Identifying single-strain growth patterns of human gut microbes in response to preterm human milk and formula

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Abstract

The intestinal microbiota of the preterm neonate has become a major research focus, with evidence emerging that the microbiota influences both short and long-term health outcomes, in the neonatal intensive care unit and beyond. Similar to the term microbiome, the preterm gut microbiome is highly influenced by diet, specifically formula and human milk use. This study aims to analyze next-generation products including preterm formula, human milk-oligosaccharide term formula, and preterm breastmilk. We used a culture-based model to differentially compare the growth patterns of individual bacterial strains found in the human intestine. This model probed 24 strains of commensal bacteria and 8 pathobiont species which have previously been found to cause sepsis in preterm neonates. Remarkable differences between strain growth and culture pH were noted after comparing models of formulas and between human milk and formula. Both formula and human milk supported the growth of commensal bacteria; however, the formula products but not human milk supported the growth of several specific pathogenic strains. Computational analysis revealed potential connections between long-chain fatty acid and iron uptake from formula in pathobiont organisms. These findings indicate that there is a unique profile of growth in response to human milk and formula and shed light into how the infant gut microbiota could be influenced.

Introduction

The infant gut microbiota undergoes significant fluxes in the domains of bacterial community structure and function during the first few years of life.[1–4] Shortly after birth,

Conflicts of interest

The authors have no conflicts of interest to disclose.

the intestine is colonized with facultative anaerobes, like Streptococcus, Enterococcus, Enterobacter, and Staphylococcus, which reduce the intestinal oxygen content and pave the way for strict anaerobes, like Bifidobacteria, Bacteroides, and Clostridia.[5–8] Several factors affect the infant microbiota composition, but the dominant influencers are gestational age and diet. The gut microbiota of preterm infants (born before 37 weeks of gestation) is characterized by limited microbial diversity and delayed colonization.[5, 9] In general, there are decreased levels of commensal, obligate anaerobes and increased levels of facultative pathobionts.[7, 10–12] Preterm infants are dominated by Enterococci, Staphylococci, and Enterbacteriacea (*Enterobacter*, *Escherichia*, and *Klebsiella spp.*) and exhibit a wider variation in microbial constituents when compared to term infants.[5, 13–18] Diet is another major driver of the composition of the infant gut microbiota.[1] The intake of human milk is associated with increased Bifidobacteria abundance, while the intake of formula correlates with increased Escherichia, Clostridia, Bacteroides, and Enterobacteriaceae.[8, 19–25] Although multiple studies have examined the microbial communities of infant stool by 16S rRNA sequencing in relationship to diet, these complex communities obscure which microbes are responding to milk or formula.[26–28]

A healthy preterm gut microbiota is supported by maternal milk rather than formula or banked donor human milk.[27] Human milk is associated with numerous benefits to the preterm neonate, including lower risks of necrotizing enterocolitis, less feeding intolerance and better lean body mass indices.[29] Studies mapping the preterm microbiota development are lacking. Large neonatal cohorts characterizing the stool microbiome of infants in the neonatal intensive care unit provide evidence of the optimal microbiome diversity and make-up,[3, 27, 30] but these analyses are unable to draw clear associations between infant diet and species growth. This is due, in part, to the frequently changing enteral diet that a preterm neonate might receive over a short course of time. For example, an infant might receive mother's own milk, donor pasteurized milk, formula, bovine or human fortification, and other supplements all within the course of a few weeks.[28, 31, 32] Additionally, the use of antibiotics is prevalent. More than 75% of very low birth weight infants admitted to neonatal intensive care units in the United States receive antibiotics in the first days of life.[33] The goal of this study was to characterize the growth of select gut microbes present in the neonatal gut to determine mechanistic associations between human milk and formula. Since alterations in the infant gut microbiota have been linked to childhood health and development, understanding how individual bacteria differentially utilize whole human milk or formula may provide insights into which microbes are driven by diet-related changes.

Experimental

Consent and Human Milk Preparation

This study was approved by the Medical University of South Carolina Institutional Review Board (#103782). Consent was obtained from 3 mothers of preterm infants who were admitted to the Shawn Jenkin's Hospital Neonatal Intensive Care Unit. Maternal details are provided in Supplemental Table 1. Mothers were educated about hygienic pumping techniques by hospital lactation specialists as part of standard practice. Expressed milk was frozen at −20°C and stored in the hospital's nutrition management center before use, which

strictly adheres to standard milk-handling protocols.[34, 35] To eliminate the impact of natural human milk microbes on the experiments in the present study, milk was pasteurized directly before use at 72°C for 15 seconds, also known as high-temperature short-time (HTST) pasteurization, which also inactivates viruses and milk cells.[36–39]

Bacterial Culture Conditions

For culturing experiments, Bifidobacteria and Lactobacilli were selected based on their isolation in infant feces.[40–50] Streptococci can be found in both human milk and feces[51–53] and Enterococcus and Klebsiella have also been identified in infant stool, particularly those born preterm.[43, 54–57] Bacteria in Table 1 were grown on agar plates (MRS agar for all *Bifidobacteria* and *Lactobacillus* strains and BHI agar for all *Streptococcus*, *Enterococcus*, and *Klebsiella* strains) and then single colonies were used to inoculate rich media (MRS for *Bifidobacteria* and *Lactobacillus* species and BHI supplemented with 2% yeast extract and 0.2% cysteine (BHIS) for all Streptococcus Enterococcus, and Klebsiella species). Bacteria were grown in rich media overnight at 37°C in an anaerobic chamber (Anaerobe Systems, AS-150, Morgan Hill, CA). After the incubation, all bacteria were examined for morphology by light microscopy at 40x (Motic AE 2000, Schertz, TX) and cultures were then examined at OD_{600nm} on a Spectronic 200 Spectrophotometer (Carolina Biological Supply Company, Burlington, NC). Sub-cultures were generated by inoculating pre-reduced chemically defined media (LDM4 for *Bifidobacteria* and *Lactobacillus* species and ZMB1 *Streptococcus*, *Enterococcus*, and *Klebsiella* species) at an OD_{600nm} of 0.1. To test bacterial growth in response to whole human milk, milk from 3 mothers of preterm infants was pooled, pasteurized at 72°C for 15 seconds, and introduced into bacterial cultures at a 1:100 dilution. Preterm formula, (Neosure Similac® 22 kcal/oz) and term formula with oligosaccharides (Similac® Pro-Advance with HMO, 2-Fl, 20 kcal/oz) was likewise introduced into bacterial cultures at a 1:100 dilution. This dilution was selected because it did not interfere with the optical density, yet significantly supported the growth of bacteria in media. Nutritional content of both preterm human milk and formula was generated from available references (Table 2).[58, 59] Growth was monitored after anaerobic incubation at 20 hr by measuring OD_{600nm}. OD_{600nm} measurements were back-calculated to standard curves made from each type of bacteria using the Quantum Tx Bacterial Cell Counter (Logos Biosystems, Annandale, VA) (see Supplemental Table 2). Each experiment was performed two independent times in triplicate.

pH assay

Bacterial supernatant pH was measured using a UV-Vis absorbance measurement as previously described.[60] Briefly, a standard curve was generated of ZMB1 or LDM4 at the pH values 2–8 in 0.5 increments. These standards were mixed with Dulbecco's Modified Eagle's Medium (DMEM) at a ratio 1 volume bacterial media: 3 volumes DMEM. DMEM contains phenol red which has an isosbestic point of 470 nm (a wavelength where the light absorption of an indicator dye is independent of solution pH and a lambda max (λ_{max}) of 560 nm.) To measure pH, bacterial cultures were centrifuged to pellet bacteria and 1 volume of bacterial culture was added to 3 volumes DMEM and the absorbance was measured at 470 nm and 560 nm. The ratio of these values was back-calculated to the standard curve to approximate pH for all cultures.

Genome Analysis

Glycosyl hydrolase families associated with human milk oligosaccharide consumption from human gut microbes were examined using the Carbohydrate-Active enZYmes (CAZy) database (www.cazy.org) as previously described.[61–64] HMO-related GH's include: GH33, GH2, GH20, GH95, GH29, GH42, GH101, GH89, GH110, GH112, and GH27. Glycosyl hydrolase families were examined in Bifidobacteria (179 genomes representing 14 different species), Lactobacilli (452 genomes representing 23 different species), Streptococci (526 genomes representing 19 different species), Enterococcus (228 genomes, representing 9 different species), and Klebsiella (884 genomes, representing 10 species). We also identified bacterial transporters involved in long-chain-fatty acid (LCFA) uptake: fadL (K06076); iron uptake: TonB (K03832), exbB (K03561), FeoB (K04759), FeoA (K04758), ECF (K16787) and FepA (K19611); as well as phosphate uptake: PiT (K16322) and pstS (K02040) using the KEGG database (KEGG: Kyoto Encyclopedia of Genes and Genomes, https://www.genome.jp). KEGG IDs were input into the gene search tool of the Integrated Microbial Genomes (IMG) database v5.0 (http://img.jgi.doe.gov)[65] and genomes were binned for analysis as previously described.[48] Bacterial transporters were identified in the following pathobionts: *E. faecalis* (416 genomes), *E. faecium* (488 genomes), *Enterococcus* spp. (16 genomes), *K. aerogenes* (102 genomes), *K. pneumoniae* (1,049 genomes), and *Klebsiella* spp. (71 genomes). Bacterial transporters were also identified in the following commensals: *B. angulatum* (4 genomes), *B. animalis* (37 genomes), *B. bifidum* (33 genomes), *B. breve* (66 genomes), *B. dentium* (7 genomes), *B. longum* (126 genomes), *B. longum subsp infantis* (36 genomes), *Bifidobacterium* spp. (10 genomes), *L. acidophilus* (23 genomes), *L. johnsonii* (18 genomes), *L. paracasei* (154 genomes), *L. plantarum* (215 genomes), *L. fermentum* (42 genomes), *L. gasseri* (15 genomes), *L. delbrueckii* (48 genomes), *L. brevis* (54 genomes), *Lactobacillus* spp. (44 genomes), *S. salivarius* (19 genomes), *S. sanguinis* (28 genomes), *S. parasanguinis* (26 genomes), *S. pyogenes* (351 genomes), *S. thermophilus* (34 genomes), *S. mitis* (41 genomes), *S. pasteurianus* (7 genomes), *S. oralis* (27 genomes) and *Streptococcus* spp. (92 genomes).

Statistics

Data are presented as mean ± standard deviation (stdev). Comparisons between groups were made with a One-way Analysis of Variance (ANOVA), using the Holm-Sidak post-hoc test to determine significance between pairwise comparisons. To determine associations between factors, a Pearson correlation was performed and the coefficient (r) was recorded. Graphs and statistics were generated using GraphPad (GraphPad Software, Inc., La Jolla, CA). A *p < 0.05 value was considered significant as indicated by *, while *n* is the number of experiments performed.

Results

To determine which gastrointestinal microbes responded to human milk and formula, we systematically examined the growth of commensal and pathobiont gut and breast milk associated microorganisms. We inoculated a chemically defined media, LDM4, with *B. longum* subspecies *infantis* ATCC 15697, *B. breve* ATCC 15698, *B. bifidum* MIMB, *B. angulatum* ATCC 27535, *B. animalis* Bi-07, *B. longum* ATCC 55813, *B. dentium* ATCC

27678 and *B. gallicum* ATCC 20093 at an OD_{600nm} of 0.1 and examined growth after 20 hr. We observed that all *Bifidobacteria spp.* had elevated growth in response to pasteurized preterm human milk compared to media alone controls (Figure 1A–H). As a comparison, we also examined the growth of *Bifidobacteria spp.* in LDM4 supplemented with preterm infant formula or term infant formula with the human milk oligosaccharide (HMO) 2'-Fucosyllactose (2-FL). We found that preterm formula enhanced the growth of 6 of the 8 strains: *B. infantis, B. breve, B. bifidum B. angulatum, B. animalis*, and *B. longum* (Figure 1A–F). Full-term formula containing HMOs only elevated the growth of 5 strains: *B. infantis, B. breve, B. bifidum, B. angulatum*, and *B. animalis* (Figure 1A–D).

Lactic acid bacteria such as Bifidobacteria are known for their ability to lower the pH and pH can serve as a functional output of microbial growth.[66] Consistent with our growth analysis, we found that all *Bifidobacteria spp.* grown with preterm milk exhibited significant reductions in pH (Figure 2A–H). Similarly, preterm formula was associated with reduced pH in 6 of the *Bifidobacteria* strains and full-term formula with HMOs reduced pH in 3 of the strains. These data indicate that the majority of *Bifidobacteria* spp. can grow with preterm milk and formula.

We observed that all *Lactobacillus* spp. (*L. acidophilus* ATCC 4796, *L. johnsonii* ATCC 33200, *L. paracasei* ATCC 25302, *L. plantarum* ATCC 14917, L. fermentum ATCC 14931, *L. gasseri* ATCC 3323, *L. delbrueckii* ATCC 11842, and *L. brevis* ATCC 27303) had increased growth in the presence of preterm human milk and preterm formula compared to media controls (Figure 3A–H). Six of the 8 *Lactobacillus* strains had elevated growth with full-term formula supplemented with HMOs. In the presence of preterm milk, *L. acidophilus, L. paracasei, L. plantarum, L fermentum, L. gasseri* and *L. delbrueckii* significantly reduced the pH compared to media controls (Figure 4A–H). Although we observed increased growth in the presence of preterm formula, we found that only 6 of the strains (*L. paracasei, L. fermentum, L. gasseri* and *L. delbrueckii*) lowered the pH in response to preterm milk. Moreover, only 3 strains reduced the pH in media supplemented with formula with HMOs. These findings suggest that lactic acid production by Lactobacilli is influenced by multiple factors and that formula supplementation does not necessarily promote pH reduction.

To assess the effects of preterm milk and formula on Streptococci growth, we grew *S. salivarius* ATCC 13419, *S. sanguinis* ATCC 49296, *S. parasanguinis* ATCC 15912, *S. thermophilus* ATCC 491, *S. pyogenes* CB1, *S. mitis* ATCC 6249, *S. pasteurianus* ATCC 49133, and *S. oralis* ATCC 35037 in another chemically defined media, ZMB1, and examined growth after 20 hr of incubation at OD_{600nm} (Figure 5A–H). We found that only 2 *Streptococcus* spp. grew with preterm human milk compared to media controls: *S. sanguinis* and *S. parasanguinis* (Figure 5B,C). Moreover, 2 strains were suppressed by preterm milk: *S. salivarius* and *S. thermophilus* (Figure 5A,D). Also, 3 *Streptococcus* strains could use preterm and full-term formula with HMOs to support their growth. Of the Streptococci examined, we found that *S. pyogenes, S. mitis, S. pasteurianus* and *S. oralis* did not respond in terms of growth to any dietary interventions. As members of the lactic acid bacteria group, Streptococci can also produce lactic acid and lower the pH.[67] When we assessed media pH (Figure 6A–H), we found that *S. sanguinis* and *S. parasanguinis*, which had

improved growth with preterm milk, lowered the pH compared to media controls (Figure 6B,C). We also found that 3 *Streptococcus* spp. reduced the pH in the presence of preterm formula and 2 strains reduced the pH in presence of full-term formula with HMOs. No changes in pH were observed in *S. thermophilus*, *S. mitis*, *S. pasteurianus* or *S. oralis* (Figure 6D,F–H). These data indicate that not all Streptococci can use human milk or formula to support their growth.

To address pathobiont species, we grew *E. faecalis* NCTC 775, *E. faecium* ATCC 8459, *E. faecium* NCTC 12204, *K. aerogenes* CB1, *K. aerogenes* ATCC 35029, *K. pneumoniae* ATCC 700607, *K. pneumoniae* ATCC 13883, and *K. pneumoniae* ATCC 35657 in ZMB1 in the presence of preterm milk or formula (Figure 7A–H). Compared to commensal Bifidobacteria, Lactobacilli and Streptococci, the *Enterococcus* and *Klebsiella* spp. only nominally used preterm milk for growth. All the pathobiont strains exhibited robust growth with preterm formula and full-term formula harboring HMOs. The highest OD_{600nm} values were largely obtained with preterm formula. *Enterococcus* spp. are part of the lactic acid bacteria group and although these species had only a minimal improvement in growth with preterm milk, all 3 *Enterococcus* spp. significantly reduced the pH in the presence of milk (Figure 8A–C). Reduced pH was also found in response to formula and HMO containing formula for all *Enterococcus* spp. In contrast to *Enterococcus* spp., *Klebsiella* strains had no effect on pH (Figure 8D–H).

Finally, we sought to determine if there was a correlation between diet (formula vs. milk) and microbial composition. One unique component of human milk is human milk HMOs (see Table 2). To degrade HMOs, bacteria must possess specific glycosyl hydrolases which enzymatically cleave HMOs. HMO degrading glycosyl hydrolase families include GH33, 2, 20, 95, 29, 42, 101, 89, 110, and 27. We profiled HMO-related glycosyl hydrolase families using the CAZy database and filtered human gut microbes using the Integrated Microbial Genome Database (IMG). We identified 179 Bifidobacteria genomes (14 different species), 452 Lactobacilli genomes (23 species), 526 Streptococci genomes (19 species), 228 Enterococcus genomes (9 species) and 884 Klebsiella genomes (10 species). Genome analysis revealed that the majority of *Bifidobacteria* spp. harbored multiple HMO-related glycosyl hydrolases, with *B. bifidum* and *B. longum* subspecies *infantis* exhibiting the largest repertoire (Figure 9A). Compared to Bifidobacteria, Lactobacilli had far fewer HMO-related glycosyl hydrolases (Figure 9B); although most species had GH2 and GH42. Streptococci had varying levels of HMO-related glycosyl hydrolases with species such as *S. mitis*, *S. oralis*, and *S. sanguinis*, which harbour the highest number of glycosyl hydrolase families (Figure 9C). Enterococcus and Klebsiella genomes had fewer HMO-related glycosyl hydrolases than Bifidobacteria; with most species possessing GH2 and GH42. To assess whether glycosyl hydrolase profiles correlated with growth in response to milk or formula, we calculated the total number of HMO-related glycosyl hydrolase families (1–11 GHs) and correlated these values with fold change growth compared to media alone controls (Figure 9E–J). Regression curves revealed a correlation between HMO-related glycosyl hydrolases and commensal growth in human milk (*p = 0.03) (Figure 9E). No correlations were identified between preterm or full-term formula and HMO-degrading glycosyl hydrolases expression in commensal organisms (Figure 9F,H). Additionally, no correlations were found between the HMO-related glycosyl hydrolases in

pathobiont microbes and the consumption of human milk or formula (Figure 9H–J). These data indicate that HMOs contribute to the growth of commensal microbes in preterm milk, but that other components also are participating in the growth of certain commensal and pathobiont organisms.

A comparison between breast milk and formula revealed that formula has significantly higher levels of linoleic acid (a long-chain fatty acid), iron and phosphate (Table 2). We speculated that these components may be driving the growth enhancement of pathobionts. To address this, we examined bacterial transporters involved in the transport of long-chain-fatty acid (fadL); iron (TonB, exbB, FeoB, FeoA, ECF and FepA) and phosphate (PiT, pstS) using the KEGG database and Integrated Microbial Genomes (IMG) database. We examined 319 Bifidobacteria genomes, 613 Lactobacilli genomes, 625 Streptococci genomes, 920 Enterococci genomes, and 1,222 Klebsiella genomes. We found that almost all Klebsiella possessed the long-chain fatty acid transporter fadL, while this gene was absent in Bifidobacteria, Lactobacilli, Streptococci and Enterococci (Figure 10A,B). We observed a similar trend for iron transporter components TonB and exbB. Some expression on iron transporters FeoB, FeoA and ECF were found in the commensal microbes, but in general, far more iron transporters were observed in pathobiont Enterococci and Klebsiella. No commensal microbes possessed the phosphate transporter PiT, while all *K. aerogenes* and *K. pneumoiae* harbored this gene. All bacteria were found to have the pstS gene. These data suggest that iron uptake in pathobionts may promote the growth of pathogens. Correlation analysis revealed a positive correlation between iron uptake related genes in pathobionts and growth in formula (*p = 0.03) (Figure 10C). We speculate that iron uptake may provide an essential nutrient for pathobiont organisms in the infant gut. Collectively, these data provide more targeted information on which microbes respond to human milk and formula.

Discussion

In this study, we sought to dissect how individual microbes respond to intact preterm milk or formula. We used intact diets because microbial communities do not just encounter single compounds in the intestinal milieu. We selected strains based on relevant clinical outcomes noted in the literature. We first examined Bifidobacteria; a group which dominates the gut of term breastfed infants and is associated with numerous health benefits including reducing feeding intolerance, lower rates of necrotizing enterocolitis and a lower the risk of asthma.[24, 68] Next, we examined Lactobacilli, another lactic acid bacterial group commonly found in infants which is associated with decreased NEC in clinical trials.[69] Streptococci are commonly found in human milk and can make up part of the preterm infant gut microbiota.[51, 70, 71] This group has been associated with childhood wheezing[72] and NEC.[73] The gut microbiota of preterm infants can harbor varying levels of pathobionts linked to NEC and late-onset sepsis such *Klebsiella* and *Enterococcus* spp.[13–18]

Using this approach, we found representative infant gut microbes from the genus Bifidobacteria and Lactobacilli exhibit robust growth in the presence of whole preterm human milk. This phenomenon was not observed with other genera, such as Streptococci, Enterococci and Klebsiella, which had minimal growth with human milk. These studies

support the dominance of Bifidobacteria and Lactobacilli in breast-fed infants and the high levels of Enterococcus and Klebsiella in formula-fed infants.[26, 67, 73–75] This work can serve as a foundation for more complex interactions with microbial communities and diet.

The composition of breast milk is influenced by multiple factors, including the phase of nursing, lactation period, genetic background of the mother, maternal diet, and gestation length.[51, 76–80] It has been shown that the breast milk from mothers who deliver full term infants significantly differs from the milk of mothers whose infants were delivered prematurely.[81] Preterm human milk is higher in fat, protein, free amino acids, and sodium in the first few weeks, but these components decrease over subsequent weeks.[81] Additionally, calcium is significantly lower in preterm milk than term milk and does not increase with time. HMO content is highly variable, but in general preterm human milk contains more lacto-N-tetraose and the HMOs are not consistently fucosylated.[82, 83] Unfortunately, the HMOs of preterm milk do not become more mature over the course of lactation and it has been speculated that there is a failure to produce mature HMOs.[83] Our data indicates that preterm milk is sufficient to increase the growth of all tested *Bifidobacteria* and *Lactobacilli* strains and does not readily promote the growth of pathobionts when strains were grown individually. It is difficult to decipher how these microbes behave as a community and future work will be needed to address how representative gut microbes respond to diet in the setting of defined communities. Despite these limitations, this work does support the notion that preterm human milk can still benefit preterm infants and supports the recommendation that preterm infants receive human milk.

It has been speculated that the development of the early gut microbiota is driven by specific dietary compounds present in human milk that support selective colonization of gut microbes.[84] The beneficial role of breast milk on the composition of gut microbiota development has largely been attributed to the presence of HMOs.[85] We demonstrate that HMO degrading glycosyl hydrolase profiles correlate with milk utilization in commensal microbes, but we did observe some outliers. For example, *B. dentium*, which only harbors 4 HMO-glycosyl hydrolases had robust growth in response to milk, while *E. faecalis*, which also has 4 HMO-glycosyl hydrolases had limited growth in response to milk. These findings suggest that other factors in milk influence certain commensal microbes. Human breast milk comprises both macronutrients (fat, proteins, and carbohydrates), micronutrients (vitamins, minerals, hormones, etc.), cellular compartments, and the milk fat globule membrane dispersed between aqueous and colloidal phases.[86] Among the macronutrients, breast milk contains carbohydrates such as a fructose and maltose[87, 88] and we have previously shown that *B. dentium* has robust growth in response to both these carbohydrates in LDM4.[89] Therefore, it is possible that these components may contribute to the growth of *B. dentium* and other minimal HMO degrading microbes.

In this study we compared preterm formula and full-term formula supplemented with the HMO 2-FL with preterm human milk. The preterm formula resembled preterm human milk in terms of commensal growth. For example, *B. infantis*, *B. breve*, *B. angulatum*, *B. animalis*, *B. bifidum*, *L. acidophilus*, *L. johnsonii*, *L. plantarum*, *L. fermentum*, *L. paracasei*, *L. gasseri*, *L. delbrueckii* and *L. brevis* had the same or higher growth with preterm formula as they did with preterm human milk. Interestingly, the full-term formula containing 2-FL

in our study supported the growth of many but not all Bifidobacteria and Lactobacilli. Preterm formula contains higher levels of protein, vitamins, minerals, specifically calcium and phosphorous, compared to term infant formula and these factors may be contributing to the more robust growth with many of our commensal microbes. A potential issue with both the preterm and full-term formula was that it also promoted the growth of Enterococcus and Klebsiella; microbes that are associated with a dysbiotic infant gut microbiota and NEC.

A nutrient that may enhance to the growth of pathobionts in this model is iron, which in 1.5x higher in formula than preterm human milk.[90] Iron deficiency anaemia related to cow's milk products and exclusive breastfeeding is prevalent, and this risk is increased due to low stores in the preterm infant.[91] Iron in human milk is thought be highly bioavailable for absorption, and is tightly regulated in low concentrations, regardless of maternal iron status or maternal iron intake.[91] Yet, iron can also selectively increase pathobiont growth and even augment the virulence of pathogenic bacteria such as *Klebsiella pneumoniae*.[92] Interestingly, iron supplementation is related to slower weight gain and head growth but higher psychomotor developmental tasks in a recent meta-analysis of exclusively breastfed infants.[93] The mechanistic influences of iron on the preterm gut and microbiome are still unclear. Deciphering which components of breast milk that promote robust growth of commensal microbes -while only nominally promoting the growth of pathobionts- is the next step to creating customized preterm diets.

One caveat of this study is that we did not determine the secretory status of these three preterm mothers. This may be important because non-secretors lack of Fucosyltransferase 2 (FUT2) and cannot produce oligosaccharides such as 2'-FL. The proportion of non-secretor of population is about 20%. As a result, the chances of having all non-secretary mothers representing our sample is <1%. We speculate that 2-Fl and other HMOs are likely present in our pooled preterm milk within the reference range values. We were unable to determine how much HMO has been added to term formula by the manufacture's website, but likely is within the range of 0.2g/dL-1g/dL per a clinical trial.[94]‡ Future studies should include the secretor status of the mother's to provide a more robust analysis of microbe-HMO interactions.

One of the observations of this study was that human milk resulted in bacterial fermentation and reductions in culture pH. Decreased bacterial pH in response to diet has been noted with non-structural carbohydrates[95] and high concentrate diets.[96] Human milk has multiple carbohydrates which could be used by bacteria to generate lactic acid, including lactose and glucose. Additionally, fermentation and lactic acid production has been demonstrated in response to HMOs present in milk and galacto-oligosaccharides that can be present in formula.[97] Our data indicates that the majority of commensal *Bifidobacteria* and *Lactobacillus* strains could ferment preterm milk and preterm formula to effectively lower the pH. Reductions in pH in the gastrointestinal tract has been speculated to limit pathogen colonization[98] and thus fermentation of milk or formula may represent an important factor in controlling the establishment of subsequent communities.

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‡This citation references a commercially-supported study.

Literature suggests that breast milk can serve as a reservoir for gut microbes. For example, Pannaraj et al. found that ~30% of the infant gut microbiota could be traced to their mother's milk.[99] This is consistent with other studies which found certain bacteria such as *Streptococcus*, *Veillonella*, *Bifidobacterium*, *Lactobacillus*, and *Staphylococcus* spp. co-occurred in mothers' milk and their infants' stool.[100–102] These studies indicate that breastmilk may transfer commensal microbes and influence the infant gut microbiota development. In contrast to full-term infants, preterm infants are often given donor milk that has been pasteurized and is therefore missing the contribution of milk-associated microbes. In this setting, gut microbes lack the competition of natural human milk microbes and this may play a role in limited microbial diversity observed in preterm infants. Co-delivery of probiotics and diet (milk or formula) may temper this phenomenon.

The method of pasteurization used in this study simulates commercial pasteurization of skim/1%/2% table milk in the dairy industry. High-temperature short time (HTST) pasteurization has been shown to better preserve the function of milk including protecting bioactive ingredients and protein integrity than Holder pasteurization (62.5°C for 30 minutes).[103] By using the HTST pasteurization method, the approach may optimally have simulated the effect of unpasteurized maternal preterm milk on bacteria, but it may not necessarily reflect how banked donor milk may affect bacterial growth. Banked donor milk uses Holder pasteurization, generally is from mothers of term infants, and has been stored for much longer periods than mother's own milk. Therefore, conclusions of these data should not be over-extended to banked donor milk or commercially sterilized human milk products.

Conclusion

The establishment of a complex intestinal microbiota during the first 1,000 days of life plays a crucial role in the long-term health and well-being of the individual.[41, 104, 105] Premature infants are at a higher risk of psychomotor and metabolic disorders (obesity, type 2 diabetes, cardiovascular diseases) in adulthood compared to full-term infants99–101.[106–108] Several health factors, including neurodevelopment, have been associated with infant growth rate; thereby raising the issue of how best to deliver proper nutrition to premature infants. Human milk remains the optimal enteral nutrition, and the many benefits of this "liquid gold" are yet to be discovered. The rapidly changing advances in preterm diets necessitate more sophisticated models that can simulate effects in the neonatal intestine, as new products are introduced. Using our in vitro model, with microbes grown individually and, in the future, grown together, we believe we have a tuneable model that has the potential to identify new strategies for manipulating bacterial communities to alter developmental trajectories. The approach here stands as an alternative to large '-omic perspectives, which are useful to reveal over-arching patterns of diet on systemic disease, but are unable to offer detailed information between specific dietary components and bacterial strains in preterm neonates. This study offers a basic model that crystallizes a species-specific mechanistic influence of infant diet on the preterm microbiome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Notes and references

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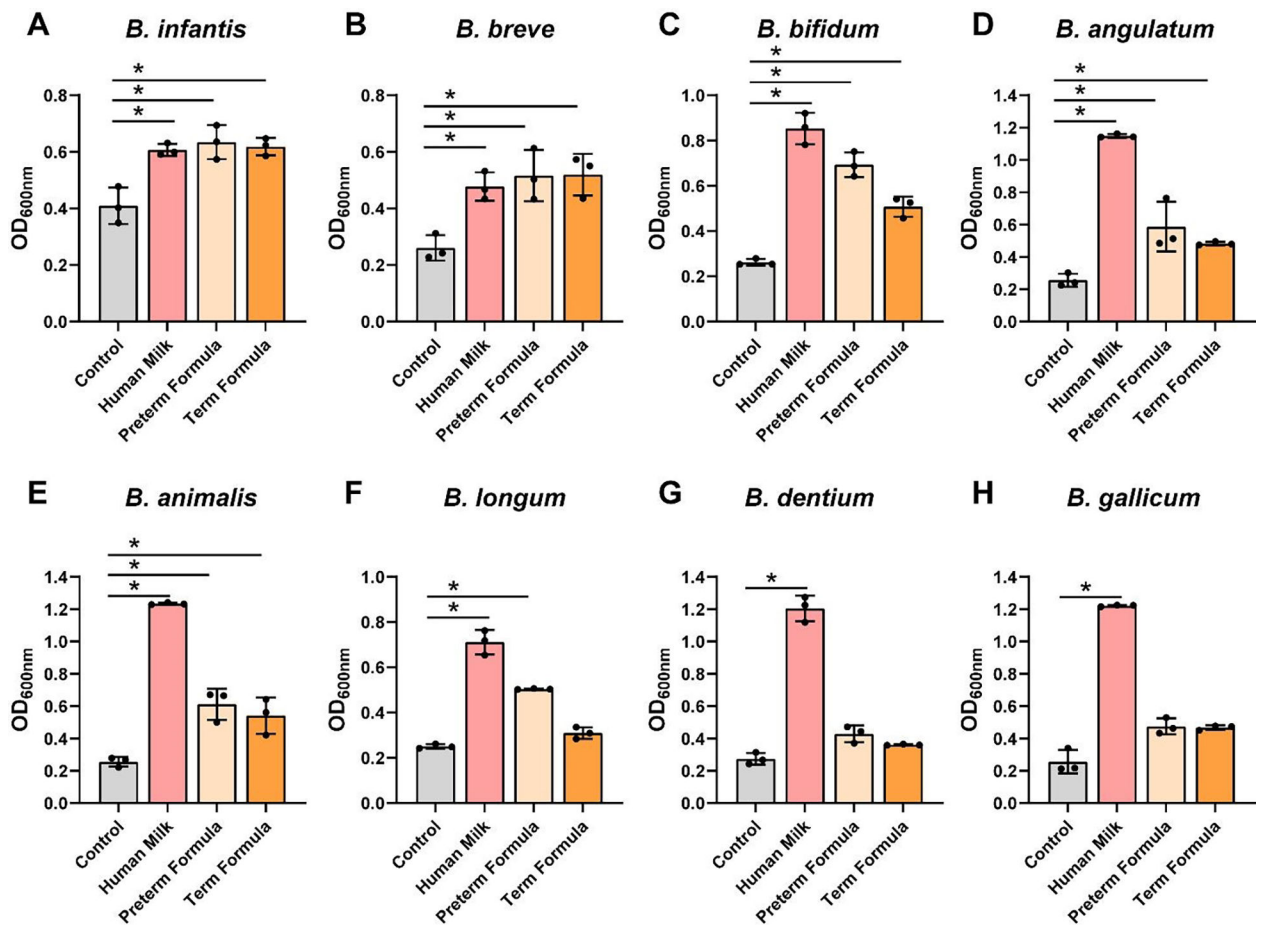
Figure 1: *Bifidobacteria* species growth with human milk, preterm and term formula.
Bifidobacteria species **A.** *B. longum* subsp. *infanti*s ATCC 15697, **B.** *B. breve* ATCC 15698, **C.** *B. bifidum* MIMB, **D.** *B. angulatum* ATCC 27535, **E.** *B. animalis* Bi-07, **F.** *B. longum* ATCC 55813, **G.** *B. dentium* ATCC 27678, and **H.** *B. gallicum* ATCC 20093 were grown anaerobically at 37°C in a chemically-defined media (LDM4) with or without pooled whole preterm breast milk (pooled from n=3), preterm formula or full-term formula supplemented with HMOs. Growth was monitored at OD_{600nm} after 20 hr. All data are presented as mean ± stdev, n=3 replicates, performed 2 independent times. One-way ANOVA, using the Holm-Sidak post-hoc test to determine significance between pairwise comparisons; *p < 0.05.
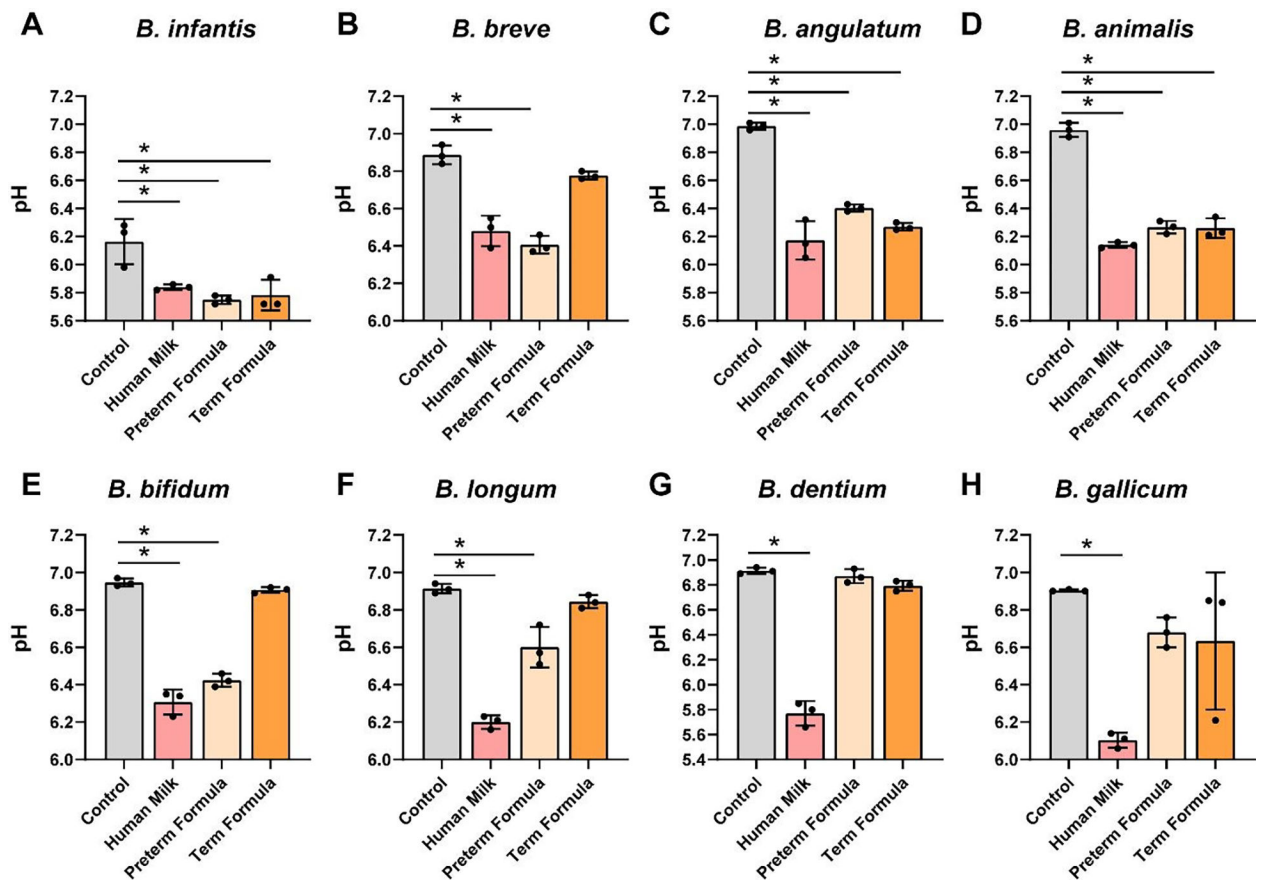
Figure 2: *Bifidobacteria* species acidification in cultures with human milk, preterm and term formula.

Bifidobacteria species **A.** *B. longum* subsp. *infanti*s ATCC 15697, **B.** *B. breve* ATCC 15698, **C.** *B. bifidum* MIMB, **D.** *B. angulatum* ATCC 27535, **E.** *B. animalis* Bi-07, **F.** *B. longum* ATCC 55813, **G.** *B. dentium* ATCC 27678, and **H.** *B. gallicum* ATCC 20093 were grown anaerobically at 37°C in a chemically-defined media (LDM4) with or without pooled whole preterm breast milk (pooled from n=3), preterm formula or full-term formula supplemented with HMOs. pH was monitored after 20 hr. All data are presented as mean ± stdev, n=3 replicates, performed 2 independent times. One-way ANOVA using the Holm-Sidak post-hoc test to determine significance between pairwise comparison; *p < 0.05.
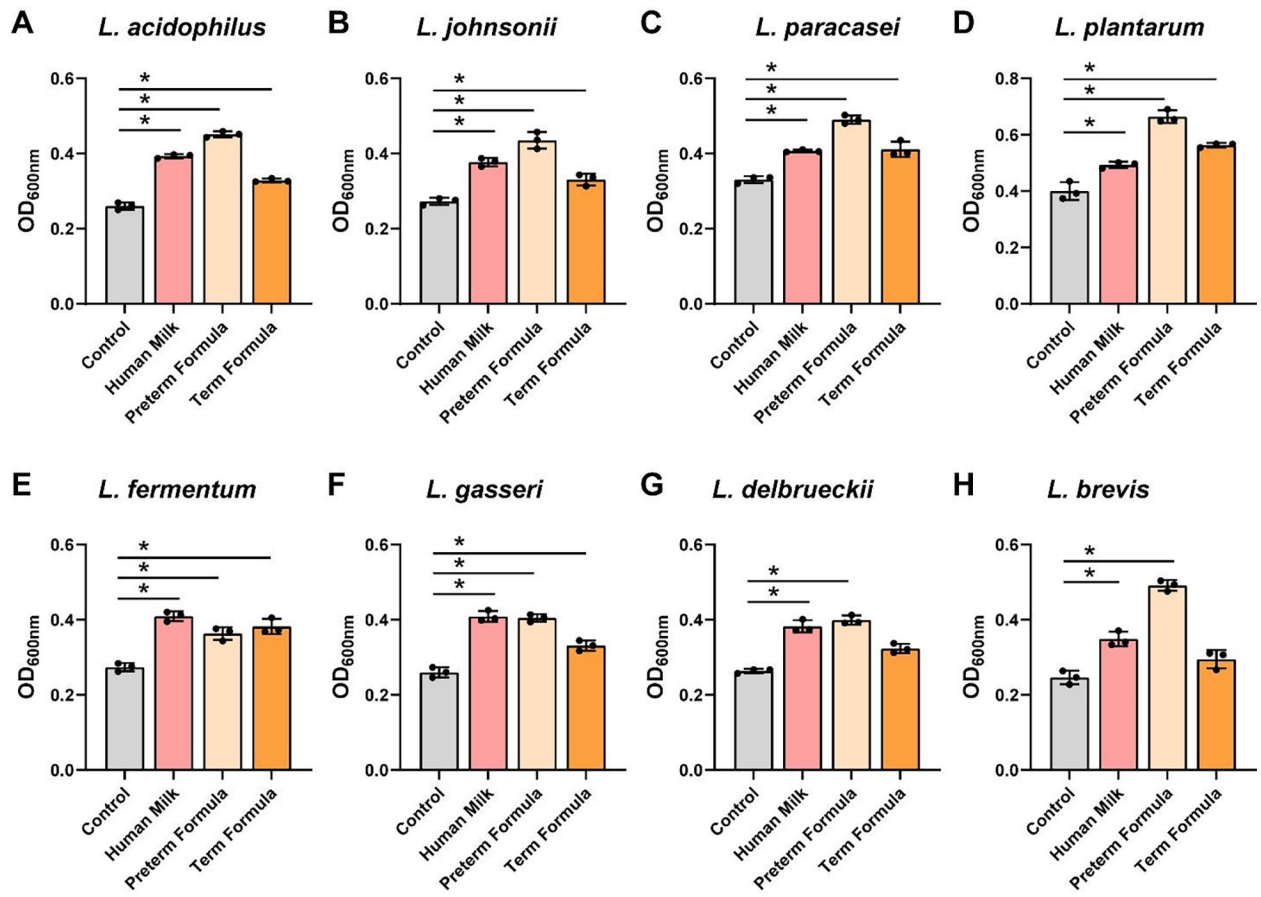
Figure 3: *Lactobacillus* **species growth with human milk, preterm and term formula.**
Lactobacillus species **A.** *L. acidophilus* ATCC 4796, **B.** *L. johnsonii* ATCC 33200, **C.** *L. paracasei* ATCC 25302, **D.** *L. plantarum* ATCC 14917, **E.** *L. fermentum* ATCC 14931, **F.** *L. gasseri* ATCC 3323, **G.** *L. delbrueckii* ATCC 11842, and **H.** *L. brevis* ATCC 27303 were grown anaerobically at 37°C in a chemically-defined media (LDM4) with or without pooled whole preterm breast milk (pooled from n=3), preterm formula or full-term formula supplemented with HMOs. Growth was monitored at OD_{600nm} after 20 hr. All data are presented as mean ± stdev, n=3 replicates, performed 2 independent times. One-way ANOVA, using the Holm-Sidak post-hoc test to determine significance between pairwise comparisons; *$p < 0.05$.
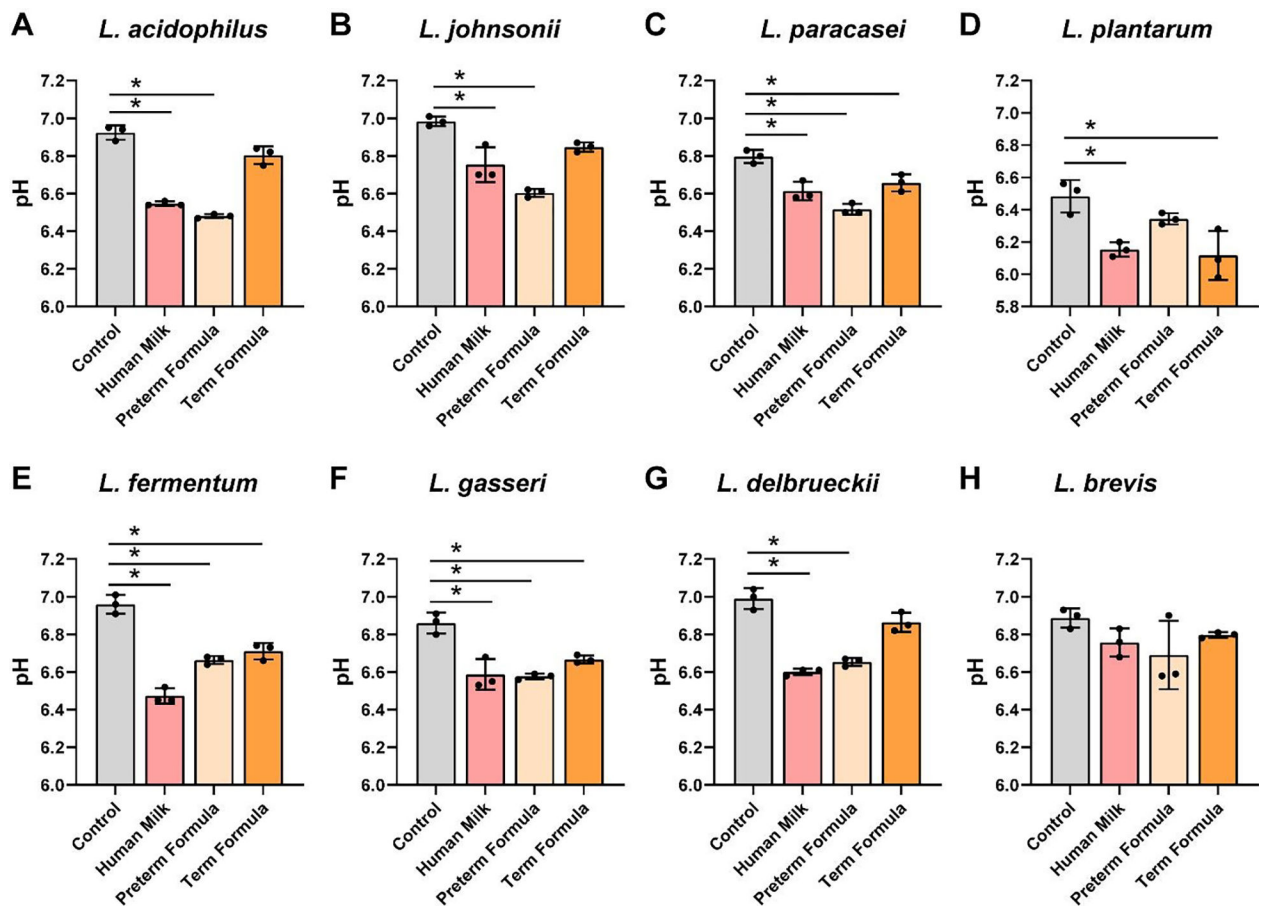
Figure 4: *Lactobacillus* species acidification in cultures with human milk, preterm and term formula.

Lactobacillus species **A.** *L. acidophilus* ATCC 4796, **B.** *L. johnsonii* ATCC 33200, **C.** *L. paracasei* ATCC 25302, **D.** *L. plantarum* ATCC 14917, **E.** *L. fermentum* ATCC 14931, **F.** *L. gasseri* ATCC 3323, **G.** *L. delbrueckii* ATCC 11842, and **H.** *L. brevis* ATCC 27303 were grown anaerobically at 37°C in a chemically-defined media (LDM4) with or without pooled whole preterm breast milk (pooled from n=3), preterm formula or full-term formula supplemented with HMOs. pH was monitored after 20 hr. All data are presented as mean ± stdev, n=3 replicates, performed 2 independent times. One-way ANOVA, using the Holm-Sidak post-hoc test to determine significance between pairwise comparisons; *p< 0.05.
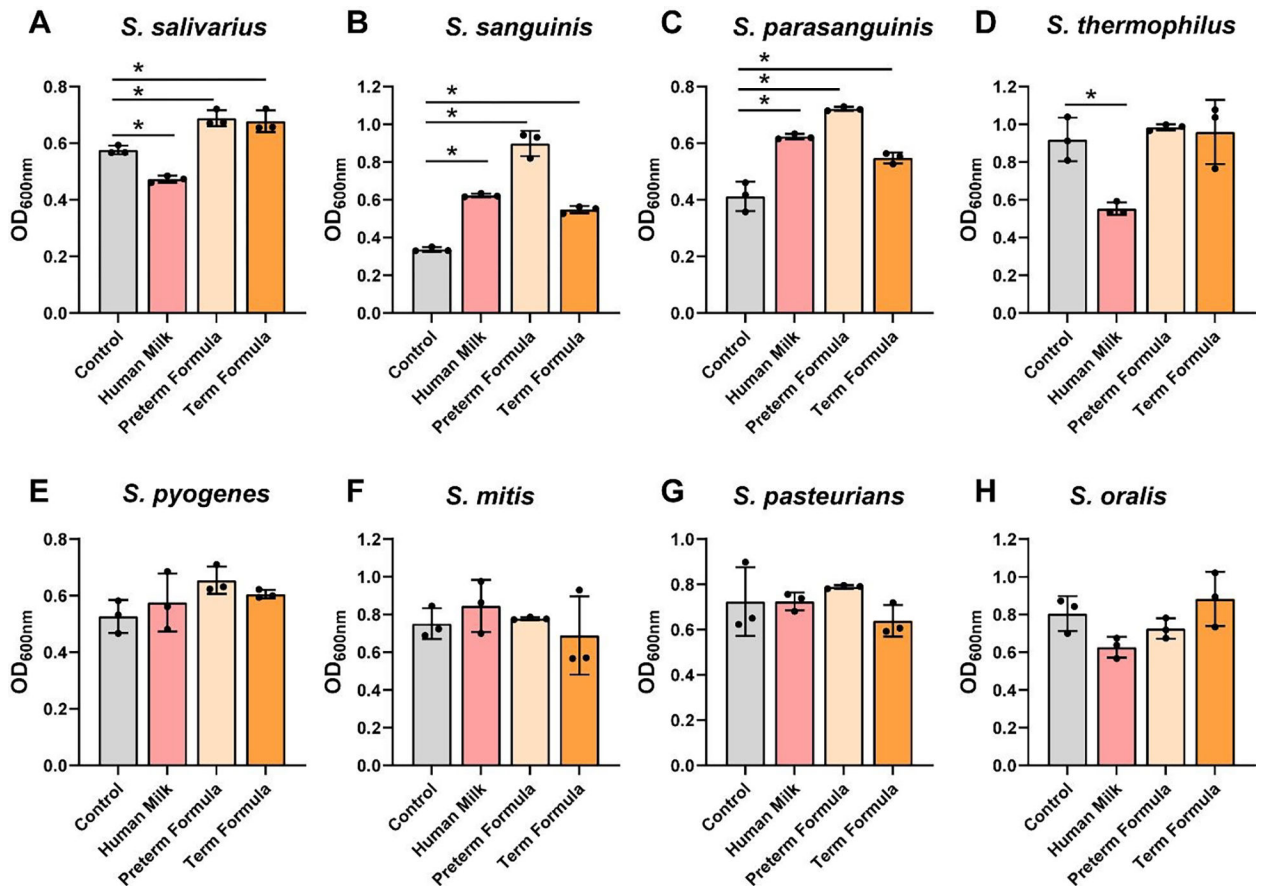
Figure 5: *Streptococcus* **species growth with human milk, preterm and term formula.**
Streptococcus species **A.** *S. salivarius* ATCC 13419, **B.** *S. sanguinis* ATCC 49296, **C.** *S. parasanguinis* ATCC 15912, **D.** *S. thermophilus* ATCC 491, **E.** *S. pyogenes* CB1, **F.** *S. mitis* ATCC 6249, **G.** *S. pasteurianus* ATCC 49133, and **H.** *S. oralis* ATCC 35037 were grown anaerobically at 37°C in a chemically-defined media (ZMB1) with or pooled whole preterm breast milk (pooled from n=3), preterm formula or full-term formula supplemented with HMOs. Growth was monitored at OD_{600nm} after 20 hr. All data are presented as mean ± stdev, n=3 replicates, performed 2 independent times. One-way ANOVA, using the Holm-Sidak post-hoc test to determine significance between pairwise comparisons; *p < 0.05.
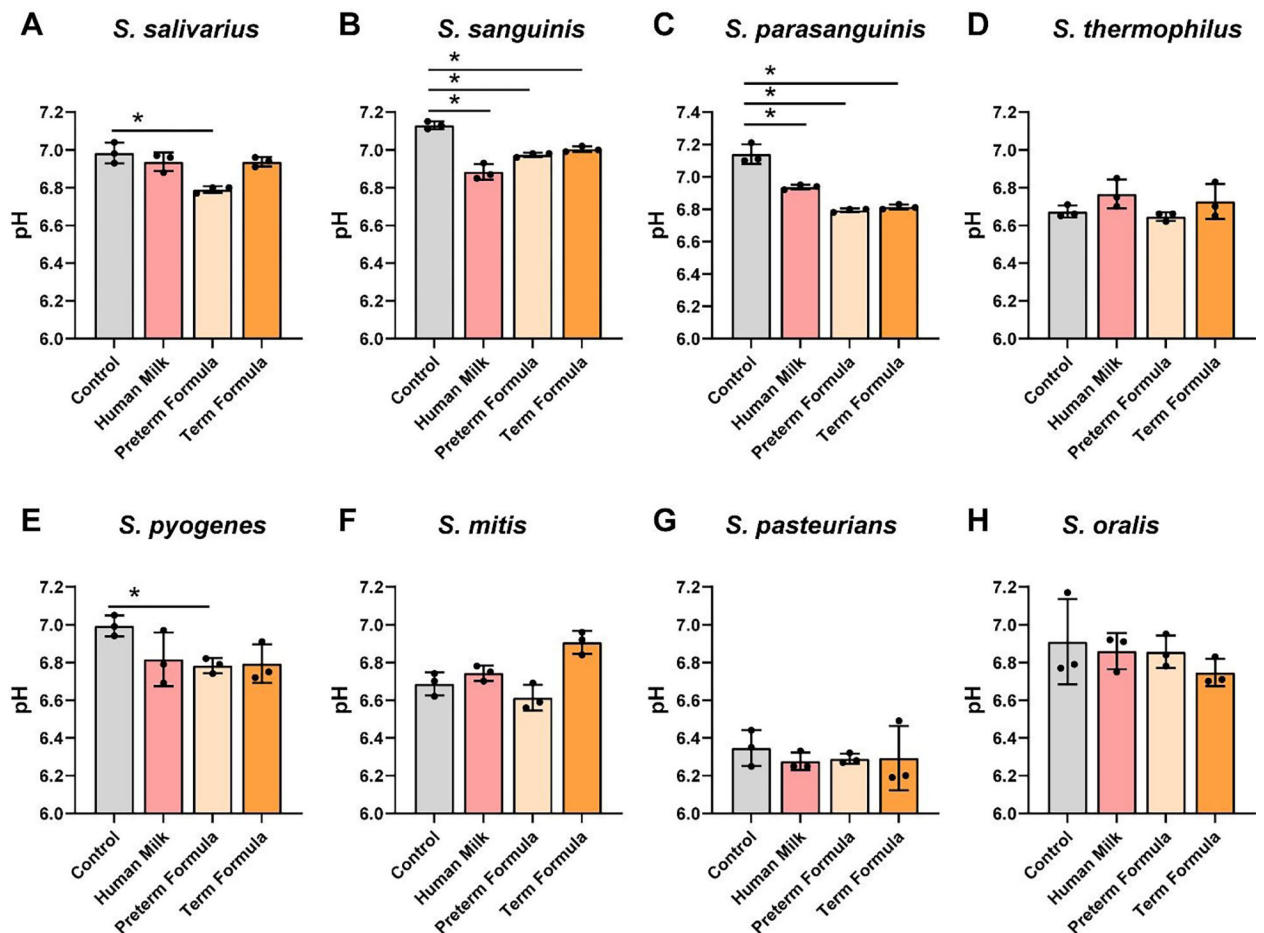
Figure 6: *Streptococcus* **species acidification in cultures with human milk, preterm and term formula.**

Streptococcus species **A.** *S. salivarius* ATCC 13419, **B.** *S. sanguinis* ATCC 49296, **C.** *S. parasanguinis* ATCC 15912, **D.** *S. thermophilus* ATCC 491, **E.** *S. pyogenes* CB1, **F.** *S. mitis* ATCC 6249, **G.** *S. pasteurianus* ATCC 49133, and **H.** *S. oralis* ATCC 35037 were grown anaerobically at 37°C in a chemically-defined media (ZMB1) with or without pooled whole preterm breast milk (pooled from n=3), preterm formula or full-term formula supplemented with HMOs. Growth was monitored at OD_{600nm} after 20 hr. pH was monitored after 20 hr. All data are presented as mean ± stdev, n=3 replicates, performed 2 independent times. One-way ANOVA using the Holm-Sidak post-hoc test to determine significance between pairwise comparisons; *$p< 0.05$.
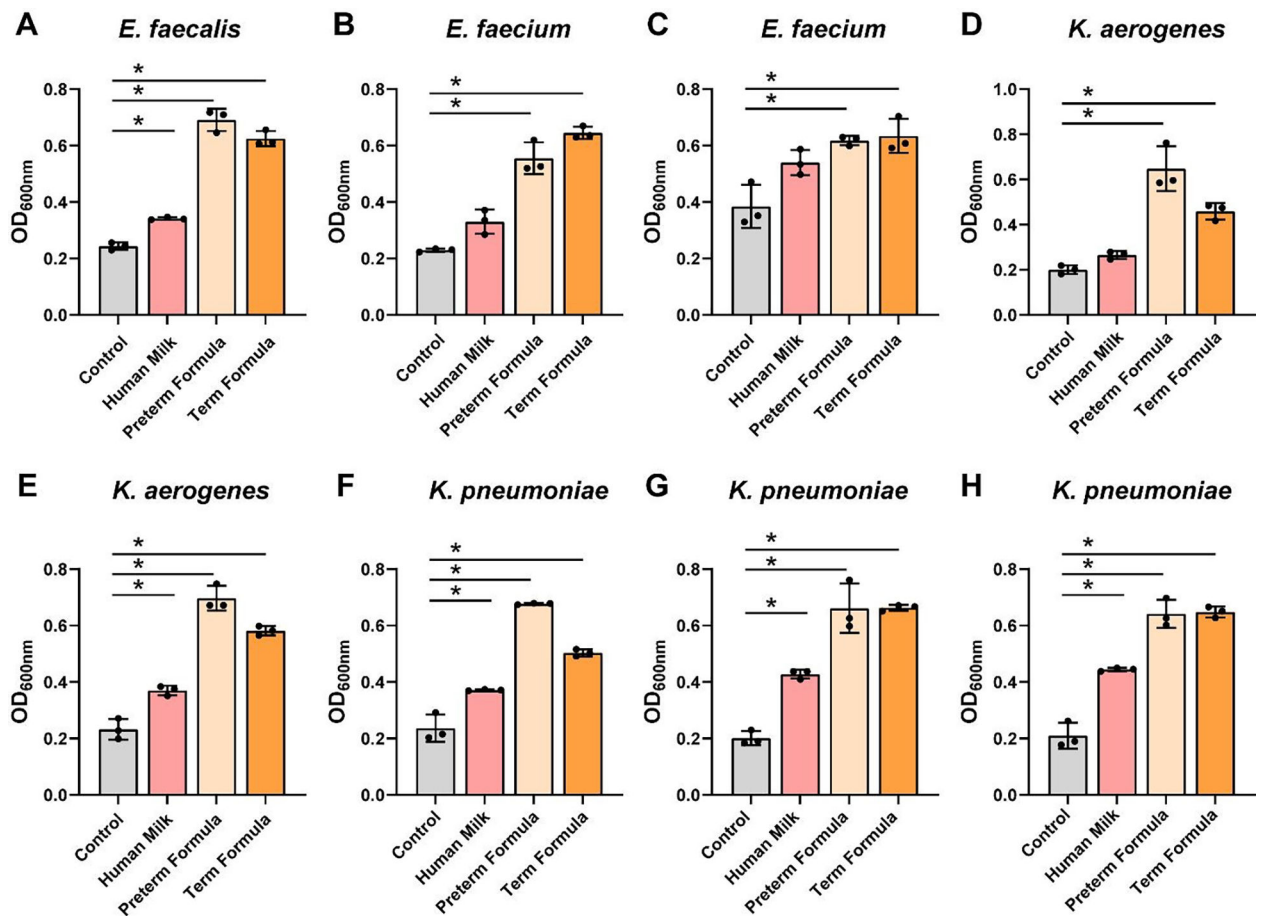
Figure 7: *Enterococcus* and *Klebsiella* species growth with human milk, preterm and term formula.
Pathobiont species **A.** *Enterococcus faecalis* NCTC 775, **B.** *Enterococcus faecium* ATCC 8459, **C.** *Enterococcus faecium* NCTC 12204, **D.** *Klebsiella aerogenes* CB1, **E.** *Klebsiella aerogenes* ATCC 35029, **F.** *Klebsiella pneumoniae* ATCC 700607, **G.** *Klebsiella pneumoniae* ATCC 13883, and **H.** *Klebsiella pneumoniae* ATCC 35657 were grown anaerobically at 37°C in a chemically-defined media (ZMB1) with or without pooled whole preterm breast milk (pooled from n=3), preterm formula or full-term formula supplemented with HMOs. Growth was monitored at OD_{600nm} after 20 hr. All data are presented as mean ± stdev, n=3 replicates, performed 2 independent times. One-way ANOVA, using the Holm-Sidak post-hoc test to determine significance between pairwise comparisons; * p < 0.05.
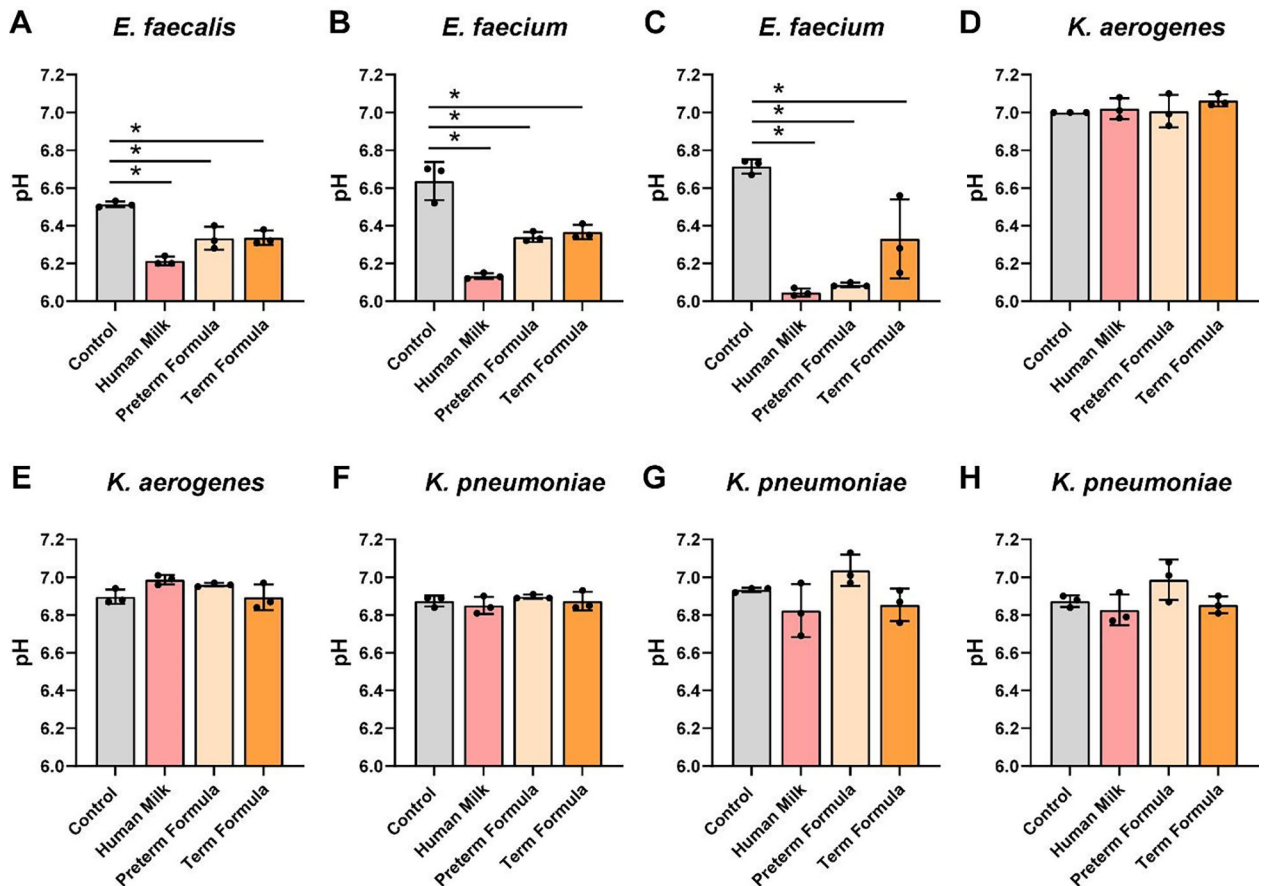
Figure 8: *Enterococcus* **and** *Klebsiella* **species acidification in cultures with human milk, preterm and term formula.**

Pathobiont species **A.** *Enterococcus faecalis* NCTC 775, **B.** *Enterococcus faecium* ATCC 8459, **C.** *Enterococcus faecium* NCTC 12204, **D.** *Klebsiella aerogenes* CB1, **E.** *Klebsiella aerogenes* ATCC 35029, **F.** *Klebsiella pneumoniae* ATCC 700607, **G.** *Klebsiella pneumoniae* ATCC 13883, and **H.** *Klebsiella pneumoniae* ATCC 35657 were grown anaerobically at 37°C in a chemically-defined media (ZMB1) with or without pooled whole preterm breast milk (pooled from n=3), preterm formula or full-term formula supplemented with HMOs. pH was monitored after 20 hr. All data are presented as mean ± stdev, n=3 replicates, performed 2 independent times. One-way ANOVA using the Holm-Sidak post-hoc test to determine significance between pairwise comparisons; *p< 0.05.
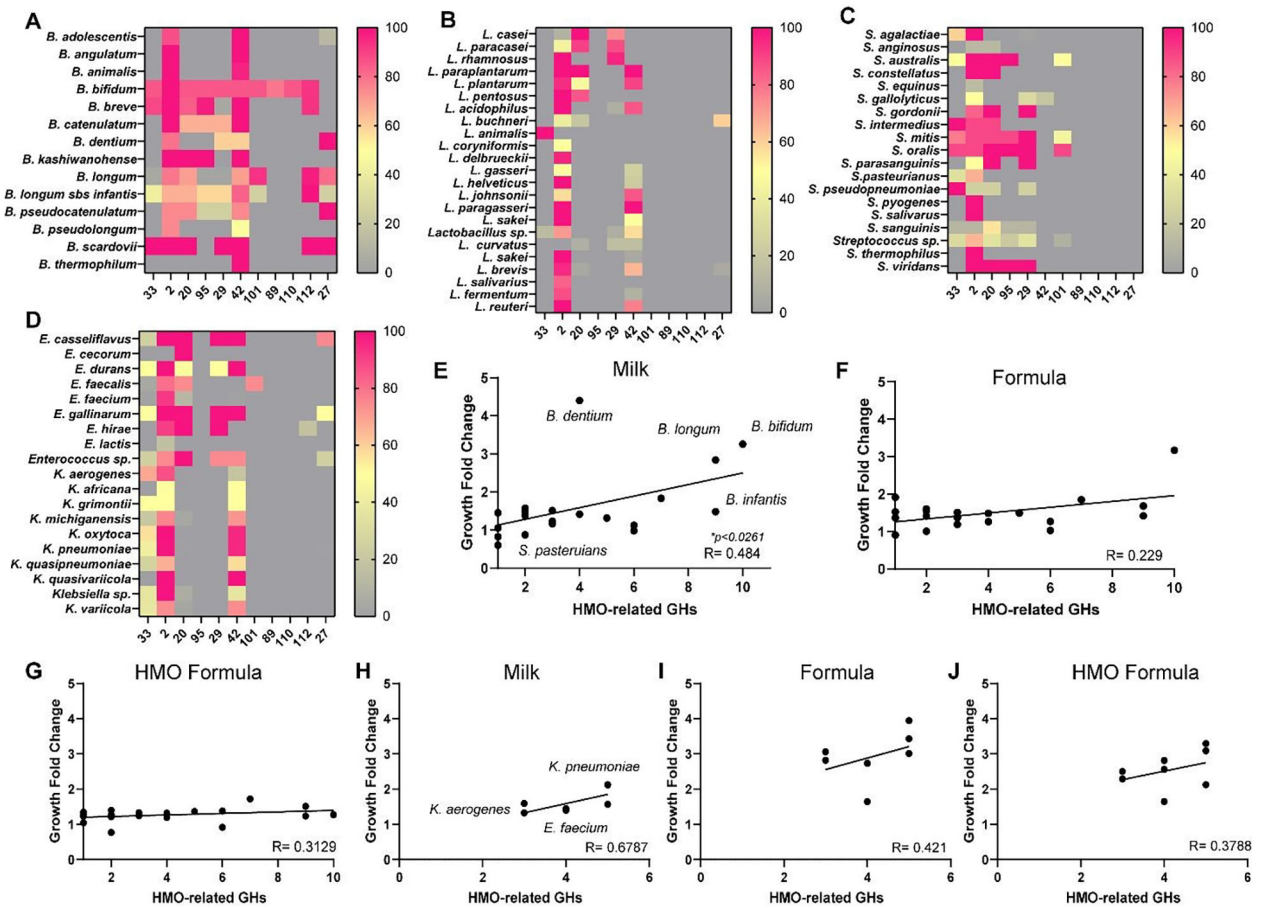
Figure 9. Heat Maps and Correlation between Growth and HMO-related Glycosyl Hydrolase Families.
Heat map of the percentage genomes that have a least one gene copy of Human Milk Oligosaccharide (HMO)-related Glycosyl hydrolase families. GH profiles were examined in **A**. *Bifidobacterium*, **B**. *Lactobacillus*, **C.** *Streptococcus*, and **D**. *Enterococcus* and *Klebsiella* species. Correlation analysis of the number of HMO-related GH families with the average fold change of growth in commensal *Bifidobacterium, Lactobacillus* and *Streptococcus* species in the presence of **E**. pooled preterm human breast milk, **F**. preterm formula, or **G**. HMO formula. Correlation analysis of the number of HMO-related GH families with the fold change of growth in pathobiont *Enterococcus* and *Klebsiella* species in the presence of **H**. human breast milk, **I**. preterm formula, or **J**. HMO formula. P values are shown in the figure.
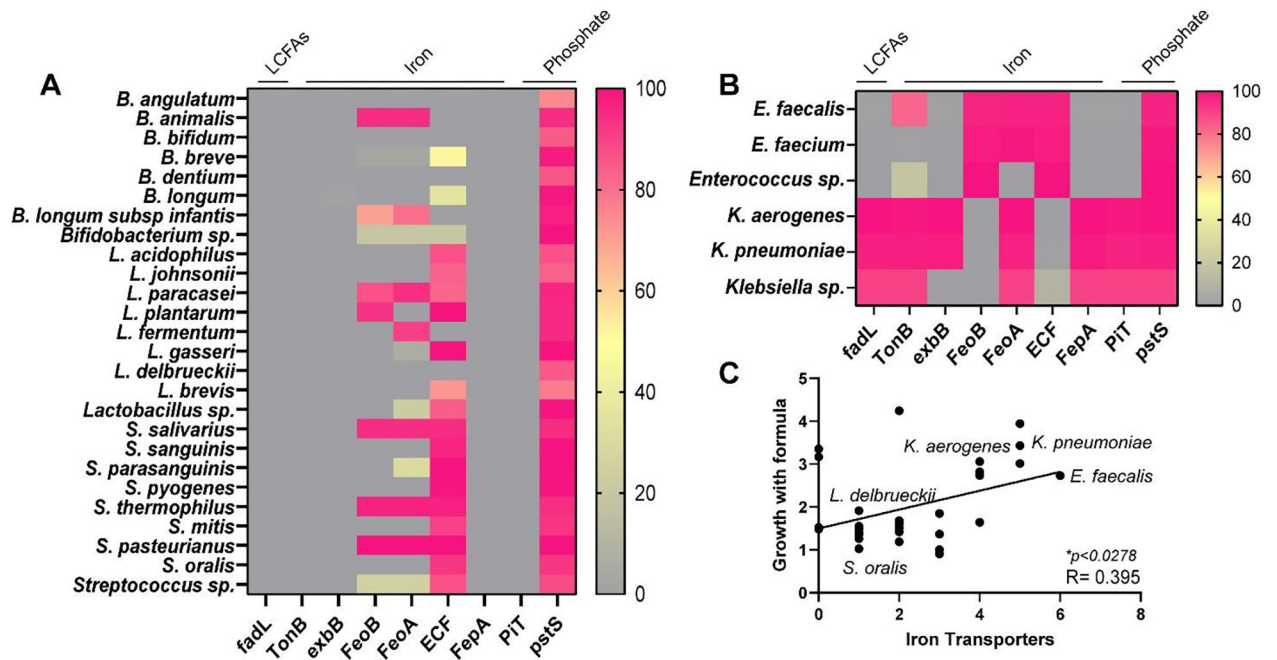
Figure 10. Heat Maps and Correlations between Growth and Long-chain Fatty Acid, Iron and Phosphate Transporters.

Heat map of the percentage genomes that have a least one gene copy of long-chain fatty acid, iron or phosphate transporters. Transporter profiles were examined in **A**. *Bifidobacterium*, *Lactobacillus*, and *Streptococcus* species, or **B.** *Enterococcus* and *Klebsiella* species. **C.** Correlation analysis of the number of iron transporters with the fold change of growth in the presence of formula. P values are shown in the figure.

Table 1:

Bacterial strains and growth conditions used in this study.

Bacteria	Designation	Rich Media	Defined Media
Bifidobacterium bifidum	MIMB	MRS	LDM4
Bifidobacterium longum	ATCC 55813	MRS	LDM4
Bifidobacterium longum subsp. infantis	ATCC 15697	MRS	LDM4
Bifidobacterium dentium	ATCC 27678	MRS	LDM4
Bifidobacterium gallicum	ATCC 20093	MRS	LDM4
Bifidobacterium angulatum	ATCC 27535	MRS	LDM4
Bifidobacterium breve	ATCC 15698	MRS	LDM4
Bifidobacterium animalis	Bi-07	MRS	LDM4
Lactobacillus brevis	ATCC 27303	MRS	LDM4
Lactobacillus gasseri	ATCC 3323	MRS	LDM4
Lactobacillus acidophilus	ATCC 4796	MRS	LDM4
Lactobacillus plantarum	ATCC 14917	MRS	LDM4
Lactobacillus johnsonii	ATCC 33200	MRS	LDM4
Lactobacillus paracasei	ATCC 25302	MRS	LDM4
Lactobacillus fermentum	ATCC 14931	MRS	LDM4
Lactobacillus delbrueckii	ATCC 11842	MRS	LDM4
Streptococcus salvarius	ATCC 13419	BHIS	ZMB1
Streptococcus sanguinis	ATCC 49296	BHIS	ZMB1
Streptococcus parasanguinis	ATCC 15912	BHIS	ZMB1
Streptococcus thermophilus	ATCC 491	BHIS	ZMB1
Streptococcus mitis	ATCC 6249	BHIS	ZMB1
Streptococcus pasteurianus	ATCC 49133	BHIS	ZMB1
Streptococcus oralis	ATCC 35037	BHIS	ZMB1
Streptococcus pyogenes	CB1	BHIS	ZMB1
Enterococcus faecalis	NCTC 775	BHIS	ZMB1
Enterococcus faecium	ATCC 8459	BHIS	ZMB1
Enterococcus faecium	NCTC 12204	BHIS	ZMB1
Klebsiella aerogenes	CB1	BHIS	ZMB1
Klebsiella aerogenes	ATCC 35029	BHIS	ZMB1
Klebsiella pneumoniae	ATCC 700607	BHIS	ZMB1
Klebsiella pneumoniae	ATCC 13883	BHIS	ZMB1
Klebsiella pneumoniae	ATCC 35657	BHIS	ZMB1

Table 2.

Nutrient comparison between milk and formula.

Macronutrients & minerals (per 100 mL)	Preterm Human Milk * (Ref values[51, 84])	Preterm formula Similac® Neosure®	Similac® ProAdvance with HMO, 2-Fl
Calories, kcal	70–80	74	68
Protein, g/dL	2.1–2.7	2.1	1.4
Fat (%)	3–4.1	4.1	3.8
Carbohydrates, g	6.7–8.5	7.5	7.1
IgA, Lactoferrin, Lysozyme g	1.3, 4–5, 3–4	-	-
HMOs, g	1.5–2	-	Quantity Not reported
Sodium, meq	0.6–1	1	0.7
Potassium, meq	1–1.7	2.7	1.8
Calcium, mg	25	78	53
Magnesium, mg	2–5	6.7	4.1
Chloride, meq	1–2	1.6	1.2
Phosphate, mg	9–18	46	28
Iron, mg	0.7–1.6	1.3	1.2
Zinc, mg	0.1–0.3	0.9	0.5
Manganese, mcg	3–6	7.4	3.4
Copper, mcg	20–40	89	61
Selenium, mcg	1.5–2.5	1.7	1.4
Iodine, mcg	3–17 *	11	10
Vitamins (per 100mL)	Preterm Human Milk (Ref values[51, 102]¥)	Preterm formula Similac® Neosure®	Similac® ProAdvance with HMO, 2-Fl
Vitamin A, IU	100–200	260	203
Vitamin D, IU	0.4–4	52	41
Vitamin E, IU	0.4–1	3	1
Vitamin K, mcg	0.2–0.3	8	5
Thiamin (B1), mcg	22–23	130	68
Riboflavin (B2), mcg	48–58	111	101
Vitamin B6, mcg	13–31	74	41
Vitamin B12, mcg	0.06–0.1	0.3	0.2
Niacin, mcg	180–230	1450	710
Folic Acid (Folacin), m	:g 8–13	19	10
Biotin, mcg	0.5	6.7	3.0
Vitamin C, mg	3.8	1 1	6
Choline, mg	<0.1	12	16
Inositol, mg	22	26	16
Panthothenic Acid, mc	g 250	595	304

Fatty Acids (Per 100ml)	Preterm Human Milk (Ref values[50, 51])	Preterm formula Similac® Neosure®	Similac® ProAdvance with HMO, 2-Fl,
Linoleic Acid, mg	400–500	750	676

*
Mature and transitional human milk is defined as 30–60 days in post-partum. Term-gestation infant milk reference values were substituted for premature gestation milk for reference values of contents (Fe, Zn, Mn, Cu, Se, vitamins, and linoleic) acid due to lack of available references ranges.

**
Highly geographically dependent.

¥
Preterm milk ranges of water-soluble vitamins are highly dependent on stage of lactation, maternal intake and preterm delivery, and term-infant ranges were used for B1, B2, B6, B12, Niacin, Folic Acid, Biotin, Vitamin C, and Panthothenic Acid using populations from the Western countries and the U.S.A. Formula nutrition is listed on manufacture's website.