Published in final edited form as:

J Pediatr Gastroenterol Nutr. 2017 August ; 65(2): 218–224. doi:10.1097/MPG.0000000000001522.

Changes in the gut microbiota after early administration of oral synbiotics to young infants in India

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Competing interests: The authors declare that they have no competing interests.

Ethics approval and Consent to participate: This study was approved by U.S. Institutional IRB and Local Ethics Committees in India. Informed parental consent was obtained at the time of infant screening and enrollment.

Authors' contribution

PP and IG conceived, designed, and led the study, with help from other authors. DC managed lab protocols, conducted initial molecular analyses, QA of samples for metagenomics analyses and wrote the first draft. RS, and SP were responsible for clinical operations, PM handled local lab protocols. RC and NS helped develop SOPs and supervised local lab protocols and were responsible for providing assistance from the reference lab at AIIMS. MP, DP and RB conducted metagenomics data analyses. FY was responsible for statistical analysis. All authors contributed to different sections and conducted edits, read and approved the final manuscript.

Legend to Supplemental Digital Content (SDC)

SDC- Methods: Protocol for bacterial community DNA extractions from infant-stool and sample preps for 454-based 16SrRNA gene sequencing.

SDC- Fig.S1a,b,c.: Illustrates intra-individual taxonomic diversity of all Synbiotic (T1-T8) and Control (C1-C3) infant stool samples at D-7 and D-60 of life, irrespective of their treatment status- at Phylum (a), Family (b) and Genus (c) levels.

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Abstract

Objectives—We examined the changes in the developing gut microbiota of Indian infants enrolled in a colonization study of an oral synbiotic (*L. plantarum* and fructo-oligosaccharides) preparation.

Methods—Frozen stool samples were available from a previously published clinical study of the synbiotic preparation administered daily for seven days to full term Indian infants delivered by C-section. 16S rRNA gene sequencing of fecal bacterial community-DNA was done in 11 infants sampled at Day-7 and Day-60 of life.

Results—All infants showed changes in bacterial diversity with age. While Firmicutes and Proteobacteria were predominant in all, Actinobacteria and Bacteroidetes were initially low at day-7. In control infants, we observed a significant increase ($p=0.012$) in the proportions of Actinobacteria at day-60. In the treated group, over the 60-day period, there was a 10-fold increase in Bacteroidetes, a somewhat smaller increase in Firmicutes, and a reduction in Proteobacteria. Compared to controls, treated infants were increasingly colonized by different Gram positive genera including Enterococcus, Lactobacillus and Bifidobacteria. Relatively less known taxa and some unassigned sequence reads added to enriched diversity observed in the treated group.

Conclusions—There was a high level of bacterial diversity among infants examined in this study. Synbiotic treatment induced an increase in overall taxa and Gram positive diversity, especially in the first week of life. Changes in the microbiota during early infancy should be used as a rationale for selecting probiotics in diverse clinical settings.

Keywords

Infant; Neonate; Gut Microbiota; Probiotics; Prebiotics; Synbiotics; *L. plantarum*; Metagenomics; 16SrRNA gene-sequencing

INTRODUCTION

While the nascent microbiome acquired during or after birth may influence infant gut development and immunity, disrupted colonization patterns early in infancy can lead to morbidity later in life (1). It is conceivable that interventions during this early infancy period could have a long lasting impact on human health and disease. While investigators in the Western world have engaged in research addressing the role of gut microbiota in allergy and other infectious and inflammatory diseases (2–5), studies in the developing world are extremely limited. Due to the early and overall high level of exposure to a relatively polluted environment in South Asia and India (where most of the one million neonatal deaths due to infection take place), changes in the infant microbiota demands critical attention. However, except for a limited number of culture-based studies (6, 7), very few reports have utilized sequence-based approaches for an in-depth analysis of the gut microbiota of infants (8).

Although many different probiotics are used rampantly in India, limited studies have described changes in gut microbiology induced by such therapy in 1–6 years old children (9, 10). No study in newborns or young infants using 16S rRNA gene-sequencing approaches has been reported to date, nor has the effect of probiotic or other interventions been examined in this population.

Utilizing samples from a previously published hospital trial (11), we examined the effect of a one-week administration of a synbiotic preparation containing *L. plantarum*, and Fructo-oligosaccharide on the developing gut microbiota of Indian infants. Changes were compared using 454-pyrosequence based 16S rRNA gene sequence analysis of fecal community DNA extracted from controls and synbiotic-treated infants sampled on Day-7 and Day-60 post-treatment. To the best of our knowledge, this is the first report on microbiota of Indian neonates using a sequencing approach, and also describes for the first time the synbiotic-mediated changes in the infant gut.

MATERIALS AND METHODS

Study design

This study utilized a subset of frozen stool samples from a previously published hospital-based clinical study of a synbiotic preparation containing *L. plantarum* (ATCC-202195) and fructo-oligosaccharide, or control (maltodextrin) in full term Indian infants (11). The study population involved newborn infants 35 wk GA and 1800 gm BW born by Caesarean section who were >12 hr and <72 hr old, had breastfeeding established, were not on antibiotics, not likely to receive antibiotics in their first seven days postpartum, and did not have major congenital anomalies. Infants were randomized in a 2:1 (Treated: Control) allocation. Administered synbiotics were dissolved in 2.0 ml of dextrose saline and given by spoon feeding. A total of seven daily oral doses were administered, some of which were at the infant's home. At study entry, there was no difference between the two groups in maternal age or in babies' sex, gestational age, birth weight, length and head circumference. Fecal samples were collected and immediately transferred to laboratory (in coolant boxes) and stored frozen (–80°C) until further processing. Informed parental consent was obtained and the study was approved by U.S. institutional IRB and local ethics committees in India.

Protocol details for the isolation of bacterial community DNA from infant-stool and sample preps for 16S rRNA gene sequencing on a Roche's 454-pyrosequencing platform has been appended as a supplemental digital content (SDC-Methods).

16S rRNA gene sequence and statistical analyses

Sequences generated by pyrosequencing were submitted to the open source software package- Quantitative Insights into Microbial Ecology (QIIME; <http://qiime.sourceforge.net>) for removal of low quality sequences (12). Sequences that met the following filtering parameters were used for analysis: (i) between 300 and 430 nucleotides long, (ii) sequences with no ambiguous bases, (iii) average base-call quality score 30, (iv) no mismatches in primers, and (v) no mismatches in barcodes. After sequences were quality controlled, chimera check was performed using the Chimera Slayer method in QIIME, and

the reads were subsequently binned by barcode. Taxonomic-based analysis was performed using the CLASSIFIER program of the Ribosomal Database Project (RDP) to assign taxonomic status to each sequence (13). The reads in each taxonomic bin were normalized to the proportion of the total number of reads per sample.

The analysis was restricted to a given taxonomic unit (Bacteria) with at least one call from a control infant, and an average of at least 3 calls per infant from either control or synbiotic treated groups during the entire study period. This filtering method was applied to focus on bacteria with some presence in the control infant, as well as reasonable presence in at least one group under comparison. The abundance levels of the bacteria were descriptively summarized using the mean proportion of the bacteria for each group at two time-points (D-7 and D-60). The generalized estimating equations (GEE) assuming Poisson distribution for count data with a log link and the total counts of bacteria called from each infant as an offset were used for modeling. In the GEE models, we considered two different variance structures including compound symmetry to account for the correlation between repeated measures from the same infant, and a simpler independent structure. Since the model with independent structure provides better performance based on its smaller QIC values (quasi-likelihood under the independence model criteria), we reported the results based on the GEE model with independent structure. Comparisons were made between the synbiotic-treated and control samples collected at D-7 and D-60, and also within treated and control infants at same time points. The Benjamini-Hochberg method was used to control for the false discovery rate (14).

Comparison of microbial communities was done by taxonomic-independent analysis. Sequences were clustered using a threshold of 97% pairwise identity for assignment into operational taxonomic units (OTU), and binned by sample using QIIME. To assess and discard overlapping OTUs, representative sequences were selected from each OTU and re-clustered keeping the 97% identity cutoff. The entire dataset was then compared to the database of non-overlapping OTUs and the single best BLAST (Basic local alignment search tool) hit was identified for all sequences in the dataset using local BLAST in the BioEdit Blastall function. Alpha-diversity analyses were performed using QIIME. Significance of alpha-diversity was measured by ANOVA test using GraphPad Prism version 7.0a. Beta-diversity was assessed by non-metric multidimensional scaling (NMDS) based on Bray-Curtis metrics (15). Adonis PERMANOVA tests using R was performed to test significance of Bray-Curtis distance.

RESULTS

Relative abundance of major Phyla in study infants

16S rRNA gene sequence data from synbiotic-treated and control infants showed abundance of four major phyla- Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria (Figure-1). In the controls, Firmicutes (28%) and Proteobacteria (64%) were predominant at D-7. However, by D-60, Proteobacteria were significantly reduced (64% to 13%, $p=0.016$) while the other three phyla showed increases in abundance. Treated infants at D-7 showed high levels of Firmicutes (48%) and Proteobacteria (51%), with relatively low proportions of Actinobacteria and Bacteroidetes. We observed a significant increase in levels of

Actinobacteria ($p=0.012$) at D-60 in treated infants. Also, there was a 10-fold increase in Bacteroidetes and somewhat smaller increase in Firmicutes (from 48% to 57%) with a reduction in Proteobacteria (51% to 31%) from D-7 to D-60 in the treated group.

Microbiota changes at the Family Level

Table-1 shows abundances of the major taxonomic families in both synbiotic and control groups. At D-7, compared to controls, synbiotic-treated infants showed a relatively high abundance of families: Streptococcaceae ($p=0.002$) and Lactobacillaceae ($p=0.10$). Enterococcaceae were significantly low (10.3% vs. 0.2%, $p<0.001$) at D-7 in the treated group. Treated infants showed higher relative increases in the proportion of Bacteroidaceae (0.9% to 10.1%, 10-fold) compared to controls (6.4% to 36.4%, 5-fold) between D-7 and D-60. We also observed Streptococcaceae to be lower in the treated group at D-60 (44% vs 12%, $p=0.13$). Although Enterococcaceae were significantly lower at D-7 in the treated compared to controls (0.2% vs. 10%, $p<0.001$), this change was reversed at D-60 with a significant increase in the treated group (29.5% vs. 1.7% $p=0.021$).

Microbiota changes discerned at the Genus level

At the genus level (Table-2), control infants at D-7 were predominant in *Escherichia/Shigella* (45%), *Enterococcus* (10%), *Clostridium* (8%), *Bacteroides* (6%), and *Staphylococcus* (5%), including some unclassified bacterial sequences (~20%). At D-60, these infants showed significant increases in *Bifidobacteria* ($p<0.001$), *Streptococcus* ($p<0.001$) and *Acinetobacter* ($p<0.001$), with an over 5-fold increase in *Bacteroides* 6% to 37%; $p=0.19$). From D-7 to D-60 there was a significant reduction in numbers of *Clostridium* 8% to 0.3%, $p<0.001$), *Escherichia/Shigella* 45% to 5%, $p=0.007$), *Staphylococcus* 5% to 0.05%, $p<0.001$) and unclassified Enterobacter 19% to 7%, $p=0.03$) in the controls.

Synbiotic treated infants at D-7 also showed a predominance of *Escherichia/Shigella* (21%), *Clostridia* (19%), *Streptococcus* (12%), *Staphylococcus* (8%), *Lactobacillus* (7%) and *Klebsiella* (5%). Unclassified Enterobacter sequences also accounted for a high proportion (23.6%). At D-60, there was a marked increase in levels of *Enterococcus* (0.25% to 30%, $p<0.001$), *Bacteroides* (0.9% to 10%; $p=0.22$) and *Bifidobacteria* (0.06% to 0.12%; $p=0.37$). However, reduced proportions of *Klebsiella* (5.2% to 0.3%, $p=0.015$), *Clostridia* (19% to 4.7%, $p<0.001$), *Lactobacilli* (7.1% to 0.6%, $p=0.021$) and *Staphylococcus* (8.2% to 0.1%, $p<0.001$) were observed in the treated infants at D-60 compared to D-7. Compared to controls, synbiotic-treated infants at D-7 showed increased abundance of *Lactobacillus* (0.004% to 7%, $p<0.001$), *Bifidobacteria* (0.004% to 0.06%, $p=0.035$), and *Streptococcus* (1.4% to 12%, $p=0.003$). However, there were marked reductions in levels of *Enterococcus* (10% to 0.25%, $p=0.001$), *Bacteroides* (7% to 0.9% and *Escherichia/Shigella* (45% to 21%; $p=0.43$) in the treated group at D-7. *Bacteroides*, *Bifidobacteria*, and *Streptococcus* were relatively low in treated infants at D-60. We also observed increases in *Clostridium* (0.03% to 5%, $p<0.001$), *Enterococcus* (1.7% to 30%, $p=0.02$), *Escherichia/Shigella* (5% to 13%; $p=0.21$), *Klebsiella* (0.005% to 0.3%, $p=0.002$), *Lactobacillus* 0.01% to 0.64%, $p<0.001$) and *Staphylococcus* (0.05% to 0.1%; $p=0.56$) in the treated group at D-60, compared to

control infants. Overall, synbiotic treatment increased colonization by many different Gram positive genera including Lactobacilli at D-7, *Enterococcus* and *Clostridia* at D-60.

Alpha and Beta diversity among study groups

Comparison of bacterial communities confirms a high degree of variability between control subjects at D-60 and probiotic-treated groups independent of time-point. Although the Adonis PERMANOVA test of Bray-Curtis distance was not statistically significant ($p=0.9130$), the NMDS plot (Figure 2) shows greater diversity between the microbial communities of control subjects at D-7 and D-60 when compared to the microbial communities of synbiotic-treated subjects at the same time points. Overlap between D-7 and D-60 demonstrates larger shared taxa between the treated groups compared to controls. Additionally, although not statistically significant, the synbiotic treatment increased diversity within subjects as measured by alpha diversity metrics. Measurement of Chao 1 index and the number of observed species show greater alpha diversity in treated subjects compared to controls (Figure 3). However, the bacterial communities of some treated infants resemble that of control at D-7, while the same applies to individuals at D-60. Thus, it seems that the high degree of intra-variability among individuals dampened the synbiotic effect. Nevertheless, these observations taken together, point toward interesting and potentially important trends. Since no clinical endpoints are being measured in this study, relevance of such observations remains to be seen.

DISCUSSION

Early colonizers of the mammalian gut can play an important role in the development of infant microbiome, in turn, driving immunity (16). Hence, the microbiota is of special interest in the care of newborn and for prevention of morbidity in early infancy and childhood. Although many clinical trials in the western world have utilized a range of probiotics to prevent or treat inflammatory and infectious conditions in neonates such as necrotizing enterocolitis, sepsis, and allergy/atopy, very little has been done in developing country settings where infections continue to be a major cause of morbidity and mortality in this age group. Also, most of the small probiotic trials conducted in the developing world have used preparations/ strains already in use in the developed world without any attention to the pre-existing or changing gut microbiota of the infants in their own setting. In this study, we utilized frozen stool samples from our clinical trial of synbiotics, where culture-based results revealed a very high rate of stool colonization by *L. plantarum* starting day 3 of dosing (11). Control infants in this study provided us with the opportunity to examine the natural development of microbiota in the growing infants. The role of prebiotic sugars, such as FOS used in the current synbiotic is well known to promote the growth of anaerobic strains. Maltodextrin used as an excipient in both synbiotic and placebo groups can also be utilized by probiotic strains for their growth. Additionally, we cannot rule out the many environmental exposures during the first sixty days of life of infants in the Indian home settings. Since these factors were present in both groups, for the purpose of this report, we are attributing the changes in the microbiota in our study to the combined effect of the probiotic strain and FOS.

In our two-month old control infants, we observed higher abundances of Firmicutes (49%) and Bacteroidetes (36%) compared to the much lower proportions seen in a 16S rDNA-microarray based study in American infants (32% Firmicutes, and 20% Bacteroidetes) (17). Infants in our study harbored only about 13% Proteobacteria compared to 46% in U.S. infants (17). On the other hand, Actinobacteria appeared to be low in both settings (<2%). In another US-based study, following infants from day 3 through 84 of life, Koenig and colleagues observed the predominance of Firmicutes, with a subsequent increase of Proteobacteria and Actinobacteria; high levels of Bacteroides were seen later in life (172–297 days) after introduction of solid foods (18).

Our study shows higher alpha diversity in the treated subjects than controls (Fig 3). The greater dispersal of Trt-7 data points seen in the NMDS plot suggests that changes at Trt-7 were greater than Ctrl-7 or Trt-60. The partial overlap of Trt-7 and Trt-60 shows sharing of some taxa whereas almost no overlap in Ctrl-7 and Ctrl-60 points toward distinct diversity between the timepoints (Fig 2). This could be due to a competitive exclusion between the species favored by the synbiotic treatment and the microbial consortia to which infants are exposed in their intimate environments. Since Lactobacilli and Bifidobacteria have previously been associated with health benefits, we are tempted to speculate similar impact of our synbiotic treatment where these species were increased. However, lacking a clear definition for a “healthy core microbiome” in a developing world setting (or for that matter in any setting), it is difficult to ascertain if overall changes induced by our synbiotic treatment indeed resulted in a healthy microbiome.

Our study infants were all hospital-born, discharged after 4–6 days of hospital stay, and were exposed to a home environment until they were 60 days old. There was no indication of acquisition of any specific microbial patterns that could be attributed to the hospital environment. Rather, these infants were all different and showed unique inter-individual diversity at every taxonomic level (SDC-1: Fig.S1 displays this unique diversity for all infants at Phyla (a), Family (b) and Genus (c) levels, irrespective of treatment status). This is similar to observations in a study by La Rosa et al. where the gut of term infants at birth showed diverse bacterial colonization, especially anaerobes. It was quite intriguing to note that in the confined NICU environment, while factors such as mode of delivery, age, antibiotics, and diet influenced the pace, they did not affect the sequence of progression. The authors described such a pre-programmed gut microbiota development to be similar to a “choreographed” event (19). Pandey et al. utilizing rDNA cloning and sequencing techniques reported absence of Bifidobacteria, and predominance of *Citrobacter* sp, *E. coli*, and *C.difficile* in fullterm hospital born (C-section) infants in India (8). While we found Bifidobacteria in low numbers, we also observed *Clostridium* to be a major genus and the presence of *Acinetobacter* and *Citrobacter*, similar to Pandey’s observations.

Although our previous culture-based (where isolation of strict anaerobes is difficult) results had shown an increase of Bifidobacteria only during month 3 and 6 (11), we were quite surprised to observe low proportions in our current sequence-based analysis. Reports on Bifidobacteria using sequencing techniques have been contradictory in the literature. Apart from extraction procedures (that can be controlled and optimized), base mismatches between the primers used in the 454 platform and some Bifidobacterial species have been

incriminated as one of the causes (20). These equivocal results are supported by a recent report from the Microbiome Quality Control Project Consortium revealing 50–150 different OTUs in a defined positive control pool that had only 20 species to start with. The authors have described the many variables in such studies including problems with primers (21, 22). However, in a very recent study, Patel and colleagues confirmed their 454 sequencing-based results on low Bifidobacteria by qPCR assays (23) pointing that there is no single approach that can be considered as gold standard at this point in time and protocols need to be tailor made based on the aim of the investigation. Since identification or quantitation of specific species was not the aim of this study, we did not conduct additional qPCR.

Although we observed overall low proportions of Bifidobacteria, a significant increase (from 0.004% to 0.06%) was demonstrated in the treated group at D-7 in our study infants. We also observed Bacteroides to be very high in abundance and ubiquitous in our study infants irrespective of treatment status. Higher abundances of Bacteroides also have been previously reported with lower counts of Bifidobacteria (23,24,25). While intriguing, we do not know if such a ratio (Bifidobacteria to Bacteroides) among strict anaerobic genera is a universal phenomenon in the human gut microbial ecology.

We were surprised to observe the inter-individual variability and how diverse and unstable the microbial milieu was. In spite of such diversity, compared to controls at D-7, infants given synbiotic containing *L. plantarum* exhibited relatively high proportions of Streptococcus (1.39 to 12.38), Bifidobacterium (0.004 to 0.058), and a remarkable increase (0.004 to 7.12, $p < 0.001$) in Lactobacillus (Table-2). Although there was a high degree of variability among all infants independent of time points, NMDS analysis showed a higher number of taxa shared among treated subjects at D-7 and D-60 indicating the impact of synbiotic treatment during the 60-day period. To the contrary, control infants at the two time points were uniquely different with very minimal sharing of taxa (Fig. 2).

The total number of studies in the literature is small, and the number of infants examined in each study is also very small (including ours which utilized 22 samples for an in-depth analysis). While larger studies are warranted and are now feasible with a lower price tag (e.g. MiSeq), our study strongly points toward an inherent diversity of microbiota in growing infants in the Indian population and the ability of specific probiotic strains to modulate the same at least over a 2 months period (26).

Rodenas and colleagues have very recently described the ability of *L. reuteri* to increase diversity and abundance of Bifidobacteria, Enterobacteriaceae and Enterococci in C-section babies but not in vaginally born infants (27). The current study also utilized samples from C-section babies. Since gut microbiota of vaginally born infants are more diverse and populated with reduced Enterobacteriaceae compared to C-section babies, it will be intriguing to examine the impact of similar synbiotics on their microbiota. The observed impact on increasing diversity in the current population may not be as distinct when we start seeding the neonatal gut that is already colonized with a more diverse group of bacteria. At the same time, if Lactobacilli or *L. plantarum* present in larger numbers even in the meconium compared to stools of babies several weeks-old (28) are natural and early colonizers, the impact may be similar or better in a more natural gut environment.

Infections and stunting in early childhood remain a daunting challenge that the global health community needs to address. Early and severe crowding of the infant gut by bacteria in these settings give rise to tropical enteropathy and under-nutrition in the first 1000 days of life. The time has come for selecting the right probiotic, for specific age groups, appropriate for specific environments, for reducing the incidence of defined, preventable disease states. A critical look at the developing microbiota and its utilization for generating the best symbiosis and optimal immunity during early childhood is indispensable in this context (16).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are thankful to the Government of Odisha, Ministry of Health & Family Welfare, for making the necessary arrangements to conduct this research study. We extend our thanks to the Indian Council of Medical Research for expediting all required reviews and approvals. This study could not have been possible without the unconditional support and faith of the parents of all newborns screened for this study.

Funding sources: This study was funded by grants U01 HD 40574 and R01 HD 53719 from the Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH, USA. Program staff was involved in study design for the clinical trial under the co-operative agreement. Funding agency had no further role on data analysis or genesis of this manuscript, although program officials provided overall guidance and monitoring.

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What is known

- Enterobacteriaceae, followed by microaerophiles, and finally strict anaerobes colonize the human gut during early infancy
- Limited number of sequence-based analyses in western countries show predominance of Firmicutes, Proteobacteria and subsequent rise in Bacteroidetes when solid food is introduced

What is new

- Utilizing 16S rRNA gene-sequence analyses, this study shows low proportion of Proteobacteria and high abundance of Bacteroidetes and Firmicutes in two-month old Indian infants
- This study also demonstrates a high level of microbiota diversity among infants in early infancy
- Synbiotic treatment for one week induces and maintains diversity for two months in life

Figure 1.

Synbiotic treated vs. Controls: Phylum proportions. Mean proportions (%) of predominant Phyla for synbiotic treated and control groups, compared at D-7 and D-60 of infant life. Compared to controls, the treated infants showed relatively high abundance of Firmicutes and low counts of Actinobacteria during the first week (D-7). Proteobacteria were predominant (> 50%) in both groups at D-7, however these were remarkably reduced in controls (< 1/5th the initial level) compared to treated at D-60. Treated infants showed increased abundance of Actinobacteria from D-7 to D-60.

Figure 2.

Non-metric multidimensional scaling (NMDS) based on Bray Curtis distance metrics. NMDS plot shows greater diversity between the microbial communities of control subjects at day 7 and day 60 when compared to the microbial communities of synbiotic-treated subjects at the same time points. Overlap between D-7 and D-60 in the treated subjects suggests shared taxa between the groups implying a limited change in diversity during this 2 month period.

Figure 3. Alpha Diversity in control and treated subjects. Synbiotic treatment increased diversity within subjects as measured by alpha diversity metrics. Although not statistically significant, measurement of Chao 1 index showed greater alpha diversity compared to controls.

Table 1

Family abundances in Synbiotic treated and control infants. Significant changes at Family level were observed within and among groups at different time points (D-7 and D-60). Streptococcaceae, Staphylococcaceae, and Lactobacillaceae (belonging to phylum Firmicutes) showed a marked increase during early synbiotic treatment (D-7). Enterobacteriaceae family significantly reduced in controls at D-60 ($p=0.002$).

Family	[%] Proportions				p-value			
	Ctrl D-7	Ctrl D-60	Tx D-7	Tx D-60	Tx-Ctrl D-7	Tx-Ctrl D-60	Ctrl D7-D60	Tx D7-D60
Bacteroidaceae	6.39	36.39	0.97	10.11	0.30	0.36	0.19	0.21
Bifidobacteriaceae	0.004	0.305	0.06	0.12	0.025	0.40	<0.001	0.36
Comamonadaceae	0.008	0.29	0.05	0.04	0.12	0.16	<0.001	0.52
Corynebacteriaceae	0.05	0.18	0.02	0.024	0.68	0.10	<0.001	1.00
Enterobacteriaceae	64.36	12.39	50.45	31.19	0.68	0.16	0.002	0.41
Enterococcaceae	10.29	1.74	0.24	29.49	<0.001	0.021	0.21	<0.001
Erysipelotrichaceae	1.41	0.054	0.008	0.09	<0.001	0.57	<0.001	0.004
Lachnospiraceae	0.016	0.12	0.002	0.93	0.09	0.16	<0.001	<0.001
Lactobacillaceae	1.13	0.01	7.12	0.69	0.10	<0.001	<0.001	0.026
Leuconostocaceae	0.057	0.55	0.09	0.05	0.72	0.16	<0.001	0.33
Micrococcaceae	0.02	0.26	0.016	0.012	0.88	0.016	<0.001	0.49
Moraxellaceae	0.02	0.17	0.038	0.018	0.68	0.13	<0.001	0.004
Peptostreptococcaceae	7.66	0.032	19.37	4.63	0.68	<0.001	<0.001	<0.001
Propionibacteriaceae	0.03	0.005	0.032	0.037	0.98	0.16	<0.001	1.00
Staphylococcaceae	5.44	0.05	8.15	0.09	0.72	0.51	<0.001	<0.001
Streptococcaceae	1.43	43.73	12.38	12.15	0.002	0.13	<0.001	1.00
Veillonellaceae	0.69	1.57	0.008	0.49	<0.001	0.16	0.035	<0.001
unclassified_Bacteria	0.03	0.06	0.064	0.12	0.11	0.48	0.19	0.40
unclassified_Firmicutes	0.008	0.01	0.029	0.05	0.31	0.16	0.22	0.33
unclassified_Lactobacillus	0.012	0.03	0.075	0.13	0.016	0.20	0.46	0.49

Table 2

Proportions of predominant genera in Synbiotic treated and control infants. *E. coli/Shigella*, Enterococcus, Clostridium, and staphylococcus were the predominant genera during early life (D-7). Lactobacilli were very low at D-7 and also at D-60 in control infants with a significant increase after synbiotic treatment ($p<0.001$). Bifidobacteria were also low at D-60, but showed significant increase only during early (first week) after synbiotic treatment ($p<0.035$).

Genus	[%] Proportions			p-value			
	Ctrl D-7	Ctrl D-60	Tx D-7	Tx D-60	Tx-Ctrl D-60	Ctrl D7-D60	Tx D7-D60
Acinetobacter	0.02	0.16	0.034	0.016	0.82	<0.001	0.004
Bacteroides	6.4	36.47	0.97	10.29	0.36	0.38	0.22
Bifidobacterium	0.004	0.31	0.058	0.124	0.035	<0.001	0.37
Citrobacter	0.004	0.07	0.03	0.012	0.18	<0.001	0.37
Clostridium XI	7.65	0.033	19.41	4.69	<0.001	<0.001	<0.001
Corynebacterium	0.049	0.18	0.024	0.025	0.67	<0.001	1.00
Diaphorobacter	0.008	0.23	0.031	0.033	0.38	<0.001	1.00
Enterococcus	10.29	1.74	0.25	29.99	0.001	0.02	<0.001
Escherichia/Shigella	44.63	5.33	21.11	12.91	0.43	0.21	0.49
Klebsiella	0.54	0.005	5.24	0.27	0.15	<0.001	0.015
Lactobacillus	0.004	0.01	7.12	0.64	<0.001	0.51	0.021
Lactococcus	0.04	0.14	0.035	0.019	0.98	<0.001	0.012
Leuconostoc	0.05	0.49	0.035	0.02	0.86	<0.001	0.37
Propionibacterium	0.032	0.005	0.032	0.037	0.98	<0.001	1.00
Rothia	0.02	0.26	0.015	0.011	0.87	<0.001	0.37
Staphylococcus	5.44	0.054	8.17	0.1	0.82	<0.001	<0.001
Streptococcus	1.39	43.67	12.38	12.34	0.003	<0.001	1.00
Veillonella	0.024	0.6	0.008	0.48	0.52	0.006	<0.001
Weissella	0.008	0.054	0.062	0.03	0.15	<0.001	0.30
unclassified_Bacteria	0.03	0.06	0.065	0.13	0.15	0.19	0.37
unclassified_Enterobacter	19.2	6.98	23.59	18.41	0.83	0.029	0.67
unclassified_Firmicutes	0.008	0.01	0.03	0.058	0.37	0.19	0.35

Genus	[%] Proportions				p-value			
	Ctrl D-7	Ctrl D-60	Tx D-7	Tx D-60	Tx-Ctrl D-7	Tx-Ctrl D-60	Ctrl D7-D60	Tx D7-D60
unclassified_Lachnospirac	0.012	0.027	0.008	0.03	0.09	0.95	<0.001	0.042
unclassified_Lactobacilla	0.012	0.033	0.075	0.14	0.025	0.21	0.48	0.49