Dissertation

The Woman - Microbiome - Child Interplay
–
A Microbial Journey through Pregnancy and Early Infancy

submitted by

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under the Supervision of

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz”.

Charlotte Julia Neumann
Graz, 20.12.2024

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All co-authors gave their approval to reuse the published manuscripts within this thesis:

Chapter 1: **The Dynamics of the Female Microbiome: Unveiling Abrupt Changes of Microbial Domains across Body Sites from Prepartum to Postpartum Phases.** [1]

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- **Eva-Christine Weiss** and **Vassiliki Kolovetsiou-Kreiner** gave medical input in conceptualizing the study and facilitated sample collection.
- **Bettina Amtmann** and **Petra Winkler** were responsible for patient recruitment and sample collection.
- **Alexander Mahnert** contributed to data analysis and interpretation
- **Victoria Haid** helped in sample processing
- **Manuela Pausan** was involved in all activities, from conceptualization to writing.
- **Christine Moissl-Eichinger** supervised all activities and wrote parts of the manuscript

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Chapter 2: **Clinical NEC prevention practices drive different microbiome profiles and functional responses in the preterm intestine.** [2]

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- **Tobias Madl** performed metabolome analysis
- **Christine Moissl-Eichinger, Lindsay Hall, Berndt Urlesberger** and **Bernhard Resch** supervised the study
- **Christine Moissl-Eichinger** supervised all activities and wrote parts of the manuscript

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Chapter 3: **First-Year Dynamics of the Anaerobic Microbiome and Archaeome in Infants' Oral and Gastrointestinal Systems** [3]

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- **Victoria Haid and Tanja Kobal** helped in sample processing
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Table of Contents

Table of Contents

Abbreviations and Definitions

BF	-	Breastfed
BV	-	Bacterial Vaginosis
CS	-	Cesarean Section
CST	-	Community State Type
DHM	-	Donor Human Milk
ELBW	-	Extremely Low Birth Weight
GIT	-	Gastrointestinal Tract
HM	-	Human Milk
HMOs	-	Human Milk Oligosaccharides
IgA	-	Immunoglobulin A
MFGM	-	Milk Fat Globule Membrane
MOM	-	Mother's Own Milk
mpost	-	maternal post-partum
mpre	-	maternal pre-partum
NBF	-	Non-Breastfed
NEC	-	Necrotizing Enterocolitis
NICUs	-	Neonatal Intensive Care Units
np	-	nonpregnant
SCFAs	-	Short Chain Fatty Acids
VLBW	-	Very Low Birth Weight (< 1,500g)

Zusammenfassung

Das menschliche Mikrobiom ist ein komplexes Netzwerk aus einer Vielzahl von Mikroorganismen, die miteinander und mit ihrem Wirt interagieren, sowie deren funktionellen Aktivitäten. Die Entwicklung beginnt in der perinatalen Phase, wobei die bedeutendsten Initiierungs- und Strukturierungsprozesse in den ersten Lebensjahren stattfinden. Da das Mikrobiom jedoch hochgradig dynamisch ist, passt es sich nicht nur an verschiedene Körperstellen und deren Umgebungen, sondern auch an Veränderungen im Laufe des Lebens an. Diese kumulierte Dissertation behandelt diese Dynamiken in drei Publikationen:

1) Eine bedeutende Veränderung, die der weibliche Körper durchlaufen kann, ist eine Schwangerschaft. Wir haben die Dynamiken untersucht, die das orale und urogenitale Mikrobiom während der Schwangerschaft und in der postpartum Phase erleben. Wir zeigten, dass sich das orale Mikrobiom während der Schwangerschaft deutlich verändert, was möglicherweise orale Gesundheitsprobleme begünstigt, sich jedoch innerhalb eines Monats nach der Geburt wieder erholt. Im Gegensatz dazu durchläuft das urogenitale Mikrobiom nach der Geburt eine stärkere Umstrukturierung, nachdem es während der Schwangerschaft eine vorteilhafte, *Lactobacillus*-dominierte Zusammensetzung angenommen hatte.
2) Die zweite Publikation befasst sich mit der Initiierung der Entwicklung des Darmmikrobioms bei Frühgeborenen mit sehr niedrigem Geburtsgewicht. Sie vergleicht die Auswirkungen verschiedener Regime, einschließlich Fütterungspraxis, Antibiotikagabe und Probiotika-Verabreichung. Wir kamen zu dem Schluss, dass eine kombinierte Strategie aus der Gabe von Muttermilch und der Verabreichung des probiotischen *Bifidobacterium longum* subsp. *infantis* NCDO 2203, eventuell zusammen mit anderen probiotischen *Lactobacillus*-Stämmen, eine vielversprechende Strategie darstellt. Diese Kombination nutzt die Synergien zwischen probiotischen Mikroben, deren Wachstum durch die präbiotischen Nährstoffe in der Muttermilch unterstützt werden, und hilft dabei, potenziell pathogene Mikroorganismen zu übertreffen.
3) Auch für die Entwicklung sowohl des oralen als auch des Darmmikrobioms im ersten Lebensjahr ist Muttermilch ein Schlüsselfaktor, dessen Einfluss den des Geburtsmodus übersteigt. Das Mikrobiom gestillter Kinder zeigten Phasen ausgeprägter Entwicklung,

während nicht gestillte Säuglinge eine frühere und graduellere Reifung aufwiesen. Dieser Effekt war im oralen Mikrobiom stärker ausgeprägt als im Darmmikrobiom. Ein weiterer Aspekt der Mikrobiomreifung war der abnehmende Einfluss spezifischer Taxa vom oralen zum Darmmikrobiom.

Abstract

The human microbiome is a complex network of trillions of microorganisms interacting with each other and their host, along with their functional activities. Its development begins in the perinatal phase, with the most significant initiation and structuring occurring during the first few years of life. However, as the microbiome is highly dynamic, it not only adapts to different body sites and their environments but also to changes throughout the lifespan. This cumulative thesis addresses these dynamics across three publications:

1) One significant change that the female body undergoes is pregnancy. We explored the dynamics that the oral and urogenital microbiomes undergo during pregnancy and in the postpartum period. We showed that the oral microbiome changes notably during pregnancy, potentially favoring oral health issues, but it recovers within one month postpartum. In contrast, the urogenital microbiome undergoes a more pronounced restructuring postpartum, after having shifted to a beneficial *Lactobacillus*-rich environment during pregnancy.
2) The second publication focuses on the initiation of gut microbiome development in premature infants with very low birth weight. It compares the effects of different regimens, including feeding practices, antibiotic use, and probiotic administration. We concluded that a combined approach of feeding human milk (HM) and administering the probiotic *Bifidobacterium longum* subsp. *infantis* NCDO 2203, likely alongside other probiotic *Lactobacillus* strains, is a promising strategy. This combination leverages the synergies between beneficial microbes supported by the prebiotic nutrients found in HM, helping to outcompete potentially pathogenic microorganisms.
3) Also, for the development of both the oral and gut microbiome in the first year of life, HM is a key driver, with its impact surpassing that of birth mode. Breastfed infants showed distinct developmental phases, whereas non-breastfed infants exhibited earlier and more gradual maturation. This effect was more pronounced in the oral microbiome than in the stool microbiome. One additional aspect of microbiome maturation was the decreasing influence of specific taxa from the oral to the stool microbiome.

Introduction

The Human Microbiome

The human microbiome is a highly complex community composed of trillions of microorganisms, including bacteria, archaea, fungi, and viruses, found throughout the human body in the same order of magnitude as human cells [4]. While the term "human microbiota" refers to the living organisms themselves, the "human microbiome" encompasses both these organisms and their "theater of activity". This includes the vast array of molecules produced by microbes, such as metabolites (e.g., toxins, signaling molecules), structural elements (e.g., proteins, polysaccharides, lipids, nucleic acids), and mobile elements like phages, viruses, as well as extracellular and "relic" DNA [5].

Microbes and their theatre of activity are found in almost every part of the body, with the large intestine being one of the most densely colonized areas [6,7]. This area has been extensively studied in recent years, especially due to the role its microbes play in nutrient digestion. These microbes metabolize nutrients that are otherwise indigestible by the human intestine, such as dietary fibers, and produce beneficial metabolic byproducts, including short-chain fatty acids (SCFAs), as well as essential vitamins like vitamin K and several B vitamins [8,9]. SCFAs are mainly produced through anaerobic microbial fermentation of e.g. dietary fibers and starch, in the large intestine, mainly to acetate, propionate and butyrate [10]. SCFAs are absorbed by colonocytes (colonic epithelial cells) in the large intestine, having anti-inflammatory properties and promoting health by e.g., mucus production [11,12]. Additionally, the gut microbiome is key in regulating the immune system and influencing behavior via the gut-brain axis [6,13,14].

Other body sites, such as the oral cavity, skin, vaginal tract, and respiratory system, also host unique microbiomes adapted to their specific environments. For example, differences in living conditions (e.g., pH, oxygen levels, and available nutrients) across these body sites result in distinct microbial communities adapted to each environment [15]. These site-specific microbiomes will be explored in further detail later.

Various factors can influence and modulate the microbiome, including diet, lifestyle and medication (particularly antibiotics) [16]. Those factors as well as probiotics, and even fecal microbiota transplants can be used to actively modulate the microbiome [17].

Not only do microbiomes vary significantly from one body site to another, but they also differ between individuals [15]. Factors like gender and life stage play critical roles in shaping the microbiome throughout a human's life. The most rapid development occurs in early childhood, as these microbial communities establish themselves. Later, puberty, with its marked hormonal changes, brings another wave of microbial transformation. For women, pregnancy triggers another reorganization within the microbiome.

The Microbiome in Women

During pregnancy, a woman's body undergoes substantial hormonal and immunological changes, which are mirrored in shifts within her microbiome [18]. Of particular focus is the vaginal microbiome as an essential to reproductive health [19,20].

The Urogenital Microbiome

The vaginal microbiome is predominantly colonized by lactobacilli, which help maintain a low pH that forms a barrier against pathogens. This barrier is crucial in preventing conditions like bacterial vaginosis (BV) and yeast infections, which can adversely affect pregnancy outcomes [21]. Pregnancy reduces bacterial diversity in the vaginal microbiome, reinforcing the predominance of *Lactobacillus* species [22]. This shift may be driven by elevated estrogen levels, which promote epithelial maturation. As a consequence, glycogen accumulates which, degraded into maltose, maltotriose and maltotetraose, further supporting the growth of lactic acid bacteria [23,24].

Microbial presence in the urogenital tract during pregnancy also impacts pregnancy outcomes. For example, urinary tract infections increase the risk of preterm birth [25]. Even the mere presence of specific microbes, like *Ureaplasma urealyticum*, can elevate this risk, independent of infection [26].

The Oral Microbiome

The oral microbiome is another area affected by pregnancy, with potential clinical implications. Many pregnant women experience bleeding gums, gingivitis, or even periodontitis [27], conditions that have been associated with adverse pregnancy outcomes [28].

These observations underscore the body's mechanisms to protect the fetus on multiple levels, including the adaptation and maintenance of a healthy, protective microbiome. However, the

extent of microbiome alternations in other body areas during pregnancy, as well as their significance, remains insufficiently understood.

Delivery and Postpartum Period

Delivery marks an abrupt end to pregnancy and ushers in major shifts throughout the body. Postpartum lifestyle changes—including disrupted sleep, increased energy and nutrient demands (especially during breastfeeding), as well as substantial hormonal changes—also influence the microbiome [29,30]. The vaginal microbiome, in particular, undergoes substantial alterations, often losing its lactobacilli predominance due to reduced estrogen levels [20]. This environment, coupled with potential wound surfaces and lochia, can increase the risk of infections, including postpartum endometritis, which occurs in 1-3% of cases [31].

To support postpartum recovery and prevent infections, it is essential to understand how the microbiome changes and how its restoration can be facilitated. Exploring the human microbiome's intricate relationship with health is a frontier in biomedical research, offering potential breakthroughs in personalized medicine and preventive healthcare.

The Infant's Microbiome

The first year of life represents a crucial window for microbiome development, setting the stage for long-term health [32]. During this period, the gut microbiome transitions from a simple, low-diversity community to a highly complex ecosystem [32]. This process is shaped by a variety of factors, including those discussed earlier and in the following.

Where Life Begins

It remains unclear to what extent a fetus encounters living microbes in the womb. Current evidence suggests that while a fetus may come into contact with bacterial components (e.g., cell wall material, DNA), colonization by living microorganisms likely begins only after birth.[33]

Birth Mode

Upon delivery, the newborn is exposed to a vast array of microbes. Vaginally delivered infants primarily encounter the vaginal and stool microbiomes, while those born via caesarean section (CS) first interact with skin and hospital-associated microbes [34,35]. These initial microbial exposures differ significantly, influencing the newborn's developing microbiome [34,36]. Those

microbiome alterations induced by CS could influence intestinal epithelial cell activation and immune system development as a downstream effect as well as certain autoimmune and metabolic disorders [34]. Still, over time, their microbiome compositions converge [37] with some differences remaining [38,39].

Human Milk and Human Milk Oligosaccharides

Human milk (HM) becomes a key driver of microbiome composition and is a superfood in multiple ways (see overview in Fig. 1). It contains immunoglobulins and anti-inflammatory compounds such as lactoferrin that are promoting health per se. Additionally, HM provides not only probiotic living microbes themselves [39] but also prebiotics [40]. Next to lactose, lactoferrin [41] and milk fat globule membrane (MFGM) with its bioactive components [42,43], the main prebiotics in HM are human milk oligosaccharides (HMOs) which are complex sugars that are indigestible by the infant's enzymes and instead are metabolized by specific microbes, *Bacteroides* and predominant *Bifidobacterium* species [44,45]. Consequently, HMOs promote the growth of *Bifidobacterium,* which degrades them into postbiotic metabolites like SCFAs and lactate [46]. These metabolites enhance gut barrier function, support immune maturation, reduce inflammation, and lower gut pH, thereby inhibiting pathogens such as *Escherichia coli* [47]. Infants who are breastfed (BF) have microbiome colonization patterns that differ significantly from those who are formula-fed (NBF), with breastfed infants showing higher *Bifidobacterium* counts [48,49]. This highlights the crucial role of HM in shaping a protective and health-promoting infant gut microbiome.

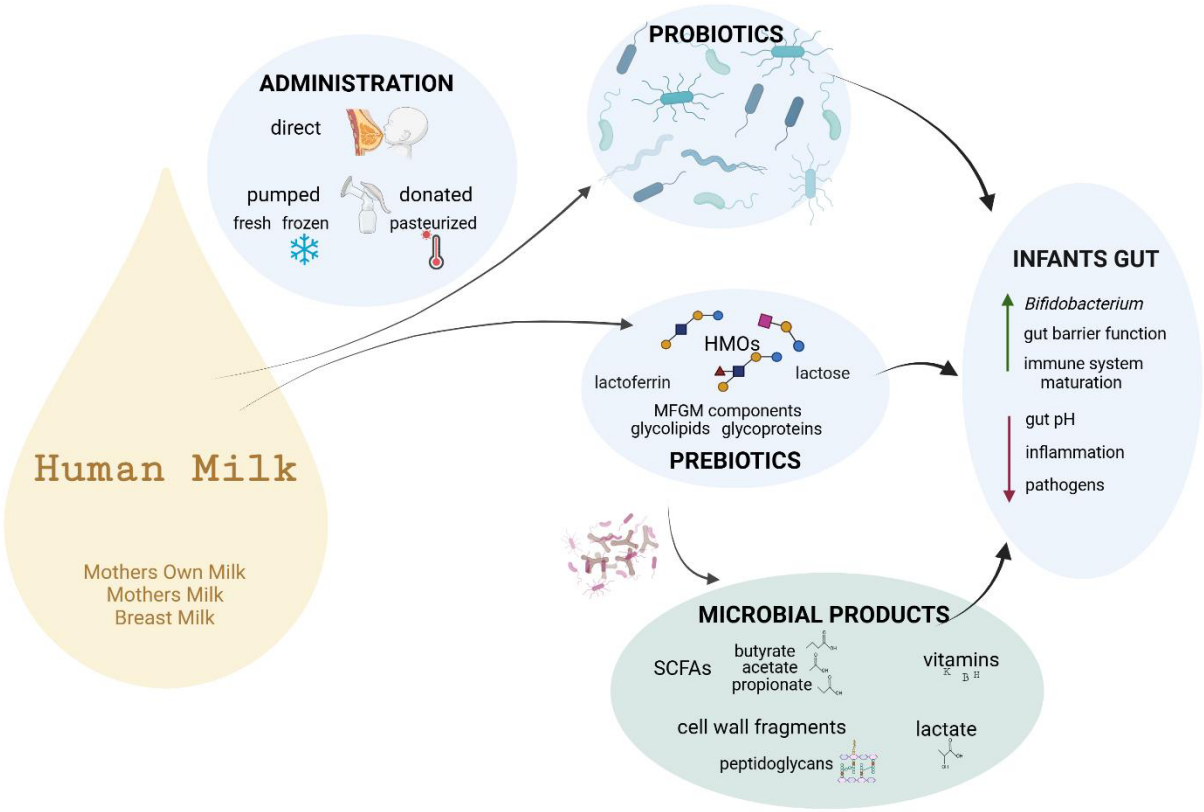

Figure 1: Human milk as a superfood; abbreviations: Human Milk Oligosaccharides (HMOs), Milk Fat Globule Membrane (MFGM) Components, Short Chain Fatty Acids (SCFAs)

Created with BioRender.com

Sources of Microbes

Microbial exposure during infancy extends beyond delivery and HM, with significant contributions from various sources such as family members, pets, food, and the surrounding environment. These diverse exposures are complemented by factors like antibiotic use, lifestyle habits, physical activity, and general health practices, all of which influence microbiome acquisition and development [50,51].

Introduction of Solid Food

The introduction of solid food marks a pivotal shift in gut microbiome composition, significantly enhancing microbial diversity and encouraging the growth of taxa commonly associated with the adult gut, such as *Firmicutes* and *Bacteroidetes*. Solid food introduction is a very diverse and highly individual process with lots of differing nuances that also impact the

microbiome development. Examples are diversity and timing of solid food introduction as well as to what extent breastfeeding is still complementary ongoing [52].

As previously mentioned, the microbiomes of different body sites develop uniquely. While the oral cavity and large intestine microbiomes, representing the start and end of the gastrointestinal tract (GIT), are connected, they acquire distinct microbial populations and follow different developmental trajectories.

The Infants' Oral Microbiome

In the oral cavity, *Streptococcus* is an early and predominant colonizer, capable of adhering to epithelial cells and producing extracellular polymers that facilitate the establishment of other microbial species [53]. Key microbial groups in the infant oral cavity include *Actinomyces*, *Streptococcus*, *Neisseria*, *Haemophilus*, and *Veillonella* [54]. Many of these microorganisms, such as *S. mitis* and *S. oralis*, produce immunoglobulin A (IgA) proteases that selectively degrade secretory salivary IgA. This ability is thought to provide an advantage for their survival in the IgA-rich environment created by HM [55]. In general, the oral cavity harbours a variety of micro-niches with varying microenvironments, such as biofilms, subgingival crevices, crypts of the tonsils and dental pockets [56]. Some of those niches just appear within the first years of life, e.g., when first teeth are emerging. As micro-niches and microbial input diversifies during the first months of life, so does the microbial diversity. Still, this maturation process of the oral microbiome continues until adulthood [57].

The Infants' Gut Microbiome

The GIT, especially the large intestine, is colonized rapidly and densely, fostering a diverse microbiome. At birth, the gut is aerobic, supporting facultative anaerobic microbes that consume oxygen and create an anaerobic environment [58–60]. This shift is critical for gut maturation and allows obligate anaerobes, such as *Bifidobacterium*, to dominate. *Bifidobacterium* is particularly important for infant health, contributing to gut barrier function and immune development [61].

The gut microbiome also harbors archaeal taxa, primarily methanogens like *Methanobrevibacter smithii*, which is considered a keystone taxon [62–65]. While archaeal signatures have been detected in young infants, the mechanisms of their colonization and development remain poorly understood and are an emerging area of research [66,67].

Microbiome Development in Preterm Infants

For preterm infants born before 37 weeks of gestation, the process of microbiome development differs significantly from that of full-term infants. Premature infants, particularly those with very low birth weight (VLBW, <1,500g), begin life in neonatal intensive care units (NICUs) under specialized conditions, including incubators, medications, and distinct feeding regimens. These circumstances create a unique and often disrupted environment for microbiome establishment. [68]. In addition, the premature gut is also physiologically immature and more permeable compared to term infants [69,70]. Permeability at tight junctions is increased, leading to a leakier gut and the mucous production is decreased, facilitating adhesion of pathobionts to the epithelium. As a consequence, the risk of local inflammation or systemic sepsis as well as other complications and diseases is increased [68,71].

Necrotizing Enterocolitis

One of those diseases that the immature gut of preterm infants is more susceptible to is necrotizing enterocolitis (NEC). This severe gastrointestinal disorder affects 7-11% of VLBW infants and is characterized by bacterial overgrowth in combination with altered microvascular blood flow. Although the pathogenesis of this disease is not fully understood, it is thought to arise from multiple factors, including microbiome disruptions [72,73]. Potentially pathogenic microbes such as *Escherichia coli*, *Clostridium perfringens* and *Klebsiella* spp. are known to be implicated in NEC, overgrowing and disrupting the gut microbiome [74]. As a consequence, bacterial endotoxins might bind to the epithelial cells and activate pathogen-associated molecular pattern receptors leading to epithelial injury and cell apoptosis and an intense inflammatory cascade [68,75].

Regimens in Neonatal Intensive Care Units

To mitigate the risk of NEC and other complications, NICUs employ several strategies targeting the microbiome, including routinely prophylactic antibiotics and antimycotics to eradicate harmful pathogens, or probiotics and HM to support healthy gut colonization by introducing and fostering beneficial bacteria. Those possible strategies differ lot between NICUs, not only between countries but also within countries. Based on current literature, data and guide treatments are missing, extrapolating efficacy from adult studies [76,77].

- *Antibiotics*

Antibiotic administration is a frequently used method to prevent infections in preterm infants. On average, 61.3% of premature neonates [78], 78.6% of VLBW infants [79] and 87.0% of extremely low birth weight infants [79] receive antibiotics during their hospital stay. There are substantial variations across NICUs with respect to type, dose and duration of administration [78,79]. Commonly-used antibiotics are ampicillin and gentamycin [79]. The high variation in antibiotic administration regimens between NICUs already implies a lack of sufficient data about the harms and benefits of different early and prolonged antibiotic therapies [80].

- *Antimycotics*

Similar to antibiotics, antifungal prophylaxis also differs a lot between NICUs. The preferred antifungals are fluconazole or nystatin especially targeting *Candida*. [81,82]

- *Probiotics*

It was shown that probiotics can decrease incidences of NEC [83–85] and accelerate microbiome maturation [86] but this is variable from strain to strain and still under dispute. Common probiotic strains used in preterm infants include *Bifidobacterium* (*B. breve*, *B. infantis*, *B. longum*) and *Lactobacillus (Lactobacillus acidophilus*, *Lacticaseibacillus rhamnosus* and *Limosilactobacillus reuteri* (both former *Lactobacillus* genus [87])) but also *Streptococcus thermophilus* or *Saccharomyces boulardii* [88,89]. It was shown that some of those probiotic species help improving gut barrier by e.g., regulating tight junction functions [90,91], decreasing permeability, restoring goblet cell numbers and increasing mucous production in mice [92]. Safety of probiotic administration is also frequently discussed. The leaky gut of premature infants could also lead to the risk of probiotic induced or -associated bacteraemia and/or sepsis, however, there are only very few documented cases to date [93–95].

- *Human Milk*

Again, preferential feeding of HM is another crucial tool in fostering the natural growth of beneficial *Bifidobacterium* species, offering both nutritional and microbial benefits. It is known that formular feeding increases the risk for NEC in preterm infants [96,97]. Therefore, feeding of HM is preferred in many NICUs. If mothers own milk (MOM) is not available, donor human milk (DHM) is a very good alternative, even though its microbial and non-microbial components and therefore efficacy are slightly altered as DHM typically undergoes pasteurization and freezing [68,98,99].

Overall, NICU protocols aim to establish a protective gut microbiome that guards against harmful pathogens, promoting better outcomes for preterm infants.

Microbiome Members other than Bacteria

Most human microbiome studies primarily focus on bacteria, often overlooking other components of the microbiome, particularly fungi and archaea. This bias is likely due to the relative abundance of bacteria, which dominate the human microbiome. Additionally, specialized methods are required to analyze fungi and archaea, from tailored DNA extraction protocols to sequencing techniques and bioinformatics pipelines (reviewed in [100]). These added requirements make comprehensive studies more complex and costly.

However, recent research suggests that fungi and archaea are widely distributed in and on the human body, colonizing not only the GIT but also other regions [101,102]. A deeper understanding of these non-bacterial components is essential, as they likely play significant roles in human health and disease.

Objectives and Summary of the Thesis

Chapter 1.: The Dynamics of the Female Microbiome: Unveiling Abrupt Changes of Microbial Domains across Body Sites from Prepartum to Postpartum Phases [1]

During pregnancy and the perinatal period, the time frame surrounding birth, a female's microbial ecosystem undergoes substantial shifts. However, the extent of these microbiome alterations, especially beyond the vaginal environment, remains largely unexplored.

In this study, we tracked a group of pregnant participants approaching delivery (pre-partum (mpre), n = 30) to their postpartum period (one-month after delivery, post-partum (mpost), n = 30). We analyzed microbiome profiles from their oral, urinary, and vaginal sites, specifically targeting bacterial, archaeal, and fungal components, and examined the urinary metabolome. These data were then compared with profiles from a control group of nonpregnant women (np, n = 29).

Our findings indicate that body site had a greater influence on microbiome composition than the pregnancy status (np, mpre, mpost). Nonetheless, the oral and urogenital (vaginal and urinary) microbiomes showed distinct shifts between the prepartum and postpartum phases. The oral microbiome, for instance, transitions during pregnancy but reverts to a nonpregnant-like state by one month postpartum. Similarly, the urinary microbiome stabilizes one month after delivery, although lactose and oxaloacetic acid levels remain elevated postpartum. By contrast, the vaginal microbiome undergoes significant reconstruction, with a marked decrease in *Lactobacillus* postpartum. This shift results in an increased prevalence of Vaginal Community State Type (CST) IV (85% of women) over CST I (40% of women).

Beyond bacterial changes, fungal and archaeal components also varied between the analysed phase, with notable differences in the archaeal genera *Methanobacterium* and *Methanobrevibacter*. During pregnancy, *Methanobrevibacter* increased in relative abundance in the oral cavity while decreasing in the vagina, while *Methanobacterium* was scarcely detected in either mpre or mpost women.

Our results highlight pregnancy and delivery as periods of significant microbiome and metabolome remodeling in the female body.

Chapter 2.: Clinical NEC Prevention Practices Drive Different Microbiome Profiles and Functional Responses in the Preterm Intestine. [2]

Infants born prematurely (<37 weeks' gestation) with very low birth weight (<1,500 g) are at high risk for necrotizing enterocolitis (NEC), a life-threatening inflammation of the gastrointestinal tract (GIT) in which the microbiome plays a partial contributing role.

We analyzed the impact of three different NEC prevention regimens on the gut microbiome (regimens are depicted in Fig. 2). Therefore, we characterized the microbiome profiles of fecal samples of 55 infants longitudinally within their first weeks of life (n= 383 samples). By using targeted 16S rRNA gene sequencing and shotgun metagenomics, we could show that the regimens do not only affect the bacterial but also archaeal, viral and fungal players of the gut microbiome. Beyond that, microbial functions, virulence factors and resistances against antibiotics could be evaluated. With addition of metabolomics, we could analyze metabolic profiles, including human milk oligosaccharides (HMOs) and short-chain fatty acids.

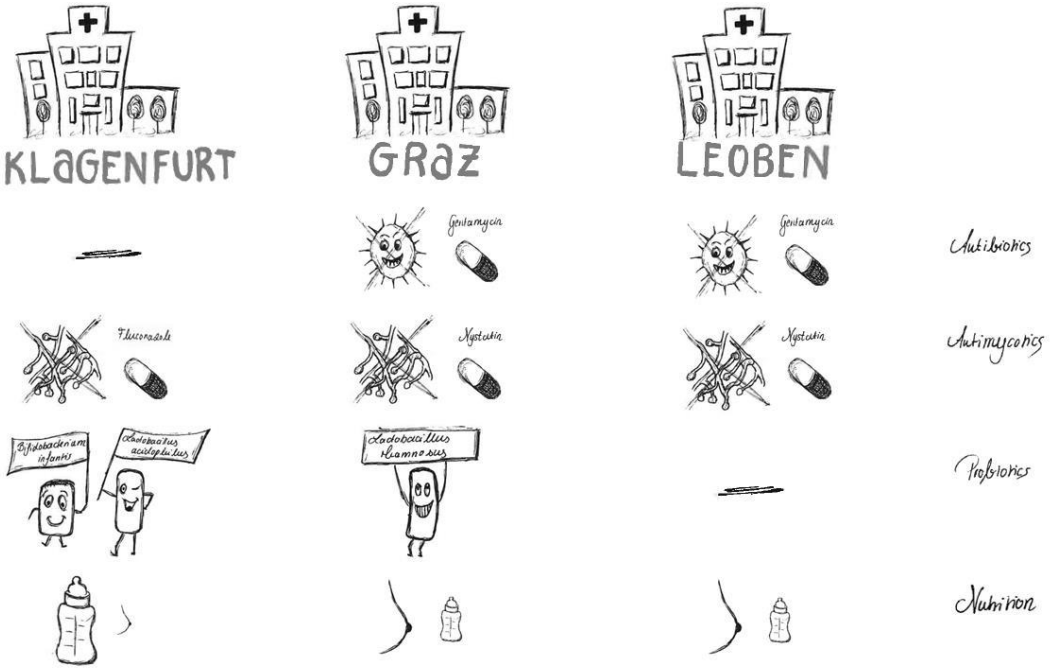

Figure 2: Graphical description of the different Necrotizing Enterocolitis (NEC) prophylaxis regimens of the three neonatal intensive care units Klagenfurt, Graz and Leoben.

We found that supplementing *Bifidobacterium longum* subsp. *infantis* NCDO 2203 as a probiotic significantly influenced the gut microbiome. This was characterized by a high relative abundance of this beneficial bacterium, and, antibiotic resistance was lower compared to

regimens without probiotic supplementation or supplementing *Lactobacillus rhamnosus* LCR 35. *Bifidobacterium longum* subsp. *infantis* NCDO 2203 also exhibited genomic potential for converting beneficial HMOs, provided that these HMOs were concurrently available, as from human milk.

Our findings demonstrate that preventive regimens can meaningfully influence the development and maturation of the gastrointestinal microbiome, promoting a resilient microbial ecosystem that reduces pathogenic risks in vulnerable preterm infants.

Chapter 3.: First-Year Dynamics of the Anaerobic Microbiome and Archaeome in Infants' Oral and Gastrointestinal Systems. [3]

The human microbiome undergoes rapid development in the first years of life, forming highly complex microbial communities. In this study, we highlighted the significant influence of breastfeeding on the development of both the oral and GIT microbiomes in infants during their first year of life. To do this, we followed 30 healthy, full-term infants over their first 12 months of life, collecting oral and stool samples monthly and analyzing them using 16S rRNA gene amplicon sequencing and metagenomic sequencing. Our analysis focused on taxonomic and functional dynamics within the microbiome, emphasizing the often-overlooked roles of archaea and anaerobic microbes.

Our findings reveal that archaea are present in the oral and GIT microbiomes from early infancy, though a stable archaeome does not yet form. Overall, *Methanobrevibacter* was the predominant genus at both body sites. We observed that breastfeeding lead to a distinct transition phase in the oral microbiome, marked by a decrease in the relative abundance of *Streptococcus* and the appearance of *Granulicatella* and other anaerobic genera. During this transition, alpha diversity increased, and beta diversity shifted significantly. This transition phase occured later in breastfed (BF) infants (at 4–6 months) compared to non-breastfed (NBF) infants, suggesting that breastfeeding supports a more defined and enduring maturation of the oral microbiome.

In contrast, the GIT microbiome develops more gradually with breastfeeding status leads to distinct differences in its development. NBF infants displayed complex microbial co-occurrence patterns from an earlier stage, while in BF infants, diversity and complexity increased more gradually between three and eight months of life. However, functional differences between BF and NBF infants GIT microbiomes were less pronounced than taxonomic differences.

Overall, our findings demonstrate that breastfeeding plays a critical role in the maturation of the infant microbiome within the first year of life.

The publications

Chapter 1.:

The Dynamics of the Female Microbiome: Unveiling Abrupt Changes of Microbial Domains across Body Sites from Prepartum to Postpartum Phases. [1]

Chapter 2.:

Clinical NEC Prevention Practices Drive Different Microbiome Profiles and Functional Responses in the Preterm Intestine. [2]

Chapter 3.:

First-Year Dynamics of the Anaerobic Microbiome and Archaeome in Infants' Oral and Gastrointestinal Systems. [3]

1st publication

The Dynamics of the Female Microbiome: Unveiling Abrupt Changes of Microbial Domains across Body Sites from Prepartum to Postpartum Phases.[1]

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Graphical Abstract

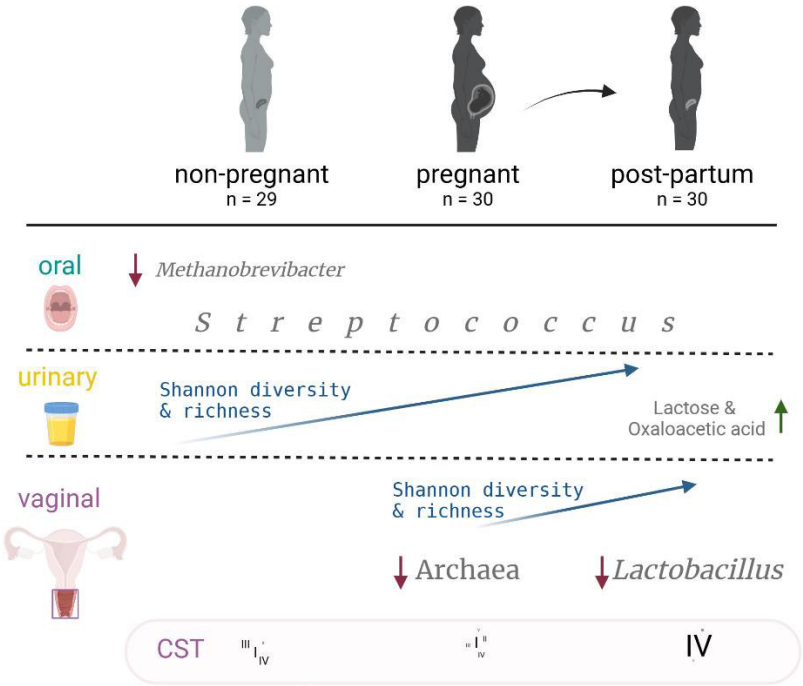

Figure 3: Graphical abstract of the major results of the 1st publication; abbreviations: Community State Types (CST); created with BioRender.com

Published in **Microbiology Spectrum** (*June 2024*)

2nd publication

Clinical NEC prevention practices drive different microbiome profiles and functional responses in the preterm intestine. [2]

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Graphical Abstract

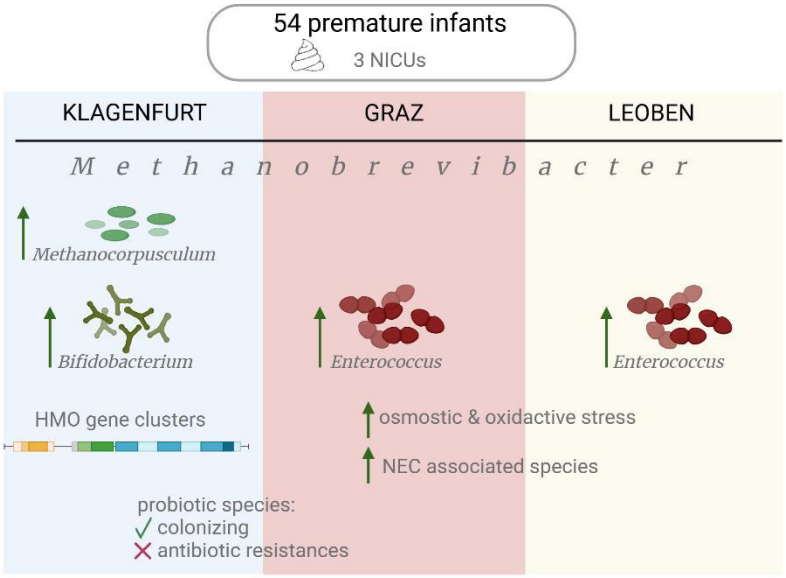

Figure 4: Graphical abstract of the major results of the 2nd publication; created with BioRender.com

Published in **Nature Communications** (*March 2023*)

3rd publication

First-Year Dynamics of the Anaerobic Microbiome and Archaeome in Infants' Oral and Gastrointestinal Systems. [3]

Charlotte J. Neumann[1], Rokhsareh Mohammadzadeh[1], Pei Yee Woh[2,3], Tanja Kobal[1], Manuela-Raluca Pausan[1,4], Tejus Shinde[1], Victoria Haid[1], Polona Mertelj[1], Eva-Christine Weiss[5], Vassiliki Kolovetsiou-Kreiner[5], Alexander Mahnert[1], Christina Kumpitsch[1], Evelyn Jantscher-Krenn[5,6,7,], Christine Moissl-Eichinger[1,7,*]*

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Graphical Abstract

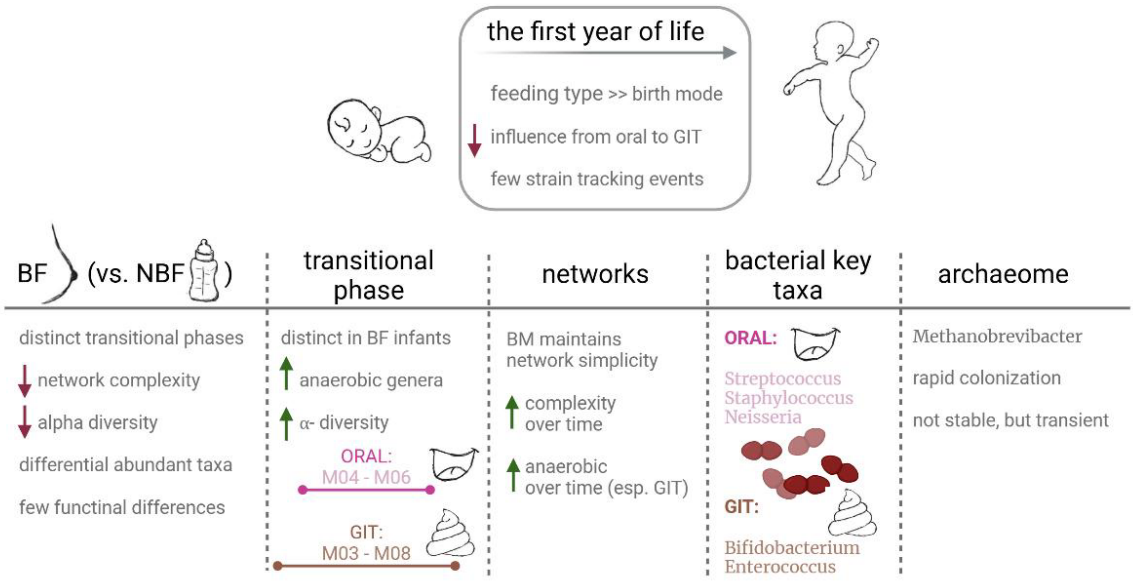

Figure 5: Graphical abstract of the major results of the 3rd publication

Created with BioRender.com

Published in **mSystems** (December *2024*)

General Discussion

Chapter 1.: The Dynamics of the Female Microbiome: Unveiling Abrupt Changes of Microbial Domains across Body Sites from Prepartum to Postpartum Phases. [1]

The human microbiome is shaped by various factors, including pregnancy and the postpartum period. In this study, we demonstrated that the female microbiome and urinary metabolome undergo significant changes from the nonpregnant to the perinatal period. Specifically, we observed that not only the bacterial but also the archaeal and fungal components of the urinary, vaginal, and oral microbiomes in 30 women shifted between pregnancy and one month postpartum, with notable differences compared to the microbiomes of nonpregnant women.

Health problems often associated with a disrupted microbiome are common during pregnancy and postpartum, emphasizing the importance of understanding the dynamic changes across different body sites during this period. This knowledge can inform prevention strategies and support interventions aimed at mitigating microbiome-associated health issues.

One such issue is bleeding gums or periodontitis, often attributed to alterations in the oral microbiome during pregnancy. In our study, we found an increase in alpha diversity (Shannon diversity and richness) of the oral microbiome, although an increase in pathogenic taxa, as reported elsewhere [103], was not observed. While bacterial-level differences in the oral microbiome were minimal, archaeal differences were more pronounced. The dominant archaeal genus, *Methanobrevibacter*, significantly increased during pregnancy and rapidly decreased postpartum, returning to pre-pregnancy levels. Elevated levels of methanogenic archaea, such as *Methanobrevibacter*, could facilitate the growth of anaerobic bacteria potentially associated with periodontitis by consuming H_2 and CO_2, which would otherwise inhibit their growth [104].

Another common concern for pregnant women is a disruption of the vaginal microbiome, often manifesting as bacterial vaginosis (BV) or fungal infections [26,105–107]. These conditions are of particular clinical interest due to their association with adverse pregnancy outcomes. A vaginal microbiome dominated by *Lactobacillus* species and characterized by low alpha diversity is critical during pregnancy for maintaining a barrier against dysbiosis. In our study, we confirmed this pattern during pregnancy. However, one month postpartum, the vaginal microbiome underwent a marked shift, transitioning from a *Lactobacillus*-dominated state to a highly

diverse community, regardless of delivery mode. Costello *et al.* showed that these disruptions persist for up to a year postpartum, with increased diversity, reduced *Lactobacillus* abundance, and a sustained loss of *L. crispatus* dominance [29]. In our dataset, 70% of postpartum women transitioned to vaginal community state type (CST) IV, characterized by low *Lactobacillus* dominance, while none exhibited CST I, dominated by *L. crispatus*. Unfortunately, our sequencing approach did not allow for species-level resolution. However, by comparing shifts in bacterial genera postpartum with those reported for CST IV and BV, we found strikingly similar patterns. Postpartum vaginal microbiome changes appear influenced by lochia, a vaginal discharge that occurs after childbirth, which inhibits *Lactobacillus* growth, and reduced estrogen levels during lactation or impaired ovarian activity [20].

Of the microbiomes analyzed in this study, the vaginal microbiome exhibited the most pronounced changes across the perinatal and postpartum periods, while the oral microbiome showed the least. The urinary microbiome also demonstrated dynamic changes, albeit to a lesser extent than the vaginal microbiome. However, due to the close proximity of the vaginal and urinary tracts, some overlap between these microbiomes was expected, as confirmed by source tracking analyses. Interestingly, the urinary microbiome appeared to have a greater effect on the vaginal microbiome than vice versa. For postpartum women, the bidirectional exchange between these microbiomes was reduced compared to nonpregnant or pregnant women. A notable finding was the significantly elevated level of lactose in postpartum urine, which could potentially support the recovery of the vaginal microbiome by promoting *Lactobacillus* growth through urinary-vaginal exchange.

We also demonstrated that the fungal component of the microbiome undergoes dynamic changes from the perinatal to the postpartum period, particularly in alpha diversity and the relative abundance of unclassified reads. However, the high proportion of unclassified reads, likely due to resolution limitations, restricted the conclusions we could draw. Metagenomic sequencing would be more suitable for investigating the fungal part of the microbiome. Nonetheless, we confirmed that the fungal microbiome across different body sites is influenced by pregnancy status.

Chapter 2.: Clinical NEC Prevention Practices Drive Different Microbiome Profiles and Functional Responses in the Preterm Intestine. [2]

In this study, we analyzed gut microbiome patterns from 383 stool samples of 55 very low birth weight (VLBW) premature infants (>1,500 g) across three Austrian neonatal intensive care units (NICUs) during the first two weeks of life. We demonstrated that the distinct necrotizing enterocolitis (NEC) prophylaxis regimens employed at each NICU led to characteristic developments not only in the bacterial component but also in the archaeal and fungal communities of the stool microbiome, along with functional potentials and metabolome patterns. This in-depth systematic comparison highlights the impact of probiotics, antibiotics, antimycotics, and feeding regimens on the initial development of the gut microbiome.

Using metagenomic sequencing, we found that while NEC regimens primarily target the bacterial gut microbiome, which accounted for 99.62% of metagenomic data, other microbiome players also exhibited NICU-specific patterns. For instance, the archaeal genus *Methanocorpusculum* predominated in one center, whereas *Methanosarcina* and *Methanobrevibacter* were dominant in the two other centers. Viral species also differed between centers, likely reflecting the bacterial differences, as most identified viruses were bacteriophages.

Probiotics, administered in two of the three centers, showed center-specific effects. *Lactobacillus* species (*Lactobacillus rhamnosus LCR 35* and *Lactobacillus acidophilus NCDO 1748*) did not achieve high relative abundances, particularly when compared to probiotic *Bifidobacterium longum* subsp. *infantis* NCDO 2203, which dominated and even outcompeted co-administered *Lactobacillus acidophilus*. These findings align with previous studies demonstrating persistent colonization of *B. bifidum* for up to 60 days after probiotic cessation [51,108], and stable colonization of the preterm gut only by *Bifidobacterium* strains but not *L. rhamnosus* [86]. Our analysis suggests successful colonization of *B. longum subsp. infantis*, although this tool alone is insufficient for confirmation. The increasing relative abundance of *Bifidobacterium* over time, despite unchanged administration doses, further supports this assumption.

Importantly, only a limited number of antibiotic resistance profiles and no virulence factors were associated with *Bifidobacterium* and *Lactobacillus*, indicating their safety as probiotics.

One key factor aiding *Bifidobacterium* colonization is its ability to metabolize human milk oligosaccharides (HMOs). Interestingly, we observed high *Bifidobacterium* levels when administered as probiotics despite the absence of HMOs, as most infants there received formula milk. This suggests that combining probiotic *Bifidobacterium* strains with HMOs, such as those found in human milk (HM), could enhance their colonization potential and health benefits. Additionally, high *Bifidobacterium* levels correlated with lower counts of other potentially pathogenic taxa. By comparing the read counts of taxa previously associated with NEC [109], we confirmed their reduced abundance in the presence of *Bifidobacterium*.

In contrast to *Bifidobacterium*, which was undetectable in the absence of probiotic administration, *Lactobacillus* species were present even when not administered, suggesting their role as natural colonizers of the infant gut. HM is a likely source of live lactobacilli, though this was unlikely for infants in our study, as HM was often donated and pasteurized. Despite the relatively low abundance of probiotic *Lactobacillus* compared to *Bifidobacterium*, its potential health benefits should not be dismissed. While it may not colonize the preterm gut as extensively as *Bifidobacterium*, it may act as a pioneer species by reducing oxygen levels in the gastrointestinal tract (GIT), thereby facilitating *Bifidobacterium* colonization [110].

Considering these findings, we propose administering a mixture of *Lactobacillus* and *Bifidobacterium* strains, combined with HM rather than formula, to maximize health benefits.

Functional analyses and metabolomics revealed that administering *Bifidobacterium* accelerated gut microbiome maturation, as evidenced by significantly reduced osmotic and acid stress, reduced respiration, and increased metabolism of complex sugars.

While antibiotics remain essential for infection prevention and treatment in vulnerable groups such as preterm infants, their prophylactic use should be carefully considered. Although antibiotics effectively reduce NEC rates [111,112], early exposure may promote antibiotic resistance and lead to adverse health outcomes, underscoring the need for balanced approaches in their use.

Chapter 3.: First-Year Dynamics of the Anaerobic Microbiome and Archaeome in Infants' Oral and Gastrointestinal Systems. [3]

The development of the human microbiome is highly dynamic during the first three years of life, gradually establishing an adult-like composition [113,114]. While this has been well-documented for the gut bacterial microbiome, our study expands the analysis to the oral microbiome, encompassing both bacterial and archaeal communities as well as their functional potential. Using monthly samples from 30 infants during their first year of life, we explored how the microbiome develops beyond the GIT.

Microbiome development in early life is shaped by various factors. Our findings reveal that feeding type exerts a stronger influence than delivery mode during the first year of life, affecting both the GIT and oral microbiomes. In breastfed (BF) infants, we identified a distinct transitional phase characterized by increased alpha diversity, shifts in beta diversity, and significant restructuring of abundant taxa. This phase was particularly pronounced in the oral microbiome but was also observed in the GIT microbiome. In contrast, non-breastfed (NBF) infants showed more gradual microbiome development, with a relatively complex and diverse microbiome already present early in life.

Network analyses highlighted these differences in microbial co-occurrence patterns. BF infants' microbiome networks displayed fewer genera occupying similar niches and lower stress-centrality compared to NBF infants. By the end of the first year, these differences diminished as both groups formed increasingly complex networks involving multiple microbial taxa. We hypothesize that the introduction of solid foods and, more significantly, the cessation of human milk (HM) are key drivers of this convergence. HM directly and indirectly influences oral microbiome development through the presence of living microbes [115,116] and milk components that affect microbial adherence to oral surfaces [117]. Additionally, Human milk oligosaccharides (HMOs), which profoundly shape the gut microbiome of BF infants, further emphasize its role in microbiome development.

We found that the oral microbiome contributes only minimally to the GIT microbiome, with a few key members transitioning from the oral cavity through the GIT to the stool. This contribution decreased over the first year of life, likely due to the maturation of the gastric barrier. In the GIT, microbial networks were more complex than in the oral cavity, dominated

by obligate and facultative anaerobic taxa. Longitudinal tracking of specific strains suggested persistent colonization of the GIT by certain taxa, although such events were surprisingly sparse.

Interestingly, despite clear compositional differences between the GIT microbiomes of BF and NBF infants, functional differences were less pronounced. Over time, functional traits diverged further, reflecting the maturation of the microbiome into an aging state.

Contrary to expectations, skin-associated bacteria such as *Staphylococcus* were not enriched in BF infants. However, archaea commonly found on human skin, such as members of the Nitrososphaeraceae family, were more prevalent in BF infants. The most abundant archaeal genus was *Methanobrevibacter*, consistent with findings from other human microbiome studies [118,119].

Archaea were detectable as early as the first months of life, with their presence steadily increasing over the first year, regardless of feeding mode or birth type. Their sporadic absence and presence suggest constant transitions from the environment into and through the GIT. These findings indicate that archaea are an integral part of the early microbiome, even in infants younger than one year.

Conclusion

The human microbiome is a complex network of microbes, including bacteria, archaea, fungi, and viruses, along with their functional activities. While many human microbiome studies primarily focus on bacterial components, often overlooking non-bacterial players due to their lower abundance and the specialized methods required to study them, the studies presented in this thesis also explore the dynamics of these non-bacterial components. This microbial composition and interplay vary significantly across body sites and evolves in response to the host's life stages.

We demonstrated that the microbiome mirrors the profound changes the female body undergoes during pregnancy and the postpartum period. While the oral microbiome undergoes distinct changes during pregnancy and reverts to a nonpregnant-like state within one month postpartum, the urogenital microbiome experiences extensive restructuring, particularly postpartum. The transition from a *Lactobacillus*-dominated vaginal microbiome during pregnancy to a highly diverse community postpartum was notable and bears clinical significance. To prevent the long-term establishment of suboptimal vaginal community state types and associated bacterial or fungal infections, interventions that promote *Lactobacillus* dominance, such as targeted probiotic administration, may accelerate the recovery of a healthier vaginal microbiome capable of combating pathogens effectively.

Microbiome adaptations during the perinatal period can be viewed as responses to changes in the female body's physiological features, such as hormonal shifts, and as mechanisms to safeguard the health of the vulnerable fetus and infant. Infants born very prematurely and with very low birth weight require exceptional support and protection.

In this context, we observed that the combination of feeding human milk (HM) and administering *Bifidobacterium longum* subsp. *infantis* NCDO 2203 during the first weeks of life offers a promising synergistic approach to support the early development of the gut microbiome in these high-risk infants. The safety of administered probiotics was confirmed with respect to antibiotic resistance and virulence factors.

Our findings also underscore the dynamic nature of the infant microbiome during the first year of life, highlighting the critical role of HM in shaping microbial communities throughout the

digestive tract. Breastfed infants experience distinct phases of microbial dynamism in the first year, whereas non-breastfed infants display more mature but steadier microbial patterns early on. Initially, the oral microbiome influences the gastrointestinal tract microbiome, but this influence diminishes as the gut microbiome stabilizes and differentiates independently over time. These observations highlight the complexities of infant microbiome development and its potential long-term health implications. Our findings underscore the benefits of breastfeeding and highlight the importance of promoting breastfeeding in public spaces to increase the frequency of HM meals provided to infants to support their microbiome development.

Our sample sizes across all three studies are relatively small, and we could not provide metagenomic analyses on all samples, which would have enabled deeper insights. While all publications provide valuable perspectives on the dynamics of the non-bacterial components of the microbiome, it is crucial to emphasize the need for advancing multi-kingdom approaches. Key priorities include improving DNA extraction methods to effectively lyse archaeal and fungal cells alongside bacterial cells, as well as enhancing non-bacterial gene catalogues and databases to enable more accurate taxonomic classification.

In summary, our findings emphasize the interdependence of maternal and infant microbiomes, advocating for integrated strategies that optimize microbial health during infancy while supporting maternal recovery postpartum.

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Original publications

Chapter 1.:

The Dynamics of the Female Microbiome: Unveiling Abrupt Changes of Microbial Domains across Body Sites from Prepartum to Postpartum Phases. [1]

Chapter 2.:

Clinical NEC Prevention Practices Drive Different Microbiome Profiles and Functional Responses in the Preterm Intestine. [2]

Chapter 3.:

First-Year Dynamics of the Anaerobic Microbiome and Archaeome in Infants' Oral and Gastrointestinal Systems. [3]

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The dynamics of the female microbiome: unveiling abrupt changes of microbial domains across body sites from prepartum to postpartum phases

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ABSTRACT The microbial ecosystem of women undergoes enormous changes during pregnancy and the perinatal period. Little is known about the extent of changes in the maternal microbiome beyond the vaginal cavity and its recovery after birth. In this study, we followed pregnant women [maternal prepartum (mpre), *n* = 30] into the postpartum period [1 month postpartum, maternal postpartum (mpost), *n* = 30]. We profiled their oral, urinary, and vaginal microbiome; archaeome; mycobiome; and urinary metabolome and compared them with those of nonpregnant (np) women (*n* = 29). Overall, pregnancy status (np, mpre, and mpost) had a smaller effect on the microbiomes than body site, but massive transitions were observed for the oral and urogenital (vaginal and urinary) microbiomes. While the oral microbiome fluctuates during pregnancy but stabilizes rapidly within the first month postpartum, the urogenital microbiome is characterized by a major remodeling caused by a massive loss of *Lactobacillus* and thus a shift from Vaginal Community State Type (CST) I (40% of women) to CST IV (85% of women). The urinary metabolome rapidly reached an np-like composition after delivery, apart from lactose and oxaloacetic acid, which were elevated during active lactation. Fungal and archaeal profiles were indicative of pregnancy status. *Methanobacterium* signatures were found mainly in np women, and *Methanobrevibacter* showed an opposite behavior in the oral cavity (increased) and vagina (decreased) during pregnancy. Our findings suggest that the massive remodeling of the maternal microbiome and metabolome needs more attention and that potential interventions could be envisioned to optimize recovery and avoid long-term effects on maternal health and subsequent pregnancies.

IMPORTANCE The perinatal microbiome is of specific interest for the health of the mother and infant. We therefore investigate the dynamics of the female microbiome from nonpregnant over prepartum to the postpartum period in urine and the oral and vaginal cavities. A specific focus of this study is put not only on the bacterial part of the microbiome but also on the underinvestigated contribution of fungi and archaea. To our knowledge, we present the first study highlighting those aspects. Our findings suggest that the massive remodeling of the maternal microbiome and metabolome needs more attention and that potential interventions could be envisioned to optimize recovery and avoid long-term effects on maternal health and subsequent pregnancies.

KEYWORDS pregnancy, perinatal, microbiome, archaea, fungi, amplicon sequencing, metabolomics, urine, vaginal, oral

The female body undergoes profound changes from conception to pregnancy and the perinatal period that affect hormonal status, metabolism, the immune system, and the microbiome (1). For example, the maternal microbiome changes during

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pregnancy, a period characterized by low-grade inflammation, increased fat storage, and insulin resistance (2). However, knowledge of the transition of the microbiome from nonpregnant to pregnant and from prepartum to postpartum is relatively sparse, particularly with respect to bacteriomes, mycobiomes, and archaeomes, and beyond vaginal and gastrointestinal body sites.

It is well known that the vaginal microbiome is altered by pregnancy. In general, a healthy vaginal microbiome is predominated by lactobacilli, which are thought to maintain low pH and produce bacteriocins that inhibit pathogen growth (3). During pregnancy, vaginal diversity decreases and the predominance of *Lactobacillus*-species increases (4), probably to protect the mother and fetus from infection (5). This rise in *Lactobacillus* may be due to increased estrogen levels during pregnancy, which causes maturation of the vaginal epithelium and consequent accumulation of glycogen. Degraded by the host, products such as maltose, maltotriose, and maltotetraose then promote the growth of lactic acid bacteria (6, 7).

Pregnancy also affects the oral and urinary microbiome. During pregnancy, the oral microbiome is of particular clinical interest, as many women suffer from bleeding gums, gingivitis, or periodontitis (8, 9), which are likely caused by microbial disturbances associated with immune modulation and hormonal changes (10). Gingival diseases are associated with adverse pregnancy outcomes (11). Similarly, the urinary microbiome of pregnant women is clinically relevant because urinary tract infections increase the risk of preterm birth (12). A previous study showed that certain microbial taxa in urine, namely, *Ureaplasma urealyticum*, were associated with preterm birth even in the absence of urinary tract infections (13).

Parturition marks a rather abrupt change for both the child and the mother, which also brings dramatic changes in microbial niches. For the newborn, birth is a starting point for microbial colonization. The transfer of the microbiome from the mother to the child plays an essential role in the development of the infant's microbiome and immune system. For the mother, childbirth is a turning point that also affects her microbiome transitioning from a pregnant to a postpartum state. In addition, the immediate postpartum period is a difficult time, characterized, for example, by drastic hormonal changes, increased energy and nutrient demands (also due to breastfeeding), sleep disturbances, or depressive symptoms (14, 15), which can affect the microbiome and *vice versa* (15).

After delivery, the vaginal microbial community shifts from a *Lactobacillus*-predominated microbiome to a *Lactobacillus*-depleted microbiome (15), likely caused by the rapid decline in estrogen levels (3). This phase is associated with diseases such as bacterial vaginosis and vulvovaginal candidiasis (16, 17). Cultivation-based studies have shown that *Gardnerella*, *Peptococcus*, *Bacteroides*, *Staphylococcus*, *Streptococcus*, and *Ureaplasma* species (3) are increased, which is associated with postpartum endometritis, which occurs in 1%–3% of all deliveries (18). It is estimated that only about 50% of women achieve a healthy *Lactobacillus*-predominated status at 1 year postpartum because *Lactobacillus crispatus* does not return to predominance (14).

Although awareness of the problematic recovery of the vaginal microbiome to the prepregnancy status quo has been raised, potentially impacting subsequent pregnancies (14), knowledge of the transition of the maternal microbiome of other body sites as well as the dynamics of the nonbacterial microbiome is still surprisingly sparse as already indicated elsewhere (19). Here, we highlight the transition of microbiomes in the oral and urogenital (urinal and vaginal) body sites of 30 women from the prepartum to 1 month postpartum and place it in context with the microbiomes of nonpregnant women. We use specific detection and analysis methods for the archaeome and mycobiome to provide a broader view of the holistic microbiome.

RESULTS

Study set-up and general description

The aim of the study was to understand the extent of imbalance of the bacterial and nonbacterial, oral, and urogenital (urinal and vaginal) microbiomes in postpartum women compared with their pregnant status or nonpregnant individuals. Thirty healthy pregnant participants were enrolled in this study to investigate the microbial changes with and after pregnancy. For this purpose, we recruited women in the third trimester (n = 30, "maternal prepartum" = "mpre"), who provided samples at this time and 1 month after delivery (n = 30, "maternal postpartum" = "mpost;" Cesarean section (CS) n = 15, vaginal delivery n = 15). The study also included nonpregnant female controls for comparison (n = 29, "nonpregnant" = "np"). The following samples were collected from all three study groups: oral swabs, noncathetered urine, and vaginal swabs. A total of 353 samples were processed by amplicon sequencing targeting the overall microbiome ("universal," targeting both archaeal and bacterial, but mainly bacterial 16S rRNA genes), archaea, and fungi. Nuclear magnetic resonance spectroscopy (NMR)-based metabolomics was performed for the urine samples. An overview on the samples, number of reads, and Amplicon Sequence Variant (ASV) numbers is shown in Fig. S1 and S2. The characteristics of the cohorts are shown in Table 1.

Bacterial and fungal microbiomes are affected by pregnancy status

Figure 1 shows that the similarity of the bacterial microbiomes of all samples could be mainly explained by the body site they are taken from (PERMANOVA, Bray-Curtis, P = 0.001, R^2 = 0.194) and, with a smaller effect size, also by perinatal status (np, mpre, and mpost; PERMANOVA, Bray-Curtis, P = 0.001, R^2 = 0.03). *Lactobacillus* was the most prominent genus, found primarily in vaginal and urinary np and mpre samples, followed by *Streptococcus* (mostly oral samples) (Fig. 1B).

Methanogenic archaea: indicators for pregnancy?

Archaeal signatures were analyzed semi-quantitatively ("universal approach") alongside bacteria and qualitatively in a separate "nested approach" specifically targeting archaea.

Overall, archaeal richness was very low in all samples from all body sites (max. four genera/six species/26 ASVs per individuum and body site; Fig. S7). A total of 25 archaeal genera, 32 species; and 352 ASVs were detected in the data set. *Methanobrevibacter* was the most prevalent archaeal genus in samples from all body sites (Fig. 2B), followed by *Methanobacterium*, which was not found in the mpost group but in all sample types, but especially in urine (Fig. 2C).

TABLE 1 Characteristics of the study cohort, statistically compared between the mpre and mpost groups and the np group as well as within the pregnant group between those who delivered via CS versus those who delivered vaginally

Characteristics	mpre and mpost (n = 30)	np (n = 29)	P value
Mean age (yr) ± SD (range)	34.5 ± 4.7 (27–44)	32.0 ± 5.7 (19–43)	0.083[a]
Mean BMI prepregnancy (kg/m^2) ± SD (range)	22.4 ± 4.1 (17.3–37.0)	22.6 ± 3.6 (17.2–31.5)	0.968[b]
	Pregnant group		
Characteristics	CS (n = 15)	Vaginal birth (n = 15)	P value
Mean age (yr) ± SD (range)	35.8 ± 5.1 (28–44)	33.1 ± 3.9 (27–39)	0.113[a]
Mean BMI at sampling (kg/m^2) ± SD (range)	27.3 ± 6.2 (21.8–47.6)	27.6 ± 3.4 (22.2–34.0)	0.436[b]
Mean BMI prepregnancy (kg/m^2) ± SD (range)	22.2 ± 4.5 (18.3–37.0)	23.0 ± 3.8 (17.3–29.4)	0.285[b]
Pregnancy days	272.7 ± 7.3 (258–286)	283.1 ± 3.5 (276–289)	<0.001[a]
Infant weight (g)	3408.6 ± 465.5 (2,510–4,250)	3486.0 ± 269.8 (3,040–4,110)	0.582[a]
Days in hospital	3.9 ± 0.7 (2–5)	3.6 ± 1.5 (1–6)	0.87[b]
Sex of the infant	F: 8; M: 7	F: 6; M: 9	0.715[c]

[a]*t*-test.
[b]Mann-Whitney U test (independent samples).
[c]Fisher's exact *t*-test

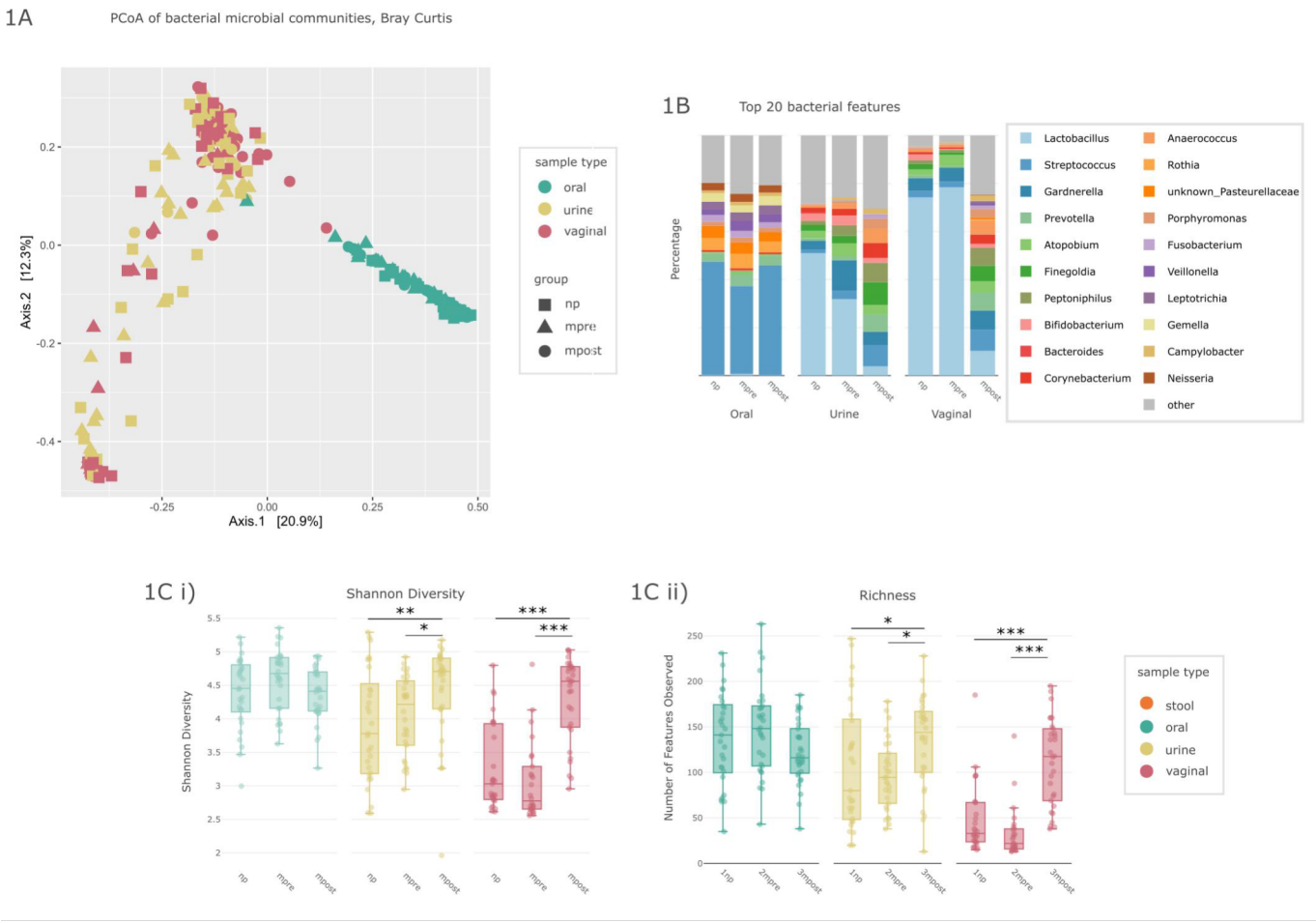

FIG 1 Beta diversity, alpha diversity, and composition of the bacterial microbiome. (A) Principal Coordinate Analysis (PCoA) of sample type (color) and group (shape) with Bray-Curtis distance matrix on ASV level, PERMANOVA. (B) Composition of the top 20 bacterial genera in relative abundance, depicted by sample type and group. (C) Alpha diversity of sample types by group on ASV level of (i) Shannon diversity and (ii) richness. Significance levels are indicated with asterisks for ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$, Kruskal-Wallis test, adjusted with Bonferroni.

The three most abundant ASVs (ASVs 1, 8, and 9) were all classified as *Methanobrevibacter smithii*, which was also the most abundant *Methanobrevibacter* species. These three ASVs accounted for 58.52% of all archaeal reads, 80.24% of *Methanobrevibacter* reads, and 85.52% of all *M. smithii* reads (Fig. 2C). It should be noted that due to insufficient databases for the classification of human-associated archaea, species classification is likely not comprehensive and therefore species information should be taken with caution (20). Classification of *Methanobrevibacter* in particular was further complicated by the fact that the two most common species, *M. smithii* and *Cand.* Methanobrevibacter intestini, cannot be distinguished using V4 16S rRNA gene amplicons (21).

The urinary archaeome did not significantly differ between the groups (Unweighted UniFrac, PERMANOVA, $P = 0.122$). We observed a decrease in Shannon diversity and richness of archaea from np to mpre and mpost (Fig. S7). Whereas the archaeal pattern in np women is more diverse and mainly *Methanobrevibacter* and *Methanobacterium* can be observed, mpre women mainly carry *Methanobrevibacter* and unknown Woesearchaeales (Fig. S8).

The vaginal archaeome carried a variety of archaeal signatures but overall had the lowest Shannon diversity and richness of archaeal genera, species, and ASVs (Fig. S7). Mpre samples contained exclusively *Methanobrevibacter* (*smithii*, ASVs 1, 8, and 9), in contrast to np and mpost samples, which contained other archaeal signatures such

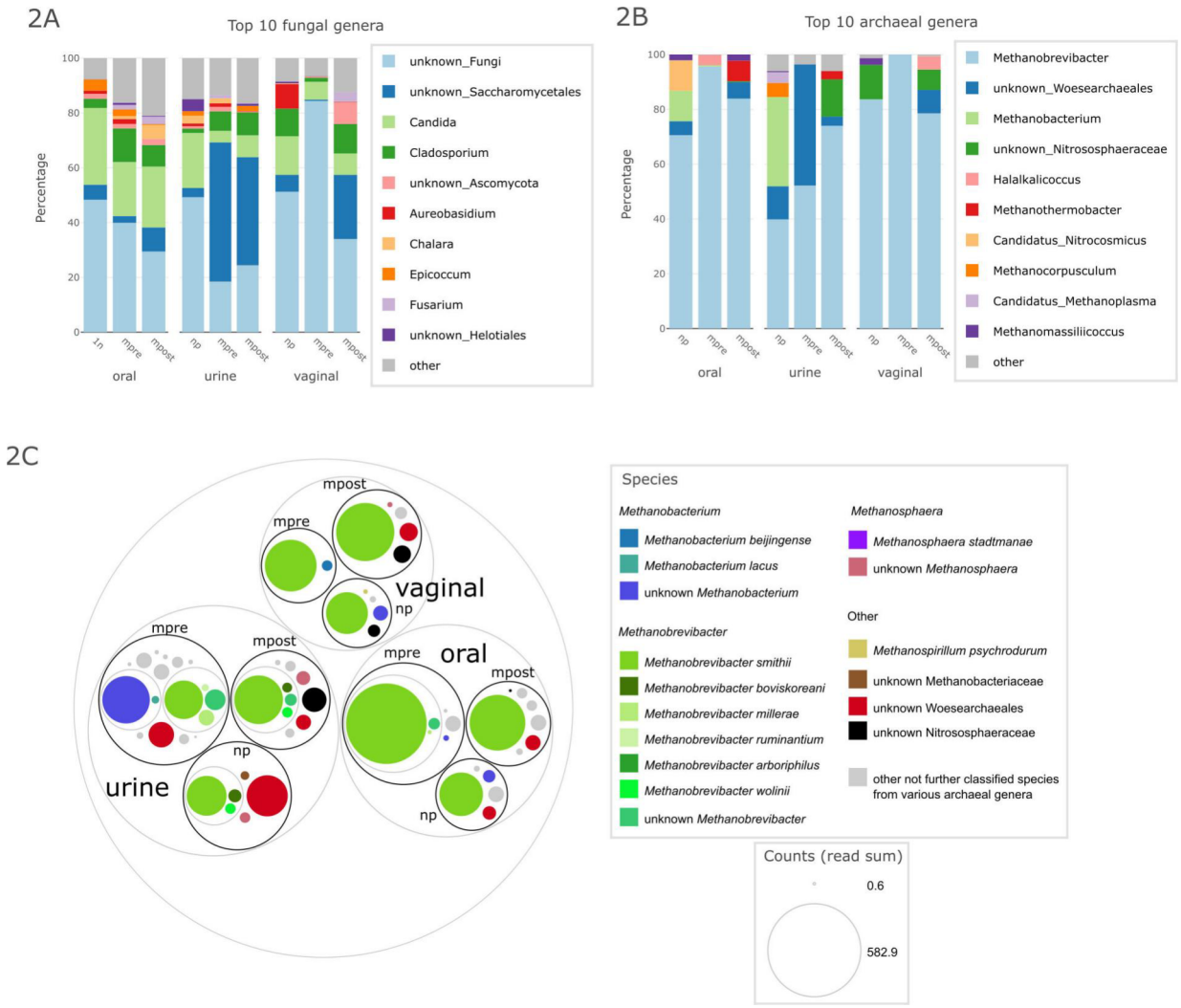

FIG 2 Relative abundance of top 10 (A) fungal genera and (B) archaeal genera, depicted by relative abundance, depicted by sample type and group. (C) Circle packing plot of archaeal species depicted by body site and group; the size of the colored dots represents counts of normalized read sums.

as unclassified Nitrososphaeraceae and Woesearchaeales, *Halalkalicoccus*, *Methanomassiliicoccus*, *Methanosphaera,* and *Methanospirillum* (Fig. S9). Interestingly, only five mpre women exhibited any archaea in their vaginal microbiome, and when they did, they were exclusively *Methanobrevibacter* (*smithii*) (Fig. S10). Although the presence of archaea in the vaginal microbiome increased after delivery, no statistically significant differences in the PCoA were observed between the groups (Fig. S5).

In contrast, the oral samples revealed a significant increase in the number (richness) and Shannon Diversity of archaeal ASVs in mpre compared with np and mpost (Fig. S7). This was observed not only in the archaea-specific amplicon approach indicating relative abundance but also in the "universal" approach for which differential abundance could be calculated. *M. smithii* was found significantly increased in mpre compared with mpost (Aldex2, $P = 0.005$, $q = 0.321$) and to np (Aldex2, $P = 0.003$, $q = 0.300$) (Fig. S4). It should be mentioned that, surprisingly, the obtained sequences indeed were classified as *M. smithii* and not as *Methanobrevibacter oralis* although classifications should be taken with caution due to incomplete databases (see above).

In fact, not a single np woman carried *M. smithii* in her oral cavity but 16 mpre women did, whereas after delivery, *M. smithii* was detected in only one mpost woman (Fig. S11). We therefore suggest that the oral niche of a pregnant woman favors the growth of the anaerobic methane-producing archaea *Methanobrevibacter* and *M. smithii* in particular.

In summary, we observed niche restriction for methanogenic archaea in the vagina in pregnancy, whereas the niche in the oral cavity was opened. This indicates that *Methanobrevibacter* species may reflect changes in the ecosystem.

The oral bacteriome and mycobiome undergo temporary changes with pregnancy

In the oral samples, alpha diversity of the bacteriome did not change substantially with pregnancy status (Fig. 1C). However, beta diversity significantly differed between the groups, as shown by PCoA plots (Unweighted UniFrac, PERMANOVA, $P = 0.001$; Fig. 1A; Fig. S12). It is noteworthy that the mpre samples clustered visibly apart from the other sample groups. We hypothesize that the differences between the groups are mainly due to numerous, highly divergent *Streptococcus* ASVs. *Streptococcus* was identified as the most abundant genus in all three groups, accounting for 43.08% of relative abundance (Fig. S3).

By performing differential abundance analysis, we did not identify a single genus, species, or ASV [with the exception for archaeal *Methanobrevibacter* (*smithii*), see above] that was differentially abundant between mpost and np (with or without BH correction; Fig. S4), indicating that a similar microbial status to prepregnancy was achieved relatively quickly after pregnancy (see Fig. 1C). However, the mpre samples showed a variety of differences (Fig. S4). However, none of these differences remained significant after BH correction, so only trends can be reported (Aldex2, $P < 0.05$, $q > 0.05$). As mentioned above, *Methanobrevibacter* signatures, particularly those of *M. smithii*, were specifically increased in mpre oral samples compared with mpost ($P = 0.0047$, $q = 0.321$) or np ($P = 0.003$, $q = 0.300$) samples. This trend was opposite to that observed for *Streptococcus*. Overall, 12 streptococcal ASVs were increased in the np group and 6 in the mpost group compared with the mpre samples ($P < 0.050$; Fig. S4). Furthermore, the relative abundance of *Streptococcus* ASVs in mpre was rather individual and less uniform than that in np and mpost, as shown by the wider ranges of the confidence intervals (Fig. S4).

It has been reported that the incidence of periodontal disease increases during pregnancy (22), possibly leading to adverse pregnancy outcomes such as preterm birth (23). In our mpre group, we did not observe a significant increase in the relative abundance of the so-called orange and red complex bacteria, which have been shown to account for the initiation and progression of periodontal disease (24) (Fig. S13 S14). It shall be mentioned though that metadata on participants' dental status was not available, not allowing further interpretation. However, *Methanobrevibacter* load or presence has been previously associated with severe periodontitis (25, 26).

Fungal diversity in the oral cavity increased from np to mpre (Fig. S15). Highly statistically significant differences (Fig. S16, Unweighted UniFrac, PERMANOVA, $P = 0.001$) between the three groups were observed by Adonis. These strong differences can be explained solely by the highly significant differences in two fungal ASVs (ASVs 8 and 9) that were not further classified. ASV8 was highly significantly increased in np compared with mpre (Aldex2, $P < 0.001$, $q < 0.001$) and with mpost (Aldex2, $P < 0.001$, $q < 0.001$), whereas ASV9 was significantly more abundant in mpre than in np (Aldex2, $P = 0.030$, $q = 0.394$). Overall, approximately 50% of all oral fungal ASVs could not be classified. The main members of the oral mycobiome were *Candida albicans* and *Cladosporium cladosporioides* followed by not further classified Saccharomycetales, which were shared between the three groups (Fig. S6).

Overall, we observed temporary fluctuation of the oral microbiome with pregnancy and rapid stabilization of the oral bacteriome and archaeome thereafter.

The urogenital mycobiome and bacteriome undergo vast changes with and after pregnancy

Due to the local proximity of the urinary and vaginal tracts, an overlap can be biologically expected. We collected noncathetered urine but still specifically asked the participants to

collect midstream urine to reduce the contamination risk during sampling. Nevertheless, the overlap was substantial: 77 genera in np, 47 in mpre, and 125 in mpost were shared (Table 2).

To numerically assess the microbial exchange between the two sites in the context of all other body sites sampled, we performed source tracking analysis (Fig. 3A; Fig. S17). In all samples, the contribution of the oral microbiome toward the urogenital tract was marginal and did not increase with pregnancy. Overall, the exchange of vaginal and urinary microbial patterns was very clear (Fig. 3A). Notably, the urinary microbiome in mpost samples contributed significantly less to the vaginal microbiome than that in mpre samples [Wilcoxon rank, mpre: 95.25% (median); mpost: 37.18% (median), P = 0.002; Fig. S17]. Similarly, we observed a trend toward an overall lower contribution of vaginal samples to urine in samples from mpost (*t*-test, np-mpost, P = 0.078; median np: 55.38%, mpre: 38.80%, and mpost: 41.54%).

Furthermore, vaginal and urine (urogenital) samples were analyzed comparatively with respect to their mycobiome and overall microbiome.

For both the vaginal and the urinary mycobiome, the groups clustered statistically significantly on the ASV level (Unweighted UniFrac, PERMANOVA, P = 0.001; Fig. S16). The vaginal mycobiome, especially in mpre samples, was predominantly made up of reads that could not be fully classified (Fig. S6 and S18). This was accompanied by a significant decrease in alpha diversity and richness in mpre (mpre-mpost; Shannon P = 0.010, richness P = 0.035) (Fig. S15) and led to a significant clustering of the groups (Kruskal-Wallis test, adjusted with Bonferroni, q < 0.001; Fig. S16). Fungal composition was quite different between groups but only in relative abundance (Fig. S6) and not in differential abundance testing with Aldex2 (Fig. S18). Similar to the vaginal mycobiome, the urinary mycobiome was predominantly unclassified fungal taxa but specifically not further classified Saccharomycetales and Candida (Fig. S6) as already described before (27). Not further classified ASVs were significantly more abundant in np than in mpre and mpost (Aldex2, np-mpre, q = 0.044; np-mpost, q = 0.161) (Fig. S18). Diversity and especially richness decreased sharply from np to mpre (Shannon, P = 0.035; richness, P < 0.001) (Fig. S15).

Significant changes in alpha diversity were also observed in bacteria (Fig. 1C, ASV level). Urogenital samples were characterized by a significant increase in Shannon diversity from mpre to mpost. Urine samples showed a continuous increase in alpha diversity from np to mpre to mpost. We observed that in some urine samples, bacterial richness was remarkably high in the np group, with some individuals carrying up to ~150 different bacterial genera. In contrast, vaginal samples had low overall richness (max. 55 different bacterial genera) and diversity. The lowest diversity was observed in vaginal mpre samples, before reaching a higher alpha diversity after birth than in the nonpregnant state (Fig. 1C).

Delivery has a substantial impact on the urogenital bacteriome driven by a massive loss of *Lactobacillus*

As indicated above, in particular, the urogenital microbiome of postpartum women differed significantly from that of mpre and nonpregnant women, in terms of alpha and beta diversity as well as microbiome composition (Fig. 1A and C). Therefore, beyond composition and diversity, we performed more detailed analyses on these urogenital microbiomes including analysis of CSTs and urine metabolomics.

TABLE 2 Shared and unique bacterial genera between vaginal and urine plotted per group

	Urine unique	Shared urine and vaginal	Vaginal unique	Total
np	470	77	18	565
mpre	302	47	9	358
mpost	174	125	56	355

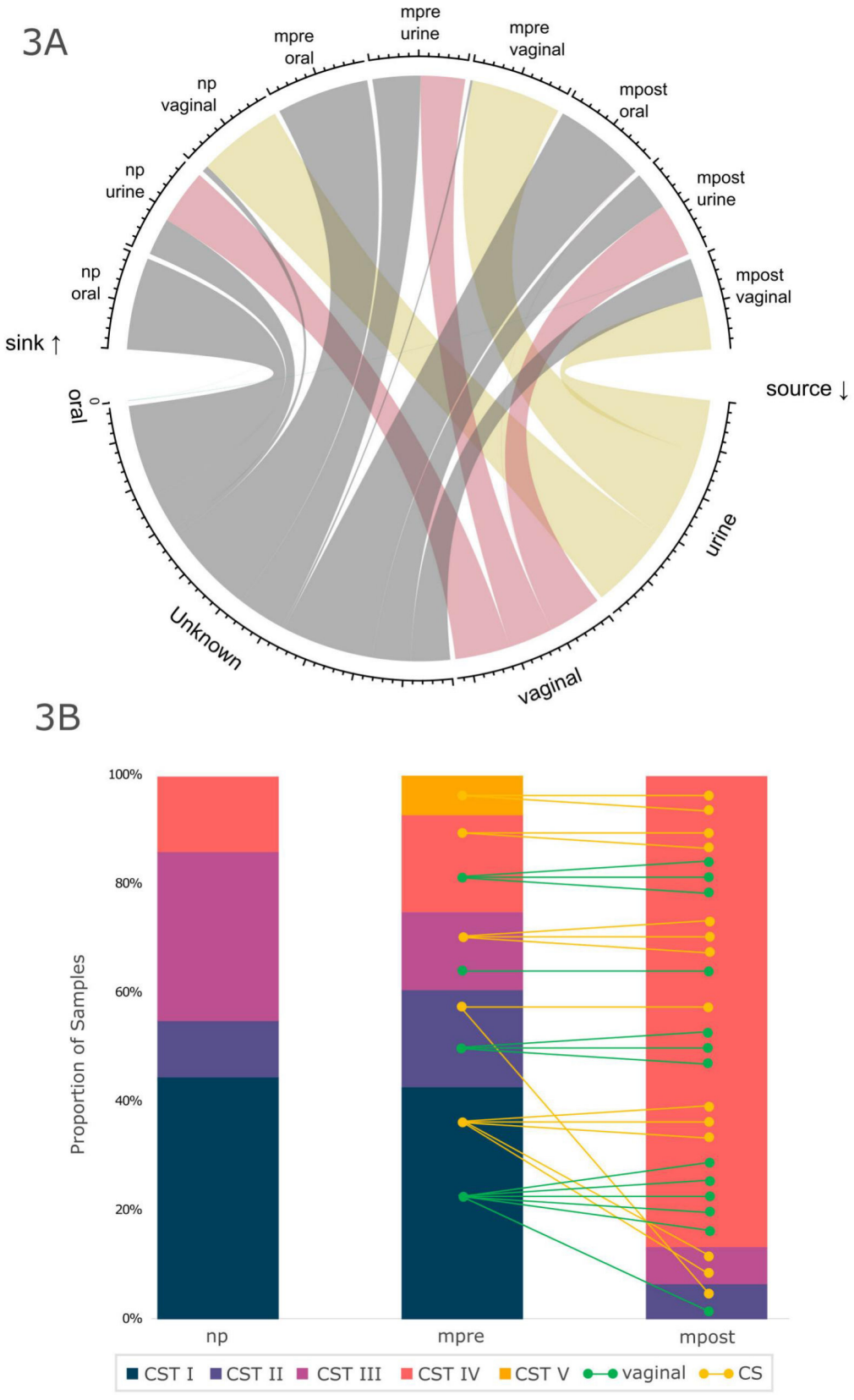

FIG 3 (A) Source tracking; comparison of oral, vaginal, and urine samples with respect to their contribution from oral, urine, vaginal, and unknown sources. (B) CST, split per group; lines indicate individuals; colors indicate delivery type; yellow, Cesarean section (CS); green, vaginal.

The vaginal microbiome in postpartum women was very different to that of np or mpre women (Fig. 1A, Unweighted UniFrac; PERMANOVA, $P = 0.001$). Postpartum, alpha diversity strongly increased and was characterized by a massive loss of *Lactobacillus* ($q < 0.001$; Fig. S4; Fig. 1C). This affected not only the three main *Lactobacillus* species representatives (*Lactobacillus crispatus*, *Lactobacillus iners*, and *Lactobacillus gasseri*) but also all other detected species (*Lactobacillus jensenii*, *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Lactobacillus amylovorus*, *Lactobacillus coleohominis*, *Lactobacillus mucosae*, *Lactobacillus ruminis*, and *Lactobacillus sanfranciscensis*). This loss of *Lactobacillus* in the postpartum vaginal microbiome has also been described elsewhere (3) and allowed colonization of the vaginal body site with new appearing bacteria. Accordingly, postpartum vaginal samples had increased abundance of anaerobic bacteria taxa such as *Prevotella*, *Atopobium*, *Streptococcus*, *Anaerococcus*, *Finegoldia*, and *Peptoniphilus*, most of which are also described as prominent members of CST IV (28) or bacterial vaginosis (BV) (29); see Table 3. Women with a CST IV vaginal microbiome may be at a higher risk for BV or other vaginal infections (30, 31).

To further analyze this interesting prepartum to postpartum shift in the vaginal microbiome, we classified the vaginal microbiomes into CSTs (Fig. 3B). The predominant CST in mpre and np was indeed CST I, predominated by *Lactobacillus crispatus* (45% in np, 43% in mpre). In 70% of the pregnant cohort, the vaginal microbiome shifted from *Lactobacillus*-predominant CSTs prepartum to CST IV postpartum. Postpartum, CST IV was predominant (23 women, 85%), whereas only five women already had CST IV prepartum. Most women with a prepartum CST other than CST IV switched to CST IV after delivery. This postpartum switch to CST IV has been described previously (3, 14). In the nonpregnant control group, only 14% of the participants had CST IV at the time of sample collection.

Of note, delivery mode (CS versus vaginal delivery) and previous deliveries had no effect on the transition of CSTs from mpre to mpost (χ^2 test, $P > 0.506$). Similarly, the delivery mode had no effect on the overall composition of the urinary microbiome postpartum (PERMANOVA; vaginal: $P = 0.188$, urinary: $P = 0.214$).

Urinary metabolic profiles mirror the transition from pre- to postpartum

Urine analysis is an important diagnostic tool in clinics, not only during pregnancy, as changes in the chemical composition of urine can indicate health problems. We subjected mpre and mpost urine samples to metabolomics analysis to assess metabolic situations and correlate with microbial features. Metabolic profiles were generated using untargeted and targeted NMR-based metabolomics.

When comparing mpre and mpost samples, a significantly higher lactose content was detected in mpost ($q < 0.001$, *t*-test after log transformation, paired samples; Fig. 4A), indicating active lactation. Postpartum women who did not breastfeed ($n = 2$) lacked

TABLE 3 Increase or decrease of specific bacterial taxa in the vaginal microbiome in CST IV [from reference (3), compared with non-CST-IV], BV [from reference (29), compared with non-BV], and the data set[a]

Organism	CST IV	BV	Data set, np to mpost
Lactobacillus	Decrease	Decrease	Decrease, ***$P < 0.001$, ***$q < 0.001$
Prevotella	Increase	Increase	Increase, ***$P < 0.001$, ***$q < 0.001$
Dialister	Increase	x	Increase, **$P = 0.0011$, *$q = 0.022$
Peptoniphilus	Increase	x	Increase, ***$P < 0.001$, **$q = 0.0018$
Atopobium	Increase	Increase	Increase, **$P = 0.0066$, $q = 0.077$
Finegoldia	Increase	x	Increase, *$P = 0.0075$, $q = 0.087$
Gardnerella	x	Increase	Increase, $P = 0.648$, $q = 0.844$
Mobiluncus	x	Increase	Increase, $P = 0.393$, $q = 0.650$
Bifidobacterium	x	Increase	Increase, $P = 0.348$, $q = 0.609$
Sneathia	x	Increase	Not present in the data set
Streptococcus	Increase	x	Increase, $P = 0.014$, $q = 0.131$

[a]*P* values and *q*-values are given for Aldex2 comparing np with mpost samples. x means that this genus is not indicated in the regarding literature.

detectable lactose in their urine. In addition, a significant increase in oxaloacetic acid was observed (Fig. 4A, $q = 0.011$), which might be explained by an altered metabolic status during breastfeeding (32). These two compounds were also significantly higher in postpartum samples compared with nonpregnant controls (lactose: $q < 0.001$; oxaloacetic acid: $q < 0.001$; *t*-test after log transformation, unpaired samples), but no other compounds were found to be significantly different. However, the urine samples from mpre women had significantly increased levels of dimethylamine, alanine, glycine, lactic acid, and other compounds, as shown in Fig. 4A.

Prepartum, none of the individuals carried the genera *Escherichia-Shigella*. *Finegoldia*, *Enterococcus*, and *Anaerococcus* were correlated with betaine and dimethylamine (Fig. 4B, i). In addition, we found significant correlations for *Varibaculum* with succinic acid (*q*

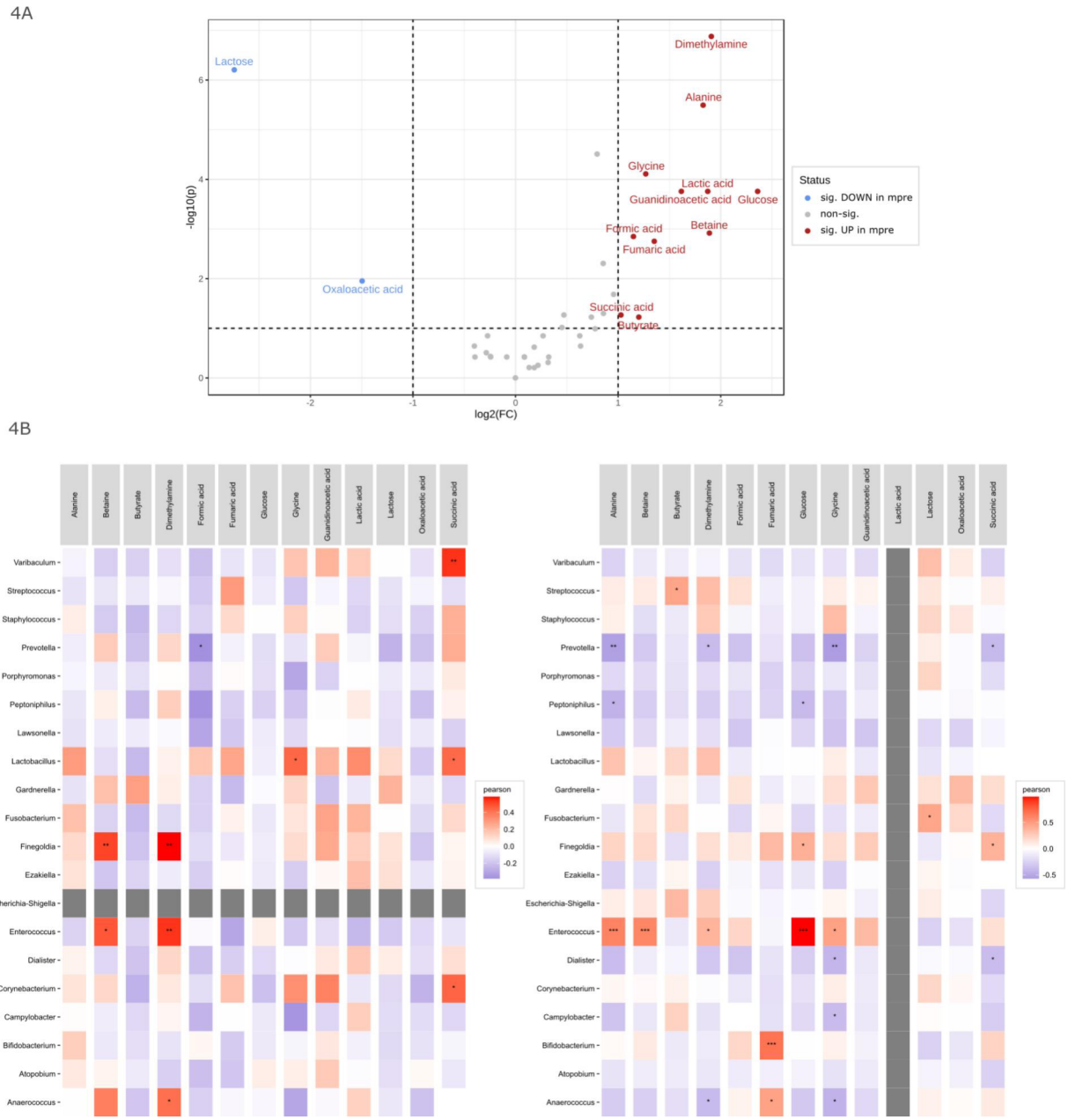

FIG 4 (A) Volcano plot of significantly differentially abundant metabolites between mpre and mpost. (B) Pearson correlation of top 20 abundant bacterial genera with 13 metabolites in urine samples which are differentially abundant between mpre and mpost. Left, mpre; right, mpost. The gray entries for Escherichia-Shigella in mpre and lactic acid in mpost reflect the absence of those features or metabolites in the subset. Significance levels are indicated with asterisks for ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$.

= 0.003), *Lactobacillus* with glycine and succinic acid, and *Corynebacterium* with succinic acid. Postpartum (Fig. 4B, ii), *Enterococcus* correlated strongly with alanine ($q < 0.001$), betaine ($q < 0.001$), and glucose ($q < 0.001$) as well as with dimethylamine ($q = 0.047$) and glycine ($q = 0.012$). Lactic acid was not detectable in any of the mpost urine samples. Fumaric acid was positively correlated with *Bifidobacterium* ($q < 0.001$) and *Anaerococcus* ($q = 0.012$). *Prevotella*, *Porphyromonas*, *Peptoniphilus*, and *Lawsonella* were weakly negatively correlated with all 13 metabolites in both mpre and mpost.

In healthy situations, the production of lactic acid lowers the pH, which prevents the growth of other microbes and promotes the acid-tolerant lactobacilli. Indeed, lactic acid was reduced in mpost samples (and B, ii), highlighting the loss of acid-producing *Lactobacillus* activity postpartum.

Although the increase of lactose and oxaloacetic acid in urine might be correlated with one of the 20 most abundant microbial genera, lactose in combination with the acid might favor the growth of lactobacilli in the long term.

DISCUSSION

In this paper, we demonstrated the dynamic transition of the maternal microbiome and urinary metabolome from the nonpregnant to the perinatal period. We revealed abrupt changes in the bacterial, fungal, and archaeal components of the microbiome in the oral and urogenital (urine and vaginal) body regions of 30 women from the pregnant state to 1 month postpartum and placed them in context to the microbiome of nonpregnant women. Understanding the normal postpartum microbiome transition and the influence of maternal (modifiable) factors could help develop prevention strategies for women who tend to develop typical microbiome-associated postpartum health problems or infections.

Pursuing a molecular approach that allowed us to capture nonbacterial components of the microbiome added interesting aspects to the microbiome transition. The overall archaeome profiles found in the urogenital tract (mainly Methanobacteriota and Halobacteriota) were consistent with the literature (33) but were not indicative of pregnancy status. Other signals reflected the pregnancy status well: we detected *Methanobacterium* almost exclusively in nonpregnant women (all four body sites examined). Even clearer evidence was provided by the major player among the archaea, *Methanobrevibacter*. The abundance of this archaeon was significantly increased in the oral cavity of pregnant women but rapidly decreased to prepregnancy levels after delivery. Indeed, alpha diversity of the oral microbiome increases with pregnancy, and an increase of pathogenic taxa has also been reported (34). Methanogenic archaea are known to effectively support fermenting microorganisms by eliminating inhibiting end products (especially H_2 and CO_2) (35). Therefore, increased methanogenic archaea could be a sign of excessive growth of bacterial anaerobes at this stage, which might be associated with periodontitis. Indeed, pregnant women are more prone to dental problems (36).

Interpretation of signals from the mycobiome was difficult due to the large number of taxonomically unclassified reads that were received. This again highlights the lack of appropriate high-throughput methods and mycobiome databases for read annotation, especially from samples outside the gastrointestinal tract. Although the mycobiome analyses in our approach were not specifically targeting the mycobiome (e.g., adjustments in cell lysis, selection of primers, or removal of host DNA could have improved results), we still obtained meaningful information: the overall mycobiome profile changed with pregnancy status, consistent with observations from previous, gut-focused studies (37). This change was also reflected by unknown Saccharomycetales that increased in urine samples with pregnancy.

Indeed, pregnant and postpartum women are at higher risk for health problems at these body sites, such as periodontitis (8, 9), bacterial vaginosis (29), or fungal infection of the vagina (13, 30, 31). Any aspects that could contribute to a rapid return to a

microbiome composition typical of a healthy, nonpregnant state could help reduce these microbiome-related complications, which could also affect maternal recovery.

In agreement with other reports (3, 14, 38, 39), we clearly demonstrated that the vaginal microbiome does not return to the nonpregnant state 1 month after delivery but that remains substantially altered, mainly due to the decrease of *Lactobacillus*. This decline has already been reported (14), and it can be explained by a decrease in estrogen in the female body due to impaired ovarian activity after delivery and during lactation (3). Decreased abundance of lactobacilli, especially in the first 6 weeks postpartum, may also be due to the lochia, which has previously been characterized as alkaline and therefore may restrict the growth of *Lactobacillus* species (3). Loss of lactobacilli is also accompanied by an increase in other, often opportunistic pathogenic bacteria, leading to the predominance of CST IV in postpartum women, which is generally associated with urogenital infections (40, 41). During the puerperal time, women are at higher risk of vaginal postnatal infections due to the large wound area, as well as other infections such as endometritis when vaginal opportunistic bacteria enter the upper genital tract, or urinary infections (14, 42). Probiotics treatment was already investigated in multiple other studies to prevent or fight vaginal infections (43–45). It needs to be further investigated how and to what extent administration of probiotics postpartum is capable to accelerate recovery of the postpartum vaginal microbiome.

Of note, the mode of delivery did not appear to affect the postpartum vaginal microbiome. Since many women in this cohort who delivered vaginally had birth tears and all women who delivered by cesarean section were treated with antibiotics, we would have expected a separation of their microbiomes, but this effect was not observed.

Source tracking analysis indicated a strong exchange between the vaginal and urinary microbiome, with the overall effect of the urinary microbiome on the vaginal microbiome being greater than *vice versa*. Thus, not only microorganisms but also important metabolic compounds, such as lactose, could be transferred. The greatly increased lactose content in postpartum urine might contribute to the recovery of lactobacilli throughout the urogenital tract. Indeed, lactose added to fecal samples could specifically increase the abundance of lactobacilli in *in vitro* experiments (46).

In addition to lactose, oxaloacetic acid was also increased in postpartum urine. Lactose is an important indicator of active lactation and might also be released from microbial metabolism of human milk oligosaccharides excreted with the urine (13). However, the origin of oxaloacetic acid is less clear. The increased excretion of unused oxaloacetic acid could reflect an increased catabolic state during lactation (32). We hypothesize that the urinary metabolome retains this signature after birth as long as the woman is breastfeeding.

In general, the female body is in an exceptional state after birth and still when the woman is breastfeeding, even months after delivery. As long as lactation hormones are produced, metabolism and physiology are altered compared with a nonpregnant and nonbreastfeeding state. Despite the fact that breastfeeding has numerous beneficial effects not only on the child but also on the mother, it might decelerate the return of the female body and its microbiome to the nonpregnant state (14). This is a very interesting research question, and longitudinal sampling of postpartum women who breastfeed and who do not breastfeed is needed to clarify this hypothesis.

Limitations

This study is based on a relatively small sample size of 30 participants per group. Still, we are able to depict meaningful aspects of the perinatal microbiome especially by highlighting the understudied fungal and archaeal part of the microbiome in three body sites of these participants.

Conclusion

In addition to the postpartum shift of *Lactobacillus* population in the vagina, which remains to be explored, additional work should be invested in the use of probiotics to improve postpartum maternal health. To avoid potentially long-term establishment of unhealthy CSTs and associated vaginal/urinary bacterial or fungal infections, we would suggest increasing *Lactobacillus* numbers in the vaginal niche, for example, by administering probiotics to naturally accelerate the establishment of a healthier vaginal microbiome that is better able to fight pathogens. Additionally, it would be important to explore why some women keep an optimal vaginal microbiome state after delivery characterized by a predominance of *Lactobacillus* species, as this could provide insights in the mechanisms that could be targeted for promoting an optimal vaginal microbiome postpartum. Overall, it remains clear that most of the research has focused on the health of the child, whereas the important recovery of the mother has not been fully elucidated, especially with regard to the nonintestinal microbiome.

MATERIALS AND METHODS

Study design

Fifty-nine participants were enrolled in this pilot study to explore the microbial changes that occur with pregnancy, by comparing the microbiome of different body sites of a cohort of healthy pregnant women (n = 30) with the microbiome of the same body sites of a cohort of healthy age-matched nonpregnant women (n = 29). All recruited participants were healthy, were 18 years of age or older, and consent to all aspects of the protocol. Participants were excluded if they had any recent genitourinary infections, if they had taken any antibiotic/probiotic treatment in the last 6 months, if they smoked or took prescription medicines, and if they had HIV or HCV. Pregnant participants were also excluded if they had multiple pregnancy, membrane rupture longer than 12 h, prepregnancy diabetes type 1 or 2, gestational diabetes mellitus, prepregnancy hypertension, or Preeclampsia/HELLP. Metadata from all participants are listed in the GitHub Repository (47). All participants were recruited at the Medical University of Graz, Styria, Austria, from 2017 to 2020.

Sample collection and processing

For pregnant participants, samples were collected at two time points, 1 to 2 weeks before delivery (prepartum) and 1 month postpartum. The following samples were collected for all three study groups: urine, vaginal swabs, and oral swabs. Samples were collected by the participants after they had been clearly instructed on how to collect and store the samples.

Approximately 20 mL of midstream urine was collected in sterile collection tubes at a not-further-specified time of the day. Vaginal and oral samples were collected using FLOQSwabs (Copan). Oral samples were collected from the cheek buccal mucosa. All samples were stored in the fridge without addition buffer added and were then transported to the lab on ice and stored at −80°C until further processing.

Genomic DNA was extracted from the urine, oral, and vaginal specimens using the QIAamp DNA Mini Kit (QIAGEN) with some modification: before the extraction with the kit, the urine samples were centrifuged at 4,400 × g for 15 min and the supernatant was removed except for 500 µL that was used to resuspend the pellet. Five hundred microliters of Lysis Buffer (sterile filtered, 20 mM Tris-HCl pH 8, 2 mM Na-EDTA, 1,2% Triton X-100) was added to the vaginal and oral swabs. To all the samples, 50 µL of lysozyme (10 mg/mL) and 6 µL of mutanolysin (25 KU/mL) were added, followed by an incubation at 37°C for 1 h. The obtained mix was transferred to Lysing Matrix E tubes (MP Biomedicals) followed by a step of mechanical lysis at 5,500 rpm for 30 s two times using the MagNA Lyser Instrument (Roche). After the mechanical lysis, the samples

were centrifuged to separate the beads from the supernatant at 10,000 × *g* for 2 min. Afterward, the DNA was extracted according to the provided instructions. The DNA was eluted in 100 µL of Elution Buffer for the vaginal and in 60 µL for the urine and oral samples. For all samples, the genomic DNA concentration was measured using Qubit HS (Thermo Fischer Scientific). Most urine samples had a DNA concentration under the detection limit (<0.1 ng).

Additional swabs and tubes filled with buffer served as negative controls. Negative controls were processed alongside during DNA extraction, PCR, sequencing, and quality control.

Statistical analyses on the metadata of the cohort were performed in SPSS using either *t*-test, Mann-Whitney U test (independent samples), or Fisher's exact *t*-test as indicated in Table 1.

PCR amplification

The obtained genomic DNA was used to amplify the V4 region of the 16S rRNA gene using Illumina-tagged primers, 515FB and 806RB (Table 4). Those primers target and partially cover the 16S rRNA gene of both bacteria and archaea, but mainly the bacterial part, and are therefore named "universal" or bacterial. In order to specifically identify the archaeal communities present in the samples, a nested PCR was performed using the primer combination 344F-1041R/519F-Illu806R as described previously (48). For the fungal communities, the ITS2 region was amplified using the primers ITS86F/ITS4. The PCR reactions were performed in triplicates in a final volume of 25 µL containing TAKARA Ex Taq buffer with MgCl2 (10×; TAKARA Bio Inc.), primers 200 nM, dNTP mix 200 µM, TAKARA Ex Taq Polymerase 0.5 U, water (LiChrosolv; Merck), and DNA template (1–2 µL of genomic DNA). The PCR amplification conditions are listed in Table 5.

Amplicon sequencing, bioinformatics, and statistical analysis

Library preparation and sequencing of the amplicons were carried out at the Core Facility Molecular Biology, Center for Medical Research at the Medical University Graz, Austria. In brief, DNA concentrations were normalized using a SequalPrep normalization plate (Invitrogen), and each sample was indexed with a unique barcode sequence (eight cycles index PCR). After pooling of the indexed samples, a gel cut was carried out to purify the products of the index PCR. Sequencing was performed using the Illumina MiSeq device and MS-102-3003 MiSeq Reagent Kit v3-600cycles (2 × 300 cycles). The obtained 16S rRNA gene amplicon data are available in the European Nucleotide Archive under the study accession number PRJEB65415.

The analysis of the 16S rRNA gene amplicon data was performed using QIIME2 (52) 2021.1-12 as described previously (53). Removal of primers and chimeras and quality filtering were performed with the DADA2 algorithm (54) for truncation (-p-trunc-len-f 200 –p-trunc-len-r 150) and denoising to generate ASVs. Taxonomic classification (55) was based on the SILVA 138 database (56) for the "universal" and archaeal approach and the UNITE database (57, 58) for the ITS approach. The obtained feature table and taxonomy file were used for further analysis. Contaminating ASVs were determined and removed by

TABLE 4 Primer pairs used for "universal," archaeal, and fungal PCRs

Approach and target	Name	Sequence (5′–3′)	Reference
PCR "Universal"	515FB	GTGYCAGCMGCCGCGGTAA	(49)
	806RB	GGACTACNVGGGTWTCTAAT	(49)
PCR Archaea I/II	344F	ACGGGGYGCAGCAGGCGCGA	(50)
	1041R	GGCCATGCACCWCCTCTC	(50)
PCR Archaea II/II	519F	CAGCMGCCGCGGTAA	(50)
	806R	GGACTACVSGGGTATCTAAT	(50)
PCR Fungi	ITS86F	GTGAATCATCGAATCTTTGAA	(51)
	ITS4	TCCTCCGCTTATTGATATGC	(51)

TABLE 5 PCR settings for the primer pairs used

Target gene	Primer pair	Initial denaturation	Denaturation	Annealing	Elongation	Final elongation	No. of cycles
"Universal" (16S rRNA gene)	515FB - 806RB	3′, 94°C	45″, 94°C	1′, 50°C	1′ 30″, 72°C	10′, 72°C	35
Archaea (16S rRNA gene)	344F-1041R	5′, 95°C	30″, 94°C	45″, 56°C	1′, 72°C	10′, 72°C	25
	519F-806R	5′, 95°C	40″, 95°C	2′, 63°C	1′,72°C	10′, 72°C	30
Fungi (ITS region)	ITS86F - ITS4	1′, 94°C	30″, 94°C	30″, 56°C	30″, 68°C	10′, 68°C	35

decontam v 1.13 (59) in R (60), running *iscontaminant* in *prevalence* mode with a threshold of 0.5. After this step, positive and negative controls were removed from the data sets. Additionally, ASVs classified as chloroplast and mitochondria were removed.

For normalization, different approaches were used for the three bacterial, fungal, and archaeal data sets based on their composition. Scaling with ranked subsampling normalization was run in QIIME2 (52) with c_{min} = 1,500 for the bacterial data set and with c_{min} = 100 for the fungal data set. The archaeal data set was normalized with total sum normalization. The number of samples that were analyzed and the number of how many samples were kept after normalization are listed in Fig. S1.

Differentially abundant taxa between the three groups were defined by q2-aldex2 (61–63) in QIIME2 (52) using standard settings, CLR (centered log-ratio) transformed in R (60), and plotted in boxplots in R [packages: ggplot2 (64), dplyr (65), and reshape (66)].

Several analysis steps were performed with Microbiome Explorer in R (67), e.g., for stacked bar plots and boxplots of alpha diversity. Statistics for alpha diversity were performed using the Kruskal-Wallis test of independent samples, adjusted with Bonferroni. PCoA were calculated and plotted in R (60) using the vegan package (68) and an Unweighted UniFrac distance matrix. Differences in beta diversity between groups were assessed with a PERMANOVA on the unweighted UniFrac distances as implemented in the vegan package (68) in R (60) accordingly.

Source tracking was performed with Sourcetracker2 (69) (standard settings) with rarefaction done at 1,500 sequences. Data were then visualized by RAWGraphs (70), based on the median of each sample set.

Sequences classified as the *Lactobacillus* genus were further classified to the species level to allow the clustering of the vaginal microbiome into CSTs. The classification was performed by classification through EzBioCloud (71). The CSTs were classified using the VALENCIA (VAginaL community state typE Nearest Centroid clAssifier) classifier (28) as described in the GitHub tutorial (72). The biom table used to run the classifier VALENCIA as well as the obtained output can be found in the GitHub Repository.

NMR metabolomics on urine samples

A subset of 87 urine samples from all three groups was analyzed in house with untargeted NMR for several metabolites as described previously (73). In short, methanol water was added to the samples and cells were lysed, lyophilized, and mixed with NMR buffer. NMR was performed on an AVANCETM Neo Bruker Ultrashield 600 MHz spectrometer equipped with a TXI probe head at 310 K and processed as described elsewhere (74). NMR data were analyzed using MetaboAnalyst (75), following the protocol for paired samples (comparison mpre to mpost; log transformation).

Significantly differentially abundant metabolites were correlated with CLR-transformed counts of bacterial genera in R (60) and plotted in heatmaps with ggplot2 (64).

Visualization and sample overview

The circle packing plot about archaeal occurrence was created with rawgraphs.io (70). All figures were aligned in Inkscape v 1.1 (URL: https://inkscape.org/en/). Codes and all data tables are available in our GitHub Repository https://github.com/CharlotteJNeumann/PerinatalMicrobiomeTRAMIC (47). An overview of the available data is displayed in two figures: Fig. S1 is following the STORM guideline and was created with drawio.com (URL: https://drawio.com). Fig. S2 displays the data available per sample and individuum.

Reproducibility

We conducted a prospective pilot study whereas sample size was not predetermined beforehand. Randomization and blinding of the investigators were not foreseen in the chosen study setup. A full study flow chart is provided in Fig. S1. Participants 13 and 17 were excluded from the study due to incomplete sampling, and all data from stool were excluded as no stool samples were collected in the mpost group, and therefore, this body site could not be sufficiently examined with the focus of this manuscript. Overall, the study is considered to be only partially reproducible, as the data are dependent on the study cohort, which was only sampled once within this study, and sampling of cohorts at the same time window cannot be repeated. However, starting from the raw sequencing data, the analysis is fully reproducible, and all required data, scripts, and details are provided.

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DATA AVAILABILITY

A STORMS (Strengthening The Organization and Reporting of Microbiome Studies) checklist (76) is available at 10.5281/zenodo.10458418. Data are available in the GitHub Repository https://github.com/CharlotteJNeumann/PerinatalMicrobiomeTRAMIC (47). The obtained 16S rRNA gene amplicon data are available in the European Nucleotide Archive under the study accession number PRJEB65415.

ETHICS APPROVAL

Research involving human participants was performed in accordance with the Declaration of Helsinki and was approved by the local ethics committees (the Ethics Committee at the Medical University of Graz, Graz, Austria) under the ethics vote number 28-524 ex15/16. The study has been registered at clinicaltrials.gov (NCT04140747).

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (Spectrum00147-24-s0001.docx). Fig. S1 to S18.

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Clinical NEC prevention practices drive different microbiome profiles and functional responses in the preterm intestine

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Preterm infants with very low birthweight are at serious risk for necrotizing enterocolitis. To functionally analyse the principles of three successful preventive NEC regimens, we characterize fecal samples of 55 infants (<1500 g, $n = 383$, female = 22) longitudinally (two weeks) with respect to gut microbiome profiles (bacteria, archaea, fungi, viruses; targeted 16S rRNA gene sequencing and shotgun metagenomics), microbial function, virulence factors, antibiotic resistances and metabolic profiles, including human milk oligosaccharides (HMOs) and short-chain fatty acids (German Registry of Clinical Trials, No.: DRKS00009290). Regimens including probiotic *Bifidobacterium longum* subsp. *infantis* NCDO 2203 supplementation affect microbiome development globally, pointing toward the genomic potential to convert HMOs. Engraftment of NCDO 2203 is associated with a substantial reduction of microbiome-associated antibiotic resistance as compared to regimens using probiotic *Lactobacillus rhamnosus* LCR 35 or no supplementation. Crucially, the beneficial effects of *Bifidobacterium longum* subsp. *infantis* NCDO 2203 supplementation depends on simultaneous feeding with HMOs. We demonstrate that preventive regimens have the highest impact on development and maturation of the gastrointestinal microbiome, enabling the establishment of a resilient microbial ecosystem that reduces pathogenic threats in at-risk preterm infants.

About eleven percent of all infants worldwide are born prematurely, i.e., before 37 weeks' gestation[1]. Very low birth weight (VLBW) preterm infants (<1500 g) are particularly vulnerable to acute and long-term clinical complications. Of particular concern is the development of necrotizing enterocolitis (NEC), a serious gastrointestinal threat that occurs in 7–11% of VLBW infants[2]. In such cases, mortality can reach up to 30%[3].

NEC is a devastating multifactorial disease that is driven in part by perturbations of the microbiome, including colonization and overgrowth of certain microbes with potentially pathogenic potential such as *Escherichia coli* or *Clostridium perfringens*[4].

Given the rapid onset of NEC, a number of neonatal intensive care units (NICUs) have developed specific NEC prophylaxis programmes that include the use of probiotics, antibiotics, and differentiated feeding protocols and that have resulted in a recent, substantial decrease in NEC rates in preterm infants[5].

Probiotic treatments are usually based on the use of *Bifidobacterium* and *Lactobacillus* species[6]. *Bifidobacterium*, in particular, is

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considered as an important member of the resident infant microbiome that is maintained into early childhood and promotes healthy infant development[7,8].

Antibiotics are administered intravenously at the first signs of infection to control early-onset sepsis. As a result, the majority of VLBW infants are exposed to antibiotics in the first few days of life and for extended periods of time[9]. A number of publications have emphasized the need for responsible antibiotic usage in such vulnerable patients, as their use is associated with the risk of infection with multi-drug resistant (MDR) microorganisms[10] and is believed to have other, largely unknown, long-term effects. Overall, antibiotic exposure is often considered as preventable[9,11].

Additionally, the use of enteral antibiotics may be effective as NEC prophylaxis. The findings of the Cochrane Neonatal Collaborative Review Group[12] suggest that oral administration of prophylactic enteral antibiotics results in a statistically significant reduction in NEC and in NEC-related deaths in low-birth-weight preterm infants. However, the risks of enteral antibiotics have not yet been quantified; thus, this strategy has never been widely adopted due to concerns about the emergence of resistant bacteria and the absorption of antibiotics from the gut[13]. However, such adverse effects have not been reported so far[14].

Human milk (HM), the gold standard for infant feeding, is a surprisingly complex synbiotic that contains probiotic bacteria and prebiotics to nourish probiotic bacteria. Prebiotic human milk oligosaccharides (HMOs) are complex carbohydrates present in large quantities in HM that are not broken down by intestinal enzymes. Therefore, they serve only as a specific substrate for certain bacteria in the infant's gastrointestinal tract (GIT), such as mainly *Bifidobacterium* (*Bifidobacterium bifidum* and *Bifidobacterium longum* subsp. *infantis*) but also *Bacteroides* (*Bacteroides vulgatus* and *Bacteroides fragilis*)[15,16]. Indeed, *Bifidobacterium* is enriched in infants fed HM[17], due to its ability to metabolize HMOs. The sophisticated, individual complexity of HM can only be partially mimicked by formula milk (FM); nevertheless, newer products also contain standardized pre- and probiotics for optimal nutrition.

Southern Austrian neonatal units have implemented various combinations of these prophylactic measures with great success, resulting in an exceptionally low average NEC rate of 2.9% in VLBW infants (2007–2016[18]).

In this work, we take the opportunity to deeply analyse the mechanism for success across these different regimens on the level of the gut microbiome and metabolome.

We recruited 55 VLBW infants in three closely neighboured hospital centers (Graz, Klagenfurt, Leoben), that differ in antibiotic treatment (enteral gentamicin or none), antifungal treatment (enteral nystatin or parenteral fluconazole), probiotic use (*Lactobacillus rhamnosus* LCR 35, Bifidobacterium *longum* subsp. *infantis* NCDO 2203 in combination with *Lactobacillus acidophilus NCDO 1748*, or none) and feeding (HM, FM). Using a multi-omics approach, we examine the composition and function of the microbiome and its metabolites in the first weeks of life to understand the importance of the interactions among dietary components, antibiotics and probiotics.

Our study differs from previous studies in that a focus is placed on different NEC-prevention protocols, not in just one but in three different clinics. This study setup also allowed us to avoid the problematic cross-contamination of probiotics into the control groups[19,20]. To understand the effects and mechanisms of the different treatments, we analyse the microbiome on a multi-kingdom level and include functional metagenomics and metabolomics, as well as genome profiling on the species level. We conclude our study with a suggestion to further improve existing protocols to support a healthy microbiome development in VLBW infants by combining effective probiotics (including *Bifidobacterium longum* subsp. *infantis* NCDO 2203) and human milk.

Results

Details on the study design are provided within the Methods. In brief, fecal samples were collected prospectively in three independent NICUs in Austria using a different NEC prophylaxis regimen (Table 1, in Methods) from preterm infants with a birthweight <1500 g. Samples were collected every other day, starting with the meconium, up until two weeks of age. The study groups do not differ statistically significantly in any observed metadata except for the length of hospital stay of the mothers after birth[21], Table 2 therein).

It should be mentioned that the three clinical situations studied here differed in a number of confounding factors, both recorded and possibly unrecorded, and we can only draw conclusions based on the overall setting in each NICU, which includes medication and probiotic regimens. However, the three hospitals are geographically very close to each other, so the patient catchment area also overlaps, and we may consider other factors to be minor compared with the medication and feeding protocols. Indeed, PERMANOVA analyses revealed that the combined variables *Bifidobacterium* administration (yes/no), feeding protocol (formula milk, FM; human milk, HM; mixed), gentamycin administration (yes/no) had the same or even stronger effect ($R^2 = 0.6763$ (time point 7), $R^2 = 0.3430$ (tp3), $R^2 = 0.2710$ (all); $p = 0.001$ (all tests)), than the grouping according to the hospital ($R^2 = 0.6763$ (time point 7), $R^2 = 0.2618$ (time point 3), $R^2 = 0.2623$ (all); $p = 0.001$ (all tests)), indicating that the major observed differences were indeed driven by the regimens (Suppl. Table 1, amplicon dataset).

Early-life therapy regimen influences microbiome composition and development across all microbial domains

Assessing the microbial composition of all infants with metagenomic analyses and 16S rRNA gene sequencing, we detected an association of

Table 1 | Prophylactic regimens of probiotics, antibiotics, antifungals and feeding protocols of the three different neonatal intensive care units (NICUs): Graz (G), Klagenfurt (K), and Leoben (L)

	NICU Graz (G)	NICU Klagenfurt (K)	NICU Leoben (L)
Probiotics	"Antibiophilus" *Lactobacillus rhamnosus* LCR 35 1×10^9 CFU/d, oral., split into 2 doses per day	"Infloran" *Bifidobacterium longum* subsp. *infantis* NCDO 2203 2×10^9 CFU/d *Lactobacillus acidophilus* NCDO 1748 2×10^9 CFU/d in combination, oral	None
Antibiotics	Gentamicin 7 mg/kg, every 12 h, oral	None	Gentamicin 7 mg/kg, every 12 h, oral
Antifungal agents	Nystatin 10,000 U/kg every 6 h, oral	Fluconazole 6 mg/kg, every 72 h (<1000 g BW), intravenous	Nystatin 10,000 U/kg every 6 h, oral
Feeding Protocol	HM favored over FM	FM and in few cases additionally pasteurized HM	HM favored over FM

CFU Colony forming units, *HM* Human milk, *FM* Formula milk.

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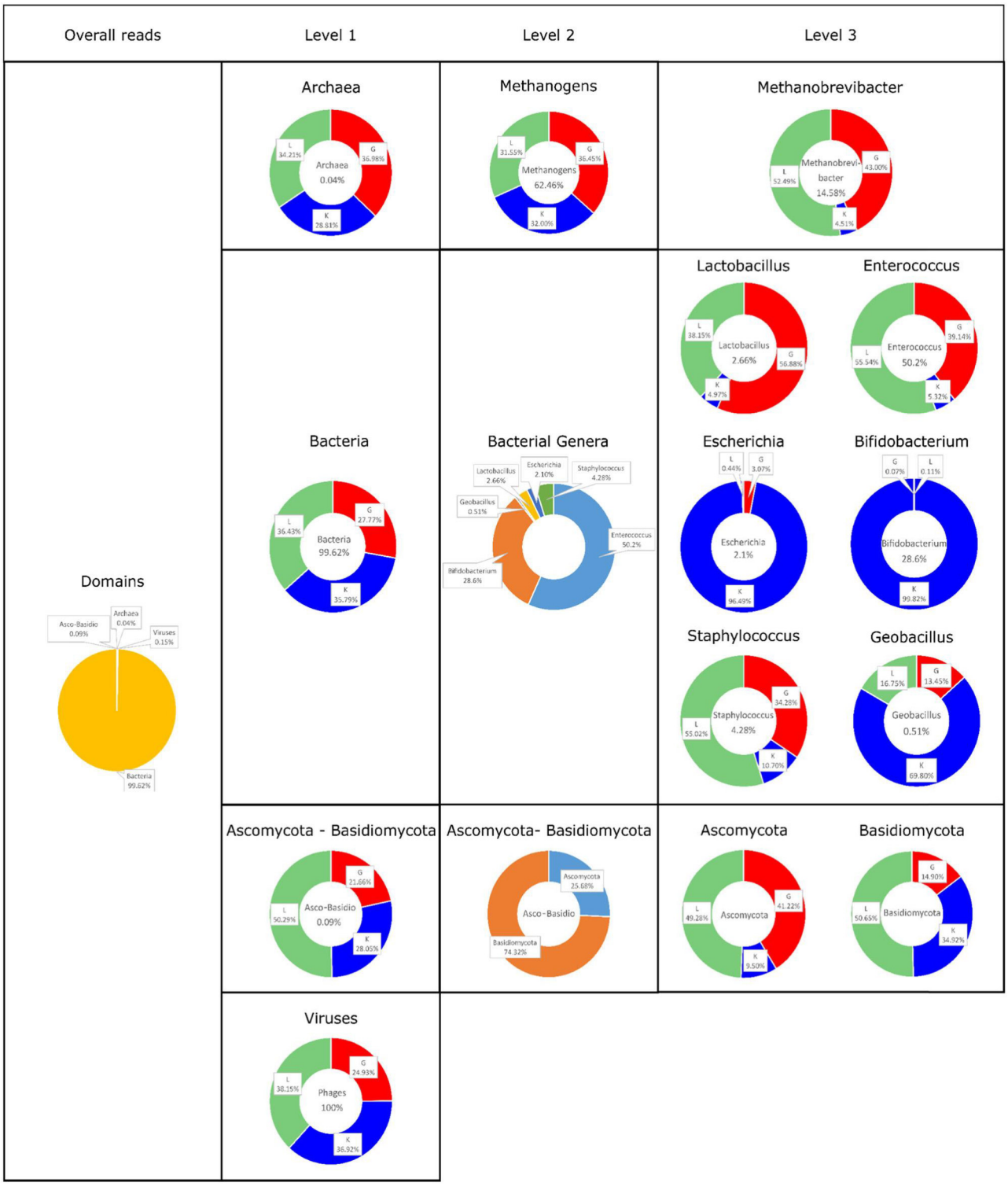
Table 2 | Distribution of overall metagenomic reads across the domains of life and between the centers on different taxonomic levels



the preventive NEC regimen on all microbial domains and groups, including bacteria (99.62% of all metagenomic reads), their phages and viruses (0.15%), but also on archaea (0.04%) and fungi (Ascomycota/Basidiomycota: 0.09%) (Table 2, Fig. 1, Fig. 2).

The role or even presence of archaeal signatures in the premature infants' gut is still unclear and underexplored. Previous publications concluded that infants generally do not carry a substantial amount of archaea until five years of age[22], and especially in preterm infants, archaea were found only in a little proportion of screened infants[23,24]. Our archaea-focused approach enabled us to successfully detect 290 different ASVs with amplicon-based analyses and 75 different archaeal species with metagenomic-based sequencing. In particular,

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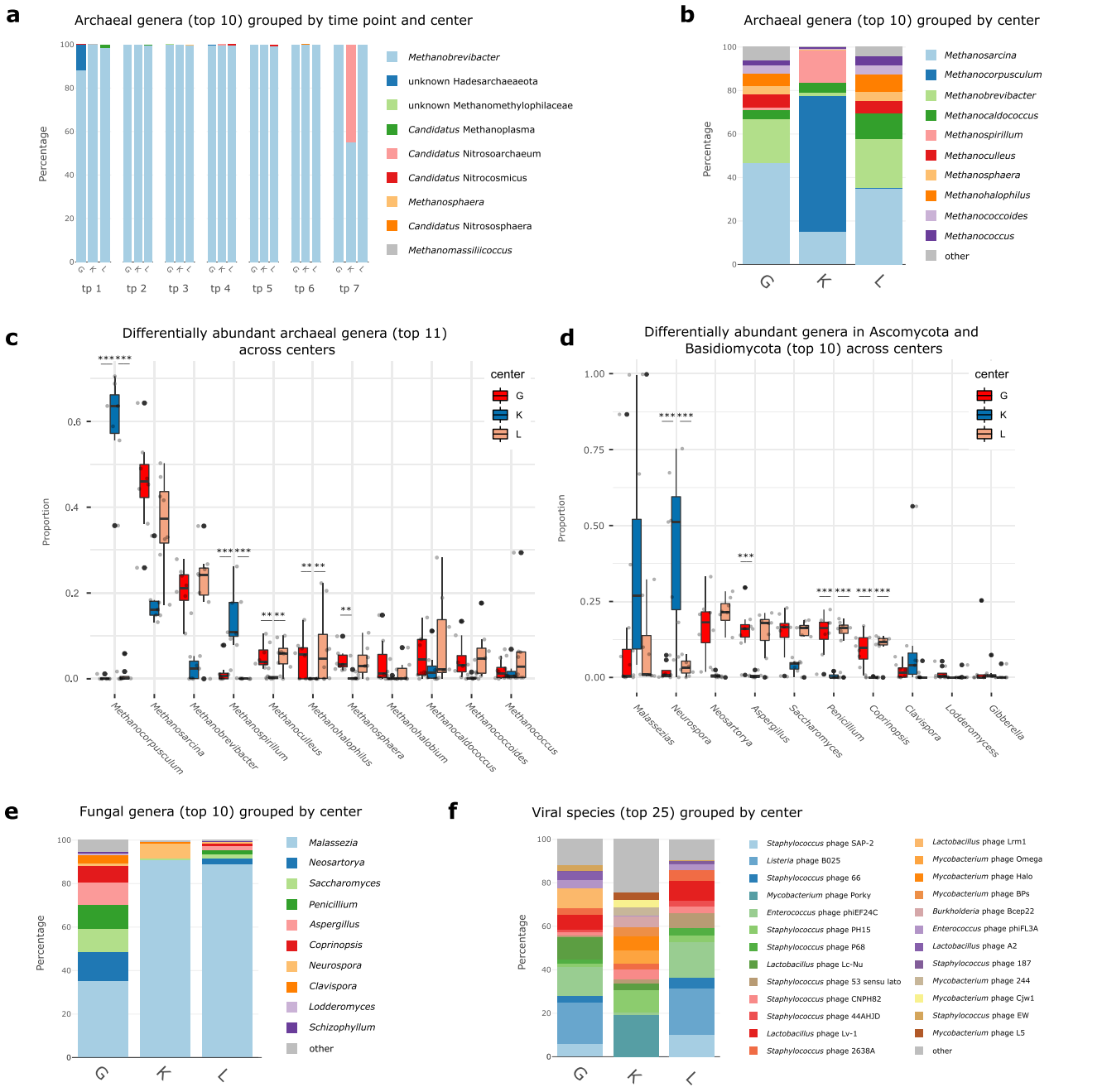

Fig. 1 | Overall distribution and abundances of microbial signatures of different domains, according to the different centers. a Stacked bar plot of relative abundances of the top ten archaeal genera in the amplicon data set, displayed per center at time points (tp) tp1–7. **b** Stacked bar plot of the top ten relative abundances of methanogenic archaeal genera in the MGS (metagenomic) dataset for tp7 per center. **c** Box plot of relative abundances of the top eleven methanogenic archaeal genera per center (MGS data, tp7, $n = 23$ biologically independent samples, DSeq2): *Methanocorpusculum*: K:G $q < 0.001$, K:L $q < 0.001$, G:L $q = 0.945$; *Methanosarcina*: K:G $q = 0.261$, K:L $q = 0.054$, G:L $q = 0.945$; *Methanobrevibacter*: K:G $q = 0.052$, K:L $q = 0.138$, G:L $q = 0.945$; *Methanospirillum*: K:G $q < 0.001$, K:L $q < 0,001$, G:L $q = 0.192$; *Methanoculleus*: K:G $q < 0.001$, K:L $q = 0.009$, G:L $q = 0.945$; *Methanosphaera:* K:G $q = 0.028$, K:L $q = 0.138$, G:L $q = 0.945$; *Methanohalobium*: K:G $q = 0.052$, K:L $q = 0.774$, G:L $q = 0.945$; *Methanocaldococcus*: K:G $q = 0.237$, K:L $q = 0.576$, G:L $q = 0.945$; *Methanococcoides:* K:G $q = 0.112$, K:L $q = 0.264$, G:L $q = 0.945$; *Methanococcus*: K:G $q = 0.371$, K:L $q = 0.964$, G:L $q = 0.945$. **d** Box plot of relative abundances of the top ten genera of Ascomycota and Basidiomycota per center (MGS data, tp7, $n = 23$ biologically independent samples, DSeq2): *Malassezia*: K:G $q = 0.021$, K:L $q = 0.095$, G:L $q = 0.792$; *Neurospora*: K:G $q < 0.001$, K:L $q = 0.001$, G:L $q = 0.856$; *Neosartorya*: K:G $q < 0.001$, K:L $q < 0.001$, G:L $q = 0.995$; *Aspergillus*: K:G $q < 0.001$, K:L $q < 0.001$, G:L $q = 0.995$; *Saccharomyces*: K:G $q = 0.289$, K:L $q = 0.035$, G:L $q = 0.995$; *Penicillium*: K:G $q < 0.001$, K:L $q < 0.001$, G:L $q = 0.995$, *Coprinopsis*: K:G $q < 0.001$, K:L $q < 0.001$, G:L $q = 0.995$; *Clavispora*: K:G $q = 0.293$, K:L $q = 0.04$, G:L $q = 0.291$; *Lodderomyces*: K:G $q = 0.056$, K:L $q = 0.834$, G:L $q = 0.006$; *Gibberella*: K:G $q = 0.056$, K:L $q = 0.785$ 0.04, G:L $q = 0.785$. **e** Stacked bar plot of the relative abundances of the top ten genera of Ascomycota and Basidiomycota in the MGS dataset for tp7 per center. **f** Stacked bar plot of the top 25 relative abundances of phage species in the MGS dataset for tp7 per center. Significance levels are indicated with asterisks for $q < 0.001$ (***), $q < 0.01$ (**), $q < 0.05$ (*) for differentially abundance testing by DSeq2, adjusted for multiple comparisons. Centers are abbreviated by G (Graz), L (Leoben) and K (Klagenfurt). For boxplots, the upper, middle and lower horizontal lines of the box represent the upper, median and lower quartile; their whiskers depict the smallest or largest values within 1.5-fold of the interquartile range. Top genera/species were calculated across all samples. Source data are provided as a Source Data file (see Github repository).

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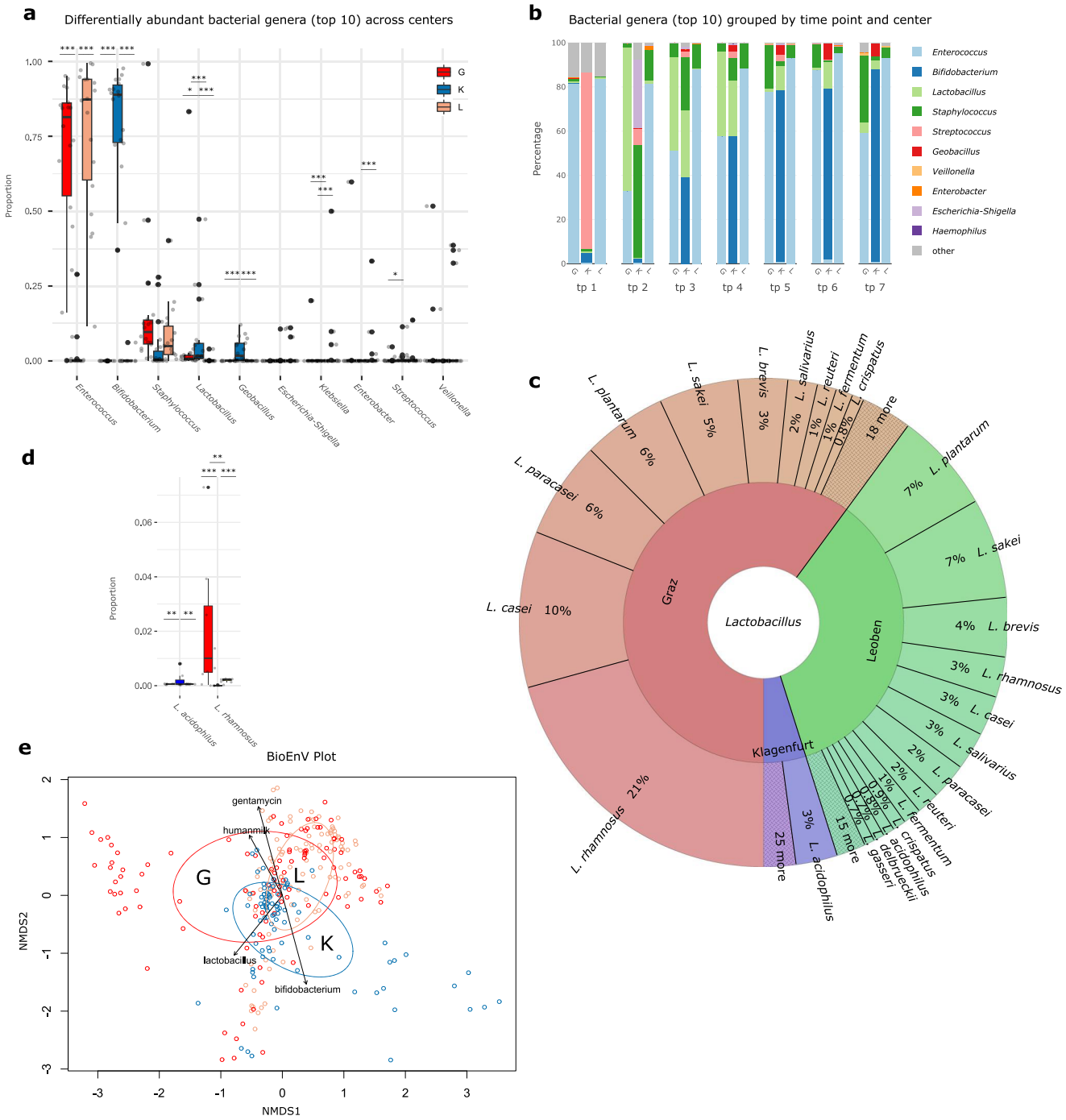

Fig. 2 | Distribution of bacterial taxa between the centers and influence of probiotic species. a Box plot of relative abundances of the top ten bacterial genera per center (amplicon data, tp7, $n = 23$ biologically independent samples, DSeq2) *Enterococcus*: K:G $q < 0.001$, K:L $q < 0.001$, G:L $q = 1$; *Bifidobacterium*: K:G $q < 0.001$, K:L $q < 0.001$, G:L $q = 1$; *Staphylococcus*: K:G $q = 0.642$, K:L $q = 1$, G:L $q = 0.274$; *Lactobacillus*: K:G $q = 0.034$, K:L $q < 0.001$, G:L $q < 0.001$; *Geobacillus*: K:G $q < 0.001$, K:L $q < 0.001$, G:L $q = 1$; *Escherichia-Shigella*: K:G $q = 1$, K:L $q = 0.979$, G:L $q = 1$; *Klebsiella*: K:G $q = 1$, K:L $q < 0.001$, G:L $q < 0.001$; *Enterobacter*: K:G $q = 0.916$, K:L $q < 0.001$, G:L $q = 1$; *Streptococcus*: K:G $q = 0.018$, K:L $q = 0.443$, G:L $q = 0.765$; *Veillonella*: K:G $q = 0.050$, K:L $q = 0.111$, G:L $q = 0.768$; **b** Stacked bar plot of relative abundances of top ten bacterial genera in the amplicon data set per center each at time points tp1–7. **c** Krona chart of the distribution of species of the *Lactobacillus* genus between the centers (MGS data, tp7). **d** Log percentages of relative abundance of probiotically administered *Lactobacillus* genera (MGS data, tp7, $n = 23$ biologically independent samples, DSeq2) *Lactobacillus acidophilus*: K:G $q = 0.003$, K:L $q = 0.002$, G:L $q = 1$; *Lactobacillus rhamnosus*: K:G $q < 0.001$, K:L $q = 0.001$, G:L $q = 0.001$ (i) *L. acidophilus* NCDO 1748 and (ii) *L. rhamnosus* LCR 35. **e** Biplot of BioEnv with correlations of the Euclidean distances for the metadata of dissimilarities between the centers (administration of gentamicin, of probiotic *Lactobacillus* or *Bifidobacterium* and human milk), amplicon data. G in red, K in blue, L in green; significance levels are indicated with asterisks for $q < 0.001$ (***), $q < 0.01$ (**), $q < 0.05$ (*) for differentially abundance testing by DSeq2, adjusted for multiple comparisons. Centers are abbreviated by G (Graz), L (Leoben), and K (Klagenfurt). For boxplots, the upper, middle and lower horizontal lines of the box represent the upper, median, and lower quartile; their whiskers depict the smallest or largest values within 1.5-fold of the interquartile range. Source data are provided as a Source Data file (see Github repository).

Methanobrevibacter was abundant, as it was detected in all infants in at least one sample and across all time points. In addition to typical human-associated archaea, *Methanobrevibacter*, *Methanosphaera*, Methanomethylophilaceae (incl. Methanomassiliicoccus), and various Nitrososphaeria (likely derived from skin sources)[25,26] (Fig. 1a, amplicon data), abundant signatures of *Methanosarcina* and *Methanocorpusculum* were additionally identified using shotgun metagenomics (Fig. 1b, MGS data), indicating that even VLWB newborns are in contact with a wide diversity of archaea. Archaea reflected the center, with, for example, *Methanocorpusculum* and *Methanospirillum* being significantly more abundant in samples from Klagenfurt (K) (*Methanocorpusculum*,DESeq2, K:G $q < 0.001$, K:L $q < 0.001$), than in Graz (G) and Leoben (L) (Fig. 1c, MGS data).

The contribution of fungal signatures to the overall microbiome was largely limited (also probably due to the application of antifungals) to Basidiomycota and Ascomycota (Fig. 1d, e, MGS data), which, however, also revealed a center-specific pattern: *Neosarorya*, *Penicillum*, *Aspergillus* and *Coprinopsis* (significantly increased in G and L with DESeq2 $q < 0.001$) were antiparallel to *Malassezia*, *Neurospora* and *Clavispora*, which were increased in K.

In order to confirm the center-specific profiles of the multiple-component microbiome data (further details, see below), a network analysis was performed based on the ten most differentially abundant genera of bacteria, methanogens, ascomycota/basidiomycota and phages (Suppl. Fig. 2 MGS data). The network revealed the formation of two separate clusters, whose nodes were mainly composed of K taxa and taxa from G and L, respectively. Those two clusters were connected with negative associations only, underlining the separating effect of the different regimens (see also PERMANOVA results, mentioned above). Some taxa were even shared by both centers (e.g., *Methanococcus*, *Candida*), suggesting an interaction between the domains.

Supplemented *Bifidobacterium longum* subsp. *infantis* outweighs natural pathobiont colonizers and co-administered *Lactobacillus acidophilus*

The bacteriomes of the infants were mainly characterized by the predominance and differential abundance of six bacterial key taxa, namely *Enterococcus, Bifidobacterium, Lactobacillus, Staphylococcus, Geobacillus* and *Escherichia* (Fig. 2a, amplicon data, tp7).

Enterococcus was found to predominate the bacterial microbiome in G and L (~77%), followed by *Lactobacillus* and *Staphylococcus*, the relative abundance of which decreased with maturation. In contrast, the K samples were dominated by *Bifidobacterium* at each time point and reached a relative abundance of > 82% at tp7 (Fig. 2b, amplicon data). Thus, G and L were dominated by a typical colonizer of the GI in preterm infants, whereas K samples showed an overall predominance of a supplemented probiotic taxon. The phenomenon of detection of *Geobacillus* signatures exclusively in K samples has been discussed previously[21] and in the next section.

Notably, 16 of all 89 phage species were strongly associated with bacterial key species, resulting in a center-specific, strongly differing phage profile (Fig. 1f, MGS data). No bacteriophages were identified for *Bifidobacterium*, *Escherichia-Shigella* and *Geobacillus*; however, other phages from key species correlated with the relative abundance of their host, exemplified by *Streptococcus* in Suppl. Fig. 3. In particular, phages targeting *Lactobacillus* (Kruskal-Wallis; G:K $q = 0.004$, G:L $q = 0.003$) and *Enterococcus* (Kruskal-Wallis; K:L $q = 0.012$) were lowest in K.

Shotgun metagenomics confirmed the significantly differential abundance of *Bifidobacterium* in the three centers that was already observed in the amplicon sequencing approach (DESeq2, K:G $q < 0.001$, K:L $q < 0.001$). Although *B. longum* subsp. *infantis* NCDO 2203 was the only *Bifidobacterium* administered in K, nine additional *Bifidobacterium* species were detected, with six species present in all K infants (*B. longum*, *B. dentium*, *B. breve*, *B. bifidum*, *B. animalis*, *B. adolescentis*). *B. longum* accounted for 95% of all reads from *Bifidobacterium*, and indeed this taxon was verified as *Bifidobacterium longum* subsp. *infantis* NCDO 2203 by genomic comparisons (see Material and Methods and Suppl. Table 3b). These analysis results support our assumption that the major *Bifidobacterium* signatures in K infants were indeed from the administered probiotic. Of note, the signatures of *B. longum* subsp. *infantis* are abbreviated as *B. infantis* in the following.

Naturally, bifidobacteria are uncommon in the premature infants' GI in the first days of life and start colonizing naturally beginning from week four and on[6,27]. Our data also indicate an absence of bifidobacteria in preterm infants who did not receive it via supplementation. Furthermore, administration of *B. infantis* NCDO 2203 resulted in very high abundances over other bacteria, including the co-administered *L. acidophilus* NCDO 1748: Although both probiotic species, *B. infantis* NCDO 2203 and *L. acidophilus* NCDO 1748, were administered in equal amounts in K, *L. acidophilus* NCDO 1748 was substantially lower in abundance than the predominant *B. infantis* NCDO 2203 (0.21% relative abundance of *L. acidophilus* NCDO 1748 at tp7 vs. 75.69% relative abundance of *B. infantis* NCDO 2203). We suggest, that the colonizing potential of *B. infantis* NCDO 2203 is higher than the one of *L. acidophilus* NCDO 1748 probably due to its different metabolic capacities. In agreement with our data, it was already shown before, that lactobacilli do not colonise the preterm gut in large numbers[28].

Probiotic administration of lactobacilli increases their natural abundance and diversity

In total, 27 species of the genus *Lactobacillus* (see details on classification issues in Methods), were detected by shotgun sequencing with different profiles between the centers (Fig. 2c). Probiotic lactobacilli were administered only in K (*L. acidophilus* NCDO 1748) and G (*L. rhamnosus* LCR 35). The presence of these lactobacilli in the infants' intestinal samples was confirmed by amplicon sequencing but also by read-mapping (Fig. 2d, Suppl. Table 3a).

Lactobacillus rhamnosus was also detected with low relative abundance (3%) in L, where it was not administered, suggesting that this species is a low abundant part of the natural infant gut microbiome of preterm infants and is possibly transmitted through breastfeeding or other sources[29]. In G, *L. rhamnosus* LCR 35 exhibited the highest abundance in G infants (21%) indicating a seven-fold increase in probiotic lactobacilli through its probiotic administration. K had the lowest absolute *Lactobacillus* abundance, with 56% of all *Lactobacillus* reads representing *L. acidophilus* NCDO 1748, the species administered there (K: 45,268 reads; G: 10,207; L: 12,431). Similar to *L. rhamnosus*, *L. acidophilus* was also detected in almost all infants in all centers, even when not supplemented as probiotics (Fig. 2d).

Although *L. acidophilus* NCDO 1748 was administered in K, it does not result in high abundances, compared to *B. infantis* NCDO 2203 administered at the same concentration. This is reflected by an *L. acidophilus* NCDO 1748 / *B. infantis* NCDO 2203 ratio of ~1:300 and *Lactobacillus*: *Bifidobacterium* ratio of ~1:200 (for genera see Suppl. Table 4).

PERMANOVA analyses confirmed the superior impact of *Bifidobacterium* ($R^2 = 0.2084$ (all samples), $R^2 = 0.1258$ (tp 3), $R^2 = 0.5716$ (tp7)) over *Lactobacillus* administration ($R^2 = 0.04733$ (all samples), $R^2 = 0.0631$, $R^2 = 0.1394$), explaining up to 57.16% and 13.94% of the observed variance, respectively (Suppl. Table 1). The dominance of *Bifidobacterium* over *Lactobacillus* could also be underlined with the BioEnv Biplot (Fig. 2e, amplicon data). This plot shows the metadata whose Euclidean distances have the maximum (rank) correlation with community dissimilarities. In particular, the administration of *Bifidobacterium* and gentamicin correlated strongly with dissimilarities between the centers, whereas the administration of lactobacilli and HM correlates to a lesser degree.

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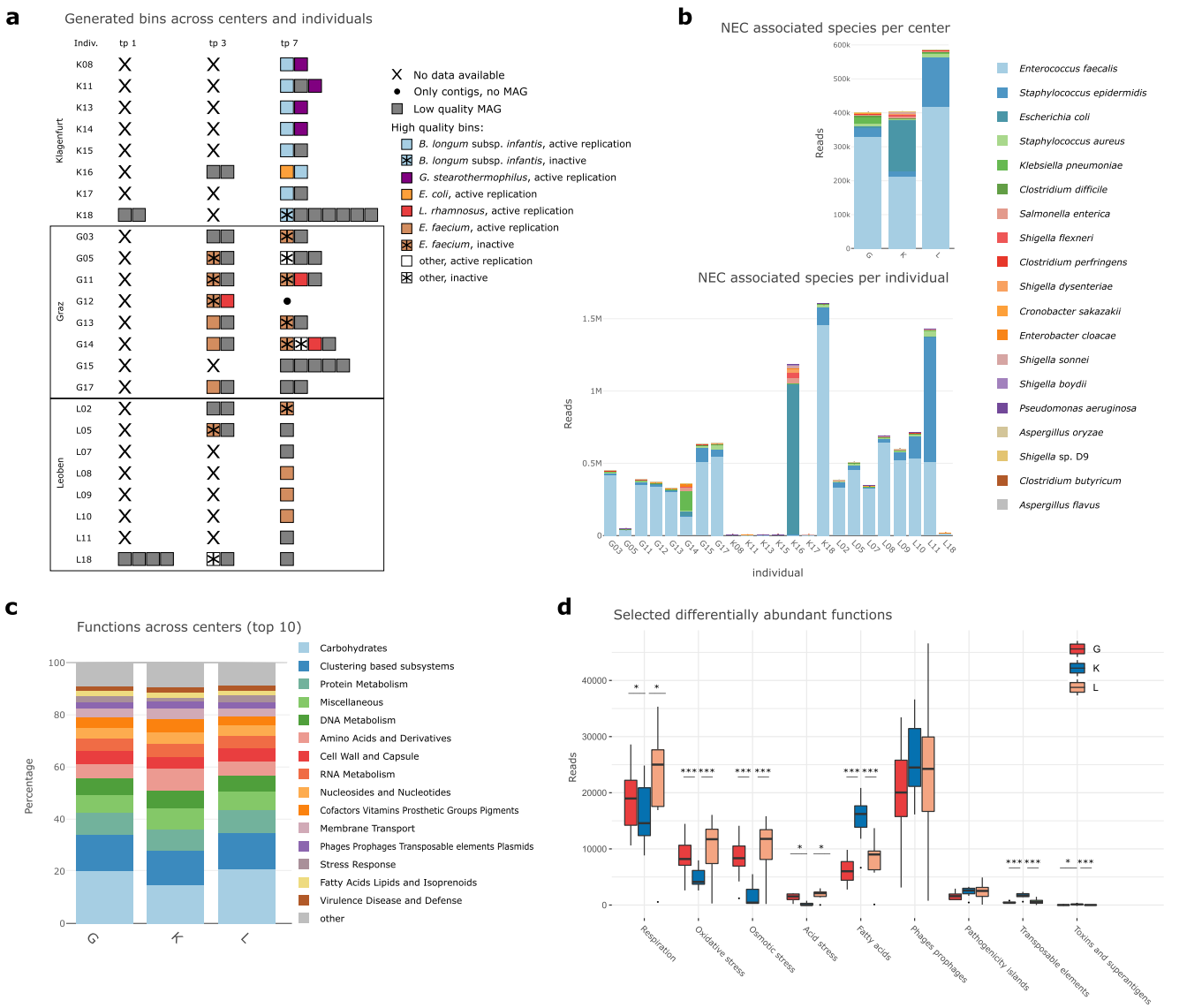

Fig. 3 | Replication values for MAGs, distribution of potentially NEC causing microbes, and specific functions. a Retrieved MAGs per center, individual and time points tp1, tp3 and tp7: availability, quality, iRep replication status [min: 1.348, max: 1.998, mean: 1.654] and taxonomic placement; **b** Read numbers of microbial species that were correlated with NEC (Necrotizing Enterocolitis) previously, per center and per individual, MGS data. **c** Stacked bar plot of relative abundances of top fifteen microbiome functions per center, MGS data; **d** reads of ten selected differentially abundant functions per center at tp7, MGS data, $n = 23$ biologically independent samples: respiration: K:G $q = 0.007$, K:L $q = 0.014$, G:L $q = 0.703$; oxidative stress: K:G $q < 0.001$, K:L $q < 0.001$, G:L $q = 0.949$; osmotic stress: K:G $q < 0.001$, K:L $q < 0.001$, G:L $q = 0.949$; acid stress: K:G $q = 0.033$, K:L $q = 0.029$, G:L $q = 0.949$; fatty acids: K:G $q < 0.001$, K:L $q < 0.001$, G:L $q = 0.949$; phages prophages: K:G $q = 0.997$, K:L $q = 0.640$, G:L $q = 0.949$; pathogenicity islands: K:G $q = 0.859$, K:L $q = 0.305$, G:L $q = 0.849$; transposable elements: K:G $q < 0.001$, K:L $q < 0.001$, G:L $q = 0.949$; toxins and superantigens: K:G $q = 0.005$, K:L $q < 0.001$, G:L $q = 0.760$;. G in red, K in blue, L in green; significance levels are indicated with asterisks for $q < 0.001$ (***), $q < 0.01$ (**), $q < 0.05$ (*) for differentially abundance testing by DSeq2, adjusted for multiple comparisons. Centers are abbreviated by G (Graz), L (Leoben), and K (Klagenfurt). For boxplots, the upper, middle, and lower horizontal lines of the box represent the upper, median, and lower quartile; their whiskers depict the smallest or largest values within 1.5-fold of the interquartile range. Source data are provided as a Source Data file (see Github repository).

Key species are reflected by MAGs and active replication of probiotic species could be inferred

Samples from tp3 (days 5–8) and tp7 (days 13–21) allowed for deep metagenomic sequencing and genome binning. Metagenome assembled genomes (MAGs) were obtained for *B. infantis* NCDO 2203 (K), *G. stearothermophilus* (K), *E. faecium* (G, L), *E. coli* (K), *L. rhamnosus* (G), *Veillonella parvula* (G, inactive), *Klebsiella oxytoca* (G, inactive) and *Escherichia flexneri* (L, inactive). Successful binning of the bacterial genomes followed the scheme of probiotic supplementation, with *B. infantis* NCDO 2203 MAGs in K samples and *L. rhamnosus* LCR 35 MAGs in G samples. Of the L samples, only MAGs of *Enterococcus faecium* were obtained (Fig. 3a, MGS data). Application of iRep[30] suggested that MAGs might correspond to actively replicating bacteria (iRep values >1), and in consequence indicate niche colonization by probiotic (*B. infantis* NCDO 2203 and *L. rhamnosus* LCR 35) or naturally predominant bacteria (*E. faecium*) (Fig. 3a, MGS data).

Notably, high-quality *G. stearothermophilus* genomes with iRep values above 1.41 were isolated from four out of eight infant samples from K (tp7) (see also ref. [21]). *G. stearothermophilus* is a frequent, most probably harmless contaminant in milk plants[31], which probably transforms to an active form during formula milk preparation and is then ingested by the infant. Thermophilic *G. stearothermophilus* grows in the temperature range of 40–70 °C, with optimal growth rates achieved at 55–65 °C[32,33]; thus, an active proliferation in the infant GI is unlikely. *Staphylococcus* MAGs could not be retrieved, despite its high abundance and identification as a key microorganism in this study (Fig. 3a).

Low level occurrence of diverse types of potentially pathogenic bacteria

Next, we searched specifically for signatures that had been associated with outbreaks or cases of NEC in previous reports[27]. In our shotgun metagenomic dataset, we identified *E. faecalis* as having the highest abundance in this dataset, while *S. epidermidis*, *E. coli* and others were found in varying amounts in samples from tp7 (Fig. 3b). While the infants from the other centers showed a more or less homogenous presence of potential NEC-causing microorganisms, the hits in K samples concentrated solely on two infants, K16 and K18 (Fig. 3b). Reads of *E. faecalis* derived mainly from K18, whereas a high number of *E. coli* signatures originated almost exclusively from K16, the only infant in this subset that developed NEC later on. Furthermore, a MAG of probably active *E. coli* could be obtained from this infant at tp7 (i.e., 14 days after birth), suggesting the possible initial bloom of *E. coli* already at this early time point before NEC is usually diagnosed. As *E. coli* MAGs were not retrieved from any other sample, our data support the potential for microbiome analyses to be used for NEC monitoring and diagnosis (Fig. 3a).

Functional profiles possibly mirror earlier gut maturity in infants following regiments with *B. infantis*

We also profiled microbiome functional characteristics at tp7 given the sufficient sequencing coverage obtained at this time point (Fig. 3c, MGS data). K samples showed an overall significantly reduced level of genes involved in osmotic stress, acid stress and respiration (DESeq2, osmotic stress, K:G $q < 0.001$, K:L $q < 0.001$; acid stress; K:G $q = 0.033$, K:L $q = 0.029$; respiration; K:G $q = 0.007$, K:L $q = 0.014$). Additionally, the GI microbiome in K was capable to degrade more complex sugars reflected by the increased relative abundance of genes involved in polysaccharide metabolism, which was also confirmed by metabolomics (Fig. 3d; see also below). Summing up those points, it seems as if already rather anaerobic and more complex metabolism takes place in K than in G and L. In general, complex metabolism and anoxic environment are characteristics of a more mature gut microbiome.

Notably, the number of genes related to transposable elements (involved in the distribution of pathogenic genomic features), were significantly higher in K samples (Kruskal-Wallis, K:G $q < 0.001$, K:L $q < 0.001$), suggesting a potentially higher bacterial pathogenesis signature in these infants.

Next, we performed NMR-based metabolomics on 111 samples from tp1, tp3 and tp7 to determine the concentration of short-chain fatty acids (SCFAs) and complex sugars in the infants' stool samples. We found a general increase in acetic acid, formic acid, valeric acid and butyric acid over time in all centers, regardless of the microbiome composition or probiotic supplementation (Suppl. Fig. 4a–d). However, unlike previous reports, we observed an unexpected spike in propionic acid at tp3 in all centers (Suppl. Fig. 4e)[34]. We hypothesize that this spike in propionic acid is related to a delayed uptake of propionate by the intestinal epithelium during maturation.

Human milk supports *Bifidobacterium* by associated HMO conversion which is impaired by formula milk feeding

Genes involved in carbohydrate metabolism were the most abundant functional features in our shotgun metagenomic dataset. Notably, the samples from K had a significantly lower proportion than the other centers (Kruskal-Wallis, K:G $q = 0.005$, K:L $q = 0{,}026$). Upon further examination, particularly genes involved in the metabolism of monosaccharides were significantly lower in K than in L (Kruskal-Wallis, $q < 0.001$) (Fig. 4a). The significantly lower availability of monosaccharides in K was confirmed by a representative, metabolomic-based quantitative assessment of glucose and fructose. However, an overall increase of both compounds was observed in all centers over time (Suppl. Fig 4f, g). Similarly, genes involved in metabolism of di- and oligosaccharides were reduced in K samples, but not significantly (Fig. 4a). In contrast, the gene proportion involved in polysaccharide metabolism was found to be significantly increased in K samples (Kuskal–Wallis-test, K:G $q < 0.001$, K:L $q < 0.001$) (Fig. 4a), indicating a higher genetic potential for the metabolism of complex sugars in K.

To answer the question regarding the genetic potential for complex HMO degradation, we searched for HMO gene clusters in the obtained MAGs and contigs. Indeed, the potential for HMO metabolism was notably higher in samples from K (Fig. 5d). The total hits with HMO gene clusters were G: 45, K: 307, L: 0, indicating a seven-fold higher numbers of HMO genes in K than in G. Moreover, only one MAG from G17 possessed the potential to convert and digest HMOs, in contrast to all infants from K with at least one MAG (Fig. 5d).

Using metabolomics, we assessed HMOs in the preterm stool samples. A total of 13 HMOs were detected measuring 111 stool samples at tp1, tp3 and tp7 (Fig. 4b). For most fucosylated HMOs (2′-fucosyllactose [2′FL], 3′fucosyllactose [3′FL], lacto-N-ducopentaoise I [LNFP1], lacto-N-fucopentaose III [LNFP3], lacto-N-difuco-hexaose [LNDFH], lactodifucotetraose [LDFT]) as well as LS-tetrasaccharide a [LSTa] and lacto-N-tetraose [LNT], at time point 7, a significantly decreased amount for K samples was detected as compared to G and L (Kruskal-Wallis; 2′FL, K:G $q = 0.091$, K:L $q = 0.033$; 3′FL, K:G $q = 0.062$, K:L $q = 0.020$; LDFP3, K:G $q = 0.003$, K:L $q = 0.009$; LNDFH, K:G $q = 0.059$, K:L $q = 0.003$; LDFT, K:G $q = 0.091$, K:L $q = 0.003$; LSTa, K:G $q = 0.003$, K:L $q = 0.003$; LSTb, K:G $q = 0.062$, K:L $q = 0.020$) (Fig. 4ci). For syalisated HMOs (such as 3′-sialyllactose [3′SL], 6′-sialyllactose [6′SL], LS-tetrasaccharide b [LSTb], LS-tetrasaccharide c [LSTc], but also lacto-N-*neo*tetraose [LNnT]), few differences between centers or time points were observed (Fig. 4cii).

We conclude that the K microbiomes have the largest potential to degrade HMOs effectively; however, this process is impaired by the lowered availability of HMOs by preferred formula feeding.

Regimens and their key taxa correlate with crucial metabolites, antibiotic resistance genes, and virulence factors

We were interested in the correlations among the six microbial key players with HMOs, carbohydrates, amino acids and short-chain fatty acids, as measured using metabolomics (Fig. 5a) (additional metabolites are shown in Suppl. Fig. 5). *Bifidobacterium* (K) showed positive correlations with several carbohydrates (galactose and fructose), and weaker ones, with certain amino acids and short-chain fatty acids. It appears under HMO-depleted conditions, *Bifidobacterium* uses galactose and fructose as alternative substrates for its carbohydrate metabolism, producing formate and acetate. In the absence of *Bifidobacterium* (G and L), the role of formate and acetate production is taken over by *Enterococcus*, indicating its importance for early SCFA production, probably from citrate or pyruvate[35]. *Staphylococcus* showed an inconsistent pattern across the centers, supporting our hypothesis that this microorganism played a smaller role for the preterm GIT. The metabolic pattern of *Lactobacillus* (G, K) could not be clearly resolved, as lactate was not included in the metabolomic approach.

The antibiotic gentamicin (used in G and L; Table 1) has a broad spectrum of activity including: *Enterobacter, Escherichia, Proteus, Klebsiella, Pseudomonas, Serratia* and *Staphylococcus*[36]. Surprisingly, the influence of gentamicin on these genera was not evident in the preterm microbiome samples, and only limited perturbations were observed. However, it should be noted that, in this study, gentamicin was administered enterally and not intravenously, which may result in an altered mode of action. Acquired resistance to the gentamicin administered was rarely detected.

Overall, clear differences were observed in the antibiotic resistance (AMR) profiles between the key genera (Fig. 5b) and centers (Fig. 5c). AMR potential of the MAGs was substantially reduced in K samples as compared to samples from G and L (G: 93 hits, K: 35 hits, L: 123 hits), once again highlighting the influence of *Bifidobacterium* on

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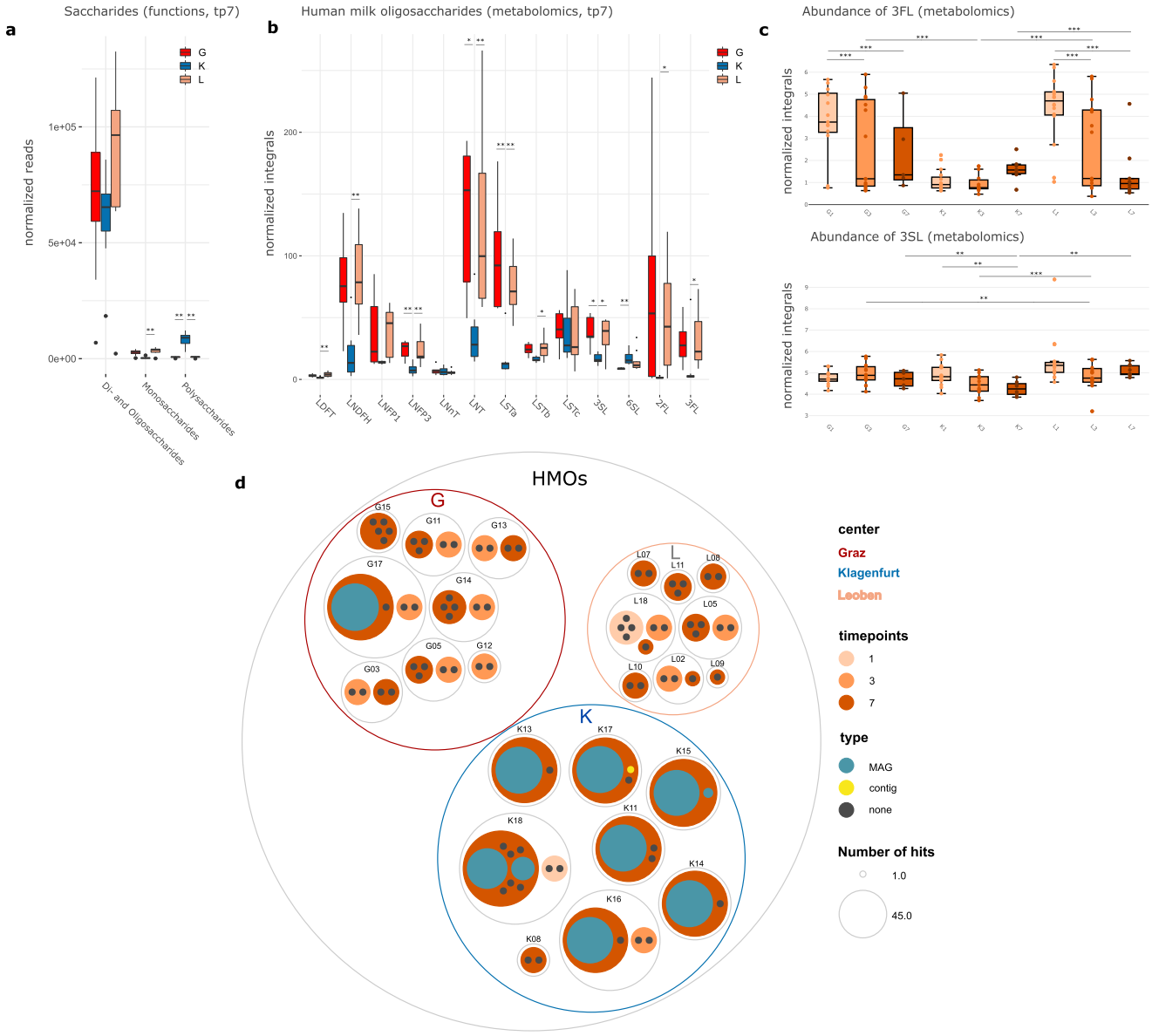

Fig. 4 | Distribution patterns of sugars and HMOs. a Differentially abundant functions (metagenomic dataset) associated with saccharides (di- and oligosaccharides, monosaccharides and polysaccharides) between the centers, tp7, $n = 23$; Monosaccharides: K:G $q < 0.001$, K:L $q < 0.001$, G:L $q = 0.949$; Di- and Oligosaccharides: K:G $q = 0.057$, K:L $q < 0.001$, G:L $q = 0.949$; Polysaccharides: K:G $q < 0.001$, K:L $q < 0.001$, G:L $q = 0.949$; **b** differentially abundant Human Milk Oligosaccharides (HMOs) between centers (metabolomic dataset, tp7, $n = 23$ biologically independent samples) LDFT: K:G $q = 0.091$, K:L $q = 0.003$, G:L $q = 1$; LNDFH: K:G $q = 0.059$, K:L $q = 0.003$, G:L $q = 1$; LNFP1: K:G $q = 1$, K:L $q = 1$, G:L $q = 1$; LNFP3: K:G $q = 0.003$, K:L $q = 0.009$, G:L $q = 1$; LNnT: K:G $q = 1$, K:L $q = 1$, G:L $q = 1$; LNT: K:G $q = 0.016$, K:L $q = 0.005$, G:L $q = 1$; LSTa: K:G $q = 0.003$, K:L $q = 0.003$, G:L $q = 1$; LSTb: K:G $q = 0.062$, K:L $q = 0.020$, G:L $q = 1$; LSTc: K:G $q = 1$, K:L $q = 1$, G:L $q = 1$; 3′SL: K:G $q = 0.019$, K:L $q = 0.018$, G:L $q = 1$; 6′SL: K:G $q = 0.003$, K:L $q = 0.413$, G:L $q = 0.088$; 2′FL: K:G $q = 0.091$, K:L $q = 0.033$, G:L $q = 1$; 3′FL: K:G $q = 0.062$, K:L $q = 0.020$, G:L $q = 1$; **c** differential abundance of the HMOs 3′-fucosyllactose (3′FL) and 3′-sialyllactose (3′SL) between centers at tp1, tp3 and tp7; $n = 109$; 3′FL: K1:K3 $q = 1.000$; K1:K7 $q = 1.000$; K3:K7 $q = 1.000$; G1:G3 $q < 0.001$; G1:G7 $q = 0.010$; G3:G7 $q = 1.000$; L1:L3 $q < 0.001$; L1:L7 $q < 0.001$; L3:L7 $q = 1.000$; G1:K1 $q = 1.000$; G3:K3 $q = 0.002$; G7:K7 $q = 1.000$; L1:K1 $q = 1.000$; L3:K3 $q = 0.002$; L7:K7 $q = 1.000$; G1:L1 $q = 1.000$; G3:L3 $q = 1.000$; G7:L7 $q = 1.000$; 3′SL: K1:K3 $q = 0.207$; K1:K7 $q = 0.021$; K3:K7 $q = 1.000$; G1:G3 $q = 1.000$; G1:G7 $q = 1.000$; G3:G7 $q = 0.830$; L1:L3 $q = 1.000$; L1:L7 $q = 1.000$; L3:L7 $q = 1.000$; G1:K1 $q = 1.000$; G3:K3 $q = 1.000$; G7:K7 $q = 0.011$; L1:K1 $q = 1.000$; L3:K3 $q < 0.001$; L7:K7 $q = 0.002$; G1:L1 $q = 1.000$; G3:L3 $q = 0.011$; G7:L7 $q = 1.000$; **d** circle packing plot displaying the numbers of hits for HMO gene clusters found in MAGs (metagenome assembled genomes) and contigs at the different time points. Each circle represents one infant. Colours of the dots indicate where the hit occurred, on MAGs (green) or if it could be only assigned to a contig (yellow) or none (grey); the size of the dots indicate the number of hits. G in red, K in blue, L in green; significance levels are indicated with asterisks for $q < 0.001$ (***), $q < 0.01$ (**), $q < 0.05$ (*) for two-sided t-test, corrected for multiple comparisons with Bonferroni. Centers are abbreviated by G (Graz), L (Leoben), and K (Klagenfurt). For boxplots, the upper, middle, and lower horizontal lines of the box represent the upper, median, and lower quartile; their whiskers depict the smallest or largest values within 1.5-fold of the interquartile range. Source data are provided as a Source Data file (see Github repository).

microbiome composition and function. Of the AMR signatures detected, most were positively correlated with the potential NEC/sepsis pathogens *Enterococcus*, *Staphylococcus* and *Escherichia*, including resistance against β-lactams and erythromycin (Fig. 5b). In contrast, AMR profiles were highly limited in the probiotic genera *Bifidobacterium* and *Lactobacillus*, underscoring the safety of probiotic administration. *Geobacillus* was also found to not encode AMR genes; therefore, it did not appear to pose an increased health risk.

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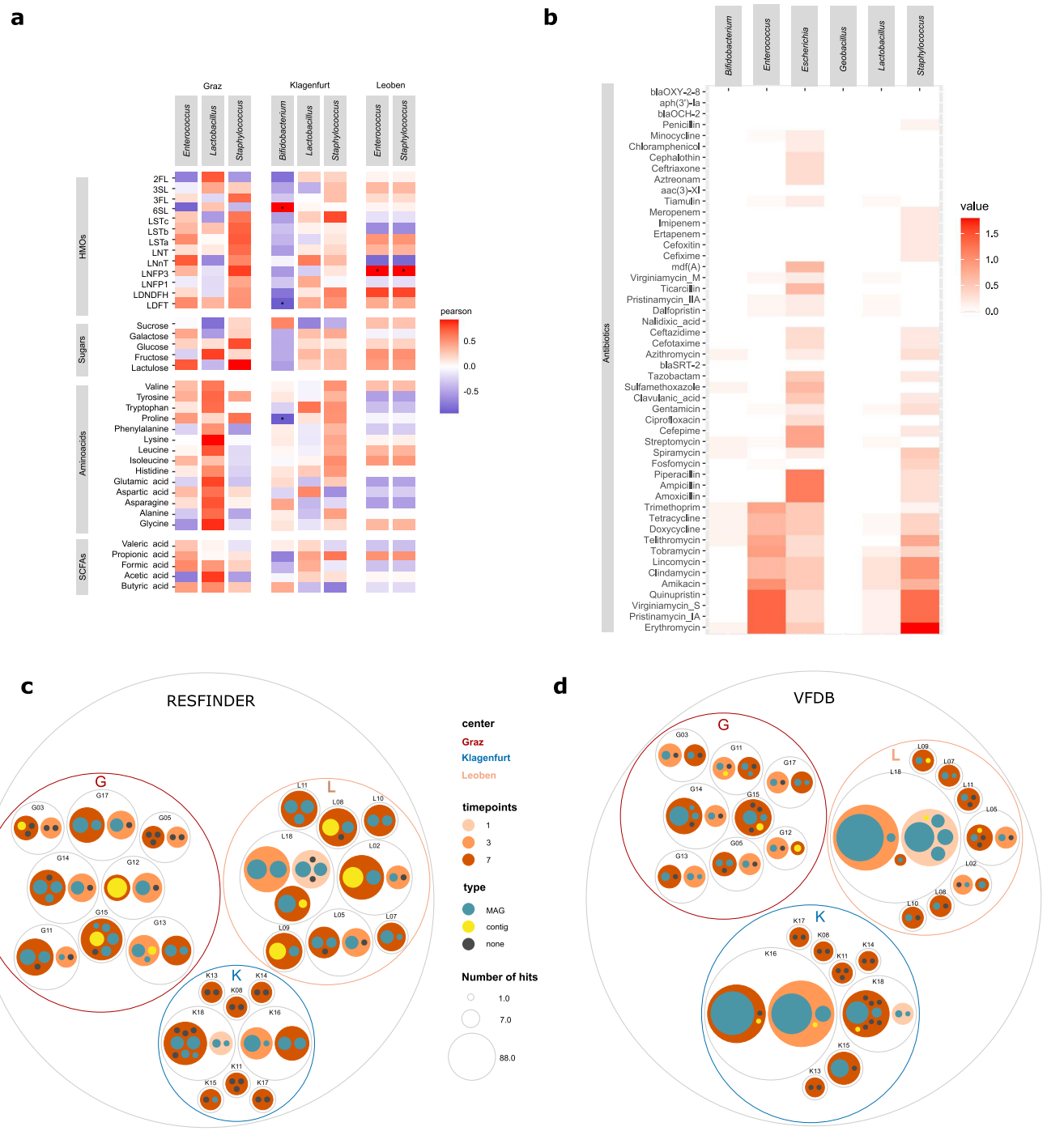

Fig. 5 | Results of taxonomic correlation analyses and distribution patterns of metabolites, antibiotic resistances and virulence factors. a Metabolites measured with NMR correlated with bacterial key genera in the three centers; metabolites of the groups of human milk oligosaccharides (HMOs), sugars, amino acids and short-chain fatty acids (SCFAs); **b** correlation of antibiotic resistance genes with bacterial key genera. Circle packing plot of **c** resistance genes and **d** virulence factors in the centers. Each circle represents an infant and is split into the three MGS sequenced time points. Colours of the dots indicate where the hit occurred, on MAGs (metagenome assembled genome) (green) or if it could be only assigned to a contig (yellow) or none (grey); the size of the dots indicates the number of hits. Significance levels are indicated with asterisks for $q < 0.001$ (***), $q < 0.01$ (**), $q < 0.05$ (*) by Pearson corrected for multiple testing by Benjamini Hochberg. Centers are abbreviated by G (Graz), L (Leoben), and K (Klagenfurt). Source data are provided as a Source Data file (see Github repository).

The virulence factor analysis results show that K infants had fewer pathogenic factors in their microbiomes than G and L infants (G: 64 hits, K: 193 hits, L: 173 hits) (Fig. 5d). Enrichment in *E. coli*-associated virulence traits was also observed in two infants in K and L (K16 and L18). Only six out of 431 hits were observed for *Staphylococcus* in six infants. We can assume that *Staphylococcus* is a key player in the context of its abundance, but not in the context of either replication or virulence factors. As expected, no virulence factors were found for the other key players, including *Bifidobacterium*, *Lactobacillus*, and *Geobacillus*.

Discussion

NEC prophylaxis and therapy have become a central aspect in the clinical management of VLBW preterm infants. Although probiotics,

human milk, and antibiotics are used by many NICUs to prevent NEC, to our knowledge, an in-depth, systematic comparison of different preventive regimens had not yet been performed. In this study, we show that NEC prophylaxis not only impacts the bacterial gut microbiome composition and function strongly, but also drives strong center-specific patterns observed in the fungal, archaeal and viral parts of the microbiome. This is particularly important with respect to archaea and phages/viruses, as NEC prophylaxes are not directly administered to change this part of the microbiome.

Our iRep analyses and the steady increase in relative abundance (Fig. 2b) suggest that *B. infantis* NCDO 2203 might be actively replicating and thus colonizing the GIT during the administration period. This could be supported by the presence of HMO gene clusters and low levels of HMOs in the stool. Nevertheless, iRep is no sufficient approach to conclude on colonization potential. Although *Bifidobacterium* does not yet appear to naturally colonize the GIT of preterm infants in large numbers, the reliable health benefits of supplemented *Bifidobacterium* have been demonstrated in multiple ways[37], including the negative correlation between *Bifidobacterium* and opportunistic pathogens[38].

Unlike bifidobacteria, a variety of lactobacilli, including *L. acidophilus* NCDO 1748 (administered in center K) and especially *L. rhamnosus* LCR 35 (administered in G), have been observed as natural colonizers of the GIT of preterm infants. In this study, we consider human milk (HM) to be the most likely source, as HM naturally contains large amounts of live lactobacilli[39]. For example, *L. rhamnosus* was successfully isolated from 8.13% of all human milk samples examined by Lubiech et al.[29] and was also detected in culture-independent assays alongside other, more frequently occurring *Lactobacillus* species (*L. salivarius, L. fermenting, L. gasseri*[17,29,40]). It should be noted, however, that the HM was pasteurized, especially if it came from a milk bank, and pasteurization may obviously reduce the chance that live lactobacilli are transmitted. As all except one infant in the metagenomic subset were born via C-section and not vaginally, vertical transfer from the maternal vaginal microbiome is untenable in this case.

The dynamics of naturally occurring and supplemented (non-natural) probiotic strains are interesting to study, as supplemented bacteria have to "invade" an ecosystem that is evolving to provide colonization resistance against other, potentially pathogenic bacteria. To avoid disrupting this process, and also considering that naturally occurring bacterial residents are likely to persist over a longer period of time[41], an ecologically oriented probiotic choice would tend to include supplementation with *L. rhamnosus* LCR 35 or another beneficial preterm *Lactobacillus* strain.

However, we and others observed that the co-administration of *B. infantis* and *L. acidophilus* in equal amounts results in the overgrowth and predominance of *B. infantis* over *L. acidophilus*[6,42]. The absence of replicating *Lactobacillus* MAGs in the presence of replicating *Bifidobacterium* MAGs leads us to hypothesize that *L. acidophilus* NCDO 1748 cannot successfully colonize the GIT of preterm infants when *B. infantis* NCDO 2203 is co-administered. On the other hand, it may be that *Lactobacillus* plays a pioneering role in anaerobic bifidobacterial colonization by removing oxygen from the GIT[43]. This supporting role of *Lactobacillus* tends to underscore the benefits of taking a multi-species probiotic approach.

Most importantly, administration of *L. rhamnosus* LCR 35 alone had no appreciable effect on the composition or function of the gut microbiome and did not result in a substantial increase in *Lactobacillus* colonization as seen before[44]. Potentially pathogenic bacteria and antibiotic resistance genes were also not substantially reduced when *L. rhamnosus* LCR 35 was administered alone.

We argue that the GIT of VLBW infants per se is not a "normal" natural habitat, and the action of *Lactobacillus* may be too mild or too slow to rapidly support the establishment of a healthy microbiome. In contrast, *B. infantis* NCDO 2203, although not naturally occurring, seems to be a strong, stable, and reliable keystone microbe of the nearly empty niche of the preterm GIT, overgrowing pathogenic threats, and microorganisms carrying antibiotic resistance genes.

Moreover, *Bifidobacterium* together with *Bacteroides* has been described as an effective converter of HMOs[16,45], producing substantial amounts of beneficial SCFAs[46]. The efficient conversion of HMOs and thus SCFA production is an extremely important process for premature infants, which is underscored by the finding that concentrations of HMOs in HM are substantially increased when the infant is born prematurely[29,47]. In fact, the bifidobacteria found in the faeces of K infants exhibited a marked genetic ability to convert HMOs. This could be related to changes in the GIT environment such as lower pH or improved colonization resistance.

This capacity, however, could not be observed in our metabolomic analyses because the K infants were fed with (HMO-lacking) FM. In contrast, in centers L and G, where HMOs were administered in the natural form of HM, the small natural proportion of microbial HMO converters was too low for observable efficient turnover. Consequently, only the simultaneous administration of HMOs and HMO-converters would result in optimal utilization of the health benefits for the infants. This highlights the importance of combining the right probiotic with the right diet.

Furthermore, clinically relevant findings from our study are related to enteral antibiotic administration, as gentamicin was administered in two NICUs, G and L. Indeed, the prophylactic enteral, but not parenteral, administration of antibiotics has been shown to substantially decrease NEC rates[12,48], which is also reflected in the low number of NEC cases observed in center G[14]. To date, no single causative microbial agent of NEC has been described; thus, the antibiotics used, such as gentamicin, must cover a broad spectrum. Our study was not designed to investigate the performance and efficiency of gentamicin in eliminating specific bacteria. However, we did not find consistent negative correlations between gentamicin administration with certain taxa or the occurrence of gentamicin resistances in the microbiome at tp7. Nevertheless, we cannot rule out a negative effect on concomitantly administered probiotic bacteria.

In several studies, intravenous antibiotic administration at a young age has been associated with adverse health outcomes later in life[49–51]. It is also proposed that antibiotic prophylaxis does not reduce NEC incidences but may rather increase the risk for high-risk premature infants of NEC[5]. Nevertheless, in these studies, antibiotics were administered enteral, not intravenously, which needs to be evaluated strictly differently. Still, the prophylactic use of antibiotics must always be weighed against the potential risk. On the one hand, prophylactic administration of antibiotics probably minimizes the outbreak of pathogenic bacteria, especially since infections in premature infants develop alarmingly rapidly, and the success of treatment is time critical. On the other hand, antibiotics could also suppress the growth of beneficial (probiotic) bacteria, which is also underlined by our study results showing that probiotic *Lactobacillus* and *Bifidobacterium* carry few AMR genes and are therefore more susceptible for antibiotics. The long-term effects of antibiotic administration at such an early, vulnerable age are difficult to predict. In general, AMR is a global threat[52] and their horizontal gene transfer to pathogenic bacteria might also be implicated in NEC.

Our study has several strengths and limitations. Overall, due to the extensive analysis performed, the study cohort was kept rather small, and the survey period was limited to the first weeks of life. Unfortunately, the early (meconium) samples could not be used for metagenomic analyses because of their exceptionally low microbial biomass, so we had to focus on tp7 for detailed functional assessments. We could not draw any conclusions about the colonization potential of the probiotically administered strains, as iRep is no sufficient tool to prove replication or colonization. Due to the study design, we cannot discern

which factor (antibiotics, probiotics, nutrition) is driving which result, as more than one of those factors changes between the centers. However, we successfully and comprehensively conducted a multicenter study in which we analysed the longitudinal composition of the microbiome of 55 VLBW preterm infants using amplicon-based and metagenomic sequencing, which also enabled us to elucidate the contribution of archaea, fungi, and phages. A large wealth of taxonomic and functional data were obtained, and analyses revealed the HMO conversion potential and the emergence of antibiotic resistance. In addition, genetically detected functions could be effectively combined with well-found metabolomic analyses.

Our study provides a solid basis for further evaluation and analyses. We found that the combination of feeding HM and administering *B. infantis* NCDO 2203 during the first weeks of life in VLBW infants could be a promising synergistic approach. Overall, all treatment regimens analysed in this study resulted in NEC rates well below the global average, confirming the very successful and strategic management of this devastating disease in our NICUs.

Methods

Study design

We conducted a prospective, triple-center cohort pilot study investigating the gut microbiome of preterm infants with a birthweight <1500 g in three Austrian neonatal intensive care units (NICUs). These centers (Klagenfurt, K; Leoben, L; Graz, G) used different regimens for NEC prophylaxis which are summarized in Table 1 and have been described in detail previously[53,21]. A detailed description of the study design is available in[53] and first results have already been published elsewhere[21].

In G, prophylaxis consisted of administration of probiotic *Lactobacillus rhamnosus* LCR 35 twice a day, nystatin, and enteral gentamicin. Probiotic bacterial species were also administered in K, namely *Bifidobacterium longum* subsp. *infantis* NCDO 2203 and *Lactobacillus acidophilus* NCDO 1748 in combination with fluconazole. In center L, no probiotic species, but enteral gentamicin and nystatin were used. Next to medication and probiotic supplementation, the feeding regimen also differed between the centers. In G and L, mainly human milk (HM) was provided. In K, enteral nutrition consisted mainly of formula milk (FM). The feeding history for each infant and time point is shown in Suppl. Fig. 1.

Between October 2015 and March 2017, stool samples were collected from preterm infants at those three centers. Inclusion criteria were birth weight <1500 g and survival in the first three weeks of life. Clinical data on the infants have been published recently[21], with no significant differences between the centers (APGAR, sex, gestational age, gestational weight), except length of hospital stay (G: 72, K: 68.5, L:58; $p = 0.04$). In case of genetic diseases, syndromes or congenital anomalies or meconium ileus, infants were excluded from the study. A total of 55 infants were included in the study (male = 33, female = 22; age 0–3 weeks). The infants' stool samples were collected every other day from meconium for the first two weeks of life, with each infant providing stool samples at seven time points (time points of samples were slightly variable (see Suppl. Table 2) due to the varying availability of fecal samples; average sampling time points were tp1: within day 1-3; tp2: day 4; tp3: day 6; tp4: day 8; tp5: day 10; tp6: day 12; tp7: day 15). A total of 383 samples and 16 negative controls were collected.

The diagnostic criteria for NEC definition were the same in all three centers and followed the AWMF guideline with Bell criteria with modifications of Walsh. NEC incidence rates are 2.2% in K, 2.7% in G, and 4.6% in L[21]. The study is registered wtihin German Registry of Clinical Trials No.: DRKS00009290 and received ethical approval from the local ethic committees (number 27-366 ex14/15) and written informed consent was obtained from the parents of the infants and participants were not compensated.

Sample processing

DNA extraction, sequencing, and metabolomics. Samples were processed as described in detail earlier[21]. In short, genomic DNA was isolated according to manufacturer's instructions using the MagnaPure LC DNA Isolation Kit III (Roche).

Targeted amplicon sequencing was performed for three different regions: one using universal primers but mainly targeting bacterial V4 16S rRNA gene sequences (515F/R926, 5′GTGYCAGCMGCCGCGGTAA3′/5′AGCCGYCAATTYMTTTRAGTTT3′), the other aimed for optimized amplification of archaeal 16S rRNA gene sequences in a nested PCR (PCR1 344F/1041R, 5′ACGGGGYGCAGCAGGCGCGA3′/5′GGCCATGCACCWCCTCTC3′; PCR2 519F/806R, 5′CAGCMGCCGCGGTAA3′/5′GGACTACVSGGGTATCTAAT3′) and the third of the ITS region of fungi (ITS86F/ITS4R, 5′GTGAATCATCGAATCTTTGAA3′/5′TCCTCCGCTTATTGATATGC3′)[54,55]. See ref. [56] for detailed primer sequences and PCR protocols. PCRs were run in triplicates and pooled subsequently. No human DNA sequence depletion, enrichment of microbial or viral DNA, or mRNA was performed. Library preparation and sequencing of the amplicons were carried out at the Core Facility Molecular Biology of the Center for Medical Research at the Medical University Graz, Austria. Sequencing was performed in paired-end run mode on an Illumina MiSeq with v3 600 chemistry and 300 bp read length[57]. Raw reads are publicly available at the European Nucleotide Archive PRJEB37883. Raw reads were processed using Qiime2 v2019.1 to v2021.2[58]. Briefly, reads that were first quality filtered with DADA2 v2019.1.0 to v2021.2.0[59] were then denoised into Amplicon Sequence Variants (ASVs). The taxonomy was assigned with a Naive-Bayes classifier based on SILVA 132 for bacterial and archaeal signatures[60,61]. Potential contaminant ASVs were removed considering the sequenced negative controls with the R package decontam v3.9 in prevalence mode, isContaminant setting, and threshold 0.5[62], (https://github.com/benjjneb/decontam/). Subsequently negative controls as well as signatures of chloroplasts and mitochondria were removed manually. As no quantification experiments were applied, relative abundance methods were applied.

The genus *Lactobacillus* has recently been taxonomically restructured[63], in which the genus was divided into 25 separate genera. In this study, we continue to refer to the amended nomenclature and dimension of the genus *Lactobacillus*, as this work is a supplement to the previously published amplicon data of this study, and we prefer to be consistent between those two publications. In addition, the taxonomic assignment of the datasets on which all other analyses are was performed using SILVA 132[60] prior to the renaming event. In this publication, we mention only two representatives of the original *Lactobacillus* genus, namely *Lactobacillus rhamnosus* (now: *Lacticaseibacillus rhamnosus*) and *Lactobacillus acidophilus*, the latter remaining unchanged. It shall be noted, that for the important probiotic representatives of the amended *Lactobacillus* genus, the new names also begin with "L" and the abbreviations of the "*L.*" genus may continue to be used[63].

An initial insight into the bacteriome of the analyzed 55 infants, based on 16S rRNA gene amplicons was provided earlier[21]. In this subsequent data analysis, we intensify the analytical assessments and include shotgun metagenomics and functional metabolomics, to substantially deepen the understanding of the development within the first weeks. Further, we include information on the archaeal, fungal, and viral part of the gut microbiome as well as on their function.

We performed shotgun metagenomic sequencing of a subset of infants for three time points (tp1, tp3, tp7). Sequencing libraries were generated with the Nextera XT Library construction kit (Illumina, Eindhoven, the Netherlands) and sequenced on an Illumina HiSeq (Illumina, Eindhoven, the Netherlands; Macrogen, Seoul, South Korea). The raw reads were quality assessed with fastqc v0.11.8[64] and filtered accordingly with trimmomatic v0.38[65] with a minimal length of 50 bp and a Phred quality score of 20 in a sliding window of 5 bp.

Both probiotics used in this study ("Antibiophilus", containing *Lactobacillus rhamnosus* LCR 35, and "Infloran", containing *Bifidobacterium longum* subsp. *infantis* NCDO2203 and *Lactobacillus acidophilus* NCDO1748) are pharmaceuticals according the Austrian regulations, and as such, their quality is strictly regulated and controlled. Therefore, we proceed on the assumption, that the probiotics contain the labeled strain purely and constant over time. However, to confirm the presence of the signatures of the probiotics in the stool of the infants, we compared their genomic information with our amplicon and metagenomic data. Therefore, amplicon sequences of interest were blasted against the respective 16S rRNA genes of the lactobacilli (*L. rhamnosus* LCR35, accession: EU184020; *L. acidophilus* NCDO1748, accession: ATCC4356), indicating a 100% identity for both. As metagenomic MAGs were available for *Bifidobacterium* from Klagenfurt samples, we used genomic information for comparison, showing 99.97% to 100% similarity (FastANI, *B. longum* subsp. *infantis* NCDO2203, accession: ATCC15696). The results are listed in Suppl. Tables 2a and 2b and on our Github repository (https://github.com/CharlotteJNeumann/preterm_shared)[66].

Samples from time points tp1 and tp3 yielded only small amounts of DNA, so that library preparation or post-sequencing quality filtering failed for most samples. Thus, we focus mainly on data from tp7 for analysis and interpretation.

The obtained reads were analyzed both in a genome- as well as gene-centric way. For the gene-centric approach, data were annotated with diamond v0.9.25[67] using blastx search against NCBInr database from 2019-07-19 and analyzed in the open-submission data platform MG-RAST according to the manual using default settings[68], on taxonomic and functional (SEED subsystems[69]) level.

For the genome-centric analysis the reads were co-assembled with Megahit v1.1.3[70] by using the default setting "meta-sensitive" into contigs which were then binned with MaxBin2 v2.2.4[71]. Potential chimers and contaminations of representative dereplicated MAGs were detected with Genome UNClutterer (GUNC v. 1.0.1)[72] using the default diamond GUNC database 2.0.4, its sensitive mode and a detailed output till species level. Genome chimerism was visualized as interactive html plots for each MAG. Finally, all outputs from GUNC were merged with those from checkM v.1.1.0[73] and all models from checkM2 v.0.1.3[74]. The bins were then de-replicated with dRep v2.0.5[75] to generate a list of representative metagenome assembled genomes (MAGs). Taxonomic classification of those MAGs was performed with GTDB-Tk v1.5.1. Replication rates were determined with iRep v1.1[76]. Indices for MAGs with the following default parameters were included: ≥75% completeness; ≤175 fragments/Mbp sequence; ≤2% contamination; ≥5 kbp scaffold length; min cov = 5; min wins = 0.98; min $r^2 = 0.9$; GC correction min $r^2 = 0.0$.

A subset of 111 stool samples for tp1, tp3 and tp7 from all three centers (corresponding to metagenomic sequencing) were analyzed with untargeted NMR (nuclear magnetic resonance spectroscopy) for several metabolites in house as described previously[77]. In short, methanol water was added to the samples, cells were lysed, lyophilized, and mixed with NMR buffer. NMR was performed on an AVANCE™ Neo Bruker Ultrashield Plus 600 MHz spectrometer equipped with a TXI probe head at 310 K and processed as described elsewhere[78].

Data analysis, statistics and visualization

Multiple analyses were performed in R v4[79] using the Microbiome Explorer package[80] using CSS normalization and DESeq2 for differentially abundance analyses: microbial composition analysis and visualization as stacked bar charts and correlation analysis of abundance of specific bacterial genera with their phages. Differential abundance was plotted in R[79] using the ggplot2 package[81] and asterisks refer to DESeq2 *q*-values (*q*-values <0.05 (*), <0.01 (**) and <0.001 (***)) which are available in our Github repository[66]. A pie chart for the *Lactobacillus* genus was created using Krona charts[82]. The BioEnv Biplot for bacterial dissimilarity of the groups was created using the vegan package[83] in R. Significance of differential abundance was calculated in SPSS v27[84] using a Kruskal-Wallis with Bonferroni correction for multiple comparisons and evaluating significance with *q*-values <0.05 (*), <0.01 (**) and <0.001 (***).

The network was created by using SparCC[85,86] within the SCNIC tool (Sparse Cooccurrence Network Investigation for Compositional data)[87,88] to calculate co-occurences from CSS normalized metagenomic observations. Apart from default settings we used 10 bootstraps to calculate p-values for the SparCC R value, filtered the dataset with activated –sparcc_filter parameter and used the recommended minimum correlation value of 0.35 to determine edges. Calculated correlations and networks were then visualized in Cytoscape v.3.9.1 in an edge-weighted spring embedded network where nodes represent taxa and edges positive and negative co-occurences according to the SparCC R values.

Permanova was used for analyzing biological and technical variations of the data. For that, the amplicon dataset was CSS normalized (RSV level), before feeding into the R-script provided by Lahti, Shetty et al.: https://microbiome.github.io/tutorials/PERMANOVA.html (microbiome::transform: "compositional", permutations=999, method= bray). Input files (all samples, tp3 samples, tp7 samples, and metadata), including script and output, are provided in the Github repository (https://github.com/CharlotteJNeumann/preterm_shared)[66].

Metabolite correlation with taxonomic information

Metabolites measured by NMR were then correlated with CLR transformed relative abundance of genera of amplicon sequencing in R[79]. As the centers differed greatly in terms of species present, this analysis was performed separately for each center. Therefore, amplicon data for each center were normalized with bestNormalized[89] and then correlated with Pearson. The list of normalizations bestNormalize chose is available in our Github repository[66]. The analysis was plotted in a heatmap using ggplot2[81]. For each center, only the genera with the highest abundance and differentially abundance representing the six key players were selected based on abundance and DESeq2 ($p < 0.001$).

Antibiotic resistance genes counts and virulence factors

MAGs and contigs were aligned against several databases in abricate (Seemann T, Abricate, Github https://github.com/tseemann/abricate) with options mincov = 70 and minid = 70, including HMO gene clusters sequences[90], EcOH[91], VFDB[92], and Resfinder[93]. To correlate antibiotic resistance gene patterns with specific genera, the taxonomy of the MAGs was assigned by GTDB-Tk[94]. The number of hits per infant and per time point is shown in a circle packing plot created with rawgraphs[95]. Data were analyzed on genus level whereas features were only depicted when the genus was represented by more than one MAG. Correlation of antibiotic resistance gene patterns with the six key species were visualized in a heatmap with R[79].

All graphs were combined and assimilated in Inkscape v1.1 (URL: https://inkscape.org/en/ RRID:SCR_014479) to obtain a uniform appearance.

Statistics & reproducibility

We conducted a prospective, triple-center cohort pilot study. As it was a pilot study, sample size was not pre-determined beforehand. Randomization and blinding of the investigators was not foreseen in the chosen study set-up, as all hospitals use different regimens and this protocol was not changed. A full study flow chart is provided in Suppl. Fig. 6. No data were excluded from the analyses. Overall, the study is considered to be only partially reproducible, as the data are dependent on the study cohort, which was only sampled once within this study, and sampling of cohorts in the same time-window cannot be repeated. However, starting from the raw sequencing data, the analysis is fully reproducible and all required data, scripts, and details are provided.

Statistics mostly focus on single time points, mostly tp7; longitudinal statistics was not performed.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The raw sequencing reads generated in this study have been deposited in the European Nucleotide Archive Database under accession code PRJEB37883. ASV-tables, sequences of MAGs and metabolomic, as well as metabolomic data and used scripts are openly available and shared via Github (https://github.com/CharlotteJNeumann/preterm_shared)[66]. All files used for the figures are listed in the Source Data File, which is also provided at Github ("list_raw_data_figures.xlsx").

Raw NMR data have been deposited in Metabolites under accession code MTBLS6866. Clinically-relevant, anonymized information on each sample (sex, hospital, birth mode, nutrition, medication etc.) is provided in the metadata table located along with the respective ASV tables in the open Github repository. Source data are provided in the open GitHub repository[66]. Source data are also provided with this paper.

Code availability

R scripts are openly available and shared via Github (https://github.com/CharlotteJNeumann/preterm_shared)[66].

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Competing interests

The authors declare no competing interests.

Inclusion and diversity statement

We worked to ensure sex balance in the selection of participants, as well as authors. We further actively worked to promote gender balance in our reference list.

Additional information

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Editor's Pick | Human Microbiome | Research Article

First-year dynamics of the anaerobic microbiome and archaeome in infants' oral and gastrointestinal systems

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ABSTRACT Recent research provides new insights into the early establishment of the infant gut microbiome, emphasizing the influence of breastfeeding on the development of gastrointestinal microbiomes. In our study, we longitudinally examined the taxonomic and functional dynamics of the oral and gastrointestinal tract (GIT) microbiomes of healthy infants (n = 30) in their first year, focusing on the often-over-looked aspects, the development of archaeal and anaerobic microbiomes. Breastfed (BF) infants exhibit a more defined transitional phase in their oral microbiome compared to non-breastfed (NBF) infants, marked by a decrease in *Streptococcus* and the emergence of anaerobic genera such as *Granulicatella*. This phase, characterized by increased alpha-diversity and significant changes in beta-diversity, occurs earlier in NBF infants (months 1–3) than in BF infants (months 4–6), suggesting that breastfeeding supports later, more defined microbiome maturation. We demonstrated the presence of archaea in the infant oral cavity and GIT microbiome from early infancy, with *Methanobrevibacter* being the predominant genus. Still, transient patterns show that no stable archaeome is formed. The GIT microbiome exhibited gradual development, with BF infants showing increased diversity and complexity between the third and eighth months, marked by anaerobic microbial networks. NBF infants showed complex microbial co-occurrence patterns from the start. These strong differences between BF and NBF infants' GIT microbiomes are less pronounced on functional levels than on taxonomic levels. Overall, the infant microbiome differentiates and stabilizes over the first year, with breastfeeding playing a crucial role in shaping anaerobic microbial networks and overall microbiome maturation.

IMPORTANCE The first year of life is a crucial period for establishing a healthy human microbiome. Our study analyses the role of archaea and obligate anaerobes in the development of the human oral and gut microbiome, with a specific focus on the impact of breastfeeding in this process. Our findings demonstrated that the oral and gut microbiomes of breastfed infants undergo distinct phases of increased dynamics within the first year of life. In contrast, the microbiomes of non-breastfed infants are more mature from the first month, leading to a steadier development without distinct transitional phases in the first year. Additionally, we found that archaeal signatures are present in infants under 1 year of age, but they do not form a stable archaeome. In contrast to this, we could track specific bacterial strains transitioning from oral to gut or persisting in the gut over time.

KEYWORDS gut microbiome, GIT, oral microbiome, infant development, early life, metagenomics, anaerobes, archaea, strain tracking, source tracking

The human microbiome is a complex ecosystem of microorganisms, undergoing substantial changes from birth to adulthood (1). Among the various microbiomes,

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the oral microbiome is one of the most complex microbiomes and comprises over 700 identified species (2, 3). The oral cavity is a primary entry point for the colonization of both oral and gastrointestinal tract (GIT), making it an accessible site for assessing microbial communities. The unique community of microbes in the oral cavity is in fact very important and any disruption in early oral colonization and the establishment of a healthy oral microbiome is linked with several oral diseases, including dental caries and periodontitis, which could start with the emergence of teeth (4), as well as increased susceptibility to systemic diseases such as cardiovascular disease, due to the presence of potential pro-inflammatory mediators present in periodontium (5).

The formation of the oral microbiome in early childhood is known to be influenced by both host and environmental factors, including genetics, delivery mode, antibiotic use during birth and early infancy feeding mode, and the characteristics of the parental oral microbiome (6). However, the process of initial acquisition and development of this complex microbiome during infancy is not fully understood.

The oral cavity is constantly exposed to oxygen on its surfaces, yet it contains numerous anoxic environments that provide habitats and favorable conditions for anaerobic metabolism and microbial growth. These include biofilms, dental pockets, subgingival crevices, and crypts of the tonsils (7). In general, facultatively anaerobic *Streptococcus* is the predominant early colonizer of the infants' oral cavity, favored by its ability to adhere to epithelial cells (8). By secreting extracellular polymers, it then paves the way for other microbes to emerge, such as *Actinomyces* species (9). The infants' oral microbiome is less diverse compared to adults but becomes more complex within the first month, with mainly *Streptococcus*, *Haemophilus*, *Neisseria*, and *Veillonella* colonizing (4, 8). Nevertheless, knowledge about colonization of non-bacterial microbial members in the oral cavity is very scarce (4).

The oral cavity and gut are connected by the continuous flow of ingested food and saliva through the GIT. Despite this connection, they host distinct microbial communities within their unique microenvironments. Research has shown that these sites harbor locally adapted strains specific to their environments (7, 10, 11), and this segregation is thought to be through various environments including gastric barrier and antimicrobial bile acids within the duodenum. However, little is known about the possible interaction and parallel development of the GIT and oral microbiomes (6, 12, 13). This is particularly true for the radical shifts in the GIT due to the oxygen depletion and the unknown interaction of both environments during this time.

The human GIT in fact harbors the most versatile microbial community. In the initial aerobic phase immediately after birth, the GIT is populated by obligately aerobic or facultatively anaerobic microbes which thrive in the presence of oxygen and are well-adapted to the aerobic environment of the newborn GIT (14–16). The shift to an anaerobic state is driven by oxygen depletion, caused by oxygen consumption by bacteria, colonocytes, or non-biological chemical processes in the cecal contents (17). This step is an essential step in GIT maturation. As oxygen levels decrease, strictly anaerobic bacteria thrive, especially as *Bifidobacterium* species begin to dominate the GIT microbiome. These microbes are adapted to a milk-based diet, using the "bifid shunt" pathway allowing for a fast growth at high lactose concentrations (18). Later, during weaning and introduction of complementary food, other microorganisms replace *Bifidobacterium* species as the dominant microbial group. Steward et al. (19) defined three distinct phases of microbiome progression: a developmental phase at months 3–14, a transitional phase at months 15–30, and a stable phase at months 31–46. These changes are influenced by numerous factors, including birth mode, gestational age, host genetics, environmental factors, and most importantly, feeding mode. The final maturation and stabilization of the GIT microbiome includes not only the settling of highly-oxygen sensitive bacteria, but also methanogenic archaea, which could be indicators for a mature microbiome situation (20).

Similar to fungi, archaea receive less attention regarding their role in the development of a healthy microbiome, although they are present in both the GIT and oral cavity,

often in substantial numbers (21, 22). Few studies have recently shown the detection of archaeal signatures in young infants (22, 23).

Herein, we conducted a longitudinal study on a birth cohort (TRAMIC, https://clinicaltrials.gov/study/NCT04140747) of 30 Austrian infants to investigate the dynamics of aerobic and anaerobic bacteria and archaea in the oral cavity and GIT. The cohort included 15 vaginally delivered infants and 15 born via C-section. Daily up to monthly monitoring of the infants' oral and GIT microbiomes was performed using shotgun metagenomic and amplicon sequencing. This allowed us to assess the development of aerobic and anaerobic microbiomes in parallel at both sites, correlating these patterns with birth mode and infant nutrition.

By elucidating the colonization patterns and ecological dynamics of obligate anaerobes and archaea in both oral and gut environments, this study aims to provide insights into a more fine-tuned early development of the infant microbiome. Understanding the factors shaping microbial colonization during infancy is fundamental for deciphering the role of the microbiome in the course of life and may lead to new strategies to promote infant health and well-being.

MATERIALS AND METHODS

Study design

A total of 32 mother-infant pairs were enrolled in the study during their prepartum visits to the Department of Gynecology at the state hospital Graz, Austria before delivery. These participants provided informed consent and obtained oral swabs and stool samples from their infants at various time intervals, commencing immediately after delivery. The primary objective of this pilot study was to investigate the anaerobic microbiome, with a specific focus on archaea, in the oral cavity and GIT of infants throughout their initial year of life.

Detailed inclusion and exclusion criteria can be found in our prior publication (24). In short, every pregnant woman included in the study was in good overall health had no tabacco or alcohol abuse, had not undergone antibiotic treatment within the past 6 months, and was 18 years of age or older. Additionally, their infants were required to be healthy, full-term singletons without any anomalies.

Metadata from all women and infants are listed in the GitHub Repository https://github.com/CharlotteJNeumann/InfantDevelopmentTRAMIC.

In sum, two women opted to discontinue their participation during the study, resulting in 30 infants successfully completing the sample collection phase. Among them, 15 infants were delivered via C-section, while the remaining 15 were born vaginally.

Sample collection and processing

Oral swabs and stool samples were gathered from all 30 infants at various time points. Stool samples were obtained by spooning the stool from the diaper, avoiding contact with the diaper whereas oral samples were collected by striking the buccal mucosa. Stool samples were collected three times during the initial days of life (S1 [first stool, Day 1], S2 [Days 2–3], and S3 [Days 3–5]). Oral samples were obtained two times during the first days of life (O1 [Day 1] and O2 [Days 3–5]). Both sample types were collected monthly until the infants reached their first birthday [Months 1 (M01) to 12 (M12)]. The collection was performed either by the study nurse at the hospital or by the mothers themselves, following clear instructions on proper collection and storage procedures.

Stool samples were obtained using sterile collection tubes, while oral samples were collected from the buccal mucosa of the cheek using FLOQSwabs (Copan, Milan, Italy). Subsequently, all samples were refrigerated, transported to the laboratory on ice and stored at −80°C until further processing.

Genomic DNA was extracted from the oral swabs utilizing the QIAamp DNA Mini Kit (QIAGEN) with slight modifications: 500 µL of Lysis Buffer (sterile filtered, 20 mM Tris-HCl

at pH 8, 2 mM Na-EDTA, and 1.2% Triton X-100) was added. To all samples, 50 µL of Lysozyme (10 mg/mL, Carl Roth) and 6 µL of Mutanolysin (25 KU/mL, Merck) were added, followed by an incubation at 37°C for 1 h. The resulting mixture was transferred to Lysing Matrix E tubes (MP Biomedicals) for mechanical lysis at 5,500 rpm for 30 s two times using the MagNA Lyser Instrument (Roche, Mannheim, Germany). Following mechanical lysis, the samples were centrifuged at 10,000 × *g* for 2 min to separate the beads from the supernatant. Subsequently, DNA extraction was performed according to the provided instructions, with the elution of DNA in 60 µL of Elution Buffer.

Stool samples were processed utilizing the QIAamp DNA Stool Mini Kit (QIAGEN) with slight modifications: approximately 200 mg of stool was combined with 500 µL Lysis Buffer (sterile filtered, 20 mM Tris-HCl pH 8, 2 mM Na-EDTA, and 1.2% Triton X-100) and homogenized. To the homogenized samples, 50 µL of Lysozyme (10 mg/mL, Carl Roth) and 6 µL of Mutanolysin (25 KU/mL, Merck) were added and incubated at 37°C for 1 h. Following the incubation, 500 µL Inhibitex was introduced to the samples, homogenized and transferred to Lysing Matrix E tubes (MP Biomedicals) for mechanical lysis at 6,500 rpm for 30 s two times using the MagNA Lyser Instrument (Roche, Mannheim, Germany). After mechanical lysis, the samples were incubated at 70°C for 5 min and then centrifuged for 10,000 × *g* for 3 min to segregate the beads from the supernatant. The resulting supernatant was then transferred to 2 mL Eppendorf tubes and the remaining steps of the DNA extraction were conducted following the kit protocol. The elution of DNA was carried out using 200 µL of Elution Buffer.

Throughout the DNA extraction procedure, negative controls and mock communities as positive controls were incorporated and processed concurrently.

PCR amplification

The genomic DNA was used to amplify the V4 region of the 16S rRNA gene employing Illumina-tagged primers, namely 515FB and 806RB (Table 1). To determine the archaeal communities, a nested PCR was performed using the primer combination 344F-1041R/519F-Illu806R, as described previously (25). PCR reactions were performed in triplicate in a final volume of 25 µL, containing TAKARA Ex Taq buffer with MgCl2 (10×; Takara Bio Inc., Tokyo, Japan), primers at 200 nM, dNTP mix at 200 µM, TAKARA Ex Taq Polymerase at 0.5 U, water (Lichrosolv; Merck, Darmstadt, Germany) and DNA template (1–2 µL of genomic DNA) and pooled after amplification. The specific conditions for PCR amplification are listed in Table 2.

Amplicon sequencing, bioinformatics, and statistical analysis

The library preparation and sequencing of amplicons were conducted at the Core Facility Molecular Biology, Center for Medical Research, Medical University of Graz, Graz, Austria. Briefly, DNA concentrations were normalized using a SequalPrep normalization plate (Invitrogen) and each sample was uniquely indexed through an eight-cycle index PCR with a unique barcode sequence. Following the pooling of these indexed samples, a gel cut was performed to purify the products from the index PCR. Sequencing was executed using the Illumina MiSeq device along with the MS-102-3003 MiSeq Reagent Kit v3-600 cycles (2 × 150 cycles). The generated 16S rRNA gene amplicon data are accessible in the European Nucleotide Archive under the study accession number PRJEB77729.

TABLE 1 Primer pairs used for universal and archaeal PCRs

Approach and target	Name	Sequence (5′–3′)	Reference
PCR Universal	515FB	GTGYCAGCMGCCGCGGTAA	26
	806RB	GGACTACNVGGGTWTCTAAT	26
PCR Archaea I/II	344F	ACGGGGYGCAGCAGGCGCGA	27
	1041R	GGCCATGCACCWCCTCTC	27
PCR Archaea II/II	519F	CAGCMGCCGCGGTAA	27
	806R	GGACTACVSGGGTATCTAAT	27

TABLE 2 PCR settings for the primer pairs used, as already described in reference (24)

Target gene	Primer pair	Initial denaturation	Denaturation	Annealing	Elongation	Final elongation	No. of cycles
Universal (16S rRNA gene)	515FB-806RB	3 min, 94°C	45 s, 94°C	1 min, 50°C	1 min 30 s, 72°C	10 min, 72°C	40
Archaea (16S rRNA gene)	344F-1041R	5 min, 95°C	30 s, 94°C	45 s, 56°C	1 min, 72°C	10 min, 72°C	25
	519F-806R	5 min, 95°C	40 s, 95°C	2 min, 63°C	1 min, 72°C	10 min, 72°C	30

The analysis of the 16S rRNA gene amplicon data were performed using QIIME2 (28) 2021.1-12 following the previously outlined methodology (29). Quality filtering was performed with the DADA2 algorithm (30) which involved merging paired-end reads, truncation (-p-trunc-len-f 200 -p-trunc-len-r 150) and denoising for the generation of amplicon sequence variants (ASVs). Taxonomic classification (31) was based on the SILVA 138 database (32) and the resultant feature table and taxonomy file were used for subsequent analysis. Contaminating ASVs were identified and eliminated via decontam v1.13 (33) in R (34), running *iscontaminant* in prevalence mode with varying thresholds (oral-bacteria: 0.3; stool-bacteria: 0.3; oral-archaea: 0.5; and stool-archaea: 0.1). Following this, positive controls (mock-communities) and negative controls were excluded from the data sets. Additionally, ASVs classified as chloroplast or mitochondria were removed as well as ASVs with ≤1 read.

For normalization, different approaches were applied for the bacterial and archaeal data sets, taking into account their respective composition. SRS (scaling with ranked subsampling) normalization was run in QIIME2 (28) applying different c_{min} for the bacterial data set (oral-bacteria: c_{min} = 8,400; stool-bacteria: c_{min} = 3,800). The archaeal data sets underwent TSS normalization (total sum normalization). The number of samples subjected to analysis and kept after normalization are listed in Fig. S1.

Several plot types, including stacked bar plots and PCA plots, were generated using MicrobiomeExplorer (35) in R (34).

Differentially abundant taxa were defined by q2-aldex2 (36–38) in QIIME2 (28). To display those taxa in boxplots (packages: ggplot2 (39), dplyr (40), reshape (34, 41), the data of relative abundance were first CLR transformed in R (34).

Alpha-diversity numbers as well as beta-diversity (PERMANOVA) were calculated with the microbiome package (42) in R (34) and plotted with ggplot2 (39) and dplyr (40).

Longitudinal linear mixed-effect models were created with q2-longitudinal (43) in QIIME2 (28) with the option "linear-mixed-effects" for Shannon diversity and "first-distances" additionally for beta-diversity.

Identification of oxygen requirements

The data sets of universal amplicon data were further investigated regarding the underlying type of respiration. This information had to be collected and entered manually. As resolution from amplicon sequencing is scarce on species level, the genus level was taken into account and physiology data were extracted from bacdive (https://bacdive.dsmz.de/). Therefore, type strain representatives were used, and the common denominator was chosen. We are aware of the problem that physiological data might differ between several species of the same genus; therefore, we handle those data with great care and only as an approximation. In the category of respiration, we assigned three groups: obligate aerobe (listed as "obligate aerobes" and "aerobes"), facultative anaerobe (listed as "microaerophile," "facultative aerobe," and "facultative anaerobe") and obligate anaerobe (listed as "anaerobes" and "obligate anaerobes").

Source tracking

Source tracking was performed to depict the potential of single ASVs of the oral microbiome (source) to be transferred to the GIT microbiome (sink). Therefore, oral and stool data sets were first merged and then TSS normalized, once for the bacterial approach and once for the archaeal approach. Source Tracking was performed with

SourceTracker2 (44) in QIIME2 (28). Rarefaction of source data (oral) and sink data (stool) and vice versa was performed as advised by SourceTracker2 (44) individually per time point. The rarefaction values are listed in a respective table on GitHub (URL: https://github.com/CharlotteJNeumann/InfantDevelopmentTRAMIC). Additionally, using the "--per_sink_feature_assignments" option in SourceTracker2 (44) on TSS-normalized data sets, we could calculate the origin source of a single taxon. The counts were log-transformed for visualization.

Network calculations and visualization

To infer genus-level associations, we employed SparCC (45) within the SCNIC tool v.0.5 (Sparse Co-occurrence Network Investigation for Compositional data) (46). SparCC was run on default settings with 1,000 permutations and the multiple testing correction method set to "fdr bh." Co-occurrence events were visualized in Cytoscape v.3.10.1 (47), where nodes represent taxa and edges represent co-occurrences according to the SparCC R values. Stress centrality and other network properties were calculated using Cytoscape. Files of stress centrality for single genera are provided on the GitHub repository (URL: https://github.com/CharlotteJNeumann/InfantDevelopmentTRAMIC).

Metagenomic data

Shotgun metagenomic sequencing

We performed shotgun metagenomic sequencing of a subset of infants for a few points (O2, S2, S3, M01, M06, and M12). Sequencing libraries were generated with the TruSeq Nano DNA Library construction kit (Illumina, Eindhoven, the Netherlands) and sequenced on an Illumina NovaSeq 6000 platform (Illumina, Eindhoven, the Netherlands; Macrogen, Seoul, South Korea).

Metagenomic data processing

The raw reads were processed using the ATLAS v.2.18.0 workflow (48). There, quality control (PCR duplicate removal, quality trimming, host removal, and common contaminant removal) was performed leading to QC reads which were then assembled into high-quality scaffolds using megahit. All parameters used for ATLAS are detailed in the config.yaml file, which is available in the GitHub repository (URL: https://github.com/CharlotteJNeumann/InfantDevelopmentTRAMIC). Genome binning was achieved with maxbin2 v.2.2.7 (49), followed by quality assessment of genome bins with checkM v.1.0.1 (50), bin refinement with DASTool v.1.1.6 (51), dereplication with dRep v.3.5.0 (26), and taxonomic classification of representative metagenomic assembled genomes (MAGs) with GTDB v 2.3.2 (27, 52, 53). Cutoffs for high-quality MAGs were set as follows: completeness >90% and contamination <5%.

Metagenomic data could only be obtained for nine oral samples in total due to the challenging nature of buccal mucosa samples, such as high presence of host DNA contamination. Therefore, no further analyses on metagenomic oral data were possible. Strain tracking was performed in inStrain v.1.5.7 (54) in ATLAS (48) with the following cutoffs: percent_genome_compare: ≥50% and popANI: ≥99.999% as indicated in the documentation of inStrain (55). Functional annotations were also run within the ATLAS pipeline (48). First, Prodigal v.2.6.3 (56) was applied for gene prediction and linclust (57) to cluster redundant genes (minid = 0.9 and coverage = 0.9) (57). The quantification of gene abundance per sample was performed using the combine_gene_coverages function via the BBmap suite v.39.01-1 (58). Employing eggnog-mapper (v.2.0.1) (59, 60) on the EggNOG database 5.0, taxonomic and functional annotations were assigned. KEGG annotations were extracted (61–63) and read counts were implemented and analyzed in R, following https://github.com/metagenomeatlas/Tutorial/blob/master/R/Analyze_genecatalog.Rmd. Annotated gene counts were normalized (size

factor normalization) and tested for differential expression between BF and NBF infants using DESeq2 (63).

Read-centric metagenome analysis

Species' relative abundances were determined using Kraken2/Bracken (64, 65). Initially, Kraken2 v.2.1.2 (64) was employed to profile the quality-filtered reads from ATLAS v.2.18.0 (48) against the Unified Human Gastrointestinal Genome (UHGG v.2.0.1) (66) database of bacterial and archaeal genomes. Subsequently, Bracken v.2.7 (65) was used with default settings to analyze the Kraken2 output and calculate the relative abundance of bacterial and archaeal species. The resulting report files were merged to generate an abundance table of microbial species for further analysis.

Additional tools used in the manuscript

ChatGPT.com and deepl.com were used for language checks, but not for interpreting the data.

An overview of the available data is displayed in two figures: Fig. S1 is following the STORM guideline and was created with drawio.com (URL: https://drawio.com). Figure S2 displays the data available per sample and individual.

Reproducibility

We conducted a prospective pilot study, whereas the sample size was not predetermined beforehand. Randomization and blinding of the investigators were not foreseen in the chosen study setup. A full study flow chart is provided in Fig. S1 and S2. Participants 13 and 17 were excluded from the study due to incompleteness. Overall, the study is only partially reproducible, as the data are dependent on the study cohort, which was only sampled once within this study, and sampling of cohorts at the same time window cannot be repeated. However, starting from the raw sequencing data, the analysis is fully reproducible, and all required data, scripts, and details are provided. The STORMS Checklist can be found in the GitHub Repository (URL: https://github.com/CharlotteJNeumann/InfantDevelopmentTRAMIC).

RESULTS

Overview on the study population and sample description

Infancy is a dynamic period for microbiome development, with the first 1,000 days of life being the most critical period (67). We highlight the dynamics of microbiome composition and co-occurrence patterns in oral and GIT microbiomes in the first year of life and their transmission patterns, with a focus on the dynamics of anaerobic microorganisms.

Oral and stool samples of 30 infants born either spontaneous (n = 15) or via C-section (CS) (n = 15) were collected at different time points (Fig. S2). Stool samples were initially collected at three time points (tps) (S1 [first stool, meconium, Day 1], S2 [meconium, Days 2–3], and S3 [Days 3–5]), while oral samples were obtained at two-time intervals (O1 [Day 1, prior to feeding, immediately after delivery] and O2 [Days 3–5]). Both sample types were collected monthly until 1 year of age (months M01 to M12). The characteristics (covariates) of the two study groups (spontaneous and CS) did not significantly differ (Chi-square test, $P > 0.5$) except for gestational age, which is significantly lower in infants born via CS (Chi-square test, $P < 0.001$). No covariates for study groups of infants that were breastfed (BF) less or longer than 6 months differed significantly except for formula feeding. The metadata of the studied cohort can be found in Tables 3 and 4; Fig. 1. For maximal resolution of the impact of the feeding regimen, the breastfeeding status was assessed for each single time point (month) individually for each infant, leading to dynamic groupings that changed over time. For example, infants who were BF at Month 3 (M03) were placed in the BF group for that time point, but once they were no longer BF, they were moved to the "non-breastfed (NBF)" group from that point onward.

TABLE 3 Perinatal and postnatal factors between spontaneous and C-section delivery

Individuals	Characteristics	Total (n = 30)	Mode of delivery		
			Spontaneous (n = 15)	C-section (n = 15)	P value
Infants	Sex				0.46
	Male	16 (53.3)	10 (62.5)	6 (42.9)	
	Female	14 (46.7)	6 (37.5)	8 (57.1)	
	Antibiotic usage during the first 12 months				0.66[b]
	Yes	6 (20.0)	4 (25.0)	2 (14.3)	
	No	24 (80.0)	12 (75.0)	12 (85.7)	
	Solid food introduction				0.72[b]
	At 6 months	12 (40.0)	7 (43.8)	5 (35.7)	
	Later than 6 months	18 (60.0)	9 (56.3)	9 (64.3)	
	Breastfeeding during the first 12 months				
	Ever breastfed	26 (86.7)	15 (93.8)	11 (78.6)	0.32[b]
	breastfed after 6 months	22 (73.3)	13 (81.3)	9 (64.3)	0.42[b]
	Formula milk feeding during the first 12 months				
	Ever formula fed	17 (56.7)	9 (56.3)	8 (57.1)	1.00
	Formula fed before 6 months	9 (30.0)	3 (18.8)	6 (42.9)	0.24[b]
	Pet at home				
	All types	11 (36.7)	5 (31.3)	6 (42.9)	0.71
	Fur pet	10 (33.3)	5 (31.3)	5 (35.7)	1.00[b]
	Gestational age (weeks) [mean ± SD]	39.3 ± 1.2	40.1 ± 0.5	38.4 ± 1.0	<0.001[a]
	Birthweight (kg) [mean ± SD]	3.4 ± 0.4	3.5 ± 0.3	3.4 ± 0.5	0.54
	Length of hospital stay (days) [mean ± SD]	3.7 ± 1.2	3.7 ± 1.5	3.8 ± 0.7	0.82
Mothers	Age at infant's birth				0.19[b]
	<31 years	8 (26.7)	5 (31.3)	3 (21.4)	
	31–35 years	10 (33.3)	7 (43.8)	3 (21.4)	
	>35 years	12 (40.0)	4 (25.0)	8 (57.1)	
	Gravida				0.23[b]
	<2	8 (26.7)	6 (37.5)	2 (14.3)	
	>2	22 (73.3)	10 (62.5)	12 (85.7)	
	Parity				0.058[b]
	<2	10 (33.3)	8 (50.0)	2 (14.3)	
	>2	20 (66.7)	8 (50.0)	12 (85.7)	
	Abortion				0.66[b]
	0	24 (80.0)	12 (75.0)	12 (85.7)	
	1	6 (20.0)	4 (25.0)	2 (14.3)	
	Pre-pregnancy weight (kg) [mean ± SD]	62.8 ± 11.9	64.1 ± 11.4	61.4 ± 12.9	0.55
	Pre-pregnancy BMI				0.23[b]
	<18.5	3 (10.0)	1 (6.3)	2 (14.3)	
	18.5–24.9	21 (70.0)	10 (62.5)	11 (78.6)	
	25.0–29.9	6 (20.0)	5 (31.3)	1 (7.1)	

[a]Significance level at $P < 0.05$.
[b]Chi-square test with more than 20% with less than five counts.

Breastfeeding is considered the most significant microbiome covariate within the first year of life (19). In line with this, in our data set, we found that the feeding type significantly impacted four and one time points for oral and GIT samples, respectively (PERMANOVA; oral: $P < 0.05$ for four tps [M03, M04, M08, and M09]; stool $P < 0.05$ for one tp [M10]) but birth mode impacted only one timepoint for both sample types [PERMANOVA; oral: $P < 0.05$ for one tp [M03]; stool $P < 0.05$ for one tp [M01]). Based on this observation, we mainly focused on the feeding types and their impact on the anaerobic microbiome in the oral cavity and GIT and their transitional phase.

TABLE 4 Perinatal and postnatal factors between infants that were breastfed less or longer than 6 months

Individuals	Characteristics	Total (n = 30)	Feeding		
			Breastfeeding less than 6 months (n = 8)	Breastfed longer than 6 months (n = 22)	P value
Infants	Sex				1.00[b]
	Male	16 (53.3)	4 (50.0)	12 (54.5)	
	Female	14 (46.7)	4 (50.0)	10 (45.5)	
	Antibiotic usage during the first 12 months				1.00[b]
	Yes	6 (20.0)	1 (12.5)	5 (22.7)	
	No	24 (80.0)	7 (87.5)	17 (77.3)	
	Solid food introduction				0.42[b]
	At 6 months	12 (40.0)	2 (25.0)	10 (45.5)	
	Later than 6 months	18 (60.0)	6 (75.0)	12 (54.5)	
	Mode of delivery				0.68[b]
	Spontaneous	26 (86.7)	3 (37.5)	12 (54.5)	
	C-section	22 (73.3)	5 (62.5)	10 (45.5)	
	Formula milk feeding during the first 12 months				
	Ever formula fed	17 (56.7)	8 (100)	9 (40.9)	0.004[a,b]
	Formula fed before 6 months	9 (30.0)	8 (100)	1 (4.5)	<0.001[a,b]
	Pet at home				
	All types	11 (36.7)	4 (50.0)	7 (31.8)	0.42[b]
	Fur pet	10 (33.3)	3 (37.5)	7 (31.8)	1.00[b]
	Gestational age (weeks) [mean ± SD]	39.3 ± 1.2	38.9 ± 1.6	39.5 ± 1.0	0.32
	Birthweight (kg) [mean ± SD]	3.4 ± 0.4	3.4 ± 0.5	3.4 ± 0.3	0.96
	Length of hospital stay (days) [mean ± SD]	3.7 ± 1.2	4.4 ± 0.7	3.5 ± 1.2	0.10
Mothers	Age at infant's birth				0.56[b]
	<31 years	8 (26.7)	1 (12.5)	7 (31.8)	
	31–35 years	10 (33.3)	3 (37.5)	7 (31.8)	
	>35 years	12 (40.0)	4 (50.0)	8 (36.4)	
	Gravida				0.64[b]
	<2	8 (26.7)	3 (37.5)	5 (22.7)	
	>2	22 (73.3)	5 (62.5)	17 (77.3)	
	Parity				1.00[b]
	<2	10 (33.3)	3 (37.5)	7 (31.8)	
	>2	20 (66.7)	5 (62.5)	15 (68.2)	
	Abortion				1.00[b]
	0	24 (80.0)	7 (87.5)	17 (77.3)	
	1	6 (20.0)	1 (12.5)	5 (22.7)	
	Pre-pregnancy weight (kg) [mean ± SD]	62.8 ± 11.9	65.3 ± 16.7	61.9 ± 10.1	0.98
	Pre-pregnancy BMI				0.86[b]
	<18.5	3 (10.0)	1 (12.5)	2 (9.1)	
	18.5–24.9	21 (70.0)	5 (62.5)	16 (72.7)	
	25.0–29.9	6 (20.0)	2 (25.0)	4 (18.2)	

[a]Significance level at $P < 0.05$.
[b]Chi-square test with more than 20% with less than five counts.

The oral cavity and GIT are rapidly exposed to strict anaerobes

Samples taken right after birth (labeled "O1" and "S1") and within the first days of life (labeled "S2", "S3," and "O2") showed that newborns get colonized rapidly by various microbes. The first obligate anaerobic bacteria detected in the oral cavity and GIT were *Rothia* (oral), *Streptococcus*, *Staphylococcus* (both oral and GIT), *Bifidobacterium*, and *Enterococcus* (GIT).

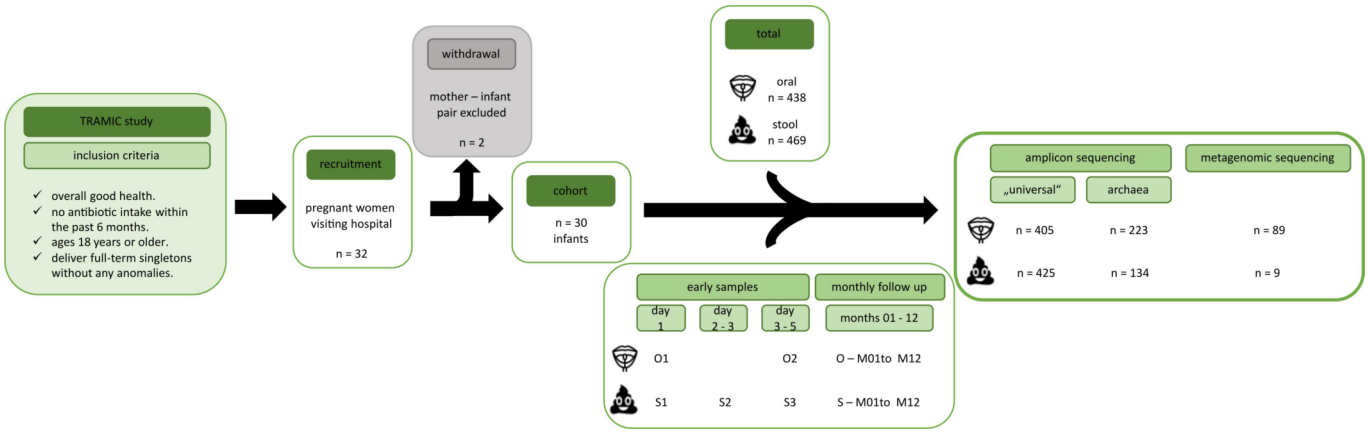

FIG 1 Graphical abstract of the study including recruitment, sample collection and genomic data received.

Interestingly, next to bacteria, archaeal signatures could also be detected in those early samples (Fig. S3). Archaeal diversity was higher in those early-stage samples with *Methanobrevibacter*, *Methanobacterium*, *Methanosphaera*, and *Methanocorpusculum* (all obligate anaerobes) being present next to unclassified Woesearchaeales (oral and GIT) and unclassified Nitrososphaeraceae (GIT). For the latter two, the oxygen requirements are unknown, as these taxa were not classified deep enough. At M01, *Methanobrevibacter* was predominant amongst archaea. As expected, samples collected at the very early stages showed different microbial profiles compared to the ones collected at M01, revealing a shift from S1/O1 to M01 (Fig. S3). It is assumed that the first samples taken immediately after birth do not reflect the inhabitant microbial community, but rather a microbial contamination given the sterile environment in the womb (68). Although the microbial ecosystem may not be fully functional at this time stage, microbial colonization can already start with exposure to the extra-uterine environment and subsequent oral-GIT transmission. However, the main analyses drawn out in this paper focus on samples collected at M01 and later, when microorganisms have started to establish.

Staphylococcus and *Streptococcus* are early but transient colonizers of the oral cavity

In the first month of life, the human skin (parents and family members) is an important source of microbial influx from the environment (8, 69). This is underlined by our data, showing high relative abundances of *Staphylococcus* (facultatively anaerobic) representing a taxon that is mainly skin- (and mucosa) associated (Fig. 2a). We did not find significant differences in the relative abundance of *Staphylococcus* between BF and NBF infants (Aldex2, all tps, $P > 0.05$, Fig. S4), indicating a general substantial transfer from skin to the oral cavity, independent of feeding mode.

To assess the connectivity and co-occurrence of microbes, we built networks for each time point by forming modules in SCNIC at the genus level (Fig. 2b; Fig. S5). From M03 on (Fig. S5), *Staphylococcus* has a very minor relative abundance and appears only sporadically in the co-occurrence networks with low centrality compared to other players (Fig. 2b and Fig. S5 for the complete networks; stress centrality BF: M02: 4, M04: 8, M06: 4; M07: 32; NBF: M01: 150, M02: 28, M05: 82, M10: 44, and M12: 28), indicating its transient colonization in the oral cavity of infants in early life.

Especially in the first month of life, the infant's early oral microbiome is predominated by facultatively anaerobic *Streptococcus* (Fig. 2a). Interestingly, the centrality of *Streptococcus* in microbial networks is surprisingly low, although the abundance is very high (>60% relative abundance) (Fig. 2b). This indicates that even if a microbe is very abundant, this does not necessarily mean that it is an important player in the networks formed by the microbial community. It appears that streptococci do not interact with

other microbes on a large scale, but rather rely on themselves and act independently. Streptococci, which are mainly involved in carbohydrate metabolism, are considered pioneer species that lead the assembly of a complex oral microbiome (70). The dominance of *Streptococcus* is higher in BF infants, reflected by both relative abundance (Aldex2, M04, M05, M06, and M09: $P < 0.05$, $q > 0.05$, Fig. S4; Fig. 2a) as well as network centrality (Fig. 2b; Fig. S5, tp M04 and M06 as an example: BF: M04 = 48,

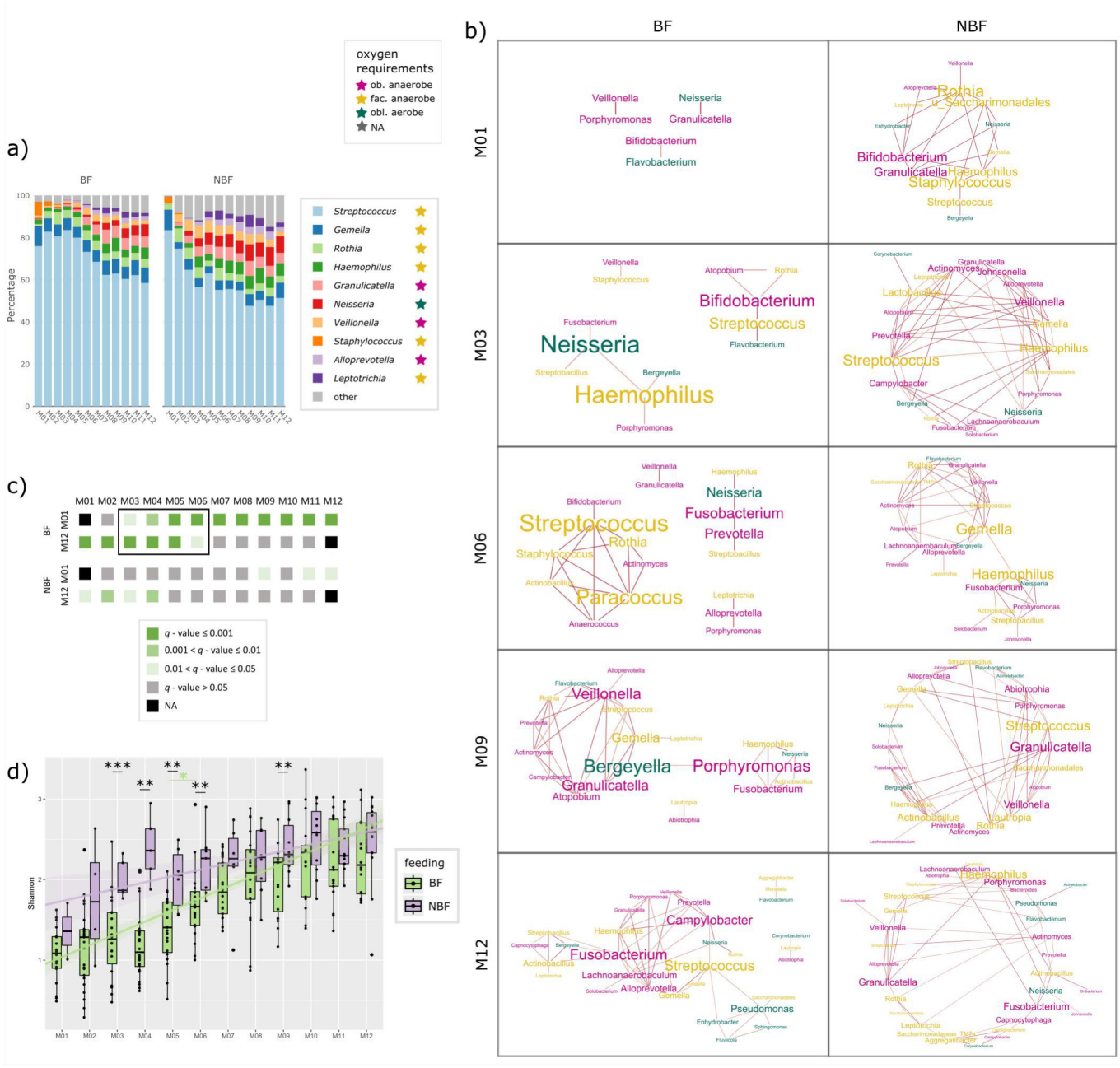

FIG 2 Panel on the oral microbiome. (a) Stacked bar chart showing the relative abundance of the ten most common bacterial genera in the oral cavity per time point (months M01 to M12). Data are shown separately for breastfed (BF) and non-breastfed (NBF) infants. The oxygen requirement of the respective genera is highlighted by colored stars: pink: obligate anaerobes, yellow: facultative anaerobes, and petrol: obligate aerobes. (b) Networks on oral samples of BF and NBF infants of selected time points (months M01, M03, M06, M09, and M1). Font size indicates stress centrality, colors indicate oxygen requirement: pink: obligate anaerobes, yellow: facultative anaerobe, and petrol: obligate aerobes. (c) Pairwise beta-diversity comparisons (PERMANOVA) from months M01 to M12 to all other time points to depict the transition phase. Gray and shaded green colors indicate *q*-values; data are separated for BF and NBF infants. (d) Shannon diversity of oral samples depicted for BF (= light green) and NBF (= lavender) infants with asterisks indicating significant differences (*q*-values) between those two groups. Significant *q*-values between tps in BF infants are indicated with light green asterisks.

M06 = 18; NBF M04 = 128, and M06 = 88). *Streptococcus* shows a decrease in relative abundance starting from M05 onwards (Fig. 2a; Aldex2, $P > 0.05$ at any tp pairwise comparison).

Distinct transitional phases of the oral microbiome in BF infants

Taking several analyses into account (alpha-diversity, beta-diversity, Aldex2, and networks), we were able to outline a time frame in which the oral microbiome changes the most and which thus represents a transition phase of the oral microbiome towards a more mature microbial community.

When the beta-diversity of all oral samples from BF infants was compared with the first (M01) and last time points (M12), it was found that the samples from M03 to M06 differed significantly from these reference points (PERMANOVA; Fig. 2c). Interestingly, this effect was way less pronounced in NBF infants, where only the first 4 months are significantly different to M12 (Fig. 2c), indicating a less defined maturation period in this group.

These observations go hand in hand with patterns we observed in alpha-diversity and allowed for defining the transitional phase of the oral microbiome. This phase seems to be less marked and more gradually processing in NBF than in BF infants. Alpha-diversity was overall significantly increasing within the first year of life (Shannon Fig. 2d and evenness and richness, Fig. S6) (Shannon, longitudinal linear mixed-effect model (LME), $P < 0.001$, Fig. S7) and was higher in NBF infants than in BF infants (LME, $P = 0.002$, Fig. S7). NBF infants' alpha-diversity was more rapidly increasing within the first 4 months of life (M01 to M04) (Fig. 2d). Highest differences in alpha-diversity between BF and NBF infants were observed at M03–M06 and M09 (Shannon diversity, *t* test: M03 $q = 0.00044$, M04 $q = 0.0013$, M05 $q = 0.0079$, M06 $q = 0.0081$, and M09 $q = 0.0096$; evenness, *t* test: M03 $q = 0.0056$, M04 $q = 0.00052$, M05 $q = 0.029$, M06 $q = 0.0098$, M09 $q = 0.0096$; richness, Wilcoxon: M05 $q = 0.0055$). For BF infants, a significant increase in both Shannon diversity and richness could be observed from M05 to M06 (Wilcoxon, $q = 0.0055$, Fig. 2d; Fig. S6).

As already pointed out above, the decrease of *Streptococcus* also started earlier in NBF than in BF infants, supporting the observation of two different dynamics patterns of microbiome maturation in BF and NBF infants.

During this transitional phase, in contrast to *Staphylococcus* and *Streptococcus* which were declining, several genera increased in relative abundance. A distinction can be made between genera such as *Gemella*, *Rothia*, and *Haemophilus* that were already abundant in the first month (all facultative anaerobes) and genera such as *Granulicatella* (obligate anaerobe), *Neisseria*, *Veillonella* (obligate anaerobe), *Alloprevotella* (obligate anaerobe), and *Leptotrichia* which were newly introduced (Fig. 2a). During the transition phase on BF infants (M03–M06), *Streptococcus* did not form any co-occurrence connections with those "new" genera *Neisseria*, *Alloprevotella*, or *Leptotrichia* at all, except with *Neisseria* at M12 (Fig. 2b). After M07, *Alloprevotella* and *Leptotrichia* began to co-occur indirectly with *Streptococcus*, primarily with *Gemella* serving as the connecting node. This suggests that *Gemella* may mediate the integration of co-occurrence between *Alloprevotella–Streptococcus* and *Leptotrichia–Streptococcus*. This integration could exemplify how the introduction of a new microbe (*Alloprevotella*/*Leptotrichia*) into the community may be facilitated by an existing microbe (*Gemella*), contributing to community maturation. Whereas niche-sharing between *Streptococcus* and the genera that were fairly abundant already at the beginning, *Gemella* and *Rothia,* were common independent of feeding mode, co-occurrence of *Streptococcus* with *Haemophilus* was exclusive to NBF infants. Additionally, *Streptococcus* showed intensive co-occurrence connections with *Granulicatella* and *Veillonella*.

These "new" bacterial genera showed a lagged increase in relative abundance in BF infants between mainly M04 and M05 (Fig. S4; Aldex2, *Granulicatella* $P = 0.003$, *Neisseria* $P = 0.012$, *Veillonella* $P = 0.003$, *Leptotrichia* $P = 0.002$). This supports our findings of a lagged transitional phase between BF and NBF infants (can also be seen in Fig. 2a).

Breastmilk maintains simplicity of oral microbial network structures

Starting around M07, after the transitional phase of the BF infants' oral microbiome had concluded, the microbiomes of BF and NBF infants became more similar in terms of alpha-diversity, beta-diversity, and differentially abundant taxa, with fewer significant differences observed on genus level. This coincides with the time when solid food typically constitutes a large portion of the infants' diet, suggesting that solid food acts as a leveling factor for the oral microbiomes of BF and NBF infants.

A notable difference between the oral microbiomes of BF and NBF infants was the overall organization of the microbial networks (Fig. 2b). From the beginning, the microbial networks in NBF infants were very complex, with multiple nodes (microbial genera) and edges (co-occurrences between genera). Several genera exhibited high-stress centrality, meaning they co-occurred with multiple genera and thus were central in the network. This structure changed only slightly over time. In contrast, the oral microbial network of BF infants was much less complex, with fewer microbial players sharing a similar niche. These differences in complexity were especially pronounced in the first month until about M06. This could be attributed to the different nutrient compositions of breast milk (BM) and formula milk, with BM being digested very efficiently, leading to a simplified microbial community.

After M06, the networks in BF infants became more complex, with higher stress centrality of individual microbes (from <50 until M06 to 800 at M11, Fig. S8) and more microbial members co-occurring (18 nodes at M06 to 31 nodes at M12). This likely reflects the introduction of solid food, which provokes a new mode of microbial (inter-)action. However, despite solid food becoming a major component of the diet of a 1-year-old child, the administration of BM, even in smaller proportions, still seemed to influence the GIT microbiome. Even at M12, clear differences between the microbial networks of BF and NBF infants were still evident (Fig. 2b).

Neisseria and its key role for the thriving of obligate anaerobes in the oral cavity

Within the first year of life, the relative abundance of facultative anaerobes decreased from 96% to 76%, with a corresponding increase in obligate anaerobes and obligate aerobes (Fig. S9). NBF infants exhibited a higher load in obligate anaerobes in their oral cavity compared to BF infants (Fig. S9). Despite the high oxygen exposure in the oral cavity, various microenvironments and mechanisms, such as biofilm formation, create suitable niches for obligate anaerobic microbes.

A key player in the infant's oral microbiome is *Neisseria*, an obligate aerobe that plays a major role in biofilm-based oral microbiome networks. In fact, *Neisseria* can protect obligate anaerobes from oxygen (71), likely by consuming it through respiration.

In our networks, *Neisseria* was present at all tps, primarily co-occurring with obligate anaerobes (e.g., *Porphyromonas*, *Fusobacterium*, and *Lachnoanaerobaculum*) and facultative anaerobes (e.g., *Haemophilus*, *Streptobacillus*, and *Leptotrichia*), but not with other obligate aerobes such as *Bergeyella* (Fig. 2b). Typically, interactions between *Streptococcus* species and *Veillonella* were found during the early stages of oral biofilm formation (72). Interestingly, in the networks of NBF infants, we also observed directed nodes between *Neisseria* and obligate aerobic genera such as *Flavobacterium* (M02) and *Bergeyella* (M07). *Neisseria* was more abundant in NBF infants (Aldex2 at M04–M07, M09, and M11 Fig. S4) and showed high centrality in the networks (Fig. 2b), indicating its prominent role in the oral microbiome of NBF infants.

Archaeal signatures are rare and probably transient in the oral microbiome of the first year of life

A high-resolution (taxonomy), highly specific nested PCR approach was used to detect the taxonomic diversity of archaea. The method was successful for 224 out of 415 oral samples which gave a high-quality amplicon result (see also overview Fig. S2).

Methanobrevibacter was indicated to be the dominant archaeal player in the oral niche (Fig. 3a). All infants harbored archaeal signatures in their oral cavity in at least one tp (Fig. 3b). The sporadic loss and emerge of these archaeal signatures in the oral cavity underline our hypothesis that archaea are transient and dependent on environmental input, and we could not define any longitudinal development pattern.

Besides *Methanobrevibacter*, unclassified Woesearchaeales could be detected in several infants and time points, followed by *Methanobacterium*, unclassified Nitrososphaeraceae [probably stemming from human skin (21)] and *Candidatus* Nitrosotenuis.

Given that a nested PCR approach is unfavorable for drawing conclusions about abundance, statistics for the archaeal genera were only performed for their presence/absence using Fisher's *t* test. No significant differences were found for feeding type or birth mode at any time point. As such, we conclude that young infants do not carry a stable oral archaeome.

The influence of the oral microbiome on the GIT microbiome decreases within the first year

To evaluate the potential of the oral microbiome as a source of microbes transferred to the GIT over the first year of life, we conducted source tracking analyses. Overall, the oral microbiome contributed minimally to the GIT microbiome with the highest contribution at tp M01 (mean 18, 27% probability) gradually decreasing over time (mean 7.63% probability at M12) (Fig. 4a).

We also calculated the origin source of individual taxa. An overview of the top 30 ASVs (Fig. 4b) showed that at M01, various ASVs found in the GIT were derived from the oral cavity (see below). This could be attributed to the GIT's limited and unstable colonization by microbes at this early stage, making it more susceptible to influence of the oral microbiome. Additionally, the gastric barrier may not be fully developed at this stage, allowing more microbial transmission from the oral cavity to the GIT.

The main representatives of this early transmission were *Bifidobacterium*, *Staphylococcus*, and *Streptococcus*. *Streptococcus* is the primary genus transferred from oral cavity to the GIT, with one dominant ASV being constantly transmitted over the first year (Fig. 4). In contrast, one *Haemophilus* ASV (ASV06) gained source tracking potential starting from M07. *Bifidobacterium* showed a notable peak at tp M07 and M08 (Fig. 4b).

It is notable that the genera of ASVs tracked from the oral cavity to the GIT generally play central roles in microbial networks or exhibit high abundance. Running source tracking in reverse mode (from GIT [source] to oral [sink]), indicated a number of ASVs shared between oral cavity and GIT: *Bifidobacterium* ASV01, *Haemophilus* ASV06 and ASV16, *Lactobacillus* ASV26, *Rothia* ASV15 and ASV24, *Staphylococcus* ASV07 and ASV27, *Streptococcus* ASV02–ASV05, and *Veillonella* ASV18 (Fig. S10 and S11).

Source tracking was further performed for the archaeal data set (nested PCR approach, based on presence/absence). It was found that the oral microbiome cannot be considered a potent source for the GIT archaeome, as only in three samples a minimal contribution 0.1% (i29_M02), 0.3% (i23_M06), and 0.3% (i05_M12) was detected.

The GIT microbiome develops more steadily throughout the first year of life than the oral microbiome

Similar to the oral microbiome, stool samples from BF infants exhibited a distinct but prolonged transition period from M03 to M08 (PERMANOVA, Fig. 5a). Again, no such obvious time frame was observed for NBF infants, highlighting fewer differences in the GIT microbiome composition between the start and end points of comparison. This is further illustrated by generally smaller Bray-Curtis distances in NBF infants compared to BF infants (Fig. S12).

This pattern is also reflected by our microbial networks and alpha-diversity. Similar to the oral microbiome, Shannon diversity, evenness, and richness of the GIT microbiome increased over time within the first year of life (LME for Shannon, $P = 0.004$, Fig. S13), with a

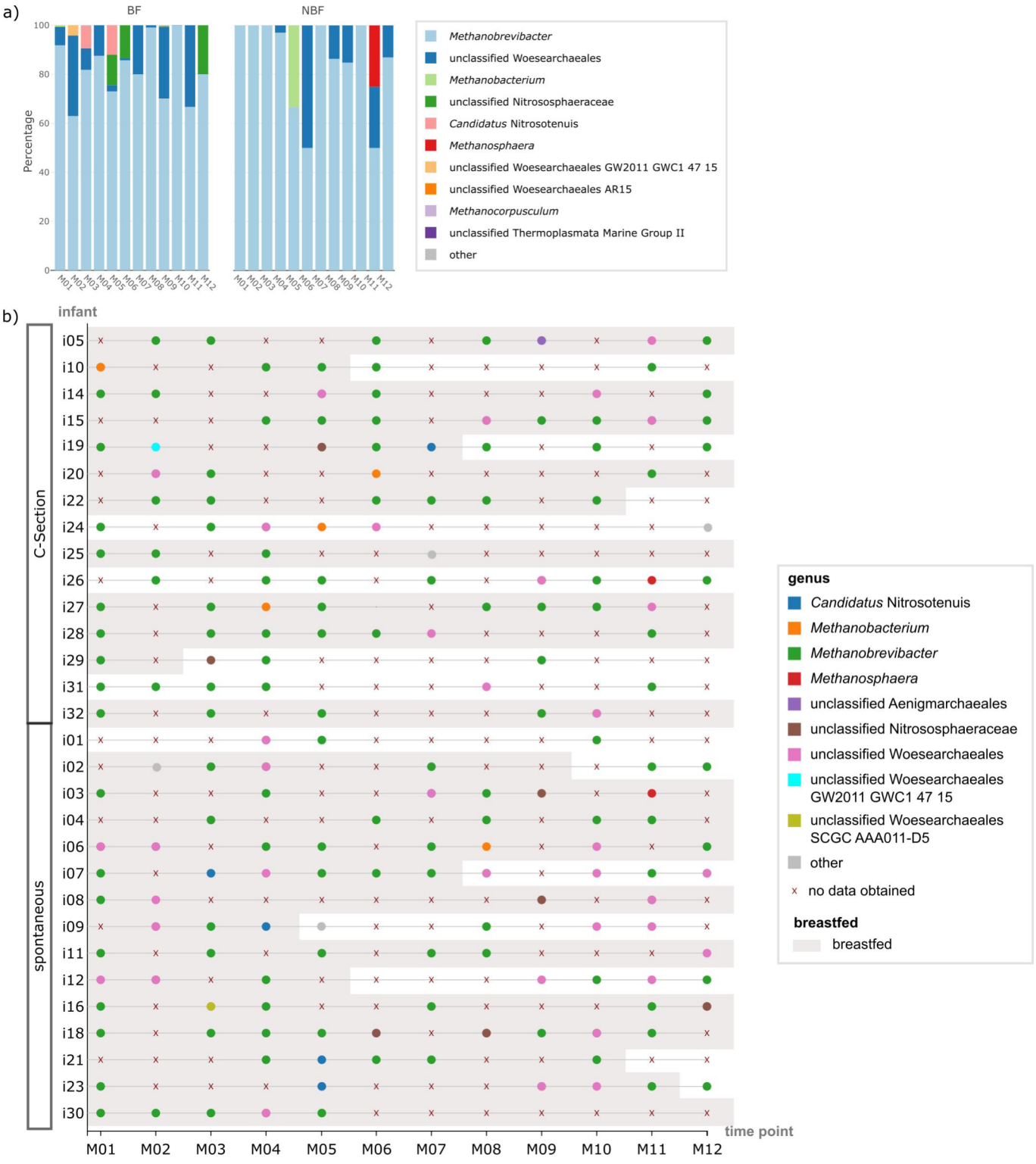

FIG 3 Panel on the oral archaeome. (a) Stacked bar chart showing the relative abundance of the ten most common archaeal genera in the oral cavity per time point (months M01 to M12); data are shown separately for breastfed (BF) and non-breastfed (NBF) infants. (b) Beeswarm plot on absence/presence of archaeal signatures in the oral microbiome per infant and time point (months M01 to M12); time points at which the infants were BF are underlaid with gray and infants are sorted by their mode of delivery.

more pronounced increase in BF infants compared to NBF infants (Shannon diversity, LME $P < 0.001$). However, these changes were not significantly different between individual tps

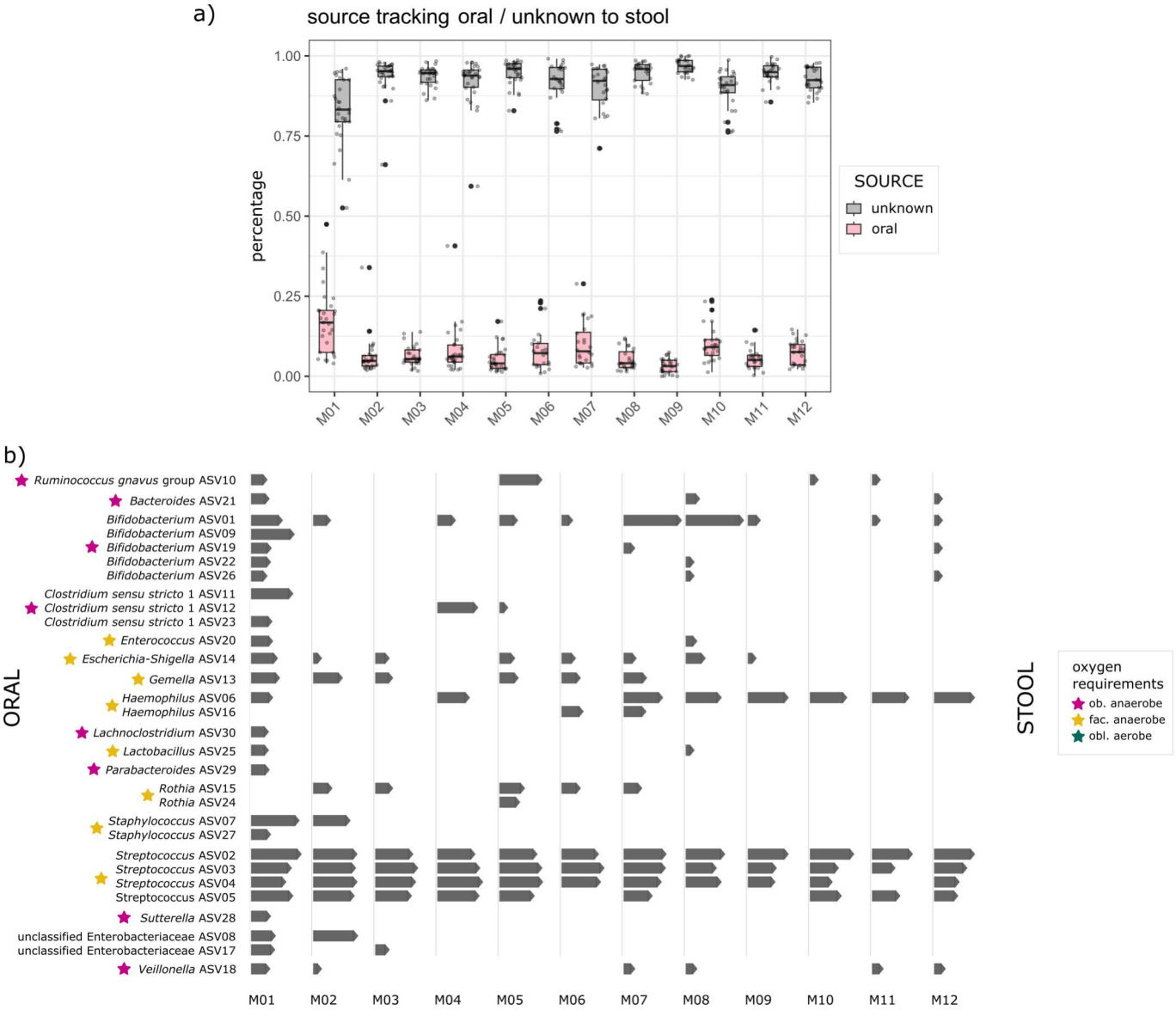

FIG 4 Source tracking from oral to GIT. (a) Source tracking probability of bacterial taxa being transferred from oral and unknown sources to the GIT as a sink, depicted per time point (months M01 to M12). (b) Source tracking of specific bacterial taxa being transferred from oral source to GIT sink. The top 30 ASVs are depicted per time point (months M01 to M12), the length of the bars indicates the log-transformed counts of a taxon. The oxygen requirement of the respective ASV is highlighted by colored stars: pink: obligate anaerobes, yellow: facultative anaerobes, and petrol: obligate aerobes.

(Fig. 5b; Fig. S14). In general, alpha-diversity and mainly Shannon diversity was again slightly higher in NBF infants than in BF infants (Fig. S13, *t* test: M04: $q = 0.029$ M05: $q = 0.044$; richness, Wilcoxon: M03 $q = 0.019$, M05: $q = 0.021$, Fig. S14).

The GIT microbiome stabilizes by establishing complex anaerobic microbial communities

Within the first year of life, a very complex microbial network was established (BF M012: nodes $n = 45$, edges $n = 78$, average number of neighbors $n = 3.467$; NBF M12: nodes $n = 38$, edges $n = 56$, and average number of neighbors $n = 3.056$). In the first month, especially for BF infants, only few bacterial genera were found to co-occur, and stress centrality in general was low (Fig. S15). As alpha-diversity increased over time, the number of genera included in networks also grew. The microbial networks in the NBF

infants were more complex from the early months with a higher number of nodes and edges, indicating more microbial interactions compared to BF infants (Fig. 5c).

The GIT microbiome was predominated by various obligately anaerobic microbes at all tps and their relative abundances were constantly increasing within the first year of life (Fig. S16, from ~50% in M01 to ~70% in M12). Initially, the human GIT contains little amounts of oxygen which is gradually consumed by microbial activity. Indeed,

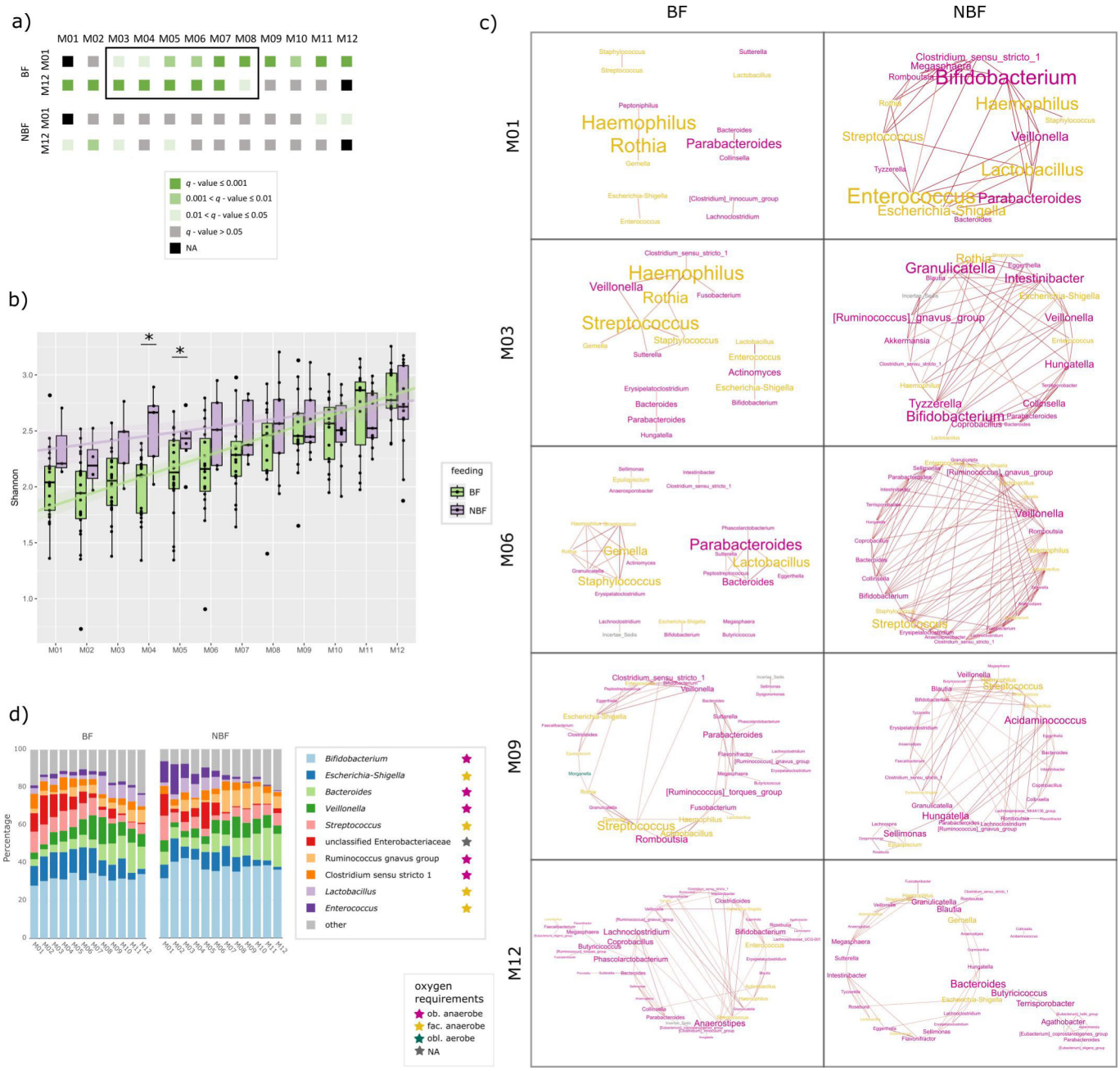

FIG 5 Panel on the GIT microbiome. (a) Pairwise beta-diversity comparisons (PERMANOVA) from months M01 to M12 to all other time points to depict the transition phase. Gray and shaded green colors indicate *q*-values; data are separated for breastfed (BF) and non-breastfed (NBF) infants. (b) Shannon diversity of stool samples depicted for BF (= light green) and NBF (= lavender) infants with asterisks indicating significant differences (*q*-values) between those two groups. No significant *q*-values between tps within one group (BF or NBF). (c) Networks on stool samples of BF and NBF infants of selected time points (months M01, M03, M06, M09, and M12) Font size indicates stress centrality, colors indicate oxygen requirement: pink: obligate anaerobes, yellow: facultative anaerobe, and petrol: obligate aerobes. (d) Stacked bar chart showing the relative abundance of the ten most common bacterial genera in the GIT per time point (months M01 to M12). Data are shown separately for BF and NBF infants. The oxygen requirement of the respective genera is highlighted by colored stars: pink: obligate anaerobes, yellow: facultative anaerobes, and petrol: obligate aerobes.

in the first month of life, some facultatively anaerobic bacteria were still detectable with central roles in bacterial networks (Fig. 5c and all networks in Fig. S16 and S17), including taxa of the genera *Escherichia-Shigella*, *Rothia*, *Haemophilus*, *Staphylococcus*, *Enterococcus*, *Lactobacillus*, and *Gemella*. This was particularly evident in BF infants, with *Haemophilus* showing particularly high-stress centrality. In contrast, we hardly found any obligate aerobes in the GIT, correlating with very low oxygen levels after initial oxygen consumption (Fig. S16).

The most prominent obligate anaerobe in the GIT was *Bifidobacterium* (Fig. 5d), consistently representing about 30% (relative abundance) at all tps. Similar to *Streptococcus* in the oral microbiome, *Bifidobacterium* was, beyond its predominant abundance, not harboring a central role in the network of the GIT microbiome, yielding only low-stress centrality except for few tps (BF: M08; NBF: M01, M02). This may be due to *Bifidobacterium's* unique metabolic ability to metabolize human milk oligosaccharides (HMOs), which limits its niche overlap with other microbes.

Interestingly, this was not the case in the GIT of NBF infants which did not receive breastmilk containing HMOs. In these infants, *Bifidobacterium* likely relied on more syntrophic interaction with other microbes. While in BF infants, *Bifidobacterium* mainly co-occurred with *Escherichia-Shigella* and *Enterococcus*, in NBF infants, it associated with a wider range of partners. Contrary to expectations, *Bifidobacterium* did not have a higher relative abundance in BF than in NBF infants' GIT microbiome (Fig. 5d).

Strain tracking of MAGs revealed several *Bifidobacterium* strains in several infants (Fig. 6). Even though MAGs of overall seven *Bifidobacterium* species could be detected (Fig. S18), only three of them, including *B. adolescentis*, *B. longum*, and *B. pseudocatenulatum,* were trackable in just one infant at two consecutive tps (Fig. 6). *Bifidobacterium longum*, on the other hand, could be tracked in four infants between S3 and M01 (Fig. 6).

Comparing BF and NBF infants, we observed only a few bacterial genera that were significantly differentially abundant between the two groups (Fig. S19). *Lactobacillus* was lower in relative abundance in NBF infants in months M08 to M11 (Aldex2, M08 $P = 0.013$, M09 $P = 0.004$, M10 $P = 0.006$, and M11 $P = 0.001$). *Intestinibacter* was significantly differentially abundant, showing higher relative abundance in M03, M04 and M08 in NBF infants (Aldex2, M03 $P = 0.015$, M04 $P < 0.001$, and M08 $P = 0.011$). This strong differential abundance of *Intestinibacter* at several time points is interesting, as *Intestinibacter* did not occur to be prominent in any other analysis. Interestingly, between M05 and M07, no genera were differentially abundant.

Persistent colonization is sparse in the first year of life

A subset of samples was subjected to shotgun sequencing, resulting in the assembly of 133 high-quality MAGs (completeness >90%, contamination <5%), derived from 65 samples from 21 infants. An overview of the samples is provided in Fig. S1. Using these data, strains of several bacterial species, in addition to *Bifidobacterium,* were tracked in several infants at various tps (Fig. 6). Surprisingly, only a few strains could be detected in an infant across more than two or three consecutive tps, which would typically indicate persistent colonization of the lower GIT by that strain. Persistent colonization was only observed for few species, including *Aeromonas caviae*, three *Bifidobacterium* species, *Blautia A wexlerae*, *Faecalibacterium prausnitzii_D*, several *Streptococcus* species, for example, *Streptococcus parasanguinis_E* and *Staphylococcus* species, for example, *Staphylococcus lugdunensis*, *Rothia*, and *Veillonella*.

Also, the archaeal profile did not reveal a steady colonization. Only 134 out of 442 stool samples and 224 out of 415 oral samples gave a high-quality amplicon output (see also overview Fig. S2). Archaeal signals were only detected in S3 for infants i20 and i21, but not from M01 to M12. Archaeal presence in the lower GIT was confirmed early in life, but a highly variable and transient pattern was observed both between infants and over time (Fig. 7a and b).

Methanobrevibacter was the most predominant archaeal genus in the GIT similar to the oral microbiome. Some infants exhibited several archaeal genera at various tps (e.g.,

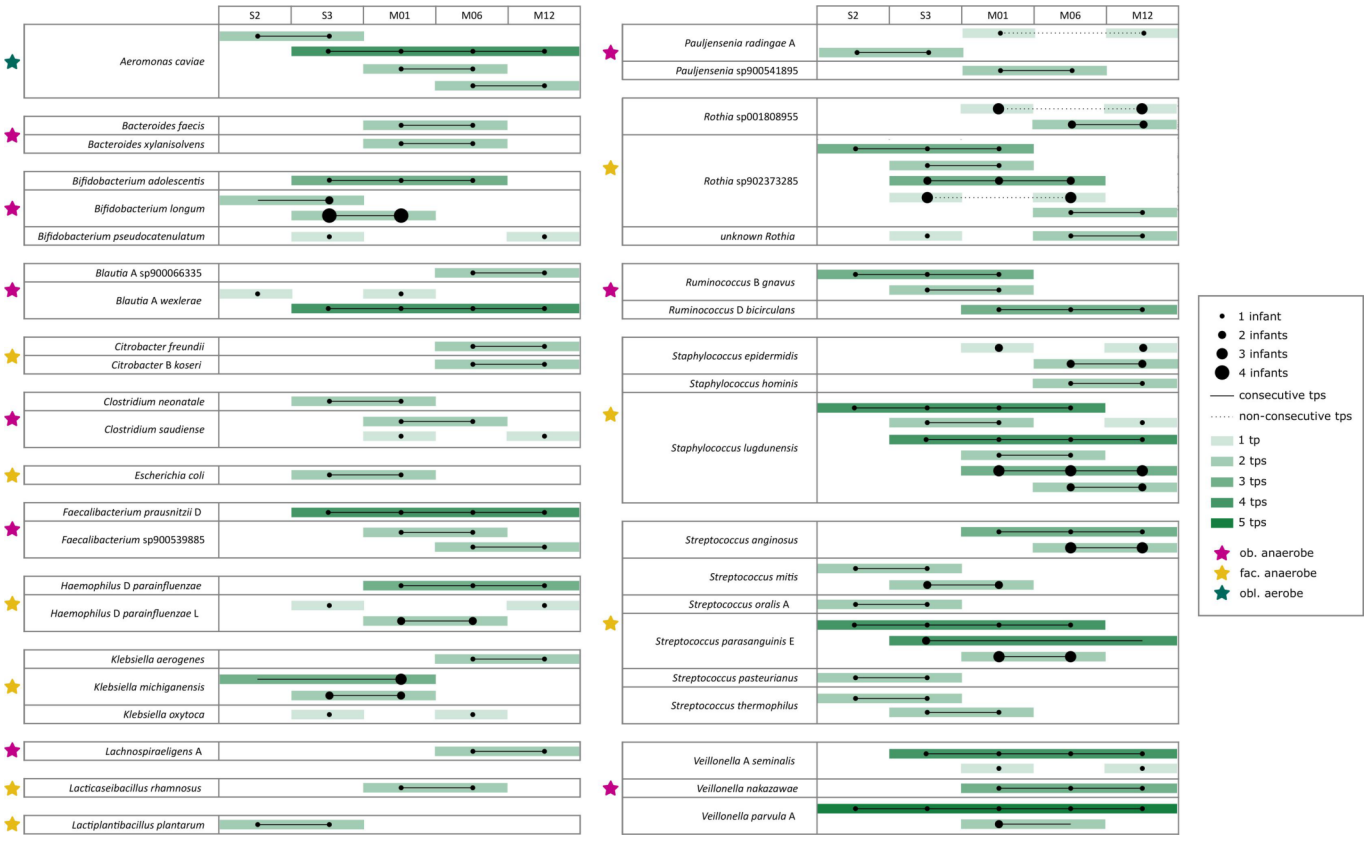

FIG 6 Species for which strains could be tracked within one or more infants (number represented by dot size) over time (number of time points represented by the intensity of green color). Stroke style indicates if the strain could be tracked at consecutive time points (line) or non-consecutive time points (dashed). The oxygen requirement of the respective strain is highlighted by colored stars: pink: obligate anaerobes, yellow: facultative anaerobes, and petrol: obligate aerobes.

i24 and i26), while others showed only one genus at single tps (e.g., i07 and i09). Statistical analysis using Fisher's *t* test revealed no significant differences in archaeal presence/absence based on feeding type or birth mode. However, *Methanobrevibacter* and *Methanosphaera* were more common in NBF infants, whereas BF infants displayed a more diverse archaeal pattern, with higher relative abundances of unclassified Nitrososphaeraceae and *Candidatus* Nitrosocosmicus, possibly due to mouth-to-skin contact during breastfeeding (Fig. 7a).

Kraken/Bracken of metagenomic sequences identified five archaeal species: *Methanobrevibacter_A_sp900766745*, *Methanobrevibacter_A_smithii*, *Methanobrevibacter_A_woesei*, *Methanosphaera_cuniculi*, and *Methanosphaera_sp900322125*, with *Methanobrevibacter_A_sp900766745* being the most predominant (Fig. S21). All 21 infants with metagenomic data, showed archaeal signatures in their GIT across 41 samples, but at very low relative abundances (<0.07%). The highest archaeal abundances were observed at M12, indicating an increase of archaea in the first year of life. We could show that infants already have archaeal signatures in their upper and lower GIT in the first month of life, but colonization takes place late or even after M12.

Differences between BF and NBF infants' GIT microbiomes are less pronounced on functional levels

Comparative, functional analyses were performed on metagenome stool samples of tps M01, M06 and M12 (Fig. 8). The very high numbers of functions that were significantly differentially abundant between the tps (DeSeq2, M01 to M06 $n = 320$ with $q < 0.05$, M06 to M12 $n = 2,218$ with $q < 0.05$, M01 to M12 $n = 2,901$ with $q < 0.05$) indicate a very high dynamic of microbial potentials in the first year of life. Microbial functions with the top

FIG 7 Panel on the GIT archaeome. (a) Stacked bar chart showing the relative abundance of the ten most common archaeal genera in the GIT per time point (months M01 to M12); data are shown separately for breastfed (BF) and non-breastfed (NBF) infants. (b) Beeswarm plot on absence/presence of archaeal signatures in the GIT microbiome per infant and time point (months M01 to M12); time points at which the infants were BF are underlaid with gray and infants are sorted by their mode of delivery; for infants i20 and i21 no archaeal data could be obtained at any time point.

20 highest log fold-change were associated mainly with growth. This was reflected by basic metabolic pathways and a high number of genes responsible for metabolism, especially energy metabolism (e.g., oxidative phosphorylation) and carbohydrate metabolism. At M06 in comparison, genes for proteins of secretion systems involved in signaling and cellular processes were overrepresented. Examples for this were transporters and signaling proteins. The aging microbiome again shifted towards metabolism when the infants were 1 year old (M12), but with more complex pathways covering the food chain down to methane. Notably, antimicrobial resistance genes could already be found at M06 and M12.

When comparing the functional potential of the GIT microbiome of BF and NBF infants at one tp, a slighter difference was observed (DeSeq2, M01 $n = 85$ with $q < 0.05$, M06 $n = 19$ with $q < 0.05$, and M12 $n = 195$ with $q < 0.05$; Fig. S20) than it was seen between tps. Additionally, differences between BF and NBF infants' GIT microbiomes are less pronounced on functional levels than on taxonomic levels.

At M01, no gene was significantly differentially abundant for NBF infants (DeSeq2, all $q > 0.05$), meaning that all 85 genes were exclusively associated with BF infants. As most of these functions are also somehow connected with metabolism, the GIT microbiome of infants that receive BM could offer a higher number of genes that are needed to metabolize this very complex food. At M06, five genes were exclusive for NBF infants: ABC transporters and proteins for genetic information processing or signaling and cellular processes. In contrast to this, proteins of secretion systems, metabolism, and signal transduction were assigned to BF infants. In the GIT microbiome of infants of one year of age (M12), the nature of genes (higher hierarchical levels) that were assigned either to BF or NBF infants are very similar, even though on the lowest hierarchical level differences were observed. It can be concluded that the GIT microbiome is fulfilling the same grand functions, but with a different taxon-dependent genetic inventory.

DISCUSSION

In infants, initial microbiome development is influenced by factors such as mode of delivery and feeding type (73). Throughout the first year of life, additional factors—including the introduction of solid foods, teething, and infants' increased mobility—shape the microbiome's structure (19, 73, 74). These changes expose infants to new microbes and create diverse environments and conditions for microbial growth, facilitating the establishment of obligate anaerobes with tense networks. We could show that birth mode impacts the initial oral and GIT colonization, but feeding has higher impacts especially later within the first year of life. Our study provides valuable insights into the early development and transition of the oral microbiome highlighting differences between BF and NBF infants. We could determine a distinct time frame in which the oral microbiome transitions most and showed that this time frame lagged between BF and NBF infants.

Breastfeeding is recognized as a significant factor influencing the GIT but also oral microbiome (75). Our results confirm that breastfeeding notably impacts microbiome composition at several time points for both oral and GIT samples. Specifically, BF infants exhibit a more defined transitional phase in their oral microbiome compared to NBF infants. This transitional phase is marked by a decrease in *Streptococcus* and the emergence of new genera such as *Granulicatella*, *Neisseria*, *Veillonella*, *Alloprevotella*, and *Leptotrichia*. It is also characterized by increased alpha-diversity of the colonizing species and significant changes in the microbial community as indicated by beta-diversity. As this transitional phase occurs earlier in NBF infants (Months 1–3) than in BF infants (Months 4–6), we can infer that breastfeeding supports a later, but more defined, maturation of the oral microbiome. By Month 7, after the BF infants' transitional phase has ended, the microbiomes of both groups become more similar in terms of alpha- and beta-diversity, as well as differentially abundant taxa. This convergence is likely influenced by the introduction of solid food, which acts as a leveling factor between BF and NBF infants' microbiomes. This aspect was already discussed before (76, 77) but still,

a)

				KEGG	baseMean	log2FoldChange	sgnf
M01	Env. Inf. Processing	Signal transduction	Two-component system	K11601			**
	Metabolism	Carbohydrate metabolism	Propanoate metabolism	K00167			***
				K17489			*
		Energy metabolism	Carbon fixation pathways in prokaryotes	K09709			*
			Oxidative phosphorylation	K02274			*
				K03890			*
				K03891			*
				K02276			*
				K02259			***
		Environmental Information Processing	Two-component system	K07669			*
		Glycan biosynthesis and metabolism	Other glycan degradation	K13714			***
		Nucleotide metabolism	Purine metabolism	K00958			***
		Xenobiotics biodegradation and metabolism	Drug metabolism - cytochrome P450	K00274			*
	NA	Unclassified: metabolism	Enzymes with EC numbers	K20902			**
	Protein families	Genetic information processing	Mitochondrial biogenesis	K14998			**
			Proteasome	K13572			**
		Metabolism	Glycosyltransferases	K25619			***
				K13671			**
				K14335			*
			Peptidases and inhibitors	K03433			*
M06	Metabolism	Glycan biosynthesis and metabolism	Lipopolysaccharide biosynthesis	K00713			***
			Peptidoglycan biosynthesis	K23170			***
		Metabolism of cofactors and vitamins	Nicotinate and nicotinamide metabolism	K15359			***
		Nucleotide metabolism	Purine metabolism	K22026			***
	Protein families	Signaling and cellular processes	Prokaryotic defense system	K19780			***
				K19779			***
				K19127			***
				K19128			***
			Secretion system	K12203			***
				K12228			***
				K12206			***
				K12218			***
				K12214			***
				K12217			***
				K12222			***
				K12213			***
				K12209			***
				K12204			***
				K12205			***
				K12687			***

b)

				KEGG	baseMean	log2FoldChange	sgnf
M06	Env. Inf. Processing	Genetic information processing	Transcription factors	K17473			***
		Membrane transport	ABC transporters	K17331			*
				K16916			**
	Metabolism	Carbohydrate metabolism	Pentose phosphate pathway	K17468			*
		Energy metabolism	Oxidative phosphorylation	K00342			***
		Xenobiotics biodegradation and metabolism	Styrene degradation	K20807			***
	NA	Signaling and cellular processes	Signaling proteins	K14061			***
		Unclassified: genetic information processing	Replication and repair	K18320			***
				K07480			***
		Unclassified: metabolism	Enzymes with EC numbers	K08313			***
	Protein families	Genetic information processing	DNA repair and recombination proteins	K13531			***
			Transcription factors	K03493			**
				K20388			*
		Signaling and cellular processes	Antimicrobial resistance genes	K04343			*
			Prokaryotic defense system	K19780			*
			Transporters	K11732			*
				K09475			***
				K18925			***
				K11081			**
		Viral protein families	Viral proteins	K21507			*
M12	Cellular Processes	Cellular community - prokaryotes	Biofilm formation Pseudomonas aeruginosa	K21006			**
	Metabolism	Biosynthesis of other secondary metabolites	Degradation of flavonoids	K26173			*
				K26174			*
		Carbohydrate metabolism	Amino sugar & nucleotide sugar metab.	K18677			*
			Fructose and mannose metabolism	K00066			***
		Energy metabolism	Methane metabolism	K14127			***
		Glycan biosynthesis and metabolism	Exopolysaccharide biosynthesis	K13665			***
		Metabolism of terpenoids and polyketides	Terpenoid backbone biosynthesis	K00587			***
	NA	Poorly characterized	Function unknown	K07161			*
		Unclassified: metabolism	Enzymes with EC numbers	K25764			*
				K18195			**
				K15538			*
				K25255			*
	Protein families	Metabolism	Glycosyltransferases	K05841			*
			Protein phosphatases & associated proteins	K21814			*
		Signaling and cellular processes	Antimicrobial resistance genes	K18844			*
			Transporters	K06311			*
				K25190			*
		Unclassified: metabolism	Others	K25263			*

FIG 8 Hierarchical KEGG functional annotation of genes of highest top 30 log fold-change rates of pairwise comparison of (i) months M01 (orange) and M06 (green) and (ii) M06 (green) and M12 (purple); negative log fold change: red, higher in (a) M01 (vs. M06) or (b) M06 (vs. M12); positive log fold change: blue, higher in (a) M06 (vs. M01) or (b) M12 (vs. M06); significant

Fig 8 (Continued)

q-value indicated by asterisks and base mean indicated by bar charts, colored by the respective time point; abbreviation env. info. processing = environmental information processing.

complete cessation of BM rather than the introduction of solid food is the major driver for aligning the microbiomes (73).

Microbial network complexity also differs significantly between BF and NBF infants. NBF infants have more complex networks from the first month, with multiple genera exhibiting high-stress centrality and consistent network structure over time. In contrast, the microbial networks in BF infants are less complex, with fewer genera sharing similar niches. This difference in complexity is most distinct in the first 6 months and can be attributed to the differing nature of BM and formula milk. Factors listed by reference (6) include transmission of bacteria only through BM (78, 79), various milk components influencing the attachment of bacteria to the oral cavity (80) and utilization of different carbohydrates in breastmilk (e.g., HMOs) and formula milk by bacteria (81, 82). After 6 months, the microbial network of BF infants becomes more complex, likely reflecting the introduction of solid food and the subsequent increased microbial colonization and co-occurrences for improved nutrient degradation.

In the first month of life, the human skin significantly contributes to the microbial influx into the oral cavity (8, 69). This is evidenced by the high relative abundances of *Staphylococcus*, a skin- and mucosa-associated facultative anaerobe. Our data showed no significant differences in *Staphylococcus* abundance between BF and NBF infants, suggesting substantial skin-to-oral transfer independent of feeding mode. However, from Month 3 onwards, *Staphylococcus* presence diminished and appeared only sporadically in the co-occurrence networks with low centrality, indicating its transient colonization in the oral cavity during early life.

In addition to the human skin, transmission from other individuals as well as environmental exposures can also be considered as the origin of the normal microbiome of the oral cavity. In fact, the predominant component of the oral microbiome, for example, *Streptococcus* is transmitted through these routes (6). This facultatively anaerobic bacterial taxon, is known for its role in carbohydrate metabolism and is in fact considered as a pioneer species in oral microbiome assembly (70). Despite its high relative abundance especially in the first 3 months (>60% in both BF and NBF infants), *Streptococcus* exhibited low network centrality, suggesting its inferior co-occurrence with other microbes and its independent functionality. Interestingly, this bacterial taxon showed a higher dominance in BF infants, as reflected by both its relative abundance and network centrality. *Streptococcus* decreases in abundance, with new microbial members emerging, marking a transitional phase in the oral microbiome.

Microbes from the oral cavity are constantly swallowed and transitioned through the GIT. Source tracking analyses showed that the contribution of the oral microbiome to the GIT microbiome was overall modest and even decreased over time. Key genera, such as *Bifidobacterium*, *Staphylococcus*, and *Streptococcus*, were identified as being transferred from the oral cavity to the GIT. The presence of these genera in both microbiomes highlights the interconnectedness of the oral and GIT microbiomes in early life. However, it is believed that the similarity of oral and GIT microbiomes decreases over time due to the development and maturation of gastric barrier, including gastric pH, motility, and enzyme production by 1 year of age (23, 83–85). Wernroth et al. (86) already highlighted that some OTUs are shared between saliva and fecal samples. They showed that one *Veillonella* OTU is mainly shared and that the similarity between saliva and stool decreased over time (86).

It should be mentioned that our methods did not allow for a distinction of living and dead microorganisms, and we cannot make definitive statements about colonization status in the early months of development, as microbial signatures might remain

(Continued on next page)

detectable throughout the GIT, although the microorganisms from the oral cavity might have died during passage.

The detection of archaea in the infant GIT as early as the first month of life provides new insights into the microbial ecology of the infant GIT. *Methanobrevibacter* is the predominant archaeal genus, with its abundance increasing over time. The presence of other archaeal genera, however, particularly skin-associated ones in BF infants, indicates a more diverse archaeal community possibly influenced by close contact during breastfeeding. Although archaea are detected at low relative abundance, their presence becomes more pronounced by Month 12 (M12), indicating a gradual establishment in the GIT microbiome.

Our findings support findings from other studies that infants under 1 year already carry archaea in their intestinal tracts (22, 23). Archaea found in human colostrum and BM (76) suggest vertical transfer from mother to child during breastfeeding. Other potential sources include cow milk, dairy products (77), and the archaeomes of other humans, exposing both BF and NBF infants to archaeal sources.

The sporadic presence and absence of archaeal signatures over time support the continuous transition of archaea from the environment into and through the infant's intestinal tract. More frequent detection of archaea in oral samples compared to stool samples implies that the input of archaea exceeds their successful colonization in the lower intestinal tract.

Next to strictly anaerobic archaea like *Methanobrevibacter*, also anaerobic bacteria played a massive role in the stabilization of microbiomes. *Veillonella* and *Alloprevotella* appeared in the oral cavity and GIT microbiomes, indicating early colonization by anaerobic bacteria. *Neisseria*, an obligate aerobe, plays a crucial role in facilitating the survival of anaerobes in the oral cavity by creating microenvironments with lower oxygen levels (71).

Interestingly, despite the increasing relative abundance of *Bifidobacterium* over time (which is expected due to the decreasing levels of oxygen), this bacterial taxon does not play a central role in microbial networks, especially in BF infants. This could be due to its unique metabolic niche of HMO conversion, highlighting the metabolic specialization and niche partitioning within the infant GIT microbiome. Notably, the strain tracking of *Bifidobacterium* in several infants over time, and therefore its persistence, highlights their colonizing potential and possible role in maintaining GIT health and stability. Other strains that we could track were *Blautia_A wexlerae* (obl. anaerobe) and *Faecalibacterium prausnitzii_D* (obl. anaerobe), both highly beneficial bacteria with anti-inflammatory properties (87) that are known to maintain GIT health by aiding in the production of short-chain fatty acids (SCFAs) (88). Further, positive tracking events could be observed for *Streptococcus parasanguinis_E* (obl. anaerobe) and *Veillonella_A seminalis* (obl. Anaerobe), which are both involved in early colonization in the oral cavity but can also be found in the GIT (72, 89). *Veillonella parvula_A* (obl. anaerobe), which like other *Veillonella* species, plays a role in maintaining a balanced GIT microbiome (89) could also be tracked over time. On the other hand, we see also bacterial species with not such a clear role, like *S. lugdunensis* (fac. anaerobe), a skin commensal whose presence in the GIT is less understood (90) and can cause severe infections, especially in hospital (91) and *A. caviae* (fac. anaerobe), an opportunistic pathogen that is rare in healthy infants (92). *S. lugdunensis* could be tracked even in several infants.

While the GIT microbiomes of BF and NBF infants differed in composition and complexity, the functional potential of these microbiomes is rather influenced by age than by feeding mode as indicated by the functional analysis of GIT metagenomes. Our results indicated a dynamic shift in gene abundance over time, with significant differences between months, but overall functional redundancy across BF and NBF infants.

Conclusion

Our findings underscore the dynamic nature of the microbiome during infancy and the significant impact of breastfeeding on microbial development throughout the entire digestive tract. We could show that the oral and GIT microbiomes of BF infants undergo distinct phases of increased dynamics within the first year of life. In contrast, the microbiomes of NBF infants are more mature from the first month, leading to a steadier development without distinct transitional phases in the first year. Additionally, we found that archaeal signatures are present in infants under 1 year of age, but they do not form a stable archaeome. While the oral microbiome initially influenced the GIT microbiome during infancy, the GIT microbiome gradually stabilized and differentiated over the first year of life. This transition was marked by a decreasing influence of the oral microbiome on the GIT microbiome composition, suggesting a maturation of the GIT microbial community independent of early oral influences. These results provide valuable insights into the often-overlooked aspects shaping the infant microbiome development.

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DATA AVAILABILITY

Data, tables, and scripts that support our findings are openly available in our GitHub Repository https://github.com/CharlotteJNeumann/InfantDevelopmentTRAMIC.
The generated 16S rRNA gene amplicon data are accessible in the European Nucleotide Archive under the study accession number PRJEB77729.

ETHICS APPROVAL

This study has been registered on clinicaltrials.gov (NCT04140747). The samples were collected under the ethical approval number 28-524 ex15/16 by the respective local ethics committees, the Ethics Committee at the Medical University of Graz, Graz, Austria and in adherence to the principles outlined in the Declaration of Helsinki.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental Figures (Neumann_infant_development_Supplementary_Figures.pdf). Figures S1 to S21.

Open Peer Review

PEER REVIEW HISTORY (review-history.pdf). An accounting of the reviewer comments and feedback.

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