# IMPACT OF INFANT FEEDING ON THE DEVELOPMENT OF PRETERM GUT MICROBIOTA

JULIANA PEREIRA MORAIS

A dissertation submitted in partial fulfillment of the requirements for the Degree of Masters in Biomedical Research

*Dissertação para obtenção do grau de Mestre em Investigação Biomédica*

at NOVA Medical School | Faculdade de Ciências Médicas of Universidade NOVA de Lisboa

September, 2019

# IMPACT OF INFANT FEEDING ON THE DEVELOPMENT OF PRETERM GUT MICROBIOTA

Juliana Pereira Morais

Supervisor: Ana Faria, Assistant Professor

Faculdade de Ciências Médicas|NOVA Medical School Universidade NOVA de Lisboa

Co-Supervisor: Cláudia Marques, Invited Assistant Professor

NOVA Medical School|Faculdade de Ciências Médicas Universidade NOVA de Lisboa

A dissertation submitted in partial fulfillment of the requirements for the Degree of Masters in Biomedical Research

*Dissertação para obtenção do grau de Mestre em Investigação Biomédica*

September, 2019

The experimental work was performed at the Nova Medical School|Faculdade de Ciências Médicas, Universidade de Lisboa, under the supervison of Professor Ana Faria and Professor Cláudia Marques. The close collaboration with different units, namely Maternidade Dr. Alfredo da Costa and Centro de Investigação em Tecnologias e Serviços de Saúde (CINTESIS) was crucial for the success of this research. The FEEDMI trial was supported by Milupa DN-ELN2017 grant awarded by the Sociedade Portuguesa de Neonatologia and ERDF through the operation POCI-01-0145-FEDER-007746 funded by the Programa Operacional Competitividade e Internacionalização – COMPETE2020 and by National Funds through FCT - CINTESIS, R&D Unit (UID/IC/4255/2013).

The candidate states that actively contributed to the experimental works as well as in the interpretation and discussion of the results presented in this dissertation. The candidate also actively contributed to the writing of the published paper presented in this thesis:

Morais J, Marques C, Teixeira D, Durão C, Faria A, Brito S, Cardoso M, Macedo I, Tomé, T, Calhau C.: FEEDMI: A Study Protocol to Determine the Influence of Infant-Feeding on Very-Preterm-Infant's Gut Microbiota. Neonaotlogy 2019;27:1–6.

## ACKNOWLEDGMENTS

My first acknowledgments go to the parents of the preterm infants who participate in this research, to the nursing team of NICU and of the Human Milk Bank. To the Portuguese Neonatal Society, for the financial support through a grant. To Sara, Dra. Manuela, Doutor Israel and Dra. Teresa Tomé, my sincere thanks.

To Professora Doutora Conceição Calhau, an example of wisdom and determination. Thank you for letting me join the group during these (almost) three years. You have given me the opportunity to learn and better understand the relationship between nutrition and gut microbiota of infants prematurely delivered (and thousands of other things).

To Professora Doutora Ana Faria and Professora Doutora Cláudia Marques for the brilliant orientation during this thesis. I will always be grateful to your support, professionalism and expertise.

To Professor Doutor Diogo and Professora Doutora Diana, thank so much for all the comprehensive advice and kind supervision. It is a great honor to work with you.

To Inês, this adventure started with you and I always carry you in my heart. Thank you for your friendship.

To Shamila, Catarina e Inês C., thank you for your smiles and supportive words.

To Professor Doutor Paulo Pereira, director of the NOVA Biomedical Research master programme, and to all my colleagues from the 1st edition of NBR. I learned a lot from all of you.

To Professora Doutora Maria de Lurdes Dapkevicius, thank you for inspiring me to do science. It all started with you.

To João Rodrigues, my friend and colleague, an example of courage and struggle. Thank you for tour wise and comforting words.

To all my crazy and lovely friends, from Viana to Azores, you make me a happier person. Specially to Pedro, my everyday friend.

To João, thank you for your friendship, support and love.

Last but not least, actually, the most important: my mother Leonor, my father Manuel and my brother Diogo. There are not enough words to express all my gratitude. Thank you for encouraging me to follow my heart and trust my gut.

## ABBREVIATIONS

| | |
|---|---|
| **BMI** | Body Mass Index |
| **DHM** | Donor Human Milk |
| **FFQ** | Food Frequency Questionnaire |
| **FISH** | Fluorescence *in situ* Hybridization |
| **FOS** | Fructooligosaccharides |
| **GOS** | Galatooligosaccharides |
| **GWG** | Gestational Weight Gain |
| **HM** | Human Milk |
| **HMO** | Human Milk Oligosaccharides |
| **HMP** | Human Microbiome Project |
| **LPS** | Lipopolysaccharide |
| **MAC** | Maternidade Dr. Alfredo da Costa |
| **MD** | Mediterranean Diet |
| **MetaHIT** | Metagenomics Of The Human Intestinal Tract |
| **MOM** | Mothers' Own Milk |
| **NEC** | Necrotizing Enterocolitis |
| **NICU** | Neonatal Intensive Care Unit |
| **NMS\|FCM** | NOVA Medical School\|Faculdade De Ciências Médicas, Universidade NOVA de Lisboa |
| **PCR** | Polymerase Chain Reaction |
| **SCFA** | Short-Chain Fatty Acids |
| **TLR** | Toll-Like Receptor |
| **TMAO** | Trimethylamine *N*-oxide |

## ABSTRACT

Background: Preterm infants are especially vulnerable to dysbiosis since their early gut microbiota is less abundant and diverse. When the first microbial colonizers reach infants' gut remains an open question. It is assumed that maternal microbiota can influence the infants' gut colonization, making it a critical player in the offspring's immune and endocrine systems, as well as in metabolic health. Infant feeding has been reported as a major factor influencing the gut microbiota. Thus, studying the preterm infant gut microbiota is a research priority to complement nutritional neonatal care.

Objective: The aim of this study was to evaluate the influence of different types of infant-feeding on the gut microbiota preterm infants. In addition, it was evaluated the preterm infants' meconium colonization and the influence of vertical microbiota transmission.

Methodology: The FEEDMI Trial is an observational longitudinal study that included very preterm infants (≤ 32 weeks of gestational age), hospitalized in the neonatal intensive care unit of Maternidade Dr. Alfredo da Costa. A total of four meconium and fecal samples from preterm infants were collected. Mothers were also asked to collect their fecal samples. Bacterial DNA present was extracted from samples and specific bacterial groups were quantified by RT-PCR.

Results: In total, 453 fecal samples were processed from 117 preterm infants and their mothers. 88% of meconium samples were colonized. *Proteobacteria* and *Firmicutes* were the most abundant phyla during the first 26$^{th}$ postnatal days of infants. Meconium microbiota of preterm infants born between 28 and 32 weeks gestation showed stronger correlations with their mothers' microbiota, as well as infants born by cesarean. Maternal factors significantly influenced the offspring's microbiota, specially the pre-gestational body mass index. Mode of delivery had a limited impact on infants' meconium with C-section promoting a greater amount of *E. coli*. Infant feeding takes time to influence the gut microbiota of preterm infants. When adjusted for gestational age, antibiotherapy and maternal diet, mothers' own milk (MOM) promoted a healthier gut microbiota with higher levels of total bacteria and *Bifidobacterium* compared to donor human milk (DHM) and formula. Nevertheless, these differences were lower in DHM than formula fed infants. It was also observed lower levels of *Firmicutes* in infants fed with formula after adjusting for the same factors.

Conclusions: The findings of this thesis suggest that infants' meconium may have bacterial DNA prior to birth and maternal factors may have a central role in this process. Furthermore, this thesis highlights the importance of human milk on gut microbiota composition of infants prematurely-delivered with MOM promoting higher levels of total bacteria and *Bifidobacterium*, which may be translated in future healthier outcomes.



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

## RESUMO

Introdução: Os bebés prematuros são especialmente vulneráveis a disbiose intestinal, uma vez que o seu microbiota é pouco abundante e diverso. O momento em que os primeiros microrganismos colonizam o intestino do recém-nascido é uma questão em aberto. Sabe-se que o microbiota materno pode influenciar a colonização do intestino dos bebés, tendo um papel crucial no desenvolvimento dos sistemas imunitário e endócrino, assim como na saúde metabólica dos mesmos. A alimentação tem sido descrita como um dos fatores mais importantes que influencia o microbiota da criança. Neste sentido, estudar o microbiota intestinal de bebés prematuros é uma prioridade para complementar os cuidados alimentares neonatais.

Objetivo: O objetivo deste estudo foi avaliar a influência dos diferentes tipos de alimentação infantil no microbiota intestinal de bebés prematuros. Mais ainda, foi analisada a colonização microbiana do mecónio destes bebés e a influência da transmissão vertical.

Metodologia: O FEEDMI é um estudo observacional e longitudinal que inclui bebés muito prematuros (≤ 32 semanas de gestação) hospitalizados nos cuidados intensivos neonatais da Maternidade Dr. Alfredo da Costa. Foram recolhidas quatro amostras de mecónio e fezes de bebés prematuros. Às mães também foi pedido que fizessem uma recolha das suas fezes e que respondessem a um questionário de frequência alimentar. O ADN bacteriano foi extraído das amostras e grupos específicos de bactérias foram quantificados por RT-PCR.

Resultados: No total, foram processadas 453 amostras fecais de 117 bebés prematuros e das suas mães. 88% das amostras de mecónio estavam colonizadas. *Proteobacteria* e *Firmicutes* foram os filos mais abundantes durante os primeiros 26 dias. O microbiota do mecónio de bebés nascidos entre as 28 e 32 mostrou ter correlações mais fortes com o microbiota das suas mães, assim com nos bebés nascidos por cesariana. A via de parto teve um efeito reduzido no mecónio dos bebés, sendo que a cesariana promoveu quantidades mais elevadas de *E. coli*. A influência da alimentação infantil no microbiota dos bebés prematuros não é imediata. Quando ajustado para idade gestacional, antibioterapia e dieta materna, o leite da própria mãe (LPM) promoveu um microbiota intestinal mais saudável com quantidades mais elevadas de bactérias totais e *Bifidobacterium*, quando comparado com o leite de dadora (LD) e formula. Contudo, estas diferenças foram inferiores nos prematuros alimentados com LD do que com formula. Também foram observados quantidades inferiores de *Firmicutes* nos bebés alimentados com fórmula, quando ajustado para os mesmos fatores.

Conclusão: Os resultados desta tese sugerem que o mecónio poderá ter ADN bacteriano antes do nascimento e fatores maternos podem ter um papel central neste processo. Mais ainda, esta tese evidência a importância do LPM na composição do microbiota intestinal de bebés nascidos prematuramente.



Palavras-chave: bebés prematuros, fatores maternos, formula, leite da própria mãe, leite de dadora, microbiota intestinal

COTENTS

## TABLE LIST

## FIGURE LIST

## INTRODUCTION

In the XVII century, Antonie van Leewenhoek described the rod, sphere, and spiral forms and movement of bacteria in his oral and fecal samples [1]. It was the first description of bacteria. The culture-based techniques allowed the identification and classification of several microorganisms in the human body, initially associated with infections and diseases. Nowadays, it is known that these microorganisms form a complex symbiotic relationship with their host. This bacterial network, together with non-bacterial members – viruses, fungi and archaea, forms a complex and dynamic ecosystem designed as microbiota [2]. Advances of the non-cultured techniques made possible the characterization and the better understanding of the encoded genes of microbes (microbiome) [2] and their role in the human health.

In the human body bacterial cells outnumber our own somatic and germ cells with an estimated count of $3.8\times10^{13}$ and $3.0\times10^{13}$ cells (1:1 ratio), respectively [3]. However, when bacterial cells are compared to nucleated human cells ($0.3\times10^{13}$, non-nucleated red blood cells is not included in the calculation) the ratio will be about 10:1 [3]. Although the number of these human-associated microbes is very variable between individuals [4], the human microbiota is composed by four dominant bacterial phyla: *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* (Figure 1) [5].

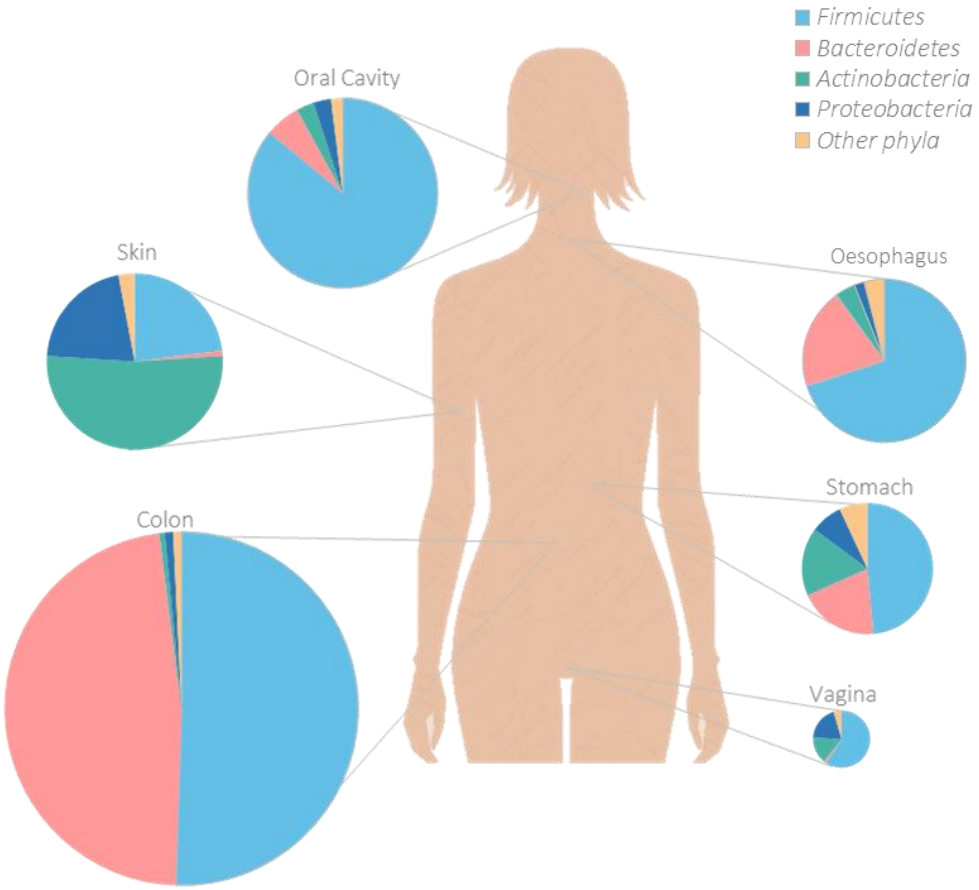


Figure 1 - Bacterial phyla composition at body human sites. Adapted from Dethlefsen *et al*, 2007 [5] and Blum, 2017 [6].

The microbes are present throughout the human body (Figure 1), mainly in the external and internal surfaces, such as skin, oral and nasal mucosa, conjunctiva, genital and urinary tracts [6], and even in mammary glands [7] and placenta [8]. But it is in the colon that inhabits the major bacterial community – the gut microbiota, representing one-half (0.2 kg) of the overall mass of the colon content [3].

Experimental and large population-scale projects focused on the potential association between gut microbiome and the human health and disease, such as Metagenomics of the Human Intestinal Tract (MetaHIT) [9] and Human Microbiome Project (HMP) [10], are essential for the understanding of this complex microbial ecosystem.

*Studying the human gut microbiota*

After the first observations of human-associated bacteria, Pasteur, Metchnikoff, Koch, Escherich, Nissle and other reference scientists, made great contributions in the microbiology field. However, it was only in 1940s with the development of methods to culture anaerobic organisms that the fascinating human-microorganism world has been gaining exponential interest. Nevertheless, it is not possible to culture the majority of bacterial species. Actually, it is estimated that only 1% of environmental bacterial are cultivated [11]. Later, the use of germ-free animals to study the influence of the gut microbiota on the host and, more recently, the development of sequencing-based approaches (non-cultured methods) provide detailed answers about the diversity, richness, composition and distribution of these microbes.

Since 1990s, it is possible to assess bacterial diversity by sequencing a small subunit ribosomal RNA gene (16S rRNA) [12]. The 16S rRNA gene is a highly conserved phylogenetic marker present in the major bacterial groups presents in the human gut [12]. Using the properly primers for amplification of single or multiple regions of the 16S rRNA by Polymerase Chain Reaction (PCR), the marker gene sequencing is a good method to determine microbial phylogenies of a sample [13]. The introduction of Next Generation Sequencing (NGS) replaced the 16S rRNA gene-based microbial profiling analysis using primers. Sequencing of the gut microbiota not only revealed new microbial species, but also microbial metabolic activities and how these correlate with human health and disease [14]. Table 1 show these and other “omics” techniques that can be used to detected and classify microbes, their genes, their products and clarify their functions.

More recently, synthetic microbial communities are being developed [11]. Using mathematical models and engineered microbes in artificial environments it is possible to study microbial interactions with each other and their responses to environmental factors [11].

Table 1 - Culture independent techniques for microbiome analysis and their interaction with the host by answering these essential questions: Who is there?; What pathways are activated?; What proteins are being produced?; How do they interact with the host?. Adapted from Ilhan, 2016 [15] and Méndez-García *et al*, 2018 [16].

| Method | Target | Technique | Information provided |
|---|---|---|---|
| Who is there? What pathways are activated?* | | | |
| Visual analysis | Encoded fluorescent nucleic acids/proteins | Fluorescence *in situ* hybridization (FISH) | Microbiome composition, function and spatial organization |
| Genomics<br>*Transcriptomics | Nucleic acids | 16S rRNA gene amplification; Shot gun (whole genome) sequencing | Amplification assembly |
| What proteins are being produced? | | | |
| Proteomics | Proteins | Mass spectrometry; Nuclear magnetic resonance Spectrometry | Protein identification and quantification |
| How do they interact with the host? | | | |
| Metabolomics | Metabolites | Mass spectrometry; Nuclear magnetic resonance Spectrometry | Identify and quantify secreted and intracellular microbial products and metabolites |

The identification of over 1000 bacterial species prove that the human gut microbiota is a dynamic and complex ecosystem [17].

*The gut microbiota is dynamic*

Contrasting to human genome, the microbiome is plastic. During the course of life the richness and diversity of bacterial composition change significantly [18]. The neonatal intestine is colonized by facultative and aerotolerant bacteria [*Proteobacteria* (*Enterobacteria*)] that will reduce oxygen content in the intestine to promote the anaerobic bacteria colonization [*Actinobacteria* (*Bifidobacterium*) and *Bacteroidetes* (*Bacteroides*)] [19]. At three years of age it is established the adult-like composition [20]. Since the very beginning, the architecture and functionally of the gut microbiota is highly dependent of host-endogenous and host-exogenous factors providing a very individual and unique microbial composition as a "microbiome fingerprint" [21]. Some of these factors include gestational age, mode of delivery, diet, exercise, antibiotic exposure, fasting, culture traditions, geography and genetic susceptibility (Figure 2) [22]. From all, diet is considered the most determinant factor for the gut microbiota [23].

*The gut microbiota is complex*

The human health success depends on these large and diverse host-bacteria community [2]. Actually, there are numerous physical and molecular mechanisms that allowed the symbiotic relationship between host and commensal microorganisms [24]. A successful commensal gut bacterial composition provide essential enzymes that digest the polysaccharides and peptides that are indigestible to human cells [24]. The role of the

gut microbiota in metabolism of dietary components through bacterial fermentation process results in the production of important and beneficial metabolites [25]. However, if there is inadequate intake of fermentable dietary components (such as fermentable fiber) the bacteria use alternative energy sources resulting in the production of metabolites that can be detrimental to human health [25].

Under the influence of the factors mentioned above these metabolites, such as short-chain fatty acids (SCFAs), trimethylamine *N*-oxide (TMAO), bile acids, gut hormones and others, may contribute to the regulation of human metabolism and immune system development and function and, consequently, to the host health or disease (Figure 2) [22,25]. Indeed, an imbalance in the composition, stability and resilience of gut microbiota (dysbiosis) is associated to the etiology and development of many diseases involving not only the gastrointestinal tract, but also other distal organs (Figure 2) [26].

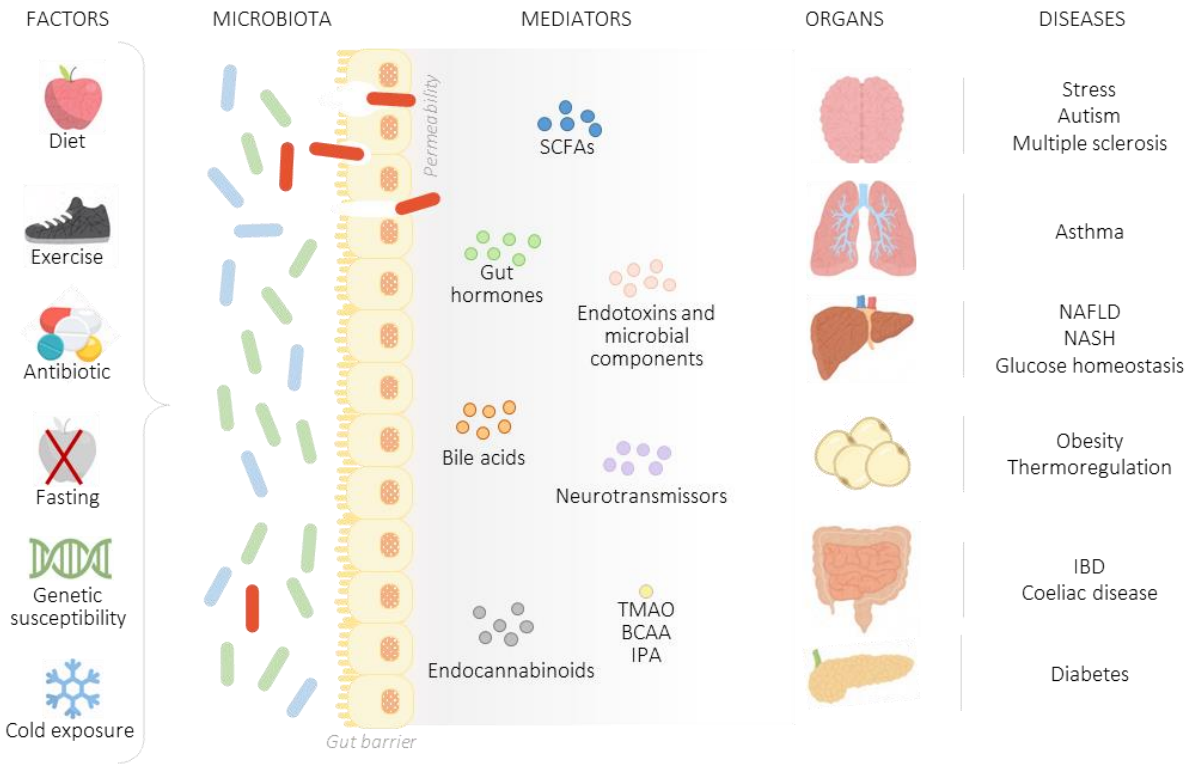


Figure 2 - Contribution of both host-endogenous and host-exogenous factors on the gut microbiota. Different bacteria produce different metabolites that can influence the disease development in the intestine and in other distal organs, through the regulation of host metabolism and immune system. SCFAs, short-chain fatty acids; TMAO, trimethylamine N-oxide; BCAA, branched-chain amino acid; IPA, indole propionic acid; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; IBD, inflammatory bowel disease. The image is an adaptation of Cani *et al*, 2019 [23] and Levy *et al*, 2017 [27].

It is imperative to understand how microbiota can be modulated with a more personalized medicine and nutrition treatments in order to prevent or delay the onset of the pathologies mentioned above. Identify

windows of opportunity for microbiota modulation can "revolutionize our approach to healthcare" [27]. In fact, a special focus is being given to pregnancy and preterm birth [27].

*Microbial colonization of the infant intestine*

The moment when the first microbial colonization occurs is an open question. Actually, the colonization process is a dogma between the "sterile womb" and "*in utero* colonization" hypotheses for more than 100 years.

Traditionally, it is accepted that vertical (maternal) and horizontal (from other persons and environment) transmission of microorganisms occurs only at birth, through vaginal canal and/or through contact with the mother's skin microbiota immediately after birth (Figure 3a). Most studies that mentioned the uterus bacterial-free used cell cultured and/or microscopy methods – methods that, although still valid, have several limitations. These studies, many from the XX century, have shown negative results for the detection of aerobic and anaerobic bacteria in amniotic fluid [28–31] and meconium (the newborn's first intestinal discharge comprising ingested or secreted material by the gastrointestinal tract during fetal life [32]) [33–35] of healthy pregnancies. However, there is no study with all samples sterile. The presence of bacteria in the amniotic fluid and placenta was limited to complications during pregnancy, such as premature labor, premature rupture of membranes and neonatal sepsis [36].

Later, Steel *et al.* (2005) studied fetal membranes of term and preterm deliveries [37]. The membrane samples were collected and placed in fixative within 30 min for FISH. Bacterial DNA was detected in term deliveries with (46% positive samples, n = 26) or without labor (73% positive samples, n = 26), preterm deliveries with labor (92% positive samples, n = 13) or without labor (83% positive samples, n = 12), and preterm delivered with prolonged premature rupture (81% positive samples, n = 22) [37]. In the same year (2005), Jiménez *et al.* (2005) reported the presence of bacteria in umbilical cord blood healthy neonates [38]. Later, two independent studies conducted by Aagaard and Mysorekar, detected bacterial content in placenta samples (n = 320 and n = 195, respectively) of women who gave birth prematurely with and without infection and also in women who had term healthy pregnancy [8,39]. However, the evidence is unclear. Lauder *et al.* (2016) did not found any difference between placental samples and negative controls [40]. A recent work "confidently" detected bacterial rRNA in placentas of 13 from 16 spontaneous preterm births and in 18 of 22 term unlabored cesareans, with no significant differences between preterm and term deliveries [41].

Bacteria communities were also detected in amniotic fluid [42,43]. However, a more recent study with 24 uncomplicated term pregnancies concluded that bacterial microbiota of amniotic fluid was indistinguishable from contaminated controls [44]. Interestingly, an Australian group wrote a critical comment about the

methodology used in that study [45]. The concerns raised were: sample processing, qPCR approach, and results analysis questioning the conclusions made [45].

Despite the growing evidence in favor of the hypothesis of colonization *in utero*, there is still no consensus among peers and several aspects have to be taken into consideration. A recent and exhaustive review pointed out several methodological limitations to studies suggesting that the microbial colonization occurs in the prenatal period, namely: (i) molecular techniques with an insufficient detection limit to analyze small microbial populations; (ii) lack of appropriate negative controls; and, (iii) lack of sterility in sample collection in clinical/hospital settings [36]. Another aspect to be considered is the contamination of samples by DNA extraction kits due to the 'kitome' present in the reagents and other components of the kits [46].

Although the placenta protects the fetus from bacterial infections, it is also the maternal-fetal communication organ (and not a barrier) providing oxygen, nutrients and, perhaps, bacteria or bacteria residue resulting from the action of antimicrobial peptides and immunoglobulins expressed in the placenta [36]. The *in utero* colonization hypothesis became stronger when animals studies suggested the maternal-fetal transfer of microbes. Using labeled *Enterococcus faecium* isolated from breast milk of healthy woman, pregnant mice were orally inoculated and then delivered the pups by C-section [38]. The labeled strain was detected in amniotic fluid [38] of these animals and in the pups' meconium [47]. External factors, such as maternal stress also influenced the gut microbiota of infant monkeys, reducing the content of *Lactobacillus* and *Bifidobacterium* [48]. In fact, the maternal transmission of microbes is a universally shared phenomena in the animal kingdom [49].

In humans, the studies are scare and the evidence is less conclusive [50]. Among 12 vaginally delivery mother-infants, 11 presented monophyletic bifidobacterial strains indicating a vertical transmission of bacteria [51]. Rautava *et al.* (2012) administered probiotic supplementation to mothers during pregnancy and the *Lactobacillus* group were detected in all placentas specimens [52]. In addition, Toll-like receptor (TLR)-related gene expression was associated with bacterial DNA in amniotic fluid and placenta [52], indicating that maternal-fetal transfer of bacteria could influence the immune development of the offspring [50]. Recently, it was reported a highly shared microbial population in a large cohort of mother and their infants (n = 415) [53]. Mechanism by which maternal bacteria pass to the fetus are not well understood [50]. However, two hypotheses are being considered (Figure 3b).

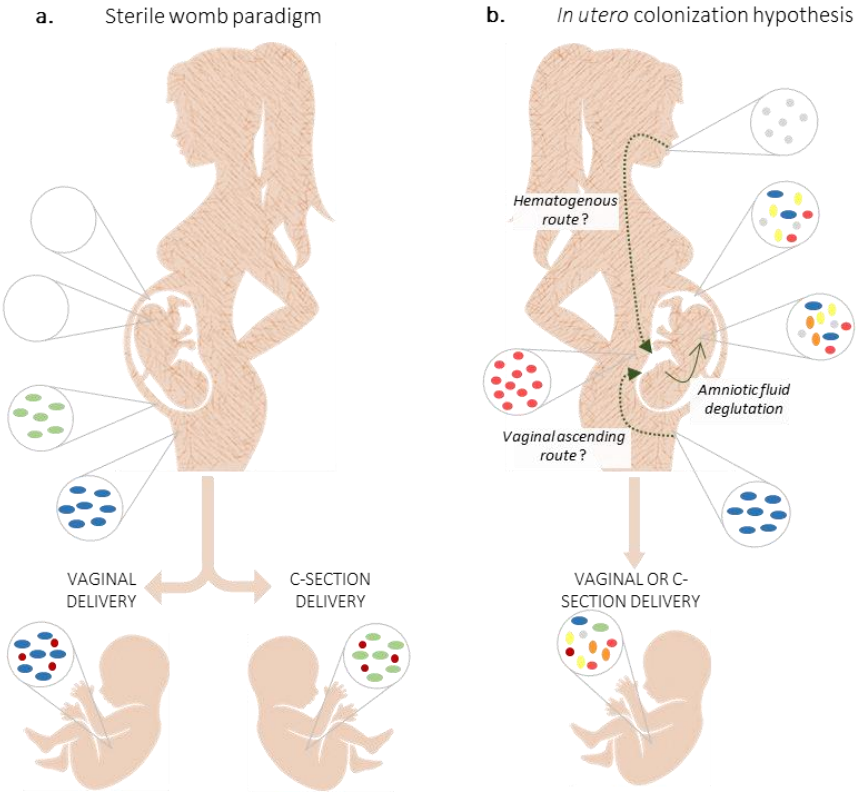


Figure 3 - Microbiota colonization of infants – two different hypothesis. a. Sterile womb paradigm defends that placenta and fetus are sterile and that gut microbiota is acquired during and after birth. Mode of delivery has been associated to transmission of specific bacteria: vaginal delivered promote a vagina-like microbiota in newborn and C-section leads to a bacterial colonization that resemble maternal skin microbiota b. The "in utero colonization hypothesis" suggests that bacterial colonization occurs prior to birth and it is influenced by maternal microbiota (oral cavity, gut and vagina). Two mechanism have been proposed to explain the mother-infant bacterial transmission: (i) the hematogenous bacterial route of bacteria from gastrointestinal tract (oral cavity and gut); and, (ii) ascension of bacteria from vaginal microbiota. Both routes argue that bacteria go into blood circulation and incorporated into the placenta decidua and, consequently, the developing fetus via amniotic fluid and cord blood [55]. The image is an adaptation of Perez-Muñoz *et al*, 2017 [37] and Milani *et al*, 2017 [55].

*Maternal influence in offspring microbiota*

Maternal factors can disrupt the *normal* infants' intestine colonization that play a critical role in the maturation of immune, endocrine and metabolic pathways and in development of disease later in life [54]. The prenatal nutrition and lifestyle are crucial for intrauterine fetal programming [55] and maternal microbial transmission seems to interact with biological systems being crucial for fetal health [50]. And even if this *in utero* colonization occurs only under certain circumstances (subclinical conditions), it is important to understand how, in order to optimize all mother, fetus and infants' health relationships. When mothers are obese, have (gestational) diabetes, increased insulin resistance and increased gestational weight gain (GWG) have an imbalanced and an unhealthy microbiota (dysbiosis) that is directly transmitted to the offspring [56]. As review

by Milani (2015), alterations in the endothelial integrity of placenta may lead to an permeable barrier and, consequently, the passage of bacteria and endotoxins, as lipopolysaccharide (LPS), into the cord blood and amnion [57]. Since fetal intestine is highly sensitivity to inflammatory mediators such as LPS, it is suggested that maternal microbes may trigger intestinal inflammation *in utero* [58].

During pregnancy the total gut bacteria abundance was reported to decrease, as well the bacterial diversity between the first and third trimesters and differences were observed between normal weight and overweight women [59,60]. To study the impact of microbial transmission, gut microbiota samples from women in the third trimester were transferred to gnotobiotic mice and it was shown that mice gained more weight, became more insulin resistant and had higher levels of inflammatory markers [59]. Furthermore, it was found that children's microbiota were most similar to their mothers' microbiota at first trimester [59]. This was the first study that used high-throughput sequencing to suggest that mother-to-child microbial transmission plays a major role, and care should be taken not only during pregnancy but also before conception. Even if the fetus is developed in a sterile environmental, it has been realized that metabolites and others molecular products of maternal gut microbiota are part of placental molecular exchange [61]. Therefore, maternal diet exposure before and during pregnancy and breastfeeding can influence the gut microbiota of their infants, with long-lasting effects [56].

Maternal microbiota metabolites of dietary components can reach the fetus via placenta or, after birth, through breast milk [61]. These molecules, or even maternal bacterial fragments, act as signals for the expansion of innate immune cells, development of intestinal epithelial cells and mucus, expression of antimicrobial peptides and secretion of antibodies into the intestinal lumen in offspring (Figure 4) [61]. In line with this, malnutrition (that includes deficiencies, excesses or imbalances in nutrients and/or energy intake) during pregnancy impairs fetal immune system through direct (for example, prolonged vitamin A deficiency leads to an impaired production of B lymphocytes [62]) and indirect (maternal immunosuppression decreased the transfer of immunoglobulins for fetus increasing the chance for infection [61]) mechanisms.

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 [63]. Furthermore, a prospective large cohort of 66 000 pregnant women reported that women adhering to a 'prudent' (characterized for high intake of vegetables, fruit, berries, nuts, whole grains, poultry and water as beverage) or a 'traditional' (rich in boiled potatoes, vegetables, lean fish and fish products, low fat milk and rice pudding) dietary patterns were at lower risk of preterm delivery compared with women with a 'Western' diet (salty and sweet snacks, white bread, desserts and processed meat products) [64].

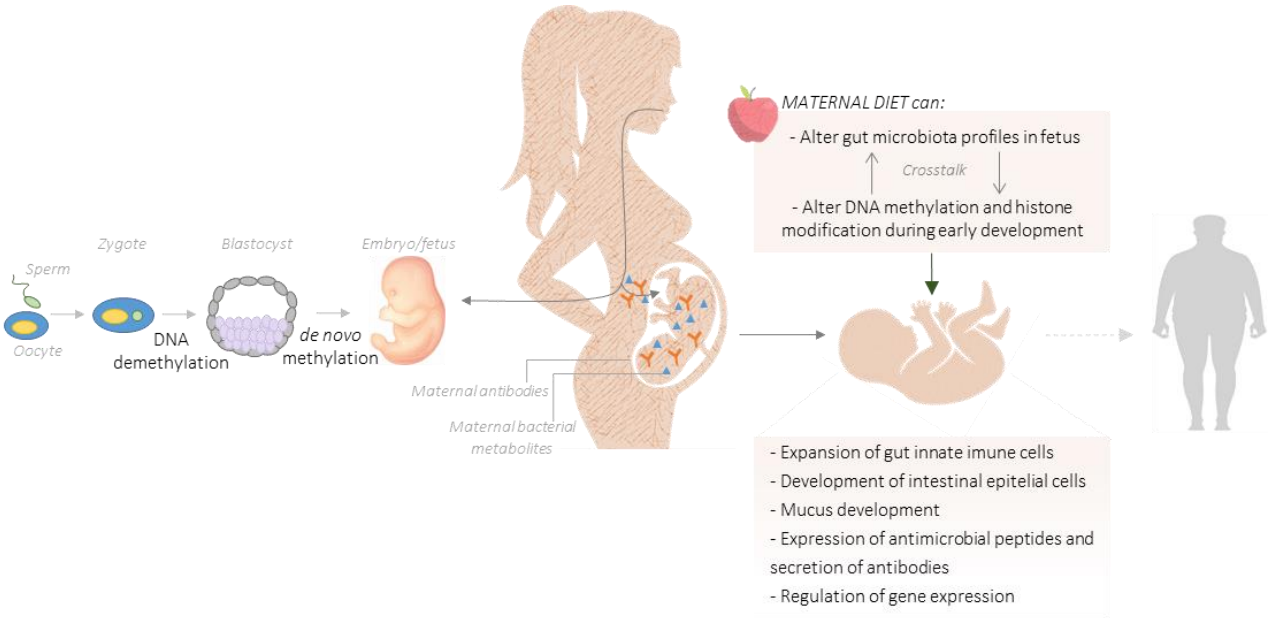


Figure 4 - Maternal diet can alter the structure and function of the gut microbiota leading to an epigenetic reprogramming processes (DNA methylation and histone modification) during early embryogenesis. In addition, it has been suggested that exist bacteria in fallopian tube and in endometrium that play a key role during conception and, posteriorly, in the embryo/fetus development [67]. However, even considering that the intrauterine environment is sterile, it is known that maternal microbiota metabolize dietary components of the diet and produce molecular signals that can reach the offspring during in utero development via the placenta. These metabolites, as well as maternal antibodies, influence the fetus innate immune system, namely in the expansion of gut innate immune cells, development of intestinal epithelial cells, mucus production, expression of antimicrobial peptides and secretion of antibodies. An unbalanced maternal diet can result in changes on adipogenesis and metabolism leading to a higher susceptibility to obesity and obesity-related metabolic diseases in adult life. The image is an adaptation of Macpherson *et al*, 2017 [63] and Li, 2018 [68].

Mediterranean Diet (MD) – characterized by high consumption of vegetables, fruit, pulses, cereals and fish; using olive oil as preferential fat source (high monounsaturated:saturated fat ratio); moderate alcoholic beverages consumption; and, low intake of meat and meat products, milk and dairy products [65] – has been associated with higher birth weight and lower risk of premature birth [66]. Moreover, MD adherence during pregnancy seems to have a protective role in fetus against the development of Metabolic Syndrome throughout life [67]. Even the studies in pregnant women is scare due to ethical reasons. To the best of our knowledge, there are no works studying the influence of MD adhesion in maternal-fetal microbial transfer. One the other hand, *in vivo* studies with high-fat diet during pregnancy (contrarily to what is encouraged in the MD) showed that female mice fed with a high-fat diet before and during pregnancy led to an increased risk of offspring obesity [68]. The impaired gut barrier integrity, increased circulation levels of LPS, increased placenta hypoxia and impaired placenta vascularization found in the high-fat group may be the reason of this association [68].

*Mode of delivery*

Regardless of when the first microbial colonization occurs, it is known that is during delivery that takes place the greatest microbial colonization of the newborn. While infants born by vaginal delivered receive a microbiota similar to the maternal vagina (through the passage on cervix and vagina), C-section delivered infants are enriched in skin microbiota, hospital staff and environment [69] (Figure 3). Infants born by vaginal delivery showed more *Lactobacillus* and *Prevotella* [70]. On the other hand, C-section delivered infants were associated with lower biodiversity, a delay in colonization by beneficial bacteria and higher colonized by *Staphylococcus*, but less colonized by *Enterococcus* [71].

Despite some contradictory works, it is assumed that the mode of delivery may play a decisive role in the development and growth of the newborns, since the bacteria present in fetal gastrointestinal tract can influence the development the immune system and therefore have relevant health consequences [72]. As mentioned by Dominguez-Bello, "Epidemiological studies, although not showing causality, have reported associations between C-section delivery and an increased risk of obesity, asthma, allergies and immune deficiencies" [73]. So, to minimalize the disruption of vertical transmission of microbiota provoked by C-section, it was developed a technique to "restore" the microbiota of these infants. A sterile gauze was pass first in the mother's vagina after the surgery and, immediately after, the same gauze was applied in the newborn (mouth, face and in rest of the body) [73]. Despite these procedure restored the infant's gut, oral and skin microbiota, it is important to ensure the costs and potential risks [74]. More recently, it was reported that the mode of delivery had a temporary and little effect on infants gut microbiota and the gestational age seems to be the main driver [75].

*The preterm infant's microbiota*

A healthy full-term vaginal delivered and exclusively breast-fed infant has been considered to be the standard for a healthy infant microbiota [76]. On the other hand, preterm infants born by C-section, fed with formula and exposed to antibiotics have abnormal patterns of colonization with lower abundance of "healthy" bacteria as *Lactobacillus* and *Bifidobacterium* [58]. Despite the very high variability between preterm infants [77], their microbiota composition is significantly different from that of the full-term infants [78]. Table 2 summarizes the principal bacteria groups in preterm infants.

Table 2 - Principal bacteria groups in preterm infants. Adapted from Underwood *et al*, 2017 [79].

| Phylum | Class | Family | Genus |
|---|---|---|---|
| *Proteobacteria* | *γ-Proteobacteria* | *Enterobacteriaceae* | *Klebsiella -/▫* |
| | | | *Escherichia -/▫* |
| | | | *Proteus -/▫* |
| | | | *Serratia -/▫* |
| | | | *Enterobacter -/▫* |
| | | | *Cronobacter -/▫* |
| | | *Pseudomonadaceae* | *Pseudomonas -/▫* |
| | | *Moraxellaceae* | *Acinetobacter -/▫* |
| *Firmicutes* | *Bacilli* | *Staphylococcaceae* | *Staphylococcus +/▫* |
| | | *Streptococcaceae* | *Streptococcus +/▫* |
| | | *Enterococcaceae* | *Enterococcus +/▫* |
| | | *Lactobacillaceae* | *Lactobacillus +/▫* |
| | *Clostridia* | *Clostridiaceae* | *Clostridium +/•* |
| | *Negativicutes* | *Veillonellaceae* | *Veillonella -/•* |
| *Bacteroidetes* | *Bacteroidetes* | *Bacteroidaceae* | *Bacteroides -/•* |
| *Actinobacteria* | *Actinobacteria* | *Bifidobacteriaceae* | *Bifidobacterium +/•* |
| | | *Propionibactebacteriales* | *Propionibacterium +/•* |

- ,Gram-negative bacteria; +, Gram-positive bacteria; ▫, facultative anaerobic; •, strict anaerobic

Preterm infants – defined as an infant born before 37 weeks of pregnancy, that includes very preterm infant born between 28 and 32 weeks and extremely preterm born before 28 weeks [80] – born to soon due to different reasons. Cervical and vascular disorders, uterine overdistension, breakdown of maternal-fetal tolerance and environmental factors as maternal age at pregnancy, maternal chronic diseases, maternal nutritional status and infection are some of the factors that can lead to premature labor [80,81].

The preterm infant presents an immature intestinal microbiota with a marked vulnerability to dysbiosis, with a change in abundance, diversity and progressive acquisition of bacteria. If, on the one hand, premature infants present an immature gut, immune system and metabolism, they also require long hospitalization leading to a colonization mainly by bacteria from the neonatal intensive care unit (NICU) and invasive procedures as intravenous access, parenteral feeding and mechanical ventilation [82]. Premature infants are exposed to endogenous factors, environmental and maternal and postnatal therapeutic conditions that constitute an imminent trigger for infection and, consequently, dysbiosis (Figure 5).

An inflammatory status in preterm infants – which can be triggered even *in utero* – can affect all organs (Figure 5). Late-onset sepsis and necrotizing enterocolitis (NEC), a very common cause of morbidity and mortality in preterm infants, were associated with microbiomes dominated by *Proteobacteria* and *Firmicutes* [71] – the dominant phyla of the preterm infant microbiota [77]. As reviewed by Staude *et al* (2018), an imbalanced gut microbiota in preterm infants, could contribute to impaired somatic growth and psychomotor development, bronchopulmonary dysplasia, retinopathy of prematurity, cardiovascular diseases and behavioral and stress responses (Figure 5) [83].

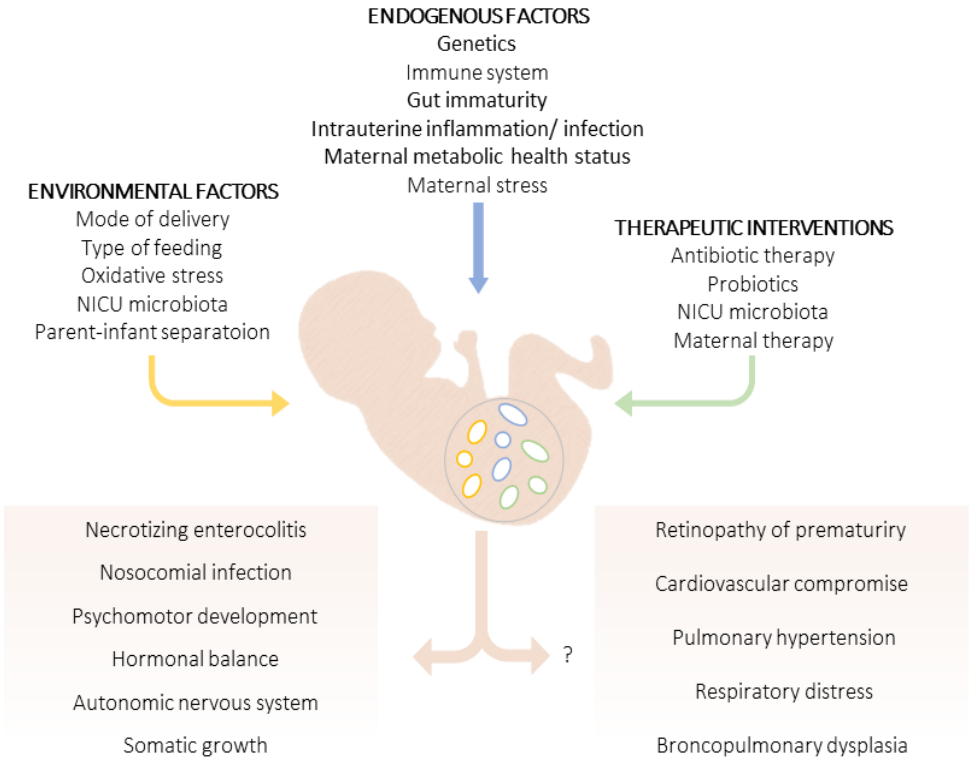


Figure 5 - Gut microbiota of preterm infants. Environmental and endogenous factors, as well as pre- and postnatal therapeutic interventions can determine the composition of gut microbiota of preterm infants. The impact of gut microbiota in NEC, nosocomial infection, psychomotor development, hormonal balance, autonomic nervous system and somatic growth have been reported. On the other hand, the association of gut microbiota and others short and long term-morbidities is unclear. Adapted from Staude *et al*, 2018 [85].

To combat this high susceptibility to infection and pathological bacteria in preterm infants, antibiotherapy is used, in most of the cases, either ante- and postpartum. However, data show that concerns should be taken when administering antibiotics due life threatening morbidities [58]. One study with 4039 preterm infants from 19 neonatal centers, showed that empirical and prolonged antibiotic therapy (≥ 5 days) was associated with increased odds of death and NEC [84]. In line with these, another study found that administration of antibiotics for 5-7 days on the first postnatal week lead to more cases of NEC, sepsis and death [85]. These preterm infants presented lower bacterial diversity and an increased abundance of *Enterobacter* [85]. 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 [77]. Perinatal antibiotic exposure also affected "strongly" the initial microbiota establishment in the preterm infants [76]. In addition, a recent study that analyzed 436 mother-child pairs 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, but the use of antibiotics in the first trimester had no effect [86].

Microbiota in early life is crucial to a healthy maturation of the immune system and other organs [83]. It is essential to identify strategies to establish a healthy early microbiota in preterm infants, since it provides antigenic stimulus for the adequate development and maturation of the immune system, intestine and even distal organs [69]. For instance, providing breast milk to preterm infants during hospitalization led them to developed a normal microbiota resembling that of term infants [87].

*The impact of the infant feeding*

In premature infants, especially in very and extremely preterm infants, an appropriated nutrition is essential to decrease the risk of adverse health outcomes and to improve cognition in adulthood [88]. According to international guidelines [88,89], which are contemplated in national recommendations [90], mothers' own milk (MOM) is always the first choice to fed preterm infants. However, MOM is not always available and sometimes it is insufficient. In that cases pasteurized donor human milk (DHM) should be administrated [91]. When Human Milk (HM – that comprises MOM and DHM) is unavailable, bovine-based preterm formulas (designed as formula hereinafter) should be used.

Breast milk composition changes through the course of gestation and lactation, and even with the time of the day [89]. Despite the protein, fat and carbohydrates source, HM is a "carrier of biochemical messages" that modulate the growth, development and the immune system of the newborn [92]. The composition of the breast milk of mothers that delivered preterm infants is significantly different that mothers that delivered at term, namely in protein and fat contents that are higher in preterm breast milk (Table 3) [93]. The most abundant proteins present in the HM are casein, α-lactalbumin, lactoferrin, secretory immunoglobulin IgA, lysozyme, serum albumin and antimicrobial peptides [92,93]. HM also contains other bioactive compounds such as essential fatty acids, enzymes, growth factors and hormones that have immune-related functions [89,92,93]. The main source of carbohydrates in HM is lactose and is the least variable macronutrients [93]. HM also contained oligosaccharides – HMOs – that are glycosylated compounds synthesized in mammary gland by glycosyltransferases. HMOs are remarkable components of breast milk due to their prebiotic effect stimulating the growth of beneficial bacteria [93].

Furthermore, breast milk is a source of probiotics providing commensal bacteria to the infant. The literature suggests that breast milk still contains $10^6$ bacteria cells per mL, with a dominance of *Staphylococcus*, *Streptococcus* (both bellowing to *Firmicutes* phylum) and *Pseudomonas* (*Proteobacteria* phylum) [94]. Two potential mechanisms can explain the presence of bacteria in breast milk. In a nutshell, mothers' skin microbiota and infants' oral can reach breastmilk through retrograde flux [95]. In addition, it has been demonstrated that dendritic cells (and macrophages) can pass intestinal epithelium through the expression of tight-junctions proteins to take up commensal bacteria and, via lymph/blood circulation, reach the mammary

gland [96]. This process showed to occur during late pregnancy and lactation in mice [97]. This may be the reason why in premature MOM the percentage of bacteria present in milk samples was lower [98]. Despite the lower total bacterial content in preterm breast milk (n = 19) compared to term breast milk (n = 13), no significantly difference were observed (Table 3) [99]. *Staphylococcus*, *Streptococcus*, *Lactobacillus*, *Enterococcus* and *Enterobacteria* were the main genera isolated in milk from preterm gestations with *Bifidobacterium* concentration significantly decreased compared to term milk [98,99].

Table 3 - Energy, macronutrient and total bacterial content composition of breast milk from mothers who delivered term and preterm; DHM and preterm infants formula [93].

| | MOM term | MOM preterm | DHM | Formula[a] |
|---|---|---|---|---|
| Energy (kcal/dL) | 65 – 70 | 78 | 50 – 115 | 80 |
| Protein (g/dL) | 0.9 – 1.2 | 2.2 | 0.6 – 1.4 | 2.6 |
| Fat (g/dL) | 3.2 – 3.6 | 4.4 | 1.8 – 8.9 | 3.9 |
| Lactose (g/dL) | 6.7 – 7.8 | 7.6 | 6.4 - 7.6 | 5.6 |
| HMO (g/dL) | 0.817 [100] | 0.857 [100] | 0.8 [101] | 0.8[b] |
| Bacterial content | 5.37 Log (gene copies $ml^{-1}$) [99] | 5.00 Log (gene copies $ml^{-1}$) [99] | $10^2$ CFU/mL [102] | n.a. |

[a] The composition of the formula described above refers to the commercial formula Aptamil Prematil, Milupa Danone®, which was used in the preterm infants included in this study; [b] The total amount oligosaccharides present in formulas is the sum of GOS (0.72 g/dL) and FOS (0.08 g/dL); n.a. – information not available.

MOM composition is also influenced by protein intake, parity, return of menstruation and nursing frequency [93]. Maternal diet also influence breast milk composition. A very recent study demonstrated the adherence to MD lead to a 10-fold higher *Lactobacillus* abundance in mammary glands of female monkeys compared to the Western diet group [7]. Likewise, maternal body mass index (BMI) showed to have influence: breast milk from obese mothers (n = 10) had more bacterial content, but it was less diverse when compared to normal weight mothers (n = 8). More, these mothers had lower content of *Lactobacillus* in colostrum and *Bifidobacterium* at 6 months [103].

Despite the rapidly increase of human milk banks, DHM is not available to all preterm infants. In Portugal, for example, there is only one human milk bank. Typically, DHM is provided by mothers who delivered at term that have excess milk production. Thus, DHM is a term and late lactation milk and require additional protein and fat acids supplementation. Moreover, DHM is submitted to Holder pasteurization (62.5°C for 30 minutes) to avoid transmission of infectious agents. Due to these reasons and to the handling and storage procedures, the DHM presents some differences in their composition in relation to preterm MOM (Table 3) [104,105]. Some compounds, such as protein, lactose, long-chain polyunsaturated fatty acids, vitamins (A, D, E, $B_{12}$, $B_9$) and some growth factors are preserved [91]. On the other hand, bioactive compounds of breastmilk are affected. B and T lymphocytes, macrophages, neutrophils, as well as lipoprotein lipase and IgM are inactivated.

The lactoferrin concentration decreased 50-75%, as well 24-74% of lysozyme and 20-30% of IgA [91]. Pasteurization procedure does not decreases the HMOs content (Table 3) [106]. However, the content of HMOs in DHM presented significantly lower amount of total HMOs in comparison to MOM [101]. The heat treatment leads to the inactivation of viruses and kills 99% of bacteria. However, Cacho *et al.* (2017) found $10^2$ CFU/mL of bacteria in 44% of DHM [102]. The most abundant genera identified were *Acinetobacter*, *Enterobacteriaceae* and *Serratia* (all bellowing to *Proteobacteria* phylum). These authors used a small amount of MOM to inoculate the pasteurized DHM, making possible to personalize DHM with MOM microbiota [102]. This could be a promising and innovative method to provide beneficial bacteria to preterm infants.

Contrarily to HM, formulas have a very standardized composition (Table 3). Preterm formulas contain all essential nutrients to provide a good growth and development to infants [88]. Although formula offers a similar percentage of total calories from fat in relation to MOM, the composition of specific fatty acids can be very different, as well the bioactive compounds [107]. Synthetic HMOs (namely, galatooligosaccharides – GOS and fructooligosaccharides - FOS) are added to formula. However, these molecules are structurally different from that naturally present in HM [108]. These differences could influence the immunogenic development of preterm infants [108]. Regarding the bacterial content, during preparation, powdered formulas may be contaminated [109]. In NICU, single doses of sterile liquid infant formula should be used [109].

It is possible to understand why the type of infant-feeding influence in different ways the development of the preterm infants' health outcomes and their gut microbiota. There are several studies showing short and long term benefits associated with HM intake in preterm infants: lower incidence of NEC, late-onset sepsis and retinopathy of prematurity; better neurological development promoting a significantly intelligence quotient in later years; lower risk of hypertension and atherosclerosis in adulthood [88]. Moreover, in preterm infants the intake of both MOM and DHM were associated with better feeding tolerance, shorter duration of hospital stay and reduced hospital costs [110].

Due to prebiotic and probiotic properties, preterm infants fed with MOM showed a greater initial diversity of gut microbiota compared infants fed non-MOM [111,112]. An effect that was maintained until 30 postnatal days [112]. On the other hand, in DHM and formula-fed infants the rate of diversity was slow over in time and at 30 postnatal days the diversity index was lower [113]. A recent study did not found differences in microbial diversity and richness between the feeding types [114]. However, significantly differences were found in bacterial profile [114]. Despite *Bifidobacteriales* showed to be higher in infants fed with MOM compared to DHM and formula infants [115], DHM showed closer microbial profiles to MOM than formulas [114]. Microbiota profile of breast-fed infant's changes to a formula-fed infant profile, with significant increase in the count of *Enterococci* and *Enterobacteriaceae*, and the appearance of *Bacteroides* and *Clostridium* [115]. Although, no significant differences were observed between MOM and formula-fed preterm infants at fourth

week of life [115]. MOM was reported to mitigate the effect of gestational age (gut immaturity) [111]. In fact, preterm infants fed with HM could develop a microbiota similar to the term infants, independently of being MOM or DHM [75].

The available data support that maternal microbiota as well as newborns postnatal microbiota contribute to a healthy or disease status of preterm infants with short- and long-term consequences. 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 [116].

## AIMS

The goal of this observational study was to evaluate the impact of different types of infant feeding (MOM, DHM and formula) on the gut microbiota of preterm infants hospitalized in NICU of Maternidade Alfredo da Costa (MAC). Furthermore, the influence of mode of delivery and mother's diet on vertical microbiota transmission were also evaluated.

Specifically, the aims of this study were:

- to characterize the preterm gut microbiota;
- to characterize the maternal gut microbiota;
- to evaluate the impact of maternal gut microbiota, antepartum factors (dietary pattern, pre-gestational body mass index, antibiotic therapy) and perinatal factors (gestational age and mode of delivery) on the bacterial colonization of preterm infants;
- to evaluated the influence of the infant feeding on preterm gut microbiota during the first 26 days of life.

## METHODOLOGY

This study protocol was approved by the Ethics Committee of Centro Hospitalar Universitário de Lisboa Central (Ref. 443/2017) and by the Ethics Committee of NOVA Medical School|Faculdade de Ciências Médicas, Universidade NOVA de Lisboa (NMS|FCM). The study was conducted in accordance to the ethical principles expressed in the Declaration of Helsinki, the Portuguese law and Good Clinical Practice guidelines.

### *Study Design*

The FEEDMI Study was an observational longitudinal study, conducted at the NICU of MAC and NMS|FCM. The study is registered in *ClinicalTrials.gov* platform, with the registration number NCT03663556. The detailed study protocol was already published [117]. An overview of the study design is described in Figure 6.

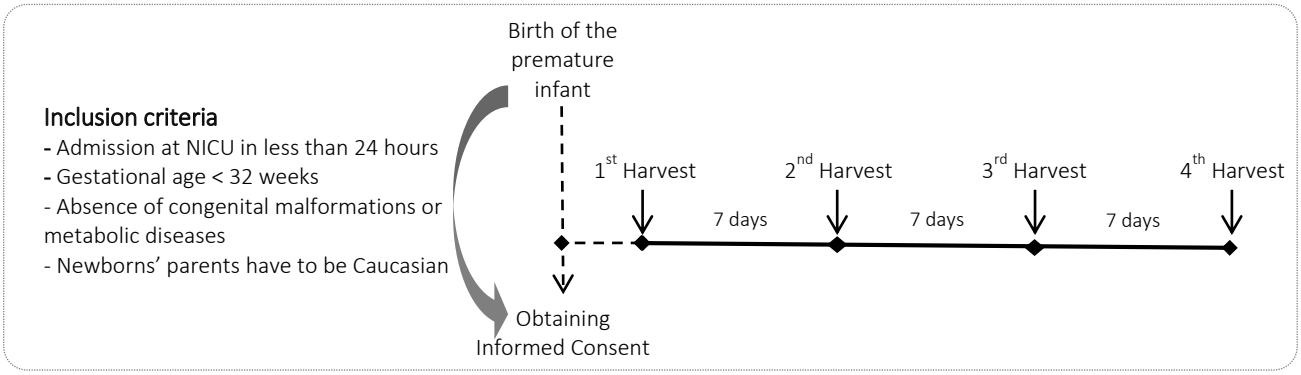


Figure 6 - Study timeline. After obtaining informed consent from legal representatives, infants will be enrolled in the study. The first stool sample will be collected within the first 24 hours after birth, followed by three subsequent collections every 7 days.

### *Participants Recruitment*

Very preterm infants (< 32 weeks gestational age) hospitalized in the NICU of MAC were recruited within the first 24 hours after birth. Inclusion criteria are displayed in Figure 6. Written informed consents were obtained for each preterm infant after explaining the entire study protocol to their legal representatives.

### *Sample Collection*

Meconium, the newborn's first intestinal discharge, and the 3 additional fecal samples of preterm infants were collected by the nursing team of MAC's Neonatology Unit. Fecal samples were collected weekly from diapers into sterile tubes. Mothers were also asked to collect their own fecal samples with an appropriate stool collection kit (EasySampler®).

*Clinical Data Collection*

Detailed clinical data were collected during the preterm infant enrollment in the study through medical records. Personal clinical data include sociodemographic information and clinical intrapartum and postpartum outcomes such as newborn's somatometry evolution, antibiotic exposure and its duration, number of total days of hospitalization, and other outcomes related to the preterm clinical evolution. Additionally, type of infant-feeding (MOM, DHM and formula) was recorded daily to select the most representative (> 50 %) type of infant-feeding received during the 7 days prior to each fecal sample collection.

*Microbiota Analysis*

Fecal sample collection is a non-invasive procedure commonly used to assess the intestinal microbiota composition. Bearing in mind meconium's tar-like texture and samples' low bacterial amount [118], DNA were extracted and purified from stool samples using NZY Tissue gDNA Isolation Kit (nzytech, Lisbon, Portugal), as previously described [119].

Different bacterial populations were analyzed by quantitative real-time PCR using LightCycler instrument (Roche Applied Science, Indianapolis, ID, USA). Specific microorganisms were assessed based on previous studies regarding preterm gut microbiota composition [111,120–122]. Two phyla (*Bacteroidetes* and *Firmicutes*), one class (*γ-Proteobacteria*), four genera (*Lactobacillus*, *Bifidobacterium*, *Bacteroides* and *Enterococcus*) and one specie (*Escherichia coli*) were analyzed of preterm infants' samples. In mothers' samples, the same bacteria groups were analyzed in addition to *Prevotella* and *Akkermansia*. Primer sequences used to target bacterial 16S rRNA genes were described in Table 4. Results on microbiota are expressed as log10 16S rDNA gene copies/10ng of DNA.

*Quality Control Analysis*

Increasing evidence has been suggested that DNA extraction kits and other laboratory reagents are commonly contaminated [123]. This contamination could have a critical impact on results, especially in samples containing low microbial biomass [123], such as preterm infants. For control purposes, a fecal collection was simulated: an empty tube (that same tubes used for collecting meconium and feces samples) was opened inside the infants' incubator and the spatula was passed through diaper; the tube was stored under the same conditions as the others; in the lab, it was added 200 mL of ultrapure water into the tube; and DNA was extracted. In addition, DNA amplification was performed in duplicated and samples with lower levels than negative controls (from extraction and PCR procedures) were discarded.

Table 4 - Primers sequences used for gut microbiota analysis. AT, annealing temperature.

| Target group | Primer sequence (5'-3') | Genomic DNA Standard | AT | Ref. |
|---|---|---|---|---|
| Total bacteria | AAA CTC AAA KGA ATT GAC GG<br>CTC ACR RCA CGA GCT GAC | *Bacteroides vulgatus*<br>ATCC 8482 | 62°C | [124] |
| *Bacteroidetes* | CAT GTG GTT TAA TTC GAT GAT<br>AGC TGA CGA CAA CCA TGC AG | *Bacteroides vulgatus*<br>ATCC 8482 | 60°C | [125] |
| *Firmicutes* | ATG TGG TTT AAT TCG AAG CA<br>AGC TGA CGA CAA CCA TGC AC | *Lactobacillus gasseri*<br>ATCC 33323 | 60°C | [125] |
| *γ-Proteobacteria* | TCGTCAGCTCGTGTYGTGA<br>CGTAAGGGCCATGATG | *E. coli*<br>ATCC 25922 | 61°C | [124] |
| *Lactobacillus* | GAG GCA GCA GTA GGG AAT CTT C<br>GGC CAG TTA CTA CCT CTA TCC TTC TTC | *Lactobacillus gasseri*<br>ATCC 33323 | 60°C | [125] |
| *Bifidobacterium* | CGC GTC YGG TGT GAA AG<br>CCC CAC ATC CAG CAT CCA | *Bifidobacterium longum*<br>ATCC 15697 | 60°C | [125] |
| *Bacteroides* | ATA GCC TTT CGA AAG RAA GAT<br>CCA GTA TCA ACT GCA ATT TTA | *Bacteroides vulgatus*<br>ATCC 33563 | 60°C | [125] |
| *Enterococcus* | CCC TTA TTG TTA GTT GCC ATC ATT<br>ACT CGT TGT ACT TCC CT TGT | *Enterococcus gilvus*<br>ATCC BAA-350 | 61°C | [125] |
| *Prevotella* | CACRGTAAACGATGGATGCC<br>GGTCGGGTTGCAGACC | Prevotella nigrescens<br>ATCC 33563 | 55°C | [125] |
| *Akkermansia* | CAGCACGTGAAGGTGGGGA<br>CCTTGCGGTTGGCTTCAGAT | *Akkermansia muciniphila*<br>ATCC BAA-835 | 60°C | [126] |
| *Escherichia coli* | GTA AGT TAC ACT ATA AAA GCA CCG TCG<br>TCT GTG TGG ATG GTA ATA AAT TTT TG | *Escherichia coli*<br>ATCC 25922 | 60°C | [127] |

*Maternal Mediterranean Diet Adherence Score*

Mothers were requested to completed a semi-quantitative Food Frequency Questionnaire (FFQ), previously validated for the Portuguese population [128,129]. In the current study, the FFQ with 86 items was used to assess food consumption during pregnancy. Response options are in a 9-point frequency scale, ranging from 'never' to '≥ 6 times per day'. Using portion sizes and frequency of consumption, daily portions (g/day) were computed for each FFQ item. Data from FFQ was used to calculate the MD adherence score using 13 items of the original questionnaire [130]. The following items were classified with 1 point if the answer to the question was "YES": olive oil as main culinary added fat/oil; olive oil ≥ 4 daily tablespoons; vegetables (including vegetable soup) ≥ 2 daily serving; fruit ≥ 3 daily servings; legumes ≥ 3 weekly servings; fish/seafood ≥ 3 weekly servings; tree nuts ≥ 1 weekly serving; and more poultry than red meat. If the answer was "NO", the item was classified with 0 points. On the other hand, the following items were classified with 1 point if the answer to the question was "NO": red/processed meat ≥ 1 daily serving; butter, cream and margarine ≥ 1 daily serving; sugar-sweetened beverages ≥ 1 daily serving; sweets and confectionary ≥ 3 weekly servings; and any consumption of wine. If the answer was "YES", the item was classified with 0 points. The MD adherence score ranged, therefore, from 0 to 13 points, where higher scores (≥ 10 points) reflect better adherence to the MD. Table 5 summarizes the portion sizes used in each item.

Table 5 - Description of the food groups and respective portion sizes used to assess the MD adherence score.

| Food Group | Description | Cut-off |
|---|---|---|
| Olive oil | Olive oil | ≥ 4 servings/day (1 serving = 10 g) |
| Vegetables | All raw and cooked vegetables | ≥ 2 servings/day (1 serving = 180 g) |
| Fruits | All fruits and 100% fruit juices | ≥ 3 servings/day (1 serving = 160 g) |
| Legumes | Bean, chickpeas, lentils, peas | ≥ 3 servings/week (1 serving = 150 g) |
| Nuts | Nuts, almonds, pistachios, walnuts, cashews | ≥ 1 servings/week (1 serving = 30 g) |
| Fish/seafood | Fresh-water and sea-water fish, preserved fish (tuna, sardines), selfish (squid, prawns) | ≥ 3 servings/week (1 serving = 150 g) |
| Red/processed meat | Beef, pork, lamb, goat, sausage, ham, bacon | ≥ 1 servings/day (1 serving = 125 g) |
| Fats and oils | Butter, cream and margarine | ≥ 1 servings/day (1 serving = 12 g) |
| Sugar-sweetened beverages | Soft drinks, fruit juices or packages nectars | ≥ 1 servings/day (1 serving = 330 ml) |
| Sweets | Cookies, croissants, doughnut, homemade cakes, chocolate (tablet or powder). Chocolate snacks. | ≥ 3 servings/week (1 serving = 80 g) |

*Statistical Analysis*

Statistical analysis was performed by SPSS software, version 25 (IBM SPSS Statistics corporation, Chicago, IL, USA). The normality of the data, at each sampling point, was checked using the Kolmogorov-Smirnov test. Comparisons between groups were performed using t-test, Mann-Whitney test or Fisher's exact test, as appropriated depending on value distribution. In order to evaluate the impact of different BMI categories and types of feeding on the evolution of gut microbiota composition, a non-parametric test (Kruskal-Wallis) was used. Univariate and multivariate linear regression analysis adjusted for gestational age, mode of delivery, gestational diabetes and antepartum antibiotic therapy were performed to investigate the association between mean Mediterranean Score (independent) and the microbes' abundance in meconium samples (dependent variables). In addition, multivariate linear regression analysis adjusted for gestational age, infants' antibiotherapy and mean Mediterranean Score were fitted to study the association between infant feeding (independent) and the microbiota composition of preterm infants at 26$^{th}$ postnatal day (dependent variables). Data are expressed as mean ± standard deviation (SD). The differences were considered statistically significant when $p < 0.05$.

## RESULTS

*Clinical characterization of the preterm infants and their mothers*

A total of 159 preterm infants were recruited consecutively from the NICU between May 2017 and April 2019. Forty-two were excluded: 14 for not meeting the inclusion criteria, 13 due to early death, 7 for having nonconformities in their fecal samples, 4 for early discharge, 3 were transferred to another hospital, and 1 for unknown perinatal factors. The remaining 117 preterm infants include 22 pairs of twins and one set of triples. The respective mothers of the eligible preterm infants (n = 93) were also enrolled in this study (Figure 7).

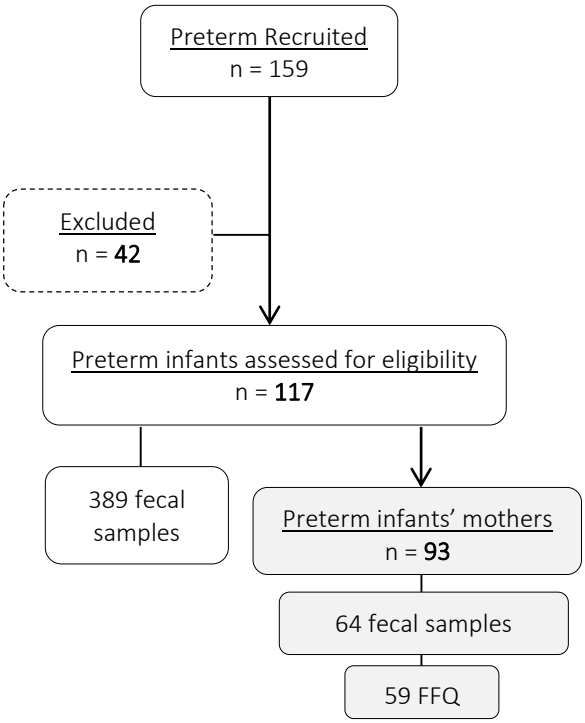


Figure 7 - Flowchart identifying study population

Preterm infants were born at gestational age between 25 and 31 weeks with birthweights ranging from 455 to 2020 g. The majority of the infants were male (56%) and delivered by cesarean-section (C-section). Clinical data of preterm infants is reported in Table 6.

Enteral feeding was introduced between the first and sixth postnatal day and the exclusive enteral feeding was achieved at 15 ± 8 postnatal days. Preterm infants were fed with their MOM, DHM and/or infant formula. Considering the most predominant type of infant feeding the one that represented more than 50% of the total feedings during the study period, 64.7% of the infants were fed with MOM. Twenty infants were fed predominantly with DHM and 13 with infant formula. In some cases, infants received mixed feeding of MOM plus DHM (design as HM, n = 6) or MOM plus formula (n = 1). Due to specific clinical conditions, a preterm infant was fed mainly by parenteral feeding, reaching exclusive enteral feeding at 48 postnatal day.

Eight infants had culture positive early onset infection and 46 had late-onset sepsis. Two of them were diagnosed with NEC at 34 and 36 days of life. The infants received a mean of 11 days of antibiotics. Six infants and 4 mother-infants pairs did not receive antibiotics, either antepartum or postnatally during the all hospitalization.

Table 6 - Clinical characteristics of preterm infants.

| Preterm infants, n = 117 | |
|---|---|
| **Sex**, n | |
| Female | 52 |
| Male | 65 |
| **Gestational age**, weeks (mean ± SD) | 28.6 ± 1.9 |
| **Extremely premature/very premature**, n | 34/83 |
| **Mode of Delivery**, n | |
| Vaginal | 45 |
| C-section | 72 |
| **Somatometry at birth** (mean ± SD) | |
| Weight, g | 1177 ± 419 |
| Length, cm | 36.8 ± 3.3 |
| Cephalic perimeter, cm | 25.7 ± 2.3 |
| **Type of Feeding**, n | |
| MOM | 75 |
| DHM | 20 |
| Formula | 13 |
| **Days of antibiotherapy** (mean ± SD) | 12 ± 10 |
| **Days of hospitalization** (mean ± SD) | 56 ± 10 |

The mean age of preterm infants' mothers was 34 years of age. The majority of mothers had normal weight before pregnancy. The mean gestational weight gain at the end of the pregnancy was 8.7 kg. Overall, 12 mothers had gestational diabetes and 50 received antepartum antibiotic therapy. Of the 59 mothers that completed the FFQ, only 9% (n = 15) showed adherence (MD score > 10) to MD during pregnancy. Mothers' characteristics are reported in Table 7.

Table 7 - Preterm infants' mothers' characteristics.

| Mothers, n = 93 | |
|---|---|
| **Age**, years (mean ± SD) | 34 ± 6 |
| **Pre-gestational BMI**, n | |
| Underweight | 1 |
| Normal weight | 58 |
| Overweight | 19 |
| Obese | 8 |
| **Gestational weight gain**, kg (mean ± SD) | 8.7 ± 5.3 |
| **Gestational Diabetes**, n | 12 |
| **Maternal Antibiotherapy**, n | 50 |
| **Mediterranean Diet Adherence**, n (yes/no) | 15/44 |
| **Rupture of membranes**, n | |
| Intrapartum | 42 |
| < 18 hours | 22 |
| > 18 hours | 16 |

*Characterization of the preterm infants' and mothers' microbiota*

In total, 389 fecal samples from preterm infants were obtained at an average postnatal age of 2 (corresponding to meconium samples), 10, 18 and 26 days. From a total of 100 meconium samples collected, only 66 were collected before 72 hours of life. Of the 66 meconium samples, bacterial DNA were not detected in 8 samples, as well as no DNA was detected in the negative control collected from a preterm infant's diaper. Meconium samples collected over 72 hours were discarded due to their greater amount of total bacteria ($p = 0.003$), reflecting an additional bacterial colonization due to external factors (NICU environment and medical and nursing contact).

In general, bacterial levels increased during the 26 days of life (Figure 8). The intestine of preterm infants showed a significant bacterial colonization after the second day of life ($p < 0.001$) (Figure 8a). *Proteobacteria* was the most abundant phylum in all time-points and, along with *Firmicutes*, increased over time ($p < 0.001$). On the other hand, *Bacteroidetes* phylum abundance decreased between the 2$^{nd}$ and 10$^{th}$ day of life ($p < 0.001$) and remained unaltered until day 26 (Figure 8b).

Of the all genera analyzed, in meconium samples *Bifidobacterium* was the most abundant genus (Figure 8c). At the 10$^{th}$ postnatal day, *Bacteroides* assumed this position. However, the *Enterococcus* content increased over time and was the most prevalent genus in fecal samples at 18$^{th}$ and 26$^{th}$ day of life ($p < 0.001$) (Figure 8c). The abundance of *Lactobacillus* and *Bifidobacterium* was the same over the time ($p = 0.201$ and $p = 0.176$, respectively). Interestingly, in extremely premature newborns' meconium (born before 28 gestational weeks) the amount of *Lactobacillus* is higher than in very preterm infants ($1.442 \pm 0.822$ vs. $1.036 \pm 1.052$, $p = 0.024$), regardless of the mode of delivery. However, between the 2$^{nd}$ and 10$^{th}$ day, extremely preterm infants had a decreased in *Lactobacillus* ($p = 0.008$) maintaining the same amount as very preterm infants until the 26$^{th}$ day. Gestational age did not influenced any other differences in infants' microbiota.

*Bacteroides* were only identified in 65% of the meconium samples and in 45-60% of the remaining fecal samples. In preterm infants colonized with *Bacteroides*, results were not different between the four time points ($p = 0.145$) (Figure 8c). The abundance of *E. coli*, the only species analyzed in this study, increased significantly over time ($p < 0.001$) (Figure 8c). Infants born by C-section presented a greater amount of *E. coli* in meconium compared to vaginal delivery infants ($2.497 \pm 1.302$ vs. $1.848 \pm 0.784$, $p = 0.042$). In addition, these infants had a lower amount of *Bacteroides* at the 10$^{th}$ postnatal day ($6.973 \pm 2.010$ vs. $2.799 \pm 0.199$, $p = 0.024$]. Besides these differences, the mode of delivery did not show other influences on infants' microbiota.

From mothers, 64 postpartum fecal samples were analyzed. Comparing to their mothers', gut microbiota of preterm infants showed a lower amount of total bacteria during the first 26$^{th}$ days of life ($p < 0.001$) (Figure 8d). The most abundant phylum in mothers' fecal samples was *Firmicutes* (Figure 8e). With the exception of

meconium samples, preterm infants presented an increased amount of *γ-Proteobacteria* compared to their mothers ($p < 0.001$). Interestingly, after birth, preterm infants' meconium had the same amount of *Lactobacillus* as their mothers ($p = 0.146$) and the content of *Bifidobacterium* was also the highest in both mothers and infants (Figure 8f).

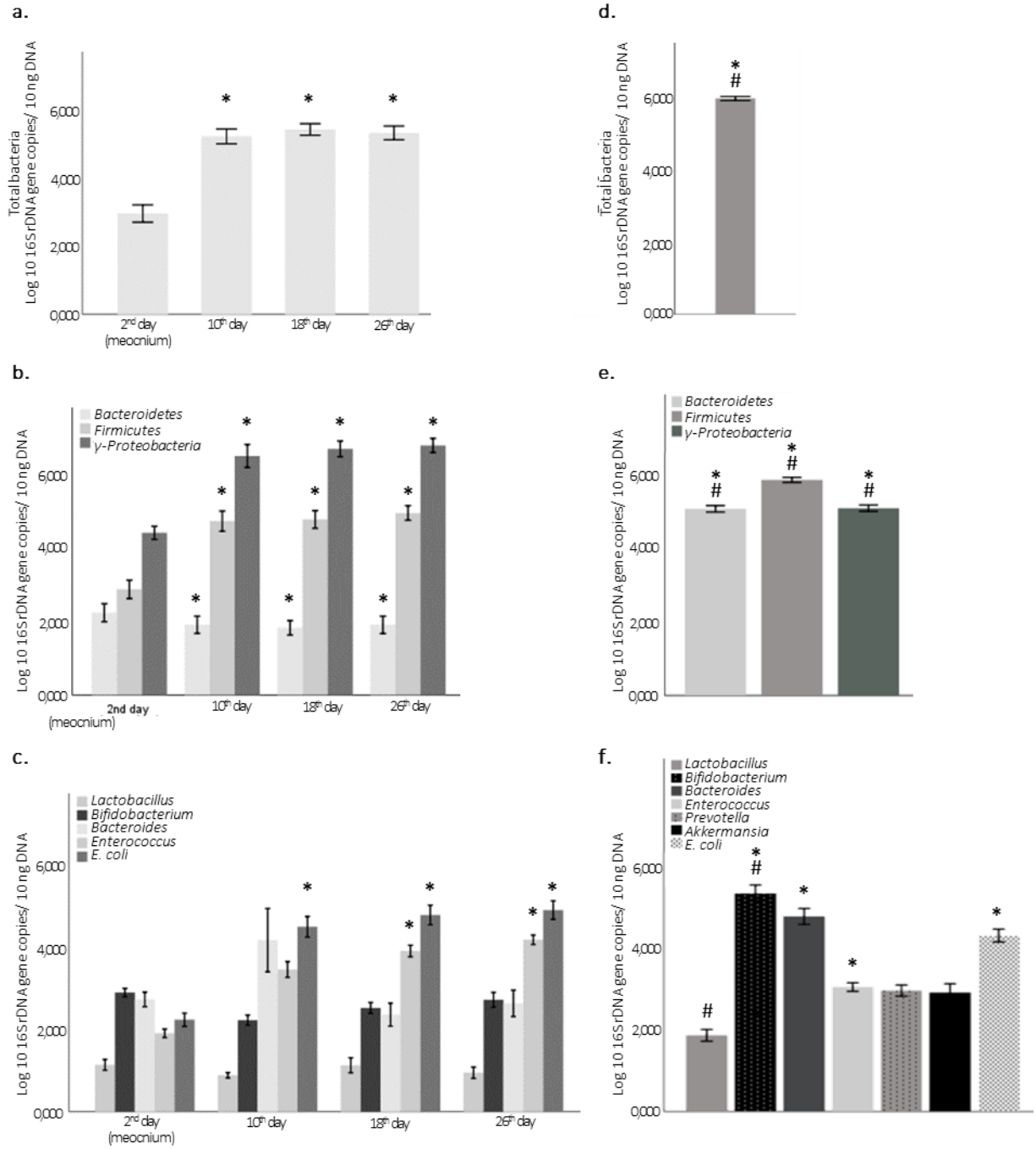


Figure 8 - Specific bacterial levels groups in meconium and fecal sample of preterm infants (a, b, c) and in their mothers fecal samples (d, f, e). * Differences in relation to meconium; # differences between mothers' samples and infants' samples at 10th, 18th and 26th days.

Considering antepartum factors, such as pre-gestational BMI, gestational weight gain and adherence to MD, no differences were observed on mothers' fecal microbiota. However, gestational diabetes promoted a lower amount of *E. coli* ($p$ = 0.020).

Fetal membrane rupture over 18 hours before delivery showed to be a major determinant of postpartum maternal microbiota. Women who had their membranes ruptured for over 18 hours presented a lower content of total bacteria, *Bacteroidetes*, *γ-Proteobacteria*, *Bifidobacterium*, *Prevotella* and *E. coli* compared to those whose fetal membranes ruptured intrapartum (Table 8). Membranes ruptured for less than 18 hours showed no differences in maternal microbiota when compared to intrapartum rupture.

Table 8 - Influence of fetal membranes ruptured intrapartum on the mothers' microbiota (mean ± SD).

| | Intrapartum | < 18 hours | > 18 hours | *p*-value [a] |
|---|---|---|---|---|
| Total Bacteria | 6.064 ± 0.545 | 5.947 ± 0.501 | 5.693 ± 0.489 | **0.008** |
| *Bacteroidetes* | 5.225 ± 0.760 | 4.659 ± 1.155 | 4.701 ± 0.696 | **0.030** |
| *Firmicutes* | 5.818 ± 0.633 | 6.094 ± 0.563 | 5.518 ± 0.511 | 0.116 |
| *γ-Proteobacteria* | 5.200 ± 0.760 | 4.810 ± 0.610 | 4.650 ± 0.740 | **0.034** |
| *Lactobacillus* | 1.909 ± 0.967 | 1.540 ± 0.997 | 1.757 ± 1.152 | 0.326 |
| *Bifidobacterium* | 5.963 ± 0.970 | 5.065 ± 1.526 | 3.646 ± 1.077 | **0.001** |
| *Bacteroides* | 4.963 ± 1.313 | 4.709 ± 1.699 | 4.405 ± 1.287 | 0.099 |
| *Enterococcus* | 3.080 ± 0.677 | 3.207 ± 0.746 | 3.062 ± 0.583 | 0.934 |
| *Prevotella* | 3.128 ± 0.957 | 2.660 ± 0.752 | 2.304 ± 1.004 | **0.007** |
| *Akkermansia* | 2.200 ± 1.551 | 2.804 ± 1.353 | 2.238 ± 1.313 | 0.093 |
| *E. coli* | 4.427 ± 1.156 | 4.377 ± 0.853 | 3.212 ± 0.957 | **0.001** |

[a] *p*-value between intrapartum rupture membranes and membranes ruptured for over 18 hours

The lower amount of specific bacteria found in mothers with membranes ruptured for more than 18 hours could be related to exposure of antibiotics, since all of these women received antibiotherapy before delivery (Table 9).

Table 9 - Fetal membranes rupture and antepartum antibiotic exposure.

| | n | | |
|---|---|---|---|
| | No-antibiotic exposure | Antibiotic exposure | *p*-value |
| Rupture of membranes | | | |
| Intrapartum | 28 | 14 | **0.001** |
| < 18 hours | 6 | 16 | |
| > 18 hours | 0 | 16 | |

In agreement, antepartum antibiotic exposure had a major impact on mothers' microbiota. Compared to mothers who did not take antibiotics before giving birth, those exposed to antibiotic therapy had less bacterial content and lower abundance of *Firmicutes*, *Lactobacillus*, *Bifidobacterium*, *Akkermansia* and *Prevotella* (Table 10).

Table 10 - Influence of antepartum antibiotic exposure on the mothers' microbiota (mean ± SD).

| | No-antibiotic exposure (n = 29) | Antibiotic exposure (n = 34) | *p*-value |
|---|---|---|---|
| Total Bacteria | 6.087 ± 0.593 | 5.857 ± 0.446 | **0.017** |
| *Bacteroidetes* | 5.193 ± 0.679 | 4.807 ± 0.888 | 0.061 |
| *Firmicutes* | 5.991 ± 0.649 | 5.686 ± 0.554 | **0.024** |
| *γ-Proteobacteria* | 5.180 ± 0.790 | 4.900 ± 0.740 | 0.163 |
| *Lactobacillus* | 2.018 ± 0.971 | 1.550 ± 0.967 | **0.035** |
| *Bifidobacteria* | 5.957 ± 1.018 | 4.628 ± 1.492 | **0.001** |
| *Bacteroides* | 5.130 ± 1.214 | 4.582 ± 1.425 | 0.055 |
| *Enterococcus* | 3.017 ± 0.657 | 3.156 ± 0.789 | 0.455 |
| *Prevotella* | 3.216 ± 0.981 | 2.597 ± 0.843 | **0.016** |
| *Akkermansia* | 3.315 ± 1.656 | 2.518 ± 1.250 | **0.031** |
| *E. coli* | 4.380 ± 1.091 | 4.113 ± 1.205 | 0.310 |

In order to verify whether antibiotic use mitigated the effects of pre-gestational BMI, gestational weight gain, adherence to MD and gestational diabetes on the maternal microbiota, only mothers who were not exposed to the antepartum antibiotics were considered for analysis and no differences were found.

*Establishment of the meconium microbiota of preterm infants*

### Mother-to-infant bacterial transmission

Altogether, 35 mother-infant pairs had fecal and meconium samples collected, respectively. The total bacteria content of infants did not correlated with mothers' microbiota. However, *Firmicutes* of the mothers' samples correlated positively and strongly with that of the infants (Figure 9c). This correlation was maintained until the 10$^{th}$ day of life, despite losing strength [ρ = 0.408 (*p* = 0.012)]. The *γ-Proteobacteria* content also showed a positive correlation between mothers' and infants' microbiota (Figure 9d). On the other hand, *Bifidobacterium* correlates negatively, but weakly, between mothers and preterm infants (Figure 9f).

Regarding the mode of delivery, among 14 vaginal deliveries, no correlation was observed between mothers' and infants' microbiota. However, among 21 C-section deliveries, microbiota of mother-infant pairs were correlated. The *Firmicutes* and *γ-Proteobacteria* content between mothers' feces and preterm infants' meconium were positively correlated, showing higher correlation coefficients than in the vaginal group [0.657 (*p* = 0.002) and ρ = 0.557 (*p* = 0.013), respectively]. *Bacteroides* was also positively and strongly correlated [ρ = 0.733 (*p* = 0.007)].

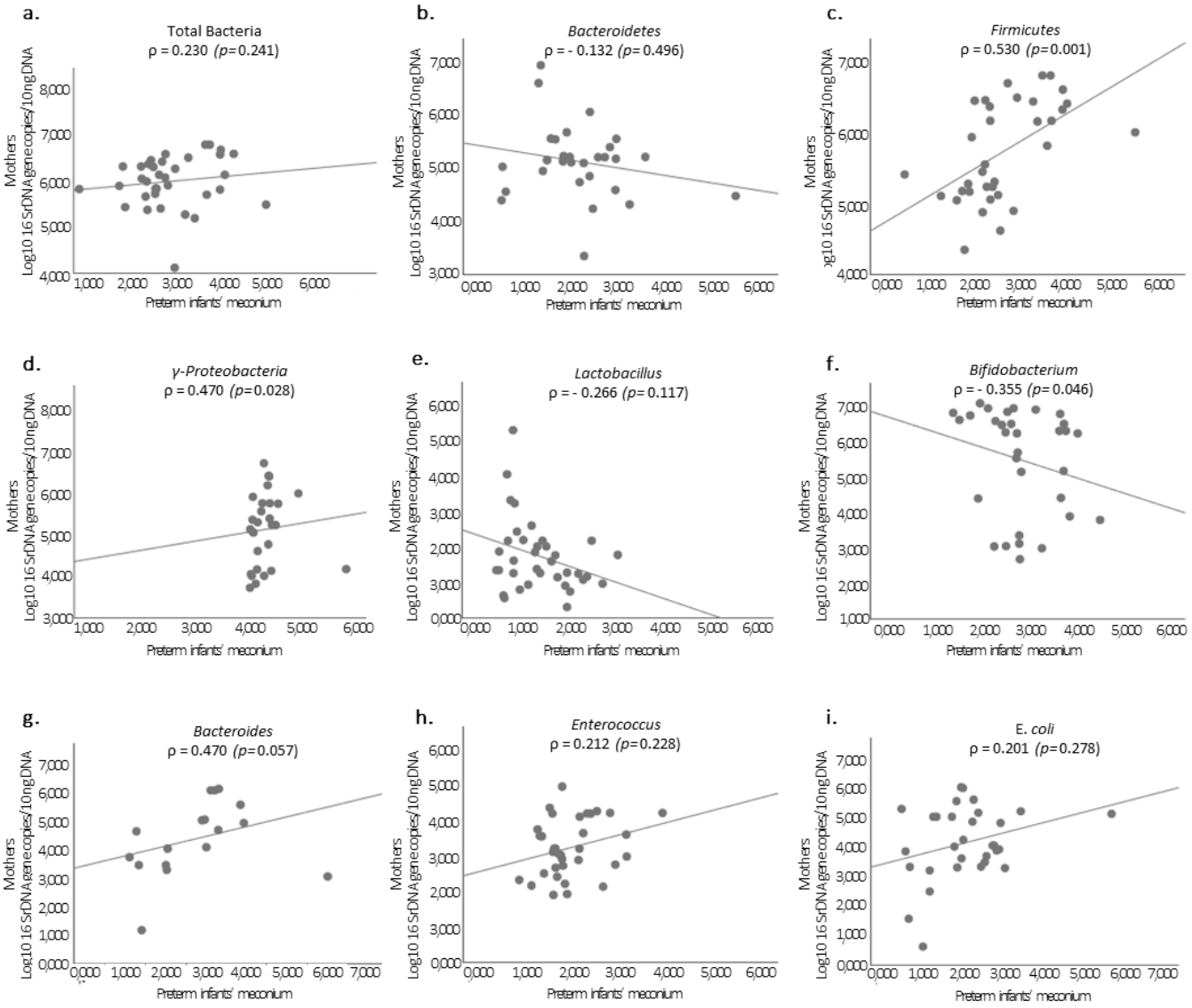


Figure 9 - Scatterplots showing the association between mother microbiota and their infants' meconium (spearman correlation). X-axis represents the bacterial content of individual infant. Y-axis represent their mothers' bacterial content.

In order to determine the effect of gestational age on bacterial transfer from mother to offspring, the same analysis was performed for infants born before and after 28 weeks of gestation. Interestingly, no correlations were found in the microbes analyzed between the mothers' feces and the extremely preterm infants' meconium (n = 11). However, in preterm infants born during the third trimester (between 28 and 32 weeks gestation) the content of *Firmicutes* and *Bacteroides* correlated positively and strongly [ρ = 0.523 ($p$ = 0.013) and ρ = 0.728 ($p$ = 0.007), respectively].

### Rupture of membranes and maternal antepartum antibiotics exposure

As observed in mothers, membrane rupture also influenced the preterm infants' microbiota right after birth. Compared with intrapartum membrane ruptured, infants born with membrane ruptured less than 18 hours had decreased levels of *Lactobacillus* ($p$ = 0.025) and increased levels of *E.coli* ($p$ = 0.023) (Figure 10).

Infants born with membrane ruptured over 18 hours, presented decreased amount of total bacteria and *Firmicutes*. However, in comparison to < 18 hours group, membranes ruptured for more than 18 hours led to an increase in *Lactobacillus* (Figure 10). These differences were not explained by antepartum antibiotherapy. It was only noted that the offspring exposed to antepartum antibiotics had lower *Enterococcus* content in their meconium (1.669 ± 0.519 vs. 2.109 ± 0.903, $p$ = 0.029).

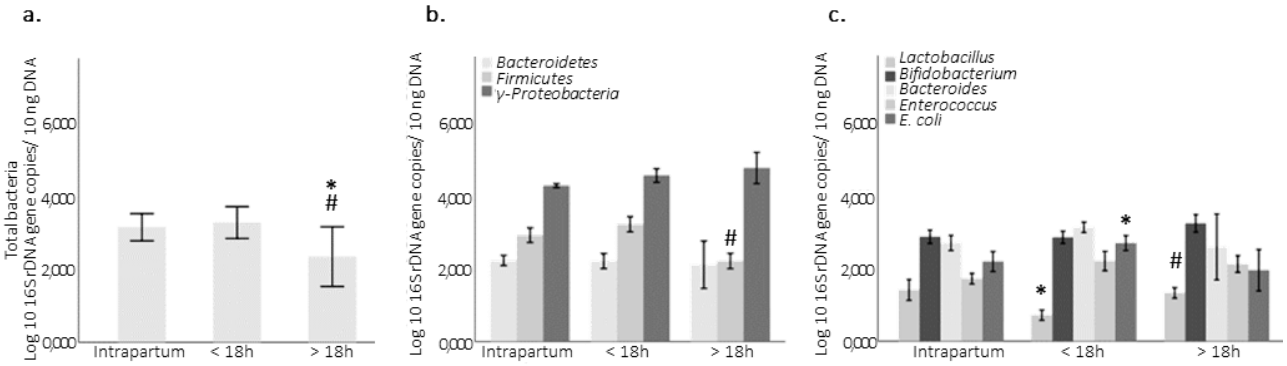


Figure 10- Influence of fetal membrane rupture on premature meconium microbiota. *differences in relation to intrapartum membrane rupture; # differences between membrane rupture for less than 18h and over 18 hours.

## Maternal diet

Maternal adherence to MD during pregnancy was not associated with the microbial composition of the meconium (Figure 11). However, the analysis was limited by the number of mothers adhering to the MD whose infant's meconium sample was collected in the correct conditions (n = 8).

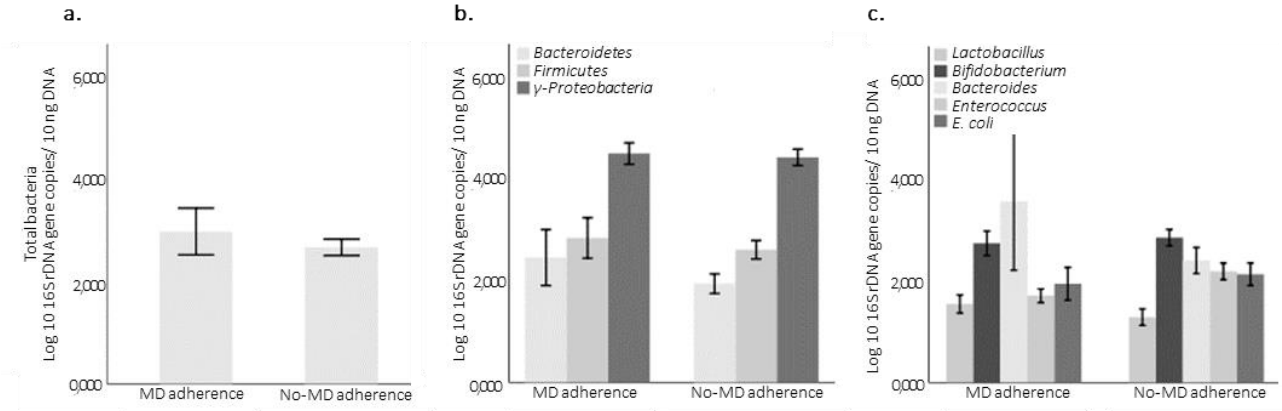


Figure 11 - Influence of maternal MD adherence on premature meconium microbiota. Mothers with MD adherence, n = 8; mothers with no-MD adherence, n = 23.

Univariate (crude) linear regression models of maternal diet (independent variable) and infants' microbiota (dependent variable) were fitted (table 11). In addition, models adjusted for gestational age and mode of delivery (model 1) as well as gestational diabetes and maternal antibiotherapy (model 2) were fitted (table 11). Maternal adherence to MD was significantly and negatively associated with Enterococcus in infants' meconium after adjusting to gestational diabetes and maternal antibiotherapy (table 11). Concerning the other bacteria analyzed, no significant associations were observed.

Table 11 - Association between Mediterranean Diet Score (independent variable) and meconium microbes (dependent variables).

| | Model 0 | | Model 1 | | Model 2 | |
|---|---|---|---|---|---|---|
| | β | *p* | β | *p* | β | *p* |
| Total Bacteria | 0.310 | 0.423 | 0.397 | 0.368 | 0.105 | 0.781 |
| *Bacteroidetes* | 0.516 | 0.267 | 0.602 | 0.200 | 0.560 | 0.262 |
| *Firmicutes* | 0.231 | 0.548 | 0.567 | 0.137 | 0.070 | 0.851 |
| *γ-Proteobacteria* | 0.075 | 0.810 | 0.187 | 0.551 | -0.059 | 0.853 |
| *Lactobacillus* | 0.261 | 0.397 | 0.261 | 0.371 | 0.403 | 0.207 |
| *Bifidobacterium* | -0.112 | 0.726 | -0.069 | 0.832 | -0.190 | 0.555 |
| *Bacteroides* | 1.174 | 0.163 | 1.089 | 0.344 | 1.239 | 0.109 |
| *Enterococcus* | -0.484 | 0.111 | -0.467 | 0.136 | **-0.616** | **0.044** |
| *Escherichia coli* | -0.189 | 0.677 | -0.096 | 0.836 | -0.295 | 0.556 |

Model 0 is univariate analysis; Model 1 is adjusted for gestational age and mode of delivery; Model 2 is adjusted for gestational diabetes and antepartum antibiotherapy

### Maternal pre-gestational BMI and weight gain during pregnancy

Regarding the maternal pre-gestational BM, no differences were also found in meconium microbiota of infants delivered by women with normal weight or overweight (Figure 12). However, some differences were detected in meconium microbiota between infants born to obese and normal weight or overweight mothers.

Preterm infants born to mothers with normal weight, overweight or obesity had the same amount of total bacterial in meconium (Figure 12a). However, obese mothers delivered infants with significantly higher amount of *Firmicutes* compared to normal weight ($p = 0.036$) and overweight mothers ($p = 0.002$) (Figure 12b). These infants had more *γ-Proteobacteria* and *Bacteroides* compared to infants of overweight mothers ($p = 0.026$ and $p = 0.048$. respectively). Surprisingly, the *Bifidobacterium* content was also higher in infants born to obese mothers compared to mothers with normal weight before pregnancy ($p = 0.044$) (Figure 12c). On the other hand, the amount of *Lactobacillus* was significantly lower (Figure 12c).

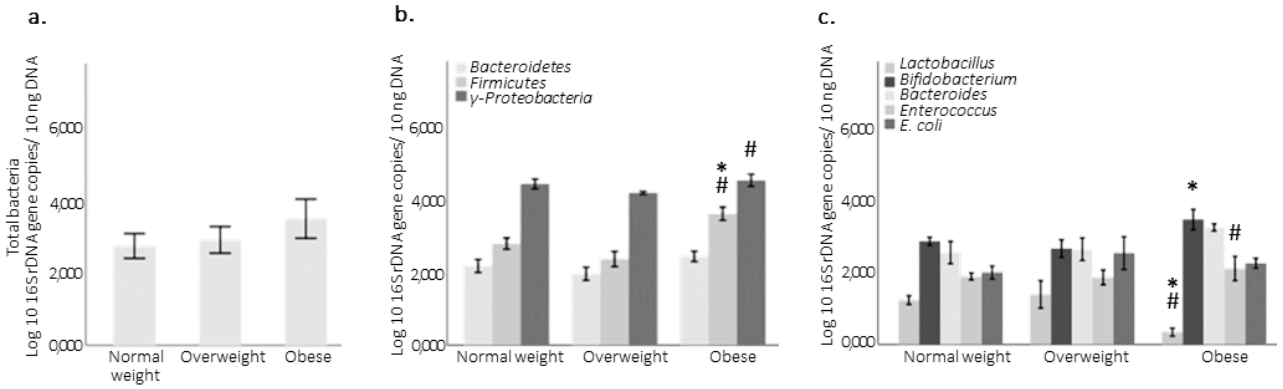


Figure 12 - Influence of maternal pre-gestational body mass index on premature meconium microbiota. Normal weight mothers, n = 33; overweight mothers, n = 15; obese mothers, n = 6; *differences between normal weight and obese mothers; #differences between overweight and obese mothers.

The weight gain during pregnancy did not affect the preterm meconium microbiota.

## Gestational Diabetes

Preterm infants whose mothers had GD also had lower *Lactobacillus* content in their meconium sample (1.256 ± 1.060 vs. 0.717 ± 0.615, $p = 0.019$). To understand if GD and obese mothers were the same, crosstab and chi-square analyses was performed (Table 10). Despite the low number of obese mothers, it was observed that the incidence of gestational diabetes differs according to the maternal pre-gestational BMI ($p = 0.007$) (Table 12). Specially, among normal weight and obese mothers ($p = 0.009$).

Moreover, six out of eight obese mothers did not follow the MD during pregnancy and the remaining 2 did not complete FFQ.

Table 12 - Gestational diabetes by maternal pre-gestational BMI.

| | n | | | | |
|---|---|---|---|---|---|
| | All | Normal weight | Overweight | Obese | *p*-value |
| Gestational diabetes | | | | | **0.007** |
| No | 73 | 53 | 16 | 4 | |
| Yes | 12 | 5 | 3 | 4 | |

### Infant feeding

Extremely preterm infants, those with less birth weight, length and cephalic perimeter, were preferably fed with MOM and DHM than formula (Table 13). During the study period, infants fed predominantly (more than 50% of the total feedings) with formula showed a greater weight gain, lower days of antibiotherapy and total days of hospitalization (Table 13) compared to MOM and DHM.

Table 13 - Clinical data of preterm infants receiving different types of infant feeding.

| | MOM (n=75) | DHM (n = 20) | Formula (n = 13) | *p*-value (DHM vs MOM) | *p*-value (Formula vs MOM) | *p*-value (Formula vs DHM) |
|---|---|---|---|---|---|---|
| **Extremely / very premature**, n | 28/47 | 5/15 | 0/13 | 0.205 | **0.004** | 0.065 |
| **Somatometry at birth** (mean ± SD) | | | | | | |
| Weight, g | 1123 ± 345 | 1173 ± 284 | 1416 ± 219 | 0.438 | **0.002** | **0.011** |
| Length, cm | 36.2 ± 3.3 | 36.8 ± 3.2 | 39.1 ± 2.0 | 0.428 | **0.003** | **0.027** |
| Cephalic perimeter, cm | 25.4 ± 2.4 | 25.3 ± 1.8 | 27.2 ± 1.5 | 0.996 | **0.007** | **0.008** |
| **Δ weight until 26th day**, g | 200 ± 190 | 200 ± 170 | 420 ± 160 | 0.671 | **< 0.001** | **0.001** |
| **Days of antibiotherapy** (mean ± SD) | 12 ± 11 | 13 ± 8 | 5 ± 3 | 0.178 | **0.010** | **< 0.001** |
| **Days of hospitalization** (mean ± SD) | 58 ± 24 | 59 ± 21 | 42 ± 12 | 0.874 | **0.026** | **0.018** |

Significant differences were found in preterm infants' microbiota regarding the feeding types during the study period (Figure 13).

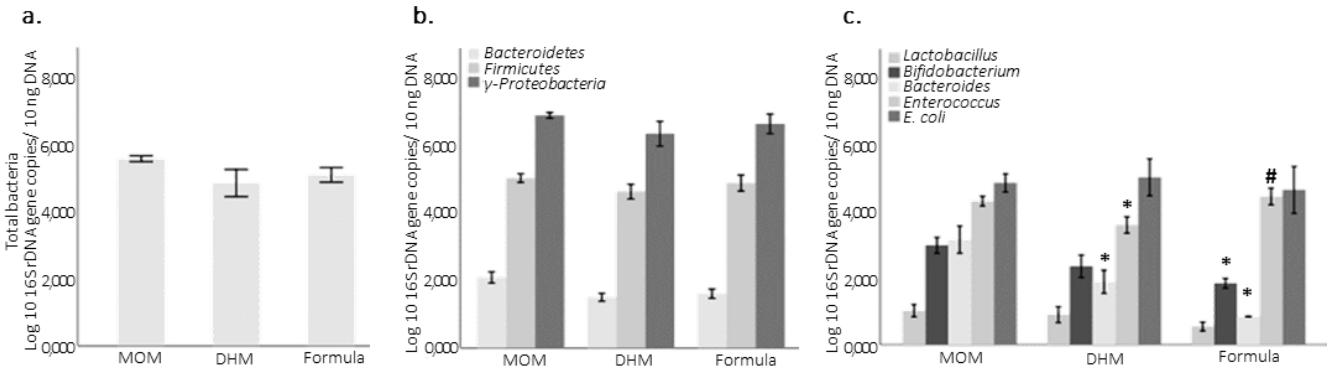


Figure 13 - Influence of feeding types on preterm infants' microbiota during the first 26 days of life. *differences in relation to MOM; # differences in relation to DHM.

MOM promoted a higher amount of *Bifidobacterium* than formula ($p$ = 0.004). However, between MOM and DHM no differences were detected in *Bifidobacterium* content ($p$ = 0.093). The same tendency was observed for *Lactobacillus* genus (Figure 13c). Preterm infants fed predominantly with MOM had higher levels of *Bacteroides* when compared to DHM group ($p$ = 0.032) and to formula group ($p$ = 0.013) (Figure 13c). DHM promoted a lower amount of *Enterococcus* when compared to MOM ($p$ = 0.023) and formula ($p$ = 0.029).

Multivariate linear regression of infants feeding (independent variable) and fecal microbiota of preterm infants at 26th postnatal day (dependent variables) adjusted for gestational age, infant's antibiotherapy received within 8 days prior to fecal collection, as well as maternal adherence to MD were fitted (table 14). DHM and formula promoted lower amounts of total bacteria and *Bifidobacterium* in preterm infants at 26th postnatal day when adjusted for gestational age (model 1), gestational age plus perinatal antibiotherapy (model 2) and gestational age, perinatal antibiotherapy plus maternal adhesion to MD (model 3) (table 14). In addition, *Firmicutes* were lower in formula fed infants when adjusted for the same factors (table 14). After adjusted to gestational age and antibiotherapy (model 2) *Firmicutes* were decreased in infants fed with DHM (table 14).

Table 14 - Association between infants feeding (independent variable) and infants' microbiota at 26th postnatal day (dependent variables).

| | Feeding type* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | DHM | | | | | | Formula | | | | | |
| | Model 1 | | Model 2 | | Model 3 | | Model 1 | | Model 2 | | Model 3 | |
| | β | *p* | β | *p* | β | *p* | β | *p* | β | *p* | β | *p* |
| *Total Bacteria* | **-0.556** | **0.023** | **-0.665** | **0.007** | **-0.670** | **0.009** | **-0.889** | **< 0.001** | **-0.934** | **< 0.001** | **-0.928** | **< 0.001** |
| *Bacteroidetes* | -0.681 | 0.112 | -0.586 | 0.157 | -0.596 | 0.142 | -0.339 | 0.375 | -0.278 | 0.450 | -0.274 | 0.460 |
| *Firmicutes* | -0.585 | 0.055 | **-0.647** | **0.029** | -0.551 | 0.070 | **-0.789** | **0.004** | **-0.829** | **0.002** | **-0.867** | **0.001** |
| *γ-Proteobacteria* | 0.104 | 0.742 | 0.030 | 0.922 | 0.061 | 0.846 | -0.438 | 0.121 | -0.486 | 0.072 | -0.499 | 0.066 |
| *Lactobacillus* | -0.935 | 0.241 | -0.995 | 0.231 | -1.178 | 0.141 | -0.825 | 0.168 | -0.844 | 0.159 | -0.741 | 0.210 |
| *Bifidobacterium* | **-2.002** | **0.003** | **-2.028** | **0.003** | **-2.005** | **0.004** | **-2.092** | **< 0.001** | **-2.109** | **< 0.001** | **-2.118** | **< 0.001** |
| *Bacteroides* | -1.087 | 0.254 | -0.753 | 0.397 | -0.774 | 0.398 | -2.044 | 0.130 | -1.997 | 0.104 | -1.989 | 0.107 |
| *Enterococcus* | 0.027 | 0.940 | 0.032 | 0.930 | -0.036 | 0.923 | -0.085 | 0.789 | -0.082 | 0.798 | -0.053 | 0.869 |
| *Escherichia coli* | 0.041 | 0.968 | 0.041 | 0.968 | -0.436 | 0.667 | -0.364 | 0.683 | -0.364 | 0.684 | -0.172 | 0.843 |

*Reference category: MOM

Model 1 was adjusted for gestational age; Model 2 is adjusted for gestational age and infant's antibiotherapy received within 8 days prior to fecal collection; Model 3 is adjusted for gestational age, infant's antibiotherapy received within 8 days prior to fecal collection and maternal diet.

In order to understand how the establishment and development of intestinal microbiota of preterm infants were affected by the feeding types received in the 8 days preceding each fecal collection, the infants were grouped according to the aforementioned predominant feeding criteria.

At 2nd postnatal day, 28 preterm infants received enteral feeding. No differences were detected on meconium microbiota in comparison with the remaining infants that did not received enteral feeding (Figure 14). Among the fed infants, no differences were also found between MOM and DHM.

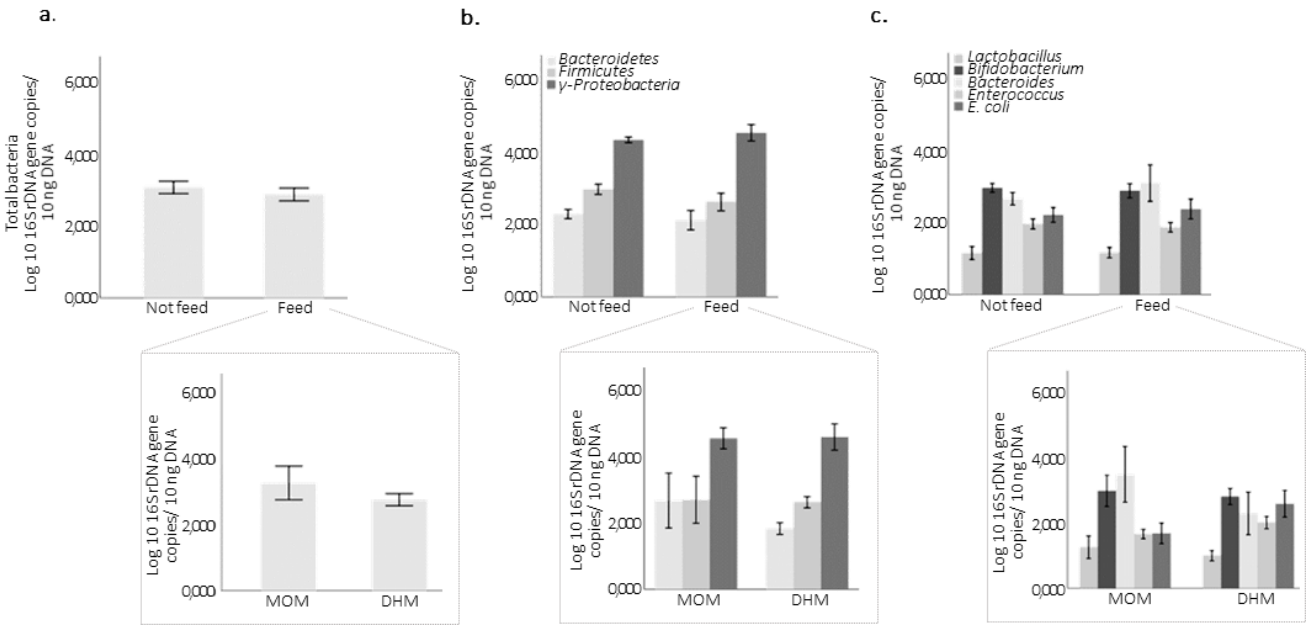


Figure 14 - Influence of enteral feed on the preterm infant meconium microbiota.

Between the 2nd and 10th days, 34 infants were fed predominantly with MOM, 26 with DHM, 10 received a mixed feeding (HM) and 7 with formula. No differences were verified in infants' microbiota at 10th day of life (Figure 15). Interestingly, in the group that received formula *Bacteroides* was not detected (Figure 15c).

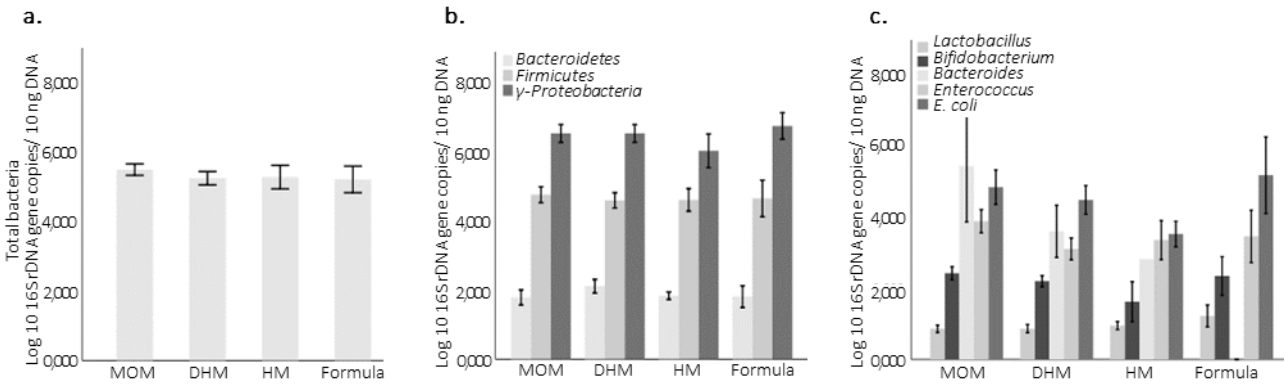


Figure 15 - Preterm infant microbiota at 10th postnatal day according to feeding type.

From the 10th and 18th day of life, formula fed infants (n = 9) presented a lower amount of total bacteria in comparison with MOM (n = 48) and HM (n = 6) groups ($p$ = 0.011 and $p$ = 0.019. respectively) (Figure 16a). Also, the formula group infants showed a decreased amount of *Firmicutes* ($p$ = 0.028) and *Bacteroides* ($p$ = 0.049) regarding the MOM group infants (Figure 16b, c).

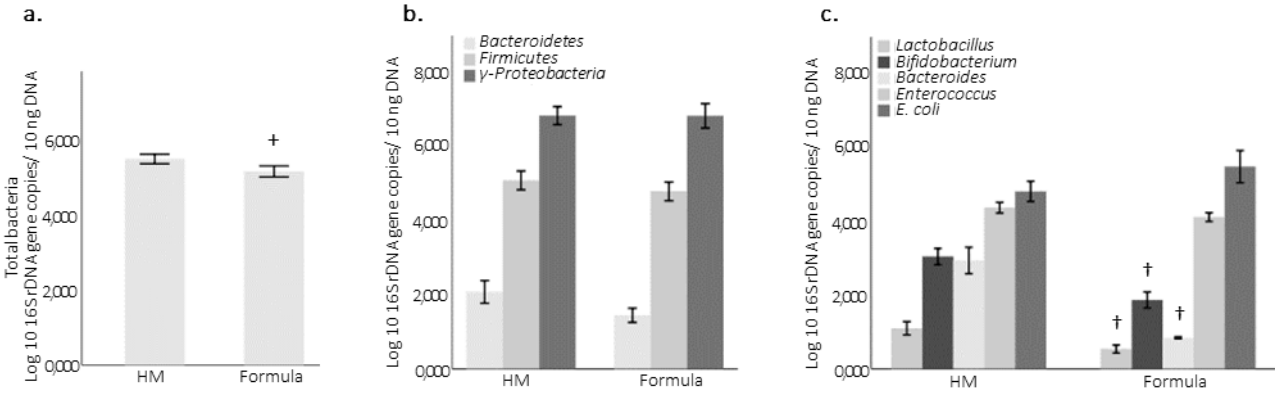


Figure 16 - Preterm infant microbiota at 18th postnatal day according to feeding type. *differences in relation to MOM.

It was between the 18th and 26th days of age that infant feeding had the greatest influence on preterm microbiota. During these period, 45 preterm infants were fed mainly with MOM, 7 received DHM, 4 HM and 17 infant formula. Because of the low number of fecal sample in the DHM and HM groups, preterm infants fed with MOM, DHM and HM were grouped in the same group – global-HM (Figure 17). Compared to global-HM infants, infants fed predominantly with formula showed lower amount of total bacteria and decreased *Firmicutes* ($p$ = 0.034), *Bacteroidetes* ($p$ = 0.005), *Lactobacillus* ($p$ = 0.018), *Bifidobacterium* ($p$ = 0.001) and *Bacteroides* ($p$ = 0.017) (Figure 17a, b and c).

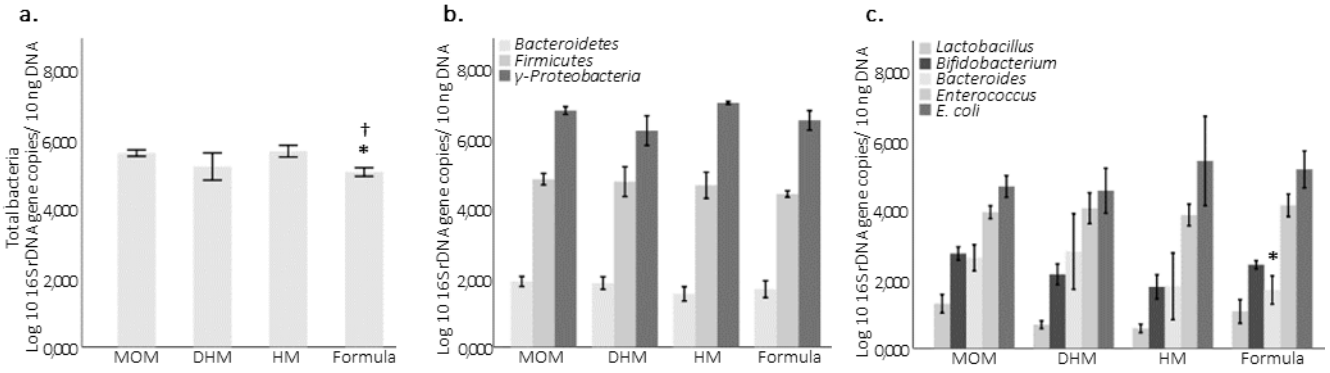


Figure 17 - Preterm infants' microbiota at 26th postnatal day according to feeding type. † Differences in relation to HM group (MOM + DHM).

## DISCUSSION

The first 1000 days of life – the period from conception to 2 years old – are described as a critical window of opportunity to a healthy growth and development [131]. Understanding the role of intrauterine environment on fetal microbiota, as well as the impact of very early postnatal factors on infants' microbiota, is essential for bacterial modulation through clinical interventions such as maternal diet, exposure to antibiotic, probiotics and prebiotics, or even fecal transplantation.

Meconium is a biological material formed during the gestation and has been considered a very useful source of information that reflects the *in utero* microbial environment [132]. Furthermore, the meconium sample collection is a non-invasive procedure making it a great candidate for routine clinical diagnostic practice [132]. The identification of specific bacteria in meconium samples provides individual-specific information to modulate the gut microbiota in order to promote health and reduce the chronic disease in adult life. Specially among the preterm infants, where it is observed a delay and immature gut microbiota, higher levels of oxidative stress and inflammation [133], they surely would benefit from these interventions.

In preterm infants' meconium, 80 to 100% of the samples presented bacterial colonized [134,135]. In this cohort of 117 infants born before 32 gestational weeks, 88% of meconium samples were colonized (range: 0.790 – 5.441 log10 16S rDNA gene copies/10ng of DNA). The first pass dejection of preterm infants is known to have very low biomass and present technical challenges [136]. Moreover, the results of these non-detections may be related to the detection limit of the equipment. The meconium analysis requires special attention. A study that collected meconium samples of healthy full-term infants within the first two hours of life showed that the samples that were immediately processed after collection had significantly less bacterial counts than samples that were stored for 4 days [47]. Nevertheless, all samples were colonized, being supportive of the *in utero* colonization hypothesis.

Meconium microbiota of preterm infants is characterized by less diversity and by altered intestinal colonization of commensal bacteria with persistent dominance of facultative anaerobic bacteria (*Enterobacteriaceae* and *Enterococcus*, from *Proteobacteria* and *Firmicutes* phylum, respectively) [134,137]. This imbalanced colonization causes a delay in the subsequent establishment of strict anaerobic bacteria, such as *Bifidobacterium* and *Bacteroides* [137–139]. Accordingly, in this study it was observed that *γ-Proteobacteria* and *Firmicutes* were the dominant bacterial groups in meconium and fecal samples, but meconium also showed higher levels of *Bifidobacterium* and *Bacteroides* than *Enterococcus*. However, the prevalence of *Bifidobacterium* in meconium samples were lower than *Enterococcus.* Several infants hosted *Bifidobacterium* also at the 10$^{th}$ postnatal day, and at the 18$^{th}$ day *Bifidobacterium* was not detected in two babies. The absence of *Bifidobacterium* was also detected in a study with 29 extremely premature infants [140]. During the study

period, the amount of *Enterococcus* increased significantly, making it the dominant genera at the 18$^{th}$ and 26$^{th}$ postnatal days.

Despite the high variation of microbiota composition between preterm infants, generally, they present a lower bacterial diversity during the first 3 months of life with dominance of *Proteobacteria* followed by *Firmicutes* [77,137]. At family level, *Enterobacteriaceae*, *Clostridium* and *Enterococcus* were identified as the most dominant [141,142]. Furthermore, the abundance of *Bifidobacterium* and *Lactobacillus* were lower at 2$^{nd}$ and 4$^{th}$ postnatal weeks [140,141], as observed in this study. Also, *Bacteroides* were reported to be lower in preterm than term infants during the first 3 months [76]. In the present study, *Bacteroides* were absent in 35-55% of fecal samples suggesting that preterm infants are not able to keep strict anaerobic bacteria [143].

The dominance of potentially pathogenic bacteria (*Enterobacteriaceae* and Clostridium species) rather than commensal bacteria (*Bifidobacterium*, *Propionibacterium*, *Bacteroides*) as observed in infants prematurely delivered (table 2 ), is associated with an increased risk of postnatal complications [79,83]. *Bifidobacterium*, as *Bacteroides*, have important saccharoclastic characteristics to metabolize HMOs providing anti-inflammatory properties to the infants [69,144]. On the other hand, the Gram-negative *Enterobacteriaceae* are a family of facultative anaerobic bacteria with proinflammatory characteristics associated to NEC development. LPS on the outer leaflet of the *Enterobacteriaceae* outer membrane is recognized by TLR-4 causing inflammation. In addition, it is known that *Enterobacteriaceae* species use products of the host inflammatory response as source of energy to growth and to compete with other gut bacteria [79].

It has been reported that mothers may be responsible for the transference of these microbes to the fetus, as well as their metabolites and other molecules that shape the offspring's innate immune system. In this cohort, 35 mother-infant pairs showed a positive correlation of *Firmicutes* and *γ-Proteobacteria* right after birth. It is during the last trimester of pregnancy that the fetus swallows large quantities of amniotic fluid [58], which may be indicative of more maternal bacterial content in meconium in infants born in this gestational period. In agreement with this, it was found that infants delivered during the third trimester (born with more than 28 gestational weeks) presented stronger correlations with their mothers, suggesting the hematogenous bacterial translocation (Figure 3b). Despite the evidence of vertical transference of microbes in infants prematurely delivered is very scare, a recent study with term infants concluded that vertical microbial transmission is a physiological process and even though infants present many microbial strains that maternal microbiota cannot explain, exists strong evidence of microbial transmission from multiple maternal sources to infants [145].

Opposing to what was observed between mothers and their corresponding infants born by vaginal delivery, bacterial correlations were stronger on preterm infants delivered by C-section. The vaginal microbiota is

known to be different from gut microbiota being dominated by *Lactobacillus* species [10]. Passaging through the vaginal canal promoted a distinct colonization that may have mitigated the effect of vertical microbial transmission during pregnancy. However, these differences were not detected possibly due to the restrict number of bacteria analyzed in this study. In fact, in this study the mode of delivery did not show a large effect on preterm microbiota, since *E. coli* was the only bacteria found to be statistically different between vaginal and C-section after birth. An effect that disappeared over time. The effect of mode of delivery in the infants' microbiota is contradictory [70,135]. In preterm infants, it was reported that *Bacteroides* were higher in vaginal delivery [76,146,147]. The presence of strict anaerobic bacteria (such as *Bacteroides*) may be indicative of the consumed available oxygen by facultative anaerobic and, consequently, of a faster intestinal development in infants born prematurely via vaginal delivery. Furthermore, it was observed that at the age of one month, preterm C-section delivered infants (n = 57) had more *Clostridium* cluster I associated with NEC (bacteria that were not analyzed in this study). However, the number of preterm infants born by vaginal delivered analyzed was low (n = 9) [71]. Recently, it was reviewed that contrary to term infants, being born either by vaginal delivery or C-section does not appear to affect the gut microbiota of preterm infants [83].

The dominance of *Lactobacillus* in vaginal microbiota is essential to produce lactic acid and, consequently, to maintain a low vaginal pH preventing dysbiosis and infection that could reach the fetus [148]. The vaginal microbiota is dynamic and changes based on gestational age [149]. Vaginal introitus and midvaginal samples collected between 24$^{th}$ and 28$^{th}$ gestational weeks showed greater richness and diversity than samples collected between 28$^{th}$ and 32$^{nd}$ weeks [149]. So, according to the theory of vaginal bacteria ascension (Figure 3b), it is possible to speculate that more *Lactobacillus* reach the fetus in 24$^{th}$-28$^{th}$ than 28$^{th}$-32$^{nd}$ weeks of gestation. In line with this, in this thesis it was observed that extremely preterm infants (born before 28 weeks) had more *Lactobacillus* compared to very preterm infants (born between 28 and 32 gestational weeks), suggesting the vertical ascension of bacteria from vaginal microbiota to placental/ amniotic fluid. All together, these findings suggest the premise that maternal bacteria from gut and vagina play a role in shaping the infants gut microbiota. However, the question remains open: do these bacteria reach the fetus alive, only in fragments or in both forms?

The rupture of fetal membranes showed a stronger influence on microbiota of preterm infants. Meconium from infants with fetal membrane ruptured less than 18 hours before birth had decreased *Lactobacillus* and increased levels of *E. coli*. On the other hand, infants with membrane ruptured over 18 hours presented decreased amount of total bacteria and *Firmicutes* when compared to intrapartum ruptured membranes. Vaginal dysbiosis in mothers with lower levels of *Lactobacillus* and increased bacterial diversity was reported as a risk factor for premature membrane rupture and preterm births [150]. After the rupture of fetal membranes, the fetus remains without the protection that it provided. Exposed to maternal vaginal bacteria,

the fetus could be at higher risk of infection. The decreased levels of total bacteria, *Firmicutes* and *Lactobacillus* observed in membrane ruptured over 18 hours may be due to antepartum antibiotic exposure, since all mothers whose membranes ruptured for more 18 hours received antibiotics, as well as 16 out of 22 with ruptured membranes for less than 18 hours.

As observed in this thesis, maternal antibiotherapy during gestation was reported to impact on both mother and offspring gut microbiota [76]. Antibiotic administration during pregnancy and/or early after birth are reported to increases risk of asthma, overweight and obesity in childhood (7-12 years of age) [50]. Maternal administration of antibiotics have been reported to reduce the infection, namely chorioamnionitis. Chorioamnionitis, an intra-amniotic infection that causes inflammation of the fetal membranes, is associated with preterm labor. In this cohort, only 6 mothers were identified with chorioamnionitis (one with intrapartum ruptured membranes, four with ruptured membranes and the other is not specified in the clinical records). Of these, only four mothers-infants pairs had their feces collected. Due to the small number of women with chorioamnionitis it was not possible to analyze this relation.

Other antepartum factors, such as maternal diet, gestational diabetes and obesity, are reported to promote offspring chronic diseases through alterations in microbiota during the gestation period [151]. The (patho)physiological mechanisms by which maternal antepartum factors affect the offspring health are not well understood [151]. Diabetes and obesity, as well as pregnancy *per se*, lead to a greater intestinal permeability that may be regulated by gut microbiota through regulation of intracellular tight junctions. A brief and simplified explanation of the mechanism by which diabetes and/or obesity may alter the health status of mothers-and-child is demonstrated in Figure 18.

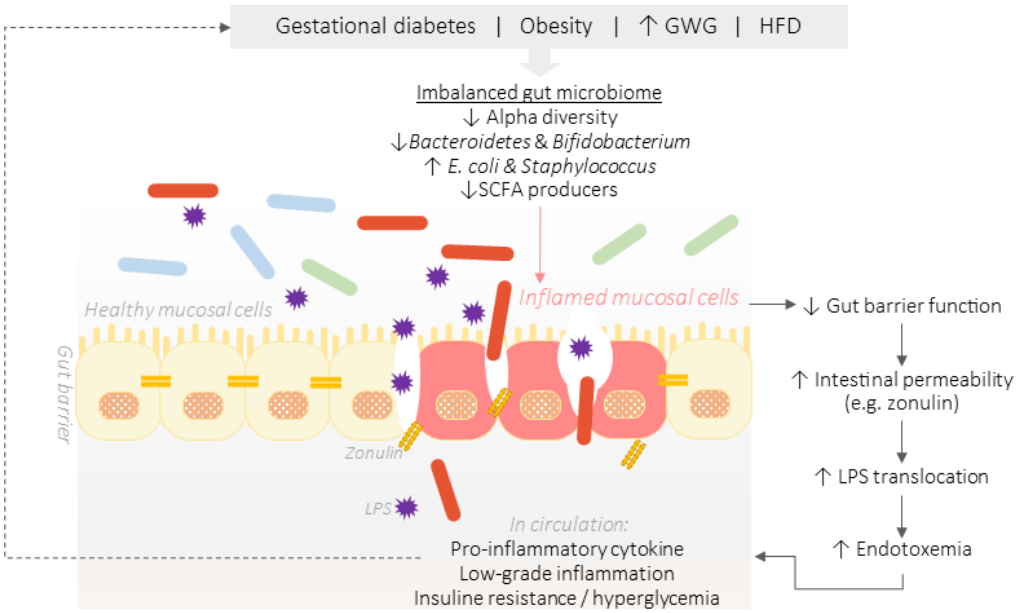


Figure 18 - Simplified mechanism by which maternal factors (gestational diabetes, obesity, increased gestational weight gain and high fat diet) may alter microbiota, inflammation and glucose metabolism. This dysbiosis may be directly transmitted to the offspring causing an impairment in development. Image adapted from Soderborg *et al*, 2016 [58] and Singh *et al*, 2017 [153].

The association between gestational diabetes and offspring health has been described. A follow-up study that recruited newborns at birth to 3 years of age, found that gestational diabetes was significantly associated with atopic dermatitis and allergen sensitization in term-delivered infants, but not in preterm infants [152]. Recent studies showed that diabetes during pregnancy can alter the microbiota of both woman and child in a similar pattern [153,154]. In 20 term newborns whose mother had gestational diabetes lower *Proteobacteria* and *Actinobacteria* and more *Bacteroidetes* were observed [153]. Furthermore, *Prevotella* and *Lactobacillus* genus were decreased [153]. Contrarily, *Lactobacillus* were found significantly more abundant in term infants delivered by mothers with gestational diabetes [154]. Short and long-term effects of gestational diabetes on microbiota of preterm infants are very scarce. Here, it was observed that *Lactobacillus* was decreased on infants born to mothers who had diabetes during pregnancy. This effect was also observed in preterm infants from obese mothers. Despite the low number of obese mothers in this study (n = 8), being obese was related to gestational diabetes status, as observed in other study [155]. However, some studies did not found associations between maternal obesity and gestational diabetes [152,156].

In fact, higher BMI categories were not only related to gestational diabetes, but also to the incidence of preeclampsia, gestational hypertension, antenatal admissions and preterm birth [155]. The incidence of preterm infants was also found higher in underweight women [155]. In addition, maternal gut microbiota is related to their BMI and weight gain during pregnancy [157]. Overweight women showed a reduced amount of *Bifidobacterium* and *Bacteroidetes* and increased *Staphylococcus*, *Enterobacteriaceae* and *E. coli,* compared to normal weigh mothers [157]. In this thesis, pre-gestational BMI did not showed differences on mothers' fecal microbiota. However, in preterm infants it was possible to see the effect of maternal BMI. While no differences were found in meconium microbiota of infants delivered by women with normal weight or overweight, obese mothers delivered infants with significantly lower *Lactobacillus* and higher amount of *Firmicutes and Bifidobacterium* than normal weight group. A study with 42 Finnish women (26 normal weight and 16 overweight/obese) found differences in infants microbiota at 1 and 6 postnatal months [158]. The overall amount of *Bifidobacterium* species were higher in infants with normal weight than overweight/obese (BMI > 25 kg/m$^2$) mothers. However, infants born from obese mothers had increased levels of *Bifidobacterium adolescentis* than normal weight group [158]. The authors discussed that *Bifidobacterium adolescentis* has been associated with inflammatory effects, contrarily to others *Bifidobacterium* species, such as *Bifidobacterium longum* and *Bifidobacterium bifidum* [158]. It would be interesting to do a deeper analysis of *Bifidobacterium* bacteria in the fecal samples of the current study, in order to determine what species could explain the increased levels of *Bifidobacterium* on infants born to obese women.

Maternal diet, namely MD, has reported to protective against the development of diseases in adults and children [66]. MD also showed to be a crucial player to healthy pregnancy outcomes and prevention of

premature birth [64,66,159]. In addition, a single-center prospective study composed by 82 women who delivered preterm infants found that low MD adherence increased the risk of intrauterine growth restriction, low weight at birth, bronchopulmonary dysplasia and NEC [160]. However, in a study with 728 women, no associations between MD adherence during pregnancy and the risk of premature delivery and fetal growth restriction were observed [161]. Regarding the gut microbiota, MD was reported to promote greater levels of *Lactobacillus*, *Bifidobacterium*, *Eubacteria*, *Bacteroides*, *Prevotella* and increased levels of fecal SCFAs [162]. Based on the literature search, no studies were found on maternal adherence to MD and its effects on preterm infants' gut microbiota. In this study, no effects were observed of MD adherence or nutrients intake on the preterm infants' or mothers' microbiota. It is important to note that almost all mothers with gestational diabetes were being followed by nutritionist and their answers may have been influenced, causing a higher PREDIMED score that may not have corresponded to what occurred during pregnancy. Furthermore, during the hospitalization of preterm infants, period in which fecal samples were collected, mothers were under stress as well as antibiotics – factors that are known to strongly influence intestinal microbiota.

Finally, increasing data suggest the infant feeding significantly influences the preterm infants' development as well as their microbiota composition [107]. In this cohort, preterm infants fed predominantly with formula showed an increased weigh gain during the first 26$^{th}$ days of life. At first glance, it seems that formula promoted a greater development and growth of preterm infants when compared to MOM or DHM. However, this may be because MOM and DHM were preferably administrated to immature preterm infants with less birth weight, length and cephalic perimeter, explaining why these infants gained less weight during the study period and had more days of antibiotic therapy. Actually, differences in the gut microbiota of infants fed with MOM, DHM or formula could support this. The present findings also demonstrate that infant feeding takes time to influence the gut microbiota of preterm infants (between the 18$^{th}$ and 26$^{th}$ day of life), as seen in others studies [71,163]. Infant feeding significantly influenced the preterm microbiota composition. Differences in the amount of total bacteria and *Bifidobacterium* were found in infant fed with MOM, which had a higher content than DHM and formula. Nevertheless, these differences were lower in DHM than formula fed infants.

DHM seemed to be more advantageous than formula in what regards benefic bacterial species. Actually, as can be seen in Figure 13, DHM promoted a gut microbiota with closer resemblance to MOM than to formula. A recent study with 69 preterm infants showed that despite the large differences between MOM (n = 34) and DHM-fed infants (n = 28), a similar profile was found in these groups compared to formula (n = 7) [114]. The dissimilarities between MOM and DHM may be due to the authors' criteria for grouping infants – at least 80% of DHM in the total feedings [114]. That infants had less influence of MOM in the DHM group than in the present study. Gregory *et al.* (2016) found that DHM resembled MOM-like microbiota but only in infants delivered after 28 gestational week of age, but not in infants born prior to 28 weeks [111]. The authors

discussed that gestational age and, consequently, gut immaturity may delay the final microbiota composition. Actually, a follow-up study showed that preterm-delivered infants at 2 years old had lower abundance of *Lactobacillus* compared to age-matched term infants, suggesting that prematurity could be a long-lasting effect [164].

Similar effects of DHM in relation to MOM on the intestinal microbiota may be related to the composition of HMOs, despite different total amount and relative composition of HMOs in DHM [101]. Furthermore, bacterial cells present in milk, even in dead or inactive form by heat treatment, may have an important role in immune system of these infants and in their microbial colonization. It is known that heat treatment, such as pasteurization, can inactive probiotic strains [165]. These non-viable microbial cells, designed as paraprobiotics (or ghost probiotics), have been reported to have immunomodulatory effects similar to living microorganisms (probiotics) [165].

On the other hand, formula fed-infants promote a distinct microbiota profile in relation to MOM [107]. However, exist a lack of congruence between the studies. Here, formula promoted lower levels of *Bifidobacterium* and *Bacteroides* when compared to MOM. These findings suggest that formula delays colonization of anaerobic bacteria and, most likely, intestinal maturation. This is an expected result since *Bifidobacterium* and *Bacteroides* species utilize HMOs that are absent in formula, unlike MOM and DHM. Despite the GOS and FOS content of formula, previous studies showed that these do not resemble the oligosaccharides from HM [108]. In this study, no infant in the formula group was exclusively formula-fed and always received some MOM or DHM. A very recent study divided 62 preterm infants into 5 groups: MOM exclusively fed infants (n = 7); predominantly formula exclusively fed (n = 8); mixed with ≥ 70% MOM and formula (n = 16); mixed with ≥ 70% formula and MOM (n = 16); and, mixed with 50% MOM and formula (n = 15) [166]. *E. coli* was always increased in all feedings containing formula and infants fed with 70% formula presented more amount of *Escherichia*, *Salmonella*, *Enterococcus* and *Enterobacteriaceae* [166]. These results suggest that even a mixed fed with at least 50% formula promote a different microbiota colonization in preterm infants.

Compared to term infants, preterm newborns presented an immature gastrointestinal tract with reduced gastric proteolytic enzymes, increased gastric pH, and decreased epithelial membrane tight junctions, diminished intestinal mucus coat and a microbiota low in abundance and in diversity (Figure 19a) [110,167]. Increasing evidence support that human milk can modify these factors creating a protection against the development of NEC or late-onset sepsis (Figure 19b) [110].

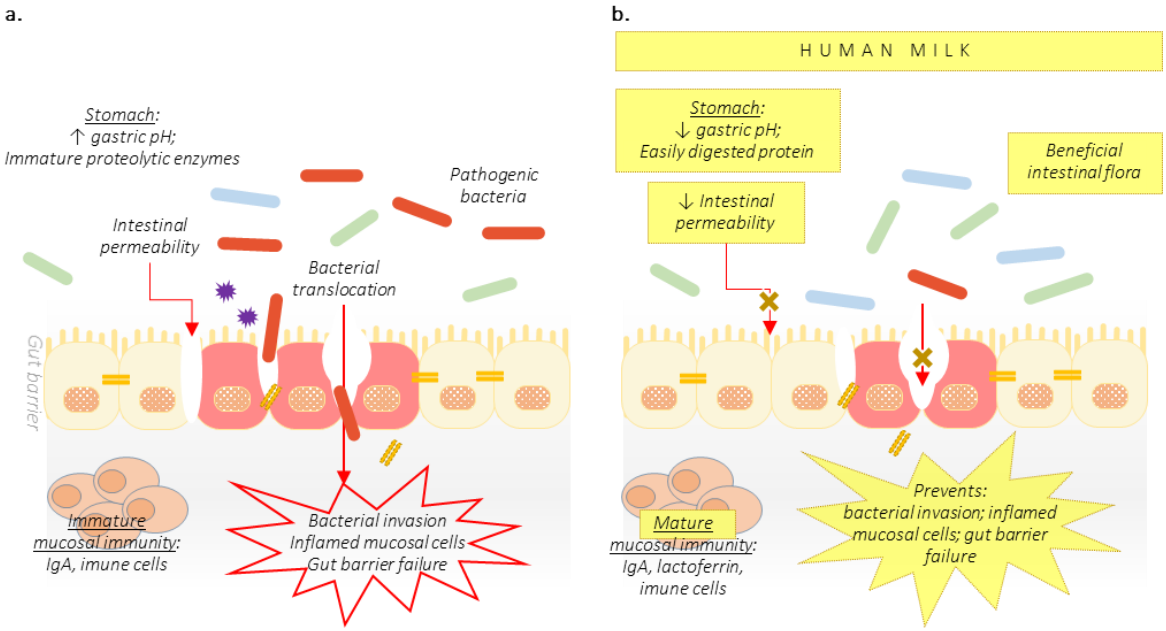


Figure 19 – Preterm infant's intestine. a. intestine of preterm infants is immature, present an imbalanced microbiota profile and it is highly susceptible for disease. b. human milk provide protection against immature gut and development of diseases. Image adapted from Maffei and Schanle, 2017 [111].

The results presented in this thesis, showed that MOM is the best type of infant feeding in the promotion of the maturation of preterm infants' gut microbiota, in concordance with other studies [107]. In a cohort of 45 breastfed premature infants it was observed that preterm infants progressed to a *Bifidobacterium*-dominated microbiota after 30 weeks postmenstrual age, even when infants born by C-section [75]. These "healthier" microbiota is characteristic of term infants and it is associated with better health outcomes. The authors concluded that *normal*-like microbiota development is possible in hospitalized preterm infants' delivery by C-section if breastmilk is administrated [75].

Studies including preterm infants' microbiota and their mothers' microbiota are very scare, since this is a particularly vulnerable study population that presents many challenges to work with. The main limitations of this study are related to fecal sample collections. Due to the high workload of the NICU nursing team, and also to the lack of daily fecal dejections of infants, several samples were collected over time, which caused loss of fecal samples. Furthermore, the collection of fecal samples from mothers was very irregular and did not respect a specific period of days due to physiological and/or emotional reasons. In addition, health related information, such as smoking, BMI was not accessible in several enrolled subjects due to the lack of information in clinical records. Exposure to probiotics and antibiotics during gestation was not questioned. Even so, the present study highlights the importance of the infant feeding, and other factors, on 117 preterm infants' gut microbiota.

## CONCLUSION

Preterm births are the leading cause of mortality among children under five worldwide [80]. The FEEDMI study confirms that preterm infant is highly affect by infant feeding, emphasizing the importance of HM. MOM promoted a healthier gut microbiota with higher levels of total bacteria and anaerobic bacteria (*Bifidobacterium*) compared to DHM and formula. Combining DHM and MOM into the same feeding could be a good strategy to prolonging HM feeding and delaying (or even suspending) formula administration in hospitalized preterm infants when their mothers' milk is not sufficient. Unfortunately, DHM milk is not enough for all preterm infants. Actually, in Portugal, only 2 out of 40 NICUs have DHM available from the only HM bank at MAC. HM donation should be promoted not only by health professionals, but also by the government, as well as the development and implementation of HM banks in the remaining NICUs. Pasteurization techniques should be optimized for minimal loss of nutritional and biological components while maintaining microbiological safety.

This thesis also provide evidence that antepartum factors affect gut microbiota composition of infants prematurely-delivered. Among the findings that almost all meconium samples were colonized, it cannot be discarded that infants' meconium may have bacterial DNA prior to birth. This highlights the relevance of maternal microbiota transmission. Furthermore, maternal obesity was associated to gestational diabetes development and that influenced the gut microbiota of the infants and their mothers. Factors closely related to preterm birth, such as premature fetal membrane rupture and maternal antibiotic prior to birth, also showed an impact in infants' microbiota.

Altogether, these results suggest that preterm infants' gut microbiota are highly susceptible to maternal, environmental and therapeutic factors. Future studies are warranted to analyze the influence of HM (MOM and DHM before and after pasteurization) bacteria composition on the preterm infants' gut microbial acquisition. Follow-up studies assessing the microbiota evolution of preterm infants are very scare. Understanding how the factors mentioned above affect the long-term development, growth and microbiota of preterm infants is essential, since pregnancy and early life are critical window of opportunity for provide effective interventions for nutritional and immunomodulatory programming. To address this need, the FEEDMI cohort may be the primary step to initiate the first prospective follow-up study about gut microbiota evolution in infants in Portugal.

## REFERENCES

1 Ursell LK, Metcalf JL, Parfrey LW, Knight R: Defining the Human Microbiome. Nat Rev 2013;70:1–12.

2 Lynch S V., Pedersen O: The Human Intestinal Microbiome in Health and Disease. New J Med 2016;375:2369–2379.

3 Sender R, Fuchs S, Milo R: Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLoS Biol 2016;14:1–14.

4 Rosner JL: Ten times more microbial cells than body cells in humans? 2014.

5 Dethlefsen L, Mcfall-ngai M, Relman DA: An ecological and evolutionary perspective on human – microbe mutualism and disease. Nature 2007;449:811–818.

6 Spor A, Koren O, Ley R: Unravelling the effects of the environment and host genotype on the gut microbiome. Nat Microbiol 2011;9:279–290.

7 Shively CA, Register TC, Appt SE, Chiba A, Tooze JA, Cook KL, et al.: Consumption of Mediterranean versus Western Diet Leads to Distinct Mammary Gland Microbiome Article Consumption of Mediterranean versus Western Diet Leads to Distinct Mammary Gland Microbiome Populations. CellReports 2018;25:47–56.e3.

8 Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J: The Placenta Harbors a Unique Microbiome. Sci Transl Med 2014;6:1–22.

9 Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al.: A human gut microbial gene catalogue established by metagenomic sequencing. Nature 2010;464:59–65.

10 Human Microbiome Project: A framework for human microbiome research. Nature 2012;486:215–221.

11 Tang L: Microbial interactions. Nature Methods 2018;16:19.

12 Hermann-bank ML, Skovgaard K, Stockmarr A, Larsen N, Mølbak L: The Gut Microbiotassay : a high-throughput qPCR approach combinable with next generation sequencing to study gut microbial diversity. BMC Genomics 2013;14.

13 Knight R, Navas J, Quinn RA, Sanders JG, Zhu Q: Best practices for analysing microbiomes. Nat Rev Microbiol 2018;16:410–422.

14 Tang L: Sequence-based identification of human-associated microbiota. Nat Milestones 2019;S10.

15 Ilhan ZE: Microbiome After Bariatric Surgery and Microbial Insights into Surgical Weight Loss. Ph. D. Disertation 2016;

16 Méndez-García C, Barbas C, Ferrer M, Rojob D: Complementary Methodologies To Investigate Human Gut Microbiota in Host Health, Working towards Integrative Systems Biology. J Bacteriol 2018;200:1–13.

17 Rajili M, Vos WM De: The first 1000 cultured species of the human gastrointestinal microbiota. FEMS Microbiol Rev 2014;38:996–1047.

18 Ottman N, Smidt H, Vos WM De, Belzer C: The function of our microbiota : who is out there and what do they do ? 2012;2:1–11.

19 Chierico F Del, Vernocchi P, Petrucca A, Paci P, Fuentes S, Pratic G, et al.: Phylogenetic and Metabolic Tracking of Gut Microbiota during Perinatal Development 2015;1–26.

20 Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al.: Human gut microbiome viewed across age and geography. Nature 2012;486:222–227.

21 Franzosa EA, Huang K, Meadow JF, Gevers D, Lemon KP: Identifying personal microbiomes using metagenomic codes 2015; DOI: 10.1073/pnas.1423854112

22 Cani PD, Hul M Van, Lefort C, Depommier C, Rastelli M, Everard A: Microbial regulation of organismal energy homeostasis. Nat Metab 2019;1:34–46.

23 Zmora N, Suez J, Elinav E: You are what you eat: diet, health and the gut microbiota. Nat Rev Gastroenterol Hepatol 2018;16:35–56.

24 Chow J, Lee SM, Shen Y, Khosravi A, Mazmanian SK: Host-Bacterial Symbiosis in Health and Disease. Adv Immunol 2010;107:243–274.

25 Rowland I, Gibson G, Heinken A, Scott K, Swann J, Thiele I, et al.: Gut microbiota functions : metabolism of nutrients and other food components. Eur J Nutr 2018;57:1–24.

26 Levy M, Kolodziejczyk AA, Thaiss CA, Elinav E: Dysbiosis and the immune system. Nat Rev Immunol 2017;17:219–232.

27 Burdett HL: Human microbiota: insights from large population studies. Nature 2019;

28 Harris JW, Brown JH: The bacterial content of the uterus at cesarean section. Am J Obstet Gynecol 1928;16:332–338.

29 Stroup PE: Amniotic Fluid Infection and the Intact Fetal Membrane.pdf. Obs Gynecol 1962;19:736–9.

30 Harwick HJ, Iuppa JB FF: Microorganisms and amniotic fluid. Obs Gynecol 1969;33:256–9.

31 Prevedourakis C, Papadimitriou G, Ioannidou A: Isolation of pathogenic bacteria in the amniotic fluid during pregnancy and labor. Am J Obstet Gynecol106:400–402.

32 Gosalbes MJ, Llop S, Vall??s Y, Moya A, Ballester F, Francino MP: Meconium microbiota types dominated by lactic acid or enteric bacteria are differentially associated with maternal eczema and respiratory problems in infants. Clin Exp Allergy 2013;43:198–211.

33 Th. Escherich: The intestinal bacteria of the neonate and breast-fed infant. Rev Infect Dis 1885;11:352–6.

34 IC H, E O, IP H: Bacterial flora of first specimens of meconium passed by fifty newborn infants. Am J Dis Child 1934;47:1279–85.

35 Hansen R, Scott KP, Khan S, Martin JC, Berry SH, Stevenson M, et al.: First-Pass Meconium Samples from Healthy Term Vaginally-Delivered Neonates : An Analysis of the Microbiota. PLoS One 2015;10:1–10.

36 Perez-Muñoz ME, Arrieta MC, Ramer-Tait AE, Walter J: A critical assessment of the “sterile womb” and “in utero colonization” hypotheses: Implications for research on the pioneer infant microbiome. Microbiome 2017;5:1–19.

37 Steel JH, Malatos S, Kennea N, Edwards AD, Miles L, Duggan P, et al.: Bacteria and Inflammatory Cells in Fetal Membranes Do Not Always Cause Preterm Labor. Pediatr Res 2005;57:404–11.

38 Jiménez E, Fernµndez L, Marín ML, Martín R, Odriozola JM, Nueno-palop C, et al.: Isolation of Commensal Bacteria from Umbilical Cord Blood of Healthy Neonates Born by Cesarean Section. Curr Microbiol 2005;51:270–4.

39 Stout MJ, Conlon B, Landeau M, Lee I, Bower C, Zhao Q, et al.: Identification of intracellular bacteria in the basal plate of the human placenta in term and preterm gestations. Am J Obs Gynecol 2013;208:226.e1-7.

40 Lauder AP, Roche AM, Sherrill-mix S, Bailey A, Laughlin AL, Bittinger K, et al.: Comparison of placenta samples with contamination controls does not provide evidence for a distinct placenta microbiota. Microbiome 2016;1–11.

41 Seferovic MD, Pace RM, Carroll M, Belfort B, Major AM, Chu DM, et al.: Visualization of microbes by 16S in situ hybridization in term and preterm placentas without intraamniotic infection. Am J Obstet Gynecol 2019;221:146.e1-23.

42 DiGiulio DB: Diversity of microbes in amniotic fluid. Semin Fetal Neonatal Med 2012;17:2–11.

43 Collado MC, Rautava S, Aakko J, Isolauri E, Salminen S: Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. Sci Rep 2016;6:23129.

44 Lim ES, Rodriguez C, Holtz LR: Amniotic fluid from healthy term pregnancies does not harbor a

detectable microbial community. Microbiome 2018;6:4–11.

45 Payne MS, Keelan JA, Stinson LF: Re: “Amniotic fluid from healthy term pregnancies does not harbor a detectable microbial community” (2018) 6:87, https://doi.org/10.1186/s40168-018-0475-7. Microbiome 2019;7:19–21.

46 Willyard C: Baby’s first bacteria. Nature 2018;553:264–66.

47 Jiménez E, Marín ML, Martín R, Odriozola JM, Olivares M, Xaus J, et al.: Is meconium from healthy newborns actually sterile? Res Microbiol 2008;159:187–193.

48 Bailey MT, Lubach GR, Coe CL: Prenatal Stress Alters Bacterial Colonization of the Gut in Infant Monkeys. J Pediatr Gastroenterol Nutr 2004;38:414–421.

49 Funkhouser LJ, Bordenstein SR: Mom Knows Best : The Universality of Maternal Microbial Transmission 2013;11:e1001631.

50 Walker RW, Clemente JC, Peter I, Loos RJF: The prenatal gut microbiome: are we colonized with bacteria in utero ? Pediatr Obes 2017;12:3–17.

51 Makino H, Kushiro A, Ishikawa E, Kubota H, Gawad A, Sakai T, et al.: Mother-to-Infant Transmission of Intestinal Bifidobacterial Strains Has an Impact on the Early Development of Vaginally Delivered Infant ’ s Microbiota. PLoS One 2013;8:e78331.

52 Rautava S, Collado MC, Salminen S, Isolauri E: Probiotics modulate host-microbe interaction in the placenta and fetal gut: A randomized, double-blind, placebo-controlled trial. Neonatology 2012;102:178–184.

53 Avershina E, Lundgard K, Sekelja M, Dotterud C, Storro O, Johnsen R, et al.: Transition from infant- to adult-like gut microbiota. Env Microbiol 2016;18:2226–2236.

54 Tamburini S, Shen N, Wu HC, Clemente JC: The microbiome in early life : implications for health outcomes. Nat Publ Gr 2016;22:713–722.

55 Barker D: Mothers, babies and disease in later life. BMJ Publ Gr 1994;673–674.

56 Soderborg TK, Borengasser SJ, Barbour LA, Friedman JE: Microbial transmission from mothers with obesity or diabetes to infants: an innovative opportunity to interrupt a vicious cycle. Diabetologia 2016;59:895–906.

57 Milani C, Mancabelli L, Lugli A, Duranti S, Turroni F, Ferrario C, et al.: Exploring Vertical Transmission of Bifidobacteria from Mother to Child. Appl Env Microbiol 2015;81:7078–87.

58 Neu J: The Microbiome and Its Impact on Disease in the Preterm Patient. Curr Pediatr Rep 2013;1:215–221.

59 Koren O, Goodrich JK, Cullender TC, Spor AA, Laitinen K, Backhed HK, et al.: Host remodeling of the gut microbiome and metabolic changes during pregnancy. Cell 2012;150:470–80.

60 Collado MC, Isolauri E, Laitinen K, Salminen S: Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women. Am J Clin Nutr 2008;88:894–899.

61 Macpherson AJ, Agüero MG De, Ganal-vonarburg SC: How nutrition and the maternal microbiota shape the neonatal immune system. Nat Immunol 2017;17:508–17.

62 Chen X, Welner RS, Kincade PW: A possible contribution of retinoids to regulation of fetal B lymphopoiesis. Eur J Immunol 2009;39:2515–24.

63 Heerwagen MJR, Miller MR, Barbour LA, Friedman JE: Maternal obesity and fetal metabolic programming: a fertile epigenetic soil. AJP Regul Integr Comp Physiol 2010;299:R711–R722.

64 Englund-ögge L, Lise A, Sengpiel V, Myhre R, Meltzer HM, Jacobsson B: Maternal dietary patterns and preterm delivery: results from large prospective cohort study. BMJ 2014;348:1–18.

65 Trichopoulou A, Kouris-blazos A, Wahlqvist ML, Gnardellis C, Lagiou P, Polychronopoulos E, et al.: Diet and overall survival in elderly people 1995;311:1457–1460.

66 Biagi C, Nunzio M Di, Bordoni A, Gori D, Lanari M: Effect of Adherence to Mediterranean Diet during Pregnancy on Children 's Health : A Systematic Review. Nutrients 2019;11:1–25.

67 Mingot DL, Gesteiro E, Bastida S, Sánchez-muniz FJ: Epigenetic effects of the pregnancy Mediterranean diet adherence on the offspring metabolic syndrome markers. J Physiol Biochem 2017;73:495–510.

68 Gohir W, Kennedy KM, Wallace JG, Saoi M, Bellissimo CJ, Britz-mckibbin P, et al.: High-fat diet intake modulates maternal intestinal adaptations to pregnancy and results in placental hypoxia , as well as altered fetal gut barrier proteins and immune markers. J Physiol 2019;12:3029–51.

69 Milani C, Duranti S, Bottacini F, Casey E, Turroni F, Mahony J, et al.: The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. Microbiol Mol Biol Rev 2017;81:1–67.

70 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 U S A 2010;107:11971–11975.

71 Itani T, Ayoub Moubareck C, Melki I, Rousseau C, Mangin I, Butel M-J, et al.: Establishment and development of the intestinal microbiota of preterm infants in a Lebanese tertiary hospital. Anaerobe 2017;43:4–14.

72 Chong CYL, Bloomfiel FH, O'Sullivan JM: Factors Affecting Gastrointestinal Microbiome Development in Neonates. Nutrients 2018;10:1–17.

73 Dominguez-Bello MG, De Jesus-Laboy KM, Shen N, Cox LM, Amir A, Gonzalez A, et al.: Partial restoration of the microbiota of cesarean-born infants via vaginal microbial transfer. Nat Med 2016;22:250–253.

74 Haahr T, Glavind J, Axelsson P, Fischer MB, Fuglsang J, Bonde U, et al.: Vaginal seeding or vaginal microbial transfer from the mother to the caesarean-born neonate: a commentary regarding clinical management. An Int J Obstet Gynaecol 2017;125:533–536.

75 Korpela K, Blakstad EW, Moltu SJ, Strømmen K, Nakstad B, Rønnest AE, et al.: Intestinal microbiota development and gestational age in preterm neonates 2018;1–9.

76 Arboleya S, Borja S, Ventura M, Margolles A, Fern N, Reyes-gavil CGDL: Intestinal Microbiota Development in Preterm Neonates and Effect of Perinatal Antibiotics 2015; DOI: 10.1016/j.jpeds.2014.09.041

77 Barrett E, Kerr C, Murphy K, O'Sullivan O, Ryan CA, Dempsey EM, et al.: The individual-specific and diverse nature of the preterm infant microbiota. Arch Dis Child - Fetal neonatal Ed 2013;98:F334–F340.

78 Hill CJ, Lynch DB, Murphy K, Ulaszewska M, Jeffery IB, O'Shea CA, et al.: Evolution of gut microbiota composition from birth to 24 weeks in the INFANTMET Cohort. Microbiome 2017;5:4.

79 Underwood MA, Sohn K: The Microbiota of the Extremely Preterm Infant. Clin Perinatol 2017;44:407–427.

80 March of Dimes, Partnership for Maternal Newborn and Child Health, Save the Children, World Health Organization: Born Too Soon: The Global Action Report on Preterm Birth. Howson CP, Kinney MV, Lawn JE, Ed 2012;

81 Romero R, Dey SK, Fisher SJ: Preterm Labor: One Syndrome, Many Causes. Science (80- ) 2015;345:760–65.

82 Strunk T, Currie A, Richmond P, Simmer K, Burgner D: Innate immunity in human newborn infants: prematurity means more than immaturity. J Matern Neonatal Med 2011;24:25–31.

83 Staude B, Oehmke F, Lauer T, Behnke J, Göpel W, Schloter M, et al.: The Microbiome and Preterm Birth: A Change in Paradigm with Profound Implications for Pathophysiologic Concepts and Novel Therapeutic Strategies. Biomed Res Int 2018;2018:1–12.

84 Cotten CM, Taylor S, Stoll B, Goldberg RN, Hansen NI, Sánchez, Pablo J. Namasivayam Ambalavanan

Benjamim DK: Prolonged Duration of Initial Empirical Antibiotic Treatment Is Associated With Increased Rates of Necrotizing Enterocolitis and Death for Extremely Low Birth Weight Infants. Pediatrics 2009;123:58–66.

85 Greenwood C, Morrow AL, Lagomarcino AJ, Altaye M, Taft DH, Yu Z, et al.: Early empiric antibiotic use in preterm infants is associated with lower bacterial diversity and higher relative abundance of Enterobacter. J Pediatr 2014;165:23–29.

86 Mueller NT, Whyatt R, Hoepner L, Oberfield S, Dominguez-Bello MG, Widen E, et al.: Prenatal exposure to antibiotics, cesarean section and risk of childhood obesity. Int J Obes 2015;39:665–670.

87 Tapiainen T, Paalanne N, Tejesvi M V, Koivusaari P, Korpela K, Pokka T, et al.: Maternal influence on the fetal microbiome in a population-based study of the first-pass meconium. Pediatr Res 2018;84:371–9.

88 Kumar RK, Singhal A, Vaidya U, Banerjee S, Anwar F, Rao S: Optimizing Nutrition in Preterm Low Birth weight infants — Consensus Summary. Front Nutr 2017;4:1–9.

89 ESPGHAN Committee on Nutrition, Agostoni C, Braegger C, Decsi T, Kolacek S, Koletzko B, et al.: Breast-feeding: A Commentary by the ESPGHAN Committee on Nutrition. J Pediatr Gastroenterol Nutr 2009;49:112–125.

90 Pereira-da-Silva L, Gomes A, Macedo I, Alexandrino AM, Pissarra S, Cardoso M: Nutrição Entérica Na Criança Nascida Pré-Termo: Revisão Do Consenso Nacional. Acta Pediatr Port 2014;45:326–339.

91 ESPGHAN Committee on Nutritio, Arslanoglu S, Corpeleijn W, Moro G, Braegger C, Campoy C, et al.: Donor Human Milk for Preterm Infants: Current Evidence and Research Directions. J Pediatr Gastroenterol Nutr 2013;57:535–542.

92 Bernt KM, Walker WA: Human milk as a carrier of biochemical messages. Acta Pediatr 1999;430:27–41.

93 Ballard O, Morrow AL: Human Milk Composition: Nutrients and Bioactive Factors. Pediatr Clin North Am 2013;60:49–74.

94 McGuire MK, McGuire MA: Got bacteria? The astounding, yet not-so-surprising, microbiome of human milk. Curr Opin Biotechnol 2017;44:63–68.

95 Jeurink P V., van Bergenhenegouwen J, Jiménez E, Knippels LMJ, Fernández L, Garssen J, et al.: Human milk: A source of more life than we imagine. Benef Microbes 2013;4:17–30.

96 Fernández L, Langa S, Martín V, Maldonado A, Jiménez E, Martín R, et al.: The human milk microbiota: Origin and potential roles in health and disease. Pharmacol Res 2013;69:1–10.

97 Perez PF, Leclerc M, Levenez F, Benyacoub J, Serrant P: Bacterial Imprinting of the Neonatal Immune System: Lessons From Maternal Cells ? Pediatrics 2007;119:e724–32.

98 Moles L, Manzano S, Ferna ÃL, Espinosa-Martos I, Ares S, Rodrı ÃJM: Bacteriological, Biochemical, and Immunological Properties of Colostrum and Mature Milk From Mothers of Extremely Preterm Infants. J Pediatr Gastroenterol Nutr 2015;60:120–126.

99 Khodayar-Pardo P, Mira-Pascual L, Collado M, Martínez-Costa C: Impact of lactation stage, gestational age and mode of delivery on breast milk microbiota. J Perinatol 2014;34:599–605.

100 Kunz C, Meyer ÃC, Collado C, Geiger ÃL, Borsch C, Rudloff S: Influence of Gestational Age , Secretor, and Lewis Blood Group Status on the Oligosaccharide Content of Human Milk. J Pediatr Gastroenterol Nutr 2017;64:789–798.

101 Marx C, Bridge R, Wolf AK, Rich W, Kim JH, Bode L: Human milk oligosaccharide composition differs between donor milk and mother's own milk in the NICU. J Hum Lact 2014;30:54–61.

102 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 Immunol 2017;8:1–12.

103 Cabrera-Rubio R, Collado MC, Laitinen K, Salminen S, Isolauri E, Mira A: The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. Am J Clin Nutr 2012;96:544–51.

104 Meier PP, Patel AL, Esquerra-Zwiers A: Donor Human Milk Update: Evidence, Mechanisms and Priorities for Research and Practice. J Pediatr 2015;18:386–392.

105 Underwood MA: Human milk for the premature infant. Pediatr Clin North Am 2013;60:189–207.

106 E. B, Coppa G V., COSCIA O, GABRIELLP, Sabatin G, Sgarrella M, et al.: Effects of Holder Pasteurization on Human Milk Oligosaccharides. Int J Immunophatology Pharmacol 2008;21:381–85.

107 Xu W, Judge M, Maas K, Hussain N, Mcgrath JM, Henderson WA: Systematic Review of the Effect of Enteral Feeding on Gut Microbiota in Preterm Infants. J Obstet Gynecol Neonatal Nurs 2018;47:451–63.

108 Ninonuevo MR, Bode L: Infant Formula Oligosaccharides Opening the Gates (for Speculation): Commentary on the article by Barrat et al. on page 34. Pediatr Res 2008;64:8–10.

109 World Health Organization in collaboration with, Nations F and AO of the U: Safe preparation, storage and handling of powdered infant formula - Guidelines. WHO Libr Cat Data 2007;

110 Maffei D, Schanler RJ: Human milk is the feeding strategy to prevent necrotizing enterocolitis! Semin Perinatol 2017;41:36–40.

111 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.

112 Cong X, Xu W, Janton S, Henderson WA, Matson A: Gut Microbiome Developmental Patterns in Early Life of Preterm Infants : Impacts of Feeding and Gender. PLoS One 2016;11:e0152751.

113 Cong X, Genomics S, Judge M, Xu W, Diallo A, Janton S, et al.: Influence of Infant Feeding Type on Gut Microbiome Development in Hospitalized Preterm Infants. Nurs Res 2017;66:123–33.

114 Parra-Llorca A, Gormaz M, Alcántara C, Cernada M, Nuñez-Ramiro A, Vento, Maximo MD P, et al.: Preterm Gut Microbiome Depending on Feeding Type: Significance of Donor Human Milk. Front Microbiol 2018;9:1376.

115 Patel K, Konduru K, Patra AK, Chandel DS: Trends and determinants of gastric bacterial colonization of preterm neonates in a NICU setting. PLoS One 2015;10:e0114664.

116 Groer MW, Luciano AA, Dishaw LJ, Ashmeade TL, Miller E, Gilbert JA: Development of the preterm infant gut microbiome: a research priority. Microbiome 2014;2:38.

117 Morais J, Marques C, Teixeira D, Durão C, Faria A, Brito S, et al.: FEEDMI: A Study Protocol to Determine the Influence of Infant-Feeding on Very-Preterm-Infant's Gut Microbiota. Neonaotlogy 2019;27:1–6.

118 Stinson LF, Keelan JA, Payne MS: Comparison of Meconium DNA Extraction Methods for Use in Microbiome Studies 2018;9:1–14.

119 Marques C, Meireles M, Norberto S, Leite J, Freitas J, Pestana D, et al.: High-fat diet-induced obesity Rat model: a comparison between Wistar and Sprague-Dawley Rat. Adipocyte 2015;3945:1–11.

120 Gale C, Logan KM, Santhakumaran S, Parkinson JRC, Hyde M, Modi N: Effect of Breast Versus Formula Feeding on Infant Body Composition: a Systematic Review and Meta-Analysis. Am J Clin Nutr 2012;95:656–669.

121 Guaraldi F, Salvatori G: Effect of Breast and Formula Feeding on Gut Microbiota Shaping in Newborns. Front Cell Infect Microbiol 2012;2:1–4.

122 Mshvildadze M, Neu J, Shuster J, Ms DT, Ms NL, Mph VM: Intestinal Microbial Ecology in Premature Infants Assessed with Non-Culture-Based Techniques. J Pediatr 2010;156:20–25.

123 Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, et al.: Reagent and laboratory contamination can critically impact sequence-based microbiome analyses 2014;1–12.

124 Gregoris TB De, Aldred N, Clare AS, Burgess JG: Improvement of phylum- and class-specific primers

for real-time PCR quantification of bacterial taxa. J Microbiol Methods 2011;86:351–56.

125 Marques C, Meireles M, Norberto S, Leite J, Freitas J, Pestana D, et al.: High-fat diet-induced obesity Rat model: a comparison between Wistar and Sprague-Dawley Rat. Adipocyte 2015;5:1–11.

126 Collado MC, Derrien M, Isolauri E, Vos WM De, Salminen S: Intestinal Integrity and Akkermansia muciniphila, a Mucin-Degrading Member of the Intestinal Microbiota Present in Infants, Adults, and the Elderly. Appl Env Microbiol 2007;73:7767–70.

127 Waitzberg DL, Pereira CCA, Logullo L, Jacintho TM, Almeida D: Microbiota benefits after inulin and partially hydrolized guar gum supplementation – a randomized clinical trial in constipated women 2012;27:123–129.

128 Lopes CM de M: Alimentação e Enfarte Agudo do Miocárdio Estudo caso-controlo de base comunitária. 4. Reprodutibilidade e Val- idação de um questionário semi-quantitativo de frequência alimentar 2000;

129 Lopes C, Aro A, Azevedo A, Ramos E, Barros H: Intake and Adipose Tissue Composition of Fatty Acids and Risk of Myocardial Infarction in a Male Portuguese Community Sample{A figure is presented}. J Am Diet Assoc 2007;107:276–86.

130 Martínez-González MA, García-Arellano A, Toledo E, Salas-Salvadó J, Buil-Cosiales P, Corella D, et al.: A 14-item mediterranean diet assessment tool and obesity indexes among high-risk subjects: The PREDIMED trial. PLoS One 2012;7. DOI: 10.1371/journal.pone.0043134

131 Robertson RC, Manges AR, Finlay BB, Prendergast AJ: The Human Microbiome and Child Growth – First 1000 Days and Beyond. Trends Microbiol 2019;27:131–47.

132 Wilczyńska P, Skarżyńska E, Lisowska-Myjak B: Meconium microbiome as a new source of information about long-term health and disease: questions and answers. J Matern Neonatal Med 2018;32:681–86.

133 Bavineni M, Wassenaar TM, Agnihotri K, Ussery DW, Lu TF, Mehta JL: Mechanisms linking preterm birth to onset of cardiovascular disease later in adulthood 2019;7:1–8.

134 Moles L, Gómez M, Heilig H, Bustos G, Fuentes S, de Vos W, et al.: Bacterial Diversity in Meconium of Preterm Neonates and Evolution of Their Fecal Microbiota during the First Month of Life. PLoS One 2013;8. DOI: 10.1371/journal.pone.0066986

135 Hu J, Nomura Y, Bashir A, Fernandez-Hernandez H, Itzkowitz S, Pei Z, et al.: Diversified Microbiota of Meconium Is Affected by Maternal Diabetes Status. PLoS One 2013;8:e78257.

136 Stinson LF, Payne MS, Keelan JA: A Critical Review of the Bacterial Baptism Hypothesis and the impact

of Cesarean Delivery on the infant Microbiome. Front Med 2018;5. DOI: 10.3389/fmed.2018.00135

137 Arboleya S, Binetti A, Salazar N, Fernández N, Solís G, Barranco AH-, et al.: Establishment and development of intestinal microbiota in preterm neonates ´. FEMS Microbiol Ecol 2012;79:763–72.

138 Magne F, Ab M, Morville P, Pochart P, Suau A: Low species diversity and high interindividual variability in faeces of preterm infants as revealed by sequences of16S rRNA genes and PCR-temporal temperature gradient gel electrophoresis pro ¢ les. FEMS Microbiol Ecol 2006;57:128–38.

139 Arboleya S, Solís G, Fernández N, de los Reyes-Gavilán C, Gueimonde M: Facultative to strict anaerobes ratio in the preterm infant microbiota. Gut Microbes 2012;3:583–88.

140 Jacquot A, Neveu D, Aujoulat F, Mercier G, Jumas-bilak E, Picaud J: Dynamics and Clinical Evolution of Bacterial Gut Micro fl ora in Extremely Premature Patients ´. J Pediatr 2011;158:390–6.

141 Barrett HL, Dekker Nitert M, Conwell LS, Callaway LK: Probiotics for preventing gestational diabetes. Cochrane Database Syst Rev 2014;

142 Chernikova DA, Madan JC, Housman ML, Zain-ul-abideen M, Lundgren SN, Morrison HG, et al.: The premature infant gut microbiome during the first 6 weeks of life differs based on gestational maturity at birth. Pediatr Res 2018;1–9.

143 LaTuga MS, Ellis JC, Cotton CM, Goldberg RN, Wynn JL, Jackson RB, et al.: Beyond Bacteria : A Study of the Enteric Microbial Consortium in Extremely Low Birth Weight Infants. PLoS One 2011;6:e27858.

144 Marcobal A, Barboza M, Sonnenburg ED, Pudlo N, Martens EC, Desai P, et al.: Bacteroides in the infant gut consume milk oligosaccharides via mucus-utilization pathways. Cell Host Microbe 2011;10:507–514.

145 Ferretti P, Pasolli E, Tett A, Huttenhower C, Bork P, Segata N, et al.: Mother-to-Infant Microbial Transmission from Different Body Sites Shapes the Developing Infant Gut Microbiome Article Mother-to-Infant Microbial Transmission from Different Body Sites Shapes the Developing Infant Gut Microbiome. Cell Host Microbe 2018;24:133–45.

146 Gregory KE, LaPlante RD, Shan G, Kumar DV, Gregas M: Mode of Birth Influences Preterm Infant Intestinal Colonization With Bacteroides Over the Early Neonatal Period. Adv Neonatal Care 2015;15:386–93.

147 Arboleya S, Martinez-camblor P, Solis G, Suárez M, Reyes-gavilan CGDL, Gueimonde M: Intestinal microbiota and weight-gain in preterm neonates 2017;8:1–5.

148 O'Hanlon DE, Moench TR, Cone RA: Vaginal pH and Microbicidal Lactic Acid When Lactobacilli Dominate the Microbiota. PLoS One 2013;8:1–8.

149 Aagaard K, Riehle K, Ma J, Segata N, Mistretta T, Coarfa C, et al.: A Metagenomic Approach to Characterization of the Vaginal Microbiome Signature in Pregnancy. PLoS One 2012;7:e36466.

150 RG B, M A-M, JR M, YS L, A S, Chan D1 LH, et al.: Establishment of vaginal microbiota composition in early pregnancy and its association with subsequent preterm prelabor rupture of the fetal membranes. Transl Res 2018;207:30–43.

151 Singh S, Karagas MR, Mueller NT: Charting the Maternal and Infant Microbiome: What Is the Role of Diabetes and Obesity in Pregnancy? Curr Diab Rep 2017;17:11.

152 Kumar R, Ouyang F, Story RE, Pongracic JA, Hong X, Wang G, et al.: Gestational diabetes, atopic dermatitis, and allergen sensitization in early childhood. J Allergy Clin Immunol 2009;124:1–18.

153 Su M, Nie Y, Shao R, Duan S, Jiang Y: Diversified gut microbiota in newborns of mothers with gestational diabetes mellitus 2018;2:1–17.

154 Wang J, Zheng J, Shi W, Du N, Xu X, Zhang Y, et al.: Dysbiosis of maternal and neonatal microbiota associated with gestational diabetes mellitus. Gut Microbiota 2018;67:1614–25.

155 Abenhaim HA, Kinch RA, Morin L, Benjamin A, Usher R: Effect of prepregnancy body mass index categories on obstetrical and neonatal outcomes. Arch Gynecol Obs 2007;275:39–43.

156 Bassols J, Serino M, Carreras-Badosa G, Burcelin R, Blasco-Baque V, Lopez-Bermejo A, et al.: Gestational diabetes is associated with changes in placental microbiota and microbiome. Pediatr Res 2016;80:777–784.

157 Santacruz A, Collado MC, GarcÃ-a-ValdÃ©s L, Segura MT, MarÃ-tn-Lagos JA, Anjos T, et al.: Gut microbiota composition is associated with body weight, weight gain and biochemical parameters in pregnant women. Br J Nutr 2010;104:83–92.

158 Collado MC, Isolauri E, Laitinen K, Salminen S: Effect of mother ’s weight on infant ’s microbiota acquisition , composition , and activity during early infancy : a prospective follow-up study initiated in early pregnancy. Am J Clin Nutr 2010;92:1023–1030.

159 Mikkelsen TB, Østerdal ML, Knudsen VK, Haugen M, Meltzer HM, Bakketeig L, et al.: Association between a Mediterranean-type diet and risk of preterm birth among Danish women : a prospective cohort study. Acta Obstet Gynecol 2008;87:325–30.

160 Parlapani E, Agakidis C, Lampoudi TK-, Sarafidis K, Agakidou E, Athanasiadis A, et al.: The Mediterranean Diet adherence by pregnant women delivering prematurely: association with size at birth and complications of prematurity. J Matern Neonatal Med 2017;13:1–8.

161 Saunders L, Guldner L, Costet N, Kadhel P, Rouget F, Monfort C: Effect of a Mediterranean Diet during

Pregnancy on Fetal Growth and Preterm Delivery : Results From a French Caribbean Mother – Child Cohort Study ( TIMOUN ) 2014;235–244.

162 Singh RK, Chang HW, Yan D, Lee KM, Ucmak D, Wong K, et al.: Influence of diet on the gut microbiome and implications for human health. J Transl Med 2017;1–17.

163 Cai C, Zhang Z, Morales M, Wang Y, Khafipour E, Friel J: Feeding practice influences gut microbiome composition in very low birth weight preterm infants and the association with oxidative stress : A prospective cohort study. Free Radic Biol Med 2019;18:1–9.

164 Gómez M, Moles L, Espinosa-Martos I, Bustos G, de Vos WM, Fernández L, et al.: Bacteriological and Immunological Profiling of Meconium and Fecal Samples from Preterm Infants: A Two-Year Follow-Up Study. Nutrients 2017;9:1–19.

165 Deshpande G, Athalye-Jape G, Patole S: Para-probiotics for Preterm Neonates—The Next Frontier. Nutrients 2018;10:1–9.

166 Zanella A, Silveira RC, Roesch LFW: Influence of own mother ' s milk and different proportions of formula on intestinal microbiota of very preterm newborns. PLoS One 2019;14:e0217296.

167 Henderickx JGE, Zwittink RD, Lingen RA Van, Knol J: The Preterm Gut Microbiota : An Inconspicuous Challenge in Nutritional Neonatal Care. Front Cell Infect Microbiol 2019;9:1–12.