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"Metagenomic trajectory of gut microbiome in the human lifespan"

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“Non aspettare di finire l'università,
di innamorarti,
di trovare lavoro,
di sposarti,
di avere figli,
di vederli sistemati,
di perdere quei dieci chili,
che arrivi il venerdì sera o la domenica mattina,
la primavera,
l'estate,
l'autunno o l'inverno.
Non c'è momento migliore di questo per essere felice.
La felicità è un percorso, non una destinazione. Lavora come se non avessi bisogno di denaro,
ama come se non ti avessero mai ferito e balla, come se non ti vedesse nessuno.
Ricordati che la pelle avvizzisce,
i capelli diventano bianchi e i giorni diventano anni.
Ma l'importante non cambia: la tua forza e la tua convinzione non hanno età.
Il tuo spirito è il piumino che tira via qualsiasi ragnatela.
Dietro ogni traguardo c'è una nuova partenza. Dietro ogni risultato c'è un'altra sfida.
Finché sei vivo, sentiti vivo.
Vai avanti, anche quando tutti si aspettano che lasci perdere.”

“Don't wait to finish university,
to fall in love,
to find a job,
to marry,
to have children,
to see them grow up,
to lose that ten kilos,
to wait for Friday night or Sunday morning,
for it to be spring,
summer,
autumn or winter.
There isn't a better moment to be happy than this,
happiness is the way, not the destination. Work as if you don't need money,
love as if you never have been hurt, and dance as if nobody can see you.
Remember that the skin wrinkles,
hairs become white and the days become years,
but the important things don't change, and your power, your belief, doesn't age.
Your spirit is the feather duster that sweeps away every web.
Behind every finishing line there is a new start. Behind every result there is a new challenge.
Until you'll live, feel alive. Go on, even when everybody expects you to give up.”

- Madre Teresa di Calcutta – Mother Teresa of Calcutta -

ABSTRACT

Co-evolving with the human host, gut microbiota establishes configurations, which vary under the pressure of inflammation, disease, ageing, diet and lifestyle.

In order to describe the multi-stability of the microbiome-host relationship, we studied specific tracts of the bacterial trajectory during the human lifespan and we characterized peculiar deviations from the hypothetical development, caused by disease, using molecular techniques, such as phylogenetic microarray and next-generation sequencing.

Firstly, we characterized the enterocyte-associated microbiota in breast-fed infants and adults, describing remarkable differences between the two groups of subjects. Successively, we investigated the impact of atopy on the development of the microbiome in Italian childrens, highlithing conspicuous deviations from the child-type microbiota of the Italian controls. To explore variation in the gut microbiota depending on geographical origins, which reflect different lifestyles, we compared the phylogenetic diversity of the intestinal microbiota of the Hadza hunter-gatherers of Tanzania and Italian adults. Additionally, we characterized the aged-type microbiome, describing the changes occurred in the metabolic potential of the gut microbiota of centenarians with respect to younger individuals, as a part of the pathophysiolology of the ageing process. Finally, we evaluated the impact of a probiotics intervention on the intestinal microbiota of elderly people, showing the repair of some age-related dysbioses. These studies contribute to elucidate several aspects of the intestinal microbiome development

during the human lifespan, depicting the microbiota as an extremely plastic entity, capable of being reconfigured in response to different environmental factors and/or stressors of endogenous origin.

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Characteristics of the human intestinal microbiota: a brief overview

Human gut is an ecological niche densely populate by microorganisms (Eckburg et al., 2005; Turnbaugh et al., 2007; Costello et al., 2009). Collectively our body is composed by 10^{13} eukaryotic cells and 10^{14} bacteria, most of them in the gastrointestinal tract (GIT), that means we are more microbes than mammal cells (Turnbaugh et al., 2007; Costello et al., 2009). Our intestinal microbial counterpart, the microbiota, is essential for many features that we have not evolved by ourselves, such as: the barrier effect against pathogens and the enhancement of the digestive efficiency. It has a crucial role in the development, education and function of our immune system and it is involved in the development and regulation of central nervous and endocrine systems (Gill et al., 2006; O'Hara et al., 2006b; Neish, 2009; Lee et al., 2010; Garrett et al., 2010; Hooper et al., 2010). Moreover, the interaction between intestinal microbiota and human host is dynamic and pivotal for the ultrastructural development of the GIT (Round et al., 2009). The dynamism of the gut microbiota consists in a predisposition to adaptation, which guarantees rapid adjustments of the metabolic potential of the microbial community in response to diet, and, simultaneously, it represents an essential component for the education of the immune system to homeostasis (Candela et al., 2012b).

Human gut microbiota: who are the actors?

Gut microbiota shows an astonishing taxonomic diversity at species level. More than 1000 different bacterial species have been identified in the human GIT (Turnbaugh et al., 2007; Garrett et al., 2010), even if, at the higher phylogenetic level, the intestinal microbiota shows a relatively low biodiversity, with only 6 of 90-100 bacterial phyla present in the human GIT (Peterson et al., 2008). In particular, two of these phyla, Firmicutes and Bacteroidetes, arrange more than 90% of the gut bacterial community. Other phyla normally well-conserved at a lower relative abundance are Actinobacteria and Proteobacteria, whereas Verrucomicrobia and Fusobacteria are generally present only in some individuals (Costello et al., 2009; Muegge et al., 2011) (**Figure 1**).

Focussing at lower taxonomic levels, each healthy human possesses a peculiar subset of hundreds of species, even if only 5-20 of them are effectively present with a relative abundance more than 1% (Qin et al., 2010). According to Turnbaugh et al. (2009a), no phylotype (groups with 97-99% of sequence identity) of the individual microbiota is present at more than 0.5% and the 70% of the phylotypes are subject-specific.

The human gut microbiome: a treasure of genes

The collective genome of the intestinal microbiota, the microbiome, has been estimated to contain more than 1 milion of genes, exceeding 150 times the number of genes of the human genome. The funtional assignment of the microbiome revealed that it is generally enriched for clusters of orthologous groups (COG) and Kyoto Encyclopedia of Genes (KEGG) categories involved in carbohydrate metabolism, short chain fatty acid production, energy process and amino acid

metabolism (Turnbaugh et al., 2009a; Gill et al., 2006; Rampelli et al., 2013b). Turnbaugh et al. (2009a) showed that the percentage of sequences assigned to carbohydrate-active enzymes (CAZymes) is greater than all the KEGG pathways. This enormous glycolityc potential confers to the gut microbiota, the ability to degrade several complex glycans that cannot be metabolized by the human host, ranging from the glycans contained in plants, to the glycans of the human mucus: a layout which complements the limited saccharolytic potential of the human genome (Koropatkin et al., 2012).

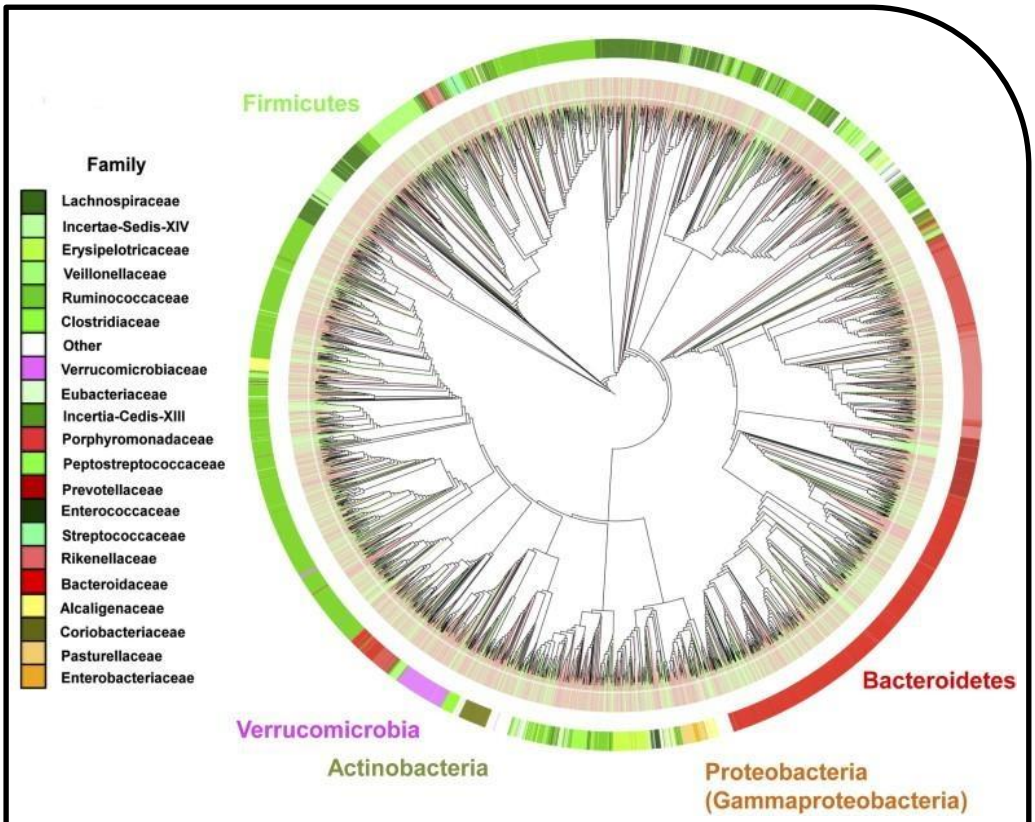

Figure 1. Phylogenetic tree and donut chart represent the bacterial groups most frequently detected in human faeces, colored by family (shade of color) and phylum (color) level. This figure is reported by Saulnier and colleagues (2011).

However, up today, metagenome means above all challenge, because more than 40% of the genes has not been assigned a function. Even if it is commonly accepted and easier to focus the studies on the shared core metagenome encoding for assigned genes involved in key metabolic functions, it will be mandatory to explore the unassigned gene fraction, including the contribution to the host genetic diversity and the impact on the human physiology, that are still unknown.

Plasticity of gut microbiota in response to diet, climate changes, geography and diseases

Through the static comparison of gut microbiota among subjects with different health status, some pioneering studies showed a core microbiome, defined as a constant and shared fraction of genes, fundamental for supporting the mutualism whit the host (Turnbaugh et al., 2009b). However, longitudinal studies underlined the dynamism of each individual microbiota (Kau et al., 2011; Maslowski et al., 2011; McNulty et al., 2011), opening perspectives about the significance of the degree of plasticity for human health and homeostasis. The most direct example of plasticity of the intestinal microbiota is provided by its ability to quickly respond to dietary changes (Walker et al., 2011; Wu et al., 2011a; Muegge et al., 2011). Evidences showed that the microbial community of the gut is able to both compositionally and functionally adapt itself to modifications in the diet in a few days (Kau et al., 2011; Maslowski et al., 2011; Walker et al., 2011). In a first dynamic work, the gut microbiota of lean individuals under caloric restriction was studied for 4 days (Muegge et al., 2011). Recording what the involved subjects ate daily, correlations of the adaptation of the microbiota to macro- and micronutrient consumption were figured out. In particular, a significant association between the

taxonomic and functional profile of gut microbiota and fiber and protein consumption was observed. A second molecular study in 14 overweight men revealed that the individual microbial community modified its taxonomic composition in response to the most important type of ingested fermentable carbohydrates. It is important to note that the adaptations consisted of a subject-specific diet-dependent fluctuation in the microbiota phylotypes (Walker et al., 2011). In a third interesting publication, short- and long-term dietary responses of the human gut microbiota were distinguished (Wu et al., 2011a). The comparison between the two dietary changes revealed that our intestinal bacterial community is composed of taxa influenced by short-term dietary changes and other bacterial groups affected only by long-term dietary habits. Taken together, all these findings support a dinamic view of the microbiota, in which our intestinal bacterial community changes in response to short- and long-term dietary habits (Thomas et al., 2011).

Another interesting point is the surprising degree of plasticity that microbiota shows in response to climate changes and geography. In a recent longitudinal work, the faecal microbiota of 15 healthy Finnish people, overseas travelers, was studied for 7 weeks. Interestingly, the results showed a clear loss in similarity between samples from the same subject (Jalanka-Tuovinen et al., 2011). Modifications in the diet more than the exposure to different environmental microbes, climate and stress, can weigh on this travel-associated plasticity of the gut microbiota. On the other hand, the impact of geographic origin on the intestinal microbiota was illustrated in a first comparative analysis among the gut microbiota of Korea, China, Japan and USA people (Nam et al., 2011). Data analysis showed that gut microbiota structures clustered for geographic origin. Significant country-related differences in the faecal microbiota were also found in a milestone comparative study between children from

Europe and Burkina Faso (De Filippo et al., 2010). In particular, children of Burkina Faso were characterized by an enrichment in Bacteroidetes and Actinobacteria, and a decrase in Firmicutes and Proteobacteria in comparison to European ones. However, in a very recent work based on a meta-analysis between public datasets, a strong clustering of the gut microbiota by study was evidenced, suggesting that technical differences between laboratories may cause significant differences in the observed diversity. Despite this, cultural habits of geographic provenience and age produced sufficient changes in the gut microbiota structure to drive the global clustering even when combining studies that used different protocols (Lozupone et al., 2013). Finally, a recent study concerning the characterization of the microbiome in urban and rural Russian population, showed distinctive sociocultural features of the gut bacterial communities across regional cohorts (Tyakht et al., 2013).

The phylogenetic and functional structure of gut microbiome is led through a strong selection of changeable individual microbiome profiles. This individual specificity is the result of the high degree of plasticity of our microbial ecosystem, in response to environmental/endogenous factors, and the peculiarity of our physiology, lifestyle and history (Garrett et al., 2010; Sansonetti et al., 2010). Considering the individual variation at 40% of the total community, the gut microbiota represents a complex dynamic system with the potential for multistability (Candela et al., 2012). In a mutualistic context, this means that the host-microbiota homeostasis could switch to different configurations, each one characterized by a high degree of biodiversity. However, some environmental stressors, such as infection, and some factors of endogenous origin, such as inflammation or ageing, can force the microbiota to shift towards a disease-associated configuration at a lower biodiversity level, compromising the mutualism and consolidating the disease (**Figure 2**).

The progressive loss of important gut microbial groups in people with a Westernized lifestyle (Kau et al., 2011), and the unbalanced microbiota of obese people (Turnbaugh et al., 2009a), provide two evident examples of deviation from the microbiota-host mutualism triggered by environmental stressors.

In the last years, many studies associated diet-dependent modifications of the intestinal microbial community with the etiology and severity of obesity (Turnbaugh et al., 2009a; Turnbaugh et al., 2009b; Diamant et al., 2011; Greiner et al., 2011). Indeed, high fat dietary habits can have a dramatic impact on the intestinal microbiota. Studies in obese patients were concordant in showing a loss of taxonomic biodiversity in the gut microbial community and heterogeneous fluctuations of taxon abundance, suggesting a wide complexity in the link between microbiota and obesity (Duncan et al., 2008; Turnbaugh et al., 2009a; Greenblum et al., 2012). In addition, the functional annotation of the obese microbiome showed a general loss even in functional biodiversity with increments in the genes involved in carbohydate, lipid and amino acid metabolism with respect to the lean-type microbiota (Turnbaugh et al., 2009a). As confirmed in recent studies in germ-free (GF) mice (Flint, 2011; Ridaura et al., 2013), the abnormal dietary habits of obese people could lead the microbiota from a healthy configuration to an obesogenic one, which supports obesity and associated comorbidities (Diamant et al., 2011; Greiner et al., 2011).

According to the “hygiene hypothesis”, the excessive sanitization typical of the Westernized lifestyle compromises the mutualistic relationship between humans and their gut bacterial community. A series of habits of our society, such as antibiotic usage, clean water and sterile food consumption and bathing, provided enormous benefits for human beings, but also favored a remarkable loss of key bacterial

groups in the human gut microbiota (Rook et al., 2005). This could result in a lack of immunological crosstalk with crucial components of the bacterial community, leading to an inappropriate activation of the immune system during infancy (Rook et al., 2005; Ehlers et al., 2010). As a consequence, in recent years we have witnessed an increase in Westernized countries of chronic inflammatory disease, such as allergy, autoimmune disorders, type 2 diabetes and inflammatory bowel diseases (Noverr et al., 2004; Rawls, 2007; Jia et al., 2008; Neish, 2009; Ehlers et al., 2010; Kau et al., 2011; Maslowski et al., 2011).

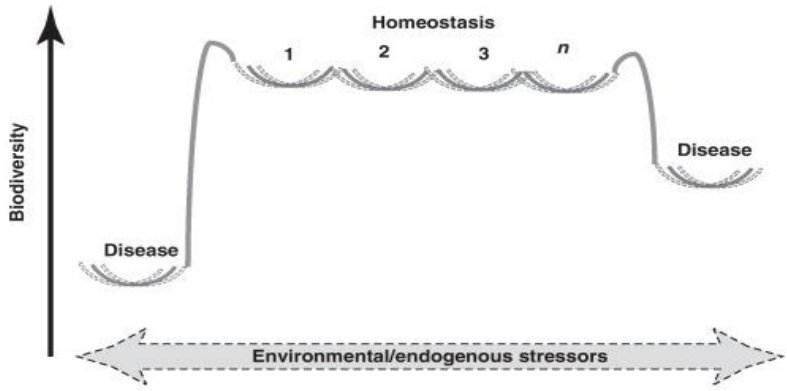

Figure 2. Dinamics of the microbiota-host mutualism: different healthy configurations are represented at a high level of biodiversity, showing the variability between and within individuals. Under some conditions, the microbiota could shift from a mutualistic profile that supports homeostasis, to a new configuration associated to the disease, characterized by a lower taxonomic and functional biodiversity. This figure is reported by Candela and colleagues (2012).

Trajectory of the intestinal microbial biodiversity: the ageing effect

It has been demonstrated in GF mice that the crucial relationship between our body and our intestinal bacterial counterpart starts during the perinatal period, affecting the synaptogenesis and modulating brain development and function (Heijtz et al., 2011). At the delivery the infant gut is colonized by bacteria of maternal origin (Dominguez-Bello et al., 2010), and during the very early infancy it is continuously inoculated by microorganisms present in the environments that the infant encounters, such as the mother's skin, the infant food, the environmental food and the faecal microbiota. Other factors of remarkable importance for the development of the infant microbiota are the living condition, the child rearing practices (Kau et al., 2011; Dominguez-Bello et al., 2011b) and the mother's milk, which favors the *Bifidobacterium*-dominated architecture of the infant-type intestinal microbial community (Sela et al., 2010). In this heterogeneous scenario, the interplay with the intestinal microbiota is pivotal for the structural development of the intestinal mucosa and maturation of the immune system (Palmer et al., 2007; Koenig et al., 2011).

The gut microbial community has notably fluctuation under stochastic bacterial exposure during infancy, but at weaning, with the introduction of a solid diet, the intestinal microbiota becomes more stable and develops an adult-like taxonomic

architecture, with a remarkable increase of phylogenetic and functional diversity (Palmer et al., 2007; Dominguez-Bello et al., 2011a).

The crosstalk with the microbes that populate our intestine remains stable during the adulthood, until a hypothetical age-related threshold, characteristic of each individual, depending on genetics, lifestyle and diet (Biagi et al., 2010; Claesson et al., 2012; Biagi et al., 2013). Ageing and its pathophysiological conditions affect the microbiota-host mutualism, inducing a reduction in the biodiversity of the microbial community with respect to younger people (Mueller et al., 2006; Biagi et al., 2012; Sim et al., 2012). What has also been reported in the elderly is a large inter-individual variability and a compromised stability of the gut microbiota, possibly led by the onset of pathophysiological ageing-related processes, i.e. the reduced intestinal motility and the deterioration of the immune system functionality (Ostan et al., 2008; Guigoz et al., 2008; Shanley et al., 2009).

In the matter of how the microbiota changes under the effect of ageing, many recent studies reported that differences in the nationality of subjects affected the impact of ageing process on the intestinal microbiota (Mueller et al., 2006; Biagi et al., 2011; Lozupone et al., 2013). These country-related peculiarities are strictly connected to dissimilarity in lifestyle and dietary habits between individuals of different provenience (Lozupone et al., 2013). In particular, the effect of age on the most represented bacterial phyla of the gut microbiota, Firmicutes and Bacteroidetes, widely varies according to nationality and age of enrolled subjects (Biagi et al., 2012). A group of bacteria belonging to Firmicutes, including genera *Eubacterium, Clostridium, Ruminococcus, Dorea, Roseburia, Lachnospira and Butyrovibrio*, were found to decrease in Japanese, Finnish and Italian elderly and centenarians (Hayashi et al., 2003; Mueller et al., 2006; Biagi et al., 2010;

Makivuokko et al., 2010), whereas an opposite trend was observed in German old adults (Mueller et al., 2006). The genus *Faecalibacterium* markedly decreased in Italian elderly and centenarians (Mueller et al., 2006; Biagi et al., 2010), but this evidence was not confirmed in other European people (Mueller et al., 2006; Claesson et al., 2011). On the other hand, an age-related increase in Bacteroidetes was found in Austrian, Finnish, German and Irish elderly (Bartosch et al., 2004; van Tongeren et al., 2005; Tihonen et al., 2008; Zwielehner et al., 2009), but not confirmed in Italian elderly and centenarians (Mueller et al., 2006; Biagi et al., 2010). Other minor components of the gut microbiota followed different trends upon ageing: Actinobacteria, i.e. the phylum that includes the *Bifidobacterium* genus, showed a notable age-related temporal instability, whereas it has been usually reported an age-related increase in facultative anaerobes, including streptococci, staphylococci, enterococci, and enterobacteria (Mueller et al., 2006; Rajilic-Stojanovic et al., 2009; Mariat et al., 2009; Biagi et al., 2010; Makivuokko et al., 2010), also known as pathobionts, i.e. bacteria present in the healthy gut microbiota in low concentration but able to thrive in inflamed conditions, nurturing the inflammation process. The increase in pro-inflammatory pathobionts and the concomitant decrease of immunomodulatory bacteria belonging to Firmicutes are hypothesized to be involved in the pro-inflammatory loop that promotes and sustains the inflammatory disorders, determining a disturbance in the host-bacteria equilibrium. Similarly, the age-related changes in the intestinal microbiota composition take part of the complex ageing process, sustaining and nurturing the overall inflammation typical of the elderly people (Biagi et al., 2013).

Finally, another important factor that influences the impact of ageing on the microbiota-host relationship is diet. In a recent publication different modulations of

the gut microbiota by dietary habits have been highlighted within elderly people of the same ethnogeographic region (Claesson et al., 2012). The results of the study argue in favor of a dietary/probiotics/prebiotics intervention to promote healthy ageing. In **Figure 3** is described the trajectory of microbial biodiversity across the human lifespan.

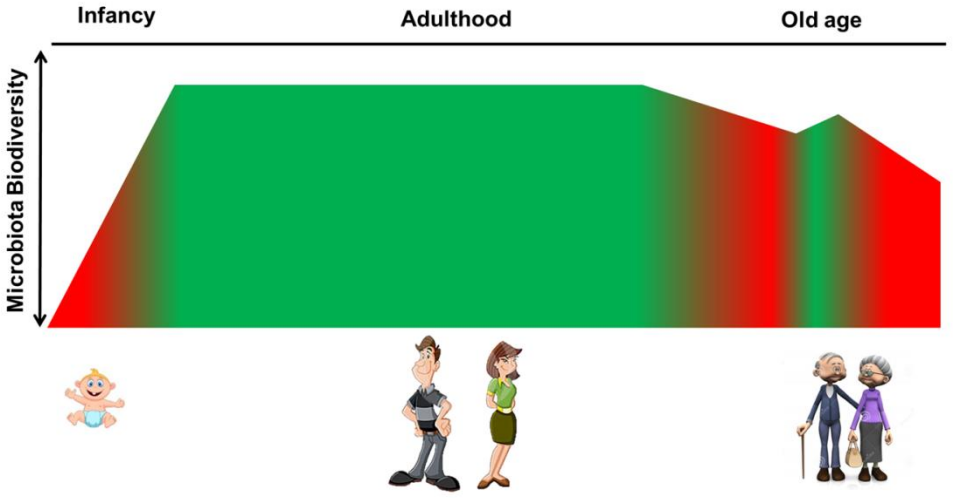

Figure 3. Every time a human baby is born, a rich and dynamic microbial ecosystem develops from a sterile environment. During the first year of life, until weaning, the gut ecosystem is prevalently colonized by opportunistic microorganisms. After weaning, the developmental changes in the gut mucosa and in the intestinal immune system, together with the introduction of a solid diet, drive to the transition to a resilient adult-like profile of the human gut microbiota, characterised by a remarkable microbial biodiversity. The ageing of the gut microbiota starts after a subject-specific 'threshold age' which depends on individual characteristics such as diet, country and, eventually, frailty. In any case, changes of diet, lifestyle and the immunosenescence of the intestinal immune system dramatically impact the microbial ecology of the human GIT. Symmetrically to what happens in the early stage of our life, the aged-type microbiota shows a low microbial biodiversity, and it is characterised by an increase in opportunistic environmental facultative aerobes. This figure has been recently presented by O'Toole PW in 2013 EFFoST Annual Meeting.

Gut microbiota and immune system during the human lifespan

The symbiotic relationship with our intestinal microbiota implies to keep the microbial community under surveillance, avoiding an excessive bacterial load on the intestinal mucosal surface (Weng et al., 2013). At the delivery, the human host meets for the first time microorganisms. With colonizing microbiota, the gut has evolved an elaborate barrier system to defend against the dissemination of microbes into sub-epithelial intestinal tissues. This barrier is composed by a surface mucus and membrane (**Figure 4**) (Weng et al., 2013). The surface mucus is secreted by the intestinal cells and composed by a layer of viscous fluid rich in mucins, non-specific antimicrobial peptides (AMPs) and secretory immunoglobulin A (sIgA). Another component of the barrier is the membrane continuity formed by the enterocytes that are attached by tight junctions with dendritic cell (DC) appendages extruding between the epithelial cells (**Figure 4**) (Weng et al., 2013). The relationship among bacterial inhabitants, intestinal barrier and gut-associated immune system ensures the generation and preservation of a stable equilibrium between the intestine and colonizing bacteria.

In the first months of human life, immunologic components of the intestinal mucosa, including M cells, the Peyer's patches rich in lymphoid elements, interstitial and intraepithelial lymphocytes, require the stimulation from colonizing bacteria (Walker et al., 2009). The aim of the story is that the human immune system has to train itself to select the correct immune responses: protective immunity against pathogens and immune tolerance to non-pathogens.

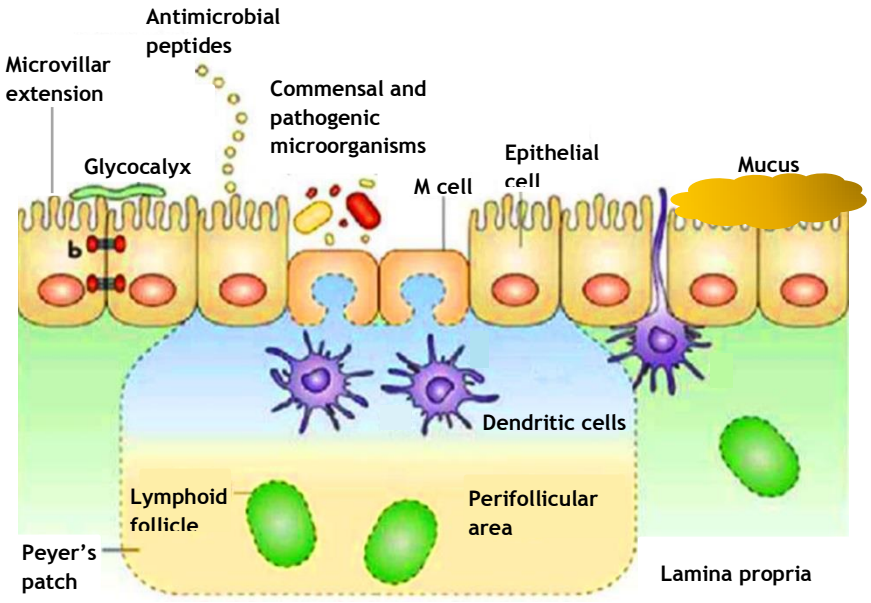

Figure 4. Human intestinal barrier (mucus layer, epithelial cells and dendritic cells) works in concert with components of the submucosal gut-associated lymphoid tissue for maintaining homeostasis. This figure is reported by Chichlowski et al. (2012) and by Weng et al. (2013).

In a healthy adult gastrointestinal ecosystem, the gut-associated lymphoid tissue (GALT) keeps microbiota under control in a low grade inflammatory status. Enterocytes actively sample microbial metabolites and bacterial quorum sensing molecules using apical channels, such as hPepT1 or OCTN2. The pattern recognition receptors (PRR) mediate sensing of microorganisms, recognizing bacterial macromolecular ligands, defined as microbial-associated molecular patterns (MAMP) (Biagi et al., 2013). However, both commensal and pathogens have MAMPs, raising the question about why pathogenic bacteria cause gut inflammation and commensal bacteria do not. Recent works propose that commensal bacteria inhibit the NF-kB pathway. In particular some *in vitro* studies showed that *Lactobacillus* spp.,

Bacteroides spp., *Escherichia coli* and the attenuated pathogenic strains of *Salmonella* spp. inhibit polyubiquitylation and the subsequent degradation of IkBa required for efficient nuclear traslocation of NF-kB and activation of the signaling pathway (Neish et al., 2000; Collier-Hyams et al., 2005; Tien et al., 2006). Specifically, in the intestinal lumen, commensal gut microbiota activates DCs via toll-like receptor (TLR)2/TLR4 signaling pathways. In the presence of tolerance signals, DCs lead the differentiation of specialized regulatory $CD4^+$ T-cells (Treg/T_H3, Tr1). Anti-inflammatory Treg cells promote tolerance via release of interleukine-10 (IL-10) to stimulate transforming growth factor beta (TGF-ß) release and thereby suppress immunoglobulin E (IgE) production (Biagi et al., 2013; Weng et al., 2013). Simultaneously, B cells drive their differentiation to IgA secreting plasma cells. The epithelial surface mucus coats with bacterial strain-specific IgA, prevents adherence of microorganisms and neutralizes dangerous toxins or enzymes (Biagi et al., 2013). On the other hand, the response of GALT to a pathogen results in the differentiation of effector T_H1, T_H2 and T_H17 cells, that induce a strong pro-inflammatory response.
Changes in the immune system responses occur during the advancement of age, in particular GALT reduces the capacity to synthesize bacterial strain-specific sIgA and the innate immune system decreases the efficiency of the defenses, in terms of α-defensins, antimicrobial peptides and mucus secretion. These immunological modifications take part of an overall process named immunosenescence, which affects the homeostatic equilibrium between microbiota and host immune system (Biagi et al., 2013). Bacteria can overgrow on the epithelial surface, engaging a high inflammatory response through the NF-kB-dependent biosynthesis of antimicrobial effectors and inflammatory cytokines and chemokines, such as tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ), IL-6, IL-8 and IL-23. In this pro-inflammatory

context, DCs drive the differentiation of effector T_H1, T_H2 and T_H17 cells that perpetuate the strong inflammatory response toward invading microbes, consolidating inflammation in the GIT (Biagi et al., 2013; Weng et al., 2013). Indeed, immunosenescence is also accompanied by an overall chronic low degree of inflammation named inflamm-ageing (Franceschi et al., 2007; Larbi et al., 2008). Inflammation favors the increase of pathobionts, creating a self-sustained pro-inflammatory loop that impacts on the entire microbial ecology of the GIT (Round et al., 2009; Winter et al., 2010). Moreover, a lower capacity to produce butyrate and other short chain fatty acids (SCFA) was observed in the gut microbiome of elderly people (Biagi et al., 2010). These are fundamental metabolites for maintaining the gastro-intestinal epithelial integrity, produced by bacteria, such as *Faecalibacterium*, *Roseburia*, *Blautia* and *Ruminococcus* (Biagi et al., 2010; Macia et al. 2012). Therefore, the decrease of these bacteria may contribute to further nurture the inflamm-ageing process in the intestine of aged-people (Biagi et al., 2013).

CHAPTER 1 – INTRODUCTION

1. *The human gut microbiome*
2. *Trajectory of the human microbiome during the lifespan*
3. ***From culturable techniques to Next-generation Sequencing***

Methods for studying gut microbiome before next-generation sequencing

Traditional culture-dependent approaches are based on selective culturing, morphological, biochemical, and physiological assays. Regarding the characterization of the gut microbiota, these techniques allow the recovering of less than 30% of the total bacterial richness (Bik et al., 2006). The majority of the biodiversity of the human microbiota remains uncultured, and needs to be characterized by molecular techniques. The principal molecular marker for genetic diversity of bacteria is the 16S rRNA gene, consisting of about 1,500 nucleotides and containing 9 regions conserved and 9 discriminating regions (V1 to V9), which are highly variable among bacterial phylotypes. Comparative sequence analysis of PCR amplicons obtained from the variable regions against assigned databases, such as the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/) allows the phylotype identification and quantification.

Otherwise, different 16S rRNA gene-based approaches are usually used, for example the PCR Denaturing Gradient Gel Electrophoresis (PCR-DGGE), a technique which allows the fingerprint of the most abundant bacterial components. Other alternative approaches are the quantitative PCR (qPCR) and the Fluorescence In Situ Hybridization (FISH), that can quantify one or few bacterial groups targeted by

specific primer sets or probes. Furthermore, several phylogenetic microarrays have been designed, such as the HTF-Microbi.Array platform (**Box 1**) (Candela et al., 2010), allowing the simultaneous quantification of many taxonomic microbial groups of the targeted ecosystem, even if this kind of approach lacks the possibility to discover unknown members.

The next-generation sequencing for characterizing the human intestinal microbiome

The automated Sanger sequencing is considered as the first step of a newer technology, that led a big revolution in study approach and data analysis: the next-generation sequencing (NGS). These newer methods are strategies that rely on a combination of template preparation, sequencing and imaging, and genome alignment and bioinformatics analysis. The major advance of NGS is the ability to produce a huge volume of data cheaply (in some cases more than billions of short reads per run). This ability has expanded the range of experiments performed with NGS, replacing the other competitor techniques. For example, phylogenetic microarray for microbial characterization has been replaced by 16S rDNA sequencing approach (Claesson et al., 2011; Claesson et al., 2012; Tyakht et al., 2013), gene-expression studies based on microarray platforms are now performed by seq-based methods, which have the advance to identify and quantify rare alternative splicing and sequence variation in genes without prior knowledge (Wold et al., 2008; Wang et al., 2009). In addition, NGS allows the sequencing of the whole genome of many organisms, and also the metagenome of many particular ecological niches, including the human gut microbiome.

BOX 1 - HTF-MICROBI.ARRAY PLATFORM

We developed and validated a DNA-microarray (HTF-Microbi.Array) for the high taxonomic level fingerprint of the human intestinal microbiota. Based on the Ligase Detection Reaction-Universal Array (LDR-UA) approach, the HTF-Microbi.Array enables specific detection and approximate relative quantification of 16S rRNAs from 31 phylogenetically related groups of the human intestinal microbiota. The members of the intestinal microbiota detected belong to *Bacteroides/Prevotella*, *Clostridium* clusters IV, IX, XIVa, XI, I and II, *Bifidobacteriaceae*, *Lactobacillaceae*, *Bacillus*, *Enterococcus*, *Enterobacteriaceae*, *Akkermansia*, *Streptococcaceae* and *Campylobacter*, demonstrating the specificity of all the probe pairs.

The sensitivity of the HTF-Microbi.Array was demonstrated in a pool of publications (Candela et al., 2010; Candela et al., 2012; Maccaferri et al., 2012; Rampelli et al., 2013a; Centanni et al., 2013b), and recently validated by comparison with 16S rDNA sequencing (Centanni et al., 2013b).

In conclusion, the HTF-Microbi.Array is a fast and sensitive tool for the high taxonomic level fingerprint of the human intestinal microbiota in terms of presence/absence of the principal groups. Moreover, analysis of the relative fluorescence intensity for each probe pair of our LDR-UA platform can provide estimation of the relative abundance of the target microbial groups within each sample.

The existing sequencing approaches, such as Illumina and 454, differ for template preparation and sequencing chemistry (**Figure 5**), however the outputs consist in reads that could be analyzed with similar bioinformatics and biostatistics methods.

Bioinformatics strategies for analyzing NGS data

In the last years NGS technologies (Shendure et al., 2008), such as 454, Illumina, SOLiD and Heli-Scope, significantly promoted the development of metagenomics by offering low-cost and ultra-highthroughput sequencing. In this new scenario, whole-genome shotgun sequencing (Metzker, 2010), is considered the best sequencing approach for an in-depth characterization of metagenomes. On the other hand, amplicon sequencing could supply information concerning community structure or metatranscriptomics. Regarding 16S rRNA gene sequencing, the QIIME pipeline allows a robust procedure, that foresees the characterization of the microbial composition by choosing operational taxonomic units (OTUs), sequence alignment, inferring phylogenetic trees and phylogenetic and taxon-based analysis of diversity within and between samples (Caporaso et al., 2010). Conversely, the world of metatrascriptomics is an expanding world, that represents even now a challenge for problems regarding the lack of a proven pipeline for sample preparation and analysis.

Metagenomics, in particular whole-genome shotgun sequencing, usually produces a huge amount of sequence data, which create tremendous challenges in data analysis. These kind of problems could be resolved by using of servers with big calculation power (Wu et al., 2011b).

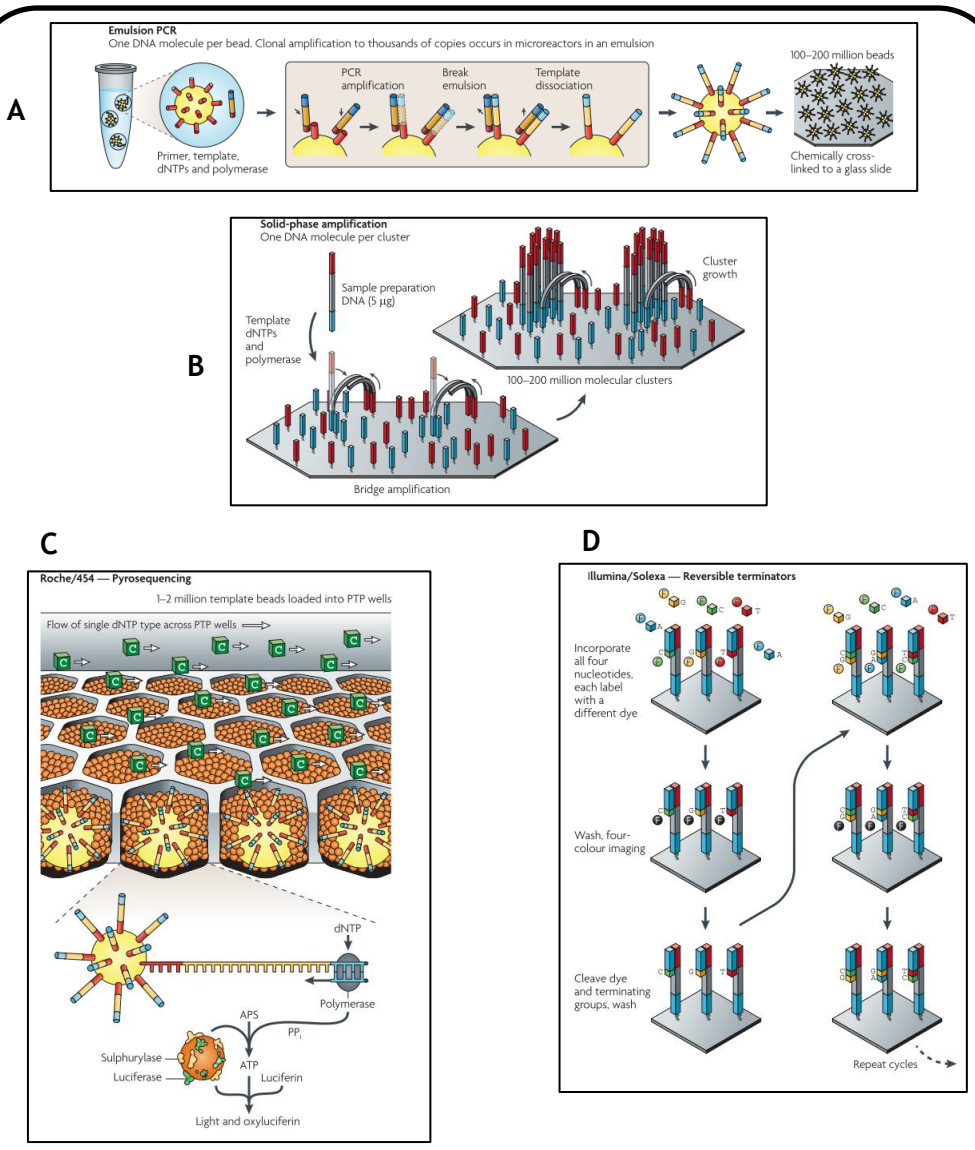

Figure 5. Template immobilization strategies in emulsion PCR (emPCR) (**A**) for 454 technology, a reaction mixture containing bead-DNA complexes is created for performing emPCR. The resulting beads, containing several thousand copies of the same template sequence can be deposited into PicoTiterPlate wells. Solid-phase amplification (**B**) for Illumina procedure, is performed in two steps: initial priming and extension of the single-stranded, and bridge amplification of the immobilized template with adjacent primers to form clusters. Pyrosequencing using Roche/454 platform (**C**), foresees the reaction between template-coated beads and additional beads coupled with sulphurylase and luciferase. Four-colour and one-colour cyclic reversible termination methods (**D**) used in Illumina technology. These figures are reported by Metzker (2010).

Other challenges derive from the high complexity of metagenomic sequence data and concretely are: (a) samples may contain hundreds or thousands of species at dramatically different abundance levels; (b) many species are unknown; (c) next-generation sequencers produce short reads with high error rate; and (d) sequence data contain experimental biases, artifacts and contaminations (Niu et al., 2011). To address these problems, many methods have been developed, such as taxonomy binning (Krause et al., 2008), use of simulated datasets (Mavromatis et al., 2007), diversity analysis (Schloss et al., 2008), ORF calling (Noguchi et al., 2006), rRNA prediction (Huang et al., 2009), sequence clustering (Huang et al., 2010), assembly (Li et al., 2002), statistical comparison (Li et al., 2010), fragment recruitment (Niu et al., 2011) and so on. The present panorama is really full of different approaches, all with several good aspects, but also many defects. For these reasons there is no a right strategy but it is quite better to perform several different analyses, which foresee the direct use of the reads and also an assembly approach (Davenport et al., 2013). The unassembled reads may be searched for the so-called environmental gene tags, i.e. gene fragments that encode conserved Pfam domains or protein family motifs (Qu et al., 2008). By direct using of shotgun reads as input data it is also possible to run MethaPhlAn (Metagenomic Phylogenetic Analysis). This tool estimates the relative abundance of microbial cells by mapping reads against a reduced set of clade-specific marker sequences (Segata et al., 2012). Genes could be identified by mapping the primary sequence reads through various distinct algorithms onto nucleotide or amino acid reference sequences deposited in the databases. This kind of approach is used by a lot of metagenome web analysis servers like MG-RAST (MetaGenomic RAST server), IMG/M (Integrated, Microbial Genome and Metagenome) and WebMGA (Web server for MetaGenomic Analysis),

(Meyer et al., 2008; Markowitz et al., 2011; Wu et al., 2011b), but also some softwares, such as MetaCV (Liu et al., 2013), utilize the same method. As a complementary approach to read mapping, short metagenome reads can also be assembled into longer contiguous sequences (contigs) using a modern short read optimized sequence assembler. Meta-Velvet (Namiki et al., 2012) is an assembler directly developed for short read data-set containing metagenome sequences and it has been used successfully in several studies. After assembly, the general procedure foresees the use of ORF prediction algorithms, which contain variants specifically appropriate for metagenome data (Hyatt et al., 2011). The advantages over read mapping include the ability to find truly novel DNA contigs encoding for example undescribed genes, and also to strictly identify genes of interest. However, this comes at the expense of the loss of the quantitative aspect of read data, as the number of reads used to build each contig is highly variable. An explicative work-flow of the processes was shown in **Figure 6**.

It is matter of fact that a metagenome approach for the characterization of the gut microbiota retrieves more information on microbial community than 16S rRNA sequencing, because it provides a view of microbiota structure in terms of species richness and distribution, as well as the functional (metabolic) potential of the community metagenome (Hugenholtz et al., 2008). However, a new bioinformatics software package, named PICRUSt, is now available, which allows the prediction of the metagenome functional content from marker gene (e.g. 16S rRNA) surveys and full genomes (Langille et al., 2013), bridging just a bit the gap between the two approaches.

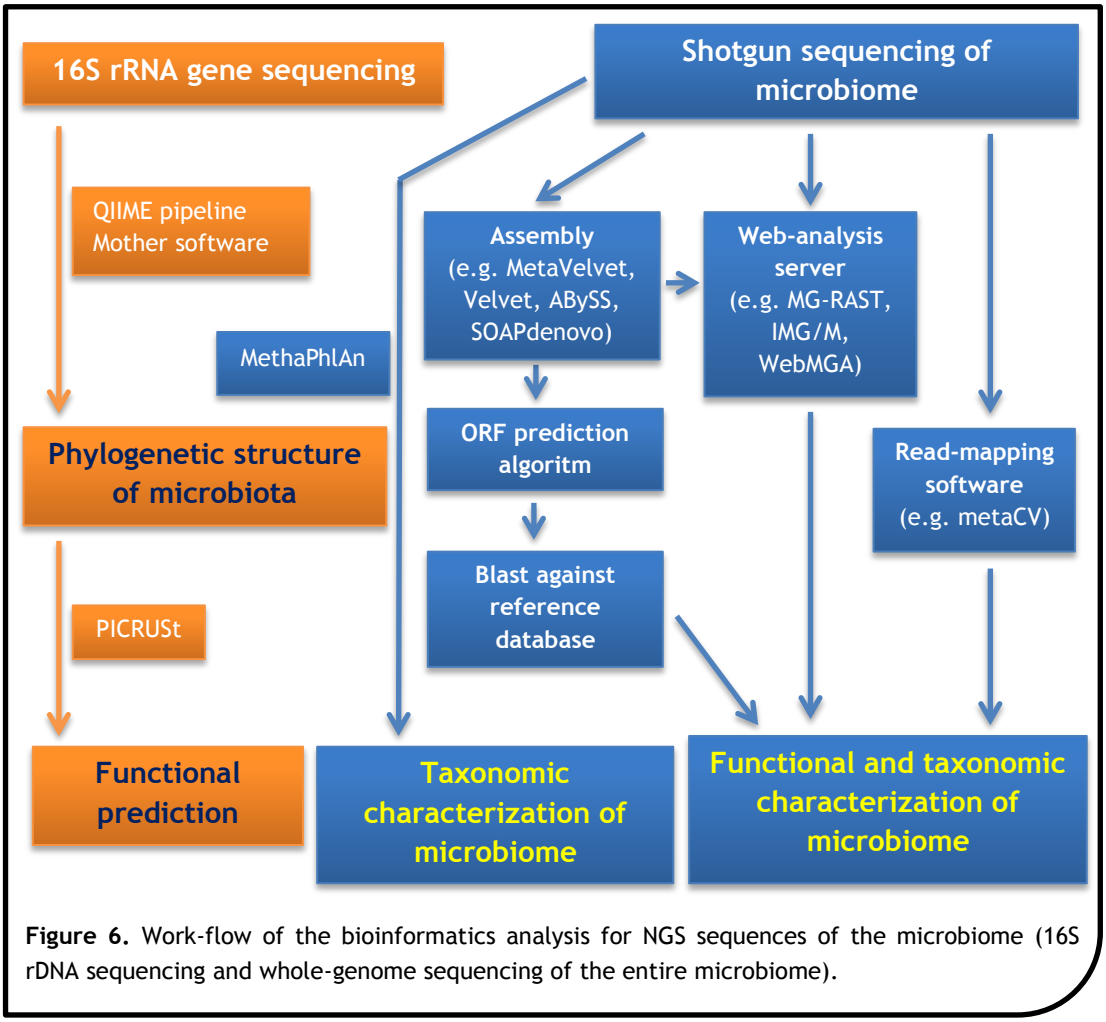

Figure 6. Work-flow of the bioinformatics analysis for NGS sequences of the microbiome (16S rDNA sequencing and whole-genome sequencing of the entire microbiome).

CHAPTER 2 – PROJECT OUTLINE

The human gut microbiome and its trajectory during the human lifespan has been thoroughly described in the last decades. In this scenario the gut microbiota is not a passive actor that responds to environmental fluctuations, but it also influences the human host through the interaction with the enterocytes and the production of specific metabolites. The result of microbiota-host crosstalk under the effect of diet, lifestyle and/or inflammation is the instauration of multiple different equilibria, that could positively or negatively affect the health status of human beings. Deviations from the canonical development produced by disease have been characterized in several studies. To monitor the intestinal microbial community seems to be the litmus test for distinguishing changes in the intestinal health status. In the light of these considerations, to characterize the variety of the microbial configurations and to evaluate dietary interventions with probiotics and prebiotics for influencing the composition and activity of gut microbiome, are assuming an increasingly greater interest.

The present study is composed of five main chapters aimed to elucidate different aspects of the interaction microbiota-host during the human lifespan.

Firstly, the relationship between gut microbiota and human host in breast-fed infants and adults was investigated through a non-invasive HT29 cell-based minimal model (Centanni et al., 2013b). In particular, after depicting the faecal microbial community of 12 breast-fed infants and 6 adults by 16S rDNA pyrosequencing, their respective HT29 cell-associated gut microbiota fractions were characterized by the

universal phylogenetic array platform HTF-Microbi.Array, both in the presence and absence of TNF-α-mediated pro-inflammatory stimulus. Interesting results revealed remarkable differences between the enterocyte-associated microbiota fractions in breast-fed infants and adults, also in pro-inflamnatory conditions. In particular, the infant-type microbiota is structured to cope with inflammation, being co-evolved to prime the early immune response by means of transient inflammatory signals from gut microorganisms.

Following a temporal line in the development of human lifespan, a second comparative study, concerning the faecal microbiota characterization of atopic children and healthy controls was performed (Candela et al., 2012). Nineteen atopic children and 12 healthy controls aged 4-14 years were enrolled and the faecal microbiota was characterized by HTF-Microbi.Array and quantitative PCR. Depleted in key immunomodulatory symbionts, the atopy-associated microbiota can represent an inflammogenic microbial consortium which can contribute to the severity of the disease.

Additionally, in order to understand the impact of lifestyle on the microbiota-host relationship, we investigated the taxonomic diversity and metabolite production of the gut microbiota from 27 Hadza hunter-gatherers of Tanzania, with respect to 16 Italian individuals. In particular, the Hadza microbiome is composed by unique features linked to a foraging lifestyle, and it is probably able to enhance the digestion of fibrous plant foods found in the diet of Hadza (Schnorr et al., 2014).

Moving to the limit of the human life, to elucidate the contribution of the gut microbiome to the complex mosaic of human longevity, we applied shotgun sequencing to total faecal bacterial DNA in a selection of samples belonging to a well-characterized human ageing cohort, including centenarians, as individuals at

the upper extremity of the human lifespan. Collectively, our data emphasize the relationship between intestinal bacteria and human metabolism, by detailing the modifications in the gut microbiome as a consequence and/or promoter of the physiological changes occurring in the human host upon ageing (Rampelli et al., 2013b).

Finally, we evaluated the impact of a bar containing the probiotics *Bifidobacterium longum* Bar33 and *Lactobacillus helveticus* Bar13 on the intestinal microbiota in the elderly (Rampelli et al., 2013a). A randomized double-blind placebo-controlled trial was performed on 32 elderly volunteers living in Italy, aged between 71 and 88 years. The consumption of probiotics-containing bars was effective in redressing some of the age-related dysbioses of the intestinal microbiota, opening the way to the development of elderly-tailored probiotic-based functional foods to counteract the age-related dysbioses of the intestinal microbiota.

CHAPTER 3

CHARACTERIZATION OF THE ENTEROCYTE-ASSOCIATED INTESTINAL MICROBIOTA IN BREAST-FED INFANTS AND ADULTS: AN EX-VIVO STUDY

1. ***Brief introduction***
2. *Materials and Methods*
3. *Results*
4. *Discussion*

It is a matter of fact that the human genome does not code for sufficient information to carry out all functions necessary to maintain health. Indeed, for several aspects of our physiology, such as nutrition (Gill et al., 2006), protection from pathogens (Lupp et al., 2007) and immunological wellbeing (Round et al, 2009; Hooper et al., 2012), we strictly depend on our symbiont microbial partner, the gut microbiota. Evolved as an integral component of the immune system, the human intestinal microbiota finely calibrates the immunological services at the different host ages (Maynard et al., 2012), supporting the process of immune education during our infancy and maintaining a balanced immune homeostasis along the adult life.

Breast-fed infants possess a peculiar gut microbiota structure which is dominated by *Bifidobacterium* and *Enterobacteriaceae* (Koropatkin et al., 2012; Jost et al., 2012). Within a critical "time window" of 8 months of life (Russell et al., 2012), the infant-type gut microbiota plays specific functions strategic for the correct maturation of the host immune system functionalities (Maynard et al., 2012), modulating the T cell differentiation process (Atarashi et al., 2011) and leading to the acquisition of the

mucosal iNKT cell tolerance (Olszak et al., 2012). These findings have been highlighted by two recent perspective surveys of gut microbiota in Danish and Swedish infants, which demonstrated that a low bacterial diversity in the early life is associated with an increased risk of immunological disorders later in life (Bisgaard et al., 2011; Abrahamsson et al., 2012). Moreover, the neonatal intestinal immune apparatus has been recently reported as highly responsive to microbial ligands (Battersby et al., 2013), being primed to establish an intense microbe-host immunological cross-talk since birth (Maynard et al., 2012). At weaning, with the introduction of solid foods, the gut microbiota progressively acquires an adult-like profile which is dominated by Bacteroidetes and Firmicutes (Dominguez-Bello et al., 2011a).

In this scenario, bacteria interacting with the gut mucosal surface have a role of primary importance in the cross-talk with the host immune system (Sansonetti, 2011). Establishing a close interaction with the epithelial apex, mucosal microorganisms enhance the level of epithelial cross-talk at the enterocyte surface, shaping the gut immunological environment (Wells et al., 2011).

Here, we used a non-invasive HT29 cell-based minimal model to characterize the enterocyte adherent gut microbiota fraction in human beings. In the light of the fact that stools are considered as representative of the mucosa-associated gut microbiota (Eckburg et al., 2005; Walter et al., 2011), our approach involved the co-incubation of freshly produced and immediately processed faecal samples with monolayers of the human enterocyte line HT29 (Rousset, 1986). The enterocyte-associated gut microbiota fraction was subsequently characterized by a dual approach based on qPCR and the phylogenetic universal array platform HTF-Microbi.Array (Candela et al., 2010; Centanni et al., 2013a).

By using our *ex vivo* HT29 cell-based model, we investigated the phylogenetic structure of the enterocyte-associated microbiota fraction of 12 breast-fed infants and 6 young adults, whose gut microbiota composition was characterized by pyrosequencing of the 16S rDNA V4 region. In order to mimic a host inflammatory response, experiments were performed in the presence or absence of a TNF-α-mediated inflammatory stimulus. This pro-inflammatory cytokine was selected because pivotal in the intestinal inflammatory processes (Yan et al., 2008).

CHAPTER 3

CHARACTERIZATION OF THE ENTEROCYTE-ASSOCIATED INTESTINAL MICROBIOTA IN BREAST-FED INFANTS AND ADULTS: AN EX-VIVO STUDY

Subject enrolment and sample collection

Twelve exclusively breast-fed genetically unrelated infants (age: 2-8 months) and 6 adults (age: 30-40 years) were recruited for this study in the Bologna metropolitan area and surroundings, Italy. All subjects were healthy and had not received antibiotics or probiotics or prebiotics for at least 3 months prior to the sampling. All participants were asked to collect one faecal sample, store it at 4°C and bring it to the research laboratory within 24 h. Stool samples from infants were collected by parents. After collection, faecal samples were immediately processed.

Faecal slurry preparation

Faecal slurries were prepared as reported by Centanni et al. (2013a). Briefly, stools were immediately diluted 1:2 in ice-cold Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) and homogenized in a Stomacher blender (VWR International PBI, Milan, Italy) for 2 min at high speed, until uniform consistency was achieved. For each subject, a 1/100 dilution of the faecal slurry containing

approximately 10^{10} bacterial cells was prepared in DMEM for the interaction with the human colonic epithelial cell line HT29.

HT29 cell culture conditions and evaluation of MUC2 transcriptional levels

HT29 cell line was grown in DMEM with 4.5 g/l glucose supplemented with 10% heat-inactivated foetal bovine serum (FBS; Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich) and 1% penicillin-streptomycin (Sigma-Aldrich), as reported by O'Hara et al. (2006a). Cells were routinely propagated in 75-cm^2 flasks (BD Falcon; Becton Dickinson, Heidelberg, Germany) at 37°C and 5% CO2 in a humidified atmosphere until they reached 90% confluence. For faecal slurry-HT29 cell interaction assays, 2.5 x 10^5 HT29 cells were seeded per well in 24-well tissue culture plates (BD Falcon), and allowed to grow to confluent monolayers. For the visualization of HT29 cell adherent faecal bacteria, 2.5 x 10^5 HT29 cells were layered on 12 mm-diameter glass coverslips in 24-well tissue culture plates (BD Falcon) and grown until confluence. Twenty-four hours before the assays, the cell medium was replaced with interaction medium (DMEM, 25 mM HEPES, 1 g/l glucose [Gibco BRL, Life Technologies, Grand Island, NY], 1% FBS) with or without the addition of 2 ng/ml human recombinant TNF-α (Thermo Scientific, Milan, Italy) (O'hara et al., 2006a; Centanni et al., 2012). According to Porath et al. (2005), the stimulation of HT29 cells with TNF-α for 24 h induces a substantial increase in mRNA levels of several cytokines and chemokines, such as TNF-α, IL-8, macrophage inflammatory protein (MIP)-2, MIP-3α, growth-regulated oncogene (GRO)-α, GRO-γ and interferon-inducible protein-10 as well as COX-2.

In control experiments, the mucus production by HT29 cells was evaluated both in the presence and absence of TNF-α. To this aim, the transcriptional levels of the

intestinal mucin gene MUC2 were determined as reported by Dolan et al. (2012) and compared. Briefly, total RNA was extracted from HT29 cells pre-treated or not with TNF-α, with the Illustra RNAspin Mini Kit (GE Healthcare, Milan, Italy), and single-stranded cDNA was synthesized using random hexamers and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). qPCR was carried out with a LightCycler SYBR Green system (Roche, Mannheim, Germany), using primers and conditions described by Dolan et al. (2012). Beta actin was used as a reference gene (Zhang et al., 2005; Rho et al., 2010). Relative mRNA expression of MUC2 was determined using the ΔΔCT method after Pfaffl correction (Pfaffl et al., 2001). According to our data, MUC2 expression was observed at basal levels in control cells and not significantly affected by TNF-α stimulation (data not shown).

Faecal slurry-HT29 cell interaction assay

Before interaction assays, interaction medium was collected from HT29 cell monolayers and replaced with 1 ml of a 1/100 dilution of freshly prepared faecal slurry. Cells were next incubated for 1 h at 37°C and 5% CO2 in a humidified atmosphere, as reported for standard bacterial adhesion assays (Candela et al., 2008). After 3 washings with PBS, 200 µl of 0.05% trypsin/0.02% EDTA (Sigma-Aldrich) were added to each well and incubated for 10 min at 37°C to detach cells and adherent bacteria (Candela et al., 2005). Wells were rinsed with 200 µl of PBS and samples were stored at -20°C for subsequent analysis. As a control, 1 ml of 1/100 dilutions of faecal slurries was incubated with or without 2 ng/ml TNF-α for 1 h at 37°C and 5% CO2 in a humidified atmosphere.

Visualization of the HT29 cell-associated microbiota fraction by fluorescence microscopy

Bacterial cells were stained as reported by Vesterlund et al. (2005). Briefly, 1 ml aliquots of 1/100 dilutions of faecal slurries were incubated with 0.2 µg/ml DAPI (Sigma-Aldrich) at room temperature in the dark for 30 min with mild shaking. After washing, stained slurry dilutions were resuspended in 500 µl of interaction medium and added to HT29 cell monolayers previously grown on glass coverslips. After 1-h incubation at 37°C and 5% CO2 (Candela et al., 2008), wells were rinsed with PBS to remove non-adherent bacteria, and HT29 cells were fixed on coverslips with 1% paraformaldehyde at 4°C for 1 h. Glass coverslips were mounted in Fluoromount-G (SouthernBiotech, Birmingham, AL) and incubated 1 h at room temperature before fluorescence microscopy observation (ECLIPSE 90i, Nikon, Melville, NY). The Nis-Elements AR 3.2 software (Nikon) was used for image acquisition. For each sample, pictures of the same area were taken at 100x magnification under both fluorescent light and phase contrast. For each coverslip, 5 different microscopic views were taken. Three independent adhesion experiments were performed for each experimental condition.

Microbial DNA extraction from faecal slurry and enterocyte-associated microbiota fraction

Total microbial DNA from faecal slurries was extracted by using QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the protocol reported by Candela et al. (Candela et al., 2010).

Genomic DNA extraction from the enterocyte-associated microbiota fraction was carried out using DNeasy Blood & Tissue Kit (QIAGEN) with a modified protocol.

Briefly, cell pellets were suspended in enzymatic lysis buffer (20 mM Tris HCl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100, 20 mg/ml lysozyme) and incubated at 37°C for 30 min. After adding 200 µl of AL buffer (QIAGEN), one 3-mm glass bead and 0.15 g of 0.1-mm zirconia beads, samples were beaten in a FastPrep-24 instrument (MP Biomedical, Irvine, CA) at 5.5 m/s for 2 min, and further processed according to the manufacturer's instructions.

DNA concentration and quality were evaluated using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE).

16S rDNA gene amplification

For the amplification of the V4 region of the 16S rDNA gene the primer set 520F (5'-AYTGGGYDTAAAGNG-3') and 802R (5'-TACNVGGGTATCTAATCC-3') (Claesson et al., 2009) was utilized. These primers were designed to include at their 5' end one of the two adaptor sequences used in the 454-sequencing library preparation protocol (adaptor A and B), linked to a unique MID tag barcode of 10 bases allowing the identification of the different samples. PCR mixtures contained 0.5 µM of each forward and reverse primer, 100 ng of template DNA, 2.5 U of GoTaq Flexi Polymerase (Promega, Milan, Italy), 200 µM of dNTPs and 2 mM of MgCl2 in a final volume of 50 µL. Thermal cycling consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 50 s, annealing at 40°C for 30 s, and extension at 72°C for 60 s, with a final extension step at 72°C for 5 min (Claesson et al., 2009). PCR amplifications were carried out in a Biometra Thermal Cycler T Gradient (Biometra, Göttingen, Germany).

Pyrosequencing of faecal slurries

The PCR products derived from amplification of the specific 16S rDNA V4 hypervariable region were individually purified with MinElute PCR Purification Kit (QIAGEN) and then quantified using the Quant-iT PicoGreen dsDNA kit (Invitrogen, Leek, Netherlands). After the individual quantification step, amplicons were pooled in equal amounts (thus creating a 10-plex and an 8-plex pool) and one more time purified by 454-Roche Double Ampure size selection protocol with Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany), in order to remove primer dimers, according to the manufacturer's instructions (454 LifeSciences, Roche, Branford, CT).

Amplicon pools were fixed to microbeads to be clonally amplified by performing an emulsion PCR following the GS-FLX protocol Titanium emPCR LIB-A (454 LifeSciences, Roche). Following this amplification step, the beads were enriched in order to keep only those carrying identical PCR products on their surface, and then loaded onto a picotiter plate for pyrosequencing reactions, according to the GS-FLX Titanium sequencing protocol. The two pools were sequenced in one eight of a plate each. Amplicon sequences were deposited in MG-RAST under the project ID 5838.

Bioinformatic analysis of 16S rDNA gene sequencing data

Sequencing reads were analyzed using the QIIME pipeline (Caporaso et al., 2010), as described in Claesson et al. (Claesson et al., 2012). Briefly, V4 sequences were filtered according to the following criteria: (i) exact matches to primer and barcode sequences; (ii) read length not shorter than 150 bp and not longer than 350 bp; (iii) no ambiguous bases (Ns); (iv) a minimum average quality score over a 50-bp rolling window of 25. For bacterial taxonomy assignment we utilized RDP-classifier (version

2.2) with 50% as confidence value threshold. Trimmed reads were clustered into OTUs at 97% identity level and further filtered for chimeric sequences using ChimeraSlayer (http://microbiomeutil.sourceforge.net/#A_CS). Alpha-diversity and rarefaction plots were computed using four different metrics: Shannon, PD whole tree, chao1 and observed species. Weighted and unweighted UniFrac distances were used to perform Principal Coordinates Analysis (PCoA) and Procrustes superimposition in order to evaluate ß-diversity and the correlation between pyrosequencing and phylogenetic microarray data. PCoA, Procrustes, heatmap and bar plots were built using the packages Made4 (Culhane et al., 2005) and Vegan (Oksanen et al., 2013) in R 3.0.0. The R packages Stats and Vegan were used to perform statistical analysis. In particular, Wilcoxon signed-rank test was used to compare infant and adult gut microbiota for α- and ß-diversity; data separation in the PCoA was tested using a permutation test with pseudo F-ratios (function adonis in the Vegan package); Fisher's exact test was used to assess the significance of differences between clusters from the hierarchical clustering analysis; Student's t-test or Mann-Whitney U test were used to carry out significant differences at phylum, group or genus level. When appropriate, P values were adjusted for multiple comparison using the Benjamini-Hochberg correction. False discovery rate (FDR) < 0.05 was considered as statistically significant. The function protest was utilized to validate the Procrustes analysis. A P value < 0.05 was considered as statistically significant.

HTF-Microbi.Array/qPCR combined approach

In order to characterize the phylogenetic structure of faecal slurries and enterocyte-associated gut microbiota fractions, the HTF-Microbi.Array/qPCR combined

approach developed by Centanni et al. (Centanni et al., 2013a) was employed. This approach is based on the association of the High Taxonomic Fingerprint (HTF)-Microbi.Array (Candela et al., 2010) - a fully validated phylogenetic microarray platform which allows the detection and quantification of up to 31 intestinal bacterial groups, covering up to 95% of the human gut microbiota – with a qPCR protocol designed to specifically quantify *Bifidobacterium* spp. In brief, for each sample the non-bifidobacterial fraction was characterized using the HTF-Microbi.Array, whereas the relative abundance of *Bifidobacterium* was estimated by qPCR. Microarray platform description and fluorescence raw data were uploaded in GEO archive under the ID GSE51177.

CHAPTER 3

CHARACTERIZATION OF THE ENTEROCYTE-ASSOCIATED INTESTINAL MICROBIOTA IN BREAST-FED INFANTS AND ADULTS: AN EX-VIVO STUDY

16S rDNA sequencing of the faecal microbiota in breast-fed infants and adults and validation of the HTF-Microbi.Array for the high taxonomic level fingerprinting of the human faecal microbiota

The faecal microbial communities of 12 breast-fed infants and 6 adults were phylogenetically characterized using 16S rDNA gene pyrosequencing. A total of 127,530 high-quality sequence reads from the 16S rDNA V4 region were obtained, with an average of 7,085 reads per subject. Reads were clustered at 97% identity in 3,471 OTUs. Rarefaction curves indicated a lower diversity in the faecal microbial community of infants with respect to adults (**Figure 7**). Comparison of community richness using several metrics showed a significantly ($P < 0.001$) lower degree of α-diversity in the infant faecal microbiota with respect to the adult one (Shannon: infants, 3.16 ± 0.91 vs adults, 6.00 ± 0.25; PD whole tree: 22.02 ± 7.42 vs 48.00 ± 5.18; chao1: 294.26 ± 108.62 vs 807.68 ± 130.32; observed species: 156.50 ± 50.56 vs 400.67 ± 44.07).

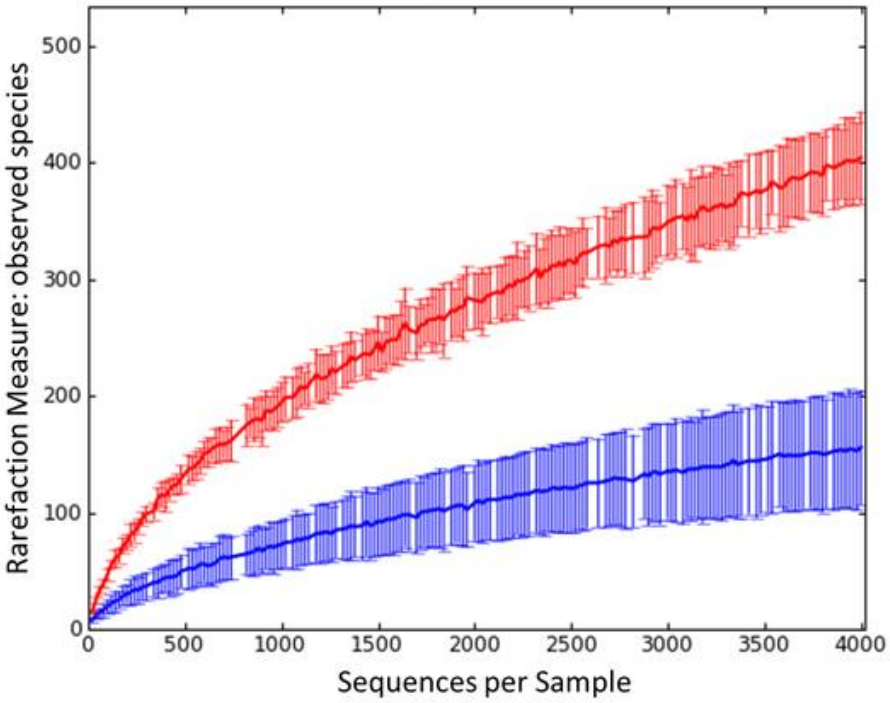

Figure 7. Rarefaction curves show the observed species in the fecal microbial communities of breast-fed infants (blue, n=12) and adults (red, n=6).

PCoA analysis of the unweighted (**Figure 8a**) and weighted (**Figure 8b**) UniFrac distances indicated a sharp ($P < 0.001$) separation between the infant and adult faecal microbial communities, with a significantly ($P < 0.001$) higher degree of interindividual variability within breast-fed infants. In particular, for breast-fed infants mean values of unweighted and weighted UniFrac distances of 0.758 ± 0.045 and 0.843 ± 0.321 were obtained, respectively, whereas for adults corresponding values of 0.693 ± 0.014 and 0.528 ± 0.153 were obtained. Moreover, clustering analysis of the OTU-based community structures resulted in a significant ($P < 0.001$) separation of infants from adults (**Figure 8c**). While the adult gut microbiota was dominated by *Bacteroidaceae* (mean relative abundance, rel. ab. ± SEM, 11% ± 3%),

Clostridiales Incertae Sedis IV (13% ± 1%), *Lachnospiraceae* (17% ± 2%) and *Ruminococcaceae* (39% ± 5%), with *Veillonellaceae* (1% ± 0.7%), *Erysipelotrichaceae* (3% ± 1%) and *Bifidobacteriaceae* (7% ± 3%) as subdominant components, the infant one was largely dominated by *Bifidobacteriaceae* (45% ± 9%), with *Bacteroidaceae* (20% ± 6%) as secondary dominant component. *Clostridiaceae* (9% ± 6%), *Lachnospiraceae* (7% ± 3%) and *Enterobacteriaceae* (4% ± 1%) were subdominant groups, whereas *Enterococcaceae* (1% ± 0.6%), *Streptococcaceae* (3% ± 1%) and *Erysipelotrichaceae* (2% ± 0.8%) represented minor components of the faecal microbial community of breast-fed infants.

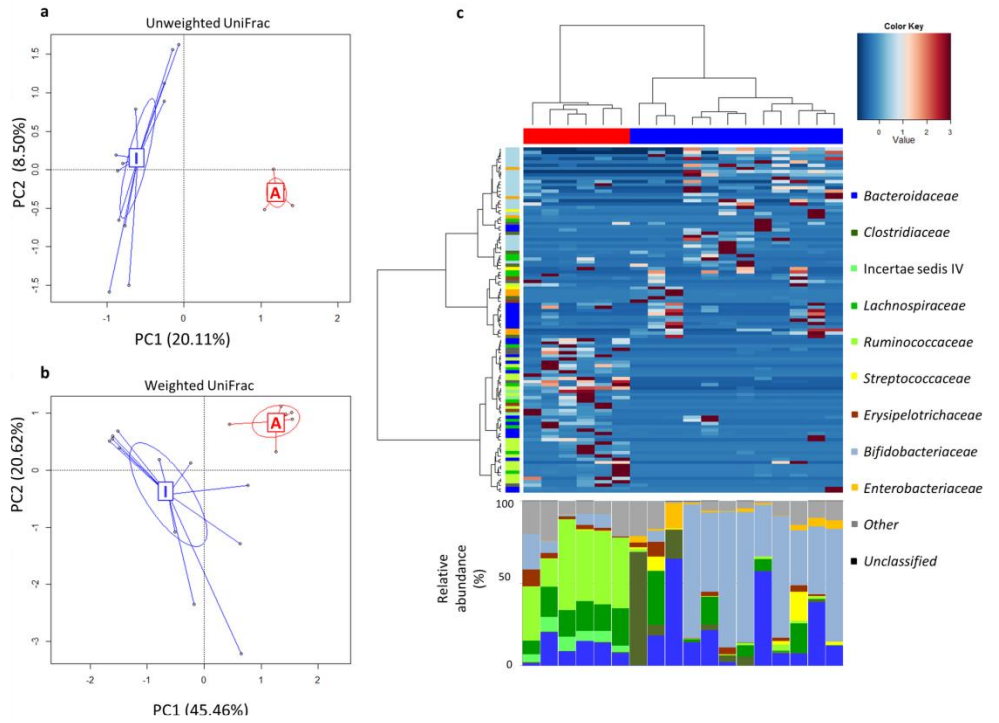

Figure 8. Unweighted (a) and weighted (b) UniFrac PCoA of the fecal microbiota from breast-fed infants (blue) and adults (red). c, Hierarchical Ward-linkage clustering based on the Spearman correlation coefficients of OTU proportion. OTUs were filtered for subject prevalence of at least 20%. Subjects are clustered in the top of panel and color-coded as in a. OTUs are clustered by the vertical tree and color-coded by family assignment. 107 OTUs confidentially classified to family level are visualized. In the bottom panel the relative abundance (%) of family-classified microbiota is shown.

In **Table 1** the significant differences at phylum and genus level between the faecal microbiota composition of breast-fed infants and adults are reported.

The comparison between the phylogenetic structure of the faecal microbial communities of breast-fed infants and adults revealed profound differences, reflecting an overall different architecture of the infant and adult gut microbial ecosystem at both phylum and family level.

	Relative abundance (mean, %)		
Phylum	**Breast-fed infants**	**Adults**	***P value**
Actinobacteria	46.0	7.6	0.024
Firmicutes	28.7	76.3	0.007
Proteobacteria	3.5	0.4	0.003
Genus			
Bifidobacterium	45.4	7.5	0.049
Alistipes	0	1.3	< 0.001
Streptococcus	2.8	0.2	0.007
Blautia	0.04	6.6	< 0.001
Lachnospiraceae; Unclassified	2.1	5.7	0.041
Roseburia	0.03	6.8	< 0.001
Faecalibacterium	0.4	19.8	< 0.001
Oscillibacter	0.01	1.5	< 0.001
Ruminococcaceae; Unclassified	0.3	3.8	0.002
Ruminococcus	0.03	8.7	0.011
Subdoligranulum	0.1	3.0	0.001
Veillonella	1.7	0.01	0.006
Erysipelotrichaceae; Unclassified	0.3	2.8	0.004
Escherichia/Shigella	2.6	0.06	0.001

Table 1. Bacterial phyla and genera showing a significantly different relative abundance in the faecal microbiota of breast-fed infants and adults.

We previously developed a combined approach based on HTF-Microbi.Array and qPCR for the high taxonomic level fingerprint of the human gut microbiota (Centanni et al., 2013a). In order to further validate this approach, we compared with the 16S

rDNA V4 pyrosequencing in providing the high taxonomic level fingerprint of the faecal microbial community of the 12 breast-fed infants and the 6 adults enrolled in our study. Procrustes similarity analysis of the weighted UniFrac distances obtained by 16S rDNA pyrosequencing and the Euclidean distances based on rel. ab. of the HTF-Microbi.Array/qPCR profiles revealed a significant relationship between the two datasets ($P < 0.001$) (**Figure 9**). These data supported the overall concordance of the two approaches in depicting the high taxonomic level fingerprint of the human gut microbiota, confirming the reliability of the HTF-Microbi.Array/qPCR combined approach.

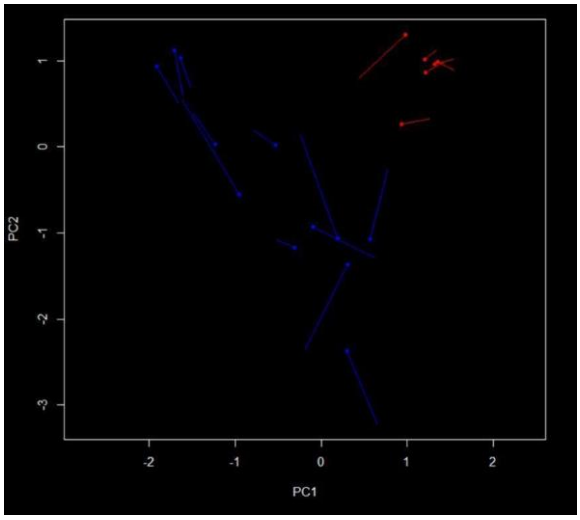

Figure 9. Procrustes analysis combining weighted UniFrac PCoA from pyrosequencing (non-circle end of lines) with PCA from HTF-Microbi.Array/qPCR data (circle end of lines) is shown.

The HT29 cell-associated microbiota fraction in breast-fed infants and adults

In order to approximate the human intestinal mucosa-associated gut microbiota fraction we developed a non-invasive ex vivo approach based on the human enterocyte line HT29. Briefly, stools were processed within 24 h after collection to obtain a homogeneous microbiota community suspension. Bacterial cells were next

incubated with monolayers of HT29 cells as performed in standard bacterial adhesion assays (Candela et al., 2008). After washing, the enterocyte-associated gut microbiota fraction was characterized by the previously validated HTF-Microbi.Array/qPCR dual approach (Centanni et al., 2013a). *Ex vivo* HT29 cell-based experiments were carried out for the 12 breast-fed infants and the 6 adults enrolled in our study (**Figure 10**). As a control, the presence of faecal bacterial cells on the HT29 cell surface was determined by DAPI fluorescence staining. Clusters of faecal microorganisms directly interacting with the HT29 cell surface were visualized (**Figure 11**).

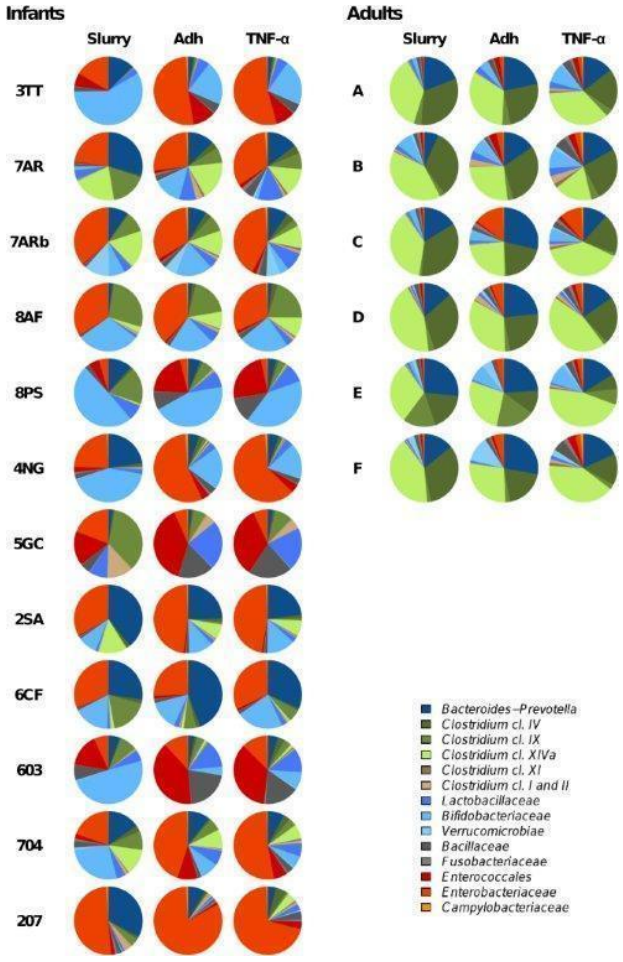

Figure 10. Pie charts of the mean values of relative abundance (%) of the major microbial groups in the fecal microbiota (Slurry) and the HT29 cell-associated fraction in the absence (Adh) or presence (TNF-α) of a TNF-α-mediated pro-inflammatory stimulus, in breast-fed infants (left panel) and adults (right panel). The relative contribution of each microbial group was determined using the HTF-Microbi.Array/qPCR combined methodology. The HT29 cell-associated fraction was evaluated after 1-h interaction of fecal slurries with HT29 cell monolayers, previously stimulated or not with 2 ng/ml of human TNF-α for 24 h.

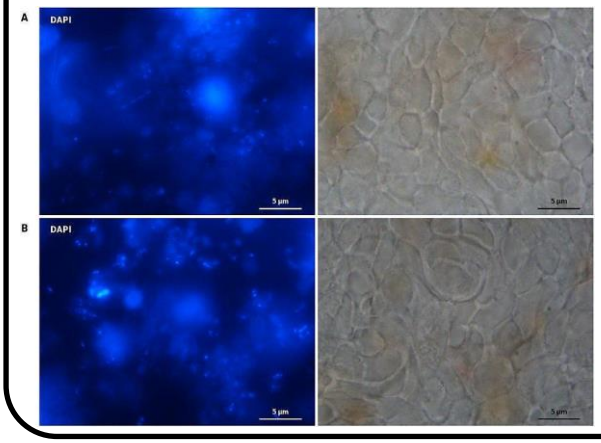

Figure 11. Fecal bacteria were stained with DAPI and allowed to interact with HT29 cell monolayers grown on glass coverslips. Representative images for an adult (A) and a breast-fed infant (B) are shown (magnification, 100x). Left panel, DAPI-stained adherent bacteria (blue) under fluorescent light. Right panel, corresponding phase contrast images showing the underlying HT29 cell layer.

According to our data, in breast-fed infants the HT29 cell-associated gut microbiota fraction showed a phylogenetic structure at high taxonomic level that generally resembled the one observed in the faecal microbiota (**Table 2**). In particular, the HT29 cell-associated gut microbiota fraction of breast-fed infants was dominated by *Bifidobacterium* (rel. ab., 14%) and *Enterobacteriaceae* (35%) with *Bacteroides-Prevotella*, *Enterococcales*, *Clostridium* clusters IX and XIVa, *Lactobacillaceae* and *Bacillaceae* as minor members (11, 11, 5, 5, 7 and 6%, respectively). Respect to the faecal microbiota, the HT29 cell-associated fraction showed a trend (FDR < 0.3) towards the enrichment in *Enterobacteriaceae*, *Enterococcales*, *Bacillaceae*, *Lactobacillaceae*, and a corresponding reduction in *Clostridium* cluster IX. A significant enrichment in the minor (rel. ab. < 1%) microbiota component *Clostridium* cluster XI was also observed.

Differently from breast-fed infants, the enterocyte-associated gut microbiota fraction in adults was remarkably different from the faecal one (**Table 2**). In particular, the HT29-cell associated fraction was significantly depleted in the dominant adult gut microbiota component *Clostridium* cluster XIVa, whose rel. ab. shifted from 38% to 29%. Moreover, concerning the other major gut microbiota

members, a tendency towards the reduction of *Clostridium* cluster IV (rel. ab. from 30% to 21%) as well as the enrichment of *Bacteroides-Prevotella* (rel. ab. from 16% to 23%) was shown. Finally, *Enterobacteriaceae*, only a minor component of the faecal gut microbiota in adults, significantly increased to a rel. ab. of 5% in the HT29 cell-associated fraction.

The comparison between the HT29 cell-associated gut microbiota fractions in breast-fed infants and adults revealed remarkable differences (**Table 2**), showing a HT29 cell-associated fraction dominated by *Bacteroides-Prevotella* and *Clostridium* clusters IV, IX, XIVa in adults, and by *Bifidobacterium* and *Enterobacteriaceae* in breast-fed infants.

	Breast-fed infants			Adults		
Microbial group	**Faecal microbiota**	**HT29 cell-associated fraction**	**FDR**	**Faecal microbiota**	**HT29 cell-associated fraction**	**FDR**
Bacteroides-Prevotella	17.4	10.8	0.282	16.1	23.5	0.096
***Clostridium* cluster IV**	1.2	1.7	0.178	30.4	21.5	0.082
***Clostridium* cluster IX**	12.2	5.4	0.298	4.6	5.2	n.s.
***Clostridium* cluster XIVa**	5.9	5	n.s.	38.4	28.7	0.006
***Clostridium* cluster XI**	0.2	0.5	0.014	0.5	0.3	0.154
***Clostridium* cluster I, II**	1.8	1.4	0.428	0.5	1.2	0.53
Lactobacillaceae	3.6	6.9	0.224	1.3	1.9	n.s.
Bifidobacteriaceae	24.1	14.4	n.s.	2.4	4	n.s.
Verrucomicrobiae	1.3	0.8	n.s.	1.3	3.3	n.s.
Bacillaceae	2.4	5.5	0.282	1.7	1.8	n.s.
Fusobacteriaceae	0.5	0.6	n.s.	1	1.1	n.s.
Enterococcales	4.3	10.9	0.428	0.9	1.6	0.406
Enterobacteriaceae	24.7	35.3	0.296	0.6	5.5	0.004
Campylobacteriaceae	0.4	0.7	0.028	0.4	0.3	n.s.

Table 2. High taxonomic level profile of the faecal microbiota and the HT29 cell-associated fraction in breast-fed infants and adults

TNF-α differently modulates the HT29 cell-associated microbiota fraction in breast-fed infants and adults

HT29 cells have been reported to show an overall TNF-α response similar to the one proper of freshly isolated intestinal epithelial cells (Bruno et al., 2005). Thus, in order to investigate whether an inflammatory response modulated the mucosa-associated gut microbiota fraction, the interaction experiments between gut microbiota and HT29 cells were repeated with cell monolayers pre-treated with TNF-α (**Figure 10**). While the inflammatory stimulus resulted in a dramatic remodeling of the HT29 cell-associated gut microbiota fraction from adults, no changes were observed for infants (**Table 3**). In particular, in adults inflammation involved a strong and significant (FDR = 0.008) reduction of the dominant gut microbiota component *Bacteroides-Prevotella* from a rel. ab. of 23% in the absence of inflammatory stimuli to 15% in the presence of TNF-α. On the other hand, the TNF-α pre-treatment prompted a concomitant significant increase of the minor microbiota component *Bacillaceae*, whose rel. ab. in the HT29 cell-associated fraction of adults shifted from 2% in the absence of TNF-α to 4% in the presence of TNF-α. Further, a tendency (FDR = 0.154) towards the increase of the major gut microbiota component *Clostridium* cluster XIVa as a response to the inflammatory stimulus was observed. In order to exclude an intrinsic effect of TNF-α on the microbiota structure, faecal microbiota suspensions were incubated with or without TNF-α in the absence of HT29 cells, and subsequently characterized by means of the HTF-Microbi.Array/qPCR dual approach. According to our findings, community fingerprints of faecal suspensions in the presence or absence of TNF-α showed a

Pearson's correlation coefficient > 0.9, indicating the absence of any significant impact of TNF-α on the community composition (data not shown).

	Breast-fed infants			Adults		
Microbial group	**- TNF-α**	**+ TNF-α**	**FDR**	**- TNF-α**	**+ TNF-α**	**FDR**
Bacteroides-Prevotella	10.8	9.3	n.s.	23.5	15.4	0.008
***Clostridium* cluster IV**	1.7	2.5	0.520	21.5	18.2	n.s.
***Clostridium* cluster IX**	5.4	5.1	n.s.	5.2	3.4	n.s.
***Clostridium* cluster XIVa**	5.0	4.8	n.s.	28.7	37.5	0.154
***Clostridium* cluster XI**	0.5	0.6	n.s.	0.3	1.3	<0.001
***Clostridium* cluster I, II**	1.4	1.3	n.s.	1.2	1.8	0.24
Lactobacillaceae	6.9	7.4	n.s.	1.9	2.1	n.s.
Bifidobacteriaceae	14.4	13.1	n.s.	4.0	5.8	n.s.
Verrucomicrobiae	0.8	1.0	n.s.	3.3	1.5	n.s.
Bacillaceae	5.5	6.3	n.s.	1.8	4.0	0.004
Fusobacteriaceae	0.6	0.8	0.570	1.1	1.7	0.132
Enterococcales	10.9	10.4	n.s.	1.6	2.5	0.430
Enterobacteriaceae	35.3	36.8	n.s.	5.5	3.8	0.360
Campylobacteriaceae	0.7	0.8	n.s.	0.3	1.1	<0.001

Table 3. TNF-α impact on the HT29 cell-associated microbiota fraction in breast-fed infants and adults.

CHAPTER 3

CHARACTERIZATION OF THE ENTEROCYTE-ASSOCIATED INTESTINAL MICROBIOTA IN BREAST-FED INFANTS AND ADULTS: AN EX-VIVO STUDY

The comparative 16S rDNA pyrosequencing study of the gut microbiota of breast-fed infants and adults demonstrated profound structural differences between the two ecosystems. Respect to adults, the breast-fed infant gut microbiota was characterized by a lower compositional diversity and a higher degree of interindividual variability. The adult gut microbiota was largely dominated by Bacteroidetes and Firmicutes, whereas the gut microbiota of breast-fed infants showed *Bifidobacterium* as a predominant component with a lower proportion of *Clostridiales*. Moreover, compared to adults, the gut microbiota of breast-fed infants was largely enriched in facultatively aerobic populations, such as *Streptococcaceae*, *Enterococcaceae* and *Enterobacteriaceae*.

We previously developed a HTF-Microbi.Array/qPCR combined approach for the high taxonomic level profiling of the gut microbiota structure, which has been proved to be a robust and rapid tool to characterize the gut microbial communities in infants and adults (Centanni et al., 2013a). Focusing its phylogenetic resolution to the level of order and cluster, the HTF-Microbi.Array is blind with respect to the astonishing

interindividual variability of gut microbiota at the species level, being specifically conceived to characterize different gut microbiota phylogenetic assets with the potential to differently modulate host physiology (Candela et al., 2010; Candela et al., 2012). In the present paper, through a Procrustes similarity analysis of the HTF-Microbi.Array/qPCR-based fingerprints of the faecal microbial communities in breast-fed infants and adults, and the ones obtained by 16S rDNA pyrosequencing, we showed a significant agreement between the two approaches, further supporting the reliability of our phylogenetic array platform for the high taxonomic level fingerprinting of the human gut microbiota.

In order to shed some light on the peculiarity of the interplay between gut microbiota and the host immune system at different ages, we developed a non-invasive minimal model based on the interaction between the human enterocyte line HT29 and microbial community suspensions obtained from fresh stools. The enterocyte-associated gut microbiota fractions from breast-fed infants and adults were compared by means of the HTF-Microbi.Array/qPCR combined approach. The mucus-secreting HT29 cells were selected since considered among the most relevant enterocyte cell lines available for in vitro reproducing physiology and immune function of the human intestinal mucosa (Rousset, 1986; Neutra et al., 1989). Moreover, remaining undifferentiated to 95% in the post-confluent state, HT29 cells have been recently indicated as a suitable in vitro model for reproducing the immature intestinal environment of a neonate (Kavanaugh et al., 2013).

Our *ex vivo* model reflects remarkable differences between the enterocyte-associated gut microbiota fractions in breast-fed infants and adults. While the enterocyte-associated microbiota in breast-fed infants showed *Bifidobacterium* and *Enterobacteriaceae* as dominant components - with *Bacteroides-Prevotella*,

Enterococcales, *Clostridium* clusters IX and XIVa, *Lactobacillaceae* and *Bacillaceae* as subdominant - in adults, the enterocyte adherent gut microbiota fraction was largely dominated by *Bacteroides-Prevotella* and *Clostridium* clusters IV and XIVa, with *Clostridium* cluster XI, *Enterobacteriaceae* and *Bifidobacterium* as minor components. These data are in general agreement with that observed by Durban et al. (2011) and Ouwehand et al. (2004) in bioptic samples collected from healthy adults and infants, respectively. While in infants, the phylogenetic structure of the enterocyte-associated gut microbiota fraction generally resembled the one observed in the faecal microbiota, in adults it showed a totally different structure, being enriched in *Bacteroides-Prevotella* and *Enterobacteriaceae* and depleted in *Clostridium* clusters IV and XIVa. This observation suggests that the gut microbiota of breast-fed infants is selected for a closer interaction with the host enterocytes, a factor that may be important for the establishment of the intense cross-talk with the host immune system which drives the process of immune education (Johnson et al., 2012). In fact, enriched in *Bifidobacterium* and *Enterobacteriaceae*, the infant-type enterocyte-associated microbiota fraction is specifically structured to drive immune education in early infancy (Candela et al., 2013). While *Enterobacteriaceae* provide genes associated with virulence functionality, which continuously boost the immunological response (Schwartz et al., 2012), the early bifidobacterial fraction - dominated by the species *B. breve* and *B. longum* (Jost et al, 2012; Turroni et al., 2012) - has been shown to exert synergic immune modulatory and protective properties (Fukuda et al., 2011; Fanning et al., 2012), allowing the promotion and maintenance of a mutualistic cross-talk between the infant immune system and the mucosa-associated gut microbiota fraction (Maynard et al., 2012).

Equipped with a vast array of Toll-like receptors (TLR), enterocytes can sense

intestinal microorganisms, resulting in the recruitment and activation of macrophages and dendritic cells. This leads to the secretion of TNF-α and other pro-inflammatory cytokines, establishing a host pro-inflammatory response (O'Mahony et al., 2008; Wells et al., 2011; Maynard et al., 2012). In the light of this, in order to assess the impact of inflammation on the microbial ecology of the microbiota-enterocyte interaction process, HT29 cell interaction experiments were repeated with TNF-α. In particular, changing the expression of enterocyte surface proteins, pro-inflammatory cytokines can modify the enterocyte microenvironment (Hess et al., 2002). For instance, it has been reported that in response to TNF-α enterocytes up-regulate the expression of polymeric Ig receptor (Kvale et al.,1988), a non-specific microbial scavenger which limits the bacterial interaction with the enterocyte surface (Phalipon et al., 2003). According to our data, the enterocyte response to the TNF-α-dependent pro-inflammatory stimulus is sufficient to compromise the enterocyte-associated gut microbiota community in adults, while it does not impair the enterocyte adherent gut microbiota of breast-fed infants, demonstrating that this microbial community structure is structured to cope with host inflammatory responses. Taken together, our findings suggest that infant- and adult-type microbiota possess different functional properties for what concerns the capacity to interact with enterocytes, suggesting the specificity of the interaction process between gut microbiota and the host immune system at different ages. Indeed, the infant-type microbiota has co-evolved to cope with the process of immune education, which is based on transient inflammatory signals from gut microorganisms, functional to prime the early immune response (Maynard et al., 2012; Johnson et al., 2012). Differently, the adult-type microbiota is structured to forge and preserve a homeostatic low-grade inflammatory status, and is

dramatically compromised by host inflammatory responses (Sansonetti et al., 2008).

In conclusion, employing our reductionist non-invasive ex vivo HT29 cell-based model, we demonstrated that the infant- and adult-type enterocyte-associated gut microbiota fractions possess different phylogenetic and functional structures strategic to respond to the specific immunological needs at different ages. Our findings open the perspective to the usage of our *ex vivo* system in non-invasive case-control screening studies aimed at characterizing disease-associated deviations from a healthy enterocyte-associated gut microbiota profile, either in adults or infants. These studies could disclose specific dysbioses in the mucosa-associated microbiota fraction that – as the first line in the interaction with the host immune system – could potentially be involved in emerging inflammatory diseases, such as allergy, inflammatory bowel disease, inflammatory bowel syndrome and obesity-associated metabolic endotoxemia.

CHAPTER 4
UNBALANCE OF INTESTINAL MICROBIOTA IN ATOPIC CHILDREN

1. ***Brief introduction***
2. *Materials and Methods*
3. *Results*
4. *Discussion*

Atopic diseases are chronic inflammatory disorders caused by an aberrant T_H2-type immune response against common and innocuous environmental antigens (Romagnani, 2006). The elaboration of cytokines, such as IL-4, IL-13 and IL-5, can contribute to disease induction (Ngoc et al., 2005). During the past decades the prevalence of atopic diseases among children in the western world has dramatically increased (Ngoc et al., 2005). Too fast for any possible shift in genetic constitution, environmental changes associated with the western lifestyle are believed to be involved (Penders et al., 2007a; Ehlers et al., 2010). The human intestinal microbial community has been indicated as a key factor to interpret the impact of the western lifestyle on the etiology of atopic diseases (Rautava et al., 2004; Penders et al., 2007a; De Filippo et al., 2010; Ehlers et al., 2010; Kau et al., 2011). The intestinal microbiota is extremely plastic in response to diet and environmental factors and, at the same time, governs many aspects of the immune function throughout the body (Lee et al., 2010). Thus, the hypothesis that specific western lifestyle-driven dysbioses of the human intestinal microbiota are involved in the bloom of atopy in western children has been advanced.

According to Gaboriau-Routhiau et al. (2009), specific members of the intestinal microbial community exert an active role in the modulation of a striking range of T cell functions, such as T_H17, T_H1, T_H2 and regulatory cell phenotype (Treg). Having a profound impact on the overall human immune status, perturbations of the intestinal microbiota have been implicated in the development and progression of inflammatory diseases, such as inflammatory bowel diseases (IBD), autoimmune disorders, allergy and type II diabetes (Neish, 2009; Maslowski et al., 2011).

On the basis of the perceived importance of the intestinal microbiota in the education of the human immune system to tolerance (Rautava et al., 2004), culture-independent perspective studies have been carried out to determine whether specific microbiota dysbioses in the early life could affect the subsequent manifestation and sensitization of atopic diseases (Kalliomaki et al., 2001; Murray et al., 2005; Penders et al., 2007a; Penders et al., 2006; Penders et al., 2007b; Vael et al., 2009; Bisgaard et al., 2011; Abrahamsson et al., 2012). These robust and extensive studies proved that the low bacterial diversity in the early life, rather than the prevalence of a specific bacterial taxon, is associated with an increased risk of subsequent atopic disease, reinforcing the “old friend hypothesis” (Blaser et al., 2009). According to this theory, the western lifestyle caused the disappearance of key bacterial groups from the intestinal microbiota, which are essential to prime the physiology of our immune system. The lack of these “old friends” during the perinatal period led to an immune system incline to inappropriate activation, which is a characteristic of the emerging chronic inflammatory diseases in the western world.

Even if the role of the intestinal microbiota in the predisposition to develop atopy in infancy has been accepted, to our knowledge, only few case-control culture-

independent studies of the gastrointestinal microbial ecology in atopic diseases have been carried out. However, by modulating the immune status throughout the body (Lee et al., 2010), an inflammogenic gut microbial community in atopic subjects could significantly contribute to the severity of the disease. Here, we performed a pilot case-control study of the atopy-associated dysbiosis of the intestinal microbiota in atopic children. Since from birth to weaning the infant intestinal microbiota is an extremely dynamic entity, which continuously fluctuates in response to factors of environmental and endogenous origin (Dominguez-Bello et al., 2011a), we enrolled children aged > 2 years, characterized by a relatively stable adult-like intestinal microbial community (Palmer et al., 2007). In particular, the faecal microbiota of 19 atopic children and 12 healthy controls aged 4-14 years was characterized by means of the previously developed phylogenetic microarray platform HTF-Microbi.Array (Candela et al., 2010) and qPCR.

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Subjects enrolled and study groups

We enrolled 19 children (referred as atopics throughout the paper) with clinical diagnosis of allergy (rhinitis, asthma, grass pollen sensitization, allergic atopic dermatitis, oral allergy syndrome, cow's milk allergy) and encountering all the following criteria: (i) delivered naturally at term, (ii) breast fed for at least 3 months, (iii) aged between 4 and 14 years, (iv) no acute diseases for at least 2 weeks, (v) no antibiotic treatment in the last 3 months. In particular, 17 children presented allergic rhinitis, in 4 cases associated with asthma. Atopic dermatitis was observed in 8 cases of which 6 associated with rhinitis and inhalant sensitization and 1 with food allergy (**Table 4**). During the visit the children underwent a clinical evaluation and skin prick test for main food or inhalant allergens. Total and specific IgE determination was performed when clinically necessary. Fresh stool samples were collected within 3 days. As controls, 12 non-allergic children who encountered the same criteria above described but without family history of atopy were enrolled.

	Clinical diagnosis of allergy					
Sample ID	RC[a]	A[b]	GPS[c]	AD[d]	OAS[e]	CMA[f]
A1	√		√	√	√	
A2	√		√	√		
A3	√		√			
A4	√	√	√			
A5	√	√	√			
A6	√		√		√	√
A7	√		√	√		
A8	√		√	√		
A9	√	√	√		√	√
A10	√	√	√			
A12	√		√	√		
A13	√		√	√		
A14	√		√			
A15				√		
A17	√		√			
A19	√		√			
A20		√	√			
A21	√			√		
A22	√		√			

[a]Rhinitis.

[b]Asthma.

[c]Grass Pollen Sensitization.

[d]Allergic Atopic Dermatitis.

[e]Oral Allergy Syndrome.

[f]Cow's Milk Allergy.

Table 4. Allergic profile of the 19 atopic children enrolled in the study

Allergometric tests

Skin prick tests were performed following established guidelines (Dreborg et al., 1993). The following allergens were tested: cow's milk, egg, soy bean, wheat, peanut, codfish, grass pollen, Dermatophagoides pteronyssinus, Dermatophagoides

farinae, and cat dander. Other allergens were tested on the basis of the child's history. Data of the skin prick tests were used to determine the presence of atopic sensitization in the definition of allergic or non-allergic atopic dermatitis. The determination of total serum IgE was performed by ELISA test; the values were assumed as normal or increased in comparison with the ones from children of the same age group (Cavagni et al., 1980). The determination of specific IgE was performed by UNICAP 1000 (Phadia) in all patients for the following allergens: cow's milk, egg, soy bean, wheat, peanut, codfish, Bermuda grass, timothy grass, D. pteronyssinus, D. farinae, and cat dander. Other allergens were tested on the basis of the child's history.

DNA extraction and PCR

Total DNA from faecal material was extracted using QIAamp DNA Stool Mini Kit (Qiagen) according to the modified protocol reported by Candela et al. (Candela et al., 2010). Final DNA concentration was determined using NanoDrop ND-1000 (NanoDrop Technologies). PCR amplifications were performed with Biometra Thermal Cycler T Gradient (Biometra). The 16S rRNA gene was amplified using universal forward primer 27F and reverse primer r1492, following the protocol described by Candela et al. (Candela et al., 2010). PCR products were purified by using the Wizard SV gel and PCR clean-up System kit (Promega), eluted in 20 µl of sterile water and quantified with the DNA 7500 LabChip Assay kit and BioAnalyzer 2100 (Agilent Technologies). All the oligonucleotide primers used for PCR reactions and probe pairs employed for the array construction were synthesized by Thermo Electron.

HTF-Microbi.Array analysis

The original HTF-Microbi.array (Candela et al., 2010) was updated to include a probe for the detection of A. muciniphila. The new probe was designed and validated as reported by Candela et al. (Candela et al., 2010). Slide chemical treatment, array production, LDR protocol and hybridization conditions were carried out as previously reported (Castiglioni et al., 2004, Consolandi et al., 2006) with probe annealing set at 60°C. The LDRs were carried out in a final volume of 20 µl with 50 fmol of PCR product.

Data analysis

All arrays were scanned and processed according to the protocol and parameters already described by Candela et al. (2010). Fluorescence intensities (IF) were normalized on the basis of the synthetic ligation control signal: (a) outlier values (2.5-fold above or below the average) were discarded; (b) a correction factor was calculated in order to set the average IF of the ligation control to 50000 (n=6); (c) the correction factor was applied to both the probes and background IF values. Reproducibility of the experiments was assessed by calculating Pearson's correlation of the fluorescence signals between the two replicates. LDR experiments showing a Pearson's correlation coefficient <0.95 were repeated. Mean data from two replicated experiments were obtained and utilized for principal component analysis (PCA), box plot analysis and calculation of the probe relative IF contribution. Non-parametric Kruskal-Wallis test was used to determine whether the contribution of each bacterial group was significantly different between atopics and controls. Two-sided t-test was applied to evaluate whether the relative percentage contribution of each bacterial group was significantly different between the two groups. Correlation

between variables was computed by Spearman rank correlation coefficient. Statistical analyses were performed by using Canoco package for Windows (Leps et al., 2003) and the R statistical software (www.r-project.org).

Quantitative PCR

qPCR was carried out in a LightCycler instrument (Roche). Quantification of the 16S rRNA gene of *A. muciniphila*, *Faecalibacterium prausnitzii*, *Enterobacteriaceae*, *Clostridium* cluster IV, *Bifidobacterium* and *Lactobacillus* group was performed with the genus-, group- or species-specific primers reported in **Table 5**. SYBR Green I fluorophore was used to correlate the amount of PCR product with the fluorescent signal. For quantification, standard curves were generated with known amounts of pCR2.1 (Invitrogen)-cloned 16S rRNA gene from *A. muciniphila* (DSM22959), *F. prausnitzii* (DSM17677), *E. coli* (ATCC11105), *Clostridium leptum* (DSM753), *Bifidobacterium animalis* subsp. *lactis* (BI-07) and *Lactobacillus acidophilus* (LA-14). Amplification was carried out in a 20 µl final volume containing 100 ng of faecal DNA, 0.5 µM of each primer and 4 µl of LightCycler-FastStart DNA Master SYBR Green I (Roche). Amplifications were done under the following conditions: (i) starting preincubation at 95°C for 10 min; (ii) amplification including 35 cycles of 4 steps each at the temperature transition rate of 20°C/s: denaturation at 95°C for 15 s, annealing at the appropriate temperature (Table 5) for 20 s, extension at 72°C for 30 s, and fluorescence acquisition at the appropriate temperature (Table 5) for 5 s; (iii) melting curve analysis.

Target microorganism	Primer set	Sequence (5' to 3')	Product size (bp)	Annealing temp (°C)	Fluorescence acquisition temp (°C)
Akkermansia muciniphila	AM1	CAGCACGTGAAGGTGGGGAC	349	63	88
	AM2	CCTTGCGGTTGGCTTCAGAT			
Faecalibacterium prausnitzii	Fprau223F	GATGGCCTCGCGTCCGATTAG	199	67	85
	Fprau420R	CCGAAGACCTTCTTCCTCC			
Enterobacteriaceae	Eco1457F	CATTGACGTTACCCGCAGAAGAAG	195	63	87
	Eco1652R	CTCTACGAGACTCAAGCTTGC			
Clostridium Cl_IV	S-*-Clos-0561-a-S-17	TTACTGGGTGTAAAGGG	588	60	85
	S-*-Clept-1129.a-A-17	TAGAGTGCTCTTGCGTA			
Bifidobacterium	bif-164	GGGTGGTAATGCCGGATG	523	60	90
	bif-662	CCACCGTTACACCGGGAA			
***Lactobacillus* group**	Lac1	AGCAGTAGGGAATCTTCCA	327	61	85
	Lac2	ATTYCACCGCTACACATG			

Table 5. Primer sets used for the 16S rRNA gene quantification of *A. muciniphila*, *F. prausnitzii*, *Enterobacteriaceae*, *Clostridium* cluster IV, *Bifidobacterium* and *Lactobacillus* group by qPCR. Amplicon size, annealing and fluorescence acquisition temperature are also reported.

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Faecal microbiota profile of atopic children and healthy controls

The faecal microbiota of 19 atopic children and 12 healthy controls living in Italy was characterized by means of the HTF-Microbi.Array platform. Hybridization experiments were performed in two replicates. Pearson's correlation coefficients ranging from 0.95 and 0.99 were achieved between the two replicates, proving the high reproducibility of the phylogenetic profiles obtained by the HTF-Microbi.Array platform. A PCA of the fluorescence signals from atopics and controls was carried out. The diagnosis of atopy was considered as a dummy environmental variable. As shown in **Figure 12a**, the principal components PC2 and PC3, which collectively represented only a minor fraction of the total variance (9.7%), resulted in the separation of samples according to the health status. In order to identify the bacterial lineages showing differences in abundance between atopics and controls, probe fluorescence signals obtained from the HTF-Microbi.Array in atopics and controls were compared by box plot analysis. Probes showing $P < 0.3$ are represented in **Figure 12b**.

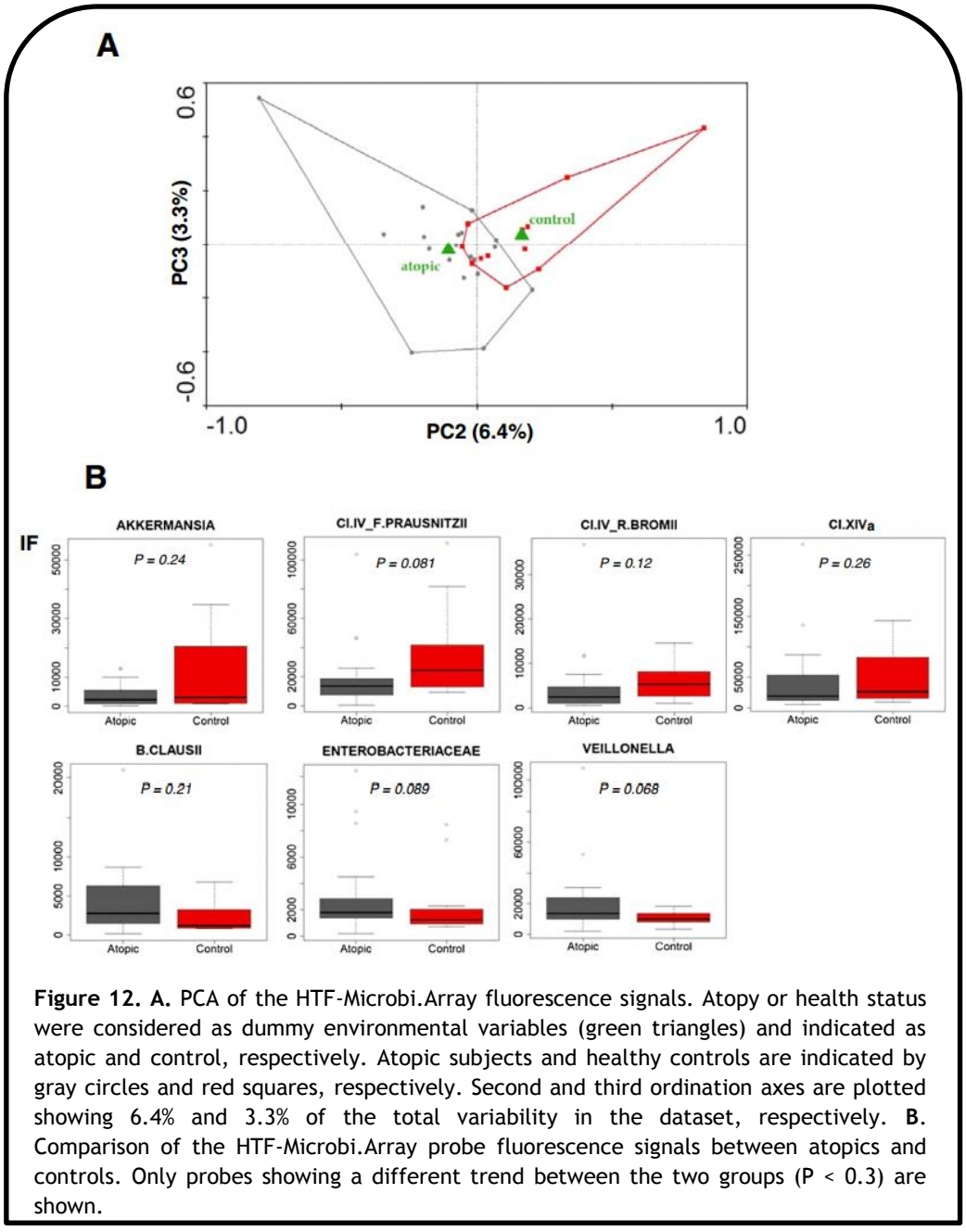

Figure 12. A. PCA of the HTF-Microbi.Array fluorescence signals. Atopy or health status were considered as dummy environmental variables (green triangles) and indicated as atopic and control, respectively. Atopic subjects and healthy controls are indicated by gray circles and red squares, respectively. Second and third ordination axes are plotted showing 6.4% and 3.3% of the total variability in the dataset, respectively. **B.** Comparison of the HTF-Microbi.Array probe fluorescence signals between atopics and controls. Only probes showing a different trend between the two groups ($P < 0.3$) are shown.

Atopic children showed a tendency towards reduction of *A. muciniphila*, *F. prausnitzii* et rel. and *Ruminococcus bromii* et rel. (*Clostridium* cluster IV), and

Clostridium cluster XIVa, and were enriched in *Enterobacteriaceae*, *Bacillus clausii* and *Veillonella parvula*.

On the basis of the HTF-Microbi.Array fluorescence data, the relative contribution of the major phyla in atopics and controls was calculated (**Figure 13**).

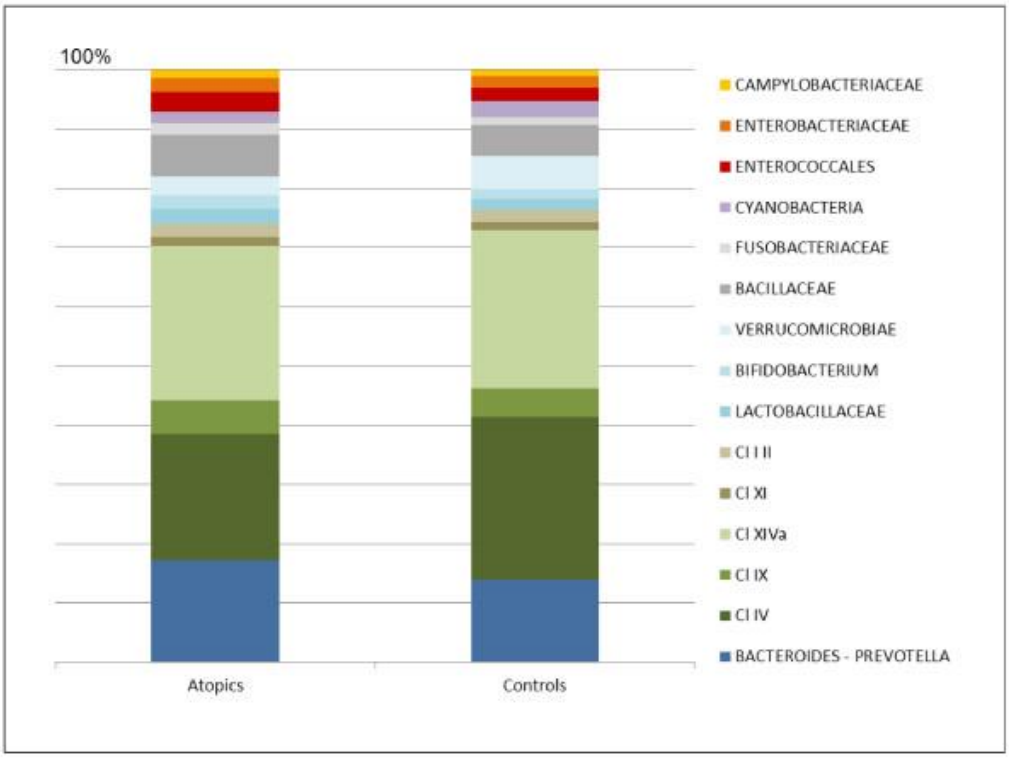

Figure 13. For each HTF-Microbi.Array probe, the relative fluorescence contribution was calculated as percentage of the total fluorescence. Sub-probes were excluded. Data represent the mean of the probe relative fluorescence contribution in atopics (n = 19) and controls (n = 12). P values derive from a two-sided t-test.

At high taxonomic level, atopics and controls showed a comparable overall phylogenetic composition of the faecal microbiota. Indeed, their microbiota resulted largely dominated by Bacteroidetes and Firmicutes, which together accounted for up to 90% of the faecal microbial community. With a relative

abundance ranging from 1 to 5%, Fusobacteria, Actinobacteria and Proteobacteria were sub-dominant components. However, focusing at lower taxonomic level, significant differences in the relative contribution of certain microbial groups were detected. In particular, atopics were characterized by a lower relative contribution of members of the *Clostridium* cluster IV (atopics, 20.9% - controls, 28.7%; P = 0.01) and a concomitant relative increase in *Enterobacteriaceae* (atopics, 2.4% - controls, 1.2%; P = 0.009) and *Fusobacteria* (atopics, 1.9% - controls, 1.2%; P = 0.001).

The abundance of *F. prausnitzii*, *A. muciniphila*, *Enterobacteriaceae*, *Clostridium* cluster IV, *Bifidobacterium* and *Lactobacillus* group in the faecal microbiota of atopics and controls was investigated by qPCR analysis of the

16S rRNA gene. As reported in **Table 6**, respect to healthy controls, atopics were significantly depleted in *F. prausnitzii*, *A. muciniphila* and members of the *Clostridium* cluster IV, and tended to be depleted in Bifidobacterium and enriched in *Enterobacteriaceae*.

	16S rRNA gene copies/µg faecal DNA		
Bacterial species / group	**Atopics**	**Controls**	**P value**
Faecalibacterium prausnitzii	6.17E+06	2.03E+07	**0.0014**
Akkermansia muciniphila	3.01E+05	5.03E+05	**0.0190**
Enterobacteriaceae	3.86E+04	1.19E+04	0.3500
***Clostridium* cluster IV**	4.46E+06	1.55E+07	**0.0035**
Bifidobacterium	1.08E+06	1.72E+06	0.0850
***Lactobacillacus* group**	3.75E+02	5.48E+02	0.6410

Table 6. qPCR quantification of *F. prausnitzii*, *A. muciniphila*, *Enterobacteriaceae*, *Clostridium* cluster IV, *Bifidobacterium* and *Lactobacillus* group in the faecal microbiota of atopics and healthy controls. For each bacterial species/group, the mean 16S rRNA copy number per µg of faecal DNA is reported.

Correlation among faecal microbiota, diagnosis of allergy and total IgE

In order to investigate whether the profile of the faecal microbial community of atopics correlated with their allergy profile, a PCA of the HTF-Microbi.Array fluorescence signals from atopics was carried out. PCA was performed by considering the types of allergic response as dummy environmental variables. No separation of the atopic children according to the specific diagnosis of rhinitis, asthma, grass pollen sensitization, allergic atopic dermatitis, oral allergy syndrome and cow's milk allergy was obtained, proving that the atopy-related dysbioses of the faecal microbiota are independent of the specific atopic outcome (data not shown).

In a subset of 10 atopy cases with clinical relevance the total serum IgE levels were determined. Total IgE ranged from 138 to 855 ku/L (geometric mean: 326 ku/L), a value above the normal for age (Cavagni et al., 1980).

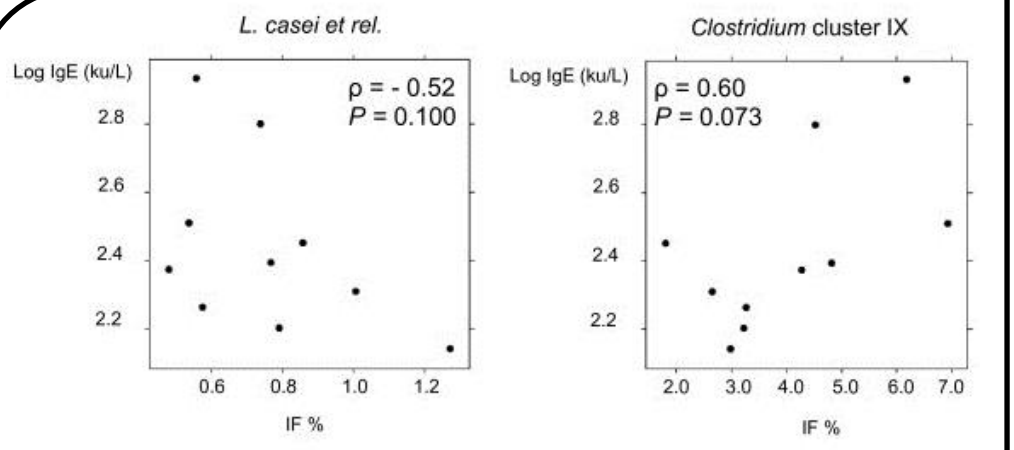

Figure 14. Spearman rank correlation between total IgE level and the abundance of *L. casei* et rel. and *Clostridium* cluster IX in the stools from a subset of 10 atopic children.

In order to investigate whether in this subset of 10 atopics IgE correlated with the relative abundance of a specific microbial group in the faeces, Spearman rank

correlation coefficients between the probe relative fluorescence signals and the IgE levels were calculated. According to our data no significant correlation was determined. However, a tendency towards an inverse correlation with IgE was obtained for *L. casei* et rel. (ρ = 0.52; P = 0.100), while *Clostridium* cluster IX abundance tended to be positively correlated with total IgE (ρ = 0.60; P = 0.073) (**Figure 14**).

CHAPTER 4
UNBALANCE OF INTESTINAL MICROBIOTA IN ATOPIC CHILDREN

1. *Brief introduction*
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At high phylogenetic level both atopics and controls showed a comparable overall microbiota profile where Firmicutes and Bacteroidetes constituted the two dominant divisions. However, focusing at lower taxonomic level, the intestinal microbiota of atopic children was characterized by a significant depletion in members of the *Clostridium* cluster IV, *F. prausnitzii*, *A. muciniphila* and a corresponding increase of the relative abundance of *Enterobacteriaceae*.

In a case-control DGGE-based study of the faecal microbiota from 20 allergic and 20 non-allergic 5-year-old Estonian children, Stsepetova *et al.* (2007) reported a less diverse composition in the faecal microbiota from atopic children but, according to the Authors, no bacterial targets could distinguish infants with or without atopy. However, the DGGE-based approach allowed to consider only the dominant fraction of the intestinal microbiota, remaining blind with respect to the whole phylogenetic complexity of the ecosystem. In an elegant 16S rDNA pyrosequencing-based dynamic study, Hong *et al.* (2010) addressed the differences in the microbiota succession between 3 infants with and 4 without eczema over 4 time points until 24 months of age. Even if age was shown to be the dominant factor mediating microbiota

changes, matched by age eczema infants were characterized by a higher abundance of the enterobacteria *Klebsiella* and *Shigella* as well as *Enterococcus*, while *Bifidobacterium* showed a higher abundance in non-eczema ones. These last data are in general agreement with the intestinal microbiota dysbioses observed in our study.

Although *Bifidobacterium* and *Lactobacillus* have been traditionally indicated as possible protective factors against atopic disease in childhood (Vael et al., 2009), we did not detect any significant differences in these health-promoting genera between atopics and controls, confirming previous findings reported by Penders *et al*. (2007a; 2007b). However, molecular studies at the species level showed a different distribution of the *Bifidobacterium* and *Lactobacillus* species between allergic and non-allergic children (Stsepetova et al., 2007; Penders et al., 2010), suggesting a potential species-specific effect of *Bifidobacterium* and *Lactobacillus* in the etiology of atopic disorders.

The atopy-related microbiota dysbioses we depicted in our cohort of 19 children were independent of their peculiar allergic profile. A subset of 10 atopics underwent clinical evaluation of total IgE level and the correlation between IgE and the relative abundance of specific microbial groups in the faeces was explored. Even if no significant correlation was determined, *L. casei* et rel. and *Clostridium* cluster IX tended to be negatively and positively correlated with IgE, respectively. Interestingly, Ogawa et al. (2006) demonstrated that orally administered *L. casei* was effective in the control of the IgE levels in human allergic reactions and, recently, Schiffer *et al*. (2011) reported that *L. casei* could inhibit the effector phase of immune inflammation *in vivo*. Finally, Penders *et al*. (2010) showed a decreased risk of atopic dermatitis in children colonized by *L. paracasei*, a member

of the *L. casei et rel.* group. Even if these studies may support the tendency towards inverse correlation between *L. casei et rel.* and IgE level we observed in our study, caution must be taken in considering these data since only a low number of children were analyzed.

Characterized by a decrease of the absolute levels of *Clostridium* cluster IV, *F. prausnitzii* and *A. muciniphila*, as well as a corresponding increase in the relative abundance of *Enterobacteriaceae*, the atopy-associated intestinal microbial community we described in this study is depleted in key immunomodulatory members of the human intestinal microbiota and possibly enriched in pro-inflammatory "pathobionts" (Chow et al., 2010). By the specific induction of T regs, members of the *Clostridium* cluster IV have been demonstrated to be strategic for maintaining the immune homeostasis (Atarashi et al., 2011). Analogously, providing a vast range of anti-inflammatory effects, *F. prausnitzii* has been considered as a crucial microorganism for gut homeostasis (Sokol et al., 2008). Finally, *A. muciniphila* is a common member of the human intestinal tract which has been recently associated with a protective/anti-inflammatory role in healthy gut (Ogawa et al., 2006). On the other hand, *Enterobacteriaceae* have been reported to prosper in the context of a host-mediated inflammatory response (Png et al., 2008). Capable to venture more deeply in the mucus layer and establish a close interaction with the epithelial surface, members of *Enterobacteriaceae* concur in the induction of a pro-inflammatory response and further consolidate the host inflammatory status. Thus, similarly to the one characterized in IBD (Sokol et al., 2008; Lupp et al., 2007; Frank et al., 2007), the atopy-associated microbiota can represent an inflammogenic microbial consortium which can contribute to the severity of the disease (Kau et al., 2011).

CHAPTER 5
THE INTESTINAL MICROBIOME OF THE HADZA HUNTER-GATHERERS: THE LIFESTYLE EFFECT

1. **Brief introduction**
2. *Materials and Methods*
3. *Results*
4. *Discussion*

Comparative studies between unindustrialized rural communities from Africa and South America and industrialized Western communities from Europe and North America have revealed specific gut microbiota adaptations to their respective lifestyles. These adaptations include higher biodiversity and enrichment of Bacteroidetes and Actinobacteria in rural communities, and an overall reduction in microbial diversity and stability in Western populations (De Filippo et al., 2010; Rinke et al., 2013). Unindustrialized small-scale rural societies are targets for understanding trends in human-gut microbiota interactions because they rely less on antibiotics and sterile cleaners, and often consume a greater breadth of unrefined seasonally available foods (Yatsunenko et al., 2012). Yet despite recent focus on rural societies, there remains a significant gap in our knowledge of the microbe-host relationship among hunter-gatherer populations. This is especially problematic because humans have relied on hunting and gathering for 95% of our evolutionary history.

To explore how a foraging subsistence strategy influences gut microbiota profiles, we collected and analyzed faecal microbiota from 27 Hadza hunter-gatherers from

two separate camp sites (see **Figure 15** for a more detailed description of Hadza ecology). Phylogenetic diversity, taxonomic relative abundance, and the short chain fatty acid profile of the Hadza microbiome were compared to those of 16 Italian adults.

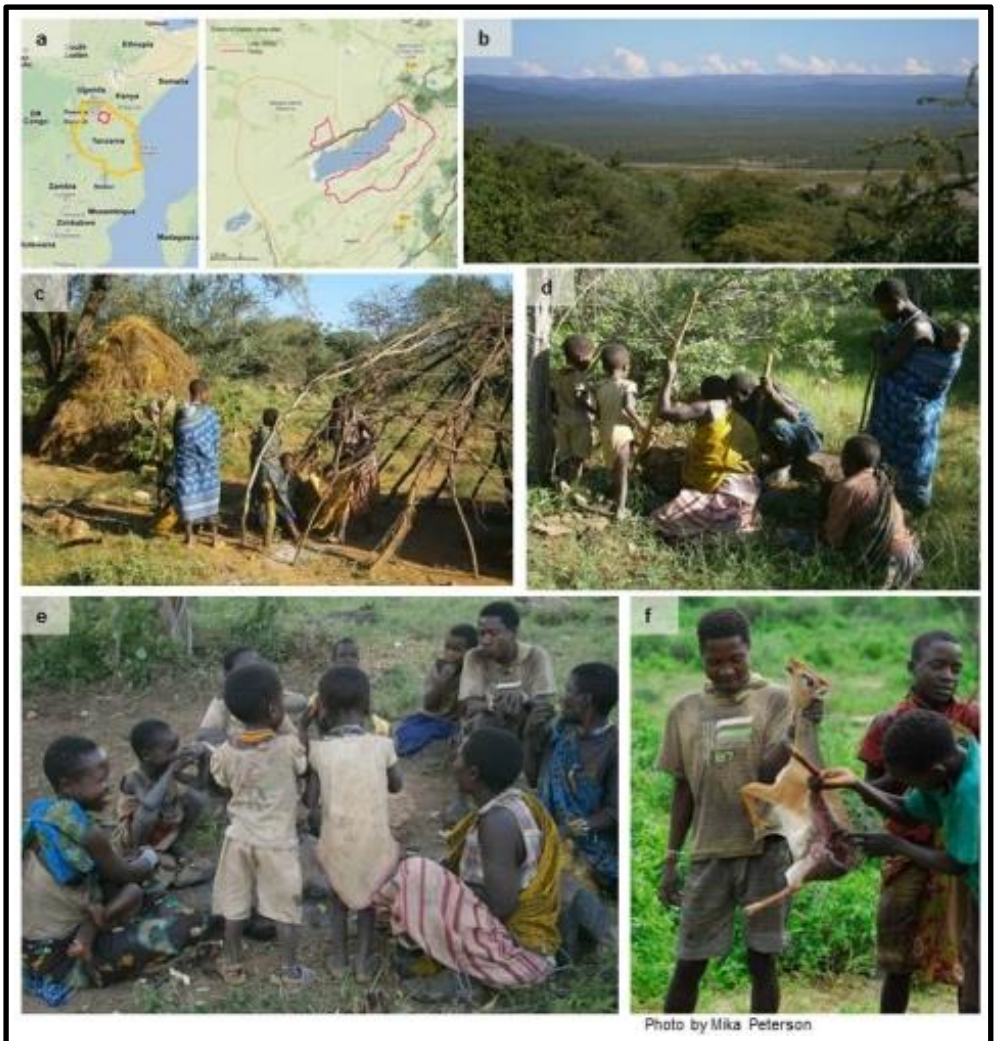

Figure 15. A glimpse into the life of Hadza. (a) Location of Hadza land in northern Tanzania; (b) top of the ridge near Dedauko camp overlooking Lake Eyasi in between two phases of the rainy season when the lake is still partially full; (c) Hadza women and children near the traditional temporary grass shelters; (d) women and children digging out tubers using fire-hardened digging sticks; (e) members of camp gather around a tuber roasting fire; (f) young men analyze the arrow entry wound for a recently killed dik-dik (Madoqua kirkii).

We compared these data with previously published data on two different rural African groups from Burkina Faso and Malawi (De Filippo et al., 2010; Yatsunenko et al., 2012) to identify gut microbiota features unique to the Hadza lifestyle. The Hadza are one of the few remaining hunting and gathering communities in the world and live in the Rift Valley ecosystem around the shores of Lake Eyasi in northwestern Tanzania. The Hadza diet consists of wild foods that fall into five main categories: meat, honey, baobab, berries, and tubers (Schoeninger et al., 2001; Murray et al., 2001; Marlowe et al., 2009). They practice no cultivation or domestication of plants and animals and receive minimal amounts of agricultural products (< 5% of calories) from external sources (Marlowe, 2010). By comparison, the diet of the Italian cohort derives almost entirely from commercial agricultural products and adheres largely to the Mediterranean diet (abundant plant foods, fresh fruit, pasta, bread, and olive oil; low to moderate amounts of dairy, poultry, fish, and red meat). Additionally, the majority of carbohydrates (based on gram amount) came from easily digestible starch (54%) and sugar (36%) while very little was derived from fiber soluble or insoluble (10%).

The Hadza live in small mobile camps with fluid membership, usually comprising a core group of approximately 30 people (Blurton et al., 1992). While the Hadza are a modern human population, they live in a key geographic region for studies of human evolution and target resources similar to those exploited by our hominin ancestors. The Hadza therefore represent the closest living approximation to a Paleolithic lifestyle. This study presents the very first characterization of the forager gut microbiota from Hadza microbiota, and will allow us to understand how the human microbiota aligns with a foraging lifestyle, one in which all human ancestors participated prior to the Neolithic transition.

CHAPTER 5
THE INTESTINAL MICROBIOME OF THE HADZA HUNTER-GATHERERS: THE LIFESTYLE EFFECT

Subject recruitment, dietary informations, amplicon sequencing and metabolomics analysis

Enrollment of Hadza volunteers was conducted at two separate camp sites of traditionally living Hadza in the Yaeda Valley region of Northwestern Tanzania. Italian subjects were recruited from the Bologna metropolitan area. All work was approved by the University of Leipzig Ethik-Kommission review board on May 29, 2012, reference number 164-12-21052012. Permission for this work was granted from the Tanzanian Commission for Science and Technology (COSTECH), permit No. 2012-315-NA-2000-80.

Dietary recalls for the Italian control cohort were collected over three 24 hour periods and included two week-days and one weekend day. Reports were then entered and analyzed using The Food Processor SQL version 10.13.0.

Total DNA from faecal material was extracted using QIAamp DNA Stool Mini Kit (QIAGEN). DNA amplification was conducted on the V4 region of the 16S rDNA gene and PCR products pyrosequenced according to the GS-FLX Titanium sequencing

protocol. qPCR for *Bifidobacterium* quantification was performed as reported by Candela et al. (2012a).

Metabolomic analysis to determine SCFAs in faecal samples was accomplished using HS-SPME GC-MS. Kendall correlation test between SCFA levels and the relative abundance of genera was achieved using the R package "Stats".

Bioinformatic analysis of 16S rDNA gene sequencing data and statistical methods

Sequencing reads were analyzed using the QIIME pipeline (Caporaso et al., 2010) as described previously (Claesson et al., 2012). Alpha-diversity and rarefaction plots were computed using four different metrics: Shannon, PD whole tree, chao1 and observed species. Weighted and unweighted UniFrac distances and Euclidean distance of genus level relative abundance were used to perform Principal Coordinates Analysis (PCoA). PCoA, and heatmap and bar plots were built using the packages Made4 (Culhane et al., 2005) and Vegan (Oksanen et al., 2013). The R packages Stats and Vegan were used to perform statistical analysis. In particular, to compare gut microbiota structure among different populations for α and ß diversity we used a Wilcoxon signed rank test. Data separation in the PCoA was tested using a permutation test with pseudo F-ratios (function Adonis in the Vegan package). Cluster separation in hierarchical clustering analyses was assessed for significance using Fisher's exact test. Significant differences in phylum or genus level abundance between Hadza and Italians, and between Hadza males and females, were assessed by Mann-Whitney U tests, and corrected for multiple comparisons using the Benjamini-Hochberg method when appropriate. FDR < 0.05 was considered as statistically significant.

Kendall correlation test between SCFA levels and the relative abundance of genera was achieved using function “cor.test” of the package “Stats” of R. Sequences from Yatsunenko *et al.*, 2012 and De Filippo *et al.*, 2010 were obtained from MG-RAST and ENA repositories, respectively, and processed and assigned following the QIIME pipeline. Bacterial co-abundance groups (CAGs) were determined as described previously (Claesson et al., 2012). Briefly the associations among the genera were evaluated using Kendall correlation test, visualized using hierarchical Ward clustering with a Spearman correlation distance metrics and used to define co-abundant genera groups. The significant associations were controlled for multiple testing using the qvalue method (FDR < 0.05; Dabney et al., 2013). Permutational MANOVA (Anderson et al., 2001) was used to determine if the CAGs were significantly different from each other. The Wiggum plot network analysis was created as previously described (Claesson et al., 2012) using cytoscape software (http://www.cytoscape.org/). Circle size represents genus abundance and connections between nodes represent positive and significant Kendall correlations between genera (FDR < 0.05).

CHAPTER 5
THE INTESTINAL MICROBIOME OF THE HADZA HUNTER-GATHERERS: THE LIFESTYLE EFFECT

1. *Brief introduction*
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Characterization of Hadza microbiota

Faecal samples from 27 Hadza, aged 8-70 years, mean age 32 years, and 16 Italians aged 20-40 years, mean age also 32 years, were collected and pyrosequenced in the V4 gene region of bacterial 16S rDNA, resulting in 309,952 high-quality reads and an average of 7208 ± 2650 reads per subject. We used several different metrics to calculate α-diversity (**Figure 16**). All measures indicate a much higher gut microbiota diversity within the Hadza than in Italian samples ($P < 0.001$).

To explore variation within the Hadza gut microbiota, we used weighted and unweighted UniFrac distances to assess differences based on camp location and sex. We found no significant difference in phylogenetic diversity or relative abundance between camps. However, unlike the Italian cohort, the Hadza gut microbiota does show significant separation by sex based on weighted UniFrac distance ($P < 0.05$). Analogous results were obtained when Euclidean and Bray-Curtis distance of genera relative abundance were considered ($P < 0.05$) (**Figure 17**). To determine a structural basis for the observed separation, we compared genera relative abundance between Hadza men and women using a Mann-Whitney U test, and found

a significantly increased abundance of *Treponema* ($P < 0.05$) in women and increased *Eubacterium* ($P < 0.05$) and *Blautia* ($P < 0.001$) in men.

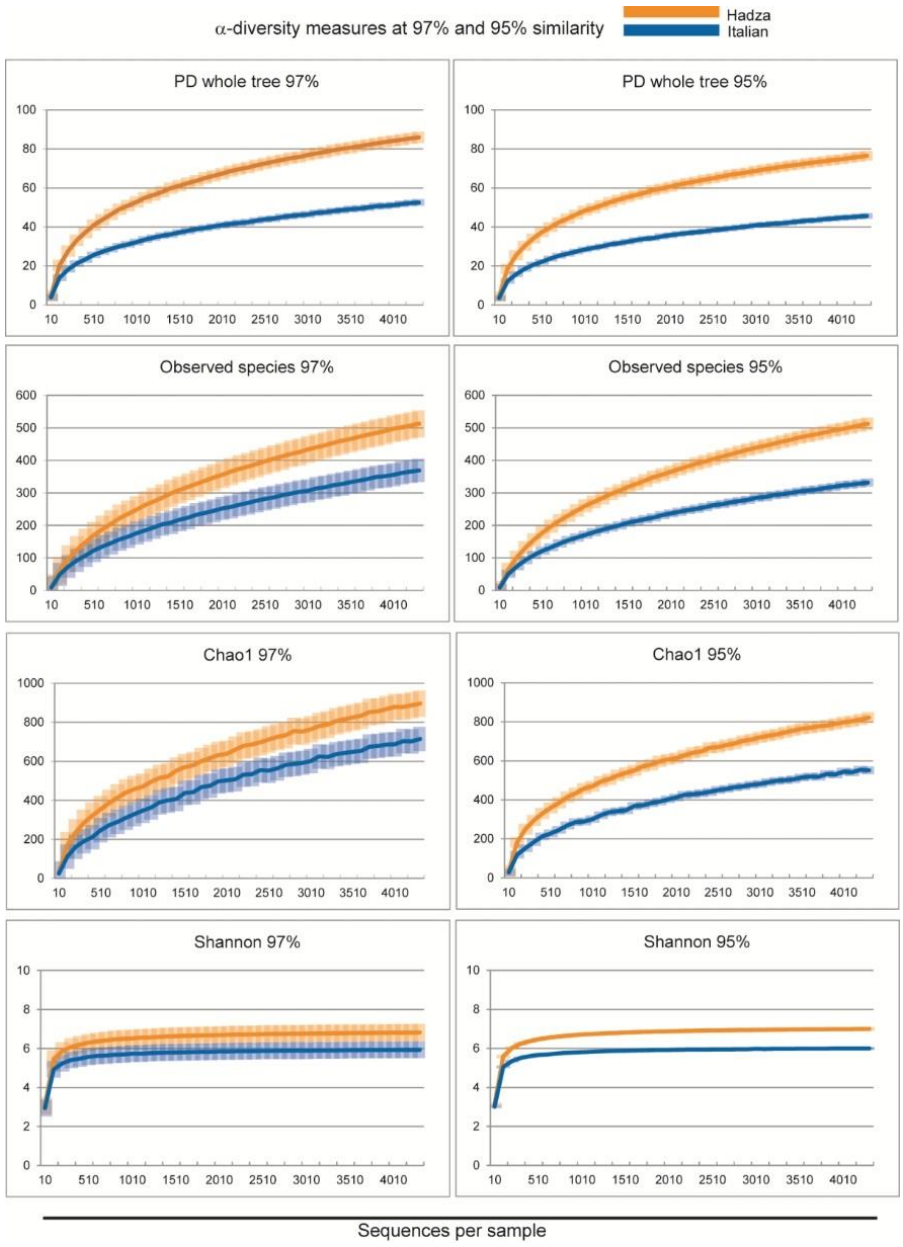

Figure 23. Rarefaction curves of different α-diversity metrics. The OTU table at 0.05 and 0.03 similarity thresholds was rarefied up to 4500 reads per sample and analyzed using various diversity metrics for both Hadza and Italian subjects.

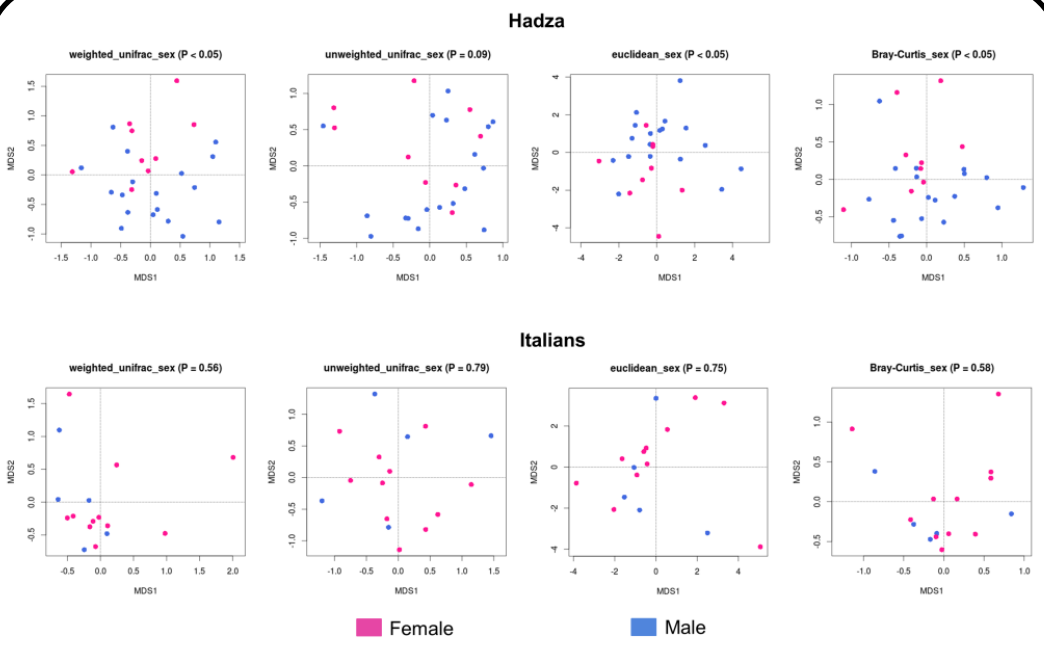

Figure 17. PCoAs based on unweighted and weighted UniFrac distances as well as Euclidean and Bray-Curtis distances show patterns of separation by sex within each subject cohort and their respective significance values. Pink, females; blue, males.

Detailed comparison to Italian controls

The Hadza and Italian gut microbiota profiles are quite distinct. Community structure visualized using PCoA of weighted and unweighted UniFrac distances reveal a sharp segregation along PCo1, indicating a strong core division in gut microbiota phylogeny between Hadza and Italian individuals ($P < 0.001$) (**Figure 18**). Mean values of unweighted UniFrac distances also reveal lower within-group variability of taxonomic diversity among Hadza than Italians ($P < 0.001$). This similarity in breadth of phylogenetic diversity among Hadza is likely a result of close proximity community living with food sharing. The Hadza and Italian samples show many notable differences in microbiota relative abundance at both phylum and genus levels.

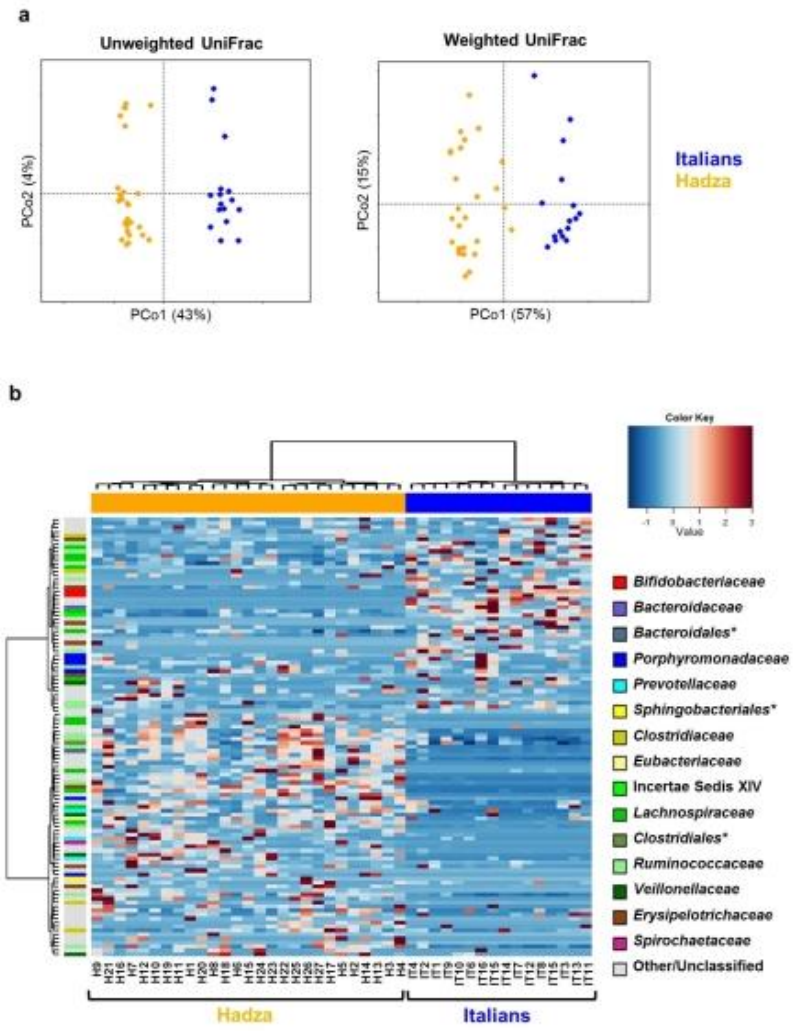

Figure 18. Unweighted and weighted UniFrac distance PCoA of the fecal microbiota from 27 Hadza (orange dots) and 16 Italians (blue dots) (**a**). Hierarchical Ward-linkage clustering based on the Spearman correlation coefficients of genus proportion (**b**). Genera were filtered for subject prevalence of at least 30% within a population. Subjects are clustered on top of the panel and color-coded as in **a**. 110 genera clustered by the vertical tree are color-coded by family assignment. *denotes unclassified OTU reported at higher taxonomic level.

All the significant differences are listed in **Table 7**. Notably, there are many unclassified genera belonging to Bacteroidetes, Clostridiales and *Ruminococcaceae* in the Hadza gut microbiota, emphasizing our still limited ability to identify

community dependent bacteria. The absence of *Bifidobacterium* in the Hadza gut microbiota was confirmed by qPCR.

a

over-represented in Hadza

Phylum	**Italians ± SEM**		**Hadza ± SEM**		**FDR**
Bacteroidetes	10.1	2.7	17.4	1.1	0.002
Bacteria unclassified	0.2	0.05	1.8	0.2	< 0.001
Proteobacteria	0.3	0.04	5.9	1.2	< 0.001
Spirochaetes	0	0	2.8	0.9	< 0.001
Genus					
Anaerophaga	0	0	0.6	0.1	< 0.001
Bacteroidales unclassified	0.07	0.02	0.8	0.1	< 0.001
Porphyromonadaceae unclassified	0.03	0.02	0.9	0.3	< 0.001
Prevotella	0.4	0.3	6.2	1.1	< 0.001
Bacteroidetes unclassified	0.2	0.07	6.6	0.8	< 0.001
Sphingobacteriales unclassified	0	0	0.6	0.1	< 0.001
Eubacterium	1.4	0.6	2.2	0.5	0.013
Robinsoniella	0.02	0	0.9	0.2	< 0.001
Clostridiales unclassified	7.4	0.8	11.1	0.6	< 0.001
Butyricicoccus	0.4	0.05	2.6	0.5	< 0.001
Hydrogenoanaerobacterium	0.07	0.03	1	0.1	< 0.001
Oscillibacter	1	0.3	3.8	0.4	< 0.001
Ruminococcaceae unclassified	3.6	0.6	8.6	0.7	< 0.001
Sporobacter	0.8	0.2	3.7	0.4	< 0.001
Veillonellaceae unclassified	0.02	0.01	0.8	0.2	< 0.001
Bulleidia	0	0	0.7	0.07	< 0.001
Catenibacterium	0.7	0.4	1.4	0.4	0.001
Firmicutes unclassified	0.3	0.1	0.8	0.1	< 0.001
Ruminobacter	0	0	1.2	0.7	0.045
Succinivibrio	0.02	0.02	2.8	0.7	< 0.001
Proteobacteria unclassified	0.02	0.01	0.5	0.2	< 0.001
Treponema	0	0	2.5	0.7	< 0.001

b

over-represented in Italians

Phylum	Italians ± SEM		Hadza ± SEM		FDR
Actinobacteria	8.3	1.5	0.1	0.02	< 0.001
Firmicutes	80.9	2.7	71.8	1.9	0.006
Genus					
Bifidobacterium	8.1	1.5	0.02	0.01	< 0.001
Bacteroides	7.1	2	0.2	0.04	< 0.001
Alistipes	0.9	0.2	0	0	< 0.001
Blautia	9.5	1.3	3.5	0.4	< 0.001
Anaerosporobacter	0.6	0.1	0.2	0.04	0.003
Coprococcus	3.6	0.7	1.4	0.2	0.005
Dorea	1.4	0.2	0.4	0.06	< 0.001
Lachnospiraceae unclassified	6.4	0.7	3.9	0.2	0.001
Roseburia	7.7	1.3	3.9	0.8	0.014
Faecalibacterium	18.5	2.4	11.8	1	0.022
Ruminococcus	8.6	1.8	2.1	0.3	0.005
Erysipelotrichaceae unclassified	1.5	0.3	0.7	0.2	0.026

Table 7. Summary of taxa differences between Hadza and Italian subjects. Bacterial phyla and genera significantly over-represented in the gut ecosystem of Hadza (**a**) and Italians (**b**). For each microbial group, the mean relative abundance (%) ± SEM and P-value of the differences between the two populations (Mann-Whitney U test) are reported.

Taken together, data from our gut microbiota comparative analysis indicate a characteristic configuration for the Hadza gut microbial ecosystem that is profoundly depleted in *Bifidobacterium*, enriched in Bacteroidetes and *Prevotella*, and comprising an unusual arrangement of Clostridiales. This arrangement is defined by a general reduction of well-known butyrate producers, members of the *Clostridium* clusters IV and XIVa, and a corresponding increase in unclassified Clostridiales and *Ruminococcaceae*. Interestingly, the Hadza gut microbiota is also characterized by a relevant enrichment in what are generally considered opportunistic microorganisms, such as members of Proteobacteria, *Succinivibrio*, and *Treponema*.

Comparison to African agricultural societies

In order to explore community-level relationships within the gut microbiota that may be unique to a foraging lifestyle, we sought associations among genera by including two previously published rural African groups with an agriculture-based subsistence and their respective Western controls: 11 Mossi children from the Boulpon village, Burkina Faso (BF) aged 5-6 years and 12 Italian children aged 3-6 years (De Filippo et al., 2010); 22 young adult members from four rural Malawian communities, Chamba, Makwhira, Mayaka and Mbiza aged 20-44 years and 17 US adults aged 24-40 years (Yatsunenko et al., 2012).

Clustering analysis shows a significant ($P < 0.001$) separation among Hadza, Malawians, BF and Western controls (**Figure 19a**). PCoA based on Bray Curtis distances of genera relative abundance confirms this separation ($P < 0.001$) (**Figure 19b**). Interestingly, PC1, which represents the 30% of the total variability, shows a clear separation between the Western controls and the African populations, while PC2, which explains a lower fraction of the total variability (19%), indicates a separation among Hadza, Malawians and BF. Separation along PC2 is also visualized between Western populations, but to a much lesser degree and with large interspersion between the U.S. and Italian children. Our data demonstrate biologically meaningful variation between the Western and non-Western gut microbiota profiles, showing that African populations with different lifestyle possess an overall more similar gut microbiota to each other than to Western populations. Caution is needed since subjects from all 6 populations are not age-matched and we cannot exclude a partial influence of a study effect on the results.

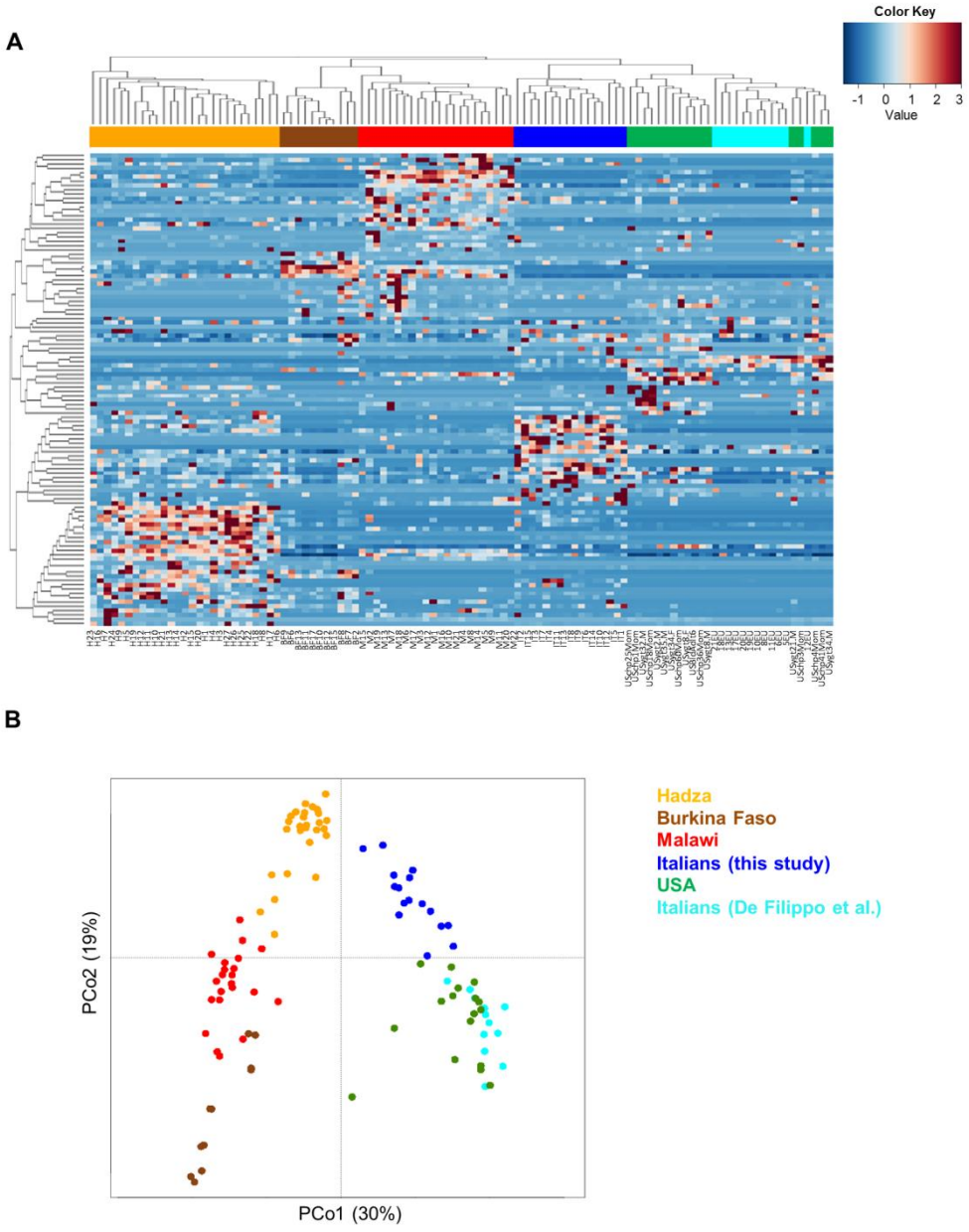

Figure 19. (**a**). Hierarchical average-linkage clustering based on Eisen's formula of correlation similarity metric of bacterial genus proportion and average linkage. Genera were filtered for subject prevalence of at least 30% of samples. Subjects are clustered in the top of panel and color-coded as in a. 107 genera are visualized. (**b**). PCoA based on Bray Curtis distances of the relative abundance of gut intestinal genera.

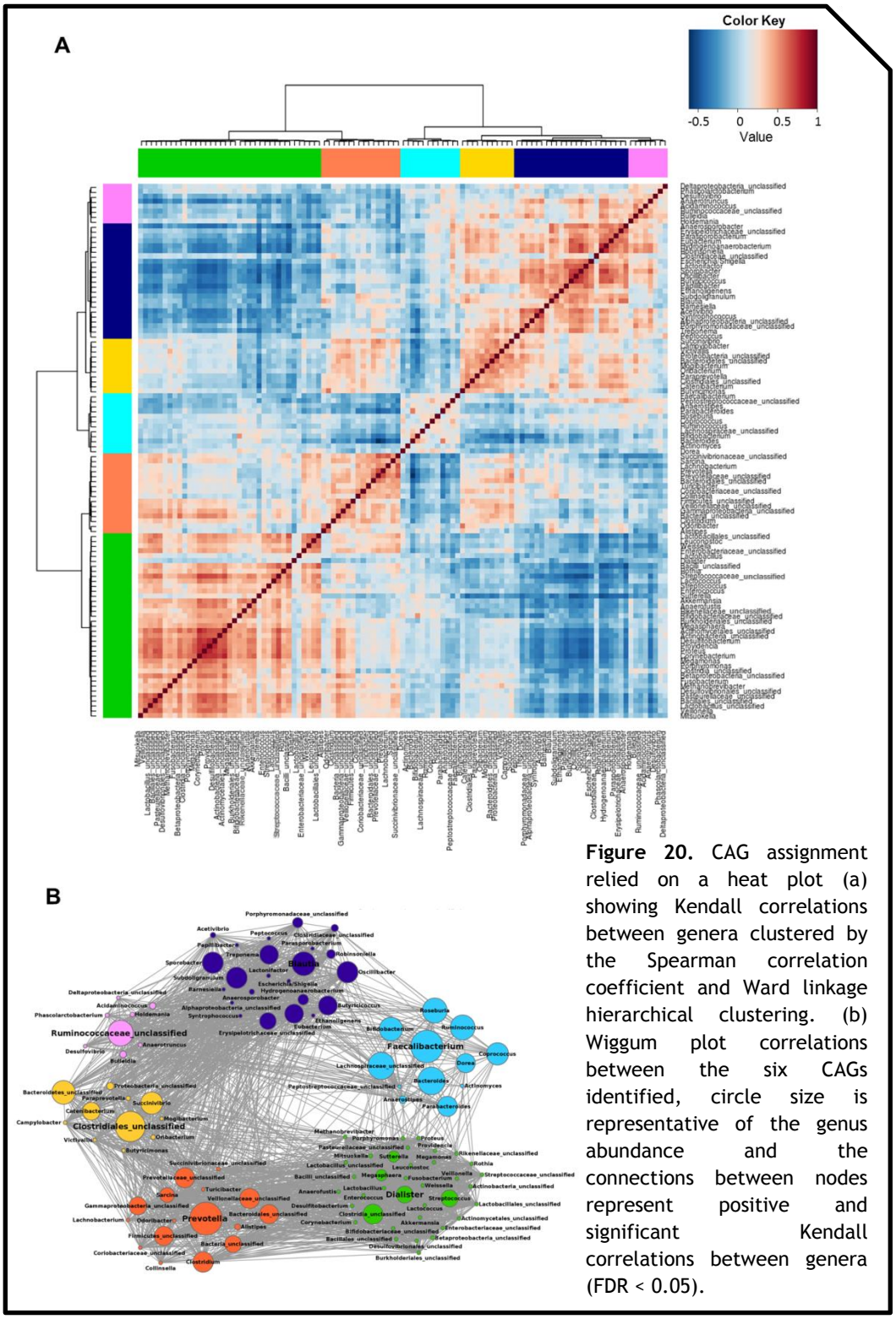

Figure 20. CAG assignment relied on a heat plot (a) showing Kendall correlations between genera clustered by the Spearman correlation coefficient and Ward linkage hierarchical clustering. (b) Wiggum plot correlations between the six CAGs identified, circle size is representative of the genus abundance and the connections between nodes represent positive and significant Kendall correlations between genera (FDR < 0.05).

To identify patterns of microbial community variation among Hadza, Malawian, BF and Western controls - Italian adults, Italian children, and US adults we determined co-abundance associations between genera and then clustered them, resulting in six co-abundance groups (CAGs) (**Figure 20**). In the context of this comparison, six CAGs define the microbial variation between populations (P<0.001). CAGs have been named according to the dominant genera in each group as follows: *Dialister*, *Faecalibacterium*, *Prevotella*, *Blautia*, *Clostridiales*_unclassified and *Ruminococcaceae*_unclassified. The Wiggum plot depicts the gut microbiota compositional relationship for each of the six populations and shows a correspondingly unique pattern of abundance of the six CAGs (**Figure 21**). Interestingly, African populations were characterized by the *Prevotella* CAG, while Western controls show a distinctive overall dominance of the *Faecalibacterium* CAG. With respect to Malawian and BF, Hadza show a peculiar enrichment of *Clostridiales*_unclassified, *Ruminococcaceae*_unclassified and *Blautia* CAGs. Given the dietary and lifestyle distinctions of each population, the CAG distribution in Hadza, Malawians, BF, and Western controls could represent predictable gut microbiota community specificity to three different modes of subsistence: foraging, rural farming, and industrial agriculture respectively. The unique CAG distribution of Hadza with respect to the other groups corresponds to the higher abundance of *Treponema* and unclassified *Bacteroidetes* and *Ruminococcaceae* co-residents in the Hadza microbiome. All Hadza we sampled share this configuration, therefore we must posit the possibility that these bacteria and their co-residents confer a structural and functional asset responding to the specific needs of the Hadza lifestyle. However, more forager and subsistence agriculture communities should be

sampled to learn what aspects of subsistence drive microbe community assimilation and whether variability is a result of environment, host selection, or both.

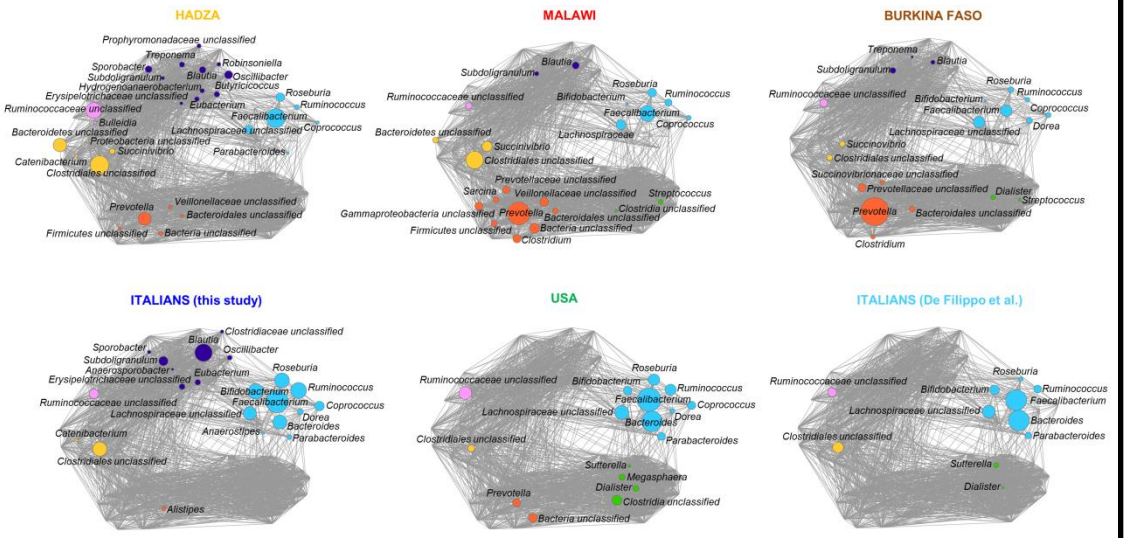

Figure 21. Distinct bacterial co-abundance groups define each population. Wiggum plots indicate pattern of variation of the six identified co-abundance groups (CAGs) in Hadza, Malawi, Burkina Faso, and Western controls. CAGs are named with the name of the most abundant genera and are color-coded as follows: *Faecalibacterium* (cyan), *Prevotella* (orange), *Dialister* (green), *Clostridiales*_unclassified (yellow), *Ruminococcaceae*_unclassified (pink), and *Blautia* (blueviolet). Each node represents a bacterial genus and its dimension is proportional to the mean relative abundance within the population. Connections between nodes represent positive and significant Kendall correlation between genera (FDR < 0.05).

Short chain fatty acid profile of Hadza and Italians

End-products of bacterial fermentation are important for microbiota-host co-metabolism and evolution. SCFAs are the dominant metabolites resulting from bacterial fermentation of plant derived substrates such as glycans and polysaccharides that pass undigested through the small intestine and into the colon. PCA analysis of the SCFA relative abundance profiles show a segregation between Hadza and Italians (P = 0.02) (**Figure 22**). The Italian samples are characterized by a significantly (P < 0.01) greater relative abundance of butyrate, while Hadza samples are enriched in propionate (P < 0.01). These differences may reflect dietary variation in both amount and type of fiber and carbohydrates consumed by Hadza and Italians, and the consequent relative depletion in butyrate producers belonging to the *Clostridium* cluster IV and XIVa in Hadza. However, because of the high degree of metabolic cross-feeding between members of the human gut microbial ecosystem, direct associative relations between bacteria presence/absence and SCFA production are not so simple. In order to investigate gut microbial networks on the basis of the observed differences in patterns of SCFA production in Hadza and Italians, we evaluated the gut microbiota genera that correlate significantly with each SCFA. Among genera with greater than 5% relative abundance in at least one of the two populations, *Bifidobacterium*, *Bacteroides*, *Blautia*, *Faecalibacterium* and *Ruminococcus* are positively (P < 0.05) correlated with butyrate, showing Kendall correlation values of 0.30, 0.31, 0.32, 0.52 and 0.30, respectively. In contrast, *Bifidobacterium*, *Blautia* and *Lachnospiraceae* show a significant (P < 0.05) negative correlation with propionate of -0.36, -0.27 and -0.24, respectively, while *Prevotella* demonstrates a positive correlation of 0.41. The absence of *Bifidobacterium* and the lower relative abundance of *Blautia*,

Ruminococcus and *Faecalibacterium* concurrent with greater relative abundance of *Prevotella* seen in the Hadza gut microbiota match a presence/absence scenario with SCFA concentrations that are enriched in propionate and reduced in butyrate with respect to Italians.

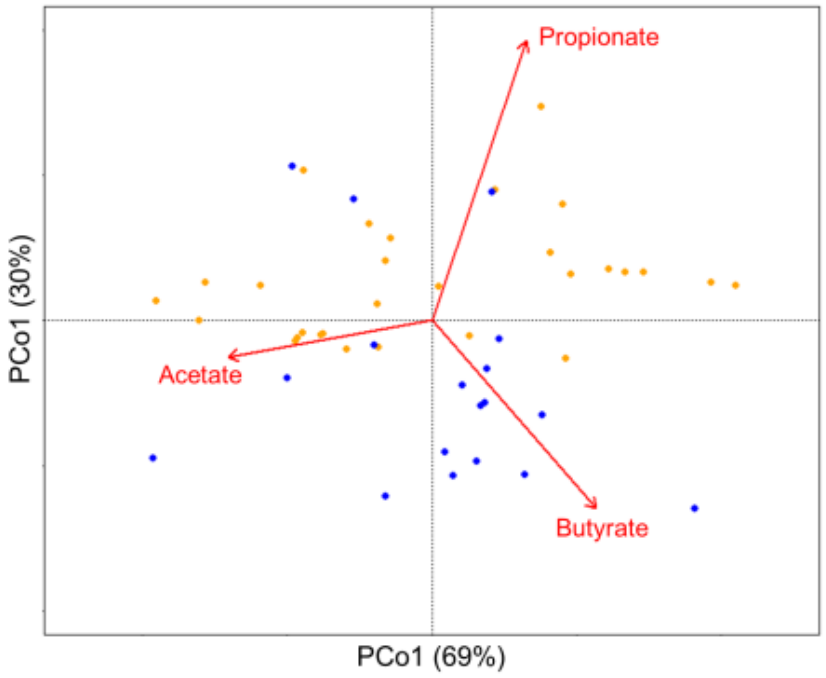

Figure 22. Comparison of metabolite production between Hadza and Italian samples. PCoA based on Euclidean distances of the profiles of short chain fatty acid relative abundance in Hadza (orange) and Italians (blue). Vectors show propionate, butyrate and acetate abundance.

CHAPTER 5
THE INTESTINAL MICROBIOME OF THE HADZA HUNTER-GATHERERS: THE LIFESTYLE EFFECT

1. *Brief introduction*
2. *Materials and Methods*
3. *Results*
4. ***Discussion***

A foraging lifestyle induces unique gut microbiota adaptations

The Hadza represent a rare example of human subsistence through hunting and gathering that persists in the same East African region where early hominins lived. The Hadza maintain a direct interface with the natural environment, deriving their food, water, and shelter from a rich biosphere blanketed in the complexity of microbial communities and interactions. In our characterization of the Hadza gut microbiota, we report several findings that we feel support the conception of the microbiome as a diverse and responsive ecosystem adapting continuously as a commensal component of the host supra-organism. Keeping this framework in mind, we interpret the gut microbiota structure as an adaptation to the Hadza foraging lifestyle.

Fiber degrading specializations of the Hadza gut microbiota

In the ligth of these consideration the Hadza gut microbiota has characteristic features that are consistent with a heavily plant-based diet. Besides the presence of several well-known fiber-degrading Firmicutes that are also shared with Italians –

e.g. members of *Lachnospiraceae*, *Ruminococcaceae*, *Veillonellaceae*, *Clostridiales* Incertae Sedis XIV and *Clostridiaceae*, the Hadza gut microbiota is enriched in *Prevotella, Treponema,* and unclassified members of Bacteroidetes, Clostridiales and *Ruminococcaceae*. These xylan-degrading *Prevotella* (Flint et al., 2008) and *Treponema* (Warnecke et al., 2007) and the abundance of still unclassified Bacteroidetes and Clostridiales, groups known for their fibrinolytic capabilities, may provide the Hadza gut microbiota with specific glycan-degrading abilities for Hadza to deal with a vast array of refractory and resistant organic materials introduced through diet.

Consistent with gut microbiota arrangements reported for other African groups (De Filippo et al., 2010; Yatsunenko et al., 2012), the Hadza gut microbiota shows a higher relative abundance of *Prevotella*, but with a correspondent reduction of *Bacteroides* in the gut ecosystem compared to the Italian control cohort. Thus, similar to what has been proposed for rural Africans consuming grain-based high-fiber diets (De Filippo et al., 2010), it is tempting to speculate that this microbe community within the Bacteroidetes phylum could harbor the necessary gut microbiota functions to deal with their especially unique, but also highly fibrous, plant food dietary constituents (Thomas et al., 2011). It would be of great interest to learn whether the shift from a largely plant-based diet to one that includes more meat, such as during the dry season, might show a concurrent change in gut microbiota structure amongst Hadza.

Sex-based variation and novel residents may be impacted by diet

We find evidence of a sex-related divergence in Hadza gut microbiota structure, which is not documented in other human groups. This divergence corresponds to the

Hadza sexual division of labor and sex differences in diet composition. In the same environment with access to the same dietary resources, Hadza men and women are differentially adapted to their particular pattern of food consumption. The potential for Hadza women's gut microbiota to respond with significant structural differences to the increased consumption of plant foods represents a profound break with traditional thinking on the limited digestive capacity of the human gut and the constraint it imposes on nutritional provisioning for reproduction and brain growth (Aiello et al., 1995; Wrangham et al., 2003). Women's foraging must adequately provision for pregnancy and lactation, which is a strong adaptive pressure for the gut microbiota to derive the most energy from consistently available plant foods. In this regard, the gut microbiota aligns with the host nutrition acquisition strategy, thus potentially buffering women from resource "gaps" that may lead to nutritional deficiencies.

The reported presence of *Treponema* in now five geographically separate extant rural human communities from this and previous studies (Hadza, BF, Malawians, South Africans, and Venezuelan Amerindians; Tito et al., 2012) supports an alternative functional role for this bacterial group whose expression in industrialized communities is normally attributed to pathogenic disease. De Filippo et al. (2010) hypothesize that the presence of *Treponema* in BF children enhance the host's ability to extract nutrients from the fibrous foods that comprise their traditional diet. While the Hadza do not eat agricultural or grain-based diets, they do rely heavily on fibrous tubers throughout the year, with women often consuming tubers for a greater percentage of daily calories than do men (Berbesque et al., 2011). These sources of fiber rich plant foods could similarly encourage a mutualistic

Treponema population whose fibrinolytic specializations would be advantageous to Hadza nutritional acquisition, particularly in women.

Medical examinations conducted on Hadza found evidence of Treponematosis from serum samples at low rates (13/215 sampled) with the highest prevalence in men of settled Hadza camps between 1966 and 1967 (Bennett et al., 1973). However, there was low but consistent prevalence for women in both settled and foraging Hadza groups with little clinical evidence of yaws, suggesting immunoregulation of *Treponema* pathogens.

Absence of bifidobacteria is a new equilibrium

The absence of Actinobacteria, particularly *Bifidobacterium*, in the Hadza gut microbiota is unexpected. Bifidobacteria are associated with breastfeeding in infants and achieve large proportions of the gut microbiota in the first few months after birth (Turroni et al., 2009). Typically, in adults, bifidobacteria commonly make up 1-10% of the gut microbiota population. Complete absence of bifidobacteria, as observed in the Hadza, has never to our knowledge been reported for any other human group. We hypothesize that the lack of bifidobacteria in adult Hadza is a consequence of the post-weaning gut microbiota composition in the absence of agro-pastoral-derived foods. Support for this hypothesis comes from the observation that other populations in which meat and/or dairy consumption is low to absent, such as vegans and Koreans, also have very low representation of Actinobacteria and *Bifidobacterium* (Nam et al., 2011; Zimmer et al., 2012). The continued consumption of dairy into adulthood could be one reason most Western populations maintain a relatively large bifidobacterial presence. Aside from bifidobacterial species of human origin, the majority of *Bifidobacterium* have been

isolated from livestock animals such as swine, cattle and rabbit (Ventura et al., 2007; Turroni et al., 2009). The Hadza neither domesticate nor have direct contact with livestock animals. Thus, as they lack exposure to livestock bifidobacteria, this raises the question of whether the necessary conditions for interspecies transfer and colonization of bifidobacteria do not occur for the Hadza (Moeller et al., 2013). Future work must focus on the gut microbiota of breast-fed Hadza infants to determine the role of bifidobacteria in the kinetics of assembly and development of the Hadza gut microbiota, and to learn whether this bacterial group is completely absent in all Hadza, including infants, or if it is definitively lost from the gut ecosystem post-weaning. It is important to note that while bifidobacteria are considered a beneficial bacterial group in Western gut microbiota profiles, their absence in the Hadza gut microbiota, combined with the alternative enrichment in "opportunistic" bacteria from Proteobacteria and Spirochaetes, cannot be considered aberrant. On the contrary, the Hadza gut microbiota likely represents a new equilibrium that is beneficial and symbiotic to the Hadza living environment. Support for the advantage of such novel gut microbiota configurations comes from the finding that gut microbiota restructuring also occurs in centenarians (Biagi et al., 2010), who are extreme examples of organismal robusticity. Additionally, these findings illustrate a need to reevaluate the standards by which we consider gut microbiota "healthy" or "unhealthy", as they are clearly context dependent.

Gut microbiota diversity is a human legacy

Gut microbiota diversity, as found in rural African populations and now in the Hadza, is almost certainly the ancestral state for humans. Adaptation to the Western lifestyle is coincident with a reduction in gut microbiota diversity, and as a

result, a decline in gut microbiota stability. Diversity and stability are factors with major health implications, particularly now that the human gastro-intestinal tract is increasingly recognized as the gateway to pathogenic, metabolic, and immunologic diseases (Blaser et al., 2009). Co-speciation between host and microbiota over millions of years has shaped both sets of organisms into mutualistic supra-organisms. Dissolving that contact through sterilization and limited environmental exposure has had a drastic effect on health and immune function of modern Westernized human groups. The Hadza gut microbiota is likely an "old friend" and stable arrangement fitting their traditional hunter-gatherer lifestyle (Rook et al., 2004).

We are only just beginning to document gut microbiota diversity across populations. In our study, more than 33% of the total Hadza gut microbiota genera remain unidentified. Such taxonomic uncertainty holds exciting prospects for discovering yet-unknown microbial genetic arrangements. This finding also underscores the importance of increasing our reference phylogenies and resolvingdeep taxonomic relationships between bacteria by sampling a wider variety of environments and extreme ecological zones (Rinke et al., 2013).

CHAPTER 6
FUNCTIONAL METAGENOMIC PROFILING OF INTESTINAL MICROBIOME IN EXTREME AGEING

Ageing is a complex multifactorial process with a major impact on the human body (Weiner et al., 2003). The ageing process seriously affects the human gut microbiota in particular, because it is accompanied by changes in the physiology of the gastrointestinal tract and associated immune system, as well as by changes in diet and lifestyle (Lovat et al, 1996; Kau et al., 2011).

The adult-like profile of the human intestinal microbiota is stably maintained over time in healthy adults (Faith et al., 2011; Lozupone et al., 2012), defining an essentially mutualistic scenario whereby, in return for a guaranteed nutrient supply, the gut microbiota provides numerous fundamental functions to the host, including vitamin and metabolite supply and colonization resistance against pathogens (Neish, 2009). The pathophysiology of the ageing process can ultimately compromise this homeostasis with a subject-specific timing, depending on the individual physiological status, diet, lifestyle and frailty (Bartosh et al., 2004; Woodmansey et al., 2004; Mueller et al., 2006; Rajilicic-Stojanovic et al., 2009; Biagi et al., 2010; Claesson et al., 2011; Biagi et al.; 2012).

Changes in microbiota composition in older people have been connected to immunosenescence and inflammageing (Franceschi et al., 2000a; Cheng et al., 2013; Biagi et al., 2013; Cevini et al., 2013). An important factor which affects the gut microbiota in ageing is diet (Yatsunenko et al., 2012). A very recent study highlighted the impact of the habitual diet on the gut microbiota of elderly people, demonstrating diet-driven alterations in varying rates of health decline upon ageing (Claesson et al., 2012). In that study, a reduced coding capacity for producing SCFAs in frail subjects correlated with lower levels of butyrate, acetate and propionate in the faecal metabolome. However, the functional aspects of the intestinal microbial community and their relation with the ageing process are not frequently investigated. Indeed, the majority of recent studies focussing on the relationship between ageing and the gut microbiota used a 16S rDNA amplicon sequencing approach, which reflects the phylogenetic structure of the microbiota but does not provide information pertaining to function. Even if microbiota compositional information allows a large number of deductions to be inferred, a metagenomics approach is ultimately required, because it provides a view of community structure in terms of species richness and distribution, as well as the functional (metabolic) potential of the community metagenome (Hugenholtz et al., 2008).

Here, we applied Illumina shotgun sequencing to 9 faecal samples, whose microbiota composition was reported in a previous study (Biagi et al., 2010), including centenarians, individuals at the upper extremity of the human lifespan (Franceschi et al., 2000a). We hypothesized that centenarians represent a valuable opportunity for increasing the temporal resolution of microbiota-ageing interactions. The particular physiology and the general inflammatory status of centenarians make these individuals very different from the rest of the population, including in terms

of gut microbiota composition (Biagi et al., 2010). In a very recent study, the metabolomic profile of centenarians was also characterized, allowing the identification of some urine metabotypes which were strongly connected with extreme ageing and some intestinal microbiota elements (Collino et al., 2013). In the present pilot work, by determining the centenarian gut microbiome we propose a wide functional description of the human gut microbiota upon ageing.

CHAPTER 6
FUNCTIONAL METAGENOMIC PROFILING OF INTESTINAL MICROBIOME IN EXTREME AGEING

Sample collection

The study employed 9 faecal samples collected for the study of Biagi et al. (2010) [8]. We utilized the same faecal genomic DNA used for previous analysis and which had been stored at -20°C.

Basic bioinformatic and statistical analysis of the metadata

Phylogenetic profiles of HITChip array data were retrieved from the study of Biagi et al. (2010). The dataset was analyzed in R version 3.0.0 (www.cran.org), using the R package "Vegan" (Oksanen et al., 2013), in order to obtain a PCoA based on Euclidean distances.

Urine metabotypes of 8 out of 9 individuals involved in our study (2 centenarians, 5 elderly people and 1 adult) had been previously characterized by ^{1}H-NMR profiling NMR (Bruker Avance III 600 MHz, Bruker Biospin, Rheinstetten, Germany) (Collino et al., 2013). The resulting spectra were analyzed in the present study using the packages "ChemoSpec" (Hanson, 2013) and "Vegan" (Oksanen et al., 2013).

Projection on Latent Structures – Discriminant Analysis (PLS-DA) plot was carried out with SIMCA software (version 13.0, Umetrics AB, Umea, Sweden).

The phylogenetic characterization of the shotgun sequences was achieved at different levels of taxonomy by using MetaPhlAn with default parameters (Segata et al., 2012). Statistical analysis was carried out using R. MetaPhlan result files were read into R, and matrices containing the relative abundances of taxa at phylum and genus level for each sample were constructed. Abundance analysis, multivariate statistical analysis, including PCoA, were computed using Cytoscape (Smoot et al., 2011), R Bioconductor package "Made4" (Culhane et al., 2005), and the R packages "stats" and "Vegan" (Oksanen et al., 2013).

Integration of the data by Procrustes and Co-inertia analyses was achieved using the functions "procrustes" and "cia" of "Vegan" (Oksanen et al., 2013) and "Made4" (Culhane et al., 2005) packages, respectively. The coefficient RV of Co-inertia Analysis describes the global similarity between datasets: the closer this value is to 1, the greater is the similarity.

Gut microbiome functional characterization: bioinformatic and statistical analysis

In order to functionally annotate the data, the shotgun reads first had to be quality filtered to remove low quality and human sequences. This step was achieved by using the human sequence removal pipeline and the WGS read processing procedure of the Human Microbiome Project (HMP) (Turnbaugh et al., 2007). The obtained reads were locally assigned for functionality at different levels of the KEGG database (Wixon et al., 2000), using MetaCV, with default parameters. This tool consists of a composition algorithm which allows classification of very short reads

into functional groups (Liu et al., 2013). The resulting table consists of a matrix, with sample IDs in the columns and annotations at different levels of KEGG database in the rows. The file produced was loaded into R and then filtered for the abundances at first and then for KEGG Orthology (KO) level of KEGG classification.

PCoA using Euclidean distances based on KO gene dataset was performed with the package "Vegan" of R (Oksanen et al., 2013) and used also for Procrustes analysis. The function "scores" of "Vegan" was used to retrieve the coordinates of the samples and KEGG KO genes in the PCoA. The values obtained were used to perform observations about their ordination along the axes. Kendall correlation test was achieved using function "cor.test" of the package "stats" of R. When appropriate, P values were adjusted for multiple comparison using the Benjamini-Hochberg correction.

CHAPTER 6
FUNCTIONAL METAGENOMIC PROFILING OF INTESTINAL MICROBIOME IN EXTREME AGEING

1. *Brief introduction*
2. *Materials and Methods*
3. ***Results***
4. *Discussion*

Meta-analysis of gut microbiota functionality, taxonomy and urine metabotypes

We previously reported substantial inter-individual variability in the faecal microbiota composition of 64 older people, comprising 21 centenarians, 22 elderly subjects genetically unrelated to the centenarians, and 21 offspring of the centenarians (Biagi et al., 2010). A group of 20 young adults had also been included as a control. Furthermore, the metabolic phenotype of the subjects was recently characterized by ^{1}H-NMR and mass spectrometry analysis of plasma and urine (Collino et al., 2013).

To investigate functional differences in the gut microbiome across the age groups studied and to characterize the metabolic trajectory of human intestinal microbiota upon ageing, we analyzed a subset of these samples: 3 centenarians, aged 99 to 102 years (mean 100.7), 5 elderly people, including 3 offspring of the centenarians, aged 59 to 75 years (mean 66.4), and one 38-year-old adult, as a young control. All the subjects were of Caucasian (Italian) ethnicity and resided in Emilia Romagna, a region in Northern Italy.

Firstly, in order to prove that the selected samples were representatives of the entire cohort they were drawn from, we performed a multivariate analysis on the previous HITChip results for the subjects selected for this study (Biagi et al., 2010; **Figure 23a**) and described their urine metabolic profiles applying PLS-DA on the ^{1}H-NMR spectra (Collino et al., 2013; **Figure 23b**). Both analyses showed that centenarians widely differed from the other subjects.

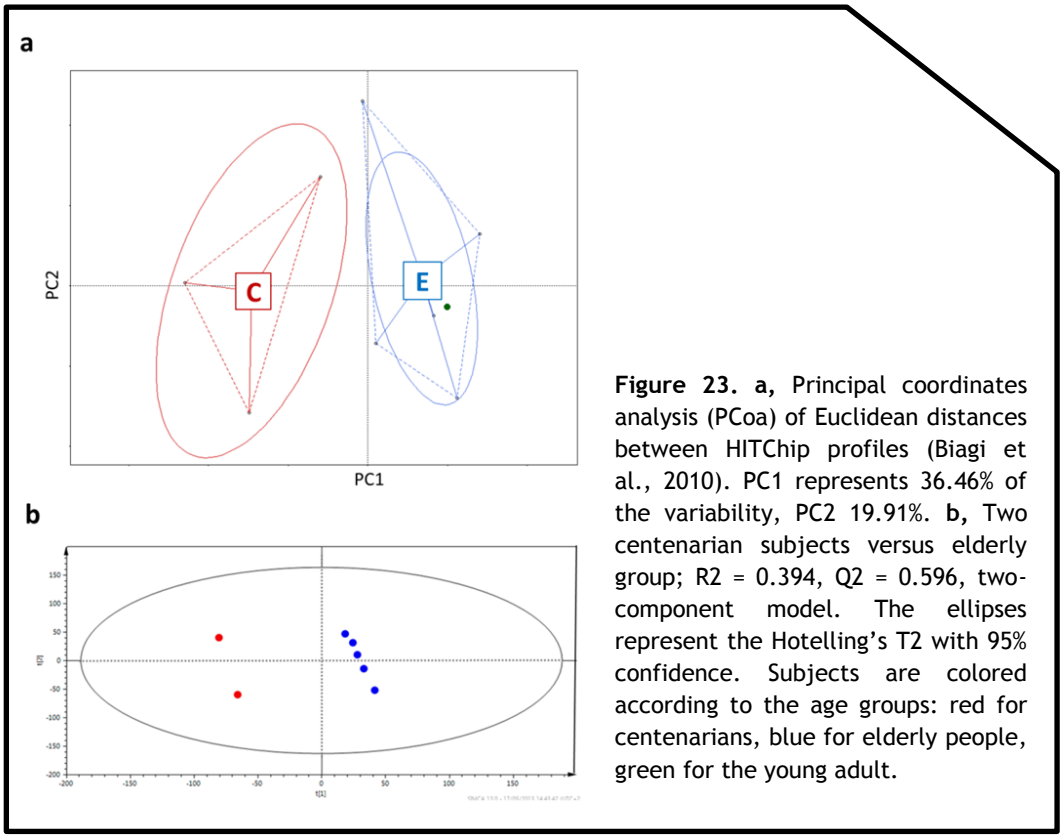

Figure 23. a, Principal coordinates analysis (PCoa) of Euclidean distances between HITChip profiles (Biagi et al., 2010). PC1 represents 36.46% of the variability, PC2 19.91%. **b,** Two centenarian subjects versus elderly group; R2 = 0.394, Q2 = 0.596, two-component model. The ellipses represent the Hotelling's T2 with 95% confidence. Subjects are colored according to the age groups: red for centenarians, blue for elderly people, green for the young adult.

By Illumina shotgun sequencing of the faecal microbial DNA from the selected individuals, we generated a total of 214.6 million paired-end reads, with an average of 23.841 million (± 0.067 SD) reads per subject.

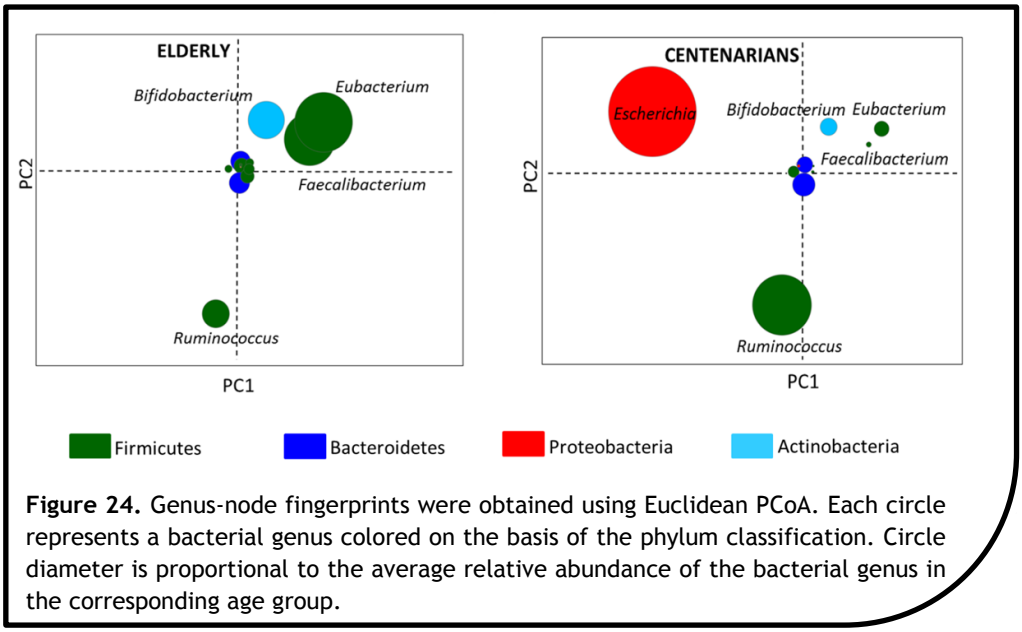

Figure 24. Genus-node fingerprints were obtained using Euclidean PCoA. Each circle represents a bacterial genus colored on the basis of the phylum classification. Circle diameter is proportional to the average relative abundance of the bacterial genus in the corresponding age group.

Shotgun sequences were processed using the MetaPhlAn pipeline and a multivariate analysis of the taxonomic results was carried out. By using the genus coordinates of the taxonomic PCoA and their mean contribution in the groups, two node graphs, respectively for centenarians and 70-year-old subjects, were obtained. **Figure 24** thus shows the gut microbiota fingerprints at the genus level in centenarians and elderly. In the center of the resulting graphs were positioned the genus-nodes that were equally abundant in all the samples while the other areas of the graph contained genus-nodes present at different relative abundance across the subjects. Thus the data indicate that the genera *Escherichia* and *Ruminococcus* were over-represented in centenarians, whereas *Faecalibacterium*, *Eubacterium* and *Bifidobacterium* were more abundant in the elderly.

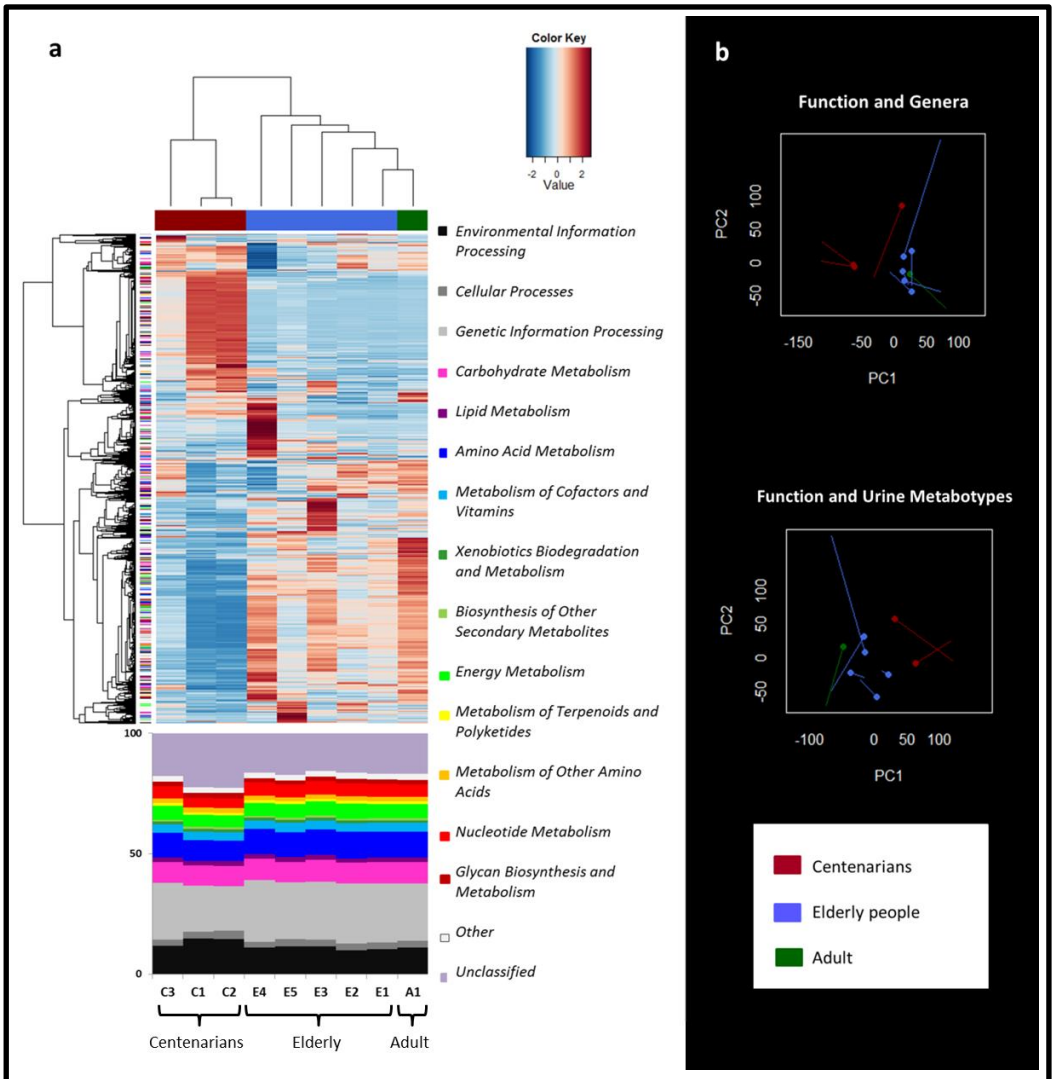

Figure 25. a, Hierarchical Ward-linkage clustering based on the Pearson correlation coefficients of the abundance of KO genes, filtered for KO gene subject presence ≥ 1 in at least 8/9 subjects. KO genes are clustered in the vertical tree and color-coded according to the first level of KEGG classification or the second level for functions concerning metabolism. 2719 KO genes confidently classified in the KEGG database are visualized. The bottom panel shows the relative abundance of the KEGG categories. **b**, Procrustes analysis combining Euclidean PCoA of functional microbiota (non-circle end of lines) with either Euclidean PCoA based on the genus dataset (circle-end of lines; upper graph) [8], or Euclidean PCoA based on the spectra of urine metabolites (circle-end of lines; lower graph) [22]. In both graphs color codes are per age group as in Figure 23.

Functional characterization of the shotgun sequence reads in the KEGG database was carried out by using MetaCV pipeline. Hierarchical Ward-linkage clustering based on the Pearson correlation of the normalized number of mapped reads per gene function at KEGG Orthology (KO) level showed a clear separation between centenarians (C1-C3) and the other subjects (E1-E5 and A1; **Figure 25a**).

When examining the functional gene distribution at high levels of the KEGG database, we identified clusters of specific genes which were characteristic of the ageing groups, even if the relative abundance of functions was distributed in a similar way across all subjects (**Figure 25a**). Procrustes analysis of the functional gene profiles and the microbiota ß-diversity or Euclidean distances of urine metabotypes was used to co-illustrate the data (**Figure 25b**). In both cases, the separation between centenarians and the other subjects occurred along the first axis. The RV coefficient obtained by co-inertia analysis between the same paired datasets, highlighted the association between the taxonomic and functional datasets (RV = 0.794), in despite of the functional and urine metabonomic results, which appeared to be not related to each other (RV = 0.380).

Relation between the functional structure of the gut microbiota and ageing

We applied the MetaCV procedure for achieving a functional characterization of the faecal microbiota at different levels of KEGG classification. Alpha-diversity was computed at KO level using the Simpson index and was not significantly different between the age groups (centenarians, 0.904 ± 0.010; the elderly, 0.892 ± 0.061). PCoA based on Euclidean distances of functional gene profiles at KO level showed an age-related resolution (**Figure 26a**). In particular, the first axis, which represented 82.5% of the data variability, described most of the ageing-related differences

(Kendall's tau coefficient = -0.778, P value = 0.002, **Figure 26b**). For this reason, we used PCo1 to assess the functional potential of the gut microbiome upon ageing. In particular, we focused our attention on the genes involved in amino acid and carbohydrate metabolisms, core genes in metabolism, in order to obtain an ordination of the pathways along PCo1. Each point of the resulting graph corresponded to the average of the coordinates of the KO genes involved in the same KEGG pathway, and the intervals coincided with the standard error of the mean (SEM; **Figure26c**). In this way, we built a sort of ranking of KEGG pathways, where negative values of PCo1 coordinates were related with ageing. We observed a polarization of the pathways related to amino acid metabolism in the extremities of the axis, and a more dense localization at positive values of PCo1 coordinates for the pathways involved in carbohydrate metabolism. In particular, the metabolism of two aromatic amino acids, tryptophan and phenylalanine, was closely associated with ageing, followed by the metabolism of other amino acids, such as tyrosine, valine and lysine. Conversely, the metabolism of other amino acids and several carbohydrates, such as glucose, galactose, histidine, pyruvate and butanoate was related to high positive values of PCo1. Notably, the last two pathways are strictly connected with SCFA production.

Functional signature of extreme ageing in the intestinal core microbiome

Since we aimed at highlighting alterations within the core microbiome structure associated with ageing, we first filtered out all the KO genes which possessed a significant (P value < 0.05) positive correlation with the proportional abundance of *Escherichia*, a pathobiont that is prevalent in the centenarian gut. The filtered genes were further analyzed for their correlation with subject age (P value < 0.05).

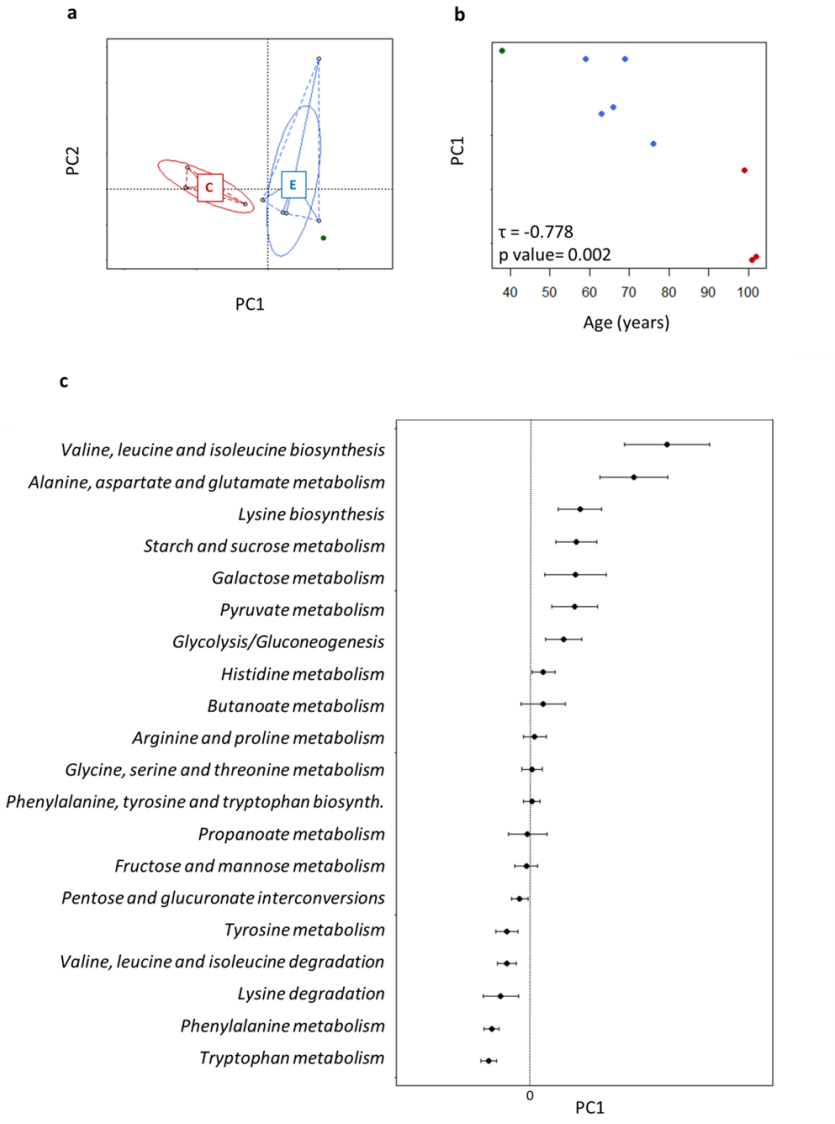

Figure 26. a, PCoA of Euclidean distances between KEGG KO profiles. Subjects are colored as in Figure 19. **b**, The position of each fecal microbiome along PC1, which described the largest amount of variation (82.5%) in the dataset, was plotted against age. Each circle is a subject colored as in Figure 23. PC1 was significantly related with age. **c**, Average (± SEM, error bar) PC1 coordinates obtained for functional cluster of KO genes, corresponding to KEGG pathways, used for building the PCoA. In this way pathways are ordered for: (1) negative value of PC1 coinciding with high concordance with ageing profile; (2) value of PC1 close to 0 coinciding with constant presence in the dataset; (3) positive value of PC1 coinciding with high concordance with the healthy adult profile.

The resulting 116 KO genes are listed with annotated functions at http://www.impactaging.com/papers/v5/n12/full/100623/Supplementary%20Tabl (Rampelli et al., 2013).

CHAPTER 6
FUNCTIONAL METAGENOMIC PROFILING OF INTESTINAL MICROBIOME IN EXTREME AGEING

In the present study we characterized the gut microbiota metagenome of extreme longevity individuals, elderly people and a young adult by Illumina shotgun sequencing. The subjects chosen typify a bigger cohort whose members had been well characterized for gut microbiota composition as well as for urine and serum metabolites (Biagi et al., 2010; Collino et al, 2013). Here we focused on the functional aspects of the gut microbiota, in order to investigate ageing-related changes of the microbiome structure and function. Confirming Biagi et al. (2010), multivariate analysis of the taxonomic dataset showed an overall increase in *Proteobacteria* and a rearrangement in *Firmicutes* in centenarians, whereas 70-year-old elderly people maintained a gut microbiota profile very similar to the one shown by the young adult. The Procrustes analysis highlighted the relationships between taxonomic and functional datasets, showing a clear separation between centenarians and younger subjects with respect to both microbiome structure and functionality. Shotgun sequencing analysis revealed important modifications in microbiota functions in centenarians, allowing us to define an aged-type microbiome

characterized by a specific functional complement different from the one present in younger subjects. As a result of taxonomic rearrangements in the gut microbiota composition, the changes in microbiome structure we detected as a function of ageing allowed us to shed some light on the mechanisms associated with bacterial community change accompanying the overall ageing process.

Examining the KO gene abundance we identified a cluster including all the centenarian subjects, separated from the clustered younger subjects. While at high level of KEGG assignments the functional structure of the microbiota was present in both clusters without significant differences, the distribution of genes at KO levels followed an age-related tendency. Among these, we observed an age-related increased abundance of genes involved in the tryptophan metabolism pathway (ko00380). This evidence is in agreement with the reduction of tryptophan found in serum of centenarians (Collino et al., 2013), although we cannot directly infer causality. Linking together the two observations, we advance the hypothesis that the potential increase of consumption of tryptophan by the gut microbiota affects its bioavailability within the host. A recent study showed patients with inflammatory diseases to have a significant depletion of serum levels of tryptophan compared to control population (Gupta et al., 2012) and Huang et al. (2002) demonstrated a clear relationship between reduced serum tryptophan levels and an increase of immune activation. In addition, the decrease of the serum level of tryptophan was associated with cognitive deficit in senile dementias (Thomas et al., 1986; Widner et al., 1999; Porter et al., 2000), and Noristani et al. (2012) demonstrated that high triptophan diet lead to a reduction of the plaque pathology in Alzheimer's disease in mouse. It is thus tempting to speculate that a microbiota-dependent reduction of tryptophan can nurture inflammageing in centenarians and could worsen the

conditions of the patients affected by cognitive deficit. Furthermore, our study revealed that the altered abundance of genes involved in phenylalanine metabolism (ko00360) and tyrosine metabolism (ko00350) was related with ageing. This evidence supports the proposal of two metabolites (phenylacetylglutamine (PAG) and p-cresol-sulfate (PCS)), as markers in urine of extreme ageing (Collino et al., 2013). Indeed, it has been extensively demonstrated that PAG and PCS derive from microbial metabolism of aromatic amino acids, including tyrosine and phenylalanine (Smith et al., 1997b).

SCFAs - mainly acetate, butyrate and proprionate - are microbiota-derived metabolites that are fundamental for the human health and wellbeing. They represent a source of energy for the gastrointestinal epithelium, stimulators for the release of mucins, immune modulators, and promoters of the integrity of the epithelial barrier (Pryde et al., 2002; Tremaroli et al., 2012). Primarily, SCFAs are produced by bacterial fermentation of dietary polysaccharides which reach the colon undigested. This process relies on the presence of a glycophilic bacterial community in the colon, and also on other bacterial groups acting in concert with these primary degraders (Chassard et al., 2006; Claesson et al., 2012; Koropatkin et al., 2012). It has been demonstrated that a reduced dietary intake of carbohydrates causes decreased numbers of butyrate and butyrate-producing bacteria such as *Roseburia* and *Eubacterium* (Duncan et al., 2007), highlighting the importance of carbohydrate availability in the large intestine for the maintenance of a fibrolytic SCFA-producing microbial community. In relation to this, our data showed a loss of genes involved in SCFA production and an overall decrease in the saccharolytic potential as a function of ageing. In particular, in the extremely aged-type microbiome we observed a decrease in starch and sucrose metabolism (ko00500),

pyruvate metabolism (ko00620), galactose metabolism (ko00052) and glycolysis/gluconeogenesis (ko00010), accompanied by a concomitant loss of fibrolytic microorganisms belonging to the *Eubacterium*, *Bifidobacterium* and *Faecalibacterium* genera. Notably, pyruvate and butanoate metabolism, which showed an inverse association with ageing, are described in KEGG database as pathways containing genes involved in SCFA production. Our data showed an overall rearrangement in the aged-type microbiome of the pathways involved in the production of SCFA via proteolytic fermentation. In particular, genes involved in lysine degradation (ko00310), implicated in butyrate and acetate production, were more abundant in centenarians, while elderly and adults were enriched in genes responsible for the metabolism of glutamate, aspartate and alanine (ko00250), which in turn are involved in the production of acetate, butyrate and propionate, respectively (Smith et al., 1997a). Nevertheless, the aged-type microbiome was characterized by an overall increase in proteolytic functions, as suggested also by the degradation of the branched-chain amino acids, valine, leucine and isoleucine (ko00280), which was strictly connected with the microbial metagenome of centenarians. Notably, the first catabolic intermediates of valine, isoleucine and leucine are the precursors of the branched-chain fatty acids that are involved in the cell membrane biosynthesis of inflammation-promoting Gram-negative bacteria (Kaneda et al., 1991; Beck et al., 2004). Interestingly, the increased abundance of proteolytic functions in the aged-type microbiome is accompanied by lower lysine, valine, leucine and isoleucine biosynthetic capability (ko00290; ko00300). All these molecules are essential amino acids, with the respective biosynthetic pathways completely lacking in mammals. In particular, lysine plays an essential role in many cellular process, including the functionality of the pyruvate dehydrogenase complex

(Jones et al., 2000; Goraça et al., 2011). Reduction in the microbiota production of essential amino acids as part of ageing could thus have a primary role in compromising the overall nutritional state of elderly subjects, leading to sarcopenia. In summary the proteolytic potential of the gut microbiota appeared to be enhanced upon ageing, even if there are changes of substrate, and a clear loss of genes involved in the metabolism of carbohydrates in the aged-type microbiota was observed. Furthermore, we found an age-related reduction of the abundance of genes in pathways involved in SCFA production. Based on differential abundance, the aged-type microbiota is structurally and functionally compromised, moving from a saccharolytic to a putrefactive metabolism. It is noteworthy that these microbiome alterations concord with the enrichment of genes belonging to pathobionts. Pathobionts are minor microbiota opportunists able to thrive in inflamed conditions, sustaining and nurturing the physiological inflammation (Biagi et al., 2013). We therefore postulate that pathobiont overgrowth can nurture a sort of pro-inflammatory loop which could worsen the health status of aged people. We can also hypothesize that in centenarians – the extreme limit of human life span - some readjustments take place within the core mutualistic microbiome functions that serve to offset detrimental activities associated with pathobiont overgrowth. In order to shed some light on this, we focused our analysis on the core microbiome changes occurring in centenarians. According to our findings, 116 genes usually present in different metagenome project databases (Cavin et al., 1998; Turnbaugh et al., 2007; Kurokawa et al., 2007; Qin et al., 2010), reflect the rearrangement of the core metabolic potential of the centenarian intestinal microbial ecosystem. This raises the question of whether these specific changes in the structure of the mutualistic counterpart of the gut microbiome reflect a new mutualistic

configuration, responding to a microbiota-host adaptation process co-evolved to support the extreme limits of human lifespan.

It is important to note that in this pilot study the functional characterization of a small number of samples, representative of a bigger cohort, allowed us to investigate the functional potential of the gut microbiota, which clearly needs further validation by increasing the sample number. Furthermore, since the subjects belong to a limited geographic area (Emilia Romagna region), and are thus similar in lifestyle and habitual dietary patterns, these data also need confirmation across populations with different environmental conditions and genetic background. Nevertheless, our microbiome study of extreme ageing contributes to assess a more inclusive portrayal of the role of the intestinal bacterial counterpart in the pathophysiology of the ageing process. An ongoing project (www.nu-age.eu) will also include analysis of different dietary regimes in the ageing human population, to allow searching for correlations between gut microbiota composition and functionality, physiological and immunological parameters, metabonomic profiles and diet.

CHAPTER 7
PROBIOTICS MODULATE THE GUT MICROBIOTA IN THE ELDERLY

Age-related factors, such as physiological changes of the gastrointestinal tract, changes in dietary habits and lifestyle and the reduction of the functionality of the immune system (Ostan et al., 2008; Shanley et al., 2009), force severe shifts in the intestinal microbiota composition, compromising its symbiotic relationship with the host. Ageing has been associated with a decrease of key microbiota components, such as members of *Clostridium* cluster XIVa (also known as *Clostridium coccoides/Eubacterium rectale* group) and *Clostridium* cluster IV (Hayashi et al., 2003; Mueller et al., 2006; Makivuokko et al., 2010). Moreover, an age-related increase in facultative anaerobes, including streptococci, staphylococci, enterococci and enterobacteria, has been widely observed (Gavini et al., 2001; Woodmansey et al., 2004; Mueller et al., 2006; Mariat et al., 2009; Rajilic-Stojanovic et al., 2009; Makivuokko et al., 2010). Finally, probably due to the frequent antibiotic usage, the prevalence of *Clostridium difficile*, a major intestinal pathogen involved in antibiotic-associated diarrhea, dramatically increases in the elderly (Simor et al., 2002). Affecting nutrition, inflammatory status and susceptibility to infection, these age-related dysbioses of the intestinal microbiota can exert a multifunctional role in

age-related pathophysiological processes. For instance, the depletion in butyrate-producing members of *Clostridium* cluster XIVa and IV can result in an overall reduction of SCFA in the GI tract. SCFAs, mostly acetate, butyrate and propionate, are microbiota metabolites which have been reported as strategic for several aspect of human biology. They represent an important energy source for the host but also regulate the immune function and concur to maintain a functional GI epithelial barrier (Neish et al, 2009; Macia et al., 2012). Consequently, the reduction of SCFA in the GI tract can involve nutritional and immunological effects, contributing to the health impairment of older people (Biagi et al., 2012). Moreover, the age-related increase in pro-inflammatory *Enterobacteriaceae* and the concomitant decrease in the key anti-inflammatory microbiota components, such as members of *Clostridium* cluster IV and *F prausnitzii,* result in the establishment of a pro-inflammatory microbial community, which can contribute to the process of inflamm-ageing (Ostan et al., 2008).

In Western countries, where elderly population is going to reach 2 billions by the year 2050 (Cohen et al., 2003; Christensen et al., 2009), the quality of life of the ageing population is becoming of primary importance and our capacity to favor an "active longevity" will be fundamental to reduce the progressive increase of the health care cost of elderly people (Hayflick et al., 2000). In the perspective to improve the health status of older people, the possibility to modulate their gut microbiota by functional foods containing probiotics and/or prebiotics has been considered (Hebuterne et al., 2003; Tuohy et al., 2007). Probiotics have been shown to be effective in promoting different aspects of health in elderly people (Tiihonen et al., 2010; Bjorklund et al., 2011; Biagi et al., 2012; Lahtinen et al., 2012). For instance, probiotic interventions have shown a therapeutic potential for the

treatment of constipation. Moreover, treatment with various probiotic *Lactobacillus* and *Bifidobacterium* strains has been proved to be effective in restoring the immune function in the elderly, limiting the effects of immunesenescence (Chiang et al., 2000; Arunachalam et al., 2000; Gill et al., 2001a; Gill et al., 2001b; Sheih et al., 2001; Takeda et al., 2007). However, even if the efficacy of probiotics in increasing the faecal *Bifidobacterium* levels in the elderly has been demonstrated (Amhed et al., 2007; Lahtinen et al., 2009; Matsumoto et al., 2009), as far as we know there is no information concerning their impact on the entire phylogenetic structure of the intestinal microbiota in aged people.

Here we investigated the impact of a a biscuit covered by chocolate including a mixture of the probiotics *Lactobacillus helveticus* Bar13 and *Bifidobacterium longum* Bar33 on the age-related dysbioses of the intestinal microbial community in 32 Italian seniors. The probiotic strains *L. helveticus* Bar13 and *B. longum* Bar33 have been selected on the basis of their adhesive and immune-regulative properties shown both *in vitro* and *in vivo* in animal models (Candela et al., 2008; Rosselli et al., 2009). The intestinal microbial community was characterized by means of the previously developed and validated phylogenetic microarray platform HTF-Microbi.Array (Candela et al., 2010).

CHAPTER 7
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1. *Brief introduction*
2. ***Materials and Methods***
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Study set-up and sample collection

Thirty-two elderly volunteers living in Italy, ex-patients of the "Villa delle Querce" nursing home Nemi (Rome, Italy), were recruited for this study. The group was composed of 19 women and 13 men, aged 71 to 88 years (mean 76), who were non-institutionalized and living in their own household. All the recruited subjects showed good physical health conditions and did not use antibiotics for one-month period before sampling. The ethics committee of the Polyclinic Umberto I of Rome approved the study (protocol number 111/10, date 02-12-2010) and a written informed consent was obtained from each subject. Subjects were randomized into two groups consuming one dose per day of a probiotics-containing bar (Barilla, Parma, Italy) consisting of a biscuit covered by chocolate that included a mixture of the probiotic strains *B. longum* Bar33 and *L. helveticus* Bar13 (Barilla culture collection) – referred to as probiotic bar throughout the text - or placebo for 1 month. A single dose of the probiotic bar contained 10^9 CFU/g of each probiotic strain. The survival of the probiotic strains *B. longum* Bar33 and *L. helveticus* Bar13 within the chocolate cream was assessed with a storage test at room temperature.

The concentration of the two probiotic strains was stable at 10^9 CFU/g per serving for 6 months. Placebo snack had taste and appearance similar to the probiotic product but contained no probiotics. For each subject enrolled in the study, faecal samples were collected before (T0) and after 1 month of intervention (T30), and immediately stored at -20°C.

DNA extraction and Polymerase Chain Reaction (PCR)

Total DNA from faecal material was extracted using QIAamp DNA Stool Mini Kit (Qiagen) according to the modified protocol reported by Candela et al. (2010). Final DNA concentration was determined using NanoDrop ND-1000 (NanoDrop Technologies). PCR amplifications were performed with Biometra Thermal Cycler T Gradient (Biometra). The 16S rRNA gene was amplified using universal forward primer 27F and reverse primer 1492r, following the protocol described by Candela et al. (2010). Per DNA sample two reactions were pooled and purified by using the Wizard SV gel and PCR clean-up System kit (Promega), eluted in 50 µl of sterile water and quantified with the DNA 7500 LabChip Assay kit and BioAnalyzer 2100 (Agilent Technologies). All the oligonucleotide primers used for PCR reactions and probe pairs employed for the array construction were synthesized by Thermo Electron.

Data analysis

All arrays were scanned and processed according to the protocol and parameters already described by Candela et al. (2010). Data storage, normalization, and probe profile extraction were performed using MATLAB (2011a, The MathWorks, Natick, MA). Mean data from two replicate experiments were obtained and utilized for

principal component analysis (PCA), box plot analysis and calculation of the probe relative IF contribution. Phylogenetic profiles of the intestinal microbiota of the 32 elderly volunteers at T0 were compared to the ones obtained from 8 healthy 30-year old young adults from a previous HTF-Microbi.Array-based descriptive study (Candela et al., 2010). Wilcoxon test was used to determine whether the contribution of each bacterial group was significantly different between young (n=8) and elderly (n=32) adults, and between treated (n=17) and control (n=15) elderly subjects. Statistical analyses were performed by using Canoco package for Windows (Leps et al., 2003) and the R statistical software (www.r-project.org). $P < 0.05$ was considered as a threshold for statistical significance.

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Comparison of the high taxonomic level phylogenetic profiles of the intestinal microbiota between elderly and young adults

The intestinal microbiota of all subjects was largely dominated by Bacteroidetes and Firmicutes, which together accounted for up to 90% of the intestinal microbial community. With a relative abundance ranging from 1 to 5%, Fusobacteria, Actinobacteria and Proteobacteria were sub-dominant components.

In order to investigate whether the 32 elderly volunteers enrolled in this study showed an age-related shift of the intestinal microbiota composition, a PCA analysis of the relative fluorescence signals at T0 (E) and from a previous HTF-Microbi.Array-based study of the intestinal microbiota in 8 healthy young adults (Rajilic-Stojanovic et al., 2007) (Y) was carried out (**Figure 27**). Dummy environmental variables were created to distinguish between young and elderly adult groups of subjects. The principal components PC1 and PC3, showing 41.7 and 13.3% of the total variability of the samples, respectively, allowed the separation of elderly from young adults. In comparison to young adults, the elderly were significantly depleted in the symbionts *Bacteroidetes*, *F. prausnitzii* et rel. and Bacillus clausii, and enriched in the

opportunistic pathogens *Clostridium* cluster XI, *Clostridium difficile*, *Clostridium* cluster I and II, *Clostridium perfringens*, *Enterococcus faecium* and *Bacillus cereus* (**Figure 28**).

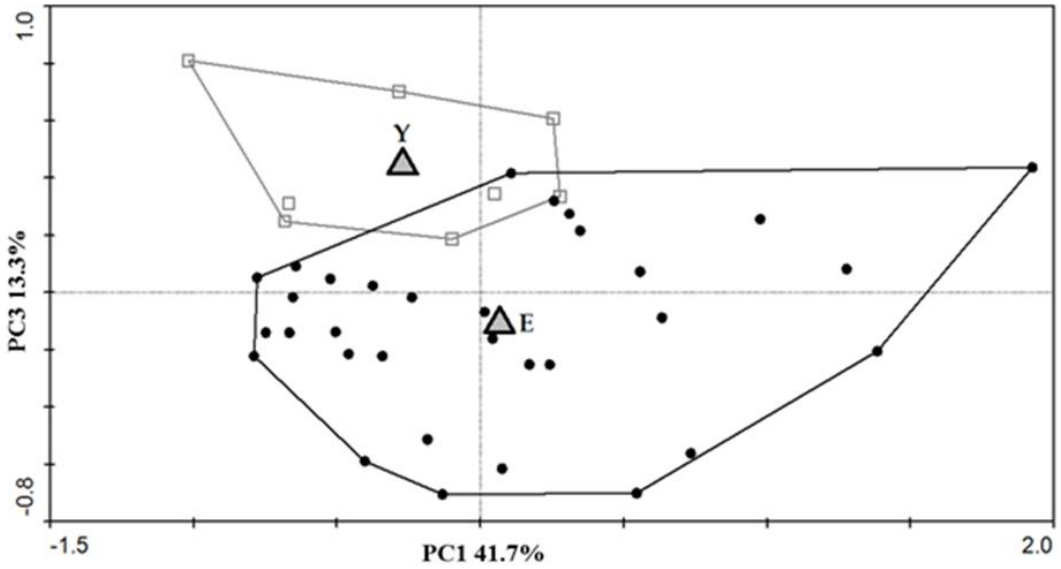

Figure 27. PCA analysis of the relative HTF-Microbi.Array fluorescence signals from the faecal microbial communities of 32 elderly at T0 and 8 healthy young adults. Dummy environmental variables (triangles) were created to distinguish between young (Y) and elderly (E). Young adults and elderly are indicated by gray squares and black circles, respectively.

The elderly were also significantly enriched in the facultatively anaerobic *Campylobacter* genus and *Enterobacteriaceae* family and, surprisingly, showed a higher abundance of some minor health-promoting components of the intestinal microbiota, such as *B. longum*, *Lactobacillus casei* and *Lactobacillus salivarius* (**Figure 28**).

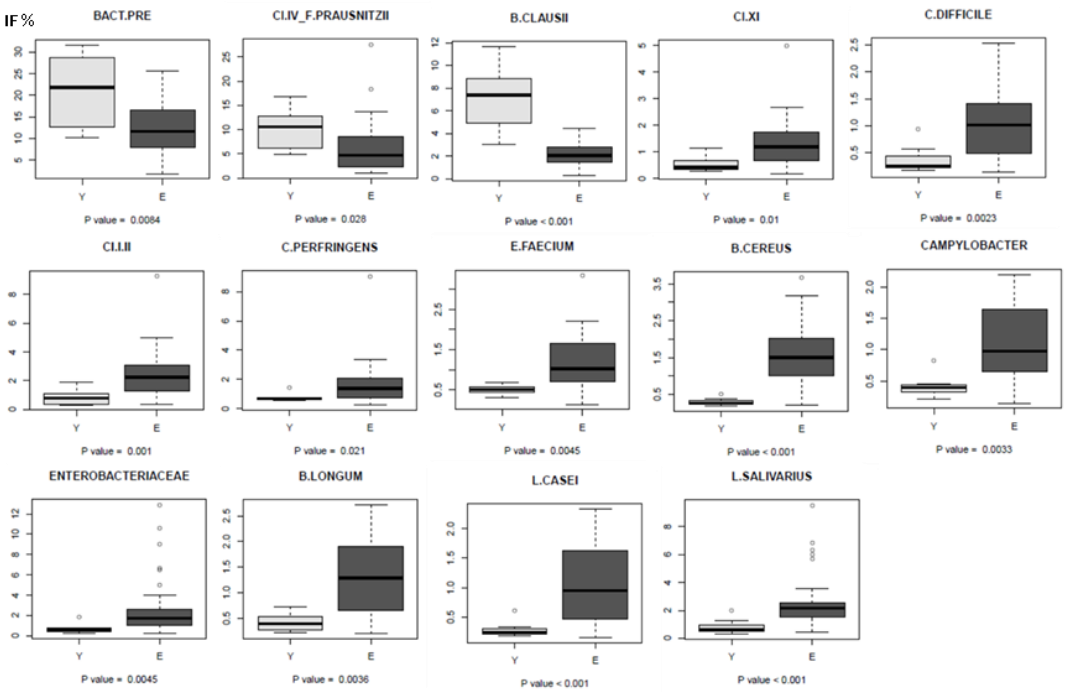

Figure 28. Box plots of the relative HTF-Microbi.Array fluorescence signals corresponding to probes showing a significantly different response between young adults (Y) and elderly (E). P values are reported for each comparison.

The representation of the HTF-Microbi.Array intestinal microbiota profiles of young and elderly adults as pie charts (**Figure 29**) highlighted the general reorganization of the intestinal microbiota occurred in the 32 Italian elderly volunteers. With respect to young adults, the elderly intestinal microbiota showed a shrinkage of the key mutualistic symbionts *Bacteroidetes*, *Clostridium* clusters IV, IX and XIVa, and an expansion of opportunistic *Clostridium* clusters I and II, *Clostridium* cluster XI and the facultative anaerobes *Proteobacteria* (*Campylobacter* and *Enterobacteriaceae)*.

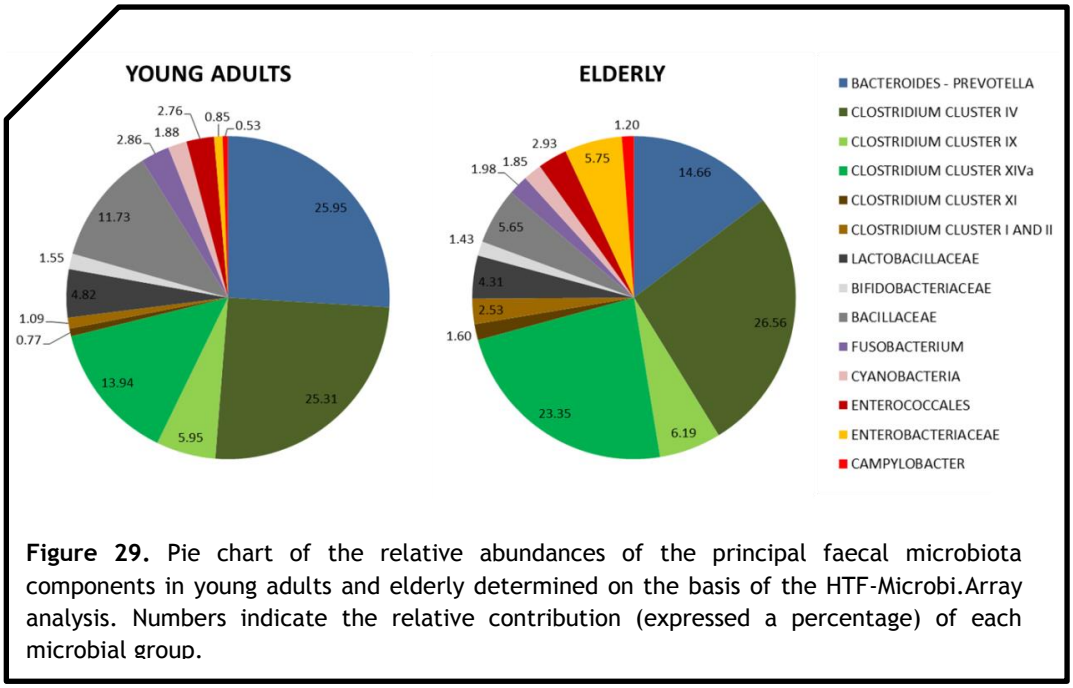

Figure 29. Pie chart of the relative abundances of the principal faecal microbiota components in young adults and elderly determined on the basis of the HTF-Microbi.Array analysis. Numbers indicate the relative contribution (expressed a percentage) of each microbial group.

Modulation of the age-related dysbioses of the intestinal microbiota by a probiotics-containing bar

We evaluated whether the intervention with a probiotic bar was effective in the modulation of the age-related microbiota dysbioses detected at T0 in the 32 elderly volunteers. To this aim, elderly volunteers were randomized in two groups: 17 were fed with the probiotic bar (Ep) and 15 with placebo (Ec). After 30 days of intervention (T30), their intestinal microbiota was characterized by HTF-Microbi.Array. According to our data, the probiotic treatment was effective in the nullification of some of the age-related dysbioses previously shown by comparing the intestinal microbiota profiles of elderly at T0 and young adults.

PROBE	Comparison *		
	E vs Y	Ep vs Y	Ec vs Y
Bacteroides/Prevotella	↓	↓	↓
Ruminococcus bromii	=	=	=
Ruminococcus albus	=	=	↓
Faecalibacterium prausnitzii	↓	=	=
Oscillospira guillermondii	=	=	=
Clostridium cluster IX	=	=	=
Veillonella	=	=	=
Clostridium cluster XIVa	=	=	=
Eubacterium rectale	=	=	=
Bifidobacteriaceae	=	=	=
B. longum	↑	↑	↑
Lactobacillaceae	=	=	=
L. plantarum	=	=	=
L. casei	↑	↑	↑
L. salivarius	↑	↑	↑
Bacillus clausii	↓	↓	↓
Bacillus subtilis	=	=	=
Fusobacterium	=	=	=
Cyanobacteria	=	=	=
Clostridium cluster XI	↑	=	↑
Clostridium diffcile	↑	=	↑
Clostridium cluster I and II	↑	↑	↑
Clostridium perfringens	↑	=	↑
Enterococcus faecalis	=	=	=
Enterococcus faecium	↑	=	↑
Bacillus cereus	↑	↑	↑
Enterobacteriaceae	↑	↑	↑
Yersinia enterocolitica	=	=	=
Proteus	=	=	=
Campylobacter	↑	=	↑

Table 8. Significant differences ($P < 0.05$) between intestinal microbiota composition of elderly at T0 and young adults (E vs Y), probiotic-treated elderly at T30 and young adults (Ep vs Y), and placebo-treated elderly at T30 and young adults (Ec vs Y) are represented by arrows indicating increase (arrowhead up) or decrease (arrowhead down) of the specific microbial group. The equal sign (=) indicates absence of significant differences.

In particular, the probiotic intake reverted the age-related increase in relative abundance of the opportunistic pathogens *Clostridium* cluster XI, *C. difficile*, *C. perfringens*, *E. faecium* and *Campylobacter* (**Table 8**). The exception was represented by the age-related decrease of *F. prausnitzii* et rel., which was nullified in both probiotic- and placebo-treated subjects (**Table 8**).

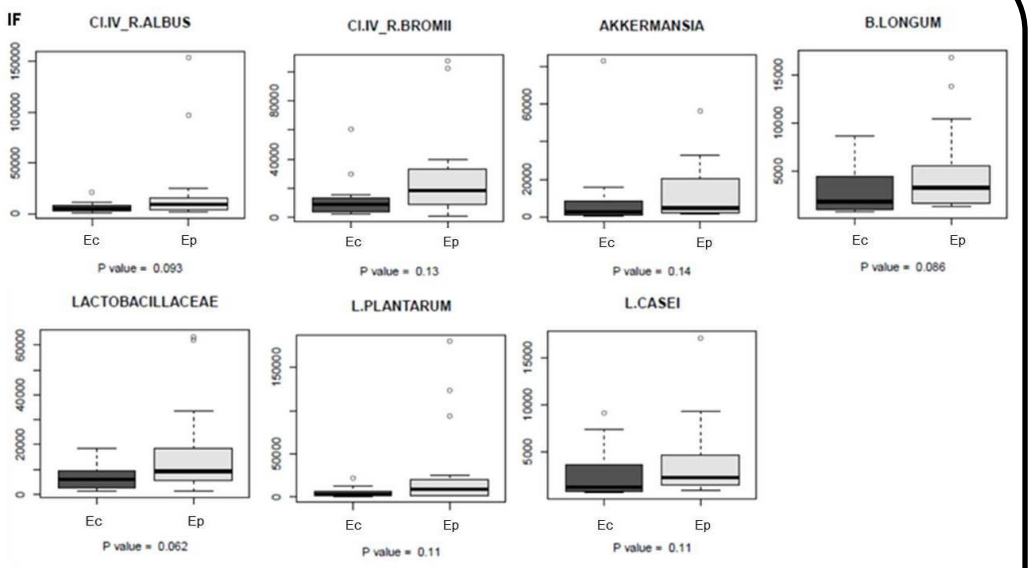

Figure 30. Box plots of the relative HTF-Microbi.Array fluorescence signals corresponding to probes showing a different response between probiotic (Ep) and placebo (Ec) treated elderly at T30. P values are reported for each comparison.

Differently, the intake of the probiotic bar did not significantly counteract the age-related decrease in relative abundance of the symbionts Bacteroidetes and *B. clausii*, as well as the increase of *Clostridium* clusters I and II, *Enterobacteriaceae* and *B. cereus*. **Figure 30** shows the comparison of the probe fluorescence signals obtained at T30 in probiotic- and placebo-treated elderly. Even if no statistically

significant differences were observed, the elderly fed with the probiotic bar demonstrated a tendency ($0.05 < P < 0.15$) toward the enrichment in *Ruminococcus bromii* et rel., *Ruminococcus albus* et rel., *Akkermansia*, *B. longum*, *Lactobacillaceae*, *Lactobacillus plantarum* and *L. casei*.

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By comparing the HTF-Microbi.Array phylogenetic profiles of the faecal microbiota of 32 Italian elderly (mean age 76 years) with the ones previously obtained from 8 Italian healthy young adults (mean age 30 years) (Candela et al., 2010), we characterized high taxonomic level microbial unbalances typical of an aged-type faecal microbiota community. With respect to healthy young adults, elderly were significantly depleted in the major symbionts Bacteroidetes, *F. prausnitzii* et rel. and *B. clausii*. This age-related shrinkage in some major microbiota mutualists could favor the expansion of opportunistic microbiota pathogens and enteropathogenic bacteria. In fact, elderly were enriched in members of *Clostridium* clusters XI, I and II, *C. difficile*, *C. perfringens*, *E. faecium*, *B. cereus* and facultatively anaerobic *Proteobacteria*, such as *Campylobacter* and *Enterobacteriaceae*. Confirming the reliability of this HTF-Microbi.Array phylogenetic fingerprint of age-related microbiota dysbioses, analogous microbiota unbalances have been reported with ageing in the literature. In particular, the age-related depletion of Bacteroidetes and *F. prausnitzii* et rel. has already been detected in Italian elderly and centenarians (Woodmansey et al., 2004; Biagi et al, 2010), and plenty of studies

report the increase in facultative anaerobes (enterobacteria and enterococci) with age (Simor et al., 2002; Woodmansey et al., 2004; Mariat et al., 2009; Rajilic-Stojanovic et al., 2009; Neish et al., 2009; Macia et al., 2012). Also the increase of the opportunistic microbiota component *C. difficile* in aged people is well documented (Cohen et al., 2003; Lahtinen et al., 2012).

In a randomized double-blind placebo controlled trial we demonstrated that one month consumption of a probiotic snack, containing a mixture of 10^9 CFU of the probiotic strains *B. longum* Bar33 and *L. helveticus* Bar13, was effective in redressing some of the age-related dysbioses of the faecal microbiota. While the placebo treated elderly showed a typical profile of an aged-type faecal microbiota community, the elderly fed with the probiotic snack partially recovered from some of these dysbioses. In particular, the probiotic treatment reverted the age-related increase in the opportunistic pathogens *Clostridium* cluster XI, *C. difficile*, *C. perfringens*, *E. faecium* and the enteropathogenic genus *Campylobacter*. Moreover, in comparison to placebo-treated elderly, the faecal microbiota of probiotic-treated subjects tended to be enriched in some major mutualistic microbiota components, such as *R. bromii* et rel. and *R. albus* et rel., the health-promoting species *Akkermansia muciniphyla* (Png et al., 2010) and the probiotics *B. longum* and *Lactobacillaceae*. These last two data probably reflect the capacity of the probiotic strains *B. longum* Bar33 and *L. helveticus* Bar13 to transiently colonize the elderly gut.

Taken together our data demonstrated the potential of a probiotic snack to counteract the age-related increase of the pathobiont components of the intestinal microbiota, which is detrimental for host longevity (Biagi et al., 2010). Pathobionts are minor microbiota components belonging to *Clostridium* clusters XI, I and II and

Proteobacteria (Pedron et al., 2008) that, in the context of an inflamed GI tract, overtake mutualistic symbionts and induce pathology. Because of their ability to prosper in an inflamed ecosystem, pathobionts can consolidate inflammation, contributing to the process of inflamm-ageing that favors frailty in aged people (Hayashi et al., 2003). Thus, by counteracting the age-related increase of pathobionts, the probiotic snack intake could help to maintain a healthy gut microbiota structure during ageing, preserving the immune homeostasis and preventing the process of inflamm-ageing. Moreover, the efficacy of the probiotic intervention in the reduction of *C. difficile* in elderly may have important clinical benefits since this microorganism is frequently involved in antibiotic associated diarrhea (lahtinen et al., 2012).

Our study opens the perspective to clinical studies specifically designed to evaluate the impact of the probiotic snack on the immune function of elderly volunteers. This information would eventually opens the way to probiotic-based dietary interventions for the modulation of the age-related dysbioses of the intestinal microbiota and to the design of elderly-tailored functional foods. Preserving the host immune homeostasis, the maintenance of a healthy intestinal microbiota profile during ageing could significantly improve the level of health among older people.

CHAPTER 8 - CONCLUDING REMARKS

In the present study we depicted trajectories of the intestinal microbiota along the human lifespan, as well as deviations associated with disease and frailty, allowing a comprehensive description of the microbiota-host relationship.

Starting with the characterization of faecal microbial community in breast-fed infant, we described the differences and the development of the enterocyte-associated microbiota in the early infancy compared to adulthood, highlighting distinct microbiota structures to respond to specific immunological needs at different age.

A first deviation from the canonical development of the gut microbiota was described in atopic children, who showed a sensible decrease of key immunomodulatory symbionts, as well as a corresponding increase in pro-inflammatory pathobionts, defining an atopic-related equilibrium, which can sustain an inflammatory status throughout the body. In this perspective, atopy treatment may be facilitated by redressing such a dysbiosis, with diet or pharmaceutical probiotics and/or prebiotics.

Additionally, exploring the intestinal microbiota in a hunter-gathering population, we demonstrate the adaptative microbiota response to specific lifestyle.

In order to investigate the final development of the human-microbiota relathionship, we explored the aged-type microbiome of centenarians in comparison to younger people, in term of metabolic potential, by detailing the modifications in

the gut microbiome as a part of the physiological changes occurring in the human host along ageing.

Finally, we demonstrated that a probiotics intervention was effective in redressing some age-related dysbioses, opening the way to the development of elderly-tailored probiotic-based functional foods to counteract the age-related dysbioses of the intestinal microbiota.

In conclusion, this work open the way to a new and dynamic vision of the gut microbiota-host mutualism, where microbiota responds adaptivily to different physiological needs during the course of human life. We described a mutualistic gut microbiome trajectory involving functional strategies to preserve homeostasis in human beings. Prolonged deviations from this trajectory, as well as a compromised adaptive response of the gut microbiota, can seriously affect the host health and consolidate the disease. It is important to note that this study is based on a limited number of microbiota configurations for depicting an exhaustive trajectory of the gut microbiome in the human lifespan. Nevertheless, our work contributes to enrich the overall portrayal of the microbiota-host relationship, opening also some perspective of specific interventions, tailored to the maintenance of a healthy trajectory of the intestinal microbiome.

CHAPTER 9 - REFERENCES

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CHAPTER 10 - ACKNOWLEDGEMENTS

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Vi voglio bene, dal cuore,

I love you, honestly,

GRAZIE

Simone

CHAPTER 11

LIST OF PUBBLICATIONS FROM THE AUTHOR

1. Schnorr S.L. and Candela M., **Rampelli S.**, Centanni M., Consolandi C., Basaglia G., Turroni S., Biagi E., Peano C., Severgnini M., Fiori J., Gotti R., De Bellis G., Luiselli D., Brigidi P., Mabulla A., Marlowe F., Crittenden A.N., Henry A.G.. **Gut microbiome of the Hadza hunter-gatherers**. Nat. Commun. 2014. Accepted for publication.

2. **Rampelli S.**, Candela M., Turroni S., Biagi E., Collino S., Franceschi C., O'Toole P.W., Brigidi P. **Functional metagenomic profiling of intestinal microbiome in extreme ageing.** AGING. 20013. Vol 5 N 12.

3. Taneyo Saa D., Turroni S., Serrazanettic D.I., **Rampelli S.**, Maccaferri S., Candela M., Severgnini M., Simonetti E., Brigidi P., Gianotti A. **Impact of Kamut® Khorasan on gut microbiota and metabolome in healthy volunteers.** Food Research International. 2014. Submitted.

4. Candela M. , Turroni S., Biagi E., Carbonero F., **Rampelli S.**, Fiorentini C., Brigidi P. **Inflammation and colorectal cancer, when microbiota-host mutualism breaks.** World Journal of Gastroenterology. 2013. Dec. Accepted for publication in the journal.

5. Centanni M., Turroni S., Consolandi C., **Rampelli S.**, Peano C., Severgnini M., Biagi E., Caredda G., De Bellis G., Brigidi P., Candela M . **The enterocyte-associated intestinal microbiota of breast-fed infants and adults responds differently to a TNF- α -mediated pro-inflammatory stimulus.** PLoS ONE. 2013 November 26, DOI: 10.1371/journal.pone.0081762.

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