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Gut Microbiota Profile of Infants with Breastfeeding and Mixed Feeding Patterns

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ABSTRACT

We explore the gut microbiota profiles of 103 stool samples collected from infants at the age of 4 and 6 months in Jakarta, Indonesia. We performed 16S rRNA gene sequencing with Illumina MiSeq to identify the diversity, structure, and composition of the gut microbiota from those stool samples. Among 103 stool samples, 55 and 48 samples were collected from infants with breastfeeding and mixed feeding patterns, respectively. We found that the most abundant bacteria were *Bifidobacteriales* from the phylum of *Actinobacteria* (43.05%), *Lactobacillales* from the phylum of *Firmicutes* (28.39%), and *Enterobacterales* from the phylum of *Proteobacteria* (13.75%). The alpha and beta diversity analysis showed that the association between feeding patterns and differences in the microbial communities was not statistically significant (p-value >0.05). Our study did not show a difference in the gut microbiota pattern between the two feeding pattern groups. This result contributed to the variety of the world gut microbiota profile data in infants.

1. Introduction

More evidences which suggest that there is a relation between human health and the gut microbiota are becoming available (Valdes *et al.* 2018; Ding *et al.* 2019). Microbiota, a part of microbiome, refers to living microorganisms on the human body, which consists of bacteria, archaea, eukaryotes, and viruses (Marchesi and Ravel 2015; Berg *et al.* 2020). There is an extraordinary number of the microbiota, the same as the number of the cells in the human body, with the majority of them living in the gut (Sender *et al.* 2016).

The formation of the gut microbiota ecosystem is a complex but continuous process, affected by internal and external determinants (Chong *et al.* 2018). The gut microbiota is essential for developing the immune system, modulation of cell proliferation, and protection against pathogenic microorganisms (Jandhyala 2015). In recent years, the gut microbiota's effect on human diseases has been a popular topic in biomedical research society (Guinane and Cotter 2013). Several studies have reported that alteration of particular microbiota composition leads to several diseases such as gastrointestinal disorder, diabetes, obesity, and even cardiovascular disease (Wang *et al.* 2011; Tilg and Moschen 2014; Davis 2016). To prevent those diseases in later life, factors that affect the development of the gut microbiota since the beginning, such as diet during pregnancy, delivery mode, infant diet, environment, antibiotic use, and host genetic, need to be noted (Stewart *et al.* 2018). This early gut microbiota development is crucial in shaping immune and metabolic functions that can have lifelong effects on health (Forno *et al.* 2008; Zeissig and Blumberg 2014; Tamburini *et al.* 2016).

Indonesia prone to the risk of both communicable and non-communicable diseases. Based on the 2019 Health SGD Profile, the number of communicable diseases such as TB, HIV, Malaria, and Hepatitis remain high. The same applied to non-communicable diseases such as stunting, obesity, diabetes, and high blood pressure (WHO 2019). For that reason, studies on gut microbiota profile in Indonesian population are crucial as the results can provide important information for diseases prevention and treatment. Some of those studies use a conventional method

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such as bacterial culture, while the others used next-generation sequencing (NGS) to get complete picture of the microbiota profile (Khine *et al.* 2020; Kamil *et al.* 2021; Surono *et al.* 2021). The gut microbiome formation in early life is a complex process that has long-term implications for a human's health (Davis *et al.* 2022). Feeding patterns significant influence, the health of children in later life. Recently, it has been reported that exclusively breastfeeding in the first six months after birth is associated with optimal cognitive and language development and well-balanced microbiota (Guo *et al.* 2023). In other studies, there were differences in the abundance of several OTUs in adolescence based on the type of feeding during the first six months of life (No infant formula versus infant formula [breastmilk + formula or only formula]) (Eshriqui *et al.* 2020). Our study aims to identify the gut microbiota profiles of infants living in Jakarta, Indonesia and determine whether there is a correlation with feeding patterns.

2. Materials and Methods

2.1. Specimen Collection

A total of 103 archived stool samples, collected at 4 and 6 months of ages from children who participated in the Breastfeeding Attitude and Volume (BRAVO) study started in July 2012 were used in this study. (Savitri *et al.* 2016) Mothers who participated in the BRAVO study were involved in children's stools collection obtained in the morning on the day of an immunization visit. In this study, 55 and 48 samples were collected from infants with breastfeeding and mixed feeding patterns. We define a mixed feeding pattern as an infant who receives mixed breast and formula feeding.

This study was approved by the Institutional Review Board of the Faculty of Medicine University of Indonesia/Cipto Mangunkusumo General Hospital (reference number: 260/UN2.F1/ETIK/III/2017). Written informed consent was obtained from the parent or guardian.The samples were transported in a cool bag to the study sites for aliquoting in 1.8 ml cryotubes. The processed samples were directly stored in a -20°C degree freezer for a maximum of 7 days before it is transferred to the central laboratory and stored at a -80°C degree temperature for further analysis.

2.2. DNA Extraction and Library Preparation

DNA extraction was conducted as manufacturer instruction (DNeasy Powersoil Kit, QIAGEN, USA). Bacterial DNA concentration obtained by extraction was measured by qPCR targeting 16S gene specific for bacterial detection. The use of 16S qPCR will specifically provide bacterial DNA concentration not total DNA obtained from extraction that is important further dilution before library preparation. The library was prepared by producing amplicon of V4 region of 16S gene tagged with specific indexed forward and reverse primer for each sample using conventional PCR (Caporaso *et al.* 2011). Bacterial DNA concentration of each sample used for library preparation was 5 ng/ul. All tagged amplicons were linearized to 75 ng for each sample followed by sample pooling in the same falcon tube. Pooled DNA was purified by using AMPure Beads (Beckman Coulter, USA). The purified pooled DNA was diluted to 4 nM before being processed for denaturation and dilution prior to injection to Cartridge. The concentration of diluted purified amplicon was measured using Qubit Fluorometric quantitation (Thermo Scienctific-USA). A 4 nM pooled library was then used for pre-processing of dilution to 20 pM and denaturation prior to injection into MiSeq Illumina cartridge v2 for 500 cycles. (Illumina, n.d.) FastQ files were extracted from instrument for further data analysis.

2.3. Data Analysis

The gut microbiota profile, including alpha and beta diversity indexes, was analyzed using Quantitative Insights Into Microbial Ecology (QIIME) version 2 (Bolyen *et al.* 2019). We looked at the sequences' quality based on some randomly selected samples and then denoised the data. We used the DADA2 pipeline for detecting and correcting Illumina amplicon sequences. This process involved filtering phiX reads, chimeric sequences, and assigning taxonomic classification. The trimming parameters consist of --p-trim-left-f m / --p-trim-left-r m, which trims off the first m bases of each sequences, and --p-trunc-len-f n / --p-trunc-len-r n, which truncates each sequence at position n, to remove low quality regions of the sequences. Based on the quality plots above, we determined the trimming parameters for denoising the data. In this step we have to be careful while choosing the trimming parameters

as we need the reads to overlap when joining both forward and reverse reads. From the demux-paired-end.qzv quality plots, the quality of initial bases from both forward reverse and reads are high, so we did not trim bases from the beginning of the reads. The quality dropped off at around position 198 bases, the position which we truncated the sequences in both reads. The taxonomic classification was based on the Greengenes database, and the output of this workflow was a classification of reads at several taxonomic levels (DeSantis *et al.* 2006). Downstream data analysis was conducted with phyloseq R package (McMurdie and Holmes 2013).

3. Results

DNA extracted from stool specimens sequenced by Illumina MiSeq resulting demultiplexed paired-end sequences with the number of reads of each sample ranging from 54 to 237,499 reads. Following the filtering process, we obtained a feature table and corresponding features sequences associated with each sample. The total feature sequences generated were 812 features and distributed in 103 samples with the minimum and maximum length 203 and 254 nucleotides, respectively. We excluded samples with a total sequence read lower than 2,000. Twelve phyla were observed and showed that *Actinobacteria* (45,03%), *Firmicutes* (35,76%), and *Proteobacteria* (14,74%) were dominant (Figure 1).

There is no difference in the microbiota pattern in the phylum level between the two feeding pattern groups. *Actinobacteria, Firmicutes*, and *Proteobacteria* were reported dominant from both groups (Figure 2). Alpha diversity measures, Observed, Shannon diversity, and Phylogenetic diversity showed no significant difference in the diversity of the bacteria abundance between breastfeeding and mixed feeding groups (p-value = 0.34; 0.28; and 0.27 respectively) (Figure 3).

In addition, for beta diversity, Jaccard distance, Bray-Curtis distance, Unweighted and Weighted Unifrac distances showed no difference among feeding pattern groups as samples from different feeding patterns spread evenly without forming groups based on the feeding pattern.

Beta diversity analysis by using PERMANOVA showed that there was no association between feeding patterns and there was no significantly different (p-value > 0.05) in the microbial community. Continuing the diversity measurement, we explored the taxonomic composition of the samples. The results showed that there was a domination of *Bifidobacteriales* (43.04%), *Lactobacillales* (28.39%), and *Enterobacteriales* (13.75%) which consist in more than three-quarters of the taxonomic abundance in the samples, followed by *Clostridiales* (4.30%), *Bacillales* (2.60%), *Bacteriodales* (2.24%), and *Verrucomicrobialles* (2.18%) (Figure 4).

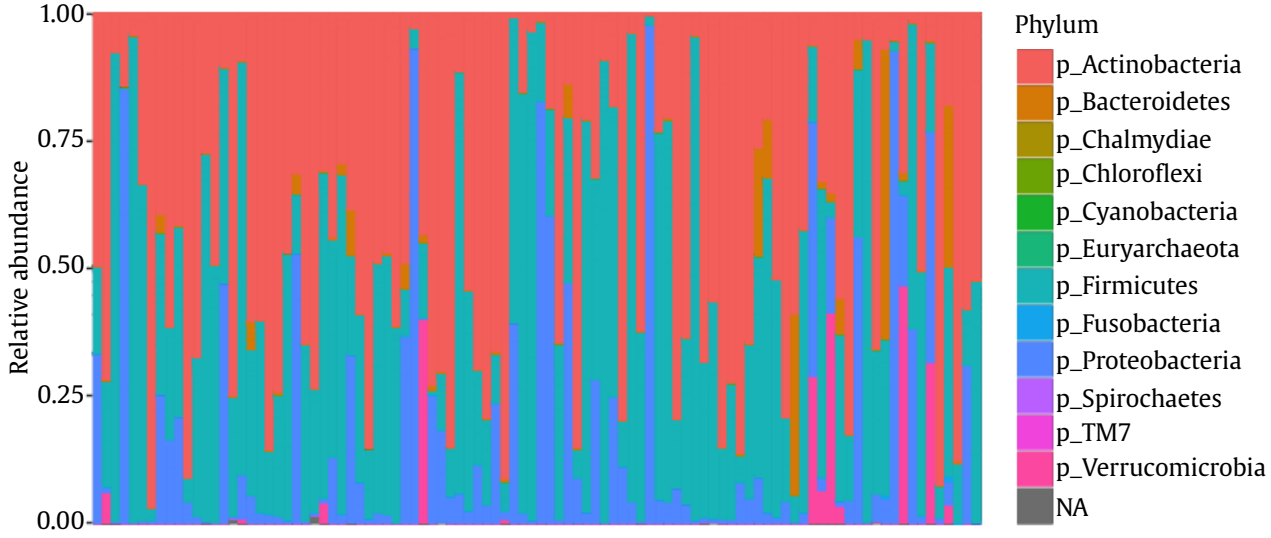

Figure 1. Relative abundance on the taxonomic phylum from all samples

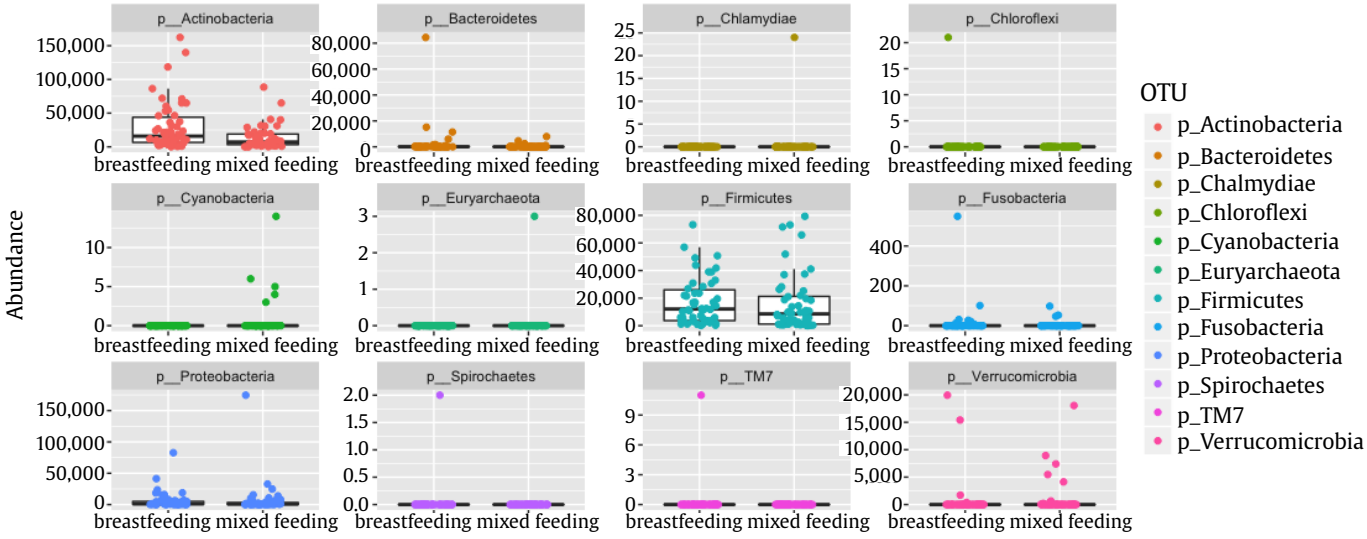

Figure 2. Abundance of the phyla observed for each feeding pattern

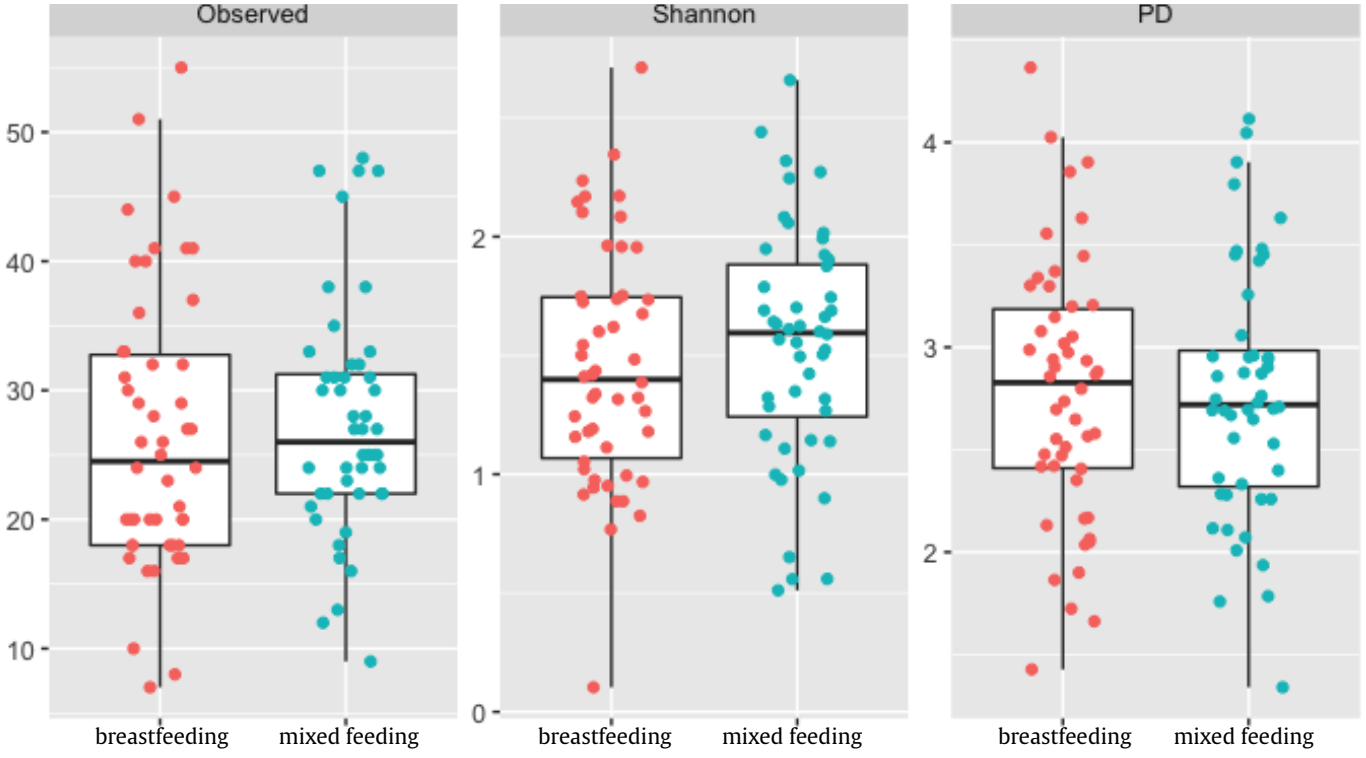

Figure 3. Alpha diversity measures between two feeding pattern groups

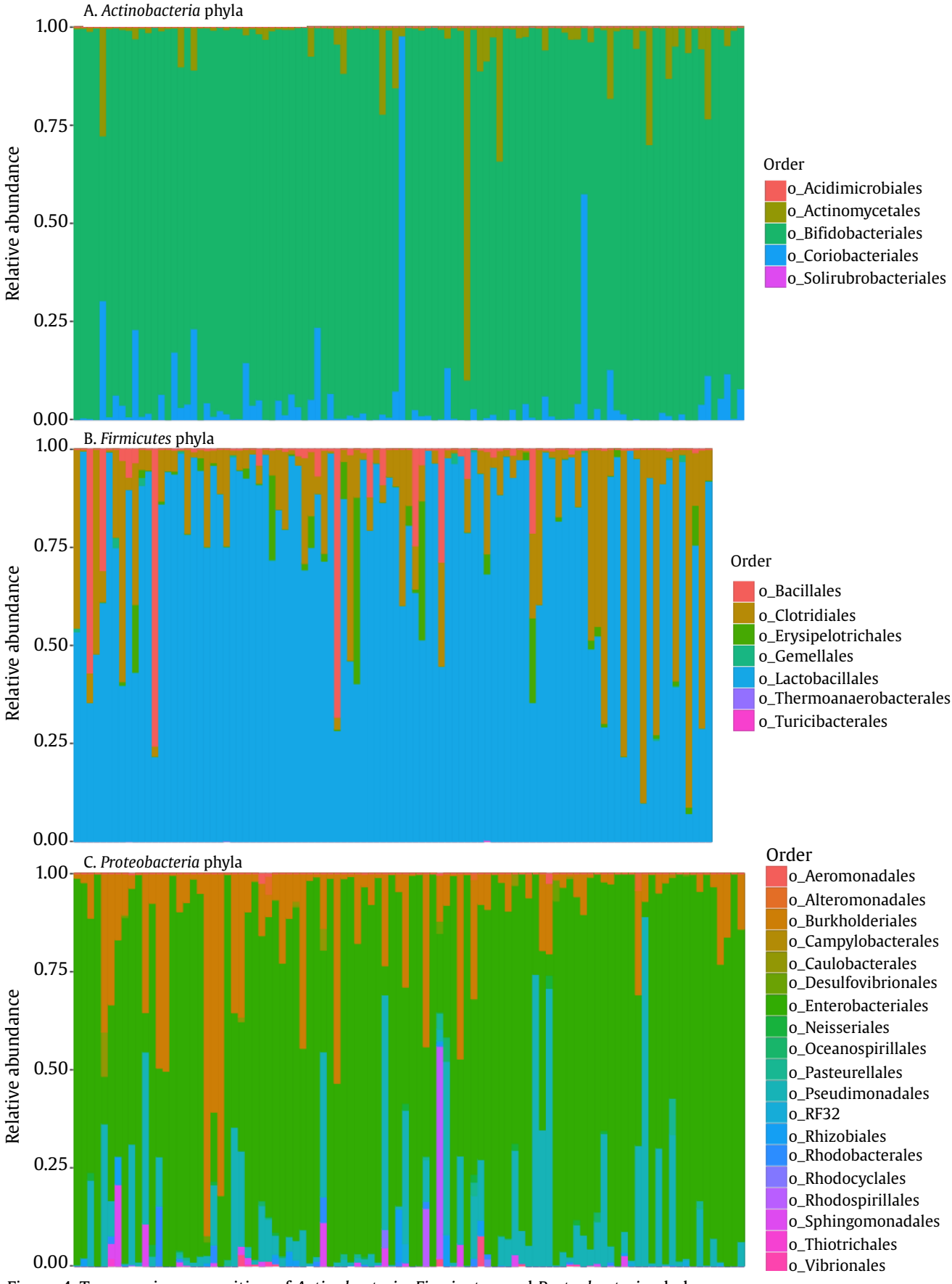

Figure 4. Taxonomic composition of *Actinobacteria, Firmicutes*, and *Proteobacteria* phyla

4. Discussion

Studies of gut microbiota composition affected by dietary habit have been conducted in various region worldwide. Children from Europe that consumed a western diet rich in sugar, starch, animal protein, and poor in fibres resulting higher abundance of Bacteroides compare to children living in rural Africa, with agrarian diet, that had a higher abundance of Prevotella (De Filippo *et al.* 2010). Furthermore, study in India also resulted similar outcome, the gut microbiota of Indian tribes with carbohydrate diet enriched in carbohydrate-metabolizing bacteria from the family of Prevotellaceae (Dehingia *et al.* 2015). Consistent with those studies, gut microbiota characterization of BaAka hunter-gatherers and Bantu, two African groups living in Central Africa, have specific compositional structure of microbial that reflects their lifestyles (Gomez *et al.* 2016).

Exposure to breast milk has been reported to have a more significant influence in shaping the configuration and function of the gut microbiota. Breastfeeding provides a specific metabolic substrate for the microbiota (Neu and Rushing 2011; Guaraldi and Salvatori 2012). Some studies reported a difference in the bacteria pattern and abundance between different feeding modes (Harmsen *et al.* 2000; Fan *et al.* 2013). On the contrary, our study showed that feeding patterns are not associated with the difference in microbial community richness and evenness based on the alpha diversity index. Alpha diversity measures the number of different species in a sample and measures the abundance of the species. In addition, results from the measurement of the beta diversity index also showed that the association between feeding patterns and differences in the microbial community is not statistically significant. Beta diversity uses to compare the differences in microbial composition in one sample group compared to another (Wagner *et al.* 2018). In this study, more than 80% of the bacteria were *Bifidobacterium, Lactobacillales*, and *Enterobacteriales*, followed by *Clostridiales, Verrumicrobialles, Bacillales*, and *Coriobacteriales*. *Bifidobacterium* has been widely known to benefit human health such as prevention of colorectal cancer, treatment of diarrhea, and reduction in the symptom of inflammatory bowel disease (Venturi *et al.* 1999; Le Leu *et al.* 2010). Bifidobacterial has been reported to correlate with human milk oligosaccharides composition, and many studies reported that this bacterial was commonly found in the feces of infants who received breastmilk (Davis *et al.* 2016; Ho *et al.* 2018). *Lactobacillales*, as a part of lactic acid bacteria (LAB), are associated with intestinal infection control and lactose digestion improvement (Gilliland 1990). A study reported no significant difference in the abundance of *Lactobacillus* between breastfeed and formula feed groups (Yang *et al.* 2019). *Enterobacteriales* has been reported to be associated with increased *in vivo* intestinal permeability in humans (Pedersen *et al.* 2018). *Enterobacteriaceae*, a family of *Enterobacteriales* has been reported to have lower abundance in less duration of breastfeeding group (Ho *et al.* 2018). In this study, we found cyanobacteria and chloroflexi in fecal samples. Other studies also found also rare Phyla (which had an average abundance of 0.01%) including Cyanobacteria, Deinococcus-Thermus, Chloroflexi, and Fusobacter among fecal samples from low birth weight (LBW) infants (Costello *et al.* 2013). Naturally, chloroflexi bacteria are found in the human oral microbiome, although in low levels (Morrison *et al.* 2023). Chlorophylaxis represents a low level (<1%) but consistent component of the human oral and skin microbiota and has also been detected in the gut of other mammals (Campbell *et al.* 2014). Our study did not show a difference in the gut microbiota pattern between the two feeding pattern groups. Recently, it was reported that at 3 months of age, Enterococcus was significantly lower in the cesarean section delivery with breast-fed group than in the formula-fed infants, although for infants delivered by vaginal delivery, the difference between feeding types (breast-fed versus formula-fed pattens) was not significant (Ma *et al.* 2022). Low diversity of gut microbiota early in life appears to be a hallmark of a healthy gut, if caused by breast-feeding, which is different from the theory in adults (Ma *et al.* 2022). Future research will be very important to investigate the feeding patterns and the mother diets during the breastfeeding period in the first six months on the diversity of microbiota and their relationship with non-communicable diseases in later life.

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