ORIGINAL ARTICLE

IMPACT OF FEEDING PRACTICE ON DIVERSITY PATTERN OF THE GUT MICROBIOME IN INFANTS

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Background: The microbiome which is developed at the time of infancy remains predominant and influences the health in childhood and then throughout life through moderating different gut metabolic activities This study was designed to look for the impact of feeding practices on the diversity of gut microbiota in infants in a Pakistani cohort. **Methods:** A cross sectional study was carried out in 46 healthy infants [23breast-fed (BF) and 23 formula-fed (FF)], aged 0–4 months, enrolled from different centers and localities in Peshawar. Infants were screened to exclude any pathological or physiological condition that can vary the gut microbial flora such as gut surgeries and the use of antibiotics. Their stool samples were collected. DNA was extracted and subjected to next generation sequencing. **Results:** The results revealed that phylum *Firmicutes* was dominant in formula-fed infants (FF=25.4±22.7, BF=4.58±5.21), p=0.001. Similarly, *Bacilli*, *Streptococcaceae,* and *Streptococcus* were significantly higher in formula-fed infants. On the other hand, *Selenomonadales* and *Streptococcus_salivarius* were significantly higher in breast-fed infants with a p-value of 0.037 and 0.029 respectively when compared with formula fed infants. **Conclusion:** The primary colonizer of the infant's gut is phylum *Firmicutes*, followed by *Bacilli*, *Streptococcaceae,* and *Streptococcus* in formula-fed infants and *Selenomonadales* and *Streptococcus_salivarius* in breast-fed infants.

Keywords: Gut microbiota; Breast-fed infants; Formula-fed infants; Next generation sequencing

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INTRODUCTION

Gut microbiota constitutes a diverse and complex microbial community that habitats more than 1000 species within 7000 strains ≥100 times more as compared to the human genome. The interaction and symbiosis between the microbial system and the gut host is very important for the optimal health of life throughout.[1,2] The gastrointestinal tract of an adult man is incredible accommodation for a large number of microorganisms. Their count is more than 100-trillions. Gut microbiota can be illustrated as a microbial unit that is present in all organs within a body. It is formed of various cell ancestries having the ability to connect with others, as well as with host. They utilize, store, and rebuild energy; this population deals with physiologically crucial chemical transformation. They are maintained and repaired by self-replication.[3] Bacterial count is highest among the three domains of life; Eukarya, Archea & bacteria, and the last domain comprises 99% of the total microbial pool, therefore the term microbiota is used for bacteria. The five major phyla in the human gut microbiome include; Bacteriodetes (*Cytophagia, Flavobacteria, Sphingobacteria, and some unclassified Bacteroidetes*)[4], Firmicutes 60% of the total pool as bacilli ((*Bacilli*, *Lactobacilli*, *Lactococci*, *Staphylococci*, *Streptococci*, *Leuconostoc*), Clostridia (Clostridial cluster, *Eubacteria*, *Roseburia* spp., *Peptococci*, *Petptostreptococci*), Erysipellotrichia (Erysipelotrichaceae), Negativicutes (such as *Veillonella*), Thermolithobacteria, and some unclassified Firmicutes)), Actinobacteria(*actinomycelate and a bifidobacteria*), Verrucomicrobia (*Akkermansia spp*) and Proteobacteria (alpha-beta-gamma and zeta -proteobacteria).[3]

Initially, it was thought that the infant gut is sterile at the time of birth but now the literature has proved that gut colonization starts intranasally and is influenced by various factors related to maternal health.[5] Bacterial colonization of the infant's gut represents the complex collection of the microbial community. This community is facilitated by various factors related to the host and environment.[5,6] It is evident from recent literature that maternal gut microbiome also has a significant impact on the infant gut colonization.[7,8] The formation of gut microbiota and its maturation comprises an active and unplanned process, having positive as well as negate synergy between the main microbial taxa.[9] The infant gut colonization is under the influence of various factors such as pre-pregnancy weight, gestational weight gain, mode of delivery, antibiotic usage, diet, type of feeding, gestational age, metabolic status, mother's age, lifestyle, family genetics, exposure to household furry pets and micronutrients deficiency.[7,8,10]

Infants feeding practice is one of the major factors that play a significant role in gut bacterial colonization as well as gastrointestinal functions. Differences in the gut flora of breast fed and formula fed

infants are widely reported.[11,12] Bifidobacterial colonies are well established in breast fed infants as mother milk provides a blend of nutrients, antimicrobial and promicrobial factors that account for the development of formal "milk-oriented microbiota". IgA present in breast milk enhances a more regulatory "tolerogenic" immune system. Human milk oligosaccharides are present in mother milk which precisely configure the growth and function of useful microflora.[13]

The microbial flora of breast fed infants displays lower heterogeneity when compared with formula fed infants. The transcriptomic analysis also revealed that the mode of feeding influences host gene expression. Transcription of genes are being modified for metabolic activities and the immunological system in breast fed infants. The presence of Bifidobacterium in breast fed infants is attributed towards lactoferrin, oligosaccharides and decreased iron content in the body.[14,15]

Formula fed infants are subjected to different bacteria, carbohydrates, micronutrients, and nutrients leading to different bacterial assemblages' patterns of the intestine. It is reported that the stool of breast fed infants has higher levels of lactobacilli and Bifidobacterium and decreased levels of potentially pathogenic bacteria. While the stools of formula fed infants contain predominantly *Staphylococcus, Clostridia, Bacteroides*, *Enterobacter,* and *Enterococci*.[16,17]

There is always a need to investigate how do the early feeding practices affect and modify the diversity of gut microbiome in early infancy as the predominant form remains the same throughout our lives. It is the feeding mode in early infancy that decide which microbes are going to be the permanent resident of the gut. Gut microbiota produces numerous metabolites like short chain fatty acids, lactates, ammonia, and hydrogen sulphide that are essential for the normal routine functions of the body. Also, this study will comparatively analyse the gut microbial diversity with European cohort.

MATERIAL AND METHODS

This was a cross sectional study, carried out in Peshawar, Khyber Pakhtunkhwa The study was approved by the Khyber Medical University Ethics board (KMU-Ethics Board) on 27th of October 2016 for 4 years under the study reference number DIR/KMU-EB/PZ/000316 and titled "Plasma zinc status concerning metabolites of gut microbiota and their diversity in breast fed and formula fed infants". This study was started in October 2017 and ended in March 2018. Based on the median difference of 14.3 mmol/kg total short chain fatty acids between breast fed and formula fed infants at 2 months of age from the study of Siigur *et al* 1993[18] and considering a margin of error of 5%, a total of 23 infants are required in each group (total n=46).

Predominantly breast-fed and formula-fed infants age 0–4 months were enrolled in this study. Written informed consent was taken from the infants' mothers/guardians. Infants were screened to exclude any pathological or physiological condition that can vary the gut microbial flora such as gut surgeries and use of antibiotics. None of the infants had diarrhoea and any other GI disorder during the fecal sampling periods.

A pre-weight stool collection pot, cool pack, pair of disposable gloves, a spatula, and a plastic bag were provided to the participant's mothers to collect the stool sample by following the standard protocol. Mothers were asked to call the researcher when the sample is ready for collection. Every faecal sample was processed within 12hrs after collection. The median time elapsed between sample production (telephone call for sample collection taken as the time of sample production). For reminder, courtesy calls were made to mothers.

Following the standard protocols by using Qigen ministool kit the DNA was extracted from the stool samples of 46 infants. Which were then subjected to next generation sequencing through illumine sequencer. To analyse the bacterial population shotgun technique was used. Standard Operating procedures of Microbiome Helper were followed to process the shotgun metagenomic data. FastQC tool was used to examine raw metagenomic reads. Knead data tool was applied to perform quality control on metagenomic sequencing data, as these sorts of experiments contain a high ratio of the host to bacterial reads, knead trim these contents of the host genome. This tool was subjected to perform in silico separation of bacterial reads from contaminated reads, i.e., from the host.

Trimmomatic tool is a flexible read trimmer for Illumina NGS. Read filtering and trimming of low-quality reads was done through it. Literature proved and also supported that it is an effectual pre-processing technique, which could correctly handle paired-end data.[19]

Bowtie2 is a memory-efficient and ultrafast tool for aligning sequencing reads to long reference reads. It supports local, gapped, and paired-end alignment modes.[20] For taxonomic profiling the processed reads were subjected to MetaPhalan2. MetaPhalan2, it is a computational tool for profiling the composition of microbial communities from metagenomic shotgun sequencing data. A unique clade-specific marker is used to estimate the relative abundance of microbiome and detect the taxonomic clades present in samples of microbiome.[21]

In this study, a total number of 46 samples were included and the average proportion of breast fed infants were 92% and similar was for formula fed infants. All the results of microbes from NGS analysis were arranged and organized on an Excel sheet. Descriptive statistics was applied for comparison between the groups. The means differences of the gut microbes between the groups were

analysed by 2 sample t-test. All the data were expressed as mean±SD. *p*-values of less than 0.05 were considered significant.

RESULTS

The microbes were compared at all levels between the groups. The mean differences were analysed at all levels. It was observed that at Phylum levels the two groups in the case of Actinobacteria % (BF: 52±35.7, FF: 62±28.2) *p*-value 0.346 and Proteobacteria % (BF: 53±36.2, FF: 45.4±37.2) *p*-value 0.550 were not significantly different. Whereas, Firmicutes % (BF: 4.58±5.21, FF: 25.4±22.7) in both groups significantly vary with p=0.001. Which means that formula fed infants harbor more Firmicutes than breast fed infants. On the other hand, Bacteroides (BF: 18.5±18.8, FF: 0.415±0.403) showed increasing trends in BFI with p=0.098. They were the main colonisers of breast fed infants (Table-1). The gut microbial analysis revealed that Phylum Actinobacteria was present abundantly in BFI whereas Firmicutes count was higher in FFI. Similarly, plenty of Bacteroides were present in BFI and very little were observed in formula-fed infants (Figure-1).

The percent distribution of Actinobacteria in BFI was 91% of the total breast-fed infants whereas 78% of the FFI were bearing Actinobacteria in their gut. Proteobacteria was found in 78% of the samples of BF group and 69% in FF. Firmicutes were found to be the predominant phylum among the FFI present in 78% of the total samples in contrary to BFI where their percent was 52% of the total samples. As far as Bacteroides are concerned 21% of the BFI harbored this phylum whereas their presence in FFI was negligible shown in the Cladogram (Figure 1 & 2). Gamma Proteobacteria were found in 78% and 65% of the BF and FFI respectively. The class Actinobacteria was detected in 90% of BF samples and 78% FFI. Furthermore, 78% of the samples had Bacilli (BF: 4.55±5.35, FF: 12.85±9.23 with *p*-value 0.007) significantly raised in FF infants (Table-2) whereas, 26% of the samples had Clostridia in the FF group. *Negativicutes* were present in 30% of the FF samples. The aforementioned figures reveal that class *Actinobacteri*a is predominant in BFI and Bacilli is predominant in FFI Cladogram (Figure 1 & 2).

Analysis of the absolute count of different orders revealed that Bifidobacterial were present in 30% and 73% of the BF group and FF group respectively. In the FF group, 65% of the samples got a higher % of *Enterobacteriales* and a low count of Coriobacteriales. The results also showed that Lactobacilli, *Clostridiales,* and *Selenomonadales* (BF: 1.37±1.20, FF: 12.1±10.5 with *p*-value 0.037) all were present in 34%, 26%, and 30% in FFI respectively and very little of these orders was observed in BFI (Figure 2). However, *Selenomonadales* were significantly higher in FF infants (Table-2).

Ninety one percent of the BF group and 73% of the FFI had *Bifidobacteriaceae* in their gut. It was also observed that *Enterobacteriacea* was present in 78% and 65% of the BF and FF samples respectively. *Ruminococcaceae* were found only in FFI. The FFI also nursed more *Streptococcaceae* (*p*-value 0.033) (Table-2) in their gut as compared to BFI i.e. about 56% of the total samples in that group. Low levels 21% and 30% of *Veillonellaceae* were observed in BFI and FFI respectively (Figure 1 & 2).

At genus level analysis it was observed that 91% of the breast-fed babies harbored Bifidobacterium in their gut. Whereas in FFI it was present in 73% of the samples. Escherichia was detected in 78% of the BF samples and 52% of the FFI. Genus streptococci was seen in the significantly higher count (p=0.0331) (Table-2) in FF group as compared to BF group. Other genera which were found in FF but not in BFI were *Leuconostoc, Subdoligranulum, Megaspheare, Blautia,* and Clostridia. Klebsiella and *Eggerthella* were present in both groups but lesser amount. Morganella, Enterococcus, and *Akkermansia* were detected only in BFI (Figure-2).

Bifidobacterium longum was the predominant species in both BF (86%) and FF (69%) infants. Sixty nine percent of the breast-fed group harboured Escherichia coli in their gut. *Streptococcus salivarius* was present in 56% of the samples from FF group (*p*-value 0.029) (Table-2). Bifidobacterium bifidum and Bifidobacterium breve were seen more in formula-fed samples than in breast-fed subject. *Akkermansia muciniphila, Prevotella copri, Eggerthella* unclassified, *Morganella morganii*, *Bacteroides fragilis* and *Streptococcus parasnguinis* were found only in BFI. *Subdoligranulum*, *Klebsiella*, *Leuconostoc lactis*, *Megaspheare*, *Ruminococcus ganavus* and *Lactobacilli ruminis* were present in FFI but were not detected in breast-fed infants. Whereas *Klebsiella pneumonia*, *Klebsiella* unclassified, *Veillonella* unclassified, and *Lactobacilli gasseri* were found in both the groups.

Table-1: Comparison of Phylum between breast-fed and formula-fed infants

Phylum	Breast fed		Formula fed		P-value
	Mean	SD	Mean	SD	
Actinobacteria %	52.3	35.7	62.0	28.2	0.346
Proteobacteria %	53.0	36.2	45.4	37.2	0.550
Firmicutes %	4.58	5.21	25.4	22.7	0.001***
Bacteroidetes %	18.5	18.8	0.415	0.403	0.098

Significant levels ($^{*}p$<0.05, $^{**}p$=<0.01, $^{***}p$<0.0001)

Table-2: Comparison of different taxonomic ranks (other than phylum) of gut microbiota

Class	Breast fed		Formula fed		*p*-value
	Mean	SD	Mean	SD	
Actinobacteria %	52.3	35.7	62.0	28.2	0.346
Gammaproteobacteria %	53.0	36.3	48.4	36.5	0.722
Negativicutes %	4.30	4.99	12.1	10.5	0.155
Bacilli %	4.55	5.35	12.85	9.23	0.007***
Order					
Bifidobacteriales %	48.1	39.0	64.6	25.1	0.330
Enterobacteriales %	47.2	37.9	48.4	36.5	0.945
Coriobacteriales %	3.51	1.97	2.91	1.85	0.770
Selenomonadales %	1.37	1.20	12.1	10.5	0.037**
Lactobacillales %	5.67	7.16	12.85	9.23	0.222
Family					
Bifidobacteriaceae %	51.9	35.5	64.6	25.1	0.205
Enterobacteriaceae %	52.9	36.3	48.4	36.5	0.727
Coriobacteriaceae %	3.51	1.96	2.92	1.85	0.771
Lactobacillaceae %	13.87	0.10	17.87	4.44	0.170
Streptococcaceae %	2.29	1.12	9.7	10.9	0.033**
Veillonellaceae %	3.09	3.89	12.1	10.4	0.070
Genus					
Bifidobacterium %	51.9	35.5	64.6	25.1	0.205
Escherichia %	48.0	34.6	42.9	35.7	0.700
Veillonella%	3.41	4.41	6.40	9.79	0.671
Lactobacillus %	13.87	0.104	14.34	8.77	0.911
Klebsiella %	19.1	17.6	29.9	26.9	0.446
Streptococcus%	1.966	0.99	9.0	10.8	0.031**
Species					
Bifiobacterium_longum %	47.6	33.5	52.7	34.7	0.661
Bifidobacterium_bifidum %	6.37	3.75	9.43	4.49	0.207
Escherichia_unclassified %	16.3	14.4	29.1	32.5	0.307
Bifidobacterium_breve %	16.7	30.2	17.1	18.7	0.976
Veillonella_unclassified %	1.29	1.38	9.1	12.1	0.530
Klebsiella_pneumonia %	24.7	16.6	37.6	35.8	0.629
Streptococcus_salivarius %	1.66	0.75	9.2	10.9	xx0.029

Significant levels (*p<0.05, **p=<0.01, ***p<0.0001)

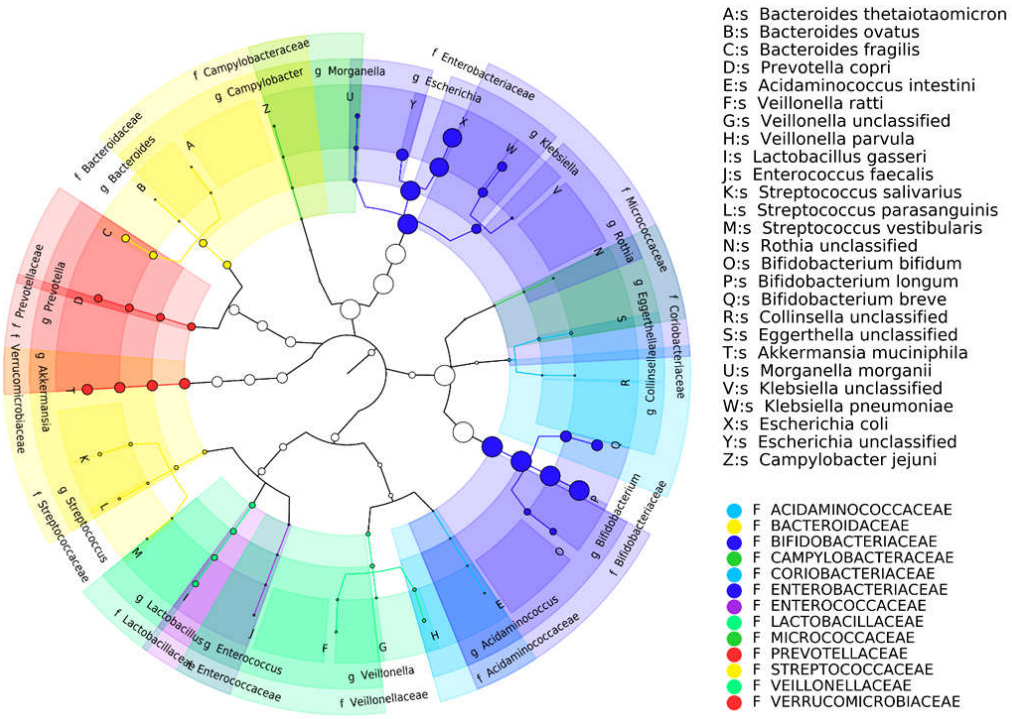

Figure-1: Cladogram of the formula-fed infants microbial taxa

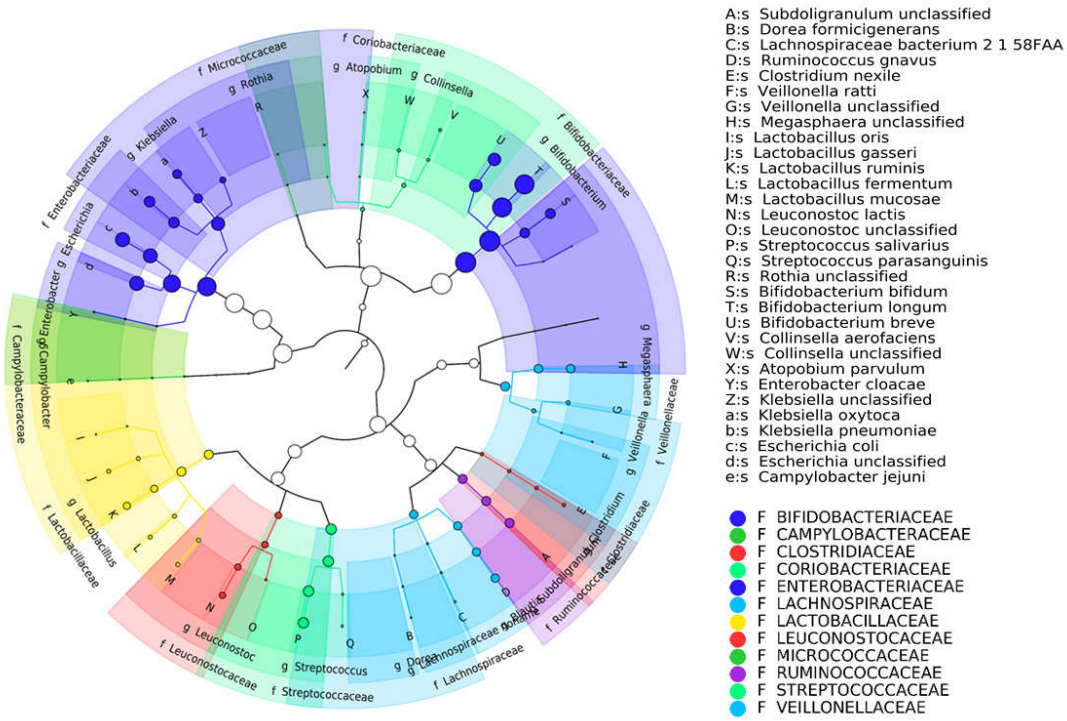

Figure-2: Cladogram of the breast-fed infants' microbial taxa

DISCUSSION

Gut microbial colonization is a dynamic and non-specific process that adapt and harbor bugs within it from the environment they are predisposed to.[22–24] In our study when the microbes at Phylum level were analysed, it was observed that *Actinobacteria, Proteobacteria* and *Firmicutes* were the predominant Phyla in both the groups, validating the results of Korean study.[25] However, these phyla were higher in FFI when compared with breast fed infants. Whereas, Bacteroides were predominant in BFI unlike reported the finding in which Actinobacteria were the predominant form of BFI[25,26] this can be attributed to the dietary and demographic variation between the test population. The findings of the present study along with earlier reports endorse that in the preweaning period, the main residents of infants gut are *bifidobacteria* followed by enterobacteria and Bacteroides.[11,27–29]

The spectrum of gut microbiota in 0-4 months old Pakistani infants of this study was different from the European infants where Bifidobacterium, Enterobacteria, and Bacteroides were detected.[11] Fallani *et al* attributed this deviation to geographical distribution that leads to variation in these microbiotas. At class level comparison between the groups, it was found that Actinobacteria, Negativicutes and Bacilli were abundant in formula fed infants, these findings were in line with the study done by Harmsen *et al*[27] approving the statement that the FFI gut microbiota is more diverse than BFI. *Gammaproteobacteria* were present in higher amount in our BFI while in Korean study the same class was abundant in FFI. The class Clostridia was present in FFI but not in BFI in the present study authenticating the data reported by Tannock.[30]

The comparison of microbes at order level yielded that *Enterobacteriales* and *Coriobacteriales* were present in equal amounts in both our study groups. However, *Bifidobacteriales*, *Selenomonadales,* and *Lactobacillales* were present in abundance in FFI as compared to BFI.

Analysis at the level of family revealed that the population of *Enterobacteriaceae* was higher in BFI analogous to earlier observation.[31] *Coriobacteriaceae* and *Lactobacillaceae* were present in equal proportion in both the groups whereas, *Bifidobacteriaceae*, *Streptococcaceae,* and *Veillonellaceae* were higher in FFI as compared to BFI.

At genus level comparison it was found that Escherichia were higher in BFI and *Veillonella* and Lactobacillus were comparable in both the groups showing compatibility with the report of.[16] Furthermore, *Bifidobacterium* and *Klebsiella* were found abundantly in formula fed infants which is in contrast with the findings of Guaraldi, Salvatori and

Guarino *et al*[16,32] who reported that Bifidobacterium is higher in BFI in comparison to FFI. On the other hand, *Streptococcus* was found in FFI in our study population as has been reported by Timmerman *et al*.[22]

Species level comparison demonstrated that a similar amount of *Bifidobacterium_bifidum* and *Bifidobacterium_breve* were present in both the groups showing agreement with an earlier report.[33] *Bifiobacterium_longum*, *Escherichia_*unclassified, *Veillonella_*unclassified %, *Klebsiella_pneumonia*, and *Streptococcus_salivarius* were present in both our groups but a higher proportion in FFI. Probiotic species (*Bifidobacterium longum*, *Streptococcus salivarius* and *Lactobacilli gasseri*) were also detected in our study population both in BF and FFI as has been reported by.[25] It was also observed that the higher number of probiotic species were present in FFI as compared to BFI this could be related to the fact that the majority of the infants' formulae in this era resembles human breast milk or probiotics are being added directly to the formula milk by the manufacturers, giving an advantage to FFI to exhibit a similar gut microbiota like that of breast fed infants.[34]

We observed that microbial taxa (*Staphylococcus*, *Actinomyces*, *Propionibacterium*, *Corynebacterium,* and *Gemella*) related to skin was not found in any of the BFI needing further investigation in a larger sample size. Maternal hygiene can be given as justification for this scenario, as the extent of hygiene measures also accounts for variant colonization of gut microbiota.[24]

CONCLUSION

This study provides a gateway for comparative analysis of gut microbial diversity with the European cohort. In our population, the primary colonizer of the infant's gut is phylum *Firmicutes*, followed by *Bacilli*, *Streptococcaceae,* and *Streptococcus* in formula-fed infants and *Selenomonadales* and *Streptococcus_salivarius* in breast-fed infants. The dissimilar spectrum of the diversity of gut microbiome in the two groups necessitates further exploration on a bigger scale. As gut microbiota produces numerous metabolites that are essential for routine functions of the body, there is a need to validate gut microbial diversity at all levels in infants. Association of the gut metabolites with the predominant members of the gut microbiome also needs further investigation.

Declaration: To the best of our knowledge this manuscript contains no material previously published by any other person. All the data provided in this manuscript is genuine and related to my research work.

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AUTHORS' CONTRIBUTION

MD: Literature search, the conceptualization of study, data collection, data analysis, data interpretation, write-up. RN: Proof reading. SF: Assisted in data analysis, data interpretation. MA: Assisted in data collection.

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