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The birth of a hologenome: how diet, genes and environment shape the bacterial and fungal gut communities in children

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La vita e i sogni sono fogli di uno stesso libro.
Leggerli in ordine è vivere, sfogliarli a caso è sognare
A. Schopenhauer

Dedicato a
tutti coloro ai quali basta uno sguardo per capirmi

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1. Introduction

1.1 The gut microbiota: structure and function

The gut microbiota is the complex of microbial communities inhabiting the human gastrointestinal (GI) tract. The GI symbiontic communities include archaea, bacteria, fungi, and viruses. The most abundance microorganisms residing in the GI tract are bacteria, with a density of approximately 10^{13}-10^{14} cells/g of fecal matter (Weinstock 2012).

The human gut microbiota is defined a "superorganism" (Fig. 1.1). Our body made up of 10% of our cells and the rest 90% comprises microbial cells (Zhao 2013), with a biomass amount of microbiota approximately of 2 kg (Qin, Li et al. 2010). It is known that microbes resident in the gut belong to more than 1000 different species encoding more than 3 million bacterial genes (microbiome), and comprising about 150 times more genes than the human genome (Qin, Li et al. 2010).

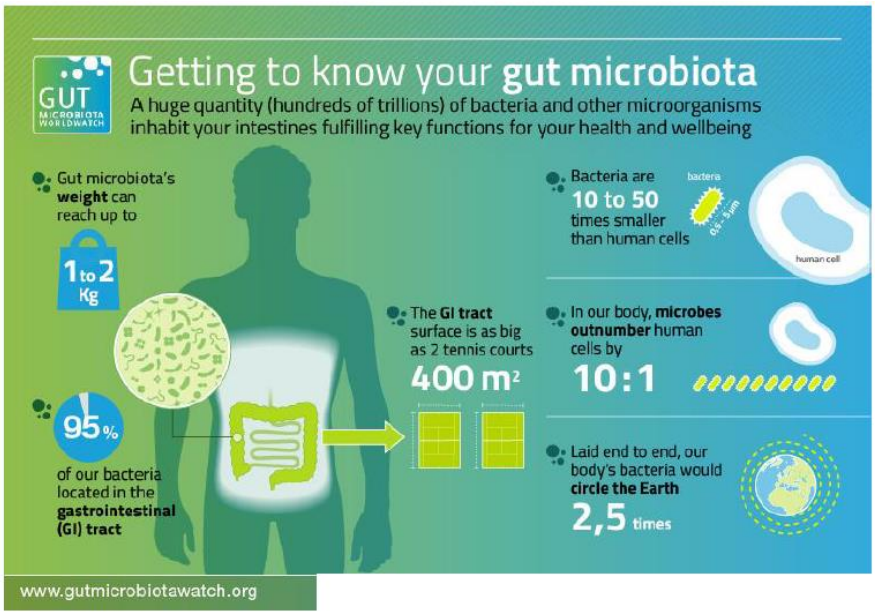

Fig. 1.1 General information about human gut microbiota (from www.gutmicrobiotawatch.org "Gut Microbiota for Health").

Under normal physiological conditions, the gut microbiota plays an important role in structural functions (Fig. 1.2), in protecting the host against pathogenic microbes (Endt, Stecher et al. 2010;Fukuda, Toh et al. 2011;Fukuda, Toh et al. 2012), in modulating immunity (Maynard, Elson et al. 2012), and in regulating metabolic processes (Tremaroli and Backhed 2012;Cani 2014), such as fermentation of indigestible dietary polysaccharides, synthesis of essential amino acids and vitamins, and metabolism of xenobiotics (Gill, Pop et al. 2006;Qin, Li et al. 2010;Yatsunenko, Rey et al. 2012;Cabreiro, Au et al. 2013).

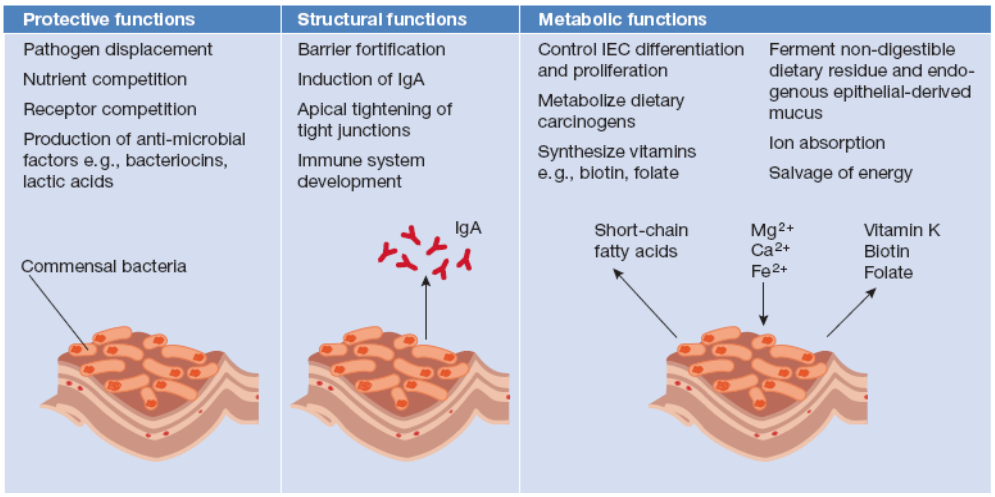

Fig. 1.2 Functional contributions of the gut microbiota (O'Hara A.M. & Shanahan F., EMBO Report 2006).

The cross talk between the host and gut microbiota in particular thorough metabolic, immune and nervous system have become the topics of study over the past decade. The symbiontic and mututalistic interaction between host and microbes is explained in the "hologenome theory of evolution" (Margulis, 1993; Rohwer et al.,2002), in which the term "hologenome" describes the sum of genetic information of the host and its symbiotic microorganisms (Zilber-Rosenberg and Rosenberg 2008). In this context, the holobiont (the host and its symbiotic microbiota) with its hologenome (genetic information of either host or microbiota), acting in consortium, should be considered as a unique unit of selection in evolution. Changes may occur either in the host genome or in the associated microbial genomes, and variation in the diverse microbial symbionts can have an important role in the adaptation and evolution of the holobiont. The association between host and symbionts affects the fitness of the holobiont within its environment, and under environmental stress, the symbiotic microbial community can change rapidly.

1.2 The microbiota composition along the gastrointestinal tract

Microbial communities are spatially distributed within the GI tract, with differential abundance from the stomach to the colon (Fig. 1.3). A variety of bacterial species passes through the GI tract strating from mouth. Few data have been collected about the stable microbial populations in the proximal esophagus. Distal esophagus is relatively limited in microbial diversity and dominated by *Streptococcus* species. Other genera such as *Prevotella*, *Actinomyces*, *Lactobacillus*, and *Staphylococcus* have been found (Pei, Bini et al. 2004). In contrast to other body sites, where greater microbial diversity is associated with human health, increased microbial diversity in the distal esophagus has been associated with chronic inflammation and dysplasia (Yang, Lu et al. 2009). The microbial diversity of the human stomach is strongly influenced by low pH of the gastric lumen that selects acid-resistant bacteria, and by presence or absence of the gastric pathogen *Helicobacter pylori*. When *H. pylori* infection occurs, the microbial diversity of the stomach is limited (Bik, Eckburg et al. 2006). The most prevalent genera in the gastric microbiota comprises a total

of 10 genera including *Prevotella*, *Streptococcus*, *Veillonella*, and *Rothia* (Andersson, Lindberg et al. 2008).

The human small intestine remains a frontier for exploration of healthy human microbiology. It is observed that diversity and complexity of bacterial communities increase in the proximal–distal direction, from the duodenum through the jejunum and ileum. *Streptococcus* seems to be a dominant genus in the duodenum and jejunum (Justesen, Nielsen et al. 1984). The 70% of the total GI microbes inhabits the colon (Ley, Turnbaugh et al. 2006). The number of bacteria in the healthy human gut likely exceed 1000 species. At the terminal ileum the prevalent species change from aerobes to anaerobes (Mondot, de Wouters et al. 2013). Significant differences in microbial composition on mucosal surfaces compared to within the lumen were observed (Li, Yang et al. 2015). At the mucosal surface microbes are in closer proximity to the intestinal epithelium and may have a greater influence on the immune system, whereas luminal/fecal microbes might be more important for energy and metabolic functions. The healthy human gut is dominated by the presence of four bacterial phyla: Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, and Verrucomicrobia. Bacteroidetes and Firmicutes account for a large majority of microbiota (Tap, Mondot et al. 2009;Forbes, Van Domselaar et al. 2016). Although some evidences suggest that the ratio of Firmicutes:Bacteroidetes is related to metabolic syndromes (Turnbaugh, Ley et al. 2006), this ratio varies significantly among healthy individuals in association with different dietary habits and geographic areas (De Filippo, Cavalieri et al. 2010;Yatsunenko, Rey et al. 2012). The phylum Proteobacteria accounts for only a small proportion of GI communities in healthy individuals, but often a higher proportion of Proteobacteria was observed in patients with GI diseases, in particular with regards to *E. coli*.

In healthy condition, considerable diversity of bacterial species exists, as well as an inter-individual microbial diversity. Several studies have recognized common core subsets within the microbiota that are relatively stable throughout large populations and that can even persist in adult age (Faith, Guruge et al. 2013). However, the human microbiota is shaped by multiple factors, such as genetics, immune system and environmental factors, including diet, life style, and the aging process.

Studies on GI colonization across the age showed that gut microbiome changes with development and maturation of its host (Cebra 1999;Hooper, Midtvedt et al. 2002;Backhed, Ley et al. 2005;Hansen, Nielsen et al. 2012). The reciprocal co-evolution between mammals and their gut microbes has lasted for hundreds of millions of years (Ley, Hamady et al. 2008). An important scientific goal is to understand the relationships between the complex gut ecosystem and its role in health and disease.

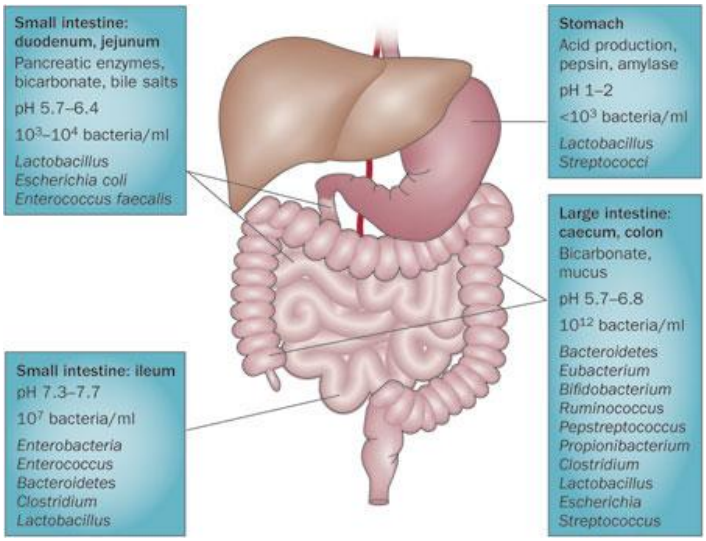

Fig. 1.3 Overview of microbial component in the different districts of GI tract (Aron-Wisnewsky J, Doré J & Clement K. *Nat Rev Gastroenterol Hepatol* 2012).

1.3 The gut microbiota: from the birth to adulthood

It was long believed that humans were sterile before birth, but recent studies have shown that the first meconium of full-term, healthy neonates contains a simple community, dominated by genera such as *Escherichia-Shigella*, *Enterococcus*, *Leuconostoc*, *Lactococcus*, and/or *Streptococcus* (Jimenez, Marin et al. 2008;Gosalbes, Llop et al. 2013). Furthermore, the mode of birth (vaginal delivery or caesarean section) may affect the composition of the early microbiota. Infants who are delivered vaginally have initial microbiota resembling those of the mothers' vaginal tracts, dominated by *Lactobacilli*, whereas those delivered by Caesarean section have initial microbiota populated by *Staphylococcus*, *Corynebacterium*, and *Propionibacterium*, largely resembling the microbiota of their mothers' skin (Palmer, Bik et al. 2007;Human Microbiome Project 2012). After the birth, the human intestine is rapidly colonized by a set of microbes. First colonizers are facultative anaerobes, which create an environment suited to colonization of strict anaerobes, such as *Bacteroides*, *Clostridium*, and *Bifidobacterium* spp. It has been observed that the gut microbiota of infants is characterized by low diversity and a relative dominance of the phyla Actinobacteria and Proteobacteria. Subsequently, the microbiota become more diverse with enrichment in Firmicutes and Bacteroidetes (Eckburg, Bik et al. 2005;Qin, Li et al. 2010;Backhed 2011). Factors known to influence colonization include genetics, gestational age, mode of birth, diet (breast milk or formula), environmental factors, such as sanitation, and antibiotic treatment (Adlerberth and Wold 2009;Marques, Wall et al. 2010) (Fig. 1.4).

As dietary richness and environmental exposures increase during the first year of life, the richness and complexity of the GI microbiota also increase (Koenig, Spor et al. 2011;Yatsunenko, Rey et al. 2012). Infancy is a distinctive stage for the microbiota also in terms of functional acquisition. The infant microbiota appears to be specialized for the acquisition of nutrients (e.g. vitamin B and amino acids) compared to that of adults (Yatsunenko, Rey et al. 2012). The first 3 years of life represent the most critical period for

child growth and development. In this period, diet changes and gut microbiota alteration has the potential to profoundly affect host health and development (Palmer, Bik et al. 2007;Agans, Rigsbee et al. 2011;Ringel-Kulka, Cheng et al. 2013). It has been observed that the GI microbiota of a 3-years-child is 40%–60% similar to that of a healthy adult, reaching an adult-like state (Palmer, Bik et al. 2007). Although the GI microbiota of children and teenagers contain many of the same taxa as adults, significant differences in proportions of *Bacteroides* and *Bifidobacterium* spp., as well as members of the class *Clostridia* have been reported.

It has been proposed that the adult GI microbiome remains stable from the third through seventh decades of life (Biagi, Nylund et al. 2010). The specific microbial diversity in healthy adult subjects plays an important role in maintaining immune homeostasis. During this relatively stable period of microbiota development, important taxa and their functions could be altered. It is known that the loss of immune gut homeostasis and leaky gut syndrome are linked with microbiota alterations and predispose to disease (Kayama and Takeda 2012). Aging and inflammation are correlated processes, and key hallmarks include decreases in GI function, host immune response and the development of chronic or low-grade inflammation. Much of these variations appear to be diet-driven, and correlate strongly with indicators of relative health, including markers of frailty and inflammation (Claesson, Jeffery et al. 2012).

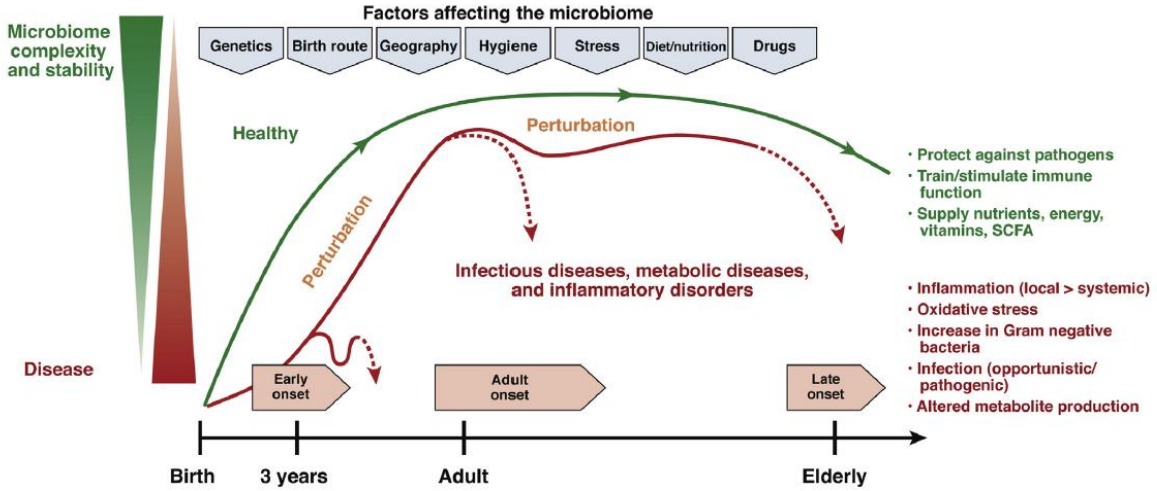

Fig. 1.4 Factors affecting the stability and complexity of the gut microbiota over time from infancy to adulthood and elderly include genetics, mode of birth, diet, geography, hygiene, stress, medication. Some of these factors can introduce perturbations affecting the complexity, resilience and stability of the microbiota, potentially introducing microbial dysbiosis. Features of an imbalanced microbiome include an increase in gram-negative bacteria linked to an environment of oxidative stress and inflammation and metabolite production. In the healthy gut, diversity, resilience and stability contribute to important physiological processes such as protection against pathogens, training of the immune system, and digestion of food to supply energy and nutrients including vitamins and SCFAs (Kostic AD, et al. *Gastroenterology* 2014).

1.4 Host-microbes coevolution, gut homeostasis and dysbiosis

Humans represent a scaffold on which diverse microbial ecosystems are established (e.g. skin, vagina and gut). During evolution, changes in the length and compartmentalization of

the digestive tract have enabled vertebrates to occupy diverse habitats and exploit different feeding strategies. Many of these innovations in gut physiology were driven by the need to optimize basic physiological and biologic functions, such as nutrient absorption (Ley, Hamady et al. 2008). Animal and human host and their microbiota have co-evolved over the millennia into a homeostatic, symbiotic relationship. Normal functioning of the digestive and immune systems depend on the presence of nonpathogenic "beneficial" bacteria. Homeostasis of the gut microbiota is characterized by the co-existence of various microbial species. Healthy status is represented by high level of gut microbial richness, while disease condition is characterized by depletion of gut microbial species (Cotillard, Kennedy et al. 2013;Le Chatelier, Nielsen et al. 2013). In an ecologic context, the biodiversity creates an ecosystem able to resist the perturbation from outside environment (Turnbaugh, Hamady et al. 2009). In fact, competitive interactions among microbial species may help in maintaining the gut microbiota stability (Coyte, Schluter et al. 2015). This phenomenon includes the concept of "resilience" that is referred to the amount perturbation that a microbial system can tolerate and the ability to restore the equilibrium state (Lozupone, Stombaugh et al. 2012). Understanding how the health microbiota forms a stable and resilient state would allow strategies able to increase the resilience in disease conditions (Fig. 1.4).

Disruption of microbial equilibrium can result in dysbiosis. Gut dysbiosis refers to an altered composition of intestinal microbial populations and it is thought to provide continuous immunological stimulation leading to immune response anomalies and increase risk of disease. Altered composition of gut microbiota has been observed in a number of GI diseases, such as Inflammatory Bowel Disease (IBD), celiac disease, irritable bowel syndrome (IBS), antibiotic-associated diarrhea, tropical enteropathy, and others (Brown, Wlodarska et al. 2015;Keely, Walker et al. 2015;D'Argenio, Casaburi et al. 2016;Distrutti, Monaldi et al. 2016;Larcombe, Hutton et al. 2016). Several evidences propose that dysbiosis of gut microbiota is not limited to gastrointestinal diseases; thereby suggesting that gut bacteria can affect the systemic immunological response. A number of studies have investigated gut dysbiosis in relation to metabolic syndromes (obesity, diabetes), chronic periodontitis, vaginosis, atopic diseases, rheumatic disease, Alzheimer's disease, autism and others (Daulatzai 2014;Blasco-Baque, Garidou et al. 2016;Johnson and Ownby 2016). However, while a breakdown in the equilibrium of the intestinal milieu may be widely recognized, it is unclear whether dysbiosis represents a cause or consequence of disease. Several studies showed differential factors contributing to dysbiosis, including host genetics (mutations in genes involved in intracellular recognition of microbes- *NOD2*, or related to immune regulatory or pro-inflammatory responses), lifestyle (diet, stress), exposure to microorganisms and medical practices (antibiotics, hygiene). Mutations in genes involved in immune regulatory mechanisms or pro-inflammatory pathways could lead to uncontrolled inflammation in the intestine. In conditions of dysbiosis, there is an abnormal shift in the microbiota composition, which results in either a reduction in the numbers of symbionts and/or an increase in the numbers of pathobionts (Fig. 1.5). The result is a non-specific inflammation, which may predispose genetically susceptible individual to inflammatory disease. It is also hypothesized that inflammation alone influences the composition of the microbiota in favor of pathobionts (Round and Mazmanian 2009).

Moreover, sanitation and overuse of drugs and antibiotics, which do not distinguish between pathogenic or symbiotic microorganisms, could adversely alter the microbiota (Round and Mazmanian 2009).

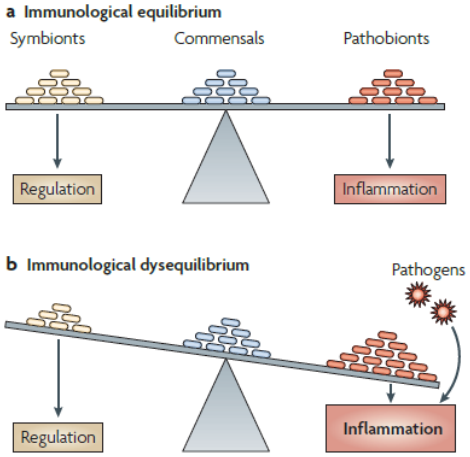

Fig 1.5 A healthy microbiota contains a balanced composition of bacteria, such as symbionts that are organisms with known health promoting functions, commensals that are permanent residents provide no benefit or detriment to the host, and pathobionts that are also permanent residents of the microbiota and have the potential to induce pathology. During dysbiosis the numbers of symbionts is decreased and the numbers of pathobionts is increased. Recent societal advances contribute to immunological dysregulation associated with dysbiosis of the microbiota (Round JL, et al. *Nat Rev Immunol.* 2009).

In this context, the "old friends hypothesis" proposed in 2003 by Rook et coworkers (Rook, Martinelli et al. 2003), and the “hygiene hypothesis” (Strachan 2000) may offer an explanation for the link between microbial exposure and development of diseases. The “old friends hypothesis” referred to the microbes evolved together with the host immune system, in hunter-gatherer era, and thus having been present during the evolution of the human immune system. They are thought to have been strictly involved with the human immune defense development. (Rook, Lowry et al. 2013). The “hygiene hypothesis” (also called the “biome depletion theory” and the “lost friends theory”) states that reduction of important microbial pattern necessary to educate the host immune system results in decreased gut microbial diversity and altered host-microbe interactions that can promote disease in susceptible host. Thus, a sterile environment or excessive hygiene, typical of westernized countries, allow to protect from exposure to dangerous pathogens, but also avoid the exposure to “old friends” health-promoting bacteria, making immature immune system, especially in childhood, and favoring development of allergies and autoimmune diseases (Fig. 1.6).

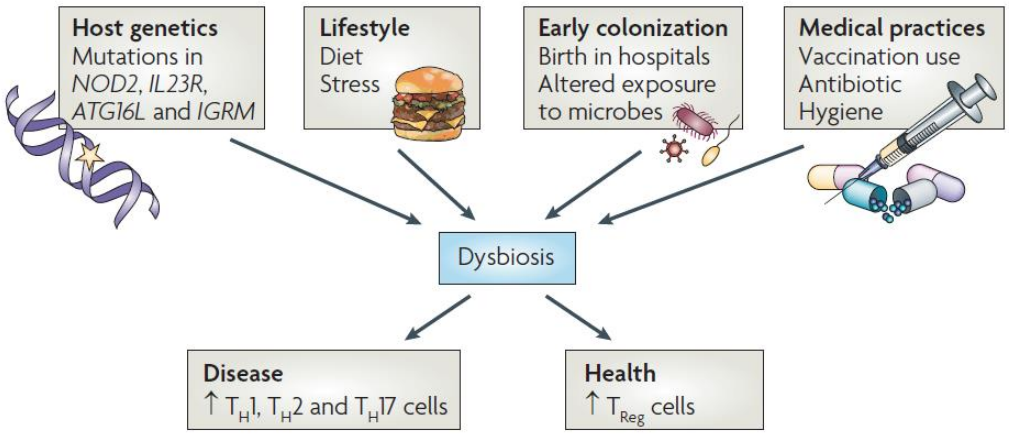

Fig. 1.6 Causes of microbial dysbiosis include genetics, lifestyle, early colonization and medical practices. The composition of gut microbiota can shape a healthy immune response (immunoregulation by production of Treg cells) or predispose to disease (inflammatory responses by T helper -Th1-Th2-Th17 cells) (Round JL, et al. *Nat Rev Immunol*. 2009).

1.5 The impact of the diet on gut microbiota

Several studies demonstrated that the mutual relationship between the gut microbiota and its host is strongly influenced by diet. Consumption of various nutrients affects the composition and structure of the microbial communities and provides substrates for microbial metabolism.

Starting from the childhood, the greatest change in the microbiota composition occurred with the introduction of solid foods. In this period of life a shift toward a more stable, adult-like microbiota occur with weaning (Koenig, Spor et al. 2011). This event introduces the potential association between diet, commensal bacteria, and health. Multiple studies have established differences in gut microbiota composition in breastfed or formula fed infants (Stark and Lee 1982;Yoshioka, Iseki et al. 1983;Penders, Thijs et al. 2006). In human milk, there are important bioactive compounds, especially indigestible glycan that contribute to absorption and digestion of nutrients, immune protection, and selectively promoting growth of *Bifidobacterium* genus (Stark and Lee 1982;Yoshioka, Iseki et al. 1983;Balmer and Wharton 1989;Le Huerou-Luron, Blat et al. 2010;Hernell 2011). *Bifidobacteria* are linked to fortification of the gut mucosal barrier, to protection against pathogens and to modulation of the gut immune system (Lievin, Peiffer et al. 2000;Ouwehand, Isolauri et al. 2002;Fukuda, Toh et al. 2011). Another difference is that aerobic organisms seem to be more prevalent in the feces of breastfed infants, whereas anaerobic and facultative anaerobic organisms, which preferentially use anaerobic glycolysis, are more commonly found in the feces of formula-fed infants (Koenig, Spor et al. 2011).

Food sources have guided the evolution of humans, and the co-evolution with symbiontic microbes leading to mutualistic relationships. A comparison of the gut microbiota between different primates and mammals found that humans clustered more closely with omnivorous primates than non-primates species (Ley, Hamady et al. 2008). Thus, variety of foods in an omnivorous diet is the most important factor of microbiota evolution.

Significant variations in microbiota composition have been associated with consumption of dietary fiber from fruits, vegetables, and other plants, compared to a diet rich in animal protein, simple sugar and lipid. In controlled dietary experiments in humans, variations in intake of resistant starch or non-starch polysaccharide altered levels of specific bacterial taxa, such as *Ruminococcus bromii* and *Eubacterium rectale* (Walker, Ince et al. 2011). These taxa were shown to selectively metabolize specific insoluble carbohydrate substrates based on *in vitro* analyses of human fecal samples (Leitch, Walker et al. 2007). Model systems have shown that an important function of the gut mcrobiota is its ability to ferment complex carbohydrates and polysaccharides which leads to production of short-chain fatty acids (SCFAs) (Koropatkin, Cameron et al. 2012). Sources of glycans for intestinal microbiota metabolism are derived not only from diet but also from mucus produced by the host.

In the last years, the study of different populations worldwide, in uncontrolled environments, help to understanding of how diet shape the microbiota composition. Several studies have showed systematic comparation of fecal microbiota from traditional populations living in non-industrialized societies to those with a westernized lifestyle (De Filippo, Cavalieri et al. 2010;Yatsunenko, Rey et al. 2012;Schnorr, Candela et al. 2014;Martinez, Stegen et al. 2015;Gomez, Petrzelkova et al. 2016). Our study (De Filippo, Cavalieri et al. 2010) showed for the first time that the gut microbiota from children living in rural African village of Burkina Faso is completely different from the microbiota of children living in the Western world, such as Italy. We demonstrated that the different dietary habits (fiber-rich diet of rural African populations versus typical western diet, high in fat, animal-protein and simple sugar) shape the gut microbiota composition. The gut microbiota of rural African children was enriched in specific bacterial genera (*Prevotella, Xylanibacter, Butyrivibrio* and *Treponema*) able to degrade fiber and to maximize the energy extraction from indigestible dietary polysaccharides, producing higher levels of SCFAs, compared to the microbiota of European children. We demonstrated that properly the diet is responsible of microbiota composition. In fact, children of 1-2 years old, belonging to both the populations, that were breast-fed, showed the same microbiota composition with predominace in *Bifidobacteria*.

More recently, the study of impact diet on gut microbiota was observed in geographically isolated populations, such as Amazonas from Venezuela, or rural populations from Malawi (Yatsunenko, Rey et al. 2012), Bangladeshi children (Lin, Bik et al. 2013), Papua New Guinea populations (Martinez, Stegen et al. 2015) versus U.S.A. residents. Moreover, evaluation of the gut microbial structures between the rural Hadza hunter-gatherers in Tanzania and urban Italians contributed to understand the differential metabolic acquisition of gut microbiota (Schnorr, Candela et al. 2014).

The MetaHIT Consortium has proposed to categorize the gut microbiota in “enterotypes” (Arumugam, Raes et al. 2011). People can be classified as having a predominance of *Prevotella*, Bacteroides, or *Ruminococcus* in the gut microbiota. These enterotypes was correlated with the diet rich in animal-protein and fat (*Bacteroides*) and diet rich in carbohydrates and simple sugars (*Prevotella*) (Wu, Chen et al. 2011). There has been considerable discussion about the enterotypes; some data sets support the existence of these categories, whereas others do not (Lozupone, Stombaugh et al. 2012), suggesting that detection of enterotypes depends on the computational approach used to analyze data sets

(Koren, Knights et al. 2013). It is suggested that a better term might be “enterogradient”, based on predominace of *Bacteroides* or *Prevotella*. It appears that these two genera largely do not exist in equal proportions in the human intestine (Faust, Sathirapongsasuti et al. 2012). A greater proportion of *Prevotella* in the human gut microbiota could be considered a marker of rural or traditional culture, whereas a greater proportion of *Bacteroides* is associated with industrialized countries (Arumugam, Raes et al. 2011).

Altogether, these studies demonstrated co-evolution between diet and microbiota in rural areas and urban societies, showing the effect of westernization on loss of traditional microbes (De Filippo, Cavalieri et al. 2010;Wu, Chen et al. 2011;David, Maurice et al. 2014;Sonnenburg and Backhed 2016), supporting the hypothesis of consequent bacterial richness depletion in relation with metabolic (obesity, insulin resistance, dyslipidemia), and inflammatory disorders.

However, diet has also been associated with other types of gut microbes, such as archaea and fungi (Dollive, Peterfreund et al. 2012;Hoffmann, Dollive et al. 2013). For example, carbohydrate consumption is associated with abundance in archeon *Methanobrevibacter* in the gut (Hoffmann, Dollive et al. 2013). *Methanobrevibacter* can increase the production of gas and SCFAs by metabolizing hydrogen, deriving by carbohydrate fermentation, and thus playing an anti-inflammatory role (Samuel and Gordon 2006). Similarly, diet might also affect intestinal fungal communities, which have been associated with the pathogenesis of IBD. Evidences showed that alteration of immune response to fungi could contribute to chronic inflammation, as occurs in Crohn’s disease (Sendid, Quinton et al. 1998;Iliev, Funari et al. 2012;Sokol, Leducq et al. 2016).

1.6 The metabolome

The gut microbiome represent an extended reservoir of metabolic capabilities able to affect significantly the host metabolism with a wide range of otherwise inaccessible metabolic capacities.

The metabolome is the set of small-molecule chemicals found within a biological sample that may include endogenous metabolites that are naturally produced by an organism (such as amino acids, nucleic acids, fatty acids, amines, sugars, vitamins, co-factors, antibiotics, etc.), as well as exogenous chemicals (such as drugs, environmental contaminants, food additives, toxins and other xenobiotics; Fig. 1.7).

In experimental animal model, Turnbaugh and collaborators defined a core gut of microbiome, representing a consistent set of functional profile (Turnbaugh, Hamady et al. 2009). Identifying biologically important variations against this core remains a challenge.

Metabolomic studies analyze complex systems, including the repertoire of the gut metabolites, using high-throughput analytical methods. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy allow robust and sensitive identification of metabolites produced by microbes and host cells in different samples (such as feces, urine, and tissue). These tools allow for reconstructing the metabolic profile of gut microbiome by analyzing the presence and quantity of thousands of metabolites simultaneously, in comparison with metabolic outcomes in the host, determining the effect of a specific treatment or perturbations.

The dietary components that escape digestion in the upper gastrointestinal tract provide most of the substrates for the gut microbiota. Fermentation of carbohydrates by bacteria leads to the production of SCFAs, such as butyrate, propionate, and acetate, promoting health and provide resistance to infection. Studies have shown that ulcerative colitis patients have fewer butyrate-producing bacteria (eg, *Roseburia hominis* and *Faecalibacterium prausnitzii*), resulting in lower levels of butyrate (Machiels, Joossens et al. 2014;Wang, Chen et al. 2014). In addition, butyrate and propionate can potentiate de novo generation of T regulatory (Treg) cells in the peripheral immune system (Furusawa, Obata et al. 2013). It has been suggested that modulation of butyrate- and propionate-producing microbes might therefore be used to treat IBD (Ursell, Haiser et al. 2014).

GI microbes can catabolize protein to amino acids and participate in the luminal conversion of amino acids to biogenic amines, immunomodulatory compounds, and other signaling molecules. Proteins from the diet may be catabolized by microbial proteinases and peptidases, together with human proteinases. The generation of free amino acids in the stomach and small intestine provide substrates for luminal conversion of amino acids. Amino acids can be converted by decarboxylation reactions

and catalyzed by GI bacteria to form different signaling molecules. L-histidine can be converted to the biogenic amine, histamine, by histidine decarboxylases produced by GI bacteria. Histamine can suppress production of inflammatory cytokines by signaling through the histamine type 2 receptor, which is present on intestinal epithelial cells (Sander, Lorentz et al. 2006;Thomas, Hong et al. 2012). In addition, bacteria can produce antimicrobial peptides or bacteriocins that promote resilience or resistance to infection by enteric pathogens (O'Shea, Cotter et al. 2012). The production of antimicrobial compounds by commensals might prevent expansion of enteric pathogens or indigenous pathobionts (Kamada, Seo et al. 2013).

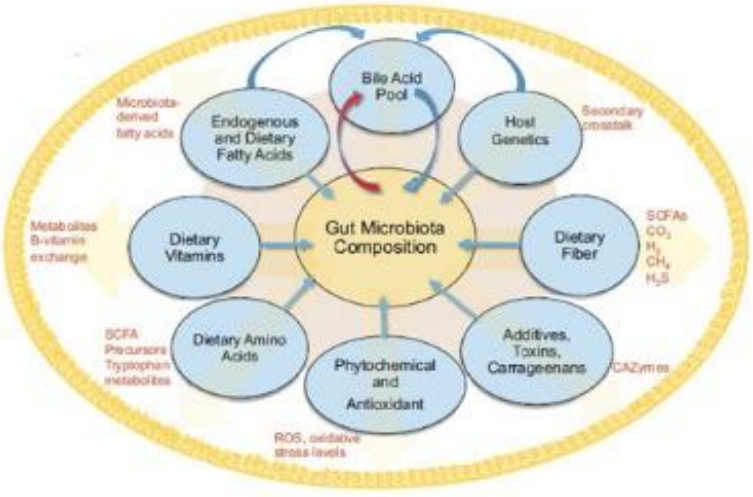

Fig. 1.7 Dietary components escaping the digestion in the GI tract provide most of the substrates for the gut microbiota (Modified from Basson A, et al. *Front Immunol.* 2016).

1.7 The gut microbiota and host immune system

Several studies indicated a mutualistic relationship between gut microbiota and host innate

and adaptive immune system. The immune system is responsible for recognizing, responding and adapting to foreign and self molecules and therefore it is important during conditions of both health and disease (Round and Mazmanian 2009).

Unlike opportunistic pathogens, which elicit immune responses resulting in tissue damage during infection, some symbiotic bacteria have been observed to prevent inflammatory disease during colonization. However, the "normal" microbiota also contains microorganisms capable to induce inflammation under particular conditions (pathobionts). Therefore, the microbiota has the potential to exert both pro- and anti-inflammatory responses, and the composition of gut bacterial communities may be intimately linked to the functioning of the immune system.

The role of the microbiota in the development of the immune system has been studied for more than a century, and major information derive by germ-free (GF) and gnotobiotic animal models. GF animals show impaired development of gut-associated lymphoid tissues (GALTs), such as Peyer's patches (PPs) and isolated lymphoid follicles (Gordon, Bruckner-Kardoss et al. 1966;Hamada, Hiroi et al. 2002;Bouskra, Brezillon et al. 2008). Notably, a greater number of IgE+ B cells and a lower number of IgA+ B cells were found in the PPs of GF mice, compared with conventionally raised mice (Durkin, Bazin et al. 1981). Colonization of GF mice by commensal microorganisms induces immune reactions in lymphoid cell called "germinal centers" and IgA development (Shroff, Meslin et al. 1995;Talham, Jiang et al. 1999).

The microbiota also have an important role in balancing effector T-cell immune responses in the GI tract (Kamada and Nunez 2013). GF mice have reduced numbers of T-helper (Th)1 and Th17 cells. Therefore, the intestinal T-cell immune response in GF animals is controlled primarily by Th2 cells (Mazmanian, Liu et al. 2005;Chung, Pamp et al. 2012). The imbalance in Th cell responses in GF mice can be reversed by reconstitution with conventional microbiota, indicating that microorganisms shape intestinal Th-cell–mediated immunity (Chung, Pamp et al. 2012).

The balance between pro-and anti-inflammatory mechanisms, important for gut immune homeostasis, is directly affected by the commensal microbes. For example, *H. pylori* infection induces Interleukin (IL)-17 responses in the GI tract. In mice, segmented filamentous bacteria (SFB) promote the accumulation of pro-inflammatory Th1 and Th17 cells in the small intestine (Gaboriau-Routhiau, Rakotobe et al. 2009). Th17 cells are a lineage of CD4+ Th cells, which defend against extracellular microorganisms and also are involved in autoimmune disease development (Littman and Rudensky 2010) (Fig. 1.8). In GF mice or mice treated with antibiotics, the numbers of Th17 cells in the small intestine is greatly reduced (Hall, Bouladoux et al. 2008;Ivanov, Frutos Rde et al. 2008). Other members of the human gut microbiota and certain bacterial pathogens are strong inducers of Th17 cell development. For example, enteric pathogens can induce a Th17-mediated response, even in the absence of commensal microbes, as observed by monocolonization of GF mice with the enteric pathogen *Citrobacter rodentium* (Kamada, Kim et al. 2012).

Anti-inflammatory responses are promoted by the generation of regulatory T cells (Treg). Several studies showed that retinoic acid (Mucida, Park et al. 2007), bacteria belonging to the *Clostridium cluster IV* and *XIVa* (Atarashi, Tanoue et al. 2013), *Faecalibacterium prausnitzii* (Qiu, Zhang et al. 2013) and SCFAs production (Arpaia, Campbell et al. 2013)

promote accumulation of Treg cells, as well as polysaccharide A of *Bacteroides fragilis*, a human commensal, facilitate the differentiation of CD4+ Th in Treg cells and IL-10 production (Round and Mazmanian 2010) (Fig. 1.8). Hence, microbes and metabolites in the gut are essential in maintaining immunological equilibrium.

Furthermore, the gut bacteria is essential for the development and function of Foxp3+ Treg cells in the intestine (Fig. 1.8). In the absence of the microbiota, the number of inducible Foxp3+ Treg (iTreg) cells is reduced significantly in the colonic lamina propria (Atarashi, Tanoue et al. 2011;Geuking, Cahenzli et al. 2011). Reduced development of iTreg cells in GF mice can be restored by recolonization with the intestinal microbiota, indicating that commensals are involved in the generation of colonic Treg cells.

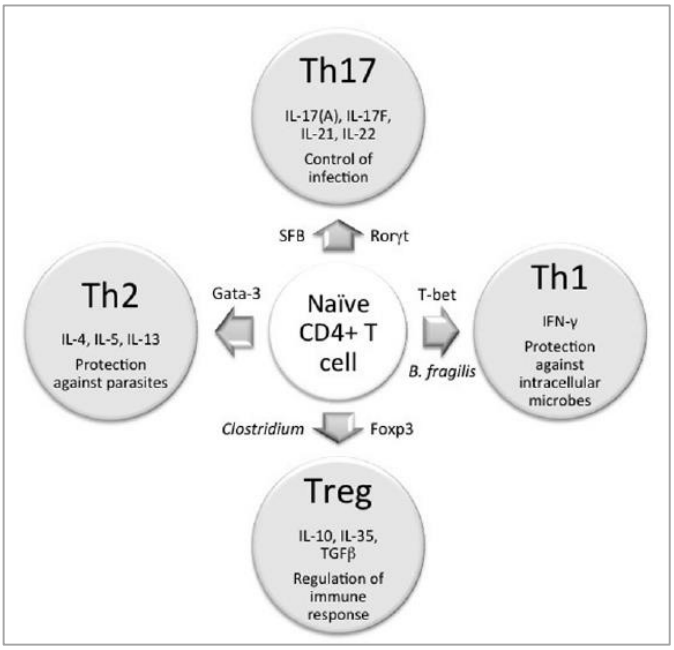

Fig 1.8 Commensal bacteria induce CD4+T cell differentiation. The differentiation of naïve CD4+T cells in Th1, Th2, Th17 lineages and Tregs. Each differentiated lineage secretes specific cytokines. Bacteria species are also able induce a particular T cell differentiation pathway. Th1 cells play an important role in eliminating intracellular pathogens, while Th2 function to control parasitic infection. The primary role of Th17 is to control infection while Tregs is to regulate immune response (Wu HJ & Wu E *Gut Microbes* 2012).

The commensal microbiota regulates the development of intestine-specific B-cell receptors, and influences immunoglobulin response within the intestinal mucosa. Within the GI tract, most IgA-producing B cells mature in PPs upon stimulation by commensal microorganisms (Macpherson, Geuking et al. 2012). Early stages of B-cell development occur not only in fetal liver and bone marrow, but also in the intestinal mucosa (Wesemann, Portuguese et al. 2013). In GF mice, the lack of microbiota-derived signals results in immature formation of germinal centers in the PPs and reduced generation of IgA-producing B cells (Fagarasan, Kawamoto et al. 2010).

Although commensal bacteria are important for the development of intestinal immune responses, excessive immune stimulation by commensals can lead to inappropriate activation of immune cells and harmful intestinal inflammation. In the colon, the epithelial surface is covered by an inner mucus layer that shields the immune system from excessive

stimulation by resident microbes and by mechanical stress, whereas large numbers of commensal bacteria colonize the outer mucus layer (Kamada and Nunez 2014). Thus, perturbations of the gut microbiota disrupt this balance primarily affecting the gut mucosa and the systemic immune response. A 'leaky gut' characterized by increased gut permeability, microbial imbalance, and impaired mucosal immunity has been identified to promote the development of immune mediated-inflammatory diseases (Forbes, Van Domselaar et al. 2016).

1.8 The gut microbiota and human diseases

The world is experiencing a progressive increase of metabolic and immune mediated diseases, with a dramatically increase in pediatric populations. It has been hypothesized that a reduced diversity of gut microbial communities may have generated an abnormal immune maturation in early childhood (Renz, Brandtzaeg et al. 2011;McDade 2012). Several immune-mediated diseases such as IBD, obesity, allergies, type I diabetes, multiple sclerosis, autism, rheumatic disease and arthritis show rising incidences in economically developed countries. In this context, a theory of "microbial deprivation syndromes of affluence" has been proposed (Renz, Brandtzaeg et al. 2011;McDade 2012;Kondrashova, Seiskari et al. 2013;West, Jenmalm et al. 2015). There is growing concern that recent lifestyle innovations, most notably the high-fat/high-sugar "Western" diet, processed and refining food, pollution, hygiene, sanitation and antibiotic treatment have altered the composition and metabolic functions of gut microbiota (Turnbaugh, Ridaura et al. 2009;David, Maurice et al. 2014).

Recent research in animal models has demonstrated an essential contribution of the gut microbiota in non-communicable diseases that have higher prevalence in westernized societies (Wen, Ley et al. 2008;Berer, Mues et al. 2011;Devkota, Wang et al. 2012;Koeth, Wang et al. 2013). These observations have led scientists to hypothesize that aberrant (dysbiotic) microbiomes and/or the loss of specific symbionts predispose westerners to non-communicable diseases (Blaser and Falkow 2009;Martinez, Stegen et al. 2015). On the other hand, non-industrialized societies are charged by a high incidence of infectious diseases, such as malaria, HIV, HBV infections, including life-threatening diarrhea (Pop, Walker et al. 2014). The importance of the gut microbiota for non-communicable diseases in westernized societies and the prevalence of infectious diseases in non-industrialized communities warrant studies that compare the microbiome in both settings.

1.9 The gut microbiota and metabolic diseases

1.9.1 Obesity

Obesity is caused by a dysregulation of energy balance where the amount of energy intake from the food exceeds the needs of the body (Turnbaugh, Ley et al. 2006). Several studies have demonstrated that altered composition of gut microbiota is correlated with obesity (Ley, Backhed et al. 2005;Ley, Turnbaugh et al. 2006;Turnbaugh, Backhed et al. 2008). One of the first studies in animal model showed that fecal microbiota of obese ob/ob mice was

enriched in Firmicutes and depleted in Bacteroidetes when compared to lean mice (Ley, Backhed et al. 2005). Similar results were confirmed in the high fat diet (HFD)-induced obese mice, in which abundance of Firmicutes was observed, together with an enrichment in Mollicutes, able to ferment simple sugars and complex carbohydrates (Turnbaugh, Backhed et al. 2008). However, the relevance of Firmicutes/Bacteroidetes ratio in human obesity did not reported in all studies (Duncan, Lobley et al. 2008;Schwiertz, Taras et al. 2010;Le Chatelier, Nielsen et al. 2013). It has been supposed that the gut microbiota may affect host metabolism by extraction of energy and nutrients from foods or by affecting the host gene expression involved in energy metabolism. Metagenomic analysis showed that the gut microbiome of obese ob/ ob mice was enriched in the degradation of dietary polysaccharides such as starch, sucrose and galactose, indicating that gut microbiota of ob/ob mice had increased capacity of food energy harvest compared with that of lean mice (Turnbaugh, Ley et al. 2006). Furthermore, GF mice with gut microbial colonization exhibited a significant increase in body fat content (Backhed, Ding et al. 2004). Fat accumulation could be promote by inhibition of AMPK-dependent fatty acid oxidation (Backhed, Manchester et al. 2007), or via suppressing the production of functional beige fat in white adipose tissue by microbiota (Suarez-Zamorano, Fabbiano et al. 2015).

Another important factor responsible for the early onset of obesity is low-grade systemic inflammation. Continuous subcutaneous infusion of lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, showed an increased body weight and adipose tissue depots, as well as the upregulation of several inflammatory cytokines (Cani, Neyrinck et al. 2007;Cani, Bibiloni et al. 2008). Interstingly, antibiotic treatment markedly reduced body weight and plasma LPS levels in both HFD-fed and ob/ob mice.

The endocannabinoid (eCB) system has been identified as another crucial modulator in the development of gut microbiota-mediated obesity. Gut microbiota alteration resulted in the change of cannabinoid 1 (CB1) receptor expression in the colon. Consequently, activation of the eCB system increased the gut permeability by decreasing the expression of two tight junction proteins. In the adipose tissues, eCB system activation also promoted adipogenesis via increasing the expression of genes involved in the adipocyte differentiation and lipogenesis (Muccioli, Naslain et al. 2010).

Several studies have demonstrated that SCFA signaling pathways are involved in the gut microbiota-mediated host metabolism (Tazoe, Otomo et al. 2008;Breton, Tennoune et al. 2016). SCFAs are the main fermentation products of dietary fibers metabolized by intestinal bacteria. G protein-coupled receptor 41 (GPR41) and 43 (GPR43) are two important SCFA receptors located in the enteroendocrine cells of intestinal epithelium. Activation of these receptors by SCFAs promoted the release of 5-hydroxytryptamine (5-HT) and peptide YY (PYY), thereby influencing the colonic mobility (Tazoe, Otomo et al. 2008). Production of circulating hormone PYY was also required for increasing gut microbiota-induced efficiency for energy harvest from the diet (Samuel, Shaito et al. 2008). Intestinal gluconeogenesis has a beneficial effect on the regulation of glucose and energy homeostasis. Butyrate directly induced gluconeogenesis by promoting the activity of glucose-6-phosphatase and the expression of phosphoenolpyruvate carboxykinase 1. In contrast, propionate-mediated induction of gluconeogenesis was dependent on a gut-brain communication axis (De Vadder, Kovatcheva-Datchary et al. 2014).

1.9.2 Type 2 diabetes

Type 2 diabetes is a metabolic disorder characterized by insulin resistance. Obesity is considered to be linked with the onset of type 2 diabetes. Recently, alteration of gut microbiota was associated with the development of diabetes (Larsen, Vogensen et al. 2010;Qin, Li et al. 2012;Karlsson, Tremaroli et al. 2013) (Fig. 1.9). Metagenomic analysis showed that the ratio of Bacteroidetes/Firmicutes and the abundance of Proteobacteria were higher in diabetic subjects, whereas *Clostridia* was significantly lower, compared with healthy controls (Larsen, Vogensen et al. 2010). In addition, in pre-diabetic subjects significantly lower abundance of Verrucomicrobia was observed suggesting that this phylum could be a potential diagnostic biomarker for the progression of diabetes (Zhang, Shen et al. 2013). In a Chinese diabetic cohort, several butyrate-producing bacteria, such as *Faecalibacterium prausnitzii*, *Roseburia intestinalis* and *R. inulinivorans* were significantly reduced, whereas opportunistic pathogens including *Clostridium symbiosum*, *Eggerthella lenta* and *Escherichia coli* were enriched compared with healthy controls (Qin, Li et al. 2012). Functional analysis indicated that pathways, related to oxidative stress resistance, methane metabolism and sulphate reduction were enriched in the gut microbiome of diabetic patients (Qin, Li et al. 2012). In another study, increased abundance of *Lactobacillus spp.* and decrease in *Clostridium spp.* were observed in the gut microbiota of European diabetic women. In general, functional pathway enriched in diabetic populations were related to starch and glucose metabolism, fructose and mannose metabolism, as well as ABC transporters and glutathione synthesis (Karlsson, Tremaroli et al. 2013).

1.9.3 Cardiovascular disease

In an animal model study, dietary supplementation with three dietary lipid phosphatidylcholine metabolites, such as choline, trimethylamine N-oxide (TMAO) and betaine resulted in the enhancement of atherosclerosis in Apoe−/− mice. In these mice, suppression of gut microbiota by broad-spectrum antibiotics completely repressed the dietary choline-mediated progression of atherosclerosis (Wang, Klipfell et al. 2011). Study in humans further confirmed that the gut microbiota played a key role in the production of plasma TMAO, significantly associated with an increased risk of major adverse cardiovascular adverse events (Tang, Wang et al. 2013) (Fig. 1.9). Zhu and collaborators demonstrated that TMAO could directly promote the platelet hyperreactivity by increasing the intracellular calcium release and thereby enhancing the potential of thrombosis risk (Zhu, Gregory et al. 2016). Targeted inhibition of microbial TMA production by a structural analog of choline, 3,3-dimethyl-1-butanol (DMB), has recently been shown to attenuate the development of atherosclerosis in Apoe−/− mice (Wang, Roberts et al. 2015).

1.9.4 Liver diseases

Altered gut microbial composition has also been demonstrated to be related with several liver diseases, such as nonalcoholic fatty liver disease (NAFLD) (Jiang, Wu et al. 2015),

hepatic encephalopathy (Bajaj, Ridlon et al. 2012) and cirrhosis (Qin, Yang et al. 2014) (Fig. 1.9).

Specific bacterial families, such as *Alcaligenaceae*, *Porphyromonadaceae* and *Enterobacteriaceae*, were significantly associated with cognitive impairment and inflammation in hepatic encephalopathy patients (Bajaj, Ridlon et al. 2012). The gut microbiota of patients with hepatitis B liver cirrhosis had depletion in bacteria important for colonic bile acid metabolism (Wei, Yan et al. 2013).

At the functional level, microbial functions of liver cirrhosis patients were enriched in ammonia production, phosphotransferase systems and membrane transport, whereas functions including carbohydrate and amino acid metabolism were enriched in healthy controls (Qin, Yang et al. 2014).

High prevalence of small intestinal bacterial overgrowth was observed in patients with cirrhosis (Gupta, Dhiman et al. 2010).

Immune responses may be involved in the etiology of microbe-associated liver diseases. In patients with hepatitis by C virus infection, the ability of anti-bacterial antibodies in regulating complement-mediated killing of gut bacteria was impaired (Lamontagne, Long et al. 2013).

Enterohepatic circulation is another key mechanism linking the gut microbiota and the liver. In the enterohepatic circulation, bile salts synthesized in the liver are secreted into the intestinal tract, where they could be metabolized by intestinal bacteria in primary and secondary bile acids (Ridlon, Kang et al. 2006). These findings suggest that bile acid may play an essential role in the regulation of gut microbiota composition, but gut microbiota can also have an active effect on the physical functions of the liver (Islam, Fukiya et al. 2011;Kakiyama, Pandak et al. 2013).

Gut microbiota-mediated metabolism of dietary choline might contribute to the development of NAFLD (Dumas et al., 2006). Moreover, inflammasome deficiency-associated dysbiosis of gut microbiota might also be a determinant factor in the progression of NAFLD (Henao-Mejia, Elinav et al. 2012).

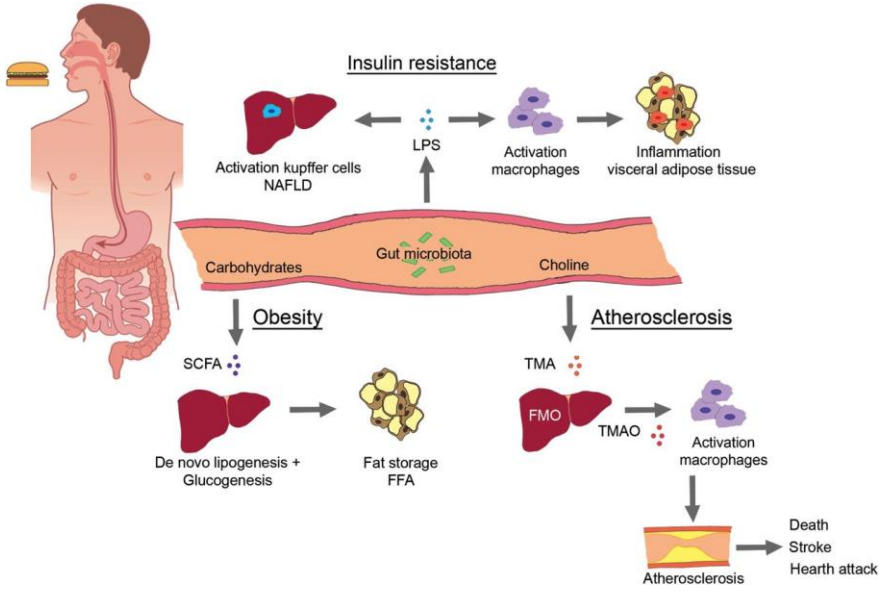

Fig. 1.9 The gut microbiota and metabolic diseases. Three major pathways through which gut microbiota can alter human cardiovascular system and metabolism (Vinjé S, et al. *Eur Heart J*. 2014).

1.10 Gut-Brain axis and neurological diseases

The "gut-brain axis" is a bi-directional communication system comprised of neural pathways, such as the enteric nervous system, vagus, sympathetic and spinal nerves, and humoral pathways, which include cytokines, hormones, and neuropeptides as signaling molecules. Recent evidences, arising from animal models, support a role of gut microbes as signaling components in the gut-brain axis (Bercik, Collins et al. 2012) (Fig. 1.10). Of increasing interest is the relationship between the GI tract and brain function and development, and their mutualistic influence. It has been reported that, on the one hand, gut microbiota is involved in activation of neuronal pathways to the brain, in activation of mucosal immune response and production of metabolites that directly affect the nervous central system. On the other hand, the stress, anxiety induced changes in GI ecosystem (Grenham, Clarke et al. 2011).

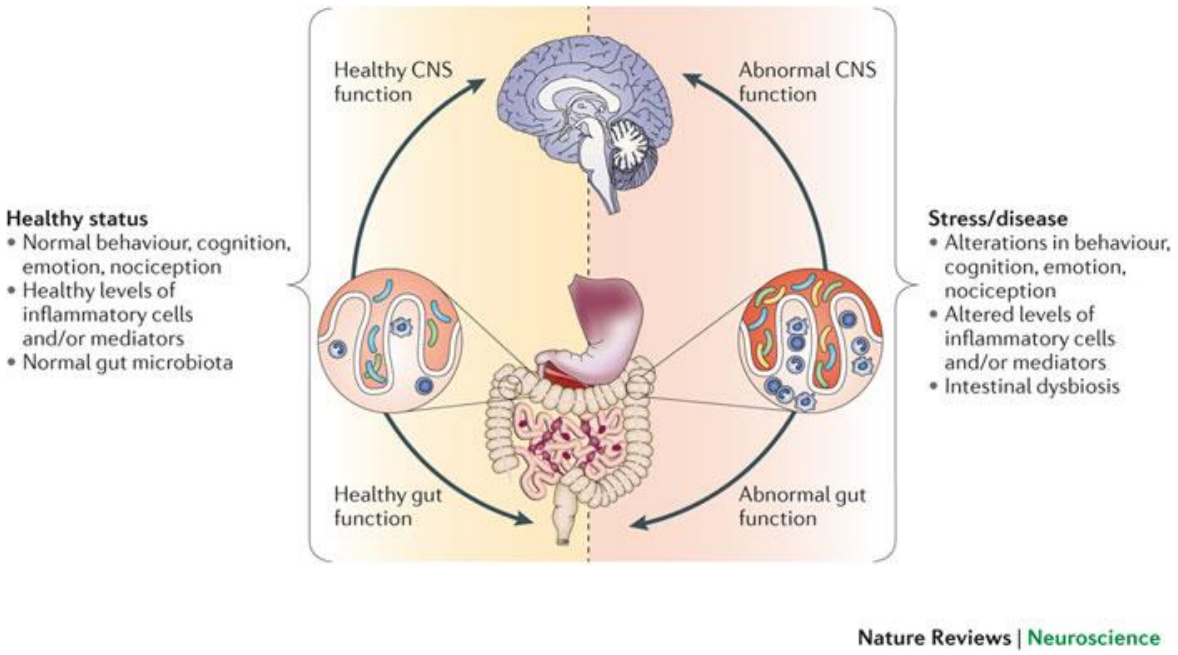

Fig. 1.10 The gut-brain axis: communication and reciprocal influence. Gut microbiota contributes to appropriate signaling along the gut–brain axis. Intestinal dysbiosis can adversely influence gut physiology, leading to inappropriate gut–brain axis signaling and consequences for central nervous system (CNS) functions, resulting in disease states. Conversely, stress and diseases can affect gut function and lead to perturbations of the microbiota (Cryan JF & Dinan TG. *Nat Rev Neurosci*. 2012).

Key experiments have shown that mice display altered and anxiety-like behavior during the early phase of acute infection with *Campylobacter jejuni* (Lyte, Varcoe et al. 1998).

Evidences indicated that blood-brain barrier (BBB) play an important role in controlling the exchange of molecules and nutrients between the brain and GI ecosystem. For example, GF mice had increased permeability of BBB compared with specific-pathogen-free (SPF) mice (Braniste, Al-Asmakh et al. 2014). Fecal transplantation from mice with normal gut microbiota into GF mice or treatment of GF mice with bacteria that produce SCFAs decreased the permeability of BBB (Braniste, Al-Asmakh et al. 2014). The gut microbiota

was also found to be crucial for the maturation and function of microglia in the brain (Erny, Hrabe de Angelis et al. 2015).
GF mice displayed increased motor activity and reduced anxiety-like behaviors when compared with the SPF mice (Diaz Heijtz, Wang et al. 2011). GF mice also had significant social impairments and were more susceptible to the restraint stress than SPF mice (Sudo, Chida et al. 2004;Desbonnet, Clarke et al. 2014). In SPF mice antibiotic treatment increased exploratory behavior and the expression of brain-derived neurotropic factor (BDNF) in the hippocampus (Bercik, Park et al. 2011).
Exposure to the social disruption stressor and early life stress could also lead to an altered gut microbial composition (O'Mahony, Marchesi et al. 2009;Park, Collins et al. 2013;De Palma, Blennerhassett et al. 2015).
These findings provide strong evidence for the existence of a "microbiota-gut-brain axis" and underscore the essential role of gut microbiota in the regulation of its homeostasis (Gareau, Wine et al. 2011;Clarke, Grenham et al. 2013).
Several neurodevelopmental disorders are associated with gut microbiota dysbiosis. Children with autism often have frequent gastrointestinal complications, and abnormal composition of gut microbiota has been reported (Bolte 1998;Song, Liu et al. 2004;Finegold, Dowd et al. 2010). In the autistic children, high levels of Bacteroidetes were found, while Firmicutes were abundant in the control subjects (Finegold, Dowd et al. 2010). Abundance of toxin-producing bacterial group of *Clostridium*, as well as lower abundance of the carbohydrate-degrading bacteria, such as *Prevotella*, *Coprococcus* and *Veillonellaceae*, were found in autistic children compare with healthy controls (Parracho, Bingham et al. 2005;Kang, Park et al. 2013). Moreover, high abundance of *Sutterella* species was found in the gut of children with autism affected from gastrointestinal dysfunction (Williams, Hornig et al. 2012).
Multiple sclerosis is immune-mediated central nervous system inflammatory disorder in which the role of gut microbiota is investigating. Mice in experimental autoimmune encephalomyelitis (EAE) model produced many features of multiple sclerosis and in GF condition showed attenuated symptoms of EAE, compared with SPF mice (Lee, Menezes et al. 2011). Bacteria-mediated signals from the gut to the brain might be transduced directly via peripheral vagal afferent nerves (Gaykema, Goehler et al. 2004;Goehler, Gaykema et al. 2005;Goehler, Park et al. 2008). Therefore, intestinal microbiota might be an important regulator in the initiation of spontaneous demyelinating autoimmune disease (Berer, Mues et al. 2011).

1.11 The gut microbiota and immune-mediated inflammatory diseases

1.11.1 Inflammatory Bowel Disease

Alteration in the commensal bacteria equilibrium was investigated principally in Inflammatory Bowel Disease (IBD). IBD comprises Crohn's disease (CD) and ulcerative colitis (UC), two chronic inflammatory disorders with multifactorial etiology (Aujnarain, Mack et al. 2013;Fiocchi 2013). Those pathologies are characterize by interplay between

genetic, environmental and immunological factors leading to a dysregulated host immune response to the intestinal microbial communities (Fig. 1.11A).

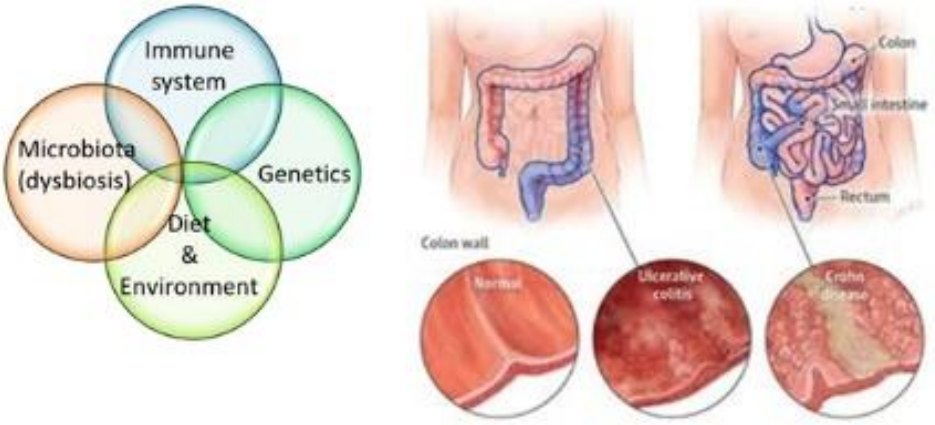

Fig. 1.11 (A) Factors involved in the etiology of IBD. (B) Different disease extension and histological characteristics between Ulcerative colitis and Crohn's disease. Ulcerative colitis typically begins in the rectum and may extend in the entire colon. UC affects only the inner layer of the bowel wall. Crohn's disease generally involved the end of the small intestine and the beginning of the colon, and may affect any part of the GI tract in a patchy pattern. CD may affect all layers of the bowel wall.

Recent evidences showed an increased incidence of IBD in children (up to 25%). Early onset-IBD is characterized by disease severity and a greater impact of genetic predisposition compared to older age of onset (Gupta, Bostrom et al. 2008;Sauer and Kugathasan 2010). Deficiency of IL-10, a well-known anti-inflammatory cytokine and its receptor genes were associated with very early-onset IBD (Glocker, Kotlarz et al. 2009;Kotlarz, Beier et al. 2012;Shah, Kammermeier et al. 2012).

CD is characterized by focal and transmural lesions, with deep ulcerations that can result in development of fistulas, abscesses, and strictures (Fig. 1.11B). In contrast, UC is characterized by mucosal inflammation in colon, beginning in the rectum and extending proximally (Fig. 1.11B). Both types of IBD often involve chronic relapsing that can be medically managed but may require surgical intervention in severe cases (Kaplan 2015).

A role of gut microbiota in IBD has been suspected since the early descriptions of potential infectious agents (Kirsner 1988), but no single agent has been proven to cause IBD. The intestinal bacterial communities in IBD has been thoroughly investigated, and several groups worldwide observed dysbiosis. In general, in the gut microbiota of CD patients a significantly lower bacterial diversity compared with that of healthy subjects was observed (Manichanh, Rigottier-Gois et al. 2006). Moreover, many IBD genetic risk variants identified by genome-wide association studies are involved in mediating host responses to gut microbiota. From the over 200 single-nucleotide polymorphisms (SNPs) associated with increased risk for IBD (Liu, van Sommeren et al. 2015), several pathways and genes have been identified that may either affect the way gut microbes are assembled or how the host is impacted by alterations in the gut microbiome. Nucleotide-binding oligomerization domain containing protein 2 (*NOD2*, also known as *CARD15*) was the first gene of the IBD

susceptibility to be identified. *NOD2* encodes an intracellular receptor for the bacterial peptidoglycan muramyl dipeptide (Hugot, Chamaillard et al. 2001;Ogura, Bonen et al. 2001). The discovery of *NOD2* and other IBD susceptibility genes (e.g. *ATG16L1*, *IRGM*, *CARD9*, and *IL23R*) underscores the importance of innate and adaptive host immune responses to gut microbiota. Furthermore, mutations that potentially alter the function or expression of these genes can affect the gut microbiota in ways that disrupt host-microbe interactions to set the stage for the onset of disease (Cadwell, Liu et al. 2008;Kaser and Blumberg 2011). Nod2-deficient mice exhibit significantly increase in *Bacteroides*, *Firmicutes*, and *Bacilli* and decreased ability to clear the pathogenic bacteria, *Helicobacter hepaticus* (Petnicki-Ocwieja, Hrncir et al. 2009). In addition, CD and UC patients with *NOD2* risk alleles (Leu1007fs, R702W, or G908R) often have decreased *Clostridium groups XIVa* and *IV* and increased *Actinobacteria* and *Proteobacteria* (Frank, Robertson et al. 2011).

In the majority of cases, however, genetic susceptibility is insufficient to cause IBD. Even among monozygotic twins, where the concordance for CD between is between 35% and 58% and for UC between 16% to 18.5% (Orholm, Binder et al. 2000;Halfvarson, Bodin et al. 2003;Spehlmann, Begun et al. 2008), the penetrance is not 100%. Thus, non-genetic factors, such as environment and gut microbiota, are important contributors to the pathogenesis of IBD.

The remarkable increase in incidence and prevalence of IBD especially in industrialized countries where changes in hygienic conditions, diet, environment, and lifestyle have taken place (Molodecky, Soon et al. 2012) suggests that the increase of IBD is more likely caused by environmental and societal factors. In this context, the increasing and promiscuous use of antibiotics may contribute to alter the gut microbiota. Changes in diet and dietary habits are among the largest modifications that occur with increased industrialization and westernization. In addition to increased daily caloric consumption, diets are higher in fat and refined sugars, and lower in complex carbohydrates and fiber. A controlled-feeding study showed that the gut microbiota composition is dramatically changed by a high fat/low-fiber compared with a low-fat/high-fiber diet (Wu, Chen et al. 2011). A study in IL-10-deficient mice showed that different types of dietary fat had different effects on gut microbiota. A diet rich in saturated milk fat, for instance, caused enrichment in Proteobacteria, particularly *Bilophila wadsworthia*, in the gut microbiota compared with isocaloric, isonitrogenous diets high in polyunsaturated fat. Furthermore, the presence of *B. wadworthia* increased the incidence and severity of spontaneous colitis in genetically susceptible IL10-deficient mice (Devkota, Wang et al. 2012). In addition, David and co-workers (David, Maurice et al. 2014) demonstrated that an animal-based diet rapidly changed the gut microbiota composition in humans and lead to increase in levels of bile-tolerant bacteria (*Alistipes*, *Bilophila*, and *Bacteroides*), including *B. wadworthia* and decrease in metabolizing plant polysaccharides bacteria, such as *Roseburia*, *Eubacterium rectale*, and *Ruminococcus bromii*.

Improvements in hygienic practices and environment conditions over the past century have also been implicated in contributing to the increase incidence and prevalence of IBD. A study in India showed that CD was positively associated with urban living and safe drinking water and negatively with the presence of cattle in the home (Pugazhendhi, Sahu et al. 2011).

Many changes in both structure and function of gut microbiota are associated with active IBD. In general, abundance of *Actinobacteria* and *Proteobacteria* and decreased abundance of *Bacteroidetes* and *Lachnospiraceae* (group IV and XIVa Clostridia) were detected in IBD patients compared with that of non-IBD controls (Seksik, Rigottier-Gois et al. 2003;Gophna, Sommerfeld et al. 2006;Frank, St Amand et al. 2007). Other studies showed alterations of gut microbiota in CD patients with increase in *Enterobacteriaceae* and *Ruminococcus gnavus*, and decrease in *Dialister invisus*, *Bifidobacterium adolescentis, Roseburia, and Faecalibacterium prausnitzii* (Willing, Dicksved et al. 2010;Joossens, Huys et al. 2011). In addition, low proportion of *Faecalibacterium prausnitzii*, a well-known anti-inflammatory commensal bacterium, was associated with a high risk of endoscopic recurrence following surgical resection in CD patients (Sokol, Pigneur et al. 2008).
The composition and abundance of both luminal and mucosa-associated microbiota vary along the GI tract (Peterson, Frank et al. 2008;Gu, Chen et al. 2013), and can be significantly impacted by intestinal inflammation in a number of different ways. Mucosal-associated microbiota can differ significantly from their luminal counterparts, the latter most likely being the major contributors to fecal microbiota.

1.11.2 Pharmacological treatment of IBD

Due to continuous efforts to unravel the pathogenesis of IBD and develop molecular targeted drugs, new IBD therapies are now available alongside conventional immunosuppressive treatments (Hirata, Ihara et al. 2016). The goal of pharmacological treatments is to prevent recurrence of active disease (relapses/flares), to achieve mucosal healing and to mantain remission (Aloi, Nuti et al. 2014). Treatments may depend on the part of the intestinal tract that is affected, as certain medications are formulated to release the active drug in different parts of the bowel.
Conventional therapies for IBD include the aminosalicylates, corticosteroids, and immunosuppressive drugs (Fig. 1.12). An increasing number of novel and alternative therapeutic approaches are in progress. New biologic therapies include the targeting of proinflammatory cytokines, enhancement or infusion of anti-inflammatory cytokines, blocking intravascular adhesion molecules, and modifying T-cell functions.
The current treatment of IBD follows a “step-up” approach (Fig. 1.12). Therapy of mild-to-moderate IBD may include induction treatment with mesalazine (5-ASA), antibiotic, or corticosteroids. Patients who respond to corticosteroids should be tapered and then switched to an immunomodulatory, such as azathioprine/6-mercaptopurine. Patients who do not respond to induction with 5-ASA might be switched to budesonide, when disease is mildly active, or to oral prednisone or anti-TNF-α /azathioprine, when disease is moderately active. The conventional therapeutic strategies could be represented by a pyramid (Fig. 1.12). When disease becomes more severe, we move up the pyramid to use "stronger" treatments. Although this pyramid gives a general framework of current therapeutic strategies by moving from "milder" treatment to "stronger" treatments, newer studies have suggested that "stronger" treatments given earlier in the disease course may lead to less bowel damage in particular for CD disease. Thus, a top-down approach is proposed (Fig. 1.12).

The first, and still most effective, molecular treatment is anti-TNFα anti-body therapy, which became available in the early 2000s. TNFα is an important cytokine in systemic inflammation, and its neutralization has been tested experimentally and clinically (Brenner, Blaser et al. 2015). Currently, anti-TNFα therapy has been approved for rheumatoid arthritis, psoriasis, ankylosing spondylitis, and both types of IBD.

In treating IBD, anti- TNFα agents successfully suppress inflammation, reverse epithelial injury and promote mucosal healing, while in CD they are often effective against fistulizing forms, which usually represent a more severe and advanced disease state. Infliximab, adalimumab, certolizumab pegol, and golimumab are the TNFα -targeting drugs currently approved for clinical use, and other drugs are also under-going clinical trials. Biologic agents are generally administered in patients who are refractory to conventional therapies. However, there is growing evidences that such agents could be used in the initial phases of the disease, typically in pediatric patients, to interrupt the inflammatory process (top-down approach; Fig. 1.12).

The successful results achieved by anti- TNFα therapy have led to the exploration of other cytokine-targeting therapies (Amiot and Peyrin-Biroulet 2015). Some of these molecules were shown to be upregulated in the serum and tissues of IBD patients, and their role was examined using gene-specific KO mouse models. However, identification of the appropriate timing and patients with the right genetic, phenotypic, and risk factors, together with more information on the safety, could favour the long-term benefits of pharmacological treatments.

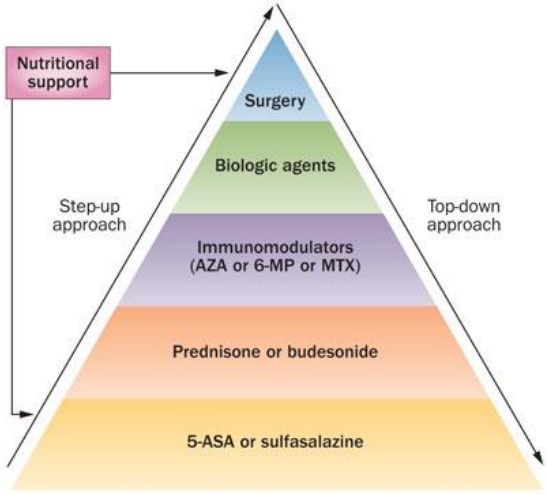

Fig 1.12 Step-up and Top-down approaches for IBD treatment. Pyramid represent different therapeutic approaches from mild to stronger and more toxic therapies (Step-up), and early aggressive treatment with immunomodulators and biologic agents (Top-down). Abbreviations: 5-ASA, 5-aminosalicylate; 6-MP, 6-mercaptopurine; AZA, azathioprine; MTX, methotrexate (Aloi M, et al. *Nat Rev Gastroenterol Hepatol*. 2014).

In pediatric patient management guideline, enteral nutrition (EN) has been proposed as support of nutritional therapy. EN is based on polymeric formula, and showed to induce clinical remission, promoting mucosal healing, improving nutritional status, decreasing pro-inflammatory cytokine levels and reducing serum inflammatory markers, especially in CD patients (Beattie, Schiffrin et al. 1994;Royall, Greenberg et al. 1995;Bannerjee, Camacho-

Hubner et al. 2004;Fell 2005). The hypothesis regarding the mechanism of action of EN include modification of gut microbiota. Several studies demonstrated the success of this therapy in induction of remission (Lionetti, Callegari et al. 2005;Leach, Mitchell et al. 2008;Shiga, Kajiura et al. 2012;Tjellstrom, Hogberg et al. 2012;D'Argenio, Precone et al. 2013).

1.11.3 Microbiota-base interventions in IBD

Regarding autoimmune and inflammatory diseases, there is a strong rationale for restoring the healthy state to gut microbiota. Several interventions on gut microbiota, including prebiotics and probiotics, have been studied especially in IBD.

Prebiotics are nondigestible constituents of food that selectively stimulate the growth or activity of one or a limited number of bacterial species already resident in the colon. Some examples of prebiotics are dietary fiber and some types of oligosaccharides (e.g. fructooligosaccharides and galactooligosaccharides).

Probiotics are live microorganisms administered to alter the gut microbiota and confer a beneficial effect on health. Potential mechanisms of probiotic action include competitive interactions, production of antimicrobial metabolites, influences on the epithelium, and immune modulation.

Few of these microbiota-based intervention have shown promise or efficacy (Shen, Zuo et al. 2014). The lack of success of these therapies is given the fact that many of these preparations contain microbial strains that may not be indigenous to the gut and cannot survive in a hostile inflammatory environment. Moreover, strong is the heterogeneity microbiota of the patients.

Recently, fecal microbiota transplantation (FMT) has been proposed as an alternative treatment for IBD. The rationale for FMT is based on the notion that transplant a microbiota from healthy subject to a host recipient (patient) would replace the functions of the diseased microbial organ, avoid inflammation, and thus restoring intestinal and immune homeostasis. FMT showed high effective for the treatment of *Clostridium difficile* colitis, but its efficacy in IBD host remains controversial (Angelberger, Reinisch et al. 2013;Rossen, Fuentes et al. 2015).

Many factors may therefore have to be taken into consideration to increase the success of FMT in IBD, such as the genetic background of donor and recipient, environmental factors, disease states, and the predisposition of gut microbiota to responde at perturbations (resilience). The recipient’s microbiota seems difficult to replace because these microrganisms have been already selected for their ability to survive in an inflammatory condition (Miyoshi and Chang 2016). In this context, it is plausible to assume that the auto-FMT, using a fecal sample collected during remission, is the most successful.

1.11.4 Rheumatic disease and arthritis

In the last decade, alteration of gut microbiota was observed in other autoimmune diseases, such as rheumatic disease and arthritis, most notably rheumatoid arthritis (RA), psoriasis, and the related spondyloarthritides (SpA), including ankylosing spondylitis (AS) and

reactive arthritis (ReA), similarly to IBD. Studies unravelling the microbiota composition in RA has gained importance.

Studies in GF animal models reveal relationships between microorganisms, mucosal immunity, and joint inflammation (Taurog, Richardson et al. 1994;Rath, Herfarth et al. 1996;Longman and Littman 2015). In fact, multiple animal models have established a biologic connection between the presence of microbiota and development of synovitis. Recent studies in humans suggest that alteration of oral and gut microbiota and an increase in leaky gut could trigger systemic joint inflammation in the context of pre-existent autoimmunity (Fig. 1.13) (Scher, Sczesnak et al. 2013;Brusca, Abramson et al. 2014;Costello, Ciccia et al. 2014;Taneja 2014;Longman and Littman 2015;Scher, Ubeda et al. 2015;Zhang, Zhang et al. 2015).

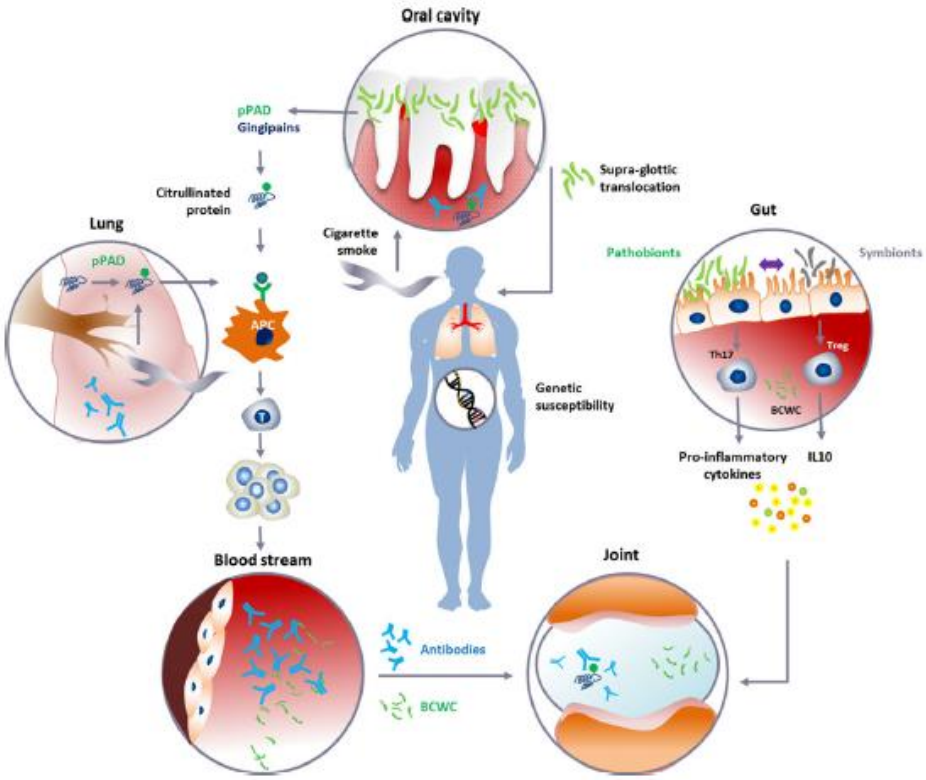

Fig. 1.13 Proposed mechanisms of pathophysiology of rheumatoid arthritis (RA) modulated by microbial dysbiosis. Microbial dysbiosis in oral cavity, lung and gut could act in concert with environmental and host genetic factors to initiate RA disease. Especially in the gut, microbial dysbiosis tilts the balance in favor of pro-inflammatory cytokine production. Infection in gut also breaks down the immune barrier, leading to release of bacterial cell wall components into the circulation. These bacterial cell wall components have been shown to elicit an immune response in the joints (Sandhya P et al. *International Journal of Rheumatic Diseases* 2016).

Unlike the case with RA, the existence of SpA has been documented for centuries, its prevalence is significantly higher in Caucasian populations, its clinical manifestations are much more heterogeneous, and genes (particularly HLA–B27) have a relatively larger effect in disease incidence. A prominent role of pro-inflammatory Th17 cells and their cytokine, IL-17 is observed. The epidemiologic relationship between intestinal microorganisms, gut inflammation, and rheumatic disease is supported by observation of infectious diarrhea in the pathogenesis of ReA, and the association between IBD and AS and PsA. A recent study

showed that HLA–B27–transgenic rats had an increase in *Prevotella* species and a decrease in *Rikenellaceae* and *Akkermansia* compared with wild type rat (Lin, Bach et al. 2014). Therefore, it is supposed that, as in RA-like disease, microbiota alterations are a consequence of host genetic predisposition or perhaps a superimposed requirement for activation of downstream immunologic events in susceptible animals.
AS patients had a higher prevalence of several bacteria, such as *Lachnospiraceae* and *Prevotellaceae* and a concomitant decrease in *Ruminococcaceae* and *Rikenellaceae* families. Scher and co-workers (Scher, Ubeda et al. 2015) defined the intestinal microbiota of PsA patients compared to that of psoriasis patients and healthy controls. While psoriasis patients and PsA patients both showed decreased levels of *Coprococcus*, PsA patients were further characterized by significantly lower levels of *Akkermansia* and *Ruminococcus*, suggesting a chronological loss of diversity that may correlate with the natural history of disease. Interestingly, studies in IBD revealed similar reductions of the *Ruminococcaceae* family and *Akkermansia* genus.
In children with enthesitis-related arthritis (ERA), a Juvenile Idiopathic Arthritis (JIA), resembling the spondyloarthropathy of the adult, the abundance of *Faecalibacterium prausnitzii* was significantly lower than that in healthy controls (Stoll, Kumar et al. 2014).
Effect of dysbiosis has been demonstrated in multiple animal models of autoimmune diseases. When the homeostatic balance in the microbial communities' composition is perturbed, a state of dysbiosis occurs and a downstream local and systemic proinflammatory response is elicited. Several evidences in humans suggest that a similar perturbation can be associated with multiple rheumatic and autoimmune disorders, which could potentially explain the source of various cytokine-induced systemic inflammatory responses. A key role is also played by epithelial cells and mucus layer, producing a protective barrier against pathobionts, and innate/adaptive immune cells residing in the lamina propria. Their fundamental role is to communicate with the microbiota to promote a state of physiologic inflammation in order to actively tolerate a constant antigenic load that promotes nutritional, metabolic, and immune benefits.

1.12 The fungal communities in the gut: the mycobiota

Despite the well-recognized role of bacteria communities in inflammatory and autoimmune diseases, eukaryotes microorganisms inhabiting the GI tract, such as fungi, could play also an important role in the stability of microbial communities in human health and disease. Yeasts were detected in human stool samples in 1917, and by the mid-20th century, their presence in the human intestine was proposed to have a saprotrophic role (Gumbo, Isada et al. 1999). The role of fungal communities in the gut, the mycobiota, has been initially studied in animals, ranging from ruminants to insects, such as wasps (Stefanini, Dapporto et al. 2012).
Fungi represent a rich and diverse microbial community in the normal human intestine. Based on cultivation studies, the total number of fungal microorganisms is estimated to be 10^0-10^2 colony forming units (CFUs)/ml in the oral cavity and increases to up 10^6 CFU/ml in the feces (Simon and Gorbach 1984;Bernhardt 1996;Bernhardt and Knoke 1997). The

available data suggest that fungal communities are stable across time (Scupham, Presley et al. 2006;Ott, Kuhbacher et al. 2008;Sokol, Leducq et al. 2016).
Recently, *Next Generation Sequencing* technologies allow deepening the composition of mycobiota in various district of human body (Rizzetto, De Filippo et al. 2014). The first culture-independent analysis of the mycobiota populating the mammalian intestine indicated that fungi belonging to four major fungal phyla, Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota, reavling diversity and abundance of fungal species in the murine gastrointestinal tract (Scupham, Presley et al. 2006). The 18S rDNA sequence is a widely used molecular target for genetic studies of fungal diversity (van Elsas, Duarte et al. 2000;Scanlan and Marchesi 2008). Similarly to bacterial 16S rDNA, 18S rDNA belongs to the gene cluster encoding for ribosomal RNA, which consists of three rRNA subunit genes, internally transcribed spacers (ITS), and intergenic sequences. The evolutionary conserved and variable regions, and the different length of ITS regions, make the 18S rRNA an ideal target for molecular phylogeny studies (van Elsas, Duarte et al. 2000;Swidsinski, Loening-Baucke et al. 2009).
Fungi are isolated from different body sites, especially *Candida albicans*, is a commensal of the normal enteric microbiota starting from birth (Ott, Kuhbacher et al. 2008).
It has been hypothesized that fungi reach the GI tract through food. Fermented foods and beverages containing eukaryotic species, such as bread, beer, and wine, are fungal sources for the host (Scanlan and Marchesi 2008). Diet is a constant and dynamic factor shaping the composition of resident microbial populations in the gut, and in turn the mucosal immunity. It is possible that differences in fungal colonization are related to differences in the host genetic makeup or differences in gut permeability.

1.12.1 Mycobiota and IBD

The different interactions between fungi, bacteria, and immune responses can significantly affect gut health and likely contribute to the pathobiology of GI disorders, especially IBD. When mucosal homeostasis breaks down in a genetically predisposed individual, the resulting immune response may lead to chronic inflammation (Zhang and Li 2014) (Fig. 1.14). Despite the well-recognized role of intestinal bacterial communities, recent evidences showed a contribution of mycobiota as the causative agents of diseases, such as IBS, IBD, and “leaky gut” syndrome (Sendid, Quinton et al. 1998;Boorom, Smith et al. 2008;Ott, Kuhbacher et al. 2008;Schulze and Sonnenborn 2009;Iliev, Funari et al. 2012;Sokol, Leducq et al. 2016).
Several years ago, antibodies against *S. cerevisiae* (ASCA) were shown to be associated with CD (Standaert-Vitse, Jouault et al. 2006), possibly indicating that fungi could play a role in the aberrant immune responses.
The immune response to the gut mycobiota is a balance between tolerance and antimicrobial defense (Mukherjee, Sendid et al. 2015). Fungi are recognized through interactions between their pathogen-associated molecular patterns (PAMPs, such as β-glucan) and host immune cells with specific pattern recognition receptors (PRRs), such as C-type lectins (for example, dectin-1, dectin-2, etc.) and Toll-like receptors (TLRs). Subsequently, antigen-presenting cells present fungal antigens, as MHC class II conjugates, which interact with T-cell

receptors on naive T cells (Romani 2011). These interactions lead to differentiation of naive T cells to different types of inflammatory T helper cells (Th1, Th2 or Th17) and regulatory T (Treg) cells in the presence of specific cytokines (Fig. 1.14). Th17 cells have been shown to interact with fungi and differentiate in the presence of IL-17A/F, IL-21, IL-22 and TNF. T-cell activation can trigger an inflammatory response, which results in the recruitment of humoral and cellular factors of innate immunity, promoting specific antibody production by B cells (Dominguez-Villar and Hafler 2011;Zelante and Ricciardi-Castagnoli 2012;Hernandez-Santos, Huppler et al. 2013).

Immune homeostasis is maintained in healthy tissues by interdependent control exerted by Th1 and Th2 cytokines and by Treg cells (Fig. 1.14). Perturbations that cause dysbiosis in the gut microbiome can disrupt immune homeostasis, resulting in unregulated immune defense and intestinal inflammation, as occurs in IBD (Mukherjee, Sendid et al. 2015).

The mechanisms by which fungi influence gastrointestinal diseases are beginning to be investigated. Some studies suggest that fungal-associated mucosal inflammation (by Th17 or Th2 cells) or tolerance (by Th1 or Treg cells) is dependent on the intracellular pathways activated by the interaction of fungal-derived molecules and PRRs.

Activation of plasma cells, *via* Th2 cells, to detect fungal cell wall antigens results in production of antiglycan antibodies (for example ASCA), which are released into the blood. The Th1 or Th17 pathways become unregulated (for example, by absence or insufficient levels of Treg cells), triggering recruitment of monocytes, monocyte-derived macrophages and neutrophils and promoting production of proinflammatory molecules. This step causes tissue damage mediated by reactive oxygen species, proteolytic peptides and enzymes, eventually manifesting as gastrointestinal diseases (Fig. 1.14).

The mechanism by which fungi aggravate the inflammatory response in gastrointestinal disease probably involves multiple steps. Aggravation can be dependent or independent of dectin and can be disease-specific. Moreover, during colonization or infection it is possible that *Candida* or other yeast, such as *Saccharmoyces cerevisiae*, release cell wall mannan, glucan and chitin, inducing the production of antiglycan antibodies (ASCA) (Sendid, Quinton et al. 1998;Standaert-Vitse, Jouault et al. 2006). Thus, the fungal and host immune system interactions could worsen the inflammatory process in gastrointestinal disease.

The ability of fungi to modulate immune cells can also be species dependent. Some studies have investigated the response of macrophages to different *Candida* spp. (*C. albicans* or *C. parapsilosis*) (Monk, Hutvagner et al. 2010;Nemeth, Toth et al. 2014). Such species-dependent induction of host immune pathways might be of particular relevance in the setting of IBD, as it is possible that the changes in immune status of affected tissues could be triggered by genus-level, as well as species-level changes in the mycobiota.

In mice, it is observed that gut inflammation promotes fungi proliferation (Jawhara, Thuru et al. 2008). Conversely, some fungi can modulate susceptibility to inflammation triggering (*Candida albicans*) or reducing it (*Saccharomyces boulardii*) (Jawhara and Poulain 2007).

Mice lacking major genes involved in fungi sensing, such as Dectin-1 or Card9, have an increased fungal microbiota load with a marked abundance in *C. tropicalis* mainly observed during active colitis (Iliev, Funari et al. 2012;Sokol, Conway et al. 2013).

In CD patients, fungal diversity is higher than that observed in healthy subjects (Ott, Kuhbacher et al. 2008). *Candida albicans*, *Aspergillus clavatus*, and *C. neoformans* were

observed increased in CD patients (Li, Wang et al. 2014). In pediatric subjects, fungal taxa, such as *S. cerevisiae*, *Clavispora lusitaniae*, *Cyberlindnera jadinii*, *C. albicans*, and *Kluyveromyces marxianus* were positively associated with CD (Chehoud, Albenberg et al. 2015). Finally, Sokol and co-workers characterized the fungal microbiota in both healthy subjects and IBD patients, observing a clear fungal dysbiosis in patients (Sokol, Leducq et al. 2016). Altogether, these findings suggest a link between fungal microbiota and IBD pathogenesis.

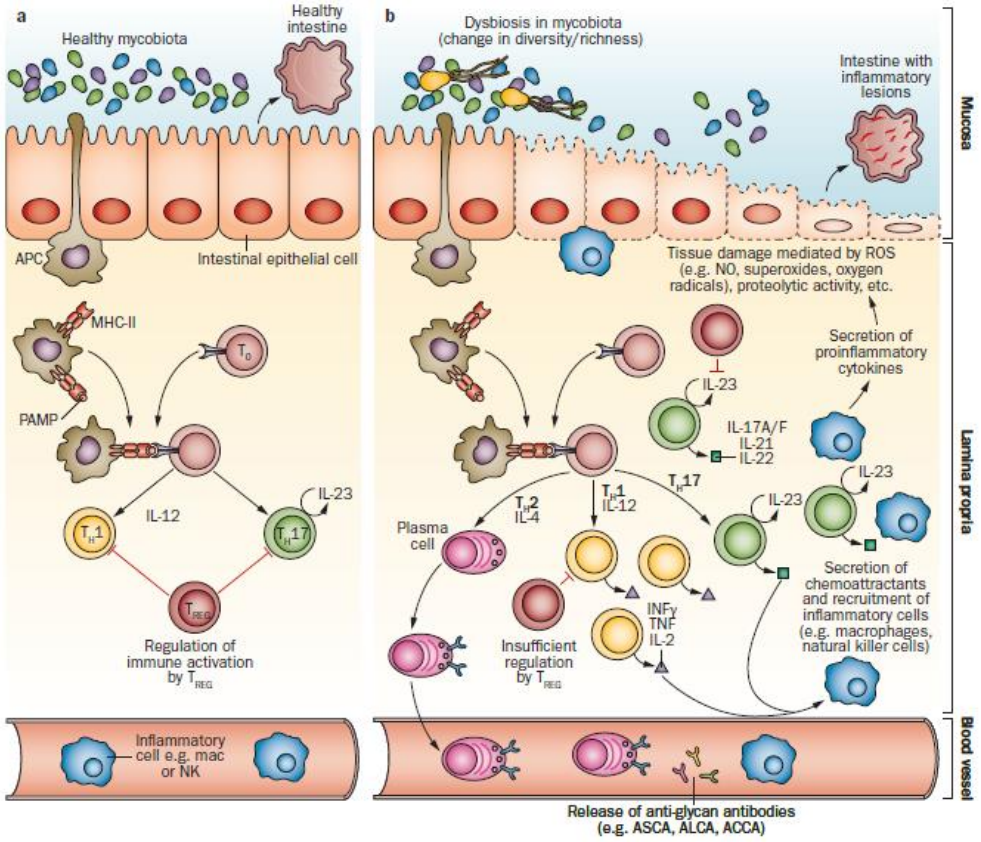

Fig. 1.14 Normal and abnormal interactions between fungi and the host immune system in the GI tract. APCs present fungal antigens as MHC class II conjugates to T-cell receptors on naive T cells. T cells then differentiate into T helper cells (TH1 or TH17), which secrete different proinflammatory and anti-inflammatory cytokines leading to recruitment of humoral and cellular factors of innate immunity. (A) In healthy tissues, immune homeostasis is maintained by interdependent control exerted by Th1 cytokines and Treg cells. (B) In IBD patients, dysfunctional regulation of Th1 or Th17 pathways triggers an unregulated inflammatory response and recruitment of innate immune cells. Activation of the Th2 pathway can lead to plasma cells detecting fungal cell wall antigens and producing antiglycan antibodies (ASCA, ALCA and ACCA). Abbreviations: ACCA, anti-chitobioside carbohydrate IgA antibodies; ALCA, anti-laminaribioside carbohydrate IgG antibodies; APC, antigen presenting cell; ASCA, anti-*S. cerevisiae* antibodies; mac, macrophage; NK, natural killer cell (Mukherjee P.K. et al *Nat. Rev. Gastroenterol. Hepatol* 2015).

1.13 Metagenomics in studying the human gut microbiota

Studies on microbial communities have been largely dependent on classical cultivation techniques. Culture of microrganisms has long provided, and still provides, one of the most detailed methods of study in microbiology. However, most intestinal microbes are anaerobic

and traditional culture methods only cultivate 10%-30% of gut microbiota (Suau, Bonnet et al. 1999;Tannock 2001;Sokol and Seksik 2010). In the mid-1970s, the discovery that portions of the gene encoding the small subunit 16S ribosomal RNA (rRNA) were highly conserved among bacteria (Woese, Fox et al. 1975), while other internal regions of the gene are highly variable, having almost entirely unique sequences in most bacteria species (Fig. 1.16), allowed the use of this gene as molecular marker discriminating bacteria at different taxa levels (Sunagawa, Mende et al. 2013).

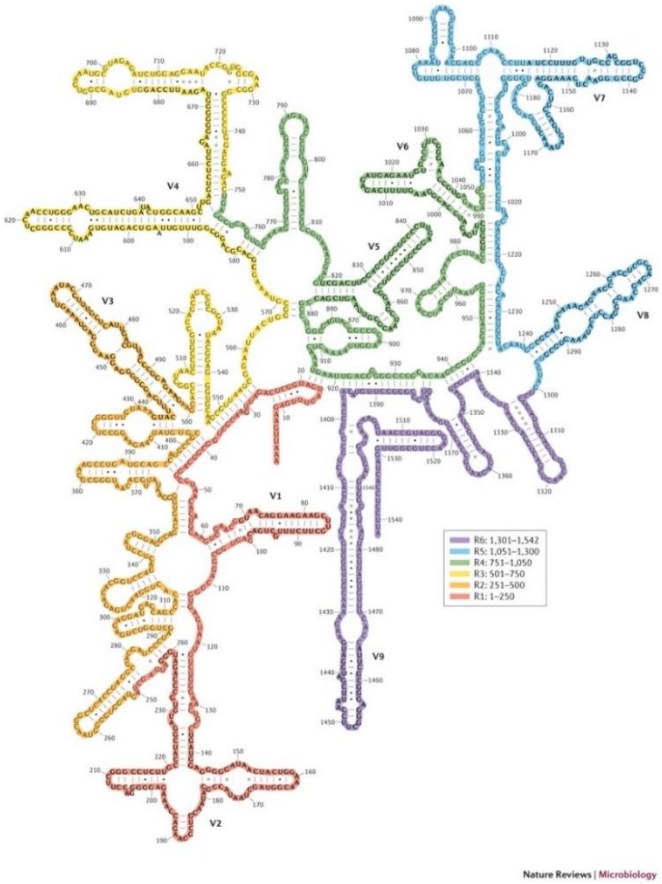

Fig 1.15 Variable regions of the 16S ribosomal RNA. Secondary structure of the 16S rRNA of *E. coli*, as generated using the xrna program. In red, fragment R1 includes regions V1 and V2; in orange, fragment R2 including region V3; in yellow, fragment R3 including region V4; in green, fragment R4 including regions V5 and V6; in blue, fragment R5 including regions V7 and V8; and in purple, fragment R6 including region V9 (Yarza P, et al. *Nat Rev Microbiol*. 2014).

Polymerase chain reaction (PCR) amplification, with universal primers, of genomic DNA from a community of microbes, followed by cloning and sequencing, provides marker genes that can be used to quantify bacterial taxa present within a sample. With the rapid development of molecular technologies such as PCR-denaturing gradient gel electrophoresis (DGGE), it has been shown that the gut microbial ecosystem is far more complex than previously thought (Eckburg, Bik et al. 2005).

In recent years, *Next Generation Sequencing* (NGS) technologies have been developed (Shendure and Ji 2008;Fuller, Middendorf et al. 2009), enabling the analysis of a large number of microorganisms in different environments and matrices, including the human gut (Human Microbiome Project 2012).

The Sanger sequencing method has advanced through 454 technologies (Margulies, Egholm et al. 2005;Sogin, Morrison et al. 2006) to Illumina (San Diego, CA) sequencing (Bentley, Balasubramanian et al. 2008;Lazarevic, Whiteson et al. 2009).

Analysis of 16S rDNA sequence and metagenomics have been used to study uncultivated gut microbial communities. These approaches allow for qualitatively defining of a given microbial community. Shotgun metagenomic sequencing can be used to understand structure and functions of microbiome (Woese and Fox 1977;Handelsman 2004;Lepage, Leclerc et al. 2013).

Metagenomics was first described in 1998 by Handelsman and Rodon (Handelsman, Rondon et al. 1998;Rondon, Raffel et al. 1999), and became another DNA sequencing approach to study the complex gut microbial community (Gill, Pop et al. 2006;Turnbaugh, Hamady et al. 2009;Qin, Li et al. 2010). Firstly, the total DNA of all microorganisms is extracted from fecal samples. Before being sequenced, total DNA samples are randomly trimmed by a "shotgun" approach. The comprehensive sequences are then analyzed to obtain either species profiles based on phylogenetic markers (16S rDNA) or genomic profiles based on whole genomes (Tringe, von Mering et al. 2005). The shotgun sequence reads are filtered to obtain the high-quality sequences for the whole genomic profile by metagenomics. Based on sequence overlaps, the filtered sequences are then assembled to form longer genomic sequence contigs. Computational methods are needed to code sequences in the contigs. Data mining and database searches applying different powerful algorithms are then used to annotate genes (Thomas, Gilbert et al. 2012). The information obtained from the sequence-based and functional metagenomics enables a more comprehensive understanding of the structure and function of microbial communities.

The use of "omic" technologies (metagenomics, transcriptomics, metabolomics, and proteomics) has revealed the complexity of gut microbial communities, providing sufficient information on: i) detection of microbial composition and diversity, ii) novel genes, iii) microbial pathways, iv) functional dysbiosis, v) antibiotic resistance genes (resistome), vi) determination of interactions and co-evolution between microbiota and host, and vii) understand the gut microbiota composition and function in health, and in diseases.

1.13.1 Statistical analysis of taxonomic and functional profiles

Metagenomic studies of the gut microbiota produce an amount of data providing the relative abundance of taxa or functional units for each sample. For interpretation of data, tools occur able to summarize and visualize the results in order to find which taxa or functional acquisitions differ significantly between metadata groups. There are many software packages that allow for visualization, exploration, and analysis of such data, using 454 or Illumina data as inputs, such as Mothur (Schloss, Westcott et al. 2009), QIIME (Caporaso, Kuczynski et al. 2010), MICCA (Albanese, Fontana et al. 2015), MEGAN (Huson, Auch et al. 2007), Galaxy (Blankenberg, Von Kuster et al. 2010). In addition, analyses of microbial communities have focused on descriptive ecologic metrics, such as alpha and beta diversity (Lozupone and Knight 2008;Morgan and Huttenhower 2012). Alpha diversity, a measure of species richness within a sample. Alpha diversity is quantified in terms of number of observed Operational Taxonomic Units (OTUs), or through richness estimators, such as

Abundance-based Coverage Estimator, Chao index, Shannon index. Beta diversity allow us to understand variability beteween samples and the microbial structure among groups, by using visualizations, such as principal coordinates analysis (PCoA) (Manton, Stallard et al. 1982), calculated on UniFrac distances (weighted –quantitative and unweighted -qualitative variants)(Lozupone, Lladser et al. 2011) or Bray-Curtis dissimilarities, based on phylogenetic distances (McMurdie and Holmes 2014). This type of visualization, known as an ordination, provides a qualitative overview of the principal factors associated with variance in the data.

Moreover, useful information are obtained by biomarker discovery evaluating which metadata are associated significantly with increases or decreases in specific taxa or functions. Tools, such as LEfSe (Segata, Izard et al. 2011), MaAsLin (Morgan, Tickle et al. 2012), MetaStats (White, Nagarajan et al. 2009), STAMP (Parks and Beiko 2010), and metagenomeSeq (Paulson, Stine et al. 2013) have been designed for this type of 16S sequence and metagenomic data analysis. These methods might detect differences in clades or activities of specific pathways in patients vs healthy controls based on different variables, such as genetic or environmental factors. By using metagenomic functional data, it is often possible to find significant shifts in the microbial community that are not visible in comparisons of strains or species. Because of the high level of variation in the gut microbiota of each individual, when the microbiomes of patients and controls are compared, many species will be present only in a few individuals, in highly varying amounts. Thus, there will be insufficient statistical power to discern whether these variations in species differ significantly between populations, and the number of species found to do so will be low. In contrast, an environmental factor that provides selective pressure can perturb many members of the community to a small but consistent degree, such that specific functional pathways differ significantly, even when no individual strain has changed sufficiently in abundance to pass the statistical threshold. These types of studies can identify changes in the balance of a community in which no single microbe dominates.

1.13.2 Metatranscriptomics and metagenomic inference

Metatranscriptomics complements metagenomic studies allowing a more dynamic view of the microbial community and its response to environmental factors.

Metagenomic data sequences provide information about microbial DNA content, and thereby the composition and potential genetic features of different microbial communities. Although metagenomes show which taxa are most abundant in communities, and differences between the functional capacities of cohorts, the metatranscriptomes determine whether the most abundant microbes are the most metabolically active, and the consequences of the observed functional differences. For example, metagenomic analysis of fecal samples from patients and healthy controls might show a large change in measured abundance of genes involved in tryptophan biosynthesis, associated with disease. Metatranscriptome analyses might show that these genes also were expressed differentially between patients and controls, supporting the association between this functional acquisition and the disease. Otherwise, metatranscriptome analyses might show that the genes were expressed in neither patients nor

controls, concluding that tryptophan biosynthesis to be discharged as a component of disease-associated changes in microbial community structure.

Metagenomic inference was developed to partially obtain Whole Genome Sequencing information based on 16S sequence data. In order to predict how taxonomic differences between microbiota of different groups affects their microbial metabolic potential, we can apply PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille, Zaneveld et al. 2013), a computational approach useful to infer the functional contribution of microbial communities on 16S rDNA sequencing data set. PICRUSt implements an extended ancestral-state reconstruction algorithm to predict which gene families are present, and then combines gene families to estimate the significant differences in the main functional classes (KEGG categories) of the composite metagenome. From a OTUs table derived by metataxonomic anlysis of 16S dataset PICRUSt first calculates the functional capacity of the genome corresponding to each OTU, producing a list of KEGG Ortholog gene identifiers by default. Functional capacity is calculated directly from corresponding reference genomes when available, and otherwise probabilistically inferred from available reference genomes based on the degree of within-clade conservation of each function (for example, is this OTU derived from an *Prevotellaceae*, and most *Prevotellaceae* have this function, so the genome corresponding to this OTU likely has this function). Finally, each function's abundance results in an inferred metagenome, or rather a set of samples with corresponding predicted functional abundances.

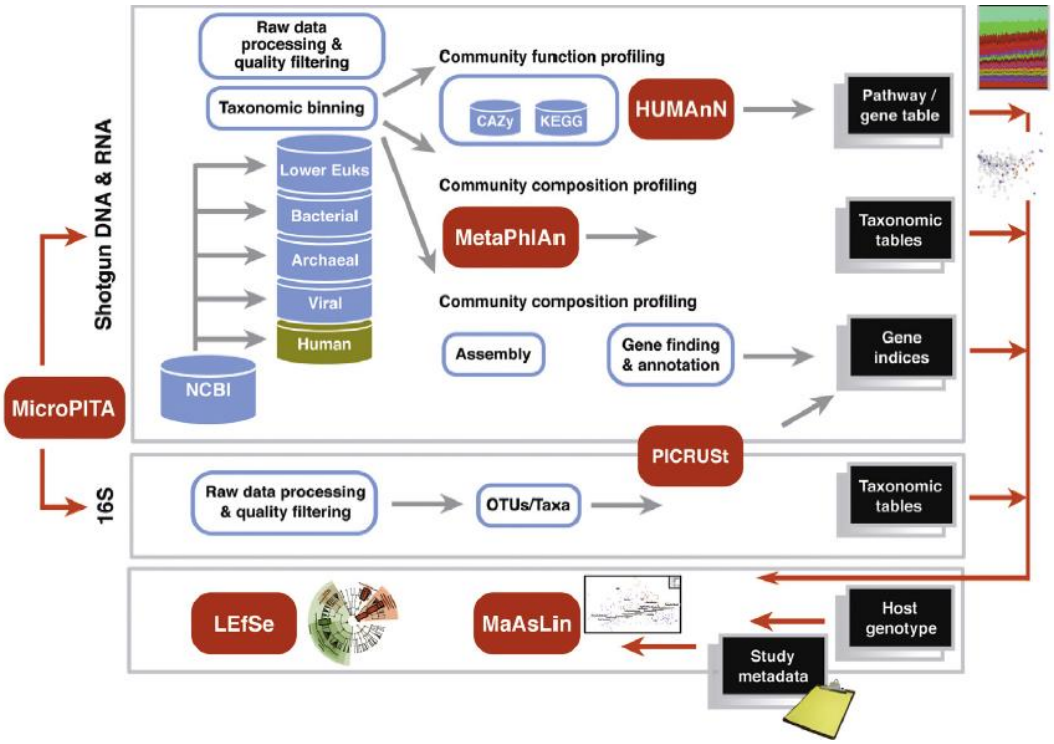

Fig 1.16 Workflow for meta'omic study of the gut microbiome proposed by Morgan X.C. and Huttenhower C. (2014). Two-stage experimental design can incorporate a large number of cost-effective 16S sequenced samples coupled with a smaller number of metagenomes or metatranscriptomes; microPITA guides the targeted selection of such samples. 16S sequencing and metagenomic data each can yield taxonomic abundance tables, the former using environments such as Quantitative Insights Into Microbial Ecology (QIIME) or MOTHUR, and the latter systems such

as MetaPhlAn. Metagenome and metatranscriptome analyses similarly can yield the abundances of gene families or pathways in the microbial community of the gut using systems such as HUMAnN or a 16S data set can be used to determine a predictive metagenome through PICRUSt. Finally, taxonomic and functional profiles can be assessed using downstream statistical tests for significant associations with clinical metadata in either a univariate (LEfSe) or multivariate (MaAsLin) manner. OTU, operational taxonomic unit; NCBI, National Center for Biotechnology Information; CAZy, Carbohydrate Active enZyme database (Morgan X.C. & Huttenhower C. *Gastroenterology* 2014).

1.13.3 Limitation of metagenomic analysis

Although metagenomics have been shown to be an exceptional and powerful technology in studying the human gut microbiome, there are still some limitations in the use of metagenomics. Firstly, it is not possible to identify microbial expression. Secondly, as metagenomics require much higher sequence coverage than 16S rDNA sequence analysis, the costs and time involved in DNA sequencing projects for gut metagenomics are far greater than those of 16S rDNA sequence analysis. Thirdly, to obtain high coverage required for metagenomics, a sufficient quantity and high quality of DNA samples are essential. Although preventive steps are performed, human contaminants are found in 50%-90% of sequences (Human Microbiome Project 2012). Different DNA extraction kits and laboratories also affect the assessment of human gut microbiota (Kennedy, Walker et al. 2014). Comparing data across studies that use different bacterial DNA extraction methods is difficult (Wesolowska-Andersen, Bahl et al. 2014). Moreover, the significant differences in microbial composition observed on mucosal surfaces compared to luminal/fecal material may not adequately reflect the totality of viable microbes within the gut, and it makes the studies can not be compared. Fourthly, the quality of the underlying functional annotations of metagenomic sequence fragments is very important. However, a significant proportion of data cannot be assigned a function due to a lack of close matches in reference databases (Qin, Li et al. 2010). Millions of sequences in each sample are required for functional gene analysis of a complex microbial community. It is difficult to identify and improve the accuracy of information derived from the relatively short gene fragments generated by NGS, due to the many bioinformatics challenges proposed by the vast metagenomic shotgun sequencing. It is also difficult to assign function unambiguously based on sequence similarity alone, which may cause misannotation (Schnoes, Brown et al. 2009). Moreover, when there are less abundant members of the microbiome or a community containing many closely related species, it may be difficult to assemble genomes (Albertsen, Hugenholtz et al. 2013). This can lead to a situation where, even if a function can be identified, it may be a challenge to assign it to specific species within the microbial community. In addition, DNA is the material used in metagenomic sequencing, and the expression of each functional gene in a sample in a given environment is very difficult to determine.

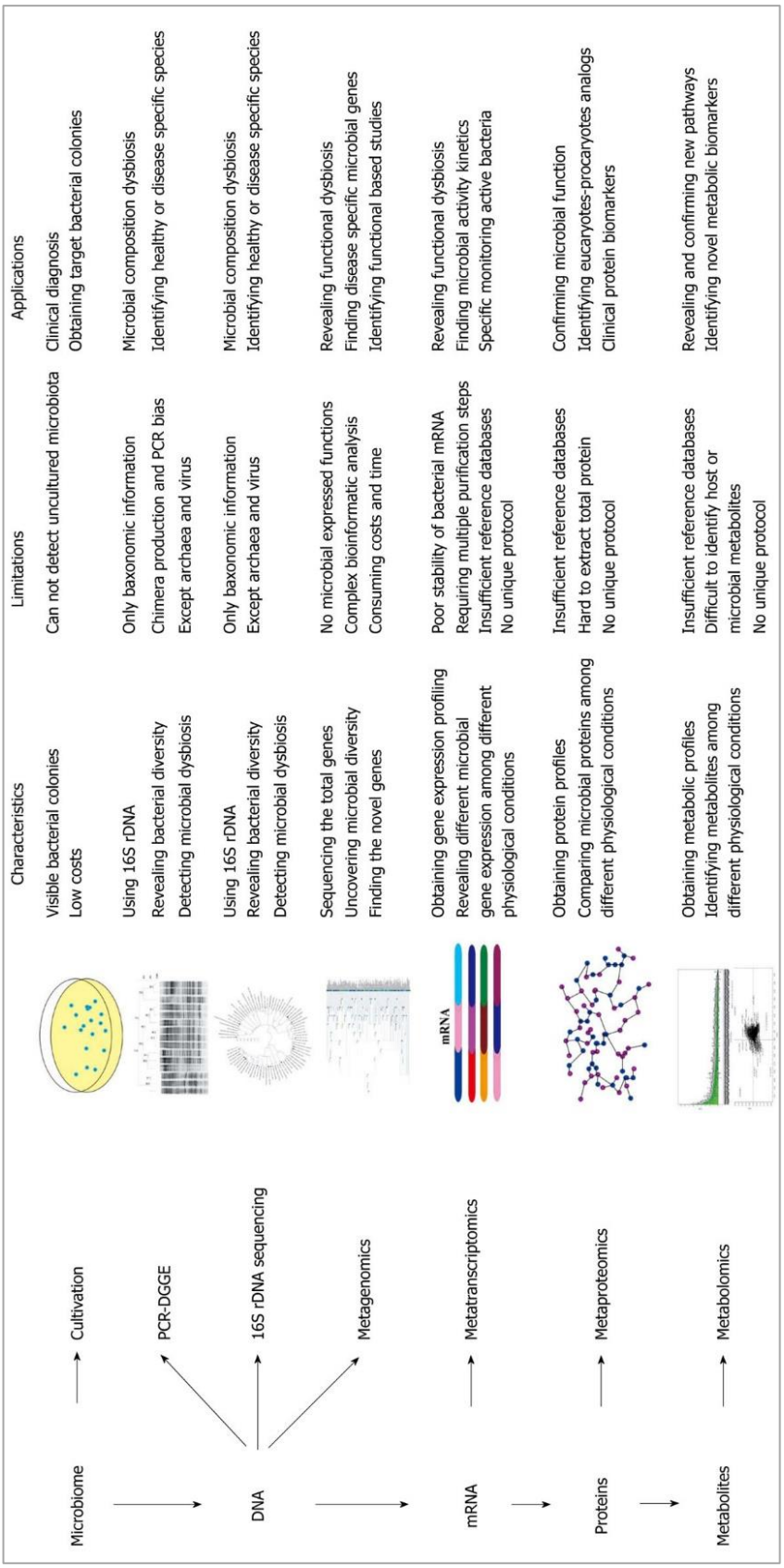

Fig 1.17 Different gut microbiome study approaches. Characteristics, limitations, and applications of different gut microbiome approaches from cultivation to metabolomics are presented (Wang W.L. et al. *World J Gastroenterol*. 2015).

2. Aim of the PhD research project

Autoimmune and inflammatory diseases are increasing in western populations of industrialized countries with a shift of disease onset to the pediatric populations. Several evidences indicate that diet globalization, processing of food, urbanization and other environmental factors, such as sanitation and antibiotic treatment are involved in modifications of gut microbiota/mycobiota composition. Alteration of gut microbial profiles could induce inflammation and immune responses, and thus promote inflammatory and autoimmune disorders, such as Inflammatory Bowel Disease (IBD) or rheumatic diseases.

This PhD project aims at: 1- dissecting the role of diet and environment on gut microbiota of healthy children from rural and urbanized environments; 2- investigating the association of bacterial and fungal microbiome alterations in pediatric patients affected by inflammatory and autoimmune disorders, dramatically increased in the western world; 3-studying the immunomodulatory potential of fungal isolates in health and disease, and their interaction with bacterial gut communities.

In particular, in order to understand the impact of diet and urbanization in shaping the gut microbiota, we investigated the microbiome of healthy children populations having different dietary habits and living in a rural African village and in urban areas (a small town and the capital city of Burkina Faso), comparing with that of children living in Italy. These pediatric populations were selected as representative populations in which the incidence of autoimmune and inflammatory diseases is very low, and as representative of the western and industrialized populations respectively.

In order to deepen the knowledge of the pathogenesis of IBD at early onset (EO), we evaluated the phenotype and course of EO-IBD (0–5 years) compared with pediatric later-onset disease (6–11 and 12–18 years). Understanding the factors that contribute to early age of onset of IBD could potentially facilitate intervention strategy development.

Then, we characterized the gut microbiota composition of children affected by Juvenile Idiopathic Arthritis (JIA), a typical immune-mediated condition that is largely increasing in western pediatric populations in order to define specific microbial "pro-arthritogenic" profiles, in association with HLA-B27 status.

Furthermore, we investigated the gut mycobiota composition in the healthy population at different age range, characterizing phenotypically the fungal isolates for traits related to adaptation in the gut environment and for potential pathogenic traits. Finally, we characterized phenotypically and immunologically fungal isolates from pediatric IBD patients in order to understand the potential role of fungal strains in etiology of IBD, and their interaction with microbiota.

3. Effect of diet and environment on gut microbiota in healthy populations living in different environments. Comparative study in children living in rural and urban Africa and in Europe

This chapter will be shortly submitted for publication as an original research article:

De Filippo C., **Di Paola M.**, Ramazzotti M., Albanese D., Pieraccini G., Banci E., Miglietta F., Cavalieri D. and Lionetti P.
Transitional hologenomes: how diet, urbanization, and environment shape the bacterial and fungal gut communities in children.

3.1 Scientific Background

Nutritional evolution in humans is a hundred-thousands year process in which genetic changes reflected environmental challenges. Animal and human hosts and their microbiota have co-evolved together over the millennia into a homeostatic, symbiotic relationship. Thus, normal functioning of the digestive and immune systems depend on the presence of non-pathogenic 'beneficial' microrganisms inhabiting our gastrointestinal tract.
Unraveling the ecology and evolutionary history of human gut microbiota has recently become possible through the advent of metagenomics. Initially, large-scale projects, such as the European Metagenomics of the Human Intestinal Tract (MetaHIT) (Qin, Li et al. 2010) and the US Human Microbiome Project (Human Microbiome Project 2012) were focused on analysis of the microbiota of volunteers from western industrialized countries, in order to discovermicrobial communities linked to the rise of new diseases such as obesity, allergies, autoimmune diseases and Inflammatory Bowel Disease (IBD). More recently studies looked beyond the Western world, relative to human population still living in rural environments and having a life-style completely different from that of western countries, are being rapidly enriched from metagenomic datasets obtained worldwide in different populations, showing that both host and environmental factors can affect gut microbial ecology over a lifetime (Borenstein, Kupiec et al. 2008;Freilich, Goldovsky et al. 2009). Dietary habits are considered one of the main factors contributing to the diversity of human gut microbiota (De Filippo, Cavalieri et al. 2010;David, Maurice et al. 2014). Our previous study showed for the first time that the gut microbiota from children living in rural African village in Burkina Faso, in an environment that still resembles that of Neolithic subsistence farmers, is completely different from the microbiota of children living in urban area of the Western world (De Filippo, Cavalieri et al. 2010), demonstrating that the different dietary habits (fiber-rich diet of rural populations versus typical western diet rich in fat, animal proteins and simple sugars) affect the gut microbiota. Since then the impact of diet on gut microbiota was observed in geographically isolated populations, such as Amazonas from Venezuela, rural populations from Malawi (Yatsunenko, Rey et al. 2012) and from Papua New Guinea (Martinez, Stegen et al. 2015).
In addition, dietary switch from carnivorous to vegetarian diets across the human evolution was associated to drastic and rapid changes in gut microbiota (David, Maurice et al.

2014;Gomez, Petrzelkova et al. 2016). The study of gut microbiota in ancestral populations, such as Hazda hunter-gatherers from Tanzania, one of the last few remaining population with diet and life-style resembling that of the Paleolithic era (Schnorr, Candela et al. 2014), showed significant differences in microbiota due to dietary fluctuations linked to seasonal and annual changes. Microbiota characterization of BaAka hunter-gatherers and Bantu agriculturalists reflect microbial gradients linked to traditional subsistence strategies (Gomez, Petrzelkova et al. 2016).

Altogether, these studies demonstrated that microbiota evolution reflects the degree of traditional lifestyle and showing the effect of westernization on loss of traditional commensal microrganisms. Attempts to understanding what benefits make both vegetarian and omnivorous diet on host's health is controversial (Wu, Chen et al. 2011). Different profiles in gut microbiota are associated with dietary pattern. A fiber and plant-derived polysaccharide-rich diet is associated with an enrichment in Bacteroidetes phylum with respect to Firmicutes (De Filippo, Cavalieri et al. 2010;Yatsunenko, Rey et al. 2012;David, Maurice et al. 2014).

It is evident that traditional microbiota are potentially a goldmine of metabolic and microbial diversity to be understood, preserved and harnessed to drive improved human health through directed dietary microbiota modulation. The multiple metabolic functions of the gut microbiota on host physiology include carbohydrate fermentation, vitamin and energy production, amino acid metabolism, xenobiotic degradation, and biotransformation of bile acids by enzymes that have important implications for the metabolism of cholesterol and glucose (Tremaroli and Backhed 2012). The fermentation of non-digestible carbohydrates stimulates the growth of bacteria producing short-chain fatty acids (SCFAs), among which the more important are the acetate, butyrate and propionate that are differentially associated to disease and health status.

Aim of the present study was assess the impact of transition from a rural village, with marginal contacts with globalized world, to a suburban small town and urban area, on dietary habits and on the gut microbiota in populations of the same African ethnic group, living in the same country. In these populations, such event is in parallel with increased wealth and greater food availability. In this study, we integrated the gut microbiota characterization of our previous study on African children population living in the rural villages of Boulpon (district of Nanoro) in Burkina Faso (De Filippo, Cavalieri et al. 2010), with microbiota of children of the same Mossi ethnicity, living in the small town of Nanoro and of children from wealth families living in Ouagadougou, the capital city. Then, we compared the gut microbiota composition of these three African populations corresponding to different levels of urbanitazion with that of a previously known Italian population, as representative of a typical western and urbanized population.

3.2 Materials and Methods

3.2.1 Enrollment of children populations and fecal sample collection

In this study, we enrolled 11 healthy children living in the rural village of Boulpon (Boulkiemde province, Burkina Faso, BR), 8 healthy children living in the small urbanized town of Nanoro (Boulkiemde province, Burkina Faso, BT), 5 children living in the capital

city of Burkina Faso, and 13 healthy children living in the urban area of Florence, Italy (EU). All children aged 2 to 6 years, had not taken antibiotics or probiotics in the 6 months prior to the sampling dates and had not been hospitalized in the previous 6 months. A detailed medical and lifestyle report was obtained from EU children's parents as well as a 4-day dietary questionnaire and an in-depth interview on African children's diet was obtained directly from their mothers.

Despite the high incidence of infectious disease, including malaria and malnutrition in the area, all children were healthy at the time of sample collection. Children living in the capital city (BC) were healthy and belonging to wealthy families. For BR and BT children, upper mid-arm measurement excluded both severe and moderate malnutrition. As representative of a healthy Western population (EU) we selected children of the same age who are generally concordant for growth, socially homogeneous and eating the diet and living in an environment typical of the developed and urbanized world. All individuals were made aware of the nature of the experiment and gave written informed consent in accordance with the sampling protocol approved by the Ethical Committee of Meyer Children Hospital, Florence, Italy. Fecal samples were collected by physicians and preserved in RNAlater (Qiagen) at −80 °C until extraction of genomic DNA.

3.2.2 Bacterial genomic DNA extraction from fecal samples

The bacterial genomic DNA extraction procedure is based on a modified protocol proposed by Zoetendal et al. (Zoetendal, Heilig et al. 2006). In brief, after dissolving about 500 mg of each fecal sample in physiological solution and homogenization by vigorous hand shaking, 600 µl of the suspension was centrifuged (10,000 g, for 10 minutes at 4°C) to obtain pellets. The pellets were dissolved in 1 ml ice-cold 1x-PBS and centrifuged at 700 g at 4°C for 1 minute. The supernatants were transferred into a 15 ml tube and were centrifuged at 9000 g at 4°C for 5 minutes. Subsequently, the pellets were suspended in 2.8 ml TE buffer by repeated pipetting. Then, 180 µl of SDS 10% (w/v) and 18 µl of proteinase K (20 mg/ml) were added. The samples were incubated for 1 hour at 37°C. Afterward, 20 µl RNase (40 µg/ml) were added and incubated at room temperature (RT) for 5 minutes. An equal volume of phenol/chloroform (50:50) was added and the samples were shaken well until the phases were completely mixed. The mixtures were centrifuged at 4500 g for 2 minutes. The upper layers were transferred into a new tube. This step was repeated again so that the interface of the two layers was clean. Then, 1/10 volume of 3 M sodium acetate pH 5.2 and two volumes of 96% ethanol were added and mixed gently. The mixtures were stored overnight at -20°C to precipitate the genomic DNA, then the samples were centrifuged at 4°C at 9000 g for 10 minutes. The genomic DNA was washed twice into 1 ml of 70% ethanol. Finally, dried samples were suspended in 300 µl of nuclease-free water (Ambion). DNA quality was assessed by gel electrophoresis and spectrophotometry measuring OD 260/280. Only samples with good DNA quality were processed.

3.2.3 Pyrosequencing

For each sample, we amplified the 16S rRNA gene using the special fusion primer set

specific for V5-V6 hypervariable regions and corresponding to primers 784F and 1061R described by Andersson et al. (Andersson, Lindberg et al. 2008), and using the FastStart High Fidelity PCR system (Roche Life Science, Milano, Italy). The 454 pyrosequencing was carried out on the GS FLX+ system using the XL+chemistry following the manufacturer recommendations.

For each sample, a PCR mix of 100 µl was prepared containing 1X PCR buffer, 5U of FastStart High Fidelity polymerase blend and dNTPs from the FastStart High Fidelity PCR system (Roche), 200 nM of primers (Eurogentec) and 100 ng of gDNA. Thermal cycling consisted of initial denaturation at 95°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 40 seconds, annealing at 55°C for 40 seconds, and extension at 72°C for 1 minute, with a final extension of 7 minutes at 72°C. Amplicons were visualized on 1.0% agarose gels using SYBR Safe DNA gel stain in 0.5X TBE (Invitrogen) and were cleaned using the HighPure Cleanup kit (Roche) according to the manufacturer's instructions.

Amplicon DNA concentrations were determined using the Quant-iT PicoGreen dsDNA reagent and kit (Invitrogen) following the manufacturer's instructions. Assays were carried out using 10 µl of cleaned PCR product in a total reaction volume of 200 µl in black, 96-well microtiter plates. Fluorescence was measured on Perkin Elmer Victor Plate reader using the 485/530 nm excitation/emission filter pair with measurement time 0.1 second. Following quantitation, cleaned amplicons were combined in equimolar ratios into a single tube. The final pool of DNA was precipitated on ice for 45 minutes following the addition of 5 M NaCl (0.2 M final concentration) and two volumes of ice-cold 100% ethanol. The precipitated DNA was centrifuged at 7,800 g for 40 minutes at 4°C, and the resulting pellet was washed with an equal volume of ice-cold 70% ethanol and centrifuged again at 7,800 g for 20 minutes at 4°C. The supernatant was removed and the pellet was air dried for 10 minutes at room temperature and then resuspended in 100 µl of nuclease-free water (Ambion). The final concentration of the pooled DNA was determined using a NanoDrop spectrophotometer (Thermo Fisher).

3.2.4 Data analysis

Pyrosequencing resulted in 194`202 filtered reads for BT (Number of reads for each BT sample, mean 24`275.25; Read length, mean 244.64), and 89`888 filtered reads for BC (Number of reads for each BC sample, mean 17`977.6; Read length, mean 291). Raw 454 files were demultiplexed using Roche's .sff file software. Data anlysis was performed together with previous data obtained for BR and EU populations (De Filippo, Cavalieri et al. 2010), and available at http://www.ebi.ac.uk/ena/data/view/ERP000133. The previous data for BR and EU showed 256`308 filtered reads for BR (Number of reads for each sample, mean 23`300.73; Read length, mean 235.73) and 303`972 filtered reads for EU Samples (Number of reads for each sample, mean 23`382.46; Read length, mean 235.95).

Reads of all data sets were pre-processed using the MICCA pipeline (version 0.1, http://compmetagen.github.io/micca/) (Albanese, Fontana et al. 2015). Forward and reverse primer trimming and quality filtering were performed using micca-preproc truncating reads shorter than 280nt (quality threshold=18). Denovo sequence clustering, chimera filtering and taxonomy assignment were performed by micca-otu-denovo (parameters -s 0.97 -c).

Operational Taxonomic Units (OTUs) were assigned by clustering the sequences with a threshold of 97% pair-wise identity, and their representative sequences were classified using the RDP software version 2.7 (Wang, Garrity et al. 2007). Template-guided multiple sequence alignment was performed using PyNAST57 (version 0.1) (Caporaso, Bittinger et al. 2010) against the multiple alignment of the Greengenes 16S rRNA gene database (DeSantis, Hugenholtz et al. 2006) filtered at 97% similarity. Finally, a phylogenetic tree was inferred using FastTree (Price, Dehal et al. 2010) and micca-phylogeny (parameters: -a template-template-min-perc 50). Sampling heterogeneity was reduced by rarefaction, obtaining 12`964 sequences per sample.

Chao1 index and Shannon entropy (indicators of alpha diversity) and UniFrac (Lozupone, Lladser et al. 2011) and Bray-Curtis dissimilarities (indicators of beta diversity) were calculated using the phyloseq package (McMurdie and Holmes 2014) of the R software suite. Exploratory analysis was performed by Principal coordinates analysis (PCoA) using the phyloseq package of the R software suite. Multiple-rarefaction PCoA plots ("jackknifed" PCoA plots) (Lozupone, Lladser et al. 2011) were computed to assess the robustness of the beta-diversity analyses.

The significance of between-groups differentiation on the UniFrac distances and Bray-Curtis dissimilarity was assessed by PERMANOVA using the adonis() function of the R package vegan with 999 permutations.

To compare the relative abundances of OTUs among the four groups, the two-sided, unpaired Wilcoxon test was computed, removing taxa not having a relative abundance of at least 0.1%, in at least 20% of the samples, and using the function mt() in the phyloseq library and the p-values were adjusted for multiple comparison controlling the family-wise Type I error rate (minP procedure).

Based on sequence abundances in each population, heatmap plots of percentage abundances, at different taxa, were obtained by using STAMP (Parks, Tyson et al. 2014), and supported by dendogram, obtained with Average Neighbour and Unweighted Pair Group Method with Arithmetic Mean (UPGMA), useful to cluster fecal samples of the children populations based on taxa abundances.

On the basis of the relative abundances, the metagenomic biomarker discovery and related statistical significance were assessed using the linear discriminant analysis (LDA) effect size (LEfSe) method (Segata, Izard et al. 2011). LEfSe uses the Kruskal–Wallis rank-sum test to identify features with significantly different abundances between assigned taxa compared to the groups, and LDA to estimate the size effect of each feature. An alpha significance level of 0.05, either for the factorial Kruskal-Wallis test among classes or for the pairwise Wilcoxon test between subclasses, was used. A size-effect threshold of 2.0 on the logarithmic LDA score was used for discriminative microbial biomarkers.

To infer the functional contribution of microbial communities on 16S rDNA sequencing data set, we applied PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille, Zaneveld et al. 2013), that implements an extended ancestral-state reconstruction algorithm to predict which gene families are present, and then combines gene families to estimate the significant differences in the main functional classes (KEGG categories) of the composite metagenome. From a OTUs table with associated Greengenes identifiers, we obtained the final output from metagenome prediction as an annotated table

of predicted gene family counts for each sample, where the encoded functions of each gene family are orthologous groups or other identifiers such as KEGG orthologs (KOs). The functional pathways discovery and related statistical significance were assessed by LEfSe.
In general, PICRUSt map the subset of 16S sequences to their nearest sequenced reference genome. To evaluate accuracy of PICRUSt, we used the Nearest Sequenced Taxon Index (NSTI), developed to quantify the availability of nearby genome representatives for each microbiome sample. NSTI is the sum of phylogenetic distances for each organism in the OTU table to its nearest sequenced reference genome, and, in general, the accuracy of PICRUSt decreased with increasing NSTI across all samples. NSTI values calculated from 16S data set of Burkina Faso and European children showed low values for BR and BT populations, and higher values for BC and EU populations, confirming that reference genome coverage is not sufficient to allow accurate PICRUSt prediction for these populations. Therefore, inference of functional profiles must be applied with caution to the most novel and diverse communities, as those of gut microbiota of Burkina Faso populations.

3.2.5 Determination of Short-Chain Fatty Acids (SCFAs) in Fecal Samples

Concentrations of fecal SCFAs were determined from 250 mg frozen fecal samples, according to the previous protocol (De Filippo, Cavalieri et al. 2010), by SPME-GC-MS using a Varian Saturn 2000 GC-MS instrument with 8200 CX SPME autosampler. Briefly, fecal samples were homogenized after addition of 1 ml of 10% perchloric acid and centrifuged at 15,000 g for 5 minutes at 4°C. Concentrations of SCFASs were determined in a 1:25 dilution of 500 µl supernatant. We used 5 µl of a mixture of deuterated acids containing 50 ng D3-propionic, 50 ng D7-butyric and 500 ng D4-acetic acids as internal standard. A calibration curve was prepared, adding the mixture of internal standards (5 µl) to scalar amounts of the acids. The Varian MS workstation software (version 6.6) was used for data acquisition and processing. The SCFAS concentration in fecal sample was expressed in µmol/g of feces. To determine statistical significance of differences observed among the four populations we used unpaired Student's t test (one tailed).

3.3 Results and discussion

3.3.1. Transition from a rural to an urban environment changes dietary habits of Burkina Faso populations

In order to investigate the effect of transition from different environments, corresponding to a different socio-economic status and food availability, on gut microbial communities, we studied fecal microbiota composition of three populations of healthy children belonging to the Mossi ethnic group (representing the largest ethnic group of Burkina Faso, accounting for the 74.9% of Burkinabè population), but living in different areas of Burkina Faso. In particular, we compared a previously analyzed population of 11 children (BR) living in Boulpon, a typical rural village of Burkina Faso (Boulkiemdé province, Nanoro department; Geographic coordinates 12°39′N 2°4′W; (De Filippo, Cavalieri et al. 2010), with 8 children (BT) belonging to families living in Nanoro, a small African town (Geographic coordinates 12°41′N 2°12′W) surrounded by rural villages, therefore corresponding to an initial

urbanization status, and 5 children (BC) from wealthy families, living in the capital city of Burkina Faso, Ouagadougou (Geographic coordinates 12°21′26″N 1°32′7″W; Fig. 3.1), about 90 km distance from Nanoro. Boulpon village consists of a cluster of huts built using wood and straw (Fig. 3.1A), in which the population live in communities based on subsistance agricolture. Nanoro is a small town with about 5`200 inhabitants, consisting in urban agglomerates of of small brick houses (Fig. 3.1B). Ouagadougou is the capital city of Burkina Faso, with a population of 1`475`223 inhabitants, and is the administrative, cultural, economic and industrial center of the nation. The industry of Ouagadougou is sector that fuels urban growth, as people move to the city from the countryside to find employment in industries of processing plants and factories. As far as concern level of economic status and health conditions, all selected children were healthy at time of the investigation. However, BR children were poor, at high risk of infectious diseases and malnutrition, BT children were of average economic conditions for Burkina Faso standard living in a dirty environment (Fig 1b), at risk of infectious disease and at low risk of malnutrition. Finally, BC children were from wealthy families living in clean modern houses, at no risk of malnutrition and at lower risk of infectious diseases when compared to the other groups of African children but certainly at higher risk of infections when compared to Italian children.
In addition, it is worth of mentioning that the capital city of Ouagadougou is very polluted by vehicles exhaust gas, especially during the dry season.

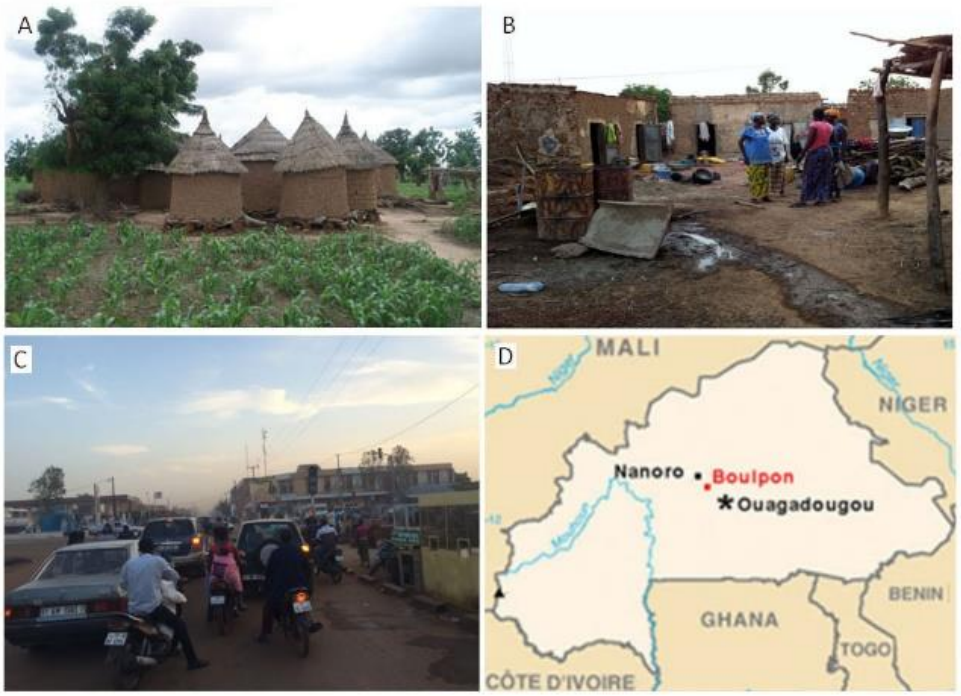

Fig. 3.1 Rural and urban environments in Burkina Faso. (*A*) Rural village in Boulpon, (*B*) urban village in Nanoro town, (*C*) Ouagadougou, the capital city of Burkina Faso, and (*D*) map of Burkina Faso.

Dietary habits of these three African populations was compared with a population of our previous study composed by 13 European children (EU) living in Florence (Italy) and characterized by a typical western diet (De Filippo et al. 2010). The age range of all groups

of children was 2-6 years, with an average of 4.6 ± 1.5 years (mean±s.e.) and the sex ratio (F:M) was 14:23 (Table 3.1). This age range was chosen in order to evaluate the acquisition of microbial biodiversity and microbiota evolution in first years of life. In fact, during human life cycle, colonization of the infant gastrointestinal tract by microbial communities is essential for microbiota–host interactions and influences health and disease status (Rodriguez, Murphy et al. 2015).

Initially, we performed an analysis of dietary habits and daily food intake for all children populations, based on dietary questionnaires and interviews to mothers, estimating average values for quantity of food ingested per day, food energy (Kcal/day), grams of protein, fat, carbohydrate, including simple sugar, and fiber (Table 3.2).

As showed in our previous study (De Filippo, Cavalieri et al. 2010), the diet of BR rural children is predominantly vegetarian, rich in fibers and plant-polysaccharides and low in fat, animal protein and simple sugar (Table 3.2A). The sources of fibers are also quite unique, as derived from locally cultivated indigenous cereals (Petit millet, *Panicum miliaceum* and Sorghum, *Sorghum vulgare*), legumes (Niebè, *Vigna unguiculata*), vegetables (Néré, *Parkia biglobosa* and Baobab leaves), fruits (especially Mango, Papaya and Bananas), and fermented products (Soumbalà from Nerè seeds). Millet and sorghum, typical cereals of Burkinabè diet, are usually still ground into flour on a grinding stone, similarly to what man began to do during the agriculture revolution in the Neolithic era, to produce a thick porridge called Tô, that is the principal dish-component of Burkinabè meals.

The BT children living in Nanoro town still eat cereals and legumes similarly to BR population, but, depending on socio-economic status, they add in their diet also rice, corn, peanuts, peanut oil, and on average once a week mutton or chicken meat from animals that are bred in the village or alternatively dried fish. Differently from BR population, in Nanoro town, cereal flour, legumes, fruit and dried fish are bought in the local market, in which products from neighboring countries can be also found (Table 3.2B).

The BC children, living in the capital city, Ouagadogou, together with a typical African diet based on cereals (millet, sorghum, rice, soja) and legumes (Niebè), eat bread, milk and dairy products as cheese and yoghurt, eggs, fruit juices, snacks, sweet bakery products and no more than 3 times per week different kinds of meat and fish, including frozen fish. Their diet therefore is very similar to that of children living in industrialized and globalized world. Many of these products are bought at supermarket (Table 3.2C).

The EU diet is a typical western diet, high in starch, simple sugar, animal protein, and fat and low in fiber, as previously described (De Filippo et al, 2010) (Table 3.2D).

Table 3.1 Characteristics of the four studied populations

Sample ID	Group	Nation	Environment	Sex	Age
2BR	BR	Burkina Faso	Rural village	M	5
6BR	BR	Burkina Faso	Rural village	F	6
7BR	BR	Burkina Faso	Rural village	M	6
8BR	BR	Burkina Faso	Rural village	M	6
9BR	BR	Burkina Faso	Rural village	M	6
10BR	BR	Burkina Faso	Rural village	F	6
11BR	BR	Burkina Faso	Rural village	M	5
12BR	BR	Burkina Faso	Rural village	M	6
13BR	BR	Burkina Faso	Rural village	M	6
15BR	BR	Burkina Faso	Rural village	M	6
17BR	BR	Burkina Faso	Rural village	F	5
				ratio (F:M) 3:8	age (average ±SD) 5.7 ±0.46
1BT	BT	Burkina Faso	Small Town	M	2
2BT	BT	Burkina Faso	Small Town	F	3
3BT	BT	Burkina Faso	Small Town	F	8
4BT	BT	Burkina Faso	Small Town	M	4
5BT	BT	Burkina Faso	Small Town	M	5
6BT	BT	Burkina Faso	Small Town	M	4
7BT	BT	Burkina Faso	Small Town	M	7
8BT	BT	Burkina Faso	Small Town	F	2
				ratio (F:M) 3:5	age (average ±SD) 4.4±2.19
1BC	BC	Burkina Faso	Capital city	M	4
2BC	BC	Burkina Faso	Capital city	F	3
3BC	BC	Burkina Faso	Capital city	F	2
4BC	BC	Burkina Faso	Capital city	F	3
5BC	BC	Burkina Faso	Capital city	F	2
				ratio (F:M) 4:1	age (average ±SD) 2.8±0.83
1EU	EU	Italy	European city	M	2
5EU	EU	Italy	European city	M	5
6EU	EU	Italy	European city	M	6
8EU	EU	Italy	European city	M	5
10EU	EU	Italy	European city	M	5
11EU	EU	Italy	European city	M	5
12EU	EU	Italy	European city	M	6
13EU	EU	Italy	European city	M	5
17EU	EU	Italy	European city	M	5
18EU	EU	Italy	European city	F	3
19EU	EU	Italy	European city	F	4
20EU	EU	Italy	European city	F	5
21EU	EU	Italy	European city	F	3
				ratio (F:M) 4:9	age (average ±SD) 4.5±1.19
Total groups				ratio (F:M)14:23	age (average ±SD) 4.6±1.55

Table 3.2 Total daily food intake in terms of protein, fat, carbohydrate and fiber in relation to the average of maximum quantity ingested per day relative to Burkina Faso and European children

(A)

BR	Composition of Edible Portion							
Dish component	**Daily Q (grams)***	**Percentage on total daily Q**	**Food Energy (Kcal)**	**Moisture (%)**	**Protein (g)**	**Fat (g)**	**Carbohydrate, total (incl. fiber) (g)**	**Fiber (g)**
Cereals and starchy component (Millet,Sorghum)	170	38%	495.4	46.38	10.1	4.06	105.53	1.09
Legumes (Niebè, Black-Eyed Peas)	70	16%	267.4	6.02	25.795	8.47	26.285	6.16
Vegetables (Nerè)	60	13%	55.2	47.1	3.36	3.84	4.26	0.612
Fruit (mango, papaya)	130	29%	48.75	115.7	0.91	0.195	12.48	6.37
Milk (cow's milk)								
Milk derivates								
Meats, meat derivates, fish								
Egg								
Oil and fats (karitè)	15	3%	129.3	0.21	0	14.67	0.09	0
Peanuts								
Sugar and honey								
Total daily food intake	**445**		**996.1**		**40.2**	**31.2**	**148.6**	**14.2**

* Average of max Quantity ingested per child per day

(B)

BT	Composition of Edible Portion							
Dish component	**Daily Q (grams)***	**Percentage on total daily Q**	**Food Energy (Kcal)**	**Moisture (%)**	**Protein (g)**	**Fat (g)**	**Carbohydrate, total (incl. fiber) (g)**	**Fiber (g)**
Cereals and starchy component (Millet,Sorghum, Rice, Corn)	195	42.4%	470	78	10.4	4.0	100.4	1.6
Legumes (Niebè, Black-Eyed Peas)	70	15.2%	222.6	4.9	21.2	7.0	21.8	4.9
Vegetables (Nerè and Baobab leaves)	50	10.9%	136	21.6	8.8	9	8	1
Fruit (mango, papaya)	100	21.7%	37.5	89.2	0.6	0.1	9.6	4.8
Milk and Milk derivatives								
Meats, meat derivatives, fish (mutton; lake fish**)**	20	4.3%	32.9	11.3	3.71	0.58	0.02	0
Egg								
Oil	15	3.3%	135	0	0	15	0	0
Peanuts	10	2.2%	60.5	0.5	1.7	5.5	2.2	0.2
Sugar and honey								
Total daily food intake	**460.0**		**1094.5**		**46.5**	**41.2**	**142**	**12.5**

* Average of max Quantity ingested per child per day

**once a week

(C)

BC	Composition of Edible Portion							
Dish component	**Daily Q (grams)***	**percentage on total daily Q**	**Food Energy (Kcal/die)**	**Moisture (%)**	**Protein (g)**	**Fat (g)**	**Carbohydrate, total (incl. fiber) (g)**	**Fiber (g)**
Cereals and starchy component (bread, millet, sorghum, corn)	160	17.1%	384	40	8.5	3.2	82.4	1.4
Legumes	30	3.2%	95.4	26.25	9.2	15	9.4	2.1
Vegetables (Nerè, Tomatoes, carrots, zucchini, aubergine)	120	12.8%	10.8	49.39	1.49	0.17	2.52	1.1
Fruit (mango, bananas, papaya)	100	10.7%	37.5	89.2	0.6	0.1	9.6	4.8
Fruit juice	110	11.8%	53.9	40.66	0.55	0.09	13.9	0
Milk and Milk derivatives (cow's milk, yoghurt, cheese)	250	26.7%	246.7	218.5	12	16.65	13.2	0
Meats, meat derivates, fish	60	6.4%	98.8	34.14	11.13	1.75	0.06	0
Egg	30	3.2%	38.4	77.1	3.72	2.61	0	0
Oil	20	2.1%	180	0	0	20	0	0
Peanuts	15	1.6%	90.7	7.5	2.55	8.25	3.3	0.3
Sugar and honey	10	1.1%	38.7	0	0	0	10	0
Snacks	30	3.2%	179.55	18.8	2.2	8.7	34.9	0
Total daily food intake	**935**		**1454.27**		**51.92**	**76.5**	**179.26**	**9.7**

* Average of max Quantity ingested per child per day

(D)

EU	Composition of Edible Portion							
Dish component	**Daily Q (grams)***	**percentage on total daily Q**	**Food Energy (Kcal/die)**	**Moisture (%)**	**Protein (g)**	**Fat (g)**	**Carbohydrate, total (incl. fiber) (g)**	**Fiber (g)**
Cereals and starchy component	160	17.3%	375	68.02	9.7	4.89	152.38	3.46
Legumes (Beans, String beans, Peas)	20	2.2%	10.9	15.4	1.2	0.1	3.7	0.9
Vegetables (carrot,potatoes, fennel, tomato, zucchini)	100	10.8%	50.9	82.3	2.3	1.1	18.4	1.6
Fruit (apple, pear, peach, grapes, bananas, mandarin)	140	15.1%	83.5	114.9	1.1	0.3	43.0	2.4
Milk and milk derivatives (cow's milk, mozzarella, parmesan, cheese)	290	31.4%	314.7	232.06	21.23	22.15	20.78	0.0
Meats, meat derivatives, fish	120	13%	157.7	83.9	26.0	4.6	6.2	0.0
Egg and derivates	30	3.2%	91.1	16.9	2.9	8.7	0.4	0.0
Oil and fats (Extra virgin olive oil and butter)	25	2.7%	210.65	1.4	0.1	23.3	0.2	0.0
Peanuts								
Sugar and Honey	10	1.1%	38.7	0.0	0.0	0.0	10.0	0.0
Snacks	30	3.2%	179.55	18.8	2.2	8.7	34.9	0.0
Total daily food intake	**925**		**1512.7**		**66.7**	**73.9**	**290.0**	**8.4**

* Average of max Quantity ingested per child per day

Considering nutritional analysis of the 4 populations we observed that, moving from rural (BR) to urban population (BT, BC and EU), the variety of food consumption was increased (Fig.3.2), and the fiber intake was progressively reduced, passing from rural to urban African populations, and finally to EU children, as well as the daily intake of fat, protein and simple sugar, and consequently the daily caloric intake (Table 3.2). The average amount of fiber in BR diet is 14.2 g/d (3.19% of total grams of daily food intake) compared with 12.5 g/d

(2.72%) in BT, 9.7 g/d (1.04%) in BC and 8.4 g/d (0.9%) in EU. The fiber intake presented an opposite trend when compared with the average daily calorie amounts (BR: 996 kcal/day; BT: 1094.5 kcal/day; BC: 1454.3 kcal/day; EU: 1512.7 kcal/day; Table 3.2).

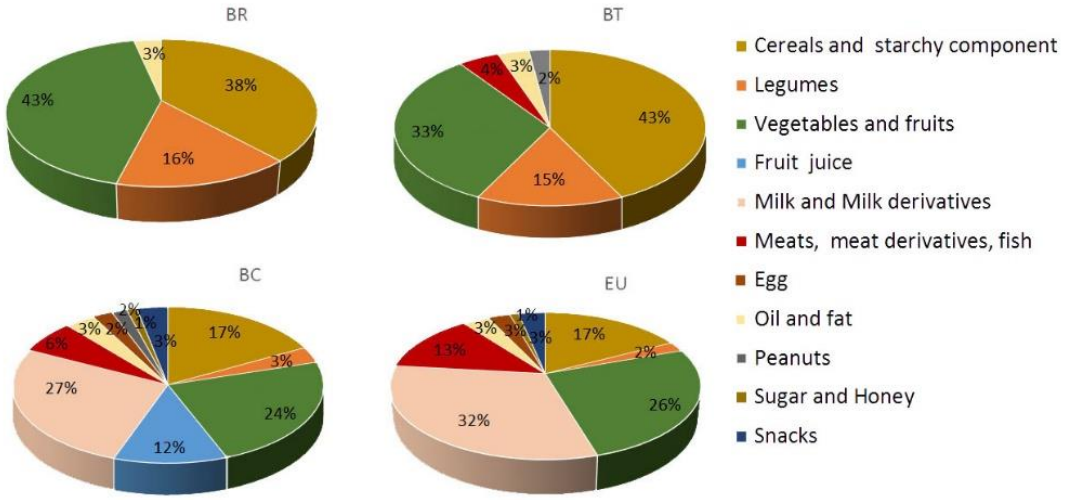

Fig. 3.2 Variety of food consumption in the four children populations. Pie charts indicate the percentages of daily foods assumed by the African and European populations. BR: children form rural village; BT: children from Nanoro town; BC: children from BF capital city; EU: children from Europe (Florence, Italy)

In our previous study (De Filippo, Cavalieri et al. 2010), comparing dietary habits of BR and EU populations, we observed a correlation between fiber intake and levels of fecal short chain fatty acids (SCFAs). Thus, considering the differences in food consumption, especially in fiber intake in BT and BC populations compared with BR, we performed metabolomics analysis by Solid-phase microextraction- Gas Chromatography- Mass spectrometry (SPME-GC-MS), measuring SCFAs levels in fecal sample of BT and BC children. The SPME-GC-MS analysis showed a significant reduction of total fecal SCFAs moving from BR to urban populations (Fig. 3.3; p-values by one-tailed Student t test) indicating a clear trend, especially for propanoic and pentanoic acids (Fig. 3.3; p-values by one-tailed Student t-test). Interestingly, EU presented significantly low levels of SCFAs, especially butyric acid, compared to the three African groups (Fig. 3.3; p-values by one-tailed Student t test). Fecal samples of BC showed a significant increase in total SCFAs levels, especially for acetic and butyric acids compared to BT and EU, but a decrease compared to BR (Fig. 3.3; p-values by one-tailed Student t test).

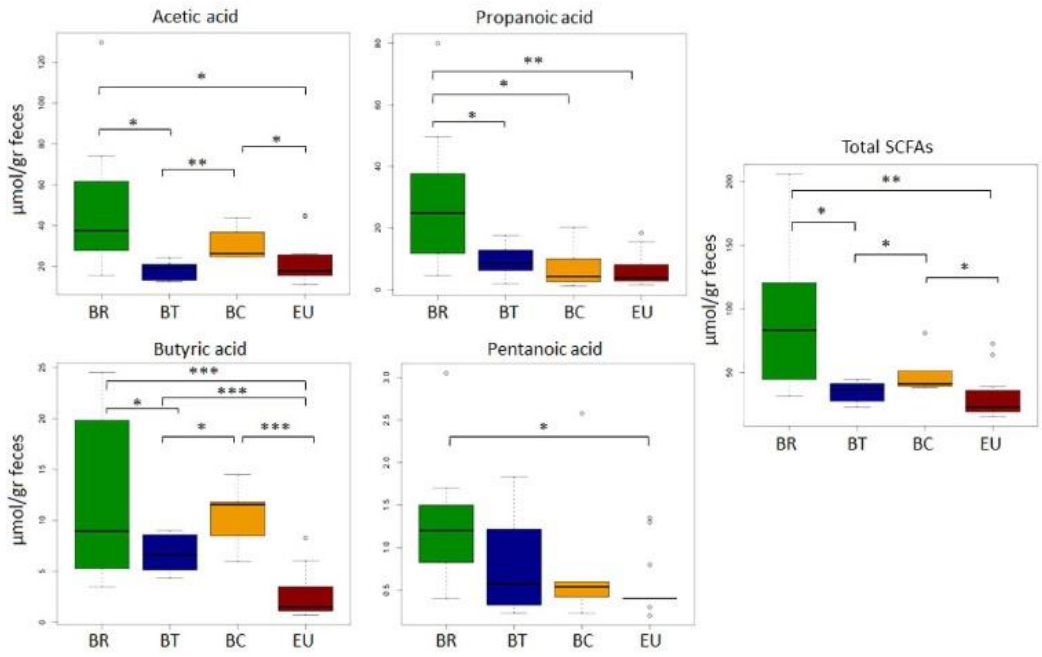

Fig. 3.3 Quantification of SCFAs levels in fecal samples from BR, BT, BC, and EU populations by SPME-GC-MS. Mean values (±SEM) are plotted. Comparison among groups by one-tailed Student t test. *p< 0.05; **p≤ 0.01; ***p≤ 0.001.

3.3.2 Microbiota characterization of Burkina Faso and European populations: taxonomic changes as an effect of diet and migration from rural to urban environments

In order to understand the correlation among SCFAs reduction observed from rural to urban populations with respect to diversity of microbial profiles, we studied the taxonomic distribution of gut microbiota of BT and BC populations through pyrosequencing, using 454 FLX sequencing of the V5-V6 hypervariable regions of 16S rRNA gene. We compared the meta-taxonomics data with the previous results obtained for BR and EU populations (De Filippo, Cavalieri et al. 2010).

The taxonomic distribution in the four populations showed a countertrend abundance of Bacteroidetes and Firmicutes, the two dominant phyla of gut microbiota, moving from BR to BT, to BC, and EU populations (Fig. 3.4A-B), with a gradual decrease of Bacteroidetes/Firmicutes ratio. Bacteroidetes were enriched especially in BR and BT (mean relative abundance 68.6% and 47.7% respectively, versus 32.6% in BC and 25.9% in EU), with a significantly higher abundance when comparing BR with BC and EU (Figure 3.4A; Wilcoxon rank-sum test). Conversely, Firmicutes were more abundant in BC and EU (57.5% and 60.2% mean relative abundance, respectively) than in BT, and significantly enriched compared with BR (Figure 3.4A; Wilcoxon rank-sum test), in according with our previous observations. Actinobacteria were more abundant in BC compared to the other African and European populations (6.74% in BC vs 3.6% in EU, 0.17% in BR, and 1.11% in BT), and significantly reduced in BR compared to the other groups (Figure 3.4A; Wilcoxon rank-sum test). An abundance in Proteobacteria was observed in BT and EU (3.8% in BT and 4.94% EU vs 2.73% in BR and 1.26% in BC), compared with other populations, although not statistically significant. Among the minor phyla of gut microbiota, Spirochaetes was

significantly increased in BR compared with BC and EU, as well as in BT compared to EU (Figure 3.4A; Wilcoxon rank-sum test). As reported in Fig. 3.4A, BR and especially BT populations showed a greater variability with respect to the relative abundance of phyla compared with BC and EU children, suggesting an inter-variability in microbiota composition. A possible explanation of such observation could be the higher exposure to an external dirty environment, lower level sanitation in both BR and BT groups of children, in addition to the higher variability of the dietary habits of the BT group.

Based on sequence abundances at phylum level in each population, the dendogram obtained with Average Neighbour and Unweighted Pair Group Method with Arithmetic Mean (UPGMA), showed a clear separation between BR and EU populations, (Fig. 3.4B) especially due to the different ratio Bacteroidetes/Firmicutes, as previousy observed (De Filippo, Cavalieri et al. 2010). In contrast, BT and BC children showed a progressive shift towards phyla distribution observed in EU children in accordance to different dietary habits and environments. Interestingly, whereas the majority of BC samples clustered together with EU, also two BT samples clustered closely to EU group, due to the Firmicutes relative abundance. In our opinion this is due to the fact that as observed by questionnaires dietary habits of BT children living in the community of Nanoro town were not uniform and some of them had a diet more similar to that of western population, whereas others still had a diet resembling that of rural areas. Thus, the majority of BT samples fell within the BR cluster, or in a subcluster interposed between BR and EU population, due to the abundance in Bacteroidetes in their microbiota (Fig. 3.4B). These results suggest that Burkina Faso populations belonging to the same Mossi ethnicity and living in the same geographic area differ in microbiota composition according to the modifications of dietary habits and kind of environment where they live.

The observed distribution of the four populations based on taxonomic assignment at phylum level was confirmed by analysis of microbial community structure (beta diversity). Considering Unweighted and Weighted UniFrac distances, and Bray-Curtis dissimilarities, Principal Coordinates (PCoA) analysis (Fig. 3.4C) showed a clear separation between BR (green dots) and EU (red dots) samples, confirming different gut microbiota composition between African and European populations (uUnifrac ADONIS and ANOSIM, p-val= 0.0001; wUnifrac ADONIS and ANOSIM p-val= 0.0001; bray-curtis ADONIS and ANOSIM p-val= 0.0001). BT samples (blue dots) result close to the BR population, while BC (orange dots) approaches the EU (Fig. 3.4C), suggesting changes of gut microbial communities in African populations, as an effect of moving from the rural to the urban condition.

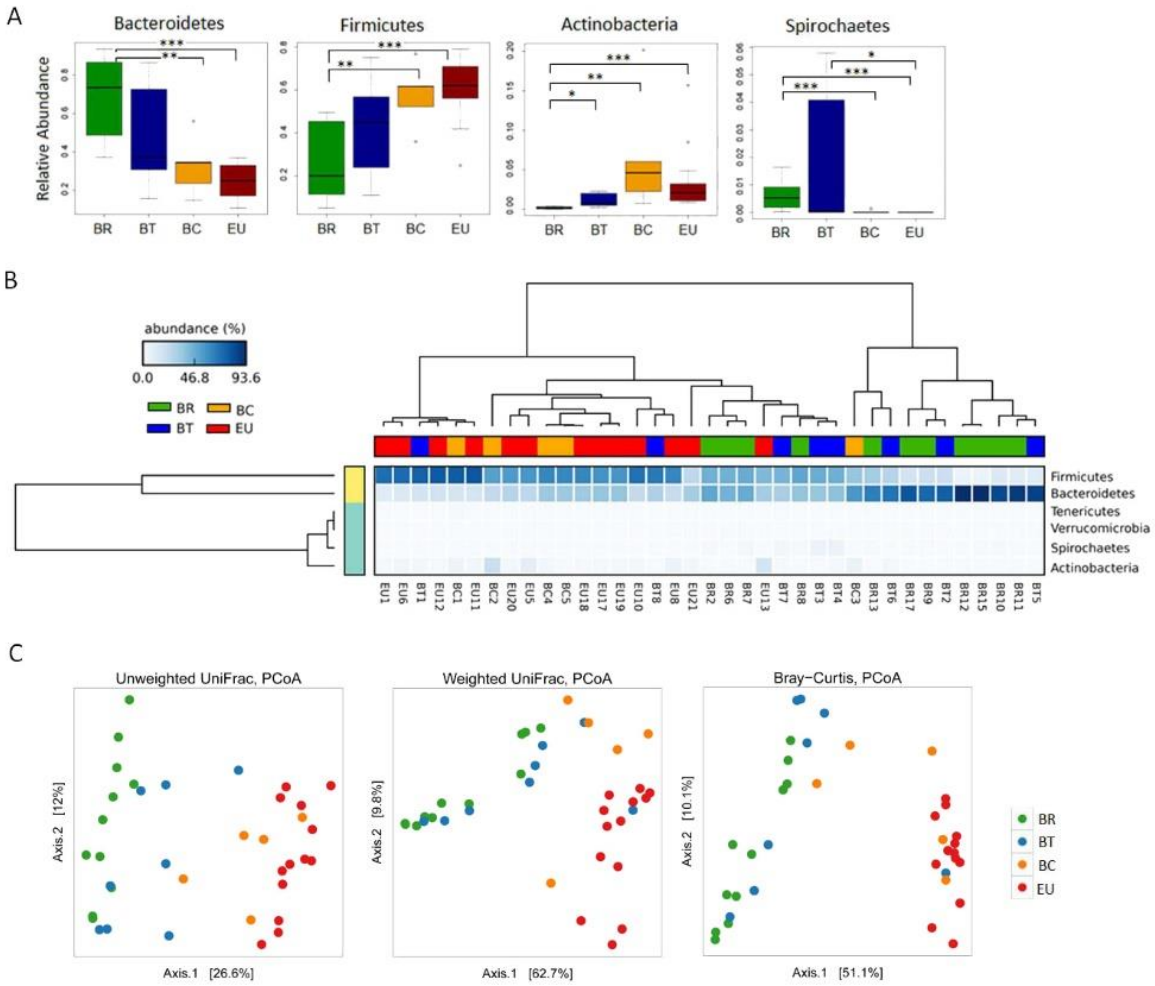

Fig. 3.4 (A) Box plot of relative abundances of the major statistically significant different phyla in African and European populations (Wilcoxon rank sum test; * p-value <0.05, **p-value <0.01; *** p-value<0.001). (B) Heatmap plot indicating the proportion (%) of sequences assigned at each phylum. Each sample, belonging to respective group, is represented by a different color: green=BR, blue=BT, orange=BC and red=EU. The blue squares indicate abundance percentage of each phylum in each sample. Dendrograms, obtained with Average Neighbour UPGMA method, are used to cluster both phyla (vertical) and fecal samples of the children populations (horizontal) based on phyla abundances. (C) Beta diversity: Principal coordinate analysis (PCoA) derived from unweighted and weighted UniFrac and Bray-Curtis distances among samples of the four populations (P=0.0001 by PERMANOVA). Colored dots representative of the four populations are as in (A) and (B). For each axis, in square brackets, the percent of variation explained was reported.

Next, we evaluated different microbial richness (alpha diversity) among populations. By observed OTUs and Chao1 index, we found a downward trend in species richness from rural BR to urban BC and to EU, whereas BT children showed a high alpha diversity index. Shannon index, estimating entropy, indicated a reduced alpha diversity in BR compared to the other populations. No statistically significant differences (PERMANOVA analysis) were obtained with the three estimators of alpha diversity, probably due to the variability observed within the African and European groups (Fig. 3.5A-C). Furthermore, measuring intra-group dissimilarities among the populations (Fig. 3.5D-E), by unweighted UniFrac distance comparison, we observed more intra-variation of gut microbiota profiles in BT and BC,

compared to BR and EU populations, even if no statistically significant. However, considering inter-group distances among African populations with respect to EU, we confirmed that microbiota composition of BR population differs significantly from EU, while distances between BT and EU, as well as between BC and EU were significantly lower than BR to EU (Fig. 3.5D-E; Pairwise comparisons using Wilcoxon rank sum test, FDR adjustment $p<0.001$), suggesting a transition of gut microbiote profiles of urban Africa populations towards profiles characteristic of western population.

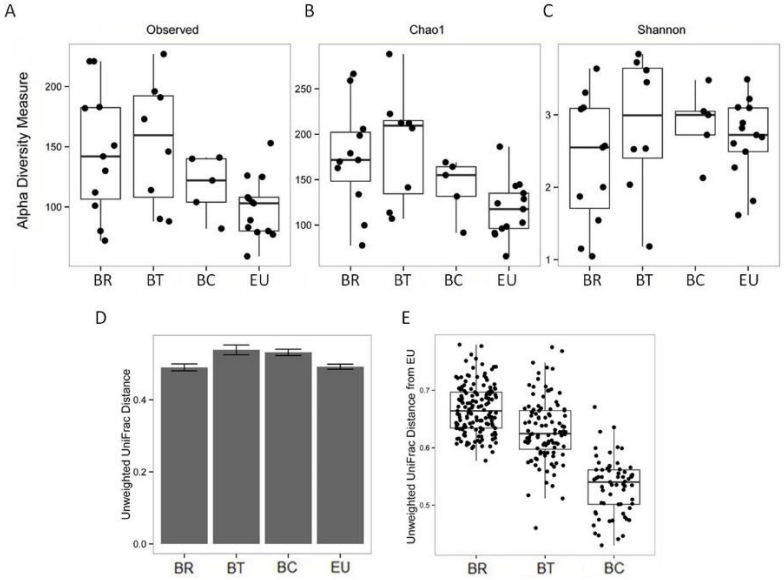

Fig. 3.5 Box plots of alpha diversity in the three African populations compared to EU, based on (A) number of observed OTUs, (B) Chao 1 index and (C) Shannon index. P=0.096 BU vs EU pairwise comparisons using Wilcoxon rank sum test. (D-E) Unweighted UniFrac distances based on observed phylogenetic differences, (D) between samples from the same population (intra-group distances) and (E) between samples from African vs EU population (inter-group distances). Pairwise comparisons using Wilcoxon rank sum test; FDR p-value adjustment method, ***$p<0.0001$.

Following the taxonomic assignment at family level, we observed that *Prevotellaceae*, representing the most abundant family in BR and BT populations (66.8% and 41.14% mean relative abundance in BR and BT respectively), were significantly enriched in rural BR compared with BC and EU with a clear reduction in BC (10.4%), and almost absence in EU (0.44%) (Fig. 3.6A; Wilcoxon rank-sum test). Conversely, several families were less abundant in BR population and progressively more abundant in urban African children (BT and BC) and in EU. Among these, *Bacteroidaceae, Bifidobacteriaceae, Porphyromonadaceae and Rikenellaceae* were significantly decreased in BR compared with BC and EU, and in BT compared with EU, as well as *Lachnospiraceae* and *Ruminococcaceae,* were significantly reduced in BR compared with BC and EU (Fig. 3.6A; Wilcoxon rank-sum test). Furthermore, we observed a significant increase in *Enterobacteriaceae* in BT population compared with EU, and in EU compared to BR (Fig. 3.6A; Wilcoxon rank-sum test).

Among the minor component of gut microbiota (relative abundance <0.05), we observed that *Coriobacteriaceae* was significantly enriched in BT compared to EU, and

Erysipelotrichaceae in BC compared with BR and EU (Fig. 3.6A; Wilcoxon rank-sum test). *Leuconostocaceae* was significantly more abundant in BR compared with EU (Fig. 3.6; Wilcoxon rank-sum test). *Spirochaetaceae* was more variables within the BT population and more abundant in BR population compared to BC and EU (Fig. 3.6A; Wilcoxon rank-sum test). *Desulfovibrionaceae* and *Sutterellaceae* were progressively and significantly enriched in urban BT, BC and EU, and almost absent in rural BR population (Fig. 3.6A; Wilcoxon rank-sum test).

Differential abundances of these families allow a clear discrimination of the microbiota profiles in the four populations, as highlighted by PCoA analysis based on UniFrac distances and Bray-Curtis dissimilarities (Fig. 3.6B). In particular, on one hand, the observed abundances of *Prevotellaceae* in BR and BT, and on other hand *Bacteroidaceae, Lachnospiraceae, Rikenellaceae and Porphyromonadaceae* in EU, explain the distances between African and European samples. The abundances in *Lachnospiraceae* and *Ruminococcaceae* explain the sample distribution of BC populations towards EU samples.

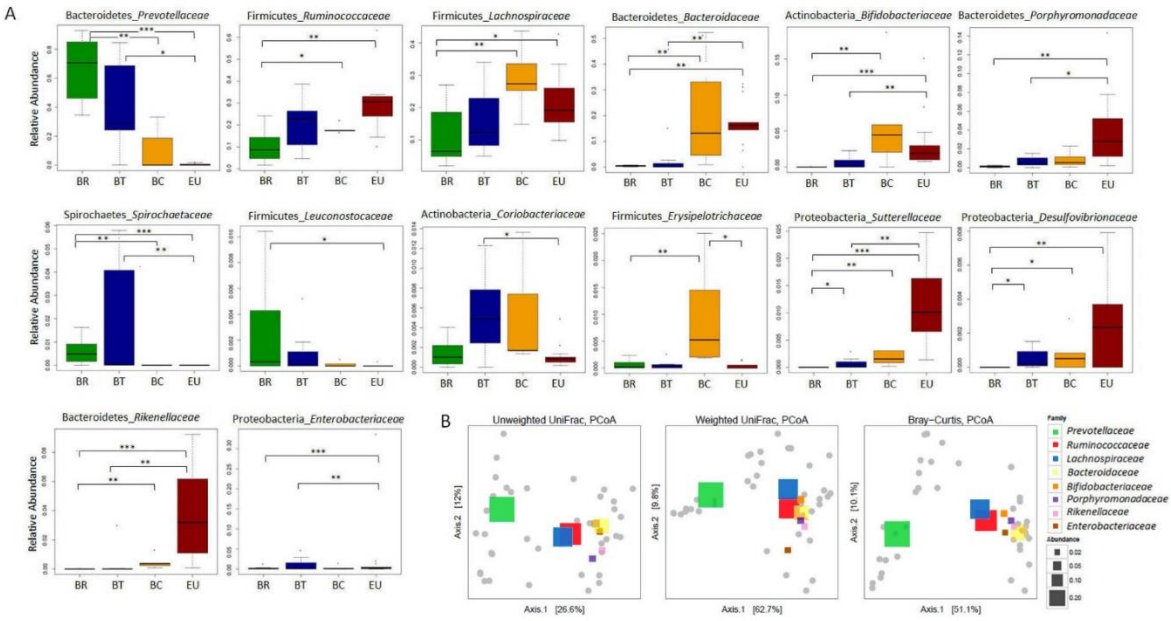

Fig. 3.6 Box plot of relative abundances of the statistically significant different families in African and European populations (Wilcoxon pairwise test; * p-value <0.05, **p-value <0.01; *** p-value<0.001). (*B*) Beta diversity and correlation with the principal abundant bacterial families. PCoA derived from unweighted and weighted UniFrac and Bray-Curtis distances among fecal samples of the four populations in relation with abundances of the principal bacterial families, represented by colored rectangles, in each population (P=0.0001 by PERMANOVA). Different sizes of rectangles indicate different order of relative family abundance. For each axis, in square brackets, the percent of variation explained was reported.

At genus level, as reported in our previous study, gut microbiota of BR was almost entirely populated by *Prevotella* (64.4% average out of total sequence amount), as confirmed by observed *Prevotellaceae* family at higher taxonomic level. BT and BC populations presented a progressive decrease in *Prevotella* abundance compared to BR (38.8% in BT and 10.3% in BC; Wilcoxon rank-sum test; BR vs BC p=0.01; BR vs EU p=0.0002; BT vs EU p=0.002;

Fig. 3.7), reflecting both the effect of transition from rural to urban environment and the reduction of dietary fiber intake. Moreover, this genus resulted absent in EU, as previously reported in our study.

By BLAST alignment, we observed that *Prevotella* sequences were attributable with 99% of identity to *P. copri*, *P. melaninogenica* and *P. stercorea*, over uncultured *Prevotella spp*. In our previous study, we found a set of sequences classified as *Xylanibacter*. Recently, *Xylanibacter* 16S rDNA was re-classified within the larger *Prevotella* genus (Sakamoto and Ohkuma 2012). Interestingly, an increase in *Treponema*, *Succinivibrio* and *Weissella* discriminated both BR and BT from BC and EU (Wilcoxon rank-sum test; p-value are respectively reported in Fig. 3.7). By BLAST alignment, we observed that *Treponema spp*. sequences were mainly attributable with 99% of identity to *T. succinifaciens*, a known carbohydrate metabolizer isolated from gut of termites and swine, and observed in other traditional human populations (Obregon-Tito, Tito et al. 2015). Interestingly, rural African population occasionally eat cooked termites. Other *Treponema* sequences were attributable to uncultured batceria and with minor percentage of identity to *T. porciunum*.

Succinivibrio, is generally associated with the bovine rumen (Bryant 1970), and was also found in higher frequency in the Hadza hunter gatherers and traditional Peruvian populations (Schnorr, Candela et al. 2014;Obregon-Tito, Tito et al. 2015) and might be involved in starch, hemicellulose, and xylan degradation, similarly to *Prevotella* and *Treponema*.

Thus, these observations confirm that the enrichment in bacteria metabolizers of plant-polysaccharides, hemicellulose, and xylan, as previous observed (De Filippo, Cavalieri et al. 2010), derived by a high-fiber diet resultant essentially by consumption of fiber rich vegetables, cereals and legumes locally cultivated, and similarly to wht reported in children and adults from Malawi and Venezuela whose diet was dominated by plant-derived polysaccharide foods (Yatsunenko, Rey et al. 2012). Passing from rural to urban microbiota, these genera decreased dramatically, until to be depleted in EU children. Interestingly, the progressive loss of these bacteria reflect the gradual reduction in fecal SCFA levels from BR to BT, BC and EU populations.

Thus, in BC the urbanization and the westernization led to loss of ancient microbial profiles typical of traditional and rural populations, such as *Prevotella,* and to the gain of bacterial species associated with a western-like diet, such as *Lachnospiraceae incertae sedis*, *Roseburia* and *Dorea* that were increased in urban BC and EU (Wilcoxon rank-sum test; p-value are respectively reported in Fig. 3.7). Morevoer, *Bacteroides* and *Bifidobacterium*, generally associated to milk and milk-derives food consumption and to childhood microbiota, were more abundant in BC and in EU compared with BR and BT, probably in relation to consumption of milk and milk-derived product in these populatons.

The majority of sequences belonging to *Bacteroides* genus, and consistently found in BC and EU metagenome, was attributable to *B. uniformis,* and in minor abundance to *B. acidifaciens*, *B. caccae*, *B. coprophilus*, *B. ovatus*, and *B. plebeius.* In BR and BT populations, although *Bacteroides* was poorly represented, we found *Bacteroides* sequences attributable with 95% of identity to *B. vulgatus.* Interestingly, a study on gnotobiotic interleukin-2-deficient mice showed that *B. vulgatus* has a possible protective role against *E. coli* induced-colitis (Waidmann, Bechtold et al. 2003), suggesting that the probable unique *Bacteroides* species found in rural BR microbiota could have a protective role against

potential pathogenic bacteria. Notably, in our previous study (De Filippo, Cavalieri et al. 2010), we showed that potential pathogenic bacteria, such as *Shigella* and *Escherichia* and other *Enterobacteriaceae*, were underrepresented in BR microbiota compared to EU, suggesting that, in accordance with the "old friend hypothesis", microbial diversity and the enrichment in SCFA-producing bacteria, in rural microbiota is able to protect against pathogenic microbes and gastrointestinal disease.

Regarding *Bifidobacterium* genus, we observed that BC and EU populations were mainly enriched in *B. longum*, and in minor part in *B. adolescentis*, *B. bifidum* and *B. breve*. We did not observe any association with age range and increase of *Bifidobacterium spp*, in accordance with the observation in other children belonging to Tunapuco traditional population (Obregon-Tito, Tito et al. 2015). BC and EU children in our study are those who consume milk and dairy products.

Finally, *Alistipes, Odoribacter* and *Barnesiella* discriminated Europeans from African populations, as representative genera of Western population, in accordance with Martinez and collaborators (Martinez, Stegen et al. 2015).

Among the minor representative genera, we observed an increase in *Odoribacter, Sutterella, Parasutterella, Bilophila* and *Clostridium XIVa*, that includes *Clostridium spp*., *Roseburia* and *Ruminococcus obeum*, in both BC and EU population (Fig. 3.7), suggesting that diet rich in protein and fat and poor in fiber influences the gut microbiota, independently from geographic origin. In fact, *Alistipes* and *Bilophila* has been previously linked to animal-based diet. It has been reported that high-fat diet induces an increase in abundance of *Biophila wadsworthia*, a member of the Desulfovibrionaceae family, which generates hydrogen sulfide via taurine metabolism, associated to inflammation, as recently observed in mice (Devkota, Wang et al. 2012).

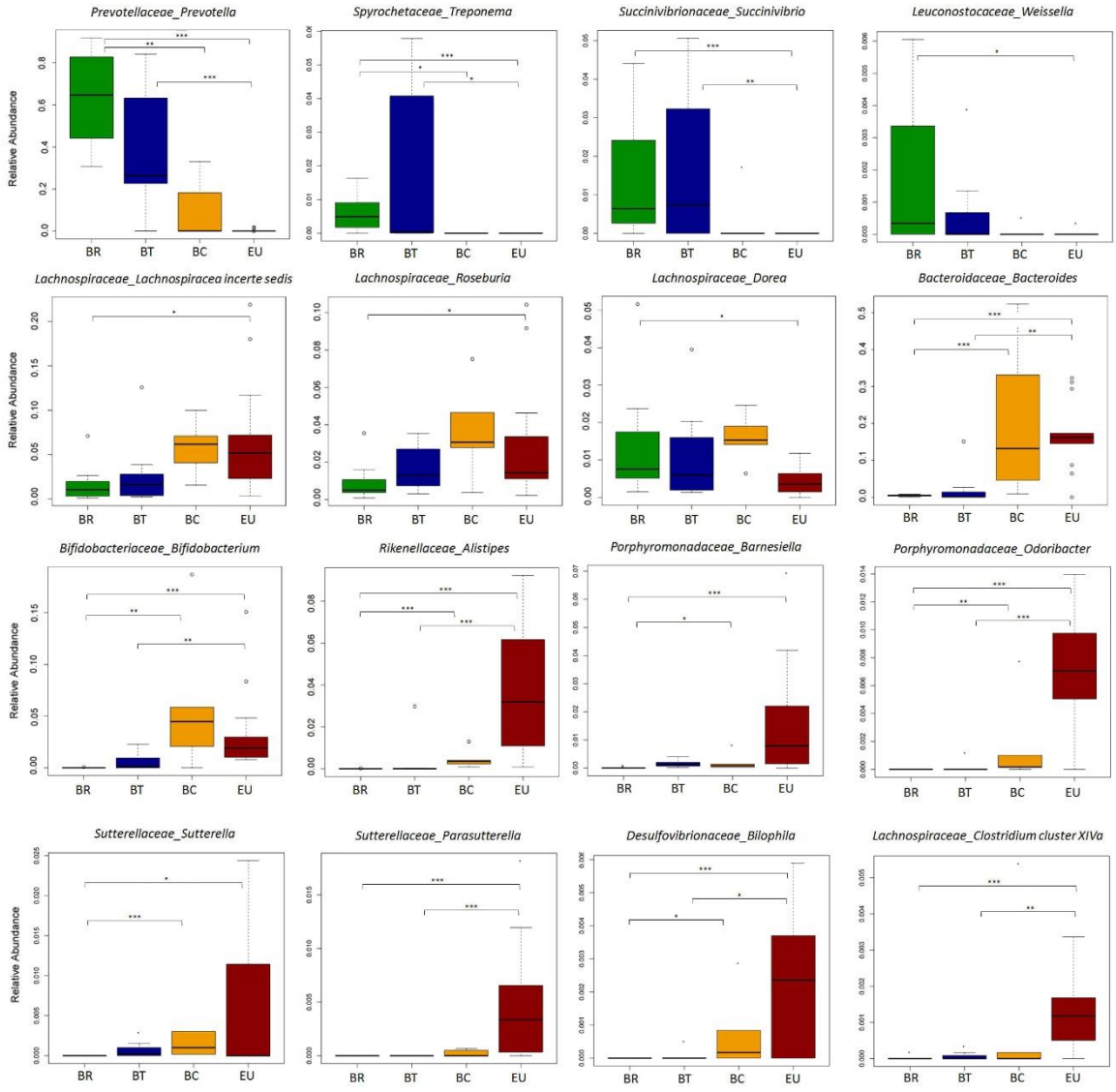

Fig. 3.7 Box plot of relative abundances of the statistically significant different genera in African and European populations (p-value by Wilcoxon pairwise test).

3.3.3 Prediction of functional metabolic profiles of gut microbiota from African and European population

In order to evaluate how the observed taxonomic differences between the gut microbiota of African and European children affect their metabolic potential, we applied PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), a computational approach useful to infer the functional contribution of microbial communities on 16S rDNA sequencing data set. We decide to use this approach although we are aware of PICRUSt imits since the accuracy of PICRUSt varies based on the availability of reference genomes for microorganisms in each environment/sample, and probably several microbial functions related to metagenome of isolated and traditional populations are still unknown, which are not yet present in the current database. PICRUSt prediction performed on samples from Burkina Faso and European children revealed significant differences in the main

functional classes (KEGG categories at level 2), deriving from acquisitions related to the different environments and to different dietary habits (Fig. 3.8). LEfSe analysis performed on PICRUSt output showed several KEGG categories differentially enriched in the African and European populations (Fig. 3.8). In particular, in BR metagenome, we observed enrichment in several functions, such as glycan biosynthesis and metabolism, energy metabolism, metabolism of terpenoids and polyketides, a source of plant phenolics, and metabolism of cofactors and vitamins, probably related to consumption of cereals, legumes, fiber and plant-polysaccharides rich food. In BC, we found enrichment in carbohydrate metabolism, mainly related to starch and sucrose rich diet, as well as biosynthesis of secondary metabolites, and function related to bacterial cell motility. In EU, we observed enrichment in function related to consumption of animal-protein and fat rich diet, such as aminoacids and lipid metabolism, and functions related to bacterial membrane transport and signal transduction. LEfSe did not show significant functional enrichment in BT metagenome, when compared to the other groups, considering KEGG categories at level 2, but at level 3 we observed enrichment of KEGG functions related to antibiotic biosynthesis (novobiocin biosynthesis) and terpenes degradation (geraniol degradation), an organic compound derived from plants.

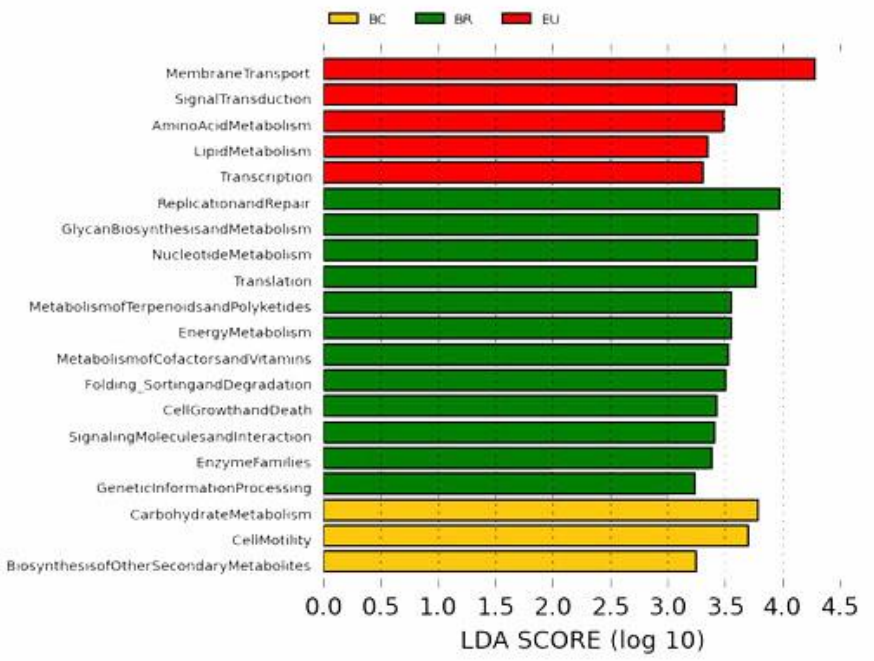

Fig. 3.8 Differences in bacterial functional classes (KEGG categories level 2). Functional pathways significantly enriched in African and European populations based on PICRUSt prediction. LEfSe results indicate a sequentially significant ranking among populations (Alpha value=0.05 for the factorial Kruskal-Wallis test among classes). The threshold for the logarithmic LDA score was 2.0.

3.3.4 Carbohydrate metabolism, glycan biosynthesis and energy metabolism

Considering KEGG categories at level 3 (Fig. 3.9), we observed that BR metagenome was enriched in functions related to glycan biosynthesis, glycosil transferases and TCA cycle, involved in complex carbohydrate metabolism, deriving from fiber and polysaccharides rich food, as well as functions related to lipopolysaccharide biosynthesis proteins and

glycosphingolipides biosynthesis, involved in building of bacterial membrane and in membrane-protein modulation contributing to cell–cell communication (D'Angelo, Capasso et al. 2013) (Fig. 3.9A). Interestingly, we found enrichment in carbon fixation pathways and oxidative phosphorylation, metabolic functions that are both related to carbohydrate metabolism and involved in releasing energy, associated with the TCA cycle pathway (Fig. 3.9B).

In BC metagenome, we observed enrichment in starch and sucrose metabolism, as well as the penthose phosphate metabolism, a metabolic pathway parallel to glycolysis that generates NADPH and 5-carbon sugars, such as ribose 5-phosphate, a precursor for the synthesis of nucleotides, and erythrose 4-phosphate (E4P), used in the synthesis of aromatic amino acids. Moreover, we found enrichment in pathways related to glycosphingolipid synthesis-lacto e neolacto series, involved in ceramide production, that plays an important role in bacterial infections (Grassme and Becker 2013) (Fig. 3.9A). Interestingly, BC metagenome was also enriched in methane metabolism (Fig. 3.9B). Fermentation of polysaccharides by colonic microorganisms can produce a number of by-products, gases, such as H_2 and CH_4 (methane) (Danielsson, Werner-Omazic et al. 2014). We could hypothesize that the presence of *Ruminococcaceae, Lachnospiraceae,* and *Clostridiaceae,* as observed in BC microbiota, can promote functional acquisition related to methane metabolism.

In EU metagenome, over the general pathway related to carbohydrate metabolism, pentose, glucoronate interconversion pathways and C5-branched dibasic acid metabolism, deriving from a simple sugar rich diet, we found enrichment in galactose metabolism, involved in conversion of galactose into glucose (Fig. 3.9B). Although legumes, grains, tubers and vegetables are galactose sources, galactose derives mainly from hydrolysis of lactose, the milk sugar. Therefore, acquisition of related function in EU metagenome arise from dairy milk derived-products. Other functions enriched in EU were the glyoxylate and dicarboxylate metabolism, related to glyoxylate cycle, involved in biosynthesis of carbohydrates from fatty acids, and butanoate metabolism. This metabolic pathwayconverge in glycolysis, aminoacid metabolism and fatty acid degradation with synthesis and degradation of ketone bodies.

Moreover, regarding energy metabolism, sulfur and nitrogen metabolism were enriched in EU metagenome. We suppose that *Bilophila,* found abundantly in EU microbiota, a well-known sulphite-reducing bacterium, could be responsible of this functional contribution. Regarding nitrogen metabolism, several enteric bacteria produce reduced nitrogen by dietary amino acids and animal-protein.

3.3.5 Functions related to aminoacid metabolism

The majority of pathways involved in amino acid metabolism were enriched in BR metagenome compared to the other populations (Fig. 3.9D), such as metabolism of glycine, serine, threonine, cysteine, methionine, D-glutamine and D-glutamate, beta and D-alanine and tyrosine, as well as the tripeptide gluthatione, derived from cysteine, glutamate and glycine, an important antioxidant molecule derived by biosynthetisis of the host but also of bacteria. These findings suggest the potential ability of BR microbiota to contribute in host

metabolic functions in conditions of poor aminoacid food intake.
The high intake of cereals and legumes in BR diet represents a source of aminoacids, especially glutamate, alanine and cysteine. An exceptionally source of glycine, alanine, and glutamic acid could be the Baobab, whose leaves are added in the principal Burkinabè dishes (Table 3.2). Similar findings were observed in Hazda, one of the last hunter-gatherer population, having Baobab leaves as part of diet (Schnorr, Candela et al. 2014). In general, plant proteins are deficient in lysine, methionine, and tryptophan, and are much less concentrated and less digestible than animal proteins. The absence of lysine and other essential aminoacids in cereals, which represent the basis of the African diet, leads to an inability to synthesize protein and ultimately could predispose to malnutrition, especially a syndrome known as Kwashiorkor, common among children in these countries (http://www.fao.org/docrep/t0395e/T0395E0c.htm).
Commensal bacteria can provide amino acids to the host from both dietary and endogenous proteins. Peptides and amino acids are used as carbon, nitrogen and energy sources by both saccharolytic and non-saccharolytic bacteria. Some saccharolytic species, such as *Prevotella*, the predominant genus in BR group, are able to derive amounts of energy from the carbon skeletons of peptides and amino acids, while others, including Enterococci and Enterobacteria, are able to ferment both carbohydrates and amino acids.
Glutammate is the principal source of nitrogen for the host. Bacteria can make the carbon skeletons of all amino acids and transaminate those carbon skeletons with nitrogen from glutamine or glutamate to complete the amino acid structures. Interestingly, the observed enrichment in beta and D-alanine metabolism represents a way to produce glucose, especially in condition of fasting. In fact, alanine can be converted to pyruvate in the liver by the glucose-alanine cycle, as a source of carbon for gluconeogenesis, in order to form glucose that can be used as energy for the muscle.
Another metabolic function found in BR population is the selenocompound metabolism, deriving from consumption of cereal grains, legumes and soybeans (Whanger 2002) (Fig. 3.9D). Selenocompounds are organometallic molecules composed by selenium, an essential element playing critical roles for the host, such as reproduction, thyroid hormone metabolism, DNA synthesis, and protection from oxidative damage and infection (Sunde and Thompson 2009).
Surprisingly, metabolism of taurine and hypotaurine was enriched in BR metagenome (Fig. 3.9D). Generally, taurine is a major constituent of bile acid, important to emulsify fat. However, taurine is also derived from cysteine, thus enrichment in cysteine metabolism found in BR could explain this functional acquisition.
In BC metagenome, we found enrichment in aromatic aminoacid (phenylalanine, tyrosine and tryprophan; Fig. 3.9D) that could derived from E4P (see carbohydrate metabolism), potentially produced by the observed pentose phosphate biosynthesis (Fig. 3.9A). Such observation is in accordance with a previous study on microbiota transplantation of obese twin in mice (Ridaura, Faith et al. 2013). In BC we also found enrichment in cyanoaminoacid and phosphonate and phosphinate metabolism (Fig. 3.9D), related to carbon-phosphorous bonds contained in several natural products (C-P compounds). Among microorganisms able to biosynthetize C-P compounds there are Actinobacteria, found abundant in BC microbiota.
In EU metagenome, we observed enrichment in metabolism of aminoacids, such as arginine,

proline, valine, leucine, isoleucine, histidine and tryptophan, and lysine biosynthesis and degradation (Fig. 3.9D). These aminoacid can originate from the animal-protein rich food, typical of western population. Good sources of lysine are high-protein foods such as eggs, meat (specifically red meat), and beans. Regarding essential branched-chain aminoacid (BCAAs, such as valine, leucine, and isoleucine) our results are in agreement with recently analyzed metagenome of an Italian population (Rampelli, Schnorr et al. 2015). It is noteworthy that, metabolic function related to BCAAs and aromatic aminoacid are enriched in obese compared to lean individuals (Newgard, An et al. 2009), and increased level in BCCA is associated with the risk of developing future type 2 diabetes (Wang, Larson et al. 2011). These findings suggest that microbiome of EU population could contribute to acquisition of metabolic conditions predisposing to metabolic syndrome, typical of western countries. Moreover, the breakdown of basic amino acids by commensal bacteria is a source of SCFAs, as demonstrated by Smith and Macfarlane (Smith and Macfarlane 1997). In general, alanine, glycine and cysteine are fermented by bacteria to acetate, propionate and butyrate. Serine is fermented to acetate and butyrate, while threonine is mainly metabolised to propionate. The main products of methionine metabolism are propionate and butyrate. Interestingly, metabolism of these aminoacids was found enriched in BR, and, together with fiber and polysaccharide fermentation, could contribute the abundance in SCFAs observed in fecal samples of BR. Regarding aminoacid metabolism enriched in EU group, lysine and arginine, and deamination of histidine produce butyrate and acetate, while BCCAs are slowly fermented by colonic bacteria. Thus, diet and the functional acquisitions of bacteria metagenome to metabolize aminoacids or fermented fiber and polysaccharides rich foods could explain the different levels of SCFAs observed in our studied populations.

3.3.6 Vitamin and cofactor metabolism

Evidences showed that commensal colonic bacteria are a significant source of a range of vitamins to the host (Hill 1997). Unlike dietary vitamins, which are mainly absorbed in the proximal part of the small intestine, the uptake of microbial vitamins predominantly occurs in the colon (Said and Mohammed 2006). Among this, the B vitamins, including thiamin, riboflavin, niacin, folate, vitamin B6, vitamin B12, biotin and pantothenic acid, cooperate collectively and individually to perform many different processes, such as the release of energy deriving from carbohydrates, proteins and fats, or regulation of immune cells (Brestoff and Artis 2013).

Regarding KEGG categories related to cofactors and vitamin metabolism, in BR metagenome we found pholate biosynthesis, riboflavin metabolism, vitamin B6 and retinol metabolism, nicotinate and nicotinamide metabolism, ubiquinone and terpenoid biosynthesis. Altogether, these functional acquisitions are related to cofactors and coenzyme involved in oxidative reactions, energy, and carbohydrate and aminoacids metabolism.

Functional acquisition in folate metabolism in BR metagenome is essential for DNA, RNA building and for metabolizing of amino acids which are required for cell division.

Riboflavin is involved in enzymatic reactions, including oxidation of pyruvate, α-ketoglutarate, and branched-chain amino acids.

Vitamin B6 is as a coenzyme involved in amino acid, glucose, and lipid metabolism.

Nicotinate, also known as niacin, and nicotinamide are precursors for generation of coenzymes, NAD+ and NADP+, which are essential for redox reactions. Ubiquinone and other terpenoid-quinone are electron carriers in oxidative phosphorylation, important to energy metabolism.

Retinol is the active form of vitamin A, a fat-soluble vitamin involved in promoting vision, strong bones and healthy skin. The body can also convert retinal and retinoic acids, compounds found in carotene, into retinol. Mango and papaya, normally present in the diet of BR population, are sources of carotenoids. A recent study showed that the bioavailability of β-carotene from papayas was approximately three times higher than that from carrots and tomatoes, generally assumed from European children (Schweiggert, Kopec et al. 2014). Thus, this finding could explain the enriched functional acquisition retinol metabolism of commensal bacteria in BR.

In BC metagenome, lipoic acid and biotin metabolism were enriched. Lipoic acid is covalently bound to specific proteins, which function as cofactors for several important mitochondrial enzyme complexes in antioxidant reactions. Biotin is a coenzyme for carboxylase enzymes, involved in the synthesis of fatty acids, isoleucine, and valine, and in gluconeogenesis.

In EU, functions related to porphyrin and chlorophyll metabolism, panthotenate and CoA metabolism, and thiamine metabolism were enriched. Porphyrins are essential cofactors of many proteins including cytochrome, haemoglobin and myoglobin. Porphyrin biosynthesis is known in proteobacteria and requires the formation of δ-aminolevulinic acid by the reaction of glycine with succinyl-CoA from the citric acid cycle.

Regarding thiamine, the best characterized form is thiamine pyrophosphate (TPP), a coenzyme involved in the catabolism of sugars and amino acids. Interestingly, thiamine metabolism is related to sulfur metabolism observed enriched in EU metagenome.

3.3.7 Lipid metabolism

Studies on animal models showed that commensal bacterial can regulate and maintain the lipid homeostasis. In gnotobiotic mice, bacterial species associated with a high-fat, high-sugar diet promote obesity (Turnbaugh, Ridaura et al. 2009). In mice living in a germ-free environment showed that they have lower fat mass compared to their conventionally reared counterparts, a phenotype that is associated with a diminished capacity for harvesting energy from ingested food and a broadly altered lipid profiles (Backhed, Ding et al. 2004;Turnbaugh, Ley et al. 2006). Moreover, alteration in lipid homeostasis in germfree mice can be partially rescued by colonization with human fecal content (Martin, Dumas et al. 2007).

Commensal bacteria might also regulate immune cell function through the fatty acidy sensor proteins in the peroxisome proliferator activated receptor (PPAR) family of nuclear receptors that recognize endogenous and exogenous lipid moieties (Odegaard, Ricardo-Gonzalez et al. 2007;Hong, Kidani et al. 2012).

Our analysis reveal differential functional acquisition related to lipid metabolism characterized the metagenome of the four studied popolations. In BR we found enrichment in arachidonic acid, fatty acid biosynthesis and lipid biosynthesis proteins (Fig. 3.9C). In

particular, bacterial biosynthesis of arachidonic acid could be involved either for phospholipids of cell membranes building or as key inflammatory intermediate.
In BC we observed enrichment in primary and secondary bile acid biosynthesis, and linoleic metabolism, an essential polyunsaturated fatty acid involved in the biosynthesis of arachidonic acid (Fig. 3.9C). Interestingly, commensal bacteria deconjugate bile acids, synthesized in the liver from cholesterol-derived precursor molecules, and convert primary into secondary bile acids (Brestoff and Artis 2013). Synthesis of bile acids is one of the predominant mechanisms for the excretion of excess cholesterol, and is involved in intestinal absorption of fat-soluble vitamins. In addition, bile acids act as signaling molecules that regulate metabolic and immune cell homeostasis.
In EU we found an enrichment in glycerophospholipid and fatty acid metabolism and synthesis and degradation of ketone bodies (Fig. 3.9C), clearly derived from lipid rich diet. In particular, synthesis and degradation of ketone bodies could be derived from glucose production from non-carbohydrate sources, and it could explain the observed enrichment in butanoate metabolism.

3.3.8 Metabolism of terpenoids and polyketides, secondary metabolism, and antibiotic biosynthesis and resistance

A wide range of natural compounds are produced by human-associated bacteria (Donia and Fischbach 2015). These molecules cover the entire spectrum of chemical classes that have been isolated from terrestrial and aquatic bacterial species and include well-characterized mediators of microbe-host and microbe-microbe interactions.
Terpenoids are a subclass of the prenyllipids (terpenes, prenylquinones, and sterols). The major microbiota-derived terpenoids are not synthesized *de novo* by the microbiota, but they are derivatives of the host-derived primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA). CA and CDCA are biosynthesized in the human liver, conjugated to taurine or glycine, and then excreted in bile. Numerous biochemical transformations of CA and CDCA are performed by gut bacteria, including deconjugation from taurine and glycine, oxidation, epimerization and esterification.
Alkaloids are biosynthetized principally by aminoacids, while polyphenols are formed by malonate/acetate pathway. Polyketides are large classes of natural organic compounds produced by living organisms (soil and aquatic bacteria) in order to give survival advantage. The biosyntheses of polyketides share striking similarities with fatty acid biosynthesis, and many of the commonly used antibiotics, such as tetracycline and macrolides, are produced by polyketide synthases.
In BR microbiome we observed enriched functions related to isoquinoline alkaloid biosynthesis, tropane, piperidine and pyridine alkaloid biosynthesis, prenyl transferases, zeatin and siderophore biosynthesis. These functions are certainly related to a high consumption of plant derived foods. In BC, we found enrichment in phenylpropanoid and carotenoid biosynthesis. On the contrary, in EU we found an enrichment in flavone and flavonol biosynthesis, stilbenoid diarylheptanoil and gingerol biosynthesis, polyketides sugar unit biosynthesis.
Then, we focused on functional acquisitions related to antibiotic biosynthesis and resistance.

In the four populations, we observed differential and progressively increased antibiotic biosynthesis functions, passing from rural to urban populations. In particular we found acquired streptomycins biosynthesis function in BR metagenome, novobiocin biosynthesis in BT, butirosine, neomycin and ansamycin biosynthesis in BC, and beta-lactamase resistance, penicillin and cephalosporin biosynthesis, and tetracyclin biosynthesis in EU (Fig. 3.9E-F). In natural environment, antibiotics production is a mechanism of competition among bacteria that provide a selective benefit for the producing strain. The most of the antibiotics derived from biomolecules and secondary metabolites produced by soil-dwelling microorganisms (Davies and Davies 2010), and microbiota of rural populations could acquired this potential for the unique scope of survival and competition among bacteria. The observed beta-lactamase resistance and the cephalosporin biosynthesis acquisitions in EU metagenome confirmed that the use of antibiotics in agriculture, in livestock and medical practice could induce functional acquisitions related to antibiotic resistance, an emerging and dramatic problem in industrialized and globalized populations.

3.3.9 Xenobiotics metabolism

In BR metagenome, we found enrichment in toluene, naftalene and ethylbenzene degradation (Fig. 3.9H). This pathway referred to aromatic organic compounds metabolism that include several compounds containing benzene ring, including phenolic compounds. As observed in a recent study (Barelli, Albanese et al. 2015) on functional acquisitions related to xenobiotic degradation by microbiota of red colobus monkeys eating several plant species in the forest of Tanzania, we could hypothesize that the presence of widespread plants and vegetables rich in tannins and phenolic compounds in rural village of Burkina Faso, require metabolic acquisition to digest xenobiotics by commensal bacteria, rather than acquired functions related to environmental contaminant degradation.

In urbanized BC and EU metagenomes, chloralkene, bisphenol, dioxin and xylene degradation and benzoate and 1,1,1-Trichloro-2,2,bis-4-chlorophenyl-ethane-DDT degradation were enriched functions, respectively (Fig. 3.9H). The degradation of these xenobiotics by microbiota of these urban populations can be considered a functional response of bacterial to exposure of toxic compounds derived by pollution (especially chloroalkene and dioxin) of industrialized and urban environment. Among benzoate compounds, sodium benzoate, a food additive with preserving role, known as E211, is most widely used in acidic foods, carbonated drinks, jams and fruit juices. Furthermore, several chlorinated organic insecticide are persistent contaminant in the environment and potentially in cultivated foods.

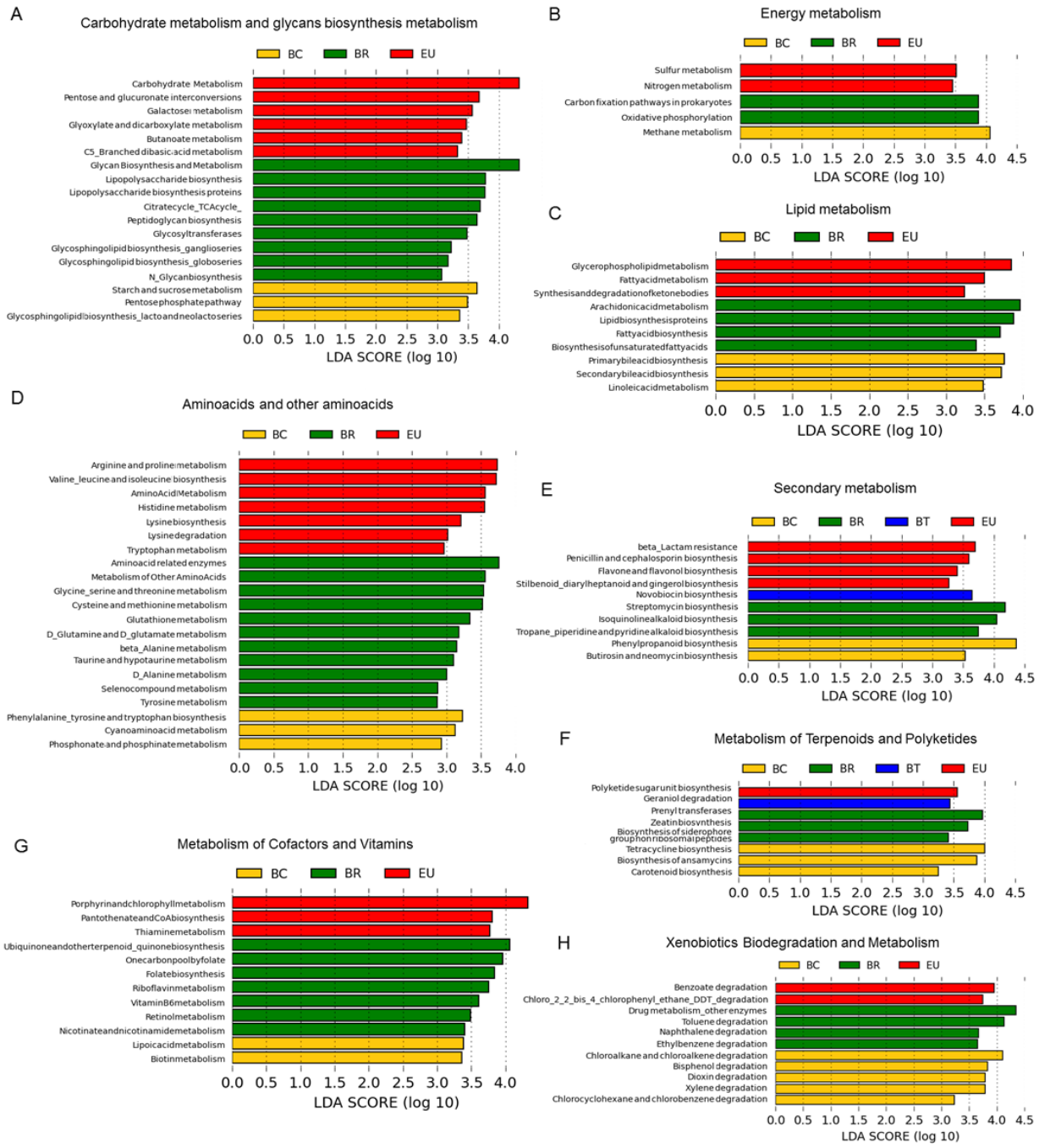

Fig. 3.9 Differences in bacterial functional classes (KEGG categories level 3). Functional pathways significantly enriched in African and European populations based on PICRUSt prediction. LEfSe results indicate a sequentially significant ranking among populations (Alpha value=0.05 for the factorial Kruskal-Wallis test among classes). The threshold for the logarithmic LDA score was 2.0.

3.4 Conclusions

Studies on traditional and culturally diverse populations living in isolation from the globalized world, and characterization of gut microbiota across geography allowed to observe relationships between environmental factors, lifestyle and intestinal microbial profiles, and to evaluate the effect of variables that correlate with microbiota diversity, such as diet, environmental exposures, and sanitation. The modification of microbiota composition that occurs in children of the same African ethnic group that moves from rural

villages to small town and then to the capital city of Burkina Faso provide useful insights on the role of the change of dietary habits and environment taking place with improvement of socio-economic resources of the family. These observations may be considered as a model of the changes that occur in human societies with the urbanization, until to reach modern societies.

Across the history, from Paleolithic to Neolithic era, humans change their lifestyle from nomadic hunter-gatherer to sedentary populations dedicated to agriculture and to food production enough to feed themselves and their families. This life-style emerged during the Neolithic revolution and continues in large parts of rural Africa, Asia and Latin America, whereas had largely disappeared in Europa and North America. Subsequently, adjacent to rural villages, humans developed small towns, where economic activity consisted primarily of trade at markets and manufacturers on a small scale. Boulpon, Nanoro and Ouagadogu represent three steps of human transition to urban environment, and the consequent dietary differentiation.

The transition from rural to urban area of populations from the same ethnic group and the same geographic region represent another piece of a puzzle, useful to complete the frame of co-evolution among diet, gut microbiota and host. Studying in deep the modification of dietary habits of an African ethnic group moving from a rural to an urban environment, we observed a gradual enrichment in food variety, with increase in animal-derived products (meat, fish and milk-derived food), processed and refined foods, and consequent increase in fat and animal protein and reduction in fiber intake. By using a metagenomics approach, we discovered key microbial markers that have remained relatively unaltered over hundreds of generations, and are reliable indicators of ancient migratory patterns, as well as microbial profiles loss in the course of urbanization, industrialization and westernization.

Interestingly, microbial profiles found enriched in microbiota of westernized populations were found associated to IBD and other autoimmune diseases, or metabolic disorders. These findings could explain the association between microbial profiles and the developing of health disorders typical of the western world, especially with early-onset. Westernization has been consistently associated with lower alpha diversity (microbial richness) and higher beta diversity (intra-individual diversity). Microbial dispersal due to environmental exposition and transmission in rural populations play a fundamental role in structuring the gut microbiome. Urbanization and westernization promote limitation in microbial dispersal due to sanitation (hygiene), medication (antibiotics overuse), together with a diet poor in fiber and rich in fat and animal-protein based products, leading to the loss of bacterial lineages able to ferment complex carbohydrates and produce the well-known anti-inflammatories SCFAs. However, we should not forget that rural children either for loss of sanitation and scarcity of food are at high risk of infectious diseases and malnutrition.

On the other hand, the importance to preserve an ancient microbiome and maintain biodiversity has considerable repercussions for human health, especially in industrialized and westernized populations, in which noncomunicable diseases are spread. Our study showed properly the gradual disappear of ancient microbes during the transition from rural to urban environment, and suggest the importance to develop strategies to preserve microbial functional acquisitions that have been destroyed in the course of urbanization of human populations.

4. Phenotype and disease course of early-onset pediatric inflammatory bowel disease

This chapter has been reprinted* from:

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Phenotype and disease course of early-onset pediatric inflammatory bowel disease.

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4.1. Scientific Background

The inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are complex polygenic disorders, resulting of an interplay between genetic, environmental, and immunological factors leading to a dysregulated immune response of the host to the intestinal microbiota (Aujnarain, Mack et al. 2013;Fiocchi 2013). Up to 25% of patients first present during childhood or adolescence, with early-onset (EO) (0–5 yr) and very early-onset (VEO) diseases (0–2 yr) increasingly reported (Marx, Seidman et al. 2002;Gupta, Bostrom et al. 2008;Nieuwenhuis and Escher 2008;Maisawa, Sasaki et al. 2013). Although data on EO forms are scattered, they seem to be characterized by a unique phenotype, extensive and aggressive, which affect the management of this age group (Gupta, Bostrom et al. 2008). Moreover, prior reports suggest that EO-IBD differ epidemiologically and are distinguished by a predominant colonic involvement and a greater impact of genetic predisposition, compared with older age of onset (Ravikumara and Sandhu 2006;Sauer and Kugathasan 2010). From this point of view, EO disease represents an exceptional model to study the initial host immune response to characterize genotype–phenotype relations and identify environmental factors influencing disease development, as well as to evaluate the natural history of the disease(Oliva-Hemker and Fiocchi 2002;Heyman, Kirschner et al. 2005;Scherr, Essers et al. 2009). Understanding the factors that contribute to early age of onset could potentially facilitate intervention strategy development, thereby influencing disease outcome and risk of long-term sequelae. Recently, an increased interest for early forms of disease came from the discovery of Crohn's-like colitis, very severe and not responding to conventional therapies, associated with a defect of the receptor of interleukin 10 (IL-10R) or IL-10 itself, in a subset of patients developing the disease within the first months of life (Glocker, Kotlarz et al. 2009;Begue, Verdier et al. 2011). Such severity and EO point to probable monogenetic diseases as opposed to the multigenetic trait observed in

the known forms of IBD. This discovery opens new horizons in the knowledge of the pathogenesis of the disease; hence, a better clinical definition of early and very early forms of disease may lay the foundation for further studies of genotype–phenotype correlation. Thus, primary aim of this study was to evaluate the phenotype and course of EO-IBD (0–5 yr) compared with pediatric later-onset disease (6–11 and 12–18 yr). Moreover, as a secondary outcome, within the 0- to 5-year group, we evaluated potential differences in presentation and behavior of VEO forms of the disease (0–2 yr) than those presenting between 3 and 5 years.

4.2 Materials and Methods

4.2.1 Patients and clinical features

Italian pediatric IBD centers belonging to the Pediatric Gastroenterology, Hepatology and Nutrition Italian Society in 2008 established a prospective registry to collect demographic, clinical, and epidemiologic data from pediatric patients with IBD. The registry started at January 1, 2009 and included patients less than 18 years with a new diagnosis of IBD. Data of all children enrolled and stored in the registry from January 1, 2009 to April 22, 2013 (the data retrieval date) were used for this study. Fourteen Italian sites of Gastronterology Unit participated to this study, including the Gastroenterology unit of Meyer Children Hospital. Institutional review board approval for the registry protocol and the informed consent and assent forms were obtained at each site before subject enrollment and data collection. Signed parental and patient informed consent and signed youth assent, when appropriate, were required from all patients enrolled. Trained investigators at each center obtained information from the medical records (electronic and paper charts). Standardized information was entered into the registry. Data were submitted quarterly to the central repository at Pediatric Gastroenterology and Liver Unit, Sapienza University of Rome for scrutiny of data quality, maintenance of data integrity, data storage, and subsequent data analysis. The study end date was defined as the date of the most recent clinic visit before April 22, 2013.

Eligible subjects included all patients with any form of IBD (UC, CD, and inflammatory bowel disease unclassified [IBDU]) who were diagnosed before 18 years of age and followed in the pediatric gastroenterology centers at the participating sites. Only new IBD cases, diagnosed after January 1, 2009 were prospectively enrolled. Diagnosis of IBD was based on clinical history, physical examination, endoscopic appearance, histologic findings, and radiologic studies, according to Porto criteria (North American Society for Pediatric Gastroenterology, Nutrition et al. 2007). The latter implied that ileocolonoscopy and upper gastrointestinal endoscopy were assumed to be performed in all patients, as well as imaging of the small bowel (except in patients with a definitive diagnosis of UC). Participating centers were also expected to take at least 2 biopsies from each segment of the gastrointestinal tract (esophagus, stomach, duodenum, terminal ileum, and all segments of the colon) and to record the endoscopic and histologic findings from each segment separately.

Allergic disorders (immunoglobulin E, skin prick, and patch tests) and infectious enteritis or colitis (*Salmonella*, *Shigella*, *Escherichia coli*, *Yersinia*, *Clostridium difficile* toxins, *Entamoeba histolytica* stool samples) were excluded during the initial work-up.

Immunological defects were also ruled out in all patients with suspected EO-IBD by routinely testing immunoglobulin levels, autoantibodies, lymphocyte phenotyping, granulocytes function, and oxidative burst. Mutations in the IL-10R and IL-10 genes were also evaluated in all children with VEO forms of IBD (<2 yr). Only patients with a final diagnosis of IBD with no other immunological (including those with confirmed IL-10R or IL-10 axis mutations), allergic, or infectious diseases were included in the registry. If the length of follow-up was less than 6 months, the data were excluded from the analysis. The information retrieved for the purpose of this study included demographic features (age, sex), family history of IBD, IBD type (CD, UC, IBDU), and disease distribution. A family history for IBD was defined by the presence of CD or UC in first-degree relatives only.

EO-IBD was defined as a diagnosis made in patients of 0 to 5 years of age. Within this group, VEO-IBD was defined as a diagnosis of IBD made between 0 and 2 years of age.

The disease location at the diagnosis and at follow-up was established by endoscopic and imaging evaluations in all patients according to the availability of individual methods for each center and reported in the registry. Disease location was described according to Paris classification (Levine, Griffiths et al. 2011). For CD, L1 was defined as an involvement of the terminal ileum and limited cecal disease, L2 an isolated colitis, L3 an ileocolonic disease, L4a an upper disease proximal to Ligament of Treitz, whereas L4b distal to ligament of Treitz and proximal to distal 1/3 ileum. For UC, proctitis (E1) was defined as an involvement limited to the rectum (i.e., proximal extent of inflammation distal to the rectosigmoid junction). Left sided UC (E2) was defined as an involvement limited to the portion of the colorectum distal to the splenic flexure. Extensive UC (E3) was defined as a disease extending proximally to the splenic flexure but distally to the hepatic flexure, whereas pancolitis (E4) included a colitis extended proximally to the hepatic flexure.

Symptoms at onset of disease included abdominal pain, diarrhea, rectal bleeding, perianal disease, and growth failure.

Disease activity at the diagnosis was scored by the Pediatric Crohn's Disease Activity Index (PCDAI)(Hyams, Ferry et al. 1991) or the Pediatric Ulcerative Colitis Activity Index (PUCAI)(Turner, Otley et al. 2007) for CD and UC, respectively. Laboratory tests included full blood count, C-reactive protein, erythrocyte sedimentation rate, perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA), anti-Saccharomyces cerevisiae antibodies (ASCA), nutritional, renal and liver function parameters. Endoscopic and imaging examinations carried out at the diagnosis and follow-up included ileocolonoscopy, esophagogastroduodenoscopy, enteroscopy, capsule endoscopy ultrasound with or without oral contrast, and magnetic resonance imaging.

Medications comprised 6-mercaptopurine (6-MP)/azathioprine, methotrexate, thalidomide, cyclosporine, biological therapy (infliximab or adalimumab), nutritional therapy, and corticosteroids (oral [budesonide, prednisone, prednisolone] and intravenous [methylprednisolone]). Surgical procedures included all intestinal resections.

Extraintestinal manifestations (EIMs) included skin, joint, and ocular manifestations; pancreatitis; osteopenia; primary sclerosing cholangitis; and growth failure.

Patient data were updated every 6 months, by entering in the registry every evaluation made by patients. Every 6 months, clinical data, PUCAI/PCDAI, medical and surgical therapy,

laboratory tests (those performed at diagnosis), imaging and/or endoscopic evaluation, and complications of the disease, were reported.
For the purpose of this study, all previous information were analyzed and compared in all patient populations. As a primary outcome of the study, recorded data of patients 0 to 5 years (EO) were compared with those with a later onset, divided in 2 groups (6–11 and 12–18 yr). As a secondary outcome, we evaluated within the population with EO-IBD the phenotype and natural history of VEO (0–2 yr) forms compared with those diagnosed in children aged 3 to 5 years.

4.2.2 Statistical methods

All data were summarized and displayed as the mean 6 SD for the continuous variables. Categorical data were expressed as frequencies and percentages. Comparison of groups was performed using Student's t test for unpaired data in two-group comparison and one-way analysis of variance with Bonferroni's test for multiple group comparison. Chi-square test with Fisher's correction was used to evaluate the differences for categorical variables wherever needed. A p-value of 0.05 or less was considered significant. Odds ratios from univariate logistic regression models were used to assess differences by age group in the prevalence of symptoms at disease onset and laboratory values at diagnosis. The Kaplan–Meier survival method was used to estimate the interval free from surgery during follow-up. Differences between curves were tested using the Log-Rank test. The GraphPad statistical package was used to perform all statistical evaluations (GraphPad Software, Inc., San Diego, CA).

4.3 Results

4.3.1 IBD subjects

From 2009 to 2013, we identified 688 patients with a diagnosis of IBD, 182 had a length of follow-up less than 6 months and were excluded from the analysis. Five hundred six children met the inclusion criteria and were enrolled in the study. Fifty-four percent were males and mean age at the diagnosis was 10.2 years (range, 0.8–18.3). Mean follow-up was 40 months (range, 6–50). Eleven percent (n=54) of patients were in the range 0 to 5 years of age, 39% (n=197) in 6 to 11 years, and 50% (n=255) in 12 to 18 years (Fig. 4.1). Table 4.1 highlights the main clinical characteristics of 506 enrolled patients.

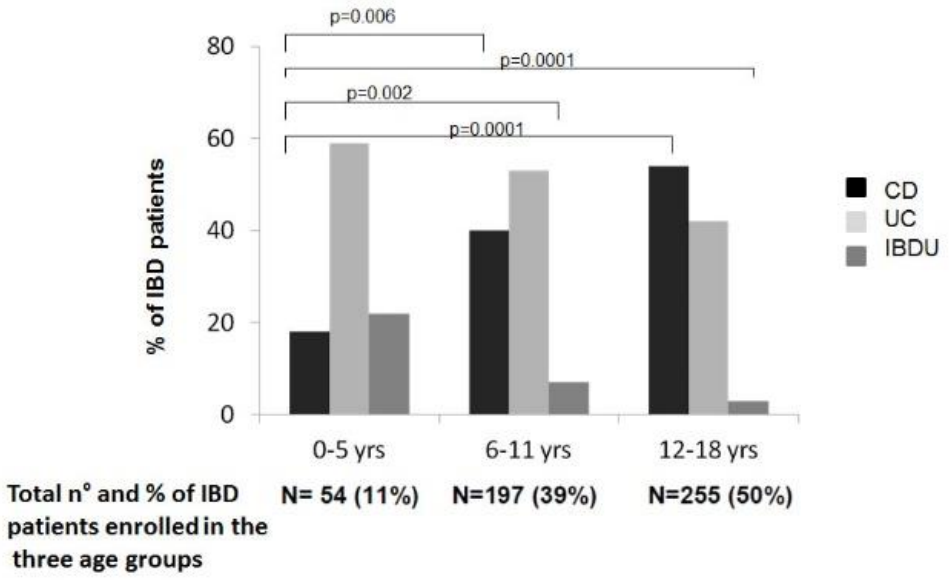

Fig. 4.1 Frequency of CD, UC and IBDU considering the different age range (0-5 years; 6-11 years; 12-18 years).

Table 4.1 Clinical Characteristics of 506 Children with IBD

	Age 0–5 yr, n = 54 (11%)	Age 6–11 yr, n = 197 (39%)	Age 12–18 yr, n = 255 (50%)	*P*
Sex (males)	23 (42%)	82 (41%)	170 (67%)	0.94[a]; <0.001[b]
Follow-up (range), mo	44 (11–50)	40 (6–48)	36 (6–46)	0.26[a]; 0.08[b]
Diagnostic delay (>6 mo)	16 (29%)	52 (26%)	72 (28%)	0.72[a]; 0.86[b]
Diagnosis				
CD	10 (18%)	78 (40%)	137 (54%)	0.006[a]; <0.0001[b]
UC	32 (59%)	105 (53%)	108 (42.5%)	0.56[a]; 0.03[b]
IBDU	12 (22%)	14 (7%)	9 (3.5%)	0.002[a]; <0.0001[b]
Family history for IBD	6 (11%)	30 (15%)	29 (11%)	0.58[a]; 0.94[b]
Disease activity (at the diagnosis)				
PUCAI (mean ± SD)	30.7 ± 17.2	33.8 ± 19.5	36.4 ± 18.1	0.54[a]; 0.52[b]
PCDAI (mean ± SD)	25 ± 6.3	21.6 ± 13.4	21.3 ± 14.3	0.53[a]; 0.23[b]

[a]0 to 5 versus 6 to 11 years.
[b]0 to 5 versus 12 to 18 years.

4.3.2 Initial classification and location of the disease

All patients had a complete ileocolonoscopy under general anesthesia or deep sedation at the diagnosis. Fifty-five percent had an esophago-gastroduodenoscopy and 48% also had an evaluation of the small-bowel (78% CD, 21% UC): of them, 52% underwent ultrasound, 43% magnetic resonance imaging, 17% capsule endoscopy, and 6% enteroscopy. Thirty-seven percent of patients had 2 or more methods for imaging the small bowel. At the diagnosis, 224 were classified as CD (44%), 245 as UC (48%), and 37 as IBDU (7%). UC was the most frequent diagnosis in EO-IBD (59%) and in 6- to 11-year-old group (53%), compared with children of 12 to 18 years of age (42.5%) (p=0.03 versus 0- to 5-yr group), whereas CD was predominant in the latter (p=0.0001 versus 0- to 5-yr group). A classification as IBDU was more common in the range 0 to 5 years (22%) compared with the other groups (7% in 6- to 11-yr and 3.5% in 12- to 18-yr group; p=0.002 and p=0.0001,

respectively; Fig. 5.1). Fifty percent of children with EO-CD presented an isolated colonic disease (L2) compared with 14% in 6- to 11-year and 16% in 12- to 18-year group (P=0.005). No significant differences were found for the other CD locations among the 3 groups, although an isolated ileal disease (L1) was rare in 0- to 5-year group (10% versus 23% in 6–11 yr and 52% in 12–18 yr), and an upper gastrointestinal involvement was more frequent in younger children (30% versus 18% in 6–11 yr and 16% in 12–18 yr). Figure 4.2 shows the CD location in all groups. Among patients with UC, 62% of patients in the 0- to 5-year range had a pancolitis, compared with 38% of 6- to 11-year (p=0.02) and 28% of 12- to 18-year range (p=0.002). No other significant differences were found for the other UC location among the three groups (Fig. 4.3).

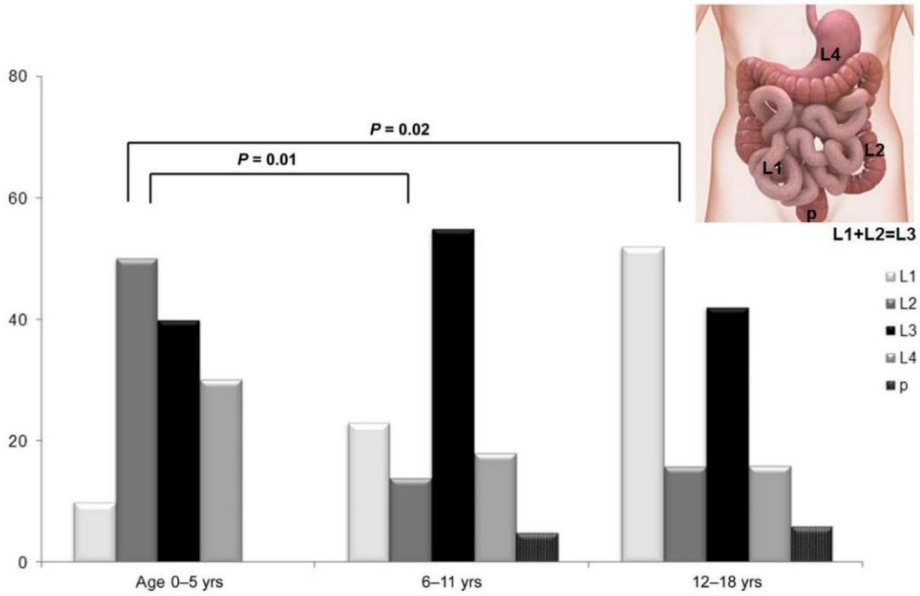

Fig. 4.2 Disease location at the diagnosis according to Paris classification in EO-CD (0–5 yr) and later-onset CD (6–11 and 12–18 yr). L1: ileum, L2: colon; L3: ileocolon; L4: upper gastrointestinal; p: perianal disease.

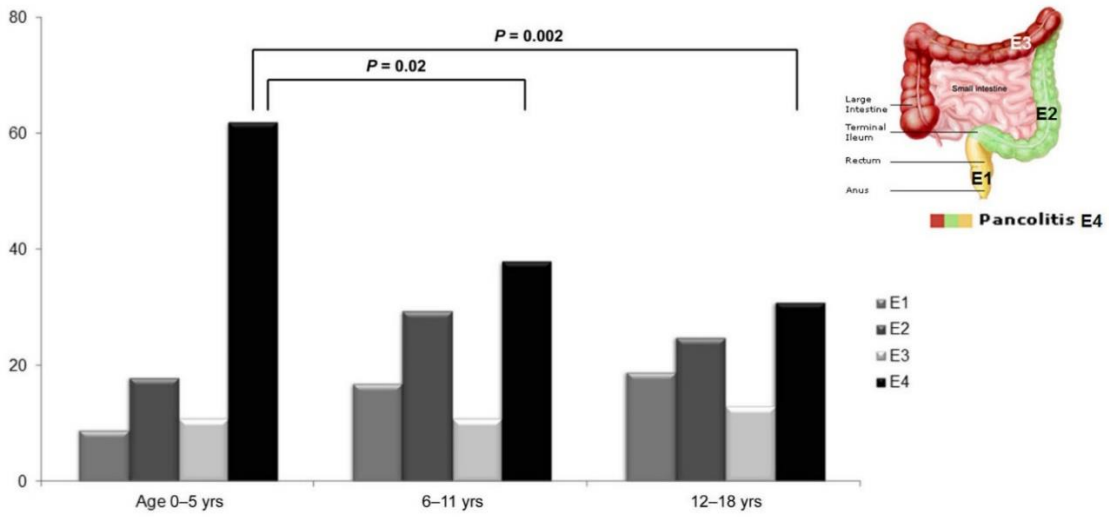

Fig. 4.3 Disease location at the diagnosis according to Paris classification in EO-UC (0–5 yr) and later-onset UC (6–11 and 12–18 yr). E1: proctitis; E2: left-sided colitis; E3: extensive colitis; E4: pancolitis.

4.3.3 Classification and Disease Extent at Follow-up

At follow-up, a change in diagnosis was reported in 2% of all patients: 40% of children in 0- to 5-year group received a change of the initial diagnosis during the follow-up, 50% of 6 to 11 years, and 10% of 12–18 years (p=0.003, 0–5 versus 12–18 yr). Among children with EO disease initially classified as IBDU, 3 (25%) were finally diagnosed as UC, 1 (8%) as CD, whereas 67% continued to be classified as IBDU. No significant differences were found for disease extent at 40-month follow-up, although a higher proportion of children with EO-CD presented an extension of the disease involving ileum and colon (L3) at the last evaluation compared with the diagnosis (60% versus 40%; p=0.65). We did not find significant changes in the CD and UC location at follow-up in older age groups.

4.3.4 Clinical Presentation

Table 5.2 shows the clinical presentation in the three age groups. EIMs were detected in 28% of patients at the diagnosis: skin involvement (n=45), axial arthropathies (n=10), peripheral arthritis/arthralgia (n=50), pancreatic involvement (n=5), and osteopenia (n=4). Growth failure at diagnosis was reported in 22% of children. No significant differences were found for the prevalence of EIMs among the three age groups. Other clinical variables at the diagnosis, such as PCDAI or PUCAI, hemoglobin, white blood cell count, erythrocyte sedimentation rate, C-reactive protein, hypoalbuminemia, positive perinuclear antineutrophil cytoplasmic antibodies, and anti-*S. cerevisiae* antibodies did not differ by age group.

4.3.5 Medical and Surgical Treatment

Fifty-seven percent of patients started corticosteroids, 35% azathioprine, 4% methotrexate, 16% nutritional therapy, 5% biologics, 2% thalidomide, and 2% cyclosporine. At the diagnosis, no significant differences were found among the three groups in the corticosteroid need, although at the latest follow-up, a significantly higher proportion of patients with EO-IBD were under steroids compared with 12- to 18-year group (p<0.05). The use of other therapies at diagnosis and follow-up (i.e., nutritional therapy, immunomodulators, and biologics) did not significantly differ among the three groups.

Table 4.2 Prevalence of Clinical Symptoms at Presentation

	0–5 yr, n = 54 (%)	6–11 yr, n = 197 (%)	Odds Ratio,[a] (95% CI)	*P*	12–18 yr, n = 254 (%)	Odds Ratio,[a] (95% CI)	*P*
Abdominal pain	27 (50)	139 (70)	0.41 (0.22–0.77)	0.005	147 (58)	0.72 (0.40–1.3)	0.29
Rectal bleeding	39 (72)	109 (55)	2.09 (1.0–4.0)	0.02	127 (50)	2.09 (1.3–4.9)	0.004
Diarrhea	33 (61)	108 (56)	1.2 (0.7–2.3)	0.44	139 (55)	1.3 (0.7–2.3)	0.45
Growth failure	9 (17)	49 (24)	0.60 (0.2–1.3)	0.27	62 (24)	0.61 (0.28–1.3)	0.28
Perianal disease	6 (11)	26 (13)	0.80 (0.3–2.0)	0.81	39 (15)	0.67 (0.27–1.6)	0.52

[a]The 0- to 5-year group is the reference group.

Figures 4.4 and 4.5 show the different therapies underwent by patients. Sixty patients underwent surgery at least once between diagnosis and last follow-up, resulting in a crude surgical rate of 12%.
No significant differences for surgical risk were reported among the three age groups (Fig. 4.6).

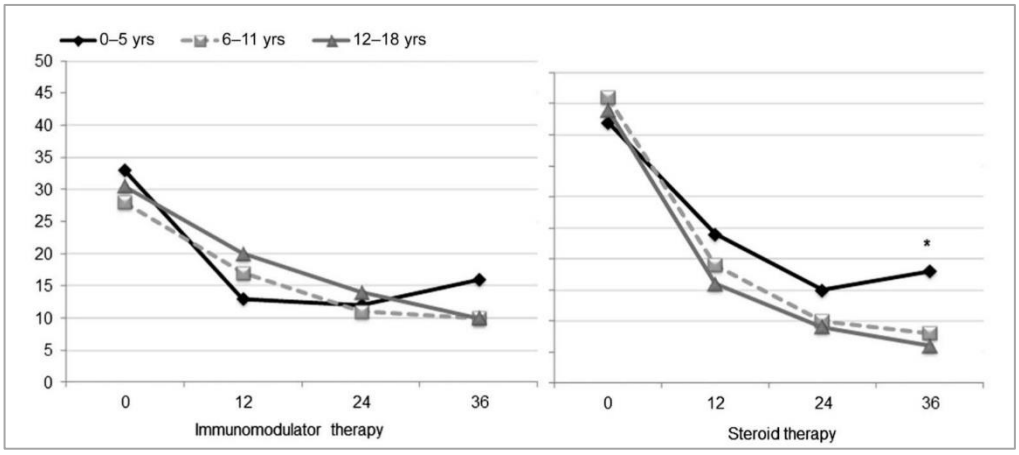

Fig. 4.4 Immunomodulator and steroid therapy at diagnosis and follow-up in 0- to 5-year, 6- to 11-year, and 12- to 18-year group. *p<0.05, 0- to 5-year versus 12- to 18-year group.

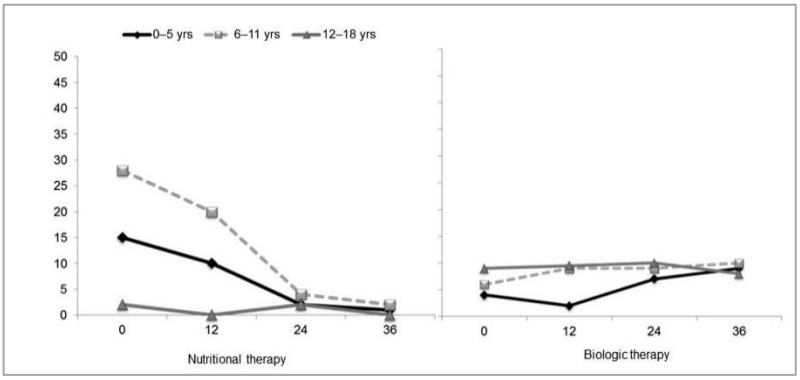

Fig. 4.5 Nutritional and biological therapy at diagnosis and follow-up in 0- to 5-year, 6- to 11-year, and 12- to 18-year group.

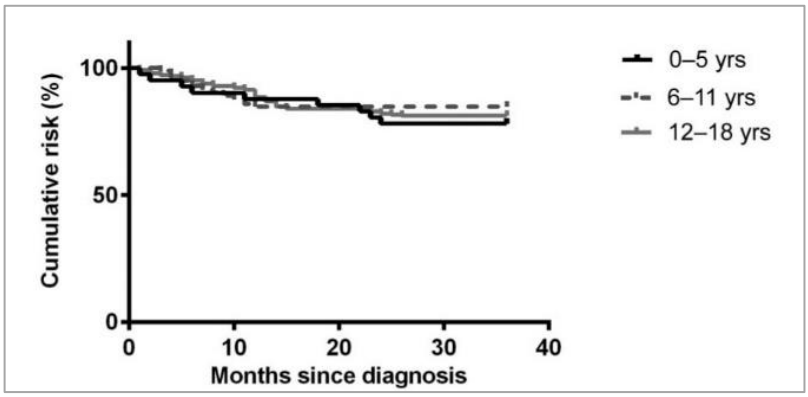

Fig 4.6 Cumulative risk of surgery in 506 children with IBD according to the age at diagnosis.

4.3.6 Subgroup Analysis of VEO-IBD

Finally, among children with EO-IBD, we made a subgroup analysis evaluating potential peculiarities in presentation and disease course of very early forms (0–2 yr) compared with diseases presenting in children of 3 to 5 years of age. The diagnosis of UC was the most common in both groups, although numerically higher in 3 to 5 years (68% versus 40%). Both groups were characterized by an extensive disease at the diagnosis, pancolonic involvement in UC, and an isolated colitis for CD. Upper gastrointestinal involvement was more common in children of 3 to 5 years than the very younger, although the difference was not significant. Other variables at the diagnosis and at follow-up, i.e., sex, family history for IBD, delay of the diagnosis (>6 months), EIMs, mean PUCAI and PCDAI at the diagnosis, C-reactive protein, and perinuclear antineutrophil cytoplasmic antibodies at the diagnosis and changes of the diagnosis at follow-up did not differ between the 2 groups (Table 4.3).

Table 4.3 Clinical Characteristics of EO- and VEO-IBD

	0–2 yr, n = 20	3–5 yr, n = 34	*P*
Sex (M)	12 (60%)	18 (53%)	0.77
Family history for IBD (first-degree relatives)	2 (10%)	4 (12%)	0.89
Delay of the diagnosis (>6 mo)	10 (50%)	11 (32%)	0.25
Diagnosis			
IBDU	6 (30%)	4 (12%)	0.14
UC	8 (40%)	23 (68%)	0.08
CD	6 (30%)	7 (20%)	0.51
Location of UC at diagnosis (Paris classification)			
E1	1 (12%)	0	0.25
E2	0	4 (17%)	0.54
E3	1 (12%)	3 (13%)	0.98
E4	6 (75%)	16 (70%)	0.95
Location of CD at diagnosis (Paris classification)			
L1	1 (17%)	0	1.0
L2	3 (50%)	1 (17%)	0.54
L3	2 (33%)	4 (66%)	0.56
L4	1 (17%)	3 (50%)	0.54
Changes of the diagnosis at follow-up	2 (10%)	2 (6%)	0.67
PUCAI (mean ± SD)	35.5 ± 12.4	38.4 ± 12.4	0.72
PCDAI (mean ± SD)	32.4 ± 14.9	34.7 ± 11.25	0.76
CRP (mean ± SD)	4.8 ± 4.7	5.7 ± 9.1	0.74
EIMs at diagnosis	1 (5%)	3 (9%)	1.0
p-ANCA	4 (13%)	7 (20%)	0.46
Surgery at follow-up	2 (10%)	3 (9%)	1.0

CRP, C-reactive protein; pANCA, perinuclear antineutrophil cytoplasmic antibodies.

4.4 Discussion

Reports of pediatric IBD have been published for several years (Sawczenko and Sandhu 2003;Griffiths 2004;Paul, Birnbaum et al. 2006). Recently, evidence for increased incidence of IBD in young children and the identification of early forms of severe disease have represented a significant step forward the understanding of the pathogenesis underlying IBD, determining renewed interest in the younger age group (Cannioto, Berti et al. 2009;Glocker, Kotlarz et al. 2009;Begue, Verdier et al. 2011;Shah, Kammermeier et al. 2012). The most relevant discovery in this field has been the recognition of forms of Crohn's-like colitis with perianal involvement and onset in the first months of life, associated with a defect of IL-10R

or IL-10 itself (Glocker, Kotlarz et al. 2009;Begue, Verdier et al. 2011). These diseases present a Mendelian pattern of heritability, differently to the multigenetic trait observed in the known forms of IBD, adding them to the group of primary immunodeficiencies resulting in severe dysregulation of the intestinal immune system (Shah, Kammermeier et al. 2012). For this reason, we did not included these forms in the registry and in this study. Our study not only adds new data on the peculiarities of EO-IBD, in terms of phenotype and disease course, but also represents a full application of the Paris classification of IBD in a large population of children. Detailed follow-up of 506 pediatric patients followed in the main tertiary referral centers for IBD in Italy has allowed us to evaluate these findings, particularly the initial clinical presentation and the disease progression. Data were collected by a structured prospective registry, including all newly diagnosed pediatric IBD patients in Italy from January 1, 2009. Our results confirm that the phenotype of pediatric IBD patients differs according to age, and that the EO disease is extensive and severe already at the presentation. Clinical data on the EO-IBD are scarce because this age group represents only a small portion of the pediatric population. Griffiths (Griffiths 2004) in a cohort of children with IBD (n=503) found 4% to be less than 5 years at diagnosis. More recently, Gupta et al (5) presented their results in 600 children with CD: 9% of them were 0 to 5 years old at the time of diagnosis. In our cohort, 11% (n=54) were less than 5 years at the time of diagnosis and 4% less than 2 years. Among the younger children, the diagnosis of UC was the most common, although a significant number of patients received a diagnosis of IBDU (22%). These data are similar to those reported in 2002 by Mamula et al. (Mamula, Telega et al. 2002) showing an indeterminate colitis in 23% of 82 children with EO-IBD. Strikingly, many of our children with IBDU maintained this diagnosis during the follow-up, suggesting the strain of making a definitive diagnosis in younger children. During the course of the disease, diagnosis changes occurred more frequently in younger than older children, although the difference was not significant. We cannot exclude that the small number of younger children has influenced the statistical significance of this result. Overall, our population of childhood-onset IBD was characterized by a widespread anatomical involvement in all age groups. CD presented with an extensive phenotype, regardless of the age at the diagnosis. EO-CD was characterized by significantly higher rates of isolated colonic CD, compared with older children. These data are consistent with that reported by Heyman et al (Heyman, Kirschner et al. 2005) from the North American Pediatric IBD Consortium Registry. In 1370 children, the prevalence of isolated colonic disease was significantly higher in younger (less than 8 yr of age) compared with older children. Paul et al (Paul, Birnbaum et al. 2006) reported data from 413 children with IBD and compared children presenting before and after the age of 5 years. In the EO group, 76% had an isolated colonic disease, 34% ileocolonic, whereas 0% had ileal involvement only. Conversely, older children mainly presented with ileocolonic involvement (48%). Also in our cohort, the proportion of patients with an ileocolonic involvement at the diagnosis was higher in older children. We also found that one-third of younger patients presented an upper gastrointestinal involvement already at the diagnosis. This result is remarkable because, to our knowledge, there are no previous studies on EO-IBD indicating the involvement of the upper gastrointestinal tract in this age group. Our study, by using the Paris classification, allowed us to accurately define this finding. Overall, 6% of our pediatric population

presented lesions of the upper gastrointestinal segment. This figure is close to prior reports in children (Vernier-Massouille, Balde et al. 2008;de Bie, Paerregaard et al. 2013). More than half of our patients underwent an upper and lower endoscopy at the diagnosis, and almost half also small-bowel imaging. The wide range of investigation may explain the high prevalence of upper gastrointestinal involvement. Nevertheless, our analysis based on the Paris classification, seem to confirm the significant differences between CD location in pediatric-onset, notably EO disease and adult onset CD (Gower-Rousseau, Vasseur et al. 2013). Overall, an extensive disease location was also demonstrated for pediatric UC, although the proportion of patients having a pancolitis already at the diagnosis was significantly higher in younger than older children. Our study not only is consistent with previous data indicating a widespread extension of pediatric UC (Van Limbergen, Russell et al. 2008;Aloi, D'Arcangelo et al. 2013;Levine, de Bie et al. 2013), but directly compares early- with later-onset cohort in the same population of pediatric patients (62% of younger children presented a pancolitis at the diagnosis compared with 38% in older group). We did not find a high overall rate of disease extension at follow-up; thus at the end of the study, the disease extent was similar to that at the diagnosis. These data are, somewhat at variance with other pediatric studies, suggesting a high rate of disease extension in pediatric UC and CD (Van Limbergen, Russell et al. 2008). One explanation could be the short follow-up of our study (40 mo). Longer, prospective studies on the disease course of EO-IBD may confirm these data. In terms of clinical presentation, rectal bleeding was the most common symptom in younger children, whereas the older age group presented with more varied symptoms. This figure can be explained by the homogeneous colonic involvement in the EO disease, while multiple locations with more variable clinical pictures were found in later-onset forms. Several data suggest that the EO-IBD present a stronger genetic impact than the later-onset disease (de Ridder, Weersma et al. 2007;Ruemmele 2010;Bianco, Zanin et al. 2013). Differently from these reports, we did not find significant differences for family history between the EO and the later-onset groups. One explanation may lie in the fact that we have only considered the first-degree relatives, not extending to the rest of the family. This could have reduced the number of patients with a family history in our cohort. Subsequent studies in this population to investigate the impact of genetic background and potential combinations of genetic and environmental influences in disease onset are therefore needed. Some data suggest a higher “severity” in terms of behavior and disease course of EO-IBD (Van Limbergen, Russell et al. 2008). We evaluated the need of corticosteroids, immunomodulators, biologics, and surgery in an attempt to identify differences in severity according to age. We did not find significant differences in medical therapeutic strategy at the diagnosis among the 3 groups, indirectly suggesting a similar response to treatment regardless of age. Although, at the maximum follow-up, a significant number of children in the 0- to 5-year age group needed steroids. This might suggest a more aggressive disease than in older children, although the use of other drugs was not different. Moreover, our data suggest a similar. However, evaluation of these results as a surrogate for severity is difficult, and confounded by various factors, particularly the variability among individual physicians in the use of these agents. Sixty patients underwent surgery at least once between diagnosis and maximal follow-up, resulting in a crude surgical rate of 12%. This figure is close to previous French and North American studies (Romberg-Camps, Dagnelie et al.

2009;Schaefer, Machan et al. 2010). We did not find differences according to age, probably the short time of follow-up influenced this result. This study has some limitations. Our registry is based on patient data from large regional referral centers, which may not allow extrapolation to the real Italian pediatric population with IBD. However, children with chronic disease tend to be referred to tertiary referral centers such as these, and unlike adults with IBD, they are rarely treated exclusively by a primary care pediatrician. In summary, our data suggest not only that the number of EO-IBD is steadily increasing, but also that the phenotype is extensive and aggressive already at the onset of the disease. The widespread location of CD and UC, notably younger patients, may suggest the use of immunomodulatory and biologic agents early in the course of the disease. Nevertheless, those therapies should be ideally targeted on those patients with a more "severe" disease, thus future studies on genetic, clinical, and serologic risk factors for a disabling course in EO-IBD are warranted. Genetic impact does not seem significant in our population, suggesting that environmental triggers play a crucial role in the pathogenesis of EO disease. Several arguments indicate that a change of the intestinal microbiota, probably secondary to changes in alimentary habits, along with particularly sterile living conditions make individuals prone to developing IBD, and this could probably be important also in younger children (Schirbel and Fiocchi 2011;Cucchiara, Stronati et al. 2012). The identification of different clinical forms of EO disease, through the analysis of genotype–phenotype correlations, the identification of specific genotypes, and particularly combinations of genetic and environmental influences in disease onset, could consent in the near future to typify the disease already at onset, allowing specific therapies for the individual patient.

5. Alteration of fecal microbiota profiles in juvenile idiopathic arthritis. Associations with HLA-B27 allele and disease status

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5.1 Scientific background

Characterization of bacterial commensal communities in autoimmune and inflammatory diseases is a topic of great interest for understanding the role and interaction of intestinal microbiota with the host immune system. It is known that the gut microbiota is shaped by several environmental factors, including dietary habits, antibiotics, infectious agents, and air pollution (De Filippo, Cavalieri et al. 2010;David, Materna et al. 2014;Salim, Kaplan et al. 2014), and in turn, that microbiota shapes the immune system, modulating homeostasis in healthy status individuals or promoting inflammation when dysbiosis occurs. Recent findings demonstrate that alteration in the equilibrium among commensal bacteria is associated not only with Inflammatory bowel disease (IBD), allergy, diabetes and celiac disease (Cheng, Palva et al. 2013), but also with rheumatoid arthritis (Vaahtovuo, Munukka et al. 2008;Sandhya, Danda et al. 2016;Scher, Littman et al. 2016). Juvenile idiopathic arthritis (JIA) is the most common chronic rheumatic disease in children, and comprises a clinically heterogeneous group of conditions characterized by chronic arthritis, synovial inflammation and erosion of bone and cartilage (Ravelli and Martini 2007). According to several factors, including the number of affected joints, JIA is divided in psoriatic, oligoarticular (up to four affected joints), polyarticular (five or more affected joints) (Oberle, Harris et al. 2014), and enthesitis-related arthritis (ERA), in which entheses (attachments of tendons and ligaments to bone) are affected. ERA accounts for 10%–20% of JIA and is considered the equivalent of spondyloarthropathy, a disease frequently characterized by clinical and subclinical intestinal involvement (Bryan and Rabinovich 2014;Oberle, Harris et al. 2014;Aggarwal and Misra 2015).

Immunological, genetic and environmental factors are involved in the pathogenesis of ERA (Stoll, Kumar et al. 2014;Gmuca and Weiss 2015). Gene variants in the Major Histocompatibility Complex, especially the HLA-B27 alleles, have been identified as predisposing factors (Gmuca and Weiss 2015).
Alterations of gut microbiota (dysbiosis) and a decrease in gut microbiota richness (Li, Wang et al. 2014;Collado, Rautava et al. 2015;Rogers 2015) are emerging as factors associated with the development of inflammatory and systemic autoimmune diseases (Yeoh, Burton et al. 2013;Longman and Littman 2015). Of great interest is the understanding of dysbiosis as a trigger or a reflection of autoimmune and inflammatory disorders (Chung and Kasper 2010;Stoll, Kumar et al. 2014), in fact, also autoimmunity can drive instability of the gut microbial ecosystem.
Studies in germ-free animal models reveal relationships between microorganisms, mucosal immunity, and joint inflammation (Taurog, Richardson et al. 1994;Rath, Herfarth et al. 1996;Longman and Littman 2015). Recent studies in humans suggest that alteration of oral and gut microbiota and an increase in leaky gut could trigger systemic joint inflammation in the context of pre-existent autoimmunity (Scher, Sczesnak et al. 2013;Brusca, Abramson et al. 2014;Costello, Ciccia et al. 2014;Taneja 2014;Longman and Littman 2015;Scher, Ubeda et al. 2015;Zhang, Zhang et al. 2015). However, although arthritis susceptibility has been linked to the gut microbiome, and it has proposed that in synovial fluids the presence of pro-arthritogenic bacterial DNA, deriving by circulating intestinal bacterial products, may promote synovial inflammation ((Kempsell, Cox et al. 2000;Gerard, Wang et al. 2001;Moen, Brun et al. 2006;Oberle, Harris et al. 2014), a causal relationship between bacterial infection and onset of rheumatological diseases has not yet been firmly demonstrated. Further studies on leaky gut syndrome could clarify the presence of bacterial products circulating and influencing the systemic immune response, also in light of the presence of bacteria in non-rheumatoid arthritis controls (Kempsell, Cox et al. 2000), and of potential microbial contamination found in amplification technique of 16S rDNA, as previous observed (Grahn, Olofsson et al. 2003). A targeted-metagenomics approach can provide an in-depth characterization of microbial communities, allowing investigation of correlations between microbiota composition and human pathologies (Lozupone, Stombaugh et al. 2012).
In this study, we characterized and compared the gut microbiota of JIA patients, affected by enthesitis-related arthritis (JIA-ERA) and polyarticular JIA (non-enthesitis-related arthritis, n-ERA), with healthy subjects (HS), in order to define specific microbial "pro-arthritogenic" profiles.

5.2 Materials and Methods

5.2.1 Sampling of subjects

We enrolled 29 JIA patients (13 males and 16 females, age range 2-18 years), 19 of whom were affected by enthesitis-related arthritis (ERA) and 10 by polyarticular JIA (n-ERA). Exclusion criteria were: acute diarrhea, infectious gastroenteritis, antibiotic treatment in the previous 3 months, and diagnosis of chronic gastrointestinal disease. A total of 29 healthy children and adolescents (11 males and 18 females; age range 2-18 years) not affected by autoimmune and inflammatory conditions, infectious gastroenteritis, or chronic gastrointestinal disease, were enrolled as controls.

We collected a fecal sample from each subject, and a second one from 17 ERA patients three months later, in order to evaluate microbiota variability. We also gathered clinical information, including: enthesitis and arthritis localization, age of onset, HLA-B27 status, family history of ankylosing spondylitis or other HLA-B27-related diseases, comorbidities, laboratory parameters (C Reactive Protein, Erythrocyte Sedimentation Rate, ANA and pANCA autoantibody positivity), and pharmacological treatments (Table 5.1). None of the patients underwent treatment with proton pump inhibitors (PPIs). Active disease was defined by the presence of active arthritis and/or enthesitis at the time of stool sampling. As reported in Table 5.1, a total of 4 JIA-ERA and 4 JIA-nERA patients had clinically active disease. Fecal calprotectin (Aomatsu, Yoden et al. 2011) was also assessed by ELISA assay for subclinical intestinal inflammation (Eurospital, Trieste, Italy). Parents or guardians gave written informed consent for fecal samples and clinical data collection of their children. The study protocol was approved by the Ethics Committee of the Meyer Children's Hospital, Florence, Italy (Protocol ref. Nov 12th, 2013), and carried out in accordance with the approved guidelines.

5.2.2 DNA extraction

Fecal samples were preserved in RNAlater (Qiagen) at 4°C for the first 48 h, and kept at −80°C until DNA extraction. Bacterial genomic DNA extraction and quality control were carried out following our previous protocol (De Filippo, Cavalieri et al. 2010).

5.2.3 Pyrosequencing

For each sample, we amplified the 16S rRNA gene using the special fusion primer set specific for V5-V6 hypervariable regions and corresponding to primers 784F and 1061R described by Andersson *et al*. (Andersson, Lindberg et al. 2008), and using the FastStart High Fidelity PCR system (Roche Life Science, Milano, Italy). The 454 pyrosequencing was carried out on the GS FLX+ system using the XL+chemistry following the manufacturer recommendations (see materials and methods in chapter 3).

5.2.4 Data analysis

Pyrosequencing resulted in a total of 2`180`826 16S rDNA reads with a mean of 29`078 sequences per sample. Average sequence lengths were 290 nt (±SD 45) and 286 nt (±SD 50) for the first and second run, respectively. Raw 454 files were demultiplexed using Roche's .sff file software, and available at the European Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/PRJEB11846) under the accession study ERP013262. Reads were pre-processed using the MICCA pipeline (version 0.1, http://compmetagen.github.io/micca/) (Albanese, Fontana et al. 2015). Forward and reverse primer trimming and quality filtering were performed using micca-preproc truncating reads shorter than 280nt (quality threshold=18). *Denovo* sequence clustering, chimera filtering and taxonomy assignment were performed by micca-otu-denovo (parameters -s 0.97 -c). Operational Taxonomic Units (OTUs) were assigned by clustering the sequences with a threshold of 97% pair-wise identity, and their representative sequences were classified using the RDP software version 2.7 (Wang, Garrity et al. 2007). Template-guided multiple

sequence alignment was performed using PyNAST57 (version 0.1) (Caporaso, Bittinger et al. 2010) against the multiple alignment of the Greengenes 16S rRNA gene database (DeSantis, Hugenholtz et al. 2006) filtered at 97% similarity. Finally, a phylogenetic tree was inferred using FastTree (Price, Dehal et al. 2010) and micca-phylogeny (parameters: -a template-template-min-perc 50). Sampling heterogeneity was reduced by rarefaction, obtaining 12,964 sequences per sample.

Chao1 index and Shannon entropy (indicators of alpha diversity) and UniFrac (Lozupone and Knight 2005) and Bray-Curtis dissimilarities (indicators of beta diversity) were calculated using the phyloseq package (McMurdie and Holmes 2014) of the R software suite. Exploratory analysis was performed by Principal coordinates analysis (PCoA) using the phyloseq package of the R software suite. Multiple-rarefaction PCoA plots ("jackknifed" PCoA plots) (Lozupone, Lladser et al. 2011) were computed to assess the robustness of the beta-diversity analyses.

The significance of between-groups differentiation on the UniFrac distances and Bray-Curtis dissimilarity was assessed by PERMANOVA using the adonis() function of the R package vegan with 999 permutations.

To compare the relative abundances of OTUs among the three groups of subjects, the two-sided, unpaired Wilcoxon test was computed, removing taxa not having a relative abundance of at least 0.1%, in at least 20% of the samples, and using the function mt() in the phyloseq library and the p-values were adjusted for multiple comparison controlling the family-wise Type I error rate (minP procedure). Further significant differences in bacterial taxa were supported by non-parametric White's test and Welch's test by using STAMP (Parks, Tyson et al. 2014), and p-values were adjusted for multiple comparisons by Storey FDR, as proposed in STAMP.

On the basis of the relative abundances, the metagenomic biomarker discovery and related statistical significance were assessed using the linear discriminant analysis (LDA) effect size (LEfSe) method (Segata, Izard et al. 2011). LEfSe uses the Kruskal–Wallis rank-sum test to identify features with significantly different abundances between assigned taxa compared to the groups, and LDA to estimate the size effect of each feature. An alpha significance level of 0.05, either for the factorial Kruskal-Wallis test among classes or for the pairwise Wilcoxon test between subclasses, was used. A size-effect threshold of 2.0 on the logarithmic LDA score was used for discriminative microbial biomarkers.

In order to predict how taxonomic differences between fecal microbiota of the three groups impact their microbial metabolic potential, we applied PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille, Zaneveld et al. 2013), a computational approach useful to infer the functional contribution of microbial communities on 16S rDNA sequencing data set. PICRUSt implements an extended ancestral-state reconstruction algorithm to predict which gene families are present, and then combines gene families to estimate the significant differences in the main functional classes (KEGG categories) of the composite metagenome. From a OTUs table with associated Greengenes identifiers, we obtained the final output from metagenome prediction as an annotated table of predicted gene family counts for each sample, where the encoded functions of each gene family are orthologous groups or other identifiers such as KEGG

orthologs (KOs). The functional pathways discovery and related statistical significance were assessed by LEfSe.

5.3 Results

5.3.1 Clinical features of JIA patients

We collected clinical features, as well as comorbidities and therapies, for each enrolled patient at the time of fecal sampling (Table 7.1). As known, due to the two different subsets of JIA, females are affected from polyarticular JIA two to four times more often than males (Oberle, Harris et al. 2014). In our cohort, most of the enrolled JIA-ERA patients were males (13/19), while all JIA-nERA patients were females (10/10). Thus, we evaluated the sex/gender as a potential variable influencing the gut microbiota composition in both JIA categories. Regarding HLA-B27 status, 47% (9/19) of JIA-ERA patients resulted HLA-B27 positive, while all JIA-nERA patients were HLA-B27 negative. Calprotectin level, a measurement of intestinal inflammation, was positive (>100 µg/g) in 10.5% (2/19) of JIA-ERA and in 20% (2/10) of JIA-nERA patients (see Methods section).

5.3.2 Microbiota characterization by 16S rDNA sequencing in the JIA groups and healthy controls

We sequenced the V5-V6 hypervariable region of 16S rRNA gene for the meta-taxonomic study of microbiota in a total of 75 fecal samples from 19 JIA-ERA patients, 10 JIA-nERA and 29 healthy subjects (HS). From 17 JIA-ERA patients a second fecal sample was collected 3 months later, to evaluate microbiota variability over time.

The taxonomic distribution in the three groups showed variations in gut microbiota composition (Fig. 5.1). Firmicutes, the dominant phylum of gut microbiota, was the most abundant in all samples (over 50% of total reads; Fig. 5.1).

Considering the 20 most abundant families, we found statistically significant differences between groups in *Ruminococcaceae, Peptostreptococcaceae, Clostridiaceae I* and *Veillonellaceae* (Fig. 5.2A). *Ruminococcaceae* was more abundant in children in both JIA categories, compared with HS (21.6% in JIA-ERA and 27.2% in JIA-nERA vs 12.0% in HS; Wilcoxon rank-sum test, JIA-ERA vs HS p=0.0004; JIA-nERA vs HS p=0.0006; Fig. 5.2A). Although there was a reduction of *Peptostreptococcaceae* and *Clostridiaceae I* in all JIA patients (ERA and nERA) compared with HS (0.3% in JIA-ERA, 0.4% in JIA-nERA and 1.1% in HS; 0.1% in JIA vs 0.5% in HS respectively), we found statistically significant differences only between JIA-ERA vs HS (Wilcoxon rank-sum test, p=0.033 for *Peptostreptococcaceae*, p=0.017 for *Clostridiaceae I* respectively, Fig. 5.2A). We observed a statistically significant predominance of *Veillonellaceae* in JIA-nERA compared with HS (1.4% in JIA-nERA vs 0.4% in HS; Wilcoxon rank-sum test, p=0.012; Fig. 5.2A).

Table 5.1 Clinical features of JIA patients and age/sex information of healthy subjects

	ERA	**n-ERA**	**HS**
Number	19	10	29
Age (years) median, range	14.3; 9 to 18	10.5; 2 to 17	13; 2 to 18
Male: Female, number	13:6	0:10	11:18
Disease duration (months) median, range	55; 2 to 93	97; 3 to 150	-
Acute disease, number (%)	4 (21%)	4 (40%)	-
Calprotectin positive, number (%)	2 (10.5%)	2 (20%)	-
High Erythrocyte sedimentation rate, number (%)	3 (15.7%)	0 (0%)	-
HLA-B27+	9 (47%)	0 (0%)	-
ANA+	4 (21%)	9 (90%)	-
Complications or comorbidities, number (%)			
No	10 (52.6%)	3 (30%)	-
Uveitis	1 (5.2%)	7 (70%)	-
β- thalassemia	1 (5.2%)	0 (0%)	-
Pectum escavatum	2 (10.5%)	0 (0%)	-
Asthma	2 (10.5%)	0 (0%)	-
Osteoporosis	1 (5.2%)	0 (0%)	-
Obesity	1 (5.2%)	0 (0%)	-
Hypothyroidism	1 (5.2%)	0 (0%)	-
Epigastric hernia	1 (5.2%)	0 (0%)	-
Cataract	1 (5.2%)	0 (0%)	-
Breast fibroadenoma	1 (5.2%)	0 (0%)	-
Treatment, number of patients (%)			
NSAIDs	18 (95%)	1 (10%)	-
Sulfasalazine	8 (42%)	0 (0%)	-
Steroids	4 (21%)	0 (0%)	-
Methotrexate	4 (21%)	0 (0%)	-
Etanercept	3 (15.7%)	4 (40%)	-
Abatacept	1 (5.2%)	5 (50%)	-
Adalimumab	2 (10.5%)	0 (0%)	-

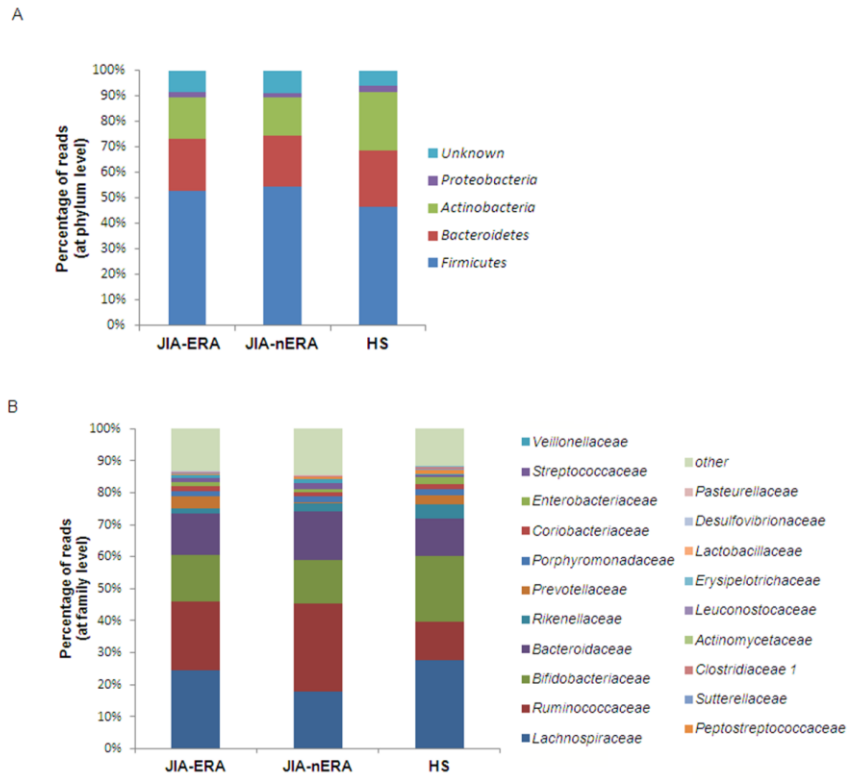


Fig. 5.1 Overview of fecal microbiota profiles in JIA and healthy subjects. Percentage of reads (A) of the main four phyla in JIA patients (ERA and n-ERA) and healthy subjects (HS) and (B) percentage of reads of the top 20 families in JIA patients and HS.

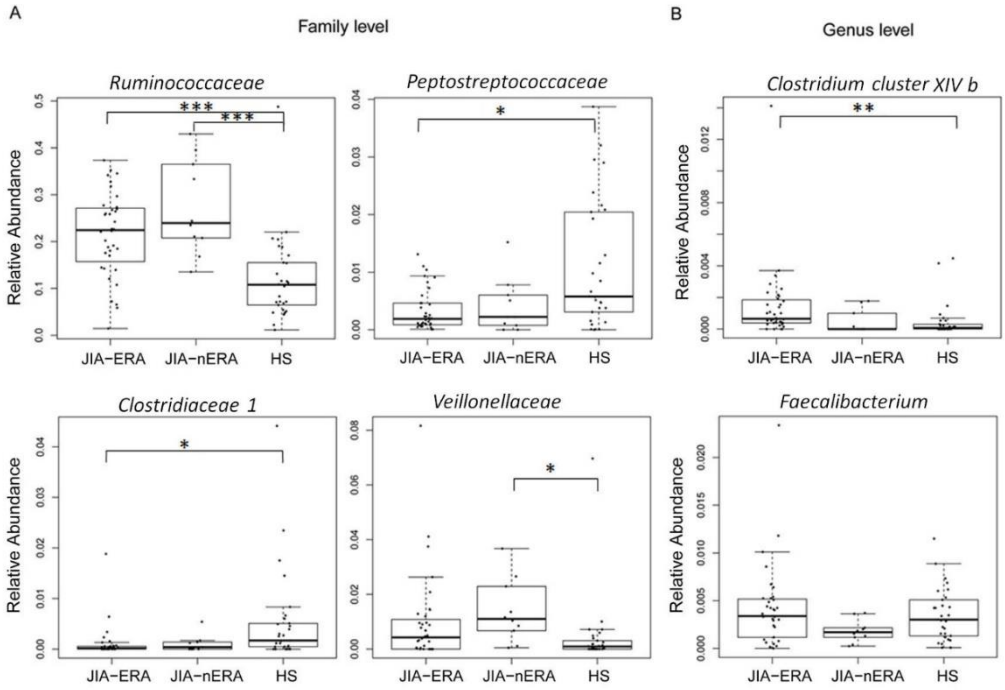


Figure 5.2 Relative abundances of fecal bacterial components in JIA and HS groups. Box plot of statistically significant different bacterial (A) families and (B) genera in JIA patients compared to HS (Pairwise comparisons using Wilcoxon rank sum test; *** p<0.001; ** p<0.01; * p<0.05 FDR

adjusted). For *Faecalibacterium* genus, statistical significance of the comparison JIA-ERA vs JIA-nERA was obtained by Welch's test without multiple testing correction.

Considering gender as potential variable influencing the gut microbiota composition, we confirmed that among the enrolled female subjects (6 JIA-ERA, 10 JIA-nERA and 18 HS), *Firmicutes* were more abundant in the JIA-nERA group compared with HS (Fig. 5.3; Wilcoxon rank-sum test $p<0.05$). Among the minor constituents of fecal microbiota, we observed an increase in *Sutterellaceae* and *Enterobacteriaceae* in JIA-ERA female patients compared with female HS (2.5% in JIA-ERA vs 1.2% in HS and 0.5% in JIA-ERA vs 0.3% in HS, respectively; Wilcoxon rank-sum test, $p<0.05$; Supplementary Figure 2B), and *Streptococcaceae* in JIA-nERA compared with HS (1% in JIA-nERA vs 0.3% in HS; Wilcoxon rank-sum test, $p<0.05$; Fig. 5.3).

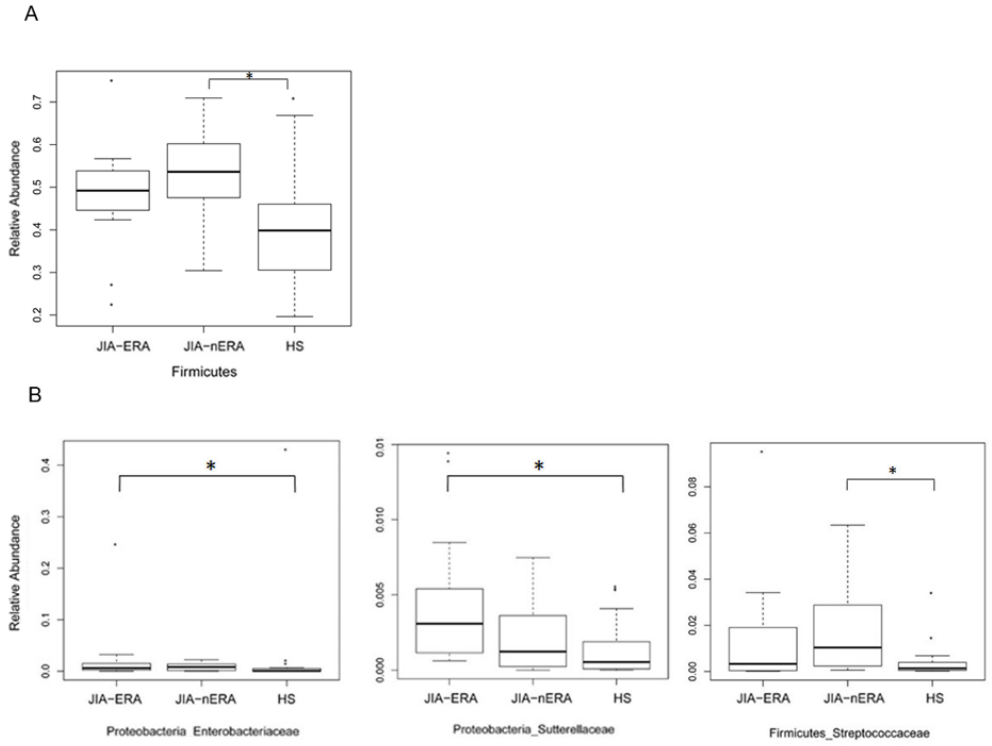


Fig. 5.3 Box plot of relative abundances of the statistically significant different bacterial (A) phylum and (B) families in JIA female patients compared with female HS (p-value by Wilcoxon rank-sum test, $p<0.05$).

At genus level, we found an abundance of *Clostridium cluster XIVb* in JIA-ERA patients compared with HS (0.23% in JIA-ERA vs 0.1% in HS; Wilcoxon rank-sum test, $p=0.007$; Fig. 5.2B). Moreover, we observed a decrease in the relative abundance in *Faecalibacterium* in JIA-nERA compared with either JIA-ERA or HS, even if not statistically significant (0.18% in JIA-nERA vs 0.35% in HS; 0.18% in JIA-nERA vs 0.41% in JIA-ERA; Fig. 5.2B).

By Linear discriminant analysis Effect Size (LEfSe), we evaluated significant differences in abundance between assigned taxa with respect to JIA patient groups. We observed differentially abundant taxa discriminating for HLA-B27 status. At family level, increased

Lactobacillaceae in HLA-B27 positive-JIA patients, and *Pasturellaceae* in HLA-B27 negative-JIA patients were found (Fig. 5.4). Of the increased genera in HLA-B27 positive JIA patients, we observed *Bilophila*, *Parvimonas* and *Oscillibacter*, while *Haemophilus* and *Eggerthella*, were differentially enriched in HLA-B27 negative patients (Fig. 5.4B). When considering only the JIA-ERA group, in addition to the five genera reported in Fig. 5.4B, *Lactobacillus*, *Clostridium cluster XI* and *Dialister* were enriched in HLA-B27 positive patients, while only *Haemophilus* discriminated for HLA-B27 negative status (Fig. 5.4C).

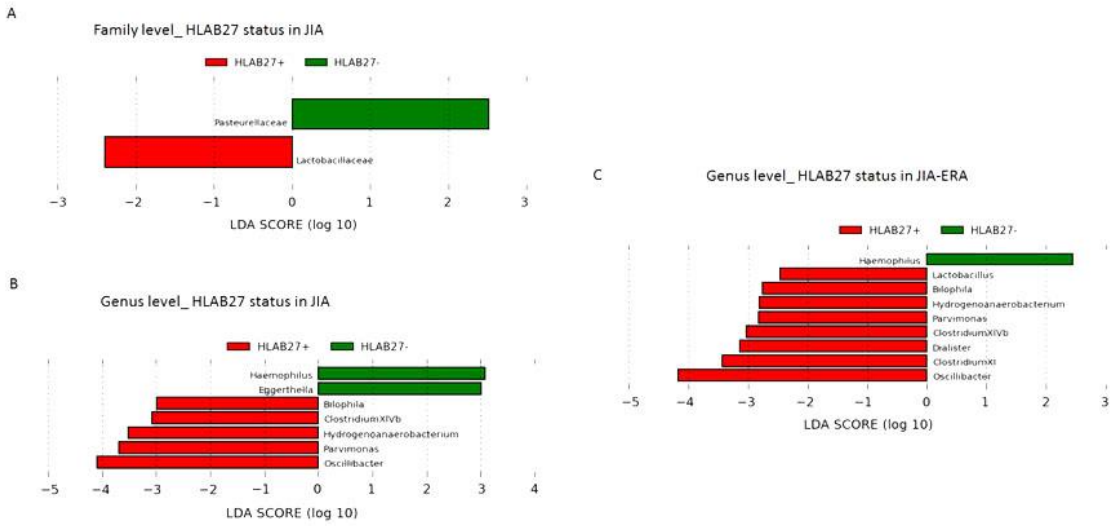


Figure 5.4 Differences in bacterial taxa per HLA-B27 status. LEfSe analysis shows a statistically significant enrichment of (A) families, and (B-C) genera, in (A-B) all JIA patients and in (C) JIA-ERA patients, considering the HLA-B27 status. LEfSe results indicate a sequentially significant ranking among groups (Alpha value=0.05 for the factorial Kruskal-Wallis test among classes). The threshold for the logarithmic LDA score was 2.0.

Further, we evaluated correlations between microbiota profiles of JIA patients and fecal calprotectin, as well as with respect to different medical treatments. We did not find any significant correlation between fecal calprotectin levels and microbiota profiles, probably due to the low number of patients with concomitant intestinal inflammation at the time of sampling. Regarding the effect of pharmacological therapies on gut microbiota of JIA patients, in our cohorts, JIA-ERA patients were treated with NSAIDs, alone or associated with sulfasalazine/methotrexate/biologics in different combinations, as reported in Table 5.1. JIA-nERA patients were mainly treated with biologic drugs, such as Abatacept and Etanercept. Despite the low number of patients stratified by pharmacological treatment, LEfSe analysis showed indications of association among different bacterial profiles and therapies (Fig. 5.5A-C), among which we observed enrichment in *Collinsella*, associated with exacerbation of joint disease (Chen, Wright et al. 2016), in JIA-ERA patients treated with combined NSAIDs and sulfasalazine therapy. However, a larger cohort of patients would be needed to strengthen our preliminary results and to understand the causality between therapies, gut microbiota profiles and clinical status.

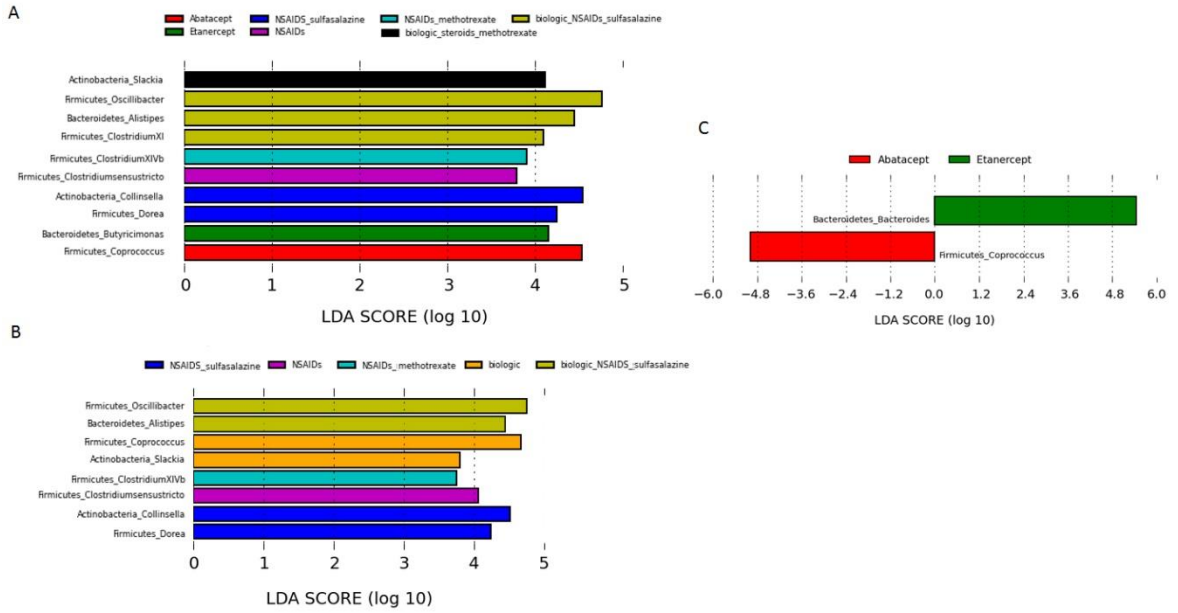


Fig. 5.5 Microbiota profile enrichment related to different therapies in JIA patients. LEfSe analysis performed in (A) all JIA patients, in (B) JIA-ERA, and in (C) JIA-nERA patients shows a statistically significant enrichment of bacterial genera correlated with different therapies. LEfSe results indicate a sequentially significant ranking among groups (Alpha value=0.05 for the factorial Kruskal-Wallis test among classes). The threshold for the logarithmic LDA score was 2.0.

In order to evaluate differences in the microbial biodiversity (alpha-diversity) among groups, we calculated the observed number of OTUs (a measurement of the total number of species present in a microbial community) and the Chao1 index (an indicator of species richness based on number of rare species), observing a significant reduction in alpha diversity in the JIA samples compared to HS (Fig. 5.6A, B; p-value<0.005 JIA-ERA vs HS and JIA-nERA vs HS). This suggests that microbiota of JIA patients is associated with biodiversity depletion, as observed in IBD patients. However, we did not find significant differences among the three groups using measures of biodiversity that take the evenness of the species distributions into account, like the Shannon entropy (Fig. 5.6C), the Dominance (1-Simpson index) and Equitability (Pielou index).

To estimate the variability of microbial communities between-sample (beta-diversity), we calculated the Bray-Curtis and unweighted UniFrac dissimilarities. Principal Coordinates analysis (PCoA) on unweighted UniFrac dissimilarities showed that both JIA samples (ERA and nERA) were more similar to each other than to HS samples (Fig. 5.6D). Bray Curtis dissimilarity confirmed the differences among samples in the three populations (PERMANOVA, p<0.001; Fig. 7.6E). Considering HLA-B27 status, PCoA, calculated on unweighted UniFrac and Bray-Curtis dissimilarity, showed that HLA-B27 positive JIA-ERA patients form subgroups with respect to other JIA-ERA samples (Fig. 5.6F-G), confirmed also by neighbor joining clustering based on Bray-Curtis dissimilarities (Fig. 5.6). However, the sample distribution of patients and healthy subjects was more marked based on disease and health status (Fig. 5.6D-E).

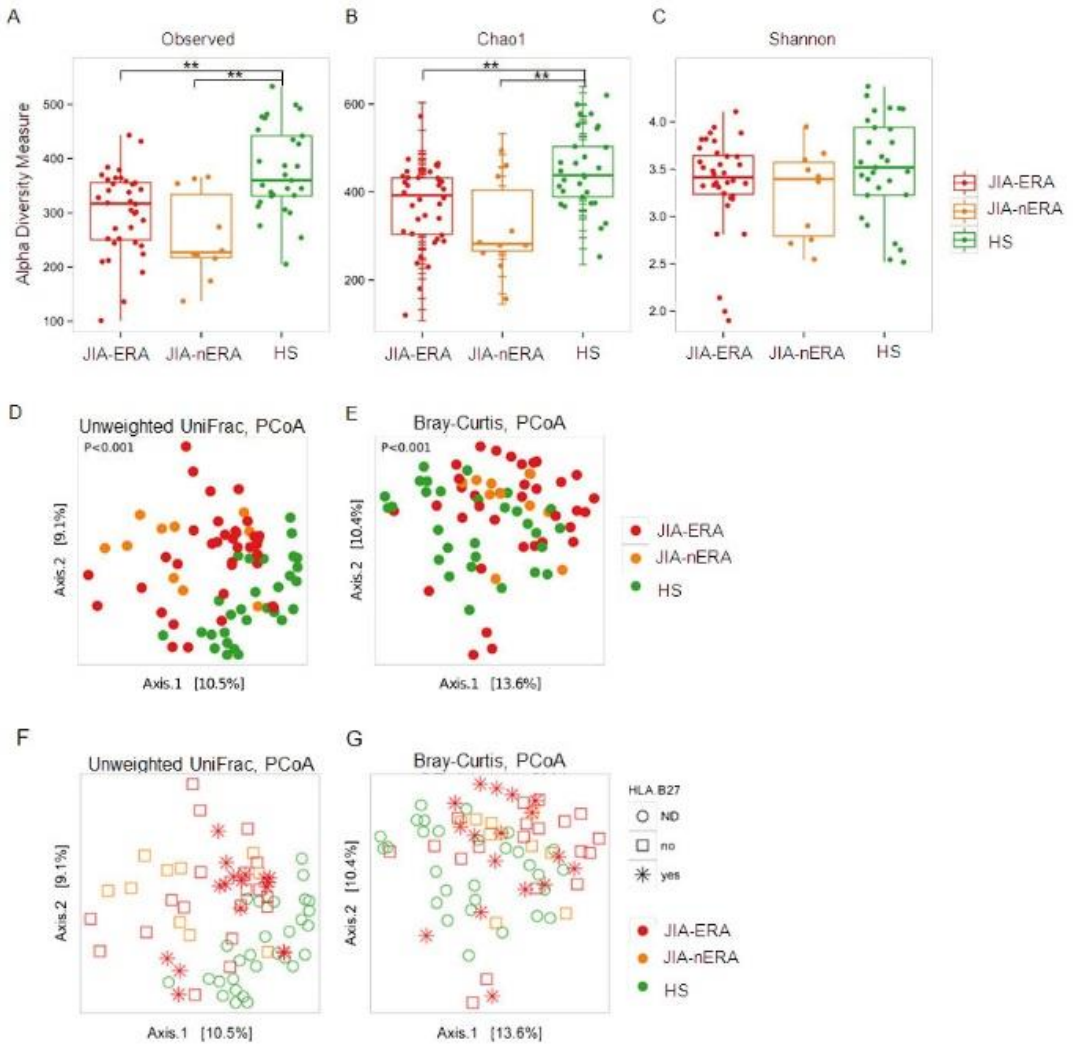


Figure 5.6 Alpha and beta diversity measures. (A-C) Box plots of (A) number of observed OTUs, (B) Chao 1, and (C) Shannon indexes in the three studied populations. Pairwise comparisons using the Wilcoxon rank sum test., **p-value <0.005, false discovery rate adjustment. (D-E) PCoA of (D) unweighted UniFrac and (E) Bray Curtis dissimilarities. (F-G) PCoA of unweighted UniFrac and Bray Curtis dissimilarities considering HLAB27 status (red asterisks). Colored dots: red=JIA-ERA, orange=JIA-nERA, and green=HS samples.

5.3.3 Fecal microbiota comparison between acute disease and remission in JIA categories

At the moment of fecal sampling, 21% of JIA-ERA patients (4/19) and 40% of JIA-nERA patients (4/10) had clinically active disease. Despite the reduced number of samples, LEfSe analysis showed significant differences in abundances of bacterial genera in samples collected during clinically active disease and during remission. In particular, *Sutterella* was increased in samples collected during active disease, while *Clostridium cluster IV* and *XVIII*, *Parasutterella* and *Odoribacter* were enriched in samples collected from patients in remission (Fig. 5.7A).

Furthermore, we calculated intra-group distances by unweighted UniFrac, comparing all JIA samples collected from patients with active disease and those in remission, versus the HS

samples. Although differences were not statistically significantly, we observed a trend indicating higher intra-group distances between JIA-ERA samples collected during active disease with respect to intra-group distances between JIA-nERA during active disease (Fig. 5.7B; intra-active JIA-ERA vs intra-active JIA-nERA). Within the JIA-ERA group, we found more variation in microbiota profile in samples collected during active disease compared to remission (Fig. 5.7B; intra-active JIA-ERA vs intra-remission JIA-ERA). On the contrary, although not statistically significantly different, the intra-distance of active disease samples in the JIA-nERA group was lower than in remission samples (Fig. 5.7B; intra-active JIA-nERA vs intra-remission JIA-nERA), indicating different microbial heterogeneity in active disease and remission in the two different JIA subsets. Comparing the samples collected from patients in remission, we observed that distances within the JIA-nERA group were greater than within the JIA-ERA group (Fig. 5.7B; intra-remission JIA-nERA vs intra-remission JIA-ERA).

Interestingly, when we compared JIA groups with HS, the intra-group distance in the HS group was lower than within both JIA groups (Fig. 5.7B) and significantly correlated with intra-distances observed in JIA-ERA patients, either active or in remission, and with intra-distance observed in JIA-nERA patients in remission (Wilcoxon rank-sum test; intra-HS vs intra-active JIA-ERA p=0.02; intra-HS vs intra-remission JIA-ERA p=0.0004; intra-HS vs intra-remission JIA-nERA p=0.03), indicating that the microbiota composition within the healthy group was significantly more homogeneous than those within the JIA groups.

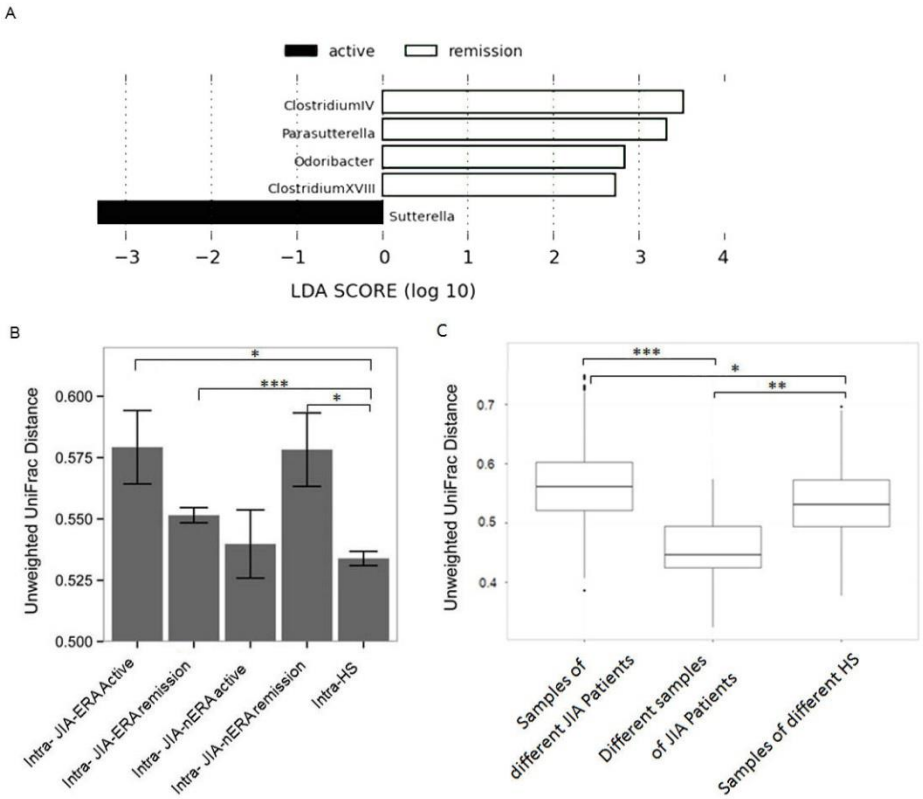


Figure 5.7 Fecal microbiota comparison in patients during acute disease and in remission. (A) LEfSe analysis shows a statistically significant enrichment of bacterial genera in JIA patients, in active disease (black) and in remission (white). LEfSe results indicate significant ranking among groups (Alpha value=0.05 for the factorial Kruskal-Wallis test among classes). The threshold for the logarithmic LDA score was 2.0. (B) Intra-group distances calculated by Unweighted UniFrac between samples collected during active disease and in remission from JIA-ERA and JIA-nERA

patients. *P*-values displayed are the results of Wilcoxon rank-sum tests. (C) Box plots of relative distances, calculated by Unweighted UniFrac, among different JIA samples and HS samples and distances of different the two samples of the same JIA-ERA patient. *P*-values displayed are the results of Wilcoxon rank sum test; * p-value <0.05, **p-value <0.01; *** p-value<0.001.

Next, we evaluated microbiota variations over time in patients of the JIA-ERA group, considering pairwise UniFrac distances between samples A and B (collected three months apart). The matrices obtained by principal coordinate analysis (PCoA), derived from pairwise unweighted and weighted UniFrac distances between samples A vs B, allowed us to explore inter- and intra-individual similarities or dissimilarities among samples, showing in most part of the cases lower distances between samples of the same patient than from different patients (Fig. 5.8). Comparing the distributions among all JIA and HS samples vs the JIA-ERA samples at two different times by Unweighted UniFrac mean distances, we confirmed that samples from the same patient are more similar than samples from different patients, as well as samples from HS, as expected (Fig. 5.7).

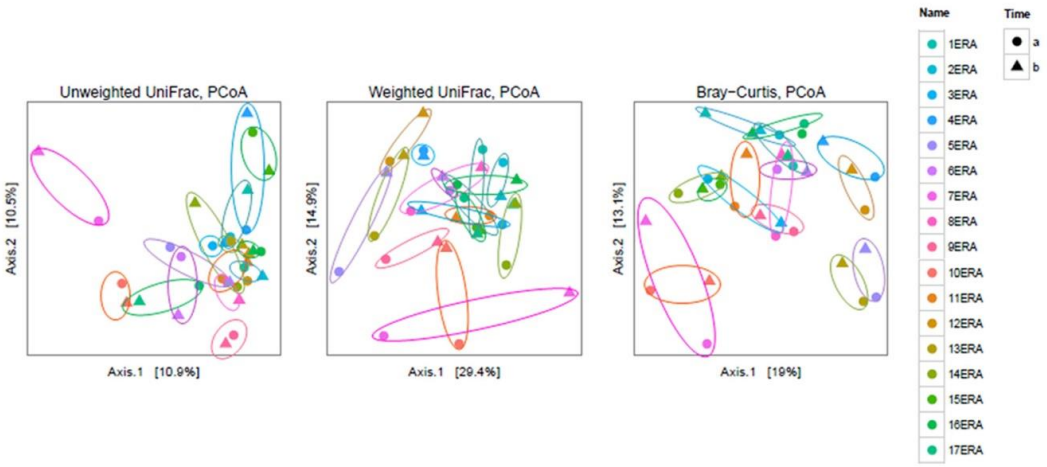
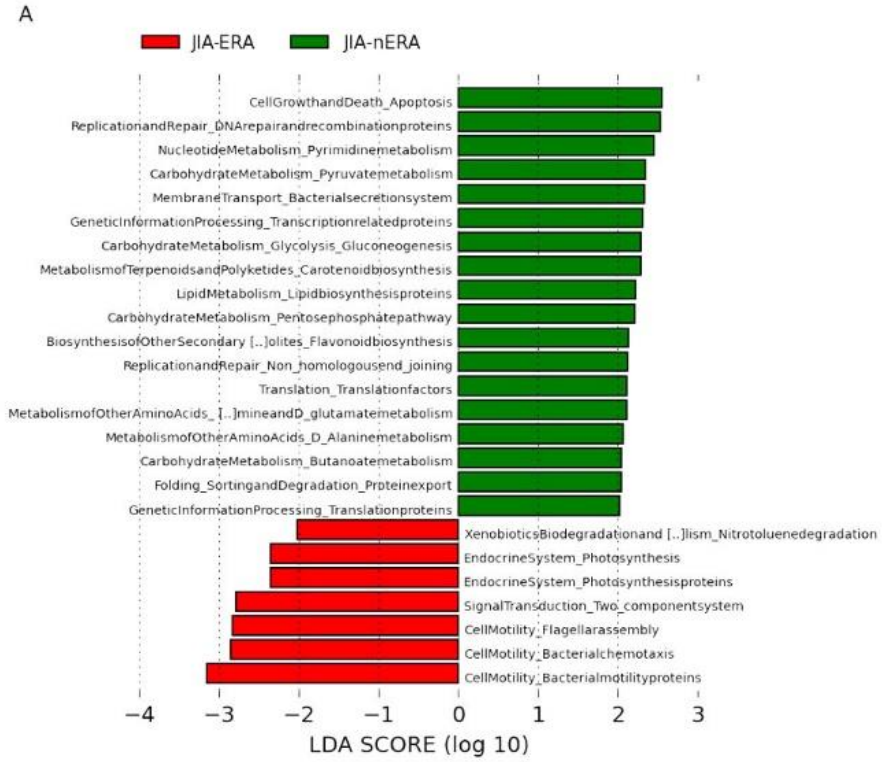


Fig. 5.8 Principal coordinate analysis (PCoA) derived from pairwise un-weighted and weighted UniFrac, and Bray-Curtis distances among JIA-ERA samples (A and B samples of the same JIA-ERA patient are represented as dot and triangle with same color, respectively). For each axis, in square brackets, the percent of variation explained was reported.

5.3.4 Metabolic function prediction

The microbiota is able to affect host physiology and metabolic functions, contributing to normal development and homeostasis of the immune system in the gut, modulating epithelial cell proliferation and protecting against pathogenic bacteria (Tremaroli and Backhed 2012;Sommer and Backhed 2013). Bacterial species are known to carry and transfer operons containing genes for different metabolic functions. Different bacterial species are enriched for certain functions and these correlations have been categorized in well-organized databases (Kanehisa, Goto et al. 2014). Therefore, in order to clarify how phylogenetic differences between the fecal microbiota of JIA patients impact their metabolic potential, we applied PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), a computational approach used to predict the functional composition of a metagenome (Langille, Zaneveld et al. 2013). LEfSe analysis performed on PICRUSt output showed differentially enriched functional classes (KEGG categories) among JIA

subgroups compared with HS (Fig. 5.9). Within the main KEGG categories, we observed significant enrichment of functions related to cell motility (Flagellar assembly, Ko:02040, Bacterial chemotaxis and Bacterial motility proteins, Ko:02030) in JIA-ERA compared with JIA-nERA and HS (Fig. 5.9A-B). Pathways related to Membrane transport (Secretion system, Ko:03070) and unclassified Cellular Processes and Signaling (Sporulation) were significantly enriched, respectively, in JIA-ERA and JIA-nERA compared with HS group (Fig. 5.9B-C). A remarkable enrichment in metabolic functions regarding carbohydrate metabolism, lipid metabolism, aminoacid metabolism, and other aminoacid metabolism identified a core of metabolic capabilities, especially in JIA-nERA metagenome compared to JIA-ERA (Fig. 5.9A) and HS (Fig. 5.9C). On the other hand, metabolism of cofactors and vitamins (folate biosynthesis and nicotinate and nicotinamide metabolism) and metabolism of other aminoacids (Glycine, Serine Threonine) significantly characterized HS metagenome compared to JIA-ERA (Fig. 5.9B).



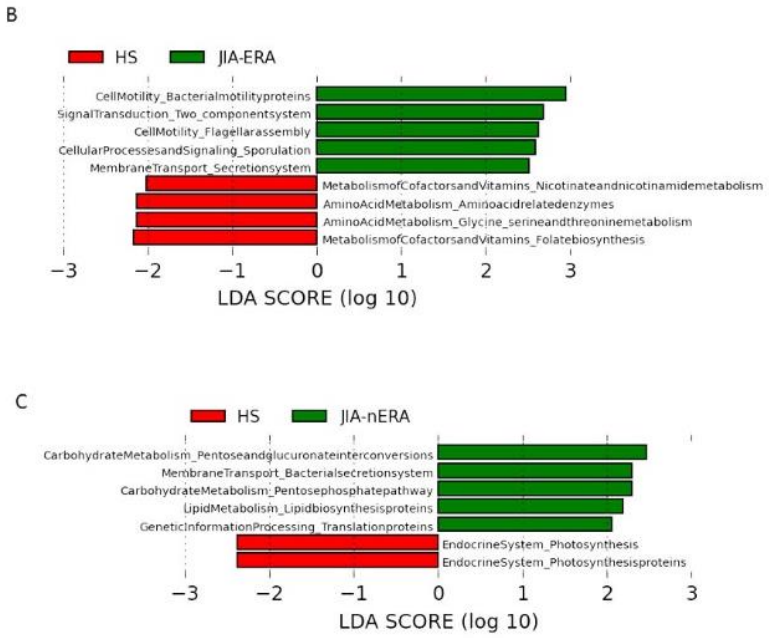


Figure 5.9 Metabolic function prediction associated to the microbiota profiles. LEfSe analysis, performed on metabolic functions inferred by PICRUSt analysis shows statistically significant enrichment of KEGG categories in (A) JIA-ERA vs JIA-nERA, (B) JIA-ERA vs HS, and (C) in JIA-nERA vs HS. LEfSe results indicate significant ranking among groups (Alpha value=0.05 for the factorial Kruskal-Wallis test among classes). The threshold for the logarithmic LDA score was 2.0.

5.4 Discussion

There are many lines of evidence that link the microbiota to rheumatic diseases. Animal models have been used to establish this possible correlation, such as the use of germ-free and gnotobiotic mice, in which animals were colonized with a specific microbial population, or through the use of antibiotics to understand the effect of microbiota modulation on rheumatic diseases. Principal limits of animal studies are the sample size and the difficulty to mimic the complex multifactorial pathogenesis of these pathologies.

In our study, we tried to define the role of microbiota in patients affected by Juvenile Idiopathic Arthritis. Alterations and decrease of microbiota richness were recently found in JIA compared with healthy controls (Stoll, Kumar et al. 2014), as well as in rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis, the latter two conditions related to spondyloarthropathies (Stebbings, Munro et al. 2002;Scher, Sczesnak et al. 2013;Costello, Ciccia et al. 2014;Gill, Asquith et al. 2015;Scher, Ubeda et al. 2015). We also observed variations in fecal microbiota composition and a reduction of microbial richness among JIA patients, affected by enthesitis-related arthritis (ERA) and polyarticular JIA (nERA), in comparison with healthy subjects. When compared to HS, in both JIA categories, we found statistically significant abundance in *Ruminococcaceae*, reduction in *Clostridiaceae* and *Peptostreptococcaceae* in JIA-ERA, and increase in *Veillonellaceae* in JIA-nERA. Of note, abundance in *Veillonellaceae* was recently found associated to ankylosing spondylitis (Costello, Ciccia et al. 2014). Conversely to our results, previous studies on ankylosing spondylitis (Costello, Ciccia et al. 2014;Scher, Ubeda et al. 2015) and on oligoarticular and

polyarticular JIA (Tejesvi, Arvonen et al. 2015) showed depletion of either *Ruminococcaceae* or *Veillonellaceae*.

The enrichment of anaerobic Gram-positive *Clostridium cluster XIVb* in JIA-ERA patients suggests a causal relation with inflammation. In fact, members of *cluster XIVb*, *C. propionicum* and *C. colinum*, (Collins, Lawson et al. 1994) were previously observed in poultry ulcerative enteritis (Berkhoff 1985). Also, studies in animal models have indicated that cell wall peptidoglycans of *Clostridium* and other anaerobic Gram-positive species can induce either chronic and erosive or transient arthritis (Severijnen, van Kleef et al. 1989;Simelyte, Rimpilainen et al. 2003). The observed decreasing trend in *Faecalibacterium* in JIA-nERA, considered an anti-inflammatory microorganism and a marker of health, has also been consistently reported in Crohn's disease patients (Sokol, Pigneur et al. 2008). Conversely to our results, a recent study showed reduction of *Faecalibacterium prasnutzii* in JIA-ERA patients (Stoll, Kumar et al. 2014).

The partial agreement of our findings with results obtained in other studies could be due to several factors, including variabilities and size of cohorts (different JIA categories, disease status, untreated or treated patients), as well as geography, environment or dietary habits of the patients, as shown by David and co-workers (David, Maurice et al. 2014).

Despite the limited possibility to generalize our results, due to the reduced number of patients in the cohorts, our results on JIA show intriguing links in terms of fecal microbiota profiles, with IBD and other autoimmune diseases associated to gastrointestinal disorders.

We are well aware that the known predominance of females in JIA-nERA introduces a potential gender effect, yet *Enterobacteriaceae* and *Streptococcaceae*, enriched in JIA-ERA and in JIA-nERA female patients, were also found to be correlated with intestinal inflammation as observed in biopsy samples of Crohn's disease patients (Gevers, Kugathasan et al. 2014) and the increase in *Sutterellaceae* observed in JIA-ERA female patients, as well as in samples collected in active disease, is also in line with increase in *Sutterella* previously found in children with autism suffering from gastrointestinal disorder (Williams, Hornig et al. 2012). Moreover, in samples collected in remission, we found abundance in *Clostridium spp. cluster IV* that have been reported to be inducers of colonic T regulatory cell (Atarashi, Tanoue et al. 2011), as well as in *Odoribacter*, known producer of Short Chain Fatty Acids (SCFAs), anti-inflammatory metabolites, and previously found reduced in IBD (Morgan, Tickle et al. 2012). However, in remission we also found enriched bacteria involved in intestinal inflammation and metabolic disorders, such as *Parasutterella*, found in a mice model of chemically induced colitis (Zhang, Wu et al. 2016), *Clostridium cluster XVIII*, encompassing *C. ramosum*, involved in diet-induced obesity (Woting, Pfeiffer et al. 2014) and *C. spiroforme*, a toxin-associated disease producer, involved in rabbit colitis (Carman and Borriello 1984).

Differentially abundant taxa, previously found in relation to rheumatoid arthritis, oral infection, intestinal inflammation or colitis, or to intestinal barrier permeability, discriminate for positivity of HLA-B27 allele, a genetic marker strongly associated with spondyloarthropathies. Among these, in HLA-B27 positive ERA patients we found *Bilophila*, a sulphite-reducing bacterium known to be involved in murine colitis (Devkota, Wang et al. 2012) and in intestinal inflammatory disorders in humans (Loubinoux, Bronowicki et al. 2002;Rowan, Docherty et al. 2009), via H_2S production promoting

intestinal inflammation. *Parvimonas* was commonly observed in periodontitis and appendicitis (Zhong, Brower-Sinning et al. 2014), and *Oscillibacter* was shown to be involved in gut barrier integrity in mice (Lam, Ha et al. 2012). In HLA-B27 negative-ERA patients, we found *Haemophilus* and *Eggerthella*, recently associated with rheumatoid arthritis (Zhang, Zhang et al. 2015;Chen, Wright et al. 2016). Moreover, when considering only the ERA group, in HLA-B27 positive patients we also showed enrichment of *Lactobacillus*, observed as potential arthritogenic agent via its cell wall pepetidoglycan (Severijnen, van Kleef et al. 1989), *Clostridium cluster XI*, that includes *C. difficile*, a well-known proinflammatory and colitis inducing-bacterium, and *Dialister*, frequently found in periodontitis and other infections (Morio, Jean-Pierre et al. 2007).

Recent studies on animal models showed association between HLA-B27 allele and other different bacterial species, including *B. vulgatus* and *Prevotella spp.* (Lin, Bach et al. 2014) and *Akkermansia muciniphila* (Asquith, Stauffer et al. 2016). In particular, *Akkermansia spp.* was suggested as potential pro-arthritogenic bacterial genus. However, the study of Stoll and collaborators (Stoll, Kumar et al. 2014) showed abundance of *Akkermansia* in a low percentage of ERA patients, but no significant association with HLA-B27 status. In our study, we did not find *Akkermansia spp.* as part of the core gut microbiota of our patients, yet we found other species correlated with HLA-B27 allele. Little is known on the geographic distribution of *Akkermansia spp.*, that could be less represented in our cohorts. Overall this could suggest that other bacterial species, in absence of *Akkermansia spp.* can discriminate HLA-B27 status, and that disease biomarkers should be based on patterns of taxonomic units or biological functions, rather than on single species.

Regarding the functional contribution of microbiota profiles, by PICRUSt prediction analysis we observed distinctive functional acquisitions among JIA subgroups and HS. A core of metabolic capabilities, regarding carbohydrate, lipid, aminoacid, cofactors and vitamins metabolism, were enriched in JIA-nERA and HS metagenomes. It is worth noting that microbiota of JIA-ERA patients is significantly enriched in functions related to cell motility, including flagellar assembly, bacterial chemotaxis and motility proteins, representing possible traits of virulence that could be associated to gut inflammation. These indications of enrichment in potentially pathogenic invasiveness-related traits in JIA-ERA metagenome could suggest a potential improved ability of microbial components to pass through the gastrointestinal barrier and migrate in other districts, also responding to nutrient gradients. Moreover, given that in mice models immunogenicity of flagellin CBir1 was observed, with consequent induction of colitis, and antibodies anti-CBir1 were found in CD patients with complicated disease (Targan, Landers et al. 2005), we cannot exclude the potential effect of flagellar-assembly proteins of some components of microbiota on host immune system of JIA.

Despite the relatively small cohort of patients in our study, the microbial profile differences in active disease and remission are corroborated by the observed intra- and inter-group distances of microbiota samples in active, remission and healthy status. As expected, our results suggest that during active disease the microbiota is strongly perturbed (major intra-group distance compared with remission). Healthy status allows a more stable microbiota ecosystem compared to disease status, as previously observed (Coyte, Schluter et al. 2015). Remission is characterized by an intermediate microbial pattern, different from both active

disease and healthy controls, likely resulting from a different trajectory to stable state and in which autoimmune reactivity and the microbial ecosystem are mutually shaped.
Although microbial profiles may differ in an individual-specific manner, the observed fecal microbiota dissimilarities in the same subject at different collection times, and among JIA categories and healthy controls suggest that continued perturbation and instability of the microbial ecosystem may contribute to inflammation.
Another aspect that should be more thoroughly investigated is the association between microbial profiles and pharmacologic therapies in autoimmune diseases. Recent studies, as previously observed in IBD patients, highlighted the effect of different therapies on microbiota, such as the rapid effect of enteral nutrition in the shaping of microbiota (Lionetti, Callegari et al. 2005) and the dysbiosis associated with antibiotic treatment (Lewis, Chen et al. 2015), as well as the effect of protonic pomp inhibitors on reduction of bacterial richness and selection of "unhealthy" microbiota (Imhann, Bonder et al. 2016). Moreover, the recent considerations suggest that the use of sulfasalazine could protect the intestinal epithelium from injury, reducing the bacterial product circulation (Rosenbaum and Asquith 2016). Despite the reduced number of patients on single or combined therapies in the cohort, our results showed indications of different microbial profiles associated to pharmacological therapies, such as NSAIDs, immunosuppressants and biologics, providing interesting clues on effect of such treatments on gut microbiota selection. For example, the enrichment of *Collinsella,* involved in exacerbation of joint disease (Chen, Wright et al. 2016), observed in JIA-ERA patients treated with NSAIDs in combination with sulfasalazine, results in contrast with hypothesis of Rosenbaum and Asquith (Rosenbaum and Asquith 2016). Our results prompt future studies on larger cohorts, including untreated newly-onset patients, addressing the effect of different pharmacological therapies on patients in active disease and remission, investigating how inflammation can indirectly modify microbiota, selecting differential microbial components, via mechanisms involved in epithelial barrier function and immune response and how pharmacological treatment contribute to perturb the gut microbial equilibrium compared to healthy status.
Finally, given the extreme inter-individual variability of microbiota in inflammatory and autoimmune diseases, further investigations on microbiota dynamics in different phases of disease should address the causes of perturbation and the restoration of microbial equilibrium, in order to adopt therapeutic strategies able to maintain microbial diversity and a stable state, essential for immune homeostasis and the host's health. Research in this direction should include therapeutic strategies able to modulate the microbiota, not only with diet and probiotics, but also evaluating new therapeutic approaches, such as fecal transplantation, recently adopted in other diseases and that have shown some effectiveness, especially during active disease. Furthermore, the understanding of the microbial functional acquisition and the relationships with epithelial barrier function and host immune response could help to identify the pro-arthtritogenic contribution of the microbiota.

6. Age and gender affect the composition of fungal population of the human gastrointestinal tract

This chapter has been reprinted* from:

Strati F., **Di Paola M.**, Stefanini I., Albanese D., Rizzetto L., Lionetti P., Calabrò A., Jousson O., Donati C., Cavalieri D., De Filippo C.

Age and Gender Affect the Composition of Fungal Population of the Human Gastrointestinal Tract. Frontiers in Microbiology. 2016 Aug 3;7:1227. doi: 10.3389/fmicb.2016.01227.

6.1 Scientific Background

The human gut is a complex ecological niche in which archaea, bacteria, protozoa, fungi, and viruses co-exist in close association with the host (Reyes, Haynes et al. 2010;Arumugam, Raes et al. 2011)(Reyes et al., 2010; Arumugam et al., 2011; Human Microbiome Project Consortium, 2012). Even if it has been estimated that the number of bacteria hugely outreaches the number of fungi in the gastrointestinal (GI) tract (Huffnagle and Noverr 2013), fungi play a relevant role in the physiology of the human host (Oever and Netea 2014;Underhill and Iliev 2014). Recent studies showed that, while the composition of the bacterial community is relatively stable over time, the fungal population inhabiting the murine gut undergoes significant changes during the animal's lifetime (Dollive, Chen et al. 2013). This brought to the conclusion that gut fungal populations are more variable than bacterial ones and that their composition may be influenced by environmental fungi (Underhill and Iliev 2014). Despite evidence that fungi inhabit the mammalian GI tract and interact with the host immune system (Romani 2011;Rizzetto, De Filippo et al. 2014;Underhill and Iliev 2014), the composition and characteristics of the mycobiota in healthy hosts have been poorly explored. The prevalent interest in describing pathogenic fungi, their phenotypes and the process by which they establish the infection is one of the major cause that brought to neglect the harmless part of the commensal fungal population. Despite this topic has been only marginally explored to date, it has been shown that mucosal fungi are able to modulate both the innate and adaptive immune responses (Romani 2011;Rizzetto, De Filippo et al. 2014;Underhill and Iliev 2014) thus supporting the need to further study the whole gut mycobiota. Furthermore, alterations of the gut mycobiota have been associated to different pathologies ranging from metabolic disorders (obesity) to

colorectal adenomas and Inflammatory Bowel Diseases (IBDs) (Luan, Xie et al. 2015;Mar Rodriguez, Perez et al. 2015;Sokol, Leducq et al. 2016). A recent study showed the association of IBDs to alteration of the gut mycobiota. In particular Sokol and colleagues showed that IBD patients bear a smaller proportion of *Saccharomyces cerevisiae* and higher of *Candida albicans* compared to healthy subjects. In addition, they highlighted the existence in Crohn's disease of interconnected alterations between bacterial and fungal communities (Sokol, Leducq et al. 2016). However, the role of the gut mycobiota in the maintenance of health it is still far from being well-understood because the studies carried out so far focused on disease-causing taxa. Nevertheless, some yeasts have been clinically prescribed for a long time because of their potential probiotic properties, suggesting a beneficial role of some fungi for host health. A great example of "beneficial" fungus is represented by *S. cerevisiae* var. *boulardii,* used for the relief of gastro enteritis (Hatoum, Labrie et al. 2012). In order to reduce the gap of knowledge concerning the gut mycobiota and its interplay with the host, we characterized the gut mycobiota composition of a cohort of healthy subjects by means of metagenomics, fungal cultivation, and phenotypic assays.

6.2 Materials and Methods

6.2.1 Study participants

Fecal samples were collected from 111 Italian healthy volunteers (49 male and 62 female, average age, 10±8.2) (Table 6.1) and analyzed within 24 hours. Written informed consent has been obtained from all the enrolled subjects or tutors in accordance with the guidelines and regulations approved by the Research Ethical Committees of the Meyer Children's Hospital and the Azienda Ospedaliera Careggi, Florence. All the subjects enrolled were non-smokers, followed a Mediterranean-based diet and they did not take antibiotics, antifungals or probiotics in the 6 months prior to sample collection. None of the participants had any history of gastrointestinal abnormalities.

6.2.2 Isolation and identification of cultivable fungal species from feces

Stool samples were diluted in sterile Ringer's solution and plated on solid YPD medium (1% Yeast extract, 2% Bacto-peptone, 2% D-glucose, 2% agar) supplemented with 25U/ml of penicillin, 25µg/ml of streptomycin (Sigma-Aldrich) and incubated aerobically at 27°C for 3-5 days. All fungal isolates grown on the selective medium were further isolated to obtain single-cell pure colonies. Genomic DNA was extracted from pure cultures of isolated colonies as previously described (Hoffman and Winston 1987). Strains were identified by amplification and sequencing of the ribosomal Internal Transcribed Spacer (ITS) region, using ITS1 (5'-GTTTCCGTAGGTGAACTTGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers, as previously described (Sebastiani, Barberio et al. 2002). Fungal isolates were identified by using the BLAST algorithm in the NCBI database (minimum 97% sequence similarity and 95% coverage with a described species).

6.2.3 Phenotypical characterization of fungal isolates

Fungal isolates were tested for phenotypical features that could be related to the ability of colonization and persistence in the human gut. Cell growth in liquid media was monitored by optical density measurement at 630nm with a microplate reader (Synergy2, BioTek, USA) after 48h of incubation under tested conditions. Three independent replicates were performed for each test.

Growth at supra optimal temperatures. Fungal isolates (~10^5 cells/ml) were grown at supra optimal temperatures in liquid YPD medium (40°C, 42°C, 44°C and 46°C).

pH impact on growth. Fungal isolates (~10^5 cells/ml) were grown at 37°C in liquid YPD medium at pH 2.0 and pH 3.0 adding hydrochloric acid/potassium chloride and citrate buffers respectively to test their ability to resist to the acidic environments encountered during GI tract passage.

Tolerance to bile acids. Fungal isolates (~10^5 cells/ml) were grown in liquid YPD medium at 37°C in the presence of three different concentrations of bile [Ox-bile, Sigma-Aldrich; 0.5%, 1% and 2% (w/v)] mimicking the physiological intestinal settings (Noriega, Gueimonde et al. 2004).

Resistance to oxidative stress. Fungal resistance to oxidative stress was evaluated by measuring the inhibition halo induced by the treatment of fungal strains (~10^7 cells/ml) grown on YPD solid medium with 0.5mM hydrogen peroxide (H_2O_2). The percentage of sensitivity to oxidative stress was calculated as the deviation of the inhibition halo diameter (Ø) from that of the environmental, oxidative stress sensitive M28-4D *Saccharomyces cerevisiae* strain (Cavalieri, Townsend et al. 2000) according to the following formula: [(Ø sample – Ø M28-4D)/Ø M284D]*100.

Invasive growth. The ability of fungal strains to penetrate the YPD solid medium was tested as previously described (Vopalenska, Hulkova et al. 2005). M28-4D and BY4742 *S. cerevisiae* strains, known to be invasive and non-invasive respectively, have been used as controls. The strain invasiveness was assigned with scores from 3 (highly invasive) to 0 (non-invasive).

Hyphal formation. Fungal cells (~10^5 cells/ml) were grown for 7 days in liquid YPD and YNB media (0.67% Yeast Nitrogen Base w/o aminoacids and $(NH_4)_2SO_4$ (Sigma-Aldrich), 2% glucose), both at 27°C and 37°C in order to evaluate hyphae or pseudohyphae formation. Formation of hyphae was inspected by optical microscope observation with a Leica DM1000 led instrument (magnification 40x and 100x).

6.2.4 Antifungal susceptibility testing

All fungal isolates were tested for susceptibility to fluconazole, itraconazole and 5-flucytosine (Sigma-Aldrich) by Minimum Inibitory Concentration (MIC) assays according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (Rodriguez-Tudela, Arendrup et al. 2008;Subcommittee on Antifungal Susceptibility Testing of the 2008). Clinical and Laboratory Standards Institute (CLSI) clinical breakpoints (CBPs) were used to evaluate the antifungal resistance (Pfaller and Diekema 2012;Castanheira, Messer et al. 2014). CBPs have not been established for non-*Candida* yeasts and non-*Aspergillus* moulds, however have been used as a proxy for the evaluation of antifungals susceptibility in such isolates.

6.2.5 DNA extraction and PCR amplification of fungal ITS1 rDNA region

DNA extraction from fecal samples (250 mg) was performed using the FastDNA™ SPIN Kit for Feces (MP-Biomedicals, USA) following manufacturer's instructions. DNA quality was checked on 1% agarose gel TAE 1X and quantified with a NanoDrop® spectrophotometer. For each sample, fungal ITS1 rDNA region was amplified using a specific fusion primer set coupled with forward primer 18SF (5'-GTAAAAGTCGTAACAAGGTTTC-3') and reverse primer 5.8S1R (5'-GTTCAAAGAYTCGATGATTCAC-3') (Findley, Oh et al. 2013) containing adaptors, key sequence and barcode (Multiple IDentifier) sequences as described by the 454 Sequencing System Guidelines for Amplicon Experimental Design (Roche, Switzerland). The PCR reaction mix contained 1X FastStart High Fidelity PCR buffer, 2mM $MgCl_2$, 200µM of dNTPs, 0.4µM of each primer (PRIMM, Italy), 2.5U of FastStart High Fidelity Polymerase Blend and 100ng of gDNA as template. Thermal cycling conditions used were 5min at 95°C, 35 cycles of 45sec at 95°C, 45sec at 56°C and 1.30min at 72°C followed by a final extension of 10min at 72°C. All PCR experiments were carried out in triplicates using a Veriti® Thermal Cycler (Applied Biosystems, USA).

6.2.6 Library construction and pyrosequencing

The PCR products obtained were analyzed by gel electrophoresis and cleaned using the AMPure XP beads kit (Beckman Coulter, USA) following the manufacturer's instructions, quantified via quantitative PCR using the Library quantification kit – Roche 454 titanium (KAPA Biosystems, USA) and pooled in equimolar way in a final amplicon library. The 454 pyrosequencing was carried out on the GS FLX+ system using the XL+ chemistry following the manufacturer's recommendations (Roche, Switzerland).

6.2.7 Data analysis

Pyrosequencing resulted in a total of 1.337.184 reads with a mean of 19.379±13.334 sequences *per* sample. Raw 454 files were demultiplexed using the Roche's sff file software and submitted to the European Nucleotide Archive with accession number PRJEB11827 (http://www.ebi.ac.uk/ena/data/view/PRJEB11827). Sample accessions and metadata are available in Supplementary Table S1. Reads were pre-processed using the MICCA pipeline (Albanese, Fontana et al. 2015). Forward and reverse primers trimming and quality filtering were performed using micca-preproc. De-novo sequence clustering, chimera filtering and taxonomy assignment were performed by micca-otu-denovo: Operational Taxonomic Units (OTUs) were assigned by clustering the sequences with a threshold of 97% pairwise identity and their representative sequences were classified using the RDP classifier version 2.8 (Wang, Garrity et al. 2007) against the UNITE fungal ITS database (Koljalg, Nilsson et al. 2013). Denovo multiple sequence alignment was performed using T-Coffee (Notredame, Higgins et al. 2000). Fungal taxonomy assignments were then manually curated using BLASTn against the GenBank's database for accuracy. High quality fungal sequences were

detected in all samples. Furthermore the sequences belonging to Agaricomycetes [unlikely to be residents of the human gut due to their ecology (Hibbett 2006)] were manually filtered out.
The phylogenetic tree was inferred by using micca-phylogeny (Price, Dehal et al. 2010). Rarefaction analysis resulted in a sequencing depth adequate to capture the ecological diversity of the samples up to saturation. Sampling heterogeneity was reduced by rarefaction. Alpha and beta-diversity estimates were computed using the phyloseq R package (McMurdie and Holmes 2013). PERMANOVA (Permutational multivariate analysis of variance) was performed using the adonis() function of the *vegan* R package with 999 permutations. Permutations have been constrained within age groups (corresponding to 0-2 y/o, 3-10 y/o, 11-17 y/o and >18 y/o) or gender to reduce possible biases related to the unequal age and gender distributions among subjects using the "strata" argument within the adonis() function. Two-sided, unpaired Welch t-statistics were computed using the function mt() in the phyloseq library and the *p*-values were adjusted for multiple comparison controlling the family-wise Type I error rate (minP procedure) (Westfall and Young 1993). Wilcoxon rank-sum tests and Spearman's correlations were performed using the R software (Team 2014) through the *stats* R package (version 3.1.2) and the *psych* R package (Revelle 2013) respectively. *p*-values have been corrected for multiple comparison by using the false discovery rate correction (Benjamini and Hochberg 1995).

6.3 Results

6.3.1 Cultivable Gut Mycobiota

The cultivable gut mycobiota of 111 healthy volunteers (Table 6.1) was investigated through isolation in selective media. Fungi were detected in more than 80% of subjects leading to the identification of 349 different isolate. Thirty-four different fungal species were detected at different frequencies of isolation (Table 6.2) among which *Aspergillus glaucus*, *Candida albicans*, *Candida deformans*, *Candida fermentati*, *Candida glabrata*, *Candida intermedia*, *Candida lusitaniae*, *Candida metapsilosis*, *Candida parapsilosis*, *Candida pararugosa*, *Candida tropicalis*, *Candida zelanoydes*, *Cryptococcus saitoi*, *Lichtheimia ramosa*, *Mucor circinelloides*, *Pleurostomophora richardsiae*, *Rhodotorula mucilaginosa*, *Trichosporon asahii*, *Yarrowia lipolytica*. These species were previously found in different human body sites, including the GI tract as commensal or opportunistic pathogens (Araujo, Pina-Vaz et al. 2007;Alastruey-Izquierdo, Hoffmann et al. 2010;Levenstadt, Poutanen et al. 2012;Gouba, Raoult et al. 2014;Lee, Billmyre et al. 2014;Rizzetto, De Filippo et al. 2014).

Table 6.1 Characteristics of the study participants

Age group (year)		Infants (0–2)	Children (3–10)		Adolescents (11–17)		Adults (≥18)	All subjects
Number of subjects		18	48		24		21	111
% with fungi		88.9	83.3		70.8		76.2	80.2
Subject ID	Gender	Age (year)	Subject ID	Gender	Age (year)	Subject ID	Gender	Age (year)
HS1	M	5	HS38*	F	25	HS75	F	1
HS2	M	5	HS39*	F	27	HS76*	M	1
HS3	M	14	HS40*	M	27	HS77	F	4
HS4	M	1	HS41*	F	24	HS78*	M	12
HS5	F	20	HS42*	F	24	HS79*	M	0.1
HS6	F	20	HS43*	M	26	HS80	F	0.1
HS7	F	20	HS44*	F	24	HS81	F	7
HS8	M	5	HS45*	F	6	HS82*	M	10
HS9	M	14	HS46*	F	6	HS83*	M	12
HS10*	F	2	HS47*	F	10	HS84	F	6
HS11	M	16	HS48*	F	2.5	HS85	F	10
HS12	M	15	HS49*	M	2.5	HS86*	M	7
HS13*	F	18	HS50*	F	1.5	HS87*	M	9
HS14	F	0.3	HS51*	F	8	HS88*	M	7
HS15*	F	11	HS52*	F	23	HS89*	M	12
HS16	M	14	HS53*	F	23	HS90	F	8
HS17	M	15	HS54	M	2	HS91	F	2
HS18	M	11	HS55*	M	2	HS92	F	12
HS19	F	3	HS56*	M	2	HS93	F	4
HS20*	F	4	HS57	F	12	HS94	F	4
HS21*	F	5	HS58	F	3	HS95	F	10
HS22*	F	15	HS59*	M	5	HS96	F	12
HS23*	F	11	HS60	F	3	HS97*	M	6
HS24	M	15	HS61*	M	2	HS98	F	16
HS25	M	7	HS62	F	4	HS99	F	3
HS26	M	3	HS63*	M	5	HS100*	M	0.1
HS27*	F	9	HS64	F	3	HS101*	M	4
HS28	M	5	HS65*	M	5	HS102	F	13
HS29*	F	16	HS66*	M	0.1	HS103*	M	7
HS30*	F	12	HS67	F	1	HS104*	M	4
HS31*	F	24	HS68	F	4	HS105	F	8
HS32*	F	32	HS69*	M	6	HS106	F	5
HS33*	F	32	HS70	F	11	HS107	M	13
HS34*	F	25	HS71*	M	1	HS108	M	4.5
HS35*	F	26	HS72	F	10	HS109	M	1
HS36*	M	20	HS73	F	4	HS110	M	12
HS37*	F	28	HS74*	M	6	HS111	M	18

*Samples analyzed also by mean of amplicon-based ITS1metagenomics.

We also isolated the environmental fungi *Aspergillus pseudoglaucus*, *Eurotium amstelodami*, *Eurotium rubrum*, *Penicillium brevicompactum*, *Penicillium paneum*, *Penicillium crustosum*, *Pichia caribbica*, *Pichia fermentans*, *Pichia kluyveri*, *Pichia manshurica*, *Rhodosporidium kratochvilovae*, *Saccharomyces cerevisiae*, *Starmerella bacillaris* and *Torulaspora delbrueckii*. Such species were previously found in fermentations, oenological samples (Chitarra, Abee et al. 2004;Butinar, Zalar et al. 2005;Scanlan and Marchesi 2008;Barata, Malfeito-Ferreira et al. 2012;Bezerra-Bussoli, Baffi et al. 2013;Tristezza, Vetrano et al. 2013;Vardjan, Mohar Lorbeg et al. 2013;de Melo Pereira, Soccol et al. 2014;Wang, Esteve-Zarzoso et al. 2014;Belda, Navascues et al. 2015) and rarely found in clinical samples (de la Camara, Pinilla et al. 1996;Kaygusuz, Mulazimoglu et al. 2003;Butinar, Zalar et al. 2005;Rizzetto, De Filippo et al. 2014). The 39.8% of subjects showed at least one *Candida albicans* isolate, which resulted in the most common yeast species found in our samples, in line with previous reports on the gut mycobiota of healthy subjects (Khatib, Riederer et al. 2001;Bougnoux, Diogo et al. 2006).

Table 6.2 Fungal isolates and frequencies of isolation

Species	%	Species	%
Candida albicans	39.8	*Rhodosporidium kratochvilovae*	0.57
Rhodotorula mucilaginosa	12.6	*Trichosporon asahii*	0.57
Candida parapsilosis	12.3	*Yarrowia lipolytica*	0.57
Torulaspora delbrueckii	6.59	*Aspergillus cristatus*	0.28
Pichia fermentans	4.29	*Candida deformans*	0.28
Penicillium brevicompactum	3.72	*Candida fermentati*	0.28
Pichia manshurica	3.43	*Candida glabrata*	0.28
Pichia kluyveri	2.86	*Candida intermedia*	0.28
Candida lusitaniae	2.58	*Candida metapsilosis*	0.28
Pennicillium crustosum	1.43	*Candida tropicalis*	0.28
Saccharomyces cerevisiae	1.14	*Candida zelanoydes*	0.28
Penicillium paneum	0.58	*Eurotium amstelodami*	0.28
Aspergillus glaucus	0.57	*Eurotium rubrum*	0.28
Aspergillus pseudoglaucus	0.57	*Lichtheimia ramose*	0.28
Candida pararugosa	0.57	*Pichia carribica*	0.28
Cryptococcus saitoi	0.57	*Pleurostomophora richardsiae*	0.28
Mucor circinelloides	0.57	*Starmerella bacillaris*	0.28

Population level analysis of the cultivable gut mycobiota revealed significant gender-related differences, with female subjects showing a higher number of fungal isolates ($p<0.005$, Wilcoxon rank-sum test; Fig. 6.1A) and fungal species ($p<0.05$, Wilcoxon rank-sum test; Fig. 6.1B) compared to male subjects (not related to individual's age) while we did not observed significant differences in the fungal population among the investigated age groups (Fig. 6.1C, D). Finally, no species per se was responsible for these differences, as indicated by the fact that we did not find significant differences between individual species abundances in male and female subjects for any investigated age group.

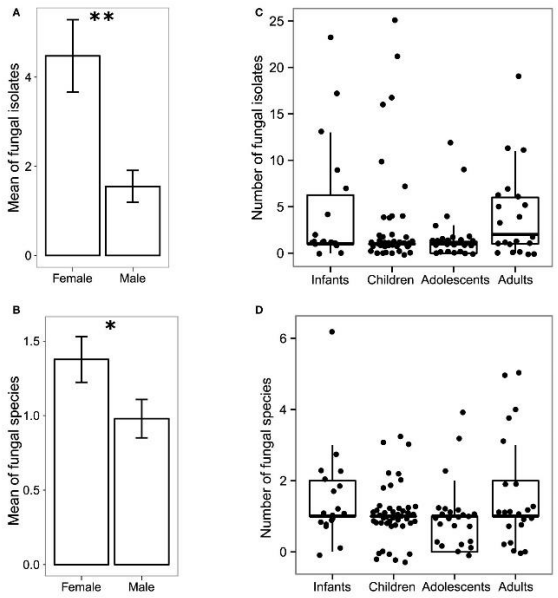


Fig. 6.1 Gender-related and age-related differences in the gut mycobiota of 111 healthy volunteers. Histogram of the mean of (A) abundances and (B) richness ± standard error of fungal isolates in female and male subjects. Box-plot representation of the (C) abundance and (D) richness of fungal isolates in different age groups i.e. infants (0–2 years old), children (3–10 years old), adolescents (11–17 years old) and adults (≥ 18 years old). $^{**}p < 0.005$,$^{*}p < 0.05$,Wilcoxon rank-sum test.

6.3.2 Fungal Gut Metagenomics

To better characterize the intestinal fungal community structure associated to our cohort of healthy subjects we further analyzed a subset of these subjects (57 subjects, 29 females and 28 males, average age 12±9.5) by means of amplicon-based ITS1 targeted metagenomics, looking at gender and age groups differences. The analysis led to the identification of 68 fully classified (to the genus level) fungal taxa and 26 taxa only partially classified (of which 2 classified to the phylum level, 5 classified to the order level, 9 classified to the class level and 9 classified to the family level). Measurements of the fungal richness within each sample i.e. the alpha-diversity (see Materials and Methods), revealed no significant differences among male and female subjects (Fig. 6.2A), differently from the above finding based on the culture-based analysis in which we observed an increased number of intestinal fungal species in females compared to males (Fig. 6.2B). Furthermore we observed that infants and children harbor a higher fungal richness compared to adults as indicated by the number of the observed OTUs ($p<0.05$, Wilcoxon rank-sum test, Fig. 6.2B). The analysis of beta-diversity identified significant differences in the composition of the gut mycobiota among gender and age groups. PCoA (Principal Coordinates Analysis) revealed that samples cluster by gender, based on the unweighted UniFrac distance and the Bray-Curtis dissimilarity ($p<0.05$, PERMANOVA; Fig. 6.2C, D) and by age groups, based on the unweighted UniFrac distance ($p<0.05$, PERMANOVA; Fig. 6.2C).

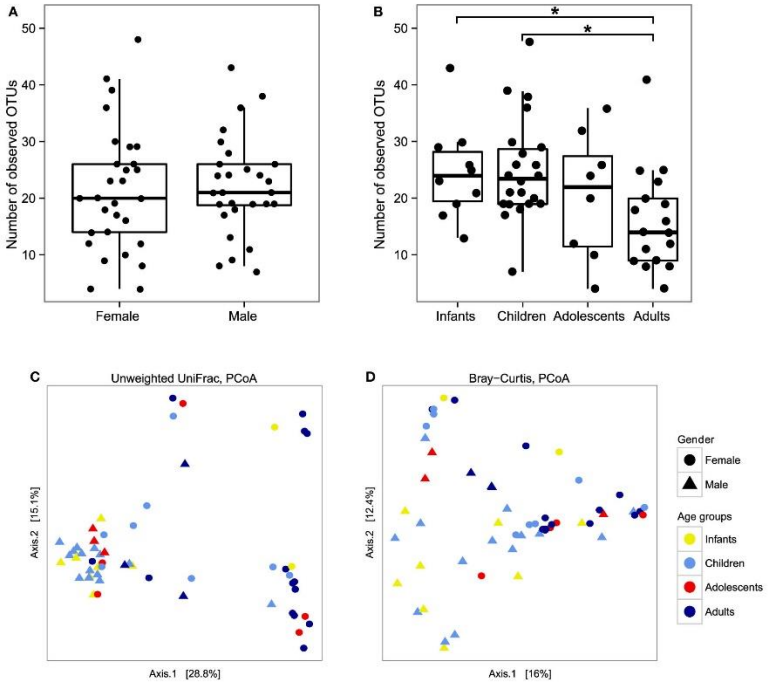


Fig. 6.2 Box-plot of fungal alpha-diversity measures using the number of observed OTUs between (A) genders or (B) age groups, and measures of fungal beta-diversity by PCoA of the between samples distances measured using (C) the unweighted UniFrac distance and (D) the Bray-Curtis dissimilarity. *$p < 0.05$, Wilcoxon rank-sum test.

We calculated PERMANOVAs constraining permutations within levels (gender or age groups) to avoid biases related to the unequal distribution of genders among age groups and

vice-versa. Genus level analysis showed *Penicillium*, *Aspergillus* and *Candida* as the most abundant genera in this subset of subjects (22.3%, 22.2% and 16.9% respectively) (Fig. 6.3). We further observed that *Aspergillus* and *Tremellomycetes_unidentified_1* were significantly more abundant in male than female subjects ($p<0.05$, Welch t-test) and in children than adults ($p<0.05$, Welch t-test). To note, the latter result could be biased by the unbalanced distribution of male and female subjects in children and adults groups (14/22 male children and 3/17 male adults). Furthermore the genus *Penicillium* was significantly more abundant in infants than adults ($p<0.05$, Welch t-test). Interestingly, we identified sequences belonging to the single-cell protozoa Blastocystis, eukaryotes abundant in the human gut microbiota (Scanlan and Marchesi 2008), only in adolescent and adult females (Fig. 6.3), that could potentially be due to exposure to pets.

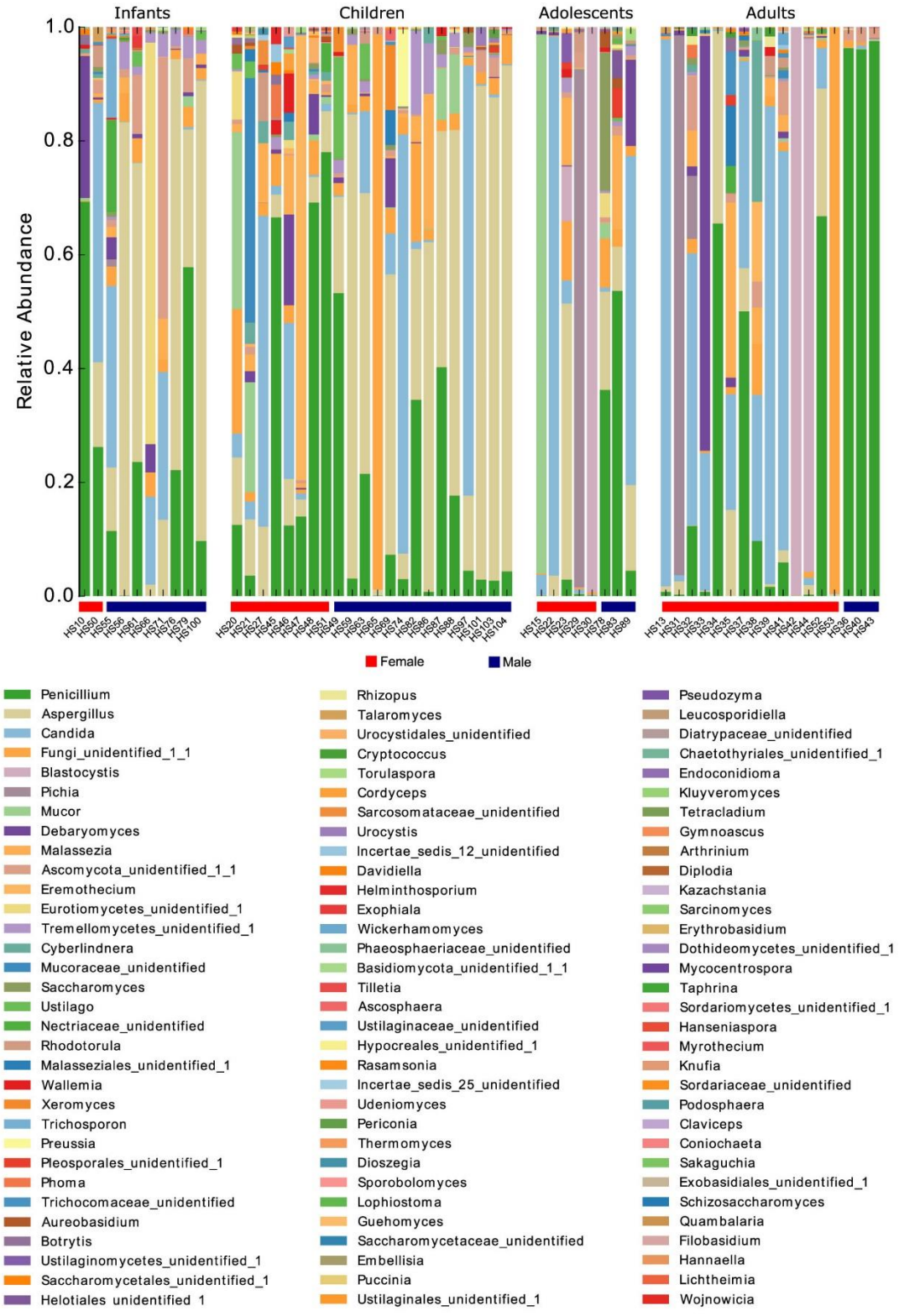


Fig. 6.3 Stacked bar-plot representation of the relative abundances at the genus level of the fecal mycobiota of healthy subjects from metagenomics analysis distributed according to individuals' life stage and gender.

6.3.3 Phenotyping the gut mycobiota

The characterization of phenotypic features of the isolates related to the ability to survive and colonize the human gut was performed to estimate if such isolates were commensals adapted to this ecological niche or passengers introduced through the diet and delivered with the feces.

We therefore investigated the isolates' resistance by a series of assays mimicking the conditions that fungal isolates face during passage through the human GI tract. In addition to the fact that the human body temperature (37°C) is higher than the optimum for most fungal species, in the GI tract fungi are also exposed to acidic and oxidative environments and to bile salts, produced by the liver and secreted into the duodenum, exposing the microorganisms to oxidative stress and DNA damage (Kandell and Bernstein 1991).

The majority of the isolates were found to tolerate acidic conditions (58.9% and 94.8% of isolates were able to grow at pH 2 and pH 3, respectively) and oxidative stress (85.7% of the isolates showed higher tolerance compared to environmental M28 *S. cerevisiae* strain), both conditions are characteristic of the gut environment. Tolerance to physiological concentrations of bile acids was also observed (89.8%, 87.5% and 85.7% of fungal isolates were able to grow in presence of ox-bile 0.5%, 1% and 2% respectively) as well as the ability to grow at supra optimal temperatures with almost all the isolates (99.4%) being able to grow at 37°C . The comparison of the growth ability of such isolates at pH 3 and at growing concentrations of ox-bile (i.e. 0.5%, 1.0% and 2.0% ox-bile) with respect to the control growth condition (37°C, no bile, pH 6.5) revealed that these stressful conditions do not significantly affect the growth ability of the fungal isolates (Fig. 6.4). By contrast, a significant growth reduction was observed when comparing the isolated grown at pH 2 with respect to the control growth condition ($p<0.0001$, Wilcoxon rank-sum test; Fig. 6.4). As expected, a progressive reduction of growth ability was observed in correspondence of incubation temperature increase (i.e. from 40°C to 46°C) for all the tested isolates ($p<0.0005$, Wilcoxon rank-sum test; Fig. 6.4).

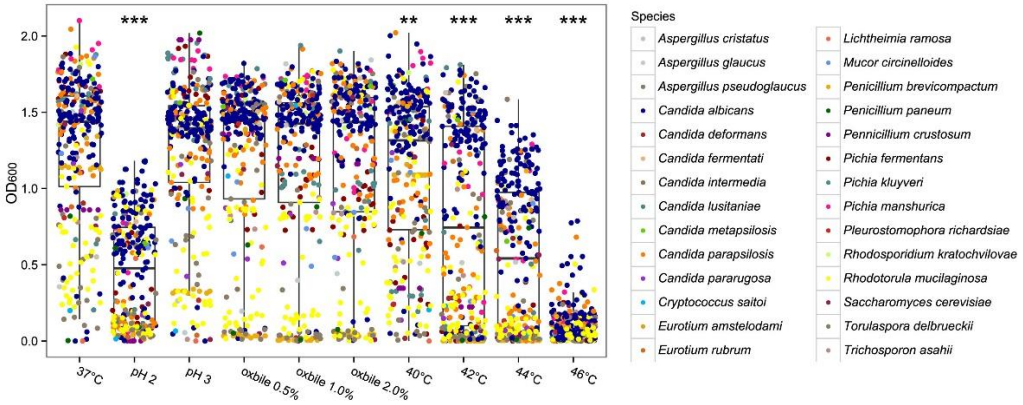


Fig. 6.4 Box-plot of the comparison of fungal isolates growth ability at 37°C (control condition) vs. different stressful conditions mimicking the gastrointestinal tract challenges. **p<0.0005, ***p< 0.0001,Wilcoxon rank-sum test.

In addition to the ability of fungal isolates to tolerate the intestinal environmental stresses, we also explored their ability to undergo phenotypic changes favoring their persistence within the human gut. Among these, we assessed the formation of hyphae and the ability to penetrate the solid growth medium, thus to adhere to host tissues. The 56.9% of fungal isolates was able to form hyphae or pseudohyphae. In addition, the morphotype switch to hyphae and pseudo-hyphae was related to the isolates' invasiveness, with hyphae and pseudohyphae-forming isolates being the most invasive (Fig. 6.5A), suggesting that such isolates may be able to adhere to or invade the host tissues. Furthermore, we observed that hyphae-forming isolates are significantly more resistant to itraconazole than pseudohyphae-forming isolates and isolates unable to form hyphae ($p<0.05$, Wilcoxon rank-sum test; Fig. 6.5B). These phenotypic traits in conditions of altered immune system or in association with intestinal dysbiosis, could represent a pathogenic potential for the host.

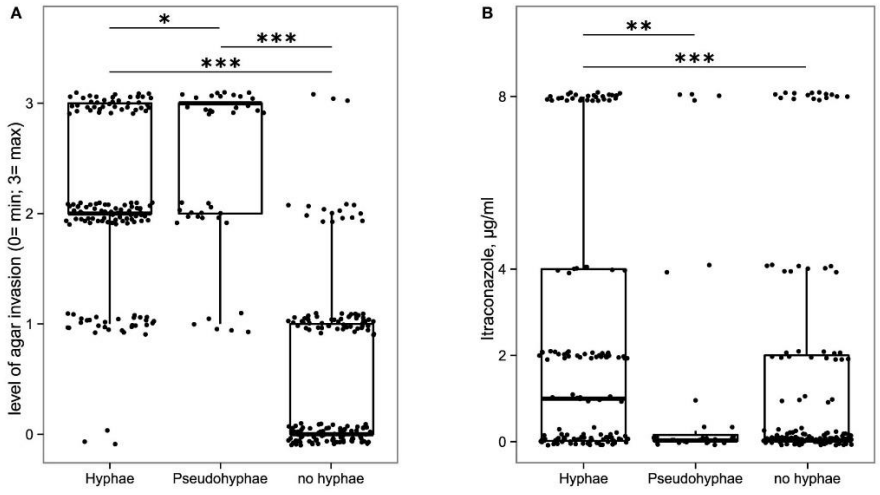


Fig. 6.5 Box-plot of fungal isolates able (or not) to produce hyphae or pseudohyphae in relationship with (A) their ability to be invasive on YPD solid medium, (B) their resistance to itraconazole. *p< 0.05, **p< 0.005,***p< 0.001,Wilcoxon rank-sum test.

It is now recognized that inappropriate antifungal use contributes to the increase in microbial antifungal resistance, complicating therapeutic intervention and the eventual eradication of pathogens (Chen, Playford et al. 2010;Arendrup, Sulim et al. 2011). Due to the relevance of such aspect and its impact on clinical studies, we tested all fungal isolates for their susceptibility to the widely therapeutically used azoles, fluconazole and itraconazole (Martin 2000) as well as the non-azole antifungal 5-flucytosine (Vermes, Guchelaar et al. 2000). A total of 31.5% of the isolates were resistant to fluconazole and, as expected, similar levels of itraconazole resistance were found (for 39.2% of the isolates the MIC was $\geq$ 1µg/ml). Previous studies have indeed suggested that cross-resistance may occur between fluconazole and other azole compounds (i.e. itraconazole) (Pfaller, Diekema et al. 2006) and we further confirmed such observations with the finding of a significant positive correlation between the isolates resistance to these two antifungals (Spearman's $r=0.43$, $p<0.05$; Fig. 6.6). Most of the isolates (99.34%) showed high susceptibility to 5-flucytosine with most MIC values $\leq$ 0.125µg/ml. Among the 9 most abundant species (at least 6 isolates per species), *C.*

albicans, *Pichia spp*. and *Rhodotorula mucillaginosa* showed the highest resistance to fluconazole, with MIC 90> 64µg/ml (Table 6.3).

Table 6.3 Antifungal activity against the most abundant fungal species

#Species (Number of tested)	Antifungal	MIC (µg/ml)		*CBPs		
		MIC_{50}	MIC_{90}	%S	%SDD	%R
Candida albicans (123)	Fluconazole	0.5	>64	65.6	0.8	33.4
	Itraconazole	2	>8	29.3	5.7	65
	5-Flucytosine	0.125	0.5	98.4	0.8	0.8
Candida lusitaniae (6)	Fluconazole	0.125	0.5	100	0	0
	Itraconazole	0.0156	0.125	100	0	0
	5-Flucytosine	0.125	0.125	100	0	0
Candida parapsilosis (40)	Fluconazole	0.5	2	92.5	0	7.5
	Itraconazole	0.031	>8	75	5	20
	5-Flucytosine	0.125	0.125	100	0	0
Penicillium brevicompactum (13)*	Fluconazole	0.125	0.125	100	0	0
	Itraconazole	0.0156	0.0156	92.5	0	7.5
	5-Flucytosine	0.125	0.125	100	0	0
Pichia fermentans (15)*	Fluconazole	32	>64	15.4	0	84.6
	Itraconazole	0.25	4	44.7	20	33.3
	5-Flucytosine	0.5	2	92.3	7.7	0
Pichia kluyveri (9)*	Fluconazole	32	32	11.1	0	88.9
	Itraconazole	0.125	0.125	88.9	11.1	0
	5-Flucytosine	0.5	0.5	100	0	0
Pichia manshurica (9)*	Fluconazole	0.25	>64	77.8	0	22.2
	Itraconazole	0.0156	>8	77.8	0	22.2
	5-Flucytosine	0.125	8	77.8	11.1	11.1
Rhodotorula mucilaginosa (41)*	Fluconazole	0.5	>64	63.4	0	36.6
	Itraconazole	0.0156	2	75.6	2.4	22
	5-Flucytosine	0.125	0.125	100	0	0
Torulaspora delbrueckii (23)*	Fluconazole	0.125	8	87	0	13
	Itraconazole	0.0156	2	69.6	4.3	26.1
	5-Flucytosine	0.125	0.125	100	0	0

**species-specific CBPs are available only for Candida and Aspergillus spp.; for those non-Candida and non-Asperigillus isolates Candida and Asperigillus' CBPs have been used as a proxy; #MIC_{50}, MIC_{90}, and CBPs have been calculated only for those species with number of isolates >5; S, sensible; SDD, Sensibility Dose-Dependent or Intermediate; R, resistant. MIC ranges: Fluconazole 0.125–64 µg/ml; Itraconazole 0.0156–8 µg/ml; 5-Flucytosine 0.125–64 µg/ml.*

Furthermore, it is worth to note that resistance to tested antifungals is positively correlated with the ability of strains to grow under stressful conditions, such as supra optimal temperature, acidic conditions, and bile salts exposure ($p<0.05$, Spearman's r correlation; Fig. 6.6).

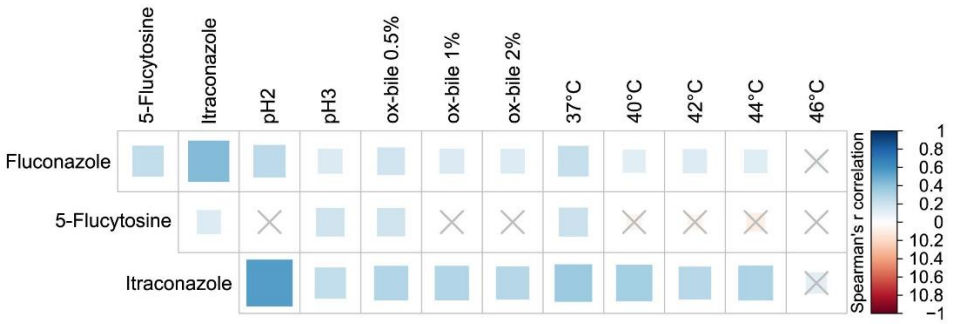


Fig. 6.6 Spearman's *r* correlation analysis between antifungals susceptibility and growth ability of the tested fungal isolates under different stress conditions. Solid squares represent the degree of correlation among the variables taken into account. Crossed squares indicate non-significant correlations; significant results with $p < 0.05$.

6.4 Discussion

The vast majority of fungal species inhabiting our body are commensals and opportunistic pathogens that could turn into potential threats depending on strain virulence traits and on the status of the host's immune system. In this perspective to discover a pathogenic infection it seems crucial to define exactly which species are normally present in a given body district. The human GI tract is known to contain variable communities of bacteria but also fungi have an important role in this ecological niche (Underhill and Iliev 2014). Nevertheless, the phylogenetic characterization of fungal microorganisms and their specific role as part of the GI niche have not yet been studied extensively.

The advent of sanitation and food globalization has reduced the possibility for humans to come across with the richness of fungal species present in traditional fermented foods. Fungal infections are an ever increasing problem either as side effects of antibiotics use, high dose chemotherapy and of the spread of immunosuppressive diseases. Estimates of global mortality rates suggest that fungi are responsible for more deaths than either tuberculosis or malaria (Brown, Denning et al. 2012). Most of this mortality is caused by species belonging to four fungal genera: *Aspergillus*, *Candida*, *Cryptococcus*, and *Pneumocystis* that are rapidly becoming resistant to most antifungal drugs (Brown, Denning et al. 2012;Denning and Bromley 2015). The information on these fungi so far derives from the study of lung infections, while little is known on the gut mycobiota composition and its role in health and disease. The knowledge on the gut mycobiota is currently limited to few studies making it difficult to assess the significance of differences found in the intestinal fungal populations of diseases such as Inflammatory Bowel Diseases due to the lack of information on what the healthy mycobiota is. Here we aimed at defining the "healthy" gut mycobiota, showing that the intestinal fungal community of a cohort of Italian healthy volunteers is a variegate ecosystem that differs in function of individuals' life stage in a gender-dependent manner. We identified 34 fungal species of different ecological origins. While the majority of our fungal isolates has been previously described as inhabitants of the mammalian gastrointestinal tract (Rizzetto, De Filippo et al. 2014), some of the isolates belong to species so far identified in environmental samples only. Environmental fungi, in particular putative food-borne fungi, have been previously observed to be able to survive the transition through the GI tract possibly being metabolically active in the gut (David, Maurice et al. 2014). The phenotypic properties of fungi isolated in this study suggested that these isolates are able to survive in the human GI tract, prompting the hypothesis of an ecological selection and potential ability to colonize this niche (David, Maurice et al. 2014). Indeed the phenotypic features of the fungal isolates identified endow such isolates with an excellent ecological fitness in the human gastrointestinal tract. We observed that approximately half of the isolates form hyphae or pseudohyphae, which are known to be involved in the adhesion to or penetration within the GI mucosa (Staab, Datta et al. 2013), consolidation of the colony, nutrient intake and formation of 3-dimensional matrices (Brand 2012). Indeed a key factor of *C. albicans* commensalism/pathogenicity is its ability to switch between different morphologies, comprising cellular, pseudohyphae and hyphae forms. As reported for *C. albicans*, the reversible transition to filamentous growth as a response to environmental cues (Sudbery 2011) and phenotypic switching is essential for mucosal fungal colonization (Vautier, Drummond et al. 2015).

Previous studies have shown that *C. albicans* over-expresses a wide range of genes involved in resistance to high temperature and pH, oxidative stress and hyphae formation during ileum and colon commensal colonization of BALB/c mice (Pierce, Dignard et al. 2013). Similarly, the fungal isolates of this study, showing resistance to oxidative, high temperature, bile acids and pH stresses may hold the potential to colonize the human gut. It is plausible that fecal fungal isolates with specific characteristics (such as high resistance to acidic pH and bile salts) survived to the gut environment, and that these traits make them able to colonize the gut. Thus, we can hypothesize a long process of evolution, selection or adaptation of environmental and food-borne strains to the human host, suggesting that pathogenic strains of commensal species can evolve through a repeated process of evolution and selection, depending on the immune status of the host. These findings encourage for in-depth, strain-level extensive studies on human gut mycobiota and the integration of such data with immunology to further establish the relevance of fungi in host physiology and host-microbe interaction. Furthermore fungi may train host's immune system simply when passengers, rather than necessarily persisting only as continuous colonizers (Rizzetto, Ifrim et al. 2016). We discovered that several fungal isolates displayed different levels of antifungal resistance. About 20 years ago, azole-sensitive *C. albicans* dominated infections, with other *Candida* species rarely observed. Actually *C. glabrata* is the second most-commonly isolated *Candida* species in the European Union and United States and has high rates of antifungal resistance (Slavin, van Hal et al. 2015). Inappropriate antifungal use has contributed to the increase in antifungal resistance, causing objective complications for the treatment of invasive fungal infections that nowadays represent a severe cause of morbidity and mortality among immunocompromised individuals, neonates and elderly (Brown, Denning et al. 2012). Recent studies indicated that fungal infections may originate from individual's own commensal strains suggesting that the ability of a commensal microorganism to promote disease is not merely a consequence of impaired host immunity (Odds, Davidson et al. 2006), suggesting that rural and other commercial uses of azole could be the culprit for the emergence of these resistant strains (Snelders, Camps et al. 2012). This underlines the risk that the increase of antifungal usage outside of the clinic could also lead to increased resistance to antifungals of individual's own commensal strains representing an important epidemiological problem in the future and remarking the importance to increase the investment in antifungal research.

It should be noted that all the samples analyzed by metagenomics resulted in high quality fungal sequences, indicating that all the fecal samples studied had fungal DNA. So far, the estimated ratio fungi/bacteria of 1:10000 (Huffnagle and Noverr 2013), discourages an approach based on whole metagenome shotgun sequencing (Underhill and Iliev 2014). We thus performed amplicon-based ITS1 metagenomics on a subset of healthy donors identifying more than 90 different fungal taxa. The first striking evidence was that metagenomics detected also sequences belonging to Agaricomycetes, among which several edible fungi, thus suggesting that dietary fungal intake is a potential confounding effect when studying the gut mycobiota. On the contrary, 34 different fungal species were isolated using the culture-based approach. Both methods detected in any case differences in the diverse groups of study. The discrepancies observed between culture-dependent and culture-independent approaches on the description of fungal populations could be attributed to the

methodological differences of the two procedures applied suggesting that several of the fungal taxa identified by the metagenomics approach are not cultivable, either because we lack the proper culture conditions or because these belong to DNA from dead cells, environmental or food-borne fungi that cannot survive the passage through the GI tract, but whose DNA is still detectable. Furthermore, the DNA extraction method used could not be suited to extract all the fungal DNA from the stool samples since the rare taxa *Yarrowia*, *Starmerella*, *Rhodosporidium* and *Pleurostomophora* have been found only by the culture-based approach. On the other hand, the culture condition that we used might be responsible for some of the discrepancies observed between the two methods. In our experience, the vast majority of human commensal fungi grows in YPD, yet other environmental derived fungi that we were not able to cultivate, might need different culture conditions from those we used in this work. Although, for example, Saccharomyces cerevisiae is often found in fermented food, it has been shown that it can survive GI tract challenges being a commensal of the human GI tract (Rizzetto, De Filippo et al. 2014) educating also adaptive immunity (Rizzetto, Ifrim et al. 2016). *S. cerevisiae* has been introduced in the human intestine through diet and fermented beverages and has accompanied human evolution for at least the past 5150 years (Cavalieri, McGovern et al. 2003). Our evidence, together with previous results, including a recent description of *S. cerevisiae* in IBS patients gut (Sokol, Leducq et al. 2016) shows that this microorganism is a potential commensal of the human intestine. The overall reduction of the amount and diversity of fungi introduced through consumption of fermented beverages suggests that the human gut mycobiota could be in dynamic change and certain potentially beneficial species could be lost as a result of modern food processing procedures, cultural changes and food globalization. Ongoing studies on microbial anthropology in human populations consuming traditional fermented foods hold the promise to shed light on the evolution of the fungal mycobiome as associated to the evolution of diet. On the contrary, the edible fungi belonging to *Agaricomycetes* cannot settle in the human gut due to their ecology (Hibbett 2006) so we filtered-out these sequences for downstream analyses to reduce statistical noises on ecological measures, improving our results on the characterization of intestinal fungal communities. We are aware that other taxa identified by our analyses having environmental and food-borne origin may not be able to settle in the human gut, however little is known about these taxa while the *Agaricomycetes* sequences that we retrieved had a very low prevalence in the dataset and mostly belonged to edible fungi such as *Boletus*, *Suillus* or *Agrocybe*.

We further observed that amplicon-based ITS1 metagenomics cannot confidently describe fungal populations at a deeper level than genus overlooking species level information provided by the fungal cultivation approach. On the other hand, metagenomics analysis detected community structure differences that fungal cultivation did not identified. However, the analysis of alpha-diversity from cultivation data on the subset of subjects used for the metagenomics analysis revealed no significant differences among genders remarking that the different sample sizes used in this work are an additional factor in the discrepancies observed between the two methods. Although the major limitation of culture-based methods for the study of microbial communities is the loss of ecological information due to the inability to cultivate most microorganisms by standard culturing techniques, fungi included,

culture-based analysis of the human gut mycobiota is fundamental to discern fungal phenotypes that would be otherwise lost by metagenomics.

In addition, population level analyses with both approaches revealed interesting cues. As occurs for the bacterial microbiota, the intestinal mycobiota is shaped by host's age, gender, diet and geographical environment (Yatsunenko, Rey et al. 2012;Hoffmann, Dollive et al. 2013;David, Maurice et al. 2014). Previous studies have shown that the development of the gut bacterial microbiota starts at birth with colonization by a low number of species from the vaginal and fecal microbiota of the mother and is characterized by many shifts in composition during infancy (Yatsunenko, Rey et al. 2012). Similarly, the mycobiota may show the same fate, but we observed an inverted trend in which the richness of the gut mycobiota of infants (0-2 years old) and children (3-10 years old) was higher than adults (≥18 years old). It has been shown that suppression of the bacterial microbiota upon treatment with antibiotics results in the outgrowth of the gut mycobiota (Dollive, Chen et al. 2013) probably as a consequence of reduced ecological competition. Similarly, a weak bacterial competition, in particular during infancy when the bacterial microbiota is less stable (Koenig, Spor et al. 2011;Lozupone, Faust et al. 2012), could be the reason why we observed an increased fungal alpha-diversity during the early stages of life or this could be due to the different interactions between intestinal fungi and diet (Hoffmann, Dollive et al. 2013;David, Maurice et al. 2014) which is peculiar during infancy. We also found that female subjects had a higher number of fungal isolates and different fungal species compared to male subjects and that female mycobiota cluster apart from male mycobiota. This may be ascribed to the role of sex hormones in modulating microbiota composition (Markle, Frank et al. 2013) and of diet in shifting the microbiota composition in a gender-dependent manner (Bolnick, Snowberg et al. 2014). Furthermore the higher relative abundance of Candida in the fecal samples from female than male subjects could be also attributed to the prevalence of Candida species in the vaginal mycobiota (Drell, Lillsaar et al. 2013) due to the anatomical proximity of the two districts. To the best of our knowledge, this is the first time that gender-related differences are described in the human gut mycobiota.

In conclusion, we can state that culture-independent approaches are very promising for future investigation of the mycobiota, but require significant improvements in the selection of markers for amplicon-based metagenomics and the reference databases. Additionally development of markers targeting pathogenicity traits, including the genes involved in host invasion or evasion of immune defenses, or markers detecting resistance to azoles or other antifungals, is required to thoughtfully apply metagenomics to fungal infections, discriminating the healthy mycobiota from an altered one. Such improvement can be achieved only through systematic sequencing efforts of the cultivable mycobiota, paralleling what happened for the prokaryotic microbiota. In our experience, currently, the combination of the two methods compensated the methodological limits intrinsic in both approaches avoiding to overlook significant differences present in the gut mycobiota of healthy subjects.

7. Genotyping and immunophenotyping of cultivable fungal isolates from pediatric patients affected by Inflammatory Bowel Disease and their interaction with bacterial communities

This chapter will be shortly submitted for publication as original research articles:

Di Paola M., De Filippo C., Stefanini I., Rizzetto L., Rivero D., Ramazzotti M., Tocci N., Lenucci M.S., Lionetti P., Cavalieri D.

Genotyping and immunophenotyping of the Crohn's disease mycobiome reveals its interactions with the bacterial communities of the gut

Di Paola M., Rizzetto L., Colombari B., Bernà L., De Filippo C., Ardizzoni A., Tocci N., Paulone S., Lionetti P., Gut I. G., Blasi E., Cavalieri D., Peppoloni S.

Candida albicans isolates with different genomic backgrounds show diverse host adaptation and differential susceptibility to phagocytosis

7.1 Scientific background

Several studied investigated the contribution of bacterial communities in the etiology of Inflammatory Bowel Disease (IBD) (Seksik, Rigottier-Gois et al. 2003;Gophna, Sommerfeld et al. 2006;Frank, St Amand et al. 2007;Ott, Kuhbacher et al. 2008;Sokol, Lay et al. 2008;Gevers, Kugathasan et al. 2014;Machiels, Joossens et al. 2014). Despite the large amount of metagenomics and clinical information, the studies on gut microbiota did not lead to discovery of the causative agents of IBD, nor allowed novel intervention strategies.

Few studies investigating the mycobiota in chronic inflammation described a more heterogeneous community, especially in patients affected by Crohn's disease (CD) compared with healthy subjects, and suggesting a contribution of altered mycobiota as the causative agents of diseases, such as Irritable Bowel Syndorme, IBD, and "leaky gut" syndrome (Sendid, Quinton et al. 1998;Boorom, Smith et al. 2008;Ott, Kuhbacher et al. 2008;Schulze and Sonnenborn 2009;Li, Wang et al. 2014;Sokol, Leducq et al. 2016). In fecal samples from IBD patients, Chehoud et al. (Chehoud, Albenberg et al. 2015) found a simplification of fungal microbiota, mainly enriched in *Candida spp.*. A study on pediatric subjects showed that fungal taxa such as *S. cerevisiae*, *Clavispora lusitaniae*, *Cyberlindnera jadinii*, *C. albicans*, and *Kluyveromyces marxianus* were positively associated with CD (Lewis, Chen et al. 2015). Finally, Sokol and co-workers (Sokol, Leducq et al. 2016) observed a clear fungal dysbiosis in CD patients, with enrichment in *Candida spp*. and reduction of *S. cerevisiae* in disease versus remission.

Studies on the mice model showed that gut inflammation promotes fungal proliferation (Jawhara and Poulain 2007). In a mice model of chemically-induced colitis, an association

between gut inflammation and an increase in *Candida* was observed (Iliev, Funari et al. 2012).

Interestingly, amongst IBDs one of the markers proposed to discriminate CD from UC was the positivity for anti-*Saccharomyces cerevisiae* antibodies (ASCA) (McKenzie, Main et al. 1990;Quinton, Sendid et al. 1998). This could be a result of colonization with *S. cerevisiae* and increase in permeability on conditions associated to a leaky gut, but previous studies showed that ASCA is produced also in response to *Candida* or other yeast. In fact, the production of ASCA appear to be induced by several types of mannans present in the fungal cell wall of different *Ascomycetes* (Sendid, Quinton et al. 1998;Standaert-Vitse, Jouault et al. 2006). Thus, the fungal and host immune system interactions could worsen the inflammatory process in gastrointestinal disease, especially in IBD, as a result of the composition of the fungal cell wall.

Candida albicans is a commensal for approximatively in 80% of our body, inhabiting our skin, GI and urogenital tracts (Rizzetto, Ifrim et al. 2016). Genome-wide surveys of the microorganisms (Costello, Stagaman et al. 2012) have suggested the presence of strong selective control on microbial communities that colonize and persist in particular environments. Adaptation to a new environment is of fundamental importance in ecology. Infectious agents adapt to escape detection by the immune system, as well as damage by safer target specifically drugs. Although *C. albicans* isolates tend to show clonal origin, recombination and cross-chromosomal rearrangements may occur and result more common under stressful environmental conditions, such as prolonged drug treatments or exposure to host immune defences, playing a significant role in promoting microevolution of this opportunistic pathogen.

Different fungal isolates differ in adhesion, filamentous growth, and virulence (Ford, Funt et al. 2015), revealing host adaptation and suggesting immune evasion strategies. Hyphal growth and biofilm formation are key factors promoting fungal escape from phagocytes (Marcil, Harcus et al. 2002;Ghosh, Navarathna et al. 2009;Orsi, Borghi et al. 2014) by impairing phagosome maturation, a key process in the control of infection and pivotal to both innate and adaptive immunity.

The scarcity of *S. cerevisiae* in the gut of healthy controls (chapter 6) found in our study (Strati, Di Paola et al. 2016) and its presence in IBD patient's gut is particularly striking.

Foods are thought to be the principal fungal sources for the host (Scanlan and Marchesi 2008). Fermented foods and beverages, such as bread, beer, and wine, are containing eukaryotes species, especially *S. cerevisiae*. The long lasting association of *S. cerevisiae* with human activities (3150 B.C.) (Cavalieri, McGovern et al. 2003) has led to the idea that its population biology and evolution (Aa, Townsend et al. 2006;Ezov, Boger-Nadjar et al. 2006;Ruderfer, Pratt et al. 2006;Legras, Merdinoglu et al. 2007;Liti, Carter et al. 2009;Dunn, Richter et al. 2012) are mainly shaped by the role of this microorganism in industrial fermentations, rather than from commensalism. Recent discovery of the role of wasp's gut as a winter reservoir and potential evolutionary niche for *S. cerevisiae* indicated how the ecological cycle of this microorganism is just beginning to be understood (Stefanini, Dapporto et al. 2012;Stefanini, Albanese et al. 2016). The possible evolution of *S. cerevisiae* in the animal GI tract is not limited to wasps or fruit-flies (Goddard and Greig 2015), but probably extends to vertebrates and finally to mammals.

The recent studies have begun to note that the mycobiota is a significant player in host-microbe interactions, and is able to shape both innate and adaptive immunity (Xu, Boyd et al. 1999;Hardison and Brown 2012;Sancho and Reis e Sousa 2012;Angebault, Djossou et al. 2013;Rizzetto, De Filippo et al. 2014;Nguyen, Viscogliosi et al. 2015;Romani, Zelante et al. 2015). It is hypothesized that differences in fungal colonization are related to differences in the host genetic makeup or in gut permeability. Furthermore, an unpaired immune response to fungi could result in altered fungal colonization/infection (Strope, Skelly et al. 2015).
In this work, we investigated the immunomodulatory features of fungal isolates from IBD patients, especially *S. cerevisiae* and *C. albicans* isolates, characterizing phenotypically and genotypically for traits related to adaptation to gut environment in order to understand the strategies related to interplay or evasion of host immune system, and the relationships between fungal and bacterial gut communities.

7.2 Materials and Methods

7.2.1 Enrolment of patients and healthy subjects

A total of 93 pediatric subjects were enrolled at the Meyer Children's Hospital (Florence, Italy). All individuals were Caucasian and the age ranged from 4-19 years (Table 7.1). For CD patients, disease activity was scored using the Pediatric Crohn's Disease Activity Index (PCDAI). For UC patients, a similar index was used to measure disease activity, the Pediatric Ulcerative Colitis Activity Index (PUCAI), as reported in materials and methods of chapter 4. Inflammatory activity was assessed in all IBD patients through clinical parameters, such as erythrocyte sedimentation rate (ESR), C Reactive Protein (CRP) and fecal calprotectin, a useful marker of mucosal inflammation (Aomatsu, Yoden et al. 2011). Anti-*Saccharomyces cerevisiae* Antibodies (ASCA), IgA and IgG levels were evaluated for each IBD patient (Table 7.1).
By calprotectin measure, mucosal inflammation of IBD patients was evaluated routinary together with endoscopy. To induce disease remission, different therapeutic regimens were adopted, such as enteral nutrition with a polymeric formula, surgery, immunosuppressant and immunomodulating therapies (such as 6-mercaptopurine and azathioprine), Infliximab (anti-TNFα Antibody), steroids (budesonide, prednisone, beclomethasone) and 5-aminosalycilates (sulfasalazine, mesalamine).
All enrolled individuals were made aware of the nature of the experiment, and all gave written informed consent in accordance with the sampling protocol approved by the Ethical Committees of the Meyer Children's Hospital and the Azienda Ospedaliera Universitaria Careggi, Florence, Italy (Ref. n. 87/10).

7.2.2 Isolation and identification of yeast species from fecal samples

Feces were collected from all pediatric subjects. A 1 ml feces aliquot was plated on Yeast Extract-Peptone-Dextrose (YPD) agar medium supplemented with chloramphenicol (1mg/ml) and incubated at 28° C. After 2-3 days, fungal colonies were observed. Yeast genomic DNA was extracted as previously described by Sebastiani and collaborators (Sebastiani, Barberio et al. 2002). Fungal isolates were identified by sequencing of the

ribosomal Internal Transcribed Spacer (ITS) region, using ITS1 (5'-GTTTCCGTAGGTGAACTTGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC -3') primers, using megaBLAST algorithm in the NCBI database. Long-term storage of the isolates was carried out in 20% glycerol at –80°C.

For host-fungal interaction studies, isolates were cultured overnight at 37°C in Sabouraud dextrose agar (SDA, Oxoid, Hampshire, UK). Then, yeast cells were washed twice with sterile Phosphate Buffered Saline (PBS, EuroClone, Wetherby, UK), counted and suspended in Roswell Park Memorial Institute (RPMI) 1640 medium at the desired concentration.

7.2.3. Statistical correlation between clinical parameters, phenotypes and fungal isolates

To evaluate variables that might influence the presence of yeast in fecal samples, such as clinical parameters, sex, age and location of the inflammation and treatment, we performed logistic regression using automated model selection. To observe correlations between yeast isolates from IBD, clinical features and ASCA, we used the Wald test. Chi Square statistics were applied to associate significant correlations with the variables mentioned above. Yates correction was applied in the case of expected frequencies less than 5. G test was applied in order to evaluate significant correlation fungal isolates between mucosal indeces. Species richness and biodiversity in yeast populations from the three groups were estimated using the Species Prediction and Diversity Estimation (SPADE) program (http://chao.stat.nthu.edu.tw). Biodiversity was estimated by comparing the Shannon and Simpson indices. Fisher Exact Test was performed in order to evaluate significant correlations between different virulence-related traits.

7.2.4 Phenotypical characterization of fungal isolates

S. cerevisiae and *C. albicans* isolates were tested for virulence related traits. We evaluated the ability of strains to rapidly adapt to environmental changes and acquire different phenotypic conformations (e.g. pseudohyphae, spores) in order to assess the potential ability of yeast cells to invade human tissues. The phenotypic characteristics of *S. cerevisiae* were compared to M28-4D and BY4742 *S. cerevisiae* laboratory strains, while for *C. albicans* strains the reference strain was SC5314 (ATCC® MYA-2876™).

Invasive growth. The ability of fungal isolates to penetrate solid rich medium (YPD, Yeast Peptone Dextrose, 1% Yeast Extract, 2% Peptone, 2% Glucose, 2% agar) was tested in order to assess the potential ability of yeast cells to invade human tissues. Yeast cells (10^4 and 10^2 cells/ml) were spotted on solid YPD plates and incubated at 28°C and 37°C. After 5 days of growth, cultures were stained with Coomassie blue as previously described (Palkova 2004). As a result, the invading cells are colored blue. M28-4D and BY4742 *S. cerevisiae* strains, known to be highly invasive and non-invasive respectively, were used as controls. SC5314 *C. albicans* strain, showing invasivity, were used as controls.

Resistance to oxidative stress. Yeast resistance to oxidative stress was evaluated by measuring the inhibition halo, treating S. cerevisiae strains (10^7 cell/ml) with tert-Butyl-hydroperoxide (tBut-OOH). After 5 days of incubation at 28° C, the inhibition halo on YPD+ 1 M tButOOH was measured. The percentage of sensitivity to oxidative stress was calculated as the deviation of the inhibition halo diameter (Ø) from that of the M28-4D *S. cerevisiae*

strain, showing the highest resistance to oxidative stress (0% sensitivity), according to the following formula: (Ø sample – Ø M284D strain) / Ø M284D strain *100.

Growth at supra optimal temperatures. *S. cerevisiae* and *C. albicans* strains were grown at supra optimal temperatures, to test a typical feature of clinically isolated and pathogen strains. Yeast cells (10^6 cell/ml) were spotted on solid YPD medium and incubated at 4° C, 40° C, 42° C, and 44° C for 3 days.

Pseudohyphal and Hyphal formation. Yeast cells (10^6 cells/ml) were grown in liquid YPD and YNB media (0.67% Yeast Nitrogen Base w/o amino acids and $(NH_4)_2SO_4$ and 2% glucose), both at 28°C and 37°C. After 7 days of incubation, pseudohyphae formation was scored for *S. cerevisiae* strains and hyphae for *C. albicans* strains. Every *S. cerevisiae* strain isolated from IBD patients and HS was tested and compared with the *S. cerevisiae* laboratory strain BY4742, known to be incapable of assuming pseudohyphal conformation. The *C. albicans* reference strain was SC5314 (ATCC® MYA-2876™).

Sporulation rate. A large amount of pre-aged *S. cerevisiae* cells, grown on YPD, was transferred onto Spo IV (sporulation medium: 2% Potassium acetate, 0.25% Yeast Extract, 0.1% glucose) and incubated at 4°C, 28°C and 37°C. After 5 and 10 days, spore formation was assessed. The SK1 *S. cerevisiae* strain was used as a positive control (100% sporulation efficiency). The sporulation percentage was calculated as a ratio of the number of asci observed and the total number of cells and asci.

pH impact on growth. S. cerevisiae strains were grown on YPD medium at pH 3.0, 4.8 and 7.4, adding citrate, acetate, and phosphate buffers respectively.

Growth on different carbon sources. C. albicans yeast cells (10^6 cells/ml) were grown in solid YP added with the following carbon sources (2%): glucose, galactose, glycerol, ethanol, sucrose, oleate, and evaluating the survival after 3 days.

Antifungal agent resistance. Antifungal drugs susceptibility was performed evaluating the growth of *C.albicans* yeast cells (10^6 cells/ml) in solid YPD added with different concentration of fluconazole and ketoconazole (0.01 mM, 0.1 mM, 0.5 mM, 1 mM and 5 mM for each drug). SC5314 was used as control. Colony formation was evaluated after 24 h, 48 h and 72 h of growth at 28°C.

7.2.5 Biofilm formation assay of *C. albicans* strains and thickness measurement

Candida strains were grown overnight in SDA plates at 37°C. Cells were washed with PBS and standardized to 1×10^5 or 1×10^6 yeast cells/ml in complete RPMI. Then, 100 µl aliquots of each yeast cell suspension were placed in 96-well polystyrene microplates and incubated at 37°C for 24 and 48 h. After incubation, the biofilm grown in each well was quantified by means of the crystal violet (CV) assay, according to previously described protocols (Orsi, Borghi et al. 2014). To evaluate the number of both yeasts and budding yeast cells released in the biofilm supernatants, biofilms were grown for 48 h as described above. One-hundred microliters of culture supernatants were then removed from each wells and the number of both yeasts and budding yeast cells counted using the chamber Burker.

To measure the biofilm thickness, 10^6 cells/ml *C. albicans* strains were seeded in 6 wells plates (Nalge Nunc International, Naperville, IL, USA), in complete RPMI and incubated for 24 h and 48 h in presence of CO_2. The cells were washed with PBS at room temperature,

fixed with 4% PFA for 30 min at 4°C and delicately washed with cold PBS to remove PFA. The plates were then placed upside down and were allowed to dry for 1 hour. The samples were finally visualized using an optical microscope equipped with Nomarski DIC optics (Nikon Instruments Inc. USA), in order to measure the biofilm thickness. The samples were moved (2 μm steps) along their vertical axis starting from the biofilm bottom to the hyphae top. Microphotographs were taken at each focal plan by means of a DS-2Mv Nikon digital camera, and the resulting photographs were analyzed [Nikon NIS ELEMENTS (version D3.1) software]. By taking into account the analyzed μm, and by putting together the acquired optical sections in a sequence, such software allowed to visualize each biofilm sample in a movie format.

7.2.6 *Candida* cell morphology studies

To examine cell morphology, $1x10^6$/ml *Candida* cells were seeded in 24-well polystyrene plates with plastic cover slips for 24 h at 37°C under 5% CO_2. Then the cells were washed three times with PBS and each strain was fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 0.1 M phosphate buffer (pH 7.2) for 18 h at 4°C. The samples were then carefully washed with 0.1 M phosphate buffer (pH 7.2). Post-fixation was carried out for 1 h at room temperature with 1% osmium tetroxide (Electron Microscopy Sciences). Initial dehydration was accomplished by placing specimens in the following series of ethanol gradients: 30% (one time for 3 min), 50% (one time for 6 min), 70% (one time for 10 min), 90% (two times for 8 min) and 100% (three times for 8 min). The samples were critical point dried (Critical Point Dryer, CPD0-10, Balzers, Lichtenstein) before being gold coated (Sputter coater K550, Emitech Ltd, Ashford, UK) and examined with a Scanning Electron Microscope (SEM, Nova-NanoSEM 450, FEI Company, OR, USA).

7.2.7 *In vitro* phagocytosis assay

The following experiments are conducted in collaboration with laboratory of Prof. Peppoloni from University of Modena and Reggio, Italy. The previously established murine microglial cell line BV2 (50) was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Defined Hyclone, Logan, UT, USA), gentamicin (50 mg/ml; Bio Whittaker, Verviers, Belgium) and L-glutamine (2 mM; EuroClone, Milan, Italy), hereafter referred to as "complete medium". Cells were detached biweekly by vigorous shaking and fresh cultures were started at a concentration of $5\ x10^5$/ml on the day before each experiment. BV2 cells were cultivated at 37°C under 5% CO_2.

To strengthen attachment of BV2 cells to the wells, Lab-Tek II chamber slides (Nalge Nunc International, Naperville, IL, USA) were pretreated with poly-L-lysine (Sigma Aldrich; 10 mg/well) for 30 min, and washed twice with PBS. BV2 cells ($2x10^6$/ml, 100 μl/well) were incubated for 15 min and then infected with a suspension of *C. albicans* cells ($4x10^6$/ml, 100 μl/well) in complete RPMI [effector to target (E:T) ratio of 1:2]. The BV2 cells were incubated for 1.5 or 3 h at 37 °C under 5% CO_2. Fifteen minutes before the end of incubation, the *C. albicans* cells were treated with Uvitex 2B (40 μl/well, Polisciences, Inc, PA, USA), a fluorescent dye that bind the chitin in the fungal cell wall. The BV2 cells were then washed

three times with PBS to remove extracellular yeasts and fixed for 30 min with 4% paraformaldehyde (PFA) (Sigma Aldrich) in PBS. BV2 cells were washed with PBS and then treated with ProLong Gold Antifade Reagent (PLGAR, Molecular Probes, Invitrogen, St. Louis, Mo, USA) in order to suppress the photobleaching effect and preserve the signals of fluorescently labeled target molecules. The remaining blue fluorescence of Candida bound to BV2 cells, as well as the unlabeled internalized fungi, was visualized by epifluorescence microscopy (Nikon Instruments). At least 200 microglial cells from each sample were examined and the percentage of cells with intracellular yeasts was defined as the ratio of the number of BV2 cells containing one or more unlabeled *Candida* to the total number of cells examined.

7.2.8 Intracellular survival assay

BV2 cells ($2x10^6$/ml, $5x10^6$/2.5 ml) were incubated in 25-cm^2 tissue-culture flasks (Nalge Nunc International, Naperville, IL, USA) for 1.5 h with the *Candida* strains ($4x10^6$/ml, 10^7/2.5 ml) in complete RPMI medium at an E:T ratio of 1:2. Cells were washed twice with PBS to remove the extracellular yeasts, suspended in complete RPMI (time 0) and seeded in twenty-four-well polystyrene plates (Nalge Nunc International, Naperville, IL, USA). At 0, 3, 6 and 20 h post-infection, the BV2 cells were lysed with 0.2% (v/v) Triton X-100 for 15 sec to release intracellular *Candida*. Serial dilutions of the lysates were then plated onto SAB plates and incubated for 24 h. The results were expressed as Survival Index (SI) at different time points. The SI was calculated as the number of CFU detected at 3, 6 and 20 h divided by the CFU number obtained at the time 0. Control experiments were carried out to verify that the treatment with Triton X-100 was not toxic for the *Candida* (data not shown).

7.2.9 Phagolysosome acidification assay

Visualization of the acidic *Candida*-containing phagosomes was performed as described previously (Orsi, Colombari et al. 2009), using the acidotropic dye LysoTracker Red DND-99 (Molecular Probes, Invitrogen) at a final concentration of 5 mM. The blue fluorescence (Uvitek 2B labelling) of bound *C. albicans* and the unlabeled internalized *Candida* (no fluorescence) were visualized by epifluorescence microscopy. Successful acidification of phagosomes containing unlabeled *Candida* was indicated by the simultaneous appearance of LysoTracker Red DND-99 (red fluorescence) and *Candida* cells within the phagosomes, resulting in red fluorescence when merging the images. For quantitative analysis, the percentage of acidic phagosomes per image was calculated as the number of red phagosomes within the phagocytic cells divided by the total number of *Candida* internalized (unlabeled yeasts).

7.2.10 *In vitro* adhesion assay and secretory activity of human epithelial cell line Caco-2

The human epithelial colorectal adenocarcinoma cell line Caco-2 was grown in 75-cm^2 tissue-culture flasks (Nalge Nunc International, Naperville, IL, USA). Just before they reach the confluence, Caco-2 cell cultures were split 1:3 by standard methods. For the culture of

the Caco-2 cells Dulbecco's Modified Eagle's Medium (DMEM; EuroClone, Milan, Italy) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine and 50 mg/ml gentamycin was used. The cells were cultivated at 37 °C under 5% CO_2. The Caco-2 fresh cultures were started at a concentration of 6.5 x10^5/ml for the adhesion experiments and at a concentration of 8 x10^5/ml for the secretion assay.
Caco-2 cells (1.3 x 10^5/200µl, 6.5 x 10^5/ml, 200 µl/well) were seeded in Lab-Tek II chamber slides, grown at 37°C to confluence (24 h) and then infected with a suspension of *Candida* (5.2 x 10^5/200 µl, 2.6 x10^6/ml, 200 µl/well) in complete RPMI (E:T ratio of 1:2). Caco-2 cells were incubated for 1.5 or 3 h at 37°C under 5% CO_2. Fifteen min before the end of incubation, *C. albicans* cells were treated with 40 µl/well of 1% Uvitex 2B, washed three times with PBS to remove unbound yeast cells and fixed for 30 min with PBS-buffered 4% PFA. The Caco-2 cells were washed twice with PBS and then treated with PLGAR. The blue fluorescence of bound *C. albicans* was visualized by epifluorescence microscopy. At least 200 epithelial cells from each sample were examined and the percentage of Caco-2 cells with adherent yeasts was defined as the ratio of the number of Caco-2 cells with one or more *C. albicans* to the total number of Caco-2 cells examined.
Caco-2 cells were seeded in 24 well-plates and were allowed to reach the confluence for 24 h. *Candida* strains (E:T ratio of 1:100) were then added and incubated for 6 h and 24 h. The presence of β-2 defensin in the culture supernatants was assessed by means of enzyme immunoassay [β-2-Defensin (Human), Phoenix Pharmaceuticals Inc., Karlsruhe, Germany].

7.2.11 Epifluorescence microscopy

Prior to visualization, Lab-Tek II chamber slides were washed with PBS and then treated with PLGAR. Epifluorescence and differential interference contrast (DIC) microscopy were performed using a Nikon Eclipse 90i imaging system equipped with Nomarski DIC optics (Nikon Instruments Inc. USA). At each time point, samples were photographed with a DS-2Mv Nikon digital camera, and the resulting microphotographs were analyzed by using the Nikon NIS ELEMENTS version D3.1 software.

7.2.12 *S. cerevisiae* population genetic analysis

A set of 129 strains was characterized in terms of allelic variation at 12 microsatellites loci (Legras, Merdinoglu et al. 2007). The chord distance Dc matrix was calculated for each strain with a laboratory-made program. The phylogenetic tree was obtained with the Neighbor-joining method from the distance matrices with the Phylip Neighbor 3.67 package and drawn up using MEGA4.0 (Tamura 2011). The tree was rooted using the midpoint method.
Sequences of analyzed samples submitted to GenBank (ID: KF261601-KF261724), were compared with *S. cerevisiae* pre-edited sequences downloaded from the Sanger institute website. The collection included strains isolated from a wide variety of sources, such as grapes, vineyards, wasps, and different types of fermentation, as well as other clinical strains (isolated in concomitance with other fungal infections). SNPs identified by alignment against the reference S288C genome were concatenated to give a unique sequence. The aligned/concatenated SNPs sequences were used to compute distances with the Kimura two

parameter model (Felsenstein 1989) using Phylip dnadist and then clustered with the Neighbor-joining method (Saitou and Nei 1987), using Phylip Neighbour.
Population ancestries were estimated by using the model-based program Structure (Pritchard et al. 2000). K=5 and K=6 were chosen as the most representative of the population structures for the three genome-mimicking gene sequences. The results of 10 independent Structure chains were combined with CLUMPP. Population structure was also evaluated using principal component analysis (PCA) and Differential Analysis of Principal Components (DAPC) using the R adegenet package. The groups' inter- and intra- genetic distances, calculated with the Kimura two parameter model, were statistically tested using the Mann-Whitney U test and Levene test.

7.2.13 Whole genome analysis of two *C. albicans* isolates

Whole genome sequencing was performed using the Illumina GAIIx and HiSeq2000 sequencing instruments. The standard Illumina protocol with minor modifications was followed for the creation of short-insert paired-end libraries (Illumina Inc.). In brief, 2.0 μg of genomic DNA was sheared on a Covaris™ E220 in order to reach the fragment size of ~500bp. The fragmented DNA was end-repaired, adenylated and ligated to Illumina specific paired-end adaptors. To obtain a library of very precise insert size (500bp) with the size deviation of +/- 25bp, the DNA with adaptor-modified ends was size selected and purified using the E-gel agarose electrophoresis system (Invitrogen). After size selection, the library was PCR amplified using 10 PCR cycles. Each library was run in a fraction of a GAIIx flowcell lane in paired end mode of 2x151bp or on HiSeq2000 in 2x101bp read length, both according to standard Illumina operation procedures. Primary data analysis was carried out with the standard Illumina pipeline. The purity of the signal from each cluster was examined over the first 25 cycles and chastity = Highest_Intensity/(Highest_Intensity + Next_Highest_Intensity) was calculated for each cycle. To remove the least reliable data from the analysis, reads were filtered according to chastity > 0.6, for all but one of the first 25 bases. If there were two bases, the read was subsequently removed.

7.2.14 Illumina quality control and SNP calling

Illumina reads were subjected to quality control (filtering and trimming) using QC Toolkit (Patel and Jain 2012). Paired reads were filtered with parameters: -l 70, (cutOffReadLen4HQ) and -s 20 (cutOffQualScore). As a result, reads with a PHRED quality score of less than 20 for more than 30% of their length were discarded. Moreover, reads were trimmed at the 3' end for bases with a PHRED quality score of less than 30. Paired reads were mapped to the reference genomes [*C. albicans SC5314*, REfSeq NZ_AACQ00000000.1, (Stanford University), and WO-1, RefSeq AAFO00000000.1 (Broad Institute)], using Burrows Wheeler Aligner (BWA http://bio-bwa.sourceforge.net/). The Genome Analysis Toolkit (GATK) was used for base quality score recalibration, Indel realignment, duplicate removal, and to perform SNP and Indel (insertion or deletion) discovery (McKenna, Hanna et al. 2010).

7.2.15 Variant imposition, gene loss prediction and Gene Ontology enrichment of lost, duplicate, truncated genes

The coding sequences of *C. albicans* strains were created using variant imposition (a.k.a. consensus calling) in order to create uniform sets of genes for further analysis. Briefly, this technique inserted variants (SNPs and Indels) produced by GATK and specific for each strain into the coding sequences of the reference strains (SC5314 and WO-1) to create accurate CDS useful for phylogenetic comparison across strains. For each strain, the alignments (BWA, then optimized by GATK) of reads versus the reference genome were analyzed and CDSs (taken from the genomic coordinates of SC5314 reference strain and reassembled in case of multiple exons) evidencing a zero coverage in at least one position were called as "lost". In fact, these genes were apparently non-functional, broken, or lacked evident similarity with the reference. We considered that only a minority of genes (around 10%) had some positions (<5) with a coverage equal to 0. Although possibly affected by technical errors, especially in genes belonging to regions with low complexity or repetitive sequences that could be difficult to align, we considered this approach a valuable source of information and that false positive genes should appear in the majority of the strains analyzed and could be either false positives or acquisitions of the reference genome. Gene Ontology Term Finder tool on Candida Genome Database (CGD; http://www.candidagenome.org/) were used for Functional Enrichment Analysis, using default parameters (therefore considering as valid results with a p-value<0.05).

7.2.16 Cell wall extraction and quantification of sugars by HPAEC-PAD

The sugar composition of *S. cerevisiae* and *C. albicans* cell walls was analysed in collaboration with Dr. M. S. Lenucci of Dep. di Scienze e Tecnologie Biologiche ed Ambientali (Di.S.Te.B.A.), Università del Salento (Italy), as previously described (Dallies, Francois et al. 1998) applying the following modifications. Briefly, about 200 mg of stationary phase cells were harvested and washed with deionized water. Cells were resuspended in 1ml of Tris-HCl 10mM (pH 8.5) and subsequently disrupted by three rounds of vortexing at maximum speed (30 s) and chilling on ice (1 min) using glass beads (0.45-0.55mm). Cell pellets were subjected to extraction with 100 µl of H_2SO_4 72% (w/w) for 1 hr at room temperature. The resulting slurry was diluted with MilliQ water, to a final volume of 1ml and heated for four hours at 100°C. The hydrolysate was then diluted to 9 ml with MilliQ water, neutralized with saturated $Ba(OH)_2$ and left overnight at 4°C to allow the precipitation of sulfate ions. After centrifugation at 3800g for 5 min. The supernatant was subjected to monosaccharide analysis with High-Performance Anion-Exchange Chromatography coupled with Pulsed Amperometric Detector (HPAEC-PAD). All samples were filtered through a 0.2mm Spartan 13 filter (Schleicher & Schuell Microscience, Dassel, Germany) prior to analysis on a Dionex HPAEC equipped with a CarboPac PA10 (4 x 50 mm) guard column and a CarboPac PA10 (4 x 250 mm) analytical column. Separation was performed as previously showed (Dallies et al., 1998). Sugars were quantified with a pulsed amperometric detector (PAD) with gold electrode. Glucosammine, galactose, glucose and mannose (for chitin, glucan and mannan content determination, respectively) were identified by comparison with reference compounds and quantified according to calibration curves obtained for each sugar.

7.2.17 Human Peripheral Blood Mononuclear Cells and Dendritic Cells preparation, fungal challenge and cytokine assays

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from fresh blood obtained from 5 pediatric CD patients and 6 healthy donors, as a control, (from Meyer children Hospital and the Transfusion Unit of the Careggi Hospital, Florence, Italy, respectively) by Ficoll-Hypaque density gradient centrifugation (Biochrom AG). The experimental plan was approved by the local Ethical Committee of the Meyer and Careggi hospitals, and informed consent was obtained from all CD patients and healthy donors. Monocytes were isolated from low density PBMCs by magnetic enrichment with anti-CD14 beads (Miltenyi Biotec). Cells were cultured in the presence of GM-CSF (800 U/ml) and recombinant IL-4 (1000 U/ml) for 6 days to allow Dendritic Cells (DC) differentiation (Netea, Ferwerda et al. 2006). DC activation was induced by fungal strains in the different life stages. Depending on the experiments, moDCs were added at different concentrations. Serial dilution of live/UV-treated yeast preparations was added to the moDCs at different stimuli:DC ratios.

For confirmation experiments, PBMCs from the same healthy subjects were used; stimulation was performed as in DC challenge experiments. All stimulations were carried out by challenging PBMCs with live fungi at 10^6 cells/ml concentration. After 24 hr or 7 days of incubation, supernatants were collected and stored at -20°C until assayed by mean of cytokine detection. Human Milliplex® assay for TNF-α, IFN-γ, IL-1β, IL-6, IL-10, IL-23, IL-12p70 and IL-17A production was performed according to the manufacturer's instructions using Luminex technology.

7.2.18 Statistical analysis of human immune response data

Principal Component Analysis (PCA) was carried out by using the dudi.mix function of the ade4 R package in order to find possible correlations between a specific immune response and the ancestral lineage of the strains and the ability to sporulate. Strains were used as cases, and the variables were represented by cytokine release from human PBMCs upon stimulation with the normalized strains (as compared to the relative cytokine amount expressed by un-stimulated PBMCs) and the strain's sporulation ability, using the percentage of sporulation after 5 days in SPOIV medium as a factor (categorized into "high sporulation"->25%, "low sporulation"-<25% and "any sporulation"-0%). Spearman's correlations among sugar cell wall and cytokine release upon fungal stimulation were performed using the R software through the stats R package (version 3.1.2). P-values have been corrected for multiple comparison by using the false discovery rate correction.

7.2.19 Bacterial DNA extraction from fecal samples and pyrosequencing

Fecal samples were preserved in RNAlater (Qiagen) at 4°C for the first 48 h, and kept at −80°C until DNA extraction. Bacterial genomic DNA extraction and quality control were carried out following our previous protocol (De Filippo, Cavalieri et al. 2010). For each sample, we amplified the 16S rRNA gene using the special fusion primer set specific for V5-V6 hypervariable regions and corresponding to primers 784F and 1061R described by Andersson *et al*. (Andersson, Lindberg et al. 2008), and using the FastStart High Fidelity

PCR system (Roche Life Science, Milano, Italy). The 454 pyrosequencing was carried out on the GS FLX+ system using the XL+chemistry following the manufacturer recommendations (see materials and methods in chapter 3).

7.2.20 Metagenomic data analysis

Raw 454 files were demultiplexed using Roche's .sff file software. Reads were pre-processed using the MICCA pipeline (version 0.1, http://compmetagen.github.io/micca/) (Albanese, Fontana et al. 2015). Forward and reverse primer trimming and quality filtering were performed using micca-preproc truncating reads shorter than 250nt (quality threshold=18). *Denovo* sequence clustering, chimera filtering and taxonomy assignment were performed by micca-otu-denovo (parameters -s 0.97 -c). Operational Taxonomic Units (OTUs) were assigned by clustering the sequences with a threshold of 97% pair-wise identity, and their representative sequences were classified using the RDP software version 2.7 (Wang, Garrity et al. 2007). Template-guided multiple sequence alignment was performed using PyNAST57 (version 0.1) (Caporaso, Bittinger et al. 2010) against the multiple alignment of the Greengenes 16S rRNA gene database (DeSantis, Hugenholtz et al. 2006) filtered at 97% similarity. Finally, a phylogenetic tree was inferred using FastTree (Price, Dehal et al. 2010) and micca-phylogeny (parameters: -a template-template-min-perc 50).

Alpha diversity was measured using richness of observed OTUs and Shannon index. Exploratory analysis was performed by Non-metric MultiDimensional Scaling (NMDS) and beta dispersion with PCoA ordination based on Bray-Curtis dissimilarities (indicators of beta diversity) by phyloseq package (McMurdie and Holmes 2014) of the R software suite. The significance of between-groups differentiation was assessed by PERMANOVA using the adonis() function of the R package vegan with 999 permutations.

To compare the relative abundances of OTUs among the three groups of subjects, the two-sided, unpaired Wilcoxon test was computed, removing taxa not having a relative abundance of at least 0.1%, in at least 20% of the samples, and using the function mt() in the phyloseq library and the p-values were adjusted for multiple comparison controlling the family-wise Type I error rate (minP procedure).

On the basis of the relative abundances, the metagenomic biomarker discovery and related statistical significance were assessed using the linear discriminant analysis (LDA) effect size (LEfSe) method (Segata, Izard et al. 2011). LEfSe uses the Kruskal–Wallis rank-sum test to identify features with significantly different abundances between assigned taxa compared to the groups, and LDA to estimate the size effect of each feature. An alpha significance level of 0.05, either for the factorial Kruskal-Wallis test among classes or for the pairwise Wilcoxon test between subclasses, was used. A size-effect threshold of 2.0 on the logarithmic LDA score was used for discriminative microbial biomarkers.

7.3 Result and discussion

7.3.1 The mycobiota of Crohn's disease patients is enriched for *S. cerevisiae* strains

We used selective media to characterize the cultivable gut mycobiota from feces from 34 pediatric Crohn's disease patients (CD), 27 ulcerative colitis patients (UC), and 32 healthy children (HC) (Table 7.1). Sequencing of rDNA ITS1-5.8S-ITS4 region allowed us to classify a total of 112 yeast isolates belonging to 20 different species (Fig. 7.1; Table 7.2). The CD group showed the highest number of isolates (N=78) belonging to 12 different species, compared to UC and HC (N=12 and N=22 isolates, respectively; Table 7.2). *S. cerevisiae*, *C. albicans* and *C. parapsilosis* composed the largest part of the CD mycobiota (27, 22 and 16 isolates belonging to the three species respectively; Fig. 7.1; Table 7.2), resulting in a significantly higher richness in CD ($p<0.0001$ Kruskal-Wallis test; Fig. 7.2).

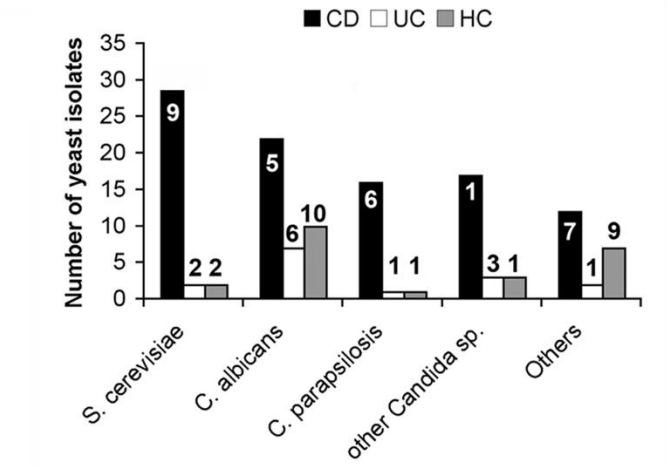


Fig. 7.1 Cultivable fecal mycobiota in IBD patients and healthy children. Distribution of yeast isolates (n =112) from fecal samples of Crohn's Disease (CD), Ulcerative Colitis (UC) patients and healthy children (HC; total subjects enrolled n=93). The number of subjects bearing at least one isolate per yeast species is reported on the bars.

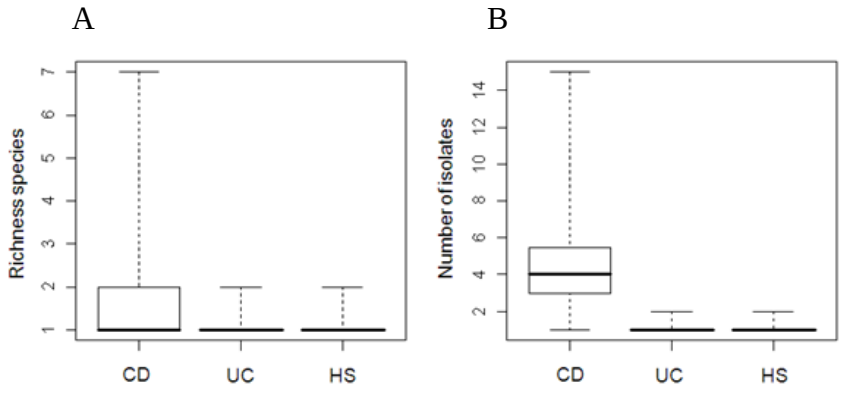


Fig. 7.2 Richness and biodiversity of cultivable mycobiota. (A-B) Differences in species richness and biodiversity of IBD and HC fecal mycobiota for the most abundant species. (A) Box plots related to species richness and (B) to the number of isolates in the three groups studied. CD= Crohn's Disease patients; UC= Ulcerative Colitis patients; HC= healthy children. Kruskal-Wallis test: Species richness, p= 0.004; number of isolates p= 9.267×10^{-8}.

Table 7.1 Clinical data of IBD patients investigated in this study

	CD	UC
Number of patients	34	27
Gender: M/F ratio	20\14	13\14
Age average (range)	15.01 (10.5-18.9)	12.4 (3.25-18.9)
LOCALIZATION (number of patients)		
ileum	13	0
colon	3	17
ileum-colon	15	0
sigma-rectum	0	8
DISEASE ACTIVITY (number of patients)		
PCDAI <10	20	
PCDAI >10	14	
PUCAI <10		23
PUCAI >10		4
Anti *S. cerevisiae* Antibody (number of patients)		
ASCA +	27	3
ASCA -	7	24
INFLAMMATION INDECES (number of patients)		
Calprotectin >100	18	17
Calprotectin <100	16	10
ESR>31	12	6
ESR <31	19	19
CRP>0,5mg/L	16	6
CRP<0,5mg/L	15	19
TREATMENTS (number of patients)		
enteral nutrition	12	0
infliximab	4	0
thalidomide	4	0
azathioprine	3	5
aminosalicylates	8	23
steroids	0	5
metotrexate	2	1
ciclosporine	2	0
6- mercaptopurine	6	5

Table 7.2 Yeast species isolated and identified by sequencing of the ITS region. For each yeast species, number of isolates and number of subjects with each yeast species isolates are indicated, as well as total species and average and standard deviation of strains for species are indicated

		N° of yeast isolates				**N° of subjects with yeast isolates**		
Phylum	**Yeast Species**	CD	UC	HC	Total	CD	UC	HC
Ascomycetes	*Saccharomyces cerevisiae*	27	2	2	31	9	2	2
	S. delbrueckii	2	0	0	2	1	0	0
	Candida albicans	22	7	10	39	5	6	10
	C. ernobii	0	1	0	1	0	1	0
	C. glabrata	0	1	0	1	0	1	0
	C. pararugosa	1	0	0	1	1	0	0
	C. parapsilosis	16	1	1	18	6	1	1
	C. tropicalis	0	0	1	1	0	0	1
	C.zeylanoides	0	0	1	1	0	0	1
	Clavispora lusitaniae	2	0	2	4	2	0	2
	Issatchenkia orientalis	1	0	0	1	1	0	0
	Pichia carribica	0	0	1	1	0	0	1
	P. kluyveri	0	0	1	1	0	0	1
	P. membranifaciens	1	0	0	1	1	0	0
	Yarrowia lipolitica	0	0	1	1	0	0	1
Basidiomycetes	*Criptococcus adeliensis*	1	0	0	1	1	0	0
	C. liquefaciens	1	0	0	1	1	0	0
	C. saitoi	0	0	1	1	0	0	1
	Rhodotorula mucilaginosa	3	0	1	4	3	0	1
	Trichosporon faecale	1	0	0	1	1	0	0
Total isolates		78	12	22	112			
Total species		12	5	11	20			
Average of strains per species		7	2	2	6			
Standard deviation of strains per species		8	2	2	11			

Stratifying CD patients according to clinical markers useful for monitoring mucosal disease activity, such as fecal calprotectin dosage, we observed that 52.9% (18/34) of the patients presented high mucosal inflammation indexes. We found yeasts in 61% (11 out of 18) of CD patients with mucosal inflammation, from which 45% (5 out of 11) we isolated *S. cerevisiae*. For UC, in 44.4% (12/27) patients we found yeasts, and in only 7.4% (2/27) of the UC with no mucosal inflammation we isolated *S. cerevisiae*.

We observed that in CD the presence of yeasts significantly correlated with mucosal inflammation indexes (G test, *G adj*. 6.280*;* p=0.012), in agreement with recent observations (Li, Wang et al. 2014).

It is well known that in CD a loss of tolerance towards commensal yeasts is expressed by ASCA marker. The clinical status and ASCA levels of the IBD patients correlate with the presence/absence of yeast isolated in feces ($p=1.42x10^{-6}$, $\chi 2$ test). Nevertheless, no significant correlation between ASCA positive (ASCA+) CD patients and the most abundant fungal species *per se* has been found, indicating that ASCA production is not promoted by a specific yeast species, but rather by strain-specific antigenic properties, as previously observed (McKenzie, Main et al. 1990).

7.3.2 *S. cerevisiae* population structure from human gut

While the identification of *Candida spp.* from fecal samples has been described in previous studies (Ott, Kuhbacher et al. 2008;Iliev, Funari et al. 2012), the isolation of a large amount of *S. cerevisiae* isolates is an intriguing finding. We thus focused on the 31 *S. cerevisiae* isolates: 27 from CD, 2 from UC patients and 2 from HC (Table 7.2).

In order to investigate the origin of these strains, we performed phylogenetic analysis based on 12 microsatellite loci (Legras, Merdinoglu et al. 2007). Neighbor-Joining clustering based on chord distances (see materials and methods) was used to assess the similarity of fecal isolates as compared to others 123 strains from a wide variety of sources (soil, insect gut, grape, wine fermentation, bakery and clinical environment) (Legras, Merdinoglu et al. 2007;Stefanini, Dapporto et al. 2012). Interestingly, we observed that the strains clustered based on the patients from which they were isolated (Fig. 7.3A). Secondly a significant fraction of gut isolates (17 out of 31), grouped in a separated cluster (Fig. 7.3A) with respect to all the other known strains. This cluster encompasses isolates (YB, YD and YE series) from three CD patients (B, D and E respectively), suggesting the existence of a population of human gut strains with common specific genomic characteristics. We defined these isolates as "human gut"-HG1 (Fig. 7.3A). We identified other 2 sub-clusters of HG strains. A second group (namely HG2; from 3 IBD patients and 2 from HC) clustered closely to strains used in bakery or isolated from the gut of wasps caught in the same geographical area of both patients and healthy subjects (Tuscany-Italy; Fig. 7.3A), suggesting an environmental/food-borne origin of these strains. A third group of four HG strains (namely HG3), encompassing only isolates from a single CD patient (YH1-YH4), co-clustered with the SK1 laboratory strain (Fig. 7.3A). This was no exception since strains isolated from the same patient resulted more genetically similar than other strains belonging to the same cluster according to subclustering, strains of the YB, YD and YE series of HG1 cluster and the YH strains of HG3 cluster (Fig. 7.3A), and to the comparison of intra-series and intra-cluster chord distances (Fig. 7.4). Both these indications suggested that the strains isolated from feces of the same patient arose from clonal expansion within the gut of each patient.

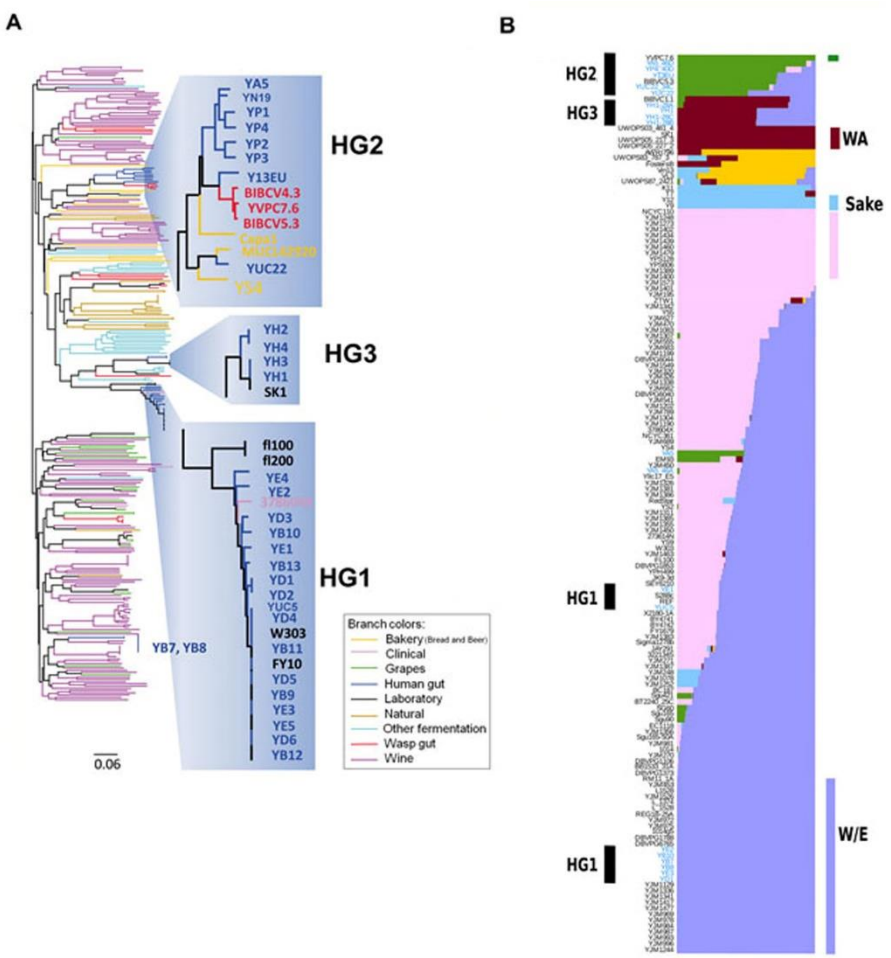


Fig. 7.3 *S. cerevisiae* population phylogeny. (A) *S. cerevisiae* strain clustering based on the genetic distances calculated with microsatellite analysis (21 selected loci). Blue shadows indicates the human gut (HG) clusters. Color code of strains: purple-wine, green-grapes, dark yellow-natural sources, yellow-bakery (bread and beer), light blue-other fermentations, pink-clinical, red-insect gut, black-laboratory, blue-fecal isolates. (B) The Strains' most probable ancestry, inferred by Structure analysis and combined with CLUMPP, performed on SNP profiles. The proportion of the most probable inferred ancestors for each strain is indicated by the colors of the horizontal bars. Vertical colored bars indicate previously identified ancestries: red-west African (WA); light blue- Sake; dark yellow- north America (NA); black-laboratory; light blue-sake; purple-wine European (WE).

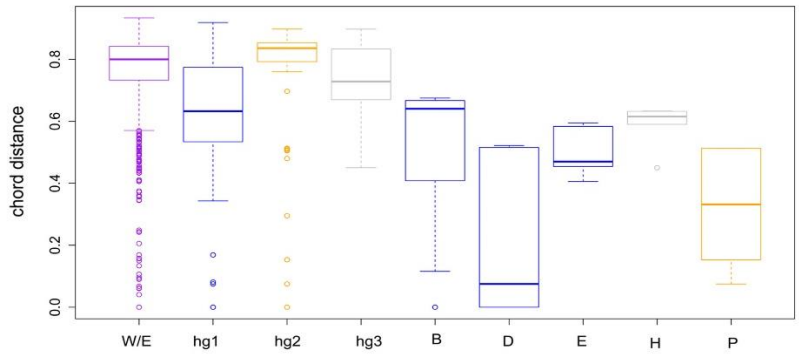


Fig. 7.4 Comparison of genetic distances (base on chord distances) among strains isolated from the same CD patient with respect to the relative clade. Wilcoxon rank sum test p-val<0.05.Note: B, D, E, P, H are different CD patients. HG= Human gut clade, W/E= wine European clade.

Notably, we observed that the HG isolates from fecal samples did not cluster with clinical strains previously sequenced (Liti, Carter et al. 2009;Strope, Skelly et al. 2015). This could be ascribed to the fact that the latter were isolated, either in the presence of infections caused by fungal pathogens or from different human body sites, constituting different environmental niche compared to gut environment. These results are in line with the previous observation (Koren, Knights et al. 2013) of a different gut microbial community with respect to different communities of other body districts. The ancestral analysis, performed on microsatellite SNPs, shows that the gut strains genome originates from different ancestors (Fig. 7.3B).

7.3.3 Phenotypic characterization of *S. cerevisiae* isolates. Sporulation ability correlate with ASCA serum level of CD patients

We studied HG strains for phenotypic differences in traits relevant for growth in the gut environment, such as invasivity, resistance to supra-optimal temperatures, oxidative stress, and sporulation (McCusker, Clemons et al. 1994;Diezmann and Dietrich 2009) (Fig. 7.5).

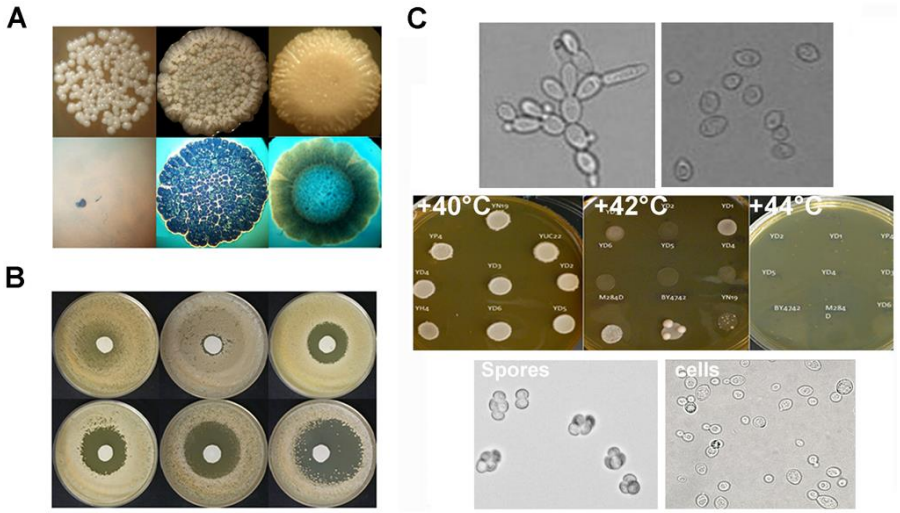


Fig. 7.5 Phenotypic characterization of *S. cerevisiae* isolates. (A) Comparison of substrate invasivity of selected strains. As a result, the invading cells are colored blue, after staining with Coomassie blue. (B) Survival to oxidative stress evaluating the inhibition halo after 5 days of incubation with tert-Butyl-hydroperoxide. (C) Pseudohyphal formation, growth assessment at high temperatures and sporulation ability.

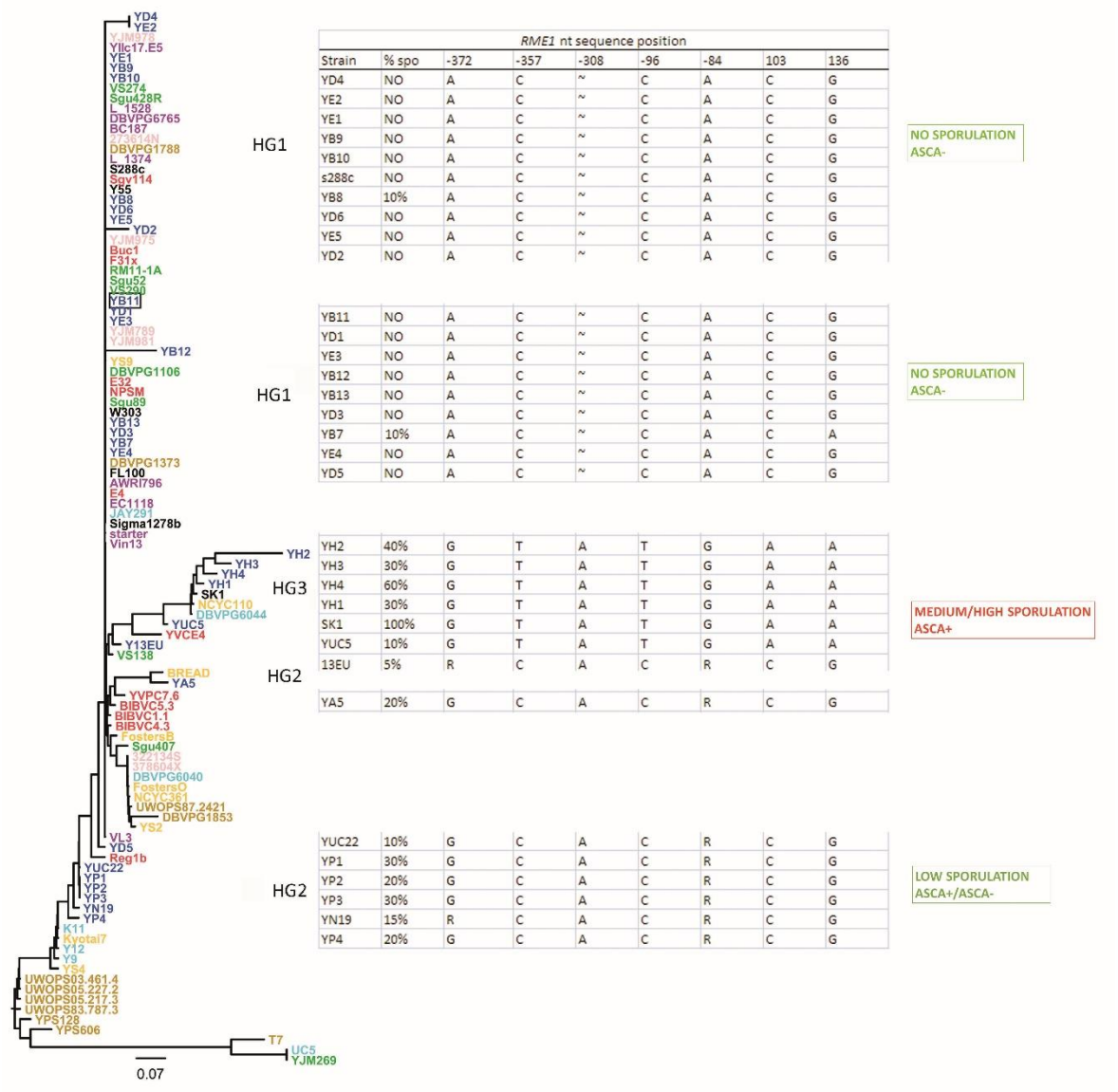
We found significant associations between fecal strain invasivity and sporulation (P=0.001, Fisher's exact test, FET), invasivity and resistance to oxidative stress (P=0.01, FET) and sporulation rate and pseudohyphal formation (P=0.022, FET).

Table 7.3 Summary of virulence–related traits tested on *S. cerevisiae* strains isolated from fecal samples of IBD patients and HC

ID PATIENT		Mucosal Inflammation	ID Strain	pseudohyphae	Invasivity	Oxidative stress (Inhibition)	4°C	40°C	42°C	44°C	pH 3.0	pH 4.8	pH 7.4	Sporulation (%) in SPO IV		
														4°C	28°C	37°C
CD	A (ASCA+)	yes	YA5	-	+	---	+	+++	+++	-	+++	+++	+++	5%	20%	10%
	B (ASCA-)	yes	YB7	-	++	--	+	+++	++	-	+++	+++	+++	-	10%	-
			YB8	-	+	--	+	+++	+++	-	+++	+++	+++	-	10%	-
			YB9	-	-	--	+	+++	++	-	+++	+++	+++	-	-	-
			YB10	-	-	--	+	+++	++	-	+++	+++	+++	-	-	-
			YB11	-	+	+++	+	+++	++	-	+++	+++	+++	-	-	-
			YB12	-	+	+++	-	+++	++	-	+++	+++	+++	-	-	15%
			YB13	-	-	---	+	+++	++	-	+++	+++	+++	-	-	-
	P (ASCA+)	yes	YP1	-	+	---	+	+++	++	-	+++	+++	+++	-	26%	20%
			YP2	-	+	-	+	+++	++	-	+++	+++	+++	5%	18%	13%
			YP3	-	+	+	+	+++	++	-	+++	+++	+++	-	33%	-
			YP4	-	+	+	+	+++	++	-	+++	+++	+++	5%	20%	-
	D (ASCA-)	yes	YD1	-	-	+++	+	+++	+++	-	+++	+++	+++	-	-	-
			YD2	-	-	+++	+	+++	+++	-	+++	+++	+++	-	-	-
			YD3	-	-	+++	+	+++	+++	-	+++	+++	+++	-	-	-
			YD4	-	-	+++	+	+++	+++	-	+++	+++	+++	-	-	-
			YD5	-	-	+++	+	+++	+++	-	+++	+++	+++	-	-	-
			YD6	-	-	+++	+	+++	+++	-	+++	+++	+++	-	-	-
	E (ASCA-)	no	YE1	-	-	+++	+	+++	++	-	+++	+++	+++	-	-	-
			YE2	-	-	+++	+	+++	++	-	+++	+++	+++	-	-	-
			YE3	-	-	+++	+	+++	++	-	+++	+++	+++	-	-	-
			YE4	-	-	+++	+	+++	++	-	+++	+++	+++	-	-	-
			YE5	-	-	++	+	+++	++	-	+++	+++	+++	-	-	-
	H (ASCA+)	yes	YH1	+++	+++	++	++	+++	+++	-	+++	+++	+++	-	30%	30%
			YH2	++	++	---	++	+++	+++	-	+++	+++	+++	-	35%	40%
			YH3	++	-	---	++	+++	+++	-	+++	+++	+++	-	20%	30%
			YH4	++	-	---	++	+++	+++	-	+++	+++	+++	18%	40%	5%
UC	5UC (ASCA-)	no	YUC5	-	-	+++	-	+++	+++	-	+++	+++	+++	-	10%	-
	22UC (ASCA-)	no	YUC22	-	-	---	++	+++	++	-	+++	+++	+++	-	10%	-
HC	19N	-	YN19	-	-	+++	++	+++	++	-	+++	+++	+++	-	15%	5%
	13N	-	13EU	-	-	+++	+	+++	++	-	+++	+++	+++	-	-	-

Only the YH strains (HG3 group) are able to form pseudohyphae, to resist to oxidative stress and showed the highest sporulation rate (>30% asci/cell at 37°C). HG1 strains (especially YD, YE and some YB) were not invasive, sensitive to oxidative stress and unable to sporulate (Table 7.3).

Interestingly, microsatellite-based clustering and ancestral lineages (Fig. 7.3) reflected the gut strains ability to sporulate. Indeed, HG1 group showed low sporulation rate (none or ranging from 0% to 10%). HG2 group showed medium/low sporulation rate (ranging from 15% to 25%). Moreover, HG3 strains showed the highest percentage of sporulation (>30%). To investigate the genetic determinants of this phenotype, we sequenced the *RME1* gene, the master regulator of sporulation that is known to have polymorphisms discriminating strains from different sources (Deutschbauer and Davis 2005;Gerke, Lorenz et al. 2009). The phylogenetic analysis based on the SNPs in *RME1* showed that strains from the same patient clustered together, supporting clonal expansion within the patient, as observed in microsatellites and ancestry analyses (Fig. 7.6). The *S. cerevisiae* isolates with medium/high sporulation capability were isolated from ASCA+ CD patients, while strains unable to sporulate were isolated from ASCA- CD patients (P<0.0001, FET). In general, sporulation ability seems to be correlated with the ASCA levels measured in CD patients.



RME1 nt sequence position

Strain	% spo	-372	-357	-308	-96	-84	103	136
YD4	NO	A	C	~	C	A	C	G
YE2	NO	A	C	~	C	A	C	G
YE1	NO	A	C	~	C	A	C	G
YB9	NO	A	C	~	C	A	C	G
YB10	NO	A	C	~	C	A	C	G
s288c	NO	A	C	~	C	A	C	G
YB8	10%	A	C	~	C	A	C	G
YD6	NO	A	C	~	C	A	C	G
YE5	NO	A	C	~	C	A	C	G
YD2	NO	A	C	~	C	A	C	G
YB11	NO	A	C	~	C	A	C	G
YD1	NO	A	C	~	C	A	C	G
YE3	NO	A	C	~	C	A	C	G
YB12	NO	A	C	~	C	A	C	G
YB13	NO	A	C	~	C	A	C	G
YD3	NO	A	C	~	C	A	C	G
YB7	10%	A	C	~	C	A	C	A
YE4	NO	A	C	~	C	A	C	G
YD5	NO	A	C	~	C	A	C	G
YH2	40%	G	T	A	T	G	A	A
YH3	30%	G	T	A	T	G	A	A
YH4	60%	G	T	A	T	G	A	A
YH1	30%	G	T	A	T	G	A	A
SK1	100%	G	T	A	T	G	A	A
YUC5	10%	G	T	A	T	G	A	A
13EU	5%	R	C	A	C	R	C	G
YA5	20%	G	C	A	C	R	C	G
YUC22	10%	G	C	A	C	R	C	G
YP1	30%	G	C	A	C	R	C	G
YP2	20%	G	C	A	C	R	C	G
YP3	30%	G	C	A	C	R	C	G
YN19	15%	R	C	A	C	R	C	G
YP4	20%	G	C	A	C	R	C	G

Fig. 7.6 Phylogenetic tree obtained by calculating the yeast strain genetic distances based on the SNP sequences of the *RME1* gene. For each HG strain, the percentage of sporification and the SNP position, including the adenine insertion (at -308 position), were reported in opposite file. Color code of strains: dark blue-fecal isolates from IBD patients and HC, purple- wine/European, green-Tuscan, dark yellow-natural sources, yellow-bakery (bread and beer), light blue-other fermentations, pink-clinical, red-insect gut, grey- west African.

7.3.4 Genetic variation analysis of *C. albicans* isolates

In order to evaluate the ability of *C. albicans* clinical strains to adapt to environmental conditions, we selected two isolates, YL1 and YQ2, from fecal samples of pediatric Crohn's disease (CD) patients. Initially we performed analysis of genome-wide variation, discovering a total of 178`336 SNPs/Indels and 168`981 SNPs/Indels for YL1 and YQ2 isolates, respectively, with nucleotide polymorphisms in coding DNA sequence (CDS) of 43%-44%, as compared to the *Candida albicans* SC5314 reference genome. Heterozygous variants ranged from 55% to 65%, with respect to the SC5314 and WO-1 *C. albicans* references genomes (Table 7.4). Specific homozygous regions for both YL1 and YQ2 were identified in chromosome 7 and in chromosome 2 and 4, respectively.

Table 7.4 Alignment statistics of YL1 and YQ2 sequenced isolates against the reference genomes of *C. albicans* SC5314 and WO-1. For each strain, the table provides the amount of reads obtained after filtering and trimming process, the mapping and coverage information, the total number of SNPs/indels with respect to the references, and the fraction of homozygous and heterozygous polymorphisms

Sequencing analysis								
		C. albicans SC5314			*C. albicans* WO-1			
ID strain	# filtered reads	# mapped	# not mapped	% mapped	# mapped	# not mapped	% mapped	% coverage
YL1	5151204	4959656	191548	96,3	4830749	320455	93,8	34,6
YQ2	9870920	9507165	363755	96,3	9203880	667040	93,2	66,2
SNP/Indels vs the references								
	C. albicans SC5314				*C. albicans* WO-1			
ID strain	# total SNP/Indels	# total homozygous	# total heterozygous	% heterozygous	# total SNP/Indels	# total homozygous	# total heterozygous	% heterozygous
YL1	178336	62260	116376	65,1	177193	61542	115635	65,3
YQ2	168981	73904	95450	56,4	171152	76188	94948	55,5
Nucleotide polymorphisms (vs *C. albicans* SC5314 reference)								
	SNP/Indel				heterozygous in CDS			
ID strain	# total	in non coding DNA sequence	in CDS	% in CDS	# total	%	SNP	Indels
YL1	178336	99806	78530	44,0	50784	64,7	48278	2506
YQ2	168981	95615	73366	43,4	40833	55,7	38796	2037

Gene Ontologies (GO) enrichment analysis showed that highly polymorphic genes in YL1 were those related to response to nutrients, external stimuli, starvation and genes, involved to filamentous growth, while highly mutated genes, common to both YL1 and YQ2 isolates, include those codifying for cell wall, adhesion and hyphal cell wall categories (Fig. 7.7).
For each sequenced genome, we identified genetic losses by comparison with the reference genome SC5314 (Table 7.5 and Materials and Methods). We found 20 lost Open Reading Frame -ORFs (7 of which were shared by YL1 and YQ2 and 6 were specific for YL1 and 7 for YQ2), that encoded for retro-transposable elements and factors involved in biofilm formation (among these, *HLA21* in both YL1 and YQ2, whereas the *ADH3* was present only in YQ2).
By comparing sequenced reads across the chromosomes, we found genomic regions with copy number variations. We identified duplication sites located especially in chromosome 2 and 6. Among the 11 duplicated ORFs present in both isolates, several were retro-elements; one of them, the *FGR24* gene, being a filamentous growth regulator. Other two members of *FGR* family (*FGR13* and *FGR14*) resulted duplicated in the YL1 genome, as well as a gene

regulated by three iron-responsive transcriptional factors (Sef1p- Sfu1p- and Hap43p), known to be involved in *C. albicans* commensalism and pathogenesis (Chen, Pande et al. 2011). The YQ2 genome showed duplication of a member of telomere-associate (TLO) gene family, known for its variability in number and position in *C. albicans* (Hirakawa, Martinez et al. 2015)(Table 7.6). Furthermore, we searched for variants resulting in loss-of-function mutations, as SNPs producing internal stop and out-frame insertions or deletions (Indels). Across the YL1 and YQ2 genomes, we found 130 and 110 truncated ORFs, respectively, while Indels resulted in out-of-frame mutations that disrupted 22 and 16 genes, respectively in the two strains. In the YL1 genome, we found prematurely stopped protein-coding genes involved in filamentous growth, cellular growth and response to stimuli. We detected as disrupted genes related to biofilm induction, such as *GPR1*, a G-protein-coupled receptor involved in filamentous growth and responsive to starvation, and *EFG1,* a key regulator of either the white/opaque switching or filamentous growth and virulence of *Candida*, whose loss of function has been related to increased commensal fitness (Hirakawa, Martinez et al. 2015). The following genes, associated with known functions (Huang 2012), were found as truncated in YL1 and YQ2 isolates. In particular, the *PHR1* (pH responsive), *CYR1* (starvation and CO_2 responsive), *SSK2* (responsive to osmotic stress), *RFG1* (Repressor of Filamentous Growth), *FLO8* (transcription factor required for hyphal formation and CO_2 induced white-opaque switching), *ALS3* (cell wall adhesion induced and required for spider biofilm), *VRG4* (a GDP-mannose transporter), *SNT1* (a transposon mutation affecting filamentous growth) and *PDX1* (a spider biofilm-repressed gene).

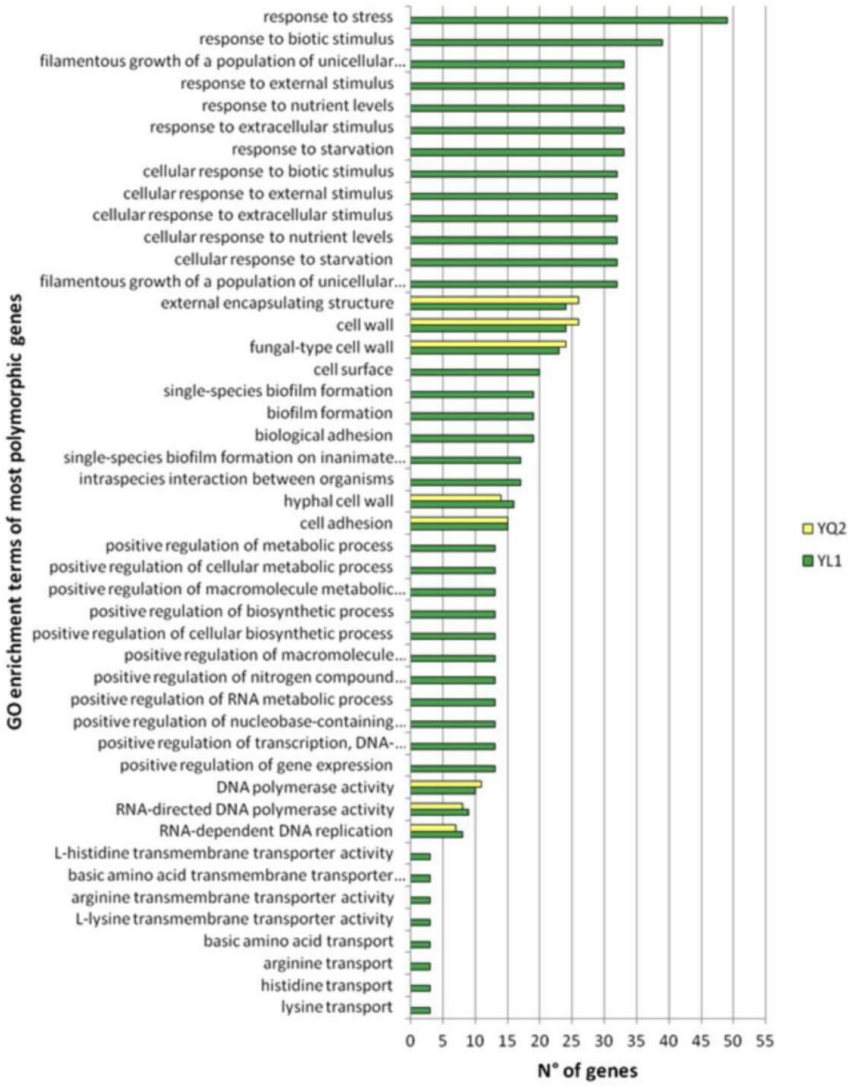


Fig. 7.7 GO enrichment analysis of the most polymorphic genes in the two *C. albicans* isolates. Gene Ontology Term Finder tool on Candida Genome Database were used for Functional Enrichment Analysis, using default parameters. Valid results were considered with a $p < 0.05$.

Table 7.5 Summary of lost genes in the whole genome sequenced isolates. Gene Ontology of lost genes for each isolate by Gene Ontology Term Finder tool on Candida Genome Database (CGD). Significant at p<0.05

	GO term		Adj P-value	FDR	Genes annotated to the term
all YQ2	RNA-directed DNA polymerase activity	GO:0003964	3.35E-05	0.00%	C7_02420C_A. ORF298. POL93
	DNA polymerase activity	GO:0034061	0.00088	0.00%	C7_02420C_A. ORF298. POL93
	nucleotidyltransferase activity	GO:0016779	0.03392	12.67 %	C7_02420C_A. ORF298. POL93
ONLY YQ2	nucleic acid binding	GO:0003676	0.08153	18.00 %	C1_12890W_A. C4_03230C_A. ORF298
	binding	GO:0005488	0.09785	9.00%	ADH3. C1_12890W_A. C4_03230C_A. ORF298
all YL1	RNA-directed DNA polymerase activity	GO:0003964	0.00132	0.00%	C7_02420C_A. POL93
	DNA polymerase activity	GO:0034061	0.01088	5.00%	C7_02420C_A. POL93
IN COMMON (>95% coverage)	nucleic acid binding		0.01863	16.00 %	C1_00270W_A. CR_09050C_A. POL93
	heterocyclic compound binding		0.06425	19.00 %	C1_00270W_A. CR_09050C_A. POL93
	organic cyclic compound binding		0.06496	14.00 %	C1_00270W_A. CR_09050C_A. POL93

Table 7.6 Summary of duplicated genes in the whole genome sequenced isolates. Gene Ontology of duplicated genes for each isolate by Gene Ontology Term Finder tool on Candida Genome Database (CGD). Significant at p<0.05

GO ENRICHMENT by Gene Ontology Term Finder tool (CGD)					
		GO term	Adj P-value	FDR	Genes annotated to the term
IN COMMON	TAR1 FGR24	molecular function unknown			
ALL YL1	FGR14	filamentous growth of a population of unicellular organisms in response to starvation	0.02549	0.00%	FGR13. FGR14
	FGR13	cellular response to biotic stimulus	0.03308	2.00%	FGR13. FGR14
		filamentous growth of a population of unicellular organisms in response to biotic stimulus	0.03555	1.33%	FGR13. FGR14
		response to biotic stimulus	0.04761	1.00%	FGR13. FGR14
		cellular response to starvation	0.05293	0.80%	FGR13. FGR14
		response to starvation	0.0571	0.67%	FGR13. FGR14
		cellular response to nutrient levels	0.06553	0.57%	FGR13. FGR14
		cellular response to extracellular stimulus	0.06706	0.50%	FGR13. FGR14
		cellular response to external stimulus	0.06899	0.44%	FGR13. FGR14
		response to nutrient levels	0.07095	0.40%	FGR13. FGR14
		response to extracellular stimulus	0.07214	0.36%	FGR13. FGR14
		response to external stimulus	0.08674	0.33%	FGR13. FGR14
ALL YQ2	TLO13	zinc ion binding	0.04857	0.25%	TLO13

7.3.5 Phenotypic analysis of *C. albicans* isolates

The ability of the two isolates to form hyphae was evaluated under standard culture conditions (i.e. YPD and YNB without amino acids and supplemented with ammonium sulphate) at 28°C and 37°C. Unlike YL1, YQ2 showed hyphal formation after 3 and 7 days, at 37°C, both in YPD and YNB media (Fig. 7.8A). Then, we evaluated the fungal growth in the presence of different carbon sources. We found that both isolates showed a significant lower growth rate in the presence of non-fermentable carbon sources, namely, 2% of

glycerol, ethanol or oleate (Fig. 7.8B). Furthermore, we exposed YL1 and YQ2 to the oxidative stress induced by 1M tert-butyl hydroperoxide. We found that the YL1 was more susceptible to the oxidative stress than YQ2 and SC5314 strain (Fig. 7.8C).

Moreover, we investigated the susceptibility to Ketoconazole and Fluconazole (0.01 mM, 0.1 mM, 0.5 mM, 1 mM and 5 mM for each drug), two fungicidal agents known to interfere with the synthesis of fungal cell membrane. We observed that both isolates showed resistance to Fluconazole, while only the YQ2 was resistant to Ketoconazole (Fig. 7.9).

We also tested the ability of the strains to form biofilm in RPMI, in presence or absence of FBS and CO_2. Both YL1 and YQ2 isolates were able to produce biofilm after 48 h of incubation, irrespectively of the presence of FBS; nevertheless, the thickness of YL1 biofilm was significantly higher than that produced by YQ2 (Table 7.7). Noteworthy, YQ2 did not produce biofilm at 24 hr in the absence of FBS (Table 7.8); also CO_2 markedly influenced the biofilm formed by YQ2 since in its absence biofilm production was delayed. Furthermore, by investigating the morphological characteristics of the biofilms grown for 48 hr under standard culture condition, we found marked differences in thickness mirrored by different cellular structures. As shown in Table 7.8, the number of both yeasts and budding yeast cells released by YL1 biofilm in the medium was significantly higher compared to YQ2. Kinetic studies showed that after 3 h both isolates form long hyphae; however, only the YL1 strain produced several budding yeast cells. At 24 hr, the YL1 biofilm showed a more dense hyphal structure with interspersed germinating yeast cells that were not present in YQ2 biofilm (Fig. 7.10).

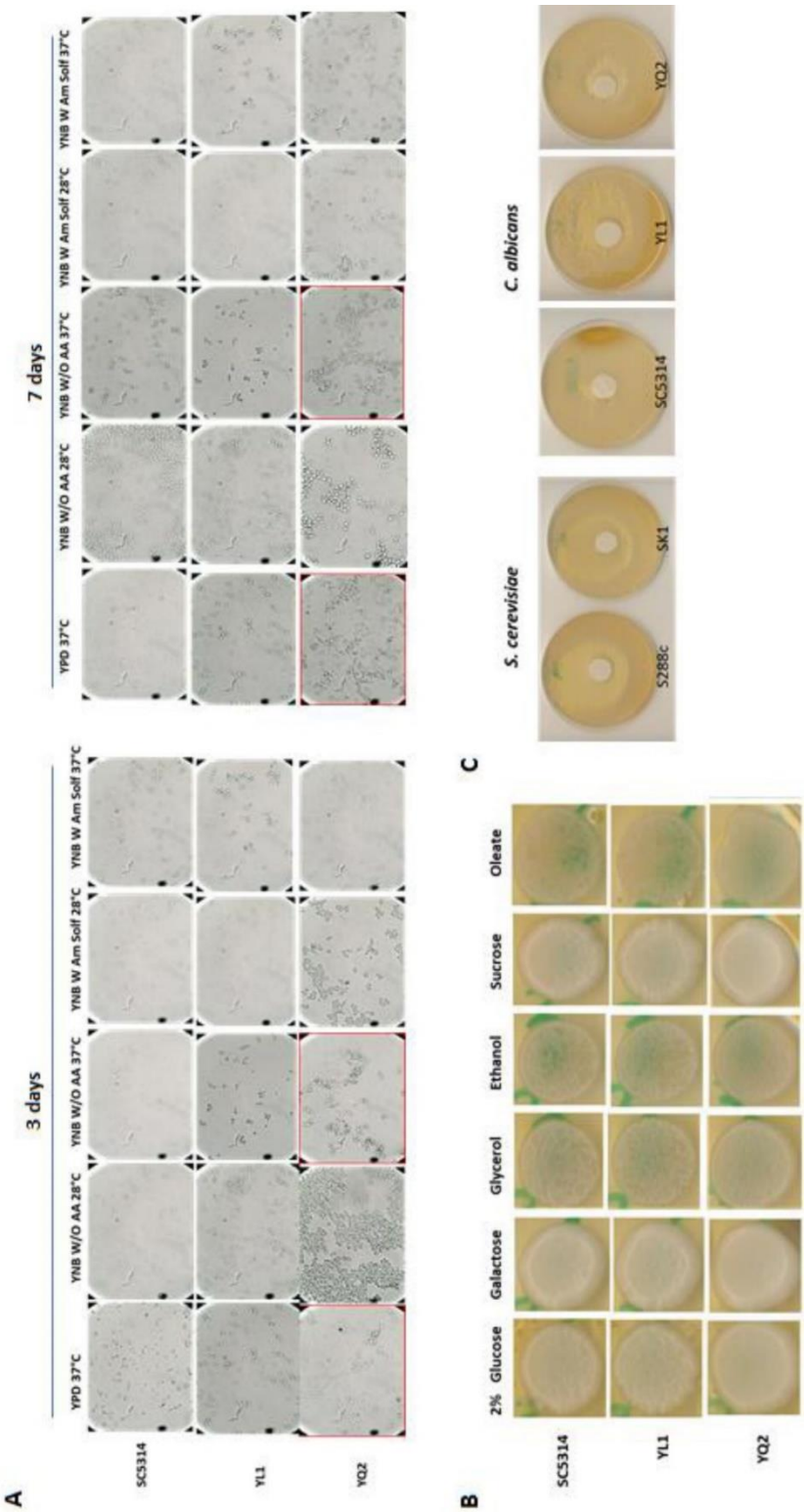


Fig. 7.8 Phenotype analysis of *C. albicans* strains in standard laboratory condition. (A) Morphotype switching of YL1, YQ2 and SC5314 was observed in standard laboratory liquid culture condition, as YPD and YNB (without amino acids and with Ammonia sulfate) at 28°C and 37°C. (B) Ability of growing in different carbon sources has been assessed by plating 104 cells on solid medium added by 2% of Glucose, Galactose, Glycerol, Ethanol or Oleate. Colony formation has been obsewrved after 3 days of growth at 28°C. (C) Survival to oxidative stress evaluating the inhibition halo after 5 days of incubation with *tert*-Butyl-hydroperoxide (1 mM).

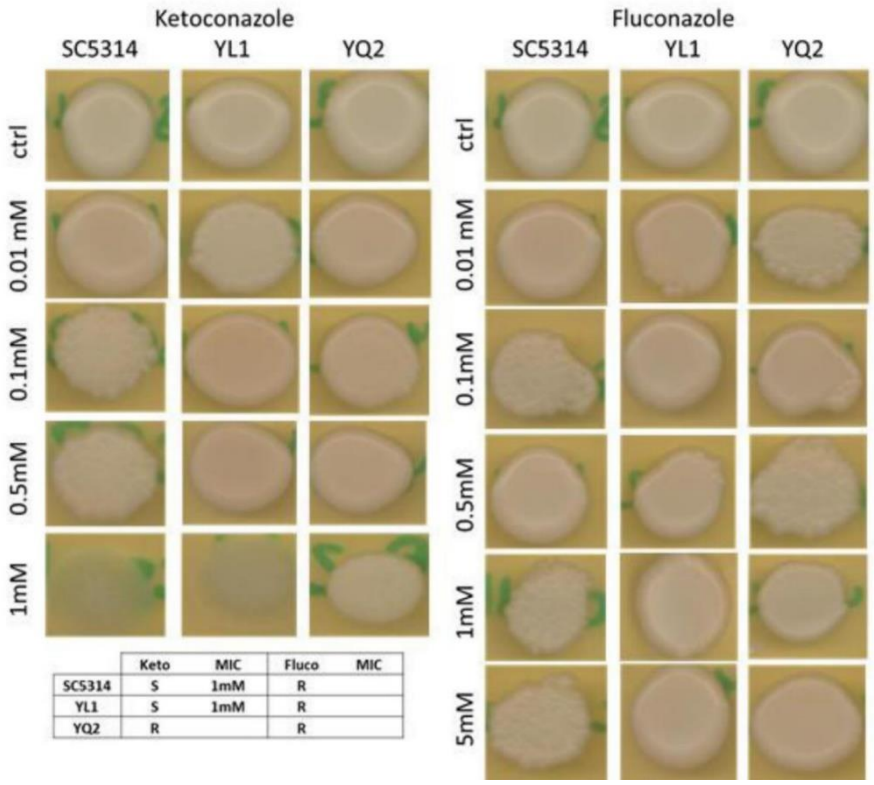


Fig. 7.9 Resistance to ketoconazole and fluconazole antifungal treatment. Antifungal drugs susceptibility was performed evaluating the growth of yeast cells (10^6 cells/ml) in solid YPD added with different concentration of fluconazole and ketoconazole (0.01 mM, 0.1 mM, 0.5 mM, 1 mM and 5 mM for each drug). SC5314 was used as control. Colony formation was evaluated after 24 h, 48 h and 72 h of growth at 28°C.

Table 7.7 Biofilm thickness of *C. albicans* isolates grown in RPMI in presence or absence of FBS

	24 h		48 h	
	RPMI +10% FBS	**RPMI**	**RPMI +10% FBS**	**RPMI**
C. albicans YL1	134	133.5*	182.6*	136.5
C. albicans YQ2	110	14	127	104.5

The asterisks indicate statistically significant differences (*p < 0.05)

Table 7.8 Biofilm structure and characteristics of *C. albicans* isolates grown for 48 h in RPMI with FBS

	Thickness (μm)	**# yeast cells released in the biofilm supernatants (x 10^4)**	**# budding yeast cells released in the biofilm supernatants (x 10^4)**
C. albicans YL1	168*	19*	6.2*
C. albicans YQ2	96.6	5	1.3

The asterisks indicate statistically significant differences (*p < 0.05)

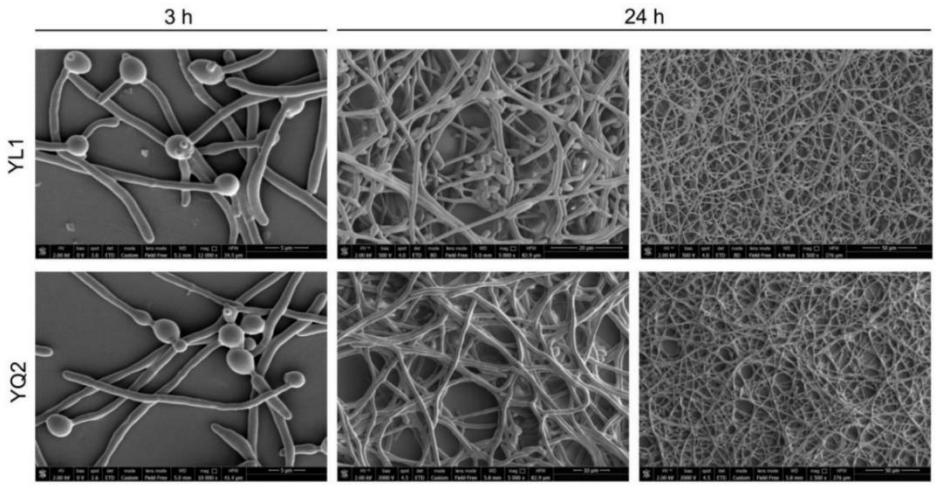


Fig. 7.10 Kinetic analysis of biofilm morphology. To examine cell morphology, *C. albicans* cells were seeded for 3 an 24 h at 37°C under 5% CO2. After fixation with 2.5 % glutaraldehyde cells were exposed for 1 h at room temperature with 1% osmium tetroxide. The samples were critical point dried before being gold coated and examined with a Scanning Electron Microscope.

7.3.6 Adhesion of *C. albicans* isolates to human epithelial cells

Thanks to the collaboration with laboratory of prof. Peppoloni from Dip. Medicina Diagnostica, Clinica e di Sanità Pubblica, Università di Modena e Reggio Emilia (Italy), we investigated the capacity of the two isolates to adhere to human epithelial cells, by using the intestinal Caco2 cell line. We found that the percentage of YL1 yeasts adherent to Caco2 cells was significantly higher, compared to YQ2 (Fig. 7.11A). It has been previously demonstrated that opportunistic pathogen yeasts can modulate host immune function by inducing the secretion of defensins, some natural antimicrobial peptides, that are highly inducible by a variety of stimuli [like inflammatory cytokines or bacterial, viral and fungal infection (Witthoft, Pilz et al. 2005) and have a broad spectrum antimicrobial activity that are fungicidal for *Candida* (Witthoft, Pilz et al. 2005;Aerts, Francois et al. 2008;Gacser, Tiszlavicz et al. 2014). We next performed experiments to determine whether Caco-2 cells challenged for 24 h with YL1 and YQ2 exhibited human beta-defensin 2 (HBD-2) production to different extent. We found that HBD-2 concentration in the supernatants of Caco-2 cells stimulated with YL1 was lower compared to the YQ2 (47 vs 67 pg/ml, respectively; Fig. 7.11B). These results indicate that YL1, although shows an higher ability to bind to intestinal epithelial cells, compared to YQ2, induces lower amounts of HBD-2 suggesting that this isolate has potentially pathogenic features.

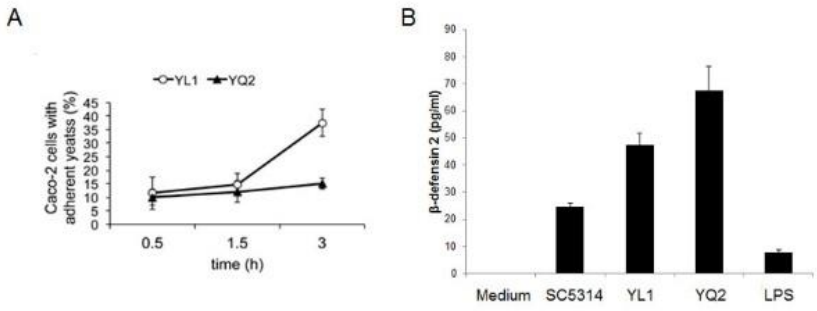


Fig. 7.11 YL1 and YQ2 showed different ability to adhere to epithelium, and produce HBD-2. (A) Caco-2 cells were seeded in Lab-Tek II chamber slides, grown at 37°C to confluence (24 h) and then infected with a suspension of *Candida* in complete RPMI for 1.5 or 3 h at 37°C under 5% CO_2. Uvitex 2B fluorescence of bound *C. albicans* was visualized by epifluorescence microscopy. At least 200 epithelial cells from each sample were examined and the percentage of Caco-2 cells with adherent yeasts was defined as the ratio of the number of Caco-2 cells with one or more *C. albicans* to the total number of Caco-2 cells examined. Data are represented as mean ± SD (N=3). *p<0.05. (B) Caco-2 cells are incubate with *Candida* strains (reference strain SC5314 and YL1 and YQ2 isolates) or LPS (10μg/ml) for 24 hr (ratio 1:100). Levels of β-defensin-2 are measured by ELISA assay.

7.3.7 Susceptibility of the *C. albicans* isolates to microglial cell-mediated antifungal activity

We performed studies to characterize the interaction of clinical isolates with microglial cells (in collaboration with laboratory of prof. Peppoloni-Università di Modena e Reggio Emilia). Microglial cells (BV2 cells) play a crucial role in preventing the outcome of experimental infection by *C. albicans* (Blasi, Mazzolla et al. 1991). In particular, such cells exert anti-candida phagocytic and killing activity and express a restricted pattern of secretory response (Blasi, Pitzurra et al. 1990;Blasi, Mazzolla et al. 1991;Blasi, Puliti et al. 1994). Using a previously established fluorescence-based assay (Peppoloni, Colombari et al. 2013), we measured the number of phagocytic cells at 1.5 and 3 hr post-infection. As shown in Fig. 7.12A, the phagocytic uptake of the two strains followed a similar pattern with time, since the percentages of microglial BV2 cells which internalized YL1 or YQ2 were not significantly different. These results indicate that the both strains were phagocytosed efficiently by microglia and to a similar extent. To examine the ability of the two isolates to survive within microglia, BV2 cells were challenged with YL1 or YQ2 strain for 1.5 h, and then fungal load was determined 0, 3, 6 and 20 h later. Initially and up to 6 h, the number of CFU recovered from microglial cells remained constant and was similar in the two groups. In contrast, at 24 h, the intracellular levels of YL1 were more than threefold higher than those observed with YQ2 ($p<0.05$) (Fig. 7.12B).

It is known that intracellular killing of microorganisms involves the fusion of lysosomes, containing microbicidal factors, with phagosomes in an acidic environment [a process known as "phagosome maturation"] (Vieira, Botelho et al. 2002) and that some pathogens are able to survive or even multiply inside of the phagocytes by inhibiting this process (Urban, Lourido et al. 2006). To investigate the events occurring after *Candida* ingestion by microglia, acidification of fungal cell-containing phagosomes was assessed (Kinchen and Ravichandran 2008). YL1 and YQ2 yeast cells were incubated for 1 and 3 hr with microglia

in the presence of LysoTracker, a marker of phagosome acidification (see Materials and Methods). Overlapping pictures obtained with different fluorescence channels were used to evaluate the successful acidification of *Candida*-containing phagosomes [simultaneous appearance of LysoTracker (red fluorescence) and unlabeled yeasts within the phagosomes] (Fig. 7.12C). To quantify the phenomenon, we determined the percentage of acidic phagosomes by counting the *Candida*-containing phagosomes within each microglial cell. As shown in Fig. 7.12D, at 1.5 h after infection, the percentage of YQ2-harbouring phagosomes was higher than that of YL1-containing phagosomes. Indeed, about 18% of the YQ2 were associated with acidic phagolysosomes, while this percentage dropped to 9% in the case of the YL1 strain. Similar results were also observed after 3 h and in both cases the differences between YQ2 and YL1 were statistically significant ($p<0.05$). Taken together, this evidence suggests that the YL1 resists to microglia-mediated intracellular killing by inhibiting phagosome maturation within these cells, indicating the pathogenic traits of fungal isolates from human gut.

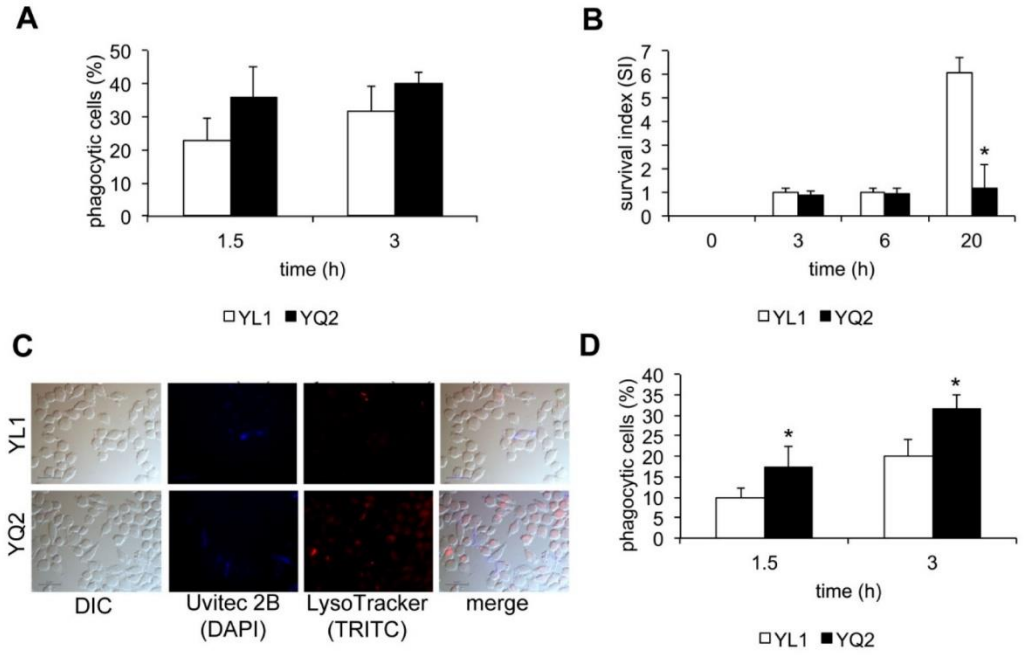


Fig. 7.12 *C. albicans* strains differently resist to phagocytic antifungal activity. (A) Susceptibility of the YL1 and YQ2 strains to phagocytosis by microglia was assessed by epifluorescence microscopy on microglia infected with the different strains. Oregon green 488 prelabelled yeast cells were exposed to BV2 cells (E : T=1 : 5) for 1.5 and 3 h. At each end point, Uvitex 2B was added for 15 min; the cultures were then washed, fixed and analysed by epifluorescence microscopy. Data are shown as mean±SD (N=3). (B) Susceptibility of the YL1 and YQ2 strains to antifungal activity by microglia. Yeast cells were exposed to BV2 cells at E : T=10 : 1. After 3, 6 and 20 h, the percent of antifungal activity was determined. Data are shown as mean±SD (N=43). *$p<0.05$, YL1 *vs* YQ2. (C) Acidification of phagolysosomes containing YL1 and YQ2 cells. Oregon green 488 prelabelled yeast cells were exposed to BV2 cells (E : T=1 : 5); then LysoTraker dye was added. After counterstaining with Uvitex 2B, samples were fixed and then visualized by epifluorescence microscopy. Representative images of acidification of phagolysosomes by means of epifluorescence. (D) Acidification of phagolysosomes containing YL1 and YQ2 cells. The results, expressed as percent of acid phagolysosomes, were calculated by evaluating the number of red-stained vacuoles among 200 yeast-containing vacuoles. Data are shown as mean±SD (N=3). *$p<0.05$, YL1 *vs* YQ2.

7.3.8 Cell wall composition of gut isolates suggests selection by intestinal environment

The sugar moieties of cell wall are the principal antigens sensed by the host immune system during host-fungal interaction (Lewis, Bain et al. 2012;O'Meara, Veri et al. 2015). We compared the cell wall sugar composition of a selection of 13 gut isolates (10 from CD, 2 from UC and 1 from HS), with a set of 10 *S. cerevisiae* strains isolated from other sources (laboratory, natural environment, wasps'gut and wine), and other 4 gut isolates, belonging to *Candida spp.* from CD patient and the SC5314 *C. albicans* reference strain (Fig. 7.13A-B).

High-Performance Anion-Exchange Chromatography (HPAEC) analyses revealed that, when grown in presence of glucose (YPD culture medium), *S. cerevisiae* gut isolates show inverse ratio mannose: galactose, with a significantly lower percentage of mannose compared to all the other strains (Kruskal-Wallis $p<0.05$, fdr corrected; Fig. 7.13C). All gut isolates, both from human and wasps, bear a significant higher galactose content (Fig. 7.13A). When compared with gut *Candida spp.* isolates, we observed a higher amount of galactose in *S. cerevisiae* gut isolates (Fig. 7.13B). The peculiar cell wall composition of HG *S. cerevisiae* strains appears to be a "gut" specific feature and could represent a selective advantage to survive and colonize the host gut.

Noteworthy, when lactose was used as sole carbon source, levels of cell wall components remain almost completely unchanged in the HG isolates, while some of the natural and wine strains showed an enrichment of the galactose and a reduction in mannose (Kruskal-Wallis test; Fig. 7.13C). This suggests an inducible induction of the genes responsible for galactose metabolism being it constitutive in gut isolates for adapting to the gut environment.

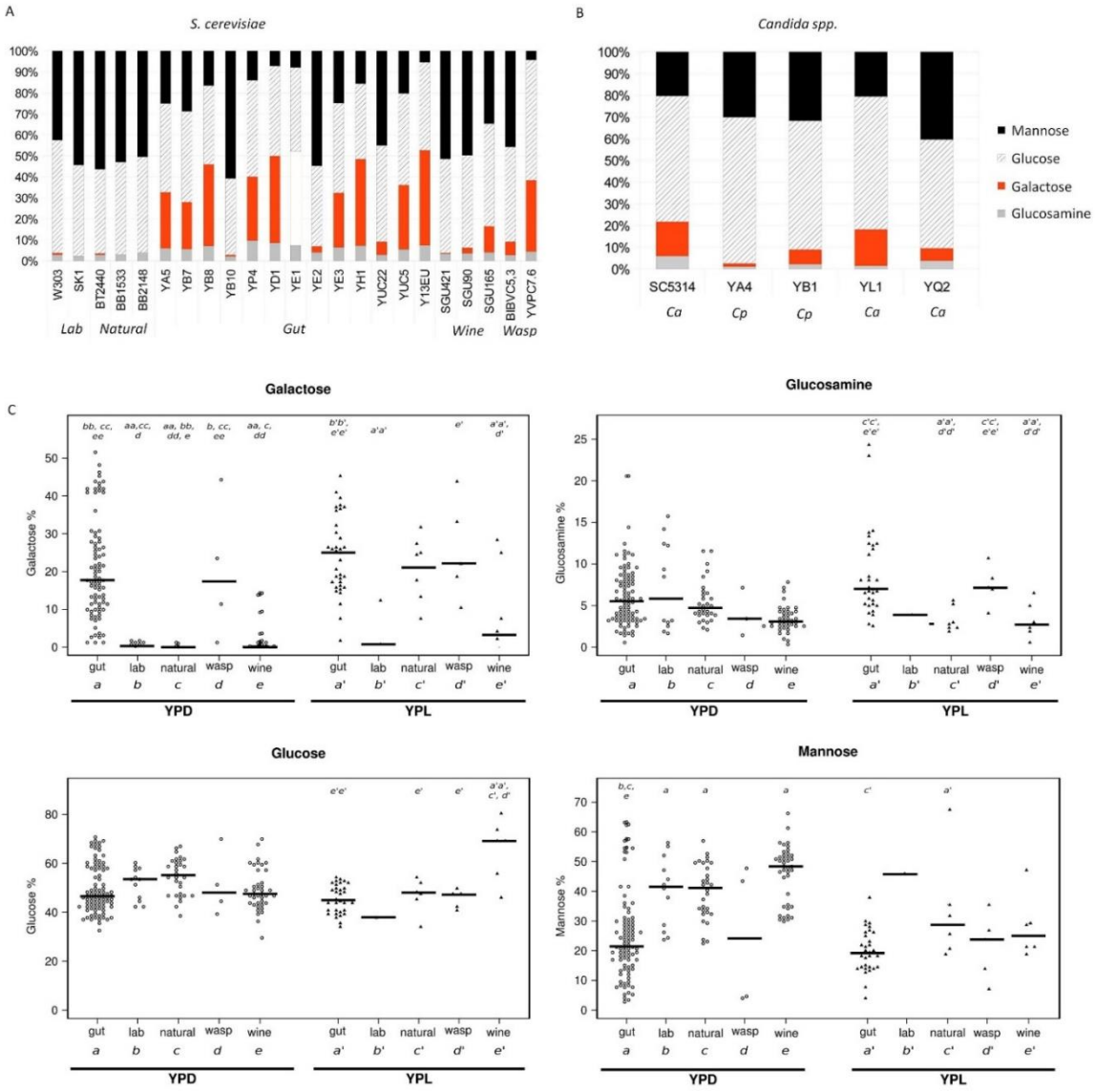


Fig. 7.13 Cell wall sugar components of HG isolates grown in presence of glucose (YPD culture medium) as carbon sources. (A-B) Percentage of mannose, glucose, galactose and glucosamine amounts in (A) *S. cerevisiae* isolates from gut and compared with strains from other sources (laboratory, natural environment, wine, and wasp's gut), compared with (B) cell wall sugar components of *Candida spp.* isolates from CD patients. *Ca= C. albcans* and *Cp= C. parapsilosis*. (C) Percentage of galactose, glucosamine, glucose and mannose amounts detected in cell wall of *S. cerevisiae* isolates grown in presence of glucose and lactate as carbon sources (YPD and YPL culture medium, respectively), and categorized based on the different origins: gut, laboratory, natural sources, wasp' gut and wine. The graphs provide average for each category and the p-values obtained by Wilcoxon rank sum test. Statistically significant differences among groups are indicated by different letters ("a"= human gut, "b"=laboratory, "c"=natural sources, "d"=wasp' gut and "e"= wine); single letter for $p<0.05$, double letter for $p<0.01$.

7.3.9 Immunomodulatory properties of cultivable fungal isolates

While responding to pathogenic organisms is a main function of the immune system, recognition and tolerance of commensal bacteria are equally important for host health (Kosiewicz, Zirnheld et al. 2011). Commensal microbes calibrate innate and adaptive immune responses and influence the activation threshold for pathogenic stimulations, in

large part by producing molecules that mediate host-microbial interactions (Donia and Fischbach 2015). A recent paper showed how differences in composition and function of gut microbial communities might contribute to inter-individual variation in cytokine responses to microbial stimulations in healthy humans. Host-microbial interactions reveal that TNF-α and IFN-γ production are associated with specific microbial metabolic pathways (Schirmer M. et al 2016). Yeast cell wall ratio between chitin and mannans has also been shown to be associated to differential production of IL-17, TNF-α and IFN-γ (Rizzetto, Kuka et al. 2010;Rizzetto, De Filippo et al. 2014;Rizzetto, Ifrim et al. 2016). To link the observed strain-specific genetic and phenotypic differences, with inflammatory cytokine production capacity of the host, we evaluated the immunoreactivity of a set of the cultivable fungal isolates belonging to *S. cerevisiae* and *Candida spp.*, measuring the cytokine profiles *in vitro* on dendritic cells (DC) and on human peripheral blood mononucleated cells (PBMCs) from healthy donors and CD patients, upon challenges with IBD and HC yeasts. A first screening was performed on *S. cerevisiae* and *Candida spp*. isolates, using monocyte derived DCs from 6 healthy donors. TNF-α IL-10, IL-1β, IL-6, IL-12p70 and IL-23 cytokines levels were measured (Fig. 7.14). We probed immune reactivity to a set of *S. cerevisiae* isolates from IBD and HC, comparing with isolates from other sources, such as 2 laboratory strains (SK1 and BY4741), 3 wild strains from Portugal, 9 isolates from human gut (2 from HC, 6 from CD and 1 from UC), 4 grape isolates from Tuscan wineyards (Fig. 7.14A). In parallel, we tested DC immune reactivity to a set of *Candida spp* isolates from IBD and healthy subjects comprising 13 *C. albicans* isolates, 4 *C. parapsilosis* isolates and 1 *C. glabrata* isolate, and comparing with the SC5314 *C. albicans* reference strain (Fig. 7.14B).

This investigation showed greater strains-specific rather than species-specific differences in the cytokine profiles. At the species level, a few generalizations were possible (Fig. 7.14C). We observed higher levels of IL-6 and IL-10 upon stimulation with *S. cerevisiae* isolates, compared to *Candida spp.* (Fig. 7.14C, Student's t test p=0.0021 *S. cerevisiae* vs *C. albicans* and p=0.011 *S. cerevisiae* vs *C. parapsilosis* for IL-6; p=0.005 *S. cerevisiae* vs *C. albicans* for IL-10). IL-1β was higher upon stimulation with *Candida spp.* compared with *S. cerevisiae* strains (Fig. 7.14C, Student's t test $p=7.76x10^{-6}$ *S. cerevisiae* vs *C. albicans* and p=0.004 *S. cerevisiae* vs *C. parapsilosis*). The TNF-α production was quite uniform in response to the different investigated isolates, belonging either to *S. cerevisiae* or *Candida spp.* with the exception of two *S. cerevisiae* gut isolates – one derived from a HC (Y13EU), one from a CD patient (YA5) - which showed a markedly low induction of this cytokine (Fig. 7.14A-B).

S. cerevisiae isolates from IBD patients showed a lower induction of IL-6, IL-1β, IL-12p70 and IL-23 with respect to the gut isolates from HC (Fig. 7.14A). Immune responses to natural strains are more variable (Fig. 7.14A). In general, the different strains isolated from diverse ecological niches showed an immune-based diversity which seems to correlate with their origin as shown by clustering the 6 cytokine profiles. The same results could be obtained by clustering only three of them, IL-10, IL-12p70 and IL-23, indicating that these cytokines mainly distinguish the immune response to the different strains (Fig. 7.14A).

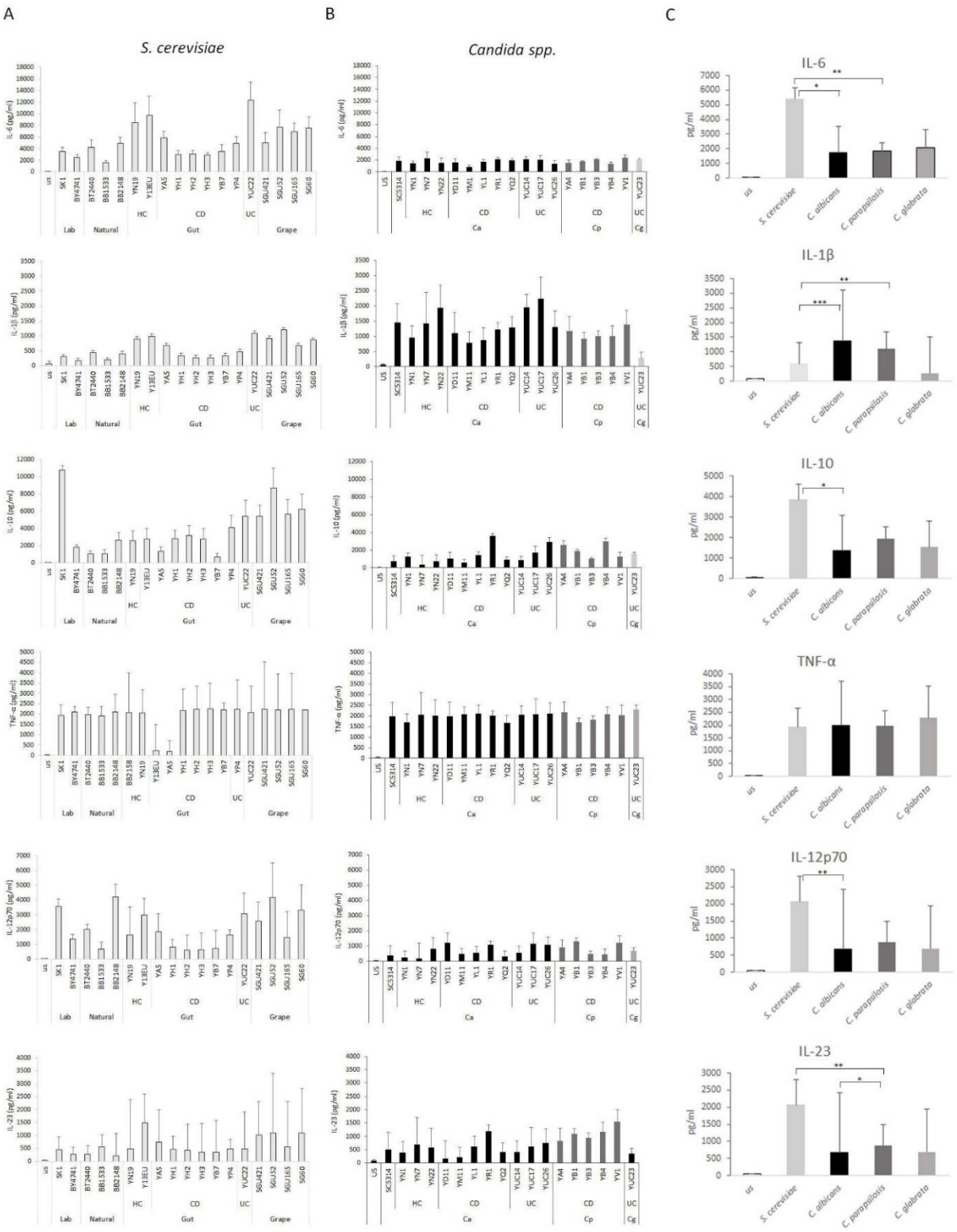


Fig. 7.14 DC immune response to *S. cerevisiae* and *Candida* spp. isolates. (A-B) Strain-specific cytokine profiles. Ability of DC from healthy donors (n=6) to discriminate among different isolates of (A) *S. cerevisiae* and (B) *Candida* spp. was tested as differential cytokine production. DCs were stimulated with cells for 24 hours and cultures supernatants used for TNF-α, IL-10, IL-1β, IL-6, IL-12p70 and IL-23 measurements. For *S. cerevisiae* isolates, different sources of isolates are indicated, such as laboratory, natural environment, gut, and grape. For both *S. cerevisiae* and *Candida spp.* from the human gut were indicated whether isolated from CD, UC patients or healthy children (HC). Ca= *C. albicans*; Cp= *C. parapsilosis*; Cg= *C. glabrata*. (C) Species-specific cytokine profiles.

Average of cytokine levels for each tested fungal species. *p<0.05; **p<0.001; ***p<0.0001 by Student's t Test.

The interesting cytokine profiles observed was confirmed by PBMCs cytokines release (Fig. 7.15). Selected strains, belonging to *S. cerevisiae* and *Candida spp.,* were tested for the ability to induce inflammatory (IL-1β, IL-6, TNF-α, IFN-γ, IL-17A) and anti-inflammatory (IL-10) responses (Fig. 7.15A-C). The different *S. cerevisiae* isolates showed diverse ability to promote IL-6, TNF-α, IL-17 and IFNγ, whose inflammatory role was counterbalanced by IL-10 induction with different extent (Fig. 7.15A-C). The results highlight the ability of *S. cerevisiae* to promote either inflammation, resistance or tolerance upon immune cell recognition.

Upon stimulation with different *Candida spp.* strains, IFNγ and TNF-α showed variations tending to parallel IL-10 profiles and to oppose IL-17 profiles (Fig. 7.15A-C). The levels of these cytokines displayed a strain-specific pattern. These screening allowed to categorize the different isolates of *Candida spp* as INF^+ or INF^- strains. While the IFNγ-mediated inflammatory response induced by the healthy isolate (YN1) seems to be well counterbalanced by IL-10 production, other responses are not (i.e YB1-induced response), as indicated by the low IL-10 production and the presence of both IFNγ and IL-17.

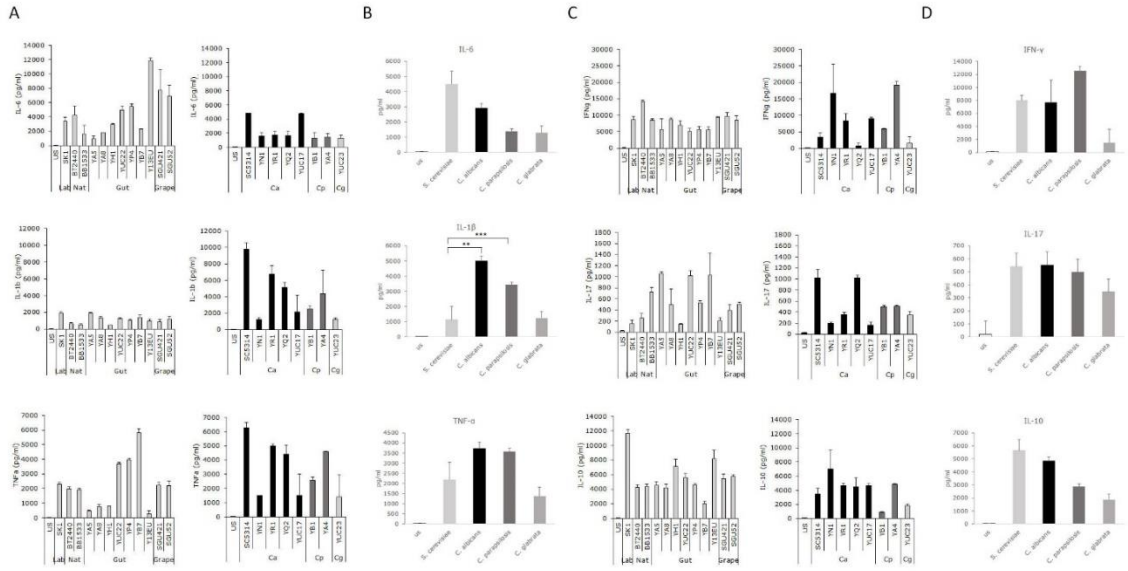


Fig. 7.15 PBMCs-primed adaptive immune response to different fungal isolates. (A-B) Selected strains *S. cerevisiae* (light grey) and *Candida spp.* (gray scales) were tested for the ability to induce inflammatory responses and specifically cytokines deriving by Th1 (IFN-γ), Th17 (IL-17) and Treg (IL-10). Healthy PBMCs from n=6 donors were stimulated with live cells for 24h or 5 days, and cytokine levels were measured. (C) Average of cytokine levels for each tested fungal species. **p<0.001; ***p<0.0001 by Student's t Test.

By comparison of healthy donors and CD patients PBMCs cytokine release (Fig. 7.16), we observed a general wide variability in immune reactivity, especially for PBMCs of patients. Generalizing, healthy subjects (HS) immune responses showed lower inter-patient variability, higher levels of IFN-γ and a trend of lower levels of IL-6 compared to CD

patients. While IL-1β, IL-17A, TNF-α and IL-10 release reflected a strain-specific pattern both in CD and HS (Fig. 7.16), ranging from high IL-17A and low IFN-γ and IL-10 (YB8 and YD1) to a pure Th1-driving response (high IFN-γ and low IL-17A and IL-10, as YE5) or high IL-10 (YH1). Therefore, unlike the healthy subjects, these results suggested that the inflammatory and clinical status of the patient strongly influence the immune response against strains. Interestingly, in some cases, CD patients (with different clinical status and mucosal inflammation; Table 7.9) response differently to own isolates compared to *S. cerevisiae* from other patients (Fig. 7.16, red triangles). PBMCs of CD patient, from which YE5 strain was isolated, the only CD patient that showed both clinical remission and lack of mucosal inflammation (Table 7.9), induced release high IFN-γ and TNF-α and low IL-17A compared to other patients in response to the same isolate (Fig. 7.16, red triangle). While, PBMCs of CD patient (H) in clinical remission, but active mucosal inflammation (Table 7.9), upon own YH1 isolate challenge, release low IL-17A and high IL-10 compared to other patients in response to the same isolate (Fig. 7.16, red triangle). Strains isolated from CD patients (B and D) in active disease and mucosal inflammation, YB8 and YD1 show high levels of IL17A and TNF-α and low level of IFN-γ and IL10 (Fig. 7.16, red triangles).

Table 7.9 Relation among and clinical features of the IBD and healthy children

***S. cerevisiae* strain**	**Subject**	**ASCA**	**Inflammation indexes**			**Intestinal mucosa status (by calprotectin dosage)**	**Clinical status**
			PCDAI	**PUCAI**	**calprotectin**		
			<10 CD remission	**<10 UC remission**	**<100 no inflammation**		
YA5	A CD patient	+	12.5		1100	Inflammation	Active
YB8	B CD patient	-	15		1300	Inflammation	Active
YP1	P CD patient	+	0		280	Inflammation	Remission
YD1	D CD patient	-	12.5		2600	Inflammation	Active
YE5	E CD patient	-	0		80	no inflammation	Remission
YH1	H CD patient	+	5		600	Inflammation	Remission
YUC22	22 UC patient	-		0	27	no inflammation	Remission
Y13EU	13 healthy child	ND					Healthy

Note: ASCA= anti-*S. cerevisiae* antibody; PCDAI and PUCAI are clinical indexes of active CD and UC disease, respectively.

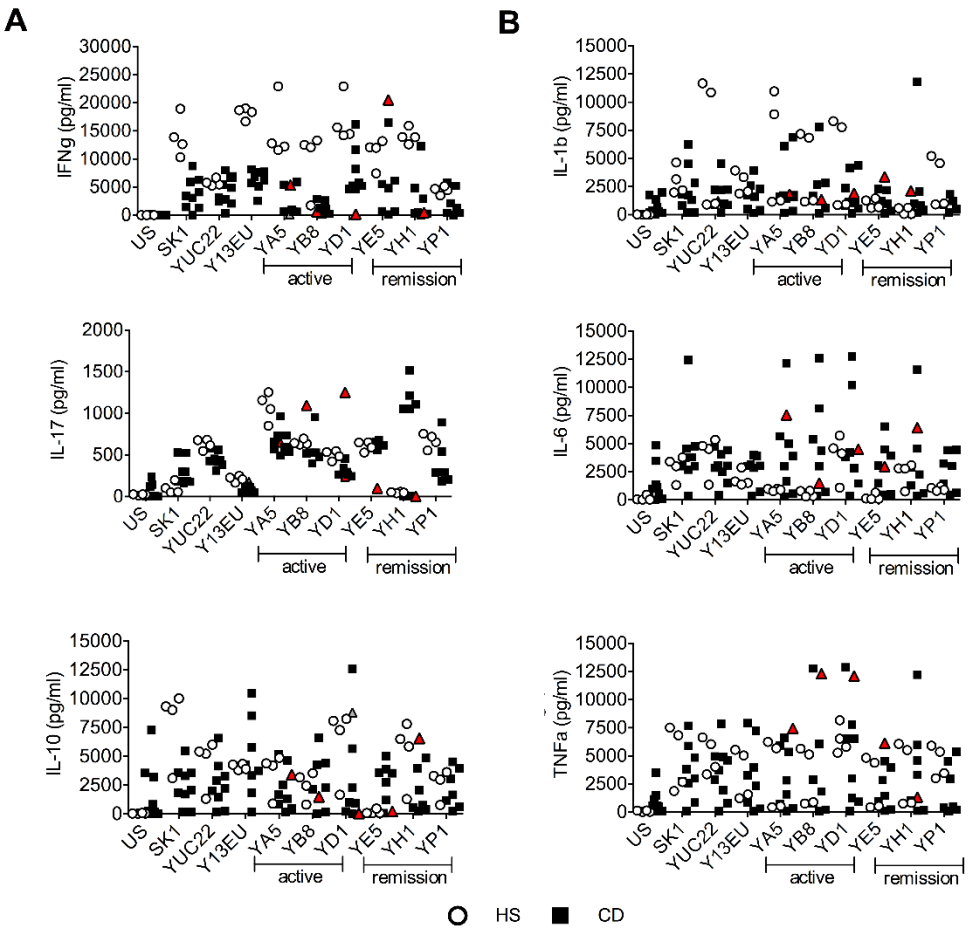


Fig. 7.16 Individual cytokine patterns for *S. cerevisiae* isolates from IBD patients (n=7) and healthy donors (n=6). PBMC T-polarizing (A) and pro-inflammatory (B) cytokine release from HC (white circles) and CD patients (black squares), upon stimulation with 9 *S. cerevisiae* selected strains (indicated in horizontal axis). In each scatter-plot, red triangles represent CD patient PBMC cytokine release after stimulation with his own isolate.

The *in vitro* assay revealed IL-17A, IFN-γ (both known to afford colonization resistance), and IL-10 (the cytokine involved in immune tolerance) as the main factors discriminating *S. cerevisiae* isolates (Fig. 7.17 A-B).

Sporulation rate allows to stratify the *S. cerevisiae* isolates for the differential cytokines induction (Fig. 7.17B). Not sporulating HG1 strains showed a tendency towards the induction of IFN-γ-mediated responses (Fig. 7.17B). HG2 strains with low sporulation efficiency induced high IL-17A production (Wilcox rank sum test $p<0.05$; Fig. 7.17B). The opposite trend was observed for the high sporulator (HG3), in which the high IFN-γ mediated-inflammatory response was counterbalanced by high levels of the immunosuppressive cytokine IL-10 (Fig. 7.17).

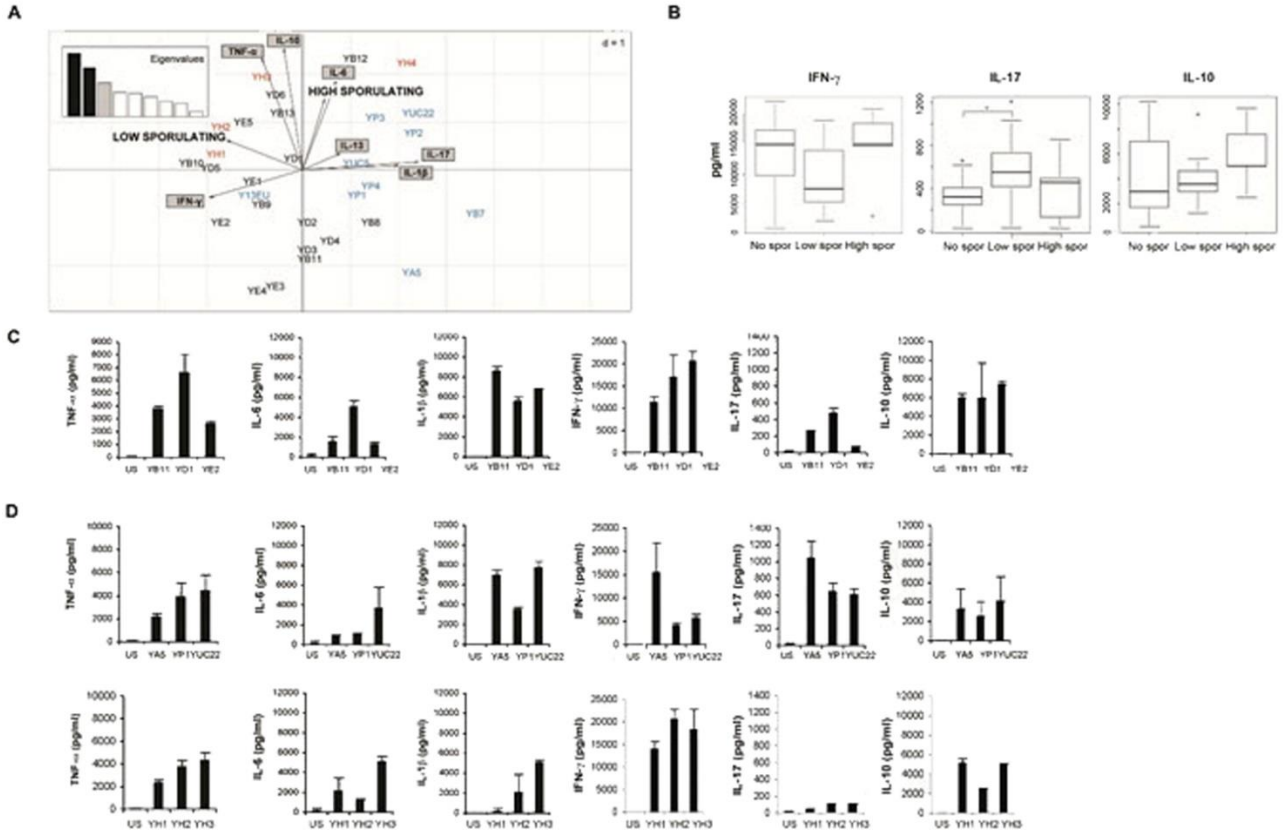


Fig. 7.17 Relationship between pro-inflammatory cytokine (TNF-α, IL-6 and IL-1β) and T-polarizing cytokine (IFN-γ, IL-17 and IL-10) release to *S. cerevisiae* strains and sporulation ability. (A) Duality diagram for the first two components obtained by analyzing correspondence between healthy human PBMCs immune reactivity to *S. cerevisiae* isolates and the ability of yeast to sporulate. The cases for analysis were the *S. cerevisiae* isolates from fecal samples of IBD patients and HC. Black labels: HG1-isolates not able to sporulate; blue labels: HG2-isolates with low sporulation frequency; red labels: HG3-isolates with high sporulation frequency. The variables are the ability to sporulate at low and high frequency and cytokines (grey rectangles). (B) Box plot of cytokine release (pg/ml) upon stimulation with yeast strains, grouped by sporulation ability. (C-E) PBMC cytokine release from healthy volunteers, upon stimulation with selected *S. cerevisiae* strains (C) not able to sporulate-HG1 (D) able to sporulate at low frequency- HG2 and (E) able to sporulate at high frequency-HG3. The means of cytokine measurements for six healthy volunteers, error bars and SD are reported.

7.3.10 Cell wall components correlated with PBMCs cytokine release

We than investigated correlations among the cell wall sugar composition of each gut strain and among the levels of cytokines produced by donors' PBMCs upon strain's challenge (Spearman r; Fig. 7.18). The analysis revealed that galactose amount positively correlated with glucosamine, and confirmed that mannose amount negatively correlated with galactose and glucosamine (Spearman r; Fig. 7.18). The pro-inflammatory IL-17 and IL-1β cytokines positively correlated among them (Spearman r; Fig. 7.18). Interestingly, the amount of glucose in the cell wall was positively correlated with the levels of IL-17A, and negatively with IFN-γ produced by challenged donor PBMCs, in concordance with the knowledge that *b*-glucans promote Th17 differentiation (Gagliardi, Teloni et al. 2010;Kashem, Igyarto et al. 2015).

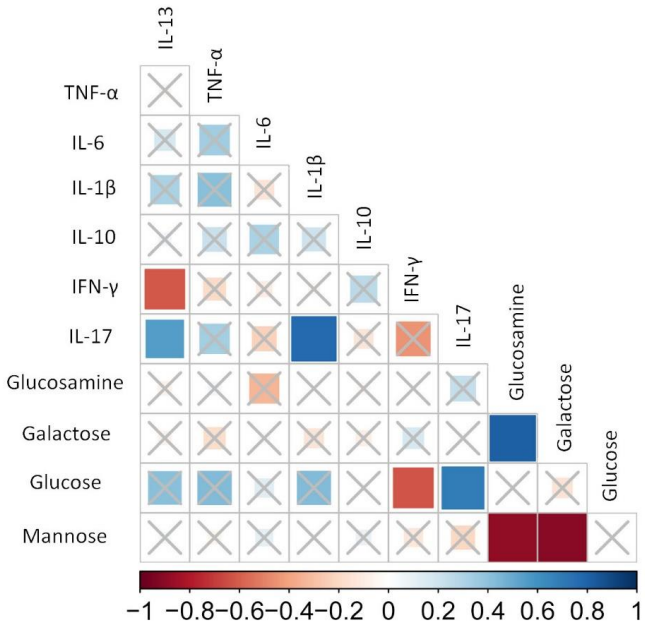


Fig. 7.18 Spearman correlation among amount of cell wall components of *S. cerevisiae* gut isolates and IL-17A, IFN-γ and IL-10 release (the intensity of colors red or blue in each rectangle indicate different rate of negative and positive correlation respectively, among the variables reported in the two coordinates, as indicated by colored scale bar; the "X" indicate no-correlation).

7.3.11 Fungal interaction with gut bacterial communities

Fungi and bacteria coexist within the gut environment and may directly interact either in mutualistic or competitive relations. The observed different strain-specific immune responses, upon stimulation with different *S. cerevisiae* and *Candida spp*. isolates, suggest that the presence/absence of yeasts could result in different bacterial community structures. We thus performed metagenomics analysis on a selection of 19 fecal samples from CD patients (Table 7.10), from which we isolated *S. cerevisiae* (n=7) or *Candida spp.* (n=5), and we compared them with fecal samples from which we did not isolated any yeasts (n=7). We sequenced the V5-V6 hypervariable region of 16S rRNA gene for the meta-taxonomic study of microbiota composition.

In order to evaluate differences in the microbial biodiversity (alpha-diversity) in presence or absence of cultivable fungi and considering the disease status, we calculated the bacterial species richness (by observed number of OTUs), finding a higher bacterial richness, when fungal isolates were absent than when we isolated *Candida spp*. or *S. cerevisiae* (Fig. 7.19A). This result could be related to the competition between fungal and bacterial communities in the gut environment. However, Shannon index, accounting evenness of the species distributions based on entropy, did not show these differences. Considering disease status, we observed an increase in bacterial species richness in samples collected in remission, considering either the number of OTUs or Shannon index, this latter resulting statistically significant (Fig. 7.19B; Wilcokon rank sum test; p value=0.003). This result is in accordance with the well-known reduction of bacterial richness in IBD gut microbiota, especially in active disease.

Table 7.10 Description of disease status and presence of yeast for each fecal sample used to metagenomic analysis

ID fecal sample	Disease status	Presence of yeast in fecal sample	Yeast species
1CHT26	active	Y+	*S. cerevisiae* (YA5)
2CHT4	active	Y+	*S. cerevisiae* (YB8)
2CHT5	active	Y+	*S. cerevisiae* (YB10)
3CHT13	remission	Y+	*S. cerevisiae* (YP)
6CHT1	active	Y+	*S. cerevisiae* (YD)
12CHT0	remission	Y+	*S. cerevisiae* (YE)
17CHT0	active	Y+	*S. cerevisiae* (YH)
1CHT14	active	Y+	*C. albicans*
1CHT34	active	Y+	*C. albicans*
23CHT13	remission	Y+	*C. albicans*
4CHT0	active	Y+	*C. parapsilosis*
24CHT8	remission	Y+	*C. parapsilosis*
23CHT0	active	Y-	-
24CHT0	active	Y-	-
2CHT0	active	Y-	-
3CHT0	active	Y-	-
3CHT3	remission	Y-	-
4CHT5	active	Y-	-
4CHT6	active	Y-	-

Note: Y+= presence of yeast isolates; Y-= absence of yeast isolates. In yeast species colomn, in brackets the isolates of *S. cerevisiae* tested for immunologic and phenotypic features are reported.

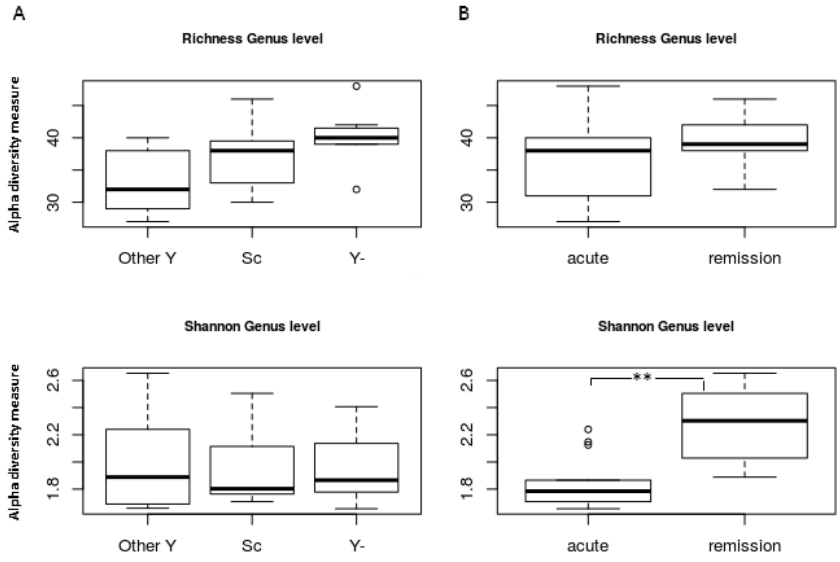


Fig. 7.19 Alpha diversity of gut microbiota in presence/absence of fungal isolates. Box plots of species richness measured by number of observed OTUs and Shannon index at genus level,

considering (A) presence/absence of fungal isolates in fecal samples (Other Y= *Candida spp*.; Sc= *S. cerevisiae*; Y- = absence of fungi), or (B) disease status of the patients (active and remission). Pairwise comparisons using the Wilcoxon rank sum test, **p<0.005.

To estimate the variability of microbial community between-sample (beta-diversity), we performed PCoA calculated on Bray-Curtis dissimilarities (Betadispersion). Although we found no statistically significant differences, we observed higher variability of bacterial communities in absence of cultivable fungi and progressively lower biodiversity in presence of other fungi, especially with *S. cerevisiae* (Fig. 7.20A), confirming the microbial competition in gut environment. However, we found a statistically significant increase in beta diversity in samples collected in active disease compared to remission (Fig. 7.20; Wilcokon rank sum test; p=0.03).

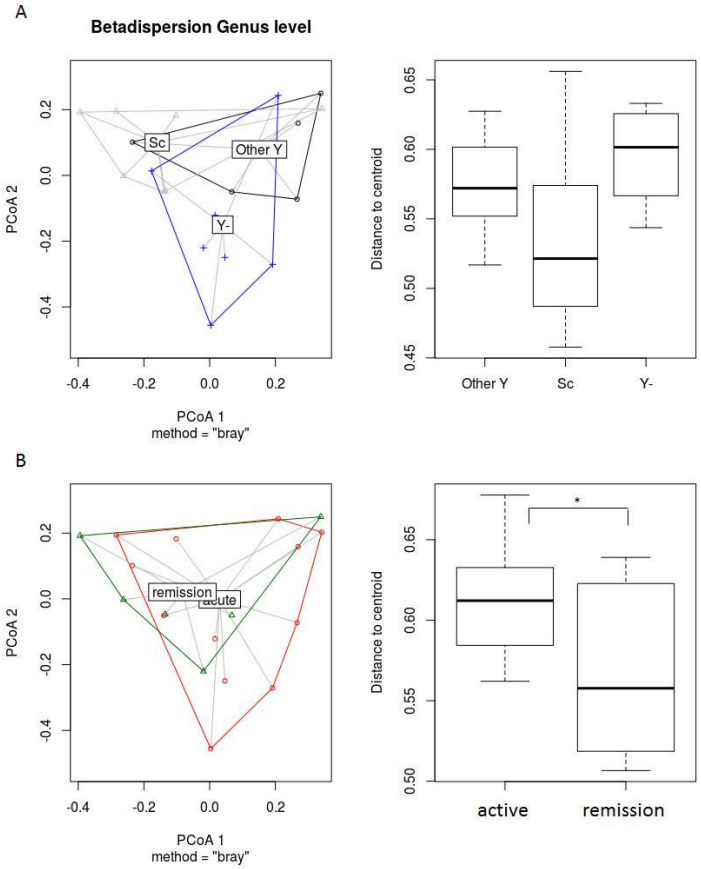


Fig. 7.20 Betadispersion analysis. Principal Coordinate analysis (PCoA) calculated on Bray-Curtis distances considering (A) presence/absence of fungal isolates in fecal samples (Other Y= *Candida spp*.; Sc= *S. cerevisiae*; Y- = absence of fungi), and (B) disease status of the patients (active and remission). The colored networks represent distances of each point from centroid. The calculated distances from centroids are reported in the boxplot on the right, respectively. Pairwise comparisons using the Wilcoxon rank sum test, *p<0.01.

The taxonomic distribution in the three groups showed variations in gut microbiota composition.

At phylum level (Fig. 7.21A), we observed that Bacteroidetes was more abundant when *S. cerevisiae* was isolated, compared to fecal samples in which we isolated other fungi (i.e. *Candida spp.*) or when we did not find any fungal isolate (Wilcoxon rank sum test, p=0.04, fdr correction). An opposed trend was observed regarding Proteobacteria that were more abundant in samples in which we did not find fungal isolates, and reduced when *S. cerevisiae* was isolated. Firmicutes were more abundant when *Candida spp.* were isolated. Considering disease status, we did not find differences at phylum level.

At family level (Fig. 7.21B), we observed abundance of *Bacteroidaceae* in fecal samples collected in remission and in which we isolated *S. cerevisiae.* While *Lachnospiraceae* and *Prevotellaceae* were found in presence of *Candida spp.*, and *Enterobacteriaceae*, *Aeromonadaceae* and *Pasturellaceae* in fecal samples, collected in active disease and in which we did not find fungi.

These results suggest that presence of fungal isolates and disease status could contribute to the shaping of bacterial microbiota composition. In fact, we found an association in remission among presence of *S. cerevisiae* and prevalence in *Bacteroidaceae*. Active disease is associated with absence of fungal isolates and prevalence of potential bacterial pathobionts, such as *Enterobacteriaceae*.

At genus level (Fig. 7.21C), we confirmed the results obtained at highest taxonomic levels. For example, abundance in *Bacteroides* were observed in fecal samples in which we isolated *S. cerevisiae*. While an increase in *Clostridium*, *Prevotella* and a decrease in *Faecalibacterium* were found in samples, in which we isolated *Candida spp*.

Interestingly, LEfSe analysis (identifying statistically different features among groups and estimating the effect size of each differentially abundant feature; see materials and methods) confirmed the abundance in Bacteroidetes phylum (Fig. 7.22A), and in particular abundance in *Bacteroides* genus, associated with presence of *S. cerevisiae*, together with *Pseudomonas* and *Phascolarctobacterium*, belonging to *Veillonellaceae* family, and involved in succinate fermentation (Fig. 7.21C). In fecal samples, in which we did not isolated any fungi, we confirmed an enrichment in Proteobacteria (Fig. 7.21A), especially *Enterobacteriaceae* families and *Plesiomonas* genus (Fig. 7.22C), known as *Aeromonas shigelloides* (name "shigelloides" is derived from the fact that many strains cross-react antigenically with *Shigella*) (Chida, Okamura et al. 2000), found responsible of diarrhea and gastroenteritis in humans (Brenden, Miller et al. 1988).

Comparing fecal samples in which we isolated *S. cerevisiae* with respect to samples in which we isolated *Candida spp.*, associated with *S. cerevisiae* isolates, we observed enrichment in *Porphyromonadaceae* family, and the well-known anti-inflammatory *Faecalibacterium* genus (Fig. 7.22B-C). In presence of *Candida spp.*, we found enrichment of *Aeromonadales*, *Clostridiaceae*, *Plesiomonas*, *Erwinia* and *Clostridium* (Fig. 7.22B-C). Comparing fecal samples in which we isolated *Candida spp.*, with respect to samples in absence of fungi, we observed enrichment in *Clostridium* genus, and *Mogibacteriaceae* family, that include genera such as *Mogibacterium* that was previously isolated from subgingival biofilm of periodontitis (Casarin, Saito et al. 2012) respectively (Fig. 7.22B-C).

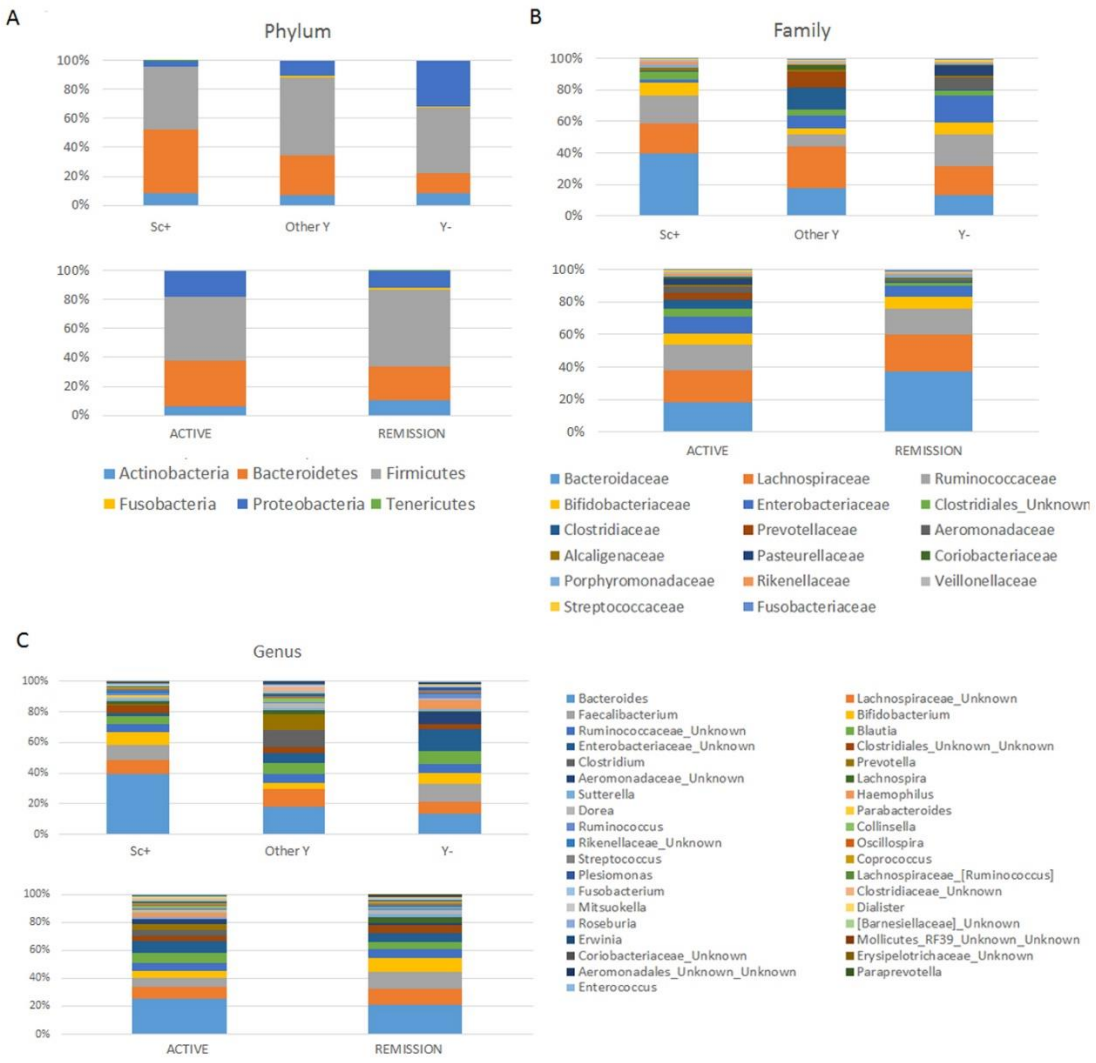


Fig. 7.21 Stacked bar-plot representation of the relative abundances at (A) phylum, (B) family and (C) genus level of the fecal microbiota of CD patients from metagenomics analysis distributed according to presence/absence of fungal isolates (Sc+= *S. cerevisiae*; Other Y= *Candida spp.* isolates; Y-= no fungal isolates) in fecal samples and disease status (active vs remission) at collection's time.

Considering disease status, we observed that *Bacteroidaceae* family and *Coprococcus*, *Paraprevotella*, *Lactococcus* and *Roseburia* genera were significantly enriched in remission status (Fig. 7.22D). However, LEfSe analysis did not find any bacterial taxa significantly enriched in active disease.

These results suggest that the presence of *S. cerevisiae* and its immunomodulatory properties, could promote a favorable gut environment for beneficial genera, such as *Faecalibacterium.* This could be the result both of production of specific metabolites or mediated by trained immunity, an innate immune mechanism through which yeasts could alter the bacterial communities composition by targeting the immune system against specific harmful bacteria (Rizzetto, Ifrim et al. 2016). On the contrary, the presence of *Candida spp.* promote enrichment in potential pathogenic bacteria, such as Proteobacteria and in particular *Enterobacteriaceae*.

However, independently by fungal isolates, significant differences in gut microbiota profiles could be found in relation to disease status. Many of these taxa have been reported in previous studies (Sokol, Cosnes et al. 2008;Willing, Dicksved et al. 2010;Morgan, Tickle et al. 2012;Gevers, Kugathasan et al. 2014;Sokol, Leducq et al. 2016), including *Lachnospiraceae*, *Enterobacteriaceae*, and *Pasteurellaceae*. Notably in IBD flare, *Coprococcus* were decreased as well as *Roseburia*, and *Faecalibacterium* (Sokol, Leducq et al. 2016).

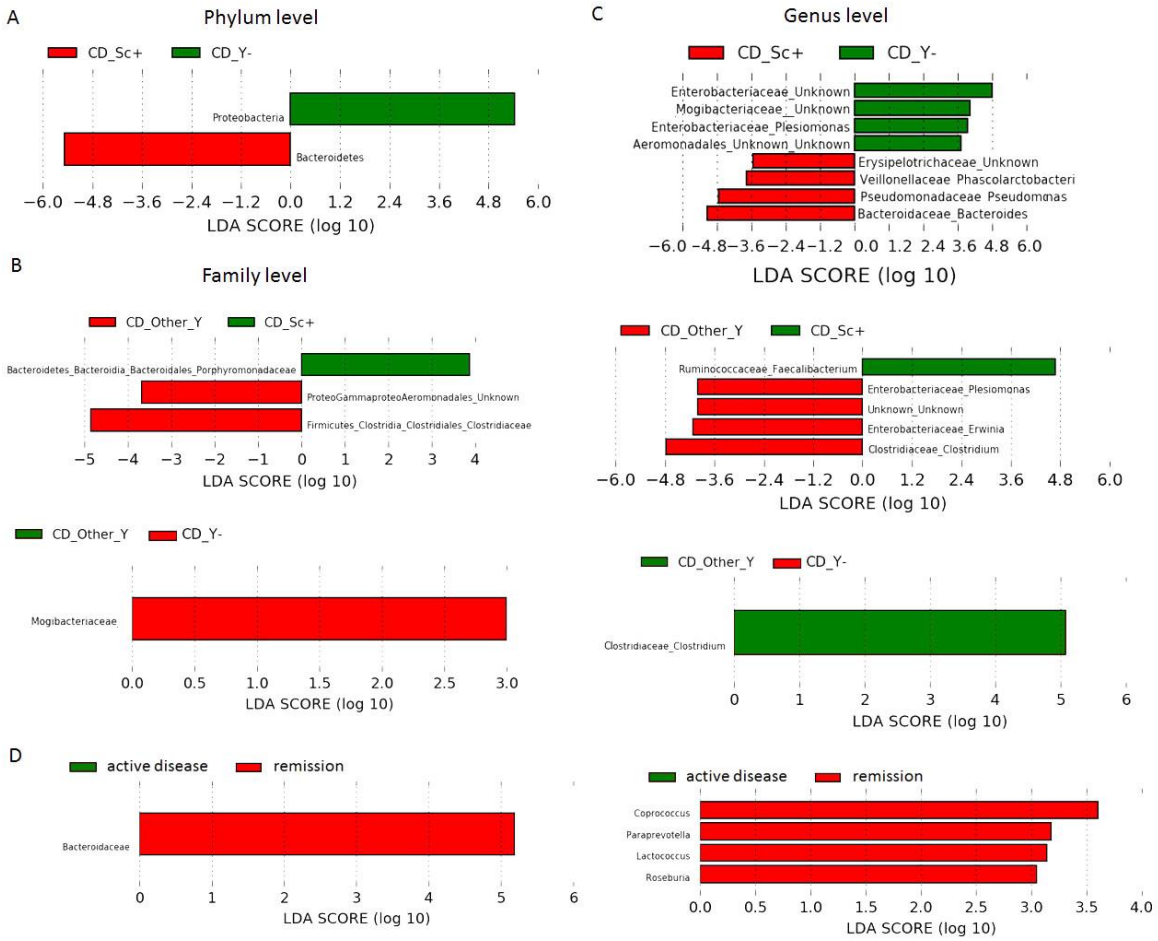


Fig. 7.22 Differences in bacterial taxa in relation with presence/absence of cultivable fungi and disease status. LEfSe analysis shows a statistically significant enrichment of (A) phylum, (B) family and (C) genera, in CD patients' fecal samples in relation with (A-C) presence/absence of fungi (CD_Y-), and specifically in presence of *S. cerevisiae* (CD_Sc+) or other fungi (CD_Other_Y), and (D) in association with active disease and remission. LEfSe results indicate a sequentially significant ranking among groups (Alpha value=0.05 for the factorial Kruskal-Wallis test among classes). The threshold for the logarithmic LDA score was 2.0.

7.4 Conclusions

Analysis of cultivable gut mycobiota showed that *S. cerevisiae* is enriched in CD patients compared to UC and healthy children. Microsatellite genotyping of *S. cerevisiae* human gut isolates, allowed identification of clusters with common specific genomic characteristics,

together with strains with an environmental/food-borne origin. The clonal expansion observed in gut environment of each CD patient suggests that a CD-specific gut environment may favor colonization and clonal expansion of fungi, such as *S. cerevisiae*, that normally are passengers, and occasionally can become colonizers. Moreover, the human gut could be considered amongst the different ecological niches of *S. cerevisiae*, so far represented by natural environments (e.g. grape, soil).

Studies on the mice model showed that gut inflammation promotes fungal proliferation (Jawhara and Poulain 2007). Our observation on clonal expansion could be a result of increase in permeability on conditions associated to a leaky gut that may favor colonization with *S. cerevisiae*.

The phenotypic characterization of *S. cerevisiae* strains showed different traits potentially associated with gut adaptation and colonization. Analysis of cell wall sugar composition showed that cell wall of HG strains is uniquely composed by high levels of galactose and glucosamine and low levels of glucose and mannose, a feature that discriminates gut-derived strains from isolates from other origins. Particularly interesting is the finding that galactose is a discriminating component of gut strains. Galactose has been reported to be crucial in cell adhesion, biofilm formation and flocculation. The ability to grow in biofilm and floc represents an advantage in response to stress condition or in immune response recognition (Smukalla, Caldara et al. 2008). Strains from environmental origin, when grown in lactate, also show enrichment in galactose, thus indicating that the genetic makeup is similar but that the expression of genes for galactose metabolism is constitutive in gut strains only, thus suggesting an "environmental imprinting" on the strains.

Regarding *C. albicans*, it is well-known its role as opportunistic pathogen. *C. albicans* morphogenetic plasticity is considered to be its most important virulence attribute (Kumamoto and Vinces 2005;Lu, Su et al. 2011). We showed that two distinct *C. albicans* isolates (YL1 and YQ2) from CD patients elaborate differential response to phagocytes and this phenomenon is dependent upon the genomic background and their ability to adapt to host environment. The whole genome sequencing of the two *C. albicans* isolates revealed that these strains present a great number of highly polymorphic genes, compared to the reference strain, involved external structure, cell wall composition, fungal-type cell wall, cell adhesion and hyphal cell wall determinants. By assessing their ability to undergo morphological switch from yeasts to hyphae, we found that the YL1 strain form biofilm (characterized by a higher density of hyphae with several interspersed germinating yeasts) more rapidly than the YQ2.

Adherence to epithelia is one of the prerequisite essential to accomplish pathogenesis of *C. albicans* in the mammalian host. Our results showed that YL1 has a higher ability, compared to YQ2, to adhere to human intestinal cells *in vitro*. Furthermore, phagocytes plays a critical role in balancing colonization/infection caused by this opportunistic yeast. Interestingly, albeit similarly internalized, the YL1 isolate, in contrast to YQ2, was able to resist to intracellular killing, and even replicate inside the microglia by inhibiting phagosome maturation in these cells.

The studies on the cell wall sugar composition have demonstrated marked differences between the two isolates in terms of mannan and glucan content. Indeed, the YQ2 strain

exhibited higher mannan concentrations in its cell wall, while YL1 had more glucan, accounting for the diversity in the yeast cell:hyphae ratio observed in the biofilm structures. The different composition of cell wall in human gut isolates in *S. cerevisiae* is associated with strain-specific differences in the cytokine pattern induced in healthy donors, thus reflecting the yeast strains ability to induce different inflammatory responses.

The discovery that cell wall glucose amount in *S. cerevisiae* strains is positively correlated with the level of IL-17A induced in challenged PBMCs is in concordance with the knowledge that the sugar portions of cell wall are the principal antigens recognized by the host immune system during host-fungal interplay, and that *β*-glucans promote Th17 differentiation. As a consequence, we can hypothesize that galactose helps yeasts to hide from immune functions and reduce inflammatory responses, that in the end would lead to strain clearance.

Increase of the intestinal permeability, dysbiosis (Koh 2013), or even impairment of the immune system are all conditions whose complicate the fungal-host interplay (Underhill and Iliev 2014). We observed that in CD the presence of yeasts significantly correlated with mucosal inflammation, in agreement with recent observations (Li, Wang et al. 2014), and that ASCA levels correlate with the presence of fecal fungi. However, it is known that ASCA production is not promoted by a specific yeast species, but rather by strain-specific antigenic properties (McKenzie, Main et al. 1990). Thus, the comparison with immunomodulatory properties of *S. cerevisiae* and *Candida spp*. isolates allowed the differentiation of pro-inflammatory response between these two fungal genera. In general, we observed differential immune response upon stimulation with both *S. cerevisiae* and *Candida spp*., but the large diversity in the immunomodulatory profiles, considering the different isolates, suggests a strain-specific pattern of cytokines. Moreover, the different strains isolated from diverse ecological niches showed an immune-based diversity which seem to correlate with their origin.

The immunological screening allowed the categorization of different isolates either belonging to *Candida spp* or *S. cerevisiae.* The different *S. cerevisiae* isolates showed diverse ability to promote proinflammtory response, *via* IL-6, IL-17 and IFN-γ, counterbalanced by IL-10 induction, suggesting the ability of *S. cerevisiae* to promote either inflammation, resistance or tolerance upon immune cell recognition. *C. albicans* isolates could be categorize as INF^+ or INF^- strains or inducers of IL-1 β and low inducer of IL-10.

By comparison of healthy donors and CD patients PBMCs cytokine release, upon *S. cerevisiae* challenge, we observed a general wide variability in immune reactivity, especially for PBMCs of patients, probably derived from clinical status of the patient and alteration status of immune system against fungal strains. The *in vitro* assay revealed IL-17A, IFN-γ (both known to afford colonization resistance), and IL-10 (the cytokine involved in immune tolerance) as the main factors discriminating *S. cerevisiae* isolates. Interestingly, in some cases, CD patients' response differently to own isolates compared to *S. cerevisiae* from other patients, as a result of altered immune response and of induction of trained immunity by fungal strain. PBMCs of CD patient with both clinical remission and lack of mucosal inflammation, upon stimulation with own *S. cerevisiae* isolate, induced high IFN-γ and TNF-α and low IL-17 compared to other patients in response to the same isolate. While, strains

isolated from patients in active disease and mucosal inflammation show high levels of IL17 and TNF-α and low level of IFN-γ and IL10.

In addition, the observed immune responses induced by gut *S. cerevisiae* strains seem to be strongly correlated with sporulation ability, and the sporulation correlated with the ASCA levels measured in CD patients. Sporulating isolates show opposite immunomodulatory ability with respect to non-sporulating strains (HG1), inducing IFN-γ-mediated responses. Strains (HG2) with medium ability to sporulate elicit IL-17-mediated inflammatory response (Th-17) and this is associated with glucose-rich cell wall. Only HG3 strains, the highest sporulators, induced tolerogenic response (high level of IFN-γ counterbalanced by IL-10 induction), showing to be able to escape immunosurveilance.

The results herein presented indicate how, from an evolutionary perspective, gut environment could serve as reservoirs and evolutionary niches for *S. cerevisiae*, in which genetic makeup could ultimately influence its immunogenicity, highlighting the need to consider the interplay between fungal cell wall and gut immune function in determining mycobiota composition.

Clonal expansion of strains with peculiar genetic characteristics and cell wall composition, derived probably by a single colonization event, could be the result of selection and adaptation to a peculiar leaky gut environment. This could be particularly relevant in understanding the role of these strains in health and disease, since the molecular mechanisms used by yeast to colonize the host, as a harmless commensal appear as a continuum with the strategies used for evading immune surveillance and can thus potentially turn a friend into a foe.

In the gut environment, yeast survival is difficult and competition is intense, if we considered coexistence with bacteria. We also evaluated the gut microbiota profiles in presence/absence of cultivable fungi. Our results showed that the presence of *S. cerevisiae* and its immunomodulatory properties promote a favorable gut environment for beneficial genera, such as *Bacteroides* and *Faecalibacterium.* While gut microbiota of CD patients enriched in *Candida spp.* or other species favor enrichment in potential pathogenic bacteria, especially Proteobacteria and *Enterobacteriaceae*.

Our *in vitro* results showed that exposure to commensal fungi can affect the immune response. The ability of these commensal to survive in different host niches suggests the acquisition of functional specializations for commensalism and disease, acting mainly on modification of the cell wall structure and composition. Such differences occur both in commensals such as *S. cerevisiae*, and opportunistic pathogens, such as *Candida albicans*. Thus, indeed, understanding of the impact of fungi on host's immune system, in health and disease, depends on strain specific phenotypic and genomic characteristics, modulating their mechanism of interaction with the host immune system. The frontiers of metagenomics and immunology studies thus should move from species level generalizations, to the profound understanding of the strain level networks of interactions both in the fungal and in the bacterial communities.

8. Summary

The comparative characterization of microbial communities (bacteria and fungi) in healthy children populations and in pediatric patients affected by autoimmune and inflammatory diseases provided insights on involvement of microbial profiles and the developing of pathologies typical of the western world.

Taking as a reference traditional pediatric populations living in rural village, we monitored the metagenomic scale of microbial changes upon transition to suburban and urban areas and shift to a globalized diet. We discovered key microbial patterns that are lost in the course of urbanization, industrialization and westernization, and a marked shift to bacterial patterns that in the microbiota of western populations appear associated to IBD and other autoimmune diseases. Analysis of the microbial functions, changed upon the transition from rural to urban life, revealed loss of functions related to dietary changes, in particular loss of functions related to fiber and polysaccharides degradation, due to the poor intake of dietary fiber and the high content in animal protein and fat, and gain of genes for antibiotic resistance, such as beta-lactamase resistance derived by antibiotics usage in livestock practice and in the medicine. Also, degradation of xenobiotics represent a functional response of symbiontic bacteria to exposure of toxic compounds derived by pollution, industrialized and urban environment, and organic insecticide potentially used in agriculture, as well as to food additive widely used to preserve foods. The importance to preserve an ancient microbiome and maintain biodiversity has considerable repercussions for human health, especially in industrialized and westernized populations, in which non-communicable diseases are spread. Our study showed the gradual disappearance of ancient microbes during the transition from rural to urban environment, and the importance to develop strategies either to support symbiont dispersion, reducing the diffusion of pathogens, or to reintroduce ancient bacteria with important functional acquisitions that have been destroyed in the course of urbanization of human populations.

Inflammatory bowel disease represent one of the major pathologies of the western world, with a growing increase in the pediatric population. In order to deepen the knowledge of the pathogenesis of IBD at early onset (EO), we evaluated the phenotype and course of EO-IBD (0–5 years) compared with pediatric later-onset disease (6–11 and 12–18 years), observing potential differences in presentation and behavior of EO forms of the disease. Understanding the factors that contribute to early age of onset of IBD could potentially facilitate intervention strategy development.

Another typical immune-mediated condition that is largely increasing in western pediatric populations is Juvenile Idiopathic Arthritis (JIA). JIA comprises a clinically heterogeneous group of conditions characterized by chronic arthritis and synovial inflammation. Among these, enthesitis related arthritis (ERA) is frequently characterized by clinical and subclinical intestinal involvement. In this PhD thesis, we observed association between alterations of gut microbial profiles in ERA samples and either HLA-B27 status or pharmacological therapies, such as anti-inflammatories, corticosteroids and anti-TNF drugs. Our results on JIA show intriguing links in terms of fecal microbiota profiles with IBD and other autoimmune diseases associated to gastrointestinal disorders. Dysbiosis could have a pro-arthritogenic role together with enriched microbial functions related to cell motility and chemotaxis, representing possible traits of virulence that could be associated to gut

inflammation. Our results suggest a potential improved ability of microbial components to pass through the gastrointestinal barrier and migrate in other districts, such as synovial fluid and joints, inducing inflammation.

However, the observed extreme inter-individual variability of microbiota in inflammatory and autoimmune diseases suggest that no a single microorganism is involved in pathogenesis of these disorders. Further investigations should address the causes of perturbation and the restoration of microbial equilibrium, in order to adopt therapeutic strategies able to maintain microbial diversity, essential for immune homeostasis and the host's health. Research in this direction should include therapeutic strategies able to modulate the microbiota, with not only diet and probiotics, but also evaluating new therapeutic approaches, such as fecal transplantation, recently adopted in other diseases, especially in *C. difficile* inducing-diarrhea, and that have shown some effectiveness during chronic inflammation. Furthermore, the understanding of the microbial functional acquisition and the relationships with epithelial barrier function and host immune response could help to understand the contribution of microbiota in chronic inflammation.

Another aspect deepen in the present PhD thesis is the characterization of fungal communities in health and disease. We try to define a "healthy" gut mycobiota, showing that the intestinal fungal communities of healthy volunteers is a variegate ecosystem that differs in function of individuals' life stage in a gender-dependent manner. As occurs for the bacterial microbiota, the intestinal mycobiota is shaped by host's age, gender, diet and geographical environment. Previous studies have shown that the development of the gut bacterial microbiota starts at birth with colonization by a low number of species from the mothers's vaginal and fecal microbiota and it is characterized by many shifts in composition during infancy. Similarly, the mycobiota may show the same fate. However, we observed an inverted trend in which the richness of the gut mycobiota of infants and children was higher than in adults, suggesting that, because during infancy the bacterial microbiota is less stable, a weak bacterial competition occurs, leading to an increase of fungi during the early stages of life. Suppression of the bacterial microbiota upon treatment with antibiotics or diet could be further factors involved in the shaping of gut mycobiota.

The majority of fungal isolates living in our gut has been previously described as inhabitants of the mammalian gastrointestinal tract, some of the isolates belong to species so far identified only in environmental samples. The fungal isolates of this study, showing resistance to oxidative, high temperature, bile acids and pH stresses, may hold the potential to colonize the human gut. It is plausible that fecal fungal isolates with specific characteristics survived to the gut environment, and that these traits make them able to colonize the gut. Thus, we can hypothesize a long process of evolution, selection or adaptation of environmental and food-borne strains to the human host, suggesting that pathogenic strains of commensal species can evolve through a repeated process of evolution and selection, depending on the immune status of the host.

To assess the potential role of fungal strains in etiology of IBD, we investigated the immunomodulatory features of fungal isolates from IBD patients. We characterized phenotypically and genotypically *S. cerevisiae* and *C. albicans* isolates, for traits related to adaptation to gut environment, in order to understand the strategies related to interplay or

evasion of host immune system, and the relationships between fungal and bacterial gut communities.

Analysis of cultivable gut mycobiota showed that *S. cerevisiae* is enriched in CD patients compared to UC and healthy children. The clonal expansion observed in gut environment of CD patients suggests that a CD-specific gut environment and leaky gut, may promote patients colonization and clonal expansion of fungi, such as *S. cerevisiae*, that normally are passengers, and occasionally can become colonizers.

Interestingly, we observed that in CD the presence of yeasts significantly correlated with mucosal inflammation, and that ASCA levels correlate with the presence of fecal fungi. However, it is known that ASCA production is not promoted by a specific yeast species, but rather by strain-specific antigenic properties. The comparison with immunomodulatory properties of *S. cerevisiae* isolates and *Candida spp*. allowed to differentiate pro-inflammatory response between *S. cerevisiae* and *Candida spp.*. Our *in vitro* results showed that exposure to commensal fungi can affect the immune response. The ability of these commensal to survive in different host niches suggests the acquisition of functional specializations for commensalism and disease, acting mainly on modification of the cell wall structure and composition. Such differences occur both in commensals such as *S. cerevisiae*, and opportunistic pathogens, such as *C. albicans*.

We also evaluated the gut microbiota profiles in presence/absence of cultivable fungi, in order to understand the bacterial-fungal interaction in the gut environment. Our findings showed that the presence of *S. cerevisiae* and its immunomodulatory properties promote a favorable gut environment for beneficial bacteria. On the contrary, gut microbiota of CD patients enriched in *Candida spp.* or other species promote enrichment in potential pathogenic bacteria belonging to Proteobacteria phylum. However, bacterial profiles resulted specific for disease status and in particular for remission, as observed in previous studies on IBD.

Altogether, these results encourage for in-depth, strain-level extensive studies on human gut mycobiota and the integration of metagenomic data with immunology to further establish the relevance of fungi in host physiology and host-microbe interaction, as well as the interaction with microbial communities.

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10. Publications

Phenotype and Disease Course of Early-onset Pediatric Inflammatory Bowel Disease

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Background: Early-onset (EO) pediatric inflammatory bowel diseases (IBD) seem to be more extensive than those with a later onset. To test this hypothesis, we examined the phenotype and disease course of patients with IBD diagnosis at 0 to 5 years, compared with the ranges 6 to 11 and 12 to 18 years.

Methods: Anatomic locations and behaviors were assessed according to Paris classification in 506 consecutive patients: 224 Crohn's disease, 245 ulcerative colitis, and 37 IBD-unclassified.

Results: Eleven percent of patients were in the range 0 to 5 years, 39% in 6 to 11 years, and 50% in 12 to 18 years. Ulcerative colitis was the most frequent diagnosis in EO-IBD and in 6- to 11-year-old group, whereas Crohn's disease was predominant in older children. A classification as IBD-unclassified was more common in the range 0 to 5 years compared with the other groups ($P < 0.005$). EO Crohn's disease showed a more frequent isolated colonic ($P < 0.005$) and upper gastrointestinal involvement than later-onset disease. Sixty-two percent of the patients in the 0 to 5 years range had pancolonic ulcerative colitis, compared with 38% of 6 to 11 years ($P = 0.02$) and 31% of 12–18 years ($P = 0.002$) range. No statistical difference for family history for IBD was found in the 3-year age groups. Therapies at the diagnosis were similar for all children. However, at latest follow-up, a significantly higher proportion of younger children were under steroids compared with older groups ($P < 0.05$). Surgical risk did not differ according to age.

Conclusions: EO-IBD exhibits an extensive phenotype and benefit from aggressive treatment strategies, although surgical risk is similar to later-onset disease. A family history for IBD is not common in EO disease.

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Key Words: children, inflammatory bowel disease, early onset

The inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are complex polygenic disorders, resulting of an interplay between genetic, environmental, and immunological factors leading to a dysregulated immune response of the host to the intestinal microbiota.[1,2] Up to 25% of patients first present during childhood or adolescence,[3] with early-onset (EO) (0–5 yr) and very early-onset (VEO) diseases (0–2 yr) increasingly reported.[4–7] Although data on EO forms are scattered, they seem to be characterized by a unique phenotype, extensive and aggressive, which affect the management of this age group.[5] Moreover, prior reports suggest that EO-IBD differ epidemiologically and are distinguished by a predominant colonic involvement and a greater impact of genetic predisposition, compared with older age of onset.[8,9] From this point of view, EO disease represents an exceptional model to study the initial host immune response to characterize genotype–phenotype relations and identify environmental factors influencing disease development, as well as to evaluate the natural history of the disease.[10–12] Understanding the factors that contribute to early age of onset could potentially facilitate intervention strategy development, thereby influencing disease outcome and risk of long-term sequelae. Recently, an increased interest for early forms of disease came from the discovery of Crohn's-like colitis, very

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severe and not responding to conventional therapies, associated with a defect of the receptor of interleukin 10 (IL-10R) or IL-10 itself, in a subset of patients developing the disease within the first months of life.[13,14] Such severity and EO point to probable monogenetic diseases as opposed to the multigenetic trait observed in the known forms of IBD. This discovery opens new horizons in the knowledge of the pathogenesis of the disease; hence, a better clinical definition of early and very early forms of disease may lay the foundation for further studies of genotype–phenotype correlation. Thus, primary aim of this study was to evaluate the phenotype and course of EO-IBD (0–5 yr) compared with pediatric later-onset disease (6–11 and 12–18 yr). Moreover, as a secondary outcome, within the 0- to 5-year group, we evaluated potential differences in presentation and behavior of VEO forms of the disease (0–2 yr) than those presenting between 3 and 5 years.

PATIENTS AND METHODS

Pediatric gastroenterologists from all the Italian pediatric IBD centers belonging to the Pediatric Gastroenterology, Hepatology and Nutrition Italian Society in 2008 established a prospective registry to collect demographic, clinical, and epidemiologic data from pediatric patients with IBD. The registry started at January 1, 2009 and included patients less than 18 years with a new diagnosis of IBD. Data of all children enrolled and stored in the registry from January 1, 2009 to April 22, 2013 (the data retrieval date) were used for this study. Fourteen sites participated to this study: Pediatric Gastroenterology and Liver Unit, Sapienza University of Rome, Rome, Italy; Gastroenterology and Nutrition Unit, Meyer Pediatric Hospital, Florence, Italy; Gastroenterology and Endoscopy Unit, G. Gaslini Institute for Children, Genoa, Italy; Pediatric Gastroenterology, University of Padua, Padua, Italy; Pediatric Gastroenterology, University of Messina; Department of Pediatrics, University of Milan, Milan, Italy; Pediatric Gastroenterology and Endoscopy, University of Messina; Pediatric Gastroenterology and Endoscopy Unit, Spirito Santo Hospital, Pescara, Pescara, Italy; Department of Pediatrics, University of Naples Federico II, Naples; Pediatric Department, Maggiore Hospital, Bologna, Italy; Pediatric Department, Giovanni XXIII Hospital, Bari, Italy; Pediatric Gastroenterology Unit, University of Turin. Turin, Italy; Department of Pediatrics, Università Politecnica delle Marche, Ancona, Italy; Department of Pediatrics, Institute of Child Health, IRCSS Burlo Garofalo, Trieste, Italy. Institutional review board approval for the registry protocol and the informed consent and assent forms were obtained at each site before subject enrollment and data collection. Signed parental and patient informed consent and signed youth assent, when appropriate, were required from all patients enrolled. Trained investigators at each center obtained information from the medical records (electronic and paper charts). Standardized information was entered into the registry. Data were submitted quarterly to the central repository at Pediatric Gastroenterology and Liver Unit, Sapienza University of Rome for scrutiny of data quality, maintenance of data integrity, data storage, and subsequent data analysis. The study end date was defined as the date of the most recent clinic visit before April 22, 2013.

Eligible subjects included all patients with any form of IBD (UC, CD, and inflammatory bowel disease unclassified [IBDU]) who were diagnosed before 18 years of age and followed in the pediatric gastroenterology centers at the participating sites. Only new IBD cases, diagnosed after January 1, 2009 were prospectively enrolled. Diagnosis of IBD was based on clinical history, physical examination, endoscopic appearance, histologic findings, and radiologic studies, according to Porto criteria.[15] The latter implied that ileocolonoscopy and upper gastrointestinal endoscopy were assumed to be performed in all patients, as well as imaging of the small bowel (except in patients with a definitive diagnosis of UC). Participating centers were also expected to take at least 2 biopsies from each segment of the gastrointestinal tract (esophagus, stomach, duodenum, terminal ileum, and all segments of the colon) and to record the endoscopic and histologic findings from each segment separately. Allergic disorders (immunoglobulin E, skin prick, and patch tests) and infectious enteritis or colitis (*Salmonella*, *Shigella*, *Escherichia coli*, *Yersinia*, *Clostridium difficile* toxins, *Entamoeba histolytica* stool samples) were excluded during the initial work-up. Immunological defects were also ruled out in all patients with suspected EO-IBD by routinely testing immunoglobulin levels, autoantibodies, lymphocyte phenotyping, granulocytes function, and oxidative burst. Mutations in the IL-10R and IL-10 genes were also evaluated in all children with VEO forms of IBD (<2 yr). Only patients with a final diagnosis of IBD with no other immunological (including those with confirmed IL-10R or IL-10 axis mutations), allergic, or infectious diseases were included in the registry.

If the length of follow-up was less than 6 months, the data were excluded from the analysis. The information retrieved for the purpose of this study included demographic features (age, sex), family history of IBD, IBD type (CD, UC, IBDU), and disease distribution. A family history for IBD was defined by the presence of CD or UC in first-degree relatives only. EO-IBD was defined as a diagnosis made in patients of 0 to 5 years of age. Within this group, VEO-IBD was defined as a diagnosis of IBD made between 0 and 2 years of age. The disease location at the diagnosis and at follow-up was established by endoscopic and imaging evaluations in all patients according to the availability of individual methods for each center and reported in the registry. For the purpose of this article, disease location was described according to Paris classification.[16] For CD, L1 was defined as an involvement of the terminal ileum and limited cecal disease, L2 an isolated colitis, L3 an ileocolonic disease, L4a an upper disease proximal to Ligament of Treitz, whereas L4b distal to ligament of Treitz and proximal to distal 1/3 ileum. For UC, proctitis (E1) was defined as an involvement limited to the rectum (i.e., proximal extent of inflammation distal to the rectosigmoid junction). Left-sided UC (E2) was defined as an involvement limited to the portion of the colorectum distal to the splenic flexure. Extensive UC (E3) was defined as a disease extending proximally to the

splenic flexure but distally to the hepatic flexure, whereas pancolitis (E4) included a colitis extended proximally to the hepatic flexure.

Symptoms at onset of disease included abdominal pain, diarrhea, rectal bleeding, perianal disease, and growth failure. Disease activity at the diagnosis was scored by the Pediatric Crohn's Disease Activity Index (PCDAI)[17] or the Pediatric Ulcerative Colitis Activity Index (PUCAI)[18] for CD and UC, respectively. Laboratory tests included full blood count, C-reactive protein, erythrocyte sedimentation rate, perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA), anti-*Saccharomyces cerevisiae* antibodies (ASCA), nutritional, renal and liver function parameters. Endoscopic and imaging examinations carried out at the diagnosis and follow-up included ileocolonoscopy, esophagogastroduodenoscopy, enteroscopy, capsule endoscopy ultrasound with or without oral contrast, and magnetic resonance imaging.

Medications comprised 6-mercaptopurine (6-MP)/azathioprine, methotrexate, thalidomide, cyclosporine, biological therapy (infliximab or adalimumab), nutritional therapy, and corticosteroids (oral [budesonide, prednisone, prednisolone] and intravenous [methylprednisolone]). Surgical procedures included all intestinal resections.

Extraintestinal manifestations (EIMs) included skin, joint, and ocular manifestations; pancreatitis; osteopenia; primary sclerosing cholangitis; and growth failure. Patient data were updated every 6 months, by entering in the registry every evaluation made by patients. Every 6 months, clinical data, PUCAI/PCDAI, medical and surgical therapy, laboratory tests (those performed at diagnosis), imaging and/or endoscopic evaluation, and complications of the disease, were reported.

For the purpose of this study, all previous information were analyzed and compared in all patient populations. As a primary outcome of the study, recorded data of patients 0 to 5 years (EO) were compared with those with a later onset, divided in 2 groups (6–11 and 12–18 yr). As a secondary outcome, we evaluated within the population with EO-IBD the phenotype and natural history of VEO (0–2 yr) forms compared with those diagnosed in children aged 3 to 5 years.

Statistical Methods

All data were summarized and displayed as the mean ± SD for the continuous variables. Categorical data were expressed as frequencies and percentages. Comparison of groups was performed using Student's *t* test for unpaired data in two-group comparison and one-way analysis of variance with Bonferroni's test for multiple group comparison. Chi-square test with Fisher's correction was used to evaluate the differences for categorical variables wherever needed. A P value of 0.05 or less was considered significant. Odds ratios from univariate logistic regression models were used to assess differences by age group in the prevalence of symptoms at disease onset and laboratory values at diagnosis. The Kaplan–Meier survival method was used to estimate the interval free from surgery during follow-up. Differences between curves were tested using the Log-Rank test. The GraphPad statistical package was used to perform all statistical evaluations (GraphPad Software, Inc., San Diego, CA).

RESULTS

Population

From 2009 to 2013, we identified 688 patients with a diagnosis of IBD, 182 had a length of follow-up less than 6 months and were excluded from the analysis. Five hundred six children met the inclusion criteria and were enrolled in the study. Fifty-four percent were males and mean age at the diagnosis was 10.2 years (range, 0.8–18.3). Mean follow-up was 40 months (range, 6–50). Eleven percent (n = 54) of patients were in the range 0 to 5 years of age, 39% (n = 197) in 6 to 11 years, and 50% (n = 255) in 12 to 18 years. Table 1 highlights the main clinical characteristics of 506 enrolled patients. Table 2 shows the prevalence of EO-IBD reported in previous studies compared with our cohort.

Initial Classification and Location of the Disease

All patients had a complete ileocolonoscopy under general anesthesia or deep sedation at the diagnosis. Fifty-five percent had an esophagogastroduodenoscopy and 48% also had an evaluation of the small-bowel (78% CD, 21% UC): of them, 52% underwent ultrasound, 43% magnetic resonance imaging, 17% capsule endoscopy, and 6% enteroscopy. Thirty-seven percent of patients had 2 or more methods for imaging the small bowel. At the diagnosis, 224 were classified as CD (44%), 245 as UC (48%), and 37 as IBDU (7%). UC was the most frequent diagnosis in EO-IBD (59%) and in 6- to 11-year-old group (53%), compared with children of 12 to 18 years of age (42.5%) ($P = 0.03$ versus 0- to 5-yr group), whereas CD was predominant in the latter ($P < 0.0001$ versus 0- to 5-yr group). A classification as IBDU was more common in the range 0 to 5 years (22%) compared with the other groups (7% in 6- to 11-yr and 3.5% in 12- to 18-yr group; $P = 0.002$ and $P < 0.0001$, respectively). Table 3 shows the initial classification of EO-IBD in our cohort compared with published data. Fifty percent of children with EO-CD presented an isolated colonic disease (L2) compared with 14% in 6- to 11-year and 16% in 12- to 18-year group ($P < 0.005$). No significant differences were found for the other CD locations among the 3 groups, although an isolated ileal disease (L1) was rare in 0- to 5-year group (10% versus 23% in 6–11 yr and 52% in 12–18 yr), and an upper gastrointestinal involvement was more frequent in younger children (30% versus 18% in 6–11 yr and 16% in 12–18 yr). Figure 1 shows the CD location in all groups.

Among patients with UC, 62% of patients in the 0- to 5-year range had a pancolitis, compared with 38% of 6- to 11-year ($P = 0.02$) and 28% of 12- to 18-year range ($P = 0.002$). No other significant differences were found for the other UC location among the 3 groups (Fig. 2).

TABLE 1. Clinical Characteristics of 506 Children with IBD

	Age 0–5 yr, n = 54 (11%)	Age 6–11 yr, n = 197 (39%)	Age 12–18 yr, n = 255 (50%)	*P*
Sex (males)	23 (42%)	82 (41%)	170 (67%)	0.94[a]; <0.001[b]
Follow-up (range), mo	44 (11–50)	40 (6–48)	36 (6–46)	0.26[a]; 0.08[b]
Diagnostic delay (>6 mo)	16 (29%)	52 (26%)	72 (28%)	0.72[a]; 0.86[b]
Diagnosis				
CD	10 (18%)	78 (40%)	137 (54%)	0.006[a]; <0.0001[b]
UC	32 (59%)	105 (53%)	108 (42.5%)	0.56[a]; 0.03[b]
IBDU	12 (22%)	14 (7%)	9 (3.5%)	0.002[a]; <0.0001[b]
Family history for IBD	6 (11%)	30 (15%)	29 (11%)	0.58[a]; 0.94[b]
Disease activity (at the diagnosis)				
PUCAI (mean ± SD)	30.7 ± 17.2	33.8 ± 19.5	36.4 ± 18.1	0.54[a]; 0.52[b]
PCDAI (mean ± SD)	25 ± 6.3	21.6 ± 13.4	21.3 ± 14.3	0.53[a]; 0.23[b]

[a]0 to 5 versus 6 to 11 years.
[b]0 to 5 versus 12 to 18 years.

Classification and Disease Extent at Follow-up

At follow-up, a change in diagnosis was reported in 2% of all patients: 40% of children in 0- to 5-year group received a change of the initial diagnosis during the follow-up, 50% of 6 to 11 years, and 10% of 12–18 years ($P = 0.003$, 0–5 versus 12–18 yr). Among children with EO disease initially classified as IBDU, 3 (25%) were finally diagnosed as UC, 1 (8%) as CD, whereas 67% continued to be classified as IBDU. No significant differences were found for disease extent at 40-month follow-up, although a higher proportion of children with EO-CD presented an extension of the disease involving ileum and colon (L3) at the last evaluation compared with the diagnosis (60% versus 40%; $P = 0.65$). We did not find significant changes in the CD and UC location at follow-up in older age groups.

Clinical Presentation

Table 4 shows the clinical presentation in the 3 age groups. EIMs were detected in 28% of patients at the diagnosis: skin involvement (n = 45), axial arthropathies (n = 10), peripheral arthritis/arthralgia (n = 50), pancreatic involvement (n = 5), and osteopenia (n = 4). Growth failure at diagnosis was reported in 22% of children. No significant differences were found for the prevalence of EIMs among the 3 age groups. Other clinical variables at the diagnosis, such as PCDAI or PUCAI, hemoglobin, white blood cell count, erythrocyte sedimentation rate, C-reactive protein, hypoalbuminemia, positive perinuclear antineutrophil cytoplasmic antibodies, and anti-*S. cerevisiae* antibodies did not differ by age group.

Medical and Surgical Treatment

Fifty-seven percent of patients started corticosteroids, 35% azathioprine, 4% methotrexate, 16% nutritional therapy, 5% biologics, 2% thalidomide, and 2% cyclosporine.

At the diagnosis, no significant differences were found among the 3 groups in the corticosteroid need, although at the latest follow-up, a significantly higher proportion of patients with EO-IBD were under steroids compared with 12- to 18-year group ($P < 0.05$). The use of other therapies at diagnosis and follow-up (i.e., nutritional therapy, immunomodulators, and biologics) did not significantly differ among the 3 groups. Figures 3 and 4 show the different therapies underwent by patients.

Sixty patients underwent surgery at least once between diagnosis and last follow-up, resulting in a crude surgical rate of

TABLE 2. Prevalence of EO-IBD in Our Cohort Compared with Previous Studies

	Population	EO-IBD, %
Aloi et al[19]	506	11
Griffiths[20]	503	4
Gupta et al[5]	600	9
Paul et al[21]	413	12
Sawczenko and Sandhu[22]	739	4
Heyman et al[10]	1370	15

TABLE 3. IBD Type at the Diagnosis in Our Cohort of EO-IBD Compared with Published Data

	CD, %	UC, %	IBDU, %
Aloi et al[19]	18	59	22
Paul et al[21]	34	66	0
Sawczenko and Sandhu[22]	31	38	24
Heyman et al[10]	35	40	27
Mamula et al[23]	33	44	23

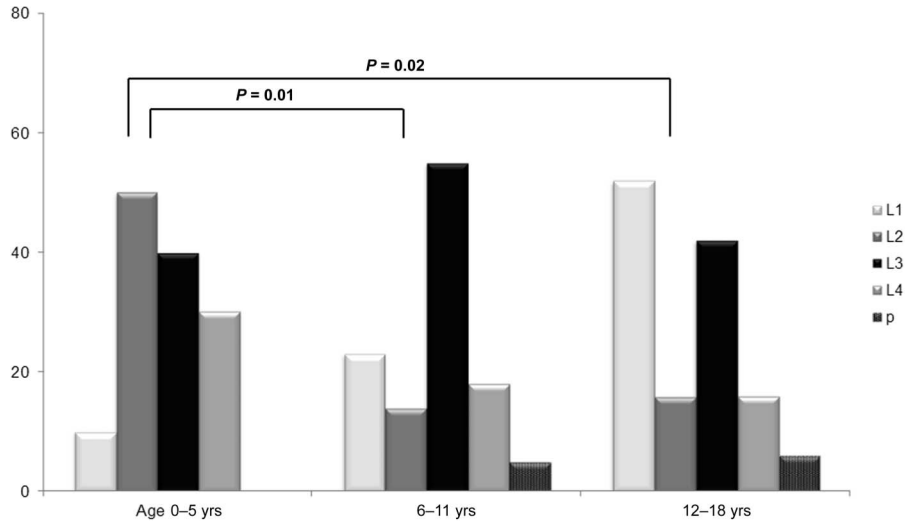

FIGURE 1. Disease location at the diagnosis according to Paris classification in EO-CD (0–5 yr) and later-onset CD (6–11 and 12–18 yr). L1: ileum, L2: colon; L3: ileocolon; L4: upper gastrointestinal; p: perianal disease.

12%. No significant differences for surgical risk were reported among the 3 age groups (Fig. 5).

Subgroup Analysis of VEO-IBD

Finally, among children with EO-IBD, we made a subgroup analysis evaluating potential peculiarities in presentation and disease course of very early forms (0–2 yr) compared with diseases presenting in children of 3 to 5 years of age. The diagnosis of UC was the most common in both groups, although numerically higher in 3 to 5 years (68% versus 40%). Both groups were characterized by an extensive disease at the diagnosis, pancolonic involvement in UC, and an isolated colitis for CD. Upper gastrointestinal involvement was more common in children of 3 to 5 years than the very younger, although the difference was not significant. Other variables at the diagnosis and at follow-up, i.e., sex, family history for IBD, delay of the diagnosis (>6 mo), EIMs, mean PUCAI and PCDAI at the diagnosis, C-reactive protein, and perinuclear antineutrophil cytoplasmic antibodies at the diagnosis and changes of the diagnosis at follow-up did not differ between the 2 groups (Table 5).

DISCUSSION

Reports of pediatric IBD have been published for several years.[20–23] Recently, evidence for increased incidence of IBD in young children and the identification of early forms of severe disease have represented a significant step forward the understanding of the pathogenesis underlying IBD, determining renewed interest in the younger age group.[13,14,24,25] The most relevant discovery in this field has been the recognition of forms of Crohn's-like colitis

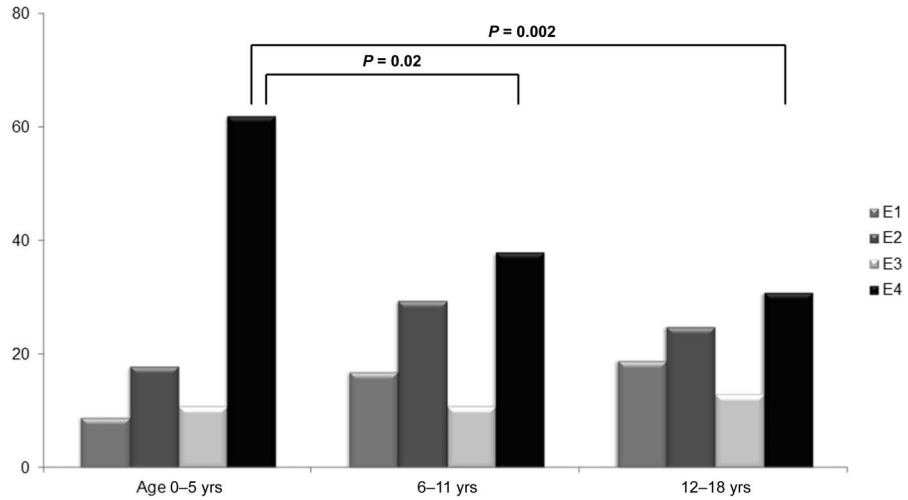

FIGURE 2. Disease location at the diagnosis according to Paris classification in EO-UC (0–5 yr) and later-onset UC (6–11 and 12–18 yr). E1: proctitis; E2: left-sided colitis; E3: extensive colitis; E4: pancolitis.

TABLE 4. Prevalence of Clinical Symptoms at Presentation

	0–5 yr, n = 54 (%)	6–11 yr, n = 197 (%)	Odds Ratio,[a] (95% CI)	*P*	12–18 yr, n = 254 (%)	Odds Ratio,[a] (95% CI)	*P*
Abdominal pain	27 (50)	139 (70)	0.41 (0.22–0.77)	0.005	147 (58)	0.72 (0.40–1.3)	0.29
Rectal bleeding	39 (72)	109 (55)	2.09 (1.0–4.0)	0.02	127 (50)	2.09 (1.3–4.9)	0.004
Diarrhea	33 (61)	108 (56)	1.2 (0.7–2.3)	0.44	139 (55)	1.3 (0.7–2.3)	0.45
Growth failure	9 (17)	49 (24)	0.60 (0.2–1.3)	0.27	62 (24)	0.61 (0.28–1.3)	0.28
Perianal disease	6 (11)	26 (13)	0.80 (0.3–2.0)	0.81	39 (15)	0.67 (0.27–1.6)	0.52

[a]The 0- to 5-year group is the reference group.

with perianal involvement and onset in the first months of life, associated with a defect of IL-10R or IL-10 itself.[13,14] These diseases present a Mendelian pattern of heritability, differently to the multigenetic trait observed in the known forms of IBD, adding them to the group of primary immunodeficiencies resulting in severe dysregulation of the intestinal immune system.[25] For this reason, we did not included these forms in the registry and in this study.

Our study not only adds new data on the peculiarities of EO-IBD, in terms of phenotype and disease course, but also represents a full application of the Paris classification of IBD in a large population of children. Detailed follow-up of 506 pediatric patients followed in the main tertiary referral centers for IBD in Italy has allowed us to evaluate these findings, particularly the initial clinical presentation and the disease progression.

Data were collected by a structured prospective registry, including all newly diagnosed pediatric IBD patients in Italy from January 1, 2009. Our results confirm that the phenotype of pediatric IBD patients differs according to age, and that the EO disease is extensive and severe already at the presentation. Clinical data on the EO-IBD are scarce because this age group represents only a small portion of the pediatric population. Griffiths[20] in a cohort of children with IBD (n = 503) found 4% to be less than 5 years at diagnosis. More recently, Gupta et al[5] presented their results in 600 children with CD: 9% of them were 0 to 5 years old at the time of diagnosis. In our cohort, 11% (n = 54) were less than 5 years at the time of diagnosis and 4% less than 2 years. Among the younger children, the diagnosis of UC was the most common, although a significant number of patients received a diagnosis of IBDU (22%). These data are similar to those reported in 2002 by Mamula et al,[23] showing an indeterminate colitis in 23% of 82 children with EO-IBD. Strikingly, many of our children with IBDU maintained this diagnosis during the follow-up, suggesting the strain of making a definitive diagnosis in younger children. During the course of the disease, diagnosis changes occurred more frequently in younger than older children, although the difference was not significant. We cannot exclude that the small number of younger children has influenced the statistical significance of this result.

Overall, our population of childhood-onset IBD was characterized by a widespread anatomical involvement in all age groups. CD presented with an extensive phenotype, regardless of the age at the diagnosis. EO-CD was characterized by significantly higher rates of isolated colonic CD, compared with older children. These data are consistent with that reported by Heyman et al[10] from the North American Pediatric IBD

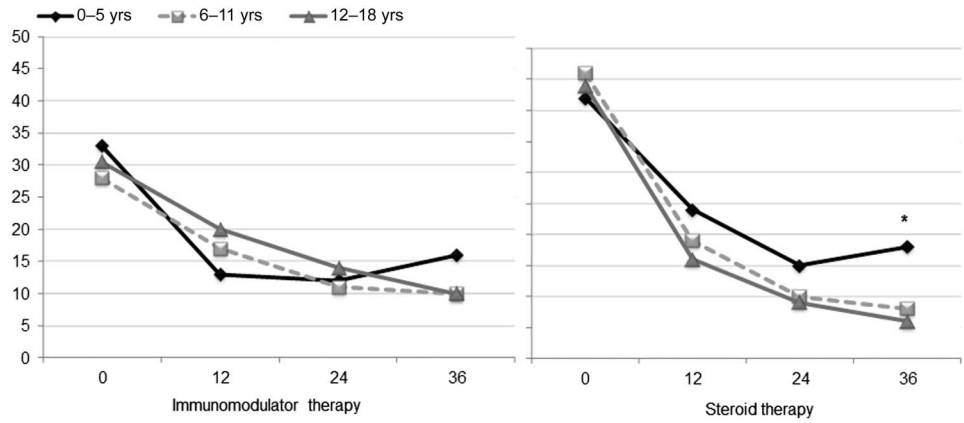

FIGURE 3. Immunomodulator and steroid therapy at diagnosis and follow-up in 0- to 5-year, 6- to 11-year, and 12- to 18-year group. *$P < 0.05$, 0- to 5-year versus 12- to 18-year group.

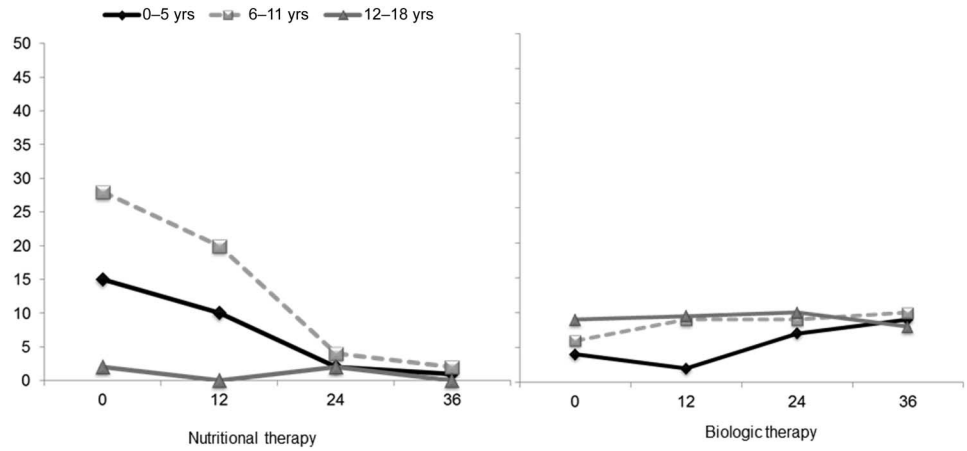

FIGURE 4. Nutritional and biological therapy at diagnosis and follow-up in 0- to 5-year, 6- to 11-year, and 12- to 18-year group.

Consortium Registry. In 1370 children, the prevalence of isolated colonic disease was significantly higher in younger (less than 8 yr of age) compared with older children. Paul et al[21] reported data from 413 children with IBD and compared children presenting before and after the age of 5 years. In the EO group, 76% had an isolated colonic disease, 34% ileocolonic, whereas 0% had ileal involvement only. Conversely, older children mainly presented with ileocolonic involvement (48%). Also in our cohort, the proportion of patients with an ileocolonic involvement at the diagnosis was higher in older children. We also found that one-third of younger patients presented an upper gastrointestinal involvement already at the diagnosis. This result is remarkable because, to our knowledge, there are no previous studies on EO-IBD indicating the involvement of the upper gastrointestinal tract in this age group. Our study, by using the Paris classification, allowed us to accurately define this finding. Overall, 6% of our pediatric population presented lesions of the upper gastrointestinal segment. This figure is close to prior reports in children.[3,26] More than half of our patients underwent an upper and lower endoscopy at the diagnosis, and almost half also small-bowel imaging. The wide range of investigation may explain the high prevalence of upper gastrointestinal involvement. Nevertheless, our analysis based on the Paris classification, seem to confirm the significant differences between CD location in pediatric-onset, notably EO disease and adult onset CD.[27]

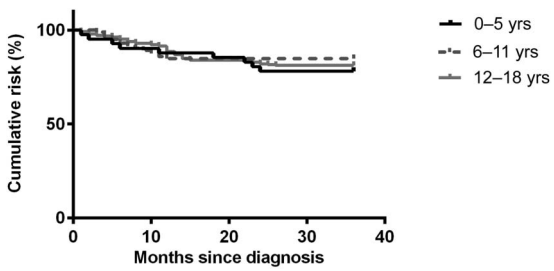

FIGURE 5. Cumulative risk of surgery in 506 children with IBD according to the age at diagnosis.

Overall, an extensive disease location was also demonstrated for pediatric UC, although the proportion of patients having a pancolitis already at the diagnosis was significantly higher in younger than older children. Our study not only is consistent with previous data indicating a widespread extension of pediatric UC,[19,28,29] but directly compares early- with later-onset cohort in the same population of pediatric patients (62% of younger children presented a pancolitis at the diagnosis compared with 38% in older group). We did not find a high overall rate of disease extension at follow-up; thus at the end of the study, the disease extent was similar to that at the diagnosis. These data are, somewhat at variance with other pediatric studies, suggesting a high rate of disease extension in pediatric UC and CD.[28] One explanation could be the short follow-up of our study (40 mo). Longer, prospective studies on the disease course of EO-IBD may confirm these data.

In terms of clinical presentation, rectal bleeding was the most common symptom in younger children, whereas the older age group presented with more varied symptoms. This figure can be explained by the homogeneous colonic involvement in the EO disease, while multiple locations with more variable clinical pictures were found in later-onset forms. Several data suggest that the EO-IBD present a stronger genetic impact than the later-onset disease.[30–32] Differently from these reports, we did not find significant differences for family history between the EO and the later-onset groups. One explanation may lie in the fact that we have only considered the first-degree relatives, not extending to the rest of the family. This could have reduced the number of patients with a family history in our cohort. Subsequent studies in this population to investigate the impact of genetic background and potential combinations of genetic and environmental influences in disease onset are therefore needed.

Some data suggest a higher "severity" in terms of behavior and disease course of EO-IBD.[28] We evaluated the need of corticosteroids, immunomodulators, biologics, and surgery in an attempt to identify differences in severity according to age. We did not find significant differences in medical therapeutic

TABLE 5. Clinical Characteristics of EO- and VEO-IBD

	0–2 yr, n = 20	3–5 yr, n = 34	*P*
Sex (M)	12 (60%)	18 (53%)	0.77
Family history for IBD (first-degree relatives)	2 (10%)	4 (12%)	0.89
Delay of the diagnosis (>6 mo)	10 (50%)	11 (32%)	0.25
Diagnosis			
IBDU	6 (30%)	4 (12%)	0.14
UC	8 (40%)	23 (68%)	0.08
CD	6 (30%)	7 (20%)	0.51
Location of UC at diagnosis (Paris classification)			
E1	1 (12%)	0	0.25
E2	0	4 (17%)	0.54
E3	1 (12%)	3 (13%)	0.98
E4	6 (75%)	16 (70%)	0.95
Location of CD at diagnosis (Paris classification)			
L1	1 (17%)	0	1.0
L2	3 (50%)	1 (17%)	0.54
L3	2 (33%)	4 (66%)	0.56
L4	1 (17%)	3 (50%)	0.54
Changes of the diagnosis at follow-up	2 (10%)	2 (6%)	0.67
PUCAI (mean ± SD)	35.5 ± 12.4	38.4 ± 12.4	0.72
PCDAI (mean ± SD)	32.4 ± 14.9	34.7 ± 11.25	0.76
CRP (mean ± SD)	4.8 ± 4.7	5.7 ± 9.1	0.74
EIMs at diagnosis	1 (5%)	3 (9%)	1.0
p-ANCA	4 (13%)	7 (20%)	0.46
Surgery at follow-up	2 (10%)	3 (9%)	1.0

CRP, C-reactive protein; pANCA, perinuclear antineutrophil cytoplasmic antibodies.

strategy at the diagnosis among the 3 groups, indirectly suggesting a similar response to treatment regardless of age. Although, at the maximum follow-up, a significant number of children in the 0- to 5-year age group needed steroids. This might suggest a more aggressive disease than in older children, although the use of other drugs was not different. Moreover, our data suggest a similar. However, evaluation of these results as a surrogate for severity is difficult, and confounded by various factors, particularly the variability among individual physicians in the use of these agents.

Sixty patients underwent surgery at least once between diagnosis and maximal follow-up, resulting in a crude surgical rate of 12%. This figure is close to previous French and North American studies.[33,34] We did not find differences according to age, probably the short time of follow-up influenced this result.

This study has some limitations. Our registry is based on patient data from large regional referral centers, which may not allow extrapolation to the real Italian pediatric population with IBD. However, children with chronic disease tend to be referred to tertiary referral centers such as these, and unlike adults with IBD, they are rarely treated exclusively by a primary care pediatrician.

In summary, our data suggest not only that the number of EO-IBD is steadily increasing, but also that the phenotype is extensive and aggressive already at the onset of the disease. The widespread location of CD and UC, notably younger patients, may suggest the use of immunomodulatory and biologic agents early in the course of the disease. Nevertheless, those therapies should be ideally targeted on those patients with a more "severe" disease, thus future studies on genetic, clinical, and serologic risk factors for a disabling course in EO-IBD are warranted. Genetic impact does not seem significant in our population, suggesting that environmental triggers play a crucial role in the pathogenesis of EO disease. Several arguments indicate that a change of the intestinal microbiota, probably secondary to changes in alimentary habits, along with particularly sterile living conditions make individuals prone to developing IBD, and this could probably be important also in younger children.[35,36] The identification of different clinical forms of EO disease, through the analysis of genotype–phenotype correlations, the identification of specific genotypes, and particularly combinations of genetic and environmental influences in disease onset, could consent in the near future to typify the disease already at onset, allowing specific therapies for the individual patient.

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Alteration of Fecal Microbiota Profiles in Juvenile Idiopathic Arthritis. Associations with HLA-B27 Allele and Disease Status

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Alteration of gut microbiota is involved in several chronic inflammatory and autoimmune diseases, including rheumatoid arthritis, and gut microbial "pro-arthritogenic" profiles have been hypothesized. Intestinal inflammation may be involved in spondyloarthropathies and in a subset of patients affected by Juvenile Idiopathic Arthritis (JIA), the most common chronic rheumatic disease of childhood. We compared the fecal microbiota composition of JIA patients with healthy subjects (HS), evaluating differences in microbial profiles between sub-categories of JIA, such as enthesitis-related arthritis (JIA-ERA), in which inflammation of entheses occurs, and polyarticular JIA, non-enthesitis related arthritis (JIA-nERA). Through taxon-level analysis, we discovered alteration of fecal microbiota components that could be involved in subclinical gut inflammation, and promotion of joint inflammation. We observed abundance in *Ruminococcaceae* in both JIA categories, reduction in *Clostridiaceae* and *Peptostreptococcaceae* in JIA-ERA, and increase in *Veillonellaceae* in JIA-nERA, respectively, compared with HS. Among the more relevant genera, we found an increase in *Clostridium cluster XlVb*, involved in colitis and arthritis, in JIA-ERA patients compared with HS, and a trend of decrease in *Faecalibacterium,* known for anti-inflammatory properties, in JIA-nERA compared with JIA-ERA and HS. Differential abundant taxa identified JIA patients for the HLA-B27 allele, including *Bilophila*, *Clostridium cluster XlVb*, *Oscillibacter*, and *Parvimonas*. Prediction analysis of metabolic functions showed that JIA-ERA metagenome was differentially enriched in bacterial functions related to cell motility and chemotaxis, suggesting selection of potential virulence traits. We also discovered differential microbial profiles and intra-group variability among active disease and remission, suggesting instability of microbial ecosystem in autoimmune diseases with respect to healthy status. Similarly to other chronic autoimmune and inflammatory diseases, different microbial profiles, as observed among different JIA subgroups compared to HS, and potential functional acquisition related to migration, could promote inflammation and contribute to the disease pathogenesis.

Keywords: juvenile idiopathic arthritis, enthesitis-related arthritis, gut microbiota, HLA-B27 allele, metagenomics

INTRODUCTION

Characterization of bacterial commensal communities in autoimmune and inflammatory diseases is a topic of great interest for understanding the role and interaction of intestinal microbiota with the host immune system.

It is known that the gut microbiota is shaped by several environmental factors, including dietary habits, antibiotics, infectious agents, and air pollution (De Filippo et al., 2010; David et al., 2014a; Salim et al., 2014), and in turn, that microbiota shapes the immune system, modulating homeostasis in healthy status individuals or promoting inflammation when dysbiosis occurs. Recent findings demonstrate that alteration in the equilibrium among commensal bacteria is associated not only with Inflammatory bowel disease (IBD), allergy, diabetes and celiac disease (Cheng et al., 2013), but also with rheumatoid arthritis (Vaahtovuo et al., 2008; Sandhya et al., 2016; Scher et al., 2016).

Juvenile idiopathic arthritis (JIA) is the most common chronic rheumatic disease in children, and comprises a clinically heterogeneous group of conditions characterized by chronic arthritis, synovial inflammation and erosion of bone and cartilage (Ravelli and Martini, 2007). According to several factors, including the number of affected joints, JIA is divided in psoriatic, oligoarticular (up to four affected joints), polyarticular (five or more affected joints) (Oberle et al., 2014), and enthesitis-related arthritis (ERA), in which entheses (attachments of tendons and ligaments to bone) are affected. ERA accounts for 10–20% of JIA and is considered the equivalent of spondyloarthropathy, a disease frequently characterized by clinical and subclinical intestinal involvement (Bryan and Rabinovich, 2014; Oberle et al., 2014; Aggarwal and Misra, 2015).

Immunological, genetic, and environmental factors are involved in the pathogenesis of ERA (Stoll et al., 2014; Gmuca and Weiss, 2015). Gene variants in the Major Histocompatibility Complex, especially the HLA-B27 alleles, have been identified as predisposing factors (Gmuca and Weiss, 2015).

Alterations of gut microbiota (dysbiosis) and a decrease in gut microbiota richness (Li et al., 2014; Collado et al., 2015; Rogers, 2015) are emerging as factors associated with the development of inflammatory and systemic autoimmune diseases (Yeoh et al., 2013; Longman and Littman, 2015). Of great interest is the understanding of dysbiosis as a trigger or a reflection of autoimmune and inflammatory disorders (Chung and Kasper, 2010; Stoll et al., 2014), in fact, also autoimmunity can drive instability of the gut microbial ecosystem.

Studies in germ-free animal models reveal relationships between microorganisms, mucosal immunity, and joint inflammation (Taurog et al., 1994; Rath et al., 1996; Longman and Littman, 2015). Recent studies in humans suggest that alteration of oral and gut microbiota and an increase in leaky gut could trigger systemic joint inflammation in the context of pre-existent autoimmunity (Scher et al., 2013, 2015; Brusca et al., 2014; Costello et al., 2014; Taneja, 2014; Longman and Littman, 2015; Zhang et al., 2015).

However, although arthritis susceptibility has been linked to the gut microbiome, and it has proposed that in synovial fluids the presence of pro-arthritogenic bacterial DNA, deriving by circulating intestinal bacterial products, may promote synovial inflammation (Kempsell et al., 2000; Gerard et al., 2001; Moen et al., 2006; Oberle et al., 2014), a causal relationship between bacterial infection and onset of rheumatological diseases has not yet been firmly demonstrated. Further studies on leaky gut syndrome could clarify the presence of bacterial products circulating and influencing the systemic immune response, also in light of the presence of bacteria in non-rheumatoid arthritis controls (Kempsell et al., 2000), and of potential microbial contamination found in amplification technique of 16S rDNA, as previous observed (Grahn et al., 2003).

A targeted-metagenomics approach can provide an in-depth characterization of microbial communities, allowing investigation of correlations between microbiota composition and human pathologies (Lozupone et al., 2012).

In this study, we characterized and compared the gut microbiota of JIA patients, affected by enthesitis-related arthritis (JIA-ERA) and polyarticular JIA (non-enthesitis-related arthritis, nERA), with healthy subjects (HS), in order to define specific microbial "pro-arthritogenic" profiles.

MATERIALS AND METHODS

Sampling of Subjects

We enrolled 29 JIA patients (13 males and 16 females, age range 2–18 years), 19 of whom were affected by ERA and 10 by polyarticular JIA (nERA). Exclusion criteria were: acute diarrhea, infectious gastroenteritis, antibiotic treatment in the previous 3 months, and diagnosis of chronic gastrointestinal disease. A total of 29 healthy children and adolescents (11 males and 18 females; age range 2–18 years) not affected by autoimmune and inflammatory conditions, infectious gastroenteritis, or chronic gastrointestinal disease, were enrolled as controls.

We collected a fecal sample from each subject, and a second one from 17 ERA patients three months later, in order to evaluate microbiota variability. We also gathered clinical information, including: enthesitis and arthritis localization, age of onset, HLA-B27 status, family history of ankylosing spondylitis or other HLA-B27-related diseases, comorbidities, laboratory parameters (C Reactive Protein, Erythrocyte Sedimentation Rate, ANA and pANCA autoantibody positivity), and pharmacological treatments (**Table 1**; Supplementary Tables S1 and S2). None of the patients underwent treatment with proton pump inhibitors (PPIs). Active disease was defined by the presence of active arthritis and/or enthesitis at the time of stool sampling. As reported in **Table 1**, a total of four JIA-ERA and four JIA-nERA patients had clinically active disease. Fecal calprotectin (Aomatsu et al., 2011) was also assessed by ELISA assay for subclinical intestinal inflammation (Eurospital, Trieste, Italy). Parents or guardians gave written informed consent for fecal samples and clinical data collection of their children. The study protocol was approved by the Ethics Committee of the Meyer Children's Hospital, Florence, Italy (Protocol ref. Nov 12th, 2013), and carried out in accordance with the approved guidelines.

TABLE 1 | Clinical features of JIA patients and age/gender information of healthy subjects (HS).

	ERA	nERA	HS
Number	19	10	29
Age (years) median, range	14.3; 9 to 18	10.5; 2 to 17	13; 2 to 18
Male: Female, number	13:6	0:10	11:18
Disease duration (months) median, range	55; 2 to 93	97; 3 to 150	–
Acute disease, number (%)	4 (21%)	4 (40%)	–
Calprotectin positive, number (%)	2 (10.5%)	2 (20%)	–
High Erythrocyte sedimentation rate, number (%)	3 (15.7%)	0 (0%)	–
HLA-B27+	9 (47%)	0 (0%)	–
ANA+	4 (21%)	9 (90%)	–
Complications or comorbidities, number (%)			
No	10 (52.6%)	3 (30%)	–
Uveitis	1 (5.2%)	7 (70%)	–
β- thalassemia	1 (5.2%)	0 (0%)	–
Pectum escavatum	2 (10.5%)	0 (0%)	–
Asthma	2 (10.5%)	0 (0%)	–
Osteoporosis	1 (5.2%)	0 (0%)	–
Obesity	1 (5.2%)	0 (0%)	–
Hypothyroidism	1 (5.2%)	0 (0%)	–
Epigastric hernia	1 (5.2%)	0 (0%)	–
Cataract	1 (5.2%)	0 (0%)	–
Breast fibroadenoma	1 (5.2%)	0 (0%)	–
Treatment, number of patients (%)			
NSAIDs	18 (95%)	1 (10%)	–
Sulfasalazine	8 (42%)	0 (0%)	–
Steroids	4 (21%)	0 (0%)	–
Methotrexate	4 (21%)	0 (0%)	–
Etanercept	3 (15.7%)	4 (40%)	–
Abatacept	1 (5.2%)	5 (50%)	–
Adalimumab	2 (10.5%)	0 (0%)	–

HLA, Human leukocyte antigen; ANA, Anti-nuclear antibody; NSAIDs, Non-steroidal anti-inflammatory drugs.

DNA Extraction

Fecal samples were preserved in RNAlater (Qiagen) at 4°C for the first 48 h, and kept at −80°C until DNA extraction. Bacterial genomic DNA extraction and quality control were carried out following our previous protocol (De Filippo et al., 2010).

Pyrosequencing

For each sample, we amplified the 16S rRNA gene using the special fusion primer set specific for V5-V6 hypervariable regions and corresponding to primers 784F and 1061R described by Andersson et al. (2008), and using the FastStart High Fidelity PCR system (Roche Life Science, Milano, Italy). The 454 pyrosequencing was carried out on the GS FLX+ system using the XL+chemistry following the manufacturer recommendations (details in Supplementary Materials).

Data Analysis

Pyrosequencing resulted in a total of 2,180,826 16S rDNA reads with a mean of 29,078 sequences per sample. Average sequence lengths were 290 nt (±SD 45) and 286 nt (±SD 50) for the first and second run, respectively. Raw 454 files were demultiplexed using Roche's.sff file software, and available at the European Nucleotide Archive[1] under the accession study ERP013262. Reads were pre-processed using the MICCA pipeline (version 0.1)[2] (Albanese et al., 2015). Forward and reverse primer trimming and quality filtering were performed using micca-preproc truncating reads shorter than 280nt (quality threshold = 18). *De novo* sequence clustering, chimera filtering and taxonomy assignment were performed by micca-otu-denovo (parameters -s 0.97 -c). Operational Taxonomic Units (OTUs) were assigned by clustering the sequences with a threshold of 97% pair-wise identity, and their representative sequences were classified using the RDP software version 2.7 (Wang et al., 2007). Template-guided multiple sequence alignment was performed using PyNAST57 (version 0.1) (Caporaso et al., 2010) against the multiple alignment of the Greengenes 16S rRNA gene database (DeSantis et al., 2006) filtered at 97% similarity. Finally, a phylogenetic tree was inferred using FastTree (Price et al., 2010)

[1]http://www.ebi.ac.uk/ena/data/view/PRJEB11846

[2]http://compmetagen.github.io/micca/

and micca-phylogeny (parameters: -a template-template-min-perc 50). Sampling heterogeneity was reduced by rarefaction, obtaining 12,964 sequences per sample.

Chao1 index and Shannon entropy (indicators of alpha diversity) and UniFrac (Lozupone and Knight, 2005) and Bray–Curtis dissimilarities (indicators of beta diversity) were calculated using the phyloseq package (McMurdie and Holmes, 2014) of the R software suite. Exploratory analysis was performed by Principal coordinates analysis (PCoA) using the phyloseq package of the R software suite. Multiple-rarefaction PCoA plots ("jackknifed" PCoA plots) (Lozupone et al., 2011) were computed to assess the robustness of the beta-diversity analyses.

The significance of between-groups differentiation on the UniFrac distances and Bray–Curtis dissimilarity was assessed by PERMANOVA using the adonis() function of the R package vegan with 999 permutations.

As a measure of species evenness, we calculated Dominance (1-Simpson index) and Equitability (Shannon diversity index divided by the logarithm of taxa number) by using PAST v 3.12 (Hammer et al., 2001).

To compare the relative abundances of OTUs among the three groups of subjects, the two-sided, unpaired Wilcoxon test was computed, removing taxa not having a relative abundance of at least 0.1%, in at least 20% of the samples, and using the function mt() in the phyloseq library and the *p*-values were adjusted for multiple comparison controlling the family wise Type I error rate (minP procedure). Further significant differences in bacterial taxa were supported by non-parametric White's test and Welch's test by using STAMP (Parks et al., 2014), and *p*-values were adjusted for multiple comparisons by Storey FDR, as proposed in STAMP.

On the basis of the relative abundances, the metagenomic biomarker discovery and related statistical significance were assessed using the linear discriminant analysis (LDA) effect size (LEfSe) method (Segata et al., 2011). LEfSe uses the Kruskal–Wallis rank-sum test to identify features with significantly different abundances between assigned taxa compared to the groups, and LDA to estimate the size effect of each feature. An alpha significance level of 0.05, either for the factorial Kruskal–Wallis test among classes or for the pairwise Wilcoxon test between subclasses, was used. A size-effect threshold of 2.0 on the logarithmic LDA score was used for discriminative microbial biomarkers.

In order to predict how taxonomic differences between fecal microbiota of the three groups impact their microbial metabolic potential, we applied PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013), a computational approach useful to infer the functional contribution of microbial communities on 16S rDNA sequencing data set. PICRUSt implements an extended ancestral-state reconstruction algorithm to predict which gene families are present, and then combines gene families to estimate the significant differences in the main functional classes (KEGG categories) of the composite metagenome. From a OTUs table with associated Greengenes identifiers, we obtained the final output from metagenome prediction as an annotated table of predicted gene family counts for each sample, where the encoded functions of each gene family are orthologous groups or other identifiers such as KEGG orthologs (KOs). The functional pathways discovery and related statistical significance were assessed by LEfSe.

RESULTS

Clinical Features of JIA Patients

We collected clinical features, as well as comorbidities and therapies, for each enrolled patient at the time of fecal sampling (**Table 1**; more details in Supplementary Tables S1 and S2). As known, due to the two different subsets of JIA, females are affected from polyarticular JIA two to four times more often than males (Oberle et al., 2014). In our cohort, most of the enrolled JIA-ERA patients were males (13/19), while all JIA-nERA patients were females (10/10). Thus, we evaluated the sex/gender as a potential variable influencing the fecal microbiota composition in both JIA categories. Regarding HLA-B27 status, 47% (9/19) of JIA-ERA patients resulted HLA-B27 positive, while all JIA-nERA patients were HLA-B27 negative. Calprotectin level, a measurement of intestinal inflammation, was positive (>100 μg/g) in 10.5% (2/19) of JIA-ERA and in 20% (2/10) of JIA-nERA patients (see Materials and Methods).

Microbiota Characterization by 16S rDNA Sequencing in the JIA Groups and Healthy Controls

We sequenced the V5–V6 hypervariable region of 16S rRNA gene for the meta-taxonomic study of microbiota in a total of 75 fecal samples from 19 JIA-ERA patients, 10 JIA-nERA, and 29 HS. From 17 JIA-ERA patients a second fecal sample was collected 3 months later, to evaluate microbiota variability over time (see Materials and Methods).

The taxonomic distribution in the three groups showed variations in gut microbiota composition (**Figure 1**; Supplementary Figure S1). Firmicutes, the dominant phylum of gut microbiota, was the most abundant in all samples (over 50% of total reads; Supplementary Figure S1A). Considering the 20 most abundant families (Supplementary Figure S1B), we found statistically significant differences between groups in *Ruminococcaceae, Peptostreptococcaceae, Clostridiaceae I* and *Veillonellaceae* (**Figure 1A**). *Ruminococcaceae* was more abundant in children in both JIA categories, compared with HS (21.6% in JIA-ERA and 27.2% in JIA-nERA vs. 12.0% in HS; Wilcoxon rank-sum test, JIA-ERA vs. HS $p = 0.0004$; JIA-nERA vs. HS $p = 0.0006$; **Figure 1A**). Although there was a reduction of *Peptostreptococcaceae* and *Clostridiaceae I* in all JIA patients (ERA and nERA) compared with HS (0.3% in JIA-ERA, 0.4% in JIA-nERA and 1.1% in HS; 0.1% in JIA vs. 0.5% in HS, respectively), we found statistically significant differences only between JIA-ERA vs. HS (Wilcoxon rank-sum test, $p = 0.033$ for *Peptostreptococcaceae*, $p = 0.017$ for *Clostridiaceae I*, respectively, **Figure 1A**). We observed a statistically significant predominance of *Veillonellaceae* in JIA-nERA compared with HS (1.4% in JIA-nERA vs. 0.4% in HS; Wilcoxon rank-sum test, $p = 0.012$; **Figure 1A**).

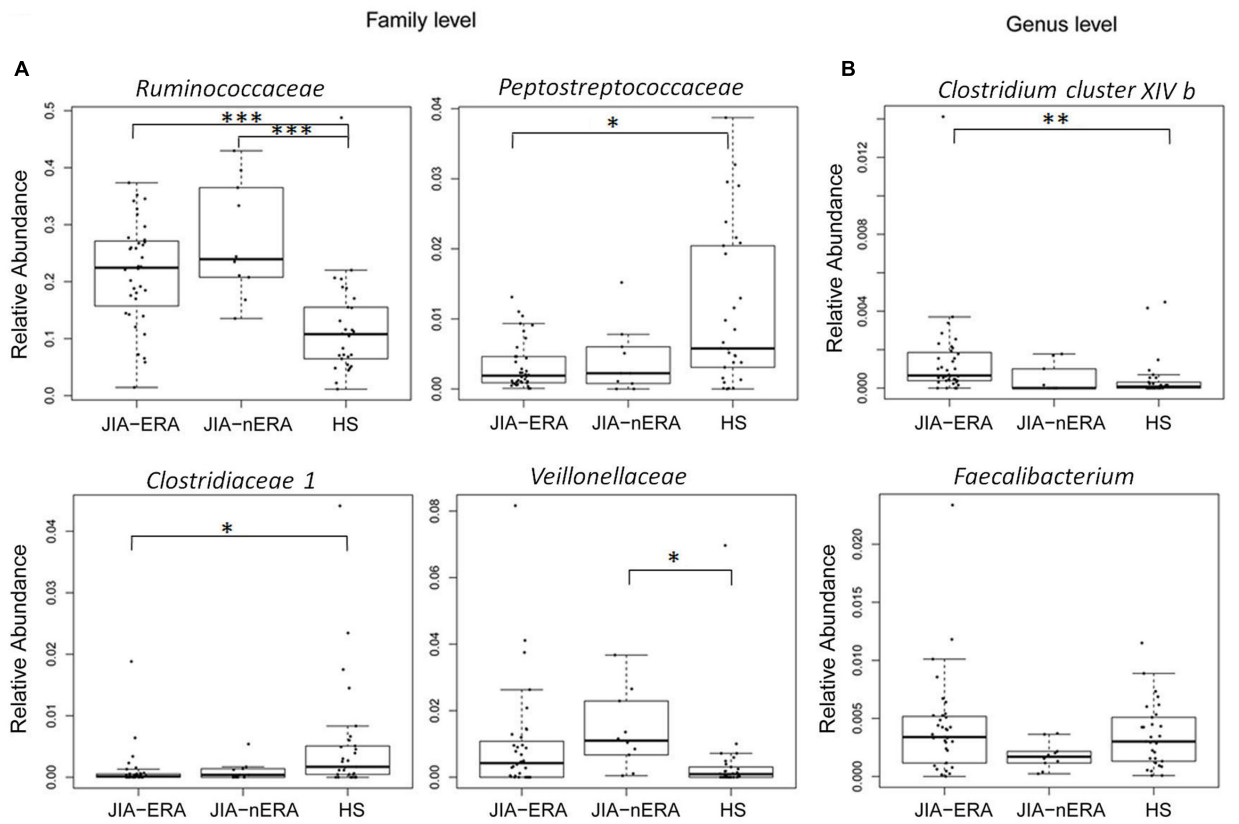

FIGURE 1 | Relative abundances of fecal bacterial components in JIA and HS groups. Box plot of statistically significant different bacterial **(A)** families and **(B)** genera in JIA patients compared to HS (Pairwise comparisons using Wilcoxon rank sum test; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ FDR adjusted). For *Faecalibacterium* genus, statistical significance of the comparison JIA-ERA vs. JIA-nERA was obtained by Welch's test without multiple testing correction.

Considering gender as potential variable influencing the gut microbiota composition, we confirmed that among the enrolled female subjects (6 JIA-ERA, 10 JIA-nERA, and 18 HS), *Firmicutes* were more abundant in the JIA-nERA group compared with HS (Supplementary Figure S2A; Wilcoxon rank-sum test $p < 0.05$). Among the minor constituents of fecal microbiota, we observed an increase in *Sutterellaceae* and *Enterobacteriaceae* in JIA-ERA female patients compared with female HS (2.5% in JIA-ERA vs. 1.2% in HS and 0.5% in JIA-ERA vs. 0.3% in HS, respectively; Wilcoxon rank-sum test, $p < 0.05$; Supplementary Figure S2B), and *Streptococcaceae* in JIA-nERA compared with HS (1% in JIA-nERA vs. 0.3% in HS; Wilcoxon rank-sum test, $p < 0.05$; Supplementary Figure S2B).

At genus level, we found an abundance of *Clostridium cluster XIVb* in JIA-ERA patients compared with HS (0.23% in JIA-ERA vs. 0.1% in HS; Wilcoxon rank-sum test, $p = 0.007$; **Figure 1B**). Moreover, we observed a decrease in the relative abundance in *Faecalibacterium* in JIA-nERA compared with either JIA-ERA or HS, even if not statistically significant (0.18% in JIA-nERA vs. 0.35% in HS; 0.18% in JIA-nERA vs. 0.41% in JIA-ERA; **Figure 1B**).

By LDA Effect Size (LEfSe; see Materials and Methods), we evaluated significant differences in abundance between assigned taxa with respect to JIA patient groups. We observed differentially abundant taxa discriminating for HLA-B27 status. At family level, increased *Lactobacillaceae* in HLA-B27 positive-JIA patients, and *Pasturellaceae* in HLA-B27 negative-JIA patients were found (**Figure 2A**). Of the increased genera in HLA-B27 positive JIA patients, we observed *Bilophila*, *Parvimonas*, and *Oscillibacter,* while *Haemophilus* and *Eggerthella,* were differentially enriched in HLA-B27 negative patients (**Figure 2B**). When considering only the JIA-ERA group, in addition to the five genera reported in **Figure 2B**, *Lactobacillus*, *Clostridium cluster XI*, and *Dialister* were enriched in HLA-B27 positive patients, while only *Haemophilus* discriminated for HLA-B27 negative status (**Figure 2C**).

Further, we evaluated correlations between microbiota profiles of JIA patients and fecal calprotectin, as well as with respect to different medical treatments. We did not find any significant correlation between fecal calprotectin levels and microbiota profiles (Supplementary Tables S1 and S2), probably due to the low number of patients with concomitant intestinal inflammation at the time of sampling. Regarding the effect of pharmacological therapies on gut microbiota of JIA patients, in our cohorts, JIA-ERA patients were treated with NSAIDs, alone or associated with sulfasalazine/methotrexate/biologics in different combinations,

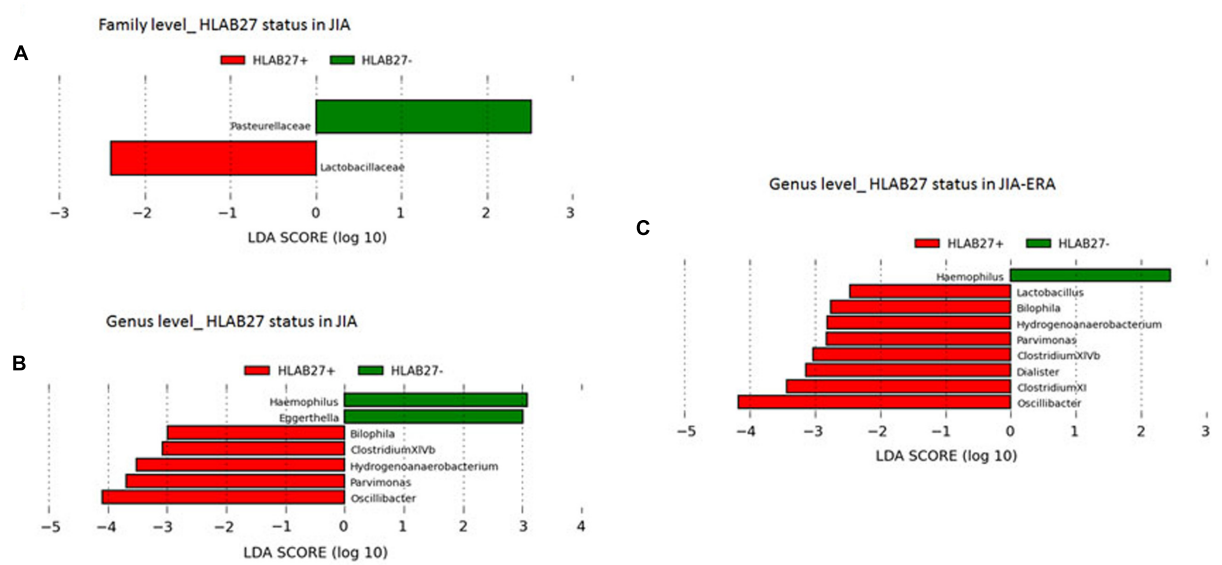

FIGURE 2 | Differences in bacterial taxa related to HLA-B27 status. LEfSe analysis shows a statistically significant enrichment of **(A)** families, and **(B,C)** genera, in **(A,B)** all JIA patients and in **(C)** JIA-ERA patients, considering the HLA-B27 status. LEfSe results indicate a sequentially significant ranking among groups (Alpha value = 0.05 for the factorial Kruskal–Wallis test among classes). The threshold for the logarithmic LDA score was 2.0.

as reported in **Table 1** and Supplementary Table S1. JIA-nERA patients were mainly treated with biologic drugs, such as Abatacept and Etanercept (**Table 1**; Supplementary Table S2). Despite the low number of patients stratified by pharmacological treatment, LEfSe analysis showed indications of association among different bacterial profiles and therapies (as reported in Supplementary data and Supplementary Figures S3A–C), among which we observed enrichment in *Collinsella*, associated with exacerbation of joint disease (Chen et al., 2016), in JIA-ERA patients treated with combined NSAIDs and sulfasalazine therapy.

However, a larger cohort of patients would be needed to strengthen our preliminary results and to understand the causality between therapies, gut microbiota profiles and clinical status.

In order to evaluate differences in the microbial biodiversity (alpha-diversity) among groups, we calculated the observed number of OTUs (a measurement of the total number of species present in a microbial community) and the Chao1 index (an indicator of species richness based on number of rare species), observing a significant reduction in alpha diversity in the JIA samples compared to HS (**Figures 3A,B**; p-value < 0.005 JIA-ERA vs. HS and JIA-nERA vs. HS; Supplementary Table S3). This suggests that microbiota of JIA patients is associated with biodiversity depletion, as observed in IBD patients. However, we did not find significant differences among the three groups using measures of biodiversity that take the evenness of the species distributions into account, like the Shannon entropy (**Figure 3C**), the Dominance (1-Simpson index) and Equitability (Pielou index; Materials and Methods; Supplementary Figure S4).

To estimate the variability of microbial communities between-sample (beta-diversity), we calculated the Bray-Curtis and unweighted UniFrac dissimilarities (see Materials and Methods). PCoA on unweighted UniFrac dissimilarities showed that both JIA samples (ERA and nERA) were more similar to each other than to HS samples (**Figure 3D**). Bray Curtis dissimilarity confirmed the differences among samples in the three populations (PERMANOVA, $p < 0.001$; **Figure 3E**). Considering HLA-B27 status, PCoA, calculated on unweighted UniFrac and Bray–Curtis dissimilarity, showed that HLA-B27 positive JIA-ERA patients form subgroups with respect to other JIA-ERA samples (**Figures 3F,G**), confirmed also by neighbor joining clustering based on Bray–Curtis dissimilarities (Supplementary Figure S5). However, the sample distribution of patients and HS was more marked based on disease and health status (**Figures 3D,E**).

Fecal Microbiota Comparison between Acute Disease and Remission in JIA Categories

At the moment of fecal sampling, 21% of JIA-ERA patients (4/19) and 40% of JIA-nERA patients (4/10) had clinically active disease. Despite the reduced number of samples, LEfSe analysis showed significant differences in abundances of bacterial genera in samples collected during clinically active disease and during remission. In particular, *Sutterella* was increased in samples collected during active disease, while *Clostridium cluster IV* and *XVIII*, *Parasutterella* and *Odoribacter* were enriched in samples collected from patients in remission (**Figure 4A**).

Furthermore, we calculated intra-group distances by unweighted UniFrac, comparing all JIA samples collected from patients with active disease and those in remission, versus the HS samples. Although differences were not statistically

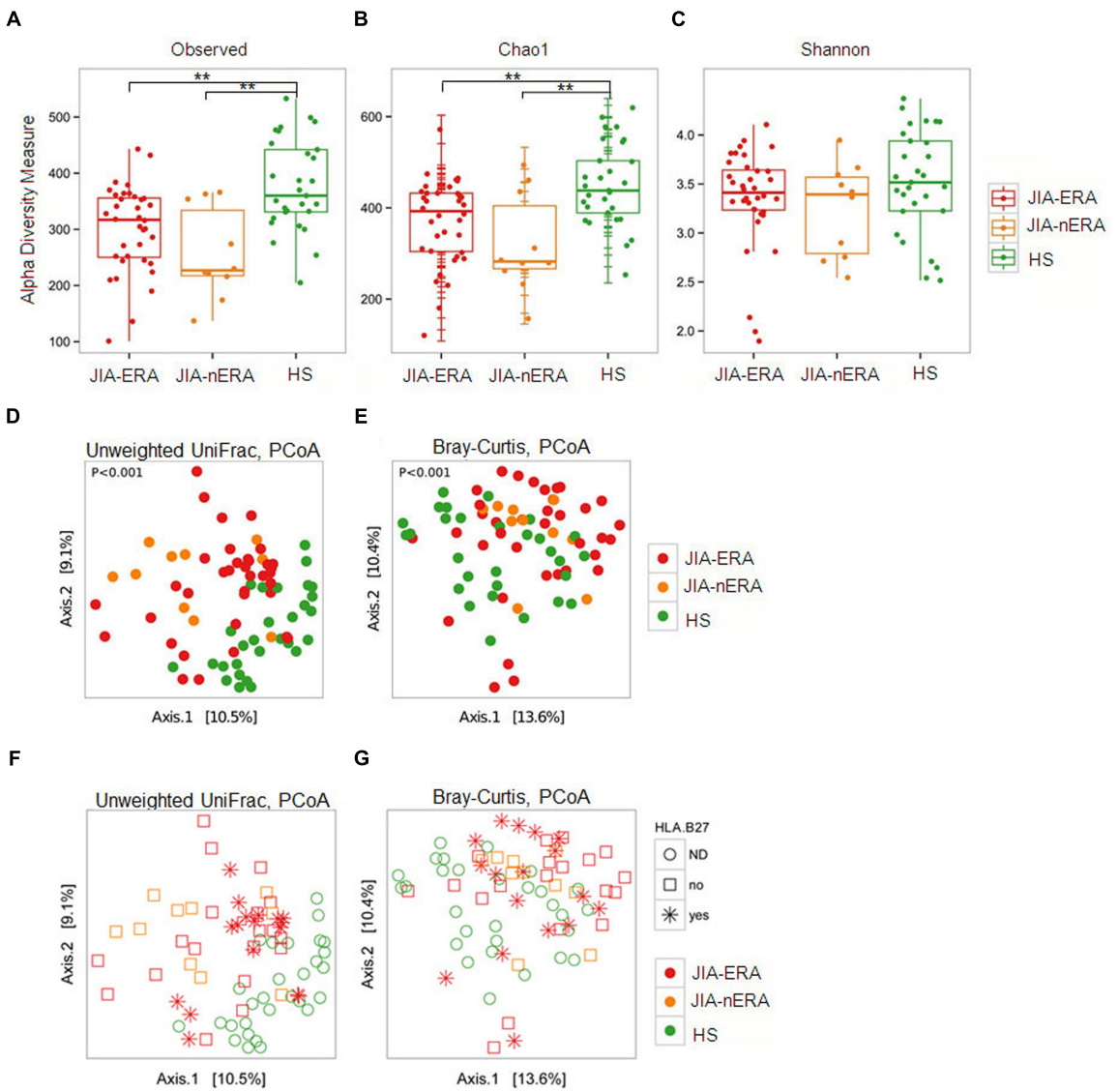

FIGURE 3 | Alpha and beta diversity measures. (A–C) Box plots of **(A)** number of observed OTUs, **(B)** Chao 1, and **(C)** Shannon indexes in the three studied populations. Pairwise comparisons using the Wilcoxon rank sum test., $^{**}p$-value < 0.005, false discovery rate adjustment. **(D,E)** PCoA of **(D)** unweighted UniFrac and **(E)** Bray–Curtis dissimilarities. **(F,G)** PCoA of unweighted UniFrac and Bray–Curtis dissimilarities considering HLAB27 status (red asterisks). Colored dots: red = JIA-ERA, orange = JIA-nERA, and green = HS samples.

significantly, we observed a trend indicating higher intra-group distances between JIA-ERA samples collected during active disease with respect to intra-group distances between JIA-nERA during active disease (**Figure 4B**; intra-active JIA-ERA vs. intra-active JIA-nERA; Supplementary Table S4). Within the JIA-ERA group, we found more variation in microbiota profile in samples collected during active disease compared to remission (**Figure 4B**; intra-active JIA-ERA vs. intra-remission JIA-ERA; Supplementary Table S4). On the contrary, although not statistically significantly different, the intra-distance of active disease samples in the JIA-nERA group was lower than in remission samples (**Figure 4B**; intra-active JIA-nERA vs. intra-remission JIA-nERA; Supplementary Table S4), indicating different microbial heterogeneity in active disease and remission in the two different JIA subsets. Comparing the samples collected from patients in remission, we observed that distances within the JIA-nERA group were greater than within the JIA-ERA group (**Figure 4B**; intra-remission JIA-nERA vs. intra-remission JIA-ERA; Supplementary Table S4).

Interestingly, when we compared JIA groups with HS, the intra-group distance in the HS group was lower than within both JIA groups (**Figure 4B**) and significantly correlated with intra-distances observed in JIA-ERA patients, either active or in remission, and with intra-distance observed in JIA-nERA patients in remission (Wilcoxon rank-sum test; intra-HS vs. intra-active JIA-ERA $p = 0.02$; intra-HS vs. intra-remission

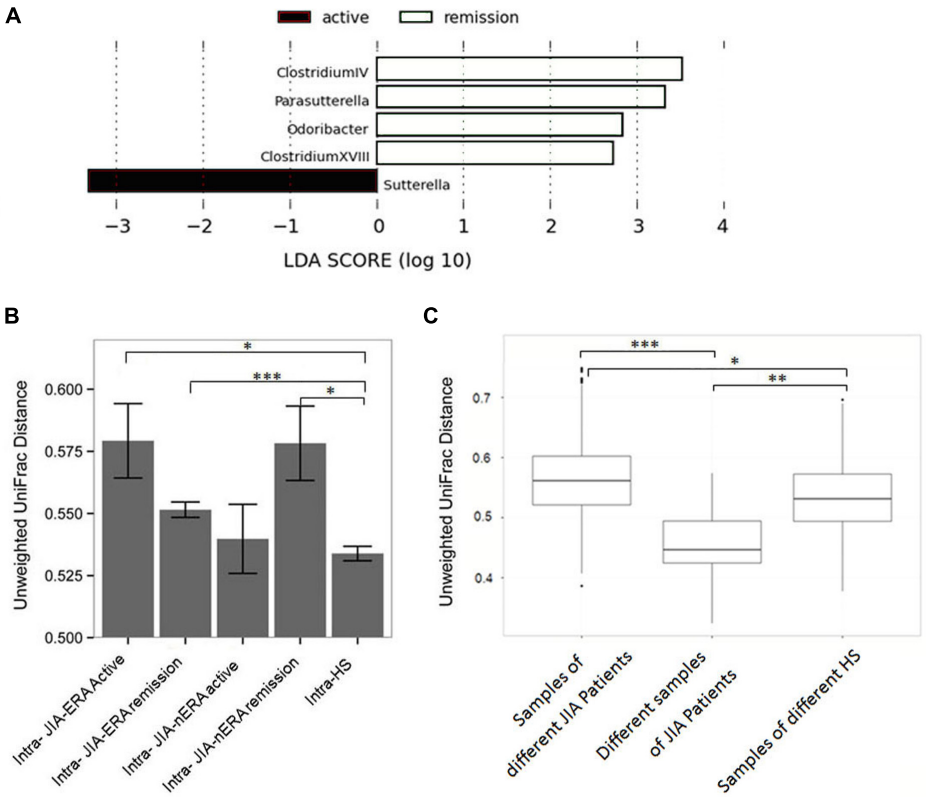

FIGURE 4 | Fecal microbiota comparison in patients during acute disease and in remission. (A) LEfSe analysis shows a statistically significant enrichment of bacterial genera in JIA patients, in active disease (black) and in remission (white). LEfSe results indicate significant ranking among groups (Alpha value = 0.05 for the factorial Kruskal-Wallis test among classes). The threshold for the logarithmic LDA score was 2.0. **(B)** Intra-group distances calculated by Unweighted UniFrac between samples collected during active disease and in remission from JIA-ERA and JIA-nERA patients. *P*-values displayed are the results of Wilcoxon rank-sum tests. **(C)** Box plots of relative distances, calculated by Unweighted UniFrac, among different JIA samples and HS samples and distances of different the two samples of the same JIA-ERA patient. *P*-values displayed are the results of Wilcoxon rank sum test; *p-value < 0.05, **p-value < 0.01; ***p-value < 0.001.

JIA-ERA $p = 0.0004$; intra-HS vs. intra-remission JIA-nERA $p = 0.03$; Supplementary Table S4), indicating that the microbiota composition within the healthy group was significantly more homogeneous than those within the JIA groups.

Next, we evaluated microbiota variations over time in patients of the JIA-ERA group, considering pairwise UniFrac distances between samples A and B (collected three months apart; see Materials and Methods). The matrices obtained by PCoA, derived from pairwise unweighted and weighted UniFrac distances between samples A vs. B, allowed us to explore inter- and intra-individual similarities or dissimilarities among samples, showing in most part of the cases lower distances between samples of the same patient than from different patients (Supplementary Figure S6). Comparing the distributions among all JIA and HS samples vs. the JIA-ERA samples at two different times by Unweighted UniFrac mean distances, we confirmed that samples from the same patient are more similar than samples from different patients, as well as samples from HS, as expected (**Figure 4C**).

Metabolic Function Prediction

The microbiota is able to affect host physiology and metabolic functions, contributing to normal development, and homeostasis of the immune system in the gut, modulating epithelial cell proliferation, and protecting against pathogenic bacteria (Tremaroli and Backhed, 2012; Sommer and Backhed, 2013). Bacterial species are known to carry and transfer operons containing genes for different metabolic functions. Different bacterial species are enriched for certain functions and these correlations have been categorized in well-organized databases (Kanehisa et al., 2014). Therefore, in order to clarify how phylogenetic differences between the fecal microbiota of JIA patients impact their metabolic potential, we applied PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), a computational approach used to predict the functional composition of a metagenome (Langille et al., 2013). LEfSe analysis performed on PICRUSt output (Supplementary Table S5) showed differentially enriched functional classes (KEGG categories) among JIA subgroups compared with HS (**Figure 5**). Within the main KEGG categories, we observed significant enrichment of functions related to cell motility (Flagellar assembly, Ko:02040, Bacterial chemotaxis and Bacterial motility proteins, Ko:02030) in JIA-ERA compared with JIA-nERA and HS (**Figures 5A,B**). Pathways related to Membrane transport (Secretion system, Ko:03070)

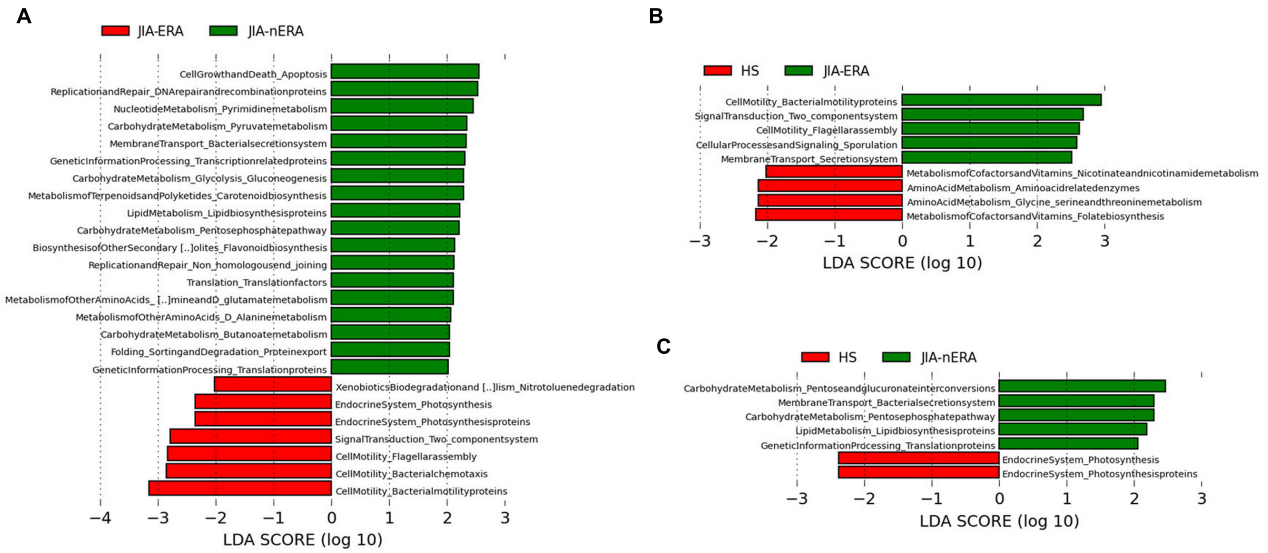

FIGURE 5 | Metabolic function prediction associated to the microbiota profiles. LEfSe analysis, performed on metabolic functions inferred by PICRUSt analysis shows statistically significant enrichment of KEGG categories in **(A)** JIA-ERA vs. JIA-nERA, **(B)** JIA-ERA vs. HS, and **(C)** in JIA-nERA vs. HS. LEfSe results indicate significant ranking among groups (Alpha value = 0.05 for the factorial Kruskal–Wallis test among classes). The threshold for the logarithmic LDA score was 2.0.

and unclassified Cellular Processes and Signaling (Sporulation) were significantly enriched, respectively, in JIA-ERA and JIA-nERA compared with HS group (**Figures 5B,C**). A remarkable enrichment in metabolic functions regarding carbohydrate metabolism, lipid metabolism, aminoacid metabolism, and other aminoacid metabolism identified a core of metabolic capabilities, especially in JIA-nERA metagenome compared to JIA-ERA (**Figure 5A**) and HS (**Figure 5C**). On the other hand, metabolism of cofactors and vitamins (folate biosynthesis and nicotinate and nicotinamide metabolism) and metabolism of other aminoacids (Glycine, Serine, Threonine) significantly characterized HS metagenome compared to JIA-ERA (**Figure 5B**).

DISCUSSION

There are many lines of evidence that link the microbiota to rheumatic diseases. Animal models have been used to establish this possible correlation, such as the use of germ-free and gnotobiotic mice, in which animals were colonized with a specific microbial population, or through the use of antibiotics to understand the effect of microbiota modulation on rheumatic diseases. Principal limits of animal studies are the sample size and the difficulty to mimic the complex multifactorial pathogenesis of these pathologies.

In our study, we tried to define the role of microbiota in patients affected by JIA. Alterations and decrease of microbiota richness were recently found in JIA compared with healthy controls (Stoll et al., 2014), as well as in rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis, the latter two conditions related to spondyloarthropathies (Stebbings et al., 2002; Scher et al., 2013, 2015; Costello et al., 2014; Gill et al., 2015). We also observed variations in fecal microbiota composition and a reduction of microbial richness among JIA patients, affected by ERA and polyarticular JIA (nERA), in comparison with HS.

When compared to HS, in both JIA categories, we found statistically significant abundance in *Ruminococcaceae*, reduction in *Clostridiaceae* and *Peptostreptococcaceae* in JIA-ERA, and increase in *Veillonellaceae* in JIA-nERA. Of note, abundance in *Veillonellaceae* was recently found associated to ankylosing spondylitis (Costello et al., 2014). Conversely to our results, previous studies on ankylosing spondylitis (Costello et al., 2014; Scher et al., 2015) and on oligoarticular and polyarticular JIA (Tejesvi et al., 2016) showed depletion of either *Ruminococcaceae* or *Veillonellaceae*.

The enrichment of anaerobic Gram-positive *Clostridium cluster XIVb* in JIA-ERA patients suggests a causal relation with inflammation. In fact, members of *cluster XIVb*, *C. propionicum*, and *C. colinum* (Collins et al., 1994) were previously observed in poultry ulcerative enteritis (Berkhoff, 1985). Also, studies in animal models have indicated that cell wall peptidoglycans of *Clostridium* and other anaerobic Gram-positive species can induce either chronic and erosive or transient arthritis (Severijnen et al., 1989; Simelyte et al., 2003). The observed decreasing trend in *Faecalibacterium* in JIA-nERA, considered to be an anti-inflammatory microorganism and a marker of health, has also been consistently reported in Crohn's disease patients (Sokol et al., 2008). Conversely to our results, a recent study showed reduction of *Faecalibacterium prasnutzii* in JIA-ERA patients (Stoll et al., 2014).

The partial agreement of our findings with results obtained in other studies could be due to several factors, including variabilities and size of cohorts (different JIA categories, disease

status, untreated, or treated patients), as well as geography, environment or dietary habits of the patients, as shown by David and co-workers (David et al., 2014b).

Despite the limited possibility to generalize our results, due to the reduced number of patients in the cohorts, our results on JIA show intriguing links in terms of fecal microbiota profiles, with IBD and other autoimmune diseases associated to gastrointestinal disorders.

We are well aware that the known predominance of females in JIA-nERA introduces a potential gender effect, yet *Enterobacteriaceae* and *Streptococcaceae,* enriched in JIA-ERA and in JIA-nERA female patients, were also found to be correlated with intestinal inflammation as observed in biopsy samples of Crohn's disease patients (Gevers et al., 2014) and the increase in *Sutterellaceae* observed in JIA-ERA female patients, as well as in samples collected in active disease, is also in line with increase in *Sutterella* previously found in children with autism suffering from gastrointestinal disorder (Williams et al., 2012). Moreover, in samples collected in remission, we found abundance in *Clostridium* spp. *cluster IV* that have been reported to be inducers of colonic T regulatory cell (Atarashi et al., 2011), as well as in *Odoribacter*, known producer of Short Chain Fatty Acids (SCFAs), anti-inflammatory metabolites, and previously found reduced in IBD (Morgan et al., 2012). However, in remission we also found enriched bacteria involved in intestinal inflammation and metabolic disorders, such as *Parasutterella*, found in a mice model of chemically induced colitis (Zhang et al., 2016), *Clostridium cluster XVIII*, encompassing *C. ramosum*, involved in diet-induced obesity (Woting et al., 2014) and *C. spiroforme*, a toxin-associated disease producer, involved in rabbit colitis (Carman and Borriello, 1984).

Differentially abundant taxa, previously found in relation to rheumatoid arthritis, oral infection, intestinal inflammation or colitis, or to intestinal barrier permeability, discriminate for positivity of HLA-B27 allele, a genetic marker strongly associated with spondyloarthropathies. Among these, in HLA-B27 positive ERA patients we found *Bilophila*, a sulphite-reducing bacterium known to be involved in murine colitis (Devkota et al., 2012) and in intestinal inflammatory disorders in humans (Loubinoux et al., 2002; Rowan et al., 2009), via H_2S production promoting intestinal inflammation. *Parvimonas* was commonly observed in periodontitis and appendicitis (Zhong et al., 2014), and *Oscillibacter* was shown to be involved in gut barrier integrity in mice (Lam et al., 2012). In HLA-B27 negative-ERA patients, we found *Haemophilus* and *Eggerthella*, recently associated with rheumatoid arthritis (Zhang et al., 2015; Chen et al., 2016). Moreover, when considering only the ERA group, in HLA-B27 positive patients we also showed enrichment of *Lactobacillus*, observed as potential arthritogenic agent via its cell wall pepetidoglycan (Severijnen et al., 1989), *Clostridium cluster XI*, that includes *C. difficile*, a well-known proinflammatory and colitis inducing-bacterium, and *Dialister*, frequently found in periodontitis and other infections (Morio et al., 2007).

Recent studies on animal models showed association between HLA-B27 allele and other different bacterial species, including *B. vulgatus* and *Prevotella spp.* (Lin et al., 2014) and *Akkermansia muciniphila* (Asquith et al., 2016). In particular, *Akkermansia* spp. was suggested as potential pro-arthritogenic bacterial genus. However, the study of Stoll and collaborators (Stoll et al., 2014) showed abundance of *Akkermansia* in a low percentage of ERA patients, but no significant association with HLA-B27 status. In our study, we did not find *Akkermansia* spp. as part of the core gut microbiota of our patients, yet we found other species correlated with HLA-B27 allele. Little is known on the geographic distribution of *Akkermansia* spp. that could be less represented in our cohorts. Overall this could suggest that other bacterial species, in absence of *Akkermansia* spp. can discriminate HLA-B27 status, and that disease biomarkers should be based on patterns of taxonomic units or biological functions, rather than on single species.

Regarding the functional contribution of microbiota profiles, by PICRUSt prediction analysis we observed distinctive functional acquisitions among JIA subgroups and HS. A core of metabolic capabilities, regarding carbohydrate, lipid, aminoacid, cofactors, and vitamins metabolism, were enriched in JIA-nERA and HS metagenomes. It is worth noting that microbiota of JIA-ERA patients is significantly enriched in functions related to cell motility, including flagellar assembly, bacterial chemotaxis and motility proteins, representing possible traits of virulence that could be associated to gut inflammation. These indications of enrichment in potentially pathogenic invasiveness-related traits in JIA-ERA metagenome could suggest a potential improved ability of microbial components to pass through the gastrointestinal barrier and migrate in other districts, also responding to nutrient gradients. Moreover, given that in mice models immunogenicity of flagellin CBir1 was observed, with consequent induction of colitis, and antibodies anti-CBir1 were found in CD patients with complicated disease (Targan et al., 2005), we cannot exclude the potential effect of flagellar-assembly proteins of some components of microbiota on host immune system of JIA.

Despite the relatively small cohort of patients in our study, the microbial profile differences in active disease and remission are corroborated by the observed intra- and inter-group distances of microbiota samples in active, remission and healthy status. As expected, our results suggest that during active disease the microbiota is strongly perturbed (major intra-group distance compared with remission). Healthy status allows a more stable microbiota ecosystem compared to disease status, as previously observed (Coyte et al., 2015). Remission is characterized by an intermediate microbial pattern, different from both active disease and healthy controls, likely resulting from a different trajectory to stable state and in which autoimmune reactivity and the microbial ecosystem are mutually shaped.

Although microbial profiles may differ in an individual-specific manner, the observed fecal microbiota dissimilarities in the same subject at different collection times, and among JIA categories and healthy controls suggest that continued perturbation and instability of the microbial ecosystem may contribute to inflammation.

Another aspect that should be more thoroughly investigated is the association between microbial profiles and pharmacologic therapies in autoimmune diseases. Recent studies, as previously observed in IBD patients, highlighted the effect of different

therapies on microbiota, such as the rapid effect of enteral nutrition in the shaping of microbiota (Lionetti et al., 2005) and the dysbiosis associated with antibiotic treatment (Lewis et al., 2015), as well as the effect of protonic pomp inhibitors on reduction of bacterial richness and selection of "unhealthy" microbiota (Imhann et al., 2016). Moreover, the recent considerations suggest that the use of sulfasalazine could protect the intestinal epithelium from injury, reducing the bacterial product circulation (Rosenbaum and Asquith, 2016). Despite the reduced number of patients on single or combined therapies in the cohort, our results showed indications of different microbial profiles associated to pharmacological therapies, such as NSAIDs, immunosuppressants, and biologics, providing interesting clues on effect of such treatments on gut microbiota selection. For example, the enrichment of *Collinsella,* involved in exacerbation of joint disease (Chen et al., 2016), observed in JIA-ERA patients treated with NSAIDs in combination with sulfasalazine, results in contrast with hypothesis of Rosenbaum and Asquith (Rosenbaum and Asquith, 2016). Our results prompt future studies on larger cohorts, including untreated newly onset patients, addressing the effect of different pharmacological therapies on patients in active disease and remission, investigating how inflammation can indirectly modify microbiota, selecting differential microbial components, via mechanisms involved in epithelial barrier function and immune response and how pharmacological treatment contribute to perturb the gut microbial equilibrium compared to healthy status.

Finally, given the extreme inter-individual variability of microbiota in inflammatory and autoimmune diseases, further investigations on microbiota dynamics in different phases of disease should address the causes of perturbation and the restoration of microbial equilibrium, in order to adopt therapeutic strategies able to maintain microbial diversity and a stable state, essential for immune homeostasis and the host's health. Research in this direction should include therapeutic strategies able to modulate the microbiota, not only with diet and probiotics, but also evaluating new therapeutic approaches, such as fecal transplantation, recently adopted in other diseases and that have shown some effectiveness, especially during active disease. Furthermore, the understanding of the microbial functional acquisition and the relationships with epithelial barrier function and host immune response could help to identify the pro-arthtritogenic contribution of the microbiota.

ADDITIONAL INFORMATION

Accession codes: Raw 454 files (.sff files) are available at the European Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/PRJEB11846) under the accession study ERP013262.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Study conception and design: RC, CDF, PL, DC, MDP. Data analysis: DA, CD, MDP, MP, MS. Interpretation of data: MDP, CDF, RC, DC, PL, IP, TG, GS, AP.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01703/full#supplementary-material

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Age and Gender Affect the Composition of Fungal Population of the Human Gastrointestinal Tract

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The fungal component of the human gut microbiota has been neglected for long time due to the low relative abundance of fungi with respect to bacteria, and only recently few reports have explored its composition and dynamics in health or disease. The application of metagenomics methods to the full understanding of fungal communities is currently limited by the under representation of fungal DNA with respect to the bacterial one, as well as by the limited ability to discriminate passengers from colonizers. Here, we investigated the gut mycobiota of a cohort of healthy subjects in order to reduce the gap of knowledge concerning fungal intestinal communities in the healthy status further screening for phenotypical traits that could reflect fungi adaptation to the host. We studied the fecal fungal populations of 111 healthy subjects by means of cultivation on fungal selective media and by amplicon-based ITS1 metagenomics analysis on a subset of 57 individuals. We then characterized the isolated fungi for their tolerance to gastrointestinal (GI) tract-like challenges and their susceptibility to antifungals. A total of 34 different fungal species were isolated showing several phenotypic characteristics associated with intestinal environment such as tolerance to body temperature (37°C), to acidic and oxidative stress, and to bile salts exposure. We found a high frequency of azoles resistance in fungal isolates, with potential and significant clinical impact. Analyses of fungal communities revealed that the human gut mycobiota differs in function of individuals' life stage in a gender-related fashion. The combination of metagenomics and fungal cultivation allowed an in-depth understanding of the fungal intestinal community structure associated to the healthy status and the commensalism-related traits of isolated fungi. We further discussed comparatively the results of sequencing and cultivation to critically evaluate the application of metagenomics-based approaches to fungal gut populations.

Keywords: commensal fungi, human gut mycobiota, antifungal resistance, fungal metagenomics, fungi-host interactions

INTRODUCTION

The human gut is a complex ecological niche in which archaea, bacteria, protozoa, fungi, and viruses co-exist in close association with the host (Reyes et al., 2010; Arumugam et al., 2011; Human Microbiome Project Consortium, 2012). Even if it has been estimated that the number of bacteria hugely outreaches the number of fungi in the gastrointestinal (GI) tract (Huffnagle and Noverr, 2013), fungi play a relevant role in the physiology of the human host (Oever and Netea, 2014; Underhill and Iliev, 2014). Recent studies showed that, while the composition of the bacterial community is relatively stable over time, the fungal population inhabiting the murine gut undergoes significant changes during the animal's lifetime (Dollive et al., 2013). This brought to the conclusion that gut fungal populations are more variable than bacterial ones and that their composition may be influenced by environmental fungi (Underhill and Iliev, 2014). Despite evidence that fungi inhabit the mammalian GI tract and interact with the host immune system (Romani, 2011; Rizzetto et al., 2014; Underhill and Iliev, 2014), the composition and characteristics of the mycobiota in healthy hosts have been poorly explored. The prevalent interest in describing pathogenic fungi, their phenotypes and the process by which they establish the infection is one of the major cause that brought to neglect the harmless part of the commensal fungal population. Despite this topic has been only marginally explored to date, it has been shown that mucosal fungi are able to modulate both the innate and adaptive immune responses (Romani, 2011; Rizzetto et al., 2014; Underhill and Iliev, 2014) thus supporting the need to further study the whole gut mycobiota. Furthermore, alterations of the gut mycobiota have been associated to different pathologies ranging from metabolic disorders (obesity) to colorectal adenomas and Inflammatory Bowel Diseases (IBDs) (Luan et al., 2015; Mar Rodriguez et al., 2015; Sokol et al., 2016). A recent study showed the association of IBDs to alteration of the gut mycobiota. In particular Sokol and colleagues showed that IBD patients bear a smaller proportion of *Saccharomyces cerevisiae* and higher of *Candida albicans* compared to healthy subjects. In addition, they highlighted the existence in Crohn's disease of interconnected alterations between bacterial and fungal communities (Sokol et al., 2016). However, the role of the gut mycobiota in the maintenance of health it is still far from being well-understood because the studies carried out so far focused on disease-causing taxa. Nevertheless, some yeasts have been clinically prescribed for a long time because of their potential probiotic properties, suggesting a beneficial role of some fungi for host health. A great example of "beneficial" fungus is represented by *S. cerevisiae* var. *boulardii,* used for the relief of gastroenteritis (Hatoum et al., 2012). In order to reduce the gap of knowledge concerning the gut mycobiota and its interplay with the host, we characterized the gut mycobiota composition of a cohort of healthy subjects by means of metagenomics, fungal cultivation, and phenotypic assays.

MATERIALS AND METHODS

Study Participants

Fecal samples were collected from 111 Italian healthy volunteers (49 male and 62 female, average age, 10 ± 8.2; **Table 1**) and analyzed within 24 h. Written informed consent has been obtained from all the enrolled subjects or tutors in accordance with the guidelines and regulations approved by the Research Ethical Committees of the Meyer Children's Hospital and the Azienda Ospedaliera Careggi, Florence. All the subjects enrolled were non-smokers, followed a Mediterranean-based diet and they did not take antibiotics, antifungals or probiotics in the 6 months prior to sample collection. None of the participants had any history of GI abnormalities.

Isolation and Identification of Cultivable Fungal Species from Feces

Stool samples were diluted in sterile Ringer's solution and plated on solid YPD medium (1% Yeast extract, 2% Bacto-peptone, 2% D-glucose, 2% agar) supplemented with 25 U/ml of penicillin, 25 μg/ml of streptomycin (Sigma-Aldrich) and incubated aerobically at 27°C for 3–5 days. All fungal isolates grown on the selective medium were further isolated to obtain single-cell pure colonies. Genomic DNA was extracted from pure cultures of isolated colonies as previously described (Hoffman and Winston, 1987). Strains were identified by amplification and sequencing of the ribosomal Internal Transcribed Spacer (ITS) region, using ITS1 (5′-GTTTCCGTAGGTGAACTTGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers, as previously described (Sebastiani et al., 2002). Fungal isolates were identified by using the BLAST algorithm in the NCBI database (minimum 97% sequence similarity and 95% coverage with a described species).

Phenotypical Characterization of Fungal Isolates

Fungal isolates were tested for phenotypical features that could be related to the ability of colonization and persistence in the human gut. Cell growth in liquid media was monitored by optical density measurement at 630 nm with a microplate reader (Synergy2, BioTek, USA) after 48 h of incubation under tested conditions. Three independent replicates were performed for each test.

Growth at Supra Optimal Temperatures

Fungal isolates ($\sim 10^5$ cells/ml) were grown at supra optimal temperatures in liquid YPD medium (40, 42, 44, and 46°C).

pH Impact on Growth

Fungal isolates ($\sim 10^5$ cells/ml) were grown at 37°C in liquid YPD medium at pH 2.0 and pH 3.0 adding hydrochloric acid/potassium chloride and citrate buffers, respectively, to test their ability to resist to the acidic environments encountered during GI tract passage.

TABLE 1 | Characteristics of the study participants.

Age group (year)		Infants (0–2)	Children (3–10)		Adolescents (11–17)	Adults (≥18)		All subjects
Number of subjects		18	48		24	21		111
% with fungi		88.9	83.3		70.8	76.2		80.2
Subject ID	**Gender**	**Age (year)**	**Subject ID**	**Gender**	**Age (year)**	**Subject ID**	**Gender**	**Age (year)**
HS1	M	5	HS38*	F	25	HS75	F	1
HS2	M	5	HS39*	F	27	HS76*	M	1
HS3	M	14	HS40*	M	27	HS77	F	4
HS4	M	1	HS41*	F	24	HS78*	M	12
HS5	F	20	HS42*	F	24	HS79*	M	0.1
HS6	F	20	HS43*	M	26	HS80	F	0.1
HS7	F	20	HS44*	F	24	HS81	F	7
HS8	M	5	HS45*	F	6	HS82*	M	10
HS9	M	14	HS46*	F	6	HS83*	M	12
HS10*	F	2	HS47*	F	10	HS84	F	6
HS11	M	16	HS48*	F	2.5	HS85	F	10
HS12	M	15	HS49*	M	2.5	HS86*	M	7
HS13*	F	18	HS50*	F	1.5	HS87*	M	9
HS14	F	0.3	HS51*	F	8	HS88*	M	7
HS15*	F	11	HS52*	F	23	HS89*	M	12
HS16	M	14	HS53*	F	23	HS90	F	8
HS17	M	15	HS54	M	2	HS91	F	2
HS18	M	11	HS55*	M	2	HS92	F	12
HS19	F	3	HS56*	M	2	HS93	F	4
HS20*	F	4	HS57	F	12	HS94	F	4
HS21*	F	5	HS58	F	3	HS95	F	10
HS22*	F	15	HS59*	M	5	HS96	F	12
HS23*	F	11	HS60	F	3	HS97*	M	6
HS24	M	15	HS61*	M	2	HS98	F	16
HS25	M	7	HS62	F	4	HS99	F	3
HS26	M	3	HS63*	M	5	HS100*	M	0.1
HS27*	F	9	HS64	F	3	HS101*	M	4
HS28	M	5	HS65*	M	5	HS102	F	13
HS29*	F	16	HS66*	M	0.1	HS103*	M	7
HS30*	F	12	HS67	F	1	HS104*	M	4
HS31*	F	24	HS68	F	4	HS105	F	8
HS32*	F	32	HS69*	M	6	HS106	F	5
HS33*	F	32	HS70	F	11	HS107	M	13
HS34*	F	25	HS71*	M	1	HS108	M	4.5
HS35*	F	26	HS72	F	10	HS109	M	1
HS36*	M	20	HS73	F	4	HS110	M	12
HS37*	F	28	HS74*	M	6	HS111	M	18

**Samples analyzed also by mean of amplicon-based ITS1metagenomics.*

Tolerance to Bile Acids

Fungal isolates ($\sim 10^5$ cells/ml) were grown in liquid YPD medium at 37°C in the presence of three different concentrations of bile [Ox-bile, Sigma-Aldrich; 0.5, 1, and 2% (w/v)] mimicking the physiological intestinal settings (Noriega et al., 2004).

Resistance to Oxidative Stress

Fungal resistance to oxidative stress was evaluated by measuring the inhibition halo induced by the treatment of fungal strains ($\sim 10^7$ cells/ml) grown on YPD solid medium with 0.5 mM hydrogen peroxide (H_2O_2). The percentage of sensitivity to oxidative stress was calculated as the deviation of the inhibition

halo diameter (Ø) from that of the environmental, oxidative stress sensitive M28-4D *S. cerevisiae* strain (Cavalieri et al., 2000) according to the following formula: [(Ø sample—Ø M28-4D)/Ø M284D]*100.

Invasive Growth

The ability of fungal strains to penetrate the YPD solid medium was tested as previously described (Vopalenska et al., 2005). M28-4D and BY4742 *S. cerevisiae* strains, known to be invasive and non-invasive, respectively, have been used as controls. The strain invasiveness was assigned with scores from 3 (highly invasive) to 0 (non-invasive).

Hyphal Formation

Fungal cells ($\sim 10^5$ cells/ml) were grown for 7 days in liquid YPD and YNB media [0.67% Yeast Nitrogen Base w/o aminoacids and $(NH_4)_2SO_4$ (Sigma-Aldrich), 2% glucose], both at 27 and 37°C in order to evaluate hyphae or pseudohyphae formation. Formation of hyphae was inspected by optical microscope observation with a Leica DM1000 led instrument (magnification 40x and 100x).

Antifungal Susceptibility Testing

All fungal isolates were tested for susceptibility to fluconazole, itraconazole, and 5-flucytosine (Sigma-Aldrich) by Minimum Inibitory Concentration (MIC) assays according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (Rodriguez-Tudela et al., 2008a,b). Clinical and Laboratory Standards Institute (CLSI) clinical breakpoints (CBPs) were used to evaluate the antifungal resistance (Pfaller and Diekema, 2012; Castanheira et al., 2014). CBPs have not been established for non-*Candida* yeasts and non-*Aspergillus* molds, however have been used as a proxy for the evaluation of antifungals susceptibility in such isolates.

DNA Extraction and PCR Amplification of Fungal ITS1 rDNA Region

DNA extraction from fecal samples (250 mg) was performed using the FastDNA™ SPIN Kit for Feces (MP-Biomedicals, USA) following manufacturer's instructions. DNA quality was checked on 1% agarose gel TAE 1X and quantified with a NanoDrop® spectrophotometer. For each sample, fungal ITS1 rDNA region was amplified using a specific fusion primer set coupled with forward primer 18SF (5′-GTAAAAGTCGTAACAAGGTTTC-3′) and reverse primer 5.8S1R (5′-GTTCAAAGAYTCGATGATTCAC-3′; Findley et al., 2013) containing adaptors, key sequence and barcode (Multiple IDentifier) sequences as described by the 454 Sequencing System Guidelines for Amplicon Experimental Design (Roche, Switzerland). The PCR reaction mix contained 1X FastStart High Fidelity PCR buffer, 2 mM $MgCl_2$, 200 μM of dNTPs, 0.4 μM of each primer (PRIMM, Italy), 2.5 U of FastStart High Fidelity Polymerase Blend, and 100 ng of gDNA as template. Thermal cycling conditions used were 5 min at 95°C, 35 cycles of 45 s at 95°C, 45 s at 56°C, and 1.30 min at 72°C followed by a final extension of 10 min at 72°C. All PCR experiments were carried out in triplicates using a Veriti® Thermal Cycler (Applied Biosystems, USA).

Library Construction and Pyrosequencing

The PCR products obtained were analyzed by gel electrophoresis and cleaned using the AMPure XP beads kit (Beckman Coulter, USA) following the manufacturer's instructions, quantified via quantitative PCR using the Library quantification kit—Roche 454 titanium (KAPA Biosystems, USA) and pooled in equimolar way in a final amplicon library. The 454 pyrosequencing was carried out on the GS FLX+ system using the XL+ chemistry following the manufacturer's recommendations (Roche, Switzerland).

Data Analysis

Pyrosequencing resulted in a total of 1.337.184 reads with a mean of 19.379 ± 13.334 sequences *per* sample. Raw 454 files were demultiplexed using the Roche's sff file software and submitted to the European Nucleotide Archive with accession number PRJEB11827 (http://www.ebi.ac.uk/ena/data/view/PRJEB11827). Sample accessions and metadata are available in **Supplementary Table S1**. Reads were pre-processed using the MICCA pipeline (Albanese et al., 2015) (http://www.micca.org). Forward and reverse primers trimming and quality filtering were performed using micca-preproc. *De-novo* sequence clustering, chimera filtering, and taxonomy assignment were performed by micca-otu-*denovo*: Operational Taxonomic Units (OTUs) were assigned by clustering the sequences with a threshold of 97% pairwise identity and their representative sequences were classified using the RDP classifier version 2.8 (Wang et al., 2007) against the UNITE fungal ITS database (Koljalg et al., 2013). *De novo* multiple sequence alignment was performed using T-Coffee (Notredame et al., 2000). Fungal taxonomy assignments were then manually curated using BLASTn against the GenBank's database for accuracy. High quality fungal sequences were detected in all samples. Furthermore, the sequences belonging to Agaricomycetes [unlikely to be residents of the human gut due to their ecology Hibbett, 2006] were manually filtered out.

The phylogenetic tree was inferred by using micca-phylogeny (Price et al., 2010). Rarefaction analysis resulted in a sequencing depth adequate to capture the ecological diversity of the samples up to saturation. Sampling heterogeneity was reduced by rarefaction. *Alpha* and *beta*-diversity estimates were computed using the phyloseq R package (McMurdie and Holmes, 2013). PERMANOVA (Permutational multivariate analysis of variance) was performed using the adonis() function of the *vegan* R package with 999 permutations. Permutations have been constrained within age groups (corresponding to 0–2, 3–10, 11–17, and >18 y/o) or gender to reduce possible biases related to the unequal age and gender distributions among subjects using the "strata" argument within the adonis() function. Two-sided, unpaired Welch t-statistics were computed using the function mt() in the phyloseq library and the *p*-values were adjusted for multiple comparison controlling the family-wise Type I error rate (minP procedure; Westfall and Young, 1993). Wilcoxon rank-sum tests and Spearman's correlations were performed using the R software (Team, 2014) through the *stats* R package (version 3.1.2) and the *psych* R package (Revelle, 2013), respectively. *p*-values have been corrected for multiple comparison by using the false discovery rate correction (Benjamini and Hochberg, 1995).

RESULTS

Cultivable Gut Mycobiota

The cultivable gut mycobiota of 111 healthy volunteers was investigated through isolation in selective media. Fungi were detected in more than 80% of subjects leading to the identification of 349 different isolates (**Supplementary Table S2**). Thirty-four different fungal species were detected at different frequencies of isolation (**Table 2**) among which *Aspergillus glaucus, Candida albicans, Candida deformans, Candida fermentati, Candida glabrata, Candida intermedia, Candida lusitaniae, Candida metapsilosis, Candida parapsilosis, Candida pararugosa, Candida tropicalis, Candida zelanoydes, Cryptococcus saitoi, Lichtheimia ramosa, Mucor circinelloides, Pleurostomophora richardsiae, Rhodotorula mucilaginosa, Trichosporon asahii, Yarrowia lipolytica*. These species were previously found in different human body sites, including the GI tract as commensal or opportunistic pathogens (Araujo et al., 2007; Johnson, 2009; Alastruey-Izquierdo et al., 2010; Kurtzman et al., 2011; Levenstadt et al., 2012; Gouba et al., 2014; Lee et al., 2014; Rizzetto et al., 2014). We also isolated the environmental fungi *Aspergillus pseudoglaucus, Eurotium amstelodami, Eurotium rubrum, Penicillium brevicompactum, Penicillium paneum, Penicillium crustosum, Pichia caribbica, Pichia fermentans, Pichia kluyveri, Pichia manshurica, Rhodosporidium kratochvilovae, Saccharomyces cerevisiae, Starmerella bacillaris*, and *Torulaspora delbrueckii*. Such species were previously found in fermentations, oenological samples (Chitarra et al., 2004; Butinar et al., 2005; Kurtzman et al., 2011; Barata et al., 2012; Bezerra-Bussoli et al., 2013; Tristezza et al., 2013; Vardjan et al., 2013; Belda et al., 2015; de Melo Pereira et al., 2014; Santini et al., 2014; Wang et al., 2014) and rarely found in clinical samples (de la Camara et al., 1996; Kaygusuz et al., 2003; Butinar et al., 2005; Rizzetto et al., 2014). The 39.8% of subjects showed at least one *C. albicans* isolate, which resulted in the most common yeast species found in our samples, in line with previous reports on the gut mycobiota of healthy subjects (Khatib et al., 2001; Bougnoux et al., 2006).

Population level analysis of the cultivable gut mycobiota revealed significant gender-related differences, with female subjects showing a higher number of fungal isolates ($p < 0.005$, Wilcoxon rank-sum test; **Figure 1A**) and fungal species ($p < 0.05$, Wilcoxon rank-sum test; **Figure 1B**) compared to male subjects (not related to individual's age) while we did not observed significant differences in the fungal population among the investigated age groups (**Figures 1C,D**). Finally, no species *per se* was responsible for these differences, as indicated by the fact that we did not find significant differences between individual species abundances in male and female subjects for any investigated age group.

TABLE 2 | Fungal isolates and frequencies of isolation.

Species	%	Species	%
Candida albicans	39.8	*Rhodosporidium kratochvilovae*	0.57
Rhodotorula mucilaginosa	12.6	*Trichosporon asahii*	0.57
Candida parapsilosis	12.3	*Yarrowia lipolytica*	0.57
Torulaspora delbrueckii	6.59	*Aspergillus cristatus*	0.28
Pichia fermentans	4.29	*Candida deformans*	0.28
Penicillium brevicompactum	3.72	*Candida fermentati*	0.28
Pichia manshurica	3.43	*Candida glabrata*	0.28
Pichia kluyveri	2.86	*Candida intermedia*	0.28
Candida lusitaniae	2.58	*Candida metapsilosis*	0.28
Pennicillium crustosum	1.43	*Candida tropicalis*	0.28
Saccharomyces cerevisiae	1.14	*Candida zelanoydes*	0.28
Penicillium paneum	0.58	*Eurotium amstelodami*	0.28
Aspergillus glaucus	0.57	*Eurotium rubrum*	0.28
Aspergillus pseudoglaucus	0.57	*Lichtheimia ramose*	0.28
Candida pararugosa	0.57	*Pichia carribica*	0.28
Cryptococcus saitoi	0.57	*Pleurostomophora richardsiae*	0.28
Mucor circinelloides	0.57	*Starmerella bacillaris*	0.28

Fungal Gut Metagenomics

To better characterize the intestinal fungal community structure associated to our cohort of healthy subjects we further analyzed a subset of these subjects (57 subjects, 29 females, and 28 males, average age 12 ± 9.5) by means of amplicon-based ITS1 targeted metagenomics, looking at gender and age groups differences. The analysis led to the identification of 68 fully classified (to the genus level) fungal taxa and 26 taxa only partially classified (of which 2 classified to the phylum level, 5 classified to the order level, 9 classified to the class level, and 9 classified to the family level). Measurements of the fungal richness within each sample i.e., the *alpha*-diversity (see Materials and Methods), revealed no significant differences among male and female subjects (**Figure 2A**), differently from the above finding based on the culture-based analysis in which we observed an increased number of intestinal fungal species in females compared to males (**Figure 1B**). Furthermore, we observed that infants and children harbor a higher fungal richness compared to adults as indicated by the number of the observed OTUs ($p < 0.05$, Wilcoxon rank-sum test, **Figure 2B**). The analysis of *beta*-diversity identified significant differences in the composition of the gut mycobiota among gender and age groups. PCoA (Principal Coordinates Analysis) revealed that samples cluster by gender, based on the unweighted UniFrac distance and the Bray-Curtis dissimilarity ($p < 0.05$, PERMANOVA; **Figures 2C,D**, **Supplementary Table S3**) and by age groups, based on the unweighted UniFrac distance ($p < 0.05$, PERMANOVA; **Figure 2C**, **Supplementary Table S3**). We calculated PERMANOVAs constraining permutations within levels (gender or age groups) to avoid biases related to the unequal distribution of genders among age groups and *vice-versa*. Genus level analysis showed *Penicillium*, *Aspergillus*, and *Candida* as the most abundant genera in this subset of subjects (22.3, 22.2, and 16.9%, respectively; **Figure 3**, **Supplementary Table S4**). We further observed that *Aspergillus* and Tremellomycetes_unidentified_1 were significantly more abundant in male than female subjects ($p < 0.05$, Welch *t*-test) and in children than adults ($p < 0.05$, Welch *t*-test). To note, the latter result could be biased by the unbalanced distribution of male and female subjects in children and adults

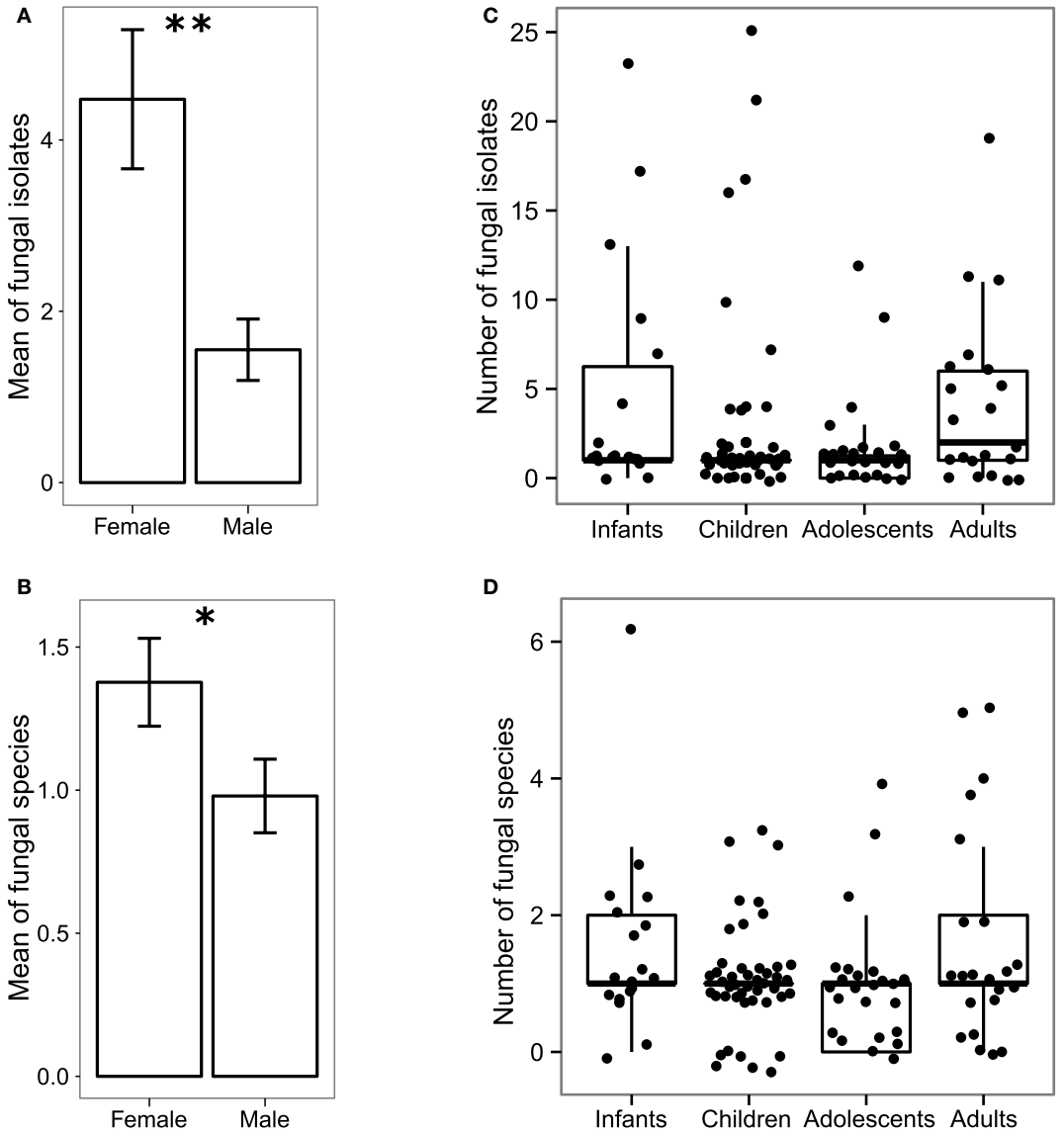

FIGURE 1 | Gender-related and age-related differences in the gut mycobiota of 111 healthy volunteers. Histogram of the mean of **(A)** abundances and **(B)** the richness ± standard error of fungal isolates in female and male subjects; box-plot representation of the **(C)** abundance and **(D)** richness of fungal isolates in different age groups i.e., infants (0–2 years old), children (3–10 years old), adolescents (11–17 years old) and adults (≥18 years old). $^{**}p < 0.005$, $^{*}p < 0.05$, Wilcoxon rank-sum test.

groups (14/22 male children and 3/17 male adults). Furthermore, the genus *Penicillium* was significantly more abundant in infants than adults ($p < 0.05$, Welch *t*-test). Interestingly, we identified sequences belonging to the single-cell protozoa *Blastocystis*, eukaryotes abundant in the human gut microbiota (Scanlan and Marchesi, 2008), only in adolescent and adult females (**Figure 3**, **Supplementary Table S4**) that could potentially be due to exposure to animals (Scanlan et al., 2014).

Phenotyping the Gut Mycobiota

The characterization of phenotypic features of the isolates related to the ability to survive and colonize the human gut was performed to estimate if such isolates were commensals adapted to this ecological niche or passengers introduced through the diet and delivered with the feces.

We therefore investigated the isolates' resistance by a series of assays mimicking the conditions that fungal isolates face during passage through the human GI tract. In addition to the fact that the human body temperature (37°C) is higher than the optimum for most fungal species, in the GI tract fungi are also exposed to acidic and oxidative environments and to bile salts, produced by the liver and secreted into the duodenum, exposing the microorganisms to oxidative stress and DNA damage (Kandell and Bernstein, 1991).

The majority of the isolates were found to tolerate acidic conditions (58.9 and 94.8% of isolates were able to grow at pH

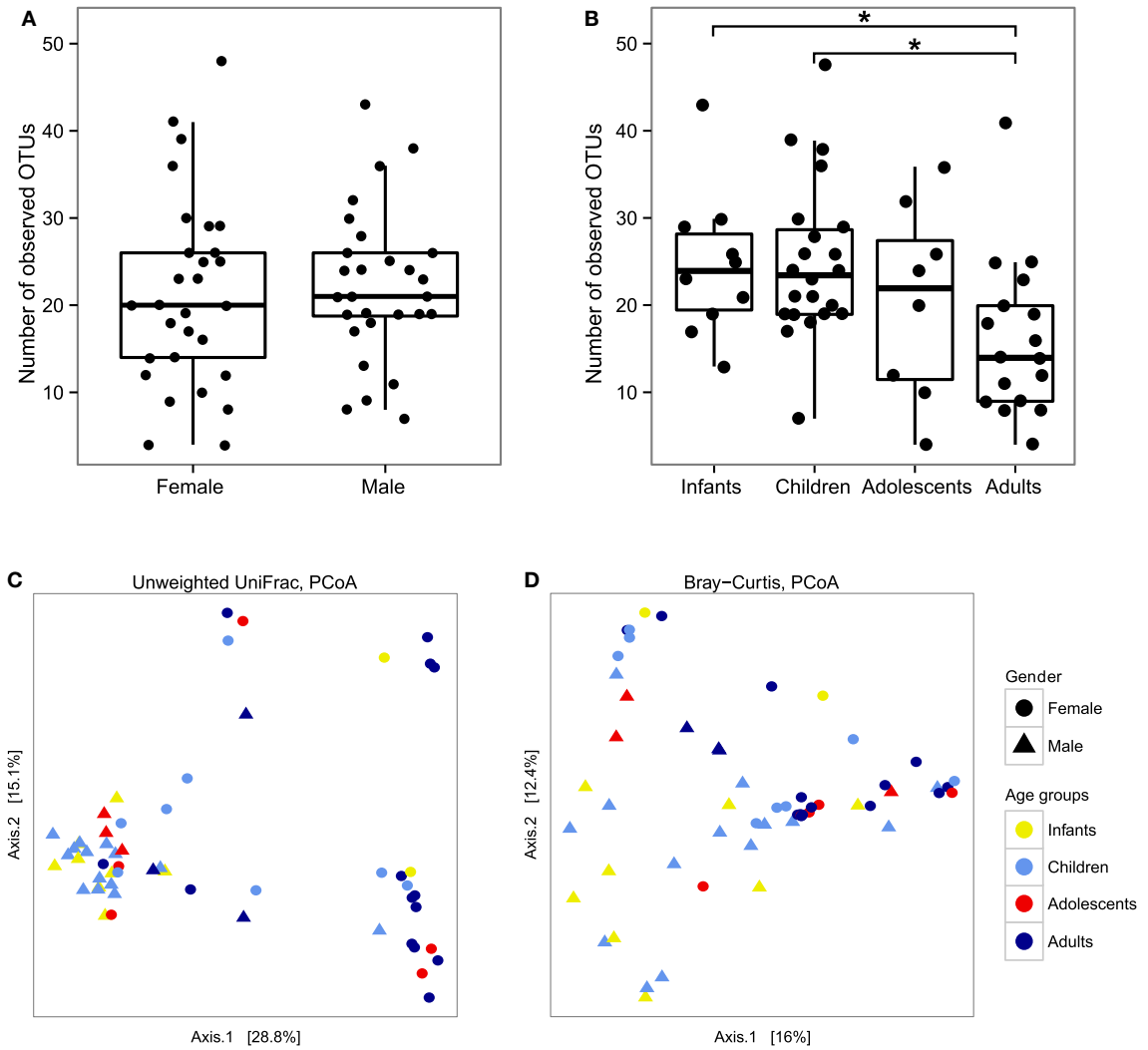

FIGURE 2 | Box-plot representation of fungal *alpha*-diversity measures using the number of observed OTUs between (A) genders or (B) age groups and measures of fungal *beta*-diversity by PCoA of the between samples distances measured using (C) the unweighted UniFrac distance and (D) the Bray-Curtis dissimilarity. *$p < 0.05$, Wilcoxon rank-sum test.

2 and pH 3, respectively) and oxidative stress (85.7% of the isolates showed higher tolerance compared to environmental M28 *S. cerevisiae* strain), both conditions are characteristic of the gut environment. Tolerance to physiological concentrations of bile acids was also observed (89.8, 87.5, and 85.7% of fungal isolates were able to grow in presence of ox-bile 0.5, 1, and 2%, respectively) as well as the ability to grow at *supra* optimal temperatures with almost all the isolates (99.4%) being able to grow at 37°C (**Supplementary Table S2**). The comparison of the growth ability of such isolates at pH 3 and at growing concentrations of ox-bile (i.e., 0.5, 1.0, and 2.0% ox-bile) with respect to the control growth condition (37°C, no bile, pH 6.5) revealed that these stressful conditions do not significantly affect the growth ability of the fungal isolates (**Figure 4**). By contrast, a significant growth reduction was observed when comparing the isolated grown at pH 2 with respect to the control growth condition ($p < 0.0001$, Wilcoxon rank-sum test; **Figure 4**). As expected, a progressive reduction of growth ability was observed in correspondence of incubation temperature increase (i.e., from 40 to 46°C) for all the tested isolates ($p < 0.0005$, Wilcoxon rank-sum test; **Figure 4**).

In addition to the ability of fungal isolates to tolerate the intestinal environmental stresses, we also explored their ability to undergo phenotypic changes favoring their persistence within the human gut. Among these, we assessed the formation of hyphae and the ability to penetrate the solid growth medium, thus to adhere to host tissues. The 56.9% of fungal isolates was able to form hyphae or pseudohyphae (**Supplementary Table S2**). In addition, the morphotype switch to hyphae and pseudohyphae was related to the isolates' invasiveness, with hyphae and pseudohyphae-forming isolates being the most invasive (**Figure 5A**), suggesting that such isolates may be able to

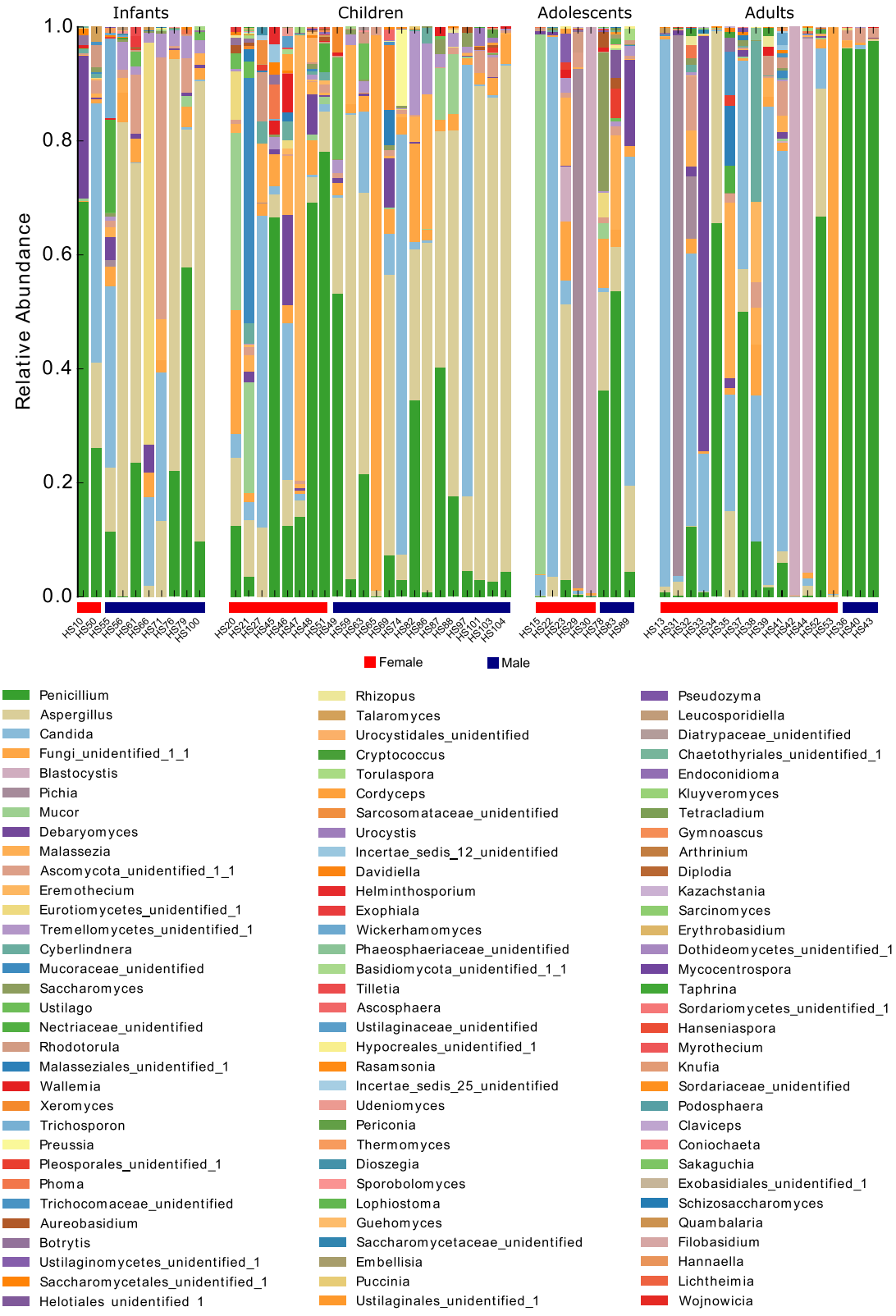

FIGURE 3 | Stacked bar-plot representation of the relative abundances at the genus level of the fecal mycobiota of healthy subjects from metagenomics analysis distributed according to individuals' life stage and gender.

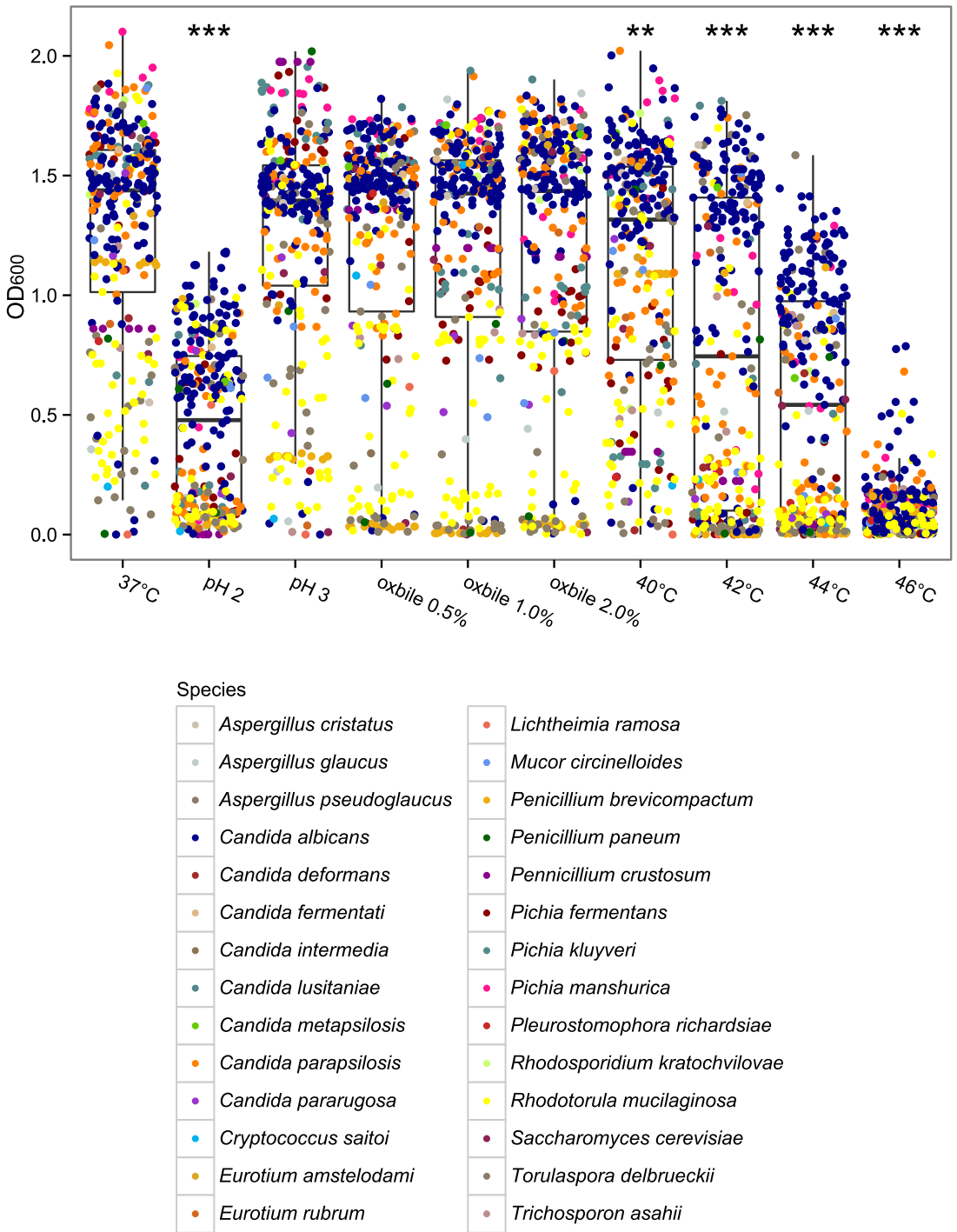

FIGURE 4 | Box-plot representation of the comparison of fungal isolates growth ability at 37°C (control condition) vs. different stressful conditions mimicking the gastrointestinal tract challenges. $^{**}p < 0.0005$, $^{***}p < 0.0001$, Wilcoxon rank-sum test.

adhere to or invade the host tissues. Furthermore, we observed that hyphae-forming isolates are significantly more resistant to itraconazole than pseudohyphae-forming isolates and isolates unable to form hyphae ($p < 0.05$, Wilcoxon rank-sum test; **Figure 5B**). These phenotypic traits in conditions of altered immune system or in association with intestinal dysbiosis, could represent a pathogenic potential for the host.

It is now recognized that inappropriate antifungal use contributes to the increase in microbial antifungal resistance, complicating therapeutic intervention, and the eventual

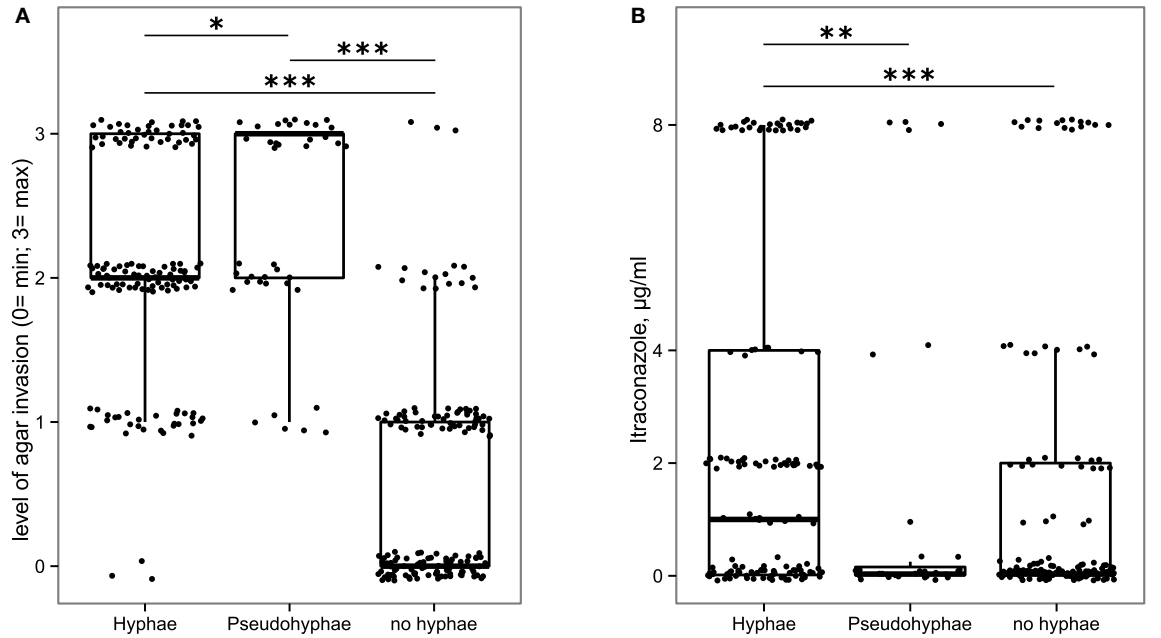

FIGURE 5 | Box-plot representation of fungal isolates able (or not) to produce hyphae or pseudohyphae in relationship with (A) their ability to be invasive on YPD solid medium, (B) their resistance to itraconazole. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, Wilcoxon rank-sum test.

eradication of pathogens (Chen et al., 2010; Arendrup et al., 2011). Due to the relevance of such aspect and its impact on clinical studies, we tested all fungal isolates for their susceptibility to the widely therapeutically used azoles, fluconazole, and itraconazole (Martin, 2000) as well as the non-azole antifungal 5-flucytosine (Vermes et al., 2000). A total of 31.5% of the isolates were resistant to fluconazole and, as expected, similar levels of itraconazole resistance were found (for 39.2% of the isolates the MIC was $\geq$1 µg/ml; **Supplementary Table S2**). Previous studies have indeed suggested that cross-resistance may occur between fluconazole and other azole compounds (i.e., itraconazole) (Pfaller et al., 2006) and we further confirmed such observations with the finding of a significant positive correlation between the isolates resistance to these two antifungals (Spearman's $r = 0.43$, $p < 0.05$; **Figure 6**). Most of the isolates (99.34%) showed high susceptibility to 5-flucytosine with most MIC values $\leq$0.125 µg/ml (**Supplementary Table S2**). Among the 9 most abundant species (at least 6 isolates per species), *C. albicans*, *Pichia* spp. and *Rhodotorula mucillaginosa* showed the highest resistance to fluconazole, with $MIC_{90} > 64$ µg/ml (**Table 3**). Furthermore, it is worth to note that resistance to tested antifungals is positively correlated with the ability of strains to grow under stressful conditions, such as *supra* optimal temperature, acidic conditions, and bile salts exposure ($p < 0.05$, Spearman's *r* correlation; **Figure 6**).

DISCUSSION

The vast majority of fungal species inhabiting our body are commensals and opportunistic pathogens that could turn into potential threats depending on strain virulence traits and on the status of the host's immune system. In this perspective to discover a pathogenic infection it seems crucial to define exactly which species are normally present in a given body district.

The human GI tract is known to contain variable communities of bacteria but also fungi have an important role in this ecological niche (Underhill and Iliev, 2014). Nevertheless, the phylogenetic characterization of fungal microorganisms and their specific role as part of the GI niche have not yet been studied extensively.

The advent of sanitation and food globalization has reduced the possibility for humans to come across with the richness of fungal species present in traditional fermented foods. Fungal infections are an ever increasing problem either as side effects of antibiotics use, high dose chemotherapy, and of the spread of immunosuppressive diseases. Estimates of global mortality rates suggest that fungi are responsible for more deaths than either tuberculosis or malaria (Brown et al., 2012). Most of this mortality is caused by species belonging to four fungal genera: *Aspergillus*, *Candida*, *Cryptococcus*, and *Pneumocystis* that are rapidly becoming resistant to most antifungal drugs (Brown et al., 2012; Denning and Bromley, 2015). The information on these fungi so far derives from the study of lung infections, while little is known on the gut mycobiota composition and its role in health and disease. The knowledge on the gut mycobiota is currently limited to few studies making it difficult to assess the significance of differences found in the intestinal fungal populations of diseases such as IBDs due to the lack of information on what the healthy mycobiota is. Here we aimed at defining the "healthy" gut mycobiota, showing that the intestinal fungal community of a cohort of Italian healthy volunteers is a variegate ecosystem

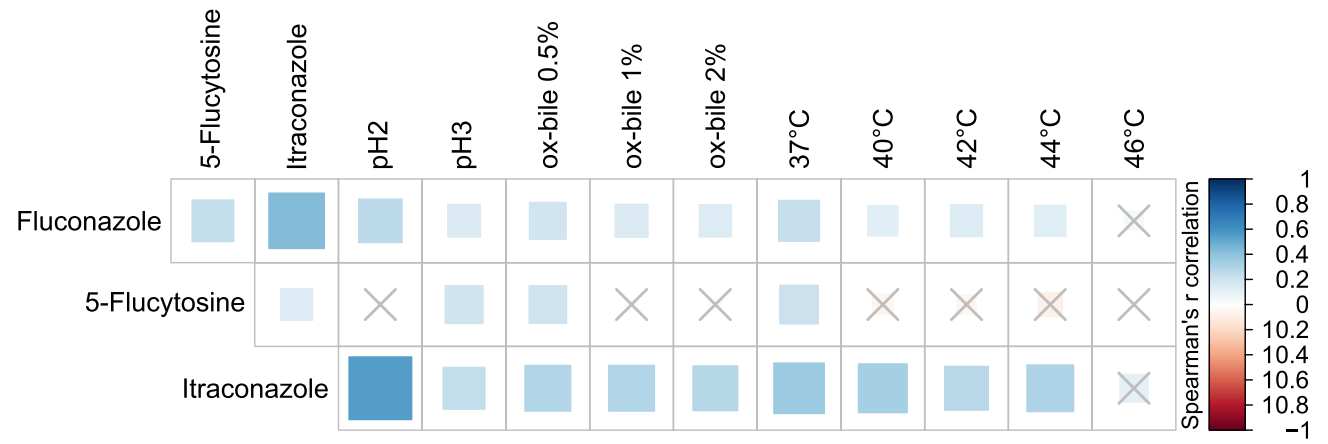

FIGURE 6 | Spearman's *r* correlation analysis between antifungals susceptibility and growth ability of the tested fungal isolates under different stress conditions. Solid squares represent the degree of correlation among the variables taken into account. Crossed squares indicate non-significant correlations; significant results with $p < 0.05$.

that differs in function of individuals' life stage in a gender-dependent manner. We identified 34 fungal species of different ecological origins. While the majority of our fungal isolates has been previously described as inhabitants of the mammalian GI tract (Kurtzman et al., 2011; Rizzetto et al., 2014), some of the isolates belong to species so far identified in environmental samples only. Environmental fungi, in particular putative food-borne fungi, have been previously observed to be able to survive the transition through the GI tract possibly being metabolically active in the gut (David et al., 2014). The phenotypic properties of fungi isolated in this study suggested that these isolates are able to survive in the human GI tract, prompting the hypothesis of an ecological selection and potential ability to colonize this niche (David et al., 2014). Indeed the phenotypic features of the fungal isolates identified endow such isolates with an excellent ecological fitness in the human GI tract. We observed that approximately half of the isolates form hyphae or pseudohyphae, which are known to be involved in the adhesion to or penetration within the GI mucosa (Staab et al., 2013), consolidation of the colony, nutrient intake and formation of 3-dimensional matrices (Brand, 2011). A key factor of *C. albicans* commensalism/pathogenicity is its ability to switch between different morphologies, comprising cellular, pseudohyphae, and hyphae forms. As reported for *C. albicans,* the reversible transition to filamentous growth as a response to environmental cues (Sudbery, 2011) and phenotypic switching is essential for mucosal fungal colonization (Vautier et al., 2015).

Previous studies have also shown that *C. albicans* over-expresses a wide range of genes involved in resistance to high temperature and pH, oxidative stress, and hyphae formation during ileum and colon commensal colonization of BALB/c mice (Pierce et al., 2013). Similarly, the fungal isolates of this study, showing resistance to oxidative, high temperature, bile acids, and pH stresses may hold the potential to colonize the human gut. It is plausible that fecal fungal isolates with specific characteristics (such as high resistance to acidic pH and bile salts) survived to the gut environment, and that these traits make them able to colonize the gut. Thus, we can hypothesize a long process of evolution, selection or adaptation of environmental and food-borne strains to the human host, suggesting that pathogenic strains of commensal species can evolve through a repeated process of evolution and selection, depending on the immune status of the host (De Filippo et al., 2014). These findings encourage for in-depth, strain-level extensive studies on human gut mycobiota and the integration of such data with immunology to further establish the relevance of fungi in host physiology and host-microbe interaction. Furthermore, fungi may train host's immune system simply when passengers, rather than necessarily persisting only as continuous colonizers (Rizzetto et al., 2016).

We discovered that several fungal isolates displayed different levels of antifungal resistance. About 20 years ago, azole-sensitive *C. albicans* dominated infections, with other *Candida* species rarely observed. Actually *C. glabrata* is the second most-commonly isolated *Candida* species in the European Union and United States and has high rates of antifungal resistance (Slavin et al., 2015). Inappropriate antifungal use has contributed to the increase in antifungal resistance, causing objective complications for the treatment of invasive fungal infections that nowadays represent a severe cause of morbidity and mortality among immunocompromised individuals, neonates and elderly (Brown et al., 2012). Recent studies indicated that fungal infections may originate from individual's own commensal strains suggesting that the ability of a commensal microorganism to promote disease is not merely a consequence of impaired host immunity (Odds et al., 2006), suggesting that rural and other commercial uses of azole could be the culprit for the emergence of these resistant strains (Snelders et al., 2012). This underlines the risk that the increase of antifungal usage outside of the clinic could also lead to increased resistance to antifungals of individual's own commensal strains representing an important epidemiological problem in the future and remarking the importance to increase the investment in antifungal research.

TABLE 3 | Antifungal activity against the most abundant fungal species.

#Species (Number of tested)	Antifungal	MIC (μg/ml)		*CBPs		
		MIC_{50}	MIC_{90}	%S	%SDD	%R
Candida albicans (123)	Fluconazole	0.5	>64	65.6	0.8	33.4
	Itraconazole	2	>8	29.3	5.7	65
	5-Flucytosine	0.125	0.5	98.4	0.8	0.8
Candida lusitaniae (6)	Fluconazole	0.125	0.5	100	0	0
	Itraconazole	0.0156	0.125	100	0	0
	5-Flucytosine	0.125	0.125	100	0	0
Candida parapsilosis (40)	Fluconazole	0.5	2	92.5	0	7.5
	Itraconazole	0.031	>8	75	5	20
	5-Flucytosine	0.125	0.125	100	0	0
Penicillium brevicompactum (13)*	Fluconazole	0.125	0.125	100	0	0
	Itraconazole	0.0156	0.0156	92.5	0	7.5
	5-Flucytosine	0.125	0.125	100	0	0
Pichia fermentans (15)*	Fluconazole	32	>64	15.4	0	84.6
	Itraconazole	0.25	4	44.7	20	33.3
	5-Flucytosine	0.5	2	92.3	7.7	0
Pichia kluyveri (9)*	Fluconazole	32	32	11.1	0	88.9
	Itraconazole	0.125	0.125	88.9	11.1	0
	5-Flucytosine	0.5	0.5	100	0	0
Pichia manshurica (9)*	Fluconazole	0.25	>64	77.8	0	22.2
	Itraconazole	0.0156	>8	77.8	0	22.2
	5-Flucytosine	0.125	8	77.8	11.1	11.1
Rhodotorula mucilaginosa (41)*	Fluconazole	0.5	>64	63.4	0	36.6
	Itraconazole	0.0156	2	75.6	2.4	22
	5-Flucytosine	0.125	0.125	100	0	0
Torulaspora delbrueckii (23)*	Fluconazole	0.125	8	87	0	13
	Itraconazole	0.0156	2	69.6	4.3	26.1
	5-Flucytosine	0.125	0.125	100	0	0

**species-specific CBPs are available only for Candida and Aspergillus spp.; for those non-Candida and non-Asperigillus isolates Candida and Asperigillus' CBPs have been used as a proxy; #MIC_{50}, MIC_{90}, and CBPs have been calculated only for those species with number of isolates >5; S, sensible; SDD, Sensibility Dose-Dependent or Intermediate; R, resistant. MIC ranges: Fluconazole 0.125–64 μg/ml; Itraconazole 0.0156–8 μg/ml; 5-Flucytosine 0.125–64 μg/ml.*

It should be noted that all the samples analyzed by metagenomics resulted in high quality fungal sequences, indicating that all the fecal samples studied had fungal DNA. So far, the estimated ratio fungi/bacteria of 1:10000 (Huffnagle and Noverr, 2013), discourages an approach based on whole metagenome shotgun sequencing (Underhill and Iliev, 2014). We thus performed amplicon-based ITS1 metagenomics on a subset of healthy donors identifying more than 90 different fungal taxa. The first striking evidence was that metagenomics detected also sequences belonging to Agaricomycetes, among which several edible fungi, thus suggesting that dietary fungal intake is a potential confounding effect when studying the gut mycobiota. On the contrary 34 different fungal species were isolated using the culture-based approach. Both methods detected in any case differences in the diverse groups of study (**Supplementary Figure S1**). The discrepancies observed between culture-dependent and culture-independent approaches on the description of fungal populations could be attributed to the methodological differences of the two procedures applied suggesting that several of the fungal taxa identified by the metagenomics approach are not cultivable, either because we lack the proper culture conditions or because these belong to DNA from dead cells, environmental or food-borne fungi that cannot survive the passage through the GI tract, but whose DNA is still detectable. Furthermore, the DNA extraction method used in this study could not be suited to extract all the fungal DNA from the stool samples since the rare taxa *Yarrowia*, *Starmerella*, *Rhodosporidium*, and *Pleurostomophora* have been found only by the culture-based approach. On the other hand the culture condition that we used might be responsible for some of the discrepancies observed between the two methods. In our experience most of the commensal fungi commonly found in the human gut can be cultivated in YPD, yet other fungi that we were not able to cultivate might need different culture conditions from those we used in this work.

Although, for example, *S. cerevisiae* is often found in fermented food, it has been shown that it can survive GI tract challenges being a commensal of the human GI tract (Rizzetto

et al., 2014) educating also adaptive immunity (Rizzetto et al., 2016). *S. cerevisiae* has been introduced in the human intestine through diet and fermented beverages and it has accompanied human evolution for at least the past 5150 years (Cavalieri et al., 2003). Our evidence, together with previous results, including a recent description of *S. cerevisiae* in IBDs (Sokol et al., 2016) showed that this microorganism is a potential commensal of the human intestine. The overall reduction of the amount and diversity of fungi introduced through consumption of fermented beverages suggests that the human gut mycobiota could be in dynamic change and certain potentially beneficial species could be lost as a result of modern food processing procedures, cultural changes, and food globalization. Ongoing studies on microbial anthropology in human populations consuming traditional fermented foods, hold the promise to shed light on the evolution of the fungal microbiota as associated to the evolution of diet. On the contrary the edible fungi belonging to Agaricomycetes cannot settle in the human gut due to their ecology (Hibbett, 2006) so we filtered-out these sequences for downstream analyses to reduce statistical noises on ecological measures, improving our results on the characterization of intestinal fungal communities. We are aware that other taxa identified by our analyses having environmental and food-borne origin may not be able to settle in the human gut, however little is known about these taxa while the Agaricomycetes sequences that we retrieved had a very low prevalence in the dataset and mostly belonged to edible fungi such as *Boletus, Suillus or Agrocybe*.

We further observed that amplicon-based ITS1 metagenomics cannot confidently describe fungal populations at a deeper level than genus overlooking species level information provided by the fungal cultivation approach (see **Figures 1B**, **2A**). On the other hand, metagenomics analysis detected community structure differences that fungal cultivation did not identified (see **Figures 2B–D**). Nevertheless, the analysis of *alpha*-diversity from cultivation data on the subset of subjects used for the metagenomics analysis revealed no significant differences among genders remarking that the different sample sizes used in this work are an additional factor in the discrepancies observed between the two methods. Although the major limitation of culture-based methods for the study of microbial communities is the loss of ecological information due to the inability to cultivate most microorganisms by standard culturing techniques, fungi included, culture-based analysis of the human gut mycobiota is fundamental to discern fungal phenotypes that would be otherwise lost by metagenomics.

However, population level analyses with both approaches revealed interesting cues. As occurs for the bacterial microbiota, the intestinal mycobiota is shaped by host's age, gender, diet, and geographical environment (Yatsunenko et al., 2012; Hoffmann et al., 2013; David et al., 2014). Previous studies have shown that the development of the gut bacterial microbiota starts at birth with colonization by a low number of species from the vaginal and fecal microbiota of the mother and is characterized by many shifts in composition during infancy (Yatsunenko et al., 2012). Similarly, the mycobiota may show the same fate, but we observed an inverted trend in which the richness of the gut mycobiota of infants (0–2 years old) and children (3–10 years old) was higher than adults (≥18 years old). It has been shown that suppression of the bacterial microbiota upon treatment with antibiotics results in the outgrowth of the gut mycobiota (Dollive et al., 2013) probably as a consequence of reduced ecological competition. Similarly, a weak bacterial competition, in particular during infancy when the bacterial microbiota is less stable (Koenig et al., 2011; Lozupone et al., 2012), could be the reason why we observed an increased fungal *alpha*-diversity during the early stages of life or this could be due to the different interactions between intestinal fungi and diet (Hoffmann et al., 2013; David et al., 2014) which is peculiar during infancy. We also found that female subjects had a higher number of fungal isolates and different fungal species compared to male subjects and that female mycobiota cluster apart from male mycobiota. This may be ascribed to the role of sex hormones in modulating microbiota composition (Markle et al., 2013) and of diet in shifting the microbiota composition in a gender-dependent manner (Bolnick et al., 2014). Furthermore, the higher relative abundance of *Candida* in the fecal samples from female than male subjects could be also attributed to the prevalence of *Candida* species in the vaginal mycobiota (Drell et al., 2013) due to the anatomical proximity of the two districts. To the best of our knowledge, this is the first time that gender-related differences are described in the human gut mycobiota.

In conclusion we can state that culture-independent approaches are very promising for future investigation of the mycobiota, but yet require significant improvements in the selection of markers for amplicon-based metagenomics and the reference databases. Additionally development of markers targeting pathogenicity traits, including the genes involved in host invasion or evasion of immune defenses, or markers detecting resistance to azoles or other antifungals, is required to thoughtfully apply metagenomics to fungal infections, discriminating the healthy mycobiota from an altered one. Such improvement can be achieved only through systematic sequencing efforts of the cultivable mycobiota, paralleling what happened for the prokaryotic microbiota. In our experience, currently, the combination of the two methods compensated the methodological limits intrinsic in both approaches avoiding to overlook significant differences present in the gut mycobiota of healthy subjects.

AVAILABILITY OF SUPPORTING DATA

Raw sequences are available in the European Nucleotide Archive (ENA) with accession number PRJEB11827 (http://www.ebi.ac.uk/ena/data/view/PRJEB11827).

AUTHOR CONTRIBUTIONS

FS designed and performed the experiments, analyzed the data, and wrote the manuscript. IS and MD performed the experiments. IS, DA, and CD supervised and contributed to data analysis. PL and AC recruited subjects and collected specimens.

IS, MD, LR, OJ, and CD critically reviewed the manuscript. DC and CDF conceived the study and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01227

Supplementary Table S1 | Correspondences between deposited metagenomics data and samples.

Supplementary Table S2 | Phenotypic characteristics and antifungals susceptibility of fungal isolates. *calculated as the deviation of the inhibition halo diameter (Ø) from that of the M28-4D *S. cerevisiae* strain, according to the following formula: (Ø sample−Ø M284D strain)/Ø M284D strain *100. #0, non-invasive; 1, poor invasive; 2, invasive; 3, very invasive. −, no growth as measured by $OD_{630} \leq 0.2$ or cfu/ml $\leq 10^5$; +, poor growth as measured by $0.2 < OD_{630} \leq 0.7$ or $10^5 <$ cfu/ml $\leq 10^6$; ++ good growth as measured by $0.7 < OD_{630} \leq 1.2$ or $10^6 <$ cfu/ml $\leq 10^7$; +++, very good growth as measured by $OD_{630} > 1.2$ or cfu/ml $> 10^7$. na, not applicable; nd, not detected.

Supplementary Table S3 | Permutational multivariate analysis of variance (PERMANOVA) tests on unweighted and weighted UniFrac distances and Bray-Curtis dissimilarity.

Supplementary Table S4 | Mean relative abundance (%) of OTUs at the genus level of fungal gut microbiota of healthy subjects from metagenomics analysis.

Supplementary Figure S1 | Mean relative abundances of the gut mycobiota in the different groups of study measured according to (A) the culture-based approach (at species level) and (B) the amplicon-based ITS1 metagenomics approach (at genus level). In panel **(B)** are shown the most abundant genera (with relative abundances >0.1%) while all the other less abundant genera were grouped together and labeled as "*others*".

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