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TITOLO DELLA TESI:

MOLECULAR CHARACTERIZATION OF THE HUMAN GUT MICROBIOTA:
THE EFFECT OF AGING

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Fact are stubborn things, but statistics are more pliable.
Mark Twain, (1835-1919)

First draw your curve, then plot your readings.
A. Bloch, in "The Murphy's Law"

ABSTRACT

Age-related physiological changes in the gastrointestinal tract, as well as modification in lifestyle, nutritional behaviour, and functionality of the host immune system, inevitably affect the gut microbiota. The study presented here is focused on the application and comparison of two different microarray approaches for the characterization of the human gut microbiota, the HITChip and the HTF-Microb.Array, with particular attention to the effects of the aging process on the composition of this ecosystem.

By using the Human Intestinal Tract Chip (HITChip), recently developed at the Wageningen University, The Netherland, we explored the age-related changes of gut microbiota during the whole adult lifespan, from young adults, through elderly to centenarians. We observed that the microbial composition and diversity of the gut ecosystem of young adults and seventy-years old people is highly similar but differs significantly from that of the centenarians. After 100 years of symbiotic association with the human host, the microbiota is characterized by a rearrangement in the Firmicutes population and an enrichment of facultative anaerobes. The presence of such a compromised microbiota in the centenarians is associated with an increased inflammation status, also known as inflamm-aging, as determined by a range of peripheral blood inflammatory markers.

In parallel, we overtook the development of our own phylogenetic microarray with a lower number of targets, aiming the description of the human gut microbiota structure at high taxonomic level. The resulting chip was called High Taxonomic level Fingerprinting Microbiota Array (HTF-Microb.Array), and was based on the Ligase Detection Reaction (LDR) technology, which allowed us to develop a fast and sensitive tool for the fingerprint of the human gut microbiota in terms of presence/absence of the principal groups. The validation on artificial DNA mixes, as well as the pilot study involving eight healthy young adults, demonstrated that the HTF-Microb.Array can be used to successfully characterize the human gut microbiota, allowing us to obtain results which are in approximate accordance with the most recent characterizations. Conversely, the evaluation of the relative abundance of the target groups on the bases of the relative fluorescence intensity probes response still has some hindrances, as demonstrated by comparing the HTF.Microb.Array and HITChip high taxonomic level fingerprints of the same centenarians.

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INTRODUCTION

1.1 The human gut microbiota

1.1.1 *Overview*

An enormous number of microorganisms are known to colonize and form complex communities, or microbiota, at various sites within the human body. In fact, humans have been proposed to be "metaorganisms" consisting of 10-fold greater numbers of bacterial than animal cells that are metabolically and immunologically integrated. The gastrointestinal tract harbors the largest and most complex bacterial ecosystem in the human body (Hattori and Taylor, 2009; Neish, 2009). An increasing gradient in bacterial concentration characterizes the human gastrointestinal tract, from stomach, to jejunum, ileum and colon, where the concentration peaks to 10^{11}-10^{12} bacterial cells per gram of stool (Ley *et al*, 2006a; Leser and Molbak, 2009).

Shaped by millennia of co-evolution, host and bacteria have developed beneficial relationships, creating an environment for mutualism. The collective genome of the gut microbial community is called "microbiome" and contains more than 100 times the number of genes in the human genome (Backhed *et al*, 2005). The microbiome endows human hosts with physiological attributes they did not evolve on their own, including enhanced metabolic capabilities, such as hydrolysis of complex plant polysaccharides, synthesis of certain vitamins and production of short chain fatty acids (SCFA) (Hooper *et al*, 2002). Other benefits provided by the gut microbiota are involved in the development and maintenance of the immune system homeostasis (Round and Mazmanian, 2009) and in the development and survival of the gut epithelium (Neish, 2009). Finally, the gut microbiota exerts a right of "first occupancy" precluding other microorganisms, particularly pathogens, from invading the occupied niches (Sansonetti and Medzhitov, 2009).

The total diversity of a healthy adult gut ecosystem is generally reported around 1,000-1,200 species-level phylogenetic types, called "phylotypes", defined as group of 16S sequences with a certain level (97-99%) of similarity (Eckburg *et al*, 2005; Rajilic-Stojanovic *et al*, 2007; Xu *et al*, 2007). However, some recent molecular studies approximated the diversity of the gut microbiota to more than 10,000 phylotypes (Frank *et al*, 2007; Tap *et al*, 2009). Only a small percentage of these gut inhabitants correspond to fully characterized bacterial isolates, whereas 75-82% is estimated to remain uncultured (Eckburg *et al*, 2005; Flint *et al*, 2007; Rajilic-Stojanovic *et al*, 2007).

This remarkable diversity (Fig. 1.1) is confined largely to very few divisions of bacteria and one member of Archaea, *Methanobrevibacter smithii*. The vast majority (90-99%) of the bacterial inhabitants belongs to Firmicutes and Bacteroidetes, with the dominant Firmicutes (50-80%) primarily composed of bacteria belonging to the *Clostridium* clusters XIVa and IV (Eckburg *et al*, 2005; Ley *et al*, 2006b; Turnbaugh *et al*, 2006; Frank *et al*, 2007; Armougom and Raoult, 2008; Dethlefsen *et al*, 2008; Tap *et al*, 2009). Other phyla represented in the human gut are Actinobacteria (3-15%), Proteobacteria (1-20%), and Verrucomicrobia (0.1%) (Frank *et al*, 2007; Andersson *et al*, 2008; Hattori and Taylor, 2009; Tap *et al*, 2009). The procaryotic phyla Fusobacteria, Cyanobacteria, Spyrochaetes, and Lentisphaerae, as well as several eukaryotic fungal species (*Candida*, *Aspergillus* and *Penicillium*), were also reported as gut inhabitants in very small percentages (Rajilic-Stojanovic *et al*, 2007).
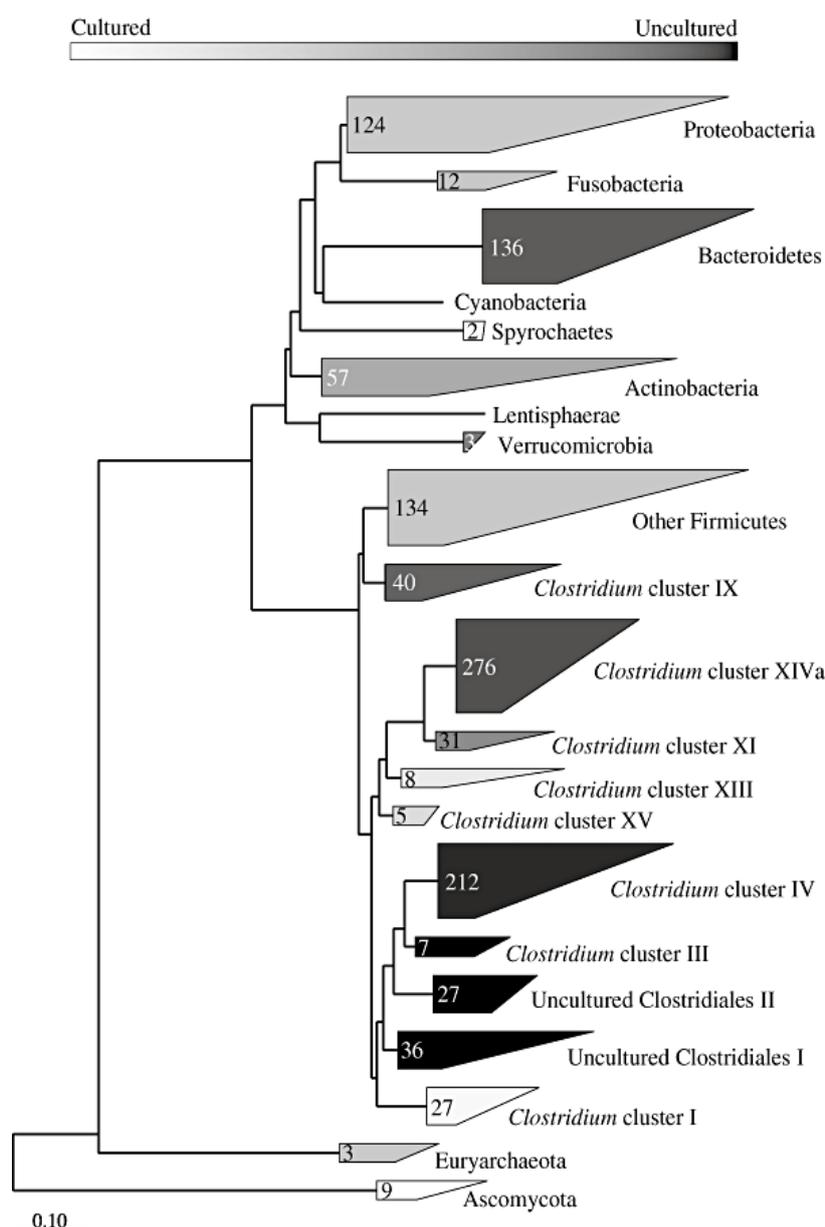
Fig. 1.1 16S rRNA-based phylogenetic tree of the distinct phylo-types that have been found in the human gastrointestinal tract. The Firmicutes phylum is divided in the different *Clostridium* clusters, as described by Collins *et al* (1994). The relative proportion of phylotypes that correspond to cultured representatives is indicated by different darkness of the filling: black fills indicates species detected in cultivation independent studies, while white indicates species detected in cultivation based studies. Numbers of distinct phylotypes are given for each phylogenetic group. (Rajilic-Stojanovic *et al*, 2007).

The adult-like structure of the gut microbiota summarized above is established after the first year of life, during which the gut ecosystem progresses from sterility to extremely dense colonization (Palmer *et al*, 2007). Through healthy adulthood, the bacterial density and diversity in the gut remains relatively stable over time, in spite of the continuous flow of intestinal content, reflecting the ability to maintain a high degree of homeostasis (Vanhoutte *et al*, 2004; Leser and Molbak, 2009). The adult microbiota shows an astonishing individual variability, and it is considered as unique as a fingerprint in terms of species and strains composition (Zoetendal *et al*, 1998; Eckburg *et al*, 2005; Ley *et al*, 2006a). Age, diet, lifestyle, and geographic origins influence the composition of the gut microbiota, but studies involving human adults with different relatedness, from genetically unrelated people to monozygotic twins, demonstrated that the impact of genotype may also be significant in shaping the gut bacterial ecosystem (Zoetendal *et al*, 2001; Lay *et al*, 2005; Mueller *et al*, 2006, Khachatryan *et al*, 2008).

Despite the remarkable host specificity in the gut community membership, a high degree of conservation in its expressed functions and metabolites has been reported (Mahowald *et al*, 2009). This suggests that the gut microbiota may be characterized by a marked "functional redundancy" to ensure that the key functions of the microbial community remain unaffected by the individual variability in terms of species composition (Gill *et al*, 2006). The existence of a "human core gut microbiome", defined as those genes which are common to the gut microbiomes of all or the majority of humans, has been hypothesized to be responsible for the functional stability of the gut microbiota (Turnbaugh and Gordon, 2009). On the contrary, a "human core gut microbiota", defined as a number of species which are common to all humans, could hardly be defined, since different combinations of species could fulfil the same functional roles (Tschop *et al*, 2009, Turnbaugh *et al*, 2009). Aside to the core, the set of genes which are present in smaller subsets of human constitutes the "human variable microbiome" (Fig. 1.2). This impressively wide variation from the core is the result of a combination of host-specific factors, such as genotype, physiological status, host pathologies, lifestyle, diet, environment, and the presence of transient populations of microorganisms that cannot persistently colonize the human gut. In return, core and variable components of the the human microbiome influence different aspects of the human health, including nutrient responsiveness and immunity (Turnbaugh *et al*, 2007).

Major alterations in the gut microbiota structure, and consequently in the gut microbiome, affect human physiology, health status and disease susceptibility. The ecological disorder of the bacterial community is called "dysbiosis", and affects the structure of the microbiota at the level of order/phylum. For instance, changes in the relative proportion of Firmicutes and Bacteroidetes are

always detected in metabolic disorders, such as obesity (Ley *et al*, 2006b; Turnbaugh *et al*, 2009; Turnbaugh and Gordon, 2009), type 1 diabetes (Wan *et al*, 2008), and inflammatory bowel diseases (IBD) (Frank *et al*, 2007; Sartor, 2008a; Sartor, 2008b). Imbalanced microbiota and bacterial overgrowth are also reported in irritable bowel syndrome (IBS), which is a very common, probably stress-related, functional gastrointestinal disorder (Kassinen *et al*, 2007; Othman *et al*, 2008).

Being involved in so many aspects of human physiology and disease, the microbiota (and microbiome) represents a new frontier of human biology and medicine, as well as a potential drug target, in which new strategies for the maintenance of human health may be found (Hattori and Taylor, 2009).
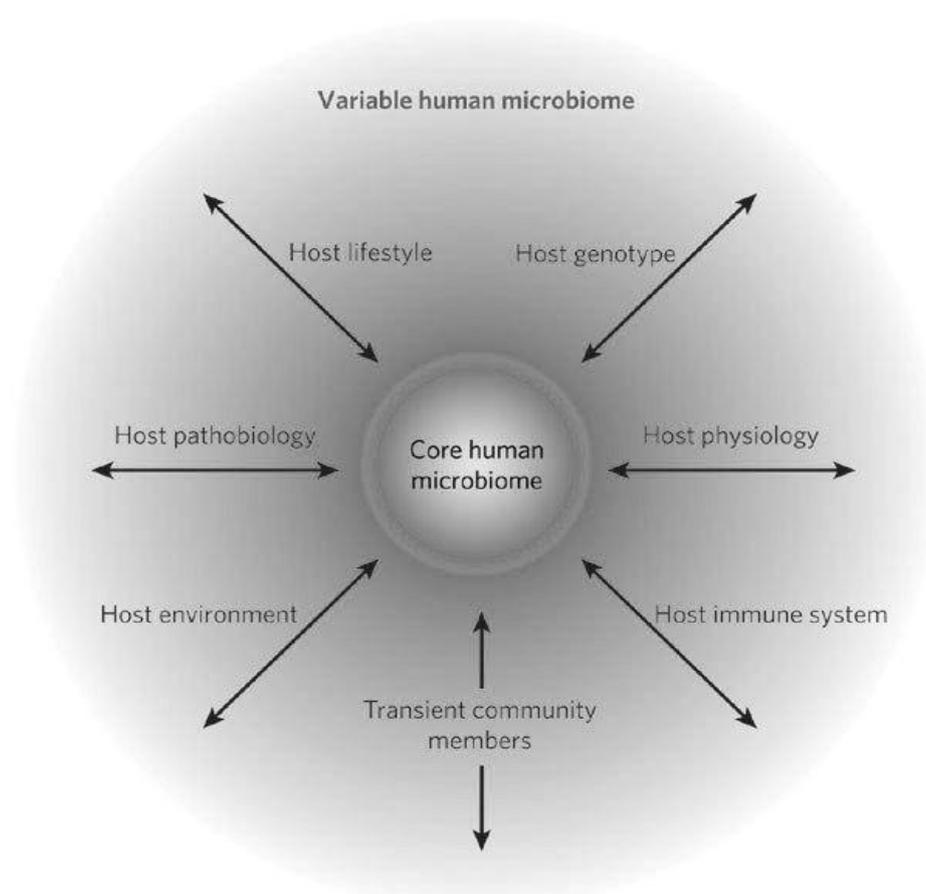
Fig. 1.2 A super-organismal view of the human microbiome. A subset of microbial genes may be found in most healthy human beings (core microbiome) whereas variable components are presents only in specific ethnic groups, age groups, geographic locations, or associated with specific dietary patterns or disease status (Turnbaugh *et al*, 2007; Preidis and Versalovic, 2009).

1.1.2 Gut microbiota and immune system

The coevolution of human host and its microbiota had a particularly strong impact on the immune system, which had to develop the ability to discriminate between the tolerated resident commensal microbiota and invasive pathogens, to whom it must respond. Immunological abnormalities observed in germ-free model animals showed how strong the impact of the microbiota is in shaping the immune system, at both structural and functional level (Macpherson and Harris, 2004). Some symbiotic bacteria of the human gut ecosystem showed to prevent inflammatory disease during colonization, whereas other components of the gut microbiota showed the potential to induce inflammation under particular conditions ("pathobionts"). Therefore, having the potential to exert both pro- and anti-inflammatory responses, the gut microbiota composition is likely to be linked to the proper functioning of the immune system (Round and Mazmanian, 2009).

In healthy condition, the cross talk between the bacteria and both the mucosal and the systemic immune system ends up in a low-level physiological inflammatory response, which is the result of the balance between tolerance and reactivity. A dysbiotic microbiota can alter this delicate and complex equilibrium, leading to a switch from physiological to pathological inflammation and immune response, characterized by unrestrained immune cell activation and pro-inflammatory cytokines production. In particular, a dysbiotic, or so-called "colitogenic" microbiota, is often associated with the pathogenesis of IBD. Since no infectious organisms have been identified as causative agents of IBD, it has been suggested that the target of the chronic inflammation may not be pathogens but overrepresented pathobionts. Although it is not clear whether dysbiosis is the cause or the effect of the disease, gut microbiota alterations may be a factor underlying the development of the disease in genetically predisposed individuals (Sartor, 2008a; Round and Mazmanian, 2009; Sansonetti and Medzhitov, 2009).

Inflammation processes induced in the gut mucosa by a dysbiotic microbiota are also connected to the pathogenesis of colorectal cancer, a major disease of the Western "over-seventy" population. Even if the complex interplay between microbiota, host and cancer still has to be completely understood, it is known that chronic inflammation supports carcinogenesis by inducing gene mutations, inhibiting apoptosis or stimulating angiogenesis and cell proliferation. Moreover, colonic microorganisms can promote DNA damages in epithelial cells by producing carcinogenic, co-carcinogenic and pro-carcinogenic molecules (Candela *et al*, manuscript in preparation).

Finally, the cross-talk between the host immune system and the gut microbiota may also be involved in the physiology of allergies and autoimmune pathologies, such as celiac disease and type 1 diabetes (MacDonald and Monteleone, 2005; Varaala *et al*, 2008; Wen *et al*, 2008; Tang, 2009). Autoimmunity is rapidly becoming a major health problem in the industrialized countries. It has

been hypothesized that modern health-care strategies, such as caesarian section, formula-based diet, improved hygiene, vaccinations, and use of antimicrobials in infants, may produce deviations in the normal development of the human microbiota. The consequent increased probability of dysbiosis can alter the development of the immune system, predisposing the individuals to various immune related diseases later in life ("hygiene hypothesis") (Round and Mazmanian, 2009).

1.1.3 Probiotics and prebiotics

The increased understanding of the impact of the gut microbiota on human health resulted in attempts of manipulate its composition by the use of probiotics and prebiotics, both in prophylactic and therepeutic perspectives.

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Guarner and Malagelada, 2003). The concept of "probiotics" dates back over 100 years, when Metchnikoff suggested that the use of living bacteria in fermented milk products could improve health by detoxifying putrefactive substances (Metchnikoff, 1907). However, only recently scientific knowledge and tools have become available to evaluate their potential in improving health, and preventing and treating diseases, allowing us to find out many "candidate probiotics" among bacteria, with different actions in different disease status (Reid *et al*, 2003; Chermesh and Eliakim, 2006).

Effectiveness and safety are prerequisites for microorganisms to be considered probiotics. Moreover, they should pass through the upper gastrointestinal tract unaffected by bile acids and proteolytic enzymes, and enter the small bowel. Their main beneficial effects are to function as the first barrier to pathogenic organisms by adherence, to produce substances that have antimicrobial effects, and to stimulate the immune processes in the host (Floch and Martin, 2005; Chermesh and Eliakim, 2006). The most employed probiotics belong to the genera *Lactobacillus* and *Bifidobacterium*, but other genera, including *Escherichia*, *Enterococcus*, *Bacillus* and *Saccharomyces,* are also used, based on documented efficacy through clinical studies (Gibson *et al*, 2004).

Prebiotics are more a recent concept, firstly defined 10 years ago. They are chemical substances, usually oligosaccharides, acting as substrates specifically for the host's autochtonous probiotic bacteria, and thus promoting their growth. Prebiotics are selected as being non-digestible by the host and non-metabolizable by non-probiotic gut bacteria, but stimulating for bifidobacteria and lactobacilli (Gibson *et al*, 2004; Hamilton-Miller, 2004).

1.2. The characterization of the gut bacterial ecosystem

1.2.1 From cultivation to molecular techniques

The characterization of the community composition is the first step in the study of a complex bacterial ecosystem. The gut microbiota has been intensively investigated by anaerobic culture techniques, but it is well known by now that culture based methods provide only an incomplete picture of the overall diversity of the microbiota (Rajilic-Stojanovic *et al*, 2007; Zoetendal *et al*, 2008). Additionally, culture based methods are laborious, time-consuming, and prone to statistical and methodological errors. Thus, in the last decade many molecular tools have been developed, allowing faster and more accurate investigations of complex microbial ecosystems.

The most efficient culture-independent strategies for exploring microbial biodiversity are based on the 16S ribosomal RNA (rRNA) gene sequence. The 16S rRNA gene (Fig 1.3) consists of about 1,500 nucleotides and contains regions conserved among all the bacteria, interspersed with 9 regions (V1 to V9) in which the sequences are variable among bacterial phylotypes (Tannock, 1999). Sequencing of the 16S rRNA genes has resulted in more than one million small subunit rRNA entries, which are available through databases such as GeneBank, EMBL, Ribosomal Database Project (RDP).
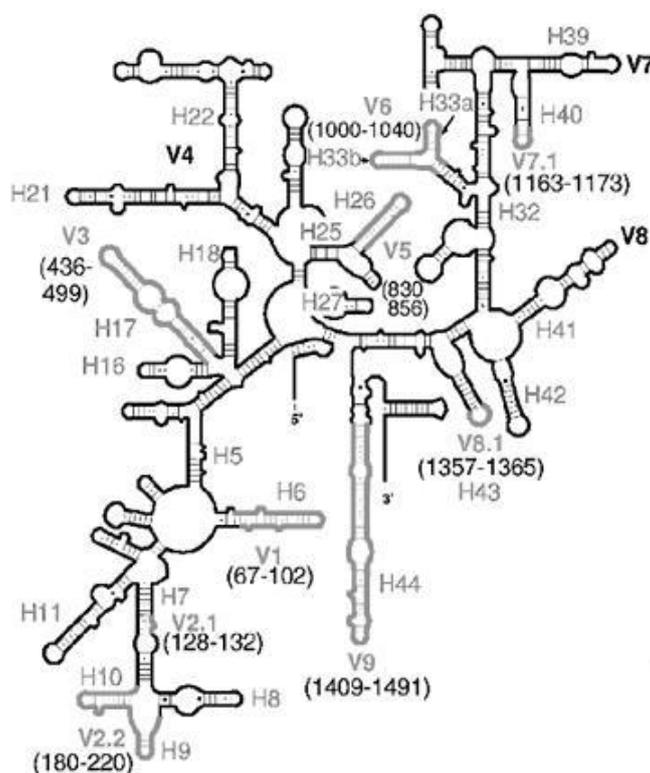
Fig. 1.3 *E. coli* 16S rRNA secondary structure. Position of the nine variable regions (V1 to V9) is indicated.

Common 16S-based approaches include cloning and sequencing of a large number of 16S rRNA genes, which gives significant information about the identity of uncultured bacteria, but is laborious, expensive and hardly quantitative. Several so-called "fingerprinting techniques" are more appropriate to study a complex bacterial community, although they are only semi-quantitative. They are used for monitoring community shifts (e.g. in response to dietary treatments), following the microbiota composition through time, or comparing individuals. The most important fingerprinting techniques are denaturing/temperature gradient gel electrophoresis (D/TGGE), single strand conformation polymorphism (SSCP) and terminal-restriction fragment length polymorphism (T-RFLP).

Frequently applied culture-independent approaches to quantify bacterial cells in environmental samples are the real time-PCR and the Fluorescent In-Situ Hybridization (FISH). These techniques use 16S rRNA-targeted primers and probes, respectively. Specific primer sets and probes have been developed to quantify bacteria belonging to various groups, such as *Eubacterium rectale - Clostridium coccoides* group (approximatively corresponding to the *Clostridium* cluster XIVa), *Clostridium leptum* group (*Clostridium* cluster IV), *Bacteroides/Prevotella* group, *Bifidobacterium*, *Lactobacillus/Enterococcus* group, *Veillonella*, *Atopobium*, and *Ruminococcus* group. When fluorescent probes are used, bacterial counts can be performed by using microscopy or flow cytometry (Zoetendal *et al*, 2004). The disadvantage of these quantitative techniques resides in the fact that primers and probes can be designed and validated only for bacterial groups which are known, and whose 16S rRNA has been sequenced. Moreover, the study consists in one experiment for each probe or primer set.

More recently, the microarrays technology has been applied to the study of the diversity of complex ecosystem. Diversity microarrays allow identification of bacterial species in unknown samples. They have recently been introduced in microbial ecology for environmental studies of phylogenetically diverse microbial groups. In the majority of the cases they are based on the 16S rRNA gene, but microarrays based on other functional genes (*rpoB, recA, gyrB, groEL,* and *atpD*) can be used to distinguish between closely related bacteria, having a resolution below the species level. For instance, members of the superfamily of γ-proteobacteria, such as *Escherichia coli, Salmonella,* and *Shigella* can be distinguished by using a *gyrB* (B subunit of DNA gyrase) based diversity microarray (Kakinuma *et al*, 2003). The first effort in developing a diversity microarray specific for the intestinal tract consisted in 60 probes, targeting 20 bacterial species predominant in human intestine (Wang *et al*, 2002).

The development of diversity microarrays during the last years has been aiming at the improvement of the coverage of the complete human intestinal microbiota. Since our knowledge of the gut

microbiota composition is becoming wider and more detailed, it is necessary to increase the number of probes spotted on the intestinal diversity microchips. In 2006, Palmer and coworkers developed a powerful diversity microarray, based on the 16S rRNA sequence, able to detect and quantify bacterial species up to 0.1% of fractional abundance. A more comprehensive and improved diversity microarray, containing more than 9,000 taxonomically specific probes, was designed by the same research group and used to profile the gut microbial community of human infants, aiming the investigation the possible origins of the infant microbiota (Palmer *et al*, 2007). More recently, a human intestinal microbiota phylogenetic microarray, based on the Affymetrix GeneChip platform, containing probes for 775 different bacterial phylotypes, was developed by Paliy *et al* (2009). The microarray was used in a pilot study, detecting differences in the microbiota composition of adults and children. In conclusion, microarray technology, with its ability to detect and measure thousands of distinct sequences simultaneously, has been recognized as a valuable tool to explore and systematically characterize complex microbial communities.

1.2.2 *The HITChip technology*

The Human Intestinal Tract Chip (HITChip) is a 16S based diversity microarray designed to comprehensively cover the diversity of the human gut microbiota reported in culture-dependent and culture-independent studies. It has been developed by Rajilic-Stojanovic *et al* (2009), on the basis of an ecosystem-specific curated database, containing 16,000 human intestinal 16S rRNA sequences. The HITChip is manifactured by Agilent Technologies and contains approximately 4,800 *in situ* synthesized, tiling oligonucleotide probes with a narrow range of melting temperatures. Each of the 1,140 targeted microbial phylotypes (<98% identity) is represented by multiple probes designed on the basis of the V1 and V6 hyper-variable regions of the 16S rRNA. The tiling approach consists in the design of series of nucleotide probes (in this case, 6 overlapping probes) which cover the entire sequence of the V1 and V6 regions, so that each phylotype is defined by a probe set, instead of a single probe. The specificity of each probe was determined by *in silico* hybridization against the human intestinal microbiota 16S rRNA database (Rajilic-Stojanovic *et al*, 2007). Probes were categorized at three levels of specificity: level 1 (phylum/order like sequence groups), level 2 (genus-like groups, >90% similarity), and level 3 (>98% similarity). Therefore, although all probes were designed to target unique phylotypes, some of them were *a posteriori* assigned to different nodes in the phylogenetic tree. Briefly, the HITChip approach consists in amplifying by universal PCR the total 16S rRNA genes from the DNA extracted from feces (or other human gut samples), than a fluorophor-labelled rRNA is obtained and hybridized on the array.

The hybridization profiles of each sample provides an identification and a relative quantification of the phylogroups present in the sample (Fig. 1.4).

HITChip was used in a pilot study involving five adults and five elderly, and confirmed previous findings that the adult fecal microbiota is highly individual specific and relatively stable over time (Rajilic-Stojanovic *et al*, 2009). This technique was also used in a clinical trial aiming the study of effects of a probiotic cocktail on people with IBS (Kajander *et al*, 2008).
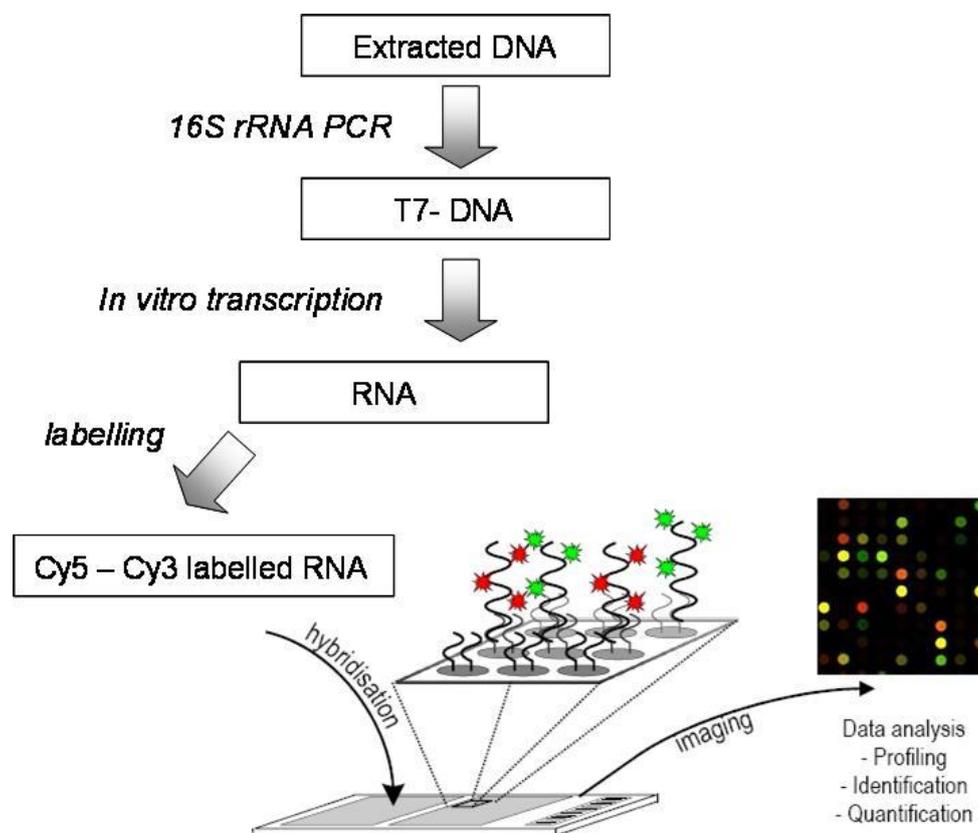
Fig. 1.4 Schematic representation of the HITChip approach for the characterization of a complex bacterial ecosystem.

1.2.3 *Pyrosequencing: a massive sequencing approach*

Even if diversity microarrays are a very straight-forward techniques to use for comparative community profiling, faster and cheaper than high-coverage sequencing, they can only detect taxa that are covered by the reference sequences. In recent years, next generation sequencing technologies have been developed, allowing the massive sequencing of a vast numbers of (partial) 16S rRNA genes from many complex bacterial ecosystems, at much lower cost than Sanger's capillary electrophoresis method (Claesson *et al*, 2009).

Pyrosequencing is a sequencing-by-synthesis method, based on the detection of the pyrophosphate release occurring at the nucleotide incorporation during the sample amplification. The technique was developed by Nyrén and Ronaghi at the Royal Institute of Technology in Stockholm (Ronaghi *et al*, 1998). The newest application of the pyrosequencing technology, the 454 FLX Sequencing System, consists in using a PicoTiterPlate device in which hundreds of thousands of beads, each carrying millions of copies of a unique single strand DNA molecules, are sequenced in parallel. Nucleotides are flowed sequentially in a fixed order across the plate, and when a nucleotide complementary to the template is flowed into a well the polymerase extends the DNA strand. The incorporation of one (or more) nucleotide results in a reaction that generates a light signal, whose intensity is proportional to the number of nucleotides incorporated (www.454.com).

Thanks to the 454 Life Science, a biotechnology company specialized in this high-throughput sequencing method, this technology is being successfully applied to many life science field, including the characterization of the gut ecosystem.

A key pyrosequencing innovation, currently used in comparative studies of microbial communities, is multiplexing. In the so-called "barcoded pyrosequencing" each sample is tagged with a unique molecular barcode (a short key sequence added during PCR) and can be sequenced together with other barcoded samples in the same run (Hamady and Knight, 2009). The applicability of the barcoded pyrosequencing to the characterization of human microbial ecosystems was demonstrated by Andersson *et al* (2008), in a comparative study of the throat, stomach and fecal microbiota, and by Dethlefsen *et al* (2008), in an analysis of the effects of antibiotic treatment on the gut microbiota diversity. More recently, Claesson and colleagues (2009) demonstrated that pyrosequencing-based composition correlates with the HITChip hybridization profile of the same samples, indicating high robustness of both approaches.

1.2.4 *The 'omics' era*

Although the techniques listed above provide an insight in the gut microbiota that was inaccessible with the use of traditional culturing methods, determining the function of all microbes remains a challenging task. One way to gain insight into potential functions and activities of microbes without the need of cultivation is the metagenomic approach.

Metagenomic is defined as the study of the collective genome of an ecosystem, with attention to both phylogenetic and functional aspects. It is performed by extracting the total DNA from a microbial community, followed by cloning the DNA fragments in a suitable host using a vector (fosmids or bacterial artificial chromosomes). This results in a metagenomic library that can be used for sequence-driven and/or function-driven analysis. The first approach is used to create a catalog of

the genetic potential that is present in an ecosystem, whereas function-driven analyses consist in the transcription and translation of the genes located on the metagenome clone, and may lead to the discovery of novel enzyme activities. However, it has to be realized that the detection of genes/functions in a metagenomic library does not necessarily mean that they are functionally important. Therefore, other meta-'omics' approaches which use RNA, proteins, and metabolites as a target (Fig. 1.5) must be used to gain insight in the activity and functionality of a microbial community (Zoetendal *et al*, 2008).
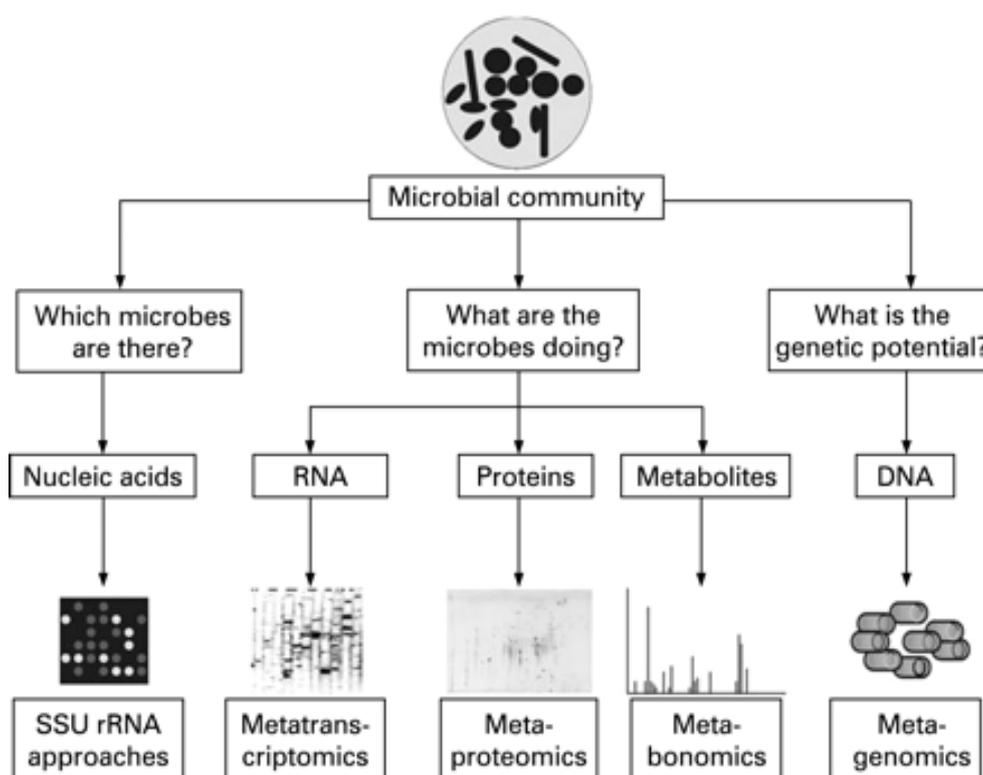
Fig. 1.5 Schematic representation of the metagenomic and other community-based 'omics' approaches. SSU rRNA, small subunit ribosomal RNA (Zoetendal *et al*, 2008).

1.3 The aging process

1.3.1 Theories of aging and perspectives

Aging has been defined as the process of intrinsic deterioration of an organism that is reflected at the population level as a decline in the production of offspring, and an increase in the death probability (Partridge and Gems, 2002). In principle, aging-associated processes are progressive and deleterious phenomena, common to all individuals of the species (Viña *et al*, 2007).

Many theories have been proposed to explain the complex phenomenon of the aging process, especially in relation to the hypothesis of aging being or not an evolutionary determined ("programmed") event. In Table 1 the various theories of aging are categorized as evolutionary, molecular, cellular and systemic (Weinert and Timiras, 2003). Evolutionary theories argue that aging results from a decline in the force of natural selection. Molecular and cellular theories are so divided depending on the level at which a factor of aging is found, whereas the system theories correlate the aging process to the different essential organ systems. However, in the recent views, aging is presented as an extremely complex, multifactorial process, that cannot be explained by a single cause. Different theories of aging should not be considered as mutually exclusive, but complementary and overlapping (Tosato *et al*, 2007). A more general definition says that aging is a stochastic process that occurs after reproductive maturation and results from the diminishing energy available to maintain molecular fidelity (Hayflick, 2000b).

A new interesting theory of aging, called "remodeling theory", has been proposed in 1995 (Franceschi *et al*, 1995; Franceschi and Cossarizza, 1995; Franceschi *et al*, 2000c) to conceptualize results emerging from studies focused on the aging of the human immune system, involving healthy centenarians. According to this hypothesis body resources are continuously optimized during the aging process, and the decline in the functionality of the immune system should be considered a dynamic process, which includes both loss and gain of functions. In this perspective, the concept of "deterioration" should be replaced by "adaptation": people who successfully age and reach longevity are those who better adapted to damaging agents and, in particular, immunological stresses. The concept of remodeling can be extended to other pathway than those involved in the immune system, to explain the aging phenomenon in its whole complexity.

Finally, it has to be reminded that, with the increasing age, individual variability also increases, and each organ, tissue, and cell type of the body may reach different level of senescence ("aging mosaic" theory), making the understanding of the aging process a complex and difficult task to be disentangled (Capri *et al*, 2008; Cevenini *et al*, 2008).

Table 1.1 The major aging theories, divided by biological level.

Theory	Description
Evolutionary	
Mutations accumulation	Mutations that affect health at older ages are not selected against
Disposable soma	Somatic cells are mantained only to ensure continued and reproductive success; after reproduction soma become disposable
Antagonist pleiotropy	Genes beneficial at younger age become deleterious at older ages
Molecular	
Gene regulation	Ageing is caused by changes in the expression of genes regulating both development and ageing.
Codon restriction	Fidelity/accuracy of mRNA translation is impaired because of an increased inability to decode codons
Error catastrophe	Decline in fidelity of gene expression with ageing results in incresed fraction of abnormal proteins
Somatic mutations	DNA damages accumulation in somatic cells
Dysdifferentiation	Gradual accumulation of random molecular damages impairs regulation of gene expression
Cellular	
Cellular senescence (Telomer theory)	Phenotypes of aging are caused by an increase in frequency of senescent cells. Senescence may result from telomer loss (replicative senescence) or cell stress (cellular senescence)
Free radicals	Oxidative metabolism produces highly reactive free radicals that subsequently damage lipids, protein and DNA
Wear-and-tear	Accumulation of normal injuries
Apoptosis	Programmed cell death from genetic events or genome crisis
System	
Neuroendocrine	Alterations in neuroendocrine control of homeostasis results in ageing-related physiological changes.
Immunological	Decline of immune functions with ageing results in decreased incidence of infectious diseases but increased incidence of autoimmunity
Rate-of-living	It assumes a fixed amount of metabolic potential for every living organism (live fast, die young)

Humans, and pet and zoo animals, are the only species in which large numbers experience aging. In the absence of human impact, aging simply does not occur in wild animals because of predation, diseases and accidents. Human aging has revealed itself because modern lifestyle allows humans to avoid or prevent many causes of death, not only in early life but long after reproducing. For instance, the improvement in the health conditions leads to the possibility to efficiently prevent and/or cure many diseases, and consequently to the reduction of the overall morbidity and mortality. Thus, aging is a consequence of the improvement in social-environmental conditions, medical cares, and quality of life, a sort of "artefact" of the human civilization (Franceschi *et al*, 2008; Hayflick, 1998; Hayflick, 2000a; Hayflick, 2000b).

It has been estimated that the elderly population in developed (western) countries, defined as over-60 years old people, will reach 2 billions by the year 2050 (Cohen, 2003), and that most babies born since 2000 will celebrate their 100th birthday (Christensen *et al*, 2009). In this perspective, it has to be considered that the aging population is the most susceptible to diseases and disability, and the most likely to be in the need of hospitalization and/or nursing care. The quality of life of this increasing share of the human population is going to become an imperative concern, also in relation of the health care cost, especially considering their high levels of health care utilization, and the high cost of medical care. Consequently, the goal of research on aging should not be the increase of human longevity regardless of the consequences, but to increase active longevity, free from disability and functional dependence (Hayflick, 2000b).

1.3.2 *Longevity*

On the scenario of the increasing aging of the human population in developed countries, longevity, defined as the attainment of the extreme limits of the human life span, is becoming a reality. Life-span is defined as the maximum numbers of years that a human being can live, whereas the life expectancy is the average number of years that a human can expect to live. The longest unambiguously documented life span is that of Jeanne Calment (France, 1875–1997), who died at the remarkable age of 122 years and 164 days (Abbott, 2004).

Longevity is considered the result of the interaction between environmental factors, genetics, epigenetics and stochasticity, each contributing for a variable amount to the overall presentation of the phenotype (Candore *et al*, 2006). In particular, researches on twins and related individuals suggested that about 25% of the total variation in adult human life span can be attributed to genetic and epigenetic factors (McGue *et al*, 1993). Another 20 to 30% may be explained by the environment, meaning in particular life style and nutrition, but also health and socio-economical conditions in childhood, adult's socio-economic position, health behavior, everyday activities,

mental and physical health (Vaupel *et al*, 1998). Today, the most addressed environmental risk factors of the western world are cigarette smoking, alcohol consumption and overweight. Psychological traits and dispositions may also have some influence on longevity: whereas subjective well-being and happiness are related to a longer life (Idler and Benyamini, 1997), stress and depression may increase the risk of mortality (Huppert and Whittington, 1995). Finally, a contribution in attaining longevity is given by stochasticity, meaning the wide variation of life span of genetically identical organisms, even if reread in a constant environment. It is possible that longevity may be achieved by different combinations of these components, that vary quantitatively and qualitatively in different geographic area, according to the population-specific gene pool and socio-economic level (De Benedictis and Franceschi, 2006).

At the basis of longevity are the adaptive mechanisms that the body set up to compensate and neutralize the adverse effects of the unrepaired damages accumulated during the whole life, leading to a progressive change in the human body composition and microenvironments (Ostan *et al*, 2008). Many studies highlighted that extremely long-living people (centenarians) show unusual and largely unexpected features for which most of the current concepts in biology and genetics are inadequate. In fact, the aging process allows the emerging of biological effects due to individual genetic differences which can be neutral or silent at younger age. This suggests that the genetic of aging and longevity, called "post-reproductive genetics", may not be ruled by the classical genetic principles (De Benedictis and Franceschi, 2006; Capri *et al*, 2008).

Centenarians may be considered the best example of extreme longevity in our species. Even if they are by definition very old people showing all the signs of a prolonged aging process, and consequently very frail, they represent a selected population in which the appearance of major age-related diseases, such as cancer and cardiovascular diseases, has been consistently delayed or escaped. Part of the centenarians, in fact, are still in quite good physical and psychological health, categorized as "group A" by Franceschi *et al* (2000c), "escapers" by Evert *et al* (2003), and "exceptionals " by Gondo *et al* (2006). However, centenarians were not the most robust subjects of their age cohort, but rather those who better adapted to the age-related changes in their body (Ostan *et al*, 2008). Although centenarians are still believed to be rare curiosity, their number is increasing dramatically: it has been estimated that the centenarians in the Italian population in the years 2005 was around 7000 (Franceschi *et al*, 2008). For this reason, studying centenarians is a matter of broad biological and medical interest.

1.3.3 *Aging and immune system*

Immune system (IS) represents an integrated, evolutionary conserved defense network, which adapts over time reflecting the history of infections experienced by the organism. In this context, antigens constitute the major pressure for the IS evolution. Lifelong exposure to a variety of infectious agents for a period longer than previously encountered during human evolution, such as the case of long living people (80-120 years old), is one of the main forces driving the aging of IS (Franceschi *et al*, 2000a; Pawelec *et al*, 2005; Ostan *et al*, 2008).

Immunosenescence can be defined as the age-associated decrease in immune competence which leads to an increased susceptibility to diseases. It is a multifaceted phenomenon, resulting from the adaptation of the body to the continuous challenge of bacterial and viral infections. Antigenic stimulation, oxidative stress, and other harmful agents are major players in this life-long remodelling of the IS (Franceschi *et al*, 1998; Larbi *et al*, 2008; Ostan *et al*, 2008).

One of the major features of human immunosenescence is thymic involution, which is at the basis of the profound alterations in the T lymphocytes observed in the elderly. Particularly, a deficient replacing of naïve T cells lost in the periphery, resulting in the inability to maintain the breadth of the T cells repertoire, and an accumulation of memory and effector T cells have been observed (Aspinall and Andrew, 2000; Ostan *et al,* 2008). The innate immunity is also affected by the immunosenescence process, with a decrease in numbers and/or functionality of natural killer cells (NK), dendritic cells, and phagocytes, which is at the basis of the increased susceptibility of elderly to infections (Ostan *et al*, 2008). In particular, high NK cytotoxicity has been associated with healthy aging and longevity (Sansoni *et al*, 1993).

The complex age-related remodelling of IS includes a profound modification within the cytokine network, consisting in a general increase in the production of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α). The result is a chronic, low-grade inflammatory condition named "inflamm-aging", characterized by activation of macrophages and expansion of specific clones (megaclones) of T lymphocytes (Fagiolo *et al*, 1993; Franceschi *et al*, 2000a; Franceschi *et al*, 2000c; Larbi *et al*, 2008). This condition goes along with a general increase in the main inflammatory markers, such as C-reactive protein and serum amyloid A. The overall inflammatory status potentially triggers the onset of the most important age-related diseases, such as cardiovascular diseases, artheriosclerosis, metabolic syndrome, type-2 diabetes, neurodegeneration, arthrosis and arthritis, osteoporosis, sarcopenia, major depression and frailty (Ostan *et al*, 2008). Chronic inflammation may also be at the basis of the pathogenesis of cancer in several organs, including stomach, liver and large intestine (Coussens and Werb, 2002; Condeelis and Pollard, 2006; de Visser *et al*, 2006).

All the age-related changes in the IS have a strong genetic component, as it was demonstrated by studies in old people and centenarians. In fact, many studies showed that the frequency of several variants of important genes involved in immune responses and inflammation are present at different frequency in long lived people with respect to young subjects. In Fig 1.6 the relevance of the genetic background in aging is summarized (Effros, 2003; Capri *et al*, 2006; Salvioli *et al*, 2006; Larbi *et al*, 2008).

Centenarians represent a cohort of selected survivors, able to counterbalance the damaging effects of immunosenescence and inflamm-aging by activating a variety of anti-inflammatory networks, such as those involving IL-10 and TGF-β1, but still benefit from the functionality of the IS necessary to resist infectious diseases (Forsey *et al*, 2003; Vasto *et al*, 2007). Thus, it is postulated that centenarians are equipped with gene variants that allow them to optimize the balance between pro- and anti-inflammatory cytokines and other mediators involved in inflammation (Carrieri *et al*, 2004; Franceschi *et al*, 2007). As a consequence, the study of centenarians is of great importance since they represent the best model to study human aging and body adaptation to the age-related stress phenomena at cellular, tissue, and systematic level (Franceschi *et al*, 1995).
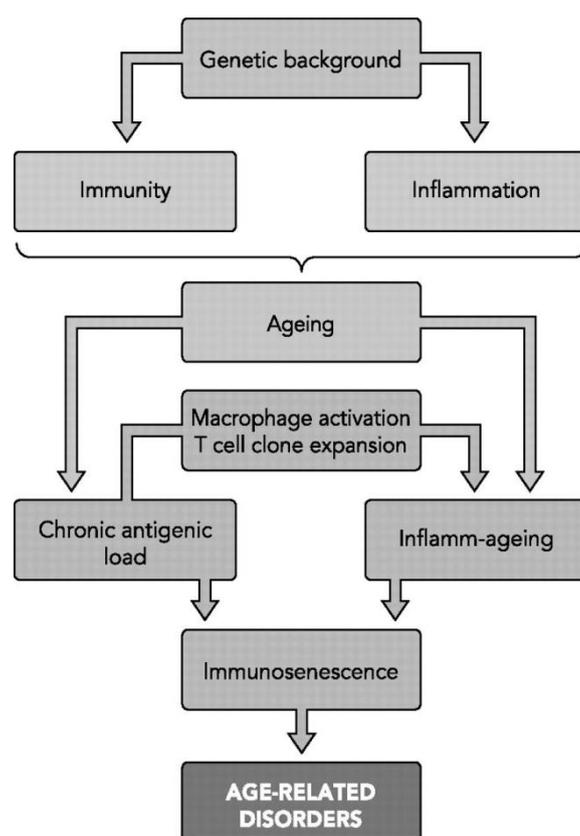
Fig. 1.6 Schematic representation of the aging of the immune system (Larbi *et al*, 2008).

1.3.4 Aging and gut microbiota

The physiology of the intestinal tract is deeply affected by the aging process, as well as the nutritional behavior and lifestyle. Increased threshold for taste and smell (Weiffenbach *et al*, 1982; Doty *et al*, 1984), coupled with swallowing difficulties (Castell, 1988) and masticatory dysfunction caused by loss of teeth and muscle bulk (Newton *et al*, 1993), can lead to the consumption of a narrow, nutritionally imbalanced diet. Atrophic gastritis, which is a common disturbance in the elderly, is associated with a decreased absorption of calcium, iron and vitamin B_{12} (Russel, 1992). Furthermore, decreased intestinal motility usually results in a slower intestinal transit, which leads to fecal impaction and constipation (Kleessen *et al*, 1997); the subsequent reduced bacterial excretion brings to fermentation and putrefactive processes in the gut (Brocklehurst, 1972; Macfarlane *et al*, 1989). The gut functionality in the elderly is also influenced by mucosal immunosenescence and chronic inflammatory status (Ginaldi *et al*, 2001; Schmucker *et al*, 2003; Guigoz *et al*, 2008).

Physiological changes in the gastrointestinal tract, as well as modification in diet, mobility, psychosocial stress and functionality of the host immune system, inevitably affect the balance of the gut microbiota, bringing to a greater susceptibility to diseases such as gastroenteritis or infection (van Tongeren *et al*, 2005; Woodmansey, 2007). Moreover, since the gut microbiota plays a role in the nutrient intake and energy homeostasis, changes in the microbiota could be also related to the loss of weight and other distinctive conditions of the elderly such as frailty, metabolic syndrome, diabetes and sarcopenia.

Although this is a field of growing interest, limited researches have been focused on the changes which occur in the gut microbiota during the aging process. Studies involving the characterization of the gut microbiota in the elderly are often hardly comparable since they can have different purposes, such as the relation between microbiota and frailty (van Tongeren *et al*, 2005), the impact of antibiotic treatments (Bartosch *et al*, 2004; Woodmansey *et al*, 2004), or the differences between the gastrointestinal health status of hospitalized and non-hospitalized patients (Bartosch *et al*, 2004; Zwielehner *et al*, 2009). Moreover, results obtained by bacterial isolation techniques (Hopkins and Macfarlane, 2002; Woodmansey *et al*, 2004) cannot be compared with results obtained with more advanced techniques for the molecular characterization of the microbiota since most of the component of the human complex ecosystems are unculturable species (Hopkins *et al*, 2001; Rajilic-Stojanovic *et al*, 2007). Consequently, the definition of the gut microbiota of healthy elderly is a challenging task, especially since Mueller and coworkers (2006) reported striking country related differences in the effect of age on the gut microbiota composition. Following, the major

findings on the topic of age-related changes in the gut microbiota composition are summarized (reviewed in: Saunier and Doré, 2002; Woodmansey, 2007).

A very well known effect of the aging process is the decrease of bifidobacteria in the gut microbiota, both in terms of abundance and species diversity (Gorbach *et al*, 1967; Mitsuoka, 1992; Gavini *et al*, 2001; Hopkins and Macfarlane, 2002; Saunier and Doré, 2002; Woodmansey *et al*, 2004; Mueller *et al*, 2006). This age-effect is magnified by antibiotic treatment, hospitalization (Bartosch *et al*, 2004; Zwielehner *et al*, 2009) and *Clostridium difficile* associated diarrhea (CDAD), which is often a major problem in hospitalized and antibiotic treated elderly patients (Hopkins *et al*, 2001). The cause of the age-related decline in bifidobacteria may be the decreased ability to adhere to the intestinal epithelium showed by *Bifidobacterium* strains isolated from elderly patients, in comparison to those isolated from younger people (Ouwehand *et al*, 1999; He *et al*, 2001). Interestingly, the viable count of *Lactobacillus*, which are other well known health promoting bacteria, were found to increase in the elderly with respect to younger adults (Mitsuoka, 1992; Makivuokko *et al*, 2010). FISH experiments showed an age-related increase of members of the *Lactobacillus-Enterococcus* group only for French and German subjects, whereas Italians and Swedish showed the opposite trend (Mueller *et al*, 2006).

Another well established age-related effect is the increase in facultative anaerobes: streptococci, staphylococci, enterococci and, above all, enterobacteria (Gavini *et al*, 2001; Woodmansey *et al*, 2004; Mueller *et al*, 2006; Makivuokko *et al*, 2010; Mariat *et al*, 2009). The enterobacteria group comprehend potentially pathogenic species, which may be the cause of infections when the host resistance mechanisms fail as a result of the aging process. Antibiotic treatment, hospitalization and CDAD are known to promote the increase of enterobacteria in the microbiota of elderly people (Hopkins *et al*, 2001; Bartosch *et al*, 2004; Woodmansey *et al*, 2004).

Isolation studies showed a decline in viable counts of Bacteroidetes with the increasing age (Hopkins and Macfarlane, 2002; Woodmansey *et al*, 2004), but this observation was confirmed by FISH only when Italian adults and elderly were compared, whereas German subjects showed an inverse relation between age and *Bacteroides* amount (Mueller *et al*, 2006). A recent real time PCR study showed an increase in *Bacteroides* relative abundance in Austrian hospitalized elderly patients with respect to healthy young adults (Zwielehner *et al*, 2009); similar results were obtained for Finnish volunteers by 16S rRNA sequencing and % G+C profiling (Makivuokko *et al*, 2010). Thus, the behavior of the *Bacteroides* population during aging seems strongly country-dependent. Moreover, according to studies using 16S rRNA-based molecular techniques, a decrease in Bacteroidetes seems to be more strictly related to frailty condition, antibiotic treatment,

hospitalization and CDAD, than to the aging process itself (Hopkins *et al*, 2001; Bartosch *et al*, 2004, van Tongeren *et al*, 2005). As Bacteroidetes and Firmicutes are the most dominant phyla in the gut, the ratio between these two groups of bacteria could be considered a more informative parameter of the overall status of the gut microbiota. In this context, Mariat and coworkers (2009) reported that the Firmicutes/*Bacteroides* ratio was lower in elderly people than in young adults.

The phylum Firmicutes raises even more problems in comparing results of isolation and molecular techniques, because isolation techniques often referred to the genus *Clostridium*, whereas the 16S rRNA based molecular techniques use the *Clostridium* clusters classification proposed by Collins *et al* (1994) which is built on the 16S rRNA sequence similarity. The 16S based phylogenetic classification is more appropriate to describe changes in a complex microbiota, as the Firmicutes phylum seems to be the most affected by the cultivation bias (Rajilic-Stojanovic *et al*, 2007). Isolation studies often focus on members of the genus *Clostridium* known to be potentially pathogenic, such as *C. perfringens* or *C. difficile*, responsible for CDAD (Hopkins and Macfarlane, 2002). Conversely, molecular characterization techniques usually consider changes in groups of bacteria which are major components of the gut microbiota, such as the *Clostridium* clusters IV and XIVa. These two groups are also very important as they contain the majority of the bacteria able to produce butyrate, a short chain fatty acid with a key role in maintaining the health of the human gut (Pryde *et al*, 2002; Louis and Flint, 2009).

In a T-RFLP study, Hayashi and coworkers (2003) reported a decrease in bacteria belonging to the *Clostridium* cluster XIVa (also called *Clostridium coccoides/Eubacterium rectale* group) in healthy Japanese elderly. This result was confirmed for Italian and Finnish people, whereas German elderly showed inverse trend (Mueller *et al*, 2006; Makivuokko *et al*, 2010). The decrease of members of the *Clostridium* cluster XIVa was also related to frailty condition, hospitalization and non-steroidal anti-inflammatory therapy (van Tongeren *et al*, 2005; Tiihonen *et al*, 2008; Zwielehner *et al*, 2009).

As for the *Clostridium* cluster IV (often referred to as *Faecalibacterium prausnitzii* group or *Clostridium leptum* group), Mueller and coworkers (2006) reported that bacteria belonging to this group significantly decrease along with the aging process in Italian subjects. There are no other studies in literature reporting changes in the *Clostridium* cluster IV during healthy aging, but it is known that hospitalization, frailty and antibiotic treatment are responsible for the decrease in the amount of *F. prausnitzii* in the gut microbiota of the elderly (Bartosch *et al*, 2004, van Tongeren *et al*, 2005). *F. prausnitzii* has recently become a matter of high interest because of its anti-inflammatory properties in the gut environment (Sokol *et al*, 2008).

In the validation study of the HITChip the gut microbiota of five adults and five elderly subjects from Northern Europe were compared. The obtained fingerprints confirmed that the gut microbiota

of young and elderly adults are different in structure and composition, especially in relation to the decrease of Bacteroidetes with the age and the increase of streptococci (Rajilic-Stojanovic *et al*, 2009).

Changes of the microbiota in terms of composition, expression of virulent factors and metabolic activity are detected by cells of the innate immune system, responsible for discriminating whether the bacterial signal represents or not a danger to the host. The gut immune system keeps under control a "physiological" level of inflammation at the mucosa microenvironment, preserving the function of the epithelium and its cross-talk with the microbiota. In old people this balance is upset because of the changes both in the microbiota structure and in the immune system activity, ending up in a localized sub-clinical inflammatory status which is typical of the intestine of the elderly.

It has been hypothesized that immunosenescence and the decline in health promoting bacteria in the gut, such as bifidobacteria, may be related. In fact, several strains of *Bifidobacterium* exhibit powerful anti-inflammatory properties; thus, they may be able to restore the balance of pro- and anti-inflammatory cytokines production, which is at the base of the immune system regulation (Isolauri *et al*, 2001; Isolauri *et al*, 2002; Matsumoto and Benno, 2006). Several immuno-stimulatory properties, such as modulation of cytokines production or adjuvant effects on T lymphocytes and NK cells activity, have been thoroughly demonstrated on the basis of *in vitro* and *ex vivo* models, for various health promoting bacteria (reviewed in: Meydani and Ha, 2000; Blum *et al*, 2002).

More recently, Ouwehand and coworkers (2008) demonstrated that the amounts of several autochthonous *Bifidobacterium* species in the gut microbiota of elderly subjects are negatively correlated with the levels of the pro-inflammatory cytokine TNF-α and the regulatory cytocine IL-10, indicating that modulation of the fecal bifidobacterial population may provide a means for influencing the inflammatory responses.

1.3.5 Probiotic and prebiotic in the elderly

The aging process is often accompanied by an increased susceptibility towards infections of the gastrointestinal tract, which has been estimated to be 400 times higher in elderly than in younger adults. This observation identifies the elderly as a potential target sub-population of particular interest for dietary modulation of the gut microbiota, for both prophylactic and therapeutic management of the gut health (Hébuterne, 2003; Tuohy, 2007). In particular, the evidences of the decline of bifidobacteria and other health promoting bacteria in the gut of aging subjects open up the possibility of reversing such trend by administration of probiotics, prebiotics or symbiotics. Up

to the moment, trials demonstrating the application of pro/prebiotics as supplement or therapy in elderly people are too limited to establish their beneficial effects.

It has been demonstrated that supplementation of probiotic *Bifidobacterium* strains significantly increases the levels of health promoting bacteria (bifidobacteria and lactobacilli) in the fecal microbiota of elderly (Amhed *et al*, 2007; Lahtinen *et al*, 2009; Matsumoto *et al*, 2009). More importantly, there are evidences about the effectiveness of *Bifidobacterium* and/or *Lactobacillus* intake in alleviating the age-related chronic constipation (reviewed in: Hamilton-Miller, 2004) and normalizing bowel movements in institutionalized elderly (Pitkala *et al*, 2007).

As for the prebiotics, inuline supplementation was reported to be able to increase the viable count of bifidobacteria in constipated elderly, while the frequency of the detection of enterobacteria decreased with the treatment. The ingestion of inuline improved constipation in 9 out of 10 subjects (Kleessen *et al*, 1997). It has been reported that fructo-oligosaccharides ingestion (Guigoz *et al,* 2002; Bouhnik *et al*, 2007), as well as the supplementation of a galacto-oligosaccharides mixture (Vulevic *et al*, 2008), are able to significantly increase the numbers of bifidobacteria at the expense of less beneficial groups. However, patients supplemented with prebiotic fibers often reported side effects, such as flatulence and abdominal pain.

Bartosch and coworkers (2005) reported that a synbiotic preparation, containing inulin and two strains of *Bifidobacterium*, was able to increase the number of total bifidobacteria and lactobacilli in elderly individuals. Similar results were obtained by Ouwehand *et al* (2009) following the supplementation of a synbiotic containing *Lactobacillus acidophilus* and lactitol; this intervention also increased the stool frequency, which is an important parameter given the high incidence of constipation in the elderly.

Even if these results seem promising, further studies are needed to determine the impact of functional foods on gut microbiota metabolism, pathogen resistance and intestinal function of elderly subjects. Moreover, therapeutic effects of pre/probiotics on age-related diseases are still to be determined.

Several *in vivo* studies have been published investigating the possibility to influence the inflammatory response and immune system through probiotic/prebiotic supplementation. Clinical trials showed that 3- and 6-weeks interventions with *Bifidobacterium lactis* can have positive effects on the immune system of old people, such as increases of NK cells activity and monocytes phagocytic capacity (Chiang *et al*, 2000; Arunachalam *et al*, 2000; Gills *et al*, 2001a, Gills *et al*, 2001b). Similar results regarding phagocytosis and NK cell activities were also described following supplementation of *Lactobacillus rhamnosus* (Sheih *et al*, 2001) and *Lactobacillus casei* Shirota (Takeda and Okumura, 2007) in elderly subject. A probiotic yogurt supplementation was also tested

on elderly affected by intestinal bacterial overgrowth, and demonstrated to be able to normalize the response to endotoxin and modulate activation markers in blood phagocytes (Schiffrin *et al*, 2009). A study focused on enterally fed elderly demonstrated that the level of the pro-inflammatory cytokine TNF-α decreased in response to supplementation of fermented milk containing a probiotic strain of *Lactobacillus*; those elderly volunteers also showed a decline in the incidence of infections (Fukushima *et al*, 2007). Significant increases of phagocytosis, NK cell activity, and production of the regulatory cytocine IL-10 were also reported following prebiotic supplementations in elderly volunteers, together with a reduction in the production of pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) (Guigoz *et al*, 2002; Schiffrin *et al*, 2007; Vulevic *et al,* 2008).

Supplementation of strains of *Lactobacillus* and *Bifidobacterium* were also positively correlated with the spermine and spermidine levels, which has been suggested to be associated with reduced inflammation (Matsumoto and Benno, 2006; Ouwehand *et al*, 2009).

Restoring the homeostasis in the host-microbiota interactions in the elderly could be a way to improve intestinal function and help in the prevention of the immunosenescence and inflamm-aging processes. Anyway, confirmation of the results summarized above, and more insights in the cross talk between gut microbiota and immune system, are needed, especially in the perspective of the development of nutritional strategies targeting the gut functionality or the mucosal-host reactivity (Guigoz *et al*, 2008). Moreover, even if some of the results seem promising, parameters need to be established to evaluate the practical significance of the effects of pro-prebiotic treatments on the immune system. An interesting decrease in the duration of respiratory and gastrointestinal "winter infections" in elderly was reported following a 3 weeks *Lactobacillus casei* supplementation (Turchet *et al*, 2003), but the study is still too isolated to draw conclusions.

2.
PROJECT OUTLINE

With its global impact on the physiology of the intestinal tract, the aging process can seriously affect the composition of the human gut microbiota. The decreased intestinal motility results in a slower intestinal transit, which leads to fecal impaction and constipation (Kleessen *et al*, 1997). The subsequent reduced bacterial excretion brings to fermentation and putrefactive processes in the gut (Brocklehurst, 1972; Macfarlane *et al*, 1989), and inevitably affects the homeostasis of the bacterial ecosystem. Moreover, the age-related decline in the functionality of the immune system (immunosenescence) (Ostan *et al,* 2008) leads to a chronic low-grade inflammatory status (inflamm-aging) which can affect the intestinal gut ecosystem, undermining the balance between the microbiota and the gut associated immune system (Franceschi, 2007a; Franceschi *et al,* 2007b; Nova *et al*, 2007; Guigoz *et al*, 2008). Finally, considering the impact of the diet on the gut microbiota composition (Flint *et al*, 2007), changes in nutritional behavior and life style of the aged people concur to the age-related unbalances of the intestinal microbial community.

The immense microbial community which constitutes the intestinal microbiota is an integral component of human physiology. With a role in host nutrition and protection against pathogens, this "microbial organ" is undeniably of primary importance for human health and well being. Thus, the hypothesis that age-related changes in the composition of this symbiont microbial community may contribute to the progression of diseases and frailty in the elderly has been ventured (Woodmansey, 2007; Guigoz *et al*, 2008).

The role of the gut microbiota in the aging process needs a deeper understanding, as well as its interaction with the host immune system. In the study presented here, we undertook to explore the age-related changes both in the inflammatory status and in the gut ecosystem composition, by using one of the state-of-the-art molecular techniques for the microbiota characterization, the HITChip (Rajilic-Stojanovic *et al*, 2009). In particular, we decided to expand the usual target populations of comparative studies, addressing not only young adults (20-40 years old) and elderly (60-80 years old), but also non-hospitalized centenarians. This approach, called the "Centenarians project", aimed at the broadening of our view of the changes which occur in the gut microbiota of adult human beings during aging, expanding our vision towards the extreme limits of the human lifespan. Gut microbiota composition and diversity, at both qualitative and quantitative levels were analyzed

by using the HITChip, and the results were confirmed and deepened by the use of real time-PCR. The results of these analyses were correlated with the inflammatory status of each subject, evaluated by immunophenotype characterization and quantification of the blood level of several pro- and anti-inflammatory cytokines.

Following the most recent demonstrations of the strong impact of inflammation on the balance between symbionts and "pathobionts" in the human gut (Round and Mazmanian, 2009), the combined approach presented here allowed us to investigate how perturbation in the gut microbiota and inflamm-aging process may affect each other.

In parallel, we overtook the development of our own phylogenetic microarray with a relatively low number of targets, aiming the description of the human gut microbiota structure at high taxonomic level. The resulting chip was called High Taxonomic level Fingerpring Microbiota Array (HTF-Microb.Array).

The gut microbiota exhibits an astonishing degree of individual variability at species level, while most of the variations which were correlated to diseases or metabolic disorders consisted in dramatic unbalances between groups of bacteria at high taxonomic groups. For instance, it is known that obesity is characterized by a higher proportion of Firmicutes and Actinobacteria with respect to Bacteroidetes (Turnbaugh *et al*, 2006; Turnbaugh *et al*, 2009), whereas IBDs are characterized by a reduction of bacterial diversity in the *Clostridium* cluster IV and XIVa*,* a decline in Bacteroidetes biodiversity, and a correspondent increase in Proteobacteria and *Bacillus* (Frank *et al*, 2007)*.* At the light of these observations we decided to focus our attention on larger phylogenetic groups of bacteria, instead of species or strains, in order to obtain a tool with the potential to characterize dramatic unbalances in the human intestinal microbiota which may be associated with specific diseases.

Due to the low number of probes, the Ligase Detection Reaction (LDR) technology (Castiglioni *et al*, 2004; Hultman *et al*, 2008) was chosen for the development of the HTF-Microb.Array. This technique is based on an enzymatic *in vitro* reaction and exploits the discriminative properties of the DNA ligase. The construction of a LDR-based array requires the design of a pair of two adjacent oligonucleotides specific for each target sequence: a probe specific for the variation ("Discriminating Probe", DS) which carries a 5'-fluorescent label, and a second probe, named "Common Probe" (CP), starting one base 3'-downstream of the DS that carries a 5'-phosphate group and a unique sequence (cZipCode) at its 3'-end. The oligonucleotide probe pairs and a thermostable DNA ligase are used in a LDR reaction with previously PCR-amplified DNA fragments. This reaction is cycled to increase product yield. The LDR products, obtained only in

presence of a perfectly matching template by action of the DNA ligase, are addressed to a precise location onto a Universal Array (UA), where a set of artificial sequences, called Zip-codes are arranged. These products, carrying both the fluorescent label and the unique cZipCode sequence, can be detected by laser scanning and identified according to their location within the array. The LDR approach is a highly specific and sensitive assay for detecting single nucleotide variations; thus, differences of a single base along the 16S rRNA gene can be employed to distinguish among different microbial lineages.

Here we describe the design procedure, validation and testing of the HTF-Microb.Array, the first application of the LDR-array technology to the gut microbial ecosystem characterization. A pilot study involving eight healthy young adults demonstrated that the HTF-Microb.Array can be used to successfully characterize the human gut microbiota, allowing us to obtain results which are in approximative accordance with the most recent characterizations.

Finally, the HTF-Microb.Array analysis and the HITChip approach were compared for validation purpose. In particular, the fecal microbiota of five of the centenarians involved in the "Centenarians project" was analyzed also with the HTF-Microb.Array, and the results were compared.

The research project presented here resulted in the production of two research articles: "Through aging and beyond: gut microbiota and inflammatory status in seniors and centenarians" (Biagi *et al*, submitted to *PloS ONE*), and "High taxonomic level fingerprint of the human intestinal microbiota by Ligase Detection Reaction - Universal Array approach" (Candela *et al*, submitted to *BMC Microbiology*).

3.
MATERIALS AND METHODS

3.1 Subjects recruitment and study groups

3.1.1 *Subjects involved in the "Centenarians project"*

Eighty-four subjects belonging to different age groups were enrolled for this study in Emilia Romagna, Italy. Group C was composed by 21 centenarians (20 women, 1 men) aged 99 to 104 years (average 100.5), whose health status was representative of this exceptional population [23]. Group E was composed by 22 elderly (11 women, 11 men) aged 63 to 76 years (average 72.7) genetically unrelated to the centenarians in group C, and they were offspring of parents who did not reach longevity (average parents death age, 59.3). Group Y was composed by 20 young adults (9 women, 11 men) aged 25 to 40 years (average 31). Group F, offspring of the centenarians belonging to group C, composed by 21 elderly people (10 women, 11 men) aged 59 to 78 (average 67.5), was also included in the analysis. Subjects of groups E, F, and Y were free living and in good physical and cognitive health conditions. Subjects affected by malignant neoplasia and/or in therapy with immunosuppressive drugs like cyclosporin, methotrexate, glucorticoids, anticoagulant drugs, and who recently (at one month) used antibiotics were excluded from the study. The study protocol was approved by the Ethical Committee of Sant'Orsola-Malpighi University Hospital (Bologna, Italy). After obtaining informed consent, a standard questionnaire to collect information regarding the health status, drugs use, clinical anamnesis, and life style was administrated. Moreover, tests to assess cognitive ability (Mini Mental State Examination, MMSE), self sufficiency and autonomy (ADL, IADL), physical ability (Handgrip Test and Chair Stand Test), arterial blood pressure measurement, and Body Mass Index (BMI) calculation were performed. Peripheral blood and feces were collected from each subject. Blood samples were not collected from 3 centenarians and 1 subject in group F because of refusal or impossibility of the subjects. Principal haemato-biochemical parameters evaluation and immunophenotypical analyses were performed on freshly collected blood samples. The resulting plasma samples were stored at -80°C for less than 3 months, and used for the cytokines pattern evaluation. Feces were stored at -80°C and analyses were performed within 3 months.

3.1.2 Subjects recruited for the validation of the HTF-Microb.Array on human feces

Eight healthy Italian subjects, 30 years old in average, were recruited for the validation of the HTF-Microb.Array n human feces. None of the subjects had dietary restriction, antibiotic therapy or functional foods supplementation for at least four weeks prior to sampling. History of gastrointestinal disorders at the moment of sampling was considered an exclusion criteria for the recruitment. Feces were stored at -80°C for less than 5 months. The study protocol was approved by the Ethical committee of Sant'Orsola-Malpighi Hospital (Bologna, Italy).

3.2 Bacterial strains and culture conditions

3.2.1 Bacterial DNA used for group specific qPCR standards

The DNA of the bacterial strains listed in Table 3.1 was used as standard for the group specific quantitative real-time PCR (qPCR) in the Centenarians project.

Table 3.1 Bacterial standards used for group specific qPCR

Target group	*Standard*
Clostridium leptum group	*Clostridium leptum* ATCC 29065
Bifidobacterium spp.	*Bifidobacterium longum* ATCC 15707
Akkermansia spp.	*Akkermansia muciniphila* ATCC BAA-835
Faecalibacterium prausnitzii	*Faecalibacterium prausnitzii* adhufec218 (Suau *et al*, 1999)
Bacteria	*Escherichia coli* ATCC 10798
Archea	*Methanobrevibacter smithii* ATCC 35061

3.2.2 Bacterial strains used for HTF-Microb.array validation

For the validation procedure of the HTF-Microb.Array genomic DNA extracted from bacterial strains listed in Table 3.2 was used. With the exception of *Lactobacillus salivarius* SV2, which is part of our strain collection, all type strains used for the validation were purchased from DSMZ and ATCC. *Salmonella cholerasuis typhimurium* and *Yersinia enterocolitica* were kindly provided by A. Essig, Department of Medical Microbiology, University of Ulm, Germany.

All *Bifidobacterium* and *Lactobacillus* strains were grown on De Man-Rogosa-Sharpe (MRS) broth (Difco, Lawrence, KS), added of 0.05% of L-cysteine, at 37°C under anaerobic conditions (Anaerocult, Merck, Darmstadt, Germany). *E. coli* was cultivated at 37°C, with shacking, in TY broth (Difco). *S. cholerasuis typhimurium* and *Y. enterocolitica* were cultivated at 30°C, with shacking, in brain-heart infusion (BHI) media (Difco).

In the case of strictly anaerobic strains, DNA was extracted from the lyophilized bacterial cells, without the cultivation step, (*Clostridium leptum, Clostridium viridae, Eubacterium siraeum, Megasphaera micrinuciformis, Proteus mirabilis, Ruminococcus albus,* and all *Bacillus* strains) or directly purchased from ATCC (*Campylobacter jejuni, Clostridium acetibutilicum, Clostridium difficile, Clostridium perfringens, Prevotella melaninogenica, Veillonella parvula, Ruminococcus productus, Bacteroides* strains, and *Enterococcus* strains).

3.3 DNA extraction

3.3.1 DNA extraction from bacterial cultures

Bacterial genomic DNA was extracted from bacterial cell cultures using a 10^9 cells pellet. DNeasy Tissue Kit (Qiagen, Duesseldorf, Germany) was used for the extraction, following the manufacturer instructions specific for Gram positive bacteria.

3.3.2 DNA extraction from lyophilized bacterial cells

Bacterial genomic DNA was extracted by resuspending lyophilized bacterial cells in 1 ml of Lysis buffer (500 mM NaCl, 50 mM Tris-HCl pH 8, 50 mM EDTA, 4% SDS). DNA extraction was carried out employing the procedure used for the total bacterial DNA extraction from feces.

3.3.3 DNA extraction from feces

Total DNA from fecal material was extracted using QIAamp DNA Stool Min Kit (Qiagen) with a modified protocol. 250 mg of feces were suspended in 1 ml of Lysis buffer (500 mM NaCl, 50 mM Tris-HCl pH 8, 50 mM EDTA, 4 % SDS). Four 3 mm glass beads and 0.5 g of 0.1 mm zirconia beads (BioSpec Products, Bartlesville, OK) were added, and the samples were treated in FastPrep (MP Biomedicals, Irvine, CA) at 5.5 ms for 3 min. Samples were heated at 95°C for 15 min, then centrifuged for 5 min at full speed to pellet stool particles. Supernatants were collected and 260 μl of 10 M ammonium acetate were added, followed by incubation in ice for 5 min and centrifugation at full speed for 10 min. One volume of isopropanol was added to each supernatant and incubated in ice for 30 min. The precipitated nucleic acids were collected by centrifugation for 15 min at full speed and washed with ethanol 70%. Pellets were resuspended in 100 μl of TE buffer and treated with 2 μl of DNase-free RNase (10 mg/ml) at 37°C for 15 min. Protein removal by Proteinase K treatment and DNA purification with QIAamp Mini Spin columns were performed following the kit protocol.

Table 3.2 Bacterial strains and bacterial genomic DNA used for HTF-Microb.Array validation procedure.

Species	Strain
Strains from private collections	
Lactobacillus salivarius	SV2
Salmonella cholerasuis typhimurium	
Yersinia enterocolitica	
Strains purchased from DSMZ	
Bacillus cereus	DSM21
Bacillus clausii	DSM2515
Bacillus subtilis	DSM704
Bifidobacterium bifidum	DSM20456
Bifidobacterium breve	DSM20091
Clostridium leptum	DSM73
Clostridium viridae	DSM6836
Eubacterium siraeum	DSM15700
Lactobacillus acidophilus	DSM20079
Lactobacillus casei	DSM20011
Lactobacillus delbrueckii	DSM20314
Lactobacillus gasseri	DSM20243
Lactobacillus pentosus	DSM20134
Lactobacillus plantarum	DSM21074
Lactobacillus ramnosus	DSM20021
Lactobacillus reuteri	DSM20016
Megasphaera micrinuciformis	DSM17226
Proteus mirabilis	DSM4479
Ruminococcus albus	DSM20455
Strains purchased from ATCC	
Bifidobacterium adolescentis	ATCC15703
Bifidobacterium longum	ATCC15707
Escherichia coli	ATCC11105
Bacterial genomic DNA purchased from ATCC	
Bacteroides fragilis	ATCC25285
Bacteroides thetaiotaomicron	ATCC29148
Campylobacter jejuni	ATCC33292
Clostridium acetobutilicum	ATCC824
Clostridium difficile	ATCCBAA1382
Clostridium perfringens	ATCC13124
Enterococcus faecalis	ATCC700802
Enterococcus faecium	ATCC51559
Prevotella melaninogenica	ATCC25845
Ruminococcus productus	ATCC23340
Veilonella parvula	ATCC10790

3.4 HITChip analysis

3.4.1 <u>16S rRNA gene amplification</u>

The total bacterial 16S rRNA genes were amplified from fecal DNA using FastStart Taq DNA polymerase, dNTPack PCR Amplification kit (Roche, Mannheim, Germany). T7prom-Bact-27-F (5'-TGAATTGTAATACGACTCACTATAGGGGTTTGATCCTGGCTCAG-3') and Uni-1492-R (5'-CGGCTACCTTGTTACGAC-3') primer set was used for amplification (Lane, 1991). PCR reactions were carried out in a final volume of 50 µl and 20 ng of DNA were used as template. Initial denaturation step was performed at 95°C for 5 min, followed by 35 cycles of 95°C (30 sec), 52°C (40 sec) and 72°C (90 sec) and a final extension of 7 min at 72°C. Per DNA sample two reactions were performed and pooled before the purification step. PCR products were purified by using High Pure PCR Clean up Micro kit (Roche) following the manufacturer instructions.

3.4.2 <u>In vitro transcription</u>

T7 RNA transcription was performed by using Riboprobe T7 RNA transcription kit (Promega, Madison, WI) on 500 ng of the T7-DNA PCR, according to the manufacturer's instructions. rATP, rCTP, rGTP and a 1:1 mix of rUTP and amino-allyl-rUTP (Ambion, Austin, TX) were used at a concentration of 0.5 mM each. Samples were incubated for 15 min at 37°C, then 1 µl of T7 RNA polymerase (Promega) was added to each tube (final volume 20 µl) and the transcription reaction was carried out for 90 min. Subsequently, DNAse treatment was performed by using Qiagen RNAse free DNAse (Qiagen), according to the manufacturer instructions. RNA purification was performed by using RNeasy Mini-elute clean-up kit (Qiagen) and the RNA yield was measured with Nanodrop ND-1000.

3.4.3 <u>Fluorescent labeling</u>

Amino-allyl-modified nucleotides were coupled with CyDye using Post-Labeling Reactive Dye (Amersham Bioscience, Piscataway, NJ), previously dissolved in DMSO. Two µg of purified RNA were used for the labeling reaction, performed in a final volume of 40 µl, in presence of 0.1 M sodium bicarbonate buffer (pH 8.7). Samples were incubated for 90 min in the dark, at room temperature. The reaction was stopped by adding 15 µl of 4M hydroxylamine and incubating for 15 min in the dark. Labeled RNA was purified and measured as described above.

3.4.4 *Hybridization and scanning*

HITChip slides were custom synthesized by Agilent Technologies (Wilmington, DE), having a 8x15K format with 8 arrays per slides. Two different RNA samples, labeled with Cy3 and Cy5, respectively, were hybridized on each array. At least 2 hybridizations on different arrays were carried out for each sample. 100 ng of each Cy3- and Cy5-labelled RNA were mixed in a final volume of 8 μl, then fragmented by adding 1 μl of 10X Fragmentation Reagent (Ambion), and incubated for 20 min at 70˚C, according to the manufacturer instruction. The fragmentation reaction was stopped by adding 1 μl of Stop Solution. The hybridization mix was prepared by adding to the RNA mixture 39 μl of pre-warmed hybridization mix (7.5 μl of 20x SSC, 1.5 μl of ultrapure 10% SDS, 30 μl of RNAse-free water). Hybridization was carried out in a rotation oven (Agilent) for 16 h at 62.5˚C. Slides were washed in 2x SSC, 0.3% SDS at room temperature for 10 min and 0.1x SSC, 0.3% SDS at 38˚C for 10 min. SDS was removed by washing the slides in 0.06x SSPE for 5 min. Microarrays were scanned by using Agilent Microarray Scanner at 2 UV lamp intensities (40 and 10% of maximum PMT voltage for red, 60 and 20% for green). Intensity values for each spot were quantified by using Agilent Feature Extraction software, version 9.5.

3.4.5 *Data analysis*

Data storage, spatial and quantile normalization and probe profile extraction were performed using a custom-designed MySQL-based relation database (www.mysql.com) and the R statistical software (www.r-project.org), as previously described (Rajilic-Stojanovic *et al*, 2009). The reproducibility of the experiments was assessed by calculating Pearson's correlation of the natural logarithm of spatially normalized signals. Hybridizations which resulted in a Pearson's correlation coefficient (Pearson's *r*) <0.98 were repeated. For each probe, signal intensity was calculated as the mean value of the normalized fluorescence intensities obtained in the 2 replicates. Phylogenetic profile of each subject was obtained by plotting probes hybridization signals grouped by specificity of probes. Hierarchical clustering of HITChip profiles was carried out using Pearson's correlation to calculate the distance among samples and Ward's minimum variance as agglomeration method.

For quantification purpose, the analysis was focused on the 129 of "level 2" phylogenetic groups, which corresponds to subsets of phylotypes with 90% or more 16S rRNA sequence similarity. The average signal intensity of all probes which are specific for one phylogenetic group was used as quantitative measure of that group in the sample. The cut-off values for positive responding probes were calculated as described by Rajilic-Stojanovic *et al* (2009). Statistics were performed on log-transformed data using the R statistical software and the Canoco package for Windows (Leps and Smilauer, 2003). To evaluate the significance of the difference between datasets, P value was

calculated by Student t-test for normally distributed data, or Wilcoxon rank sum test for not-normally distributed data. P value <0.05 was considered as threshold for statistical significance.

Simpson's reciprocal index of diversity (Simpson, 1949), was used to express the diversity of microbial communities, calculated using the equation $\lambda = 1/\Sigma \ Pi2$, where Pi is the proportion of the ith taxon. The proportion of each taxon was calculated as the proportion of each probe signal compared to the total signal. Simpson's reciprocal index of diversity takes into account both the number of taxon present in a sample and their abundance in the community. A higher value of Simpson index corresponds to a more diverse community.

3.5 Real time PCR analysis

Quantitative real time PCR (qPCR) analysis was carried out in a Applied Biosystems 7300 Fast Real-Time PCR System in a 96-well format and using SYBR Green chemistry (Power SYBR Green PCR Master Mix, Applied Biosystems, Foster City, CA) and 0.2 µM of each primer. The total volume of qPCR reaction was 25 µl, employing 1 µl of DNA sample or standard as a template. The forward (F) and reverse (R) primers are listed in Table 3.3. The previously described thermocycling conditions were used (Kaufmann *et al*, 1997; Kullen *et al*, 2000; Marteau *et al*, 2001; Baker *et al*, 2003; Matsuki *et al*, 2004; Rinttila *et al*, 2004; Collado *et al*, 2007). Samples were assayed in duplicate in at least 2 independent runs and the results were analyzed using Applied Biosystems 7300 Fast Real-Time PCR System SDS Software (version 1.4.0). Melting curve analysis was performed after the PCR to confirm specificity of amplification. The amount of 16S copies of specific bacterial genera or groups in the fecal samples was determined by comparing the Ct (threshold cycle) values of samples to those of the standard curves.

Standards were prepared by amplifying the 16S rDNA from a representative species from each bacterial group targeted by qPCR. The amplified fragments were subsequently purified by the using the QIAquick PCR purification kit (Qiagen), quantified by using the NanoDrop ND-1000 and diluted appropriately for use as standards. Statistical analysis of qPCR data was carried out with log-transformed data. Non-detected values were imputed with the half of the theoretical detection limit. Nonparametric tests were used since data were not normally distributed. Kruskall-Wallis test was used to determine the statistical differences among the age groups. Mann-Whitney U-test was used for pair-wise comparisons. P value < 0.05 was considered as a threshold for statistical significance.

Table 3.3 Group specific primer set used in qPCR

Specificity	Primer set	Ref.
Bacteria	**F** 5'-AGAGTTTGATCCTGGCTCAG-3' **R** 5'-GGCTGCTGGCACGTAGTTAG-3'	Kullen *et al,* 2000
Clostridium leptum group	**F** 5'-GCACAAGCAGTGGAGT-3' **R** 5´-CTTCCTCCGTTTTGTCAA-3´	Matsuki *et al,* 2004
Bifidobacterium genus	**F** 5'-GATTCTGGCTCAGGATGAACGC-3' **R** 5'-CTGATAGGACGCGACCCCAT-3'	Kaufmann *et al,* 1997 Marteau *et al*, 2001
Akkermansia muciniphila	**F** 5'-CAGCACGTGAAGGTGGGGAC-3' **R** 5'-CCTTGCGGTTGGCTTCAGAT-3'	Collado *et al*, 2007
Faecalibacterium prausnitzii	**F** 5'-CCCTTCAGTGCCGCAGT-3' **R** 5´-GTCGCAGGATGTCAAGAC-3´	Rinttila *et al*, 2004
Methanobrevibacter smithii	**F** 5'-CCGACGGTGAGRGRYGAA-3' **R** 5-YCCGGCGTTGAMTCCAATT-3'	Baker *et al*, 2003

3.6 Immunological characterization

3.6.1 *Immunophenotyping*

The identification of the major lymphocyte subsets (B lymphocytes, T lymphocytes, virgin T lymphocytes, memory T lymphocytes and NK cells), some of which considered important for the immunological risk phenotype, was performed using the following combination of monoclonal antibodies: CD5/CD19 to identify B and autoreactive B lymphocytes, CD3/CD4/CD8 to identify T lymphocytes subsets, CD28/CD95/CD4 and CD28/CD95/CD8 to identify effector T helper and cytotoxic lymphocytes, CD45RA/CD4/CCR7 and CD45RA/CD4/CCR7 to identify naive, central memory, effector memory and terminal effector T helper and cytotoxic lymphocytes and CD4/CD25/CD8 to identify activated T lymphocytes. The phenotypical analysis of peripheral blood lymphocytes on whole blood samples lysis was performed as previously described (Cossarizza *et al*, 1990). To quantify the main lymphocyte sub-populations, a cytometric approach with fluorochrome-labelled monoclonal antibodies (mAbs) directly conjugated to fluorescent molecules (fluorescein isothiocyanate, FITC; phycoerythrin, PE; phycoerythrin-cy-crhome, PE-Cy7) staining was used. mAbs, including the appropriate isotype controls, were purchased from BD Biosciences Pharmingen (San Diego, CA). Phenotypic analyses of cytotoxic and helper subsets were performed placing an electronic gate on CD8+ and CD4+ cells and evaluating the expression of CD45RA

versus CCR7, i.e. naïve (CD45RA+CCR7+), central memory (CD45RA-CCR7+), effector memory (CD45RA-CCR7-), and terminal effector (CD45RA-CCR7+). Similarly, the expression of CD25 was analyzed on CD8+ and CD4+ gate. Data were acquired using a FACScalibur (BD Biosciences) flow cytometer and analyzed with FlowJo (Tree Star Inc., Ashland, OR) software. A minimum of 30,000 cells per sample was acquired.

3.6.2 *Plasmatic cytokines evaluation*

Levels of IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, IFN-γ, and TNF-α trimer in plasma samples were measured in duplicate by multiplex sandwich ELISA technology (Human Cytokine Array 1, SearchLight, Aushon Biosystems, Billerica, MA) according to the manufacturer's instructions. The concentration of each analyte in the array was detected by biotin-streptavidin reaction and quantified by a SearchLight CCD Imaging System. The lowest detectable concentrations were the following: 0.1 pg/ml for IL-1α, 0.1 pg/ml for IL-1β, 0.4 pg/ml for IL-2, 0.2 pg/ml for IL-6, 0.4 pg/ml for IL-8, 0.2 pg/ml for IL-10, 0.3 pg/ml for IL-12p70, 0.1 pg/ml for IFN-γ, 0.6 pg/ml for TNF-α. Samples, standards, and reagents were dispensed in the plates by a standardized technique employing a robotic liquid handling system with 16 channels (Microlab® STAR, Hamilton Robotics, Reno, NV). Plasmatic TGF-β1 concentration was determined in duplicate by ELISA using a commercial kit (DRG Instruments GmbH, Marburg, Germany) according to the manufacturer's instructions. The analytical sensitivity was 1.2 pg/ml. The interassay coefficient of variation was 7.5%. Concentration of TGF-β1 was detected and quantified by a Synergy HT Multi-Detection Microplate Reader (Bio-Tek® Instruments, Winooski, VT).

3.6.3 *Inflammation score*

Aiming the evaluation of the inflammatory status of each subject, an inflammation score was calculated as inspired by previous studies (Duncan *et al*, 2003; Recasens *et al*, 2005). The score was composed of 8 markers: white blood cell count, C reactive protein, number of central memory helper and cytotoxic T lymphocytes (CD45RA-CCR7+), number of effector helper and cytotoxic T lymphocytes (CD28-), IL-6 and IL-8 plasmatic levels. The inflammation score ranging from 0 to 8, was calculated by adding 1 unit for each value greater than the median of the study samples for each inflammatory marker. Subjects with at least 5 markers greater than the median, with an inflammation score between 5 and 8, were considered highly inflamed while IS between 0 and 4 was considered as indication of low inflammatory status.

3.7 HTF-Microb.Array design

3.7.1 Target selection and consensus extraction

A database of 16S rRNA sequences was created by integration of the 16S rRNA database of the ARB Project (release February, 2005) (Ludwig *et al*, 2004), with the database of the Ribosomal Database Project (RDP; release September, 2007) (Cole *et al*, 2007; Wang *et al*, 2007). A phylogenetic tree was obtained in the ARB software, by using the neighbour-joining algorithm for the sequence alignment. The tree was used for the rational selection of phylogenetic groups of bacteria belonging to the human intestinal microbiota which correspond to nodes of the phylogenetic tree (Appendix 1). Group specific consensus sequences were extracted, with a cut-off of 75% for base calling. Nucleotides which occurred at lower frequencies were replaced by the appropriate IUPAC ambiguity code.

3.7.2 Probe design

Multiple alignment step of the selected sequences was performed in ClustalW (Chenna *et al*, 2003). Since the taxonomic classification of the 30 groups selected for the probe design varied from species to phylum level, careful grouping of the sequences was performed for the multiple alignment step: (a) for higher level probes, only family/phylum consensus sequences were used as a negative set for probe design; (b) for genus/species level probes, only sequences belonging to other families/phyla were selected. All the LDR probe pairs were designed in collaboration with the Institute for Biomedical Technologies, Milan, Italy, using the tool ORMA (Severgnini *et al*, 2009). Both DS and CP were required to be between 25 and 60 bases pair, with a Tm of 68±1°C, and with maximum 4 degenerated bases. In-silico check versus a publicly available database (i.e. RDP) was then performed for assessing probe pair specificity.

3.8 LDR/Universal Array approach

3.8.1 Universal Array construction

The construction of the Universal Array and the Ligase Detection Reactions (LDR) were performed at the Institute for Biomedical Technologies, Milan, Italy. All the oligonucleotide probe pairs were synthesized by Thermo Electron (Ulm, Germany).

Phenylen-diisothiocyanate (PDITC) activated chitosan glass slides were used as surfaces for the preparation of Universal Arrays (Gerry *et al*, 1999), comprising a total of 49 Zip-codes. Spotting was performed by using a contact-dispensing system (MicroGrid II Compact, BioRobotics,

Huntingdon, UK). The printed slides were processed according to the manifacturer's protocols. Eight arrays per slide were generated. Hybridization controls (cZip 66 oligonucleotide, complementary to zip 66, 5'-Cy3-GTTACCGCTGGTGCTGCCGCCGGTA-3') were used to locate the submatrixes during the scanning. The entire experimental procedure for both the chemical treatment and the spotting is described in detail in Consolandi *et al* (2006).

3.8.2 *16S rRNA gene amplification*

The 16S rRNA gene was amplified using universal forward primer 16S27F (5'-AGAGTTTGATCMTGGCTCAG-3') (Edwards *et al*, 1989) and reverse primer R1492 (5'-TACGGYTACCTTGTTACGACTT-3') (Weissburg *et al*, 1991), following the protocol described in Castiglioni *et al* (2004), except for the use of 50 ng of starting DNA and 0.5U of DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland). Briefly, PCR reactions were carried out in a final volume of 25 µl and 50 ng of DNA were used as template. Initial denaturation step was performed at 95°C for 10 min, followed by 30 cycles of 94°C (45 sec), 60°C (45 sec) and 72°C (90 sec) and a final extension of 10 min at 72°C. PCR products were purified by using High Pure PCR Clean up Micro kit (Roche) following the manufacturer instructions. PCR products were purified by using a Wizard SV gel and PCR clean-up System purification kit (Promega), according to the manufacturer's instructions, eluted in 20 µl of sterile water, and quantified with the DNA 7500 LabChip Assay kit and BioAnalyzer 2100 (Agilent Technologies).

3.8.3 *LDR and hybridization*

A schematic representation of the LDR/Universal Array approach is presented in Fig. 3.1.

LDR and hybridization of the products on the Universal Arrays were performed according to the protocol described by Castiglioni *et al* (2004), except for the probe annealing temperature, which was set at 60 °C. The LDRs were carried out in a final volume of 20 µl with different quantities of purified PCR products. All LDRs for specificity tests were performed on 50 fmol of initial PCR product, for having no issues related to target. Sensitivity tests were performed with decreasing PCR product concentration from 75 to 0.7 fmol. Relative abundance tests were performed on 1 fmol *E. coli* PCR amplicon, mixed with human genomic DNA, at decreasing concentrations, from 4%, down to 0.02%. LDR experiments on the eight faecal samples were performed on 50 fmol of PCR product. 4U of Pfu DNA ligase (Stratagene, La Jolla, CA) were used for each reaction.

Hybridizations were performed on a final volume of 65 µl, containing the 20 µl of the LDR, 16 µl of 20X SSC buffer, 0.1 mg of Salmon sperm DNA. After heating at 94°C for 2 min and chilling on ice, the hybridization mix was applied to the slide, using Press-to-Seal silicon isolators (Schleicher

& Schuell BioScience, Dassel, Germany) to separate the 8 arrays. Hybridization was carried out in the dark at 60°C for 1 h. Then the slide was washed in pre-warmed 1X SSC, 0.1% SDS for 15 min, and dried by centrifugation.

3.8.4 *Data analysis*

All arrays were scanned with ScanArray 5000 scanner (Perkin Elmer Life Sciences, Boston, MA), at 10 μm resolution. In the experiments, the fluorescent images were obtained with different acquisition parameters on both laser power and photo-multiplier gain, in order to avoid saturation. Fluorescence Intensities (IF) were quantified by ScanArray Express 3.0 software, using the "Adaptive circle" option, letting diameters vary from 60 to 300 μm. No normalization procedures on the IFs have been performed. To assess whether a probe pair was significantly above the background (i.e. was "present" or not), we performed a one-sided t-test. The null distribution was set as the population of "Blank" spots (e.g. with no oligonucleotide spotted). For each zip-code, we considered the population of the IFs of all the replicates (n=4) and tested it for being significantly above the null-distribution. In case one replicate in the test population was below 2.5 times the distribution mean, this was considered an outlier and was discarded from the analyses. Data analyses were performed using the software Matlab, in collaboration with the Institute for Biomedical Technologies, Milan, Italy.

Fig. 3.1. (Following page) Main features of LDR method coupled with a universal microarray. After hybridization of a discriminating probe and a common probe to the target sequence (16S rRNA gene), ligation occurs only if there is perfect complementarity between the two probes and the template (A). The reaction is thermally cycled, generating single-stranded DNA fragments bearing a 5' Cy3 fluorescent moiety and a 3' czip code sequence. The cycling allows more common probe (and the corresponding czip code) to ligate to the discriminating probe, given a fixed amount of PCR target. (B) The LDR product is hybridized to a universal microarray, where unique zip code sequences have been spotted. (Castiglioni *et al*, 2006)
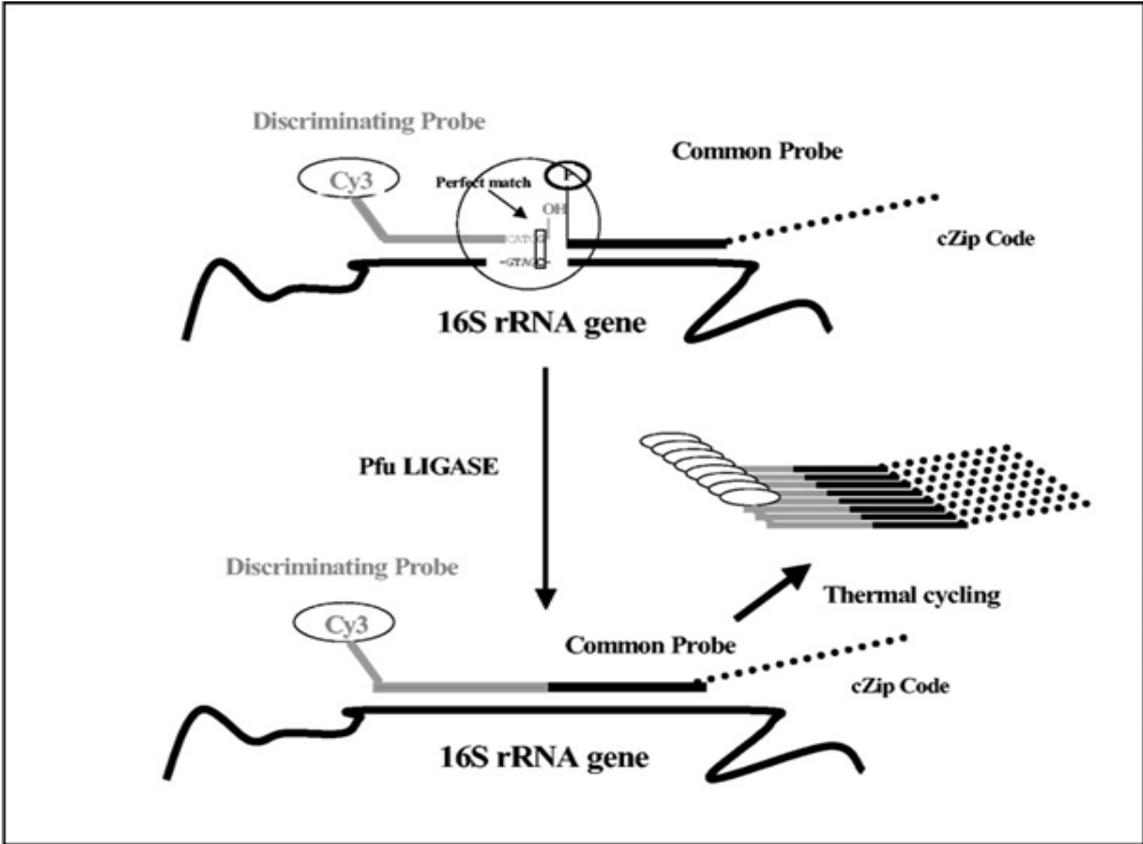
A
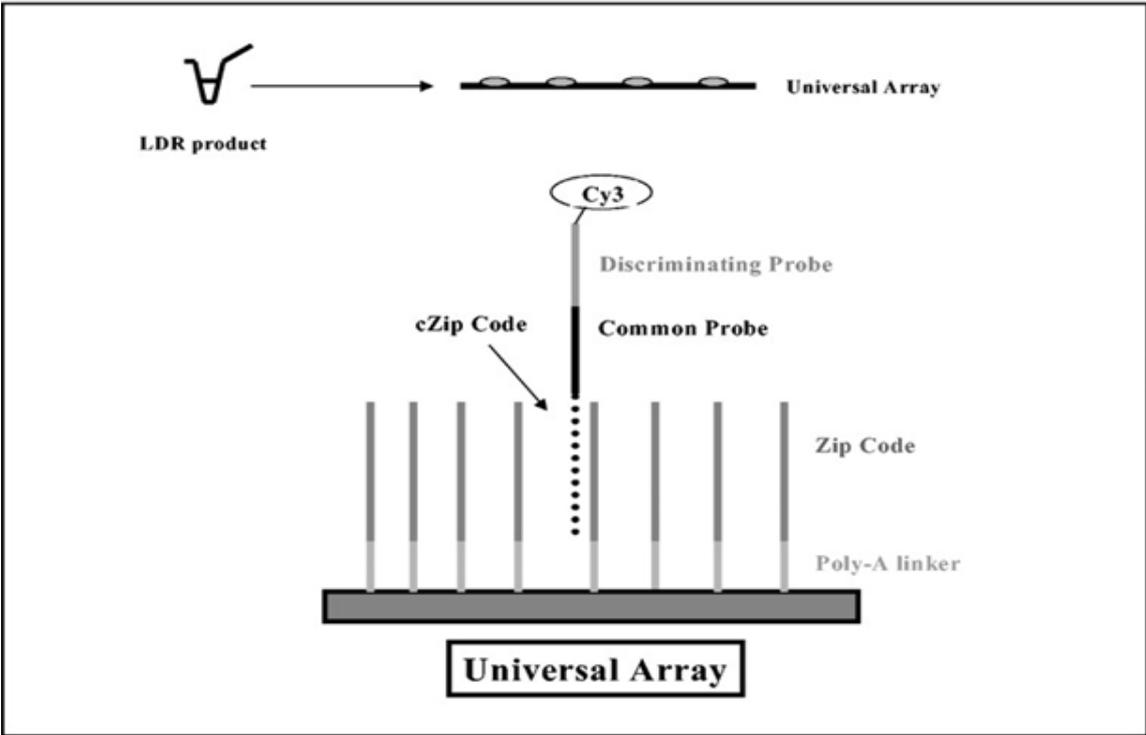
B

4.
RESULTS

4.1 The "Centenarians project": characterization of the gut microbiota

4.1.1 HITChip profiling of centenarians, elderly and young adults

The fecal microbiota of centenarians (group C), elderly (group E) and young adults (group Y) was characterized using the recently developed diversity microarray HITChip (Rajilic-Stojanovic *et al*, 2009). Hybridization was performed in at least 2 replicates for each sample, and reproducibility was tested by calculating Pearson's correlation coefficient (Pearson's r) and Relative Standard Deviation (RSD). For each subject a phylogenetic fingerprint of the intestinal microbiota was obtained. Pearson's r ranged between 0.982 and 0.999, with a mean value of 0.993, showing that the HITChip profiles were highly reproducible. RSD ranged between 1.8% and 13.2%, with a mean value of 5.1%.

Hierarchical clustering with heat map of the level 2 average hybridization signals (Fig. 4.1) showed that centenarians grouped together, whereas no separation of subjects belonging to groups E and Y was observed. The proportion of centenarians in cluster 3 (67%) is significantly higher than in the other clusters (χ2 test, P value = 0.001). Clusters 1 and 2 contain similar proportions of each age groups (18% C, 44% E, 39% Y, for cluster 1; 22% C, 38% E, 38% Y, for cluster 2). According to the heat map result, the gut microbiota of subjects in cluster 3 is characterized by higher amounts of Proteobacteria and Bacilli, and decreased amounts of *Clostridium* cluster XIVa bacteria. Finally, Bacteroidetes showed remarkably lower intensities in cluster 1, which is composed by mixed subjects, highlighting that the decrease in Bacteroidetes amount in the gut microbiota does not seem to be related to the aging.

Multivariate analysis was carried out using the age groups C, E, and Y as nominal environmental variables, and the log-transformed average hybridization signal for the 129 level 2 phylogenetic groups as "species" variables. Ordination plot in Fig. 4.2 shows the redundancy analysis (RDA), which focuses on the major carriers, accounting for more than 10% of the difference among the groups of samples. The differences shown in RDA are highly significant (P value = 0.002) as assessed by Monte Carlo Permutation Procedure (MCPP). 6.1% of the total variation in the dataset could be related to the environmental variable. Most of this variation (5.2%) is plotted on the first axis that separates the centroid of group C from the other two groups E and Y. E and Y centroids are plotted on a line along the second axis, which shows only 0.9% of variability. The analysis

indicated that the differences in the microbiota fingerprint between centenarians and all the other subjects are greater than the differences between elderly and young adults. Samples of groups C and Y form almost separate clusters in RDA, whereas the group E cluster overlap with both of them.

Confirming the heatmap results, RDA showed that the fecal microbiota of centenarians is enriched in many facultative anaerobes, mostly belonging to the Proteobacteria (*Escherichia coli et rel., Haemophilus, Klebsiella pneumoniae et rel., Leminorella, Proteus et rel., Pseudomonas, Serratia, Vibrio*, and *Yersinia et rel*.), and Bacilli (*Bacillus, Staphylococcus*). On the other side of the plot, higher amounts of many bacterial groups belonging to the *Clostridium* cluster XIVa seemed to characterize the fecal microbiota of samples of groups E and Y. Interestingly, the arrows corresponding to the phylogroups *Faecalibacterium prausnitzii et rel*. and *Clostridium leptum et rel*., both belonging to the *Clostridium* cluster IV, pointed in opposite directions, indicating that *F. prausnitzii* group decreased in centenarians whereas *C. leptum* group increased.

Gender and BMI were also used as environmental variables in multivariate analyses, but they did not show any relation with the gut microbiota composition of the subjects (data not shown).

The diversity of the microbial communities in the different age groups was assessed. The diversity discovered by the HITChip was expressed as Simpson reciprocal index of diversity. Simpson indices obtained for group C (127.0 ± 54.2) were significantly lower than those obtained for E (149.4 ± 40.5, $P = 0.02$) and Y (162.8 ± 35.1, $P = 0.002$), indicating that the microbiota of centenarians is significantly less diverse than that of elderly or adults. On the contrary, the difference between Simpson indices of E and Y was not statistically significant.

Correlation matrices were obtained for each groups of samples in order to investigate the inter-individual variability in the different age groups (Fig. 4.3). Pearson correlation coefficients were calculated comparing the hybridization profile of each subject with all the subjects of the same group. Mean Pearson correlation coefficients of 0.36, 0.47 and 0.47 were obtained for groups C, E, and Y, respectively. This analysis pointed out that centenarians are significantly less correlated to each other for their gut microbiota composition, if compared to subjects belonging to groups E ($P < 0.0001$) and Y ($P < 0.0001$).

Fig. 4.1 (following page) Hierarchical clustering, with heat map, of the gut microbiota profiles of centenarians, elderly and young adults. Subjects belonging to the groups C, E, and Y are indicated by black circles, grey squares and white squares, respectively. Darkness of the spot corresponds to the bacterial abundance in the sample. Pearson correlation of level 2 phylogeny and Ward's clustering method were used. Level 2 phylogenetic groups members of the Proteobacteria (P), Bacilli (B), *Clostridium* cluster IV (C.IV) and XIVa (C.XIVa) are indicated. The two phylogroups members of the *Clostridium* cluster IV located within the *Clostridium* cluster XIVa cluster (bottom) are *Faecalibacterium prausnitzii et rel*. and *Papillibacter cinnamovorans et rel*.
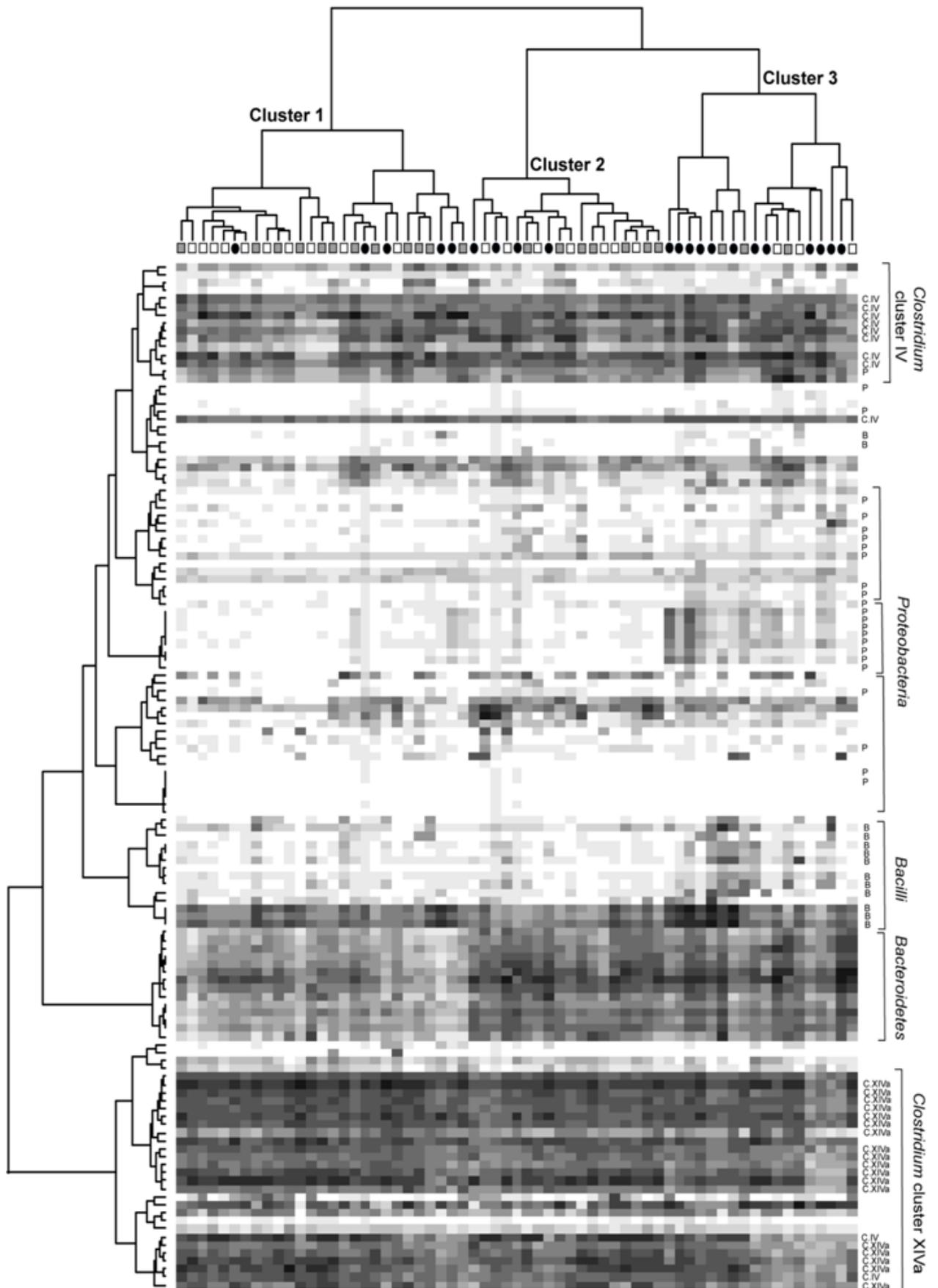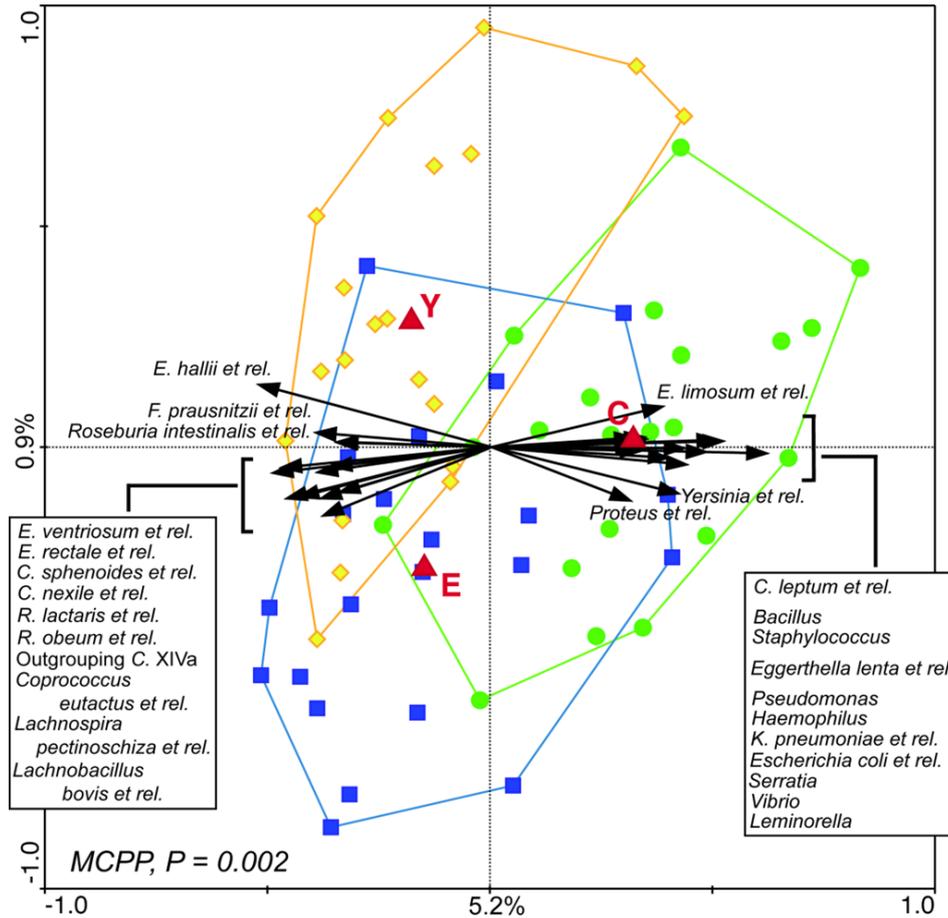
Fig. 4.2. Triplot of the RDA of the microbiota composition of centenarians, elderly and young adults. Subjects belonging to group C, E, and Y are indicated by green circles, blue squares and yellow diamonds, respectively. Constrained explanatory variables (C, E, and Y) are indicated by red triangles. Responding bacterial subgroups that explained more than 10% of the variability of the samples are indicated by black arrows. First and second ordination axes are plotted, showing 5.2% and 0.9% of the variability in the dataset, respectively. Log transformed data were used for the analysis. Bottom-left, P value obtained by MCPP is reported. Abbreviations: C., *Clostridium*; E., *Eubacterium*; F., *Faecalibacterium*; R., *Ruminococcus*; K., *Klebsiella*.
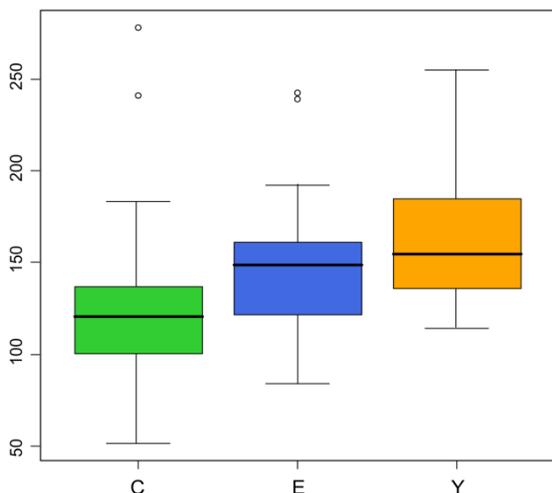
Fig 4.3. Boxplot of the Simpson indices obtained for the gut microbiota profiles of subjects in groups C (green), E (blue), and Y (orange).The box for each group represents the interquartile range (25–75th percentile) and the line within this box is the median value. Bottom and top bars indicate the 10th and 90th percentiles, respectively. Outlier values are indicated (circles). Median values are also reported for each bacterial group at the bottom of the plots.
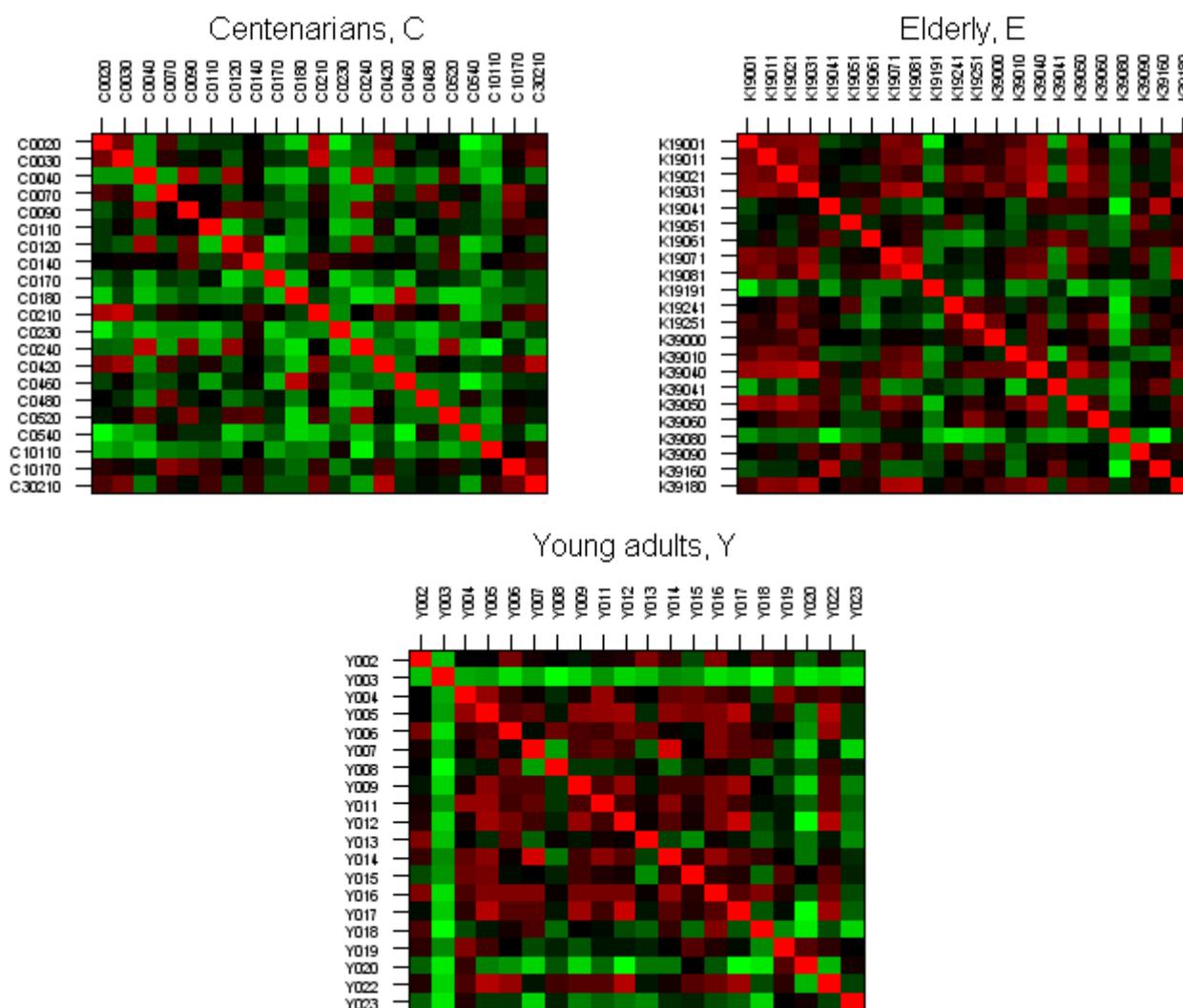
Fig. 4.4. Correlation matrices obtained for groups of subjects C, E and Y. Pearson correlation coefficient obtained when the gut microbiota profile of each subject was compared to all the others in the same group are represented in a scale from red (Pearson's *r* = 1, high similarity) to lime green (Pearson's *r* = -1, low similarity).

4.1.2 *Quantitative differences between the gut microbiota of centenarians, elderly and young adults*

The relative contributions of the major phyla (level 1 phylogenetic groups, corresponding to the phylum/order level) in the fecal microbiota of the subjects in groups C, E and Y are plotted in Fig. 4.5. Bacteroidetes and Firmicutes contributed to the fecal microbiota for the 93% in the case of centenarians, and 95% in both groups E and Y. Bacteroidetes contributed for 20, 16, and 19% to the total microbiota of subjects belonging to groups C, E and Y, respectively. The Firmicutes/Bacteroidetes ratios obtained for groups C, E and Y were 3.6, 5.1, and 3.9, respectively. To evaluate the significance of this difference, the sum of the hybridization signals of the probes

which referred to the Bacteroidetes and the Firmicutes was calculated for each subject. The differences among groups of samples, in both the Bacteroidetes proportions and the Firmicutes/Bacteroidetes ratios, were not statistically significant (P = 0.73, and P = 0.55, respectively).

Clostridium cluster IV contributed for the 22% to the microbiota of all age groups. qPCR for *C. leptum* group, which is comparable to *Clostridium* cluster IV, showed no significant difference between centenarians and other age groups (Table 4.1), confirming the HITChip result. Conversely, qPCR showed that E have significantly more *C. leptum* group bacteria when compared to Y (P = 0.05), although no statistical difference in HITChip signals was observed. The relative contribution of *Clostridium* cluster XIVa is clearly lower in centenarians (34%) than in the other groups (49%, group E; 44% group Y). By comparing the sum of the hybridization signals of the probes which referred to the *Clostridium* cluster XIVa in the C and E groups, and in the C and Y groups, P values of 0.001 and 0.02 were obtained, respectively. The proportion of Bacilli is significantly higher in centenarians (12%), if compared to groups E (5%, P = 0.05) and Y (5%, P = 0.03). Centenarians also tended to have a higher proportion of Proteobacteria (2.6%) with respect to E (1.2%, P = 0.06) or Y (1.2%, P = 0.07).

Table 4.1 Quantitative PCR results for bacterial groups, expressed as average amount of 16S rRNA copies per µg of fecal DNA. Ratios of bacterial 16S DNA counts between groups C, E and Y are indicated. P values <0.05 are reported.

Bacterial group	Average values (16S copies/ug fecal DNA)			Ratio			P values		
	C	E	Y	C/E	C/Y	E/Y	CvsE	CvsY	EvsY
Clostridium leptum group	1.99E+07	3.02E+07	1.60E+07	0.7	1.3	1.9	/	/	0.05
Faecalibacterium prausnitzii	2.01E+07	6.99E+07	3.80E+07	0.3	0.5	1.8	0.02	/	/
Bifidobacterium spp.	6.57E+08	6.87E+08	9.50E+08	1	0.7	0.7	/	0.02	/
Akkermansia	2.75E+06	1.45E+06	1.22E+06	1.9	2.2	1.2	/	/	0.01
Archaea	1.98E+06	1.48E+06	4.58E+05	1.3	4.3	3.2	/	/	/
Universal (total bacteria)	1.78E+09	1.39E+09	1.38E+09	1.3	1.3	1	/	/	/
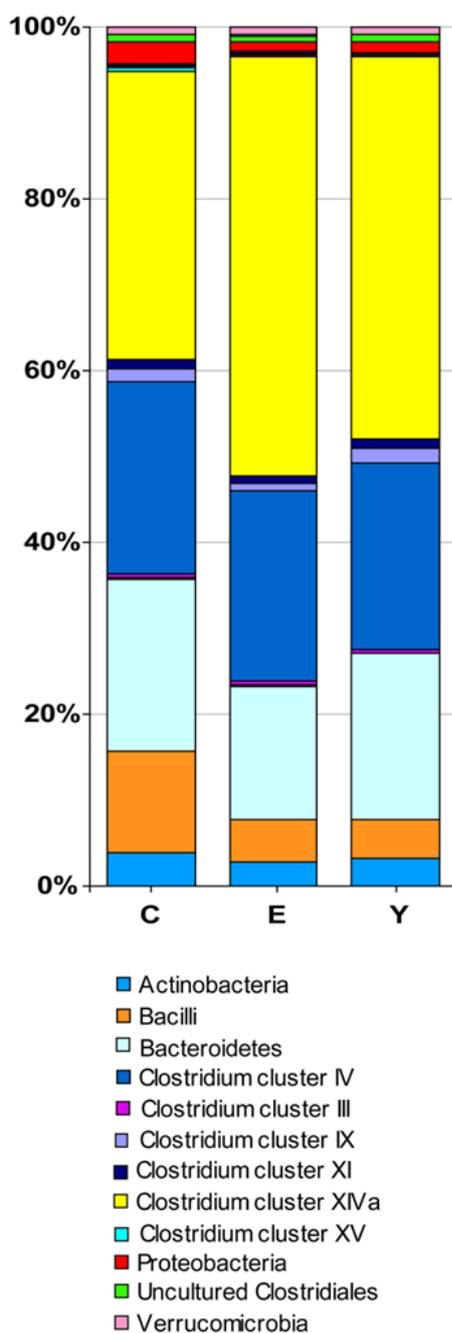
Fig 4.5. Relative contribution of the level 1 phylogroups to the fecal microbiota of subjects in groups C, E, and Y. In the legend, phylogroups which contribute for at least 0.5% to one of the profiles are indicated.

In order to have a more detailed view of the age-related changes in the core gut microbiota composition, analysis was focused on the level 2 phylogenetic bacterial groups (genus-like level) having 100% of prevalence (defined as percentage of positive samples in each study group) in all the age groups. The core microbiota was constituted principally of Bacteroidetes and Firmicutes, mostly *Clostridium* clusters XIVa and IV. Bacterial groups with significantly different abundance in groups C, E and Y, as well as several bacterial groups showing a tendency (P values = 0.05-0.08) of increased or decreased abundance in the different age groups, are listed in Table 4.2. In accordance with the RDA result (Fig. 4.2), the vast majority of the significant differences in bacterial groups were found when group C was compared to E and Y. Ratio of the average hybridization signals obtained for groups C and E, and C and Y, is reported to highlight the increase or decrease of each level 2 phylogenetic group at the different stage of life. Most of the bacteria that significantly decreased in group C with respect to both E and Y (highlighted in grey in Table 2) belonged to *Clostridium* cluster XIVa. In *Clostridium* cluster IV, *Papillibacter cinnamovorans et rel.*, and *F. prausnitzii et rel.* showed a significant decrease in centenarians, while *C. leptum et rel., Sporobacter termiditis et rel., Anaerotruncus colihominis et rel., Clostridium orbiscindens et rel.* showed a significant increase. The decrease of *F. prausnitzii* in centenarians with respect to the other two age groups was confirmed by qPCR analysis (Table 4.1).

Considering that the relative proportion of *Clostridium* cluster IV in the total microbiota remained unaffected by the age of the subjects (Fig. 4.5, Table 4.1), the analysis of level 2 phylogenetic groups suggests a rearrangement in the composition of this bacterial cluster, in terms of genera or species, in centenarians.

Confirming the RDA results (Fig. 4.2), the average amount of all the bacteria listed above did not significantly differ between E and Y. Differences between E and Y were found only for the groups *Acquabacterium* (P = 0.05), *Collinsella et rel.* (P = 0.04), and *Dialister et rel.* (P = 0.02). These groups, belonging to Proteobacteria, Actinobacteria and *Clostridium* cluster IX, respectively, are not highly represented in the human gut. Moreover, for all the bacterial groups which showed significant differences between centenarians and all the other subjects, the HITChip signal ratios C/E and C/Y were often very similar (Table 4.2), suggesting that the changes in the composition of the gut microbiota are not linearly correlated with the age.

In addition to the qPCR analyses of *Clostridium* cluster IV and *F. prausnitzii*, performed to confirm the HITChip results, we quantified bifidobacteria and *Akkermansia*, which in previous studies had shown to decrease in the old age (Table 4.1). qPCR revealed that the amount of total bifidobacteria was significantly lower in C when compared to Y (P = 0.023). However, no statistical difference was detected in the *Bifidobacterium* signals in the HITChip. This discrepancy is likely due to the lower quantitative sensitivity of the HITChip, with respect to the qPCR, which detected a difference of only 0.3 log unit between groups C and Y. The amounts of *Akkermansia* spp. were comparable between centenarians and other age groups, both in qPCR and HITChip analysis. However, by qPCR, subjects in group Y were observed to harbour significantly less *Akkermansia* than group E, both in means of prevalence and amount of 16S copies.

We also quantified by qPCR the archea, which are not targeted by the HITChip. The amount of archaeal DNA did not show any significant difference between centenarians and the other age groups (Table 4.1). Interestingly, centenarians tended to harbour archaea more frequently than the elderly or young adults (prevalence 65%, 36% and 45%, respectively), although the difference was not significant.

Table 4.2. Bacterial groups that were found to differ significantly between centenarians (C), elderly (K) and young adults (Y). Ratio calculated between the average relative abundance of each phylogroup in the HITChip in C and K, and C and Y are reported. Shadowed, are the bacterial groups which showed C/K and C/Y ratio <1, indicating a decrease in the subjects of group C. P values are reported for each difference. Several bacterial groups with P values ranging from 0.05 to 0.08 are also listed.

Phylum/order	Level 2 phylogenetic group	Ratio		P value	
		C/K	C/Y	C vs K	C vs Y
Clostridium cluster XV	*Eubacterium limosum et rel.*	16.2	14.5	< 0.001	0.01
Proteobacteria	*Klebsiella pneumoniae et rel.*	5.3	6.7	0.002	< 0.001
	Vibrio	5.4	5.4	< 0.0001	< 0.0001
	Enterobacter aerogenes et rel.	1.9	2.1	0.03	0.04
Actinobacteria	*Eggerthella lenta et rel.*	1.8	2.7	0.02	< 0.001
Bacilli	*Bacillus*	1.4	2	0.01	0.04
Clostridium cluster IV	*Clostridium leptum et rel.*	1.8	1.8	0.006	0.005
	Sporobacter termiditis et rel.	1.5	1.6	0.05	0.04
	Anaerotruncus colihominis et rel.	1.4	1.5	*0.08*	0.01
	Clostridium orbiscindens et rel.	1.4	1.3	0.03	*0.08*
	Faecalibacterium prausnitzii et rel.	0.5	0.5	0.01	0.006
	Papillibacter cinnamovorans et rel.	0.7	0.7	*0.06*	0.04
Clostridium cluster XIVa	*Clostridium colinim et rel.*	0.4	0.6	*0.06*	0.05
	Clostridium sphenoides et rel.	0.5	0.6	< 0.001	0.003
	Eubacterium hallii et rel.	0.7	0.5	0.03	0.004
	Eubacterium rectale et rel.	0.5	0.5	0.001	0.004
	Eubacterium ventriosum et rel	0.4	0.4	< 0.001	< 0.001
	Lachnobacillus bovis et rel.	0.6	0.8	0.007	0.03
	Outgrouping *Clostridium* cluster XIVa	0.7	0.6	0.02	0.01
	Roseburia intestinalis et rel.	0.5	0.5	0.006	0.03
	Ruminococcus lactaris et rel.	0.6	0.7	0.002	0.01
	Ruminococcus obeum et rel.	0.6	0.6	0.003	0.01

4.1.3 The effect of genetic: microbiota comparison between centenarians and their offspring

In order to assess if the microbiota of centenarians can be related to that of their offspring, the HITChip analysis was performed on samples from group F, composed of 21 offspring subjects of centenarians. The average age in group F was 67.5 years. The HITChip profile of each centenarian was compared to that of his/her offspring, and to the profile of randomly selected subjects belonging to the group E, and Pearson's correlation indices were calculated. A mean Pearson's r of 0.42 ± 0.18 for the C-F pairs and 0.44 ± 0.20 for the C-E pairs were obtained. The result demonstrates that the gut microbiota composition of parents and offspring shows no significant similarity at the old age ($P = 0.79$).

The average Simpson diversity indices obtained for group F was 151.7 ± 49.3. The microbiota of elderly belongings to groups E and F did not differ in diversity as assessed by Simpson index ($P = 0.87$). Also the proportions of the level 1 phylogenetic groups in groups E and F were comparable (data not shown).

RDA analysis showed that samples of the F and E groups were mixed and the centroids were close to each other, whereas the centroid of group C was plotted distant from both of them (Fig. 4.6). No significant differences in the overall microbiota composition were found between groups F and E (MCPP, $P = 0.39$). The statistically significant differences in bacterial groups between C and E (Table 4.2) were also found to differ significantly between C and F (data not shown). In general, the microbiota of elderly people in group F seemed more similar to the microbiota of subjects in the age-matched group E, than to that of their centenarian parents.

Phylotypes belonging to the major bacterial phyla of the human gut microbiota, such as *Clostridium* cluster IV and XIVa and Bacteroidetes, were present in all study subjects. On the other hand, some bacterial groups which are less represented in the human gut ecosystem, such as Fusobacteria and Proteobacteria, showed higher prevalence (the percentage of positive samples in each study groups) in centenarians and their offspring than in E or Y groups (Table 4.3). The most noticeable differences in the prevalence concerned *Campylobacter, Helicobacter* and *Fusobacterium.* This trend is mainly limited to opportunistic or potentially pathogenic bacterial groups, and it is likely to reflect the microbial exposure in the shared living environment and/or the close contacts between family members, since most of the subjects in group F (13 out of 21, defined as group FC) lived with their centenarian parent. Several of the phylogroups listed in Table 4.3, *Asteroplasma et rel., Peptostreptococcus micros et rel., Fusobacterium, Alcaligenes faecalis et rel., Campylobacter, Desulfovibrio et rel., Helicobacter, Leminorella, Moraxellaceae, Proteus et rel., Brachyspira*, showed higher prevalence values in group FC than in group FN, a subset of group F including the offspring subjects who did not live with their parents.

Table 4.3. Bacterial groups which showed differences in terms of prevalence between groups C, F, K, and Y. Prevalence values of subsets of group F, FC (offspring who lived with their centenarian parents) and FN (offspring who do not live with their centenarian parents).

Phylum/order	Level 2 phylogenetic group	Prevalence (%)				
		C	F	(FC, FN)	K	Y
Actinobacteria	*Corynebacterium*	90	90	(83, 100)	50	40
	Micrococcaceae	90	76	(83, 67)	59	35
Asteroplasma	*Asteroplasma et rel.*	48	38	(42, 33)	32	20
Bacilli	*Staphylococcus*	100	100	(100, 100)	91	85
Clostridium cluster XI	*Peptostreptococcus anaerobius et rel.*	76	100	(100, 100)	59	55
	Peptostreptococcus micros et rel.	52	52	(67, 33)	18	15
Fusobacteria	*Fusobacterium*	28	43	(50, 33)	5	5
Proteobacteria	*Alcaligenes faecalis et rel.*	71	81	(92, 67)	50	35
	Bilophila et rel.	90	86	(83, 89)	68	70
	Campylobacter	29	33	(50, 11)	5	5
	Desulfovibrio et rel.	90	90	(100, 78)	77	50
	Escherichia coli et rel.	95	81	(75, 89)	77	70
	Haemophilus	90	76	(75, 79)	68	55
	Helicobacter	33	33	(50, 11)	5	5
	Leminorella	95	76	(83, 67)	64	65
	Moraxellaceae	48	38	(58, 11)	18	10
	Oceanospirillum	57	76	(75, 78)	36	30
	Proteus et rel.	90	76	(83, 67)	41	35
	Pseudomonas	90	90	(83, 100)	77	45
	Serratia	100	95	(92, 100)	77	70
	Yersinia et rel.	95	86	(83, 89)	72	60
Spirochaetes	*Brachyspira*	19	38	(50, 22)	9	0
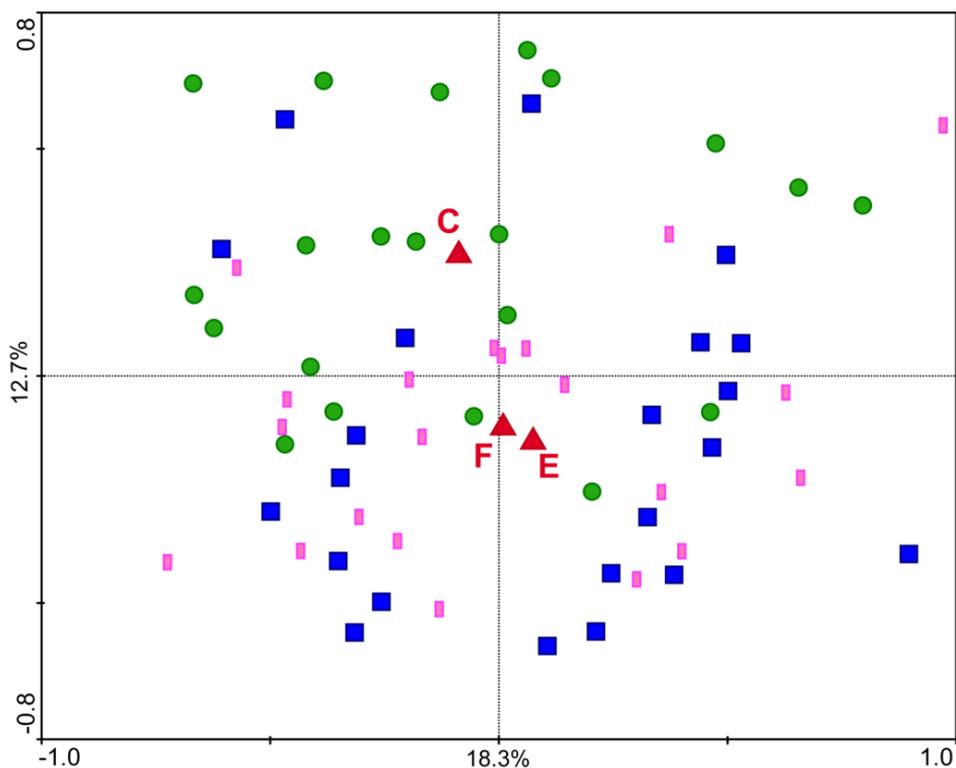
Fig. 4.6. Score plot of the PCA, centered by species and grouped by samples, of the microbiota composition of centenarians (C, green circles), elderly (E, blue squares), and offspring of the centenarians (F, pink boxes). Explanatory variables are indicated by red triangles. First and second ordination axes are plotted, explaining together the 31% of the variability in the considered dataset. Log transformed data were used for the analysis.

4.2 The "Centenarians project": immunological profile

4.2.1 *Immunophenotyping analysis*

In order to assess how the immune system changes in the age groups, we performed a cytofluorimetric quantification of the major lymphocytes populations in the peripheral blood of all subjects, with particular attention to the T-cell compartment. The percentages of naïve, central memory, effector memory, terminal effector cells were measured by flow cytometry in the main lymphocyte population (T helper and T cytotoxic cells). To identify these lymphocyte subsets an electronic gate was set on $CD4^+$ and $CD8^+$ T cells and the expression of CD45RA versus CCR7, CD28, and CD25 was analyzed on each main T-cell subset. Since the immunophenotype of subjects belonging to groups E and F did not differ for any of the analyzed lymphocytes population (data not shown), group S (seventy years old people) was defined, comprehending F and E subjects, and used for further analysis. Regarding B and T lymphocytes, no difference was found among age groups (data not shown). The other results are showed in Table 4.4.

The percentage of naive cytotoxic and helper T lymphocytes significantly decreased with age, whereas central memory T helper and T cytotoxic lymphocytes significantly increased. For effector memory T cytotoxic lymphocytes a significant increase in group Y in comparison to C and S was observed. Terminal effector helper T lymphocytes decreased significantly in C in comparison to both S and Y. Effector cytotoxic and helper T lymphocytes (CD28⁻) increased significantly in groups S and C with respect to Y. On the contrary, activated T cytotoxic lymphocytes were significantly higher in C with respect to both S and Y groups.

4.2.2 *Evaluation of the inflammatory status*

In order to explore the inflammation level of the subjects involved in this study, plasmatic levels of the major pro- and anti-inflammatory cytokines were evaluated (Table 4.5). The pro-inflammatory cytokines IL-6 and IL-8 increased in C with respect to S and Y. In the case of IL-6 the increase was significant (C*vs*S, P = 0.003; C*vs*Y, P = 0.05). On the contrary, IL-1α and TNF-α levels were significantly lower in C in comparison to Y (IL-1α, P = 0.03; TNF-α, P = 0.03). IL-1β and IFN-γ levels did not change significantly in the different age groups. Regarding anti-inflammatory cytokines (TGF-β1 and IL-10), no significant differences were observed among the age groups. Moreover, the plasmatic levels of IL-2 and IL-12 which play an important role in activating the immune response, showed a decrease in C with respect to S and Y, which was significant in the case of IL-2 (C*vs*Y, P = 0.04).

The calculation of the inflammation score (Table 4.6) highlighted that most of the centenarians in this study (69.2%) were characterized by highly inflamed status, whereas in groups S and Y the majority of the subjects fell in the "low inflammation" group (S, 66.7%; Y, 75%). The difference in the proportion of highly inflamed subjects was significant when groups C and S (χ^2 test, P = 0.05), and C and Y (χ^2 test, P = 0.03) were compared.

Table 4.4 Immunophenotypical analysis of the main lymphocyte subsets involved in immunosenescence. Naïve, central memory, effector memory, terminal effector, effector (CD28-) and activated T lymphocytes are expressed as percentage of T helper and T cytotoxic lymphocytes. Data are expressed as mean percentage ± S.E.M.

Lymphocyte subsets		*Mean (%)*			*P value*		
		C	*S*	*Y*	*C vs S*	*S vs Y*	*C vs Y*
Naïve T lymphocytes (CD45RA+CCR7+)	T helper	15.3±2.5	22.5±1.3	33.1±2.0	0.008	<0.0001	<0.0001
	T cytotoxic	12.9±1.4	15.7±1.2	33.9±2.3	/	<0.0001	<0.0001
Central Memory T lymphocytes (CD45RA-CCR7+)	T helper	34.8±3.5	21.5±1.9	13.7±1.1	<0.001	0.009	<0.0001
	T cytotoxic	11.4±1.2	9.8±1.5	5.0±0.6	/	0.03	<0.0001
Effector Memory T lymphocytes (CD45RA-CCR7-)	T helper	42.2±2.8	40.8±1.7	37.8±2.2	/	/	/
	T cytotoxic	47.3±3.5	42.5±2.0	33.4±2.3	/	0.007	0.002
Terminal Effector T lymphocytes (CD45RA+CCR7-)	T helper	7.6±1.1	15.2±1.6	15.4±0.7	0.03	/	<0.0001
	T cytotoxic	28.5±3.9	32.0±2.6	27.7±2.2	/	/	/
Effector T lymphocytes (CD28-)	T helper	10.7±2.0	9.5±1.6	3.5±1.2	/	< 0.001	0.001
	T cytotoxic	48.2±5.4	49.8±3.7	28.6±4.4	/	0.002	0.02
Activated T lymphocytes (CD25+)	T helper	26.6±2.6	23.8±1.5	29.2±1.8	/	0.03	/
	T cytotoxic	13.3±2.9	6.6±0.7	5.5±0.4	0.003	/	0.001

Table 4.5. Plasmatic levels of pro- and anti-inflammatory cytokines.

Cytokines	*Mean ± S.E.M.*			*P value*		
	C	*S*	*Y*	*C vs S*	*S vs Y*	*C vs Y*
IL-6 (pg/ml)	61.4±18.9	24.6±6.2	20.9±4.3	0.003	/	0.05
IL-8 (pg/ml)	30.4±7.8	22.0±4.2	21.9±4.1	/	/	/
IL-1α (pg/ml)	1.7±0.6	3.0±0.6	3.0±0.7	/	/	0.03
TNF-α (pg/ml)	12.2±3.6	22.9±6.4	17.8±4.5	/	/	0.03
IFN-γ (pg/ml)	8.1±1.9	15.5±3.0	13.9±2.7	/	/	0.08
IL-1β (pg/ml)	1.3±0.3	1.7±0.3	1.5±0.2	/	/	/
TGF-β1 (ng/ml)	5.6±0.8	6.2±0.9	5.5±0.7	/	/	/
IL-10 (pg/ml)	1.7±0.2	2.1±0.3	2.3±0.6	/	/	/
IL-2 (pg/ml)	18.8±4.6	30.0±4.6	27.2±5.4	/	/	0.04
IL-12p70 (pg/ml)	4.4±1.4	6.8±1.7	7.3±2.1	/	/	/

Table 4.6. Mean inflammatory score values in C, S, and Y group, and percentage of subjects in the two different inflammation categories. *P value<0.05. Distribution was analyzed using Chi Square test.

Age group	Mean inflammatory score	Inflammatory score distribution	
		low inflammation (0-4)	high inflammation (5-8)
C	4.8	33.3%	67.7%
S	4.0	69.2%*	30.8%*
Y	3.3	75%*	25%*

4.3 The "Centenarians project": correlation between cytokines level and gut microbiota.

In order to individuate correlations between the microbiota composition and the cytokines pattern, log-transformed results of pro-inflammatory cytokines quantification and HITChip profiling of the gut microbiota were used in a multivariate analysis, using cytokines quantification and the age groups as environmental variables. RDA shows that 8.9% of the total variability of the gut microbiota can be related to the pro-inflammatory cytokines pattern (Fig. 4.7). Relations shown in the plot are statistically significant, as established by the MCPP (P = 0.014). In accordance with previous analyses (Fig. 4.2), the centroid of group C is plotted distant from both the S and Y centroid, highlighting the similarity in the gut microbiota asset and relation with the inflammatory status between elderly and young adults.

Several bacteria belonging to the phylum Proteobacteria seemed to be positively correlated with IL-6 and IL-8. IL-8 was correlated with *Alcaligenes faecalis et rel.*, *Leminorella*, and *Proteus et rel.*, while IL-6 was correlated with *Escherichia coli et rel., Haemophilus, Klebsiella pneumoniae et rel., Pseudomonas, Serratia, Yersinia et rel.*, and *Vibrio*. IL-8 and IL-6 were correlated also with *Bacillus* (Bacilli), *Egghertella lenta et rel.* (Actinobacteria), and *Eubacterium cylindroides et rel.* (*Clostridium* cluster XIVa). On the other side, *Eubacterium hallii et rel., Eubacterium ventriosum et rel., Eubacterium rectale et rel., Clostridium nexile et rel.*, and Outgrouping *Clostridium* cluster XIVa (all belonging to the *Clostridium* cluster XIVa) are inversely correlated with IL-6 and IL-8.
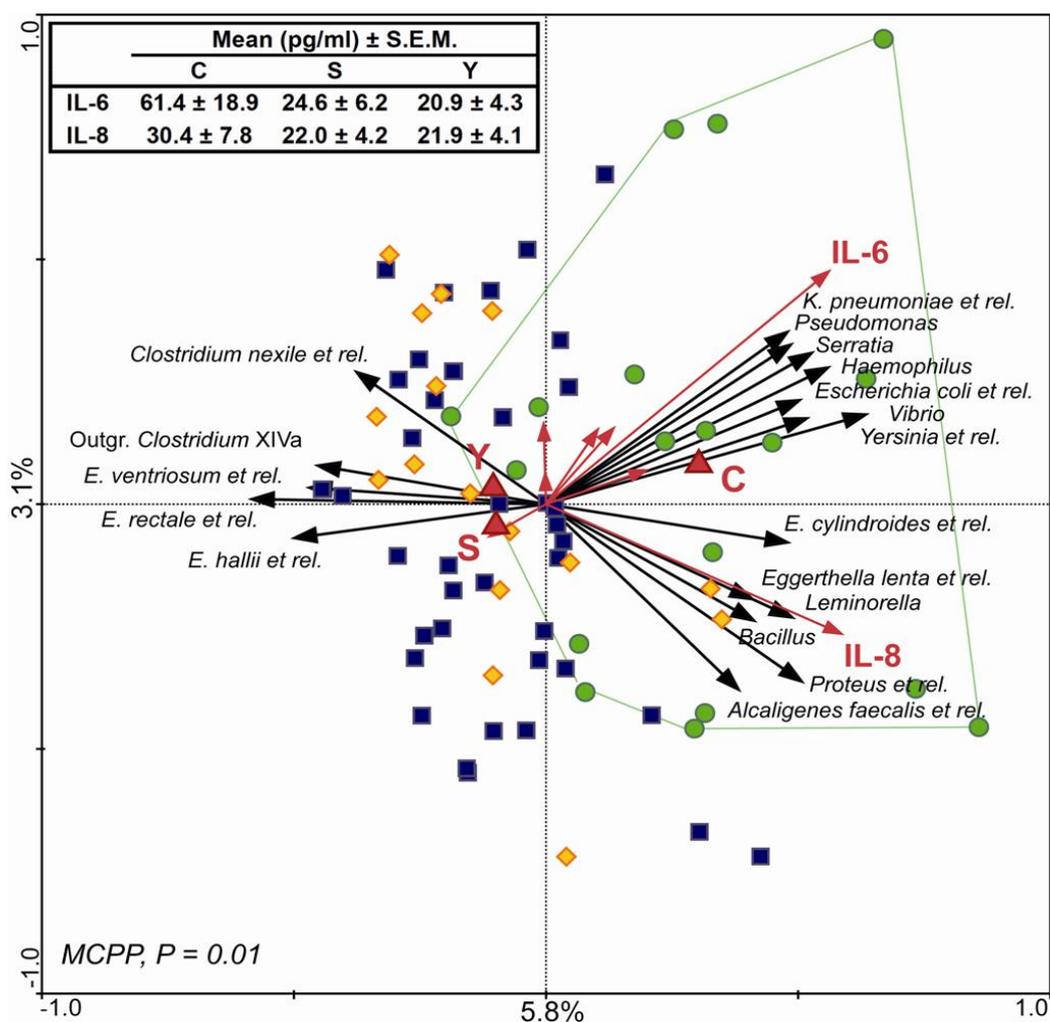
Fig. 4.7. Triplot of the RDA showing the relation between the microbiota composition, the pro-inflammatory cytokines levels and the age of the subjects. Cytokines level (red arrows) and age groups (C, S, and Y, red triangles) are used as linear and nominal environmental variables, respectively. Samples belonging to C, S and Y groups are indicated by green circles, blue squares and yellow diamonds, respectively. Responding bacterial subgroups that explained more than 20% of the variability of the samples are indicated by black arrows. First and second ordination axes are plotted, showing 5.8% and 3.1% of the variability in the dataset. Red arrows which are not labelled corresponds to (clockwise, starting from the left) TNF-α, IFN-γ, IL2, IL1α, IL12p70, and IL1β. Bottom-left, P value obtained by MCPP is reported.

Pearson's correlation analysis of the log transformed level 2 HITChip data and cytokines revealed significant Pearson's r for IL-6 and IL-8 (Table 5). All the bacterial groups which exhibited a slight positive correlation with either IL-6 or IL-8 (ranging between 0.41 and 0.55) belonged to the phylum Proteobacteria. Interestingly, only one bacterial group, *Ruminococcus lactaris et al.*(*Clostridium* cluster XIVa), has been found with a slight negative correlation with IL-8 (-0.44, P = 0.0001).

Table 4.7. Relevant Pearson's correlations between microbiota components and the pro-inflammatory cytokines IL6 and IL8.

Bacterial group	IL-6		IL-8	
	Pearson's r	**P value**	**Pearson's r**	**P value**
Alcaligenes faecalis et al.	/	/	0.43	00.02.00
Escherichia coli et rel.	0.46	< 0.0001	0.35	0.003
Haemophilus	0.47	< 0.0001	0.24	0.04
Klebsiella pneumoniae et rel.	0.47	< 0.0001	/	/
Leminorella	0.26	0.02	0.41	< 0.0001
Proteus	0.32	0.007	0.55	< 0.0001
Pseudomonas	0.45	< 0.0001	/	/
Serratia	0.45	< 0.0001	0.23	0.05
Vibrio	0.45	< 0.0001	0.28	0.02
Yersinia	0.48	< 0.0001	0.33	0.005
Ruminococcus lactaris et rel.	-0.32	0.006	-0.44	0.0001

4.4 Design and construction of the HTF-Microb.Array.

4.4.1 *Target selection and probe design*

The rational selection of the HTF-Microb.Array targets was carried out using a phylogenetic approach. To this aim we implemented the 16S rRNA database of the ARB Project (release February, 2005) with the 16S rRNA gene database of the RDP available at the time and a phylogenetic tree was constructed. Based on the tree nodes, 30 phylogenetical groups of the human intestinal microbiota were rationally selected as the target group for the HTF-Microbi.Array (Appendix 1). The selected groups belonged to different phylogenetic levels (species, genus, family, cluster, or group of species indicated by the warding "*et rel.*"). The entire list of the array targets is represented in Tab. 4.8. For part of the division *Firmicutes*, the target selection was carried out based on the classification proposed by Collins *et al* (1994). *Clostridium* cluster I and II, *Clostridium* cluster IX, *Clostridium* cluster XI, and *Clostridium* cluster XIVa were selected. For the *Clostridium* cluster IV, four subgroups of species were defined: *Ruminococcus albus et rel., Ruminococcus bromii et rel., Faecalibacterium prausnitzii et rel.*, and *Oscillospira guillermondii et rel.* Within the *Firmicutes* division, the family *Lactobacillaceae*, and the groups *Bacillus clausii et rel., Bacillus subtilis et rel., Bacillus cereus et rel., Enterococcus faecalis et rel.*, and *Enterococcus*

faecium et rel. were also selected. Other selected groups were the *Bacteroides/Prevotella* cluster (division *Bacteroidates*), the family *Bifidobacteriaceae* (division *Actinobacteria*), the family *Enterobacteriaceae* and the genus *Campylobacter* (division *Proteobacteria*). For clusters or families, relevant species, genera or subgroups of species were selected to design "sub-probes". The genus *Veillonella* was selected for *Clostridium* cluster IX, the species *Eubacterium rectale* for *Clostridium* cluster XIVa, *Clostridium difficile* for *Clostridium* cluster XI, and *Clostridium perfringens* for *Clostridium* cluster I and II. The group *Bifidobacterium longum et rel*. was chosen for the family *Bifidobacteriaceae*, and the genera *Yersinia* and *Proteus* for the *Enterobacteriaceae*. Specificity and coverage of each candidate probe was assessed by using the tool Probe Match of the RDP database. The probe pairs selected for the HTF-Microbi.Array were required to perfectly match the sequences of the positive set and to possess at least a mismatch at the 3' end of the discriminating probe respect to the entire negative set. The designed probes pairs had an average melting temperature (Tm) of 67.8 ± 0.9 °C (n=60) and an average length of 35.6 ± 4.9 nucleotides. Sixteen out of the 30 probe pairs were characterized by having no degenerated bases, whereas only one probe pair (i.e. the one for *Clostridium* cluster I and II) had 4 and 3 ambiguous bases on DS and CP, respectively (Appendix 2).

4.4.2 *Validation of LDR probe pair specificity*

The specificity of the designed LDR probe pairs was tested by using 16S rRNA PCR amplicons from 28 microorganisms members of the human intestinal microbiota. Amplicons were prepared by amplification of genomic DNA. Proving the specificity of the HTF-Microbi.Array, all the 16S rRNA amplicons were properly recognized in separate LDR hybridization reactions with the entire probe set of the array. Two replicated independent LDR-UA experiments were performed with an optimal reproducibility. For each of the 16S rRNA template only group-specific spots, and spots corresponding to the hybridization controls showed positive signals ($P<0.01$) (Appendix 3). As a negative control, we performed two independent PCR-LDR-UA experiments using double distilled water, instead of genomic DNA, as sample. As expected, no positive signal was detected. The ratio between the signal intensities of the specific probes and the blank intensity (SNR_S) averaged 206.9 ± 185.7, whereas the ratio between all the other probes and the blank intensity (SNR_{ns}) averaged 2.1 ± 1.4. Therefore, the ratio between specific and non-specific probes resulted more than 100 fold on average.

Table 4.8 Probe set of the HTF-Microbi.Array. For each probe is indicated the spot number (N.), the phylogenetic level, the phylogeny of the target group (cluster, order, division), and the ecology in the gastrointestinal ecosystem [mutualistic (M), opportunistic (O), pathogen (P)]. The relative abundance in a healthy gut ecosystem of the principal microbial groups is also indicated. B., *Bifidobacterium*; L., *Lactobacillus*; Cl, *Clostridium* cluster.

PROBE	*N.*	*PHYLOGENETIC LEVEL*	*CLUSTER*	*DIVISION*	*ECO*
Bacteroides/Prevotella	16	Cluster	*Bacteroides/Prevotella*	Bacteroidetes	M
Ruminococcus bromii	38	Sub cluster	Cl IV	Firmicutes	M
Ruminococcus albus	39	Sub cluster	Cl IV	Firmicutes	M
Faecalibacterium prausnitzii	40	Sub cluster	Cl IV	Firmicutes	M
Oscillospira guillermondii	41	Sub cluster	Cl IV	Firmicutes	M
Clostridium IX	37	Cluster	Cl IX	Firmicutes	M
Veillonella	20	Genus	Cl IX	Firmicutes	M
Clostridium XIVa	22	Cluster	Cl XIVa	Firmicutes	M
Eubacterium rectale	19	Species (*et rel*)	Cl XIVa	Firmicutes	M
Bifidobacteriaceae	25B	Family	*Bifidobacterium*	Actinobacteria	M
B. longum	3	Species (*et rel*)	*Bifidobacterium*	Actinobacteria	M
Lactobacillaceae	21B	Family	*Lactobacillaceae*	Firmicutes	M
L. plantarum	33	Species (*et rel*)	*Lactobacillaceae*	Firmicutes	M
L. casei	12	Species (*et rel*)	*Lactobacillaceae*	Firmicutes	M
L. salivarius	14	Species (*et rel*)	*Lactobacillaceae*	Firmicutes	M
Bacillus clausii	32	Species (*et rel*)	*Bacillaceae*	Firmicutes	M
Bacillus subtilis	8	Species (*et rel*)	*Bacillaceae*	Firmicutes	M
Fusobacterium	15	Genus	*Fusobacteriaceae*	Fusobacteria	M
Cyanobacteria	42	Family	Cyanobacteria	Cyanobacteria	M
Clostridium XI	36	Cluster	Cl XI	Firmicutes	O
Clostridium difficile	18	Species (*et rel*)	Cl XI	Firmicutes	O
Clostridium I and II	35	Cluster	Cl I and II	Firmicutes	O
Clostridium perfringens	17	Species (*et rel*)	Cl I and II	Firmicutes	O
Enterococcus faecalis	9	Species (*et rel*)	*Enterococcales*	Firmicutes	O
Enterococcus faecium	10	Species (*et rel*)	*Enterococcales*	Firmicutes	O
Bacillus cereus	7	Species (*et rel*)	*Bacillaceae*	Firmicutes	P
Enterobacteriaceae	23B	Family	*Enterobacteraceae*	Proteobacteria	O/P
Yersinia enterocolitica	4	Species (*et rel*)	*Enterobacteraceae*	Proteobacteria	O/P
Proteus	5	Genus	*Enterobacteraceae*	Proteobacteria	O/P
Campylobacter	6	Genus	*Campylobacteraceae*	Proteobacteria	P

4.4.3 Evaluation of the LDR sensitivity and relative abundance detection level

In order to define the detection limits of the HTF-Microbi.Array, LDR-UA experiments were carried out with different concentrations of an artificial mix of 16S rRNA amplicons from 6 members of the human intestinal microbiota. The 16S rRNA amplicons from *Bacillus cereus*, *Lactobacillus casei*, *Bifidobacterium adolescentis*, *Ruminococcus albus*, *Prevotella*, *Y. enterocolitica* were all specifically recognized in a range of concentrations from 0.7 to 75 fmol ($P<0.01$), demonstrating the high sensitivity and specificity of the array (Fig. 4.8). Subsequently, in order to evaluate the relative abundance detection level of the HTF-Microbi.Array, LDR-UA experiments were performed on hybridization mixes containing low quantities of *Escherichia coli* PCR products and increasing amounts of human genomic DNA. This is a fundamental issue in the case of single species present in the gut microbiota at very low fractional abundance. According to our data, 1 fmol of *E. coli* amplicon was sufficient ($p<0.005$) to be detected in all the tested conditions (from up to 6.3 μg of human gDNA). Considering the PCR product as a ~ 1700 bp amplicon, 1 fmol corresponds to 1.2 ng and, thus, the sensitivity limit results 0.02%.

4.5 Validation of the HTF-Microb.Array on human fecal microbiota.

4.5.1 Characterization of the faecal microbiota of eight healthy young adults

The HTF-Microbi.Array was applied in a pilot study for the characterization of the faecal microbiota of eight young adults. For all subjects faecal DNA was extracted, total bacterial 16S rRNA amplified, and two separate LDR-UA experiments were carried out. For each sample a profile of presence-absence probes response was obtained. The cluster analysis of the phylogenetic fingerprints showed that, with the exception of subject n. 2, samples from the same subject clustered together, demonstrating a good reproducibility of the microbiota fingerprints obtained using the HTF-Microbi.Array (Fig. 4.9).

Fig. 4.8. (following page) LDR-universal array experiments carried out on a complex mix of 16 rRNA amplicons obtained from six members of the human intestinal microbiota: *B. cereus* (B.c.), *L. casei* (L.c.), *B. adolescentis* (B.a.), *R. albus* (R.a.), *Prevotella* (Pr.), *Y. enterocolitica* (Y.e.). Amplicons were tested in a concentration ranging from 0.7 to 75 fmol. Green bars indicate the fluorescence intensity (IF) of each probe. Blue stars over the fluorescence bars indicate the probes that gave a positive response with a P value <0.01. Red dots indicate that one or two replicates out of four for each ZipCode were excluded because having an IF < 2.5 times the average of the spots. Blue bar corresponds to the hybridization positive control signal. Black, red and purple bar correspond to the negative controls signals.

(B.c-L.c-B.a-R.a-Pr-Y.e) 16S rDNA **0.7 fmol**

(B.c-L.c-B.a-R.a-Pr-Y.e) 16S rDNA **1.5 fmol**

(B.c-L.c-B.a-R.a-Pr-Y.e) 16S rDNA **3 fmol**

(B.c-L.c-B.a-R.a-Pr-Y.e) 16S rDNA **6 fmol**

(B.c-L.c-B.a-R.a-Pr-Y.e) 16S rDNA **12 fmol**

(B.c-L.c-B.a-R.a-Pr-Y.e) 16S rDNA **25 fmol**

(B.c-L.c-B.a-R.a-Pr-Y.e) 16S rDNA **50 fmol**
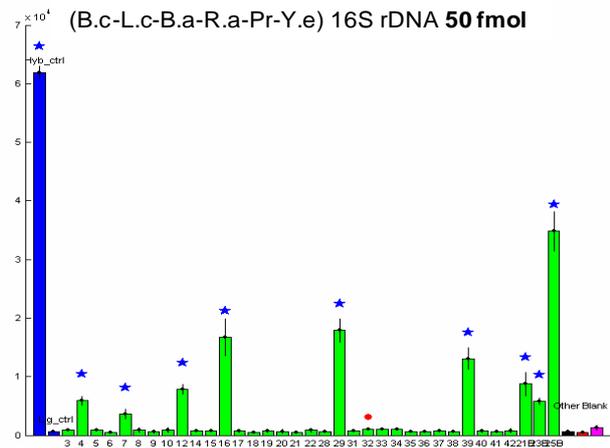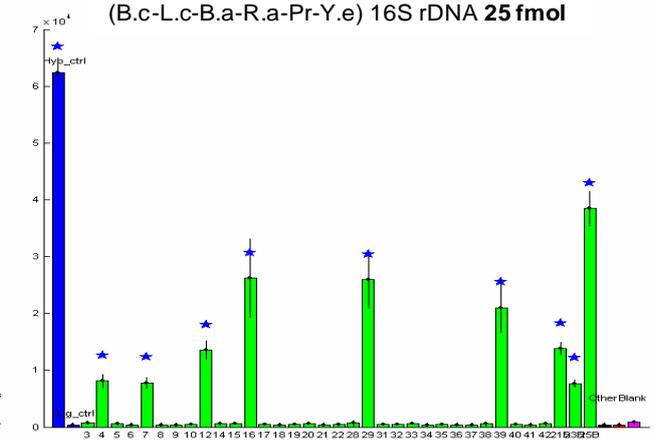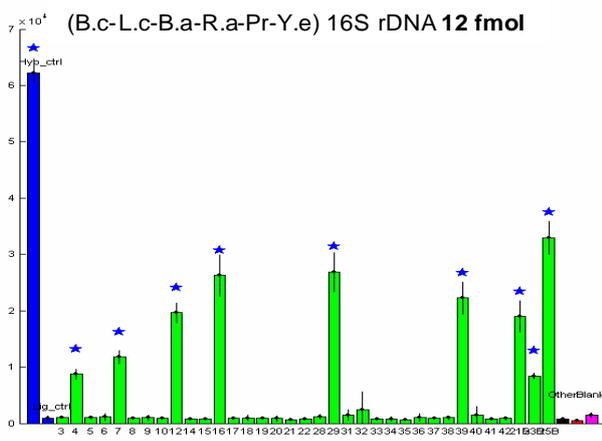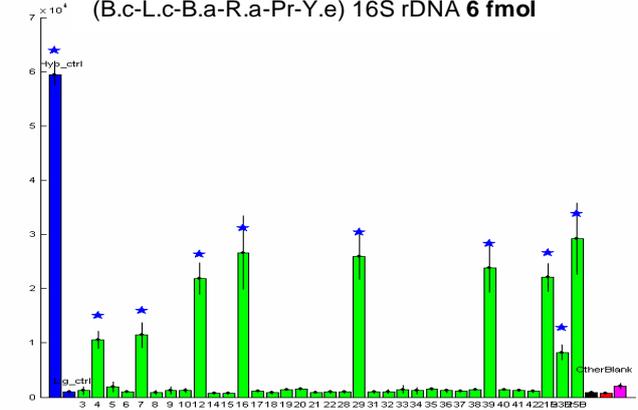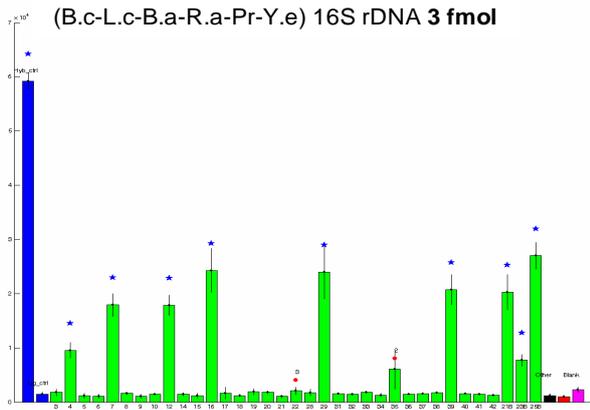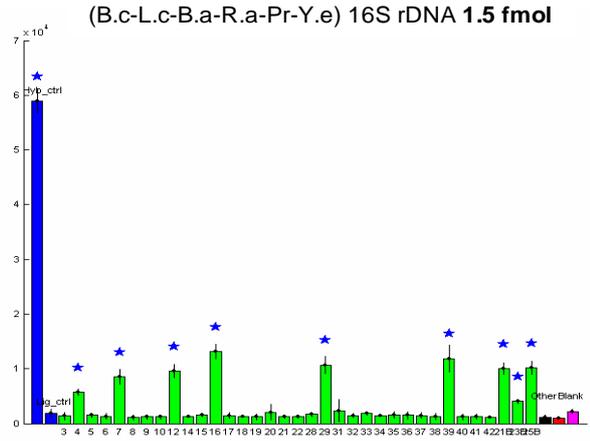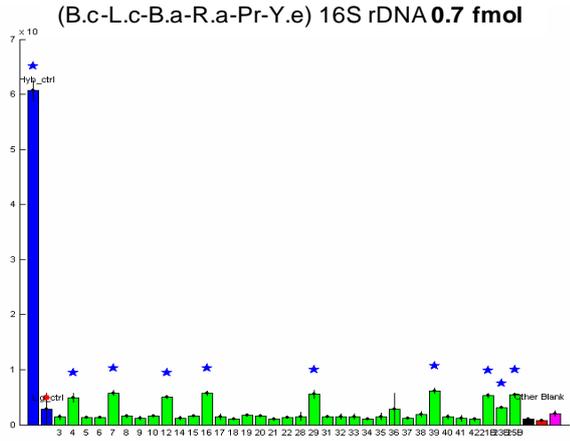
As expected, the major mutualistic symbionts of the human intestinal microbiota, such as *Bacteroidetes* and the members of the *Clostridium* cluster IV and XIVa, were represented in the faecal microbiota of all the subjects. With the exception of *B. clausii et rel.*, minor mutualistic symbionts such as *Actinobacteria*, *Lactobacillaceae*, *B. subtilis et rel.*, *Fusobacterium*, and *Cyanobacteria* were detected only in different sub-fractions of the subjects. In particular, subjects n. 17, 15, 4, and 1 were characterized by the presence of *Fusobacterium*. Subjects n. 4, 15 and 17 possessed *B. subtilis et rel.*, while subjects n. 4, 1, 9, 16 and 5 harboured *Cyanobacteria* in their faecal microbiota. On the other hand, only a fraction of the subjects, clustering on the left side of the map, presented opportunistic pathogens in their faecal microbiota. Subjects n. 17, 15 and 4 presented both *Proteus* and *E. faecalis et rel.*, while in subject n. 15 members of the *Clostridium* cluster I and II and *Yersinia enterocolitioca et rel.* were also detected.

For each subject the relative fluorescence intensity (IF) contribution of each HTF-Microbi.Array probes, in terms of percentage of the total IF, was also calculated (Fig. 4.10). The mean of IF data from both the LDR-UA experiments were considered. Even if all subjects were characterized by a specific individual profile, a common trend can be found by comparing the comprehensive relative IF contribution of probes targeting major mutualistic symbionts (*Bacteroides/Prevotella*, *Clostridium* clusters IV, IX, and XIVa), minor mutualistic symbionts (*Bifidobacteriaceae, Lactobacillaceae, B. clausii et rel., , B. subtilis et rel.*, *Fusobacterium*, and *Cyanobacteria*), and opportunistic pathogens (*Clostridium* clusters I and II, IX, *E. faecalis et rel.*, *E. faecium et rel., B. cereus et rel., Enterobacteriaceae, Yersinia enterocolitica et rel., Proteus, Campylobacter*). In particular, for all subjects the highest relative IF contributions were obtained for major mutualistic symbionts. The contribution of *Bacteroides/Prevotella* ranged between 8-37%, whereas the contribution of *Clostridium* clusters IV, IX, and XIVa ranged between 17-34%, 3-15%, and 5-29%, respectively. Differently, minor mutualistic symbionts were characterized by lower values of relative IF contributions. *Bifidobacteriaceae* contributed for the 0.5-3.1%, *Lactobacillaceae* for the 1.5-9.4%, *B. clausii et rel.* for the 4-13%, *B. subtilis et rel.* for the 0.6-2.5%, *Fusobacterium* for the 1.2-4.4%, and *Cyanobacterium* for 0.6-4.5%. As expected, opportunistic pathogens showed together the lowest relative IF contribution in all the subjects under study (from 5 to 10%).

Fig. 4.9. (Following page) Cluster analysis of the phylogenetic fingerprint of 16 faecal samples from 8 young adults. Response of each of the HTF-Microbi.Array probes for what concerns presence/absence of the target group is shown: positive response in red (P<0.01), negative responses in blue (P>0.01). Gray lines below the samples indicate adjacent replicated LDR of the same sample.
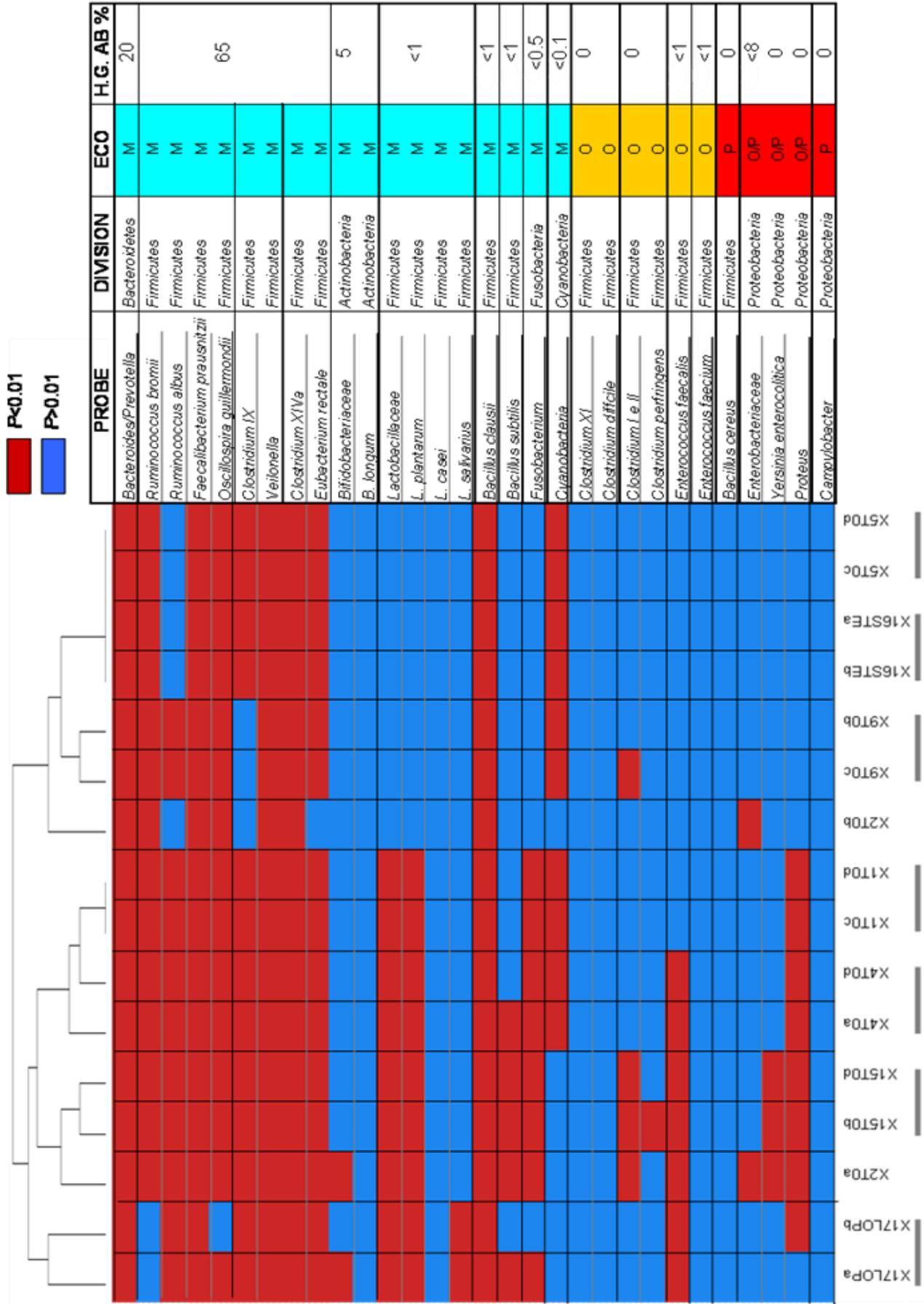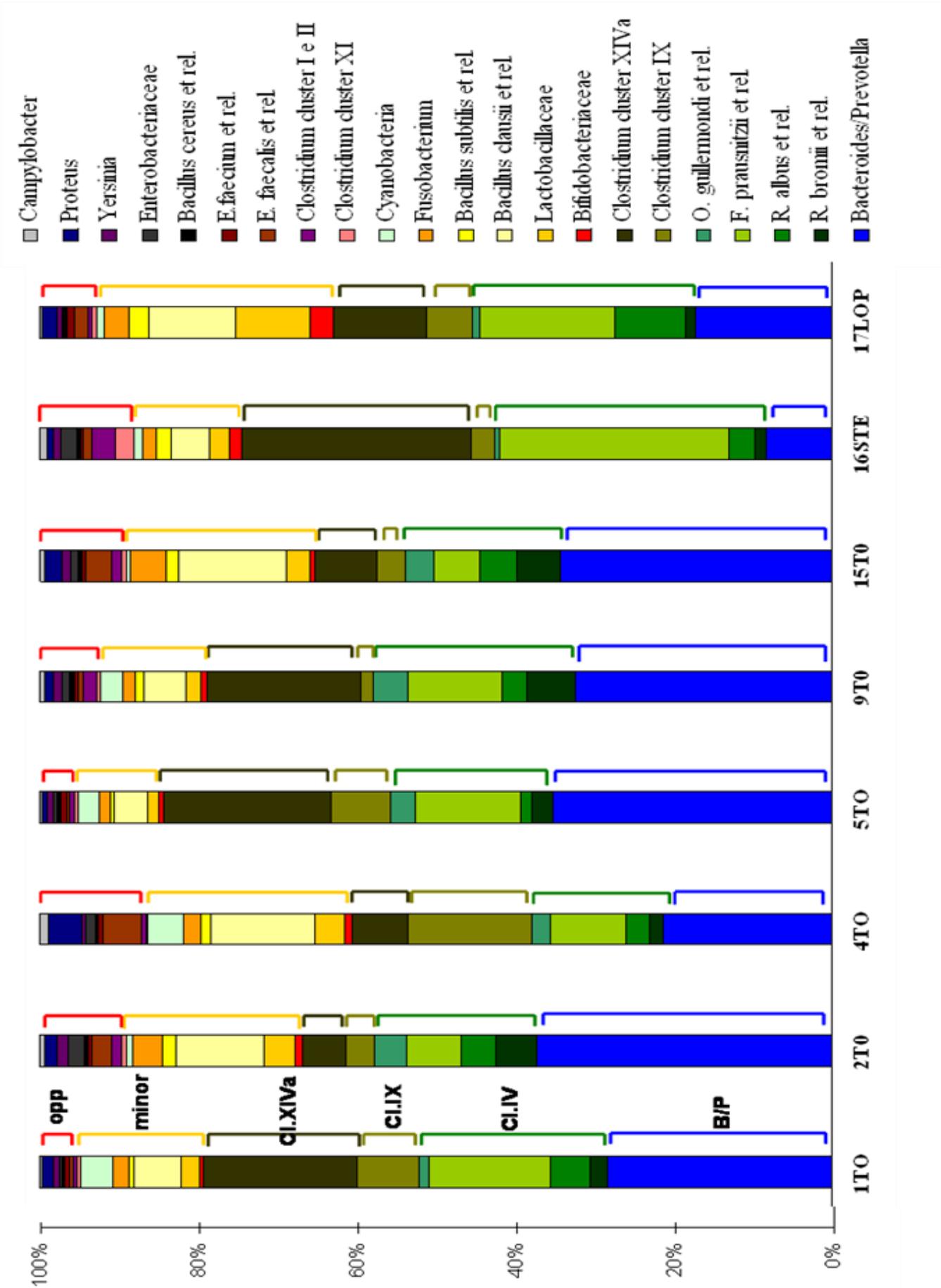
Fig. 4.10. (Previous page) IF relative contribution of targeted groups to the total microbiota. For each sample the entire HTF-Microbi.Array probe set was considered and their relative IF contribution was calculated as percentage of the total IF. Sub-probes were excluded and, for each subject, data from two separate LDR-universal array experiments were taken onto consideration. The averaged IF from both the LDR-Universal Array experiments was considered. The principal intestinal groups of major mutualistic symbionts are indicated: *Bacteroides/Prevotella* (B/P) blue, *Clostridium cluster* IV (Cl.IV) green, *Clostridium cluster* IX (Cl.IX) brown, *Clostridium cluster* XIVa (Cl.XIVa) dark brown. *Lactobacillus*, *B. clausii*, *B. subtilis*, *Fusobacterium* and *Cyanobacteria* are grouped as minor mutualistic symbionts (minor) indicated in yellow. Opportunistic pathogens (opp) are indicated in red.

4.6 Comparison of the two microarray approaches.

The fecal microbiota of six of the centenarians involved in the "Centenarians project" was analyzed by using HTF-Microb.Array, in order to compare the two microarray approaches presented here. Following the procedure showed in Fig. 4.5 and 4.10, the average relative IF contribution, in terms of percentage of the total IF, was calculated and compared to the average relative contribution of the level 1 phylogenetic groups obtained for the same six centenarians with the HITChip analysis (Fig. 4.11). Because of the completely different approach used for the design of the two diversity microarrays, approximations were performed to make the results comparable. The IF contribution of the *Entorobacteriaceae* probe was approximated to the one of the Proteobacteria in the HITChip profile, even if the phylum Proteobacteria comprehends many more families. Similar approximation was performed to compare the IF contribution of the *Bifidobacteriaceae* probe and the contribution of the phylum Actinobacteria. The contribution of Bacilli in the HITChip profiling was considered equal to the sum of the IF contribution of the probes *B. clausi et rel., B. cereus et rel., B. subtilis et rel.*, and *Lactobacillaceae* on the HTF-Microb.Array. The contribution of *Clostridium* cluster IV was considered equal to the sum of the IF contribution of the four probes in which this *Clostridium* cluster had been split during the HTF-Microb.Array design (*F. prausnitzii et rel., O. guillermondii et rel., R. bromii et rel., R. albus et rel.*).

The HTF-Microb.Array seems to underestimate the presence of bacteria of the *Clostridium* cluster XIVa with respect to the HITChip profiling. On the contrary, the other main *Clostridium* clusters (IV, IX, and XI) seem slightly overestimated, probably as a consequence of the previous observation. The relative IF contributions of *Enterobacteriaceae*, *Bacteroides/Prevotella*, and *Bacilli* appear to be comparable to the relative contribution of Proteobacteria, Bacteroidetes and *Bacilli* on the HITChip profiling. The relative contribution of Actinobacteria to the HITChip profile

is slightly higher than the IF relative contribution of the *Bifidobacteriaceae* probe on the HTF-Microb.Array, due to the fact that the gut microbiota contains more Actinobacteria families alongside to the *Bifidobacteriaceae*. Finally, the contribution of the Fusobacteria is clearly overestimated by the HTF-Microb.Array analysis, when compared to the HITChip profiling.
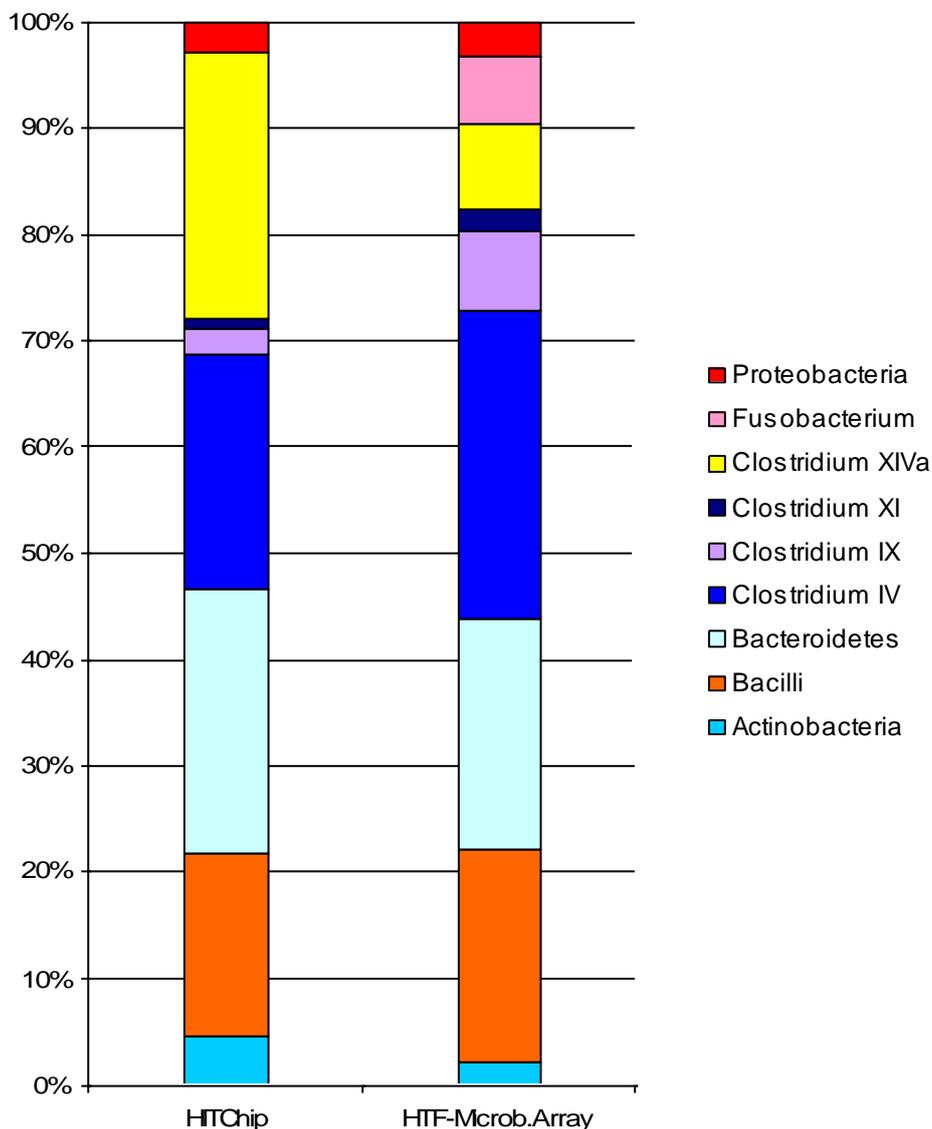
Fig 4.11. Relative contribution of the level 1 phylogroups to the fecal microbiota obtained by HITChip profiling, and relative IF contribution to the total IF obtained by HTF-Microb.Array analysis, of six centenarians.

5.
DISCUSSION

5.1 The "Centenarians project"

5.1.1 General remarks

The "Centenarians project" aimed the study of the age-related changes in both the gut microbiota and the inflammatory status during the whole adult life, starting from young adults (30 years old in average), through elderly (70 years old), to the extreme limit of the human lifespan, represented by a group of centenarians.

The exceptionality of this comparative study resides in the introduction of this third, extremely aged, population, alongside to young and elderly adults, the two age groups addressed by the studies published until now (Bartosch *et al*, 2004; Woodmansey *et al*, 2004; Mariat *et al*, 2009; Rajilic-Stojanovic *et al,* 2009). This peculiarity allowed us to highlight that changes in the gut ecosystem, in terms of composition and diversity, do not follow a linear relation with age. In fact, the difference between the gut microbiota of young adults and elderly, separated by more than 40 years in average, is remarkably small if compared to the difference observed between centenarians and elderly, separated by less than 30 years of life.

Young and elderly adults show a very comparable overall structure of the gut microbiota, which confirms the most recent characterizations, with Bacteroidetes and Firmicutes highly dominant (contributing for approximately 95% to the microbiota), and smaller fractions of Actinobacteria, and Proteobacteria (Ley *et al*, 2006; Andersson *et al*, 2008; Tap *et al*, 2009). The diversity values of the gut microbiota of elderly and young adults were found comparable (average Simpson index 149.3 and 162.8, respectively), and fits the range of diversity expected for healthy adults (Simpson index = 150-200) (A. Salonen and W. de Vos, personal communication).

On the other hand, centenarians come into view as a separate population, whose microbiota shows an organization which significantly differs from the adult-like pattern, and a low diversity in terms of species composition (Simpson index = 127). Bacteroidetes and Firmicutes still dominate the gut microbiota of extremely old people (93%), but a change in the relative proportion of Firmicutes subgroups was found, with a decrease in the contributing *Clostridium* cluster XIVa, an increase in Bacilli and a rearrangement of the *Clostridium* cluster IV composition. Moreover, the gut microbiota of centenarians is enriched in Proteobacteria, a group containing many of those bacteria recently redefined as "pathobionts", referring to minor opportunistic components of the human gut

ecosystem that, under some circumstances, e.g. inflammation, may escape surveillance, overtake mutualistic symbionts and induce pathology (Sansonetti and Di Santo, 2007; Round and Mazmanian, 2009).

The structure of the gut microbiota is well known to be relatively stable through adulthood (Vanhoutte et al, 2004; Leser and Molbak, 2009). Our findings suggest that this stability may last longer than expected, and that the ageing process starts to affect the gut microbiota later than 65 years old, which is the usual threshold age for being defined as "elderly".

Up to now, only few studies were published focusing on the molecular characterization of the gut ecosystem in the elderly. In general, these studies reported compositional differences comparing groups of young adults (20-50 years old) to elderly with a wide interval of age, ranging from 60 to 95 years old approximately (Bartosch *et al*, 2004; Mueller *et al*, 2006; Mariat *et al,* 2009). Conversely, thanks to a narrower range of age for the recruited elderly (63-78 years old), and the introduction of the "third point" of the analysis (centenarians), we could provide a higher resolution on the effects of ageing on the gut microbiota. This approach indicates that the threshold for an "aged" microbiota should be moved to the age of 75-80 years.

Recently, Rajilic-Stojanovic *et al* (2009) used the HITChip technology to highlight differences between the fecal microbiota of 5 young adults and 5 elderly from Northern Europe, aged 71 years in average. An age-related increase in the number of Bacilli, and a decrease in bacterial groups belonging to the Bacteroidetes were reported. Interestingly, these differences between elderly and young adults were not confirmed by our HITChip study, where only centenarians showed significantly higher proportions of Bacilli, and the Bacteroidetes population remained unchanged among the age groups. A fascinating explanation of this discrepancy may reside in the demographic and geographic differences between the study population, also defined as country specificity, which has been reported to strongly affect the age-related changes in the gut microbiota composition (Mueller *et al*, 2006).

5.1.2 *The gut microbiota of centenarians*

Our experimental data provide a view of a centenarian gut ecosystem characterized by a lower relative contribution of *Clostridium* cluster XIVa, than in younger people. The general decrease in the abundance of *Eubacterium rectale - Clostridium coccoides* group, corresponding to the *Clostridium* cluster XIVa, has already been described as an effect of the ageing process (Hayashi *et al*, 2003; Mueller *et al*, 2006; Zwielehner *et al,* 2009).

On the other hand, our results showed that the proportion of *Clostridium* cluster IV in the total microbiota is comparable in all the subjects, but the proportions of different phylotypes within the

cluster differ significantly between the age groups, suggesting a rearrangement in the composition of *Clostridium* cluster IV in centenarians.

In particular, our results showed a rearrangement in the population of butyrate producer bacteria in centenarians, with respect to the microbiota of younger people. Butyrate is a short chain fatty acid (SCFA) mainly produced in the gut by Firmicutes of the *Clostridium* clusters IV and XIVa (Barcenilla *et al*, 2000; Pryde *et al*, 2002; Louis and Flint, 2009). Butyrate producers are receiving a growing interest in the gut ecology, as this SCFA represents a major energy source for the enterocytes and may be implicated in the protection against inflammatory bowel diseases (IBD) (Thibault *et al*, 2009). In our study, several butyrate producers were found in lower amounts in centenarians than in the other age groups. These included *Ruminococcus obeum et rel., Roseburia intestinalis et rel., Eubacterium ventriosum et rel., Eubacterium rectale et rel.,* and *Eubacterium hallii et rel.* (belonging to the *Clostridium* cluster XIVa), and *Papillibacter cinnamovorans et rel.,* and *Faecalibacterium prausnitzii et rel.* (belonging to the *Clostridium* cluster IV). Conversely, the butyrate producers *Anaerotruncus colihominis et rel.,* and *Eubacterium limosum et rel.* increased in centenarians. Interestingly, *E. limosum*, a species with anti-inflammatory effects (Kanauchi *et al*, 2006), shows the highest mean differences between centenarians and both elderly (ratio 16.2) and young adults (ratio 14.5).

F. prausnitzii is a species of particular interest for the gut inflammation processes, able to exert also a butyrate-independent anti-inflammatory effect (Sokol *et al*, 2008, Sokol *et al*, 2009), and it is already known to decrease in the elderly (Mueller *et al,* 2006, Zwielehner *et al*, 2009).

Another feature of the centenarians gut ecosystem is the increase in facultative anaerobes, such as bacteria belonging to the groups *Fusobacterium, Bacillus, Staphylococcus, Corynebacterium, Micrococcaceae*, and many members of the phylum Proteobacteria. Such opportunistic species, especially Enterobacteriaceae, thrive in an inflamed environment (Pédron and Sansonetti, 2008; Sansonetti and Medzhitov, 2009), and are known to increase both in elderly and people affected by IBD (Guigoz *et al*, 2008; Sartor, 2008).

The decrease in the amount of health promoting bacteria, in particular bifidobacteria, is another well reported effect of age (Woodsmansey *et al*, 2004; Mueller *et al*, 2006). In our study, we detected a significant decrease of bifidobacteria in centenarians when compared to young adults, only by using specific real time PCR. Discrepancies between results obtained by different methods used for quantification of bifidobacteria in feces have been reported, not only when viable counts are compared with results obtained by molecular techniques (Sghir *et al*, 2000; Hopkins *et al*, 2001), but also when two different molecular techniques are compared (Tap *et al*, 2009).

Interestingly, we found increased levels of the mucin degrading *A. muciniphila* in aged people if

compared to the young adults, contrasting the earlier results obtained by Collado *et al* (2007), who demonstrated that *A. muciniphila* counts decline with the age. Again, the country-specificity of the age-related changes in the gut microbiota may be the cause of this discrepancy, as well as differences in the diet of the aged people, that may favour populations of bacteria able to degrade mucins.

The centenarians involved in this study, representative of this exceptionally aged population, were obviously very frail and inflamed (Baggio *et al*, 1998; Passeri *et al*, 2003). This could explain why some of the differences found between centenarians and elderly, such as the decrease in *F. praunitzii* and the increase in *Enterobacteriaceae*, were reported also between elderly with high and low frailty score (Van Tongeren *et al*, 2005).

5.1.3 *Gut microbiota and inflamm-aging*

The fecal microbiota of centenarians shows all the features of a partially compromised ecosystem, whose balance with the immune system is likely to be upset. The immune system of these very old people deteriorates under the effect of the immunosenescence, a progressive age-related remodelling of the immune functions, where several functions are reduced, others remain unchanged, or even increase (Franceschi *et al*, 1995). The careful immunophenotyping we performed, allowed us to confirm that the major, expected age-related changes of the T cells compartment are present in the enrolled subjects, including centenarians (Fagnoni *et al*, 2000; Zanni *et al*, 2003; Alberti *et al*, 2006; Nasi *et al*, 2006; Koch *et al*, 2008), suggesting that the population here considered represents a suitable sample to investigate the possible relationship between gut microbiota composition and immune/inflammatory status. One of the major characteristics of immunosenescence is the progressive development of a chronic, low grade inflammatory status called inflamm-ageing (Franceschi *et al*, 2000a; Franceschi, 2007a; Franceschi *et al*, 2007b). In our study population, the proportion of centenarians showing a high inflammation score was significantly higher than in the other age groups, confirming the inflamm-aging hypothesis.

The parallel analysis of the gut microbiota composition and the inflammatory parameters shows that the increase in pro-inflammatory cytokines in the peripheral blood correlates with changes in the gut microbiota profile of centenarians. In particular, the increase of IL-6 and IL-8 was linked with the enrichment in Proteobacteria, whereas it seemed to be correlated with a decrease in the amount of some butyrate producing bacteria, such as *E. rectale, E. hallii*, and *E. ventriosum*. This observation supports the hypothesis that the age-related changes of gut microbiota composition, particularly evident in centenarians, may either contribute to inflamm-aging (proliferation of opportunistic Proteobacteria to the detriment of symbionts *Firmicutes* and *Bacteroidetes)* or be

affected by the systemic inflammatory status. This hypothesis is supported by recent findings demonstrating that the inflammation shifts the balance between symbionts and pathobionts, on behalf of the seconds (Round and Mazmanian, 2009).

However, an important characteristics of centenarians is that, despite their inflammatory status, they reached the extreme limit of human lifespan and escaped or delayed the major age-related diseases which share an inflammatory pathogenesis. This brings to the hypothesis that the inflammatory status may have been counterbalanced by other physiological events, in order to help the survival of this exceptional individuals. As for the gut microbiota, we demonstrated that there is a remodelling in the composition of the Firmicutes population in centenarians, in which some bacterial groups decrease and other increase. Among the decreasing Firmicutes, we found many species known to have anti-inflammatory properties (*F. prausnitzii*, and several butyrate producers), but other bacteria with unknown anti-inflammatory properties may be among the increasing ones. For instance, the remarkable increase of *E. limosum* in centenarians may be of particular interest, opening the way to fascinating hypotheses. According to the literature, *E. limosum* may positively contribute to the health status of an aged and inflamed intestine, thanks to its anti-inflammatory properties (Kanauchi *et al*, 2006), and its ability to convert dietary isoflavonoids into other phytoestrogens, such as genistein and daidzein (Hur and Rafii, 2000; Possemiers *et al*, 2008). Phytoestrogens are being intensively studied because of their potential protective role against the development of breast, prostate and colon cancer, and coronary heart diseases (Adlercreutz, 2002). Even if it remains speculative, a positive effect of *E. limosum* on the gut health may be possible according to the literature.

5.2 The HTF-Microb.Array

5.2.1 *Design and validation of the tool*

The HTF-Microb.Array is based on the Ligation Detection Reaction – Universal Aray (LDR-UA) approach, which is a fast and sensitive tool for the characterization of complex microbial communities (Castiglioni *et al*, 2004; Hultman *et al*, 2008). The use of this molecular technique allows overcoming the major limitations of DNA microarrays whose discriminative power is based on hybridization. In fact, *i*. optimization of the hybridization conditions for each probe set is not required; *ii*. problems due to the secondary structures of the target DNA are minimized, *iii*. steric hindrances of differentially sized nucleic acid hybrids formed on the array after the hybridization are decreased (Peplies *et al*, 2003). The final probe set of the HTF-Microb.Array allows a high taxonomic level fingerprint of the human intestinal microbiota, with a good coverage of the major

and minor components, as well as some of the most important pathogens and opportunistic bacteria (Jin *et al*, 2005). The LDR probes were designed by choosing discriminating (DS) oligonucleotides whose 3'end allowed the perfect discrimination of the target species from the non-target ones on the basis of our 16S rRNA sequence database. Definition of accurate and specific negative sets of gut microbiota sequences by ORMA tool allowed the selection of maximally discriminative probe pairs. Probe specificity was confirmed on the entire known 16S rRNA gene sequences environment by the RDP Probe Match tool. This requirement is fundamental, since the primer set used for the PCR amplification was the "universal" 16S rRNA primer set designed by Edwards *et al* (1989).

The HTF-Microb.Array recognized without ambiguity the 16S rRNA amplicons obtained from 28 members of the intestinal microbiota belonging to *Bacteroides/Prevotella*, *Clostridium* clusters IV, IX, XIVa, XI, I and II, *Bifidobacteriaceae*, *Lactobacillaceae*, *Bacillus*, *Enterococcus*, *Enterobacteriaceae* and *Campylobacter*, demonstrating the specificity of all the probe pairs. The sensitivity of the HTF-Microbi.Array was evaluated by using different concentrations of an artificial mix of 16S rRNA amplicons obtained from 6 microorganisms members of the human intestinal microbiota. All PCR products were specifically recognized in a concentration ranging from 75 to 0.7 fmol, showing high array sensitivity. The efficiency of the HTF-Microbi.Array in the detection of a particular target in a complex DNA environment was also determined. According to our data, the array is able to detect a specific DNA target down to 0.02% of the total 16S rRNA, which is comparable to the values obtained by Rajilic-Stojanovic *et al.* (2009) and Palmer *et al.* (2006). Thus the HTF-Microb.Array shows the potentiality to sense low abundant species of the gastrointestinal microbiota, enabling the detection of the 16S rRNA of a peculiar target group present at a fractional abundance <0.1% in an artificial mixture.

5.2.2 *Validation of HTF-Microb.Array on human fecal samples*

The HTF-Microb.Array was used in a pilot study to characterize the faecal microbiota of eight young adults. Cluster analysis of the presence-absence probes profiles enabled the identification of a reproducible microbiota fingerprint for each subject at high taxonomic level. As expected, the intestinal microbial community of the volunteers in the study resembled the typical fingerprint of healthy adults (Rajilic-Stojanovic *et al*, 2007). According to our data, the faecal microbiota of the enrolled subjects was dominated by major mutualistic symbionts. In fact, members of *Bacteroidetes, Clostridium* clusters IV, IX and XIVa were all represented in 100% of the subjects. On the other hand, minor mutualistic symbionts, such as *Lactobacillaceae*, *Bacillus subtilis et rel.*, *Fusobacterium* and *Cyanobacteria,* were detected in 55, 37, 50, and 63 % of the subjects, respectively. Opportunistic pathogens, such as *Enterococcus faecalis et rel.,* members of the

Clostridium clusters I and II and of the *Enterobacteriaceae*, were represented only in 43, 25 and 12% of the subjects, respectively. Most importantly, enteropathogens such as, *Clostridium difficile*, *Clostridium perfringens*, *Enterococcus faecium et rel.*, *Bacillus cereus et rel.*, and *Campylobacter* were never detected. A discrepancy between our data and the literature is the relatively low prevalence of the health promoting *Bifidobacteriaceae* in our samples (only 13% of samples). However, the low prevalence of bifidobacteria is a typical bias for several phylogenetic DNA microarrays (Palmer *et al*, 2007; Rajilic-Stojanovic *et al*, 2009). Probably this is due to the intrinsic low efficiency of amplification of the bifidobacterial genome with universal primer sets for the 16S rRNA gene (Hattori and Taylor, 2009). Surprisingly, a high prevalence was obtained for the minor mutualistic symbiont *Bacillus clausii et rel.*, 100% of samples, and the opportunistic pathogen *Proteus*, 50% of samples. For each subject the relative fluorescence intensity (IF) contributions of the probes were calculated, obtaining an approximate evaluation of the relative abundance of the principal microbial groups of the faecal microbiota. In general agreement with previous metagenomic studies (Eckburg *et al*, 2005; Andersson *et al*, 2008; Peterson *et al*, 2008; Claesson *et al*, 2009; Hattori and Taylor, 2009) and SSU rRNA phylogenetic microarray investigations (Palmer *et al*, 2007; Rajilic-Stojanovic *et al*, 2009), mutualistic symbionts such as *Bacteroidetes, Clostridium* clusters IV, IX and XIVa largely dominated the faecal microbiota, contributing for the 65 to 80% of total microbiota, depending on the subject. Differently, with an overall contribution ranging from 10 to 30%, minor mutualistic symbionts such as *B. clausii et rel., Bifidobacteriaceae*, *Lactobacillaceae*, *B. subtilis et rel.*, *Fusobacterium*, and *Cyanobacteria* were largely subdominant. Opportunistic pathogens represented only a small fraction of the intestinal microbiota. Even if subjects under study showed a common trend when the ratio between the relative IF of major, minor and opportunistic components were considered, differences in the relative IF contribution of single probes were detectable and subject specific profiles were identified. For instance, subject n. 1 showed a higher relative fluorescence for probes targeting major mutualistic symbionts and a lower relative fluorescence for minor mutualistic symbionts and opportunistic pathogens than subjects n. 4 and 15. On the other hand, subjects n. 15 and 17 were characterized by a lower ratio *Bacteroidetes*/*Firmicutes* with respect to all the other subjects. Differences in relative IF contribution within samples could represent an useful approximation of differences in relative abundances of the targeted groups in the faecal microbiota, able to reflect compositional changes at high taxonomic level such as those occurring in the case of metabolic syndromes or IBD.

5.3 Comparison of the two microarray approaches

In order to compare the two diversity microarray approaches presented here (HITChip and HTF-Microb.Array), the fecal microbiota of five of the centenarians involved in the "Centenarians project" was analysed also with the HTF-Microb.Array tool. Because of the differences in the design of the two arrays, several approximation, described in details in the Results section, has been done to make the results as comparable as possible. The obtained profiles showed an overall similarity, with the exception of the underestimation of the *Clostridium* cluster XIVa and the overestimation of the Fusobacteria contribution in the HTF-Microb.Array. The underestimation of the *Clostridium* cluster XIVa may be due to the fact that in the HTF-Microb.Array this cluster is represented by only one probes, whereas in the HITChip the contribution of each cluster is calculated as the sum of all the probes hybridizing with members of that cluster. This problem may be solved by splitting the *Clostridium* cluster XIVa in several probes as it was done with the *Clostridium* cluster IV.

With the correction of these deficiencies, the HTF-Microb.Array may represent a fast tool for the detection of high taxonomic level variations in the human gut microbiota, such as those occurring as a consequence of pathologies, metabolic syndromes or antibiotic/prebiotic treatments. However, caution must be taken when microarray based methods for the relative quantification of bacterial groups in complex microbial communities are used. In fact, biases are introduced at several levels of the experimental procedure: DNA extraction and purification, PCR amplification of the 16S rRNA gene, and interspecies variation of the rRNA gene copy number (Palmer *et al*, 2006).

6.
CONCLUDING REMARKS

The study presented here is focused on the application and comparison of two different microarray approaches for the characterization of the human gut microbiota: the HITChip and the HTF-Microb.Array. The first has been developed and validated on a small group of patients by the Molecular Ecology Group, at the University of Wageningen, The Netherlands, whereas design, construction and validation of the second one are reported here.

Here we confirmed that the HITChip is a powerful and reliable tool for the gut microbiota fingerprinting, and for the relative quantification of the microbiota components at different phylogenetic levels. The statistical analysis allowed us to obtain a large number of information about the impact of the aging process on the gut microbiota composition, diversity and variability, and to venture hypothesis about the functional role of these changes.

Conversely, the validation of the HTF-Microb.Array on a group of healthy adults showed that this different microarray approach is a fast and sensitive tool for the high taxonomic level fingerprint of the human gut microbiota in terms of presence/absence of the principal groups. The evaluation of the relative abundance of the target groups on the bases of the relative fluorescence intensity probes response still has some hindrances, as demonstrated by comparing the HTF.Microb.Array and HITChip high taxonomic level fingerprints of the same centenarians. However, considering all the possible biases typical of the microarray technology (i.e. DNA extraction/purification, PCR, copy number variations), analysis of the fluorescence intensities may represent an useful approximation to estimate the relative abundance of the targets groups within each sample. Focusing the phylogenetic resolution at division, order and cluster levels, the HTF-Microb.Array showed the potential to characterize the high order taxonomic unbalances of the human gut microbiota associated with specific diseases.

The study of the impact of the aging process on the human gut microbiota structure and functionality is of great importance in the perspective of both prevention and therapy of age-related diseases. Up to now, limited researches have been focused on this topic, and the available literature is poor and scattered. In the study presented here we explored the age related changes in both gut ecosystem composition, by using one of the newest diversity microarray approaches, the HITChip, and inflammatory status. The choice to focus our analysis on three age-groups (young adults, elderly and centenarians), instead of two (young adults and elderly, usually defined as "over-65")

which is the usual approach, allowed us to provide a complete view of the age related changes in the gut microbiota composition during the whole adult life.

Our results showed a remarkable similarity between the the gut microbiota composition of young adults and elderly, suggesting that this ecosystem is even more stable than expected during most of the human life in healthy conditions. On the contrary, a deep rearrangement in the gut bacterial ecosystem occurs in centenarians: the microbiota of this exceptionally old population showed alteration in the composition of the Firmicutes, especially those *Clostridium* clusters known to comprehend bacterial species with health promoting, i.e. anti-inflammatory, properties. Moreover, the gut microbiota of centenarians is enriched in those bacteria which are known to thrive in an inflamed environment, and probably nurture it, such as several members of the Proteobacteria. These observations, and the analysis of the correlation between the gut microbiota composition and several pro-inflammatory markers, suggest that the rearrangement in the composition may have a functional meaning in relation to the inflamm-aging process. In fact, changes in the gut microbiota composition may be caused by and/or contribute to the overall inflammatory status which is typical of the old age. The results presented here also suggest that the rearrangement of the gut bacterial ecosystem in terms of relative abundance of several groups of species may be part of the complex physiological remodelling which is at the base of the longevity of these exceptional individuals.

"Since people are going to be living longer and getting older,
they'll just have to learn how to be babies longer."
Andy Warhol, (1928-1987)

Picture: Quirino Maestrello, "Reminescenze di mio padre".

7.

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8.
ACKNOWLEDGMENTS

9.
ABOUT THE AUTHOR

Elena Biagi was born in December 4th, 1982, in San Giovanni in Persiceto, Bologna, Italy. After achieving high school diploma at the Istituto Tecnico Agrario "A. Serpieri", Bologna, in 2001 with a final grade 100/100, she was admitted at the first level degree program in Biotechnology at the University of Bologna. She graduated in 2004 with a final grade 110/110 *cum laude*, with a dissertation entitled "Physiological aspect of rifaximin-resistance in a probiotic strain of *Bifidobacterium infantis*". She continued her studies in the same field, obtaining a Master Degree in Molecular and Industrial Biotechnology in 2006 with a final grade 110/110 *cum laude*. The dissertation topic was "Analysis of the intestinal microbiota composition using the newly developed diversity microarray - HITChip", and summarized the results obtained during the six month stage at the Molecular Ecology group, Laboratory of Microbiology, University of Wageningen, The Netherlands, under the supervision of Mirjana Rajilic-Stojanovic, Ph.D., and Professor Willem de Vos. In January 2007 she started her Ph.D. studies in Molecular, Cellular and Industrial Biology, at the Department of Pharmaceutical Sciences of the University of Bologna, under the supervision of Professor Patrizia Brigidi, working on the characterization of the human gut microbiota, and on the stdy of adhesive properties of probiotic strains of *Bifidobacterium*. During the three years of the PhD program she supervised three students. Moreover, she was admitted to attend the ABS-VLAG course "The Light in the Intestinal Tract Tunnel", in Helsinki, Finland (March 11-14th, 2009), and the CBM Summer School in Personalized Medicine on "Nutrition and Health", Trieste, Italy (July 15-17th, 2009).

Fellowships and Awards

June 13th, 2009. 28th National Congress SIMGBM, Spoleto, Italy. **Best Poster in Microbial Biotechnology**. Poster title: "Gut microbiota and longevity: HITChip analysis of centenarians".

March, 2006. **Fellowship for Thesis Project Abroad**, University of Bologna, 1st place.

September, 2001. **Youth Merit Award**, Rotary Club Bologna, as best high school graduate of the year at Istituto Tecnico Agrario "A. Serpieri" (High School).

Lab training

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Laboratory of Microbiology, Molecular Ecology group, Microbiology Department, Wageningen University, Wageningen, The Netherlands

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March – September, 2006

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> › Thesis Project: "Analysis of the intestinal microbiota composition using the newly developed diversity microarray - HITChip"
> › Supervisors: Professor Willem de Vos; Mirjana Rajilič-Stojanovič, MSc; Hauke Smidt, PhD.

July, 2005 – February, 2006

Laboratory of Microbial Biotechnology, Department of Pharmaceutical Sciences, University of Bologna, Bologna, Italy

> › Research interests: "Characterization of women vaginal microflora in healthy and non-healthy condition, by DGGE and Real Time analyses"
> › Tutor: Prof. Patrizia Brigidi

April – October, 2004

Laboratory of Microbial Biotechnology, Department of Pharmaceutical Sciences, University of Bologna, Bologna, Italy

> › Thesis Project: "Genetic and physiological aspect of rifaximin-resistance in a probiotic strain of *Bifidobacterium infantis*"
> › Tutor: Prof. Patrizia Brigidi; Supervisor: Dr. Beatrice Vitali, PhD.

Pubblications

Biagi E, Nylund L, Candela M, Bucci L, Ostan R, Pini E, Nikkila J, Monti D, Satokari R, Franceschi C, Brigidi P, and De Vos WM. Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. Submitted to *PloS ONE*.

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Vitali B, Pugliese C, **Biagi E**, Candela M, Turroni S, Bellen G, Donders GGG, and Brigidi P. **2007**. Dynamics of vaginal bacterial communities in women dveloping bacterial vaginosis, candidiasis or no infection analysed by PCR-denaturing gradient gel electrophoresis and real-time PCR. *Appl Environ Microbiol* 73, 5731-5741

Posters

Biagi E, Cruciani F, Bucci L, Franceschi C, de Vos WM, and Brigidi P. Gut microbiota and Longevity: HITChip analysis of centenarians. 28th National Congress SIMGBM, Spoleto, PG, Italy. June 11-13th, 2009.

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APPENDICES

Probes	Sub-probes	Probe number	Targeted bacteria
Bacteroides/Prevotella		16	*Bacteroides acidofaciens*
			Bacteroides barnesiae
			Bacteroides caccae
			Bacteroides coprocola
			Bacteroides dorei
			Bacteroides eggerthii
			Bacteroides finegoldii
			Bacteroides fragilis
			Bacteroides helcogenes
			Bacteroides intestinalis
			Bacteroides massiliensis
			Bacteroides nordii
			Bacteroides ovatus
			Bacteroides plebeius
			Bacteroides splanchnicus
			Bacteroides stercoris
			Bacteroides thetaiotaomicron
			Bacteroides uniformis
			Bacteroides vulgatus
			Prevotella albensis
			Prevotella bivia
			Prevotella brevis
			Prevotella bryantii
			Prevotella buccae
			Prevotella buccalis
			Prevotella corporis
			Prevotella dentalis
			Prevotella denticola
			Prevotella disiens
			Prevotella intermedia
			Prevotella loescheii
			Prevotella melaninogenica
			Prevotella nigrescens
			Prevotella oralis
			Prevotella oris
			Prevotella oulorum
			Prevotella pallens
			Prevotella ruminicola
			Prevotella salivae
			Prevotella shahii
			Prevotella tannerae
			Prevotella veroralis
Clostridium cluster IV	*Ruminococcus bromii et rel.*	38	*Clostridium leptum*
			Ruminococcus bromii
	Ruminococcus albus et rel.	39	*Ruminococcus albus*
			Ruminococcus callidus

			Ruminococcus flavefaciens
	Oscillospira guillermondii et rel.	40	*Clostridium orbidescens*
			Clostridium viride
			Oscillospira guillermondii
			Papillibacter cinnaminovorans
			Termitobacter aceticus
	Faecalibacterium prausnitzii et rel.	41	*Acetanaerobacterium elongatum*
			Anaerofilum agile
			Anaerofilum pentosovorans
			Clostridium cellulosi
			Clostridium methylpentosum
			Ethanologenbacterium harbin
			Eubacterium desmolans
			Eubacterium siraeum
			Faecalibacterium prausnitzii
			Linmingia china
Clostridium cluster IX		37	*Acetonema longum*
			Acidaminococcus fermentans
			Allisonella histaminiformis
			Anaeroarcus burkinensis
			Anaeroglobus geminatus
			Anaeromusa acidaminophila
			Anaerosinus glycerini
			Anaerospora hongkongensis
			Anaerovibrio lipolytica
			Anaerovibrio lipolyticus
			Centipeda periodontii
			Dendrosporobacter quercicolus
			Dialister invisus
			Dialister pneumosintes
			Megamonas hypermegale
			Megasphaera cerevisiae
			Megasphaera elsdenii
			Megasphaera micrinuciformis
			Mitsuokella jalaludinii
			Mitsuokella multiacidus
			Pectinatus cerevisiiphilus
			Pectinatus frisingensis
			Pectinatus portalensis
			Phascolarctobacterium faecium
			Propionispira arboris
			Propionispora hippei
			Propionispora vibrioides
			Schwartzia succinivorans
			Selenamonas flueggei
			Selenomonas dianae
			Selenomonas infelix
			Selenomonas lacticifex
			Selenomonas noxia
			Selenomonas ruminantium
			Selenomonas sputigena

			Sporomusa acidovorans
			Sporomusa aerivorans
			Sporomusa malonica
			Sporomusa ovata
			Sporomusa paucivorans
			Sporomusa silvacetica
			Sporomusa sphaeroides
			Sporomusa termitida
			Succiniclasticum ruminis
			Succinispira mobilis
			Zymophilus paucivorans
	Veillonella	20	*Veillonella atypica*
			Veillonella caviae
			Veillonella criceti
			Veillonella dentocariosa
			Veillonella dispar
			Veillonella monpellierensis
			Veillonella parvula
			Veillonella ratti
			Veillonella rodentium
Clostridium cluster XIVa		22	*Acetitomaculum ruminis*
			Anaerostipes caccae
			Bryantella formatexygens
			Butyrivibrio crossotus
			Butyrivibrio fibrisolvens
			Butyrivibrio hungatei
			Clostridium algidixylanolyticum
			Clostridium aminophilum
			Clostridium aminovalericum
			Clostridium amygdalinum
			Clostridium bolteae
			Clostridium celerecrescens
			Clostridium clostridiiforme
			Clostridium coccoides
			Clostridium fimetarium
			Clostridium fusiformis
			Clostridium hathewayi
			Clostridium herbivorans
			Clostridium horoticum
			Clostridium hylemonae
			Clostridium indolis
			Clostridium methoxybenzovorans
			Clostridium nexile
			Clostridium phytofermentas
			Clostridium polysaccharolyticum
			Clostridium populeti
			Clostridium proteoclasticum
			Clostridium saccharolyticum
			Clostridium scindens
			Clostridium sphenoides

	A5		*Clostridium symbiosum*
			Clostridium xylanolyticum
			Clostridium xylanovorans
			Desulfomaculum guttoideum
			Dorea formicigerans
			Eubacterium cellulosolvens
			Eubacterium contortum
			Eubacterium eligens
			Eubacterium hallii
			Eubacterium oxidoreducens
			Eubacterium pectinii
			Eubacterium plexicaudatum
			Eubacterium ramulus
			Eubacterium ruminantium
			Eubacterium uniforme
			Eubacterium ventrosum
			Eubacterium xylanophilum
			Hespellia porcina
			Hespellia stercorisuis
			Johnsonella ignava
			Lachnobacterium bovis
			Lachnospira multiparus (multipara)
			Lachnospira pectinoschiza
			Parasporobacterium paucivorans
			Pseudobutyrivibrio ruminis
			Pseudobutyrivibrio xylanovorans
			Roseoburia cecicola
			Roseoburia intestinalis
			Ruminococcus gnavus
			Ruminococcus lactaris
			Ruminococcus torques
			Ruminococcus hansenii
			Ruminococcus hydrogenotrophicus
			Ruminococcus obeum
			Ruminococcus productus
			Ruminococcus schinkii
			Shuttleworthia satelles
			Sporobacterium olearium
			Syntrophococcus sucromutans
	Eubacterium rectale	19	*Eubacterium rectale*
Bifidobacteriaceae		25B	*Bifidobacterium aerophilum*
			Bifidobacterium adolescentis
			Bifidobacterium angolatum
			Bifidobacterium animalis
			Bifidobacterium asteroides
			Bifidobacterium bifidum
			Bifidobacterium boum
			Bifidobacterium breve
			Bifidobacterium catenolatum
			Bifidobacterium choerinum
			Bifidobacterium coryneforme

			Bifidobacterium cuniculi
			Bifidobacterium dentium
			Bifidobacterium gallicum
			Bifidobacterium gallinarum
			Bifidobacterium indicum
			Bifidobacterium magnum
			Bifidobacterium merycicum
			Bifidobacterium minimum
			Bifidobacterium pseudocatenolatum
			Bifidobacterium psychroaerophylum
			Bifidobacterium pullorum
			Bifidobacterium ruminantium
			Bifidobacterium saeculare
			Bifidobacterium scardovii
			Bifidobacterium simiae
			Bifidobacterium subtile
			Bifidobacterium thermoacidophilum
			Bifidobacterium thermophilum
			Bifidobacterium urinalis
			Gardnerella vaginalis
			Metascardovia tsurumii
			Parascardovia denticolens
			Scardovia inopinata
	Bifidobacterium longum et rel.	3	*Bifidobacterium longum biovar infantis*
			Bifidobacterium longum biovar longum
			Bifidobacterium longum biovar suis
			Bifidobacterium pseudolongum
Lactobacillaceae		21B	*Lactobacillus acidifarinae*
			Lactobacillus acetotolerans
			Lactobacillus acidophilus
			Lactobacillus amylolyticus
			Lactobacillus amylophilus
			Lactobacillus amylotrophus
			Lactobacillus amylovorus
			Lactobacillus antrumi
			Lactobacillus coleohominis
			Lactobacillus crispatus
			Lactobacillus delbrueckii
			Lactobacillus fermentum
			Lactobacillus fornicalis
			Lactobacillus frumenti
			Lactobacillus gallinarum
			Lactobacillus gasseri
			Lactobacillus gastricus
			Lactobacillus gastricus
			Lactobacillus hamsteri
			Lactobacillus helveticus
			Lactobacillus iners
			Lactobacillus ingluviei

Lactobacillus intestinalis
Lactobacillus jensenii
Lactobacillus johnosii
Lactobacillus kalixi
Lactobacillus kefiranofaciens
Lactobacillus kefirgranum
Lactobacillus kitasatonis
Lactobacillus kitasoi
Lactobacillus mucosae
Lactobacillus oris
Lactobacillus panis
Lactobacillus pontis
Lactobacillus psittaci
Lactobacillus reuteri
Lactobacillus secaliphilus
Lactobacillus sobrius
Lactobacillus suntoryeus
Lactobacillus thermotolerans
Lactobacillus ultunense
Lactobacillus vaginalis
Lactobacillus brevis
Lactobacillus buchneri
Lactobacillus faeni
Lactobacillus ferintoshensis
Lactobacillus ferraginis
Lactobacillus fructivorans
Lactobacillus hammesii
Lactobacillus hilgardii
Lactobacillus homohiochii
Lactobacillus kefiri
Lactobacillus lindneri
Lactobacillus namurensis
Lactobacillus parabrevis
Lactobacillus parabuchneri
Lactobacillus paraferraginis
Lactobacillus parakefiri
Lactobacillus sanfranciscensis
Lactobacillus spicheri
Lactobacillus vermiforme
Lactobacillus zymae
Lactobacillus backi
Lactobacillus bifermentas
Lactobacillus composti
Lactobacillus coryniformis
Lactobacillus curvatus
Lactobacillus durianis
Lactobacillus fuchuensis
Lactobacillus graminis
Lactobacillus harbinensis
Lactobacillus malfermentas
Lactobacillus oligofermentans

			Lactobacillus perolens
			Lactobacillus rossii
			Lactobacillus sakei
			Lactobacillus siligionis
			Lactobacillus suebicus
			Lactobacillus vaccinostercus
			Pediococcus acidilactici
			Pediococcus damnosum
			Pediococcus parvulus
			Pediococcus pentosaceus
	Lactobacillus plantarm et rel.	33	*Lactobacillus arizonensis*
			Lactobacillus collinoides
			Lactobacillus crustorum
			Lactobacillus farciminis
			Lactobacillus kimchii
			Lactobacillus letivazi
			Lactobacillus mindensis
			Lactobacillus paracollinoides
			Lactobacillus paralimentarius
			Lactobacillus paraplantarum
			Lactobacillus plantarum
			Lactobacillus versmoldensis
	Lactobacillus casei et rel.	12	*Lactobacillus casei*
			Lactobacillus manihotivorans
			Lactobacillus pantheris
			Lactobacillus paracasei
			Lactobacillus rhamnosus
			Lactobacillus sharpeae
			Lactobacillus zeae
	Lactobacillus salivarius et rel.	14	*Lactobacillus agilis*
			Lactobacillus algidus
			Lactobacillus animalis
			Lactobacillus aviarius
			Lactobacillus cypricasei
			Lactobacillus equi
			Lactobacillus mali
			Lactobacillus mobilis
			Lactobacillus murinus
			Lactobacillus nageli
			Lactobacillus ruminis
			Lactobacillus saerimmeri
			Lactobacillus salivarius
Bacillus clausii et rel.		32	*Bacillus clausii*
			Bacillus gibsonii
			Bacillus halodurans
			Bacillus horikoshii
			Bacillus okuhidensis
			Bacillus racemilacticus
			Sporolactobacillus inulinus
			Sporolactobacillus laevis
			Sporolactobacillus laevolactis

		Sporolactobacillus myxolacticus
		Sporolactobacillus racemicus
		Sporolactobacillus racemilacticus
Bacillus subtilis et rel.	8	*Bacillus amyloliquefaciens*
		Bacillus atrophaeus
		Bacillus licheniformis
		Bacillus mojavensis
		Bacillus pumilus
		Bacillus sonorensis
		Bacillus subtilis
		Bacillus vallismortis
Fusobacterium	15	*Fusobacterium canifelinum*
		Fusobacterium equinum
		Fusobacterium gonidiaformans
		Fusobacterium mortiferum
		Fusobacterium naviforme
		Fusobacterium necrogenes
		Fusobacterium necrophorum
		Fusobacterium nucleatum
		Fusobacterium periodonticum
		Fusobacterium russii
		Fusobacterium simiae
		Fusobacterium ulcerans
		Fusobacterium varium
Cyanobacteria	42	*UNICYANO probe (Castiglioni B et al., 2004)*
Clostridium cluster XI	36	*Acidaminobacter hydrogenoformans*
		Alcaliphilus transvaalensis
		Alkaliphilus metalliredigenes
		Anaerococcus hydrogenalis
		Anaerococcus lactolyticus
		Anaerococcus octavius
		Anaerococcus prevotii
		Anaerococcus tetradius
		Anaerococcus vaginalis
		Anaerovorax odorimutans
		Anoxynatronum sibericum
		Caldanaerocella colombiensis
		Caminicella sporogenes
		Clostridium aceticum
		Clostridium alcalibutyricum
		Clostridium alcaliphilum
		Clostridium aminobutyricum
		Clostridium aminovorans
		Clostridium bartlettii
		Clostridium bifermentans
		Clostridium elmenteitii
		Clostridium filamentosum
		Clostridium firmicoaceticum
		Clostridium ghoni
		Clostridium glycolicum

Clostridium halophilum
Clostridium hastiforme
Clostridium hiranonis
Clostridium irregularis
Clostridium litorale
Clostridium litoseburense
Clostridium mangenotii
Clostridium mayombei
Clostridium paradoxum
Clostridium purinolyticum
Clostridium sordelli
Clostridium sticklandii
Clostridium subatlanticum
Clostridium thermoalcaliphilum
Clostridium ultunense
Eubacterium angustum
Eubacterium brachy
Eubacterium infirmum
Eubacterium minutum
Eubacterium nodatum
Eubacterium pyruvativorans
Eubacterium saphenum
Eubacterium sulci
Eubacterium tenue
Eubacterium yurii
Filifactor alocis
Filifactor villosus
Finegoldia magna
Frigovirgula patogoniensis
Fusibacter paucivorans
Gallicola barnesae
Guggenheimia bovis
Helcoccus kunzii
Micromonas micros
Natronincola histidinovorans
Peptoniphilus asaccharolyticus
Peptoniphilus indolicus
Peptoniphilus lacrimalis
Peptostreptococcus anaerobius
Peptostreptococcus hareii
Peptostreptococcus ivoricus
Peptostreptococcus micros
Pseudoramibacter alactolyticus
Soehngenia saccharolytica
Sporoanaerobacter acetigenes
Tepidibacter formicigenes
Tepidibacter thalassicus
Tindallia californiensis
Tindallia magadii
Tissierella praeacuta

Clostridium difficile	18	*Clostridium difficile*

Clostridium clusters I and II	35	*Anaerobacter polyendosporus*
		Clostridium absonum
		Clostridium acetireducens
		Clostridium acetobutylicum
		Clostridium acidisoli
		Clostridium akagii
		Clostridium algidicarnis
		Clostridium argentinense
		Clostridium aurantibutyricum
		Clostridium autoethanogenum
		Clostridium barati
		Clostridium beijerinckii
		Clostridium botulinum
		Clostridium bowmanii
		Clostridium butyricum
		Clostridium cadaveris
		Clostridium caliptrosporum
		Clostridium carboxidivorans
		Clostridium carnis
		Clostridium celatum
		Clostridium cellulovorans
		Clostridium chartatabidum
		Clostridium chauvoei
		Clostridium chromoreductans
		Clostridium cochlearium
		Clostridium colicanis
		Clostridium collagenovorans
		Clostridium corinoforum
		Clostridium diolis
		Clostridium disporicum
		Clostridium estertheticum
		Clostridium fallax
		Clostridium favososporum
		Clostridium felsineum
		Clostridium fragidicarnis
		Clostridium frigoris
		Clostridium gasigenes
		Clostridium grantii
		Clostridium haemolyticum
		Clostridium histolyticum
		Clostridium homopropionicum
		Clostridium intestinalis
		Clostridium kainantoi
		Clostridium kluyveri
		Clostridium lacusfrykellense
		Clostridium laramiense
		Clostridium limosum
		Clostridium ljungdahlii
		Clostridium longisporum
		Clostridium magnum
		Clostridium maleniminatum

			Clostridium neonatale
			Clostridium novyi
			Clostridium oceanicum
			Clostridium paraputrificum
			Clostridium pasqui
			Clostridium pasteurianum
			Clostridium peptidovorans
			Clostridium proteolyticum
			Clostridium proteolyticus
			Clostridium puniceum
			Clostridium putrefaciens
			Clostridium putrificum
			Clostridium quinii
			Clostridium ragsdalei
			Clostridium roseum
			Clostridium saccharobutylicum
			Clostridium saccharoperbutylacetonicum
			Clostridium sardiniensis
			Clostridium sartagoformum
			Clostridium scatologenes
			Clostridium septicum
			Clostridium sporogenes
			Clostridium subterminale
			Clostridium tertium
			Clostridium tetani
			Clostridium tetanomorphum
			Clostridium thermopalmarium
			Clostridium thiosulfatireducens
			Clostridium tunisiense
			Clostridium tyrobutyricum
			Clostridium uliginosum
			Clostridium vincentii
			Eubacterium budayi
			Eubacterium combesii
			Eubacterium moniliforme
			Eubacterium multiforme
			Eubacterium nitritogenes
			Eubacterium tarantellus
			Sarcina maxima
			Sarcina ventriculi
	Clostridium perfringens	17	*Clostridium perfringens*
Enterococcus faecalis et rel.		9	*Enterococcus caccae*
			Enterococcus faecalis
			Enterococcus haemoperoxidus
			Enterococcus moraviensis
			Enterococcus rottae
			Enterococcus silesiacus
Enterococcus faecium et rel.		10	*Enterococcus azikeevi*
			Enterococcus canis
			Enterococcus durans

		Enterococcus faecium
		Enterococcus hirae
	A13	*Enterococcus mundtii*
		Enterococcus porcinus
		Enterococcus pseudoavium
		Enterococcus ratti
		Enterococcus sanguinicola
		Enterococcus villorum
Bacillus cereus et rel.	7	*Bacillus anthracis*
		Bacillus cereus
		Bacillus mycoides
		Bacillus pseudomycoides
		Bacillus thuringensis
		Bacillus weihenstephanenis
Enterobacteriaceae	23B	*Citrobacter amalonaticus*
		Citrobacter farmeri
		Citrobacter koseri
		Citrobacter rodentium
		Citrobacter sedlakii
		Enterobacter cowanii
		Escherichia albertii
		Escherichia coli
		Escherichia fergusonii
		Escherichia senegalensis
		Escherichia vulneri
		Hafnia alvei
		Photorabdus luminescens
		Roultella planticola
		Salmonella agona
		Salmonella blockley
		Salmonella bongori
		Salmonella bovis
		Salmonella chingola
		Salmonella dublin
		Salmonella enterica
		Salmonella enteritidis
		Salmonella give
		Salmonella houten
		Salmonella matopeni
		Salmonella paratyphi
		Salmonella shomron
		Salmonella typhi
		Salmonella typhimurium
		Salmonella waycross
		Salmonella weltevreden
		Shigella boydii
		Shigella dysenteriae
		Shigella flexneri
		Shigella sonnei
Yersinia enterocolitica et rel.	4	*Yersinia aldovae*
		Yersinia aleksiciae

		Yersinia bercovieri
		Yersinia enterocolitica
		Yersinia frederiksenii
		Yersinia intermedia
		Yersinia kristensenii
		Yersinia pestis
		Yersinia pseudotubercolosis
		Yersinia massiliensis
		Yersinia mollaretii
		Yersinia rohdei
		Yersinia ruckerii
Proteus	5	*Proteus hauseri*
		Proteus mirabilis
		Proteus myxofaciens
		Proteus penneri
		Proteus vulgaris
Campylobacter	6	*Campylobacter coli*
		Campylobacter concisus
		Campylobacter curvus
		Campylobacter faecalis
		Campylobacter fetus subsp fetus
		Campylobacter fetus subsp venerealis
		Campylobacter gracilis
		Campylobacter helveticus
		Campylobacter hominis
		Campylobacter hyoilei
		Campylobacter hyointestinalis
		Campylobacter insulaenigrae
		Campylobacter jejuni
		Campylobacter lanienae
		Campylobacter lari
		Campylobacter mucosalis
		Campylobacter rectus
		Campylobacter showae
		Campylobacter sporotum
		Campylobacter upsaliensis

Appendix 2. _HTF-Microbi.Array probe list._ Table of the 30 designed probe pairs.
Sequences (5' -> 3') for both DS and CP are reported, as well as major thermodynamic parameters
(melting temperature, length, number of degenerated bases)

Table A2.1. Sequences (5' -> 3') for each discriminating probes (DS). In bold, the discriminating
nucleotide.

Zip code	Probe Name	Discriminating oligo (DS)
3	*Bifidobacterium longum et rel.*	GTATGGGATGGGGTCGCGTCCTATCAGCTTGA**C**
4	*Yersinia enterocolitica et rel.*	GAGTAGGGCTACACACGTGCTACAATGGCAGATACAAAG**T**
5	*Proteus et rel.*	GGTCTTGAACCGTGGCTTCTGGAGCTAACGCGTTAA**A**
6	*Campylobacter*	GCTAGTTGGTRAGGTAATGGCTTACCAAGGCTATGACGCWTA**A**
7	*Bacillus cereus et rel.*	ACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAG**C**
8	*Bacillus subtilis et rel.*	GCAGCGAAACCGCGAGGTTAAGCCAATCCCA**C**
9	*Enterococcus faecalis et rel.*	GGAAGTACAACGAGTCGCTAGACCGCGAGGTCA**T**
10	*Enterococcus faecium et rel.*	CGCTTCTTTTTCCACCGGAGCTTGCTCCACC**G**
12	*Lactobacillus casei et rel.*	GGGTCGTAAAACTCTGTTGTTGGAGAAGAATGGTCGG**C**
14	*Lactobacillus salivarius et rel.*	GTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAA**A**
15	*Fusobacterium*	GGGGAAGCCAGCYTACTGGACAGATACTGACGCTRA**A**
16	*Bacteroides/Prevotella*	CATTAAGYATYCCACCTGGGGAGTACGCCGGCAA**C**
17	*Clostridium perfringens et rel.*	CTACACTTGACATCCCTTGCATTACTCTTAATCGAGGAA**A**
18	*Clostridium difficile et rel.*	GAACGCTGGCGGCGTGCCTAACACATGCAAGT**T**
19	*Eubacterium rectale et rel.*	CATTGCTTCTCGGTGCCGTCGCAAACGCA**G**
20	*Veillonella*	GGTGGGAACTCATGAGAGACTGCCGCAGACAA**T**
25B	*Bifidobacteriaceae*	TAGGGGAGACTGGAATTCCCGGTGTAACGGTGGAATG**T**
23B	*Enterobacteriaceae*	GGGACCTTCGGGCCTCTTGCCATCGGATG**T**
21B	*Lactobacillaceae*	AAGAACACCAGTGGCGAAGGCGGCTSTCTGG**T**
22	*Clostridium* cluster XIVa	CCGCGTGAGYGAAGAAGTATTTCGGTATGTAAAGCTCT**A**
32	*Bacillus clausii et rel.*	CCTAGAGATAGGGCTTTCCCCTTCGGGGGACAA**A**
33	*Lactobacillus plantarum et rel.*	CTACAATGGATGGTACAACGAGTTGCGAACTCGCGAGA**A**
35	*Clostridium* cluster I and II	GCGTAAAGGGWGCGTAGGYGGATNTTTAAGTGRGATGTGAAATA**A**

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36	*Clostridium* cluster XI	CGATGAAGGCCTTCGGGTCGTAAAGCTCTGTC**C**
37	*Clostridium* cluster IX	GAGCGAACGGGATTAGATACCCCGGTAGTCCT**G**
38	Cl IV: *Ruminococcus bromii et rel.*	GAACCTTACCAGGTCTTGACATCCAACTAACGAAGTAGAGATRC**A**
39	Cl IV: *Ruminococcus albus et rel.*	GAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAACA**T**
40	Cl IV: *Faecalibacterium prausnizii et rel.*	GTAAAGGGAGCGCAGGCGGGANGGCAAGT**T**
41	Cl IV: *Oscillospira guillermondii et rel.*	GGCYTTCGGGTTGTAAACTTCTTTTAAGGGGGAAGARCAGA**A**
42	*Cyanobacteria*	CCCAGACTCCTACGGGAGGCAGCAGT**G**

Table A2.2. Sequences (5' -> 3') for each common probes (CP). In bold, the discriminating nucleotide.

Zip code	Probe Name	Common probe (CP)
3	*Bifidobacterium longum et rel.*	GGCGGGGTAACGGCCNACCGTGGCT
4	*Yersinia enterocolitica et rel.*	GAAGCGAACTCGCGAGAGCAAGCGGACC
5	*Proteus et rel.*	TCGACCGCCTGGGGAGTACGGCCGC
6	*Campylobacter*	CTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAG
7	*Bacillus cereus et rel.*	TGGCACCTTGACGGTACCTAACCAGAAAGCCACGG
8	*Bacillus subtilis et rel.*	AAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGA
9	*Enterococcus faecalis et rel.*	GCAAATCTCTTAAAGCTTCTCTCAGTTCGGATTGCAGGCTG
10	*Enterococcus faecium et rel.*	GAAAAAGARGAGTGGCGAACGGGTGAGTAACACGTGG
12	*Lactobacillus casei et rel.*	AGAGTAACTGTTGTCGGCGTGACGGTATCCAACCAG
14	*Lactobacillus salivarius et rel.*	GCGGCTCTCTGGTCTGTAACTGACGCTGAGG
15	*Fusobacterium*	GCGCGAAAGCGTGGGTAGCAAACAGGATTAGATACC
16	*Bacteroides/Prevotella*	GGTGAAACTCAAAGGAATTGACGGGGGCCCGC
17	*Clostridium perfringens et rel.*	TCCCTTCGGGGACAAGGTGACAGGTGGTGCAT
18	*Clostridium difficile et rel.*	GAGCGATTTACTTCGGTAAAGAGCGGCGGACGG
19	*Eubacterium rectale et rel.*	TAAGTATTCCACCTGGGGAGTACGTTCGCAAGAATGAAACTC
20	*Veillonella*	GCGGAGGAAGGCGGGGATGACGTCAAATC
25B	*Bifidobacteriaceae*	GTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCT

23B	*Enterobacteriaceae*	GCCCAGATGGGATTAGCTWGTWGGTGGGGTAACG
21B	*Lactobacillaceae*	CTGTAACTGACGCTGAGGCTCGAAAGCATGGGTAGC
22	*Clostridium* cluster XIVa	TCAGCAGGGAAGAWAATGACGGTACCTGACTAAGAAGCNC
32	*Bacillus clausii et rel.*	AGTGACAGGTGGTGCATGGTTGTCGTCAGCTCG
33	*Lactobacillus plantarum et rel.*	GTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTG
35	*Clostridium* cluster I and II	CCCGGGCTYAACYTGGGTGCTGCATTYCAAAC
36	*Clostridium* cluster XI	TAARGGAAGAWAATGACGGTACYTTAGGAGGAAGCCCCG
37	*Clostridium* cluster IX	GCCGTAAACGATGGRTACTAGGTGTAGGAGGTATCG
38	Cl IV: *Ruminococcus bromii et rel.*	TTAGGTGCCCTTCGGGGAAAGKTGAGACAGGTG
39	Cl IV: *Ruminococcus albus et rel.*	CAGTGGCGAAGGCGGCTTACTGGGCTTTAACTG
40	Cl IV: *Faecalibacterium prausnizii et rel.*	GGAAGTGAAATCTATGGGCTCAACCCATGAACTGCTTTCAAAAC
41	Cl IV: *Oscillospira guillermondii et rel.*	GACGGTACCCCTTGAATAAGCCACGGCTAACTACG
42	*Cyanobacteria*	GGGAATTTTCCGCAATGGGCGAAAGCCTGACGG

Table A2.3. Major thermodynamic parameters of each probe pair (DS and CP): length, melting temperature (Tm), number of degenerated bases (Deg).

Zip code	Probe Name	DS Length (nt)	CP Length (nt)	Tm DS	Tm CP	Deg DS	Deg CP
3	*Bifidobacterium longum et rel.*	33	25	68.1	68.3	0	1
4	*Yersinia enterocolitica et rel.*	40	28	67.6	67.2	0	0
5	*Proteus et rel.*	37	25	67.8	69.2	0	0
6	*Campylobacter*	43	37	67.9	67.8	2	0
7	*Bacillus cereus et rel.*	40	35	64.5	69.1	0	0
8	*Bacillus subtilis et rel.*	32	38	68.2	66.6	0	0
9	*Enterococcus faecalis et rel.*	34	41	68	67.5	0	0
10	*Enterococcus faecium et rel.*	32	37	68.2	68.3	0	1
12	*Lactobacillus casei et rel.*	38	36	67.7	67.9	0	0
14	*Lactobacillus salivarius et rel.*	40	31	65.5	68.3	0	0
15	*Fusobacterium*	37	36	68.9	67.9	2	0

16	*Bacteroides/Prevotella*	35	32	69.1	68.2	2	0
17	*Clostridium perfringens et rel.*	40	32	64.5	68.2	0	0
18	*Clostridium difficile et rel.*	33	33	68.1	68.1	0	0
19	*Eubacterium rectale et rel.*	30	42	67.1	67.4	0	0
20	*Veillonella*	34	29	66.9	67.2	0	0
25B	*Bifidobacteriaceae*	38	39	68.8	68.7	0	0
23B	*Enterobacteriaceae*	30	34	68.5	68	0	2
21B	*Lactobacillaceae*	32	36	68.2	69	1	0
22	*Clostridium* cluster XIVa	39	40	66.1	68.1	1	2
32	*Bacillus clausii et rel.*	33	33	68.1	68.1	0	0
33	*Lactobacillus plantarum et rel.*	38	45	67.7	67.3	0	0
35	*Clostridium* cluster I and II	44	32	67.8	67.6	4	3
36	*Clostridium* cluster XI	33	39	68.1	67.6	0	3
37	*Clostridium* cluster IX	33	36	68.1	67.3	0	1
38	Cl IV: *Ruminococcus bromii et rel.*	45	33	67.7	67.5	1	1
39	Cl IV: *Ruminococcus albus et rel.*	46	33	67.2	68.1	0	0
40	Cl IV: *Faecalibacterium prausnizii et rel.*	30	44	67.8	67.3	1	0
41	Cl IV: *Oscillospira guillermondii et rel.*	42	35	67.4	67.9	2	0
42	*Cyanobacteria*	27	33	67.3	68.1	0	0

Appendix 3. *Results of the probe specificity tests.*

Twenty-eight bacterial DNA targets were chosen to validate the probe pairs. For each DNA analyzed are reported: probe pair showing significant positive signals, SNRs, SNRns (see main text for acronym definitions). Results are reported for each duplicate experiment. Where needed (i.e. more than one probe pair was present), data are the average of the positive signals (for both SNR and P values)

Test	*Positive signal*	*SNR ns*	*SNR s*	*P values specificity*
Bacteroides fragilis ATCC25285	*Bacteroides/Prevotella*	0.85	30.81	9.35E-05
		0.53	21.45	7.39E-04
Bacteroides thetaiotaomicrom ATCC29143	*Bacteroides/Prevotella*	0.45	61.44	2.56E-04
		1.66	347.24	9.10E-06
Lactobacillus gasseri DSM20243	*Lactobacillaceae*	0.3	5.58	4.98E-03
		1.56	20.59	6.58E-03
Prevotella melaninogenica ATCC25845	*Bacteroides/Prevotella*	1.54	480.24	6.02E-08
		0.9	266.63	3.74E-09
Bacillus subtilis DSM704	*Bacillus subtilis et rel.*	7.93	637.39	1.56E-09
		5.62	350.1	1.47E-05
Escherichia coli ATCC11105	*Enterobacteriaceae*	3.27	555.04	8.65E-08
		2.59	222.39	4.50E-07
Proteus mirabilis DSM4479	*Proteus et rel.,* *Enterobacteriaceae*	2.42	703.22	7.74E-09
		2.03	497.1	1.97E-09
Bifidobacterium bifidum DSM20456	*Bifidobacteriaceae*	2.67	289.39	4.78E-11
		2.23	407.1	2.40E-08
Lactobacillus casei DSM20011	*Lactobacillaceae,* *Lactobacillus casei et rel.*	2.59	125.13	1.01E-04
		2.26	134.78	5.92E-04
Yersinia enterocolitica	*Yersinia enterocolitica et rel., Enterobacteriaceae*	1.53	231.33	1.01E-05
		2.89	340.2	1.61E-06
Bacillus cereus DSM31	*Bacillus cereus et rel.*	2.83	193.85	1.53E-06
		2.49	196.82	4.16E-03
Bifidobacterium adolescentis ATCC15703	*Bifidobacteriaceae*	4.1	732.95	3.95E-10
		2.9	338.59	5.59E-07
Lactobacillus ramnosus DSM20021	*Lactobacillaceae,* *Lactobacillus casei et rel.*	2.4	101.76	1.41E-03
		4.23	177.7	4.62E-07
Lactobacillus delbrueckii DSM20074	*Lactobacillaceae*	3.77	210.11	2.24E-08
		3.1	121.93	6.27E-08
Lactobacillus pentosus DSM20314	*Lactobacillaceae*	3.05	131.65	4.58E-09
		1.63	58.3	5.32E-07

Lactobacillus acidophilus DSM20079	*Lactobacillaceae*	2.39	68.49	8.70E-05
		2.66	78.5	5.88E-06
Lactobacillus reuteri DSM20016	*Lactobacillaceae*	3.17	150.57	4.66E-09
		1.74	83.6	1.98E-07
Lactobacillus plantarum DSM21074	*Lactobacillus plantarum et rel., Lactobacillaceae*	2.12	197.32	3.79E-09
		2.09	148.35	2.77E-08
Clostridium difficile ATCCBAA1382	*Clostridium* cluster XI, *Clostridium difficile et rel.*	1.12	238.87	4.88E-04
		0.8	126.38	1.96E-03
Campylobacter jejuni ATCC33292	*Campylobacter*	0.7	19.89	5.29E-03
		0.91	28.44	5.69E-03
Veillonella parvula ATCC10790	*Veillonella,* *Clostridium* cluster IX	1.12	205.66	1.57E-04
		0.99	140.95	1.39E-04
Bifidobacterium breve DSM20091	*Bifidobacteriaceae*	2.22	570.01	6.22E-05
		1.69	289.07	2.72E-04
Bifidobacterium longum ATCC15707	*Bifidobacterium longum et rel., Bifidobacteriaceae*	1.76	341.94	1.64E-03
		0.66	134.86	4.26E-02
Ruminococcus productus ATCC 23340	*Clostridium* cluster XIVa	0.64	4.21	1.41E-03
		1.06	17.16	1.24E-06
Lactobacillus salivarius SV2	*Lactobacillus salivarius et rel., Lactobacillaceae*	0.89	12.23	4.34E-04
		0.65	7.27	2.69E-05
Enterococcus faecalis ATCC700802	*Enterococcus faecalis et rel.*	3.12	306.51	1.09E-03
		2.27	217.16	6.56E-03
Clostridium leptum DSM73	*Ruminocuccos bromii et rel., Clostridium* cluster IV	2.28	88.89	5.52E-07
		1.13	39.86	2.00E-07
Ruminococcus albus DSM20455	*Ruminocuccos albus et rel., Clostridium* cluster IV	1.46	47.05	2.50E-07
		1.41	32.01	4.37E-06

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