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Impact of different types of infant-feeding on evolution of preterm gut microbiota

Impacto de diferentes tipos de alimentação infantil na evolução do microbiota intestinal do pré-termo

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Abstract

The importance associated with microbiota and metabolic imbalances has been increasing. Premature infants, given their susceptibility, require proper care and effective procedures. The aim of this study was to evaluate the impact of different feeding profiles on the gut microbiota preterm infants hospitalized at Maternidade Dr. Alfredo da Costa. For this purpose, a study was designed that gathered a total of four meconium and stool samples collected from premature infants with a periodicity of 7 days. Additionally, the mother was asked to collect her own feces and to respond to a food frequency questionnaire. Subsequently, the bacterial DNA present in the fecal samples was extracted, purified and quantified by real-time PCR. A total of 28 stool samples of preterm infants (n=6) and their mothers (n=4) were analyzed. It was found that *Bacteroides* were not presented in the samples of preterm infants, but *E.coli* content was much higher than *Lactobacillus* and *Bifidobacterium*. This study highlights the influence that maternal milk and the intake of formulas in the first days of life have on the evolution of the gut microbiota of preterm infants.

Key-words: intestinal microbiota, preterm infants, breast milk, donor human milk, formula.

Resumo

A importância associada ao microbiota e ao desequilíbrio metabólico tem vindo a crescer. Os bebés pré-termo, dada a tua a sua susceptibilidade, requerem cuidados próprios e procedimentos eficazes. O objetivo deste estudo foi avaliar o impacto de diferentes perfis alimentares no microbiota intestinal de bebés pré-termo hospitalizados na Maternidade Dr. Alfredo da Costa. Para este fim, foi desenhado um estudo que reuniu um total de quatro recolhas de amostras de mecónio e fezes de cada prematuro, com uma periodicidade de sete dias. Às mães também foi pedido que fizessem uma recolha das suas fezes e que respondessem a um questionário de frequência alimentar. Posteriormente, o DNA bacteriano presente nas amostras fecais foi extraído, purificado e quantificado por PCR em tempo real. Um total de 28 amostras de fezes dos prematuros (n=6) e das suas mães (n=4) foram analisadas. Verificou-se que nos bebés prematuros não havia *Bacteroides. No* entanto, na amostra presente, o contudo de *E. coli* foi muito maior do que *Lactobacillus* e *Bifidobacterium*. Este estudo destaca a influência que o leite materno e a ingestão fórmulas nos primeiros dias de vida têm na evolução do microbiota intestinal dos bebés pré-termo.

Palavras-Chave: microbiota, prematuros, leite materno, leite de dadora, formula

Contents

1. Introduction

It is well-known that the microbiota plays very important roles in the metabolic homeostasis. It is often cited that there are 10^{14} microbial cells, mostly in the gut, and 10^{13} human cells in the body. However, the theory that the human body contains ten times more microbial cells than human cells is not accepted by all scientists(1). In a recent study, which estimated the number of microbial cells, human cells and their ratio in the body, it was found that the bacterial and human cell ratio in a standard man (a man between 20-30 years of age, weighing 70 kg and 170 cm in height) is about 1:1(2). That should replace the 10:1 values that are stated in the literature. Although this number is much smaller than previously thought, the importance of the microbiota does not diminish. The interaction between the host and this biosystem results in the production of bacterial metabolites(3), which influence metabolic, biochemical and physiological processes(4,5), regulating: the absorption of energy from food(6); fat storage(6,7); the development of tumor cells(3); levels of circulating leptin; insulin resistence(8); and the immune system, which may lead to increased expression of inflammatory factors(7).

Until very recently, it was believed that intrauterine environment was sterile and the presence of bacteria in meconium and/or amniotic fluid functioned as a marker of pathological infections usually associated with preterm birth(9). Although the origin of the intrauterine microbiota is not known, it was found an unique microbial communities in amniotic fluid and placenta even when no membrane rupture has occurred, which may provide the initial gut colonisation(10–12). Bacteria could be isolated from meconium of healthy neonates and this bacterial composition is

affect (both qualitatively and quantitatively) by the mother(13). To study the impact of microbial transmission, gut microbiota samples from women in the third trimester were transferred to germ-free mice and it was shown that mice gained more weight, became more insulin resistant and had higher levels of inflammatory markers. Furthermore, it was found that the children's microbiota were most similar to their mothers' microbiota at first trimester(14). This evidence suggested that the microbial transmission of the mother to the child has a preponderant role, being that care should be taken not only during pregnancy but also before conception. If the time before birth is important, the mode of delivery has significant differences on the newborn's microbiota(11,15,16), even not all studies confirm this(12). Others factors may effect colonization process, such as antibiotic exposure during and after pregnancy. One study that gathered 10 premature infants, found that only one single infant was dominated by *Bifidobacterium*, since it was also the only baby that did not receive antibiotic treatment during the first four weeks of life(17). In addition, a recent study that analyzed 436 mother-child dyads followed until 7 years of age, found that children born from mothers who were given antibiotics during the second or third trimester of pregnancy had an 84% higher risk of obesity at age 7 years, but the use of antibiotics in the first trimester had no effect(18). The type of infant-feeding also plays a major role in the development of the preterm microbiota, not only because of the functional and morphological immaturity of the gastrointestinal tract, but also because they require prolonged hospitalization and often develops a 'neonatal intensive care unit microbiota'. The education and maturation of the intestinal immune system are the results of dietary adequacy, providing all macro and micronutrients, antioxidants and immunomodulatory factors. Breast milk modulates the infant gut

microbiota because of its richness in oligosaccharides, nucleotides, fatty acids, immunoglobulins, cytokines, immune cells, lysozyme, lactoferrin and commensal bacteria(19). Breast milk is not only a food rich in probiotics, but also in prebiotics because of the high content in oligosaccharides. The HMOs – human milk oligosaccharides – are the third largest component of human milk and because they are not digestible by the baby, they shape the composition of the intestinal microbiota through selective consumption by commensal bacteria. There are several benefits associated with milk intake, such as: decreased rates of late-onset sepsis; reduces the incidence of NEC and retinopathy of prematurity; decrease re-hospitalization in the first year of life; improved neurodevelopmental outcomes; and decreased rates of metabolic syndrome and blood pressure(20). When the mother's own milk is not available, donor human milk (DHM) should be the first alternative option. It is important to note that DHM is not the same as mother's own milk, since the DHM must to be pasteurized and stored at - 20 °C. Still, DHM is always preferred over the formulas milk. Even though the DHM and formula contain many ingredients similar to an infant's own mother's milk, the effects are not the same. Formula-fed infants decreased the diversity if the genus *Bifidobacterium* and this was associated with increased adiposity at 18 months, which is not the case with breastfed infants(21).

2. Study aims

The aim of this study was to evaluate the impact of the introduction of different types of infant feeding (breast milk, DHM and formulas) on the intestinal microbiota of preterm infants hospitalized as the Neonatal Intensive Care Unit

(NICU) of the Maternity Dr. Alfredo da Costa. In this way, it was also evaluate how the mode of delivery and the feeding behavior of the mother during pregnancy can influence the effect of feeding profile on the preterm microbiota.

3. Material and Methods

3.1. Ethics Statement

The study was approved by the Health Ethic Committee of Hospital Central Lisbon Center (Ref. 443/2017). Samples and clinical information were obtained after informed written consent by the study infants' mothers.

3.2. Study design

In this observational prospective study, a sample of newborn preterm infants was recruited to evaluate the impact of different types of infant feeding on the composition of the intestinal microbiota. The recruitment of the participants was done respecting the order of entry in the NICU in Maternidade Dr. Alfredo da Costa, since 25 of May of 2017. After that, a total of four meconium and stool samples were collected from premature infants. Stool sampling kits (EasySampler® - Complete Stool Collection kit) were delivered to each mother to collect faeces as soon as possible after delivery. Mothers also were invited to respond to a semi-quantitative food frequency questionnaire (validated by Hygiene and Epidemiology Department of the Faculdade de Medicina Universidade do Porto)(22).

An overview of the study procedures is shown in Figure 1.

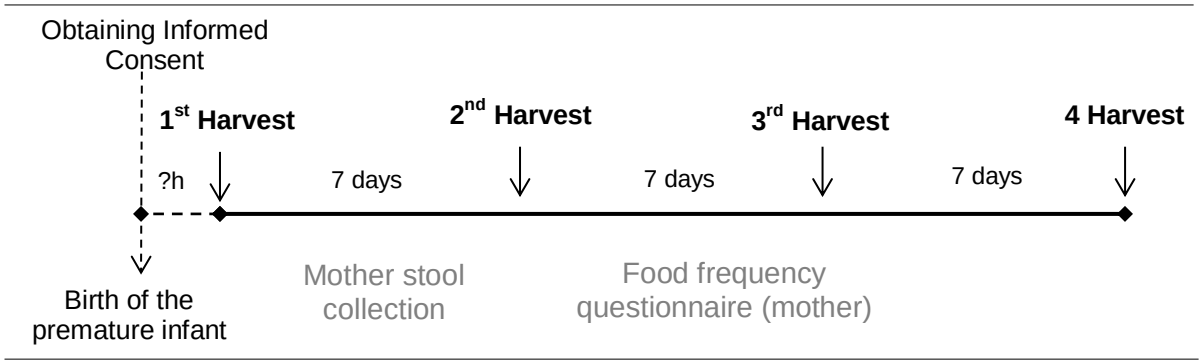

Figure 1. Study procedures. After informed consent is obtained, the infant entered the study and four stool samples were taken as NIUC. After the fourth harvest, the baby left the study. At the same time, the mother collected her feces and answered a food frequency questionnaire.

3.3. Participants recruitment

Preterm infants hospitalized in the NICU in Dr. Alfredo da Costa Maternity and their mothers were recruited. Written informed parental consent was obtained for each preterm infant before birth (if the mother was hospitalized in maternal-fetal care unit before delivery) or after birth (if the mother had not been previously admitted in the maternity). To be eligible for enrolment, preterm infants must have been admitted to the NICU in less than 24 hours of life, have been born with less than 32 weeks with absence of malformations or metabolic diseases.

3.4. Sample collection

Meconium, the newborn's first intestinal discharge that represents material ingested or secreted by the gastrointestinal tract during fetal life, including amniotic fluid, epithelial cells and mucus(11), and the remaining 3 fecal samples of the preterm infants were collected by the nursing team of the Neonatology Service of the maternity. Fecal samples were collected weekly from diapers into sterile tubes.

Mother's fecal samples were collected by themselves with an appropriate kit previously provided. Samples were kept refrigerated waiting for being stored at -20°C until further analysis.

3.5. DNA extraction, purification and amplification

DNA was extracted and purified from meconium and fecal samples (170-200 mg) using NZY Tissue gDNA Isolation Kit (nyztech, Lisbon, Portugal)(23). First, faeces were homogenized in TE buffer (10 mM Tris/HCl; 1 mM EDTA, pH 8.0) and centrifuged at 4000 x *g* for 15 min. The supernatant was discarded and 350 µl of buffer NT1 was added to the precipitate. Then, samples were incubated at 95 °C for 10 min and it was again centrifuged at 11,000 x *g* for 10 min; 25 µl of proteinase K were added at 200 µl of the supernatant and incubated at 70 °C for 10 min. All subsequent steps were followed according to manufacturer's instructions. DNA purification and quantification was assessed with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Amplification of the DNA was performed using real-time polymerase chain reaction (RT-PCR) in sealed 96-well microplates using LightCycler FastStart DNA Master SYBR Green kit and a LightCycler instrument (Roche Applied Science, Indianapolis, ID, USA). The mixtures of PCR reactions had a total of 10 µl (5 µl of 2 x FastStart SYBR Green, 0.2 µl of each primer, 3.6 µl of water and 1 µl of DNA). *Bacteroides, Lactobacillus, Bifidobacterium* and *Escherichia coli* were quantified through RT-PCR by using specific 16S rRNA gene targeted primers. Primer sequences and PCR amplification reaction conditions are described in Table 1.

Table 1. Primers sequences used for gut microbiota analysis. AT, annealing temperature.

Target group	Primer sequence (5'-3')	Genomic DNA Standard	AT	Reference
Proteobacteria	CIA GTG TAG AGG TGA AAT T CCC CGT CAA TTC CTT TGA GTT	*E. coli* ATCC 25922	61.5°C	(24)
Escherichia coli	GTA AGT TAC ACT ATA AAA GCA CCG TCG TCT GTG TGG ATG GTA ATA AAT TTT TG	*E. coli* ATCC 25922	60°C	(25)
Firmicutes	ATG TGG TTT AAT TCG AAG CA AGC TGA CGA CAA CCA TGC AC	*Lactobacillus gasseri* ATCC 33323	60°C	(23)
Staphylococcus	GAT GTG CGA AAG CGT GGG GAT GAA CTG AGA ACA ACT TTA TGG GA	*S. aureus* ATCC 12600	60°C	(16)
Clostridium	AAA GGA AGA TTA ATA CCG CAT AA ATC TTG CGA CCG TAC TCC CC	*C. perfringens* AF316589	60°C	(26)
Enterococcus	CCC TTA TTG TTA GTT GCC ATC ATT ACT CGT TGT ACT TCC CT TGT	*Enterococcus gilvus* ATCC BAA-350	61°C	(23)
Lactobacillus	GAG GCA GCA GTA GGG AAT CTT C GGC CAG TTA CTA CCT CTA TCC TTC TTC	*Lactobacillus gasseri* ATCC 33323	60°C	(23)
Actinobacteria	TAC GGC CGC AAG GCT A TCR TCC CCA CCT TCC TCC G	*Bifidobacterium longum* ATCC 15697	61.5°C	(24)
Bifidobacterium	CGC GTC YGG TGT GAA AG CCC CAC ATC CAG CAT CCA	*Bifidobacterium longum* ATCC 15697	60°C	(23)
Bacteroides	ATA GCC TTT CGA AAG RAA GAT CCA GTA TCA ACT GCA ATT TTA	*Bacteroides vulgatus* ATCC 33563	60°C	(23)

3.6. Statistical analysis

Data are expressed as mean ± standard deviation (SD). The composition of fecal microbiota of the preterm infants and their respective mothers are presented as $\log_{10}$ 16S rRNA gene copies/10 ng DNA. To compare differences in the microbial composition of preterm infants between the two harvest, the Wilcoxon test was used. The Mann-Whitney test was used to evaluate the influence of the mode of delivery in the microbial composition. In order to evaluate the impact of different types of feeding on the evolution of gut microbiota composition, a non-parametric test (Kruskal-Wallis) was used. The differences were considered statistically significant when $p<0.05$. All data were analyzed using SPSS software (24.0 version).

4. Preliminary results*

4.1. Characteristics of the infants and their mothers

A total of six preterm infants were enrolled in the study from May 25 until June 12, with the last harvest on July 4. Of these, only one was female and two are twins. Infants were born with a mean gestational age of 29 weeks and with 1.233 ± 0.293 kg. Feeding of premature babies is very heterogeneous depending not exclusively on their clinical conditions, but also on the availability of breast milk. However, enteral feeding of preterm infants only varies between breast milk, DHM of formula. In this sample only two babies were born by vaginal delivery and the others were born by C-section, which are the same ones that were fed predominantly with formula. Relevant demographic and clinical data recorded for each infant are described in Table 2.

Table 2. Demographic and clinical characteristics of the preterm infants recruited in this study. GA, gestational age; VD, vaginal delivery; C-section, cesarean section.

Infant	Gender	GA (week)	Delivery mode	Birth weight (kg)	Sample Collection (days of life)
1	Female	28	C-section	1.320	17; 26
2	Male	30	C-section	1.005	18; 25
3	Male	30	C-section	1.335	18; 25
4	Male	30	VD	1.530	16; 23
5	Male	25	VD	0.745	17; 24
6	Male	29	C-section	1.460	15; 22

The mothers of the 6 preterm infants were previously admitted to the maternal-fetal unit at risk of preterm labor. At this time, the mothers were invited to participate in the study. During the time of study it was not possible to find the twin's mother, so she did not collect her stool sample and did not answers the food frequency questionnaire, counting a total of 4 mothers include in the study. The

* The present work describes only the beginning of what is a research project that is now starting. In this sense, the number of premature infants recruited until now has been low, which limits the statistical analysis of the data

mothers were between the ages of 25 and 38 years old, were all Caucasian and resident in Lisbon.

4.2. Microbiota composition of fecal samples

A total of 24 stool samples were collected from 6 preterm infants during their first three weeks of life. Although it was initially proposed to analyze four fecal samples of each preterm infant, the analysis of the first and second collection was not possible since the nursing team collected small amounts of feces. Thus, only the fecal samples corresponding to the third and fourth collections (that corresponds to the 2nd and 3rd weeks of life) were analyzed. Only the abundance of *Bacteroides, Lactobacillus, Bifidobacterium* and *E. coli* were quantified and analyzed in fecal samples of premature infants and their mothers, since that real-time quantification process for the remaining primers presented in Table 1 was not optimized in time for the present work. *Bacteroides* were not presented in the fecal samples of preterm infants. On the other hand, their mothers presented high levels of *Bacteroides* (4.33 ± 1.22). The mean abundance of *Lactobacillus, Bifidobacterium* and *E. coli* at third harvest was 0.52 ± 0.86, 0.55 ± 0.85 and 4.49 ± 1.54, respectively. Figure 2 presents the evolution of the abundance of *Lactobacillus*, *Bifidobacterium* and *E. coli* of each baby between the third and fourth collection. From the second to the third weeks, no significant differences were found in the microbial composition of preterm infants.

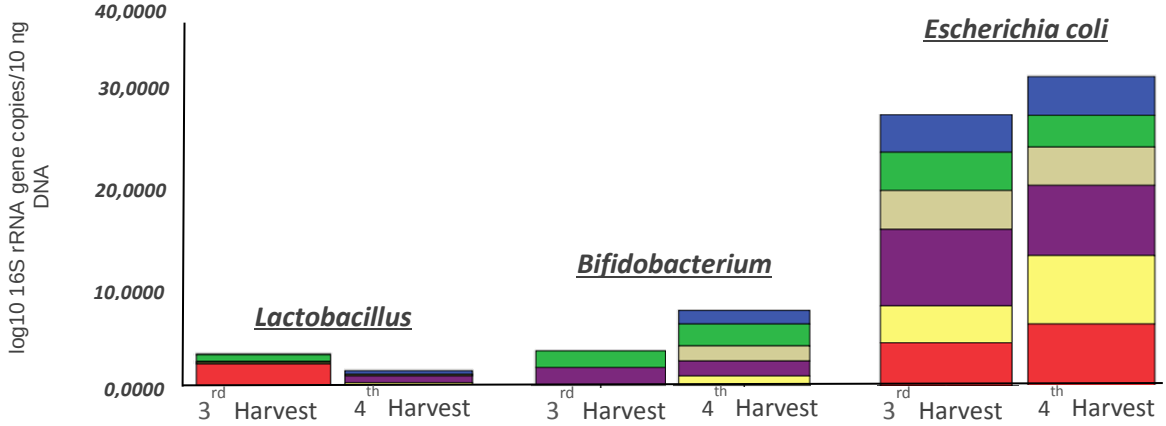

Figure 2. Gut microbiota of the feces samples ofpreterm infant at third and fourth harvest.

4.3. Influence of mode of delivery on gut microbiota

The microbial composition of neonates at 4th harvest (mean of days of life = 24.2) born by vaginal delivery (n=2) or by C-section (n=4) are described in Table 3. The babies delivery by caesarean section had more abundance of *E. coli*, *Bifidobacterium* and finally *Lactobacillus, and no Bacteroides*. This trend is seen in two preterm babies born by vaginal delivery. Thus, delivery mode in these preliminary results had no a significant impact on the bacteria analysed. However, *Lactobacillus* seems to be more abundant in preterm babies born by a vaginal delivery. In addition, *E. coli* seems to be more abundant in preterm babies born by C-section delivery.

Table 3. Abundance of *Lactobacillus*, *Bifidobacterium* and *E. coli* in neonates born by vaginal delivery or by C-section.

	Vaginal Delivery	**C-section**
Lactobacillus	0.44 ± 0.27	0.12 ± 0.14
Bifidobacterium	1.17 ± 0.37	1.22 ± 0.89
E. coli	6.94 ± 1.12	4.29 ± 1.37

4.4. Effects of different type of feeding on the gut microbiota

Enteral feeding was introduced between postnatal day 1 to day 4, depending on the infant's clinical condition. 66.7% of the total infants were fed predominantly with formula, although all babies were given formula during the study period. The feeding profile of preterm infants was categorized according to the type of feeding predominated (≥50%) in the previous seven days of sample collection. In this way, three feeding profiles were drawn: infants who were fed formula, n=4; with fortified breast milk, n=1; or breast milk preceding formula, n=1. There are no associations between bacterial composition and the feeding profile ($p > 0.05$).

Table 4 shows how the microbial composition was distributed among the 3rd and 4th harvest according to the feeding profile of preterm infants.

Table 4. Bacteria abundance according to different feeding profiles.

		Feeding Profile		
		Formula (n =4)	**Fortified breast milk** (n =1)	**Breast milk preceding formula** (n =1)
Lactobacillus	3rd harvest	0.73 ± 1.03	0.08	0.14
	4th harvest	0.12 ± 0.14	0.63	0.26
	Δ	**-2.9 ±1,01**	**0.55**	**0.12**
Bifidobacterium	3rd harvest	0.40 ± 0.79	1.69	0.00
	4th harvest	1.22 ± 0.89	1.43	3.65
	Δ	**0.65 ± 0.69**	**-0.26**	**3.65**
E. coli	3rd harvest	3.92 ± 0.24	7.60	3.67
	4th harvest	4.29 ± 1.37	7.02	6.05
	Δ	**0.68 ± 1.56**	**-0.58**	**2.38**

In order to verify the difference in *Lactobacillus*, Bifidobacterium and *E. coli* abundance between the 3rd and 4th harvest, the variance it was calculated. In formula-fed infants, the *Lactobacillus* amount decreased, contrasting with Bifidobacterium and *E. coli. However*, these variations are not significant. In preterm infants fed with fortified breast milk, the amount of *Bifiobacterium* and *E. coli* decreased, and *Lactobacillus* increased. In infants initially fed with breast milk and then formula, the abundance of three bacteria increased between the 3rd harvest and 4th harvest.

5. Discussion and conclusion

The meconium and feces collection are the simplest and least invasive way of analyzing microbial composition. The microbiota composition of the full-term infants is significantly different from that of the preterm infant(27) - “preterm”, as WHO defined, is an infant born before 37 weeks of pregnancy, and “very preterm”

a baby born between 28 and 32 weeks(28). The premature, given the circumstances, presents a marked vulnerability to dysbiosis, with a change in abundance, diversity and progressive acquisition of the intestinal microbiota. The microbiota of the preterm infant were dominated by *Proteobacteria* (46%), *Firmicutes* (45%), *Bacteroides* (7%) and *Actinobacteria* (2%)(17). Late-onset sepsis and necrotizing enterocolitis (NEC) were associated with microbiomes dominated by *Proteobacteria* and *Firmicutes,* a very common cause of morbidity and mortality in preterm infants(16). The abundance of *Bifidobacterium* and *Lactobacillus* genera were very low in these subjects. However, it is important to note that there is a very high variability between preterm infants(17). Several factors may justify this variability in the composition of the intestinal microbiota of newborns, such as the mother's diet and lifestyle before and during pregnancy, mode of delivery, antibiotic intake and type of feeding.

Maternal diet shapes microbiota profile

Fetal and neonate life are characterized by an enormous capacity to respond to environmental factors, altering the levels of gene expression through epigenetic modifications. If we think that during the gestational period the only environment that fetus knows is that of the mother, she carries a giant responsibility in the development and growth of her child. Fetal exposure to excess blood lipids, particularly saturated fatty acids, can activate proinflammatory pathways, which could impact substrate metabolism and affect organ development and the response to the postnatal environmental factors, since the epigenetic regulation of gene expression is characterized by covalent modifications to DNA and chromatin that alter gene expression independent of gene sequence(29). A recent and very well-structured review lists a number of pathological conditions associated with

teratogenicity due to maternal obesity, gestational diabetes and/or excessive weight gain throughout pregnancy, such as the development in later years of obesity, diabetes, non-alcoholic fatty liver disease and metabolic-syndrome(21). Differences in the microbiota acquisition, composition and activity during early infancy are associated with the pregnancy BMI and weight gain of mothers during pregnancy(30). In the current study, not all mothers of preterm infants included in this study answered to food frequency questionnaire, which made it impossible to analyze the data.

The mode of delivery matters

While babies born by vaginally delivered receive a microbiota similar to the maternal vagina, C-section delivered infants are enriched in skin microbiota, that is associated with a higher incidence of diseases in adulthood(15). As already been cited “with the trend of increasing C-section deliveries, there has been an epidemic of both autoimmune disease such as type 1 diabetes, Crohn’s disease, multiple sclerosis and allergic diseases, such as asthma, allergic rhinitis, and atopic dermatitis”(31). Compared with vaginal delivery, C-section was associated with lower biodiversity, a delay in colonization by beneficial bacteria and higher colonized by *Staphylococcus*, but less colonized by *Enterococcus*(16). Babies born by C-section were more highly colonized by *Clostridium* at one month of life, that was involved in NEC(16). The mode of delivery may play a decisive role in the development and growth of a premature infant, since the bacteria present in fetal gastrointestinal tract can influence the development of the microbiota, the immune system and therefore have relevant health consequences(11). Since it was not possible to make the analysis of the samples corresponding to the first

two collections, the major impact of the mode of delivery remained to be evaluated. However, at 24 days of life the infants evaluated in this study presented higher amounts of *E. coli*, regardless of the way of delivery.

The type of infant feeding affects the composition of the intestinal microbiota

The literature suggests that breast milk still contains 10^9 bacteria per liter(32). Breast milk induces a less diverse microbiota dominated by *Bifidobacterium* compared with formula feeding that is associated with an abundance of *Enterobacteriaceae*, *Bacteroides* and *Clostridum*(32). Breast milk should always be the food of choice since it contains immunomodulatory nutrients (oligosaccharides) and growth factors(8). Studies have reported the presence of bioactive compounds, such as IgA, lactoferrin, β-lactoglobulin and α-lactalbumin, which directly or indirectly affect microbial development. Exposure to a diversity of commensal species regulate local microbial growth, modulate the morphology and function of enterocytes, reduce activation of proinflammatory cascade, and affect gene expression(4). The numerous benefits associated with breast milk microbiome and the mechanism by which bacteria reach the mammary gland need to be further studied. However, there are three hypotheses that may explain the migration of bacteria into breast milk: increased permeability of the gastrointestinal tract during pregnancy, allowing the passage of bacteria by bloodstream into the mammary gland; direct contamination by the mother's skin and oral secretions from the infant; active migration of bacteria with the aid of dendritic cells(33).

In certain circumstances, administration of pasteurized DHM is necessary and, as described, promotes a microbiome partially identical to that stimulated by breast milk(34). The heat treatment this milk leads to the inactivation of viruses and the largest thermosensitive bacteria. B and T lymphocytes, macrophages, neutrophils,

as well as lipoprotein lipase and IgM are also inactivated. While 40% of lactoferrin, 75% of lysozyme and 70-80% of IgA and IgG are preserved, fatty acids are practically unchanged, as the oligosaccharides, proteins and most of vitamins and minerals(35). In preterm infants the intake of both breast milk and DHM were associated with lower rates of NEC, better feeding tolerance, shorter duration of hospital stay, and reduced hospital costs(36). In a NICU the use of formulas is very frequent because mothers cannot always produce enough milk and the milk bank lacks donors. Once dietary supplementation begins, microbiota profile of breast-fed infant's changes toward formula-fed infant profile, with significant increase in the count of *Enterococci* and *Enterobacteriaceae*, and the appearance of *Bacteroides* and *Clostridium*(5). Although no significant differences were observed between breast milk and formula-fed term infants after one week of life, there was a significant difference after two and three weeks of life. Formula-fed infants had higher fat-free mass throughout in first year of life(37).

Knowledge of the external factors responsible for derivation and the characterization of microbial composition in preterm infants are essential in order to explore interventions that contribute to the overall health and quality of life of these children and future adults. To assess the impact of different feeding profiles on the gut microbiota of preterm infants, as well as the impact of other variable already mentioned, it will be necessary to gather a large number of infants.

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