Chapter 1: General introduction

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1.1 Introduction: early life microbiota

Bacterial communities colonizing humans have been seen as mutualistic symbioses with their hosts. The microorganisms inhabiting the gastrointestinal tract (GIT) of humans were originally overlooked as they caused no manifest disadvantage. During the past 20 years we have constantly faced the evidence that the microbiota plays a crucial role in the development of the GIT (Wilks *et al.,* 2007). The bacterial composition of gut microbiota in early life stimulates a range of important functions, from postnatal intestinal development to the maintenance of the mucosal barrier and in nutrient absorption mechanisms (Jandhyala *et al.,* 2015). The exact timing of bacterial colonization of intestinal infant microbiota is unknown and in the past decades it has been speculated that possibly the gut is virtually sterile until birth. However the combination of bacterial culture techniques with bio-molecular methods revealed that the amniotic cavity harbors a far larger diversity of microorganisms than previously suspected, including uncultivated and uncharacterized taxa (DiGiulio *et al.,* 2008). Recent studies concerning human placental tissue described the presence of a "vibrant placental microbiome" showing peculiar specific metabolic functions (Aagaard *et al.,* 2014). Nevertheless, the interaction between the metabolic functions of the placenta and the developing fetus microbiota are still unknown. Multiple studies on meconium suggest that bacterial transmission from the mother to the fetus is a normal occurrence during human pregnancies. Jiménez *et al.* (2008) reported that traditional cultures of meconium in 21 healthy term babies, gathered within 2h of birth and prior to feeding, contained Gram-positive and Gram-negative bacteria. In a recent study, the meconium of 14 preterm infants was compared with postnatal fecal samples. *Firmicutes* were identified as the predominant bacteria group detected in meconium, while fecal samples of the first days mainly contained Proteobacteria (Moles *et al.,* 2013). The in-utero colonization remains a topic still debated and it is commonly accepted that the microbiota colonization starts in the first days of life. The infant microbial gastrointestinal tract colonization is a process of paramount relevance and interactions between the microbiota and the hosts have important consequences for the adult life (O'Toole *et al.,* 2010). The acquisition and the diversity of the gut microbiota composition have been widely studied in term neonates (Gosalbes *et al.,* 2013; Koenig *et al.,* 2011) and the whole microbial complexity sets the stage for the lifelong, relatively stable adult microbiome (Turnbaugh *et al.,* 2009) (**Figure 1**). The bacterial ecological structure in early life is influenced by numerous shaping factors such as delivery mode, surrounding environment, maternal

microbiota and infant nutritional intake, although their actual contribution to the infant microbiota development remains unclear (Romano-Keeler *et al.,* 2015).

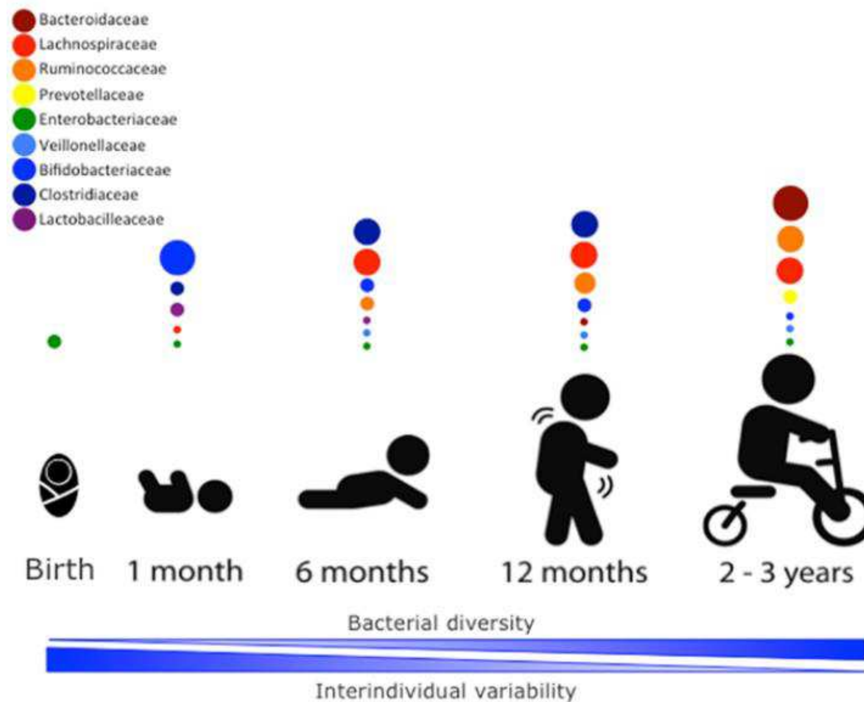

Figure 1: Prevalent bacterial families showed in colored circles. The size of the circle is proportional to the relative abundance of the bacterial taxa at each growth stage. A newborns microbiota is first colonized by *Enterobacteria*. Subsequently, strict anaerobic bacteria dominate the GIT community. During the first month, *Bifidobacterium* is the predominant genus within the gut, but with the introduction of solid foods (4–6 months) *Clostridium*-related families sharply increase (e.g. *Lachnospiracea*, *Clostridiaceae*, and *Ruminococcaceae*). Starting from 2–3 years of age, the microbiota is mainly composed of *Bacteroidaceae*, *Lachnospiraceae* and *Ruminococcaceae*, and it remains rather stable into adulthood. (Arrieta *et al.,* 2014).

1.2 Effect of delivery mode

Theoretically infant intestinal colonization begins with oral inoculation by maternal vaginal and fecal microbiota. The intestinal microbiota of a vaginally delivered newborn closely resembles the microbiota of the mother's vagina (Dominguez-Bello *et al.,* 2010). During natural birth, the baby passes through the vaginal birth canal and is inoculated by maternal intestinal bacteria (Makino *et al.,* 2013). This first inoculum is composed of Gram-negative and Gram-positive bacteria, aerobes, and anaerobes. At birth, the infant GIT is an aerobic environment, which gradually becomes anaerobic in few days (Johnson *et al.,* 2012; Jost *et al.,* 2012). The earliest colonizing bacteria are mainly facultative anaerobes (e.g. *Escherichia* and *Enterococcus*), which

establish an anaerobic environment. This step fosters the shift to obligate anaerobes, including *Clostridia*, *Bacteroidetes*, and *Bifidobacteria* spp. (Yatsunenko *et al.,* 2012). These first microorganisms act as pioneers until the microbiota diversity increases (Koenig *et al.,* 2011). Caesarean delivery is common in preterm infants and has been shown to result in delayed colonization; environmental bacteria rather than vaginal species, for example Clostridium, Staphylococcus, Propionobacterium, and Corynebacterium, initially dominate the gut ecology. C-section delivered babies present a deficiency of strict anaerobes with lower numbers of *Bacteroides* and *Bifidobacterium* when compared to vaginally delivered infants (Biasucci *et al.,* 2010). The mode of delivery exerts an influence on the diversity and function of the infant microbiota, which can persist after birth. Jakobsson *et al.* (2014) reported that full-term cesarean delivered infants lacked or showed delayed gut colonization by *Bacteroidetes* phylum members by up to 1 year and presented a lower total microbial diversity.

1.3 Feeding

Breast milk has been regarded as the gold standard that fulfills the balanced nutritional requirements of a newborn and fosters the growth of neonates. The World Health Organization (WHO) promotes the exclusive breastfeeding of infants until the 6th month of life and the supplemented breastfeeding up to 12 months (WHO, 2003). Breast-fed infants showed an increased abundance of Lactobacilli and Bifidobacteria, compared to formula fed ones (Fernandez *et al.,* 2013). Formula fed infants presented increased levels of facultative and obligate anaerobes, in particular *Bacteroides*, *Clostridium coccoides* group members, and *Enterobacteriaceae* compared to breast-fed newborns (Fallani *et al.,* 2010). The genotypization of *Lactobacillus*, *Staphylococcus*, and *Bifidobacterium* spp. isolated from the breast milk of mothers and from fecal samples of their babies revealed the presence of identical strains. These results suggested the crucial role of breast milk as a source of GIT microbial colonization (Martin *et al.,* 2012). The overall microbiota composition and, in particular, the shift of the Lactobacilli species have also been reported during weaning (Koenig *et al.,* 2011). The introduction of solid food has been correlated with the reduction of the inter-variability between subjects (Roger and McCartney, 2010). Moreover, it is commonly accepted that the microbiota reaches the adult-like shape at the second year of life (Magne *et al.,* 2006; Roger and McCartney, 2010).

1.4 Environment

The familial environment has also been considered as a shaping factor in the development of the intestinal microbiota. A work conducted on infants with older siblings showed that these babies have greater proportion of *Bifidobacterium* spp. and lower total counts of bacteria per gram of feces (Penders *et al.,* 2006). The sources of gut microbiota variation are also influenced by cultural traditions and geographical locations. Fallani *et al.* (2010) observed that in European babies the *Bifidobacterium* genus was prevalent (40% of total detectable bacteria), followed by *Bacteroides* (11.4%) and *Enterobacteria* (7.5%). Northern European babies harbored higher proportions of bifidobacteria in feces, while southern infants were colonized by a more diverse microbiota with more *Bacteroides* members. Another study assessed the different microbial compositions between Finnish and German infants. Finnish infants showed a higher prevalence of *Bifidobacterium* compared to the German group, and lower rates of *Akkermansia muciniphila*, *Clostridium histolyticum* and *Bacteroides–Prevotella* group members (Grześkowiak *et al.,* 2012). The microbiota of children is composed of a few bacterial genera and species, but these groups are extremely variable between subjects. These inter-individual variations are greater among children than adults and diminish together with the increase of microbial population complexity with age (Fallani *et al.*, 2010). In general it is commonly accepted that the familial environment is the principal source of colonizing gut microorganisms during the first year of life.

1.5 Physiological effects of microbial manipulation

Usually, the administration of drug-based therapies influences the microbiota of the subject to some extent (Dethlefsen *et al.,* 2008). Antibiotic therapy during infancy was related to increased proportions of Enterobacteria and Enterococci, and a decrement of bifidobacteria species (Tanaka *et al.,* 2009). Further, such therapies with antibiotics influence the development of the intestinal microbiota, frequently decrementing the phylogenetic diversity (Koenig *et al.,* 2011). Antibiotic therapy of mothers during the prenatal or the breastfeeding period correlated with lower rates of *Bacteroides*, *Atopobium*, and a diminished presence of total bacteria (Fallani *et al.,* 2010). The use of probiotics is another possible external source that could modify the infant microbiota. During infancy, the microbiota is not stable and the composition of bacterial populations may not support the colonization by a new probiotic member. Theoretically the consumption of probiotics by the mother could impact the development of the infant microbiota. Several studies conducted on healthy term babies until the second year of life assessed that the employment of Bifidobacteria or Lactobacilli for probiotic purpose promoted the weight and length gain (Gibson *et al.,* 2009; Scalabrin *et al.,* 2009). Probiotics have also been administered

in preterm babies (Underwood *et al.,* 2009), undernourished children (Sazawal *et al.,* 2010) or for the treatment of specific pathologies for example NEC (Necrotising Enterocolitis) (Deshpande *et al.,* 2010) and the probiotic use seemed to be well tolerated by infants. However, it is usually recommend to use tested probiotics for the treatment of specific conditions in infants (Braegger *et al.,* 2011).

1.6 Long-term effects of specific microbiota colonization

The initial microbial colonization and the resulting metabolic features exert an influence on the microbiota development. For example, cesarean delivered babies seemed to be correlated with the insurgence of type 1 diabetes, celiac disease and asthma (Rautava *et al.,* 2012). Moreover, decreased counts of *Bifidobacterium* spp. during early infancy (6 and 12 months) have been correlated with the infant obesity insurgence (Kalliomäki *et al.,* 2008).

1.7 Sampling strategy

Studies exploring the gut microbiota diversity are typically conducted on stool samples because those specimens are noninvasive. The gut microbiota is distributed differently throughout the gastrointestinal tract. The inhomogeneous community composition is due to the different physicochemical conditions (e. g. pH, redox potential, nutrient supplies, water content, host secretions) that exert selective pressures on microorganisms (Booijink *et al.,* 2007). Despite the common knowledge that the microbiota distribution is not homogeneous throughout the GIT, the exact differences in diversity have still not been assessed (Gerritsen *et al.,* 2011). It has been postulated that the stool microbiota is a combination of mucosal bacteria and the luminal populations (Eckburg *et al.,* 2005). Investigations assessing the structure of microbiota in adult stool showed a structured community from the outside of the stool, the portion closest to the mucosa, toward the center that contains luminal bacteria. It was observed that this structure could be perturbed in patients with idiopathic diarrhea (Swidsinski *et al.,* 2008). However, the microbial composition did not differ when comparing the front and end of a stool pellet, thus a section of a stool pellet is an accurate and representative sample of the whole stool (Mai *et al.,* 2010). The majority of publications on the microbiota ecology are based on results from stool samples, but a major goal will be to supply more detailed knowledge on how the fecal microbiota differs from the distinct niches throughout the GIT. Another important aspect for the downstream analyses is the storage of samples because the consistency and scientific accuracy of experiments rely on correct conservation. A study on sputum specimens focusing on the effect of storage showed that microbial profiles could be sharply altered with an overall reduction of bacterial diversity when samples were left at room temperature for 24 hours compared to prompt

cold storage (Nelson *et al.,* 2010). It is also notable that freeze-thaw cycles lyse cells which can be very critical especially for culture based experiments, thus in order to preserve the complexity of the whole microbiota it would be useful to assess the cultures from freshly collected materials (Sharma *et al.,* 2006). However, another work showed that the storage of stool samples for up to two weeks at room temperature does not significantly modify the bacterial community composition in molecular based approaches when the total extracted DNA is used to explore the whole microbiota (Lauber *et al.,* 2010). Overall, preservation at -80 ºC and avoidance of continuous freeze-thaw cycles is endorsed to minimize the loss of bacterial diversity.

1.8 Methods for studying the human microbiota

During the past years, investigations on the infant gut microbial populations were culture-based and the research was sharply limited by a lack of knowledge on growth requirements of most of anaerobic bacteria. The accuracy of robust culture-based results strictly correlated to the use of the correct growth conditions (e.g. media, temperature, oxygen content). It is thought that only a small percentage (10–50%) of the entire gut microbiota is cultivable (Goodman *et al.,* 2011). Culture-based approaches are often employed in studies coupled with culture-independent techniques (e.g. fluorescent in situ hybridization (FISH) or flow cytometry (FCM)). However, those “classical” approaches are usually unsuitable for the human microbiota characterization. Another approach routinely used allows the assessment of the Microflora Associated Characteristics (MACs) between different subject groups by means of evaluating the differences in the composition or presence/absence of some specific microorganisms. The concept of MACs was described in 1978 (Midtvedt *et al.,* 1985), and of the most studied MACs is represented by short chain fatty acids (SCFAs) metabolizers. The variation of bacterial population rates could lead to alterations in the quantity and quality of fecal SCFAs. The analyses of MACs compositions have been used in ecological studies between coeliacs compared with controls (Tjellström *et al.,* 2007), probiotic or antibiotic treated infants and their relatives compared to healthy subjects (Bezirtzoglou *et al.,* 2011). This approach has also been used to identify microbiota variations in allergy diseases (Thompson-Chagoyan *et al.,* 2011). Together with the advent of bio-molecular techniques researchers developed culture-independent, DNA-based approaches to overcome the limitations associated with culture-based approaches. The most popular DNA-based techniques employed in the past were temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE) (Cani *et al.,* 2008). These systems promote the separation of amplicons usually of the 16S ribosomal RNA gene (rRNA) on the basis of their GC content and generate different migration patterns. The 16S

rRNA gene is present in all eubacteria and archaea. This gene is composed of conserved and variable regions, which allows phylogenetic identification of microorganisms present in an environment (O'Toole *et al.,* 2010). The advance of these rapid techniques allowed the overview of the whole composition of bacterial communities. The downstream analyses of the TGGE and DGGE are characterized by bands excision and sequencing. These methods provide only limited phylogenetic information because they can be influenced by PCR bias. Another technique that has been used to explore the infant gut microbiota is the dot-blot hybridization (Malinen *et al.,* 2003). This approach involves the RNA isolation and immobilization and evaluates quantitatively and qualitatively the similarity to the target by means of oligonucleotide-labeled probes. The advantage of this method is that it is not influenced by PCR bias, however the main limitation is the resolution of results for they are partial and focused on single populations rather than on the entire microbial complexity. Moreover, the quality of the results is linked to the generation of reference sequences to design specific probes. The FISH approach has also been used and provided respected results, but it shared the same technique limitations as the dot-blot hybridization (Duncan *et al.,* 2008; Manichanh *et al.,* 2006). Quantitative PCR (qPCR) is routinely used; it quantifies the accumulation of amplification products by means of measurement of labeled probes or fluorescent reaction mixtures (Overturf *et al.,* 2009). Some studies have combined different techniques, for instance qPCR together with dot-blot hybridization to quantify bacterial populations as well as identify the different species (Zwielehner *et al.,* 2009). The combination of qPCR and FISH yielded significant and more comprehensive results (Collado *et al.,* 2010). Microarrays were the next step of the culture-independent technologies for the phylogenetic studies (Palmer *et al.,* 2007). The base concept of microarrays is similar to the above-mentioned approaches, but the great innovation consists on the possibility of hybridizing larger numbers of sequences at the same time. This approach allows extensive data generation from the single read. During the last decades the gut microbiota research has been focused exclusively on the bacterial 16S rRNA gene (Frank *et al.,* 2008). As reported by Franck *et al*. (2008), the investigation of the gut microbiota has faced the "metagenomic era," with the advent of the DNA sequencing-based techniques. The 16S rRNA gene sequencing provides the gene sequence composition in bases and direct information on the species identity of bacterial populations present. DNA sequencing performed on a larger scale revealed information on the whole microbiota of a specific environment. The initial sequencing-based techniques applied the cloning of the entire 16S rRNA gene into a plasmid and its introduction into a bacterial host, usually *Escherichia coli*, followed by Sanger sequencing. This old technique was laborious and expensive even if allowed the microorganism identification at

the species level (Strausberg *et al.,* 2008). Today high-throughput sequencing is the most common approach used because of the speed for sequence data generation and for the precise insight into the microbiota composition (Mardis *et al.,* 2008). High-throughput sequencing technologies, such as those provided by Roche/454 and Illumina, have been commonly performed for gut microbiota studies. Even if those techniques are really powerful they are not without limitations. First of all, amplicons are susceptible to PCR bias. Furthermore these analyses require bio-informatic competences to analyze the total amount of data obtained and the associated platforms are expensive. It must also be considered that these technologies provide qualitative information on the populations present in a particular ecological niche, but to generate quantitative data it is important to perform qPCRs. Currently the human microbiome is taken in consideration by two large consortia; Metagenomics of Human Intestinal Tract (MetaHIT) and the Human Microbiome Project (HMP). Another technique available is the shotgun sequencing, where the metagenomic bacterial DNA is first fragmented into short fragments and sequenced randomly (Hattori *et al.,* 2009). This approach applies the sequencing of random DNA amplicons and not of targeted regions. It could generate interesting information regarding the functional potential of bacterial populations and of the identity of microorganisms harbored in a particular niche. It could be also possible generate entire genomic sequences (Kurokawa *et al.,* 2007). In the near future other novel high-throughput sequencing technologies will occur. Examples may be the Ion torrent (Schadt *et al.,* 2010), SMRT (Pacific Biosystems) (Levene *et al.,* 2003), SOLid (Applied Biosystems) (Shendure *et al.,* 2005), and nano pore sequencers (Clarke *et al.,* 2009). The aim of those techniques is to produce higher numbers of reads in shorter time and at lower cost. It is important to remark how those technologies will update the studies involving the human gut microbiota. However, even if technologies are advancing there is a limitation that has to be faced. A main goal is to obtain a comprehensive insight of representative bacterial populations in fecal sample. This topic is more important for infants, actually the microbiota of babies is assessed by collecting stool samples and by extracting the DNA. The primary limitations of this approach are that fecal samples tend to well represent the bacteria present in the lower colon but not those of the stomach and upper intestine. Despite this limitation, fecal samples are routinely employed to identify the complexity of bacterial populations inhabiting the colon and, to date, fecal-based assessments remain the prevalent approach.

1.9 DNA extraction optimization

The major challenge that has to be assessed when bio-molecular techniques are employed for gut microbiota studies is the acquisition of consistent data whilst not underestimating the whole complexity of bacterial populations or single groups. The first step to generate accurate bio-molecular results is represented by the DNA extraction. This process is variable and crucial for DNA-based microbial community analyses in order to supply reproducible results. For decades many protocols for the isolation of purified DNA from different specimens have been tested. Different extraction methods have been developed; some of them are based on enzymatic lysis (lysozyme, mutanolysin, and Proteinase K) or use strong chaotropic agents or physical methods (sonication, freezing-thawing or repeated bead-beating). The possible combination of protocol steps is critical because the prevalent objective is to minimize the DNA degradation while maximizing the recovery of DNA. The storage condition is a critical point.. It has been demonstrated that applying different storage times to human fecal samples, ranging from short-term storage to 2 weeks at room temperature prior to completely freezing at −20°C or −80°C, had no significant influence in modifying or reducing the bacterial distribution (Lauber *et al.,* 2010), however freeze/thaw cycles may alter the microbiota structure (Bahl *et al.,* 2012). The water content of fecal samples, typically ranging from about 70% for formed stool to >85% for liquid stools (Bliss *et al.,* 1999), or sample homogenization have been evaluated to possess little effect on the relative abundance of single bacterial taxa, but inappropriate DNA extraction protocols could introduce strong bias into microbiome data (Santiago *et al.,* 2014). The fecal microbiota is composed by more than 30 different phyla, the most representative belong to *Firmicutes*, *Bateroidetes*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* (Rajilić-Stojanovic *et al.,* 2007). One of the problems that has to be faced during DNA extraction is represented by the rigidity of the bacteria cell wall. The gut-residing bacteria are prevalently Gram-positive (Lagier *et al.,* 2012) and those types of microorganisms are harder to lyse compared to Gram-negative ones. The archaea domain is also present within the human gut and the predominant genus in human adults is represented by methanogenic *Methanobrevibacter* spp. with a variable prevalence (Hoffmann *et al.,* 2013). Other taxa present in the GIT can form endospores (e.g. *Bacillus*, *Clostridium*, and some bacteria from the *Firmicutes* phylum), which are the strongest cellular structures known. Among the different DNA extraction protocols, the mechanical disruption of cells has been described to be more efficient compared to other techniques. The repeated bead-beating is very important especially for the lysis of Gram-positive bacteria and archaea and allows the highest ecological diversity recovery when compared with enzymatic protocols with lysozyme and/or mutanolysin (Salonen *et al.,* 2010). It has also been

described that the combination of bead-beating with a freeze-thaw cycle protocol for cell lysis had positive effects on the DNA extraction of Gram-positive bacteria and fungi (Sergeant *et al.,* 2012). Variations caused by the lysis efficiency between protocols could lead to under- or overestimation of some genera belonging to the phyla of *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (Wesolowska-Andersen *et al.,* 2014). The major challenge is identifying a quality-controlled DNA extraction method suitable for all bacteria and archaea. Another notable fact is that too strong lysis protocols could partially degrade the first released DNA and probably lead to an underestimation of Gram-negative bacteria (Hugon *et al.,* 2013). In order to recover high quality purified DNA, an additional extraction step such as chromatographic purification, chloroform extraction, treatment with activated carbon or sample dilution may be required. This aspect is especially important for fecal specimens because the presence of polysaccharides, bile salts, lipids, may be PCR-inhibiting (Schrader *et al.,* 2012).

1.10 16S rRNA gene primers: a crucial choice.

The aim of profiling the whole complexity of microbial communities is one of the prevalent goals for microbiologists exploring different ecosystems. However, our knowledge of the bacteria and archaea domains within the human gut is uncompleted because most of those microorganisms are not easily cultured under laboratory conditions. For this reason the phylogenetic classification of bacteria and archaea is based on genetic and phenotypic information , which usually is limited to cultured strains (Yarza *et al.,* 2014). Bio-molecular techniques as DGGE (Denaturing gradient gel electrophoresis) (Muyzer *et al.,* 1993), T-RFLP (Terminal restriction fragment length polymorphism) (Liu *et al.,* 1997), FISH (fluorescent in situ hybridization) (Wagner *et al.,* 1998), were the first methods exploited for studies focused on microbial communities until the onset of high-throughput sequencing technology. The development of next-generation sequencing technology such as Roche 454 and Illumina has increased our knowledge regarding uncultured bacteria (Tamaki *et al.,* 2011). The first use of the 16S rRNA gene sequence for phylogenetic analysis was established in 1985 (Lane *et al.,* 1985). The 16S rRNA gene sequence is the most common marker gene used for profiling bacterial populations due to the fact that it contains both highly conserved regions for primer design and hyper-variable regions for phylogenetic characterization (Tringe *et al.,* 2008). The whole 16S rRNA gene sequence is represented by nine hyper-variable regions that are interspersed by nine highly conserved regions (Wang *et al.,* 2009). As supported by sequencing technology, to date it is possible to study ecological niches by only using partial sequences of the 16S rRNA gene. The main limitation in the use of all bio-molecular techniques is the selection of suitable primers to

study the composition of the bacterial phylogeny. An initial study has described that the use of different primer sets might result in different DGGE patterns (Yu *et al.,* 2004). More recent studies performing high throughput technologies have also confirmed that the use of different primer couples could lead to either an under- or over-estimation of some species in a microbial population (Tringe *et al.,* 2008; Hamady *et al.,* 2009). qPCR represents a valid alternative to the classical cultivation approaches. The concept of "universal" PCR primers has been evaluated although it is unlikely to design a primer set that can equally amplify all the bacterial and archaeal populations. Several broad-range 16S rRNA gene primer sets and probes have been designed to target all bacteria and archaea present in different specimens (Ott *et al.,* 2004, Takai *et al.,* 2000). The effective quality of PCR assays is usually established by testing predominant genera or species. Nevertheless, since it is not feasible to empirically verify all bacterial and archaeal strains, it is impossible to prove if the "universal" PCR primer sets really include the whole bacterial complexity. For the NGS sequencing several different primers have been designed and recently a primer set for the simultaneous analysis of bacteria and archaea with a good species recovery has been described (Takahashi *et al.,* 2014). The limitation linked to the "universal" 16S rRNA primer sets is that they are designed by exploiting synthetic microbial communities. As a consequence final results are strongly influenced by the taxa that are chosen to conduct the experiments. Furthermore, the use of different bio-molecular techniques and the amplification of diverse sub-regions of 16S rRNA gene may result in a dissimilar structure of the microbial community. The main objective for the near future will be to design specific primer sets that allow the maximum recovery of all the bacterial and archaeal species present in different environments. This goal may be of paramount importance considering the specific contest of the baby gut microbiota.

1.11 References

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1.12 PhD research steps: some considerations

The general introduction was the brief description of the state of the art. My research project was aimed to ecologically describe the hydrogenotrophic populations in infants until the second year of life. The investigation was targeted on some bacteria and archaea involved in the hydrogen metabolism, namely Sulphate Reducing Bacteria (SRB), acetogens and methanogenic archaea. Research was performed on fecal samples of healthy babies with the intent of detecting some differences in the colonization pattern related to the delivery and feeding mode. The doctoral period was initially characterized by testing different DNA extraction kits in order to obtain both quantitatively and qualitatively adequate amount of DNA from fecal samples. Having chosen the appropriate extraction protocol, the second step was mainly devoted to testing different qPCR primer couples available from the literature. This phase was stressful and time consuming due to the fact that those molecular tools seemed to be inappropriate for the quantification of hydrogenotrophs in infant specimens. The major difficulties occured with the quantification and description of methanogenic archaea and SRB due to their low levels. Acetogens were detected as predominant among H_2-consuming microorganisms and it has been possible to describe the *Blautia* genus and the *Lachnospiraceae* family as prevalent within this group. Another notable finding was the recovery of *R. gnavus* in the 88% of babies analyzed, not correlating with delivery mode and feeding conditions. During the last period of the PhD I focused my attention on the archaeal populations in piglets fed pectin-containing milk or a control diet. The ambition of this study was to provide some new information on the physiological significance of some fibers used in human nutrition. The phases of my research lead to results that will be explained in detail within each chapter.