Effect of feeding methods on intestinal microbiota of Chinese infants: abridged secondary publication

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KEY MESSAGES

1. Breastmilk-fed infants have fewer pathogenic bacteria and more beneficial bacteria in intestinal microbiota than formula-fed infants.
2. Direct and expressed breast milk feeding results in significantly fewer pathogenic bacteria in infants at 6 weeks of age.
3. Breastfeeding regardless of feeding mode (direct or expressed) is a modifiable factor that affects the infant gut microbiome and has potential short-term and long-term consequences for infant health later in life.

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Introduction

Infant gut microbiota plays an important role in intestinal homeostasis, development of the immune system, and protection against pathogens.[1] Bifidobacteria and Lactobacilli are the most important health-beneficial bacteria, whereas staphylococci and clostridia are potential pathogenic bacteria.[2] Disruption of the intestinal microbiota is associated with inflammatory bowel disease, necrotising enterocolitis, diabetes, obesity, cancer, allergies, and asthma.[3]

Breastmilk promotes a healthy gut microbiota by providing selective metabolic substrates for beneficial bacteria.[4] Exclusively breast-fed infants have more beneficial bacteria (such as Bifidobacterium and Lactobacillus) and fewer pathogenic bacteria, compared with formula milk–fed infants.[5] Nonetheless, infants in different geographical regions with different ethnic background may possess different types of gut microbiome profile. Studies from Asia or Chinese populations are limited.

There is a growing trend in expressed breast milk feeding among mothers of healthy term infants in developed countries. In Hong Kong, 84.6% of mothers had given expressed breast milk at some point; 14.6% and 20% of mothers had fed expressed breast milk within 1.5 and 3 months after birth, respectively.

The intestinal microbiota plays a critical role in infant development, and the type of feeding and mode of delivery may cause perturbations of microbial profiles. This study aims to compare infant microbiota profile between those fed directly at the breast, those fed expressed breast milk, and those fed formula milk. Methods of breast milk feeding may have short-term and long-term consequences for infant health later in life.

Methods

A total of 218 women with singleton pregnancies were recruited between August 2018 and December 2019. Recruitment was halted since January 2020 because home visits for stool collection was stopped during the COVID-19 pandemic. Participants were excluded if their infants were <37 weeks of gestation, had an Apgar score of <8 at 5 minutes, had a birthweight of <2500 g, had any severe medical conditions or congenital malformations, had been in special care unit for >48 hours, had been in neonatal intensive care unit, received a prescribed antibiotic, or had pre-existing gastrointestinal and immunodeficiency disease.

Infant feeding was recorded by a standardised questionnaire at recruitment and before the scheduled home visit at 6 weeks. Infant feeding was classified as direct breastfeeding, expressed milk feeding (>70% of feeding is expressed breastmilk), and formula milk feeding.

Infant stools were collected at a scheduled home visit when infants reached 6 weeks of age. Participants were instructed to place the diaper

with stool sample in a collection bag and keep it in a refrigerator until collection by trained research assistant using a container and a spoon. Containers were labelled with a unique number and date, time, and place of collection and placed in a cooler. The sample was immediately transported to laboratory with ice blocks and frozen at -80°C.

Total genomic DNA was extracted from 200 mg of faecal sample using QIAamp PowerFecal DNA Kit (Qiagen) according to the manufacturer instructions. The genomic DNA and its quality were quantified and checked. The 16S DNA library was prepared based on the Illumina protocol for 16S Metagenomic Sequencing Library Preparation. The 16S amplicons were generated using primers that span the hypervariable regions V3-V4 of the bacterial 16S rRNA gene with overhang adapters attached, with 25 cycles of polymerase chain reaction (PCR). Five μL of amplicon from each sample was used to generate indexed library using Nextera XT Index Kit v2, with eight cycles of PCR. These enriched libraries were validated by Qubit and quantitative PCR for quality control analysis. The indexed libraries were pooled in equimolar amounts. The pooled library was then denatured and diluted to the optimal loading concentration. Sequencing was performed using the MiSeq PE300 platform.

Using QIIME2 version 2019.7, reads were assembled, demultiplexed, and filtered against the SILVA reference database release 132. Chimeric filtering was performed using DADA2. For subsequent analyses, data were rarefied to 28 020 sequences per sample. Microbiota alpha diversity was assessed using Chao1, abundance-based coverage estimator (ACE), and observed amplicon sequence variants (ASVs) indices for species richness, whereas the Shannon index, Simpson index, and Faith's phylogenetic diversity (PD) were used as diversity estimation. Intestinal microbiota of infants were compared between the three feeding status groups using non-parametric Kruskal–Wallis test and Wilcoxon rank-sum test. Microbiota community structures were compared using permutational analysis of variance on unweighted unique fraction (UniFrac) distance and weighted UniFrac distance matrices with 9999 permutations. Beta diversity distances were visualised using principal coordinate analysis. Differentially abundant genera in breastfed, expressed milk–fed, and formula milk–fed samples were tested using the analysis of composition of microbiomes. The percentage of Bifidobacterium and differentially abundant taxa were compared using the Kruskal–Wallis test and Wilcoxon rank-sum test.

Results

As of December 2019, 218 women were recruited and their infants were classified as directly breastfed (n=84), expressed milk fed (n=50), and formula milk fed (n=84). There was significant difference between the three groups in terms of maternal age, maternal education, and family income, intention to return to work post-partum, partner's infant feeding preference, and mode of birth (Table). There was no significant difference between the three groups in terms of parity and length of residence.

In alpha diversity analyses, compared with breastfed infants, formula milk–fed infants had a higher Chao1 (55.0 vs 40.0, P=8.3e-10), ACE (55.0 vs 40.0, P=8.6e-10), and observed ASVs (53 vs 39, P=4.7e-09) abundance in gut microbiota. This indicates higher species richness in formula milk–fed infants. A similar difference of species abundance was found between the formula milk group and the expressed milk group in terms of Chao1 (55.0 vs 43.5, P=1.12e-05), ACE (55.0 vs 43.5, P=1.15e-05), and observed ASVs (53 vs 41, P=4e-05). However, there was no significant difference in species abundance between breastfed infants and expressed milk–fed infants in terms of Chao1 (40.0 vs 43.5, P=0.33), ACE (40.0 vs 43.5, P=0.34), and observed ASVs (39 vs 41, P=0.32).

Shannon index, Simpson index, and Faith's PD were used to determine the microbial diversity. The species diversity was significantly higher in formula milk–fed infants than in breastfed infants (Shannon index: P=0.0137, q=0.021; Simpson index: P=0.1020, q=0.1525; Faith's PD: P=0.0002, q=0.0005) and expressed milk–fed infants (P=0.0044, q=0.0132; P=0.0175, q=0.0522; and P=0.0156, q=0.023, respectively), but there was no significant difference between breastfed and expressed milk–fed infants (P=0.6048, q=0.6; P=0.3758, q=0.38; and P=0.174, q=0.17, respectively).

Principal coordinate analysis was used to determine the varieties of community structure of individual samples from the three groups. The overall beta diversity of the community structure of microbiota was evaluated using weighted UniFrac and unweighted UniFrac analyses. Both beta diversity indices indicated that the structure of microbiota significantly differed: weighted UniFrac (overall P=0.0011) and unweighted UniFrac (overall P=0.0001). In pairwise comparisons of the three groups using weighted UniFrac, formula milk–fed infants were strongly associated with a distinct community structure from breastfed infants (pseudo F=4.0, P=0.0038, q=0.0057) or expressed milk–fed infants (pseudo F=4.7, P=0.0005, q=0.0015). There was no significant difference between breastfed infants and expressed milk–fed infants using weighted UniFrac (pseudo F=1.2, P=0.2911, q=0.2911); this indicates that both feeding methods resulted in similar relative abundance of microbiota.

At the phylum level, >90% of the bacterial sequences were assigned to Proteobacteria,

TABLE. Characteristics of participants

Characteristic	Total (n=218)*	Direct breastfeeding (n=84)*	Expressed breast milk feeding (n=50)*	Formula milk feeding (n=84)*	P value
Maternal age, y					0.03
18-29	51 (23.4)	12 (14.3)	11 (22.0)	28 (33.3)	
30-34	107 (49.1)	45 (53.6)	29 (58.0)	33 (39.3)	
≥35	60 (27.5)	27 (32.1)	10 (20.0)	23 (27.4)	
Maternal education					<0.001
Below university degree	128 (58.7)	41 (48.8)	21 (42.0)	66 (78.6)	
University degree or above	90 (41.3)	43 (51.2)	29 (58.0)	18 (21.4)	
Monthly family income, HK$					0.002
<20 000	31 (14.2)	12 (14.3)	3 (6.0)	16 (19.1)	
20 000-34 999	64 (29.4)	20 (23.8)	10 (20.0)	34 (40.5)	
≥35 000	123 (56.4)	52 (61.9)	37 (74.0)	34 (40.5)	
Length of residence in Hong Kong, y					0.07
<10	25 (11.5)	13 (15.5)	1 (2.00)	11 (13.1)	
≥10	58 (26.6)	24 (28.6)	11 (22.0)	23 (27.4)	
Since birth	135 (61.9)	47 (56.0)	38 (76.0)	50 (59.5)	
Intention to return to work post-partum					0.007
No	81 (37.2)	29 (34.5)	11 (22.0)	41 (48.8)	
Yes	137 (62.8)	55 (65.5)	39 (78.0)	43 (51.2)	
Parity					0.081
Primiparous	88 (40.4)	26 (31.0)	23 (46.0)	39 (46.4)	
Multiparous	130 (59.6)	58 (69.1)	27 (54.0)	45 (53.6)	
Partner's infant feeding preference					<0.001
Breastfeeding	87 (39.9)	51 (60.7)	31 (62.0)	5 (6.0)	
No preference	92 (42.2)	32 (38.1)	13 (26.0)	47 (56.0)	
Formula milk and mixed feeding	39 (17.9)	1 (1.2)	6 (12.0)	32 (38.1)	
Mode of birth					0.03
Spontaneous vaginal	157 (72.0)	65 (77.4)	41 (82.0)	51 (60.7)	
Assisted vaginal	9 (4.1)	5 (6.0)	2 (4.0)	2 (2.4)	
Planned caesarean	32 (14.7)	9 (10.7)	5 (10.0)	18 (21.4)	
Emergency caesarean	20 (9.2)	5 (6.0)	2 (4.0)	13 (15.5)	

* Data are presented as No. (%) of participants

Bacteroidetes, Firmicutes, Actinobacteria, Verrucomicrobia, Fusobacteria, Patescibacteria, and Epsilonbacteraeota. The relative abundance of Proteobacteria (41.5%±22.6%; range, 0.1%-90.8%), Bacteroidetes (19.4%±22.6%; range, 0%-83.3%), Firmicutes (19.7%±15.9%; range, 0.6%-80.2%), and Actinobacteria (18.2%±16.8%; range, 0%-87%) were the most dominant gut microbiota in the three groups of infants (Fig 1). The breastfed and expressed milk–fed infants exhibited similar relative abundance among all these phyla. However, gut microbiota that were significantly higher in formula milk–fed infants than in breastfed infants and expressed milk–fed infants were Firmicutes (21.05% vs 12.81% vs 10.8%, P<0.0005) and Verrucomicrobia (2.22% vs 0% vs 0%, P<0.05).

Given the limited association of feeding mode with the main objective taxa, analysis of composition of microbiomes was applied to test differentially abundant taxa caused by feeding practice. Two families and ten genera including *Enterobacteriaceae*, *Enterococcaceae*, *Staphylococcus*, *Haemophilus*, *Enterococcus*, *Corynebacterium 1*, *Veillonella*, *Acinetobacter*, *Serratia*, *Clostridium sensu stricto 1*, *Cutibacterium*, and *Gemella* were found to be different (Fig 2). Relative to formula milk feeding, breastfeeding and expressed milk feeding were associated with increasing relative abundance of

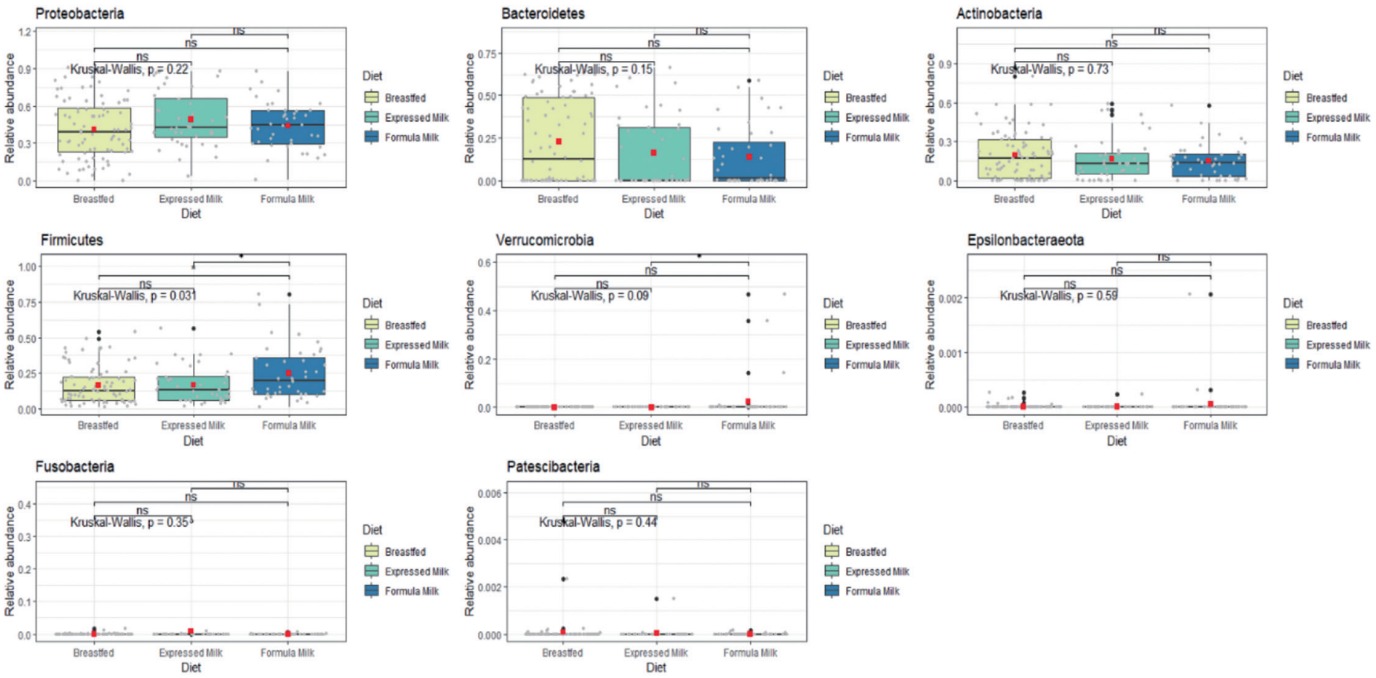

FIG 1. Relative abundance of dominant phyla across diet groups

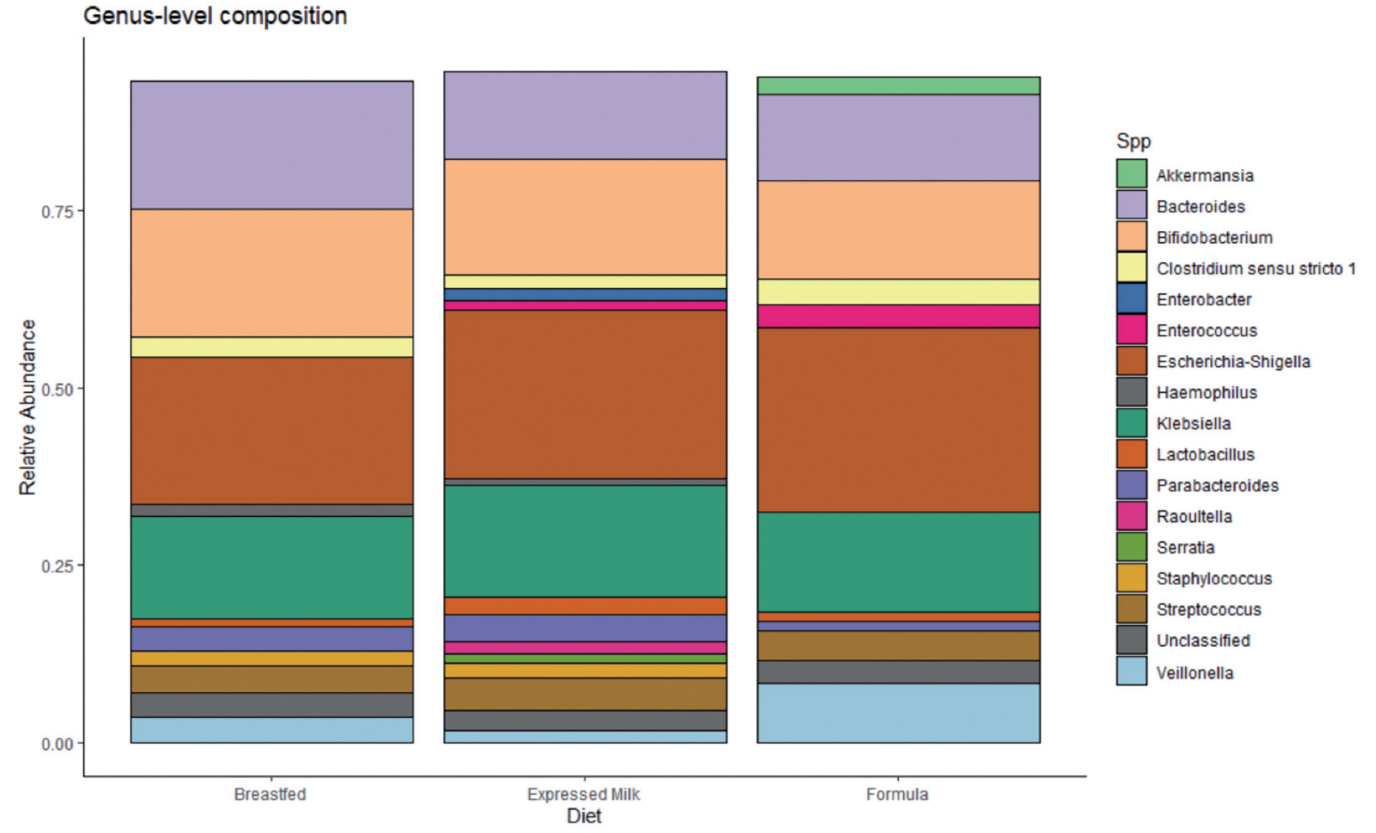

FIG 2. Relative abundance comparison of analysis of composition of microbiomes identified differentially abundant taxa

Staphylococcus (0.05% vs 0.9% vs 1.0%, P=9.10E-20). However, some decreased taxa were found in the formula milk group including *Haemophilus*, *Corynebacterium*, *Serratia*, *Cutibacterium*, and *Gemella* (Fig 2). Most notably, formula milk–fed infants had a significant increase of *Veillonella* (3.10% vs 0.51% vs 0.19%, P=3.1e-07) and *Enterococcus* (1.09% vs 0.02% vs 0.02%, P=2.37e-11) [Fig 2].

Relative to formula milk–fed infants, breastfed infants and expressed milk–fed infants showed no difference in the relative abundance of most tested genera (Fig 2). *Acinetobacter* (a type of potentially pathogenic bacteria associated with antibiotic resistance) was found more abundant in expressed milk–fed infants than breastfed infants and formula milk–fed infants (0.25% vs 0.03% vs 0.013%, P=0.0016, Fig 2).

Discussion

Most of microbiota compositions were similar in breastfed and expressed milk–fed infants. Nonetheless, there were some minor genera (*Acinetobacter* and *Serratia*) that might be associated with expressed milk feeding. *Acinetobacter* (a type of potentially pathogenic bacteria associated with antibiotic resistance) was found more abundant in expressed milk–fed infants than breastfed infants and formula milk–fed infants. Pumping breast milk may be associated with these minor genera difference. It is possible that habitual pumping or the process of storage (freezing and thawing) may alter the composition of minor genera differences in the gut microbiome. Breastmilk feeding practice does not alter the infant microbial profile regardless of feeding mode; gut microbiota of breastfed and expressed milk–fed infants is more commonly colonised by aerobic organisms, whereas that of formula milk–fed infants are enriched with anaerobic organisms such as Bacteroides and Veillonella. Future research is warranted to examine the antibiotic resistance genes between these microbial communities and to determine how they are impacted by breast milk feeding practices.

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