Influence of own mother’s milk and different proportions of formula on intestinal microbiota of very preterm newborns

Adriana Zanella¹, Rita C. Silveira¹, Luiz F. W. Roesch², Andréa L. Corso¹, Priscila T. Dobbler², Volker Mai³, Renato S. Procianoy¹*¹

1 Unidade de Neonatologia do Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil, 2 Centro Interdisciplinar de Pesquisas em Biotecnologia-CIP-Biotec, Campus São Gabriel, Universidade Federal do Pampa, São Gabriel, Rio Grande do Sul, Brazil, 3 Department of Epidemiology, College of Public Health and Health Professions and College of Medicine, Emerging Pathogens Institute, University of Florida, Gainesville, Florida, United States of America

Abstract

Objective

To determine the differences in preterm infants’ stool microbiota considering the use of exclusive own mother’s milk and formula in different proportions in the first 28 days of life.

Methods

The study included newborns with GA ≤ 32 weeks divided in 5 group according the feeding regimen: 7 exclusive own mother’s milk, 8 exclusive preterm formula, 16 mixed feeding with >70% own mother’s milk, 16 mixed feeding with >70% preterm formula, and 15 mixed 50% own mother’s milk and preterm formula. Exclusion criteria: congenital infections, congenital malformations and newborns of drug addicted mothers. Stools were collected weekly during the first 28 days. Microbial DNA extraction, 16S rRNA amplification and sequencing were performed.

Results

All groups were similar in perinatal and neonatal data. There were significant differences in microbial community among treatments. Approximately 37% of the variation in distance between microbial communities was explained by use of exclusive own mother’s milk only compared to other diets. The diet composed by exclusive own mother’s milk allowed for greater microbial richness (average of 85 OTUs) while diets based on preferably formula, exclusive formula, preferably maternal milk, and mixed of formula and maternal milk presented an average of 9, 29, 23, and 25 OTUs respectively. The mean proportion of the genus Escherichia and Clostridium was always greater in those containing formula than in the those with maternal milk only.
Conclusions
Fecal microbiota in the neonatal period of preterm infants fed with exclusive own mother’s milk presented increased richness and differences in microbial composition from those fed with different proportions of formula.

Introduction
The intestinal microbiota is very important for human metabolism, development and behavior [1,2]. Despite several studies on the subject and its connection with high complexity diseases [1,3,4], the studies were based on culture, genetic profile and/or the use of small sample sizes, which makes it clear that the variables responsible for shaping the intestinal microbiota have not been satisfactorily examined [1,5,6]. It is known that the development of infant microbiota depends on medical and dietary factors [1,7], but it is not yet known how such factors influence the microbial overall composition and their associations with the human body [1]. The human body has millions of microorganisms that work in partnership with our own cells to influence the quality of our lifelong health [8,9]. The composition of the childhood intestinal microbiome is influenced by factors such as the type of birth, gestational and postnatal age, ingestion of antibiotics, environment, nutritional exposures, and breastfeeding, which should be emphasized as an important variable for the assembly of the intestinal microbiota [5,8,10,11,12]. La Rosa et al have shown that the gut microbiota of premature infants admitted to Neonatal Intensive Care Units progresses and bacterial population changes in composition along the time [6]. Despite the influence of breast milk versus formula in the assembly of the gut microbiota, the true impact of own mother’s breast milk on the composition of the intestinal microbiome of premature infants is not fully understood [8].

The immature intestinal microbiota of premature newborns is influenced by factors such as postnatal age, gestational age, birth weight and nutritional exposures [8,13,14,15]. In the specific case of breastfeeding, this seems to disguise the influence of birth weight, which suggests a protective function against the intestinal immaturity of the premature newborn at the onset of life [8]. These findings suggest not only the existence of a microbial mechanism underlying the body of evidence that elucidates that breast milk promotes the intestinal health of the premature newborn [16], but also the dynamic interaction of host and dietary factors that help in the colonization and enrichment of specific microbes during the establishment of its intestinal microbiota [8].

Therefore, the aim of this study is to describe the intestinal microbiota of very low birth weight infants in the first 28 days depending on use of mother’s own milk or use of formula in different proportions.

Material and methods
This study used a convenience sampling strategy with patients recruited from the Neonatology Section of Hospital de Clínicas de Porto Alegre (HCPA), Brazil from May 2014 to January 2017. Pregnant women with gestation age ≤ 32 weeks that provided written informed consent were enrolled at hospital admission for their delivery. The study protocol was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre (HCPA), and the guardians signed an informed consent form. Exclusion criteria were: 1) HIV or congenital infections, 2) mothers with substance abuse and 3) neonatal congenital malformations. Infants’ weekly stool
samples were collected from diapers beginning with the first stool until the 4th week of life. All samples were immediately stored in liquid nitrogen until DNA extraction.

Newborns were divided in five groups according to the feeding: exclusive own mother’s milk (LME), exclusive formula (PFL), mixed 50% own mother’s milk and 50% formula (MFLM), mixed with formula and 70% or more of own mother’s milk (PLM), and mixed with own mother’s milk and 70% or more of formula (PFL). The newborns received daily the same diet for up to 28 days. The different amounts of formula and own mother’s milk was offered separately, at different times of the day, so that at the end of the day the ratio was maintained. The ratio of breast milk to formula was the limitation of the amount of breast milk available. The preterm formula used was Pre Nan. This formula does not contain neither probiotics nor prebiotics.

Obstetrical data and neonatal data were collected prospectively.

DNA extraction and the 16S rRNA library preparation

The laboratory technique as described in detail previously [16].

Microbial DNA was isolated from samples using the QIAamp Fast DNA Stool Mini Kit (Qia-gen, Valencia, CA, USA) following manufacturer’s instructions with modifications. All DNA samples were kept at -80˚C until use. The V4 region of the 16S rRNA gene was amplified and sequenced using the PGM Ion Torrent platform (Thermo Fisher Scientific, Waltham, MA, USA) with the barcoded bacterial/archaeal primers 515F and 806R [17]. PCR amplification was carried out using barcoded primers linked with the Ion adapter “A” sequence (5’-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3’) and Ion adapter P1 sequence (5’-CCTCTCTAGTGCCAGTGCTGAT-3’) to obtain a sequence of primer composed for A-barcode-806R and P1-515F adapter and primers. Each of the 25μL of PCR mixture consisted of 2U of Platinum Taq DNA High Fidelity Polymerase (Invitrogen, Carlsbad, CA, USA), 4μL 10X High Fidelity PCR Buffer, 2 mM MgSO4, 0.2 mM dNTP’s, 0.1 μM both the 806R barcoded primer and the 515F primer, 25μg of Ultrapure BSA (Invitrogen, Carlsbad, CA, USA) and approximately 50 ng of DNA template. PCR conditions used were: 95˚C for 5 min, 35 cycles of denaturation at 94˚C for 45s; annealing at 56˚C for 45s and extension at 72˚C for 1 min; followed by a final extension step at 72˚C for 10 min.

The resulting PCR products were purified with the Agencourt AMPure XP Reagent (Beckman Coulter, Brea, CA, USA) and the final concentration of the PCR product was quantified by using the Qubit Fluorometer kit (Invitrogen, Carlsbad, CA, USA) following manufacturer’s recommendations. Finally, the reactions were combined in equimolar concentrations to create a mixture composed by 16S gene amplified fragments of each sample. This composite sample was used for library preparation with Ion OneTouch 2 System with the Ion PGM Template OT2 400 Kit Template (Thermo Fisher Scientific, Waltham, MA, USA). The sequencing was performed using Ion PGM Sequencing 400 on Ion PGM System using Ion 318 Chip v2 with a maximum of 40 samples per microchip.

Sequence processing

The 16S rRNA raw sequences were analyzed following the recommendations of the Brazilian Microbiome Project [18]. Briefly, the OTU (Operational Taxonomic Unit) table was built using the UPARSE pipeline [19], in which the reads were truncated at 200 bp and quality filtered using a maximum expected error of 0.5. Filtered reads were dereplicated and singletons were removed. After clustering sequences into OTUs, with a similarity cutoff of 97%, chimeras were checked to obtain representative sequences for each microbial phylotype. Taxonomic classification was carried out using SINTAX [20] against the Ribosomal Database Project.
(RDP) database [21] with a confidence threshold of 80%. Sampling effort was estimated using Good’s coverage [22].

**Statistical analysis.** All clinical data was analyzed using the software SPSS 21.0 at the significance level of 5%. Quantitative variables with normal distribution were described through means/SD and compared by ANOVA with Tukey test. Quantitative variables with asymmetric distribution were described through median/interquartile range and compared by the Kruskall-Wallis test with Dunn Test. For comparison among proportions Pearson’s chi-square test was used in conjunction with residue analysis adjusted.

For all 16S rRNA downstream analysis, the data set was filtered, keeping only OTUs that were present in at least 30% of the samples in each treatment and rarefied to the same number of sequences [23]. The biom file was imported into R environment [24] and estimations of alpha and beta diversity were calculated using the “phyloseq” package [25], and further plotted using the “ggplot2” package.

To accesses the main differences among treatments in this study, a Permutational Multivariate Analysis of Variance (PERMANOVA) [26] as performed using a binomial dissimilarity matrix among samples and the `adonis` function implemented in the vegan package [27]. Differences between treatments were accessed through the STAMP software [28]. P-values were obtained by the two sided White’s non-parametric t-test followed by the Benjamini-Hochberg FDR correction. A p-value < 0.05 together with effect size filter (difference between proportions effect size < 1.00) was applied to determine the most important taxa that differed between treatments.

Analysis along the 28-day period was performed using meta-analysis of effect sizes reported at multiple points using general linear mixed mode [29].

**Results**

A total of 175 samples from 62 preterm newborns divided in five groups (7 in LME, 8 in FLE, 16 in PLM, 16 in PFL, and 15 in MFLM) were collected and analyzed. The five groups were similar in respect to maternal and obstetrical data (Table 1).

Neonatal data are shown in Table 2.

**Overall microbial community differences among diets**

The microbial community differences at OTU level (97% similarity cutoff for grouping definition) found among fecal samples from preterm newborns’ fed with five different diets during a period of 28 days are presented in Fig 1. The ordination analysis revealed significant differences in microbial community structure among treatments suggesting the feeding of different diets was responsible for the assembly of preterm gut community. Those differences were further confirmed by the permutational multivariate analysis of variance (Table 3).

The overall analyses (all samples) indicate that approximately 31% of the variation in distances among treatments was explained by the different diets provided in each treatment. Pair-wise comparisons revealed that diets based on maternal milk assembled microbial communities with large variation within group (e.g. greater differences among microbial communities from subjects feed with maternal milk) while diets based on formula created more similar microbial communities (Fig 1A and 1B) (Table 3). The use of maternal milk was responsible for the greatest variation observed between diets. The highest variation between treatments was observed among the samples under exclusive own mother’s milk and samples under exclusive formula. Approximately 37% of the variation in distance between microbial communities could be explained by the treatment with maternal milk only (LME) compared to the diet based preferentially in formula (PFL).
Besides, the greatest variations in distances between treatments were observed in those comparisons involving the use maternal milk only. Approximately 37% of the variation in distance between microbial communities could be explained by the treatment with maternal milk only compared to the diet based preferentially in formula (Table 3).

In agreement with the ordination analysis, alpha diversity measurements indicated significant differences (p-value < 0.001 according to the Kruskal-Wallis test) among the richness of OTUs within treatments (Fig 2). The diet composed by maternal milk only (LME) allowed for

### Table 1. Characteristics of the subjects.

| Variables                                | Exclusive breast milk (n = 7) | Exclusive Formula (n = 8) | Predominance of breast milk (n = 16) | Predominance of formula (n = 16) | Mixed (n = 15) | p-value |
|------------------------------------------|-----------------------------|--------------------------|--------------------------------------|---------------------------------|----------------|---------|
| Maternal age (years)—mean ± SD          | 24.0 ± 9.1                  | 26.1 ± 6.4               | 31.6 ± 5.5                           | 24.6 ± 7.4                     | 28.0 ± 7.5     | 0.052   |
| C-section—n(%)                           | 7 (100)                     | 6 (75)                   | 13 (81.3)                            | 11 (68.8)                      | 12 (80)        | 0.556   |
| Rupture of membranes (hours)—median (P25-P75) | 0 (0–3)                    | 0 (0–0.4)                | 0.04 (0–37)                          | 6.1 (0–96.5)                   | 0 (0–3)        | 0.246   |
| Rupture of membranes ≥18 hours—n(%)     | 0 (0.0)                     | 1 (12.5)                 | 4 (25)                               | 7 (43.8)                       | 2 (13.3)       | 0.062   |
| Maternal antibiotic—n(%)                | 3 (42.9)                    | 5 (62.5)                 | 11 (68.8)                            | 14 (87.5)                      | 8 (53.3)       | 0.188   |
| Preeclampsia—n(%)                       | 0 (0.0)                     | 1 (12.5)                 | 0 (0.0)                              | 0 (0.0)                        | 0.119          |         |
| Yes                                      | 5 (71.4)                    | 1 (12.5)                 | 7 (43.8)                             | 6 (37.5)                       | 4 (26.7)       |         |
| No                                       | 2 (28.6)                    | 6 (75)                   | 9 (56.3)                             | 10 (62.5)                      | 11 (73.0)      |         |
| Eclampsia                                | 0 (0.0)                     | 1 (12.5)                 | 0 (0.0)                              | 0 (0.0)                        | 0.062          |         |
| Streptococcus—n(%)                      | 1 (14.3)                    | 2 (25)                   | 4 (25)                               | 5 (31.3)                       | 6 (40)         | 0.944   |

Statistically significant association by the test of the residuals adjusted to 5% of significance

The mean, the median standard deviation and the interquartile range were used, using Analysis of Variance (ANOVA) in conjunction with the Tukey test was applied. In case of asymmetry, the Kruskal-Wallis test in set with Dunn were used. Categorical variables were described by absolute and relative frequencies. In the comparison of proportions, Pearson’s chi-square test was used in conjunction with residue analysis adjusted

https://doi.org/10.1371/journal.pone.0217296.t001

### Table 2. Neonatal data according to study group.

| Variables                                | Exclusive breast milk (n = 7) | Exclusive Formula (n = 8) | Predominance of breast milk (n = 16) | Predominance of formula (n = 16) | Mixed (n = 15) | p-value |
|------------------------------------------|-----------------------------|--------------------------|--------------------------------------|---------------------------------|----------------|---------|
| Birth Weight (g)—mean ± SD              | 912.1 ± 291.5ab             | 1684 ± 430.1b            | 1460 ± 575.4ab                       | 1459 ± 413.3ab                  | 1332 ± 372ab   | 0.021   |
| Gestational age (weeks)—mean ± SD       | 27.7 ± 2.7a                 | 30.6 ± 1.7b              | 29.9 ± 2.4ab                         | 30.5 ± 1.6a                     | 29.4 ± 1.7ab   | 0.031   |
| NEC-n (%)                                | 1 (14.3)                    | 0 (0.0)                  | 1 (6.3)                              | 1 (6.3)                        | 2 (13.3)       | 0.778   |
| Use of antenatal corticosteroid—n (%)    | 6 (85.7)                    | 7 (87.5)                 | 16 (100)                             | 15 (93.8)                      | 15 (100)       | 0.185   |
| Early onset sepsis—n (%)                 | 0 (0.0)                     | 0 (0.0)                  | 0 (0.0)                              | 0 (0.0)                        | 1 (6.7)        | 0.527   |
| Use of Antibiotics on the first week—n (%) | 6 (85.7)                | 3 (37.5)                  | 12 (75)                               | 13 (81.3)                      | 8 (53.3)       | 0.110   |
| Late onset sepsis—n (%)                  | 2 (28.6)                    | 3 (37.5)                 | 7 (43.8)                             | 5 (31.3)                       | 6 (40.0)       | 0.937   |
| Use of Antibiotics on the 2nd week—n (%) | 7 (100)                     | 5 (62.5)                 | 10 (62.5)                            | 10 (62.5)                      | 41 (66.1)      | 0.396   |

Statistically significant association by the test of the residuals adjusted to 5% of significance

For the Weight at birth and Gestational age the mean and standard deviation were used. We compare the averages through Analysis of Variance (ANOVA) in conjunction with the Tukey test. In case of asymmetry the Kruskal-Wallis test in conjunction with Dunn were used. Categorical variables were described by absolute and relative frequencies. In the comparison of proportions, Pearson’s chi-square test was used in conjunction with residue analysis adjusted.

https://doi.org/10.1371/journal.pone.0217296.t002
Fig 1. Beta diversity comparisons among microbial communities. **A** and **B**. Graph **A** represents clusters of microbial communities. Each point represents an individual sample, with colors indicating feeding treatments. Graph **B** represents measurement of multivariate dispersion for each treatment. FLE = exclusive formula; LME = exclusive own mother’s milk; MFLM = 50% formula and 50% own mother’s milk; PFL = ≥70% formula; PLM = ≥70% own mother’s milk.

https://doi.org/10.1371/journal.pone.0217296.g001
greater microbial richness (average of 85 OTUs). On the other hand, the diet preferably based in formula (PFL) presented the smallest richness (average of 9 OTUs) (Fig 2). The average number of OTUs found within the other diets was similar. The diets based in formula only (FLE) and preferably maternal milk (PLM) presented an average of 29 and 23 OTUs respectively and the diet based in a mixture of formula and maternal milk presented an average of 25 OTUs.

Identification of the main taxonomic differences among diets

Once overall differences among microbial communities found in fecal samples from preterm newborns fed with different diets during 28 days were detected, the next step was to identify the microbial taxa responsible for that difference. To detect differences among treatments at genus level, a pairwise differential abundance based on a two-sided White’s non-parametric t-test followed by the Benjamini-Hochberg FDR correction was performed. As LME treatment presented the greatest difference in microbial community among diets, the pairwise comparisons were performed between LME and the other diets (Fig 3).

The mean proportion of the genus *Escherichia* was always greater in treatments containing formula (FLE, PLM, MFLM and PFL) than in the treatment with maternal milk only. Particularly, the diet based on maternal milk presented an increased abundance of *Acinetobacter*, *Bradyrhizobium*, *Caulobacter*, *Corynebacterium* and *Paenibacillus*, *Burkholderia*, *Faecalibacterium*, *Sphingomonas* and the unknown genus from the *Microbacteriaceae* family as compared with the other treatments. Compared to the diet based on maternal milk, the diet preferably based on maternal milk (PLM) and the diet composed by a mixture of formula and maternal milk increased the abundance of *Clostridium* and *Escherichia*. The fecal samples from newborns fed with a diet preferable based on formula (PFL) presented greater abundance of
Escherichia, Salmonella, Enterococcus and the unknown genus from the Enterobacteriaceae family when compared to the LME treatment (Fig 3).

**Discussion**

The variables influencing the composition of the intestinal microbiota are topic of multiple studies. The assumption is that, once they are known and understood, new strategies can be developed to maintain a state of health [30,31,32]. In this study, we found global differences in the microbial community among the types of milk administered to preterm infants, showing that the greatest microbial richness was found in those who were exclusively fed with own mother’s milk. Approximately 37% of the variation in the distance between microbial communities was explained by treatment with breast milk exclusively, in comparison with diets based preferably on formula.

Knowing the total number of bacteria according to type of feeding allow us an understanding of certain situations which facilitate the development of diseases [8,31,32,33]. In our study, fecal samples of premature infants fed different diets were decisive in the diversity of the microbial community during the 28-day period. All infants were premature and were
separated according to the type of diet fed. Feeding them own mother’s milk exclusively allowed for greater microbial richness (mean of 85 OTUs). The formula-based group had the lowest richness (mean of 9 OTUs). These diets based on the exclusive offer of formula and, preferably, breast milk, showed an average of 29 and 23 OTUs respectively; and the diet based on a mixture of formula and breast milk presented an average of 25 OTUs.

Gregory et al studied 30 preterm infants (10 in each group) during the first 60 days of life fed with maternal breast milk, pasteurized donor human milk and preterm infant formula. It was found that those fed with maternal breast milk presented higher diversity [8]. Cacho et al showed that, in vitro, incubation of own mother´s milk with donor breast milk in a certain percentage and for 4 hours, may restore the maternal milk microbiome [31]. We did not mix human milk with formula. We offered them individually in different proportions throughout the day. Our data showed that this unmixed administration of breast milk and formula does not determine the restoration of human milk microbioma.

In our study the average proportion of the *Escherichia* and *Clostridium* genus were always higher in treatments containing formula than in treatment which provided breast milk exclusively. This finding has been reported previously [6]. A possible explanation for the results is found in the transmission carried out from the mother to the child and the external environment after the birth, as has already been indicated in other studies [33,34]. Besides that, there are factors like lactoferrin and glycoproteins in human milk that are protective against pathogen bacteria [35, 36].

In spite of several studies on the topic and the subject’s relation with diseases of high complexity [1,3,4], such studies were restricted to the enumeration of such diseases based on culture, genetic profile (16S) and the use of small samples, which makes it clear that the variables

Fig 3. Differential microbial abundance among microbial communities detected in samples from preterm babies fed with different diets during 28 days. p-values were obtained by the two-sided White’s non-parametric t-test followed by the Benjamini-Hochberg FDR correction. A p-value of < 0.05 together with effect size filter (difference between proportions effect size < 1.00) was applied to determine the most important taxa that differed between treatments. Only statistically significant differences are shown.

https://doi.org/10.1371/journal.pone.0217296.g003
that shape the intestinal microbiota have not been satisfactorily examined [1,5,6]. It is a known fact that the development of the microbiota in infants depends on medical and dietary factors (1,7), but it is not yet known how such factors influence the general composition of the microbiota, and how those factors cooperate with each other [1]. Studies based on fecal samples of infants and their mothers contribute for monitoring of each chronological and functional stage during the first year of life [1,34].

Besides contributing to the microbial richness, breast milk also favors the prevention of sepsis, necrotizing enterocolitis (NEC) and other diseases [29,31,33,37]. NEC is one of the main causes of morbidity and mortality in neonatal intensive care units, with most cases occurring among premature infants [32,37]. In another study of our group, we provide evidence of an association between NEC and distortions in the normal development of the microbiota and low diversity in NEC cases [32]. Within this study we found that increased diversity and breast feeding correlate with reduced incidence of NEC [32]. Mai et al reported a microbiota that predisposed to late onset sepsis in preterm infants that was closer to that we found in preterm newborns that were not fed exclusively with own mother’s breast milk [38].

Other studies emphasize the influence of diet according to the ethnic and/or geographic characteristics as variables that influence the development of newborn intestinal microbiota [39,40,41]. In our study, these issues were not addressed because they were not the focus of the study, leaving open perspectives for innovative studies.

The microbiome found in breast milk contributes in the short and long term to the prevention of colonization by pathogens, as it stimulates the production of reactive antibodies and establishes a healthy intestinal microbiome capable of preventing long-term morbidities such as obesity, type 2 diabetes, chronic intestinal inflammation, autoimmune disorders, allergies, irritable bowel syndrome and allergic gastroenteritis [31,42,43,44]. Our study confirms that the intestinal microbiota of preterm infants presents differences according to their diet—whether breast milk or formula—and emphasizes the importance of breast milk in the maintenance of microbial richness of the newborn’s microbiota. Escherichia and Clostridium have been associated to NEC as well as a high proportion of Proteobacteria with few numbers with Firmicutes [45].

Some situations, however, crossed this research, such as the loss of fecal samples; the difficulty of mothers in breastfeeding preterm infants with breast milk exclusively due to socioeconomic difficulties, for example; and the low fecal volume produced by premature infants. Another important point to consider when analyzing the intestinal microbiota of the newborn, we analyze only the stools and we do not know the microbiota of the proximal colon neither of the small bowel. We could not adjust microbial analysis for birth weight and gestational age because the number of subjects in exclusive breast milk group was not big enough. It is very difficult to have extreme premature newborns fed exclusively with breast milk during the whole period in NICU hence we were not allowed to study different variables. Our objective was to compare exclusive own mother’s milk feeding with different proportion of formula feeding. We studied just extreme premature newborns, all of them included in the same category of prematurity, and those small differences in birth weight and gestational age probably do not have major repercussion on microbiota.

Many studies have focused on the issue of breastfeeding, particularly regarding the newborns’ microbiota, to identify its benefits for the development and prevention of diseases throughout their lives. Considering the importance of the topic, this study aimed to describe the intestinal microbiota of preterm newborns according to their nutritional habits establishing modifications of the intestinal microbiota according to the type of enteral diet administered.
Based on our data, it is noticed that global differences of the microbial community are found among the types of diets administered to preterm infants, showing that the greatest microbial richness was found in those who received exclusive own mother’s milk in comparison with those that received different proportion of formula.

**Acknowledgments**

This study was supported by a grant from Bill and Melinda Gates Foundation, CNPQ and DECIT/Ministério da Saúde do Brasil.

**Author Contributions**

**Conceptualization:** Rita C. Silveira, Renato S. Procianoy.

**Data curation:** Renato S. Procianoy.

**Formal analysis:** Adriana Zanella, Rita C. Silveira, Luiz F. W. Roesch, Priscila T. Dobbler, Volker Mai, Renato S. Procianoy.

**Funding acquisition:** Renato S. Procianoy.

**Investigation:** Andréa L. Corso, Volker Mai, Renato S. Procianoy.

**Methodology:** Rita C. Silveira, Luiz F. W. Roesch, Priscila T. Dobbler, Volker Mai, Renato S. Procianoy.

**Project administration:** Andréa L. Corso, Renato S. Procianoy.

**Resources:** Adriana Zanella, Luiz F. W. Roesch, Priscila T. Dobbler, Renato S. Procianoy.

**Software:** Luiz F. W. Roesch, Priscila T. Dobbler.

**Supervision:** Rita C. Silveira, Andréa L. Corso, Renato S. Procianoy.

**Validation:** Luiz F. W. Roesch, Renato S. Procianoy.

**Visualization:** Renato S. Procianoy.

**Writing – original draft:** Adriana Zanella, Rita C. Silveira, Luiz F. W. Roesch, Andréa L. Corso, Volker Mai, Renato S. Procianoy.

**Writing – review & editing:** Adriana Zanella, Rita C. Silveira, Luiz F. W. Roesch, Andréa L. Corso, Volker Mai, Renato S. Procianoy.

**References**

1. Bäckhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P, et al. Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. Cell Host Microbe. 2015; 17: 690–703. https://doi.org/10.1016/j.chom.2015.04.004 PMID: 25974306

2. Cabreiro F, Gems D. Worms need microbes too: microbiota, health and aging in Caenorhabditis elegans. EMBO Mol Med. 2013; 5:1300–1310. https://doi.org/10.1002/emmm.201100972 PMID: 23913848

3. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. Nature. 2012; 486:207–214. https://doi.org/10.1038/nature11234 PMID: 22699609

4. Li J, Jia H, Cai X, Zhong H, Feng Q, Sunagawa S, et.al. MetaHIT Consortium. An integrated catalog of reference genes in the human gut microbiome. Nat Biotechnol. 2014; 32: 834–841. https://doi.org/10.1038/nbt.2942 PMID: 24997786

5. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci USA. 2010; 107: 11971–11975. https://doi.org/10.1073/pnas.1002601107 PMID: 20566857
6. Subramanian S, Huq S, Yatsunenko T, Haque R, Mahfuz M, Alam M, et al. A Persistent gut microbiota immaturity in malnourished Bangladeshi children. *Nature*. 2014; 510: 417–421. https://doi.org/10.1038/nature13421 PMID: 24891687

7. La Rosa PS, Warner BB, Zhou Y, Weinstock GM, Sodergren E, Hall-Moore, et al. Patterned progression of bacterial populations in the premature infant gut. *Proc. Natl. Acad. Sci. USA*. 2014; 111: 12522–12527. https://doi.org/10.1073/pnas.1409497111 PMID: 25114261

8. Gregory KE, Samuel BS, Houghteling P, Shan G, Ausubel FM, Sadreyev RI, et al. Influence of maternal breast milk ingestion on acquisition of the intestinal microbiome in preterm infants. *Microbiome*. 2016; 4:68. https://doi.org/10.1186/s40168-016-0214-x PMID: 28034306

9. Houghteling P, Walker WA. Why is initial bacterial colonization of the intestine important to the infant’s and child’s health? *J Pediatr Gastroenterol Nutr*. 2015; 60: 294–307. https://doi.org/10.1097/MPG.0000000000000597 PMID: 25313849

10. Zeissig S, Blumberg RS. Life at the beginning: perturbation of the microbiota by antibiotics in early life and its role in health and disease. *Nat Immunol*. 2014; 15: 307–10. https://doi.org/10.1038/ni.2847 PMID: 24645687

11. Brooks B, Firek BA, Miller CS, Sharon I, Thomas BC, Baker R, et al. Microbes in the neonatal intensive care unit resemble those found in the gut of premature infants. *Microbiome*. 2014; 2: 1. https://doi.org/10.1186/2049-2618-2-1 PMID: 24468033

12. Ardeshr A, Narayan NR, Mendoza-Lagares G, Lu D, Rauch M, Huang Y, et al. Breast-fed and bottle-fed infant rhesus macaques develop distinct gut microbiotas and immune systems. *Sci Transl Med*. 2014; 6: 252ra120. https://doi.org/10.1126/scitranslmed.3008791 PMID: 25186175

13. Boix-Amorós A, Collado MC, Mira A. Relationship between Milk Microbiota, Bacterial Load, Macronutrients, and Human Cells during Lactation. *Front Microbiol*. 2016; 7: 492 https://doi.org/10.3389/fmicb.2016.00492 PMID: 27148183

14. Khodayar-Pardo P, Mira-Pascual L, Collado MC, Martínez-Costa C. Impacto flactation stage, gestation alage and mode of delivery on breast milk microbiota. *J.Perinatol*. 2014; 34: 599–605. https://doi.org/10.1038/jp.2014.47 PMID: 24674981

15. Cabrera-Rubio R, Mira-Pascual L, Mira A, Collado MC. Impact of mode of delivery on the milk microbiota composition of healthy women. *J. Dev. Orig. Health Dis*. 2016; 5: 54–60. https://doi.org/10.1017/S2040174415001397 PMID: 26260404

16. S204017441 500139 7 PMID: 24646587

17. Roesch LF, Silveira RC, Corso AL, Dobbler PT, Mai V, Rojas BS, et al. Diversity and composition of vaginal microbiota of pregnant women at risk for transmitting Group B Streptococcus treated with intrapartum penicillin. *PLoS One*. 2017; 12(2):e0169916. https://doi.org/10.1371/journal.pone.0169916 PMID: 28178310

18. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J*. 2012; 6: 1621–1624. https://doi.org/10.1038/ismej.2012.8 PMID: 22402401

19. Pylro VS, Roesch LF, Morais DK, Clark IM, Hirsch PR, Totola MR. Data analysis for 16S microbial profiling from different benchtop sequencing platforms. *J. Microbiol. Methods*. 2014; 107: 30–37. https://doi.org/10.1016/j.mimet.2014.08.018 PMID: 25193439

20. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods*. 2013; 10: 996–998. https://doi.org/10.1038/nmeth.2604 PMID: 23955772

21. Edgar R. SINTAX: a simple non-Bayesian taxonomy classifier for 16S and ITS sequences. 2016. https://doi.org/10.1101/0714161

22. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, et al. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* 42, D633–D642. 2014. https://doi.org/10.1093/nar/gkt1244 PMID: 24288368

23. Lemos LN, Fulthorpe RR, Trippett EW, Roesch LFW. Rethinking microbial diversity analysis in the high throughput sequencing era. *J. Microbiol. Methods*. 2011; 86: 42–51. https://doi.org/10.1186/2049-2618-2-1 PMID: 24468033

24. R Development Core Team. R: A Language and Environment for Statistical Computing. 2008. Available at: http://www.R-project.org

25. McMurdie PJ, Holmes S. phyloseq: an R Package for reproducible interactive analysis and graphics of microbiome census data. *PLOS ONE*. 2013; 8:e61217. https://doi.org/10.1371/journal.pone.0061217 PMID: 23630581

26. Anderson MJ. A new method for non-parametric multivariate analysis of variance. *Austral Ecol*. 2001; 26: 32–46
27. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O’Hara RB, et al. Vegan: Community Ecology Package. 2015. R package vegan, version 2.2–1.
28. Parks DH, Tyson GW, Hugenholtz P, Beiko RG. STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics*. 2014; 30: 3123–3124. https://doi.org/10.1093/bioinformatics/btu494 PMID: 25061070
29. Musekiwa A, Manda SO, Mwambi HG, Chen DG. Meta-Analysis of Effect Sizes Reported at Multiple Time Points Using General Linear Mixed Model. *PLoS One*. 2016; 11(10):e0164314. https://doi.org/10.1371/journal.pone.0164314 PMID: 2779661
30. Yasmin F, Tun HM, Konya TB, Guttmann DS, Chari RS, Field CJ, et al. Cesarean Section, Formula Feeding, and Infant Antibiotic Exposure: Separate and Combined Impacts on Gut Microbial Changes in Later Infancy. *Front Pediatr*. 2017; 5:200. https://doi.org/10.3389/fped.2017.00200 PMID: 29018787
31. Cacho NT, Harrison NA, Parker LA, Padgett KA, Lemas DJ, Marcial GE, et al. Personalization of the Microbiota of Donor Human Milk with Mother’s Own Milk. *Front Microbiol*. 2017; 8:1470. https://doi.org/10.3389/fmicb.2017.01470 PMID: 28824595
32. Dobbler PT, Procianny RS, Mai V, Silveira RC, Corso AL, Rojas BS, et al. Low Microbial Diversity and Abnormal Microbial Succession Is Associated with Necrotizing Enterocolitis in Preterm Infants. *Front Microbiol*. 2017; 8:2243. https://doi.org/10.3389/fmicb.2017.02243 PMID: 29187842
33. Gritz EC, Bhandari V. The human neonatal gut microbiome: a brief review. *Front. Pediatr*. 2015; 3:17. https://doi.org/10.3389/fped.2015.00017 PMID: 25798435
34. Murphy K, Curley D, Pirmohamed M, Carrol ED. The role of Clostridium difficile in the paediatric and neonatal gut—a narrative review. *Eur J Clin Microbiol Infect Dis*. 2016; 35:1047–57. https://doi.org/10.1007/s10096-016-2639-3 PMID: 27107991
35. Lees EA, Miyajima F, Carrol ED. The role of Clostridium difficile in the paediatric and neonatal gut—a narrative review. *Eur J Clin Microbiol Infect Dis*. 2016; 35:1047–57. https://doi.org/10.1007/s10096-016-2639-3 PMID: 27107991
36. Stearns JC, Zulyniak MA, de Souza RJ, Campbell NC, Fontes M, Shaikh M, et al. Ethnic and diet-related differences in the healthy infant microbiome. *Genome Med*. 2017; 9:32. https://doi.org/10.1186/s13073-017-0421-5 PMID: 28356137
37. Fallani M, Amari S, Uusijarvi A, Adam R, Khanna S, Aguilera M, et al. Determinants of the human infant intestinal microbiota after the introduction of first complementary foods in infant samples from five European centres. *Microbiology*. 2011; 157:1385–92.2. https://doi.org/10.1099/mic.0.042143-0 PMID: 21330436
38. Fallani M, Young D, Scott J, Norin E, Amari S, Adam R, et al. Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. *J. Pediatr. Gastroenterol. Nutr.* 2010; 51:77–84. https://doi.org/10.1097/MPG.0b013e3181d1b11e PMID: 20479681
39. Goulet O. Potential role of the intestinal microbiota in programming health and disease. *Nutr. Rev*. 2015; 73:32–40. https://doi.org/10.1093/nutrrev/nuv039 PMID: 26175488
40. Wallace JG, Gohir W, Sloboda DM. The impact of early life gut colonization on metabolic and obesogenic outcomes: what have animal models shown us? *J. Dev. Orig. Health Dis*. 2016; 7:15–24. https://doi.org/10.1017/S2040174415001518 PMID: 26399435
41. Lemas DJ, Yee S, Cacho N, Miller D, Cardel M, Gurka M, et al. Exploring the contribution of maternal antibiotics and breastfeeding to development of the infant microbiome and pediatric obesity. *Semin. Fetal Neonatal Med*. 2016; 21:406–409. https://doi.org/10.1016/j.siny.2016.04.013 PMID: 27424917
42. Harts LE, Bradshaw W, Brandon DH. Potential NICU environmental influences on the neonate’s microbiota: a systematic review. *Adv Neonatal Care*. 2015; 15; 324–335 https://doi.org/10.1097/ANC.0000000000000220 PMID: 26340035