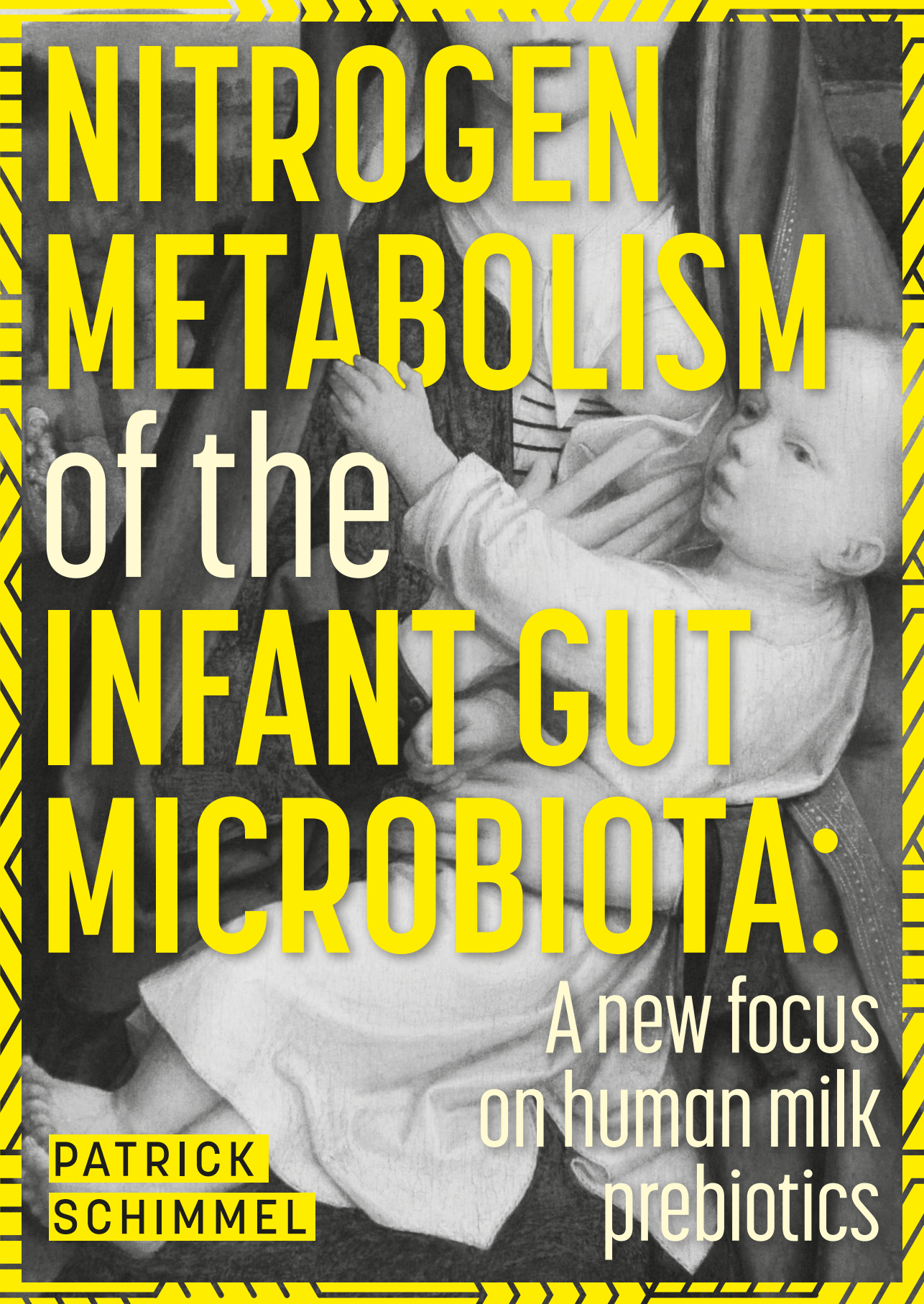

NITROGEN METABOLISM of the INFANT GUT MICROBIOTA:

A new focus on human milk prebiotics

PATRICK SCHIMMEL

PROPOSITIONS

1. Fundamental bacterial studies are still crucial for understanding how a microbial community functions.
   (this thesis)

2. The end of the amplicon sequencing era is near.
   (this thesis)

3. Revising our methods of sharing scientific data is required to understand interindividual variance in clinical studies.

4. Especially in a pandemic, science should integrate outwards communication strategies.

5. Financial rewards for research groups to finish a PhD trajectory are an erroneous motivation for supervision.

6. There is a racist hiding in everyone and education is the only tool to deal with it.

7. The largest fiasco of modern culture is the underappreciation of modern music.

Propositions belonging to the thesis, entitled
Nitrogen Metabolism of the Infant Gut Microbiota:
A new focus on human milk prebiotics

Patrick Schimmel
Wageningen, 6 December 2022

NITROGEN METABOLISM of the INFANT GUT MICROBIOTA:

A new focus on human milk prebiotics

Patrick Schimmel

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NITROGEN METABOLISM of the INFANT GUT MICROBIOTA:

A new focus on human milk prebiotics

Patrick Schimmel

Thesis

submitted in fulfilment of the requirements for the degree of doctor
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Prof. Dr A.P.J. Mol,
in the presence of the
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‘The highest function of ecology is the understanding of consequences.’

– FRANK HERBERT

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CHAPTER 1

GENERAL INTRODUCTION AND THESIS OUTLINE

The human gastrointestinal (GI) tract is densely populated by bacteria. This microbiota is established soon after birth, during a dynamic period in which changes in microbiota composition occur rapidly [1, 2]. During the crucial first months of life the infant microbiota plays a role in maturation of the immune system [2, 3], the gut itself, prevents pathogens from invading [4], and is there to deal with indigestible and recalcitrant nutrients [5, 6]. Perturbations in this process have been associated with health issues during later life [7-9]. The community is at that time built up of facultative anaerobes such as members of the *Enterobactericeae* spp. and *Staphylococcus* spp., while shortly after, the community transitions towards one dominated by anaerobes with the genus *Bifidobacterium* being most dominant [1, 10-12]. This infant gut community has a lower species richness compared to the adult gut community and is considered to be less resilient towards perturbations [13]. Describing factors that stimulate a beneficial community composition of the infant gut is therefore a current research interest, with the main focus being the early life diet.

DIET AND ITS IMPACT ON THE EARLY LIFE GUT MICROBIOTA

Diet is the main influencer in establishment of the gut microbiota. The exclusive milk-consuming period in early infancy provides a unique observation window into the effects of dietary intake (including prebiotics) on the manifestation of the gut microbiome. Especially, since the bifido- dominated community lasts until the weaning of the infant [12]. As a unique human milk compound that reflects the long-lasting co-evolution between humans and their microbiota: human milk oligosaccharides (HMOs) have been identified as a prebiotic for an infant gut microbiota in which bifidobacteria thrive [14, 15]. Not surprisingly, it has been established that the breast-fed gut microbiome significantly differs from the community's composition of formula-fed children [16, 17]. Traditionally, strictly formula-fed children have distinct differences when looking at the species level, including an overrepresentation of *Bacteroides*, *Clostridium* spp. and opportunistic pathogens [18, 19]. In **Chapter 4** we add our own study of the infant gut microbiome, replicating some of the key pillars of the research field. The strong relation between diet and infant gut microbiota composition leads to hypotheses on more human milk components acting as prebiotics. Human milk nitrogen (HMN) is of interest, since a supply of amino acids is important for the fast growing infant body [20]. We have studied the relationship between the infant gut microbiota, its

functioning and the level of amino acids and protein in **Chapter 5**. Especially since, average intake of protein-related nitrogen in a human milk diet is lower compared to formula. Bacterial niches among the early life gut microbes, concerning potential non-protein nitrogenous prebiotics is therefore a research focus in this thesis.

BREASTFEEDING AND ITS NITROGEN

Human milk is a complex biofluid formed by evolution to nourish and protect infants. It is considered the golden standard of infant feeding [21, 22]. The human milk contents are fluctuating in a specific pattern over the first months and it is tailored to the infant's needs in that way [21, 23-25]. Besides milk protein, the postnatal infant is supplied with non-protein nitrogenous compounds that include urea, creatine, creatinine, polyamines and free amino acids, combining for around 25% of total nitrogen intake in the exclusive breast-feeding period [23]. It is to be expected that among the early colonizers bacterial species are adapted to deal with these nitrogenous compounds like they evolved in relation to human milk carbohydrates. Especially, when considering the low average protein intake of human milk, compared to later life stages [26, 27]. Therefore, Non-protein HMN forms an understudied fraction of the human milk, yet many nitrogenous compounds are bioactive in relevant processes for the infant host development and are crucial for bacterial survival [28-30].

AIM OF THESIS

It is very clear that disturbances in intestinal colonization during early infancy have long-lasting effects on health [31-34]. Thus, we should improve our understanding of the driving factors behind this colonization, so that we can protect this critical process. A big factor in determining the outcome of the intestinal colonization is diet and neonates ingest milk until a transition to solid food occurs. Therefore, the ecology of gut microbes should be studied in the light of HMN. In **Chapter 2** we have described a novel relationship between HMN, specifically urea, and a successful bacteria from that environment. Furthermore, **Chapter 3** gives a broad perspective on how bacteria are involved in cycling of HMN during the early life. While prebiotics are extensively studied among carbohydrates and fibers, they are far more elusive among nitrogenous

compounds. On the other side is the microbiota which has been widely implied in host health status. Describing early life nitrogen metabolism could therefore prove an essential next step in infant gut microbial ecology. This knowledge can then be applied in specializing diet towards the infant's needs. Subsequently, this could lead to further development on infant formulae, infant gut interventions and infant health care.

TERMINOLOGY OF THIS THESIS

Biotics The classes of different biotics applications include probiotics, prebiotics, synbiotics and postbiotics. Modulation of microbiota composition by biotics requires fundamental understanding of the microbiota, its members and its processes [35].

Prebiotics Prebiotics are defined as substrates that are selectively utilized by microorganisms that confer a health benefit [36].

Probiotics Live microorganisms which when administered in adequate amounts confer a health benefit on the host [36].

Commensal Commensals are in this thesis microbes that reside in the human host or at mucosa without harming human health.

Metagenome The collection of genomes and genes from the members of a microbiota [37].

HMN Human milk nitrogen is the collection of all nitrogen sources in human milk (this thesis).

Microbiome This term refers to the entire habitat, including all microbial life, their genomes, viruses, and the surrounding environmental conditions [37].

Microbiota The assemblage of microorganisms that inhabit the human body, their genomes and metabolites, and their stage of operation, is called the microbiota [38].

THESIS OUTLINE

▶ In **Chapter 2** we describe utilization of urea by a member of the *Bifidobacterium* genus and common infant gut symbiont.
Title: Breast milk urea as a nitrogen source for urease positive *Bifidobacterium infantis*

▶ In **Chapter 3** we reviewed are currently known interactions and relations between HMN and the infant gut microbiota.
Title: A breastfed infant's gut microbiota: in pursuit of nitrogen

▶ In **Chapter 4** we studied a Belgian cohort for infant gut microbiota development.
Title: Proof of principle study replicating microbial clusters in connection to birth mode and diet in the early life intestine

▶ In **Chapter 5** we studied the effect of adapting formula to contoin a lower protein concentration and its effect on the infant gut microbiota.
Title: Effect of a low protein diet on the early life gut microbiota: a follow-up to the Proteus study

▶ **Chapter 6** is the general discussion to this thesis.

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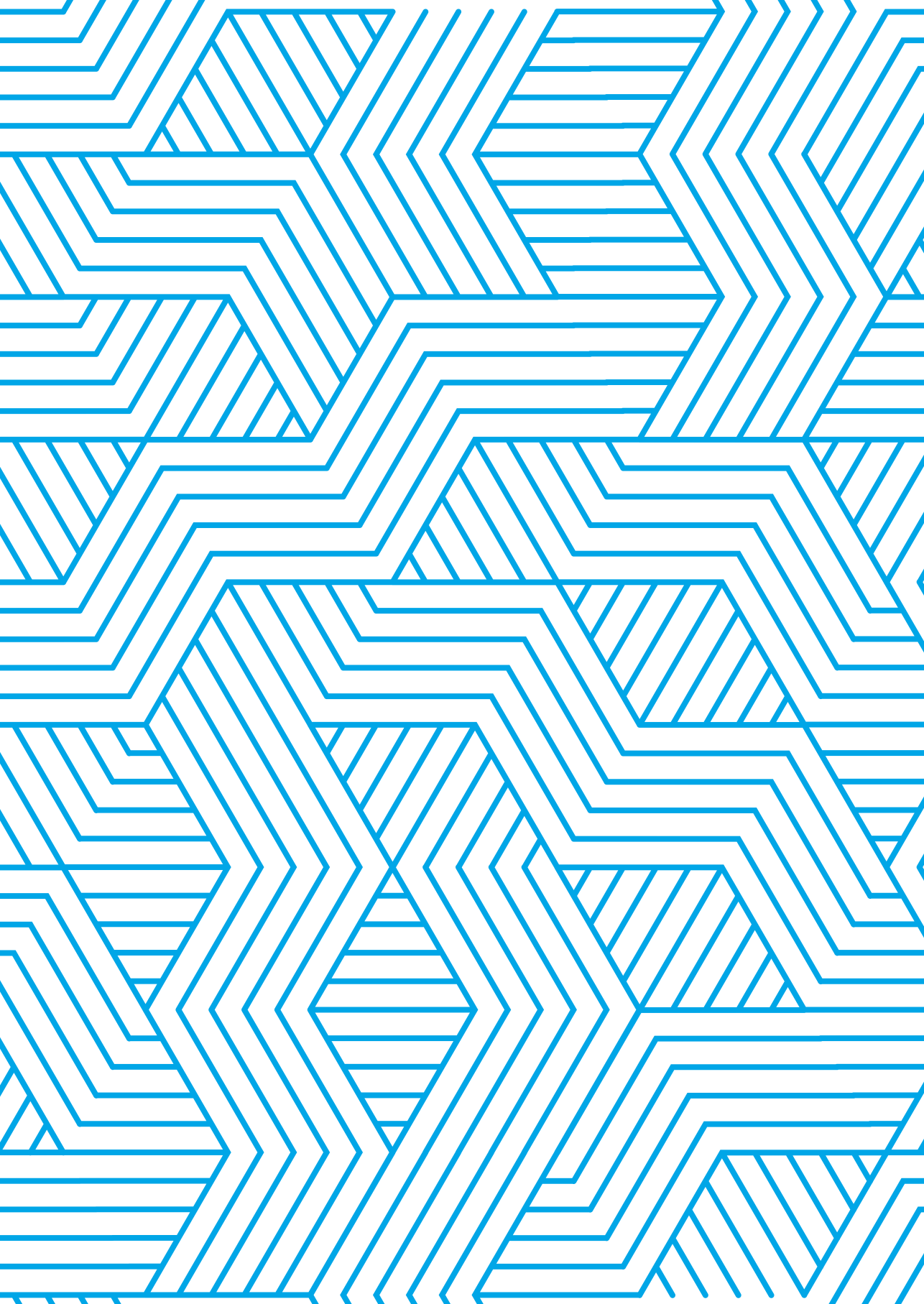

CHAPTER 2

BREAST MILK UREA AS A NITROGEN SOURCE FOR UREASE POSITIVE *BIFIDOBACTERIUM INFANTIS*

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ABSTRACT

Human milk stimulates a health-promoting gut microbiome in infants. However, it is unclear how the microbiota salvages and processes its required nitrogen from breast milk. Human milk nitrogen sources such as urea could contribute to the composition of this early life microbiome. Urea is abundant in human milk, representing a large part of the non-protein nitrogen (NPN). We found that *B. longum* subsp. *infantis* (ATCC17930) can use urea as a main source of nitrogen for growth in synthetic medium and enzyme activity was induced by the presence of urea in the medium. We furthermore confirmed the expression of both urease protein subunits and accessory proteins of *B. longum* subsp. *infantis* through proteomics. To the same end, metagenome data were mined for urease-related genes. It was found that the breastfed infant's microbiome possessed more urease-related genes than formula fed infants (51.4:22.1; 2.3-fold increase). Bifidobacteria provided a total of 106 of urease subunit alpha alignments, found only in breastfed infants. These experiments show how an important gut commensal that colonizes the infant intestine can metabolise urea. The results presented herein further indicate how dietary nitrogen can determine bacterial metabolism in the neonate gut and shape the overall microbiome.

IMPORTANCE PARAGRAPH

Human milk stimulates a health-promoting microbiome in infants. Urea is abundant in human milk, making up a large part of the non-protein nitrogen. We wanted to explore if human milk urea could be used by the microbiota. Considering bacterial nitrogen metabolism for further understanding why breastfeeding is so beneficial, is the next step. In that fashion, this study indicates that nitrogen sources in human milk are potentially selecting for a healthy gut microbiome, which is important for infant feeding and health considerations.

Keywords: urease, infant gut, microbiota, *Bifidobacterium*, urea, human milk

INTRODUCTION

When human life begins, the gut microbiota develops dynamically [1, 2]. Notably, this microbiota can aid with digestion and thus supports the infant's nutritional needs [3-5]. Moreover, the microbiome is crucial in establishing health for the infant by supplying amino acids and vitamins and contributing to gut maturation and immune development [6-12]. This vital balance originates in the early moments of life and the bacteria responsible are promoted by dietary factors named prebiotics [1, 13, 14]. In recent years, research has therefore focused on describing early microbial colonization patterns and mechanisms of human milk utilization by early life gut symbionts [15-18]. Human milk and all of its components are likely to have evolved to promote symbiosis between host and microbiome. However, it is currently unclear how the microbiota salvages nitrogen from breast milk. Human milk nitrogen comes in many forms and is for example represented by milk proteins, peptides and free amino acids, but also human milk oligosaccharides hold nitrogen that could be utilized by the infant gut microbiota [17, 19-25]. Interestingly, this complex bio-fluid holds some nitrogen sources that are waste products of human metabolism, like urea [19, 26, 27].

Urease is the first enzyme ever isolated [28]. As such, the enzyme urease (*EC* 3.5.1.5) has been studied in various bacteria and ecosystems and has been found to play a crucial role in bacterial survival [29- 31]. Urease is a nickel or iron co-factored multimeric enzyme that catalyses the hydrolysis of urea into carbon dioxide and ammonia (NH_3) [32-34]. In bacterial pathogens, e.g. *Helicobacter pylori*, *Proteus mirabilis*, urease functions as a virulence factor [31, 35-37]. It functions as such by managing the environment around the bacterial cell, through neutralization of the acidic microenvironment by protonation of ammonia, resulting in ammonium (NH_4) [30, 38]. Ureolytic activity is seen with non- pathogenic bacteria as well [31, 39]. However, in both pathogens and non-pathogens the underlying mechanism and ecological function are often unclear [29, 31, 40, 41]. The resulting ammonium has been suggested as the main nitrogen source of choice for amino acid synthesis by gut bacteria through glutamate dehydrogenase activity [10, 42-44]. Therefore, bacterial urease can fulfil a function in gut nitrogen cycling to serve the bacterial needs.

Nitrogen availability, specifically urea, both host- and diet-derived, can greatly affect the human gut microbiota [45-49]. For diet, the biological norm is that for approximately the first six months of human life, infant feeding consists solely of human milk because of its effect on microbiota composition and overall health [1, 14, 50-52]. Human milk

composition varies inter-individually and over time from colostrum to late lactation [21]. It holds a collection of non-protein nitrogen (NPN) sources (25% of total N), including urea [19, 21, 27, 53, 54]. Urea constitutes 15% (3-6mM) of this NPN, while others claim that it actually represents 15% of total nitrogen in human milk [23, 26, 31, 54-57]. The relevance of urea nitrogen salvation for the infant gut, although not intensively studied, has often been suggested [11, 23, 56, 58-60]. There are, nonetheless, a few indications of urease activity by bacteria in the infant lower gut [61-64]. In an early study, labelled 15N urea turned up in microbial protein and in infant serum amino acids, indicating a function of urea in microbial biosynthetic processes and showing utilization out of a dietary source [61, 62]. Notably, in an infant fed with a breast milk-like diet, utilization of urea increased significantly compared to the infants fed with formula [56]. Finally, a recent metaproteomics study showed enrichment of nickel transport systems in the infant gut assigned to *Bifidobacteriaceae*, which are potentially important for activation of urea metabolism [65].

Early studies, observed urea degradation among gut commensals without further characterization of the process [39, 63]. Interestingly, several genomes of *Bifidobacterium* spp. possess urease genes [58, 66]. Of particular interest was the discovery of a full urease cluster in the common infant gut colonizer *Bifidobacterium longum* subsp. *infantis* (*B. infantis*, ATCC15697; *Blon_0104-Blon_0115*, Figure 1A) [58]. As biomarkers for infant health, urease is likely to fulfil a different niche function for *Bifidobacterium,* compared to a function in virulence in model organisms. It is hypothesized here that *Bifidobacterium longum* subsp. *infantis* urease is a growth factor for the species.

In this paper we further characterized urease activity as a growth factor *in vitro* for *Bifidobacterium longum* subsp. *infantis*, a potentially beneficial bacteria and a common infant gut colonizer [50, 58, 67]. This specific trait might explain why *B. infantis* is an efficient colonizer of the infant gut and how it is adapted to the human milk diet. Public metagenome data of the infant gut was studied to investigate the relationship between diet and potential urease activity of the microbiome. We expect *Bifidobacterium* to interact with human milk urea. Both aspects increase our understanding of how breastfeeding might stimulate a beneficial microbiome.

MATERIALS AND METHODS

METAGENOME MINING FOR UREASE GENES

An infant gut metagenome database comprised of 3 publicly available datasets along with dietary metadata was studied for occurrence of urease-related genes [2, 68, 69]. The study holds a total of 92 samples obtained from 74 infants with a total of 10442761 DNA reads (S1,2). These three studies were selected since they all contain both breastfed and formula-fed infants. Included infants were solely breastfed or solely formula-fed and were 6 months or younger. Antibiotic-treated, diseased or preterm infants were excluded from the study. The reads were previously quality filtered in different methods by the authors of before mentioned publications. To extract predictive functional data from metagenomes, protein aligners have been developed (e.g. DIAMOND, [70, 71]). Using DIAMOND, the raw shotgun reads were translated into protein and mapped against UniProt databases (Swiss- Prot and TrEMBL, Release 2019_01), to create a functional profile. The complete UniProt database was in this case included to counter the lack of inclusions of *Bifidobacterium* protein sequences and genes in the SwissProt database alone. Quality cut-offs for DIAMOND alignment were set at 60% coverage of the read length and 60% alignment identity for the protein alignments. Alignment results were filtered for urease-related hits. These included urease protein subunits, urea transporters, urease accessory proteins and urease activity regulatory genes. Protein hits were normalized per 1 million high quality reads. This method yielded occasionally multiple successful assignments per read. Hits with the highest reported identity scores were considered for taxonomic analysis. One sample from the Schwartz *et al.* [68] dataset was excluded since it contained a far higher rate of urease genes than the other samples combined from that study. We suspect an artefact resulting from our methods or that the infant was suffering from an infection, which warranted exclusion. Included samples list and dietary metadata are available in the supplementary data (Table S1). A phylogenetic tree for urease subunit alpha genes related to the infant gut was constructed using ARB software package (max. likelihood; Phylip PROML), based on the 25 most dominant taxa in study by Stewart et al. [50, 72]. Original tree file was uploaded at Open Science Framework repository (OSF, https://osf.io/2hu4m). From NCBI Nucleotide database, full genome sequence annotations of *Bifidobacterium* were checked for urease gene clusters.

BACTERIAL STRAINS AND GROWTH CONDITIONS

Bifidobacterium longum subsp. *infantis* (ATCC 17930/DSM20218/JCM1260) and *Bifidobacterium breve* (ATCC 15698/DSM20091) were anaerobically grown on TOS-

propionate agar medium (Sigma-Aldrich, St. Louis, USA), specialized for enumeration of *Bifidobacterium* species. Plated cultures were incubated at 37°C and plates were stored at 4°C for long-term storage. Plates cultures were used to inoculate liquid pre-cultures by swiping colonies in an anaerobic tent (Coy Vinyl Anaerobic Chamber, max. 5% H_2/N_2). For liquid culturing, a nitrogen-limiting medium with excess in carbon source (lactose 2%; pH 6.2): yeast extract (1g/L); potassium dihydrogen phosphate (3g/L); dipotassium hydrogen phosphate (4.8g/L), magnesium sulphate (0.2g/L), sodium propionate (15g/L) and L-cysteine hydrochloride (0.5g/L) to counter the strain's auxotrophy for the amino acid. Serum bottles were supplemented with filter-sterilized vitamin mix and trace metals (100x; originally designed for *Lactobacillus lactis* [73]) Nitrogen treatments included urea (0.6g/L; 10mM), tryptone, (protein digest, w/w: 12.7% N, 2.2g/L, Oxoid, Basingstoke, UK), ammonium sulfate ($(NH_4)_2SO_4$) or no added nitrogen source. Nickel chloride (NiCl; 0.1-1 mM) was included as it is required by active urease enzyme. Test cultures were subsequently inoculated with 0.1mL of late logarhytmic phase pre- culture (24h). Anaerobic serum bottles possessed either a headspace of N_2 or CO_2/N_2 (80%/20%) at 1.7 atm. The basal growth media was tested in a pH range and with a bicarbonate buffer (CO_2/N_2; 20 mM Na_2CO_3). Liquid pre-cultures contained only CO_2/N_2. Growth was evaluated by measuring optical density at 600nm (OD600; OD600 DiluPhotometer™, IMPLEN, Germany). Acidification was observed by measuring pH (ProLine B210). For statistical analysis, an unpaired *t* test was used in SPSS Software (V24). *P* values < 0.05 were considered statistically significant.

UREA COLORIMETRIC ASSAY

Urea levels were determined by a colorimetric urea assay (MAK006; SigmaAldrich) according to manufacturer's instruction. The coloured result of a coupled enzyme reaction was measured at 570 nm on a Synergy™ Mx microplate reader (Biotek, Winooski, USA). Culture supernatants were diluted 100x prior to analysis to fit the kit's standard curve. Data were normalized to observed background effects due to interference of compounds in the media, like e.g. ammonium, NAD+/NADP+ and pyruvate.

UREASE ENZYMATIC ASSAY

Bacterial cultures (1 mL) were retrieved and centrifuged (10 mins, 20,000xg) before supernatants were separated from pellets. Cell pellets were suspended in sodium phosphate buffer (10 mM) before analysis. Supernatants were filtered (0.2 µm) before use in enzymatic assay. Sonication of bacterial cell pellets was performed using a MS72 probe on a Bandelin Sonopuls Sonicator (Bandelin Electronic, Berlin, Germany).

Cells were lysed using 20s sonication at 30% amplitude with 30s intervals on ice and repeating this 4 times. Enzymatic activity of supernatants and cell lysates was then assessed using urease activity assay kit (MAK120, Sigma Aldrich, St. Louis, USA) as reported by the manufacturer, derived from the Berthelot method. Exposure to urea lasted for 10 minutes. After 30mins of colour formation, absorbance at 670nm was measured, preceded by 2s medium shake on a Synergy™ Mx microplate reader (Biotek, Winooski, USA).

PROTEOMICS

Samples were taken from mono-cultures of *B. infantis* (ATCC17930) with urea during late exponential growth phase. Cell lysis was achieved through bead beating in a FastPrep-24™ 5G instrument (MP Biomedicals) for 6 times 30 seconds at 6.5m/s with cooling after every bead step. Protein concentration was assessed using Pierce BCA protein assay (ThermoFisher Scientific, Waltham, USA; S8). Subsequent protein digestion was performed overnight using dithiothreitol (DTT, 2mM), iodoacetamide (IAA, 4mM) and trypsin (1:50 of a 1µg/µL solution) at 37°C. Cleanup was performed through SPE columns (Solid Phase Extraction; ThermoFisher Scientific, Waltham, USA) with acetic acid (100mM in 95% acetonitrile) to be finally dissolved in the eluent ammonium formate (10mM). Samples were analyzed in duplicate by nano-LC-HRMS/MS as described by Meiring *et al.* [74]. An Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, USA) was used, connected to a Q-Exactive Plus Mass spectrometer (ThermoFisher Scientific, Waltham, USA). Peptides were trapped on a 100µm inner diameter trap column packed using ReproSil-Pur C18-AQ, 3 µm resin (Dr. Maisch, Ammerbuch, Germany) at 5µL/min in 100mM acetic acid. Afterwards the peptides were eluted at 100mL/min in a 90 minutes extended gradient from 10-40% acetic acid solvent (in 95% acetonitrile) to a 20-cm IntegraFrit column (50µm inner diameter, Reprosil-Pur C18-AQ 3 µm, New Objective, Woburn, USA). The acquired spectra were analyzed using Thermo Proteome Discoverer in combination with Mascot (ThermoFisher Scientific, Waltham, USA). The reference database comprised of protein sequences from *Bifidobacterium longum* subsp. *infantis from Uniprot* (ATCC15697) since the genome of *B. infantis* strain ATCC17930 is not publically available.

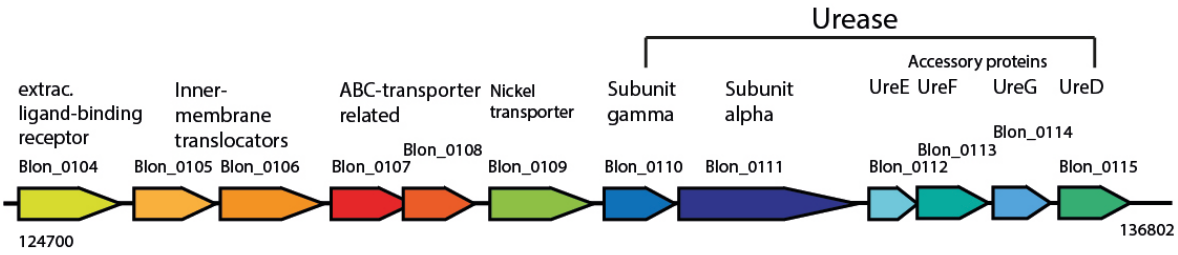
RESULTS

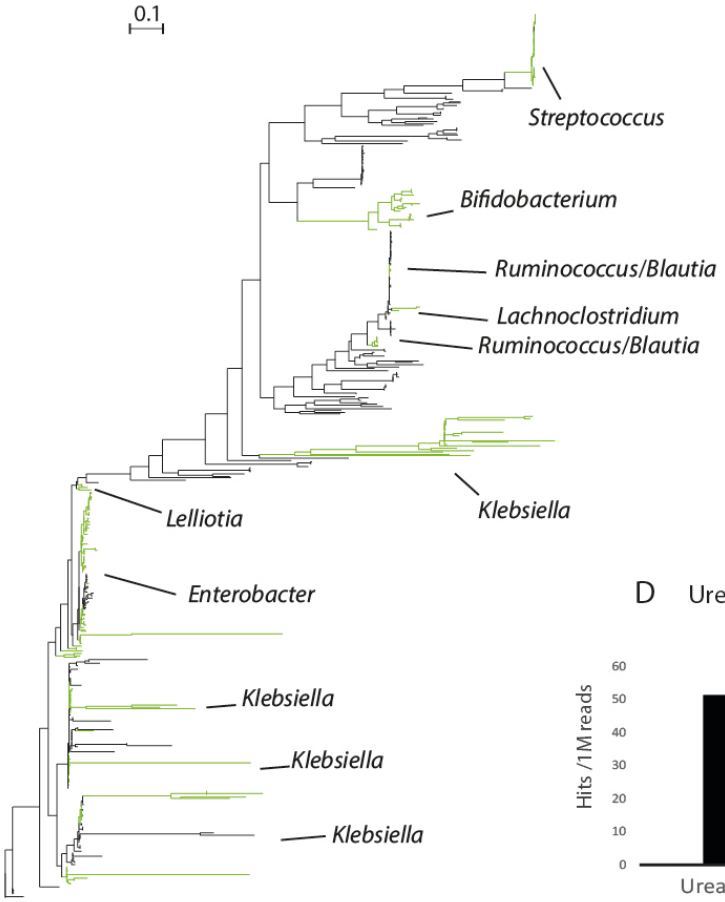
OCCURRENCE OF UREASE GENE CLUSTER IN *BIFIDOBACTERIUM* SPP.

Bifidobacterium whole genome annotations were scanned for urease gene clusters as present in *B. infantis* (Figure 1A) [17]. *Bifidobacterium* urease genes are currently annotated across 7 species that are related to the human gut. Namely, the *B. longum* subspecies *longum, infantis* and *suis; B. subtile*; *B. kashiwanohense* and *B. scardovii* (Table S3). Either a full gene cluster or no gene cluster was found to be present every time. One exception occurred with *B. subtile*, where one accessory protein gene was missing from the cluster. Interestingly, of *B. infantis* whole genome sequences only 50% of the cases had a urease gene cluster annotated (Table S3). Meanwhile, no urease gene clusters are currently annotated in several other common *Bifidobacterium* infant gut colonizers like *B. breve* and *B. bifidum* spp.

UREASE GENES FOUND IN INFANT GUT METAGENOMES

We studied bacterial urease gene cluster occurrence in the infant gut of which *B. infantis* holds an example (Figure 1A). A phylogenetic tree was constructed of urease alpha subunit genes that are expected to occur in the infant (Figure 1B). Interestingly, all *Bifidobacterium* genes cluster together. Furthermore, many genes, belonging to other genera have been traced to the metagenomes included in this study (Figure 1B, green highlights). In total, the functional profile of the metagenomes yielded a total of 27 taxa for urease protein subunit alpha hits (Figure 1C). The most dominant genus holding urease genes was *Enterobacter*, representing 27.8% (326) of alignments. The other most dominant found genera were *Streptococcus* (15.4%), *Klebsiella* (13%), *Escherichi*a (12.4%). *Bifidobacterium* was the fifth most abundant genus with 9% (Figure 1C). The genus was represented by *B. longum* subspp., *B. kashiwanohense* and *B. scardovii*. Interestingly, *Bifidobacterium* urease genes were only found in breastfed infants, while *Escherichia* hits occurred mostly in samples belonging to strictly formula fed infants (breastfed 6%, formula-fed 41%). Sequences traced to *Enterobacter* were present in both diet types 28,34% and 25,13% in breastfed and formula-fed infants respectively. *Klebsiella* showed a similar pattern (12,63%; 15.08%). After data normalization a 2.3-fold increase of urease-related hits was observed for breastfed infants compared to formula-fed infants (Figure 1D). A 1.5-fold increase was observed when measuring urease protein subunits separately.





C Genera classification subunit alpha hits (%)

	Breastfed	Formula-fed
Total	974	199
Bifidobacterium	10.78	0
Klebsiella	12.63	15.08
Streptococcus	17.56	5.03
Escherichia	6.47	41.71
Enterobacter	28.34	25.13
Yokenella	0.51	0.50
Leclercia	1.44	1.01
Citrobacter	2.77	3.52
Salmonella	0.10	0
Raoultella	0.72	1.51
Haemophylus	5.03	0
Blautia	0.51	0
Ruminococcus	0.41	0
Pedobacter	0.20	0
Lelliotia	2.16	1.51
Paraburkholderia	0.10	0
Cedecea	0.10	0
Superficiaebacter	0.21	0.50
Acinetobacter	8.52	0
Azotobacter	0.31	0
Uliginosibacterium	0.10	0
Pseudomona	0.41	4.02
Lachnospiraceae	0.10	0
Firmicutes	0.10	0
Vibrio	0.10	0
Pantoeeia	0.31	0
Elstera	0	0.50

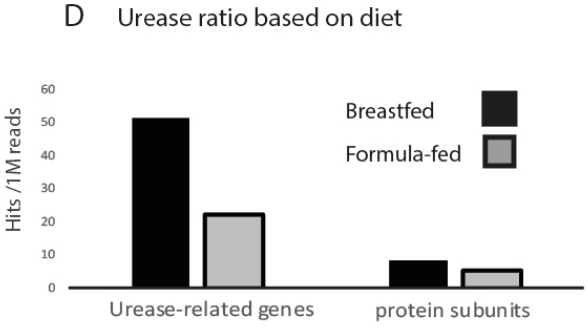


Figure 1. (A). Urease gene cluster as found in B. infantis (Sela et al. 2008) [58]. (B). Phylogenetic tree of urease subunit alpha genes expected in the human gut. Nodes of genes found in study labeled green. (C). Genera of origin of the urease gene hits in the metagenomes, retrieved from all urease protein subunit alpha hits surpassing the alignment quality threshold (%). (D). Normalized ratio of reads that aligned with urease proteins for all bacterial species based on infant diet (per 1 million reads).

UREA SERVES AS A NITROGEN SOURCE FOR *B. LONGUM* SUBSP. *INFANTIS*

We tested the ability of *B. infantis* to use urea as the main nitrogen source by comparing it with growth on a protein digest (tryptone) or no added nitrogen. Growth on urea as the main nitrogen source was observed for *B. infantis* and not for *B. breve*, which was included as a non-urease expressing control (Figure 2AB, 0.4 compared to 4.1; OD600; 32h; p≤0.05, S4&5). Differences in growth were reflected by different pH across the culture types (S4). Growth on $(NH_4)_2SO_4$ was checked to confirm the strain's ability to process the ammonium output resulting from degrading urea (S7; headspace: N_2). An active urease complex produces carbon dioxide (CO_2). Growth was therefore compared to conditions with CO_2 present. Growth increased in the presence of CO_2 compared to conditions with only N_2 present, but the presence of urea still increased the growth of *B. infantis* relative to the basal medium (Figure 2B). Thus, CO_2 absence is not a trigger for *B. infantis* to become urease active.

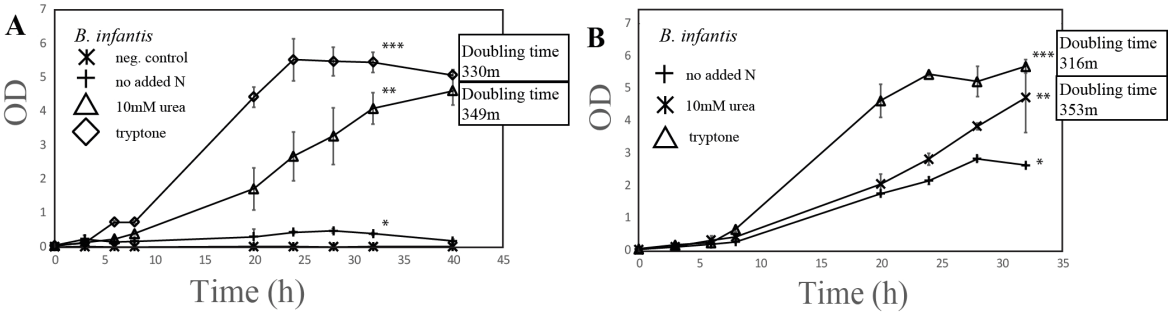


Figure 2. (A). Growth of B. infantis in nitrogen limiting media, headspace 100% N2 (OD600). (B). Growth of B. infantis in nitrogen limiting media, headspace 80% N2 /CO2 , *P values < 0.05. Doubling time in minutes.

UREASE PROTEINS CONFIRMED AND ACTIVITY IS LINKED TO THE PRESENCE OF UREA

Presence of urease-related proteins was confirmed with high reliability for several proteins deriving from the *B. infantis* urease gene cluster (S8). This included urease subunit alpha, beta/gamma and accessory proteins UreE, UreG (Figure 1A, S8). This thus included Uniprot accessions: B7GT16- 18 and 20. The sequence coverage can be considered low in some cases (4-35%). However, the occurrence of unique peptides (3-7) caused that the matches can be regarded as very confident through classification in the Proteome Discoverer software (S8). Urease activity of *B. infantis*, was only detected upon cultivation with urea as the nitrogen source (Figure 3, $p<0.05$, S5). At 20h no activity was observed in basal medium and basal medium supplemented with tryptone. No clear and reliable activity measurements were obtained, when the supernatant was analyzed. Degradation of 10mM urea was confirmed at 24h, which corresponds with the exponential growth phase (Figure S6).

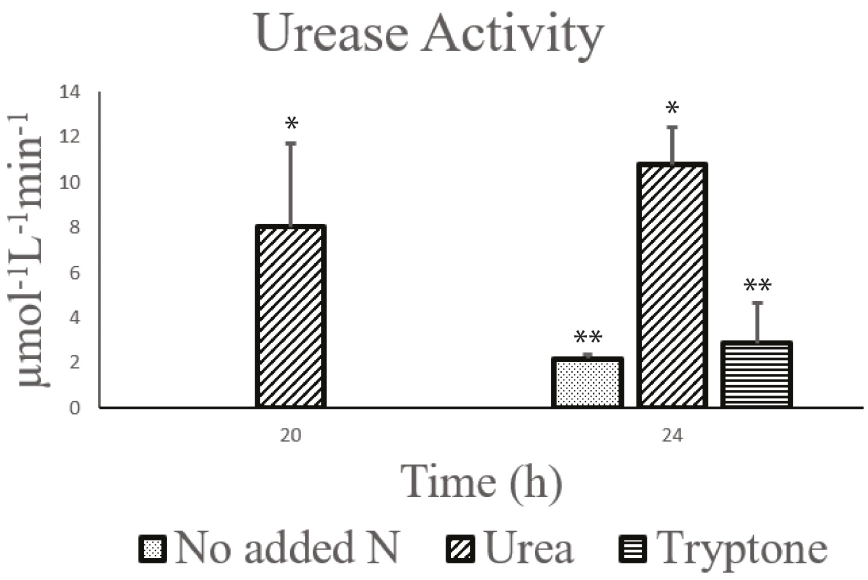


Figure 3. Urease enzyme activity (ammonium production/µmol/L/min) measured on two timepoints with the Berthelot method, $P < 0.05$ (Figure S5, Supporting information).

DISCUSSION

Since the discovery of a urease gene cluster in *B. infantis* [58], urea nitrogen salvaging by the infant gut microbiota was highlighted as of potential importance to the settling microbiota. Our results indicate once more, the adaptation of *B. infantis* towards the human milk diet and gives rise to speculation on the role of urea in breastfeeding. Together with the metagenome data this study illustrates a new mechanism by which urea in breast milk can shape a microbial community and thus strengthens the hypothesis that urease could be used as a growth factor for a potentially beneficial strain.

METAGENOME STUDY

Bifidobacterium urease represents a significant part of the microbiota that is potentially utilizing urea in breastfed infants (Figure 1BC). Notably, some *Bifidobacterium* hits belonged to *Bifidobacterium callitrichos*, *Bifidobacterium primatium*, *Bifidobacterium biavatii, Bifidobacterium tissieri* that are not associated with the human infant gut, but rather with the primate gut that is likely to have urea available as well [75-77]. This indicates that there might be more *Bifidobacterium* urease genes associated with the human infant gut that are not described. The constructed tree shows a likelihood of alternate evolutionary paths of urease activity due to large spreading within phylogenetic groups, while in the meantime *Bifidobacterium* clustered together and thus showing similarity. The functional profile showed that *Streptococcus* urease genes are dominantly found in the breastfed infant (Figure 1C) and their urease activity is connected to biosynthetic pathways and provides intracellular benefits due to pH regulation [31, 78, 79]. It can now be hypothesized that *Bifidobacterium* urease fulfills a similar role, especially considering the highly acidic environment that is the infant colon. Interestingly *Escherichia* (*E. coli*) shows a reverse pattern compared to *Bifidobacterium*. The genus appeared more in formula-fed infants compared to breastfed infants, while their urease activity has been mostly associated with pathogenesis [80]. However, this might be due to the lack of prebiotics in the formula products, of which no data was available. Moreover, several other pathogenic genera, e.g. *Citrobacter*, *Klebsiella* were found [81-83]. Urea metabolism has been intensively studied for these pathogenic genera, which could result in an underrepresentation of *Bifidobacterium* urease proteins in the database, a common bias and obstacle when constructing functional profiles [84]. Also, *Bifidobacterium* spp. have one locus containing an urease gene cluster annotated, while others like *Escherichia coli* can have multiple [85]. Proper validation or normalization for such biases, should be developed. Nonetheless, beneficial bacteria, e.g. *B. infantis*, that possess urease

activity might compete with pathogens for urea as a nitrogen source. Bifidobacteria might be dominant in breastfed infants due to their capacity to degrade HMOs and through this mechanism, it could limit the possibility of other urease positive species occurring in that same environment. Moreover, it might be acting as a detoxifier, since urea clearance from the gut is important in gut health [86]. This could make *Bifidobacterium* urease a target for therapeutics. How dietary urea would favour *B. infantis* or other *Bifidobacterium* spp. over these potential pathobionts needs to be further investigated.

The functional profile showed that breastfeeding selects for genes related in urea metabolism and thus bacteria involved in urea utilization (Figure 1D). However, this pattern was not observed consistently for urease protein subunits when looking at the included studies separately (Figure S2). This data nonetheless provides a fair representation of the total level of genomic capabilities for a specific process, compared to an approach that included assembly or binning. Short genetic fragments of low abundance species that lack sufficient sequence coverage are now included because of recent advances in fast translated searches [70, 84, 87].

UREA IN THE INFANT GUT

It is expected that urea is a microbiome modulator in the infant gut and that urease producing microbes profit in a comparable way as for example how host-derived urea in uremic patients selects for urease active bacteria [47, 48]. Urea could be present at low levels in the lower gastrointestinal tract of the infant due to earlier ureolytic activity along the gastrointestinal tract or because of inter- individual variation between maternal factors that affect milk composition [11, 19, 21, 23, 31, 88].

Urea can be supplied by the human body, or by other bacteria through amino acid fermentation as well [10, 11, 31, 43, 61-64]. Notably, lower protein diets have been shown to change urea kinetics in adults, shifting towards more host-derived urea utilization [64, 89]. A similar pattern could exist for the infant gut. Host-derived urea can anyhow explain the level of urease genes found in formula- fed reference group. Moreover, infants fed formula were not completely excluded from dietary urea, since it occurs in formula as a trace compound due to its cow milk origin [54]. The hypothesis that milk urea serves a biological purpose through possibly selecting for specific bacteria, is nonetheless underlined by the described results.

UREA UTILIZATION BY B. *INFANTIS*

We provide evidence for the ability of *B. infantis* (ATCC17930) to utilize urea as a main nitrogen source. The strain is able to express necessary gene products, which were confirmed through proteomics albeit with low coverage. This can be explained by the use of the related proteome database, which can lower coverage from a methodological point of view. While being comprised of fairly large proteins, which can lower coverage on its own, it is expected that the urease enzyme is embedded in the membrane [90]. This might lead to lesser efficiency with the described protein extraction method due to enzyme being lost with the cell debris. The likelihood of the strain being able to produce active enzyme, was confirmed by the presence of the accessory activator UreE in the proteomics data [34]. This accessory protein is thought to be involved in nickel-chelation which is essential for urease activation in other bacteria [91, 92]. The lack of UreD and UreF occurrence cannot be explained, especially since all other data from this study suggests urease activity in *B. infantis*. Possibly this accessory protein is not required for active enzyme or it is produced in another stage of the culture experiment. Accessory protein UreF was scarcely detected, but never surpassed the low reliability threshold. Interestingly, according to enzymatic assay, only the presence of urea made *B. infantis* active in degrading urea, which indicates some form of metabolic switching and regulation according to the enzymatic assays (Figure 3A). Similar observations were made for other bacteria hosted by humans that have proven to be urease active and that specifically transcription was regulated by UreR in the presence of urea [42, 93]. The fact that the presence of urea is required for *B. infantis* was furthermore observed in *Streptococcus thermophilus,* while also indicating that aspartate metabolism and glutamate metabolism might be linked to urease activity [79, 94]. Since we tested urease activity in pure strain cultures, there is a chance that the organism changes it activity in the presence of other species in a community. Further investigation into the regulation of the urease gene cluster in *B. infantis* is therefore definitively warranted. Due to presence of genes encoding a transporter system on the cluster (Figure 1A), it is to be expected urease activity is at least partially intracellularly and thus related to the cell lysate as was shown in this study. Urease activity in the supernatant has not been reliably measured using the enzymatic assay, but cannot be excluded. It has been shown that CO_2 availability promotes growth of *B. infantis*, yet lack of CO_2 is not a trigger to become ureolytic in this organism, since growth occurs under both conditions. The promoting effect of CO_2 indicates the capnophilic nature of this organism. Despite the importance of bifidobacteria for infant health, the effect of CO_2 on the overall metabolism of the genus is poorly investigated and is surely related to the effect of urease activity by these bacteria. Urease activity can furthermore be

a means of pH regulation by intestinal bacteria for the acidic environment that the breastfed infant's gut traditionally is [30, 38, 78, 95]. These results cannot exclude that *B. infantis* benefits from urease activity in low pH and even regulates the pH (S4).

CONCLUSIVE WORDS

Human milk shapes our microbiome from the moment we are born. The authors hypothesize that urea in breast milk functions as an important nitrogen source for beneficial bacteria in the early life gut microbiota and provide the first evidence for this. To our knowledge this is the first time that a direct link has been made between *Bifidobacterium* and urease activity and meanwhile its relevance in the infant gut is supported by metagenomic data. This study further characterizes a mechanism by which a common gut symbiont utilizes urea as a nitrogen source from human milk. It is proposed here that urea degradation by *B. infantis* is another relevant means to acquire nutrients from breast milk. Further research is needed to clarify the function of *B. infantis* urease activity in the infant gut ecosystem.

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The study was designed by P.S., L.K., R.B., J.K. and C.B.; P.S. performed the study in the lab; P.S. collected and generated the data from the metagenomes; P.S. performed the proteomics study; P.S. performed data analysis and figure generation; P.S. and C.B. wrote the manuscript. The manuscript was checked by P.S. L.K., R.B., J.K. and C.B. All authors contributed to critical revisions and approved the manuscript. First of all, the authors would like to thank Dr. Bernd Stahl[2] for being an initial inspiration to this project. Secondly, the authors would like to thank Athul Sundaresan[1] for his contributions in the lab as part of his thesis project. Finally, the authors would finally like to thank Heleen de Weerd[2], Joost Gouw[2] and Gido Jehoel[2] for their help and advice with the metagenomics data generation, proteomics processing and analysis. R.B. and J.K. are employees of Danone Nutricia Research. P.S., L.K., C.B. received funding from Danone Nutricia Research.

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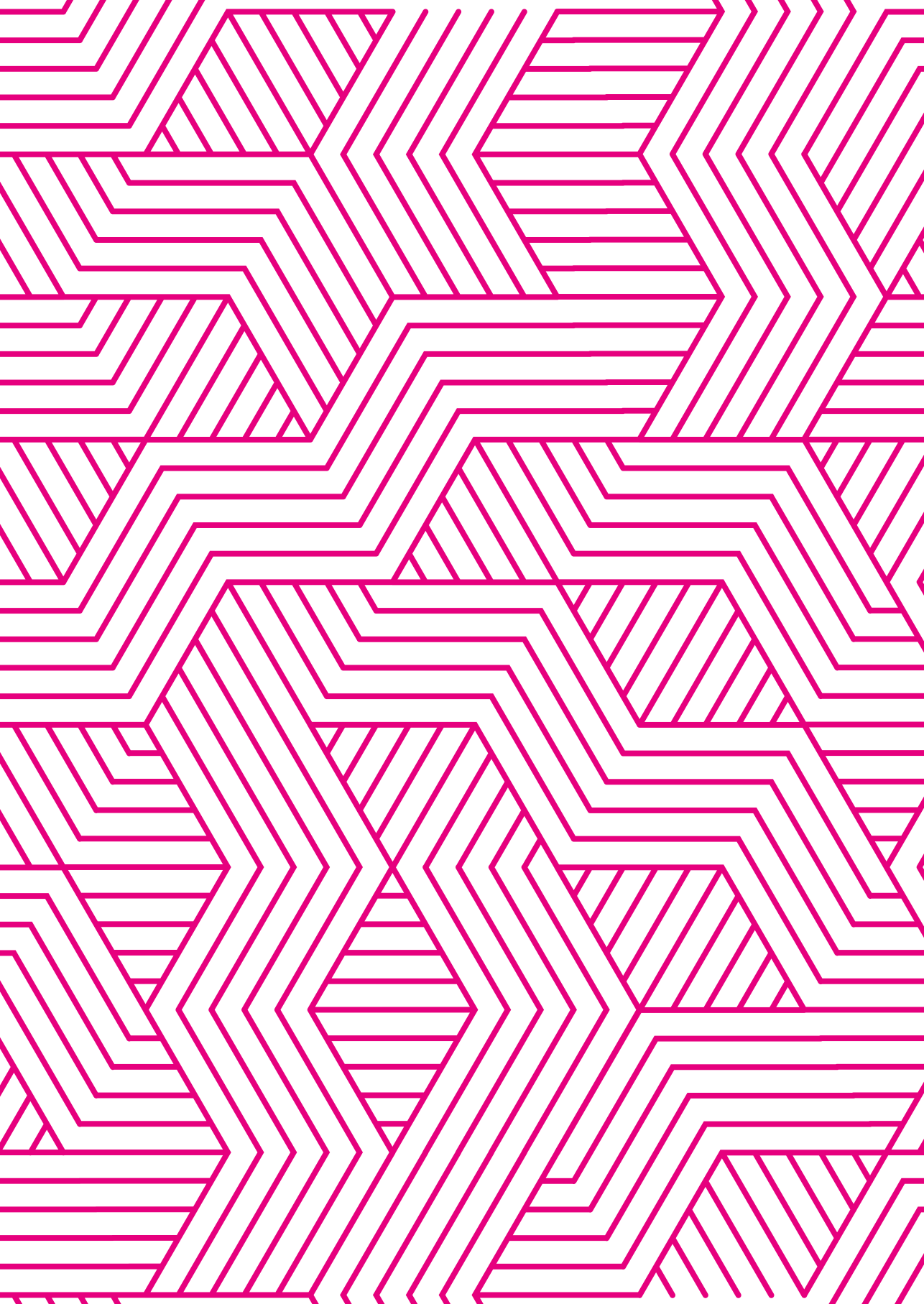

CHAPTER 3

A BREASTFED INFANT'S GUT MICROBIOTA: IN PURSUIT OF NITROGEN

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ABSTRACT

Diet shapes our gut microbiome from the day we are born. The contribution of dietary non-protein nitrogen to normal and healthy nitrogen cycling in the infant gut is scarcely described. Herein we review *in vitro and in vivo* findings that show the impact human milk nitrogen (HMN) has on the gut microbiota that settles in early life. We describe that several non-protein nitrogen sources, that include creatine, creatinine, urea, polyamines and free amino acids, are key in establishing the *Bifidobacterium*-dominated microbiome and thus are bifidogenic. Furthermore, several parts of HMN-related metabolism is associated with a healthy infant gut and commensal microbiota. We illustrate an overlap and great diversity in accessibility of HMN by large parts of the infant gut microbiota. This review nonetheless shows the importance of research on HMN and its effects on the activity and composition of the infant gut microbiota and subsequent early life infant health.

Keywords: human milk, nitrogen, breastfeeding, infant gut, bacterial, metabolism, microbiome, health

INTRODUCTION

The human gut holds a microbial cell population, or microbiome, that through its presence and activity supports human immunology and prevents disease occurrence [1]. This support by the microbes in our gut starts immediately after birth and is efficiently stimulated by human milk (HM) [2, 3]. The early life scenario provides a unique study opportunity, to apprehend the effect of diet on microbiota. Even though the definition of a healthy infant's microbiota is still gathering support, we understand that breastfeeding is promoting a *Bifidobacterium*-dominated microbiome in healthy infants [3, 4]. It cannot be ignored however that variations in microbiota composition, geographically and inter- individually are high [2, 5]. Nonetheless breastfed infants are considered a low-risk group for many health threats [6, 7], thus making breastfeeding the golden standard for infant feeding. This guided research towards identifying prebiotics in HM that promote a beneficial microbiota. Fundamentally, to understand metabolism in the breastfed infant's gut and more applicably to improve infant feeding alternatives. Therefore, a recent research focus was the bacterial acquisition of carbon in the infant gut. Human milk oligosaccharides (HMOs), a dominant carbon source in HM, are selectively utilized by *Bifidobacterium* spp. and are therefore promoting *Bifidobacterium* spp. in the infant gut environment [8-10]. HMOs are not the only nutrient class in HM and the bacteria have likely adapted to other molecules and nutrients present. Moreover, nitrogen is a fundamental need for bacteria to survive in any environment. This suggests that implementing complex oligosaccharides in food alone will not lead to a complete resemblance of a breastfed microbiota [5, 11].

An equally crucial pinnacle of survival for bacteria in the infant gut is not well documented, namely the acquisition of nitrogen. Meanwhile, it is known that microbial communities are profoundly affected by nitrogen availability [12, 13]. Breast milk is a complex bio-fluid and holds an orchestrated nitrogen content. While containing relatively low amounts of protein compared to the diet in later life, it holds various other sources of nitrogen including waste products of human metabolism, amino acids (AAs), polyamines and vitamins [6, 14-22]. The evolution of HM and a co-evolving microbiome make it likely that the microbiota of the breastfed infant is adapted to low protein concentrations and to utilize this specific nitrogen supply and by these means gains a competitive advantage. The rapidly developing human infant is in high demand of nitrogen as well, sketching a vital and delicate balance between host and gut microbiota.

Many gut symbionts can incorporate ammonium (NH_4) since most gut bacteria possess the capability to produce a glutamate dehydrogenase and use ammonium in their biosynthetic pathways. Not surprisingly, this pathway has proven to be one of the most dominant microbial pathways expressed in the infant gut microbiome [2]. This is furthermore important as detoxification, since high levels of ammonium can be detrimental to both microbiota and host [23]. It can therefore be hypothesized that this is the most common source of nitrogen available in the gut and that microbial metabolism centres around ammonium. Ammonium itself is interestingly enough only present in trace amounts compared to other HMN sources [24]. We suspect that the infant gut microbiota catabolizes other nitrogenous compounds to acquire and share ammonium.

This review is focused on microbiome interactions with dietary nitrogen in the infant gut, during the first months of life, in particularly with HMN sources. Since HM provides a tailored nutrient input, hypotheses can be formed on how nitrogen is salvaged by the microbiome. A surge in expressional and metaproteomic studies provides information on which bacteria are actively metabolizing in the infant gut. This data contributes to our understanding which bacterial metabolic pathways are active in early life. Especially since, a functional characterization of nitrogen metabolism is often lacking, while its importance for understanding the gut microbiota has been suggested [25]. By linking the bacterial genomes, microbiomes *in vitro* findings of active symbionts to dietary input, new avenues for scientific experimentation can be found. Bacterial auxotrophy will be discussed and potential metabolic networks for nitrogen will be described. These nitrogen sources include the mother's secretory metabolites, free AAs, polyamines, nitrate and nitric oxide (NO) and nitrogen from HMOs which are all featured in HM.

Sizeable efforts have been contributed to describing the composition of the infants' gut microbiota through 16S rRNA sequencing [2, 5, 26] and later through functional profiling with metagenomics, metaproteomics and metatranscriptomics [2, 27-30]. Potential functions and their relative occurrence have led to theories on what the microbiota is doing in early life. Furthermore, descriptive studies of infant gut bacteria are included to study the potential of interacting with HMN sources. Here, we reviewed these different types of studies. Combined, they provide information on the bacterial genera *Bifidobacterium*, *Enterococcus*, *Escherichia*, *Bacteroides, Enterobacter* and *Streptococcus* on their interactions with HMN during the first 6 months.

BREAST MILK DERIVED SECRETORY NITROGEN-CONTAINING METABOLITES

Secretory products, such as urea, creatinine and creatine, deriving from the mother's metabolism end up in the breast milk [13, 14, 17]. Currently there is no known biological reason for the occurrence of urea, creatinine and creatine in HM. However, gene content related to nitrogen metabolism of the infant gut microbiota has been shown to be responsive to diet [25, 31]. If a supportive microbiome is dependent on these compounds through breast milk, related nitrogen metabolism could prove crucial in understanding the settlement of the microbiome. Especially, considering the sensitive development of the gut microbiota during infancy.

THE POTENTIAL ROLE OF UREA IN THE EARLY LIFE MICROBIOTA

Interestingly, urea is the most abundant non-protein nitrogen compound in HM. Urea constitutes up to 15% of total nitrogen in HM [15, 18, 22, 32]. The human body is incapable of degrading urea through endogenous enzyme production. Urea is a product from human liver metabolism that constitutes of two amine groups connect by a carbonyl group and is for the largest part secreted through urine production. Bacterial Urea Nitrogen Salvation (UNS) has often been suggested as important for infant gut nitrogen cycling [15, 31, 33, 34]. Urease (EC3.5.1.5) is the first bacterial enzyme ever described [35]. It is widely spread across the bacterial kingdom and is especially known as a virulence factor for human pathogens. Regulation and activation of this enzyme has been studied in those pathogens. For example, nickel chelation is required for enzyme activation when studying urease activity in pathogen model systems like *Helicobacter pylori* and *Klebsiella aerogenes* [36, 37]. On the contrary, urease has been indicated as a health-associated factor [38]. As the input metabolite, urea can derive from bacterial metabolism due to bacterial arginase activity [39]. In *Bifidobacterium infantis* (*B. infantis*) it seems that urease activity is limited to the presence of urea [31]. Urease is involved in microbial amino acid metabolism, yet has also been indicated as a pH regulator as part of an acid response, for intestinal bacteria [40-42].

Bacteria removing urea from the infant gut can be crucial to infant health since liver and kidney systems are underdeveloped compared to adult life [43, 44]. The health of the human host is determinant of the urea availability in the gut and that subsequently increases urease activity [38, 45, 46]. Clearly, Urea will be available to the infant gut colonizers during early life, due to it being multi-sourced. Besides originating in breast milk, it is likely to also be secreted into the gut lumen by the neonatal host. Notably, in the early days of infant gut studies, it was shown that the urea faecal output is lower than the estimated input through breastfeeding, indicating urea processing in

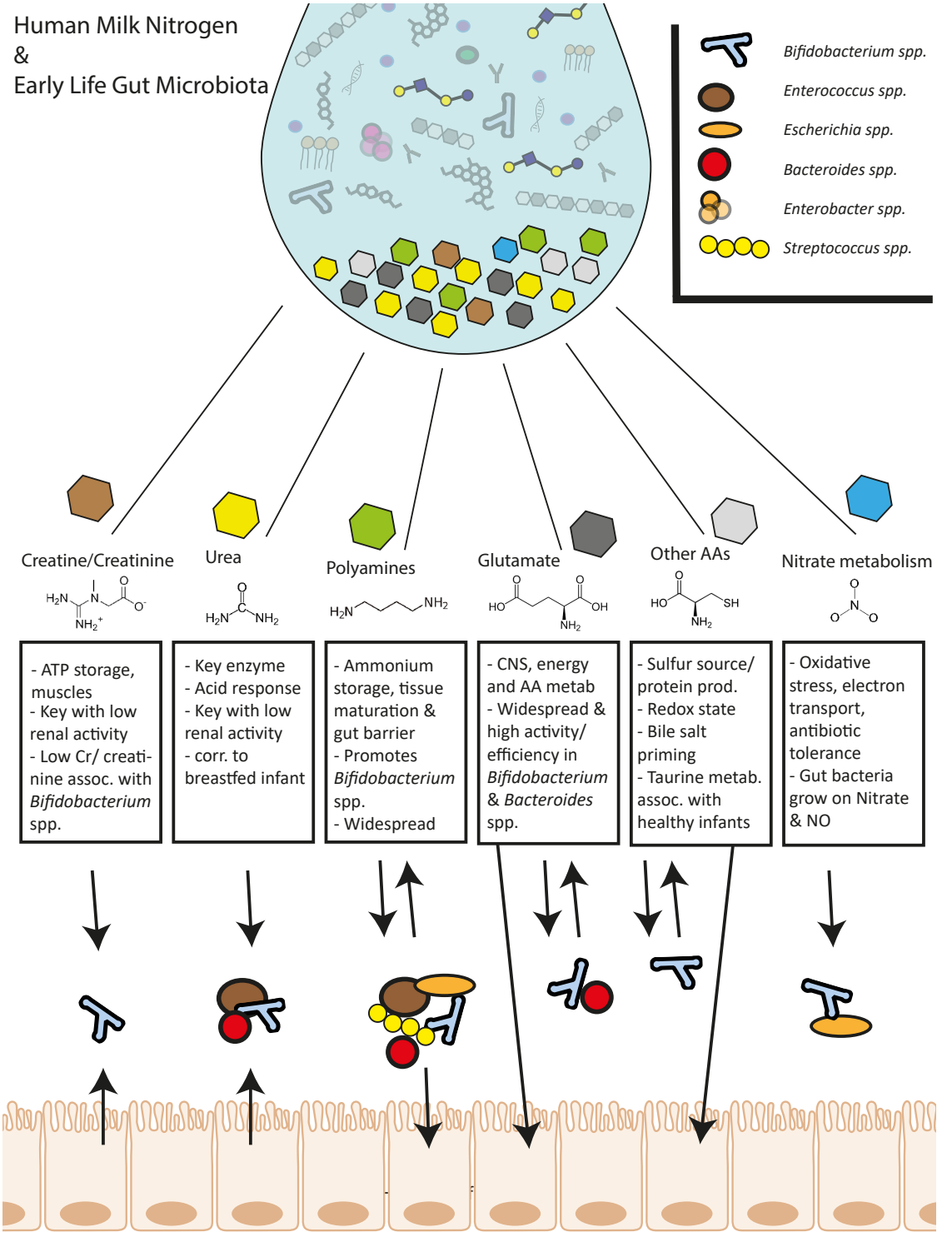

Figure 1. A representation of the important bacterial processes surrounding human milk nitrogen (HMN) and which bacterial genera are involved, concerning 6 nitrogen sources: Urea, Creatine (Cr) / Creatinine, Polyamines, Glutamate/Glutamine, Other Amino Acids (AAs), Nitrate. Abbreviations: assoc. = associated; corr. = correlated; metab. = metabolism; prod. = production; antib. = antibiotic.

the gut. Furthermore, 15N isotope studies in humans showed assimilation of urea into AAs [47, 48]. Interestingly, microbial nickel transporters were found to be abundant during early life [30], which are required as a cofactor for urease enzyme activation. Furthermore, there is evidence that the potential of urease activity relates to the microbiome of breastfed infants. As such, urease genes and the enzymatic activity are more abundant in exclusively breastfed infants [5, 32].

Interestingly, urease activity is linked to amino acid metabolism and thus the acquisition of nitrogen for synthesis in bacterial gut species. Specifically, urease activity was shown in *Streptoccus thermophilus* to be associated with amino acid synthesis and cell growth [49, 50]. Furthermore, *Bifidobacterium* spp. have shown to be urease active [31, 51]. However, it is also clear that not all *Bifidobacterium* (spp.) are able to access urea as nitrogen source. Urease-active bifidobacteria might however prevent pathogens from utilizing this urea. The opportunistic pathogen group of *Enterococci* spp. have been associated with luminal urease activity for example [52]. Notably, *Enterococci* spp. colonization of the infant gut negatively correlates with infant health status [29]. *Enterobacteriaceae* is an interesting genus when considering urease activity [53], as well as *Escherichia* spp. that occur in the early life human gut [54, 55]. Finally, *Bacteroides* can be the genus that profits from urea in mature HM. For example, *Bacteroides koreensis sp. nov*. and *Bacteroides kribbi sp. nov.*, two new members of the genus *Bacteroides* [56]. Importantly, urease activity might be a way to survive in an acidic environment when fatty acid production is high. How nitrogen is further cycled by bacteria is still unclear, although the resulting ammonium should be a suitable nitrogen source for many other microbes in the environment. Thus, both beneficial bacteria and potentially infectious or harmful bacteria are potentially competing over breast milk urea. This competition over urea with commensals prevailing might be crucial for a healthy microbiome.

THE POTENTIAL ROLE OF CREATININE IN THE EARLY LIFE MICROBIOTA

Both creatine and creatinine are transported into HM. Measured creatinine concentrations in HM vary greatly, but it was early established in the ±41-65µM range [57]. Infant formula often contains higher levels of creatinine, due to its origins in cow's milk [57, 58]. Thus, just like urea, creatinine origins in both breast milk and the human host as a secretory product of muscle use and repair [59, 60]. It is stored as phosphocreatine locally, to provide plenty of phosphate for human energy metabolism and specifically ATP [61]. It is most abundant in skeletal muscle as a reservoir for activity outbursts by the host. During muscle metabolism creatine turns into creatinine in an endogenously irreversible reaction. This creatinine will then be secreted from the

host and functions as a biomarker in urine and blood. Clearing breast milk creatinine by the early life gut microbiome could impact neonatal gut health.

During life, creatinine will likely be available to the gut microbiome, especially during periods of low kidney functioning. Just as during early life, when kidney and other secretive functions of the human body are hindered or still in development [43, 62-65]. However, it is unclear how much creatinine is secreted in the infant gut by the host. Creatinine degradation occurs through bacterial creatininase activity wherever creatinine is available [59, 66, 67]. This occurs via three routes 1) a combined effort of creatinine iminohydrolase (creatinine deaminase; EC 3.5.4.21) and a cytosine aminohydrolase (cytosine deaminase; EC 3.5.4.1); 2) creatinine amidohydrolase (creatininase; EC 3.5.2.10); 3) and finally a less characterized route via creatol and mehylguanadine [59]. A major product of creatinine degradation via creatinine deaminase is 1-methylhydantoin, an intrinsic hydroxyl radical scavenger or antioxidant [68, 69]. The use of ^{13}-C-labelling to prove that creatinine is oxidized by mammals into creatol and 5-hydroxy-1-methylhydantoin [69]. Specific genes involved in this metabolism are hardly characterized in common infant gut symbionts. However, the infant gut microbiota gets a chance of interacting with this nitrogen source in early life.

For every HMN source, we studied its potential in selecting for a health-promoting microbiota. Creatinine is negatively correlated with abundance of *Bifidobacterium* during the first month of life, indicating a role for the genus in a detoxing effect [70]. This was confirmed when applying a mixture of *Bifidobacterium* spp. and *Lactobacillus* spp. led to lower creatinine levels in broiler chickens, although the opposite has also been shown [71, 72]. Therefore, the fate of creatinine in infant gut nitrogen cycling remains elusive. Perhaps it diffuses into the intestinal tract, to be degraded by the gut microbiome or to be secreted through urine. In the gut, through bacterial metabolism more creatine can become available for the bacteria, so creatine should be considered a potentially important part of HMN.

Creatine (Cr) is provided by HM in concentrations around 77µM, while others established it at slightly lower concentration [57, 73]. In HM there was no observed difference between Cr concentrations at 1-2 weeks postpartum and 5-6 weeks. Interestingly, formula seems to hold higher concentrations of creatine (>4x), while others are almost derived of it [73]. The milk source of these formulae is explaining these levels, with for example cow's milk being rich in Cr. Guanidinoacetate (GAA) is the precursor of Cr in the human body. Edison *et al.* 2013 [73] established however that HM is not a

clear route of supplementing infants with GAA. Interestingly, calculated from intake and an estimated size of the Cr pool in infants, 90% of creatine should derive from endogenous production.

Creatine functions in bioenergetics for neurological and muscle cells by maintaining ATP levels [59, 74]. A Cr pool exists in the human body to maintain homeostasis in this concern, together with creatine phosphate can accumulate to approximately 100g [57, 59]. As mentioned, creatine is linked to the availability of creatinine as well. Creatine kinase (EC 2.3.7.2) transform Cr into phosphocreatine, a crucial step in human body energy homeostasis. Creatine kinase is traditionally an indicator of health [75] and that might be impacted by bacterial Cr metabolism in the neonatal gut. Furthermore, there are a few indications that high Cr is associated with infants fed formula [76, 77]. Bacteria that are capable of degrading Cr in the human gut have been identified [78]. First indications of association of creatine with microbiome composition also exist [79]. It was shown that Cr negatively correlates with *Bifidobacterium* spp. and positively correlates with *Klebsiella* spp., further indicating the potential for a microbiome dominated by bifidobacteria to have a higher potential for clearing Cr [80].

Furthermore, probiotic supplementation of *Bifidobacterium lactis* has been shown to alter Cr amounts in rats indicating an improved energy metabolism for the host [81]. Finally, in the aging mice gut, Cr availability led to an increase in creatine degradation occurrence in the functional profile of the gut [82].

POLYAMINES AND THEIR IMPACT ON THE INFANT GUT MICROBIOTA

Polyamines (PAs) are, under physiological pH, polycationic substances, rich in nitrogen that function in human cell growth and tissue maturation. These biogenic amines can therefore be in high demand for rapidly growing tissues, like the developing gastrointestinal tract of the newborn [21, 83, 84]. Moreover, PAs are suggested to be involved in immune system maturation, functioning and modulation of gut permeability [85-88]. In HM occurring PAs are spermine, spermidine, putrescine and cadaverine all have two or more amino groups [89]. PAs can derive from either host and bacterial metabolism or diet. The fact that PAs are rich in nitrogen makes it an interesting nutrient source for the colonizing microbiome. In HM, polyamine concentrations increase postpartum [19, 20, 90] with concentrations collapsing from the second month onwards [19, 21, 91]. Gòmez-Gallego *et al.* investigated polyamine concentrations in HM of healthy mothers. The most abundant was spermine (6.1μM), followed by spermidine (4.2μM) and finally, in lesser amounts, putrescine (0.7μM),

with slight variation due to geographic locations in spermidine and putrescine. It was however, established early that concentrations vary greatly between mothers [19]. Buts *et al*. (1995) established a similar polyamine profile in HM samples (spermine, 3.1μM; spermidine, 2.2μM ; and putrescine, 0.24μM) with a total polyamine concentration of 5.57μM (+/- 0.18μM) [92].

According to current knowledge, most PAs are however adsorbed in the upper intestine in support of growth processes of the human body [93]. In the infant GI-tract however, optimal adsorption might be developing in combination with a large supply through HM, causing polyamines to reach the colon. PAs occur in the colon of healthy adults within the range of 0.5 to 1mM [94]. There, PAs are adsorbed into the human body through the colonic mucosa [95]. In the colon, bacteria are suggested to be largely responsible for the PAs present there. Bacteria can produce a wide range of PAs, including spermidine, homospermidine, sym-homospermidine, norspermidine, putrescine, cadaverine and 1,3-diaminopropane, while there are also bacteria that are not able to produce any [96, 97].

Synthesis of polyamines is mainly regulated and activated by ornithine carboxylases [98]. These carboxylases catalyse the de-carboxylation of ornithine to produce putrescine. Followed by several methods of elongation, for example spermidine synthase (EC 2.5.1.16), while in the meantime adding one or two more amine groups originating from other nitrogenous compounds (Figure 1). For a long time, biosynthesis of polyamines was mainly studied in *Escherichia Coli (E. coli)* of which related spp. also occur in the infant gut, albeit as a minor component [26]. More common microbiome members in the first two months of life, e.g. *Streptococcus* spp. and *Enterococcus* spp. have been shown to produce polyamines from AAs [99]. This enzyme is broadly studied in *Bacteroides* spp. [100, 101]. More recently, activity of a carboxyspermidine decarboxylase (*casdc*) was described in *Bacteroides thetaiotaomicron*, a representative of a genus highly present in many life stages, including infancy. This *Bacteroides* strain produces the polyamine spermidine in a polyamine free medium and moreover this that activity provided a growth benefit for the strain under these conditions [102]. This provides an indication of polyamine metabolism potentially selecting for bacteria in a complex microbial environment. In HM however, the longer polyamines are more dominant than its precursors (Figure 1).

Polyamines might also provide a health benefit through gut microbiome modulation and first evidence has been provided [103]. In mice, supplementation of PAs in formula led to a significant increase of *Bifidobacterium* spp., *Bacteroides* spp., *Clostridium* spp.

and *Verrucomicrobia* spp. (as "Akkermansia- like bacteria") in the large intestine. *Bifidobacterium* spp. were even higher than breastfed pup-mice. Interestingly, polyamine supplementation led to promotion of autophagy in human cell lines, an indicator of gut health which inhibited propagation of SARS-CoV-2 [104]. Since autophagy plays a role in gut barrier maintenance, polyamine as a part of our diet can have a health impact [105]. There is no current evidence of extracellular degradation of polyamines by gut bacteria. Within the *Bifidobacterium* genus the capability to synthesize and transport polyamines to the intracellular environment has been reported [106].

With spermidine *in vitro*, spermidine is absorbed by the bacteria and in some cases is processed (*Bifidobacterium scardovii*) [106]. No known homologs were detected, indicating a possibility for novel methods of polyamine processing in *Bifidobacterium* spp. Spermine, the longest naturally occurring polyamine in HM, was taken up by more *Bifidobacterium* strains in the study by Sugiyama *et al.* [106]. Again, the system of choice for this type of transport, a *potRABCD* transport system, was not found in the genus [106, 107]. Moreover, extracellular concentrations of spermine were increasing during growth phase of, amongst others, *B. infantis*. Also, *Bifidobacteirum adolescentis* (*B. adolescentis*) was proven to export spermidine into the supernatant *in vitro* [99]. The production of putrescine from ornithine seems to be prevalent in many species of the *Bacteroides* genus. For example, *Bacteroides fragilis* has been noted as possessing this capability with the goal to produce y-aminobutyric acid (GABA) [108, 109]. Most studies have shown that culture conditions tend to be highly specific for strains, leading to low replicability. However, this is a promising mechanism by which the infant gut microbiota promotes health during early life. Especially, since it is known that polyamine supplementation in formula counteracts allergy occurrence and gut permeability issues [88]. Bacterial metabolism concerning polyamines has hardly been considered. According to literature reviewed here, polyamines are bifidogenic. It makes this part of HMN interesting for future study.

AA METABOLISM & THE GABA SHUNT

Both bacterial protein fermentation and human digestion can lead to the liberation of amino acids (AAs). Before that, proteases produced by the mother digest human milk protein [110]. This review highlights that the commonly released AAs are at the core of bacterial metabolism and their survival [111]. Where there are AAs, bacteria multiply. This review proposes that the infant gut is not any different. If the first meals to pass through the newborn's GI-tract are developed to kick-start bacterial metabolism, AAs might be key.

The role of Glutamate and glutamine for the infant gut microbiota

The amino acid glutamate, however nonessential, plays a role in many important metabolic processes, including the citric acid cycle, protein synthesis and acts furthermore as a precursor for several bioactive compounds [112, 113]. Glutamate is the most dominantly occurring free amino acid in HM only closed by its close relative glutamine. Glutamate as a dominant dispensable free amino acid occurs in the range of 960.1-1529.0μM, reaching peak supply at 4 months into the lactational period. However a higher concentration of 4.5mM has been argued [6, 14, 114, 115]. Free glutamate is during this period proportional to the amount of glutamate supplied through protein, in which glutamate is also the most dominant AA [116]. This level of free glutamate is significantly higher than the level found in the average cow's milk-based formula [115, 117]. Interestingly, it is also higher than the 30 mg/kg bw per day acceptable daily intake (ADI) set by the European Food Safety Authority for infants, due to neurotoxicity concerns. Breastfeeding being the infant feeding method of choice makes it likely potential health benefits underly the presence of these levels of glutamate. Bacterial interaction in the infant gut with glutamate is likely a very prominent part of infant gut nitrogen cycling.

Glutamate catabolism is achieved through glutamate dehydrogenase (GDH) or glutamate decarboxylase (GAD). The GDH enzyme leads to the assimilation of ammonia into AAs, with glutamate as the starting point. Glutamine synthetase (GS) catalyses the reaction of glutamate to glutamine [112, 118]. Glutamate metabolism can be part of bacterial stress responses, including acid responses [113]. Glutamate decarboxylase is involved in acid stress response, causing decarboxylation of glutamate to γ-aminobutyrate (GABA). GABA is the main inhibitory neurotransmitter, while glutamate, its precursor is the main excitatory neurotransmitter [119, 120]. Bacteria can then export it through a GadT2 Glutamate/GABA antiporter, after which is absorbed into the human body, providing benefits for health and development in the nervous system [121-123]. Notably, the human body can convert glutamate through GAD-activity, hence the high demand of glutamate for the infant body and its developing nervous system. Interestingly, a ferredoxin dependency occurs in the GABA-shunt via dependency of the glutamate synthase [124]. This enzyme catalyses the production of glutamate from 2-oxoglutarate and glutamine as nitrogen source. However, Ferredoxin metabolism is not commonly studied in the infant gut. Physiologically, an external supply of glutamate could replace a need for endemic production of this amino acid. In the developing human body glutamate serves a wide array of purposes in neurology and energy homeostasis. Bacteria-wise, a metaproteomics study by Xiong *et al.* [27]

showed that among conserved functional groups was glutamate dehydrogenase, in all studied infants. The importance of glutamate is furthermore indicated by the fact that it is almost completely metabolized first pass in infant pigs. Such a scenario is likely in the human infant gut as well [125].

There is evidence for glutamate metabolism by bifidobacteria [126]. All included *Bifidobacterium* strains (*Bifidobacterium breve*, *Bifidobacterium longum* subspp, *Bifidobacterium pseudolongum*, *B. adolescentis*) showed both synthethase and dehydrogenase activity for all, although at different levels [126]. Interestingly, lower Km values for the glutamate synthetase of *Bifidobacterium* spp. compared to almost every other included strain except a *Lactobacillus* sp., indicating that *Bifidobacterium* is an efficient genus in detoxifying the infant gut from ammonia. Indicating a key role for *Bifidobacterium* spp. in glutamate cycling, the purified GS of *B. bifidum* reacted optimally in an acidic environment, which was not the case for the non-*Bifidobacterium* spp. [127]. This would match the infant gut's acidic conditions. Glutamine and glutamate cycling by *Enterococcus* and *Streptococcus* spp. has been studied, but in the small intestine of ruminants or in the bird gut [128, 129]. Finally, the production of GABA from glutamate as an important metabolic route should be investigated. *E. coli* was one of the organisms in which it was shown that GABA metabolism conferred acid resistance in bacteria [130]. Recently, the production of GABA has been contributed to more common infant gut symbionts like *Bacteroides* spp. [109].

Other predominant amino acids in human milk

Taurine or aminoethylsulfonate (a C2 sulfonate) is a sulfur-containing amino acid in HM that the human body extracts from a solid diet. Taurine is the third most dominant free amino acid in HM [16, 17, 131, 132]. Moreover, the human body can synthesize taurine but not degrade it, making plenty of the amino acid available for microorganisms as well. A large portion of taurine is secreted from the human body in the form of taurine-conjugated bile salts [133]. This requires the release of taurine by bile salt hydrolases by bacteria. One of those genera is *Bacteroides* [134, 135]. Fermentation of taurine is dependent on the cleavage of the inert sulfonate C-S bond [136]. A process not commonly described for anaerobic human gut bacteria. Even though it is closely linked to the production of H_2S, toxic to the human body. Moreover when it does occur, like *e.g* in *E. coli*, it concerns a process involved in aerobic growth [137, 138]. There is a diverse range of strategies for cleaving the C-S bond, yet not all are anaerobically feasible. Among the known pathways is a thiamine pyrophosphate dependent Sulfoacetyldehyde acetyltransferase system (Xsc) which

occurs under both aerobic and anaerobic conditions [136, 139]. Further anoxic options are still poorly understood. One of the more remarkable is the description of an IseG-dependent system, where also L-alanine can be produced, in opportunistic pathogen *Bilophila wadsworthia* and *Desulfovibrio piger* [139]. Collard *et al.* [140] went further and described taurine and its related physiological processes as key contribution to resistance to new infections. In another study taurine alone did not alter immune responses in the lamina propria [141]. However, it did affect gene expression in epithelial cells and more interestingly it showed the importance of a taurine trained microbiota. That led to a greater systemic resistance against *Klebsiella pneumoniae* and lower oxygen availability [141]. The relation to oxygen makes it so that the lack of a taurine-trained microbiota could lead to more potential for opportunists to settle, like *Enterococcus* spp., a common paediatric infection source in the neonatal period. Targeting of taurine utilizers can benefit options for infant health care and nutrition. In another study, a healthy infant control group showed significant higher levels of taurine metabolism through a metagenomic approach, confirming a role in the healthy infant's metabolism [142-144].

The biosynthesis of the amino acid cysteine serves to incorporate inorganic sulfur into organic matter [145]. Cysteine then plays a crucial role in many catalytic sites of subsequent protein and protein folding by being part of disulfide bonds [146]. Interestingly, bifidobacteria as dominant colonizers of the of the gut seem to be auxotrophic for the amino acid [147]. *B. bifidum* (multiple strains) has shown reduced growth only when cysteine is lacking in an evaluation of auxotrophy across AAs occurring in HM [147]. Cysteine metabolism has also been indicated as a key metabolite that inhibits gut related oxidative stress [148, 149]. Conclusively, both these AAs need to be considered in relation to infant feeding and the infant gut microbiota (Figure 1).

BREAST MILK DERIVED NITRIC OXIDE, NITRATE & NITRITE

Nitric oxide (NO) is involved in physiological processes in the gut that can determine an individual's health [25, 150, 151]. Nitric oxide is synthesized from L-arginine by a nitric oxide synthase (NOS), and is involved in vasodilation, neurotransmission, the immune system, gene ex-pression and regulation. Interestingly, NO is involved in interactions between bacterium and host. Furthermore it seems to be important for the oxidant and antioxidant status of human breast milk during lactation period [152]. In the infant gut NO synthesis by bacteria, which handles oxidative stress, electron transport or antibiotic tolerance, can potentially be harmful as well [153]. Notably, it is

involved in triggering of lactation [154]. Meanwhile, HM NO concentrations peak in the first week postpartum [155]. NO might function as a antimicrobial metabolite in HM, on that is lacking from formula products [156].

In healthy adults 1/3 of dietary nitrate ends up in the lower intestine, but only up to 1% ends up in the faeces [157]. Nitrate can provide a growth advantage for strains belonging to the genera *Escherichia*, *Bifidobacterium* spp. and *Lactobacillus* spp. under anaerobic conditions and low oxygen conditions (Figure 1) [158]. Furthermore, nitrate is an electron acceptor when oxygen is limiting, a realistic scenario in the infant gut a few weeks postpartum [158-161]. *E. coli* even possesses three nitrate reductase that are active under anaerobic conditions. [158, 162]. Nitrite can be toxic at higher concentrations and is therefore excreted to the environment. Interestingly, a study by Tiso & Schechter (2015) showed that *in vitro* culturing of infant gut symbionts producing large amounts of fatty acids and the subsequent acidification drives nitrite disproportionation to NO [158]. An environment dominated by *Bifidobacterium* spp. could theoretically produce levels of NO that affect gut health and integrity. However, to our current knowledge, *Bifidobacterium* spp. do not possess enzymes to do so.

THE ROLE OF NITROGEN FROM HUMAN MILK OLIGOSACCHARIDES AND OTHER GLYCOCONJUGATES FOR THE INFANT GUT MICROBIOME

Human milk oligosaccharides (HMOs) are indigestible carbohydrate structures in amount and complexity unique to the milk composition of us humans. HMOs are the third most dominant carbohydrate source (after lactose and fat) available to the microbiome occurring as high as 15g/L [163, 164]. Especially so, since the infant is incapable of degrading these complex polysaccharides and only small amounts will be absorbed intact and thus they will reach the colon largely unscathed [165, 166]. So far proven prebiotics such as GOS and FOS have been used in infant nutrition. In recent years, the first synthetic HMOS structures are available. The oligosaccharides are the main prebiotics used to enforce formula products, so that they better promote *Bifidobacterium* spp. [10, 166-168]. HMOs, besides being a major carbon source for the infant gut microbiota, were shown as a nitrogen for those same bacteria [169]. The simplest HMOS are derivatised lactoses such as galactosyllactoses and fucosyl and sialyllactoses. The usual composition of detected HMOs follow the formula Lx/y-z (with L:lactose; x:Gal-GlcNac disaccharide units; y:fucoses and z:sialic acids) [170]. Nitrogen is provided via (*N*-Acetylglucosamine) GlcNac and sialic acid [171, 172]. Neutral HMOs following this formula were detected between 8 kDa and 10 kDa which means that the majority of HMO-structures contains nitrogen with GlcNAc.

It was also determined that acidic HMOS of 3,5 kDa and higher provide additional nitrogen from sialic acids beyond the core GlcNAc nitrogens [173, 174]. On a different resolution, the HMOs lacto-N-tetraose (LNT) and lacto-N- neotetraose (LNnT) contain this nitrogen, which are respectively featured in Type I and Type II HMOs [163]. For some bacterial species GlcNac is even a strict requirement [175]. GlcNAc nitrogen is likely utilizable by more infant gut colonizers in the early stages of infancy. N-Acetylneuraminic acid (Neu5Ac) nitrogen can also prove to be a key nitrogen source as it's the predominant sialic acid in HM [171, 176]. Furthermore, HM is not only rich in HMOS glycoconjugates,since other components in HM are rich in glycosylation (partly highly individual features) which exhibits certain biological functions, digestive survival and offers furthermore like the indigenous mucins of the host to function as substrate for saccharolytic bacterias. In particular glycolipids and glycoproteins need to be considered [177-179].

Several bacteria thriving in the breastfed infant's gut have been shown to degrade HMOs or parts of them. This activity is thus also potentially liberating some nitrogen sources, although that specific focus is rare. They are specifically interesting because there are strong indications that HMOs reach the infant gut largely undigested. Upper small intestinal enzymes do not have a significant impact on the HMO structure [165]. James *et al.* [163] established that *Bifidobacterium breve* (*B. breve*), among many *Bifidobacterium* spp. holds the capability to degrade LNT, LNnT via different pathways. HMO utilization by *Enterobacteriaceae* has also been studied [180]. The study showed that none of the Enterobacteriaceae strains grow on 6-siallylactose (6-SL), and LNnT. Although the importance of LNnT for bacteria is apparent, the role and effect of the included nitrogen on bacterial metabolism is clearly under established. Meanwhile, there are several bacterial species from the genus *Bacteroides* and probiotic *A. muciniphila* that have been shown to interact with LNT and LnNT [181, 182]. Since these bacteria specifically are promoted by breastfeeding, the relationship between the unique structure of HMOs and their prevalence is confirmed. The obvious suggestion is that HMOs are a preferred substrate with for *Bifidobacterium* spp., meanwhile making the nutrients and the nitrogen less available for less beneficial bacteria. The bonus of having almost exclusive access to nitrogen embedded in HMOs should not be underestimated for bacteria in an increasingly competitive infant gut.

DISCUSSION

Although it's early on, plenty of evidence has been displayed here showing that from the early onset of life, the gut microbiota is involved in catabolic and synthetic activities involving HMN. The neonatal microbiota is highly susceptible to outside influences, like the diet. This makes the early life gut microbiota a very suitable platform of study for the impact of nitrogen on the microbiota and on subsequent health. This review focused on the relationship between the settling infant gut microbiota and the non-protein part of HMN. Moreover, it is becoming clear that the human host stands to benefit from this early life bacterial nitrogen cycling. We can also conclude that more data is needed, quantitatively and qualitatively, *in vivo* and *in vitro*, on an -omics scale, yet also an *in vitro* approach with early life microbiota members. Metabolomic, (meta-)proteomic and metagenomic data from clinical studies can help elucidate what the bacteria are doing in the infant gut, while breastfeeding ensues. For example, ever since the first attempt of Klaassens *et al.* [183] to use metaproteomics to functionally characterize the infant gut microbiota, meta-studies have become increasingly impactful in describing bacterial activity in the gut. On the other hand, studying how important (*Bifidobacterium*) species react *in vitro* to HMN can provide evidence as well [31, 181]. This review provides further evidence on the fact that many aspects of breastfeeding are tailored to suit the infant's and gut microbiota's early life needs. The many relations between HMN and bifidobacteria can explain why the genus is successful in the infant gut.

For some nitrogen sources, bacterial species seem to have somewhat exclusive access. While for others, like urea, many of the early life symbionts of the breastfed infant have the capability to process it. As the main non-protein nitrogen source in HM, this component might prove a key metabolite in establishing the early life microbiota. The knowledge of specific bacteria degrading, and processing creatinine, creatine, or polyamines is far more elusive. For example, for creatine and creatinine metabolism genes are hardly found or described in the common infant gut symbionts. Nonetheless both metabolites/nutrients are involved in host health and the impact of these nitrogen sources should be investigated more in depth to explain the role of HM. This will help develop formula products in such a way that they promote a health-inducing microbiota in a similar fashion. As has been hopefully indicated by this review, many of the described nitrogenous compounds can be the result of existing interspecies networks between common infant gut symbionts. Clinical studies specifically focused on dietary nitrogen and infant gut microbiota can therefore elucidate which processes

matter the most *in vivo*. In contract, future *in vitro* studies should determine if and under what conditions these bacteria produce or consume the HMN. Furthermore, focus should lie on determining if nitrogen cycling is part of metabolic interspecies networks, to what extent competition over HMN occures and if certain interactions exist with human cells in that environment.

There is more nitrogen in HM, besides the non-protein part there is of course protein. Lactoferrin (LF) is among the most detected proteins in the early life gut, indicating its availability to the microbiota and showing the potential for this (±700 AAs) protein to have a beneficial effect in lowering pathogen colonization [184, 185]. Products of the bacterial colonists of the infant gut could be crucial vitamins. Vitamins are at the centre of human health and are a product of our diet and bacterial metabolism [111, 186]. HMN could ensure that bacteria are producing vitamins at the right place and at the right time. Is that dependent on the right nitrogen source as input? In general, the HMN supply seems fit for promoting microbial growth and making sure the gut is colonized in the early life stages. This is accomplished by the presence of GABA-shunt metabolites. The role of these free AAs in HM has once again been confirmed. Namely, much of the early life gut symbionts seem to possess the potential to process glutamine and glutamate, the two main free AAs in HM. The free AAs seem to be there to promote general microbial growth when the gut is still relatively low on microbial mass. This nitrogen is perhaps cycled into vitamins and neurogenic compounds that can have a profound effect on the development of the infant.

Nitrogen input can steer a microbiome in a certain direction and affects the output towards a human host. For example, when looking at the expression levels of a *B. longum* strain in breast milk compared to glucose medium and formula, A nitrogen regulatory protein (N-II EP) e.g. is up-regulated [187]. Indicating underlying regulation and response to the presence of HMN. Determination of HMN concentrations that are suitable for infant formulae is difficult due to the lack of consensus of HM concentrations [21]. Concentrations of HMN sources described are largely dependent on time of day, lactation stage or type, maternal diet, among others [188, 189]. Clearly, the GABA shunt and urea provide a crucial research window in studying infant gut nitrogen cycling and its relationship to bacterial survival and health. The involved metabolites surround one of the most conserved and active metabolic pathways in the infant gut and its products are crucial for both bacterium and host.

CONCLUSIONS

We have only just started to unravel the digestive microbial processes in early life, that lead to a healthy infant gut. It is clear however there is much knowledge to be gained from studying the interaction between nitrogen and the settling infant gut microbiota. This review provides an overview on the current relationship between HMN and the most prevalent gut symbionts during early life.

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CONFLICTS OF INTEREST

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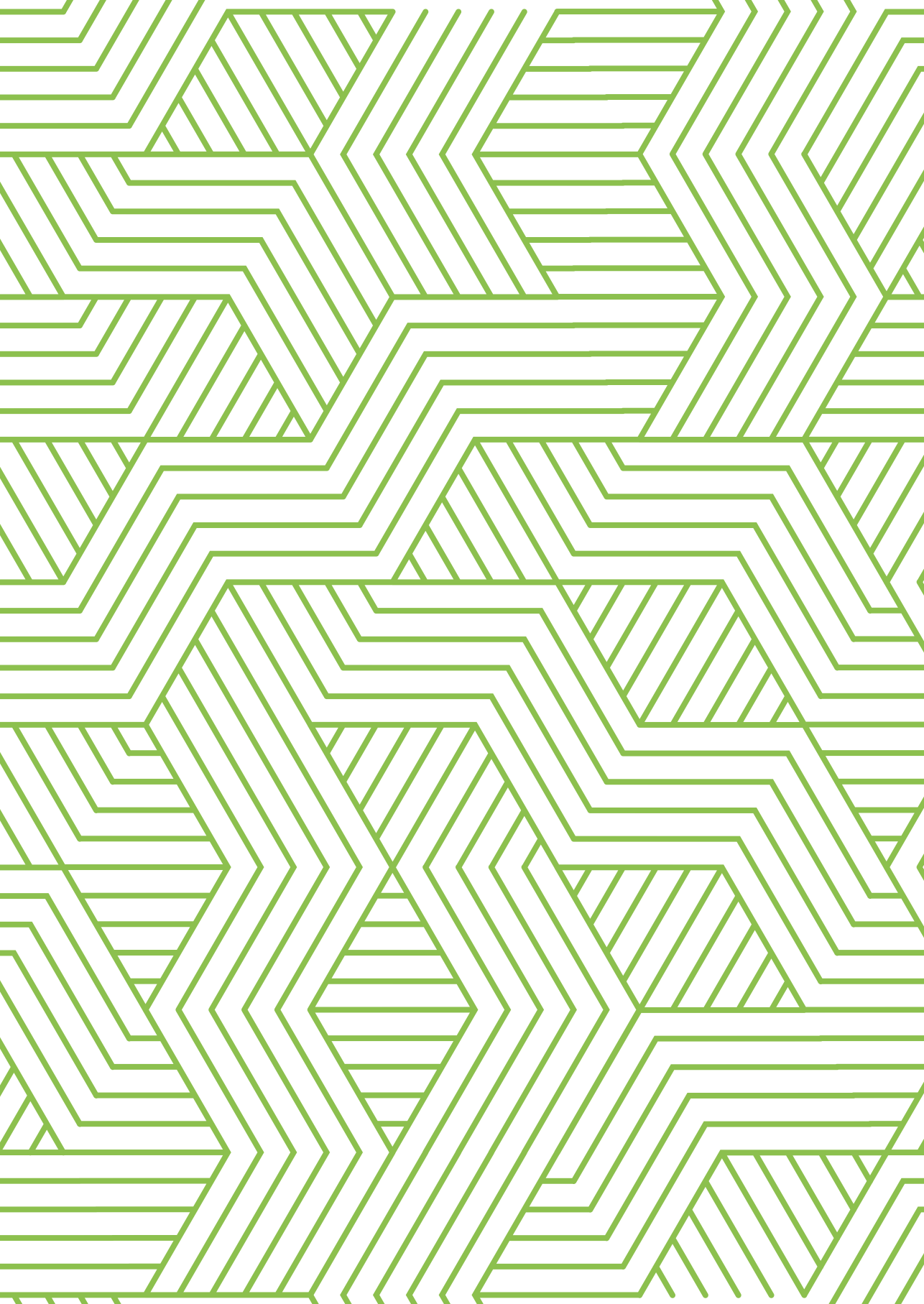

CHAPTER 4

PROOF OF PRINCIPLE STUDY REPLICATING MICROBIAL CLUSTERS IN CONNECTION TO BIRTH MODE AND DIET IN THE EARLY LIFE INTESTINE

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ABSTRACT

The human gut ecosystem starts developing at birth and is influenced by many factors during early life. In this study we make use of a Belgian cohort of 64 children, followed until the age of 6 years, to analyze different phases of microbiota development. We analyzed fecal samples taken before weaning (age 1 month), shortly after weaning (age 6 months), when milk feeding has been discontinued completely (age 1 year), and at the age of 6 years. We performed 16S rRNA gene amplicon sequencing on the collected fecal samples and analyzed the compositional data in relation to dietary metadata and birth mode. Human and formula milk feeding promotes a microbiota dominated by either *Bacteroides or Bifidobacterium,* respectively. Into later life stages, the microbiota composition follows distinct microbiota clusters, related to abundance dynamics of certain bacterial groups. Furthermore, it becomes apparent that a formula diet leads to early maturation of the infant gut microbiota. Despite other clinical variables within the infant cohort, they did not significantly contribute to the microbiota patterns we observed. Our data provide a proof of principle study of the importance of diet to the development of the microbiota in early life that replicates earlier findings in other cohorts.

Keywords: infant gut, microbiota, *Bifidobacterium*, *Bacteroides*, diet, birth mode, human milk

INTRODUCTION

The seeding and development of the human gut microbiota has been implicated to be involved in health outcomes during early and later life. Examples of diseases related to a disrupted microbiota homeostasis are asthma, allergy, eczema, obesity and inflammatory bowel disease [1-3]. Over recent years, there have been many reports on the microbiota of infants with the use of multiple methods and samples taken at a multitude of ages [4-11]. However, limited data is available on longitudinal cohort studies of the gut microbiota of children. The development of the gut ecosystem starts at birth when a child encounters the mothers' vaginal and faecal microbiota as it exits the birth canal [12]. In contrast, children born through caesarian section are colonized by bacteria they encounter from their surroundings, family and caretakers in the hospital environment, whereas the vaginally-born infants' gut microbiome resembles that of the mother's vagina [13]. Later in infancy the bacteria found in vaginally born infants were *Bacteroides* spp., *Bifidobacterium* spp., *Parabacteroides* spp. and *Escherichia/Shigella* spp. [14]. Bokulich *et al.* found that alpha diversity between birth modes started deviating greatly only from the age of 8 months, showing an enduring effect on the gut microbiota composition [15]. Initially, a diverse consortium of bacteria colonizes the infant gut, but only a part of the initial inoculum has the capacity to permanently inhabit its new environment. During the following period, facultative anaerobic bacteria from the genus *Enterobacteriaceae* [15], *Bacillus* [16] *Enterococcus, and Streptococcus* [14] are commonly detected in the infant gut. Bacteria detected then resemble those in colostrum [17]. Sometimes strictly anaerobic genera such as *Bacteroides* spp., *Veillonella* spp. and *Bifidobacterium* spp. already occur at the age of one week [18]. Once an anaerobic environment is ensured, the microbiota becomes more stable. This relative stability is maintained throughout milk feeding [14, 16, 19, 20]. Human milk and infant formula both contain oligosaccharides indigestible to humans. Gut bacteria specialized in utilizing these oligosaccharides as growth substrate therefore often become dominant in the infant gut microbiota. The most well established degraders of human milk oligosaccharides (HMOs) are members of the genus *Bifidobacterium.* Some bifidobacteria are highly specialized in metabolizing HMOs [21] and *Bifidobacterium* spp. are therefore often dominant in the infant microbiota [2, 14]. However, *Bacteroides* spp. have also been shown to degrade HMOs [22]. A few years ago, a *Bacteroides*-dominated infant microbiota was discovered [13, 20, 23]. The *Bacteroides*-dominated microbiota has been described in relation to birth mode. Vaginally born infants have been reported to have higher levels of *Bacteroides* spp., or C-section born infants have been described to be depleted of *Bacteroides* spp. [15, 20].

Vatanen *et al.* describe that the microbiota of children from Estonia and Finland is dominated by *Bacteroides* during infancy. This contrasts with the microbiota of Russian infants, which is first initially occupied by *Bifidobacterium* and only later transitions to *Bacteroides* becoming more prevalent [24]. Another example is breastfed infants having lower microbial diversity than formula fed infants [15] and their microbiota is often enriched in more typical glycan degraders such as *Bifidobacterium* spp. and *Lactobacillus* spp, whereas the formula fed microbiota was enriched in *Clostridia* spp. and *Enterobacteriaceae* spp. [14, 15]. Because human milk has such a profound effect on microbiota composition, a major shift in microbiota composition occurs when milk feeding is discontinued. Typical adult-associated bacteria like *Roseburia* spp., *Bilophila* spp., *Anaerostipes* spp. and *Clostridium* spp. increase significantly in abundance [14]. This indicates clusters of microbiota composition occurring in early life between infants with different lifestyles. This fragmented and diverse development of the early life gut microbiota is also studied here in a longitudinal cohort. In an approach to discover bacteria associated with aging of the gut microbiota, by far the most important species were *Faecalibacterium prausnitzii* and a species of *Ruminococcus*, which were positively correlated with age [25]. In terms of alpha and beta diversity parameters, it seems that the gut microbiota reaches an adult-like composition at the age of about 3-4 years [5].

We have access to a comprehensive sample collection of faecal samples from a Cohort of Belgian children, taken at crucial time-points (1 month, 1 year, 6 years) from groups of children with different diets. This provides a longitudinal cohort with some typical aspects of human gut microbiota development in early life. We investigated the effects of birth mode, feeding mode and duration on microbiota composition, with a focus on developmental dynamics and maturation. 16S rRNA amplicon sequencing was performed on collected fecal material and a bio-informatics analysis on the compositional data and metadata was conducted.

METHODS

CLINICAL TRIAL SETUP AND SAMPLES

The faecal samples analysed in this study originate from a trial (NTR3505) to investigate whether intervention with prebiotics during the first year of life has an impact on prevalence of atopic diseases at 6 years of age. The study was approved via the medical ethics committee of Wageningen University (METC-WU), and both parents gave written consent for inclusion. The study included 64 children (inclusion 2004-2006), at high risk for atopy (parental history), followed until the age of 6 years. It was performed in a randomized; controlled, double-blind parallel-group way. It was tested if the incidence of allergic disease can be reduced through the use of hydrolyzed infant formula with short-chain galacto- oligosaccharides and long-chain fructo-oligosaccharides (GOS/FOS) together with pectin-derived acidic oligosaccharides (pAOS). Infants either received a hypo-allergenic formula with or without a mixture of GOS/FOS and pAOS until 1 year of age. Mothers were encouraged to breastfeed as long as possible. Describing the infant gut microbiota was a part of the study design, however the specific bioinformatics methodology was considered post-hoc. Our data describes several lifestyle variables in relation to the gut microbiome and its development in this European infant cohort (Figure 1).

BACTERIAL 16S RRNA GENE AMPLICON SEQUENCING

Samples were prepared for Illumina Miseq sequencing using a two-step protocol to amplify the 16S rRNA and to barcode the samples. Bacterial 16S rRNA gene fragments were amplified by using universal primers covering the V3-V4 region of the bacterial 16S rRNA gene. The forward primer consisted of the S-D-Bact-0341-b-S-17 primer (5'-CCTACGGGNGGCWGCAG-3') [26] added to the 3' end of the Unitag1 barcoding adapter (5'-GAGCCGTAGCCAGTCTGC-3'). The reverse primer consisted of the S-D-Bact-0785-a-A-21 primer (5'-GACTACHVGGGTATCTAATCC-3') [26] added to the 3' end of the Unitag2 barcoding adapter (5'-GCCGTGACCGTGACATCG-3'). The PCR was performed in a volume of 50µL containing 10µl of 5× HF green buffer (Finnzymes, Vantaa, Finland), 1µL dNTP mix (Promega Benelux B.V., Leiden, The Netherlands), 0.5µL of Phusion Hot Start II DNA polymerase (2U/µl; Finnzymes), 2.5µL of the reverse primer mix and the forward primer (both 10µM), 1µL template, and 32.5µL nuclease free water. The PCR program was 98°C for 30 seconds to activate the enzyme, then 25 cycles of 98°C for 10 seconds, 56°C for 20 seconds, 72°C for 20 seconds, and then a final extension at 72°C for 10 minutes.

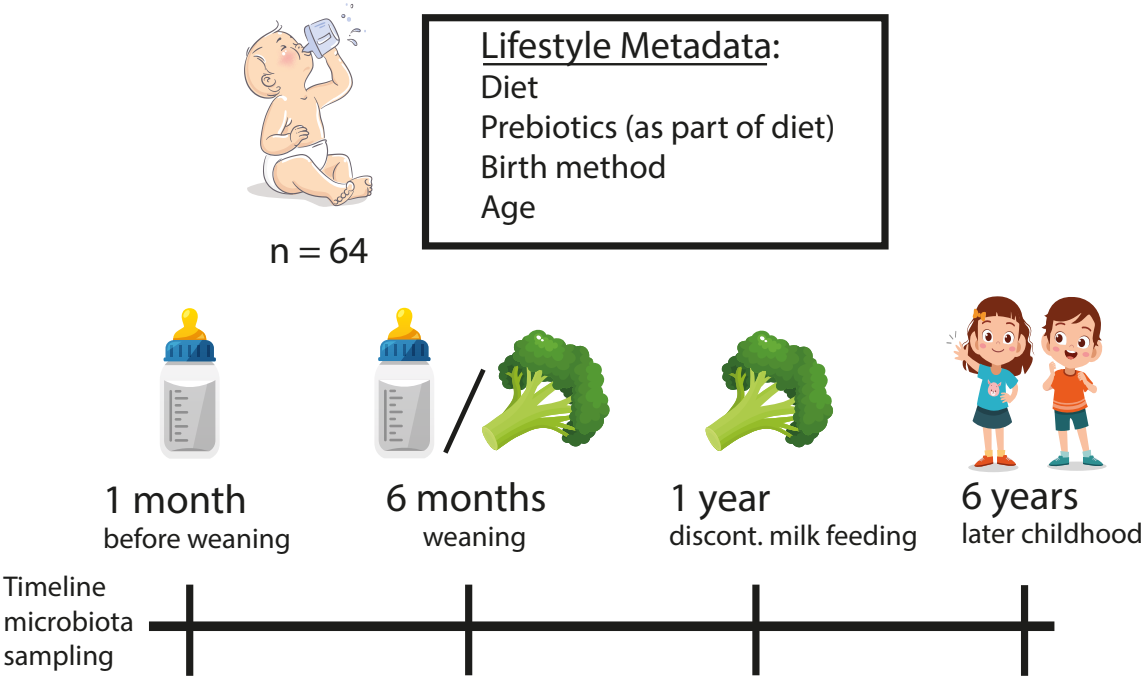

Figure 1. NTR3505 study setup & metadata (birth mode, feeding, age, prebiotic supplementation).

The PCR products were analysed on a 1.2% FlashGel (Lonza, Basel, Switzerland) to verify product formation. If a clear band was visible, 5µL of PCR product was used in a second PCR with 8 nucleotide sample specific barcodes, which were added to the Unitag1 and Unitag2 sequences. This second PCR mixture contained 20µL HF green buffer (Finnzymes), 2µL dNTP mix (Promega), 1µL Phusion Hot Start II DNA polymerase (Finnzymes), 62µL nuclease free water, 5µL forward barcoded Unitag1 primer (10µM), 5µL reverse barcoded Unitag2 primer (10µM) and 5µL product of the first PCR. The PCR program started with an activation step at 98°C for 30 seconds, followed by 5 cycles of 98°C for 10 seconds, 52°C for 20 seconds, 72°C for 20 seconds, and finished with an extension step at 72°C for 10 minutes. Product formation was verified on a 2.2% Flashgel (Lonza). The PCR product was purified using the Highprep PCR clean-up magnetic beads (Magbio, London, UK). The concentration of the cleaned PCR product was measured with the Qubit dsDNA BR Assay Kit in the Qubit

2.0 device (Thermo Fischer, Waltham, MA, USA). Finally, the samples were pooled equimolarly with 48 samples per library, including 2 mock communities as an internal standard. Then the libraries were concentrated with the Highprep PCR beads (Magbio). The samples were analysed on the Illumina HiSeq sequencing platform in Rapid Run mode. (Illumina, San Diego, CA, USA).

DATA ANALYSIS

The raw sequencing data were analysed with the in-house NG-tax pipeline [27]. Briefly, paired-end libraries were filtered to contain only read pairs with perfectly matching barcodes. These barcodes were used for demultiplexing. Operational Taxonomic Unit (OTU) picking was performed with an open reference approach and a customized SILVA 16S rRNA gene reference database [28]. ClustalW was used to generate an alignment of OTU sequences and corresponding dendogram. Samples with less than 1000 reads were filtered from the OTU table. Alpha and beta diversity metrics were calculated using scripts from the Quantitative Insights Into Microbial Ecology (QIIME) v1.8.0 package at 99% similarity [29]. Ordination analyses were performed with the Canoco 5.0 software package [30]. Age types were determined by Partitioning Around Medoids (PAM) clustering, based on a weighted UniFrac [31] distance matrix. The clustering analysis was performed in R version 3.2.3. Statistical analyses were performed in SPSS version 22.

RESULTS

ORDINATION ANALYSES REVEAL TAXA THAT CHARACTERISE MICROBIOTA AGING

First we performed a Principal Component Analysis (PCA) based on the microbiota composition of all samples from all time points (Figure 2). The analysis shows that a large proportion of the variance in microbiota composition can be explained by age. This is illustrated by the 1-month and 6-year samples positioned on the extremes of the first principal component. There is a large overlap between 1m and 6m samples, yet a fraction of the 1m samples do not overlap with 6m samples. These samples are characterized by a high abundance of *Escherichia-Shigella* spp., whereas the 1m samples that overlap with 6m samples are characterized by *Bifidobacterium* spp. The later time points are characterized by increasing numbers of *Faecalibacterium* spp. and *Blautia* spp.

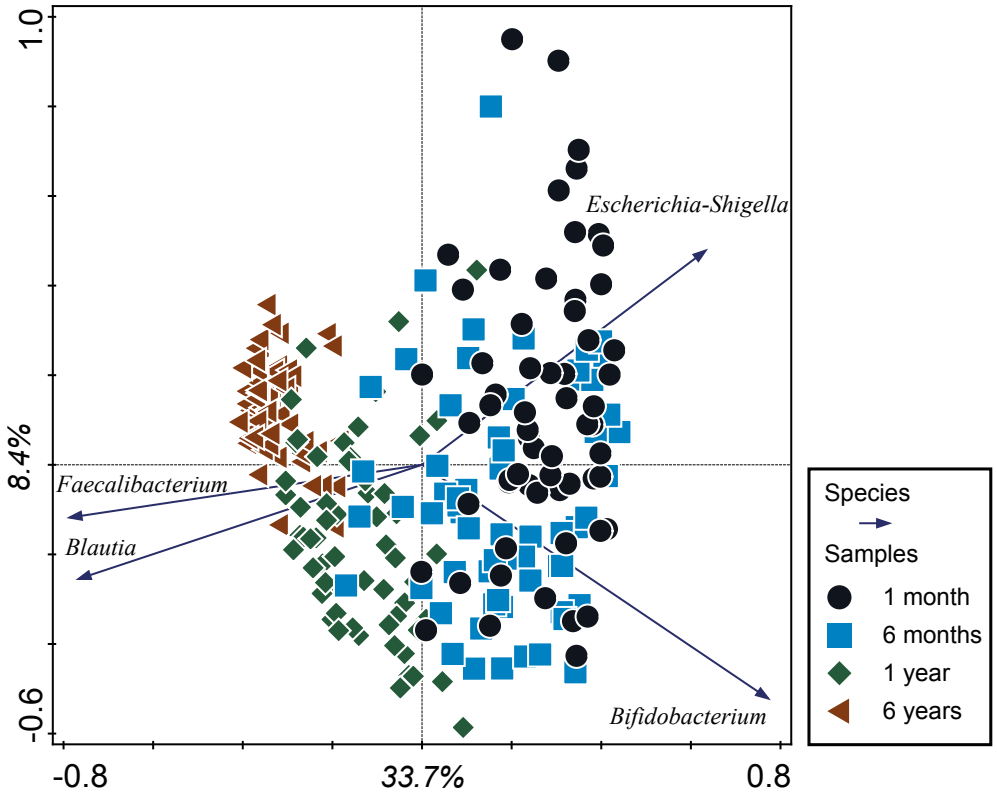

Figure 2. Principal component analysis based on microbiota composition for all samples, labelled per timepoint.

That the factor age is driving microbiota composition is further confirmed by a Redundancy Analysis (RDA). Age explains 21.8% of the observed variation in microbiota composition (Figure 3). It becomes apparent that increasing numbers of *Blautia* spp. and *Faecalibacterium* spp. are associated with increasing age between 1m and 1y. *Erysipelotrichaceae* spp, *Ruminoccaceae spp*, *Ruminococcus* spp and *Clostridiales Family XIII Incertae Sedis* are typical for the 6y microbiota. We compared the Spearman correlations of these taxa with age to their position on the X-axis of the PCA and RDA (Table 1). The Spearman correlations show an age effect, which is influenced strongly by the increase towards age 6y. The taxa X-axis values of the RDA also reflect

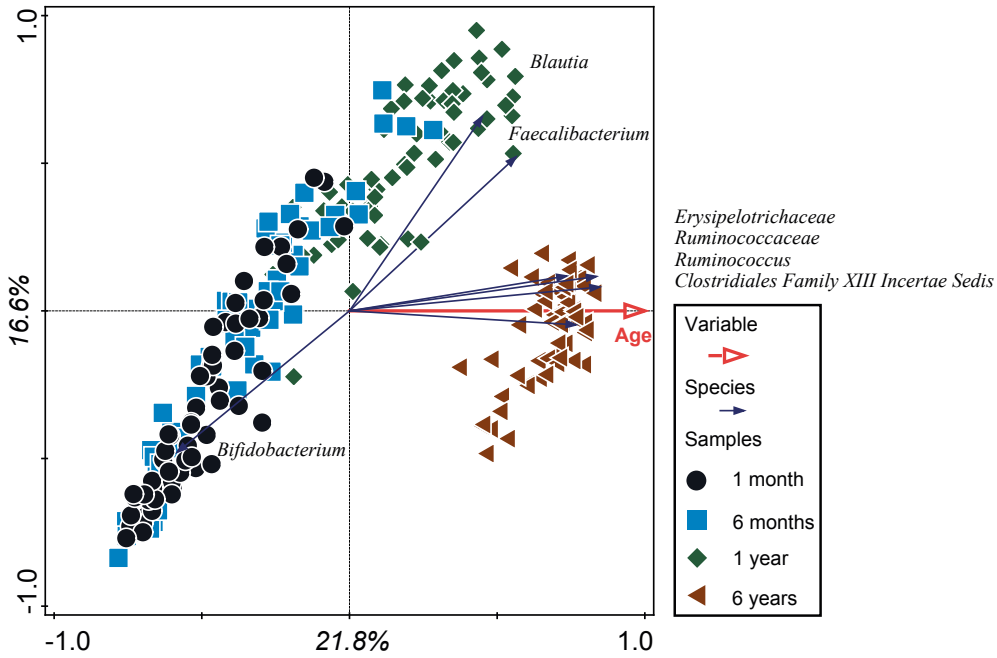

Figure 3. Redundancy analysis (RDA) on all included samples based on microbiota composition, with the main variable: age on the X-axis.

the increase in abundance towards 6y, because age is used as an explanatory value in the analysis. The PCA X-axis values rather reflect microbiota maturity independently from the value of age. As such, taxa that have a higher PCA X-axis value as compared to the Spearman correlation, such as *Blautia* spp., *Dorea* spp. and *Faecalibacterium* spp. are typical for 1y samples. Similarly, the taxa that show higher RDA X-axis values than Spearman correlations are typical for the 6y samples, e.g. *Christensenellaceae* spp., *Clostridiales Family XIII Incertae sedis* spp., *Alistipes* spp., *Erysipelotrichaceae* spp., *Ruminococcus* spp. and *Ruminococcaceae* spp.

Taxon	RDA	PCA	Age correlation
Bifidobacterium	-0.584	0.773	-0.586
Escherichia-Shigella	-0.460	0.633	-0.595
Veillonella	-0.459	0.528	-0.529
Streptococcus	-0.417	0.566	-0.628
Staphylococcus	-0.307	0.483	-0.666
Anaerostipes	0.399	-0.700	0.723
Blautia	0.450	-0.767	0.641
Peptostreptococcaceae Incertae Sedis	0.457	-0.427	0.514
Coprococcus	0.534	-0.512	0.608
Lachnospiraceae	0.535	-0.695	0.708
Faecalibacterium	0.564	-0.787	0.756
Turicibacter	0.616	-0.437	0.514
Pseudobutyrivibrio	0.628	-0.631	0.666
Roseburia	0.631	-0.689	0.717
Dorea	0.661	-0.684	0.662
Subdoligranulum	0.682	-0.656	0.682
Alistipes	0.694	-0.620	0.675
Ruminococcaceae Incertae Sedis	0.712	-0.660	0.726
Christensenellaceae	0.720	-0.484	0.657
Erysipelotrichaceae	0.736	-0.653	0.715
Clostridiales Family XIII IncertaeSedis	0.768	-0.577	0.663
Ruminococcaceae	0.836	-0.750	0.827
Ruminococcus	0.848	-0.735	0.806

Table 1. A comparison of Spearman correlations with age and their positions on the X-axis of the PCA and RDA.

MICROBIOTA DIVERSITY DOES NOT INCREASE DURING WEANING

The availability of samples taken from the same infant at different time points allowed us to study the differences in alpha diversity over time. When alpha diversity is assessed as OTU richness, there is no significant difference between 1m (32.9) and 6m (35.3) samples (paired t-test, p=0.163, Table 2). The difference in Simpson's diversity between 1m (25.2) and 6m (27.7) samples is also not significant (paired t-test,

p=0.115). However, when alpha diversity is measured as phylogenetic diversity, the difference between 1m (1.345) and 6m (1.564) is significant (paired t-test, p=0.001). This indicates that while the microbiota of the individuals is similar in richness and evenness, the microbial composition has changed. From 6m to 1y there was a large increase in richness (35.3 vs. 42.1, paired t-test, p<0.001). Simpson's diversity increased from 27.7 to 41.6 (paired t-test, p<0.001), as well as phylogenetic diversity (1.551 vs. 2.027, paired t-test, p<0.001).An even larger difference in alpha diversity was observed between the samples taken at 1y and 6y. Richness increased from 52.1 to. 88.6 (paired t-test, p<0.001), Simpson's diversity from 41.6 to 71.9 (paired t-test, p<0.001) and phylogenetic diversity from 2.037 to 3.241 (paired t-test, p<0.001).

						Difference with previous age			
					Std.	95% C.I.			
Diversity index	Age	Mean	Minimum	Maximum	Deviation	Mean	Lower	Upper	p-value
Simpson Index	1m	25.2	13.8	44.4	7.0	-	-	-	-
	6m	27.7	12.7	52.7	8.4	2.1	-0.5	4.8	0.115
	1y	41.6	24.0	65.1	9.1	14.2	11.4	17.1	<0.001
	6y	71.9	53.3	96.4	11.0	30.1	26.5	33.6	<0.001
OTU richness	1m	32.9	17	58	8.8	-	-	-	-
	6m	35.3	16	64	9.9	2.4	-1.0	5.7	0.163
	1y	52.1	29	80	10.9	17.2	13.8	20.5	<0.001
	6y	88.6	67	118	12.9	36.1	31.9	40.3	<0.001
Phylogenetic diversity	1m	1.35	0.68	2.28	0.36	-	-	-	-
	6m	1.56	0.86	2.58	0.38	0.21	0.09	0.34	0.001
	1y	2.04	1.34	2.66	0.32	0.48	0.36	0.59	<0.001
	6y	3.26	2.43	4.67	0.52	1.20	1.07	1.34	<0.001

Table 2. Diversity metrics across all the timepoints for different models and the differences with the previous study timepoint.

THERE ARE DISTINCT EARLY LIFE MICROBIOTA CLUSTERS

The age gradient observed in the PCAs also becomes apparent in a Principal Coordinates Analysis (PCoA) based on weighted UniFrac distances (Figure 4a). The samples taken at the 1m and 6y time points are on the outer ends of the first axis. The neighbouring time points show overlap, yet to a lesser extent between the 1y and 6y samples. To determine this overlap and further elaborate on stages in microbiota development we performed PAM-clustering based on weighted UniFrac distances. This resulted in four unique clusters (Figure 4b). An area plot shows the distribution of age over the different clusters (Figure 4c). There are two early life clusters, cluster 1 and 2. Cluster 1 consists of mainly 1m samples combined with about half of the 6m samples. Cluster 2 contains a lower number of samples, but also consists of mainly 1m and a few 6m samples. Cluster 3 can be considered an intermediate cluster, consisting of 6m and 1y samples with a few 1m and 6y samples. Cluster 4 contains almost all of the 6y samples, combined with about half of the 1y samples; therefore we named it the matured cluster.

THE EARLY LIFE MICROBIOTA IS MOSTLY DOMINATED BY EITHER *BACTEROIDES* SPP. OR *BIFIDOBACTERIUM* SPP.

The most common early life cluster, cluster 1, is dominated by *Bifidobacterium*. On average *Bifidobacterium* makes up 41.5% of the microbiota in cluster 1 (Figure 5). Cluster 2 however, is not dominated by *Bifidobacterium*; it only has an average relative abundance in this cluster of 11.0%. Instead, the dominant genus in cluster 2 is *Bacteroides*, with an average abundance of 40.0%. In cluster 1 *Bacteroides* is not at all common because it only makes up 2.2% of the total microbiota. The second most common genus in cluster 1 is *Streptococcus,* with an average abundance of 12.3%. That makes it significantly more abundant in cluster 1 than in cluster 2, which has an average of 2.6%. Also common in the early life microbiota are the facultative anaerobic *Proteobacteria*. In our study, the most abundant group in both clusters is *Escherichia-Shigella*, with comparable abundances of 14.4% for cluster 1 and 11.3% for cluster 2. The phylogenetic diversity of both early life clusters is very similar with values of 1.364±0.372 for cluster 1 and 1.367±0.194 for cluster 2.

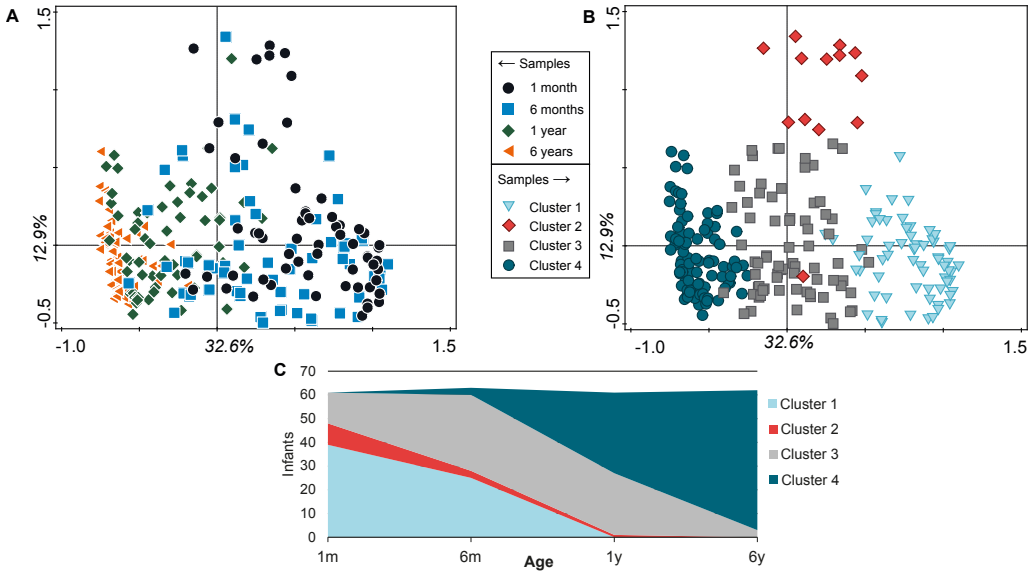

Figure 4. (a) Principal Coordinates Analysis (PCoA) based on Weighted Unifrac; (b) PAM-clustering with 4 clusters; (c) area-plot showing age-distribution within the clusters.

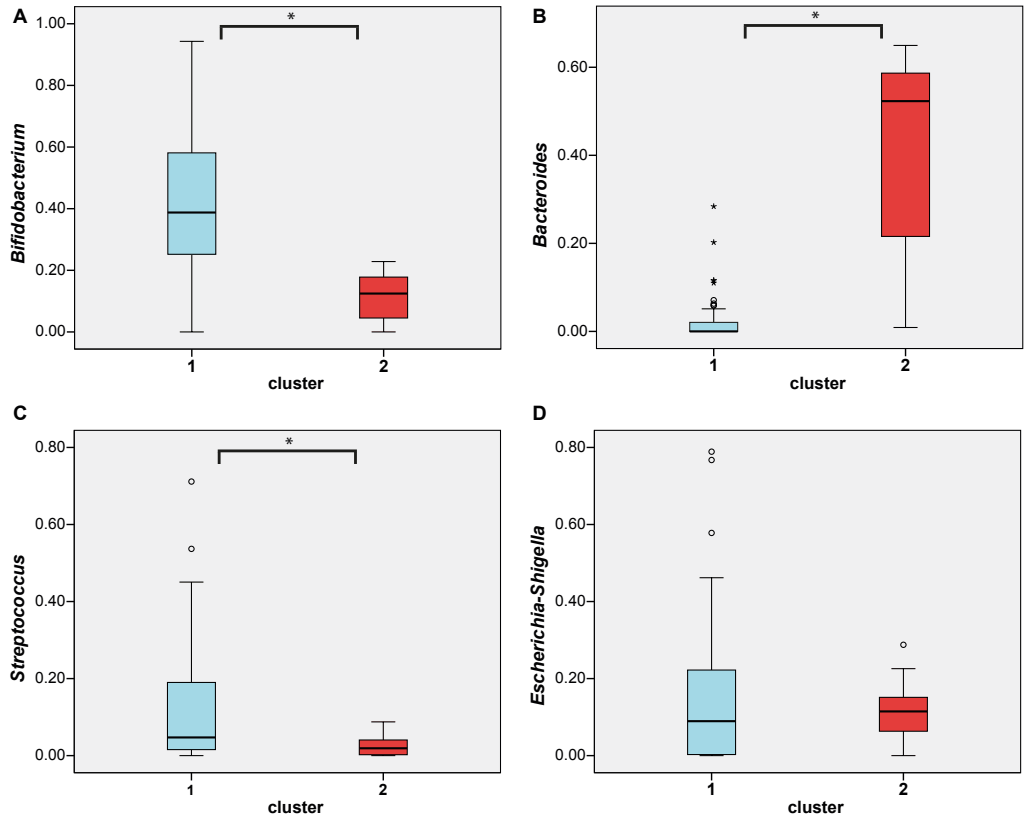

Figure 5. Boxplots depicting bacterial difference (relative abundance) between the clusters.

NO *BACTEROIDES*-DOMINATED MICROBIOTA IN INFANTS DELIVERED THROUGH CAESAREAN SECTION

The samples taken at 1m can be found in cluster 1, 2 and 3. Because delivery mode has previously been correlated with infant microbiota composition, we investigated whether the distribution of delivery mode was similar over the different clusters (Figure 6). At 1m 63.9% of the infants can be found in cluster 1, 14.8% in cluster 2 and 21.3% in cluster 3. The distribution of the clusters is very similar in infants born through vaginal birth, but differs in infants born through caesarean section. This different distribution is caused by the absence of infants from cluster 2 amongst the C-section born children. The number of infants however makes it impossible to perform a valid statistical test.

			Cluster			Total
			1	2	3	
Birth mode	Caesarian	Count	8	0	2	10
		Expected Count	6.4	1.5	2.1	
	Vaginal	Count	31	9	11	51
		Expected Count	32.6	7.5	10.9	
Total		Count	39	9	13	61

Figure 6. Birth mode metadata occurrence across the clusters.

THERE ARE FOUR REPEATED DEVELOPMENTAL TRAJECTORIES

The clustering analysis allows us to study microbiota maturation by following how individuals move through the clusters as age progresses. The most commonly observed progression is repeated 10 times (trajectory A). In trajectory A, infants are in cluster 1 at 1m, in cluster 3 at 6m and reach the matured cluster 4 at age 1y (Figure 7a). Trajectory B and C are the second most often repeated with 8 individuals each (Figure 7b & 7c). In both trajectories infants are in cluster 1 at both 1m and 6m. In trajectory B infants go through intermediate cluster 3 at 1y before reaching cluster 4 at 6y. In contrast to trajectory C, where the intermediate cluster is skipped and infants reach cluster 4 at the age of 1y. Trajectory D is observed in six individuals that are found in cluster 1 at 1m, cluster 3 at 6m and 1y before arriving in cluster 4 at 6y (Figure 7d). For individuals found in cluster 2 at 1m, there are only two repeated sequences

A

Trajectory A				
1m	6m	1y	6y	#infants
1	3	4	4	10

B

Trajectory B				
1m	6m	1y	6y	#infants
1	1	3	4	8

C

Trajectory C				
1m	6m	1y	6y	#infants
1	1	4	4	8

D

Trajectory D				
1m	6m	1y	6y	#infants
1	3	3	4	6

E

Start in cluster 2				
1m	6m	1y	6y	#infants
2	3	4	4	3
2	3	4	4	2
2	1	4	4	1
2	2	4	4	1
2	4	4	4	1

F

Others				
1m	6m	1y	6y	#infants
3	1	4	4	2
1	1	4	3	1
1	3	3	3	1
1	4	3	4	1
3	1	3	4	1
3	2	3	4	1
3	3	4	4	1
3	3	3	4	1

Figure 7/8. Trajectory formulations and their occurrence rate across the infants.

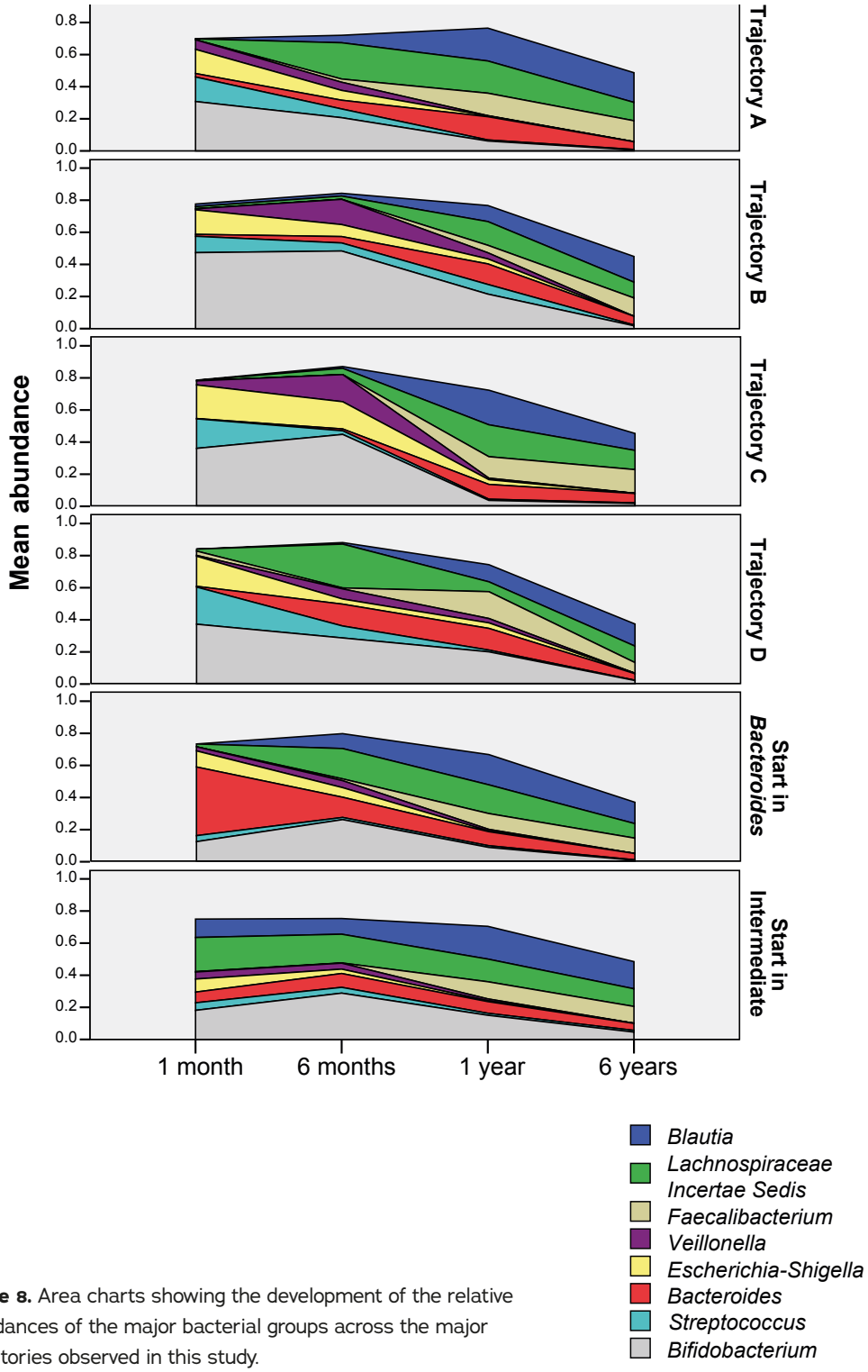

Figure 8. Area charts showing the development of the relative abundances of the major bacterial groups across the major trajectories observed in this study.

(Figure 7e). With three individuals, the most repeated trajectory starting with cluster 2 is similar to trajectory A except for start. Notably, In trajectory B and C there is an increase in *Veillonella* spp. from 1m to 6m, whereas in A and D there is an increasing abundance in *Lachnospiraceae Incertae Sedis* spp. (Figure 8). The levels of *Bacteroides* in infants that are in cluster 2 at 1m restore to levels seen in other trajectories.

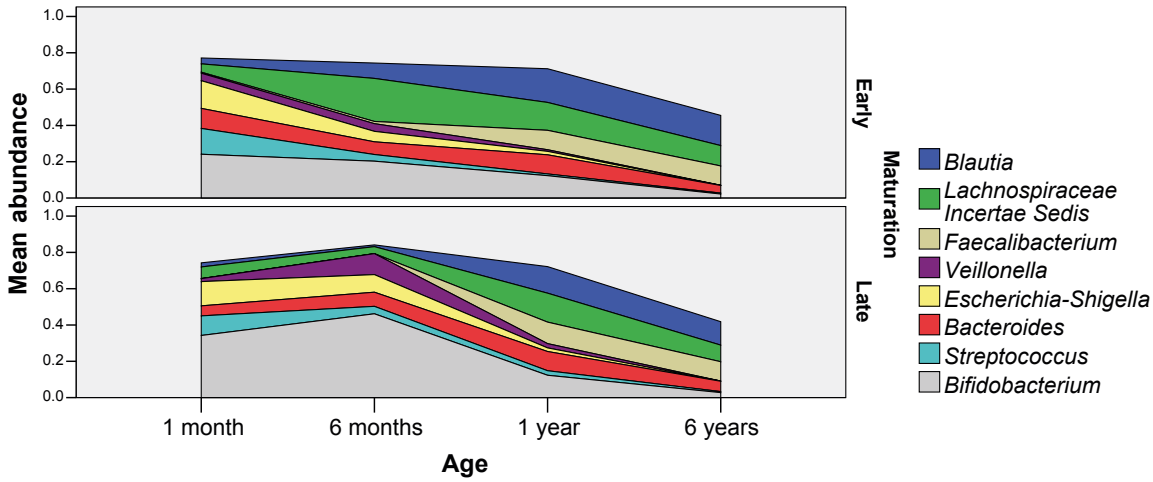

Figure 9. Area charts depicting relative abundance of major bacterial groups when separating infants into 'early' and 'late' maturation.

			Maturation		Total
			Early	Late	
Feeding mode	BF	Count	2	14	16
		Expected Count	8.9	7.1	
	FF	Count	21	9	30
		Expected Count	16.7	13.3	
	Mix	Count	12	5	17
		Expected Count	9.4	7.6	
Total		Count	35	28	63

Figure 10. The differences in maturation rate between feeding mode and the expected values if it was random.

			Maturation		
			Early	Late	Total
Weaned at 6m	No	Count	21	19	40
		Expected Count	22.2	17.8	
	Yes	Count	14	9	23
		Expected Count	12.8	10.2	
Total		Count	35	28	63

Figure 11. The differences in maturation rate between timing of weaning and the expected values if it was random.

			Maturation		
			Early	Late	Total
Oligos	No	Count	14	4	18
		Expected Count	10,0	8,0	
	Yes	Count	21	24	45
		Expected Count	25,0	20,0	
Total		Count	35	28	63

Figure 12. The differences in maturation rate with our without oligos in the diet and the expected values if it was random.

FORMULA FED INFANTS ARE MORE LIKELY TO HAVE EARLY MICROBIOTA MATURATION

We studied whether mode of feeding had influence on microbiota maturation. We regarded having a cluster 3 microbiota composition at the age of 6m as 'early maturation' and having a cluster 1 or 2 microbiota composition as 'late maturation'. Early maturation is characterized by decreasing numbers of *Bifidobacterium* spp. and *Veillonella* spp. from 1m to 6m and increasing numbers of *Blautia* and *Lachnispiraceae Incertae Sedis* spp. (Figure 9). The abundance dynamics are highly intra-individual but overall we found different dynamics towards maturation. At 6m 44.4% of the infants had a 'late maturation' microbiota type (Figure 10). In breastfed infants the

percentage of 'late maturation' was 87.5%. In formula fed and mixed feeding infants we found that 70.0% and 70.6% (respectively) had 'early maturation' as opposed to 55.6% among all the infants (Pearson Chi-Square test, $p<0.001$). We did not observe a significantly different distribution of microbiota maturation between infants that had started weaning and those that did not (Figure 11, Pearson Chi-Square test, $p=0.520$). To test whether the presence of oligosaccharides in the nutrition of infants has an effect of on microbiota maturation, we divided our subjects in two groups. First a group that received oligosaccharides, either through human milk or prebiotics supplemented formula, and second a group that received formula without prebiotics (Figure 12). We found that the group that did not receive oligosaccharides more often had a more mature microbiota composition (Pearson Chi-Square test, $p=0.025$). Clearly, we observed that early maturation is more common amongst formula fed infants, but late maturation is more common amongst prebiotics supplemented formula fed infants.

DISCUSSION

This study describes the microbiota development from a group of 64 children over the first 6 years of life. With these data we independently reproduced known concepts of microbiota development and come up with new findings, using a European cohort. Our dataset revealed two distinct types of early life microbiota composition; a typical *Bifidobacterium*-dominated [4, 32] and a more recently discovered *Bacteroides*-dominated infant microbiota [13, 20, 23]. The *Bacteroides*-dominated microbiota has been described previously in relation to birth mode. Vaginally born infants have been reported to have higher levels of *Bacteroides* and C-section born infants have been described to be depleted of *Bacteroides* [15, 20]. In our dataset none of the C-section born infants had a *Bacteroides*- dominated microbiota and as such this study supports previous findings. All the infants in our study were receiving either human or formula milk at 1m and 6m. It is at these time-points that the large majority of infants have a *Bifidobacterium*- or *Bacteroides*-dominated microbiota. Species from both genera are known to be well-equipped to metabolize the complex carbohydrates in milk [33, 34]. Because of their shared preference for these carbohydrates, *Bacteroides* spp. and *Bifidobacterium* spp. compete for dominance during the period that infants receive human milk. However, since we only report up to the phylogenetic family and genus level, the potential for human milk substrate utilization can still differ greatly between infants. For example, the *Bifidobacterium* spp. possess varied HMO

utilization pathways [35]. Moreover, the relative abundance of the aforementioned genera can vary quite a lot between individuals. As such, the OTUs associated with either *Bacteroides* or *Bifidobacterium* in our study show differences between the early life microbiota clusters.

It has been described that the microbiota of children from Estonia and Finland is dominated by *Bacteroides* spp. during infancy [24]. This contrasts with the microbiota of Russian infants, which is first dominated by *Bifidobacterium* spp. and only later transitions to *Bacteroides* domination. Therefore, it appears that the dynamics of *Bacteroides* in microbiota of Belgian infants rather resembles that of their Estonian and Finnish counterparts. The *Bacteroides* spp. we find in cluster 2 are almost all related to *Bacteroides dorei* and *Bacteroides vulgatus.* When exploring associations of infant gut microbiota and food sensitization, a *Enterobacteriaceae*/*Bacteroidaceae* ratio was used as a measure for microbiota maturity [36]. Similarly, to this dataset, if the microbiota of infants is dominated by *Bacteroides* spp. at early infancy, their abundance might not reflect microbiota maturity as well as in cohorts without *Bacteroides*-dominated infants. Notably, on another cohort of Belgian children, this ratio was hypothesized to be a natural regulator of the Th1/Th2 balance, with the risk of early colonization by Bacteroides leading to a Th2 dominance and higher risk of asthma development [37].

The clustering analysis performed on samples of different ages allowed us to determine different phases in microbiota development. Subsequently analyzing the trajectories that individuals follow through the clusters that represent a maturation state led to the discovery of four distinct trajectories. Due to the number of samples, we were not able to make correlations of metadata variables with specific trajectories. However, the pooling of trajectories into either early or late maturation led to the finding that formula feeding is associated with early maturation in this cohort, thereby confirming previous observations by *Bäckhed et al.* [14]. If there is a difference in maturation among infants fed formula with and without prebiotics supplementation is not clear from this study.

Most infants have already reached the relatively stable microbiota state associated with a milk diet at the 1m timepoint. In a Singaporean study that also used a clustering approach to determine stages in microbiota development, but included samples taken at day 3, there is a cluster that is dominated by the family *Enterobacteriaceae,* especially *Klebsiella* spp. [38]. The study also finds a cluster with high levels of *Firmicutes*, in particular *Streptococcus.* We find *Streptococcus* to be the second most abundant genus

in the *Bifidobacterium*-dominated microbiota and it rather seems to cooccur with *Bifidobacterium* than to compete with it. From the age of 3 weeks, the majority of infants in the Singaporean study had a *Bifidobacterium*-dominated microbiota similar to ours, indicating that our first time-point is probably too late to detect a *Enterobacteriaceae*-dominated infant microbiota cluster.

A study by Subramanian *et al.* analyzes the contribution of specific taxa to the microbiota age of Bangladeshi infants [25]. By calculating the correlation with age and analyzing the X-axis position in ordination plots, we find similar microbial groups. We also find that *Faecalibacterium* spp., *Ruminococcus* spp., *Bifidobacterium* spp., *Dorea* spp., *Staphylococcus* spp. and *Streptococcus* spp. are strongly correlated with age. An important difference is that we also find correlations for *Veillonella* spp., which might be a geographical effect.

Similarly to Bäckhed *et al.*, we find increasing alpha diversity over time, combined with reduced beta-diversity [14]. We do not find increased diversity in terms of richness and Simpson's index from 1m to 6m, even though 23 out of 63 infants had already started weaning at 6m. We did find increased phylogenetic diversity from 1m to 6m, indicating that although richness and evenness were similar, the phylogenetic distance between OTUs within the 6m samples increased. This demonstrates the suitability of phylogenetic diversity as compared to more classical diversity indices for this type of analysis.

At 6m, we did not find a difference in maturation state between infants that had already been weaned and infants that had not. Weaning often leads to transformation of the microbiota composition, since the solid foods that are introduced resemble the adult diet more than either human milk or formula [39, 40]. However, we did find that infants receiving milk with oligosaccharides, either breastmilk with HMOs or formula with added prebiotics possessed a pre-mature microbiota composition as compared to infants receiving formula without prebiotics. It seems that in this study, discontinuation of human and formula milk with their associated oligosaccharides is more important than the introduction of solid foods. An observation also similar to that of Bäckhed *et al.* [14].

In conclusion, this study describes two types of infant gut microbiota ecosystems. This study features both the traditional *Bifidobacterium*-dominated infant microbiota and the microbiota dominated by *Bacteroides.* In our dataset, there were no C-section

delivered infants that had a *Bacteroides* microbiota. Differential microbiota maturation was influenced by feeding mode. It seems that any type of milk diet and the oligosaccharide prebiotics delay microbiota maturation. The next step will be to study the health effects of both infant microbiota types; early and late microbiota maturation and whether the presence of specific bacteria promotes or reduces infant health status.

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The study was performed by L.K., J.K. and C.B.; L.K. collected the data; P.S. & L.K. performed data analysis and figure generation; P.S., L.K. and C.B. wrote the manuscript. The manuscript was checked by P.S. L.K., C.V., K.D., J.K. and C.B. All authors contributed to critical revisions and approved the manuscript. First of all, the authors would like to thank all clinical personnel involved in the Op Pad Study.

CONFLICT OF INTERESTS

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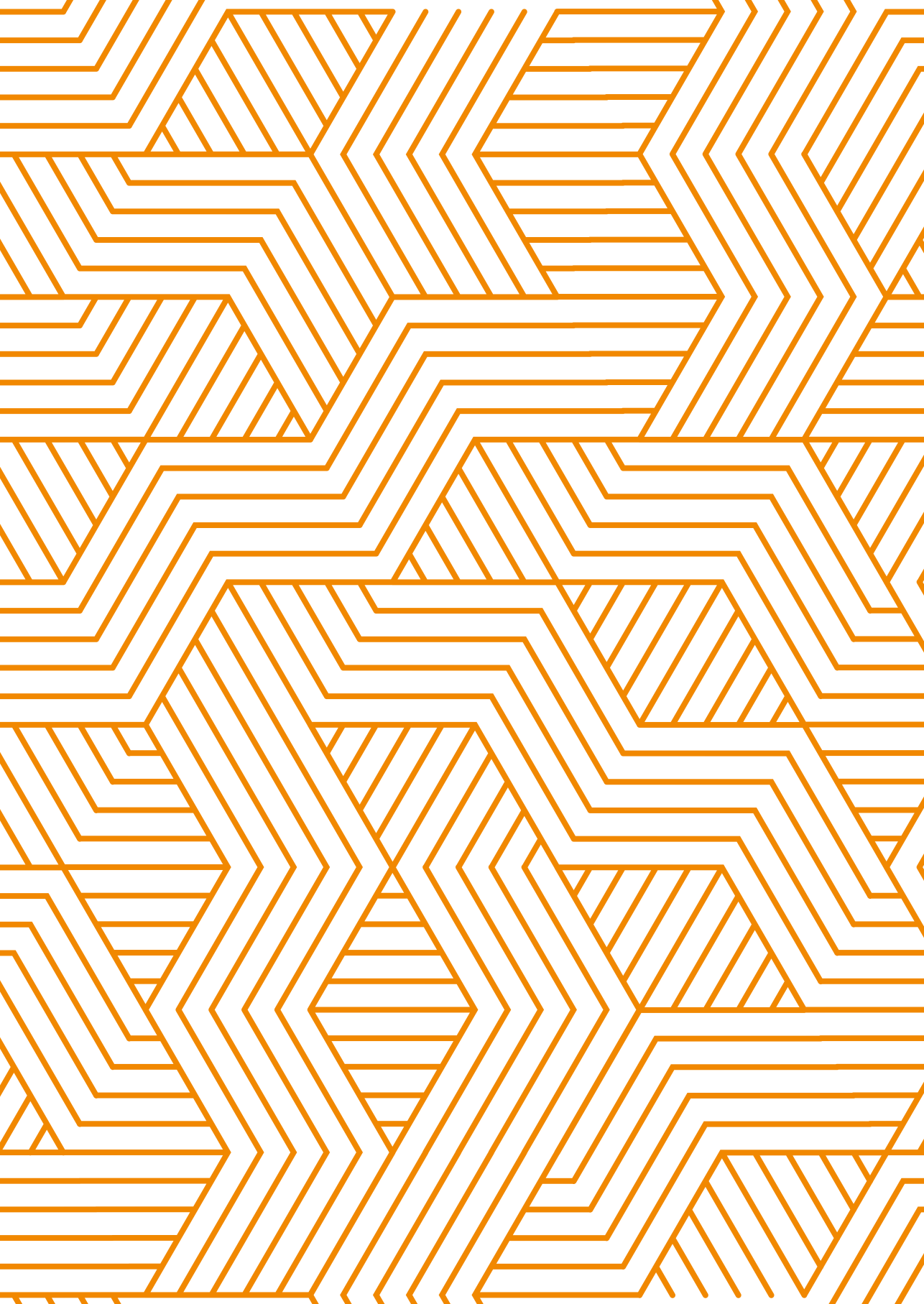

CHAPTER 5

EFFECT OF A LOW PROTEIN DIET ON THE EARLY LIFE GUT MICROBIOTA: A FOLLOW-UP TO THE PROTEUS STUDY

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ABSTRACT

Establishing the infant gut microbiota through early life diet is pivotal in human health. Nitrogen content in the infant's diet is currently largely overlooked. Meanwhile, supplying infants with too much protein has been shown to negatively impact the infant's health status. This study acts as a follow- up to Kouwenhoven *et al*. (2019, Proteus cohort) [1], in which a modified low-protein (mLP; in this study: I, intervention) formula was compared to designed control formula (in this study: C, control) in relation to growth and development of the infant. Herein, microbiota composition is assessed at baseline (1 month) and at 4 months of age. We investigated whether lowering protein content in formula impacts microbiota composition and development of the microbiota. Discriminant analyses showed that the breastfeeding regime leads to the most distinct microbial signature compared to both formula groups. However, the intervention product significantly affected the some signatures of the microbiome composition between baseline and 4 months of age. Specifically, it increased the abundance of the *Bifidobacterium* genus compared to the control formula group. Moreover, the beta diversity measurements showed a decrease between the 1 month timepoint and the 4 month time point in the intervention product, as we tend to see in breastfed infants [2].

Keywords: infant gut, microbiota, *Bifidobacterium*, diet, protein, birth mode, human milk, formula

INTRODUCTION

In early life, a settling gut microbiota impacts our health for years to come. Evidence is accumulating that cessation of breast milk is the single, most contributing factor driving the maturation and modification of the infant gut microbiome towards a health-promoting state [2-6]. However, breastfeeding is not always available which raises the need for the development of infant formula to suit their health needs. Notably, many studies highlighted the difference in microbial composition throughout infancy in breastfed infants versus formula-fed infants [6-8]. For example, there is a decrease in relative abundance of *Bifidobacteriaceae* in formula-fed infants. This coincided with an overall increase in *Staphylococcaceae*, *Clostridiaceae*, *Enterococcaceae*, and *Bacteroidaceae* and with a higher bacterial diversity (alpha diversity) [2, 4, 9-11].

Breast milk provides newborns with important nitrogenous compounds, and amino acids, such as glutamate, other free amino acids, urea and other non-protein nitrogen (NPN) sources, which might be of impact on the microbiota composition [12, 13] Furthermore, recent evidence shows that microbial metabolism in the human gut may be altered by the application of biotics strategies for eliminating undesirable nitrogenous metabolites by shifting complete body metabolism [51,52]. Especially under the conditions of intestinal stress, liver disease and low renal function [14]. Low protein concentration of human milk can be a factor that impacts an infants' growth and can prevent obesity and diabetes later on [15-17]. In contrast, overfeeding infants with protein has been linked to negative health effects in later life, including occurrence of non-communicable diseases and obesity [18-21]. This indicates nitrogen composition of the infant's diet is potentially relevant in establishing the infant gut microbiota and in nitrogen homeostasis for the human body. Ultimately, breast milk provides the optimal microbiota and more often leads to healthy infants [2, 9, 19, 22]. Reduction of protein intake may affect microbiome composition and subsequently affect the risk of overweight and obesity. Here we provide longitudinal data that shows the impact on infant gut microbiota composition of lowering protein content and changing the ratio of free amino acids in formula. Meanwhile, comparing it to breastfeeding and integrating a key clinical outcome, namely blood urea nitrogen, of Dutch infants in the Proteus cohort. In addition, through metagenomics functional profiling, we showed the impact of these diet on the bacterial gene catalog and predicted cycling of nitrogen.

MATERIALS AND METHODS

SAMPLE COLLECTION

The stool samples used for analyses originated from a double-blind, randomized controlled trial (ProtEUs study; NL4677) [1]. The study was a collaboration between the Amsterdam UMC and Dr. von Hauner Children's Hospital in Münich. Healthy, formula-fed and term-born infants were randomly allocated to a modified low protein (mLP) infant formula (protein content 1.7g/100 kcal) or a control (2.1g/100 kcal). A group of breastfed infants served as the reference group. Exclusion criteria of the ProtEUs study were illnesses, current or previous conditions, interventions, congenital diseases or malformations and being part of a twin. It was also examined whether or not the infants tolerated standard cow milk-based infant formula. Included in this study was only the fecal material from Dutch infants monitored at Amsterdam UMC. Finally, for both timepoints combined 286 samples and 137 complete infant pairs were longitudinally (at 1 month and 4 months of age) included for microbiome analysis before later mentioned quality filtering.

UREA SERUM MEASUREMENTS

Blood of 83 infants included in this microbiome study was taken at 4 months of age. Venous blood was sampled in a 2.5-mL serum tube and a 0.5-mL heparin tube. After centrifugation (10 min; 1800 × g; 20˚C), these tubes were divided into six aliquots to protect the samples. The aliquots were stored at -80 degrees and only once thawed before analysis. Blood parameters were analyzed using the Beckman Coulter AU5800/AU680 in serum. The concentrations of urea, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were assessed using a kinetic UV test [23].

16S RRNA GENE AMPLIFICATION AND SEQUENCING

DNA was isolated from faecal material using the Maxwell® RSC Instrument Promega following an in-house standardized protocol. The Maxwell 16 Tissue LEV Total RNA Purification Kit (cat no. AS 1220) was used in the Maxwell instrument. Isolated DNA was amplified using the 515F/806R PCR primers, targeting the V4 hypervariable region of 16S rRNA in bacteria and archaea [24, 25]. A purification step was performed using CleanNA beads and the purified DNA quantities were measured in the Qubit 2.0 Fluorometer. Finally, samples were barcoded and sent for sequencing in the Illumina HiSeq2000 machine (Novogene. Cambridge UK). Metagenomes were sequenced at a deep shotgun rate at Novogene (Singapore) and BGI Europe (Kopenhagen, Denmark; 6Gb).

MICROBIAL COMMUNITY ANALYSIS

The sequence data was processed using our in-house Ng-Tax pipeline [26]. Four major tasks executed by this pipeline were: quality filtering, demultiplexing, ASV and taxonomic assignment at 97% identity by comparison to the SILVA 138.1 database. The OTU table was rarefied to 2458 sequences to perform diversity and relative abundance analyses. GGplot2 was used the generate the stacked bar plots after selection of the most abundant families. DNA concentration and quality were determinant of inclusion of in data analysis. A cut-off was set at 50ng/μL and 10000 reads.

DIVERSITY MEASUREMENTS

The alpha diversity indices Richness, Simpson and Shannon were calculated after rarefaction and log- and square root transformation with the plot-anova- diversity function using the microbiomeSeq package. Beta diversity was measured using Bray-Curtis dissimilarity. The statistical test was conducted using a multivariate analysis of variance with permutation (PERMANOVA) with the adonis function of the Vegan package.

ORDINATION

To assess quantitative community structures, Principal Coordinate Analysis (PCoA) was performed. The distance metrices Weighted Unifrac, Unweighted Unifrac and Brays-Curtis were used to construct the dissimilarity matrix. Additionally, a Redundancy analysis (RDA) with computed eigenvalues was performed. The Phyloseq package was used in both cases.

STATISTICAL ANALYSES

Statistical analyses were conducted in R using the packages microbiome (v.1.16.0, [27]), phyloseq (v.1.38.0) [28], vegan (v.2.5-7) [29], microbiomeutilities (v.1.00.16), (SIAMCAT v.1.14.0) [30], ALDEx2 (v.1.26.0), permute (v.0.9-7), microViz (v.0.9.0) [31], PERMANOVA (v.0.2.0), glmnet (v.4.1-3) and ggplot2 (v.3.3.5) and maaslin2 [32]. Equal variance and normality assumptions were not met. Hence, non-parametric statistical tests with multiple testing correction for p-values (Benjamini- Hochberg) were used. Unless otherwise specified, significance was assumed at $P < 0.05$. LEfSe was applied to look for significant discriminants between the treatment groups in this study [33].

MICROBIAL COMMUNITY VARIATION AS EXPLAINED BY DIET AND TIME

Associations of metadata variables diet and time on the inter-individual microbiota community composition were determined using Bray-Curtis dissimilarity measures. To determine the mean difference of relative abundant bacterial groups per diet, Kruskal–

Wallis tests with a post-hoc Dunn tests were applied. Statistical differences in the proportions of categorical variables (diets) among groups were evaluated using pairwise chi-squared tests. Finally, mixed models for univariate comparisons were applied to look at the relative abundance for the most abundant genera over time per diet.

MACHINE LEARNING

Machine learning was first performed using the SIAMCAT pipeline (https://siamcat.embl.de/[30]). Data was filtered on abundance (0.05 cut-off threshold). Yielding 128 and 132 samples at 1 month and 4 months of age respectively. The data was accordingly checked for multicollinearity and the detection limit was set at 1^{e}-06. Next, the data was mean normalized using the 'log.unit' method. The k-fold cross validation was set to five with two resamples, delivering a different training set every run, to prevent prediction biases. Lasso, RandomForest and Elastic Net were used to check model performance. Secondly, the datadist function of the rms package was used to determine the root mean square as estimator of the imperfection of the fit of the estimator to the data. A cubic spline model with four parameters with three knots each was then fitted to the data and the linearity of the fits was predicted. Subsequently bootstrapping and calibration were performed. C-scores were finally calculated to quantify model performance.

FUNCTIONAL PROFILING OF METAGENOMES

Kneaddata genomic filtering tool (https://huttenhower.sph.harvard.edu/kneaddata) was used to filter out human reads from the metagenomes. The 36 metagenomes (n=11/36; Supplemental Table 2) were randomly selected but passed previous DNA quality filtering. They were analyzed for the relative abundance of gene ortholog groups and biochemical pathways using HUMAnN2 ((http:// huttenhower.sph.harvard.edu/humann2 [34]) with their standardized workflow.)the HUMAnN2 output tables (genefamilies, pathabundance, pathcoverage) were (humann2_join_tables), normalized to counts per million (cpm) and relative abundance (humann2_renorm_table), before being stratified. The genefamilies tables were regrouped (humann2_regroup_table) to KEGG Orthology (KO) and enzyme classification number (EC).

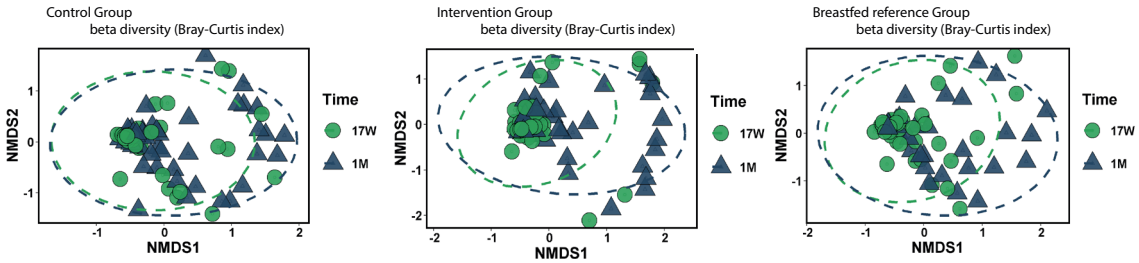
DATA AVAILABILITY

Raw sequencing data, metadata for dietary intake of the first month, urea serum levels and study assignment will be made available at a public repository upon submission.

RESULTS

DECREASING BETA DIVERSITY IN THE BREASTFEEDING GROUP AS WELL AS THE INTERVENTION GROUP AFTER 4 MONTHS

A hallmark for infant gut microbiota development is tracking community. Beta diversity of the I and BF group significantly decreased between one and four months of age, whereas the beta diversity of the control formula-fed group did not. The accompanying test statistics for the breastfed group were a P-value of 0.03 with 74 degrees of freedom and an F-value of 4.1987. For the intervention group, the P-value was 0.003 with 99 degrees of freedom and an F-value of 8.9127 (Figure 1). No significant differences were found between any groups and over time for the alpha-diversity using different indices (Supplemental Figure 1).



	Df	SumsOFSqs	MeanSqs	F.Model	R2	Pr(>F)
Diet I	1	0.3617	0.36173	8.9127	0.08336	0.003 **
Residuals	98	3.9774	0.04059		0.91664	
Total	99	4.3392			1.00000	
Diet BF	1	0.2579	0.257906	4.1987	0.05439	0.03 *
Residuals	73	4.4840	0.061425		0.94561	
Total	74	4.7419			1.00000	
Diet C	1	0.0137	0.013707	0.36859	0.00399	0.807
Residuals	92	3.4213	0.037188		0.99601	
Total	93	3.4350			1.00000	

Figure 1. Beta diversity (Bray-Curtis) plots comparing the 1M (1 month of age) and 4M (4 months of age) samples and significance values ($p<0.05$). Top from left to right: Control, Intervention and the Breastfed reference group. Bottom: Statistical information of the beta diversity differences within the diets between 1M and 4M.

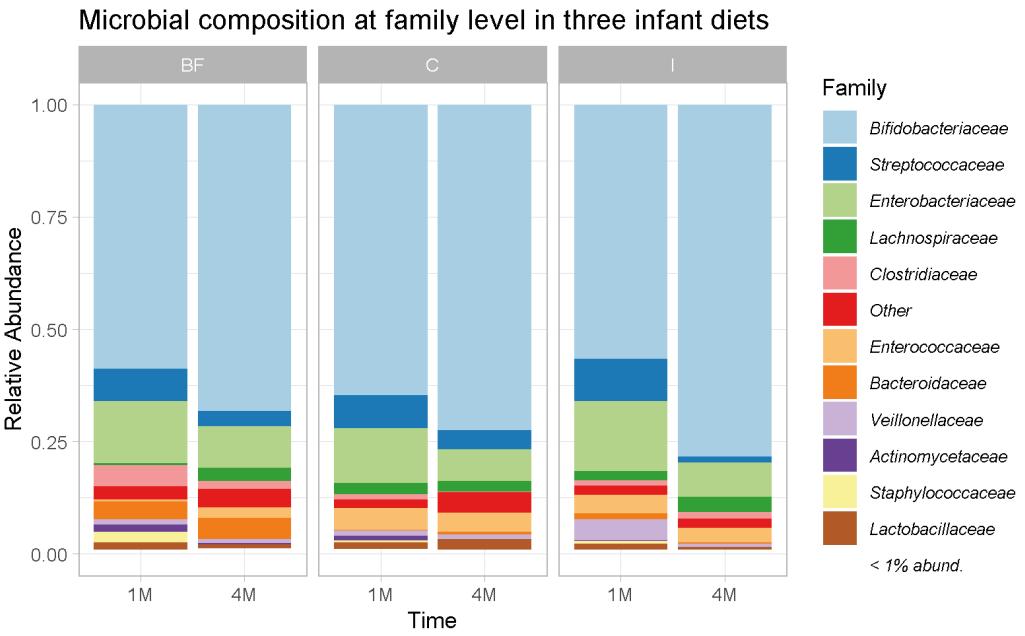


Figure 2. Relative abundance plots of the microbiota composition across time and diet. BF: Breastfed infants, C: infants that received the control formula, I: infants that received the intervention product.

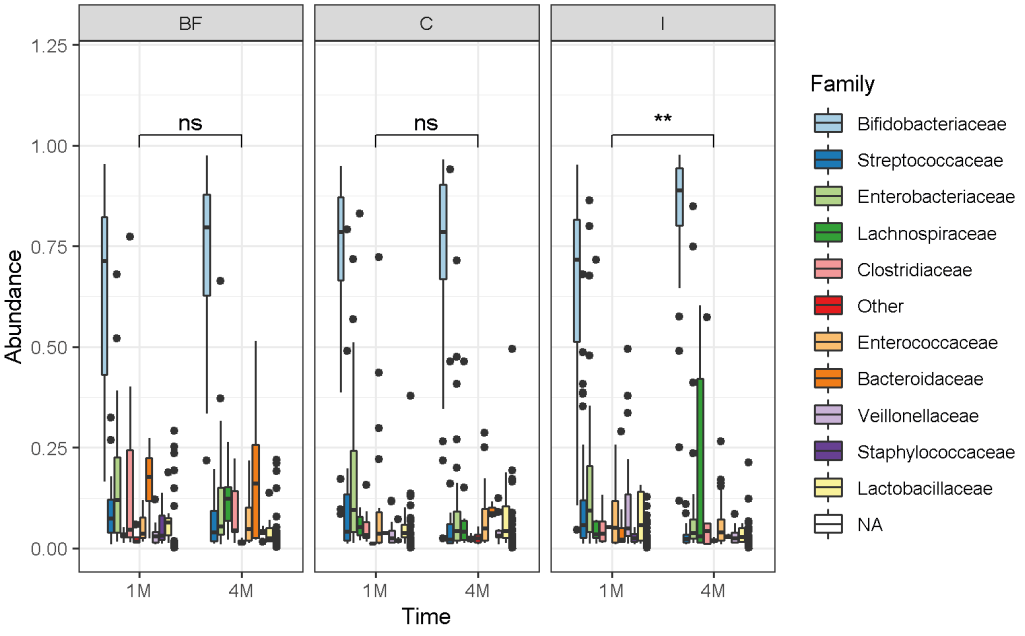


Figure 3. Wilcoxon plot showing shifts in relative abundance of the then most abundant bacterial families in the study ($p<0.05$).

THE EFFECT OF DIET ON RELATIVE ABUNDANCE COMPOSITION: LOW PROTEIN FORMULA PROMOTES *BIFIDOBACTERIUM* SPP.

The relative abundance plots show that the intervention group is more bifidogenic compared to the breastfed and the control formula group (Figure 2; Supplemental Table 1). This was statistically confirmed using univariate mixed models comparing the two timepoints within a diet group (I: p=0.017). A significant decrease of *Streptococcaceae* was also observed in BF (p=0.045) and I (p=0.015); A significant decrease in *Lactobacillus* was observed in BF (p=0.047); *Staphylococcaceae* significantly decreased in all groups compared to one month of age (BF; p=0.0156, I; p=0.018, C; p=0.025); *Veillonellaceae* significantly decreased in I (p=0.035) and *Lachnospiraceae* significantly decreased in I compared to the baseline (p=0.017), but significantly increased in BF over the longitude of this study (p=0.031).

A heatmap was constructed to depict a higher resolution of differences between the treatments at both timepoints (Supplemental Figure 2). What stands out is a higher abundance of *Enterococcus* spp. (4-5% over 0.53%) and *Streptococcus* spp. (8-10% over 6.83%) in both formula groups compared to the breastfeeding group, and higher *Veilonella* spp. abundance (4.97 over 1.17%) in the intervention group compared to the breastfeeding group at one month of age compared to the breastfeeding group. When applying a Wilcoxon-test on the 10 most abundant families in the dataset it was furthermore found that the intervention product was the only diet group that delivered a significant difference between one and four months of age (Figure 3).

MINOR GUT MICROBIOME DIFFERENCES IN THE FIRST FOUR MONTHS OF LIFE BETWEEN HUMAN MILK AND FORMULA DIETS.

The microbiome was also studied in relation to LDA Effective Size (LEfSe). Figure 4 shows that the breastfeeding group possesses the most discriminants, with many bacterial families and genera contributing (LDA score log10 > 2.0) to the differences between the diet groups. Notably at 4 months of age, *Bifidobacterium* spp. largely contribute to what sets the intervention group with lower protein apart, showing the promoting effect of the modified low protein formula for promoting this crucial bacterial group. The *Streptococcaceae* are a strong linear determinant of the control formula group (C) at 4 months of age which is typical for regular formula-fed infants. *Bacteroides* spp. are still the main factor setting breastfed infants apart at both time points. It is well known that breastfeeding contributes to the occurrence of *Bacteroides* spp. A PERMANOVA was performed to further investigate the top contributing taxa within the diet across the longitude of this study (Supplemental Figure 3). This

has shown that within all three diet groups *Bifidobacteriaceae* increase between 1 month and 4 months of age. Furthermore, it shows that *Streptococcaceae* are a major contributing taxa at 1 month of age. But, less so within the control formula diet with regular protein levels (C), since it remains more abundant, and thus is a contributing taxa at 4 months age.

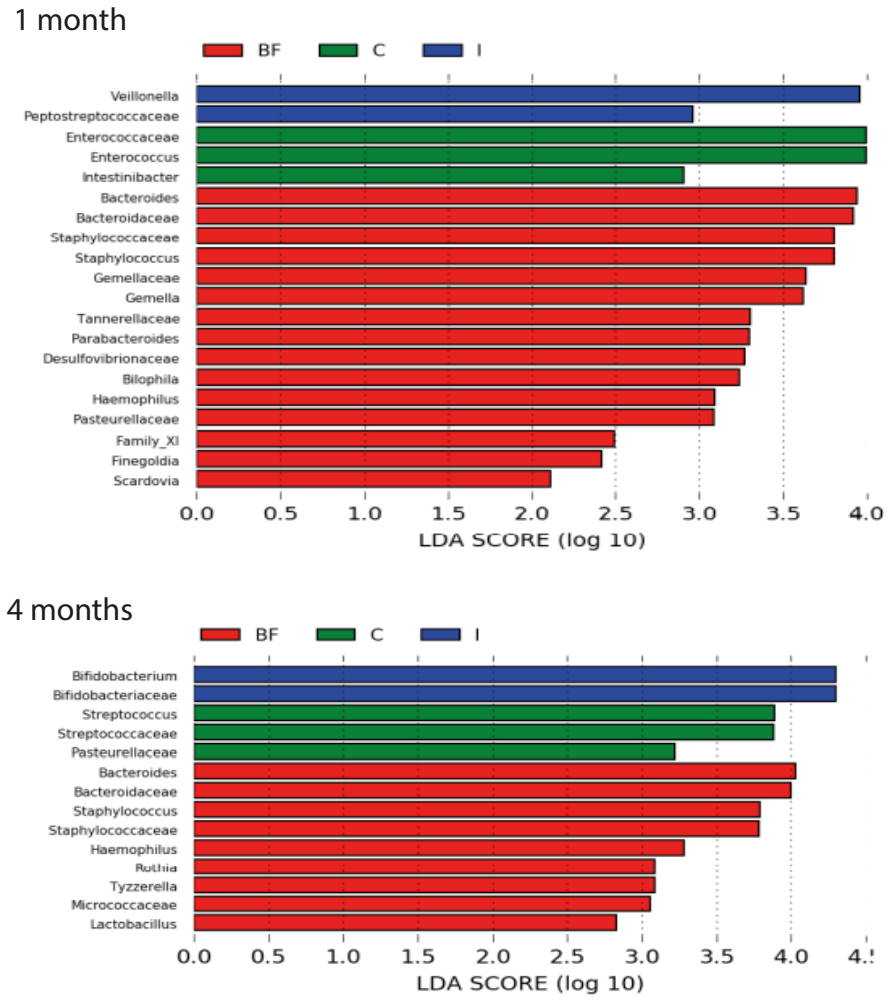


Figure 4. LEfSe analysis showing the most discriminant taxa across the diet types comparing 1 diet versus the others (LDA Score > 2.0); top: 1 month, bottom: 4 months. BF: Breastfed infants, C: infants that received the control formula, I: infants that received the intervention product.

MACHINE LEARNING MODEL INDICATES HIGHER SIMILARITY BETWEEN DIET TYPES AT 4 MONTHS OF AGE.

Predictive properties of the LASSO model were considered accurate for the breastfed group against both formulas combined at 1 month of age. The mean prediction area under the curve (AUC) was 0.867 for the Receiver Operating Characteristic curve (ROC). Eighteen variables were selected as model-building parameters. These included *Actinomycetaceae, Akkermansiaceae, Bacteroidaceae, Bifidobacteriaceae, Clostridiaceae, Coriobacteriaceae, Enterobacteriaceae* and *Enterococcaceae* but also less abundant families. Figure 6 shows the largest contribution of some of these families to the model, when comparing the BF- group with the I and C diets combined. Because of the study set-up it was not surprising that the model failed to make adequate predictions for C and I at 1 month of age. There, the mean predictive AUC was lower than expected by pure chance (0.458). This confirms that I and C were adequately randomized. In addition to LASSO, elastic net and RandomForest were performed. Their performance was similar. However, the LASSO model cannot discriminate between the modified low protein formula and breastfeeding for the samples at four months of age. For samples at that timepoint, mean predictive properties of the trained model lowered to 0.515 for BF vs C and I, corresponding with the close clustering of the microbiomes. This shows that prediction results were close to random assignment. Furthermore, out-of-sampling performance was predicted for the cubic spline models. Before bootstrapping, the C-value of 0.803 indicated high algorithmic performance. Bootstrapping did not lower the C-value significantly (0.771). This slight drop in model performance suggests we may expect to be able to predict BF from I/C with reasonable accuracy in a new sample of infants drawn from a similar population using just the major components (Figure 6).

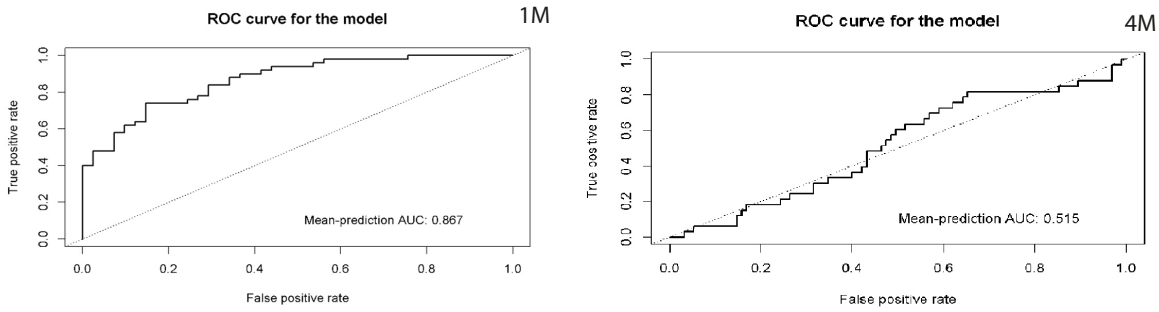


Figure 5: ROC curve illustrating LASSO model performance at one months of age and four months of age. The left figure illustrates good algorithmic performance, while the right figure does not perform better than what would be expected by pure chance.

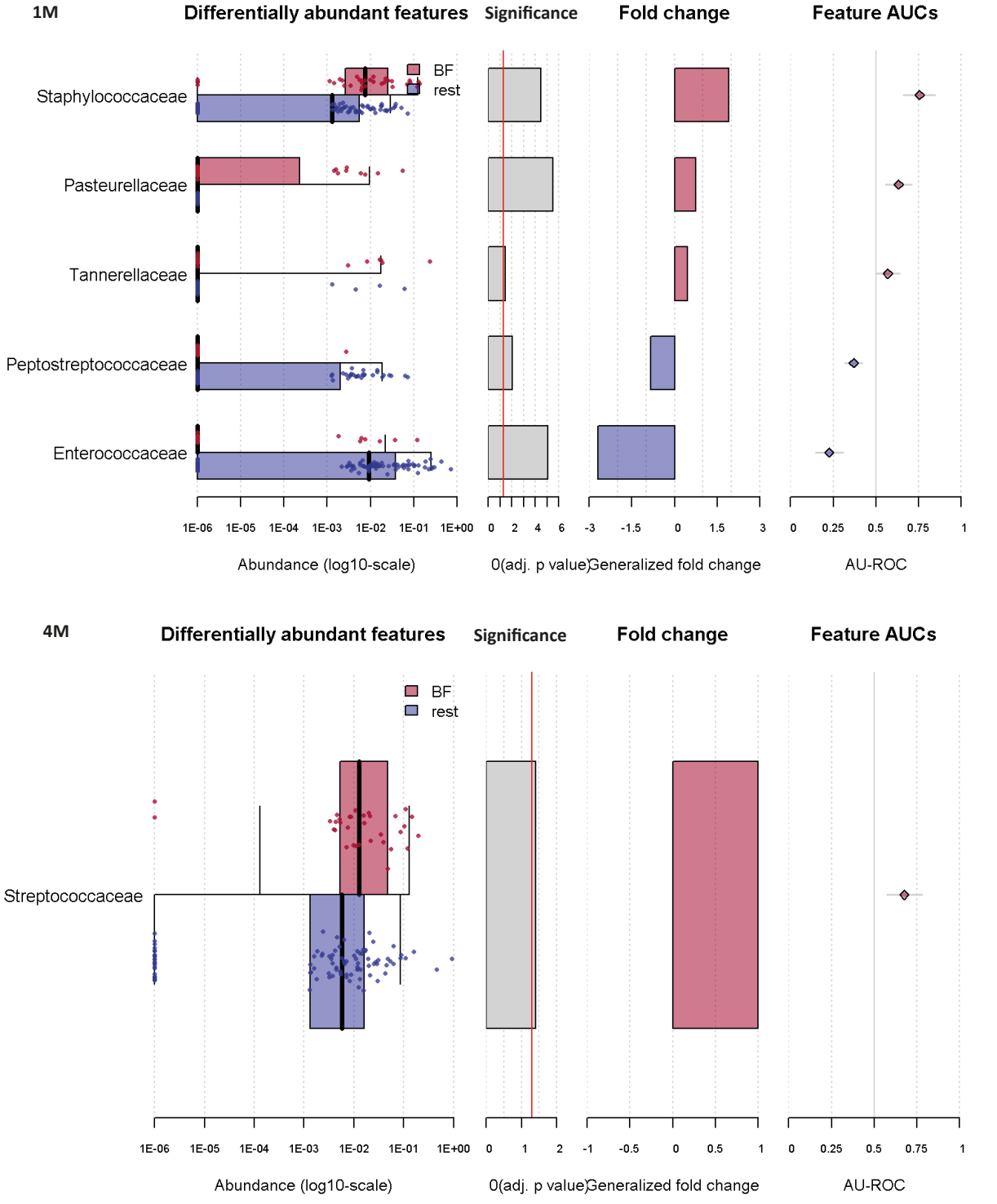


Figure 6: Differently abundant features, significance, fold-change and feature AUC related to the machine learning protocol; top: 1M; bottom: 4M.

LOWER LEVELS OF BLOOD SERUM UREA IN CHILDREN THAT RECEIVED THE INTERVENTION PRODUCT CORRELATED TO THE MICROBIOME COMPOSITION

Of all infants included in the study blood serum was analyzed at 4 months of age. Urea levels in blood were significantly lowered by the application of the intervention formula (Figure 7). The blood urea level was significantly lowered to close to the level of the breastfed infants ($p<0.05$).

To study the statistical relationship of this result to the microbiome composition, *adonis* (vegan package) was applied to search for a general correlation between the microbiome composition and urea concentration. No clear relation was observed. MaAslin analysis was performed to determine any significant taxa associated with urea level. At the genus level, only one genus showed significance in a relation to urea serum concentrations, namely the genus *Rothia* (Figure 8l, Supplemental Table 3). However, at the ASV level at trend correlation occurred (ASV_125655102, Figure 8r). In theory this constitutes specific bacteria strongly relating to either low or high urea concentrations. BLAST reveals, that this ASV is likely belongs to *Bifidobacterium longum* sp.

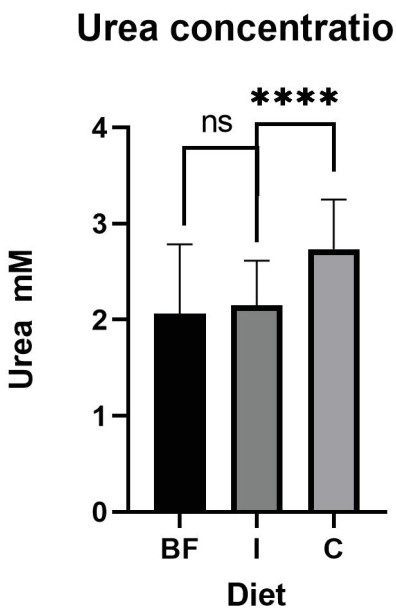
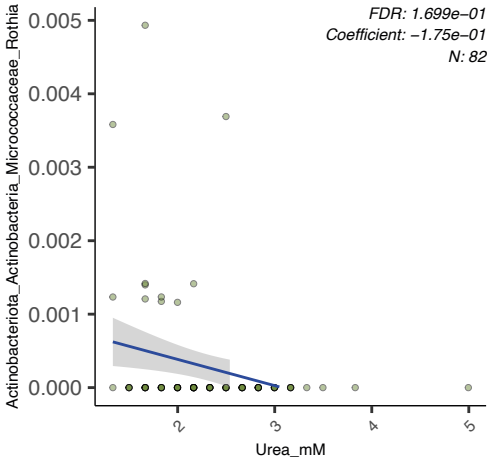


Figure 7. Blood urea levels of infants included in the microbiome study (I:C = $p<0.0001$).



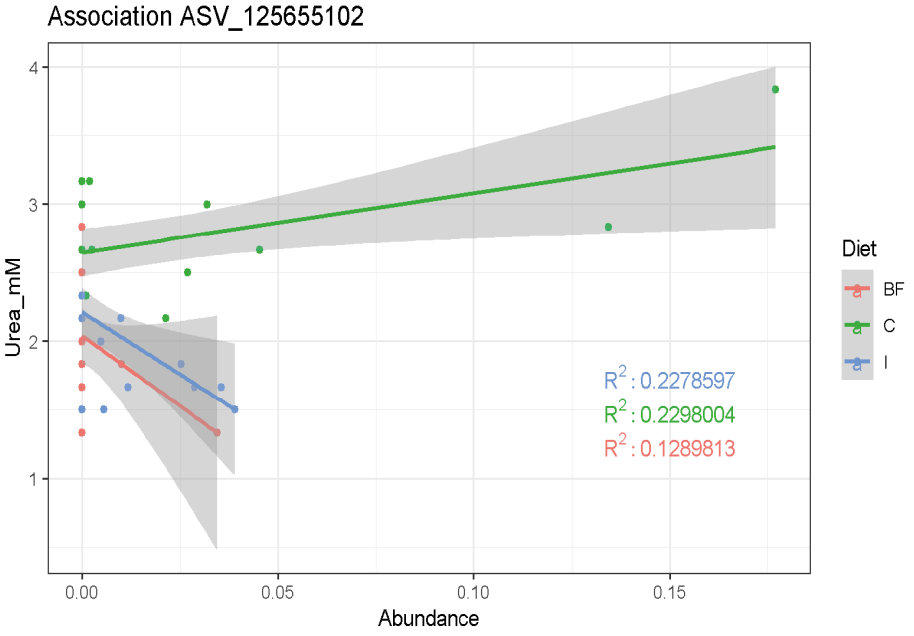


Figure 8. Top: MaAslin2 correlation on genus level towards urea concentrations, genus: *Rothia*; Bottom: Trend of individual example ASV (*Bifidobacterium longum* sp.) in relation to urea level.

METAGENOMICS FUNCTIONAL PROFILE: ANALYSIS OF UREASE-RELATED GENES AND FUNCTIONAL REDUNCANCY IN AMINO ACID METABOLISM OF THE MICROBIOME

Included here is a partial dataset (n=11/36, Supplemental Table 2). Unfortunately, due to not meeting quality control requirements, samples of infants at 4 months of age that received the intervention product were not included. HUMAnN2 was used to explore the functional profile of the bacterial gene capacity of the microbiome from the different diets and timepoints. Figure 9 shows a formula-fed infant with a relatively high abundance of *Bifidobacterium*- related urease genes. Beyond this infant, the

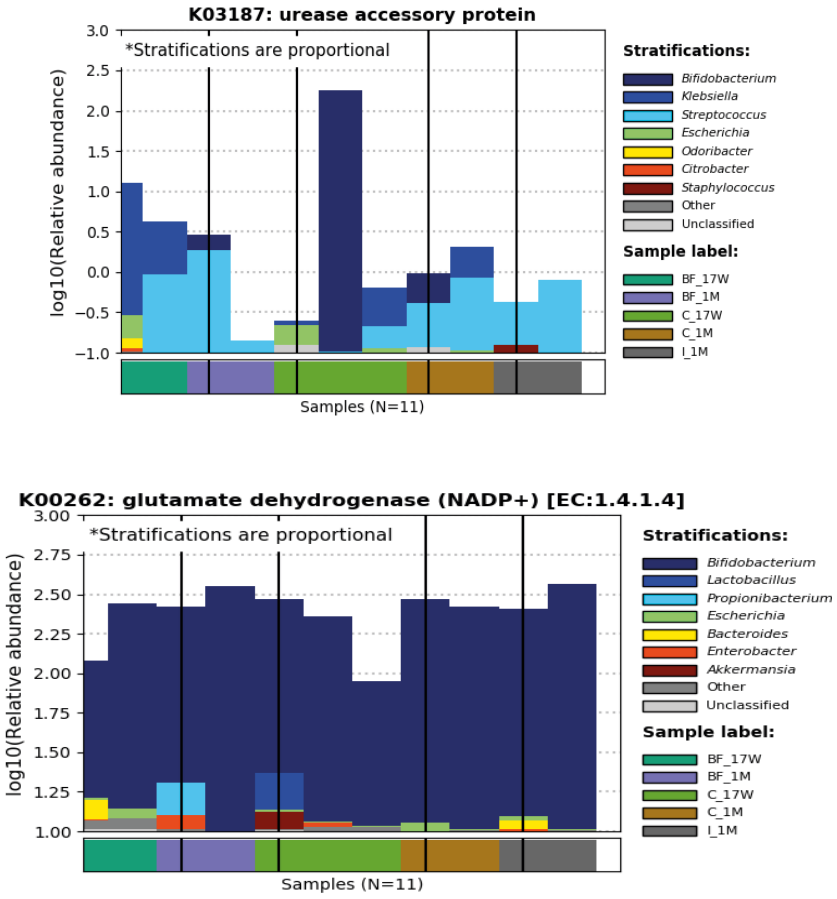


Figure 9. Partial data of metagenomic dataset belonging to the Proteus study, HUMAnN2 , log10 relative abundance of gene occurrence related to urease activity and free amino acid metabolism across the diets and timepoints: 1M (1 month) & 17W (4 months). KO identity can be found at: https://www.genome.jp/kegg/ko.html.

three infants with the most urease-related gene abundance are breastfed, but also one breastfed infant that is almost derived of this particular urease-related gene. Specifically, this *Bifidobacterium* gene encoding for a urease accessory protein involved in activating the urease enzyme is found in all three diet types [36]. Furthermore, the *Streptococcus* spp. version of the gene is tracked in eight out of eleven infants included in this analysis. Notably, the taxonomic assignment of the genes is a good representation of what genera are expected to be involved in urease activity according to our review in **Chapter 3**. Furthermore, we observe *Bifidobacterium*-related glutamate dehydrogenase genes making up the largest proportion for that gene. This is not surprising as the genus dominates the compositional data.

DISCUSSION

This study analyzed the impact of diet on the early life gut microbiota, with a focus on the effect of protein level on microbiota composition and function. In this study all infants are healthy and receive proper feeding throughout, which leads to a large overlap in microbiota composition, mainly because of the typical early life type dominated by *Bifidobacterium* spp. in Western Europe [4]. Interestingly, the beta diversity measurements showed a decrease between the 1 month timepoint and the 4 month time point in the intervention product, as we tend to see in breastfed infants [2]. Another notable effect of the I group is that the relative abundance models showed a significant increase in the relative abundance of *Bifidobacterium* spp. over time. This indicates that the modified low protein (I) diet, is more bifidogenic compared to breastmilk and the control.

The microbiomes of infants included in this study follow many hallmarks of infant gut microbiota development. For example, we observed the decrease in beta diversity in the breastfed group, which has been previously observed [2, 6, 35]. Interestingly, the intervention low protein product, showed a similar pattern, which could indicate a more selective effect on a certain part of the infant gut microbiota. Generally, the relative abundances of bacterial genera in these exclusively healthy infants did not differ greatly among the diets, underlining the current state of formula feeding. The level of variation in microbiota composition was reflected by the outcomes of several diversity metrics and principal component analyses (Figure1, Supplemental Figure 4). Ideally, the first two eigenvalues should capture most of the variation in the (dis)

similarity matrix. In this case, PCo1 explains 41.06% and 39.59% of the dissimilarity respectively. PCo2 captures 13.11% and 12.46%. The cumulative variation at 1 month of age equals 54.17% and 52.05% at 4 months, again showing the overall decrease in variation between the diets.

The changes in microbiota composition within this cohort, in and between diet groups can be a result from different diets. Higher protein concentration can make it more likely for the infant gut microbiota to interact with exogenous protein. In reverse, the level of free AAs can impact the bacterial composition by the existence of auxotrophy amongst bacteria for these AAs [14, 37]. Making the diets isocaloric by the application of lactose, might be an explanation for the increase in *Bifidobacterium* spp. in the intervention group. Lactose is built from galactose and glucose and is cleaved by the enzyme activity of lactase in the intestines of neonates [29]. This cleavage is commonly performed by *Bifidobacterium* spp., but *Lactobacillus* spp., *Bacillus* spp., *Escherichia coli* (*E. coli*), *Streptococcus thermophilus* (*S. thermophilus*), and *Enterobacter aerogenes* are also equipped with lactase genes [30]. For the breastfed and intervention group, a significant decrease in relative abundance was detected for *Streptococcus*. For the breastfed group, the HMOs present in breastmilk can be degraded by *Bifidobacterium* spp., which gives them a selective advantage. HMOs are fermented by *Bacteroides* spp., and *Bifidobacterium* spp. [31-33], which complies with our compositional results where we see *Bifidobacterium* spp. increasing in the breastfed group.

There are plenty of studies relating diet to microbiome composition and specifically comparing formula-fed infants and breastfed infants. However, the crucial period for studying the establishment of a health-promoting microbiome might be the first month of life. Most infants have already reached the relatively stable microbiota state associated with milk feeding at the 1m timepoint (Supplemental Figure 5, showing beta diversity plots & amount of breastfeeding during the first month). However, the applied depth in this microbiome study is insufficient to reliably study shifts within *Bifidobacterium* spp. The application of plant-oligos GOS/FOS in modern formula products, results in an increase of *Bifidobacterium* spp. in the infant gut, albeit different species [55, 56]. Different intervention groups in this study likely contain different *Bifidobacterium* spp., This would require increased depth, or any long-read sequencing the sequencing method, or a switch of hypervariable region to study this effect in the right resolution (e.g. ITS region). In the future, scientists could retrieve this kind of information from functional metagenomics databases. Logically, the HUMAnN2 pipeline also includes taxonomic assignment step. However, currently there is a lack of a sufficiently broad

spectrum of annotated *Bifidobacterium* spp. genomes to reliably assign metagenomic reads to lower phylogenetic levels during functional profiling [54].

In this study we applied machine learning to study the level of discrimination between the diet groups. We observed that the total formula-fed group, including the intervention group, could not be as easily distinguished from the breastfed group at 4 months of age. A valid discussion point highlights that the study size is too small to apply machine learning algorithms. However, our applied cross-validation method reduces the risk of a small data set [50]. Nonetheless, the application of machine learning for this microbiome study furthermore underlined the function of the modified low-protein formula, in establishing a health-promoting microbiome.

Considering the similarity of the gut microbiomes in this study, which included solely healthy infants it is not surprising no general correlation exists with the blood urea level. It is still likely the microbiome is involved in regulating urea levels in the infant host, but further research showing actual urease activity levels is warranted. Urea can be generated by bacteria from degrading protein, peptides and amino acids [40-42]. The urea can then be transported inside colonocytes and exert both beneficial and deleterious effects on these epithelial cells depending on their luminal concentrations. Some of the bacterial metabolites in colonocytes are released in the blood circulation and exert various effects on and in peripheral organs and tissues [13, 14]. We mined the compositional data for relations between microbiota composition and the urea levels. We highlighted an ASV (*Bifidobacterium longum* sp.) that shows a strong linear relation with the level of urea. However, a reversed pattern is observed for the control diet, namely an upwards trend: More abundance, but also slightly higher urea (R^2=0.2298004). Nonetheless, this method may provide interesting insight into specific bacterial species affecting the urea levels through their activity. The lower urea in the intervention group can indicate an improved functioning of the microbiome or the human host in removing urea from circulation. It warranted a search into the functional capacity of the infant gut microbiome to contribute to this effect. Therefore, we decided to functionally analyze the infant's gut microbiota to determine a potential relationship between bacterial activity and the diet types in this cohort.

From our metagenomics dataset it is clear that potential urease activity can be very diverse between infants in this cohort. One formula-fed infant has a great abundance of urease genes associated with *Bifidobacterium* spp., which would be the opposite indication of our theory in Schimmel *et al.* [12]. Interestingly, *Streptococccus* spp.

appear to be the main facilitator of urease activity in this cohort across the diets. Notably, *Streptococcaceae* are, in our machine learning approaches, a significantly different bacterial family at 4 months of age. In line with our observations of lower blood urea in breastfed infants, it contributes to the machine learning models by defining the breastfed microbiome as having higher abundance of *Streptococcaceae*. Counterintuitively, we see *Streptococcaceae* as discriminant of the control formula group in Figure 4. Therefore, it remains to be investigated if *Streptococcaceae* have a relevant contribution to urea processing in the infant gut. The level of glutamate dehydrogenase genes is constant across the diet, indicating an equal capacity of producing ammonium, which is the preferred nitrogen source for gut bacteria, from the main free amino acid in human milk and the formulae. The level of glutamate dehydrogenase genes assigned to *Bifidobacterium* spp. has led to the hypothesis that free HM-derived amino acids are mainly utilized by *Bifidobacterium* spp.and could explain why we see further promotion of the genus in the intervention formula, where glutamate replaces part of the dietary protein supply. Evidence has shown that *Bifidobacterium* spp. are very efficient in processing glutamate which might be why they are so successful in the early life gut [54]. Moreover, it is another reason the modified low protein formula included for the Proteus cohort, promotes *Bifidobacterium* spp. more efficiently. To further apply functional profiling to this microbiota study and bind meaningful conclusions to it, a larger sample set is required.

Another potential health implication potentially deriving from changes in the microbiome is the blood urea level in the included infants. Bacteria removing urea from the infant gut can be crucial to infant health since liver and kidney systems are underdeveloped compared to adult life [38, 39]. Especially since, a high protein level can increase gut urea, as a product of fermentation of amino acid and peptides [40-42]. *Bifidobacterium* spp. have been linked to lowering urea in human hosts [12, 43-45]. Before this study, we hypothesized a role for the microbiota in regulating the urea levels in infants through urease activity. That hypothesis is not faulted, however this study shows no clear signal in composition and in function that explains the lowered level of urea in especially the intervention infants. However, the significant increase in *Bifidobacterium* spp. and specifically *Bifidobacterium longum* spp. in that diet group can indicate that group's role in maintaining urea homeostasis. There are however plenty of other aspects that can be impacted by diet that possess feedback on infant health. First of all, the exact species, or even strain-level composition of the bifidobacteria can matter. All things considered, variation and specialization in this genus is high [12, 46, 47]. Diet can furthermore impact fecal structure (Bristol stool

scale) or fecal pH [48, 49], which are indicators of gut health not included for the Proteus cohort. Further health impact of lowering formula protein for infants should be considered and studied.

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CONFLICTS OF INTEREST

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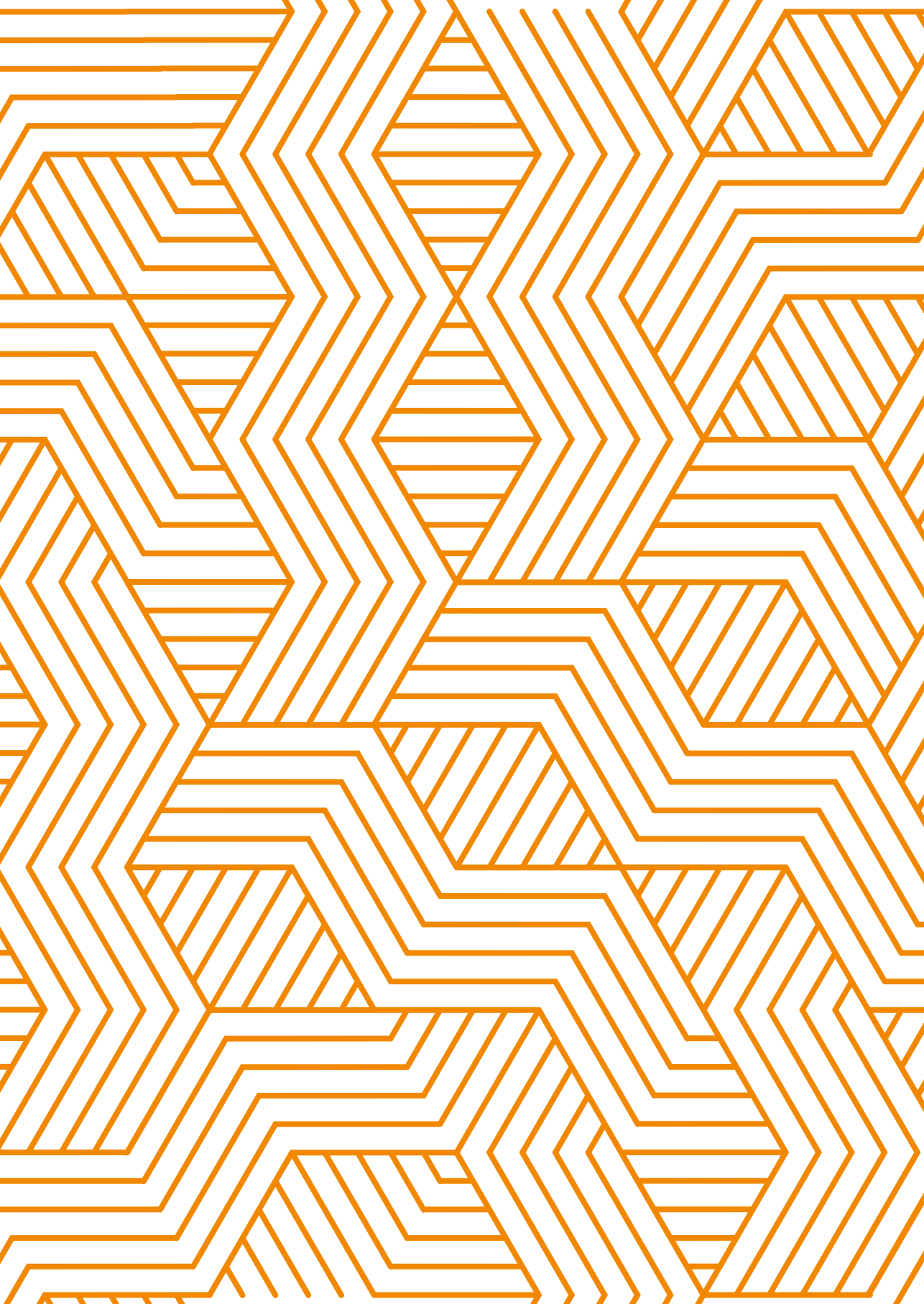
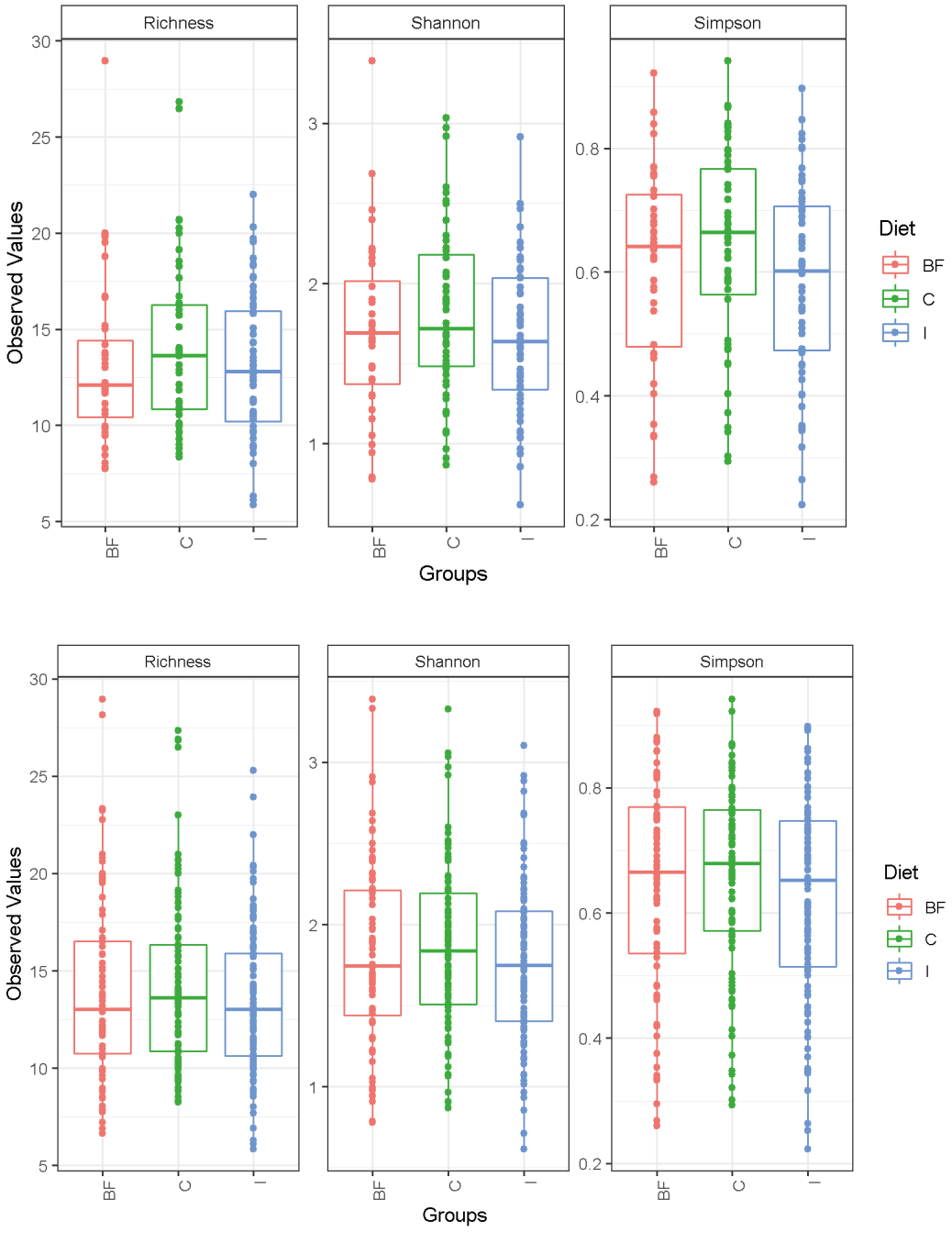


SUPPLEMENTAL FIGURES AND TABLES



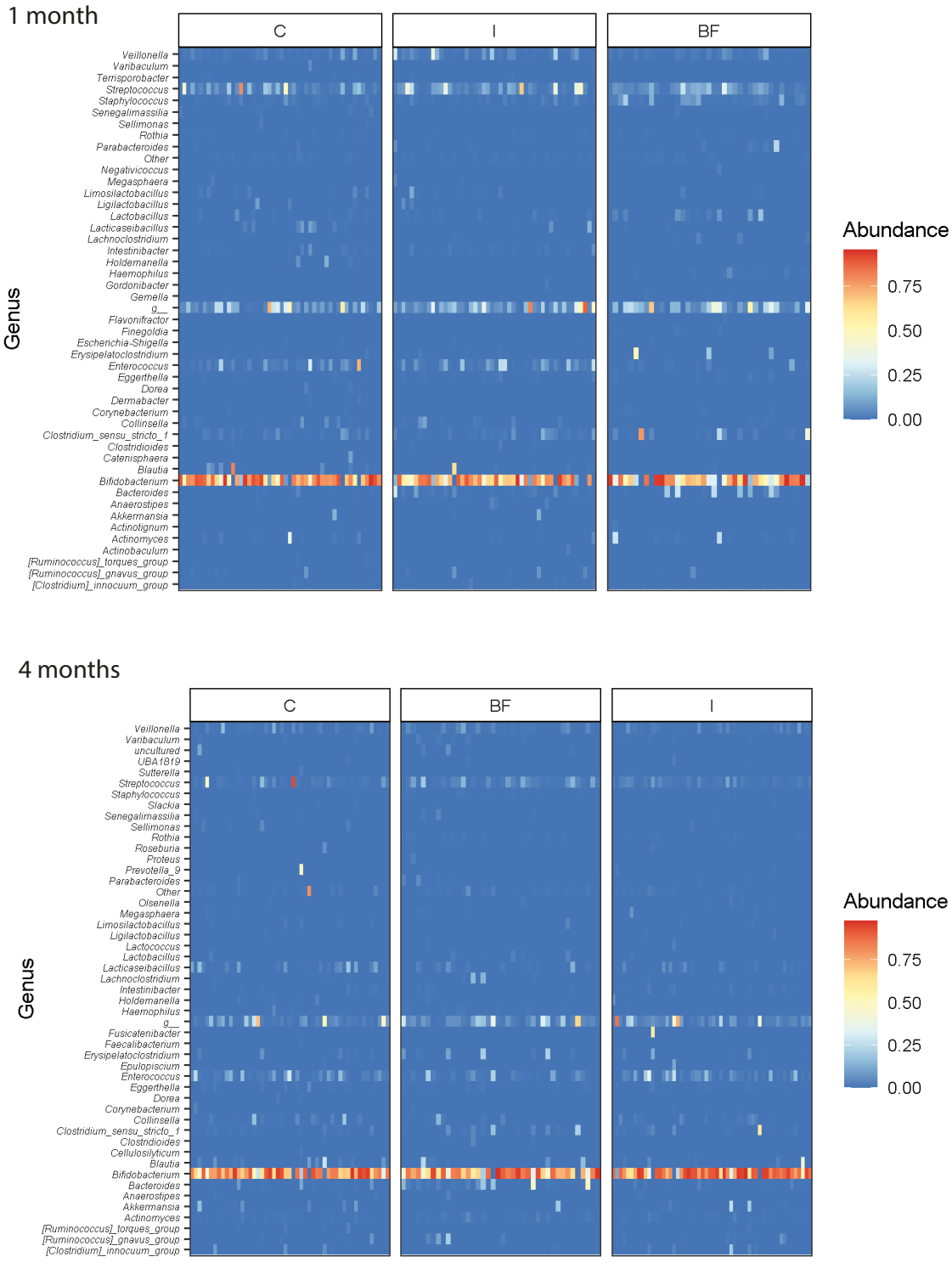
Supplemental Figure 1. Alpha diversity indices, Richness, Shannon and Simpson for the two timepoints (1 month of age; 1M; 4 months of age; 4M). BF: Breastfed infants, C: infants that received the control formula, I: infants that received the intervention product.

Family T1, T2	BF	C	I	Mean	BF	C	I	Mean
Bifidobacteriaceae	59.03	63.9	56.64	60.19	69.11	71.53	76.41	72.35
Enterobacteriaceae	13.59	11.80	15.54	13.64	8.81	6.87	7.97	7.85
Streptococcaceae	6.83	8.20	9.54	8.18	3.58	4.07	1.59	3.08
Enterococcaceae	0.53	4.71	4.23	3.19	2.29	3.97	4.17	3.46
Veilonellaceae	1.18	1.29	4.97	2.48	1.33	1.37	1.10	1.26
Clostridiaceae	4.13	1.18	1.33	2.21	1.59	0.29	1.34	1.07
Lachnospiraceae	0.71	2.57	2.05	1.78	3.35	2.85	3.17	3.12
Bacteroidaceae	3.86	0.10	1.31	1.78	4.21	0.59	0.21	1.67
Lactobacillaceae	1.48	1.53	1.36	1.46	1.13	2.33	0.67	1.38
Staphylococcaceae	3.01	0.53	0.62	1.39	0.08	0.01	0.02	0.03
Other	5.61	4.19	2.40	4.07	4.58	6.21	3.37	4.72

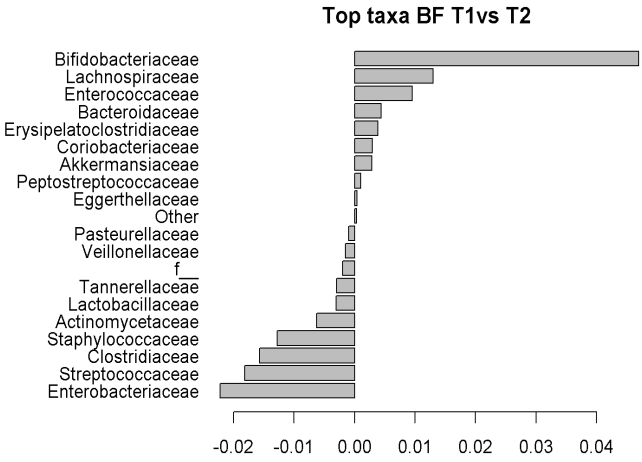
Supplemental Table 1. Table showing percentile values of relative abundance coming from analysis per diet. BF: Breastfed infants, C: infants that received the control formula, I: infants that received the intervention product.

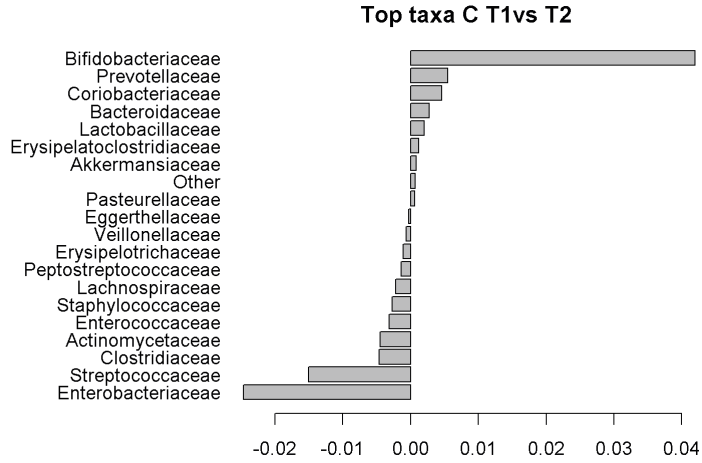
Study ID (DNA processing ID)	Metadata Code	Study Inclusion ID	Isolated DNA Concentration (Qubit)
1	C_1M	101025	181.6
3	I_1M	101026	305.4
8	C_4M	p101025	284.9
10	C_1M	101064	377
14	C_4M	p101042	293
16	C_4M	p101016	279.4
24	BF_1M	901007	266.7
27	BF_4M	P901042	144.5
46	BF_1M	901005	215
50	I_1M	p101015	300
55	BF_4M	p901028	329.3

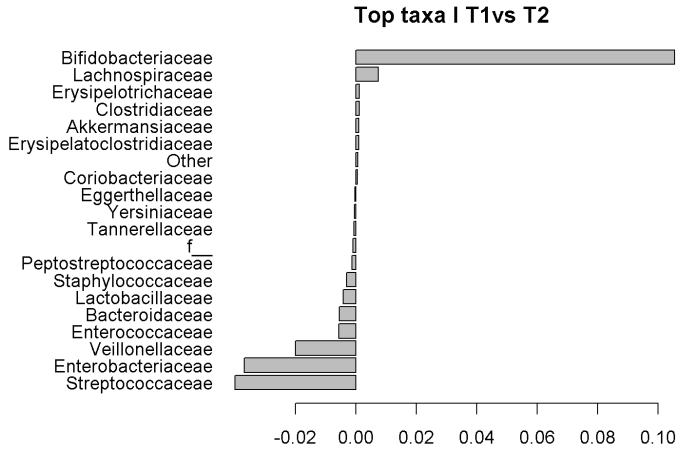
Supplemental Table 2. Metadata of included metagenomic DNA for functional profile analysis.



Supplemental Figure 2. Heatmaps showing relative abundance of all samples across diets over the two timepoints (1 month of age; 1M; 4 months of age; 4M). BF: Breastfed infants, C: infants that received the control formula, I: infants that received the intervention product.



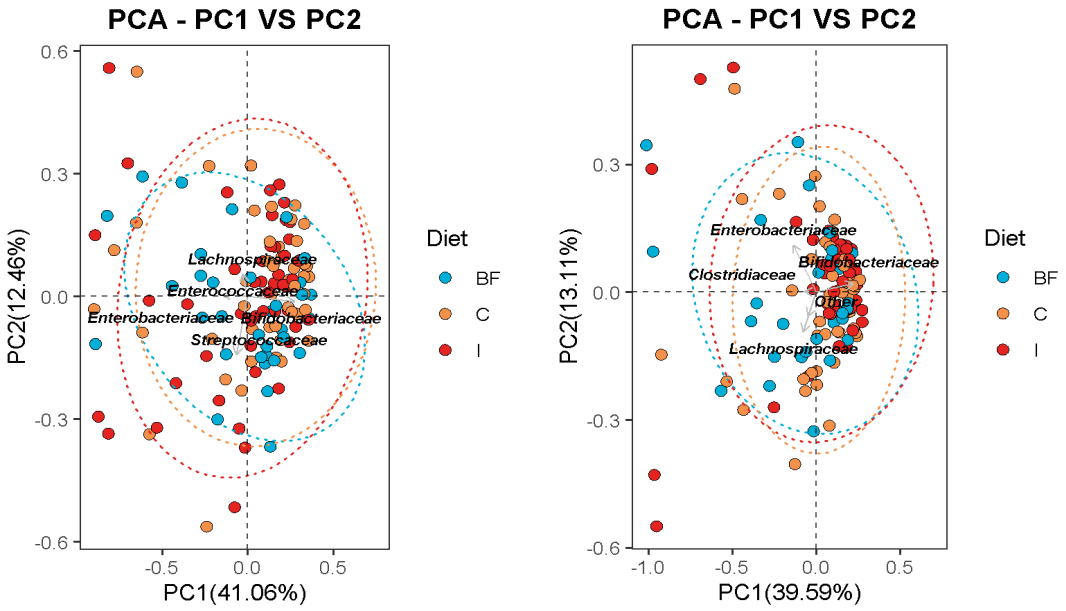




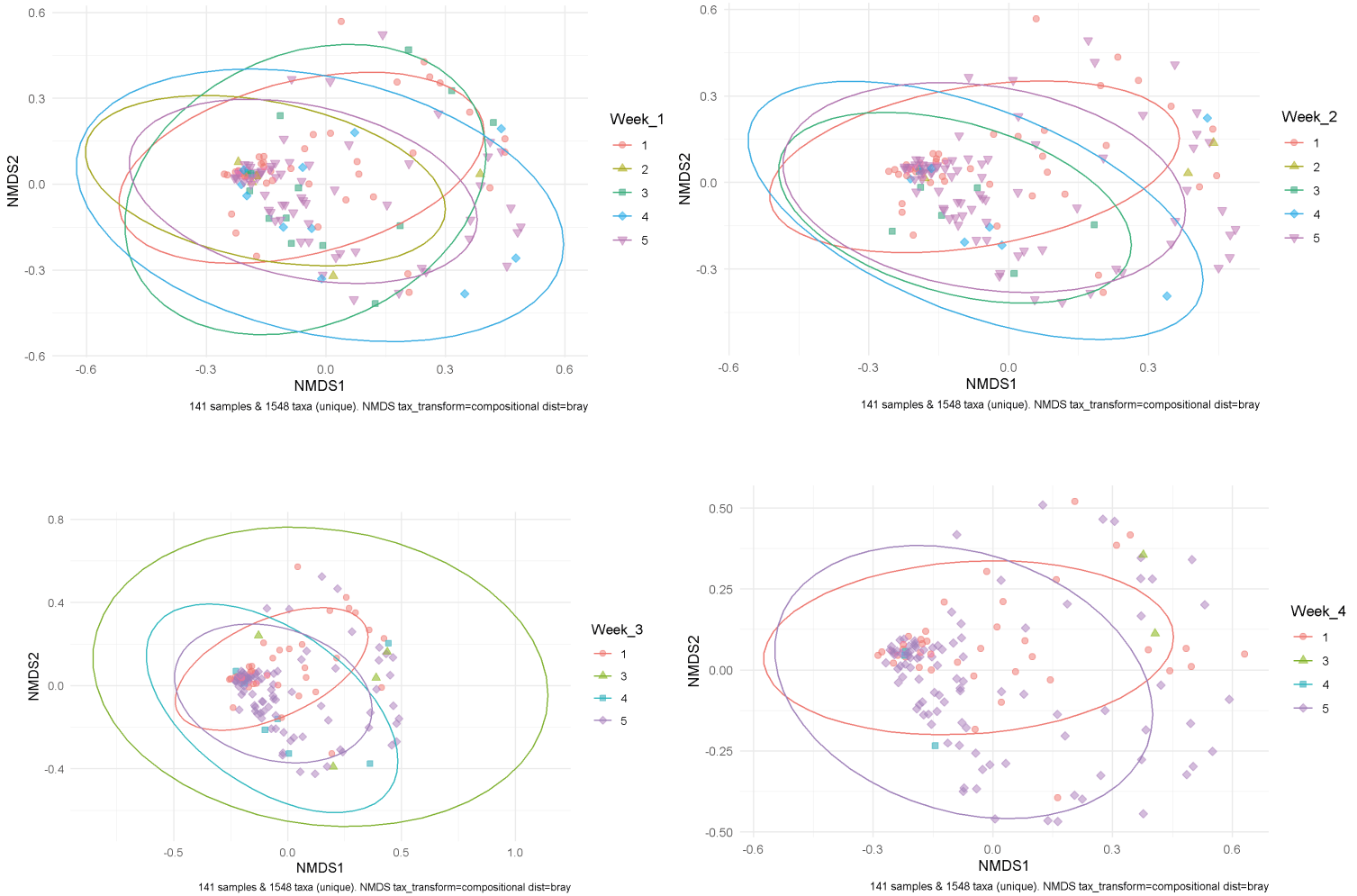
Supplemental Figure 3. PERMANOVAs, discriminant analysis between study timepoints within diet. BF: Breastfed infants, C: infants that received the control formula, I: infants that received the intervention product.

feature	metadata	value	coef	stderr	N	N.not.0	pval	qval
Actinobacteriota_Actinobacteria_Micrococcaceae_Rothia	Urea_mM	Urea_mM	-0.174679929295146	0.0651856090909634	82	11	0.00894282373700998	0.16991365100319
Proteobacteria_Gammaproteobacteria_Enterobacteriaceae_g__	Urea_mM	Urea_mM	-0.350342819581104	0.279000323750299	82	75	0.212877169415734	0.62127744961944
Firmicutes_Bacilli_Staphylococcaceae_Staphylococcus	Urea_mM	Urea_mM	-0.162171380109389	0.105695739281142	82	11	0.128895780471859	0.62127744961944
Firmicutes_Bacilli_Streptococcaceae_Streptococcus	Urea_mM	Urea_mM	-0.3496895108699	0.249845702622778	82	69	0.165492410445226	0.62127744961944
Firmicutes_Bacilli_Enterococcaceae_Enterococcus	Urea_mM	Urea_mM	0.40861775287928	0.337005869406992	82	53	0.228891691965057	0.62127744961944
Firmicutes_Clostridia_Lachnospiraceae_.Ruminococcus._gnavus_group	Urea_mM	Urea_mM	0.24520604008845	0.170978451899473	82	10	0.155431339629802	0.62127744961944
Firmicutes_Clostridia_Peptostreptococcaceae_Intestinibacter	Urea_mM	Urea_mM	0.217974158556114	0.149064126350527	82	15	0.147580703627612	0.62127744961944
Firmicutes_Clostridia_Lachnospiraceae_Blautia	Urea_mM	Urea_mM	0.25471630902756	0.237985710877705	82	14	0.287702489769959	0.683293413203652
Firmicutes_Bacilli_Lactobacillaceae_Lacticaseibacillus	Urea_mM	Urea_mM	0.213557139913838	0.276131703895379	82	28	0.441571661990919	0.762714688893406
Firmicutes_Bacilli_Erysipelatoclostridiaceae_Erysipelatoclostridium	Urea_mM	Urea_mM	-0.163501120688747	0.185069036498604	82	11	0.379634931676171	0.762714688893406
Actinobacteriota_Coriobacteriia_Eggerthellaceae_Eggerthella	Urea_mM	Urea_mM	0.0997435717627048	0.12661684596334	82	18	0.433165514644235	0.762714688893406
Bacteroidota_Bacteroidia_Bacteroidaceae_Bacteroides	Urea_mM	Urea_mM	-0.0426510023614632	0.248258762078991	82	21	0.864028144928921	0.951859074656228
Actinobacteriota_Actinobacteria_Bifidobacteriaceae_Bifidobacterium	Urea_mM	Urea_mM	-0.0197317896174208	0.124387373718353	82	80	0.874358762347457	0.951859074656228
Actinobacteriota_Actinobacteria_Actinomycetaceae_Varibaculum	Urea_mM	Urea_mM	-0.0424012362390106	0.0963985049556717	82	10	0.661229110783286	0.951859074656228
Actinobacteriota_Actinobacteria_Actinomycetaceae_Actinomyces	Urea_mM	Urea_mM	0.0207990726235406	0.183618330526734	82	47	0.910097579491784	0.951859074656228
Proteobacteria_Gammaproteobacteria_f___g__	Urea_mM	Urea_mM	-0.00946800247594816	0.156335686921862	82	14	0.951859074656228	0.951859074656228
Firmicutes_Negativicutes_Veillonellaceae_Veillonella	Urea_mM	Urea_mM	0.0971395390904415	0.271151426303141	82	44	0.721101091044392	0.951859074656228
Firmicutes_Clostridia_Clostridiaceae_Clostridium_sensu_stricto_1	Urea_mM	Urea_mM	0.0228114598583048	0.243222929073212	82	26	0.92551184044287	0.951859074656228
Actinobacteriota_Coriobacteriia_Coriobacteriaceae_Collinsella	Urea_mM	Urea_mM	0.0854777909349533	0.255252497517174	82	22	0.738595511026298	0.951859074656228

Supplemental Table 3. Significance results of correlation analysis of family level microbiome composition versus infant blood urea levels.



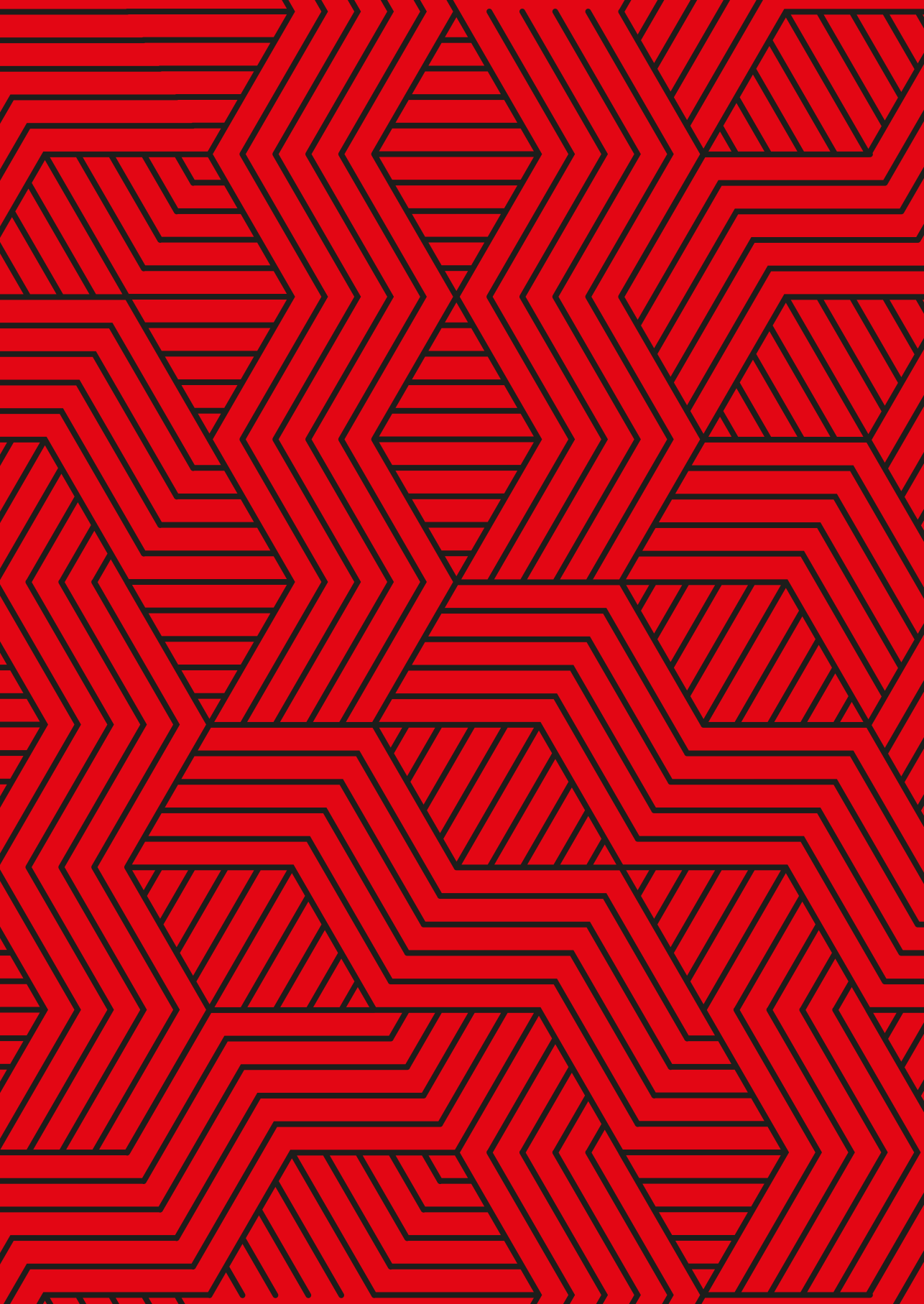
Supplemental Figure 4. Principal Component Analysis showing the variation between the diets and individual samples. Left: 1 month of age, right: 4 months of age, BF: Breastfed infants, C: infants that received the control formula, I: infants that received the intervention product.

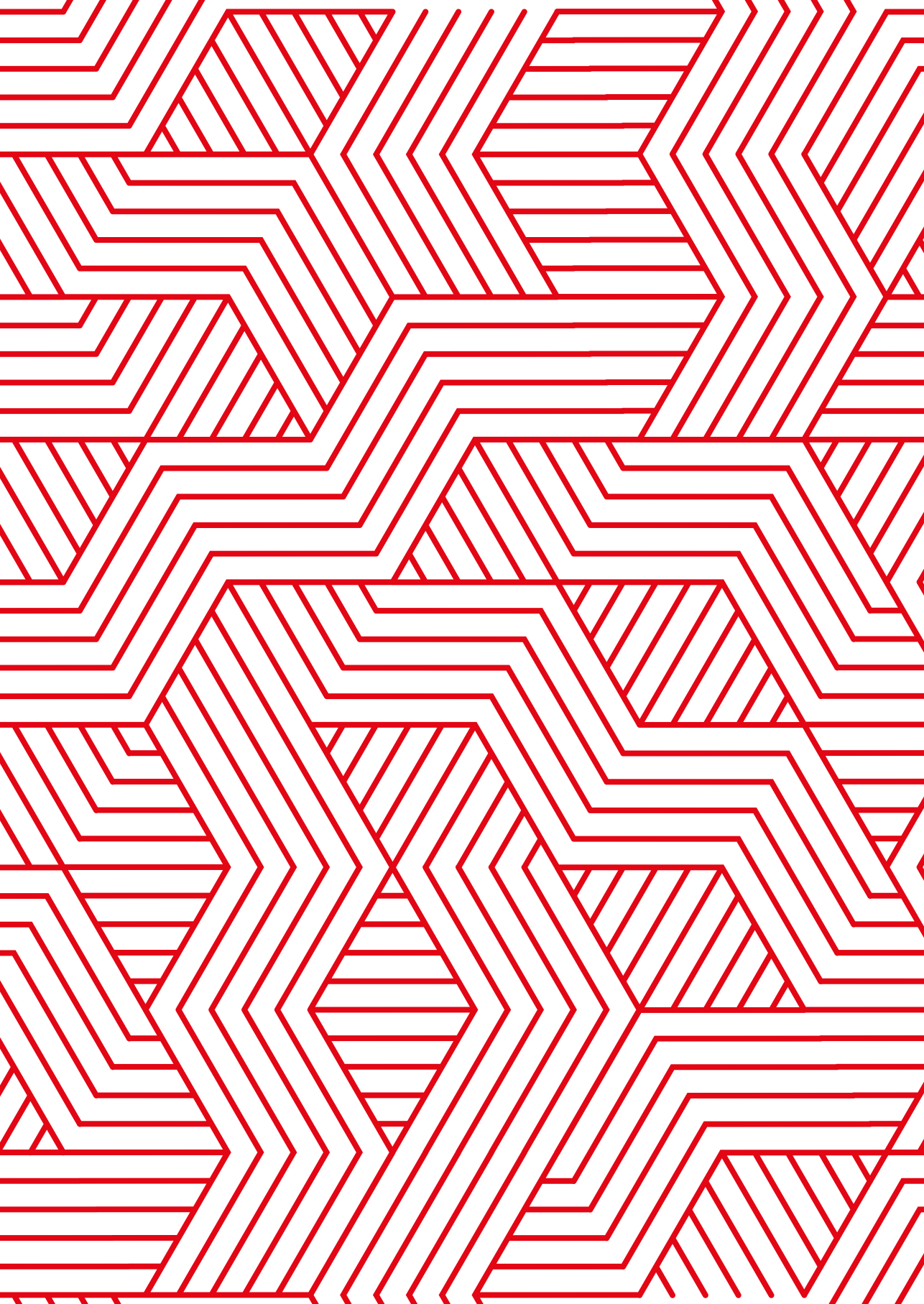


Supplemental Figure 5. NMDS beta-diversity plots showing the organization of the individual samples at 1 month of age by the level of breastfeeding during the first four weeks of life. From level 1 (low breastfeeding <20%) to level 5 (high breastfeeding, 100%).

‘Life finds a way’

—MICHAEL CRICHTON





CHAPTER 6

GENERAL DISCUSSION

The infant diet is crucial in establishing a gut microbiome that supports healthy development in early life. The settlement of the intestinal microbiota from birth towards the transition to solid food is a well-orchestrated process which is the result of co-evolution between microbes and humans. The great scientific challenge is not only to investigate the complexity of both human milk and the gut microbiota but also to connect them both. Being able to study the microbiome through (complex) *in vitro* and bio-informatics approaches increases our understanding of its functioning. Furthermore, this is achieved by applying clinical data on the differences and relations between infants, diet types, health statuses and the intestinal bacteria. Looking at diet, the research field concerning the infant gut microbiome was mostly focused on the prebiotic human milk oligosaccharides (HMOs). Science has established their role for the infant gut microbiota, which is providing an important carbon and energy source. In this thesis we discuss both a top-down (genomics) and bottom-up (*in vitro*) approach towards describing the early life gut microbiota, specifically in relation to the available nitrogen sources in the early life gut. Hence, this thesis adds knowledge on the interaction between dietary nitrogen in early life and the composition and functioning of the gut microbiota during that crucial phase.

THE INFANT GUT MICROBIOTA: A FOUNDATION FOR HEALTH

The infant gut is the primary example of a (largely) sterile human environment being colonized by "a plethora of microbial communities" [1]. This orchestrated process can be defined by stochastic processes and host-microbe coevolution. Early life diet, after initial colonization of facultative anaerobes, is one of the main drivers for the bacterial part of the microbiota during the first postnatal months towards a more strictly anaerobic community [2, 3]. It has been indisputably shown that specific factors in human milk support selective colonization. The major example is that *Bifidobacterium* spp. and other glycan-degrading bacteria have adapted to dealing with specific glycan components of this human secretory fluid. Notably, other maternal factors responsible for the establishment of the early infant gut bacteria include mode of delivery, host genetics, gestational age, maternal diet and health status, antibiotic usage, pet presence and hygiene [4-10].

As described several times in this thesis, bifidobacteria represent the norm for the western infant gut microbiota and this group of beneficial bacteria is already being

exploited by food industries as probiotics. If you compare 'older' scientific contributions with modern Northern European studies, you see the contributions of *Bifidobacterium* spp. growing. A previous 'extinction' of bifidobacteria can be assessed in the light of changing Western diets, including the shift to formula milk instead of human milk in the last century [11-13]. Improved formula products have countered that effect and led to a more recent increase in *Bifidobacterium* spp. in formula-fed infants compared to formulae not containing bifidogenic compounds [14, 15]. Of course, we have no data available on the microbiota composition of humans in Medieval days, or perhaps the Stone Age. I would speculate, in current times with our dietary practices, there is a potential that bifidobacteria are overestimated and overselected, since we mostly added formula components that are described as bifidogenic [14,15]. It should be defined that not every formula diet increases bifidobacteria beyond the breastfeeding reference [216]. In **Chapter 5** we describe the bifidogenic effect of an experimental infant formula with lowered protein content. This increase in *Bifidobacterium* spp. might be due to other *Bifidobacterium* spp. present in the infants compared to the ones in the breastfeeding reference, for which we lack the genomic resolution. Furthermore, a potential technical overestimation might be caused by the fact that *Bifidobacterium* spp. are gut lumen-based bacteria that experience a higher gut flowthrough compared to for example bacterial species occupying niches in the mucus layer. This can lead to an overestimation of the abundance of *Bifidobacterium* spp. in human fecal material. However, if we look at the occurrence of bifidobacterial across mammals, we see a broad occurrence across lifespans, body sites and specialization for different hosts, which indicates the importance of the genus [50, 211].

A myriad of clinical trials provided (mainly bacterial) data on the infant's gut microbiome, which has led to the realization that certain developmental trajectories come with a lower risk of certain disease patterns [8, 16, 17]. That includes microbiotas dominated by bifidobacteria. On the other hand, discrepant microbiota composition have been described in relation to a lower health status or occurrence of diseases, has led to labelling some microbiota compositions as dysbiotic. Dysbiosis is an imbalance present in a person's microbiome contributing to the health status of that person [18]. However, prospective cohorts, that include longitudinally tracking infants for longer periods of life in terms of their gut microbiota and health status in later life are still scarce [212-214]. Nonetheless, it has become increasingly clear that there is a role of the microbiome in laying a foundation for a healthy life unto adulthood. Novel experiments concerning the infant gut microbiota and its contribution to health will lead to pivotal discoveries that can impact functional food or therapies in a medical setting.

NITROGEN AND THE IMPACT ON THE MICROBIOTA AND BACTERIAL SURVIVAL

From many environments where bacteria 'inhabit' and 'cohabit' with higher organisms we know that nitrogen is a main determinant of the outcome [19-24]. Nitrogen availability for bacteria is absolutely essential since nitrogen (atom number 7) is the atomic building block of the molecular building blocks; the nucleic acids and subsequently the amino acids. Any independent living organism therefore has to have some extent of nitrogen metabolism to maintain their cells and nucleic acids, which in turn form proteins that are essential for almost any function; as enzymes, messengers, structural components and nutrient storage. Bacteria are no different. However, a fundamental part of studying microbial physiology and ecology concerns why certain microorganisms prefer certain nutrients over others. Interestingly, human milk is relatively high in non-protein nitrogen (**Chapter 3**). This indicated some nitrogen sources of interest that can impact bacterial survival and success of colonization in the infant gut.

Because of the necessity of nitrogen, bacteria possess core nitrogen metabolism pathways in one form or another, since it is a strict requirement for life. In contrast, nitrogen can also be an insidious pollutant when applied in large volumes to an environment. For example, as plant fertilizer in agricultural settings, which has led to a 'nitrogen crisis' in the Netherlands [234]. There is one constant: bacteria in all environments process nitrogen for their survival. The core of bacterial nitrogen metabolism pathways encircles amino acids that are commonly available in many environments [25, 26]. However, for many microorganisms the preferred nitrogen source is ammonium, and we have measured ammonium in many of our cultivations as a determinant for nitrogen cycling. Ammonium is likely the preferred nitrogen source due to physico-chemical constraints of larger nitrogen sources, e.g. the amino acids [27]. However, having access to more constrained nitrogen sources can lead to a significant competitive advantage. Unsurprising, for many bacterial model systems efficient catabolism on amino acids and survival was observed [28-30]. Interestingly, the key human milk nitrogen (HMN) sources glutamate and glutamine have been indicated as internal sensors of nitrogen availability in bacterial cells [31]. Finally, in **Chapter 3** we highlighted a differentially regulated nitrogen regulatory protein in breastfed infants. The system that this specific protein is part of has been highlighted as important in tuning the amino acid transport systems and catabolism components, in order that essential nitrogenous nutrients are retained [27]. With that in mind, it

is in fact surprising how little has been described of nitrogen metabolism in the early life gut compared to for example the soil and plant environment and even the adult human gut [19, 22, 23, 87, 217]. This thesis underlines the importance of considering nitrogen for its impact on the human microbiome in **Chapters 2**, **3** and **5**.

In **Chapter 3**, our extensive review covers the relationships between HMN and the early life microbiome. Our main focal points included the free amino acids, urea, creatine/creatinine and nitrogen from HMOs. Our literature study shows that there is plenty of evidence of catabolic activity by the microbiome on HMN. Our focus was on urea, as the main constituent of the non-protein nitrogen content of human milk. Urea and secondly urease, are indicated as key factors for establishment of the human gut microbiome, as it is a widespread enzyme and as it is featured prominently in *Bifidobacterium* spp. genomes. Interactions of the bacteria with creatine, creatinine and polyamines is far more elusive, but warrant interest for further research. Therefore, in **Chapter 2** we describe an interaction between a dominant HMN source and a common infant gut symbiont. Our work shows the activity of an infant gut bacterium that is part of the dominant genus *Bifidobacterium* to utilize and grow on urea. Urease activity is crucial for bacterial survival, due to the fact it supplies ammonium and counters low pH. This is observed in many bacterial model systems [32-34]. Physiologically, the gut microbiota furthermore possesses specific responses to nitrogen starvation [35]. Indicatively for this research field, this has been mostly described for *Escherichia coli*, a common model organism for bacterial infection, gut colonization and bacterial metabolism [218]. In the next years I believe, we should aim to describe nitrogen acquisition and processing under relevant ecological conditions that include the presence of HMN, low pH and limited nitrogen. This will be the case especially for common commensals that inhabit the infant gut, since they are most likely to interact with the diet. Not surprisingly, microbial communities have been shown to respond to long-term nitrogen application by showing changes in community composition [36]. We have also observed such shifts in **Chapter 5**, albeit minor, in microbiota composition. In this study, we observed longitudinal compositional shifts in the intestine of the newborn in response to lower protein concentrations and increasing amino acids concentrations in the infant's diet. The application of amino acid-based formulae in managing allergy and disease has been investigated and is more broadly applied [37-39]. These formulae provide health benefits for infants that cannot tolerate standard formula or human milk. Taking all of this together, I believe that HMN is determining the outcome of the infant gut microbiota composition and activity and subsequently infant health.

CO-EVOLUTION BETWEEN HUMANS AND EARLY LIFE MICROBES

Coevolution and co-diversifaction are processes where two or more species, through corresponding selection share a parallel evolutionary history [40, 41]. Cospeciation is the result of many years of coevolution between the human gut microbiome and species from the bacterial domain. Specific species occur in the human gut compared to occurring in a primate gut, for example. This indicates that coevolution is still ongoing and can be affected by our genetic background, our individual health status, but interestingly also, our current dietary habits as a species [42, 43]. All of this has raised fundamental questions on our perception of our microbiome, which at times is more of a philosophical question than a microbiological one [44]. It is about setting definitions that encompass a broad and correct understanding, whilst not making it too complex or too specific. This requires philosophical discussions on how we view the microbiome: as an organ of the human body or as a more independent entity. Nonetheless, applying a solid philosophical stance can contribute to building a solid foundation of terminology for microbiology and its ecology. Currently the definition of what constitutes a microbiota is still very much in development, as the research field realizes the importance of clear definitions and includes new insight into the respective definitions. To achieve this, I believe the current approach of reflecting on past definitions and developing simpler, broader definitions is an important step [44, 45]. Especially, considering the increasing importance of outwards communication for science, which will be discussed later. The definition of the microbiota should encompass the assemblage of all microbial life, not just bacteria and archaea, within a defined stage of operation and it does according to a recent definition update [45]. The microbiome is all microbial life including everything that contributes to its stage of operation, including the environmental conditions, microbial structural elements and microbial metabolites [45]. More and more the interaction between bacteria and other microbial forms that inhabit our gut are being described, including the mycobiome and virome [46, 47]. In the future, focusing on microbes that have shown to have coevolved and specifically cospeciated with us for studying and manipulating infant health will be an important approach.

The scale and complexity of our microbiome is extraordinary. Therefore, the efforts of describing the human microbiome, its function and its relation to health, will be a dominant scientific field for decades to come. Understanding coevolution of the infant gut microbiota as a whole will provide insight to what drives selection in that

environment and to what extent metabolic activity matters in the gut, a place where it will be hard for scientist to directly observe. Hence, the push for developments in more complex *in vitro* culturing systems, including human cell lines, will be discussed later [48]. I speculate that nitrogen absorption by the infant's growing body can make nitrogen scarce for the gut microbes. On the other hand, the human host might depend on certain metabolic transformations with nitrogen that occur through bacterial metabolism, for e.g. production of essential amino acids. *Bifidobacterium* spp. have been in focus when searching for infant gut probiotics and with good reason. Their importance in helping establish a healthy gut is nearly uncontested [49]. *Bifidobacterium* spp. show strong signs of a history of co-evolution alongside humans and their gut [50]. This thesis underlines that there is likely not only adaptation to carbohydrate availability but to nitrogen availability as well. The statement that nitrogen affects a microbial community is not groundbreaking, however the evidence for it is lacking and strikingly limited compared to research on infant gut carbon metabolism. We have established that HMOs, as carbohydrates, are available to the microbiota and that a certain part of that carbohydrate supply is accessible by some gut bacteria while not by [219, 220]. For nitrogen this is not clearly established [25, 26, 221]. Therefore, focusing on nitrogen may change our perception of the best diet for the young infant. Coevolution can be part of the reason why we should assume that nitrogen is important in early life diet considerations. I believe it is likely, the infant gut microbiota, and especially *Bifidobacterium* spp. have specifically adapted, as they have to the carbohydrate supply, to the nitrogen supply of human milk.

THE IMPORTANCE OF CULTURING AND FUNDAMENTAL GUT SYMBIONT STUDIES

With the arrival of genomics and later on more sophisticated top-down approaches used for studying bacterial communities in a wide range of environments, it has become harder to see the relevance of bottom-up, more fundamental microbiology approaches, like culturing and isolation of microbes [51]. Both **Chapter 2** and **Chapter 3** highlight the importance of microbial physiology for describing bacterial settlement in the early life gut, albeit only to counteract the biases coming from genomics approaches [51]. These biases for example include considering gene content over actual bacterial activity and considering fecal relative abundances as representative for the overall actual microbiota composition. In (clinical) microbiology, pure bacterial cultures are essential

for e.g. the study of virulence or antibiotic susceptibility and moreover for full genome construction to extract potential metabolic activity [51-53]. Therefore, they are crucial for understanding how individual members of a bacterial community function and interact with their surroundings. To be able to break down a bacterial community to essential metabolism, a bottom-up approach of looking at bacterial communities is required. Only in pure or small-community cultures can a close approximation of controlled conditions be achieved, by which more causal relationships of a bacterium's ecological niche can be described.

The apparent decline of more classical anaerobic microbiologists that was described by Lagier *et al.* [52], can explain what we see in the 'current state of microbiology'. Namely, that the field of microbial ecology is currently suffering from a tidal wave of high-throughput sequence data for which researchers have a hard time fully processing. One can imagine that this is currently leading to a decrease in the considered importance of more fundamental culture studies. In other words, more data does not directly lead to a more fundamental understanding of what is metabolically happening in the human gut. However, we are also in need of high-throughput data to counter the biases we may introduce with culturing experiments. For example, bacteria that are well-known to science, model systems, from the infant gut or anywhere else, might be among bacteria well-suited to life in a laboratory and the conditions we have set. We might overlook more fastidious bacterial strains from the microbiota. The century-old conundrum of the unculturable part of the population also comes to mind for this general discussion. Ever since the rise of sequencing techniques or fluorescent *in-situ* hybridization (FISH), the combination with culturing was deemed important for not missing environmentally relevant bacteria [53]. Nonetheless, the current lack of integration of genomic data and culture practices is a large obstacle to efficiently study the human microbiome.

Now, with increasing understanding of the complexity of the gut microbiome, effort should be put into devising laboratory conditions that more closely simulate the habitat the samples originate from. More accurate metabolic predictions and models derived from bioinformatics data can improve the efficiency by which we isolate and culture [54]. This will eventually help culture the yet unculturable. Furthermore, it is apparent that we are collecting more high-throughput data than the research field can analyze [51, 207]. On the other hand, predicting actual metabolic activity from genomic information can put microbiology in acceleration [222]. Not only 'classical' cultivation approaches can ben benefit from the integration of genomics data with

laboratory studies. Approaches that help us better study the gut microbiome and its bacterial strains *in vitro* are those that include the use of human cell lines, advanced chemostat culturing techniques and/or 'gut on a chip' approaches [54, 55, 207]. These still have to overcome significant gaps, culture-wise and technically. These gaps include consistent human cell line functioning and having all relevant bacterial functions induced as well, to make sure it is a representable technique for studying the interactions between microbes and humans [54]. For now, the most direct evidence of the impact of bacterial metabolism on health is mainly derived from clinical studies and intervention studies [208].

To improve success of culturing, you need to be aware of the ecology and physiology of the organism, which is a seemingly impossible conundrum. So, gut microbiologists should always be wary of any effects that occur because of a specific culture setting. During the making of this thesis, we observed plenty of nitrogen metabolism pathways impacting experimental design and conditions that we not always anticipated. A bacterium's functioning in a specifically controlled environment (e.g. well-defined medium, pure culture and chemostats), has been surprising at times. It shows the complexity and flexibility of infant gut bacteria. One of those outcomes was the occurrence of γ-aminobutyric acid (GABA) in the supernatant of our cultures and others [68, 72]. Because of its apparent function in human health, I believe GABA will be crucial for describing infant gut nitrogen metabolism.

GABA AS A KEY NITROGENOUS COMPOUND FOR EARLY LIFE

During our cultivation attempts, we monitored the production or consumption of specific nitrogen sources. A metabolite with high potential for neuroactivity and other health implications in the host produced by a certain part of the microbiota is GABA [56-61]. GABA is the major inhibitory neurotransmitter in the brain. Recent interest towards this metabolite comes during a peak in research interest for the so-called Gut-Brain axis [62]. With current knowledge of the impact of the microbiome on our wellbeing it seems more and more likely that the activity of bacteria in your gut can impact your neurological homeostasis and mental wellbeing [63, 64]. New evidence has revealed the presence of the glutamate decarboxylase-encoding (GAD) genes, a key enzyme to produce GABA, in prominent human intestinal bacterial genera [65]. Since GABA is not a key component in human milk, we might be reliant on the

infant gut microbiome to supply us with it. Especially, if the endogenous production is insufficient in early life, with a central nervous system still in development where glutamate is endogenously transformed into GABA [58, 59]. The adult host maintains a balance between excitatory and inhibitory neurotransmitters, glutamate and GABA respectively, through endogenous enzyme activity. This balance is changing for different body sites and might be affected by gut metabolism, both from the side of the host and of the bacteria [66, 67]. Recent research has suggested that gut bacteria can produce nitrogenous metabolites with high neuroactive potential (neurotransmitters) beside GABA, including tryptamine, serotonin and dopamine [68, 69]. For most gut-derived neurotransmitters, the function and benefits for the bacterial cells and the regulation of their production within the infant gut ecosystem, or finally, their interaction with intestinal tissue, remains largely unexplored. For example, whether the level of GABA in the human gut, affects the level in other areas of the CNS needs to be further elucidated. Perhaps it only affects the enteric nervous system (ENS) and impacts gut health in that manner [70]. However, there are indications that GABA is involved in bacterial spore formation and pH regulation and energy metabolism in both humans and bacteria [71-76].

GABA can be produced by bacteria via two pathways. A series of enzymes can convert arginine, ornithine, and agmatine to putrescine and finally GABA, which in turn is an intermediate for the production of succinate, called the GABA-shunt. This can be perceived as a strategy to harvest nitrogen under nutrients-limiting conditions [77]. On the other hand, GABA can be produced via the glutamate decarboxylase (GAD) system converting glutamate to GABA while producing CO_2, which might be relevant for the ecology of certain anaerobes in the infant gut [78]. Combined with amino acid cross-membrane transport, this system was described as an acid resistance mechanism [65, 72, 79]. As is clear from previous scientific studies, GABA metabolism has mainly been studied in the model organism *E. coli*. In general it has been shown as a bacterial metabolite in the gut, since it is significantly lowered in germ-free mice [80]. However, current evidence also seems to indicate, that gut-derived GABA does not directly impact brain GABA levels in germ-free mice and that also in humans a more local effect is suspected, since it is yet unclear if it passes the human Blood-Brain barrier [57, 80, 215]. In the infant gut it has been indicated as being related to breastfed infants and that the production of GABA is performed by several *Bifidobacterium* spp. [81]. Moreover, the activity was described for lactic acid bacteria, primarily applied in the development of GABA-containing fermented food [82]. Interestingly, recent metagenomic-based studies in humans have revealed a strong negative correlation

between depression and bacterial GABA metabolism by the gut microbiota [69], thus instigating further investigation of this relationship.

We are currently investigating the role of GABA in the ecology of key infant gut commensals as we saw certain nitrogen and pH-based conditions led to different levels of GABA production and consumption. Interestingly, this short impression also gives rise to thoughts that GABA-metabolism is of impact on short-chain fatty acids (SCFA) production, which is a recent research focus and from which we know that it is of impact on infant health [83-86]. Summarizing, there are several indications that GABA levels to which bacteria contribute, is part of normal development of human health in early life.

THE IMPACT OF NITROGEN ON HUMAN HEALTH

Human milk provides protection, growth and development to the infant in ways we cannot completely fathom yet. Assuming that every aspect of early life diet and the microbiome matters to human health and in relation to each other, will help us to faster understand this foundational aspect of human life. This thesis underlines that nitrogen composition of the diet can steer the microbiota in a direction where it potentially promotes human health from the moment we are born. Therefore, gut nitrogen cycling through bacterial gut activity is important for future early life studies. It can establish what requirements for infants are in terms of nitrogen in diet. Beyond bacterial processing, a part is lost as (bacterial) biomass in feces, another part is undigested and a part of nitrogen supply is processed by the human host [24, 30]. Moreover, the role of disbalance in nitrogen concerning creatine, urea, nitrite, in unhealthy subjects leads to the hypothesis nitrogen cycling in the gut is key to steering a healthy gut [87-90]. In all large life forms, the microbiota produces indispensable amino acids for the hosts, although that contribution has been described as limited in large mammals [87]. The impact of too high protein in early life is that it increases the risk of overweight for the infant and for later in life [53]. Therefore, childhood obesity that sets on at infancy can be considered a major public health issue. Infant feeding may play a prophylactic role lowering the risk for obesity for later life [93]. It has been shown that a formula diet traditionally possesses up to 70% more protein than human milk [91]. Current formula products have per advice lowered protein levels and human milk is the golden standard for neonates [93, 98]. Notably, infants

that received formula early on in their lives are in a higher-risk group for allergies and disease onset [92-96]. However, recent standards and research into formula protein levels have indicated that protein levels closer to human milk are desirable for infant health and formulae following that concept have thus been developed [97-100].

You could speak of 'detoxing' for several nitrogen sources for gut bacteria common to the infant gut. The infant secretes waste products that would otherwise be processed via liver and renal system into the gut [87]. The infant gut microbiota might be helping the infant remove some of these waste products by being metabolically active in the colon. Since they are waste products of a human host, it is preferable to have low concentrations of e.g. urea, cycling back into the host [87, 89]. Especially so, since the infant's renal function compared to an adult is still in development [209]. In **Chapter 3**, we discuss the potential health effects of bacterial metabolism concerning HMN and among those the metabolites that the infant needs to secrete or have removed. Already known was the duplex role of urea and urease in gut health and disease [89]. Urease functions as both a health factor and a virulence factor amongst microbes. Urease activitity contributes in removing urea for the host and supports opportunistic pathogens and their colonization in the gut [32, 89]. However, direct causal relationships are very hard to define without *in vitro* laboratory studies, due to the lack of evidence on actual activity in relation to any ecological circumstances. Especially, if we want to consider involvement of the gut bacteria for human health. Furthermore, bacteria are involved with the production and degradation of neurotransmitters, neuroactive components or their precursors. Indicating the importance for the emergence of the new research field concerned with the 'gut-brain axis'. However, the bioavailability of gut-derived neurotransmitters across the human host needs to be assessed. For early life, this might provide crucial understanding on the role of the infant gut microbiota for infant health and cognitive development.

Another clear interaction between bacteria in the gut, intestinal nitrogen cycling and human health is bacterial vitamin production. Vitamins are an integral part of our diet and are, besides being typically found in fruits and vegetables, a product of bacterial metabolism. B-class vitamins are involved in our immune regulation and gut health [101-103]. Commensal bacteria are both providers and consumers of B-class vitamins and vitamin K [104-106]. B vitamins from diet, are absorbed in the small intestine. However, bacteria-derived B vitamins are absorbed mainly in the colon, where the largest part of the gut microbiota resides [107]. Once again, *Bifidobacterium* spp. stand out as a bacterial group that seems very well capable of producing vitamins to our benefit (B1,

B3, B6, B9, B12) [102, 107, 108]. However, they are not the only ones as *Bacteroides* spp., *Clostridium* spp., *Lactobacilli* spp. *Ruminococcus* spp. have also been shown to produce multiple B-class vitamins [107]. However, how bacterial vitamin production is connected to or impacted by other parts of bacterial nitrogen cycling remains elusive.

Not only do catabolic nitrogen sources itself function in establishing a healthy gut, but they also act as precursors for other bacterial metabolites. Among the bacterial products are SCFAs and organic acids that are substrates for the colonic mucosa and peripheral tissues [109-111]. Actually, ammonia directly affects the energy metabolism of colonic epithelial cells, since is it being used by colonic glutamine synthetase which is highly active there [112]. Notably, a dichotomy exists within gut microbes in ecological strategy for access to nitrogen. This is either a preference of exogenous protein or endogenous nitrogen sources, the latter interestingly mimicking the non-protein nitrogen sources in human milk [20]. Therefore, we can assume that the microbiota we are supposed to have in our gut from the moment we are born is adapted to dealing with these endogenous nitrogen sources, since they are present in human milk. This includes urea, glutamate, glutamine, creatine and creatinine, as major constituents of the non-protein nitrogen part of human milk that can also be secreted by the infant host [26, 87, 113, 114]. Furthermore, having a gut microbiota that is able to adequately process these components can have an impact on host health by lowering the urea and creatine load for the human body [87, 89, 115]. Thus, researchers need to investigate if bridging the gap between formulae and human milk on the levels of these human milk components is required. Particularly, in the case of urea, where studies in pigs and humans have shown that approximately 25% of urea synthesized in liver will enter the gut lumen via enterocytes [87]. Especially, since the infant is not equipped to deal with (or produce) essential nitrogenous metabolites all by itself. Conclusively, an infant's wellbeing is dependent upon the ability to regulate nitrogen metabolism across spatiotemporal scales, with help from the microbiome, from the moment we are born.

MICROBIOME AND THE AGE OF OMICS

Amplicon sequencing, and specifically that of the 16S ribosomal RNA (rRNA) regions that is typically present in all prokaryotes, and later on shotgun sequencing methods for bacterial life, have led to a tidal wave of genomics information. This information overflow holds great challenges for microbiologists, computational experts

and bioinformaticians. Nonetheless it has provided great insight into 'who is there'. However, it could be stated that microbial ecology itself is driven by technological advances more than anything else. In the 1990s, 'fingerprinting' of environmental microbiomes started with the use of PCR- amplified DNA fragments or amplicons [116]. Ironically, the field of microbiome research is past its infancy, into a phase of critical development and reflection. Maturity some might say. It has become increasingly clear that the composition and functionality of the human microbiome is involved in the spectrum of health and disease. Especially so in the gut, where many studies have been dedicated to describing microbiome signatures related to e.g. inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), allergies and diabetes [17, 117, 118]. However, functionality might overrule the importance of compositional data in determining the role of the microbiome in medical conditions. Considering the human microbiome has potentially 150-fold more genes than the human genome [119], this is not surprising. This second genome of human body cannot be adequately studied by traditional methods, e.g. amplicon sequencing, when describing the microbiota. In **Chapter 4** and **5**, we described relative abundance of bacteria in the infant gut microbiome. With an ever increasing frequency, the validity and information density of amplicon sequencing data is being discussed [120-122]. Science is of course bound by budgets and (not just financial) costs. However, discussions in this thesis have often mentioned inadequate depth to answer research questions, especially when relating bacterial metabolism to microbiota shifts. Therefore, I believe that the end of the amplicon sequencing era is near, especially when the applications of -omics approaches become more efficient, more developed, and more widely supported. Still, more is not always better, the value of amplicon sequencing stands or falls with if they are underlined with good research questions, to prevent the description of false correlations and conclusions. It is widely discussed that the application of amplicon sequencing and specifically that of the 16S rRNA, heavily impacts compositional results [123, 124]. The biases are introduced by selection of hypervariable region (V1-V5), sequencing pipeline, bioinformatics pipeline and its settings, and study sizes [225, 266]. In current times, studying the fecal microbiome with relative abundance data, does not provide sufficient information to efficiently tackle issues of multifactorial disorders and diseases or any other state of dysbiosis. Especially, since dysbiosis can occur in the functional aspect of the gut microbiota.

For some phylogenetic groups there is an obstacle with the resolution of amplicon-based microbiome studies. Other regions for *Bifidobacterium* spp., namely the hypervariable 16S rRNA internal transcribed spacer (ITS) region has been suggested

to provide a higher result resolution for this genus [125]. Which makes us able to explain, with more detail, why certain *Bifidobacterium* spp. thrive in the infant gut under certain conditions. Furthermore, a bacterial species, or strain even, is a very fluid concept and a concept that is very responsive to the environment [126, 127]. On the bacterial strain level, a high level of genomic variation exists amongst members of the gut microbiota [128, 129]. Furthermore, their genetic expression can change based on changes in the environment or the bacterial species they coinhabit the infant gut with [130, 131]. Thus, when studying the gut microbiota on the resolution of species level, some of the more subtle relations between diet and occurrence of certain bacteria can be lost. To reliably study the infant gut microbiota in relation to diet and health, scientists must apply next-generation sequencing technologies that provide a higher resolution fit for dealing with that level of complexity.

For a bacterium, efficiency in processing dietary components can equal being very abundant in the gut, and thus provide a competitive advantage over other bacteria. For example, in **Chapter 3**, we mention the efficiency of *Bifidobacterium* spp. in processing glutamate during *in vitro* fermentations at acidic pH, at a higher rate than some of its fellow bacterial microbiota members. If that would be the case for many of the genus's interaction with HMN and or carbohydrates, it is no surprise they are as dominant in numbers as they seem. Their niche is the infant gut supplied with digested human milk, due to their cospeciation history. Efficiency as a concept is functional and can only be extracted from higher data levels that the 'omics' era supplies. Metagenomics, metatranscriptomics and metaproteomics, in this order, provide a higher level of information for showing bacterial interactions with the diet. They can explain what the niche of certain bacteria actually entails by describing active functions of the gut microbiota. They look beyond the functional redundancy and provide insight into which organisms are performing the relevant nutrient cycling steps that directly and indirectly support our health. Moreover, currently more consideration is going to non-bacteria members of the microbiome (virome and mycobiome). Unfortunately, using amplicon sequencing for taxonomic studies in those kingdoms, is less developed as for bacteria [210]. Integration of bacterial databases with fungal and viral databases in -omics approaches can lead to discoveries on the interaction of the kingdoms. This is especially crucial the coming years, with costs and time decreasing for scientists to generate these more advanced datasets. Moreover, multiple omics-type data sets are on the rise, including, glycomics, lipidomics, microbiomics and phenomics [132]. 'Integrated Omics' can lead to some perspective on how each microbial domain and kingdom contributes in relation to one another in the gut in early life. Moreover, it can

help us better relate different types of datasets towards each other, by making certain observations in line with 'a certain relative abundance leads to a certain level of activity of a bacterial species'. This kind of integration can also support science that only has access to one data type when studying environmental microbiomes.

A NITROGEN-RELATED FUNCTIONAL STUDY OF THE INFANT GUT MICROBIOTA

In the following paragraph, I would like to present some research outcomes that are not part of a previous chapter, but I want to highlight nonetheless. Here I illustrate an example of using previously published metagenomics data (Bäckhed *et al.*) to functionally profile the infant gut microbiota for nitrogen metabolism [133]. The goal is to show the power of functionally profiling metagenomics datasets in elucidating how the microbiome is involved in cycling nitrogenous metabolites in our gut [134]. As discussed before, -omics approaches that show actual activity are recommended to be used in the future as well. Improved tools and approaches to study gene content from metagenomics data greatly improve our insight into the gut microbiota over conventional compositional data and plenty of data is available [133, 135-137].

Below, we show two identical randoms selection of samples from this dataset, to illustrate the diversity and complexity of nitrogen metabolism in the infant gut. This data was processed in a similar fashion as described in **Chapter 5**, bundled in relevant nitrogen pathways. Figure 1 depicts the profile for the metabolic route from glutamate (the main free amino acid in human milk) to ornithine. This is considered a crucial bidirectional step in linking amino acid and carbohydrate metabolism in both humans and bacteria and occurs through ornithine aminotransferase activity [138, 139]. Figure 1 shows only one example of the metabolic flexibility of glutamate, and not covers it functioning as a deamination source for ammonium production or how it regulates the urea cycle in the human host through the production of n-acetylglutamate [139]. Interestingly, the infants that are exclusively breastfed at 4 months of age possess elevated gene content of this part of amino acid metabolism compared to the 12 month old infants that receive mixed feeding. Namely, one in every 998 genes average is assigned to this pathway for the infants at 4 month of age that are breastfed, compared to one in 623 genes at 12 months of age in infants that received a mixed diet. In the neonate (Figure 1 top, B_BF) it is observed that there are a few infants that

have a lower relative gene content involved in glutamate and ornithine metabolism. Together, this potentially indicates the effect of breastfeeding and later on the effect of moving away from the free amino acids in human milk. Since, in the mixed feeding phase, the infant gut microbiota shifts to being more capable of degrading protein, since the diet at that time likely includes solid food and more formula milk [133, 227, 228]. Notably, the plots are dominated by metagenomic reads assigned towards pathogens (e.g. *Klebsiella pneumoniae*). This is something we also observed in **Chapter 2**. There we debated if it was due to overrepresentation of those bacterial species in the functional database since they are bacterial model systems for nitrogen metabolism and it would therefore increase the chance of assignment to that species. It raises questions on the development and choice of functional database for analyzing this kind of data set. The level of mislabeled genes, not towards function, but towards host organism, might be higher during metagenomic analyses, than we would expect with merely compositional data. This is due to the lower development time and thus smaller functional databases, due to the lack of isolates and reference genomes [140, 141]. Especially, compared to the density of public amplicon sequence data [141]. Moreover, for some relevant infant gut bacterial species, it is difficult to find sufficient genome annotations that add to the gene databases.

In the cohort represented below, the breastfed infants were dominantly colonized by *Bifidobacterium* spp. [133]. However, the functional profile plots show a more even distribution across multiple genera, indicating a higher diversity than the described microbiota composition of that study. Moreover, the large proportion of genes being assigned to other bacterial species, or even as unclassified, raises the idea that we are not aware of many of the microbial functional diversity present in the infant gut. Interestingly, Figure 1, depicts the shift across *Bifidobacterium* spp. from *Bifidobacterium longum* subspp. towards *Bifidobacterium breve* spp. at the end of the first year of life. In general, shifts over time, as described by Bäckhed *et al.* can be observed here as well, which empowers the method we applied [133]. For *Bifidobacterium* spp., this method already provides you with a higher resolution for this important genus. Figure 1 (bottom), shows another example closely linked to how nitrogen metabolism can impact human health, by depicting the functional profile of thiamine (B_1) synthesis. The role of this exemplary vitamin and how its cycled in the gut remains elusive, but it has been shown to impact the metabolism of *Bacteroides thetaiotaomicron*, a common inhabitant of the human gut [142]. Nonetheless, thiamine impacts human health and deficiencies in humans is of interest [143, 144]. It is furthermore often included as a supplement for bacterial culturing where it likely acts as a cofactor in bacterial

metabolism [145]. It has to be noted here that, the relative abundance of thiamine synthesis genes is higher, than the glutamate-related pathway in Figure 1 (top). This indicates that science is perhaps underestimating contribution of vitamin metabolism by bacteria for infant health.

It can be speculated that this data represents functional redundancy within the infant gut microbiota for nitrogen metabolism pathways. On the species level, they show a more diverse pattern than the average bacterial relative abundance plot shows representing the early life gut. Clearly, the potential for species beyond the *Bifidobacterium* spp. to interact and produce important nitrogenous metabolites can be derived from this example. Levels of potential functionality in this dataset are stable across diets and even for the mothers. These are not the mothers of the included infants due to random selection of the data. An interesting next step would be to correlate and link these results to individual clinical parameters, similarly as was discussed in **Chapter 5**. Nonetheless, our results underline the role of the gut microbiota for the production of essential amino acids and vitamins for the growing infant. Furthermore, it underlines the importance and value of diversity in a gut microbiome. From this data, it could be derived that changes in microbiota composition would not matter, due to functional redundancy. There seem to be plenty of bacterial species that can fill an ecological niche concerning glutamate, ornithine and thiamine, according to this example. Underlying health consequences may only appear when looking at a larger scale, pathway or any higher level of functional abundances [133].

THE FUTURE OF -BIOTICS AND THE LEVEL OF CONTROL IT IMPLIES.

Studying interactions between early life diet, and specifically human milk, is the foundation on which this thesis is built. Because of the crucial role of human milk in early life health, the improvement of replacement feeding, or formulae, and the search for biotics is a crucial step for early life research. The full scope of biotic concepts includes live or dead organisms and dietary metabolites that, when applied to an animal body confer a health benefit [146]. In a sense, application of biotics is a measure of stimulating parts of a highly complex natural process. That counts for every type of biotics application, from prebiotics to synbiotics. Yet, to quote the 1993 Hollywood release Jurassic Park: 'Life finds a way'. I am not insinuating here that bacteria will

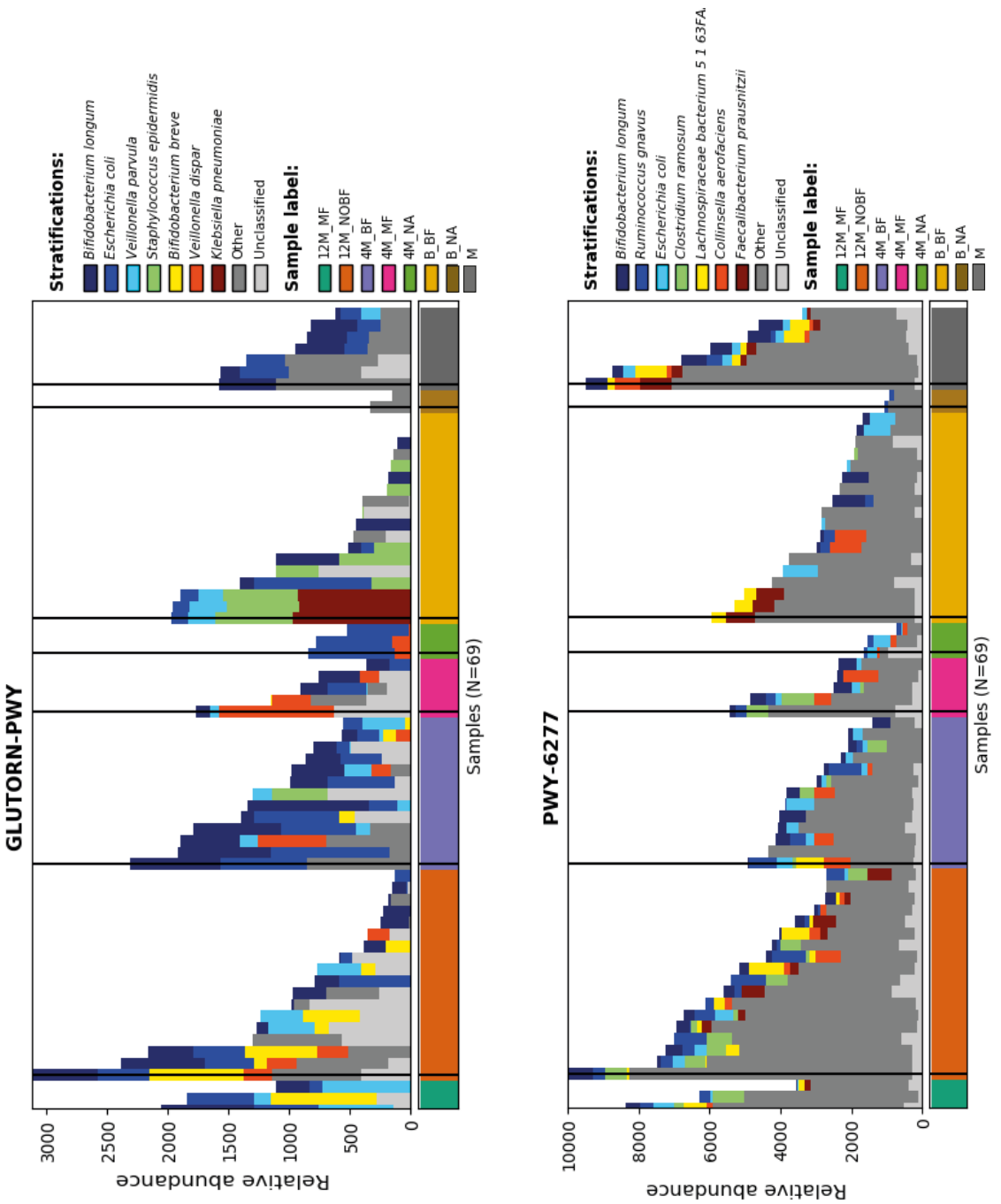


Figure 1. Top: Example of amino acid metabolism pathway abundance, glutamate-ornithine pathway for the top 7 bacterial species, across diet type and host. Bottom: Example of Thiamine, vitamin synthesis pathway abundance (PWY-6277), plotted for the top 7 bacterial species, across diet type and host. Data was process as described in Chapter 5, with the HUMAnN2 tool [134]. Relative abundance of gene occurrence is in count per millions (cpm). PWY identity can be found at: https://metacyc.org/, Diet types: BF: Breastfed/ Human milk diet; MF: Mixed feeding; NOBF: no human milk in diet; NA: no dietary metadata acquired. Host types: B: neonate, under 1 month of age, 4M: 4 month old infant; 12M: 12 month old infant; M: mother.

not let themselves be controlled by science and food technology. However, just as the movie, I am implying that the level of control we are trying to apply on our microbiome is not possible with our current approaches and levels of understanding. We cannot and probably never can account for everything. We should counter naivety and we should prevent being over-optimistic about the current applications of biotics in diet. Nonetheless, the (historic) examples and current applications show great effects and future potential [147, 148]. However, I am convinced we will encounter the proverbial 'dangerous dinosaur' that escapes our perceived control. Whether it is a patient that we cannot treat with our latest microbiota transplant technology or we didn't account for a bacterium's ecological niche under a very specific condition. For developing infant nutrition we should increase our understanding of the functioning of the (infant) gut microbiota and all individual aspects, e.g. the genetic background and diet, that come with it.

Currently, there are four main biotic concepts through which the microbiota is modulated: prebiotics, probiotics, synbiotics, and postbiotics. A prebiotic is "a substrate that is selectively utilized by host microorganisms conferring a health benefit" [146]. These currently include lactulose, inulin derivatives, and galactooligosaccharides amongst others [158]. Probiotics are defined as follows: "live microorganisms which when (orally) administered in adequate amounts confer a health benefit on the host" [229]. Upon survival of the upper intestinal tract, alive probiotics can be involved in the production of health-promoting components. Gut bacteria, in general, have been widely applied in food products, mainly through LAB (Lactic-Acid Bacteria and bifidobacteria) in commercialized milk products although most have not been defined as probiotics by published science. Probiotics as a way of modulating the infant gut microbiota have been linked to treating diseases occurring in neonates, including atopic dermatitis, necrotizing enterocolitis (NEC) and early onset of obesity [159-161]. Furthermore, probiotics have been described to be involved in direct immune-modulation and interact with enteric neurons [230]. Synbiotics are composed of combinations of pre- and probiotics that can have synergistic effects by ensuring delivery and survival of the selected probiotic strain into the gut [162]. Synbiotics have also been shown to be able to treat allergy occurrence in infants [163, 164]. Finally, the application of postbiotics are a result of our further understanding of gut microbiota functioning since they represent knowledge on the impact of bacterial activity and interaction with the human host. Their feasibility and safety needs to be established, but that can be stated for all four biotic types [161, 165]. Postbiotics are currently defined as a "preparation of inanimate microorganisms and/or their

components that confers a health benefit on the host" [168]. Thus, postbiotics is a food concept resulting from previously metabolically active bacteria providing health benefits through direct and indirect mechanisms [166-168]. The components can include SCFAs, secreted and extracellular polysaccharides and other cell lysates that are present before oral administration and that have a clear relation to health [109, 169, 170]. Arguably, sufficient reasoning exists to see the potential health benefits of postbiotic application [169]. However, the actual health benefits during clinical application have not always been clearly observed [171]. Therefore, fundamentally understanding the impact of certain constituents of human milk and specifically how it impacts bacterial metabolism, will greatly increase our effectiveness in applying prebiotics for infant formula and health. This will echo on into probiotic, synbiotic and postbiotic applications, because of being able to better estimate the success chance of a certain bacterial strain in a given microbiota.

The application of -biotics in our diets can increase our health status tremendously over the coming decades. The application of probiotics as food additives that are generally recognized as safe (GRAS) is becoming widespread [147, 148]. There are however also, risks and potential side-effects to note. These include changing health parameters involved with a changing gut microbiota, like bloating or constipation [149]. Interestingly, biotics are also suggested for treatment of both [150-155]. In both perspectives, it shows the requirement of careful individual treatment when applying biotics. Some scientists claimed caution was needed for the application of *Lactobacillus* spp. and *Bifidobacterium* spp. [156]. This was because the application of these probiotics during infancy was not associated with an impact in gut microbiota composition and SCFA levels. In contract, higher occurrence of mucosal-associated diseases occurred later in life in infants that received probiotics in this particular study [156]. In another evaluation, probiotic translocation and infection across the human host was assessed. It was concluded that probiotic translocation is difficult to induce and subsequent detrimental effects are rare in healthy subjects. I hypothesize that a potential disadvantage of increasingly widespread application of (pro-)biotics is the effect it can have on the microbial diversity associated with us humans as a species. Currently, no evidence suggests that this is the case. One could state that if a large proportion of society would possess a very similar microbiota, we are very inflexible against dietary changes, and likely less effective against fighting disease and infection.

The key lies in human milk composition from healthy mothers and to try and grasp what human milk promotes in terms of bacteria, metabolism and in the human host.

This thesis gives further rise to the idea that applying HMN in -biotics can help impact the infant gut microbiota. This thesis in general and **Chapters 2** and **3** specifically highlight a broadening of what human milk prebiotics could encompass. Should we not consider every molecule that is being processed by the microbiota with a selective effect and with a beneficial effect for the host, a prebiotic? In this context, human milk, as the whole secretory fluid, can be debated a prebiotic mixture, or even as a synbiotic drink. Rightfully so, the International Scientific Association for Probiotics and Prebiotics (ISAPP) widened the definition in 2016 to include non-carbohydrates, to broaden the scope beyond carbohydrate fibers [146]. Since many of its components never act alone on a bacterial population, and a microbiota should be viewed in the light of its operating environment. In **Chapter 2** specifically, we provided evidence for urea, a common human milk constituent to be a prebiotic, with the benefit for the host having a microbiota that is catabolically active with removing urea and stimulating potential beneficial bacteria.

The application of -biotic strategies in diet for infants is extra relevant. This thesis underlines the general consensus that the neonatal phase and the infant gut microbiota are crucial in inducing long-term health. Creating the environment for the development of a health-inducing microbiota is important for ensuring the child's future well-being, and -biotics can provide such conditions. This thesis provides indication for the future success of prebiotic application in **Chapter 2** and **Chapter 5**. We hypothesize that the lowering of protein in the Proteus cohort, replacing it with key free amino acids, may have led to the minor shift in the microbiota composition and that thus, free amino acids are a prebiotic in human milk. The effects in **Chapter 5** cannot however be seen separate from other differences between the included experimental formulae (e.g. lactose).

Another method of indirectly applying microbiome therapy as part of the dietary strategy is Fecal Microbiota Transplant (FMT) [172-174]. FMT is a method to directly transplant the recipient's gut microbiota from another healthy subject to normalize the composition and implement a therapeutic benefit. Application of FMT has been traced back to the 4th century [175], but only in recent years modern applications have been investigated. It can be perceived as literally transferring a complete microbiota composition towards a new host. With in exception some aspects that are fixed in the new host, like host genetics and dietary habits that also impact the microbiota [174, 175]. Although, scientists and medical specialists should consider the effect of the oxygen load the microbiota transplant receives during transfer [176]. It can greatly

impact bacterial survival and can potentially decrease effectiveness of the therapy [176]. A highly interesting test-case of FMT applied in infants is the study by Korpela *et al.* [177]. There they have shown that the deviation of caesarean-born infants is corrected by applying FMT from the mother early in life. These infants then continue to have a naturally born development of their microbiome, specifically correcting the lack of *Bacteroides* spp. and lagging *Bifidobacterium* spp. development typical for caesarean-born infants. There were no complications. However, FMT without fully grasping the ecology on which the microbiota is acting, with all aspects of diet, host and the bacteria and other microbes itself, will always provide a certain, albeit low, health risk. This risk would manifest itself through the potential for introducing further dysbiosis to the microbiota or the that one aspect of the health-promoting microbiota, in its widest definition, is lacking in the new host. Another risk would manifest itself through pathogen transfer [231], with the potential of a microbe manifesting itself as a pathogen even when it did not in the fecal donor. Nonetheless, in the dynamic period that is the early life settlement of the microbiota, underlined by a clear dietary pattern for most, pre, pro-, post and synbiotics will have a tremendous role to play in the future of infant feeding.

THE FUTURE OF INFANT FEEDING

The main reason for our research, aimed at infants having insufficient access to human milk, or which do not have a healthy start to life, is the development of infant feeding. Among public health experts, nutritionists, pediatricians, it is accepted that breastfeeding is optimal. The WHO stated, just over a decade ago: "while breastfeeding is a natural act, it is also a learned behavior." (WHO, 2011) [178]. The same can be said however of formula feeding. The improvements made on formulae has instilled confidence in parents in the Western World to feed their infants formula, even when breastfeeding as a practice would be available. Thus, social and economic contexts impact how babies are fed. Interestingly, in 2014, likely scenarios were constructed for the future of infant feeding [178]. It was targeted at North America, it nonetheless provides interesting food for thought. We seem to be on track towards the 'Milk & Honey' scenario, in which the formula market is thriving and lower breastfeeding rates occur due to believe in the formula products. The breastfeeding rates are not considered low in a recent study [179], but the shifting balance between the two main diet options could lead to negative health effects on biologically fragile infants. Since,

a donor milk system to fill all requirements of parents is not prevalent in society, the development of formula is crucial. In contrast, the larger milk conglomerates of the globe, operate on a thin edge, on the one side supporting breastfeeding, while on the other hand depending on developing formula products in a market that seems ever more demanding. With what we know on the importance of breastfeeding, there is a risk it becomes obsolete as a practice. Is lactation becoming vestigial for our species? The development of formula (e.g. inclusion of oligosaccharides as prebiotics) is proving otherwise and another long-term goal appears. Formulae can function as a medium of guaranteeing infant health in the future, yet breastfeeding should remain the golden standard for infant feeding for at least the first 6 months of life.

Cow's milk production is currently under pressure due to environmental concerns. Interestingly, the use of goat's milk as base for formula production is currently considered [180, 181]. Environmental impact or the carbon footprint from large scale goat milk production is currently being discussed and evidence suggests that it might be similar [223, 224]. Like cow's milk, goat's milk would need to be fortified to provide optimal nutrition [180]. Interestingly, there are compositional differences that could have an effect on microbiota settlement in early life. Goat milk holds lower alpha s1-casein, a protein present in ruminant milk, but not in human milk. In contrast, it possesses higher levels of nucleotides, polyamines and essential amino acids, including taurine [181-183]. This thesis has highlighted their importance in **Chapters 3** and **5**. Not surprisingly, the gut microbiota composition of infants receiving goat milk-based formula differs from that of infants fed regular cow milk formula or a human milk reference [184]. However, it was also shown that the level of *Lachnospiraceae* spp. was more similar comparing the microbiota of infants fed goat milk formula and breastfed infants [184]. All in all, goat milk possesses some potential benefits for application into infant formulae that need to be further assessed. In the meantime, several other alternatives have been described in research. Namely, soy and rice milk alternatives [235], which have been indicated as cheaper alternatives from infant's with cow milk's allergy than extensively hydrolyzed formulae. Although it must be noted that cow's milk and soy allergies can co-occur [235]. Moreover, it was shown that a percentage (<14%) of infants that had cow milk formula replaced with soy formula early in life, developed soy allergy [236]. I speculate that with the current dietary trends, vegan plant-based formula options will receive increased attention.

Perhaps the future of infant milk diets lies in 'breast-on-a-chip' techniques or other cell-culture techniques that might even be applied on a factory scale. Besides formula

development, there are several initiatives emerging that are trying to develop methods for producing human milk through human cell line induction, amongst others. This approach, however currently does not account for the indications that human milk from the infant's actual mother holds benefit over donor human milk [185, 186], to which the human cell line produced milk would be comparable. Specifically, this does not include the microbial signature present in human milk [186]. Not to mention flavor components involved in the development of feeding behavior in children [232, 233]. Also, human milk delivers a complex repertoire of components that affect human life and development in other ways, including hormones and microRNAs [187]. Moreover, part of this repertoire is dependent on the mother's genetic background [188-190], which would need to be applied in this type of human milk production. Finally, the biotechnological production of HMOs from any type of cell still has optimization to achieve for efficient production, although it is gaining ground [191, 192]. However, this thesis underlines that formula development and infant health support is most benefited by mimicking human milk factor by factor.

SCIENCE AND MODERN COMMUNICATION

The COVID-19 Pandemic showed the great importance of fundamental science and also its great flaws and challenges [193, 194]. Scientific validity was questioned, criticized and merged into political discussion [195]. To ensure tribute of sciences towards a broader discussion, clear communication is warranted and tools and strategies to communicate scientific outcomes need to be developed [193, 196]. Because of its nuance and self-criticism, it is often difficult or even impossible to adequately summarize in just a couple of sentences for a non-scientific audience. Moreover, modern communication needs to integrated not only from science onto society, but between scientists as well [196]. Open science strategies can and have been contributing to that [197]. Sharing data on early findings, integrating multiple databases, resources and ideas can lead to a better evaluation of scientific discovery and can subsequently lead to clearer communication into society. It could also stimulate collaboration and lower competition within scientific fields [198]. Especially since, as is the nature of humans, science is also competition. I speculate that this competition is costing us societal development and an effective response during a pandemic. Despite everything, the pandemic showed us the importance of science integrating communication.

When quickly summarizing development of modern media and its communication strategies, anno 2022, you could conclude it is 'quick and dirty' [199]. Modern communication on large media platforms is defined by catchphrases: putting large amounts of information into quickly digestible headliners. I believe, science does not benefit from 'quick and dirty', it is defined by nuance and counter- discussion. Communicating scientific findings in a short and catchy way, will never pay tribute to science. Because of the modern communication methods in modern media, the pandemic put pressure on the current frameworks of science communication and even science evaluation [195]. Furthermore, it showed the importance of the speed by which science communicates and shares data and observation with peers and then the governing world.

Communicating and publishing scientific information plays a vital role for science, if society wants to benefit from it [197]. Therefore, In the Netherlands, several universities have decided to include a Science Communication studies into their study portfolio. They have implemented study trajectories focused on communicating sciences towards a non-scientific crowd. This gives scientists and future scientists more tools and more platforms to communicate science quickly and correctly to a broader audience. People educated in this field should then create awareness and prowess for understanding scientific communication in society and this should be one of the lessons of the COVID-19 global pandemic. As a part of this, education for critical thinking and evaluating information will be important and not only on universities. This is important because of the ever-increasing flow of information, which requires people to be critical on the information they receive. However, it stands without reason: a scientist does not become a communications expert overnight, yet, the two cannot be seen separate ever since the COVID-19 outbreak.

I believe, the outwards communication strategy is not a sole responsibility of scientists, but we could have adapted our communication streams towards the general public, now that this was suddenly a larger part of our audience [193, 194]. Something that was generally not the case before the Covid19-pandemic. For the future, scientific communication and information concerning general health and environment needs to be prepared for inclusion into large- scale media outlets and for social media. I believe this can benefit from structuring it with discipline as follows: 1) What do we know? 2) then, what don't we know? 3) and finally, how will new scientific discovery contribute to the problem at hand. This structure was in my opinion lacking between the scientific community and media outlets. There is also a lesson here as a young

scientist. Sticking to this 'discipline' will lead to clear communication with fellow scientists, supervisors and students as well, which I believe will eventually lead to better scientific development and more impactful discovery. This will further underline the importance of science and will train the non-scientific audience for dealing efficiently with this type of complex information. If we desire science to steer society forward, the future of science and communication is dependent on both development of scientists themselves as well as underlining the importance of science with clear communication.

REFLECTIONS ON THE RESEARCH FIELD OF THE INFANT GUT MICROBIOTA

A PhD Thesis is a place for reflection on your scientific field. Microbiome research is a thriving, abundant and rapidly developing research field, that sometimes seems like it is outrunning itself. However, it appears obvious that there is plenty of future applications of microbiome research into food and health. Firstly, I believe, functional -omics approaches should be broadly applied in (clinical) microbiome studies. The occurrence of functional redundancy in nitrogen metabolism points to a common microbiome hypothesis: It doesn't matter who's there, but more importantly what everyone is doing. Therefore, microbiologists are focusing on studies on individual species to understand the microbiome and its ecology. This thesis underlines the importance of such studies. Secondly, the relevance of other bacterial groups should be considered in contrast to *Bifidobacteriaceae* only, which dominate infant gut microbiomes in Western Europe, which might lead to overestimation of their importance in contributing to our health. We must not forget that the infant gut microbiota is still a developing system and not constant within the first year of life, with significant longitudinal succession and development over time [8]. Especially, in the adult gut microbiota, it is not only about bifidobacteria. Furthermore, clinical study setup, sample processing and bioinformatics analysis have to develop alongside new microbiome considerations. To prevent asking identical questions repeatedly in relation to microbial compositional data, inclusion of microbiologists in clinical design needs to be further standardized. For example, statistical power calculations for clinical approaches are determined a priori based on an acceptable error level [200]. However, in clinical microbiome studies there are plenty of examples of different levels of individuality and variance across patients compared to other clinical parameters [200-203]. Subsequently, this then requires a different power analysis to adequately

test hypotheses in microbiome science. Moreover, science is in need of updating its sharing and communication strategies to overcome these challenges. Still, a clinical gut microbiota investigation, will never be like answering a yes-or-no question. Naturally, methods and scientific habit are determinant of the outcome and interpretation of results. In any case, linking clinical outcomes in microbiome research towards health applications is still a promising field of research and has led to thrilling discoveries.

Recent discoveries make scientists and society wonder: Are we moving towards a 'Microbiome reset' for all patients? Will we in the future, provide every patient with an ultimate microbiome, functionality-wise or a synbiotic mixture that levels out the globe's gut health? There are inherent risks involved with this loss of diversity, as is the case in the microbiota of the aging human [204]. Not to mention the interindividual variation in microbiome research, which will make it unlikely that one treatment fits all [205, 206]. Microbiome research will most likely lead to the realization that there is power in microbial diversity and resetting everyone's gut microbiota to a perceived as ideal pattern will perhaps provide health problems of its own. Fundamental understanding of microbial ecology and improved data prowess are the keys to the future. It is clear, microbiome research has its work cut out for itself.

CONCLUDING REMARKS

This thesis contributes to the research field of the early life gut microbiota from different angles and this general discussion reflects that. The aim of this thesis was to describe if and how human milk nitrogen (HMN) affects the infant gut microbiota and its development. Our findings describe the early life microbiome and highlight key understudied aspects of bacterial nitrogen metabolism in the early life gut. This contribution is not only relevant from a microbial ecology perspective but also from a societal perspective for developing infant replacement feeding and therapeutic gut strategies. The findings in this thesis confirmed previous knowledge in the development of the infant gut microbiome in the first six months of life. However, the main revelation from the thesis is a new focus on nitrogen in the human milk diet and the microbiome during this fascinating period for gut microbiology. We looked for new applications for metagenomic data while studying the infant gut microbiota. These approaches have allowed us to experience a sneak peek at relationships between a previously considered as less relevant part of the human milk diet.

All findings in this thesis point to one foundation of gut microbiology: diet is one of the main factors determining the gut microbiota composition and activity in early life. And we should consider all aspects of it. Even if that on its own is not a new piece of information, this thesis describes a couple of new aspects that highlight why the diet is so important in early life. In doing so, the work described here is not only relevant for a fundamental microbiology perspective, but perhaps even more so from a nutrition and health perspective. Specifically, this thesis highlights the importance of urea and other non-protein nitrogen parts of human milk for early life studies. In this thesis, a relatively small adaptation to the infant's dietary nitrogen led to measurable and significant differences. "Nature finds a way" is the inspiring quote I used in this discussion and it refers to what ecology is in its essence. Microbial nature has found a way to interact with us at a fundamental level so that we are now dependent on it for our health and well-being. In the meantime, we have adapted to live with microbes that have inhabited our globe for billions of years. Moreover, the microbes have likely adapted to our developments as a species and it has pushed our own natural evolution towards being able to control them for our wellbeing. The coming decades will show if we can. A true symbiotic relationship that needs to be described by science. Are bacteria showing us the way? Most likely, and now we need to show them the right environmental conditions through our diet from the moment we are born.

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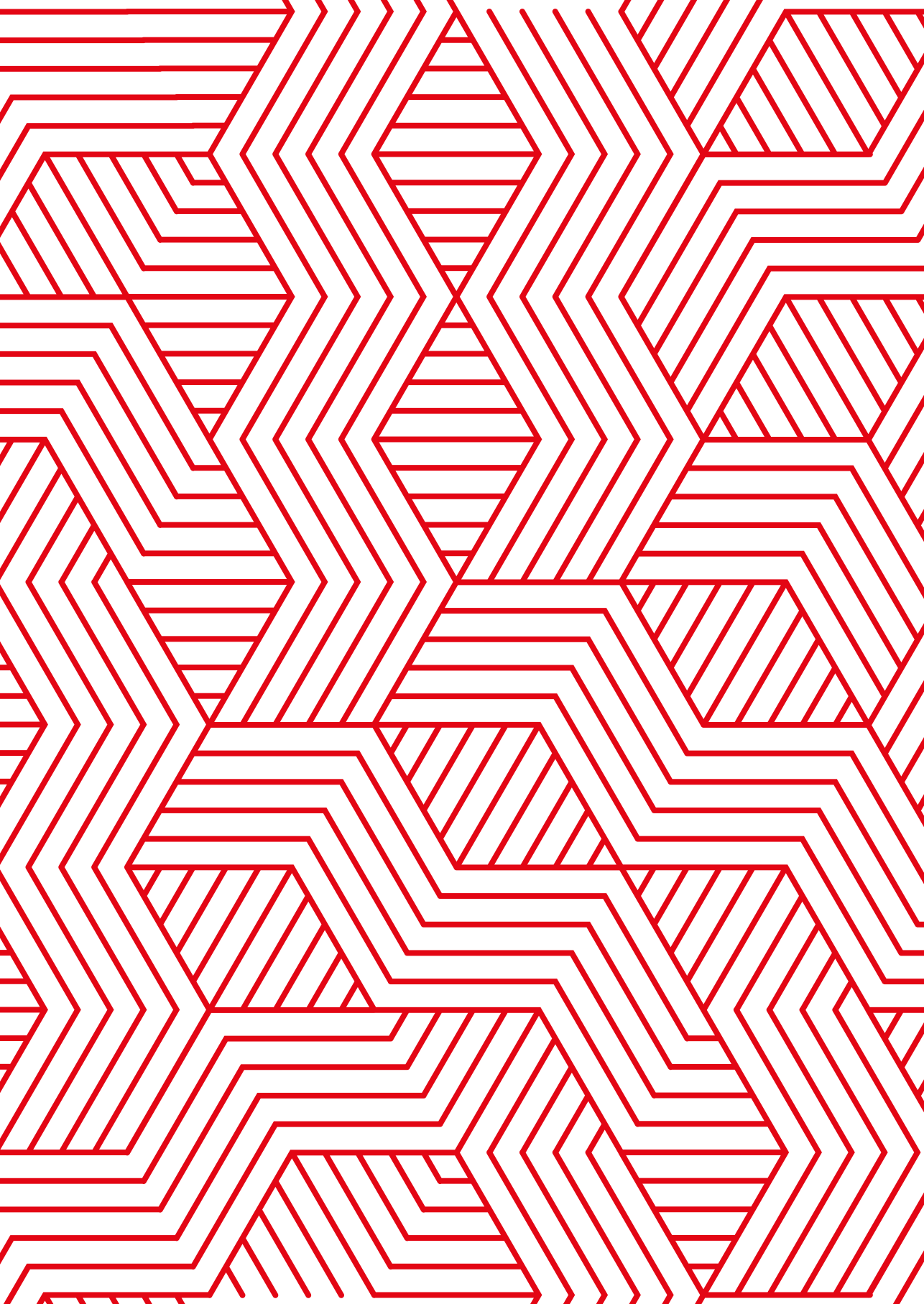

APPENDICES

- English summary of thesis
- Dutch summary of thesis
- Acknowledgements
- About the author
- List of publications
- Overview of completed training activities
- About the cover
- Colophon

ENGLISH SUMMARY OF THESIS

Colonization by the commensal gut microbiota in the infant gastrointestinal (GI) tract is a dynamic process. Upon first encounter of the infant with nutrients and environmental bacteria crucial interplay between two world starts. The breastfed infant gut microbiome, in most Western societies, largely contains beneficial bifidobacteria. Since, before weaning, breast milk is the sole source of macro- and micronutrients, and protein intake at this time relatively low. The case of the human milk oligosaccharides (HMOs) has shown us the great impact of prebiotics on health. This thesis adds the focus to nitrogenous compounds of human milk that contribute to the microbiota composition, which is a new avenue of research. For example, urea and amino acids are a constant and dominant factor in human milk. We hypothesize that human milk nitrogen (HMN) is overlooked as a category of prebiotics and is of significant impact on health in early life.

To promote settlement of beneficial microbes in our guts, we need to study how they utilize the human milk diet. This thesis includes in **Chapter 2** a laboratory study that studies the interactions of a potential probiotic gut microbe present in the infant gut with the main non-protein nitrogen compound in human milk: urea. Explorations into the potential impact of HMN are included by reviewing (**Chapter 3**) all known relations and using metagenomic data to describe the potential interactions that could occur (**Chapter 6**). What are the ecological niches in this community dominated by bifidobacteria concerning urea and other parts of the non-protein HMN? This output can help improve infant feeding as well as providing a more broad understanding of the microbial ecology of the infant gut.

We also studied the microbiota and its development in relation to diet in **Chapter 4** and **5**. We describe the interesting relationship between and a ratio of occurrence of *Bacteroides* and *Bifidobacterium* spp. The trajectories of development indicate that infants fed formula possess an early maturation of the microbiome. With that, **Chapter 4** provides a proof-of-principle on development of the microbiota in the infant gut and its relation to diet. In **Chapter 5** we provide new evidence for the importance of formula protein levels for the development of the infant gut microbiota and show the bifidogenic capacity of a formula that in terms of nitrogen is closer to human milk. Furthermore, comparing the intervention group of the Proteus cohort with breastfed and regular formula-fed groups shows similar composition, opening the way for the application of formulae with altered nitrogen content.

DUTCH SUMMARY OF THESIS

Kolonisatie door een behulpzaam microbioom van ons darmkanaal is een dynamisch proces. Tijdens de eerste ontmoeting van een baby met nutriënten en bacteriën uit de omgeving, begint een cruciaal samenspel tussen twee werelden. Het microbioom van de borst gevoede baby, in de Westerse samenleving bestaat voornamelijk uit behulpzame bifidobacteriën. Dit is met name omdat, voor de overgang naar vast voedsel, de aangeraden voedingsbron humane melk is, met een relatief lage eiwit-inhoud en al haar typische macro- en micronutriënten. Het onderzoek naar de Humane Melk Oligosachariden (HMOs) heeft ons geleerd wat het positieve effect op de gezondheid van de pasgeborenen kan zijn, als prebiotica. Deze thesis voegt een nieuwe focus toe op de inhoud van humane melk, namelijk stikstofhoudende non-eiwitten die potentieel als prebiotica kunnen functioneren en een impact hebben op de samenstelling van het microbioom. Als voorbeeld ureum of vrije aminozuren die een constante factor zijn in moedermelk. We begrijpen ondertussen dat veel van de mogelijke bacteriële stikstofproducten en bronnen betrokken zijn bij de ontwikkeling van ons lichaam, en specifieker ons darmstelsel en zenuwstelsel. Wij brengen als hypothese voort dat deze stikstofbronnen uit humane melk (HMN) verborgen prebiotica zijn en een significante impact kunnen hebben op de gezondheid van de pasgeborenen.

Om de kolonisatie van behulpzame bacteriën te bevorderen in onze darmen, moet de wetenschap bestuderen hoe bacteriën omgaan met nutriënten uit de humane melk. In **Hoofdstuk 2** hebben wij een laboratoriumstudie beschreven die de interactie tussen een bacterie die vaak voorkomt in borst gevoede baby's en de meest aanwezige non-eiwit stikstofverbinding in humane melk: ureum. Een ontdekkingsreis langs wetenschappelijke literatuur (**Hoofdstuk 3**) over de mogelijke interacties tussen andere HMN-bronnen uit andere laboratoriumonderzoeken en DNA-gerelateerd onderzoek. Wat zijn de ecologische niches van de bacteriën die ons helpen met het verteren van de stikstof in onze borstvoeding. Dit onderzoek kan daarom helpen met het begrijpen van hoe stikstof rond gaat in onze darm en daarna ons jonge lichaam.

We hebben ook de samenstelling van het microbioom bestuurd in relatie tot het dieet van de pasgeborenen in **Hoofdstuk 4 en 5**. We hebben de relatie beschreven tussen Bacteroides bacteriën. (een andere groep darmbacteriën) en de hiervoor genoemde bifidobacteriën. Verder is er beschreven dat de ontwikkeling van baby's die een poedermelkvariant ontvangen leidt tot een vroege maturiteit (volwassenheid) van het microbioom leidt. Daarnaast beschrijven wij **(Hoofdstuk 5)** dat de eiwitconcentratie in het

dieet in poedermelkproducten een effect kan hebben op de microbioomcompositie. Specifiek laten wij zien dat een poedermelkproduct met een lagere eiwit concentratie, en dus meer lijkt op de natuurlijke borstvoeding van een gezonde moeder, kan leiden tot meer bifidobacteriën. In dit Proteus-cohort laten wij daarmee het belang zien van de aanpassingen van poedermelkvarianten op het niveau van stikstof. Daar liggen in de toekomst velen mogelijkheden voor het verbeteren van voeding voor pasgeborenen.

ACKNOWLEDGEMENTS

Just like with our microbiome, you are not alone in pulling off a PhD degree. So to everyone I have met and worked with, thank you and all the best in your future careers. I hope we will meet again in academia and continue to impact science like I know we can.

Then, first off: I would like to thank my promotors **Clara Belzer** and **Jan Knol**, Dear **Clara** and **Jan**, Thank you for all your support during this PhD Project. Thanks for all the lively discussions about science, about the world to put that science in. Guiding me back on the right track every time I got lost in my own enthusiastic endeavors. Thanks for calling me the 'mad and chaotic' scientist at exactly the right time to keep me focused. And there is plenty more to list. All of your insight into science and media, business policy, scientific writing and many other aspects will help me for decades to come. To say the least we met some hardship along the way, but I am confident in saying that always had a very efficient and pleasant working relationship. I think the results definitely show that, thank you.

My sweet **Margo**: we only met until later during my PhD, but it couldn't have been at better timing for me and my life. You reinvigorate me on a daily basis, with your kind words and loving enthusiasm. I have read the following countless times, but it makes me think of you. "I would burn the world and use my soul for tinder to hear her laugh again." (From *Wheel of Time* of course). The last few months have been challenging: getting married, moving, emigrating, new science, your business rising to new levels and to finally being on different continents. Together, we managed easily and the highs always outweighed the lows. The design of this thesis is top notch because of you, as everybody will see. On to many more adventures together. You push me to greater heights. Love you dearly.

Thanks to **my parents**, that, on top of everything else, are very proud of me. This thesis also belongs to all the freedom and silent trust you have given me over the years. Lieve ouders, dank voor al die jaren blind vertrouwen en vrijheid. Dankzij jullie ga ik nu mijn dromen achterna.

A big thanks to all my **PhD Defense committee members**, for what I am sure will be a lively discussion with some great lessons. I hope, us too, will meet again in the exciting world of scientific collaboration.

Now for my **paranymphs**, which I both got to know as among the most excellent scientists I know and will probably ever meet.

My dear friend **Carrie**: Thanks for being there through all the hardship and struggles that seem to be usual business with getting a doctorate, but are in fact not business as usual. I think in the beginning we were mostly friends of friends, since we did not experience our MSc thesis together. I believe we connected strongly on our sometimes absurd sense of humor and our strong opinions on the going of things. Laughing together was a natural thing. So, we quickly grew close, and could not wish for a better companion on walks (with or without the dog), rants, bad movie nights or just drinking (All hail the drinking group leader!). Never forget, you are a tough competitor that can get anything done. Thank you for what is sure to be a long-lasting friendship that I value deeply. It is an honor to have you as my Paranymph.

Prokopis, you are my true scientific sparring partner of the laboratory of Microbiology. You've been my longest running office mate. My dearest Malaka. And we did not only match on the science level, I think when the world news went absolutely in a bad direction, we had longlasting and very valuable discussions about that. We always ended with a laugh though. I believe strongly that we will meet or collaborate again in the world of science, and I will definitely visit Cyprus someday. After 4 years, you have me finally convinced. It is an honor to have you as my Paranymph.

OFFICE MATES:

I was blessed to have some amazing office mates for great discussion on my research field and just life in general. Thank you for being my friend and helpful contributor over the years. I will closely watch your progress in the academic world and hope you succeed.

Zhuang - I was happy to have you join my office for the later part of my PhD Project, Zhuang. I got to know you as a very outgoing, charming and relaxed guy, who knows what he wants, a rare quality. And although I kept butchering the pronunciation of your name, you still accepted me. Keep it up! We might meet each other plenty more in dietary fiber research.

Ioannis – My second-dearest Malaka, You were dearly missed, but I am sure you contributed greatly at Danone Nutricia. It is very unfortunate that we didn't have more chance to work together during our overlapping stays at MIB, but I was happy to have got to know you during my stay. I Am sorry that I almost killed you while go-karting. But I let you win with the arcade basketball in return of course. To see you with your little one really made me and many other colleagues happy!

Catarina – I always felt like we were water and fire. But in a good way, enforcing each other. However that resulted in a great amount of philosophical walks, scientific

chats and a lot, a lot of laughs. We could have fun discussing very serious stuff as well. And you always surprised with your opinions and that is quality I admire and love in my friends. You have been all over the place recently and that made me notice how much your presence is missed. But I believe you are a nomad, so please, stay the way you are, keep exploring the world and science. I sincerely hope we meet again and can stay in touch!
Gerben - You were dearly missed in our department after leaving before I finished. We couldn't have had enough goodbye parties and gifts for you, if I am very honest. This combined says it all, really. Not just for the science, but even more so for the endless reflections on life and the social aspect that you brought. You have taught me plenty of invaluable lessons about science and its community, that I will treasure, probably forever. I hope and I believe your adventure and further career in Denmark will be a success.

OTHER COLLEAGUES:

Sharon, thanks for sharing in all the hard work and in the laughs. I always appreciated your company greatly. Furthermore, I hope we can continue chatting on video games and your progress as a postdoc at MIB, which I am sure will be a great success, but I am very curious where you and also **Isma**, will end up. I think the work you did on your publications is actually astonishing. We discussed technical aspects of our work in great length and that helped me out greatly. I am happy to have had you as a colleague at MIB.
Oh **Jannie**, where to start. All the crazy dances, crazy moments, the collaboration, everything always led to a lot of laughs. You truly contributed greatly and showed me and more people in the lab: a PhD Candidacy can be tremendous fun. That is something that I will carry with me and remember. It was an honor playing you in your defense video, but honestly not one actor in that video would do you credit. You are an incredible hard worker, with great determination that I believe still underestimates herself. Not many at MIB can measure with your success and self-motivation. I greatly enjoyed working with you on the cultivation projects and it is a shame that we didn't get to exploit that collaboration more. I see you flourishing in your new position which I see is a great fit for you. You are a great peoples, skills and resources manager, besides being a scientist. Thanks, thanks so much for all your great contributions to discussion with me, and most of all the hilarious moments, memes (you are the meme queen of MIB by far). I am happy to have you and **Ruud** as friends.
Ineke. First off, you are invaluable to all of us. The countless times I have sent you an email with a basic practical question and your quick response. Even it is was a silly question Thank you for that alone. Besides this everyone knows you as a happy spirit in the lab and that greatly impacted the atmosphere there. You and your partner in

crime **Laura** made sure the lab was running smoothly. And **Laura,** Thanks for all the moments laughing at the lab radio together. You added another level of fun to my time at MIB. And it goes beyond saying, thanks for all the help and advice in the lab.

Dear **Nancy**, Thank you for all the times that your sweetness helped to put things in a different perspective. It happened more times than you know. Besides that, you are a great scientist, with lots of great ideas. You work meticulously and I am sure the rest of your PhD will turn out great. Never change. I hope to see you at your defense soon.

Hauke, I think there was something to learn from every PI in our group, but from you especially. I got to witness your leadership during the Daily Board meetings that we were both present at. There were lessons in that for me that I will cherish. Moreover, thank you for all the lively discussion at the MolEco meetings, they developed me into the scientist I am today. Good luck with UNLOCK and all the ongoing projects. I am sure we will meet again.

First of all, **Erwin**, thank you for bringing me into contact with Dr. Isaac Cann and essentially granting me this opportunity to work in Illinois. Also, I vividly remember all our conversations about cult 80ies horror movies and metal music. Those are the kind of conversations with colleagues that make everything worthwhile. We will surely meet again.

Janneke. I have endless admiration for your endeavors in science, communication and sports all at the same time. I think this is very much underestimated still. I always found it fascinating as well. I think I cheered when I heard you won your first marathon race and tracking your achievements with the 'Alternatieve Elfstedentocht'. I am going to miss the talks as friend but also the talks about cycling and skating too. Us talking about the glory days of Janneke Elzinga and Jumbo Visma. It is very unfortunate that I will be missing the moment you will be joining their ice skating team.

Martha is the one person that always takes time to ask you: How are you doing? She can put a smile on many people's faces. And that's what we need in science, more smiling. Thanks for that.

Carina, you are a personality that has always fascinated me, an innate drive and broad interest and activities make you an interesting addition the scientific community. It is a shame we were not able to organize the PhD trip together in together. Thanks for the great talks during many occasions, about my migraines, your flying adventures and good luck with the start-up(s) and for some of your Poker money.

Also a grateful word to our chair **Thijs** Ettema. I was an active witness of the changes happening at MIB when you arrived as our new Chair. There were lots of issues typical for a scientific group growing and thriving as MIB did. I noticed you tackled them head on and some habits were not always easy to get rid of, but I have seen certain

aspects change for the best. I valued your strong opinion in matters concerning the future of MIB. Moreover, I felt very much heard and listened to during my time in the Daily Board and as a PhD Board member and I believe that was very important to me. I think you are a great example to many young scientists and I was glad to be a part of the changes at MIB.

Dear **Marie,** you are truly everybody's friend at MIB, your warm and kind spirit always lightened the mood, while others, including me were ranting on about the work, the workload or other colleagues (everybody does that let's admit it). However, I think there are very few people with a bad word to say about you. You are a person that faces every day's challenges with a smile and a happy thought. I think that is very admirable and a good quality to have in science. I will also vividly remember your presentation and your skill with that. As a parttime fiction writer, I enjoyed your storytelling greatly.

TJ, my dear Toajun, you are hands down the most energetic and uplifting colleague I've met at MIB. Many thanks for that alone. Moreover, thanks for all the laughs and a smile that is almost as contagious as some of the coronaviruses. I am truly happy for you that you have found a spot in the same exciting environment as I have. I truly hope that we can find a way to collaborate while we are here across the pond.

Dear **Belen,** thank you for sticking around when you didn't want to do a PhD anymore. Just kidding, I think the department is lucky to have you as a happy spirit always in for a chat. It is much appreciated.

As my supervisor of my thesis, you were the final push of me knowing for certain I wanted to be active in science. And now we are here, Thank you **Jie**!

Detmer, first of all thanks for referring me into the direction of other people at the Laboratory of Microbiology. Who knows where I would've been without that. Secondly, thanks for the discussion on marine topics over the years, I still feel connected to that research field because of it. That is of high value to me.

Dear **Chen**, all the weekends in the lab where you were blasting Chinese songs, you really opened my eyes to Chinese music. I hope I didn't bother you too much with my 'hard-rock' songs. I hope you will see a good end to your PhD candidacy as well.

My Southampton buddies: **Thomas**, **Dani** and my other colleagues there, and last but not least **Franklin**. I was moving around a lot in 2022, but this was definitely one of the highlight. Franklin, I got to witness you and your newly formed group and took great lessons from it. For me personally it was a great benefit to be able to jump into a new topic, whilst having the opportunity to finish some great work in a new and exciting environment. I am sure we will keep discussing what we can do with metabolomics and phages, things I wouldn't have thought to be working on 1 year ago.

With both of you, **Heidi & Anja**, I almost feel like apologizing to you for what feels

like an endless flow of questions and problems I have thrown your way. Thanks for all the availability and help. MIB wouldn't run the same way without you.
Thanks for all my fellow **DB and PhD Board** members. An inspiring environment where I really felt like I could contribute. An experience that will help me in my future career. Thanks for all the lovely and lively discussions in there. I would like to specifically mention the People I have been intensively involved with to set up the PhD Board: **Timon, Nicolas, Jolanda, Wen, Lot, Zhuang, Peter** and everybody else involved in the idea-making and formulation process of what I think is a crucial step for MIB.
Dear **Max & Thijs**, remember that Jazz club in the States? You are two of the most broadly interested and societally invested PhD candidates I have met. Thanks for that lovely memory.
Thanks **Joep** for saving me from that crazy 'escaped patient' in Manhattan. Easily one of the most exciting moments of my PhD. (Many thanks for all the fun and lively chats during Thursday Fries).
Thanks to the **few lunatics** that showed up at my house on a Friday night to threaten me because I was left-winged and a scientist. You have only strengthened my resolve.
Also….
Menia, Ran, Dani, Diana, Peter, Diana, Burak, Guillaume, Patricia, Hannie, John, Nico, Emmy, Anastasia, Costas, Annelies, Valentina, Marina, Valentina, Caifang, Hugo, Gosse, Kate, Nicolas, Ton, Enrique, Caroline, Felix, Victor and probably a few more, thank you for being a part of this journey. I wish you all the best in your careers and personal lives (let's not forget about those).
A note to the '**PhD Survivors'**: Keep on surviving. I hope to see each and every one of you involved in science and development of the world. Some of you are already and others will be dearly missed.

MY DEAR STUDENTS:

Even though your names could not be on every single publication, all of you left your mark on my thesis, and I am happy to have got to know you all and wish you all the best in your future careers.
Berna: I got to you know as a student, that I needed to contain rather than set loose. Your self-motivation and sometimes pressure to discover is something that you can use for the rest of your life. Your skill in approaching bioinformatics and statistics is tremendous and helped me even change my perspective on those approaches. I think we had a great project and I hope it will soon be out in the world. I am happy to hear that you are applying for PhD positions since I believe you are an excellent candidate.
Endria: From all my students, you bloomed the most during your thesis. I saw growth

and a lot of scientific potential when you did your BSc project. I also saw an interesting character, that has an interesting way of looking at things. Most importantly, you helped me confirm hypotheses about GABA metabolism in bifidobacteria, which although not a part of this thesis, ended up being one of my most exciting projects. It was so very good to see you work with my paranymph Prokopis now on GABA metabolism.

Nienke: I believe that when you entered our lab, you were not sure what to think of science or working in a group like MIB. More and more you came out of your shell and you worked efficiently and showed great interest to learn. It all starts with that. Thank you for time in our lab.

Ziyu: Dear Ziyu, I hope you are doing well. I know you had a great time in our lab, because you told me very openly on many occasions. That gave me confidence as a supervisor. Your self-motivation to sit in a Covid-lockdown hotel room and do your thesis defense says it all. Thank you very much for your contributions.

Athul: it seems like ages ago, that I learned of your endless drive and motivation to prove yourself. Almost wanting to do too much, but wanting to succeed mostly. And succeed you did, you took a challenging project and made it your own, with ease. Good to see you are active in the probiotics industry.

OTHER COLLABORATORS:

Dr. Jonathan Swann – Dear **Jon,** Thanks for welcoming me with open arms into a great project that will soon bear fruit. Thanks also, for inviting me to Southampton for an experience that I will treasure for a lifetime. Communication with you always felt natural and very friendly, I cherish that.

Dr. **Bernd** Stahl – Thank you, sincerely for all the reviewing of my review and I can confidently say: your endless ideas are an inspiration on its own. You made me look differently at human milk and kept expanding my knowledge on it, which greatly helped me finish my thesis.

Roger Bongers – My urease sparring partner at Danone Nutricia, I think this project and patent wouldn't be there without and that is well-deserved. The chats were always pleasant and I am very sorry that due to the pandemic or collaborative work in Utrecht never came to be. Happy to see you continue the work which I think will be crucial for infant feeding applications.

Sebastiaan Tims – I think it was very good for us Danone PhD Candidates to have you around in our meeting, as an example, as another discussion partner. Thanks for all the clear communication and help navigating us through the Danone systems. Thanks for the recent compliments about my work.

Heleen de Weerd – Thanks for the big help with the metagenomics and other bioinformatics challenges. I would not have been the (developing) bioinformatician I am today without your help. Especially the help in the beginning of my project was very useful and crucial. Good luck in Edinburgh.
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Thanks to the **Dr. Cann lab, Dr. Isaac Cann** and **Dr. Roderick Mackie** for accepting me as a member and my constant blabbing about thesis stuff I was still doing. We will achieve great things together.

Finally I would like to thank everybody else that I met along this exciting journey. See you!
And if I am have forgotten someone, rest assured it was not on purpose and I surely hope we will meet again. The experience of a PhD is all the people you meet and then the exchange of ideas, so stay connected. Unfortunately the Covid19-pandemic disturbed that greatly, nonetheless this hold some of my greatest memories and that is mostly thanks to you.

See you out there in the scientific world!

ABOUT THE AUTHOR

Patrick Schimmel was born on the 10th of May in Zevenaar, the Netherlands. Patrick was raised in Duiven and got his Gymnasium degree at the Candea College. Later he moved to Wageningen in 2008, to get his BSc in Biology at the Wageningen University. He combined his study with being a soccer player, music journalist and writer. Patrick continued his study by starting his MSc degree in Biology at the Wageningen University in 2012. During that time Patrick moved back to Arnhem, since that city truly feels like home to him. With a major in Molecular Ecology and a minor in Business and Marketing. During that time, Patrick developed a surging interest in the interplay of bacterial life with all other life domains and how bacteria can help us face societal challenges. For his MSc Thesis, he studied the interactions between bacteria and microalgae in relation to biofuel production, and some of this work was published in 2021 (Lian *et al.*, see publication list). For his MSC internship Patrick lived on Svalbard, Norway to study the microalgae in the wild during an experience that shaped him to be the scientist that he is today. In both occasions this happened under the supervision of Dr. Detmer Sipkema at the Laboratory of Microbiology at the Wageningen University. In Norway he was supervised by Dr. Tove Gabrielsen of UNIS, Norway. Patrick then started his PhD Project in 2018 at the Laboratory of Microbiology in Wageningen under supervision of Clara Belzer and Jan Knol. Together, they tried to change the view on bacterial nitrogen metabolism of the infant gut and approach the ecology of bifidobacterial in a new fashion. The research described in this thesis is the product of that collaboration. Moreover, Patrick worked with many other scientists in the gut microbiota field, coming from medical centers, business and nearby universities.

Patrick has now started his Post-Doctoral research at the University of Illinois at Champaign-Urbana in the lab of Dr. Isaac Cann. Patrick will continue his exploration into the gut microbiome by working on bacterial polysaccharide utilization and their associated bacterial sensors in the human gut and their application in personalized nutrition.

LIST OF PUBLICATIONS

Patrick Schimmel, Lennart Kleinjans, Roger S. Bongers, Jan Knol, Clara Belzer; Breast milk urea as a nitrogen source for urease positive *Bifidobacterium infantis*; FEMS microbiology, 2021

Patrick Schimmel, Bernd Stahl, Jan Knol, Clara Belzer, A breastfed infant's gut microbiota: in pursuit of nitrogen; Under revision, 2022

Patrick Schimmel, Lennart Kleinjans, Carl Vael, Kristine Desager, Jan Knol, and Clara Belzer; Proof of principle study replicating microbial clusters in connection to birth mode and diet in the early life intestine; Accepted after Revision, PLOS ONE, 2022

Patrick Schimmel[*], Stefanie Kouwenhoven[*], Berna de Vries, Jacqueline Muts, Johannes B. van Goudoever, Jan Knol, Clara Belzer; Effect of a low protein diet on the early life gut microbiota: A follow-up to the Proteus study; Manuscript in preparation.

Jie Lian, **Patrick Schimmel**, Selene Sanchez-Garcia, Rene H. Wijffels, Hauke Smidt, Detmer Sipkema; Different co-occurring bacteria enhance or decrease the growth of the microalga Nannochloropsis sp. CCAP211/78; Microbial Biotechnology, 2021

CO-AUTHOR AFFILIATIONS

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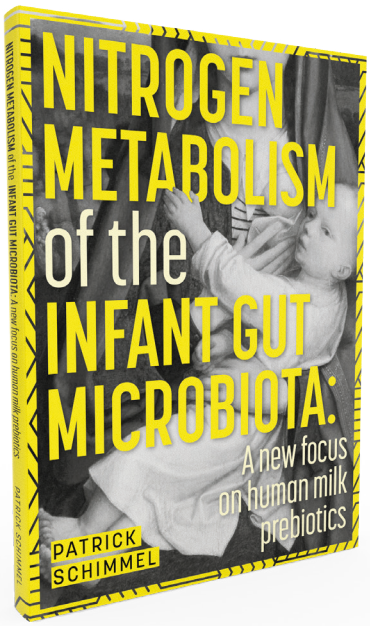
OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Name of the course/meeting	Organizing institute(s) & Place	Year
Discipline specific activities		
Gut Day 2018	Microbiology, WUR, Wageningen, the Netherlands	2018
System & Synthetic Biology Symposium,	SSB-WUR, Wageningen, the Netherlands,	2019
Stable Isotope Methods in Nutrition Research	VLAG, Wageningen, the Netherlands	2019
Intestinal microbiome of humans and animals	VLAG, Wageningen, the Netherlands	2019
Microbial Ecology symposium	NIOO/WEES, Wageningen, the Netherlands	2019
Gut Day 2019	AMC, Amsterdam, the Netherlands	2019
Annual KNVM meeting	KNVM,Arnhem, the Netherlands	2019
World Microbiome Forum	ASM/FEMS, USA/EU	2021
Datacamp MIB Account, Self-learning R	MIB, Datacamp	2019-2022
PreProPed 2021	DanoneNutricia Research & others, Online	2021
General Courses:		
VLAG PhD week	VLAG, Baarlo, the Netherlands	2018
Research data management	WGS, Wageningen, the Netherlands	2018
PhD workshop carousel	WGS, Wageningen, the Netherlands	2018
Scientific Publishing	WGS, Wageningen, the Netherlands	2018
Competence assessment	WGS, Wageningen, the Netherlands	2018
Illustrator for Scientists	WUR, MIB/Kevin Bonham, Wageningen, the Netherlands	2018
H_2S NOGEPA 0.8 Safety instruction	WUR/Vinnie de Wilde, Wageningen, the Netherlands	2018
Bioinformatics with Linux and Python	WIMEK/SENSE, Martin Jones, Wageningen, the Netherlands	2020
Other:		
Preparation of research proposal	MIB, Wageningen, the Netherlands	2018
PhD Trip to USA	MIB, United States, East Coast.	2019
Weekly group meetings	MIB, Wageningen, The Netherlands	2018-2022
Monthly Danone Nutricia Research Meetings	Danone Nutricia Research, Utrecht, the Netherlands	2018-2022
Quarterly Danone Nutricia Research Meetings	Danone Nutricia Research, Utrecht, the Netherlands	2018-2022
Daily board PhD representative	MIB, Wageningen, the Netherlands	2019-2022



ABOUT THE COVER

Breastfeeding as the golden standard for infant feeding required art from the golden ages of Dutch art. As an art enthusiast, I saw a mix of irony, conspicuity and beauty. The paintings included in this thesis show the Virgin Mary breastfeeding Jesus and sending him on his developmental way. Society is on a path to perhaps move away from breastfeeding, due to societal and formula product development. Classic institutions, like the church, saw value in direct feeding by the mother. This is reflected in these paintings that are further processed and designed by Margo Togni (margotogni.nl). The images are officially and legally acquired from the collection of the Rijksmuseum (see colofon). At the time, the artist and people viewing this painting for the first time were likely unaware of any biological reasons behind the importance of human milk. We are currently still on a path of discovery of the relation between human milk and infant health. However, I believe, we should not forget our classical biological standards and how far breastfeeding has brought us along our evolutionary trajectory. I hope the thesis cover reflects that.

COLOPHON

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Cover and lay-out design: Margo Togni | www.margotogni.nl
Cover artwork front: PRISMA ARCHIVO / Alamy Stock Photo. *Attributed to Gerard David (1460-1523). Flemish painter. Madonna and Child, ca.1500. National Gallery. Oslo. Norway.*
Cover artwork back: Rijksmuseum Amsterdam. *Maria met kind, Vereniging Rembrandt.*
Print: Ridderprint | www.ridderprint.nl