GN=IGHM PE=1 SV=1;<br>>tr\|A0A075B6N9\|A0A075B6N9_HUMAN Ig mu chain C region (Fragment) OS=Homo sapiens GN=IGHM PE=1 SV=2 |
| 7 | >tr\|C9JF17\|C9JF17_HUMAN Apolipoprotein D (Fragment) OS=Homo sapiens GN=APOD PE=4 SV=1;<br>>sp\|P05090\|APOD_HUMAN Apolipoprotein D OS=Homo sapiens GN=APOD PE=1 SV=1 |
| 8 | >sp\|P00760\|TRY1_BOVIN Cationic trypsin OS=Bos taurus PE=1 SV=3;<br>>sp\|P00760\|TRY1_BOVIN Cationic trypsin OS=Bos taurus GN=Trp1 PE=1 SV=3 |
| 9 | >sp\|P04264\|K2C1_HUMAN Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 |
| 10 | >sp\|P08779\|K1C16_HUMAN Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4 |
| 11 | >sp\|P13645\|K1C10_HUMAN Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 |
| 12 | >sp\|P13647\|K2C5_HUMAN Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 |
| 13 | >sp\|P35527\|K1C9_HUMAN Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 |
| 14 | >sp\|P35908\|K22E_HUMAN Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 |
| 15 | >tr\|G3X807\|G3X807_BOVIN Histone H4 (Fragment) OS=Bos taurus PE=3 SV=1;<br>>sp\|P62803\|H4_BOVIN Histone H4 OS=Bos taurus PE=1 SV=2 |
| 16 | >tr\|F5H265\|F5H265_HUMAN Polyubiquitin-C (Fragment) OS=Homo sapiens GN=UBC PE=4 SV=1;<br>>tr\|J3QS39\|J3QS39_HUMAN Ubiquitin (Fragment) OS=Homo sapiens GN=UBB PE=4 SV=1 |
| 17 | >tr\|G5E5H7\|G5E5H7_BOVIN Uncharacterized protein OS=Bos taurus GN=PAEP PE=3 SV=1 |
| 18 | >sp\|P00450\|CERU_HUMAN Ceruloplasmin OS=Homo sapiens GN=CP PE=1 SV=1;<br>>tr\|E9PFZ2\|E9PFZ2_HUMAN Ceruloplasmin OS=Homo sapiens GN=CP PE=4 SV=1 |
| 19 | >sp\|P01008\|ANT3_HUMAN Antithrombin-III OS=Homo sapiens GN=SERPINC1 PE=1 SV=1 |
| 20 | >sp\|P01009\|A1AT_HUMAN Alpha-1-antitrypsin OS=Homo sapiens GN=SERPINA1 PE=1 SV=3 |
| 21 | >sp\|P01011\|AACT_HUMAN Alpha-1-antichymotrypsin OS=Homo sapiens GN=SERPINA3 PE=1 SV=2;<br>>tr\|G3V595\|G3V595_HUMAN Alpha-1-antichymotrypsin (Fragment) OS=Homo sapiens GN=SERPINA3 PE=4 SV=3 |
| 22 | >sp\|P01023\|A2MG_HUMAN Alpha-2-macroglobulin OS=Homo sapiens GN=A2M PE=1 SV=3 |
| 23 | >sp\|P01024\|CO3_HUMAN Complement C3 OS=Homo sapiens GN=C3 PE=1 SV=2 |
| 24 | >sp\|P01833\|PIGR_HUMAN Polymeric immunoglobulin receptor OS=Homo sapiens GN=PIGR PE=1 SV=4 |

| | Fasta header |
|---|---|
| 25 | >sp\|P01876\|IGHA1_HUMAN Ig alpha-1 chain C region OS=Homo sapiens GN=IGHA1 PE=1 SV=2 |
| 26 | >sp\|P02768\|ALBU_HUMAN Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2;<br>>tr\|B7WNR0\|B7WNR0_HUMAN Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=1 |
| 27 | >sp\|P02787\|TRFE_HUMAN Serotransferrin OS=Homo sapiens GN=TF PE=1 SV=3 |
| 28 | >sp\|P02788\|TRFL_HUMAN Lactotransferrin OS=Homo sapiens GN=LTF PE=1 SV=6;<br>>tr\|E7ER44\|E7ER44_HUMAN Lactotransferrin OS=Homo sapiens GN=LTF PE=1 SV=1 |
| 29 | >sp\|P02794\|FRIH_HUMAN Ferritin heavy chain OS=Homo sapiens GN=FTH1 PE=1 SV=2;<br>>tr\|G3V192\|G3V192_HUMAN Ferritin OS=Homo sapiens GN=FTH1 PE=1 SV=1 |
| 30 | >sp\|P04745\|AMY1_HUMAN Alpha-amylase 1 OS=Homo sapiens GN=AMY1A PE=1 SV=2;<br>>sp\|P19961\|AMY2B_HUMAN Alpha-amylase 2B OS=Homo sapiens GN=AMY2B PE=1 SV=1 |
| 31 | >sp\|P05109\|S10A8_HUMAN Protein S100-A8 OS=Homo sapiens GN=S100A8 PE=1 SV=1 |
| 32 | >sp\|P05155\|IC1_HUMAN Plasma protease C1 inhibitor OS=Homo sapiens GN=SERPING1 PE=1 SV=2;<br>>tr\|E9PGN7\|E9PGN7_HUMAN Plasma protease C1 inhibitor OS=Homo sapiens GN=SERPING1 PE=3 SV=1 |
| 33 | >sp\|P05164\|PERM_HUMAN Myeloperoxidase OS=Homo sapiens GN=MPO PE=1 SV=1 |
| 34 | >sp\|P06702\|S10A9_HUMAN Protein S100-A9 OS=Homo sapiens GN=S100A9 PE=1 SV=1 |
| 35 | >sp\|P07355\|ANXA2_HUMAN Annexin A2 OS=Homo sapiens GN=ANXA2 PE=1 SV=2;<br>>tr\|H0YN42\|H0YN42_HUMAN Annexin (Fragment) OS=Homo sapiens GN=ANXA2 PE=1 SV=1 |
| 36 | >sp\|P08727\|K1C19_HUMAN Keratin, type I cytoskeletal 19 OS=Homo sapiens GN=KRT19 PE=1 SV=4;<br>>tr\|C9JM50\|C9JM50_HUMAN Keratin, type I cytoskeletal 19 (Fragment) OS=Homo sapiens GN=KRT19 PE=1 SV=1 |
| 37 | >sp\|P15085\|CBPA1_HUMAN Carboxypeptidase A1 OS=Homo sapiens GN=CPA1 PE=1 SV=2;<br>>tr\|C9JUF9\|C9JUF9_HUMAN Carboxypeptidase A1 OS=Homo sapiens GN=CPA1 PE=4 SV=1 |
| 38 | >sp\|P15144\|AMPN_HUMAN Aminopeptidase N OS=Homo sapiens GN=ANPEP PE=1 SV=4 |
| 39 | >sp\|P25311\|ZA2G_HUMAN Zinc-alpha-2-glycoprotein OS=Homo sapiens GN=AZGP1 PE=1 SV=2;<br>>tr\|C9JEV0\|C9JEV0_HUMAN Zinc-alpha-2-glycoprotein OS=Homo sapiens GN=AZGP1 PE=3 SV=1 |
| 40 | >sp\|P30740\|ILEU_HUMAN Leukocyte elastase inhibitor OS=Homo sapiens GN=SERPINB1 PE=1 SV=1 |
| 41 | >sp\|P47989\|XDH_HUMAN Xanthine dehydrogenase/oxidase OS=Homo sapiens GN=XDH PE=1 SV=4 |
| 42 | >sp\|P55259\|GP2_HUMAN Pancreatic secretory granule membrane major glycoprotein GP2 OS=Homo sapiens GN=GP2 PE=2 SV=3 |
| 43 | >sp\|P60174\|TPIS_HUMAN Triosephosphate isomerase OS=Homo sapiens GN=TPI1 PE=1 SV=3;<br>>sp\|Q5E956\|TPIS_BOVIN Triosephosphate isomerase OS=Bos taurus GN=TPI1 PE=2 SV=3 |
| 44 | >sp\|P63258\|ACTG_BOVIN Actin, cytoplasmic 2 OS=Bos taurus GN=ACTG1 PE=1 SV=1;<br>>sp\|P63261\|ACTG_HUMAN Actin, cytoplasmic 2 OS=Homo sapiens GN=ACTG1 PE=1 SV=1 |
| 45 | >sp\|Q08380\|LG3BP_HUMAN Galectin-3-binding protein OS=Homo sapiens GN=LGALS3BP PE=1 SV=1;<br>>tr\|K7EP36\|K7EP36_HUMAN Galectin-3-binding protein (Fragment) OS=Homo sapiens GN=LGALS3BP PE=1 SV=1 |
| 46 | >sp\|Q13228\|SBP1_HUMAN Selenium-binding protein 1 OS=Homo sapiens GN=SELENBP1 PE=1 SV=2 |
| 47 | >sp\|Q9UGM3\|DMBT1_HUMAN Deleted in malignant brain tumors 1 protein OS=Homo sapiens GN=DMBT1 PE=1 SV=2 |

In case protein groups consisted of multiple proteins, the two proteins with highest protein existence (PE) value were selected.

**Table S3.6** Bacterial oxidative stress proteins from opportunistic pathogens including *Enterococcus* spp., *Escherichia* spp. and *Klebsiella* spp.

| | **Fasta header** |
|---|---|
| 1 | >tr\|D5CGU0\|D5CGU0_ENTCC Superoxide dismutase OS=Enterobacter cloacae subsp. cloacae (strain ATCC 13047 / DSM 30054 / NBRC 13535 / NCDC 279-56) GN=ECL_05067 PE=3 SV=1; >tr\|A0A023V7A4\|A0A023V7A4_CITFR Superoxide dismutase OS=Citrobacter freundii CFNIH1 GN=CFN |
| 2 | >tr\|A6LCS3\|A6LCS3_PARD8 Catalase OS=Parabacteroides distasonis (strain ATCC 8503 / DSM 20701 / NCTC 11152) GN=BDI_1740 PE=3 SV=1; >tr\|Q5LG24\|Q5LG24_BACFN Catalase OS=Bacteroides fragilis (strain ATCC 25285 / NCTC 9343) GN=katA PE=3 SV=1 |
| 3 | >tr\|A6T7U8\|A6T7U8_KLEP7 Catalase OS=Klebsiella pneumoniae subsp. pneumoniae (strain ATCC 700721 / MGH 78578) GN=katE PE=3 SV=1 |
| 4 | >sp\|A6T9H9\|KATG_KLEP7 Catalase-peroxidase OS=Klebsiella pneumoniae subsp. pneumoniae (strain ATCC 700721 / MGH 78578) GN=katG PE=3 SV=1 |
| 5 | >tr\|A6TA04\|A6TA04_KLEP7 Superoxide dismutase OS=Klebsiella pneumoniae subsp. pneumoniae (strain ATCC 700721 / MGH 78578) GN=sodB PE=3 SV=1; >tr\|D5CBR7\|D5CBR7_ENTCC Superoxide dismutase OS=Enterobacter cloacae subsp. cloacae (strain ATCC 13047 / DSM 30054 / |
| 6 | >tr\|D5CC12\|D5CC12_ENTCC Catalase OS=Enterobacter cloacae subsp. cloacae (strain ATCC 13047 / DSM 30054 / NBRC 13535 / NCDC 279-56) GN=ECL_02433 PE=3 SV=1; >sp\|P21179\|CATE_ECOLI Catalase HPII OS=Escherichia coli (strain K12) GN=katE PE=1 SV=1 |
| 7 | >tr\|V5VFT2\|V5VFT2_ACIBA Catalase OS=Acinetobacter baumannii GN=P795_10275 PE=4 SV=1; >tr\|F0KKY1\|F0KKY1_ACICP Hydroperoxidase II OS=Acinetobacter calcoaceticus (strain PHEA-2) GN=katE PE=4 SV=1 |
| 8 | >sp\|P0A0J3\|SODM1_STAA8 Superoxide dismutase [Mn] 1 OS=Staphylococcus aureus (strain NCTC 8325) GN=sodA PE=1 SV=1 |
| 9 | >sp\|P0AE08\|AHPC_ECOLI Alkyl hydroperoxide reductase subunit C OS=Escherichia coli (strain K12) GN=ahpC PE=1 SV=2; >tr\|Q32IW2\|Q32IW2_SHIDS Alkyl hydroperoxide reductase, C22 subunit OS=Shigella dysenteriae serotype 1 (strain Sd197) GN=ahpC PE=4 SV=1 |
| 10 | >tr\|Q32FB5\|Q32FB5_SHIDS Superoxide dismutase OS=Shigella dysenteriae serotype 1 (strain Sd197) GN=sodB PE=3 SV=1; >sp\|P0AGD3\|SODF_ECOLI Superoxide dismutase [Fe] OS=Escherichia coli (strain K12) GN=sodB PE=1 SV=2 |
| 11 | >sp\|P13029\|KATG_ECOLI Catalase-peroxidase OS=Escherichia coli (strain K12) GN=katG PE=1 SV=2 |
| 12 | >sp\|Q2FYU7\|CATA_STAA8 Catalase OS=Staphylococcus aureus (strain NCTC 8325) GN=katA PE=2 SV=2 |
| 13 | >sp\|Q5HNZ5\|SODM_STAEQ Superoxide dismutase [Mn/Fe] OS=Staphylococcus epidermidis (strain ATCC 35984 / RP62A) GN=sodA PE=3 SV=1 |
| 14 | >sp\|Q5HPK8\|CATA_STAEQ Catalase OS=Staphylococcus epidermidis (strain ATCC 35984 / RP62A) GN=katA PE=3 SV=1 |
| 15 | >sp\|Q5HRY1\|AHPC_STAEQ Alkyl hydroperoxide reductase subunit C OS=Staphylococcus epidermidis (strain ATCC 35984 / RP62A) GN=ahpC PE=3 SV=1 |
| 16 | >sp\|Q838I4\|SODM_ENTFA Superoxide dismutase [Fe] OS=Enterococcus faecalis (strain ATCC 700802 / V583) GN=sodA PE=3 SV=1 |
| 17 | >sp\|P37689\|GPMI_ECOLI 2,3-bisphosphoglycerate-independent phosphoglycerate mutase OS=Escherichia coli (strain K12) GN=gpmI PE=1 SV=1 |
| 18 | >sp\|P77212\|RCLA_ECOLI Probable pyridine nucleotide-disulfide oxidoreductase RclA OS=Escherichia coli (strain K12) GN=rclA PE=2 SV=2 |
| 19 | >tr\|Q838J4\|Q838J4_ENTFA OsmC/Ohr family protein OS=Enterococcus faecalis (strain ATCC 700802 / V583) GN=EF_0453 PE=4 SV=1 |

In case protein groups consisted of multiple proteins, the first proteins with highest protein existence (PE) value were selected.

# CHAPTER 4

## From mum to bum: an observational study protocol to follow digestion of human milk oligosaccharides and glycoproteins from mother to preterm infant

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*This chapter has received minor editing after publication.

## Abstract

The nutritional requirements of preterm infants are challenging to meet in neonatal care, yet crucial for their growth, development and health. Aberrant maturation of the gastrointestinal tract and the microbiota could affect the digestion of human milk and its nutritional value considerably. Therefore, the main objective of the proposed research is to investigate how the intestinal microbiota of preterm and full-term infants differ in their ability to extract energy and nutrients from oligosaccharides and glycoproteins in human milk. This pilot study will be an observational, single-center study performed at the neonatal intensive care unit at Isala Women and Children's Hospital (Zwolle, The Netherlands). A cohort of thirty mother–infant pairs (preterm ≤ 30 weeks of gestation, $n$ = 15; full-term 37–42 weeks of gestation, $n$ = 15) will be followed during the first six postnatal weeks with follow-up at three- and six-months postnatal age. We will collect human milk of all mothers, gastric aspirates of preterm infants and feces of all infants. A combination of 16S rRNA gene amplicon sequencing, proteomics, peptidomics, carbohydrate analysis and calorimetric measurements will be performed. The role of the microbiome in infant growth and development is often overlooked, yet offers opportunities to advance neonatal care. The "From Mum to Bum" study is the first study in which the effect of a preterm gut microbiota composition on its metabolic capacity and subsequent infant growth and development is investigated. By collecting human milk of all mothers, gastric aspirates of preterm infants and feces of all infants at each timepoint, we can follow digestion of human milk from the breast of the mother throughout the gastrointestinal tract of the infant, or "From Mum to Bum".

## Introduction

Human milk is strongly recommended in infant feeding[24,342]. Besides its nutrient composition, human milk educates the neonatal immune system by promoting selective tolerance toward dietary and microbial components[40,343–345]. Human milk digestion starts with maternal enzymes in the breast that are subsequently accompanied by infantile enzymes in the mouth and stomach upon ingestion[51,346]. Further down the gastrointestinal tract, the microbiota in the colon fulfills an essential role in extracting nutrients from a considerable amount of food components that are otherwise indigestible, such as oligosaccharides and glycoproteins in human milk[258,289]. The process of human milk digestion is pivotal for development of the gastrointestinal tract, microbiome and immune system[11,17,24,68].

Digestion and absorption of human milk is impaired in preterm infants, having considerable consequences on their growth and development[11,146]. Besides physiological immaturity of the gastrointestinal tract, aberrant microbiota development impedes human milk digestion in preterm infants[11,146]. Preterm infants typically have a decreased microbial diversity compared to full-term infants, which has been shown to play a role in achieving weight gain[125,135,347]. Moreover, a differential microbiota composition may affect the abundance of the microbial gene pool encoding for proteins involved in metabolism of macronutrients, which subsequently would alter the metabolic activity and energy harvest[11,52,217,289,291]. Microbial digestive proteins have already been shown to vary with gestational and postnatal age in preterm infants[137,295]. Most convincing, however, are studies in preterm infants showing associations between the gut microbiota, growth and development in early life[52,215]. For example, various microbiota phases in preterm infants—each characterized by distinct metabolic functions—were significantly associated with preterm infant growth[52]. More specifically, levels of the genera *Bacteroides*, *Enterobacteriaceae* and *Streptococcus* at early age could be associated with weight gain of preterm infants at one month of age[215].



With advances in neonatal care, the survival rates of preterm infants born at younger gestational ages have increased[9]. This imposes new clinical challenges such as meeting the unique nutritional requirements[39,51]. In fact, more than half of hospitalized preterm infants are being discharged with ongoing severe postnatal growth failure[52,348]. Growth impairment in the neonatal period is common and increases susceptibility to infections and impaired cognitive development[213,214]. The role of the microbiome in this process is often overlooked, yet offers opportunities to advance neonatal care. Therefore, the metabolic capacity of the preterm gut microbiota and its subsequent role in infant growth and development should be investigated.

### The "From Mum to Bum" study

The new "From Mum to Bum" pilot study is well suited to investigate this and broadens our previous clinical set-up of the EIBER study. In the EIBER study, gastric aspirates and feces were collected from preterm and full-term infants with the main objective being to investigate the colonization and development of the gut microbiota[137,144,146,327]. The EIBER study has

enabled us to study maturation of the gastrointestinal tract and the microbiota in the early life of preterm infants, as well as the relationship between microbiota composition and antibiotic treatment[137,144,146,327]. More specifically, gastrointestinal and beneficial microbial proteins involved in gastrointestinal maturity were associated with gestational and postnatal age[137,146]. In the new observational, single-center study at the neonatal intensive care unit (NICU) at Isala Women and Children's Hospital (Zwolle, The Netherlands), we aim to achieve a targeted approach to compare the microbiota's functionality of preterm and full-term infants with regard to the digestion of human milk components. To this end, a group of mother–infant pairs will participate during the first six postnatal weeks with follow-up at three- and six-months postnatal age. The group will consist of fifteen mothers delivering vaginally and preterm (≤ 30 weeks of gestation) and fifteen mothers delivering vaginally and full-term (37–42 weeks of gestation). By collecting human milk of all mothers, gastric aspirates of preterm infants and feces of all infants at each timepoint, we can follow the digestion of human milk from the breast of the mother throughout the gastrointestinal tract of the infant, or "From Mum to Bum". Previously, a similar set-up was used, in which the comparison of human milk and corresponding infant feces showed that human milk oligosaccharides (HMOs) are important for shaping the gut microbiota of infants[349–351]. In the current study, gastric aspirates of preterm infants are included in sample collection, which will provide additional information on human milk digestion from a host perspective. Moreover, our study aims to integrate 16S rRNA gene amplicon sequencing, proteomics, peptidomics and carbohydrate analysis. With the integration of these methods, we can assess how the intestinal microbiota of preterm and full-term infants differ in their ability to extract energy and nutrients from oligosaccharides and glycoproteins in human milk. In fact, the combination of genomics and proteomics has been key in understanding that the bacterial digestive proteins of preterm infants vary with gestational age[137]. Moreover, the collection of multiple types of samples at each timepoint provides longitudinal data that allow us to follow microbial composition and host/microbial protein development during the first six postnatal months. Moreover, we will include calorimetric measurements to assess intestinal functionality.

## Aim and hypothesis

The main objective of the proposed research is to investigate how the intestinal microbiota of preterm and full-term infants differ in their ability to extract energy and nutrients from human milk. We expect that differences in the gut microbiome of preterm infants will mainly be emphasized with regard to the digestion of HMOs and glycoproteins from human milk, since *Bifidobacterium* spp. are equipped with genes encoding for enzymes that digest these components and are lower in abundance in preterm infants[11,69,146].

Other aims are to: (1) identify the composition of the microbiota in early life and its development over time; (2) assess the bifidogenic effect of human milk; (3) establish if there is a relationship between preterm microbiota composition, weight gain and growth in early life; and (4) explore the relationship between preterm microbiota composition and registered clinical variables.

## Materials and methods

### Study design and setting

The "From Mum to Bum" study is an observational, single-center pilot study that will include a cohort mother–infant pairs followed from birth until six months postpartum. The mother–infant pairs will comprise mothers delivering preterm and full-term. The cohort will be recruited at the obstetrics department and at the NICU of Isala Women and Children's Hospital, as well as at several midwifery practices. Isala Women and Children's Hospital is one out of nine hospitals with a level III NICU in the Netherlands.

### Sample size calculation

No published data are available to contribute to the estimation of the desired sample size. Therefore, a non-probabilistic, convenience sampling method will be applied over a period of two years. Based on the hospital's statistics, it is expected that fifteen preterm mother–infant pairs, who fulfill the inclusion criteria and not the exclusion criteria, could be recruited within two years. The full-term mother–infant pairs group will be of equal size.



### Recruitment criteria

Subjects are eligible if they fulfill all the inclusion criteria, but not the exclusion criteria. Screening takes place when an infant is (to be) admitted to the NICU because of (suspected) preterm birth. Full-term subjects are recruited by midwives on a voluntary basis during pregnancy. Potential subjects are screened with respect to the inclusion and exclusion criteria. Written informed consent is obtained before inclusion in the study.

#### *Inclusion criteria for preterm mother–infant pairs*

The inclusion criteria for preterm mother–infant pairs are: (1) mothers who deliver ≤ 30 weeks of gestation and of whom the infants are admitted to the NICU at Isala Women and Children's Hospital; (2) the infant is born vaginally; (3) the infant has a nasogastric tube; and (4) there is an intention to breastfeed.

#### *Inclusion criteria for full-term mother–infant pairs*

The inclusion criteria for full-term mother–infant pairs are: (1) mothers who deliver between 37 and 42 weeks of gestation, of whom infants are born either in a hospital after an uncomplicated pregnancy or at home; (2) the infant is born vaginally; (3) there is an intention to breastfeed; and (4) both mother and infant are healthy, which is defined as not receiving any medication except vitamins.

#### *Exclusion criteria for (pre)term mother–infant pairs*

Mother–infant pairs will be excluded if they do not meet the inclusion criteria. Other exclusion criteria include: (1) major congenital malformations (of the gastrointestinal tract) of the infant; (2) high probability of death within six weeks postpartum; (3) expected discharge from the

NICU or transfer to another hospital during the first postnatal week; and (4) there is no intention to breastfeed and/or the infant does not receive any human milk after the first week postpartum.

## Sampling procedures

### *Data collection timeline*

The study will have a duration of six weeks and a follow-up at three and six months postpartum. Samples will be collected weekly on the last day of the week during the first six weeks. Follow-up will occur on the last day of week 12 and week 24 (Fig. 4.1A). Sample collection comprises: (1) human milk; (2) gastric aspirate (only in preterm infants); and (3) feces of the infant (Fig. 4.1B). In case of discharge from the hospital, human milk and feces will be collected at home and frozen at −20 °C. Home collections will be transported by courier to Isala Women and Children's Hospital.

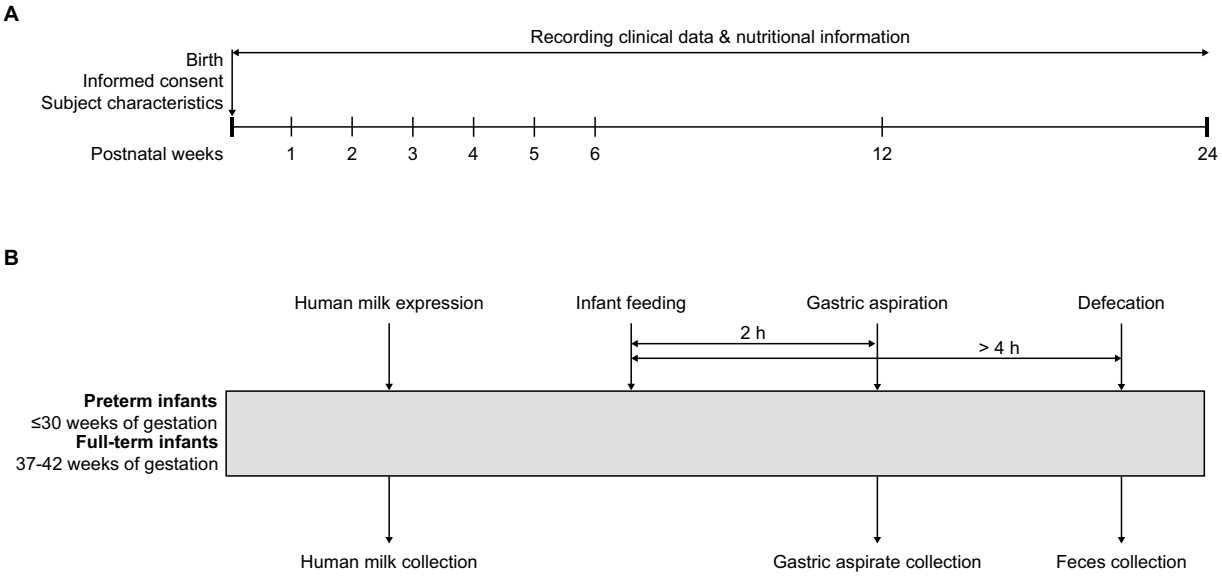


**Figure 4.1** Sampling and data collection scheme. Scheme of (A) sampling points over the first six months and (B) one sampling point. While human milk and feces will be collected in full-term and preterm infants, gastric aspirates will only be collected in preterm infants during hospital stay. Clinical data will be monitored at every sampling point throughout the duration of the study.

### *Human milk*

Human milk samples will be collected if the infant is exclusively fed with human milk or mixed fed. Before feeding the infant, 4 mL of expressed human milk will be collected by manual or mechanical expression. The sample will be stored at −20 °C until transfer to −80 °C for later analysis. Breastfeeding the infant will always be prioritized, and mothers will be encouraged to breastfeed their infants at all times as soon as the infant is able to drink from the breast; otherwise, gavage feeding of expressed human milk will take place. The amount of mother's human milk will be registered in the Case Report Form (CRF). In case of insufficient human milk expression, infants will receive additional infant formula to complete the amount. If the

mother cannot express human milk at all after the first week postpartum, mother–infant pairs will be excluded. No donor milk bank will be available at the NICU during the study period. For infants below 1800 g, human milk will be supplemented with human milk fortifier and vitamins according to the NICU protocol. In those cases, we will continue sample collection according to the protocol.

### *Gastric aspirates*

Preterm infants (≤ 30 weeks of gestation) admitted to the NICU will receive a nasogastric tube for gastrointestinal feeding as per usual. Generally, the contents of the stomach will be aspirated two hours after feeding to empty the stomach and to prepare it for next feedings. From this gastric aspirate, 1 mL will be collected and frozen at −80 °C for later analysis. If no stomach content is available, this will be reported and other samples will be collected according to the protocol.

### *Feces*

Fecal samples will be collected from the first stool passed at least four hours after feeding. With a scoop attached to the sampling bottle, at least one scoop of feces will be collected. These samples will be stored at −20 °C and transferred to −80 °C for later analysis.

### *Clinical data collection*

After birth, clinical data of preterm and full-term infants will be registered and will comprise the gestational age, date of birth, mode of delivery, birth weight and parental data. During the hospital stay, the investigator will register the study parameters of the preterm infant weekly in a CRF at days of sampling and whenever applicable. The study parameters will include the date and time of measurement, body weight, length, head circumference, feeding regimen, feeding intolerance, morbidities, medication and respiratory support information. The feeding regimen data will include the volume of human milk, the volume of formula and data on nutritional support including parenteral and enteral feeding. In case of enteral feeding, human milk intake will be corrected for enteral feeding.

During home sampling at follow-up of the preterm infant group and for the full-term group in general, feeding information will be registered in online questionnaires that will be sent at the planned time of home sampling. Feeding information will include the volume of human milk and formula given to the infant at each sampling point.

## Primary outcome

The main objective of the proposed research is to investigate how the intestinal microbiota of preterm and full-term infants differ in their ability to extract energy and nutrients from human milk. As such, the primary outcome will be the combination of quantitative differences between preterm and full-term infants in (1) HMO-degrading bacteria; (2) bacterial HMO-degrading enzymes; (3) human- and bovine-derived proteins; and (4) intestinal absorption capacity. This

will be assessed in human milk, gastric aspirates and fecal samples collected during the first six postnatal weeks and during follow-up at three and six months.

## Secondary outcomes

- Microbiota composition in early life and its development over time, assessed using 16S rRNA gene amplicon sequencing and quantitative PCR (qPCR).
- The effect of (corrected) human milk intake on the relative abundance of *Bifidobacterium* spp.
- The relationship between preterm microbiota composition and weight gain in early life assessed by means of 16S rRNA gene amplicon sequencing and anthropometrics (weight, length and head circumference).
- The relationship between preterm microbiota composition and registered clinical variables.

## Sample and data processing

### *Total carbohydrates and human milk oligosaccharides*

Chemical analyses will be used to assess the compounds present in human milk, gastric aspirates and feces. Specifically, the identity and quantity of carbohydrates present in human milk, gastric aspirates and feces will be analyzed by gel permeation chromatography (GPC) as described by Chia et al.[352]

HMOs will be measured by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-$MS^2$) as described by Mank et al.[353] Pre-treatment of samples for this method will depend on the type. Human milk and gastric aspirates will be processed according to Mank et al.[353] Briefly, samples will be thawed on ice and vortexed. Quantities of 15 µL of internal standard α-L-arabinopentaose (0.05 mM) will be added to 135 µL human milk or gastric aspirate. The solution of the sample and the internal standard will be further diluted 1:11 (v/v) through the addition of 150 µL Pierce Water, LC-MS Grade (ThermoFisher Scientific, Waltham, United States, Cat. No. 51140). Subsequently, 450 µL of diluted sample will be transferred to a 500-µL Amicon Ultra centrifugal filter with 3-kDa cutoff and ultrafiltration (UF) will be performed at 14,000× g for 1 h. Subsequently, 300 µL of UF permeate will be transferred to a LC-MS screw top vial for LC-MS analysis. The protocol will be slightly adapted for fecal samples, as suggested by Mank et al., and would include "additional microfiltration steps or SPE (...) in addition or as an alternative to 3-kDA ultrafiltration."[353] Acquired data will be processed as described by Mank et al.[353] Processed data will be used for data analysis.

### *Metaproteomic and peptidomic analysis*

The metaproteome of human milk, gastric aspirate and feces will be characterized using LC-MS/MS according to the methods outlined by Zwittink et al.[137]. For peptidomics, the samples will be prepared and analyzed according to Dallas et al.[354].

Metaproteomics and peptidomics data will be processed with MaxQuant[311] and further processed in Perseus[312] as described previously[137]. Label-free quantification (LFQ) intensities will be $log_{10}$-transformed. Intensity-Based Absolute Quantification (iBAQ) intensities will be used to measure the relative abundance of proteins. Functional profiles of proteins will be generated by assigning protein IDs to KEGG Orthology (KO) identifiers using the KEGG Brite database. Processed data will be used for data analysis.

### *Microbiota analysis*

16S rRNA gene amplicon sequencing be used to assess the microbiota composition and relative abundance in feces. Quantities of 0.13 g of feces will be weighed into a 2.0 mL screw cap tube filled with 0.25 g of 0.1 mm zirconia beads and three 2.5 mm glass beads. Negative controls will be included and consist of FastPrep tubes with beads. Furthermore, 300 µL of Stool Transport and Recovery Buffer (S.T.A.R. buffer, Roche Diagnostics, Almere, The Netherlands, Cat. No. 03335208001) will be added and bead-beaten three times at 5.5 ms for 60 s with 15 s pause (FastPrep-24 5G bead beating grinder and lysis system, MP Biomedicals, Irvine, United States). Subsequently, samples will be incubated for 15 min at 95 °C at 100 rpm, after which they will be centrifuged (4 °C, 5 min, 14,860 rpm) and the supernatant will temporally be stored at 4 °C. The process will then be repeated with 200 µL S.T.A.R. buffer. In case the first step does not yield supernatant, 300 µL S.T.A.R. buffer will be added. A total of 250 µL of recovered supernatant will be used for DNA extraction with Maxwell 16 Tissue LEV Total RNA Purification Kit (Promega, Wisconsin, United States Cat. No. AS 1220).

Isolated DNA will be PCR-amplified with barcoded V4 primers (515F: GTGYCAGCMGCCGCGGTAA[355]; 806R: GGACTACNVGGGTWTCTAAT[356]). Next, PCR products will be purified with the CleanPCR kit (CleanNA, Waddinxveen, The Netherlands, Cat. No. CPCR-0050) according to the manufacturer's protocol. DNA will be quantified with the Qubit dsDNA BR Assay Kit (ThermoFisher Scientific, Waltham, United States, Cat. No. Q32850) on DeNovix DS-11 FX (DeNovix, Wilmington, United States) and pooled into libraries at an equimolar concentration of 200 ng. The pooled products will be purified with the CleanPCR kit according to the manufacturer's protocol and sequenced with the Illumina HiSeq platform.

Sequencing data will be annotated with the SILVA reference database using our in-house NG-Tax pipeline with default settings[357]. In short, NG-Tax will perform read filtering, Amplicon Sequence Variant (ASV)-picking and taxonomic assignment[357,358]. The processed data will be used for data analysis.

A subset of bacterial families and genera of interest will additionally be quantified using a SYBR-based real-time qPCR. The subset of microorganisms will be selected based on reported core microbiota in preterm infants and on their involvement in the degradation of components in human milk[68,69,128,137]. The subset of bacterial families and genera will include the *Enterobacteriaceae* family and the *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterococcus* and *Lactobacillus* genera. Primer

sequences will be used to target the family- or genus-specific regions of the bacterial 16S rRNA gene (Table 4.1). Instead of genus-specific primers, phylogenetic cluster XIVa will be selected as target for the *Clostridium* genus as the 16S rRNA gene shares great homology between strains[359]. The selected cluster is among the most abundant *Clostridium* phylogenetic clusters that have been identified in the human gastrointestinal tract[360].

**Table 4.1** Overview of primer sequences to target specific regions of the bacterial 16S rRNA gene for taxa within the preterm core microbiota. The subset of selected microorganisms is based on reported core microbiota in preterm infants and on their involvement in the degradation of components in human milk. References of primer sequences and associated methodology are included.

| Target | | Name | Sequence (5'–3') | Amplicon Length (bp) | Tm | Reference |
|---|---|---|---|---|---|---|
| 16S | Forward | BACT_1369F | CGG TGA ATA CGT TCY CGG | 142 | 56 | Suzuki et al.[361] |
| | Reverse | PROK_1492R | GGW TAC CTT GTT ACG ACT T | | | |
| *Bacteroides-Prevotella-Porphyromonas* | Forward | - | GGT GTC GGC TTA AGT GC CAT | 140 | 68 | Jian et al.[362] |
| | Reverse | - | CGG AYG TAA GGG CCG TGC | | | |
| *Bifidobacterium* spp. | Forward | - | TCG CGT CYG GTG TGA AAG | 243 | 58 | Jian et al.[362] |
| | Reverse | - | CCA CAT CCA GCR TCC AC | | | |
| *Clostridium* cluster XIVa | Forward | - | CGG TAC CTG ACT AAG C | 429 | 55 | Jian et al.[362] |
| | Reverse | - | AGT TTY ATT CTT GCG AAC G | | | |
| *Enterobacteriaceae* spp. | Forward | En-lsu-3F | TGC CGT AAC TTC GGG AGA AGG CA | 428 | 60 | Matsuda et al.[363] |
| | Reverse | En-lsu-3'R | TCA AGG ACC AGT GTT CAG TGT C | | | |
| *Enterococcus* spp. | Forward | g-Encoc-F | ATC AGA GGG GGA TAA CAC TT | 337 | 55 | Matsuda et al.[364] |
| | Reverse | g-Encoc-R | ACT CTC ATC CTT GTT CTT CTC | | | |
| *Lactobacillus* spp. | Forward | F_alllact_IS | TGG ATG CCT TGG CAC TAG GA | 92 | 58 | Haarman et al.[365] |
| | Reverse | R_alllact_IS | AAA TCT CCG GAT CAA AGC TTA CTT AT | | | |
| | Probe | P_alllact_IS | TAT TAG TTC CGT CCT TCA TC | | 68 | |

### *Calorimetry*

The energy contained within human milk and feces will be measured using bomb calorimetry, as described earlier[366–370]. Intestinal absorption capacity will by defined by the energy difference between nutritional intake and fecal losses, which is a widely accepted method and semi-quantitative marker for intestinal function in clinical practice[366]. Human milk will be used to measure nutritional intake and feces will be used to measure the energy excreted in feces. Analyses will be performed according to Hosoi et al. for human milk and Wierdsma et al. for feces[366,368].

## Data availability

Once available, the mass spectrometry data will be deposited to the ProteomeXchange Consortium[319] via the PRIDE partner repository. Sequencing data will be made available via the European Nucleotide Archive.

## Ethics approval and consent to participate

The protocol for the "From Mum to Bum" study was approved by the board of the Medical Ethics Committee (METC) of Isala Women and Children's Hospital (Zwolle, The Netherlands) in May 2019 as a study not falling under the scope of the Medical Research Involving Human Subjects Act (WMO). The study was registered under the number 190503 with the Research Manager of METC Isala Women and Children's Hospital and began recruiting in August 2020. This study will be conducted according to the principles of the Declaration of Helsinki (64$^{th}$ WMA General Assembly, Fortaleza, Brazil 2013), the Personal Data Protection Act (UAVG), the "Gedragscode Gezondheidsonderzoek" and the "Code Goed Gedrag".

## Data management

The privacy of the participants will be guaranteed at all times. The data of participating infants will be pseudonymized with personal codes. Samples and registered data will be collected in the CRF using this code. The document linking codes to participants' data will only be accessible for the researchers of this study. The investigator is responsible for designing and updating the CRF and other data collection forms. All documents pertaining to the conduct of the study must be kept by the investigator for a period of 15 years.

## Results

### Data analysis and assessments

Subjects with missing values will be excluded prior to data analysis. Data will be analyzed using the statistical program R and RStudio software[315], as well as dedicated in-house R scripts and available packages.

#### *Carbohydrates and oligosaccharides*

Preterm and full-term infants will be compared with regard to the quantity of total carbohydrate and the quantity of HMOs in their respective postnatal week, as well as within one age group between sample types. In addition, temporal dynamic plots will be used to assess the quantity of total carbohydrates and HMOs over the first six postnatal weeks.

#### *Metaproteomics and peptidomics*

Proteins and peptides will be compared between the preterm and full-term groups in their respective postnatal week, as well as within one age group between sample types, using Perseus' volcano plots[312]. The quantities of proteins and peptides of interest will be further analyzed with temporal dynamic plots over the first six postnatal weeks.

#### *Microbiota data*

16S rRNA gene amplicon sequencing data over time will be analyzed in terms of composition, diversity and richness. Descriptive statistics such as summaries and graphics will be used to describe the basic features of the colonization and development of the gut microbiota of the subjects. The diversity and richness of the microbiota within and between individuals will be analyzed at various phylogenetic levels using the Wilcoxon test or Mann-Whitney test, respectively. Differences in microbial composition, diversity and richness between time points will be assessed using a repeated measure Analysis of Variance (ANOVA) if the data are normally distributed or a Kruskal–Wallis test if the data are skewed. qPCR data will be used to assess the microbial load in each sample.

#### *Calorimetry*

Measured energy (kcal/100 g) and intestinal absorption capacity (as a percentage of nutritional intake) will be compared between preterm and full-term groups in their respective postnatal week as well as within one age group over the first six postnatal weeks.

#### *Relationships between data*

Metaproteomic and 16S rRNA gene amplicon sequencing will be further analyzed in relation to clinical variables. Considering all measured variables, principal component analysis (PCA) will be used to assess the captured variation between groups. Moreover, this technique allows us to examine potential clusters and outliers. Next, redundancy analysis (RDA) will be used

to estimate the relationship between quantitative and qualitative variables including 16S rRNA gene amplicon sequencing data, metaproteomics data and clinical variables. Forward and reverse automatic stepwise model selection for constrained ordination will be performed to build a model with variables that significantly explain variation in the data. Additionally, correlation network analyses will be performed between the relative abundance of intestinal bacteria, human/bacterial proteins and the clinical variables.

## Discussion

The "From Mum to Bum" study is a new clinical pilot study investigating how the intestinal microbiota of preterm and full-term infants differ in their ability to extract energy and nutrients from oligosaccharides and glycoproteins in human milk. It capitalizes upon the set-up of our previous clinical trial (EIBER) and broadens it by including mother's human milk in the sample collection. The inclusion of human milk is crucial to advance the understanding of the digestion of human milk, from the breast of the mother throughout the gastrointestinal tract of the infant. The microbiome plays a central role in this study as it is often overlooked in nutritional neonatal care[11]. The "From Mum to Bum" study is the first study in which the metabolic capacity of the preterm gut microbiota and subsequent infant growth and development is investigated. We aim to unravel microbial degradation of oligosaccharides and glycoproteins present in human milk along the gastrointestinal tract. The proposed research is innovative in terms of the collection of samples obtained at multiple sites along the gastrointestinal tract. Human milk, gastric aspirates and feces have previously been studied in relation to microbial human milk digestion, but our study is the first to combine all three types of samples. Previously, intact HMOs and glycan digestion products have been quantified and characterized in human milk and/or feces[261,371–376]. Others have characterized and compared peptides in human milk and gastric aspirates[346,354]. However, these studies have not used a combination of human milk, gastric aspirates and feces in preterm infants. Another innovative aspect is the investigation of the microbial metabolic capacity in relation to anthropometric data, which only few studies have focused on[52,215]. Moreover, we will be able to follow this process during the first six postnatal weeks.

We acknowledge a few limitations of this study. First, the single-center set-up of the study may compromise the feasibility of recruiting solely preterm infants that are born vaginally. The mode of delivery has been identified to strongly influence microbiota composition in (preterm) infants[121,228]. Selecting infants with the same mode of delivery, therefore, eliminates differences in microbiota composition due to confounding factors. Yet, more frequently than full-term infants, preterm infants are born via caesarean section and this group may, thus, not be represented by the cohort within this study [11]. Additionally, preterm infants are a heterogeneous group with many clinical variables acting as confounding factors. Selecting for mode of delivery does not exclude the effects of other confounding factors. Second, the sample size is based on a non-probabilistic, convenience sampling method but it remains unknown whether this sample size is large enough to capture heterogeneity in microbiota composition amongst preterm infants. Third, the collection of data from full-term infants relies heavily upon the compliance of participating parents. Questionnaires need to be filled out weekly by the parents to inquire about infant feeding practices. Additionally, human milk and feces need to be collected weekly and stored in the correct way to allow for microbiota analysis. Storage conditions, including temperature, have been shown to influence human milk peptidome and fecal microbiota composition[98,100,377,378]. Lastly, the absorption of proteins from human milk in the small intestine cannot be measured directly, although it may influence metabolic activity of the microbiota in the colon.

With increasing survival rates at lower gestational ages, the feeding of preterm infants with unique nutritional requirements has become a new clinical challenge[9,39,51]. We expect that insights from this study can be used to tailor nutritional care to preterm infants in such a way that optimal growth and development can be enforced, which is beneficial for short- and long-term health.

## Conclusion

In summary, the "From Mum to Bum" study aims to investigate how the intestinal microbiota of preterm and full-term infants differ in their ability to extract energy and nutrients from human milk. By collecting human milk of the mother and gastric aspirates and feces of the infant, we can determine human milk composition, gastric digestion by the infant and fermentation by the intestinal microbiota of the infant. This may aid in the optimization of current feeding regimens and could contribute to reductions in morbidity, mortality and healthcare costs. Additionally, the innovative methods from this study could be used to study the digestion of bovine milk components and thereby contribute to developments in preterm infant formulas tailored to fit the needs of this group of infants.

# CHAPTER 5

## The first fungi: mode of delivery determines early life fungal colonization in the intestine of preterm infants

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## Abstract

The role of intestinal fungi in human health and disease is becoming more evident. The mycobiota composition and diversity of preterm infants is affected by interactions with bacteria and clinical variables. In this study, we aimed to characterize the composition and the diversity of the preterm infant mycobiota and the effect of clinical variables on it in the first six postnatal weeks. Preterm infants ($n$ = 50) and full-term infants ($n$ = 6) admitted to Isala Women and Children's Hospital (Zwolle, The Netherlands) who were born during 24–36 or 37–40 weeks of gestation, respectively, were included in this study. Feces were collected during the first six postnatal weeks ($n$ = 109) and their mycobiota composition and diversity were characterized by ITS2 amplicon sequencing. Composition analyses identified fungi and other eukaryotic kingdoms, of which Viridiplantae was most abundant. Of the fungal kingdom, Ascomycota and Basidiomycota were the first and second most prominent phyla in early life of all infants. *Candida* was the most abundant genus in the first six weeks of life and increased with gestational and postnatal age. Fungal phylogenetic diversity remained stable in the first six postnatal weeks. The individuality and the mode of delivery were identified as significant predictors for the variation in the mycobiota composition. Vaginally delivered infants were enriched in *Candida* spp., whereas infants delivered through emergency C-section were characterized by *Malassezia* spp. These results indicate that fungi and other eukaryotic kingdoms are detected in the intestine of preterm and full-term infants in the first six postnatal weeks. Similar to the microbiota, colonization of the preterm intestine with fungi is determined by clinical variables including individuality and mode of delivery.

## Introduction

The human gastrointestinal tract harbors bacteria, fungi, archaea, protozoa and viruses that together form the microbiota[58]. Most research has emphasized the relationship between the bacterial part of the microbiota and its link to health or disease[379–381]. By comparison, little is known about the fungal part of the microbiota, which collectively is called the "mycobiota". The necessity to investigate the microbiota beyond bacteria is becoming more evident, as "interkingdom" interactions in the intestine can affect ecosystem dynamics and immune homeostasis[58,382].

The initial fungal colonization occurs during early life and the process is very similar to that of the microbiota. The acquisition of the first fungi may occur by vertical transmission from mother to infant, in which *Candida* spp. is most extensively studied in this regard[156,162]. After birth, the mycobiota composition and diversity is affected by variables very similar to those affecting the microbiota. They include gestational age, mode of delivery, hospital environment, antibiotic exposure and diet[54,155,157,159].

The mycobiota composition and diversity of preterm infants may be considerably different compared to full-term infants due to aberrant circumstances in early life. Apart from their direct impact, those aberrant circumstances may affect the mycobiota indirectly through interkingdom interactions[58,190,207]. The preterm infant gut mycobiota, in contrast to healthy full-term infants, is often dominated by a single species[157]. Yeasts, and more specifically *Candida* spp., are typically one of those predominant species in preterm infants up to a postnatal age of six months[136,157]. Within the *Candida* genus, opportunistic pathogens *Candida albicans* and *Candida parapsilosis* are highly prevalent and persistent in preterm infants[157]. Other dominant genera identified in preterm infants include *Aspergillus*, *Davidiella*, *Debaryomyces, Penicillium* and *Saccharomyces*[157]. In addition, fungi of the Saccharomycetales order and species of the *Cladosporium* and *Cryptococcus* genus have been identified in stools of extremely low birth weight and preterm infants[59,136].

While many intestinal fungi are commensal and may confer health benefits, fungal overgrowth may lead to infections that are associated with considerable morbidity and mortality rates[155,176,177]. Preterm infants are particularly prone to invasive, systemic candidiasis that affects approximately 10% of preterm infants and has an associated mortality rate of 20%[178,383]. The susceptibility to fungal overgrowth in preterm infants correlates to predisposing clinical factors including a naïve immune system, bacterial dysbiosis following exposure to a hospital environment, antibiotic treatment and use of parenteral nutrition[155].

In this study, we aimed to characterize the composition and diversity of the preterm infant mycobiota and the effect of clinical variables on it during the first six postnatal weeks. We investigated the fecal mycobiota of infants born with varying degrees of prematurity during the first six postnatal weeks.

## Materials and Methods

### Ethics declaration

The board of the Medical Ethics Committee (METC) of Isala Women and Children's Hospital (Zwolle, The Netherlands) 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.

### Study description

The samples from this study derive from the EIBER study; a single-center, observational study involving full-term and preterm infants admitted to the neonatal intensive care unit (NICU) or the pediatric ward of Isala Women and Children's Hospital in Zwolle, The Netherlands. The two objectives of the EIBER study were to investigate colonization and development of the gut microbiota and to understand the relationship between microbiota composition and antibiotic treatment duration[137,144,146,327].

The preterm infants were fed with mother's own milk when available, which was increasingly supplemented with human milk fortifier (Nenatal BMF, Nutricia, The Netherlands) starting at an intake of 100 mL/kg/day according to standard practice in Dutch NICUs. Whenever human milk was insufficient or not available, preterm infants were (mixed) fed with preterm formula (Nutrilon Nenatal Start, Nutricia, The Netherlands). Data on the percentage of human milk and formula feeding are available (Table S5.1). No donor milk bank was available at the NICU during the study period.

As part of the EIBER study, fecal samples of preterm and full-term infants were collected immediately after birth and during postnatal weeks 1, 2, 3, 4 and 6. Previously, these samples have been used to assess the composition and functionality of the preterm microbiome by means of 16S rRNA gene amplicon sequencing[144,327] and metaproteomics[137,146].

### Sample selection

Fecal samples were selected based on the following criteria:

- Gestational age was between 24 and 40 weeks.
- Mothers did not receive antibiotic treatment during labor until six weeks thereafter.
- Infants received at least one antibiotic treatment.

The selection criteria were formulated to yield an as homogeneous as possible group. Infants were excluded if the mother received antibiotic treatment in the period of 48 h before birth until six weeks after birth. After infant selection, samples of some infants were unavailable or insufficient in volume at specific timepoints to conduct DNA extraction (Table S5.2). This resulted in a total of 116 fecal samples from 57 infants for DNA extraction (Fig. S5.1).

### DNA extraction

DNA extraction was performed on feces. First, 0.13 g of feces were weighed into a 2.0 mL screw cap tube filled with 0.25 g of 0.1 mm zirconia beads and three 2.5 mm glass beads. The weighed samples were stored at −80 °C until further processing. Every run included randomly selected fecal samples as well as a negative control consisting of one empty FastPrep tube with beads. Then, 300 µL of Stool Transport and Recovery Buffer (S.T.A.R. buffer, Cat. No. 03335208001, Roche Diagnostics) were added and bead-beaten three times at 5.5 ms for 60 s with 15 s pause (FastPrep-24 5G bead beating grinder and lysis system, MP Biomedicals). Subsequently, samples were incubated for 15 min at 95 °C at 100 rpm after which they were centrifuged (4 °C, 5 min, 14,860 rpm) and supernatant was stored at 4 °C. The process was then repeated with 200 µL S.T.A.R. buffer. In the case the first step did not yield supernatant, 300 µL of S.T.A.R. buffer were added. Subsequently, 250 µL of recovered supernatant were used for DNA extraction with Maxwell 16 Tissue LEV Total RNA Purification Kit (Cat. No. AS 1220, Promega). Isolated DNA was checked for quality with Nanodrop and quantified with Qubit dsDNA BR Assay Kit (Cat. No. Q32850, ThermoFisher Scientific) on DeNovix (DS-11 FX, DeNovix).

### Mock community

The Mycobiome Genomic DNA Mix (MSA-1010, ATCC) was used as mock community and was included in each sequencing library. The DNA-based mock community samples were derived from the same stock and were used as technical replicates. Species in the Mycobiome Genomic DNA Mix included *Aspergillus fumigatus* (ATCC MYA-4609D-5), *Cryptococcus neoformans* var. *grubli* (ATCC 208821D-2), *Trichophyton interdigitale* (ATCC 9533D-5), *Penicillium chrysogenum* (ATCC 10106D-5), *Fusarium keratoplasticum* (ATCC 36031D-5), *Candida albicans* (ATCC 10231D-5), *Candida glabrata* (ATCC 2001D-5), *Malassezia globose* (ATCC MYA-4612D-5), *Saccharomyces cerevisiae* (ATCC 201390D-5) and *Cutaneotrichosporon dermatis* (ATCC 204094D-5).

### Amplification and sequencing

Fecal samples, mock communities and negative controls were sent to Novogene (Cambridge, United Kingdom). Isolated DNA was measured for DNA purity and concentration with Nanodrop and Qubit 2.0, respectively, and integrity was visually inspected by agarose gel electrophoresis. Subsequently, samples were PCR-amplified with primers targeting the Internal Transcribed Spacer (ITS) 2 region (ITS3-2024F GCATCGATGAAGAACGCAGC, ITS4-2409R TCCTCCGCTTATTGATATGC) according to Novogene's protocol. Quality control of the PCR-amplified samples was performed by visual inspection of amplified PCR products after gel electrophoresis on agarose gel. Next, PCR products were mixed, purified and randomly assigned to a library. Libraries were prepared with NEBNext Ultra IIDNA Library Prep Kit (Cat No. E7645, New England Biolabs). After quality control of the library, ITS amplicon metagenomic sequencing was performed on the Novaseq6000 platform with 250 paired-end reads and a sequencing depth of 30,000 raw tags/sample. The samples were sequenced in two independent sequencing runs, in which mock communities were included in each library as technical replicates. DNA Mocks 1–4 and 5–8 were present as technical replicates in the first and second libraries,

respectively. Raw sequencing data were checked for distribution of sequencing quality and error rate. Raw sequences with barcode and primer removed and supporting metadata were deposited in the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under the accession number PRJEB48004.

## Bioinformatics

### *Preparing a theoretical mock community*

As quality control, a theoretical mock community was prepared and used to compare to the sequencing output of the Mycobiome Genomic DNA Mix. To this end, fasta sequences of the ITS region of fungal species in the Mycobiome Genomic DNA Mix were retrieved from the nucleotide database of NCBI. ITS sequences of each fungus were trimmed by aligning them with the primers in the MUSCLE alignment tool of MEGA X (version 10.1.8)[384]. A fake mock was then created with our in-house Python code (available at: https://gitlab.com/wurssb/gen_fake_mocks) by importing trimmed sequences as well as a file containing a barcode and a file containing proportions of species (10.0% each).

### *Taxonomic assignment with Qiime2*

Raw reads were processed according to the Q2-ITSxpress workflow[385]. Raw reads without barcodes and primers were imported in Qiime2. Subsequently, the conserved regions around the ITS gene were trimmed with ITSxpress[386], which has been shown to improve accuracy of taxonomic classification[387]. The sequence variants were then identified in the unmerged, trimmed sequences with Dada2[388]. Next, the Qiime classifier was trained using the UNITE database (version 8.3, all eukaryotes) with highest number of reference sequences (RefS) as compared to representative sequences (RepS)[389]. Fungal ITS classifiers were trained on the UNITE database on full reference sequences. Subsequently, sequence variants were classified with the trained classifier.

## Data analysis

### *Pre-processing*

Data were imported in R version 3.6.3[315] with the *Qiime2R* package (version 0.99.6)[390] to make a phyloseq object. Before pre-processing, 10,596 taxa were identified in 129 samples with 9,216,861 reads in total. The average number of reads per sample were 71,449 with a minimum of 5 and a maximum of 138,138, showing high variability between samples. Pre-processing of the data included various steps, of which the first was filtering ASVs on kingdoms. Non-fungal kingdoms were removed and consisted of Alveolata, Chromista, Eukaryota kgd Incertae sedis, Metazoa, Stramenopila and Viridiplantae (Fig. S5.2A and B). However, unassigned sequences at kingdom level were retained. Next, as part of further downstream processing, 834 singletons (ASVs of which the sum of reads is equal to one) were removed. Subsequently, samples with reads below 1000 were omitted. Eight samples were omitted with reads below 1000 for further analyses (Fig. S5.1). This resulted in a total of 121 samples, namely 109 fecal samples from 56 infants, 8 mocks, 1 theoretical mock and 3 negative controls (Fig. S5.1, Table S5.1). Infants were

categorized according to their gestational age into extremely preterm (24–27 weeks of gestation, *n* = 18 infants), very preterm (28–31 weeks of gestation, *n* = 15 infants), late preterm (32–36 weeks of gestation, *n* = 17 infants) and full-term (37–40 weeks of gestation, *n* = 6 infants). For each infant, the human milk intake was corrected for enteral feeding. To this end, the fraction of human milk intake was multiplied by the fraction of enteral feeding.

### *Composition plots*

For the number of reads, the reads from the ASV table were used to generate composition plots. For the relative abundance composition plots, the data were first transformed to compositional data with the *transform* function from the *microbiome* package (version 1.8.0)[391]. Composition plots were visualized and customized with the *ggplot2* package (version 3.3.3)[317].

### *Mock community check*

Quality control was based on mock communities, in which compositions of DNA mocks were compared to each other and to the fake mock. First, normality of the number of reads and of the relative abundance was checked visually with the *ggqqplot* function from the *ggpubr* package (version 0.3.0)[318] and quantitatively with *shapiro.test* function from the *stats* package (version 3.6.3)[315]. The null hypothesis (normal distribution) was rejected in both cases as the *P*-values were smaller than 0.05.



First, compositions of DNA mocks were compared to each other based on the number of reads and the relative abundance. The number of reads between the deviating DNA Mock 1 and other DNA mock samples were compared with the *kruskal.test* from the *stats* package. Although the number of reads of DNA Mock 1 were lower, this did not yield a significant difference compared to other DNA mock samples ($P = 0.76$, Fig. S5.3A). Next, technical replicates of the DNA mock communities were correlated with a Pearson correlation matrix using *pairs.panels* from the *psych* package (version 1.9.12)[392]. Correlation coefficients of the DNA Mock Technical Replicates 2–8 ranged between 0.85 and 1.00 indicating reproducibility of sequencing runs ($P \leq 0.001$, Fig. S5.4). As DNA Mock 1 was in the same library as the DNA Mocks 2–4, we deemed our data reliable.

Second, compositions of DNA mocks were compared to the fake mock. The same genera were identified in the DNA mock communities and the fake mock. However, some genera were over- or under-represented in the DNA mock communities. In the DNA mock communities, the mean relative abundances of *Fusarium*, *Candida* and *Cutaneotrichosporon* were 0.22 ± 0.05, 0.16 ± 0.05 and 0.16 ± 0.07, respectively (Fig. S5.3B). Compared to a theoretical relative abundance of 0.1 of each genus, they were the three most overrepresented genera in the DNA mock communities compared to the fake mock. On the other hand, compared to a theoretical relative abundance of 0.1 of each genus, *Trichopython*, *Aspergillus* and *Malassezia* were the most underrepresented with relative abundances of 0.03 ± 0.01, 0.03 ± 0.01 and 0.01 ± 0.01, respectively (Fig. S5.3B). Mean relative abundances of over- and under-represented genera in DNA mocks were not significantly different from the theoretical mock community (Mann–Whitney test, *P*-values not shown).

### *Hierarchical clustering*

Data were rarified on the minimum sum of reads (1184) using the *rarefy_even_depth* function of the *microbiome* package. Distance was calculated with the *distance* function of the *phyloseq* package (version 1.30.0)[393] using unweighted UniFrac and sample-wise comparisons. The *dendextend* package (version 1.13.4)[394] was used for generating the hierarchical cluster plot.

### *Phylogenetic diversity*

Phylogenetic diversity was calculated on rarified data with the *pd* function of the *picante* package (version 1.8.2)[395]. Significance was determined with the *compare_means* function of the *ggpubr* package with default settings except *P*-values were adjusted using BH correction. The plot was generated using the *ggplot2* package.

### *Redundancy analysis*

Dimension reduction analysis was performed to identify clinical variables that significantly explained variation in the mycobiota composition. To this end, compositional data were transformed with centered log ratio (CLR) using the *transform* function of the *microbiome* package. Next, core members of the mycobiota were defined with *core_members* from the *microbiome* package with detection set to 1/1000 and prevalence set to 25/100. Detrended correspondence analysis was performed with *decorana* from the *vegan* package (version 2.5-6)[316] to determine the correct dimension reduction method. Redundancy analysis (RDA) was performed with the *vegan* package, using Aitchison distance, defined as the Euclidean distance between CLR-transformed compositions[396,397]. CLR-transformed ASV relative abundances were not scaled. Samples with missing values of explanatory variables were omitted, leaving 95 samples as input. After running the first RDA, variance inflation was checked with *vif.cca* from the *vegan* package to omit clinical variables with VIF ≥ 10. Next, RDA was repeated, now with forward and reverse automatic stepwise model selection for constrained ordination with *ordistep* from the *vegan* package with settings $p_{in} = 0.05$, $p_{out} = 0.1$ and 999 permutations. Resulting *P*-values were adjusted with *p.adjust* from the *Stats* package using BH correction.

### *Permutational multivariate analysis of variance*

Permutational multivariate analysis of variance (PERMANOVA) was performed with *adonis* from the *vegan* package to test for community-level differences between group centroids. CLR-transformed compositional data of the core mycobiota were used for this analysis. Permutations were set to 999 and Euclidean was used as dissimilarity matrix. Gestational age category was tested with PERMANOVA. Subsequently, homogeneity of variances was checked with *vegdist* and *betadisper* from the *vegan* package. For the gestational age categories, the outcome failed to reject the null hypothesis of homogeneous multivariate dispersions, and this predictor was therefore concluded to have homogenous multivariate dispersions.

### *Linear discriminant analysis Effect Size*

Linear discriminant analysis Effect Size (LEfSe) was performed to assess differences between mode of delivery groups (vaginal delivery, planned C-section and emergency C-section) at phylum, class, order, family, genus and species level. For this analysis, only fecal samples from preterm infants were selected ($n$ = 96). The samples per mode of delivery groups were as follows: vaginal delivery $n$ = 54; planned C-section $n$ = 28; and emergency C-section, $n$ = 14. The *phyloseq2lefse* function as provided on the *Rrumen* package GitHub[398] was used on compositional data to generate the input file for Huttenhower lab Galaxy server (https://huttenhower.sph.harvard.edu/galaxy/root). The alpha value for the two-tailed non-parametric Kruskal–Wallis test was set to 0.01 and the logarithmic LDA score for discriminative features to 3.5. For multi-class analyses, the one-against-all method was selected.

## Results

### Composition of fungal taxa in the preterm infant intestine over the first six postnatal weeks

Besides fungi, other eukaryotic kingdoms were observed and included Alveolata, Eukaryota kgd Incertae sedis, Chromista, Metazoa, Stramenopila and Viridiplantae (Fig. S5.2A). These kingdoms comprised 4283 taxa and 23.1% of total observed reads. For further analyses, the fungal and unassigned kingdoms were retained, after which the relative abundance of the fungal kingdom ranged between 95.3% and 99.2% during the first six postnatal weeks with the remainder being unassigned (Fig. S5.2B). After pre-processing the data, the remaining fecal samples ($n$ = 109) were further assessed for mycobiota composition. The first and second most abundant phyla in feces were Ascomycota and Basidiomycota, respectively (Fig. 5.1). Mean Ascomycota relative abundance varied between a minimum of 82.1% and a maximum of 91.7% (± 28.8% and ± 8.9% SD, respectively) in the first six postnatal weeks. Mean relative abundance of Basidiomycota gradually increased until the fourth week from 3.5% to 17.0% (± 4.2% and ± 28.6% SD, respectively), after which it decreased in the sixth week to 5.4% (± 11.5% SD). Both phyla were consistently the most dominant in preterm and full-term infants in all postnatal weeks. In twenty samples, Basidiomycota abundance was higher than the highest average of 17.0%. However, this could not be related to gestational or postnatal age.

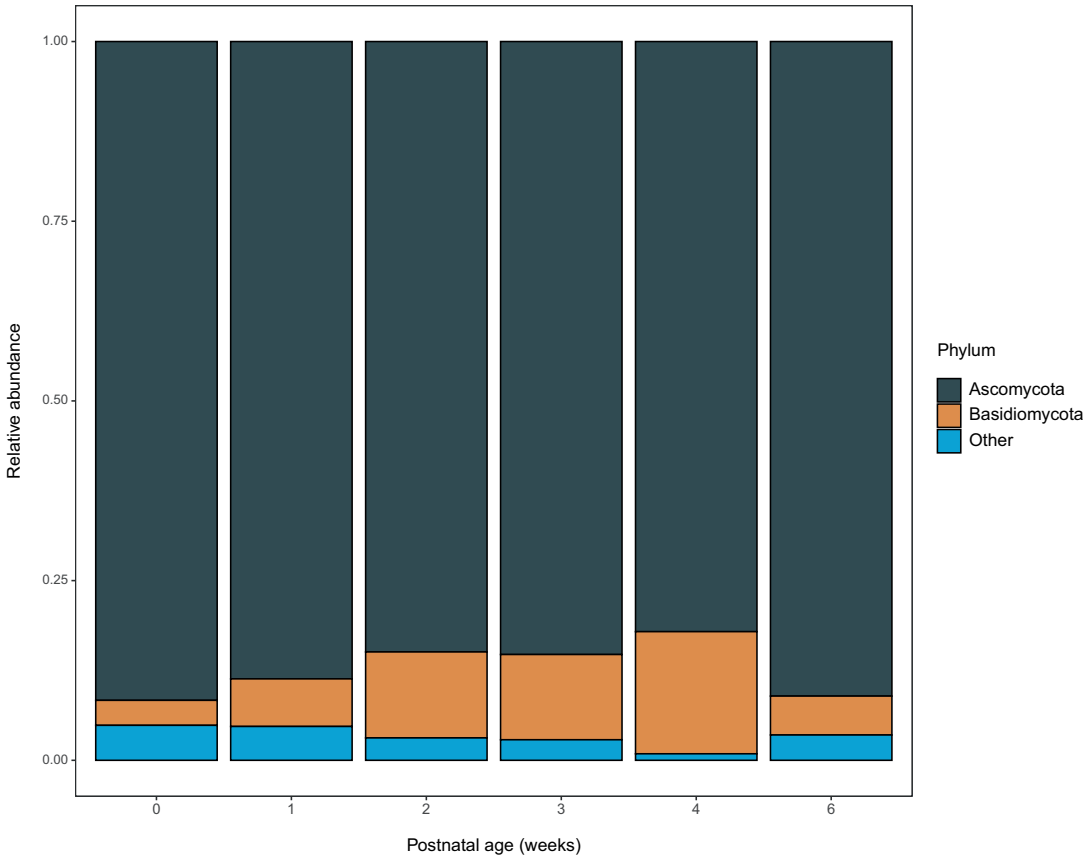


**Figure 5.1** Relative abundance of the two most abundant fungal phyla in feces of preterm and full-term infants during the first six postnatal weeks.

## The relative abundance of *Candida* spp. increases with gestational and postnatal age

Within the Ascomycota phylum, *Candida* spp. was consistently the most abundant genus in the first six weeks. The genus was observed in all samples of preterm and full-term infants (Fig. 5.2). On average, it comprised approximately one third of observed genera in the first week (35.2% ± 40.0%) and up to more than two thirds in the last week (68.6% ± 36.3%), albeit with high variability between samples (Fig. S5.5A and Fig. 5.2). The total number of reads for this genus increased over time from 21,871.6 reads in meconium to 60,094.6 reads at Postnatal Week 6 (Fig. S5.6). Of the *Candida* species, *C. albicans* was predominant with relative abundances ranging between 88.7% ± 21.5% (Week 1) and 96.5% ± 7.3% (Week 6) (Fig. S5.7).

Relative abundance of *Candida* spp. gradually increased both with gestational age category as well as postnatal age (Fig. S5.5B). In extremely preterm infants, colonization with *Candida* spp. was most stochastic due to high standard deviations. The relative abundance of this genus increased from 0.39 in the first week to 0.56 in the sixth week in extremely preterm infants (± 0.38 and ± 0.41 SD, respectively), whereas *Candida* spp. increased from 0.02 in the first week to an abundance as high as 1.00 in full-term infants (± 0.02 and ± 0.00 SD, respectively, Fig. S5.5B).



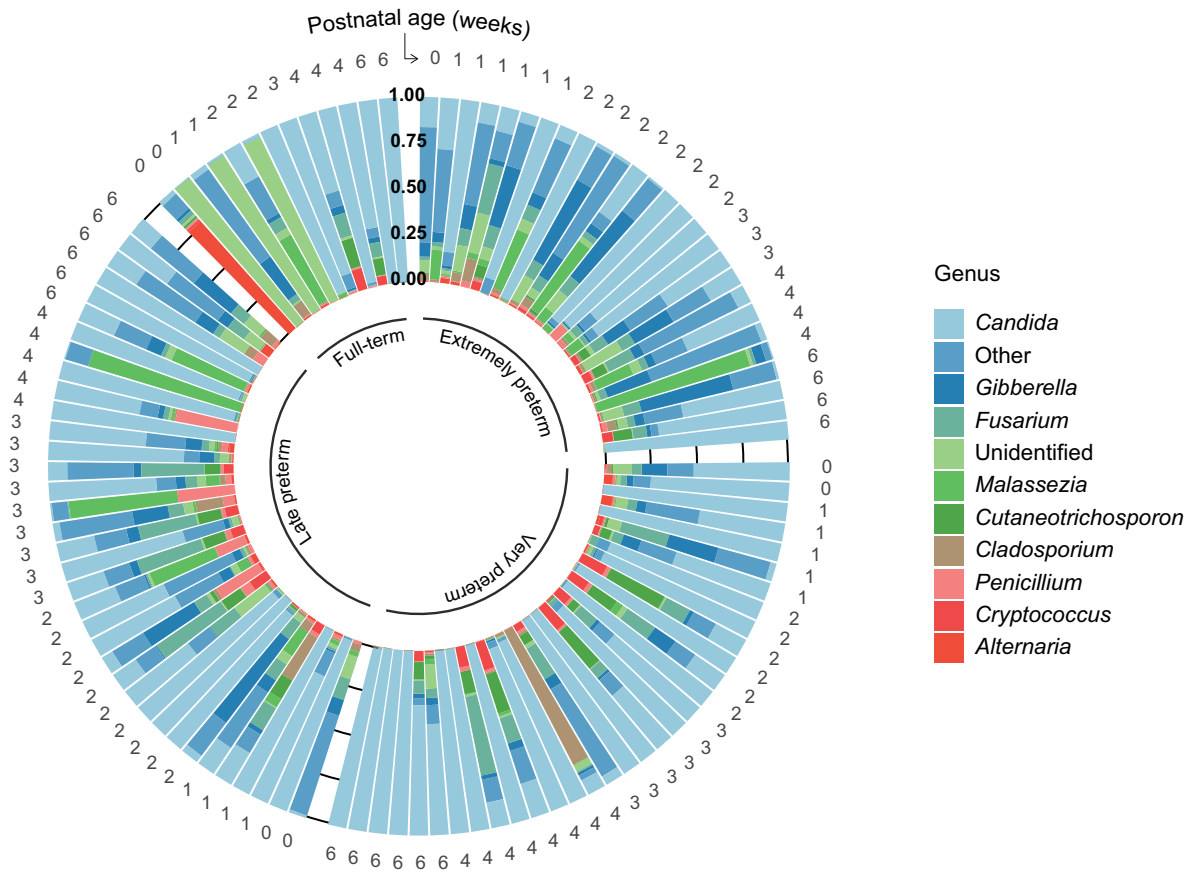


**Figure 5.2** Relative abundance of the ten most abundant genera in every fecal sample of preterm and full-term infants. The postnatal age in weeks is displayed on the outer circle; the gestational age categories are displayed on the inner circle. The horizontal lines indicate the relative abundance in quartile percentages. Genera not belonging to the ten most abundant ones are merged under "Other".

## Phylogenetic diversity of the mycobiota remains stable in the first six postnatal weeks

Diversification of the mycobiota was investigated by performing phylogenetic diversity analyses in each gestational age group over the first six postnatal weeks (Fig. 5.3). Median phylogenetic diversity decreased from the first week onwards in extremely and very preterm infants, although none of these changes were statistically significant. Late preterm infants and full-term infants, who are physiologically most similar, were stable in phylogenetic diversity in the first two postnatal weeks. The number of samples in later postnatal weeks in the full-term infant group were too limited to be conclusive. Interestingly, phylogenetic diversity decreased significantly in the fourth postnatal week compared to the preceding Postnatal Week 3 in late preterm infants.

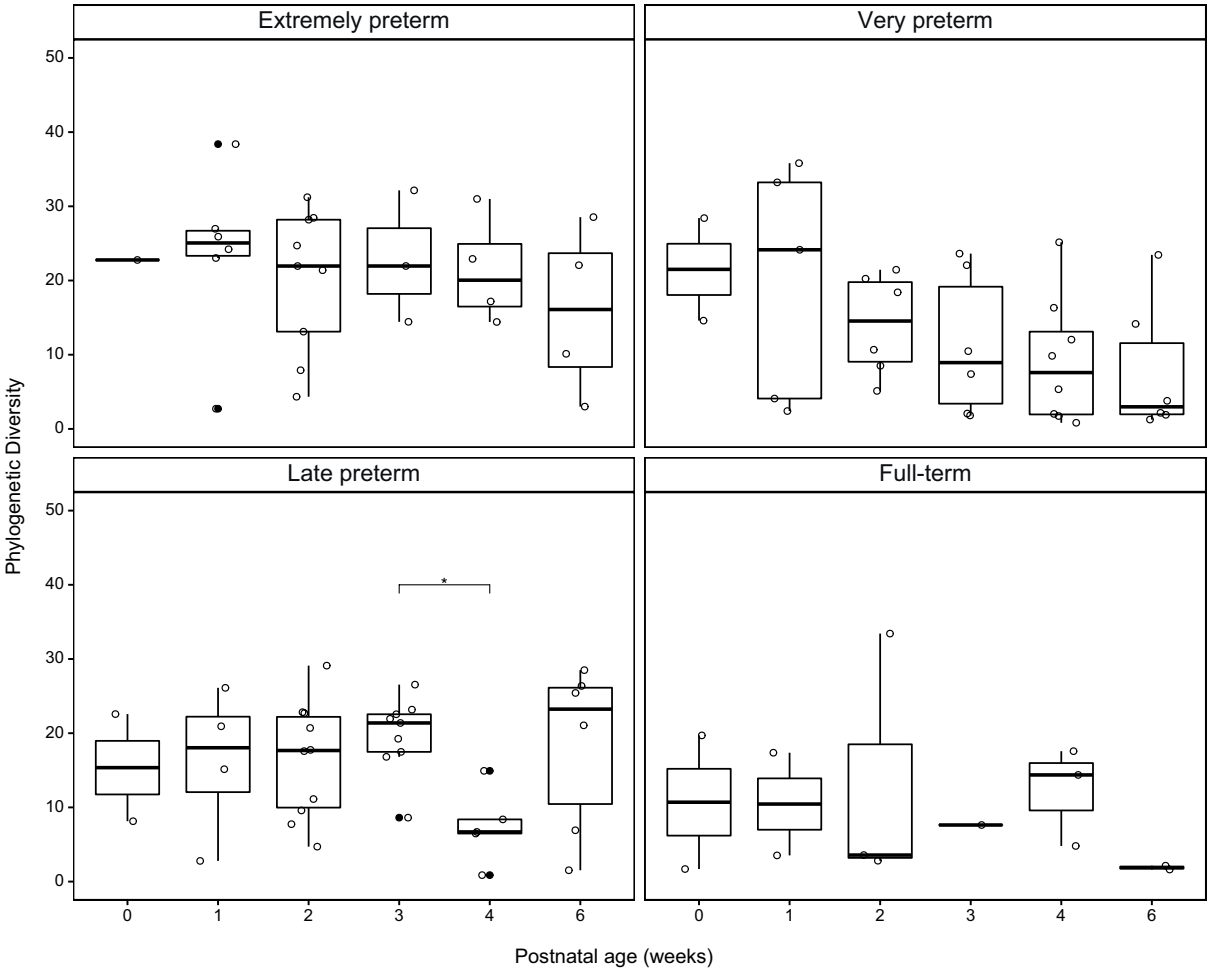


**Figure 5.3** Phylogenetic diversity of preterm and full-term infants during the first six postnatal weeks. Individual data points are displayed as open circles, whereas outliers are filled circles. $^{*}P_{adj} \leq 0.05$.

## Individuality and mode of delivery significantly explain variation in mycobiota composition

To investigate which clinical variables explained variation in the fecal mycobiota composition of preterm and full-term infants, PERMANOVA and redundancy analysis were performed. The

differences between centroids of gestational age groups were assessed by PERMANOVA and differences were statistically significant ($P = 0.005$, Table S5.3). Therefore, gestational age groups were used to categorize infants in hierarchical clustering and redundancy analysis. Hierarchical clustering was performed to investigate relatedness of samples in their respective gestational age categories. Samples of all gestational age categories did not cluster based on unweighted UniFrac distance of the mycobiota (Fig. S5.8). Results of hierarchical clustering were rather random and could indicate that individual variability is high as well as the need for a larger number of samples per gestational age category.

Subsequently, redundancy analysis was performed to investigate the effect of clinical variables on the variation of the mycobiota composition. The mode of delivery, gestational age, birth weight, individuality, duration of the second and third antibiotic treatment and body weight contributed to explaining the variation in mycobiota composition before automatic stepwise model selection. After automatic stepwise model selection, individuality and mode of delivery were predictors significantly explaining variation in the mycobiota composition ($P_{individuality} = 0.005$, $p_{MoD} = 0.005$) (Table S5.4). However, these predictors lost their significance after adjusting the $P$-value ($P_{adj.\ individuality} = 0.238$ and $P_{adj.MoD} = 0.238$). Subsequently, the effect of individuality was removed to further investigate the effect of other clinical variables (Fig. 5.4). Here, mode of delivery did not significantly explain variation in the mycobiota composition.



## Vaginal and caesarean delivery enrich for vaginal-like and skin-like fungi in preterm infants

Being significant initially in the redundancy analysis, the mode of delivery was hypothesized to influence mycobiota seeding. Vaginal delivery in particular is known to vertically transfer *Candida* spp. As such, we investigated the effect of mode of delivery on the mycobiota composition solely in preterm infants with Linear discriminant analysis Effect Size (LEfSe) (Fig. 5.5A and B). Each type of delivery mode was characterized by specific taxa, with no overlap in taxa characteristic for planned and emergency caesarean (C-)sections (Fig. 5.5B). Instead, vaginally delivered infants indeed were enriched with the *Candida* genus. On the other hand, the Malasseziomycetes class and lower taxonomic levels were mainly characteristic for infants delivered through emergency C-section. Interestingly, the vaginally delivered and emergency C-section infants shared fungi within the Saccharomycetes class but not for lower taxonomic levels. Infants delivered with a planned C-section were, among others, enriched in the Microascales order and *Cladosporium* genus.

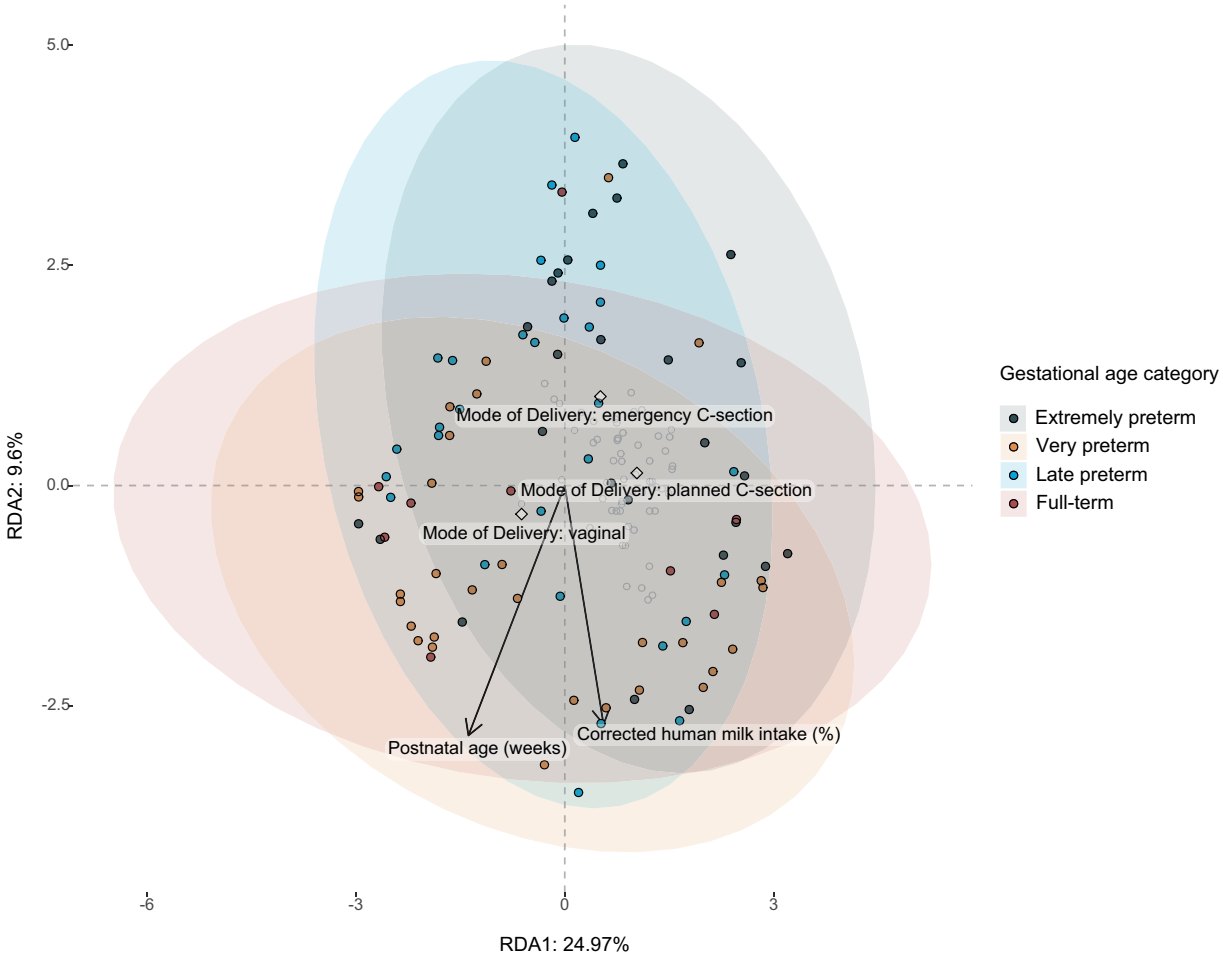


**Figure 5.4** Redundancy analysis on the fecal mycobiota of preterm and full-term infants during the first six postnatal weeks. Continuous clinical variables are indicated with arrows, whereas the centroids of categorical clinical variables are indicated with diamonds. Mode of delivery was significant after automatic stepwise model selection ($P = 0.005$) and its centroids are displayed; centroids of other clinical variables were left out for clarity. Colored points indicate individual fecal samples colored within its respective gestational age category. The effect of gestational age categories on the mycobiota composition was verified with PERMANOVA analysis ($P = 0.005$, Table S5.3).

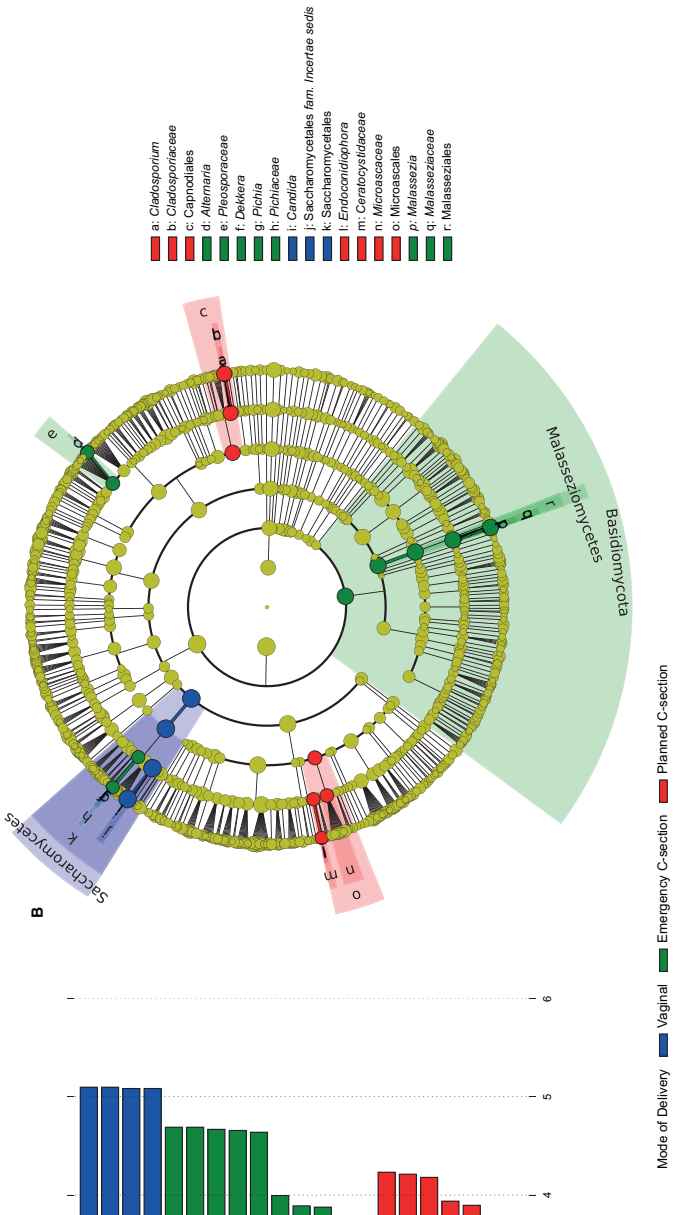


**Figure 5.5** Linear discriminant analysis Effect Size results on the mode of delivery in preterm infant feces ($n$ = 96). The fungal taxa that were significantly different in abundance between the mode of delivery groups are displayed in (A) a histogram of the LDA scores and (B) a cladogram.

## Discussion

Our findings show that preterm and full-term infants are colonized by various eukaryotic kingdoms, of which fungi were most prominent. Fungal diversity remained stable in the first six postnatal weeks and the genus *Candida* was the most abundant. Its abundance was additionally shown to increase with gestational and postnatal age. Although gestational age was important for the mycobiota composition, samples did not cluster based on gestational age categories. Instead, individuality and mode of delivery were significant predictors for mycobiota variation. Vaginally delivered infants were characterized by high abundance of *Candida* spp., whereas infants delivered through emergency C-section were characterized by *Malassezia* spp. Although the mycobiome is gaining more attention recently, this is the first time that the effect of clinical variables on the gut mycobiota composition is described for preterm infants with varying degrees of prematurity. We speculate these findings are relevant for clinical practice and will gain traction in the near future.

Interestingly, many other eukaryotic kingdoms were observed besides fungi. After fungi, the next most abundant kingdom was Viridiplantae. This kingdom has been observed more often in infants and has been suggested to be remnants from plant material ingested by the mother[136,399]. In fact, green algae are part of this kingdom and are used to generate supplements such as docosahexaenoic acid (DHA, omega-3)[400]. Omega-3 is essential for fetal neurodevelopment and is recommended in pregnancy and during breastfeeding[400,401]. We therefore hypothesize that parts of this eukaryotic DNA may end up in human milk and is thus transferred to the infant. Moreover, mother's own milk was increasingly supplemented with human milk fortifier (Nenatal BMF) as part of standard neonatal care practices in Dutch NICUs, starting at 100 mL/kg/day enteral feeding. Fortification of human milk is necessary to meet the nutritional needs of the preterm infant. Human milk fortifier contains—besides protein, minerals and vitamins—DHA, which might well be the origin of the detected Viridiplantae.

Within the fungal kingdom, the phyla Ascomycota and Basidiomycota were the most abundant in the infant intestine, which has also been observed previously[61]. Moreover, the abundance of *Candida* spp. increased with gestational and postnatal age. Similar to findings of James et al.[157], we observed the *Candida* genus and the species *C. albicans* were most dominant in preterm infants. Interestingly, the abundance of the *Candida* genus was reported in lower abundance by James et al.[157] Most preterm infants of that study did not receive antibiotic treatment after the second day of life, which suggests antibiotic treatment may have enriched *Candida* spp. in the preterm infants described herein. However, other confounding factors including the sampling period, mode of delivery, gestational age and postnatal age should be accounted for in future studies to assess the effect of antibiotic treatment on mycobiota development.

While previous research highlighted the abundance of *Candida* spp.[157], we were additionally able to show that vaginal delivery promotes colonization with *Candida* spp. Vertical transfer of this genus has been described and is therefore very likely to occur in infants described herein[156,162]. Although *Candida* spp. is commensal in most cases, the genus may also cause disease in immunocompromised hosts. Preterm infants often experience overgrowth of an opportunistic

pathogenic fungus after antibiotic treatment, typically invasive systemic candidiasis[178–181]. Invasive systemic candidiasis in preterm infants can lead to considerable morbidity and mortality rates[182–184]. It is most often caused by *C. albicans*, which interestingly was the most abundant *Candida* species during all postnatal weeks. It might transition from commensal to opportunistic pathogen in response to perturbations in the microbiota and weakening of the immune system or of the physiologic barriers[181,185–188]. Factors that might trigger the transition include long-term or repeated use of broad-spectrum antibiotics, use of central venous catheters, parenteral nutrition and a naïve immune system[180,182,185]. Indeed, antibiotics may promote overgrowth by *Candida* spp. through induction of genetic changes leading to increased fitness of *C. albicans* in the gut[189]. All infants in our cohort received at least one antibiotic treatment, were predominantly colonized by *Candida* spp. and the most abundant species was *C. albicans*. However, candidiasis was not observed in the current cohort. Hence, the mycobiome may act as reservoir for opportunistic pathogens in immunocompromised hosts such as preterm infants, which may be triggered by specific environmental influences such as antibiotic treatment[161].

Individuality and mode of delivery were observed as significant predictors for mycobiota variation. Similar to our results, infant mycobiomes from anal swabs exhibited high intra-individual variation and were concluded to be individualized[54]. Moreover, mode of delivery has previously been shown to shape the mycobiome composition in human milk as well as on skin, oral and anal body sites of infants[54,165]. As hypothesized before[155], we observed that vaginally delivered infants were enriched in *Candida* spp., whereas infants delivered through (emergency) C-section were characterized by *Malassezia* spp. *Malassezia* spp. are commonly identified on the skin of adults and infants and therefore have been hypothesized to be vertically transmitted from parent to infant upon skin contact[402,403]. In C-section infants, the gut microbiota composition has already been described to be more similar to mother's skin microbiota[121]. Our data support the hypothesis of vertical transmission of fungi and thereby underpin the importance of the mode of delivery in bacterial and fungal colonization. However, *Malassezia* spp. were not characteristic for infants born through planned C-section. It remains unknown what has contributed to these differences as most studies lack distinction between types of caesarean delivery.

The question remains if the observed fungi are residents of the gut or rather transients. Fungi are present in relatively low concentrations of $10^5$–$10^6$ cells per gram of fecal matter, although these numbers may be an underestimation[56,59,60]. Even though they are smaller in cell counts, fungal cells are 10-fold longer and 100-fold larger in volume than bacterial cells. Hence, the fungal biomass and the metabolites they produce cannot be compared with the microbiota by solely considering cell counts[61]. It is plausible that fungi are able to perform bioactive functions in the preterm gut, as metabolic, trophic and protective functions have been described[58]. The same cohort of preterm and full-term infants has been studied previously by metaproteomics[137,146]. Here, we did detect *Candida*-derived proteins sporadically in gastric aspirates and feces. With advances in technology, we may now identify more proteins to better approximate fungal activity in the intestinal tract of infants. Therefore, future studies should elucidate activity of the mycobiota by investigating fecal proteomes of infants with state-of-the-art techniques that enable to identify more proteins.

While our study identified prominent fungi in the intestine of preterm infants over time and assessed which clinical variables influence the mycobiota composition, we acknowledge the relatively small number of particularly full-term infants and the lack of longitudinal data for some of the infants described in this study. This should be considered when interpreting the data and the significant outcomes, particularly when studying the differences in mycobiota composition per gestational age category. Additionally, the fungal load was not assessed by means of quantitative PCR (qPCR) due to insufficient sample material, which is needed to put the results into perspective of the intestinal ecosystem. Furthermore, sequencing the mycobiota has its challenges. Such challenges include the lack of a standardized and reliable method of mycobiota sequencing, as well as a more comprehensive fungal database coverage compared to bacterial databases[62,404,405]. Therefore, some taxa may have been over- or under-represented in the results described herein. Future studies should focus on developing standardized and reliable methods to allow scalability[62]. Subsequently, this may advance research of interkingdom interactions that are currently limited. These interactions are expected to be of great importance in a key body site where crosstalk and interactions with host immunity result in systemic manifestations of either health or disease[62].

## Conclusion

Our findings indicate that fungi and other eukaryotic kingdoms can be detected in the intestine of preterm and full-term infants in the first six postnatal weeks. While intestinal fungi have been characterized in preterm infants before, this is the first time it was assessed in relation to clinical variables in preterm infants. The mycobiota shows great similarities with the microbiota in how individuality, mode of delivery, and gestational and postnatal age drive its development in preterm infants. As mycobiome research is gaining traction, future studies should focus on bridging the gap between the bacterial and fungal kingdoms in the intestine. Such insights could refine the healthcare of this vulnerable group of infants.

## Supplementary information

**The first fungi: mode of delivery determines early life fungal colonization in the intestine of preterm infants**

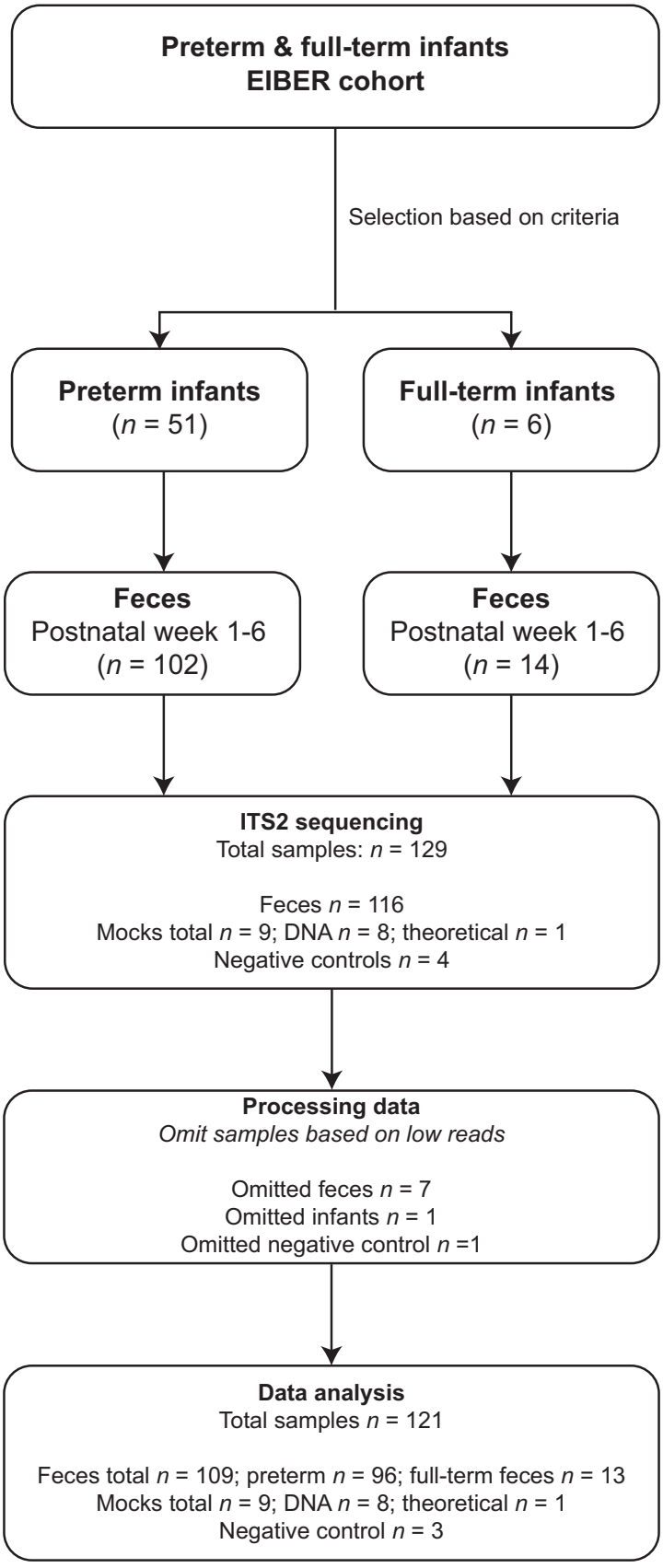


**Figure S5.1** Overview and workflow diagram of this study. Preterm and full-term infants were part of the EIBER study, in which feces were collected in the first six postnatal weeks with the exception of Week 5. Samples of the current study were selected based on the sample selection criteria and were used for ITS2 sequencing.

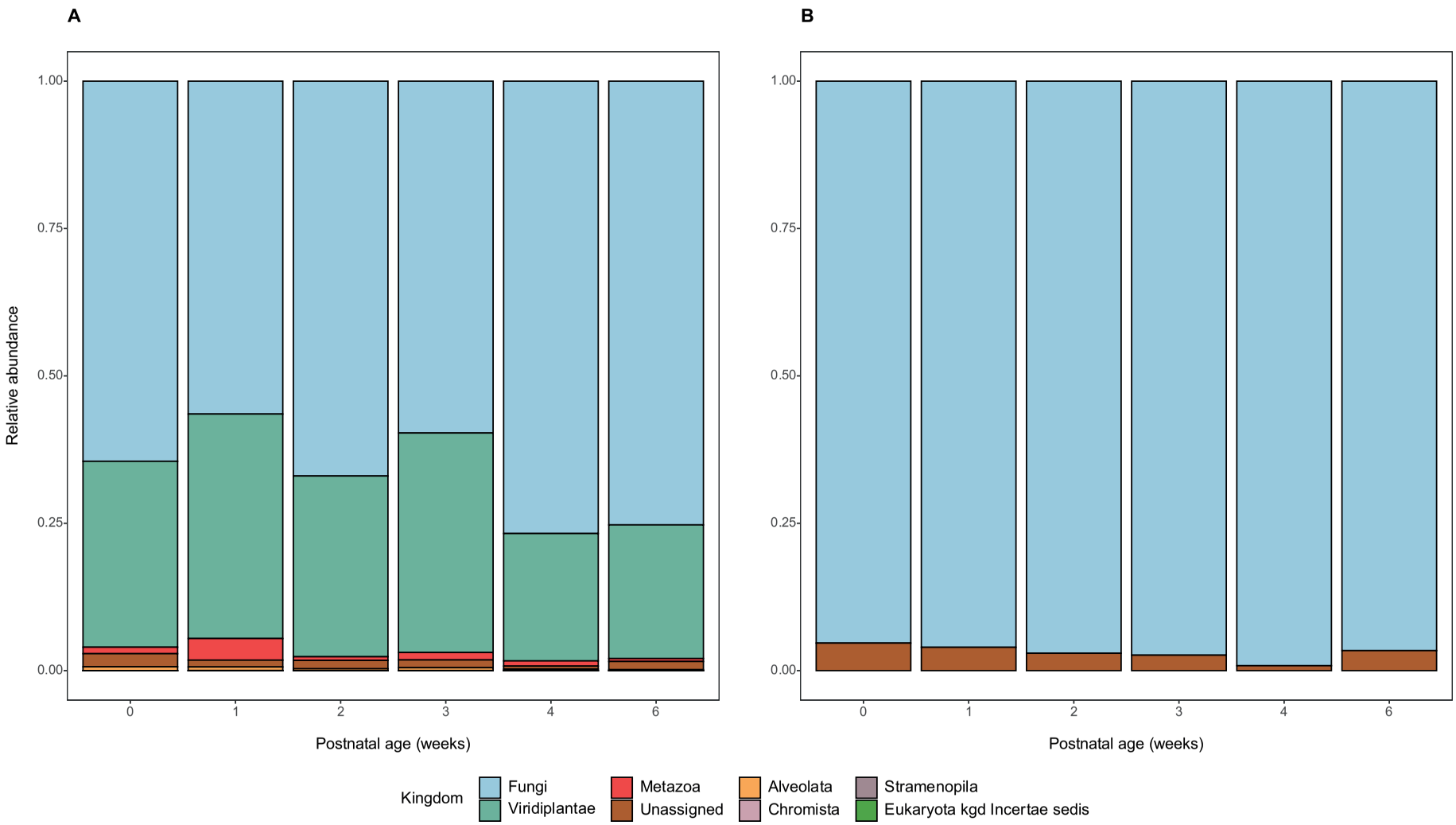


**Figure S5.2** Relative abundance of all kingdoms identified in feces of preterm and full-term infants in the first six postnatal weeks. The data are displayed (A) before and (B) after pre-processing.

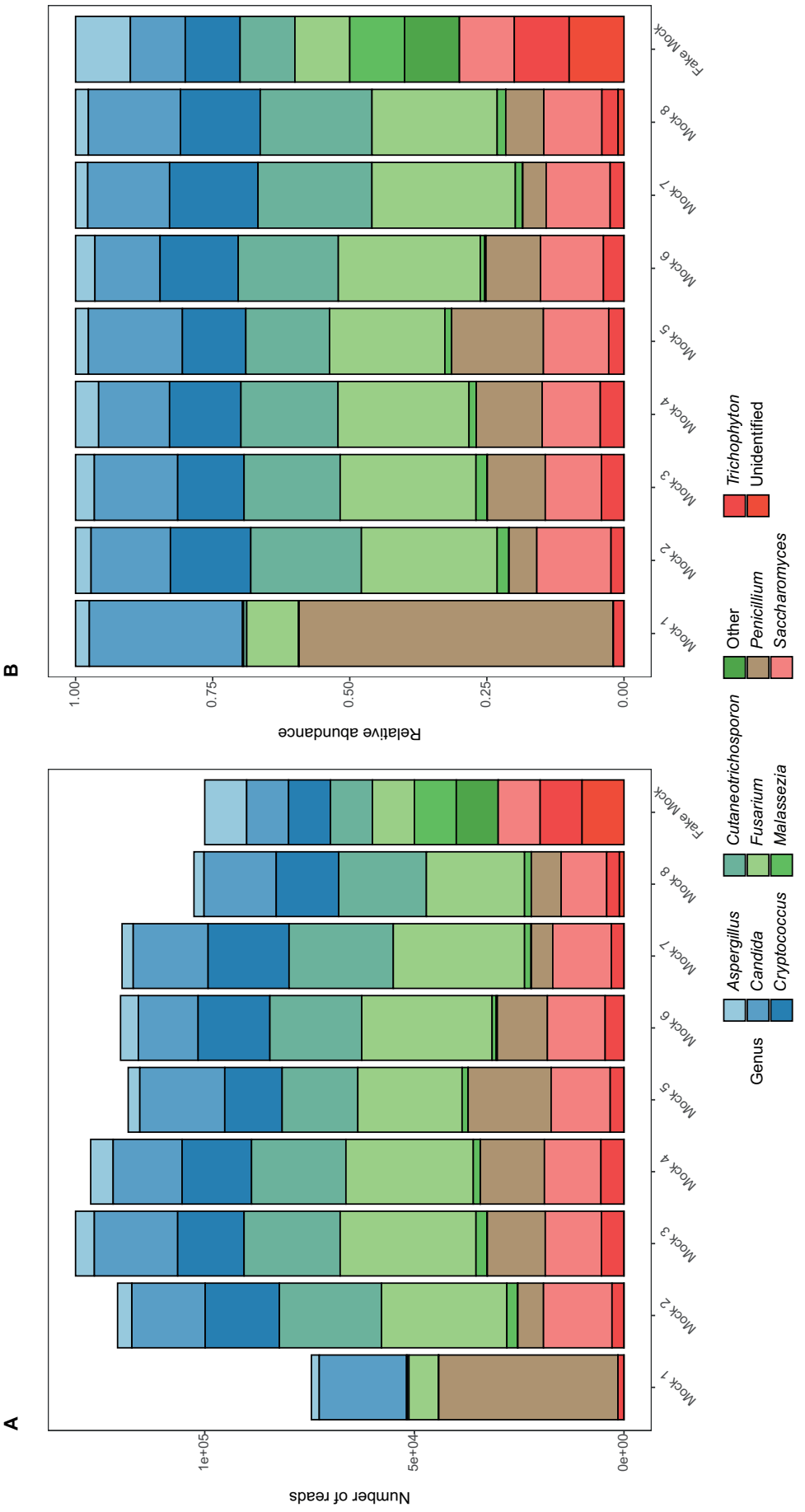


**Figure S5.3** DNA and fake mock community composition plots of the ten most abundant genera. The DNA and fake mock communities are shown as (A) number of reads and (B) relative abundance.

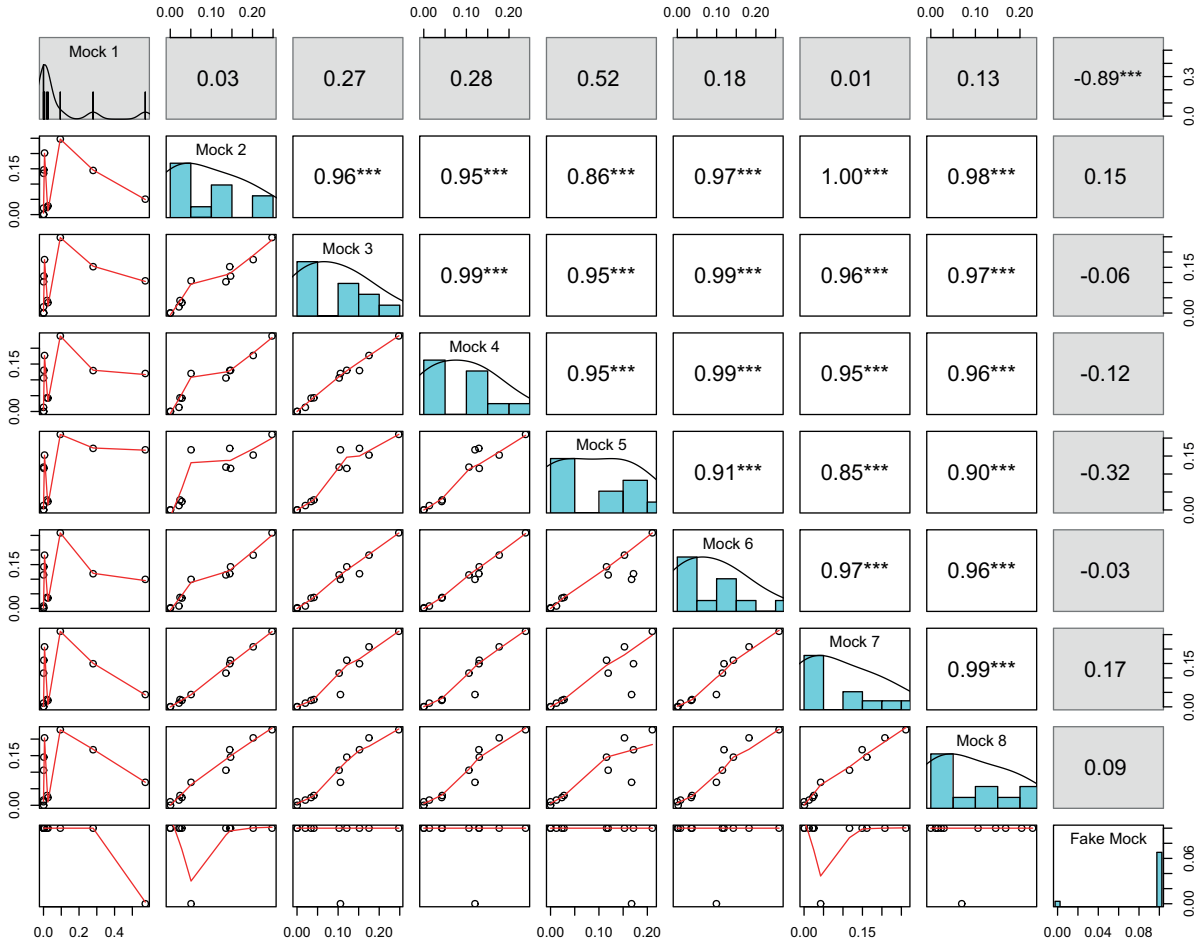


**Figure S5.4** Correlation matrix of DNA and fake mock communities. The correlation coefficients of Mocks 2–8 were used for quality control, whereas correlation coefficients of Mock 1 were not considered due to deviant absolute reads (indicated in grey). Asterisks indicate significance levels with $^{***}P \leq 0.001$.

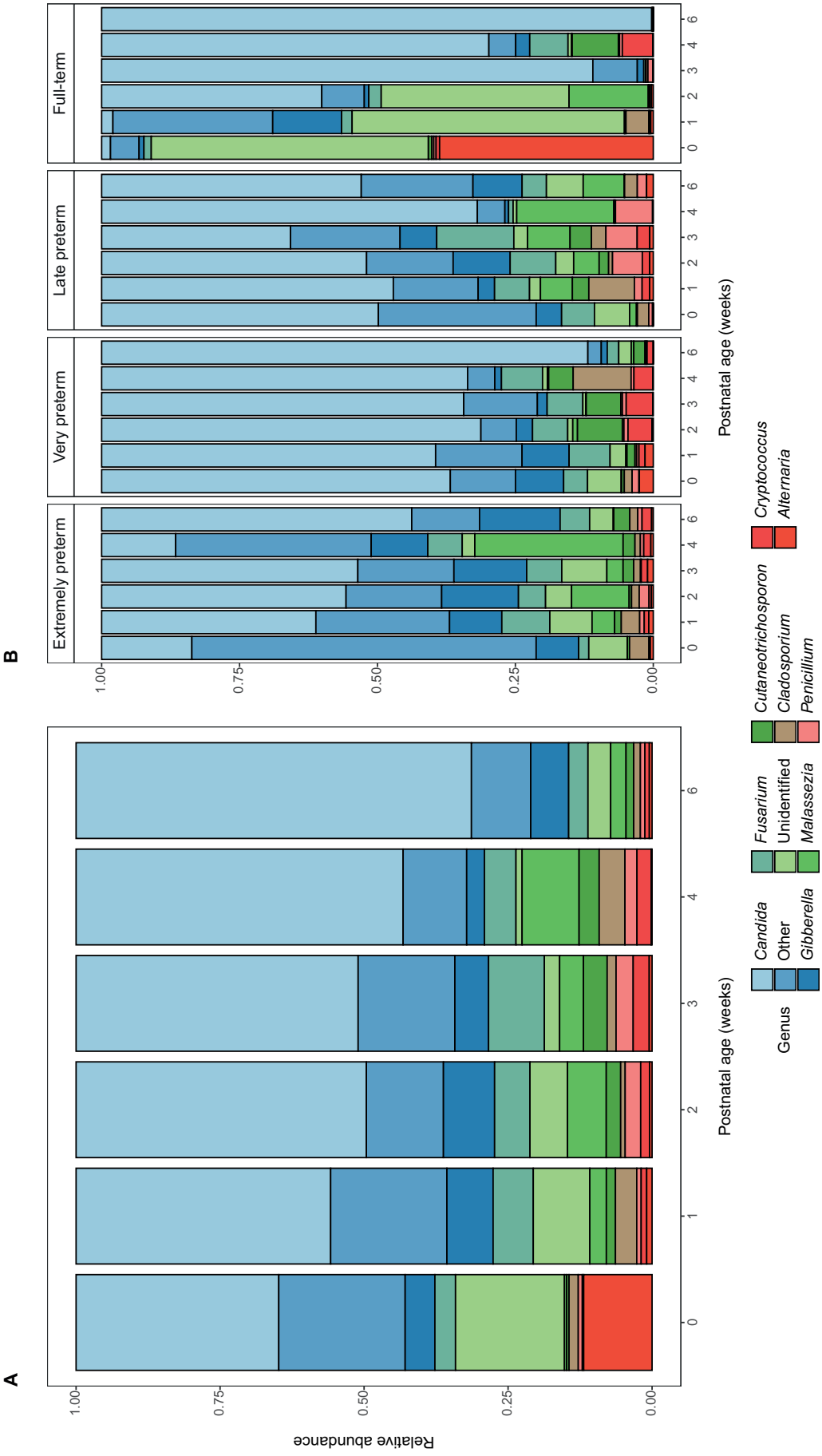


**Figure S5.5** Relative abundance of the ten most abundant genera in feces of preterm and full-term in the first six postnatal weeks. Feces of (A) all preterm and full-term infants together and (B) preterm and full-term infants in their gestational age categories.

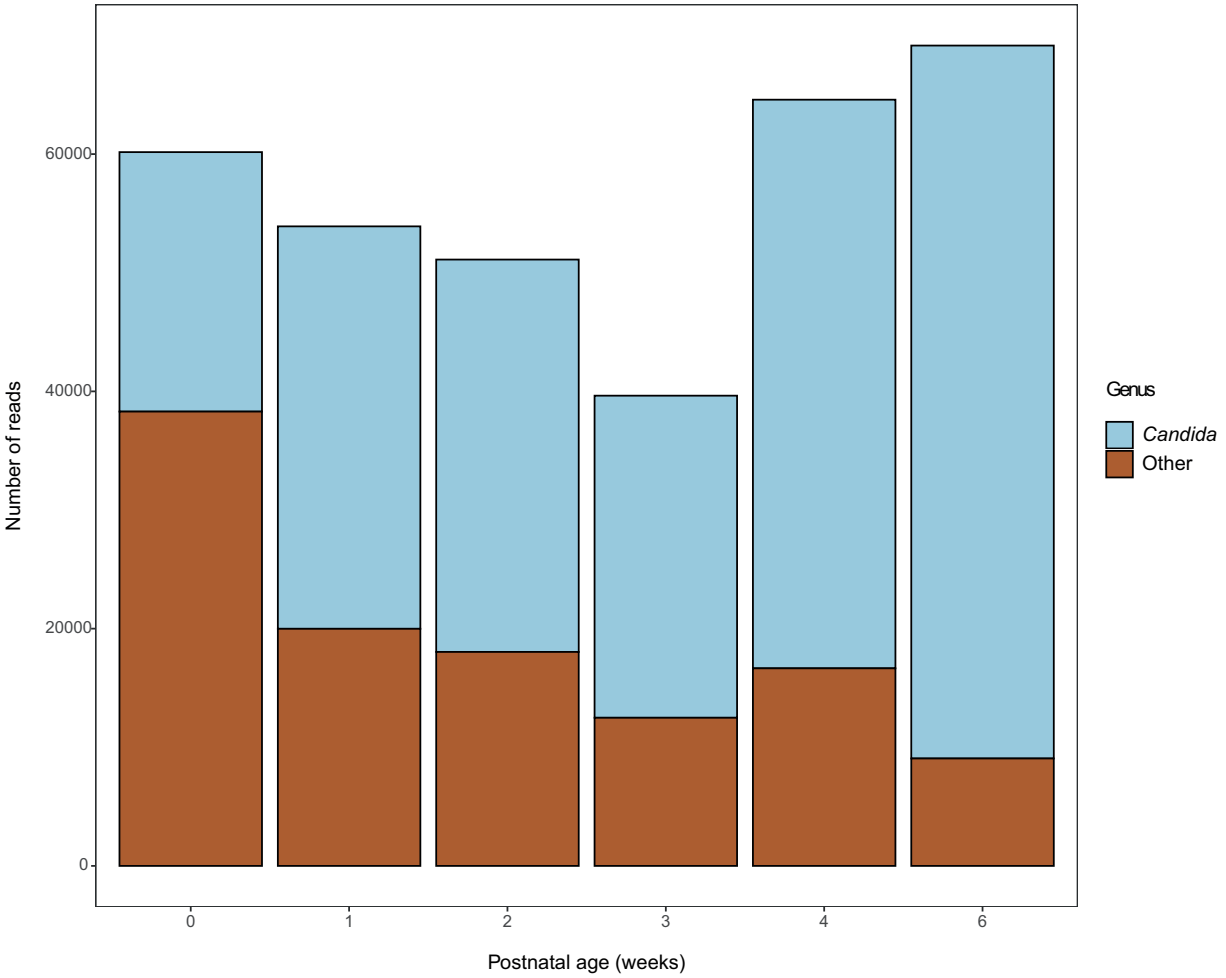


**Figure S5.6** Number of reads of *Candida* spp. relative to the other genera identified in feces of all preterm and full-term infants together in the first six postnatal weeks.

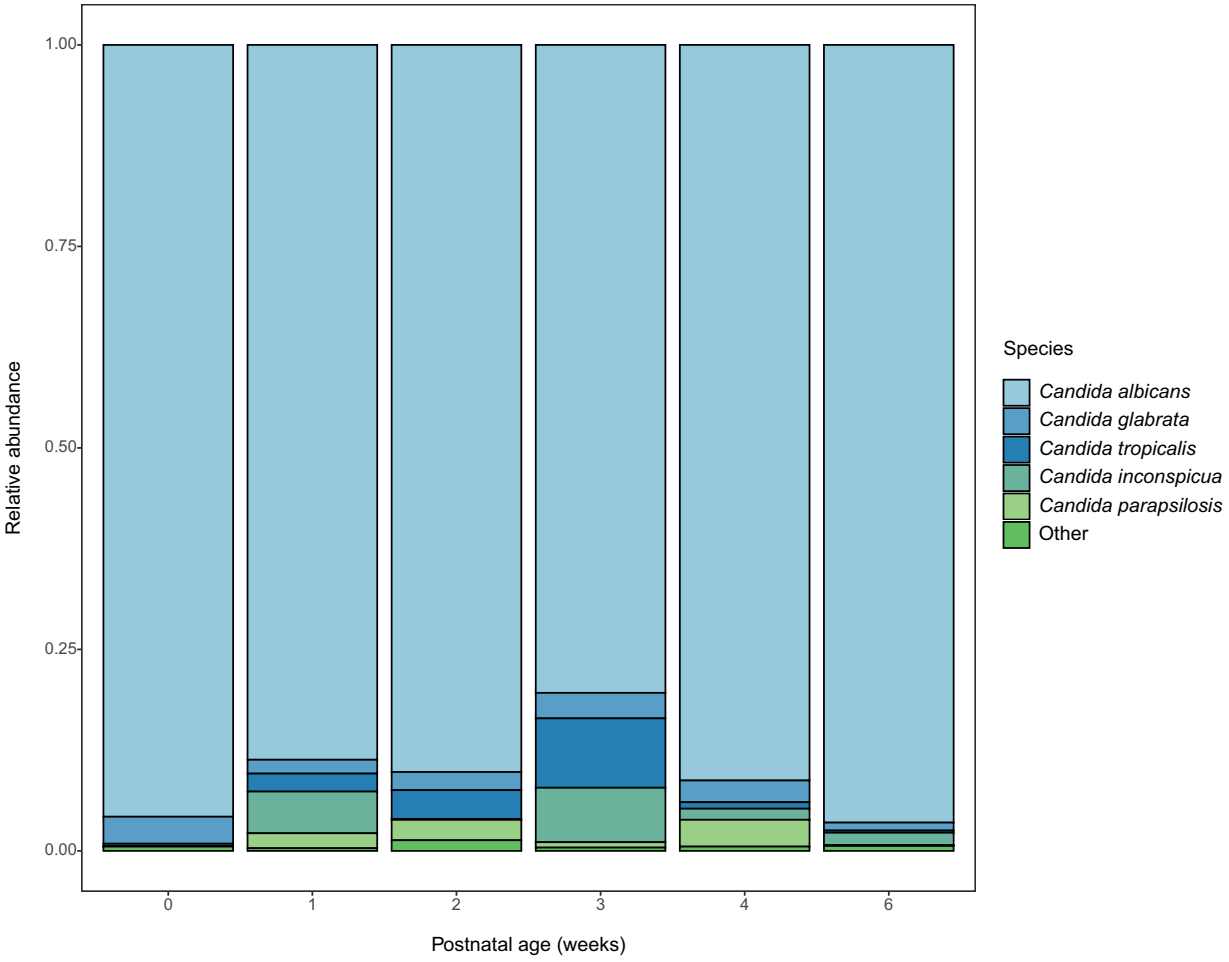


**Figure S5.7** Relative abundance of the five most abundant species within the *Candida* genus for all preterm and full-term infants in the first six postnatal weeks.

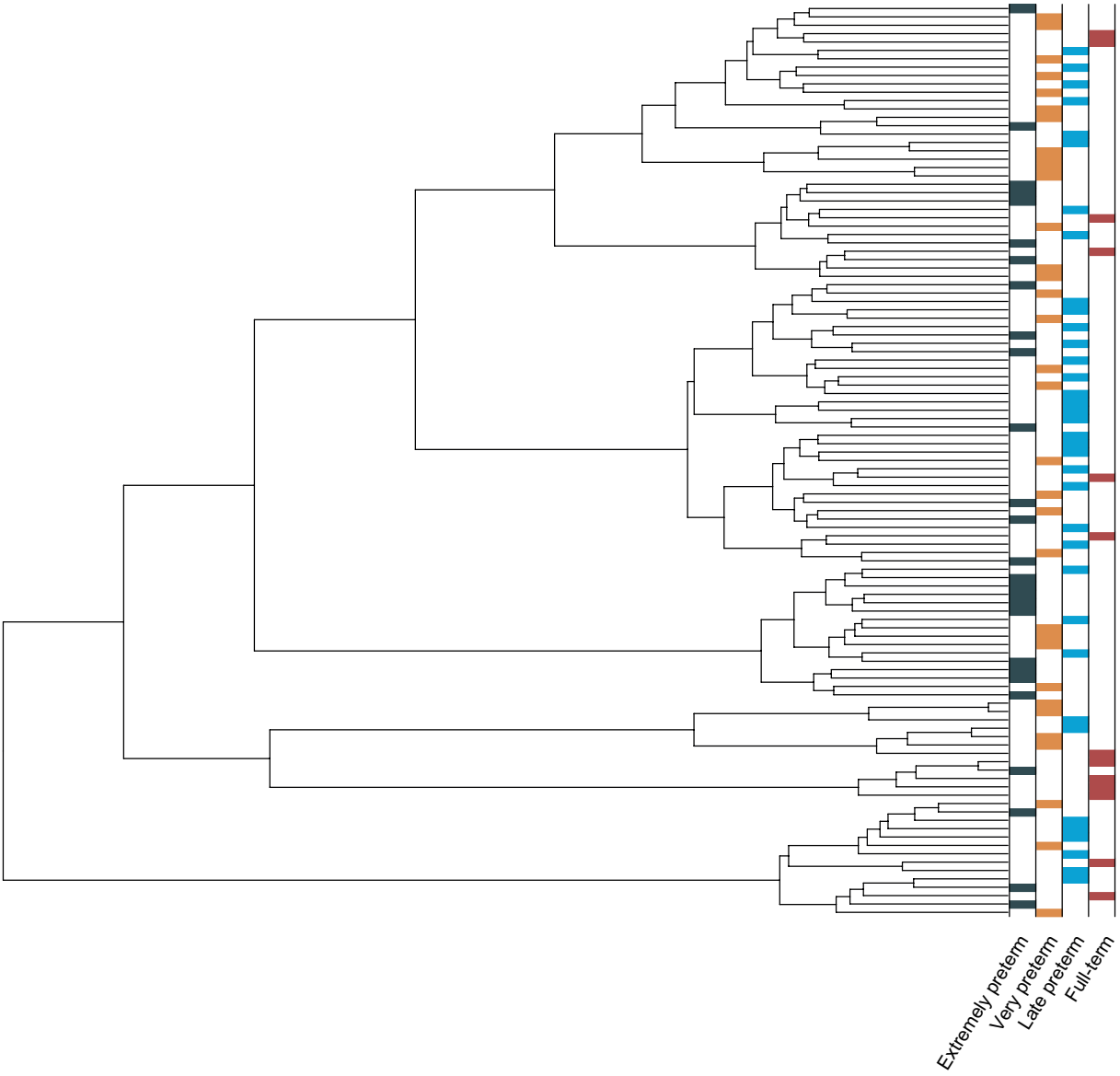


**Figure S5.8** Hierarchical cluster dendrogram of feces of preterm and full-term infants in their gestational age categories. The distance is based on unweighted UniFrac, and clustering was performed with Ward. The bars indicate a sample belongs to the gestational age category when colored.

**Table S5.1** Characteristics of the infants used for data analysis.

| | Total infants | Total samples[a] | Gestational age (mean ± SD) | Birth weight (gram ± SD) | Female (%) | C-section (%) | Enteral feeding (%)[a] | Human milk (%)[a,b] | ≥ 2 antibiotic treatments (%) | Antimycotics (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| All | 56 | 109 (7, 17, 28, 19, 20, 18) | | | | | | | | |
| EP | 18 | 27 (1, 6, 9, 3, 4, 4) | 26.3 ± 0.9 | 855.2 ± 207.7 | 44.4 | 66.7 | 31, 76, 76, 95, 100 | 25, 65, 76, 95, 100 | 77.8 | 61.1 |
| VP | 15 | 33 (2, 5, 6, 6, 8, 6) | 29.2 ± 1.1 | 1387.3 ± 260.1 | 46.7 | 46.7 | 54, 91, 100, 88, 100 | 47, 75, 83, 85, 50 | 40.0 | 26.7 |
| LP | 17 | 36 (2, 4, 10, 9, 5, 6) | 33.8 ± 1.1 | 2254.6 ± 506.0 | 35.3 | 23.5 | 78, 98, 100, 100, 100 | 54, 48, 45, 36, 25 | 11.8 | 41.2 |
| FT | 6 | 13 (2, 2, 3, 1, 3, 2) | 38.0 ± 1.3 | 3279.6 ± 555.0 | 16.7 | 33.3 | 100, 100, 100, 100, 100 | 38, 78, 90, 91, 44 | 0.0 | 33.3 |

Characteristics of the selected infants from the EIBER cohort of whom feces were used to perform data analysis: 56 infants in total, of whom 50 are preterm and 6 are full-term. EP: extremely preterm, VP: very preterm, LP: late preterm, FT: full-term. Feces of the first six postnatal weeks were collected with exception of Postnatal Week 5. [a] The percentage at Postnatal Weeks 1, 2, 3, 4 and 6, respectively. [b] The percentage of human milk was corrected for the amount of enteral feeding.

**Table S5.2** Scheme of samples available per infant for characterization of the intestinal fungal community.

| Infant ID | Gestational age (weeks) | Gestational age group | Week 0 | Week 1 | Week 2 | Week 3 | Week 4 | Week 6 | Number of samples |
|---|---|---|---|---|---|---|---|---|---|
| **A001** | **27** | **EP** | 0 | 1 | 1 | 0 | 0 | 0 | **2** |
| **A003** | **25** | **EP** | 0 | 1 | 1 | 0 | 0 | 0 | **2** |
| **A007** | **28** | **VP** | 0 | 0 | 0 | 0 | 0 | 1 | **1** |
| **A008** | **28** | **VP** | 0 | 0 | 1 | 1 | 0 | 0 | **2** |
| **A012** | **27** | **EP** | 0 | 0 | 1 | 0 | 0 | 0 | **1** |
| **A015** | **31** | **VP** | 1 | 1 | 0 | 0 | 1 | 1 | **4** |
| **A019** | **26** | **EP** | 0 | 0 | 0 | 0 | 0 | 1 | **1** |
| **A020** | **28** | **VP** | 1 | 0 | 1 | 0 | 1 | 1 | **4** |
| **A021** | **28** | **VP** | 0 | 0 | 0 | 1 | 1 | 1 | **3** |
| **A022** | **27** | **EP** | 0 | 0 | 0 | 0 | 0 | 1 | **1** |
| **A028** | **30** | **VP** | 0 | 1 | 0 | 0 | 0 | 0 | **1** |
| **A030** | **26** | **EP** | 0 | 0 | 1 | 0 | 0 | 0 | **1** |
| **A031** | **26** | **EP** | 0 | 1 | 0 | 0 | 0 | 0 | **1** |
| **A032** | **24** | **EP** | 0 | 1 | 0 | 0 | 0 | 0 | **1** |
| **A037** | **29** | **VP** | 0 | 0 | 1 | 0 | 1 | 0 | **2** |
| **A038** | **30** | **VP** | 0 | 1 | 1 | 0 | 1 | 0 | **3** |
| **A041** | **26** | **EP** | 0 | 1 | 0 | 0 | 0 | 0 | **1** |
| **A043** | **27** | **EP** | 0 | 0 | 1 | 0 | 1 | 1 | **3** |
| **A044** | **28** | **VP** | 0 | 1 | 0 | 0 | 1 | 0 | **2** |
| **A047** | **27** | **EP** | 0 | 0 | 1 | 0 | 0 | 0 | **1** |
| **A050** | **30** | **VP** | 0 | 0 | 1 | 1 | 0 | 0 | **2** |
| **A051** | **30** | **VP** | 0 | 0 | 1 | 1 | 1 | 0 | **3** |
| **A056** | **30** | **VP** | 0 | 1 | 0 | 0 | 0 | 0 | **1** |
| **A063** | **25** | **EP** | 0 | 0 | 1 | 1 | 0 | 0 | **2** |
| **A068** | **27** | **EP** | 0 | 0 | 0 | 1 | 1 | 0 | **2** |
| **A074** | **26** | **EP** | 0 | 0 | 1 | 0 | 0 | 0 | **1** |
| **A076** | **27** | **EP** | 0 | 1 | 0 | 0 | 0 | 1 | **2** |
| **A082** | **25** | **EP** | 1 | 0 | 1 | 0 | 0 | 0 | **2** |
| **A095** | **27** | **EP** | 0 | 0 | 0 | 0 | 1 | 0 | **1** |
| **A097** | **27** | **EP** | 0 | 0 | 0 | 1 | 1 | 0 | **2** |
| **A102** | **29** | **VP** | 0 | 0 | 0 | 0 | 0 | 1 | **1** |
| **A103** | **29** | **VP** | 0 | 0 | 0 | 1 | 1 | 1 | **3** |
| **A108** | **28** | **VP** | 0 | 0 | 0 | 1 | 0 | 0 | **1** |
| **B202** | **33** | **LP** | 0 | 0 | 1 | 0 | 0 | 1 | **2** |
| **B211** | **32** | **LP** | 0 | 0 | 1 | 1 | 0 | 1 | **3** |

| Infant ID | Gestational age (weeks) | Gestational age group | Week 0 | Week 1 | Week 2 | Week 3 | Week 4 | Week 6 | Number of samples |
|---|---|---|---|---|---|---|---|---|---|
| **B212** | **32** | **LP** | 0 | 0 | 1 | 0 | 0 | 0 | **1** |
| **B214** | **40** | **FT** | 0 | 0 | 0 | 0 | 1 | 0 | **1** |
| **B216** | **37** | **FT** | 0 | 1 | 0 | 1 | 1 | 1 | **4** |
| **B217** | **34** | **LP** | 1 | 0 | 0 | 0 | 0 | 0 | **1** |
| **B233** | **39** | **FT** | 1 | 0 | 0 | 0 | 1 | 0 | **2** |
| **B234** | **36** | **LP** | 0 | 0 | 1 | 0 | 1 | 1 | **3** |
| **B237** | **33** | **LP** | 0 | 0 | 0 | 1 | 1 | 0 | **2** |
| **B245** | **34** | **LP** | 0 | 1 | 1 | 1 | 0 | 1 | **4** |
| **B246** | **34** | **LP** | 0 | 0 | 1 | 0 | 0 | 0 | **1** |
| **B248** | **34** | **LP** | 0 | 0 | 0 | 1 | 1 | 1 | **3** |
| **B254** | **32** | **LP** | 0 | 0 | 1 | 1 | 0 | 0 | **2** |
| **B266** | **35** | **LP** | 0 | 0 | 1 | 1 | 1 | 1 | **4** |
| **B270** | **39** | **FT** | 0 | 0 | 1 | 0 | 0 | 1 | **2** |
| **B280** | **37** | **FT** | 1 | 1 | 1 | 0 | 0 | 0 | **3** |
| **B285** | **34** | **LP** | 1 | 1 | 1 | 1 | 0 | 0 | **4** |
| **B300** | **34** | **LP** | 0 | 1 | 0 | 1 | 0 | 0 | **2** |
| **B308** | **33** | **LP** | 0 | 0 | 1 | 0 | 0 | 0 | **1** |
| **B310** | **40** | **FT** | 0 | 0 | 1 | 0 | 0 | 0 | **1** |
| **B313** | **35** | **LP** | 0 | 1 | 0 | 0 | 0 | 0 | **1** |
| **B316** | **33** | **LP** | 0 | 0 | 0 | 1 | 0 | 0 | **1** |
| **B318** | **34** | **LP** | 0 | 0 | 0 | 0 | 1 | 0 | **1** |
| | | | | | | | | **Total:** | **109** |

Available samples are indicated with "1", while unavailable/insufficient samples are indicated with "0". EP: extremely preterm, VP: very preterm, LP: late preterm, FT: full-term.

**Table S5.3** PERMANOVA analysis.

Table S5.3A

| | Df | Sum of Squares | Mean Squares | F Model | R2 | Pr(>F) |
|---|---|---|---|---|---|---|
| Gestational age category | 3 | 3202 | 1067.4 | 2.05 | 0.06 | 0.005** |
| Residuals | 91 | 47,502 | 522.0 | | 0.94 | |
| Total | 94 | 50,705 | | | 1.00 | |

Table S5.3B

| | Df | Sum Squares | Mean Squares | F value | Pr(>F) |
|---|---|---|---|---|---|
| Groups | 3 | 57 | 19.06 | 0.85 | 0.47 |
| Residuals | 91 | 2029 | 22.29 | | |

(A) The results of PERMANOVA analysis based on gestational age categories; and (B) the results of the associated check for assumed homogeneity of variances. $^{**}P \leq 0.01$.

**Table S5.4** Tables of RDA data.
Table S5.4A

| | **Df** | **AIC** | **F** | **Pr(>F)** | **$P_{adj}$** |
|---|---|---|---|---|---|
| Mode of Delivery | 2 | 594.9 | 3.902 | 0.005** | 0.238 |
| Individuality | 53 | 597.9 | 1.440 | 0.005** | 0.238 |

Table S5.4B

| | **RDA1** | **RDA2** |
|---|---|---|
| Eigenvalue | 134.6732 | 51.7781 |
| Proportion explained | 0.2497 | 0.0960 |
| Cumulative proportion | 0.2497 | 0.3457 |

(A) The ANOVA table and (B) accumulated constrained eigenvalues. Scaling 2 for species and site scores. Species are scaled proportional to eigenvalues. Sites are unscaled: weighted dispersion equal on all dimensions. General scaling constant of scores: 15.0059. $^{**}P \leq 0.01$, *P*-values were adjusted with Benjamini-Hochberg.

# CHAPTER 6

## General discussion and future perspectives

The concordant maturation of the gastrointestinal tract and the microbiome is pivotal for growth and health of the (preterm) infant. As a result of preterm birth, there is a discrepancy in early life between the maturation status of the gastrointestinal tract and the process of microbial colonization. Multiple factors interfere with the microbial maturation, among which gestational and postnatal age, mode of delivery, antibiotic use and feeding regimens. Consequently, the disrupted infant's and microbiota's maturation status affect their metabolic, protective and trophic functions. This facilitates pro-inflammatory responses in preterm infants who already are predisposed to inflammation and infections. In fact, preterm birth and its associated complications cause high mortality rates in children under the age of five years[1,9]. Hence, preterm birth still is a major societal issue with an impact on the health of the infant and on associated healthcare costs[2,3].

Neonatal support in early life of preterm infants offers the opportunity to orchestrate the maturation of the immature gastrointestinal tract and the colonizing microbes. Orchestrating these developmental processes creates intestinal homeostasis and forms the basis for long-term health and well-being. It is thus key to understand how the gastrointestinal tract of preterm infants, and the bacteria and fungi (microbes) therein, mature in order to support the infants in their developmental processes. The work within this thesis therefore aimed to elucidate how maturation of the host and its microbes is affected by nutrition. To this end, we have described maturation of the gastrointestinal tract and of the intestinal microbes in preterm infants. Additionally, we related these processes to nutrition, clinical variables and to infant growth, development and health.

## Integrating the intestinal bacteria and fungi in a clinical setting

Preterm infants experience a different start of life compared to full-term infants (**chapter 2**). In the previous chapters, our findings have emphasized multiple clinical variables influencing the microbiota's proteome and mycobiota's composition in early life (**chapter 3** and **chapter 5**). These findings are relevant to the clinical setting, in which the intestinal bacteria and fungi are expected to be integrated in future neonatal care. Before they can be used as therapeutic target, it is important to understand which clinical variables affect the microbiota and mycobiota development.

Gestational and postnatal age, antibiotic treatment duration and diet are known to influence the microbiota composition. In our work we showed that they also influence the microbial proteome, that is the microbiota's functional profile (**chapter 3**). Consequently, shifts in the microbiota-associated functional responses may occur during early life of preterm infants[295,307]. A better scientific understanding of these microbial functional responses after clinical interventions or in disease outcomes are needed to incorporate microbiome markers in neonatal care. Such an approach could help to identify commonalities and correlations between preterm infants that remain healthy and those that develop disease. Moreover, the microbial functionality could be accounted for in individual clinical approaches, that are needed regarding the observed heterogeneity of preterm infants (**chapter 3** and **chapter 5**). In light of prevention, it would be interesting to identify specific microbial activity patterns in infants who develop necrotizing enterocolitis (NEC).

In addition to the bacteria described in this thesis, we have unraveled the development of the fungal community in the preterm intestine in relation to clinical variables. In **chapter 5**, we described developmental patterns of the fungal community in which *Candida* spp. typically increased with gestational and postnatal age. While *Candida* spp. were commonly identified, no cases of candidiasis were registered within the selection of studied infants. Such fungal and bacterial infections are commonly tested and diagnosed with blood cultures. However, poor sensitivity of blood cultures may lead to underdiagnosis in infants[406,407]. Hence, comparing blood culture outcomes and compositional data allows to assess the mycobiota as therapeutic application to increase sensitivity in early detection of infant fungal infections. Besides age, the mode of delivery was a significant driver of the variation in the intestinal fungal composition (**chapter 5**). As hypothesized, the findings within **chapter 5** confirm that vaginally delivered preterm infants were characterized by the vaginal-like fungal genus *Candida* spp., while caesarean (C-)section delivered preterm infants were characterized by the skin-like fungal genus *Malasezzia* spp.[155] Above all, preterm infants are more frequently born via C-section, suggesting the colonization with *Malasezzia* spp. would be promoted. Our work underpins the importance of the mode of delivery in bacterial and fungal colonization by examining the relationship between acquisition of vaginal-like or skin-like microbiota depending on the mode of delivery (**chapter 5**)[121]. In an attempt to converge the C-section infants' microbiota toward a vaginal-like microbiota, vaginal microbial transfers have been performed[237]. This procedure of "vaginal seeding" has solely been investigated for the intestinal bacteria in full-term infants and not in preterm infants hitherto. Therefore, the practice of vaginal seeding is highly questionable and even more so in preterm infants. The risk for infectious exposures are high, especially in preterm infants[238]. Before such procedures can be applied in a clinical setting, the benefit to risk ratio and the effect of vaginal seeding on the intestinal fungal community have to be thoroughly assessed [238,408,409].



## Confounding factors explain heterogeneity in preterm infants

Being born at varying gestational ages and being exposed to a myriad of clinical interventions (**chapter 2**), preterm infants are a heterogenous group in which high inter-individual differences were identified in the microbiota's proteome and mycobiota's composition (**chapter 3** and **chapter 5**). High inter-individual differences are frequently identified in the preterm infant microbiota and mycobiota[54,137,138]. In fact, individuality significantly explained variation in the intestinal fungal composition (**chapter 5**). I would therefore advocate to include individual-based approaches in future microbiome studies, besides trends and associations between the microbiome and clinical variables.

Gestational age is one of the major confounders for the results described in this thesis. As acknowledged in **chapter 3**, gestational age significantly explained the variation in the infant's fecal metaproteome. Generally, the health and maturation status of the infant—and, thus, the type of care and duration of it—strongly depend on gestational age. This may explain the variable developmental patterns of bacteria and fungi in preterm infants compared to full-term infants, which was most evident in the youngest gestational age group (**chapter 3** and **chapter 5**). Moreover, we showed that birth weight correlated significantly to higher proportions of

bifidobacterial proteins in **chapter 3**. As a consequence of a higher gestational age, infants are more likely to have a higher birth weight, a more mature gastrointestinal tract and a higher bifidobacterial abundance. In a similar way, maturation status of preterm infants—for which body weight was found to be a marker—was linked to the developmental patterns of the microbiota, in which a shift occurred from a microbiota dominated by *Staphylococcus* spp. to one dominated by *Enterobacteriaceae* as infants gained more weight[410].

## The microbiome is an inconspicuous challenge in nutritional management

Nutrition in early life—that is, human milk—has been a cornerstone within the work of this thesis. The extent to which human milk can confer benefits depends on the maturation status of the infant's gastrointestinal tract and of the microbiota. In the previous chapters, we emphasized the importance of gastrointestinal and microbial maturity for human milk digestion (**chapter 2–4**). Despite its relevance to infant health, the intestinal microbiota is often overlooked in nutritional neonatal care (**chapter 2**). Therefore, it is crucial to understand how human milk is digested by the preterm host and its microbes. To this end, we took a dichotomous approach in studying the interaction between the gastrointestinal tract and the microbiota. On the one hand, the maturation of the gastrointestinal tract was studied from a host perspective with its implications for the microbiota's functionality in early life of preterm infants (**chapter 3**). On the other hand, we designed a clinical study in **chapter 4** to investigate gastrointestinal maturation from the microbiota's perspective.

Our findings show that the gastrointestinal tract and the microbiota from the preterm infants of our cohort were immature and could not digest human milk to the same extent as full-term infants (**chapter 3**). We have shown low enzyme activities of pepsin and proteases, but these were likely less active due to high gastric pH observed in the first two postnatal weeks. Indeed, the pH was inversely correlated to the activity of the main gastric protease pepsin (**chapter 3**), indicating that pepsin is less active in preterm infants due to a higher gastric pH[411]. Similar findings have been described for gastric digestion of human milk by preterm infants[287,288,412]. Interestingly, although shown to be active, we could not detect pepsin in the metaproteomes of gastric aspirates. Noteworthy, the data described in **chapter 3** do not resolve if the observed and active proteases derive from the infant itself or from the mother. Human milk is a source of enzymes, although the higher gastric pH of preterm infants may inactivate some, as described before[285,286]. Also, less proteins from human milk oligosaccharide (HMO)-degrading *Bifidobacterium* spp. were detected in preterm infants compared to full-term infants (**chapter 3**). One should, however, consider the relatively small number of full-term infants included in this study when interpreting the data, particularly when studying the differences per gestational age category. Nevertheless, the link between gastric aspirates and feces in human milk digestion is useful in assessing the role of the microbiota herein.

In continuation of the work in **chapter 3**, we have initiated an observational, single-center clinical study to investigate gastrointestinal maturation from the microbiota's perspective (**chapter 4**). Previous studies have compared human milk and feces to study the effect of human milk on the microbiota[349–351]. The "From Mum to Bum" study adds gastric aspirates of preterm infants to the sample collection, which has already proven useful to study gastrointestinal function and maturation with regard to human milk digestion (**chapter 3**). The design of this new study will give even more detailed insights into the digestion of human milk across the gastrointestinal tract. Although human milk digestion has been studied along the gastrointestinal tract of preterm and full-term infants, this has not been linked to the microbiota as described in **chapter 4**[413]. The main objective will be to identify how the intestinal microbiota of preterm and full-term infants differ in their ability to extract energy and nutrients from human milk. The "From Mum to Bum" study relies heavily upon the (compliance of) parents to collect samples and nutritional data, and could become a limitation of the study upon incorrect execution thereof. Correct sampling methods and storage conditions namely have been shown to influence the human milk peptidome and microbiota composition of human milk and feces[98,100,377,378,414]. Besides that, sampling is bound by ethical considerations and, while likely more representative for the microbiota than feces, retrieving a direct sample of the colon is invasive. The same considerations hold for the full-term counterparts of whom the gastric aspirates are missing.

The results from our new clinical study will uncover a vast amount of information that enables us to study gastrointestinal digestion from multiple perspectives. Moreover, it allows us to correlate microbial activity to anthropometric outcomes, which in my opinion is the next step of the work described in this thesis. The extent to which the microbiota influences metabolism, growth and development is worth to be investigated considering its therapeutic application in preterm infants. The incentive hereof, however, has already been shown in **chapter 3**, where we correlated anthropometric outcomes to bifidobacterial abundance. Previously, distinct microbial taxa and features have been associated to anthropometric outcomes in preterm infants, underpinning the microbiota's ability to induce growth restriction[52,215,415]. Also, few studies have indicated that postnatal growth failure related to disrupted gut microbiota maturation in preterm infants[416,417]. Based on our work, my hypothesis is that differences in microbiota development changes the metabolic profile of the microbiota. This in turn impacts nutrient efficiency and intestinal homeostasis that both contribute to infant growth (**chapter 2**). Previous findings confirm this hypothesis, where disruptions in the gut microbiota of preterm infants were associated to distinct microbial metabolic functions[137,295,307,416,417]. The outcomes of our new clinical study (**chapter 4**) may provide therapeutic directions by pinpointing microbes that stimulate infant growth by being involved in lipid metabolism, endocrine functions and production of compounds that stimulate intestinal homeostasis[294]. The commonalities and discrepancies in the microbial functionality between preterm and full-term infants allow us to identify metabolic "gaps" that could be complemented by means of pre- and probiotics.

Protein supplementation is standard practice in preterm infants since they require high protein intake for adequate growth and development. As observed in **chapter 3** and hypothesized in **chapter 4**, human milk protein digestion is hampered in preterm infants. Although not

investigated within this thesis, the combination of protein supplementation and gastrointestinal immaturity results in a protein surplus in the colon where bacteria ferment specific amino acids into branched-chain fatty acids (BCFA)[418]. Generally, this so-called "putrefaction" is presumed detrimental to the host's health by negatively affecting mucosal cells[284,418]. Given the undesired consequences, an interesting therapeutic target would be to divert bacterial putrefaction to carbohydrate digestion. The therapeutic potential of diverting putrefaction requires assessment which macronutrient—that is, proteins and carbohydrates—is used preferentially by intestinal bacteria, and in which macronutrient ratio such diversion occurs. A promising strategy for assessing preferential use of macronutrients by intestinal bacteria would be a controlled set-up in bioreactors. In such a set-up, the protein of choice would first need to undergo a gastric digestion step. Afterwards, multiple bioreactors would have varying (pre-digested) protein concentrations, of which all would be inoculated with the same preterm infant feces. A prebiotic mixture of non-digestible carbohydrates could then be "fed" to the bioreactor, before and after which sampling allows to assess microbial community composition, activity and its metabolic output including produced sugars, short-chain fatty acids (SCFAs) and BCFAs. Within this set-up, the bacterial degradation of non-digestible carbohydrates in the intestine is expected to be energetically more favorable over the degradation of (pre-digested) proteins[419]. Once assessed thoroughly, a specific ratio of protein and non-digestible carbohydrates could be applied in nutritional strategies to prevent BCFA production and to support mucosal health optimally. Another nutritional strategy could be the synbiotic combination of non-digestible carbohydrates with bacteria specified toward their fermentation.

## The immature intestinal barrier is compromised in preterm infants

The intestinal barrier of preterm infants is well known to be immature or "leaky"[27,28]. Despite the fact that the intestinal barrier is compromised, metaproteomic studies in preterm infants have identified proteins related to intestinal mucosal barrier development and protection[295,307]. We showed that those mucosa-associated proteins—involved in establishing a stable mucus layer—were less abundant in preterm infants compared to full-term infants during the first six postnatal weeks (**chapter 3**). These findings indicate that preterm infants have a less thick and stable mucus layer, which subsequently might lead to an impaired intestinal barrier.

Intestinal homeostasis and barrier function depend on a balanced microbiome, in which commensal and pathogenic bacteria and their metabolites interact with components of the intestinal barrier. The type and abundance of bacterial ligands in the intestine are pivotal for homeostasis, as each Toll-like receptor (TLR) binds specific bacterial ligands and depending thereon, elicits immunological responses. A culmination of clinical interventions and developments are needed for the microbial balance to be disturbed and, thus, for the infant to become susceptible to infections. Speculatively, the disrupted microbiome in preterm infants (as seen in **chapter 3**) causes shifts in Microbial Associated Molecular Patterns (MAMPs) and the microbiota's metabolic output. Subsequently, the type and amount of activated of Toll-like receptors (TLRs) changes, which results in tight junction alterations. Generally, the ligands and metabolites of *Bifidobacterium* spp. and *Lactobacillus* spp. stimulate TLR2 that strengthens the

intestinal barrier by inducing the expression and localization of tight junction proteins[420–426]. For example, SCFAs and indole-3-lactic acid, produced by *Bifidobacterium* spp. and *Lactobacillus* spp., alter cytokine secretions that regulate tight junction protein expression[425,426]. Contrastingly, the bacterial endotoxin lipopolysaccharides (LPS) on the cell membrane of Gram-negative bacteria are recognized by TLR4, upon which pro-inflammatory processes and tight junction permeability are initiated[153,427] . Interestingly, the activation of TLR4 is antagonized by apical activation of TLR9, which recognizes the CpG motif that is frequently observed in *Bifidobacterium* spp.[153]

These mechanisms support the hypothesis that the deviating microbiome of preterm infants causes a shift in MAMPs and TLR activation, which triggers downstream pro-inflammatory processes and compromises the intestinal barrier[153,417]. Such mechanisms increase the risk for nosocomial infections. In point of fact, NEC has been associated to a microbiota composition dominated by *Enterobacteriaceae*, which overstimulate TLR4 with LPS[153]. Probiotic treatment of the preterm microbiota may thus be designed to accommodate appropriate TLR signaling or cytokine secretion to upregulate tight junction proteins[428]. Probiotic strains of the *Lactobacillus* and *Bifidobacterium* genera or constituents thereof may be particularly useful to strengthen the intestinal barrier[334,423,424,429–431]. Multiple meta-analyses suggest beneficial effects of probiotic administration against NEC in preterm infants, despite others not observing such effects[432,433].

## Respiratory support relates to microbial oxidative stress

Our previous work showed that respiratory support may introduce oxygen into the intestinal lumen and may thereby sustain the abundance of facultative anaerobes and delay the colonization with beneficial, obligate anaerobes[137]. The facultative anaerobes have a competitive advantage as they tolerate oxygen by employing oxidative stress proteins, as shown in the same cohort of preterm infants in **chapter 3**. Other studies additionally report a shifted ratio of facultative to obligate anaerobic bacteria in preterm infants, as well as in C-section infants[137,253434]. Our hypothesis on the effect of respiratory support on the composition of the microbiota was confirmed in our targeted follow-up study. In a cohort of the Amsterdam Academic Medical Center (Amsterdam, The Netherlands), feces were collected of preterm infants receiving a constant oxygen supply either at low percentages ("constant", $fiO_2$ 21-25%) or at increasing percentages ("high", $fiO_2$ > 25%) or no respiratory support (control) in the first four weeks after birth. Preliminary data of the microbiota suggest that respiratory support lowered the abundance of obligate anaerobes. Moreover, the ratio between facultative to obligate anaerobic bacteria increased with higher percentages of oxygen supply. The intestine may become further oxygenated as a consequence of an impaired and permeable intestinal barrier. In line with our hypothesis, the gut barrier proteins in **chapter 3** were inversely associated to bacterial oxidative stress proteins of facultative anaerobic bacteria. The relationship between respiratory support and the microbiota should be further explored in our follow-up study by investigating the functional responses of the intestinal bacteria to oxygen supply. As such, the percentage of oxygen supply could be correlated to the previously described digestion and barrier defense markers, as well as bacterial oxidative stress proteins (**chapter 3**). Furthermore, I would propose to determine the associations between oxygen supply and: (1) the ratio of facultative to obligate

anaerobes; (2) the HMO-degrading capacity of the microbiota; and (3) the clinical outcomes in which an impaired intestinal barrier is implicated.

Like described before, it is in fact the obligate anaerobic bacteria that stimulate the intestinal barrier function and retain intestinal homeostasis[71,73,303,332]. In this way, the preterm intestinal barrier is not strengthened and the risk for nosocomial infections increases[435]. In case an infection is suspected, antibiotics are prescribed prophylactically that further contribute to the impaired intestinal barrier, as antibiotic type and duration have considerable impact on the microbiota. For instance, it increases the abundance of *Enterococcus* spp. at the expense of *Bifidobacterium* spp.[144] Yet, current advantages of antibiotics are still greater than the disadvantages although it gives rise to an antibiotic resistome, which could complicate neonatal care on the long term[245,436,437]. Hence, to understand which future directions should be taken in antibiotic stewardship, we need to continuously assess the effect of antibiotic treatment type and duration in NEC and sepsis. Instead of prophylactic antibiotic treatment, nutritional interventions with antioxidant provision could hypothetically be established within neonatal care given the situation outlined above. Modulation of the redox potential, and thereby the microbiota of preterm infants, may aid in mitigating the risk of nosocomial infections by inducing microbial shifts affecting colonization resistance and oxidative stress[253,434,435].

## Human milk offers protection to the preterm infant

While exposed to a plethora of clinical procedures affecting the microbiome, not all preterm infants develop NEC, sepsis or other complications in practice. Most likely, the protective benefits from human milk have a mitigating effect on the immaturity of the preterm infant. First, immunoglobulins confer protective functions on the infant's naïve immune system. Our findings confirmed the survival of (maternal) immunoglobulins as indicated by the persistent presence of them in the metaproteomes of gastric aspirates and feces of (pre)term infants (**chapter 3**). Congruently, immunoglobulins—particularly the secretory component, IgM and IgG—have previously been reported to remain intact throughout the gastrointestinal tract of preterm infants[324,438]. The work within this thesis described the identification of fragments of IgA, IgM and IgG in gastric aspirates and feces, although their persistence was not assessed. Second, many other bioactive proteins involved in innate immune responses are present in human milk and have been identified in preterm infants[286,295,307,322,323,325,336]. One of those components is lactotransferrin, a key player in the innate immunity. It remained undigested in the gastrointestinal tract of (pre) term infants, thereby conferring functional benefits to the host and its microbiota (**chapter 3**). The ascribed antimicrobial activity of lactotransferrin depends on sequestration of the essential nutrient iron, and direct interaction with the microbe's LPS or (lipo)teichoic acids causing cell lysis[439,440]. The interaction of lactotransferrin with LPS also modulates TLR4 interactions, having anti-inflammatory consequences[440]. The described mechanisms of lactotransferrin thereby could protect against neonatal infections, although evidence of meta-analyses was of low quality[340,341,441]. Third, proteolytic enzymes were detected and active in preterm infant feces as described before (**chapter 3**). The combination of metaproteomics and enzyme activity analyses as applied in **chapter 3**, or other "–omic" methods such as transcriptomics or metabolomics,

remains essential in future studies to fully assess the activity of detected proteins. Although it remains unclear if the enzymes derive from mother or infant, it is likely that at least some derive from human milk as the gastrointestinal tract and enzyme production of the preterm infant are immature[325].

Notwithstanding the mitigating effect of human milk, morbidity rates are high in preterm infants. With many proven benefits, human milk remains a cornerstone for neonatal care and infant health[24]. Unfortunately, human milk feeding can be challenging after delivering prematurely. In case maternal milk is not sufficiently available, donor milk is the recommended alternative for enteral feeding. Compared to infant formula, donor milk protects against NEC, improves feeding intolerance and reduces cardiovascular risk at later age[442,443]. In spite of the indisputable benefits, I find it questionable if donor milk feeding is optimal for the preterm infant's safety and growth due to few remaining concerns. First, the safety of donor milk is guaranteed by strict guidelines for screening, storage and handling procedures, but there is no consensus in guidelines between donor milk banks[443]. For (microbiological) safety, the donor mother and her milk are screened, and the donor milk is pasteurized[38,443]. However, one should be aware that donor milk may still contain unknown or unscreened hazardous components for the preterm infant[443]. Second, the nutritional and biological value of donor milk are compromised upon storage and processing[443]. Pasteurization is most commonly used to process donor milk, for which alternative methods are currently under investigation[444]. Third, donor milk does not meet the nutritional requirements of preterm infants. As described in **chapter 2**, mother's own milk adapts with premature delivery and through lactation, thereby (partly) accommodating the needs of the preterm infant. The nutrient, HMO and microbiota composition of human milk changes with preterm delivery and over time[261,445,446]. Donor milk often derives from mothers delivering at term and might be pooled and, thus, is not specified toward the needs of the preterm infant[38,442,443]. For these reasons and the fact that donor milk banks are not widely available yet, infant formula may be considered safer and may allow for more consistent delivery and greater amounts of nutrients[442,443].



## Prematurity is a chronic condition

Early in life there is a window of opportunity in which the bacteria, fungi and antigens interact with the immune system via the Gut-Associated Lymphoid Tissue (GALT)[67]. In this chapter we emphasized that a balanced interaction between the gastrointestinal tract, the immune system and the intestinal microbes is pivotal for health in (preterm) infants. Balanced interactions within this window of opportunity prevent inappropriate inflammatory responses toward antigens and commensals on a short and long term[53,67,175]. The aberrant microbiota's proteome and mycobiota's composition of preterm infants (**chapter 3** and **chapter 5**) may alter the development of the immune system although the effect of the mycobiota is not as clear hithertho[32,86]. In fact, the aberrant microbiome in infants has been associated to a chronic pro-inflammatory state, although the preterm microbiome composition is not detectable at a later age *per se*. In those cases, the risk for allergies, asthma, overweight, obesity, type 2 diabetes and inflammatory bowel disease (IBD) increased[13–16,32].

The microbiome may additionally influence cognitive and psychosocial outcomes via the microbiota-gut-brain axis[447]. With crucial brain development occurring after birth, preterm infants are prone to brain injury and white matter injury specifically. This type of injury causes developmental issues of the white matter and increases the risk for cognitive and psychosocial deficits in preterm infants[448,449]. The microbial and neural development occur in parallel, implying a neurodevelopmental period in which disrupted microbiota development has consequences for brain function and behaviour[447,450]. Given the interactions between bacteria and fungi, evidence for the role of fungi in neurological disorders is growing[451]. Mechanisms by which the gut microbiota modulates brain function and behavior comprise metabolic, endocrine, immune and neuronal pathways[447,450]. Growing evidence suggests that the gut microbiota modulates brain function and behavior, which is often identified through associations between the disruptive effect of antibiotics, psychiatric disorders and neurodevelopmental outcomes[452–457]. The microbiota functional profiles, as described in **chapter 3**, were affected by antibiotic treatment duration and could impact the microbiota-gut-brain axis of the preterm infants[458]. Long-term follow up of preterm infants is needed to assess the effect of such a disrupted microbiota development on neurodevelopmental outcomes. Conversely, efforts are being made to exploit the neurodevelopmental period by modulating the microbiota with nutrition[459,460]. An example of this is a study investigating the effect of a synbiotic intervention on white matter injury in preterm infants[461].

## Challenges and opportunities

All results described within this thesis derive from observational studies. Inherent to those studies are a few challenges. One of those is the representativeness of preterm infants in the EIBER study (**chapter 3** and **chapter 5**) and the "From Mum to Bum" study (**chapter 4**). Both are single-center studies and, thus, subjects from other hospitals may not be well represented by these cohorts. In fact, the hospital environment and the neonatal intensive care unit (NICU) have been shown to influence microbiota and mycobiota composition, of which its effects sustained throughout the first year of life[131,141,161,222,224]. Moreover, our findings indicated that preterm infants are a heterogenous group regarding the composition of the microbiota and mycobiota (**chapter 3** and **chapter 5**). This is explained by many confounding factors known to influence microbiota and mycobiota composition and diversity. Careful selection of inclusion and exclusion criteria helps to specify a sub-population of infants, but inevitably avoids investigation of these confounding factors. One of those examples is the exclusion of infants delivered via C-section (**chapter 4**), while mode of delivery has been identified to strongly influence microbiota and mycobiota composition in (preterm) infants (**chapter 5**)[121,228]. As such, challenges remain to obtain a homogeneous and representative group of subjects. Besides that, power calculations to determine the minimum number of participants remain challenging in clinical microbiome studies, which is especially the case in observational studies[462]. In **chapter 4**, sample size is based on a non-probabilistic, convenience sampling method. It remains unknown whether this sample size is large enough to capture heterogeneity in microbiota composition amongst preterm infants.

Additional challenges lie in specific methods described in this thesis. The methods described in this thesis allowed us to study the functionality of the microbiota (**chapter 3** and **chapter 4**) as well as to characterize the mycobiota of preterm infants (**chapter 5**). The functionality of the microbiota was investigated by metaproteomics, which currently encounters challenges regarding depth and coverage of metaproteomic databases[20]. Yet, also the sample preparation highly depends on the type of sample, in which feces of breastfed infants may require additional steps to precipitate fats. Besides that, bioinformatic data processing require additional efforts[305,306]. The biggest challenges, however, remain for mycobiota characterization (**chapter 5**), which still is in its infancy. There is no consensus yet on standard and reliable methods for mycobiota sequencing, such as the choice of a target region. Furthermore, taxonomic assignment of fungi is challenging as the fungal database coverage is more comprehensive compared to bacterial databases[62,404,405]. Therefore, some taxa may have been over- or underrepresented in the results described in **chapter 5**. Standardized and reliable methods are needed to allow scalability, which is necessary for comparing results of studies and, thus, establishing a benchmark for a "healthy" mycobiota[62]. In this process, the fungal load should be assessed by means of quantitative PCR (qPCR) to put results into perspective of the intestinal ecosystem. The lack of investigation of the bacterial and fungal load, that is absolute quantitation, and the comparison thereof remains a gap in our knowledge as of yet. Such information would be essential in assessing the biological implications of the intestinal bacterial and fungal community. Importantly, an absolute-abundance-based approach has been proven useful for interkingdom ecological inferences[191]. For that matter, quantitative and relative methods both have limitations and complementary approaches should be used to obtain full insight in composition, dynamics and functionality of the intestinal microbial community.



Aside from the methods' challenges, they offer opportunities to gain insight into the functioning of the host and their microbiota as described in **chapter 3** and **chapter 4**. Measuring and reporting both human and microbial proteins in gastric aspirates and feces, as described for the first six postnatal weeks in **chapter 3**, is novel and allowed us to gain insight into the gastrointestinal maturation and its implications for the microbiota. Additionally, metaproteomic results were related to enzyme activity analyses. In **chapter 4** we described a study design in which we will continue to use the integration of these perspectives to gain even more insight into human milk digestion. Moreover, metaproteomics or metagenomics should be employed to hypothesize if the observed intestinal fungi in **chapter 5** are residents with relevant bioactivities for the host or solely transients. The integration of techniques and perspectives is becoming increasingly important to understand interactions between the host, microbes and nutrition in the intestine.

Although described separately in this thesis, future studies should focus on bridging the gap between the bacterial and fungal kingdoms in the intestine. The work in this thesis should be considered an incentive to further explore the potential of intestinal fungi and their interactions with the intestinal bacteria and the immune system of (preterm) infants. Most convincingly, a causal role of fungi has already been established in mice with regard to microbiota composition and host immune development[192]. Furthermore, in preterm infants the fungal and bacterial load were inversely associated, suggesting that interkingdom interactions are pivotal in microbiome

development and community dynamics[191]. Most notably, *Enterococcus* spp. were reported to inhibit *Klebsiella* spp., and both bacterial genera were inhibited by the fungal genus *Candida* spp.[191]. Our findings suggest concordant patterns, as proteins from *Enterococcus* spp. and *Klebsiella* spp. decreased, while *Candida* spp. abundance increased with higher gestational and postnatal age in infants of the same cohort (**chapter 3** and **chapter 5**). The interkingdom interactions are expected to be of great importance in the intestine as key body site where crosstalk with host immunity results in systemic manifestations of either health or disease[62]. Archaea and viruses additionally need to be accounted for to obtain a holistic ecological perspective of the human intestine. Researching such interkingdom interactions in the (infant) intestine would be especially relevant in a clinical setting, as bacteria and fungi may interact synergistically in mixed biofilms to increase antimicrobial recalcitrance and protection against host immune responses[195–199]. Subsequent formation of mixed biofilms on indwelling medical devices—such as nasogastric enteral feeding tubes—could become a reservoir of microbes and antibiotic resistance genes that are introduced into the preterm gastrointestinal tract upon feeding, thereby forming a risk of microbial dysbiosis and infections[463–465].

## Conclusion and future perspectives

The research described in this thesis contributes to current knowledge of the preterm gastrointestinal maturation and its intestinal microbes during early life of preterm infants, as well as the clinical influences on their development. Our findings confirmed previous knowledge on the immature status of the gastrointestinal barrier and the microbiota, in which we showed that human milk-degrading bacteria were less active, and host intestinal barrier proteins were less abundant in the preterm intestine. We also revealed the effect of mode of delivery on the intestinal fungi for the first time, with vaginal-like fungi predominant in vaginally born infants and skin-like fungi in C-section born infants. Furthermore, we obtained insights into the bacterial and fungal differences across all degrees of prematurity, as well as between preterm and full-term infants.

All findings described in this thesis emphasize the importance of the intestinal barrier interface as key body site where gastrointestinal epithelium, microbes and immune system interact. These complex interactions drive growth, development and health of the preterm infant. As such, not only is the work described herein relevant from a microbiological perspective, but even more so from a clinical perspective. First, the gained insights from this thesis could complement current nutritional neonatal care by considering the microbiome. By those means, the infant's need for human milk, its intestinal maturation status and its microbiome provide directions to adapt feeding strategies. The outcomes of our new clinical study will pinpoint bacteria and beneficial metabolites worthwhile to be stimulated in order to complement the microbial activity. Once thoroughly assessed, also the ratio of protein and carbohydrates could be finetuned. In a similar way, preterm infant formula composition may be adapted based on the maturation status. Second, the results in this thesis imply potential therapeutic targets for microbial modulation. By supporting microbiome development, the preterm infant could be supported in optimal barrier functioning, growth and development. For example, antioxidant

supplementation would reduce oxidative stress in infants receiving high amounts of oxygen through respiratory support. Additionally, probiotic treatment of the preterm microbiota may be designed to strengthen the intestinal barrier and prevent inflammation. While promising, probiotic administration remains tricky business to pick the right strain or combination thereof, as well as the dosage and duration[432,466]. Therefore, the therapeutic potential of prebiotics or postbiotics needs further investigation in preterm infants, as they are generally considered a safer alternative[467]. Probiotics should then only be administered upon consent of the parents and in combination with routine culture methods to monitor their safety. Although it is not realistic to prevent nosocomial infections completely by modulating the microbiome, it may complement the preterm microbiota. By those means, alterations in microbial composition and metabolism allow to break the cycle of the deviating microbiota, impaired intestinal barrier and inflammatory processes.

Based on the research described in this thesis, I conclude that the microbiome and nutrition hold promising applications for preterm infant care that help to orchestrate maturation of the preterm gastrointestinal tract. Microbiota modulation offers hope for future improvements in preterm infant care that pave the way for systemic and lifelong effects. Before nutritional therapies targeting the microbiome can be implemented in preterm infant care, the mechanisms by which microbes are involved in preterm infant health need to be thoroughly assessed. As such, the preterm infant gut microbiome remains a research priority.

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*If we knew what it was we are doing,*
*it would not be called research, would it?*
*Albert Einstein*

# APPENDICES

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With love,

Jannie

## About the author

Jannie Gertrudis Elisabeth Henderickx was born on June 16th 1994 in Weert, The Netherlands. In 2012, she graduated *cum laude* from her high school education at Heerbeeck College in Best, after which she continued her bachelor's and master's education in Nutrition and Health at Wageningen University & Research (WUR, Wageningen). In 2015, she obtained her *cum laude* bachelor's degree, followed by her master's degree in 2017. Jannie specialized in Molecular Nutrition and Toxicology and developed a keen interest for the interaction between nutrition, health and the intestinal microbiota. During her master thesis at the Laboratory of Microbiology, she studied the meconium microbiota composition and subsequent microbiota development during early life of preterm infants under supervision of dr. Romy D. Zwittink and dr. Clara Belzer (WUR). This research was followed up by a research grant proposal Jannie wrote in collaboration with dr. Clara Belzer. For her internship, Jannie joined Winclove Probiotics (Amsterdam). Under supervision of dr. Jacoline Gerritsen, she worked on an *in vitro* gastrointestinal model to assess survival of probiotics strains in infants. In October 2017, she returned to the Laboratory of Microbiology to start her scientific career as doctorate student at the Molecular Ecology group under guidance of prof. dr. Jan Knol and dr. Clara Belzer. In her PhD research, as described in this thesis, she investigated the maturation of the gastrointestinal tract and of the intestinal microbes in preterm infants with their implications for infant growth, development and health. The research described in her thesis is a culmination of collaborations with researchers and neonatologists from Danone Nutricia Research (Utrecht, The Netherlands), Isala Women and Children's Hospital (Zwolle, The Netherlands) and Amsterdam Academic Medical Center (Amsterdam, The Netherlands). Besides her research activities during her PhD, Jannie has been involved in initiating and guiding a thesis ring for bachelor and master students. Moreover, she has been an active member of the Microbiology Seminar Committee throughout her PhD. Currently, Jannie works as a postdoctoral researcher at the Center of Microbiome Analyses and Therapeutics at Leiden University Medical Center (Leiden, The Netherlands).

# List of publications

**Henderickx JGE**, Zwittink RD, van Lingen RA, Knol J & Belzer C. The preterm gut microbiota: an inconspicuous challenge in nutritional neonatal care. *Front Cell Infect Microbiol.* 2019;9:85. doi:10.3389/fcimb.2019.00085

**Henderickx JGE**, Zwittink RD, Renes IB, van Lingen RA, van Zoeren-Grobben D, Groot Jebbink LJ, Boeren S, van Elburg RM, Knol J, & Belzer C. Maturation of the preterm gastrointestinal tract can be defined by host and microbial markers for digestion and barrier defense. *Sci Rep.* 2021;11(1):12808. doi: 10.1038/s41598-021-92222-y

**Henderickx JGE**, d'Haens EJ, Hemels MAC, Schoorlemmer ME, Giezen A, van Lingen RA, Knol J, & Belzer C. From mum to bum: an observational study protocol to follow digestion of human milk oligosaccharides and glycoproteins from mother to preterm infant. *Nutrients.* 2021;13(10):3430. doi: 10.3390/nu13103430

**Henderickx JGE**, de Weerd H, Groot Jebbink LJ, van Zoeren-Grobben D, Hemels MAC, van Lingen RA, Knol J & Belzer C. The first fungi: mode of delivery determines early life fungal colonization in the gastrointestinal tract of preterm infants. *Microbiome Res Rep,* 2022;1:7. doi: 10.20517/mrr.2021.03

# Overview of completed training activities

| Discipline specific activities | Organizing institute | Country | Year |
|---|---|---|---|
| *International congress* | | | |
| World of microbiome: pregnancy, birth and infancy (**poster**) | GEMS | Italy | 2019 |
| *National congress* | | | |
| Fall meeting microbial ecology | KNVM | NL | 2017 |
| MIB Centennial Symposium | Microbiology | NL | 2017 |
| Gut day (**poster**) | WUR | NL | 2018 |
| Gut day (**oral**) | AMC | NL | 2019 |
| Scientific spring meeting (**poster**) | KNVM | NL | 2019 |
| Expert Meeting Infant & Pregnancy Microbiome | Base Clear | NL | 2020 |
| *Courses* | | | |
| Advanced Proteomics | VLAG | NL | 2019 |
| Intestinal microbiome of humans and animals | VLAG | NL | 2019 |
| Multivariate analysis | PE-RC | NL | 2020 |
| *Project meetings* | | | |
| Early life team meeting | Microbiology | NL | 2017-2021 |
| WUR Microbiology meeting | Microbiology | NL | 2020-2021 |
| WUR/Nutricia Research GBM quarterly meeting | Nutricia | NL | 2020-2021 |

| General courses | Organizing institute | Country | Year |
|---|---|---|---|
| Applied statistics | VLAG | NL | 2018 |
| Chemometrics | VLAG | NL | 2018 |
| Competence assessment | WGS | NL | 2018 |
| Introduction to R | VLAG | NL | 2018 |
| PhD Week | VLAG | NL | 2018 |
| *Research Data Management* | WGS | NL | 2018 |
| Part 1 | | | |
| Part 2 | | | |
| Part 3 | | | |
| Start to supervise BSc and MSc students | WGS | NL | 2018 |
| WGS PhD Workshop Carousel | VLAG | NL | 2018 |

| Other activities | Organizing institute | Country | Year |
|---|---|---|---|
| Development MOOC "Nutrition and Health: Human Microbiome" | Microbiology | NL | 2017 |
| Preparation of research proposal | Microbiology | NL | 2017 |
| Journal Club | Microbiology | NL | 2017-2019 |
| MIB seminars | Microbiology | NL | 2017-2021 |
| Molecular Ecology group meetings | Microbiology | NL | 2017-2021 |
| PhD/postdoc meeting | Microbiology | NL | 2017-2021 |
| PhD Trip | Microbiology | USA | 2019 |

A

## Colophon

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