Effects of exclusive breastfeeding on infant gut microbiota: a meta-analysis across studies and populations

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Abstract

Literature regarding the differences in gut microbiota between exclusively breastfed (EBF) and non-EBF infants is meager with large variation in methods and results. We performed a meta-analysis of seven studies (a total of 1825 stool samples from 684 infants) to investigate effects of EBF compared to non-EBF on infant gut microbiota across different populations. In the first 6 months of life, overall bacterial diversity, gut microbiota age, relative abundances of Bacteroidetes and Firmicutes and microbial-predicted pathways related to carbohydrate metabolism were consistently increased; while relative abundances of pathways related to lipid, vitamin metabolism and detoxification were decreased in non-EBF vs. EBF infants. The perturbation in microbial-predicted pathways associated with non-EBF was larger in infants

delivered by C-section than delivered vaginally. Longer duration of EBF mitigated diarrhea-associated gut microbiota dysbiosis and the effects of EBF persisted after 6 months of age. These consistent findings across vastly different populations suggest that one of the mechanisms of short and long-term benefits of EBF may be alteration in gut microbes.

Key words: exclusive breastfeeding, gut, microbiota, infant, meta-analysis

Background

Establishment of the gut microbiota in early life has substantial impact on subsequent health [1]. Common sources of the infant's intestinal microorganisms are from the mother's skin, vagina, stool and from breastfeeding [2–5]. There is a close relationship between the infant's gut microbiota and the mother's breast milk microbiota and human milk oligosaccharides (HMO) composition [6–9]. Indeed, recent evidence has shown that breast milk microbiota can directly seed the infant gut microbiota and the effects of breastmilk on infant gut microbiota is dose-dependent [5]. The microbiota in breast milk changes over time during lactation and has been shown to be different between exclusive breastfeeding (EBF) vs. non-EBF mothers [10,11]. Gut microbial abundances in breast-fed infants, especially bifidobacterial species, are correlated with the mother's HMOs and HMO-related catabolic activity [3,12,13]. Infant gut microbiota have been shown to be different between breast fed vs. formula-fed infants [14–20] and change rapidly during the transition from breastfeeding to formula [21].

EBF in the first 6 months of life provides a multitude of health benefits [22,23]. For example, EBF has been shown to be strongly protective against diarrhea morbidity and mortality [24] and

decreases long-term risk of diabetes and obesity as compared to non-EBF or formula-fed infants [25–27]. We hypothesize that the numerous benefits of EBF may be in part due to its effects on the infant gut microbiota. Several recent studies have identified varying differences in gut microbial composition or diversity between EBF and non-EBF infants [5,28–30] or gradients in the gut microbiota composition or diversity across EBF, non-EBF and non-breastfed (non-BF) infants [5,14,29,31,32]. However, some other studies have found no significant differences in gut microbial communities between EBF and non-EBF infants [3,33]. In addition, mode of delivery has been variably reported to have no effect [34] or a significant effect [30] or a potential interaction effect with breastfeeding [31] on the infant gut microbiota. The wide variation in reported results together with heterogeneity in feeding category definitions, study designs, study populations, and especially in data processing and analysis methods make these findings difficult to synthesize and interpret.

In this study, we applied appropriate and robust statistical methods to analyze gut microbiome data and performed meta-analyses pooling estimates from seven microbiome studies (a total of 1825 stool samples from 684 infants) to investigate the effects of EBF compared to non-EBF on infant gut microbiota in the first 6 months of life across different populations. In addition, we evaluated effect modification by infants' mode of delivery. We aimed to synthesize existing data and to examine consistency or inconsistency in findings across studies and populations. Where data were available, we also examined persisting effects of EBF occurring in the first 6 months on the gut microbiota of infants after 6 months of life as well as interaction effects between diarrhea and EBF on the infant gut microbiota.

Results

Gut microbial diversity is increased in non-EBF vs. EBF infants ≤ 6 months of age

In infants ≤ 6 months of age, across the seven included studies, non-EBF consistently associated with increased gut microbial alpha diversity (standardized Shannon index) compared to EBF adjusting for infant age at sample collection (pooled standardized diversity difference (DD)= 0.34 standard deviation (sd), 95% confidence interval (95% CI)= [0.20; 0.48], pooled p-value<0.0001) (Figure 1a, Figure 1b). In a subset of five studies that also contained non-BF infants (Bangladesh [35], Canada [31], USA (California-Florida (CA-FL)) [5], USA (California-Massachusetts-Missouri (CA-MA-MO)) [18], and USA (North Carolina (NC)) [29]), gut microbiome diversity (standardized Shannon index) was significantly increased in infants with less breastfeeding after adjusting for age of infants at sample collection (pooled standardized DD=0.39 sd, 95% CI=[0.19; 0.58], pooled p-value=0.0001) (Figure 1c). Results were consistent utilizing three other commonly used alpha diversity indices (Phylogenetic diversity whole tree, Observed species, Chao1) (all pooled p-values <0.05) (Figure 1d, Figure 1e).

In sensitivity meta-analyses excluding estimates from either the USA (NC) study [29] (which contained a small number of infants ≤ 6 months old), or the Haiti study [3] (which included samples from HIV-uninfected infants born to HIV-infected and HIV-uninfected mothers) or the Vitamin D Antenatal Asthma Reduction Trial (VDAART) study [18] in the USA (CA-MA-MO) (which contained samples from infants at high risk of asthma and allergies, half of whom were randomized to high-dose antenatal vitamin D supplementation), the results remained similar (Supplementary figure 1).

In a subset of four studies (Canada [31], Haiti [3], USA (CA-FL) [5], and USA (CA-MA-MO) [18]) with available data on mode of delivery, the increase in microbial diversity associated with non-EBF was similar in the meta-analysis stratified on vaginally-delivered infants and on cesarean-delivered infants (Supplementary figure 2).

Gut microbiota age is increased in non-EBF vs. EBF infants ≤ 6 months of age

A Random Forest (RF) model was used to predict the infant age in each included study based on relative abundances of the shared gut bacterial genera of the seven included study (Supplementary table 1). The model explained 95% of the variance related to chronologic age in the training set and 65% of the variance related to chronologic age in the test set of Bangladesh data (Supplementary figure 3). The predicted infant age in each included study based on relative abundances of the shared gut bacterial genera using this Random Forest model was regarded as gut microbiota age.

In infants ≤ 6 months of age, a consistent (6/7 studies) increase of gut microbiota age was observed in non-EBF as compared to EBF infants after adjusting for infant age at sample collection (pooled standardized microbiota age difference (MD)= 0.33 sd, 95%CI= [0.09; 0.58], pooled p-value= 0.0074) (Figure 2a, Figure 2b). In sensitivity meta-analyses excluding either of the three studies mentioned above [3,18,29], the association remained unchanged (Supplementary figure 4). In the subset of four studies with available data on mode of delivery [3,5,18,31], meta-analysis stratified on vaginally-delivered infants and on cesarean-delivered infants showed similar overall increase in microbiota age in non-EBF vs. EBF infants (Supplementary figure 5).

The trend of increasing gut microbiota age in infants with less breastfeeding after adjusting for age of infants at sample collection was also observed in a subset of five studies containing a non-BF group [5,18,29,31,35] (pooled standardized MD= 0.35 sd, 95%CI= [0.09; 0.61], pooled p-value =0.0079) (Figure 2c).

Gut microbial composition is altered in non-EBF vs. EBF infants ≤ 6 months of age

Across the seven included studies, there was a large heterogeneity in the difference (log(odds ratio)) of gut bacterial taxa relative abundances between non-EBF vs. EBF infants after adjusting for age of infants at sample collection. Notably, a decrease in relative abundance of Proteobacteria in non-EBF vs. EBF infants was observed in the four studies in North America but the opposite was observed in the other three studies in Haiti, South Africa and Bangladesh (Figure 3a). However, there was also remarkable consistency across studies. At the phylum level, there was an overall significant increase in the relative abundances of Bacteroidetes (consistent in all seven studies) and Firmicutes (consistent in 6/7 studies) in non-EBF vs. EBF infants (all pooled p-values <0.05 and false discovery rate (FDR) adjusted pooled p-values <0.1) (Figure 3a, Supplementary table 2). There was also a consistent trend of increasing relative abundances of these two phyla across EBF, non-EBF and non-BF in the subset of five studies with a non-BF group [5,18,29,31,35] (all pooled p-values <0.05 and FDR adjusted pooled p-values <0.1) (Supplementary table 3).

At the order level, the relative abundances of Bacteriodales (7/7 studies) and Clostridiales (6/7 studies) were consistently increased in non-EBF infants (all pooled p-values <0.05 and FDR adjusted pooled p-values <0.1) (Supplementary figure 6, Supplementary table 2). At the family

level, the relative abundances of Bacteroidaceae (7/7 studies) and Veillonellaceae (6/7 studies) were consistently increased in non-EBF infants (all pooled p-values <0.05 and FDR adjusted pooled p-values <0.1) (Figure 3b, Supplementary table 2). At the genus level, there were increases in the relative abundances of *Bacteroides* (7/7 studies), *Eubacterium, Veillonella* (6/7 studies), and *Megasphaera* (5/7 studies) in non-EBF infants (all pooled p-values <0.05, FDR adjusted pooled p-value of *Eubacterium* <0.1) (Supplementary figure 7, Supplementary table 2). In sensitivity meta-analyses excluding either of the three studies mentioned above [3,18,29], results remained similar (Supplementary table 4, Supplementary table 5, Supplementary table 6).

In the subset of four studies with available data on mode of delivery [3,5,18,31], the results of meta-analysis stratified on vaginally-delivered infants were similar to those of meta-analysis stratified on cesarean-delivered infants from the phylum to family level. Phylum Proteobacteria (particularly family Enterobacteriaceae) was markedly and significantly reduced in non-EBF infants, especially among infants delivered by cesarean (Supplementary figure 8, Supplementary figure 9, Supplementary figure 10, Supplementary table 7). At the genus level, relative abundance of *Acidaminococcus* was significantly higher in vaginally-delivered non-EBF vs. vaginally-delivered EBF infants, whereas, relative abundances of *Proteus* and *Anaerotruncus* were significantly lower in cesarean-delivered non-EBF vs. cesarean-delivered EBF infants (all FDR adjusted pooled p-values <0.1) (Supplementary figure 11, Supplementary figure 12, Supplementary table 7).

Gut microbial-predicted functions are perturbed in non-EBF vs. EBF infants ≤ 6 months of age

Across the seven included studies, although the difference (log(odds ratio)) of relative abundances of gut bacterial KEGG functional pathways between non-EBF vs. EBF infants was largely heterogeneous, important consistencies were found. At KEGG level two, there was no pathway significantly different between non-EBF and EBF group after adjusting for multiple testing (Supplementary figure 13, Supplementary table 8). At KEGG level three, the relative abundances of 24 pathways were significantly different between non-EBF and EBF infants (pooled p-values <0.05) and nine of these remained significant after adjusting for multiple testing (FDR adjusted pooled p-values <0.1) (Figure 4a). Specifically, after adjusting for age of infants at sample collection, in the non-EBF group vs. the EBF group, there were higher relative abundances of several pathways related to carbohydrate metabolism, viz. Fructose and Mannose Metabolism (7/7 studies), Pentose and Glucuronate Interconversions, and Pentose Phosphate Pathway (6/7 studies), as well as Fatty Acid Biosynthesis and Biosynthesis of Ansamycins pathways (6/7 studies). In addition, in non-EBF infants, there were lower relative abundances of some pathways related to lipid metabolism, lipid homeostasis and free radical detoxification (Fatty Acid Metabolism (6/7 studies) and Peroxisome (6/7 studies)), Vitamin 6 Metabolism (7/7 studies) and Ribosome Biogenesis in Eukaryotes (5/7 studies) (all pooled p-values <0.05 and FDR adjusted pooled p-values <0.1) (Figure 4a, Supplementary table 8). Sensitivity meta-analyses excluding either of the three studies mentioned above [3,18,29] showed similar results (Supplementary table 9).

In the subset of four studies with available data on mode of delivery [3,5,18,31], meta-analysis stratified by mode of delivery showed remarkable heterogeneity between vaginally-delivered infants and cesarean-delivered infants. In vaginally-delivered infants, there were six pathways

related to carbohydrate and lipid metabolism significantly perturbed between non-EBF vs. EBF infants after adjusting for multiple testing (all FDR adjusted pooled p-values <0.1) (Figure 4b, Supplementary table 10). Whereas, in cesarean-delivered infants only, there were a much larger number of pathways (35 pathways) of many cellular and metabolic processes (such as cell growth and death, membrane transport, replication and repair, carbohydrate, lipid, amino acid, vitamin, energy metabolism) significantly perturbed between non-EBF vs. EBF infants after adjusting for multiple testing (all FDR adjusted pooled p-values <0.1). In addition, the perturbation of these pathways were mostly consistent across four included studies (Figure 4c, Supplementary table 10).

Duration of EBF in the first 6 months alters infant gut microbiota after 6 months of age

Using data from the Bangladesh study only, which included 996 stool samples collected monthly from birth to 2 years of life in 50 subjects, we found that shorter duration of EBF (less than 2 months vs. more than 2 months from birth) was associated with a larger increase in gut microbiota age. This association was significant from 6 months to 15 months of age (microbiota age difference (MD)= 1.64 months, 95%CI=(0.23, 3.05), p-value=0.0233) (Figure 5a).

From 6 months to 2 years of age, after adjusting for age of infants at sample collection, shorter duration of EBF (less than 2 months vs. more than 2 months from birth) was associated with lower relative abundance of the phylum Actinobacteria and higher relative abundance of Firmicutes (all FDR adjusted p-values <0.1) (Supplementary table 11). At the family level, infants with shorter duration of EBF had lower relative abundances of Bifidobacteriaceae, and Enterococcaceae and higher relative abundances of Lactobacillaceae, Coriobacteriaceae,

Prevotellaceae, Clostridiaceae, Erysipelotrichaceae, and Lachnospiraceae (all FDR adjusted p-values <0.1) (Figure 5b, Supplementary table 11).

Breastfeeding mitigates diarrhea-associated gut microbiota dysbiosis in infants from 6 months to 2 years of age

Again, using data from the Bangladesh study, we found that, after adjusting for age of infants at sample collection, diarrhea at the time of sample collection (vs. no diarrhea) was associated with reduced gut microbiota age in infants who received less than 2 months of EBF (MD= -1.17 months, 95% CI= (-2.11;-0.23), p= 0.0146) (Figure 5c). Diarrhea in infants who received less than 2 months of EBF was also associated with reduced gut microbial diversity as showed by Shannon index (diversity difference =-0.58, 95%CI=(-0.83,-0.34), p<0.0001) (Figure 5d) and the three other common alpha diversity indices (Supplementary figure 14). In contrast, no diarrhea-associated differences in gut microbiota age or microbial diversity was observed in infants who received more than two months of EBF (all p-values for heterogeneity tests <0.05).

Diarrhea at the time of sample collection was also associated with major perturbation in the gut bacterial composition of infants who received less than 2 months of EBF with a significant increase in the relative abundance of family Streptococcaceae and a significant decrease in the relative abundances of Bifidobacteriaceae and Coriobacteriaceae (all p-values <0.05 and FDR adjusted p-values <0.1). These changes in microbial composition were not observed in infants who received more than 2 months of EBF (Figure 5e, Supplementary table 12). Diarrhea at the time of sample collection was associated with an even more striking perturbation in the gut bacterial composition in infants who were not concurrently being breastfed, with a large

outgrowth of Streptococcaceae and a tremendous decrease in the relative abundance of Bifidobactericeae (all p-values <0.05). These perturbations were almost absent in infants receiving breast milk at the time of diarrhea (Figure 5f, Supplementary table 12). The incidence of diarrhea was not different between breastfeeding statuses.

Discussion

Our study analyzed data from seven microbiome studies and performed meta-analysis pooling estimates across studies with a total of 1825 stool samples of 684 infants from five countries. We found remarkably consistent differences between non-EBF vs. EBF infants in gut microbial diversity, microbiota age, microbial composition and microbial predicted functional pathways. The infants' mode of delivery modified these effects. We also found notable interaction effects between breastfeeding and diarrhea on infant gut microbiota. With large datasets combined from different populations, our results are more robust and generalizable than from a single study.

Prior studies have reported increased bacterial species richness or diversity in non-EBF vs. EBF[28] and/or trends of increased bacterial diversity across EBF, non-EBF and non-BF. [29,31,32] However, studies report different indices, analyze data in different ways and some do not account for age of infants at the time of stool sample collection, which is associated with breastfeeding status and infant gut microbiota. Our results showed a significant and consistent increase in all four commonly-used alpha diversity indices in non-EBF vs. EBF infants ≤ 6 months of age after adjusting for age of infants at sample collection. We also showed a consistent increase in gut microbiota age in non-EBF infants vs. EBF infants before 6 months of age across studies. At first glance these results may seem paradoxical but we hypothesize that the

increase in diversity and microbiota age associated with non-EBF in the first 6 months may be out of step with age-appropriate development. A more stable, less diverse gut microbiota, associated with EBF, may be necessary in the early months of development.

There was substantial heterogeneity across studies and populations in gut bacterial taxonomic composition and gut bacterial metabolic pathway composition differences between non-EBF vs. EBF infants in the first 6 months of life. For example, the decrease in relative abundance of Proteobacteria in non-EBF vs. EBF infants was observed in four studies in North America but the opposite was observed in other studies in Bangladesh, Haiti and South Africa. This heterogeneity may be due to dietary differences or differences in formula ingredients in the non-EBF group across different populations. In addition, the gut microbiota of EBF infants, which is largely seeded by their mothers' breastmilk microbiota and HMOs [3,5,9,12], might also be influenced by the mother's diet or other exposures, which might also be different across populations [10]. Infant ethnicity has been reported to influence the infant gut microbiota [36]. In addition, variation in the region of 16S RNA gene targeted between studies may also contribute to heterogeneity. Despite this expected variation, our results revealed some important consistencies across populations. Our results showed a consistent increase in the relative abundance of Bacteroidetes in non-EBF vs. EBF infants in all seven studies as well as an overall significant increase in relative abundance of Firmicutes. More specifically, our results showed a persistent increase in relative abundances of genera *Bacteroides*, *Eubacterium* and *Veillonella* in non-EBF vs. EBF infants. While *Bifidobacterium* is the most common bacterial genus in gastrointestinal tract of young infants, *Bacteroides* and *Eubacterium* are the most common bacterial genera in the gastrointestinal tract of adults [37,38]. Though these genera may be part of

normal gut bacterial community, the increase in abundance of gut *Bacteroides* has been shown to be associated with higher body mass index (BMI) in young children [39] and *Veillonella* can be associated with different types of infection [40]. Our results also showed a consistent increase in relative abundances of major microbial predicted pathways related to carbohydrate metabolism as well as a consistent decrease in relative abundances of crucial pathways related to lipid metabolism/homeostasis, free radical detoxification and metabolism of cofactors and vitamins in non-EBF infants [41]. These findings may provide insight into biological mechanisms for the higher risk of obesity, diabetes and other adverse health outcomes in children who were not breastfed or non-exclusively breastfed in early months of life [25–27].

Interestingly, our results revealed notable heterogeneity regarding the perturbation in microbial-predicted functional pathways associated with non-EBF stratified by mode of delivery. The remarkably larger number of perturbed pathways of different cellular and metabolic processes in infants of cesarean deliveries may suggest that gut microbiota in these infants are more vulnerable to the effects of non-EBF. We also observed that non-EBF infants had a much lower abundance of Proteobacterial species than EBF infants after cesarean delivery. It appears that when gut microbiota are depleted with Bacteroidetes, as characteristically in early infancy after cesarean delivery, formula-feeding further depletes Proteobacteria [42]. These findings may shed light on the mechanisms for the higher risk of adverse health outcomes in infants delivered by cesarean section [43–45] and emphasize the importance of EBF in cesarean-delivered infants. Differences in breastfeeding practices by mode of delivery may also account for these findings [46].

Effects of EBF in the first 6 months of life were still evident on the infant gut microbiota between 6 months to 2 years of age. Shorter duration of EBF was associated with increased gut microbiota age as well as earlier and larger increases in relative abundances of many bacterial families other than the beneficial family Bifidobacteriaceae. In contrast, longer EBF maintained a more stable bacterial composition in the early months of life and was associated with higher relative abundance of Bifidobacteriaceae. These findings again support our hypothesis that early changes in gut microbiota associated with non-EBF may be disproportional to immunological and biological maturity of infants in early months. That is, EBF nourishes a stable gut bacterial taxa composition that may be beneficial for the infants who are still immature in early month of life. Our results are also consistent with published literature that exposures in early life can affect the establishment of the gut microbiota in older children and adults [36,47–49] and may help explain the mechanism for the short and long-term health effects of EBF in early months of life.

Another particularly intriguing finding from our analysis is the protective effect of EBF on the infant gut microbiota during diarrheal episodes. Diarrhea has been previously shown to cause perturbations in the gut microbiota [35,50]. Our analysis revealed that diarrhea was associated with a loss of microbial diversity, microbiota age and the relative abundance of Bifidobacteriaceae as well as an increase in the relative abundance of Streptococcaceae. Incredibly, these changes were almost completely abolished in infants who received more than 2 months of EBF as well as in those who were being breastfed at the time of diarrhea. These findings highlight the importance of longer duration of EBF in the first 6 months of life and continuation of breastfeeding after 6 months of life in maintaining a homeostatic gut microbiota that may be more resistant to outgrowth of pathogenic microbes that lead to diarrhea. Taken together, our findings suggest a

key role for gut commensal bacteria in mediating the protective effects of breastfeeding on diarrhea morbidity and mortality.

In terms of methodology, our study applied appropriate and robust methodological approaches for the analysis of microbiome data and meta-analysis across microbiome studies. Standardization of alpha diversity indices and predicted microbiota age from each study makes the estimates of these measures comparable between studies. Zero-inflated beta GAMLSS models allow proper examination of relative abundances of bacterial taxa and predicted functional pathways, which range from zero to one and are generally zero-inflated as well as adjustment for confounding covariates and handling longitudinal or cross-sectional data. The estimates from zero-inflated beta GAMLSS models are log(odds ratio) of relative abundances and thus are comparable between studies. All effect estimates in our analyses were adjusted for variation in age of the infant at sample collection which might largely influence the infant gut microbiota composition as well as breastfeeding status but was not routinely accounted for in analysis [3,29] or was partially accounted by study design (collecting samples at similar infant age) [31,33] in some published studies. There have been some published meta-analyses for microbiome data [51–56] but none of these addressed between-group-comparison pooled effects when combining data from many studies as done here. The use of random effect meta-analysis models pooling estimates from studies allows examination of study-specific effects, the heterogeneity between studies, and the overall pooled effects across studies.

This study has some limitations. First, definitions of EBF and non-EBF were not identical across seven included studies. Specifically, for the five studies in Bangladesh, Canada, CA-FL, North

Carolina and CA-MA-MO, EBF was defined as ingestion of only breastmilk without formula or solid food and non-EBF as ingestion of breastmilk plus formula and/or solid food. Whereas, the two studies in Haiti and South Africa followed World Health Organization (WHO) guidelines with EBF as feeding only breastmilk and non-EBF as feeding breast milk plus anything other than breast milk including traditional medicines or water. This difference might contribute to some of the variation in results across studies. Second, several of the studies were of a relatively small size or included other potential issues. Specifically, the Haiti study included only 48 infants, of which half were born to HIV-infected mothers, which has been shown to influence the infant gut microbiota [3]. The North Carolina study included a very small number of samples in infants ≤ 6 months old (n=21). The VDAART trial (CA-MA-MO) study included samples from infants who were at high risk for asthma and allergies half of whom were randomized to vitamin D supplementation in pregnancy. However, sensitivity meta-analyses excluding these studies showed similar findings to the overall meta-analysis, suggesting that our results are robust. Finally, all of the results pertaining to samples collected after 6 months of age were from a single study (Bangladesh) and thus should be replicated in other cohorts. However, this study included nearly 1,000 samples collected monthly from birth to 2 years of life in 50 subjects with detailed meta-data [35].

Conclusion

Our meta-analysis revealed robust findings across populations that may help elucidate the effects of EBF on the infant gut microbiota. Non-EBF or shorter duration of EBF in the first six months of life was associated with higher gut microbial diversity, higher microbiota age, bacterial composition more closely resembling the adult microbiota, higher composition of bacterial

functional pathways related to carbohydrate metabolism and lower composition of bacterial functional pathways related to lipid metabolism, detoxification, cofactor and vitamin metabolism. The perturbation in microbial functional pathways associated with non-EBF was larger in cesarean-delivered infants than in vaginally-delivered infants. Furthermore, EBF, especially longer than two months from birth, was associated with a more stable gut bacterial taxa composition and reduced diarrhea-associated microbial dysbiosis. The early and large change associated with non-EBF in the infant gut microbiota may be disproportional to age-appropriate immunological and biological development of the infant. Altogether, our results highlight a consistent role of EBF to maintain a homeostatic developmental trajectory of the infant gut microbiota and shed light on the mechanisms of the short and long-term benefits of EBF in the first six months of life.

Methods

Data sources and study population

Processed and partially processed 16S rRNA gene sequence data of stool samples were obtained from seven previously published studies [3,5,18,28,29,31,35]. Of the included studies, three were from the US, one from Canada, one from Haiti, one from South Africa and one from Bangladesh. There were five studies with three breastfeeding categories (EBF, non-EBF and non-BF) and two studies with two breastfeeding categories (EBF and non-EBF). The total number of samples of infants ≤ 6 months of age included in the overall meta-analyses was 1151 (EBF=547, non-EBF=436, non-BF=168). There were four studies with available information regarding the infants' mode of delivery included in meta-analyses stratified by mode of delivery with a total number of samples of 670 (vaginal deliveries =484, cesarean deliveries =186). The Bangladesh

study contained 674 samples from 6 months to 2 years of age. In total, 1825 samples of 684 infants were used in our analyses. A summary of the included studies, data characteristics and prior data processing are presented in Table 1.

Data processing

Sequence data from each included study were processed separately. To achieve necessary data consistency for meta-analyses in this study, OTU picking was performed at 97% similarity using QIIME version 1.9.1 [57] with the Greengenes database (version 13.8) [58]. Alpha rarefaction was done in QIIME using default options. Rarefaction depth was selected as the highest depth that retained all study samples. Taxonomic relative abundances from phylum to genus levels and alpha diversity indices were calculated based on rarefied OTU tables. Metagenomic functional compositions of stool bacterial communities were predicted based on the normalized OTU tables using PICRUSt [59] and relative abundances were then calculated for the resulting KEGG (Kyoto Encyclopedia of Genes and Genomes) functional pathways [41].

Statistical analysis

For each study, mean alpha diversity indices were calculated for each sample at the selected rarefaction depth. Random Forest (RF) modeling of gut microbiota maturity has been widely used to characterize development of the microbiota over chronological time [5,35,49]. Adapting the approach from Subramanian et al. [35], relative abundances of 36 bacterial genera (Supplementary table 1) that were detected in the data of all seven included studies were regressed against infant chronological age using a RF model on the training dataset of the Bangladesh study. The RF model fit based on relative abundances of these shared bacterial genera was then used to predict

infant age on the test data of the Bangladesh study and the data of each other included study. The predicted infant age based on relative abundances of these shared bacterial genera in each study is referred to as gut microbiota age in this paper. Alpha diversity indices and microbiota age from each study were standardized to have a mean of 0 and standard deviation of 1 to make these measures comparable across studies. Generalized additive mixed model (GAMM) as well as linear mixed effect model with subject random intercept (for longitudinal data) or linear model (for non-longitudinal data) adjusted for infant age at the time of stool sample collection were used to further examine the curves of standardized alpha diversity indices and standardized gut microbiota age over age of infants as well as the difference between groups in each study.

For each study, the summary tables of bacterial taxa and pathway relative abundances were filtered to retain only the taxa and pathways that had an average relative abundance of at least 0.005% and were detected in at least 5% of the number of samples in that study. Relative abundances of bacterial taxa and bacterial KEGG metabolic pathways were examined using Generalized Additive Models for Location Scale and Shape (GAMLSS) with zero inflated beta family (BEZI) and (mu) logit links and other default options as implemented in the R package gamlss [60]. This approach allows proper examination of microbiome relative abundance data, which range from zero to one and are generally zero-inflated, as well as adjustment for covariates (e.g. infant age at sample collection) and handling of longitudinal data by including a subject random effect. In each study, to roughly test for trends across three breastfeeding categories (EBF, non-EBF and non-BF), breastfeeding was coded as a continuous variable in the models.

To examine overall effects while addressing heterogeneity across studies, random effect meta-analysis models with inverse variance weighting and DerSimonian–Laird estimator for between-study variance were used to pool the adjusted estimates and their standard errors from all included studies. Meta-analyses were done for only bacterial taxa and pathways whose adjusted estimates and standard errors were available in at least 50% of the number of included studies. All statistical tests were two sided. P-values <0.05 were regarded as significant and false discovery rate (FDR) adjusted p-values <0.1 were regarded as significant after adjusting for multiple testing.

Code availability

The R code used to generate the results in this paper are available at https://github.com/nhanhocu/metamicrobiome_breastfeeding.

Data availability

All data used in the analyses of this study are included in these published articles and their supplementary information files (references [3,5,18,28,29,31,35]). The data from the Bangladesh study were downloaded from: https://gordonlab.wustl.edu/Subramanian_6_14/Nature_2014_Processed_16S_rRNA_datasets.html. The data from six other studies were obtained directly from the investigators. All processed microbiome datasets and relevant meta-data from all included studies as well as intermediate saved outputs used to generate the results in this paper are available at https://github.com/nhanhocu/metamicrobiome_breastfeeding.

Competing interests

The authors declare that they have no competing interests.

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Authors' contribution

N.T.H and L.K conceived the study. F.L, K.A.L, H.M.T, B.B, P.S.P, L.F.W, J.M.B, J.E.S, M.B.A, A.L.T, S.T.W, M.A.A.P, A.A.L, A.L.K, H.B.J, G.M.A provided data. N.T.H performed the data analysis with inputs from F.L and L.K. N.T.H, F.L, H.B.J, G.M.A and L.K prepared the manuscript with inputs from all authors. All authors read and approved the final manuscript.

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Figure 1. Meta-analysis: the effects of non-exclusive vs. exclusive breastfeeding on gut microbial diversity in infants ≤ 6 months of age.

a: Gut microbial alpha diversity (standardized Shannon index) by breastfeeding status by age of infants at stool sample collection from each of seven included studies. Fitted lines and 95% confidence intervals (95% CI) were from Generalized Additive Mixed models (GAMM).

b: The difference in gut microbial alpha diversity (standardized Shannon index) between non-exclusively breastfed (non-EBF) vs. exclusively breastfed (EBF) infants ≤ 6 months of age from each study and the pooled effect across seven included studies (meta-analysis).

c: The trend effect of gut microbial alpha diversity (standardized Shannon index) across EBF, non- EBF and non-BF infants ≤ 6 months of age from each study and the pooled effect across five included studies (meta-analysis). Data from the Haiti and South Africa studies were not

included as there was no non-BF group in these two studies. In each study, to roughly test for trends across breastfeeding categories, breastfeeding was coded as a continuous variable in the models (EBF=1, non-EBF=2 and non-BF=3).

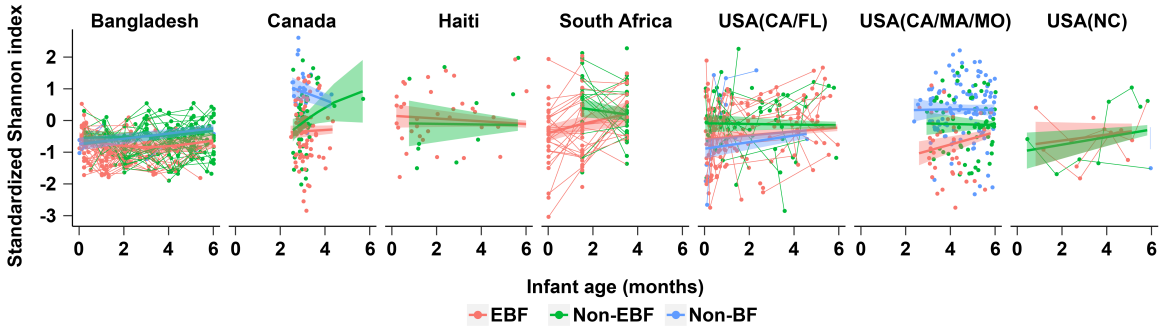
d: Pooled estimates and 95% CI for the difference in gut microbial alpha diversity (four common alpha diversity indexes (all standardized)) between non-EBF vs. EBF infants ≤ 6 months of age across seven studies.

e: Pooled estimates and 95% CI for the trend effect of gut microbial alpha diversity (four common alpha diversity indexes (all standardized)) across EBF, non- EBF and non-BF infants ≤ 6 months of age.

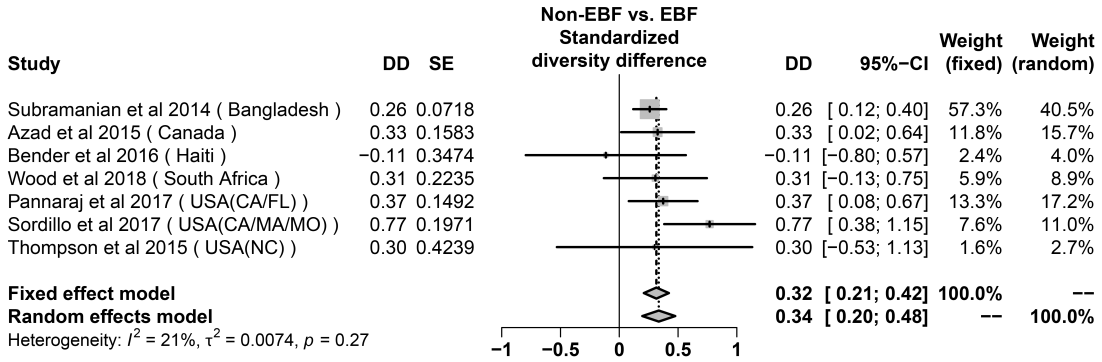
Estimates for diversity difference or trend and corresponding standard errors from each study were from linear mixed effect models (for longitudinal data) or linear models (for non-longitudinal data) and were adjusted for age of infants at sample collection. Pooled estimates of standardized diversity difference or trend and their 95% CI were from random effect meta-analysis models based on the adjusted estimates and corresponding standard errors of all included studies. Pooled estimates with pooled p-values<0.05 are in red and those with false discovery rate (FDR) adjusted pooled p-values <0.1 are in triangle shape.

EBF: exclusive breastfeeding; non-EBF: non-exclusive breastfeeding; non-BF: non-breastfeeding; USA: United States of America; CA: California; FL: Florida; MA: Massachusetts; MO: Missouri; NC: North Carolina; DD: Diversity difference; SE: Standard error.

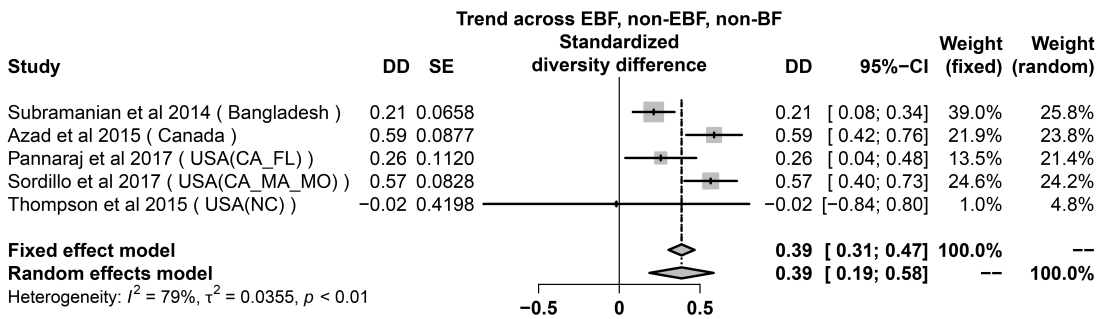
a


Bangladesh
Canada
Haiti
South Africa
USA(CA/FL)
USA(CA/MA/MO)
USA(NC)
Standardized Shannon index
Infant age (months)
EBF
Non-EBF
Non-BF

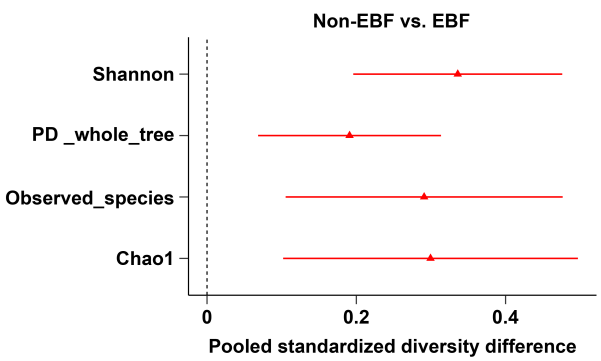
b


Non-EBF vs. EBF
Standardized diversity difference
Study
DD
SE
DD
95%−CI
Weight (fixed)
Weight (random)
Subramanian et al 2014 (Bangladesh) 0.26 0.0718 0.26 [0.12; 0.40] 57.3% 40.5%
Azad et al 2015 (Canada) 0.33 0.1583 0.33 [0.02; 0.64] 11.8% 15.7%
Bender et al 2016 (Haiti) −0.11 0.3474 −0.11 [−0.80; 0.57] 2.4% 4.0%
Wood et al 2018 (South Africa) 0.31 0.2235 0.31 [−0.13; 0.75] 5.9% 8.9%
Pannaraj et al 2017 (USA(CA/FL)) 0.37 0.1492 0.37 [0.08; 0.67] 13.3% 17.2%
Sordillo et al 2017 (USA(CA/MA/MO)) 0.77 0.1971 0.77 [0.38; 1.15] 7.6% 11.0%
Thompson et al 2015 (USA(NC)) 0.30 0.4239 0.30 [−0.53; 1.13] 1.6% 2.7%
Fixed effect model 0.32 [0.21; 0.42] 100.0% --
Random effects model 0.34 [0.20; 0.48] -- 100.0%
Heterogeneity: I^2 = 21%, τ^2 = 0.0074, p = 0.27
−1
−0.5
0
0.5
1

c


Trend across EBF, non-EBF, non-BF
Standardized diversity difference
Study
DD
SE
DD
95%−CI
Weight (fixed)
Weight (random)
Subramanian et al 2014 (Bangladesh) 0.21 0.0658 0.21 [0.08; 0.34] 39.0% 25.8%
Azad et al 2015 (Canada) 0.59 0.0877 0.59 [0.42; 0.76] 21.9% 23.8%
Pannaraj et al 2017 (USA(CA_FL)) 0.26 0.1120 0.26 [0.04; 0.48] 13.5% 21.4%
Sordillo et al 2017 (USA(CA_MA_MO)) 0.57 0.0828 0.57 [0.40; 0.73] 24.6% 24.2%
Thompson et al 2015 (USA(NC)) −0.02 0.4198 −0.02 [−0.84; 0.80] 1.0% 4.8%
Fixed effect model 0.39 [0.31; 0.47] 100.0% --
Random effects model 0.39 [0.19; 0.58] -- 100.0%
Heterogeneity: I^2 = 79%, τ^2 = 0.0355, p < 0.01
−0.5
0
0.5

d


Non-EBF vs. EBF
Shannon
PD _whole_tree
Observed_species
Chao1
0
0.2
0.4
Pooled standardized diversity difference

e

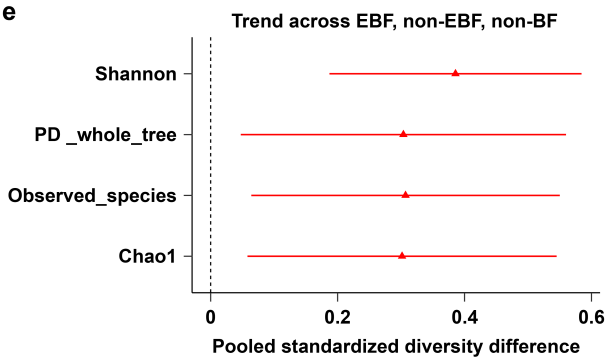

Trend across EBF, non-EBF, non-BF
Shannon
PD _whole_tree
Observed_species
Chao1
0
0.2
0.4
0.6
Pooled standardized diversity difference

Figure 2. Meta-analysis: the effects of non-exclusive vs. exclusive breastfeeding on gut microbiota age in infants ≤ 6 months of age.

a: Gut (standardized) microbiota age of infants ≤ 6 months of age by breastfeeding status by age of infants at stool sample collection from each of seven included studies. Fitted lines and 95% confidence intervals (95% CI) were from Generalized Additive Mixed models (GAMM).

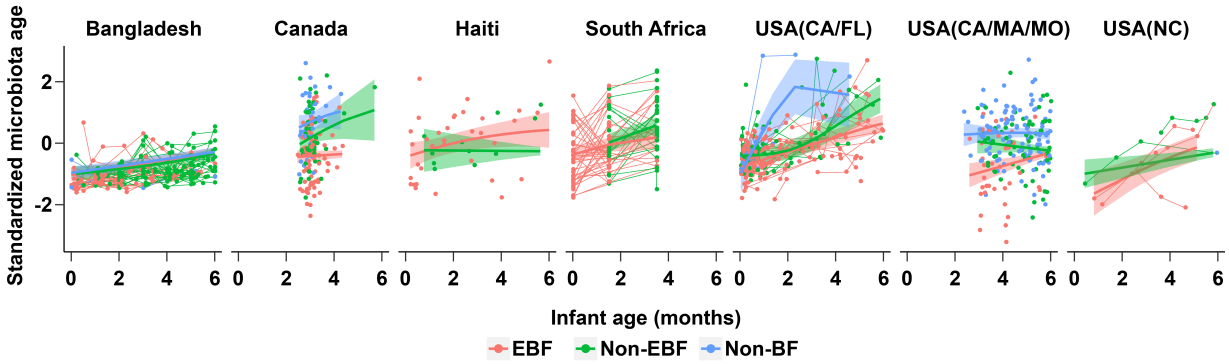
b: The difference in gut (standardized) microbiota age between non-exclusively breastfed (non-EBF) vs. EBF infants ≤ 6 months of age from each study and the pooled effect across seven included studies (meta-analysis).

c: The trend of gut (standardized) microbiota age across EBF, non-EBF and non-BF infants ≤ 6 months of age from each study and the pooled effect across five included studies (meta-analysis). The Haiti and South Africa studies were not included as there was no non-BF group in these two studies. In each study, to test for trend across breastfeeding categories, breastfeeding was coded as a continuous variable in the model (EBF=1, non-EBF=2 and non-BF=3).

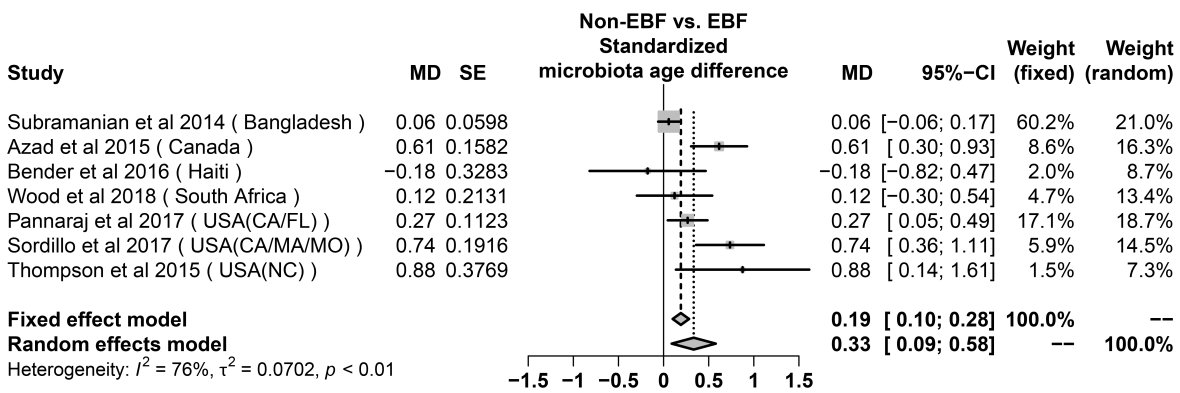
Estimates for (standardized) microbiota age difference or trend and corresponding standard error from each study were from linear mixed effect models (for longitudinal data) or linear models (for non-longitudinal data) and were adjusted for age of infants at sample collection.

EBF: exclusive breastfeeding; non-EBF: non-exclusive breastfeeding; non-BF: no breastfeeding; USA: United States of America; CA: California; FL: Florida; MA: Massachusetts; MO: Missouri; NC: North Carolina; MD: Microbiota age difference; SE: Standard error.

a


Bangladesh
Canada
Haiti
South Africa
USA(CA/FL)
USA(CA/MA/MO)
USA(NC)
Standardized microbiota age
Infant age (months)
EBF
Non-EBF
Non-BF

b


Non-EBF vs. EBF
Standardized microbiota age difference
Study MD SE MD 95%−CI Weight (fixed) Weight (random)
Subramanian et al 2014 (Bangladesh) 0.06 0.0598 0.06 [−0.06; 0.17] 60.2% 21.0%
Azad et al 2015 (Canada) 0.61 0.1582 0.61 [0.30; 0.93] 8.6% 16.3%
Bender et al 2016 (Haiti) −0.18 0.3283 −0.18 [−0.82; 0.47] 2.0% 8.7%
Wood et al 2018 (South Africa) 0.12 0.2131 0.12 [−0.30; 0.54] 4.7% 13.4%
Pannaraj et al 2017 (USA(CA/FL)) 0.27 0.1123 0.27 [0.05; 0.49] 17.1% 18.7%
Sordillo et al 2017 (USA(CA/MA/MO)) 0.74 0.1916 0.74 [0.36; 1.11] 5.9% 14.5%
Thompson et al 2015 (USA(NC)) 0.88 0.3769 0.88 [0.14; 1.61] 1.5% 7.3%
Fixed effect model 0.19 [0.10; 0.28] 100.0% --
Random effects model 0.33 [0.09; 0.58] -- 100.0%
Heterogeneity: I^2 = 76%, τ^2 = 0.0702, p < 0.01
−1.5 −1 −0.5 0 0.5 1 1.5

c

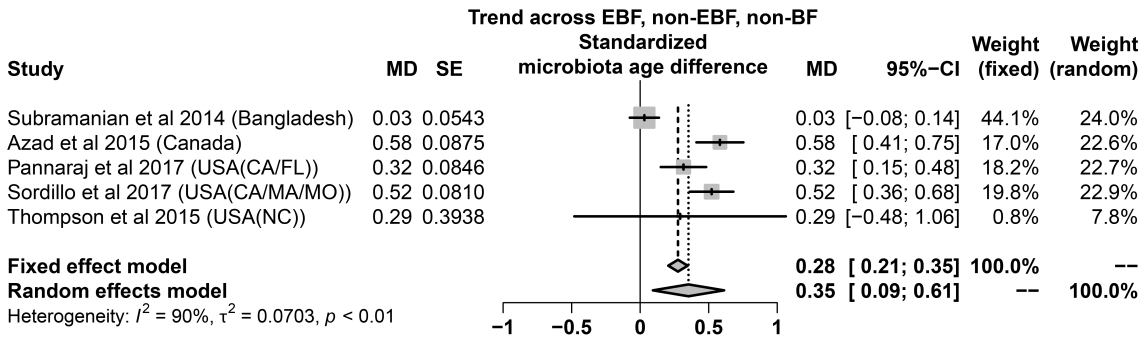

Trend across EBF, non-EBF, non-BF
Standardized microbiota age difference
Study MD SE MD 95%−CI Weight (fixed) Weight (random)
Subramanian et al 2014 (Bangladesh) 0.03 0.0543 0.03 [−0.08; 0.14] 44.1% 24.0%
Azad et al 2015 (Canada) 0.58 0.0875 0.58 [0.41; 0.75] 17.0% 22.6%
Pannaraj et al 2017 (USA(CA/FL)) 0.32 0.0846 0.32 [0.15; 0.48] 18.2% 22.7%
Sordillo et al 2017 (USA(CA/MA/MO)) 0.52 0.0810 0.52 [0.36; 0.68] 19.8% 22.9%
Thompson et al 2015 (USA(NC)) 0.29 0.3938 0.29 [−0.48; 1.06] 0.8% 7.8%
Fixed effect model 0.28 [0.21; 0.35] 100.0% --
Random effects model 0.35 [0.09; 0.61] -- 100.0%
Heterogeneity: I^2 = 90%, τ^2 = 0.0703, p < 0.01
−1 −0.5 0 0.5 1

Figure 3. Meta-analysis: the effects of non-exclusive vs. exclusive breastfeeding on relative abundances of gut bacterial taxa in infants ≤ 6 months of age.

a: Gut bacterial phyla: heatmap of log(odds ratio) (log(OR)) of relative abundances of all gut bacterial phyla between non-exclusively breastfed (non-EBF) vs. exclusively breastfed (EBF) infants for each study and pooled estimates across all seven studies with 95% confidence intervals (95% CI) (forest plot).

b: Gut bacterial families: heatmap of log(OR) of relative abundances of all gut bacterial families between non-EBF vs. EBF infants for each study and pooled estimates across all seven studies with 95% CI (forest plot).

All log(OR) estimates of each bacterial taxa from each study were from Generalized Additive Models for Location Scale and Shape (GAMLSS) with zero inflated beta family (BEZI) and were adjusted for age of infants at sample collection. Pooled log(OR) estimates and 95% CI (forest plot) were from random effect meta-analysis models based on the adjusted log(OR) estimates and corresponding standard errors of all included studies. Pooled log(OR) estimates with pooled p-values<0.05 are in red and those with false discovery rate (FDR) adjusted pooled p-values <0.1 are in triangle shape. Missing (unavailable) values are in white. Bacterial families with unassigned name are numbered.

USA: United States of America; CA: California; FL: Florida; MA: Massachusetts; MO: Missouri; NC: North Carolina.

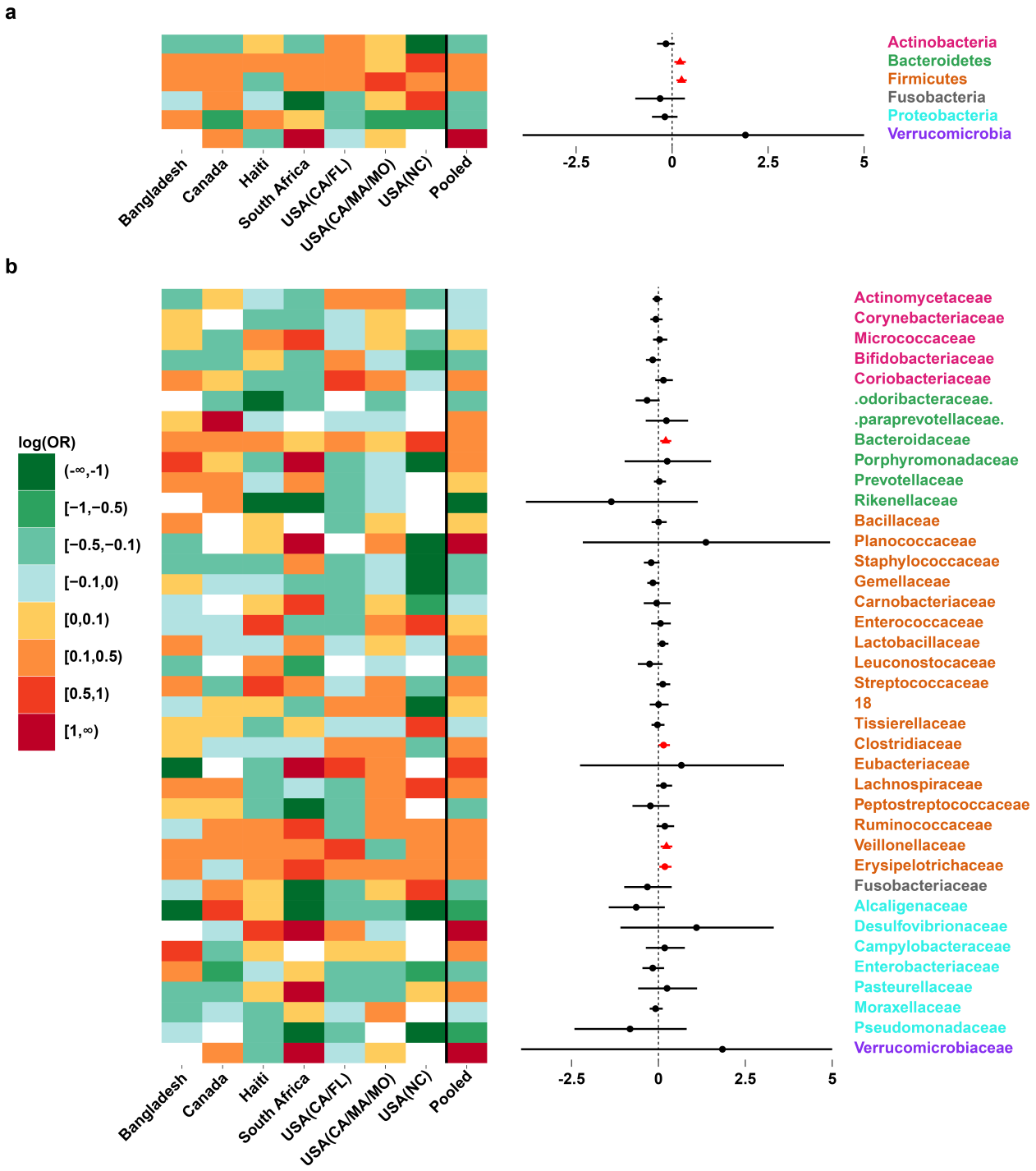

a
Bangladesh
Canada
Haiti
South Africa
USA(CA/FL)
USA(CA/MA/MO)
USA(NC)
Pooled
-2.5
0
2.5
5
Actinobacteria
Bacteroidetes
Firmicutes
Fusobacteria
Proteobacteria
Verrucomicrobia
b
log(OR)
(-∞,-1)
[−1,−0.5)
[−0.5,−0.1)
[−0.1,0)
[0,0.1)
[0.1,0.5)
[0.5,1)
[1,∞)
Actinomycetaceae
Corynebacteriaceae
Micrococcaceae
Bifidobacteriaceae
Coriobacteriaceae
.odoribacteraceae.
.paraprevotellaceae.
Bacteroidaceae
Porphyromonadaceae
Prevotellaceae
Rikenellaceae
Bacillaceae
Planococcaceae
Staphylococcaceae
Gemellaceae
Carnobacteriaceae
Enterococcaceae
Lactobacillaceae
Leuconostocaceae
Streptococcaceae
18
Tissierellaceae
Clostridiaceae
Eubacteriaceae
Lachnospiraceae
Peptostreptococcaceae
Ruminococcaceae
Veillonellaceae
Erysipelotrichaceae
Fusobacteriaceae
Alcaligenaceae
Desulfovibrionaceae
Campylobacteraceae
Enterobacteriaceae
Pasteurellaceae
Moraxellaceae
Pseudomonadaceae
Verrucomicrobiaceae

Figure 4. Meta-analysis: the effects of non-exclusive vs. exclusive breastfeeding on relative abundances of gut microbial KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways in infants ≤ 6 months of age.

a: Meta-analysis of all infants in all seven included studies: heatmap of log(odds ratio) (log(OR)) of relative abundances of gut microbial KEGG pathways at level 3 between non-exclusively breastfed (non-EBF) vs. exclusively breastfed (EBF) infants for each study and pooled estimates of all seven studies with 95% confidence intervals (forest plot).

b: Meta-analysis of vaginally born infants in four studies: heatmap of log(OR) of relative abundances of gut microbial KEGG pathways at level 3 between non-EBF vs. EBF infants for each study and pooled estimates of four studies with 95% confidence intervals (forest plot). Only four studies with available birth mode information (Canada, Haiti, USA(CA_MA_MO) and USA(CA_FL)) are included.

c: Meta-analysis of C-section born infants in four studies: heatmap of log(OR) of relative abundances of gut microbial KEGG pathways at level 3 between non-EBF vs. EBF infants for each study and pooled estimates of four studies with 95% confidence intervals (forest plot). Only four studies with available birth mode information (Canada, Haiti, USA(CA_MA_MO) and USA(CA_FL)) are included.

All log(OR) estimates of each pathway from each study were from Generalized Additive Models for Location Scale and Shape (GAMLSS) with zero inflated beta family (BEZI) and were adjusted for age of infants at sample collection. Pooled log(OR) estimates and 95% confidence intervals (95%CI) (forest plot) were from random effect meta-analysis models based on the adjusted log(OR) estimates and corresponding standard errors of all included studies. Pooled log(OR) estimates with pooled p-values<0.05 are in red and those with false discovery rate

(FDR) adjusted pooled p-values <0.1 are in triangle shape. Only pathways with FDR adjusted pooled p-value <0.1 are shown.

USA: United States of America; CA: California; FL: Florida; MA: Massachusetts; MO: Missouri; NC: North Carolina.

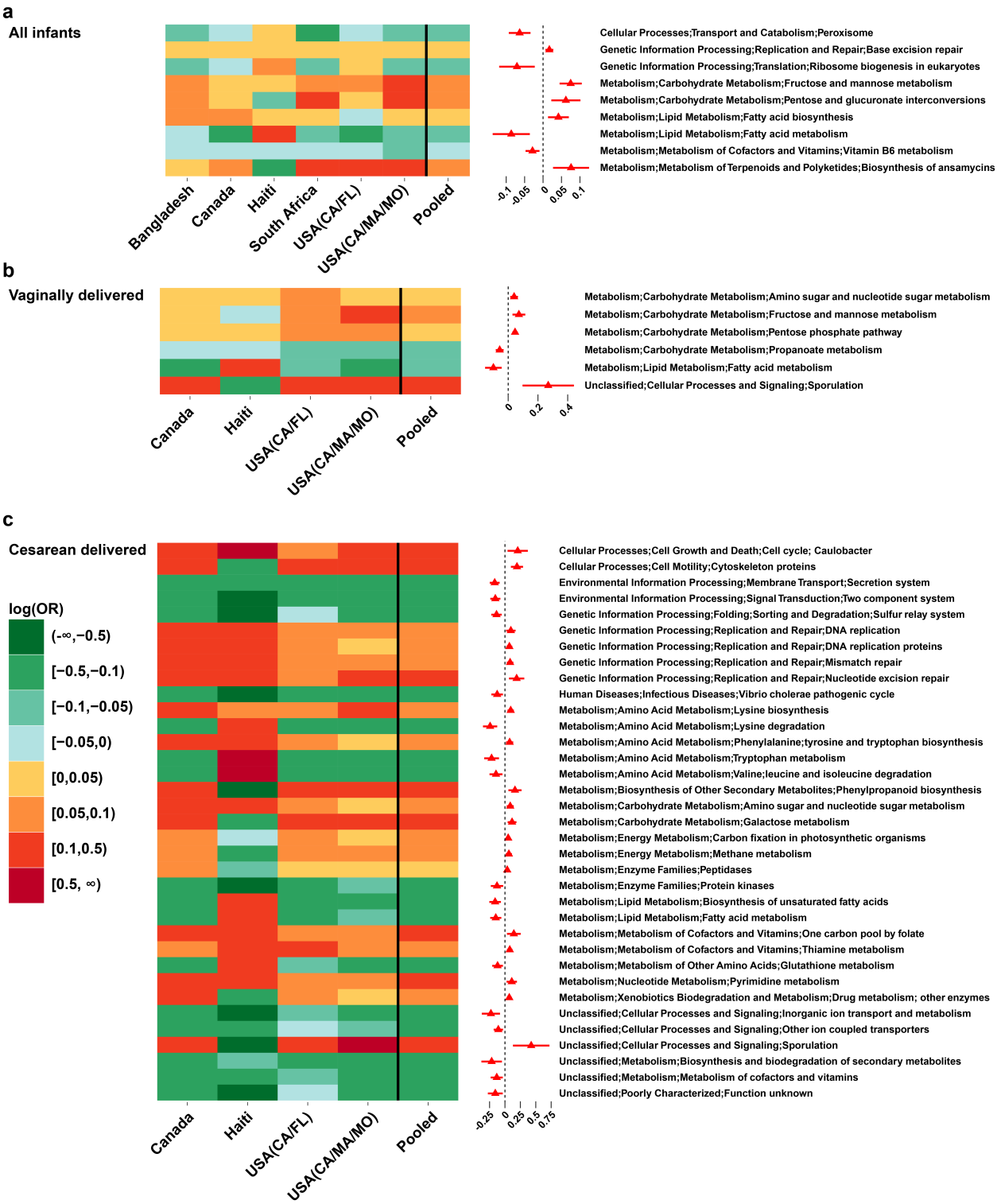

a
All infants
Bangladesh
Canada
Haiti
South Africa
USA(CA/FL)
USA(CA/MA/MO)
Pooled
Cellular Processes;Transport and Catabolism;Peroxisome
Genetic Information Processing;Replication and Repair;Base excision repair
Genetic Information Processing;Translation;Ribosome biogenesis in eukaryotes
Metabolism;Carbohydrate Metabolism;Fructose and mannose metabolism
Metabolism;Carbohydrate Metabolism;Pentose and glucuronate interconversions
Metabolism;Lipid Metabolism;Fatty acid biosynthesis
Metabolism;Lipid Metabolism;Fatty acid metabolism
Metabolism;Metabolism of Cofactors and Vitamins;Vitamin B6 metabolism
Metabolism;Metabolism of Terpenoids and Polyketides;Biosynthesis of ansamycins
-0.1
-0.05
0
0.05
0.1
b
Vaginally delivered
Canada
Haiti
USA(CA/FL)
USA(CA/MA/MO)
Pooled
Metabolism;Carbohydrate Metabolism;Amino sugar and nucleotide sugar metabolism
Metabolism;Carbohydrate Metabolism;Fructose and mannose metabolism
Metabolism;Carbohydrate Metabolism;Pentose phosphate pathway
Metabolism;Carbohydrate Metabolism;Propanoate metabolism
Metabolism;Lipid Metabolism;Fatty acid metabolism
Unclassified;Cellular Processes and Signaling;Sporulation
0
0.2
0.4
c
Cesarean delivered
log(OR)
(-∞,−0.5)
[−0.5,−0.1)
[−0.1,−0.05)
[−0.05,0)
[0,0.05)
[0.05,0.1)
[0.1,0.5)
[0.5, ∞)
Canada
Haiti
USA(CA/FL)
USA(CA/MA/MO)
Pooled
Cellular Processes;Cell Growth and Death;Cell cycle; Caulobacter
Cellular Processes;Cell Motility;Cytoskeleton proteins
Environmental Information Processing;Membrane Transport;Secretion system
Environmental Information Processing;Signal Transduction;Two component system
Genetic Information Processing;Folding;Sorting and Degradation;Sulfur relay system
Genetic Information Processing;Replication and Repair;DNA replication
Genetic Information Processing;Replication and Repair;DNA replication proteins
Genetic Information Processing;Replication and Repair;Mismatch repair
Genetic Information Processing;Replication and Repair;Nucleotide excision repair
Human Diseases;Infectious Diseases;Vibrio cholerae pathogenic cycle
Metabolism;Amino Acid Metabolism;Lysine biosynthesis
Metabolism;Amino Acid Metabolism;Lysine degradation
Metabolism;Amino Acid Metabolism;Phenylalanine;tyrosine and tryptophan biosynthesis
Metabolism;Amino Acid Metabolism;Tryptophan metabolism
Metabolism;Amino Acid Metabolism;Valine;leucine and isoleucine degradation
Metabolism;Biosynthesis of Other Secondary Metabolites;Phenylpropanoid biosynthesis
Metabolism;Carbohydrate Metabolism;Amino sugar and nucleotide sugar metabolism
Metabolism;Carbohydrate Metabolism;Galactose metabolism
Metabolism;Energy Metabolism;Carbon fixation in photosynthetic organisms
Metabolism;Energy Metabolism;Methane metabolism
Metabolism;Enzyme Families;Peptidases
Metabolism;Enzyme Families;Protein kinases
Metabolism;Lipid Metabolism;Biosynthesis of unsaturated fatty acids
Metabolism;Lipid Metabolism;Fatty acid metabolism
Metabolism;Metabolism of Cofactors and Vitamins;One carbon pool by folate
Metabolism;Metabolism of Cofactors and Vitamins;Thiamine metabolism
Metabolism;Metabolism of Other Amino Acids;Glutathione metabolism
Metabolism;Nucleotide Metabolism;Pyrimidine metabolism
Metabolism;Xenobiotics Biodegradation and Metabolism;Drug metabolism; other enzymes
Unclassified;Cellular Processes and Signaling;Inorganic ion transport and metabolism
Unclassified;Cellular Processes and Signaling;Other ion coupled transporters
Unclassified;Cellular Processes and Signaling;Sporulation
Unclassified;Metabolism;Biosynthesis and biodegradation of secondary metabolites
Unclassified;Metabolism;Metabolism of cofactors and vitamins
Unclassified;Poorly Characterized;Function unknown
-0.25
0
0.25
0.5
0.75

Figure 5. The continued effects of exclusive breastfeeding in the first 6 months of age on the infant gut microbiota up to 2 years of age.

Data from Bangladesh study only.

Upper panel: The impact of duration of exclusive breastfeeding (EBF) on the gut microbiota of infants up to 2 years of age. **a**: the impact of duration of EBF shorter than 2 months vs. longer than 2 months from birth on infant gut microbiota age. **b**: the impact of duration of EBF shorter than 2 months vs. longer than 2 months from birth on infant gut bacterial family composition. Number of infants=50 (duration of EBF≤2 months=30, duration of EBF>2 months=20). Number of samples 0-2 years of age=996 (duration of EBF≤2 months=580, duration of EBF>2 months=416)

Lower panel: EBF mitigates diarrhea-associated gut microbiota dysbiosis in infants from 6 months to 2 years of age. **c**: the effects of diarrhea (vs. no diarrhea) around the time of stool sample collection on gut microbiota age in infants with duration of EBF shorter than 2 months vs. infants with duration of EBF longer than 2 months. **d**: the effects of diarrhea (vs. no diarrhea) around the time of stool sample collection on gut microbial diversity (Shannon index) in infants with duration of EBF shorter than 2 months vs. infants with duration of EBF longer than 2 months. **e**: the effects of diarrhea (vs. no diarrhea) on bacterial taxa composition at the family level around the time of stool sample collection in infants with duration of EBF shorter than 2 months vs. infants with duration of EBF longer than 2 months. **f**: the effects of diarrhea (vs. no diarrhea) on bacterial taxa composition at the family level around the time of stool sample collection in infants receiving no breastfeeding at the time of diarrhea vs. infants receiving breastfeeding at the time of diarrhea.

Number of samples 6 months to 2 years of age= 674 (duration of EBF ≤2 months= 378 (diarrhea=29, no diarrhea=349); duration of EBF >2 months=296 (diarrhea=19, no diarrhea=277; with breastfeeding=616 (diarrhea=45, no diarrhea=571); without breastfeeding=44 (diarrhea=2, no diarrhea=42)).

Fitted lines and 95% confidence intervals (95%CI) were from Generalized Additive Mixed models (GAMM). Dashed lines demarcate time periods tested. * indicate statistical significance.

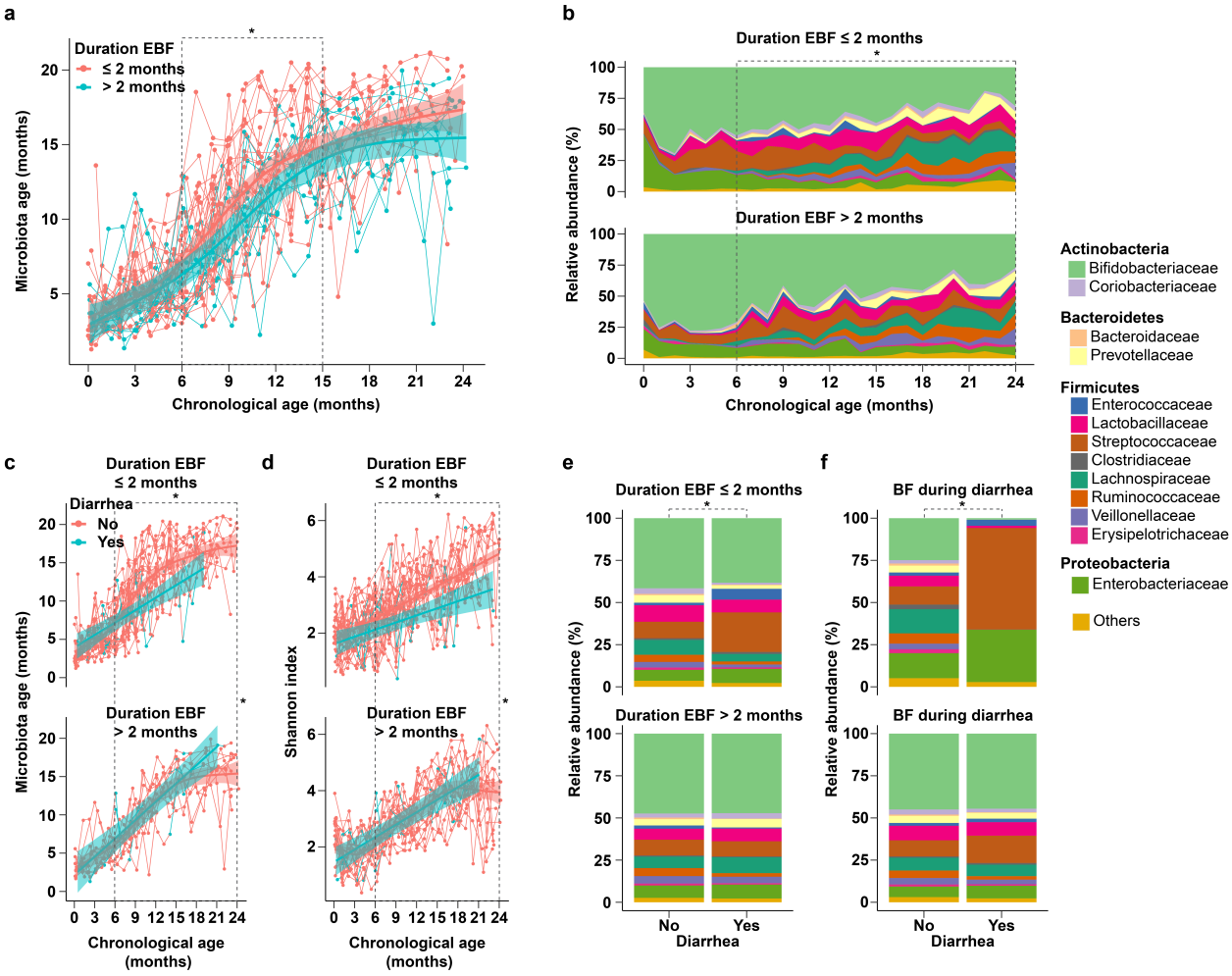


Table 1. Summary of included studies.

Data origin (study population), reference	Study design, sample size (≤6 months of age)	Available breastfeeding categories and definition (used in this paper), number of samples (n)	Target region of 16S rRNA genes /sequence platform	Results about the effects of breastfeeding on infant gut microbiome and statistical methods used in published paper	Starting files used and data processing done in this project	Note
Bangladesh (Subramanian et al. 2014) #*	Longitudinal monthly stool sample collection during the first 2 years after birth of 50 healthy Bangladeshi children (25 singletons, 11 twin pairs, 1 set of triplets). Number of samples ≤6 months of age: 322	Three categories: (at the time of sample collection). - EBF: fed breast milk without formula or solid food (n=138) - Non-EBF: fed breast milk plus either formula or solid (n=178) - Non-BF: fed formula or solid food without breast milk (n=6)	V4 /Illumina MiSeq	No analysis on breastfeeding status	Assembled 16S reads used for OTU picking (.fna file), mapping and meta-data files. - Open OTU picking with UCLUST with 97% similarity using the Greengenes database (version 13.8)	Total number of all samples from birth to 2 years of age =996 (EBF=152, non-EBF=794, non-BF=50)
Canada (Azad et al. 2015)#$	One time sample collection of 167 infants around 3 months of life (a subset of the Canadian Healthy Infant Longitudinal Development (CHILD) national population-based birth cohort). Number of samples ≤6 months of age: 167	Three categories: (at the time of sample collection) - EBF: fed breast milk without formula or solid food (n=86 (vaginally born=68, C-section born=18)) - Non-EBF: fed breast milk plus either formula or solid (n=48 (vaginally born=36, C-section born=12)) - Non-BF: fed formula or solid food without breast milk (n=33 (vaginally born=26, C-section born=7)))	V4/Illumina MiSeq	- diversity (Chao1, Shannon): trend test significant (EBF<Non-EBF<non-BF) - composition: (Kruskal–Wallis test): EBF>Non-EBF>non-BF: Proteobacteria, Actinobacteria; EBF<Non-EBF<non-BF: Bacteroidetes, Firmicutes (Clostridiales)	- Bacterial taxa relative abundance summary tables from phylum to genus levels; alpha diversity summary tables; predicted KEGG pathway abundance summary tables; metadata file. (Prior sequence data processing was done by the collaborative group using similar procedures as of this project).	The effect of infant age on breastfeeding status and gut microbiome was not accounted in the analysis (relatively accounted by study design (stool sample collection at similar age))
Haiti (Bender et al. 2016)$	One time stool sample collection of 48 HIV negative infants with age varied from 0 to 6 months whose mothers were HIV negative (n=25) or HIV positive (n=23). Number of samples ≤6 months of age: 48	Two categories: (at the time of sample collection) - exclusive breastfeeding (EBF): fed only breast milk (n=37 (vaginally born=32, C-section born=5)) - non-EBF: fed breast milk plus anything other than breast milk (n=11 (vaginally born=10, C-section born=1))	V4 /Illumina MiSeq	PERMANNOVA test: no significant difference between EBF and non-EBF	- Assembled 16S reads used for OUT picking (.fna file), mapping and meta-data files. - Open reference OTU picking with UCLUST with 97% similarity using the Greengenes database (version 13.8)	The effect of infant age on breastfeeding status and gut microbiome was not accounted in the analysis (relatively accounted by study design(stool sample collection at similar age))

South Africa (Wood et al. 2018)	Longitudinal stool sample collection of 72 healthy infants of HIV negative mothers at birth, 6 and 14 weeks Number of samples ≤6 months of age: 143	Two categories: (at the time of sample collection) - EBF: fed breast milk, exclusively, except for prescribed medicine (n=86) - non-EBF: fed breast milk plus any other foods, including traditional medicines and water (n=57)	V4 /Illumina MiSeq	- Alpha diversity (Chao1): non-EBF >EBF (Wilcoxon's test) - Bacterial composition (DESeq2 package (negative binomial generalized linear models)): - EBF > non-EBF: *S. lactarius*, *Actinomyces*, *Atopobium* - non-EBF > EBF: *Streptococcus. luteciae*, Bacteroides (OTU)	- Assembled raw FASTQ file. - Converting FASTQ file to FASTA and QUAL files. Open reference OTU picking with UCLUST with 97% similarity using the Greengenes database (version 13.8)	The effect of infant age on breastfeeding status and gut microbiome was accounted by study design (stool sample collection at the same age)
USA (California and Florida) (Pannaraj et al. 2017)#$	Longitudinal stool sample collection of 113 healthy full-term infants at 0 to 7 days, 8 to 30 days, 31 to 90 days, 91 to 180 days. Number of samples ≤6 months of age =230	Three categories: (at the time of sample collection) - EBF: fed only breast milk (n=150 (vaginally born=99, C-section born=48)) - non-EBF: fed breast milk plus either formula or solid (n=68 (vaginally born=53, C-section born=15)) - Non-BF: fed formula and/or solid without breast milk (n=12 (vaginally born=9, C-section born=2)) 9 samples with unknown feeding category; 4 samples with unknown birth mode	V4 /Illumina MiSeq	Bacterial composition (Random Forest model): - EBF > non-EBF: Proteobacteria, Bacteroides. - non-EBF >EBF: Firmicutes, Actinobacteria	- Assembled 16S reads used for OTU picking (.fna file), mapping and meta-data files. - Open reference OTU picking with UCLUST with 97% similarity using the Greengenes database (version 13.8)	The effect of infant age on breastfeeding status and gut microbiome was not accounted in the analysis
USA (Massachusetts, Missouri, and California) (Sordillo et al. 2017)#$	One time stool sample collection of 228 infants at age 3 to 6 months, who were enrolled in Vitamin D Antenatal Asthma Reduction Trial (VDAART), a clinical trial of vitamin D supplementation in pregnancy to prevent asthma and allergies in offspring. Number of samples ≤6 months of age: 220	Three categories: (at the time of sample collection) - EBF: fed breast milk without formula or solid food (n=38 (vaginally born=28, C-section born=10)) - Non-EBF: fed breast milk plus either formula or solid (n=66 (vaginally born=45, C-section born=21))) - Non-BF: fed formula or solid food without breast milk (n=116 (vaginally born=72, C-section born=44))) 8 samples with unknown feeding category	V3-V5 /Pyrosequencing (Roche 454 Titanium)	No direct comparison between EBF and non-EBF (EBF and non-EBF was compared with formula fed)	- Bacterial taxa relative abundance summary tables from phylum to genus levels; alpha diversity summary tables of different rarefaction depth; predicted KEGG pathway abundance summary tables; metadata file. (Prior sequence data processing was done by the collaborative group using similar procedures as of this project).	The effect of infant age on gut microbiome was accounted in the analysis
USA (North Carolina) (Thompson et al. 2015)#	Longitudinal stool sample collection of 6 healthy full term infants with varied age. Number of samples ≤6 months of age: 21	Three categories: (at the time of sample collection) - EBF: fed breastmilk without formula or solid food (n=12) - Non-EBF: fed breastmilk plus either formula or solid (n=8)	V1-2 /Roche GS FLX Titanium	(non-parametric test ANOSIM (Analysis of Similarities)) - Trend in species richness and diversity: EBF <non-EBF<Non-BF Relative abundance: - Bacterial composition: EBF	Assembled 16S reads used for otu-picking (.fna file), mapping file. - Open OTU picking with UCLUST with 97% similarity using the	The effect of infant age on breastfeeding status and gut microbiome was not accounted

	- Non-BF: fed formula or solid food without breast milk (n=1)	>non- EBF: Actinobacteria (phylum), *Bifidobacterium* (genus) - non- EBF >EBF : Bacteroidetes (phylum), Clostridiales (order), Lachnospiraceae (family), *Blautia* and *Faecalibacterium* (genus)	Greengenes database (version 13.8)	(neither in the design nor in analysis).

Studies with three breastfeeding categories (exclusive breastfeeding (EBF), non-exclusive breastfeeding (non-EBF), non-breastfeeding (non-BF)) used for trend tests across three categories.

$ Studies with available birth mode information used for meta-analysis stratified by birth mode.

* This study contains data from 6 months to 2 years of age, which was used for the analysis from 6 months to 2 years of age. Data from this study was downloaded from the authors' website:

https://gordonlab.wustl.edu/Subramanian_6_14/Nature_2014_Processed_16S_rRNA_datasets.html. Data from six other studies were obtained directly from the investigators.