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Establishment of gut microbiota in term and preterm infants: evaluation by culture-independent methods

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Esame finale anno 2017

May you grow up to be righteous

May you grow up to be true

May you always know the truth

And see the lights surrounding you

May you always be courageous

Stand upright and be strong

May you stay

May you stay forever young

Introduction

Evolution of human beings as super-organisms is the result of a mutualistic relationship with the huge microbial community residing in the human gastrointestinal (GI) tract, which is better known as the human gut microbiota (GM) [1,2].

GM is made of a huge number of diverse microbial species. Recently, an integrated catalogue of the human faecal microbial metagenome, including almost 9.9 million microbial genes [3], has been created by combining data from 1200 subjects in the United States, Europe and China [4,5]. This reference catalogue can be accessed freely online (*http://meta.genomics.cn*) and represents a fundamental resource for further investigation over human GM.

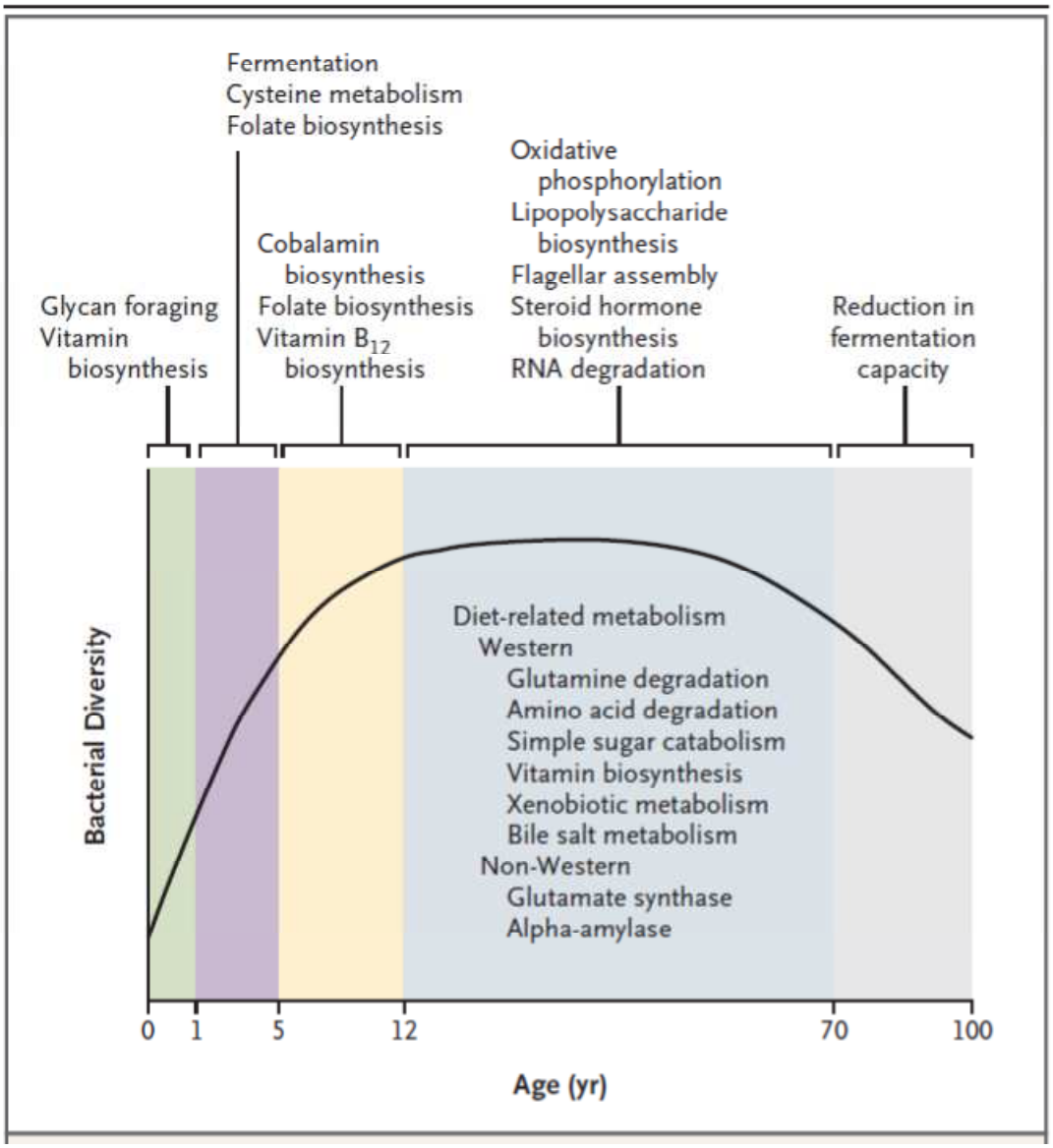
GM of healthy adults is highly resilient and stable over time. In adult life, GM reaches a state of homeostasis, which is the ability of the microbial ecosystem to maintain a fluctuating equilibrium among bacterial communities, the gut, and the immune system of the host [6].

Imbalances in the composition and function of GM, also known as GM dysbiosis, have been linked to a large number of diseases: actually, GM has been recognized to influence several biological processes, such as immune maturation and homeostasis, host cell proliferation, vascularisation, neurologic signalling, pathogen burden, gut endocrine function, bone mineralization and energy biogenesis. In addition, human GM is involved in the biosynthesis of vitamins, steroid hormones and neurotransmitters, and in the metabolism of aminoacids, dietary components, bile salts, drugs and xenobiotics [7]. Finally, the shaping of human GM during the whole lifespan has been proposed as a potential determinant of healthy aging [8].

GM of each individual acquires its final and unique structural and functional layout through a complex process which begins *in utero* and reaches its climax in infancy [9], when infants are exposed to several external factors which modify profoundly their GM. From early childhood, the rapid rate of expansion in bacterial diversity observed during the first year of life slows down and GM

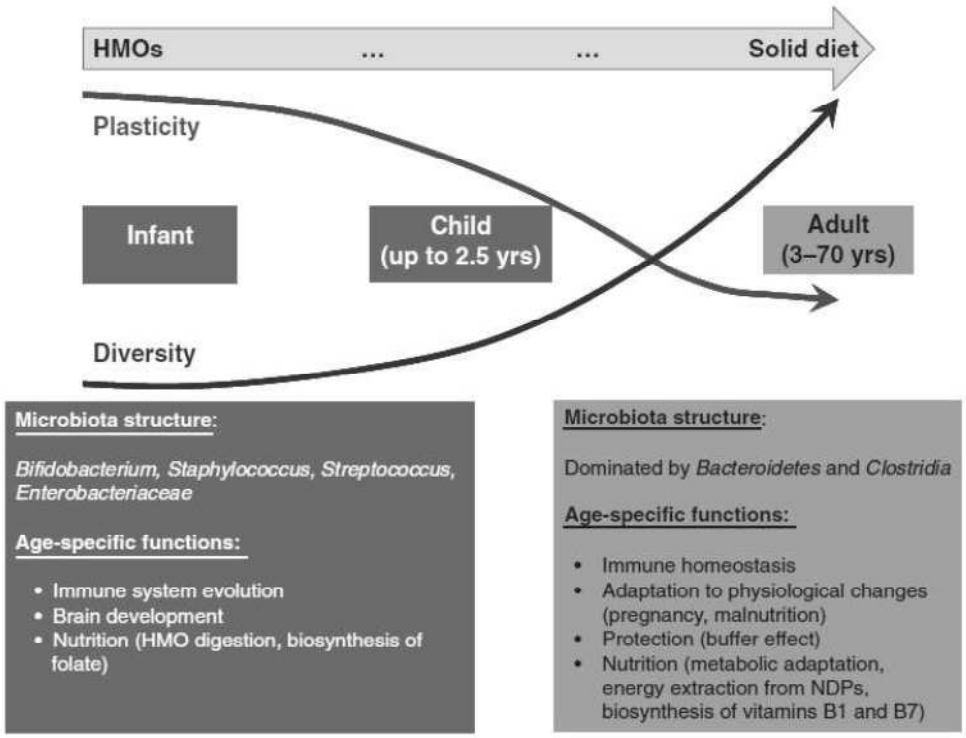
gradually acquires its adult shape (Figure 1). Specifically, during toddlerhood, the introduction of complementary feeding and the maturation of the immune system start to shape GM towards its adult profile, which is further influenced, but to a lesser extent, by hormonal and sexual development, social behaviour, and adult-like diet and lifestyle during childhood and adolescence [10].

Figure 1. Temporal development of gut microbiota in humans [7].



Changes in GM along the course of human life are age-specific, as they are aimed at providing the host with an ecosystem finely calibrated for each stage of life (Figure 2) [1,11].

Figure 2. Trajectory of gut microbiota development from birth to adult life [11].
HMOs = human milk oligosaccharides; NDPs = non-digestible polysaccharides.



Maternal influences on GM

The infant GM is highly dependent from maternal influences, such as maternal genetics, environmental exposure, and diet before and during pregnancy, as well as during breastfeeding [12].

Recent data confirm that bacterial colonization of the human gut begins well before birth: several studies have now demonstrated the presence of bacteria, during healthy pregnancies, in placental tissue, cord blood, amniotic fluid, foetal membranes and meconium [13–16].

Actually, our understanding of the shaping of human GM has been recently revolutionized by the introduction of culture-independent molecular assays

aimed at detecting and classifying GM and at characterizing its genes and gene products (Figure 3 [7]).

Figure 3. Culture-independent tools for analyzing microbiota [7]

Table 1. Tools for Analyzing Microbiota.

Approach	Data	Platform	Pros and Cons
Biomarker sequencing (e.g., 16S rRNA gene or internal transcribed spacer region)*	Community composition	Next-generation sequencing	Is cost-effective, is semiquantitative, permits resolution of genus level and in some cases species level; shorts reads may make accurate classification difficult
Metagenomics	Generation of draft genomes, functional capacity, growth dynamics	Next-generation sequencing	Has capacity for strain-level reconstruction, is quantitative, allows for functional annotation with pathway predictions; is currently very costly, has community coverage that may be relatively shallow in more complex assemblages
Metatranscriptomics (RNA sequencing)	Gene expression	Next-generation sequencing	Highly expressed genes are more likely than others to be detected, depletion of human transcripts is possible, requires immediate preservation or processing of fresh or snap-frozen intestinal specimens
Metaproteomics	Protein expression	Liquid or gas chromatography–mass spectrometry	Primarily detects dominant proteins; makes removal of host-derived proteins impossible
Metabolomics	Metabolic productivity	Liquid or gas chromatography–mass spectrometry or magnetic resonance spectroscopy	Is semiquantitative; can be targeted or untargeted; detects metabolites that are platform- and database-dependent; detects metabolites that may originate from microbes, diet, or host

* The term rRNA denotes ribosomal RNA.

The introduction of these techniques has allowed to recognize that many infants are already exposed to microbes via a non-sterile amniotic fluid [17,18]: this results in bacterial colonization of the foetal gut, as demonstrated by microbial DNA found in the meconium of preterm infants [19] and by the similarities in microbiota profile observed between the amniotic fluid and the meconium itself [20].

Recently, a placental-specific microbiome profile has also been characterized [13], suggesting that the placenta may serve as an antenatal source of commensal bacteria for the infant. It is also plausible that length of gestation influences the quality and quantity of bacteria which are transferred from the mother to the foetus, contributing since before birth to differences in microbiota profiles between term and preterm infants [21].

Mode of delivery

A recent study performed in healthy term infants (TIs) who were followed up during the first year of life has confirmed that mode of delivery and feeding patterns have a dominant role in driving the assembly of the adult-like GM [9].

GM in healthy infants born to vaginal delivery (VD) is built up through the oral inoculation of bacteria from the vagina and the maternal gut during labour and delivery. GM of these infants at birth is thus dominated by *Lactobacillus* and

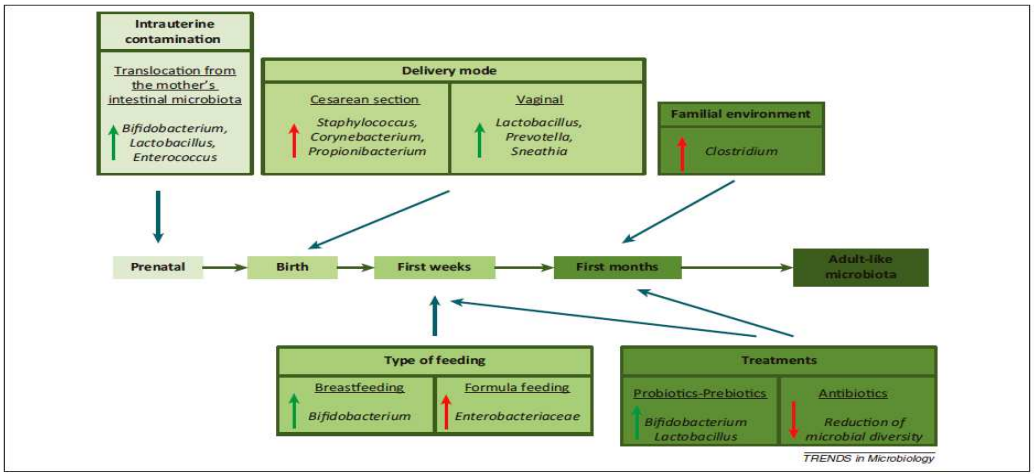
Prevotella. On the contrary, the acquisition of GM in infants born to caesarean section (CS) occurs later, is dependent from environmental sources (such as maternal skin) and is characterized by a lower diversity and by different bacteria (*Staphylococcus, Corynebacterium* e *Propionibacterium*) [6,22,23]. The differences in GM composition are more pronounced for infants born to elective CS compared to those born to in-labour CS [24] and apparently persist through childhood [25,26]. A low diversity in GM, such as that seen in infants born to CS, has been linked to an increased risk of several diseases, including late-onset sepsis [27] and necrotizing enterocolitis [28]. In addition, the timing of colonization of the neonatal gut has been related in animal models to the ability to affect immune function [12].

Further development of GM after birth is guided by a complex interaction between the microbiota itself, the host's immune system and the environment. It is still unclear how this process exactly works, but several studies have shown a great inter-individual variability in composition and temporal patterns of GM establishment during the first year of life [29].

Interestingly, gut colonization patterns established within the first week of life (guided by the so-called "pioneer bacteria" or "early settlers") are believed to shape the composition of future GM [30]. For this reason, all the events which occur during the first weeks/months of life are considered to be fundamental in laying the foundations for a healthy GM.

Beyond intrauterine contamination and mode of delivery, feeding type, gestational age (GA) and antibiotic/probiotic use are considered the major determinants of GM in the neonatal period (Figure 4) [6,26].

Figure 4. Impact of external factors on infant gut microbiota. Green arrows show beneficial modification; red arrows show modification considered as negative for healthy development [6].



Feeding type

Healthy TIs, who are born to VD and are exclusively breastfed, are thought to have the most beneficial GM composition.

Human milk (HM) represents nature's first functional food [31], as it provides a mix of nutrients, bacteria and functional compounds (such as oligosaccharides, proteins with antimicrobial activity, and fatty acids) which exert several biological functions, including the establishment of GM [32,33].

According to recent data, the number of bacteria in HM is huge: in a recent study, HM median bacterial count was 10^6 bacterial cells/ml [34]. HM bacterial community contains over 350 prokaryotic genes, the dominant phyla being Proteobacteria and Firmicutes, and the most represented genera *Pseudomonas*, *Staphylococcus* and *Streptococcus* [35].

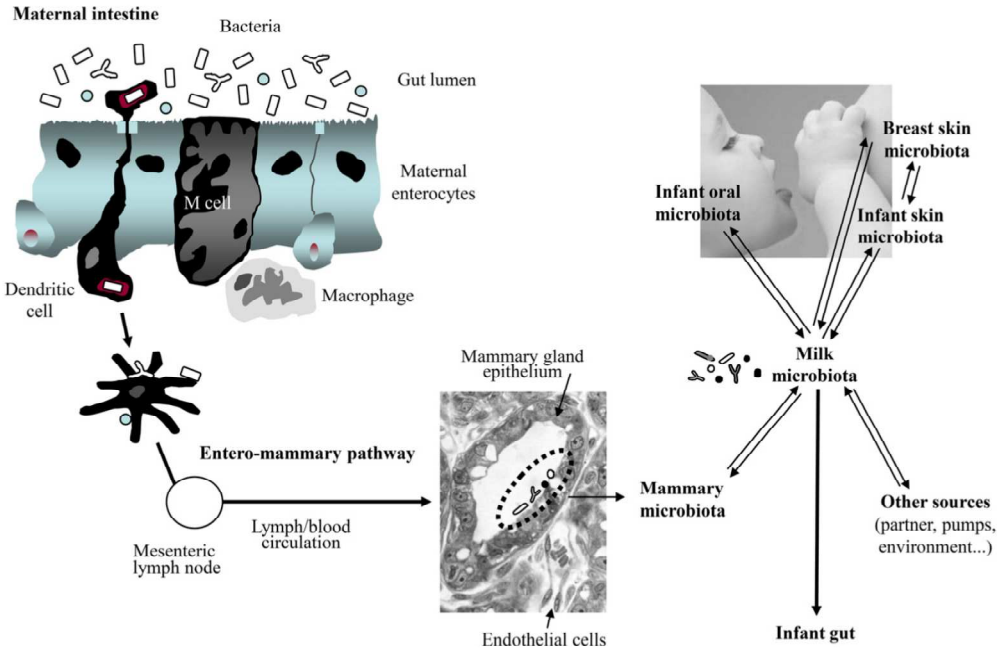
The exact mechanism through which bacteria reach the mammary gland and are excreted into breast milk is still debated [36]. One hypothesis is that HM mainly contains bacteria derived from the contamination with the mother's skin and/or the infant's mouth. The other hypothesis involves the so-called "entero-mammary pathway" [36,37], according to which some bacteria could migrate from

the maternal GI tract to the mammary gland during late gestation and lactation, through a mechanism involving gut monocytes.

Facultative anaerobic or prevalently aerobic strains are among the most important components of HM microbiota, with a dominance of *Streptococcus* and *Staphylococcus*, together with skin-derived or environmental bacteria, such as *Propionibacterium* or species belonging to Enterobacteriaceae, and probiotic genera such as *Bifidobacterium* and *Lactobacillus* [38]. Next generation sequencing also allowed the detection of obligate anaerobic, gut-associated genera, such as *Bacteroides*, *Blautia*, *Dorea* and *Faecalibacterium* [39], which, if alive, could act as pioneers in shaping the infant's GM towards its adult profile.

It is plausible that the infant's mouth, being the transition point for HM to reach the GI tract, represents another fundamental actor in shaping GM features, both indirectly, through the contamination of HM, and directly through swallowing of saliva: the oral microbiota shows some peculiar characteristics, as it is usually dominated by *Streptococcus* and *Staphylococcus* in healthy breastfed TIs, while other bacterial taxa, such as *Gemella*, *Actinomyces* and *Veillonella*, represent minor colonizers [40].

Figure 5. Potential sources of the bacteria present in human milk [36].



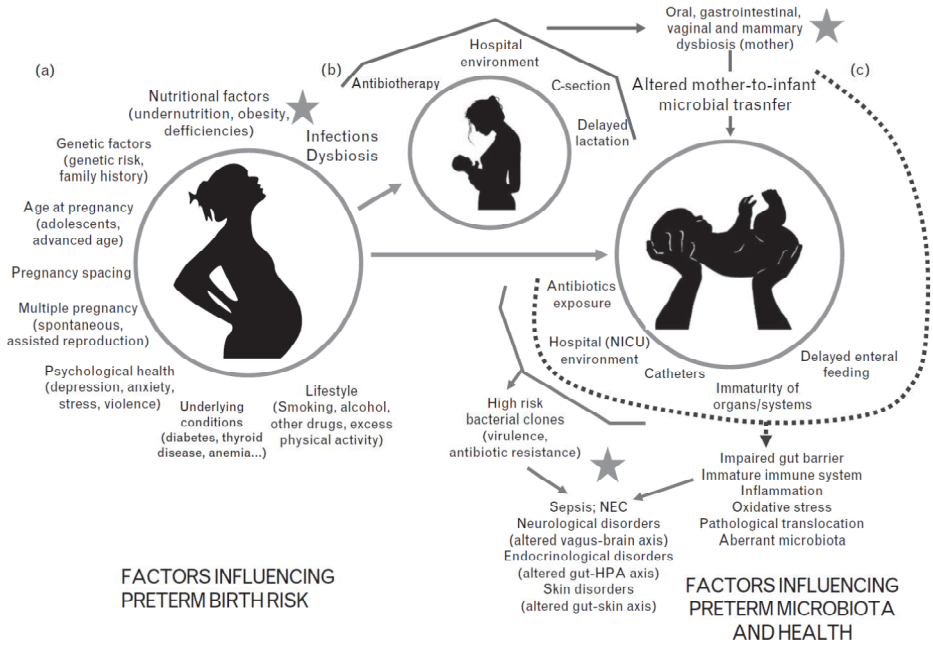
Regardless their origin (Figure 5 [32]), HM bacteria and bioactive components confer to the infant's GM several peculiar microbial features (high abundance of *Bifidobacteria* and *Enterobacteria*), which distinguish it from GM of formula-fed infants [41]. Interestingly, even small amounts of formula given to breastfed infants appear to determine a shift in GM composition towards an "exclusive- formula" pattern [42], which is characterized by higher diversity and higher representation of bacteria such as *Escherichia coli*, *Clostridium difficile*, *Bacteroides*, *Prevotella* and *Lactobacillus* species.

Preterm birth

Preterm birth constitutes a challenge for both obstetricians and neonatologists. Multiple mechanisms are thought to lead to spontaneous preterm birth but, despite this, its exact trigger remains unknown in at least half of the cases. For this reason, prevention of spontaneous preterm labour and accurate management of preterm infants at and after birth represent a clinical and research priority.

Preterm birth can be the result of maternal microbial dysbiosis and infection; furthermore, due to intrinsic immaturity and environmental factors, preterm infants always experience a certain grade of dysbiosis which appear to be dependent upon GA and clinical conditions (Figure 6 [43]).

Figure 6. Overview of environmental factors that may condition the establishment of gut microbiota (GM) in preterm infants, including maternal factors during pregnancy (A), post-partum (B), and hospital-related factors known to affect neonatal GM establishment during the first weeks of life. Stars represent the stages when dietary strategies for GM modulation are feasible [43]. HPA = hypothalamic-pituitary-adrenal; NEC = necrotizing enterocolitis; NICU = neonatal intensive care unit.



The most immature is the infant and the most severe are his/her clinical conditions, the most disrupted his/her GM will be compared to the GM of a healthy TI. This comparison is particularly relevant as we know that preterm infants are exposed during the early stages of their lives to a series of medical interventions which can impact on their short and long-term health status. In this perspective, all the interventions which interfere with the establishment of a healthy GM can further impair the already unstable clinical conditions of these infants, leading to serious medical complications such as necrotizing enterocolitis and sepsis. On the other side, improving the knowledge of the features of dysbiosis associated with preterm birth can help in identifying potential

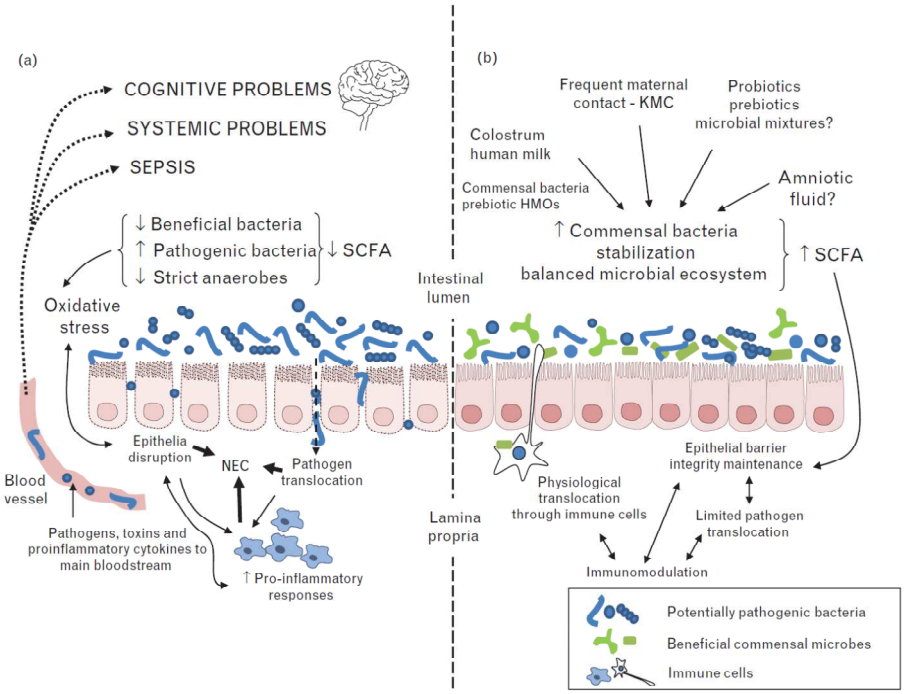
interventions aimed at restoring GM equilibrium (Figure 7 [43]), in order to improve these infants' clinical outcome.

Figure 7. Differences in gut microbiota between preterm and healthy term infants, and health consequences of dysbiosis at intestinal and systemic level.

(a) Environmental factors conditioning variations in gut microbiota composition and health consequences at intestinal (NEC) and systemic level (cognitive and systemic problems, and sepsis).

(b) Environmental and nutritional strategies with potential to modulate preterm gut microbiota.

HMO = HM oligosaccharides; KMC = kangaroo mother care; NEC = necrotizing enterocolitis; SCFA = short-chain fatty acids.



It has been shown that in preterm infants, compared to term babies, gut colonization is delayed, bacterial diversity is generally low, and there is a considerable individual variation in bacterial composition. Preterm GM is generally characterized by an increased number of Enterobacteriaceae (including *E. Coli*, *Klebsiella*, *Enterobacter*), Enterococcaceae, Lactobacillaceae and

Staphylococcaceae, and a lower and delayed representation of Bifidobacteriaceae. This abnormal intestinal colonization may alter the barrier, nutritional and immunological functions of the host-microbiota relationship, thus increasing susceptibility to disease [44–46].

Despite growing literature about GM features in very (<32 weeks GA) and extremely (<28 weeks GA) preterm infants, little is still known about infants who are born at 32-34 and 35-36 weeks gestation, who are respectively defined as "moderately" and "late" preterm infants. Even if they have a much lower risk of medical complications than more premature infants, they still experience, compared to full TIs, higher rates of infant morbidity and mortality, as well as higher risks of childhood disabilities [47].

When preterm birth occurs at 32-36 weeks gestation, it interrupts physiological development of pulmonary and gastrointestinal functions [48]. Moderately and late preterm infants experience a delay in the full establishment of coordinated latch, suckling, swallowing and breathing [49], incomplete oesophageal peristalsis [50], and altered gastric emptying [51]. The achievement of these developmental milestones is essential for establishing complete feeding tolerance and satisfying the high nutritional needs of the growing preterm infant. In addition, impairment in GI function, as well as difficulties in achieving compete and exclusive breastfeeding [52], have the potential to impair the establishment of a healthy GM.

The features of GM in moderately preterm infants (MPIs) have been investigated in few studies, which have led to inconclusive results. In this context, it has been suggested that gut colonization with Bifidobacteriaceae [53] does not begin before 33 weeks gestation, that the relative abundance of these bacteria is low and GM is dominated by members of the Enterobacteriaceae, similarly to preterm infants of lower GA [54].

Antibiotics and probiotics

The use of prenatal and neonatal antibiotics has been linked in several studies to the disruption of GM. Antibiotics given to the mother during the last trimester of pregnancy, during CS, and/or during breastfeeding have been linked to neonatal GM dysbiosis [55]. In addition, the use of *intrapartum* antibiotic prophylaxis for maternal group B *Streptococcus* infection has been shown to modify GM in otherwise healthy TIs [56]. Similarly, the use of antibiotics during the neonatal period is considered one of the main interventions which could affect negatively GM, especially in preterm infants [57]. In this perspective, growing literature is attempting to identify preventive interventions, such as the use of probiotics [58–60], prebiotics [61] and functional nutrients [62], aimed at restoring a healthy profile of GM.

Aims of the study

The present research project was developed in order to add further knowledge regarding the mechanisms guiding the establishment of GM in term and preterm infants. Specifically, the aim of the study was to characterize the establishment of GM, in relation to the microbiota of saliva and mother's milk, in term and preterm infants, with a focus on GM features in moderately preterm infants.

Methods

Study details and ethics

The study was conducted in collaboration with the Department of Pharmacy and Biotechnology of the University of Bologna and is part of the Cluster Research Project named PROSIT ("*PROmozione della Salute del consumatore: valorizzazione nutrizionale dei prodotti agroalimentari della tradizione ITaliana*" – study code CTN01_00230_413096).

The study protocol was approved by the independent Ethical Committee of Sant'Orsola-Malpighi Hospital (study IDs: 25/2014/U/OSS and 53/2014/U/Tess). For each enrolled infant, parents and/or legal guardians were asked to provide a written informed consent before entering the study protocol.

Patients

Newborns were recruited at the Neonatal Unit of Sant'Orsola-Malpighi Hospital (AOU Bologna), if fulfilling the following characteristics:

- Group A: TIs (GA ≥37 weeks), born to VD, and exclusively breastfed, who were not exposed to any antibiotic/probiotic before, during and after delivery. Infants who developed any medical condition requiring hospital admission were excluded from the study.
- Group B: MPIs (GA 32 – 34^{+6} weeks) who were admitted to the Neonatal Intensive Care Unit (NICU).
- Group C: very preterm infants (GA <32 weeks) and/or very low birth weight (VLBW) infants (birth weight [BW] <1500 g) who were admitted to the NICU.

Each infant/mother pair's demographic and clinical characteristics were collected in a specific case report form.

Sample collection

For each infant/mother pair, the following biological samples were collected:

Group A

At 20 days of life (DOL):

- Infant stools
- Own mother's milk (OMM)
- Neonatal oral swabs, pre and post breastfeeding

Group B

At DOL 1, 2-4, 7, 14, 21, and, after that, once each month until the introduction of complementary feeding:

- Infant stools
- OMM, when available
- Neonatal oral swab

Additional samples were collected at the beginning of breastfeeding:

- Infant stools
- OMM
- Two neonatal oral swabs (before and after the infant's contact with the breast)

Group C

At DOL 1, 2-4, 7, 14, 30, and, after that, once each month until 3 months term-equivalent age (which roughly corresponds to the timing of the introduction of complementary feeding in these patients):

- Infant stools
- OMM, when available

Regardless study group, sample collection methods were the following:

- Stools were collected directly from diapers and immediately placed into sterile plastic tubes.

- OMM was collected with the aid of a breast pump into sterile plastic tubes; prior to collection, mothers were asked to wash the nipple and mammary areola with soap and water.
- Oral samples were obtained by gently swabbing a sterile cotton-tipped applicator on the inside of the infant's cheek.

All the samples were delivered promptly to the Laboratory at the Department of Pharmacy and Biotechnology of the University of Bologna for the analyses.

Microbiota analyses (from Biagi E, Quercia S, Aceti A et al. Bacterial sharing between the ecosystems of mother's milk and infant's mouth and gut. Submitted to Frontiers in Microbiology – March 2017)

1. Total bacterial DNA extraction

Total bacterial DNA was extracted from stools using the DNeasy Blood & Tissue Kit (QIAGEN, 273 Hilden, Germany) with a modified protocol, as previously described [8]. Two-hundred-fifty mg of stools were resuspended in 1 ml of lysis buffer (500mM NaCl, 50 mM Tris–HCl pH 8, 50 mM EDTA and 4% SDS) and treated with 3 bead-beating steps in a Fast Prep instrument (MP Biomedicals, Irvine, CA) at 5.5 movements per sec for 1 min. Samples were then heated at 95°C for 15 min. Solid particles were centrifuged at full speed for 5 min at 4°C, then 260 µl of 10M ammonium acetate were added and the samples incubated for 5 min in ice. Debris was pelleted by 10 min of centrifugation at full speed at 4°C, the supernatants were collected and 1 volume of isopropanol was added. Samples were incubated in ice for 30 min. DNA was collected by 15 min of centrifugation at full speed at 4°C and the pellet washed with 70% ethanol. The pellet was then dissolved in 100 µl of TE buffer and treated with 2 µl of DNase-free RNase (10 mg/ml) for 15 min at 37°C. After incubation, 200 µl of AL buffer (QIAGEN) and 15 µl of proteinase K were added and heated at 70°C for 10 min. DNA was further purified using QIAamp Mini Spin columns (QIAGEN) following the manufacturer's instructions.

For HM samples, 2 ml of HM were centrifuged at full speed for 10 min at 4°C and then the same protocol described for stool samples was applied.

For oral swabs, the cotton swab was suspended in 500 µl of PBS, vortexed for 1 min and sonicated for 2 min. These 2 steps were repeated twice, and then 2 cycles of bead-beating with FastPrep at 5.5 movements per sec for 1 min, with 200 mg of glass beads, were applied. Cotton residues were removed and the debris pelleted by centrifugation at 9000 g for 5 min. The supernatant was discarded and the pellet resuspended in 180 µl of enzymatic lysis buffer (QIAGEN). Samples were then treated according to the DNeasy Blood&Tissue kit (QIAGEN) instructions, following the protocol for Gram positive bacteria. Extracted DNAs were quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

2. <u>16 rRNA gene amplification and sequencing.</u>

For each sample, the V3-V4 region of the 16S rRNA gene was PCR amplified in 25 µl final volume containing 5 µl of microbial DNA (diluted to 5 ng/µl for faecal samples, undiluted for milk and oral swab), 2X KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Resnova, Rome, Italy), and 200 nM of S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 primers carrying Illumina overhang adapter sequences. Thermocycler was programmed as follows: initial denaturation at 95°C for 3 min, 25 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, and a final extension step at 72°C for 5 min [8]. Amplicons of about 460 bp were purified with a magnetic bead-based clean-up system (Agencourt AMPure XP; Beckman Coulter, Brea, CA) and sequenced on Illumina MiSeq platform using a 2×300 bp paired end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA). Briefly, indexed libraries were prepared by limited-cycle PCR using Nextera technology and further cleaned up with AMPure XP magnetic beads (Beckman Coulter). Libraries were pooled at equimolar concentrations (4nM), denatured and diluted to 6 pM before loading onto the MiSeq flow cell.

Bioinformatics and statistics

Raw sequences were processed using a pipeline combining PANDAseq [63] and QIIME [64]. Sequencing reads were deposited in the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA; BioProject ID PRJNA378341). High-quality reads were binned into operational taxonomic units (OTUs) according to the taxonomic threshold of 97% using UCLUST [65]. Taxonomy was assigned using the RDP classifier against Greengenes database (May 2013 release). Chimera filtering was performed by discarding all singleton OTUs. Alpha rarefaction was analyzed by using Chao1, PD whole tree, observed species, and Shannon index metrics. Beta diversity was estimated by computing weighted and unweighted UniFrac distances.

Statistics was performed using R software (https://www.r-project.org/) and the libraries vegan and made4. Weighted and unweighted UniFrac distances were used for Principal Coordinates Analyses (PCoA), and the significance of separation was tested by permutational multivariate analysis of variance using the function “adonis” of the vegan package. Alpha diversity was quantified by computing the Simpson diversity index (SDI) using the function “diversity” of the vegan package and the normalized OTU counts for each sample. Mann-Whitney U test was used to assess significant differences between groups of samples. P values were corrected for multiple comparisons using the Benjamini-Hochberg method. $P<0.05$ was considered as statistically significant. Correlation between datasets was tested by using the Kendall method.

Results

Patients' recruitment

During the study period, eighty-one infants and their mothers were enrolled in the study:

- Thirty-six in Group A (TIs)
- Fifteen in Group B (MPIs), for whom a faecal sample obtained at DOL 21 was available. Among them, complete longitudinal data (stools, HM, and saliva) of 7 infants were also evaluated (microbiota analysis of the remaining infants is ongoing).
- Thirty in Group C (very preterm and/or VLBW infants). Sample collection in this group is ongoing.

Group A

All the infants were born to VD, and had not been exposed to any antibiotic/probiotic before, during or after delivery. All of them had been exclusively breastfed since birth. Mean GA was 39^{+5} (range 37-41^{+4}), with a BW ranging from 2530 to 4090 g.

The following samples were collected at DOL 20:

- 36 OMM samples.
- 36 infants' stool samples.
- 71 infants' oral swabs (35 pairs of samples pre and post-breastfeeding, and 1 unpaired pre-breastfeeding sample).

Group B

The 15 infants belonging to this group were born at a mean GA of 33^{+2} weeks (range 32-34^{+6}), with a BW ranging from 1210 to 2310 g. There were 9 twins and 6 singleton infants. All of them, except one, were born to CS, and all the mothers had received antibiotics before or during delivery. All the infants were admitted to the NICU at birth. Twelve of them received antibiotic treatment with ampicillin during the first days of life.

As for feeding, 6 infants were fed exclusive HM (4 infants were fed OMM, while a couple of twins received exclusive donor milk). Two infants received exclusively formula, and the remaining 8 received mixed feeding (HM + formula). Nine infants started breastfeeding during the study period, at a mean age of 14 days of life (range 7-21 days).

For each infant, a stool sample collected at DOL 21 was analyzed for comparison with TIs GM.

In addition, for the 7 infants whose longitudinal data were complete (P11, 12, 21, 22, 31, 32, 40), the following samples were analyzed:

- 22 OMM samples.
- 48 infants' stool samples.
- 57 infants' oral swabs.

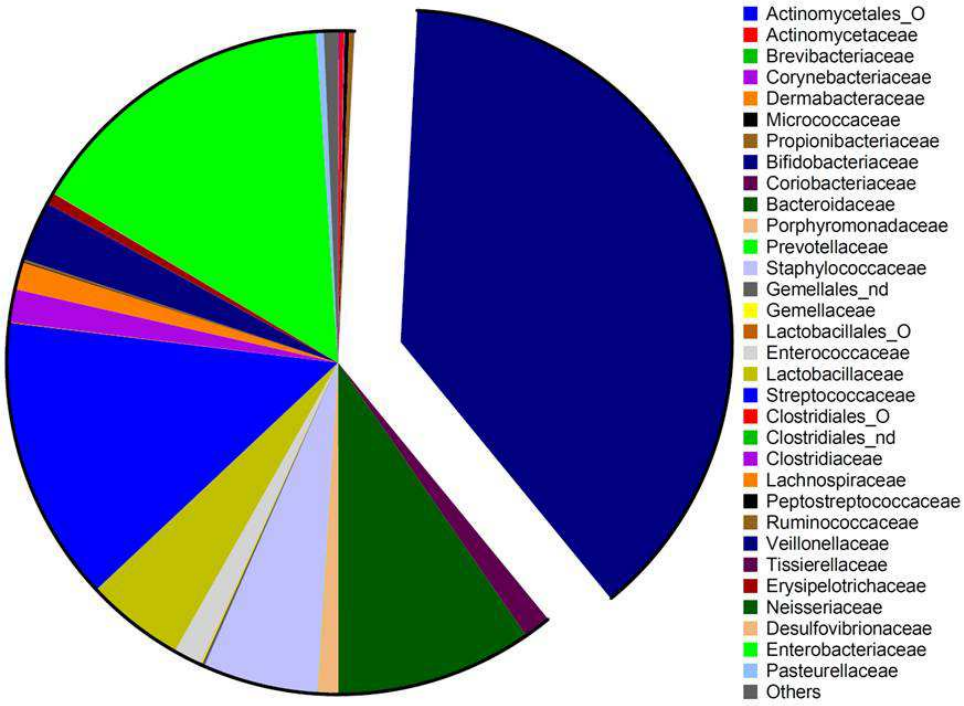
Microbiota analysis

Group A

Stools

Faecal microbiota of healthy TIs (Figure 8) was largely dominated by members of the family Bifidobacteriaceae (average relative abundance [ARA] 38.3%), followed by Enterobacteriaceae (ARA 15.4%), Streptococcaceae (ARA 13.9%), Bacteroidaceae (ARA 9.6%), Staphylococcaceae (ARA 5.4%), and Lactobacillaceae (ARA 4.8%). *Bifidobacterium* represented the dominant genus in 67% of samples.

Figure 8. Relative abundances of different microbial families found in the stools of term infants.



Mother's milk

Streptococcaceae, which are peculiar of HM, were the most represented bacterial family (ARA 24.5%); in addition, a discrete representation of Bifidobacteriaceae (ARA 11.5%), which are common in infants' stools, and

Staphylococcaceae (ARA 11.1%), which are instead common skin and mouth inhabitants, was found in HM. Furthermore, HM contained some anaerobic bacterial families which are usually found in the adult GI tract, such as Lachnospiraceae (ARA 10.3%), Ruminococcaceae (ARA 5.4%), and Bacteroidaceae (ARA 4.4%).

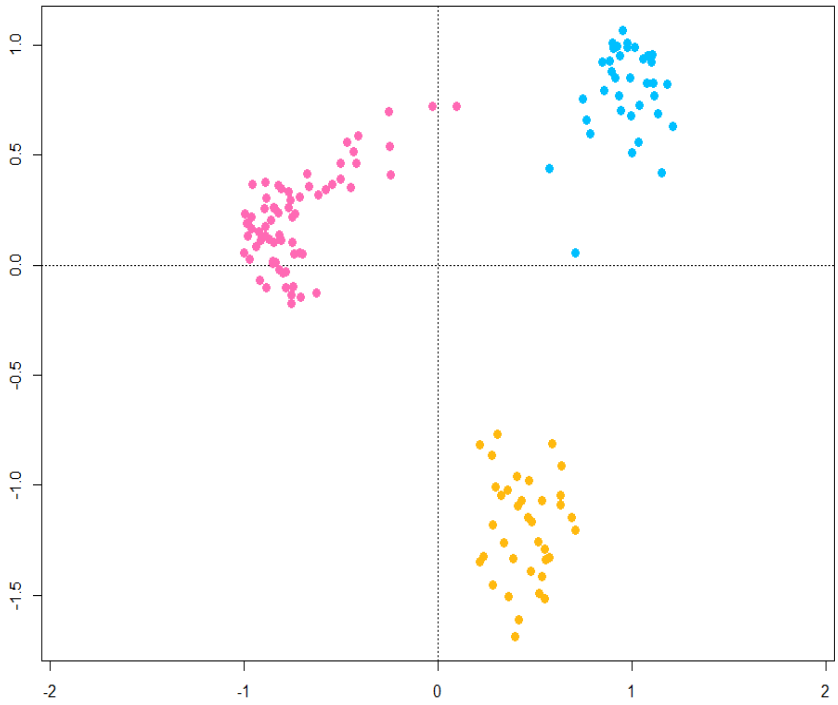
Saliva

Microbiota from oral swabs was largely dominated by the family Streptococcaceae (ARA 69.8%), with *Streptococcus* being the dominant genus in 94% of the samples.

Relationship among the three microbial ecosystems

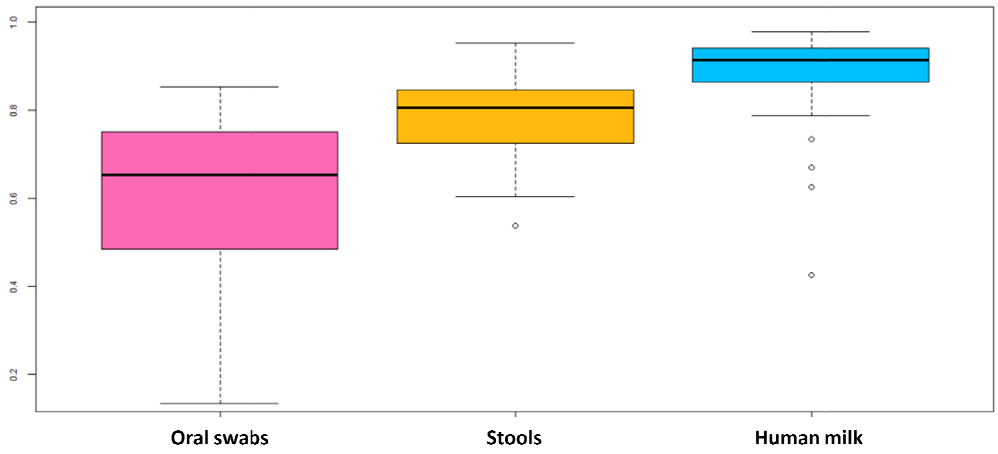
As shown in Figure 9, microbiota from infant's saliva, infant's stools and mother's milk clustered separately, as can be expected since the three body sites are different in terms of pH, oxygen status, and nutrients availability.

Figure 9. PCoA based on unweighted UniFrac distances of the microbiota of mother's milk (light blue), infant stools (yellow), and infants mouth (pink).



SDI was calculated for each ecosystem (Figure 10): HM microbiome was significantly more diverse (SDI 0.88 ± 0.11) than both faecal and oral microbiome ($p<0.0001$ for both comparisons).

Figure 10. Simpson diversity index for each ecosystem: human milk (blue), infant stools (yellow), and infant saliva (pink).



Within-samples variability of HM was lower compared to those of faecal samples according to both unweighted (0.68 ± 0.03 vs. 0.80 ± 0.04, respectively) and weighted (0.25 ± 0.07 vs. 0.50 ± 0.20) UniFrac metric ($p<0.0001$ for both comparisons), meaning that HM microbiota was more "homogeneous" in terms of bacterial species than GM.

Saliva unweighted UniFrac distances (0.80 ± 0.03) were as high as faecal ones; however, when the weighted UniFrac distances were calculated, saliva showed the lowest within-samples variability (0.14 ± 0.06), suggesting that the variability between oral samples resided in the subdominant, non-Streptococcaceae fraction of the ecosystem.

In order to explore potential migration patterns from one ecosystem to the others, the characteristics of the OTUs shared between at least two ecosystems were evaluated, taking into account only OTUs which accounted for at least 0.1% of the ecosystem diversity.

OTUs assigned to *Staphylococcus* spp. were shared by the three ecosystems in 80% (pre breastfeeding) and 83% (post breastfeeding) of cases, and by stools and milk in 91% of cases. OTUs assigned to *Streptococcus* spp. were shared by the three ecosystems in more than 80% of cases, and by stools and saliva in 86% (pre breastfeeding) to 91% (post breastfeeding) of cases. *Streptococcus infantis* was shared between stools and HM in almost all the cases (97%), and by the three ecosystems in 63% of cases. Quite interestingly, the *Streptococcus* OTUs shared between two or three ecosystems were also those which were dominant in the saliva microbiota.

HM and infant's saliva shared an OTU assigned to unclassified members of the family Gemellaceae in 51% of cases. In addition, the majority of OTUs shared between HM and stools belonged to the *Bifidobacterium* genus: *Bifidobacterium breve* (46%), *Bifidobacterium bifidum* (51%), and *Bifidobacterium longum* (74%). On the contrary, *Bifidobacteria* were almost absent in the oral ecosystem (ARA 0.4%).

Group B

Stools – DOL 21 evaluation and comparison with term infants

Data from 15 MPIs were evaluated and compared with data from the 36 TIs.

At DOL 21, faecal microbiota of MPIs (Figure 11) was largely dominated by members of the family Enterobacteriaceae, followed by Bifidobacteriaceae. Compared to TIs (Table A), there was a similar ARA of Streptococcaceae and Staphylococcaceae, slightly lower ARA of Lactobacillaceae, and higher ARA of Clostridiaceae and Veillonellaceae. Bacteroidaceae were almost absent in the stools of MPIs.

Both in term and preterm infants' stools, there was a high interindividual variability in the proportion of the eight most abundant bacterial families (Table A - Figure 12-13).

Figure 11. Relative abundances of different microbial families found in the stools of moderately preterm infants.

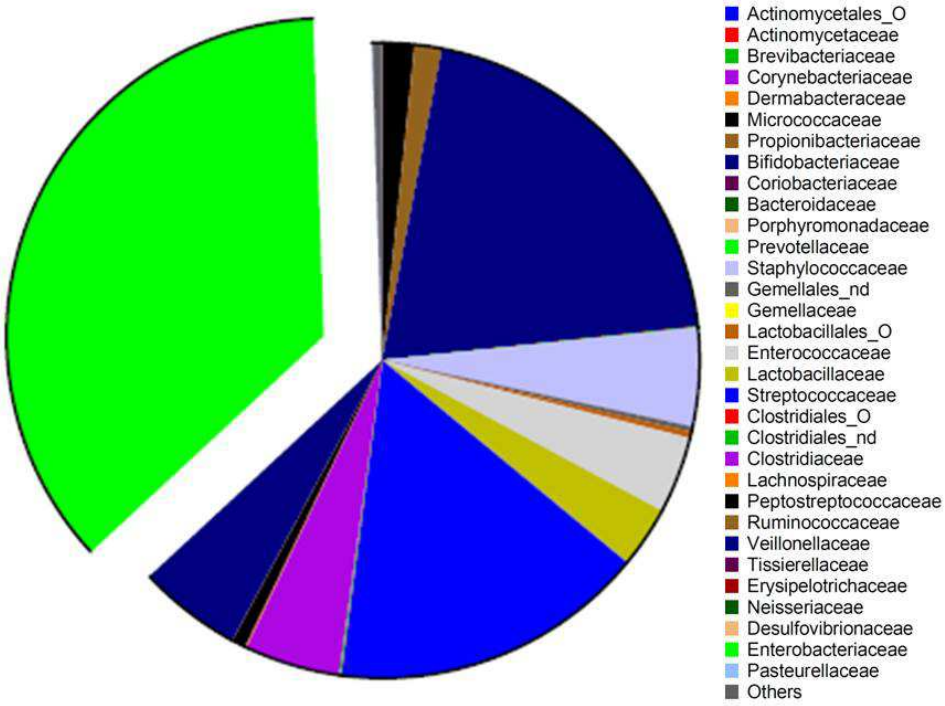


Table A. Average relative abundance (range) of the most represented bacterial families in stools of term and moderately preterm infants at 20-21 days of life.

	Preterm infants	Term infants
Enterobacteriaceae	36% (0.1-80.6)	15.4% (0.01-60)
Bifidobacteriaceae	20% (0.04-41.3)	38.3% (0.03-90.3)
Streptococcaceae	16% (1.6-54.1)	13.9% (0.02-39.9)
Staphylococcaceae	5% (0-19.2)	5.4% (0-20.9)
Lactobacillaceae	3.1% (0.02-16.3)	4.8% (0-33.7)
Clostridiaceae	4.9% (0.05-26.4)	1.6% (0-21.3)
Veillonellaceae	5.3% (0-36.5)	2.9% (0-27.5)
Bacteroidaceae	0.02% (0-0.2)	9.6% (0-45.4)

Figure 12. Proportion of the eight most abundant bacterial families in gut microbiota for each term infant.

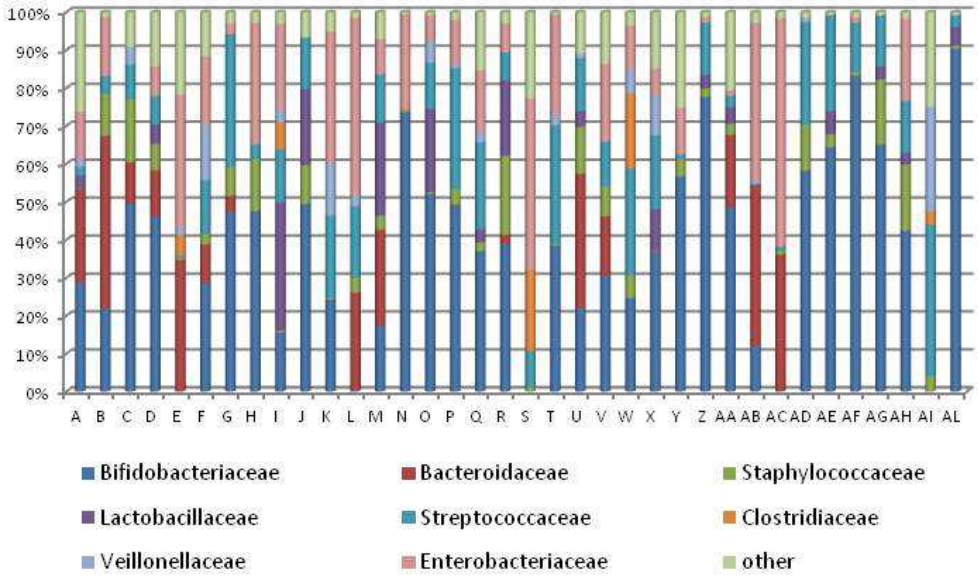
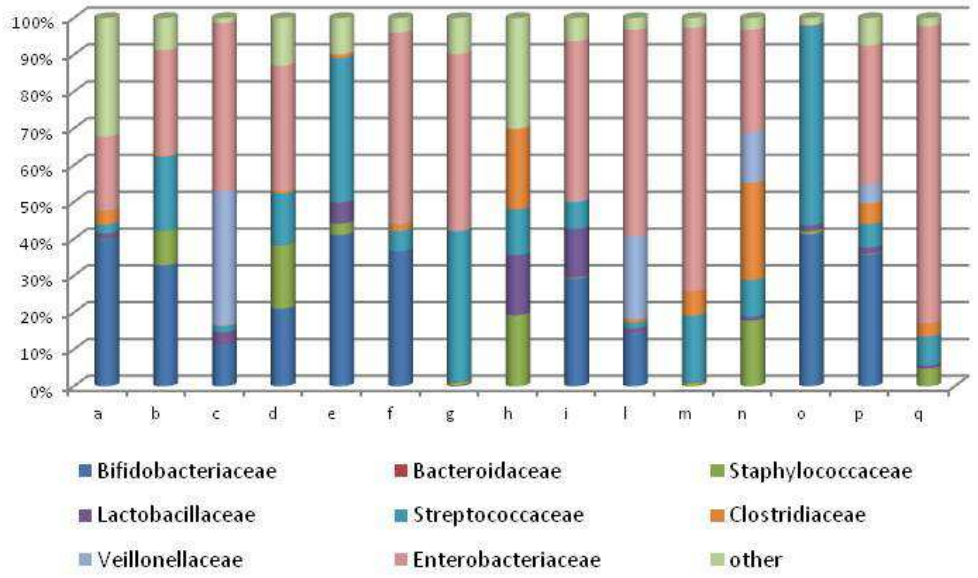


Figure 13. Proportion of the eight most abundant bacterial families in gut microbiota for each moderately preterm infant.



Mother's milk

Longitudinal data from 7 patients and their mothers were examined.

HM samples were available for two mothers (the mother of a couple of twins [M20] and the mother of a singleton baby [M40]). The remaining two mothers of two twin couples [M10 and M30] were unable to provide a complete set of HM for study purposes, due to a reduced milk production over time.

Regardless sampling time, HM microbiota of mothers of MPIs was quite different compared to that of TIs, as it was largely dominated by members of the family Staphylococcaceae (ARA 28.2%), followed by Streptococcaceae (ARA 19.02%) and Enterococcaceae (ARA 5.6%). The average abundance of Bifidobacteriaceae was much lower than in HM from mothers of TIs (ARA 4 vs. 11.5%). On the contrary, a discrete amount of members of the family Corynebacteriaceae (ARA 3.7%) was found in MPIs' HM.

MPIs' HM also contained adult gut-specific bacterial families, but in lower proportion compared to term HM (Lachnospiraceae: ARA 3.9 vs. 10.3%; Ruminococcaceae: ARA 2.7 vs. 5.4%; Bacteroidaceae: ARA 1.6 vs. 4.4%).

Saliva

Oral swabs data were available for all the infants. Regardless sampling time, the characteristics of microbiota in MPIs saliva were similar to those of TIs, with a dominance of Streptococcaceae (ARA 59.1%), followed by Micrococcaceae (ARA 9.7%), Pseudomonadaceae (ARA 7.7%), and Staphylococcaceae (ARA 5.2%).

However, it is interesting to note that the abundance of Streptococcaceae was very low on DOL 1 (ARA 4.8%), but their dominance of the infant's saliva microbiota was already established by DOL 4 (ARA 55.4%) or DOL 7 (ARA 68.2%). Distribution of the abundance of Micrococcaceae was also peculiar, as almost all the infants had a low abundance of Micrococcaceae at birth, followed by a sudden but transient increase over time.

Establishment of GM in MPIs – Relationship with microbiota in HM and saliva

For graphical purposes, the analysis of longitudinal data was restricted to the twelve most abundant bacterial families (see Table B).

Table B. Bacterial families which the highest average relative abundances in stools, human milk, and saliva.

Stools	**Mother's milk**	**Saliva**
Enterobacteriaceae	Staphylococcaceae	Streptococcaceae
Bifidobacteriaceae	Streptococcaceae	Micrococcaceae
Streptococcaceae	Enterococcaceae	Pseudomonadaceae
Staphylococcaceae	Bifidobacteriaceae	Staphylococcaceae
Lactobacillaceae	Lachnospiraceae	
Clostridiaceae	Corynebacterium	
Veillonellaceae		

Longitudinal data regarding human milk from single mothers showed some peculiar changes through the course of lactation. Two sets of HM were evaluated; one from the mother of a couple of twins (M20, Figure 14), and the other from the mother of a singleton baby (M40, Figure 15).

Figure 14. Longitudinal data of human milk microbiota from the mother of a couple of moderately preterm twins (M20 – P21 and P22; x axis shows days of life, y axis represents relative abundance of bacterial families).

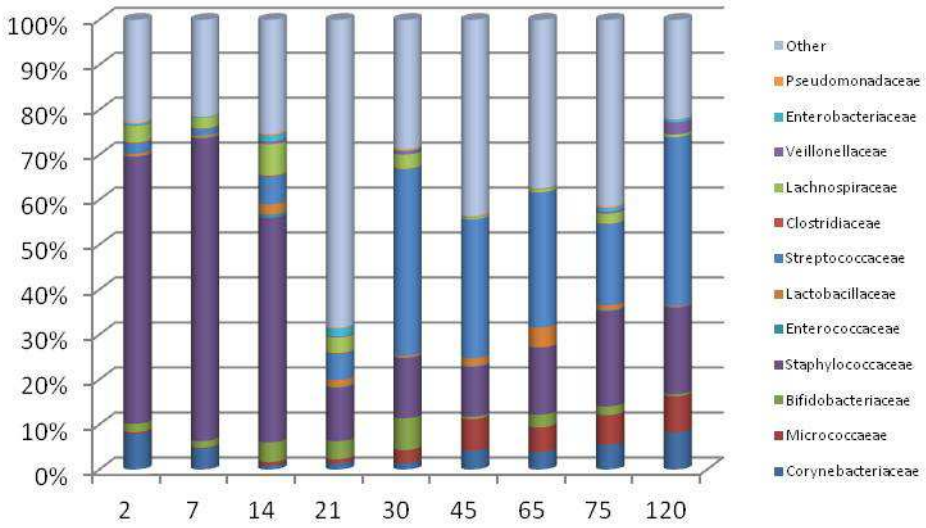
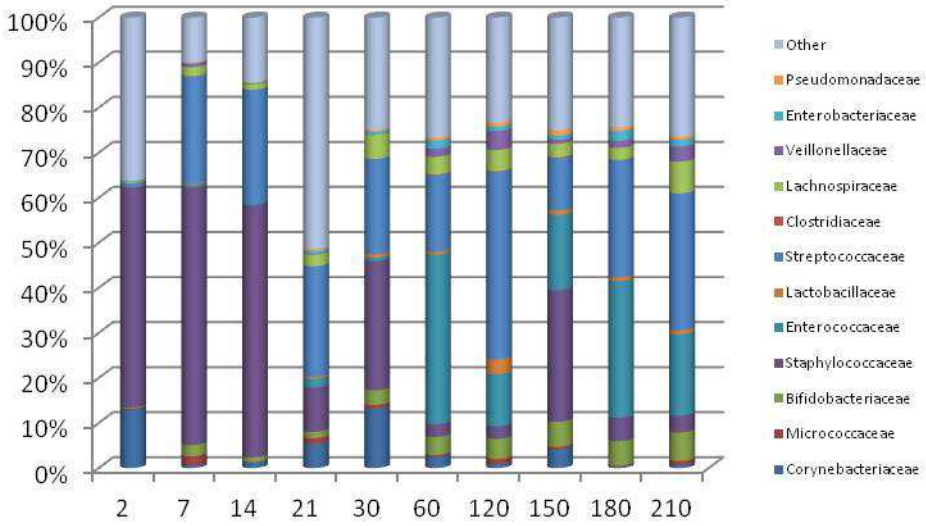


Figure 15. Longitudinal data of human milk microbiota from the mother of a singleton moderately preterm infant (M40 – P40; x axis shows days of life, y axis represents relative abundance of bacterial families).



Both HM sets showed an initial prevalence of Staphylococcaceae, whose relative abundance tended to diminish during the course of lactation. On the other side,

the relative abundance of Streptococcaceae tended to increase over time, especially for M20. Both these changes seemed to begin in conjunction with the first contact of the infants with their mother's breast (DOL 21 for one of the twins, between DOL 7 and 14 for the singleton baby). The proportion of Bifidobacteriaceae in both sets remained very low during the entire course of lactation.

The two HM sets also showed some peculiar differences: HM from M20 had a discrete proportion of Micrococcaceae, which increased after DOL 30, and which were present only in very small amounts in HM from M40. On the contrary, HM from M40 showed a sudden increase in the proportion of Enterococcaceae from DOL 60; Enterococcaceae were almost absent in HM from M20.

Figure 16 represents PCoA based on unweighted and weighted UniFrac distances of microbiota of the two HM sets (M20 and M40) over time, while Figure 17 focuses on HM samples taken from M40. It is interesting to note how HM microbiota at various time points clusters in both unweighted and weighted analyses: all the HM samples taken from DOL 60 onwards tend to cluster together in both analyses, with no apparent relationship with samples taken previously. The only exception is the 150-DOL sample, which is distant from the others in the weighted analysis. This is due to a transient but significant change in HM microbiota at DOL 150 compared to previous and later ones, characterized by a much higher proportion of Staphylococcaceae and a much lower proportion of Streptococcaceae.

Figure 16. PCoA based on unweighted and weighted UniFrac distances of microbiota of two human milk sets (M20 and M40) over time. Symbols represent the two human milk sets, colours the different time points.

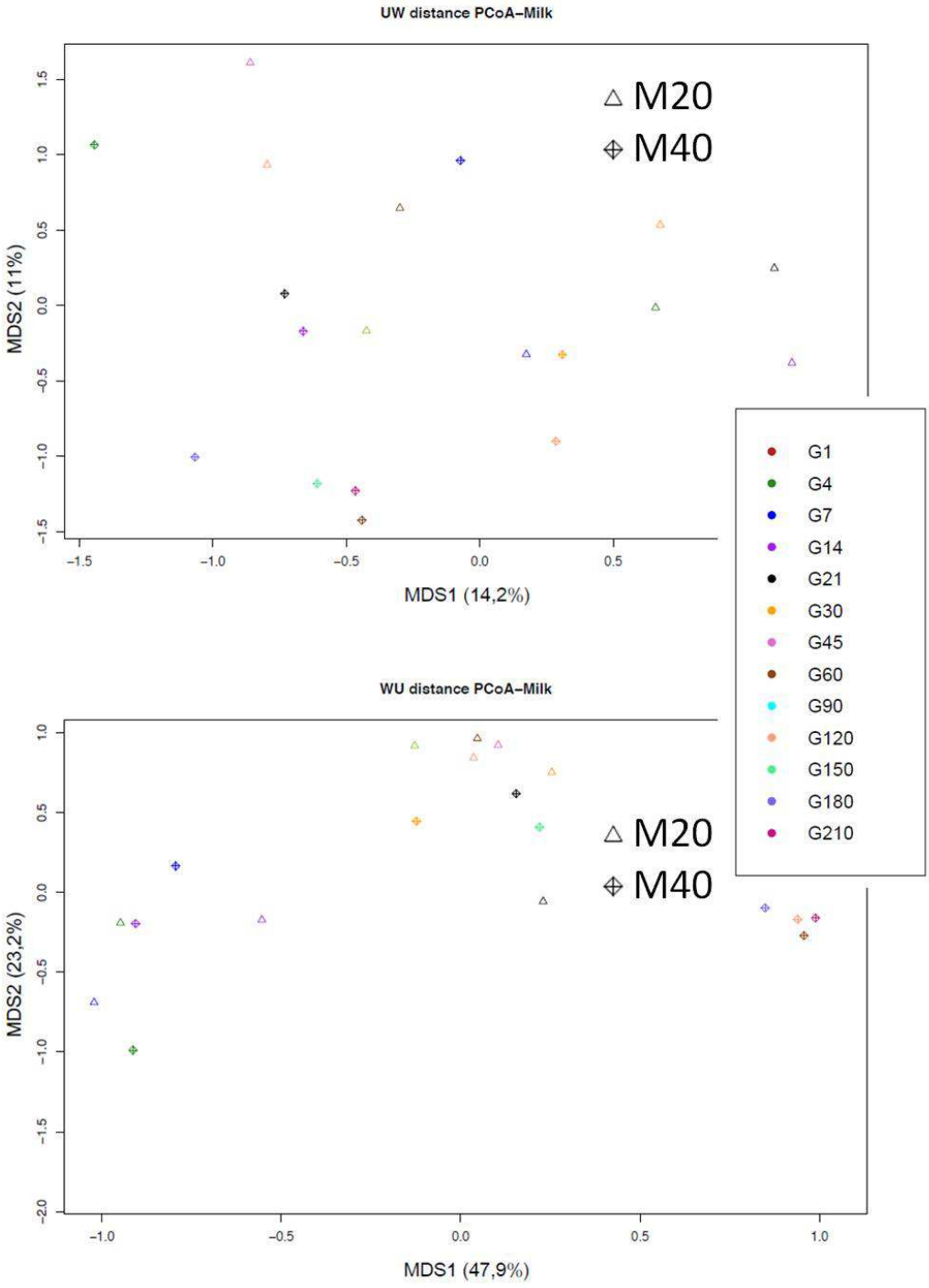
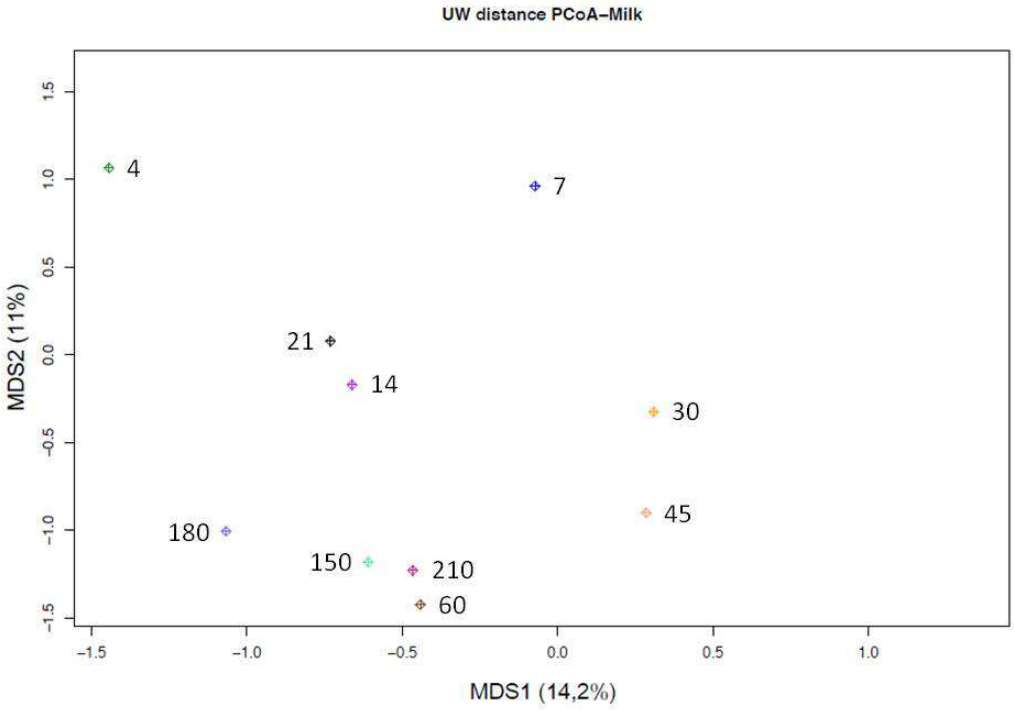
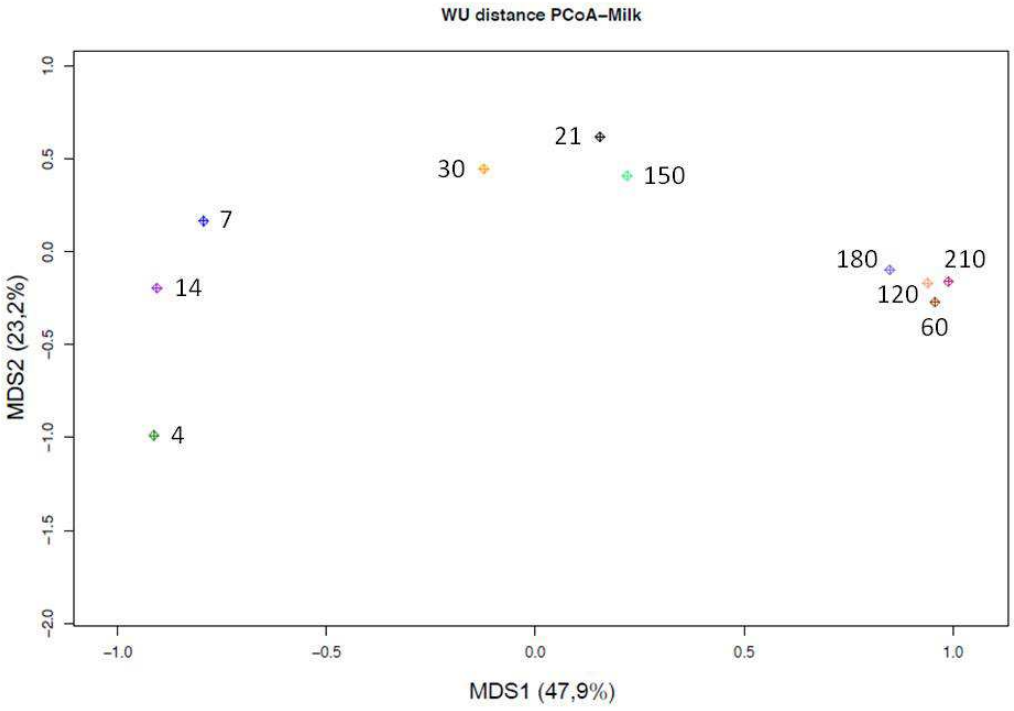


Figure 17. PCoA based on unweighted and weighted UniFrac distances of microbiota of human milk from M40 over time. Symbols represent the two human milk sets, colours the different time points.





Longitudinal data obtained from oral swabs were available for all the infants. The common tract among all of them was the very low proportion of Streptococcaceae on DOL 1, followed by a sudden and stable increase of these bacteria over time. Micrococcaceae were the second most abundant bacterial family in saliva: it is interesting to note that, in all the patients, the amount of Micrococcaceae in saliva increased after the first-second week of life, and decreased at DOL 150-210. The relationship between the amount of Micrococcaceae in HM and saliva was variable: the amount of Micrococcaceae in the saliva of P21 and P22 increased significantly since DOL 14-30 and, since DOL 30, a sudden increase of these bacteria was documented also in the HM from their mother. P40 showed a similar increase in saliva Micrococcaceae since DOL 7, but this was not linked to any increase of these bacteria in his mother's milk.

Individual patients' samples showed some peculiar differences in the proportion of the other, subdominant bacterial families, which could not be attributable to any known clinical characteristic. Differences in saliva microbiota at a given sampling time were present also within twin couples (Figure 18). There were no apparent differences between samples taken immediately before and after the first contact of the infants with their mothers' breast.

Figure 19 represents PCoA based on unweighted and weighted UniFrac distances of saliva microbiota over time. As shown by green boxes, saliva microbiota clustered in a peculiar way on DOL 1 both in unweighted and weighted analysis. In addition, also the three samples taken on DOL 210 clustered together in the two analyses (red boxes).

Figure 18. Longitudinal data of saliva microbiota from three infants (P21, P22, P40. x axis shows days of life, y axis represents relative abundance of bacterial families).

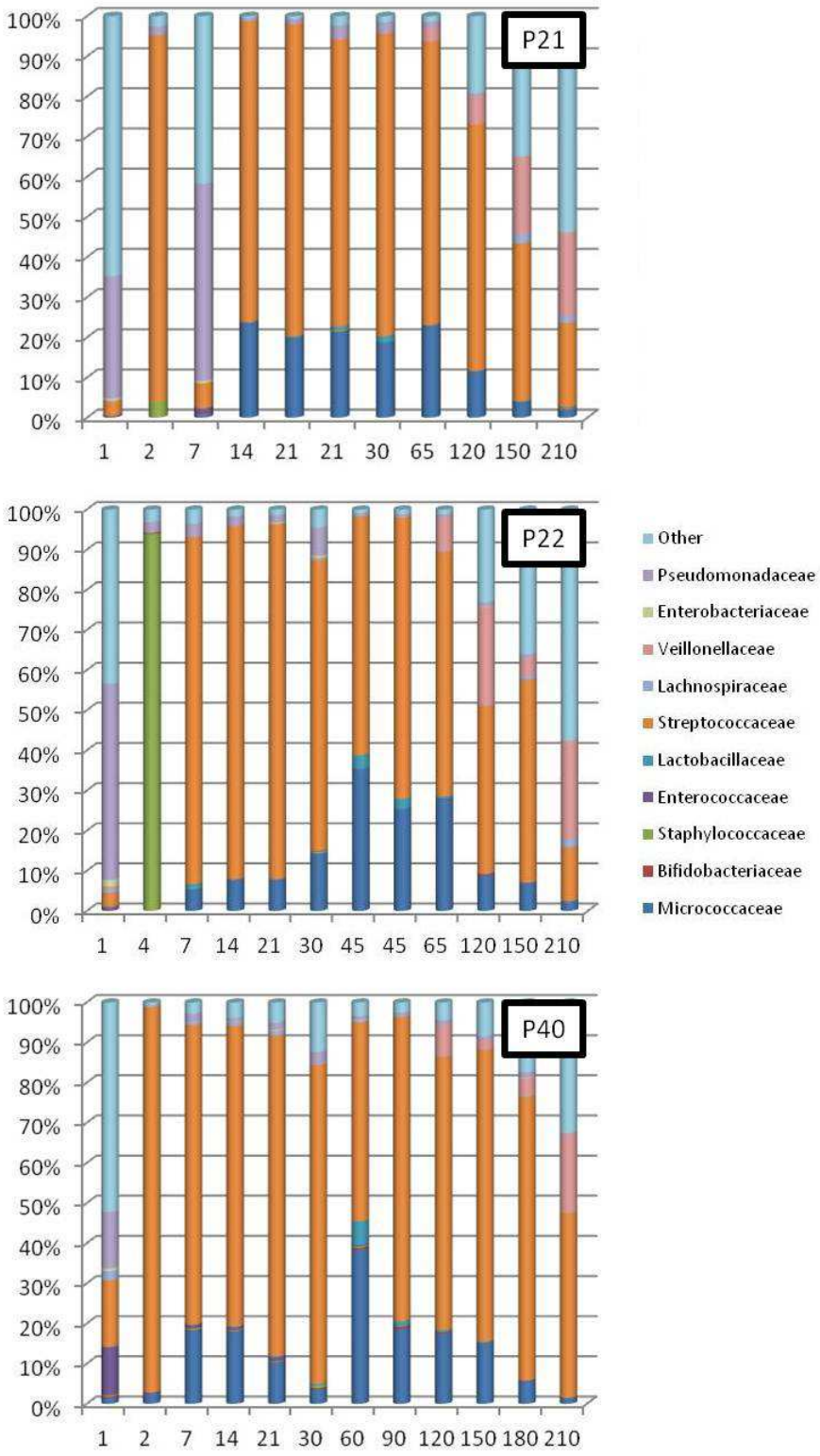
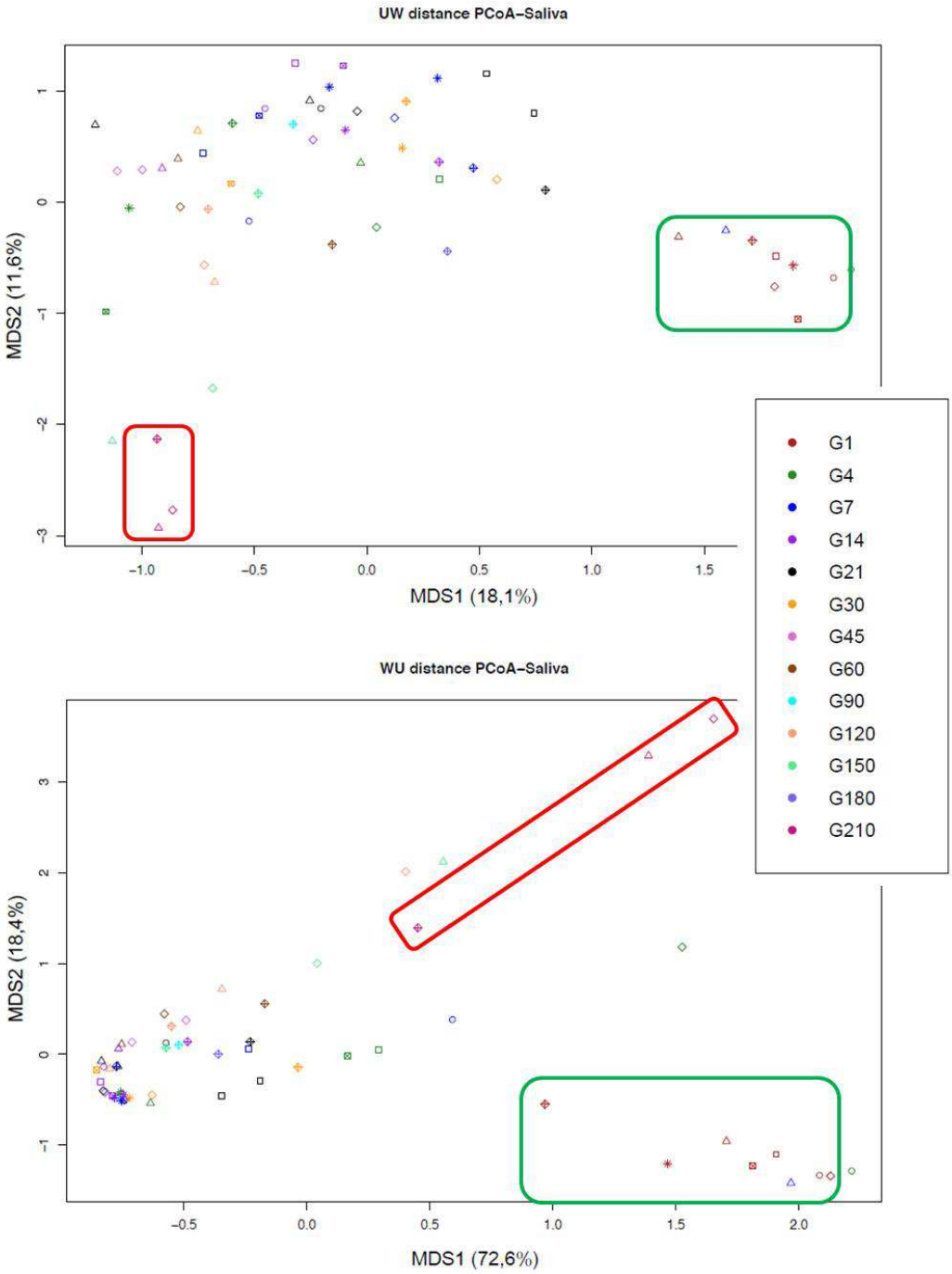


Figure 19. PCoA based on unweighted and weighted UniFrac distances of saliva microbiota over time. Symbols' colours represent the different time points (DOL-1 samples are inside green boxes, DOL-210 samples inside red boxes).



Stools

Longitudinal data about GM were available for all the infants. P21, P22, and P40 completed the longest follow up (from birth to DOL 210).

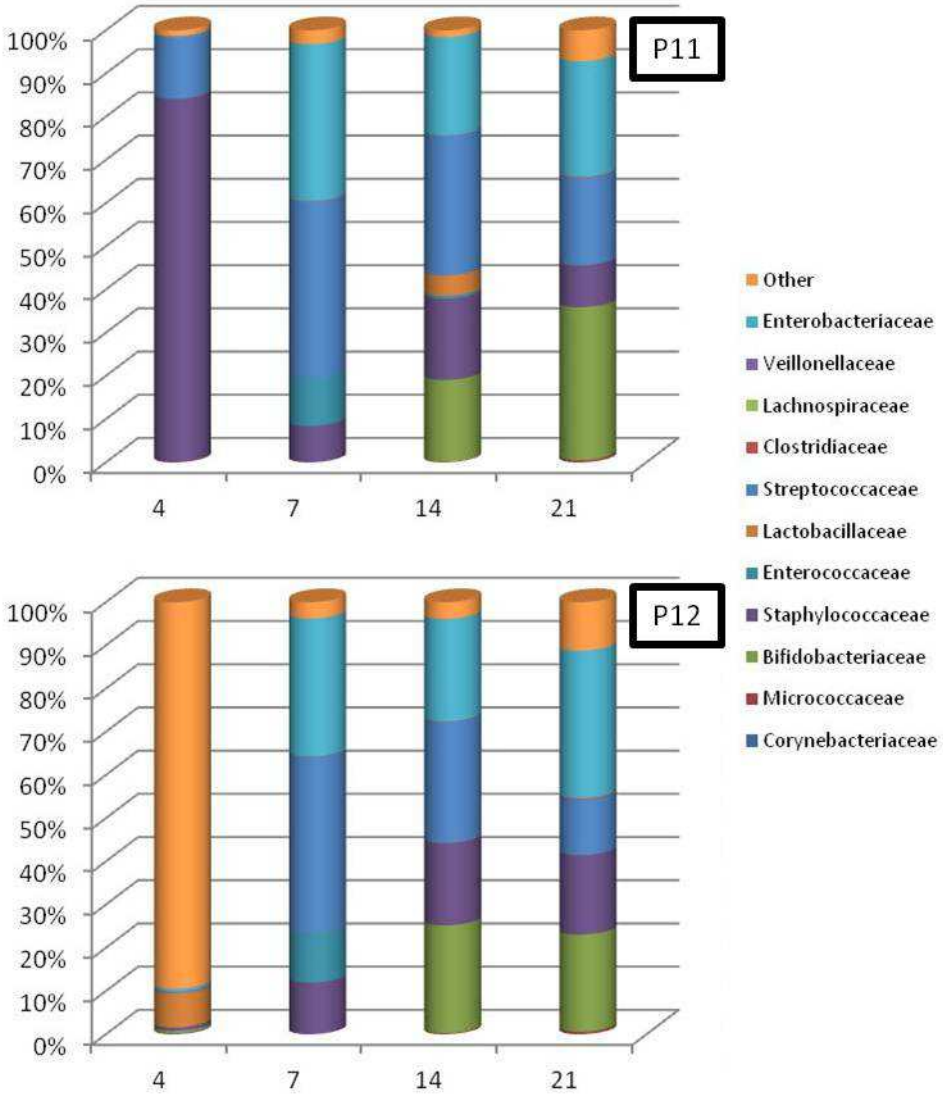
There were some similarities and some remarkable differences among patients and also within twin couples.

Twins P11 and P12 showed some differences in GM since DOL 1, which could not be linked to any clinical difference between the two infants: on DOL 4, P11 stools were abundant in Staphylococcaceae (84%), while stools of P12 were dominated by members of the family Oxalobacteraceae, and Staphylococcaceae represented less than 1% of the overall bacterial diversity (Figure 20). The relative abundance of Enterobacteriaceae was not very high (mean 21.5% over time in P11 and 22.5% over time in P12).

Quite interestingly, the differences in GM between P11 and P12 reflected a different saliva microbiota on DOL 4: specifically, in P12, Staphylococcaceae represented only the 0.5% of the overall saliva microbiota, which was made almost exclusively by Pseudomonadaceae (48.3%), followed by Oxalobacteraceae (14.7%) and Paenibacillaceae (12.2%). The abundance of Streptococcaceae was very low (2%). On the contrary, in P11 on DOL 4 almost half of the saliva microbiota was already constituted by Streptococcaceae (48.4%), followed by a quite high proportion of Staphylococcaceae (42.9%).

In both twins, a discrete amount of Bifidobacteriaceae (relative abundance 19-25%) appeared in GM since DOL 14. Bifidobacteriaceae were almost absent in both twins' saliva. Unfortunately, HM from the twins' mother was not available for analysis; none of the twins had been in contact yet with their mother's breast on DOL 14.

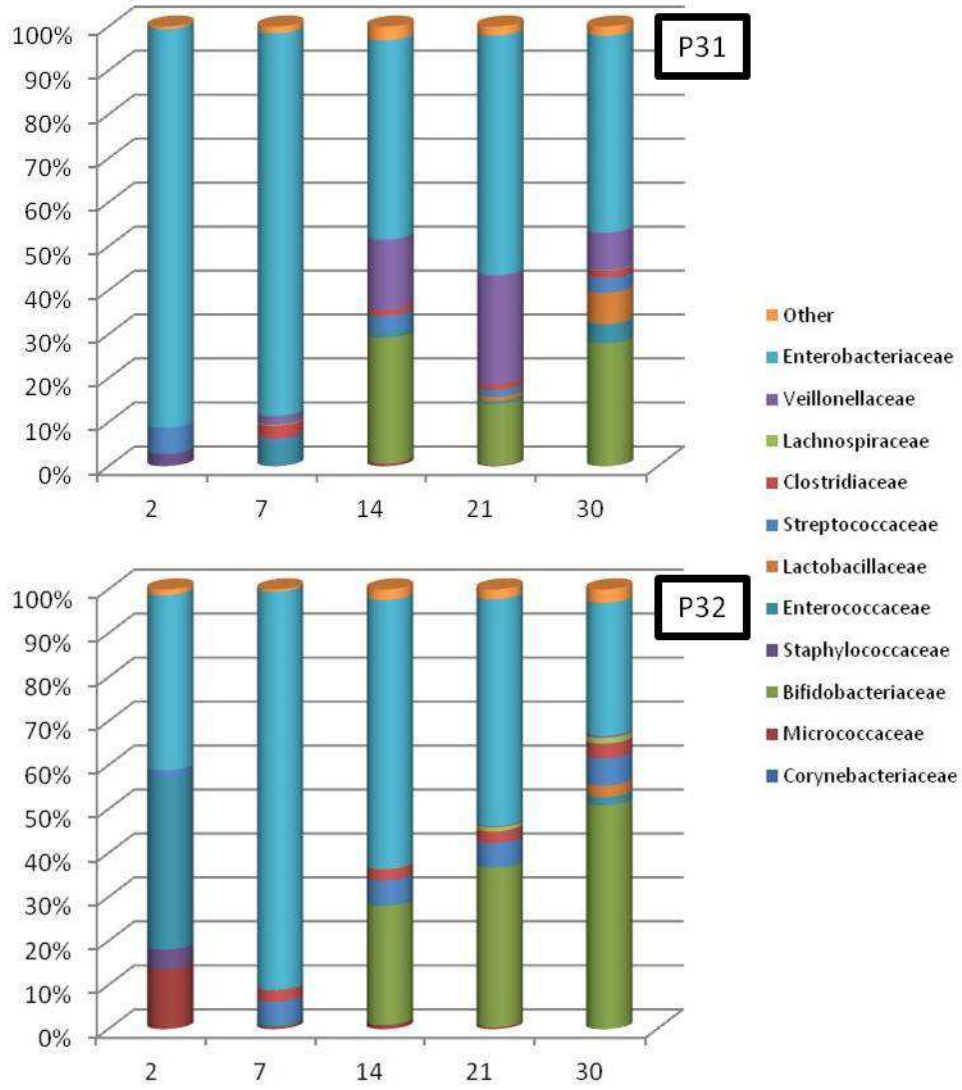
Figure 20. Longitudinal development of gut microbiota diversity in two twins (P11 and P12; x axis shows days of life, y axis represents relative abundance of bacterial families).



GM of another couple of twins (P31 and P32 - Figure 21) showed some peculiar differences compared to GM of P11 and P12, as it was largely dominated by Enterobacteriaceae in the first week of life (on DOL 7, 87.1% in P31 and 90.6% in P32). Similarly to P11 and P12, Bifidobacteriaceae appeared in a discrete amount since DOL 14 of life; both twins had started breastfeeding between DOL 7 and 14, but unfortunately the mother was unable to provide HM samples for analysis. Bifidobacteriaceae were almost absent in both twins' saliva.

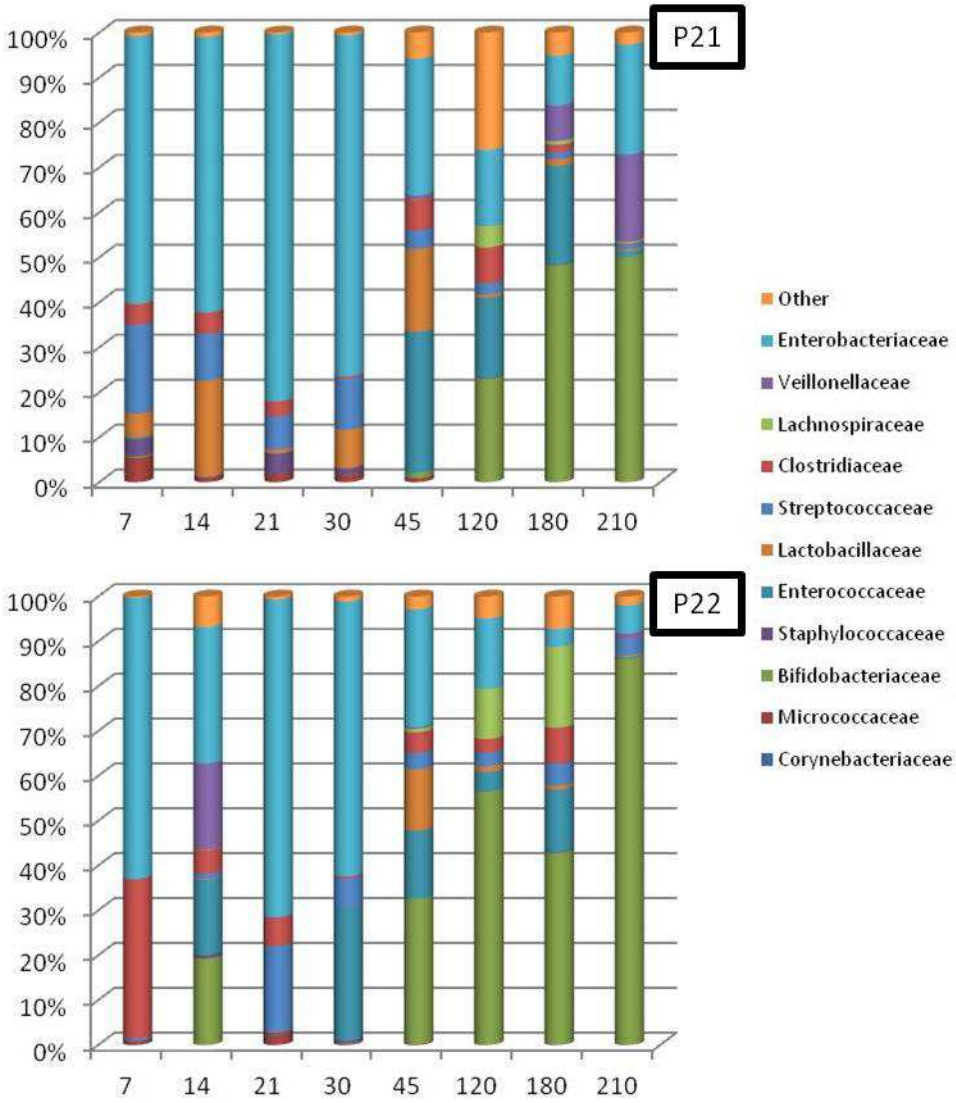
GM of P31 and P32 was different in terms of relative abundance of Veillonellaceae (mean 10.2% in P31, 0.2% in P32) and Micrococcaceae (mean 0.2% in P31, 3.1% in P32). Differences between the two twins could not be attributed to differences in saliva microbiota: relative abundance of Veillonellaceae was very low in both infants, and mean abundance of Micrococcaceae was similar (4.5% in P31, 2.4% in P32).

Figure 21. Longitudinal development of gut microbiota diversity in two twins (P31 and P32; x axis shows days of life, y axis represents relative abundance of bacterial families).



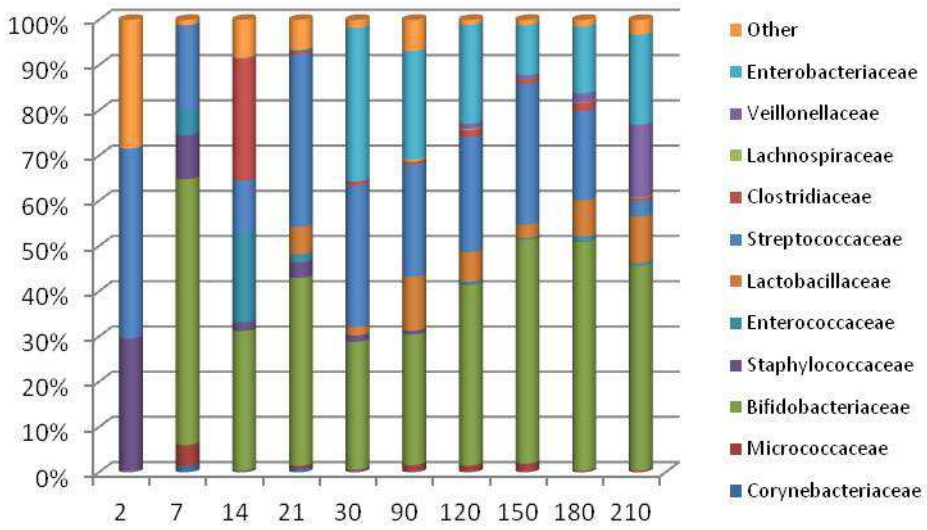
Other twins (P21 and P22) were quite similar in terms of developmental pattern of GM over time: similarly to P31 and P32, GM was dominated by Enterobacteriaceae in the first month of life; however, significant colonization with Bifidobacteriaceae did not occur before the second month of life (Figure 22). In addition, GM of P21 and P12 appeared to be more diverse compared to GM of the previous two couples of twins, with several subdominant bacterial families reaching at least 3% mean relative abundance over time (P21: Enterococcaceae 9.1%, Streptococcaceae 7.3%, Lactobacillaceae 7.2%, Clostridiaceae 3.7%, Veillonellaceae 3.6%; P22: Enterococcaceae 10%, Clostridiaceae 7.9%, Streptococcaceae 5.3%, Lachnospiraceae 3.8%). Quite interestingly, despite the discrete amount of Micrococcaceae found in saliva and HM of the twins, the abundance of these bacteria in stools was extremely low for both of them.

Figure 22. Longitudinal development of gut microbiota diversity in two twins (P21 and P22; x axis shows days of life, y axis represents relative abundance of bacterial families).



P21 and P22, as well as P40, completed the longest follow up (from birth to DOL 210): it is quite interesting to note that, in these three infants, intestinal dominance of Bifidobacteriaceae was established at very different time points (not before DOL 120 in the twins, and already by DOL 7 in P40 – Figure 23).

Figure 23. Longitudinal development of gut microbiota diversity in a singleton infant (P40; x axis shows days of life, y axis represents relative abundance of bacterial families).



Bacterial diversity of GM in P40 was peculiar, as GM was dominated by Bifidobacteriaceae since DOL 7 (mean 37.4% over time), followed by Streptococcaceae (24.7%), and Enterobacteriaceae (12.6%).

Similarly to all the other MPIs, Bifidobacteriaceae were very low in HM from P40's mother and almost absent in his saliva. The high proportion of Enterobacteriaceae in P40's stools, especially since DOL 30, did not relate to a relevant abundance of these bacteria in HM or saliva. The proportion of Micrococcaceae in P40's GM was very low, despite the high abundance in his saliva.

Discussion

The building of GM in both term and preterm infants is crucial to educate the infants' immune system to the balance between tolerance and reactivity which is needed to maintain health through life [7,66]. For this reason, adding further knowledge about how neonatal GM is shaped by the interaction with the mother and the environment would be extremely helpful in order to better define the influence of microbiota on neonatal outcome and thus to identify potential interventions aimed at limiting negative effects of dysbiosis in high-risk patients, such as preterm infants.

Nutrition is known to strongly and directly affect clinical outcomes, both in term and preterm infants: as for term infants, several studies suggest a direct effect of breastfeeding on various aspects of neurological and white matter development [67]. More importantly, HM feeding has been linked to improved clinical outcome [68,69], including better neurodevelopment [70], also in very preterm infants.

During the neonatal period, nutrition represents one of the most important factors which guide the establishment of a healthy GM. It is well known that HM has its own peculiar microbiota, but the exact mechanism through which HM microbial diversity is built is still a matter of research: even if a controversial entero-mammary pathway has been proposed for some bacteria [37], it is plausible that HM microbiota is also subject to other environmental influences.

According to the results of the present study, and in line with previous literature [38], HM produced by mothers of healthy term infants is characterized by a "core" of few bacterial families which represent at least half of the microbial community [32,71,72]. The relative amount of these dominant bacterial families and the abundance of other bacteria vary across studies and could be dependent on geographical location [73,74]. In the present study, HM microbiota showed a slight dominance of members of the family Streptococcaceae, followed by Bifidobacteriaceae and Staphylococcaceae, and by lower amounts of members of Lachnospiraceae, Ruminococcaceae and Bacteroidaceae. This composition of HM microbiota probably reflects different sources of colonization: the infant's saliva

during breastfeeding for Streptococcaceae, maternal skin and the environment for Staphylococcaceae, and maternal gut through the so-called entero-mammary pathway [39] for the last three bacterial families.

As reported by previous studies, Bifidobacteriaceae are the most abundant bacterial family in healthy term infants' GM, which is thus referred to as the "milk-oriented" microbiota [75]. The origin of these bacteria in HM does not need to be necessarily attributed to a complex entero-mammary pathway [37]; actually, recent observations suggest that each individual is surrounded by an unique microbial cloud, which could allow the migration of specific bacteria between different body sites and, possibly, different individuals [76].

It is interesting to note that HM microbiota in term infants had the highest diversity compared to oral and gut microbiota, which means that HM microbiota was the richest among the three. However, within-samples variability of HM was lower compared to those of faecal samples, which also means that HM microbiota was more "homogeneous" in terms of bacterial species than GM. This observation suggests that the mammary gland might act as an environmental filter which allows survival and proliferation of a certain number of selected bacterial families in the majority of individuals. This is in line with the "niche-based" bacterial community assembly proposed by Costello et al. in the context of the metacommunity theory [77]. According to this theory, human beings can be viewed as made of several habitats suitable for bacteria, which are spatially distinct areas and contain each a peculiar community of microorganisms [78].

In order to identify potential migration patterns from one ecosystem to the others, the characteristics of the OTUs shared among the three ecosystems were explored. Members of the families Streptococcaceae and Staphylococcaceae were found to be shared by at least two ecosystems in the vast majority of the patients; interestingly, there was a substantial identity between the Streptococcaceae found in the infant's mouth and in HM. This observation, taken together with the very high abundance of Streptococcaceae in the infants' mouth, confirms the hypothesis according to which the infant's saliva could have

a seeding effect, during breastfeeding, on HM microbiota. Recent data suggest that the interaction between HM and neonatal saliva is extremely important, not only for sharing beneficial bacteria, but also because it creates an unique synergism which boosts early innate immunity [79].

In addition, the very high proportion of cases in which the same *Streptococcus* and *Staphylococcus* OTUs were shared by all the three ecosystems within single infant/mother pairs calls for some speculation about the existence of a biological or ecological role of these bacteria in the building of infants' GM. Although sharing does not necessarily prove the direction of bacterial migration, we might speculate that, for these bacteria, the infant's mouth can be the principal source of contamination, during breastfeeding, for both HM and the gut.

Several OTUs assigned to the genus *Bifidobacterium* (*B. breve*, *B. bifidum*, *B. longum*) were shared in at least half of HM and stool samples taken from single infant/mother pairs: this observation supports the hypothesis of a vertical transfer of these bacteria via breastfeeding [72]. In this perspective, HM acts as a reservoir of these bacteria, which are crucial for infant's health since they are involved in the metabolism of HM oligosaccharides [80,81], for the infant's GM. Bifidobacteriaceae were almost absent in the infant's saliva, which was expected due to the unfavourable aerobic oral environment. However, thanks to their ability to tolerate oxygen exposure [82], it is plausible that Bifidobacteriaceae can transit safely through the oral cavity without actively colonizing it.

To the best of our knowledge, this is the first study aiming at investigating the establishment of GM in a very homogeneous cohort of healthy term infants, by evaluating at the same time microbiota of stools, saliva, and mother's milk.

The characteristics of GM in term infants were similar to those described in the literature, with a dominance of Bifidobacteriaceae, followed by Enterobacteriaceae and Streptococcaceae. Interestingly, GM showed the highest weighted and unweighted UniFrac distances, confirming the wide interindividual variability in GM microbial features. On the contrary, within-samples variability of saliva microbiota was the lowest when the weighted UniFrac distances were

calculated, confirming that saliva microbiota is “homogeneous” as for the dominance of Streptococcaceae, but very variable in terms of the other subdominant families.

In the present study, the evaluation of GM establishment in term infants had two main limitations: the first was the absence of a maternal stool sample, which could have allowed a deeper evaluation of a potential entero-mammary pathway for some bacteria. The second was the lack of the evaluation of microbial changes occurring in microbiota of HM, stools and saliva during the first days of life.

On the contrary, data obtained in the present study from moderately preterm infants are quite helpful at shedding some light over developmental microbiota trajectories over time.

Recent literature, focused on the establishment of GM in both healthy term infants and very low birth weight infants, has highlighted significant dissimilarities between these two groups of infants, which can possibly impact on their tremendously different clinical outcome [41]. The establishment of GM begins *in utero* in both groups. However, the shaping of GM during the first days/weeks of life follows two completely separate paths, as these two groups of infants experience very different environmental and clinical influences. Although moderately preterm infants do not usually have the same risk of adverse clinical outcomes such as infants born very preterm, they often experience mild gastrointestinal and pulmonary impairment, which require a few-week admission to the NICU and the consequently inevitable separation from the mother.

For this reason, it is plausible that the establishment of GM in moderately preterm infants would be more affected by environmental factors than by the mutual relationship with the mother, thus resembling more the GM of more preterm infants. However, at present there is virtually no knowledge about the features of GM in moderately preterm infants.

According to the results of the present study, microbiota from gut, saliva, and HM of infants born moderately preterm is different in terms of microbial composition compared to those of term infants.

Specifically, at DOL 21, gut microbiota of moderately preterm infants was largely dominated by Enterobacteriaceae, while the contribution of Bifidobacteriaceae to its overall diversity was less pronounced than in term infants. Furthermore, GM of moderately preterm infants had a higher proportion of Clostridiaceae and Veillonellaceae compared to that of term infants, while Bacteroidaceae were almost absent. Similarly to term counterparts, in moderately preterm infants there was a high interindividual variability in the most abundant bacterial families.

When HM microbiota data from moderately preterm infants was analysed regardless sampling time, there were also several substantial differences with term infants. Similarly to term infants' one, HM microbiota of moderately preterm infants was largely dominated by Staphylococcaceae and Streptococcaceae. However, the relative abundance of Staphylococcaceae was much higher than in term infants, whereas the proportion of Bifidobacteriaceae was very low. There was also a lower representation of adult-gut specific bacteria such as Lachnospiraceae, Ruminococcaceae and Bacteroidaceae. Overall, these differences with term infants can be interpreted in the frame of a completely different environmental exposure of moderately preterm infants, who are generally separated from the mother at birth and admitted to the NICU.

The separate longitudinal analysis of the two HM sets showed some similarities and some differences in HM microbiota development. The most interesting finding was that, in both sets, the relative abundance of Staphylococcaceae decreased, while that of Streptococcaceae increased over time. The shift between the dominance of Staphylococcaceae vs. Streptococcaceae appeared to be temporally related to the first contact of the infants with their mothers' breasts. This observation seems to confirm the hypothesis according to which, when breastfeeding starts, the infant's saliva can have a seeding effect of HM

microbiota composition. The proportion of Bifidobacteriaceae in both HM sets remained very low during the entire sampling time, even if a discrete amount of Bifidobacteriaceae was found in the stools of all the three infants.

As for saliva microbiota, this was largely dominated by members of the family Streptococcaceae, similarly to what is documented in term infants. However, these bacteria were absent from the oral cavity on DOL 1, due to the dominance of other bacteria of environmental origin. Saliva microbiota of all the infants also had a discrete representation of Micrococcaceae: the relative abundance of these bacteria showed a peculiar behaviour in all the infants, with a sudden increase at a certain sampling time, followed by a sort of plateau and a subsequent sudden decrease (generally at 5-6 months of life). Micrococcaceae are common skin inhabitants [83]: we can speculate that the fluctuations of their abundance in the saliva of moderately preterm infants might be related to the characteristics of preterm infant feeding, which change over time. As suggested by data from the included infants who had the longest follow up, Micrococcaceae increased in saliva when infants were first breastfed; later on, when infants started to receive complementary feeding, and thus the contact with maternal skin for breastfeeding was reduced, the abundance of Micrococcaceae in saliva decreased dramatically.

Beyond the common dominance of Streptococcaceae and the peculiar behaviour of Micrococcaceae, which were similar in all the infants, there were several differences in the abundance of the saliva subdominant bacterial families among infants, and even between twins, which could not be attributable to any known clinical characteristics.

The longitudinal analysis of GM in moderately preterm infants confirmed the high interindividual variability of its features; in addition, the relationship of the characteristics of GM with oral and HM microbiota was variable and often unpredictable on the basis of clinical and environmental factors. Furthermore, Bifidobacteriaceae, which were dominant in term infants' stools, appeared at very different time points in the faeces of moderately preterm infants. Despite

the abundance of Micrococcaceae in saliva, none of the infants showed a similar abundance of these bacteria in stools.

Even if the number of samples analysed so far is quite low, we can affirm that GM of moderately preterm infants appears to be much more similar to that of smaller preterm infants [84] than to term infants' one, with a dominance of members of the family Enterobacteriaceae and other bacteria of environmental origin, followed lately and variably by Bifidobacteriaceae.

These data are in accordance with the very few reports of GM analysis in moderately/late preterm infants: in the study by Arboleya et al. [54], GM of two moderately preterm infants was evaluated at DOL 10, showing that Enterobacteriaceae were the dominant bacterial family in both moderately preterm and VLBW infants. A single study evaluated exclusively moderately and late preterm infants, and focused on colonisation by Bifidobacteria [53]. According to the results of that study, colonisation by members of the family Bifidobacteriaceae was influenced by both gestational and postmenstrual age and did not occur before 33 weeks corrected age. This observation was not confirmed by the data from the present study, where colonisation by Bifidobacteriaceae occurred at very different time points and without any apparent relationship with the abundance of these bacteria in HM or with the beginning of breastfeeding.

Conclusions

According to the results of the present study, we can state that microbiota from HM, saliva, and stools in both term and moderately preterm infants is highly variable and is able to adapt to the changing environment following paths which, at present, are quite difficult to identify. One example of this behaviour is probably represented by infant feeding (i.e. contact with mother's breast, beginning of complementary feeding), which seems to guide a shift in microbial composition of HM and saliva in moderately preterm infants. We can thus speculate that the establishment of microbiota in infants is a dynamic process,

specifically designed in order to adapt to the changing environmental conditions. The rules of this adaptation are far to be understood, but probably are guided primarily by the peculiar biological characteristics of each body site (mouth, mammary gland, gut), which tries to adapt rapidly and independently to the changing environment in order to maintain a microbiota as healthy as possible. The retrieval of a few number of bacterial families shared among term infants in HM can be viewed in this perspective. When adverse external stimuli prevail over this ability to adapt, microbiota tends towards a variable grade of dysbiosis: in this perspective, preterm birth disrupts, at least partially, the ability to create such a microbial “niche” in HM, as the preterm infant is largely exposed, in the first weeks of life, to several environmental stimuli (separation from the mother, hospital environment, drugs) which facilitate dysbiosis.

Beyond few similarities, it is striking to note that microbiota of moderately preterm infants is completely different from that of term infants. The biological role of these differences, however, is unknown, and deserves further evaluation. In addition, while term infants appear to constitute a quite homogeneous group in terms of microbiota features, the seven moderately preterm infants analysed so far are extremely heterogeneous, and this heterogeneity applies also to twins with identical clinical and environmental exposures. We can thus speculate that every infant has his/her own microbiota fingerprint, and adapts the features of this fingerprint in his/her own peculiar way, trying to reach a delicate balance between positive and negative external influences.

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