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A cross-sectional study of diverse bacterial and fungal communities in different body habitats in Sardinian centenarians

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Abstract

Microbes in the human body co-evolved with the host during the aging process, adapting to the aging-related niche alternation, however, the full view of the human microbiota variation related to aging is still unknown. Besides, the gut microbiota has been proposed as a promising determinant for human health. Using centenarians as a model for extreme aging may help to understand the correlation of gut microbiota with healthy-aging and longevity. By recruiting the young, elderly and centenarians in Sardinia, Italy, we obtained an integrated view of the spatial distribution of microbiota in the human body across a wide age range and determined the compositional and functional differences in gut microbiota associated with populations of different age in Sardinia. We found that the distribution and correlation of bacteria and fungi community in Sardinians were driven by body sites. In each different age groups, both the bacterial and fungal communities in the skin were significantly different in structure, but not in the oral. The gut bacterial communities in the centenarians clustered separately from the young and elderly which had overlapped clustering, while the fungal communities in gut can't be separated by the age groups. Moreover, our data revealed that gut microbiota of the healthy elderly and young Sardinians also shared similar metabolic functional profiles, while a distinct cluster is found in centenarians. Within the centenarian group, the gut microbiota is correlated with health status of the host. The centenarians have higher diversity of core microbiota

species and microbes genes compared with that in young and elderly. The enrichment of *Methanobrevibacter* and *Bifidobacterium* were detected in Sardinian centenarians, which were also verified in a bigger centenarian cohort in Bologna, Italy. Moreover, potential metabolic functional analysis revealed that the gut microbiota in the centenarian group had significantly lower capability to digest complex carbohydrates but had enhanced fermentation capability via glycolysis. Gene pathways involved in amino acid biosynthesis are lower abundance, while menaquinol biosynthesis is higher abundance in the centenarians compared with that of the young and elderly. Our study indicates that the critical role aging plays in shaping human microbiota is habitat dependent, further suggesting the diverse degree of niche alternation caused by aging in different body habitats, emphasizing the importance of integrating the potential confounding factors into the microbiota studies. Sardinian centenarians' specific gut microbiota may hold promising clues for the future research to identify the possible causative relationship between gut microbiota and longevity in human.

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Chapter Ⅰ. Introduction

1. Longevity in Sardinia

Sardinia, a large island located in the Mediterranean sea, 120 Km from the Italian coast, is famous for the high prevalence and low female/male ratio of centenarians in Europe. Based on the data in 2001, the prevalence of centenarians in Sardinia was 16.6 per 100,000 inhabitants, greater than the observed 10 per 100,000 inhabitants in other European countries. Furthermore, the female/male ratio was 2:1, substantially lower than the ratio in other population (generally more than 5:1) [1]. Besides, from 1999 to 2011, the prevalence of centenarians in Sardinia increased from 13.6 to 20.5 per 100,000 inhabitants. The high prevalence of centenarians, consistent lifestyle, high endogamy and low immigration rates, make Sardinia an ideal geographic area for studying longevity and healthy aging. The AKEA (AKEA is an acronym extracted from the Sardinian traditional wisk A KENT ANNOS that means approximatively "Health and life up to 100") project began studying Sardinian centenarians in the 1990s, providing an enormous amount of information about longevity and healthy aging in this population [1-6]. The AKEA project demonstrated that longevity is a complex biological phenotype determined not only by genetic, epigenetic but is also influence by environmental factors such as diet, lifestyle, and even geographic location. Many of these same factors also affect the microbiota in humans. It has been suggested that the human microbiota, especially the gut microbiota, are tightly linked

to human health and disease. So the microbiota in centenarians is worth fully investigated to integrate our understanding of longevity and healthy aging.

2. Human microbiota and next generation sequencing (NGS)

In the past decades, the development of nucleic acid sequencing technologies especially the high-throughput NGS provided a novel culture-independent and high resolution strategy for microbial community investigation [7]. With the powerful NGS, human microbiota research has become one of the fastest growing scientific fields.

2.1 NGS

NGS, referred to as Next Generation Sequencing, as opposed to the first generation technology Sanger sequencing, was first commerciallay released in 2005 with the release of the 454 Roche and then followed by Solexa/Illumina, SOLiD and Ion Torrent. NGS shares two main advantages for microbiota studies: First, they are culture independent, which is critical for environment samples which including around 98% un-cultureable microbes. The library preparation relies on extracting DNA directly from the environment. Second, they are high throughput, with millions of sequencing reactions and detection running in parallel. There are several NGS-based strategies for microbiome sequencing, such as Amplicon sequencing, Shotgun Metagenomic Sequencing, Metatranscriptomics (RNA sequencing). Sometimes, multi-omics studies such as Metaproteomics and Metabolomics also are integrated to facilitate the and strengthen the analysis. Among the different NGS

approaches, Amplicon sequencing and Shotgun Metagenomic Sequencing are the most extensively applied NGS methods for human microbiota study.

2.1.1 Amplicon Sequencing

Amplicon Sequencing relies on PCR amplification and sequencing of a single informative marker such as 16S rRNA gene and ITS (Internal Transcribed Spacer) gene to analyze the genetic variation in this targeted genomic region. It's widely used for phylogenic and taxonomic studies in diverse environmental samples.

As a highly targeted approach, Amplicon Sequencing can efficiently identify the genetic variation with high coverage and low cost. Besides, the multiplexing of amplicons per reaction is high throughput and time saving with relatively easy laboratory handing. Amplicon Sequencing has an inherent bias due to PCR amplification and intragenomic heterogeneity. More importantly, potential function information is cannot be obtained using this methodology.

2.1.1.1 16S rRNA sequencing for bacterial community

The 16S rRNA gene, which is approximately 1,500 bp long and contains nine variable regions interspersed between conserved regions, is used as the standard for classification and identification of bacteria. Variable regions of 16S rRNA are frequently used in phylogenetic classifications such as genus in diverse microbial populations. Several primer pairs binding at the conserved regions are commonly used to amplify different fragments, for example, the 27F-1492R for the full length of 16S,

27F-534R for V1 to V3, 319F-806R for V3 to V4. The target 16S rRNA region to sequence is an area of debate, which might vary depending on experimental design. Evidence has shown that the V1-V3 regions have good resolution for the skin microbiota, while for the gut microbiota, the V3V4 region is a good choice [8]. There are several public databases for 16S rRNA such as GreenGenes and SILVA which are comprehensively used in microbiota studies [9, 10].

2.1.1.2 ITS sequencing for fungi community

ITS refers to the spacer DNA situated between the small-subunit rRNA and large-subunit rRNA genes in the chromosome. It is widely sequenced for phylogenic analysis of fungi [11]. There are two ITS regions in fungi, ITS1 and ITS2. 18SF and 5.8S-1R primers for ITS1 are frequently used to classify the fungi in the human body. UNITE is a database for ITS-based molecular identification of fungi [12]. Besides ITS, 18S rRNA gene is also used for the detection of fungi. While evidence showed that ITS gene had greater genus-level taxonomic resolution compared with 18S rRNA gene [13].

2.1.2 Shotgun metagenomic sequencing

Shotgun metagenomic sequencing is the massively parallel sequencing of random DNA fragments from all the genes in a microbial community. It is widely used for full characterization of the microbiota in a diverse community with functional annotation. With full coverage of all the genes from a community, the Shotgun metagenomic

sequencing can obtain an accurate taxonomic and functional profile in a single experiment covering multi-kingdoms. The resolution is high and can reach the strain level even the SNP level. Meanwhile, Shotgun metagenomic sequencing also has limitations: the cost is high; the input DNA is larger; laboratory work is complex; a large amount of data generated also need more extensive analysis.

2.2 Human microbiota

The term "microbiome" was coined in 2001 by Nobel Prize-winning microbiologist Joshua Lederberg, "to signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease."[14] Till now, nearly all the body space that contact with the environment were detected with microbes: the oral, the skin, the digestive system, the urinary system, the mammary gland and the genital system even the uterus with a baby in [15-17]. The human body and microbes organize into a super meta-organism. In this complex, the microbes and host can live in symbiosis. The resident microbes include not only bacteria but also other micro-organisms such as fungi and archaea, which maintain the balance of the ecosystem of the human body through cooperation and completion [18, 19]. Dysfunction of any members of this community may affect the health of the human body and may cause disease [20, 21]. The distribution of microbes in the human body is largely and primarily determined by the body habitats with different ecological

niches [15]. However, our microbial habitats are not isolated from one another but interconnected as a unit. So it's critical that we utilize an integrated "whole-body" view of the multi-kingdoms microbial communities.

2.2.1 Skin microbiota

Skin, as the largest organ of the human body, also is the first line of defense against pathogens, composed of several distinct habitats with different moisture contents, pH and nutrients for microbes. Skin commensal microbes including bacteria, fungi and viruses, live in symbiosis with the host, the disruption of microbiota balance in the skin is associated with cutaneous disorders like atopic dermatitis, psoriasis, rosacea, acne etc [22-24]. Generally, *Propionibacterium*, *Staphylococcus* and *Corynebacteria* are the dominant bacterial species and *Malassezia* is the dominant fungi in the skin [13]. Skin microbiota is affected by the topography of the skin, gender, age, environment and immune status [13, 25-31]. Previous studies have found that skin microbial communities are stable over time in the healthy adults at the kingdom, phylum, species, and even, strain level, despite constant exposure to the external environment and other individuals [25]. However, it is poorly known how the skin microbiota changes over human lifespans. Understanding the variation in the skin microbiota during human ageing is critical to investigate if aging contributes to a healthy skin microbial community and if alterations influence host health.

2.2.2 Oral microbiota

The oral cavity itself harbors a complex microbial community and is a major gateway for microbe entry to the human body via digestive tracts. The oral cavity is also a heterogeneous ecological system containing distinct niches with significantly different microbial communities [32]. The core microbes in the oral cavity include *Proteobacteria*, *Firmicutes* and *Bacteroidetes*, with *Prevotella*, *Veillonella*, *Neisseria* and *Streptococcus* as the predominant genera [33]. *Candida* and *Cladosporium* are the most frequently obtained fungi genera in the oral cavity [34]. Previous studies have found several factors associated with oral changes in microbiota: age, population, diet and health condition of the oral cavity [33, 35-37]. Diseases like periodontitis, teeth reduction, caries and even systemic disorders such as stroke, diabetes and cancer are known to be associated with alternations in the oral microbiota [33, 37-40].

2.2.3 Gut microbiota

The gut microbiota is the most comprehensively studied microbial community in the human body and has the strongest association with human disease and health. Gut microbiota integrates diet, life-style and other environmental factors with the genetic and immune factor to modulate the host metabolism and immune responses [41, 42]. The gut microbiota dominanted by *Firmicutes* and *Bacteroidetes*, followed by *Proteobacteria* and *Actinobacteria*, and is a very complex, dynamic and highly personalized ecosystem [43]. The gut microbiota is stable, however, it can be rapidly

altered by diet intervention [44-47]. The alternation of composition and function of gut microbiota is related to a wide range of diseases, from gastroenterological disorders to metabolic diseases. Tracing the changes of the gut microbiota during the life time of an individual is essential for the understanding of the development of disease and the process of aging. With the development of gut microbiota related therapies, such as the successful use of faecal microbiota transplantation (FMT) in treating *Clostridium difficile* infection, the gut microbiota shows great potential for the intervention of disease.

2.3 The Human microbiome project

After the completion of the first complete draft of the human genome in 2003, it was realized that the human genome is only part of understanding human health and disease. Importantly, with the development of NGS, the cost of the sequencing has decreased rapidly allowing for the simultaneous sequencing of multiple communities. In 2008 the National Institutes of Health (NIH) funded the first phase of the Human Microbiome Project. The study characterized the human microbiome across several different sites on the human body including nasal passages, oral cavity, skin, gastrointestinal tract, and urogenital tract in 300 healthy subjects aged from 18 to 40. Furthermore, longitude samples were collected to look at changes in individual microbiomes over time. The HMP established a population-scale framework to develop metagenomic protocols, resulting in a broad range of quality-controlled

resources and data including standardized methods for creating, processing and interpreting distinct types of high-throughput metagenomic data available to the scientific community [48]. The work on HMP has yielded fundamental insights into the human microbiome, opening a new era of the human microbiome research. In the next ten years, enormous research about human microbiota was launched, including research on other population such as MetaHIT in European, enriched our understanding of microbiome, but still, it's the beginning.

3. Gut microbiota in human health and disease

Given the diverse functions of gut microbiota, the relevance of gut microbiota to human health and disease has been found in recent years. Chronic diseases such as obesity and inflammatory bowel disease (IBD) to infectious diseases such as Clostridium difficile infection (CDI) are examples of microbiota linked human disease [49-51]. Gut microbiota is also involved in the development and progression of other human diseases such as cancers, metabolic diseases, respiratory diseases, mental or psychological diseases, and autoimmune diseases [52-56].

3.1 Development of gut microbiota in human

Over a lifetime, each individual undergoes a dynamic process of gut microbiota development. Especially in the early-in-life and later-in-life stages, the host-microbiota relationship is complex and dynamic [57]. The colonization of microbes in the infants starts when the fetus is in the uterus [58]. After delivery, with

comprehensive contact with the environment, infants establish gut microbiota rapidly. Several factors were found to contribute to the establishment of the gut microbiota in infants, such as host genetics, gestational age, delivery mode, feeding, environment exposure and medication [59, 60]. The initial gut microbiota was dominated by *Bifidobacterium*, *Lactobacillus* and *Veillonella* heavily influenced by the milk consumption [59]. With solid food introduction at about one half year of age, the gut microbiota starts to transit toward an adult-like microbiota dominated by *Bacteroides* and *Firmicutes* [59]. The alternation continues to three years old, and then the gut microbiota enters into a relatively stable community stage [44, 57]. In adults, the alternation of the gut microbiota is associated with several clinical conditions, such as diabetes, obesity and IBD [49, 51, 56, 61]. During the aging process, the physiological change of the host impacts diet preference and immunological status leading directly or indirectly to the alternations of the gut microbiota. Age related signatures of gut microbiota have been demonstrated in several studies, for example, the decrease of *Faecalibacterium* and *Ruminococcus* associated with aging [57, 62-64]. Furthermore, age-related compositional change of gut microbiota is correlated with clinical variables including dietary and health factors [41]. The loss of diversity in core microbiota groups was found to be associated with increased frailty and decreased cognitive performance, but not significantly associated with chronological aging [65]. It is unknown if gut dysbiosis associated with ageing is a cause or consequence of aging and senescence-associated inflammatory disorders.

3.2 Factors contributing to gut microbiota

3.2.1 Genetics

Genetic differences between host species are reflected in the similarity of the gut microbiota among phylogenetically related animals [66]. In humans, the gut microbiota of monozygotic twins is significantly more similar than that of dizygotic twins [67]. In the microbiome genome-wide association studies (mGWAS), the association between the lactase gene (LCT) and the abundance of *Bifidobacterium* was validated in several cohorts [68]. Future studies are likely to reveal more host genetic variants and their influence on the gut microbiota. Additionally, different populations with genetic variations also have diverse life-style and dietary preferences, emphasizing the importance of studying gut microbiota at the population level.

3.2.2 Host immunity

The gut microbiota and the host immune system have complex interaction [69]. Gut microbiota plays a fundamental role in the induction, training, and function of the host immune system [70, 71]. In return, the immune system protects of the host against potential harmful infections from the gut microbes, meanwhile, the host must be tolerant toward the harmless commensal microbes. Reduced microbial exposure and alteration of microbial communities in certain body sites are found associated with autoimmune and allergic disease [72]. Several important effects of the microbiota on the host immune system have been determined by studies of germ-free mice, for

instance, the extensive deficits in the development of the gut-associated lymphoid tissues [73], the greatly reduced numbers of IgA-producing plasma cells and lamina propria $CD4^+$ T cells [74, 75]. Consequently, the altered immune status is also found associated with increased epithelial permeability and systemic microbial translocation which was found in HIV patients [76]. The dysbiosis of gut microbiota, leading to decreased resistance to pathogen colonization and increased pathological immune responses which cause inflammation reaction. However, the causative relationship between inflammation and dysbiosis, and the mechanism by which they interact to, remain areas of active investigation.

3.2.3 Diet and nutritional status

Diet and nutrition are the major modulators in the gut ecosystem. Long-term diet influences the structure and function of the gut microbiota and was linked with gut microbial enterotypes [45, 77]. Acute changes in diet can also lead to rapid alterations in the gut microbiota [47]. Intervention target at diet which is easy to modify is highly practicable. The western diet with high fat and sugar causes alterations in the genetic composition and the metabolic activity of gut microbiota, compared with the gut microbiota in the hunter-gatherer populations with a diet rich in protein and low in sugar [78, 79]. Such diet-induced changes to gut microbiota are now suspected of contributing to growing epidemics of chronic illness in the developed world, including obesity and IBD. Interestingly, high-level adherence to a Mediterranean diet

which is rich in olive oil, fresh vegetables, fruits, and fermented food beneficially impacts the gut microbiota and the associated metabolome [80, 81]. Diet as a non-negligible factor contributed to the population level gut microbiota variation [46, 82], further research should make more effect to find its association with gut microbiota and health.

3.2.4 Other environmental factors

Other environmental factors, such as geographic location, ethnicity and life-style are usually associated with variations in host genetics and diet and can influence the gut microbiota [78, 79, 83]. A recent study examined the genotype and microbiome data from 1,046 healthy individuals, and demonstrated that the gut microbiome is not significantly associated with genetic ancestry but is associated with the genetically unrelated individuals who share a household [84]. Over 20% of the inter-person microbiome variability is associated with factors related to diet, drugs and anthropometric measurements. This study indicates that the environment dominates over host genetics in shaping human gut microbiota. Although there are heritable bacteria taxa, the gut microbiota in human determined by the environment is a positive signal for gut microbiota intervention.

3.3 “Beneficial” versus “harmful” Microbes

With the rapid advances in the understanding of the human microbiota, parallel efforts have been made to start microbe-based intervention prevent disease and improve

health. The fecal microbiota transplantation (FMT) has already proven to be a highly effective therapy for *Clostridium difficile* infection [85], while this therapy is based on a microbial-population level, there is still possibility that undetected and potentially pathogenic viruses, bacteria, or parasites and other biological materials from donors to recipients. As a complex microbial ecosystem, the gut microbiota consists of a large number of microbes with high genetic diversity. Therefore, strain-level dissection of the contribution of the gut microbiome to heath and diseases is critical for at this stage. Take *Akkermansia muciniphila* for examples, it has been shown to have a protective effect against obesity in both humans and mice [86, 87], regarding as a promising probiotics for against metabolic disorders. Meanwhile, recent research also found the increasing of *Akkermansia* abundance in Parkinson's disease, Alzheimer's disease and multiple sclerosis patients [88-91]. Caution should be made in the design and use of microbial interventions for human therapeutic use.

However, it is an over simplification to classify microbes as "beneficial" and "harmful" microbes. Their function it highly dependent on multiple host and microbial factors, including the abundance, strain, site of colonization, as well as the age, genetic background, environmental factors and immune status of the host.

3.3.1 "Beneficial" microbes

"Beneficial" microbes, also refers as "Probiotics" can be defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit

on the host" (Food and Agriculture Organization of United Nations; World Health Organization e FAO/WHO, 2001). Certain strains of *Bifidobacterium*, *Lactobacillus*, and *Akkermansia*, such as *Bifidobacterium bifidum*, *Lactobacillus rhamnosus* and *Akkermansia muciniphila* are widely regarded as probiotics. Former studies have found that these microbes have immune-modulatory properties and potential to synthesis health-promoting metabolites in vitro and in vivo, in humans and animal models [92-94]. Noticeably, the aging-dependent and disease-associated decline of these microbes have also been found both in animal models and human [57, 63, 95-97]. With the accumulating of knowledge of the "beneficial" microbes, we can use them as the novel source of probiotics for future therapies.

3.3.2 "Harmful" microbes

Meanwhile, there exists a contrasting assortment of "harmful" microbes, which generally considered ''harmful'' for the host or associated with dysbiosis. For example, pathobionts are typically kept at low levels within the healthy gut and do not cause problems in immune-competent hosts; however, the outgrowth of these organisms can contribute to dysbiosis and cause disease [98, 99]. However, Pathobionts also have beneficial functions. Certain strains of *Escherichia coli* are highly virulent pathogens that cause acute hemorrhagic diarrhea and haemolytic uremic syndrome but are also a normal inhabitant in the gut microbiota, with the function of stimulating the innate immune system to tolerate and survive the pathogens [100, 101].

3.4 Gut microbiota and host immunity

The microbiota plays a fundamental role in the induction, training, and function of the host immune system. In return, the immune system has largely evolved to maintain the symbiotic relationship of the host with these highly diverse and dynamic microbes. During early life, with a relatively immature immune system and provided with maternal milk containing live microbes, metabolites, IgA, and immune cells as well as cytokines, neonates become colonized by microbes, initiating the induction and training of the immunity system [102].

Gut-associated lymphoid tissues: Studies performed in germ-free mice revealed the critical role microbiota plays in immune maturation [103, 104]. The immunological abnormalities in germ-free animals including hypoplastic PEYER'S PATCHES that contain few germinal centers, as well as greatly reduced numbers of IgA-producing plasma cells and lamina propria CD4+ T cells [75]; the spleen and lymph nodes are relatively structure-less, with poorly formed B- and T-cell zones [73];

IgA-producing B cells: In the intestine, microbe-associated molecular patterns (MAMPs), including lipopolysaccharide, lipid A, peptidoglycan, flagella and microbial RNA/DNA, can be mediated through antigen uptake by M cells and goblet cells to dendritic cells (DCs), along with a direct trans-epithelial luminal sampling from DCs. DCs induce the differentiation of CD^4-expressing T cells into the T follicular helper (T_{FH}) cell subset. CD40 ligand (CD40L) and IL-21 from T_{FH} cells induce the expression of activation-induced cytidine deaminase (AID) in B cells and

promote IgA class-switch recombination which mostly taken place in Peyer's patches [105]. While T-cell-independent IgA class-switch recombination occurs predominantly in the lamina propria and isolated lymphoid follicles (ILFs), where B-cell activating factor (BAFF) and its homologue APRIL, which are derived from dendritic cells, promote the induction of AID expression in B cells [106]. Meanwhile, Paneth cells which are enriched in the crypts, secreting AMPs, which can cross-link with the mucus layer [70].

T_H17 cell, T_{Reg} cells and Innate lymphoid cells (ILC): Segmented filamentous bacteria (SFB) and other commensal microorganisms activate DCs and macrophages to initiate the differentiation of naive CD4+ T cells into RORγt-expressing T cells (belonged to ILC3s), it accumulates and further differentiate into IL-17-expressing homeostatic T_H17 cells in the lamina propria of the small intestine. This process can be stimulated by IL-1β, IL-6 and IL-23 [107]. These homeostatic T_H17 cells then stimulate epithelial cells to enhance the integrity of the intestinal mucosal barrier by producing pro-inflammatory cytokines: IL17A, IL17F and IL22 [108]. T_H17 produced IL-22 to aid in IEC barrier function by induction of regenerating islet-derived protein 3γ (REGIIIγ) in intestinal epithelial cells. Alternatively, stimulated by IL12, DCs can induce T_H1 cells. *Clostridium* spp. clusters IV and XIVa, polysaccharide A $(PSA)^+$ *Bacteroides fragilis* and other microbiota stimulate intestinal epithelial cells, T cells, and lamina propria DCs and macrophages to promote the development and/or the activation of forkhead box P3 $(FOXP3)^+$regulatory T cells [69].

With the establishment of the commensal relationship between the microbiota and host immunity, the intestinal immune system can tolerate the microbes, while simultaneously remaining vigilant against the potential threats posed by these microbes. Gut microbiota compositional changes can be induced by antibiotic treatment, dietary changes and gastrointestinal pathogens and even aging [47, 50, 109, 110]. Ingestion of gastrointestinal pathogens represents a threat to intestinal homeostasis. Side effects of the immune response to pathogens can lead to tissue damage and alteration of the composition of the microbiota. In some cases, this can lead to dysbiosis. The robust proinflammatory immune response caused by pathogenic invasion, can lead to disruption of the intestinal barrier and an altered microbiota, favoring the efficient colonization and survival of the pathogen [98, 111].

The immune system is not only closely related to the symbiotic microbiota but is also exquisitely sensitive to the nutritional status of the host. Evidence has shown that the interaction between diet, immunity and gut microbiota in both human and animal models [80, 112-114]. In addition to profoundly regulating the composition and function of gut microbiota, food-derived metabolites also can directly regulate the immune cell, for example, the SCFA, folate, Vitamin and AHR [115-117]. Conversely, immunodeficiency can alter the gut microbiota composition and thereby the metabolic capacity of both the flora and the host [118]. Therefore, an understanding of the role of gut microbiota in immune responses requires the integration of the diet as well.

3.5 Gut microbiota and host metabolism

As a moderator between diet and host metabolism, the gut microbiota participates in host metabolism. In the symbiotic gut system, gut microbiota depends on the host to obtain food for energy, while at the same time, the microbiota return metabolites to the host that the human body utilizes. Carbohydrates are the most important source of energy for both human and gut microbes; its fermentation is a core activity for the human gut microbiota [119]. While human enzymes cannot digest most of the complex carbohydrates, such as cellulose and resistant starches, those fibers are fermented by gut microbes in the colon, to be digested into simple oligosaccharides or monosaccharides which can be used as resources for glycolysis to yield energy for the growth of microbes and to produce end products, including short chain fatty acids (SCFAs). Colonocytes in the gut are the first host cells that take up SCFAs, which depend largely on butyrate for their energy supply [120]. SCFAs provide ~10% of the daily caloric requirements in humans [121]. SCFAs also function as anti-inflammatory agents by signal transformation [116]. Moreover, SCFAs are involved in most of the functions for gut microbiota since they also epigenetically modify the host epithelial cells and stimulate the epithelial cells to release molecules to facilitate the brain-gut cross-talk [122]. However, because energy resources mainly depend on carbohydrates, the human and gut microbes also form a competitive relationship. The combined exchange of and competition for energy between humans and their gut microbiota is the basis that maintains the homeostasis of the gut. In

addition to the carbohydrate metabolic pathway, gut microbiota are also involved in the metabolism of proteins and the production of several related metabolites: branched SCFAs, ammonia, sulfur-containing metabolites such as hydrogen sulfide and methanethiol, and neuroactive compounds such as tryptamine, serotonin, phenethylamine, and histamine [123]. Gut bacteria can regulate the amino acid metabolism by utilization of several amino acids originating from both food and endogenous proteins. In turn, gut bacteria also provide amino acids to the host. Other metabolites such as bile acid, vitamins, polyphenols, polyamines and methylamines are also critical for human health [124]. As we known, obesity and other associated metabolic diseases are intimately linked with diet. Besides, alternation in the gut microbiota was also observed in those diseases [56, 125]. Gut microbiota can modulate the dietary impact on the host metabolism. Gut microbiota transplantation from obese human into germ-free mice has shown that the obese phenotype can be transferred to recipient mice [126]. In humans, the insulin sensitive feature can be transferred by fecal microbiota transplantation to insulin in-sensitive participants [127, 128]. Further studies are needed to determine the causative linkage between gut microbiota alteration and metabolic disease.

3.6 Diet-Gut microbiota interaction and intervention

Diet is an important factor in modulating the composition and function of gut microbiota in humans and other mammals [45-47, 129]. As the major components in

diets of human, the type and amount of carbohydrate, protein, and fat present in the diet have been widely found to influence the composition of the gut microbiota in the host [95, 130, 131]. Dietary fiber as a carbohydrate that can't be digested by humans, its fermentation is one of the main functions of the microbiota, providing the principle energy source for gut bacteria. Previous studies have associated the dietary fibers with the gut microbiota composition and host metabolism [132]. Dietary fiber intervention resulted in a higher abundance of *Bifidobacterium* and *Lactobacillus* as well as fecal butyrate concentration compared with placebo/low-fiber comparators [133]. The perturbation of amino acid metabolism was also found related to the alternation of gut microbiota in Type 2 Diabetes and insulin-resistance [134, 135]. Moreover, high dietary fat can induce increased of inflammatory responses [136, 137]. Considering the critical function diet plays in shaping the gut microbiota, it's essential to explore the mechanism of how diet modulates the microbiota and host metabolism.

However, even though acute changes in diet can have an impact on microbiota, long term dietary habits are the dominant force in determining the major compositional feature of the individual's gut microbiota [45, 47]. Therefore it is possible to reprogram the gut microbiota by dietary change. Considering the complexity and individual specific features of gut microbiota, diet intervention should be personalized.

Dietary interventions, as a comparatively easier approach than manipulation, have enormous therapeutic application potential for human health and diseases. Dietary

restriction (DR), a form of dietary intervention, has proven health promoting and may impact longevity in different model organisms and humans [138, 139]. Alterations in the gut microbiota were also found in the DR subject, although the contribution of these changes to the health of the host remains to be elucidated [140].

3.7 Gut microbiota is a reservoir for antibiotic resistance gene

Antibiotic resistance for pathogenic bacteria is a global threat to human health both individually and as a population. Bacteria can acquire antibiotic resistance genes by the mobilization and transfer of resistance genes from a donor strain. Gut microbiota, including billions of bacteria, is a reservoir for antibiotic resistance gene (ARG), which offers ample opportunities for the horizontal transfer of genetic material, including antibiotic resistance genes. Therefore, more concern should be taken to the ARGs in the human gut microbiota. Development of antibiotic resistance in microbes is largely contributed by the usage of antibiotics in medicine and agriculture. Furthermore, different populations display diverse pattern of ARG profile [141]. With the facilitate of gut microbiota metagenomic sequencing, we can get the gut microbiome information which can further be extracted to get the ARG profile for a defined population. Monitoring the population level of ARG in gut microbiota is essential for the controlling of the ARG spread.

4. Aging and microbiota

Aging is a highly complex process affecting a wide array of physiological, genomic,

metabolic, and immunological functions [142]. Aging associated changes in the physical and cognitive status of the host lead to life style alternations. Consequently, they impact the niche that microbes inhabit. Aging accompanied by the alternation of the immune response state directly interfaces with the microbiota and enhances susceptibility to infection [143, 144]. For instance, the elderly are marked susceptibility to dermatologic disorders caused by infection [145]. With aging, the microbiota in humans co-evolves with the host to adapt to the niche alternation. Interestingly, many clinical issues accompanied with aging, such as Bowel disorder, cardiovascular disease, constipation and Parkinson's disease, are also closely correlated with perturbations in gut microbiome composition and functions [49, 88, 89, 96, 146, 147]. Given that the gut microbiome is closely associated with several features of gut barrier integrity, intestinal inflammatory balance, immune and metabolic health, and gut-brain axis [69, 148, 149], these old-age-related clinical issues could clearly contribute to the increased the susceptibility to various infectious and gut-associated diseases by causing alterations in the microbiota of elderly people . Investigation into the baseline of aging-related microbiota alternation in different body habitats enhances our understanding of the biological insights of aging and microbiota, ultimately making it possible to promote healthy aging through the intervention of the microbiota in human.

4.1 Aging related changes in gut microbiota

Since the aging population is a global issue, prolonging a healthy lifespan has become a vital challenge for modern medical research. Gut microbiota, widely regarded as associated with health and aging, has become a new focus for research on aging. With the aging process, the natural decline in physical function which, along with dietary change and immune response changes, may cause gradual alterations of gut microbiota. Aging related alternation in gut microbiota was observed in different cohorts [57, 150, 151]. Early data from the ELDERMET cohorts showed the core microbiota of the elderly was distinct from the young adults, and later, demonstrated the association of the gut microbiota in the elderly with diet and health [65, 152]. Their research indicated that the chronological aging is not the cause of gut microbiota alternation, but the loss of diversity in core microbiota groups is associated with increased frailty in the elderly [62]. Recently, a survey of gut microbiota in a large healthy cohort in China showed that the gut microbiota of healthy elderly Chinese is similar to that of the healthy young [150], further emphasizing the important role health plays in shaping the gut microbiota. While there are also certain signatures of aging related to the alternation of gut microbiota that is shared between populations, such as the reduction in *Faecalibacterium* and *Bifidobacterium* abundance and the increase in *Enterobacteria* abundance. Gut microbiota composition alternations cause functional shifts which lead to the change in energy utilization and the abundance of bacterial metabolites production. Functional annotation of the gut

microbiota in the aging process is critical for the utilizing future therapeutic modulation of gut microbiota to promote healthy aging. Although alternations in the composition and function were observed, till now we still do not have a clear understanding of the causative relationship between the altered gut microbiota and decreased heath during the aging. It has been hinted that gut microbiota homeostasis is crucial for healthy aging and hence restoration of this homeostasis might be supportive for human longevity [153]. Further longitudinal research following the aging process is eagerly needed to help our understanding of aging and gut microbiota.

4.2 Gut microbiota character of longevity

Evidence shows that gut microbiome impacts host longevity in animal models such as *Caenorhabditis elegans* and *Turquoise Killifish* which have short and easily monitored lifespan [154, 155]. Moreover, longitudinal study in a large human cohort has also found the association between the distinct metabolomics signatures and longevity of humans [156]. Thus human gut microbiota may act as the modulator between longevity and the genetic and environmental factors by affecting the host metabolism. During a life time, gut microbiota undergoes a co-evolution with the human host, adapting to the progressive changes of the host gut environment, the longevity is a successful outcome of human host and gut microbiota symbiosis. Centenarians represent a population with an extremely long lifespan, and have been

used by several groups as a model to study aging and gut microbiota [57, 63, 64, 151, 157, 158]. These studies have focused on the compositional feature of gut microbiota in centenarians with younger groups as controls. As a dynamic ecological system, gut microbiota composition is various for individuals. The variation may cause by different populations with diverse genetic, dietary, environmental factors even with different methodologies of processing the samples and data. Certain compositional features of centenarians' gut microbiota have been found, including accumulation of some subdominant species. Among the variations, some seem universal, such as the decrease in abundance of *Faecalibacterium*, it shows an aging dependent decrease in trajectory. Other changes are unique to the defined population; for example, the enrichment of *Roseburia* in Chinese centenarians but a decrease in Italian and Japanese centenarians [57, 63, 151]. The variation of the changes may be caused by the different populations with different genic backgrounds, diets, and lifestyle. Noticeably, metabolic function of gut microbiota in centenarians has not yet been explained yet.

5. Hypothesis and objectives

Sardinia is famous for the high prevalence and low female/male ratio of centenarians in Europe. While the reason for the specific longevity in Sardinia is not clear, the gut microbiota may hold a clue. Centenarians as a model to study the aging and gut microbiota are highly accessible in Sardinia. Besides, the consistent lifestyle, high

endogamy and low immigration rate in Sardinia make the population there being an idea cohort to study the population specific variations in human microbiota. What's more, more evidence showed that the Mediterranean diet has a beneficial impact on the health. The typical Mediterranean diet in Sardinia may also contribute to the longevity for Sardinians, so investigate the correlation among diet, gut microbiota and longevity is Sardinia is meaningful.

The human body and microbes that inhabitants in organize into a super meta-organism. In this complex, the microbes and host live in symbiosis. The resident microbes include not only bacteria but also other micro-organisms such as fungi and archaea, which maintain the balance of the ecosystem of the human body through cooperation and completion. Dysfunction of any members of this community may affect the health of the human body even cause some disease. The distribution of microbes in the human body is largely and primarily determined by the body habitats with different ecological niches. However, our microbial habitats are not isolated from one another but interconnected as a unit. So it's critical that we integrated "whole-body" view of the multi-kingdoms microbial communities.

Former research has found that aging is associated with alternation of the microbiota in the body habitats [31, 57]. With aging, microbiota in human co-evolved with the host to adapt to the niche alternation. Investigation into the baseline of aging-related microbiota alternation in different body habitats enhances our understanding of the biological insights of aging and microbiota, ultimately making it possible to promote

healthy aging through the intervention of the microbiota in human.

Based on this information about Sardinian and human microbiota, the hypothesises of this study are:

1. Host aging is associated with the changes in the microbiota distributed in the human body.
2. Sardinia centenarians share defined unique features of gut microbiota, both in composition and function, which may facilitate the longevity.
3. Centenarians with different health status have diverse gut microbiota.

The Objectives of this thesis are:

1. Determine the compositional and functional profiles of the Sardinian population.
2. Compare the variation of the gut microbiota between Sardinian centenarians and the healthy elderly and young age groups.
3. Identify specific gut microbiota features in centenarians.
4. Find the correlation of the gut microbiota with healthy status parameters in the centenarians.

Chapter Ⅱ. Material and methods

1. Cases enrolment and clinic information collection

We recruit 65 subjects in Sardinia, Italy, as a part of the AKEA Study. The ethical approval was provided by the Institutional Local Ethics Committee, Azienda Sanitaria Locale n.1 of Sassari, Italy.

Subjects are divided into three age groups:

1. Healthy young group
2. Healthy elderly group
3. Centenarian group

Exclusion criteria including (for the Young and Elderly group):

a. with a history of chronic medical conditions (diabetes, hypertension)
b. with chronic dermatologic diseases (psoriasis, atopic dermatitis, vitiligo urticarial)
c. use antimicrobial medication (antibiotic or antifungal treatments) half year before sampling. All subjects provided informed consent. Clinical history, status, medical history and anthropometric measurements were collected. The clinical and nutritional data were collected. All subjects provided informed consent. Clinical history, status, medical history and anthropometric measurements were collected. Briefly, the MNA to assess the malnutrition, the MMSE to evaluate the cognitive status, FIM to assess the disability,

healthy parameter and medication records are also carried

2. Samples collection and Meta-DNA extraction

2.1 Skin samples

To maximize microbial load, no prior cleaning of the skin is needed before sample collection. The skin samples will be collected by the professional staff with sterilized swabs (Catch-All™ Sample Collection Swabs) at four different sites, including forehand, two palms and umbilical area. 5 cm^2 area of skin is gently rubbed using swab (soaked in sterilized enzyme lysis buffer) for 10 times. Then suspend the swab samples in 200ul enzyme lysis buffer. Keep the samples on ice when shipping to the lab. Negative control specimens are collected by exposing swabs to the room air and then processing with the samples. Extract the skin meta-genomic DNA according to the DNeasy blood and tissue extraction kit's manual instructions with some modification. In brief, the samples in lysis buffer were incubated in 37℃ for 2h. 0.4 g of 5 mm zirconia beads (Sigma) were added, and the samples subjected to a bead beating step using Biosan for max 3,000rpm, 30min. 25µl of proteinase K was added, incubated in 56℃ for 30min. Then samples were heated at 95℃ for 5 min followed by ice for 1min, and performed following the DNeasy blood and tissue extraction kit protocol. Elute the DNA by 200µl TE buffer. Final DNA concentration was determined by using NanoDrop ND-1000 (NanoDrop Technologies)

2.2 Oral sample

Participants were asked to avoid eating or drinking for one hour prior to oral sampling. Subject was asked to let saliva collected in the mouth for at least 1 minute. The subject then asked to drool into the labeled 50 mL collection tube. This process may be repeated multiple times in order to collect larger volumes of saliva (2-5 mL). For the centenarians, it's difficult to collect the saliva so the oral washing samples were collected instead. After low temperate shipping the samples to the lab, centrifuge at 7,500rpm for 10min, and then discard the supernatant, extract the DNA from the sediment following the same protocol with skin samples.

2.3 Stool sample

Fecal samples were collected by the participants at home. Participants were provided with a stool specimen collection tube. After passing stools, the spoon was used to collect about 1g stool of sample by scraping off the outer layer of solid feces and collecting the central part into the tube. Samples were immediately frozen at home at -20℃ and collected by laboratory personal within 6 weeks. Long term storage of samples was in a -80℃ freezer located at University of Sassari. Stool meta-genomic DNA was extracted according to the manual instructions for the QIAamp DNA Stool Mini Kit (QIAGEN) with some modifications. In brief, 200mg of stool was suspended in 1.4mL of buffer ASL and 0.4 g of 5 mm zirconia beads (Sigma) were added. Then each sample was subjected to a bead-beating step using Biosan for a maximum of

3,000 rpm for 30min. Samples were heated at 95℃ for 5 min, and then centrifuged for 5min at 13,000 rpm to pellet stool particles. Next, 1.2mL of the supernatant was collected and the InhibitEX tablet was added, followed by incubation at RT for 1 min and centrifugation at 13,000 rpm for 3 min, then 15ul of proteinase K and 200μl of buffer AL was added to 200μl supernatant and incubated at 70℃ for 10 min. 200μl of absolute ethanol was then added to the mixture, vortexed, and loaded on QIAamp Mini spin columns. The columns were washed with buffer AW1 and buffer AW2, as per the QIAamp DNA Stool Mini Kit instructions. The DNA was eluted with 200μl TE buffer. Finally, the DNA concentration was determined by using NanoDrop ND-1000 (NanoDrop Technologies).

3. Library construction and sequencing

3.1 16S rRNA and ITS1 library

Procedures for 16S rRNA and ITS1 library generation were performed as previously described [159]. Briefly, the V3V4 region of the 16S rRNA gene and ITS1 gene were amplified using an improved dual-indexing approach. Primers for 16S rRNA amplification were 5’-ACTCCTACGGGAGGCAGCAG-3’, 5’-GGACTACHVGGGTWTCTAAT-3’, for ITS1 amplification the primers were 5’-GTAAAAGTCGTAACAAGGTTTC-3’, 5’-GTTCAAAGAYTCGATGATTCAC-3’. 16S and ITS1 libraries were respectively normalized and pooled with SequalPrep™ Normalization Plate Kit (Invitrogen) prior to sequencing on Illumina MiSeq platform.

3.2 Shotgun metagenomic library

Illumina libraries were prepared with 100ng of input DNA, using KAPA Hyper Prep Kit (Kapa Biosystems) following the manufacturer's instructions. Libraries were quality checked by KAPA Library Quantification Kit (Kapa Biosystems) and 2100 Bioanalyzer (Agilent). Qualified libraries were transported on dry ice to BGI-Shenzhen for paired-end metagenomic sequencing which was performed on an Illumina HiSeq X10 PE150 platform (with average insert size 350 bp). Sequence read quality was first filtered by the in-house pipeline at BGI-Shenzhen. 6.2×10^9 clean reads (Q20 percentage 95%) were generated for 59 samples.

4. Bioinformatics and statistical analysis

4.1 Bioinformatics for 16S and ITS1 sequencing

Raw sequence reads were first trimmed by removal of the barcodes and linker sequence. Then VSEARCH was used for truncation of the reads not having an average quality of 15 based on the phred algorithm. Reads with less than 75% of their original length were removed [160]. Further read processing was performed using QIIME (version 1.9.1) [161]. Reads were clustered into operational taxonomic units (OTUs) at 97% of similarity using the open-reference strategy by uclust based on the Greengenes database (2013_8 version) for 16S and UNITE fungal ITS database (version 7.2)[9, 12]. The first cluster seed was chosen as the representative sequences

for the OTUs. Uclust for 16S rRNA and BLAST for ITS1 were used for taxonomic assignment of representative sequences. Alignment of representative sequences was performed using PyNAST for 16S rRNA and MUSCLE for ITS1. Aligned sequences were filtered and phylogenetic trees constructed using FastTree. Rarefaction OTU Table 3.were generated. OTU counts were binned into genus-level taxonomic groups according to the taxonomic assignments described earlier. MEGAN6 Community Edition was used to visualize the microbiota taxonomy profile by bubble charts and PCoA plots [162].

4.2 Bioinformatics for shotgun metagenomic sequencing

Raw reads were trimmed then filtered and mapped to the human genome (hg19) with BWA under default settings [163]. The filtered, clean reads were used as input for further analysis. The profiles of microbiota composition were predicted using MetaPhlan2, and gene family profiles and pathway profiles were predicted using HUMANN2 [164, 165]. The gene family profile was normalization by reads per kilobase, annotated to UniProt Reference Cluster (UniRef90). Further pathway mapping was performed using the MetaCyc metabolic pathway database. The gene family profile was regrouped to the orthologous groups using the KEGG and EggNOG database. We also used the Integrated catalog of reference genes in the human gut microbiome (IGC) as a reference for mapping our clean reads, which is a published, high-quality reference catalog generated from 1267 fecal samples and 1070

people around the world, including Danish, Spanish, Chinese and Americans. We used Samtools to determine the matching results [166]. The annotation results were compared with HUMANN and IGC methods to validate the data analysis.

Antibiotic resistance genes were predicted following the DeepARG pipeline [167]. Briefly, the low quality reads were removed using TRIMMOMATIC, then, reads were merged into one large file by VSEARCH and are submitted for classification to the deepARG algorithm. The alignment algorithm identity was set as 50% with E-value of $1e^{-10}$. The percent coverage was set to 50% and the probability used was 0.8. The abundance of the ARGs was normalized to the 16s rRNA abundance in each of the sample.

4.3 Statistical analysis

All statistical analysis were performed using R software (version 3.4.2). Multivariate analyses of community diversity including PCoAs and NMDS were performed using ade4, ape and vegan and visualized using ggplot2. Bray-Curtis distance matrix was used as a similarity index. ANOVA and Kruskal-Wallis tests were used to determine whether significant differences existed between multiple groups in STAMP [168]. Welch's two-sided t test was used for the analysis of two groups. Discrimination among groups was detected by MRPP，Adonis and Anosim methods using 999 permutations to test the significance. The association between the health status and species within the compositional profiles of the microbiota was determined by the

envfit function in vegan package. Significance was tested by 999 permutations. The Mantel test was used to test the correlation between two distant matrices. The correlation network was constructed by Cytoscape with App MetScape 3 [169]. The correlation matrix was visualized by Hmisc package in R.

Chapter Ⅲ. Results: Diverse bacterial and fungal communities in different body habitats in Sardinians across age

1. 16S rRNA gene and ITS1 gene amplicon sequencing for different body habitats in the Sardinian population

To quantify and compare community structure and relationship between bacteria and fungi among all body habitats in different age groups, a total of 65 Sardinians were recruited. Clinical parameters of the subjects were collected (Table 3.1).

Table 3.1 Statistics of the clinical characteristics in the three age groups.

Parameters	Centenarians(n=22)	Elderly(n=24)	Young(n=19)
	Mean ± SD (Range)		
Age(yrs)	102.0±1.5(99-107)	77.2±5.9(68-88)	24.9±3.5(21-33)
Female (%)	72.7%	56.60%	41.2

Values are presented as Mean±SD with the range in parentheses.

For each subject, the skin (including four different sites: Left palm, Right palm, Forehead and Umbilicus), oral and fecal samples were collected. The workflow is shown in Figure 3.1

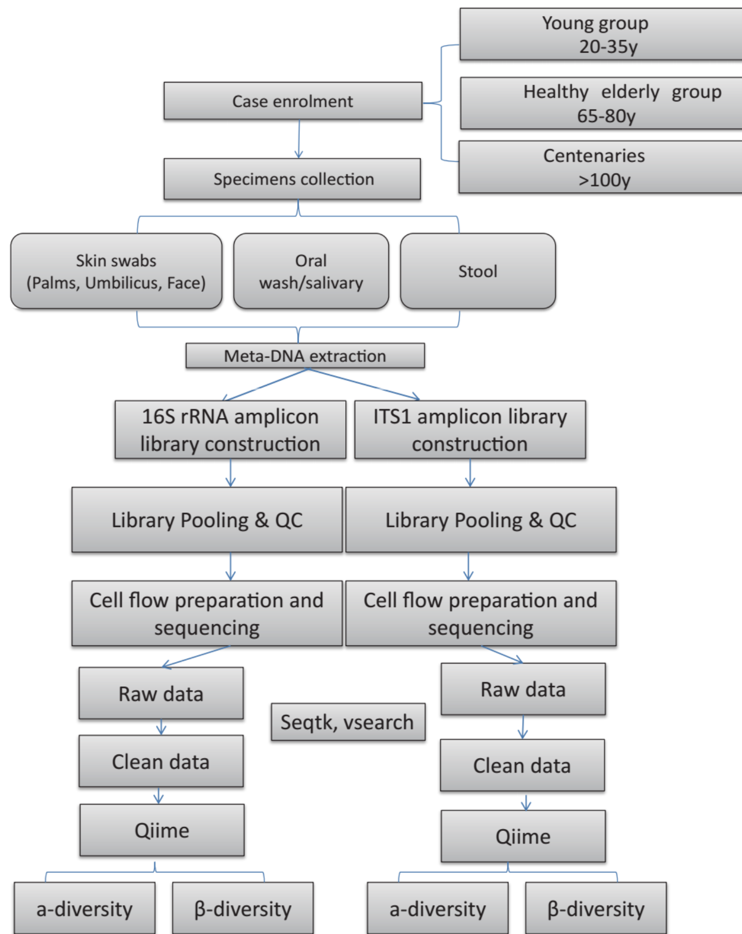


Figure 3.1 Design and workflow of the study

A total of 379 16S rRNA and 377 ITS1 amplicon libraries were constructed and successfully sequenced on an Illuminal Miseq platform. More than 9.6 million reads were generated for 16S rRNA sequencing, 11.2 million reads for ITS1 sequencing. On average, 25,341 clean reads for 16S rRNA sequencing and 29,813 clean reads for ITS1 were sequenced for each sample.

Considering the inter-individual variation of the sequencing depth, 16S rRNA and ITS1 reads were randomly resampled to keep the depth of reads for each sample

constant. Specifically, 5,000 reads for 16S rRNA data sets and 10,000 reads for ITS1 data sets were used. Across all body habitats, we detected members of 30 bacterial phyla assigned to 562 bacterial genera and 6 fungi phyla assigned to 691 fungi genera. Rarefaction analysis at the genus level of the taxonomy in MEGAN showed the taxonomic richness detected in the samples (Figure 3.2). The curves were levelling off from a straight line at the depth of 5,000 reads for 16s and 10,000 for ITS1, indicating repeated sampling of the same taxon. The taxonomy rarefaction curve indicated that most of the taxonomic richness was accounted for with the sequencing reads.

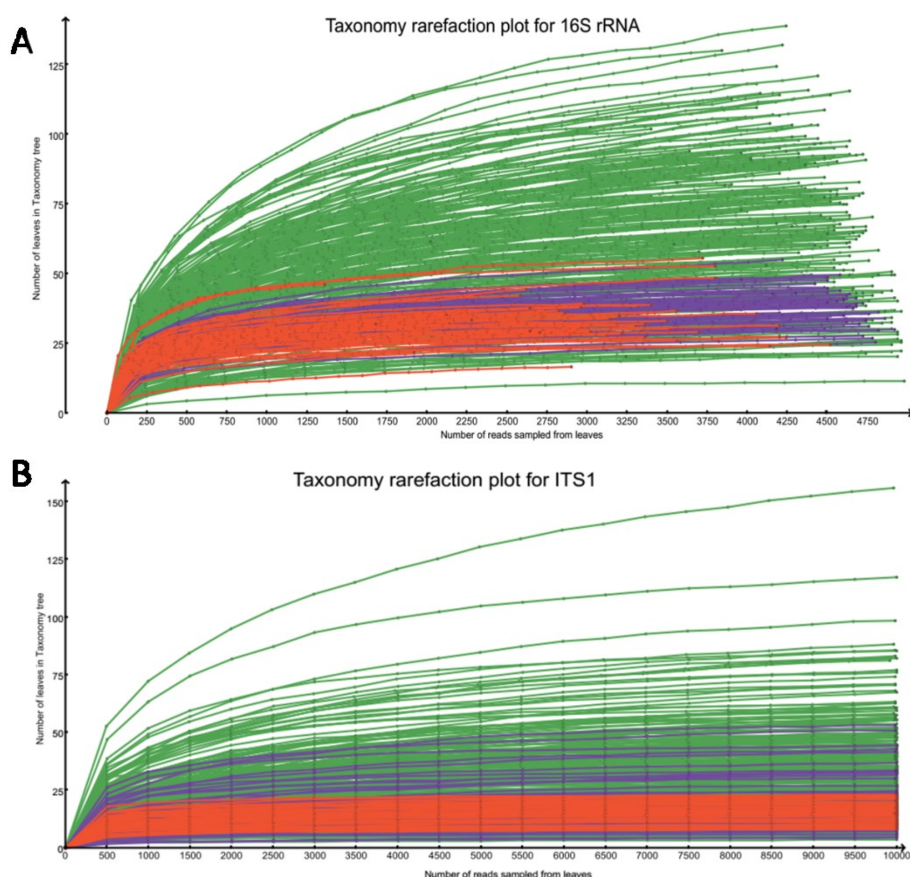


Figure 3.2 The taxonomy rarefaction curve created in Megan. The taxonomy

rarefaction curve was performed by repeatedly sampling subsets from normalized reads and computing the number of leaves to which taxa have been assigned. 16s rRNA sequencing (A); ITS1 sequencing (B).

2. Full view of Sardinians microbiota community compositional profile across different habitats

In order to visualize the global microbiota community signatures for both bacteria and fungi in a babble chat, we used the bubbles that represent different dominant genera to show the compositional profile of each subject (Figure 3.3). The profiles showed that the bacteria had compositional patterns determined by body habitats. As seen in our data set the dominant bacteria in the skin were *Propionibacterium*, *Staphylococcus* and *Corynebacterium*, while the dominant fungi was *Malassezia*. The dominant bacteria in the oral cavity were *Streptococcus*, *Veillonella*, *Prevotella*, *Rothia* and *Actinomyces*. The dominant fungi in the oral cavity were *Malassezia*, *Candida* and *Saccharomyces*. The dominate gut bacteria were *Bacteroides*, *Feacalibacterium*, *Blautia*, *Coprococcus* and *Bifidobacterium*. *Penicillium* and *Saccharomyces* were the dominant fungi in the gut.

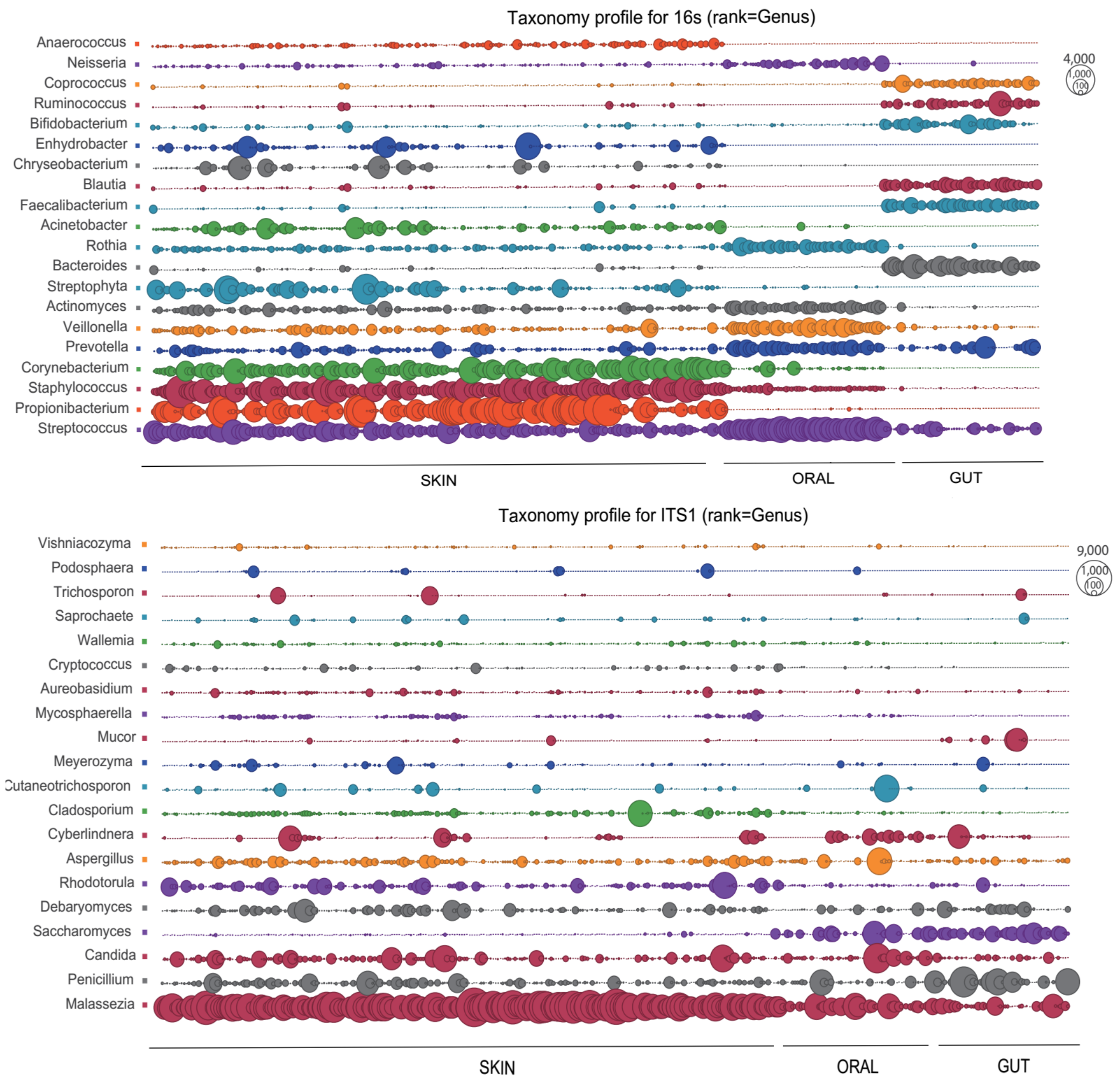


Figure 3.3 Global Sardinian microbiota composition profile surveyed by 16S rRNA and ITS1 sequencing. Bubble charts for bacterial and fungal genus for 3 different body habitats. The bubble charts show the top 20 bacterial and fungal genera assigned to 338 samples for 16S and 354 samples for ITS1 sequencing (excluded the samples didn't have enough reads number). The size of the bubbles is square-root-scaled (scale in grey) and refers to the normalized number of reads assigned to each genus (listed in the Y axis) in each of the sample (listed in the X axis). The label line under the X axis marks the sample type.

3. α diversity of the microbiota communities for each body habitat in three age groups

α diversity representing the mean species diversity in a habitat at a local scale is an important character of the community. Shannon diversity index and species richness are frequently used to evaluate α diversity in gut microbiota research.

3.1 Bacteria and fungi have various α diversity in different age groups across body habitats

Shannon diversity index was used to predict the α diversity of genera in each sample. We found that the Shannon diversity for bacterial and fungal communities from different body habitats in three age groups varied dramatically (Figure 3.4). Overall, the bacterial had higher Shannon diversity compared with fungal at the same body habitat for all age groups. The skin samples had a higher diversity of fungal community compared with oral and gut samples which had a higher diversity of the bacterial community. For each age group, the forhead had lower microbiota community diversity than palms and umbilicus. Meanwhile, we observed that the elderly had higher bacterial and fungi community diversity in the skin than the young. The centenarians had the skin community diversity between the elderly and young. The variation of skin microbiota community diversity between individuals in the young group was larger than that for the elderly. The fungal communities in oral cavity and gut had similar low diversity in three age groups, except for the oral cavity

of the young. The elderly group had a more complex bacterial community in gut compared with the young and centenarian group. The centenarians had the lowest gut bacterial community diversity.

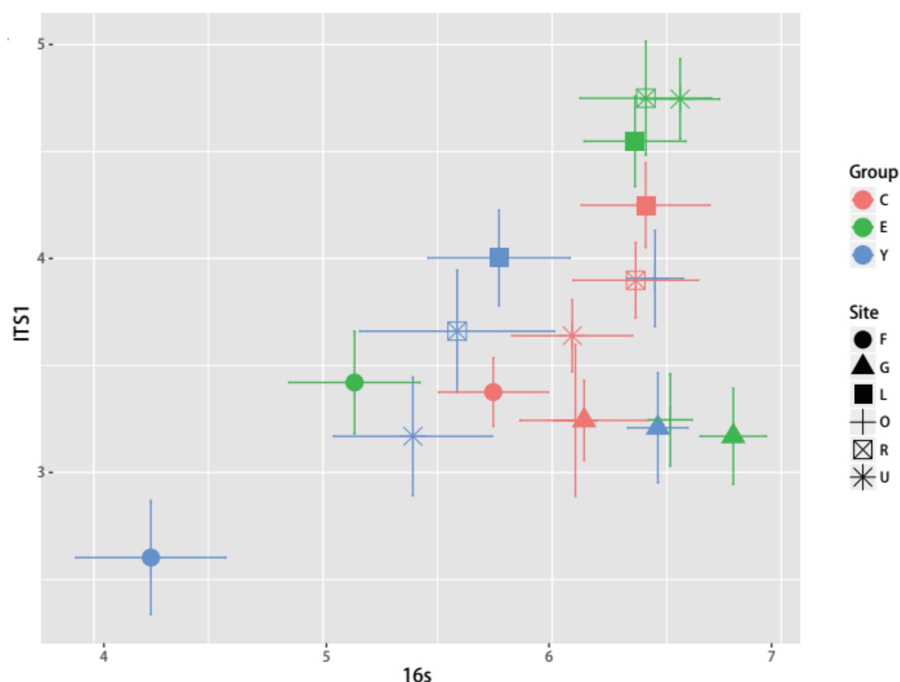


Figure 3.4 Microbiota diversity surveyed by 16S rRNA and ITS1 sequcencing in Sardinians across body habitats. Median Shannon diversity of fungal and bacterial OTUs for 6 different body habitats in three age groups. Error bars represent the median absolute deviation with color representing each age group.

3.2 Correlation between bacterial and fungal α diversity is body habitat dependent

To explore the relationship between bacteria and fungi in the same body habitats for each individual, the Shannon diversity index for 16S and ITS1 for each individual in each body habitats was performed and is displayed in Figure 3.5. We used linear

regression to investigate the potential relationship between bacterial and fungal community diversity. The Shannon diversity for the bacterial and fungal community in the gut and oral cavity was not linearly correlated, but a significant association was observed in the skin. This indicated that for the skin, the individual had a higher diversity of in the bacterial community and was associated with a higher diversity of the fungal community. While for the gut and oral, the diversity of bacterial and fungal community was independent.

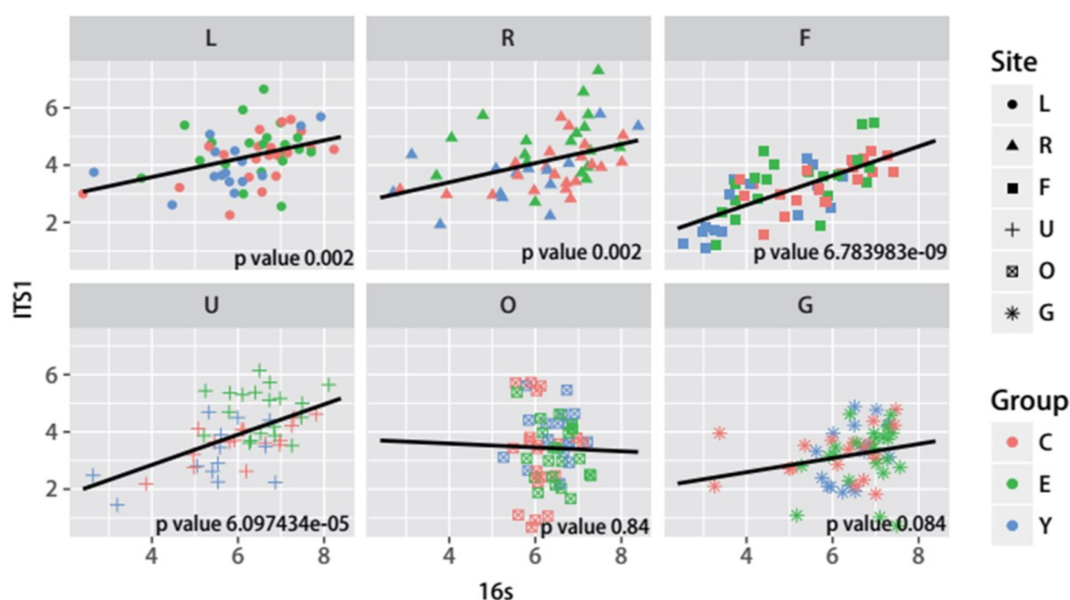


Figure 3.5 Correlation between bacterial and fungi α diversity in different body habitats. The association between 16S and ITS1 Shannon diversity for all the samples. The samples are differently shaped for each body habitat and colored by age groups. The p value of the linear regression analysis is shown.

4. β diversity of microbiota communities for each body habitat in three age groups

β diversity is another characters of the community referred to as the species differentiation among habitats. The variation can be tested by several approaches, such as using the ordination method (including PCoA, PCA and MMND) and using ANOVA (including Adonis, Anosim and MRPP). In our study, we detected that the microbiota communities display not only habitat specific variation but also age related features.

4.1 Body habitats variation of microbiota communities

4.1.1 Different grouping of the bacterial and fungi communities in each body habitat

Principal coordinates analysis (PCoA) based on the Bray-Curtis distance of the microbiota compositional profile revealed that the primary clustering of the bacteria in the body was driven by body habitats Figure 3.6. Compared with skin which was rather discretely clustered, the gut and oral were densely clustered. The Adonis test showed that the grouping of bacterial communities by each body habitat was statistically significant (R^2 =21%, p=0.001), also each subject showed a significant grouping of bacterial communities (R^2 =25%, p=0.001). Fungal communities in PCoA were less distinctively separated by different body habitats than bacterial communities, however, the Adonis test showed that the grouping of fungal communities was statistically significant both in each body habitat (R2 =15%, p=0.001) and in each

individual (R2 =30%, p=0.001).

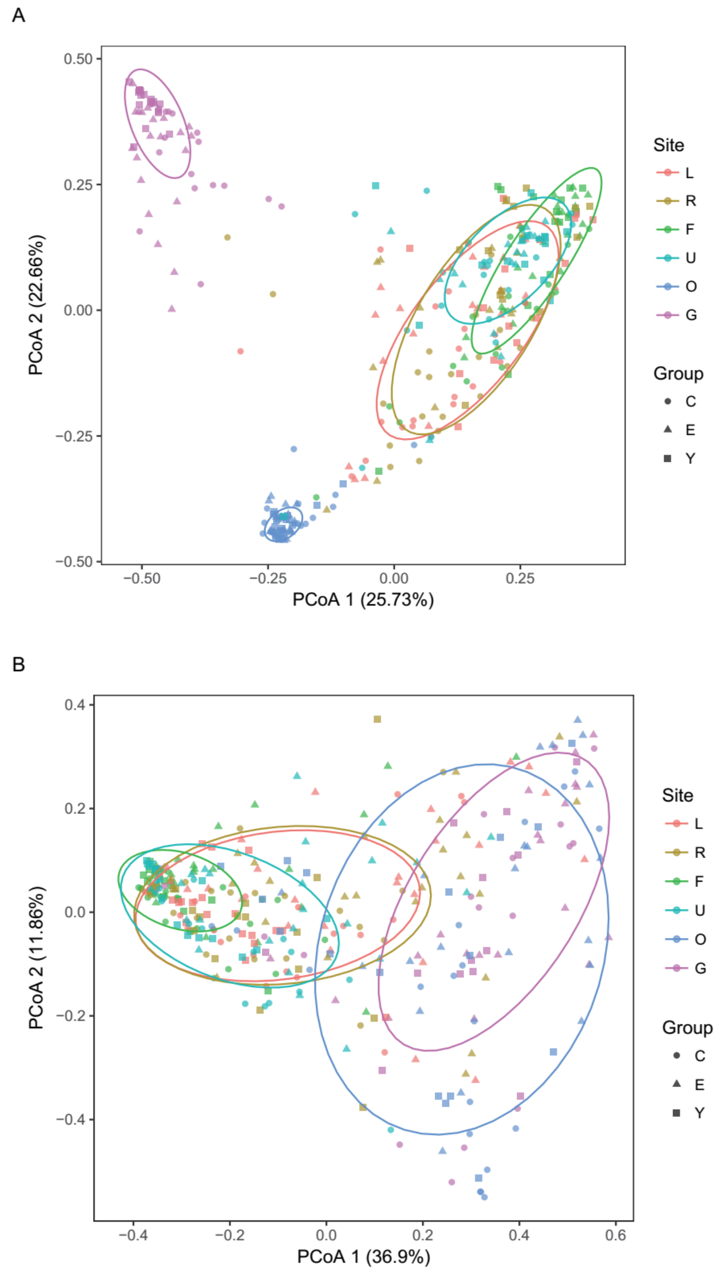


Figure 3.6 Variability of the bacterial and fungal community dissimilarities based on Bray-Curtis distance for each individual among the body habitats shown on the PCoA. Each sample was marked as plots in symbols and colors indicated for different habitats and age groups. A: bacterial communities, B: fungal communities

The compositional differences of bacterial and fungal communities among each individual for the same body habitat was assessed in Figure 3.7. The distance within the defined body habitat reveals different degrees of community composition variation. Consistent with the variation of the Shannon diversity for each body habitat, the variation of bacterial community composition within the oral and gut were also smaller compared with that of skin, while the fungal community composition variation within oral and gut were larger compared with skin.

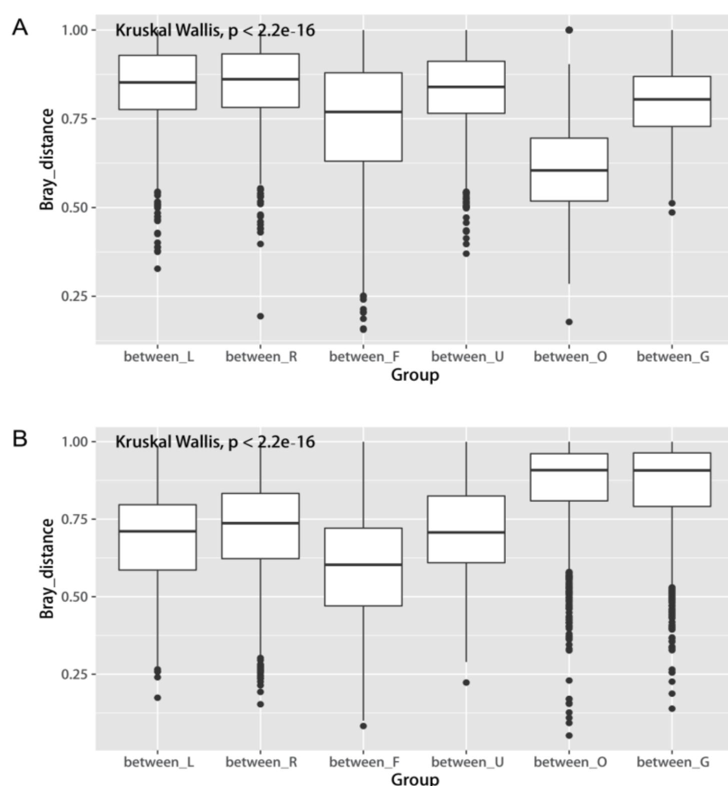


Figure 3.7 Similarities of the bacterial and fungal community structure in different body habitats. A: Boxplot of Bray-distance of bacterial communities within the same body habitat survey by 16S rRNA sequencing. B: Boxplot of Bray-distance of fungal communities within the same body habitat survey by ITS1 sequencing.

4.1.2 Body habitats specific enrichment of bacterial and fungal taxa

To further identify the species that were significantly distributed in the different body habitats (skin, oral and gut), we used the linear discriminant analysis (LDA) effect size (LEfSe) to determine the differentially distributed species with abundance over 0.1% in all subjects. We identified 43 bacterial species and 17 fungi species with abs LDA score >2.0 among habitats. We visualized the results in a cladogram in Figure 3.8. The results revealed that the most abundant bacterial phylum in the skin body habitats were *Proteobacteria, Actinobacteria* and *Cyanobacteria*. *Bacteroidetes* and *Firmicutes* were the dominant phyla in the gut, and *Fusobacteria* and *TM7* enriched in the oral cavity.

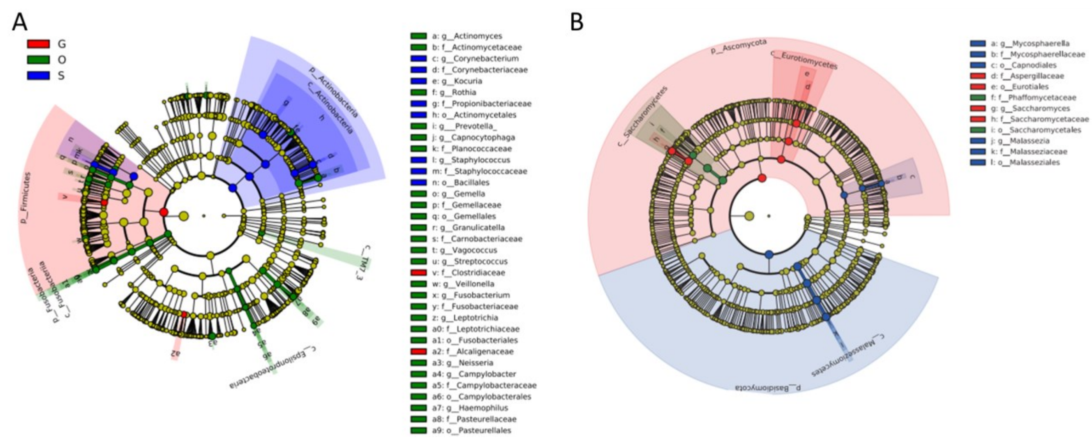


Figure 3.8 The cladogram of the taxa detected by LEfSe. Bacterial community (A); Fungal community (B). The LEfSe use Kruskal-Wallis test (p<0.05) among each body habitat; Threshold on the logarithmic LDA score for discriminative features is 2.0; Strategy for multi-class ananlysis for bacterial is All-against-all (more strict), for fungi is one-against-all (less strict). Three classes: G indicates gut, O indicates oral and S

indicates skin.

For the fungi communities, the phylum Ascomycota and Basidiomycota were significantly distributed in different body habitats, respectively enriched in gut and skin. The plot of the two phyla is shown in Figure 3.9. At the genus level, the Saccharomyces was abundantly enriched in the gut and oral cavity but not in the skin, while, Malassezia and Mycosphaerella were enriched in the skin but had lower abundance in the gut and oral cavity.

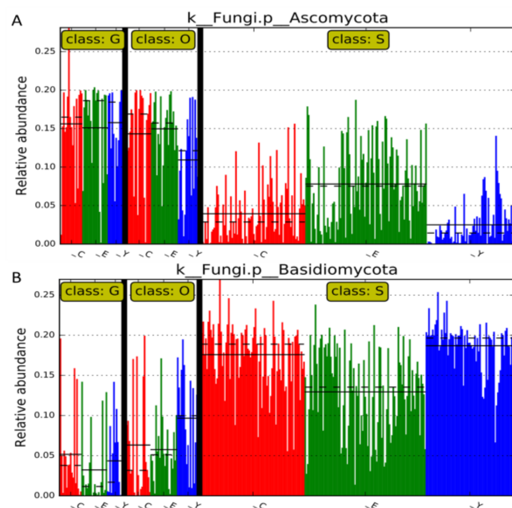


Figure 3.9 The plot of the phylum with statically significant difference among body habitats. Ascomycota (A); Basidiomycota (B).Class G indicates the gut, O indicates the oral and S indicated the skin. Subclass C indicates the centenarian, E indicates the elderly and Y indicates the young.

4.1.3 Correlation of the microbiota communities among different body habitats

The Mantel test was used to explore the correlation of the microbiota communities

among different body habitats in Table 3.2. Skin habitats showed significant positive association among fungi community distance. No correlation was detected between the skin and the gut nor the gut and oral fungal community distance. This indicated that individuals that shared similar fungal communities in the skin habitat also shared similar fungal communities in other skin habitats. Associations could only be detected for bacterial communities between palms and forehead but not with umbilicus. The gut, oral cavity and skin showed no correlation between each other for the bacterial communities.

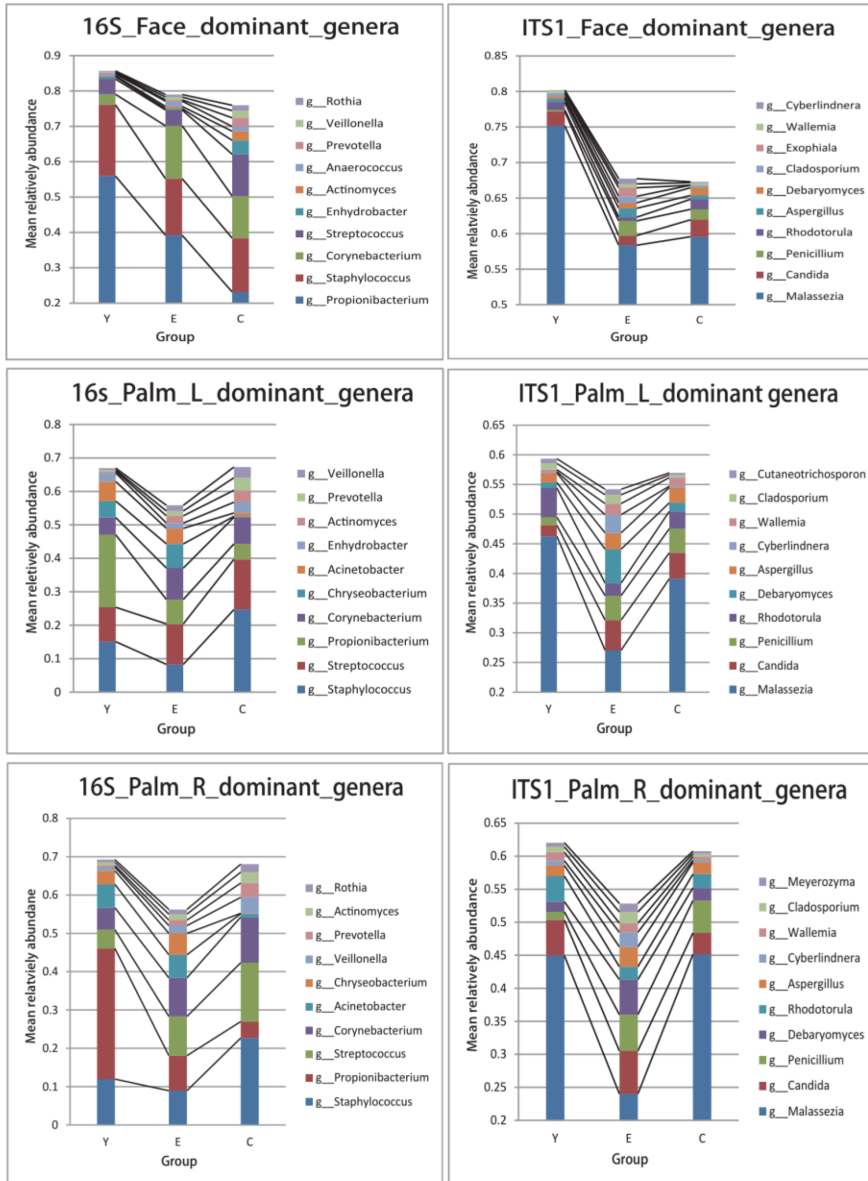
Table 3.2 Correlations between the bacterial communities' distances and fungal communities' distances evaluated by Mantel test based on Bray-Curtis distance.

Habitat 1	Habitat 2	16S Bray-Curtis distance		ITS1 Bray-Curtis distance	
DM1	DM2	Mantel r statistic	p-value	Mantel r statistic	p-value
L	R	0.649	0.001	0.661	0.001
G	O	0.083	0.288	-0.006	0.903
F	U	-0.011	0.878	0.483	0.001
F	L	0.283	0.001	0.447	0.001
G	U	0.085	0.366	-0.057	0.394
U	R	0.081	0.348	0.275	0.001
F	O	0.065	0.358	0.020	0.729
R	O	0.012	0.866	0.020	0.661

4.2 Age related variation of microbiota community in each body habitat

4.2.1 Diverse bacterial and fungal community compositional signatures for different age groups in each habitat

To seek the bacterial and fungal community compositional signatures, we investigated the mean relative abundance of the most dominant bacterial and fungi genera in different age groups in each body habitat Figure 3.10. Except for the oral bacterial communities in which the dominant genera profile was similar among three age groups, the other communities in each habitat had different dominant genera profile for three age groups. The dominant bacterial and fungal genera in palms had decreased in the elderly compared with the young. Interestingly, centenarians had increased abundance compared with the elderly. In the gut, the abundance of the dominant bacterial genera decreased constantly with aging, indicating that the proportion of the sub-dominant genera in centenarians was increased. The dominant fungal genus in the skin habitats was *Malassezia* which was highly enriched in the young but decreased in the elderly, while in the centenarians *Malassezia* had increased compared with the elderly. The dominant fungal genera profile in the forehead was similar for elderly and centenarian but different from the young. In the oral, the relative abundance ratio of the dominant fungal genera for elderly was only 35% while the young was 56% and centenarians was 62%, indicating that the elderly had a high prevalence of sub-dominant genera in the oral cavity.


16S_Face_dominant_genera
Mean relatively abundance
Group
Y
E
C
g__Rothia
g__Veillonella
g__Prevotella
g__Anaerococcus
g__Actinomyces
g__Enhydrobacter
g__Streptococcus
g__Corynebacterium
g__Staphylococcus
g__Propionibacterium
ITS1_Face_dominant_genera
Mean relatviely abndance
g__Cyberlindnera
g__Wallemia
g__Exophiala
g__Cladosporium
g__Debaryomyces
g__Aspergillus
g__Rhodotorula
g__Penicillium
g__Candida
g__Malassezia
16s_Palm_L_dominant_genera
Mean reletively abundance
g__Veillonella
g__Prevotella
g__Actinomyces
g__Enhydrobacter
g__Acinetobacter
g__Chryseobacterium
g__Corynebacterium
g__Propionibacterium
g__Streptococcus
g__Staphylococcus
ITS1_Palm_L_dominant genera
Mean relatively abundance
g__Cutaneotrichosporon
g__Cladosporium
g__Wallemia
g__Cyberlindnera
g__Aspergillus
g__Debaryomyces
g__Rhodotorula
g__Penicillium
g__Candida
g__Malassezia
16S_Palm_R_dominant_genera
Mean relatviely abundane
g__Rothia
g__Actinomyces
g__Prevotella
g__Veillonella
g__Chryseobacterium
g__Acinetobacter
g__Corynebacterium
g__Streptococcus
g__Propionibacterium
g__Staphylococcus
ITS1_Palm_R_dominant_genera
Mean relatively abundance
g__Meyerozyma
g__Cladosporium
g__Wallemia
g__Cyberlindnera
g__Aspergillus
g__Rhodotorula
g__Debaryomyces
g__Penicillium
g__Candida
g__Malassezia

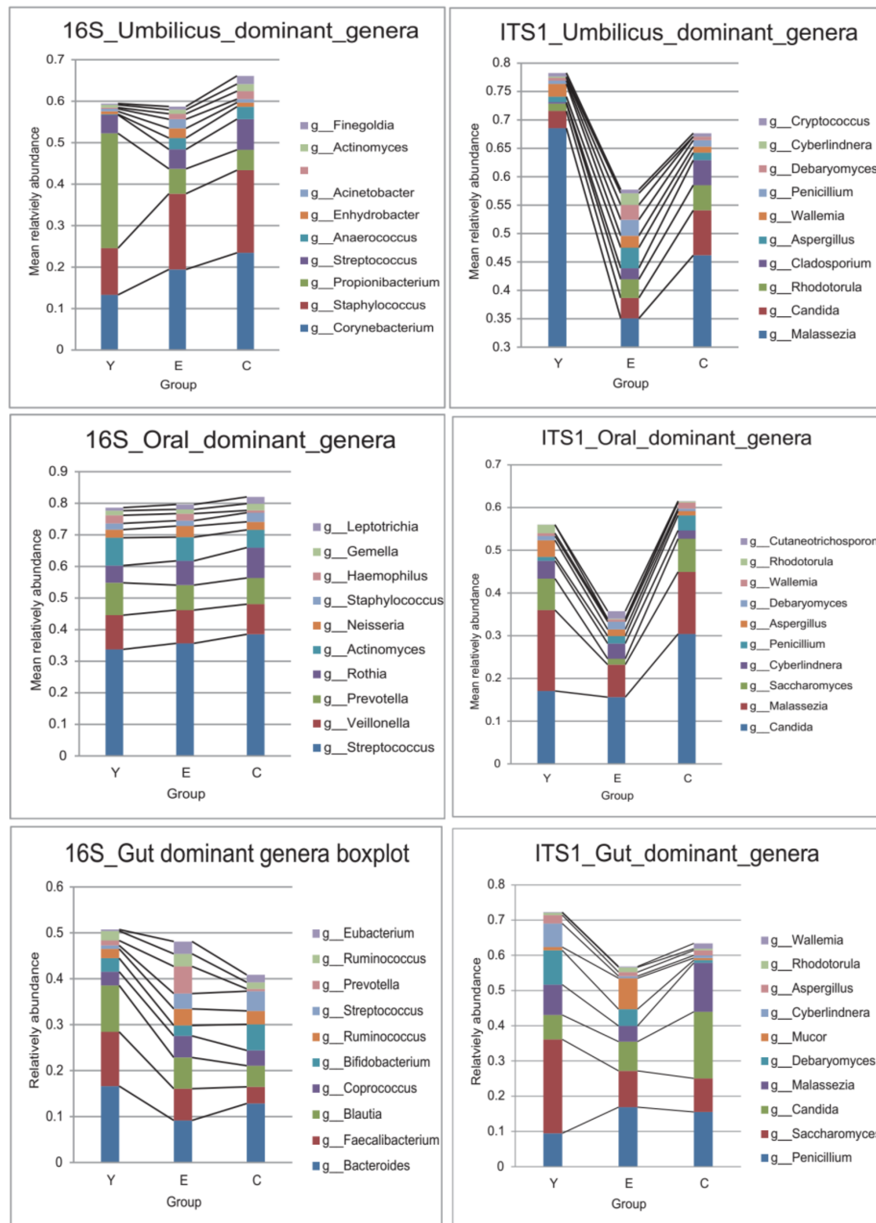


Figure 3.10 The compositional features of microbiota among three age groups. Boxplot shows the relative abundance of the top 10 dominant genera in each age group for bacteria and fungi respectively. Group Y indicates young group, E indicates elderly group and C indicates the centenarian group.

4.2.2 Clustering of bacterial and fungal communities for different age groups in each body habitat

Anosim and MRPP tests were applied to test statistically whether the microbiota communities were significantly different between three age groups (Table 3.3&3.4). The test revealed that three age groups showed different degrees of sub-clustering in certain habitats. For example, skin and gut bacterial communities were clearly clustered by age group but for the oral communities the age group separation was not clear. No significant difference among the three age groups was detected for fungal communities in the oral and gut habitats (Anosim test, R<0.05 and P>0.05). Beta diversity of bacterial communities on the skin and in the gut for each individual was significantly different among the three age groups, while the oral bacterial communities did not show age dependent clustering. For the fungi communities on the skin habitats, the beta diversity showed significant differences among age groups, even so, the group dissimilarities were not sharp (0<R<0.1).

Table 3.3 Evaluate the difference between three age groups for bacterial and fungal communities in each habitat by Anosim test based on the Bray-Curtis distance. The number of permutations is 999.

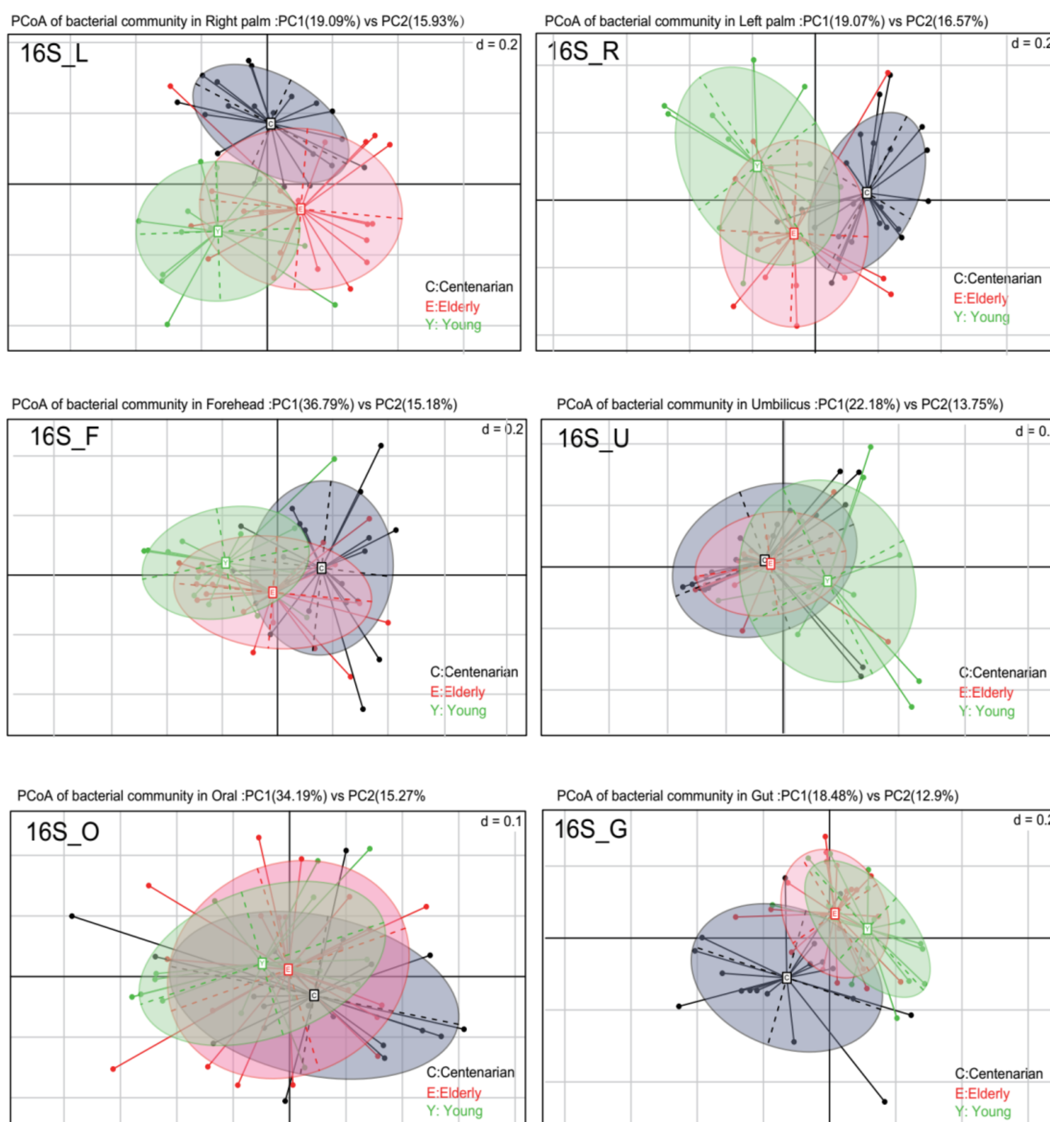
ANOSIM	16S_L	16S_R	16S_F	16S_U	16S_O	16S_G	ITS1_L	ITS1_R	ITS1_F	ITS1_U	ITS1_O	ITS1_G
Sample size	55	53	60	54	59	58	64	63	63	56	61	58
Test statistic	0.225	0.208	0.128	0.127	0.019	0.176	0.091	0.083	0.088	0.080	0.029	0.040
P-value	0.001	0.001	0.001	0.003	0.185	0.001	0.002	0.009	0.003	0.015	0.125	0.087

Table 3.4 Evaluate the difference between three age groups for bacterial and fungal communities in each habitat by MRPP test based on the Bray-Curtis distance. The number of permutations is 999. Observe-delta represents the difference within the group. Expect-delta represents the difference between groups. Group_Delta represents the within groups distance for each age group.

MRPP	16S_L	16S_R	16S_F	16S_U	16S_O	16S_G	ITS1_L	ITS1_R	ITS1_F	ITS1_U	ITS1_O	ITS1_G
Observed delta	0.75	0.76	0.65	0.76	0.54	0.76	0.66	0.67	0.54	0.65	0.84	0.85
Expected delta	0.79	0.79	0.67	0.78	0.55	0.79	0.68	0.7	0.57	0.67	0.84	0.85
C_delta	0.7486	0.7579	0.7292	0.7828	0.5976	0.8187	0.6347	0.6121	0.563	0.6478	0.8288	0.8495
E_delta	0.8059	0.7978	0.6179	0.7378	0.5275	0.7641	0.7617	0.7728	0.6085	0.7067	0.848	0.8599
Y_delta	0.6814	0.7212	0.5766	0.7663	0.5022	0.6782	0.5457	0.6161	0.428	0.5716	0.8011	0.7895
p-value	0.001	0.001	0.001	0.001	0.008	0.001	0.001	0.001	0.001	0.001	0.071	0.054

PCoA was used to visualize bacterial and fungal community structure for different age groups in Figure 3.11. We found that the age group clustering was matched with the Anosim test. More specifically, we observed that for the bacterial communities, the symmetric palms showed similar patterns: young and centenarians had clearly separated clusters, while the elderly cluster overlapped with both the young and centenarian groups, indicating that the palm bacterial community structure shifted with aging. In the face and umbilical sites, the clustering of bacterial communities for age groups was slightly different: elderly and centenarians clustered closely but more

distinct from the young. For the fungi communities in all skin habitats, young and centenarians showed close clustering, whereas the elderly clustered separately. The bacterial and fungal communities for oral habitats did not display any age group clustering. In the gut, we found that the young and healthy elderly had similar clusters for bacterial communities which were distinct from that of the centenarians. The fungi communities in the gut were not be separated by the age groups.



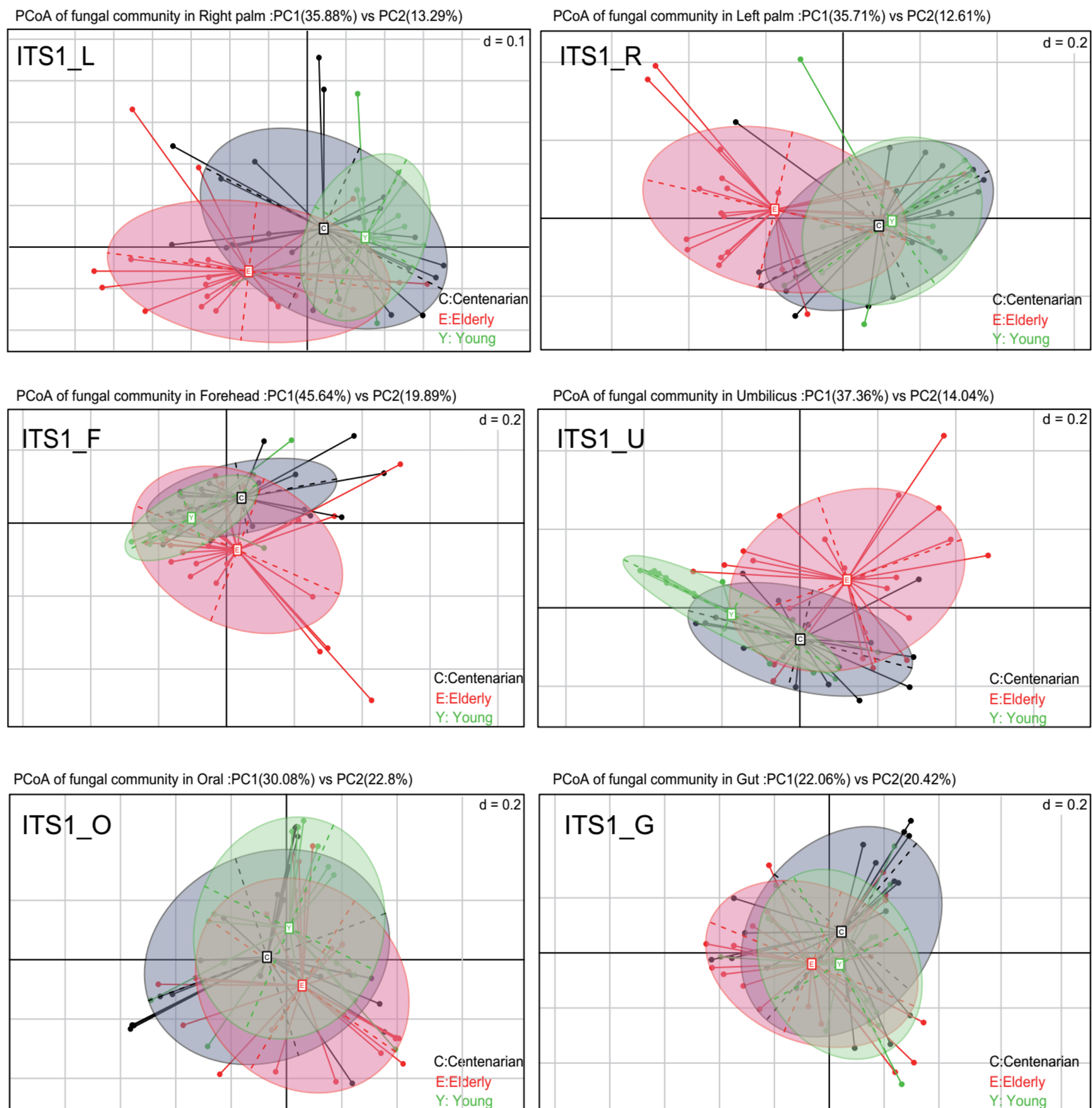


Figure 3.11 PCoA visualized the dissimilarities of the bacterial and fungal community compositional profile for each individual in three age groups for each habitat based on Bray-Curtis distances of the relative abundance of each genus. Individuals were identified as spot filled with black (centenarian), red (elderly) and green (young) in scatter plot. Each age group was clustered by ellipses and labeled by C (centenarian), E (elderly) and Y (young).

4.2.3 Dominant genus correlated with the similarities of microbiota community in oral and gut

Except for gut bacterial community, the microbiota structure of oral cavity and gut didn't display significant clustering by age groups, we further explored the contribution of the dominant genera to the ordination of the subject in PCoA (Figure 3.12) to determine the driven force for similarities of the microbiota structure for each individual. We observed that for gut bacterial communities, young and elderly individuals clustered tightly, and the clustering was positively correlated with *Bacteroides* and *Faecalibacterium*. Interestingly, a group of centenarians clustered separately, influenced by the contributed of *Bifidobacterium*. For the fungal community in the gut, the individuals showed three sub-clusters: one was positively driven by *Candida*, one was positively driven by *Penicillium* and the other was negatively driven by both *Candida* and *Penicillium*. In the oral habitat, bacterial community similarities were driven by all the dominant genera, among them, *Streptococcus*, *Veillonella*, *Neisseria* and *Prevotella* showed the highest fitness. The fungal communities in the oral habitat also formed three sub-clusters: one was driven by *Malassezia*, one was strong positively driven by *Candida* and the other was negatively driven by the two.

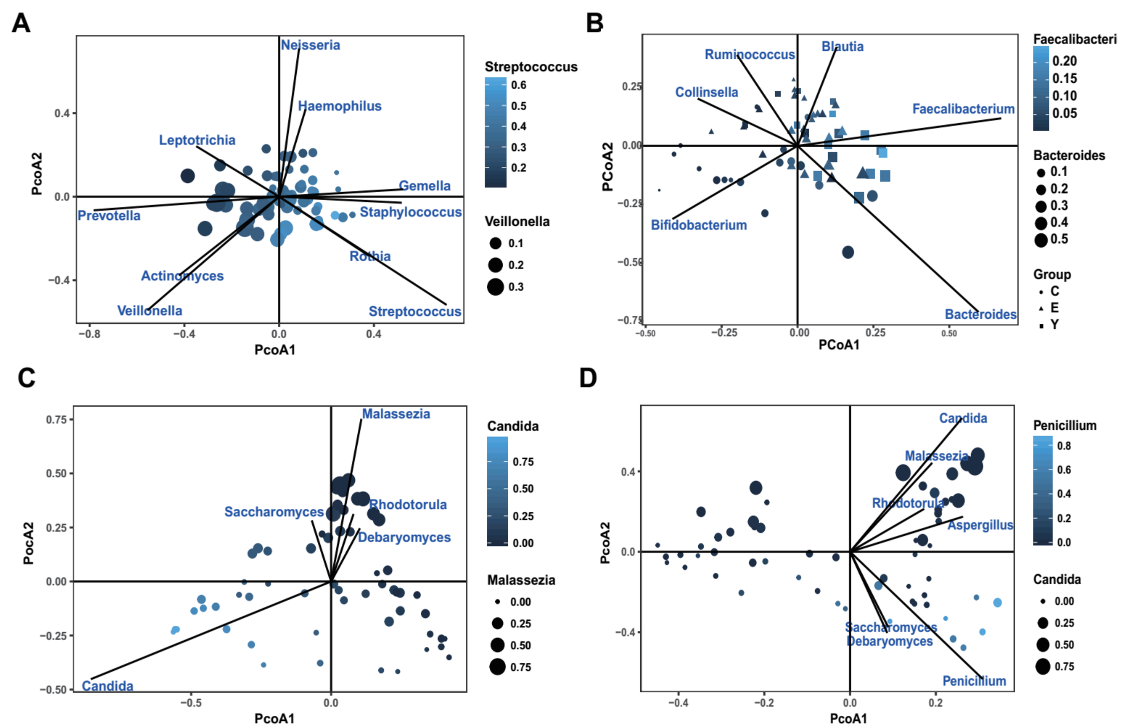


Figure 3.12 Driving forces of the bacterial and fungal community structure in oral and gut. Identify the bacterial and fungal genera with a significant contribution (Permutational correlation test, 999; $p < 0.005$) to the individual ordination in PCoA based on the Bray-Curtis distances of the relative abundance of each genus. Samples are indicated as dots in plot, the color and size were identified by the relative abundance of the top two dominant genera. The ten dominant genera that significantly contributed to variation and their relative lengths which present the fitness are shown.

4.2.4 Variation of the relative abundance of taxa among age groups in different habitats

In comparing the genera with significant differences in abundance between the three age groups in Figure 3.13, we found that more bacteria were significantly distributed between age groups compared with fungi. Besides, fungi showed accumulation in the

elderly compared with the young and centenarian in the skin. Bacteria displayed diverse features for the enrichment of genera. For instance in the gut, *Blautia, Roseburia* and *Feacalibacterium* exhibit an age dependent decrease pattern. *Prevotella* and *Clostridium* were enriched in the elderly but were lower in the young and centenarian groups. *Bilophila*, *Butyricimonas* and *Parabacteroides* had similar abundance in the young and elderly but were enriched in centenarians. Moreover, in the skin, the *Propionibacterium* decreased with aging. In the palm habitats, *Streptococcus, Selenomonas*, *Ruminococcus* and *Veillonella were* enriched with aging, *Staphylococcus* had the the lowest abundance in the elderly but were especially high in the centenarian group. In the face habitat, *Corynebacterium* had similar abundance in the elderly and centenarians but was significantly lower in abundance in the young. *Veillonella*, *Actinomyces*, *Leptotrichia* and *Fusobacterium were* only enriched in centenarian. The relative abundance of *Malassezia* was statistically different among the age groups, the elderly had the lowest relative abundance of *Malassezia*. *Saccharomyces* and *Debaryomyces* were decreased with aging in the gut. *Rhodotorula* was significantly enriched in the young and had similar abundance in the elderly and centenarians oral habitats. In the umbilical site of elderly, high abundance of *Penicillium* and *Naganishia* were observed.

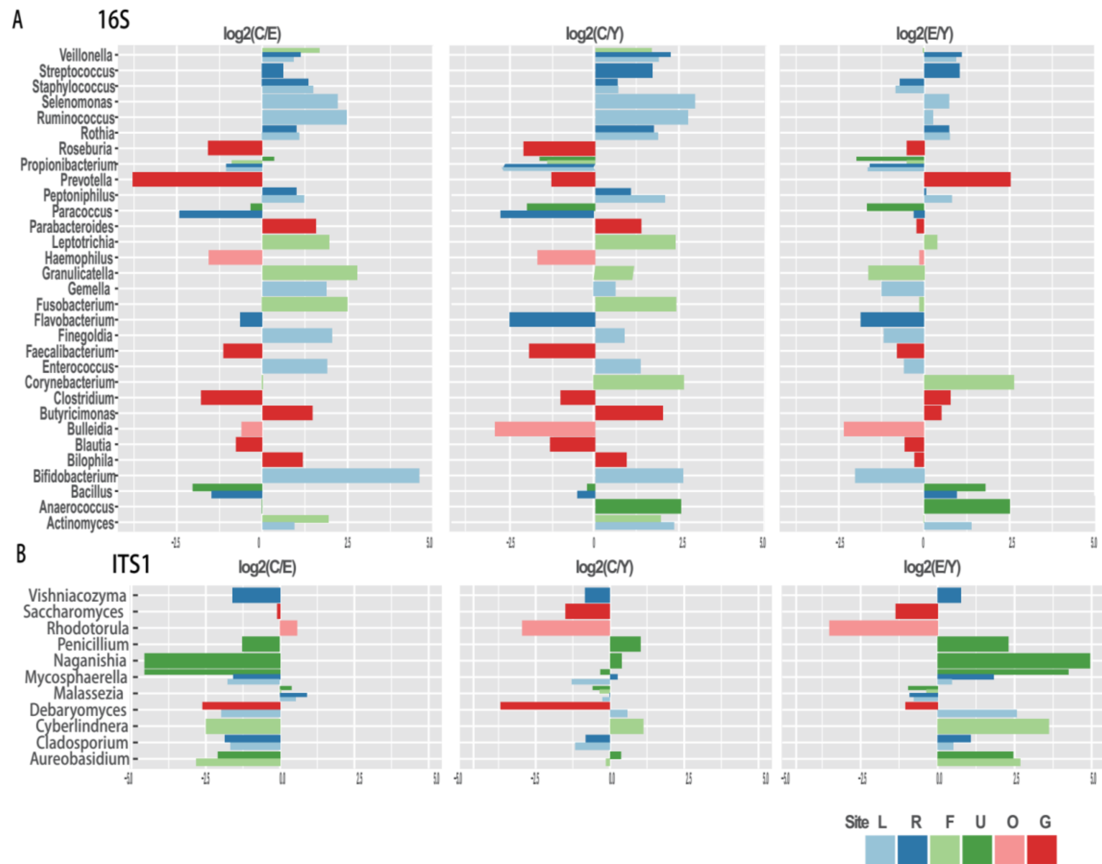


Figure 3.13 Boxplot compared the relative abundance of bacterial and fungal genera that were significant differently distributed in three age groups. Only genera that detected have abundance >0.1% and with significant variation between age groups (detected by ANOVA analysis followed by Tukey-kramer test, $p<0.05$) were plot. Color coded by different body habitat sites.

5. Correlations between bacteria and fungi in each habitat

The correlation between bacterial and fungal community dissimilarities for each individual within habitats was investigated using the Mantel-test Table 3.5. A significantly positive correlation between bacterial and fungal community

dissimilarities was detected in palms, showing that two individuals with similar bacterial communities were associated with similar fungal communities in palms. While for other habitats, the correlation was not significant.

Table 3.5 The correlation between bacterial and fungal community dissimilarities for each individual within habitats was investigated by Mantel-test based on Bray distance matrix derivate from the bacterial and fungal compositional profile.

Habitats	Mantel r statistic	p-value
L	0.3331	0.001
R	0.33803	0.001
F	0.12436	0.122
U	0.03653	0.696
O	0.05528	0.356
G	0.12172	0.051

Pearson's correlation revealed that for different niches, the correlation of the bacterial and fungal taxa was diverse (Figure 3.14). In the skin bacteria and fungi exhibit more significant inner and inter correlation. While in the oral cavity, only the bacterial genera had a significant inner correlation. For the gut, inner and inter bacteria and fungi correlation was not abundant but the correlation between fungi and bacteria was

stronger than that in the oral. We also observed that the correlation between the same taxa in different niches can be distinct. For instance, *Candida* and *Malassezia* had a significant negative correlation in the skin and oral but they didn't show a significant correlation in the gut. The strongest positive correlation occurred between the *Meyerozyma* and *Rhodotorula* in the gut. *Ruminococcus* and *Wallemia*, *Dialister* and *Trichosporon* also exhibit significant positive correlation. In the oral sites, *Rhodotorula* showed significant positive correlation with *Cryptococcus*, but without a significant correlation with *Meyerozyma* which was observed in the gut. A strong negative correlation was found between *Prevotella* and *Streptococcus* in oral but only a week positive correlation was observed in the skin.

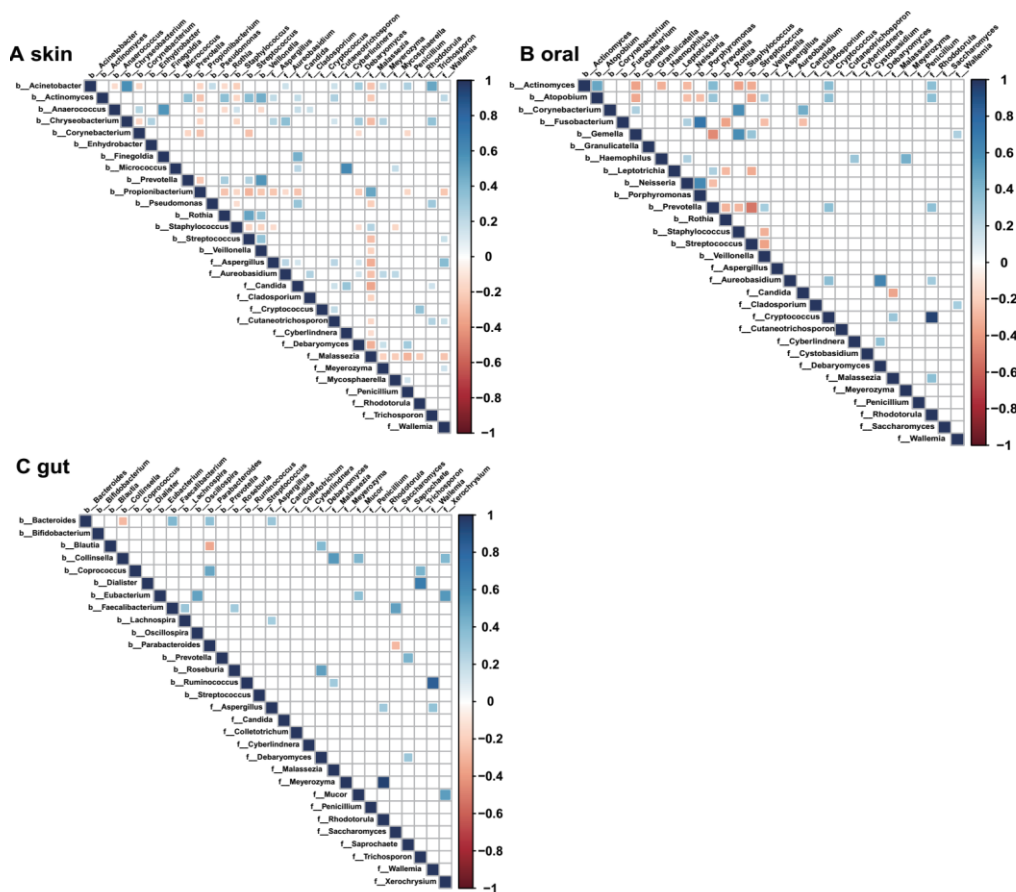


Figure 3.14 Correlation between fungal and bacterial genera in different body habitats. The colors of the squares represent correlation strength between the relative abundance of genera in each individual. Only significant correlation was plotted ($p<0.05$). Only shown the most dominant 15 bacterial and fungi genera. "f_" indicated the fungi and "b_" indicated the bacteria.

6. Correlations between gut microbiota and clinic parameter

Since gut microbiota is closely related with human health and disease, we further investigated the correlation of the clinical parameters and gut microbiota. The clinical parameters including MMSE (Mini-Mental State Examination), MNA (Mini Nutritional Assessment), FIM (Functional Independence Measure), Age, weight , and the number of drugs taken. We found that the clinical parameters were significantly correlated with the separation of the individuals in the PCoA for the gut bacterial community but not significantly correlated with the fungal community. The clinical parameters significantly correlated with the bacterial community in PCoA are shown in Figure 3.15. Compared with the young and the elderly who shared similar clusters in PCoA, Centenarians' clusters shifted toward a direction correlated with increased age and medication and lower scores of FIM, MMSE and MNA. The centenarians with higher levels of FIM, MMSE and MNA score tend to have more similar gut microbiota composition with the young and the elderly. We also observed that MNA

was positively associated with FIM and MMSE, emphasizing the important role diet may play in maintaining the healthy aging.

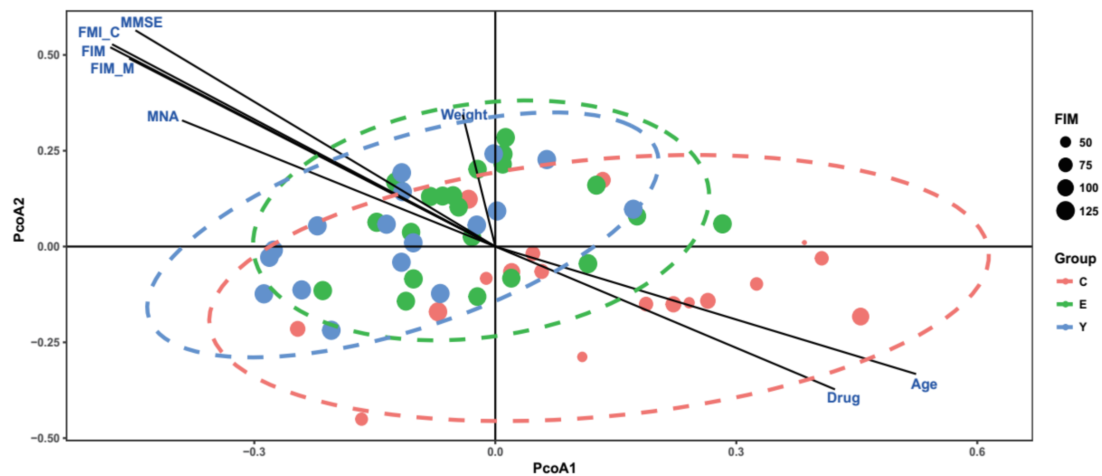


Figure 3.15 Correlation between the clinical parameters and gut microbiota composition in PCoA. Clinical parameters with significant correlations with the ordination of the diagram were visualized as arrows. ($p<0.05$, permutations test, N=999). The length and direction of each arrow indicates the parameter's strength with the ordination configuration.

Chapter Ⅳ. Results: Compositional and functional profiles of gut microbiota in Sardinian centenarians

1. Cohort characteristics

We recruited a cohort including three age groups in Sardinia: young (N=18, age from 21 to 33), healthy elderly (N=25, age from 68 to 88) and centenarians (N=21, age from 99 to 107). The clinical characteristics are shown in Table 4.1.

Table 4.1 Statistics of the clinical characteristics and health measurements in three age groups.

Parameters	Centenarians(n=19)	Elderly(n=23)	Young(n=17)
	Mean±SD (Range)		
Age(yrs)	101.8±1.4(99-107)	76.7±5.9(68-88)	25.5±3.5(21-33)
Female (%)	76.5%	56.6%	41.2%
Weight (kg)	57.1±5.7（43-73）	68.7±14.3(42-103)	63.2±3.5(44-95)
BMI (kg/m^2)	23.5±2.1(17.9-28.1)	25.9±4.1 (19.5-36.9)	22.8±3.7(16.1-40.1)
MMSE (0,30)	15.8±6.7(5-26)	26.6±3.0(22-30)	30(30-30)
MNA (0,30)	18.9±3.7(8-26)	24.1±2.0(18-28)	24.3±1.3(20.5-28)
FIM (0,126)	77.5±21.1(31-123)	123.7±1.9(119-126)	126(126-126)

* Total number of subjects is 59 excluding individuals with unqualified stool samples (N=6).

Values are presented as Mean±SD with the range in parentheses.

BMI: Body Mass index; MMSE: Mini-Mental State Examination; MNA: Mini Nutritional Assessment; FIM: Functional Independence Measure

2. Survey of Sardinian gut microbiota by shotgun metagenomic sequencing

A total of 59 qualified stool samples were used to extract microbial DNA for DNA library construction and shotgun metagenomic sequencing on Hiseq X10. On average

5.8 Gb data (approximately 41.3 million high-quality clean reads) were generated per sample. Human contamination was removed (on average, up to 14% of the total reads) before further processing. The taxonomic compositional profile was generated by MetaPhlAn2 that relied on unique clade-specific marker genes identified from 3,000 reference genomes. We verified our results using the IGC database. The workflow is shown in Figure 4.1.

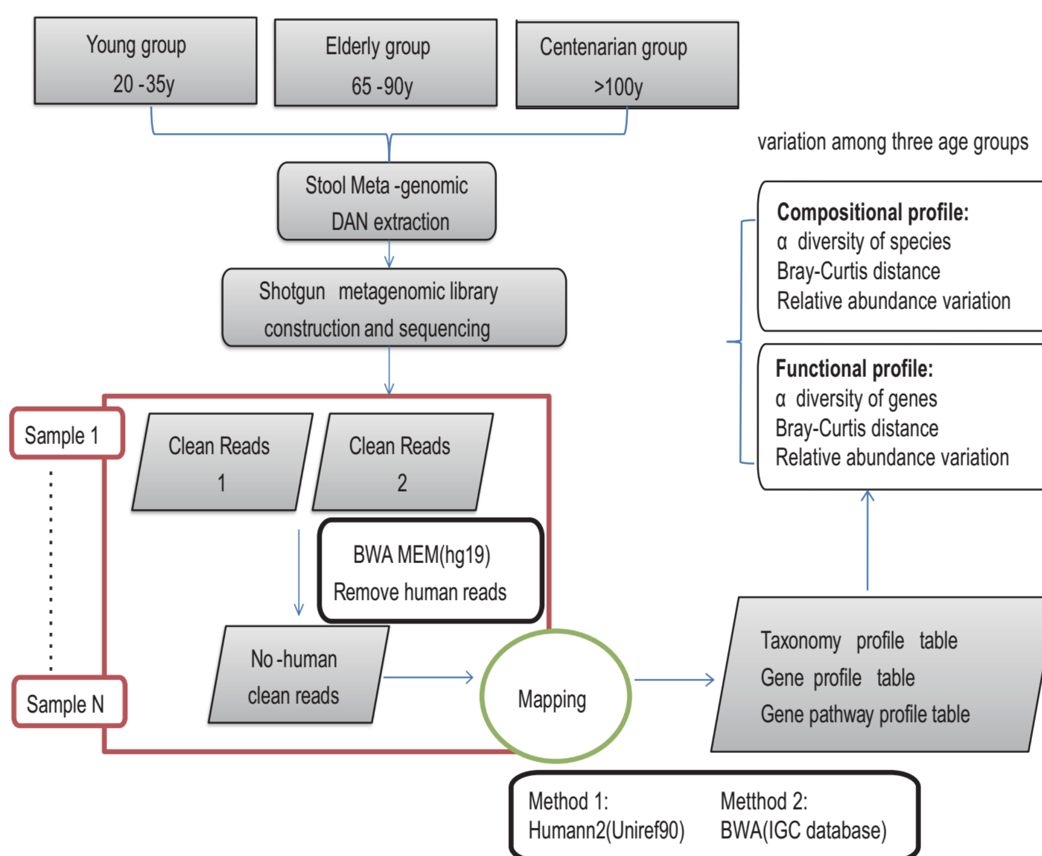


Figure 4.1 Workflow to analyze gut microbiota in the Sardinian population.

3. Gut microbiota compositional profile in Sardinian across age

3.1 Comparison of gut bacterial communities by shotgun metagenomic sequencing and 16S rRNA sequencing

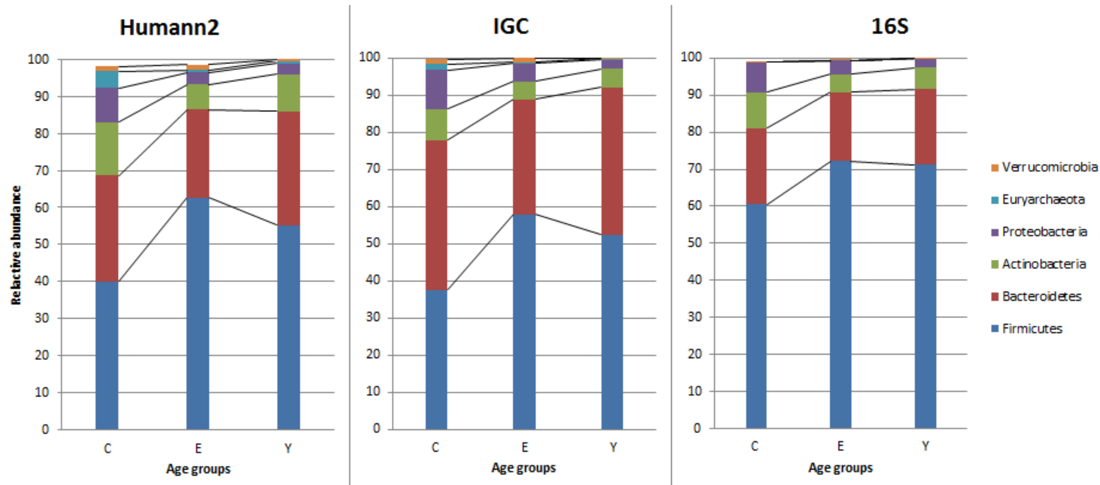


Figure 4.2 Comparison of the dominant phyla between shotgun metagenomics sequencing and 16S rRNA sequencing datasets.

Examination of the gut microbiota compositional profiles at the phylum level for three age groups revealed that the gut microbiota for Sardinians are dominated by *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*, which corresponds with previous research [15]. Comparing the dominant phyla between shotgun metagenomic sequencing (with two different databases) and 16S rRNA sequencing datasets (Figure 4.2) reveals that the proportion of dominant phyla display variation between the three datasets. For example, the dominant phyla, Firmicutes are less frequently detected in shotgun metagenomics sequencing compared with 16sRNA gene sequencing, when using either the Humann2 or IGC databases. On the other

hand, *Bacteroidetes*, *Euryarchaeota* and *Verrucomicrobia* are more frequently detected. Similar results were also found by others [170]. Since sample preparation for DNA extraction is the same, the variation may be caused by the bias of two sequencing methods. Although there is variation between different databases, the patterns for the phyla with each age group is similar, therefore we used the Humann2 database to further explore the structure of the gut microbiota in different age groups.

3.2 Achaea and virus in gut detected by shotgun metagenomic sequencing

Shotgun metagenomic sequencinig can detect not only bacterial composition but also the presence of Achaea and viruses in the gut. Methanobrevibacter smithii, the dominant *Archaea* in the human gut ecosystem was frequently detected in Sardinians, the mean relative abundance reached 1.8%. We detected viruses belonging to *Caudovirales* which are double strand DNA virus. The mean relative abundance of *Siphoviridae* represented 0.58% and is the most abundance virus species detected, followed by *Myoviridae* represented by 0.056%. Bacterial phage belongs to *C2likevirus* was most frequently detected genus.

3.3 Compositional variation of gut microbiota among different age groups

3.3.1 Phylum level variation among the three age groups

Firmicutes has a higher abundance in the elderly group compared with that of the young group, and the abundance is even greater in the centenarian group when

compared to the elderly group (Kruskal-Wallis test, χ^2 = 12.893, df = 2, p-value = 0.0016). The variation of abundance for *Firmicutes* among three age groups was tested using ANOVA (Figure 4.3).

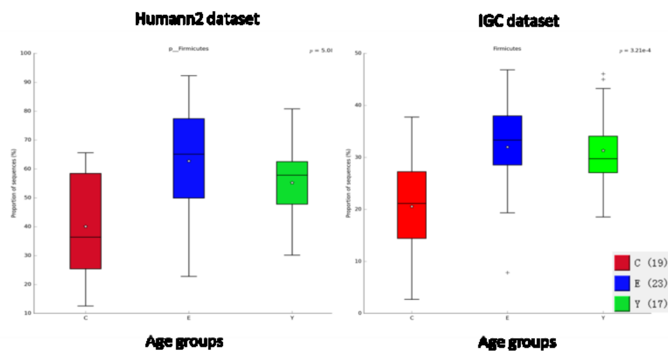


Figure 4.3 Boxplot of the abundance of *Firmicutes* among three age groups

Proteobacteria is enriched in the elderly and especially enriched in centenarians when compare to the young individuals (Kruskal-Wallis test, χ^2 = 9.0686, df = 2, p-value = 0.0107). As the most abundant two phyla in the gut, the *Firmicutes/Bacteroidetes* proportion (F/B) is an important index for the structure of gut microbiota. The F/B ratio was significantly lower in the centenarians compared with the elderly (Figure 4.4).

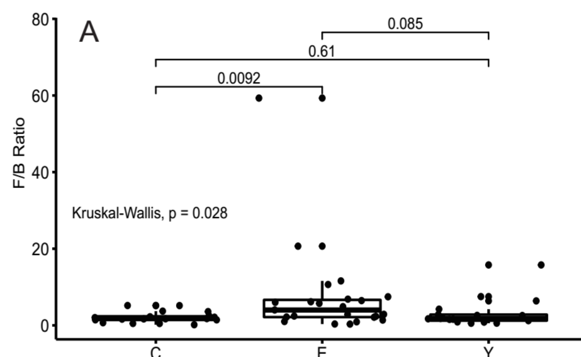


Figure 4.4 F/B ratio in the gut microbiota among three age groups.

3.3.2 Genus level variation among the three age groups

To explore gut microbiota composition in detail, we determined the relative abundance of each genus of gut microbiota for the three age groups (Figure 4.5). The relative abundance of the dominant genus was sharply rearranged in centenarians compared with that of young and healthy elderly individuals. Furthermore, the total amount of the sub-dominant genus showed an age dependent pattern, in which the centenarians group was significantly enriched in the sub-dominant genus.

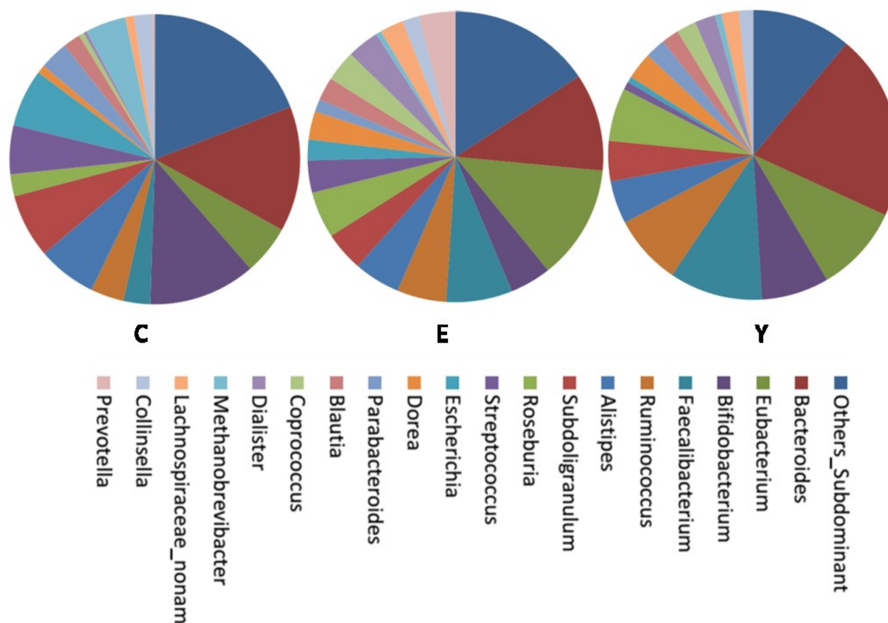


Figure 4.5 Frequency of the top 20 genera. Other low-abundant genera are summed into one group labeled " Others_Subdominant".

Comparing the taxonomy compositional profile within each age group, we found several genera that were significantly different in abundance and prevalence among the three age groups. The dominant genera that were significantly distributed among

the age groups are shown in Figure 4.6 (ANOVA followed by Tukey-Kramer post-hoc test, p<0.05).

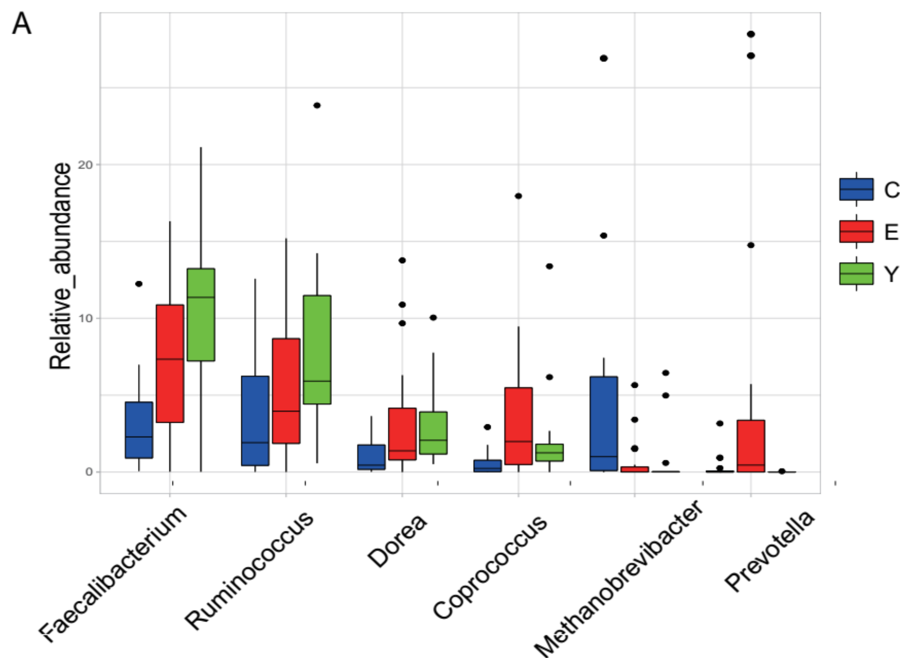


Figure 4.6 Statically significant different genera distribution in three age groups. Boxplot with standard deviation of the mean value of relative abundance of genera in different age groups is shown. Only the dominant genera with significant difference (p value<0.05) between age groups detected by ANOVA followed by Tukey-Kramer test are shown.

For instance, the relative abundance of *Faecalibacterium* and *Ruminococcus* are lower in the elderly compared with the young, and yet even lower in the centenarians. A low relative abundance *for Corprococcus* and *Dorea* were also observed in our centenarian cohort compared with the young and elderly. A high abundance of *Prevotella* is observed in 8/23 of our elderly individuals (Figure 4.7). *Methanobrevibacter*, a dominant *Archaea* in the human gut ecosystem, has a high

frequency in centenarians (Figure 4.7). *Pyramidobacter* was also found to be only enriched in centenarians.

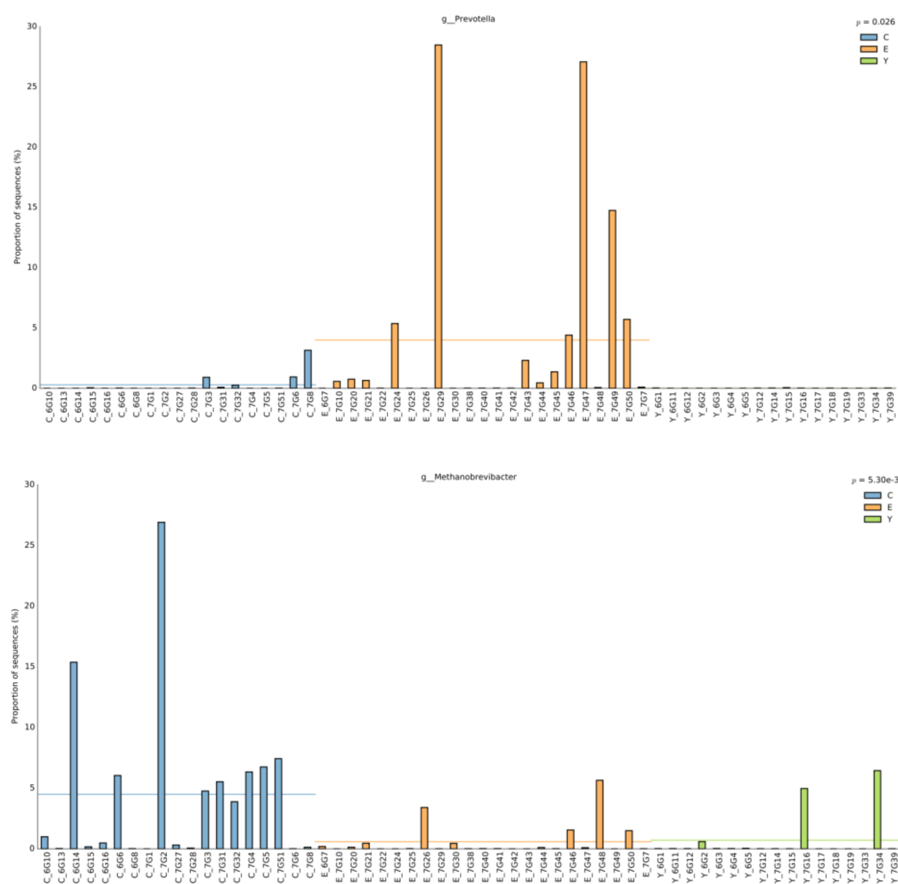


Figure 4.7 The abundance of *Prevotella* and *Methanobrevibacter* in three age groups. Three age groups were indicated by different color: blue for centenarian group (C), orange for the elderly group (E) and green for the young group (Y). Variations among three age groups were tested by ANOVA, with p value <0.05.

To further investigate the similarity of the community structure of the gut microbiota for each individual among the three age groups, Principal Coordinates Analysis

(PCoA) based on the Bray-Curtis distance matrix of the relative abundance of genus was used to visualize the distribution and cluster of the subjects. The individuals were clustered by three age groups with an ellipse of 95% confidence. We found that the three age groups clustered separately: the elderly group was positioned similar to the young group but both of them were distinct from the centenarian group (Figure 4.8). The elderly group cluster overlapped with the young group but showed a slight shift, while the centenarians, had some subjects with a profile similar to those of the young and elderly, but the cluster shifted in a different direction from that of the elderly. Analysis of similarities (ANOSIM) test using Bray-Curtis distance revealed that no significant differenence in the composition of gut microbiota at the genus level was evident between young and elderly (R-value=-4.602e-05, p-value =0.464). However significant differenence between centenarian and young (R-value=0.1792, p-value =0.001), and significant differenence between centenarian and elderly was oberved (R-value=0.1707, p-value =0.001). Multiple Response Permutation Procedure (MRPP) analysis revealed that the delta of the young was 0.65, while that of the elderly was 0.74 and the centenarian was 0.76 (p = 0.001, A =0.03). This analysis shows that within group distance is larger in the elderly and centenarian groups, and the inter-group distance is significantly greater than that of the inner group. We discovered that the distribution of the individuals in the PCoA was driven by the dominant genera (Figure 4.9).

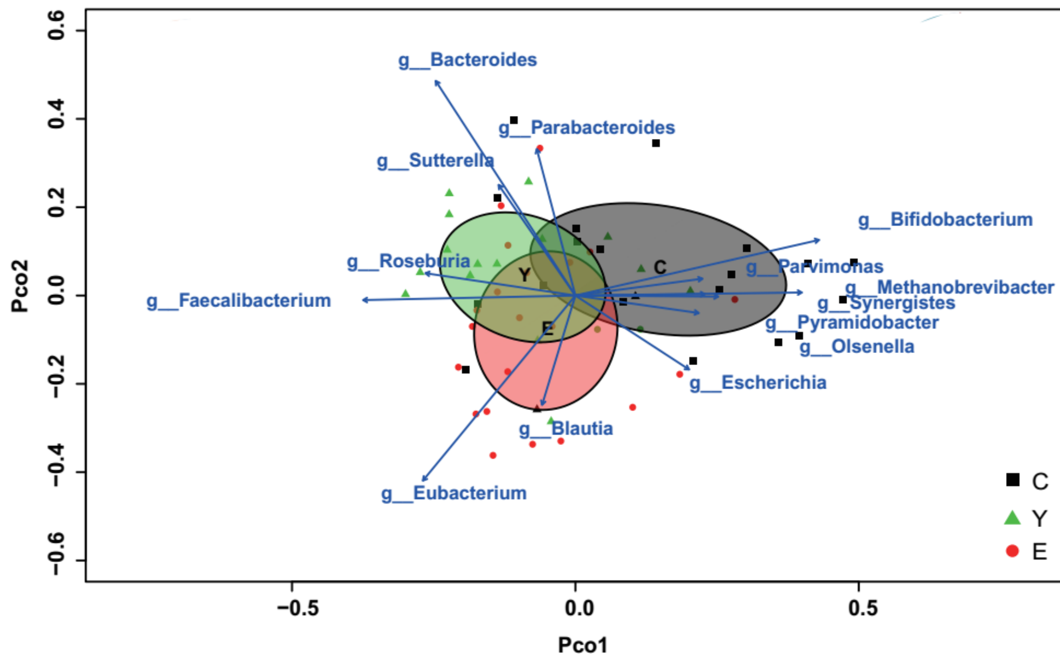


Figure 4.8 Gut microbiota composition for three age groups at the genus level in PCoA. Ellipses around the centroid are plotted; the age group is labeled as C for the centenarian, E for elderly and Y for young. The genus that significantly correlated with the ordination in PCoA are shown as arrows (Permutation test, p<0.01), the length of the arrow indicated for the Goodness of fit statistic: Squared correlation coefficient. Bray-Curtis distance was used for PCoA.

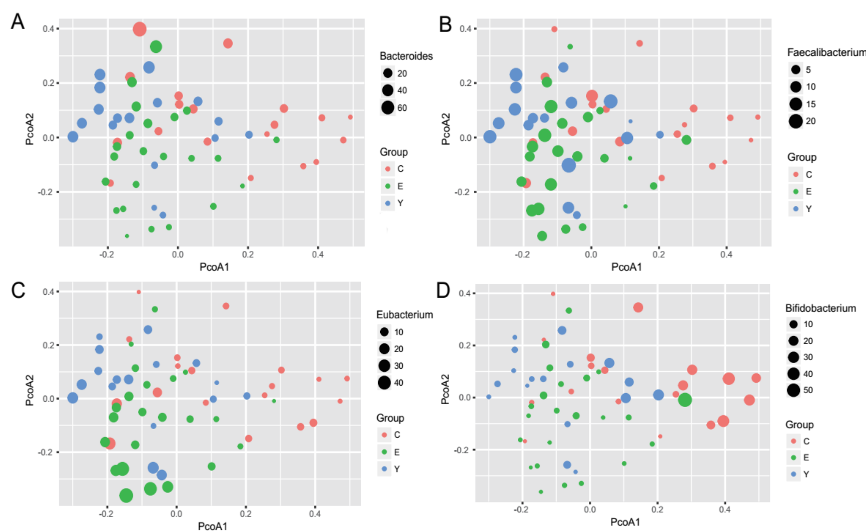


Figure 4.9 Relative abundance of the dominant genera in PCoA. Relative

abundance of the dominant genera shows that the similarity of the individuals is driven by the relative abundance of the dominant genere: Bacteroides (A); Faecalibacterium (B); Bifidobacterium (C) and Eubacterium (D). The relative abundance of the four domiant genera was shown by the size of the plots in each PCoA. The age group is labeled as C for the centenarian, E for elderly and Y for young with a different color.

The genera significantly contributing (permutation correlation test, p value<0.01) to the ordination of the samples are shown in Figure 4.8. *Faecalibacterium, Bacteroides, Roseburia*, *Sutterella and Parabacteroides* were positively correlated and significantly contributed to the cluster of the young group, while *Eubacterium* and *Blautia* were positively correlated and significantly contributed to the cluster of the elderly. The enrichment of *Bifidobacterium, Methanobrevibacter*, *Pyramidobacter, Synergistes* and *Escherichia* were detected and positively correlated with the cluster of centenarians.

The heatmap of the relative abundance of the genera that significantly correlated with the cluster of age groups is displayed in Figure 4.10. Interestingly, eight centenarians formed a separate group in the hierarchical cluster with high abundance of *Bifidobacterium, Methanobrevibacter and Echerichia.*

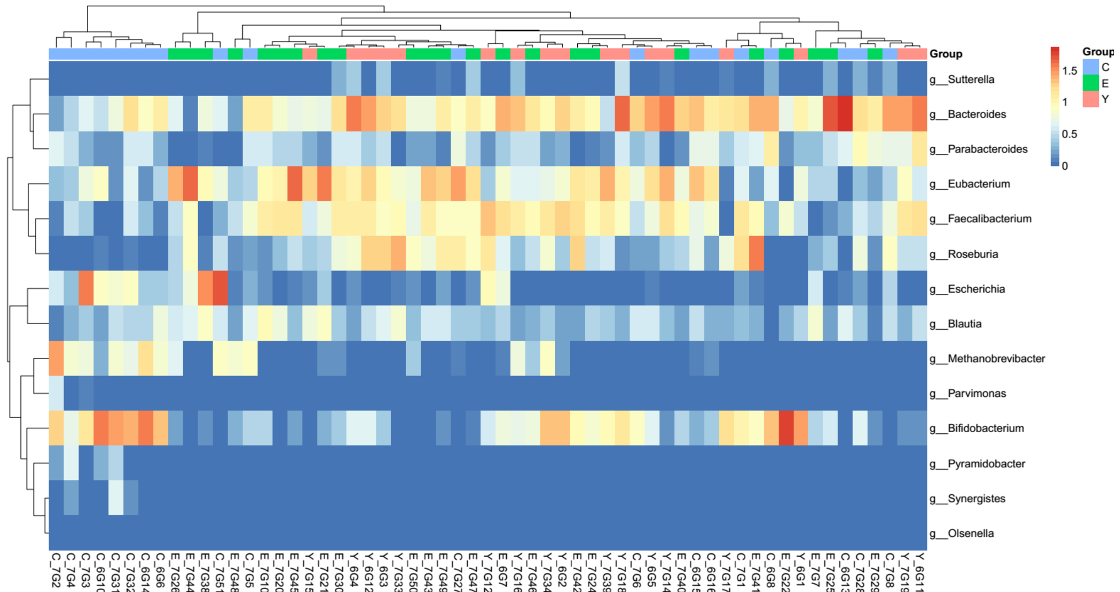


Figure 4.10 Heatmap of the relative abundance of genera that are significantly correlated with the samples separation in PCoA in three age groups. The relative abundance was base 10 logarithm as input, complete linkage clustering was used.

3.3.3 Species level variation among the three age groups

Survey the taxonomic composition at the species level, we found the α diversity of the gut microbiota was not significantly different among age groups. Compared with young and elderly individuals who shared a similar species α diversity, the centenarian group had higher α diversity but was not significant (Figure 4.11).

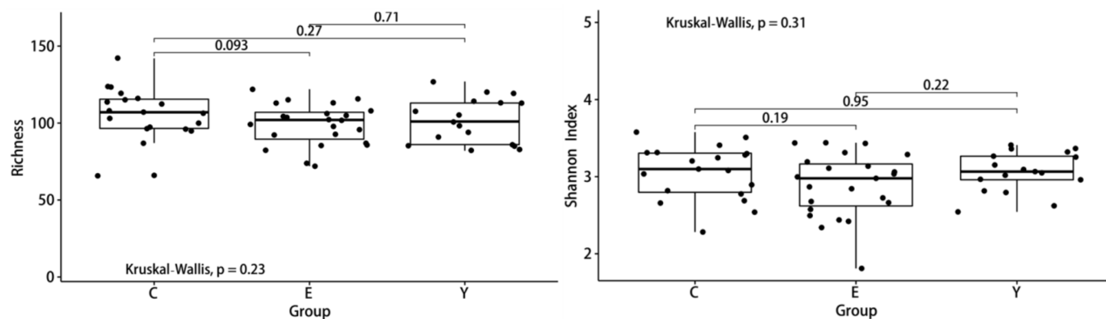


Figure 4.11 Species richness and Shannon index for three age groups. Boxplot with standard deviation of the mean value of Species richness and Shannon index of in different age groups is shown. The age group is labeled as C for the centenarian, E for elderly and Y for young. The variation among three age groups is detected by Kruskal-Wallis test.

The dominant species that were significantly different among the three age groups are shown in Figure 4.12.

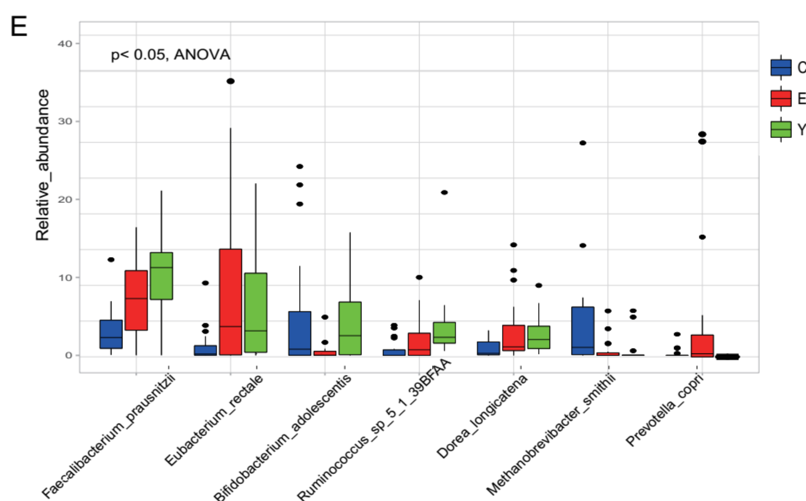


Figure 4.12 Statically significant different species distribution in three age groups. Boxplot with standard deviation of the mean value of relative abundance of species in different age groups. Only the dominant species with significant difference (p value<0.05) between age groups detected by ANOVA followed by Tukey-Kramer test are shown.

3.4 Core microbiota in different age groups of Sardinians

The core microbiota referred as a group of microbes shared among individuals, it's found associated with the function of the microbiota and healthy status of the host [152, 171]. Studies in the elderly population observed the age related loss of diversity in the core gut microbiota [62, 152]. In our dataset, we found that at the genus level the richness of the core microbiota (shared by 50% individuals) among three age groups is similar, while at the species level, the centenarians show an increase of the core microbiota richness compared with young and elderly (Figure 4.13).

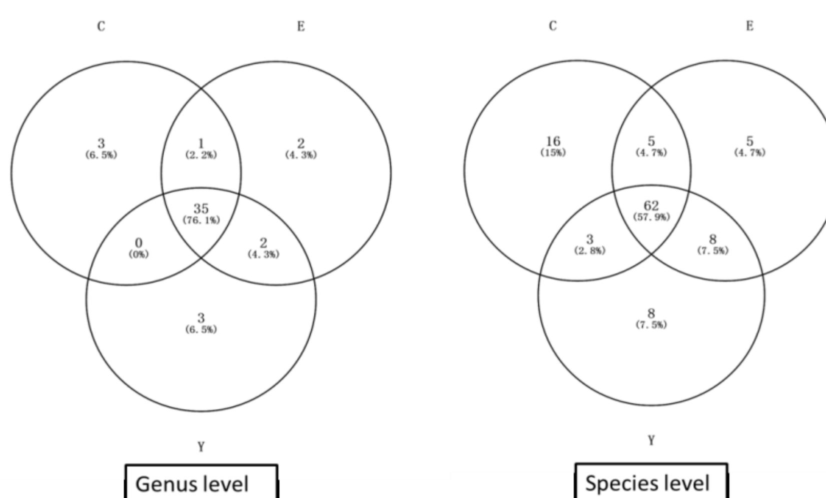


Figure 4.13 Core microbiota in different age groups. Vienn diagram representation of the core microbiota at the Genus level and the Species level. Percentages are shown in ().

The relative abundance of the centenarian specific core microbe distribution in three age groups are shown in Figure 4.14. We observed the enrichment of several species

belonging to *Bacteroides, Bifidobacterium, Clostridium, Erysipelotrichaceae* and *Lactobacillus* in centenarians.

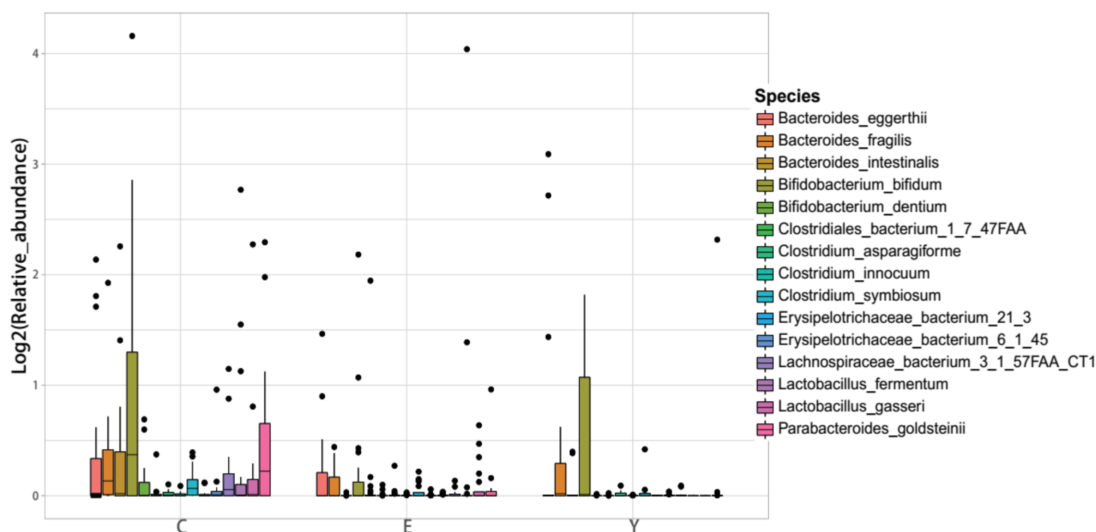


Figure 4.14 Distribution of the relative abundance of centenarian unique core microbiota in three age groups. The relative abundance of centenarian unique core microbiota in three age groups is represented using Box plots for centenarians (C), elderly (E), and young (Y).

3.5 Co-abundance network of taxa in gut microbiota for three age groups in Sardinia

Correlation analysis of the genus in the gut microbiota (Figure 4.15) revealed that *Bacteroides* as the most abundant genus in the gut formed a positive network with dozens of other sub-dominant genus. *Bacteroides* showed no correlation with *Faecalibacterium, Bifidobacterium* nor *Eubacterium.* Meanwhile, *Bifidobacterium* and *Eubacterium* show a strong negative correlation. Besides, *Feacalibacterium*,

Roseburia and *Coprococcus* show a positive correlation, corresponding to the same pattern in three age groups. Interestingly, there are positive association among *Lactobacillus*, *Methanobrevibacter* and *Streptococcus*, which all show a correlation with dairy product consumption [172, 173].

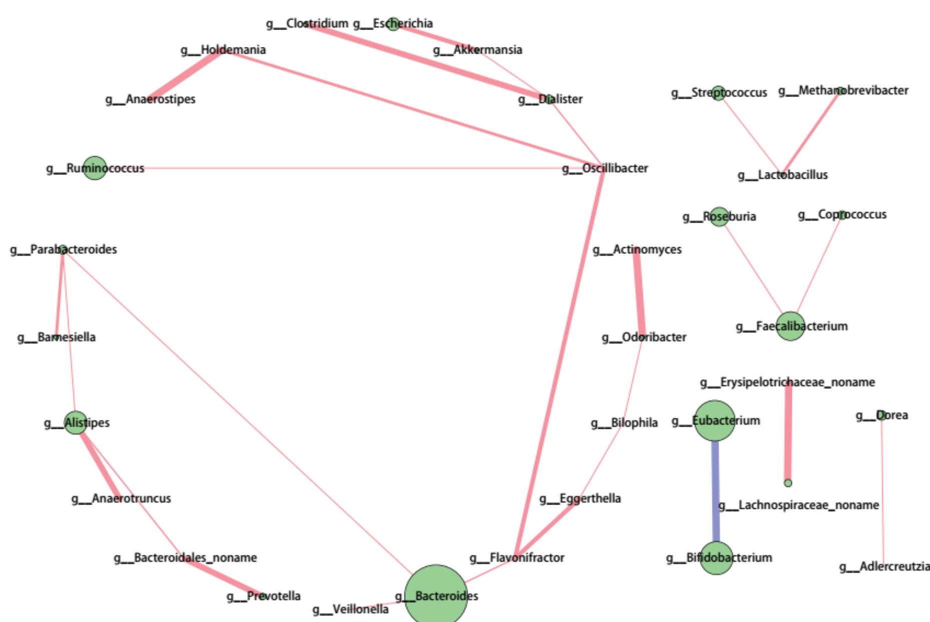


Figure 4.15 Co-abundance network of taxa in gut microbiota for Sardinian in age groups. Genera were plotted as circle nodes in the network. The size of the circles indicated the mean abundance in the Sardinian population. The edge represents the Pearson's correlation between each genus. Only the absolute correlation that >0.3 is shown. The thickness of the edge line indicates the proportion to the strength of the correlation; the colors represent positive and negative correlations.

The patterns of co-abundance networks of the core microbes in the gut microbiota

were obtained by calculating the associations among core species. The networks show several separated clusters of co-abundance groups, which represented the main basic gut microbiota structure in Sardinians (Figure 4.16). As the most abundant species, *Faecalibacterium prausnitzii* and *Eubacterium rectale* did not form a cluster with other species. Different species of *Bacteroides* formed a cluster separated from the core cluster. Interestingly, *Subdoligranulum* correlated with a group of species that formed the core co-abundance cluster. *Methanobrevibacter smithii* and *Bifidobacterium adolescentis* are not only significantly enriched in centenarians, but also are significantly correlated within all the age groups.

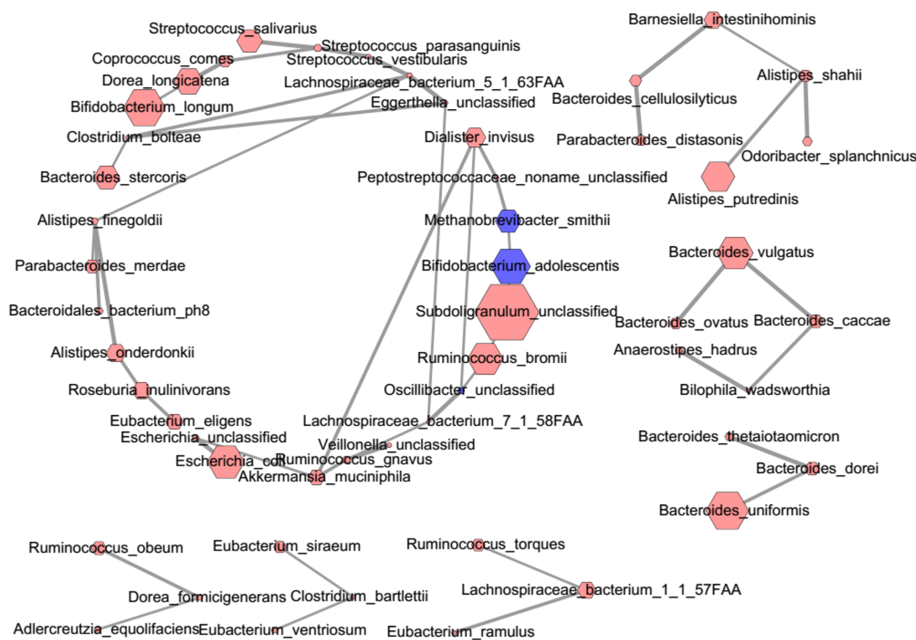


Figure 4.16 Network of the core species shared by young, elderly and centanarians. Core species shared by the three age groups in Sardinians were plotted as circle nodes in the network. The size of the circles indicated the average species

abundance in the Sardinian population. The edge represents the Pearson's correlation between each pathway. Only the absolute correlations that >0.3 with p>0.01 are shown.

4. Compared gut microbiota in Sardinian with other populations

The variation of gut microbiota has previously been studied for different geographical populations [174]. In our study, we compared the findings of the Italian study with our findings and found that at the phylum level, the Sardinians have a different profile compared with urban individuals from Bologna, Italy [78]. Interestingly, *Protebacteria* has low abundance in Bologna and a Dutch cohort [170] but are shared by the Sardinian and Hadza cohorts [78] and are enriched in the Sardinian centenarians. The abundance of *Bacteroidetes* is similar for Sardinian and Hadza populations, having higher abundance when compared with Bologna and Dutch populations. Meanwhile, the abundance of *Actinobacteria* is extremely low in the Hadza population but is higher abundance in Sardinian, Bologna and Dutch populations (Figure 4.17)

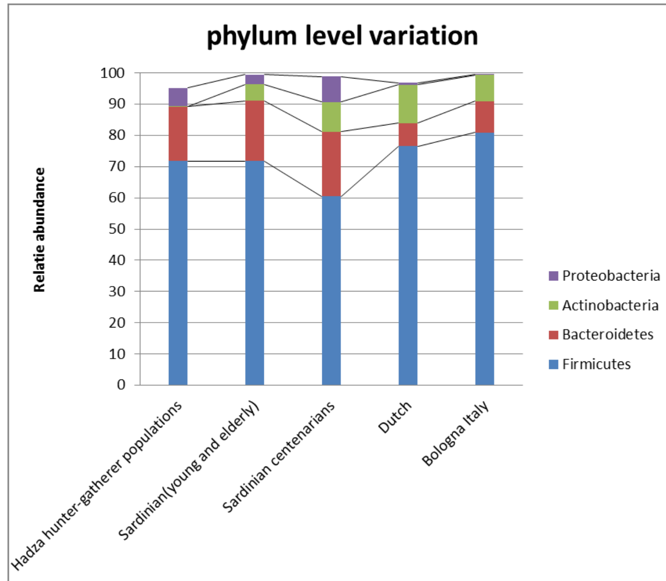


Figure 4.17 Relative abundance of dominant phylum in different populations. The Hadza hunter-gatherer population and Bologna data were generated by 16s rRNA V4 sequencing [78]; the Sardinian dataset was generated by 16s rRNA V3V4 sequencing in the present study; and the Dutch dataset was generated by 16s rRNA v3V4 sequencing [170].

Comparing the Bologna results on gut microbiota in centenarians [63] with their data, we found certain features that are shared with two cohorts. At the phylum level, we found that centenarians in both cohorts accumulated *Proteobacteria* and *Actinobacteria* (Figure 4.18).

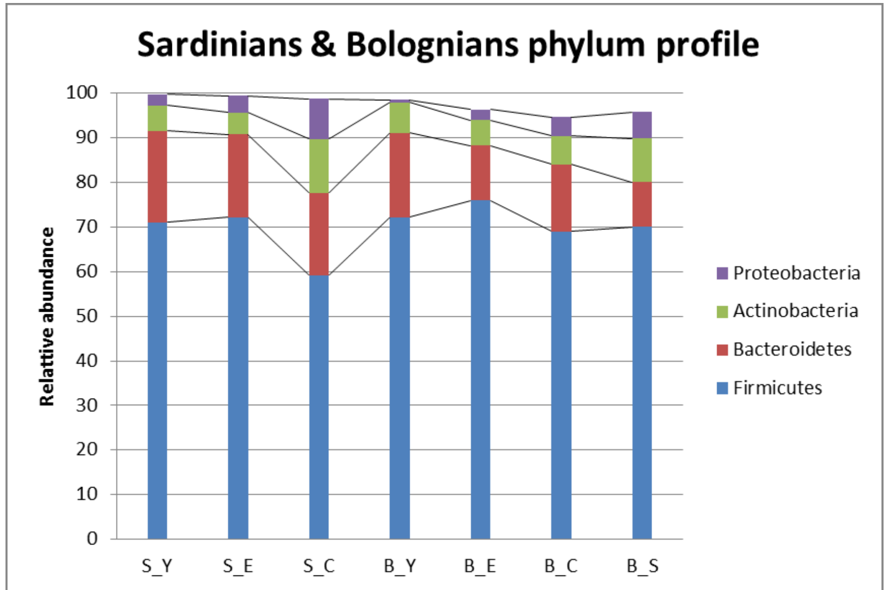


Figure 4.18 Relative abundance of dominant phylum in Sardinian and Bolognian cohorts. Both data were generated by 16s rRNA V3V4 sequencing.

In the PCoA plot (Figure 4.19), the young and elderly in both studies are similarly clustered for both populations, and the centenarians clustered separately.

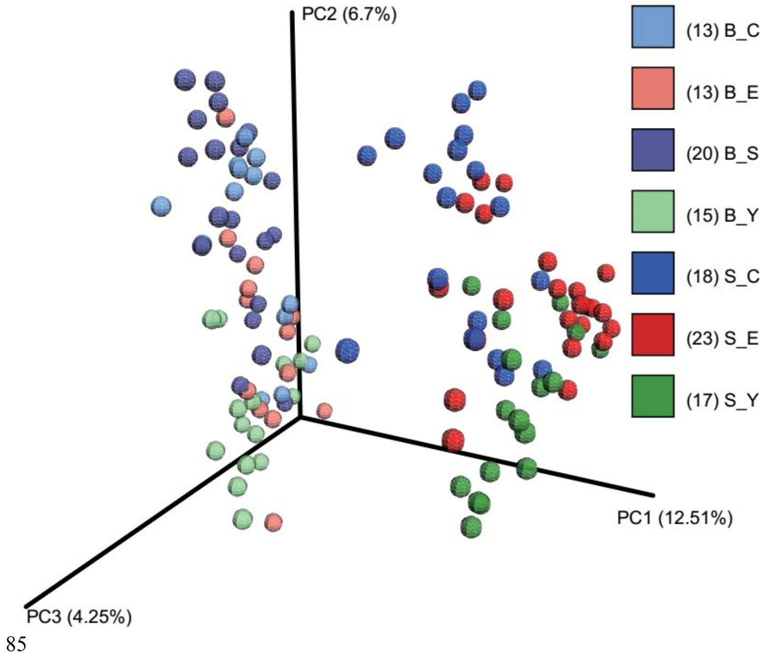


Figure 4.19 The bacterial community similarities for different age groups in PCoA for Sardinians and Bolognians

At the genus level, our Metagenomic sequencing detected the enrichment of *Bifidobacterium* and *Methanobrevibacter*. Both genera were found enriched in Bologna centenarians, while, the enrichment of *Akkermansia* reported in Bologna centenarians [63] is not high enriched in Saridnian centenarians (Figure 4.20).

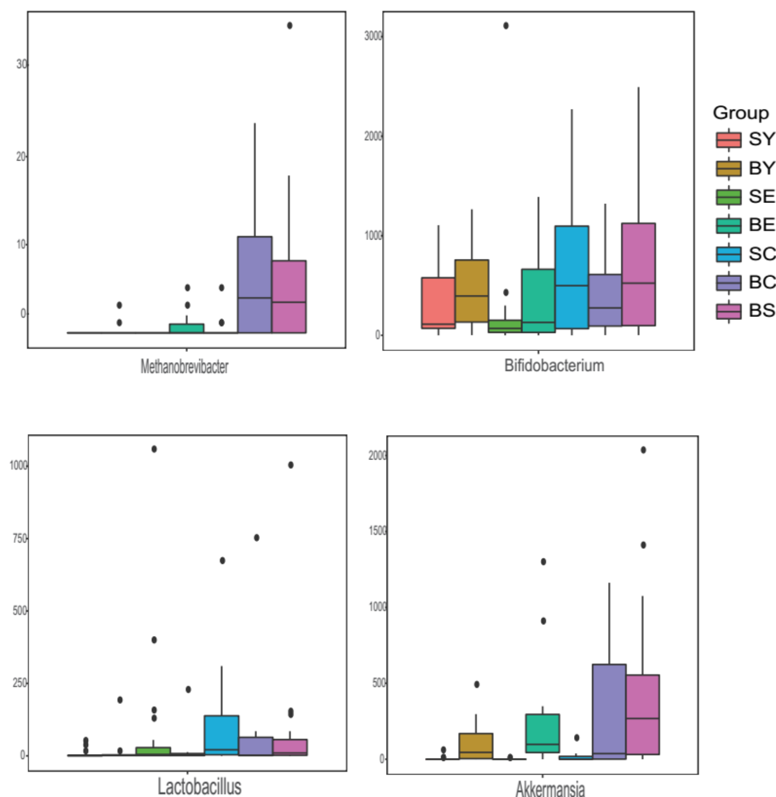


Figure 4.20 Comparison of the relative abundance for several genera in different age groups for Sardinian and Bolognian cohorts.

5. Gut microbiota functional profiles in Sardinian cohorts of different ages

To determine if metabolic activities in gut microbiota shift with aging, metagenomic sequencing data were processed by Humann2 pipeline using the UniRef90 database. The relative abundance of gene families and gene pathways were obtained. We detected 384,425 gene families assigned to 1,924 species. A total of 463 gene pathways were rebuilt, which were calculated from the constituent gene family abundance for each individual. After normalizing and regrouping the gene family profile into a Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology profile, we obtained the mean relative abundance profile for each of the KEGG orthology (KO) in the three age groups.

5.1 Validate the functional annotation of gut microbiota from two databases.

For two dataset, the Shannon diversity and richness of KO both show significant increases in the centenarian group consistent with the increase of Shannon diversity of genus in gut microbiota, while the diversity in the young and healthy elderly was not significantly different (Figure 4.21). Considering that the two datasets have similar results, we next used the Humann2 dataset to obtain functional annotation.

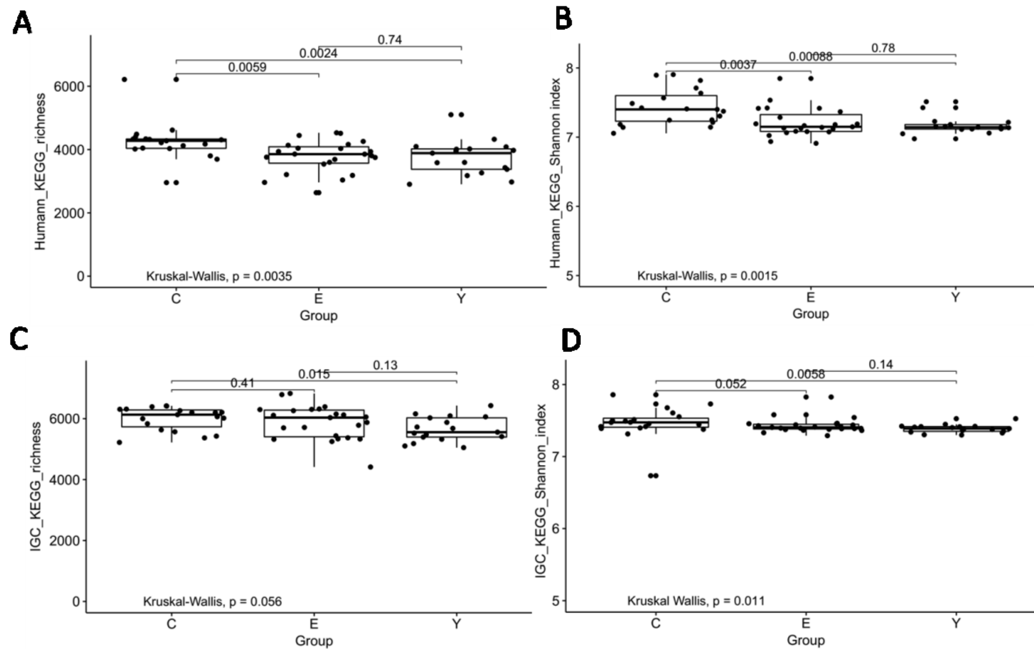


Figure 4.21 α diversity of the KEGG orthology comparison between different age groups in two datasets. Variation detected by Kruskal-Wallis Test. Mean value of Richness in Humann2 (A); Mean value of Shannon diversity index in Humann2 (B). Mean value of Richness in IGC (C); Mean value of Shannon diversity index in IGC (D).

5.2 Functional variation in the gut microbiota among different age groups

We compared the mean relative abundance for each KO between different age groups to see how aging affects the gut microbiota gene abundance in each group (Figure 4.22). We observed that the majority of KO present in gut microbiota are in low proportions (<0.02%). Furthermore, the young and elderly share similar abundance patterns. When compared with the elderly, the centenarians have a lower abundance of most of the dominant KO.

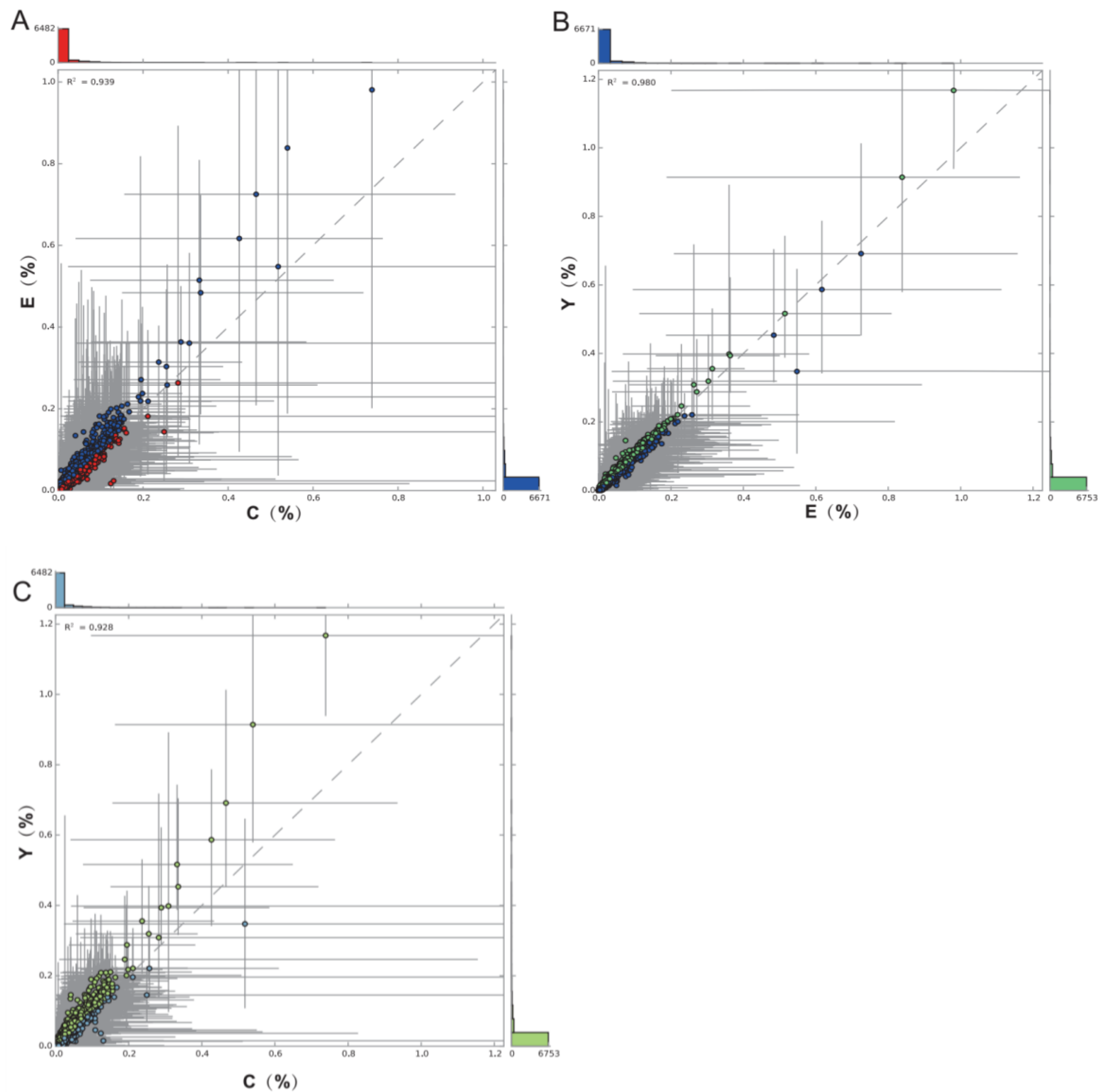


Figure 4.22 Relative proportion of all KEGG orthologs detected within gut microbiota in different age groups. Centenarians compared with healthy elderly (A); Healthy elderly compared with young (B); Centenarians compared with young (C). Points on either side of the grey dashed y = x line are enriched in one of the two groups. Welch's two side T test was used to determine if the observed difference was significant. Confidence intervals for each KO are displayed and are calculated using the Wilson score method.

Gene pathway profile similarities assessed among individuals by Nonmetric multidimensional scaling (NMDS) revealed that, consistent with the taxonomic and KEGG KO profiles, the inter-individual differences increased with aging. The elderly group shared a similar gene profile with the young group but differed strikingly with the centenarian group (Figure 4.23).

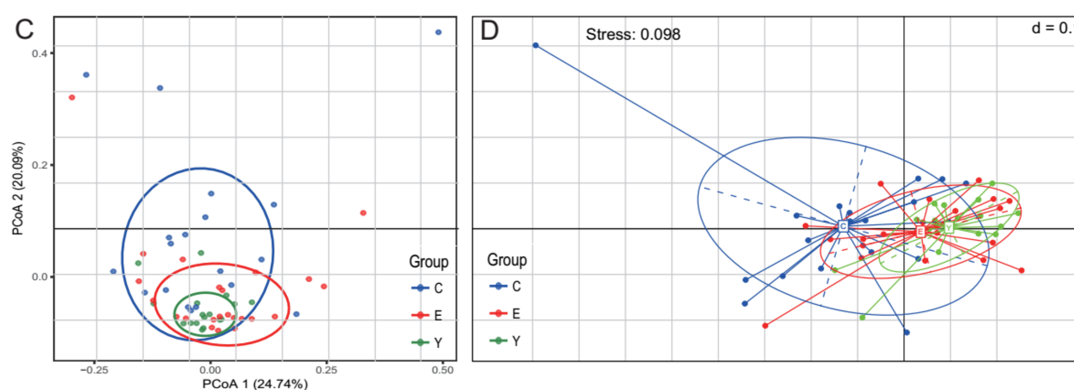
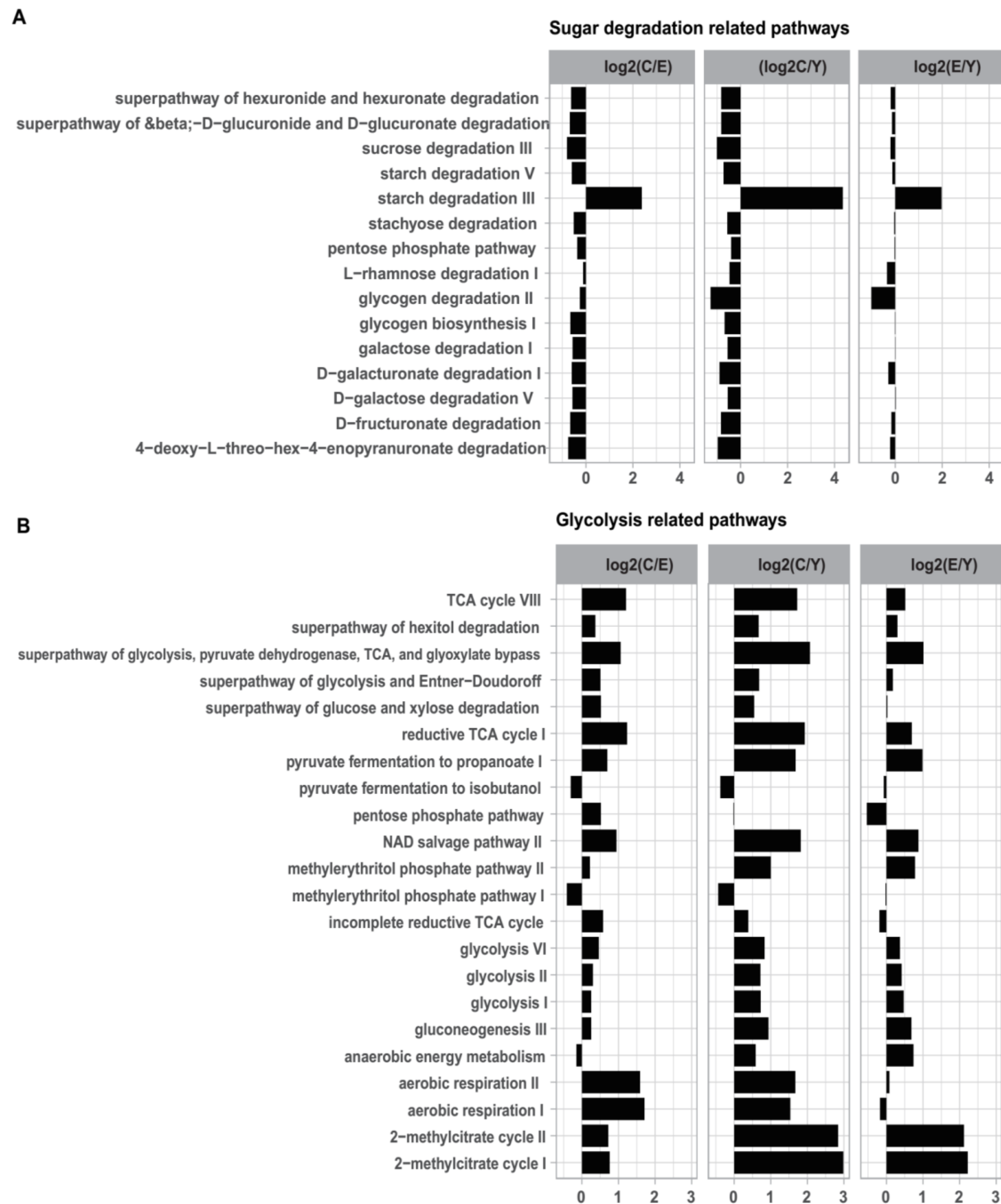


Figure 4.23 Dissimilarities of the functional profile for gut microbiota among the three age groups. Dissimilarities of the functional profiles based on the relative abundance of KEGG KO using PCoA (A); Dissimilarities of the functional profiles based on the relative abundance of gene pathways using non-metric multidimensional scaling (NMDS) (B).

Although most of the gene pathways we detected were shared by all the age groups, the dominant pathways were conserved in all individuals. For example, the gene pathway for Nucleotides Biosynthesis and Cell Wall Biosynthesis are highly abundant in all age groups. Based on the ANOVA test, we detected 115 pathways out of 463 pathways that have significant variation among the three age groups. Gene pathways

for metabolic functions for each age group are shown in Figure 4.24.



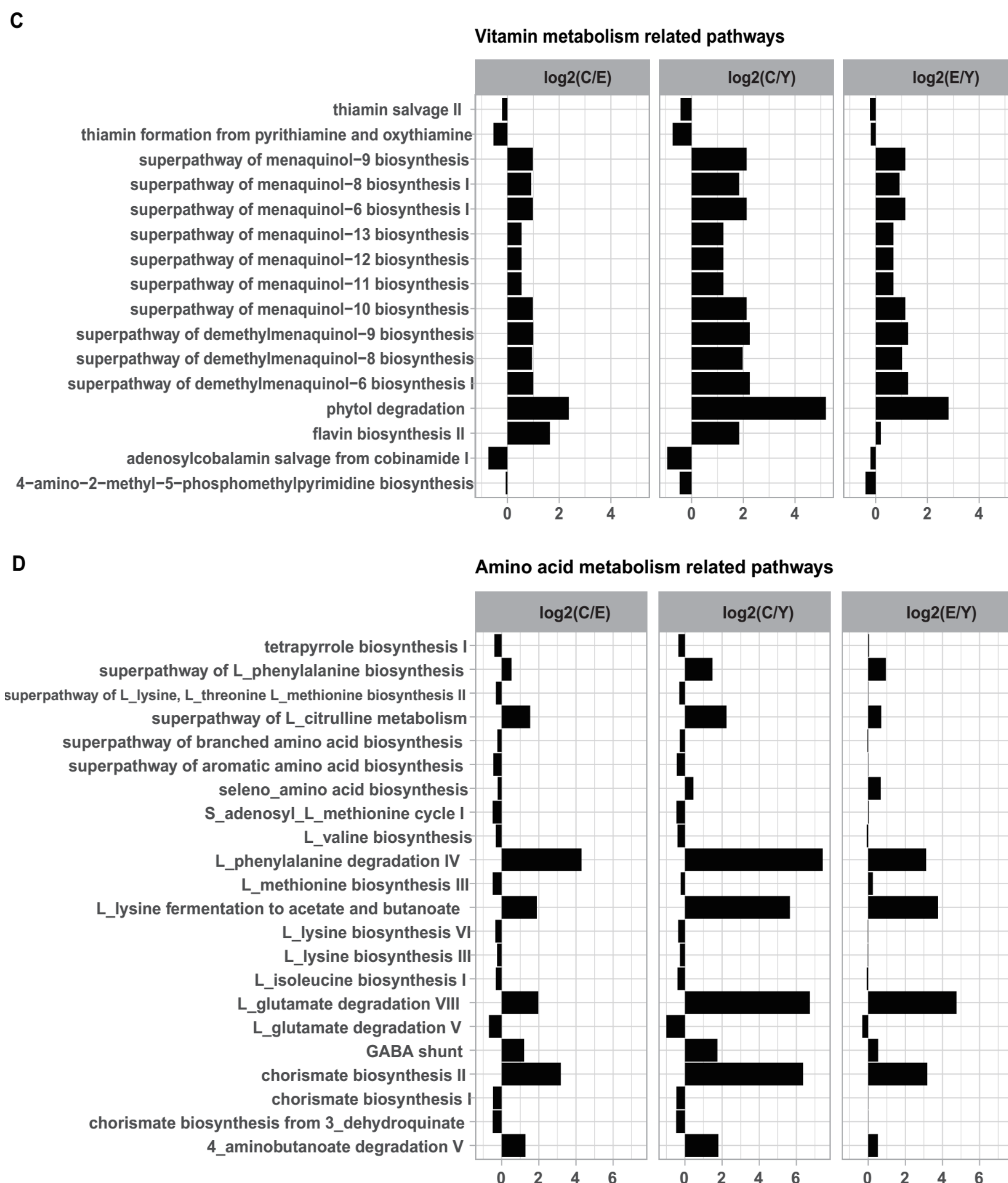


Figure 4.24 Functional signatures of gut microbiota in different age groups. Relative abundance of the gene pathways that are significantly different in the three age groups. The centenarian groups compared with the elderly group (C vs E), and the centenarian groups compared with the young group (C vs Y), and the elderly compared with the young group (E vs Y) are shown in each panel. The length of the

bar indicates the base 2 logarithm value of the relative abundance ratio between each age group. Gene pathways are grouped in related pathways: (A) Sugar degradation related pathways. (B) Glycolysis related pathways. (C) Vitamin metabolism related pathways. And (D) Amino acid metabolism related pathways. Gene pathway variation between age groups are detected by ANOVA followed by Tukey-Kramer test (p value<0.05).

As a core function of gut microbiota, carbohydrate metabolism-related pathways were abundantly detected with significant variation in distribution among the different age groups (Figure 4.24 A&B). Our results revealed that the relative abundance of the pathways for polysaccharide degradation were similar for the elderly and young groups but significantly decreased in centenarians, except for the starch degradation III pathway that is only utilized by the *Archaea* (Figure 4.24A) Interestingly, in centenarians we detected a high prevalence of gene pathways involved in glycolysis and the related Tricarboxylic Acid Cycle (TCA) and related respiration pathways in gut microbes (Figure 4.24B). Additionally, in centenarians, we also detected high abundance of KO for the Phosphotransferase System (PTS) and the major facilitator superfamily (MFS) system transporters, which can facilitate the transfer of sugar into the cytoplasm of bacteria (Figure 4.25).

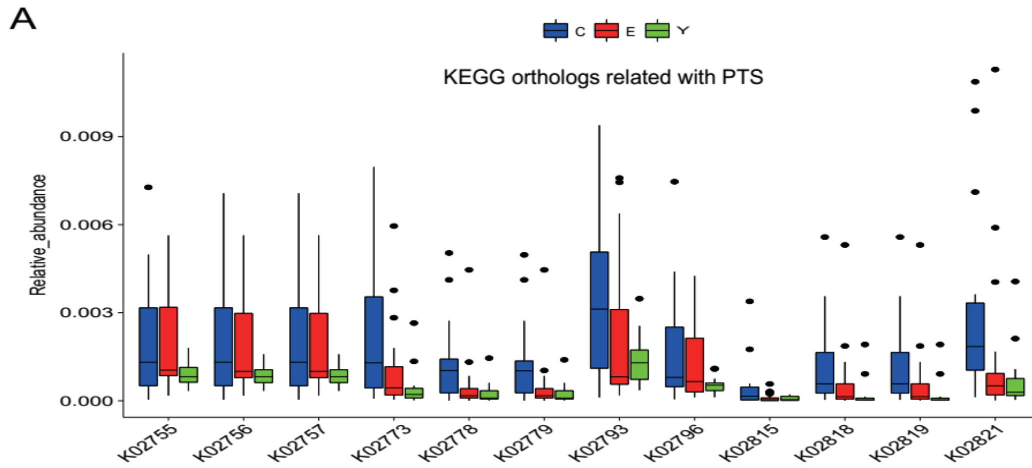


Figure 4.25 Relative abundance frequency of KEGG orthologs in three different age groups.

Gene pathways related to vitamin metabolism are shown in Figure 4.24C. We found the centenarian group displayed a significant enrichment of menaquinone (vitamin K2) gene pathways compared with the elderly group. We further noticed that menaquinone related pathway abundance also showed an increase in the elderly group compared with the young group, which indicates a possible age-dependent increase pattern. Moreover, in our study, the riboflavin (vitamin B2) synthesis pathway was also highly enriched, with the age-dependent *Archaea* increase in centenarians. The synthesis of thiamine (vitamin B1) appeared to decrease with aging.

The results for protein and amino acid metabolism related pathways are shown in Figure 4.24D. As expected, the centenarians but not the healthy elderly exhibited a decline in most of the amino acid biosynthesis pathways compared with the young

group participants. For instance, L-Lysine, L-isoleucine, and L-methionine related pathways were decreased in centenarians. Additionally, some pathways were enriched, in the centenarians, such as the L-phenylalanine metabolism related pathways, the Chorismate biosynthesis pathway, and the L-lysine fermentation pathway.

5.3 Co-abundance of functional pathway define the metabolic potential of different age groups

To further explore the correlation among differentially distributed pathways, we calculated the Pearson correlation among each pathway. The network of the pathways is shown in Figure 4.26, The correlation network among gene pathways for different age groups indicates that over abundant gene pathways in the young and healthy elderly groups were positively correlated, while for the centenarians, the enrichment pathways were not tightly correlated with gene pathways that were enriched in the young and healthy elderly, but displayed a strong negative correlation with defined pathways that were enriched in the elderly. The gene pathways belonging to *Achaea* were also positively associated with each other and showed co-abundance in centenarians corresponding to the enrichment of *Achaea* in taxonomy composition.

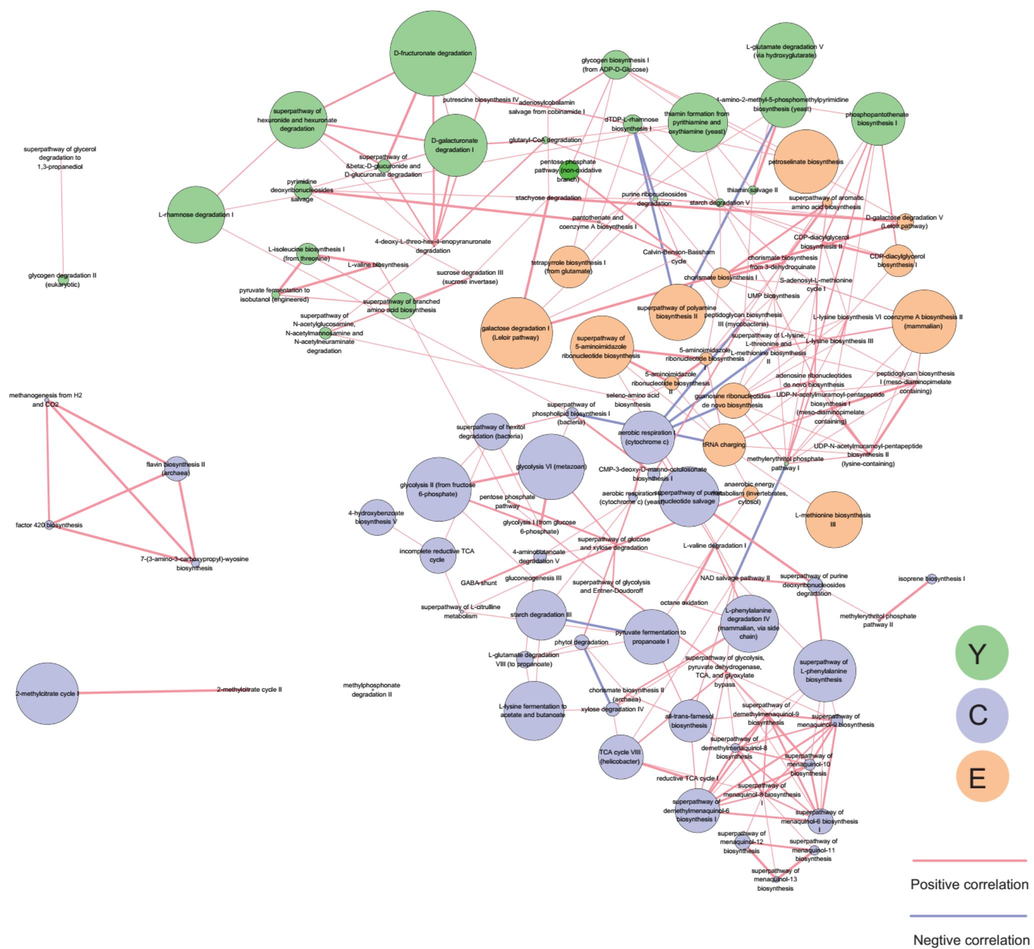


Figure 4.26 Network of the gene pathways that are significantly distributed in three age groups. Gene pathways with significantly different abundance between age groups were plotted as circle nodes in the network. The size of the circles indicated the average pathway abundance in the three age groups. The color of the circles represents an enrichment of the pathways in each age group. The edge represents the Pearson's correlation between each pathway. Only the absolute correlation that is >0.3 is shown. The thickness of the edge line indicates the proportion of the strength of the correlation; the colors represent positive and negative correlations.

The correlation matrix of the gene pathways is presented in Figure 4.27. The matrix showed that significantly distributed pathways mainly separated into two negatively correlated parts. Within those two groups, we observed a correlated cluster of several pathways; for instance, the amino acid metabolism related pathways were closely correlated and further positively correlated with polysaccharide degradation pathways. Manaquinol biosynthesis and glycolysis related pathways were also significantly positively correlated.

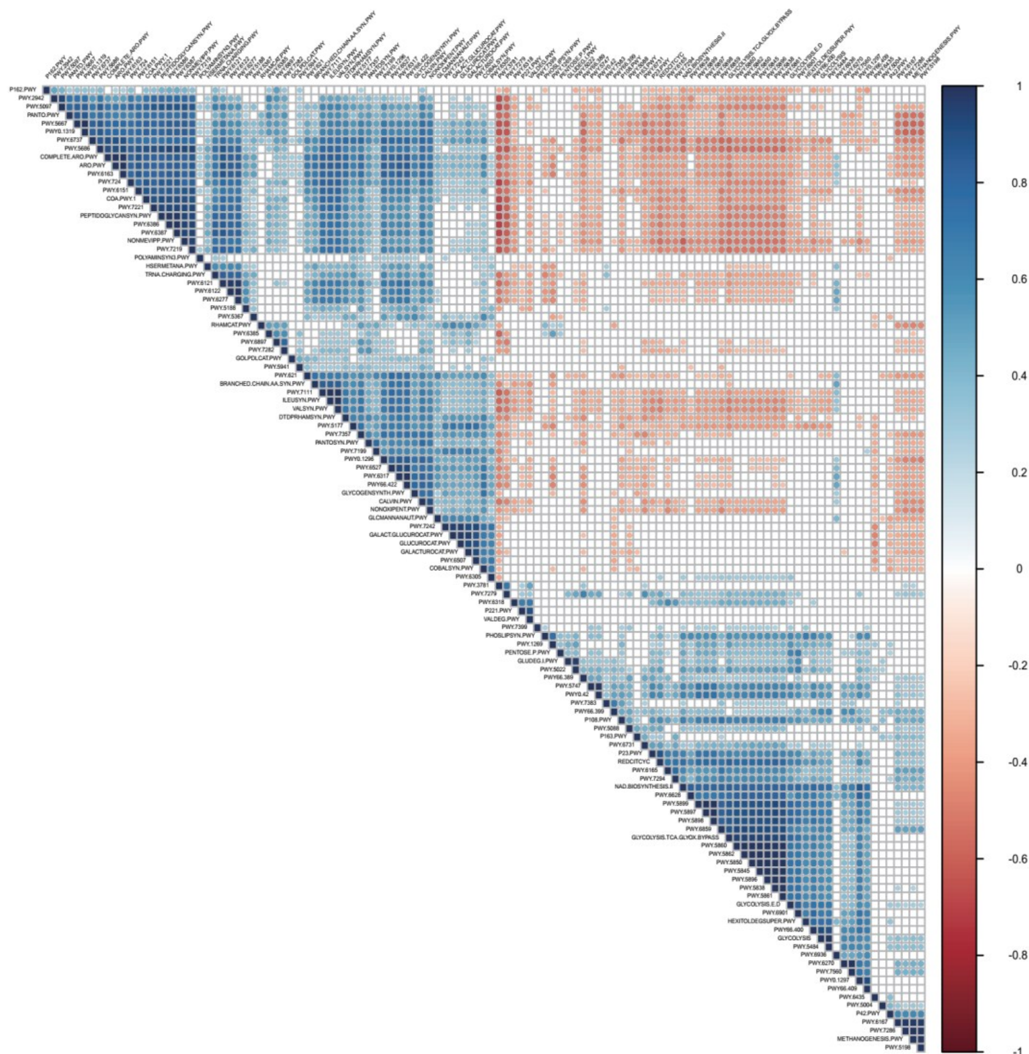


Figure 4.27 Correlation Matrix among gene pathways that have significantly

different abundance in three age groups. Pearson correlation was used to calculate the correlation between pathways. Only the corresponding correlation coefficient that was significant was plotted ($p<0.05$). Circles show the absolute value of corresponding correlation coefficients. Color of coefficients added on the bar: blue = positive correlation; red = negative correlation. The pathways were clustered by hierarchical clustering.

6. Association among gut microbiota composition, gut microbiota function and host clinical parameters

The statistics of clinical and health measurements for 59 subjects within the three age groups are shown in Table 4.**2.** On average, the centenarians in our cohort scored poorly for diverse health parameters, including Mini-Mental State Examination (MMSE), Mini Nutritional Assessment (MNA) and Functional Independence Measure (FIM) compared with the healthy elderly, who had similar scores with the young. Furthermore, MMSE, MNA and FIM of the centenarians varied significantly.

Table 4.2 Statistics of the clinical characteristics and health measurements in the three age groups.

Parameters	Centenarians(n=19)	Elderly(n=23)	Young(n=17)
	Mean ± SD (Range)		
Age(yrs)	101.8±1.4(99-107)	76.7±5.9(68-88)	25.5±3.5(21-33)
Male (%)	76.50%	56.60%	41.2
Weight(kg)	57.1±5.7（43-73）	68.7±14.3(42-103)	63.2±3.5(44-95)
BMI(kg/m^2)	23.5±2.1(17.9-28.1)	25.9±4.1 (19.5-36.9)	22.8±3.7(16.1-40.1)
MMSE(0,30)	15.8±6.7(5-26)	26.6±3.0(22-30)	30(30-30)
MNA(0,30)	18.9±3.7(8-26)	24.1±2.0(18-28)	24.3±1.3(20.5-28)
FIM(0,126)	77.5±21.1(31-123)	123.7±1.9(119-126)	126(126-126)

* Total number of subjects is 59 excluding individuals with unqualified stool samples (N=6).

Values are presented as Mean±SD with the range in parentheses.

BMI: Body Mass index; MMSE: Mini-Mental State Examination; MNA: Mini Nutritional Assessment; FIM: Functional Independence Measure

6.1 Association between the compositional and functional profile for gut microbiota

Using Mantel test to evaluate the correlation between individual specific gut compositional profile distance matrix and KEGG KO functional profile distance

matrix, we found that Mantel statistic r is 0.718 with p value 0.001, indicating there is a strong positive association between the compositional and functional profile for gut microbiota.

6.2 Clinical parameters associate with gut microbiota composition

The correlation between health parameters and microbiota composition were examined using Mantel tests (Table 4.3). Calculating the correlation between the similarities of gut microbiota composition measured by Bray-Curtis distance and Body Mass index (BMI), MNA, MMSE, FIM and Drug parameters we observed. Significant associations between the composition of gut microbiota and MMSE, MNA, FIM and Drug were detected respectively. No association was observed between the gut microbiota composition and chronological age, BMI.

Table 4.3 The Mantel tests of correlation between clinical parameters and microbiota composition in the three age groups.

Parameters	Mantel r statistic	p-value
Age	0.0019	0.484
BMI	-0.0391	0.704
Drug	0.2827	0.001
MNA	0.2585	0.001
MMSE	0.2931	0.001
FIM	0.3739	0.001

BMI: Body Mass index; MMSE: Mini-Mental State Examination; MNA: Mini Nutritional Assessment; FIM: Functional Independence Measure; Drug: The number of medicine the subject taking. The Correlation method is spearman, the Number of permutations is 999.

As the young and elderly had relatively consistent clinical parameters we measured, in order to further explore the correlation of the health parameters with gut microbiota in centenarians, we determined the correlation of the health parameters with the gut microbiota composition (Figure 4.28). We found that in centenarians the FIM score is significantly associated with the separation of the individuals in the PCoA. That indicates centenarians with similar FIM scores tended to have similar gut microbiota composition. We also observed that age is not associated with FIM, which further suggests that health in centenarians is not related with chronological age. The MNA was detected as positively related with FIM, emphasizing the potential importance of diet for maintaining healthy aging.

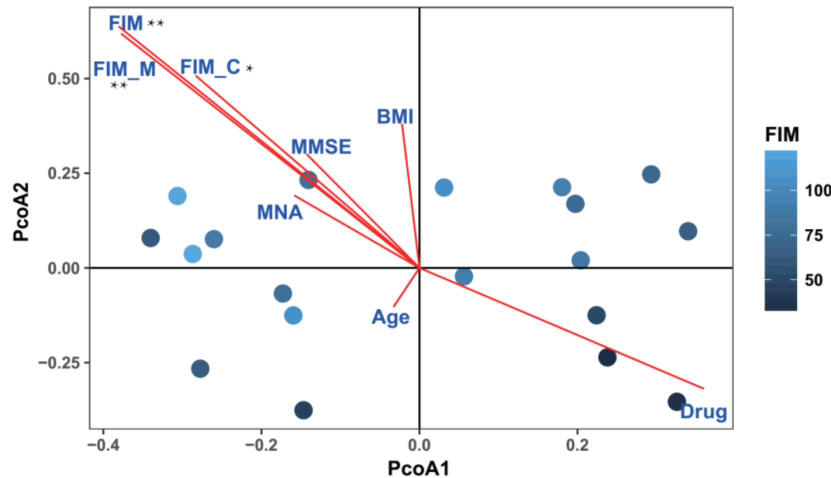


Figure 4.28 Gut microbiota in centenarians correlate with clinical parameters. PCoA based on the Bray-Curtis distance was plotted for the bacterial microbiota composition of centenarians at the species level. The colour of the spots was ranked by FIM index. Clinical parameters including FIM (FIM-C: cognitive FIM, FIM-M: motor FIM), MMSE, MNA, BMI, Age and Drug were used as factors to show correlation with the ordination configuration. The lengths of factors indicate the level of the correlation. Permutation was used to test the significance of the fitness, the number of permutation was 999. Significant codes: 0.001 '**', 0.01 '*'.

7. The prevalence of ARG (Antibiotic Resistance Genes) in the gut microbiota of Sardinians across age

7.1 The prevalence of ARGs in the gut microbiota of the three age groups in Sardinia

Following the pipeline of DeepARG, we obtained the ARG distribution profile for each subject. In total, 578 ARG subtypes belonging to 30 ARG types were detected in

the three age groups. ARGs type resistant to glycopeptide, multidrug, tetracycline and macrolide lincosamide streptogramin (MLS) are highly enriched in our cohorts, with abundance ranging from 0.83-0.9 copy of ARG/cell. Among these, MLS has the smallest variation in each subject while tetracycline has the widest variation. MACB coding for MLS resistance was the most enriched ARG gene in our cohorts with mean abundance 0.52 copy of ARG/cell, followed by UPPP and BCRA genes coding for bacitracin resistance. The enriched ARGs within gut microbiome in our cohorts were shown in Figure 4.29.

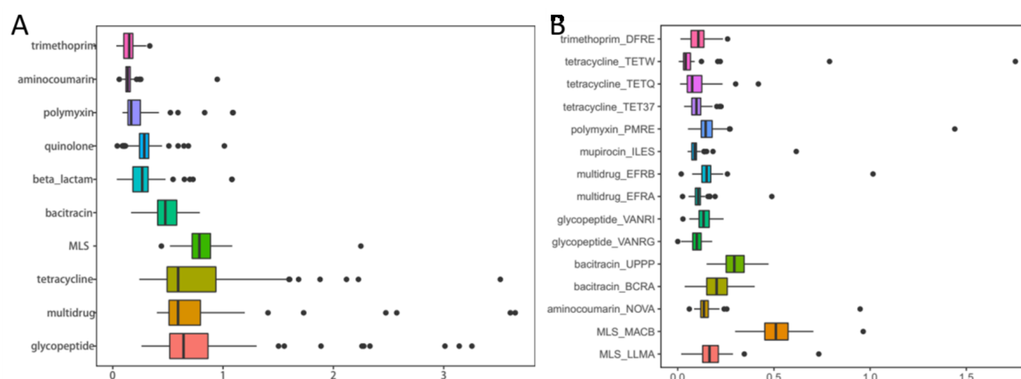


Figure 4.29 Enriched ARGs within gut microbiome in the three age groups in Sardinia. Top10 enriched ARG Type (A); Top15 enriched ARG Subtype (B). The abundance of ARGs was normalized and expressed as copy of ARG/cell.

7.2 ARGs distribution profile feature in different age groups

Compared the abundance of the enriched ARGs among all three age groups in Figure 4.30, we found that the centenarians highest accumulation of ARGs, while

interestingly, the elderly had least ARG enrichment when compared with the young group. For the top 10 enriched ARG types, on average, each centenarian have over 5.9 copies of different ARG types per gut microbe compared with each elderly individual having 4.4 copies, each young individual having 4.8 copies. The ARG subtype gene shows a similar patter with as ARG types.

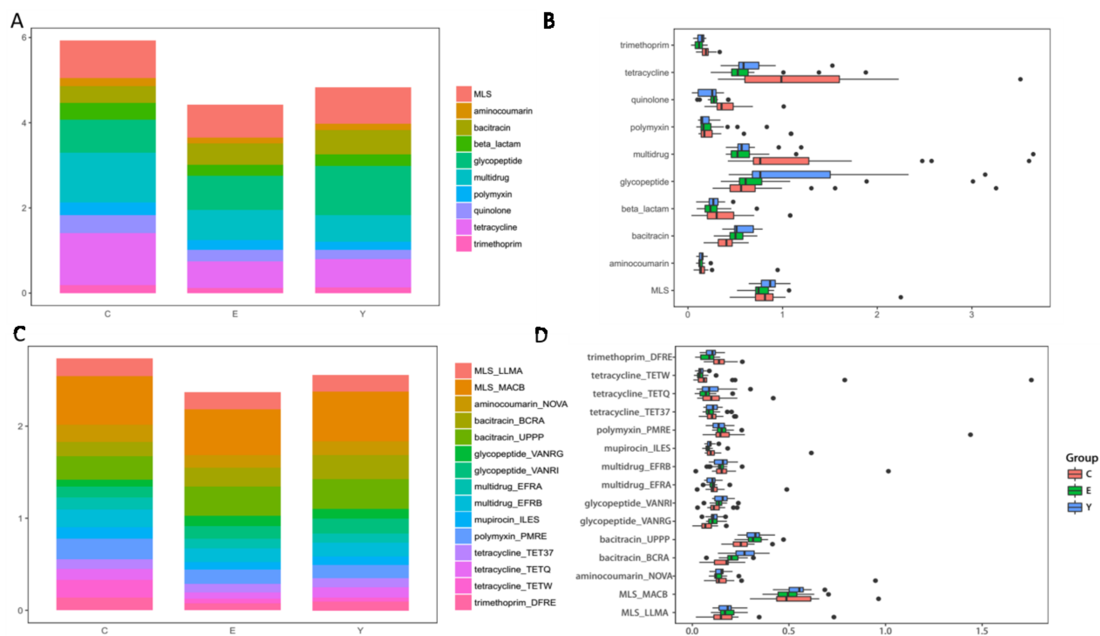


Figure 4.30 ARGs abundance in three different age groups. Barplot of the top10 enriched ARG Types (A); Boxplot of the top15 enriched ARG types (B). Barplot of the top10 enriched ARG subtypes (C); Boxplot of the top15 enriched ARG subtypes (D).

PCoA plot was used to display the similarities of the ARG distribution profiles for each individual among three age groups (Figure 4.31). The clustering pattern shows that the young and healthy elderly share similar ARG profiles, while the centenarian

group has a distinct ARG profile with wide variation among individuals.

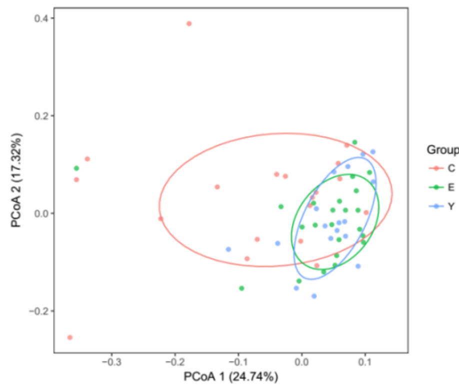


Figure 4.31 PCoA plot of the ARGs subtype profiles in different age groups

The boxplot in Figure 4.32 shows the relative abundance of the ARG type which has significantly less variation among three age groups. Tetracycline and multidrug resistance ARGs are highly enriched in the centenarians although a with wide variation among individuals. Noticeably, the glycopeptide and bacitracin resistance ARGs shows an age dependent decline in accumulation. The centenarian group has the lowest enrichment while the young group has the highest abundance. Meanwhile, the glycopeptide resistance ARGs has wide variation among individuals in the young group.

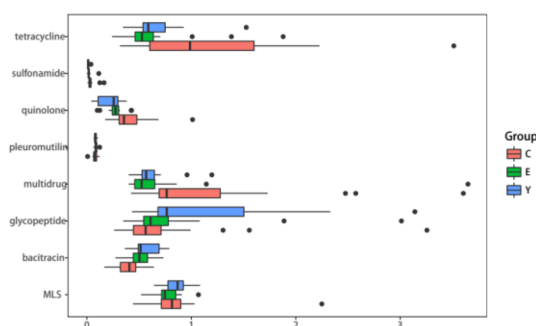


Figure 4.32 ARGs distribution in different age groups. (ANOVA, $p<0.05$)

7.3 ARG distribution correlated with gut microbiota composition and function in the three age groups in Sardinia

Considering that the clustering of the ARG distribution profiles for the three age groups in PCoA is similar to the clustering of gut microbiota composition and function profiles in our cohorts, we further explored the correlation of the ARG distribution with gut microbiota composition and function by the Mantel test (Table 4.4). A significant positive correlation was detected between the ARG profile and gut microbiota composition, and also between ARG profile and gut microbiota function.

Table 4.4 Mantel test of the correlation between the ARG profile and gut microbiota composition and function profile

Distance matrix 1	Distance matrix 2	Mantel statistic r	P value
Gut species composition	ARGs Type	0.4395	0.001
Gut species composition	ARGs Subtype	0.6039	0.001
Gut KEGG KO function	ARGs Type	0.5374	0.001
Gut KEGG KO function	ARGs Subtype	0.7171	0,001

ChapterⅤ. Discussion

Surveying the full view of the microbiota across age in different body habitats, our results indicated that for each body habitats, the diversity of bacterial and fungal is dramatically different. Both α and β diversity of fungi are lower than that of bacteria in all body habitats. The skin had higher fungi α diversity but lower bacterial α diversity compared with oral and gut. While the skin had higher fungal β diversity but lower bacterial β diversity compared with gut and oral. Interestingly, fungal α diversity was positively correlated with bacterial α diversity in the skin, but independent of each other in the oral and gut. The variation of α and β diversity for human microbiota may be caused by the differences of the ecological niche in each body habitat. The skin habitat may provide more a compatible environment for the fungi but not for bacteria, so the colonization of the fungi in the skin is easier leading to universal colonization of different fungi (higher α diversity) and finally making the skin community more similar for each individual (lower β diversity). On the contrary, the bacteria but not fungi had higher fitness in the oral and gut niche.

We demonstrated that the distribution of microbiota in human was largely determined by body habitats and individuals, consistent with former research [15, 25]. Besides, with an integrated view of microibota in different habitats, we found that the microbiota correlation between different body sites was diverse. In four skin sites, the fungal communities were correlated but bacterial communities were correlated only

between the sites commonly in contact with foreign surfaces, such as palms and forehead. Interestingly, significant positive association between bacterial and fungal communities were detected only in palms, similar results also found in another study where the palms showed the greatest positive correlation [175], the reason why palms are distinct from other body habitats is currently unknown. For the gut and oral, the specific mucosal niche might account for the microbiota community differences between skin habitats. Further studies that utilize strain and SNP level of resolution for microbiota community genomic analysis are needed to explore the correlation of microbes among different body habitat [176].

We surveyed three typical biographical habitats for skin microbiota in our study: the forehead represents a sebaceous niche with high environment exposure; two symmetric palms represent dry niches with high-exposure and perturbation; umbilical represents another dry niche though with lower exposure. We detected a positive association between the Shannon diversity of fungal and bacterial communities in each individual in all four skin sites, suggesting that the biological niches in the skin have a similar affect for bacterial and fungal communities, similar results were observed in a Hongkong population [175]. For the bacterial communities in the skin, we observed that the three age groups displayed different compositional profiles, consistent with the former study in Japan but different from a study in China [27, 31]. We found that the elderly had more complicated skin bacterial communities compared with young and centenarians, similar results were also observed for the fungal

communities. Bacterial communities in skin displayed an age related cluster pattern, especially for the palms. Unexpectedly, fungal communities were more similar between young and centenarian but distinct from the elderly. So the variation of the diversity for the skin communities in different age group was not only related with the age but also could associate with additional factors such as host physiology and environment exposure. The forehead with high sebaceous gland activity was highly enriched in lipophilic microorganisms. *Propionibacterium* was especially enriched in the young group. A significant decrease of *Propionibacterium* with aging was detected, which could correlate with the decrease of the sebaceous gland activity in all skin sites with aging, consistent with prior studies [13, 31]. The high abundance of potential pathogenic genera *Staphylococcus* and *Streptococcus* in centenarians' palms suggested a higher risk of infection for centenarian was possible. Various groups have explored the direct interactions of skin inhabitants with the host immune system, immunosenescence in centenarians may associate with this risk [177-179]. Further attention should be paid to the potential infection in centenarians. The dominant *Malassezia* was observed to significantly decline in the elderly compared with young, this is different from a former research study in a Toronto population where the elderly had the highest abundance of *Malassezia* [180]. As a lipophilic microorganism, the decrease of *Malassezia* in the elderly skin may be explained by the decrease in the level of skin lipids in aging people, however, the centenarian group showed a higher abundance of *Malassezia* compared with that of the elderly, which suggested that the

alternation of lipids level in the skin was not the only determinant of *Malassezia* colonization.

Evidence shows that specific microbes in the oral cavity are associated with oral health and disease, and even linked with other systemic disease [37] [39]. With the aging process, although physiologic changes may cause dental problems and tooth loss, we found that both the bacterial and fungal communities in the oral cavity did not display distinct clustering for different age groups, suggesting that the oral microbita is not age dependent in Sardinians. Meanwhile, we found the clustering for the bacterial communities for different individuals was driven by the dominant genera, indicating that the relative abundance of the dominant genera determine the similarities of their community structure in the oral cavity. Similar "salivary type" microbes driven by dominant genera was also observed in another Italian study and a Japanese study [33, 181]. Variation of the oral microbial associated structure with aging in a Chinese cohort was not observed in our cohort [32].

Among those microbial communities distributed across the body, the gut microbiota is the most comprehensively studied microbial community and has the strongest association with human disease and health [21]. Besides, numerous recent studies indicate the gut microbiota has a major impact on the host metabolic status and can regulate host lifespan in animal models [148, 154, 155, 182]. Studying the gut microbiota in centenarians which represent a population with an extremely long lifespan, may contribute to identifying features that correlate with aging and longevity.

As a first step in exploring the potential association between longevity and gut microbiota in human, we used 16s rRNA, ITS1 and metagenomic sequencing to get a full picture of the taxonomic and functional profile of gut microbiota in Sardinians. Our data confirmed the results found in HMP cohort, that indicated the basic fungal and bacterial community characters in gut：such as the fungal diversity was lower than bacterial diversity, the independent relationship between the α diversity of the bacterial and fungal community, and the dominant fungal genera that included *Saccharomyces*, *Malassezia* and *Candida* [18]. But the Sardinian population showed a higher prevalence of *Penicillium* which means relative abundance in each individual make up more than 10% of gut micorbes compared with less than 4% in the HMP cohort. Other dominant fungal genera such as *Cladosporium* detected in HMP cohort was lower in the Sardinia cohort. Besides, for both 16s rRNA and shotgun metagenomic sequencing, we observed that young and elderly shared similar gut bacterial community structure, consistent with previous studies [62, 150], but distinct from most of the centenarians.

For bacterial communities we detected by 16s rRNA sequencing, we identified the aging dependent decline of *Feacalibacterium* and *Roseburia*. The enrichment of *Bilophila, Butyricimonas* and *Christensenella* in centenarians was also detected in our study and others [63]. The *Clostridium* which was reported to be increased with aging was found increased in our elderly group compared with the young but significantly decreased in the centenarians compared with the elderly. Moreover, *Parabacteroides*

was detected enriched in our centenarian cohort but was decreased in a Chinese centenarian cohort [158]. *Prevotella* is a dominant taxon in subjects classified as "enterotypes 2" which are associated with a carbohydrate rich diet [45, 77]. The reason *Provetella* can only be detected highly enriched in the elderly group in Sardinia is still unknown. *Bifidobacterium* was detected decreased in another Italian centenarian cohort [63, 183], while in our cohort the mean relative abundance of *Bifidobacterium* reached 5.6% of the total genera in centenarians, compared with 2.9% in the young and 2.3% in the elderly. What's more, *Bifidobacterium* was positively contributed to the similarities of the centenarian gut bacterial communities. *Bifidobacterium* is believed to have health-promoting properties as a probiotic for human [184]. *Bifidobacteria* naturally occur in a range of ecological niches that are either directly or indirectly connected to the human gastrointestinal tract. The significant enrichment of *Bifidobacterium* was detected in our centenarians' plams and face, which may be correlated with the high prevalence of *Bifidobacterium* in the gut of Sardinian centenarians.

Fungi detected by ITS1 sequencing in the gut can be divided into two types: resident and no-resident. *Penicillium* and *Debaryomyces* which are found on fermented foods but cannot grow in the gut niche [185], have a high prevalence in Sardinians compared with the HMP population from Houston and Texas (USA) and may correspond to the high consumption of cheese, ham and sausage in Sardinian [18]. Besides, *Debaryomyces* was significantly enriched in the young group, displayed an

aging dependent decrease in our cohort. *Saccharomyces* also presumably originates in food, and is enriched in yeast-containing foods such as bread and beer. The young group also have a significantly higher abundance of *Saccharomyces* compared with the elderly and centenarians. The variation of *Debaryomyces* and *Saccharomyces* may be partly caused by the diet preference for different age groups. Previous study observed a decreased proportion of *Saccharomyces* and an increased proportion of *Candida* in IBD patient compared with healthy controls [61]. Further study will need to determine the role of that fungi plays in the gut during aging. *Candida* and *Malassezia* belong to resident fungi. However, the resident fungi also had a wide variation of relative abundance between individuals, although the variation between age groups was not significant. Research has shown that diet can module the fungi communities in the murine gut [186], while in our cohort different age groups didn't display a clear age-dependent cluster. Intriguingly, we found the dominant genera with a significant contribution for the gut fungi community similarities was divided into two negatively associated groups: one including *Debaryomyces, Saccharomyces* and *Penicillium* represented the food original non-resident species; the other group included *Candida* and *Malassezia* representing resident species. The clustering of subjects dominated by non-resident fungi may be associated with a diet of enriched fungi, further suggesting that caution should be made in metagenomic studies because separating live from dead, and resident from non-resident microbes can be difficult.

Preliminary evaluation of the association between fungi and bacteria revealed that the

associations were site-specific and population-specific. For instance, lipophilic *Malassezia* and *Propionibacterium* showed a significant strongly positive association in the skin, reflecting their similar nutrition requirement. While in the mucous habitats, the correlation was not clear because *Propionibacterium* was not a dominant genus. Furthermore, in the skin, *Actinomyces* was positively correlated with *Staphylococcus* and *Streptococcus*, whereas in oral, the correlation was negative. Also for different populations the co-occurrence pattern is different. In our study, a strong negative association was detected between *Prevotella* and *Streptococcus* in oral, consistent with an Italian study, while in a Japanese study the correlation was positive [33, 181]. In the gut, the association relationship we observed, such as the positive association between *Faecalibacterium* and *Saccharomyces*, was also found in a USA study [186]. The correlation we detected based on the co-occurrence didn't necessary mean the true biological interaction between the species. Our results provide a framework for future investigation of the interactions between pathogenic and commensal microbes in maintaining human health and contributing to disease pathogenesis. Further cultivation assays are needed to gain insight into the mechanisms of these correlations.

Using shotgun metagenomic sequencing, we found the variation between the different age groups of the main structure of the gut microbiota is large. Previous studies had already associated F/B ratio with BMI, obesity and the production of short chain fat acid (SCFA), and aging [171, 187-190]. It has been suggested that a higher F:B ratio

may be associated with an increased energy harvest in animal studies [127]. The F/B ratio in centenarians showed an interesting trait: significantly lower compared with the elderly group, but similar with young. The low ratio of F/B in centenarians may lead to the decline of energy harvesting capabilities in centenarians. Although at the species level, the gut microbiota α diversity is not statistically different among the three age groups, the core microbiota richness of centenarian is higher than that of young and elderly, indicating that Sardinian centenarians shared a wide array of specific species among individuals. The loss of diversity in the core microbiota has been associated with increased frailty in the elderly [62], which was not found in Sardinian centenarians. The clusters of the gut microbiota compositional profiles for different age groups revealed that the elderly had a similar cluster to that of the young. Our cluster data is similar with data from a study of gut microbiota in a large healthy cohort in China [150]. However, our data is different from the results of a cohort in Italy in which all age groups showed good separation [63]. For the centenarians, the cluster shifted sharply from that of the young and elderly, which suggests that most of the centenarians have a gut microbiota profile different from the gut microbiota profiles of the young and elderly.

Compositional changes in the gut microbiota ultimately cause functional changes of the gut microbiota [157]. Here we used our Sardinian cohort to identify the functional profile of gut microbiota in centenarians and compared the profiles with the elderly and young to identify specific functional profiles associated with different age groups.

Interestingly, the centenarian group has significantly higher α diversity of KOs compared with young and elderly, representing the highly functional redundancy in centenarians, similar with the Hadza hunter-gatherers population [78, 79]. The reduced diversity of the gut microbiome is considered to be related with a low dietary fiber intake diet and a disturbed gut eco-system [191, 192]. Consistent with the gut microbiota compositional profile, the young and elderly shared similar gut microbiota functional genetic profiles. Centenarians had genetic profiles that clustered distinctly from the young and elderly. There are two possible explanations for the shifting of the clusters of the centenarians' gut microbiota compositional and functional profiles. The first is a critical point: the health status of the centenarians. Previous research based on the elderly population suggests that the health status of the elderly closely correlates with the gut microbiota [41, 65, 152]. Our research also demonstrated that the diverse health status of centenarians correlated with the gut microbiota. Most of the centenarians in our study had lower parameters for health status, which may associate with the variation in the gut microbiota. Future investigation of the gut microbiota in centenarians will be facilitated by recruiting enough subjects to divide them into different subgroups based on health status. This will aid in statistically determining the differences between gut microbiota in healthy centenarians and centenarians with fragility-related changes. Similarly, in the future we will examine elderly with lower health status to confirm the correlation between gut microbiota and health status. The second possible explanation is that the centenarian subjects had a

unique gut microbiota different from that of the normal population. In our study, although with limited samples size, we found centenarians share part of the core microbiota that is unique to centenarians. Another limitation of the cross-sectional nature of our research is that it was not possible for our study to establish the dynamic model of gut microbiota alterations with aging through time. Further follow-up research using longitudinal cohorts is imperative to address this question.

Functional annotation of gut microbiota in centenarians reflects unique energy metabolic patterns that are distinct from those of the young and elderly. We found that, in centenarians in Sardinia, different microbes seem to have the potential to develop an aging adaptation metabolic pattern especially for exploiting carbohydrate. Diminished physical activities and energy expenditure are associated with the aging process and may cause the decline of energy requirements for humans [193, 194]. In Sardinian centenarians, we found that gut microbiota has lower gene pathway abundance involved in complex carbohydrate digestion, which correlates with the significantly lower prevalence of *Ruminococcus* and *Faecalibacterium* in the gut compared with young and elderly. Furthermore, the enrichment of gene pathways related to the utilization of energy by microbes via glycolysis correlated with the enrichment of *Enterococcus, Lactobacillus*, and *Escherichia* in centenarians [119]. Moreover, the strong negative correlation between glycolysis and sugar degradation pathways we observed also supported those results. Former research has shown that *M.smithii* can cooperate with the *Bacteroides* to enhance fermentation and decrease

the level of TMA which has been shown to correlate with clot-related events such as heart attacks and strokes [195, 196]. Previous research has shown the Mediterranean diet was negatively related to urinary TMAO levels [81]. Related gene families such as coenzyme M and F420 were also detected as significantly enriched in the centenarian group in our study (Figure 5.1) [197].

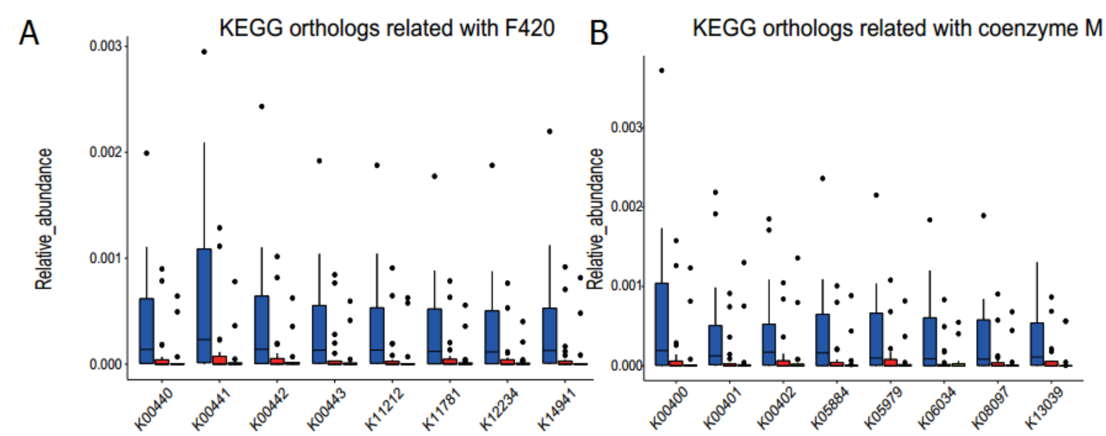


Figure 5.1 Bar graph with standard deviation shows the mean value of relative abundance frequency of KEGG orthologs in three different age groups. F420 (A); Coenzyme M (B)

Moreover, *Desulfovibrio* also has the ability to use hydrogen as electron donors for sulfate reduction. *M.smithii* and *Desulfovibrio* are abundant in the centenarian group in our study, which may explain the increased ability for centenarians to eliminate the fermentation product that limits the glycolysis reaction. Gut microbiota in the centenarians performs in a way that can be described as CR mimetic, which is a process that mimics the effects of CR by targeting carbohydrate metabolic pathways affected by CR, but without actually restricting caloric intake [139]. The enrichment

of probiotics such as *Bifidbacterium* and *Lactobacillus* was also detected in the centenarians. *Bifidbacterium* and *Lactobacillus* have the potential to produce metabolites beneficial to human health, such as amino acids and vitamins. In the centenarian group, the enrichment of some low-abundance pathways that may function to facilitate longevity and good health were also observed, such as menaquinol biosynthesis, flavin biosynthesis and Phytol. They can produce essential metabolites such as vitamins that can compensate the aging-related decline of microbial products. For instance, menaquinol is mainly present in fermented foods such as cheese and natto (fermented soybeans) [198]. It is interesting to note that all members of the longevity cohort, such as Sardinians, Japanese, and Korean populations traditionally eat fermented foods [1, 57, 64]. Research also shows that menaquinol is important for bone and heart health [199-201]. Overall, it appears that gut microbes may develop a cooperative relationship with centenarians, which acts as a positive factor for longevity. In Figure 5.2, we summarized compositional and functional features in the centenarians gut microbiota, which may be related to the longevity and healthy aging in Sardinian centenarians.

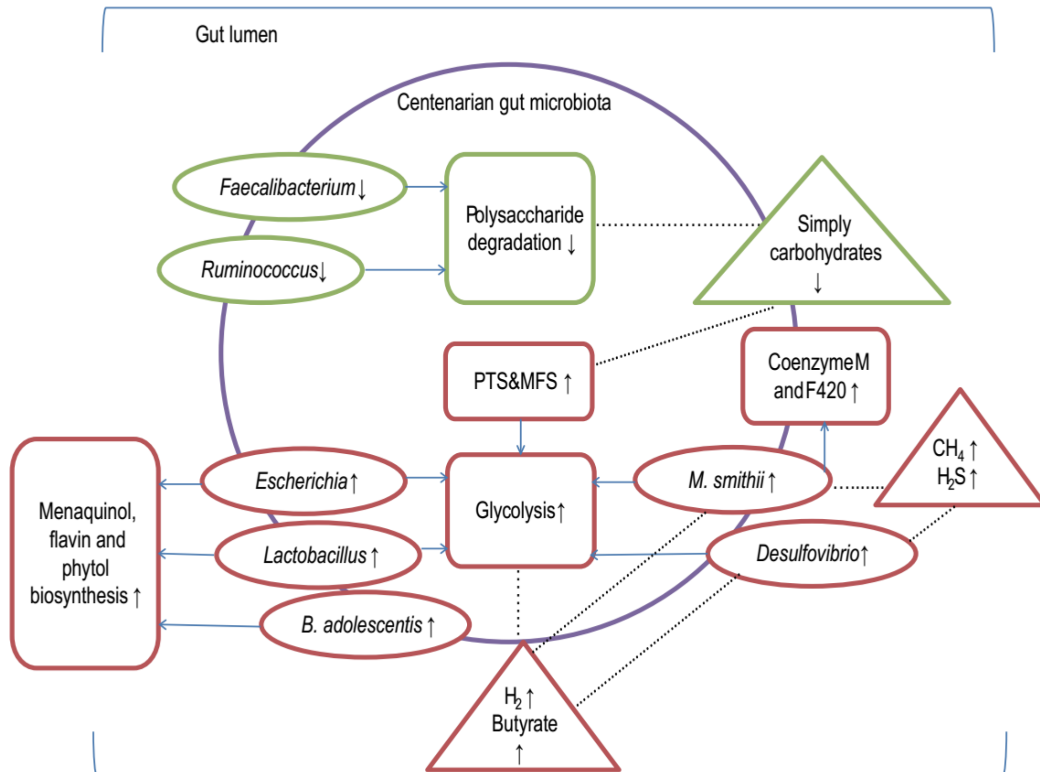


Figure 5.2 Compositional and functional features in the centenarians gut microbiota. Observed high abundance (red) or low abundance (green) of compositional (ellipse) and functional (round rectangle) features. Potential metabolites (triangle) released by the gut microbes. Proposed possible contributions of the gut microbes to metabolites (dotted cure) and metabolic functions (arrow)

Population specific compositional features of centenarians' gut microbiota have been found in former studies [63, 64, 151, 153]. As a dynamic ecological system, gut microbiota has various compositions for individuals. The variation may be caused by different populations with diverse genetic, dietary, environmental factors, or even the use of different methodologies of processing samples and data [47, 67, 174, 202, 203].

Within a defined cohort, different age groups also display diverse gut microbiota features [57, 62, 63, 65, 158]. Among the observed gut microbiota features found in previous studies and our study reported here, some seem universal, such as the lower abundance of *Faecalibacterium, Ruminococcus* and *Coprococcus* in centenarians. Other features are unique to defined population; for example, the enrichment of *Methanobrevibacter* and *Bifidobacterium* in centenarians as was detected in our centenarian cohort but not in other centenarian cohorts [63, 151, 158]. *M. smithii,* the dominant species in *Methanobrevibacter*, was reported to correlate with the consumption of milk products [172]. The high prevalence of *M. smithii* in Sardinia centenarians' gut microbiota may be linked with a dairy rich diet. *B. adolescentis* and *B. longum* are the most abundant species belonging to *Bifidobacterium.* We detected in our cohort, noticeably, only *B. adolescentis* which was highly enriched in centenarian and young compared with elderly. The enrichment of *Bifidobacterium* was also observed in Romagna, Italy semi-supercentenarians (>105 years of age) but not in other centenarian groups. Meanwhile, *Escherichia coli* which was constantly reported enriched in centenarians was also found in our dataset. *Akkermansia* was found with low abundance in centenarians in Sichuan, China, but enriched in the semi-supercentenarians but not in the centenarians (100 to 105 years of age) in Romagna, Italy. In Sardinia centenarians, the abundance of *Akkermansia* is not statically different from the elderly and young. Overall, the independent studies on gut microbiota in centenarians reveal that the gut microbiota in long-living peoples share

the features of declining dominant genera but also accumulate diverse sub-dominant species.

Our study analyzed the gut microbiota composition at the species level and metabolic function at the community level, further analysis is needed to build a more complete picture of aging, healthy aging and longevity with respect to the microbiota and metabolic function. When we talk about gut microbiota intervention, we should identify the microbes at the strain level because even individual bacterial species can contain many functionally different strains with significant genetic diversity. Resolution at the strain level must be conducted to assess the contribution of the gut microbiota to metabolic function [204]. Furthermore, although we detected specific gut bacterial gene pathways that correlate with longevity or aging, without detecting the metabolites in the human system, the analysis is only suggestive. One of the limitations of the study is that the metagenomic sequencing analysis is at the genomic level, without separating the viable microbes from the dead or the resident from the transient. Fortunately, some metabolites can be identified by nuclear magnetic resonance-based metabolomics of urine and other samples [153]. Also, metatranscriptomics can detect gut microbiota at gene expression level [205]. Such multi-omics approaches should be integrated into the further studies. Last, if we want to demonstrate the causative role the strains play in longevity, we should start follow-up mechanistic studies in gnotobiotic models. Eventually, such strains can have the potential to become biomarkers for longevity and targets for intervention.

Conclusion and prospect

Investigation of the bacterial and fungal communities in different body habitats in a population across age to identify the microbiota variation is the primary step in characterizing the factor influencing the diversity and distribution of human microbiota, especially the aging. In the first phase, our study indicated that the distribution of the microbiota in the human body is not only determined by the body habitats but also age-related, revealing the critical role aging plays in shaping the microbiota in human. Skin microbiota was age-related, three age groups showed dissimilarities in composition. While for oral microbiota, the variation between age groups was not significant. For the gut, bacterial communities in Sardinians were correlated with health status and age groups of the host. But the fungal communities in the gut can't be separated by age groups. Compared with the elderly and young who shared similar gut microbiota compositional and functional profiles, Sardinian centenarians have a distinct gut microbiota profile that correlates with health status. Specific enrichment of species was found in Sardinian centenarians. For the first time we demonstrated the taxonomic compositional and metabolic functional profile of gut microbiota in Sardinians, and further found that centenarians display specific gut metabolic pathway patterns, especially in the energy-harvesting related pathways. The findings of the specific taxonomic composition and metabolic pathways of gut microbiota in centenarians could be helpful in future research to determine the

relationship between gut microbiota and longevity and finally provide support for gut microbiota intervention. At the first step, we should use the animal model to study the relationship between the centenarian enriched species such as the *M.smiith* and *B.adolescentis*, also further identify their roles in host metabolism. Moreover, to further verify the "fragile" gut microbiota pattern we found in the centenarians, a cohort from Sardinian which presents the individual with the same age distribution with the elderly group but with lower health status is also need be recruited. Besides, this study eventually gave way to a second phase of exploring how the aging process shapes the microbiota.

Further integrating the surveillance of habitat environmental factors alternation into the microbiota research, we can define the correlation of aging with habitats niche alternation and microbita adaptation. For instance, for the skin microbiota, with the measurement of clinical skin parameters including the sebum level and pH of the skin surface, skin thickness and pore area; for the gut microbiota, with the record of full diet information; for the oral microbiota, with the proteomics study of the salivary. Moreover, with comparison of this healthy microbiota profile with the "unhealthy" disease related microbiota profile also can reveal how the microbiota changes cause disease. Meanwhile, the longitudinal study should also be launched to verify the aging related alternation pattern of microbiota.

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