INFANT GUT MICROBIOTA COLONIZATION AND FOOD IMPACT

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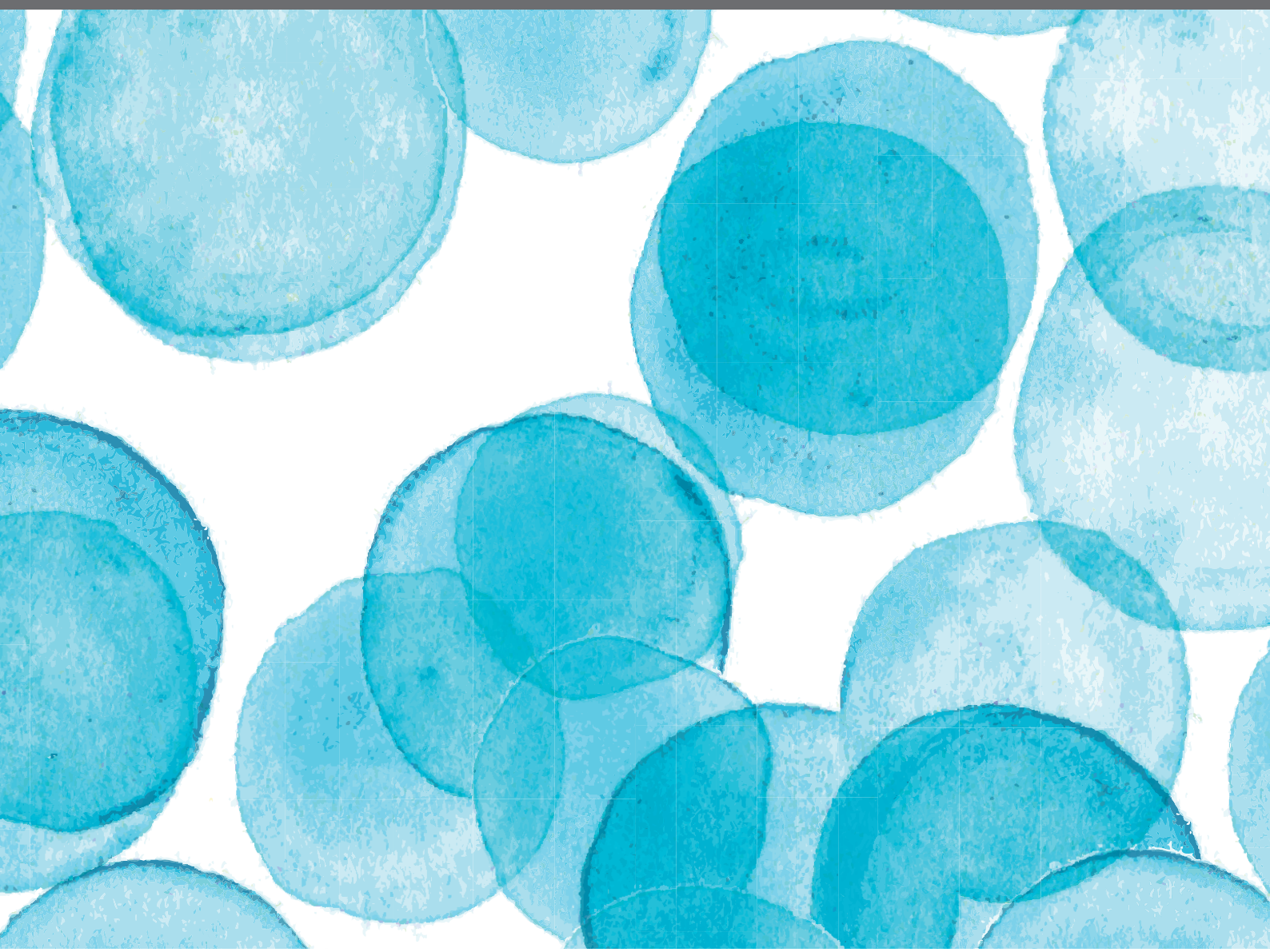

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INFANT GUT MICROBIOTA COLONIZATION AND FOOD IMPACT

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Preterm Gut Microbiome Depending on Feeding Type: Significance of Donor Human Milk

Anna Parra-Llorca[1], María Gormaz[1,2], Cristina Alcántara[3], María Cernada[1,2], Antonio Nuñez-Ramiro[1,2], Máximo Vento[1,2†] and Maria C. Collado[3*†]*

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Preterm microbial colonization is affected by gestational age, antibiotic treatment, type of birth, but also by type of feeding. Breast milk has been acknowledged as the gold standard for human nutrition. In preterm infants breast milk has been associated with improved growth and cognitive development and a reduced risk of necrotizing enterocolitis and late onset sepsis. In the absence of their mother's own milk (MOM), pasteurized donor human milk (DHM) could be the best available alternative due to its similarity to the former. However, little is known about the effect of DHM upon preterm microbiota and potential biological implications. Our objective was to determine the impact of DHM upon preterm gut microbiota admitted in a referral neonatal intensive care unit (NICU). A prospective observational cohort study in NICU of 69 neonates <32 weeks of gestation and with a birth weight ≤1,500 g was conducted. Neonates were classified in three groups according to feeding practices consisting in their MOM, DHM, or formula. Fecal samples were collected when full enteral feeding (defined as ≥150 cc/kg/day) was achieved. Gut microbiota composition was analyzed by 16S rRNA gene sequencing. Despite the higher variability, no differences in microbial diversity and richness were found, although feeding type significantly influenced the preterm microbiota composition and predictive functional profiles. Preterm infants fed MOM showed a significant greater presence of Bifidobacteriaceae and lower of Staphylococcaceae, Clostridiaceae, and Pasteurellaceae compared to preterm fed DHM. Formula fed microbial profile was different to those observed in preterm fed MOM. Remarkably, preterm infants fed DHM showed closer microbial profiles to preterm fed their MOM. Inferred metagenomic analyses showed higher presence of *Bifidobacterium* genus in mother's milk group was related to enrichment in the Glycan biosynthesis and metabolism pathway that was not identified in the DHM or in the formula fed groups. In conclusion, DHM favors an intestinal microbiome more similar to MOM than formula despite the differences between MOM and DHM. This may have potential beneficial long-term effects on intestinal functionality, immune system, and metabolic activities.

Keywords: preterm infant, their mother's own milk, donor human milk, formula milk, intestinal colonization, microbiota

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Abbreviations: BPD, bronchopulmonary dysplasia; DHM, donor human milk; FM, special preterm formula; HM, human milk; LOS, late onset sepsis; MOM, mother's own milk; NEC, necrotizing enterocolitis; NICU, neonatal intensive care unit; PVL, periventricular leukomalacia; ROP, retinopathy of prematurity.

INTRODUCTION

In preterm infants, neonatal microbial dynamics and alterations in early gut microbiota may precede and/or predispose to diseases such as NEC or LOS (The European Perinatal Health Report, 2010). In the newborn period differential microbial colonization clearly relates to weeks of gestation and mode of delivery (Lehtonen et al., 2017), but also to infant nutrition (Collado et al., 2015).

Human milk is the gold standard for infant nutrition in the first 12 months of life for term and preterm newborn infants (American Academy of Pediatrics, 2012). Beyond nutritional components, HM contains important bioactive compounds such oligosaccharides, cytokines, immunoglobulins, microbes, and proteins among others that directly influence the developing infant and shape the intestinal microbiota colonization. Those bioactive compounds are considered not only protective but also stimulate the development and maturation of the immature immune system (Agostoni, 2010; Ballard and Morrow, 2013). Moreover, breastfeeding practices have been associated with a risk reduction of NEC and LOS in preterm infants (Meinzen-Derr et al., 2009; Collado et al., 2012) and an improvement in growth and cognitive development and modulating metabolic and inflammatory conditions in later childhood and adulthood (Ballard and Morrow, 2013; Belfort et al., 2016). However, often mothers who deliver preterm are not able to successfully breastfeed (Wilson et al., 2017). In the absence of MOM, DHM has become the preferred alternative for preterm infants (ESPGHAN Committee on Nutrition et al., 2013). Despite the beneficial effects of DHM, little is known about its effect upon preterm gut microbiota colonization and its potential biological implications. Most HM bank guidelines recommended Holder pasteurization (62.5°C for 30 min) in order to inactivate viral and bacterial agents (Human Milk Banking Association of North America, 2000; Arslanoglu et al., 2010; National Institute for Health and Clinical Excellence, 2010; Peila et al., 2016). However, HM pasteurization causes the loss of several of the structural and functional properties of HM (Baro et al., 2001). Moreover, pasteurization also significantly reduces the cellular and bacterial constituents, enzymatic activities, and IgA, lactoferrin and lysozyme contents (Untalan et al., 2009; Christen et al., 2013; Espinosa-Martos et al., 2013; Sousa et al., 2014). Contrarily, other components with biological relevance such as oligosaccharides, nucleotides, and polyunsaturated and long chain fatty acids (LCPUFA) are preserved (Bertino et al., 2008; Coscia et al., 2015). As a consequence, pasteurization is still a matter of debate (Bertino et al., 2009; ESPGHAN Committee on Nutrition et al., 2013; Corpeleijn et al., 2016; Madore et al., 2017).

In this scenario, we hypothesized that DHM would promote a specific microbiota profile similar to the observed in the preterm infants who receive MOM. To pursue this objective we analyzed the impact of different nutritional approaches upon the gut microbiota composition of preterm infants born at ≤32 weeks of gestation.

MATERIALS AND METHODS

Study Design

We conducted a prospective, observational unicentric cohort study including consecutively admitted preterm infants born at ≤32 weeks of gestation and birth weight ≤1,500 g in the Division of Neonatology of the University and Polytechnic Hospital La Fe (Valencia, Spain) during a 12-month period. The study protocol was approved by the hospital IRB (Comité de Ética e Investigación Médica) and parents approved and signed informed consent in all cases.

Patients' Characteristics

Inclusion and exclusion criteria are shown in **Table 1**. Demographic, perinatal, clinical, and analytical data were recorded and matched according to the type of feeding (**Table 2**). Administration and duration of antibiotic therapy was also collected.

Fresh DHM was collected and immediately frozen at −20°C until Holder pasteurization process (62.5°C for 30 min followed by fast cooling). After treatment, pasteurized DHM was frozen until the distribution to patients. The Division of Neonatology protocol involves strong support to breastfeeding and offering DHM as a supplement to preterm infants born below ≤32 weeks or ≤1,500 g birth weight. Type of feeding was a parents' decision. The infants were fed at least with an 80% of either MOM or DHM and table intakes of 150 cc/kg/day.

The nutritional intake was prospectively monitored but never influenced by this observational study.

Fecal Samples, DNA Extraction, and 16S rDNA Sequencing

Fecal samples were directly collected from the diaper when full enteral feeding (defined as ≥150 cc/kg/day of MOM, DHM, or formula) was achieved. Samples were frozen and stored at −80°C for later analysis.

Total fecal DNA was isolated using the MasterPure Complete DNA & RNA Purification Kit (Epicentre, Madison, WI,

TABLE 1 | Inclusion and exclusion criteria for preterm infants receiving different types of nutrition and whose microbiota was studied.

Inclusion criteria	Exclusion criteria
BW ≤ 1.500 g and/or GA ≤ 32 weeks	GA > 32 weeks
Enteral intake (≥ 150 mL/kg/day)	Parents refuse to participate/sign informed consent
The principal nutrient received (MOM, DHM, or formula) represents 80% of the intake	Mixed breastfeeding
DHM from just one donor to one premature or maximum of two different donors	Chromosomopathies
No additional treatments that could alter the microbiota (e.g., probiotics) or oxidative status (e.g., vitamins C, A, and E)	Major malformations or surgery of the digestive tract

GA, gestational age; BW, birth weight; MOM, mother's own milk; DHM, donor human milk.

TABLE 2 | Perinatal characteristics and confounders of preterm infants receiving different types of nutrition and whose microbiota was studied.

	MOM (*n* = 34)	DHM (*n* = 28)	*p*-Value
GA weeks, mean (SD)	28.85 (1.9)	29.78 (2.42)	0.09
Antenatal steroids full course (%)	97.1	92.8	0.44
Type of delivery (%)			
Vaginal	58.8	39.3	0.126
Cesarean section	41.2	60.7	
Birth weight (g), mean (SD)	1,228 (301)	1304.3 (262)	0.3
Race (%)			
Caucasian	85.3	67.8	0.1
Non-Caucasian	14.7	32.1	
Apgar 1 min (median; 5–95% CI)	7.3 (2.15)	7.1 (1.81)	0.68
Apgar 5 min (median; 5–95% CI)	9.02 (1.3)	8.6 (1.4)	0.24
Age (days) at sample collection, mean (SD)	9.7 (7.03)	8.7 (6.2)	0.52
Chorioamnionitis (%)	76.4	89.2	0.19
Mechanical ventilation	11.7	14.3	0.76
Non-invasive ventilation	75	85.3	0.3
Persistent ductus arteriosus	29.4	25	0.69
Antibiotic therapy (%)	38.2	39.2	0.93

United States) according to the manufacturer's instructions with modifications that included a bead-beater step and enzyme incubation to increase DNA extraction as described elsewhere (Boix-Amoros et al., 2016). Total DNA concentration was measured using a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, United States) and normalized to 5 ng/μL for 16S rDNA gene (V3–V4 region) amplification using Nextera XT Index Kit. Amplicons were checked with a Bioanalyzer DNA 1000 chip and libraries were sequenced using a 2 × 300 bp paired-end run (MiSeq Reagent kit v3) on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain). Controls during DNA extraction and PCR amplification were also included and sequenced.

Bioinformatics and Statistical Analysis

Data were obtained using an *ad hoc* pipeline written in RStatistics environment (R Core Team, 2012) and data processing was performed using a QIIME pipeline (version 1.9.0) (Caporaso et al., 2010). Chimeric sequences and sequences that could not be aligned were also removed from the data set. The clustered sequences were utilized to construct operational taxonomic units (OTUs) tables with 97% identity and representative sequences were taxonomically classified based on the Greengenes 16S rRNA gene database (version 13.8). Sequences that could not be classified to domain level, or classified as cyanobacteria and chloroplasts, were removed from the data set. Subsequently, alpha diversity indices (Chao1 and Shannon, Species richness estimates and diversity index, respectively), beta diversity based on UNIFRAC unweighted distance (phylogenetic) and Bray–Curtis distance (non-phylogenetic), and PERMANOVA with 999 permutations was used to test significance between groups. The DESeq2 method was used to determine differential abundances of specific bacteria between feeding groups. Calypso software[1] version 7.36 was used with total sum normalization for the statistical analysis, and also, cumulative sum scaling (CSS) normalization for multivariate test. Furthermore, predictive inferred functional analysis was performed using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) approach as described (Langille et al., 2013). Linear discriminant analysis effect size (LEfSe) (Segata et al., 2011) was used to detect unique biomarkers in relative abundance of bacterial taxonomy and specific functions (KEGG pathways). A size effect cut-off of 3.0 on the logarithmic linear discriminant analysis (LDA) score was used. The 16S rRNA gene sequence data generated is available through GenBank Sequence Read Archive Database under project accession number PRJEB25948.

RESULTS

Patients' Characteristics

A total of 69 preterm infants ≤32 weeks of gestation pertaining to the MOM (*n* = 34), DHM (*n* = 28), and formula (*n* = 7) groups were recruited. No differences for prenatal demographic characteristics or confounders during the hospitalization between patients in the three feeding-type groups were found (**Table 2**). Individual information is available in Supplementary Table S1.

A small group of preterm neonates (*n* = 7) fed with formula milk was also included. The low number is explained because the protocol of our NICU recommends for all preterm ≤32 weeks of gestation and ≤1,500 g MOM and DHM as an alternative and therefore preterm fed with formula render exceptional. Characteristics of the formula group were as follows: 33 ± 2 weeks of gestation; 1,702 ± 321.6 g birth weight; 57.1% male; 14.3% born by vaginal delivery (85.7% by cesarean section); 57.1% received antenatal steroids.

Impact of DHM on Preterm Microbiota

Significant differences were found in preterm microbiota composition according to feeding type. We found lower relative abundance of Firmicutes (30.9 vs 45.5%, *p*-value = 0.029) and higher abundance of Actinobacteria (20.1 vs 10.2%, *p*-value = 0.040) in MOM compared to DHM group (**Figure 1A**). At family level, higher abundance of Bifidobacteriaceae (19.5 vs 9.0%, *p*-value = 0.027) and lower abundance of Clostridiaceae (3.7 vs 11.2%, *p*-value = 0.029) were observed in MOM compared to DHM (**Figure 1B**). At genus level, higher levels of *Bifidobacterium* (19.5 vs 8.98%, *p*-value = 0.027) and unclassified Enterobacteriaceae (29.77 vs 18.48%, *p*-value = 0.060) and lower levels of *Citrobacter* (2.60 vs 9.83%, *p*-value = 0.060), and unclassified Clostridiaceae (3.46 vs 9.43%, *p*-value = 0.062) were

[1] http://cgenome.net/calypso/

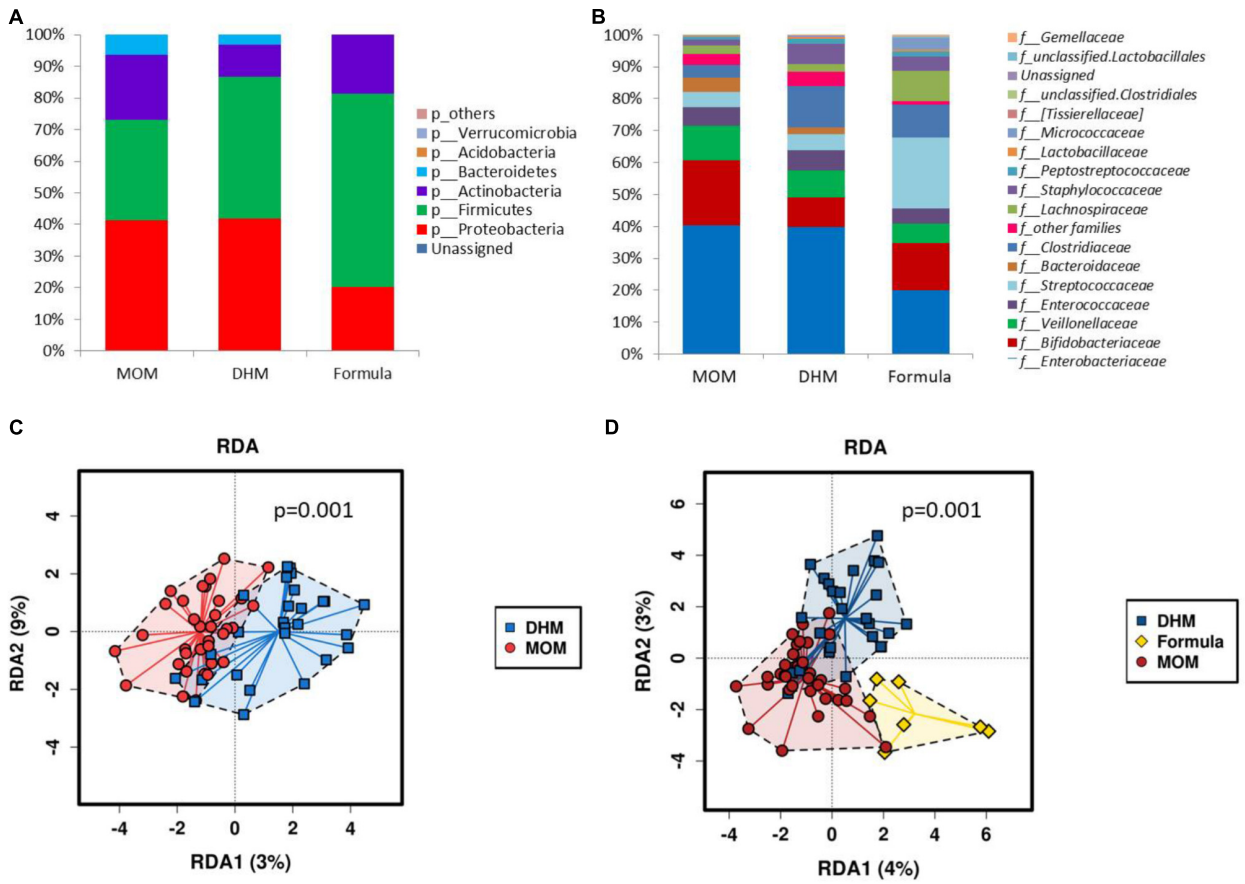


FIGURE 1 | Microbial relative abundances (%) at phylum **(A)** and family level **(B)** of preterm gut microbiome according to diet (MOM, DHM, and formula). RDA plots of the preterm microbiota grouped by infant feeding type: MOM vs DHM **(C)** and MOM, DHM, and formula **(D)**.

observed in fecal samples of MOM as compared to the DHM group.

The effect of the diet on the preterm gut microbiota was explored by applying the multivariate method PERMANOVA with 999 permutations on the phylogenetic distances (p-value = 0.09 for unweighted UNIFRAC distance) and Bray–Curtis distance (non-phylogenetic; p-value = 0.0046). Furthermore, multivariate redundant discriminant analysis (RDA) based on the observed OTUs showed statistically significant differences in microbial composition between MOM and DHM groups (p = 0.001) (**Figure 1C**).

To explore the variation of the microbial community composition between MOM and DHM, we performed LEfSe tests to detect differences in relative abundance of bacterial taxa across fecal samples (**Figure 2**). At the family level, Bifidobacteriaceae family was significantly enriched in MOM compare to DHM samples (LDA = 4.90, p-value = 0.025) while Staphylococcaceae (LDA = 4.63, p-value $\leq$0.042). Pasteurellaceae family was enriched in DHM (LDA = 4.358, p-value = 0.050). Specific enriched features at genus and OTUs levels are shown in **Figure 2**.

DESeq2 test was used to identify differential abundances of specific bacteria between feeding groups. Actinobacteria phylum was higher in MOM compared to DHM group (20.07 vs 10.25%, p-value = 0.0044, FDR = 0.013). The abundance of *Staphylococcus* (p-value <0.0001, FDR < 0.0001), *Clostridium* (p-value <0.0001, FDR = 0.0013), *Serratia* (p-value <0.0001, FDR = 0.0022), *Coprococcus* (p-value = 0.0021, FDR = 0.012), *Aggregatibacter* (p-value = 0.015, FDR = 0.059), and *Lactobacillus* (p-value = 0.056, FDR = 0.18) was significantly higher in DHM group than MOM group. However, *Bacteroides* (p-value <0.0001, FDR = 0.0044), *Acinetobacter* (p-value <0.0001, FDR = 0.002), and *Haemophilus* (p-value = 0.0014, FDR = 0.009) were significantly higher in the MOM than in DHM group.

Impact of Formula vs Human Milk Groups on Preterm Microbiome

Despite the low number of formula fed preterm infants, we analyzed the differences in microbiome between MOM, DHM, and FM groups. A multivariate RDA based on the observed OTUs showed statistically significant differences in microbial composition between groups (p = 0.001) (**Figure 1D**). Significantly higher relative abundance of Firmicutes (p-value = 0.027, FDR = 0.016) was observed in FM group compared to MOM and DHM

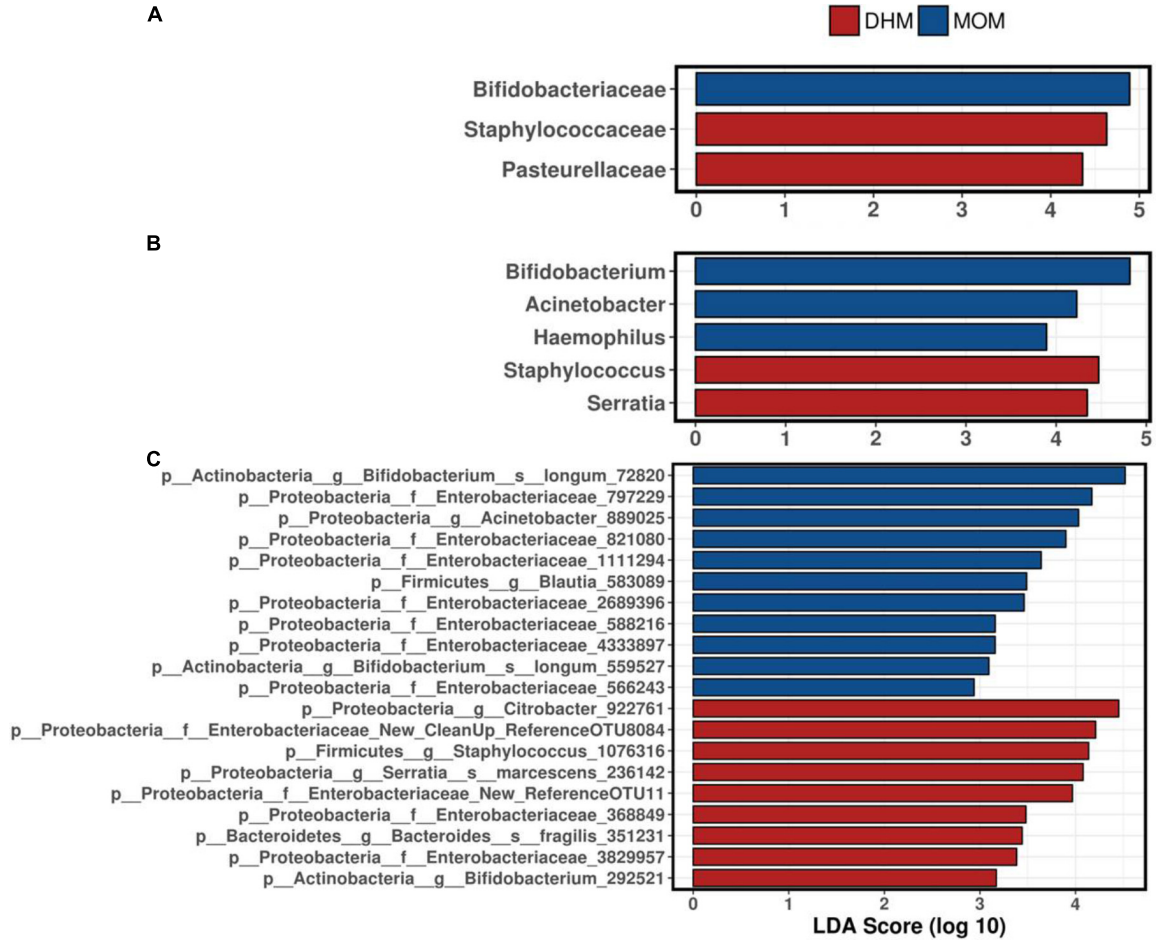


FIGURE 2 | Linear discriminant analysis (LDA) combined with effect size measurements (LEfSe) revealed specific microbes at family **(A)**, genus **(B)**, and OTUs **(C)** between MOM and DHM feeding groups.

(**Figure 1A**). At genus level, significant higher abundance of *Blautia* (p-value <0.001, adjusted p-value = 0.033, FDR = 0.033), *Streptococcus* (p-value = 0.0024, FDR = 0.054), *Acidaminococcus* (p-value = 0.0093, FDR = 0.099), *Rothia* (p-value = 0.0059, FDR = 0.088), and *Dorea* (p-value = 0.011, FDR = 0.099) were observed in the FM group compared to the MOM and DHM groups.

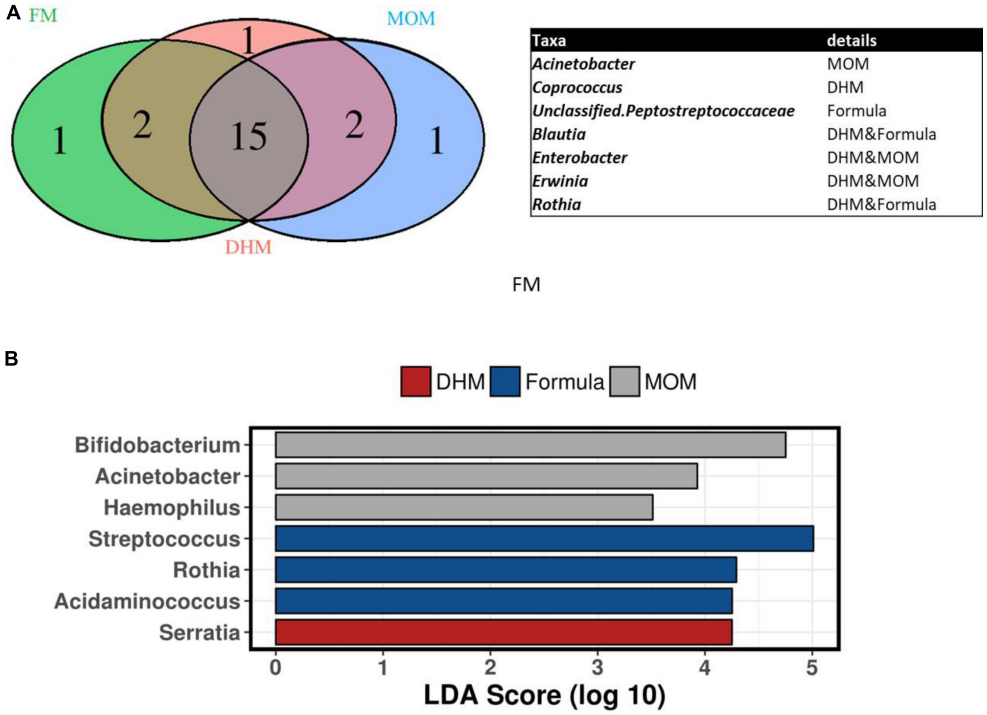
Preterm core microbiome was composed by a total of 15 shared genus independently of feeding-type diet (**Figure 3A**). *Acinetobacter* genus was exclusively present in MOM group; while *Coprococcus* genus was present in DHM and unclassified Peptostreptococcaceae genus in formula group.

Linear discriminant analysis effect size test showed that *Rothia*, *Streptococcus*, and *Acidaminococcus* genus were significantly enriched in formula group compared to MOM and DHM group, while *Bifidobacterium*, *Acinetobacter*, and *Haemophilus* genus were enriched significantly in MOM compared to DHM, representing a hallmark for breast fed preterm gut microbiota (**Figure 3B**).

We also applied discriminant analysis of principal components (DAPC) identifying specific feeding-type related clusters of preterm microbiota (**Figure 4**). These microbial shifts were attributed to subtle changes in the abundance of several bacterial OTUs. *Clostridium*, *Bifidobacterium*, unclassified Enterobacteriaceae and *Veillonella* related OTUs were the strongest indicator of the presence of distinct microbial clusters.

Functional Assignment of the Preterm Microbiota

Inferred metagenomic PICRUSt prediction revealed significant differences in the main functional classes (Kyoto Encyclopedia of Genes and Genomes, KEGG categories at level 2), deriving from functional acquisitions associated with different diets (multivariate RDA test, p = 0.007) (**Figure 5A**). Moreover, no different metabolic profile was found (RDA test p-value >0.05) when MOM and DHM were compared, while formula functional profile was significantly different from those observed in MOM (RDA test p-value = 0.024) and DHM (RDA test p-value = 0.002).



Taxa	details
Acinetobacter	MOM
Coprococcus	DHM
Unclassified.Peptostreptococcaceae	Formula
Blautia	DHM&Formula
Enterobacter	DHM&MOM
Erwinia	DHM&MOM
Rothia	DHM&Formula

FIGURE 3 | Venn diagram of shared genus between group **(A)** and linear discriminant analysis LEfSe test at genus level between MOM, DHM, and formula preterm microbiota **(B)**.

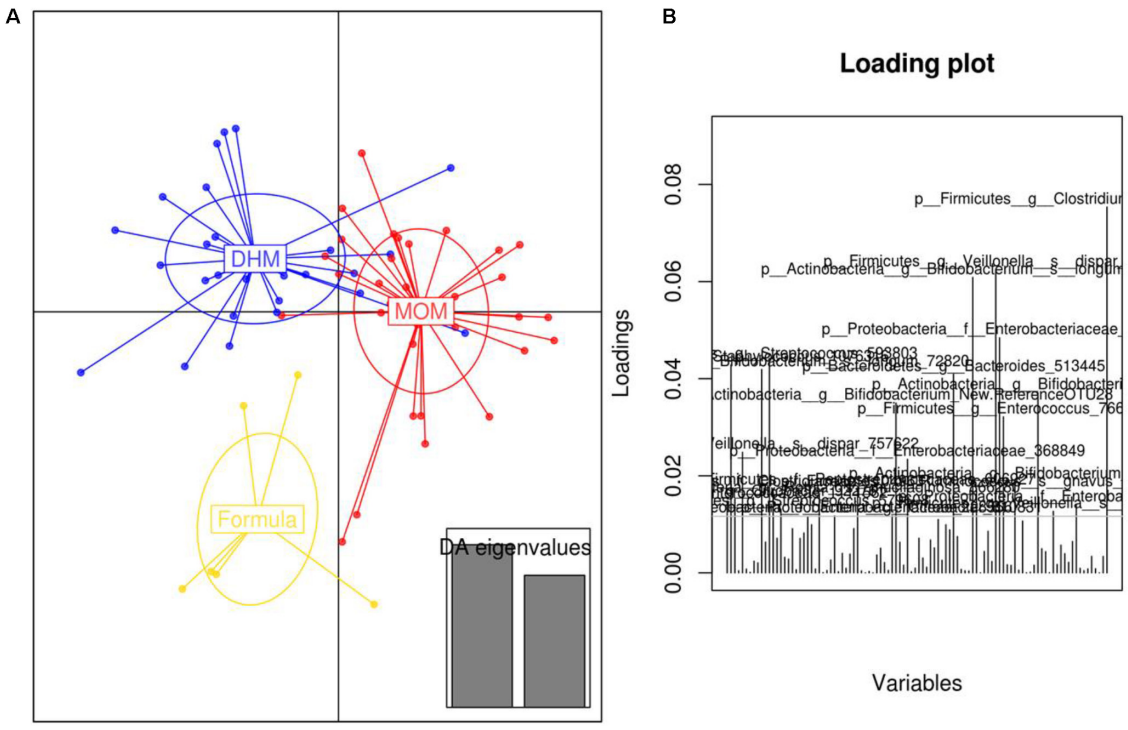


FIGURE 4 | Discriminant analysis of principal components (DAPC) plot **(A)** at OTUs level revealed distinct clustering of the MOM (*red*), DHM (*blue*), and formula-fed group (*yellow*). Canonical loading plot **(B)** showing differentially abundant bacterial genera. The individual peaks show the magnitude of the influence of each variable on separation of the groups (0.05 threshold level).

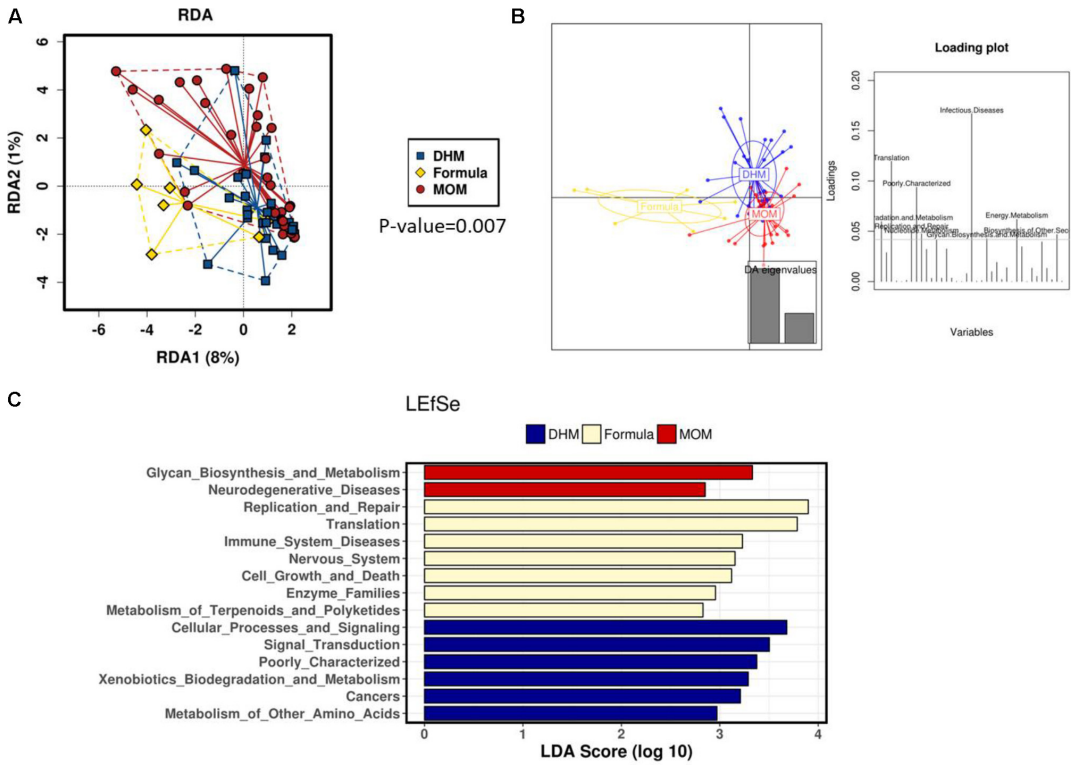


FIGURE 5 | RDA plot **(A)**. Discriminant analysis of principal components (DAPC) plot and loading plot **(B)** at KEGG pathways. The individual peaks in the loading plot show the magnitude of the influence of each variable on separation of the groups (0.05 threshold level). LEfSe test **(C)** revealed distinct clustering of the MOM (*red*), DHM (*blue*), and formula-fed group (*yellow*).

The DAPC to identify specific clusters of functional activity (KEGG level 2 and 3) of the gut microbiome in preterm groups according to type of diet (**Figure 5B**) suggesting a distinct KEGG activities DHM and MOM microbiome activities are similar than those observed in the FM group.

Linear discriminant analysis effect size analysis performed on PICRUSt output showed several KEGG (level 2, **Figure 5C** and level 3, Supplementary Figure S1) categories differentially present in the MOM, DHM, and formula groups. MOM functional profile is mostly represented by bacterial secretion system, lipopolysaccharide (LPS) biosynthesis, and biosynthesis. In particular, we observed a deprivation in functions involved in complex carbohydrate metabolism, deriving from HMO present in breast milk, such us Glycan biosynthesis and metabolism in the formula group (p-value = 0.019) compared to the other MOM and DHM groups. This glycan pathway was not different between MOM and DHM profiles (p-value >0.05). Interestingly, we observed a reduced LPS biosynthesis (p-value <0.0001) and LPS biosynthesis proteins (p-value <0.0001) in formula fed infants compared to MOM and DHM (Supplementary Figure S2). However, the most predominant function in DHM group was the two-component regulatory system followed by other functions related to amino acid metabolisms, fatty acids metabolisms (butanoate metabolism) and to sulfur metabolism and sulfur relay system and also, nitrogen metabolism in DHM group (Supplementary Figure S2). Methane metabolism pathway is also enriched in formula group as compared to MOM and DHM groups (Supplementary Figure S2). Furthermore, we also observed in formula group an enrichment of KEGG functions related to sugar metabolisms as galactose metabolism, and amino sugar and nucleotide sugar metabolisms compared to the observed ones in MOM and DHM (Supplementary Figure S2).

DISCUSSION

In recent years, nutritional practices have shifted toward encouraging breastfeeding practices in preterm neonates (Keunen et al., 2015; O'Connor et al., 2016). In the absence of MOM, DHM has become the preferred nutritional alternative and a formula feeding remains the last option when the others are not available. The setting up of milk banks has rendered DHM the most widely prescribed alternative to MOM (Underwood, 2013).

In preterm infants, our results have demonstrated that the feeding type has an important impact on gut microbial composition in preterm infants ≤1,500 g. We found that MOM and DHM microbial profiles were different. MOM fed babies showed a significantly enriched and greater presence of Bifidobacteriaceae and lower of Staphylococcaceae,

Clostridiaceae, and Pasteurellaceae compared to DHM fed babies. At genus level, higher levels of *Bifidobacterium* and unclassified Enterobacteriaceae and lower unclassified Clostridiaceae were observed in fecal samples from MOM group compared to DHM preterm group. It has been reported that preterm infant receiving MOM had a higher abundance of Clostridiales, Lactobacillales, and Bacillales compared to both the DHM and formula groups (Gregory et al., 2016). Both these groups had higher abundance of Enterobacteriales. After controlling for gender, postnatal age, weight, and birth gestational age, the diversity of gut microbiota increased over time and was constantly higher in infants fed MOM relative to infants with other feeding types. Finally, in the formula microbial profile was distinct than those observed in MOM and DHM, suggesting that DHM favors an intestinal microbiome more similar to MOM despite the differences between MOM and DHM.

In accordance to the microbiota shifts, we observed that KEGG profiles in DHM and MOM were similar than those microbial profile observed in formula. MOM functional profile is mostly represented by bacterial secretion system, LPS biosynthesis and biosynthesis protein which would be mainly related to the presence of Gram-negative bacteria. Interestingly, we observed a significant reduction on the LPS biosynthesis and proteins in formula fed infants compared to MOM and DHM (without difference between them). These data would suggest the potential link between LPS and immune response as reported previously (Cullen et al., 2015). Recent study has been shown that variation on the microbial LPS produced by specific microbiota groups as Enterobacteriaceae and *Bacteroides* spp., could either stimulate or actively inhibit inflammatory pathway and also, have a role on the autoimmune diseases risk (Vatanen et al., 2016). In our context, MOM and DHM modulate a preterm gut microbiota toward an enrichment in *Bifidobacterium* spp. and also, *Bacteroides* spp. that may promote the specific LPS signaling and its contribution to the immune system. Furthermore, we observed a significant reduction in functions derived from the presence of HMO and involved in complex carbohydrate metabolism (e.g., glycan biosynthesis) in the FM group as compared with the MOM and DHM groups. These differences could be explained by the abundance of HMO metabolizers as *Bifidobacterium* and *Bacteroides* spp. in preterm gut fed with MOM and DHM compared to formula fed preterm.

We also found enrichment on the functions related to the fatty acids metabolism and to sulfur and nitrogen metabolism in DHM and MOM groups. Several enteric bacteria and oral bacteria produce reduced sulfur and nitrogen and maybe some specific bacteria, as Deltaproteobacteria, *Clostridium* spp., *Veillonella* spp., *Rothia* spp., would be responsible for this functional contribution as they were enriched in DHM group although it was also observed in MOM group. In formula metagenome, we observed enrichment of KEGG functions related to sugar metabolisms as galactose metabolism, involved in conversion of galactose into glucose could arise from consumption of infant formula and/or dairy products, and amino sugar and nucleotide sugar metabolisms. In general, minor differences were observed in the functional profiles between MOM and DHM suggesting the potential effect of DHM in mimicking the microbiome functionality of own maternal milk feeding. These results would open new possibilities in future research where bigger studies should be carried out.

Two important factors influence the differences found in preterm gut microbiota depending on feeding type (MOM and DHM). The first one would be related to the timing of milk extraction in relation to gestational age and to the lactation stage. While MOM is the biological product of a prematurely interrupted gestation, DHM is composed mainly by donated mature milk from mothers who completed term gestations and were extracting milk for several weeks thereafter. Preterm milk has higher amount of proteins, fats, and energy (Underwood, 2013; Gidrewicz and Fenton, 2014). Hence, depending on the staging of lactation they can be also a great variability among donators of components such as essential fatty acids or amino acids indispensable not only for an adequate nutrition but also for promoting microbiota colonization. Although donor milk pooling tries to avoid these circumstances, a recent study has shown a shortage of docosahexaenoic acid or lysine in DHM. Targeted supplementation would be needed not only to optimize nutritional properties of DHM but also to improve bacterial colonization (Ballard and Morrow, 2013). The second factor would be related with the pasteurization procedure that inevitably alters essential thermolabile milk components. Hence, differences in the microbiota would be at least partially explained by the different composition in relation to nutritional parameters and bioactive compounds such as immune markers, microbiota, oligosaccharides, neurotrophic, and growth factors among others (Bertino et al., 2008; Untalan et al., 2009; Ballard and Morrow, 2013; Christen et al., 2013; Espinosa-Martos et al., 2013; Sousa et al., 2014; Coscia et al., 2015; Valentine et al., 2017). Altogether these findings could explain the similarities and differences in the microbiota profile between preterm infants fed DHM or MOM and influence health outcomes in preterm infants.

Finally, the number of formula fed infants included in this study was limited (n = 7) due mainly because milk bank provides with DHM to almost all preterm babies attended in our NICU (>90%) and therefore it is difficult to recruit preterm babies on formula feeding and randomization was not ethically acceptable. Despite these limitations, our results reveal a substantial impact of DHM feeding on the structure of the intestinal microbial community composition.

CONCLUSION

Feeding type modulates the preterm microbiome composition. DHM feeding had an impact on preterm microbiota that could have potential beneficial long-term effects on intestinal

functionality, immune system, and metabolism. However, available pasteurization methods cause changes that may blunt many of the positives aspects derived from the use of MOM. Therefore, further studies are stringently need to understand the complex links between microbiome and DHM host, its impact on health programming, and to develop sensitive methods capable of providing promptly after birth preterm infants with HM as similar as possible to their MOM when the latter is not yet available.

AUTHOR CONTRIBUTIONS

AP-L, MG, MC, MCC, and MV planned the experiments. AP-L, MG, MC, AN-R collected the samples and clinical data. AP-L, CA, and MCC performed the microbiological analysis and analyzed data. AP-L wrote first draft and all authors commented criticized, and reviewed the manuscript. All authors accepted the final version the manuscript.

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SUPPLEMENTARY MATERIAL

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Inefficient Metabolism of the Human Milk Oligosaccharides Lacto-*N*-tetraose and Lacto-*N*-neotetraose Shifts *Bifidobacterium longum* subsp. *infantis* Physiology

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Human milk contains a high concentration of indigestible oligosaccharides, which likely mediated the coevolution of the nursing infant with its gut microbiome. Specifically, *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) often colonizes the infant gut and utilizes these human milk oligosaccharides (HMOs) to enrich their abundance. In this study, the physiology and mechanisms underlying *B. infantis* utilization of two HMO isomers lacto-*N*-tetraose (LNT) and lacto-*N*-neotetraose (LNnT) was investigated in addition to their carbohydrate constituents. Both LNT and LNnT utilization induced a significant shift in the ratio of secreted acetate to lactate (1.7–2.0) in contrast to the catabolism of their component carbohydrates (~1.5). Inefficient metabolism of LNnT prompts *B. infantis* to shunt carbon toward formic acid and ethanol secretion. The global transcriptome presents genomic features differentially expressed to catabolize these two HMO species that vary by a single glycosidic linkage. Furthermore, a measure of strain-level variation exists between *B. infantis* isolates. Regardless of strain, inefficient HMO metabolism induces the metabolic shift toward formic acid and ethanol production. Furthermore, bifidobacterial metabolites reduced LPS-induced inflammation in a cell culture model. Thus, differential metabolism of milk glycans potentially drives the emergent physiology of host-microbial interactions to impact infant health.

Keywords: bifidobacteria, human milk oligosaccharides, lacto-*N*-tetraose, lacto-*N*-neotetraose, host-microbial interactions, microbiota

INTRODUCTION

Breastfeeding is critical for infant development and health in the absence of formula milk substitutes. Human milk contains a high concentration of indigestible oligosaccharides, as well as other nutritive molecules that promote growth experienced early in life (1–5). Human milk oligosaccharides (HMOs), are indigestible carbohydrates soluble in human milk, and are composed

of five monosaccharides: D-glucose (Glc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc), L-fucose (Fuc), and *N*-acetylneuraminic acid (Neu5Ac or sialic acid) with a varying degree of polymerization and branching (6–9). These oligosaccharides are not directly metabolized by the infant; however, commensal bifidobacteria have coevolved within the nursing infant gut to utilize HMO (10, 11).

Bifidobacterium longum subsp. *infantis* (*B. infantis*) colonizes the nursing infant and is typically overrepresented within the infant gut microbiome (12–15). The *B. infantis* genome encodes an array of glycosyl hydrolases, oligosaccharide transporters, and catabolic enzymes that enable HMO utilization (10, 16–19). Both glycosyl hydrolases and membrane-spanning transporters feed milk oligosaccharides and their derivatives into the bifidobacterial fructose-6-phosphate phosphoketolase (F6PPK) central fermentative pathway. The F6PPK is believed to be unique to the genus *Bifidobacterium,* which generates ATP from hexoses via substrate-level phosphorylation resulting in the secretion of endproducts to recycle co-factors (20–23). Bifidobacteria initially converts one mole of fructose-6-phosphate to one mole of erythrose 4-phosphate and one mole of acetyl-phosphate via F6PPK (EC 4.1.2.22). A transaldolase (EC 2.2.1.2) and transketolase (EC 2.2.1.1) converts erythrose 4-phosphate and one mole of fructose-6-phosphate to two moles of xylulose-5-phosphate, which are converted into two moles of acetyl-phosphate and glyceraldehyde 3-phosphate via xylulose-5-phosphate phosphoketolase activity (EC 4.1.2.9). Acetyl-phosphate is dephosphorylated into acetate by an acetate kinase (EC 2.7.2.1), accompanied by a single ATP per acetyl-phosphate (24). In addition, glyceraldehyde 3-phosphate is oxidized to pyruvate accompanied by the production of a single ATP. Pyruvate is converted to lactate by lactate dehydrogenase (EC 1.1.1.27) along with recycling NAD^+ from NADH (25). For every 2 moles of hexose entering the F6PPK pathway, 3 moles of acetate and 2 moles of lactate are produced (i.e., ratio of 1.5). Based on transcriptomic evidence, it is likely that *B. infantis* catabolizes HMO-derived monosaccharides through the F6PPK pathway to generate ATP (26). This potentially links bifidobacterial physiology (i.e., flux through the F6PPK pathway) with infant nutritional and health outcomes as *B. infantis* benefits their developing host (27, 28).

In general, all *B. infantis* strains examined to date efficiently utilizes HMOs pooled from several donor mothers with the exception of one (29–34). The tetrasaccharides lacto-*N*-tetraose (LNT) and lacto-*N*-neotetraose (LNnT) are highly abundant oligosaccharides secreted in human milk (2, 6). LNT (Galβ1-3GlcNAcβ1-3Galβ1-4Glc) is classified as a type I HMO, which incorporates lactosyl coupled to a lacto-*N*-biose (LNB) (Galβ1-3GlcNAc) residue. In contrast, LNnT (Galβ1-4GlcNAcβ1-3Galβ1-4Glc) is an isomer of LNT and classified as a type II oligosaccharide, linking the terminal lactosyl with *N*-acetyllactosamine (LacNAc) (Galβ1-4GlcNAc). These isomers are identical aside from a sole glycosidic linkage (i.e., β1-3 vs. β1-4) thus leading to the hypothesis that this structural variation is responsible for differential phenotypes in bifidobacterial utilization of these major HMOs.

MATERIALS AND METHODS

Bacterial Strains and Propagation

Bacterial strains used in this study are summarized in **Table 1**. Bifidobacterial strains were propagated in De Man Rogosa Sharp (MRS, Oxoid, Hampshire, England) medium supplemented with 0.05% (wt/v) L-cysteine hydrochloride (Sigma-Aldrich, St. Louis, MO) (35) at 37°C under anaerobic conditions (Coy Laboratory Products, Grass Lake, MI). Bacterial strains were routinely verified using the bifidobacterial-specific phosphoketolase assay (36) and bifidobacterial-specific PCR targeting the 16S rRNA gene sequence using the previously developed Bif164-F (5′-GGGTGGTAATGCCGGATG-3′) and Bif662-R 5′-CCACCGTTACACCGGGAA-3′) (37). In addition, the PCR-based *Bifidobacterium longum*/*infantis* ratio analysis (BLIR) was performed to verify subspecies as previously described (38).

Microplate Growth Assay

In order to evaluate growth phenotypes in a 96-well format, overnight cultures were inoculated 1% (v/v) to modified MRS media (mMRS; a defined carbohydrate substrate and no acetate). Carbohydrate substrates used in this study include glucose (Sigma-Aldrich Co. St. Louis, MO), galactose (Sigma-Aldrich Co. St. Louis, MO), lactose (Sigma-Aldrich Co. St. Louis, MO), L-fucose (Sigma-Aldrich Co. St. Louis, MO), *N*-acetylglucosamine (GlcNAc) (Tokyo Chemical Industry Co, Tokyo Japan), lacto-*N*-tetraose (LNT) (Elicityl-oligotech, Crolles, France), and lacto-*N*-neotetraose (LNnT) (Elicityl-oligotech, Crolles, France) at a final concentration of 2% (wt/v) as the sole carbon source. Carbohydrate sources were incorporated into culture media in non-limiting concentrations. The growth assay was conducted anaerobically at 37°C for 48 h by assessing optical density at 600 nm ($OD_{600\ nm}$) in an automated PowerWave HT microplate spectrophotometer (BioTek Instruments, Inc. Winooski, VT) placed within the anaerobic chamber. Each strain was evaluated in biological triplicates with three technical replicates. Inoculated mMRS media in the absence of carbohydrate substrates served as the negative control. Bacterial growth kinetics were calculated using Wolfram Mathematica 10.3 Student Edition with the equation below as described in Dai et al. (39).

$$\Delta OD\,(t) = \Delta ODasym\{\frac{1}{1+\exp\left[ktc-t\right]} - \frac{1}{1+\exp\left[ktc\right]}\}$$

TABLE 1 | List of strains used in this study[a].

Strain	Species	Origin
ATCC 15697	*B. longum* subsp. *infantis*	Human infant feces
UMA299	*B. longum* subsp. *infantis*	Human infant feces
UMA300	*B. longum* subsp. *infantis*	Human infant feces
UMA301	*B. longum* subsp. *infantis*	Human infant feces

[a]*UMA, University of Massachusetts Amherst Culture Collection; ATCC, American Type Culture Collection.*

ΔOD_{asym} is the growth level at stationary phase with k representing the growth rate and tc is the inflection point indicating the time to reach the highest growth rate.

Characterization of Microbial Metabolic Endproducts

Endproducts from bacterial fermentation were quantitated by HPLC. Bacterial strains were initially propagated as described above. Cell-free supernatants from microplate growths were obtained at early stationary phase and filtered through a 0.22 μm filter (Sartorius Corp, Bohemia, NY) following centrifugation and stored at −20°C until further analysis. Organic acids were quantified using a Schimadzu HPLC system equipped with a Refractive Index Detector 20A, (Schimadzu Corp., Kyoto, Japan). Separation was carried out using an Aminex HPX-87H column (7.8 mm ID × 300 mm, Bio Rad Laboratories, Hercules, CA) at 30°C in a mobile phase of 5 mM H_2SO_4 at flow rate of 0.6 ml/min with 20 μl of injection volume. Standards including organic acids (i.e., acetic acid, lactic acid, formic acid), ethanol, and carbohydrates (i.e., glucose, galactose, lactose, and GlcNAc) were acquired from Sigma-Aldrich Co. (St. Louis, MO). Metabolite concentrations were calculated from standard curves derived from external standards for six concentrations (0.5, 1, 5, 10, 20, and 50 mM). Metabolite profiling was carried out in triplicate and each measurement was performed in duplicate. The metabolite profiling for each strain subsisting on the panel of carbohydrates were analyzed using MetaboAnalyst 3.0 (http://www.metaboanalyst.ca) (40). The sugar consumption in percentages was calculated by dividing the amount of mono- and di-saccharide consumed after fermentation by the concentration of carbohydrate source prior to fermentation. The carbon recovery in percentages was calculated by dividing the total amount of carbon recovered in the metabolites by the total amount of carbon present in pre-fermentation minus total carbon of carbohydrate source after fermentation.

Quantitative Real-Time PCR Analysis

B. infantis gene expression was performed by quantitative real-time PCR (qRT-PCR) on a relative basis. One ml samples were harvested at mid-exponential phase ($OD_{600\ nm}$ ∼ 0.4–0.6 varied depending on carbohydrate source), pelleted at 12,000 × g for 2 min, and stored in 1 ml Ambion RNAlater (Life Technologies, Carlsbad, CA). RNA extraction and cDNA conversion was performed as previously described (26). Briefly, samples were centrifuged at 12,000 × g for 2 min to collect the cell pellet. The pellet was washed twice with PBS buffer to remove residual RNAlater and centrifuged at 12,000 × g for 2 min. Total RNA was extracted using Ambion RNAqeous-Mini kit (LifeTechnologies, Carlsbad, CA) according to the manufacturer's instructions. Cells suspended in lysis buffer were transferred to the Lysing Matrix E tubes (MP Biomedicals LLC, Solon, Ohio) to disrupt cell walls through beadbeating at 5.5 m/s for 30 s twice using FastPrep 24 bead beader (MP Biomedicals, Santa Ana, CA). Total RNA was eluted in 50 μl of EB solution and immediately subjected to DNase treatment with the Ambion Turbo DNA-free (Invitrogen, Vilnius, Lithuania) using 1 μl of DNase I for 30 min. Subsequently, total RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. The resultant cDNA was quantified by a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Agawam, MA). The qRT-PCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Singapore) with PowerUP SYBR Green Master Mix (Applied Biosystems, Foster City, CA) using 200 ng of input cDNA. The reaction conditions were informed by manufacturer recommendations and optimized for the specific target locus. qRT-PCR primers were designed using the Primer3 software (Table S1; http://frodo.wi.mit.edu). The gene Blon_0393, encoding a cysteinyl-tRNA synthetase was used as an endogenous control as previously (16, 41). Growth on lactose (2% wt/v) served as the reference condition for gene expression. Results were expressed as fold change relative to the reference. These experiments were conducted in triplicates and triplicate technical measurements were performed. Following DNase treatment, the absence of genomic DNA was confirmed using total RNA as template by qRT-PCR (i.e., endogenous control reaction).

Statistical Analyses

The relationships between asymptotic $OD_{600\ nm}$, growth rates and metabolites were characterized with principal components analysis (PCA) and hierarchical clustering with Ward's method and Euclidean distances using R (R.3.4.0). The outliers were determined according to their distance to the average within biological replicates were omitted to maintain at least biological triplicates. When no growth was observed in sugars, the values were assigned as "0" for PCA function(prcomp) analysis and PCA plots were drawn using ggbiplot in R. Growth kinetics, metabolite concentrations, fold change in gene expressions of cell culture for *B. infantis* ATCC 15697 were subjected to one-way analysis of variance (ANOVA) and Tukey's HSD test for multiple comparisons between carbohydrate source. The fold change in gene expression for *B. infantis*, growth kinetics, and metabolites between strains were analyzed with two-way ANOVA. The simple effects and main factor effects were determined with Tukey's HSD test for multiple comparisons of carbohydrate sources for the same strain and between strains for a defined carbohydrate source.

Bioinformatic Analysis of Transcriptome Data

Transcriptomic data (i.e., raw reads) of *B. infantis* ATCC 15697 while growing on lactose, LNT, and LNnT was retrieved from a previously performed RNA-seq study (26) publically deposited in the NCBI Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE58773 (and personal communication with Danielle Lemay). This data was uploaded to the Massachusetts Green High Performance Computing Cluster (MGHPCC) that was used for all computational/statistical analyses unless specifically noted. The RNA-seq reads were aligned to the reference *B. longum* subsp. *infantis* ATCC 15697 genome (NC_011593.1). Coding

regions of the ATCC 15697 genome were subjected to this analysis. Total and unique gene reads aligning to a specific genomic locus (i.e., locus tag), as well as calculated raw counts was obtained for differential expression analysis.

Differential Gene Expression

In order to identify and quantify the magnitude of differentially expressed genes, the R package DESeq2 was used to analyze the raw count data (42). Genes with a mean count <200 was removed from analysis by pre-filtering. DESeq2 applies the Wald test for statistical analysis. Adjusted $p \leq 0.05$ were defined as statistically significant.

Anti-inflammation Assay Performed in a Cell Culture Model

Caco-2 cells (ATCC HTB 37) lines were routinely cultured in High Glucose Dulbecco's Modified Eagle Medium (DMEM) (Corning, Manassas, VA) supplemented with $NaHCO_3$ (Sigma-Aldrich, St. Louis, MO), 1% non-essential amino acids (Gibco, Dublin, Ireland), 100 U/ml penicillin-streptomycin (Gibco, Dublin, Ireland), 10% (v/v) fetal bovine serum (Seradigm VWR, Radnor, PA), and 7 mM HEPES (Gibco, Dublin, Ireland). Caco-2 cells were routinely grown in 20-cm Petri plates and subcultured at 80% confluence and maintained at 37°C in a humidified atmosphere of 5% (v/v) CO_2 in air.

The cells were differentiated at passages 30–32 and collected by dissociation of a 90% confluent stock culture with 0.25% trypsin/EDTA (Gibco, Dublin, Ireland). For the inflammation assay, Caco-2 cells were seeded in 24-well plate at a concentration of 1–2 $\times$ 10^5 cells/cm^2 and were differentiated for 17 days with the medium changed every 2–3 days. Replicate supernatants collected from *B. infantis* ATCC 15697 growing on lactose, LNT, and LNnT were mixed in equal volume and added into the DMEM at the final concentration of 15% (v/v). One hundred microliter of acetic acid, lactic acid, and formic acid controls were mixed with DMEM. Subsequently, media was added to each well in triplicates and incubated at 37°C in a 5% CO_2 atmosphere for 2 h. For negative and positive controls, triplicates were seeded with only DMEM. After incubation, 10 μl of 5 mg/ml lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO) in PBS was added into the wells and incubated for 24 h for treatments. LPS alone was used for negative control. PBS alone served as the carrier solution and a reference control. After incubation, the cells were detached from the plate surface by incubation with trypsin/EDTA, suspended in 500 μl of RNAlater solution and stored at −80°C until RNA extraction.

The relative gene expression of interleukin-8 (IL-8) linked with LPS-induced inflammation expression was quantified using qRT-PCR. Total RNA was extracted using the Ambion RNAqeous-Total RNA extraction kit (Invitrogen, Vilnius, Lithuania) according to the manufacturer's instructions. Cells suspended in lysis buffer were transferred to Lysing Matrix D tubes specific for eukaryotic cell and tissues culture (MP Biomedicals LLC, Solon, Ohio) and were subject to a speed of 5.5 m/sec for 30 sec twice using the FastPrep 24 bead beader (MP Biomedicals, Santa Ana, CA). The total RNA was eluted in 50 μl of EB solution and immediately subjected to DNase treatment with the Ambion Turbo DNA-free (Invitrogen, Vilnius, Lithuania) using 1 μl of DNase I for half-hour. Total RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) according to manufacturer's instructions. The resultant cDNA was quantified in a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Agawam, MA). The qRT-PCR analysis was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Singapore) with PowerUP SYBR Green Master Mix (Applied Biosystems, Foster City, CA) using 200 ng of cDNA with primers GAPDH-F (5′-GTCGCTGTTGAAGTCAGAGG-3′) and GAPDH-R (5′-GAAACTGTGGCGTGATGG-3′) for endogenous control and primers IL-8-F (5′-GACCACACTGCGCCAACAC-3′) and IL-8-R (5′-CTTCTCCACAACCCTCTGCAC-3′) (43). The PCR cycling conditions were applied as recommended by the manufacturer and tailored specifically to the target genes. Additional markers of inflammation were tested using primers IL-10-F (5′-GGTTGCCAAGCCTTGTCTGA-3′), IL-10-R (5′-AGGGAGTTCACATGCGCCT-3′) (44), and TNF-α-F (5′-TCAACCTCCTCTCTGCCATC-3′), TNF-α-R (5′-CCAAAGTACACCTGCCCAGA-3′) (45).

RESULTS

B. longum subsp. *infantis* Exhibits Divergent Growth Phenotypes During Utilization of the Milk Oligosaccharides Lacto-*N*-tetraose and Lacto-*N*-neotetraose

In order to understand *B. infantis* metabolism of HMOs, the type strain ATCC 15697 was subjected to growth on purified HMO species and constituent mono- and di-saccharides. Accordingly, *B. infantis* ATCC 15697 grew vigorously on lactose ($OD_{600\,nm,\,asym} = 1.27 \pm 0.12$, $k = 0.56 \pm 0.03\ h^{-1}$) as well as galactose ($OD_{600\,nm,\,asym} = 1.20 \pm 0.13$, $k = 0.61 \pm 0.02\ h^{-1}$) (**Figure 1**). The HMO species LNT was utilized as a sole carbohydrate source to a similar extent ($OD_{600\,nm,\,asym} = 1.19 \pm 0.24$, $k = 0.51 \pm 0.02\ h^{-1}$) as these two constituent residues (**Figure 1**). Interestingly, the structural isomer LNnT promoted a more moderate growth profile ($OD_{600\,nm,\,asym} = 0.85 \pm 0.09$, $k = 0.57 \pm 0.04\ h^{-1}$) ($p < 0.05$) (**Figure 1**). The *B. infantis* type strain did not grow on the HMO constituents GlcNAc and fucose as a sole carbohydrate source. It is noteworthy that growth on glucose was inconsistent in terms of final $OD_{600\,nm}$; therefore, a comparison to the other carbohydrates was limited. The significant difference between LNT and LNnT utilization ($p < 0.05$) suggests that these HMOs are metabolized via differential mechanisms that vary in efficiency (**Figure 1A**). Differences in growth rates between the HMO species were not observed which indicates an equivalent preference for LNT and LNnT (**Figure 1B**). *B. infantis* ATCC 15697 exhibited similar growth rates for lactose, galactose, and LNnT ($p > 0.05$). Although growth efficiencies on galactose and LNT are similar (**Figure 1A**), *B. infantis* ATCC 15697 prefers galactose to LNT as indicated by growth rate ($p < 0.05$). In aggregate, the single structural difference between LNT and LNnT is directly linked to the efficiency by which *B. infantis*

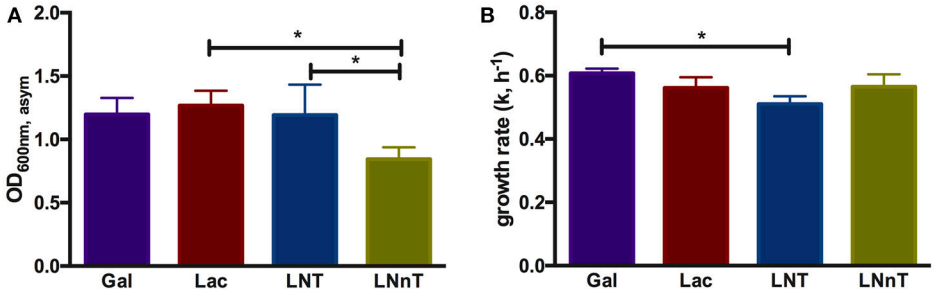

FIGURE 1 | *B. longum* subsp. *infantis* ATCC 15697 growth kinetics while utilizing milk carbohydrates. The final asymptotic $OD_{600\ nm}$ **(A)** and growth rate (k, h^{-1}) **(B)** of *B. infantis* ATCC15697 subsisting on mMRS medium containing 2% (wt/v) galactose (Gal), lactose (Lac), lacto-*N*-tetraose (LNT), or lacto-*N*-neotetraose (LNnT). The growth kinetics was calculated with Wolfram Mathematica 10.3. The data depicts mean ± *SD* of three independent experiments. The single asterisk (*) indicates the significant differences between carbohydrate utilizations evaluated by one-way ANOVA and Tukey's multiple comparison ($p < 0.05$).

utilizes these HMO species. Previous studies indicate that ATCC 15697 grows on both LNT and LNnT to achieve a $OD_{600\ nm} > 0.8$, however these studies did not report the specific asymptotic growth (i.e., efficiency) or growth rate (i.e., preference) when growing on these two HMO species (17, 26, 46, 47). Thus it is significant that ATCC 15697 exhibits clear differences in utilization between LNT and LNnT when accumulating biomass to $OD_{600\ nm}$ values >0.8.

Metabolic Endproducts Are Differentially Secreted Dependent on the Milk Oligosaccharide Substrate Lacto-*N*-tetraose or Lacto-*N*-neotetraose

Fermentative endproducts were profiled to detail the metabolic consequences of HMO carbohydrate flux through the F6PPK pathway. During hexose fermentation, acetic acid and lactic acid are typically secreted in a theoretical ratio of 1.5. In contrast, formic acid production is not expected under most conditions tested to date (20, 47). The absolute concentrations of lactic acid, acetic acid, formic acid, ethanol, and the ratios between these metabolites are depicted in **Figure 2**. *B. infantis* ATCC 15697 produces similar concentrations of lactic acid while growing on galactose, lactose, and LNT (42.5 ± 5.7, 47.8 ± 5.7, and 46.5 ± 2.5 mM respectively) ($p > 0.05$, **Figure 2A**). A much lower lactic acid concentration, however, was secreted while utilizing LNnT (29.5 ± 5.9 mM) compared to the metabolism of other carbohydrates ($p < 0.05$). Interestingly, formic acid and ethanol concentrations were significantly higher while growing on LNnT (16.3 ± 4.1 and 2.5 ± 1.1 mM respectively). This is contrasted with the relatively smaller concentrations while growing on LNT, lactose, and galactose ($p < 0.05$, **Figures 2C,D**).

LNT metabolism resulted in the highest concentration of secreted acetic acid (84.8±4.4 mM, **Figure 2B**) and significantly differed from LNnT metabolism ($p < 0.05$). In general, fermentative endproduct concentrations are expected to be positively correlated with the final biomass (48), as more carbohydrates processed by the F6PPK pathway results in more organic acids secreted. Therefore, it is expected that lactic acid and acetic acid concentrations will be higher when greater biomass is achieved (47). However, acetic acid concentrations while utilizing galactose, lactose, and LNnT did not significantly differ from each other ($p > 0.05$). Overall, these data support the hypothesis that *B. infantis* deploys a different mechanism while utilizing LNnT than LNT.

The Ratio of Secreted Endproducts Indicate an Alternative Pathway for Lacto-*N*-neotetraose Metabolism

Bifidobacteria, including *B. infantis*, catabolize 2 moles hexose to secrete 2 moles of lactic acid and 3 moles of acetic acid via the F6PPK pathway (**Figure 3**). This theoretical yield (i.e., acetate: lactate ratio of 1.5) was achieved during growth on galactose and lactose (1.56 ± 0.01 and 1.58 ± 0.01, respectively, **Figure 2E**). During HMO metabolism, LNT and LNnT utilization shifted the ratio toward greater acetic acid production (1.84 ± 0.01 and 2.08 ± 0.14, respectively, $p < 0.05$, **Figure 2E**). This is likely due to the deacetylation of the GlcNAc residue, at least in part. Notably this ratio significantly diverges between LNT and LNnT utilization with the latter experiencing a stronger shift ($p < 0.05$). If both HMO isomers increased the relative proportion of acetic acid via GlcNAc deacetylation, the higher ratio during LNnT metabolism is due to either decreased lactic acid production and/or increased acetic acid production from acetyl-CoA conversion.

LNnT metabolism was characterized by a significant increase in formic acid and ethanol production despite lower biomass. Accordingly, the ratio of formic acid to lactic acid was significantly higher during LNnT metabolism ($p < 0.05$, **Figure 2F**). Similarly, LNnT metabolism increased the formic acid to acetic acid ratio significantly ($p < 0.05$, **Figure 2G**). The theoretical formic acid to acetic acid ratio is 2:5 during more than 50% conversion of acetyl-CoA to acetic acid (25). This ratio was approached during LNnT metabolism (**Figure 2G**). This means, in part, that pyruvate is shunted toward acetyl-CoA resulting more formic acid and acetic acid production rather than lactic acid. Accordingly, ethanol to lactic acid ratio during LNnT fermentation differed significantly from LNT, as well as the other carbohydrates ($p <$ 0.05,

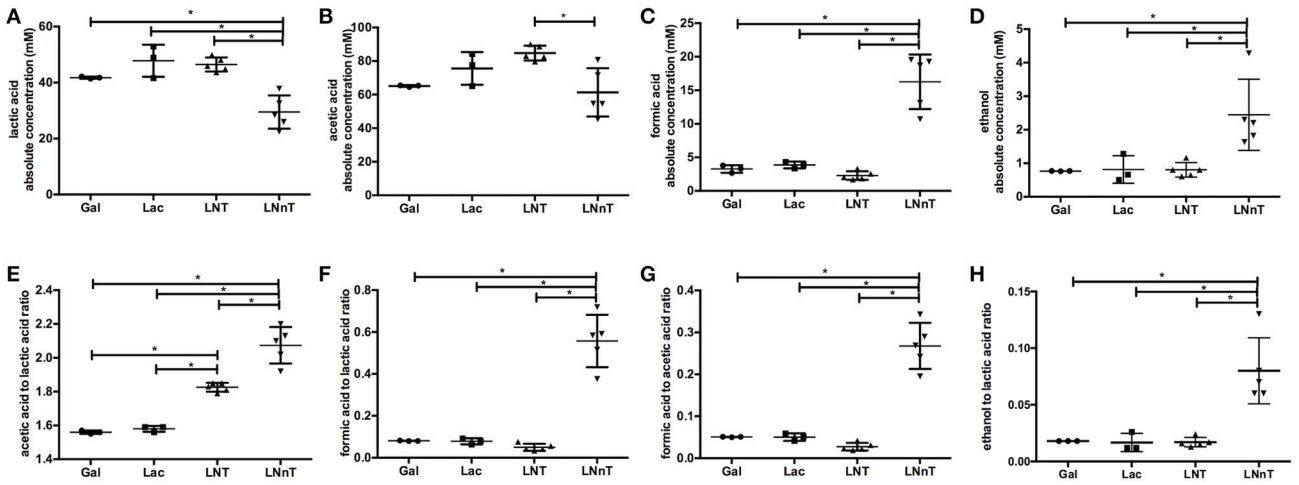

FIGURE 2 | *B. longum* subsp. *infantis* ATCC 15697 fermentative endproducts while utilizing milk carbohydrates through the F6PPK pathway. Absolute concentrations of lactic acid **(A)**, acetic acid **(B)**, formic acid **(C)**, and ethanol **(D)**. In addition, acetic acid to lactic acid ratio **(E)**, formic acid to lactic acid ratio **(F)**, formic acid to acetic acid ratio **(G)**, and ethanol to lactic acid ratio **(H)**. All panels represent *B. infantis* ATCC15697 growing on mMRS medium containing 2% (wt/v) galactose (Gal), lactose (Lac), lacto-*N*-tetraose (LNT), or lacto-*N*-neotetraose (LNnT). Averages from independent biological replicates (triplicate or more) are shown with bars representing standard deviations of the means. The values for organic acid production are expressed in millimolar (mM) absolute concentration. A single asterisk (*) denotes significant differences between metabolite production evaluated by one-way ANOVA and Tukey's multiple comparison test ($p < 0.05$).

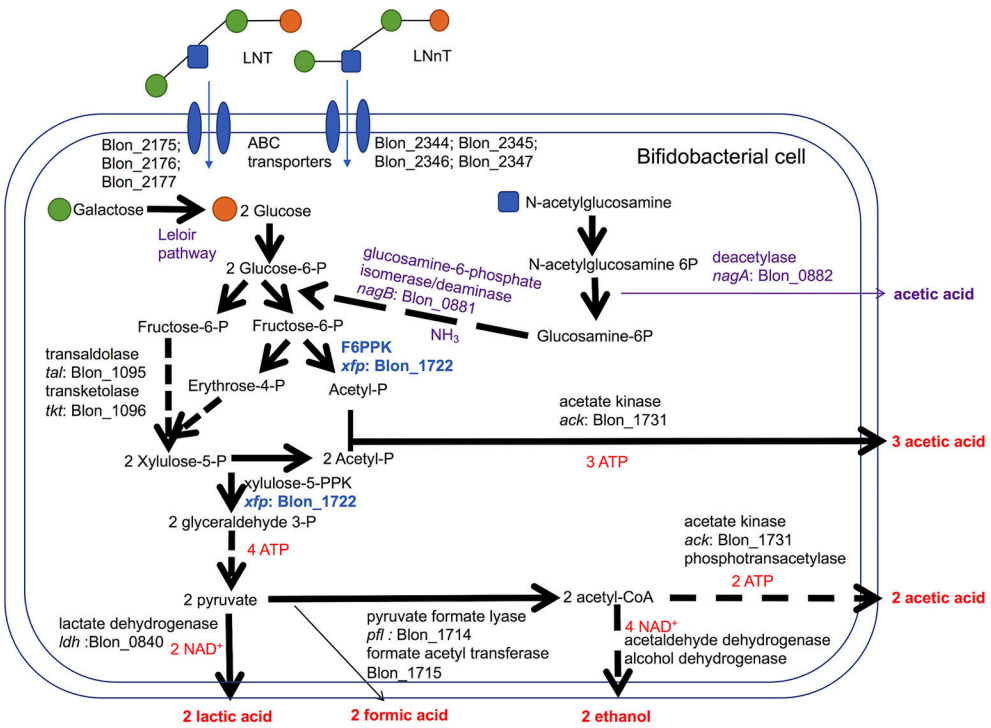

FIGURE 3 | *Bifidobacterium longum* subsp. *infantis* metabolic pathways for utilization of lacto-*N*-tetraose (LNT) and lacto-*N*-neotetraose (LNnT) and their constituent monosaccharides. LNT and LNnT translocate through the cell membrane facilitated by ABC transporters. Intracellular glycosyl hydrolases process HMO into constituent monosaccharides to enter the central fermentative pathway. This pathway involves the characteristic fructose-6-phosphate phosphoketolase (F6PPK) activity denoted in blue. Genes encoding intracellular metabolic enzymes are depicted next to arrows according to their locus tag in the ATCC 15697 genome. Solid arrows are direct conversions with dashed arrows depicting the sequential actions of multiple enzymes. Predicted catabolic operations that feed into the F6PPK pathway and their corresponding products are denoted as purple. Stoichiometric coefficients of secreted metabolites, ATP, and NAD^+ produced during metabolism are labeled in red. Experimental observations depicted in **Figures 1**, **2** including stoichiometry are incorporated.

Figure 2H). The theoretical ratio is 1:1 when 50% of acetyl-coA is converted to ethanol. The higher ethanol to lactic acid ratio in LNnT indicates that ethanol production occurs for regenerating NAD^+. The theoretical ratio has not been reached (∼0.08), thus this explains that acetyl-CoA was mostly converted to acetic acid rather than ethanol to increase ATP production instead of NAD^+ recycling. This indicates a clear metabolic shift toward these endproducts while subsisting on LNnT.

Oligosaccharide Transport Gene Expression Remains Similar Regardless of Lacto-*N*-tetraose and Lacto-*N*-neotetraose Substrates

As with other bifidobacteria examined to date, *B. infantis* ATCC 15697 encodes several family 1 solute binding proteins (F1SBPs), ATP-binding domains, and permeases that assemble into ABC transporters with predicted affinity for oligosaccharides (10, 16, 49). The expression of four F1SBPs and their cognate ABC permeases during LNT and LNnT utilization was evaluated to test the hypothesis that transport contributes to the differential metabolic phenotypes (**Figures 4A,B**). These F1SBPs were previously identified to bind glycans that incorporate HMO moieties (16). Three F1SBPs (Blon_0883, Blon_2344 and Blon_2347) and four ABC permeases (Blon_2175, Blon_2176, Blon_2345 and Blon_2346) were induced more than 2-fold during the growth on LNT or LNnT as the sole carbon source relative to lactose ($p < 0.05$). Only Blon_2347 expression differed significantly between the metabolism of the two HMO species ($p < 0.05$, **Figure 4A**). Interestingly, both LNT and LNnT induced the F1SBP Blon_0883, although its adjacent permease proteins Blon_0884 and Blon_0885 were not induced ($p > 0.05$). Main effect analysis via two-way ANOVA indicates that Blon_2347 exhibits the strongest induction among the four F1SBP genes regardless of substrate ($p < 0.05$, **Figure 4A**). Among the permeases, it is notable that the highest relative expression occurred in transcription of Blon_2346, followed by Blon_2345 ($p < 0.05$, **Figure 3B**). These genes are located in a 40-kb catabolic cluster dedicated specifically to HMO metabolism (10). This indicates that *B. infantis* deploys HMO cluster transporters while utilizing both LNT and LNnT which differs significantly from its corresponding component, lactose. The differential metabolic phenotypes between LNT and LNnT are not linked to the expression of these transport genes, indicating that it is likely a function of intracellular catabolic operations.

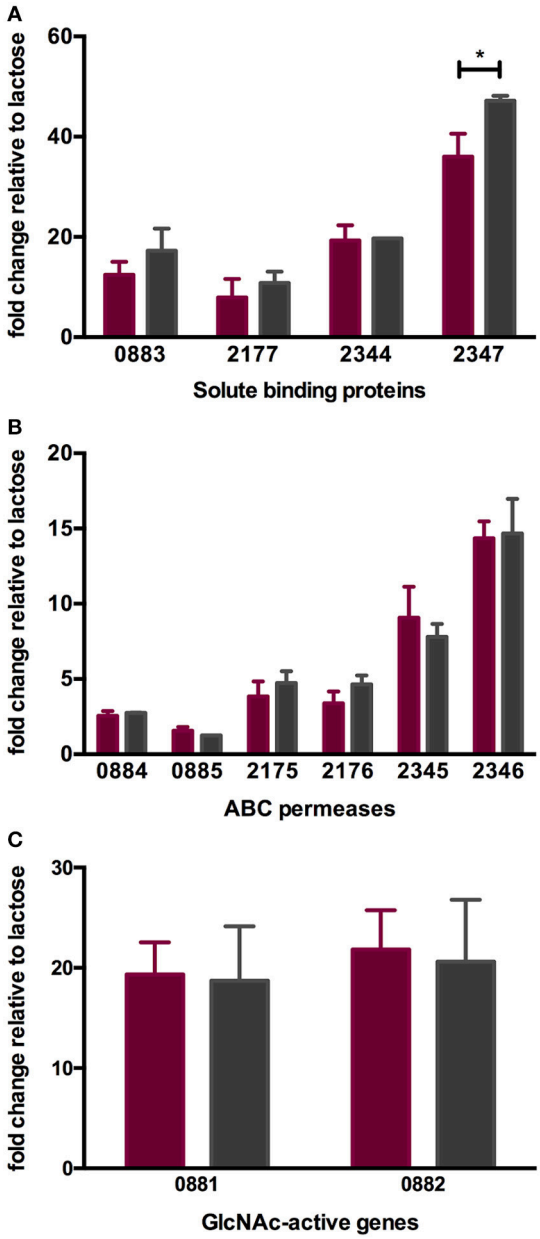

FIGURE 4 | ATCC 15697 gene expression while subsisting on milk oligosaccharides as a sole carbon source. The x-axis represents gene locus tags of Family 1 solute binding proteins **(A)**, ABC permeases **(B),** and GlcNAc-active genes **(C)**. The y-axis depicts the fold changes in gene expression relative to the control lactose as measured by qRT-PCR. LNT and LNnT are denoted in purple and gray. The error bars show standard deviations of three biological replicates except for LNnT. *$p < 0.05$ evaluated by one-way ANOVA and Tukey's multiple comparison.

B. infantis Upregulates *N*-acetylglucosamine Metabolic Genes While Utilizing Lacto-*N*-tetraose and Lacto-*N*-neotetraose

During HMO hydrolysis, GlcNAc is liberated from the oligosaccharide and likely subjected to deamination and deacetylation before entering the F6PPK pathway (**Figure 3**). This is catalyzed by GlcNAc-6-phosphate deacetylase (*nagA*; Blon_0882, EC 3.5.1.25) and glucosamine-6-phosphate isomerase/deaminase (*nagB*; Blon_0881, EC 3.5.99.6). Both Blon_0881 and Blon_0882 exhibited significant upregulation while growing on LNT and LNnT relative to lactose ($p < 0.05$). Specifically, LNT induced fold changes of 19.34 ± 3.21 and 21.84 ± 3.90 of Blon_0881 and Blon_0882 respectively, whereas LNnT prompted a similar induction measured at 18.71 ± 5.43 and 20.61 ± 6.19 (**Figure 4C**). This upregulation is interpreted

as consistent with GlcNAc catabolism providing evidence that deacetylation occurs during LNT and LNnT utilization. Significant differences in the expression of these GlcNAc genes were not detected between LNT and LNnT metabolism. This expression profile could reflect the growth rate similarity between LNT and LNnT as depicted in **Figure 1B**.

The *B. infantis* Transcriptome Diverges During Lacto-*N*-tetraose and Lacto-*N*-neotetraose Metabolism

The *B. infantis* ATCC 15697 transcriptome while utilizing HMOs was previously characterized by RNA-seq (26). Given the differential metabolism observed in the current study, specific pathways predicted to be relevant to LNT and LNnT utilization were examined in greater depth according to differential gene expression beyond normalized counts. Accordingly, raw reads were retrieved and subjected to differential expression analysis (i.e., 2-fold change) for HMO utilization cluster genes, galactose catabolic genes (i.e., Leloir pathway), GlcNAc-related genes, glycosyl hydrolases, and the F6PPK pathway as listed in Table S2.

Genes involved in galactose metabolism (Blon_2171, Blon_2172, and Blon_2174) and adjacent ABC transporters (Blon_2175, Blon_2176, and Blon_2177) are strongly upregulated by both LNT and LNnT compared to lactose ($p < 0.05$) (**Figure 5**). Moreover, LNnT prompted stronger induction of these genes relative to LNT ($p < 0.05$). Similarly, F1SBPs and permeases localized to the HMO utilization cluster (i.e., Blon_2344-2352) were upregulated by both LNT and LNnT relative to the lactose control ($p < 0.05$). In addition, Blon_2344, Blon_2347, and Blon_2352 were significantly upregulated during LNT fermentation when compared to LNnT ($p < 0.05$).

The GlcNAc utilization genes Blon_0881 (*nagB*) and Blon_0882 (*nagA*) were significantly upregulated while *B. infantis* utilizes both LNT and LNnT relative to lactose ($p < 0.05$, **Figure 5**). However, there is no significant difference between LNT and LNnT metabolism ($p > 0.05$). This is consistent with the qRT-PCR gene expression analysis.

The key enzyme fructose-6-phosphate phosphoketolase (*xfp*, Blon_1722) has been postulated to be highly expressed regardless of carbohydrate substrate (26). Interestingly, F6PPK pathway genes are downregulated by LNT relative to lactose and LNnT ($p < 0.05$, **Figure 5**). In addition, LNnT showed strong upregulation of those genes compared to lactose ($p < 0.05$), except for Blon_1722, Blon_1096, and Blon_1368 that did not significantly differ ($p > 0.05$). This is interesting as the biomass and growth rate achieved with LNT or lactose did not significantly differ ($p > 0.05$, **Figure 1**). Despite the potential for greater flux through the central fermentative pathway as per the transcriptome, LNnT prompted less biomass production ($p < 0.05$). Furthermore, lactate dehydrogenase (*ldh;* Blon_0840, EC 1.1.1.37), converts pyruvate to lactate to recycle cofactors and was significantly induced by LNnT relative to lactose and LNT ($p < 0.05$). The relationship between *ldh* expression and LNnT inducing lower lactic acid concentrations is unclear. This is potentially indicative of variation between the physiological state of the cells during sample collection (i.e.,

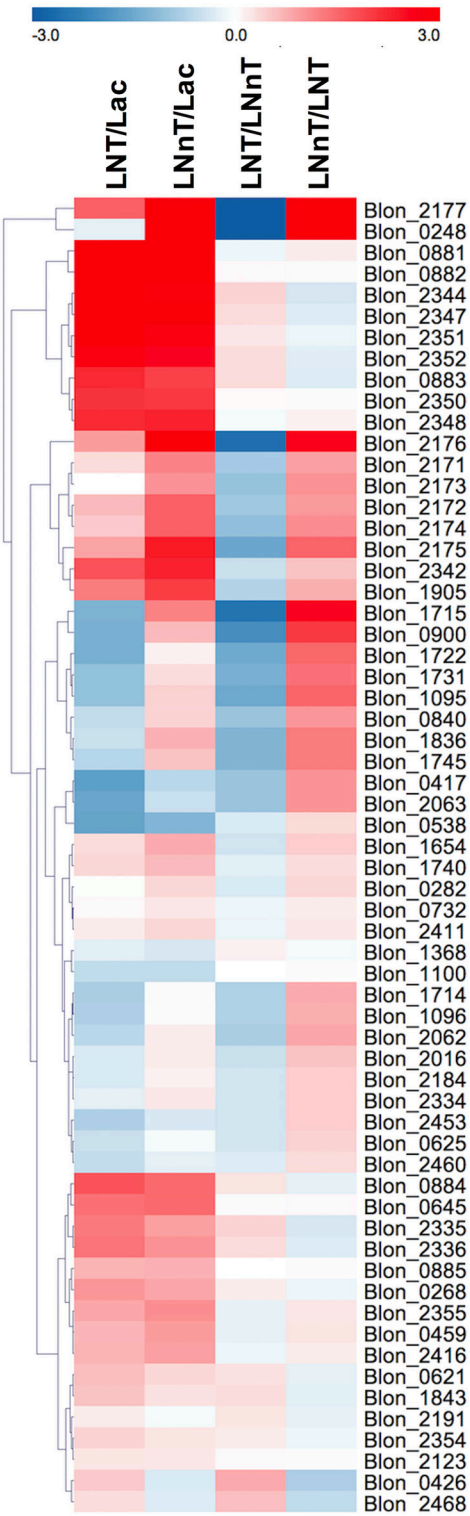

FIGURE 5 | Relative gene expression within the global transcriptome depicted as log2-fold change. The log2-fold change gene expression from independent biological duplicates was performed from raw reads using the R package DESeq2. The 2-fold change expressions were subjected to hierarchical clustering using Euclidean distance and depicted as z-scores. Gene annotations and predicted functions are reported in Table S2.

mid-exponential or stationary phase). Accordingly, high levels of lactic acid was observed at the beginning of fermentation of oligofructose by *B. animalis* and replaced by formic acid at later stages (50).

Interestingly, and potentially underlying differential metabolism, acetate kinase (*ack*; Blon_1731, EC 2.7.2.1) was more strongly upregulated while consuming LNnT relative to lactose and LNT ($p < 0.05$). Acetate kinase catalyzes substrate-level phosphorylation in the F6PPK pathway that is both involved in conversion of phosphoketolase to acetyl-P and conversion of acetyl-coA to acetate, and thus reflects relatively higher acetic acid secretion during LNnT fermentation.

As LNnT utilization is characterized by increased formic acid production, putative genes involved in this pathway were interrogated. It is noteworthy that this metabolic process is incompletely characterized in the bifidobacteria. Formate acetyl transferase (Blon_1715, EC 2.3.1.54) and pyruvate formate lyase (Blon_1714, EC 1.97.1.4) potentially converts pyruvate to acetyl-coA and produces formic acid. Accordingly, Blon_1715 is highly expressed during LNnT utilization relative to lactose and LNT ($p < 0.05$). Conversely, LNT metabolism is not characterized by increased formic acid production and prompted a downregulation of both Blon_1714 and Blon_1715 relative to lactose and LNnT ($p < 0.05$). Although F6PPK genes were upregulated by LNnT relative to lactose, the strongest change was observed in formate acetyl transferase (2-fold change = 1.20). Again, this is consistent with increased formic acid production during LNnT metabolism to provide evidence that differential phenotypes exhibited between LNnT and LNT is regulated at the gene expression level, at least in part.

Twenty five key glycosyl hydrolases (GHs) were selected for further analysis (**Figure 5**) (10, 17, 51). A total of 13 loci significantly differ between LNT and LNnT metabolism ($p < 0.05$). Among β-galactosidases, Blon_2016 (GH family 42), and Blon_2334 (GH family 2) were downregulated during LNT utilization relative to lactose and LNnT ($p < 0.05$). This is interesting because Blon_2016 was shown to have specificity to type I HMOs such as LNT (52) and Blon_2334 was shown to be constitutively expressed in the utilization of pooled HMOs and other complex oligosaccharides (26, 51). β-glucosidase Blon_1905 was significantly upregulated by both LNT and LNnT compared to lactose ($p < 0.05$) with expression during LNnT growth significantly higher than LNT ($p < 0.05$).

Salient to general HMO metabolism, an α-L-fucosidase Blon_0248 (GH family 29) was significantly upregulated by LNnT compared to lactose and LNT ($p < 0.05$). Interestingly, another GH 29 α-L-fucosidase (Blon_0426) is strongly upregulated by LNT whereas it is downregulated by LNnT ($p < 0.05$). Other fucosidases Blon_2335 and Blon_2336 were strongly upregulated by LNT rather than LNnT ($p < 0.05$). The glycosyl hydrolases, Blon_0625 and Blon_2460 were downregulated in both HMO species compared to lactose, with a significantly stronger downregulation observed in LNnT than LNT ($p < 0.05$). Blon_2468, an endo-β-*N*-acetylglucosaminidase, which generally releases *N*-glycans from human milk glycoproteins was upregulated by LNT while it was downregulated by LNnT ($p < 0.05$).

B. longum subsp. *infantis* Exhibits Growth Phenotype Variance While Utilizing Lacto-*N*-tetraose and Lacto-*N*-neotetraose in a Strain-Dependent Manner

In order to evaluate potential phenotypic variation within *B. infantis*, three strains in addition to ATCC 15697 were subjected to growth on glucose, galactose, lactose, LNT, and LNnT as a sole carbon source (Table S3). Both *B. infantis* UMA299 and UMA300 utilized GlcNAc as a sole carbohydrate substrate in contrast to ATCC 15697. None of the *B. infantis* strains tested utilized fucose as a sole fermentative substrate. UMA299 exhibited lower growth on LNT ($OD_{600\,nm,\ asym} = 0.69 \pm 0.09$, $k = 0.57 \pm 0.05\ h^{-1}$) and LNnT ($OD_{600\,nm,\ asym} = 0.71 \pm 0.06$, $k = 0.68 \pm 0.06\ h^{-1}$) compared to constituent carbohydrate residues within HMOs ($p < 0.05$, Table S3). This is significant, as UMA299 does not utilize pooled HMOs efficiently in contrast to other *B. infantis* strains (31). This is likely due to the absence of two F1SBP transporter genes (Blon_2344 and Blon_2351) in its HMO catabolic cluster (30). Interestingly, and despite limited growth, UMA299 exhibited a higher preference for LNnT with significantly lower growth rate on LNT ($p < 0.05$). In addition, UMA299 exhibited a $OD_{600\,nm,\ asym}$ of 0.42 ± 0.09 on soluble GlcNAc with a growth rate of $0.16 \pm 0.01\ h^{-1}$ which is significantly lower compared to other carbohydrates tested ($p < 0.05$).

B. infantis UMA300 utilized LNnT efficiently ($OD_{600\,nm,\ asym} = 1.30 \pm 0.12$, $k = 0.51 \pm 0.05\ h^{-1}$) which is to the same extent as LNT ($OD_{600\,nm,\ asym} = 0.99 \pm 0.20$, $k = 0.71 \pm 0.05\ h^{-1}$). The strain utilized galactose to similar cellular concentrations as LNT and LNnT and inefficiently utilizes lactose and GlcNAc (Table S3). In terms of growth rate (k), UMA300 experienced the highest rate on LNT and galactose with a significantly lower growth rate on LNnT ($p < 0.05$). This provides additional evidence that the two HMO species are utilized by divergent mechanisms by *B. infantis* strains. Despite achieving highest biomass concentrations on LNnT, UMA300 preference as determined by growth rate did not vary appreciably between LNnT and lactose ($p > 0.05$). UMA300 low growth rate on GlcNAc suggests that this aminosugar is not preferred relative to the other carbohydrates tested ($p < 0.05$).

In contrast to UMA300, *B. infantis* UMA301 exhibited vigorous growth on lactose ($OD_{600\,nm,\ asym} = 1.29 \pm 0.05$, $k = 0.56 \pm 0.05\ h^{-1}$), followed by galactose ($OD_{600\,nm,\ asym} = 1.22 \pm 0.05$, $k = 0.63 \pm 0.06\ h^{-1}$), and LNT ($OD_{600\,nm,\ asym} = 1.01 \pm 0.17$, $k = 0.44 \pm 0.06\ h^{-1}$) all of which does not differ significantly ($p > 0.05$, Table S3). UMA301, however, achieved significantly lower biomass concentrations on LNnT ($OD_{600\,nm,\ asym} = 0.86 \pm 0.04$, $k = 0.40 \pm 0.01\ h^{-1}$) concomitant with a lower growth rate. This suggests a clear preference for LNT rather than LNnT between these two HMO tetrasaccharides. As with ATCC 15697, UMA301 does not utilize GlcNAc as a sole carbohydrate source.

Consistent with the growth, ATCC 15697, UMA299, and UMA301 consumed galactose and lactose >40%, whereas UMA300 consumed lactose at 25% ($p < 0.05$, Figure S1A).

Similarly, UMA299 consumed GlcNAc to a greater extent than UMA300.

In order to determine phenotypic variation as a function of carbohydrate source and *B. infantis* strain, principal component analysis (PCA) and hierarchical clustering was performed. This analysis incorporated both final asymptotic $OD_{600\ nm}$ and growth rate data for strains growing on individual substrates (**Figure 6**). The first principal component (PC1) explains 40.8% variation in $OD_{600\ nm,\ asym}$ values, whereas PC2 captures 37.7% variation (**Figure 6A**). The scores of each component grouped as strains clustered closely (i.e., within the normal probability). This indicates that the growths are consistent among biological replicates regardless of fermentative substrate. Arrows oriented toward the same direction denote that growth on a particular carbohydrate is correlated with that PC component with the angle between arrows indicating a similar response profile. LNT and galactose utilization vectors emanated toward similar directions that is negatively correlated with both PCs (**Figure 6A**). These two vectors aligned along PC1 where ATCC 15697 and UMA301 growths clustered. UMA300 growth, however, aligned positively with PC1 in the same direction as the LNnT utilization vector. This indicates that UMA300 utilized LNnT well relative to other carbohydrates and strains. This is consistent with strain-dependent utilization, as UMA300 had a higher final $OD_{600\ nm,\ asym}$ during LNnT utilization compared to other strains (**Figure 7D**). Interestingly, the LNnT and lactose utilization vectors were oriented in opposing directions suggesting utilization differences between them. GlcNAc and glucose utilization had a similar alignment and positively correlated with both components where UMA299 clustered, which is consistent with its limited ability to utilize HMO efficiently.

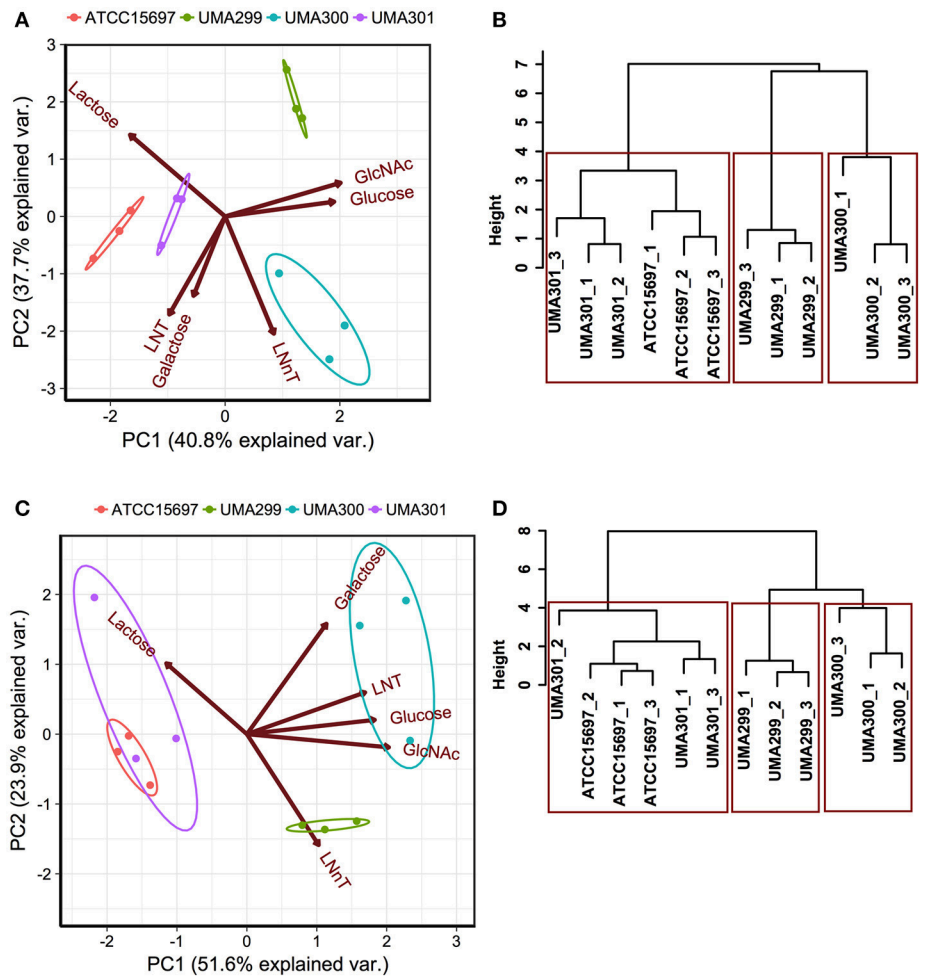

FIGURE 6 | Strain dependent variation of human milk oligosaccharide utilization. Panels **(A,C)** depict the 2D-principal component analysis (PCA) performed on the final asymptotic OD_{600} and the growth rate (k, h^{-1}) respectively. The arrows in PCA plot represent the correlation of variables with the principal components (PC1 and PC2). Points represent the scores of each component grouped as biological replicates. The ellipses encompassing each strain capture 68% of the normal probability of the scores within corresponding strains. Panels **(B,D)** show the hierarchical clustering dendrogram of strains on the final asymptotic $OD_{600\ nm}$ and the growth rate (k, h^{-1}) using ward method and Euclidean distance. The y-axis measures closeness of either individual strains or clusters. The boxes show the 95% of clustering and closeness of the strains.

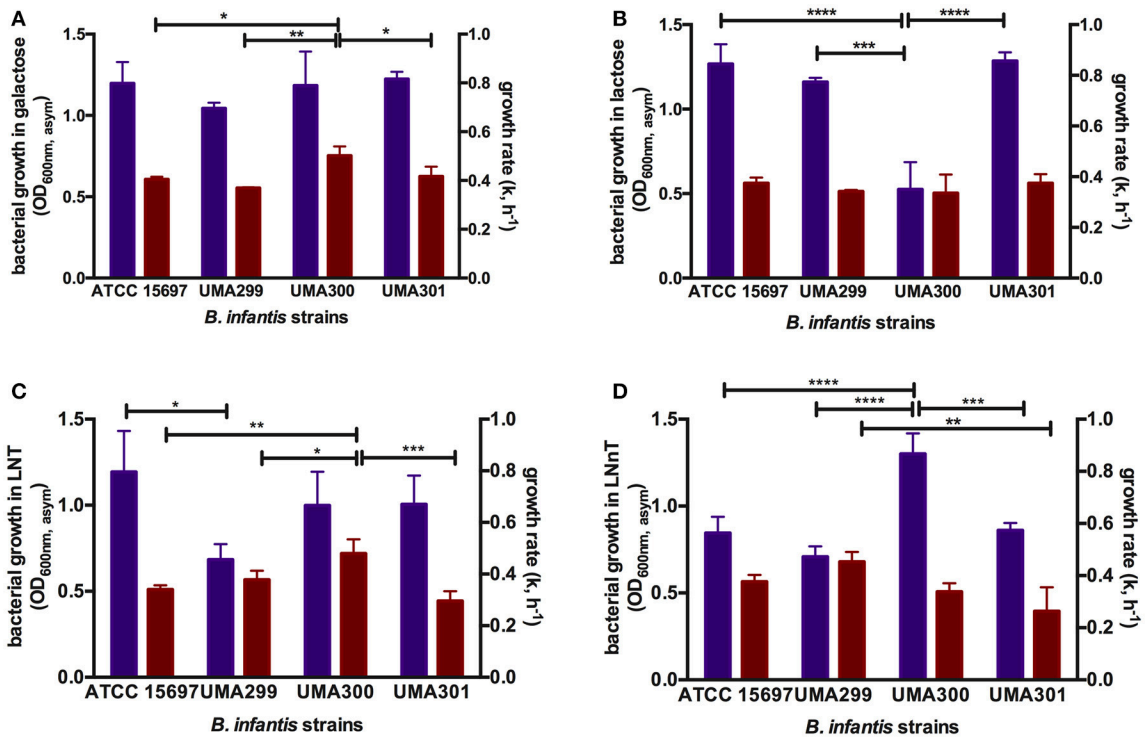

FIGURE 7 | Growth kinetics of *B. longum* subsp. *infantis* strains subsisting on milk carbohydrates. The final asymptotic $OD_{600\ nm}$ and growth rate (k, h^{-1}) of *B. infantis* strains while growing on mMRS medium containing 2% (wt/v) galactose **(A)**, lactose **(B)**, lacto-*N*-tetraose (LNT) **(C)**, and lacto-*N*-neotetraose (LNnT) **(D)**. The growth kinetics was calculated with Wolfram Mathematica 10.3 and represents the mean ± *SD* of three independent experiments. Purple and red bars indicate bacterial growth and growth rate respectively. The asterisks indicate the significant differences between strains evaluated by one-way ANOVA and Tukey's multiple comparison. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, and ****$p <$ 0.0001. The growth kinetics for glucose and GlcNAc were not included as not all strains consume these monosaccharides.

Hierarchical clustering was employed to determine quantitative similarities between strains. The height between the lines indicates the distances between the strains and their biological replicates (**Figure 6B**). The PCA and hierarchical clustering were concordant, and indicated that ATCC 15697 and UMA301 clustered together with the height of <4 (**Figure 6B**). Interestingly, this reflects the phylogenic relationship between ATCC 15697 and UMA301 (30).

In addition to final biomass achieved, PCA of growth rates observed on multiple substrates resolved onto PC1 to encompass 51.6% variability for each strain and positively correlated with all carbohydrates except lactose (**Figure 6C**). This is interesting as *Bifidobacterium* strains were previously determined to possess a preference for lactose over glucose (41), with lactose often used as a positive control and propagation of bifidobacterial strains. ATCC 15697 and UMA301 co-clustered and negatively correlated with PC1. These two strains exhibited similar utilization rates for all substrates tested. Interestingly, UMA300 growth rates on galactose, LNT, glucose, and GlcNAc were similar and distinctly clustered from other strains (**Figure 6C**) with significantly higher values on these carbohydrate sources compared to other strains ($p < 0.05$, **Figure 7**). Hierarchical clustering using the Ward method validated the PCA (**Figure 6D**). Hierarchical clustering of the growth rate is also consistent with the same analysis of the final biomass ($OD_{600\ nm,\ asym}$), which yielded a similar distance topology. Accounting for the empirical evidence in aggregate, *B. infantis* utilization of LNT and LNnT diverges in a strain-dependent manner.

B. longum subsp. *infantis* Strains Differentially Metabolize Lacto-*N*-tetraose and Lacto-*N*-neotetraose

A comparative analysis of the fermentative endproducts lactic acid, acetic acid, formic acid, and ethanol was conducted on the panel of *B. infantis* strains. These endproducts are secreted as a result of carbon flux through the F6PPK pathway with data reported as a heatmap with hierarchical clustering analysis (**Figure 8A**). As expected, acetic acid and lactic acid production clustered more closely with each other than the ethanol and formic acid production as these two endproducts are secreted regardless of substrate (**Figure 8A**).

The metabolic profiling of ATCC 15697 and UMA301 while fermenting galactose, lactose, and LNT closely clustered together with high acetic acid and lactic acid and less formic acid and ethanol produced (**Figure 8A**). Interestingly, UMA299 produced higher concentrations of formic acid and ethanol when utilizing

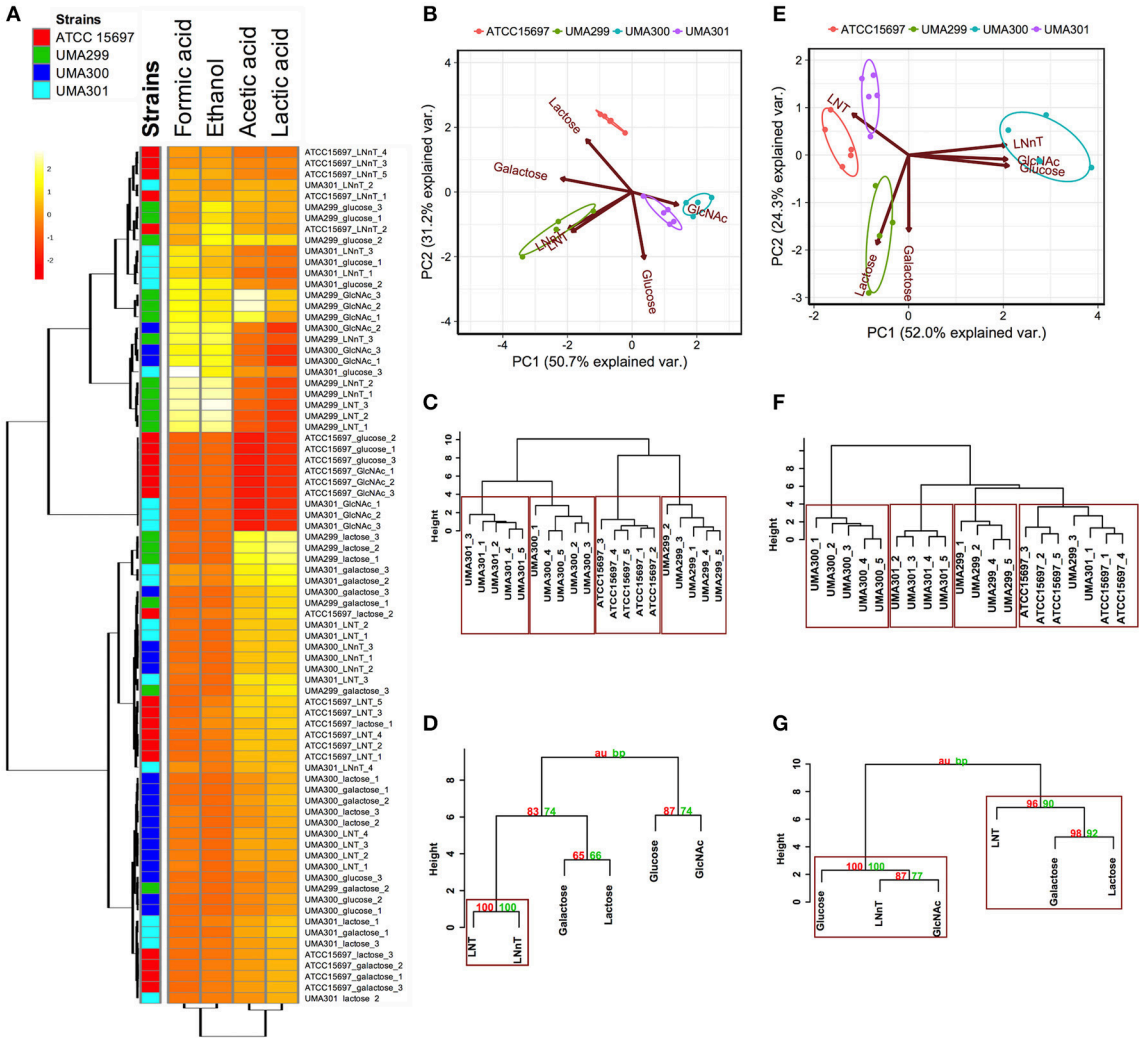

FIGURE 8 | Analysis of *B. longum* subsp *infantis* strains secreted fermentative endproducts while utilizing milk carbohydrates. Panel **(A)** shows metabolites secreted by *B. infantis* strains for each carbohydrate clustered by Euclidean distance calculated with MetaboAnalyst 3.0. The scaling was performed by mean-centering and dividing by the standard deviation of each metabolite. The red denotes lower concentrations of the metabolite with yellow approaching higher concentrations. Panels **(B,E)** display the 2D-principal component analysis (PCA) plot depicting acetic acid to lactic acid ratios and acetic acid to lactic acid to formic acid ratios respectively. The arrows in PCA plot represent the correlation of the variables with the principal components (PC1 and PC2). Points represent the scores of each component grouped as biological replicates. The ellipses for each strain incorporate 68% of the normal probability of the scores for corresponding strains. Panels **(C,F)** are hierarchical clustering dendrogram of strains according to acetic acid to lactic acid ratios and acetic acid to lactic acid to formic acid ratios using the Ward method and Euclidean distance. The y-axis measures the closeness of either individual strains or their calculated clusters. Panels **(D,G)** represent hierarchical clustering based on *p*-values calculated with multiscale bootstrapping between acetic acid to lactic acid ratio and acetic acid to lactic acid to formic acid ratio. The y-axis measures the closeness of either individual substrates or clusters according to two metrics: Approximately Unbiased *p*-value (AU in red) and Bootstrap Probability value (BP in blue). Clusters exhibiting AU values >95% are highlighted by rectangles.

GlcNAc, glucose, LNT, and LNnT relative to other carbohydrates (**Figure 8A**). This indicates that UMA299 utilizes LNT via a different metabolic trajectory relative than other strains. This is consistent with its final biomass and general phenotype as an atypical HMO consumer (**Figure 7C**).

ATCC 15697, UMA301, and UMA299 LNnT metabolism was linked to higher formic acid and ethanol concentrations. UMA300, in contrast, exhibited a significantly different metabolic profile that is closely linked to LNT with less formic acid and ethanol production than the other strains (**Figure 8A**). This did not differ appreciably from galactose, lactose, and glucose metabolism. This suggests that UMA300, unlike other strains, preferentially converts pyruvate to lactic acid rather than acetyl-CoA regardless of the substrate with the exception of GlcNAc.

The carbon recovery observed in secreted metabolites after utilization of mono- and di-saccharides are depicted in Figure S1B. UMA299 recovered almost 100% carbon for all sugars except galactose. All strains recovered <100%

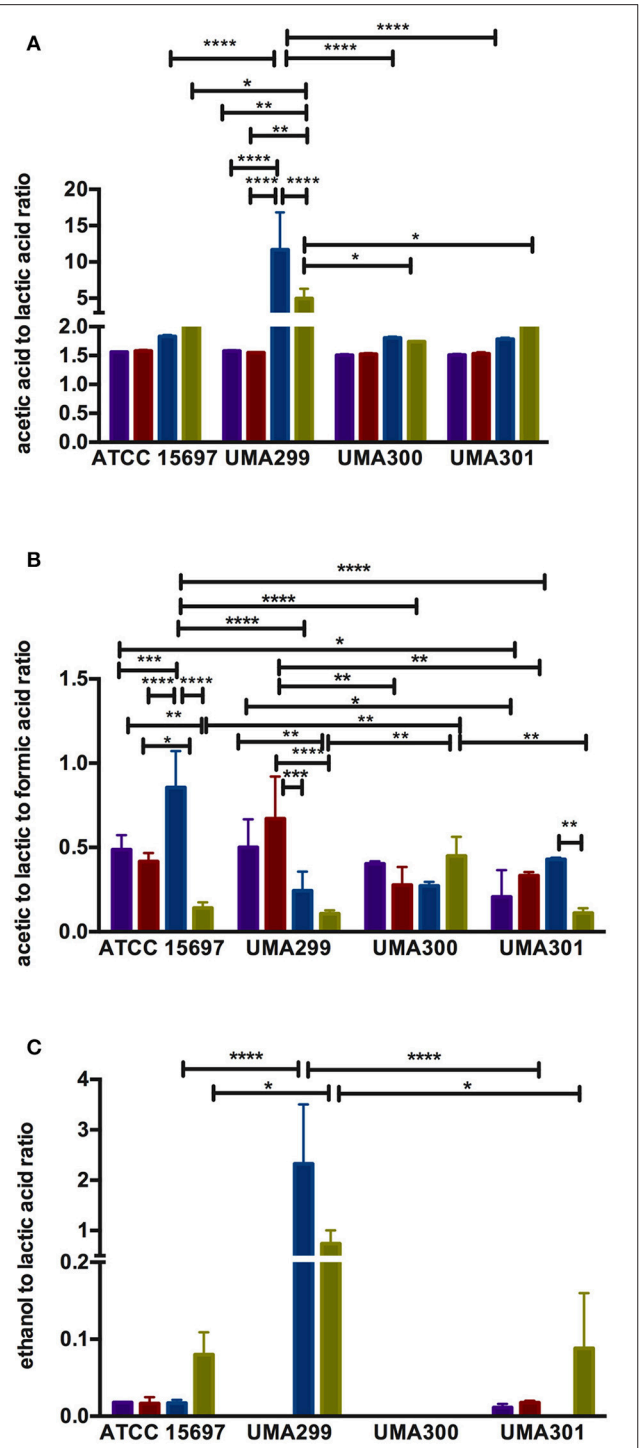

FIGURE 9 | Endproduct ratios of *B. longum* subsp. *infantis* strain fermentative endproducts while utilizing the milk carbohydrates. Acetic acid to lactic acid ratio **(A)**, acetic acid to lactic acid to formic acid ratio **(B)**, and ethanol to lactic acid ratio **(C)**. Colors indicate the following carbohydrate substrates: Purple, galactose; red, lactose; dark blue, lacto-*N*-tetraose; green, lacto-*N*-neotetraose. Averages from independent biological replicates (at least triplicate) are shown with bars representing standard deviation from the mean. The asterisks indicate the significant differences evaluated by two-way ANOVA and Tukey's multiple comparison. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, and ****$p < 0.0001$.

carbon while fermenting galactose. ATCC 15697, UMA299, and UMA301 achieved almost 100% carbon recovery while utilizing lactose. Interestingly, UMA300 exhibited 138% of carbon recovery as determined by secreted metabolite following growth on lactose. This was significantly higher than ATCC 15697 and UMA301 ($p < 0.05$). This might be due to hydrolysis of the remaining lactose into glucose and galactose in the post-fermentation medium with UMA300 potentially exhibiting a preference for utilizing monosaccharides over lactose.

The acetic acid:lactic acid ratios secreted after *B. infantis* strains fermented substrates were compared (**Figures 8B–D**). Accordingly, PC1 and PC2 encompass 50.7 and 31.2% of the variation for these ratios (**Figure 8B**). UMA300 and UMA301 displayed a positive correlation with PC1 whereas ATCC 15697 and UMA299 were negatively correlated. This is due to higher ratio values of strains on a particular carbohydrate with each strain clustering distinctly on PC2. The hierarchical clustering of strains was consistent with the PCA (**Figure 8C**). This is because UMA299 showed a significantly higher ratio in both LNT and LNnT metabolism relative to other strains ($p < 0.05$, **Figure 9A**). In addition, the direction of the LNT and LNnT vectors are negatively oriented with both components with a highly similar magnitude and direction in the PCA (**Figure 8B**). This explains the close clustering of LNT and LNnT based on Euclidean distance (<0.95) whereas the HMO carbohydrate constituents segregated away from both HMO species (**Figure 8D**). The agglomerative clustering of strains revealed no differences for LNT and LNnT. This is potentially due to GlcNAc deacetylation increasing acetic acid production during HMO utilization.

Formic acid production underlies divergent mechanisms for LNT and LNnT metabolism (**Figures 8E–G**). Accordingly, the PCA performed on the acetic acid:lactic acid:formic acid ratios clearly indicates that LNT and LNnT fermentation proceeds via distinctive metabolic routes as these vectors are oriented in opposing directions along PC1 (captures 52.0% variation) (**Figure 8E**). This suggests that *B. infantis* strains shunt LNnT catabolism toward conversion of pyruvate to acetyl-CoA rather than lactic acid to subsequently secrete formic acid.

UMA300 was positively positioned along PC1 whereas ATCC 15697 and UMA301 were primarily explained by PC2. Interestingly, ATCC 15697 and UMA301 do not cluster together as depicted in **Figure 8F** despite exhibiting phenotypic and phylogenetic similarities. However, if the significance of height in the hierarchical clustering is increased, they cluster together, and only UMA300 stands alone. This is due to UMA300 not significantly secreting formic acid except during GlcNAc fermentation (**Figure 8A**). *B. infantis* ATCC 15697 and UMA301 produced significantly more formic acid during LNnT fermentation than LNT that resulted in a decrease of the ratio ($p < 0.05$, **Figure 9B**).

The ethanol to lactic acid ratio was dependent on the carbohydrate source and strain (**Figure 9C**). UMA300 did not produce ethanol regardless of the substrate whereas UMA299 exhibited a higher ratio for both LNT and LNnT relative to other strains ($p < 0.05$). Interestingly, UMA301 had limited ethanol production during LNT fermentation while the ratio increased in LNnT utilization. This means that all strains

except UMA300 utilize LNnT along a similar metabolic route. Thus LNnT utilization by ATCC 15697 and UMA301 might involve NAD^+ regeneration through ethanol production whereas recycling NAD^+ during LNT utilization most likely occurs as pyruvate is converted to lactic acid.

Human Milk Carbohydrate Utilization Mitigates Lipopolysaccharide-Induced IL-8 Expression in Caco-2 Epithelial Cells

It is known that metabolism of specific carbohydrates may influence bifidobacterial interactions with intestinal epithelia under certain conditions. Pooled HMO-grown bifidobacteria reduce inflammatory markers compared to glucose or lactose-grown bifidobacteria (53, 54). These previous studies examined adhesive properties of bifidobacteria and bacterial translocation in exponential growth instead of cell-free supernatants collected at stationary phase. Differential metabolism of LNT and LNnT inspired the hypothesis that host-microbial interactions may be influenced in a milk oligosaccharide-dependent manner. In order to address this, metabolites present in spent media subsequent to *B. infantis* growth on lactose, LNT, and LNnT were evaluated for their ability to mitigate inflammation. Specifically, it was hypothesized that higher acetic acid and formic acid concentrations secreted from LNnT metabolism will differentially influence inflammation. Gene expression of the cytokine marker of inflammation IL-8 was measured in Caco-2 cells following lipopolysaccharide-induced inflammation (**Figure 10**). Accordingly, spent media from all three fermentations significantly reduced IL-8 expression compared with the negative control ($p < 0.05$). However, there was no significant difference between the three milk carbohydrates. In addition, the markers of inflammation IL-10 and TNF-α were assayed and yielded inconsistent and thus inconclusive results. Although *B. infantis* metabolism of these human milk carbohydrates protects against inflammation, it is unclear to what extent that *B. infantis* alone is responsible for the anti-inflammatory effect. In addition, purified acetic acid, lactic acid, and formic acid were tested, however, the results were inconclusive due to variation between biological replicates.

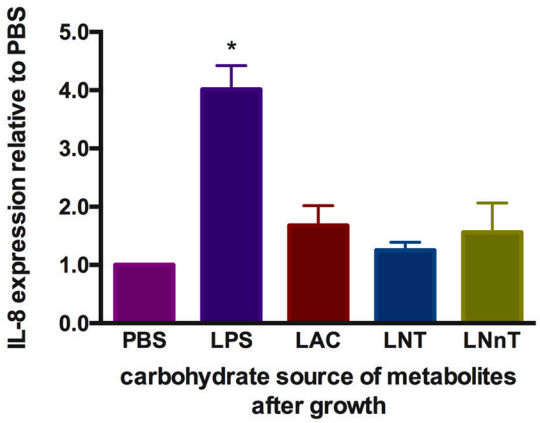

FIGURE 10 | Gene expression of inflammatory marker Interleukin-8 in Caco-2 epithelial cells exposed to spent media following milk oligosaccharide fermentation. The y-axis represents the fold change in IL-8 expression relative to phosphate buffer solution (PBS). The x-axis depicts the sources of *B. infantis* metabolites which are used to treat Caco-2 cells after lipopolysaccharides (LPS) induction. The error bars show standard deviations of biological duplicates, each measured with three technical replicates. The single asterisks (*) indicate the significant differences evaluated by one-way ANOVA and Tukey's multiple comparison ($p < 0.05$).

DISCUSSION

Bifidobacterium longum subsp. *infantis* evolved to utilize glycans secreted in human milk to generate ATP as well as provide substrates for anabolic processes. Accordingly, its genome incorporates a 40-kb locus dedicated to human milk oligosaccharide utilization that is conserved in all *B. infantis* strains isolated to date (10, 30). The activities encoded by the HMO gene cluster allocate degradation products to be further metabolized prior to entering the F6PPK pathway, the characteristic fermentative pathway unique to the *Bifidobacterium* genus (20). HMOs evade digestion during gastrointestinal tract transit and thus are available to *B. infantis* to translocate intracellularly (16, 29). The F6PPK pathway terminates invariably in the extracellular secretion of acetic acid and lactic acid, with formic acid and ethanol generated to a lesser extent under specific conditions (20–23). The potential for *B. infantis* to differentially metabolize purified HMO species has not been fully tested. The HMO tetrasaccharides LNT and LNnT differ by a β1-3 and β1-4 linkage between galactose and *N*-acetylglucosamine at the non-reducing terminus respectively. Accounting for this structural variance, we hypothesized that LNT and LNnT are differentially metabolized after initiating distinct transcriptomic cascades to process these HMOs.

Previous research conducted on *B. infantis* provided the preliminary observations to generate this hypothesis (17, 26). In this current study, the model HMO-consuming strain *B. infantis* ATCC 15697 exhibits higher growth efficiency (i.e., asymptotic final OD) while metabolizing LNT rather than LNnT. This occurred in the absence of a preference for LNT over LNnT extrapolated from their similar growth rates. Thus ATCC 15697 may experience enhanced fitness when encountering LNT in the infant gut, although this remains to be tested in an *in vivo* system. Moreover, ATCC 15697 diverges in the metabolic fate of carbons during LNT or LNnT utilization. The ratio of secreted acetic acid to lactic acid (AA:LA) is considerably higher for LNT and LNnT than other carbohydrates. Importantly, LNnT promotes a significantly higher AA:LA ratio relative to LNT. The deacetylation of GlcNAc via deacetylase activity (EC 3.5.1.25, **Figure 3**) likely contributes to the increase in relative concentrations of acetic acid during LNT metabolism.

Of particular importance is that LNnT metabolism significantly increases formic acid production. This is not observed during LNT metabolism and constitutes a major metabolic shift solely attributable to the isomeric composition of LNnT. Thus the AA:LA ratio increases during LNnT fermentation likely due to GlcNAc deacetylation and a simultaneous decrease in lactic acid in shunting pyruvate

toward formic acid production. The conversion of pyruvate to acetyl-CoA and subsequently to acetic acid during LNnT utilization results in formic acid and ethanol production. Modulation of acetic acid production produces higher levels of ATP during LNnT fermentation. This is consistent with the relative inefficiency LNnT is utilized for biomass as limited ATP restricts cellular growth. Bifidobacteria are known to increase formic acid secretion during inefficient metabolism of unfavorable substrates (25, 50, 55–57). It is noteworthy that previous studies observed cellular growth considerably lower than the accumulated biomass generated on LNnT in the present study.

In addition, increased ethanol production during LNnT metabolism recycles NAD^+ following reduction of acetyl-coA. Since formic acid is generated at the expense of lactic acid, recovering NAD^+ is critical as lactic acid production recycles cofactors and does not yield ATP (23). On a molar basis the 2-carbon pathway terminating in ethanol recoups double the amount of NAD^+ than the 3-carbon arm (i.e., pyruvate to lactic acid). Whereas it is clear that LNnT shifts metabolism toward formic acid and ethanol production, the molecular mechanisms underlying these alternative pathways remains incompletely understood. It is clear, however, that the terminal β1-4 linkage in LNnT prompts this divergent physiological response.

In an effort to determine the contribution of global gene expression to LNT and LNnT metabolism, previously generated RNA-seq data was analyzed in addition to targeting key loci with qRT-PCR. A previous study of *Bifidobacterium breve* UCC2003 concluded that there are overlapping metabolic transcriptional networks with some critical features that are unique between LNT and LNnT metabolism (58). It is noteworthy that *B. breve* evolved the capacity to hydrolyze HMO extracellularly and imports degradation products. There is limited evidence that *B. infantis* is able to do so, which indicates a fundamental physiological difference between the two species. As *B. infantis* captures HMO from its extracellular environment, the complement and expression of transport proteins may catalyze or restrict metabolism of a given HMO species. In this study, the F1SBP Blon_2347 expression differed between the two HMO species, whereas Blon_2177 and Blon_2344 were expressed regardless of the specific HMO. Interestingly, the global transcriptome exhibited a different expression profile for these particular genes. Additional studies are required to resolve the conflict between expression of transporters predicted to be active on type I glycans (i.e., LNT) (16) that were observed to be induced by the type II LNnT. Further characterization of the functional interactions between transport systems and HMO substrates may be essential to address these discrepancies.

The aminosugar residue GlcNAc is a constituent of LNT, LNnT, and all HMOs with a degree of polymerization $\geq$4. Prior to entering the F6PPK pathway, GlcNAc is processed by two enzymes putatively encoded within the ATCC 15697 genome. This includes GlcNAc-6-P deacetylase (*nagA*; Blon_0882) that deacetylates GlcNAc prior to deamination by glucosamine-6-P isomerase (*nagB*; Blon_0881). *B. infantis* expresses both of these proteins when grown on pooled HMO as reported in a previous study (51). In this study, both LNT and LNnT upregulated these loci supporting the postulate that HMO-bound GlcNAc metabolism contributes to skewing the AA:LA ratio. The elevated AA:LA ratio was observed for pooled HMOs and LNT in a previous study that focused primarily on galactooligosaccharides (47). It is important to note that GlcNAc-bound in HMO may not be fully catabolized through the F6PPK pathway as GlcNAc could serve as a substrate in anabolism including peptidoglycan and other biosynthetic processes (17). Interestingly, ATCC 15697 does not utilize GlcNAc when supplied as sole carbon source in the media in contrast to other *B. infantis* strains. This may be due to a genetic or regulatory variation inherent to the strain. Moreover, hexosaminadase genes were expressed similarly regardless of the particular HMO isomer. These enzymes liberate GlcNAc from galactose through hydrolyzing the β1-3 linkage that is present in both LNT and LNnT.

The terminal galactose, contrastingly, is connected to GlcNAc via a β1-3 linkage in LNT and a β1-4 linkage in LNnT. Thus it was unexpected that a type I glycan-active (e.g. LNT) β-galactosidase (Blon_2016) is downregulated by LNT and not LNnT ($p < 0.05$) (52). This may be due to constitutive expression of other β-galactosidases that cleave the terminal galactose *in vivo*. It is significant that both HMO isomers upregulate two genes that are predicted to feed galactose into the F6PPK pathway. This includes gal-1-P uridylyltransferase (*galT*; Blon_2172) and Uridine 5′-diphospho-glucose-4-epimerase (UDP-glc epimerase, *galE*; Blon_2171) that are localized adjacent to HMO transporters on the ATCC 15697 chromosome. Furthermore, LNnT induces these genes to a greater extent than LNT. This provides mechanistic detail for the physiological differences between LNnT and LNT fermentation.

Interestingly, LNT and LNnT upregulates α-L-fucosidase gene expression despite lacking fucosyl moieties within their respective oligosaccharide structure. Accordingly, LNT strongly upregulates two fucosidases localized to the HMO catabolic cluster. This suggests that there is overlapping regulatory systems or *B. infantis* recognizes LNT and LNnT as signaling molecules to prepare for metabolizing fucosylated HMOs. HMO tetrasaccharides is utilized early in fermenting pooled HMOs prior to fucosylated glycans (19).

Constituent monosaccharides bound in HMO are transformed into substrates catabolized through the F6PPK pathway. Accordingly, LNnT upregulates several genes in this central metabolic pathway likely to satisfy energy demands from a more inefficiently metabolized oligosaccharide. It is interesting that both acetate kinase and lactate dehydrogenase are upregulated by LNnT relative to LNT or lactose. The former is expected given the physiological evidence for increased acetic acid secretion. Lactate dehydrogenase upregulation may be a consequence of fully activating a central metabolic regulon to maintain NAD^+/NADH homeostasis. There is a significant link between formic acid production and transcriptional processes. LNnT strongly induces formate acetyl transferase (*pfl*; EC 2.3.1.54), which catalyzes formic acid and acetyl-CoA production from pyruvate. In contrast, LNT downregulates this gene as well as pyruvate formate lyase, the latter of which appears to be constitutively expressed during LNnT fermentation. This represents a strong mechanistic association between the LNnT

structure and *B. infantis* genomic features to drive the metabolic phenotype.

As bacterial strains of a given taxon may exhibit profoundly dissimilar phenotypes, three additional *B. infantis* strains were examined. *B. infantis* UMA301 exhibits a very similar metabolic response to LNT and LNnT relative to ATCC 15697. Interestingly, these two strains are closely related phylogenetically (30), which suggests that metabolic signature may be a function of phylogenetic divergence for *B. infantis* HMO utilization. Similarly to ATCC15697, LNnT shifts UMA301 metabolism toward formic acid and increases the ratio of acetic acid to lactic acid secretion.

In contrast, UMA300 efficiently utilizes both LNnT and LNT and does not produce formic acid and ethanol on either substrate to the same extent as ATCC 15697 and UMA301. This is likely a function of UMA300 processing LNnT in a efficient manner as LNT, which obviates the need for a metabolic shift. The inefficient HMO-consumer UMA299 exhibits a metabolic response congruent with this unique phenotype among *B. infantis* examined to date (30). The limited capacity to utilize HMO has been attributed to genetic defects within its HMO genomic cluster and provides a control strain linking genotype with metabolic phenotypes. As a result of inefficient growth on LNT and LNnT, UMA299 increases the AA:LA ratio, and formic acid/ethanol production. This is consistent with the hypothesis that diminished capacity for utilizing HMO promotes higher acetic acid concentrations compared to other carbohydrates despite achieving low optical density.

A cell culture approach was used to further develop a model of host-microbial interactions that incorporates inefficient metabolism of specific HMOs. Cell-free supernatants from *B. infantis* HMO fermentations were evaluated for their anti-inflammatory properties on Caco-2 cells. Given the parameters tested, it appears that *B. infantis* reduces inflammation regardless of milk carbohydrate source. Specific anti-inflammatory molecules presented or secreted by *B. infantis* remains hypothetical. Moreover, the extent to which other HMO structures diminish inflammatory outcomes is not understood.

In conclusion, LNT and LNnT utilization increased the AA:LA ratio in all strains. In instances where LNT or LNnT was inefficiently utilized, carbon was shunted toward formic acid and ethanol secretion. A fully integrated mechanistic model underlying this phenotype remains incompletely developed. Thus there is a scientific need to investigate all purified HMO species, additional *B. infantis* strains, as well as other bifidobacterial species to ascertain linkages between HMO structure and physiological responses. This will further refine the metabolic model by which bifidobacteria utilize HMO to colonize the nursing infant colon. In addition to fundamental biological research, there are broad implications to infant nutrition and health. There is accumulating evidence that rationally designing interventions to enhance infant nutrition will require judicious selection of HMOs. This could involve incorporating a single HMO species or a mixture. Clearly a specific HMO tetrasaccharide has different metabolic consequences depending on the *B. infantis* population. There is the potential for strain-level effects to influence the emergent properties of the infant gut microbiome community. Accounting for variation between bifidobacteria, HMO structures, the biology of the infant, and their hosted microbiome communities may enable delivery of precision nutrition and increase impact of the intervention.

AUTHOR CONTRIBUTIONS

EÖ and DS designed experiments. EÖ performed the experiments and drafted the manuscript through several iterations. DS conceived the study, revised, and approved the manuscript.

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SUPPLEMENTARY MATERIAL

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Early Dietary Patterns and Microbiota Development: Still a Way to Go from Descriptive Interactions to Health-Relevant Solutions

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Early nutrition and growth in the initial years of life are important determinants of later body weight and metabolic health in humans, and the current epidemic of obesity involving children requires a better understanding of causal and protective mechanisms and components in infant foods. This review focuses on recent evidence implicating feeding modes (e.g., breast milk and formula milk) and dietary transitions toward complementary foods in the progression of microbiota maturation in children. The literature exploring body weight outcomes of microbiota changes induced by diet in early life is limited. Representative studies addressing the use of probiotics in pregnant women and infants are also examined. Methodological and geo-cultural variations make it difficult to avoid (apparently) controversial findings. Most studies indicate differences in the microbiota of formula versus breastfed infants, but some do not. Duration of breastfeeding delays the maturation of the microbiota toward an adult-like profile. However, the effect size of the early feeding pattern on microbial function was found to be very small, and absent after the third year of life. There are several interesting mediators whereby milk composition can affect infants' microbiota and their optimization is a desirable strategy for prevention. But prevention of what? Although there are few correlative evaluations relating microbiota and body weight in early life, studies demonstrating a cause–effect relationship between diet-induced changes in early microbiota development and subsequent metabolic health outcomes in humans are still missing.

Keywords: milk, formula, obesity, children, probiotic, prebiotic, maternal, complementary food

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INTRODUCTION

Associations between microbiota composition and obesity in humans have been repeatedly confirmed, although the cumulating effects of diseases, medications, genetic, and environmental factors make it difficult to dissect causative from adaptive microbial changes in adults. Life course studies would help to identify primary and secondary events and critical time windows. The transition from birth to adulthood results in profound modifications of the microbiota, which is characterized by a dominance of Firmicutes and Bacteroidetes in adults, as opposed to infants, in whom Actinobacteria and Firmicutes are dominant, Proteobacteria are more represented, and Bacteroidetes are nearly or entirely absent, especially in newborns (1, 2). Early nutrition predicts the later obesity risk and drives microbiota development. Considering the relevance of early life in gut colonization and microbiota

stabilization, it is plausible to hypothesize that early life dysbiosis could be prevented or corrected by optimizing nutritional patterns. This minireview summarizes studies examining the longitudinal development of the gut microbiota from infancy to early childhood in relation to breastfeeding, complementary foods, probiotic interventions, and the potential link with childhood obesity.

MICROBIOTA DEVELOPMENT OVER FEEDING TRANSITIONS

There are few longitudinal studies covering the period from birth to infancy or early childhood. In a Swedish cohort (3) of 98 mothers undergoing gut microbiota analysis at 2 days of delivery, and their full-term infants studied in the first week of life, at 4 and 12 months of age, a 72% correspondence in MetaOTUs in mother–newborn pairs was observed after vaginal delivery, compared with 41% after C-section, indicative of vertical transmission. In spontaneously delivered newborns, the gut was initially colonized by relatively aerobic bacteria (*Enterococcus*, *Escherichia/Shigella*, *Streptococcus*, and *Rothia*), followed during lactation by anaerobic bacteria involved in lactate metabolism (*Bifidobacterium*, *Lactobacillus*, *Collinsella*, *Granulicatella*, and *Veillonella*), and in complementary feeding period by bacteria involved in fiber and carbohydrates degradation, and short-chain fatty acid production (*Bacteroides*, *Bilophila*, *Roseburia*, *Clostridium*, and *Anaerostipes*), resembling maternal microbiota. Consistently, the repeated characterization of 192 breastfed infants (4) from 1 week to 6 months of age to primarily addresses the effects of gestational age and delivery mode, showed a progressive reduction in genera that are abundant at 1 week toward genera that become abundant at subsequent ages, including (from 8 weeks) those that are core to enterotypes, such as *Prevotella*, *Blautia*, and *Ruminococcus*. On a functional level (3), genes for carbohydrate uptake, vitamin metabolism, and essential amino acid transport were enriched in newborns microbiome, while functions involved in lactose transport were most abundant in 4-month-old infants, and those implicated in degradation of complex sugars, starch, pectin, and in methionine degradation, lysine biosynthesis, and carbohydrate, leucine, and tryptophan metabolism, were enriched at 12 months. The subsequent period of life was examined in 264–311 Danish children (5) studied at 9, 18, and 36 months, showing that microbial composition at 9 months maintains an influence of the preceding milk-based diet, whereas dramatic changes occur between 9 and 18 months, followed by fewer adaptations later. This suggested that the period necessary for microbiome stabilization is longer than previously claimed. The progression from 9 to 36 months was primarily characterized by a reduction in *Bifidobacterium* (*longum* and *breve*), *Lactobacillus* spp., *Enterobacteriaceae*, *Clostridium coccoides*, and by an increase in Bacteroidetes-related species, *Clostridium leptum*, *Eubacterium hallii*, *Roseburia* spp., and *Bifidobacterium* (*adolescentis* and *catenulatum*). In addition, the initial establishment of human enterotypes, expressed as *Prevotella*/*Bacteroides* ratio, was observed between 18 and 36 months of age, reflecting the transition toward an adult-like microbiota, adapting to an adult-type diet.

Overall, despite a large number of intervening factors, nutrients seem to be dominant regulators of gut microbiota development across the first 3 years of life.

MICROBIOTA DEVELOPMENT AND BREAST OR FORMULA FEEDING IN EARLY INFANCY

In the study by Bäckhed et al. (3), 70% of infants were exclusively breastfed at the age of 4 months, showing increased levels of *Lactobacillus* (*johnsonii/gasseri* and *paracasei/casei*), and *Bifidobacterium longum*, compared with formula-fed infants, in whom *Clostridium difficile*, *Granulicatella adiacens*, *Citrobacter* spp., *Enterobacter cloacae*, *Bilophila wadsworthia*, and *Bifidobacterium adolescentis* were more dominant. At a functional level, formula feeding was associated with enrichment in functions typical of the adult microbiota, whereas exclusive breastfeeding was characterized by functions of oxidative phosphorylation and vitamin B synthesis (3). However, the feeding pattern explained only 1.3% of the functional variation. In a Finnish study, a higher number of IgG-secreting cells was observed in 3-month-old infants undergoing exclusive compared with non-exclusive breastfeeding (6), suggesting functional immune-related effects.

The above findings on microbiota composition were partly confirmed or extended by studies in Swedish infants aged 6 days, 3 weeks, 2 and 6 months (1), in Finnish 3, 6, and 12-month-old (6), in Canadian 4-month-old (2), and in North American 3-month-old infants (7), showing that formula-fed infants had increased richness of species, with overrepresentation of *C. difficile* (1, 7), whereas breastfed infants had higher relative abundance of *Bacteroides* (7) and *Bifidobacterium* (6), and lower abundance of *Clostridium*, *Peptostreptococcaceae*, and *Verrucomicrobiaceae*, including *Akkermansia* genus (2), *Lachnospiraceae incertae sedis*, *Streptococcus*, *Enterococcus*, and *Veillonella* (7). Results from other studies (4, 7, 8) are partially at variance with these findings. In a UK cohort (4), infants were stratified in subgroups receiving breastfeeding until 2, 4, and beyond 4 months (feces at 1, 4, 8, and 24 weeks), observing no influence of the feeding pattern on microbiota composition, with the exception of 24-week-old infants delivered by C-section, in whom the abundance of five genera was enhanced by breastfeeding protraction beyond 4 months, suggesting that breastfeeding may compensate for the C-section-dependent deficiency in selected bacteria. Unexpectedly, *Bifidobacterium* abundance did not differ in any groups. Likewise, in a French study (8), absolute bacteria and *Bifidobacterium* abundances were measured on feces collected during 3 subsequent periods in which 11 infants received exclusive breastfeeding (2–11 weeks), mixed feeding (6–21 weeks), and exclusive formula feeding (7–42 weeks), showing no difference across them, due to formula–milk integration or breast-milk exclusion. These two studies do not exclude a long-term impact of early breastfeeding on microbiota development.

Different methodologies, types of formula used, and cohort characteristics (e.g., inclusion of preterm infants, differing frequencies of delivery modes, and antibiotic use), depending on the focus of investigation, might explain some discrepancies between

studies. Furthermore, the use of relative or absolute bacterial counts may impact physiological interpretations.

In summary, there is evidence that in the first few months of life, breastfeeding favors the development of health-promoting microbes, whereas formulas stimulate growth of disease-related bacteria, but there are also studies showing marginal or no effects of such dietary patterns on microbiota. Even when a strong impact of early diet on microbiota composition was found, microbial functional properties seemed to be marginally affected (3), challenging the mechanisms that may link microbiota functions and health.

MICROBIOTA MATURATION AND DURATION OF BREASTFEEDING

One relevant question to understand mechanisms relating milk–microbiota interactions with health outcomes is whether the duration of breastfeeding has long-lasting impact on microbiota development. In 227 Danish infants (2 cohorts, 9) characterized at 9 and 18 months of age, duration of exclusive breastfeeding in 9-month olds was positively correlated with the abundance of bacteria that utilize milk-derived oligosaccharides or lactate (*Bifidobacteria*, *Veillonellaceae*, and *Pasteurellaceae*), and negatively associated with the abundance of species that utilize plant-derived complex carbohydrates and resistant starch from solid foods, i.e., *Lachnospiraceae* (*Dorea*, *Coprococcus*, *Blautia*, *Pseudobutyrivibrio*, and *Roseburia* genera), *Ruminococcaceae* (*Ruminococcus*, *Anaerotruncus*, *Oscillibacter*, *Clostridium* IV, and *Butyricicoccus* genera), *Erysipelotrichaceae*, *Peptostreptococcaceae*, and *Eubacteriaceae*.

Interestingly, children fed formula since birth underwent premature microbiota maturation (3), whereas continued breastfeeding at 9–12 months delayed this process, with persistence of high relative levels of *Bifidobacterium* (spp. and *longum*), *Lactobacillus*, *Collinsella*, *Megasphaera*, and *Veillonella* (5, 9), and low levels of butyrate-producing taxa, such as *Clostridia* (*leptum* and *coccoides*), *E. hallii*, and *Roseburia* spp., and of *Desulfovibrio* spp., *Akkermansia muciniphila*, and Bacteroidetes species. Coherently, daily breast-milk intake at 9 months was quantitatively correlated with microbiota composition (9), and alpha diversity was lower at increasing durations of exclusive breastfeeding.

In summary, the effects of breastfeeding on microbiota composition are protracted to the second semester of life but become limited and vanish at 18 and 36 months of age (5, 9). Selected differences were abolished already at 12 months of age (6). Once confirmed, these findings convey fundamental mechanistic insight: if breastfeeding impacts later health through the microbiota (to be demonstrated), the mediating mechanism in tissues should be settled during the first year of life, and studying the microbiota as a function of breastfeeding in later life may not elucidate that causal link.

MILK COMPONENTS AFFECTING INFANT'S MICROBIOTA

One potential source of variability and discrepancy between studies may be the composition of breast milk or formula due to, e.g., geo-cultural differences, maternal factors, industrial production, and distribution. Properties whereby breast milk may influence child's microbiota establishment include the vertical transmission of bacteria, the oligosaccharides-stimulated bacterial growth, and the exposure to immune modulators. In a UK study (10), 10 mother–infant pairs underwent collection of maternal milk and feces at 1, 3, 6, and 12 weeks of newborns life. The milk microbiota showed high diversity and changes in dominant microbes over time. Intriguingly, milk microbiota composition accounted for the 70–88% of infant's microbial abundance, and identical strains of *Bifidobacterium breve* and *Lactobacillus plantarum* were observed in maternal milk and respective infant gut. Milk bacteria can originate from maternal skin, neonatal oral cavity, or maternal gut (enteromammary pathway) and are influenced by delivery mode (11), with higher bacterial diversity and richness in response to vaginal compared with C-section delivery. Considering that samples were collected after 1 month of breastfeeding, the influence of neonatal oral bacteria is a possible explanation, since the newborn microbiota (at least in the gut) is by itself influenced by delivery mode.

Breast milk is rich in oligosaccharides. Oligosaccharides are present in formulas, explaining some of the above controversies (7, 12), although the relative prevalence of these sugars is different (13). In a study relating milk oligosaccharides and microbiota of 3-month-old infants (7), 141 oligosaccharides were identified in breast milk. The authors found that each oligosaccharide predicted multiple microbial changes, and each of the affected microbial abundances was predicted by different oligosaccharides. Gut *Bifidobacterium*, *Bacteroides*, *Enterococcus*, *Veillonella*, and *Rothia* were impacted. Corroborating a cause–effect relationship, infants receiving oligosaccharides-supplemented formula (14) showed a fecal *Bifidobacteria* abundances and composition similar to breastfed infants, exceeding those observed in formula-fed infants.

Breast milk is a source of secretory-IgA, leading to antigen-specific gut immune protection. Mice studies (15) indicated that an early gut exposure to maternal secretory IgA modulates microbiota composition and prevents translocation of aerobic bacteria from the neonatal gut into draining lymph nodes. The effect was persistent and greater in adults, in which the expression of genes implicated in inflammatory diseases by intestinal epithelial cells was also modified. Moreover, maternal secretory IgAs were shown to be protective against colonic damage caused by an epithelial-disrupting agent. These findings reveal mechanisms through which breastfeeding and secretory IgA may protect intestinal health. A Finnish study (6) reported positive associations between levels of soluble cluster of differentiation 14 (sCD14) in maternal colostrum and circulating immunoglobulin-secreting cells in 3 (IgG cells) and 12-month-old offspring (IgA and IgM cells), supporting the role of breast milk sCD14 in influencing infants' immune intestinal and humoral responses.

In summary, the characterization of milk-related effects on the microbiota may hold important preventive implications, considering that both breast milk and formula compositions can be theoretically manipulated. Identifying the desirable health-related microbial profile in infants would make it possible to design interventions that optimize milk quality. To this

end, much effort is required to prove cause–effect relationships between early-life bacteria and later health outcomes.

BREASTFEEDING–MICROBIOTA INTERACTION AS CAUSE OF OBESITY

Longer duration of breastfeeding is dose-dependently associated with a decrease in later overweight risk (16). Consistently, duration of breast-milk consumption was negatively associated with the overall energy intake (5, 17), and breastfed infants were leaner than formula-fed children at 9 and 18 months in Danish cohorts (5, 18). Breast milk promoted *Bifidobacteria* development in that study and might underlie these findings. Indeed, low *Bifidobacterium* and high *Staphylococcus aureus* abundances at 6 and 12 months of age predicted weight and obesity in 7-year-old Spanish children (19). Considering that the effects of breastfeeding versus formula feeding on microbiota are not lasting beyond 1–3 years (5, 6, 9), it is possible that weight differences emerged earlier, as weight gain during the first 6 months of life is particularly predictive of later obesity, or that later-developing bacteria, colonizing the gut during complementary feeding, may have contributed to aggravate childhood obesity, as discussed below.

COMPLEMENTARY FEEDING: INTERACTION WITH THE MICROBIOTA AND OBESITY

Longitudinal studies covering the transition of microbiota from lactation toward solid food and adult-like diets are limited. A thorough 7-day food questionnaire was collected in the Danish cohorts, suggesting that the progression of infant diet toward family foods, enriched in meat, milk, cheese, and animal fats was associated with a reduction in *Bifidobacteriaceae* and *Enterococcaceae*, in favor of *Lachnospiraceae* and *Sutterellaceae* abundances (9). However, these cohorts did not address microbiota composition before the ninth month of age, and longer observation periods are required to expand the above knowledge.

There is no clear association between the timing of complementary food introduction and childhood overweight or obesity, but some evidence suggests that its introduction at 4 months or earlier, compared with 4–6 months or longer, may increase the risk of childhood overweight (20, 21). Coherently, age at introduction of complementary foods did not correlate with microbial abundances or alpha diversity at 9 months (9). The composition, rather timing of complementary foods has been more clearly related to later obesity risk (22). In particular, high intakes of energy and dairy protein in infancy could be associated with an increase in body weight and fatness. Consistently, correlative results indicated that changes in the microbiota induced by the progression from early infant to family foods are mostly driven by protein and fiber dietary contents (9). However, the microbial categories that changed due to family food (*Bifidobacteriaceae*, *Enterococcaceae*, *Lachnospiraceae*, and *Sutterellaceae*), did not correspond to bacteria that were predictive of weight gain. Indeed, the increase in body mass index between 9 and 36 months (5) was predicted by positive changes in Firmicutes, *C. leptum*, and *E. hallii*, i.e., butyrate-producing groups contributing to energy harvest, and negative changes in *Methanobrevibacter smithii* and *Enterobacteriaceae*. Interestingly, these bacteria are the same predicted by breastfeeding duration in earlier life phases, and not by complementary diet characteristics.

PROBIOTICS AND MICROBIOTA DEVELOPMENT

Few interventions have been conducted to examine the effects of probiotics on infant's microbiota or immunity development. A recent double-blind, randomized, placebo-controlled study (23) was conducted in 106 newborns assigned to standard whey-based formula, containing 10^8 colony-forming units/g of *Bifidobacteria* (*bifidum*, *breve*, and *longum*), or to control formula (placebo) in a 12-month intervention, followed by 24 months of follow-up to examine microbiome–metabolome outcomes. The conclusion was that supplementation of *Bifidobacteria* to infant diet can modulate the occurrence of specific bacteria, i.e., *Bacteroides* and *Blautia* spp. and metabolites during early life, with no detectable long-term effects. In a 4-week double-blind, placebo-controlled trial (6), 96 mothers were randomized to receive placebo or 10^{10} colony-forming units of *Lactobacillus rhamnosus GG* before delivery. The treatment was protracted in respective infants until 6 months of age, with follow-up visits at 3, 6, and 12 months. The study, mostly oriented to immune-related aspects, demonstrated a significant interaction between probiotics and breastfeeding in relation to the amounts of immunoglobulin-secreting cells observed in infants' circulation and suggested that *Lactobacilli* may potentiate the beneficial effects of maternal milk on the offspring's immunity. Similarly, in a randomized, double-blind, placebo-controlled trial, an oral synbiotic preparation (*L. plantarum* plus fructooligosaccharide) administered for 7 days to 4,556 rural Indian newborns following breastfeeding, showed efficacy on neonatal sepsis prevention, suggesting that host–probiotic interactions enhance local gastrointestinal mucosal and systemic host immunity (24).

CONCLUSION

Factors affecting early microbiota are numerous and heterogeneous between studies, depending on their focus. They include delivery mode, gestational age, geographical variations in dietary habits, and antibiotic use. Due to their potentially compensatory or synergistic interaction in modulating microbiota development, it is difficult and not necessarily plausible to statistically correct for them. Geographical provenance of cohorts was underlined in this review, since cultural heterogeneity is a potentially important source of actual heterogeneity. Different methods are used to quantify microbial abundance either in relative or absolute terms, which are not of interchangeable interpretation. One reflects the balance between species, in which potentially favorable may overrule the effects of unfavorable bacteria and *vice versa*; the other informs on the independent load of given taxa. The comparison between sequencing techniques goes

beyond the scope of this review. The common use of data filtering, and exclusion of large portions of less abundant or frequent taxa, not always clearly justified, may obscure the value of physiologically relevant species and be source of interpretative heterogeneity between studies.

Notwithstanding the above, a majority of reports support a role of early dietary patterns to regulate microbiota composition. Continued breastfeeding appears to delay the transition toward an adult-like microbiota, whereas formula feeding or shorter breastfeeding accelerate this process. It seems important to account for different species of the same genus (e.g., *Bifidobacterium*), which dominate in different life periods. Some controversies remain on the relevance of breastfeeding versus formula feeding, since not all studies could identify a difference in microbiota, especially in very young infants. Complementary food protein and fiber contents, but not age at introduction, were related to microbiota characteristics. There is convincing evidence that milk composition represents an important modulator of infants' microbiota and intestinal health, and optimization of this interaction is a promising preventive perspective. Some studies have been carried out using pre- or probiotics to influence the development of infants' microbiota, but their clinical benefits and safety remain to be clearly established.

The most important gap of knowledge relates to the lack of studies including health outcomes. Most studies are descriptive, and some show correlations with body weight, but no cause–effect mechanisms are actually demonstrated. The evidence that early dietary patterns result in a limited functional effect size in metagenome analysis, and that breastfeeding effects on microbiota are no longer seen after the third year of life, or earlier, prompt for studies demonstrating direct cause–effect relationships linking early dietary–microbiota interactions, and specific taxa, with short- and long-term body organ development and health-related consequences.

AUTHOR CONTRIBUTIONS

PI conceived, drafted, and reviewed the manuscript. ES contributed to the critical revision of the manuscript, and with PI approved its final version for publication.

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MINI REVIEW
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Microbial Therapeutics Designed for Infant Health

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Acknowledgment of the gut microbiome as a vital asset to health has led to multiple studies attempting to elucidate its mechanisms of action. During the first year of life, many factors can cause fluctuation in the developing gut microbiome. Host genetics, maternal health status, mode of delivery, gestational age, feeding regime, and perinatal antibiotic usage, are known factors which can influence the development of the infant gut microbiome. Thus, the microbiome of vaginally born, exclusively breastfed infants at term, with no previous exposure to antibiotics, either directly or indirectly from the mother, is to be considered the "gold standard." Moreover, the use of prebiotics as an aid for the development of a healthy gut microbiome is equally as important in maintaining gut homeostasis. Breastmilk, a natural prebiotic source, provides optimal active ingredients for the growth of beneficial microbial species. However, early life disorders such as necrotising enterocolitis, childhood obesity, and even autism have been associated with an altered/disturbed gut microbiome. Subsequently, microbial therapies have been introduced, in addition to suitable prebiotic ingredients, which when administered, may aid in the prevention of a microbial disturbance in the gastrointestinal tract. The aim of this mini-review is to highlight the beneficial effects of different probiotic and prebiotic treatments in early life, with particular emphasis on the different conditions which negatively impact microbial colonisation at birth.

Keywords: probiotics, prebiotics, gut microbiota, infant, health

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INTRODUCTION

From birth through to the initial stages of weaning, intestinal microbial composition has a significant impact on infant gut health. Recent advances in culture-independent sequencing technologies has allowed for the identification of key microbial species involved in the initial colonization process, including those facultative anaerobes such as *Streptococcus*, *Staphylococcus*, and *Enterobacter* spp. (1, 2). Mode of delivery and feeding regime are two important factors which influence microbial colonization at birth (**Figure 1**). Host genetics may also impact development

Abbreviations: GI, gastrointestinal; FT, full term; PT, preterm; VLBW, very low birth weight; GOS, galacto-oligosaccharides; FOS, fructo-oligosaccharides; SCFAs, short chain fatty acids; NEC, necrotizing enterocolitis; MS, metabolic syndrome; HFD, high-fat diet; ASD, autism spectrum disorder; MIA, maternal immune activation; NGPs, next generation probiotics; LBPs, live biotherapeutic products.

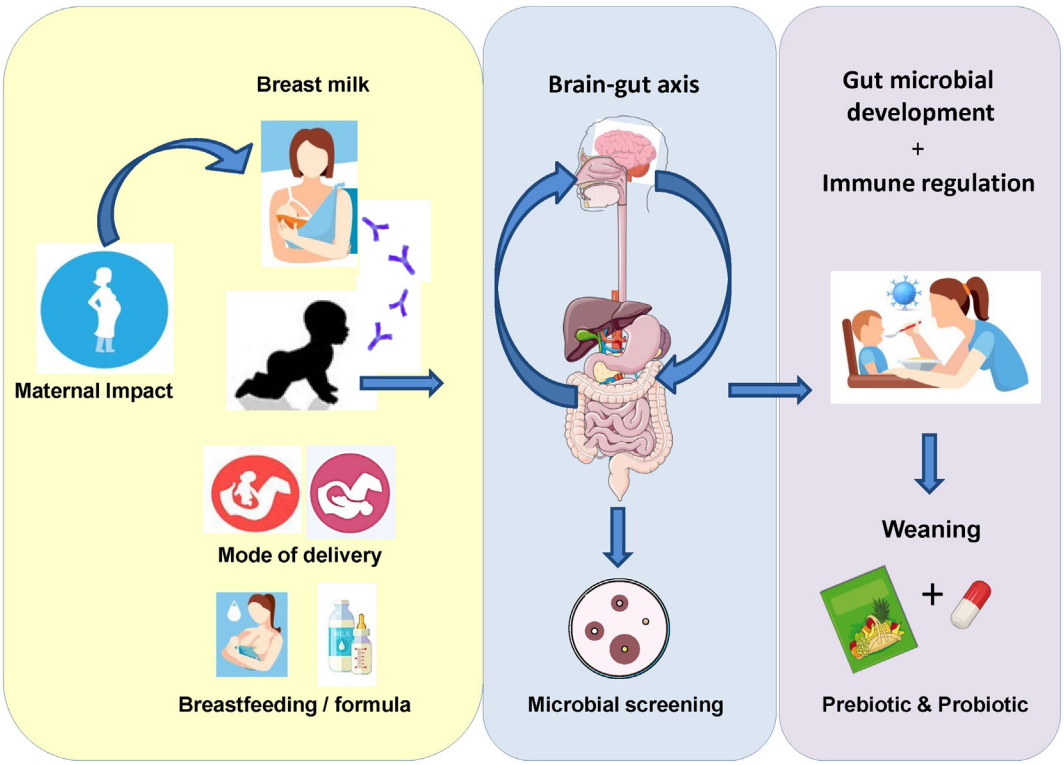

FIGURE 1 | Initial exposure to the microbial environment surrounding the infant can have a significant impact on gut microbiota development. External factors, such as maternal health status, mode of delivery, gestational age, and feeding regime, can impact the colonization and flux of microorganisms during this critical period in life. Subsequently, multiple studies have begun to focus on how these factors can affect the gut microbiome in early life. Moreover, in order to improve the health status of the infant gut, current focus is on the effect of probiotics and prebiotics in terms of their potential multifaceted health benefits. The current mini-review outlines a number of studies where either pro- or pre-biotics were utilized as a microbial therapeutic to improve infant health.

of the gut microbiome, with recent studies focusing on similar microbial patterns between monozygotic twin pairs and their fraternal siblings (3, 4). Indeed, the duration of breast feeding and introduction of formula feed can play a significant role in shaping the gut microbiome (5–7). Thus, it is imperative that we understand how the introduction of particular microbial species and prebiotic additives may restore balance and ameliorate the effects associated with gastrointestinal (GI) disorders.

GI Microbial Development at Birth

Many studies have begun to focus on the development of the infant gut microbiome over time (8–10). A study by our group found that in full-term (FT) cesarean delivered infants, an increased fecal abundance of Firmicutes and lower abundance of Actinobacteria was evident after the first week of life; however, the gut microbiota of preterm (PT) infants displayed a significantly greater abundance of Proteobacteria compared to the FT infant group. Interestingly, the gut microbiota profile of FT cesarean delivered infants resembled that of the vaginally delivered infants at 8 weeks of life (1). In terms of gestational age at birth, the PT infant gut has previously been characterized by delayed microbial colonization, with reduced levels of anaerobic taxa such as *Bifidobacterium* and *Bacteroides* (11, 12). Indeed, gut microbiota development in PT infants has been found to correlate with the infant's postconceptual age (13). Moreover, Stewart et al. (14) described the impact of delivery mode on the PT gut microbiome and found no significant change in microbial diversity during the first 100 days of life.

Maternal–Infant Transmission

The acquisition of microbial strains may occur through multiple different pathways. For example, the administration of the probiotic *Lactobacillus rhamnosus* GG in a small subset of pregnant woman (between 30 and 36 weeks gestational age) found that maternal–infant transmission was successful and identified the strain in the feces of the infant cohort 6 months after birth (15). Indeed, maternal–infant transmission of mothers' lactobacilli predominantly occurs when the infant is delivered vaginally (16, 17). It is understood that the vaginal tract harbors these lactobacilli to reduce the pH of the intestinal milieu and prevent the growth of potentially pathogenic microorganisms in the infant gut.

Interestingly, a number of studies have begun to examine microbial communities present within breastmilk. Nine genera have previously been identified as part of the "core" breastmilk microbiome, including *Streptococcus*, *Staphylococcus*, *Serratia*, *Pseudomonas*, *Corynebacteria*, *Ralstonia*, *Propionibacterium*, *Sphingomonas*, *and Bradyrhizobiaceae* (18). Several other studies

have also identified the horizontal transfer of *Lactobacillus*, *Staphylococcus*, *Enterococcus*, and *Bifidobacterium* spp., from breastmilk to the infant gut (19–21). In a more recent study, Murphy et al. (22) reported the presence of 12 dominant genera in the breastmilk of lactating mothers. Results from this study described a number of frequently shared taxa, including *Bifidobacterium*, *Lactobacillus*, *Staphylococcus*, and *Enterococcus*, common in both breastmilk and infant feces during the first 3 months of life. Moreover, culture-dependent analysis identified *Bifidobacterium breve* and *Lactobacillus plantarum* present in both breastmilk and infant feces. Indeed, similar studies have also identified genomic patterns of *Bifidobacterium* and *Lactobacillus* spp. present in breastmilk and corresponding infant feces (23–25).

Recent studies have also begun to focus on microbial colonization which may occur *in utero*. Isolation of microorganisms from the umbilical cord blood of cesarean delivered infants (26), as well as the detection of bacteria in "first-pass" meconium (27, 28), suggest that the fetus may be colonized at a low abundance prior to exiting the womb. Moreover, research focused on the placental microbiome has found significant correlations between the placental and oral microbial communities (29). However, supporting evidence for the existence of a distinct placental microbiome is currently lacking (30). Thus, microbial colonization of the infant gut may be strongly influenced by the maternal microbiome, originating from several different niches, including the vaginal tract, breastmilk, and possibly the placenta.

Throughout the remainder of this mini-review, a number of studies will be discussed regarding prebiotic and probiotic treatments to prevent and/or treat conditions linked to GI health in early life.

PREBIOTICS

Human Milk Oligosaccharides (HMOs)

The current definition of prebiotics defined by Gibson et al. (31) describes "selectively fermented ingredients that result in specific changes, in the composition and/or activity of the GI microbiota, thus conferring benefit(s) upon host health."

Human breastmilk is a natural prebiotic source which contains essential nutrients and growth factors required for development of a healthy gut microbiome. Selective proliferation of healthy intestinal bacteria is thought to be just one of the multiple benefits of exclusive breast feeding, in addition to the nutrient supply of HMOs and glycoconjugates it provides (32). As HMOs are not digested by the infant themselves, they reach the colon intact and act as an essential substrate for the growth of beneficial *Bifidobacterium* and *Bacteroides* spp. Breastfed infants have also been found to harbor gut microbial taxa with genes involved in the phosphotransferase system for carbohydrate uptake, in addition to harboring an increased abundance of microbial species commonly used as probiotics, such as *L. johnsonii*/*L. gasseri*, *L. paracasei*/*L. casei*, and *B. longum* (33). Moreover, Hill et al. (1) found that prolonged breast feeding (>4 months) had a significant effect on the microbial composition of cesarean delivered FT infants at 24 weeks of life, in comparison to vaginally delivered infants, suggesting that breastmilk may prove to be even more beneficial in caesarian delivered infants.

Prebiotics and Weaning

It is well known that the infant gut microbiome does not fully develop until an infant reaches 2–3 years of age. Therefore, it is important that we recognize the changes occurring in the infant gut during this transition from early infant feeding to solid foods. Indeed, the World Health Organisation (34) states that the appropriate age for complementary feeding is "6 to 23 months of age"; however, this can change in exceptionally difficult circumstances [e.g., very low birth weight (VLBW) infants]. The following studies investigate the role of diet and the introduction of galacto- and fructo-oligosaccharides (GOS and FOS) for improving gut microbiota development in early life.

In terms of diet, a recent study investigated the impact of different foods on the gut microbiota profile of a Danish infant cohort. Results from this study found strong correlations between microbial taxa present and the dietary intake of foods high in protein and fiber; specifically meats, cheeses, and Danish rye bread (35). Interestingly, breastmilk/early infant feeding was correlated with the presence of *Bifidobacteriaceae*, *Enterococcaceae*, and *Lactobacillaceae*, whereas *Lachnospiriaceae* abundance was positively correlated with protein intake and negatively correlated with *Bifidobacteriaceae*. Moreover, *Pasteurellaceae* abundance was positively correlated with fiber and health conscious food choices (high in vegetable fats, fruits or fish, but low in sugar). Findings from this study suggest that the transition from breast feeding to "family-like" foods rich in fiber and protein significantly affects development of the infant gut microbiome (35). Digestion of these foods provides a variety of fermentable substrates necessary for the growth of colonic bacteria and thus further investigation into the by-products of predigested foods may provide valuable information to positively modulate the infant gut throughout weaning.

With respect to prebiotic supplementation of infant formulae, recent studies have investigated the use of GOS and FOS to reduce pH and produce a similar short-chain fatty acid (SCFA) profile to that of exclusively breastfed infants (36). Indeed, where infant formulae have been supplemented with GOS/FOS, a higher abundance of *B. longum* was found in the infant gut (37, 38). In addition, Haarman and Knol (38) found that infants consuming a standard formula (without prebiotic supplement) possessed a higher abundance of *Bifidobacterium catenulatum* and *Bifidobacterium adolescentis*, resembling a more adult-like microbiota. Alternatively, prebiotic inulin-type fructans and FOS can be found readily available in foods such as cereals, chicory, and bananas, which are recommended for infants during weaning. These previously mentioned studies, and others (**Table 1**), provide evidence for the beneficial use of prebiotics, GOS, and FOS, to help maintain a well-balanced microbial progression from infancy to early adulthood.

Although synbiotics, a combination of both a probiotic and a prebiotic (51), were not discussed in this review, the beneficial effects of bovine milk oligosaccharides and *Bifidobacterium* spp. on the infant gut have been noted in two human interventions (**Table 1**).

TABLE 1 | (A) Prebiotics effective in altering the intestinal microbiota in human infant studies, (B) probiotic strains effective in altering the intestinal microbiota in a number of human infant studies, and (C) synbiotics effective in altering the intestinal microbiota in a number of human infant studies.

(A) Infant study	Prebiotic	Duration	Microbial shift	Outcome	Reference
Healthy PT–FF	FOS	2 weeks	↑ *Bifidobacterium Bacteroides* ↓ *E. coli*	Improved stool frequency	(39)
Healthy FT–FF	GOS + FOS	4–5 weeks	↓ Clostridia	Improved stool frequency	(40)
Healthy PT + FT–FF	GOS + FOS	24 weeks	↕ *Bifidobacterium Clostridium*	Increase in sIgA	(41)
Healthy FT–FF	GOS, beta-palmitate + acidified milk	135 days	↕ *Bifidobacterium Clostridium*	Adequate growth. Increasing anthropometric parameters	(42)
Healthy FT–FF	GOS + FOS	6 weeks	↑ *Bifidobacterium*	Increase in acetate, butyrate, propionate. Reduced fecal pH	(43)
Healthy FT (>1 year age)–FF	GOS, FOS + inulin	8 weeks	↕ *Bifidobacterium Clostridium perfringens*	Increase in total organic acids. Lactacte, acetate, proprionate, butyrate	(44)
(B) Infant study	**Probiotic**	**Duration**	**Microbial shift**	**Outcome**	**Reference**
Healthy FT–FF	*L. rhamnosus* GG	24 weeks	↑ Lactobacilli	Increased length and weight. Improved growth	(45)
Low birth weight PT–BF + FF	*Bifidobacterium breve, Bifidobacterium longum* ssp. *infantis, B. longum* ssp. *longum*	6 weeks	↑ *Bifidobacterium* ↓ *Clostridium Enterobacteriaceae*	Promoted the formation of a healthy gut microbiota. *B. breve* suggested more suitable for PT infants	(46)
Late PT infants–FF	*Clostridium butyricum*, *Bifidobacterium*	1 week	Not reported	Proliferation of T lymphocytes. Clinical evaluation for *C. butyricum*	(47)
Healthy FT–FF	*Bifidobacterium animalis* ssp.*lactis, Streptococcus thermophiles.*	~1 year	Not reported	Lower frequency of reported colic or irritability. Lower frequency of antibiotic use	(48)
(C) Infant study	**Synbiotic**	**Duration**	**Microbial shift**	**Outcome**	**Reference**
Healthy FT–FF	*B. animalis* ssp. *lactis* + bovine milk oligosaccharide	48 weeks	↑ *Bifidobacterium Lactobacillus* ↓ *Clostridium Staphylococcus*	Supports normal growth. Fecal IgA and pH similar to breastfed infant	(49)
Healthy FT–FF	*B. animalis* ssp. *lactis* + bovine milk oligosaccharide	24 weeks	↑ *Bifidobacterium*	No differences in anthropometric measurements. Lower fecal pH	(50)

↑, *increased levels;* ↓, *decreased levels; FF, formula fed; BF, breastfed; CS, cesarean section; VD, vaginally delivered; PT, preterm; FT, full term; GOS, galacto-oligosaccharides; FOS, fructo-oligosaccharides.*

PROBIOTIC INTERVENTION

Health Benefits of Probiotics

Probiotics, described as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (52–55), have been investigated as potential prophylactics and/or treatments to re-establish gut homeostasis (**Table 1**). Moreover, the metabolism of indigestible oligosaccharides and plant polysaccharides by probiotic microorganisms, such as *Bifidobacterium* spp., contributes to the production of microbial bioactive molecules, such as SCFAs (56).

Subsequently, probiotic treatment is being extensively studied in different conditions associated with a disturbance in the gut. Necrotizing enterocolitis (NEC), childhood obesity and autism will be discussed next to highlight the link between a microbial disturbance in the gut and the beneficial use of probiotic prophylaxis in early life. The following conditions have been chosen due to their significant prevalence in current literature.

Necrotizing Enterocolitis

Necrotizing enterocolitis, where portions of the bowel undergo necrosis, is the second most common cause of mortality in PT infants. Subsequent studies focused on improving health outcomes have found an increased abundance of Proteobacteria prior to and throughout the condition, including potentially pathogenic organisms such as *Salmonella* and *Escherichia coli* (57, 58). In a longitudinal study examining gut microbiota development in PT twins, a twin pair discordant for NEC was discovered. Results from this study found clear changes attributable to antibiotic exposure and NEC development, with reduced microbial diversity and an increase in *Escherichia* spp. preceding NEC (59).

Probiotic prophylaxis has thus been investigated to examine whether this form of treatment could improve the quality of

life in PT infants. In a comprehensive review by AlFaleh and Anabrees (60), it was found that enteral administration of probiotics reduced incidence of severe NEC, and NEC-related mortality, with the majority of infants being administered a combination of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* spp. *via* breastmilk. Interestingly, the administration of bovine lactoferrin, in combination with *L. rhamnosus* GG, was found effective in reducing incidences of NEC in VLBW infants (61). In addition, the routine use of *Lactobacillus reuteri* DSM 17938 (BioGaia®) was found to be highly successful in reducing rates of NEC in infants at highest risk (birth weights ≤ 1,000 g) (62).

Metabolic Syndrome (MS) and Obesity in Childhood

Metabolic syndrome, described by WHO in 1998, relates to any case of insulin resistance found in the presence of at least two of the following risk factors; hypertension, obesity, high triglyceride levels, or reduced high-density lipoprotein cholesterol levels. To examine the effectiveness of probiotics in preliminary animal trials against MS, *L. paracasei*, *L. rhamnosus*, and *Bifidobacterium animalis* subsp. *lactis* were found to improve glucose–insulin levels and hepatic steatosis in a high-fat diet (HFD)-induced murine model (63). However, in a systemic review on the use of early probiotic intervention in human clinical trials, inadequate evidence was found to support the use of the probiotic *L. rhamnosus* GG or *L. paracasei* F19, when administered to both mothers and infants, in the prevention of childhood MS (64).

To tackle the prevalence of childhood obesity, scientists have begun to unravel the link between diet, the gut microbiome and consequent energy intake and adiposity. Turnbaugh et al. (65) examined the hypothesis that particular communities of microorganisms could be involved directly with obesity in an obese mouse model. Results from the study found an increased capacity to harvest energy from diet, with an increased ratio of Firmicutes to Bacteroidetes (65). Further metagenomic analysis revealed that the microbiome of mice fed a high-fat/high-sugar (Western) diet, was significantly enriched in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways involved in the fermentation of simple sugars (66). Thus, with the aim of reducing HFD-induced weight gain in humans, animal studies are examining the antiobesity effects of different probiotics (67, 68). In-depth analysis of these animal studies may provide opportunities for the introduction of probiotics to help reduce weight gain in early life.

Autism

The cause for autism spectrum disorder (ASD), a syndrome characterized by a deficit in social and communicative interactions, is yet unclear; however, recent studies have revealed a link between symptomatic cognitive dysfunctions and GI distress through a connection in the central nervous system, coining the term "brain–gut axis." There is now evidence that probiotics alleviate GI distress in various murine models which mimic the symptomatic traits of ASD (69, 70). Studies have found that a member of the *Bacteroides* spp., *Bacteroides fragilis*, acts as a natural anti-inflammatory, capable of inhibiting inflammatory responses in a chemically induced murine model of experimental colitis (71, 72). Moreover, a maternal immune activation (MIA) model, which challenges the immune system and promotes inflammatory factors in pregnant dams, induces key features of ASD and thus serves as an appropriate murine model in testing *B. fragilis* as a potential therapeutic (70). Hsiao et al. (70) demonstrated the ability of *B. fragilis* to correct the levels of a MIA-induced serum metabolite which was found at significantly higher concentrations in MIA-offspring. Overall, *B.fragilis* improved gut permeability, as well as correcting ASD-related behavioral abnormalities. This suggests that a microbe-mediated therapy, such as *B. fragilis*, may alleviate various behavioral disorders during childhood.

CONCLUSION

Throughout this mini-review, we have discussed the introduction of microbial therapeutics, in addition to prebiotic supplementation, to highlight the health benefits for their use in relieving GI distress in early life.

With regards to infant formulae, prebiotic supplementation with a mixture of GOS/FOS can help mimic the composition of breastmilk and promote the development of *Bifidobacterium* in the infant gut, in particular *B. longum*.

In terms of the clinical use of probiotics, it is crucial that we develop standardized treatments which take into account the age group of a specific human cohort, in addition to health status of the group in question. In this respect, the appropriate dose of a probiotic must be determined. Moreover, it is vital that we re-evaluate the safety of alternative probiotics, coined "next-generation probiotics" (NGPs). A preliminary evaluation on the safe use of a *Bacteroides xylanisolvens* isolate has recently been reported (73), in addition to the beneficial effects of *Faecalibacterium prausnitzii* (74, 75), and bacterial strains belonging to the *Eggerthellaceae* family, which produce metabolites with anti-inflammatory and cardioprotective properties (76). However, guidelines outlined by the European Food Safety Authority, and the Food and Agriculture Organization of the United Nations, have made it difficult to introduce these bacteria as food supplements. In terms of economic potential, further research is required to upscale these NGPs for food and/or pharmaceutical industries. The industrial challenges may be overcome through high throughput selection of bacterial strains with the capacity to grow well in selective media and tolerate the presence of oxygen. In other words, the technological robustness of the strain in question must be tested, in addition to the suitable anaerobic media and encapsulation methods required to retain probiotic viability under good manufacturing practices. Moreover, *in silico* screening of bacterial genomes will ensure the safety of these strains through the detection of antibiotic resistance and virulence genes. Alternatively, live biotherapeutic products (LBPs) may create an opportunity to introduce NGPs to the market (77). An LBP has recently been described as "a biological product that: (1) contains live organisms, such as bacteria; (2) is applicable to the prevention, treatment, or cure of a disease or condition of human beings; and (3) is not a vaccine" (78). In addition, O'Toole et al. (77) described future testing of LBPs as biological medicinal products, thereby providing new

opportunities to introduce NGPs as well characterized drugs to the market.

Overall, we conclude that additional studies are necessary to investigate the influence of prebiotics and probiotics in early life. It is important that we consider the mixed microbial communities present within foods and select those which will survive and adapt readily in an industrial environment (79, 80). More importantly, we suggest if new probiotics and prebiotics are to be considered health beneficial in the European market, the necessity for comprehensive, randomized controlled trials is vital. The current approach requires further strategy to provide consumers with valid information toward the use of probiotic and prebiotic supplementation in early childhood.

AUTHOR CONTRIBUTIONS

RPR, CS, and CAR conceived the manuscript. CW drafted the manuscript. All authors reviewed the final version of the manuscript and approved it for publication.

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Diet, Environments, and Gut Microbiota. A Preliminary Investigation in Children Living in Rural and Urban Burkina Faso and Italy

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Diet is one of the main factors that affects the composition of gut microbiota. When people move from a rural environment to urban areas, and experience improved socio-economic conditions, they are often exposed to a "globalized" Western type diet. Here, we present preliminary observations on the metagenomic scale of microbial changes in small groups of African children belonging to the same ethnicity and living in different environments, compared to children living on the urban area of Florence (Italy). We analyzed dietary habits and, by pyrosequencing of the 16S rRNA gene, gut microbiota profiles from fecal samples of children living in a rural village of Burkina Faso ($n = 11$), of two groups of children living in different urban settings (Nanoro town, $n = 8$; Ouagadougou, the capital city, $n = 5$) and of a group of Italian children ($n = 13$). We observed that when foods of animal origin, those rich in fat and simple sugars are introduced into a traditional African diet, composed of cereals, legumes and vegetables, the gut microbiota profiles changes. Microbiota of rural children retain a geographically unique bacterial reservoir (*Prevotella*, *Treponema,* and *Succinivibrio*), assigned to ferment fiber and polysaccharides from vegetables. Independently of geography and ethnicity, in children living in urban areas these bacterial genera were progressively outcompeted by bacteria more suited to the metabolism of animal protein, fat and sugar rich foods, similarly to Italian children, as resulted by PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), a predictive functional profiling of microbial communities using 16S rRNA marker gene. Consequently, we observed a progressive reduction of SCFAs measured by gas chromatography–mass spectrometry, in urban populations, especially in Italian children, respect to rural ones. Our results even if in a limited number of individuals point out

that dietary habit modifications in the course of urbanization play a role in shaping gut microbiota, and that ancient microorganisms, such as fiber-degrading bacteria, are at risk of being eliminated by the fast paced globalization of foods and by the advent of westernized lifestyle.

Keywords: microbiota, diet, environment, urbanization, Africa, children, short chain fatty acids

INTRODUCTION

Microbial colonization of the gastrointestinal (GI) tract is a fundamental process in human life cycle since microbiota–host interactions influence health and disease (Qin et al., 2010; Collado et al., 2012; Human Microbiome Project Consortium, 2012; Tremaroli and Backhed, 2012; Rodriguez et al., 2015). Animal and human hosts and their microbiota have co-evolved together over the millennia into a homeostatic and symbiotic relationship.

Dietary habits are one of the main factors contributing to the diversity of human gut microbiota (De Filippo et al., 2010; David et al., 2014). Dietary changes and gut microbiota alterations have the potential to profoundly affect host health and development (Palmer et al., 2007; Agans et al., 2011; Ringel-Kulka et al., 2013).

In infants, GI colonization is influenced by several factors including genetics, gestational age, mode of birth, diet, environment, sanitation, and antibiotic treatment (Adlerberth and Wold, 2009; Marques et al., 2010; Khodayar-Pardo et al., 2014). During the first year of life, dietary richness and environmental exposures increase and in parallel, the richness and complexity of the GI microbiota also increase (Koenig et al., 2011; Yatsunenko et al., 2012). Early life is a distinctive stage for the microbiota also in terms of functional acquisition (Yatsunenko et al., 2012). However, microbial colonization in children, following dietary and environmental changes, is still being completely uncovered.

In the last few years, researchers have gone beyond the Western world, to investigate human populations still living in rural environments and having a lifestyle completely different from that of developed countries. Metagenomic datasets obtained worldwide in different populations show that both host and environmental factors, especially diet, can affect gut microbial ecology over a lifetime (Borenstein et al., 2008; Freilich et al., 2009).

Our previous study showed for the first time that gut microbiota from children living in a rural African village in Burkina Faso, an environment resembling that of Neolithic subsistence farmers, is completely different from the microbiota of children living in the urban Western world (De Filippo et al., 2010). We demonstrated that in children different dietary habits (a fiber-rich diet of rural populations versus a typical Western diet rich in fat, animal proteins, and simple sugars) affect the gut microbiota.

A fiber and plant-derived polysaccharide-rich diet in children and adults is associated with a human gut microbiota enriched in Bacteroidetes phylum compared to Firmicutes (De Filippo et al., 2010; Yatsunenko et al., 2012; David et al., 2014). The fermentation of non-digestible carbohydrates stimulates the growth of bacterial producers of short-chain fatty acids (SCFAs) that are associated with disease and health in different ways (Tan et al., 2014).

Since the publication of our study, the impact of diet on gut microbiota has been observed and reported in different geographically isolated populations, such as Amazonas from Venezuela and rural populations in Malawi (Yatsunenko et al., 2012) and Papua New Guinea (Martinez et al., 2015). In addition, there are reports about drastic and rapid changes in gut microbiota when adults make a dietary switch from carnivorous to vegetarian diets (David et al., 2014; Gomez et al., 2016). The study of gut microbiota in ancestral populations, such as Hazda hunter-gatherers from Tanzania, one of the last few remaining populations with a Paleolithic type diet and lifestyle (Schnorr et al., 2014), showed significant differences in microbiota due to dietary fluctuations linked to seasonal changes. The microbiota of BaAka hunter-gatherers and Bantu agriculturalists is characterized by microbial gradients linked to traditional subsistence strategies (Gomez et al., 2016).

Altogether, the studies on traditional and culturally diverse populations living in isolation from the globalized world showed the degree of an individual's traditional lifestyle and can explain the evolutionary processes of human gut microbiota.

We hypothesized that in the same country the phenomenon of urbanization, and consequently the "Westernization" may affect dietary habits of traditional populations, resulting in modification of gut microbiota profiles. The aim of the present preliminary investigation was to assess the impact of dietary habits on the gut microbiota of small groups of African children who live in a rural village, with marginal contacts with the globalized world, respect to children living in suburban small town and in an urban area. For such populations who live in the same country and belong to the same ethnic group, such a transition is in parallel with increased wealth and greater food availability.

We integrated the data on the gut microbiota characterization of our previous study on children living in the rural villages of Boulpon (district of Nanoro) in Burkina Faso (De Filippo et al., 2010), with data on microbiota from children of the same Mossi ethnicity who live in the small town of Nanoro and from those of wealthy families living in Ouagadougou, the capital city. We then compared the composition of the gut microbiota of these three African populations, corresponding to different levels of urbanization, with that of previously known Italian children, as representative of a typical Western and urbanized population.

MATERIALS AND METHODS

Enrollment of Children Populations and Fecal Sample Collection

In this study, we enrolled 11 healthy children living in the rural village of Boulpon (Boulkiemde province, Burkina Faso, BR), 8 healthy children living in the small town of Nanoro (Boulkiemde province, Burkina Faso, BT), 5 children living in the capital city of Burkina Faso, and 13 healthy children living in the urban area of Florence, Italy (EU). All children aged 2–8 years, had not taken antibiotics or probiotics in the 6 months prior to the sampling dates and had not been hospitalized in the previous 6 months. A detailed medical and lifestyle report was obtained from EU children's parents as well as a 4-day dietary questionnaire and an in-depth interview on African children's diet was obtained directly from their mothers. Additional data were collected for all children (Supplementary Table 1) including ethnicity, environment in which they live, and mode of birth (natural or cesarean). All children were breastfeed as infants, except one EU children (1EU) who was formula-fed.

Despite the high incidence of infectious disease, including malaria and malnutrition in the area, all children were healthy at the time of sample collection. BC children were healthy and belonging to wealthy families. For BR and BT children, upper mid-arm measurement excluded both severe and moderate malnutrition. As representative of a healthy Western population, we selected children of the same age who are generally concordant for growth, socially homogeneous and eating the diet and living in an environment typical of the developed and urbanized world. This study was carried out in accordance with the recommendations of the Ethical Committee of Meyer Children Hospital, Florence – Italy. All children's parents were made aware of the nature of the experiment and gave written informed consent in accordance with the sampling protocol approved by the Ethical Committee of Meyer Children Hospital, Florence, Italy and in accordance with the Declaration of Helsinki.

Fecal samples of African and Italian children were collected in the morning, 1–2 h after the first meal, by physicians or parents and preserved in RNAlater (Qiagen) at −80°C until extraction of genomic DNA. All samples were processed in the same way as reported in the successive paragraph in the laboratory of University of Florence (Supplementary Materials).

Bacterial Genomic DNA Extraction from Fecal Samples

The bacterial genomic DNA was extracted as previously reported (De Filippo et al., 2010). The procedure was based on a modified protocol (Supplementary Materials) proposed by Zoetendal et al. (2006).

Pyrosequencing

For each sample, we amplified the 16S rRNA gene using the special fusion primer set specific for V5–V6 hypervariable regions and corresponding to primers 784F and 1061R described by Andersson et al. (2008), and using the FastStart High Fidelity PCR system (Roche Life Science, Milano, Italy). The 454 pyrosequencing was conducted outsourcing by DNAVision Agrifood S.A. (Liège, Belgium) on the GS FLX+ system using the Titanium chemistry following the manufacturer recommendations (Supplementary Materials).

Data Analysis

Sequence data of BT and BC samples are available at http://www.ebi.ac.uk/ena/data/view/PRJEB19895, under the accession number PRJEB19895. Data of BR and EU samples are available at http://www.ebi.ac.uk/ena/data/view/ERP000133, under the accession number ERP000133, as previously reported (De Filippo et al., 2010). Raw 454 files were demultiplexed using Roche's sff file software. Reads of all data sets were pre-processed altogether using the MICCA pipeline (v. 1.5) (Albanese et al., 2015). *De novo* sequence clustering, chimera filtering and taxonomy assignment were performed by micca-otu-de novo (parameters -s 0.97 -c), after trimming and quality filtering (Supplementary Materials).

Operational taxonomic units (OTUs) were assigned by clustering the sequences with a threshold of 97% pair-wise identity, and their representative sequences were classified using the RDP software version 2.7 (Wang et al., 2007). Template-guided multiple sequence alignment was performed using PyNAST57 (v. 0.1) (Caporaso et al., 2010) against the multiple alignment of the Greengenes 16S rRNA gene database (DeSantis et al., 2006) filtered at 97% similarity. Sampling heterogeneity was reduced by rarefaction, obtaining 12,964 sequences per sample.

Alpha (Chao1 index and Shannon entropy) and beta diversity [UniFrac-(Lozupone et al., 2011) and Bray–Curtis dissimilarities] were calculated using the Phyloseq package (McMurdie and Holmes, 2014) of the R software suite. Principal coordinates analysis (PCoA) using the phyloseq package of the R software suite was performed. The significance of between-groups differentiation on the UniFrac distances and Bray–Curtis dissimilarity was assessed by PERMANOVA using the adonis() function of the R package vegan with 999 permutations.

To compare the relative abundances of OTUs among the four groups, the two-sided, unpaired Wilcoxon test was computed, removing taxa not having a relative abundance of at least 0.1%, in at least 20% of the samples, and using the function mt() in the phyloseq library and the *p*-values were adjusted for multiple comparison controlling the family-wise Type I error rate (minP procedure).

Heatmap plots of percentage abundances at phylum level were obtained by using STAMP (Parks et al., 2014), and supported by dendogram, obtained with Average Neighbor and Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

Bacterial species were assigned, based on Basic Local Alignment Search Tool nucleotide (BLASTn) software in the National Center for Biotechnology Information (NCBI) database, considering the highest percentage of identity (Query cover 100–99% and Identity 99 or 95%). Expectation value (E-value) was used to select significant BLAST hits, keeping only outcomes with the lowest E-value (minimal E-value of 10^{-3}). To infer the functional contribution of microbial communities on 16S rDNA sequencing data set, we applied PICRUSt (Phylogenetic

Investigation of Communities by Reconstruction of Unobserved States; Langille et al., 2013). The functional pathways discovery and related statistical significance were assessed by using the linear discriminant analysis (LDA) effect size (LEfSe) method (Segata et al., 2011). An alpha significance level of 0.05, either for the factorial Kruskal–Wallis test among classes or for the pairwise Wilcoxon test between subclasses, was used. A size-effect threshold of 2.0 on the logarithmic LDA score was used.

Determination of Short-Chain Fatty Acids (SCFAs) in Fecal Samples

Concentrations of fecal SCFAs were determined from 250 mg frozen fecal samples, according to the previous protocol (De Filippo et al., 2010), by solid-phase microextraction- gas chromatography-mass spectrometry (SPME-GC-MS) using a Varian Saturn 2000 GC–MS instrument with 8200 CX SPME autosampler. The SCFAs concentration in fecal sample was expressed in μmol/g of feces. To determine statistical significance of differences observed among the four populations we used unpaired Student's *t*-test (one tailed).

RESULTS AND DISCUSSION

Change of Dietary Habits in Burkina Faso Children Living in Rural and Urban Areas

In order to investigate the effect of diet modifications, corresponding to different socio-economic conditions and food availability, on gut microbial communities, we studied dietary habits of three populations of healthy children, living in different areas of Burkina Faso. All children belong to the Mossi ethnic group (the largest in Burkina Faso comprising 74.9% of the Burkinabè population). The age range of all groups of children was 2–8 years, with an average of 4.6 ± 1.5 years (mean ± SE). Age and gender characteristics of each group are reported in Supplementary Table 1.

We compared a previously analyzed population (De Filippo et al., 2010) of 11 children (rural, BR) living in Boulpon, a typical rural village of Burkina Faso (Boulkiemdé province, Nanoro department; geographic coordinates 12°39′N 2°4′W) with 8 children (town, BT) living in Nanoro (geographic coordinates 12°41′N 2°12′W), a small African town surrounded by rural villages, corresponding to an initial urbanization status, and 5 children (Capital city, BC) from wealthy families, living in the capital city of Burkina Faso, Ouagadougou (geographic coordinates 12°21′26″N 1°32′7″W; **Figure 1**), about 90 Km from Nanoro (**Figure 1**).

The Boulpon village consists of a cluster of huts built using wood and straw (**Figure 1A**), in which the population live in communities based on subsistence agriculture. Nanoro is a small town with about 5,200 inhabitants, consisting of urban agglomerates of small brick houses (**Figure 1B**). Ouagadougou is the capital city of Burkina Faso, with a population of 1,475,223 inhabitants and is the administrative, cultural, economic and industrial center of the nation (**Figure 1C**). The industry of Ouagadougou is the sector that fuels urban growth, as people move to the city from the countryside to find employment in processing plants and factories.

All study children were healthy at the time of the investigation. However, BR children were poor, at high risk of infectious diseases and malnutrition, while BT children lived in average economic conditions for Burkina Faso which meant a dirty environment (**Figure 1B**), and were at risk of infectious disease but at low risk of malnutrition. The BC children were from wealthy families living in clean, modern houses, at no risk of malnutrition and at lower risk of infectious diseases when compared to the other groups of African children, but certainly at higher risk of infections when compared to Italian children. It is worth of mentioning that the capital city of Ouagadougou is very polluted by vehicle exhaust, especially during the dry season.

The dietary habits of these three African populations were compared to a population of our previous study comprised of 13 European children (EU) living in Florence (Italy) and having a typical Western diet (De Filippo et al., 2010).

Initially, we analyzed the dietary habits and daily food intake of all our pediatric populations, based on dietary questionnaires and interviews with mothers and care givers, estimating average quantities of food ingested per day, food energy (kcal/day), grams of protein, fat, carbohydrates, including simple sugars, and fiber (Supplementary Table 2).

As shown in our previous study (De Filippo et al., 2010), the diet of BR children is predominantly vegetarian, rich in fibers and plant-polysaccharides and low in fat, animal protein, and simple sugars (Supplementary Table 2A). The sources of fibers are also quite unique, as derived from locally cultivated indigenous cereals (millet, *Panicum miliaceum* and sorghum, *Sorghum vulgare*), legumes (Niebè, *Vigna unguiculata*), vegetables (Néré, *Parkia biglobosa* and baobab leaves), fruits (especially mango, papaya, and bananas), and fermented products (Soumbalà from Nerè seeds; Supplementary Figure 1). Millet and sorghum, typical cereals of the Burkinabè diet in these rural villages, are frequently still ground into flour on a flat grinding stone, similarly to what humans did during the agriculture revolution in the Neolithic age, to produce a thick porridge called Tô, that is the principal dish-component of Burkinabè meals.

The BT children living in Nanoro town still eat cereals and legumes, similarly to BR children but, depending on their socio-economic status, their diet also contains rice, corn, peanuts, peanut oil, and about once-a-week mutton or chicken from animals bred in the village or dried fish. Unlike the BR population, cereal flour, legumes, fruit, and dried fish can be bought in the local market in Nanoro town, where products from neighboring countries can also be found (Supplementary Table 2B).

The BC children, living in the capital city of Ouagadougou, eat a typical African diet of cereals (millet, sorghum, rice, soya) and legumes (Niebè), but also bread, milk and dairy products such as cheese and yogurt, eggs, fruit juices, snacks, sweet bakery products and different kinds of meat and fish, including frozen fish no more than three times *per* week. Their diet, therefore, is very similar to that of children living in an industrialized and globalized world. Many of these products are bought at supermarkets (Supplementary Table 2C).

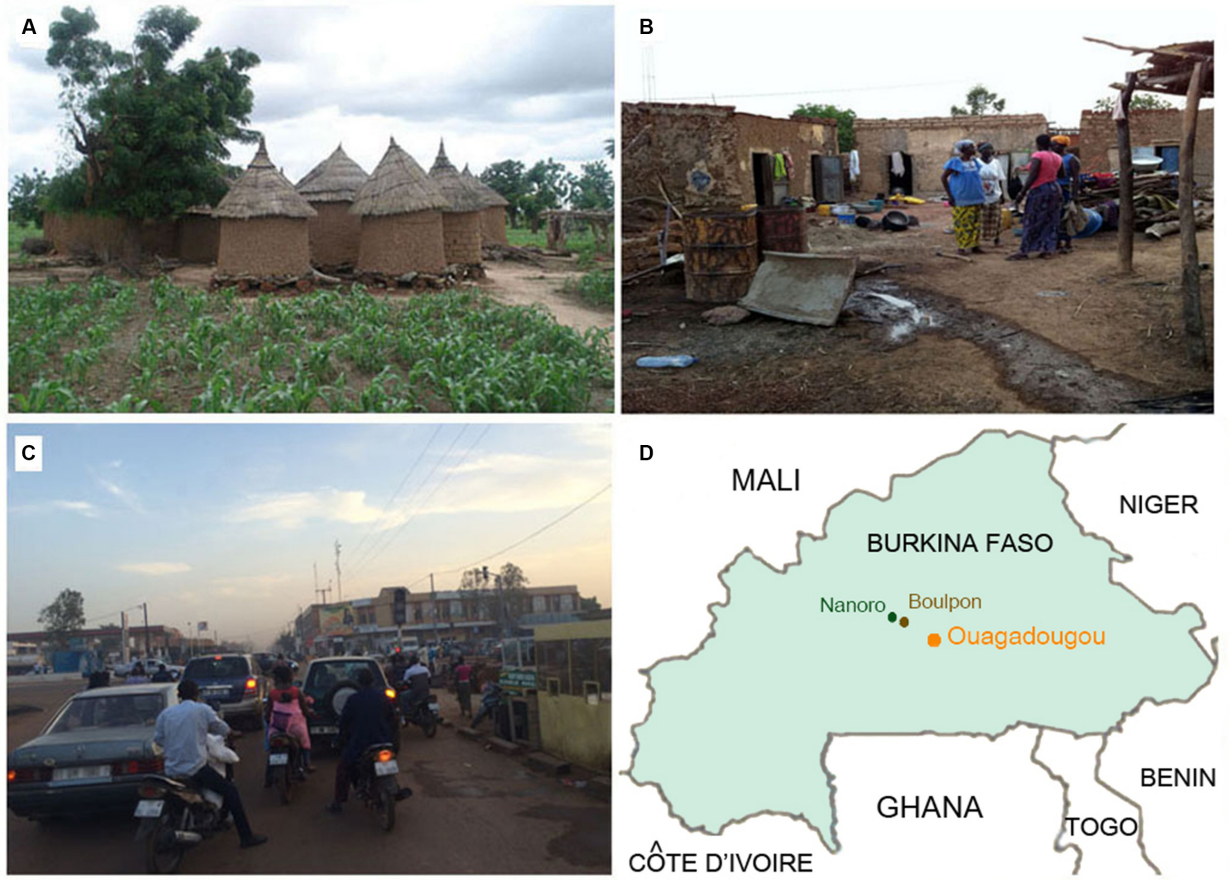

FIGURE 1 | Rural and urban environments in Burkina Faso. **(A)** Rural village in Boulpon, **(B)** urban village in Nanoro town, **(C)** Ouagadougou, the capital city of Burkina Faso (personal photographs of Prof. P. Lionetti), and **(D)** map of Burkina Faso.

The Italian children in our study eat a typical Western diet, high in starch, simple sugars, animal protein, and fat and low in fiber (Supplementary Table 2D), as previously described (De Filippo et al., 2010).

Our nutritional analysis of the four populations of children showed that passing from rural (BR) to urban centers (BT, BC, and EU), the variety of food consumed increased (**Figure 2**), as well as the daily intake of fat, protein and simple sugars, and consequently the daily caloric intake, whereas the fiber intake was progressively reduced (Supplementary Table 2). The average amount of fiber in the BR diet is 14.2 g/day (3.19% of total grams of daily food intake) compared with 12.5 g/day (2.72%) in the BT diet, 9.7 g/day (1.04%) in the BC, and 8.4 g/day (0.9%) in the EU diet. Fiber intake diminished as the average daily calorie intake increased (BR: 996 kcal/day; BT: 1094.5 kcal/day; BC: 1454.3 kcal/day; EU: 1512.7 kcal/day; Supplementary Table 2).

Despite the limitation of the small sample number, our groups of African children clearly reflect different dietary habits in populations of the same ethnic group and belonging to the same geographic area, but living either in rural or urban areas, and having different socio-economic conditions.

Fiber Intake Is Correlated with Levels of Fecal Short Chain Fatty Acids

In our previous study (De Filippo et al., 2010) comparing dietary habits of BR and EU populations, we observed a correlation between fiber intake and fecal levels of SCFAs. Thus, considering the differences in food consumption, especially in fiber intake, in BT and BC populations compared with BR, in the present study we analyzed metabolomics by SPME-GC-MS, to measure SCFA levels (Supplementary Table 3). We observed a significant reduction in total fecal SCFAs in children who live in urban centers with respect to BR children (**Figure 3**; *p*-values by one-tailed Student's *t*-test; Supplementary Table 4). We found a clear trend, especially for propionic and valeric acids (**Figure 3**; *p*-values by one-tailed Student's *t*-test; Supplementary Table 4). Fecal samples of BC showed a significant increase in total SCFAs, especially for acetic and butyric acids compared to BT and EU, but a decrease compared to BR (**Figure 3**; *p*-values by one-tailed Student's *t*-test; Supplementary Table 4). Interestingly, the EU children's fecal samples contained significantly low levels of SCFAs, especially butyric acid, compared to the three African groups (**Figure 3**; *p*-values by one-tailed Student's *t*-test; Supplementary Table 4).

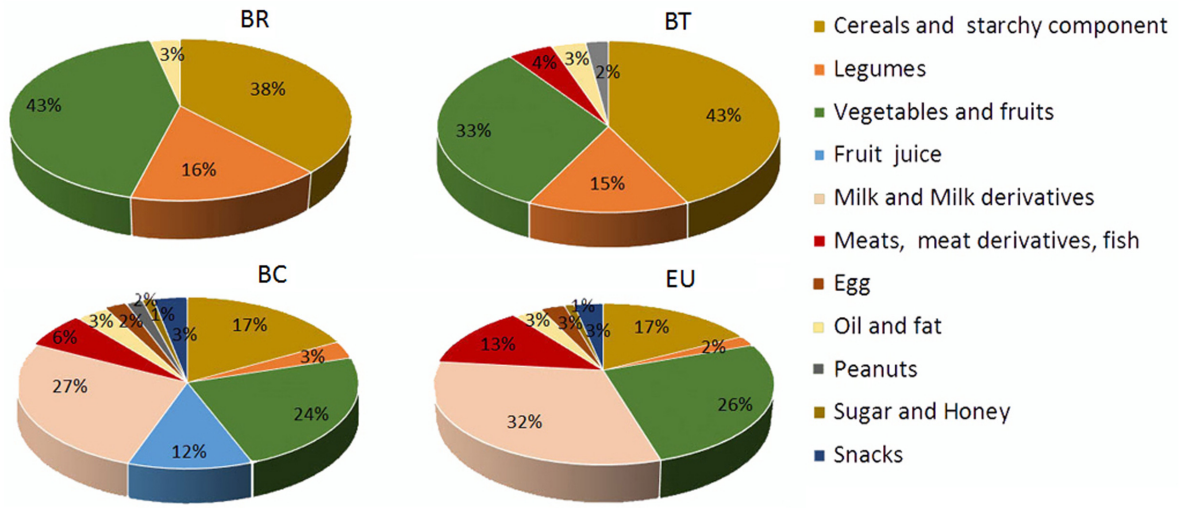

FIGURE 2 | Variety of food consumption in the four children populations. Pie charts indicate the percentages of daily foods assumed by the African and European populations. BR: children form rural village; BT: children from Nanoro town; BC: children from capital city of Burkina Faso; EU: children from Europe (Florence, Italy).

Microbiota Characterization of Different Populations of African Children Compared to Europeans: Taxonomic Changes as an Effect of Diet and Environment

We compared the meta-taxonomic data of BT and BC populations, obtained by pyrosequencing (454 FLX, Roche) of the V5–V6 hypervariable regions of the 16S rRNA gene with the previous results obtained with the same methodologies for BR and EU populations (De Filippo et al., 2010).

We evaluated microbial richness (alpha diversity) among populations. Observed OTUs and the Chao1 index indicated a downward trend (although not significant according to PERMANOVA analysis) in species richness from rural BR to BC and EU children, whereas BT children showed a high alpha diversity index. The Shannon index, estimating entropy, indicated reduced alpha diversity in BR children compared to the other populations (Supplementary Figure 2).

At phylum level, the rural populations had a higher ratio of Bacteroidetes to Firmicutes, but this ratio gradually diminished in fecal samples from children in more urban settings (**Figure 4**). Bacteroidetes were abundant especially in BR and BT children (68.6 and 47.7% respectively) compared to 32.6% in BC and 25.9% in EU children with a significantly higher abundance when comparing BR with BC and EU (**Figure 4A**; Wilcoxon rank-sum test; Supplementary Table 5A). Conversely, Firmicutes were more abundant in BC and EU children (57.5 and 60.2%, respectively) than in BT, and significantly higher than in BR children (**Figure 4A**; Wilcoxon rank-sum test; Supplementary Table 5A).

Actinobacteria were more abundant in BC compared to the other African and European populations (6.74% in BC vs. 3.6% in EU, 0.17% in BR, and 1.11% in BT), and significantly reduced in BR compared to the other groups (**Figure 4A**; Wilcoxon rank-sum test; Supplementary Table 5A). An abundance of Proteobacteria was observed in BT and EU (3.8% in BT and 4.94% EU vs. 2.73% in BR and 1.26% in BC; Supplementary Figure 3), compared with other populations, although not statistically significant.

Among the minor phyla of gut microbiota, Spirochaetes was significantly increased in BR compared with BC and EU children, as well as in BT compared to EU children (**Figure 4A**; Wilcoxon rank-sum test). Tenericutes and Verrucomicrobia were relatively increased in the BT population compared with the other groups (Supplementary Figure 3).

BT populations showed a greater variability in the relative abundance of phyla (**Figure 4A**) compared with BC and EU children, suggesting an inter-individual variability in microbiota composition. One possible explanation for this phenomenon could be the increased variety of food consumption with the addition of dietary products of animal origin to a typical rural Africa vegetarian diet.

Based on sequence abundances at phylum level in microbiota of each population, the dendogram, obtained with the Average Neighbor and Unweighted Pair Group Method with Arithmetic Mean (UPGMA), showed a clear separation between BR and EU populations (**Figure 4B**), especially due to the different ratio Bacteroidetes/Firmicutes, as previously observed (De Filippo et al., 2010). In contrast, BT and BC children showed a progressive shift toward the phyla distribution observed in EU children in accordance with their different dietary habits and environments.

Whereas most BC children clustered together with EU, two out of eight BT samples clustered closely to the EU group, due to the relative abundance of Firmicutes. We think this is due to the fact that BT children living in Nanoro did not have a uniform diet, with two of them having a more Westernized diet and the

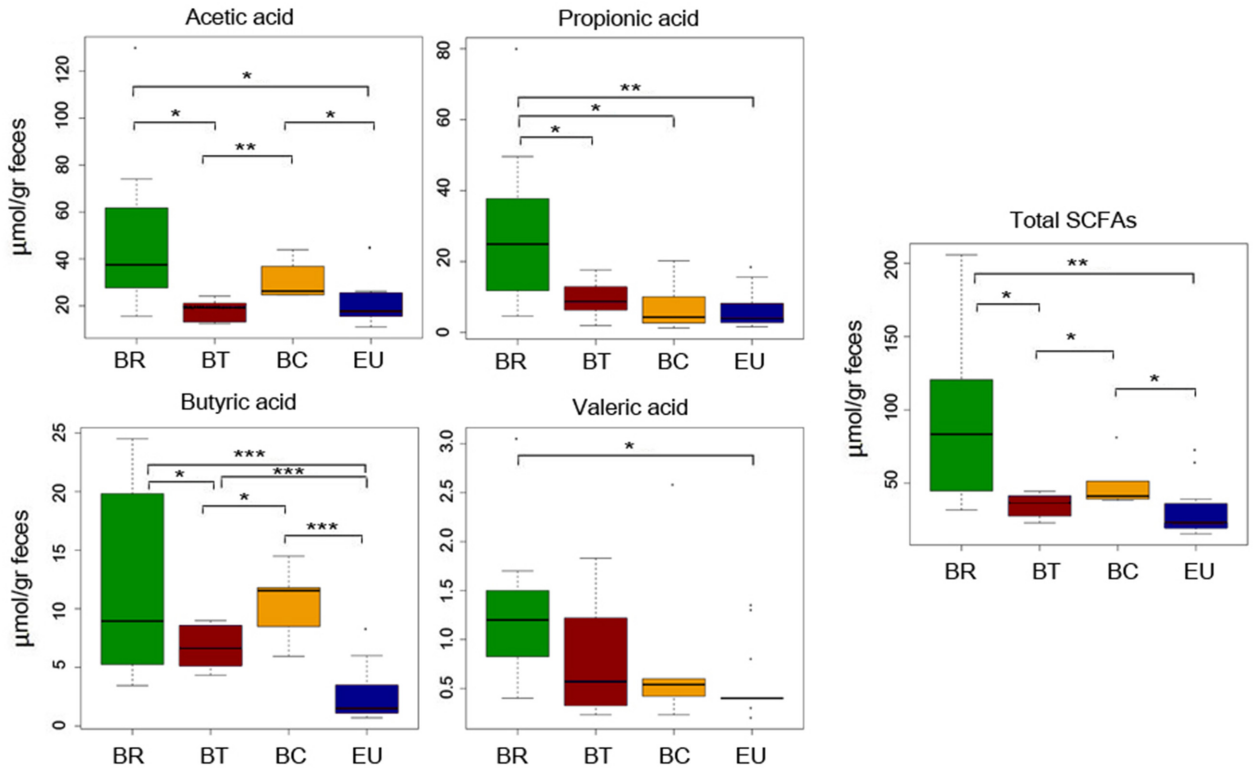

FIGURE 3 | Quantification of SCFAs levels in fecal samples from African and EU populations by SPME-GC-MS. Mean values (± SEM) are plotted. Comparison among groups by one-tailed Student's *t*-test. $^{*}p < 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

others eating very similarly to the rural area diet, as revealed in the questionnaire we administered. However, the majority of BT children fell within the BR cluster, or in a sub-cluster interposed between BR and EU population, due to the abundance in Bacteroidetes (**Figure 4B**).

Although the limited number of samples have to be considered, these results, confirmed also by dendograms obtained at family and genus levels (Supplementary Figure 4), suggest that Burkina Faso children of the Mossi ethnic group who live in the same geographic area have different gut microbiota composition, according to the ways in which their diets and the environment where they live, have changed.

Following the taxonomic assignment at family level, we observed that *Prevotellaceae,* the most abundant family in BR and BT populations (66.8 and 41.14% mean relative abundance respectively), were significantly more abundant in rural BR children compared with BC and EU with a clear reduction in BC (10.4%), and almost absent in EU children (0.44%) (Supplementary Figure 5; Wilcoxon rank-sum test; Supplementary Table 5B). Conversely, several bacterial families were less abundant in the BR population and progressively more present in urban African (BT and BC) and EU children. Among these, *Bacteroidaceae, Bifidobacteriaceae, Porphyromonadaceae,* and *Rikenellaceae* were significantly decreased in BR compared with BC and EU, and in BT compared with EU children, as were *Lachnospiraceae* and *Ruminococcaceae* significantly reduced in BR compared with BC and EU children (Supplementary Figure 5; Wilcoxon rank-sum test; Supplementary Table 5B).

We also observed a significant increase in *Enterobacteriaceae* in the BT population compared with EU, and in EU compared to BR (Supplementary Figure 5; Wilcoxon rank-sum test; Supplementary Table 5B). *Spirochaetaceae* was more variable within the BT population and more abundant in the BR population compared to BC and EU (Supplementary Figure 5; Wilcoxon rank-sum test; Supplementary Table 5B). *Desulfovibrionaceae* and *Sutterellaceae* were progressively and significantly more abundant in BT, BC and EU children, and almost absent in the BR population (Supplementary Figure 5; Wilcoxon rank-sum test; Supplementary Table 5B).

The observed distribution of the four populations based on taxonomic assignment at phylum level (**Figure 4B**) was confirmed by analysis of microbial community structure (beta diversity). Considering Unweighted and Weighted UniFrac distances and Bray–Curtis dissimilarities, PCoA analysis (**Figure 5A**) showed a clear difference between BR and EU samples, confirming the different gut microbiota composition between African and European populations (PERMANOVA analysis; unweighted-UniFrac, $p = 0.0001$; weighted-UniFrac $p = 0.0001$; Bray–Curtis $p = 0.0001$). The BT sample distribution was close to the BR population, while BC was similar to the EU (**Figure 5A**), suggesting a progressive change in gut microbial communities in African populations, as an effect of transition

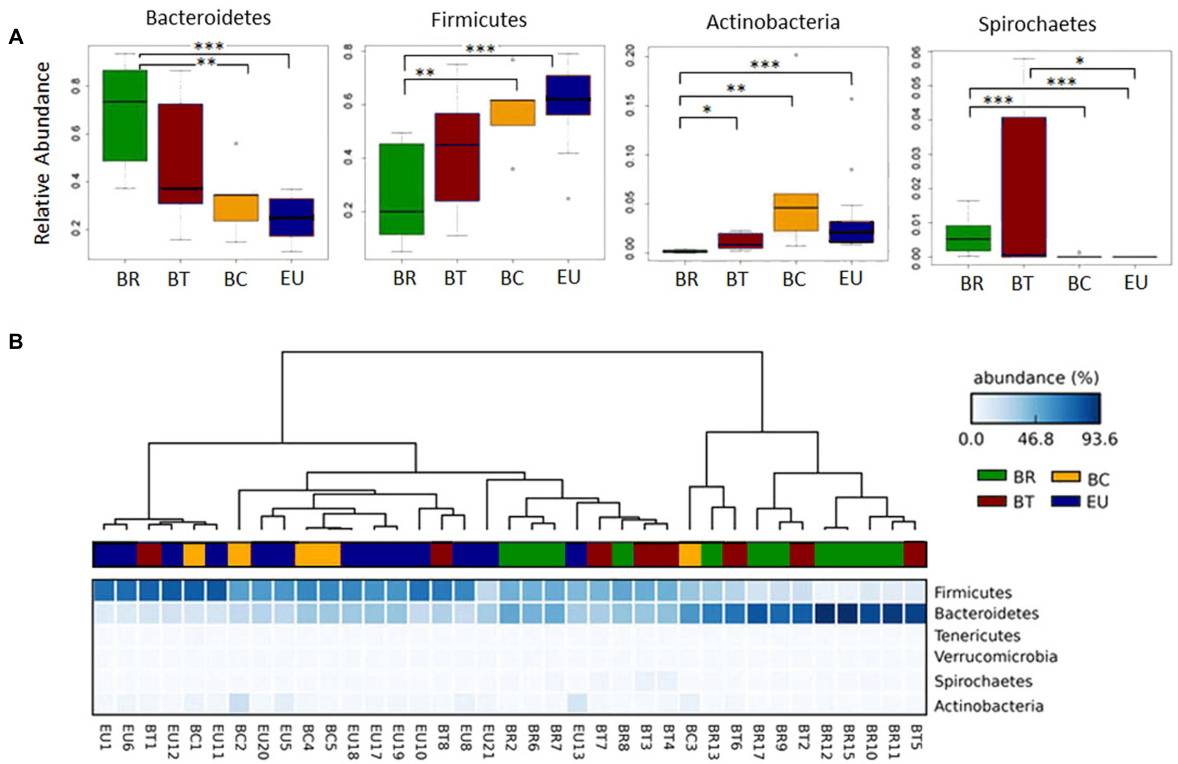

FIGURE 4 | Clustering of African and European populations based on microbiota composition. **(A)** Box plot of relative abundances of the statistically significant different major phyla in African and European populations (Wilcoxon rank sum test; **p*-value < 0.05, ***p*-value < 0.01, ****p*-value < 0.001). **(B)** Heatmap plot indicating the sequence abundances (percentage; blue-scale squares) assigned at each phylum in each sample. Dendrograms, obtained with Average Neighbor UPGMA method, are used to cluster each fecal sample of the children populations (horizontal) based on phyla abundances. Each sample, belonging to respective group, is represented by a different color: green = BR, brown = BT, yellow = BC, and blue = EU.

from the rural to the urban environment, improvement in socio-economic status and modification of dietary habits.

The different abundances of some bacterial families allow a clear discrimination of the microbiota profiles in the four populations, as highlighted by PCoA analysis based on UniFrac distances and Bray–Curtis dissimilarities (**Figure 5B**). The abundance of *Prevotellaceae* in BR and BT populations and of *Bacteroidaceae, Lachnospiraceae, Rikenellaceae, Porphyromonadaceae,* and *Enterobacteriaceae* in the EU children illustrates the variety between African and European samples. The abundance of *Lachnospiraceae* and *Ruminococcaceae* indicates how BC samples come close to EU microbiota.

Urbanization and Improved Socio-economic Conditions Led to the Loss of Microbial Profiles Typical of Rural Populations

According to our previous study, at genus level, the gut microbiota of BR children was almost entirely populated by *Prevotella* (64.4% average out of total sequence amount). BT and BC populations presented a progressive decrease in its abundance compared to BR (**Figure 6**; 38.8% in BT and 10.3% in BC; Wilcoxon rank-sum test; Supplementary Table 5C), reflecting the reduction of dietary fiber intake and consequently SCFAs levels in fecal samples. It is worth noting that this genus was almost absent in EU children's gut microbiota.

Sequence alignments by BLASTn indicated that *Prevotella* sequences were attributable with 99% of identity (see section "Materials and Methods") to *P. copri*, *P. stercorea* and *P. melaninogenica*, over uncultured *Prevotella* spp. In our previous study, we found a set of sequences classified as *Xylanibacter*. Recently, *Xylanibacter* 16S rDNA was re-classified within the larger *Prevotella* genus (Sakamoto and Ohkuma, 2012).

Interestingly, an increase in *Treponema*, *Succinivibrio,* and *Weissella* distinguished both BR and BT from BC and EU populations (**Figure 6**; Wilcoxon rank-sum test; Supplementary Table 5C). BLASTn alignment showed that *Treponema* spp. sequences were mainly attributable with 99% of identity to *T. succinifaciens*, a known carbohydrate metabolizer isolated from the gut of termites and swine, and observed in other traditional human populations (Obregon-Tito et al., 2015). Interestingly, rural Burkina Faso populations occasionally eat cooked termites, and prepare foods on insect-contaminated surfaces, thus supporting the idea that termites and the fiber degrading microbes they harbor are likely to colonize the human gut.

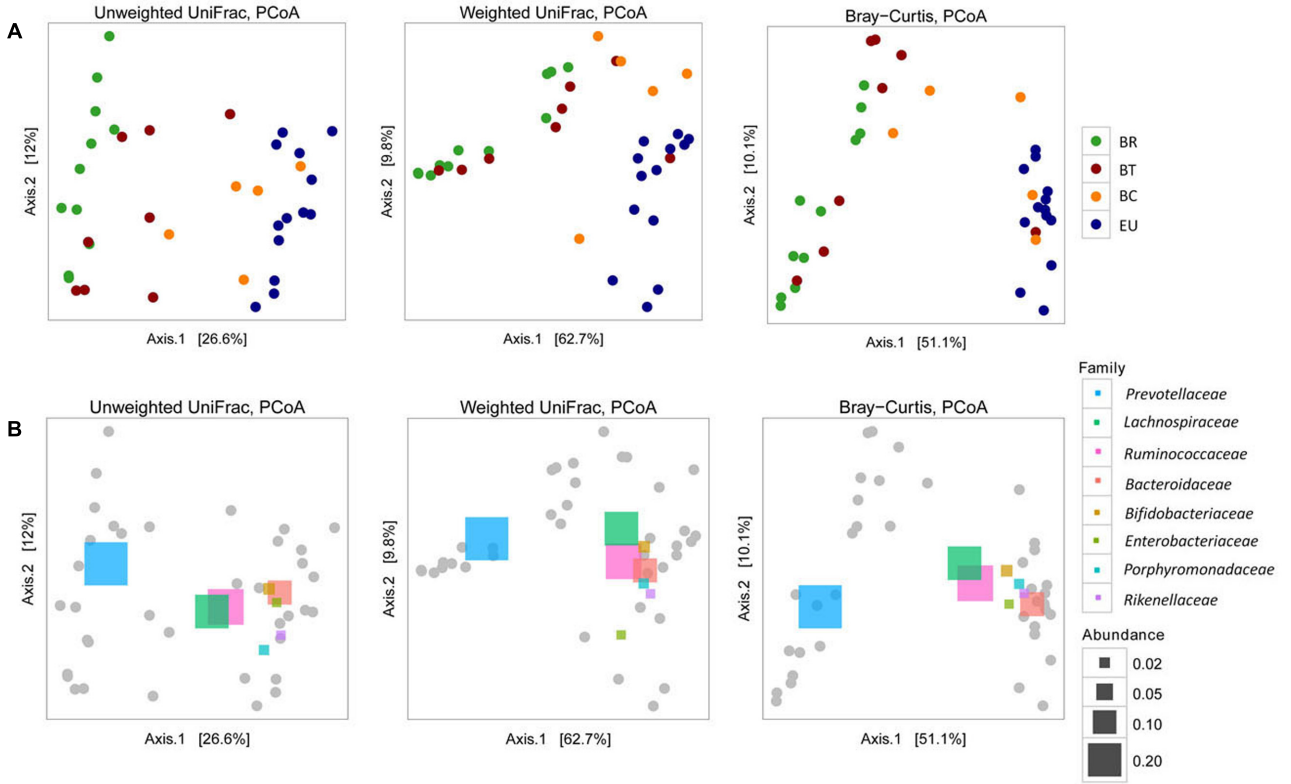

FIGURE 5 | Beta diversity **(A)** Principal coordinates analysis (PCoA) derived from unweighted and weighted UniFrac and Bray–Curtis distances among samples of the four populations (p = 0.0001 by PERMANOVA). Colored dots representative of the four populations are as in **Figure 4**. For each axis, in square brackets, the percent of variation explained was reported. **(B)** Beta diversity and correlation with the principal abundant bacterial families, represented by colored rectangles (p = 0.0001 by PERMANOVA). Different sizes of rectangles indicate the size of relative family abundance. For each axis, in square brackets, the percent of variation explained was reported.

Succinivibrio is generally associated with bovine rumen (Bryant, 1970), and was also found in higher frequency in the Hadza hunter gatherers and traditional Peruvian populations (Schnorr et al., 2014; Obregon-Tito et al., 2015) and might be involved in starch, hemicellulose, and xylan degradation, similarly to *Prevotella* and *Treponema*.

These observations confirm that the abundance of bacteria metabolizers of plant-polysaccharides, hemicellulose and xylan (De Filippo et al., 2010), are derived from high-fiber diets similarly to what is reported in children and adults from Malawi and Venezuela whose diets are dominated by plant-derived polysaccharide foods (Yatsunenko et al., 2012). When individuals do not live any more in rural areas but in urban environments, these genera decrease dramatically, to the point of being depleted in EU microbiota. Interestingly, the progressive loss of these bacteria reflects the gradual reduction in fecal SCFAs levels from BR to BT, BC, and EU populations.

Conversely, *Lachnospiraceae incertae sedis*, *Roseburia,* and *Dorea* were reduced in BR and BT children, but increased in BC, and variously represented in EU (**Figure 6**; Wilcoxon rank-sum test; Supplementary Table 5C). Thus, urbanization and a Westernized diet led to the loss of ancient microbial profiles typical of traditional and rural populations in BC children, such as *Prevotella*, and to the increase in bacterial genera associated with a Western-like diet (Wu et al., 2011; David et al., 2014; Martinez et al., 2015).

Bacteroides and *Bifidobacterium*, were more abundant in BC and EU children compared with BR and BT children (**Figure 6**; Wilcoxon rank sum test; Supplementary Table 5C). Enrichment of *Bacteroides* was related to lipids, cholesterol and amino acids intake, and dairy consumption, as previously observed (Wu et al., 2011; Martinez et al., 2015). *Bifidobacterium*, generally associated to infant microbiota and with milk and milk-derived food consumption, is probably related to their increased consumption of milk and dairy products respect to BR and BT children.

Finally, *Alistipes,* and *Barnesiella* distinguished Europeans from Africans (**Figure 6**; Wilcoxon rank sum test; Supplementary Table 5C), in accordance with a previous study showing enrichment of these bacterial genera in Western populations (Martinez et al., 2015).

Among the minor genera, we observed an increase in *Bilophila, Sutterella, Parasutterella, Odoribacter,* and *Clostridium cluster XIVa* (that includes *Clostridium* spp., *Eubacterium, Ruminococcus, Coprococcus, Dorea, Lachnospira, Roseburia,* and *Butyrivibrio*), in both BC and EU children (**Figure 6**; Wilcoxon rank sum test; Supplementary Table 5C).

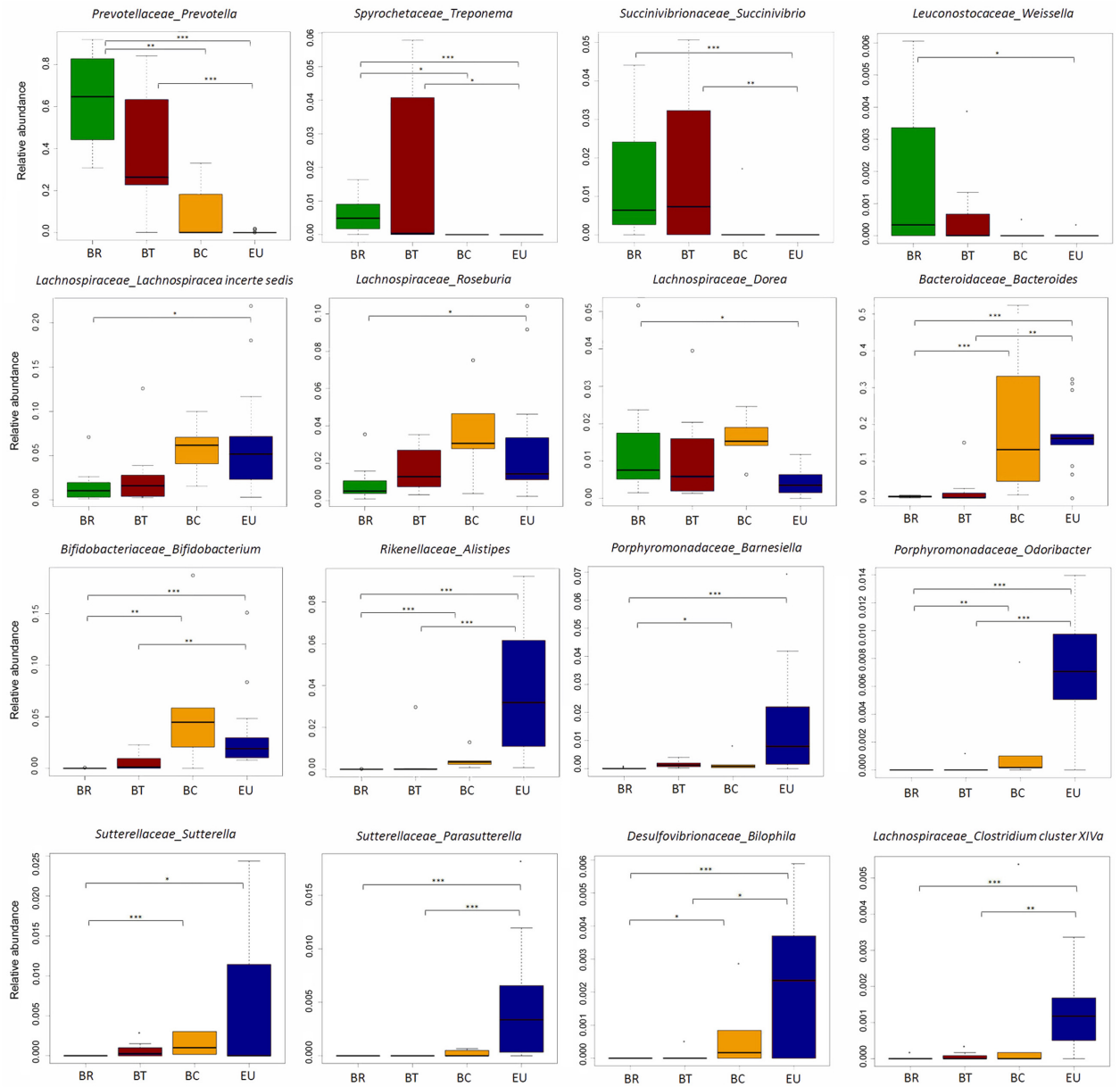

FIGURE 6 | Disappearance of ancient microbial pattern and acquisition of "Western" microbial profiles passing from rural to urban environments in Burkina Faso populations. Box plot of relative abundances of the statistically significant different genera in African groups in comparison with European population (Wilcoxon pairwise test; *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001).

Our results suggest that diets rich in protein and fat and poor in fiber influence the gut microbiota, independently of geographic origin. *Alistipes* and *Bilophila* have been previously linked to an animal protein-rich diet (Wu et al., 2011; David et al., 2014; Martinez et al., 2015). It has been reported that high-fat diets induce an increase in the abundance of *Bilophila wadsworthia*, a member of the *Desulfovibrionaceae* family, which generates hydrogen sulfide through taurine metabolism. An abundance of *Bilophila* and its metabolism has been associated with inflammation, as recently observed in a mouse model (Devkota et al., 2012). Members of *Sutterella* genus are known to be resistant to bile acids, and their role as commensals in human GI tract or their association with dysbiosis in some human diseases, such as Inflammatory Bowel Disease, autism, arthritis and celiac disease, remains partly controversial (Mukhopadhya et al., 2011; Williams et al., 2012; Cheng et al., 2013; Hansen et al., 2013; Wang et al., 2013; Lavelle et al., 2015; Di Paola et al., 2016).

Clostridium XIVa and *Odoribacter,* well-known butyrate-producer bacteria (Morgan et al., 2012; Lopetuso et al., 2013; Van den Abbeele et al., 2013), contribute to SCFAs

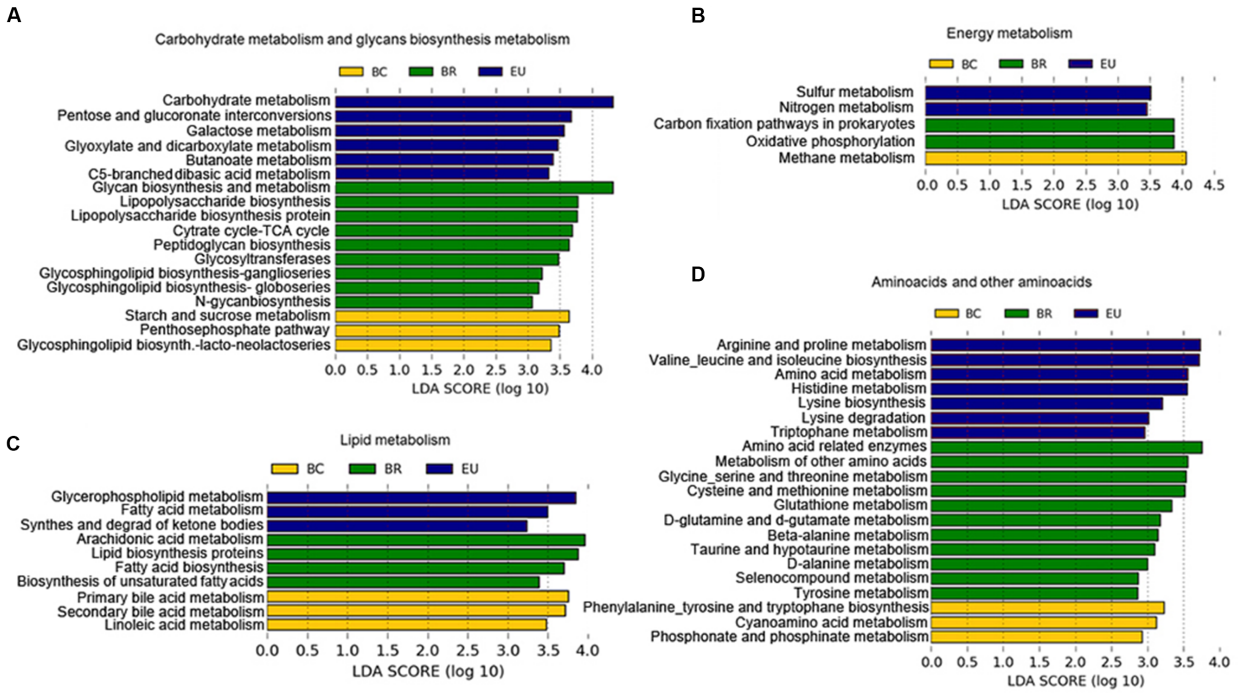

FIGURE 7 | Differences in the most representative bacterial functional classes (KEGG categories level 3). **(A–D)** Functional pathways significantly enriched in African and European populations based on PICRUSt prediction. LEfSe results indicate a sequentially significant ranking among populations (Alpha value = 0.05 for the factorial Kruskal–Wallis test among classes). The threshold for the logarithmic LDA score was 2.0.

production in BC and EU children. However, their least abundance in the gut microbiota in comparison with *Prevotella* enrichment in microbiota of BR could explain the differences in SCFAs levels found in fecal samples of rural and urban populations.

Functional Metabolic Profiles of Gut Microbiota from African and European Children Reflect Different Dietary Habits and Environments

To evaluate how the observed taxonomic differences between the gut microbiota of African and European children affect their metabolic potential, we applied PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013), a computational approach useful for inferring the functional contribution of microbial communities on the 16S rDNA sequencing dataset. Despite the limitation of the functional inference approach, we evaluated accuracy of PICRUSt, by using the Nearest Sequenced Taxon Index (NSTI), developed to quantify the availability of nearby genome representatives for each microbiome sample (Supplementary Materials and Methods and Table 6).

The PICRUSt prediction revealed significant differences in the main functional classes [Kyoto Encyclopedia of Genes and Genomes (KEGG) categories at levels 2 and 3], deriving from functional acquisitions associated with different environments and different dietary habits in the four study populations (Supplementary Figure 6). LEfSe analysis (Segata et al., 2011) performed on PICRUSt output showed several KEGG categories differentially present in the African and European populations (Supplementary Figure 6). In particular, considering KEGG function categories at level 3 (**Figure 7**), among the most representative metabolic functions enriched in the BR metagenome, we observed functions involved in complex carbohydrate metabolism, deriving from foods rich in fiber and polysaccharides, such as glycan biosynthesis, glycosyl transferases, and tricarboxylic acid (TCA) cycle (**Figure 7A**). These metabolic functions could explain the microbiota abundance of plant polysaccharides-bacteria degraders and the highest levels of SCFAs observed in fecal samples of BR with respect to other urban populations.

In the BC metagenome, we observed enrichment of KEGG categories involved in starch and sucrose metabolism and methane metabolism (**Figure 7B**), related to the fermentation of polysaccharides (Danielsson et al., 2014). We could hypothesize that the presence of known fermenting bacteria such as *Ruminococcaceae, Lachnospiraceae* and *Clostridiaceae,* observed in BC microbiota, can promote functional acquisition related to methane metabolism.

In the EU metagenome, we found enrichment of KEGG functions deriving from a simple sugar-rich diet. The acquisition of functions related to galactose metabolism, involved in conversion of galactose into glucose (**Figure 7A**), could arise from consumption of dairy products. Other functions enriched in the EU gut microbiota were the biosynthesis of carbohydrates

from fatty acids and to sulfur and nitrogen metabolism (**Figure 7B**). Several enteric bacteria produce reduced sulfur and nitrogen by dietary amino acids and animal protein. We supposed that bacteria such as *Bilophila,* found abundantly in EU microbiota, a well-known sulfite-reducing bacterium, could be responsible for this functional contribution.

In the BC and EU metagenome, we observed enrichment in functions related to consumption of a diet with high fat and animal protein, such as lipid and amino acid metabolism.

Among the KEGG functional categories, in BC metagenome, we observed enrichment of primary and secondary bile acid biosynthesis (**Figure 7C**). Commensal bacteria deconjugate bile acids, synthesized in the liver from cholesterol-derived precursor molecules, and convert primary into secondary bile acids (Brestoff and Artis, 2013).

In the BR metagenome, PICRUSt analysis showed several enriched amino acid metabolism (**Figure 7D**), suggesting a potential ability of BR microbiota to contribute to host metabolic functions in conditions of poor amino acid food intake. The absence of essential amino acids in food, especially in cereals, which are the basis of the African diet, leads to an inability to synthesize protein and ultimately can lead to malnutrition, especially through a syndrome known as Kwashiorkor, which is common among children in these countries. However, the high intake of cereals and legumes in the BR diet could be a source of amino acids, especially glutamate, alanine, and cysteine. Interestingly, one exceptional source of glycine, alanine, and glutamic acid could be the Baobab, whose leaves are added to main Burkinabè dishes (Supplementary Table 2). Similar findings have been observed in the Hazda, one of the last hunter-gatherer populations, who eat Baobab leaves (Schnorr et al., 2014).

In the BC metagenome, we found enrichment of aromatic amino acid (phenylalanine, tyrosine, and tryptophan; **Figure 7D**), and in the EU, of metabolism of arginine, proline, valine, leucine, isoleucine, histidine and tryptophan, and lysine biosynthesis and degradation, that may originate from the animal protein-rich food, typical of the Western diet (**Figure 7D**). In particular, concerning essential branched-chain amino acid (BCAAs, such as valine, leucine, and isoleucine), our results are in agreement with a recently analyzed metagenome of an Italian population (Rampelli et al., 2015).

The breakdown of basic amino acids by commensal bacteria is a source of SCFAs, as demonstrated by Smith and Macfarlane (1997). In general, alanine, glycine, and cysteine are fermented by bacteria to acetate, propionate, and butyrate. Serine is fermented to acetate and butyrate, while threonine is mainly metabolized to propionate. The main products of methionine metabolism are propionate and butyrate. Interestingly, we found that metabolism of these amino acids was enriched in our BR group, and, together with fiber and polysaccharide fermentation, could contribute to the abundance of SCFAs we observed in their fecal samples (**Figure 3**).

Regarding the more prevalent amino acid metabolism in our EU group, lysine, arginine and deamination of histidine produce butyrate and acetate, while BCAAs are slowly fermented by colonic bacteria. Thus, diet and the functional acquisitions of the bacterial metagenome for metabolism of amino acids or foods rich in fermented fiber and polysaccharides could explain the different levels of SCFAs observed in our studied populations.

CONCLUSION

The first years of life are fundamental for acquiring the gut microbial biodiversity, following dietary changes, and are essential for microbiota–host interactions that will later influence the health and disease status in adulthood (Rodriguez et al., 2015).

With respect to our previous work (De Filippo et al., 2010) and other studies investigating dietary habits and gut microbiota composition in traditional populations (Yatsunenko et al., 2012; David et al., 2014; Schnorr et al., 2014; Martinez et al., 2015; Gomez et al., 2016), this preliminary study although performed in a limited groups of children point out the modification of microbiota composition that occurs in African children belonging to the same ethnicity but living in different environments recapitulating processes occurring over a longer time in the co-evolution of diet, gut microbiota and the host.

The gradual enrichment of food variety, with an increase in animal protein (meat, fish, and dairy products), processed and refined foods, the increase in fats and reduction in fiber intake, occurring during the Westernization of dietary habits, drastically changes the microbial profiles and functions, especially bacterial lineages able to ferment complex carbohydrates and produce the well-known anti-inflammatory SCFAs, as observed in urban African children. Despite the limited number of samples from children of each African and Italian population, the dramatic differences among groups presented in this study provide preliminary insights into how improved socio-economic level and exposure to globalized foods could rapidly endanger ancient microbial communities able to ferment dietary fiber, and replaced by other bacteria more suited to metabolism of animal protein, fat and simple sugar. Although the limitations of the approach of functional inference by PICRUSt must be considered, as well as the limited number of individuals analyzed, the concordance of observation on the microbiome and the SCFAs profiles, suggest that the observed differences cannot be explained by chance, but rather from variation in fundamental microbial processes.

These key microbial and metabolic markers that have remained relatively unaltered over 100s of generations in rural Burkina Faso can be considered as reliable indicators of "pristine" microbial patterns, suggesting that dramatic microbial profile losses could occur during the course of urbanization, industrialization, and Westernization.

It is important to remember that children in rural areas of developing countries are at high risk of infectious disease and malnutrition, either because of lack of sanitation and/or the scarcity of food. This has key implications on policies related to mitigation of malnutrition and famine, as well as health assessment and protection of migrants. On the other hand, the gradual disappearance of "old friends" during the transition from rural to urban environments may also be a danger to the health of industrialized and Western populations,

in which non-communicable diseases are widespread, as well-reported by hygiene hypothesis (Rook and Brunet, 2005). Our results provides, in a preliminary way, a vital piece to the puzzle of the co-evolution of diet, gut microbiota and host, and highlight the importance of developing strategies to preserve microbial functional acquisitions, especially in childhood, that have been lost during the course of urbanization and the economic development of human populations, and that might have important health implications.

ADDITIONAL INFORMATION

Data deposition: data were submitted to European Nucleotide Archive (ENA) and available at http://www.ebi.ac.uk/ena/data/view/ERP000133, http://www.ebi.ac.uk/ena/data/view/PRJEB19895.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Study conception and design: CDF, PL, DC, and MDP; data analysis: MDP, CDF, MR, DA, GP, and EB; interpretation of data: CDF, MDP, PL, DC, and FM.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.01979/full#supplementary-material

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Personalization of the Microbiota of Donor Human Milk with Mother's Own Milk

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The American Academy of Pediatrics recommends that extremely preterm infants receive mother's own milk (MOM) when available or pasteurized donor breast milk (DBM) when MOM is unavailable. The goal of this study was to determine whether DBM could be inoculated with MOM from mothers of preterm infants to restore the live microbiota (RM). Culture dependent and culture independent methods were used to analyze the fluctuations in the overall population and microbiome, respectively, of DBM, MOM, and RM samples over time. Using MOM at time = 0 (T0) as the target for the restoration process, this level was reached in the 10% (RM-10) and 30% (RM-30) mixtures after 4 h of incubation at 37°C, whereas, the larger dilutions of 1% (RM-1) and 5% (RM-5) after 8 h. The diversity indexes were similar between MOM and DBM samples, however, different genera were prevalent in each group. Interestingly, 40% of the bacterial families were able to expand in DBM after 4 h of incubation indicating that a large percentage of the bacterial load present in MOM can grow when transferred to DBM, however, no core microbiome was identified. In summary, the microbiome analyses indicated that each mother has a unique microbiota and that live microbial reestablishment of DBM may provide these microbes to individual mothers' infants. The agreement between the results obtained from the viable bacterial counts and the microbiome analyses indicate that DBM incubated with 10–30% v/v of the MOM for 4 h is a reasonable restoration strategy.

Keywords: mother's own milk, restoration, personalization, donor breast milk, bacterial load, human milk microbiome

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INTRODUCTION

The benefits of human milk for preterm infants include immune and nutritional protection against infection, decreased necrotizing enterocolitis (NEC), and other morbidities (Wight, 2001). In the absence of mother's own milk (MOM), the American Academy of Pediatrics recommends using DBM over formula in very preterm infants (Moretti, 2012). Thus, the current practice at the

Abbreviations: DBM, donor breast milk; MOM, mother's own milk; NICU, Neonatal Intensive Care Unit; RM, restored microbiota pasteurized donor milk.

University of Florida, UF Health Neonatal Intensive Care Unit (NICU), is to provide premature infants of gestational age less than 30 weeks with DBM if the MOM supply is low or not available.

Numerous components of human milk are thought to be beneficial for the infant (Newburg et al., 2005). Accumulating data suggests that microbes that are indigenous to human milk may not be contaminants and play a beneficial role for the infant (Jeurink et al., 2013). Up to 200 different bacterial species have been found in human milk. Hunt et al. (2011) studied milk samples from 16 healthy women collected at three different time points. A common group of nine bacterial genera was present in all samples, but in different concentrations among the subjects. Each individual demonstrated a unique milk microbiome that was stable over time (Hunt et al., 2011). Collectively, these data highlight a personalized collection of milk microbes from each mother that is optimized for the health of her own infant.

Donor breast milk used in most NICUs is pasteurized due to safety concerns (Landers and Updegrove, 2010). Pasteurization of DBM kills 99% of bacteria and may also inactivate a large proportion of the bioactive components. Since DBM is pooled and pasteurized, it lacks the unique live maternal milk microbiome, which may be of benefit to the infant (for a review see Jost et al., 2015). The majority of mothers of very preterm infants are able to express small amounts of their own milk and although it may be of insufficient volume to meet the daily nutritrional requirements of their infant, it can still provide lasting health benefits. Our objective was to encourage the NICU mothers to continue pumping and use a small amount of MOM to inoculate the pasteurized DBM to add back the potentially beneficial naturally occurring microbes. We hypothesized that fresh MOM can be mixed with DBM to improve the quality of DBM by cultivating the beneficial milk microbiome to reflect MOM. Studies were performed to determine optimal dilutions and incubation times to obtain a microbial content most similar to that of the MOM.

MATERIALS AND METHODS

Subjects

The study cohort consisted of twelve mothers who provided a breast milk sample between December 2014 and February 2016. Mothers had delivered an infant at less than 32 weeks gestation, weighing less than 1500 g at birth, and who were expressing over 100 mL of breast milk per day and producing at least 45 mL with each expression session. This pilot study was approved by the Institutional Review Board (IRB201400527) at the University of Florida and mothers provided written informed consent. Exclusion criteria included mothers who had delivered an infant with a chromosomal abnormality or who was severely ill and mothers who were currently taking antibiotics. Data collected included gender, gestational age at birth, gestational age at breast milk collection, birth weight, race, maternal age, parity, mode of delivery, maternal BMI, maternal infections, maternal medications, maternal antibiotics, and Medicaid eligibility. Baseline demographics are summarized in **Table 1**.

Milk Collection

Each mother pumped one sample of 45 mL of breast milk (MOM) into a sterile container using a new breast pump kit (Symphony Breast Pump Kit, Medela LLC, McHenry, IL, United States) and hospital grade electric breast pump (Symphony Breast Pump, Medela LLC, McHenry, IL, United States). Prior to sample collection, mothers were provided the verbal instructions regarding hand hygiene during milk expression and techniques for breast pump cleaning per NICU protocol. Immediately following collection, the samples were placed on ice and delivered to the laboratory for processing. The pasteurized DBM was obtained frozen from the Human Milk Banking Association of North America (HMBANA) milk bank (see http://rmchildren.org/mothers-milk-bank/donate-milk/collection-and-storage/ for details on the collection guidelines for donors) and were thawed immediatly prior to each restoration process.

Restoration of the Live Microbiome of DBM

The restoration strategy was to add increasing amounts of MOM (1, 5, 10, and 30% v/v) into pasteurized DBM. As controls, pure DBM and MOM were included. Once blended, the milk mixtures were incubated at 37°C. Samples were taken at time 0, 4, and 8 h. For viable bacterial counts, samples were analyzed immediately. For microbiome analyses, 2 mL were centrifuged at 12,000 rpm

TABLE 1 | Demographics of mothers and infants.

Infant demographics ($n = 12$)	
Gestational age at birth (weeks)	27 ± 2.67
Birth weight (grams)	951.83 ± 397.02
Post-menstrual age at sample collection (weeks)	31 ± 2.86
Gender	
Male	75% ($n = 9$)
Female	25% ($n = 3$)
Maternal demographics	
Maternal age (years)	27 ± 4.95
Medicaid eligible	50% ($n = 6$)
Delivery	
C-section	42% ($n = 5$)
Vaginal	58% ($n = 7$)
Maternal BMI	28.35 ± 5.44
Maternal antibiotics	92% ($n = 11$)
Breastfeeding or attempts prior to milk sample	25% ($n = 3$)
Mother/Infant	
Race	
Caucasian	58% ($n = 7$)
African American	25% ($n = 3$)
Hispanic	17% ($n = 2$)

for 5 min at 4°C, the supernatant removed, and pellets stored at −80°C until the DNA was extracted. The pH and physical appearance of the samples at each time point was also recorded.

Culture Dependent Bacterial Analysis

Viable cell counts were determined by plating serial dilutions of each sample on selective and non-selective agar plates at time 0, 4, and 8 h (T0, T4, and T8, respectively). Based on the most common groups of bacteria cultivated from human milk (Wight, 2001; Martín et al., 2003; Reviriego et al., 2005; Jost et al., 2013; Menon and Williams, 2013), the following media were used: acidified Man, Rogosa and Sharp (MRS) agar for lactic acid bacteria (Fisher Scientific Company), Berens agar (BSM agar) for *Bifidobacterium* (Sigma–Aldrich) amended with BSM supplement Mupirocin Lithium Antibiotic (Fisher Scientific Company), Mannitol salt agar (MSA) for *Staphylococcus* (Fisher Scientific Company), Nutrient rich agar for facultative aerobes including *Streptococcus* (Sigma–Aldrich), and MacConkey agar for enterobacteria (Fisher Scientific Company). All plates were incubated at 37°C for 48–72 h. MRS and BSM agar plates were incubated in jars enclosing a burning candle to create a reduced oxygen environment while MacConkey and Nutrient agar plates remained incubated aerobically.

DNA Isolation, Library Construction and Sequencing

DNA was extracted from milk samples and preserved at −80°C using the PowerFecal® DNA isolation kit (MoBio Lab, Inc. United States) with the following modification: the pellet was homogenized in 750 μL of bead solution, then 100 μL of Protease from *Streptomyces griseus* 20 mg/mL (Sigma–Aldrich, Steinheim, Germany) was added (Marcial et al., 2017) The mixture was incubated at 37°C for 15 min, then the samples were processed according to the manufacturer's protocol. In the elution step, the DNA was collected in 70 μL of water and quantified. The DNA concentration was standardized to 1 ng/μL before the amplification of the V4 region using primers 515F/806R barcoded for Illumina HiSeq platform (Caporaso et al., 2012). To reduce variability and potential bias from potential sources of DNA contamination, all samples were processed with the same batch of DNA extraction kits as well as PCR reagents.

Bioinformatics and Statistical Analysis

Clustering of Operational Taxonomic Units (OTUs) at 97% similarity was performed with the subsampled open-reference OTU picking method (Rideout et al., 2014) with no removal of singletons. The Greengenes reference dataset version 13.8 (DeSantis et al., 2006) was used as the reference for OTU picking and for taxonomy assignment with uclust (Edgar, 2010). OTUs identified as mitochondrial DNA or as chloroplasts were removed from further analyses using R studio.

Community structure was analyzed in R with phyloseq (McMurdie and Holmes, 2013) and plotted with ggplot2 (Wickham, 2009). Differences in taxonomic profiles were analyzed by Welch's *t*-test (for two groups) or by ANOVA (for multiple groups) with Tukey-Kramer *post hoc* tests with STAMP (Parks et al., 2014) and PAST (Hammer et al., 2001).

RESULTS

Mother's Own Milk Shows a High Variability in the Number of Culturable Bacteria

At baseline $t = 0$, the amount of four bacterial populations were quantified in MOM and used as the target goal for each individual mother. It was found that the amount of bacteria in MRS plates (lactic acid bacteria including *Lactobacillus*) were between 10^3 and 10^5 CFU/mL with most of them being at 10^4 CFU/mL (**Figure 1B**). The bacterial load in MSA and nutrient broth were between 10^3 and 10^6 CFU/mL with an equal distribution in concentrations among the MOMs (**Figures 1A,C**). Only four MOM samples (30% of samples) grew on MacConkey agar at concentrations between 10^1 and 10^2 CFU/mL (data not shown). *Bifidobacterium* colonies were not recovered on Berens agar under our experimental conditions. The viable counts were also determined in DBM. Around 10^2 CFU/mL were counted in nutrient broth for half of the DBM samples (**Figure 1C**).

Restoration of the Live Microbiome of DBM with Mother's Own Milk

As described in the methods section, each of the twelve samples of MOM were inoculated in DBM at 1% (RM-1), 5% (RM-5), 10% (RM-10) and 30% (RM-30). Samples, including incubated DBM and MOM, were taken across three time points. The goal of this experiment was to determine the cultivable bacterial load of each sample to establish the minimum time and dilution required to reach the initial bacterial concentration found in MOM.

For each growth media, the concentration of bacteria was determined and ratios were calculated using MOM concentration at time 0 as the target concentration (represented as 1) (**Figures 1D–F**). On average, all RM samples increased in bacterial concentration over time (**Figures 1A–C**). A good correlation was found between the size of the inoculum and the amount of bacterial growth while the initial concentration of bacteria did not affect the outcome.

For MSA media, which targets mostly *Staphylococcus,* after 4 h of incubation at 37°C, 75% of the RM-10 reached a ratio of 0.6 compared to the MOM original bacterial load while all RM-30 reached a ratio of 1. For MRS media, which targets mostly lactic acid bacteria, 33% of the RM-10 reached a ratio of 1 compared to MOM while 58% of RM-30 reached the same ratio after 4 h. In Nutrient agar, which is a general purpose media targeting non-fastidious organisms, after 4 h of incubation 42% of samples in RM-10 reached a ratio of 1 compared to MOM while 83% reached a ratio of 1 in the RM-30 samples. After 8 h of incubation the bacterial load in all growth media tested (MSA, MRS, and Nutrient agar) for RM-10 and RM-30 went over the initial concentration of MOM (**Figure 1**). In contrast, the two highest milk dilution ratios (RM-1 and RM-5) were less than

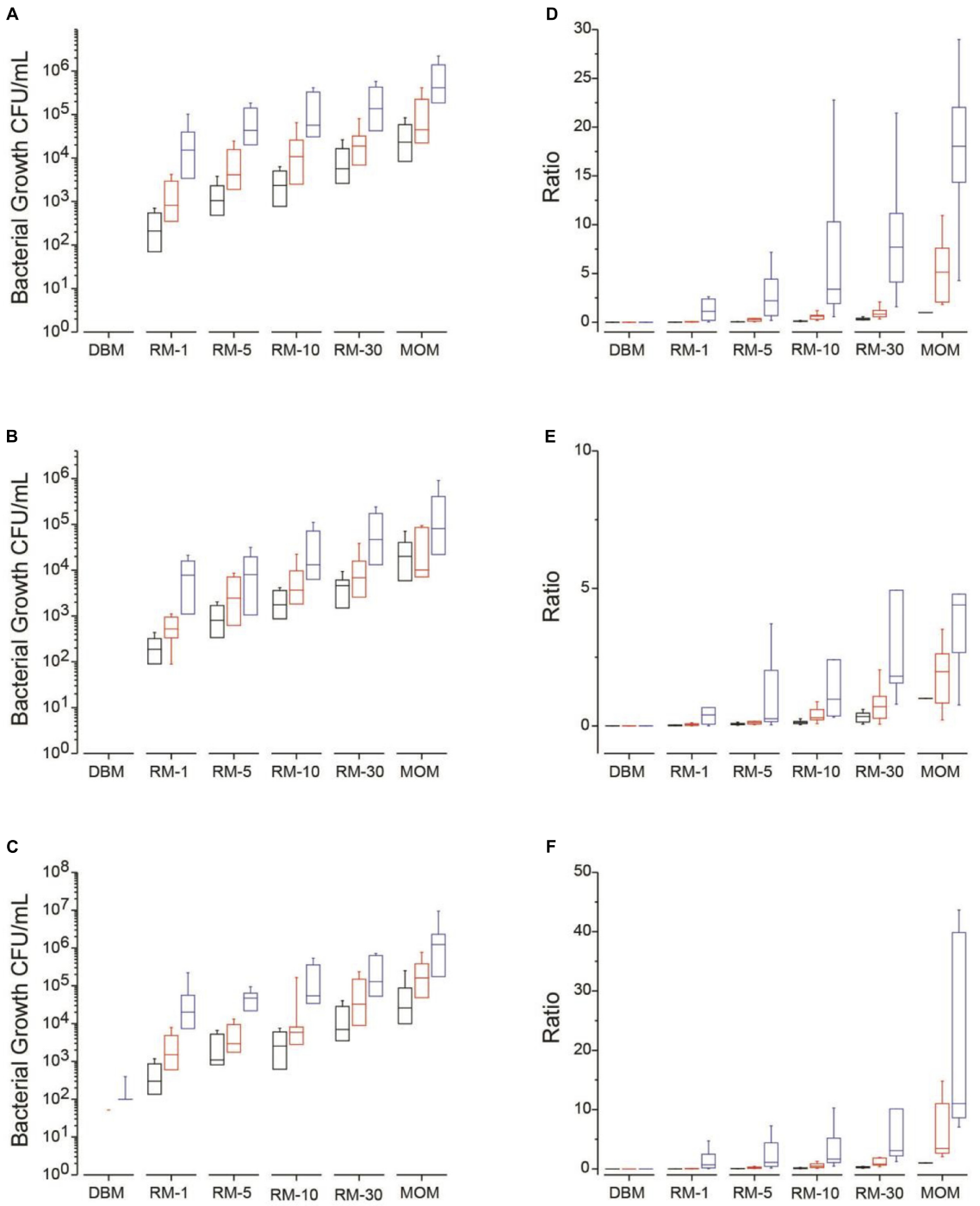

FIGURE 1 | Median bacterial growth (CFU/mL) for each milk sample (DBM, RM-1, RM-5, RM-10, RM-30, and MOM) on different media types at time = 0 (T0, black boxes) and incubated over 4 h (T4, red boxes) and 8 h (T8, blue boxes) at 37 °C. Bacterial growth was determined on **(A)** MSA, **(B)** MRS and **(C)** on Nutrient media. Mean ratios of bacterial growth of RM samples to MOM **(D–F)**. The ratio was calculated for each RM sample by dividing the CFU/mL in each RM sample to its corresponding MOM sample at T0 (MOM = 1). The ratios were calculated at each time point in **(D)** MSA, **(E)** MRS, and **(F)** Nutrient media.

0.5 at 4 h of incubation and then at 8 h of incubation reached a ratio > 1, exceeding the MOM bacterial load (**Figure 1**).

Since microbial growth may result in changes in pH as well as physical changes to the milk, the overall appearance (i.e., phase separation and curdling) and pH was monitored throughout the incubation period. Visual inspection of the milk samples did not reveal changes during the incubation period. Analysis of pH indicated that MOM samples were more alkaline (pH 7.5 ± 0.11)

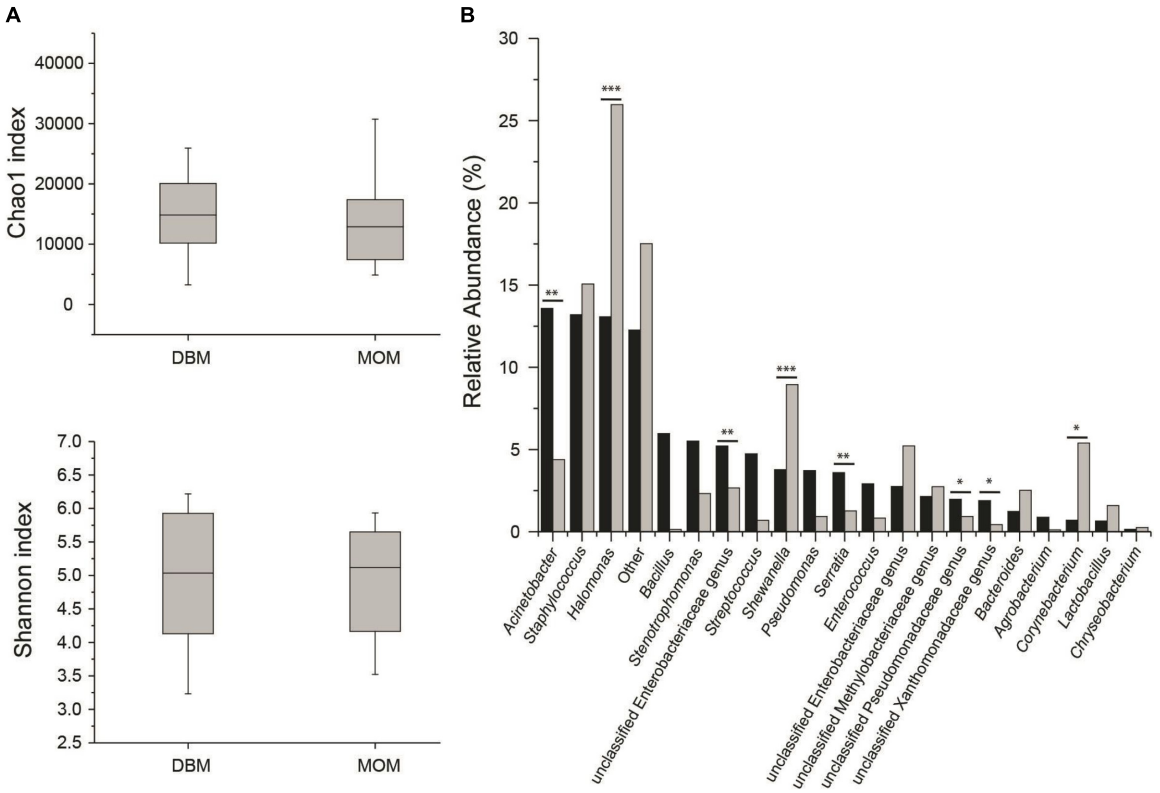

FIGURE 2 | (A) Alpha diversity determinations of DBM and MOM at T0 using Chao1 and Shannon indexes. **(B)** Summary of the most abundant genera found in DBM (black) and MOM (gray) samples. Statistically significant values are indicated $^{**}p < 0.05$ and $^{***}p < 0.01$. Differences at $^{*}p < 0.1$ are also indicated.

than pasteurized DBM (pH 6.5 ± 0.15) at T0. The incubation of MOM milk for 8 h resulted in a significant decrease ($p = 0.047$) in pH to 7 ± 0.5 while no significant changes were observed in DBM samples or RM samples over time.

Donor Milk versus Mother's Own Milk Have a Similar Diversity Index

Illumina sequencing of the V4 region of the bacterial 16S rRNA was performed on all milk samples. After quality control, a total of 15,575,142 sequences were obtained with a mean of 75,976 sequences per sample. The rationale of this microbiome analyses was to have an unbiased view of the changes in the microbial community during the restoration process.

First, we compared the community structure of DBM and MOM at T0. The alpha diversity expressed as Chao1 and Shannon index was similar between the two sets of samples (**Figure 2A**). It cannot be concluded, however, that due to the similarity in alpha diversity between DBM and MOM that the bacterial load is the same since DBM is pasteurized. The relative abundance of genera was compared between the two samples (**Figure 2B**). *Acinetobacter, Staphylococcus, Halomonas, Bacillus, Stenotrophomonas, unclassified Enterobacteriaceae genus, Streptococcus, Shewanella, Pseudomonas, Serratia, Enterococcus,* unclassified Enterobacteriaceae genus, unclassified Methylobacteriaceae genus, unclassified Pseudomonadaceae genus, unclassified Xanthomonadaceae genus and *Bacteroides* constituted 85% of the sequences found in DBM. In MOM the most abundant genus were *Halomonas, Staphylococcus, Shewanella, Corynebacterium*, Enterobacteriaceae genus, *Acinetobacter*, unclassified Methylobacteriaceae genus, unclassified Enterobacteriaceae genus, *Bacteroides, Stenotrophomonas* and *Lactobacillus*. The statistical analyses showed that *Halomonas* ($p < 0.01$) and *Shewanella* ($p < 0.01$) were more abundant in MOM than in DBM samples. Similarly, *Staphylococcus, Corynebacterium*, and *Lactobacillus* were more abundant in MOM, yet did not reach statistical significance ($p < 0.1$) when compared to that of DBM. On the contrary, *Acinetobacter,* an unclassified Enterobacteriaceae genus, and *Serratia*, showed a significantly higher relative abundance ($p < 0.05$) in DBM.

Fluctuations in the Alpha Diversity of RM Samples during Microbial Restoration

Next, the fluctuations in the microbial community as a result of the restoration process was determined. As described earlier, the working hypothesis is that the restoration process will result in the expansion of the microbial population without loss of diversity. The expansion of MOM was used as a positive control to determine microbial populations that will be able to expand *in vitro*. The Shannon index was utilized to determine the species richness across time between DBM, MOM, and the RM samples

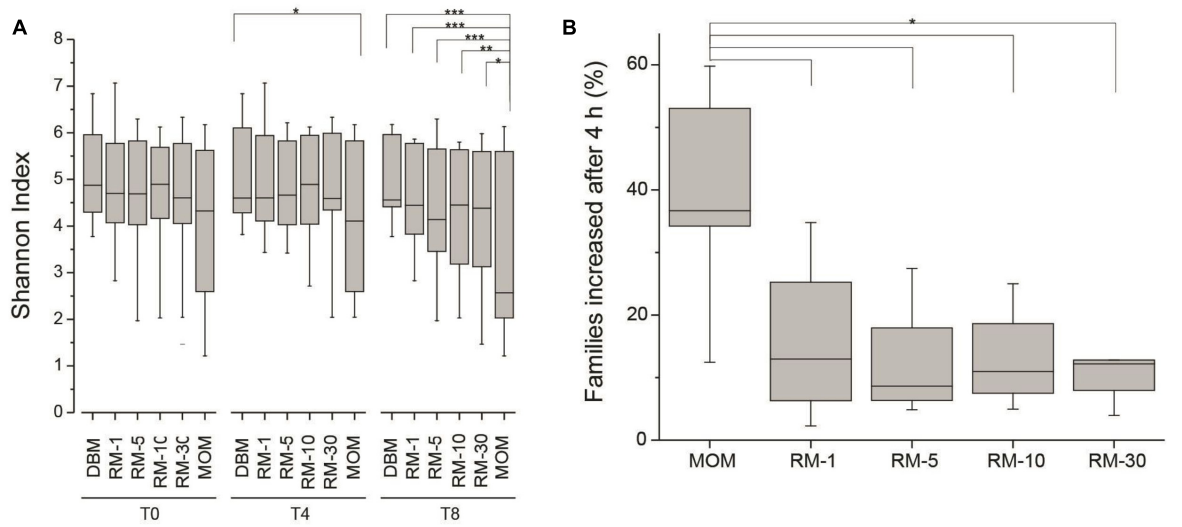

FIGURE 3 | (A) Alpha diversity assessment in RM samples (RM-1, RM-5, RM-10 and RM-30, across time points (0, 4, and 8 h). DBM and MOM samples were included in the analyses. It was found that DBM and MOM are significantly different from each other at T4 and T8. The alpha diversity of the 30% dilution of MOM at T4 resembles that of MOM at T0. Statistically significant values are indicated as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. **(B)** Summary of the expansion of bacterial families after 4 h of incubation. The expansion of bacterial families was determined for each sample by substracting the relative abundancy at T4 from the relative abundancy at T0. Statistically significant values are indicated as $*p < 0.001$.

(**Figure 3A**). It was found that after 4 h of incubation the MOM samples had a significant decrease ($p < 0.01$) in alpha diversity only when compared to DBM and RM-1. In contrast, after 8 h of incubation, there is a significant decrease ($p < 0.001$) in diversity when comparing MOM to all other samples (DBM and RM). However, even when a decrease in diversity was observed in RM-10 and RM-30, they did not reach statistical significance ($p = 0.07$ and $p = 0.14$, respectively). The change in diversity of MOM and RM-10 or RM-30 suggests the differential growth of few bacterial species.

The analysis of the bacterial richness of the RM allowed for the assessment of the expanding microbiome to determine if the RM samples, over time, become similar to MOM at T0. A multidimensional scaling (MDS) plot was generated to visualize the variability of the microbial community in each MOM and their derived RM samples (**Figure 4**). It was expected that the DBM samples of each individual set should remain clustered together since DBM is pasteurized and should have negative to little bacterial growth. In most cases, the DBM samples remained clustered together, such as MOM sample 4, 5, 6, 7, 8, 10, 11, and 12 (shown as circles in **Figure 4**) whereas, in DBM used for MOM samples 2 and 9 has a small difference in bacterial richness over time. Overall, these MDS plots allowed the visualization of fluctuations of the microbial population in the different RM samples over time. In some mothers, it was observed that the different RM samples migrated toward MOM at T0, suggesting that the RM sample became more similar to MOM at T0 (**Figure 4**). However, some exceptions were observed. For example, all samples derived from MOM 3 cluster together, indicative that the restoration process was not successful. Mothers 11 and 12 show migration of all the dilutions moving away from MOM at all time-points and toward DBM instead. Mothers 11 and 12 shared the same sample of DBM so it is possible that live bacteria still found within this DBM sample are hindering the expansion of MOM-derived microbiota.

RM Samples Do Not Share a Common Expanding Microbiome

Next, we determined if a core microbiome was expanding in all the RM samples. Based on the culture dependent results where the target bacterial load (of the culturable population) was reached after 4 h and a decrease in diversity was observed in the microbiome after 8 h of incubation, the next series of analyses were performed on all the samples (DBM, RM, and MOM) only after 4 h of incubation.

For these analyses the relative abundance of each bacterial family was obtained. To identify families that increase in abundance after 4 h, the bacterial load from T0 was subtracted from the bacterial load from T4 across all samples. If a family increased in concentration within the negative control (DBM), it was excluded in the results. It was found that out of the 120 ± 40 families identified within MOM samples, an average of 37 ± 12 were able to increase concentration after 4 h of incubation. Interestingly, in all RM samples, 23–38% of the families were able to increase in concentration (**Figure 3B**).

All bacterial genera within the families that increased in concentration after 4 h of incubation were analyzed. Some genera showed some statistical trends like *Gemella*, unclassified Gemellales genus, *Salinicoccus, Gallionella,* unclassified Proteobacteria genus 1, unclassified Proteobacteria genus 2, *Shewanella, Alicyclobacillus, Pediococcus,* and *Lactobacillus*, however, no significant differences ($p < 0.2$) were observed (data not shown). In contrast, *Staphylococcus* showed a significant

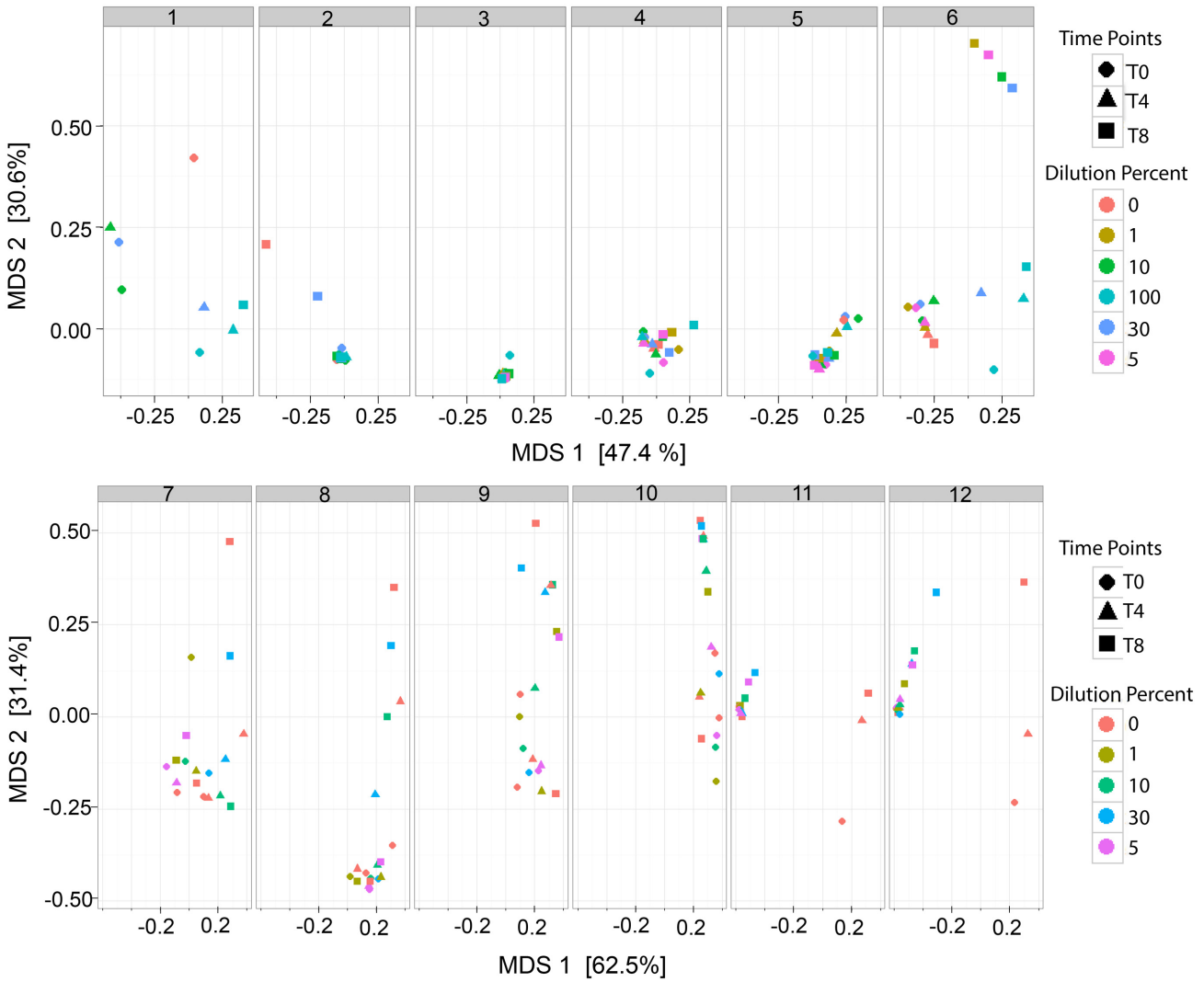

FIGURE 4 | Multidimensional scaling (MDS) plots of RM samples. Two dimensional clustering determined by the distance of each sample to show individual clustering in each MOM derived subset. Each time point (T0, T4, and T8) is signified by a shape (Circle, Triangle, and Square, respectively). Each dilution is signified as a color. For MOM 1 and MOM2 samples RM-1 and RM-5 were not available. For MOM1, only DBM at T0 (DBM = 0 and T0 = circle) is shown.

increase in concentrations ($p < 0.01$) (data not shown). In summary, a common core of microbes that expand in RM samples was not identified, with the exception of *Staphylococcus*.

Mother's Own Milk Samples Cluster by the Birth Mode of Delivery

Unable to determine a common expanding microbiome among RM samples, the data was stratified and analyzed considering the birth mode of delivery. The principal component analysis using MOM samples at T0, clustered into c-section and vaginal delivery with the exceptions of mothers 6 and 7 (**Supplementary Figure S1**). The distribution of the 15 most abundant genera among samples is shown in **Supplementary Figure S2**.

The microbiome of MOM samples divided by mode of delivery (c-section $n = 5$ and vaginal $n = 7$) were analyzed using a Welch's *t*-test (Supplementary Table S1). It was found that *Erwinia* ($p = 0.03$) and *Pseudomonas* ($p = 0.03$) were more prevalent among c-section samples while *Halomonas* ($p = 0.02$), *Lactobacillus* ($p = 0.04$), *Prevotella* ($p = 0.04$), *Ruminococcus* ($p = 0.04$), unclassified Clostridiaceae genus ($p = 0.03$), and unclassified Enterococcaceae genus ($p = 0.02$) were found at higher concentrations in vaginal delivery samples (Supplementary Table S1). Other genera that had *p*-values greater than 0.05 and less than 0.13 were also taken into consideration for further analyses. These results indicate that the differences in genera observed between the mode of delivery of MOM might explain the inability to identify a core microbiome that expand in RM samples.

The data from RM samples was stratified by mode of delivery and the genera that were differentially found between c-section and vaginal delivery were analyzed (Supplementary Table S1).

The relative increase in abundace after 4 h of incubation was tested using ANOVA and Tukey's *post hoc* tests. It was found that most genera did not change significantly. *Agrobacter* showed a significant difference ($p < 0.05$) between vaginal RM-10 and c-section RM-10. Interestingly, 3 unclassified genera (unclassified Clostridiaceae genus, unclassified Enterococcaceae genus 2, and unclassified Methylophilaceae genus) increased in relative abundace in all c-section RM samples but not in vaginal delivery birth RM samples (data not shown). These results suggest that each MOM has a unique diverse bacterial load, having no core microbes.

DISCUSSION

In this work we show that by using a small amount of MOM to inoculate pasteurized DBM, it is possible to reestablish the potentially beneficial naturally occurring microbes. MOM contains irreplaceable immune modulating factors including commensal bacteria (Wold and Adlerberth, 2002; Jost et al., 2013). Feeding preterm infants MOM has been shown to decrease NEC and sepsis with even small amounts of MOM providing some protection (Furman et al., 2003; Schanler, 2005; Meinzen-Derr et al., 2009; Corpeleijn et al., 2012, 2016; Abrams et al., 2014; Chowning et al., 2016). The microbiome found in MOM may provide short term and long term benefits to infants including preventing colonization by pathogens, stimulating production of cross-reactive antibiodies, and possibly establishing a healthy intestinal microbiome which may prevent long term morbidities including obesity, type 2 diabetes, chronic intestinal inflammation, autoimmune disorders, allergy, irritable bowel syndrome, and allergic gastroenteritis (Goulet, 2015; Lemas et al., 2016; Wallace et al., 2016). Unfortunately, many mothers of preterm infants are unable to produce sufficient amounts of breast milk to sustain 100% of their infant's nutritional needs (Smith et al., 2003; Lee and Gould, 2009). The RM may offer a personalized and beneficial alternative to DBM when MOM is limited. Since MOM contains a unique and unchanging microbiome (Hunt et al., 2011; Cacho and Neu, 2014), providing infants their own mother's milk may be beneficial, especially for infants born preterm, at risk for infection and other premature specific morbidities.

Our culture dependent approach indicated that the main bacterial groups that were tested, namely *Staphylococcus*, lactic acid bacteria (and other that can grow in MRS agar), along with other facultative aerobes (including *Streptococcus*), can be propagated into DBM, however, a large variability in bacterial load was observed between mothers. Previous studies examining bacteria present in breast milk have required aseptic sample collection of milk by mothers to limit skin flora and potential contaminants (Martín et al., 2003, 2007; Collado et al., 2009; Hunt et al., 2011; Jost et al., 2013; Khodayar-Pardo et al., 2014). In contrast, we aimed to examine the typical microbes that the preterm babies consistently received from their mother's milk during routine NICU expression practices and therefore, mothers performed routine NICU protocols for hand hygiene techniques and equipment cleaning. Consistent with Heikkilä and Saris (2003) and Martín et al. (2003), our results reveal that *Staphylococcus*, facultative aerobes, lactic acid bacteria, and few Gram negatives can be cultured from breast milk. We were not able to isolate *Bifidobacterium* in culture which may be due to the sensitive anaerobic nature of *Bifidobacterium*, having not provided the proper anaerobic environment or handling techniques to facilitate growth. In addition, it is possible that *Bifidobaceterium* is negligible in breast milk from mothers delivering preterm infants as indicated from the study by Khodayar-Pardo et al. (2014), which showed less *Bifidobacterium* compared to milk from mothers of term infants. Small numbers of Gram negative bacteria grew on MacConkey agar (results not shown), which is consistent with previous research indicating that approximately 30–70% of breast milk samples contained Gram negative bacteria (Botsford et al., 1986; Landers and Updegrove, 2010; Keim et al., 2013). Small amounts of Gram negative bacteria in breast milk may be important in preparing the immune system to utilize toll-like receptor mediated tolerizing mechanisms to prevent an exaggerated response to future Gram negative bacteria (Madara, 2004).

Interestingly, pasteurized DBM obtained from HMBANA showed that 10^2 CFU/mL of bacteria were still present in nutrient broth (facultative anaerobes including *Staphylococcus* and *Streptococcus*) in 44% of the DBM samples. This is consistent with previous studies describing the presence of staphylococcal species in DBM as well as spore forming bacteria such as *Bacillus cereus* (Crielly et al., 1994; Landers and Updegrove, 2010; Decousser et al., 2013; Akindolire et al., 2015; Dewitte et al., 2015). Although microbial diversity indexes were similar between DBM and MOM, the most abundant genera differed. The most prevalent genera in MOM were *Halomonas, Shewanella, Corynebacterium, Staphylococcus*, and *Lactobacillus*, while the most common genera in DBM were *Acinetobacter*, unclassified Enterobacteriaceae, and *Serratia*. These differences may reflect variances in the mothers who provided breast milk samples and the sanitation or method used for collection. DBM is typically obtained from mothers who are breastfeeding term infants who are often more than 6 months old. In contrast, the MOM in this study was obtained from mothers of preterm, hospitalized infants who because of their infant's prematurity, were unable to breastfeed and were thus dependent on mechanical breast milk expression to obtain milk for their infants.

We found that *Halomonas* was present in greater abundance than *Staphylococcus* in comparison with other studies in MOM. Although *Halomonas* has not been previously described in breast milk from mothers delivering preterm, *Staphylococcus* is a well-known predominant phyla in breast milk (Hunt et al., 2011; Urbaniak et al., 2012, 2016; Jost et al., 2013). We also found *Shewanellaceae* was high in MOM ($p < 0.05$) compared to DBM. The main genera found to be of great abundance in MOM across different studies are *Staphylococcus, Streptococcus,* Proteobacteria groups, and *Propionibacteria* (Hunt et al., 2011; Kumar et al., 2016). However, *Bifidobacterium, Bacteroides, Parabacteroides* and Clostridia groups have also been identified to be a part of the breast milk microbiota (Sinkiewicz and Ljunggren, 2008; Kumar

et al., 2016). Although *Bifidobacterium* was identified from our sequencing results, it was not shown to be prevalent. These differences in the predominant genera being *Halomonas* and *Shewanellaceae* as opposed to *Staphylococcus* and *Streptococcus* may be due to the fact that (except for rare occasions in three patients) mothers in our study did not breastfeed their infants. They were dependent on mechanical breast pumps for milk removal, thus the mother's breasts were not routinely exposed to oral microbes from the infant's mouth. Other reasons for these variations may be attributed to younger gestational age at delivery, stage of lactation, milk collection strategies and geographic variations in our study compared to others. In DBM, the most abundant genera were *Acinetobacter,* unclassified Enterobacteriaceae genus, and *Serratia.*

Results of this study suggest that the optimal restoration strategy to reach a microbial content most similar to MOM was a mixture of RM-10 incubated for 4 h. Using the bacterial load and microbial content of MOM at T0 as the target, we were able to successfully restore the microbiome in the RM-10 and RM-30 mixtures after 4 h of incubation, whereas larger dilutions of RM-1 and RM-5 reached the target level after 8 h. Although it did not reach the microbial content of MOM, RM-10 was able to reach 60% of the bacterial load of MOM. In contrast, RM-30 exceeded the target goal of MOM in the majority of incubated samples. This may be clinically undesireable since potentially pathogenic bacterial strains may grow to possibly harmful levels. Overall, our results demonstrate that inoculation with an amount of MOM as small as 1% can populate DBM with the mother's potentially beneficial bacteria.

The alpha diversity of MOM and the larger dilutions of RM (RM-10 and RM-30) decreased as incubation time increased. In contrast, the diversity of DBM and smaller dilutions of RM (RM-1 and RM-5) remained similar to their original levels as incubation time increased. This trend of MOM toward decreased diversity suggests replication of only a few microbial species. Breast milk studies show that microbial diversity is associated with a healthy lactating milk microbiome as opposed to the milk microbiome of a mother with mastitis where *Staphylococcus* or *Streptococcus* species predominate (Delgado et al., 2008). This decreased diversity indicates that in order to preserve microbial diversity similar to that found in MOM, a 4 h incubation time compared to an 8 h incubation time may be optimal, which confirms the culture based results favoring a 4 h incubation time over 8 h for the RM.

Birth mode of delivery has been shown to affect the microbiota of breast milk in the majority of studies, suggesting a difference in the milk microbiome between infants born via c-section and those born vaginally (Azad et al., 2013; Gregory et al., 2015; Liu et al., 2015; Brumbaugh et al., 2016; Dominguez-Bello et al., 2016; Lee et al., 2016; Nagpal et al., 2016; Rutayisire et al., 2016). In our study, we observed a differential clustering of microbiomes from the breast milk of c-sections versus vaginally delivering mothers. Although it is clear each individual mother's milk microbiota has bacterial variability, c-section and vaginal deliveries cluster with one another. Further analysis illustrated that bacterial genera most prevalent in breast milk from vaginal deliveries were *Halomonas, Lactobacillus, Prevotella,* unclassified Clostridiaceae genus, *Clostridium, Comamonas,* and *Dorea.* Those genera most prevalent in breast milk from cesarean deliveries were *Erwinia, Pseudomonas, Ruminococcus,* unclassified Enterococcaceae genus*, Agrobacterium, Citrobacter, Enterococcus, Klebsiella,* unclassified Bacilli genus, unclassified Bradyrhizobiaceae genus, and unclassified Methylophilaceae genus. Further statistical analysis does not show a common microbiota across breast milk regardless of c-section or vaginal delivery, concluding that the microbiota of breast milk is variable between each mother.

Based on our results, restoration of the live microbiome of DBM with MOM appears to be a promising and innovative method to provide preterm infants with beneficial breast milk bacteria. It is well known that breast milk changes over time to meet specific needs of infants which are attributed to stages of lactation, gestational age, infant feeding, and the health status of the breastfeeding dyad (Daly et al., 1996; Kent, 2006; Hassiotou et al., 2013). If mothers of preterm infants are able to express even minimal amounts of breast milk, restoration of the microbiome in DBM may allow their infants to receive milk more specific to their individual needs based on the stage of lactation and gestational age, thereby potentially improving their overall health. We used non-culture based techniques to take a snapshot of the full range of bacteria present in fresh preterm milk and pasteurized donor milk. The main limitation of our study is its small sample size and the use of antibiotics during the peripartum period. Nevertheless, it addresses the concept that the live microbiota donor human milk can be effectively restablished by MOM. In addition, mothers were not required to clean their breasts prior to breast milk sampling so their samples may have contained a higher level of skin colonizing microbes. Another limitation of the study is that safety parameters were not assessed. Future studies will need to include the analyses of potential pathogenic bacterial groups that may proliferate in the RM samples in the NICU environment.

In summary, we have shown that each mother has a unique milk microbiota and that the live microbiome in DBM can be restored with these unique bacteria using small amounts of MOM. This is a novel approach to possibly improving the bioactivity of DBM by adding specific MOM microbes in small quantities to personalize her own infant's milk. Personalizing DBM may benefit the mother–infant dyad and contribute to a more robust infant intestinal microbiome. The agreement between the results obtained from the viable bacterial counts and the microbiome analyses indicate that DBM incubated with 10 percent of the MOM for 4 h is a reasonable restoration strategy. Future studies should include larger samples sizes, activity of the microbes in RM in comparison to DBM and MOM samples, and clinical evaluation of the safety and efficacy.

ETHICS STATEMENT

This pilot study was carried out in accordance with the recommendations of the Institutional Review Board (# 2014 00527) at the University of Florida with written informed consent from all subjects. All subjects gave written informed

consent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Board at University of Florida.

AUTHOR CONTRIBUTIONS

The authors' responsibilities were as follows: NC, LP, JN, and GL designed the research; NC, NH, KP, GM, LC, and NL performed the research; DL contributed new reagents/analytic tools; NC, NH, LC, LP, and GL analyzed the data; NC, NH, LP, JN, and GL evaluated the data; NC, NH, LP, JN, and GL wrote the manuscript; NC, NH, LP, JN, and GL had primary responsibility for the final content; All authors read and approved the final manuscript. Medela, AG was not involved in the implementation, data collection, statistical analysis, interpretation of date, or manuscript preparation and writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01470/full#supplementary-material

FIGURE S1 | This is a principal component analysis (PCA) of the microbiota of MOM samples at T0. The circles represent moms that delivered through C-Section and the squares are those that delivered through vaginal births.

FIGURE S2 | Heatmap showing the top 15 genera of each DBM sample, with the exception of replicates DBM 1, 2 and 11 (DBM 1 and 2 being a replicates of 3 and DBM 11 being a replicate of 12), and each MOM sample.

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Abundance and Diversity of Hydrogenotrophic Microorganisms in the Infant Gut before the Weaning Period Assessed by Denaturing Gradient Gel Electrophoresis and Quantitative PCR

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Delivery mode (natural vs. cesarean) and feeding type (breast vs. formula feeding) are relevant factors for neonatal gut colonization. Biomolecular methods have shown that the ecological structure of infant microbiota is more complex than previously proposed, suggesting a relevant presence of unculturable bacteria. It has also been postulated that among unculturable bacteria, hydrogenotrophic populations might play a key role in infant health. Sulfate-reducing bacteria (SRB), acetogens, and methanogenic archaea use hydrogenotrophic pathways within the human colon. However, to date, few studies have reported detection of hydrogenotrophic microorganisms in newborns, possibly because of limitations on available group-specific, culture-independent quantification procedures. In the present work, we analyzed 16 fecal samples of healthy babies aged 1–6 months by means of quantitative PCR (qPCR) targeting the 16S rRNA or metabolic functional genes and by denaturing gradient gel electrophoresis (DGGE). qPCR data showed quantifiable levels of methanogens, SRB, and acetogens in all samples, indicating that the relative abundances of these microbial groups were not affected by delivery mode (natural vs. caesarian). DGGE revealed a high prevalence of the *Blautia* genus within the acetogenic bacteria despite strong interindividual variability. Our preliminary results suggest that hydrogenotrophic microorganisms, which have been a neglected group to date, should be included in future ecological and metabolic studies evaluating the infant intestinal microbiota.

Keywords: hydrogenotrophs, babies, gut microbiota, *Blautia*, quantitative PCR

INTRODUCTION

Hydrogenotrophic microorganisms inhabiting the gut microbiota of humans and non-human animals are involved in a mutualistic relationship. Three major types of H_2-consuming microorganisms colonize the human colon, namely, methanogenic archaea, sulfate-reducing bacteria (SRB), and acetogens (1). The common limitation in characterizing these low-abundance microbial communities is

related to difficulty in their cultivation, but DNA-based techniques allow detection of microorganisms that are not easily cultured.

Microbiota development is strongly influenced by delivery mode (natural vs. cesarean) and feeding mode (breast vs. formula) (2). To date, only limited and fragmented information is available concerning the influence of delivery and feeding mode on the occurrence of hydrogenotrophic microorganisms. Breastfeeding has been positively associated with CH_4 production but negatively associated with CH_3SH and H_2S (3). On the other hand, soy-based formula has been linked to high CH_4 and H_2S levels (3). In an early study, methanogens were detected in 10 out of 40 children (aged 3 months to 10 years), but their levels could not be precisely measured because the 16S rRNA gene density was below the assay's lower detection limit (4). By using quantitative PCR (qPCR), Palmer et al. (5) detected archaeal 16S rRNA gene sequences in 7 out of 14 samples from 1-year-old infants. Using a PCR assay targeting the *mcrA* gene, a functional gene encoding the methyl coenzyme M reductase, Mihajlovski et al. (6) reported the presence of methanogens in a newborn. Archaeal presence also has been identified in feces samples collected from birth to age 2.5 years (7). Within the archaeal group, *Methanobrevibacter smithii* is the dominant species detected in the human gut microbiota (8). Of interest, *M. smithii* was detected in all fecal specimens collected from 16 children up to the second year of life, including one sample gathered from a 2-week-old infant (9). Previous studies found no evidence of a possible correlation between intestinal archaeal colonization in newborns and dietary habits (10), but a potential, direct mother-to-child transmission has been hypothesized (5).

Concerning SRB, Fite et al. (11) reported their occurrence in 10 infants (aged 12 months) by means of qPCR targeting the 16S rRNA gene. SRB were also detected in 40 newborn stool samples collected from the neonatal period until age 24 months (12). Furthermore, the presence of SRB was reported in 15% of 12 children aged 3 months to 10 years, based on results with a real-time PCR protocol targeting the adenosine-5′-phosphosulfate reductase gene (4). More recently, De Palma et al. (13) described increased values for SRB from age 7 days to 4 months in 20 healthy babies with at least one relative who had celiac disease, as assessed by fluorescent *in situ* hybridization. The distribution of SRB has also been studied using primers targeting the *dsrA* gene, which encodes the alpha subunit of the dissimilatory sulfite reductase (DSR). The ubiquity of DSR in all known SRB microorganisms and its highly conserved nucleotide sequence make this gene suitable for evaluating the quantity and diversity of SRB in the human gut (14). In a recent paper, Pham et al. (15) investigated the occurrence of lactate-utilizing bacteria in 40 term infants, aged 2 weeks to 6 months, delivered either by vaginal birth or a C-section. In this study, SRB were enumerated by both plate count and qPCR. SRB were detected in 71% of infants at 2 weeks by means of plate count, and their levels increased with age. SRB were detected in 33, 23, 19, and 23% of babies at 2 weeks, 1 month, 3 months, and 6 months, respectively, when evaluated by qPCR.

Acetogens are obligate anaerobic bacteria that use the acetyl-CoA (Wood–Ljungdahl) pathway to synthesize acetyl-CoA from CO_2 and H_2 (16). Cultivation-based studies have estimated that the number of acetogens in adult human feces ranges from 10^2 to 10^8 colony-forming units/g (17). This group consists of metabolically versatile and phylogenetically diverse microorganisms. To date, more than 100 acetogenic species representing 22 genera have been isolated, most belonging to the genera *Acetobacterium* and *Clostridium* (18). In addition, some acetogenic microorganisms isolated from human feces are members of the *Clostridium* cluster XIVa, also known as the *C. coccoides* group (19), which contains a large number of butyrate-producing species (20). Moreover, some species belonging to *Ruminococcus* and *Clostridium* have been reclassified within the genus *Blautia* (21). The characterization of the polyphyletic distribution of acetogens and the identification at the species level using 16S rDNA-based molecular approaches is problematic because of their heterogenic composition. Recently, two functional genes—*fhs*, which encodes formyl tetrahydrofolate synthetase (22), and *acsB*, which encodes acetyl-CoA synthase (23)—have been identified as reliable molecular tools for acetogenic diversity studies. To date, acetogenic bacteria in infant fecal specimens have not been investigated.

Archaeal and bacterial diversity can be characterized using the previously described 515F/806R primer set targeting the V4 region of the 16S rRNA gene (24). However, more recent studies (25, 26) indicate that concentrations around 10^4 copies per microliter of this target are required to obtain reliable amplicon sequencing data for each bacterial population. In infant fecal samples, Archaeal and SRB levels are likely to fall below this cutoff and, consequently, result in no or reduced PCR product, thereby not allowing the comprehensive profiling of gut microbiota.

The aim of the present study was to assess the presence of hydrogenotrophic populations in the infant gut microbiota of 16 babies aged 1–6 months by means of qPCR and denaturing gradient gel electrophoresis (DGGE). In addition, we evaluated whether or not delivery mode influenced the dynamics of microbial colonization.

MATERIALS AND METHODS

Subjects

Sixteen term infants were investigated (age range: 1–6 months; mean: 2.96 months; SD: 1.35 months), and one fecal sample was collected for each subject. Babies were delivered either vaginally ($n = 8$) or by cesarean section ($n = 8$). No antibiotic treatment was provided during the 4 weeks before analysis. Samples were obtained as part of a previous study performed by Coppa et al. (27). Fecal samples were collected in 2009 at the Department of Pediatrics of the General Hospital of Ascoli Piceno and at the Neonatal Intensive Care Unit, Department of Pediatrics, of the University of Turin. The current study was conducted in compliance with the Helsinki Declaration; each mother signed an informed written consent. The Ethics Committee of the "Ospedali Riuniti" University Hospital, Polytechnic University of Marche, Ancona (Italy), reviewed and approved the study.

DNA Extraction

Stool samples were stored at −80°C until used. The samples were thawed at room temperature, and total DNA was extracted from 50 mg (wet weight) of feces using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Switzerland) according to the manufacturer's

instructions (28). Genomic DNA was eluted with 100 µl of elution buffer and its quality verified by agarose gel electrophoresis.

To verify the absence of environmental contamination, a negative control reaction was included in the extraction step. Test sample was replaced by DNA-free water, which underwent the same extraction process that was used for stool specimens. DNA concentration was determined using the Qubit HS dsDNA fluorescence assay (Life Technologies, Carlsbad, CA, USA). Purified DNA was stored at −20°C until used.

Quantitative PCR

The hydrogenotrophic populations were investigated by means of qPCR with previously described primers (22, 23, 29–32), as reported in **Table 1**. Six different qPCR assays were performed to specifically quantify total archaea and methanogens (16S rRNA and *mcrA* genes, respectively), SRB (*dsrA* and *aps* genes), and acetogens (*acsB* and *fhs* genes). Additional qPCR reactions were carried out to quantify the *Blautia* genus and the *Clostridium* cluster XIVa, which also includes relevant acetogenic *Blautia* spp (33, 34). and total bacteria (35) (**Table 1**). All qPCR assays were performed in the StepOnePlus™ Real-Time PCR System (Applied Biosystems Japan, Tokyo, Japan) by using the KAPA SYBR® FAST qPCR Kit Master Mix 2X (Biolab Scientifics Instruments SA, Switzerland) or the KAPA Probe® FAST qPCR Kit Master Mix 2X (Biolab Scientifics Instruments SA, Switzerland). Tenfold serial dilutions of genomic DNA isolated from reference strains were included in each experiment to generate the standard curves; all samples including the negative control for DNA extraction and a no-template control for PCR to confirm the absence of the qPCR water contamination were processed under the conditions reported in **Table 1**.

PCR–DGGE

Primers for DGGE analysis of the acetogens group are not available, so we used the primer pair targeting the *acsB* gene described by Gagen et al. (23) and modified the ACS_f primer by adding the GC-clamp. The PCR products obtained with these primers were loaded onto 8% (w/v) polyacrylamide gels (37.5/1, acrylamide/bis-acrylamide) with 20–70% linear DNA-denaturing gradients. Electrophoresis was carried out at 100 V and 60°C for 18 h in an INGENYphor 2 × 2 System (INGENYphor, Goes, Netherlands). We also used the primer pairs described by Maukonen et al. (36) to amplify the V6 region of the 16S rRNA gene of the *C. coccoides–Eubacterium rectale* group, a bacterial cluster that also includes some acetogenic microorganisms, among them *Blautia* spp. The PCR products obtained with the CcocF-GC-CcocR primers (36) were analyzed by DGGE following the methodological conditions described by Sagheddu et al. (37). Predominant bands were excised, re-amplified, sequenced (BMR Genomics, Padova, Italy), and then aligned with the GenBank database (http://www.ncbi.nlm.nih.gov/) by BLAST (38) and the blast algorithm and the Ribosomal Database Project by the Sequence Match tool (39). PCR-DGGE profiles were analyzed by Fingerprinting II SW software (Bio-Rad Laboratories, Hercules, CA, USA). Dendrograms were generated based on the Pearson's correlation coefficient by means of the Unweighted Pair Group Method with Arithmetic Mean algorithm (UPGMA).

TABLE 1 | Conditions and thermal protocols for the assessment of quantitative PCR.

Group	Gene	Primer set	Primers and probe, final concentration	Thermal protocol	Standard curve	Reference
Archaea	16S rRNA	ARC787F ARC1059R ARC915F (probe)	400 nM (primers), 100 nM (probe)	94°C 10 s, 60°C 20 s, 45 cycles	g DNA *Methanobrevibacter smithii* DSM 861	(29)
Archaea	*mcrA*	qmcrA-F qmcrA-r-d	400 nM	95°C 10 s, 60°C 40 s, 45 cycles	g DNA *M. smithii* DSM 861	(30)
Sulfate-reducing bacteria (SRB)	*dsrA*	DSR 1-F DSR-R	300 nM	95°C 10 s, 60°C 60 s, 35 cycles	g DNA *Desulfovibrio piger* DSM 749	(32)
SRB	*aps*	aps3F aps2R	400 nM	95°C 10 s, 60°C 40 s, 40 cycles	g DNA *D. piger* DSM 749	(31)
Acetogens	*fhs*	FTHFS_f FTHFS_r	700 nM	95°C 10 s, 55°C 20 s, 72°C 40 s, 40 cycles	g DNA *Blautia producta* DSM2950	(22)
Acetogens	*acsB*	ACS_f ACS_r	500 nM	95°C 10 s, 52°C 20 s, 72°C 30 s, 40 cycles	g DNA *B. producta* DSM2950	(23)
Blautia genus	16S rRNA	g-Blau-F g-Blau-R	200 nM	95°C 10 s, 60°C 50 s, 35 cycles	g DNA *B. producta* DSM2950	(33)
Clostridium XIVa group	16S rRNA	ErecF–ErecR	400 nM	95°C 10 s, 60°C 50 s, 35 cycles	g DNA *Eubacterium rectale* DSM 17629	(34)
Total bacteria	16S rRNA	Uni 331 F—Uni 797 R	400 nM	95°C 10 s, 60°C 40 s, 40 cycles	g DNA *E. coli* DSM 18039	(35)

Statistics

Data normality was checked using the Shapiro–Wilk test, and homoscedasticity was assessed using Levene's test. Since qPCR data were not normally distributed, they were log-transformed prior to statistical analysis. Differences among the two tested groups were analyzed by means of *t*-test for independent groups (R version 3.1.2) (R Core Team, 2014) (40).

RESULTS

qPCR Quantification of Hydrogenotrophic Microorganisms

The cohort of 16 infants under investigation included two different experimental groups based on delivery mode. The groups were as follows: samples 1–8, C-section delivered and samples 9–16, vaginally delivered babies (**Table 2**).

Quantitative PCR assays were performed to evaluate the numbers of acetogens, *Clostridium* cluster XIVa, *Blautia* spp., total archaea, methanogens, SRB, and total bacteria in fecal samples. For these bacterial groups, qPCR data indicated that fecal levels were very similar across all infants (**Table 2**). We found a higher abundance of acetogens, as assessed both by *acsB* and *fhs* gene quantification, compared with both methanogenic archaea and SRB. In addition in our samples, the abundances of *Clostridium* cluster XIVa and *Blautia* spp. were much more homogeneous, suggesting that most of the *Clostridium* cluster XIVa members were represented by species within the *Blautia* genus. No statistically significant difference was found between experimental groups for any bacterial group tested.

TABLE 2 | Quantification of hydrogenotrophic populations, *Blautia* spp., and *Clostridium* XIVa cluster by quantitative PCR.

Microbial populations	Infant group		*P*-value
	C-section delivered (gene copies/g of wet feces)	Vaginal delivered (gene copies/g of wet feces)	
Total archaea			
16S rRNA gene	4.52 (0.56)	4.56 (0.45)	0.327
Methanogens			
mcrA	4.84 (0.28)	4.78 (0.47)	0.429
Sulfate-reducing bacteria			
dsrA	3.43 (0.38)	3.28 (0.32)	0.543
aps	3.15 (0.28)	3.56 (0.31)	0.499
Acetogens			
acsB	8.61 (0.85)	8.72 (0.74)	0.474
fhs	9.49 (0.42)	9.52 (0.51)	0.805
Blautia			
16S rRNA gene	7.75 (0.88)	8.01 (0.75)	0.339
***Clostridium* XIVa**			
16S rRNA gene	9.3 (1.56)	9.75 (1.27)	0.466
Total bacteria			
16S rRNA gene	11 (0.72)	10.99 (0.55)	0.147

Results are expressed as the log-transformed mean values of the gene copies per gram of wet feces for each group of infants; SDs in brackets. The last column reports the P value obtained by t-test for independent groups ($P < 0.05$).

PCR-DGGE Analysis of Acetogenic Bacteria in the Infant Gut

Acetogens emerged as the dominant functional bacterial group among hydrogenotrophic microorganisms in the infant gut, so we attempted to assess the composition of the acetogenic bacterial community through PCR-DGGE analysis targeting the *acsB* gene. This analysis produced DNA bands that could not be identified at the species level although they were all assigned to the genus *Blautia* (data not shown).

Although the *Clostridium* cluster XVIa is a phylogenetic group that includes many butyrate producers and some acetogenic microorganisms, considering the abundance of the genus *Blautia* in our samples, we attempted to improve the taxonomic classification of the *Blautia* species occurring in our stool samples by using PCR primers targeting the whole *Clostridium* cluster XIVa. The resulting DGGE profiles were markedly different among infants and strongly influenced by interindividual variability rather than by the delivery and feeding conditions (**Figure 1**). Taxonomic assignment indicated that the genus *Blautia* was prevalent in all fecal samples, with dominant species represented by *Blautia luti*, *Blautia producta*, *Blautia wexlerae*, *Blautia hansenii*, *Lachnoanaerobaculum orale*, *Dorea formicigenerans*, and *Ruminococcus gnavus*, as reported in **Table 3**. Of interest, in one sample, a band corresponding to *Hungatella effluvii* was identified (**Table 3**). We detected the presence of *B. wexlerae* in eight fecal samples (50%), *B. luti* in two (12.5%), *B. producta* in seven (43.75%), and *B. hansenii* in three (18.75%). The distribution of *Blautia* spp. was not correlated with the delivery mode, thus confirming the qPCR results.

DISCUSSION

Hydrogenotrophic populations are present in the infant intestinal ecosystem, but their detection in infant fecal samples is hampered by their low relative abundance and by experimental limitations related to the recovery of purified high-quality DNA from infant fecal samples. Previous studies conducted on adult humans reported that 30–60% of Western people harbor methanogenic bacteria in their gut (41). More recently, some studies have described higher percentages of adult carriers (42). Methane production during childhood has been demonstrated to start around the third year of life and then to increase with age (43). Stool sample analyses, however, have revealed that methane is produced by 15.3% of 6-month-old infants at concentrations higher than 2 ppm (44). An early study estimated that approximately 10^8 methanogenic microorganisms per gram of dry weight of stool are needed to generate enough methane to be detectable by breath analysis (45). Additionally, direct competition among methanogens, SRB, and acetogens may occur for the common substrate H_2. As reported, ~50% of healthy humans carry intestinal SRB, and several studies have described their presence at different ages in the gut lumen (4, 46) and the distal gut mucosa (14). In addition, SRB are postulated to be positively associated with inflammation (47). In contrast, few studies have assessed the presence and distribution of acetogenic bacteria in the human gastrointestinal tract.

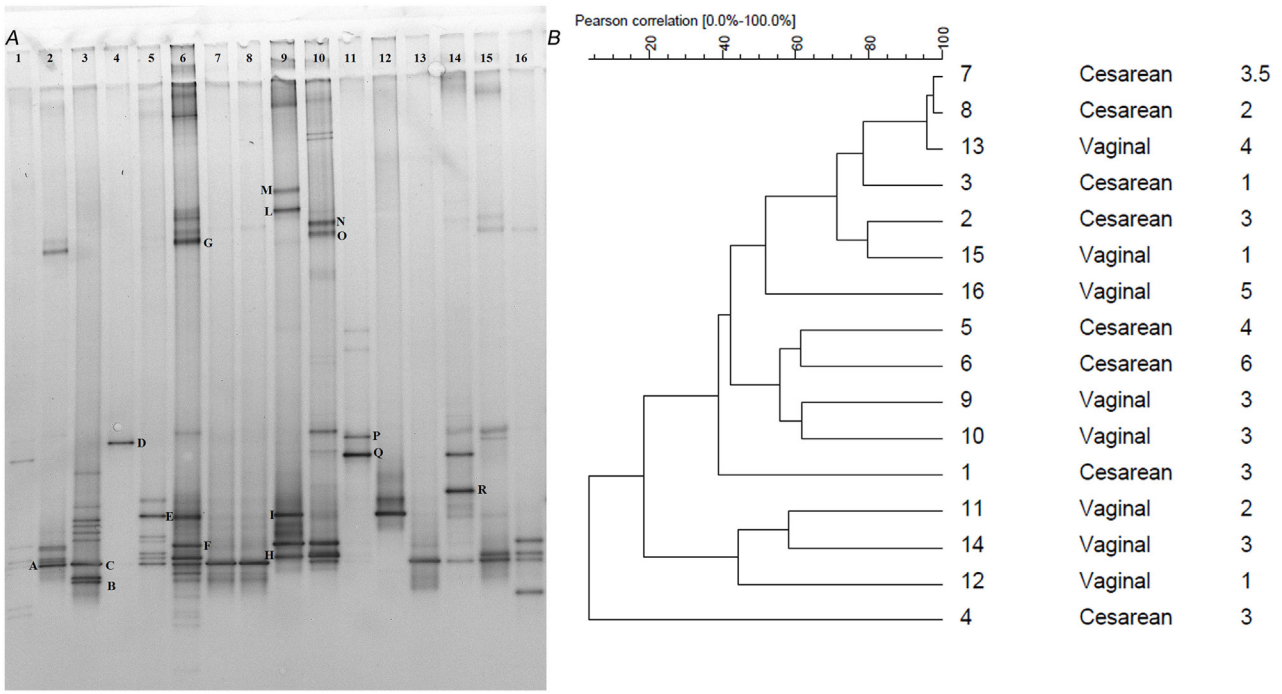

FIGURE 1 | **(A)** PCR-denaturing gradient gel electrophoresis (DGGE) profiles of 16 samples obtained using the primers CcocFGC-CcocR. Samples are subdivided by delivery mode (n = 1–8 C-section; n = 9–16 vaginal). Bands with letters were sequenced after re-amplification, and the corresponding identities were obtained by alignment in GenBank as reported in **Table 3**. **(B)** Dendrogram constructed on DGGE patterns by software analysis measured by the Pearson's correlation coefficient with the UPGMA algorithm. Columns, respectively, indicate delivery mode and infant age expressed in months.

TABLE 3 | Identification of bacteria belonging to the *Clostridium* XIVa group based on denaturing gradient gel electrophoresis (DGGE) profiles (see also **Figure 1A**).

Identification	Bands[a]	Accession number	% Similarity
Ruminococcus gnavus	A, C	NR_036800, NR_118690	100
Hungatella effluvii	R	NR_133762.1	100
Lachnoanaerobaculum orale	D, Q	NR_118086	99
Blautia luti	I, L, M, N, O	NR_114315.1	99
Blautia producta	F, P	NR_113270	99
Blautia wexlerae	H, G	NR_044054	100
Blautia hansenii	B	NR_104687.1	99
Dorea formicigenerans	E	NR_0044645.2	100

[a]*Bands are lettered as indicated on the DGGE gel shown in* ***Figure 1A****.*

A deep understanding of the diversity of bacteria inhabiting the infant gut microbiota is relevant for human gut ecology and future nourishment research. The major result of our study was that all infants harbored detectable levels of methanogens, acetogens, and SRB, as evaluated using specific qPCR for the 16S rRNA gene or the functional metabolic genes *mcrA*, *acsB*, *fhs*, *dsrA*, and *aps*. We detected about 10^4–10^5 copies/g of feces of the 16S rRNA (total archaea) and *mcrA* genes (methanogens), respectively, 10^3–10^4 copies/g of feces of the *dsrA* and *aps* genes (SRB), and 10^8–10^9 copies/g of feces of the *acsB* gene, and 10^9–10^{10} copies/g of feces of the *fhs* gene (acetogens), respectively. Considering the numbers of total bacteria present in our samples (about 10^{10}–10^{11} 16S rRNA gene copies/gram of feces), hydrogenotrophic microorganisms and in particular acetogens seem to represent a not negligible fraction of the infant gut microbiota.

The consistent recovery of hydrogenotrophic populations in all samples probably depended on the DNA extraction method performed or primer pairs used for the qPCR analyses. As Dridi et al. emphasized (9), the DNA extraction method exerts great influence on the recovery of archaea, suggesting that mechanical lysis with beads could overcome the presence of the K-resistant proteinase cell wall of methanogenic archaea. As in our study, Fite et al. (11) and Hopkins et al. (12), using a bead beating extraction protocol, recovered the presence of SRB in all fecal samples of babies tested. With a different DNA extraction system, Stewart et al. (4) reported the presence of methanogens and SRB, respectively, in 25 and 15% of fecal samples using previously described primers targeting the 16S rRNA (48, 49) and the *aps* (50) gene, respectively. With the same DNA extraction protocol, Palmer et al. (5) detected about 10^3–10^6 archaeal 16S rRNA gene copies/gram of wet feces in 7 out of 14 fecal samples using universal primers for the 16S rRNA gene (51, 52). These inconsistencies suggest that further efforts are needed to develop reliable molecular tools for hydrogenotrophic microorganism investigations. Notably, a recent review by Carbonero et al. (53) concluded that the occurrence of the functional genes *acsB* and *fhs* is not automatically associated to reductive acetogenesis. On the contrary, *mcrA* and *dsrA* genes seem to be reliable markers of both relative abundance and activity of methanogenic and SRB communities, respectively.

Comparison between the two DGGE analyses revealed that the 16S rRNA gene better described the complexity of the acetogenic distribution compared to the functional gene *acsB*. The DGGE analyses of the 16S rRNA gene allowed for identification of a greater number of acetogenic bacteria species compared to the DGGE for the *acsB* gene. In contrast, it was not possible to perform the DGGE analyses for the SRB and methanogens because their levels fell below the detection limit of PCR-DGGE, which ranges from 10^4 to 10^8 colony-forming units/ml (54). As a result, the presence of these microorganisms was quantifiable by qPCR, but conventional PCR did not produce any amplification fragment. In a recent review, Flint et al. (55) pointed out the necessity of investigating functional rather than phylogenetic marker genes. The study of metabolism-related genes will provide additional information allowing a comprehensive analysis of the gut microbiota. Unfortunately, in our case, only the DGGE analysis of the 16S rRNA gene gave positive results.

In this study, we failed to detect *Blautia* spp. 16S rRNA gene sequences by DGGE in only 1 of the 16 stool specimens, and in 2 out of 16, the band corresponding to the *Blautia* genus was faint; nevertheless, the identification of this genus was possible by qPCR. The *Blautia* genus was recognized in 93.75% of infants by DGGE and in all samples by qPCR in babies under age 6 months. Our results are in agreement with those reported by Kurakawa et al. (33), who described *Blautia* spp. predominance in the human intestinal *Clostridium* XVIa group regardless of age in three groups (32 children aged 3.2 ± 0.1 years, 32 healthy adults aged 39 ± 11 years, 32 healthy elderly aged 82 ± 6 years) (33). In recent work, Touyama et al. (56) described the presence of *B. wexlerae* and *B. luti* in the fecal samples of 12 healthy Japanese adults. The present study underlines the real problem of the possible underestimation of neglected hydrogenotrophic populations.

Another interesting finding was the recovery of a band corresponding to *H. effluvii*. This microorganism was recently isolated from an industrial treatment plant (57), and to the best of our knowledge, this study is the first time that it has been recovered in infant human samples. The difficulty of detecting these microorganisms is higher in infant specimens compared to adult samples, mainly because of lower levels of these microorganisms in infant specimens.

This pilot study also presents some limitations that require mention. First is the small number of recruited infants, and further studies on larger cohorts of babies are needed to define the putative role of hydrogenotrophic microorganisms in the infant gut microbiota. Since our study involved only a restricted number of samples, the major inferences that can be drawn from our results are related to the methodological approaches which allowed us to detect hydrogenotrophic populations at a quantifiable level in all infant fecal samples. The possibility of enumerating and identifying hydrogenotrophic species would be a major goal. To achieve such an aim, the punctual setting of DNA extraction protocols and the development of highly specific and efficient sets of primers with a good coverage would represent a crucial step to gain deeper insight into their actual ecological impact.

ETHICS STATEMENT

The current study was conducted in compliance with the Helsinki Declaration; each mother signed an informed written consent. The Ethics Committee of the "Ospedali Riuniti" University Hospital, Polytechnic University of Marche, Ancona (Italy), reviewed and approved the study.

AUTHOR CONTRIBUTIONS

VS drafted the manuscript, collected samples, and performed DNA extraction and quantitative PCR. VP jointly led the study and revised the manuscript. FM performed the denaturing gradient gel electrophoresis and the cluster analysis and revised the manuscript. LM conceived and designed the study and revised the manuscript. All the authors read and approved the final manuscript.

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Mechanisms Affecting the Gut of Preterm Infants in Enteral Feeding Trials

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Large randomized controlled trials (RCTs) in preterm infants offer unique opportunities for mechanistic evaluation of the risk factors leading to serious diseases, as well as the actions of interventions designed to prevent them. Necrotizing enterocolitis (NEC) a serious inflammatory gut condition and late-onset sepsis (LOS) are common feeding and nutrition-related problems that may cause death or serious long-term morbidity and are key outcomes in two current UK National Institutes for Health Research (NIHR) trials. Speed of increasing milk feeds trial (SIFT) randomized preterm infants to different rates of increases in milk feeds with a primary outcome of survival without disability at 2 years corrected age. Enteral lactoferrin in neonates (ELFIN) randomizes infants to supplemental enteral lactoferrin or placebo with a primary outcome of LOS. This is a protocol for the mechanisms affecting the gut of preterm infants in enteral feeding trials (MAGPIE) study and is funded by the UK NIHR Efficacy and Mechanistic Evaluation programme. MAGPIE will recruit ~480 preterm infants who were enrolled in SIFT or ELFIN. Participation in MAGPIE does not change the main trial protocols and uses non-invasive sampling of stool and urine, along with any residual resected gut tissue if infants required surgery. Trial interventions may involve effects on gut microbes, metabolites (e.g., short-chain fatty acids), and aspects of host immune function. Current hypotheses suggest that NEC and/or LOS are due to a dysregulated immune system in the context of gut dysbiosis, but mechanisms have not been systematically studied within large RCTs. Microbiomic

Abbreviations: ELFIN, enteral lactoferrin in neonates trial; GCMS, gas chromatography mass spectrometry; HMOs, human milk oligosaccharides; iFABP, intestinal fatty acid-binding protein; LCMS, liquid chromatography mass spectrometry; LOS, late-onset sepsis; NEC, necrotizing enterocolitis; NICU, neonatal intensive care unit; NIHR, National Institutes for Health Research; PN, parenteral nutrition; RCT, randomized controlled trial; SCFA, short-chain fatty acids; SEM, structural equation modeling; SIFT, speed of increasing milk feeds trial; TLf, talactoferrin (recombinant); VOC, volatile organic compound.

analysis will use next-generation sequencing, and metabolites will be assessed by mass spectrometry to detect volatile organic and other compounds produced by microbes or the host. We will explore differences between disease cases and controls, as well as exploring the actions of trial interventions. Impacts of this research are multiple: translation of knowledge of mechanisms promoting gut health may explain outcomes or suggest alternate strategies to improve health. Results may identify new non-invasive diagnostic or monitoring techniques, preventative or treatment strategies for NEC or LOS, or provide data useful for risk stratification in future studies. Mechanistic evaluation might be especially informative where there are not clear effects on the primary outcome (ISRCTN 12554594).

Keywords: lactoferrin, preterm infant, gut microbiota, metabolome, nutrition, late-onset sepsis, necrotizing enterocolitis, mechanistic evaluation

INTRODUCTION

Preterm delivery is associated with an increased risk of mortality and serious morbidities. Among these late-onset sepsis (LOS) and necrotizing enterocolitis (NEC) are of particular concern. Adverse neurodevelopmental outcome is a major cause of long-term morbidity associated with preterm birth and along with other serious medical problems represents a major cost to health-care services and society. The UK National Institutes for Health Research (NIHR) Health Technology Assessment (HTA) programme has funded two large randomized controlled trials (RCTs) of nutrition and feeding in preterm infants born <32 weeks gestation: the speed of increasing milk feeds trial (SIFT) (ISRCTN 76463425) and the enteral lactoferrin in neonates (ELFIN) trial (ISRCTN 88261002). These trials are the largest interventional trials in preterm infants conducted in the UK and Europe with almost 5,000 proposed recruits from more than 50 neonatal units. In the planning stage, it was anticipated that infants might be eligible for recruitment to both as the timing of the trials was predicted to overlap. There was, therefore, an explicit intention to enable and support recruitment to both trials and avoid unnecessary duplication of trial processes and procedures.

In the SIFT trial, 2,804 preterm infants in the UK were randomized to one of two different rates of increase in milk feeds: increasing by 18 or 30 ml/kg/day.[1] Recruitment commenced in August 2013 and completed ahead of schedule in June 2015. The primary outcome is survival without moderate or severe disability at 2 years corrected age, and follow-up and data collection will not complete until 2018. The SIFT protocol allowed for publication of key outcomes at hospital discharge including LOS and NEC. In the ELFIN trial, 2,200 UK infants are being randomized to either receive supplemental enteral bovine lactoferrin or placebo (sucrose).[2] ELFIN is anticipated to complete toward the end of 2017. Both SIFT and ELFIN are pragmatic trials with key neonatal morbidities as primary outcomes, but neither included mechanistic evaluation.

This publication describes the protocol for a mechanistic evaluative study of both trials—the mechanisms affecting the gut of preterm infants in enteral feeding trails (MAGPIE) study. This will examine patterns of gut microbiota and metabolites and will be conducted in a subset of infants recruited to the main trials. Prospective recruitment to MAGPIE will only be possible for infants in the ELFIN study, although research ethics permissions exist to analyze samples from around 100 babies in the SIFT study. Participation does not affect the main trial protocols or conduct and will use safe and non-invasive collection of specimens (urine and stool) from ~480 infants from 10 sites. In addition, in those infants who undergo surgery, the MAGPIE study will aim to retrieve gut tissue from pathology archives after all necessary routine clinical tests are completed.

SCIENTIFIC BACKGROUND

Prematurity is a major cause of mortality and serious long-term morbidity with an enormous burden on health care and educational systems with total costs to the public sector of approximately £UK 3 billion per year in the UK (1). NEC, a serious inflammatory bowel disease, and LOS are responsible for more deaths after the first week of life in extremely preterm infants than any other single pathology (2). NEC is associated with significant mortality (<20–40%) and affects <10% of infants born <32 weeks gestation (3–5) and occurs in first few weeks (6). In the UK, there are at least 6,000–8,000 births per year <32 weeks (7). LOS will affect <20–30% of these infants, of whom 1 in 10 may die (8). National data on cause of death in preterm infants are not routinely collected, but extrapolating data from one health region (2) suggests there are at least 250 deaths from NEC and LOS alone per year in England, although the true figure may be higher because postmortems are not always performed and clinical coding can be inaccurate. This number of deaths is similar to that for all childhood cancers (9), but unlike childhood cancer there is very little mechanistic work in preterm infants despite the worldwide rate of preterm births continuing to increase (10). NEC and LOS are both associated with significant morbidity in survivors including worse cognitive outcome and a twofold increase in the risk of cerebral palsy, and very high health-care costs: costs of surgery for NEC and prolonged intensive care are in excess of £UK 100,000 per case (5). In the US, it has been estimated that total costs related to the treatment of NEC and its consequences

[1] www.npeu.ox.ac.uk/sift.
[2] www.npeu.ox.ac.uk/elfin.

may be in excess of $US 5 billion per year (5). In addition, the long-term costs to society, the individual, and their families due to lifelong physical and mental impairment are substantial.

The SIFT and ELFIN trials do not include evaluation of disease pathophysiology. The practicalities of conducting trials in multiple sites over prolonged time periods means a pragmatic balance has to be reached between the detail and complexity of data collection. In SIFT, there were 56 recruiting neonatal units (primarily neonatal intensive care units, NICUs) but at least an additional 150 neonatal units required research ethics and governance approvals to collect and report trial-related data. Over 100 neonatal units who looked after SIFT babies will therefore be required to report data on the primary outcome of disability-free survival at 2 years corrected age. In the ELFIN trial, there are 35 recruiting sites (primarily NICUs) but data collection from an additional ~100 neonatal units is required in order to collect data until discharge to home. Collecting even limited biological samples from all recruited infants would be extremely challenging and expensive.

The interventions explored in SIFT and ELFIN both act *via* effects on the gut and are therefore likely to involve interactions with gut microbiota (2, 11–14). MAGPIE will use the opportunity provided by two large RCTs to explore some of the putative actions of the interventions as well as potentially explore disease mechanisms where NEC or LOS occur. MAGPIE will do this by sampling stool and urine from the infants and use emerging technologies including next-generation sequencing of gut bacteria, and urine and stool mass spectrometry, as windows into host and bacterial metabolism, respectively. The aim is to understand some of the mechanisms of actions of the interventions and diseases and provide new data in the areas of diagnosis, monitoring, and therapeutics.

Overview of the SIFT and ELFIN Trials

Speed of increasing milk feeds trial and ELFIN trials are funded by the HTA and managed by the National Perinatal Epidemiology Unit Clinical Trials Unit, Oxford, UK.[3] Both trials recruit very preterm infants (<32 weeks gestation) in the first few days after birth while receiving care on a neonatal unit. The trial interventions complete prior to hospital discharge, although follow-up for SIFT continues until 2 years corrected age. SIFT recruited 2,804 infants and is powered to detect a clinically important difference in disability-free survival at 2 years, as well as having adequate power to detect a difference in the rate of the key short-term outcomes NEC and LOS. Infants were enrolled when stable, tolerating some milk (but less than 30 ml/kg/day), and when the attending clinician was ready to start increasing the amount of milk feeds. Infants were randomized to increases in milk feeds of either 18 or 30 ml/kg/day. Infants from multiple pregnancies (twins, triplets, etc.) were corandomized to the same treatment arm. It was anticipated that the speed of milk feed increases would result in full feeds (defined as at least 145 ml/kg/day) being achieved about 4 days later in the slower arm. This affects the duration of central venous access and use of parenteral nutrition, although this was not mandated in the SIFT protocol and therefore is likely to affect two key risk factors for LOS. The speed of milk feed increases may also affect exposure to breast milk in the first few days of life, a further risk factor for both LOS and NEC. SIFT is a trial of comparative clinical effectiveness and recognizes the possibility that there might be competing outcomes, i.e., there may be opposing impacts on NEC and LOS between the two trial arms (15). Hence, the use of disability-free survival as an outcome that takes this possibility into account. In addition, if there are no differences in key clinical outcomes (including neurological development), then data collected for health economic analyses may determine which regime is adopted in clinical practice.

The ELFIN trial is evaluating whether supplemental bovine lactoferrin added to milk feeds affects the rate of sepsis and is powered to detect a clinically important reduction in the primary outcome (LOS) from 18 to 13% and will recruit 2,200 infants. Infants are randomized to receive either bovine lactoferrin (150 mg/kg/day) or placebo 150 mg/kg/day (sucrose) both added to milk feeds. The RCT is blinded using masked pots containing the investigational medicinal product (IMP). Unlike SIFT, infants from multiple pregnancies are randomized independently. An internal pilot phase started in June 2014 in five sites, with recruitment to the main trial commencing in July 2015. The trial is currently in progress and anticipated to complete recruitment toward the end of 2017. Further details of the trial are available (16).

Existing Mechanistic Evaluation of NEC and LOS in a Prospective Trial Setting

Despite the major contribution of NEC and LOS to neonatal mortality and serious morbidity, there are few large interventional studies in preterm neonates exploring biological mechanisms. This is partly because of the challenges faced by adequately powered interventional studies that typically require sample sizes of >1,000 infants to detect realistic effects on NEC or LOS. Undertaking large clinical studies with mechanistic evaluation in vulnerable preterm neonates presents many complexities practically, logistically, and ethically. There are particular issues with biological sampling, especially of blood from small infants, for example, a 500-g infant has only 40 ml of total circulating blood (17). NICUs are extremely busy environments and clinical needs take precedence over activities that are purely research orientated, e.g., collection of stool samples. While NEC and LOS are major causes of morbidity, they only affect a minority of infants, with an incidence of 5–10 and 20%, respectively, in the highest risk infants (<32 weeks): a typical NICU may only admit 100–150 such infants a year. Thus, collaboration between multiple NICUs is essential for performing research with sufficient statistical power. Securing ethics and R&D approvals, collecting samples and accessing freezers close to NICUs, and storing and transporting biological samples across multiple hospital sites present many logistic challenges. In addition, the timing of onset of NEC or LOS is highly variable and unpredictable meaning that considerable "over-sampling" is required to ensure appropriate informative sampling relative to disease onset.

Perhaps because of these challenging reasons, few RCTs powered to explore differences in NEC or LOS in preterm infants have involved biological sampling on a large scale. The largest ever UK probiotic trial PiPS (3) explored whether the probiotic

[3] https://npeu.ox.ac.uk/trials.

strain was detectable in the stool by qPCR at 2 weeks of age and 36 weeks corrected gestational age (while infants were still receiving the probiotic) and noted (a) in 11% of probiotic-treated infants the administered probiotic strain could not be detected in their stools and (b) 49% of those receiving placebo had the administered probiotic strain in their stools. Broader impact on the microbiome in this trial is awaited. These results differ from those in a large Australian probiotic trial in preterm infants [ProPrems (18)] where microbiome analysis was undertaken in 43 unblinded infants and showed (a) 8 who were in the active intervention group all carried probiotic strains and (b) only 3 out of 35 infants who were in the placebo group carried any of the probiotic strains in stool (19). Again further microbiomic impact of colonization status is awaited. Other trials have used standard culture to evaluate presence of probiotic strains in the stool of infants in an RCT, demonstrating significant differences in detection rates above or below 27 weeks gestation and significant detection in the "placebo" recipients (20, 21). None of these nested studies has to date published further microbiomics, metabolomics, or other data.

Existing Mechanistic Evaluative Research of Feeding Rates and Supplemental Lactoferrin

Recent Cochrane systematic reviews of trials of early enteral feeding strategies for preterm infants have explored the role of trophic feeds, timing of introduction, and rate of increase in feed volume (22, 23), but none of these studies incorporated detailed mechanistic evaluation of gut function or microbiota. This might be important because alterations of gut microbes appear to be one of the key mechanisms involved in NEC or LOS pathogenesis (12, 13, 24–28). However, whether abnormal gut microbial communities are causative, or whether they simply reflect other processes involved in disease initiation is uncertain, and so can only be adequately explored in prospective trials. In the absence of a consistent causative agent in NEC studies, recent data demonstrate a specific microbial signature of high diversity and dominance of bifidobacteria that may be protective (28).

Breast milk is a complex biological fluid and differs from artificial formula milk in many respects. Components that differ include protein quality (including human lactoferrin), peptides and free amino acids, lipids (including long-chain polyunsaturated fatty acids), carbohydrates including human milk oligosaccharides (HMOs), cells, cytokines, and growth factors (e.g., insulin-like growth factor 1, epidermal growth factor, insulin, etc.). HMOs cannot be digested by the host, but rather act as growth substrates for specific *Bifidobacterium* spp. (29, 30). In the context of the SIFT trial, delayed initiation or slower increases in milk feeds reduce exposure to breast milk, which may affect gut epithelial development, function, and the pattern of gut microbiota.

At the time of funding MAGPIE, the only existing published RCT in preterm infants ($n = 450$) using bovine lactoferrin showed reductions in the incidence of LOS for a range of bacteria, both Gram-negative and Gram-positive, as well as fungi (31). The study did not include any mechanistic evaluation using biological samples. Subsequently, other studies have confirmed a reduction in LOS (32, 33), and further analyses have suggested an effect on NEC (34, 35). Recent reviews have highlighted the potential mechanisms of action for lactoferrin (36). Lactoferrin, a member of the transferrin family, is a key component of the mammalian innate immune response (37). It is the major whey protein in human colostrum and is also present in tears and other secretions, as well as being released from secondary granules in poly-morphonuclear leukocytes (38). Concentrations in human colostrum are especially high (39). However, preterm infants ingest little milk in the first few days, and this may be further inhibited by the delayed lactogenesis frequently seen in women who deliver preterm. Lactoferrin intake in preterm infants is therefore probably far lower than in healthy term neonates.

Lactoferrin has broad microbiocidal activity by mechanisms such as cell membrane disruption, iron sequestration, inhibition of microbial adhesion to host cells, and prevention of biofilm formation (16, 31, 40, 41). Development of resistance to lactoferrin is unlikely as it would require multiple simultaneous mutations. Lactoferrin remains a potent inhibitor of viruses, bacteria, fungi, and protozoa after millions of years of mammalian evolution (38). Lactoferrin has prebiotic properties, creating an environment in the gut that might promote the growth of beneficial bacteria and reduce colonization with potentially pathogenic organisms (42). It has direct intestinal immunomodulatory and anti-inflammatory actions mediated by modulating cytokine expression, mobilizing leukocytes into the circulation, and activating T-lymphocytes (37). Lactoferrin enhances proliferation and differentiation of enterocytes, closure of enteric gap junctions, and suppresses free radical activity when iron is added to milk.

Although the structure of lactoferrin is broadly similar across mammals (43), bovine lactoferrin differs from human lactoferrin, so while there are good theoretical reasons why it might have beneficial effects, the precise mechanisms of actions in preterm infants may differ or may not be present (40, 44–46). A recombinant form of lactoferrin [talactoferrin (TLf)] was also available in the US and has been used within an RCT as prophylaxis against infection, showing a reduction in fecal staphylococci load to almost undetectable levels in infants receiving TLf, and an associated reduction in coagulase-negative staphylococci infections. The authors also demonstrated a TLf-modulated reduction in fecal Enterobacteriaceae postulating that this may be a possible mechanism for a reduction in NEC since proteobacteria have been associated with NEC development (47).

Recent Studies of NEC and LOS Using Microbiomic and Metabolite Data

Necrotizing enterocolitis and LOS are complex multifactorial diseases. In particular, a pathological finding of NEC is likely to represent the final common pathway of a wide array of pathophysiological processes (48–51). Recent studies show that NEC and LOS are associated with abnormal gut microbial patterns including lack of diversity, presence of "marker" bacteria, and alterations in bacterial community structures (24, 28, 52, 53). However, a key feature of most recent publications is the lack of a specific or consistent gut microbiomic signature between studies. Since 2011, when the use of next-generation sequencing came to the forefront of microbiome research, specific bacterial taxa have been associated with NEC onset, particularly those from

the Proteobacteria phylum such as *Enterobacter*, *Escherichia*, *Sphingomonas*, and *Klebsiella* spp., although the studies produce different findings and many have limited power due to their small size.

However, even large studies exploring NEC produce inconsistent findings. In a single NICU in Denmark $n = 163$ preterm infants were studied (21 with NEC) and 482 samples analyzed: there were no clear differences between NEC and control infants, although Gram-positive bacteria appeared more common in NEC cases using culture, a result not confirmed in molecular analyses (54). In one of the largest studies to date, Warner et al. recruited $n = 166$ infants (46 with NEC) and analyzed 3,587 stools and found increased relative abundance of Gamma-proteobacteria and reduced Negativicutes (48). Independent of specific bacteria, it has also been shown that the overall load of bacteria (52) or the presence of fungi have no clear association with NEC onset (24, 52). Thus, while the microbiome is undoubtedly important to the pathogenesis of NEC, a specific microbe is unlikely to be causative. This is in accordance with a recent microbiome and metabolome investigation by Stewart et al., where instability of the microbiome and a lack of bifidobacteria were significant risk factors for NEC and for the generation of NEC-associated metabolites (28). Indeed, metabolites associated with NEC were not correlated to any specific bacteria, but were negatively correlated with *Bifidobacterium*. To this end, several recent papers have used multi-omic technologies to go beyond "who is there" and determine the microbe–host interaction and overall functional profiles. These studies support the notion that different microbiome communities can yield more comparability at the protein and metabolite level, demonstrating the promise for mass spectrometry-based techniques (55–57).

Methodological Issues Associated with Microbiomic and Metabolite Analytical Platforms

In exploratory studies, the use of two independent small molecule (metabolite) profiling techniques such as liquid chromatography mass spectrometry (LCMS) and gas chromatography mass spectrometry (GCMS) may improve the detection of metabolites of interest. GCMS will detect volatile organic compounds (VOCs) that appear to have important consequences for preterm gut development and maturation such as short-chain fatty acids (SCFAs), and the GCMS protocol we will employ in MAGPIE has been extensively optimized to ensure appropriate capture of VOCs. Likewise, it is important to employ an LCMS method optimized for the high-throughput processing of preterm samples (28, 57, 58). While GCMS methods are facilitated by spectral reference databases for the identifications of compounds, LCMS requires standard compounds to be ran to confirm otherwise putative identifications from databases that are typically based on mass to charge. While validated tools exist for the identification of metabolites in untargeted LCMS experiments such as Mummichog (59), where appropriate and available matching to standard references to confirm identifications is important. In addition, the choices of columns (LC) and fibers (GC) can have profound effects on the metabolite profiles and the detected compounds, so it is important that the methods used are validated in these sample types. Recent validation work identifying peroxidation metabolites in urine from preterm infants provides a good example of this approach (60, 61).

Furthermore, the 16S rRNA gene sequencing has been extensively applied by several groups to preterm stool research as previously described. While this technique will only allow classification of bacterial hits to genus level, it has proven to be an effective technology for microbial ecology-based research. Tools such as Tax4Fun (62) and PICRUSt (63) also exist for predicting the bacterial metagenome based on the bacteria identified. While this inference is predicted, such tools offer an important means of investigating the functional capacity of the microbiome, which can be further linked to metabolomic data. Therefore, combining these different approaches may allow investigators to discern "who is there" and "what they are doing (microbe and host)."

However, all of the above techniques have limitations, and there are many other factors that are not explored with these methods, including genomic, epigenetic, transcriptomic, proteomic, and posttranscriptional modification of proteins. However, there is already strong data to show that the approaches we aim to use in MAGPIE will provide important data. Differences in the presence of VOCs have already been linked with the development of NEC (64) and may also relate to the emergence of LOS (65, 66). In addition, organisms causing blood culture-positive sepsis in preterm infants were frequently detected within the gut prior to LOS onset, and typically as an abundant member of the gut microbial community (24).

The MAGPIE aims to explore potential interactions between microbes and metabolites critical for development of gut immune function expanding work from our group and others (67, 68). Additional studies highlight the importance of host–microbe interactions by demonstrating the pivotal role of SCFAs and other compounds in inducing differentiation of gut regulatory T cells (69–71), a pathway of major importance in preterm infant NEC and LOS.

The MAGPIE may provide insights into the effects of early gut microbe/host interactions in establishing gut health and immune function. We will build on our existing work that has optimized techniques to understand gut inflammation in the presence of suppressed or dysregulated immune systems, e.g., inflammatory bowel disease (72). These will utilize specialized immunohistochemical analysis of resected diseased and non-affected tissue and use computer-aided learning techniques, digital quantification of bright field chromogenic staining to explore implicated causal biological pathways, especially if there are differences between trial arms in SIFT and ELFIN. We will compare diseased cases with controls either recruited to the main trials or from recent cohorts in our hospitals and examine cell surface markers and cytokines that might link microbial and metabolomic changes, as well as exploring the potential for transcriptomic analysis.

AIMS AND OBJECTIVES

The aim of the MAGPIE study is to explore differences in gut microbiota and metabolic correlates between trial intervention arms (feed rate, lactoferrin) and dynamic changes in the period preceding disease onset (NEC or LOS). We aim to determine effects in both the stool and urinary metabolome, because these

may reflect changes in either bacterial or host metabolism, or both. Specifically, we will determine changes in the bacterial community and overall metabolome profiles using both LCMS and GCMS, and using computational tools will determine correlations between the microbiome such as predicted gene orthologs and resulting metabolite compounds. Our specific aims are to test the following hypotheses:

1. Trial interventions will result in detectable differences in gut microbiota that will be directly related to metabolic function;
2. Infants who develop NEC or LOS will have differences in gut microbiota and metabolic profile in the period preceding disease onset compared to control infants;
3. There will be detectable differences in gut tissue inflammatory response between surgically resected gut tissue affected by NEC and control tissue.

We will achieve these aims by determining the following outcomes:

1. Gut microbial diversity (e.g., Shannon Diversity Index) and differences in the proportions of key bacterial taxa measured using 16S next-generation sequencing in stool samples collected after enrollment on days 1–3, 7, 10–14, and 21 (±1) days.
2. The association between gut microbiota and the stool metabolome using mixed-effect models, structural equation modeling (SEM), and ordination analyses. Stool metabolome is measured using GCMS and/or LCMS in samples collected on days 1–3, 7, 10–14, and 21 (±1) days.
3. Pattern of gut microbiota prior to the onset of NEC or LOS measured using up to seven daily stool samples in the period immediately prior to disease compared to samples from control cases who do not develop disease.
4. The gut tissue inflammatory response in surgically resected gut tissue affected by NEC and in control tissue (either non-affected tissue from the same infant or tissue from an infant requiring gut resection who does not have disease) will be determined by immunohistochemistry. This will be measured after trial completion by retrieving samples from hospital pathology archives.

Our analytical models will also explore the effects of clinical risk factors for NEC and LOS such as gestation and markers of illness severity, and the effects of exposure to interventions such as breast milk and antibiotics, and consider other key outcomes such as time to full feeds, age at discharge, and growth, e.g., predischarge weight gain.

PROCEDURES—SUMMARY

We will

1. Recruit at least 480 infants from up to 10 neonatal units in the UK enrolling infants to ELFIN.
2. Identify stored samples from infants recruited to SIFT (one site only).
3. Collect a daily stool and urine sample from MAGPIE infants until hospital discharge (average duration 40–50 days) or transfer back to local neonatal unit.
4. Retrieve any residual resected gut tissue of enrolled infants who undergo intestinal surgery.
5. Identify samples which are the most:
   a. *informative*—based on trial intervention and disease presence;
   b. *comprehensive*—consistency of daily sampling;
   c. *representative*—balanced for trial intervention and other key factors, e.g., gestation, breast milk exposure.
6. Samples to be analyzed will include
   a. *all diseased cases: infants who meet the SIFT and ELFIN internationally agreed predefined case definitions of confirmed NEC or LOS* following review at Blinded Endpoint Review Committee;
   b. *non-disease cases*: infants who do not develop NEC or LOS, selected using matching algorithms to ensure trial intervention and risk factor coverage.
7. The samples analyzed will focus on the early postnatal period when trial intervention differences will be greatest: e.g., day 0—3, 6–8, 9–11, 13–15, and 20–22 in ~25–50 infants per trial intervention arm. While we aim for daily sampling we recognize this will not always be achieved due to the complexity of the NICU working environment, combined with the fact that many sick infants do not pass a stool every day. We will also analyze samples at additional time points as necessary to ensure a daily sample is analyzed for up to 7 days before diagnosis in all diseased cases and match these to non-disease controls.
8. Analyze samples for
   a. *gut microbiota* using next-generation sequencing on the MiSeq (Illumina) platform to determine gut microbial patterns;
   b. *stool VOC* using "headspace" GCMS;
   c. *stool metabolomic profile* using LCMS to determine patterns in the metabolome;
   d. *urine metabolomic profile* using LCMS and assays for inflammatory proteins, e.g., intestinal fatty acid-binding protein (iFABP) where sufficient samples and informative cases exist.
9. Determine changes due to trial interventions and changes preceding disease onset. We will explore dynamic changes in the gut community structure: proportions of key bacterial operational taxonomy units, presence of specific pathogenic strains, diversity, richness, and stability of communities.
10. Analyze resected gut tissue using optimized immunohistochemistry to determine gut immune response where NEC develops. If sufficient samples exist, explore how trial interventions (feed rate, lactoferrin) and changes in gut microbiota or metabolome may relate to histological findings.
11. Store residual samples in a Human Tissue Act (UK) and research ethics approved Newcastle University Biomedicine Biobank (the "Great North Neonatal Biobank") for use in future studies.[4] HTA license no. 12534, Ethics approval 15/NE/0334, IRAS 161883.

[4]http://www.ncl.ac.uk/nbb/.

Standard Operating Procedures (SOPs)—Sample Collection, Storage, and Transport

Samples will be collected, anonymized, and analyzed according to established SOPs already developed by members of the project team. In brief, stool samples are collected from the nappy/diaper using a clean disposable plastic spoon and placed in a glass pot with a lid. Samples will be collected at routine clinical nursing care times. Urine samples are collected according to standard NICU procedure, which typically involves collecting urine passed spontaneously onto sterile cotton wool ball, squeezed out using a sterile syringe, and aliquoted into two 2 ml cryovials. Samples are labeled and placed in a −20°C freezer (generally immediately or within an hour of collection) located on the NICU. Samples are then transferred frozen in batches from local hospitals to central laboratories where they will be stored at −80°C prior to microbiomic and metabolomic analyses. Transport of samples will take place every 6–8 weeks meaning the duration of local storage at −20°C is around 1–8 weeks, on average 3–4 weeks. We will record sample storage duration at −20 and −80°C and conduct analysis throughout the study period in a chronological fashion so that we can adjust, if required, for any confounding introduced by varying storage durations.

Short-term storage of stool samples does not significantly affect the microbial communities, although measurement of storage time of samples is important to avoid potential bias (73–75). We aim to account for the amount of time in storage for DNA extractions to minimize bias. To determine any storage effects, we will conduct quality control by comparing gold standard immediate DNA extracts from <10 samples to the same number following storage of stool for 12 and 18–24 months.

Any gut tissue resected during surgery for either NEC or other conditions (e.g., spontaneous intestinal perforation) will be retrieved from paraffin blocks located in NHS pathology archives after all clinical tests have been complete. Samples will be transported to the central laboratory for immunohistochemical analyses. It is possible that only 10–12 infants undergo surgery for NEC (or other conditions) and have residual tissue available. We will therefore consider the use of control tissue from other preterm infants available locally within one hospital (Newcastle Hospitals NHS Foundation Trust) where we have research ethics permission to analyze historical samples.

Enrollment, Consent, and Data Collection

Parents can be approached for written informed consent at any time after enrollment to ELFIN is complete, but this will usually be within the first 3 days, and frequently will occur at the same time as consent to ELFIN. Written information will explicitly state the intention to share and use data collected for ELFIN. Research ethics approval allows the collection of stool and urine samples and storage on the NICU prior to signed consent for MAGPIE, but any such samples will be destroyed if consent is not obtained. We will use data collected for SIFT and ELFIN by electronic encrypted data transfer from the clinical trials unit (NPEU) and supplement this with additional items for MAGPIE that include specific antibiotic type, use of prophylactic antifungal and probiotic strain/brand if these are used, and date and time of stool and urine collection.

Case Definitions of Disease (NEC or LOS)

We will use the internationally accepted definitions used by SIFT and ELFIN, which are subsequently confirmed at Blinded Endpoint Review Committees conducted by at least two senior clinician investigators blinded to trial interventions at the NPEU.

Late-Onset Sepsis

Microbiological culture of potentially pathogenic bacteria (including coagulase-negative staphylococci species but excluding probable skin contaminants) or fungi from fluid sampled aseptically more than 72 h after birth from blood or CSF, or clinically suspected sepsis (meeting three objective clinical criteria) AND intention for treatment for 5 or more days with intravenous antibiotics. If the infant died, was discharged, or was transferred prior to the completion of 5 days of intravenous antibiotics, this condition would still be met if the intention was to treat for 5 or more days.

Necrotizing Enterocolitis

Necrotizing enterocolitis may be diagnosed at surgery, at postmortem or clinically and radiologically: at least one of the following clinical signs present: bilious gastric aspirate or emesis, abdominal distension, or occult or gross blood in stool AND at least one of the following radiological features: pneumatosis intestinalis, hepatobiliary gas, or pneumoperitoneum. Infants who satisfy the definition of NEC but at surgery or postmortem have a "focal gastrointestinal perforation" will not be coded as having NEC.

All diseased cases meeting the predefined case definition of NEC or LOS will have samples analyzed (expected total ~70–100 infants). Samples from up to 200 non-diseased cases will be selected to ensure sufficient coverage of intervention arms using matching algorithms and coverage of other clinical risk factors and outcomes. We will also analyze samples from non-diseased cases to determine microbiomic and metabolomic profiles between trials arms.

Laboratory Procedures—Stool and Urine

We will analyze bacterial DNA extracted from stool samples using our well-established protocols and 16S ribosomal RNA methods that are effective tools to explore the diversity of bacterial communities (24, 76, 77). We will conduct metabolomic profiling of stool and urine. Extraction of samples will be optimized for detection of SCFAs and samples processed using non-targeted and targeted high-resolution LCMS to generate metabolomic profiles that may indicate functional changes in the host and the gut microbiota. Our targeted approach will investigate known gut flora fermented products of complex carbohydrates, including SCFAs, acetates, amino acids, and carbohydrate fragments, which may be present in stool samples. Analysis of the stool metabolome reflects changes in gut microbial activity and may impact on host gut function such as changes in permeability. Our non-targeted approach will allow us to compare metabolomic differences between trial arms in order to define a metabolite

pattern associated with sample groups that can be correlated with information on microbial diversity, health, or disease.

We have optimized a LCMS method based on C18 reverse phase chromatography coupled to a Q-Exactive high-resolution mass spectrometer validated on 100 mg of stool and has demonstrated the robust and reproducible detection of 10^3 metabolites (28, 58). Identification of significant metabolites will be based on data-dependent tandem MS/MS and confirmed using standards. Analysis (see Statistical Analysis) will use modeling techniques to explore relationships between the microbiome and metabolome, and study interventions and disease. Examination of the urinary metabolome is more reflective of changes to the overall host (infant) metabolic state but may also reflect differences in absorption of compounds from the gut. Determining the metabolomic profiles between and within patients to complement the stool microbiome sequencing data will enable exploration of how the host, gut microbes, trial interventions, and other clinical factors may interact, and any downstream functional effects such as feed tolerance or growth. To supplement the metabolomic data, we will store samples so future studies can analyze urine and stool samples using assays for proteins such as calprotectin and iFABP that may provide additional diagnostic or mechanistic information (78–81).

Volatile organic compounds from stool samples will be analyzed by GCMS using well-established protocols for extracting and analyzing headspace gases. These methods are based on a CARB/PDMS SPME fiber, a Combipal sampler with a Peltier-cooler, and a Perkin Elmer Clarus 600 Gas Chromatograph with Clarus 600T Mass Spectrometer (82). The method is validated on as little as 50 mg of sample: sufficient to analyze ~40 compounds, which includes 8 different acids, particularly SCFAs, branched and linear, alcohols, and esters. Interpretation of fragment patterns will be undertaken against the current mass spectral NIST library, followed by manual visual inspection (82).

Laboratory Procedures—Resected Gut Tissue

The MAGPIE study provides a unique opportunity to explore potential gut actions of feeding rate and lactoferrin in a small number of cases if NEC occurs. This may include immunomodulatory and anti-inflammatory actions mediated by modulating cytokine expression, mobilizing leukocytes into the circulation, and activating T-lymphocytes. Breast milk and lactoferrin enhance proliferation and differentiation of enterocytes and closure of enteric gap junctions (36). Tissue-based analyses will take place in two domains: (1) exploring the aberrant innate and adaptive immune mechanisms and (2) validating immune pathways or biomarkers identified by microbiomic or metabolomic profiling.

Immunohistochemistry will be performed using paraffin blocks cut into 4 μm sections for staining using a Discovery XT auto-stainer (Ventana Medical Systems, Inc., Tucson, AZ, USA). Slide images will be acquired using the Vectra 3.0 Automated Quantitative Pathology Imaging System (Perkin Elmer, Waltham, MA, USA), using techniques optimized by our group at Newcastle. Antigens of interest will be quantified digitally. Leukocyte infiltrates associated with NEC will be identified using antibodies to several epitopes, for example, markers of inflammatory cell subsets, cellular proliferation, cytotoxic granule expression, transcription factors, or cytokines. Many of these antibodies have been previously optimized for intestinal tissue by the team at Newcastle, and new antibodies will be optimized as required informed by data generated in other work strands of this project. Staining of "healthy" resection margins from the same patient will be undertaken where possible and control analyses will be performed by use of matched non-NEC control tissue collected from MAGPIE infants, supplemented if needed by further samples from the Newcastle upon Tyne Hospitals biorepository (e.g., cases of spontaneous neonatal perforation). Targeted exploratory histology will validate immune mechanisms highlighted by metabolomics or microbial assays, and *in situ* hybridization may also be performed to identify transcription of relevant immune pathways identified by VOC, microbiomic, or metabolomic profiling.

Sample Size Estimates

Sample size requirements for each element of this study have been evaluated and are based on both published data and practical/logistical aspects from our existing work and that from other published studies. These considerations include

1. The duration, estimated start time, and recruitment rate for ELFIN, along with the estimated start date for MAGPIE and logistical issues in establishing recruitment from 10 hospital sites.
2. The trial efficacy and disease event rate will not be known until the main trial completes. The incidence of NEC (Bell stage 2 or greater) is expected to be 5–10% and for LOS <20%, and some infants will have both diseases. Recruiting 480 infants will identify between 70 and 100 "disease" cases, 10–20 cases requiring gut surgery, and provide well sampled "non-diseased" infants with varying clinical risk factors, exposures, and outcomes.
3. VOC analysis may identify several individual compounds; in our recent paper publishing the methodology, we show that on average 31.3 ± 10.5 (mean and SD) VOCs were identified per sample (82). At a power of 80%, and two-sided significance at the 5% level, we would need 50 infants per trial intervention group to show an increase in 5 VOCs. The SD for each sample was 2.9 ± 1.3 compounds and on average 90% of the VOC abundances showed a coefficient of variation smaller than 30%. Our data also showed in a study of 13 infants, which VOC number in healthy neonates significantly increased with age (0.49 extra VOCs per day 95% CI 0.12–0.86), a trend not seen in those who developed NEC (64).
4. Microbiomic data complexities means that the sample size necessary to evaluate the actions of different interventions and the incidence of disease is dependent on effect size, the number of interacting factors, and their correlation. For a power of 80% to detect a 50% difference in community profile patterns arising from a categorical descriptor of microbial community variation, using a two-sided test at a significance level of 5%, the study needs ~200 samples. In our previous studies (1) 12 twin pairs analyzed (gut microbial profiling) using PLS-DA showed highly significant correlations between pairs (53) and (2) examination of 136 samples from 32 patients ($n = 20$ NEC

or LOS) showed significant differences ($p = 0.002$) in microbiomic patterns between diseased and healthy individuals (24).

5. Using immunohistochemical data from our group (83), and assuming that any differences between disease and control will be greater than those in healthy individuals, and using a two-sample *t*-test, our proposed sample size of 20 would give 80% power to detect a difference of 0.66 (66%) in cells/crypt cross section at a significance level of 5% and an approximated SD of 0.5 (50%).
6. Mixed-effects models, which we propose for modeling the direct effects of the microbiome on disease risk, are economical with power because the residual variance of these models is smaller since some of it is accounted for in the random effect. Using the method by Cohen (84), we calculate that a GLM would have a power of 82% with 10 predictors and 50 for a "large" effect size ($f^2 = 0.35$, $R^2 = 0.51$) at the 5% level of significance, and a power of 47% for a "medium" effect size ($f^2 = 0.15$, $R^2 = 0.36$) at the 5% level of significance. With $n = 100$ the calculated powers are 99% for a large effect size and 74% for a medium effect size. Including random effects in the GLM would increase power at the same effect size or conversely permit smaller effect sizes at the same power. The power analysis of an SEM is altogether more complex because it relies on the goodness-of-fit criteria selected for the model. Power at the model level may be low when there are few model degrees of freedom even for a reasonably large sample size; requiring greater than 100 samples if there are fewer than 20 model degrees of freedom (85). We will supplement these models where needed using a Bayesian SEM approach.

Statistical Analysis

There are three major modeling issues that have to be addressed in this analytical pathway:

(i) the multivariate nature of the microbiome and metabolome, and data generated from immunohistochemistry;
(ii) the longitudinal/developmental component in the neonate, which will lead to repeated measures of disease state and microbiome on the same individuals, and
(iii) the interdependence and interactions between different predictors, which may have both direct and indirect effects on incidence and progression of disease.

The analytical approach used in this study will focus on modeling the relationship between putative risk factors, trial interventions, and incidence of NEC and LOS from longitudinal data capturing variation in both risk factors and disease through time, and the any differences in non-diseased cases between trial arms. We will use a progressive modeling strategy based on combining multivariate analyses of micro- and metabolomic immunological data, with mixed-effect modeling of outcome in relation to drivers of disease; and finally, mixed-effect SEM to quantify the importance of interacting factors and drivers. Specifically, we will

(i) quantify the impacts of individual risk factors in causing disease;
(ii) quantify the effects of direct and indirect risk factors on disease;
(iii) quantify the dynamics of pathogens, the neonatal microbiome, the immune response, and their impact on outcomes, and
(iv) identify the impacts of trial interventions and clinical management on the drivers and disease.

ANTICIPATED RESULTS FROM ANALYSIS OF MICROBIOME AND METABOLOME

We will use multivariate ordination techniques to summarize and visualize the major trends in variation in microbial community composition of infants' stool collected. The analyses will identify those taxa most closely associated with microbiome change through time. We will use canonical ordination to quantify the impacts of other covariates (diet, age, and interventions) on the microbiome composition. We will identify key microbial taxa in the ordination space that capture the trend in variation in relation to disease and use these with the results of the ordination as input variables for the subsequent mixed-effect and SEM analyses below. To ensure the results are not spurious, the observed differences in metabolomic profiles will be validated by permutation testing in MetaboAnalyst 3.0 (86). Where appropriate, significance of categorical variables were determined using the non-parametric Mann–Whitney test for two category comparisons or the Kruskal–Wallis test when comparing three or more categories (87) and all *p*-values were adjusted for multiple comparisons with the false discovery rate algorithm (88).

MixOmics (89) will be implemented to determine the correlation between the relative abundance of the dominant bacterial taxa from 16S rRNA gene sequencing and the intensity of metabolites of interest by sparse partial least squares regression (90). Longitudinal and network analyses will facilitate investigations into the directionality of any observations. Tax4Fun (62) will be used to infer the metabolic potential of the microbiome based on the 16S data. Model-based integration of metabolite observations and species abundances will then be applied to determine biologically feasible correlations between the inferred bacterial KEGG orthology and the resulting metabolites of interest (91). This will determine which metabolites are likely to be bacterial derived. It is important to note that in the course of data generation, new tools are likely to emerge that can perform distinct analyses on multi-omic data. Where appropriate we will incorporate such tools where they are likely to (1) outperform existing tools and/or (2) allow novel analyses to be performed.

Quantifying the Direct and Indirect Impact of Risk Factors on Disease

We will use repeated measure mixed-effect modeling to quantify the direct effects of microbiome on risk of disease while adjusting for clinical factors. We will use case as the random effect and adjust for autocorrelation in the response using appropriate correlation structures in the model. The applicants have used this approach extensively to investigate the epidemiology of food-borne pathogens (92). We will use an SEM approach to quantify direct and

indirect effects of risk factors on the incidence of disease. We will develop a set of models that characterize the relationship between clinical factors, the changes in gut microbial community types, developmental stage (postnatal and postmenstrual age), and comorbidities associated with disease onset and then challenge the model with the data derived from the laboratory and clinical data collection (93).

Plan for Interim Statistical Analyses

The MAGPIE study funder (NIHR) and the project team agreed that it would be appropriate to conduct a futility-type analysis to ensure there are measurable impacts on the microbiome after ~150 (of the total planned 480) infants have been recruited, and prior to commencing further analysis of the microbiome and metabolome. The ELFIN study is blinded so any laboratory and statistical analysis of anonymized samples while recruitment continues will be conducted by investigators blinded to trial interventions. Blinding to trial intervention arm will also be maintained while gut tissue is examined.

For the interim analyses we will

1. Select samples taken before disease onset at around 7–10 days after commencing the trial IMP (lactoferrin or placebo) in up to 60 infants per trial group so as to compare and assess microbial community changes between trial groups. Because other risk factors are also associated with disease (e.g., gestation, postnatal age, etc.), we will use progressive model building that investigates microbial community dynamics in relation to these covariates.
2. Use multivariate ordination and classification approaches to quantify trial intervention group differences in gut microbial communities. This will (i) quantify the variation in microbial community composition before disease onset and (ii) develop suitable covariates describing microbial community variation for inclusion in statistical analysis as risk factors for disease.
3. Use unconstrained ordination to investigate trends in community composition and to identify those microbial taxa from the NGS data that contribute most to community variation. These axes may be used as covariates in the subsequent analysis.
4. Use constrained ordination to investigate the impact of trial intervention group on community variation prior to disease onset.
5. Use divisive classification approaches to identify classes of microbial community among the cases. This will create a suite of categorical descriptors of community composition. Higher level community descriptors (diversity, evenness, rate of change in ordination score) will be derived in case the overall structure of the community is an important driver.
6. In the interim analyses, we will determine whether there is any effect on the total microbial community differences or on individual bacterial taxa using MANOVA and consider a difference in the microbial community of $p < 0.05$ to be proof an effect. If the p value is between 0.05 and 0.1, we will consider this to be strong evidence of a likely effect, where statistical significance might subsequently be achieved in an analysis with larger numbers and/or greater numbers of diseased or high risk cases and will complete the study as planned. Regression models and SEM will adjust for potential confounding variables such as age, sex, gestation, and feed and antibiotic exposures.

SUMMARY

The MAGPIE study provides a unique opportunity to explore the interaction between nutritional and feeding interventions on gut microbiota in early life in preterm infants, and within the context of large randomized trials. Next-generation sequencing and metabolomic techniques produce large datasets that require complex modeling in order to present findings of clinical relevance. While these new "omic" techniques have provided unique insights into complex multifactorial diseases such as NEC, current studies are limited by their observational nature and are at significant risk from type I errors due to residual confounding and/or reverse causation. Prospective trials are needed to determine direct causation due to clinical interventions such as milk feed rate or enteral lactoferrin supplementation. Nesting mechanistic evaluation within these trials using non-invasive and safe sampling increases the value of large RCTs. This may produce important new data of diagnostic and therapeutic relevance or identify differences in responses between groups of infants (i.e., exploring in what setting interventions may be most or least efficacious), as well as potentially providing information about risk stratification for future studies. NEC and LOS remain extremely prevalent and serious diseases in preterm infants and both are associated with considerable mortality and morbidity, as well as generating significant health-care resource costs. Studies such as MAGPIE will improve our understanding of the basic biology of health and disease and the role of early life gut microbiota colonization in a high risk group of patients where mechanistic understanding is limited.

ETHICS STATEMENT

This study received research ethics approval from East Midlands—Nottingham 2 Research Ethics Committee 16/EM/0042.

AUTHOR CONTRIBUTIONS

NE, JB, JD, AE, EJ, JK, CAL, CVL, WM, CP, SR, MS, CS, and SC all made substantial contributions to the study design, drafted and/or revised this manuscript, and approved it for publication. All authors agreed to be responsible for its integrity.

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Colonization and Succession within the Human Gut Microbiome by Archaea, Bacteria, and Microeukaryotes during the First Year of Life

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Perturbations to the colonization process of the human gastrointestinal tract have been suggested to result in adverse health effects later in life. Although much research has been performed on bacterial colonization and succession, much less is known about the other two domains of life, archaea, and eukaryotes. Here we describe colonization and succession by bacteria, archaea and microeukaryotes during the first year of life (samples collected around days 1, 3, 5, 28, 150, and 365) within the gastrointestinal tract of infants delivered either vaginally or by cesarean section and using a combination of quantitative real-time PCR as well as 16S and 18S rRNA gene amplicon sequencing. Sequences from organisms belonging to all three domains of life were detectable in all of the collected meconium samples. The microeukaryotic community composition fluctuated strongly over time and early diversification was delayed in infants receiving formula milk. Cesarean section-delivered (CSD) infants experienced a delay in colonization and succession, which was observed for all three domains of life. Shifts in prokaryotic succession in CSD infants compared to vaginally delivered (VD) infants were apparent as early as days 3 and 5, which were characterized by increased relative abundances of the genera *Streptococcus* and *Staphylococcus*, and a decrease in relative abundance for the genera *Bifidobacterium* and *Bacteroides*. Generally, a depletion in Bacteroidetes was detected as early as day 5 *postpartum* in CSD infants, causing a significantly increased Firmicutes/Bacteroidetes ratio between days 5 and 150 when compared to VD infants. Although the delivery mode appeared to have the strongest influence on differences between the infants, other factors such as a younger gestational age or maternal antibiotics intake likely contributed to the observed patterns as well. Our findings complement previous observations of a delay in colonization and succession of CSD

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infants, which affects not only bacteria but also archaea and microeukaryotes. This further highlights the need for resolving bacterial, archaeal, and microeukaryotic dynamics in future longitudinal studies of microbial colonization and succession within the neonatal gastrointestinal tract.

Keywords: fungi, succession, delivery mode, infant gut microbiome, amplicon sequencing, microbial colonization, quantitative real-time PCR

INTRODUCTION

The human microbiome contributes essential functionalities to human physiology and is thought to play a crucial role in governing human health and disease (Greenhalgh et al., 2016). A growing body of evidence suggests that chronic diseases such as allergies (Abrahamsson et al., 2012, 2014), type 2 diabetes (Delzenne et al., 2015), obesity (Turnbaugh et al., 2006), and metabolic syndrome (Vrieze et al., 2012) are associated with a disequilibrium in the microbiome of the human gastrointestinal tract (GIT).

The initial microbiome colonization process is crucial for the development and maturation of the GIT as well as the immune system of the developing infant (Björkstén, 2004; Caicedo et al., 2005; Rautava and Walker, 2007; Eberl and Lochner, 2009; Houghteling and Walker, 2015). During vaginal delivery, a subset of the maternal bacterial community is supposedly transferred to the infant; in contrast, early-stage microbiome profiles from infants delivered by cesarean section (C-section) are typically not as reflective of the mothers' vaginal or gastrointestinal environment (Dominguez-Bello et al., 2010; Bäckhed et al., 2015; Nayfach et al., 2016). Based on spatio-temporal studies in humans (Abrahamsson et al., 2014), it has been suggested that various disturbances in the initial microbiome colonization process as early as 1 month after birth may increase chronic disease susceptibilities over the course of human life (Arrieta et al., 2014; Cox et al., 2014; Houghteling and Walker, 2015). It has been previously observed that the delivery mode is the most important factor in determining the early colonization pattern(s) (Biasucci et al., 2008; Dominguez-Bello et al., 2010; Jakobsson et al., 2014; Rutayisire et al., 2016), although other factors, such as diet (breast milk vs. formula milk; Le Huërou-Luron et al., 2010), gestational age (term delivery vs. preterm delivery; Barrett et al., 2013), or the maternal intake of antibiotics (Sekirov et al., 2008) have also been observed to have effects on this process.

Even though the colonization and succession within the GIT have been studied extensively, the focus has mostly been directed to the bacterial domain. However, such a constrained view may lead to an underestimation of the contribution of the archaeal and eukaryotic domains, in particular microeukaryotes, such as unicellular parasites or yeasts, and could ultimately lead to incomplete conclusions (Horz, 2015).

Within the archaeal domain, methanogenic archaea (mainly those belonging to the order Methanobacteriales) have been estimated to comprise between 10^8 and 10^{10} cells per gram dry weight of stool (Miller and Wolin, 1986) and are considered almost ubiquitous inhabitants of the intestinal microbiome with a presence in up to 95.7% of all adult humans (Dridi et al., 2009). Methanogenic archaea are functionally important due to their ability to consume molecular hydrogen, which is both an end product and a concentration-dependent inhibitor of bacterial fermentation (Thauer et al., 2008). Consequently, methanogens drive the effective degradation of organic substances and play an important role in interspecies hydrogen transfer through maintaining syntrophic relationships with bacterial populations (Hansen et al., 2011). Additionally, gut methanogens have been linked to energy metabolism and adipose tissue deposition of the human host (Samuel et al., 2007), and the ability of certain archaea to produce methane may play a role in the pathogenesis of several intestinal disorders (Roccarina et al., 2010). Despite these observations, the simultaneous presence of archaea and bacteria has been ignored in the majority of studies on the gastrointestinal microbiome to date and details about neonatal colonization by archaea remain limited. Previous studies have detected archaea transiently and almost exclusively in the first few weeks of life, and considerably less in samples collected after the fifth week of life (Palmer et al., 2007). Archaea have been sporadically detected in the vaginal environment before, although exclusively in women with bacterial vaginosis (Belay et al., 1990). As archaea are mainly inhabitants of the human GIT, but also colonize the skin surface (Probst et al., 2013) as well as the oral cavity (Nguyen-Hieu et al., 2013), a transfer from mother to infant by fecal-oral or oral-oral route seems thereby most probable.

Eukaryotes and microeukaryotes, which form part of the human microbiota, have been shown to exert immunomodulatory effects on the host (Weinstock, 2012; Rizzetto et al., 2014). Furthermore, infections by parasitic eukaryotes have been linked to decreased allergic and autoimmune disease prevalence (Weinstock, 2012) and have been used for therapeutic interventions in that context (McFarland and Bernasconi, 1993; Williamson et al., 2016). However, the role of microeukaryotes within the human GIT microbiome and the resulting impact on the human host remain so far unresolved (Andersen et al., 2013). It has been previously reported that the overall microeukaryotic diversity of the adult human GIT is low but largely temporally stable (Scanlan and

Abbreviations: BMI, body mass index; Cp, Crossing point; CSD, cesarean section delivery; C-section, cesarean section; FNR, Luxembourg National Research Fund; CART-GIGA, Center of Analytical Research and Technology—Groupe Interdisciplinaire de Génoprotéomique Appliquée; GIT, gastrointestinal tract; IBBL, Integrated BioBank of Luxembourg; ISBER, International Society for Biological and Environmental Repositories; OTU, operational taxonomic units; pam, partitioning around medoids; PCoA, Principal Coordinate Analysis; qPCR, quantitative real-time PCR; RDP, Ribosomal Database Project; VD, vaginally delivered.

Marchesi, 2008), whereas other research suggested that the adult GIT microbiome harbors a complex microeukaryotic community with the most abundant taxa by far being fungi (Hamad et al., 2012). To date, a single study followed the initial colonization of the GIT by microeukaryotes using 18S rRNA gene amplicon sequencing in four newborn infants (Pandey et al., 2012), but failed to detect any microeukaryotes at the timepoints analyzed. However, this study might have been substantially limited by its sample collection as well as the applied sequencing technique.

In our present work, a longitudinal study was conducted to describe the colonization and succession of the three domains of life within the GIT of newborns. More specifically, we investigated the microbiome changes during the first year of life among eight vaginally delivered (VD) infants and seven infants delivered by C-section (CSD). The latter are statistically at a higher risk of developing metabolic disease such as obesity (Mueller et al., 2015) and/or related diseases like type 2 diabetes (Nguyen and El-Serag, 2010), as well as allergic diseases such as atopic eczema (Abrahamsson et al., 2012) and asthma (Abrahamsson et al., 2014) in childhood and/or adulthood. Fecal samples were collected from all infants (VD and CSD) at six time points between day 1 and 1 year *postpartum* and, using quantitative real-time PCR (qPCR), we determined the sizes of prokaryotic (bacteria and archaea) and fungal populations, the relative quantities of archaea and validated the amounts of four selected bacterial genera and two phyla in the collected samples. Additionally, targeted high-throughput 16S and 18S rRNA gene amplicon sequencing was conducted on the isolated DNA. After processing and filtering of the resulting data, we compared the prokaryotic and microeukaryotic community structures in relation to the delivery mode and a multitude of other recorded maternal/neonatal characteristics. The resulting data provides a detailed overview of the neonatal colonization and succession patterns of members of all three domains of life.

MATERIALS AND METHODS

Sample Collection, Processing, and Biomolecular Extraction

Study Context

In the context of the national COSMIC study, pregnant women were recruited in Luxembourg starting in 2012. The 15 pregnant women included in the presented study were aged between 24 and 42 years and gave birth in the maternity department of the Centre Hospitalier de Luxembourg (CHL). This study was carried out in accordance with the recommendations of good clinical practices established by the "International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use" with written informed consent from all subjects in accordance with the Declaration of Helsinki. The protocol and informed consent form was approved by the Luxembourg "Comité National d'Ethique de Recherche" in 2011 (reference number 201110/06).

Sample and Data Collection

To mitigate pre-analytical confounders, fecal samples were immediately snap-frozen in liquid nitrogen or placed on dry ice following collection and were stored at −80°C until further processing. Fecal samples were scheduled to be collected at day 1, 3, 5, 28, 150, and 365. The medical histories of both parents and medication intake of the mother were recorded, as well as weight, date of birth, gender, mode of delivery, and gestational age of the infant. Additional data, which was collected subsequently for all infants included weight, type of milk fed, medication intake including antibiotics and time point at which solid food was introduced. If an infant received formula at a specific point in time, it was considered as receiving combined feeding for the entire remainder of the study, as even short-term formula-feeding has been shown to cause profound and long lasting shifts to the gastrointestinal microbiome composition (Guaraldi and Salvatori, 2012). Hospitalization in the neonatal care unit and administration of antibiotics to infants immediately *postpartum* as well as birth prior to 34 weeks of gestation were exclusion criteria. Samples and associated data were collected and stored at the Integrated BioBank of Luxembourg (IBBL) following ISO17025:2005 standards and the International Society for Biological and Environmental Repositories (ISBER) best practices.

DNA Extraction from Fecal Samples

Pre-processing of all fecal samples (150–200 mg of weighed material) was carried out according to Shah et al. (in press; subsection 3.2, steps 1–4). After high-speed centrifugation, DNA was extracted from the resulting interphase pellet using the PowerSoil® DNA isolation kit (MOBIO Laboratories, Belgium). The method was optimized for mechanical disruption with bead-beating to ensure a realistic representation of microbial communities (Walker et al., 2015). DNA quality and quantity were determined on 1% agarose gels, by NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA) and Qubit 2.0 fluorometer (Thermo Fisher Scientific, USA). The extracted DNA was stored at −80°C until qPCR validation and sequencing library construction.

DNA Analyses and Sequencing

Quantitative Real-Time PCR

Extracted DNA was diluted, when applicable, to a concentration of 5 ng/μl and amplified in duplicates, using previously published primers targeting prokaryotes, archaea, or specific fungi as well as specific bacterial genera and phyla (**Table 1**), which were ordered and received from Eurogentec (Belgium). The reaction mixture contained 1 μl template DNA, 5 μl of Mastermix (iQ SYBR Green Supermix; Bio-Rad Laboratories, USA), and 500 nMol of each primer, in a final reaction volume of 10 μl. Genomic DNA isolated from *Salmonella* Typhimurium LT2 and *Saccharomyces cerevisiae* BY4743 was used to prepare standard curves for the universal prokaryotic and fungal primers, respectively. A sample pool, comprised of 1 μl of undiluted DNA from each of the 65 samples, was used to prepare standard curves for all assays. All standard curves were prepared with a total of at least five successive 10-fold dilutions. qPCR was performed on a LightCycler 480 (Roche Diagnostics, Germany) with an initial denaturation step of 1 min at 95°C followed by primer-specific cycling times (**Table 1**), a single fluorescence acquisition step at the end of each extension step and a final melting curve. Crossing

TABLE 1 | Primer pairs and conditions of quantitative real-time PCR.

Main target (target gene)	Designation	Oligonucleotide sequence (5′-> 3′)	Annealing temperature (°C)	Cycling	References
Fungi (18S rRNA)	Fungi2F	F: ATT-GGA-GGG-CAA-GTC-TGG-TG	55	60 cycles: 15 s at 95°C, 10 s at 55°C, 25 s at 72°C	Einsele et al., 1997
	Fungi2R	R: CCG-ATC-CCT-AGT-CGG-CAT-AG			
Staphylococcus (tuf)	TStaG422-F	F: GGC-CGT-GTT-GAA-CGT-GGT-CAA-ATC-A	55	45 cycles: 20 s at 95°C, 30 s at 55°C, 1 min at 72°C	Martineau et al., 2001
	TStag765-R	R: TAT-HAC-CAT-TTC-AGT-ACC-TTC-TGG-TAA			
Haemophilus (P6)	HI-IV	F: ACT-TTT-GGC-GGT-TAC-TCT-GT	55		van Ketel et al., 1990
	HI-V	R: TGT-GCC-TAA-TTT-ACC-AGC-AT			
Universal archaea (16S rRNA)	ARC787F	F: ATT-AGA-TAC-CCS-BGT-AGT-CC	60		Yu et al., 2005
	ARC1059R	R: GCC-ATG-CAC-CWC-CTC-T			
Lactobacillus (16S rRNA)	Lac774F	F: GCG-GTG-AAA-TTC-CAA-ACG	60	45 cycles: 15 s at 95°C, 30 s at 60°C, 1 min at 72°C	Hermann-Bank et al., 2013
	Lac989R	R: GGG-ACC-TTA-ACT-GGT-GAT			
Streptococcus (16S rRNA)	Strep488F	F: CTW-ACC-AGA-AAG-GGA-CGG-CT	60		Hermann-Bank et al., 2013
	Strep824R	R: AAG-GRY-CYA-ACA-CCT-AGC			
Firmicutes (16S rRNA)	Lgc353	F: GCA-GTA-GGG-AAT-CTT-CCG	60		Fierer et al., 2005
	Eub518	R: ATT-ACC-GCG-GCT-GCT-GG			
Bacteroidetes (16S rRNA)	798cfbF	F: CRA-ACA-GGA-TTA-GAT-ACC-CT	61	45 cycles: 15 s at 95°C, 20 s at 61°C, 30 s at 72°C	Bacchetti De Gregoris et al., 2011
	cfb967R	R: GGT-AAG-GTT-CCT-CGC-GTA-T			
Universal prokaryotes (16S rRNA)	926F	F: AAA-CTC-AAA-KGA-ATT-GAC-GG	61		Bacchetti De Gregoris et al., 2011
	1062R	R: CTC-ACR-RCA-CGA-GCT-GAC			

point (Cp) values were calculated using the second derivative method within the Roche LightCycler 480 software version 1.5. Absolute copy numbers of prokaryotic 16S and fungal 18S rRNA genes were calculated using the Cp values and the reaction efficiencies based on the standard curves obtained from defined DNA samples and extractions yields were estimated from these numbers. Relative concentrations of specific taxa compared to all 16S rRNA genes were calculated using Cp values and the standard curves obtained for the sample pool. Only samples where the target was positively detected in both duplicate reactions were considered for further analyses.

16S/18S rRNA Gene Amplicon Sequencing

Specific sets of primers targeting 16S and 18S rRNA genes were chosen for the amplification and subsequent sequencing to broadly cover bacterial, archaeal and eukaryotic diversity. The bacterial and archaeal community structures of the 65 samples were resolved by amplifying the V4 region of the 16S rRNA gene using the universal primers 515F and 805R (515F_GTGBCAGCMGCCGCGGTAA; 805R_GACTAC HVGGGTATCTAATCC; Herlemann et al., 2011; Hugerth et al., 2014a). This primer pair covers the bacterial domain, including the phylum Actinobacteria and additionally resolves the archaeal domain.

The eukaryotic community structures for 63 samples were analyzed by amplifying the V4 region of the 18S rRNA gene using primers 574*F and 1132R (574*F_CGGTAAYTCCAGCTCYV; 1084r_CCGTCAATTHCTTYAART; Hugerth et al., 2014b). Two samples did not yield sufficient amplicons (CSD infant 7 collected on days 1 and 3).

The KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, USA) was used for amplification with 25 cycles and according to the service provider's standards. Paired-end sequencing with 2 × 300 nt was performed on an Illumina MiSeq platform with the V3 MiSeq kit at the Center of Analytical Research and Technology—Groupe Interdisciplinaire de Génoprotéomique Appliquée (CART-GIGA; Liège, Belgium).

16S rRNA and 18S rRNA Gene Sequencing Data Processing

The raw 16S rRNA gene amplicon sequencing data were processed using the LotuS software (version 1.35) with default parameters (Hildebrand et al., 2014). After clustering the reads into operational taxonomic units (OTUs) at 97% identity level, they were classified and taxonomically assigned using the Ribosomal Database Project (RDP) classifier version 2.10.1 (Wang et al., 2007). OTUs with a confidence level below 0.8 at the domain level were discarded. The amplicon sequences belonging to the 100 most abundant OTUs were additionally manually curated for unspecific amplification. As only few archaeal reads were detected, the overall quality of the archaeal reads were manually assessed using the FASTQC results[1]. As the paired-end 18S rRNA gene amplicon reads obtained in this study did not overlap, a specifically tailored workflow was used to process the raw 18S rRNA gene amplicon sequencing data[2]. For the classification step and the taxonomic assignment, the PR2

[1]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

[2]https://github.com/EnvGen/Tutorials/blob/master/amplicons-no_overlap.rst

database (Guillou et al., 2013) was used according to Hu et al. (2016).

16S rRNA and 18S rRNA Gene Sequencing Data Analysis

In order to exclude sequencing artifacts for both prokaryotic and eukaryotic datasets, we removed OTUs that were represented by <10 reads in all of the sequenced samples, thereby examining the dominant phylotypes for all three domains of life throughout this study. One sample was excluded from further analyses as its read count (4,141 reads) was far below the average read count of 213,469.5 ± 84,713.4 reads (average ± standard deviation) for all 16S rRNA gene sequencing datasets and was thereby yielding <5,000 16S rRNA gene amplicon reads that are necessary for assessing bacterial diversity (Lundin et al., 2012; Kozich et al., 2013; Song et al., 2013; Sebald et al., 2016; Hill-Burns et al., 2017). As the complexity of the microeukaryotic community structure is largely undetermined and no previous recommendations exist, no cutoff for the number of 18S rRNA gene amplicon reads was applied. All statistical analyses and visualizations were performed using the R statistical software package (version 3.2.0; R Development Core Team, 2008). Per-sample normalization, calculations of richness, diversity (Shannon's diversity index), evenness (Pielou's evenness index), dissimilarity index (distance to the most mature sample, calculated using Soerensen's similarity index of presence/absence of taxa at each individual time point compared to samples collected at the last individual time point) and non-parametric estimation of minimum community richness according to Chao (1984) were performed using the "vegan" package[3]. For the calculations of diversity and evenness indices for microeukaryotes, only samples with a total of more than 10 reads were considered. Differential analysis of relative OTU abundances based on read count data for the 16S rRNA gene amplicon sequencing dataset was done using the "DESeq2" package (Love et al., 2014), which allows testing for differential abundance using negative binomial generalized linear models and multiple-testing adjustment by controlling the false discovery rate (Benjamini and Hochberg, 1995). Adobe Illustrator (version 19.1.0) was used for labeling axes and creating multi-plot graphs.

Various neonatal characteristics that were previously shown to have an impact on the microbiome (e.g., delivery mode, fed milk type, gestational age, maternal antibiotic, and probiotic intake, positive screening for Group B *Streptococcus* (*Streptococcus agalactiae*) colonization of the mother) were compared between samples using the Wilcoxon rank sum test or Kruskal–Wallis test where applicable and comparisons with $P < 0.05$ were considered statistically significant. Principal coordinate analysis (PCoA) graphs were generated using the Jensen-Shannon distance as implemented in the R package "phyloseq" (McMurdie and Holmes, 2013) and clusters were defined using the partitioning around medoids (pam) function contained in the R package "cluster" (Maechler et al., 2016).

[3]https://cran.r-project.org/web/packages/vegan/index.html

Workflow for DNA Mock Extracts and Control Samples

As negative controls for the qPCR quantifications and 16S/18S rRNA amplicon sequencing, sample-free "DNA mock extracts," i.e., 2 extraction controls that underwent the same DNA extraction protocol without initial input, and a no template control, were prepared and subjected to qPCR analyses and amplicon sequencing together with the study samples.

In order to exclude any biases by low-yield samples (Salter et al., 2014; Jervis-Bardy et al., 2015), a control fecal sample from a single healthy female adult individual was collected and preserved under the same conditions as described. This control stool sample was extracted using the same DNA extraction protocol and created a dilution series ranging from 2 to 0.002 ng/μl. The four DNA dilution samples were 16S rRNA gene amplicon sequenced using the same primer pair as for the collected study samples.

RESULTS

Cohort Characteristics

Sixty-five fecal samples were collected between September 2012 and April 2014 at the CHL from eight healthy VD and seven healthy CSD infants at six time points (samples collected around days 1, 3, 5, 28, 150, and 365). The birth weights as well as the gestational ages of the infants were similar, while the ratios of genders, the maternal age and the maternal postnatal body mass index (BMI) differed between both groups,

TABLE 2 | Neonatal and maternal characteristics ($n = 15$).

	Total cohort ($n = 15$)	VD ($n = 8$)	CSD ($n = 7$)[a]
INFANT CHARACTERISTICS			
Female gender	7 (46.7%)	5 (62.5%)	2 (28.6%)
Gestational age at delivery (weeks)	38.7 ± 1.8	39 ± 1.5	38.3 ± 2.1
Birth weight (g)	3273 ± 416	3311 ± 543	3230 ± 236
MATERNAL CHARACTERISTICS			
Positive group B *Streptococcus* screening	3 (21.4%)	3 (37.5%)	0
Age	33.6 ± 4.6	32.5 ± 4.4	35 ± 4.8
Postnatal body mass index	24 ± 4.3	21.8 ± 2.7	26.8 ± 4.6
ETHNICITY			
Caucasian	12 (85.7%)	7 (87.5%)	5 (83.3%)
African	2 (14.3%)	1 (12.5%)	1 (16.7%)
Perinatal antibiotic intake[b]	11 (78.6%)	6 (75%)	5 (83.3%)
Penicillin[c]	6 (42.9%)	6 (75%)	0
Cephalosporin	4 (28.6%)	0	4 (66.7%)
Clindamycin	1 (7.1%)	0	1 (16.7%)
Probiotic use during pregnancy	2 (14.3%)	1 (12.5%)	1 (16.7%)

[a] *2 C-section infants are twins.*
[b] *Considering all antibiotics administered to the mother 12 h prior and after the delivery.*
[c] *As ampicillin belongs to the penicillin group, ampicillin and penicillin intake were both categorized as "penicillin."*
Study groups are defined according to delivery mode (VD: n = 8; CSD: n = 7). CSD, C-section delivery; VD, vaginal delivery.

with the CSD group comprising more male infants as well as mothers with a higher average age and postnatal BMI (**Table 2**). Three mothers who gave birth vaginally screened positively for Group B *Streptococcus*, whereas all mothers giving birth by C-section were screened negatively. Clinical healthcare guidelines in Luxembourg recommend that mothers who were screened positively for Group B *Streptococcus* should be treated intravenously with antibiotics prior to birth. Although mothers undergoing C-section were preferentially treated with antibiotics prior to birth, the majorities of both cohorts received antibiotic treatment (**Table 2**). Two of the three mothers who did not receive any antibiotics prior to birth chose to take probiotics during their pregnancies, whereas none of the other mothers recorded any probiotic supplementation. Out of eight VD infants, four were fed purely with maternal breast milk, while two others received formula milk and the remaining two were fed a combination of formula and breast milk. Out of the seven CSD infants, five were purely fed breast milk and the remaining two received a combination of breast milk and formula (**Supplementary File 1**, **Table S1**). According to the self-assessment of mothers that were purely breastfeeding, both the frequency and duration of feeding were not significantly different between VD and CSD infants. Introduction of solid food occurred in average around day 150 for all infants.

Assessment of Bacterial, Fungal, and Archaeal Load Using Real-Time PCR

Specific qPCR assays using previously published primers were used to obtain quantitative information on the individual taxonomic groups of interest (**Table 1**). Absolute yields of extracted DNA were quantified and prokaryotic and fungal DNA, as well as the relative quantities of archaea were calculated based on the ratio between the relative concentrations obtained for the universal prokaryotic primer pair and the relative concentrations obtained for archaea (**Figure 1**). The detection of organisms in the "DNA mock extracts" reflecting the three domains of life was negative for the archaea- and fungi-specific primer sets whereas the universal prokaryotic primer set resulted in the detection of a minimal amount of DNA close to the qPCR detection limit (average concentration of 0.002 ng/μl measured for the "DNA mock extracts" as opposed to 0.3 ng/μl measured for meconium samples, i.e., the earliest fecal material excreted by infants, which had the lowest observed concentrations amongst all study samples). Therefore, the mock extracts and subsequent analyses did not indicate the presence of reagent-derived contaminants.

The qPCR-based quantification of prokaryotic DNA was successful for 64 out of 65 samples, with yields ranging from 0.2 ± 0.4 ng of DNA per mg of stool (average ± standard deviation) in the meconium samples of day 1, to 16.6 ± 6.4 ng of DNA per mg of stool on day 365. Generally, the prokaryotic load of both cohorts increased considerably after the introduction of food. The DNA yields were dependent on the collection time point, and the greatest differences were observed between day 1 and all other collection time points (**Figure 1A**; for all significant differences between collection time points, see **Supplementary File 1**, **Table S2**). Moreover, at day 5 significantly lower extraction yields ($P = 0.03$; Wilcoxon rank sum test) were observed for samples derived from infants whose mothers received antibiotics prior to birth (**Supplementary File 1**, **Figure S1**).

The presence of archaea was detected in 91% of all samples (59 out of 65 samples) and the concentration of archaeal DNA relative to the mean of all samples ranged from 5.5 ± 7.8 on day 1 to 0.5 ± 0.4 on day 365. Generally, more samples were found to be positive in VD (97% of VD infant samples) than in CSD infants (86% of CSD infant samples) and archaeal presence was as well-detected in the samples from the very first time points (**Figure 1B**).

Presence of fungal organisms was detected in 37% (24 out of 65 samples) of all samples, ranging from 0.0007 ± 0.0005 ng of fungal DNA per mg of stool on day 3 to 0.002 ± 0.002 ng of fungal DNA per mg of stool on day 365, with generally

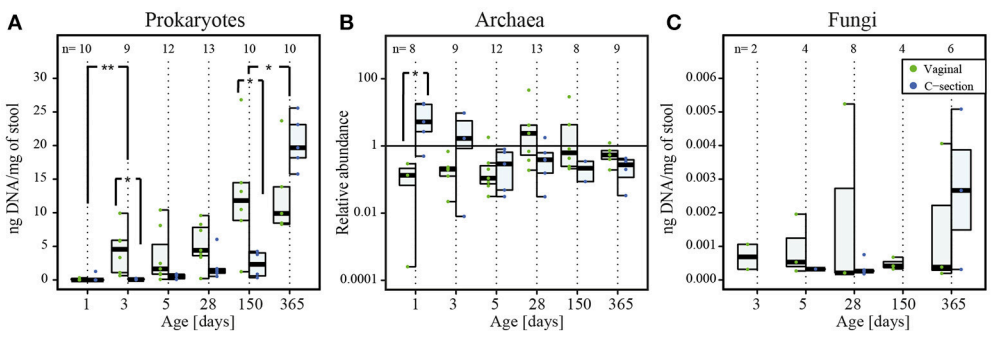

FIGURE 1 | Detection of prokaryotes, archaea and fungi in infant stool during the first year of life. (A) Absolute quantification of 16S rRNA gene copy numbers for prokaryotic DNA (ng DNA per mg of stool), **(B)** relative quantification of archaeal read counts. **(C)** Absolute quantification of 18S rRNA gene copy numbers for fungal DNA (ng DNA per mg of stool) by quantitative real-time PCR and over the course of the first year of life. The numbers of samples per collection time point are provided at the top of the graph. For the purpose of clarity, only significant differences between subsequent time points are shown in the figure; for all significant differences between collection time points, see **Supplementary File 1**, **Tables S3**, **S4**. Significant differences obtained by Wilcoxon rank sum test between consecutive time points are represented by asterisks (* when $P < 0.05$; ** when $P < 0.01$). CSD, C-section delivery; VD, vaginal delivery. Fecal samples originating from VD infants are represented on the left side of each barplot and by green points, samples from CSD infants are represented on the right side of each barplot and by blue points.

more samples being positive for fungi in VD (43% of VD infant samples) compared to CSD infants (31% CSD infant samples). Fungi were detected earliest at day 3 in VD and at day 5 in CSD infants. The fungal DNA yield tended to increase over time, even though the magnitude of the increase was smaller compared to prokaryotes (**Figure 1C**).

Validation of GIT Microbiome Profiles in Low-Yield Samples

The absolute quantification of prokaryotic 16S rRNA gene copy numbers in all samples showed that the earliest samples contained significantly less microbial DNA compared to all other visits (**Figure 1**, **Supplementary File 1**, **Table S2**).

Analyzing the 16S rRNA gene sequencing data obtained for the dilution series of the human adult fecal control sample, we observed that the undiluted sample, reflecting the concentration of most samples in the study (**Figure 1**), and all three dilutions, simulating low-yield samples, showed highly comparable diversity and evenness indices (**Supplementary File 1**, **Figure S2A**). For richness, the undiluted sample and both 10- and 100-fold diluted samples had highly comparable results, while the 1,000-fold dilution caused a slight decrease. This loss of observed richness is also reflected in a slightly increased dissimilarity index for the 1,000-fold diluted sample compared to the undiluted sample. Considering the observed taxonomic composition with decreasing DNA concentration, all three dilutions showed high resemblance to the undiluted sample, while the 100- and 1,000-fold dilutions showed slightly over-estimated relative abundances for *Roseburia* spp. and *Collinsella* spp. and a slight under-estimation for *Bacteroides* spp. (**Supplementary File 1**, **Figure S2B**). However, in each case, a similar taxonomic profile to the one in the undiluted sample was observed and potential reagent contaminants or sequencing artifacts did not have a significant effect on the taxonomic composition in the low-yield samples. These data indicated that the chosen approach allowed the comparison of samples with low extraction yields to those with higher yields.

Generated Amplicon Sequencing Data

After the 16S rRNA gene sequencing and following the primary data processing and filtering, a total of 13,136,451 reads were retained and used for the subsequent analyses. With 205,000 ± 90,000 reads per sample (average ± standard deviation), a total of 1,053 unique OTUs were identified. One out of the 65 samples was excluded from further 16S rRNA gene sequencing analysis due to poor coverage (sample collected at day 3 for VD infant 8).

For the processed 18S rRNA gene amplicon sequence data, only OTUs reflecting the microeukaryotic members of the microbiome were considered. To achieve this, we manually curated the dataset of initially 3,376,004 reads by removing classified OTUs that belonged to the following clades containing multicellular organisms: Metazoa (total of 3,302,231 reads), Chlorophyta (total of 4,611 reads), Streptophyta (total of 7,414 reads), and Agaricomycetes (7,038 reads). After filtering out OTUs that were represented by <10 reads, a total of 60,476 reads (average of 960 ± 1,540 reads per sample) and 152 microeukaryotic OTUs were retained for the subsequent analyses.

Prominent Bacterial, Archaeal, and Microeukaryotic Taxa

In order to resolve which specific taxa were present during neonatal GIT colonization, we first identified the most common and abundant OTUs in the 16S rRNA gene amplicon sequencing data, which belonged to the phyla Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, and Verrucomicrobia (**Figure 2A**). Bacterial genera present in all samples ("core populations") included *Bifidobacterium* spp., *Escherichia/Shigella* spp., *Bacteroides* spp., *Streptococcus* spp., and *Enterococcus* spp., with the first three genera also being the bacterial taxa represented by the most reads out of the total of sequencing reads in all samples (**Supplementary File 2**).

As our qPCR results suggested the presence of archaea in most samples, their classification was scrutinized by 16S rRNA gene sequencing. Two OTUs belonging to the domain archaea were identified. OTU 1128 was assigned to the genus *Methanosphaera* and comprised a total of 25 reads in a single sample (day 1 for a VD5, 0.02% of reads; **Supplementary File 2**). Despite being low in abundance, reads of OTU 1128 (*Methanosphaera* sp.) were of good quality and allowed us to confidently ascertain the presence of this organism in this sample (**Supplementary File 1**, **Figures S3A,B**). Meanwhile, OTU 693, assigned to the genus *Methanobrevibacter*, was found in four samples represented by 1–11 reads but showed insufficient sequence quality for a confident classification (**Supplementary File 1**, **Figures S3C,D**).

Overall, microeukaryotic taxa were less frequent in the individual samples compared to bacterial taxa, with fewer OTUs and without specific "core" OTUs, which were detected in all samples. The most represented fungal phyla in all samples belonged to the phyla Basidiomycota and Ascomycota (**Figure 2B**), with the genus *Saccharomyces* and the class Exobasidiomycetes having been detected in more than 40% of the samples (**Supplementary File 3**).

Interestingly, meconium samples already presented a relatively large diversity of different prokaryotic and microeukaryotic populations. For prokaryotes, a total of 674 OTUs were detected in the 10 collected meconium samples (minimum of 109 OTUs, maximum of 347; **Supplementary File 4**). OTUs that were detected in all meconium samples included *Escherichia/Shigella* spp. and *Bifidobacterium* spp., which were also two of the taxa with the highest read counts over all samples. *Enterobacter* spp., *Staphylococcus* spp., *Streptococcus* spp., *Veillonella* spp., *Bacteroides* spp., *Prevotella* spp., *Clostridium sensu stricto* spp., *Delftia* spp., and *Blautia* spp. were also detected across all meconium samples. For the microeukaryotic community, a total of 45 OTUs were detected in the 9 sequenced meconium samples (**Supplementary File 5**). The most frequently detected OTU (in 77.8% of meconium samples) belonged to *Exobasidiomycetes* spp., while *Saccharomyces* spp., represented by the two most dominant OTUs with the highest relative abundances, were detected in more than half of the meconium samples.

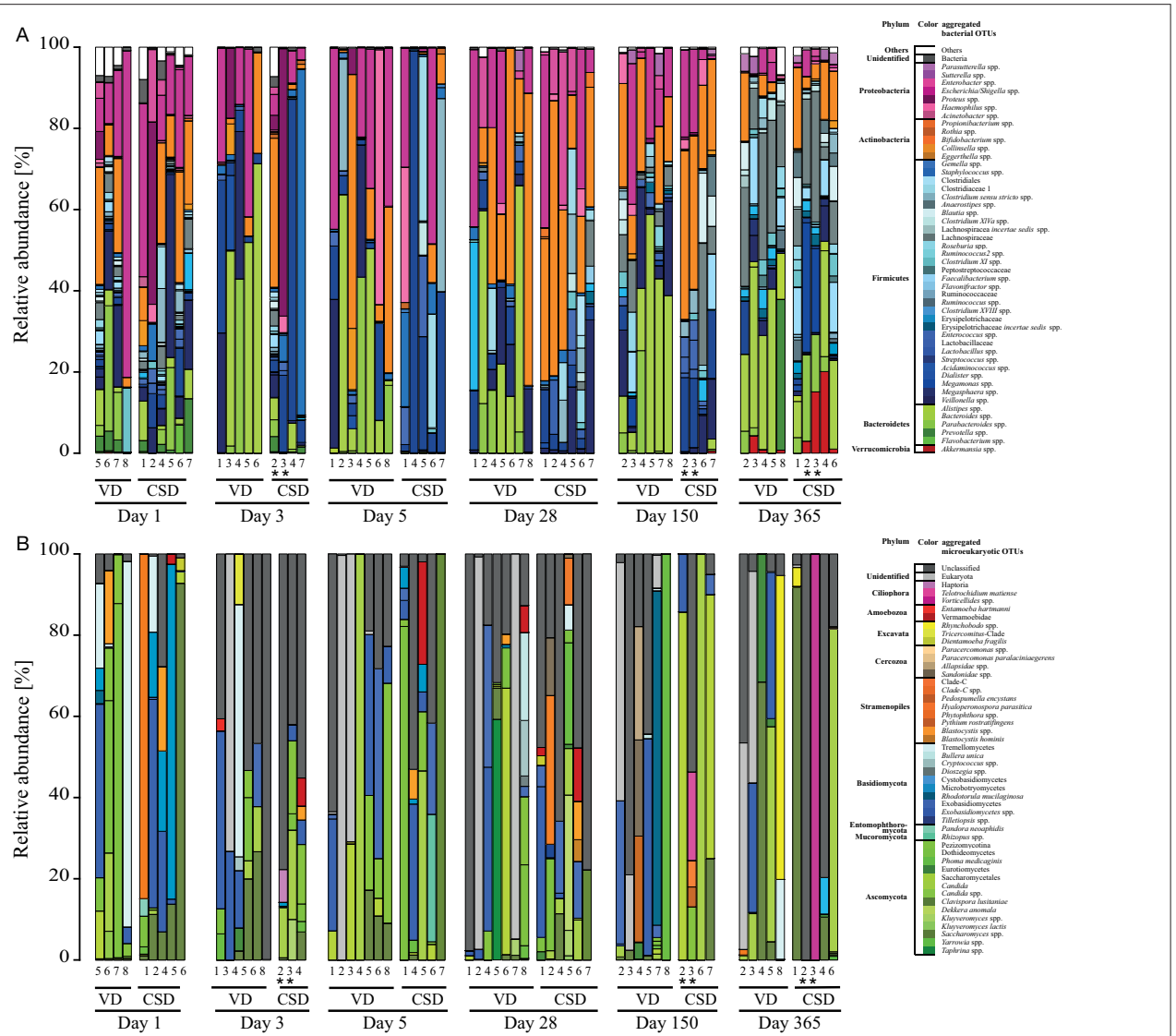

FIGURE 2 | Prokaryotic and microeukaryotic microbiome compositions in infants over the first year of life. Barplots of relative abundances of the 49 most abundant taxa per sample for **(A)** prokaryotes and **(B)** microeukaryotes for both delivery modes. All OTUs with the same taxonomy were regroupedd into the same taxa, whereas taxa that did not belong to the 49 most abundant were regrouped under "Others." Sequences were classified to the highest taxonomic level that could be confidently assigned. Aggregated OTUs are color-coded according to the phylum they belong to. Numbers below the barplots are representative of the different infants in the study. CSD, C-section delivery; VD, vaginal delivery. *Twins.

Colonization and Succession

As the amount of microbial DNA in the infants' stool increased with time, we analyzed whether the increase in microbial biomass was accompanied by a change in community characteristics such as richness or diversity. Based on the 16S and 18S rRNA gene amplicon data, we calculated overall richness, diversity, evenness, and dissimilarity indices for the prokaryotic (bacterial and archaeal; **Figures 3A–D**) and microeukaryotic (**Figures 3E–H**) datasets over the entire cohort. Non-parametric estimation of community richness for the individual time points according to Chao (1984) for prokaryotes and microeukaryotes showed comparable trends to the estimation of richness based on the numbers of different OTUs (**Supplementary File 1**, **Figure S4**). Given the sparseness and low abundance of archaeal OTUs detected by 16S rRNA gene amplicon sequencing, the observed patterns regarding prokaryotic diversity were mostly driven by bacterial taxa.

A significantly higher bacterial richness (number of different OTUs) was observed for the meconium samples compared to all other collection time points (**Figure 3A**, **Supplementary File 1**, and **Table S3**). In general, the inter-individual variability in richness was high on the first two sampling dates. The lowest richness of any sample was observed on day 3 *postpartum* and the overall median richness was lowest on day 5. The median

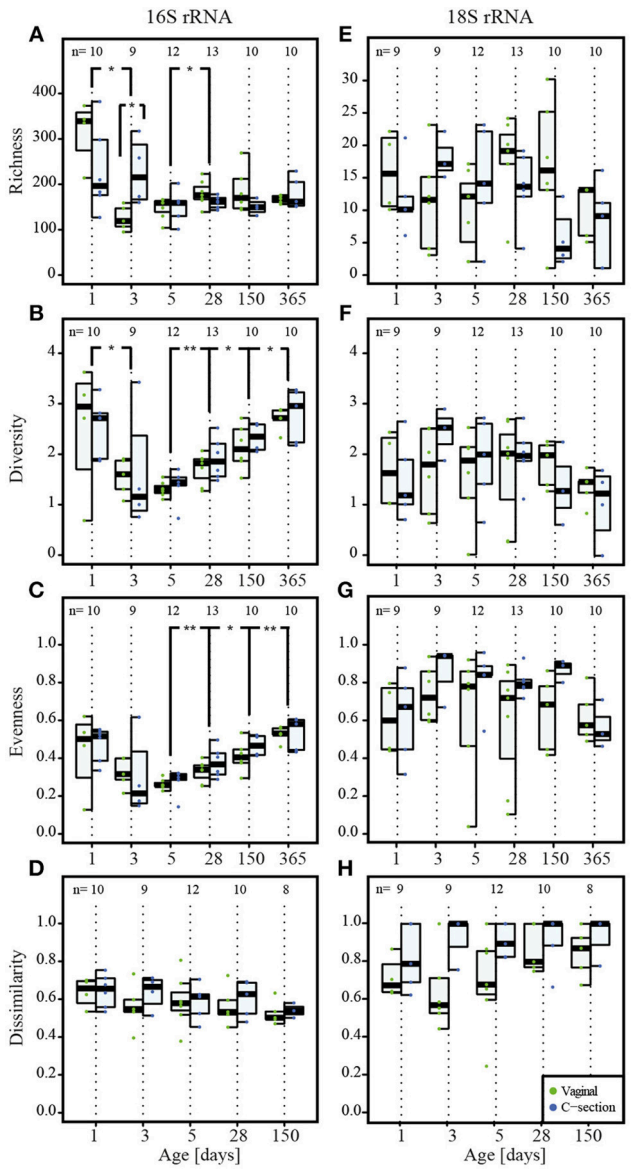

FIGURE 3 | Colonization of prokaryotes and microeukaryotes. Depiction of **(A,E)** richness (number of OTUs), **(B,F)** diversity (Shannon's diversity index), **(C,G)** evenness (Pielou's evenness index) and **(D,H)** dissimilarity index reflecting the distance to the most mature sample (Soerensen's similarity index of presence/absence of taxa at each individual time point compared to the most mature microbial community structures represented by samples collected at the last individual time point) for prokaryotes and microeukaryotes, respectively. Dominant phylotypes for prokaryotic and microeukaryotic datasets were considered. The numbers of samples per collection time point are provided at the top of the graph. For the purpose of clarity, only significant differences as assessed by Wilcoxon rank sum test between subsequent time points are shown in the figure; for all significant differences between collection time points, see **Supplementary File 1**, **Table S3** for the prokaryotic and **Table S4** for the microeukaryotic datasets. Significant differences between consecutive time points are represented by asterisks (* when $P < 0.05$; ** when $P < 0.01$). CSD, C-section delivery; VD, vaginal delivery. Fecal samples originating from VD infants are represented on the left side of each barplot and by green points, samples from CSD infants are represented on the right side of each barplot and by blue points.

richness increased subsequently and stabilized between day 28 and 150 (**Figure 3A**). The observed microeukaryotic richness tended toward a lower median richness at the end of the first year and showed a high level of variability throughout the first year of life (**Figure 3E**; **Supplementary File 1**, **Table S4**).

Shannon diversity and evenness metrics (**Figures 3B,C**, respectively) showed comparable trends for prokaryotic OTUs, i.e., a decrease in diversity and evenness with a concomitant decrease in variation in both diversity and evenness between individuals until day 5 *postpartum*. This was followed by a gradual increase for the subsequent collection time points. The observed microeukaryotic diversity and evenness (**Figures 3F,G,** respectively) followed no discernible trends compared to the bacterial data and exhibited constantly high levels of inter-individual variation. When linking samples according to the type of milk the infants received per time point, it became apparent that at day 5 and 28, infants that received combined feeding and formula-fed infants had a significantly lower microeukaryotic diversity compared to breast milk-fed infants ($P = 0.01$ at day 5 and $P = 0.03$ at day 28; Kruskal–Wallis test).

We calculated the Soerensen distance between the community structure at each time point and the community structure of the same individual in the most mature sample, i.e., usually the sample collected at 1 year, and compared the distances as a measure for maturity. For the prokaryotic dataset, the distances to the most mature sample exhibited a decreasing trend over time (**Figure 3D**). The observed patterns suggested a gradual development toward the 1 year samples, with day 150 exhibiting significantly more similarities to the most mature samples compared to the samples collected at day 1 ($P = 0.009$; Wilcoxon rank sum test). The same trend was observed for the Spearman correlation between the different time points (**Supplementary File 1**, **Figure S5A**), with samples of day 150 being significantly more correlated to the most mature microbiome than samples of day 1 ($P = 0.004$; Wilcoxon rank sum test). In contrast, the distances to the most mature microbial composition for the microeukaryotic microbiota (**Figure 3H**) as well as the Spearman correlation (**Supplementary File 1**, **Figure S5B**) displayed high variability among infants and between time points, and remained variable over time without reaching a certain level of maturity in regard to the 1 year samples.

Comparison of Microbiome Community Profiles of VD and CSD Infants

Absolute quantification of 16S rRNA gene counts by qPCR showed that CSD infants carried significantly lower bacterial loads and thereby a decreased colonization density at day 3 and day 150 ($P = 0.03$ and $P = 0.04$ respectively; **Figure 1A**; Wilcoxon rank sum test). At the same time, CSD infants had microbial community structures with a significantly higher richness compared to VD infants at day 3 ($P = 0.02$; Wilcoxon rank sum test; **Figure 3A**).

To provide an overview of the development of the microbiome of the eight VD (34 samples) and the seven CSD infants (30 samples), the 16S and 18S rRNA gene amplicon data were

represented by an ordination of their respective Jensen–Shannon distances (**Figure 4**), a method that is commonly used for human microbial community structure analyses (Koren et al., 2013). Clusters on the PCoA plots were defined by partitioning around medoids (Maechler et al., 2016). For the prokaryotic community structure, samples collected at 1 year clustered together independently of delivery mode (Cluster I in **Figures 4A,B**), whereas most samples collected for CSD infants around days 3 and 5 *postpartum* were located in Cluster II (**Figure 4B**). In order to identify cluster-specific taxa, we compared the taxa in both clusters using DESeq2, resulting in 52 OTUs that were significantly different in their DESeq2-normalized read numbers between both clusters (**Supplementary File 6**). Among the top 10 OTUs with the smallest adjusted *P*-values ranging from $1.41*10^{-18}$ to $3.06*10^{-04}$, 6 OTUs belonged to the genus *Streptococcus* and always one OTU belonged to the genera *Proteus*, *Haemophilus,* and *Rothia*, which all exhibited increased abundances in Cluster II; and one OTU classified as *Bifidobacterium* spp. which was more abundant in Cluster I.

Similar to the 16S rRNA gene sequence data, the 18S rRNA data exhibited two clusters (**Figures 4C,D**). One cluster (Cluster III) comprised all samples except for the samples belonging to three VD infants (Cluster IV), while the microeukaryotic community composition of one VD infant transitioned between both clusters (**Figure 4C**). When comparing the taxonomic compositions in samples between both clusters (III and IV) using the Wilcoxon rank sum test and adjusting for multiple testing, eight OTUs, with six unclassified OTUs and two OTUs classified as *Candida* spp., were detected to be differentially abundant in both clusters with *P*-values ranging between $5.94*10^{-10}$ and $2.63*10^{-02}$ (**Supplementary File 7**). These OTUs were increased in their abundances in samples belonging to Cluster IV, but were most often missing or decreased in abundance in samples from Cluster III. Additionally, samples that fell into Cluster IV, were collected from vaginally delivered infants that were either undergoing weaning, were fed with formula milk or received a mixed combination of breast and formula milk but were not exclusively breast-fed at the given time point.

Depletion of Bacteroidetes in CSD Infants

The most profound difference between CSD and VD infants was observed for the Firmicutes/Bacteroidetes ratio. While both phyla were approximately equally abundant in the VD infants (**Figure 5**), the corresponding ratio was significantly higher for CSD infants at days 5 ($P = 0.006$), 28 ($P = 0.005$), and 150 ($P = 0.01$; Wilcoxon rank sum test) while the proportional abundance

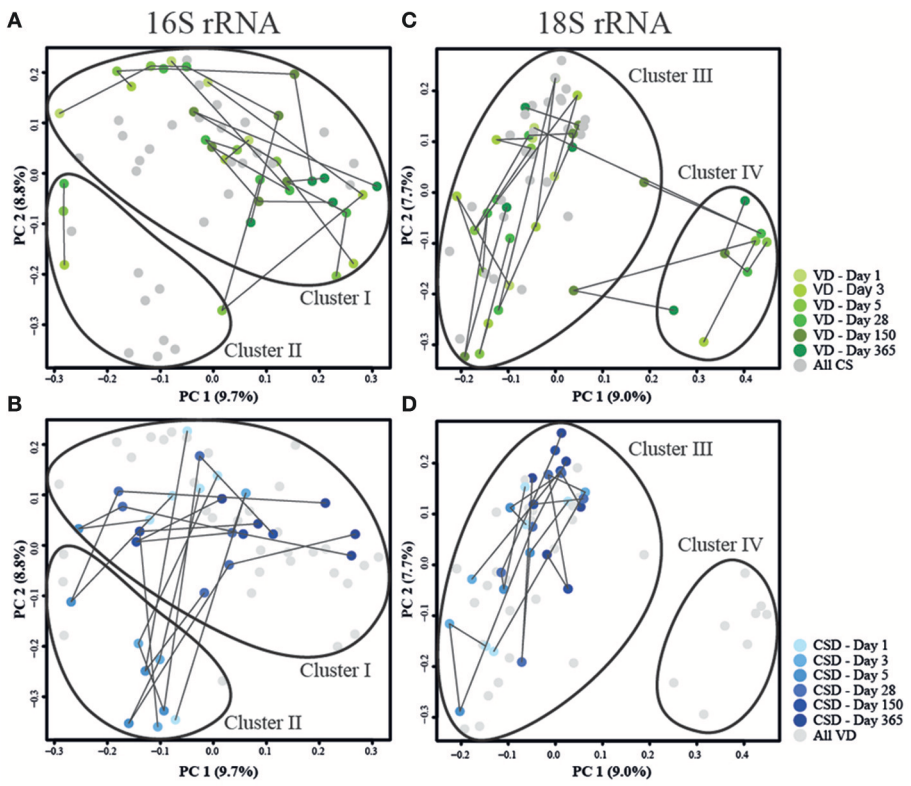

FIGURE 4 | Principal coordinate analyses of Jensen–Shannon distances for prokaryotic and microeukaryotic rRNA gene amplicon sequencing data. Depiction of **(A,C)** data from VD infants in green and **(B,D)** CSD infants in blue for prokaryotes and microeukaryotes, respectively. Sampling time points are represented by shadings, with lighter colors depicting an earlier sampling time point. Lines connect samples which originated from the same infant according the order of sampling. Samples that are the focus of the corresponding other sub-panel are shaded in gray. Cluster delineations were added manually after computing the cluster membership of each sample using the partitioning around medoids (pam) function contained in the R package "cluster" (Maechler et al., 2016).

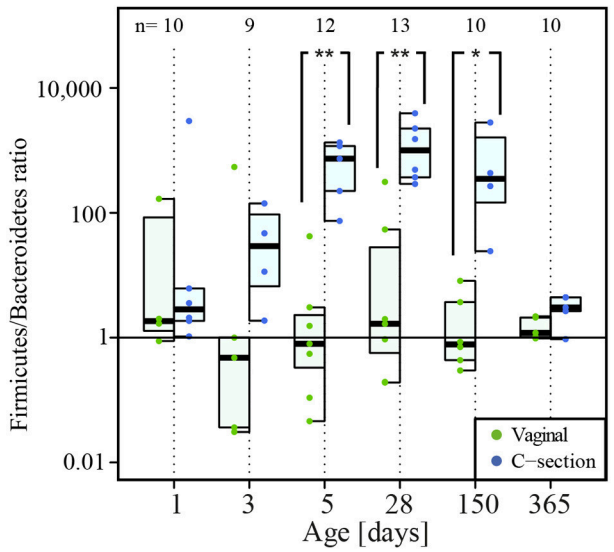

FIGURE 5 | Firmicutes/Bacteroidetes ratio over time. The numbers of samples per collection time point are given at the top of the graph. Significant differences obtained by Wilcoxon rank sum test and according to delivery mode are represented by asterisks (*when $P < 0.05$; ** when $P < 0.01$). CSD, C-section delivery; VD, vaginal delivery. Fecal samples originating from VD infants are represented on the left side of each barplot and by green points, samples from CSD infants are represented on the right side of each barplot and by blue points.

for the phylum Bacteroidetes was significantly decreased in samples from CSD infants over most of the sampling time points (day 5: $P = 0.006$, day 28: $P = 0.003$, day 150: $P = 0.01$, day 365: $P = 0.04$; Wilcoxon rank sum test; **Supplementary File 1**, **Figure S6A**). At the same time, there was a concomitant increase in Firmicutes at day 5 in CSD infants ($P = 0.01$; Wilcoxon rank sum test). Preceding the drastic decrease in Bacteroidetes at day 5, there was already a significant difference at day 3 between infants born at different gestational ages, whereby full term (≥39 weeks) infants showed a higher relative abundance of Bacteroidetes when compared to late preterm (34–36 weeks) and early term (37–38 weeks) born infants ($P = 0.05$; Kruskal–Wallis test; **Supplementary File 1**, **Figure S7**).

In addition, we also more specifically analyzed richness, evenness, and diversity within the Bacteroidetes phylum (**Figure 6**). We observed a significant decrease in the Bacteroidetes richness in CSD infants at day 28 compared to VD infants ($P = 0.01$; Wilcoxon rank sum test; **Figure 6A**). The relative abundance of the genus *Bacteroides*, which made up more than 10% of the reads in most VD infants at days 28 and 150, exhibited a significant decrease in abundance associated with a delayed colonization in CSD infants ($P = 0.04$ at day 28 and 0.01 at day 150; Wilcoxon rank sum test; **Supplementary File 1**, **Figure S6B**). Due to this significant decrease in relative abundance of *Bacteroides* spp. compared to earlier and later time points in CSD infants and the subsequent shift in dominance inside the Bacteroidetes phylum, the diversity and evenness inside this phylum at day 28 were significantly increased ($P = 0.005$ for both; Wilcoxon rank sum test; **Figures 6B,C**). The different measures of diversity and evenness within the Firmicutes phylum did not show any significant differences between both delivery modes.

Additional Differences in Prokaryotic Community Structure in CSD Infants

We further aimed to determine whether other bacterial taxa also showed different changes in CSD infants compared to VD infants during the first year of life. We identified taxa that were differentially abundant according to delivery mode at each collection time point. After filtering the resulting 88 differentially abundant OTUs according to a cumulative read count above 10,000, we retrieved 29 OTUs with a positive fold change in CSD infants compared to VD infants and four OTUs that exhibited a negative fold change (**Supplementary File 8**). The same analysis was performed at the genus level and resulted in three genera with a negative fold change and 20 with a positive fold change in CSD compared to VD infants (**Supplementary File 9**).

The fecal microbiome of CSD infants was associated with increased proportional abundances of, amongst others, OTUs assigned to the genera *Haemophilus* spp., *Streptococcus* spp., *Enterobacter* spp., *Propionibacterium* spp., *Staphylococcus* spp., and the genus *Lactobacillus* over the first year of life. Furthermore, the microbiome of CSD infants contained lower proportions of *Bacteroides* spp. and *Parabacteroides* spp.

In order to validate that CSD infants harbored substantially different relative abundances of certain prokaryotic populations compared to VD infants at certain time points, we amplified specific target regions of the genera *Staphylococcus* spp. and *Streptococcus* spp. (at days 3 and 5), *Haemophilus* spp., and *Lactobacillus* spp. (at days 3 and 28) and the two phyla Firmicutes and Bacteroidetes (at days 5 and 28), to calculate their relative abundances. Validation by qPCR was done on samples that were collected on days on which the differences in relative abundances between both delivery modes were most pronounced. All targeted differences between CSD and VD children obtained in the previous differential analysis could be confirmed by qPCR analysis for the specific collection time points (**Figure 7**).

DISCUSSION

Detection of Prokaryotic and Microeukaryotic Communities in Meconium

A number of recent studies indicate that meconium samples are not sterile but contain complex bacterial communities (Jiménez et al., 2008; Gosalbes et al., 2013; Ardissone et al., 2014). In this context, the previously accepted dogma of intrauterine sterility has been questioned. According to our results based on qPCR analyses as well as 16S and 18S rRNA gene amplicon sequencing, representatives of all three domains of life were present in meconium samples. Given that DNA yield out of meconium samples was limited (**Figure 1**), it might be possible that this microbial DNA might not be derived from the samples but may in fact represent contaminants of the reagents used for

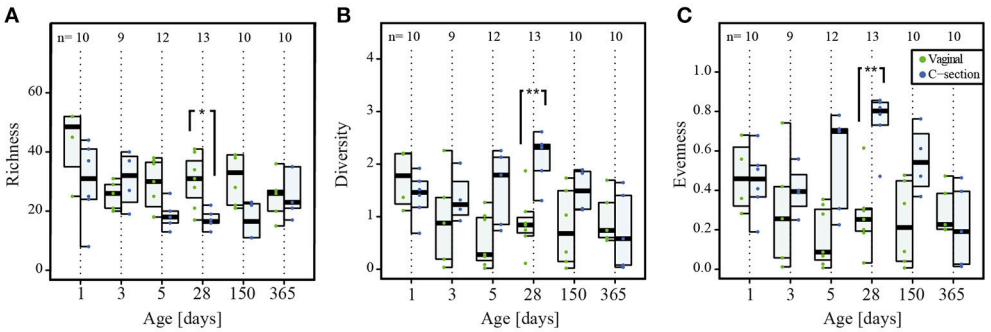

FIGURE 6 | Colonization by Bacteroidetes phylum. Per collection time point depiction of **(A)** richness (number of OTUs), **(B)** diversity (Shannon's diversity index), and **(C)** evenness (Pielou's evenness index) of the phylum Bacteroidetes. The numbers of samples per collection time point are provided at the top of the graph. Significant differences as assessed by Wilcoxon rank sum test and according to delivery mode are represented by asterisks (* when $P < 0.05$; ** when $P < 0.01$). CSD, C-section delivery; VD, vaginal delivery. Fecal samples originating from VD infants are represented on the left side of each barplot and by green points, samples from CSD infants are represented on the right side of each barplot and by blue points.

DNA extraction (Salter et al., 2014; Jervis-Bardy et al., 2015). However, according to simultaneously conducted analyses, even a 1,000-fold dilution of DNA extracted from an adult stool sample did not considerably change the taxonomic composition compared to the undiluted as well as the 10- to 100-fold diluted samples (**Supplementary File 1**, **Figure S2B**). From these results, we deduce that potential reagent contaminants did not have any significant impact on the overall community structure observed in our study. Moreover, the fact that we observed a significantly increased prokaryotic richness and diversity in meconium samples (**Figures 3A,B**) stood in stark contrast to the results from the dilution series, which revealed a decreased richness along with a stable diversity in the low-yield samples due to several taxa being diluted out of the adult stool sample during the 1,000-fold DNA dilution process (**Supplementary File 1**, **Figure S2A**). Additionally, the sequencing of all "DNA mock extracts" yielded very low coverage, while the detection of representatives of all three domains of life by qPCR could be considered negative as well. Taking these results into account, we suggest that the detection of taxa inside the meconium samples is not an artifact but has to be considered genuine. Whether the neonatal GIT was colonized prenatally or whether detected microbial populations were acquired perinatally could not be assessed in the context of our study.

The bacterial richness was significantly higher in meconium samples than at later time points. Samples from the first day were also highly diverse and the taxa were evenly distributed compared to subsequent collection time points, which suggests that these samples captured the potential early pioneering microbiota, most of which did not stably colonize the GIT thereafter. The richness decreased during the following days as the initial colonizers took hold in the GIT. Some of the taxa detected in the meconium samples may have been present in later samples but were not captured due to the masking by the dominant taxa. At day 1, the most abundant bacterial taxa in all infants were *Escherichia/Shigella* spp., *Bifidobacterium* spp., *Enterobacter* spp., *Staphylococcus* spp., *Streptococcus* spp., *Prevotella* spp., and *Veillonella* spp., which have all been previously described in meconium samples as being pioneering genera of the human GIT (Gosalbes et al., 2013; Ardissone et al., 2014; Hansen et al., 2015). The latter four are either present predominantly on skin (Dominguez-Bello et al., 2010), in colostrum or are typical inhabitants of the oral cavity (Cabrera-Rubio et al., 2012). Pioneering bacterial colonizers of the microbiome are usually facultative anaerobes, such as *Escherichia* spp. (Jiménez et al., 2008), as also observed in our study. These pioneers shape the gastrointestinal microbiome environment, promoting the subsequent colonization by strict anaerobes such as *Bacteroides* spp., *Clostridium* spp., and *Bifidobacterium* spp., which were already detected in samples collected on day 1 in our study. Overall, the earliest bacterial colonizers detected in all meconium samples included both facultative and strict anaerobic taxa suggesting that the GIT rapidly transitions toward an anaerobic environment after birth. *Bifidobacterium* spp., which was the taxon with the highest read counts across all samples, are important for neonatal health and are known to have beneficial effects for the host through their breakdown of dietary carbohydrates, the products of which directly feed into host metabolism (Davis et al., 2011). *Bifidobacterium* spp. are colonizers of the vaginal microbiome and are supposedly transferred to the infant during vaginal delivery (Dominguez-Bello et al., 2010). However, while in line with previous findings (Jakobsson et al., 2014), no significant difference in *Bifidobacterium* spp. abundances between VD and CSD infants could be detected for meconium samples, suggesting that other routes of transmission are also very likely during neonatal colonization. Additionally, the growth of this specific taxon is promoted selectively by prebiotic oligosaccharides present in the maternal colostrum and breast milk (Zivkovic et al., 2011; Yu et al., 2013).

Results from the quantitative real-time PCR assay suggested that archaea, even if low in abundance, were amongst the earliest colonizers of the neonatal GIT microbiome. The only methanogenic archaeon that was identified using the 16S rRNA gene amplicon sequencing was *Methanosphaera* spp., which was exclusively detected in VD infant 5 at day 1. This human

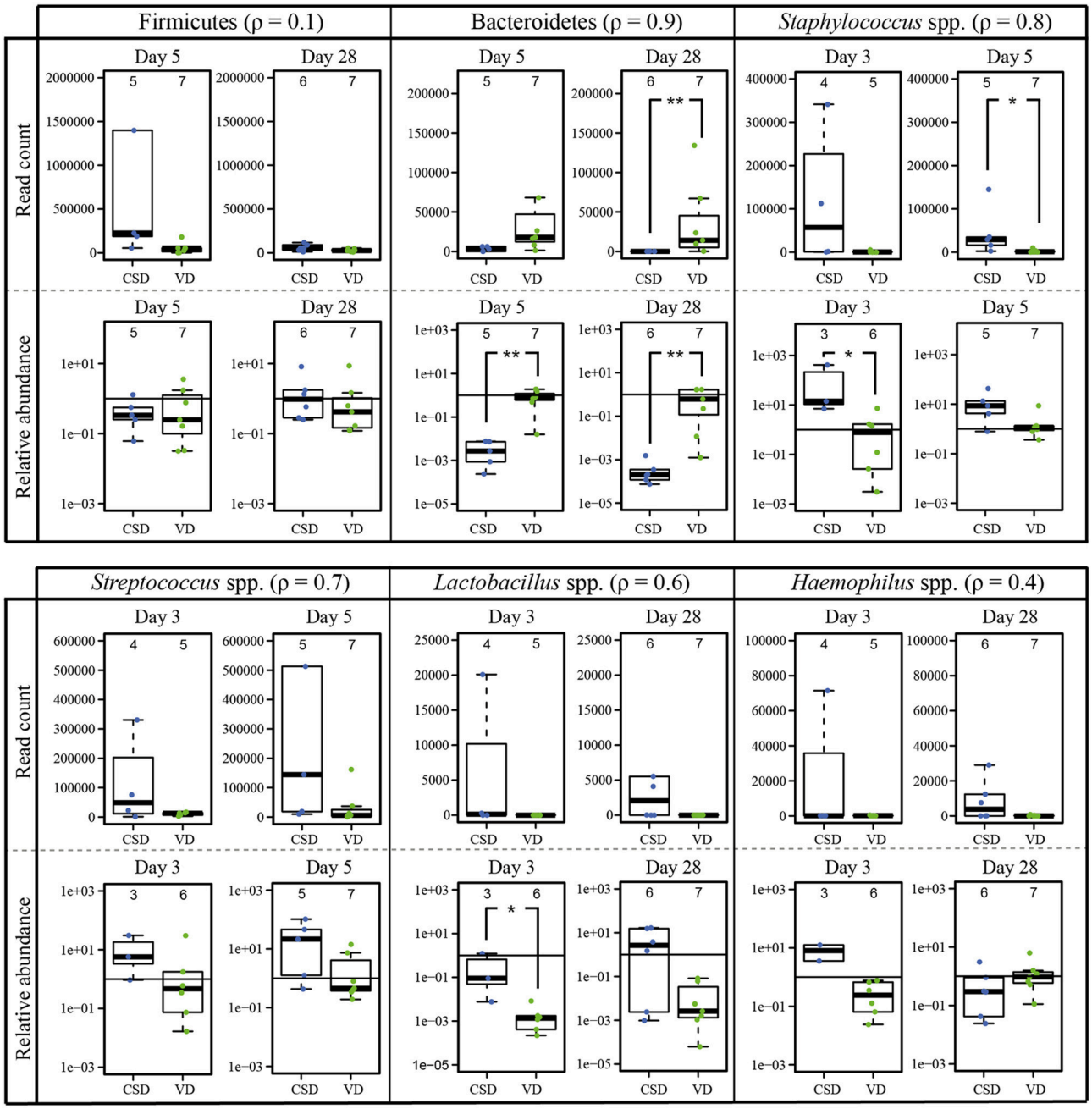

FIGURE 7 | qPCR validation of 16S rRNA gene sequencing data based differences according to delivery mode. Comparison of the DESeq2-normalized 16S rRNA read numbers and relative abundances (given on log scale) measured by qPCR for two phyla and four genera that were found to be significantly different between birth modes. For each comparison the Spearman correlation coefficient (ρ) was calculated and figures next to the taxa. The numbers of samples per collection time point are given at the top of each barplot. Significant differences according to a Wilcoxon rank sum test for delivery mode are represented by asterisks (* when $P < 0.05$; ** when $P < 0.01$). CSD, C-section delivery; VD, vaginal delivery. Fecal samples originating from CSD infants are represented on the left side of each barplot and by blue points, samples from VD infants are on the right side of each barplot and by green points.

archaeal commensal has a highly restricted energy metabolism (Fricke et al., 2006), which makes it a specialized member of the gastrointestinal microbiome. Archaea have been shown to be ubiquitous members of the adult GIT microbiome (Dridi et al., 2009), were sporadically detected in the vaginal environment (Belay et al., 1990), and were shown to colonize the skin surface (Probst et al., 2013) and the oral cavity (Nguyen-Hieu et al., 2013). As the presence of archaea was also apparent in CSD infants

and also in samples collected at day 1 in our study, we can postulate that transmission paths besides vaginal transmission, such as fecal-oral, oral-oral, or by skin contact most probably occur perinatally.

The earliest microeukaryotic colonizers included *Exobasidiomycetes* spp. and 2 OTUs classified as *Saccharomyces* spp., which were detected in meconium from CSD infants, whereas *Dothideomycetes* spp. and *Pezizomycotina* were detected mostly in VD infants. A recent study found *Saccharomyces* spp. and *Dothideomycetes* spp. to be present in more than half of the analyzed adult stool samples (Mar Rodríguez et al., 2015), which make them common taxa of the human GIT microbiome. As the vaginal tract is largely colonized by yeasts such as *Saccharomyces* spp., vaginal delivery is supposedly linked to neonatal colonization by yeasts through vertical transmission from the mother's vaginal microbiome or through horizontal transmission from the environment and hands of family members as well as health care workers (Lupetti et al., 2002; Bliss et al., 2008).

If pioneering microbiota, including representatives from all three domains of life, have the potential to colonize the GIT microbiome prenatally (Greenhalgh et al., 2016), according to our results, birth still marked the time point of extensive microbial colonization, which further defined microbial succession. Clearly, more work needs to be undertaken on meconium and the crucial first hours of life to ascertain the different sources of the pioneering microbiota.

Colonization and Succession within the Neonatal GIT Microbiome by Prokaryotes and Microeukaryotes during the First Year of Life

The progressive nature of neonatal GIT colonization and succession by prokaryotes was apparent through an increase in absolute prokaryotic DNA load (**Figure 1**), overall alterations to community compositions (**Figure 2**) as well as changes in richness, diversity and evenness (**Figure 3**). A general trend regarding the prokaryotic community members is that their structure matures over the course of the first year of life. This maturation was reflected by increases in diversity and evenness over time and shortly after an initial decrease from day 1 to 5 after birth. The fact that diversity and evenness keep increasing over time has already been reported in previous studies (Yatsunenko et al., 2012; Jakobsson et al., 2014). However, in our study, significant differences in diversity and evenness between subsequently sampled time points were observed as early as between days 5 and 28 (**Figures 3B,C**). The prokaryotic richness stabilized between days 28 and 150 (**Figure 3A**). Similarly, the dissimilarity index, reflecting the distance of the taxonomic composition of each sample to the last collected sample per child, showed a decreasing trend (**Figure 3D**), highlighting that the microbiome composition gradually changed from a neonatal profile toward the most mature composition available by 1 year of age.

A previous study, focusing on neonatal colonization, has found archaea to be transiently and almost exclusively present in the first few weeks of life during their sample collection, which was conducted until around 17 months (Palmer et al., 2007), whereas archaea are considered core members of the adult GIT microbiome (Dridi et al., 2009). While archaea could not be identified confidently by amplicon sequencing in our study after the first day, the qPCR assays using an archaea-specific primer set suggested that they were indeed present in 90% of all samples, opposing previous results and highlighting their potential importance in the maintenance of inter-species community networks (Hansen et al., 2011). Although the 16S rRNA gene amplifying primer used for sequencing covered both domains bacteria and archaea, the nature of GIT microbiome profiles, with bacteria making up the large majority of the composition, likely caused a lack of primer availability for archaea, potentially explaining why this domain was more extensively detected with qPCR using the archaea-specific primers rather than using the more generic 16S rRNA gene primers used for the amplicon sequencing. In the future, dedicated archaeal and bacterial primer sets may be used to allow better resolution of the archaea.

When considering the microeukaryotic community, no clear successional patterns were discernible. In line with previous studies involving culture-independent analyses of the GIT microbiome, most detected fungal taxa belonged to the phyla Ascomycota and Basidiomycota (Ott et al., 2008; Scanlan and Marchesi, 2008). In contrast to previous reports on adult GIT microbiota (Scanlan and Marchesi, 2008), identities and abundances of detected microeukaryotic taxa fluctuated strongly throughout the first year of life. Similarly, richness, diversity and evenness indices did not follow discernible trends over time (**Figures 3E–G**). However, we found a more rapid microeukaryotic diversification in infants who were fed exclusively breast milk between days 5 and 28 as well as a separation of samples on the PCoA plot that were collected from vaginally delivered infants either undergoing weaning, that were fed with formula milk or that received a mixed combination of breast and formula milk but were not exclusively breast-fed at that time point (**Figures 4C,D**). These findings suggest a possible link between the infants' feeding regimes and early changes to microeukaryotic community development in the human GIT. When considering the intra-individual dissimilarity index in addition to the apparent large inter-individual variation, our findings indicated that the microeukaryotic community members were more dynamic compared to their prokaryotic counterparts (**Figure 3H**). A previous study in the mouse GIT observed similar results with fungal populations varying substantially, while bacterial populations remained relatively stable over time (Dollive et al., 2013). Typically, only a small number of common genera, such as the genus *Saccharomyces*, and a large number of spurious taxa that have been barely reported previously have been described to form part of the human GIT microbiome (Suhr and Hallen-Adams, 2015). The specific characteristics of these rare taxa suggest that they do not persist inside the GIT microbiome but are likely more transient in nature when compared to bacteria (Suhr and Hallen-Adams, 2015). Also, fewer microeukaryotic species and individual microeukaryotes are found in the human GIT than bacteria, potentially explaining why the microeukaryotic community may be less robust in

comparison to bacteria (Underhill and Iliev, 2014). Furthermore, according to our results, the general lack in successional patterns with regards to the microeukaryotes suggested that either the neonatal GIT would not allow any durable colonization by microeukaryotes, including known common microbiome members such as *Blastocystis* spp. or *Dientamoeba fragilis* (Scanlan et al., 2014), that the required ecological niches did not exist in the GIT during the first year of life or that those microeukaryotes never actually stably colonize the GIT as suggested before by Suhr and Hallen-Adams (2015).

Prokaryotic Differences in Colonization and Succession between CSD and VD Infants

Diversity and evenness measures were not significantly different between CSD and VD infants (**Figures 3B,C**), in contrast to the results from another recent study (Jakobsson et al., 2014). However, a difference between VD and CSD infants was observed early on in terms of the prokaryotic richness, which was significantly increased in CSD infants (**Figure 3A**). This finding could reflect the different pioneering taxa between both delivery groups. Furthermore, we found that generally lower amounts of DNA were extracted from stool of CSD infants compared to VD infants using the same extraction protocol, suggesting a delay in the acquisition of prokaryotic biomass in the GIT of CSD infants. While the DNA yields quickly increased over time for VD infants, CSD infants showed a slower acquisition of a similar colonization density, which could be explained by either a delay in exposure to bacteria or the inoculation by fundamentally different microbial taxa, which could be less adapted to the human GIT and therefore exhibited lower growth rates.

In addition to differences in microbial loads during the first days after birth (**Figure 1A**), we identified apparent differences in early prokaryotic succession. For instance, several samples taken from CSD infants during days 3 and 5 were found to share similarities in community structure (Cluster II) that were not typically observed in samples from VD children (**Figures 4A,B**). These similarities included increased relative abundances of *Streptococcus* spp. and *Staphylococcus* spp. (**Supplementary File 6**). These taxa are typically found in the oral cavity and on the skin surface and are supposedly transferred from mother to infant through skin contact in CSD infants (Dominguez-Bello et al., 2010). Furthermore, these samples showed significantly decreased relative abundances of *Bacteroides* spp. and *Bifidobacterium* spp., whose colonization has been shown to be delayed in CSD infants (Adlerberth et al., 2006; Penders et al., 2006; Sufang et al., 2007; Biasucci et al., 2008; Dominguez-Bello et al., 2010). Interestingly, allergic diseases have been previously associated with a low prevalence of *Bacteroides* spp. and *Bifidobacterium* spp. (Björkstén et al., 1999; Watanabe et al., 2003), and low levels of *Bifidobacterium* spp. together with significantly increased levels of *Staphylococcus* spp. have been associated with childhood obesity (Kalliomäki et al., 2008). Generally, the genus *Bifidobacterium* is associated with an enhanced epithelial barrier function (Cani et al., 2009). These findings are in line with the statistically higher risks of CSD infants of developing obesity (Mueller et al., 2015) or allergic diseases (Abrahamsson et al., 2012, 2014). Although the differences observed in our study were compelling, whether the observed microbiome signatures in CSD infants are directly causally linked to disease development later in life has yet to be established in larger infant cohorts with longer-term follow-up. After day 150, the observed differences between CSD and VD infants became less pronounced. This observed trend could have been driven by weaning the infants from an exclusive milk diet and/or the introduction of solid food around the same time. Previous studies showed that through the introduction of new and diverse nutrients, the microbiome quickly changes toward a more adult-like profile, thereby decreasing early differences in profiles caused by delivery mode or other maternal and neonatal characteristics (Fallani et al., 2011; Koenig et al., 2011).

Although the delivery mode appeared to have the strongest influence on differences between the infants, other factors may also contribute to the observed patterns. Most notably, reduced gestational age, higher maternal age, a higher maternal BMI, and specific maternal antibiotic treatments are commonly observed in the context of CSD (van Schalkwyk et al., 2010; Al-Kubaisy et al., 2014; Delnord et al., 2014; Klemetti et al., 2016). For example, gestational age may have been an additional factor driving the early Bacteroidetes depletion. Already at day 3, Bacteroidetes were significantly decreased in five infants that were born late preterm (34–36 weeks) or early term (37–38 weeks) compared to four full term infants ($\geq$39 weeks; **Supplementary File 1**, **Figure S7**). Known effects of preterm delivery on neonatal microbiome colonization include reduced levels of strict anaerobes such as *Bifidobacterium* spp. and *Bacteroides* spp. (Arboleya et al., 2012, 2016) and a slower microbial succession (La Rosa et al., 2014), all of which were observed in our study for samples collected from CSD infants. Another factor may have been maternal perinatal antibiotics intake which was associated with significantly lower amount of prokayotic DNA at day 5 (and a similar trend at days 1 and 28; **Supplementary File 1**, **Figure S1**). Importantly, the antibiotic intake of the mother may have effects on the GIT microbiome of the infant, either directly, e.g., transfer from maternal blood via the blood-placental barrier prior to birth (Pacifici, 2006), or indirectly, e.g., transfer of antibiotics via breast milk *postpartum* (Zhang et al., 1997). As antibiotic administration is recommended in case of delivery by C-section, this could be yet another factor that had a negative influence on the observed delay in colonization and succession in CSD infants while even potentially inhibiting the succession rate in VD infants to a certain extent. As the present study cohort was limited in size, the obtained data cannot be considered representative of a more general situation such that other environmental factors may have non-negligent influences on the composition of the developing microbiome as well.

Besides shifts in the early successional patterns and factors that could enhance the observed delay in colonization, we also observed fundamental differences in the taxonomic composition of CSD infants compared to VD infants and over all time points, such as a significantly decreased relative abundance of Bacteroidetes (**Supplementary File 1**,

Figure S6A), which remained prominent even at 1 year. The most drastic difference in microbiome composition was an elevated Firmicutes/Bacteroidetes ratio observed in CSD infants between days 5 and 150 (**Figure 5**). An elevated Firmicutes/Bacteroidetes ratio has been previously linked to an increased energy harvesting capacity by the host and its potential contribution to the development of metabolic disorders such as diabetes, obesity, or metabolic syndrome in adulthood (Turnbaugh et al., 2006), although more recent findings seem to suggest that evidence for the implication of the Firmicutes/Bacteroidetes ratio in human health may be weaker than previously assumed (Sze and Schloss, 2016). The differential analysis detected statistically significant alterations of additional bacterial taxa in CSD infants over all time points, of which several were also validated by qPCR (**Figure 7**, **Supplementary Files 8**, **9**). As already highlighted previously, CSD infants harbored lower proportions of *Bacteroides* spp. and *Parabacteroides* spp., which again point out that CSD infants were subject to a delayed rate of colonization for the phylum Bacteroidetes and more specifically the genera *Bacteroides* and *Parabacteroides*. Taxa commonly derived from skin, the oral cavity and the environment exhibited an enrichment in CSD infants. These taxa included *Haemophilus* spp., *Streptococcus* spp., *Enterobacter* spp., *Propionibacterium* spp., and *Staphylococcus* spp., which have been previously found to be enriched in CSD infants, supposedly through skin microbiome transfer from mother to the newborn after birth (Dominguez-Bello et al., 2010; Bäckhed et al., 2015). Interestingly, CSD infants in our study were also enriched in the genus *Lactobacillus*. As *Lactobacillus* spp. are usually dominant in the vaginal microbiome, they are supposedly transferred from mother to infant during vaginal delivery, thereby being deficient and delayed in CSD infants (Grönlund et al., 1999; Adlerberth et al., 2006; Dominguez-Bello et al., 2010; Rutayisire et al., 2016). Other routes of colonization however also include the administration of breast milk (Bäckhed et al., 2015).

General Delay in Colonization Rates in CSD Infants

Overall, archaea and fungi were more often detected by qPCR in VD infants compared to CSD infants, and the yield of fungal DNA was lower in CSD infants compared to VD infants, except at 1 year and after introduction of solid food in all infants. These findings indicate that the previously described delay in colonization and succession observed for bacteria in CSD infants may actually affect all three domains of life, adding valuable information to our current knowledge regarding neonatal colonization of the GIT microbiome.

The initial microbiome colonization process is especially crucial for the early stimulation and maturation of the immune system (Rizzetto et al., 2014; Houghteling and Walker, 2015; Mueller et al., 2015), such that the observed delay of all three domains of life in CSD infants may result in an altered immunostimulatory effect, which in turn may potentially have long-lasting effects in relation to human health. Whether the early disturbance and delay of the colonization and succession processes in CSD infants could potentially exacerbate or contribute to the higher risk of CSD infants to develop certain diseases, therefore requires additional immunological data. However, what has been observed so far is that due to the close contact between the developing GIT, the underlying immune system and the colonizing bacteria, the early microbiome acts as an important interface in the neonatal development of the immune system (Björkstén, 2004; Caicedo et al., 2005; Rautava and Walker, 2007; Eberl and Lochner, 2009). Substantial shifts of neonatal taxonomic compositions or disruptions of natural colonization and succession processes may thereby lead to changes in the long-term developmental processes and subsequent altering of immune development. Additionally, the timing of colonization plays an important role in neonatal immune programming. Previous studies on mouse models observed that a delayed microbial GIT colonization of germ-free mice caused long-term changes in the immune system (Sudo et al., 1997; Rautava and Walker, 2007; Eberl and Lochner, 2009; Hansen et al., 2012; Olszak et al., 2012). These recent findings suggest that the composition and timing of early neonatal colonization in CSD infants are important factors influencing the microbial education of the developing immune system, which could result in long-term persistent alterations in systemic gene expression and increased disease predispositions.

CONCLUSIONS

Here, we describe for the first time the colonization of the neonatal human GIT resolved to all three domains of life. We demonstrate that bacteria but also archaea and microeukaryotes, predominantly fungi, are detectable in meconium samples and are thereby among the earliest colonizers of the neonatal GIT microbiome.

In contrast to the patterns observed for prokaryotes, microeukaryotic abundances fluctuated strongly over time, suggesting that the microeukaryotic community did not reach a stable colonization state during the first year of life. Based on our results, the milk-feeding regime appeared to impact the early microeukaryotic colonization and diversification process. An important question in this context is whether a diverse microeukaryotic microbiome is more resilient to disturbances and beneficial for the host as it has been proposed for bacterial constituents of the GIT microbiome.

As for the differences in colonization and succession between VD and CSD infants during the first year of life, our findings highlight that CSD infants experience a delay in colonization and succession affecting all three domains of life, generally complementing and further extending previous observations. Substantial shifts in the community compositions started as early as day 5 and were potentially caused by differences in time of incidental exposure to bacteria from the environment in CSD infants. We further suggest a potential link to earlier gestational age and maternal antibiotics intake. Given that the early microbiome supposedly shapes the immune system, our observations that CSD infants exhibited a different succession pattern early on raises the possibility that disturbances to the microbiome in the early stages of neonatal development might have long-lasting health effects. Although major differences between VD and CSD infants were less apparent at 1 year of age, the question whether differences in the early stimulation of the immune system by either the VD or the CSD microbiomes

may change the infants' response to later perturbations, such as during the introduction of solid food, will require further in-depth studies. In order to answer these questions, high-frequency sampling of GIT microbiota along with resolving crucial immune characteristics over longer periods of time should be undertaken.

Further additional work is required to determine at which stage of infant development the GIT microbiome acquires a mature archaeal community, as well as when the transition between the highly dynamic early microeukaryotic microbiota and the stable adult microeukaryotic community occurs. Open questions in this context revolve around which role these two domains play with respect to neonatal host metabolism, how they influence the host's immune system and how they influence the GIT microbiome through providing specific metabolic functions.

Our findings provide an important account of the neonatal colonization and succession within the human GIT microbiome by bacteria, archaea, and microeukayotes. In particular, our findings highlight the need for studying all three domains of life in future longitudinal studies of microbial colonization and succession within the human GIT to finally understand how the individual taxa affect host physiology and how differences in colonization and succession of all three domains may contribute to the development of diseases later in life.

AUTHOR CONTRIBUTIONS

LW carried out the qPCR assays, data processing, comparative analyses of the 16S and 18S rRNA gene amplicon sequencing data and data interpretation, participated in the biomolecular extractions and wrote the manuscript. AHB, AH, CL, Cd, and PW were involved in data analysis and interpretation. AHB contributed toward biomolecular extractions and writing the manuscript. AH participated in the design of the study and in the sample and data collection. EM, SN, and CL participated in data analysis and critical revision of the manuscript. LH and AA participated in the data processing of the 18S rRNA gene amplicon sequencing data and provided important advice. LB and JB participated in the sample collection and processing. Cd and PW conceived and coordinated the study and participated in its design and in writing the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00738/full#supplementary-material

Figure S1 | Impact of maternal antibiotic intake prior to birth on the yield of prokaryotic DNA from neonatal stool samples.

Figure S2 | Analysis of 16S rRNA gene amplicon data from a DNA dilution series.

Figure S3 | Quality of the archaeal sequencing reads.

Figure S4 | Microbial richness during colonization and succession of the infant GIT.

Figure S5 | Spearman correlations between samples from each time point compared to the individual most mature microbial community profiles represented by samples collected at the final time point per infant.

Figure S6 | Differences between delivery modes in relation to relative abundances of Bacteroidetes and *Bacteroides spp.*

Figure S7 | Relative abundances of Bacteroidetes in children born at different gestational ages.

Table S1 | Milk feeding regime of the infants prior to the different sample collection time points.

Table S2 | Results of Wilcoxon rank sum test comparing the yields measured for the prokaryotic and fungal DNA at different collection time points.

Table S3 | Results of Wilcoxon rank sum tests for prokaryotic diversity, evenness, richness, and dissimilarity indices at different collection time points.

Table S4 | Results of Wilcoxon rank sum tests for microeukaryotic diversity, evenness, richness, and dissimilarity indices at different collection time points.

Supplementary File 1 | Figures S1–S7 and Tables S1–S4.

Supplementary File 2 | Number of reads obtained from the 16S rRNA gene amplicon sequencing data at OTU level and in all samples.

Supplementary File 3 | Number of reads obtained from the 18S rRNA gene amplicon sequencing data at OTU level and in all samples.

Supplementary File 4 | Sequencing reads obtained from the 16S rRNA gene amplicon sequencing data at OTU level in meconium samples.

Supplementary File 5 | Sequencing reads obtained from the 18S rRNA gene amplicon sequencing data at OTU level in meconium samples.

Supplementary File 6 | List of OTUs with significantly different relative abundances between Clusters I and II.

Supplementary File 7 | List of OTUs with significantly different relative abundances between Clusters III and IV.

Supplementary File 8 | List of OTUs resulting from the differential analysis (DESeq2) that were significantly differentially abundant across all collection time points and comparing VD and CSD infants.

Supplementary File 9 | List of genera resulting from the differential analysis (DESeq2) that were significantly differentially abundant across all collection time points and comparing VD and CSD infants.

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Bacterial Diversity of the Gastric Content of Preterm Infants during Their First Month of Life at the Hospital

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Studies focused on the stomach microbiota are relatively scarce, and most of them are focused on the adult population. The aim of this work is to describe the bacterial communities inhabiting the gastric content (GC) of preterm neonates. For that purpose, GC samples were collected weekly from a total of 13 preterm neonates during their first month of life within their hospital stay. Samples were analyzed by using both culture-dependent and -independent techniques. The former allowed the isolation of bacteria belonging mainly to the genera *Enterococcus*, *Staphylococcus*, *Streptococcus*, *Serratia*, *Klebsiella*, and *Escherichia*. The cultured dominant species in the GC samples during all the hospitalization period were *Enterococcus faecalis* and *Staphylococcus epidermidis.* Multilocus sequence typing (MLST) analysis revealed the presence of high-risk clonal complexes associated with the hospital environment, which may colonize enteral feeding tubes. Similarly, the 16S rRNA sequencing showed that *Streptococcus*, *Staphylococcus*, *Lactobacillus*, *Enterococcus*, *Corynebacterium*, and *Propionibacterium* were the dominant genera present at 75% of the gastric samples. However, the genera *Serratia*, *Klebsiella*, and *Streptococcus* were the most abundant. Own mother's milk (OMM) and donor milk (DM) were collected after their pass through the external feeding tubes to assess their bacterial content. OMM and DM had a similar bacterial pattern to GC. Based on these data, the GC of preterm neonates is dominated by *Proteobacteria* and *Firmicutes* and harbors high-risk bacterial clones, which may colonize enteral feeding tubes, and therefore the feeds that pass through them.

Keywords: preterm infants, stomach, gastric content, microbiome, microbiota

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INTRODUCTION

The early colonization of the infant digestive tract is a complex process that has relevant consequences for health throughout the life span (1–7). Exposure to a myriad of microorganisms during the perinatal and neonatal periods is followed by a crucial sequence of active events leading to immune tolerance and homeostasis (8). The discrimination between commensal bacteria and invading pathogens is essential to avoid an inappropriate immune stimulation and/or host infection.

The dysregulation of these tight interactions between host and microbiota can be responsible for important health disorders, including inflammation and sepsis.

Although full-term, vaginally delivered, and breast-fed infants are considered the ideal for the correct development of the gastrointestinal microbiota, in practice, there are many factors that may affect the acquisition, composition, and evolution of the infant gut microbiota, including gestational age, mode of delivery, diet, environment, or medical treatments (9–12). The initial colonization process is particularly challenging in preterm newborns, because of organ immaturity, higher rates of C-section deliveries, frequent use of antibiotics, and the stay at the hospital's neonatal intensive care unit (NICU) (13). Under such conditions, it is not strange that preterm infants are frequently associated with an abnormal intestinal colonization pattern (14, 15), a fact that increases susceptibility to disease (16–18). Globally, the intestinal microbiota of preterm infants exhibits a significantly reduced bacterial diversity, an abundance of microorganisms usually related to hospital environments, and a reduced proportion of strict anaerobes with respect to facultative ones (19–23).

So far, gut has been the subject of most studies concerning gastrointestinal colonization, while studies dealing with the stomach's microbiota are relatively scarce, and most of them are focused on the adult population with or without *Helicobacter* infection (24–31). In comparison to term neonates, the preterm's stomach is characterized by the absence of periodical or rhythmic motility, a slow gastric emptying, and a relatively high gastric pH (5.5–7.0), facts that can be determinant in the establishment of the gastric microbiota (32–34). In addition, enteral feeding tubes (NEFTs) are often required to feed these babies but also contributed to the colonization by NICU-associated microorganisms (35, 36). As a consequence, the pass of any nutritional source [either own mother's milk (OMM), donor milk (DM), or preterm formula] through the tubes may sweep along bacteria and have a strong impact on the infant intestinal colonization (36).

In this context, the objectives of this work were, first, to describe the bacterial communities inhabiting the gastric content (GC) of preterm neonates using culture-dependent techniques; such approach included the characterization of the bacterial lineages in the case of species frequently involved in preterm sepsis; and second, to apply the culture-independent techniques to describe the microbiome of a subset of samples.

MATERIALS AND METHODS

Study Design

Thirteen preterm infants (≤32 weeks of gestation and/or ≤1,500 g of weight) of 31 recruited in a previous study (36) born at the Hospital Universitario 12 de Octubre of Madrid (Spain) were randomly selected for this study. Preterm infants with malformations, metabolic diseases, or severe conditions were excluded. Relevant demographic and clinical data such as antibiotherapy, enteral and parenteral nutrition, use of nasogastric tube, need of mechanical ventilation, hospital stay, birth weight, gestational age, gender, or delivery mode are described in **Tables 1** and **2**.

Following the routine NICU feeding protocols, all infants were preferably fed with their OMM and, when this was not possible, with pasteurized human milk from the Milk Bank Unit DM. When the weight of the infants was ≥1,500 g and both types of human milk were unavailable, they received adapted preterm formula. In general, the feeding patterns of the recruited infants were very heterogeneous, a fact that prevented the formation of well-defined feeding groups.

Continuous nasogastric feeding was ordinarily administrated with a pump and intermittent feeding by gavage or pump. The syringe barrels were used as reservoirs that were connected through an external feeding tube (EFT). Feeding tubes were routinely replaced every 24 h, which means that different feed types could pass through the same tube during such a period.

None of the infants received antiacid treatment during their participation in the study.

Samples Collection

Gastric content samples (~2 mL) were collected weekly by aspiration, using a sterile syringe, through the NEFT inserted into each baby's stomach before new milk administration when intermittent feeding.

TABLE 1 | Demographic characteristics of the infants included in the study.

Infant	Gestational age (weeks)	Gender	Birth weight (g)	Delivery mode	Sample collection (days of life)	
					Culture	16S rRNA
1	30	M	1,550	Cesarean section	0, 14, 21	0, 14
2	27	F	1,080	Cesarean section	0, 7, 14, 21, 28	0, 21
3	30	M	2,030	Cesarean section	0, 7, 14, 21	14
4	30	M	1,760	Vaginal	0, 7, 14, 21	14
5	32	F	1,310	Vaginal	0, 14, 21	14
6	26	F	920	Vaginal	7, 14, 21	21
7	29	F	1,040	Cesarean section	7, 21, 28	21
8	24	M	740	Vaginal	7, 14, 21	21
9	25	M	720	Vaginal	14, 21	21
10	28	M	1,100	Cesarean section	14, 21	21
11	31	F	1,430	Vaginal	0, 7, 14, 21	21
12	27	M	950	Cesarean section	7, 14, 21	28
13	24	M	870	Cesarean section	21	21
Mean (95% CI)	27.9 (26.3–29.5)		1,192 (950–1,435)			

TABLE 2 | Clinical characteristics of the infants included in the study.

Infant	Antibiotherapy (days)	Parenteral nutrition (days)	Enteral feeding tube (days)	Mechanical ventilation (days)	Hospital stay (days)	*N* episodes of sepsis
1	3	5	38	0	42	–
2	9	3	48	0.5	60	–
3	3	0	26	2	27	–
4	0	0	26	0	27	–
5	3	0	21	0	28	–
6	33	8	97	26	102	2
7	7	5	45	0	47	–
8	27	13	112	35	116	1
9	38	10	144	140	144	1
10	7	14	62	10	73	–
11	3	4	35	0	37	–
12	3	8	62	0.04	70	–
13	29	12	90	37	102	–
Mean (95% CI)	12.7 (4.4–20.9)	6.3 (3.3–9.3)	62.0 (39.1–84.9)	0.5 (0–26)[a]	67.3 (44.2–90.4)	

[a]*Median (IQR).*

The feeding (OMM and/or DM) samples collected were the last fraction obtained after their passage through the EFT, immediately before entering the nasogastric tube at the connector, as previously reported (36).

All samples were collected 12 h after the replacement of the nasogastric feeding tubes and stored at −20°C until analysis.

Culture Analysis

Adequate dilutions of GC samples were spread onto Man, Rogosa and Sharpe (MRS; Oxoid, Basingstoke, UK) and MRS supplemented with L-cysteine (0.5 g/L) (Sigma, St. Louis, MO, USA) (MRScys) for isolation of lactic acid bacteria, MacConkey (BioMérieux, Marcy l'Etoile, France) for isolation of *Enterobacteriaceae*, Baird Parker (BioMérieux) for isolation of staphylococci, Sabouraud Dextrose Chloramphenicol (SDC; BioMérieux) for isolation of yeasts, and Brain Heart Infusion (BHI; Oxoid), Wilkins-Chalgren (WC; Oxoid), and Columbia Nalidixic Acid Agar (BioMérieux) as general media for isolation of other bacterial groups. Plates were aerobically incubated at 37°C for up to 48 h, with the exception of SDC plates, which were incubated at 32°C for 96 h, and WC and MRScys plates, which were incubated (85% nitrogen, 10% hydrogen, and 5% carbon dioxide) in an anaerobic workstation (Mini-MACS Don Whitley Scientific Limited, Shipley, UK) at 37°C for 48 h.

Bacterial Identification

Bacterial identification was performed as previously reported (36). The identifications were confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Vitek MS, Biomerieux) at the facilities of ProbiSearch, SL. (Tres Cantos, Spain).

Genetic Diversity of *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Escherichia coli* Isolates

The genetic diversity of all the isolates belonging to the species *E. faecalis*, *S. aureus*, and *K. pneumoniae* was assessed by pulsed-field gel electrophoresis (PFGE) in a CHEF DR II apparatus (Bio-Rad, Birmingham, UK). To separate *Sma*I-digested fragments of enterococci and staphylococci, different protocols were applied (2–28 s for 24 h and 5–15 s for 10 h, and then 15–60 s for 13 h). The chromosomal DNA of *K. pneumoniae* isolates was digested with *Xba*I enzyme, and the electrophoresis conditions were 1–40 s for 20 h. The analysis of PFGE profiles was performed using the UPGMA method based on the Dice similarity by the Phoretix 5.0 software.

Multilocus sequence typing (MLST) schemes were applied for PFGE-unrelated strains of *E. faecalis* and *S. aureus*,[1] *E. coli*,[2] and *K. pneumoniae*.[3]

DNA Extraction from the Gastric Samples

Gastric content samples were thawed at room temperature and centrifuged at 13,000 rpm and 4°C for 10 min. Then, the pellets were washed with TE buffer and centrifuged under the same conditions. DNA extraction protocol was carried out as previously described (23). To detect similarities and, more interesting, differences between GC and infant's feeding, the bacterial composition of some OMM and DM samples was also assessed. DNA extraction of those samples was made following the same protocol.

Next-Generation Sequencing Analysis

PCR amplifications were performed using primer 27F-DegL (5′-GTTYGATYMTGGCTCAG-3′) in combination with an equimolar mixture of two reverse primers, 338R-I (5′-GCWGC CTCCCGTAGGAGT-3′) and 338R-II (5′GCWGCCACCCGTA GGTGT-3′), generating ~345 bp amplicons from the V1 to V2 hypervariable regions of 16S rDNA genes. Barcodes used for Illumina sequencing were appended to 3′ and 5′ terminal ends of PCR amplicons to allow separation of forward and reverse sequences. Subsequently a bioanalyzer (2100 Bionalyzer, Agilent)

[1]www.mlst.net.

[2]http://mlst.ucc.ie/mlst/dbs/Ecoli.

[3]http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html.

was used to determine the concentration of every sample in the region of interest.

Barcoded PCR products from all samples were pooled at approximately equal molar DNA concentrations and run on a preparative agarose gel. The correct sized band was excised, and the DNA was purified. One aliquot of pooled, purified, barcoded DNA amplicons was sequenced on an Illumina MiSeq pair-end 2 bp × 250 bp protocol (Illumina Inc., San Diego, CA, USA) at the Unidad de Genómica of the Fundación Parque Cientifico de Madrid (Spain).

Raw sequences were processed according to their quality using the program TRIMOMMATIC by filtering those reads that showed a window of 50 bp with an average of quality values below 25. MOTHUR v 1.33.0 and UCHIME programs were used to eliminate chimeras and ambiguous bases. Resulting reads of quality controls were assembled and classified taxonomically by comparison with databases Greengenes, Ribosomal Database Project, and SILVA using a Bayesian classification method and a level of similarity of at least 97%.

Statistical Analysis

The statistical analysis was performed using R 2.15.3 (R-project).[4] When data were not normally distributed, median and interquartile ranges (Q1 and Q3) were calculated for all sampling times, and mean and 95% confidence interval (95% CI) were used for normal distributed data. The Kruskal–Wallis test for non-normal data or one-way ANOVA test when data were normally distributed were used to evaluate the differences between sampling times. In all cases, P values of <0.05 were considered to be significant.

RESULTS

Characteristics of the Preterm Population

The 13 infants enrolled in this study had a mean gestational age of 28 weeks (ranging from 24 to 32 weeks) and a mean birth weight of 1,192 g (from 720 to 2,030 g) (**Table 1**). Approximately half of the infants ($n = 7$) were born by cesarean section (**Table 1**). All of them, except one, received antibacterial prophylaxis for, at least, the first 3 days of life and eight infants needed mechanical ventilation (**Table 2**). All infants were fed either with their OMM, DM, and/or preterm formula by nasogastric feeding tube for, at least, 21 days after delivery (mean of 62 days) (**Table 2**).

Culture-Based Analysis of the GC Samples

A total of 38 GC samples collected during the first month of life were analyzed by culture-based methods: 6 samples from the first day of life (day 0), 8 from the first week of life (day 7), 11 from the second week of life (day 14), 11 from the third week of life, and 2 from the fourth week of life (day 28). Samples were very heterogeneous in texture and color, ranging from milky to mucous.

No microorganism could be isolated from 25% of the samples (particularly from those collected at day 0). When bacterial growth was detected, total bacterial counts oscillated between 4.20 and 5.95 $\log^{10}$ CFU/mL at day 0 and between 6.00 and 8.13 $\log^{10}$ CFU/mL at day 28 after birth in BHI media (Table S1 in Supplementary Material). The dominant bacterial genera detected were *Enterococcus*, *Staphylococcus*, and *Lactobacillus*, among Gram-positive bacteria, and *Klebsiella*, *Serratia*, and *Escherichia*, among Gram-negative ones. All of them showed a trend to increase, in both concentration and frequency, from birth onward, but only the genus *Enterococcus* showed a statistically significant increase during the study period ($P = 0.027$) (**Table 3**).

A total of 241 isolates, belonging to 22 different species, were identified. The number of species per sample ranged from 1 to 17 species, and the bacterial profiles showed a high interindividual variability (**Figure 1**). Globally, the dominant species were *E. faecalis* and *Staphylococcus epidermidis*, followed by *Serratia marcescens*, *E. coli*, and *K. pneumoniae*.

MLST Clones

MLST analysis revealed the presence of five different ST clones among the *E. faecalis* isolates. Most of the infants (67%) were colonized by the clone ST 64, while clone ST 56 was detected in two infants (6 and 9) and clones ST21, 34, and 40 only in one infant each (2, 7, and 10, respectively) (**Figure 1**). All these clones belonged to high-risk clonal complexes and are associated with hospital environments. In relation to *S. aureus*, ST378 and ST34 were isolated in GC of only two infants (1 and 2, respectively), but both clones are also associated with high-risk clonal complexes. The ST393, ST1978, and ST69 of *E. coli* were detected in gastric samples from four infants; two of them were colonized by the same clone (ST393). Finally, clones ST641 and ST505 of *K. pneumoniae* were detected in GC of two infants (3 and 11, respectively), and, again, both clones belonged to high-risk clonal complexes (**Figure 1**).

Metagenomic Analysis of the GC and Milk Samples

DNA of good quality was obtained from 15 gastric samples of 13 infants. Two of them from the first week after birth (day 0), four from the second week of life (day 14), eight from the third week of life (day 21), and one from the fourth week of life (day 28). After processing the quality of the reads, a total of 3,613,468 sequences were analyzed that represented an average of 240,898 ± 32,493 sequences per sample. A high interindividual variability was detected among the samples. The Shannon Index ranged between 1.158 and 2.563 independently to the sample collection time with a mean of 1.863 ± 0.476 (**Figure 2**).

Three phyla predominated in the analyzed samples, *Proteobacteria* (49.61% ± 14.69), *Firmicutes* (30.11% ± 8.86), and *Actinobacteria* (15.66% ± 9.44) (**Figure 3**). Other phyla were detected but with a relative abundance below 1%. Similarly, to the culture results, the most abundant genera observed in GC samples with 16S rRNA sequencing were *Corynebacterium*, *Streptococcus*, *Staphylococcus*, *Lactobacillus*, *Enterococcus*, and *Serratia*. However, 11 other genera represented that ≥1% of the

[4] www.r-project.org.

TABLE 3 | Microbial genera isolated from gastric content of preterm neonates.

Genus	Day 0 (n = 6)		Day 7 (n = 8)		Day 14 (n = 11)		Day 21 (n = 11)		Day 28 (n = 2)		P value*
	N samples, n (%)	Bacterial counts, median (IQR)	N samples, n (%)	Bacterial counts, median (IQR)	N samples, n (%)	Bacterial counts, median (IQR)	N samples, n (%)	Bacterial counts, median (IQR)	N samples, n (%)	Bacterial counts, median (IQR)	
Staphylococcus	1 (17)	5.18	6 (75)	5.29 (1.27–5.99)	7 (64)	5.81 (3.35–6.23)	5 (45)	4.02 (2.30–5.85)	1 (50)	5.85	0.245
Enterococcus	0 (0)	–	2 (25)	4.66 (3.23–6.09)	5 (45)	4.19 (3.40–4.74)	8 (73)	6.25 (4.26–6.63)	2 (100)	3.58 (3.45–3.71)	0.027
Streptococcus	0 (0)	–	0 (0)	–	0 (0)	–	1 (9)	5.78	0 (0)	–	0.653
Lactobacillus	1 (17)	2.70	1 (13)	2.65	0 (0)	–	4 (36)	4.47 (3.43–5.02)	1 (50)	8.85	0.139
Bifidobacterium	0 (0)	–	0 (0)	–	1 (9)	2.00	0 (0)	–	0 (0)	–	0.653
Klebsiella	1 (17)	5.88	1 (13)	1.70	2 (18)	6.30 (6.17–6.42)	0 (0)	–	1 (50)	8.18	0.292
Serratia	1 (17)	2.70	2 (25)	6.05 (4.70–7.40)	1 (9)	6.88	2 (18)	6.14 (5.66–6.62)	1 (50)	9.06	0.575
Escherichia	0 (0)	–	0 (0)	–	2 (18)	3.33 (3.01–3.64)	1 (9)	5.22	1 (50)	2.78	0.269

*Friedman test.

communities were observed across samples (**Figure 4**). The genera *Serratia*, *Klebsiella*, and *Streptococcus* were most abundant in GC with a median relative abundance of 15.68, 9.59, and 9.07%, respectively (**Table 4**).

Gastric content samples were collected through the nasogastric tubes by syringe aspiration. Since this route was shared with OMM or DM samples, analyses of feeding samples by sequencing the 16S rRNA gene were also performed. Globally, a high degree of similarity between GC and milk samples was observed at the genus level, although the bacterial pattern of OMM samples were more similar to GC than DM (**Figure 4**). However, the statistical analysis did not show any significant difference (**Table 4**) between these three types of samples.

DISCUSSION

In this work, the microbiota and microbiome of the GC of a population of preterm infants during their first month of life at the NICU were studied. Traditionally, the human stomach was considered to be an inhospitable environment for microorganisms, with limited bacterial colonization and survival, because of the acidic conditions, fast peristalsis, and other antimicrobial factors. This led to the assumption that the human stomach did not harbor a complex microbiota. Initially, cultivation of gastric juice or mucosal biopsies identified several members of the *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Fusobacteria* phyla in relatively low abundance (37). Bacterial viable counts in gastric luminal material usually ranges from non-detectable to 10^6 CFU/mL (38), but the values may be strongly dependent on different factors, such as the part of the stomach, the actual gastric pH, diet, fastening time, or ethnicity (38, 39). The spectrum of values obtained in this work was also wide and within the range cited above.

The discovery of the genus *Helicobacter*, and the subsequent interest in the mechanisms by which these organisms adapt to the gastric environment, fueled research on the gastric microbiome (24–29, 31). The results of these studies have shown that the stomach's microbiome is far more complex than initially expected and confirmed the dominance of this niche by members of the phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Fusobacteria*, in addition to some belonging to the phylum *Bacteroidetes*. *Streptococcus*, *Lactobacillus*, *Veillonella*, and *Prevotella* seem to be the dominant genera in healthy hosts, while *Helicobacter* was the most abundant in the human stomach of subjects who tested positive for this organism by using conventional clinical approaches. A vast number of microorganisms ($>10^{10}$ CFU) may enter the human stomach every, day and as a consequence, a clear differentiation between truly resident from transient (swalled) microbial species is difficult. However, microbiome analysis has revealed that bacterial sequences in the stomach are not simply a random sampling of bacterial sequences from oral, upper respiratory tract, or esophageal communities, a fact suggesting that the presence of distinct bacterial communities adapted to the specific gastric environment (24, 40, 41).

In contrast to adults, there is an almost complete lack of data in relation to the microorganisms present in the GC of preterm neonates. Obviously, there are many environmental and medical

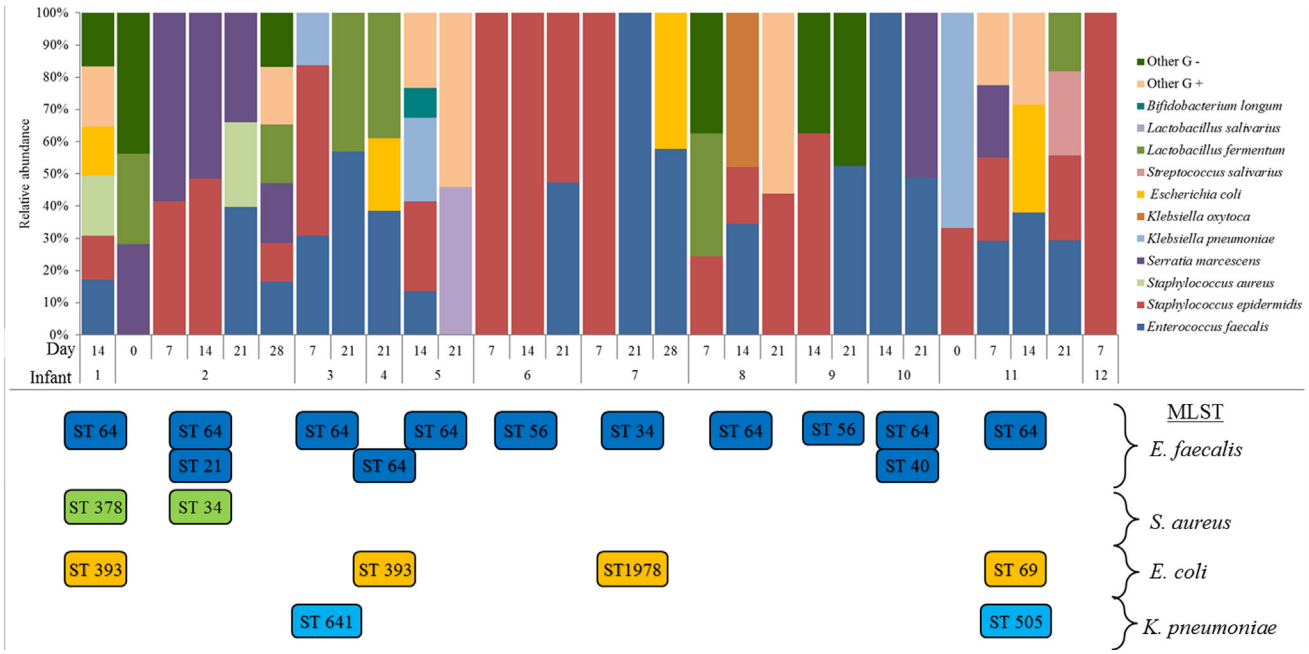

FIGURE 1 | **Bacterial species isolated from the gastric content samples analyzed in this study**. ST clones of *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* detected by MLST genotyping in each infant and at each sampling time are shown below the graph.

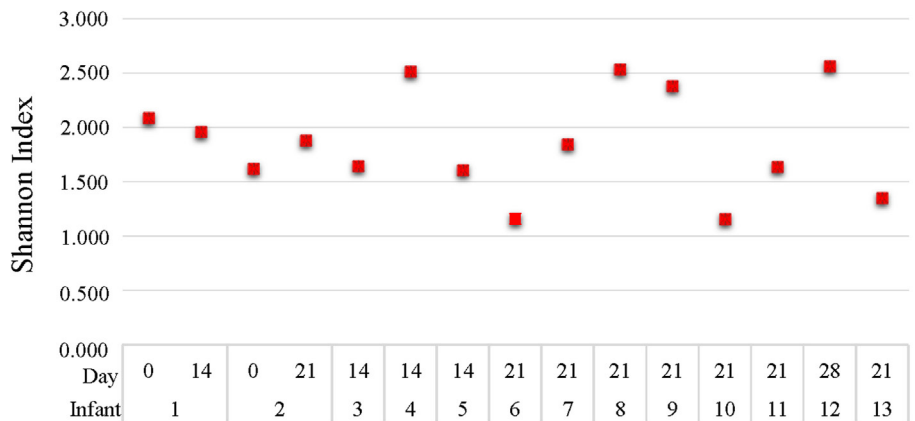

FIGURE 2 | **Shannon diversity index of gastric content samples analyzed with 16S rRNA**.

differences among adults, term neonates, and preterms, and in addition, there are notable anatomical and physiological differences among the adult, the term neonate, and the preterm neonate stomachs; some of these factors may be determinant in the microbial composition of the preterm GC (32–34).

The culture approach used in this study allowed the isolation and characterization of a relatively ample collection of bacterial isolates belonging to hospital-associated species. Sepsis is one of the main causes of concern in the NICU, and therefore, hospital-adapted high-risk clones that exhibit antibiotic resistance and contain virulence factors are of the uppermost relevance (42, 43). The structure and main characteristics of the *E. faecalis*, *S. aureus*, *E. coli*, and *K. pneumoniae* populations were investigated, and, for these four species, high-risk clones linked to nosocomial infection were detected (e.g., *E. faecalis* ST64). In a previous study involving the same preterm population, a high proportion of antibiotic-resistant high-risk clones were detected in their fecal samples, suggesting a high degree of similarity between the fecal and the gastric microbiotas of preterm neonates (36). It has been reported that gastric hypochlorhydria in adults leads to an increased presence of intestinal bacteria in gastric samples (31).

Preterm infants are routinely tube-fed until they are physiologically ready for coordination of sucking, swallowing, and breathing, which often occurs at 33–36 weeks of postmenstrual age (44, 45). Therefore, any type of feed must be applied through the same feeding device as long as it is placed in a given neonate. Few studies have considered the role of neonatal NEFTs as a site of bacterial colonization and, consequently, as a source of bacteria

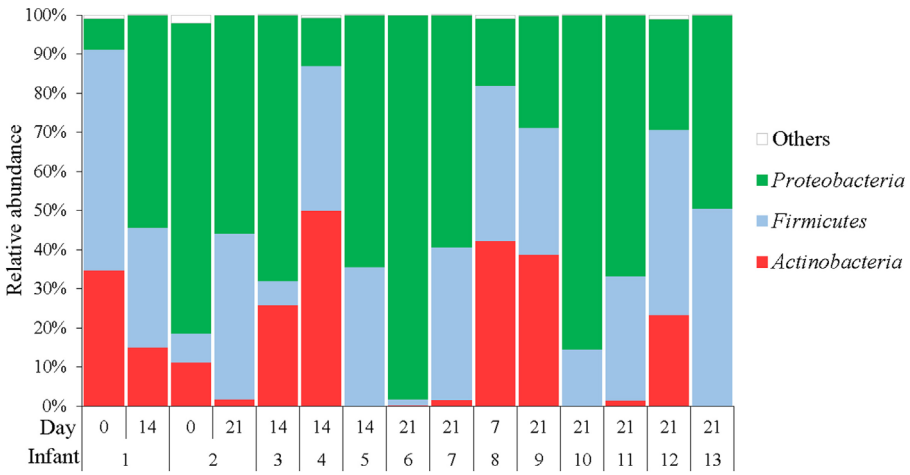

FIGURE 3 | Relative abundances of operational taxonomic units at phylum level accounting for >1% of the total bacterial community are shown for each sample.

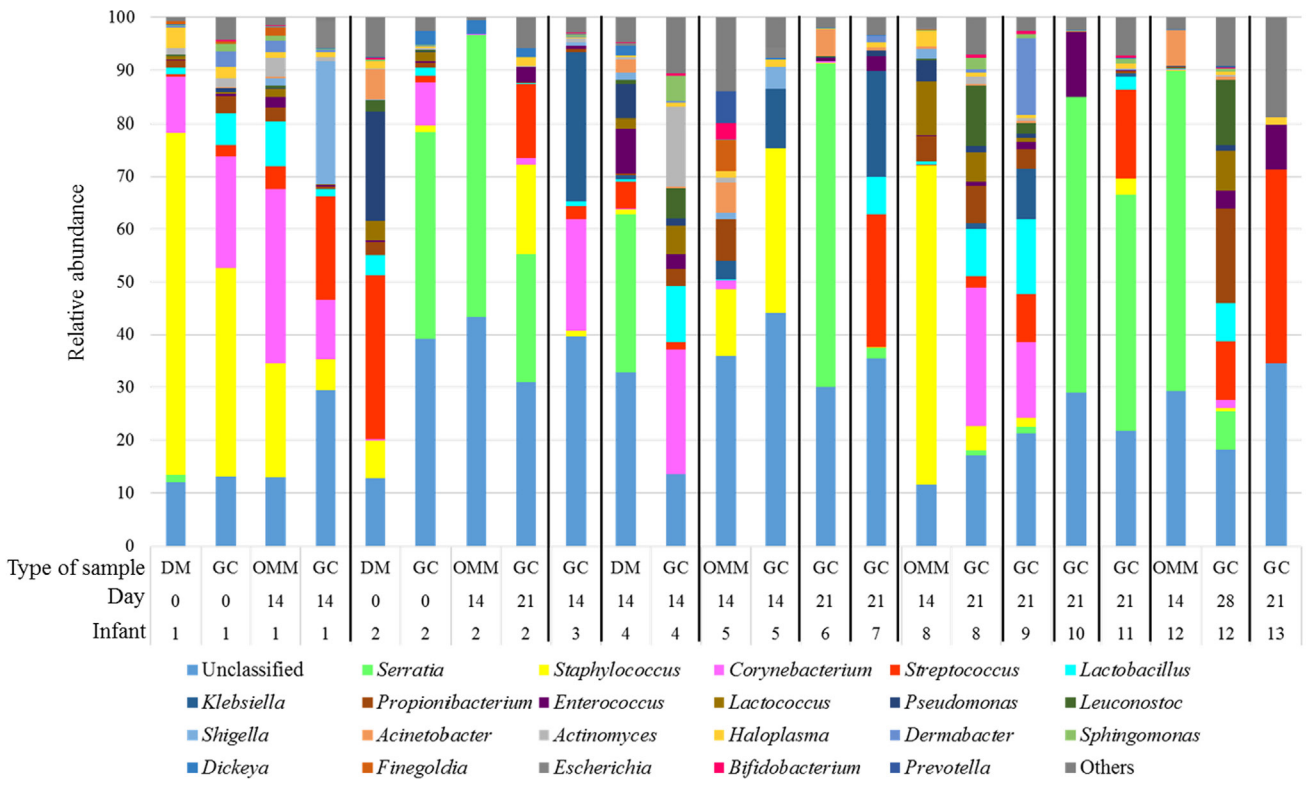

FIGURE 4 | Relative abundances of operational taxonomic units at genus level accounting for >1% of the total bacterial community are shown for gastric content (GC), own mother's milk (OMM), and donor milk (DM) samples.

for preterm infants and the influence of the feeding regime on the pattern of colonization of such devices. However, such studies have revealed the consistent presence of staphylococci (*S. epidermidis*, *S. aureus*), enterococci (*E. faecalis*, *Enterococcus faecium*) and *Enterobacteriaceae* (*K. pneumoniae*, *S. marcescens*, *Enterobacter cancerogenus*, *Enterobacter cloacae*, *E. coli*, and so on), including clones harboring antibiotic resistance genes, from the inner wall of most enteral feeding tubes analyzed so far (35, 36, 46). In a previous work (36), we detected the same bacterial genotypes of different genera in OMM, DM, and formula milk after their passage through the EFT and the fecal samples of different infants. SEM analysis of the internal surfaces of some sets of NEFTs revealed that complex microbial biofilms were formed when such devices were placed for at least more than 12 h.

TABLE 4 | Relative abundance of bacterial genera represented ≥0.5% of the communities detected in gastric content (GC) and human milk samples.

Phylum	Genus	GC, median (n = 15)	Own mother's milk (OMM), median (n = 5)	Donor milk (DM), median (n = 3)	*P* value 1	*P* value 2
Actinobacteria	*Actinomyces*	0.58	2.16	0.36	0.197	0.735
	Bifidobacterium	0.46	1.62	0.20	0.558	0.127
	Corynebacterium	11.21	0.98	0.28	0.602	0.311
	Dermabacter	0.54	2.16	0.32	0.439	0.414
Bacteroidetes	*Prevotella*	0.28	5.87	0.13	–	0.221
	Chryseobacterium	–	0.22*	0.84*	–	–
Firmicutes	*Propionibacterium*	0.92	3.63	1.38	0.514	0.484
	Enterococcus	1.04	0.18	0.27	0.131	0.341
	Gemella	3.32*	–	0.09*		0.485**
	Lactobacillus	4.16	0.33	1.26	0.115	0.312
	Lactococcus	4.24*	5.79*	20.14*	0.515**	0.170**
	Leuconostoc	1.97	0.34	0.67	0.305	0.380
	Staphylococcus	2.41	17.12	7.11	0.833	0.112
	Streptococcus	9.07	0.10	5.10	0.122	0.062
	Finegoldia	0.42	1.43	0.65	0.655	–
	Peptoniphilus	0.41	1.96	0.11	1.000	–
Proteobacteria	*Agrobacterium*	0.06*	0.40*	1.12*	0.604**	0.180**
	Dickeya	0.14	1.39	0.98	0.296	0.117
	Pseudomonas	0.89	0.12	13.59	0.683	0.307
	Sphingomonas	0.71	0.12	0.27	0.229	0.157
	Acinetobacter	0.36	3.14	4.14	0.089	0.079
	Klebsiella	9.59	1.84	0.79	0.380	–
	Leclercia	1.39	0.05	0.22	0.134	0.739
	Serratia	15.68	56.86	15.68	0.133	–
	Shigella	0.57	1.34	0.79	0.655	0.770
Tenericutes	*Haloplasma*	0.78	1.29	1.44	0.086	–

P value 1: Kruskal–Wallis was used to compare GC and OMM.
P value 2: Kruskal–Wallis was used to compare GC and DM.
**Mean of the relative abundance.*
***ANOVA test.*

Aspiration of GC through the NEFTs was probably the source of bacteria proliferating in feeding systems and the source of bacteria that contaminated the milk samples. This would explain why, despite interindividual variability, the microbiome profiles of GC and milk samples were so similar in this study.

The 16S rRNA sequencing analysis revealed the dominance of two phyla (*Firmicutes* and *Proteobacteria*) in our gastric samples, which is in agreement with the results of a previous study (34). However, in the cited work, *Firmicutes* was the most abundant phylum, accounting for 50% of the reads, while *Proteobacteria* was the dominant one in our samples (33%). All the *Firmicutes* genera detected by Milisavljevic et al. (34) were also found in our samples, while there was no complete agreement regarding the bacterial genera belonging to the phylum *Proteobacteria*. In relation to the phylum *Actinobacteria*, *Corynebacterium* was the most abundant genus in both works. The presence of facultative anaerobic bacteria (streptococci, staphylococci, lactic acid bacteria, and enterobacteria, etc.) is a distinctive feature in the gastrointestinal tract of neonates, while strict anaerobes dominate in the adult gastrointestinal tract. This fact can explain why anaerobic bacteria are also dominant in the adult stomach (24–29, 31).

Globally, the results of this study show that GC of preterm neonates is dominated by Proteobacteria and Firmicutes and harbors high-risk bacterial clones, which may colonize enteral feeding tubes. Later, the preterm gastrointestinal tract is reinoculated with such bacteria when milk or preterm formula are administered through the same system. Therefore, future strategies to reduce bacterial contamination with high-risk clones of enteral feeding systems while preserving the potential transfer of beneficial bacteria should be devised. They may include a replacement of enteral feeding tubes as frequent as possible, the frame of a feasible NICU's management, and/or the precoating of the internal surfaces of the tubes with probiotic bacteria specifically targeted for the inhibition of sepsis-related microorganisms.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethical Committee of the Hospital Universitario 12 de Octubre with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethical Committee of the Hospital Universitario 12 de Octubre.

AUTHOR CONTRIBUTIONS

LM, MG, EJ, GB, JA, AM, DE, LF, RC, and JR conceived and designed the experiments; LM, MG, EJ, JA, and RC performed the experiments; GB, AM, and DE recruited all the volunteers and collected the samples and the clinical data; LM, MG, EJ, JA, LF, and RC analyzed the data; LF, RC, and JR contributed with reagents/materials/analysis tools. All the authors wrote and read the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fnut.2017.00012/full#supplementary-material.

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Fecal Short-Chain Fatty Acid Variations by Breastfeeding Status in Infants at 4 Months: Differences in Relative versus Absolute Concentrations

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Our gut microbiota provide a number of important functions, one of which is the metabolism of dietary fiber and other macronutrients that are undigested by the host. The main products of this fermentation process are short-chain fatty acids (SCFAs) and other intermediate metabolites including lactate and succinate. Production of these metabolites is dependent on diet and gut microbiota composition. There is increasing evidence for the role of SCFAs in host physiology and metabolic processes as well as chronic inflammatory conditions such as allergic disease and obesity. We aimed to investigate differences in fecal SCFAs and intermediate metabolites in 163 infants at 3–5 months of age according to breastfeeding status. Compared to no exposure to human milk at time of fecal sample collection, exclusive breastfeeding was associated with lower absolute concentrations of total SCFAs, acetate, butyrate, propionate, valerate, isobutyrate, and isovalerate, yet higher concentrations of lactate. Further, the relative proportion of acetate was higher with exclusive breastfeeding. Compared to non-breastfed infants, those exclusively breastfed were four times more likely (aOR 4.50, 95% CI 1.58–12.82) to have a higher proportion of acetate relative to other SCFAs in their gut. This association was independent of birth mode, intrapartum antibiotics, infant sex, age, recruitment site, and maternal BMI or socioeconomic status. Our study confirms that breastfeeding strongly influences the composition of fecal microbial metabolites in infancy.

Keywords: short-chain fatty acids, lactate, succinate, breastfeeding, infants, gut microbiota

INTRODUCTION

Gut microbiota have coevolved over millennia in a largely symbiotic relationship with the host. For the majority of time, human milk has been the sole source of nutrition for infants, providing essential nutrients for infant growth, as well as bioactive components to stimulate the gut microbiome. Both breastfeeding and gut microbial composition have been associated with a number of

health outcomes during infancy and later life (1). In addition to providing a wide range of pathogen exclusion and immune and biosynthesis functions (2), a major role of gut microbiota is the metabolism of dietary fiber and other complex macronutrients that escape digestion in the small intestine.

The main products of nutrient breakdown by microbes are short-chain fatty acids (SCFAs), the predominant ones being acetate, butyrate, and propionate and to a lesser degree branched-chain fatty acids (BCFAs), valerate, isobutyrate, and isovalerate (3). Less well studied are lactate and succinate, intermediate metabolites in the microbial production of SCFAs (4). In adults, the majority of the SCFAs are rapidly absorbed or used as an energy source by colonocytes (5). There is increasing evidence that gut microbial metabolites have wider systemic effects in the host through their action as signaling molecules and involvement in regulators of gene expression (6, 7). SCFAs have been linked to appetite suppression by activating free fatty acid receptors in the intestine and increasing circulating anorectic gut hormones (3). They have also been shown to play an important role in the activation and differentiation of immune cells and have been implicated in inflammatory and allergic disease (3, 8, 9).

The quantity and relative proportion of metabolites produced in the colon of adults has been closely linked to diet, as well as microbiota composition, diversity, and activity (10). Infancy represents a rapid period of gut microbial development, which is shaped by early-life events such as birth mode, antibiotic administration, and importantly, infant feeding (11–13). Human milk provides optimal nutrition for infants in the first 6 months of life and contains significant amounts of carbohydrates that escape digestion in the small intestine, identified as human milk oligosaccharides (HMOs), which are the preferred substrates for certain gut microbiota in the production of SCFAs (14). Studies have shown that infants who are exclusively breastfed have lower microbial diversity, with a predominance in Proteobacteria and Actinobacteria (most notably bifidobacteria), whereas formula-fed infants tend to have a more diverse microbiota with increased abundance of Clostridia and *Bacteroides* species (15–17).

Gut microbiota dysbiosis and subsequent changes to metabolite profiles may be particularly important in infancy, which presents a critical window of opportunity in programming of future metabolic and immune health. Understanding how diet in early life can shape gut microbiota-associated metabolites is therefore of interest. While studies have previously reported differences in fecal SCFAs by infant diet (18–24), many had a small sample size, and few studies have reported on differences in relative proportions of SCFAs or intermediate metabolites. In addition, limited studies to date have assessed the impact of other birth factors, implicated in early microbiota development, on these associations (21).

We aimed to investigate differences in fecal SCFAs and intermediate metabolites in infants at 3–5 months of age according to breastfeeding. Specifically, we investigated whether fecal total and individual SCFAs, lactate, and succinate (measured as both absolute concentrations and relative proportions) differed according to breastfeeding status at the time of metabolite measurement and duration of exclusive breastfeeding. A secondary aim was to investigate whether these associations were independent of birth mode and intrapartum antibiotics use, as well as other early-life factors.

MATERIALS AND METHODS

Study Design and Covariates

The study included an unselected subset of 163 infants from the Canadian Healthy Infant Longitudinal Development national population-based birth cohort (25) (www.canadianchildstudy.ca) whose mothers were enrolled between November 2009 and February 2012 and who had fecal samples available for analysis collected between 3 and 5 months of age.

Mothers reported infant feeding practices using standardized questionnaires administered at 3, 6, and 12 months postpartum. Questionnaires asked mothers to record if they had ever breastfed their child, if they were currently breastfeeding their child, and the age of the child when breastfeeding ceased, if applicable. Mothers were also asked questions regarding formula feeding initiation and cessation. From these questionnaires, variables on feeding habits between birth and fecal sample collection were calculated. These included whether infants had ever been breastfed, duration of exclusive breastfeeding (never, <3 months, and ≥3 months), and breastfeeding status at the time of fecal sample collection (exclusively breastfed, partially breastfed, and not breastfed).

Mode of birth [classified as vaginal, elective, or emergency cesarean section (CS)] and maternal intrapartum antibiotic prophylaxis (IAP) were extracted from hospital records. This was used to create a four-category variable for birth mode/IAP exposure: vaginal birth (no IAP), vaginal birth IAP, elective cesarean, and emergency cesarean, which we have previously reported on in association with infant gut microbiota (11). Information on other covariates including maternal age, ethnicity and education, infant sex, gestational age, and birth weight were obtained from hospital records or through standardized questionnaires completed by mothers. Maternal weight status [body mass index, weight (kilograms)/height (square meter)] was calculated from height and weight data taken from hospital records or measured at the 1-year postpartum clinic visit. There was a small amount of missing data for some variables (see **Table 1**).

Sample Collection, Preparation, and Nuclear Magnetic Resonance (NMR) Analysis

Samples were analyzed using NMR spectroscopy, which allows for the simultaneous measurement of a wide range of metabolites in a sample and has been successfully applied to metabolite measurement in fecal samples (26). Fecal samples (fresh or refrigerated for a short period) were collected using a standard protocol at a home visit at a mean age of 3.7 months (SD 0.47). Samples were refrigerated during transport and stored at −80°C until analysis.

Approximately 100 mg of fecal sample was homogenized and quickly transferred to an Eppendorf tube. One milliliter of ice cold water was added to the fecal powder and vortexed vigorously for 5 min followed by sonication at 4°C for 20 min. The samples were further subjected to vortex shaking at 250 rpm for 20 min. The fecal water extract thus obtained was centrifuged

TABLE 1 | Study characteristics.

Characteristic	
Mean (SD)	
Maternal age (years) (n = 162)	32 (4.7)
Gestational age (weeks) (n = 155)	38.9 (1.5)
Birth weight (g) (n = 158)	3,462 (503)
Age at stool sample collection (months), *n* (%)	3.65 (0.47)
City of birth, *n* (%)	
Edmonton	52 (32)
Vancouver	95 (58)
Winnipeg	16 (10)
Infant sex, *n* (%)	
Male	90 (55)
Female	73 (45)
Maternal ethnicity, *n* (%) (*n* = 160)	
Caucasian	119 (74)
Asian	24 (15)
Other	17 (11)
Birth mode, *n* (%) (*n* = 158)	
Vaginal—no IAP	78 (49)
Vaginal—IAP	31 (20)
Cesarean section—emergency	29 (18)
Cesarean section—elective	20 (13)
Breastfeeding status at fecal sample collection, *n* (%) (*n* = 158)	
None	44 (28)
Partial	66 (42)
Exclusive	48 (30)
Ever breastfed, *n* (%)	
No	17 (10)
Yes	146 (90)
Exclusive breastfeeding duration, *n* (%)	
Never	51 (31)
<3 months	54 (33)
≥3 months	58 (36)

N = 163 unless otherwise specified.
IAP, intrapartum antibiotic prophylaxis.

at 15,000 × *g* for 1 h at 4°C, and the supernatant was carefully aspirated into a fresh Eppendorf tube. This supernatant was centrifuged again at 15,000 × *g* for 1 h at 4°C to precipitate any particulate fecal matter (that might have been introduced during the first separation), and the clear extract was transferred into a clean Eppendorf tube. The resultant fecal water was stored at −20°C until further analysis.

A 570 µL aliquot of fecal water was placed in a 1.5-mL Eppendorf tube followed by the addition of 70 µL of D_2O and 60 µL of standard buffer solution [585 mM $NaHPO_4$ (pH 7.0), 11.667 mM disodium-2,2-dimethyl-2-silapentane-5-sulfonate (DSS), and 0.47% NaN_3 in H_2O]. The samples (700 µL) were then transferred to a regular NMR tube for subsequent NMR spectral analysis.

All ^{1}H-NMR spectra were collected on a Varian 500 MHz Inova spectrometer equipped with a 5-mm HCN Z-gradient pulsed-field gradient cyrogenic probe. ^{1}H-NMR spectra were acquired at 25°C using the first transient of the Varian tnnoesy pulse sequence, which was chosen for its high degree of selective water suppression and quantitative accuracy of resonances around the solvent. Water suppression pulses were calibrated to achieve a bandwidth of 80 G. Spectra were collected with 128 transient and 8 steady-state scans using a 4-s acquisition time (48,000 complex points) and a 1-s recycle delay.

Before spectral analysis, all free induction decays were zero-filled to 64,000 data points and line broadened 0.5 Hz. The methyl singlet produced by a known quantity of DSS was used as an internal standard for chemical shift referencing (set to 0 ppm) and for quantification. All ^{1}H-NMR spectra were processed and analyzed using the Chenomx NMR Suite Professional software package version 8.1 (Chenomx Inc., Edmonton, AB, Canada). The Chenomx NMR Suite software allows for qualitative and quantitative analysis of an NMR spectrum by manually fitting spectral signatures from an internal database to the spectrum. Typically 90% of visible peaks were assigned to a compound, and more than 90% of the spectral area could be routinely fit using the Chenomx spectral analysis software. Most of the visible peaks were annotated with a compound name.

Statistical Analysis

Fecal metabolites were analyzed as absolute concentrations (micromoles per gram of feces) and as relative proportions (%) of total SCFAs/BCFAs (labelled as SCFAs for short, and taken as the sum of acetate, butyrate, propionate, valerate, isobutyrate, and isovalerate). Median concentrations of total SCFAs (acetate, butyrate, propionate, valerate, isobutyrate, and isovalerate) and individual SCFAs as well as lactate and succinate were compared according to breastfeeding duration and breastfeeding at time of fecal sample collection using Kruskal–Wallis test (non-parametric ANOVA, with Bonferroni posttest for multiple comparison). Median relative proportions of SCFAs were also compared using Kruskal–Wallis test (with Bonferroni posttest).

Given the skewed nature of the data, fecal metabolites were categorized into a binary variable (high and low) using the median as a cut point, and associations with breastfeeding at stool collection were tested using logistic regression. Models were adjusted for birth mode and IAP exposure, age of stool sample collection as well as maternal and infant factors found to be associated with breastfeeding in our study (infant sex, city of birth, maternal education and BMI). All analysis was conducted using IBM SPSS (version 24). Statistical significance was considered when $P \leq 0.05$.

RESULTS

Fecal samples were collected from 163 infants between the ages of 3–5 months (mean age 3.65 ± 0.47 months). Subject characteristics of our sample are shown in **Table 1**. At fecal sample collection, 30% were exclusively breastfed and 42% of infants were partially breastfed. Twenty-two (14.2%) infants had started solid foods. The majority of infants (69%) were born vaginally, while almost a third of infants (31%) were born by CS. Twenty-eight percent of vaginally delivered infants received IAP, and all cesarean infants received IAP as is standard practice in Canada.

Breastfeeding exclusivity at fecal sample collection differed by city of birth (**Table 2**). Males were less likely to be exclusively breastfed than females (25 versus 37%), as were infants born to overweight or obese mothers and mothers with a lower level of

TABLE 2 | Associations between breastfeeding and subject characteristics.

Characteristic	Breastfeeding status, *N* (%)			
	None	Partial	Exclusive	*P* value
City of birth				
Edmonton	19 (37)	25 (49)	7 (14)	0.002
Vancouver	18 (20)	35 (38)	39 (42)	
Winnipeg	7 (47)	6 (40)	2 (13)	
Infant sex				
Male	22 (25)	44 (50)	22 (25)	0.06
Female	22 (31)	22 (31)	26 (37)	
Maternal ethnicity (*n* = 155)				
Caucasian	31 (27)	50 (44)	33 (29)	0.96
Asian	6 (25)	10 (42)	8 (33)	
Other	5 (29)	6 (35)	6 (35)	
Maternal education (*n* = 155)				
Less than University	29 (46)	23 (37)	11 (18)	<0.001
University or higher	14 (15)	42 (46)	36 (39)	
Maternal BMI (kg/m^2) (*n* = 155)				
Normal (<25)	20 (22)	35 (39)	36 (40)	0.02
Overweight or obese (≥25)	23 (36)	29 (45)	12 (19)	
Birth weight (*n* = 153)				
≤4 kg	38 (29)	54 (41)	41 (31)	0.45
>4 kg	5 (25)	11 (55)	4 (20)	
Gestational age (*n* = 150)				
Early term (<38 weeks)	4 (20)	9 (45)	7 (35)	0.78
Term (38–40)	33 (30)	45 (41)	32 (29)	
Late term (41+ weeks)	6 (30)	10 (50)	4 (20)	
Birth mode (*n* = 153)				
Vaginal—no IAP	24 (32)	26 (34)	26 (34)	0.34
Vaginal—IAP	5 (17)	16 (55)	8 (28)	
Cesarean section—emergency	8 (29)	11 (39)	9 (32)	
Cesarean section—elective	6 (30)	11 (55)	3 (15)	

Breastfeeding status at fecal sample collection. N = 158 unless otherwise specified.
P value Pearson chi-square test.
IAP, intrapartum antibiotic prophylaxis.

education. Breastfeeding also differed according to birth mode, with exclusive breastfeeding at the time of fecal collection being highest in vaginally delivered infants not exposed to IAP and lowest in elective CS infants (34 and 15%, respectively), although this difference was not statistically significant (chi-square $P = 0.16$).

Absolute Concentrations of SCFAs and Intermediate Metabolites and Breastfeeding Status

Concentrations (micromoles per gram of feces) of SCFAs, lactate, and succinate in all infants are presented in **Table 3**. Total SCFA concentration was 142.0 μmol/g with the most abundant SCFA being acetate followed by propionate and butyrate.

In univariate analysis, infants who had ever been breastfed had lower concentrations of total SCFAs, acetate, butyrate, propionate, valerate, isobutyrate, and isovalerate and higher concentrations of lactate and succinate than those who had never been breastfed (**Table 3**). Compared to those not being breastfed at time of fecal metabolite profiling, those who were exclusively breastfed had significantly lower total SCFAs concentrations and

TABLE 3 | Median concentrations of short-chain fatty acid (SCFA) and intermediate metabolites according to breastfeeding status and duration.

Metabolite (μmol/g) [interquartile range (IQR)]	All infants (*n* = 163)	Breastfeeding status[a] (*n* = 158)			Ever breastfed (*n* = 163)		Exclusive breastfeeding duration (*n* = 163)		
		None (*n* = 44)	Partial (*n* = 66)	Exclusive (*n* = 48)	No (*n* = 17)	Yes (*n* = 146)	Never (*n* = 51)	<3 months (*n* = 54)	≥3 months (*n* = 58)
Total SCFA[b]	142.0 (101.6–203.8)	177.8 (125.7–241.6)	161.4 (126.1–233.6)	99.4*** (63.8–130.5)	185.0 (135.1–239.7)	138.9 (96.3–199.3)	176.0 (134.1–239.9)	173.6 (122.5–235.5)	96.9*** (60.4–133.4)
Acetate	116.5 (74.9–165.6)	135.6 (86.2–190.5)	128.8 (81.0–179.3)	79.4*** (52.7–121.3)	132.8 (109.4–193.9)	115.0 (73.2–164.7)	146.5 (97.2–200.8)	126.5 (87.0–185.6)	75.9*** (50.5–123.6)
Butyrate	6.6 (1.9–12.5)	11.7 (5.8–18.0)	7.1* (3.7–11.6)	1.6*** (0.44–7.2)	11.5 (6.1–15.8)	6.1* (1.7–12.0)	9.2 (4.8–13.9)	7.8 (3.7–16.3)	1.8*** (0.47–7.2)
Propionate	14.5 (4.6–28.4)	19.7 (13.7–32.7)	19.6 (9.6–34.1)	4.3*** (1.7–11.1)	22.1 (17.4–34.8)	12.7* (4.3–27.2)	22.5 (13.5–34.9)	17.2 (10.9–35.3)	4.7*** (1.9–13.1)
Valerate	1.2 (0.42–2.3)	1.9 (1.2–2.8)	1.3 (0.60–2.4)	0.39*** (0.15–1.2)	1.7 (0.72–2.8)	1.2 (0.41–2.3)	1.7 (0.66–2.8)	1.6 (0.81–2.8)	0.41*** (0.19–1.3)
Isobutyrate	0.67 (0.22–1.7)	1.3 (0.69–2.2)	0.59 (0.26–2.0)	0.20*** (0.07–0.72)	1.6 (0.77–3.3)	0.57** (0.20–1.7)	1.2 (0.50–1.2)	0.83 (0.40–2.2)	0.20*** (0.08–0.69)
Isovalerate	1.0 (0.34–2.4)	1.9 (1.2–3.5)	1.3* (0.43–2.8)	0.33*** (0.09–0.91)	3.0 (1.8–3.9)	0.92*** (0.30–2.2)	1.6 (0.79–3.1)	1.6 (0.80–3.1)	0.36*** (0.10–0.95)
Lactate	3.7 (1.5–18.5)	2.3 (1.3–3.2)	4.7* (1.5–12.4)	7.2*** (2.2–32.7)	2.4 (1.4–2.7)	4.7 (1.6–20.2)	3.5 (1.6–15.7)	2.5 (1.2–8.2)	6.6 (2.2–29.9)
Succinate	10.4 (3.1–29.6)	4.3 (1.6–17.7)	21.8*** (4.2–49.7)	8.4 (3.4–30.6)	3.3 (1.9–18.7)	11.9 (3.4–34.5)	7.9 (2.7–24.5)	11.3 (3.5–31.8)	10.6 (3.2–37.1)

Values are presented as median and IQR in micromoles per gram of feces. Comparisons by non-parametric Mann–Whitney U test (two groups) or Kruskal–Wallis test (three groups, with Bonferroni posttest for multiple comparison) versus none or never breastfeeding category.
****P < 0.001.*
***P < 0.01.*
**P < 0.05.*
[a]Breastfeeding status at stool sample collection.
[b]Total SCFAs represent the sum of acetate, butyrate, propionate, valerate, isobutyrate, and isovalerate.

lower concentrations of all individual SCFAs (**Table 3**). Lactate was significantly higher in those exclusively breastfed versus those not breastfed (7.2 versus 2.3 μmol/g; $P < 0.001$). Those who were partially breastfed had similar concentrations of total SCFAs, acetate, and propionate compared to those not breastfed at the time of fecal metabolite profiling or concentrations falling midway between those not breastfed and those exclusively breastfed (seen for butyrate, valerate, isobutyrate, isovalerate, and lactate). Similar differences were observed according to duration of exclusive breastfeeding with those breastfed for ≥3 months having significantly lower concentrations of SCFAs than infants who had never been breastfed (**Table 3**). Similar results were observed when data were restricted to infants who had not started solid foods (Table S1 in Supplementary Material) and vaginally delivered infants without exposure to IAP (Table S3 in Supplementary Material).

Relative Proportions of SCFAs and Breastfeeding

In all infants, acetate made up the largest proportion of the SCFAs (80%) followed by propionate (10%) and butyrate (5%) with isovalerate being the least abundant (**Table 4**). In univariate analysis, infants exclusively breastfed at time of fecal metabolite profiling had significantly higher relative proportions of acetate and lower proportions of butyrate, propionate, isobutyrate, and isovalerate compared to those not breastfed (**Table 4**; **Figure 1**). Similar results were seen for those exclusively breastfed ≥3 months versus those never exclusively breastfed (**Table 4**). Compared to infants who had never been breastfed, breastfed infants also tended to have higher proportions of acetate and lower proportions of butyrate, propionate, isobutyrate, and isovalerate although differences were only significant for the latter two metabolites. Similar trends were observed when data were restricted to infants who had not started solid foods (Table S2 in Supplementary Material) and infants vaginally delivered without exposure to IAP (Table S4 in Supplementary Material), although loss in statistical significance was observed in some instances.

The higher relative proportion of acetate seen in exclusively breastfed infants is in contrast to the lower overall concentration of acetate in fecal samples of these infants as illustrated in **Figure 2**.

Odds of High SCFAs and Intermediate Metabolites According to Breastfeeding, Adjusted for Birth Mode, and IAP

Exclusively breastfed infants had 86% lower odds of having high absolute concentrations of total SCFAs (concentrations greater than the group median) compared to those not breastfed at metabolite profiling [odds ratio (OR) 0.14, 95% CI 0.06–0.38; **Table 5**]. Odds of high total SCFAs was not significantly lower in those partially breastfed versus not breastfed (OR 0.92, 95% CI 0.42–2.05), although partial breastfeeding was associated with reduced odds of having high butyrate, valerate, isobutyrate, and isovalerate. Exclusively breastfed infants also had significantly reduced odds of having high acetate, butyrate, propionate,

TABLE 4 | Relative proportions of total short-chain fatty acid according to breastfeeding status and duration.

Metabolite% [interquartile range (IQR)]	All infants ($n = 163$)	Breastfeeding status[a] ($n = 158$)			Ever breastfed ($n = 163$)		Exclusive breastfeeding duration ($n = 163$)		
		None ($n = 44$)	Partial ($n = 66$)	Exclusive ($n = 48$)	No ($n = 17$)	Yes ($n = 146$)	Never ($n = 51$)	<3 months ($n = 54$)	≥3 months ($n = 58$)
Acetate	79.9 (72.7–87.9)	77.3 (71.0–81.3)	79.4 (68.5–87.2)	86.7*** (77.0–95.5)	77.5 (73.2–81.7)	80.5 (72.3–89.4)	78.8 (71.0–86.0)	77.2 (70.1–83.9)	85.5* (75.6–94.9)
Butyrate	4.9 (1.5–7.7)	6.5 (4.5–8.2)	5.0 (2.1–7.6)	1.6*** (0.43–5.8)	6.4 (4.9–6.9)	4.0 (1.2–8.0)	5.6 (3.1–6.9)	6.2 (2.3–8.5)	1.9* (0.48–6.1)
Propionate	10.1 (4.4–16.8)	13.5 (7.1–19.0)	12.5 (5.4–19.5)	4.6*** (2.2–10.6)	13.4 (7.4–16.8)	9.9 (4.1–16.3)	12.4 (6.9–19.7)	13.9 (5.6–19.4)	5.7** (2.6–12.4)
Valerate	0.79 (0.41–1.46)	0.97 (0.63–1.6)	0.87 (0.36–1.4)	0.48** (0.24–1.3)	0.83 (0.60–1.5)	0.79 (0.36–1.4)	0.88 (0.41–1.6)	0.93 (0.60–1.4)	0.5 (0.25–1.3)
Isobutyrate	0.42 (0.18–0.91)	0.69 (0.39–1.2)	0.4 (0.23–1.0)	0.28*** (0.10–0.66)	0.85 (0.53–1.5)	0.37** (0.17–0.87)	0.64 (0.27–1.2)	0.48 (0.29–1.1)	0.27** (0.10–0.65)
Isovalerate	0.75 (0.25–1.5)	1.1 (0.82–1.8)	0.75* (0.29–1.6)	0.34*** (0.14–1.0)	1.56 (1.0–2.6)	0.64*** (0.22–1.4)	0.98 (0.42–1.8)	0.95 (0.59–1.8)	0.35** (0.17–1.0)

Values are presented as median relative proportions (%) and IQR. Comparisons by non-parametric Mann–Whitney U test (two groups) or Kruskal–Wallis test (three groups, with Bonferroni posttest for multiple comparison) versus none or never breastfed category.

****$P < 0.001$.*

***$P < 0.01$.*

**$P < 0.05$.*

[a]Breastfeeding status at fecal sample collection.

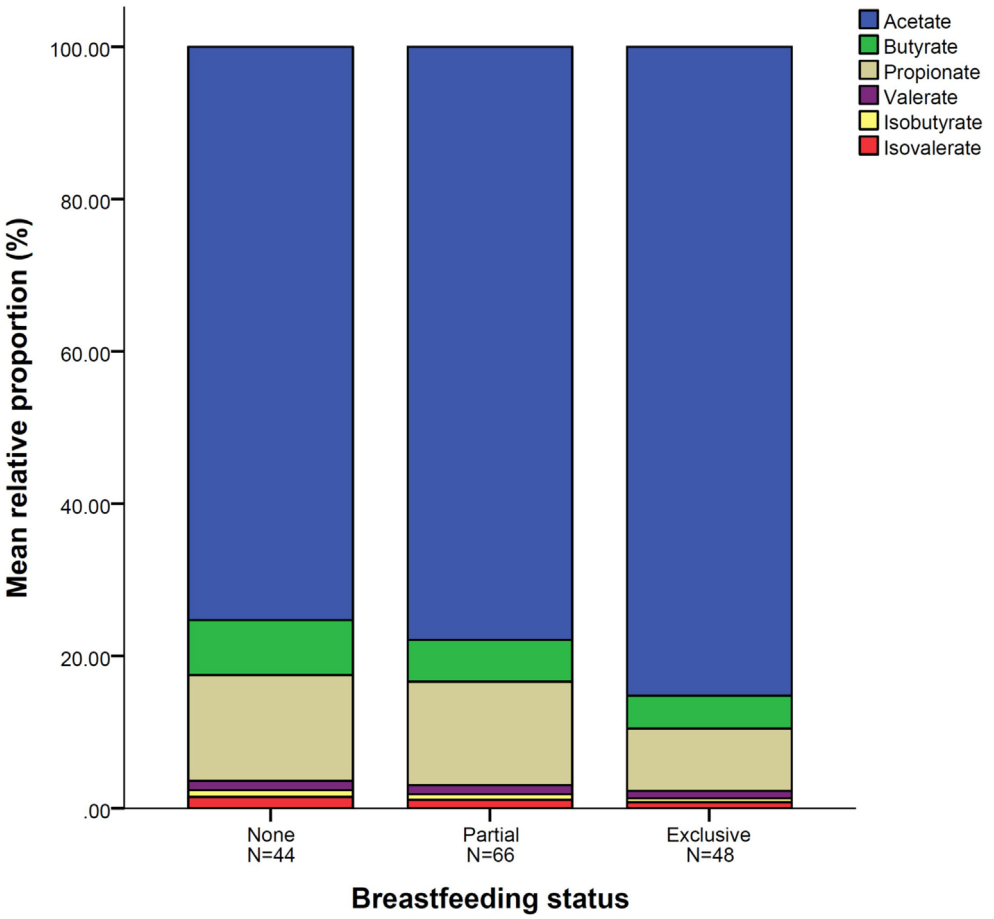

FIGURE 1 | **Mean relative proportion of short-chain fatty acid according to breastfeeding status**. *N* = 158. Breastfeeding status at fecal sample collection.

valerate, isobutyrate, and isovalerate compared to those not breastfed (**Table 5**). Similar results were seen in these infants following adjustment for birth mode and IAP, as well as age at stool collection, sex, city of birth, maternal education, and BMI (**Table 5**).

Exclusively breastfed infants were seven times more likely to have high concentrations of lactate, and partially breastfed infants four times more likely than those not breastfed (OR 7.81, 95% CI 2.99–20.37 and OR 4.56, 95% CI 1.88–11.04, respectively; **Table 5**). These associations remained after adjustment for covariates. Partially breastfed infants, but not exclusively breastfed infants, had significantly higher odds of high succinate (OR 3.45, 95% CI 1.53–7.79).

Infants exclusively breastfed were over four times more likely to have high relative proportions of acetate compared to those not breastfed (OR 4.26, 95% CI 1.76–10.36; **Table 6**). This association was independent of birth mode and IAP, as well as other covariates (aOR 4.50, 95% CI 1.58–12.82). In line with this, odds of high butyrate, propionate, valerate, isobutyrate, and isovalerate were significantly lower in exclusively breastfed infants. Associations with valerate did not remain after adjustment with all covariates. Partial breastfeeding was associated with reduced odds of high butyrate (OR 0.34, 95% CI 0.15–0.80), and isovalerate (OR 0.36, 95% CI 0.16–0.81) although these associations were no longer significant after additional adjustment for maternal education and BMI.

DISCUSSION

In this subsample of 163 Canadian infants aged between 3 and 5 months from a general population birth cohort, we found strong associations between breastfeeding exclusivity and infant fecal metabolites. Exclusive breastfeeding was associated with lower absolute concentrations of total SCFAs, acetate, butyrate, propionate, valerate, isobutyrate, and isovalerate, yet higher concentrations of lactate. Further, the relative proportion of acetate was higher with exclusive breastfeeding. Compared to non-breastfed infants, those exclusively breastfed were four times more likely (aOR 4.50, 95% CI 1.58–12.82) to have a higher proportion of acetate relative to other SCFAs in their gut. This association was independent of birth mode, intrapartum antibiotics, infant sex, age, recruitment site, and maternal BMI or socioeconomic status.

Our results are similar to that of Edwards et al. who also found a predominance in relative proportions of acetate (76%) in breastfed infants at 4 weeks of age (19). Higher relative abundance

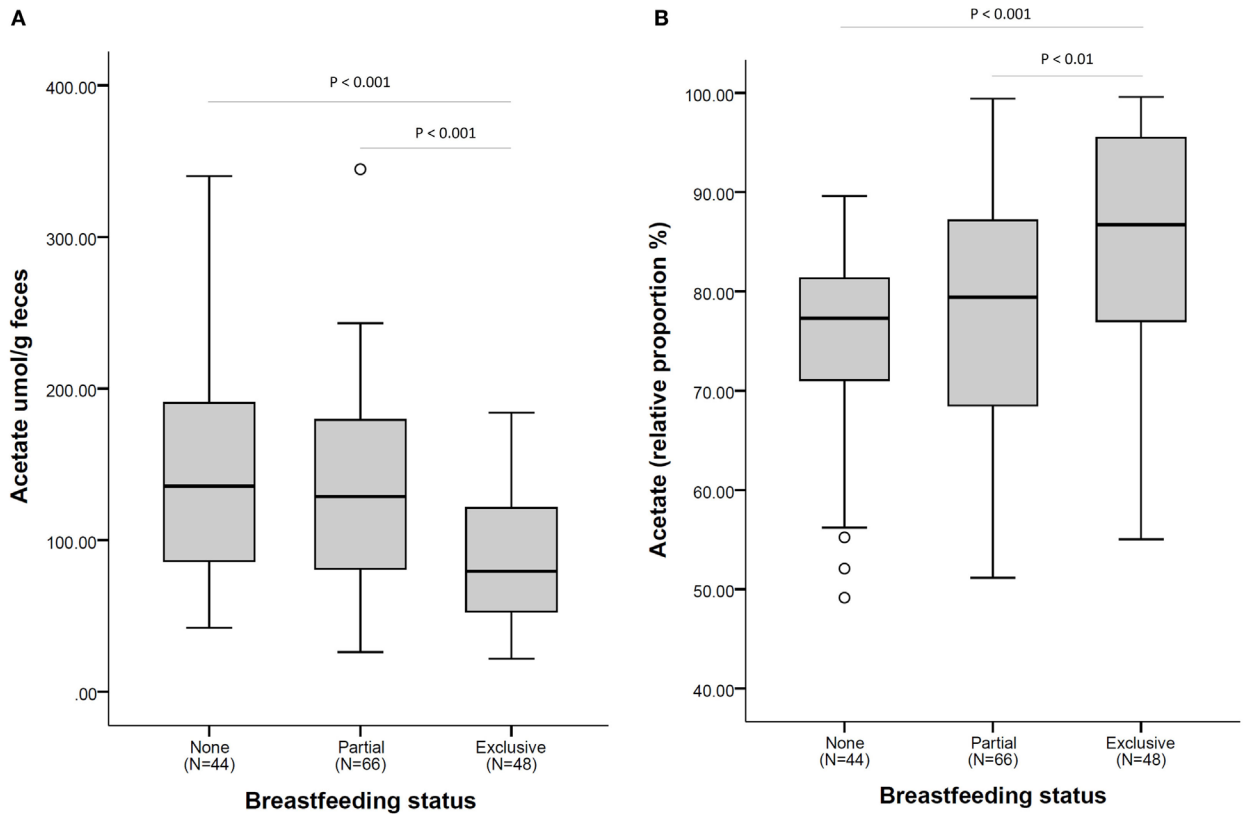

FIGURE 2 | Acetate fecal concentration (A) and as a relative proportion of total short-chain fatty acid (B), according to breastfeeding status at fecal sample collection. *N* = 158. Comparisons by non-parametric Kruskal–Wallis test (with Bonferroni posttest for multiple comparison). Box plots present the group median (thick black line), upper quartile (top of box), and lower quartile (bottom of box). Whiskers present the maximum and minimum values excluding outliers (denoted by circles).

of acetate in exclusively breastfed infants may in part be due to the presence of HMOs in breast milk, not present in formula. HMOs, which represent the third largest component of breast milk, are soluble carbohydrates that are undigested by the host and provide substrates for select gut microbiota (27). *Bifidobacterium* species have been shown to be one of a narrow selection of gut bacteria that are able to metabolize HMO (28) and subsequently are overrepresented in the microbiota of breastfed infants compared to formula-fed infants (11, 16, 29). Results from our group by Azad et al. found the *Bifidobacteriaceae* family to be enriched with breastfeeding (11). Bifidobacterium have been shown to metabolize HMO to produce acetate and lactate (30, 31). Martin et al. observed that increases in bifidobacteria counts corresponded to increases in fecal acetate (12). Although evidence is limited, higher acetate in breastfed infants may afford protection against intestinal pathogens and allergic disease (3). Fukudo et al. demonstrated that acetate, produced by bifidobacteria, improved intestinal defense and protected against *Escherichia coli* O157:H7 in mice (30). Thornburn et al. have proposed that acetate-mediated inhibition of histone deacetylases, demonstrated in an adult mouse model, increases transcription of the Foxp3 gene that may promote Treg-suppressing airway inflammation and inducing oral tolerance. In infants, Arrieta et al. found that reduced fecal acetate at 3 months was associated with allergic disease in later infancy (32).

We also observed lower absolute concentrations of SCFAs in exclusively breastfed infants, which are consistent with other published studies. In 111 fecal samples analyzed by NMR, Martin et al. found lower concentrations of SCFAs in breastfed infants at 3 and 6 months born to overweight or obese mothers (20). In a small study on 4 infants using GC and LC mass spectrometry, valerate and isovalerate concentrations were higher in formula-fed infants, the latter over 40 times higher than breastfed infants (18). A study of 67 infants not only found lower fecal SCFA concentrations in breastfed infants but also observed that the addition of milk (formula or cows milk) to the diet of breastfed infants was sufficient to change the SCFA profile (22). We observed similar results, in that partially breastfed infants had SCFA and lactate concentrations more similar to exclusively formula-fed infants than infants exclusively breastfed.

The higher absolute concentrations of SCFAs observed in formula-fed infants may be a consequence of greater bacterial diversity observed in these infants in comparison to breastfed fed infants (11) and thus increased capability to metabolize substrates present in the gut. Indeed, previous results from our group found breastfeeding exclusivity to be inversely associated

TABLE 5 | Associations between breastfeeding status and high metabolite concentrations, adjusting for birth mode and IAP, and other covariates.

Breastfeeding status	Number of infants with high metabolite concentration, *n/N* (%) (*n* = 158)	*P* value for trend*	Odds of high metabolite concentration			
			Unadjusted OR (95% CI) (*n* = 153)	Model 1 aOR (95% CI) (*n* = 153)	Model 2 aOR (95% CI) (*n* = 153)	Model 3 aOR (95% CI) (*n* = 148)
Total SCFA						
None	28/44 (63.6)	<0.001	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Partial	41/66 (62.1)		0.92 (0.42–2.05)	0.89 (0.40–2.01)	0.79 (0.34–1.83)	0.73 (0.30–1.75)
Exclusive	9/48 (18.8)		0.14 (0.06–0.38)	0.14 (0.05–0.36)	0.13 (0.05–0.38)	0.13 (0.04–0.39)
Acetate						
None	25/44 (56.8)	0.01	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Partial	43/66 (65.2)		1.38 (0.62–3.05)	1.45 (0.64–3.30)	1.27 (0.54–3.00)	1.33 (0.54–3.28)
Exclusive	14/48 (29.2)		0.32 (0.13–0.75)	0.29 (0.12–0.71)	0.30 (0.11–0.79)	0.30 (0.11–0.86)
Butyrate						
None	33/44 (75.0)	<0.001	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Partial	37/66 (56.1)		0.37 (0.15–0.87)	0.37 (0.16–0.90)	0.35 (0.14–0.87)	0.42 (0.16–1.11)
Exclusive	13/48 (27.1)		0.11 (0.04–0.28)	0.11 (0.04–0.29)	0.12 (0.04–0.35)	0.13 (0.04–0.39)
Propionate						
None	33/44 (75.0)	<0.001	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Partial	40/66 (60.6)		0.50 (0.22–1.17)	0.45 (0.18–1.09)	0.42 (0.17–1.05)	0.48 (0.18–1.25)
Exclusive	8/48 (16.7)		0.07 (0.03–0.20)	0.68 (0.02–0.18)	0.06 (0.02–0.19)	0.07 (0.02–0.24)
Valerate						
None	32/44 (72.7)	<0.001	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Partial	33/66 (50.0)		0.32 (0.14–0.75)	0.31 (0.13–0.74)	0.35 (0.14–0.86)	0.42 (0.16–1.07)
Exclusive	12/48 (25.0)		0.11 (0.04–0.28)	0.10 (0.04–0.27)	0.13 (0.04–0.36)	0.15 (0.05–0.45)
Isobutyrate						
None	34/44 (77.3)	<0.001	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Partial	31/66 (47.0)		0.25 (0.11–0.59)	0.23 (0.09–0.55)	0.18 (0.07–0.47)	0.23 (0.09–0.63)
Exclusive	15/48 (31.3)		0.13 (0.05–0.34)	0.13 (0.05–0.35)	0.12 (0.04–0.33)	0.15 (0.05–0.45)
Isovalerate						
None	34/44 (77.3)	<0.001	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Partial	34/66 (51.5)		0.30 (0.13–0.72)	0.30 (0.12–0.71)	0.25 (0.10–0.63)	0.33 (0.13–0.86)
Exclusive	11/25 (22.9)		0.08 (0.03–0.23)	0.09 (0.03–0.23)	0.08 (0.03–0.22)	0.09 (0.03–0.29)
Lactate						
None	10/44 (22.7)	<0.001	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Partial	36/66 (54.5)		4.56 (1.88–11.04)	4.51 (1.82–11.18)	5.75 (2.16–15.26)	5.37 (1.90–15.15)
Exclusive	32/48 (66.7)		7.81 (2.99–20.37)	8.81 (3.27–23.72)	13.94 (4.39–44.24)	12.29 (3.64–41.46)
Succinate						
None	15/44 (34.1)	0.15	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Partial	41/66 (62.1)		3.45 (1.53–7.79)	3.25 (1.42–7.41)	3.03 (1.30–7.02)	3.21 (1.31–7.88)
Exclusive	24/48 (50.0)		2.07 (0.88–4.90)	2.03 (0.85–4.84)	1.85 (0.72–4.72)	1.93 (0.71–5.22)

Breastfeeding status at fecal sample collection. High metabolite concentration denotes values above the median. Model 1: adjusted for birth mode and IAP; model 2: adjusted for birth mode and IAP, age of stool collection, sex, and city of birth; model 3: adjusted for birth mode and IAP, age of stool collection, sex, city of birth, maternal BMI, and education. OR, odds ratio; SCFA, short-chain fatty acid; IAP, intrapartum antibiotic prophylaxis.

**Pearson chi-square P value for linear trend (univariate).*

with both microbiota richness and diversity (11). Our finding may also be a result of differences in the composition or absorption of breast milk versus formula milk and thus differences in availability of substrates. Further, higher concentrations of branch-chain fatty acids, valerate, isobutyrate and isovalerate, derived from the metabolism of amino acids indicate reduced protein absorption or excess protein intake [potentially due to higher protein content of formula versus human milk (33)] in formula-fed infants. The availability of these substrates likely drive the higher abundance of proteolytic bacteria such as Bacteroides and Clostridia seen in formula-fed infants as reported in our study by Azad et al. (11). Higher concentrations of proteolytic metabolites in formula-fed infants may also be due to reduced carbohydrate availability in the absence of HMOs and hence to greater derivation of energy from protein metabolism. Chow et al. demonstrated that when fermentable carbohydrates were not present in fecal cultures from both breast and formula-fed infants, metabolites indicative of protein fermentation were mainly produced; their production was reduced after the addition of various fermentable carbohydrate substrates similar to HMOs. Increased fecal SCFAs seen in formula-fed infants may have metabolic consequences. Several studies have reported greater concentrations of fecal SCFAs in overweight adults and children compared to lean counterparts (34–38) and correlations with other metabolic risk factors (38). Although causality is yet to be established, the authors of these studies hypothesize that higher SCFAs may reflect an increased capacity of the gut microbiota to harvest energy from the diet.

TABLE 6 | Associations between breastfeeding status and high relative proportions of SCFA, adjusting for birth mode and IAP, and other covariates.

Breastfeeding status	Number of infants with high relative proportion of metabolite, *n/N* (%) (*n* = 158)	*P* value for trend*	Odds of high relative proportion of metabolite			
			Unadjusted OR (95% CI) (*n* = 153)	Model 1 aOR (95% CI) (*n* = 153)	Model 2 aOR (95% CI) (*n* = 153)	Model 3 aOR (95% CI) (*n* = 148)
Acetate						
None	15/44 (34.1)	<0.001	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Partial	35/66 (53.0)		2.25 (1.02–5.00)	2.25 (0.99–5.09)	2.18 (0.94–5.09)	2.12 (0.86–5.23)
Exclusive	34/48 (70.8)		4.26 (1.76–10.36)	4.38 (1.78–10.83)	5.28 (1.96–14.26)	4.50 (1.58–12.82)
Butyrate						
None	32/44 (72.7)	<0.001	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Partial	34/66 (51.5)		0.34 (0.15–0.80)	0.33 (0.14–0.78)	0.35 (0.14–0.87)	0.51 (0.19–1.35)
Exclusive	16/48 (33.3)		0.17 (0.07–0.42)	0.17 (0.07–0.43)	0.17 (0.06–0.47)	0.22 (0.07–0.65)
Propionate						
None	27/44 (61.4)	0.001	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Partial	37/66 (56.1)		0.79 (0.36–1.73)	0.79 (0.35–1.79)	0.68 (0.29–1.59)	0.72 (0.29–1.77)
Exclusive	13/48 (27.1)		0.26 (0.11–0.63)	0.24 (0.10–0.60)	0.21 (0.08–0.56)	0.25 (0.09–0.71)
Valerate						
None	25/44 (56.8)	0.04	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Partial	32/66 (48.5)		0.64 (0.29–1.39)	0.58 (0.26–1.30)	0.65 (0.28–1.50)	0.84 (0.35–2.06)
Exclusive	17/48 (35.4)		0.38 (0.16–0.91)	0.39 (0.16–0.92)	0.44 (0.17–1.12)	0.58 (0.21–1.58)
Isobutyrate						
None	30/44 (68.2)	0.002	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Partial	33/66 (50.0)		0.45 (0.20–1.01)	0.42 (0.19–0.97)	0.37 (0.16–0.88)	0.53 (0.21–1.33)
Exclusive	17/48 (35.4)		0.26 (0.11–0.62)	0.26 (0.11–0.64)	0.22 (0.08–0.58)	0.30 (0.11–0.85)
Isovalerate						
None	32/44 (72.7)	<0.001	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Partial	31/66 (47.0)		0.32 (0.14–0.73)	0.33 (0.14–0.77)	0.33 (0.14–0.78)	0.45 (0.18–1.09)
Exclusive	13/48 (27.1)		0.14 (0.05–0.35)	0.13 (0.05–0.34)	0.13 (0.05–0.35)	0.16 (0.06–0.47)

Breastfeeding status at fecal sample collection. High metabolite proportion denotes values above the median. Model 1: adjusted for birth mode and IAP; model 2: adjusted for birth mode and IAP, age of stool collection, sex, and city of birth; model 3: adjusted for birth mode and IAP, age of stool collection, sex, city of birth, maternal BMI, and education.
OR, odds ratio; SCFA, short-chain fatty acid; IAP, intrapartum antibiotic prophylaxis.
**Pearson chi-square P value for linear trend (univariate).*

In addition to SCFAs, we also measured concentrations of lactate and succinate. Both are important intermediates in the production of SCFAs (3). Succinate is primarily converted to propionate through the succinate pathway. Lactate, produced by many colonizers of the infant gut including bifidobacteria and lactobacilli, can also be converted to propionate through the acrylate pathway but is also an important substrate used in cross-feeding by lactate-utilizing bacteria in the production of SCFAs as demonstrated by Pham et al. (24). Similar to other studies (18–20, 22), we observed higher lactate concentrations in exclusively breastfed infants, which is indicative of the predominance of lactate-producing bacteria, *Lactobacillus* and bifidobacteria, in the gut of breastfed infants. Lactate production in the gut affects luminal pH, and stool from breastfed infants typically has a lower fecal pH compared to formula-fed infants (5.8 versus 6.3–7.10) (22, 39). Studies in adults have suggested that accumulation of lactate in the gut may have undesirable health consequences including increased risk of ulcerative colitis; however, in infancy, it may be an important mechanism in preventing overgrowth of pH-sensitive pathogenic bacteria, such as Enterobacteriaceae and Clostridia (40, 41). Lactate has also been shown to be important in modulating immune and inflammatory processes and maintaining gut barrier function through stimulation of enterocyte proliferation (42). We are the first to report on differences in fecal succinate in infants. Although understudied, succinate may be an important signaling molecule and has been shown to activate GPR91 on dendritic cells and thus may play a role in modulation of gut immunity and inflammation (6, 43). Unlike the other associations we observed, differences in succinate were only apparent in those partially breastfed. This observation may in part be explained by the higher abundance of Bacteroidetes observed in partially breastfed infants reported in our study infants by Azad et al (11). Bacteroidetes utilize the succinate pathway in the formation of propionate (4, 44).

One of the strengths of our study is the use of infants from a well-characterized general birth cohort, affording detailed information on birth and early-life characteristics. This, along with our relatively large sample size, enabled us to account for prenatal and postnatal factors shown to impact early gut microbiota development (most importantly birth mode and perinatal antibiotic exposure) in our estimates of associations between fecal metabolites and breastfeeding. While we adjusted for IAP, shown to be significantly associated with gut microbiota dysbiosis in early life (11), we did not additionally adjust for postnatal infant antibiotic use due to missing data in our sample. However, it has been shown that IAP is a good proxy for perinatal antibiotic use in infancy (11). One limitation of our study was the indirect measurement of luminal metabolites *via* analysis of fecal samples. Concentrations

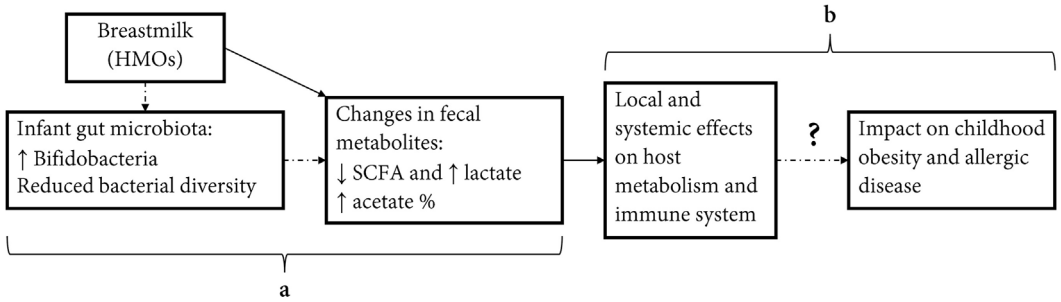

FIGURE 3 | Effect of infant diet on fecal short-chain fatty acids (SCFAs) and potential effect on host metabolic and immune programming. Human milk feeding causes changes in infant fecal metabolites, potentially through effects on gut microbiota composition (a). Changes in concentrations and relative proportions of SCFA and intermediate metabolites exert local effects on the gut environment and act as signaling molecules effecting host metabolism and immune system. These actions may have important programming effects on inflammatory-mediated diseases in childhood, including obesity and allergic disease (b).

of metabolites in feces are a function not only of production but also of absorption, utilization by other microbes and stool transit time. Few studies have estimated whether fecal SCFA output is a reliable proxy for luminal content, particularly in early childhood although it is estimated that 95% of the SCFAs produced in the gut are rapidly absorbed with only 5% being excreted in the feces (45). In a study on healthy adults, Vogt and Wolever found fecal acetate to be inversely correlated to acetate absorption, suggesting that fecal acetate concentration may reflect intestinal absorption rather than production (46). However, given that the potential of analyzing fecal metabolites is to provide a biomarker to predict future disease risk, analysis of fecal samples provides a non-invasive and cost-effective method for use in epidemiological cohort studies. Our sampling was also only at one time point in infancy, and therefore, our data do not provide information on trends in metabolites over time in relation to diet.

In conclusion, exclusive breastfeeding has been associated with a number of beneficial health outcomes in early childhood including reduced infections, allergic disease, and improved metabolic markers (1). Recent research has highlighted the importance of our gut microbiota and associated metabolites as a potential causal factor or mediator in the programming of later disease states. Our study confirms that breastfeeding is strongly associated with gut metabolites, which may be an important mediator in the protective effect of breastfeeding on later-onset diseases (**Figure 3**). Given differences seen in the study between metabolites measured as absolute concentrations and relative proportions, we encourage future studies to report on both of these measures in relation to health outcomes.

ETHICS STATEMENT

Written informed consent was obtained from parents at enrollment. This study was approved by the University of Alberta, University of British Columbia and University of Manitoba, and McMaster University Human Research Ethics Boards.

AUTHOR CONTRIBUTIONS

AK, SB, MA, CF, AH, PM, and DW conceptualized the research question. SB analyzed the data and drafted the manuscript. SB and AK interpreted findings and edited the manuscript. MA created breastfeeding measures for use in the analysis, contributed to interpretation of findings, and edited the manuscript. DW conducted NMR analysis on the fecal samples. JS, MS, AB, PM, PS, and ST directed the design and implementation of the CHILD study from which these samples were drawn. All authors revised and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fnut.2017.00011/full#supplementary-material.

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Gut Colonization by Methanogenic Archaea Is Associated with Organic Dairy Consumption in Children

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The gut microbiota represents a complex and diverse ecosystem with a profound impact on human health, promoting immune maturation, and host metabolism as well as colonization resistance. Important members that have often been disregarded are the methanogenic archaea. Methanogenic archaea reduce hydrogen levels via the production of methane, thereby stimulating food fermentation by saccharolytic bacteria. On the other hand, colonization by archaea has been suggested to promote a number of gastrointestinal and metabolic diseases such as colorectal cancer, inflammatory bowel disease, and obesity. Archaea have been shown to be absent during infancy while omnipresent in school-aged children, suggesting that colonization may result from environmental exposure during childhood. The factors that determine the acquisition of methanogenic archaea, however, have remained undefined. Therefore, we aimed to explore determinants associated with the acquisition of the two main gastrointestinal archaeal species, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*, in children. Within the context of the KOALA Birth Cohort Study, fecal samples from 472 children aged 6–10 years were analyzed for the abundance of *M. smithii* and *M. stadtmanae* using qPCR. Environmental factors such as diet, lifestyle, hygiene, child rearing, and medication were recorded by repeated questionnaires. The relationship between these determinants and the presence and abundance of archaea was analyzed by logistic and linear regression respectively. Three hundred and sixty-nine out of the 472 children (78.2%) were colonized by *M. smithii*, and 39 out of the 472 children (8.3%) by *M. stadtmanae*. The consumption of organic yogurt (odds ratio: 4.25, CI95: 1.51; 11.95) and the consumption of organic milk (odds ratio: 5.58, CI95: 1.83; 17.01) were positively associated with the presence of *M. smithii*. We subsequently screened raw milk, processed milk, and yogurt samples for methanogens. We identified milk products as possible source for *M. smithii*, but not *M. stadtmanae*. In conclusion, *M. smithii* seems present in milk products and their consumption may determine archaeal gut colonization

in children. For the first time, a large variety of determinants have been explored in association with gut colonization by methanogenic archaea. Although more information is needed to confirm and unravel the mechanisms in detail, it provides new insights on microbial colonization processes in early life.

Keywords: microbiota, gut, infant, child, archaea, dairy products, milk, *M. smithii*

INTRODUCTION

The human gut contains a complex and diverse ecosystem consisting of hundreds of microbial species that are acquired during the first years of life (van Best et al., 2015). Although a myriad of bacterial species have been studied within the human infant gut, important colonizers that are often disregarded are the methanogenic archaea (Horz, 2015). At present, five methanogenic archaea species and two halophilic archaea have been isolated from human feces from which only the *Methanobrevibacter smithii* (*M. smithii)*, *Methanosphaera stadtmanae* (*M. stadtmanae),* and *Methanomassiliicoccus luminyensis* (*M. luminyensis)* have been detected more than once (Miller et al., 1982; Miller and Wolin, 1985; Dridi et al., 2012a; Khelaifia et al., 2013, 2017; Khelaifia and Raoult, 2016). A previous study from Dridi et al. showed that the most dominant archaeal gut inhabitant is *M. smithii* with a prevalence of 88% in children of 0–10 years of age. In contrast, *M. stadtmanae* and *M. luminyensis* tend to colonize the child's gut less frequently with a prevalence of 11 and 1%, respectively (Dridi et al., 2012b).

Upon colonization, methanogenic archaea are responsible for producing the majority of methane in the gut by reducing carbon dioxide into methane in the presence of hydrogen (Roccarina et al., 2010). The hydrogen in the gut is mainly the result of bacterial fermentation, and accumulation of hydrogen subsequently inhibits this process of breaking down food components for energy. Therefore, reduction of hydrogen levels by methanogens stimulates food fermentation by saccarolytic bacteria (Horz and Conrads, 2010; Gaci et al., 2014). On the other hand, colonization by archaea has been suggested to be potentially detrimental for host health due to alterations in gut metabolism and syntrophic interactions with other microbes (Cavicchioli et al., 2003; Conway de Macario and Macario, 2009; Nakamura et al., 2010; Roccarina et al., 2010; Gill and Brinkman, 2011). For instance, in previous studies higher levels of archaea and excreted methane were found in patients with gastrointestinal and metabolic diseases such as colorectal cancer, inflammatory bowel disease, irritable bowel syndrome, constipation, and obesity (Haines et al., 1977; Pimentel et al., 2003; Kim et al., 2012; Blais Lecours et al., 2014; Triantafyllou et al., 2014; Mbakwa et al., 2015; Vandeputte et al., 2016).

Methanogenic archaea have been shown to be absent during infancy while omnipresent in school-aged children and their presence seems to increase with age (Dridi et al., 2012b). The latter suggests that colonization may result either through exposure to sources of archaea during childhood, or through factors shaping the gastrointestinal ecophysiology to make the gut more favorable for archaeal colonization during this time period. The factors that determine the acquisition of methanogenic archaea, however, have remained undefined. Although the rumen of beef cattle have been shown to be a carrier for *M. smithii* and *M. stadtmanae* (Carberry et al., 2014), these human gut colonizers have not been identified in selected food products so far (Brusa et al., 1998). Moreover, no study has conducted a comprehensive analysis on potential lifestyle and dietary determinants of human gut colonization by these methanogenic archaea. Therefore, we aimed to explore a wide variety of potential determinants associated with the acquisition of the two main archaeal species, *M. smithii* and *M. stadtmanae,* in children. To this end, we used extensive data on determinants prospectively gathered through repeated questionnaires within the KOALA Birth Cohort Study in combination with data on presence and abundance of *M. smithii* and *M. stadtmanae* obtained from fecal samples in children of 6–10 years.

MATERIALS AND METHODS

Study Population

This study was conducted within the KOALA Birth Cohort Study in the Netherlands. The study design has been described in detail elsewhere (Kummeling et al., 2005). In summary, 2,834 pregnant women were recruited between October 2000 and December 2002. Pregnant women with a conventional lifestyle ($N = 2343$) were recruited from an ongoing cohort study on pregnancy-related pelvic girdle pain in the Netherlands. In addition, pregnant women with an "alternative" lifestyle ($N = 491$) were recruited through organic shops, anthroposophical doctors and midwives, anthroposophical under-five clinics, Steiner schools and magazines for special interest groups. The "alternative" lifestyle was expected to differ from the "conventional" lifestyle in vaccination practices, use of antibiotics, dietary habits (breastfeeding, organic foods, and vegetarian diet) and child rearing practices (Kummeling et al., 2005).

Parents of a subgroup of children ($N = 1{,}204$) were approached for the collection of a fecal sample of their child at age 6–10 (**Figure 1**). Fecal samples were provided by 669 children. Transport time exceeded 4 days for 197 samples, which were therefore excluded from analyses. As a result, quantitative real-time PCR analysis was performed on 472 fecal samples.

Informed consent was given by all parents, and the study was approved by the Medical Ethics Committee of Maastricht University and the National Ethical Committee for Medical Research.

Fecal Sampling

Parents received a stool sample kit consisting of a feces collection tube with a spoon attached to the lid (Sarstedt, Nürnbrecht, Germany) together with an instruction form

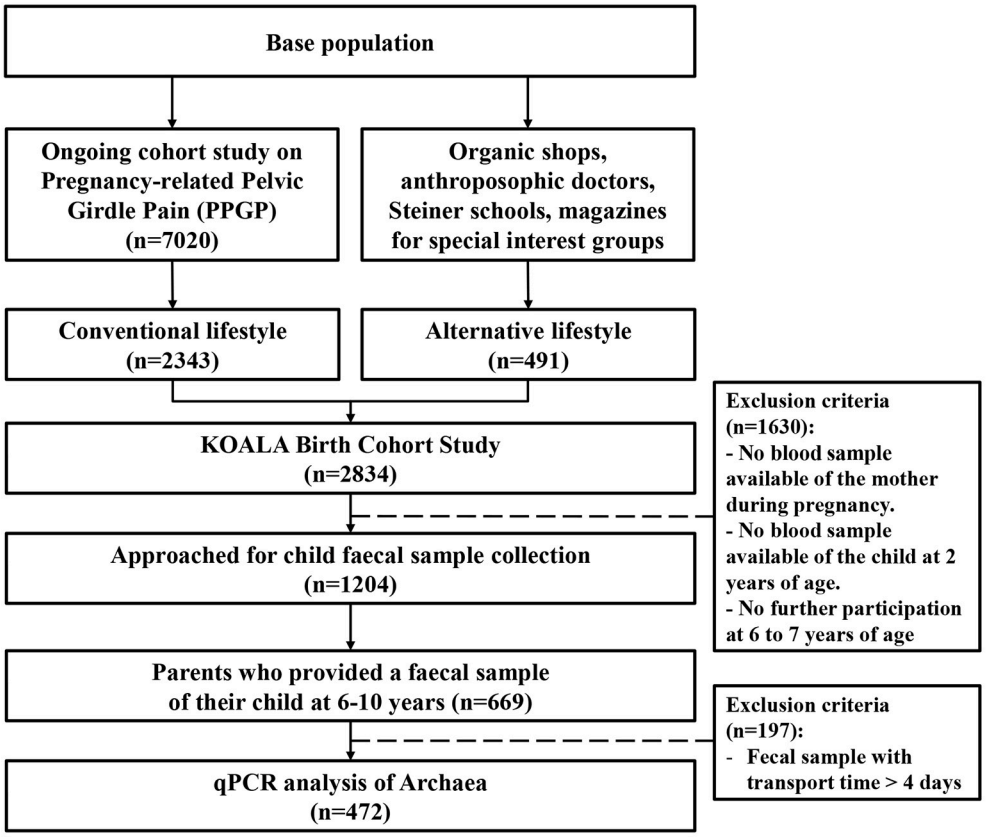

FIGURE 1 | Flowchart illustrating how the population under study (n = 472) was obtained from the KOALA Birth Cohort Study population.

on fecal collection. After collection, the fecal sample was sent to the Department of Medical Microbiology at the Maastricht University Medical Center+ by mail. Upon arrival fecal samples were 10-fold diluted in Peptone Water (Oxoid CM0009) containing 20% (vol/vol) glycerol (Merck, Darmstadt, Germany) and stored at −80°C until further analysis.

Fecal DNA Isolation and Quantitative Real Time PCR

Repeated-Bead-Beating (RBB) plus a column-based purification method was used to isolate DNA as described in detail elsewhere (Zoetendal et al., 2006; Salonen et al., 2010). Afterwards, DNA concentration and purity were determined by the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). For the enumeration of *M. smithii* and *M. stadtmanae* all fecal samples were subjected to 5′-nuclease based real-time PCR assays. Primers and probes employed for PCR and the amplification and quantification process as performed on an Applied Biosystems Prism 7,000 sequence detection system (Applied Biosystems) have been described in detail elsewhere (Mbakwa et al., 2015). In short, quantification of the abundance of *M. smithii* and *M. stadtmanae* in fecal samples was achieved by comparing the cycle threshold (Ct) values to a standard curve. The standard curves were constructed by subjecting serial 10-fold dilutions of positive plasmid constructs containing the target sequences of *M. smithii* and *M. stadtmanae* to the same qPCR assays. The lower limit of detection was 3.81 $\log_{10}$ copies/g feces for *M. smithii* and 4.82 $\log_{10}$ copies/g feces for *M. stadtmanae*. Further details on qPCR assays and conditions can be found elsewhere (Mbakwa et al., 2015).

Determinants

Information on potential determinants was collected by repeated questionnaires in the KOALA Birth Cohort Study. Information was requested from 34 weeks of gestation until 6–7 years of age. Questionnaires covered multiple topics including dietary habits (food frequency questionnaires, FFQ), lifestyle characteristics, hygiene, child rearing practices, diseases, and medication use. Determinants of interest were selected based on findings from previous research on the establishment of the microbiota and the availability of information in repeated questionnaires.

Determinants selected for analysis were: age of the child at time of fecal sample (continuous in years), recruitment group (conventional cohort, alternative cohort), child's gender, maternal education (low: primary school, preparatory vocational or lower general secondary school; middle: vocational, higher general secondary or pre-university; high: higher vocational

or academic education), maternal diet during pregnancy (conventional; organic/biodynamic), place, and mode of delivery (vaginal delivery at home; vaginal delivery in hospital; artificially induced delivery in hospital; cesarean section in hospital), gestational age (continuous [weeks]), birth weight (continuous [grams]), regularly cleaning of pacifier in boiling water (no, yes), duration of pacifier use (never, short [until 7 months], long [at least until 12 months]), first time exposure to antibiotics during the first 2 years of life (Never, at 0–3 months, at 3–7 months, at 7–12 months, at 12–24 months), number of siblings at 1 year of age (0, 1, ≥2), regular stay at daycare during the first 2 years of life (never; at host parent or daycare; host parent and daycare combined), vitamin D/AD supplementation during the first 2 years of life (no; yes), number of siblings at 4–5 years of age (0; 1; ≥2), type of pets kept during life of the child (none; dog; cat; other; combination), exposure to farm (animals) during the last 12 months at 6–7 years of age (never; pigs; chicken/pigeons; ox/goat/sheep/horse; children's farm visits; combination), antibiotic use within 1 year prior to fecal sampling (no; yes), child's diet at 6–7 years (conventional; >25% organic/biodynamic), vegetarian diet of child (no; yes), total energy intake (continuous [kcal]), carbohydrate intake (continuous [en%]), protein intake (continuous [en%]), animal protein intake (continuous [en%]), fiber intake (continuous [grams]), regular intake of organic raw vegetables (no; yes), regular intake of organic fruit (no, yes), regular milk consumption (at least once per week; no, yes), regular organic milk consumption (at least once per week; no, yes), regular cheese consumption (at least once per week; no, yes), regular organic cheese consumption (at least once per week; no, yes), regular yogurt consumption (at least once per week; no, yes), regular organic yogurt consumption (at least once per week; no, yes).

Statistical Analysis

Characteristics of the present study population ($N = 472$) and the total KOALA birth cohort ($N = 2{,}834$) are presented as median plus range for continuous variables and as frequency (n, %) for categorical variables.

Multivariable logistic regression models were used to assess the association between potential determinants and the presence of *M. smithii* and *M. stadmanae*, respectively. Multivariable linear regression models were used to assess the association with *M. smithii* abundance. Variable selection for the regression models was based on purposeful selection as described by Hosmer and Lemeshow (2000). A multivariable model was fitted including all determinants identified from univariable models with a $p < 0.25$. This model was further reduced to only include variables with either a p-value lower than 0.10 or variables that affected the parameter estimate (β) of other variables in the model by more than 20%, as recommended by Bursac et al. (2008). This model was subsequently refitted with variables that were not statistically significant (i.e., $p > 0.25$) in univariable analyses. Afterwards, the model was reduced as described before ($p < 0.10$), but only for the added variables. As a result, all variables included in the main effects model were statistically significant or made an important contribution based on the presence of other variables in the model (Bursac et al., 2008). After completion of purposeful selection the resulting main effects model was additionally adjusted for the following covariables of prior interest: age at time of fecal sampling (years), gender (male, female), total energy intake (kcal), recruitment group (conventional, alternative), and BMI (z-score). For determinants with more than 30 missing values a missing value group was constructed. Models were checked for multicollinearity through Variance Inflation Factor (VIF) scores. If VIF scores exceeded 10, determinants causing multicollinearity were separated into independent models to obtain effect estimates without multicollinearity for the affected determinants. To limit the number of spurious associations, results from multivariable analyses were corrected for multiple comparisons by adjusting α by Benjamini-Hochberg procedure at a false discovery rate (FDR) level of $q = 0.05$ (Benjamini and Hochberg, 1995).

In secondary analyses, the impact of the number of consumed organic dairy products, and organic as well as raw vegetable and fruit products on the presence and abundance of *M. smithii* and *M. stadtmanae* was examined. For this purpose the individual variables for organic dairy (cheese, milk, and yogurt) and organic fruit and raw vegetables were grouped into index variables as follows: number of different organic dairy products (0; 1; 2; 3), number of different organic raw vegetable and fruit products (0; 1; 2). Since organic dairy products were significantly associated with the presence of *M. smithii*, analyses were extended to yogurt, milk and cheese, irrespective of organic or conventional origin.

For all statistical analyses IBM SPSS version 23 (SPSS Inc., Chicago, IL) was used.

Verification of Archaeal Presence in Milk

Raw Bovine Milk in Tanker Trucks

To validate findings from this study, publicly available data from another study was used to identify archaea in 975 raw milk samples collected from 899 tanker trucks (Kable et al., 2016). These individual trucks arrived at two dairy processors in San Joaquin Valley of California for product manufacturing during three seasons between October 2013 and September 2014. The sequences of the 16S rRNA V4 regions were obtained via the Qiita database (https://qiita.ucsd.edu) under study ID 10485, and further analyzed within Qiime (Caporaso et al., 2010)

Metagenomic DNA Isolation from Dairy Products

Processed milk and yogurt samples were collected from biodynamic, organic and non-organic brands from the supermarket (Supplementary Table 1). Raw unprocessed milk was obtained from a local farmer via a standard tap-procedure (Brunimat). Metagenomic DNA was extracted from dairy products with the PowerFood™ Microbial DNA Isolation kit (MoBio Laboratories Inc.) according to manufacturer's instructions as evaluated in detail elsewhere (Quigley et al., 2012). In short, cell lysis with chaotrophic agents and bead-beating plus a column-based extraction was used to isolate DNA.

RESULTS

Fecal samples from 472 children with a median age of 7.2 (6.0–12.0) years were analyzed. Of these, 369 (78.2%) out of 472 were colonized by *M. smithii*, while 39 (8.3%) out of 472 children were colonized by *M. stadtmanae*. Due to the low colonization rate of *M. stadtmanae,* analyses on its abundance were not performed in this study. Supplementary Table 2 shows participant characteristics of the KOALA Birth Cohort Study ($n = 2834$) and the study population ($n = 472$) for all selected determinants. In general, the distribution of participant characteristics of the study population was similar to the population of the entire KOALA Birth Cohort Study.

Methanobrevibacter smithii Presence and Abundance

The multivariable regression models (**Table 1** and Supplementary Table 3) showed a positive association between organic yogurt and organic milk consumption and *M. smithii* colonization. Children who regularly consumed organic yogurt were more than four times as likely to be colonized by *M. smithii* as compared to children who did not consume organic yogurt [$OR_{adjusted}$: 4.25 95% Confidence Interval (CI95): 1.51; 11.95], whereas children consuming organic milk were even over five times more likely to be colonized by *M. smithii* as compared to children who did not consume organic milk ($OR_{adjusted}$: 5.58, CI95: 1.83; 17.01). We subsequently performed secondary analyses to examine the effect of the number of different organic dairy products being consumed on *M. smithii* colonization (**Table 2** and Supplementary Table 4). These analyses showed a statistically significant increasing trend between the number of dairy products consumed and the chance of colonization by *M. smithii* (**Table 2**, $p = 0.002$).

For all other potential determinants that have been examined in the present study, no relationship with *M. smithii* colonization was found. Moreover, when examining the relationship between *M. smithii* colonization and the consumption of dairy products, irrespective of organic or conventional origin, we did neither find an association for milk nor for yogurt and nor for cheese consumption.

In contrast to the analyses on the presence of *M. smithii*, none of the determinants assessed in this study were positively associated with the abundance of *M. smithii* (Supplementary Tables 5, 6).

In models for both *M. smithii* presence and abundance the model adjustment by a priori selected covariates did not meaningfully alter parameter estimates. The minimal effect of these covariates on parameter estimates indicates that models for *M. smithii* were robust.

Methanosphaera stadtmanae Presence

Multivariable regression analyses on the presence of *M. stadtmanae* showed no significant results after FDR correction (Supplementary Table 7). However, after adjustment for a priori selected covariables birth by cesarean section (OR: 6.89, CI95: 2.09; 22.67), first exposure to antibiotics at 13–24 months (OR: 3.38, CI95: 1.34; 8.50) and organic fruit intake (OR: 4.73, CI95: 1.64; 13.62) were associated with an increased *M. stadtmanae* presence (**Table 3**). In secondary analyses to examine the effect of the number of organic fruit and vegetable products consumed on the presence of *M. stadtmanae* no significant trend was found ($p = 0.321$).

In models for *M. stadtmanae* presence minor differences in model estimates were found after adjustment for a priori selected covariates. These differences may indicate underlying relationships between potential determinants and a priori selected covariates or model instability of *M. stadtmanae* models.

TABLE 1 | Final multivariable logistic regression model showing the association between potential determinants and the presence of *Methanobrevibacter smithii*.

	***M. smithii* presence**		
	Adjusted main effects model ($N = 419$)[a]		
Determinant	**OR (CI95%)**	**FDR crit.[b]**	***p*-value**
Regular intake of organic milk[c,d]			
No	Ref.		
Yes	5.58 (1.83; 17.01)	0.006	**0.003[f]**
Regular intake of organic yogurt[c,d]			
No	Ref.		
Yes	4.25 (1.51; 11.95)	0.006	0.006
Diet of child[e]			
Conventional (≤25% organic)	Ref.		
Organic (incl. biodynamic; >25% organic)	0.36 (0.17; 0.79)	0.011	**0.010[f]**
Fiber intake (g)[e]	0.95 (0.88; 1.03)	0.028	0.250

[a] *Model adjusted for: age at fecal sampling (years), gender (male/female), recruitment group (conventional/alternative), total energy intake (kcal), and BMI (z-score).*
[b] *Critical FDR cut-off level as determined by Benjamini-Hochberg procedure.*
[c] *Missing value category omitted (included in FDR correction).*
[d] *Due to multicollinearity both regular intake of organic milk and regular intake of organic yogurt were included in separate models, which included all other variables as listed in this table.*
[e] *Parameter estimates presented for the model containing organic milk intake. Parameter estimates did not change substantially when the variable "organic milk intake" was replaced by the variable "organic yogurt intake" (data not shown).*
[f] *Significant association after correction for FDR by Benjamini-Hochberg procedure, also indicated in bold.*

Verification of Archaeal Presence in Milk

To evaluate the contribution of milk and yogurt to gut colonization by archaea, we re-analyzed 16S sequence-data of raw milk from a previous study (Kable et al., 2016) as only the bacterial results of these samples from 899 tanker trucks were reported in the original publication. The relative abundances showed that the vast majority of all recovered archaeal sequences could be assigned to the genera *Methanobrevibacter* (87.70%) and *Methanosphaera* (3.63%) compared to the other 10 (≤1.70%; **Figure 2**). In addition, 947 out of the 975 milk samples (97.13%) were positive for Methanobrevibacter while Methanosphaera sequences were present in 348 of the milk samples (35.69%). To further assess the absolute counts of methanogens in dairy products, we subsequently screened unprocessed raw milk, pasteurized milk, and pasteurized yogurt

TABLE 2 | Secondary multivariable logistic regression model estimating the impact of the number of organic dairy products on the presence of *Methanobrevibacter smithii*.

M. smithii presence			
Adjusted main effects model (*N* = 406)[a]			
Determinant	**OR (CI95%)**	**FDR crit.[b]**	***p*-value**
First exposure to antibiotics (during first 2 years of life)			
Never	Ref.		
At 0–7 months	0.61 (0.33; 1.13)	0.020	0.115
At 8–12 months	1.20 (0.58; 2.49)	0.035	0.631
At 13–24 months	0.78 (0.39; 1.57)	0.030	0.490
Regular intake of organic products (cheese, milk, and yogurt)			
Trend (0, 1, 2, 3)	2.12 (1.31; 3.43)	0.005	**0.002[c]**
Diet of child			
Conventional (≤25% organic)	Ref.		
Organic (incl. biodynamic; >25% organic)	0.30 (0.14; 0.65)	0.010	**0.002[c]**

[a] *Model adjusted for: age at fecal sampling (years), gender (male/female), recruitment group (conventional/alternative), total energy intake (kcal) and BMI (z-score).*
[b] *Critical FDR cut-off level as determined by Benjamini-Hochberg procedure.*
[c] *Significant association after correction for FDR by Benjamini-Hochberg procedure, also indicated in bold.*

TABLE 3 | Final multivariable logistic regression model showing the association between potential determinants and the presence of *Methanosphaera stadtmanae*.

M. stadtmanae presence			
Adjusted main effects model (*N* = 420)[a]			
Determinant	**OR (CI95%)**	**FDR crit.[b]**	***p*-value**
Birthweight (g)[c]	1.001 (1.000; 1.002)	0.019	0.028
Place and mode of delivery[c]			
Natural birth at home	Ref.		
Natural/artificial birth at hospital	1.61 (0.67; 3.88)	0.034	0.288
Caesarean section	6.89 (2.09; 22.67)	0.003	**0.002[f]**
First exposure to antibiotics (during first 2 years of life)[c]			
Never	Ref.		
At 0–7 months	0.54 (0.16; 1.83)	0.038	0.323
At 8–12 months	0.70 (0.21; 2.36)	0.050	0.566
At 13–24 months	3.38 (1.34; 8.50)	0.013	**0.010[f]**
Regular intake of organic milk[d,e]			
No	Ref.		
Yes	3.52 (0.97; 12.73)	0.022	0.056
Regular intake of organic yogurt[d,e]			
No	Ref.		
Yes	1.690 (0.49; 5.86)	–	0.409
Regular intake of organic fruit[c]			
No	Ref.		
Yes	4.73 (1.64; 13.62)	0.006	**0.004[f]**
Animal protein intake (en%)[c]	0.77 (0.65; 0.92)	0.009	**0.004[f]**

[a] *Model adjusted for: age at fecal sampling (years), gender (male/female), recruitment group (conventional/alternative), total energy intake (kcal) and BMI (z-score).*
[b] *Critical FDR cut-off level as determined by Benjamini-Hochberg procedure.*
[c] *Parameter estimates presented for the model containing organic milk intake. Parameter estimates did not change substantially when the variable "organic milk intake" was replaced by the variable "organic yogurt intake" (data not shown).*
[d] *Missing value category omitted (included in FDR correction).*
[e] *Due to multicollinearity both regular intake of organic milk and regular intake of organic yogurt were included in separate models, which included all other variables as listed in this table.*
[f] *Significant association after correction for FDR by Benjamini-Hochberg procedure, also indicated in bold.*

samples (Supplementary Table 1). *M. smithii* and *M. stadtmanae* were quantified by qPCR on isolated DNA of these samples. Although *M. smithii* could not be detected in yogurt samples, we found significant levels of *M. smithii* in milk (**Figure 3**). The average absolute counts in processed milk was highly similar for biodynamic (2.63 log10 DNA copies/ml), organic (2.88 log10 DNA copies/ml) and conventional milk (2.94 log10 DNA copies/ml). However, raw milk (3.73 log10 DNA copies/ml) showed higher *M. smithii* counts compared to processed milk. For *M. stadtmanae*, no detectable levels could be measured in any of the samples. In conclusion, we identified milk as a possible source off *M. smithii*.

DISCUSSION

To the best of our knowledge, this is the first large-scale explorative study investigating the associations between a wide range of potential determinants and intestinal colonization by methanogenic archaea in school-aged children. We found that consumption of organic yogurt and milk products is related to colonization of *M. smithii* in particular. Moreover, we identified *M. smithii* in raw and processed milk, which could therefore be considered as potential sources for archaeal colonization.

The difference in metabolic activity might be a possible explanation for the large difference in the prevalence of the two main intestinal archaea. *M. smithii* is responsible for producing the majority of methane in the gut by reducing carbon dioxide using hydrogen as the primary electron donor. Alternatively, *M. smithii* can use formate as a direct substrate to produce methane (Roccarina et al., 2010). In contrast, *M. stadtmanae* is only able to produce methane from hydrogen (Gaci et al., 2014). Moreover, colonization by *M. smithii* and *M. stadtmanae* might originate from different environmental sources, which could explain their different prevalence. Factors that play a role in the acquisition of methanogenic archaea are largely unknown. However, the environment is assumed to play a crucial role in gut colonization. This view is strengthened by a twin study that indicated that not genetic factors, but shared and unique environmental factors were of importance for the occurrence of methanogens in humans (Florin et al., 2000). The prospective design and long-term follow-up allowed us to examine a wide range of environmental, dietary and life-style associated determinants throughout childhood. Out of the determinants present during infancy, caesarean section compared to vaginal delivery at home, first exposure to antibiotics in the 2nd year of life compared to

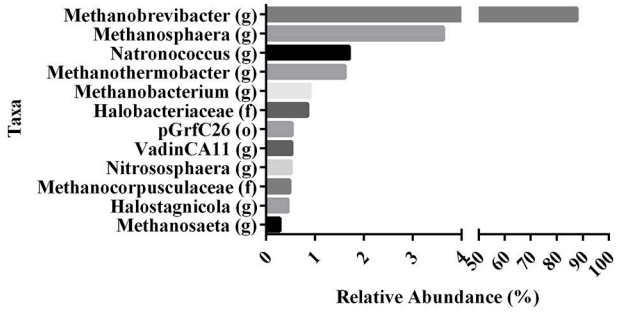

FIGURE 2 | Variation in the relative abundances of dominant archaea taxa in raw milk of re-analyzed 16S sequence-data from Kable et al. (2016). Data represent the abundances of the various archaeal taxa (found at 0.1% or greater) as proportion of the total archaeal populations in raw tanker milk. Relative abundances of OTU's rarified at 15,000 sequences per sample are indicated at their highest identified taxa. (o), order. (f), family. (g), genus.

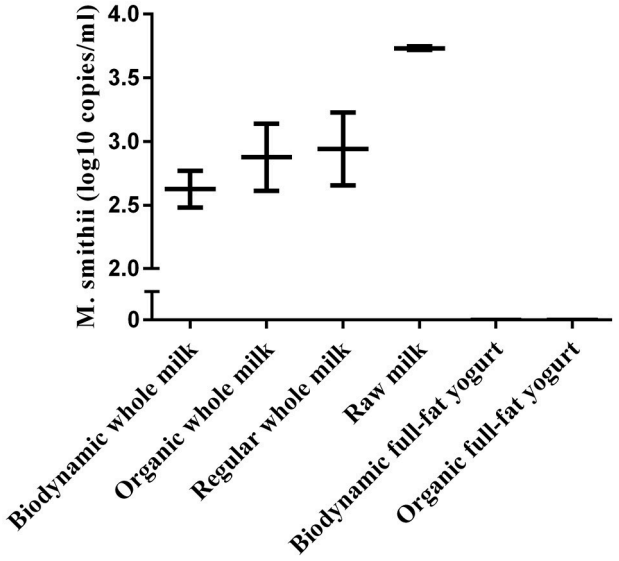

FIGURE 3 | Absolute counts of *M.smitthii* in whole or full-fat dairy products ($n = 2$–3). Replicates *(n)* denotes repeated isolation and quantification of the product. Average counts were calculated from archaea positive samples only.

no antibiotic treatment during the first 2 years and organic fruit consumption were associated with increased odds of colonization by *M. stadtmanae*. These findings on *M. stadtmanae* in the present study need however to be interpreted with great care. Due to the small group of children colonized by *M. stadtmanae* ($n = 39$), models for *M. stadtmanae* were prone to instability. Therefore, these factors need to be verified in other studies with, preferably, larger sample sizes. In particular, the association with birth mode is in contrast with previous findings that intestinal colonization by methanogenic archaea in neonates and infants is very rare (Palmer et al., 2007). However, it could be postulated that the pioneer species associated with caesarian section delivery drive a subsequent colonization pattern that is more favorable for the establishment of *M. stadtmanae* at a later stage. As such, it is important to study the co-occurrence and co-exclusion of archaea and bacterial taxa in future studies, in order to identify microbial networks that might favor archaeal colonization.

Although one previous study found females to be more often colonized by methanogenic archaea than males (Florin et al., 2000), the current view is that there is no association between methanogenic archaea and gender (Dridi et al., 2011b). In this study, we assessed fecal samples of 244 (51.7%) males and 228 (48.3%) females. Of all males under study 187 (76.6%) were colonized by *M. smithii*, while 182 (79.8%) females were colonized by *M. smithii*. Furthermore, 19 (7.8%) males were colonized by *M. stadtmanae*. This was similar to the colonization by *M. stadtmanae* in females, as 20 (9.6%) females were colonized. Moreover, we also did not find an association between gender and either *M. smithii* or *M. stadtmanae*.

So far, only one study assessed potential carriers of archaea in food, but yogurt and milk have not been assessed herein (Brusa et al., 1998). The present study suggests an association between dietary factors and archaeal colonization, as organic yogurt and milk were significantly associated with the presence of *M. smithii*. Due to the high correlation between organic milk and yogurt intake, we were however not able to disentangle whether only organic milk or organic yogurt or both were truly responsible for the association with *M. smithii* during multivariable analyses. Cheese was however not associated with *M. smithii* colonization, which is in line with previous findings where no *M. smithii* or *M. stadtmanae* were detected in different types of cheese (Brusa et al., 1998). Analyses on the number of dairy products consumed, used to address multicollinearity, indicated that the consumption of multiple different dairy products might be associated with an increased *M. smithii* presence.

A well-known carrier of *M. smithii* and *M. stadtmanae* is the rumen of beef and cows (Carberry et al., 2014; Cersosimo et al., 2016). Therefore, it is likely that products derived from cows, such as dairy products, may contain some of these taxa, which was reflected in our results. Moreover, these specific methanogenic archaea have also been found in soil which could be the route of origin to cows as revealed from the Integrated Microbial Next Generation Sequencing database (Lagkouvardos et al., 2016). The archaeal presence in soil might be the underlying reason that especially organic products were associated with colonization. Organic cows have more outdoor access compared to conventionally farmed cows and could therefore have increased archaea uptake. Additionally, drugs for organic cows are less prescribed whereas conventional cows more often receive antibiotics to prevent microbial infections (Zwald et al., 2004). Methanogens are susceptible to antibiotics such as bacitracin, a commonly used antibiotic in cattle, which might eliminate them (Dridi et al., 2011a). Although the screening of multiple dairy products indicated the presence of *M. smithii* in both conventional and organic dairy products, the latter could alternatively explain the lack of association of consumption of conventional dairy products with *M. smithii* colonization within our cohort study.

As the culture-independent techniques applied in the present study do not distinguish viable microbes from cell-free DNA

originating from lysed microorganisms, we cannot completely exclude that dairy products only contain archaeal DNA instead of viable archaea. However, we used the concentrated pellet of the microbial cells for downstream analyses of milk products, thereby minimizing the detection of circulating cell-free DNA. It is therefore likely that a living fraction of archaeal cells has been measured.

For milk, the typical neutral pH is viable for both *M. smithii* and *M. stadtmanae*, which favor an optimal pH of 6.9–7.4 whereas yogurt has a more acidic environment (Miller and Lin, 2002; Ledenbach and Marshall, 2009; Dridi et al., 2011b), which might explain why we could not detect methanogenic archaea in yogurt samples. In addition, methanogens have been discovered in a wide variety of extreme environments with temperatures until at least 100°C (Elias et al., 1999; Tung et al., 2005). The latter might indicate that these microbes could resist heat treatments and pasteurization performed for milk products. All in all, this strengthens the possibility that viable methanogenic archaea could indeed be present in milk products, even after thermal processing.

To get definite prove that milk products are a source of archaea, future studies using either culture-based methods or molecular methods that enable differentiation between intracellular and cell-free DNA, such as the use of propidium monoazide (PMA) as a membrane impermeable DNA intercalating dye (Janssen et al., 2016), are warranted. Several studies already identified living bacteria with an implemented PMA-assay in both human feces and processed milk (Bae and Wuertz, 2009; Soejima et al., 2012; Quigley et al., 2013; Cangelosi and Meschke, 2014).

In conclusion, dairy products, in particular organic milk products, may play an influential role in the colonization of the gut by *M. smithii* in children. Moreover, *M. smithii* seems to be present in both raw and commonly consumed milk products. For the first time, a large variety of determinants have been explored in association with gut colonization by methanogenic archaea. Although more information is needed to confirm and unravel the mechanisms of archaeal colonization in more detail, it may provide new targets for prevention of diseases associated with the presence of methanogenic archaea.

AUTHOR CONTRIBUTIONS

Jv performed the analyses on the cohort, and wrote partly the manuscript together with Nv. Nv designed the laboratory experiments, performed laboratory isolation, and qPCR techniques on milk products, analyzed the genomic data, and wrote partly the final manuscript as submitted. CM performed the laboratory isolation of fecal DNA and qPCR techniques, data processing, and approved the final manuscript as submitted. JP contributed to the design of the study and collection of the data, supervised the laboratory work, interpreted the data, critically reviewed the manuscript, and approved the final manuscript as submitted. MM contributed to the design of the study and collection of the data, interpreted the data, critically reviewed the manuscript and approved the final manuscript as submitted. CT and IA contributed to the design of the study and collection of the data, critically reviewed and revised the manuscript and approved the final manuscript as submitted. PS and MH critically reviewed and revised the manuscript and approved the final manuscript as submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00355/full#supplementary-material

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PERSPECTIVE
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First Foods and Gut Microbes

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The establishment of the human gut microbiota in early life has been associated with later health and disease. During the 1st months after birth, the microbial composition in the gut is known to be affected by the mode of delivery, use of antibiotics, geographical location and type of feeding (breast/formula). Consequently, the neonatal period and early infancy has attracted much attention. However, after this first period the gut microbial composition continues to develop until the age of 3 years, and these 1st years have been designated "a window of opportunity" for microbial modulation. The beginning and end of this window is currently debated, but it likely coincides with the complementary feeding period, marking the gradual transition from milk-based infant feeding to family diet usually occurring between 6 and 24 months. Furthermore, the 'first 1000 days,' i.e., the period from conception until age 2 years, are generally recognized to be of particular importance for the healthy development of children. While dietary changes are known to affect the adult gut microbiota, there is a gap in our knowledge on how the introduction of new dietary components into the diet of infants/young children affects the gut microbiota development. This *perspective* paper summarizes the currently very few studies addressing the effects of complementary diet on gut microbiota, and highlights the recent finding that transition to family foods greatly impacts the development of gut microbial diversity. Further, we discuss potential impacts on child health and the need for further studies on this important topic.

Keywords: infant gut microbiota, breast feeding, weaning, gut microbial diversity, complementary diet, transition, SKOT cohorts, family foods

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WHAT DO WE KNOW ABOUT COMPLEMENTARY FEEDING AND GUT MICROBIOTA DEVELOPMENT?

The differential effect of breastfeeding as compared with formula feeding or mixed feeding on the composition of the gut microbiota is well established (Penders et al., 2006; Fallani et al., 2010; Bergström et al., 2014; Jost et al., 2015). However, what happens to the gut microbiota when the first solid foods are introduced and gradually replace the milk-based diet has only scarcely been addressed. Longitudinal studies with repeated sampling in a limited number of infants have indicated that microbial composition changes significantly around the period of introduction to solid foods and cessation of breastfeeding/formula feeding (Favier et al., 2002; Wang et al., 2004; Amarri et al., 2006; Roger and McCartney, 2010; Koenig et al., 2011; Thompson et al., 2015). We have previously monitored the fecal microbiota of 330 Danish children participating in the study designated 'SKOT,' at age 9, 18, and 36 months. It was evident that *Lactobacillaceae*, *Bifidobacteriaceae, Enterococcaceae,* and *Enterobacteriaceae* abundance decreased while *Lachnospiraceae*, *Ruminococcaceae,* and *Bacteroidaceae* species increased during the period

from 9 to 18 months, i.e., during the period characterized by transition from milk-based feeding to family diet (Bergström et al., 2014). This was largely in agreement with a European study including 531 infants from five different countries demonstrating that consistent compositional changes (decrease in *Bifidobacteriaceae*, *Enterobacteriaceae*, *Clostridiaceae* while increase in *Ruminococcaceae* and *Lachnospiraceae* species) occurred from 6 weeks of age until 4 weeks after the introduction of solid foods, irrespectively of differences in geographic location, use of antibiotics, mode of delivery (vaginal or C-section) and milk feeding practices (Fallani et al., 2011). Recently Thompson et al. (2015) found increased gut microbial diversity of both exclusively breastfed infants ($n = 4$) and mixed-fed infants ($n = 5$) after introduction of solid foods.

The general challenge in longitudinal studies is to differentiate between the effects caused by advancing age (leading to increased exposure to environmental microbes and increased maturity of the gut) and effects induced directly by dietary changes. In order to address this issue, we recently combined a comprehensive analysis of the complementary diet with 16S rRNA gene sequence analysis of the fecal microbiota in two independent Danish cohorts of infants aged 9 months, participating in the previously mentioned SKOT study, and demonstrated that gut microbial diversity correlated significantly with the child's progression toward family foods in both cohorts (Laursen et al., 2016). A Principal Component Analysis of the complete infant dietary records at 9 months, including 23 food groups (recorded in g/day/kg body weight) was performed. The first principal component was defined as "Family Food," since it described the transition from early infant foods, with high content of breastmilk, formula and porridge (low loadings), to family foods introduced during late infancy (high loadings), with high content of meat, milk, cheese, animal fat and rye bread (type of bread commonly used in complementary feeding in Denmark) (**Figure 1A**). We found separately in both cohorts (Laursen et al., 2016), as well as for the compiled data set ($n = 217$), that the family food parameter was strongly associated with the Shannon index, a commonly used measure of alpha diversity (e.g., within-sample complexity) of the gut microbiota (**Figure 1B**). Other measures of alpha diversity, namely microbial richness (Observed genera) and evenness (Pielou's evenness index) were strongly associated with the progression to family foods, indicating that both the number of different microbes (richness) and the balance between these (evenness) are affected by this progression (**Figure 1C**). These associations seem to be driven both by the cessation of breastfeeding and by the introduction of food items with higher fiber and protein content, such as rye bread, cheese and meat products, as evidenced from a hierarchical clustering of correlation coefficients between dietary factors and alpha diversity metrics from the compiled data set (**Figure 1C**). Further, progression toward family foods at the of age 9 months was negatively associated with abundances of *Bifidobacterium*, *Enterococcus, Enterobacteriaceae* (*Escherichia*, *Kluyvera*) and to a lesser degree with *Clostridiaceae* (*Clostridium sensu stricto*), but positively associated with abundances of several different bacterial genera (**Figure 1D**), most of them within *Lachnospiraceae* (*Blautia*, *Roseburia*, *Pseudobutyrivibrio*, *Dorea*, *Coprococcus*, *Lachnospiraceae incertae sedis*) and *Ruminococcaceae* (*Faecalibacterium* and *Ruminococcus*), confirming the previous observations from the SKOT cohort (Bergström et al., 2014) as well as other study populations (Fallani et al., 2011; Thompson et al., 2015). Indeed, the relative abundance of species within *Lachnospiraceae* and *Ruminococcaceae* increases when dietary fibers from rye bread (arabinoxylans) or protein from dairy/cheese (casein) and meat are included in the diet (Walton et al., 2012; Zhu et al., 2015). Thus, the introduction of these food components may provide selective advantages for specific microbes to establish in the gut, which will increase alpha diversity. However, we also observed in our cohort studies that the duration of exclusive breastfeeding (range 0–6 months) was more determining for microbial diversity at the age of 9 months than the time of first introduction of solid foods (range 3–6 months), reflecting the dominating impact of breastfeeding (Laursen et al., 2016). To further elucidate the influence of breastfeeding in relation to the effect of complementary diet, we performed a sub-analysis, stratifying the infants by breastfeeding status (defined as at least 1 breastfeeding per day at age 9 months), and correlating the dietary parameters with gut microbial alpha diversity (**Figure 1E**). Protein, fiber and progression toward family foods were positively correlated with the Shannon index in both subsets, indicating that these associations occurred independently of breastfeeding. Interestingly, while meat, cheese and rye bread represented the specific dietary groups most strongly correlated to diversity in the complete dataset ($n = 217$) and in the subset of infants no longer breastfed ($n = 145$), porridge (primarily oatmeal, the most common type of porridge used in complementary feeding in Denmark) was most strongly correlated to diversity in the subset of infants still breastfed at 9 months ($n = 72$). Oatmeal is rich in dietary fibers such as β-glucans, which can be utilized by gut microbes (Hughes et al., 2008). Our results suggest that while infants are still breastfed, porridge is the dietary factor contributing most to gut microbial diversity, whereas when infants are no longer breastfed, consumption of cheese, meat and rye bread is more important. Formula intake was positively associated with diversity in the breastfed infant subset (**Figure 1E**), supporting the previous observations that mixed-fed infants have higher microbial diversity than exclusively breastfed infants (Thompson et al., 2015). Oppositely, formula feeding was negatively associated with diversity in the infants that were no longer breastfed, indicating that the substitution of formula with solid foods such as meat, cheese and rye bread further increase microbial diversity (**Figure 1E**). Together, these results suggest that effects on gut microbial composition and diversity during the complementary feeding period is not solely mediated by weaning/cessation of breastfeeding, but indeed affected by the introduction of new specific complementary foods with higher protein and fiber content.

The development of the intestinal microbiota is affected by a number of factors including geography, lifestyle and oligosaccharide content of the breastmilk. Although the microbial succession in the infant gut thus greatly varies among individual infants, the general picture emerging

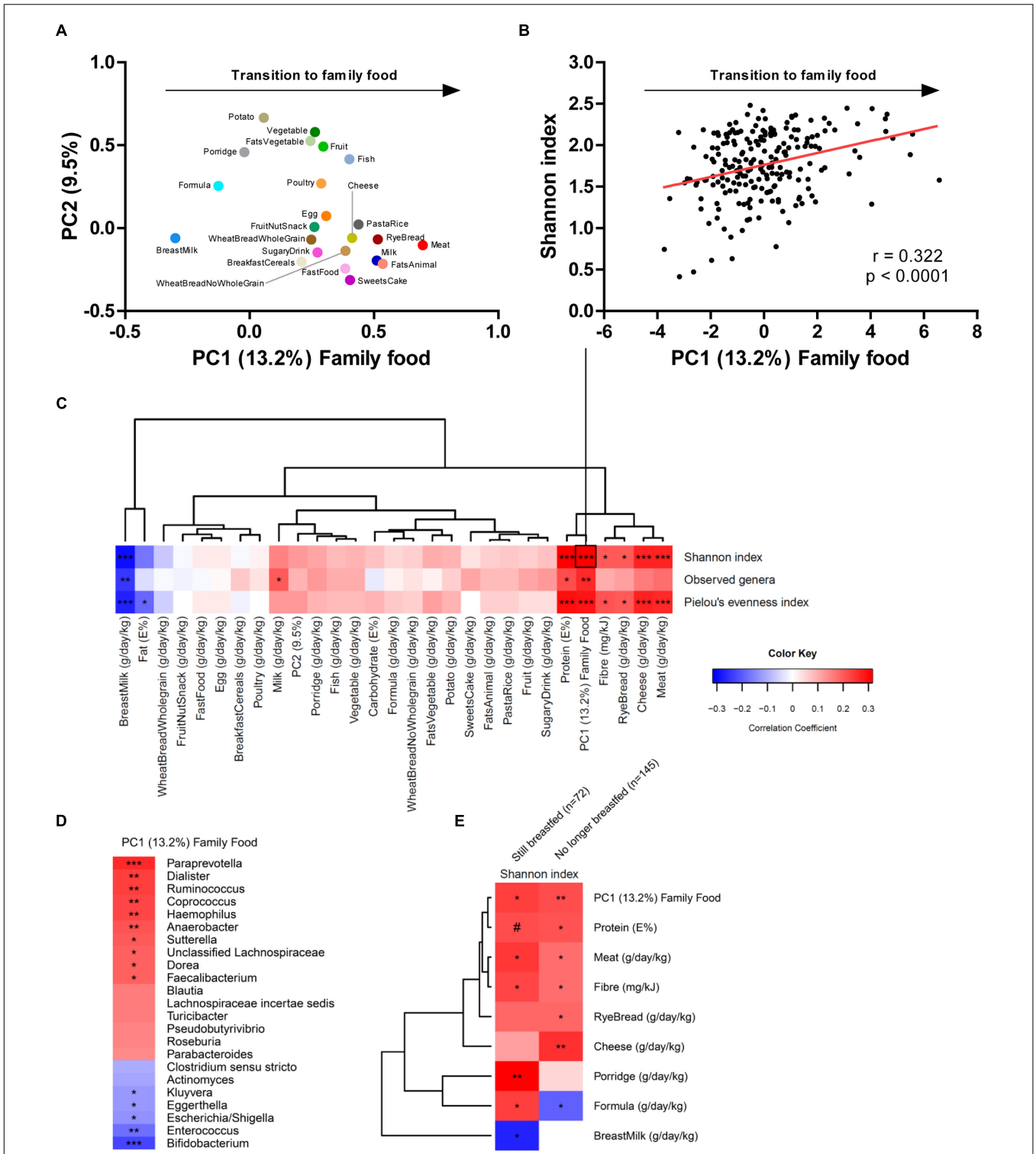

FIGURE 1 | (A) PCA loading plot of the 23 food groups (grams per day per kilogram body weight), with the fist principal component defined as family food, describing the transition from early infancy foods to family foods. **(B)** Pearson's correlation between family food and the Shannon index of alpha diversity. **(C)** Heatmap illustrating hierarchical clustering of correlation coefficients between dietary parameters and alpha diversity metrics. **(D)** Heatmap illustrating correlation coefficients between family food and gut microbial genera showing a *p*-value below 0.1. **(E)** Heatmap illustrating hierarchical clustering of correlation coefficients between dietary parameters and Shannon index in partially breastfed (n = 72) and weaned (n = 145) infants aged 9 months. Color key applies to panel **(C–E)** and represents correlation coefficients of Spearman's rank/Pearson's correlations. Statistical significance is indicated by $^{\#}p = 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. The figure is based on previously published data (Laursen et al., 2016).

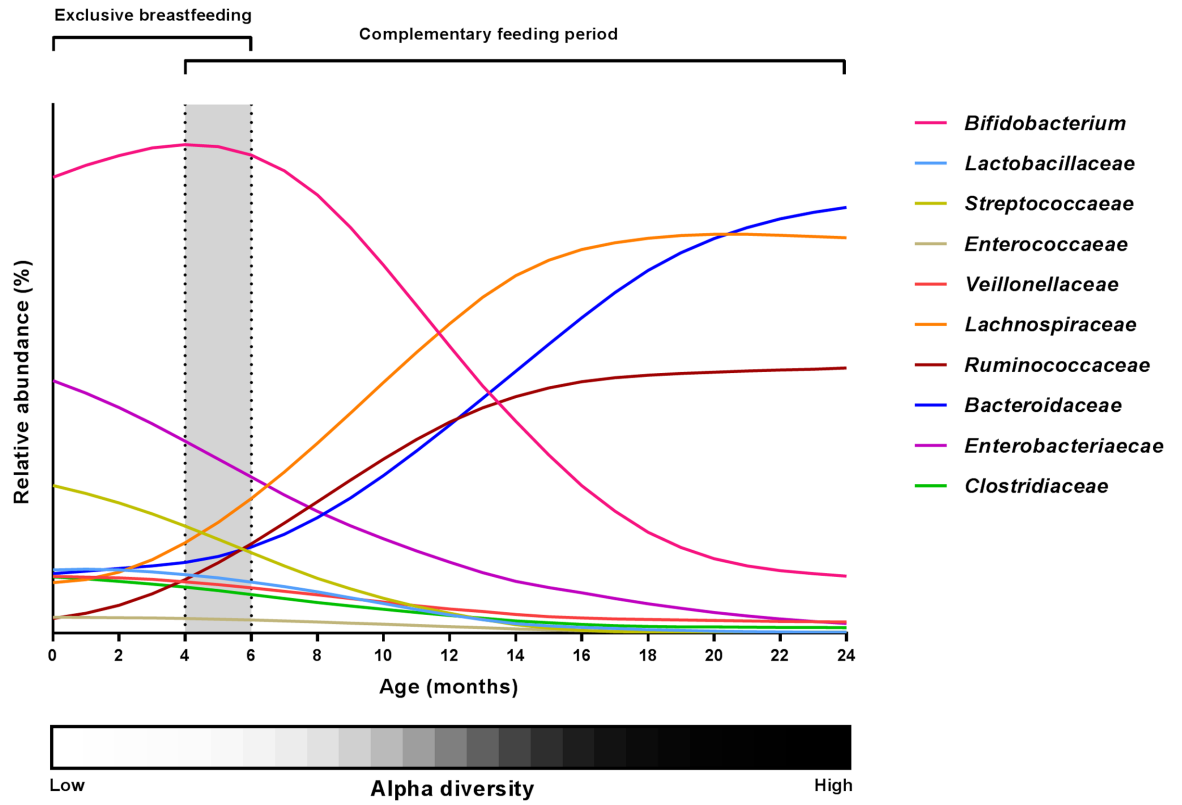

FIGURE 2 | Changes in the major gut microbial taxa during the first 24 months of life. Relative abundance estimates and curve shapes are based on average values from available literature (Arrieta et al., 2014; Bäckhed et al., 2015; Kostic et al., 2015; Laursen et al., 2016; Yassour et al., 2016). Individual abundances were normalized to an abundance sum for all bacterial families at each month, and curve smoothing was performed with GraphPad Prism. Dashed lines and gray background indicate the period of introduction to solid foods. Alpha diversity development is shown by a grayscale gradient (white = low diversity, black = high diversity).

(**Figure 2**) is that breast milk keeps the gut microbiota in a state characterized by a high relative abundance of *Bifidobacterium* (with lower abundance of other breast milk associated bacteria such as *Veillonellaceae, Enterococcaceae*, *Lactobacillaceae*, *Enterobacteriaceae,* and *Streptococcaceae*) accompanied by low diversity, and that these characteristics are affected only to a limited degree by mixed feeding and introduction of the first solid foods as long as the child is still partially breastfed (Roger and McCartney, 2010; Bäckhed et al., 2015; Laursen et al., 2016). However, as complementary feeding progresses, the gut microbial composition changes (increase in the diverse groups of *Lachnospiraceae* and *Ruminococcaceae* species and decrease of *Bifidobacterium*, *Enterobacteriaceae*, *Enterococcaceae, Lactobacillaceae*, *Veillonellaceae, Clostridiaceae*) and total diversity increases (Favier et al., 2002; Roger and McCartney, 2010; Laursen et al., 2016), probably as a result of the increased fiber and protein content of the diet (Laursen et al., 2016). Finally, cessation of breastfeeding/formula feeding and complete transition to family food marks another important event in gut microbial development (Favier et al., 2002; Roger and McCartney, 2010; Bäckhed et al., 2015), characterized by an increased abundance of *Bacteroidaceae, Lachnospiraceae,* and *Ruminococcaceae* and a further reduction in *Bifidobacterium* as well as increased alpha diversity (Roger and McCartney, 2010; Bergström et al., 2014; Bäckhed et al., 2015).

WHAT DOES IT MATTER FOR CHILD HEALTH?

The consistently observed increase in gut microbial alpha diversity during the time period coinciding with complementary feeding (Yatsunenko et al., 2012; Kostic et al., 2015; Avershina et al., 2016) and recent evidence showing that this increase is driven by transition from breastfeeding to family foods (Laursen et al., 2016), raise some key questions: Is the low diversity sustained by breastfeeding beneficial for infant health? Does the increase in microbial diversity observed during complementary feeding characterize healthy gut microbiota development?

Many studies have linked low gut microbial diversity to diseases in adulthood as recently reviewed (Mosca et al., 2016). Conditions associated with low gut microbiota diversity range from gastrointestinal pathologies such as Crohn's disease, ulcerative colitis, irritable bowel syndrome and colorectal cancer to metabolic disorders including obesity, type 2 diabetes, as well as neurological conditions such as autism (Mosca et al.,

2016). Recent studies reveal that an important determinant of high microbial diversity in adult fecal samples is a long intestinal transit time (Vandeputte et al., 2015; Falony et al., 2016; Roager et al., 2016). In this context, we have recently proposed that a high microbial richness and diversity may not imply *per se* a healthy gut microbial ecosystem, as longer transit times are significantly associated with increased bacterial protein degradation, which results in potentially detrimental bacterial metabolites (Roager et al., 2016). During the complementary feeding period, transition to family foods implies increased protein and fiber intake, which increases microbial diversity (Laursen et al., 2016). While the implications of this transition on intestinal transit time are not known, bowel movement frequency is generally reported not to change significantly during the weaning period (Tunc et al., 2008).

In infants, the major determinant of a low microbial diversity is probably breast milk (Azad et al., 2013a; Thompson et al., 2015; Laursen et al., 2016), which selects efficiently for specific bacterial species capable of degrading particular oligosaccharides present in breastmilk, the so-called human milk oligosaccharides (HMOs). HMO-degrading species include *B. bifidum, B. longum* ssp. *infantis* and to a lesser degree *B. breve* (Sela and Mills, 2010), while, noteworthy, bifidobacterial species common in the adult gut (e.g., *B. adolescentis*) do not share the capacity to degrade HMOs (Sela and Mills, 2010). *In vitro* studies suggest that the infant-type bifidobacteria play an important role in the maturation of the child's immune system (Underwood et al., 2014). In line with this, breastfeeding promotes several health effects that are likely to be caused by an improved immune response, such as a reduced incidence of childhood infections (Victora et al., 2016). This suggests that the predomination of infant-type bifidobacteria, implying a low bacterial diversity during breastfeeding, is beneficial for child health. In contrast, some health disorders have been linked to a reduced microbial diversity in early life. Examples include development of eczema (Forno et al., 2008; Wang et al., 2008; Abrahamsson et al., 2012; Ismail et al., 2012) and asthma (Abrahamsson et al., 2014), which have been linked to low microbial diversity in very early life (age 1 week – 4 months). Additionally, in children aged 18–24 months, low microbial diversity has been shown to be a risk factor for development of type 1 diabetes (Giongo et al., 2010; Kostic et al., 2015). Importantly, the low microbial diversity in these studies was not coupled to *Bifidobacterium* abundance and no negative effects of breastfeeding on prevalence of asthma, allergies or development of type 1 diabetes have been reported (Victora et al., 2016). Thus, even though early life low microbial diversity seems to be associated with diseases later in childhood, this is not likely to be caused by *Bifidobacterium* predominance or prolonged breastfeeding. Importantly, the microbial taxa that are absent or otherwise driving low diversity in the above-mentioned studies and their causality to diseases remain to be identified. Some studies suggest that an insufficiently developed microbial community in the gut can be "repaired," e.g., it has recently been demonstrated that an immature gut microbiota of stunted children aged 6 months, which is within the aforementioned window of opportunity, may at this age be repaired by introduction of adult-like microbes such as *Ruminococcus gnavus* and *Clostridium symbiosum*, both belonging to the *Lachnospiraceae* (Blanton et al., 2016), a microbial family greatly increasing during introduction of family foods (Laursen et al., 2016). The normally observed development across human populations is an increasing microbial diversity with age during the first 3 years of life (Yatsunenko et al., 2012) and probably characterizes a healthy development. While proteins and fibers were found to increase gut microbial diversity (Laursen et al., 2016), a high-protein intake in the complementary feeding period may increase the risk of obesity later in life (Lind et al., 2017), and defining recommendations for fiber intake in early life is equally challenging (Aggett et al., 2003; Zalewski et al., 2015). Consequently, even though gut microbial diversity increases with protein and fiber intake during the complementary feeding period, there is no evidence to support that excessive intake of these macronutrients in early childhood is beneficial for child health.

In summary, we want to emphasize that findings regarding the diversity of the intestinal microbiota in adults cannot be extrapolated to children, since the factors governing the co-development of the microbial ecosystem and the immune system in infanthood are completely different from the factors of importance for adult health. A low diversity of the infant gut microbiota in early life must thus be expected to characterize a healthy gut, if caused by breastfeeding. However, later progression into a diverse gut microbiota during childhood seems to be a fundamental part of the natural succession of the gut ecosystem and characterizes healthy development. One of the main challenges remaining is to identify more accurately the best timing of breastfeeding cessation and of introduction of family foods. This timing is influenced by many other factors than the microbiota, and is likely to be determined by the level of maturation of the infant's immune system.

WHAT IS NEEDED TO EXPAND OUR KNOWLEDGE ON COMPLEMENTARY DIET AND GUT MICROBES?

Until now, only a few studies have performed dietary assessment during the complementary feeding period and compared this with gut microbiota development. To decipher the effects of specific food components on gut microbial development, detailed assessments of the complementary diet constituents are needed. If food frequency questionnaires (FFQ) are used, they should be validated before application to minimize the risk of over/underestimation because they are prone to recall bias. Additionally, it should be considered that FFQs are generally developed to depict long-term or habitual diets, which may not be appropriate during a period of rapid dietary changes. Twenty-four hour dietary recalls are less prone to recall bias, but do not capture day-to-day variations in the diet. The most detailed and accurate results are obtained from food records for periods of 5–7 days since they collect actual intake information from a specific short-term period and are not associated with recall bias (Shim et al., 2014). Validated food records that minimize the risk of under/overestimation were used in our SKOT cohort

studies, and because they were conducted during the time period of fecal sample collection, they allowed us to directly assess the impact of the complementary diet on gut microbiota composition (Laursen et al., 2016). Ideally, longitudinal studies should include several diet recordings across the complementary feeding period to capture the individual dynamics during this period of multiple dietary changes.

To capture changes in the gut microbiota, either 16S rRNA amplicon or shot-gun based community sequencing can provide detailed information of the entire microbial community, while shot-gun based sequencing additionally provides direct information on the functional capacity of the bacterial population. However, while these methods generate a large amount of useful data, they lack enough sensitivity for low abundant microbial species. Therefore, complementing these techniques with quantitative PCR approaches and culture-based approaches provide better sensitivity, but are more laborious and time consuming. To minimize confounding effects on the gut microbiota in cross sectional studies, accurate standardization of the age of infants at which the samples are taken is highly desirable. Additionally, it is important to consider adjustments for other factors known to affect infant gut microbiota, including antibiotic intake, C-section, childcare/daycare, geographical location, family environment and exposure to pets and siblings (Yatsunenko et al., 2012; Azad et al., 2013b; Laursen et al., 2015; Thompson et al., 2015; Martin et al., 2016; Yassour et al., 2016). In order to establish causal relationships between specific components of the complementary diet and gut microbial composition, intervention studies are required. This could be done by performing short-term intervention studies, where the effect of specifically designed differences in dietary composition can be tested. Inclusion of the above-mentioned factors in the design of future studies will enable us to expand our knowledge on the impact of the complementary diet on the infant gut microbiota.

SUMMARIZING REMARKS

The very first nutrition most infants receive, breastmilk, keeps the microbiota in a state characterized by low diversity and *Bifidobacterium* domination, which is likely to be beneficial for child health. Introduction of complementary feeding and the transition to family foods increase gut microbial diversity and is accompanied by an increase of adult-associated microbes belonging to the families *Lachnospiraceae* and *Ruminococcaceae*. This increase in diversity occurs across different human populations and is likely to characterize a normal and healthy development of the gut microbiota, since perturbation of the process has been linked to an increased risk of diseases later in life. An important remaining challenge is to determine the optimal age for increasing intestinal microbial diversity by reduction of breastfeeding and introduction of family foods in order to optimize effects on gut microbiota, immune maturation and later health outcomes.

ETHICS STATEMENT

The present perspective uses data from previous publications based on the SKOT cohorts, in which 311 (SKOT I) and 184 (SKOT II) Danish children were followed for the first 3 years after birth, with the overall aim of investigating relationships between early diet, growth development, and later disease risks, especially obesity and metabolic syndrome. The study protocols were approved by the Committees on Biomedical Research Ethics for the Capital Region of Denmark (H-KF-2007-0003 and H-3-2010-122).

AUTHOR CONTRIBUTIONS

ML, MB, KM, and TL conceived the idea for this perspective, and all contributed to the outline. All four authors were significantly involved in the studies resulting in the data presented in **Figure 1**. ML drafted the manuscript and the figures. All authors contributed to the writing, and approved the final manuscript.

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Intestinal Microbiota and Weight-Gain in Preterm Neonates

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The involvement of the gut microbiota on weight-gain and its relationship with childhood undernutrition and growth has been reported. Thus, the gut microbiota constitutes a potential therapeutic target for preventing growth impairment. However, our knowledge in this area is limited. In this study we aimed at evaluating the relationship among early microbiota, growth, and development in preterm infants. To this end we assessed the levels of specific microorganisms by qPCR, and those of short chain fatty acids by mean of gas-chromatography, in feces from 63 preterm newborns and determined their weight-gain during the first months. The statistical analyses performed indicate an influence of the intestinal microbiota in weight-gain, with the levels of some microorganisms showing a significant association with the weight-gain of the infant. The levels of specific microbial groups during the first days of life were found to affect weight gain by the age of 1 month. Moreover, clustering of the infants on the basis of the microbiota composition at 1 month of age rendered groups which showed differences in weight z-scores. Our results suggest an association between the gut microbiota composition and weight-gain in preterm infants at early life and point out potential microbial targets for favoring growth and maturation in these infants.

Keywords: newborn, premature-infant, weight-gain, colonization, microbiota, probiotics

INTRODUCTION

Microbial colonization of the neonatal gut provides a stimulus necessary for the development of the intestine and the physiological homeostasis (Hooper and Macpherson, 2010; Sommer and Bäckhed, 2013). The early neonatal period represents the most important moment for this microbiota-induced maturation of the host, being a key determinant for later health (El Aidy et al., 2013). This neonatal colonization is affected by several factors such as gestational age, mode of delivery, antibiotic use, or feeding habits (Faa et al., 2013).

From the pioneering studies showing a reduction of Bacteroidetes and an increase of Firmicutes in obese animals (Ley et al., 2005), and the mechanistic works demonstrating the involvement of the gut microbiota in the regulation of food energy harvesting, fat storage and modulation of the endocrine function (Bäckhed et al., 2004; Cardinelli et al., 2015), the role of the microbiota in weight-gain has attracted increasing attention (Cardinelli et al., 2015). The modulation of the gut microbiota offers a potential therapeutic target for both, weight-management (Dror et al., 2017) and prevention of growth impariment (Blanton et al., 2016). Most data on the relationship between

microbiota and weight-gain come from animal models or from studies in adults. Epidemiologycal data in infants are also available, evidencing associations between microbiota composition and later obesity (Koleva et al., 2015). However, these studies have focused on full-term infants and this interaction has not been explored in other populations, such as preterm babies, where weight-gain may be an indicator of maturation and positive outcome.

Preterm infants present an immature immune system and gut barrier, which leads to an increased disease risk. Moreover, the process of microbiota establishment is altered in these infants, who harbor more *Enterobacteriaceae* and potential pathogens and less commensals than full-terms (Arboleya et al., 2012, 2015). In these infants the nutritional goal is achieving a weight-gain and body composition approximating the fetal intrauterine growth (Brennan et al., 2016). For this reason, the adjustment of the preterm infant growth to the growth-tables, calculated from data on patients born at various gestational ages, is widely used to estimate newborn size and postnatal evolution (Fenton and Kim, 2013). This adjustment would be a positive nutritional outcome but it is difficult to achieve. Thus, developing strategies to enhance growth and maturation of preterm infants could be of clinical interest. Given the relationship between microbiota and weight-gain, the gut microbiota establishment process may constitute an adequate target.

The present study aims at evaluating the potential relationship between early microbiota development and weight-gain in preterm neonates.

MATERIALS AND METHODS

Subjects and Samples

Sixty-three preterm infants born at gestational ages between 28 and 33 weeks were recruited at the Neonatology Units of Cabueñes Hospital and the University Central Hospital of Asturias (Northern Spain). None of the infants had necrotizing enterocolitis or culture positive early onset infection. All infants received mixed feeding (infant formula and some breast-milk administration during the study period). The infants were discharged from the hospital after an average stay of 38 days of hospitalization.

Fecal samples were collected at 2, 10, and 30 days-of-age and weights determined monthly up to 3 months. Weight data were used to calculate weight-gain at different sampling times and weight Z-scores were determined using the growth curves obtained by Fenton (Fenton and Kim, 2013). Fecal samples were immediately frozen and sent to the laboratory for analyses. The Regional Ethical Committee of Asturias Public Health Service approved the study and informed written consent was obtained from the parents.

Intestinal Microbiota and SCFA Analyses

The absolute levels of the different bacterial populations analyzed, including the main representatives of the predominant phyla in the infant gut (*Bifidobacterium*, *Streptococcus*, *Staphylococcus*, *Enterococcus*, *Bacteroides*-group, *Enterobacteriaceae*, *Lactobacillus*-group, *Weissella,* and total bacteria), were determined by quantitative PCR using primers and conditions previously described (Arboleya et al., 2012). When a sample resulted negative for a certain microbial group, the value of the detection limit obtained for the corresponding primer pair (ranging between 10^3 and 10^4 cells per gram depending on the bacterial group) was assigned. For the preparation of standard curves, pure cultures of appropriate strains were used as previously reported (Arboleya et al., 2012).

Analysis of the main short-chain-fatty-acids (SCFA) (acetate, propionate, and butyrate) was carried out in supernatants of homogenized feces by using a 6890N gas chromatograph (Agilent Technologies Inc., Palo Alto, CA, USA) connected to a FID and a MS 5973N detectors (Agilent) as previously reported (Arboleya et al., 2012).

Statistical Analyses

Multiple mixed linear models were used to investigate the relationship between the microbial levels and fecal SCFA with weight-gain, adjusting by possible confounders. Backward stepwise analyses based on the Aikaike Information Criterion (AIC) were employed to determine whether the variables were included in predictive models. A forest plot was used in order to show the effect sizes in both the so-labeled univariate and the multivariate models (always adjusting by infant and gestational ages). With the goal of investigating different groups of subjects based on microbiota measures, a Euclidean cluster was performed. Dendrogram was used to determine the number of groups and standard robust Welch tests. Standard parametric robust Welch tests or non-parametric Kruskal-Wallis test, depending on the data distribution, was used to check equality among groups. Analyses were performed with R software, the conventional p-level of 0.05 was used in the interpretation of results.

RESULTS

The study included 28 males and 35 females (birth weights from 1,085 to 1,580 g). Twenty-two were vaginally delivered (VD) whilst 41 were born by caesarean section (CS). During the 3 months of duration of the study 35 of the infants received antibiotics at some time, most of them during the first month. The characteristics of the study population and the evolution of the intestinal microbiota in the total cohort and in the cohort subdivided according to delivery mode are reported in Supplementary Tables 1 and 2, respectively. In general, bacterial levels, and the concomitant production of SCFA, increased along the study, which is in good agreement with previously reported data (Arboleya et al., 2012, 2016).

After the first few days, infants' weight increased, fitting well into a linear structure (Supplementary Figure 1). Initial exploratory analyses were conducted by using generalized mixed linear effects model with infant as random effect and gestational age as spline. The model showed a large effect of time (infant age) and gestational age, which together explained 80.5% of variance (determination coefficient, $r^2 = 0.805$). Therefore, to further assess these associations the model was adjusted for these two variables and analyzed by multivariate regression to

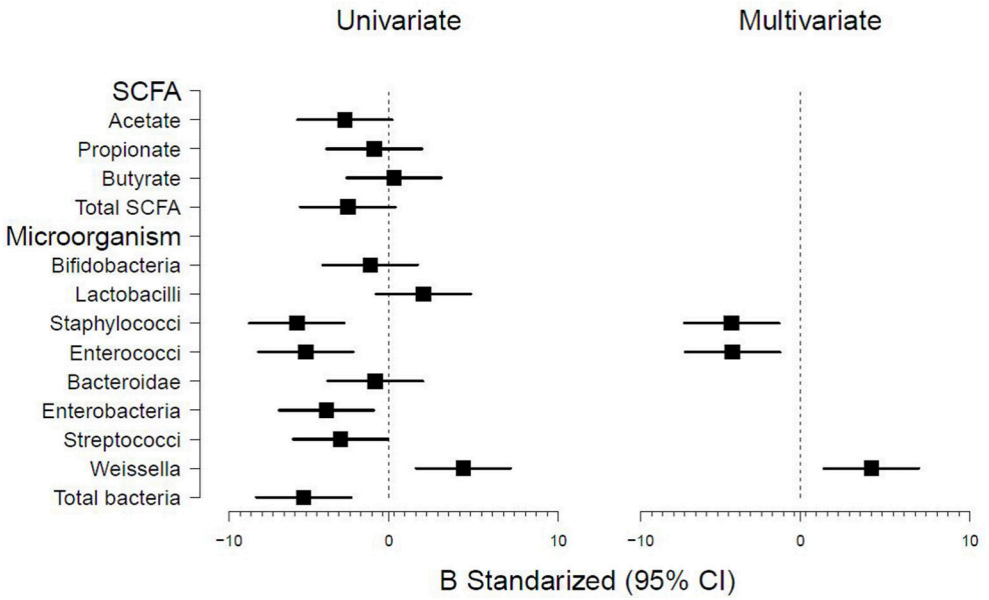

FIGURE 1 | Forest plot showing the microbiota-related variables measured and association with infant weight determined by univariate and multivariate generalized linear models. Models were adjusted by infant and gestational age.

determine the effects of the microbiota-related variables. The results indicated an association of the intestinal microbiota with weight-gain in preterm infants (**Figure 1**). A significant effect of the levels of *Staphylococcus* ($p < 0.001$), *Enterococcus* ($p < 0.001$), *Enterobacteriaceae* ($p = 0.011$), *Streptococcus* ($p = 0.047$), *Weissella* ($p < 0.001$), and total bacteria ($p = 0.002$) was observed. In the full multivariate model (using backward elimination stepwise regression based on AIC) three variables remained significant; *Enterococcus* ($p = 0.004$), *Staphylococcus* ($p = 0.005$), and *Weissella* ($p = 0.004$) (**Figure 1**). The other microbial groups analyzed did not show any significant effect. Similarly, none of the SCFA analyzed showed a significant effect.

To evaluate the ability of the early microbiota-related variables for predicting weight-gain, the association of the microbiota variables at 2 and 10 days-of -age with the weight gained at 30 days of age (as percentage of birth-weight) was studied. Gestational age was found to be the major contributing factor, explaining a 55% of variance ($r^2 = 0.55$). After adjusting for gestational age and birth weight (both together explaining 56% of variance), the levels of *Enterobacteriaceae* at 2 and 10 days-of-age, *Streptococcus* and total bacteria levels at 2 days, and those of *Bacteroides-group* at 10 days were found to have a significant effect upon weight gain (**Table 1**), increasing a 17.6% the percentage of variance explained, up to a total of 73.5%. None of the other microorganisms was found associated with weight-gain at 1 month, although a trend was observed for bifidobacterial levels (estimate 3.23 ± 1.64, $p = 0.054$).

Finally, the microbiota data obtained at 1 month of age were used for performing a cluster analysis, which allowed identifying three microbiota-driven infant groups (Supplementary Figure 2). These groups were not significantly different ($p > 0.05$) regarding gestational age, birth weight in grams or weight Z-score at birth

TABLE 1 | Microbiota-related variables at 2 and 10 days of infant age that were associated with weight-gain at 30 days of life (measured as percentage with regard to birth-weight).

Variable	Multivariate[a]		
	Estimate	Standard error	*p*
Enterobacteriaceae at 2 days	15.892	2.701	< 0.001
Streptococcus at 2 days	7.080	2.217	0.003
Total bacteria at 2 days	−19.159	3.288	< 0.001
Enterobacteriaceae at 10 days	−3.316	1.174	0.007
Bacteroides-group at 10 days	5.234	1.982	0.011

[a] *Adjusted for gestational age and basal weight.*

(data not shown). However, infant weight at 3 months of age showed a statistically significant difference among clusters ($p = 0.033$). At this later time infant weight in group 3 (3003 ± 681 gr) was significantly lower than in the other two infant groups, that did not show statistically significant differences between them (3988 ± 1163 gr for group 1 and 3883 ± 1344 gr for group 2). Similarly, at 3 months of age Z-scores differed among groups ($p = 0.013$) with group 3 (-2.37 ± 2.24) differing significantly from groups 1 and 2 (-0.62 ± 1.08 and -0.90 ± 1.90, respectively).

When the statistical models used in this study were adjusted for the potential confounders "delivery mode" or "antibiotic exposure" significant effects were not observed, which indicates that these variables are not influencing the associations observed. Actually, in our study delivery mode did not show a large effect on the analyzed microorganisms, since only the *Bacteroides*-group was found to be statistically different between vaginal and C-section babies, with reduced levels in the latter group at 10 and 30 days of life (Supplementary Table 2).

DISCUSSION

An altered intestinal microbiota during the first year of life, with reduced levels of bifidobacteria and increased number of *Staphylococcus aureus*, has been observed in infants developing obesity later on (Kalliomäki et al., 2008). Particular microbial species, such as *Bacteroides fragilis*, have been related with higher BMI z-scores (Vael et al., 2011; Scheepers et al., 2014). However, this evidence is still limited and focuses in full-term infants and obesity prevention, whilst nothing is known on the relationship between microbiota and weight-gain in preterm infants.

Our results indicate an association between the intestinal microbiota and weight-gain. The levels of some microorganisms, such as *Staphylococcus* and *Enterococcus*, were negatively associated with weight-gain whilst others such as *Weissella* did it positively. This last microorganism belongs to the *Leuconostoccaceae* family from the *Lactobacillales* order (Fusco et al., 2015), being then related to *Lactobacillus*, some of whose strains were found to maintain growth of infant mice during chronic undernutrition (Schwarzer et al., 2016). Since lactobacilli are often used as probiotics, their ability, as well as that of non-pathogenic *Weissella* strains, to promote growth and maturation in preterm infants should be further explored.

Moreover early microbiota was associated with later weight-gain, suggesting a potential effect of the early microbiota in weight-gain in preterm infants. Especially, *Enterobacteriaceae* and *Streptococcus* levels at 2 days of age and *Bacteroides*-group levels at day 10 showed a positive association with weight gain at 1 month of age. To this regard, the species *B. fragilis* has been repeatedly associated to increased BMI (Vael et al., 2011; Scheepers et al., 2014) in infants, pointing out at these microorganisms as potential players in the association intestinal microbiota-weight gain. When infants were clustered on the basis of their microbiota at 30 days of age, differences among the clusters were obtained for infant weight and weight Z-scores at 3 months of age, again suggesting a relationship between microbiota and weight-gain. To this regard, certain oligosaccharides have been reported to promote a microbiota-dependent increase in body-mass (Charbonneau et al., 2016), and transplanting the microbiota of undernourished children into germ-free mice resulted in growth impairment (Blanton et al., 2016). Similar relationships between intestinal microbiota and growth have also been reported in infants (Gough et al., 2015), and delayed colonization by specific commensals has been related with altered adiposity (Dogra et al., 2015). All these reports suggest the involvement of the gut microbiota in infants' weight-gain and development.

Neither the delivery mode, nor the antibiotic exposure, showed a significant effect in any of the statistical models used in this study. These facts point out that these important potential confounders are not influencing the observed associations between microbiota and weight-gain. To this regard, in the present study the delivery mode was found to have a limited impact on intestinal microbial levels; *Bacteroides* was the only group significantly affected by delivery mode, presenting lower counts in babies born by C-section than in those vaginally delivered. The reduced levels observed for this microbial genus is in agreement with previous studies carried out in a similar preterm infants group (Arboleya et al., 2015). In relation to this, reduced levels of *Bacteroides* in full-term babies born by C-section have also been reported (Jakobsson et al., 2014), although in full-term babies the impact of C-section seem to be larger (Dominguez-Bello et al., 2010) than in our cohort of preterm infants.

Our results underline the interest of exploring the intestinal microbiota as a potential target for favoring growth and maturation in preterm infants. However, the meaning of weight-gain in terms of infant maturation, disease risk, and later weight is a matter of concern (Wang et al., 2016) that requires of further, larger, and longer observational and intervention studies.

AUTHOR CONTRIBUTIONS

PM, GS, MS, NF, CD, and MG. designed the study; GS, MS, and NF recruited the infants and collected the data and samples; SA, CD, and MG. performed the microbiological analyses; PM, GS, and MG analyzed the data; all authors participated in the data interpretation, revised the manuscript and approved its final version. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

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Trophic Interactions of Infant Bifidobacteria and *Eubacterium hallii* during L-Fucose and Fucosyllactose Degradation

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Fucosyllactoses (2′- or 3′-FL) account for up to 20% of human milk oligosaccharides (HMOs). Infant bifidobacteria, such as *Bifidobacterium longum* subsp. *infantis*, utilize the lactose moiety to form lactate and acetate, and metabolize L-fucose to 1,2-propanediol (1,2-PD). *Eubacterium hallii* is a common member of the adult gut microbiota that can produce butyrate from lactate and acetate, and convert 1,2-PD to propionate. Recently, a Swiss cohort study identified *E. hallii* as one of the first butyrate producers in the infant gut. However, the global prevalence of *E. hallii* and its role in utilization of HMO degradation intermediates remains unexplored. Fecal 16S rRNA gene libraries ($n = 857$) of humans of all age groups from Venezuela, Malawi, Switzerland, and the USA were screened for the occurrence of *E. hallii*. Single and co-culture experiments of *B. longum* subsp. *infantis* and *E. hallii* were conducted in modified YCFA containing acetate and glucose, L-fucose, or FL. *Bifidobacterium* spp. ($n = 56$) of different origin were screened for the ability to metabolize L-fucose. Relative abundance of *E. hallii* was low (10^{-5}–10^{-3}%) during the first months but increased and reached adult levels (0.01–10%) at 5–10 years of age in all four populations. In single culture, *B. longum* subsp. *infantis* grew in the presence of all three carbohydrates while *E. hallii* was metabolically active only with glucose. In co-culture *E. hallii* also grew with L-fucose or FL. In co-cultures grown with glucose, acetate, and glucose were consumed and nearly equimolar proportions of formate and butyrate were formed. *B. longum* subsp. *infantis* used L-fucose and produced 1,2-PD, acetate and formate in a ratio of 1:1:1, while 1,2-PD was used by *E. hallii* to form propionate. *E. hallii* consumed acetate, lactate and 1,2-PD released by *B. longum* subsp. *infantis* from FL, and produced butyrate, propionate, and formate. Beside *B. longum* subsp. *infantis*, *Bifidobacterium breve,* and a strain of *B. longum* subsp. *suis* were able to utilize L-fucose. This study identified a trophic interaction of infant *bifidobacteria* and *E. hallii* during L-fucose degradation, and pointed at *E. hallii* as a metabolically versatile species that occurs in infants and utilizes intermediates of bifidobacterial HMO fermentation.

Keywords: *Eubacterium hallii*, trophic interactions, bifidobacterium, fucose, fucosyllactose

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INTRODUCTION

Human milk oligosaccharides (HMOs) are one of the major glycan source of the infant gut microbiota. Primary components of HMOs are D-glucose, D-galactose, L-fucose, N-acetylglucosamine, and sialic acid. Lactose constitutes the reducing end of HMOs, its galactose moiety can be fucosylated or sialylated to form 2′- or 3′-fucosyllactose (2′-FL or 3′-FL), or 3′- and 6′-sialyl-lactose (3′-SL or 6′-SL). Lactose can also be elongated with units of N-acetyllactosamine (Gal-β1-4GlcNAc) with its simplest form being Lacto-N-neotetraose (LNnT) (Kunz et al., 2000). The composition of HMOs is individually different and remains stable during different lactation phases (Niñonuevo et al., 2008; de Leoz et al., 2012). FLs can account for up to 20% of all HMOs (Niñonuevo et al., 2008; de Leoz et al., 2012).

Infant bifidobacteria species, such as *Bifidobacterium longum* subsp. *infantis* and *Bifidobacterium bifidum*, are adapted to degrade HMOs (LoCascio et al., 2010; Rockova et al., 2012; Turroni et al., 2014) and constitute a big proportion of the infant intestinal microbiota immediately after birth (Avershina et al., 2013; Jost et al., 2014). The degradation of HMOs relies on a complex network of extracellular solute binding proteins, transporters, and intra- or extracellular glycosyl hydrolases (GH). *B. longum* subsp. *infantis* carries out intracellular degradation while *B. bifidum* metabolizes HMOs extracellularly (Garrido et al., 2015). Both *B. longum* subsp. *infantis* and *B. bifidum* possess several α-fucosidases that release L-fucose from FL (Sela et al., 2012; Garrido et al., 2015). L-fucose can then be either used by the strain itself or can be released for bacterial cross-feeding (Garrido et al., 2015; Bunesova et al., 2016).

L-fucose is a desoxyhexose that is a common component of many N- and O-linked glycans and of glycolipids produced by mammalian cells (Becker and Lowe, 2003). L-fucose utilization has been investigated in depth in pathogens such as *Escherichia coli, Shigella* spp. and *Bacillus cereus* (Staib and Fuchs, 2014). These bacteria employ a fucose isomerase FucI, a fucose aldolase FucA, and a fucose kinase FucK to form L-lactaldehyde which can be further metabolized to 1,2-propanediol (1,2-PD). Recently, we could show that strains of *B. longum* subsp. *infantis* and *B. longum* subsp. *suis* metabolize L-fucose to 1,2-PD presumably by a pathway that employs non-phosphorylated intermediates similar to *Campylobacter* and *Xanthomonas* spp. (Bunesova et al., 2016). Genes related to L-fucose degradation were located on two genomic regions, one is part of the HMO degradation cluster H1 of *B. longum* subsp. *infantis* DSM 20088 (LoCascio et al., 2010), while region 2 contained a gene encoding a putative fucose permease (Bunesova et al., 2016).

In the intestine, 1,2-PD is a precursor of propionate by a pathway that employs a glycerol/diol dehydratase as a key enzyme (Reichardt et al., 2014). The frequent detection of genes encoding glycerol/diol dehydratases in fecal metagenomes of adults suggested that 1,2-PD conversion significantly contributes to intestinal propionate formation (Reichardt et al., 2014; Engels et al., 2016). One species with the ability to convert 1,2-PD to propionate is *Eubacterium hallii* which is a common commensal in adults (Engels et al., 2016). *E. hallii* formed similar amounts of propionate in the presence or absence of glucose and did not utilize glucose if 1,2-PD was present (Engels et al., 2016). *E. hallii* can also grow and form butyrate using either glucose, or acetate and lactate as substrates (Duncan et al., 2004).

We hypothesized that a trophic interaction between *E. hallii* and *B. longum* subsp. *infantis* can yield short chain fatty acids (SCFAs) butyrate or propionate from lactate and acetate, or from 1,2-PD, respectively. Both butyrate and propionate are important for gut microbiota/host homeostasis as they interact with the host epithelium and impact the immune system. Butyrate is a main energy source of colonocytes, impacts cell proliferation and differentiation, and lowers the risk of colitis and colorectal cancer (Wong et al., 2006; Plöger et al., 2012). Propionate acts as a precursor for gluconeogenesis in the liver and also impacts cell differentiation with potential health-promoting impact on intestinal inflammation, and cancer development (Reichardt et al., 2014).

Bifidobacterium is the predominant genus of the gut microbiota of breast fed infants. Little data exists on the occurrence of *E. hallii* in early life. Recently, a Swiss cohort study identified *E. hallii* as one of the first butyrate producers in the infant gut (Pham et al., 2016). However, the global prevalence of *E. hallii* and its role in the metabolism of L-fucose sourced from HMOs remains unexplored.

It was therefore the aim of this study to investigate the occurrence and abundance of *E. hallii* in populations of different age and origin, and to prove trophic interactions of *E. hallii* and *B. longum* subsp. *infantis* during growth in the presence of glucose, L-fucose, and FL. We also screened further *Bifidobacterium* spp. and strains ($n = 56$) to investigate whether species other *B. longum* subsp. *infantis* or subsp. *suis* are able to metabolize L-fucose.

METHODS

Bacterial Strains and Culture Conditions

E. hallii DSM 3353 obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) was cultivated in modified YCFA medium (mYCFA) containing 30 mM acetate as described by Duncan et al. (2004) with slight modifications (**Table 1**). All components except L-cysteine-HCL (Sigma-Aldrich) were solubilized in deionized water, and pH was adjusted to pH 7.6 with NaOH. The medium was flushed with CO_2 and boiled. When the color changed from blue to pink, L-cysteine-HCl (0.01%, w/v) was added. The medium was transferred to Hungate tubes flushed with CO_2, and tubes were sealed and autoclaved. Stab cultures of *E. hallii* that were frozen at −20°C in mYCFA agar (1.5% (w/v) agar) were used as stock cultures. For each experiment, a fresh agar stock was thawed; 1 ml of liquid YCFA medium was added and thoroughly shaken before being transferred to 8 ml liquid mYCFA medium. After incubation at 37°C for 24 h, the culture was transferred at least once to fresh mYCFA broth before the experiment. *Bifidobacterium* spp. (**Table 2**) were obtained from the culture collections of the Laboratory of Food Biotechnology of ETH Zurich, the Department of Microbiology, Nutrition, and Dietetics, of the Czech University of Life Sciences Prague, or

TABLE 1 | mYCFA medium composition.

Component	Addition
Amicase	1% (w/v)
Yeast extract	0.25% (w/v)
Sodium bicarbonate	0.5% (w/v)
Glucose (replaced with L-fucose and FL)	1% (w/v)
Mineral solution [3% (w/v) potassium dihydrogen phosphate, 6% (w/v) sodium chloride, 0.6% (w/v) magnesium sulfate, 0.6% calcium chloride (w/v)]	15% (v/v)
Vitamin solution [0.01% (w/v) biotin, 0.01 (w/v) cobalamin, 0.03% p-aminobenzoic acid (w/v), 0.05% folic acid (w/v), 0.15% pyridoxamine (w/v)]	0.1% (v/v)
Volatile fatty acid mix [56.6% (v/v) acetic acid, 20% (v/v) butyric acid, 13.3% (v/v) propionic acid]	0.31% (v/v)
Hemin (0.5 mg ml^{-1})	0.02%
Resazurin (1 mg ml^{-1})	0.1%
L-cysteine hydrochloride monohydrate	0.1%

from DSMZ. Stock cultures of bifidobacteria were maintained at −80°C in 30% glycerol. To prepare working cultures, bifidobacteria were streaked on Wilkens–Chalgren medium (Oxoid) supplied with soya peptone (5 g L^{-1}, Biolife, WCSP), Tween 80 (1 mL L^{-1}, Sigma-Aldrich), and fresh, filter-sterilized L-cysteine-HCl (0.5 g L^{-1}). Single colonies were picked and were grown in liquid WCSP supplied with fresh, filter-sterilized L-cysteine-HCl (0.5 g L^{-1}) at 37°C for 24 h. For preparation of co-culture experiments, 100 μL of *B. longum* subsp. *infantis* overnight culture grown liquid WCSP were added to mYCFA medium, and the culture was incubated at 37°C for 24 h. Unless otherwise stated, mYCFA containing 55 mM glucose was used to routinely cultivate *E. hallii* and *B. longum* subsp. *infantis*.

Single and Co-Culture Studies in the Presence of Different Substrates

Growth kinetics were assessed in mYCFA medium supplied with glucose (50 mM, mYCFA_glc, Sigma-Aldrich), L-fucose (40 mM, mYCFA_fuc, Sigma-Aldrich), or FL (6 mM 2′-FL and 6 mM 3′-FL, mYCFA_FL, Glycom A/S). Trace amounts of glucose were added to mYCFA_fuc to enforce initial growth (Bunesova et al., 2016). Hungate tubes containing 9 ml mYCFA_glc, mYCFA_fuc, or mYFA_FL were inoculated with overnight cultures of *E. hallii* and *B. longum* subsp. *infantis* (0.25 mL each). For comparison, *E. hallii* and *B. longum* subsp. *infantis* were also grown in single cultures. Samples were taken after 0, 4, 8, 12, 24, and 48 h of incubation for substrate and metabolite analysis, and for DNA isolation. Bacterial growth was evaluated by measuring the optical density at 600 nm (OD_{600}). Additionally, 16S rRNA gene copies of *E. hallii* and *B. longum* subsp. *infantis* were determined in the co-cultures as outlined below. Growth was investigated in at least independent triplicates with the exception of *B. longum* subsp. *infantis* growth in mYCFA_glc and mYCFA_fuc, which was only investigated in duplicates. Therefore, standard deviations are not shown in the respective graphs.

Screening of Bifidobacteria Strains for L-Fucose Utilization

Overnight bifidobacteria cultures grown in liquid WCSP were washed and resuspended in phosphate buffered saline (PBS). Bifidobacteria (50 μL) were inoculated in 950 μL API medium supplied fresh, filter-sterilized L-cysteine-HCl (0.5 g L^{-1}) and with 30 mM glucose, or with 30 mM L-fucose and trace amounts of glucose. Cultures were incubated at 37°C for 48 h. Growth and utilization of the carbohydrate source was judged by color change of the medium from blue to yellow. For selected strains, L-fucose utilization and metabolite formation were assessed by HLPC-RI as outlined below.

Analysis of Substrate Utilization and Metabolite Formation

Glucose and L-fucose consumption, and the formation of 1,2-PD, lactate, acetate, formate, butyrate, and propionate was measured using high performance liquid chromatography (Merck-Hitachi) equipped with an Aminex HPX-87H column (300 × 7.8 mm; BioRad) and a refractive index detector (HPLC-RI). Samples were centrifuged at 13,000 *g* for 5 min at 4°C. Supernatants (40 μL injection volume) were eluted with 10 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1} at 40°C. Sugars, SCFAs, 1,2-PD, and lactate (all Sigma-Aldrich) were quantified using external standards.

Propanal and propanol were quantified with ion chromatography with pulsed amperometric detection (IC-PAD) on a ICS-5000^{+} system (Thermo Scientific) equipped with a quaternary gradient pump, a thermoautosampler, and an electrochemical detector with a cell containing an Ag/AgCl reference electrode and a disposable thin-film platinum working electrode tempered at 25°C. Analytes were separated on a IonPac ICE-AS1 4 × 250 mm ion-exclusion column with guard column (Thermo Scientific) operated at 30°C using isocratic conditions (0.1 M methanesulfonic acid; 0.2 mL min^{-1}) for 36 min. The injection volume was 10 μL. Electrochemical data was obtained using a triple potential waveform consisting of regeneration/detection, oxidation, and reduction potentials: $E_1 = 0.3$ V ($t_1 = 0.31$ s), $E_2 = 1.25$ V ($t_2 = 0.34$ s, $t_{int} = 0.02$ s), $E_3 = -0.4$ V ($t_3 = 0.39$ s). Currents were measured and integrated with respect to time (t_{int}). Propanal and propanol (Sigma-Aldrich) were quantified using external standards.

DNA Isolation and Quantification *E. hallii* and *B. longum* subsp. Infantis in Infant Feces and in Co-Cultivation Studies

Genomic DNA was isolated from 0.5 mL fermented mYCFA using the FastDNA SPIN Kit for Soil (MP Biomedicals). Genomic DNA from stool samples ($n = 368$) collected as part of an infant cohort study, and of Swiss children and adult had been isolated as described before (Vanderhaeghen et al., 2015; Pham et al., 2016). The abundance of *E. hallii* was determined using primers EhalF (5′- GCGTAGGTGGCAGTGCAA -3′) and EhalR (5′- GCACCG RAGCCTATACGG-3′) (Ramirez-Farias et al., 2009). *B. longum* subsp. *infantis* was quantified using primer pair F (5′-TCGCGT CYGGTGTGAAAG-3′), and R (5′-CCACATCCAGCRTCCAC-3′) (Rinttilä et al., 2004). Primers Eub338F (5′-ACTCCTACG

TABLE 2 | L-fucose utilization and 1,2-PD formation of strains of bifidobacteria.

Species	Strain code	Origin	L-fucose utilization	1,2-PD formation
B. breve	DSM 20213	Intestine of infant	+	nd
	TPY 10-1	Kenyan infant feces, 6 m	+	+
	BSM 1-2	Kenyan infant feces, 6 m	+	+
	TPY 5-1	Kenyan infant feces, 6 m	+	+
	N4-BM5-i12	Swiss mother breast-milk	+	+
	N4-NF3-i1	Swiss infant feces, 1 w	+	+
	N4-NF3-i3	Swiss infant feces, 1 w	+	+
	N4-NF4-i1	Swiss infant feces, 2 w	+	+
	BR03	Probiotic drops	+	nd
	OL2	Czech infant feces, 10 w	+	nd
	TA1	Czech infant feces, 10 w	+	nd
B. longum subsp. *infantis*	DSM 20088	Intestine of infant	+	+
	DSM 20090	Intestine of infant	+	nd
	BRS 8-2	Kenyan infant feces, 6 m	+	nd
	TPY 12-1	Kenyan infant feces, 6 m	+	+
	TPY 8-1	Kenyan infant feces, 6 m	–	–
	BV	BIOPRON probiotic product	+	nd
B. longum subsp. *longum*	DSM 20219	Intestine of adult	–	–
	TA2	Czech infant feces, 10 w	–	–
	N2-MF1-i1	Swiss adult feces	–	–
	N18-MF4-i8	Swiss adult feces	–	–
	2ToBifN	Czech infant feces	–	–
	MA2	Czech infant feces	–	–
	BL13	Czech adult feces	–	–
B. longum subsp. *suis*	DSM 20211	Pig feces	–	–
	5/9	Calf feces	–	–
	022II	Calf feces	–	–
	BSM 11-5	Kenyan infant feces, 6 m	+	+
B. kashiwanohense	DSM 21854	Infant feces, 1.5 y old	–	–
	PV 20-2	Kenyan infant feces, 6 m	–	–
	TPY11-1	Kenyan infant feces, 6 m	–	–
B. bifidum	DSM 20456	Feces breast fed infant	–	–
	DSM 20239	Feces breast fed infant	–	–
	DSM 20082	Adult feces	–	–
	DSM 20215	Adult feces	–	–
	BRS 26-2	Kenyan infant feces, 6 m	–	–
	BSM 28-1	Kenyan infant feces, 6 m	–	–
	BRS300	Kenyan infant feces, 6 m	–	–
B. thermophilum	RBL67	Infant feces	–	–
B. animalis subsp. *lactis*	N1-MF3-i7	Intestine of adult	–	–
B. adolescentis	JK3	Czech infant feces, 3 w	–	–
	JK10	Czech adult feces	–	–
	JK17	Czech adult feces	–	–
	1MBif	Czech adult feces	–	–
B. catenulatum	DSM 16992	Human feces	–	–
	20ToBifN	Czech infant feces	–	–
	10VoBif	Czech infant feces	–	–
B. pseudocatenulatum	N18-NF4-i5	Swiss infant feces, 1 m	–	–
B. dentium	DSM 20436	Dental caries	–	–
	VBif10D2	Czech infant feces	–	–
B. angulatum	DSM 20098	Human feces	–	–

(Continued)

TABLE 2 | Continued

Species	Strain code	Origin	L-fucose utilization	1,2-PD formation
B. minimum	DSM 20102	Sewage	–	–
B. pseudolongum subsp. *pseudolongum*	DSM 20099	Pig feces	–	–
	DSM 20095	Chicken feces	–	–
B. pseudolongum subsp. *globosum*	DSM 20092	Rumen	–	–
	PV8-2	Kenyan infant feces, 6 m	–	–

*Utilization was tested in API medium supplied with 5 g L^{-1} L-fucose and trace amounts of glucose. Color shift of the API medium after 48 h of incubation indicated L-fucose utilization (+). 1,2-PD formation was verified by HPLC-RI for selected samples (**Table 3**). nd, not determined; m, months; w, weeks.*

GGAGGCAGCAG-3′) and Eub518R (5′- ATTACCGCGGCT GCTGG-3′) were employed to quantify total bacteria 16S rRNA genes (Fierer et al., 2005). Reactions were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems) and the Kapa SYBR FAST qPCR mastermix (Biolab Scientifics Instruments SA). Thermal cycling started with an initial denaturation step at 95°C for 3 min, followed by 40 cycles consisting of denaturation at 95°C for 3 s, annealing at 60°C for 10 s, and elongation at 72°C for 25 s. To verify specificity of amplification, melting curve analysis and agarose gel electrophoresis for amplicon size control were performed. To generate standards, PCR amplicons were cloned into PGEMT Easy Vector and heterologously expressed in *E. coli* according to instructions of the supplier (Promega). Standard curves were prepared from ten-fold dilutions of linearized plasmids harboring the 16S rRNA gene of interest. Linear detection range was between, log 2.3 and log 8.3 gene copies for *E. hallii* 16S rRNA genes, between log 2.9 and log 8.9 gene copies bifidobacteria 16S rRNA genes, and between log 3.0 and log 8.0 gene copies for total bacteria 16S rRNA genes. A factor of 5.5 and 4 for *Eubacterium* spp. and *B. longum* subsp. *infantis*, (rrnDB, http://rrndb.mmg.msu.edu; Větrovský and Baldrian, 2013), respectively, was applied to calculate the numbers of cells accounting for several 16S rRNA gene copies per genome.

16s rRNA Gene Amplicon Libraries Screens

16S rRNA gene sequencing datasets published by Yatsunenko et al. (2012) were downloaded from MG-RAST (MG-RAST ID 401). The 489 datasets with known age of the donor contained in total $1.0*10^9$ sequences and on average $2.1*10^6$ sequences. All reads were aligned against the Silva database (version 123.1, Quast et al., 2013) using MALT in semiglobal alignment mode (Herbig et al., 2016) and only matches with a percent identity >97% were reported. Alignments were then used to assign reads on the Silva taxonomy. A read was placed on the lowest taxon so that at least 90% of the alignments were covered by that taxon (majority vote 90%). Unaligned reads were extracted and placed on the Silva taxonomy by using the rdp classifier with a cutoff of 0.8.

Identification of L-Fucose Utilization Related Genes in *B. breve* Genomes

Genomes of *Bifidobacterium breve* DSM 20213 (PRJDB57) and UCC 2003 (CP000303.1) were screened for genes encoding proteins related to L-fucose metabolism using BlastP and the corresponding proteins of *B. longum* subsp. *infantis* DSM 20088 region 1 and 2 for the query (Bunesova et al., 2016, see also below).

Screening of Shotgun Sequencing Datasets for Presence of Proteins Encoding L-Fucose Utilization Regions 1 and 2

Metagenomic datasets ($n = 111$) published by Yatsunenko et al. (2012) were downloaded from MG-RAST (MG-RAST ID 98). The datasets contained in total 16,318,166 sequences and on average 147,010 sequences per sample. The smallest and largest datasets contained 19,587 and 478,588 sequences, respectively, with an average sequence length of 358 bases. All datasets were aligned using DIAMOND blastx in sensitive mode (Buchfink et al., 2015) against a modified bacterial RefSeq database (Pruitt et al., 2007). The modified RefSeq database was composed by adding 24 protein sequences of *B. longum* subsp. *infantis* DSM 20088 and *B. breve* DSM 20213 fucose utilization regions 1 and 2 (Bunesova et al., 2016, WP_003830405.1, WP_003830403.1, WP_003830401.1, WP_003830400.1, WP_065457149.1, WP_014484327.1, WP_003829769.1, WP_003829768.1, WP_025300063.1, WP_013141362.1, WP_003829764.1, WP_012578562.1, WP_012578563.1, WP_012578564.1, WP_012578565.1, WP_012578566.1, WP_012578567.1, WP_012578568.1, WP_013141357.1, WP_012578533.1, WP_012578534.1, WP_012578535.1, WP_012578536.1, WP_012578537.1) to the standard RefSeq database. Reads were assigned to the best aligning protein sequences which had a bitscore >50. Reads assigned to the 24 proteins sequences of interest were reported.

Statistical Analysis

A sigmoidal non-linear regression model [Weibull, 5 Parameter model, f = if(x<=x0-b*ln(2)^(1/c); y0; y0+a*(1-exp(-(abs(x-x0+b*ln(2)^(1/c))/b)^c))) implemented in SigmaPlot 13, Systat Software] was chosen to fit curves to log% abundance data of *E. hallii* in the different populations. Student's paired *t*-test with two-tailed distribution was used to identify significant differences in metabolite formation, $OD_{600\ nm}$, and cell counts between treatments. A $p < 0.05$ was considered significant.

RESULTS

Age and Geographical-Dependent Occurrence of *E. hallii*

We screened 489 previously obtained 16S rRNA gene amplicon libraries from Malawi, Venezuela and USA (Yatsunenko et al., 2012) for the occurrence of *E. hallii*. Concurrently, we determined *E. hallii* relative abundance in a Swiss infant cohort study which followed the fecal microbiota of 40 infants for the first 2 years of life (Pham et al., 2016) using qPCR, and compared to Swiss teenagers and Swiss adults (Vanderhaeghen et al., 2015; together $n = 368$; **Figure 1**). Minimum detection limits were $\sim 10^{-4}$ and 10^{-5}% relative abundance for 16S rRNA gene libraries and qPCR screenings, respectively.

In populations from Venezuela, the USA, and Malawi, *E. hallii* occurred in all infant samples of 1 year of age or younger

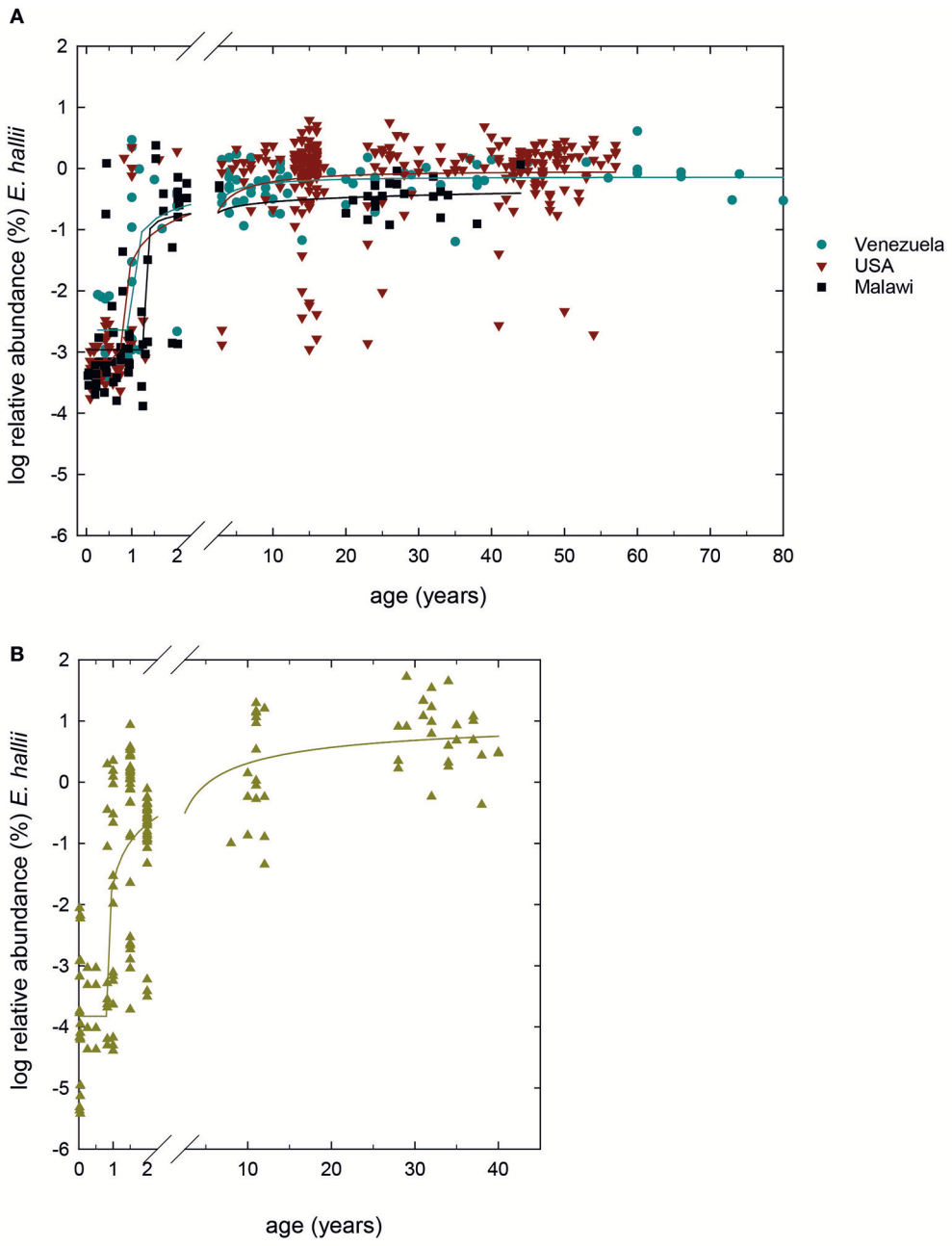

FIGURE 1 | Global occurrence of *E. hallii*. (A) Log % abundance of *E. hallii* in populations from USA, Malawi and Venezuela extracted from 16S rRNA gene amplicon libraries (Yatsunenko et al., 2012) ($n = 489$). **(B)** Log% abundance of *E. hallii* in feces of donors of different age groups from Switzerland ($n = 348$). A sigmoidal non-linear regression model was chosen to fit curves to log % abundance data of *E. hallii* in the different populations.

at means of log −2.0 ± 1.2, −2.9 ± 1.0, and −2.9 ± 0.8%, respectively (**Figure 1A**). Relative abundance increased after 1–1.5 years in donors from Venezuela (100% occurrence), the USA (95% occurrence), and Malawi (100%). In Swiss infants, occurrence levels of *E. hallii* fluctuated between 13 and 40% until 1 year, and increased to 85% at 2 years of age (log −2.9 ± 0.8%; **Figure 1B**). Between 5 and 10 years, relative abundance of *E. hallii* reached adult levels in all four populations (Malawi: log −0.4 ± 0.3%, Venezuela: log −0.2 ± 0.3%, USA: log −0.1 ± 0.6%, Switzerland: log 0.8 ± 0.5%).

Growth of *E. hallii* and *B. longum* subsp. *Infantis* in Single- and Co-Cultures

To investigate trophic interactions between *B. longum* subsp. *infantis* and *E. hallii* in the presence of glucose, L-fucose and FL, *B. longum* subsp. *infantis,* and *E. hallii* were grown in single- and co-cultures in mYCFA.

In single culture, *B. longum* subsp. *infantis* reached a final optical density ($OD_{600\ nm}$ 1.9) in the presence of glucose, and a lower maximal $OD_{600\ nm}$ when grown with L-fucose and FL (1.5 ± 0.1 and 1.5, respectively) after 48 h of incubation (**Figure 2A**). In mYCFA_glc −25.6 mM glucose was used, and 55.4, 9.9, and 13.1 mM acetate, lactate, and formate were formed (**Figure 2D**). In mYCFA_fuc, *B. longum* subsp. *infantis* used −28.3 ± 2.8 mM L-fucose and produced nearly equimolar amounts of 1,2-PD, acetate and formate (25.5 ± 4.8, 22.4 ± 1.0, and 26.7 ± 0.5 mM, respectively; **Figure 2G**). Lactate was produced at low amounts (2.0 ± 1.0 mM), and mean carbon recovery was 86%. In the presence of FL, *B. longum* subsp. *infantis* formed acetate (33.6 mM), lactate (16.8 mM), 1,2-PD (7.8 mM), and low amounts of formate (3.1 mM; **Figure 2J**).

In single culture, *E. hallii* grew rapidly to $OD_{600\ nm}$ of 1.7 ± 0.2 after 12 h of incubation in mYCFA_glc (**Figure 2B**). From 1 mol glucose and 0.5 mol acetate, ∼1 mol butyrate and 0.5 mol formate were produced (**Figure 2E**). Maximum optical density in mYCFA_FL and mYCFA_fuc was significantly lower than with glucose with $OD_{600\ nm}$ 0.9 and 0.5 after 12 and 9 h of incubation, respectively (**Figure 2B**). With L-fucose and FL, *E. hallii* formed 2.7 ± 1.0 and 6.0 ± 1.1 mM butyrate, respectively (**Figures 2H,K**). No acetate utilization and formate production were detected.

For the co-cultures, qPCR was used in addition to optical density measurements to monitor the growth of both strains (**Figure 3**). Cell counts were calculated based on 16S rRNA genes corrected for several 16S rRNA gene copies per genome. In mYCFA_glc, cell counts of both strains increased by log 2.8 cells ml^{-1} during the first 8 h of incubation, after which growth slowed down (**Figure 3A**). Highest $OD_{600\ nm}$ was reached after 24 h of incubation (**Figure 2C**). The amount of acetate used, and butyrate and formate formed in the co-cultures was similar to *E. hallii* single cultures (**Figure 2F**), and only low amounts of lactate (0.6 ± 0.7 mM) were detected after 48 h (**Figure 2F**).

Co-cultures grown in mYCFA_fuc reached a maximum OD of 1.1 ± 0.1 after 48 h of incubation (**Figure 2C**). During the first 12 h, *B. longum* subsp. *infantis* and *E. hallii* grew exponentially with increased cell counts of log 3 and log 2.7 cells mL^{-1}, respectively (**Figure 3B**). In co-cultures, *B. longum* subsp. *infantis* utilized only ∼35% of L-fucose compared to single cultures (10.4 ± 8 mM). The co-cultures formed propionate (8.2 ± 3.5 mM), butyrate (7.4 ± 0.8 mM), and formate (10.9 ± 1.7 mM; **Figure 2I**). Butyrate formed by the co-cultures was significantly ($p < 0.05$) higher compared to butyrate formation of *E. hallii* single culture in mYCFA_fuc (7.4 ± 0.8 vs. 2.7 ± 1.0 mM). 1,2-PD, propanal, and propanol were not detected during co-culture fermentations, and there was no apparent accumulation or consumption of acetate or lactate (**Figure 2I**).

When grown in co-cultures in mYCFA_FL, cell counts of *B. longum* subsp. *infantis* and *E. hallii* increased by 3.2 and 2.8 log during the first 8 h of incubation (**Figure 3C**), and maximal OD was reached after 24 h of incubation (**Figure 2C**). Acetate (18.2 ± 1.6 mM), propionate (9.7 ± 5.1 mM), and butyrate (5.4 ± 3.1 mM), and formate (19.7 ± 5.0 mM) were detected after 48 h of incubation (**Figure 2L**). 1,2-PD and lactate were only present at low levels <1 mM.

Bifidobacterium Production of 1,2-PD from L-Fucose

To investigate whether L-fucose utilization was a trait limited to *B. longum* subsp. *infantis,* or was also present in other infant-, adult-, or animal-associated bifidobacteria, we screened $n = 56$ *Bifidobacterium* strains (**Table 2**) for growth in API medium supplied with 30 mM L-fucose and trace amounts of glucose. All isolates were capable of growing in API medium supplied with 30 mM glucose indicating the suitability of the assay. Only strains of *B. longum* subsp. *infantis* and subsp. *suis,* and of *B. breve* were identified as being able to metabolize L-fucose and to form 1,2-PD. L-fucose was used and 1,2-PD was formed in a ratio of ∼1:1 by all L-fucose-utilizing strains (**Table 3**). Additionally, lactate, acetate, and formate were produced leading to carbon recoveries between 87 and 109% (**Table 3**).

Genes Related to L-Fucose Utilization in *B. breve* Genomes

We previously identified two genomic regions that encompass genes potentially involved in L-fucose utilization of strains of *B. longum* subsp. *infantis* and *B. longum* subsp. *suis* (Bunesova et al., 2016). The genome of *B. breve* DSM 20213 (PRJDB57) possessed a set of genes with slightly differing genome organization to *B. longum* subsp. *infantis* and *B. longum* subsp. *suis* (**Figure 4**). Region 1 encompassed a gene encoding an α-fucosidase (GH family 95) with 78% homology in 607/783 AA to BLON_2335 of *B. longum* subsp. *infantis* DSM 20088. Region 1 also contained genes encoding a putative L-fuconate dehydratase (96% homology in 284/293 AA to *B. longum* subsp. *infantis* DSM 20088 L-fuconate dehydratase, region 1), a L-fucose dehydrogenase (93% homology in 245/262 AA, region 1), L-fuconolactone hydrolase (50% homology in 132/264 AA, region 2), and a 4-hydroxy-tetrahydrodipicolinate synthase (89% homology in 265/296 AA, region 1). *B. breve* region 2 contained another L-fuconate dehydratase (97% homology in 417/426 AA, region 2), L-fucose dehydrogenase (94% homology in 249/263 AA, region 2), a putative fucose permease (62%

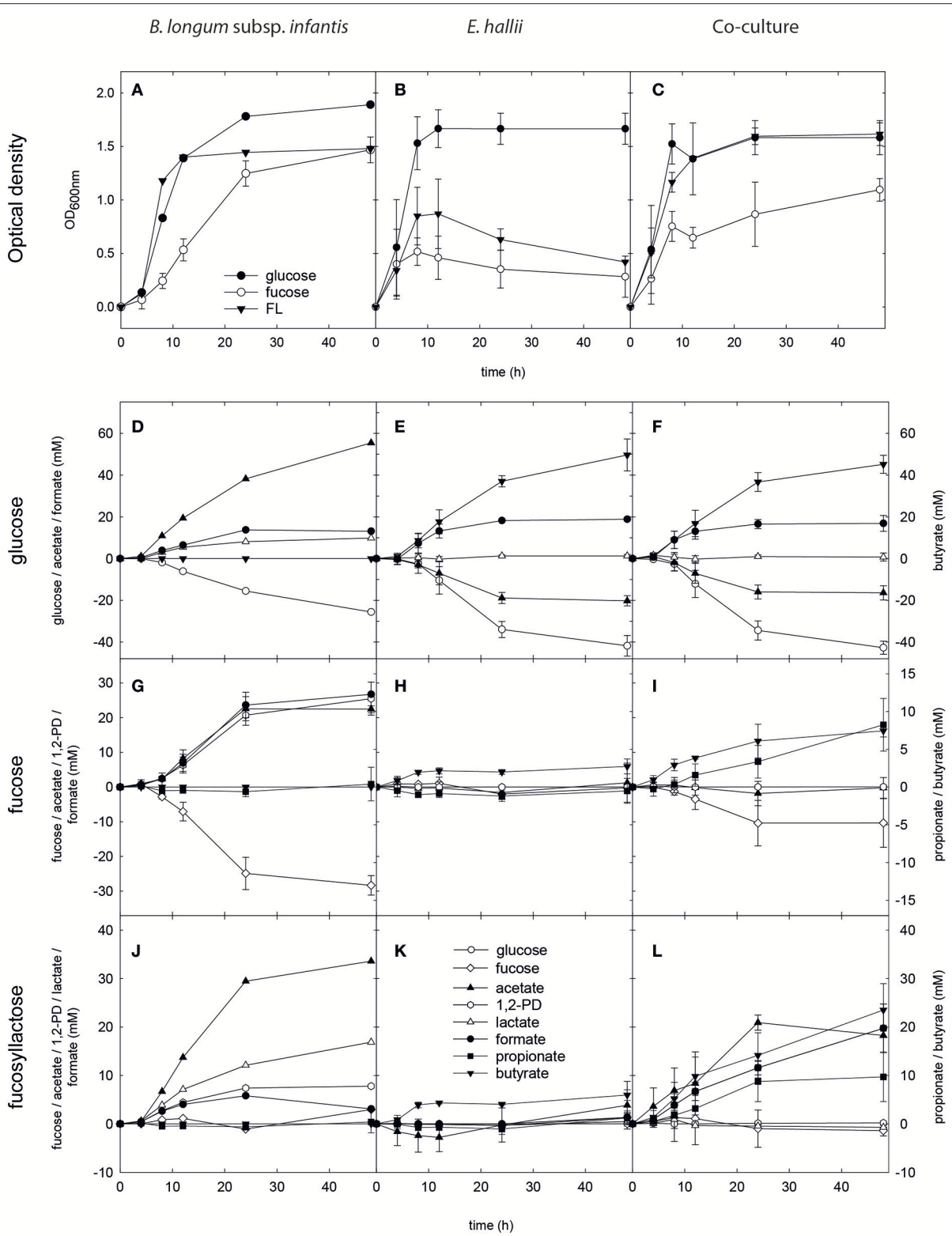

FIGURE 2 | Growth, substrate utilization, and metabolite formation of *E. hallii* and *B. longum* subsp. *infantis* in single and co-culture. Optical density (OD_{600nm}) of *B. longum* subsp. *infantis* **(A)**, *E. hallii* **(B)**, and the *B. longum* subsp. *infantis* / *E. hallii* co-culture **(C)** during growth in modified YCFA supplied with glucose, L-fucose or FL. Substrate utilization and metabolite formation of *B. longum* subsp. *infantis* **(D,G,J)**, *E. hallii* **(E,H,K)**, and the *B. longum* subsp. *infantis* / *E. hallii* co-culture **(F,I,L)** in mYCFA supplied with glucose **(D–F)**, L-fucose **(G–I)**, or FL **(J–L)**.

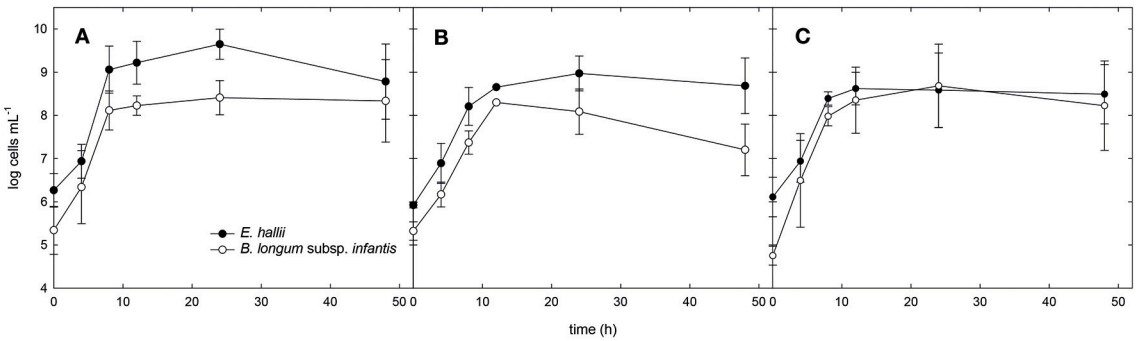

FIGURE 3 | Cell counts of *B. longum* subsp. *infantis* and *E. hallii* during growth in co-culture. Cell counts of *B. longum* subsp. *infantis* and *E. hallii* during growth in co-culture in mYCFA_glc **(A)**, mYCFA_fuc **(B)**, and mYCFA_FL **(C)**.

TABLE 3 | Substrate utilization, metabolite formation and carbon recovery.

		Substrate consumption (mM)	Metabolite production (mM)				Carbon recovery (%)
Species	**ID**	**L-fucose**	**Lactate**	**Formate**	**Acetate**	**1,2-PD**	
B. breve	TPY 10-1	−14.9 ± 4.5	10.2 ± 5.2	6.3 ± 6.0	9.2 ± 6.7	13.8 ± 4.0	109
	BSM 1-2	−8.5 ± 1.1	5.3 ± 4.4	4.2 ± 3.0	3.1 ± 2.0	9.3 ± 1.3	105
	TPY 5-1	−12.7 ± 0.5	6.5 ± 2.3	5.4 ± 0.9	6.5 ± 4.1	11.4 ± 1.4	94
	N4-BM5-i12	−8.3 ± 3.9	4.6 ± 2.5	4.0 ± 0.5	2.5 ± 3.4	6.8 ± 1.9	87
	N4-NF3-i1	−11.7 ± 3.1	7.2 ± 1.5	6.6 ± 6.7	5.5 ± 5.0	11.2 ± 2.8	99
	N4-NF3-i3	−5.9 ± 1.4	6.1 ± 1.5	0.6 ± 1.1	1.4 ± 0.9	5.7 ± 1.7	102
B. longum subsp. *infantis*	DSM 20088	−3.9 ± 0.7	3.8 ± 0.4	−0.1 ± 0.2	0.8 ± 1.4	3.9 ± 0.8	109
	TPY 12-1	−3.8 ± 1.5	1.4 ± 0.5	1.2 ± 1.1	1.6 ± 0.3	3.8 ± 1.1	91
B. longum subsp. *Suis*	BSM 11-5	−11.5 ± 1.0	5.8 ± 1.4	4.8 ± 4.2	3.3 ± 0.9	11.2 ± 1.3	91

L-fucose utilization and lactate, formate, acetate, and 1,2-PD formation of selected bifidobacteria in API medium supplied with 30 mM L-fucose and trace amounts of glucose after 48 h of incubation at 37°C.

homology in 262/461 AA, region 2), a L-fuconolactone hydrolase (96% homology in 246/255 AA, region 2), and a 4-hydroxy-tetrahydrodipicolinate synthase (69% homology in 206/296 AA, region 1; **Figure 4**). *B. breve* UCC 2003 had an identical genomic set-up to *B. breve* DSM 20213 (data not shown).

Coding Potential for Bifidobacteria Fucose Utilization in Fecal Metagenomes

Bifidobacteria were the predominant taxa in feces of infants from Venezuela, Malawi, and the USA during the first 2 years of life based on 16S rRNA gene sequencing data (**Figure 5**). For 111 samples, metagenomic data of the same donor data were also available (Yatsunenko et al., 2012). Metagenomes were screened for the presence of proteins presumably involved in bifidobacterial L-fucose utilization (**Figure 4**). At the threshold of detection allowed by sequencing coverage, the majority of fecal metagenomes of infants under 2 years of all three populations possessed the coding potential for bifidobacterial fucose utilization (**Figure 5**). All infant metagenomes from Venezuela (n = 11, 0.25–2 years) had coding potential for bifidobacteria L-fucose utilization. Eighty-seven and Seventy-one percent of proteins of both regions were recovered for *B. longum* subsp. *infantis* and *B. breve* respectively; all infants were positive for *B. longum* subsp. *infantis* while *B. breve* L-fucose utilization was not detected in 1 infant. Similarly, all infant metagenomes from Malawi (n = 18, 0.05–1.53 years) had coding potential for bifidobacteria L-fucose utilization. On average, 93 and 74% of the proteins of *B. longum* subsp. *infantis* and *B. breve* were detected, respectively. Of the 44 fecal metagenomes of US American infants (0.08–1.6 years), 16 and 28 were negative and positive for bifidobacteria L-fucose utilization, respectively. *B. breve* assigned proteins were detected in all children while *B. longum* subsp. *infantis* L-fucose utilization related proteins were not detected in one infant. The recovery of proteins of region 1 and 2 was 47 and 23% for *B. longum* subsp. *infantis* and *B. breve*, respectively. Bifidobacterial L-fucose utilization related proteins were also detected in feces of a 5 and a 6 year old US American child (**Figure 5**).

DISCUSSION

E. hallii is a metabolically versatile species that can contribute to intestinal butyrate and propionate formation (Duncan et al., 2004; Engels et al., 2016). In adults, *E. hallii* is a regular constituent of the gut microbiota (Engels et al., 2016). As shown in this study, *E. hallii* persistently occurred in the first months

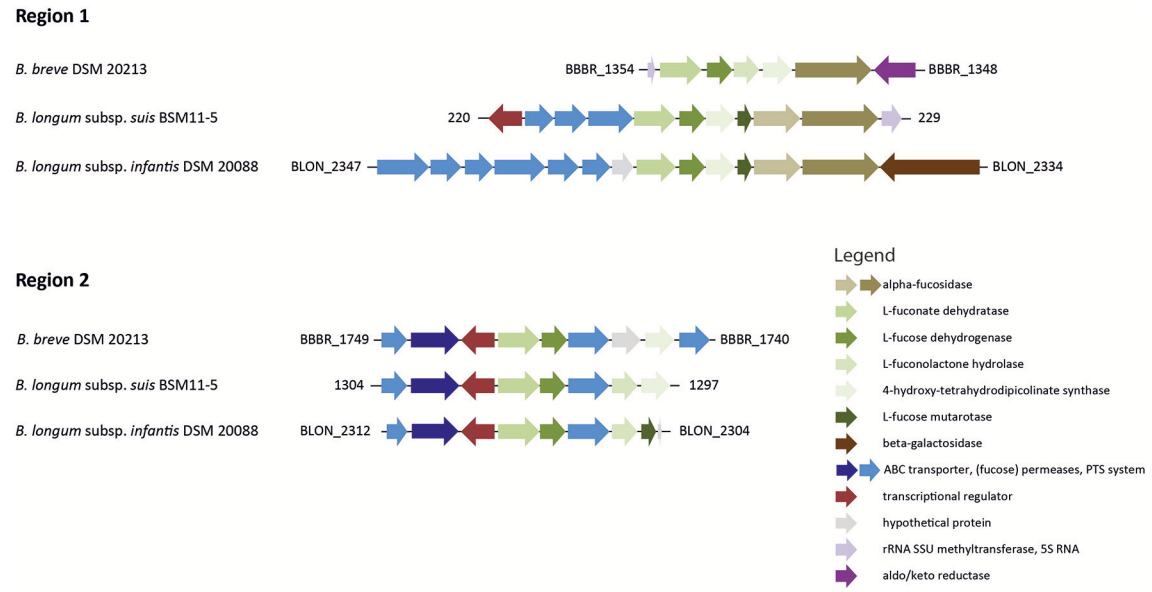

FIGURE 4 | Genomic regions encompassing genes putatively involved in L-fucose degradation in *B. longum* and *B. breve*. The gene cluster of region 1 also contained genes encoding the α-fucosidases BLON_2335 and BLON_2336 and is part of the *B. longum* subsp. *infantis* HMO utilization operon H1 (adapted from LoCascio et al., 2010; Bunesova et al., 2016 not drawn according to scale).

after birth at low abundance of the fecal microbiota and reached adult levels at ∼5–10 years of age independent of geographical donor origin. Thus, *E. hallii* is a commensal occurring very early in life which might contribute to metabolic interactions starting at 1–2 years of age when abundance markedly increased in all populations.

B. breve and *B. infantis* subsp. *longum* were identified as species capable of metabolizing fucose. Both are two of the most representative species found in breast-milk fed infants (Avershina et al., 2013; Matsuki et al., 2016), and fecal metagenome analysis indicated the coding potential for bifidobacterial fucose utilization in children under 2 years. Trophic interactions of *E. hallii* and *B. longum* subsp. *infantis* and/or *B. breve* during L-fucose and FL utilization can therefore be considered infant specific (**Figure 6**). Interestingly, general occurrence and the presence of bifidobacteria capable of L-fucose utilization appeared to be higher in Venezuelan and Malawian than in American infants. This might reflect differences in feeding practice, as in Venezuela and Malawi, all analyzed infants below 2 years were breast-fed, while in the USA, 71% received formula (Yatsunenko et al., 2012).

In the infant gut, *E. hallii* can utilize lactate and acetate produced by bifidobacteria during the degradation of hexoses. Bifidobacteria metabolize hexoses via the "bifid shunt" with fructose-6-phosphoketolase as the key enzyme. Glucose (1 mol) theoretically yields 1.5 mol acetate, 1 mol lactate, and 2.5 ATP (de Vries and Stouthamer, 1967, 1968). However, this ratio depends on whether the intermediate pyruvate is cleaved to acetyl phosphate and formate, or whether it is reduced to lactate (Palframan et al., 2003). *B. longum* subsp. *infantis*, *B. longum* subsp. *suis*, and *B. breve* also metabolized the desoxyhexose L-fucose. In mYCFA, nearly equimolar proportions of acetate, formate and 1,2-PD and only little lactate were formed while in API medium, lactate was produced at the expense of formate and acetate. The ratio of acetate, formate, and lactate formed varies for species, substrate source and carbohydrate supply (limitation or excess; Macfarlane and Gibson, 1995; Palframan et al., 2003). Here, ratios differed for the same species when supplied with the same carbohydrate (30 mM L-fucose). In this study, mYCFA was prepared strict anaerobically while API medium was only facultative anaerobic, which could have also impacted pyruvate metabolism. It was shown before that the presence of oxygen changed final metabolites formed by *B. longum subsp. infantis* (González et al., 2004).

Both bifidobacteria and *E. hallii* were able to produce formate during the degradation of FL (**Figure 6**). Methanogens, which can produce methane from formate and CO_2, are usually not detected in infants (Vanderhaeghen et al., 2015). As feces collected as part of a cohort study following 16 infants from 2 weeks to 2 years of age (Pham et al., 2016, unpublished data) contained no or only very low levels of formate, it can be assumed that the formate produced during HMO degradation is further utilized. Formate together with CO_2 can also be used by acetogenic microbes such as *Blautia* spp. to produce acetate via the Wood-Ljungdal pathway. Little literature exists on formate cross-feeding within the infant gut microbiota. However, as one of the important intermediate metabolites, the effect of formate and formate utilization on infant gut health should be investigated in further studies.

We identified for the first time the ability of *B. breve* strains to metabolize L-fucose to 1,2-PD. We previously

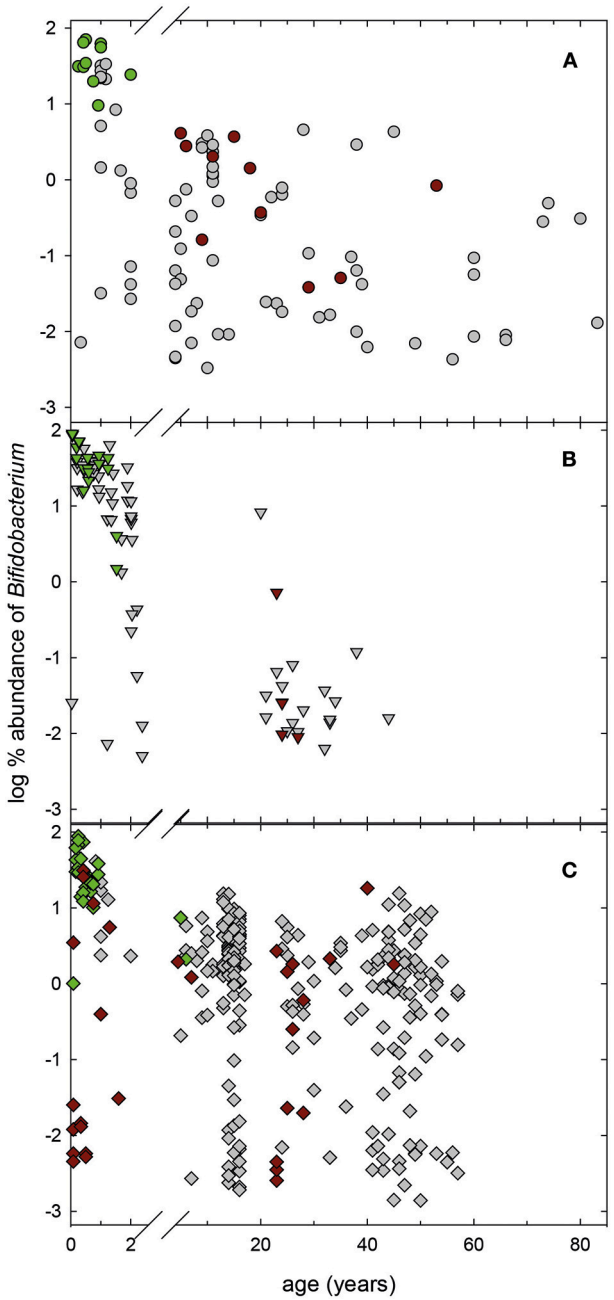

FIGURE 5 | Relative abundance of the genus *Bifidobacterium* in infant fecal samples. Relative abundance of *Bifidobacterium* in 16S rRNA gene libraries of infant feces collected from Venezuela **(A**, $n = 98$**)**, Malawi **(B**, $n = 84$**)**, and USA **(C**, $n = 307$; Yatsunenko et al., 2012**)**. For samples with available metagenomes (Yatsunenko et al., 2012), the presence of genes encoding bifidobacterial L-fucose utilization related proteins. Green symbols, samples that were positive for bifidobacteria L-fucose utilization related proteins, red symbols, samples that were negative, gray samples, no metagenomes available.

suggested a *Bifidobacterium* L-fucose utilization pathway based on genome comparison which has been also identified by a recent study investigating FL degradation by a strain of *B. longum* subsp. *longum* (Bunesova et al., 2016; Garrido et al., 2016). Similar to strains of *B. longum* subsp. *infantis* and *B. longum* subsp. *suis*, *B. breve* harbored two genomic regions which encompassed genes putatively encoding enzymes involved in L-fucose degradation with non-phosphorylated intermediates. In contrast, the L-fucose-negative *B. kashiwanohense* only possessed region 1 (Bunesova et al., 2016), suggesting that both regions are necessary for L-fucose metabolism. In addition, two of the identified genes encoding fucose permease (FucP) and a 4-hydroxy-tetrahydrodipicolinate synthase, were upregulated when *B. breve* was grown in co-culture with mucin degrading *B. bifidum* (Egan et al., 2014). *B. bifidum* releases L-fucose during growth with mucin similar to the release of fucose from FL (Turroni et al., 2014; Garrido et al., 2015; Bunesova et al., 2016), which then can be imported and metabolized by other species such as *B. breve* or L-fucose-utilizing strains of *B. longum*.

In adults, *B. adolescentis* is a predominant *Bifidobacterium* species, and cross-feeding between *B. adolescentis* and *E. hallii* has been reported before (Belenguer et al., 2006). *E. hallii* was thereby able to form butyrate from lactate and acetate that was produced when *B. adolescentis* grew in the presence of starch, or to use mono- or dissacharides released by *B. adolescentis* from fructooligosaccharides. We also observed substrate-dependent routes of metabolic cross-feeding. In the presence of L-fucose, propionate was the main metabolite formed by *E. hallii*. In co-cultures with *B. longum* subsp. *infantis*, the formation of propionate, butyrate, and formate from FL was observed. Here, butyrate and formate could have been produced from lactate and acetate, or directly from glucose released by *B. longum* subsp. *infantis*, while propionate again was derived from L-fucose.

FL is a major component of HMOs (de Leoz et al., 2012). Therefore, it can be implied that a substantial proportion of L-fucose is metabolized to propionate if *E. hallii* is present. However, not all women are able to secrete α-(1-2)-fucosylated HMOs due to mutations that render the responsible fucosyltransferase FUT2 inactive. About 20% of the European and African population carry an inactive FUT2 (Kelly et al., 1995; Liu et al., 1998). The activity of FUT2 and with that the presence of fucosylated HMOs has been linked to differences in the establishment of the infant gut microbiota (Lewis et al., 2015). The bifidobacteria community of infants born to non-secretor mothers was reported to establish later, to carry reduced numbers of bifidobacteria, and to contain a lower percentage of bifidobacteria capable of degrading 2-FL compared to infants of secretor mothers (Lewis et al., 2015). Thus, non-secretor mothers' milk lacks a significant source for intestinal propionate formation. It could therefore be speculated that the SCFA profile of infants of secretor- and non-secretor mothers is different. However, to date, no large scale study compared the fecal SCFA profile of infants born to secretor and non-secretor mothers.

Fucosylated oligosaccharides are not only a carbon source for the intestinal microbes but also play an important role in host-microbe interactions (Pickard et al., 2014). On one hand, fucosylated HMOs protect against invasion of enteric pathogens acting as a decoy for epithelial attachment sites (Morrow et al., 2005). On the other hand, L-fucose released from fucosylated oligosaccharides has been related to virulence and

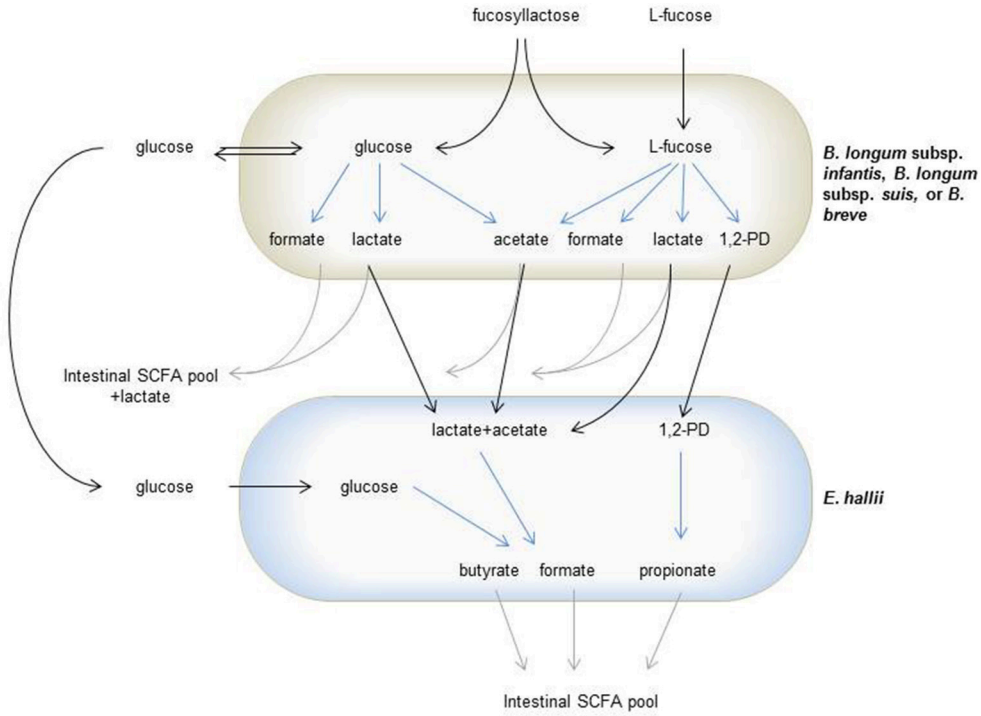

FIGURE 6 | Trophic interactions of L-fucose utilizing bifidobacteria and *E. hallii* during degradation of glucose, L-fucose or FL. Gray arrows indicate glucose, L-fucose or FL derived degradation metabolites fed in the intestinal SCFA pool, black arrows indicate sugars or metabolites cross-fed between bifidobacteria and *E. hallii*, blue arrows indicate metabolite formation.

host colonization of enteric pathogens. It was suggested that L-fucose released by *Bacteroides thetaiotamicron* was utilized by *Salmonella enterica* serovar Typhimurium during colonization of a mouse model (Ng et al., 2013). Likewise, L-fucose released by *B. thetaiotamicron* increased colonization of an enterohaemorrhagic *E. coli* (EHEC) in comparison to mutants with a knock-out of a fucose sensing two-component system (Pacheco et al., 2012). This fucose sensing two-component system regulates virulence and metabolic gene expression of EHEC (Pacheco et al., 2012). The ability to utilize L-fucose provided *Campylobacter jejuni* with a competitive advantage during the colonization of birds or pigs compared to fucose-negative mutants (Muraoka and Zhang, 2011; Stahl et al., 2011). Through utilization of L-fucose, the infant bifidobacterial community therefore could enhance colonization resistance toward enteric pathogens.

CONCLUSION

L-fucose utilization was identified here as a trait of only infant-derived bifidobacteria. Trophic interactions of L-fucose-utilizing infant bifidobacteria and *E. hallii* yielded different SCFAs from L-fucose or FL pointing at *E. hallii* as a metabolically versatile species utilizing intermediate metabolites of HMO fermentation. The utilization of L-fucose by the infant bifidobacterial community might enhance colonization resistance toward fucose-dependent enteric pathogens.

AUTHOR CONTRIBUTIONS

VB and CS designed experiments; VB, CS, VTP, and HR conducted experimental work; VB, CS, VTP, and H-JR analyzed data; CL, NB provided financial support; CS wrote manuscript with the help of all authors.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Sensitive Quantitative Analysis of the Meconium Bacterial Microbiota in Healthy Term Infants Born Vaginally or by Cesarean Section

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For decades, babies were thought to be born germ-free, but recent evidences suggest that they are already exposed to various bacteria *in utero*. However, the data on population levels of such pioneer gut bacteria, particularly in context to birth mode, is sparse. We herein aimed to quantify such bacteria from the meconium of 151 healthy term Japanese infants born vaginally or by C-section. Neonatal first meconium was obtained within 24–48 h of delivery; RNA was extracted and subjected to reverse-transcription-quantitative PCR using specific primers for *Clostridium coccoides* group, *C. leptum* subgroup, *Bacteroides fragilis* group, *Atopobium* cluster, *Prevotella, Bifidobacterium*, *Lactobacillus*, *Enterococcus*, Enterobacteriaceae, *Staphylococcus, Enterococcus*, *Streptococcus*, *C. perfringens*, and *C. difficile*. We detected several bacterial groups in both vaginally- and cesarean-born infants. *B. fragilis* group, Enterobacteriaceae, *Enterococcus*, *Streptococcus,* and *Staphylococcus* were detected in more than 50% of infants, with counts ranging from 10^5 to 10^8 cells/g sample. About 30–35% samples harbored *Bifidobacterium* and *Lactobacillus* (10^4–10^5 cells/g); whereas *C. coccoides* group, *C. leptum* subgroup and *C. perfringens* were detected in 10–20% infants (10^3–10^5 cells/g). Compared to vaginally-born babies, cesarean-born babies were significantly less often colonized with *Lactobacillus* genus (6% vs. 37%; $P = 0.01$) and *Lactobacillus gasseri* subgroup (6% vs. 31%; $P = 0.04$). Overall, seven *Lactobacillus* subgroups/species, i.e., *L. gasseri* subgroup*, L. ruminis* subgroup*, L. casei* subgroup*, L. reuteri* subgroup*, L. sakei* subgroup*, L. plantarum* subgroup, and *L. brevis* were detected in the samples from vaginally-born group, whereas only two members, i.e., *L. gasseri* subgroup and *L. brevis* were detected in the cesarean group. These data corroborate that several bacterial clades may already be present before birth in term infants' gut. Further, lower detection rate of lactobacilli in cesarean-born babies suggests that the primary source of lactobacilli in infant gut is mainly from maternal vaginal and–to a lesser extent–anal microbiota during vaginal delivery, and that the colonization by some important *Lactobacillus* species is delayed in babies delivered via cesarean-section.

Keywords: C-section, dysbiosis, gut bacteria, intestinal microbiota, *Lactobacillus*, meconium, RT-qPCR

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INTRODUCTION

Gut microbiota plays a fundamental role in various aspects of health and numerous diseases, but the multifarious dynamics of early-life intestinal microbiota colonization are only now beginning to come to light. For decades, it had been believed that the fetus as well as the intrauterine environment is universally sterile, and the earliest microbial exposure and colonization of infant gut begins during delivery and promptly thereafter by the acquisition of maternal (vaginal, anal, and skin microbiota) and environmental bacteria. However, some recent studies have elegantly reported the presence of bacteria in placenta (Aagaard et al., 2014; Collado et al., 2016), amniotic fluid (Oh et al., 2010; Collado et al., 2016), and umbilical cord blood (Jimenez et al., 2005), thereby manifesting that the fetus is already exposed to a variety of bacteria *in utero*. Several studies have also detected various bacteria in the meconium of healthy babies (Gosalbes et al., 2013; Hu et al., 2013; Del Chierico et al., 2015; Hansen et al., 2015; Collado et al., 2016), further evidencing that the gut microbial colonization may already begin before birth. However, quantitative data on the types and particularly the population levels (i.e., the absolute count) of bacteria that may be present in the meconium is still sparse. Also, the information on how the composition of '1st-day flora' differs between infants born vaginally or by cesarean section is limited. Given that the early-life microbiota plays important role in infant's physiological, metabolic and immunological development (Matamoros et al., 2013; Arrieta et al., 2015; Kerr et al., 2015) and is strongly impacted by birth mode (Penders et al., 2006; Tsuji et al., 2012; Backhed et al., 2015; Nagpal and Yamashiro, 2015; Bokulich et al., 2016; Nuriel-Ohayon et al., 2016), the numeric data about the population levels of bacteria present in the first intestinal discharge of vaginally-and cesarean-born newborns could be very informative and relevant. In this milieu, we herein aimed to quantify the bacteria present in the meconium of healthy term infants born vaginally or by C-section. Bacteria were quantified by using a highly sensitive culture-independent reverse-transcription-quantitative-PCR (RT-qPCR) based analytical method that targets the bacterial 16S rRNA molecules (Matsuda et al., 2009). We applied this method because it provides quantitative data; whereas 16S rRNA gene-based sequencing methods are largely qualitative and do not provide numerical data of bacterial population levels. Moreover, it is highly sensitive, whereas DNA-targeted PCR methods have relatively lower sensitivity and hence may overlook several subdominant but important bacteria, especially in neonatal stages when the microbiota is underdeveloped. Another advantage is that it provides information on viable cells, whereas the results of DNA-based analyses might also include background DNA (e.g., free bacterial DNA, or DNA of dead bacteria) that may be present in prenatal niches such as amniotic fluid and placenta and also in meconium. We have previously validated that the counts obtained by this RT-qPCR method are equivalent to the bacterial counts enumerated by culturing and fluorescent *in situ* hybridization (FISH) methods and that the detection sensitivity of RT-qPCR is approximately 100- to 1000-fold higher than that of other molecular methods such as qPCR and terminal restriction fragment length polymorphism (t-RFLP) (Matsuda et al., 2007, 2009; Kubota et al., 2010; Kurakawa et al., 2015b). Therefore, owing to these advantages, we specifically implemented RT-qPCR for the present study. Herein we demonstrate the quantification of bacteria that are present in the first meconium of healthy term Japanese infants born vaginally or by C-section.

MATERIALS AND METHODS

Subjects

The details of infants enrolled in this study are provided in **Table 1**. The study included 151 healthy full-term infants [134 vaginally-born (VG); 17 cesarean-born (CS)] whose mothers were recruited at the Gonohashi Obstetrics and Gynecology Hospital, Tokyo. The samples used in this study were part of a large cohort of Japanese infants enrolled in a longitudinal study monitoring the development of intestinal microbiota during the first 3 years of life, as reported in detail elsewhere (Tsuji et al., 2012). However, the meconium bacterial carriage was not previously investigated in detail, particularly in context to the delivery mode. In the present study, none of the babies were exposed to any type of formula-feed up to the point of first meconium; and all the infants as well as their mothers were apparently healthy with no indications of disease or intrauterine infection. All 17 cesarean cases were elective (planned) cesareans. The study design was approved by the ethical committee of Yakult Central Institute, Tokyo. Prior written informed consent was obtained from the parents or legal representatives of infants.

Sample Collection

A spoonful (0.5–1.0 g) of the first intestinal discharge (obtained from the first diaper) was collected fresh in a fecal collection tube (Sarstedt AG & Co., Numbrecht, Germany) containing 2 ml RNA*later* (Ambion, Austin, TX, USA). Out of total 151 samples, 148 were passed within 24 h after birth whereas the rest three samples were discharged between 24 and 48 h. As described elsewhere (Tsuji et al., 2012), stool samples were also collected at age 3 and 7 days, 1, 3, and 6 months, and 3 years for follow-up analyses. All samples were collected at the hospital by following

TABLE 1 | General characteristics of the 151 babies enrolled in the study including birth mode, gender, sampling time, birth-weight and height, and antibiotic exposure.

	Vaginally-born	Cesarean-born
Number of infants	134	17
Male:Female	71:63	11:6
Sampling time (days after birth)	1 ± 0.3	1 ± 0.4
Birth-weight (kg)	2.98 ± 0.36	2.97 ± 0.32
Height (cm)	48 ± 1.8	48 ± 1.5
Antibiotics (birth to sampling)	3	0
Formula-feed (birth to sampling)	0	0

routine and standard clean techniques such as the use of sterile sample collection tube and spatula, and handler's mouth masked, hands sanitized and gloved, and head capped while retrieving the sample. Immediately after collection, samples were stored in the refrigerator (3–4°C) anaerobically by using Anaero Pouch-Anaero (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) and were sent immediately in a cooling box with refrigerants and anaero-packs to the research lab where these were stored at 3–4°C in a Biosafety Category II microbiology laboratory until further processing.

Sample Processing, RNA Extraction and RT-qPCR

Primary Treatment

The samples were subjected to pretreatment according to the methods described previously (Matsuda et al., 2009). Briefly, the fecal samples were weighed and suspended in nine volumes of RNA*later* to make a fecal homogenate (100 mg feces/ml). In preparation for RNA extraction, 1ml of PBS(−) was added to 200 μl of fecal homogenate. The fecal homogenate was centrifuged at 4°C at 13,000 *g* for 10 min, the supernatant was discarded, and the precipitating pellet was stored at −80°C until RNA extraction.

RNA Extraction

RNA extraction was done by using previously described methods (Matsuda et al., 2009). Briefly, the thawed sample was resuspended in a solution containing 346.5 μl of RLT buffer (Qiagen Sciences, Germantown, MD, USA), 3.5 μl of β-mercaptoethanol (Sigma–Aldrich Co., St. Louis, MO, USA), and 100 μl of Tris-EDTA buffer. Glass beads (BioSpec Products, Inc., Bartlesville, OK, USA) (300 mg; diameter, 0.1 mm) were added to the suspension, and the mixture was subjected to a vigorous vortex procedure for 5 min using a ShakeMaster Auto apparatus (catalog no. BMS-A15; Bio Medical Science Inc., Tokyo, Japan). Acid phenol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (500 μl) was added, and the mixture was incubated for 10 min at 60°C. After phenol-chloroform purification and isopropanol precipitation, the nucleic acid fraction was suspended in 0.2 ml of nuclease-free water (Ambion, Inc.).

Reverse-Transcription-Quantitative-PCR (RT-qPCR)

Bacterial counts of various bacterial groups including total bacteria, *Clostridium coccoides* group, *C. leptum* subgroup, *Bacteroides fragilis* group, *Atopobium* cluster, *Prevotella*, *Bifidobacterium*, *Lactobacillus* subgroups and species, Enterobacteriaceae, *Enterococcus*, *Staphylococcus, Streptococcus*, *C. perfringens*, and *C. difficile* were analyzed by using a sensitive quantitative analytical system based on 16S or 23S rRNA molecules-targeted RT-qPCR, as per the methods described previously (Matsuda et al., 2009, 2012; Sakaguchi et al., 2010; Tsuji et al., 2012). Briefly, RT-qPCR was performed with a Qiagen OneStep RT-PCR kit (Qiagen GmbH, Hilden, Germany). Each reaction mixture (10 μl) was composed of 1X Qiagen OneStep RT-PCR buffer, 0.5X Q-solution buffer, each deoxynucleoside triphosphate at a concentration of 400 μM, a 1:100,000 dilution of SYBR green I (BioWhittaker Molecular Applications, Rockland, ME, USA), 0.4 μl of Qiagen OneStep RT-PCR enzyme mixture, and 5 μl of template RNA. Each primer set was added at a concentration of 0.6 μM. The reaction mixture was incubated at 50°C for 30 min for reverse transcription. The continuous amplification program consisted of one cycle at 95°C for 15 min, followed by 45 cycles at 94°C for 20 s, 55/60°C for 20 s, and 72°C for 50 s. The fluorescent products were detected in the last step of each cycle. A melting curve analysis was performed after amplification to distinguish the targeted PCR products from the non-targeted ones. The melting curve was obtained by slow heating at temperatures from 60 to 95°C at a rate of 0.2°C/s with continuous fluorescence collection. Amplification and detection were performed in 384-well optical plates with an ABI PRISM® 7900HT sequence detection system (Applied Biosystems, Foster, CA, USA). Standard curves for the corresponding standard bacterial strains were generated by using C_q (quantification cycle) values and the corresponding cell counts, which were determined microscopically with the DAPI (4′,6-diamidino-2-phenylindole) staining method as previously described (Matsuda et al., 2009). To determine the target bacterial populations in the fecal samples, 1/20,000, 1/200,000, and 1/2,000,000 portions of the RNA solution were subjected to RT-qPCR. The C_q-values in the linear range of the assay were applied to the analytical curve generated in the same experiment to obtain the corresponding bacterial count in each nucleic acid sample; this count was then converted to the count per sample. The details of analytical validation of specificities and sensitivities of these assays have been reported elsewhere (Matsuda et al., 2009, 2012; Sakaguchi et al., 2010). Briefly, the specificity of each primer set was confirmed against the total RNA fractions extracted from 10^5 cells of the corresponding standard bacterial strain (Matsuda et al., 2009) by using RT-qPCR. The amplified signal was considered positive (+) at $>10^4$ standard cells, positive/negative (±) at 10^4 to 100 standard cells, and negative (−) at <100 standard cells. The amplified signal was also defined as negative (−) when the corresponding melting curve had a peak different from that of the standard strain. To determine the detection sensitivity, RNA fractions were extracted from culture samples of corresponding reference strain in the early stationary phase (24 h), and bacterial counts were determined microscopically by DAPI staining. Serial RNA dilutions corresponding to bacterial counts ranging from 10^{-3} to 10^5 cells were assessed by RT-qPCR assay. The range of RNA concentrations at which there was linearity with C_q-value was confirmed ($R^2 > 0.99$). The details of the 16S or 23S rRNA gene targeted primers and the corresponding primer sequences, annealing temperatures and the minimum detection limits have been provided in the Supplementary Table S2.

Statistical Analyses

The results of bacterial count ($\log_{10}$ cells per gram of feces) are expressed as mean ± standard deviation. The detection rate was expressed as the percentage of infants in which the specific bacterium was detected. During analysis in the spread-sheets to calculate the mean bacterial count and the detection

rate, the cells were left blank if the specific bacterium was not detected (i.e., below the detection limit) in a sample. The count of genus *Lactobacillus* was expressed as the sum of the counts of six subgroups (*L. casei* subgroup, *L. gasseri* subgroup, *L. plantarum* subgroup, *L. reuteri* subgroup, *L. ruminis* subgroup, and *L. sakei* subgroup) and two species (*L. brevis* and *L. fermentum*). The comparisons of fecal bacterial counts by the mode of delivery were calculated by unpaired Student's *t*-test. Differences in the detection rate of bacteria were calculated by Fisher's exact probability test. $P < 0.05$ was considered statistically significant.

RESULTS

We detected several bacterial groups in the first meconium samples of both VG and CS infants. There was no significant difference in the birth weight, height or other generic parameters according to the birth mode (**Table 1**). The count and detection rate of intestinal bacteria are presented in **Table 2**. Overall, the meconium of 95% infants were found to harbor one or more types of bacteria; whereas the meconium of remaining 5% infants appeared to be sterile, i.e., no bacteria were detected in these samples. *B. fragilis* group, Enterobacteriaceae, *Enterococcus,*

TABLE 2 | Count and detection rate of major bacterial groups in the meconium of vaginally- and cesarean-born babies.

	Vaginally-born $n = 134$		Cesarean-born $n = 17$	
	Count[1]	Detection rate[2]	Count[1]	Detection rate[2]
Total bacteria[§]	6.8 ± 2.0	96.3	7.0 ± 1.9	88.2
Obligate anaerobes:				
Clostridium coccoides group	4.8 ± 1.6	20.1	4.8 ± 0.5	17.6
Clostridium leptum subgroup	4.8 ± 1.1	9.0	4.1 ± 0.3	23.5
Bacteroides fragilis group	5.3 ± 2.0	56.7	5.0 ± 2.3	52.9
Prevotella	5.2	1.5	ND	ND
Bifidobacterium	5.7 ± 1.7	32.1	5.7 ± 1.6	47.1
Atopobium cluster	6.0 ± 1.2	3.7	4.8	5.9
Clostridium perfringens	2.9 ± 0.9	11.2	2.8 ± 1.2	11.8
Clostridium difficile	3.8	0.7	ND	ND
Facultative anaerobes:				
Enterobacteriaceae	7.0 ± 1.8	53.7	5.9 ± 1.6	58.8
Enterococcus	5.0 ± 1.8	56.0	4.9 ± 1.8	64.7
Staphylococcus	5.4 ± 1.5	61.9	5.9 ± 1.7	41.2
Streptococcus	4.4 ± 1.2	52.2	3.9 ± 0.6	52.9
Lactobacillus[#]	3.7 ± 0.9	36.6	5.0	5.9*

[1]*Mean ± SD of Log_{10} cells/g feces.*
[2]*Detection rate (%) was expressed as the percentage of infants in which the specific bacterium was detected.*
[§] *Total bacteria are expressed as the sum of all the other bacteria listed in the table.*
[#]*The count of genus Lactobacillus was expressed as the sum of the counts of six subgroups (L. casei subgroup, L. gasseri subgroup, L. plantarum subgroup, L. reuteri subgroup, L. ruminis subgroup, and L. sakei subgroup) and two species (L. brevis, L. fermentum).*
**P = 0.012, vs. vaginally-born (Fisher's exact probability test).*
ND, not detected.

TABLE 3 | Count and detection rate of *Lactobacillus* subgroups and species in the meconium of vaginally- and cesarean-born babies.

	Vaginally-born $n = 134$		Cesarean-born $n = 17$	
	Count[1]	Detection rate[2]	Count[1]	Detection rate[2]
L. gasseri subgroup	3.6 ± 0.9	30.6	5.0	5.9*
L. ruminis subgroup	2.9	1.5	ND	ND
L. casei subgroup	3.6	0.7	ND	ND
L. reuteri subgroup	3.1 ± 0.6	5.2	ND	ND
L. sakei subgroup	4.6 ± 0.6	3.0	ND	ND
L. plantarum subgroup	4.5	0.7	ND	ND
L. brevis	3.1	1.5	3.2	5.9
L. fermentum	ND	ND	ND	ND

[1]*Mean ± SD of Log_{10} cells/g feces.*
[2]*Detection rate (%) was expressed as the percentage of infants in which the specific bacterium was detected.*
**P = 0.04, vs. vaginally-born (Fisher's exact probability test).*
ND, not detected.

Streptococcus, and *Staphylococcus* were most prevalent members (detected in more than 50% infants), with counts ranging from 10^5 to 10^8 cells/g sample, followed by bifidobacteria, lactobacilli, *C. coccoides* group, *C. leptum* subgroup, *C. perfringens*, *Atopobium* cluster, and *Prevotella* (**Table 2**). Of total 151 infants, *C. difficile* was detected only in one baby (in VG group).

Compared to VG infants, the meconium of CS infants were significantly less often colonized with *Lactobacillus* genus (6% vs. 37%; $P = 0.01$) (**Table 2**). Further examination of eight *Lactobacillus* subgroups and species revealed that *L. gasseri* subgroup was the most prevalent subgroup in VG infants (detected in 31% babies) whereas rest of the subgroups and species were detected only in less than 6% babies (**Table 3**). The detection rate of *L. gasseri* subgroup was significantly lower in CS babies compared to those born vaginally (6% vs. 31%; $P = 0.04$). Overall, total seven *Lactobacillus* subgroups/species, i.e., *L. gasseri* subgroup, *L. ruminis* subgroup, *L. casei* subgroup, *L. reuteri* subgroup, *L. sakei* subgroup, *L. plantarum* subgroup, and *L. brevis* were detected in the meconium samples of VG group, whereas only two members, i.e., *L. gasseri* subgroup and *L. brevis* were detected in CS group ($P = 0.04$). Other than lactobacilli, no significant difference was observed in the carriage of any other bacterial group in the meconium samples. However, the count of *Enterobacteriacea* was insignificantly lower in CS babies compared to VG babies ($P = 0.058$) (**Table 2**). Also, the overall ratio of facultative vs. obligate anaerobes (in proportion to total bacterial count) was significantly lower in CS babies than those delivered vaginally ($P = 0.005$) (**Figure 1A**); and accordingly, the overall proportional bacterial composition appeared to be slightly different in VG and CS babies (**Figure 1B**). Follow-up analysis of lactobacilli revealed that, compared to VG infants, the detection rate of *Lactobacillus* genus and several subgroups and species remained significantly or insignificantly lower in CS infants at different time-points during the first 6 months of life (**Figure 2**); but these differences tended to disappear by age 3 years.

DISCUSSION

To our knowledge, this is the largest birth cohort studied demonstrating a quantitative profile of the meconium bacterial microbiota in babies delivered vaginally or via C-section. Several remarkable studies have recently reported the presence of bacteria not only in meconium but also in placenta, amnion, and umbilical cord blood (Gosalbes et al., 2013; Hu et al., 2013; Del Chierico et al., 2015; Collado et al., 2016); but these were primarily based on DNA-based sequencing methods which, while being elegantly elaborative, may overlook several subdominant bacteria (e.g., $\leq 10^4$ cells/g feces), particularly during the earliest phase of life when the microbiota is immature. In this context, our data should fortify these evidences with important information of population levels of not only the predominant but also the subdominant bacteria that may inhabit the infant gut during critical developmental stages. We detected bacteria in the meconium of 95% babies while rest 5% samples appeared to be sterile (i.e., no bacteria detected). However, previous studies on term infants reported the presence of bacteria in 66% (using 16S rRNA gene-targeted FISH method) (Hansen et al., 2015) and 77% (using 16S/23S rRNA gene-targeted qPCR) (Martin et al., 2016) of total babies studied. This difference can most possibly be ascribed to differences in the detection sensitivities of the methods used, and hence could corroborate the advantage of high sensitivity of our RT-qPCR assays. We detected several bacterial clades, *albeit* with varying detection rates and numbers, in the meconium of both VG and CS infants (**Tables 2** and **3**), which clearly hint that the intrauterine environment harbors several bacterial communities which may provide the early inoculum for intestinal microbiota colonization. Further, these data show that the facultative anaerobes thrive–and hence are the first settlers–in the primitive gut, thereby pointing toward a relatively aerobic intestinal environment (Backhed et al., 2015; Del Chierico et al., 2015; Bokulich et al., 2016; Collado et al., 2016; Martin et al., 2016). This also suggests that the pioneer microbiota core is comprised predominantly of facultative anaerobes such as Proteobacteria, Staphylococci, Streptococci, Enterococci etc.; whereas obligates belonging to Firmicutes, Bacteroidetes, and Actinobacteria take over the predominance later on during infancy and early childhood, as also evident from the follow-up data of this cohort (Supplementary Table S1). This also concurs well with a recent study by our colleagues who reported that the 1st-day microbiota of healthy infants is by and large predominated by Enterobacteria or Staphylococci, but this predominance is mostly, rapidly transitioned to *Bifidobacterium*-dominated microbiota within a few days after birth (Matsuki et al., 2016).

Early-life microbiota colonization is strongly impacted by birth mode (Penders et al., 2006). During vaginal delivery, the infant is seeded with certain bacteria from the birth canal; but C-section bypasses this seeding thereby resulting in a different spectrum of pioneering microbiota (Dominguez-Bello et al., 2010; Backhed et al., 2015; Bokulich et al., 2016; Nuriel-Ohayon et al., 2016). Therefore, to determine whether–and how prominently–these differences exist in the first-pass samples, we compared the meconium microbiota of vaginally- vs. cesarean-born infants. The lower detection rate of *Lactobacillus* genus and the *L. gasseri* subgroup in the meconium of CS babies was intriguing. To know how long this difference persisted, we followed up the *Lactobacillus* carriage, and interestingly–*albeit* not surprisingly–VG and CS infants were found to exhibit dissimilar degrees of *Lactobacillus* detection rate at several time-points during the first 6 months of life, with detection rate being relatively lower in CS infants (**Figure 2**). It might also be possible that some vaginal materials are also transmitted from mother to baby during vaginal delivery that may have promoted the growth and activity of lactobacilli in vaginally-born babies; however, we were not able to verify this issue because of the unavailability of the maternal samples in this study. Interestingly, by using same RT-qPCR assays, we recently reported that the vaginal microbiota of healthy Japanese women of reproductive age is predominated numerically by *Lactobacillus* communities among

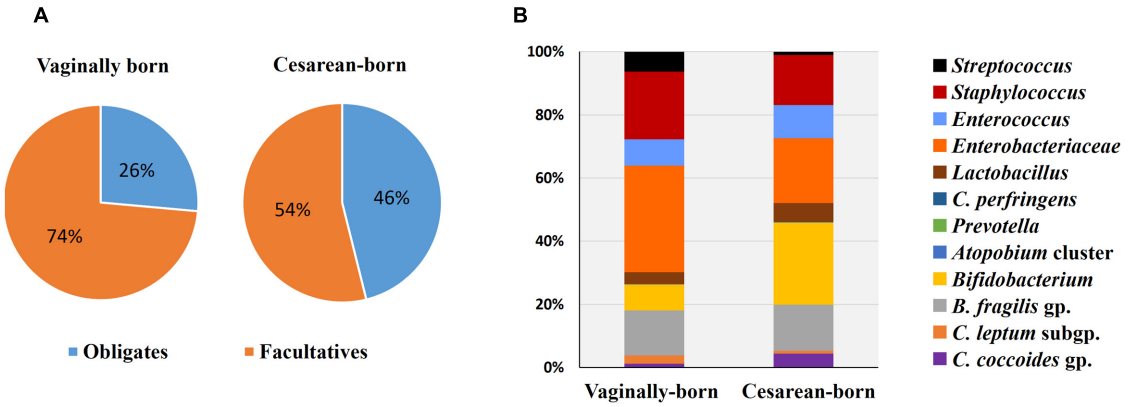

FIGURE 1 | Comparison of proportional ratio of obligatory anaerobic vs. facultative anaerobic bacteria (A), and relative proportions of different gut bacteria **(B)** in the meconium samples of vaginally- vs. cesarean-born infants. Proportions were calculated by using the original arithmetical number of the bacterial count and are expressed as the percent of numerical value of the total bacterial count. Obligates: *Clostridium coccoides* group, *C. leptum* subgroup, *C. perfringens*, *Bacteroides fragilis* group, and *Bifidobacterium*. Facultatives: *Lactobacillus*, Enterobacteriacea, *Enterococcus*, *Staphylococcus*, and *Streptococcus*.

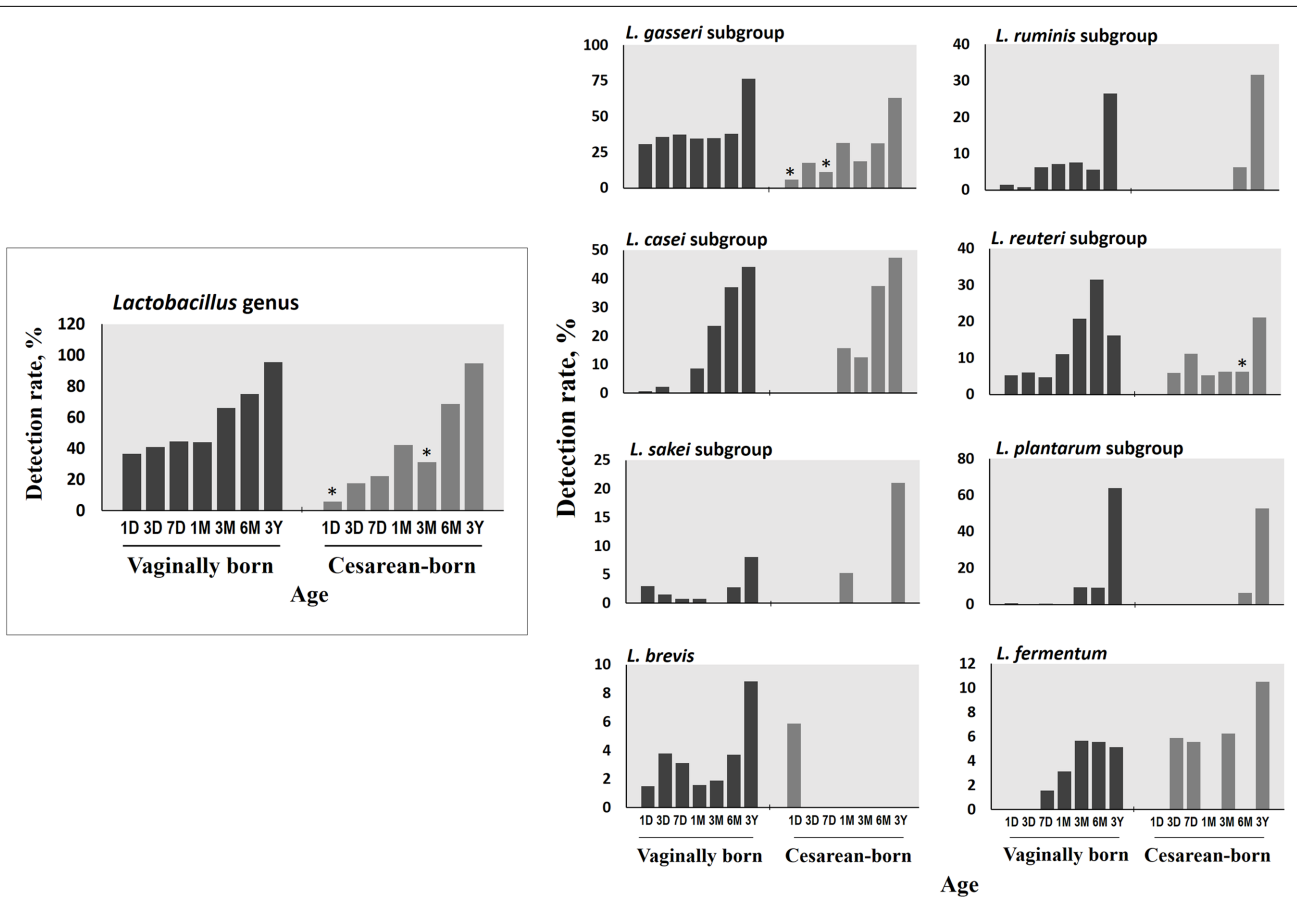

FIGURE 2 | Differences in the detection rate of *Lactobacillus* genus, subgroups and species between vaginally- and cesarean-born babies at different time-points during the first 3 years of life. Detection rate (%) was expressed as the percentage of infants in which the specific bacterium was detected. The count of genus *Lactobacillus* was expressed as the sum of the counts of six subgroups (*L. casei* subgroup, *L. gasseri* subgroup, *L. plantarum* subgroup, *L. reuteri* subgroup, *L. ruminis* subgroup, and *L. sakei* subgroup) and two species (*L. brevis*, *L. fermentum*). *$P < 0.05$ vs. vaginally-born at same-point (Fisher's exact test). Age (*x*-axis): 1, 3, and 7 days, 1, 3, and 6 months, 3 years.

which *L. gasseri* subgroup is a major constituent (Kurakawa et al., 2015a). Also, lactobacilli are rarely detected in amniotic fluid and placenta (DiGiulio et al., 2010; Aagaard et al., 2014). Collectively, these studies suggest a prominent mother-to-baby transmission of vaginal *Lactobacillus* communities during vaginal delivery, while indicating a delayed sampling of lactobacilli in CS babies' gut. However, given the limitation of large difference between the numbers of vaginally- and cesarean-born infants, this finding needs to be corroborated in studies with comparable number of subjects in the two groups. Also, given that *L. gasseri* subgroup comprises several beneficial commensal species that can elicit probiotic health benefits such as anti-pathogenic activity, bacteriocin production, immune-modulation, and maintenance of gut homeostasis (Selle and Klaenhammer, 2013); studies are needed to investigate the impact, if any, of its lower/delayed colonization in CS babies. As for the clinical milieu, this delayed sampling does not seem to directly indicate any disease risk, because all these infants were apparently healthy (at least until age 3 years). Nevertheless, differences in *Lactobacillus* carriage in the 'first flora' justify further research and should be an exciting theme for future studies about viable therapeutic targets (probiotics, prebiotics, etc.) in context to the gut dysbiosis, particularly in cesarean-born and/ or preterm babies.

While the birth mode did not have a significant effect on the carriage of other bacteria (apart from lactobacilli), we noticed differences in the ratio of facultative vs. obligatory anaerobes and also in the relative proportions of different bacteria between VG and CS infants (**Figure 1**). Assuming VG babies as healthy controls, this might contemplate some sort of imbalanced microbiota in CS babies (Arboleya et al., 2012) and might also hint that the trajectory of C-section-associated gut dysbiosis may already instigate as early as the 1st day of life. Slightly higher count of facultative anaerobe community belonging to Enterobacteriaceae in VG babies might indicate the transmission of maternal perianal bacteria during birth, because enterobacteria are typical fecal bacteria and defecation during parturition is not uncommon (de Muinck et al., 2011). We have previously reported that the carriage of *B. fragilis* group, bifidobacteria and several other bacteria remain inconsistently lower in CS babies until age 6 months, but these differences begin to appear after 3–7 days of age (Tsuji et al., 2012). Together, these reports suggest that while the infant microbiota is definitely, strongly influenced by delivery mode, these differences (with the exception of lactobacilli) are not noticeable at meconium stage and become prominent thereafter (Backhed et al., 2015; Bokulich et al., 2016). This is perplexing and calls for further research, because if the initial dose and pool of microbes in VG and CS babies is similar, then what is it that drives the salient differences in the carriage of certain bacteria (such as *B. fragilis* group, bifidobacteria etc.) during subsequent stages. Nevertheless, our data show that irrespective of birth mode, the prenatal microbiome is characterized by specific founding bacterial clades which subsequently become more diverse with age as exposure to various environmental and dietary factors increases (**Figure 2**; Supplementary Table S1). While the source(s) of these bacteria in the meconium remain largely unclear, they are speculated to be of intrauterine origin and suggestive of swallowed amniotic fluid (Ardissone et al., 2014; Collado et al., 2016). Given that the meconium bacterial composition could reveal several features of maternal health (Hu et al., 2013) and might also be associated with several infantile diseases (Gosalbes et al., 2013), further research on the characteristics of these bacteria and their interaction with the host may lead to novel interventions and therapies for newborn's health.

Our study did have some limitations most important of which was our inability to evaluate the influence of prenatal factors such as maternal diet, antibiotics, stress etc. because of the paucity of information. Also, the influence of maternal microbiota (e.g., fecal, vaginal, and skin microbiota) was not studied because maternal samples were not collected in this open observational study. Even though all samples were from first stools, the time of sampling may have varied in each case and hence might have some influence on the bacterial composition in babies who passed their first stool at an earlier vs. later time. However, because 148 out of total 151 samples were collected within the first 24 h of life, it is highly implausible that sampling time could have any significant bias on the results. While the possibility of environmental contamination during sample collection cannot be completely ruled out; we followed all standard and potential laboratory procedures to avoid as much as possible the chances, if any, of contamination. Moreover, the finding that 5% samples were found to be sterile (no detectable bacteria) also corroborates the sterility during sample collection. While no baby received any kind of formula-feed up to the point of first defecation, some of the babies might have been breast-fed during this short period. However, because of lack of precise information, we could not distinguish such infants to exclude the possibility of contamination with breast-milk's bacteria. Nevertheless, given that all the mothers and babies were apparently healthy, the data could be extrapolated to the general population.

CONCLUSION

In summary, our data demonstrate the quantitative picture of bacterial pool dwelling in neonate's first meconium, and corroborate the evidence that the colonization of gut microbiota may begin already before birth, i.e., *in utero*. Furthermore, delayed/lower colonization of lactobacilli and relatively different proportions of facultative vs. obligatory anaerobes in the meconium of CS babies indicate that the elements of gut dysbiosis associated with birth mode may start building up as early as the 1st day of life. Given that early-life microbiota acquisition is crucial for newborn's growth and development, our data underscore the need for broader studies to elucidate the sources, routes and significance of these microbial clades in the prenatal niches, as well as to investigate the impact of these primitive microbial exposures on newborn's long-term health.

AUTHOR CONTRIBUTIONS

RN, HT, KN, and YY: conception and design of study. RN and HT: performed experiments. HT and KK: coordinated sample

collection. RN and HT: analyzed data. RN, HT, KN, and YY: interpreted data. RN: drafted manuscript. RN, HT, TT, KN, KK, SN, and YY: edited and revised manuscript. RN, HT, TT, KN, KK, SN, and YY: approved final version of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01997/full#supplementary-material

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