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Analysis of gut microbiome and diet in African population transitioning to western lifestyle

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Indice

Summary

Urbanization probably represents the global change with the highest impact on human lifestyles. The rural-to-urban transition produced drastic changes in social, food and human health care dynamics. These changes also reflect a remodelling of the intestinal microbial communities, with a loss of bacterial richness and diversity and the related encoded functions. As consequence to dietary and lifestyle changes following rising of social-economic status, disease epidemiology is also radically changing with an increase in the incidence of non-communicable diseases. The bacterial fraction of the transitional microbiomes was investigated, but the shaping of intestinal fungal communities following this human transition are almost unexplored. Fungi have accompanied human evolution, being part of our diet since the advent of the Neolithic revolution and the production of fermented beverages. Ecological and evolutionary approaches and recent technological innovations in metagenomics are currently making it possible to understand and deepen the mechanisms underlying host-fungi interaction. However, since the fungal component in humans has been neglected for a long time compared to bacteria, it is crucial to increase knowledge and improve technologies to overcome the existing challenges in studying the mycobiota.
Here, are shown changes of gut mycobiota composition and lifestyle of families from Burkina Faso living in areas at different levels of urbanization (rural, semi-urbanized and urban areas), thus following a Western-related gradient. These changes were compared with that of Italian families, as representative of Western world. These variations were also studied within family members (i.e., mother, father, and children). We observed that families living in rural areas showed a greater intestinal mycobiota complexity, in terms of diversity and composition, compared to the urbanized families of Burkina Faso and Italy. We also found that the mycobionts differed within family members only in rural and in semi-urbanized areas. Furthermore, a differential abundance gradient of the 33 detected fungal ASVs was associated to rural- to-urban transition. We identified 12 fungal species affected by urbanization gradient. These species are known for their immunogenic activity and overall, the impact on human health. These results shed light on a not yet well-explored transitional mycobiota demonstrating that the level of urbanization plays a crucial role in the diversity, composition, and assembly of intestinal fungal communities, producing effects also within family members, and suggesting a legitimate role of fungal community in the hypothesis of hygiene and implication on human health.

L'urbanizzazione rappresenta probabilmente il cambiamento globale con il maggiore impatto sullo stile di vita umano. La transizione dalle campagne alle città ha prodotto cambiamenti drastici nelle dinamiche sociali, alimentari e di assistenza sanitaria. Questi cambiamenti riflettono a loro volta un rimodellamento delle comunità microbiche intestinali, che si traducono in una perdita di ricchezza e diversità batterica e delle relative funzioni codificate. Come conseguenza dei cambiamenti nella dieta e nello stile di vita, a seguito del miglioramento dello status socioeconomico, anche l'epidemiologia delle malattie sta cambiando radicalmente con un aumento dell'incidenza delle malattie non trasmissibili. La frazione batterica dei microbiomi di popolazioni umane in transizione è stata studiata; tuttavia, la composizione delle comunità fungine intestinali resta quasi inesplorata. I funghi hanno accompagnato l'evoluzione umana, facendo parte della nostra dieta fin dall'avvento

della rivoluzione neolitica e della produzione di bevande fermentate. Gli approcci ecologici ed evolutivi e le recenti innovazioni tecnologiche nel campo della metagenomica stanno attualmente consentendo di comprendere e approfondire i meccanismi alla base dell'interazione ospite-funghi. Tuttavia, poiché la componente fungina nell'uomo è stata e resta tutt'ora trascurata rispetto alla controparte batterica, risulta cruciale uno sforzo finalizzato all'aumento delle conoscenze e al miglioramento delle tecnologie per superare le sfide esistenti nello studio del micobiota.
Qui vengono riportati i cambiamenti della composizione del micobiota intestinale e dello stile di vita di famiglie del Burkina Faso che vivono in aree soggette a diversi livelli di urbanizzazione (aree rurali, semi-urbanizzate e urbane), seguendo così un gradiente legato all'Occidente. Questi cambiamenti sono stati confrontati con quelli delle famiglie italiane, rappresentative del mondo occidentale. Queste variazioni sono state studiate anche all'interno dei membri della famiglia (madre, padre e figli). Abbiamo osservato che le famiglie che vivono in aree rurali mostrano una maggiore complessità del micobiota intestinale, in termini di diversità e composizione, rispetto alle famiglie urbanizzate del Burkina Faso e dell'Italia. Abbiamo anche riscontrato che i micobionti differiscono all'interno dei membri della famiglia solo nelle aree rurali e semi-urbanizzate. Inoltre, un gradiente di abbondanza differenziale dei 33 ASV fungini rilevati è stato associato alla transizione rurale-urbana. Abbiamo identificato 12 specie fungine interessate dal gradiente di urbanizzazione. Queste specie sono note per la loro attività immunogena e per l'impatto complessivo sulla salute umana. Questi risultati fanno luce su un micobiota di transizione non ancora ben esplorato, dimostrando che il livello di urbanizzazione gioca un ruolo cruciale nella diversità, nella composizione e nell'assemblaggio delle comunità fungine intestinali, producendo effetti anche all'interno dei membri della famiglia e suggerendo un ruolo legittimo della comunità fungina nell'ipotesi dell'igiene e delle implicazioni sulla salute umana.

Introduction

The human gut microbiome represents a complex ecosystem in which different types of microorganisms such as bacteria, fungi, archaea, and viruses coexist, influencing host metabolism, cognitive activities and overall, the human well-being (Gilbert et al., 2018; Kho et al., 2018).
Although being part of one of the largest branches of the Tree of Life, Fungi are often neglected because of their low abundance compared to bacteria, and due to the methodological challenges associated to their detection as extensively reported in Chapter 1. Speaking about genomes, compared to bacteria, fungi contain complex genetic features such as multiple chromosomes, expanded repeated regions and larger genome size, all of which introduce inaccuracies during sequence classification. It has been predicted that 2.2-3.8 million fungal species inhabit planet earth and according to published estimates only a small percentage (approximately 5–10% depending on the habitat) of the total fungal diversity is known and catalogued (Hawksworth and Lücking, 2017); most likely, the same is true for yeasts. Based on the 2000 yeast species recorded thus far, approximately 150,000 fungal species have been listed (Lücking et al. 2021). The label "yeast" refers to any fungus that reproduces asexually through budding or fission, developing single-cell stages, and does not have sexual structures enclosed in a fruiting body (Kurtzman & Sugiyama, 2015). This wide definition includes dimorphic lineages that generate mycelial growth in their sexual phases, as well as biotrophic pathogens and black yeasts. As a result, they do not constitute a monophyletic but rather represent a fungal lifestyle that is shared by several distinct lineages and implies a spectrum of phenotypic traits; however, several exceptions and comments to the imprecise border between yeasts and the dimorphic filamentous fungi that form yeast-like stages as well as yeast lineages that display strictly filamentous growth are reported. Yeasts are classified in the phylum Ascomycota, primarily in the subphyla *Saccharomycotina* (budding yeasts) and *Taphrinomycotina* (which also includes fission yeasts), as well as three subphyla of Basidiomycota, namely *Ustilaginomycotina*, *Pucciniomycotina*, and *Agaricomycotina* (Li et al., 2021).

As shown in Chapter 2, since fungi are averagely larger than bacteria, they expose a bigger surface, approximately 100 times greater than bacterial cells, thus their importance in engaging with the host immune system, if not equal, should not be underscored (Naglik et al., 2017; Patin et al., 2019). This evidence, together with insights on deleterious effects of fungi in dysbiosis (Iliev & Leonardi, 2017) and in diseases onset (Köhler et al., 2017), as well as beneficial role towards host immunity (Rizzetto et al., 2010; Rizzetto et al., 2016; Jiang et al., 2017), suggest that fungi play a fundamental role in shaping the human holobiont (Lai et al., 2019). Yeasts resident in the intestinal tract constantly interact with the immune system, however they do not necessarily represent a threat for the host inducing an inflammatory response. Several studies have better defined the role of yeasts in host-microbe interaction (Hall & Noverr, 2017; Di Paola et al., 2020). The exposure of non-pathogenic yeasts such as *S. cerevisiae* and *C. albicans* induces the activation of human monocyte cells causing a Th17-mediated differential response (Rizzetto et al., 2010; Rizzetto et al., 2014). The role of yeasts in human immune system homeostasis implicates a level of complexity that goes beyond the commensal interaction, and that the relationship between commensal yeasts and their human host has crossed several coevolution steps. Recently, it has been suggested that trained

immunity is a process that gives an evolutionary benefit to the recipient. It is described as a long-term functional change of innate immune cells that results in a stronger reaction to a subsequent independent immune assault (Netea et al., 2020). *S. cerevisiae* has been shown to elicit trained immunity in human monocytes in a strain-dependent way, resulting in higher release of TNF and IL-6 following secondary activation with TLR ligands. Furthermore, owing to the varying chitin composition of the cell wall, the strain used had a strong influence on immune system activation (Rizzetto et al., 2016). According to these studies, the role of yeasts seems crucial in the modulation of the human immune system, and it is evident that this mechanism is dependent on the strain-specific cell wall variability.

Previous research (Di Paola et al., 2020) found genetic and phenotypic variations in isolates separated from intestine and non-gut habitats (e.g., natural sources, fermentation). The role of *Saccharomyces* spp. in health and disease has been investigated, but major issues remain unexplored (Sokol et al., 2017; Liguori et al., 2016). A disease-specific gut environment, on the other hand, may favour yeast strain growth through the emergence of unusual characteristics that are likely to influence the yeast's fitness and interactions with the host, as seen in IBD conditions. Some *S. cerevisiae* strains may be introduced into the gastrointestinal tract with the food and may be capable of colonizing the host under certain circumstances, such as the existence of a leaky gut or an unresponsive host immune system. In addition, opportunistic pathogenic fungi, such as *Candida albicans*, are frequently found as commensals on human mucosal surfaces, but migrate into the host when epithelial barriers are disrupted, or the host immune system is compromised. Pathogenicity may have originated from commensalism through the acquiring of features that allowed it to colonize and ultimately invade the host. Within the genus *Candida* is collected the largest number of commensal or pathogenic species associated with human species (Romo & Kumamoto, 2020; Witherden et al., 2017; Huffnagle & Noverr, 2013; Huseyin et al., 2017; ten Oever & Netea, 2014). Representatives of this genus can exhibit several virulence factors such as adherence, biofilm formation and secretion hydrolytic enzymes that both increase their persistence within host as well as cause host cell damage (Silva et al., 2012); due to these features, the scientific community has typically conducted research from a pathogenic standpoint (Kojic & Darouiche, 2004; Spellberg et al., 2005; Thompson et al., 2010). Even so, *Candida spp.* may advantageously affect the host by modulating the development of mucosal immunity, which protects against other fungal diseases. (Atarashi et al., 2015; Ifrim et al., 2015; Markey et al., 2018).

During human evolutionary history, the process of urbanization has promoted a set of conduct aimed at reducing exposure to pathogenic organisms, increasing the use of pharmaceuticals as well as sanitation measures, and establishing food sterilization workflows. As a result, in addition to the known benefits, this phenomenon has had negative impacts on the human gut microbial ecology, neutralizing possibly beneficial microorganisms and their associated activities. (Zuo et al., 2018). This microbial depletion on a broad time scale has been illustrated by David Strachan's hygiene hypothesis (Strachan, 1989), which has been conceptually enriched by Blaser and Falkow through the incorporation of the concept of disappearing microbiota, referring to the loss of specific ancestral microorganisms within the human core microbiota due to Western-related habits (Blaser

& Falkow, 2009). In recent times, the depletion of intestinal bacterial populations has been linked to behavioral changes brought about by the recent COVID-19 epidemic. In this instance, the hygiene hypothesis had been correlated to a deficiency in the microbiota structure and a substantially greater frequency of COVID-19-related adverse effects. (Finlay et al., 2021). In terms of socioeconomic and health care settings, non-Western populations constitute the ideal context in which researchers can investigate the effect of urbanization on the human microbiota in real time.

At present, urbanization rate in Sub-Saharan Africa is the highest globally; while the Westernization of lifestyle is already pervasive in metropolitan areas, people in rural areas are still close to a traditional lifestyle but starting to be exposed to the increasing contamination of Western-like factors. This pattern emphasizes changes in socioeconomic status as well as a shift away from subsistence farming and toward more specialized employment options. Disease prevalence is also changing dramatically. Several studies indicate that by the year 2030, the incidence of noncommunicable diseases (NCDs) including cancer, chronic renal and respiratory conditions, and dietary-related disorders may outnumber infectious diseases in Sub-Saharan Africa because of shifting trends in urban lifestyle and nutrition (Kiawi et al., 2006; Frank et al., 2014; Dake et al., 2016). Burkina Faso, a West African country, is still in the initial phases of its demographic transition. The main urban area consists of Ouagadougou's capital city and its outlying area; here, typical urban lifestyle changes have taken place already (Bosu, 2015). On the flip side of the spectrum, Burkina Faso's rural areas continue to maintain a traditional rural lifestyle characterized by Neolithic-like subsistence farming and with minimal interaction with occidental societies.

Globalization has resulted in major shifts in human lifestyle, and in nutritional terms these changes are recapped by the "globesity" concept, due to radical diet disruption and the negative impacts on human health (Costa-Font & Mas, 2016). Conversely, the traditional Burkinabe diet has been related to beneficial impacts on local communities, such as an upward trend in the amounts of short-chain fatty acids (SCFAs) resulting from the gut bacterial community activities (De Filippo et al., 2010). The impacts of urbanization on gut microbiome makeup have also been emphasized in two different rural communities of Papua New Guinea (Martinez et al., 2015) and in rural and urban South African individuals. (Kabwe et al., 2020). However, as discussed in Chapter 4, diet is only one of the variables that globalization affects; this phenomenon is altering local and global dynamics, resulting in macro- and micro-urban changes that impose a different degree of urbanization. As a result, research on the gut microbiome's adaptation to urbanization has linked contemporary habits to bacterial diversity loss and a proclivity to develop typical Western conditions such as obesity and autoimmune conditions. (Zuo et al., 2018; Blaser, 2017; Vangay et al., 2018).

Even though fungi and yeasts have been observed in human faeces specimens dating back as early as 1917 (Anderson, 1917), and that yeasts in the human intestine have been thought playing a saprotrophic role since the mid-20th century (Gumbo et al., 1999), most microbiota research has primarily concentrated on bacterial communities. In the human gut, they are estimated to make up between 0,01% and 0.1% of the total microorganisms (Qin et al., 2010; Auchtung et al., 2018), yet this assessment might underestimate their actual richness. Multiple technical challenges have restricted the implementation of metagenomic approaches to the study of the fungal component

of the microbiome, including (i) sample storage and preparation, (ii) fungal genomic DNA extraction, (iii) a lack of standardized wet lab protocols, (iv) a scarcity of properly annotated yeast and fungal reference genomes in public databases, and (v) a lack of fungi-specific bioinformatic pipelines. A decade of metagenomic-based studies has provided a survey of the fungal phyla within the human mycobiota, and all current research regarding the mycobiota composition acknowledge that the most represented yeasts belong to the genera Candida and Saccharomyces, both of which belong to the Ascomycota phylum. (Underhill & Iliev, 2014; Iliev & Leonardi, 2017; Limon et al., 2017; Runge & Rosshart, 2021; Begum et al., 2022; Belvoncikova et al., 2022). Within the genus *Saccharomyces* is featured an assortment of wild and domesticated yeast species, the latter of which are widely recognized to be associated to human activities and to industrial applications, such as food and beverage fermentation (Sicard & Legras, 2011). Specifically, the widespread presence of *S. cerevisiae* in the human GI tract would not be unexpected since it has been purposely ingested by humans worldwide for thousands of years via bread, beer, and other traditional fermented foods and beverages (McGovern et al., 2004; Cavalieri et al., 2003), whose consumption influences its abundance (Sun et al., 2021). Because of the increased interest in the subject, it is becoming clear how fungal communities are tightly adapted to the human gut (Strati et al., 2016b), the way they have been influenced by environmental variables, and how their disequilibrium has severe repercussions for human health. (Strati et al., 2016a; Strati et al., 2017).

Thus, to date, the investigation of the influence of urbanization on bacterial microbiome has significantly increased, however, the knowledge related to the mycobiota remains virtually untapped. At present, few research efforts have been centered on the identification and description of the eukaryotic components of African individuals' GI (Hamad et al., 2019; Kabwe et al., 2020; Nel Van Zyl et al., 2022). In chapter 3, to investigate the role of urbanization on the fungal communities of the human gut, mycobiomes within family members, stratified for age and gender, from Italy and Burkina Faso areas have been compared; it is in fact critical to assess the impact of environmental factors on the gut mycobiome across a larger sample of participants to determine the implications on host-microbiota dynamics and health. Only a few studies have already described the microbiota and dietary habits of rural and urban populations (Becquey et al., 2010; De Filippo et al., 2017; Sié et al., 2018; Casari et al., 2022), but an in-depth assessment of the gut mycobiota of the whole family or households, as focal points of a shared lifestyle and diet, living in environments with different stages of urbanization and from different geographical areas was still lacking and it is therefore one of the most innovative aspects of this thesis.

The aim of the present work is to understand how the effect of urbanization, expressed by the transition of human populations from a rural to an urban environment, influence the structure of the intestinal mycobiota. For this purpose, the dataset of the previous surveys on the individuals of Burkina Faso has been expanded to a wider cohort, collecting faecal samples within the family members living in three different environments, representing three phases of the demographic transition process from rural to urban environment, and comparing them with an Italian cohort of healthy families as Western controls.

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Chapter 1

The hidden part of the microbiome: Ascomycetes Yeasts transition from passengers, colonisers and invaders in humans

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Abstract

The human microbiota is increasingly considered as a key player in health and disease. The fungal component of the microbiota, the mycobiota, is often neglected because of its low abundance compared to bacteria, and due to the limitations in its detection at different taxonomic levels by using metagenomic approaches borrowed from bacteriome analyses. Here, we review recent data on identification of yeasts of the Ascomycota phylum in the human body districts, and their role in the regulation of the host immune balance in health and disease. Additionally, we show how yeasts, more than bacteria, provide a fingerprint of the environmental microbiota transient into the human gut. Finally, we show how their status as passengers or colonisers is related with the integrity of the gut barrier related to the risk of multiple disorders. Thus, the study of this less known component of the microbiota in different districts of the human body could unravel the rules of the transition from passengers to colonisers and invaders, as well as their dependence on the innate component of the host's immune response.

1. INTRODUCTION

Human microbiota is a complex ecosystem consisting of microorganisms from all kingdoms of life, namely bacteria, archaea, unicellular eukaryotes, including yeasts and protozoa, multicellular eukaryotes, such as fungi and helminths, and viruses (Norman et al., 2014; Virgin, 2014). Despite fungi and yeasts have been detected in human stool samples as far back as 1917, and since the mid-20th century the presence of yeasts in the human intestine was proposed to have a saprotrophic role, many microbiota studies have focused on bacterial communities (Gumbo et al., 1999; Gorbach et al., 1967). To date, the fungal component of the microbiota, the mycobiota, has gained recognition as a fundamental part of the microbial communities inhabiting several human body districts. In the past decade, thanks to advances in Next Generation Sequencing (NGS) techniques, and especially through amplicon-based approaches targeting the ribosomal DNA and the internal transcribed spacer (ITS) region, the primary genomic biomarker of fungi, as well as the shotgun sequencing (Shaffer et al., 2022), our understanding of the prevalence and diversity of the fungal communities associated with ecological niches in human bodies has been expanded (Clemente et al., 2012; Erturk-Hasdemir & Kasper, 2013; Tremaroli & Bäckhed, 2012; HMPC, 2012). The mycobiota (Ghannoum et al., 2010; Iliev et al., 2012) has been neglected for a long time mostly because of its low abundance compared to bacteria. In the human gut, they are estimated to make up to approximately 0.1% of the total microorganisms (Qin et al., 2010; Auchtung et al., 2018), yet this assessment might underestimate their actual richness. Several technical challenges have hampered the application of metagenomic approaches to the investigation of the mycobiome, mainly (i) sample preparation, (ii) fungal genomic DNA extraction (iii) paucity of properly annotated yeast and fungal reference genomes in public databases. Moreover, since fungi are averagely larger than bacteria, they expose a bigger surface, approximately 100 times greater than bacterial cells, thus their importance in the interaction with the host immune system, if not equal, should not be underscored (Naglik et al., 2017; Patin et al., 2019). This evidence, together with insights on deleterious effects of fungi in dysbiosis (Iliev & Leonardi, 2017) and in diseases onset (Köhler et al., 2017), as well as beneficial role towards host immunity (Rizzetto et al., 2010; Rizzetto et al., 2016;

Jiang et al., 2017), suggest that fungi play a fundamental role in shaping the human holobiont (Lai et al., 2019).

Almost 50 years of culture-based and a decade of metagenomic-based studies have outlined an overview of the fungal phyla within the human mycobiota, and all the recent reviews concerning the mycobiota composition in accordance with the fact that the most represented yeasts are *Candida* and *Saccharomyces* genera belonging to *Ascomycota* phylum (Underhill & Iliev, 2014; Iliev & Leonardi, 2017; Limon et al., 2017; Runge & Rosshart, 2021; Begum et al., 2022; Belvoncikova et al., 2022). This review is a survey of the literature on mycobiota composition and functions, and on Ascomycetes yeasts as predominant components of the human fungal community, focusing on diverse perspectives: (i) characterization and role of *Ascomycota* yeasts in human health and disease, (ii) the multiple interactions between fungi and host immune system, and (iii) the possible evolutionary mechanisms subtending the fungi-host relationships. For this overview, we searched into PubMed database using keywords such as human mycobiota, *Saccharomyces*, *Candida*, gut, oral, skin, genitourinary, respiratory, lung, health, disease, host immune system, dysbiosis, evolution, composition, colonisation, metagenomic analysis, next generation sequencing. No exclusion criteria on the date and type of article were applied, but up-to-date studies were preferred.

2. THE HUMAN MYCOBIOTA: RICHNESS AND DIVERSITY IN THE HUMAN BODY SITES DEPEND ON GENDER AND AGE.

Here we reviewed the current knowledge about the *Ascomycota* yeasts residents in the most characterised human body districts, such as the oral tract, lungs, gut, genitourinary tract, and skin (Figure 1). While the bacterial microbiota is relatively stable over time, richness, and diversity of fungal communities in the human host are lower in absolute terms (Nash et al., 2017). Evidence suggests that the mycobiota is highly variable not only between individuals but also within the same person during the lifespan (Scanlan & Marchesi, 2008; Findley et al., 2013; Strati et al., 2016, Hallen-Adams & Suhr, 2017). One of the first studies on characterization of mycobiota in the healthy human gut showed also marked differences in richness and diversity according to gender in addition to age, with younger individuals and females having a higher fungal richness compared to adults and male subjects, respectively (Strati et al., 2016). These gender differences could be reflected by the observation that different body sites Different organs are populated by different patterns of fungal species, and overall, the species diversity and fungal abundance differs significantly among them. For instance, *Basidiomycota* (prevalently species belonging to the genus *Malassezia*) colonise preferentially the skin surfaces of both males and females, while the female reproductive tract and gut are mostly inhabited by *Ascomycota*, especially species belonging to the genus *Candida*. Additionally, several studies showed the presence of fungal communities in breast milk suggesting a specific mother-offspring transmission of the mycobiome (Boix-Amorós et al., 2019; Saxena et al., 2018; Shivaji et al., 2022). Thus, we can say that studies on gender medicine and gender microbiomes should pay a special attention to the fungal component of the microbiome.

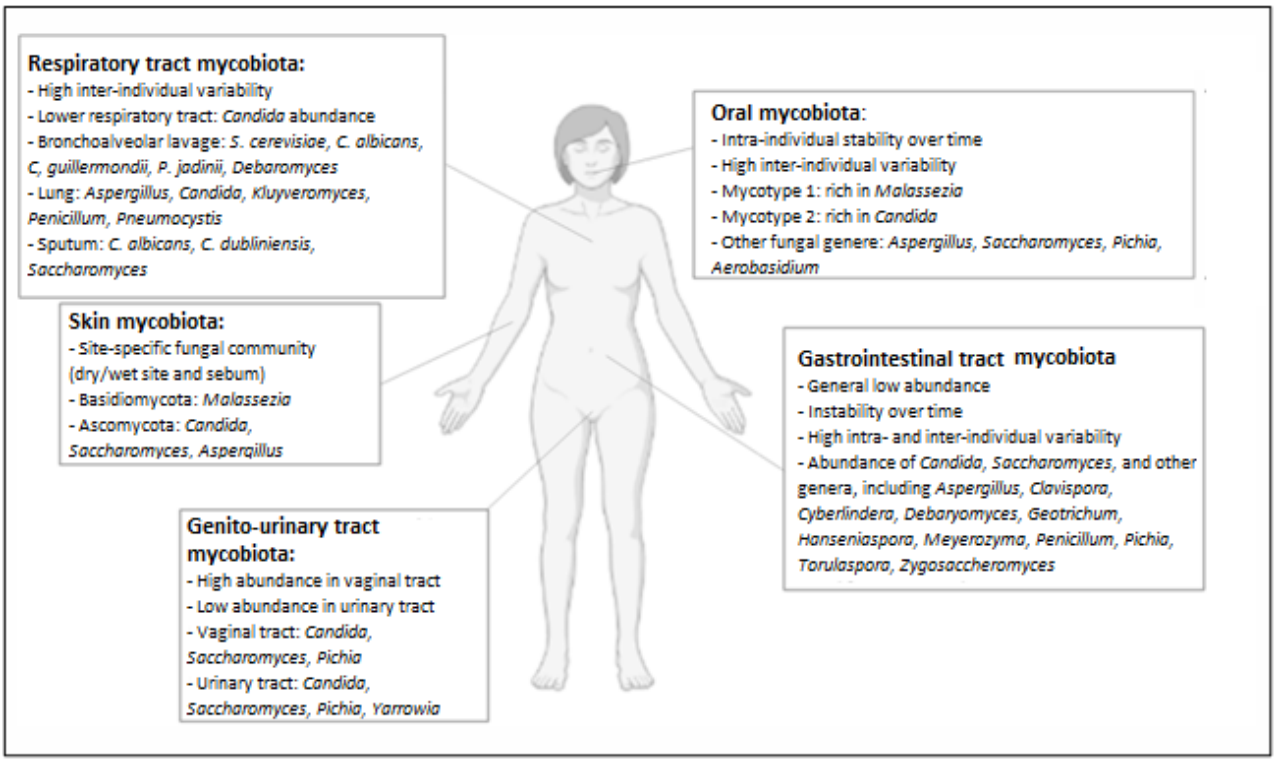

FIGURE 1. Overview of mycobiota characterization in the major studied human body sites. The schematic representation describes the inter- and intra-individual fungal variability in health condition, highlighting the Ascomycetes fungal populations identified in the different anatomical districts.

2.1 Oral tract

The mouth is one of the first human body sites where presence of Ascomycetes yeasts has been described, more than 60 years ago (Krasner et al., 1956). Since fungi are difficult to grow in ordinary laboratory culture media, uncultivable species have eluded detection and the predominant species belonging to the genus *Candida* have stolen the spotlight for decades (Young et al., 1951; Diaz et al., 2017), until the implementation of metagenomics through NGS techniques that showed the presence of a manifold oral fungal community (Bandara et al., 2019; Dupuy et al., 2014; Ghannoum et al., 2010). Other studies have shown that, while bacterial diversity in the oral cavity is one of the highest in the human body sites, the mycobiota of a single individual is relatively stable over time, but with higher inter-individual variations. This result suggests a common and a variable group of fungi in the oral cavity (Diaz & Dongari-Bagtzoglou, 2021; Monteiro-da-Silva et al., 2014). To date, oral mycobiota studies suggested that *Candida* is the most represented fungal genus within *Ascomycota* phylum (Li et al., 2019; Diaz et al., 2017; Charlson et al., 2012), but other Ascomycetes yeasts genera, namely *Aureobasidium*, *Saccharomyces* and *Pichia*, were described as part of oral mycobiota (Ghannoum et al., 2010; Auchtung et al., 2018; Monteiro da Silva et al., 2014; Khadija et al., 2021; Stehlikova et al., 2019). Two different studies on saliva and oral mucosa samples suggested the existence of two possible community types (called mycotypes) based on abundance of *Candida,* on one hand, and *Malassezia* species, on other hand (Abusleme et al., 2018; Hong et al., 2020). Moreover, each mycotype seems to be associated with specific clinical and bacteriome profiles. Besides these two genera, metagenomic studies suggest that the rest of diversity is almost certainly acquired from the environment, mostly from ingested food (Diaz & Dongari-Bagtzoglou,

2021). The first evidence of a pathogenic oral mycobiota comes from studies on HIV positive (HIV^+) patients. These patients can frequently develop a pharyngeal candidiasis, where the overgrowth of *Candida albicans* leads to an opportunistic infection (Klein et al., 1984). It is well known that, under certain conditions, *C. albicans* can shift from commensal to pathogenic status. In the oral tract, the balance of this transition is regulated not only by the host immune system (Cassone & Cauda, 2012), but also by the fungal-fungal interaction. Specifically, in oral mycobiota of HIV^+ patients an inverse correlation was observed between the pathogenicity of *C. albicans* and the abundance of *Pichia* species. The inhibitory effects of *Pichia* on *Candida* could be due to a secretion of a mycotoxin (Mukherjee et al., 2014). Numerous other studies have suggested a role of the genus *Candida* in oral dysbiosis and in disease. Increased abundance of *C. albicans* and *C. dubliniensis* species correlated with dental caries in early childhood (Ghasempour et al., 2011; Kneist et al., 2015). Increase abundance of *C. glabrata* resulted in tongue infections and the interactions between *C. glabrata* and hyphae of *C. albicans* established oropharyngeal candidiasis, as well as abundance of *C. parapsilosis*, *C. tropicalis* and *C. crusei* (Tati et al., 2016). Significantly higher abundances of the genus *Candida* were observed in patients with erosive oral lichen planus (Li et al., 2019). In a recent study alteration of the oral mycobiota caused by smoking tobacco showed a decreased oral fungal diversity and an increase of *Pichia* genus correlated with the severity of oral lesions (Sajid et al., 2022).

2.2 Respiratory tract

Despite the respiratory tract being constantly exposed to environmental and airborne fungi and being inevitably in contact with oral mycobiota (van Woerden et al., 2013; Nguyen et al., 2015), lungs in healthy individuals have been considered sterile until less than 30 years ago (Cabello et al., 1997). Bacterial colonisation was thought to occur only during disease (Marsland & Gollwitzer, 2014). Before culture-independent approaches, fungal communities have been studied only in lower respiratory tract (LRT) diseases, revealing the presence of multiple *Candida* species (BAUM, 1960; Corley et al., 1997; el-Ebiary et al., 1997; Meersseman et al., 2009). During the NGS era, a few studies investigated the mycobiota of respiratory tract in health status. However, these studies reached a consensus on three main points: (i) in health condition, fungi are present in the human respiratory tract; (ii) in the respiratory tract fungi are highly variable between individuals; (iii) many diseases affecting lungs are associated to decreased fungal diversity (Tipton et al., 2017; Nguyen et al., 2015). To date, the published literature showed a clearer overview of predominant fungal species in the different sites of the respiratory tract, mainly belonging to *Ascomycota* and *Basidiomycota* phyla. From bronchoalveolar lavage samples, Ascomycetes yeasts, such as *Saccharomyces cerevisiae*, *Candida albicans*, *Meyerozyma guilliermondii (Candida guilliermondii)*, *Pichia jadinii* and *Debaryomyces* spp. were found (Cui et al., 2015; Charlson et al., 2012; Martinsen et al., 2021). In lungs, *Candida* spp., *Kluyveromyces* spp. and *Pneumocystis* spp. were predominant (Delhaes et al., 2012; Charlson et al., 2012). In sputum, *Candida albicans*, *Candida dubliniensis* and *Saccharomyces* spp. were also identified (Ali et al., 2019). A recent study on patients with non-inflammatory respiratory tract disorders found no substantial differences in the core lung mycobiota (mainly characterised by *Malassezia*, *Candida* and *Cryptococcus* genera) with respect to previous studies, except for a lower abundance of *Candida* spp. (including the absence of *C. albicans*)

and a higher diversity of the total oral microbiota (Rubio-Portillo et al., 2020). The observed discrepancies among studies are probably due to the type of collected biological samples.The predominance of *Candida* spp., as observed in some studies, could be explained by the frequent use of sputum as a representative sample of the LRT condition (Aliouat-Denis et al., 2014; Soret et al., 2020; Willger et al., 2014; Delhaes et al., 2012), while these types of samples would carry fungi for the upper respiratory tract, where *Candida* spp. is prevalent. Regarding the differences in total lungs microbiota diversity, it is known that inflammation is associated with reduced diversity of the microbial community compared to a non-inflammatory disease condition (Enaud et al., 2018; Huffnagle & Noverr, 2013; Richardson et al., 2019; Soret et al., 2020). An overview of the culture-independent studies (Krause et al., 2017) on mycobiota of lower respiratory tract in disease confirms that the genus *Candida* is predominant in most of the studies on cystic fibrosis, lung transplant, HIV infected, intensive care unit (ICU), bronchiectasis, asthma and immunocompromised patients (Bousbia et al., 2012; Charlson et al., 2012; Bittinger et al., 2014; Willger et al., 2014; Cui et al., 2015; Kramer et al., 2015; Krause et al., 2016; Mac et al., 2018; Cui et al., 2014; van et al., 2013; Zinter et al., 2019).

2.3 Gastrointestinal tract

The gastrointestinal tract (GIT) is the most studied human body site concerning the characterization of the microbial communities. Multiple studies across years demonstrated that fungi are normal inhabitants of the GIT (Nilsson et al., 2006; Hube, 2004; Scupham et al., 2006; Iliev et al., 2012; Dollive et al., 2013; David et al., 2014; Hoffmann et al., 2013; Underhill & Iliev, 2014). The presence of fungal communities has been detected in the gut of at least 70% of healthy adults (Raimondi et al., 2019; Schulze & Sonnenborn, 2009). However, there is not a broad consensus in defining a healthy gut mycobiota. Multiple factors, including low abundance and diversity of fungi in the gut, temporal instability of mycobiota across lifespan, and high variability both inter- and intra- individual across time affect the gut mycobiota composition (Hallen-Adams & Suhr, 2017; Nash et al., 2017). Fungi represent only up to 0,1% of the total gut microorganisms (Qin et al., 2010; Arumugam et al., 2011; Auchtung et al., 2018). The concentration of fungi along the gut seems to be relatively stable, with an average of 10^6 cells per gram (Sender et al., 2016; Huseyin et al., 2017), compared to the bacterial community, whose concentration increases from stomach to the colon (Jiang et al., 2017; Donaldson et al., 2016). Therefore, the ratio between fungi and bacteria might be higher in the upper GIT than in the colon (Richard & Sokol, 2019). Several studies showed that the biodiversity of human mycobiota is also lower and characterised by greater unevenness than the bacterial microbiota (Raimondi et al., 2019; Qin et al., 2010; Breau et al., 2022; Nash et al., 2017). Moreover, the fungal biodiversity seems to increase from the stomach to the lower GIT (Hallen-Adams & Suhr, 2017). Fungi also produce a wide range of metabolites with the potential to affect host and intercellular communication (Enaud et al., 2018). Thus, gut mycobiota could play a key role in host's homeostasis and disease as already described for the bacteriome (Sam et al., 2017; Mims et al., 2021; Leonardi et al., 2022). Historically, mycobiota studies were based on *in vitro* cultures (Finegold et al., 1974), but the development of culture-independent methods allowed the discovery of a much more diverse fungal community due to unculturable fungal species (Gouba et al., 2013; Browne et al., 2016), both in health and disease (Huseyin et al., 2017). One of the first studies that relied on

culture-independent methods to assess the composition of human gut mycobiota detected fungal species in 88% of the 17 individuals sampled (Scanlan & Marchesi, 2008). The first comprehensive culture-independent study of a large cohort of individuals through both targeted and untargeted metagenomic approaches have been conducted within the Human microbiota Project (HMP) (Nash et al., 2017). Results from 370 stool samples confirmed the lower diversity of the fungal community compared to bacteria, and the high variability both inter- and intra-volunteer. Moreover, in these samples yeast represented 8 of the 15 most abundant found genera. *Ascomycota* was the most present phylum, with a prevalence of genera *Saccharomyces* and *Candida* (found in 96.8 and 80.8% of the samples, respectively), followed by *Basidiomycota* (70% and 30% respectively). Other findings, both in mice (Dollive et al., 2013) and in humans (Underhill & Iliev, 2014), showed that the mycobiota of a single subject is no more similar to itself over time compared with that of another individual, but several fungal species persist across a majority of samples from different individuals. These clues suggest that a human core gut mycobiota may exist. The fungal species characterising the core mycobiota were already identified in previous studies through culture-dependent methods (Gouba et al., 2013; Gouba et al., 2014; Strati et al., 2016; Scanlan & Marchesi, 2008; Agirbasli et al., 2005) and results of the HMP project studies confirmed them. Ascomycetes yeasts are the predominant fungi, especially the genera *Candida* and *Saccharomyces* (Borges et al., 2018; Chen et al., 2011; Hamad et al., 2012; Motooka et al., 2017; Pandey et al., 2012; Kabwe et al., 2020; Botschuijver et al., 2017). Other ascomycetes yeasts described in human gut mycobiota were belonging to the genera *Debaryomyces*, *Meyerozyma*, *Torulaspora*, *Pichia*, *Clavispora*, *Cyberlindnera*, *Hanseniaspora*, *Geotrichum*, *Galactomyces* and *Zygosaccharomyces* (Hallen-Adams & Suhr, 2017; Mar et al., 2015; Raimondi et al., 2019). Most of the studies have focused on fungal community diversity and richness and characterization of fungal species in gut diseases, especially in Inflammatory Bowel Disease (IBD). Here we present a brief overview of gut Ascomycetes yeasts composition in several gut diseases, as described in previous reviews in a more comprehensive way (Mahmoudi et al., 2021; Gouba & Drancourt, 2015; Iliev & Leonardi, 2017; Chu et al., 2018; Chin et al., 2020; Zhang et al., 2020; Lai et al., 2019; Richard & Sokol, 2019; Huseyin et al., 2017; Mukherjee et al., 2015; Wu et al., 2021; Li et al., 2019). In general, differences within the fungal community were found when IBD patients were compared with healthy subjects, but not between Crohn's disease and ulcerative colitis (Ott et al., 2008; Sokol et al., 2017; Chen & Wang, 2022). For the first time, in 1988, antibodies against *S. cerevisiae* in Crohn's disease patients' blood were found, but not in Ulcerative Colitis (UC) patients (Main et al., 1988). Anti-Saccharomyces cerevisiae antibody (ASCA) recognises cell wall peptidomannans of this yeast (Sendid et al., 1998). Subsequent studies showed that other yeasts of the genus *Candida*, such as *C. albicans* and *C. tropicalis*, displayed interactions with ASCA (Sokol et al., 2017; Chehoud et al., 2015; Hoarau et al., 2016; Liguori et al., 2016)]. Specifically, in IBD, increased abundances of *C. albicans*, *C. glabrata* and *C. tropicalis* were observed, and the relative abundance of *C. albicans* was found differently correlated with the remission and relapse. Relative abundance in *S. cerevisiae* is still a matter of debate. Sokol and collaborators (Sokol et al., 2017) found an enrichment of *Candida* spp. and a reduction of *S. cerevisiae* in Crohn's disease (CD) flare compared to remission. In our previous study (Di Paola et al., 2020) we isolated *S. cerevisiae* and *Candida spp* from faecal samples of pediatric IBD patients. *S. cerevisiae* was more abundant in

CD patients compared to ulcerative colitis and healthy controls. Liguori and co-workers (Liguori et al., 2016) observed that *S. cerevisiae* was enriched in CD patients' non-inflamed gut mucosa. On the other hand, Chiaro and collaborators (Chiaro et al., 2017) reported that *S. cerevisiae* can exacerbate DSS-induced colitis and affects gut barrier permeability by inducing overproduction of uric acid. In obese and overweight individuals fungal gut dysbiosis was observed (Borges et al., 2018; Mar et al., 2015), as well as in irritable bowel syndrome (IBS) patients. In IBS, the principal changes were observed especially for the genera *Saccharomyces* and *Candida (Santelmann & Howard, 2005; Botschuijver et al., 2017; Hong et al., 2020)*. The gut mycobiota in type II diabetes displayed different fungal composition at the phylum level, as well as at the genus level, compared to healthy individuals, with an increase of the genera *Candida* and *Meyerozima*, and a decrease of *Saccharomyces*, *Clavispora* and *Wickerhamomyces* (Jayasudha et al., 2020; Bhute et al., 2017). An increased abundance of the genus *Candida* was reported in type I diabetic patients (Gosiewski et al., 2014; Soyucen et al., 2014). Gut microbial alterations are associated not only with gastrointestinal and metabolic disorders, but also with diseases affecting other distal organs. Differences in gut mycobiota composition have been investigated in various chronic conditions, ranging from GI to liver, skin, and cardiovascular diseases, especially colorectal cancer (Coker et al., 2019), alcoholic liver disease (Yang et al., 2017; Szabo, 2018; Lang et al., 2020), chronic kidney disease (Hu et al., 2022), atopic dermatitis (Mok et al., 2021), atherosclerosis (Chacón et al., 2018). Mycobiota composition can influence the gut–brain axis through immune and non-immune mediated crosstalk systems. Studies in mice models and humans suggest a contribution of commensal fungi, in particular an increase in *Candida albicans* and/or *Candida parapsilosis* in neurological disorders, such as Parkinson's disease (De Pablo-Fernandez et al., 2022), Rett syndrome (Strati et al., 2016), autism spectrum disorders (Strati et al., 2017), schizophrenia (Severance et al., 2012; Zhang et al., 2020) and multiple sclerosis (Shah et al., 2021; Gargano et al., 2022).

2.4 Genitourinary tract

First studies on the fungal community in woman reproductive tract date back to 1929 (Carter et al., 1959). The vaginal bacteriome is dominated by *Lactobacillus* species (HMPC, 2012; Li et al., 2012), and the low-pH environment caused by their lactic acid production inhibits the growth of most of the filamentous species, resulting in a selective fungal community (Underhill & Iliev, 2014). Nonetheless, using culture-dependent methods, researchers have investigated the vaginal fungal community in healthy volunteers and patients with diabetes (Nowakowska et al., 2004). Fungi were recovered by culture in 20–60% of the samples. Without exception, the predominant member of the fungal community was *Candida albicans* (often >70%) (Barousse et al., 2004; Nowakowska et al., 2004; Goldacre et al., 1981; Holland et al., 2003). Other epidemiological studies indicated a greater abundance of *Candida*, especially *C. albicans*, making up 85–95% of isolates (Sobel, 1986; Landers et al., 2004). In the past 10 years, thanks to the advent of NGS technologies, several culture-independent studies showed that the vaginal mycobiota is mainly composed of yeasts belonging to *Ascomycota*, followed by Basydiomicota, for a total of 22 genera (Bradford & Ravel, 2017). The most ubiquitous natural coloniser is the genus *Candida* and, namely, the species *C. albicans*, followed by *C. glabrata*, and other *Candida* species inclding *C. krusei*, *C. tropicalis*, *C. parapsilosis*, *C.*

dubliniensis and *C. guillermondi* (Drell et al., 2013; Zheng et al., 2013; Guo et al., 2012; Liu et al., 2022). The load rate of *Candida* among healthy adults ranges from 30 to 70% (Huffnagle & Noverr, 2013). Other non-*Candida* Ascomycetes yeasts found are *Saccharomyces cerevisiae* and *Pichia kudriavzevii* (Papaemmanouil et al., 2011; Drell et al., 2013). Several factors, such as antibiotic use, pregnancy, viral infection (HIV and HPV) and recurrent vulvovaginal candidiasis have been associated with alterations of the vaginal fungal community structure, commonly leading to *Candida* spp. colonisation that could increase up to 40% during pregnancy due to estrogen levels and glycogen production (Farr et al., 2015; Guo et al., 2012). Regarding urine and the urinary tract, there are not enough studies yet to determine a proper urinary mycobiota. Before the advent of culture-independent analyses, the uninfected urinary tract had been assumed to be a sterile environment (Ackerman & Underhill, 2017). Nevertheless, some culture-dependent studies have shown the presence of *Candida* and *Saccharomyces* species both in healthy donors and patients (Pearce et al., 2014; Thomas-White et al., 2016; Hilt et al., 2014; Khasriya et al., 2013; Nickel et al., 2016). Moreover, a recent culture-independent characterisation of 504 urine samples from 202 females revealed the presence of 13 fungal species from 8 genera, including the Ascomycetes yeasts *Candida*, *Saccharomyces*, *Pichia* and *Yarrowia* (Nickel et al., 2020).

2.5 Skin

Since the skin surface is the primary barrier between our body and the environment, it is exposed to an extremely high number of different microorganisms. Nonetheless, it is populated by commensal species, both bacteria and fungi, which help to maintain homeostasis and mediate lipid and urea degradation (Zhu et al., 2020; Ratanapokasatit et al., 2022; Boxberger et al., 2021), as well as the development and activation of the host immune system (Jo et al., 2017). Deep sequencing approaches have demonstrated that skin harbours a unique bacterial and fungal microbiota (Grice et al., 2009; Costello et al., 2009; Findley et al., 2013; Tagami, 2008; Grice & Segre, 2011). Fungal community represents around 10% of all the skin microbiota and consists of more than 150 genera (Findley et al., 2013; Li et al., 2018). Mycobiota of the skin is site-specific, depending mostly on the dry or sebaceous nature of the microenvironment (Leung et al., 2016; Grice et al., 2009). As the skin is a self-renewing organ, dead cells are continuously shed, providing an environment for saprophytic microbial growth. The most common fungi colonising the human skin belongs to *Basidiomycota*, especially *Malassezia* species, which represent on average at least 57% of the total mycobiota of all skin areas (Li et al., 2018; Paulino et al., 2006; Zhang et al., 2011). Among ascomycetes yeasts, *Candida* is the most abundant genus, particularly *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. orthopsilosis*, followed by *Saccharomyces cerevisiae* (Leong et al., 2019; Huffnagle & Noverr, 2013; Zhu et al., 2020; Ward et al., 2018). In skin of infants, different abundances of *S. cerevisiae*, *C. tropicalis*, *C. parapsilosis*, *C. albicans* and *C. orthopsilosis* were influenced by the delivery mode (Ward et al., 2018). The skin microbiota study provides insights on the interactions between pathogenic and commensal fungal communities, and how these interactions can result in beneficial or pathologic outcomes. Fungal species often considered colonisers of the healthy skin, such as *Malassezia*, can become causal agents of diseases. Cutaneous inflammatory conditions, such as psoriasis, atopic dermatitis, pityriasis versicolor, folliculitis,

seborrhoeic dermatitis, dandruff and rosacea have been associated with dysbiosis of the cutaneous microbiota (Castelino et al., 2014; Zeeuwen et al., 2013).

3. MOST REPRESENTATIVES ASCOMYCETES YEASTS IN HUMAN MYCOBIOTA

As shown in the previous sections, several studies investigating the composition of human mycobiota both in health and in disease reached the consensus that the most representative genera in terms of abundance and distribution are Ascomycetes yeasts, in particular *Saccharomyces* and *Candida*. Here we provide an overview on these genera to introduce their relevant characteristics and relationships with the human host.

3.1 *Saccharomyces* species

The genus *Saccharomyces* includes a wide population of wild and domesticated yeast species, these latter well-known to be related to human activities and to industrial applications, such as food and beverage fermentation (Sicard & Legras, 2011). Domestication have contributed to genomic evolution of *Saccharomyces* species (Gallone et al., 2016; Dujon & Louis, 2017).The intensive research on population diversity and genome evolution of this genus (Dunn & Sherlock, 2008; Morales & Dujon, 2012; Piatkowska et al., 2013; Hewitt et al., 2014; Dujon & Louis, 2017; Peris et al., 2018; Liti et al., 2009; Schacherer et al., 2009; Shahait et al., 2022; Peter et al., 2018) and last taxonomic reannotation led to the identification of 8 different species, namely *S. cerevisiae*, *S. paradoxus*, *S. mikatae*, *S. jurei*, *S. kudriavzevii*, *S. arboricola*, *S. eubayanus* and *S. uvarum,* and 2 natural hybrids, namely *S. bayanus* and *S. pastorianus* (Alsammar & Delneri, 2020). Last evidence indicated a large biodiversity of *Saccharomyces* species in the natural environment. Investigation of the natural ecological niches allowed us to discover wild environments, such as soil, bark, leaves and insect guts in which *Saccharomyces* species inhabit (Libkind et al., 2011; Peter et al., 2018; Sampaio JP, 2017; Stefanini et al., 2012; Stefanini et al., 2016). In the last decades, growing interest is placed in the evolutionary process that drives genotypic and phenotypic diversity between yeast species and populations to allow adaptation to different niches. Recently, the ecology of *S. cerevisiae* has also been extended to the study of the human gut (Nash et al., 2017). The prevalence of *S. cerevisiae* in the human GI tract would not be surprising since it has been purposely ingested by humans worldwide for thousands of years in bread, beer, and other fermented foods and beverages (McGovern et al., 2004; Cavalieri et al., 2003), and its abundance is indeed related to the consumption of fermented products (Sun et al., 2021). It is also the most abundant species in early life (at 1–2 years of age), which is a crucial window of infant dietary shift from breastmilk to solid food (Schei et al., 2017). The role of *Saccharomyces* spp. in health and disease has been investigated, but major issues remain (Sokol et al., 2017; Liguori et al., 2016). In particular: (i) are there differences in terms of abundance of strains between health and disease conditions? (ii) are *Saccharomyces* strains from the human gut genotypically and phenotypically adapted to survive and colonise the gut environment or are they transient? (iii) how related are strains with gut environment and host immune system? In the gut environment, yeast survival is difficult and fungi-bacteria and fungi- fungi competitions for the same niche are high. We cannot exclude that the

presence of *Saccharomyces* in the gut is due to food ingestion. Our previous study (Di Paola et al., 2020) provided evidence of genetic and phenotypic differences between strains isolated from gut and non-gut environments (e.g., natural sources, fermentation). However, a disease-specific gut environment may favour expansion of yeast strains, through the onset of peculiar features that are likely to affect the yeast's fitness and the interaction with the host, as observed in IBD conditions. In our previous study (Di Paola et al., 2020) we screened the *S. cerevisiae* strains isolated from faecal samples of Crohn's disease patients. Genetic and phenotypic differences (e.g., cell wall composition) among strains reflected the strain-specific differences in eliciting host immune reactivity (Liti, 2015; Goddard & Greig, 2015). It is possible to hypothesise that some *S. cerevisiae* strains may be a passenger introduced in the gut with the diet and could be capable of colonising the host in certain conditions, such as the presence of a leaky gut or in case of non-responsive host immune system. Otherwise, opportunistic fungal pathogens, such as the species *C. albicans*, commonly occur as commensals on mucosal surfaces of humans, but invade the host when epithelial barriers are disturbed, or the host immune system is impaired. Pathogenicity possibly evolved from commensalism, by acquisition of traits suited to colonise and then invade the host. These issues will be dealt with later in the paragraph entitled: "Fungi: transients or colonisers?".

3.2 *Candida* species

The genus *Candida* comprises the highest number of different human-related species (Romo & Kumamoto, 2020; Witherden et al., 2017; Huffnagle & Noverr, 2013; Huseyin et al., 2017; ten Oever & Netea, 2014), both pathogens commensal members of the healthy mycobiota, able to colonise the host since the birth (Hallen-Adams & Suhr, 2016; Mukherjee et al., 2014; Nash et al., 2017; Reef et al., 1998; Kondori et al., 2019). As above reported, different species are commonly found on the skin, GI tract genitourinary tract (Kühbacher et al., 2017; Odds, 1987; Neville et al., 2015; Fidel, 1998; Barousse, 2004). An extensive review on their survival strategies within human host has been made by M. Polke and colleagues (Polke et al., 2015). *Candida* species can exhibit several virulence factors such as adherence, biofilm formation and secretion hydrolytic enzymes that both increase their persistence within host as well as cause host cell damage (Silva et al., 2012). Due to these reasons, the scientific community has traditionally focused on disease-related studies (Odds, 1987; Kojic & Darouiche, 2004; Naglik et al., 2003; Odds, 1994; Spellberg et al., 2005; Thompson et al., 2010). Nevertheless, *Candida spp.* are also able to exert beneficial effects for the host, by shaping the development of mucosal immunity protecting from fungal infections (Atarashi et al., 2015; Ifrim et al., 2015; Markey et al., 2018). Out of approximately 200 known *Candida* species (Brandt, 2002; Yapar, 2014), at least 15 cause opportunistic infections in humans. Among these *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* were found (Kapitan et al., 2018; Yapar, 2014). In the last twenty years, the number infections identified as due to non-*C. Albicans Candida* (NCAC) species has increased significantly (Kauffman et al., 2000; Ruan & Hsueh, 2009; Hani et al., 2015; Staniszewska, 2020; Maubon et al., 2014). The apparent increased involvement NCAC species in human candidiasis may partly be related to improvements in diagnostic methods (Liguori et al., 2009) or their inherently higher level of resistance to antifungal drugs (Gonza'lez et al., 2008; Tortorano et al., 2021). While *C. tropicalis*, *C. parapsilosis* and *C. krusei* can be found both as part of human mycobiota (Pfaller et al., 2011; Roilides et al., 2003; Trofa et al., 2008) different

environmental niches (Carruba et al., 1991; Yang et al., 2012; Nielsen et al., 2005; Gadanho & Sampaio, 2005; Medeiros et al., 2008; Suh et al., 2008), *C. albicans* and *C. glabrata* are thought to be predominantly associated with host (Gabaldón & Carreté, 2015; Brandt, 2002), even if recent evidence suggests that latter seems to have environmental reservoirs (Gabaldón & Fairhead, 2018). *C. albicans* represents by far the most studied yeast related to humans. It is an opportunistic pathogen that is transmitted vertically from the mother, frequently inhabits the oral, vaginal GI tract mucosa healthy individuals as a harmless commensal (d'Enfert, 2009; Miranda et al., 2009; Zhai et al., 2020; Nash et al., 2017; Prieto et al., 2016; Mishra & Koh, 2018). Specific conditions, such as an unbalanced microbiota, a suppression immune system, and an impaired mucosal barrier can predispose for invasive infections (Kumamoto et al., 2020). Depending on environment, *C. albicans* can switch reversibly between unicellular yeast (which can be additionally divided in white, grey opaque phenotypes), pseudohyphae and true hyphae forms (Sudbery et al., 2004; Noble et al., 2016). Although both hyphal morphologies are necessary for virulence (Jacobsen et al., 2012), there is a consensus upon fact that yeast cells are best suited for dissemination and hyphal cells for tissue invasion (Gow et al., 2002; Jacobsen et al., 2012). Morphology transitions are related to different factors, ranging from physiological and chemical nature environment to necessity mating or immune evasion (Miller & Johnson, 2002; Pande et al., 2013; Morschhäuser, 2010; Mayer et al., 2013), and are always correlated with huge changes in gene expression profile (Mayer et al., 2013). In absence risk factors, infections and overgrowth of *C. albicans* are usually not severe. Oral, skin and vaginal candidiasis are very common human individuals (Kapitan et al., 2018). For example, around 75% women experience at least one-episode vulvovaginal candidiasis during their reproductive age (Yano et al., 2019). Life-threatening systemic *C. albicans* infections can arise when fungus enters bloodstream, mostly from host's GI tract (Miranda et al., 2009; Gouba & Drancourt, 2015; Zhai et al., 2020; Odds et al., 2006; Kumamoto, 2011) when immune defences are compromised for different possible reasons (Koh et al., 2008; Papon et al., 2020). Through blood, infection can disseminate among almost all organs (Pappas et al., 2018), the fungus is able to tune the type of infection thanks to its high genetic variability adaptation capability (dEnfert et al., 2020). Systemic infections can easily occur, mostly associated with predisposing conditions, immunodepression or following nosocomial infection (Dadar et al., 2018).

4 FACTORS SHAPING THE MYCOBIOTA COMPOSITION

The scientific consensus upon the high variability of the mycobiota in the human body, as observed both inter and intra-individuals across time, suggests a crucial impact of several factors in shaping the composition of the fungal communities (Figure 2). Colonisation of the human body by fungi begins immediately after birth (Ward et al., 2018). Mother to child transfer (vertical transmission) is the initial source of fungi. The early mycobiota is influenced by the gestational age of a newborn, birth weight, delivery mode and feeding. During the human's life, the mycobiota composition is influenced by environment (horizontal transmission) and by a large number of endogenous and exogenous factors, including age, gender, diet, bacteriome, antibiotic and antifungal therapy. In the next sections, we will explore the main factors.

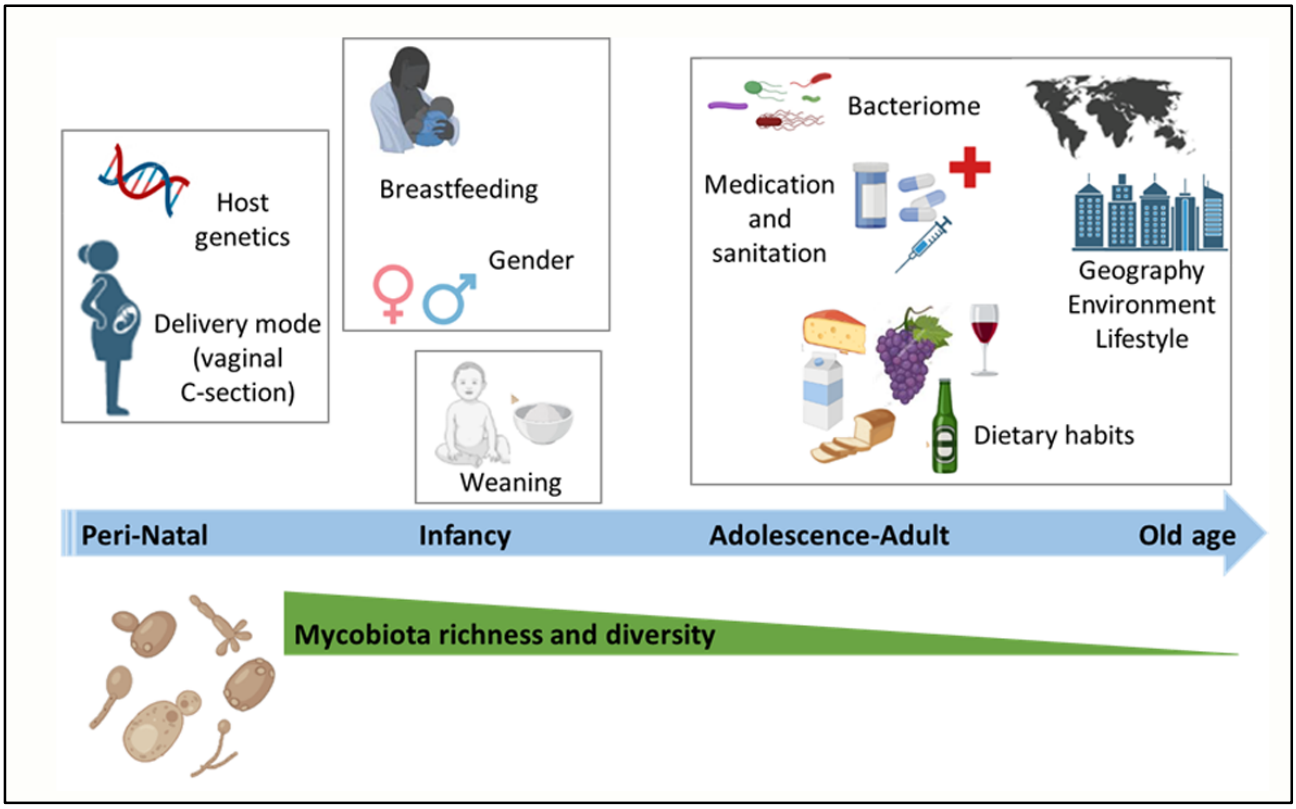

FIGURE 2. Factors affecting the human mycobiota across lifespan. Primary fungal community colonisation begins at birth and the major factors contributing to the infant mycobiota composition are host genetics, gender, delivery mode and feeding. Richness and diversity of mycobiota increase during infancy and decrease in young adults. During adulthood, other factors, including dietary habits, lifestyle, geography, medication and sanitation contribute to influence the human mycobiota composition.

4.1 Host genetics

Similarly to the microbiota, host genetics is able to shape the mycobiota composition and plays a key role in the severity and susceptibility to fungal infection (Maskarinec et al., 2016; Duxbury et al., 2019; Pana et al., 2014). Host-pathogen interaction can be strongly influenced by genetic variations arising in some key genes (Merkhofer & Klein, 2020). Gene polymorphisms are often associated with an increased incidence of opportunistic fungal diseases, and it's known that also epigenetic events are involved in disease progression (Martin & Fry, 2018; Dolinoy & Jirtle, 2008; Goodrich et al., 2017). Among the genetic factors that can lead to a host's susceptibility, mutations in gene coding for host receptors (PRRs e.g., TLR, CLR, NLR, RLR, MBL, Dectins), SNPs in gene coding for crucial mediators of immune response (TNF, INF, Immunoglobulins, Interleukins) and mutations in gene controlling inflammation, phagocytosis and metabolism (eg. ZNF341, STAT1/3, GATA2, NLRP3, PTX3, CARD9) represent an element of predisposition to fungal invasion. Indeed, immunodeficiency-causing mutations are strongly associated with impaired of mucosal immunity and overgrowth of fungal species, especially *Candida* genus, in multiple body sites (Lai et al., 2018). Mutations affecting the T helper 17 cell responses (Patel & Kuchroo, 2015), or in genes involved in fungal sensing (e.g., dectin-3- Clec4d), as well as a defects in mannose binding lectin (MBL) (Hammad et al., 2018) induce blooming of various *Candida* species (Bak-Romaniszyn et al., 2011; Nedovic et al., 2014). Hyphae of *C. albicans* are able to activate the inflammasome (Lev-Sagie et al., 2009; Jaeger et al., 2016), and

polymorphisms in the NLRP3 inflammasome or mutations in CLEC7A (Dectin-1) or CARD9 are associated with susceptibility to vulvovaginitis. A comprehensive compilation of immune system genetic polymorphisms associated with susceptibility to fungal infections can be found in the review by Naik et al. (Naik et al., 2021). Although the identification of monogenic susceptibility variants has made it possible to elucidate the pathways that are triggered in defence of both mucosal and systemic fungal infections, taken individually these variants are unable to explain the spectrum of susceptibility to mycosis that is recorded among human populations. For this reason, recent studies have begun to investigate the relationships between genetic susceptibility to fungal infection and human ancestry (Domínguez-Andrés & Netea, 2019; Hughes et al., 2008). In spite of the fact that this research field is still in its infancy, the possibility of personalised therapeutic approaches against invasive fungal diseases is becoming increasingly concrete.

4.2 Gender and age

Evidence of gender and age-related effects on mycobiota were reported for the first time in our study on healthy individuals, both children and adults (Strati et al., 2016). Females showed high fungal richness and biodiversity compared to males, with a higher prevalence of *Candida* species. Gender-dependent differences seems to be due to sex hormones, as observed in mice model (Markle et al., 2013) or differences in dietary habits between female and male (Bolnick et al., 2014). Increased levels of *Candida* during ovulation, pregnancy or following the use of oral contraception (hormonal therapies) could be associated with the window of opportunity to invade the host determined by a mild reduction of immune defences (Farr et al., 2015; Guo et al., 2012, Lasarte et al 2013, Salinas-Muñoz et al., 2018). The production of estradiol during the menstrual cycle has an anti-inflammatory effect, temporarily lowering the immune system during the phase of interaction between sperm and egg cells. The immune system lets its guard down when a woman is ovulating, increasing the likelihood of a sperm cell surviving in the reproductive tract. As a side effect, women who are ovulating, or on birth control pills, are more prone to yeast, bacterial and viral infections (Relloso M et al., 2012). Thus, we can probably say that the mycobiome is the most gender-related part of the microbiome. Unlike microbiota (Yatsunenko et al., 2012; Koenig et al., 2010; Lozupone et al., 2012), in humans the fungal community diversity decreases ranging from childhood to adult age (Chehoud et al., 2015; Strati et al., 2016; Jo et al., 2016). The age-related changes in fungal communities appears to be more relevant in the first months of life (Schei et al., 2017; Wampach et al., 2017; Ward et al., 2017). Studies revealed that newborn infants between 1 and 4 months of age have a gut mycobiota dominated by the orders *Malasseziales* and *Saccharomycetales*, whereas *Saccharomycetales* (*Saccharomyces* and *Candida* genera) preveal from older infants to adulthood (Fujimura et al., 2016). It has been hypothesised that a high percentage of the maternal mycobiota could be transferred to the newborn, consistently with pioneer works mainly based on *Candida* (Waggoner-Fountain et al., 1996; Bliss et al., 2008). Recent studies confirmed that newborn babies receive *C. albicans* strains from their mothers during vaginal delivery (Bliss et al., 2008; Schei et al., 2017). Caesarean-section-born children had a different stool mycobiota than vaginally delivered children (Wampach et al., 2017; Ward et al., 2017). Also, the initial colonisation of the skin by fungi is shaped by the delivery mode (Wampach et al., 2017; Ward et al., 2018). Compositional changes of the skin mycobiota were observed in several studies from

childhood to adulthood (Grice & Segre, 2011; Jo et al., 2016), and similar patterns have been documented in other body sites (Peters et al., 2017; Strati et al., 2016; Ackerman & Underhill, 2017). *C. albicans* was significantly more abundant on the skin of infants who were born vaginally (Ward et al., 2018). Vaginal delivery is shown to promote oral yeast carriage (Azevedo et al., 2020), but the relative abundance of certain species, such as *Candida orthopsilosis*, is significantly higher in C-section-born infants (IKEBE et al., 2006; Ward et al., 2018). Interestingly, infant feeding (formula-fed or breast-fed) doesn't seem to affect the mycobiota composition (Oba et al., 2020; Azevedo et al., 2020; Darwazeh & Al-Bashir, 1995), although human breast milk contains approximately 3.5 x 10^5 fungal cells per ml (Boix-Amorós et al., 2017). Unlike childhood, mycobiota composition in older healthy individuals was less studied (Barrera-Vázquez & Gomez-Verjan, 2019), although it was investigated in elderly diseased subjects (Ahmad et al., 2020; Nagpal et al., 2020; Alonso et al., 2018; Jayasudha et al., 2020). The most comprehensive work was performed in 100 elderly participants aged 65–81 years with hypertriglyceridemia. This study showed that *Penicillium*, *Candida*, and *Aspergillus* were the three most representative genera (Ahmad et al., 2020).

4.3 Diet

Diet is probably the most important and recurrent factor that determines the composition and shifts in the human mycobiota, especially in the gut (Shankar, 2021; Graf et al., 2015; David et al., 2013). The impact of diet in shaping the mycobiota has been studied since 1974, when Finegold S. M. and colleagues (Finegold et al., 1974) compared the culturable fungi from stools of individuals that followed either Western or Japanese diets . Since then, diet-related studies have focused on the gut mycobiota. A lot of fungal species are introduced in our body through food and beverages and could potentially become more than transient colonisers (Belvoncikova et al., 2022; Hoffmann et al., 2013; David et al., 2013; Sun et al., 2021). Indeed, fungi and especially Ascomycetes yeasts are commonly associated with several food products, such as *Saccharomyces*, *Candida, Pichia, Galactomyces, Hanseniaspora, Debaryomyces, Brettanomyces, Zygosaccharomyces* and *Wickerhamomyces* species that have been found in baking goods (Dangi et al., 2017; Li et al., 2019), fruits (Vadkertiová et al., 2012; Tournas et al., 2006), fermented and acidophilus milk (Reed & Nagodawithana, 1990; Griffin et al., 2020; Bell et al., 2018), cheese (Bintsis, 2021), fermented beverages like wine, beer and sake (Copetti, 2019; Jolly et al., 2013), different meats and soy sauce (Copetti, 2019). The ingestion of these species through food has been shown to alter the gut mycobiota composition. For instance, *Candida* species were found to be positively associated with recent consumption of high amounts of carbohydrates and negatively with a diet high in proteins or fatty acids (Hoffmann et al., 2013), while *S. cerevisiae* was found reduced in the gut after a decreased intake of bread and beer (Auchtung et al., 2018). Moreover, the amount of the genus *Candida* in the gut was observed in association with a plant-based diet (David et al., 2013). A recent study comparing faecal samples of Indian and Japanese individuals showed that polysaccharides in the Indian plant-rich diet led to an abundance of *Candida* in their gut (Pareek et al., 2019). Related to diet, body weight was observed as a determinant factor of the gut mycobiota composition. Gut fungal communities differ between overweight, obese, and lean subjects. Predominant Ascomycetes yeasts in overweight individuals were *Candida* and *Pichia*, whereas *Candida* and *Nakaseomyces* were found more

abundant in obese individuals (Borges et al., 2018; Rodríguez et al., 2015). Finally in murine models, mice fed with a high-fat diet showed significant differences in gut mycobiota composition (Heisel et al., 2017).

4.4 Lifestyle, culture, and geography

Other factors affecting the human mycobiota composition and closely related to diet are lifestyle, culture, residence, and geography. In the last decades, several studies addressed the impact of these factors on gut bacterial communities (Angebault et al., 2013; Clemente et al., 2015; Filippo et al., 2010; Martínez et al., 2015; Yatsunenko et al., 2012). The main evidence showed that modern Western populations have a reduced gut and skin microbiota diversity compared to that of traditional, rural and agrarian populations. Regarding the mycobiota, here we report some findings of different mycobiota composition according to different lifestyle, culture, and geography. In Wayampi Amerindian populations, in French Guiana, a relatively rich fungal diversity was observed, although a significantly lower prevalence of *C. albicans* was found compared to Western industrialised societies (Angebault et al., 2013). In a community - dwelling elderly of Asian people a low abundance of oral *Candida* species was found (Zakaria et al., 2017).

4.5 Bacteriome

The coexistence and mutual influence of bacterial and fungal communities are well-known in all ecological systems. Bacterial communities' impact on mycobiota has been studied especially regarding *Candida* colonisation in the human gut and genitourinary tract. Antibiotic treatments favour fungal overgrowth in both these sites (Fan et al., 2015; Spinillo et al., 1999; Xu et al., 2008; Zaborin et al., 2014). Apart from dynamics competition between bacteria and fungi, several studies showed that bacteria impact on fungal growth through production of fungistatic acids (Mortensen & Clausen, 1996; Noverr et al., 2004; Cottier et al., 2015) as well as reactive oxygen species (Fitzsimmons & Berry, 1994) and biosurfactants (Velraeds et al., 1998; Hogan & Kolter, 2002; Gibson et al., 2009) that inhibit the growth or the hyphal formation of *Candida* species. At the same time, other studies focus on the bacterial ability to produce metabolites that enhance the growth and pathogenesis of fungi (Xu et al., 2008; Gale & Sandoval, 1957; Neely et al., 1986; ADAM et al., 2002), showing synergistic interactions between these two kingdoms into the human bodies. Finally, also indirect activation of the host immune system against bacteria can lead to an impairment of *Candida* colonisation (Lamas et al., 2016; Zelante et al., 2013). More studies are needed to deepen microbiota-related bacterial influences on other yeasts non- *Candida* species.

5. METHODS TO CHARACTERISE THE MYCOBIOTA: IDENTIFICATION AND TECHNOLOGICAL ISSUES

As mentioned above, many questions regarding mycobiota remain to be addressed. Firstly, the scientific community is still in doubt whether a proper core mycobiota exists. Secondly, further studies are needed to fully understand all the possible factors that drive the fungal presence in human hosts. Therefore, it is crucial to increase our knowledge and improve our technologies in order to overcome the challenges upon the fungal communities inhabiting the environmental ecosystem and our body. Many methodologies applied for investigation of the bacteriome are not consistent for studying the fungal community. As a result, non-standardized techniques, technical

issues, restricted availability of reference data, and (Suhr & Hallen-Adams, 2015). Culture-dependent approaches have traditionally been employed to investigate microorganisms' diversity, but these techniques have well-known limitations that also apply to fungi. For instance, many species are undetected because appropriate culture conditions are unknown or hard to reproduce. Moreover, once the fungal strains have been isolated, identification is not yet complete, but it requires further steps. Culture methods are time-consuming and unsuitable for high-throughput analysis. However, culturomic approaches have the undeniable benefit of allowing access to the fungus itself, its viability, metabolites, phenotypical and functional characterisation and other host-adaptation features (Strati et al., 2016). As a result, culture-dependent approaches are still useful and of great interest (Borges et al., 2018; Hamad et al., 2017) especially in the light of several studies that have highlighted how some fungal strains can't be properly identified by culture-independent method, resulting in the underrepresentation of some species potentially because of cell wall structure or inadequacy of the chosen PCR primers and/or barcode sequence (Huseyin et al., 2017; Hamad et al., 2017). The use of DNA as an identifying marker in culture-independent approaches avoids some of the aforementioned issues, but it strongly relies on the choice and efficiency of DNA recovery methods, and it also introduces new limits and hurdles. Fungi, unlike bacteria, have a strong and complex cell wall rich in glucans and chitin (Aimanianda et al., 2009; Valiante et al., 2015; Gow et al., 2017) and for this reason efficient cell wall destruction is indispensable for fungal DNA extraction. Several bead-beating stages followed by enzymatic cell lysis are required for successful mycobiota analysis from any sample matrix (Huseyin et al., 2017). Following DNA extraction, different approaches can be used to detect and identify fungi, such as PCR and sequencing analysis, according to metabarcoding, or whole-genome shotgun (WGS) metagenomics sequencing. The choice of the barcode sequence and sequence-specific primers is crucial in metabarcoding. Similarly to bacterial metabarcoding, the usual fungal barcode is the rRNA gene locus, which includes the genes for 18S rRNA, 5.8S rRNA and 28S rRNA separated by the internal transcribed spacers (ITS1 and ITS2). It seems to better discriminate higher taxonomic ranks than the 16S rRNA gene (De et al., 2017; Nilsson et al., 2019). After exploring fungal rRNA genes, Schoch and colleagues in 2012 identified the ITS as the universal DNA barcode identifier for fungi (Schoch et al., 2012; Nilsson et al., 2008), although currently it is still not clear which of the two ITS components have the better resolution in strain prediction. Recent findings demonstrated that both regions suffer from amplification bias resulting in a uneven representation of synthetic fungal communities (Ali et al., 2019; Bokulich & Mills, 2013; Bellemain et al., 2010; Tedersoo & Lindahl, 2016): ITS1-based PCR appears to favour *Basidiomycota*, whereas *Ascomycota* seem to be favoured by ITS2-based PCR (Bellemain et al., 2010). Hoggar et al. recommend the selection of the ITS2 region in human mycobiota investigation after comparing four primers set targeting the small subunit (SSU) rRNA (18S), ITS1, ITS2 and large subunit (LSU) rRNA (26S) genomic regions (Hoggard et al., 2018). In addition, Nilsson and colleagues propose a set of fungus-specific primers with their superior coverage of the fungal kingdom, targeting the ITS2 sub-region with degenerate forward primers gITS7ngs and reverse primer ITS4ng (Nilsson et al., 2019). Besides primers choice, length variation among ITS at fungal species, spanning from 200 bp to 800 bp, has a strong impact on PCR efficiency, as well as sequencing technologies (Tang et al., 2015; De et al., 2017). Moreover, not only this region is present in multiple copies within one species (Kiss, 2012) but intragenomic variation within a

single species, such as numerous paralogous or non-orthologous copies, may lead to an overestimation of global fungal diversity (Schoch et al., 2012). Since ITS copies number has highly interspecific variation, an accurate determination of fungal abundance is hard to reach, and caution must be taken when attempting to make quantitative comparisons between diverse species in mixed populations. Using *in silico* pipelines, Stielow et al. confirmed the already known TEF1α as a secondary barcoding marker for fungi and proposed genes topoisomerase I (TOP1) and phosphoglycerate kinase (PGK) as promising ascomycetes identifiers, based on the analysis of complete sequenced genomes (Stielow et al., 2015). Other suggested secondary markers for fungal DNA amplification are the intergenic spacer (IGS), RNA polymerase II (RPB1 and RPB2), β-tubulin II (TUB2), and the minichromosome maintenance complex component 7 (MCM7) protein (Meyer et al., 2019; Větrovský et al., 2016; Morrison et al., 2020; Park et al., 2011; Huang et al., 2012; Schoch et al., 2012). The selection of one or more reference genes is crucial for standardisation and promotion of large-scale investigations, but in some cases primer bias in targeted sequencing can be overcomed by opting for the shotgun metagenomic approach. Because of its non-specificity, the whole genome sequencing (WGS) is the most unbiased technique, but also the most sensitive to host DNA contamination, which can easily represent most of the sequenced reads in samples collected from soft tissues and biological fluids (HMPC, 2012). This problem is particularly relevant for the study of mycobiota, since fungi represent a very small fraction of the total microorganisms and high sequencing depth is required. Currently, it emerges that low fungal abundance in human samples is impeding the broad use of metagenomic WGS in human samples, a finding that is unrelated to DNA extraction techniques and reflects really low total in vivo fungal abundance (Nash et al., 2017). Shotgun metagenomics is a highly effective method for describing pathways and discovering novel functions, but it is far more expensive than metabarcoding and computationally more challenging (Morgan & Huttenhower, 2014). However, the recent development of long-read sequencing technologies by PacBio and Oxford Nanopore is making it possible to reconstruct more complete fungal genomes thanks to covering critical genomic regions (like the highly repetitive ones). Regardless of the used methodologies (assembly-based or assembly-free (Quince et al., 2017) to classify sequenced reads, probably the most concerning analytic challenge for mycobiota investigations is still the poorly cured fungal databases. Despite the fact that fungi are one of the largest branches of the Tree of Life, the number of high-quality fungal sequences in curated databases, such as SILVA for rRNA sequences (Pruesse et al., 2007) or UNITE for ITS sequences (Abarenkov et al., 2010) is still significantly lower than that of available bacterial rRNA sequences. This lack results in a substantial amount of unclassified reads, which might be addressed by producing additional high-quality metagenomic and whole-fungal genome assemblies (Nash et al., 2017; Mac et al., 2019). Furthermore, confunding redundancies in fungal taxonomy make identification even more complex: sexual (telomorph) and asexual (anamorph) forms of the same fungus are frequently classified as separate taxa, with different approved names at the phylum level (Halwachs et al., 2017). Although a general consensus on wet lab practices is still lacking, what arguably afflicts mycobiota investigations most is the lack of standardisation of the pipelines employed for sequencing data analysis. To improve mycobiota investigation, major efforts will be needed in the identification and validation of targeted and shared practices in all analytical steps.

6. HOST-FUNGI EVOLUTION AND COEVOLUTION

Since fungi diverged from animals, about 1 billion years ago (Taylor & Berbee, 2006; Hedges et al., 2004), they have become one of the most important sources of genetic diversity on Earth (Wainright et al., 1993; Peay et al., 2016). Moreover, they coevolved with animals both as parts of holobiont ecosystems. Fungi could establish different types of relationships with animals, ranging from mutual to commensal and pathogenic microorganisms, including numerous species with potential to pose threats to multiple ecosystems (Seyedmousavi et al., 2018; Gnat et al., 2021). Nonetheless, fungi are drastically understudied, such that they have been referred to as the "hidden kingdom" (Fisher et al., 2020). Only in the last 5 years, several publishing and research groups have raised the attention on the urge to deepen the knowledge about fungal biology in order to tackle the threats coming from emerging pathogens (Konopka et al., 2019; Fisher et al., 2016; Microbiology, 2017). One of the reasons why this kingdom has been historically understudied could be the fact that humans, and mammals in general, are biologically less prone to lethal fungal infections with respect to other classes of animals (Kwon-Chung & Bennett, 1992; Canny & Gamble, 2003; Connole et al., 2000; Pollock, 2003). Systemic diseases in humans were considered rare until the middle of the last century, and nowadays they usually don't occur unless in cases of impaired immune functions, exposure to large inocula, immunosuppressive drugs, integument compromise by catheters and surgery, and disruption of microbiota (Edwards, 1991; Gupta & Kogan, 2004; Deshaw & Pirofski, 1995; Ward et al., 1979; Fessel, 1993; Fisher et al., 2000). The relatively low abundance of diseases caused by fungi, despite their ubiquitous presence in every environment, suggests that mammals were evolutionarily selected to defend themselves against these microorganisms. Specifically, different authors have suggested that the most important characteristics of mammals in avoiding lethal fungal infections are the combination of a high basal temperature and an advanced immune system (Kwon-Chung & Bennett, 1992). Indeed, fungal diseases have a greater impact in terms of frequency and lethality for a huge variety of ectothermic animals (Berger et al., 1998; Daszak et al., 1999; Fisher et al., 2012), and mammals can succumb from usually non-lethal fungal infections, when they took place in cooler body sites or time of the year (Perfect et al., 1980; Blehert et al., 2009). The importance of host-fungi interactions in balancing the rates and causes of mortality among different classes in the animal kingdom have been stressed to the point that an evolutionary hypothesis was proposed (Casadevall, 2005), discussed (Casadevall, 2012) and recently revisited (Casadevall & Damman, 2020) about the emergence of mammals over reptiles at the Cretaceous-Paleogene (K–Pg) boundary (formerly known as Cretaceous-Tertiary boundary). In that era an asteroid impact (Schulte et al., 2010) triggered a mass extinction followed by deforestation (Vajda et al., 2001) and ubiquitary fungal bloom (Vajda & McLoughlin, 2004). The up-to-date hypothesis, called "fungal infection-mammalian selection" (FIMS) by the authors Casadevall and Damman (Casadevall & Damman, 2020), states that, despite their more energetically expensive bodies and lifestyle, mammals survived the catastrophe better than reptiles because their endothermy would have protected them from fungal diseases. In contrast, the rapid global cooling would have negatively selected ectothermic animals in terms of reproduction and sustenance, as well as deadly fungal infections that proliferate at low temperatures. In this scenario, mammals began to dominate the current Cenozoic era together with the ubiquitary presence of microorganisms, including fungi. Since several different types of stable relationships between mammals (in particular humans) and fungi are known, it is reasonable to infer that both the groups

have evolved in a way that, on one hand, enabled fungi to survive in the hostile mammal microbiota environment, and, on other hand, mammals to tolerate their presence both as commensal and opportunistic dwellers. In the following sections, we will provide an overview of the genetic features for the survival in humans of ascomycetes yeasts, which are the most representative fungi in our microbiota, and also the immunological mechanisms that allow us to control and prevent their spreading.

6.1 Fungi: transients or colonisers?

Currently there is still not sufficient evidence of the existence of a core mycobiota in each human body district (Sharon et al., 2022). The observed inter- and intra- individual variability of the mycobiota could suggest a high functional redundancy among fungal residents, or could be due to technical artefacts arising from the low absolute abundance of fungi compared to bacteria. Some authors have attempted to discern between what is considered core mycobiota, and a transient fungal colonisation that most likely comes from the environmental species. A small-scale study by Auchtung and colleagues (Auchtung et al., 2018) sought to identify a possible core mycobiota of the human GI tract analysing stool samples of healthy volunteers. For this investigation, the authors considered that, in other mycobiota studies, low longitudinal stability across samples from a single person was recurrent and that most commonly detected fungal genera are also present in food and the oral cavity (Hallen-Adams & Suhr, 2017; Oh et al., 2014; Ghannoum et al., 2010). Since the alleged core gut mycobiota could be obscured in metagenomic analysis from diet-related fungi, the authors conducted an experiment where volunteers of diverse geographical areas and with different dietary habits followed four controlled diets as representative of major food groups and relatively low fungal load. An extremely low fungal abundance was found compared to the bacterial community (from 0.001 to 0.01%). *Candida* albicans and *Saccharomyces* were the unique Ascomycetes yeasts present in each sample. Furthermore, following a *S. cerevisiae*-free diet, the *Saccharomyces* operational taxonomic unit (OTU) dropped below the limit of detection within a few days. Moreover, they tried to verify whether *Candida* species found in the human gut derive from the swallowing of saliva. They found that when a healthy adult volunteer increased the frequency of cleaning of teeth, the abundance of *C. albicans* in stool was lowered 10-fold to 100-fold. Therefore, the authors concluded that in healthy adults of Western society there are few or no fungal species that colonise the GI tract. Conversely, a more recent study by van Tilburg Bernardes and colleagues (van Tilburg Bernardes et al., 2020) sought to determine the role of colonising fungi using a gnotobiotic mice model. Results showed that in absence of bacteria, fungi can be cultivated from faecal samples several weeks after initial inoculation. The authors concluded that fungi are gut dwellers and that they don't require bacterial communities to engraft in the mouse gut. Moreover, when bacteria and fungi coexist, the fungal concentration was lower, but the fungal community was rich, revealing not only interkingdom competition/antagonism, but also beneficial relationships. Other studies focused on specific body sites to assess the nature of human mycobiota. Kramer and colleagues (Kramer et al., 2015) showed that the mycobiota of the upper respiratory tract was dominated by transient species, and that there is not enough scientific evidence to distinguish between resident and transient components of airway mycobiota. Concerning oral mycobiota, there are indications of both resident and transient fungi (Santus et al., 2021).

Ascomycetes yeasts of *Candida* and *Saccharomyces* genera have been found in several studies, but only *Candida* is recognized to date as an active coloniser of the mouth, especially *Candida albicans* that has been identified in both culture-dependent and independent studies (Zaura et al., 2009; Monteiro-da-Silva et al., 2014; Dupuy et al., 2014). As far as the gut mycobiota is concerned, studies in mice and humans showed that the gut ecosystem is populated by both a transient mycobiota, and a resident community of fungi (Santus et al., 2021). The Human Microbiome Project data showed that the most abundant ascomycetes yeasts are *Saccharomyces* (Shankar, 2021; Fröhlich-Wyder et al., 2019; Heitmann et al., 2018; Nielsen, 2019), *Candida* and *Cyberlindnera* (Buerth et al., 2016). These genera are components of several fermented food and beverages, such as *Saccharomyces*, or food additives, such as *Cyberlindnera* (Nash et al., 2017). As already mentioned, *Candida* is the most abundant fungal genus of oral mycobiota, and almost certainly the main colonisers of several human body districts, including the gut ecosystem. In general, the scientific consensus agrees that the great intra- and inter-individuals variability observed in the human gut mycobiota (HMPC, 2012), suggests that these most representative fungal genera could be introduced in through food, since fungi are usually present in most of the human diets (Tournas & Niazi, 2017; Fiers et al., 2019; Graves & Hesseltine, 1966; Raimondi et al., 2019). However, studies on in vivo mouse models through a culture-independent approach (Scupham et al., 2006; Aykut et al., 2019; Heisel et al., 2017; Qiu et al., 2015; Rosshart et al., 2019; Yeung et al., 2020) showed a different scenario. On one hand, *Candida* and *Saccharomyces* genera were found in mice faeces but not in chow, and on the other hand, their abundance was a lot higher in the faeces, or a high percentage (80-90%) of the taxa identified in the faeces were not present in chow (Iliev et al., 2012; Scupham et al., 2006; Qiu et al., 2015; Heisel et al., 2017). Altogether these studies suggest that investigation of the core gut mycobiota needs to be further investigated. The discrepancies found can be almost certainly ascribed to the different models used (human and mouse), to the culture-dependent or non-culture-dependent approaches, or to the sequencing and analysis methods.

6.2 Fungal evolution towards pathogenesis

Despite being the cause of more than a billion infections in humans every year (Bongomin et al., 2017) and constituting one of the largest eukaryotic kingdoms with over two hundred orders (Hawksworth & Lücking, 2017; Li et al., 2021), fungi are still highly understudied (Microbiology, 2017). One of the main causes of this negligence lies in the opportunistic behaviour of pathogenic fungi that make them predominantly harmful to immunocompromised hosts owing to congenital or acquired deficiency (Fisher et al., 2020; Crum-Cianflone, 2016; Koehler et al., 2020). Because of a long history of coevolution between mammals and fungi, major systemic fungal infections are very unusual in the healthy population, despite being routinely exposed to commensal mycobiota and environmental fungi. Within the large number of identified fungal species, only a few are known to be true pathogens, although it is becoming evident that probably fungi (included the harmless ones, such as *S. cerevisiae*) can be opportunistic or accidentally pathogens in the presence of a non-immune competent host. Pathogenicity has evolved several times within the fungal kingdom through different mechanisms and genetic variations. Genes associated with fungal pathogenicity show a degree of heterogeneity not only at the species level, but also at the strain level. However, little is known regarding the extent of this variability.

Indeed, pathogenic fungi are not a monophyletic group and the characteristics they have developed have probably evolved to survive in conditions independent of successful infection of the human host (Bowman et al., 1996; Rizzetto & Cavalieri, 2011). It has been proposed that the traits associated with pathogenicity are the same ones that have enabled them to survive in nature by adapting to a wide range of even extreme conditions (Casadevall & Pirofski, 2007; Gostinčar et al., 2018). For example, it has been noted that ascomycetes tend to be more thermotolerant and present in different environments than other fungal phyla. This may explain at least part of the abundance of human pathogens, but also commensals, within *Ascomycota* (Robert et al., 2015; Rokas, 2022). However, even though the human host environment resembles fungi natural habitat, once associated with human host fungi adaptive strategies may diverge from their natural habit as demonstrated by comparison between *S. cerevisiae* natural and clinical strains where the latter show increased levels of heterozygosity and pseudohyphal development and a reduction of sexual reproduction (Magwene et al., 2011; Strope et al., 2015). These microorganisms have developed pathogenicity as a consequence of complex interactions between themselves, their host and the environment (Casadevall, 2017). The use of an ecological-evolutionary approach, coupled with recent technological innovations, is currently making it possible to understand and deepen the mechanisms underlying fungal pathogenicity. To be able to infect humans, fungi must possess four characteristics: thermal resistance to human body temperature (including fever), avoidance or breakthrough of surface barriers, tissue lysis and penetration and immune defence resistance (Köhler et al., 2017). These criteria are only met by a small number of species which differ considerably in terms of adaptation to pathogenic lifestyle. In the context of Ascomycetes yeasts, *Candida* genus include the most prevalent and harmless human commensals. However, this genus includes the most common human pathogens such as the previously cited *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. krusei* and the emerging *C. auris*. These species are responsible for a large number of diseases, ranging from minor mucosal infection such as vulvocandidiasis and oropharyngeal thrush to potentially fatal diseases, including disseminated hematogenous and invasive candidiasis (Pfaller & Diekema, 2007). Thanks to its major ability of adaptation to human host, *C. albicans* is the dominant species among commensals and pathogens accounting for almost half of disseminated illness and an even higher proportion of mucosal infections. Its pathogenicity is mainly linked to morphological plasticity that enables it to reversibly switch from yeast to hyphal growth (Chow et al., 2021). Recently, the chromatin- mediated epigenetic regulation of this switching mechanism has been reported by Iracane et al. (Iracane et al., 2021) that highlight the role of environmental stimuli in triggering pathogenic behaviour. It is interesting to note that cryptic species, such as *C. albicans* and *C. dubliniensis*, even though they share several phenotypic traits and do not have major disparities in the gene content (Jackson et al., 2009), are quite different in terms of virulence. However, looking at orthologous genes' expression levels, a systematic comparison highlighted main differences in 15 genes involved in glycolysis that are less expressed in *C. dubliniensis*. In the same study (Singh-Babak et al., 2021), after genetically engineering *C. dubliniensis* to express higher levels of these genes, an increase in virulence of the species was observed. Thus, recent evidence shows both gene variation and regulation of expression levels are mechanisms capable of inducing and modulating pathogenicity among fungi. As previously mentioned, temperature is a selective pressure factor to which fungi are able to respond promptly.

It has been assumed that current climate change, and the consequent global warming, are selecting thermotolerant fungi able to become human pathogens (Garcia-Solache & Casadevall, 2010; Nnadi & Carter, 2021). This is the case of the recently discovered *C. auris*, which has an environmental reservoir (Arora et al., 2021). This species has caused simultaneous nosocomial infections in more than 30 countries in the last 10 years, probably representing the first pathogen selected by global warming (Casadevall et al., 2021). With the same mechanism, in the last three decades also *C. glabrata* infections have become more frequent (Gabaldón & Carreté, 2016), thanks to its ability to tolerate temperatures superior to 37°C, to withstand various types of stress including prolonged starvation and to have great adherence to host tissue. Nevertheless, from an evolutionary point of view, this species represents an outlier since it is more linked to *S. cerevisiae* than to other *Candida* species. These two species in fact belong to the same Saccharomycetaceae clade which is characterised using the classic genetic code code (Mühlhausen & Kollmar, 2014) (in contrast to the *Candida* CTG clade in which CUG encodes the amino acid serine instead of leucine (Santos et al., 2011)) and furthermore both possess a duplicated genome as the inheritance of an ancient common ancestor (Wolfe & Shields, 1997). Along with *C. glabrata, C. krusei* is the other clinical isolated species not included in the CTG clade, but little is known about the virulence mechanisms of this species although the number of nosocomial infections has increased in recent years (Gómez-Gaviria & Mora-Montes, 2020). To date it's well known that there are important physiological differences between *C. glabrata* and *C. albicans* since they do not even share a large number of protein-coding genes; it has been noticed, that even in the case in which they share some orthologous genes the functional divergence may be of such magnitude that it leads to completely different outcomes in terms of virulence, as reported for instance by MacCallum and colleagues (MacCallum et al., 2006) in relation to null mutations in the transcription factor ACE2 resulting in reduced virulence in *C. albicans* and increased virulence in *C. glabrata*. However, *C. glabrata* ability to reshape its metabolism in response to phagocytosis is similar to what is known for *C. albicans* (Kaur et al., 2007), in contrast to its closest relative *S. cerevisiae* which does not appear to be able to provide as robust a response (Lorenz et al., 2004). *S. cerevisiae* has long been considered a non-pathogenic organism, however, a comparison of environmental and clinical strains showed that the latter are mosaic strains characterised by heterogeneous gene content due to outcrossing between different subpopulations (Magwene, 2013; Cavalieri, 2010). Clinical strains in fact generally show a reduced capacity for sexual reproduction, increased pseudohyphal development and copper resistance (Strope et al., 2015). Since *S. cerevisiae* lacks homologous genes for adhesin and shows lower adhesion levels to human tissue compared to *Candida* species it has been proposed that it can behave like an opportunistic pathogen only when host barriers integrity has been formerly compromised (Pérez-Torrado et al., 2015). Due to the increasing number of immunocompromised individuals and as a consequence of climate change and globalisation, we are witnessing a progressive increase in the number of emerging pathogenic fungi. It therefore becomes crucial to understand the ecology and evolutionary history of these organisms in order to systematically classify them. A better understanding of the molecular mechanisms underlying fungal pathogenicity and virulence could make it possible not only to predict the emergence of new pathogens but also to develop targeted therapies.

6.3 Fungi and host immune system

Healthy people live surrounded by environmental fungi and harbour a rich mycobiota without frequently incurring into harmful infections. This is possible thanks to a strong immune system that has evolved to respond to fungi and tolerate them. Since fungi are ubiquitous and range across several mechanisms of interactions with other organisms, nearly all immunity types of the host are involved in the process of achieving balance with the fungal community. The immune homeostasis is exerted on barrier surfaces, such as mucous membranes and constantly shape immunity. A proper immune response relies on the host ability to discern self from non-self, starting from the detection of fungal cell components by the innate immune system, followed by a cascade of signalling pathways that leads to activation of gene expression and, ultimately, giving rise to adaptive immunity (Figure 3). Although mycobiota have been neglected for a long time, in the last ten years numerous studies have focused on the impact of fungal species on the host immune system and vice versa (Romani, 2011; Cui et al., 2013; Underhill & Iliev, 2014; Hohl, 2014; Rizzetto et al., 2014; Netea et al., 2015; Underhill & Pearlman, 2015; Wheeler et al., 2017; Lionakis et al., 2017; Iliev & Leonardi, 2017; Lai et al., 2019; Zhang et al., 2020; Santus et al., 2021). These comprehensive reviews deepeneed all the aspects around the immunological response towards fungal communities, and therefore the following subsections will provide a brief overview of the current knowledge, focusing on the most important features that allow human bodies to both tolerate and control the mycobiota.

6.3.1 Fungal recognition by host immune system

The response to fungi begins with recognition of fungal presence by innate immunity, followed by downstream signalling pathways that drive innate, adaptive and humoral responses. Fungi are recognised by host immune cells' pattern recognition receptors (PRRs) that sense pathogen-associated molecular patterns (PAMPs) of fungi (Figure 3A). Fungal PAMPs are mostly components of the cell wall such as glucans, mannans and the chitin monomer N-acetylglucosamine (Hatinguais et al., 2020; Erwig & Gow, 2016; Doering, 2009; Lee & Sheppard, 2016; Netea et al., 2008). The most important PRRs that can identify fungi are Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and galectin-3, extensively reviewed elsewhere (Romani, 2011; Underhill & Pearlman, 2015; Wheeler et al., 2017; Iliev & Leonardi, 2017; Zhang et al., 2020). Through differential recognition by several types of PRRs, the immune system can discriminate between commensal, passenger, opportunistic and pathogenic fungi in order to orchestrate a proper reaction and maintain homeostasis between all the microbial populations. The importance of PRRs in immune homeostasis is underlined by the effects on mutations in these receptors, which lead to increased infections, tissue burden and exacerbated inflammation (Iliev et al., 2012; Ifrim et al., 2014; Netea et al., 2002). Among PRRs, CLRs such as dectin-1, dectin-2, dectin-3, Macrophage inducible Ca_2^+-dependent lectin receptor (Mincle) and the mannose receptor likely play the most central role in fungal recognition, activating several pathways including the Syk-Card9 pathway, which leads to different signaling cascades and an inflammatory reactions (Brown & Gordon, 2003; Smeekens et al., 2014; Plato et al., 2015; Taylor et al., 2007; Geijtenbeek & Gringhuis, 2009; Dambuza & Brown, 2015; Sancho & Reis, 2012; Drummond & Brown, 2013). Also TLRs are essential parts of the fungal sensing, specifically TLR2 and TLR4 for cell wall components, whereas TL3, TLR7 and TLR9 (which are expressed intracellularly) for exogenous RNA and DNA (Elieh et al., 2018;

Bourgeois & Kuchler, 2012). Sensitivity to fungal infections is strongly influenced by mutations in both CLR (Taylor et al., 2007; Saijo et al., 2007; Gross et al., 2006; Whitney et al., 2014) and TLR genes (Bochud et al., 2008; Carvalho et al., 2008; Netea et al., 2002). Although fungal NLR ligands are not fully defined yet, the NLRP3 inflammasome process has been shown to influence the defence against mycobiota (Gross et al., 2009). PRRs on immune cells initiate downstream intracellular events that eventually lead to the clearance or the tolerance of fungal cells within the body. The choice between innate or adaptive immune response depends on the cell type involved (Patin et al., 2019). In addition, humoral immune mechanisms, such as the complement and specific antibodies against fungi, play an important role in antifungal immunity (Netea et al., 2015; Speth et al., 2008; Casadevall & Pirofski, 2012).

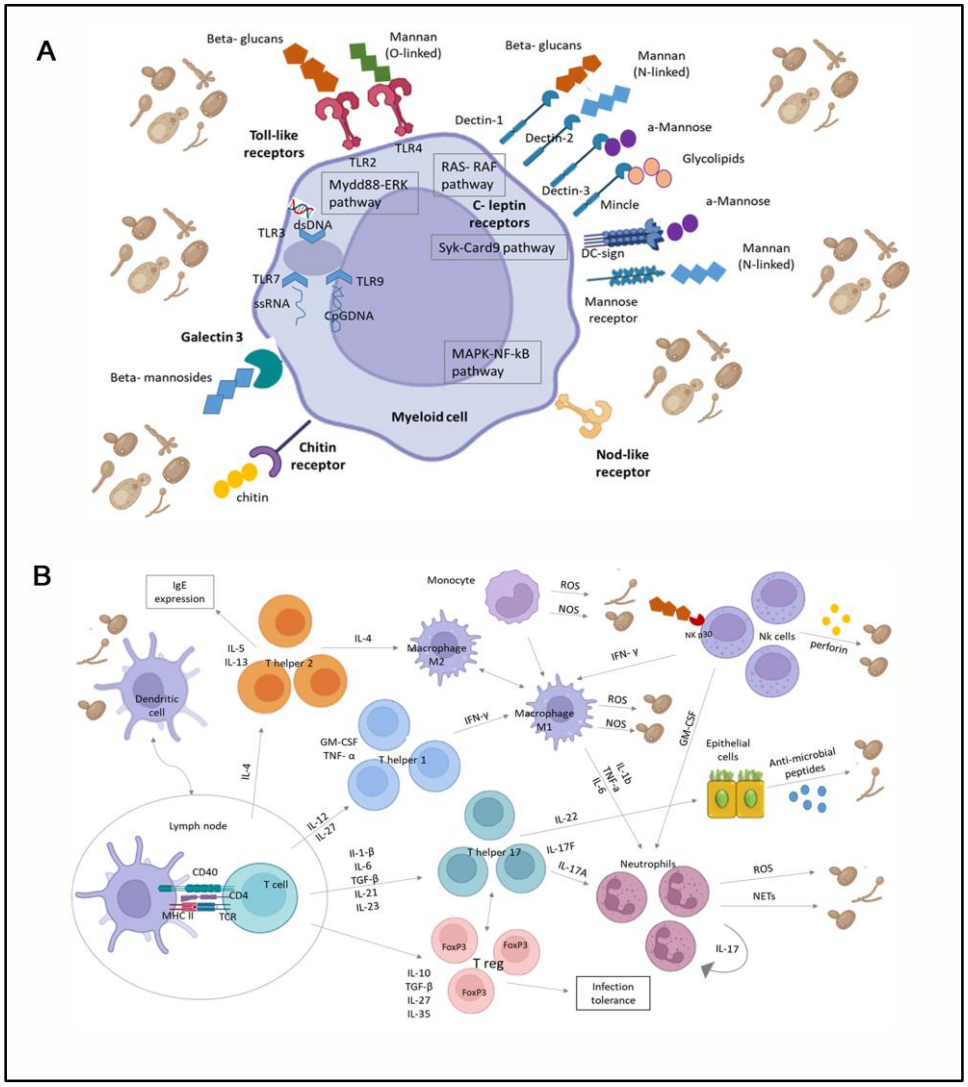

FIGURE 3. Fungi and host immune system. (A) Schematic representation of fungal recognition mechanisms by innate immunity. The principal pattern recognition receptors (PPRs) and the pathogen-associated molecular patterns (PAMPs), mainly of the fungal cell wall, are reported. (B) Schematic representation of fungal recognition mechanisms by innate and adaptive immunity. Innate immune response is the first essential step for fungal clearance. Neutrophils and natural killer (NK) cells can kill fungi through different mechanisms including oxidative bursts after phagocytosis or through neutrophil extracellular traps (NETs), secretion of antifungal molecules that prevent yeast to hyphal transition and initiation of adaptive responses. Also, monocytes and macrophages can kill fungi, although less efficiently than neutrophils and NK cells. Dendritic cells, after exposure with fungal PAMPs, module cytokines production, as well as activation markers, migrating to lymph nodes and priming T cells to initiate an adaptive response.

6.3.2 Innate response

Innate immune response is the first essential step for fungal clearance and initiation of adaptive responses through cell-cell crosstalk. Several studies deeply reviewed all the known aspects of this defence mechanism (Salazar & Brown, 2018; Underhill & Pearlman, 2015; Drummond et al., 2014; Dambuza et al., 2017). Therefore, here we will provide a brief overview to summarise the most important features regarding the factors that drive the shift between innate and adaptive responses (Figure 3B). Among all immune cells, neutrophils are shown to be the most effective in fungal clearance for their ability in killing them through oxidative bursts after phagocytosis or through neutrophil extracellular traps (NETs), secrete antifungal molecules and prevent yeast to hyphal transition (Wozniok et al., 2008; Ermert et al., 2013; Warnatsch et al., 2017; Brown, 2011; Branzk et al., 2014; Missall et al., 2004; Urban et al., 2009), and also the most crucial because of the increased risk of invasive fungal infections due to neutropenia (Koh et al., 2008; Uzun et al., 2001) or mutations in genes associated with neutrophil activity (Lehrer & Cline, 1969; Winkelstein et al., 2000; Swamydas et al., 2016). Also, monocytes and macrophages can kill fungi, even if less effectively than neutrophils (Hünniger et al., 2014). They exert an important antifungal activity in peripheral organs (Ngo et al., 2014) and fungal infections are enhanced after their depletion (Qian et al., 1994) or mutations involving the chemokine receptor CX3CR1 (Lionakis et al., 2013; Lionakis & Levitz, 2018). Other major contributors to the immune response to fungi come from epithelial cells, which release antimicrobial peptides and represent the first barrier for environmental fungi (Kolls et al., 2008), natural killer cells (NK), which are primarily active against hyphal forms trough cytotoxicity (Mody et al., 2019; Li et al., 2013). They have also been shown to regulate other immune cells through cytokines production (Campbell & Hasegawa, 2013; Schmidt et al., 2017), other innate lymphoid cells (Huang et al., 2015; Mear et al., 2014; Gladiator et al., 2013) and innate-like lymphocytes such as γδ T cells (Albacker et al., 2013; Cohen et al., 2011). Finally, dendritic cells (DCs), considered as a bridge between innate and adaptive immunity, play a fundamental role in shaping the proper immune reaction towards fungi (Steinman & Hemmi, 2006; Paul, 2011). After exposure with fungal PAMPs, DC module cytokines production, as well as activation markers, migrating to lymph nodes and priming T cells to initiate an adaptive response (Kashem et al., 2015; Ramirez-Ortiz & Means, 2012; Roy & Klein, 2012) (Figure 3B). The most studied signalling pathway of immune activation is the “spleen tyrosine kinase - caspase recruitment domain-containing protein 9” (SYK-CARD9), which is downstream of fungal PAMP recognition by CLRs and induce a large range of cytokine gene expression (Geijtenbeek & Gringhuis, 2009; LeibundGut-Landmann et al., 2007; Hardison & Brown, 2012). The organisation of innate and adaptive responses to fungi is tuned by the production of cytokines by immune cells, already comprehensively reviewed elsewhere (Ward & Vyas, 2020; Becker et al., 2015; Underhill & Pearlman, 2015).

6.3.3 Adaptive response

The central role of cytokines produced by immune cells after fungal recognition is especially highlighted in the activation of the adaptive immunity, a mechanism that involves primarily DCs, although macrophages could also be part of it. DCs primes naïve CD4+ and CD8+ T cells through fungal antigen presentation and release various panels of cytokines to drive the differentiation of T

cell subsets (Iwasaki & Medzhitov, 2010) (Figure 3B). Although CD8+ T cells have been shown to participate in antifungal response (Leibundgut-Landmann et al., 2008; Hernández-Santos et al., 2013; Lindell et al., 2005; Nanjappa et al., 2012), especially in case of CD4+ deficiency (Nanjappa et al., 2012), the pivotal role in antifungal adaptive response is played by T helper cells (Th), which derive from CD4+. Once differentiated, the principal effector function of Th cells is the release of cytokines that allow the host to properly respond to the fungal presence. As well as for the innate form, antifungal adaptive immunity has already been extensively reviewed (Wüthrich et al., 2012; Verma et al., 2014; Speakman et al., 2020), thus here we will summarise the most important Th responses in the context of fungal presence. Th1 immunity is mainly directed towards defence against fungal pathogens through the production of IFN-γ, TNF-α, and GM-CSF, which lead to the classical pathway of macrophages activation and antibody class switching to IgG2, as well as the enhancement of phagocytosis and activity of antigen presenting cells (Beaman, 1987; Snapper & Paul, 1987; Shalaby et al., 1985; Schroder et al., 2004; Novak & Koh, 2013; Subramanian et al., 2013). Th2 immunity has shown to be mostly implicated in allergic response to fungi through production of IL-4, IL-5 and IL-13, that drive an alternative activation to macrophages, and induction of IgE expression, therefore resulting in a detrimental influence on host health during fungal infections (Zhang et al., 2017; Müller et al., 2013; Voelz et al., 2009; Cenci et al., 1993), although a protective role of Th2 cells has been described in Pneumocystis pneumonia (Nelson et al., 2011; Perez-Nazario et al., 2013). Similarly, Th9 cells have been associated with enhanced fungal asthma, increased severity of chronic airway hyperreactivity and impairments in gut fungal dissemination and inflammation (Li et al., 2018; Kerzerho et al., 2013; Renga et al., 2018). Th17 immunity plays, together with Th1, a central role in fungal response. Th17 differentiation and expansion are prompted by IL-6, IL-21 and IL-23 (Nurieva et al., 2007; Korn et al., 2007; Aggarwal et al., 2003), while both IL-1 and TGF-β have been shown to produce controversial effects (Mangan et al., 2006; Das et al., 2009; Hirahara et al., 2010). This T cell subset is identified by the production of IL-17A, IL-17F and IL-22, and their antifungal activity is carried out by two main mechanisms: in systemic infections by the recruitment of neutrophils, and in mucosal infections by the induction of antimicrobial peptides (AMPs) and β-defensins from epithelial cells and keratinocytes (Hernández-Santos & Gaffen, 2012; De et al., 2010; Conti et al., 2011). Moreover, studies have shown that Th17 cells enhance the production of IgA (Cao et al., 2012; Hirota et al., 2013) and that Th17 cells induced by gut *C. albicans* could become cross-reactive to other fungi in different body districts, indicating that a single member of the mycobiota can elicit a systemic antifungal response, with potential risks for inflammatory and autoimmune diseases (Bacher et al., 2019). Regulatory T cells (Treg), which are composed of different subsets, contribute to an appropriate regulation of the antifungal response to limit damage to the host. They exert this role by (i) secretion of inhibitory cytokines such as IL-10, TGF-β or IL-35, (ii) repression of IL-2 production and (iii) down-modulation of antigen presenting cells (Goodman et al., 2012). It is still not fully understood how they are generated in response to fungal infections, but some studies have shown that FoxP3+ Tregs promote immune response against *Candida* in mice (Feng et al., 2011), whereas a fungal stimulation of human peripheral blood mononuclear cells (PBMCs) stimulate a Treg but not an effector T cell response, highlighting the tolerogenic potential of the firsts through an inflammation dampening mechanism (Bacher et al., 2014). Therefore, other studies are required to clarify the role of Tregs in the context

of fungal commensalism and pathogenesis. Finally, although early studies concluded that humoral immunity is not necessary for antifungal response (Hurd & Drake, 1953; Monga et al., 1979; Carrow et al., 1984), more recent works showed that immunoglobulins directly target antigens of the fungal cell wall (Casadevall & Pirofski, 2012) and exert a protective immune response both binding to the fungal surface (a role mostly played by IgA) and enhancing the microbicidal potential of effector cells (McClelland et al., 2010; Brena et al., 2011; Nabavi & Murphy, 1986; Cao et al., 2012). Nevertheless, the investigation of B cells during fungal infections is still in its early stages.

6.3.4 Immune system adaptive mechanisms: fungal-mediated immune enhancing and trained immunity

Yeasts resident in the intestinal tract constantly interact with the immune system, however they do not necessarily represent a threat for the host inducing an inflammatory response. Several studies have better defined the role of yeasts in host-microbe interaction (Hall & Noverr, 2017; Di et al., 2020). The exposure of non-pathogenic yeasts such as *S. cerevisiae* and *C. albicans* induces the activation of human monocyte cells causing a Th17-mediated differential response (Rizzetto et al., 2010; Rizzetto et al., 2014). The yeast *C. albicans* is extensively studied for its ability to switch from a commensal to a parasitic microorganism. *C. albicans* is commonly tolerated by the host as a commensal organism as it does not activate innate immune responses that cause inflammation; however, following dysbiosis and/or immune suppression, *C. albicans* can proliferate, inducing hyphae development and thus activating innate immune responses with consequent inflammatory process (Hall & Noverr, 2017). The continuous recruitment of immune cells by *C. albicans* into the vaginal mucosa causes an increased activity of neutrophils, damaging the mucosa and causing symptomatic candidiasis (Fidel et al., 2004; Bradford & Ravel, 2017). The role of *S. cerevisiae* has also been widely discussed since the presence of circulating ASCAs is associated with the incidence of several human chronic inflammatory and autoimmune diseases (Kaul et al., 2012; Mankaï et al., 2013; Shor et al., 2012; Barclay et al., 1992). According to these studies, *S. cerevisiae* shows negative immunogenic activity capable of threatening human health; however, *S. boulardii* is used as a probiotic for the treatment of gastrointestinal disorders showing traits of a mutualistic symbiont (Szajewska et al., 2016; Abid et al., 2022). Given this controversial evidence, the role of yeasts has not been completely resolved yet and probably the immunogenic activity drastically depends on the strain-specific wall structure rather than taxonomic species (Briard et al., 2021; Marakalala et al., 2013). However, the role of yeasts in the human immune system homeostasis implicates a level of complexity that goes beyond the commensal interaction, and that the relationship between commensal yeasts and their human host has crossed several coevolution steps. It has been proposed that trained immunity represents a mechanism that provides the host an evolutionary advantage. It is defined as a long-term functional modification of innate immune cells which leads to a greater response to a second unrelated immune challenge (Netea et al., 2020) (Figure 4). Trained immunity was initially referred to bacteria only, exerted through Lipopolysaccharides (LPS) recognition (Netea et al., 2011), but then also fungi have been shown to participate (Quintin et al., 2012) and its role during mucosal diseases has been recently reviewed (Yu et al., 2021). Systemic infection of mice with a non-pathogenic strain of *C. albicans* PCA-2 conferred protection against subsequent infection with lethal concentrations of the *C. albicans* pathogenic

strain CA-6 and/or *Staphylococcus aureus* (Bistoni et al., 1986). These mechanisms were not regulated by the adaptive immune response but by a T-independent functional reprogramming operated by fungal cell wall β-glucans (Bistoni et al., 1988; Quintin et al., 2012). The PCA-2 strain was able to induce increased levels of granulocyte/monocyte colony-stimulating factor (GM-CSF), tumour necrosis factor-α (TNF-α), interleukin 1 (IL-1) and interferon-γ (IFN-γ) in mice model, and these levels persisted for several days after infection (Vecchiarelli et al., 1989). *S. cerevisiae* has been proven to induce trained immunity in human monocytes in a strain-dependent manner, resulting in increased production of TNFα and IL-6 upon secondary stimulation with Toll-like receptors (TLRs) ligands. Moreover, the differential activation of the immune system strictly depended on the strain used due to the different chitin content of the cell wall (Rizzetto et al., 2016). Dectin-1 was depicted as a target receptor involved in the fungal wall, through recognition of β-glucans (Moerings et al., 2021; Walachowski et al., 2017). Exposure of mice lacking functional T and B lymphocytes to *C. albicans* and fungal cell wall β-glucans resulted in protection against *C. albicans* reinfection through a process that directly involved the Dectin-1 receptor pathway and the Raf-1 mediator leading to histone H3K4 trimethylation resulting in a functional epigenetic reassortment of monocytes (Quintin et al., 2012). According to these studies, the role of yeasts seems crucial in the modulation of the human immune system, and it is evident that this mechanism is dependent on the strain-specific cell wall variability. Adaptive traits of the innate immune system have been proved also for the invertebrates (Kurtz, 2005; Conrath et al., 2015; Gourbal et al., 2018). These insights are out of the scope of this review but given the importance of the trained immunity in the fungi-host research field from a conceptual and applicative point of view, we highlighted some interesting examples. In particular, the activity of β-glucans in increasing the immune activity of shrimps towards pathogen infections has suggested their use in aquacultures (Amparyup et al., 2016; Roy et al., 2020). Trained immunity mechanisms were also described in insects. Strains belonging to the species *S. cerevisiae* and *C. albicans* were proven to enhance the immune response against a *C. albicans* reinfection in the insect model Galleria mellonella (Bergin et al., 2006). *G. mellonella* also showed immune enhancement following the immune system exposure with the cell wall of *A. fumigatus* (Fallon et al., 2011; Mowlds & Kavanagh, 2008; Mowlds et al., 2008; Mowlds et al., 2010) and *Candida glabrata* (Huang et al., 2020). Similarly, also in the future foundresses of the social wasp *P. dominula* an immune enhancing mechanism against generic pathogen *E. coli* following exposure with *S. cerevisiae* was highlighted (Meriggi et al., 2019). Fungal-mediated immune priming was also depicted in *Drosophila melanogaster* following treatment with the entomopathogenic fungus *Beauveria bassiana* (Pham et al., 2007). These immune mechanisms are considered a crucial evolutionary strategy for insect survival (Cooper & Eleftherianos, 2017). This also highlights the evolutionary convergence in the mechanisms of tolerance and defence towards the resident fungal community or pathogens. Moreover, these studies provide evidence that helps in understanding the molecular mechanisms underlying adaptive traits of innate immunity.

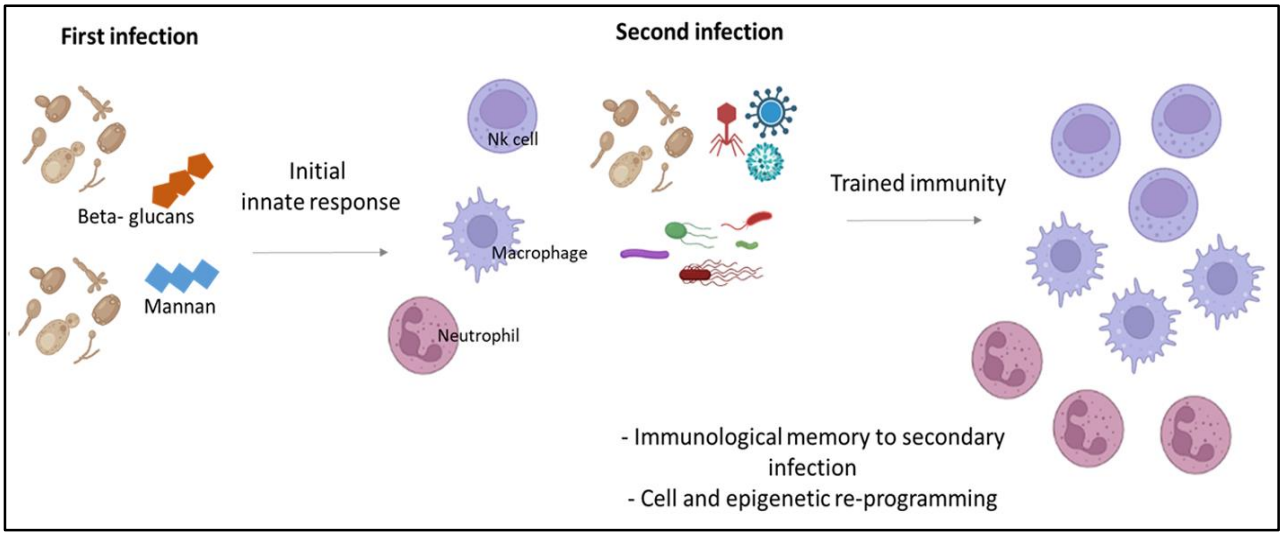

FIGURE 4. Trained immunity. A primary fungal infection (represented by exposure to fungal cell wall components) induces an initial innate immune response. The innate immune cells (natural killers, macrophages, neutrophils) are epigenetically reprogrammed during exposure to microorganisms, allowing an enhanced response upon a second infection by fungi, bacteria or virus.

7. CONCLUSIONS

Fungi have accompanied human evolution, being part of our diet since the advent of the Neolithic revolution and the production of fermented beverages. Ecological and evolutionary approaches and recent technological innovations in metagenomics are currently making it possible to understand and deepen the mechanisms underlying host-fungi interaction. Furthermore, culture-dependent approach and the use of fungal laboratory strains allowed the understanding of cellular and molecular mechanisms in health and disease statuses. However, since the fungal component in humans has been neglected for a long time compared to bacteria, it is crucial to increase knowledge and improve technologies to overcome the existing challenges in studying the mycobiota. Within the fungal kingdom, Ascomycota is the more present phylum interacting with the human host, either as transient commensals or colonisers, both beneficial and pathogenic. Ascomycota yeasts, such as *Sacchormyces* species, can act as probiotics promoting our health or they can be pathogens capable of threatening humans and animals. At the same time, *Candida* species are known to colonise multiple body sites, both as commensals and pathogens; moreover, the dimorphic fungus *Candida albicans* can shift from a usually commensal yeast form to pathogenic pseudohyphal and hyphal forms, depending on several environmental factors. The balance between pathogenic and commensal yeasts has been explored especially within the different districts of the human body. Over time, this has allowed the characterisation and anatomical mapping of human fungal communities, also referred to as human mycobiota. This multitude creates a close relationship with the host influencing its physiology, cognitive activity and overall, their health. Cell wall components play a central role in the modulation of host immune response ranging from innate to cell-mediated and humoral adaptive forms. Recent discovery of immune enhancing mediated by fungal trained immunity reveals a new concept in immunity, helping us to understand the beneficial role of yeasts. The findings discussed in this review show the importance of investigating the interactions between the yeast component of the microbiota and their host. Only using evolution as a magnifying lens together with modern systems level analyses we will understand their transition from passengers

to colonisers and invaders and unravel the subtle networks discriminating the role of the mycobiota in health from disease.

AUTHOR CONTRIBUTIONS

DC conceived the work. SN and SR gathered data from the literature. DC, SN, SR, NM and MDP wrote and revised the manuscript. MDP and SN created figures. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

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Chapter 2

Human conventional and plasmacytoid dendritic cells differ in their ability to respond to Saccharomyces cerevisiae

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Abstract

Saccharomyces cerevisiae is a commensal yeast colonizer of mucosal surfaces and an emerging opportunistic pathogen in the mucosa and bloodstream. The role of *S. cerevisiae* has been largely characterized in peripheral blood mononuclear cells and monocyte-derived dendritic cells, where yeast cells induce the production of inflammatory cytokines through the interaction with mannose receptors, chitin receptors, DC SIGN, and dectin1. However, the response of blood-circulating dendritic cells (DCs) to *S. cerevisiae* has never been investigated. Among blood DCs, conventional DCs (cDCs) are producers of inflammatory cytokines, while plasmacytoid DCs (pDCs) are a specialized population producing a large amount of interferon (IFN)-α, which is involved in the antiviral immune response. Here we report that both human DC subsets are able to sense *S. cerevisiae*. In particular, cDCs produce interleukin (IL)-6, express activation markers, and promote T helper 17 cell polarization in response to yeasts, behaving similarly to monocyte-derived DCs as previously described. Interestingly, pDCs, not cDCs, sense fungal nucleic acids, leading to the generation of P1-pDCs (PD-L1+CD80–), a pDC subset characterized by the production of IFN-α and the induction of a Th profile producing IL-10. These results highlight a novel role of pDCs in response to *S. cerevisiae* that could be important for the regulation of the host microbiota–immune system balance and of anti-fungal immune response.

INTRODUCTION

In the last years, a crucial role of microbiota is emerging in the development of pathologies involving the immune system, such as allergies, inflammatory disorders, and autoimmune diseases.
Microbiota is the set of microbes, including viruses, bacteria, and fungi, living in the host organism. Microbiota is essential for the protection against pathogens, the synthesis of molecules, and the metabolism of substances derived from diet (1). In a healthy condition, all of these microbes are balanced with each other and are able to induce immune tolerance. However, an alteration in the number and/or localization of different microbes (dysbiosis) may promote diseases associated to immune dysregulation. The microbiota influences the immune system through the interaction with innate immune cells (2). In particular, dendritic cells (DCs) interact with microorganisms through a set of pattern recognition receptors, such as Toll-like receptor (TLR), and initiate the adaptive immune response by activating naïve T lymphocytes through the release of cytokines and the expression of co-stimulatory molecules (3). Blood DCs can be divided into conventional (cDCs) and plasmacytoid DCs (pDCs) (4, 5). pDCs predominantly recognize viruses and are IFN-α producers (6–9), while cDCs are involved in the recognition of several microorganisms, including bacteria and viruses, and produce inflammatory cytokines, such as interleukin (IL)-6 (10). However, bacteria and viruses are not the unique microbes composing the microbiota. The fungi kingdom is an important component of the microbiota, called mycobiota. Interestingly, the composition of the mycobiota is particularly unstable compared with the rest of the microbiota (11). Thus, fungi derived from dietary or environmental sources may contribute to mycobiota diversity and may strongly influence innate immunity. The most common genus of fungi originating from food and environment and harboring

the gastrointestinal tract is *Saccharomyces.* Many members of this genus are considered very important in food production, such as *Saccharomyces cerevisiae*, the bakers' and brewers' yeast (11). However, *S. cerevisiae* is also an etiologic agent of opportunistic fungal infection in immunocompromised patients (12), and the ability to colonize and give rise to disease depends on the host immune response. Although immune response to *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, and *Malassezia* have been largely characterized (13), little is known about the interactions between *S. cerevisiae* and host defense cells. Previous studies reported that the wall components of *S. cerevisiae* activate human monocyte-derived DCs through mannose receptor, DC SIGN, dectin-1, and chitin receptor, thus leading to the production of IL-6 and other inflammatory cytokines (14–16). However, the ability of blood-circulating human DCs to respond to *S. cerevisiae* has never been investigated. Here we challenged human blood DC subtypes with *S. cerevisiae*, and we found a differential response of cDCs and pDCs, characterized by IL-6 and IFN-α production, with a consequent IL-17 and IL-10 induction in *in vitro*-differentiated Th cells, respectively. Thus, the exposition of *S. cerevisiae* as well as the prevalence of specific DC targets *in situ* may influence the development and the progression of diseases associated to microbiota-dependent immune dysregulation or fungal opportunistic infection.

RESULTS

The Laboratory Strain of Saccharomyces cerevisiae SK-1 Promotes Activation of Human Blood DCs

In order to test blood DCs' response to S. cerevisiae, human pDCs and cDCs were purified from the peripheral blood of healthy donors (Supplementary Figures S1, S2) and stimulated with a laboratory strain of S. cerevisiae, called SK-1. Specifically, we cultured DCs in the presence of SK-1 at multiplicity of infection (MOI) of 5 (colony-forming unit SK-1/DC). We used as positive control Resiquimod (R848), which is an imidazoquinoline compound known to be a potent immune activator of both pDCs and cDCs because it is an agonist of TLR7 and TLR8, expressed by pDCs and cDCs, respectively. Unstimulated DCs were used as negative control. We evaluated the levels of CD80 and CD86, two molecules binding CD28 on T cell surface and inducing T cell activation and proliferation, and the expression of programmed death-ligand 1 (PD-L1), which is a co-inhibitory molecule known to reduce T cell proliferation through the binding with PD-1 on T cell surface. Our results showed that SK-1 induces a significant increase of CD80 and CD86 expression by pDCs and cDCs (Figure 1A). Surprisingly, we also found a significant increase of PD-L1 expression induced by SK-1 in both DC subsets, especially in pDCs (Figure 1A). Consistently, the increase of CD80+ , CD86+ , and PD-L1+ cells in DCs is associated with an overall high median fluorescence intensity (MFI) (Figure 1B). In addition, we measured IFN-a and IL-6 production by SK-1-stimulated pDCs and cDCs, respectively. We found that SK-1 induces IFN-a production by pDCs (Figure 1C) and IL-6 production by cDCs (Figure 1D) compared with unstimulated DCs.

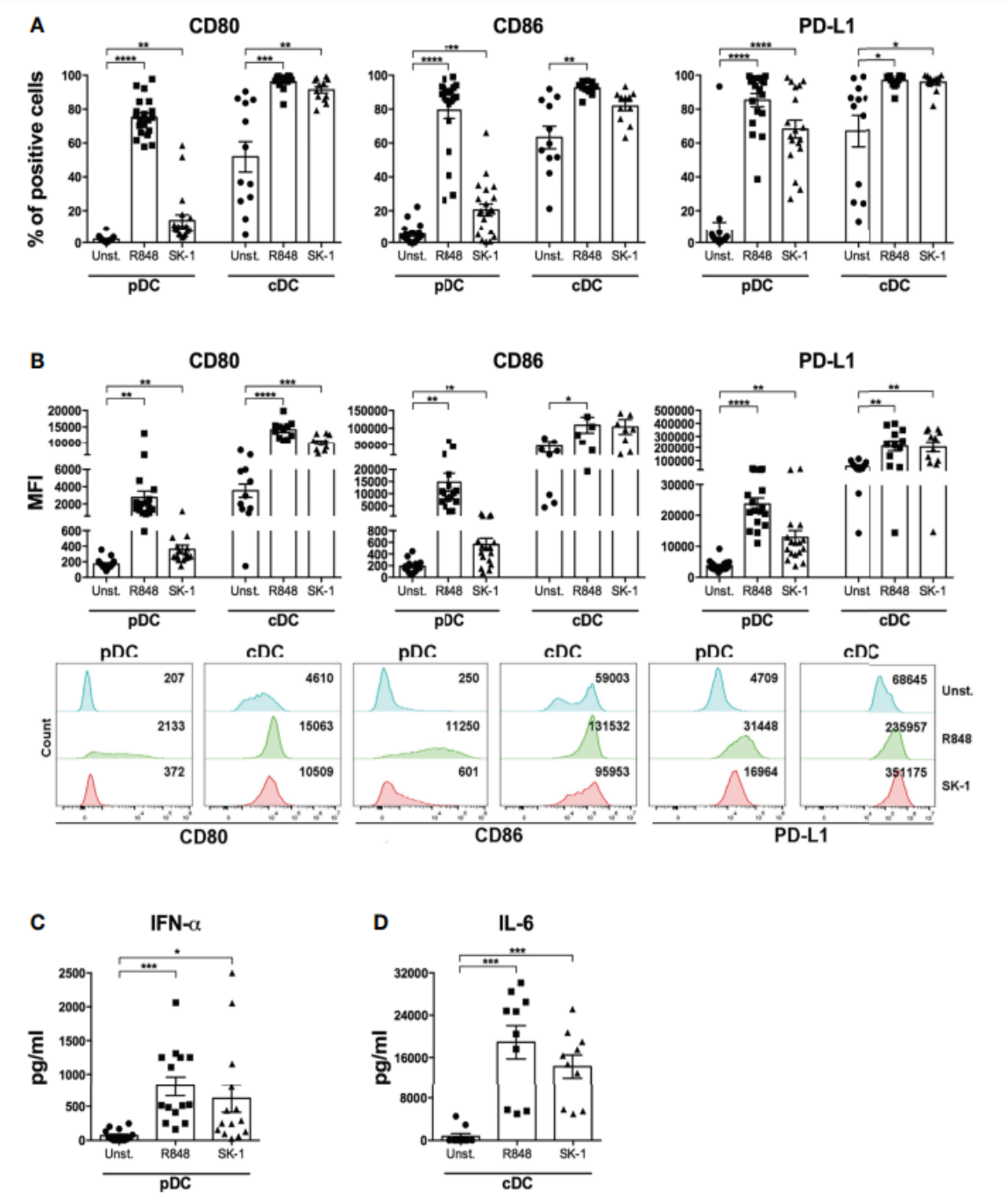

Figure 1. The laboratory strain of Saccharomyces cerevisiae SK-1 promotes the activation of human blood dendritic cells. Human pDCs and cDCs purified from the peripheral blood of healthy donors were cultured for 48 h without stimulation (Unst.), with R848 (1 mg/ml) as positive control, or with the laboratory strain of Saccharomyces cerevisiae SK-1 at multiplicity of infection = 5 (colony-forming unit SK-1/DC). The expression of molecules CD80, CD86, and PD-L1 was analyzed by flow cytometry, and the percentage of positive cells (A) and median fluorescence intensity (B) were reported. The levels of IFN-a (C) and IL-6 (D) were measured in the culture supernatants by ELISA. Data are the mean of 11 independent experiments, each from different donors. Error bars represent SE

SK-1 Induces the Differentiation of P1 Subpopulation of Human pDCs

To further gain insight into pDC activation by SK-1, we cultured pDCs in the presence of SK-1 at different MOI values. After 48 h of culture, we firstly evaluated the formation of cell clusters, which reflects pDC activation and pDC viability by optical microscopy. Our results showed that stimulation with SK-1 leads to cell cluster formation (Figure 2A). As expected, pDCs stimulated with the positive control (R848) form cell clusters, whereas unstimulated pDCs do not form any cell cluster (Figure 2A). Moreover, we found that different doses of SK-1 lead to a progressive increase of CD80, CD86, and PD-L1 within viable pDCs in a dose-dependent manner (Figure 2B). We found that SK-1 at MOI 5, not at MOI 10, induces the highest levels of IFN-a (Figure 2C), which is likely due to the lethal effect of a high dose of SK-1 (data not shown). These results confirm that SK-1 (MOI 5) promotes pDC activation and viability with the concomitant IFN-a production. Recently, it has been observed that the differential expression of the surface molecules PD-L1 and CD80 defines three specific pDC subpopulations with distinct functions: P1 (PD-L1+ CD80-), P2 (PD-L1+ CD80+), and P3 (PD-L1- CD80+) (17). Given the high expression of PD-L1 induced by SK-1 in pDCs, we hypothesized that the P1-pDC subpopulation is preferentially induced upon stimulation with the laboratory strain of S. cerevisiae. Thus, we analyzed pDC subpopulations in our experimental conditions by flow cytometry. Our results showed that SK-1 is able to induce P1- pDC subpopulation (Figures 2D, E). In particular, we observed a dose-dependent induction of the percentage of P1-pDCs at increasing doses of SK-1. The positive control R848 induces all three subpopulations (Figures 2D, E), as previously demonstrated (17). The fluorescence-minus-one experiment demonstrates the specificity of PD-L1 staining (Supplementary Figure S3). Moreover, it has been reported that IFN-a-producing pDCs are mostly P1-pDCs (9). Therefore, we investigated whether the induction of P1-pDCs obtained upon stimulation with SK-1 was associated to IFN-a production in the same experimental conditions. To address this relationship, we correlated the levels of IFN-a and the percentage of P1-pDCs in several pDC-SK-1 cultures. Interestingly, we found a positive correlation between P1-pDCs and IFN-a (Figure 2F), suggesting that SK-1 induces PD-L1+ CD80- pDCs, which, in turn, produce IFN-a.

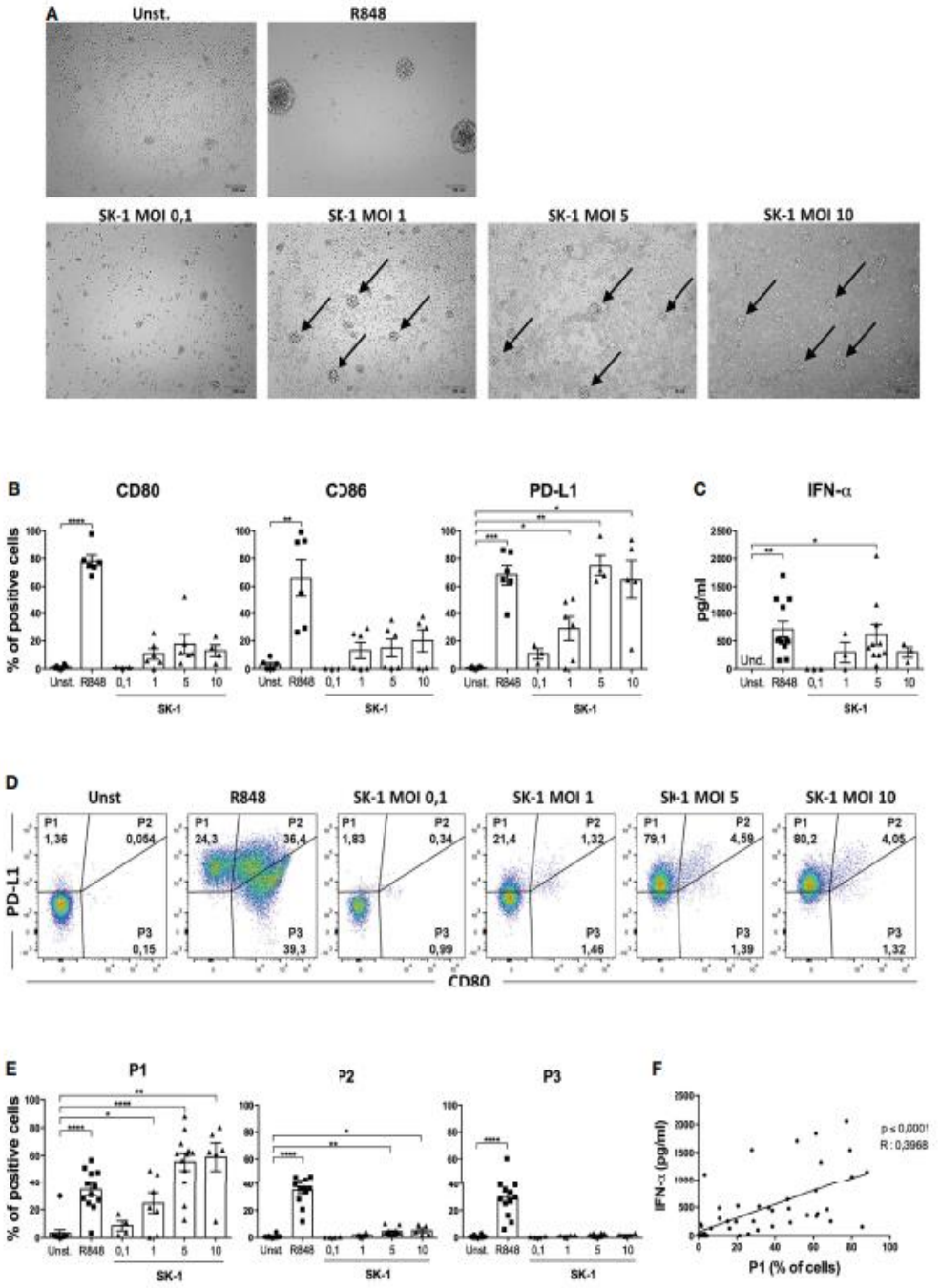

Figure 2. SK-1 induces the differentiation of the P1 subpopulation of human plasmacytoid dendritic cells (pDCs). Human pDCs purified from the peripheral blood of healthy donors were cultured for 48 h without stimulation (Unst.), with R848 (1 mg/ml) as positive control, or with the laboratory strain of Saccharomyces cerevisiae SK-1 at different multiplicity of infection values (0.1–1–5–10; colony-forming unit SK-1/pDC). Photos by optical microscopy show cell clusters, indicated by arrows, formed by pDCs upon activation. The pictures are representative of three independent experiments, each from a different donor (A). The expression of molecules CD80, CD86, and PD-L1 was analyzed by flow cytometry and reported as the percentage of positive cells (B). Levels of IFN-a were measured in the culture supernatants by ELISA assay (C). The expression of PD-L1 and CD80 was analyzed by flow cytometry. Representative plots show the percentage of three pDC subpopulations (D), and the graphs show the corresponding quantification of more experiments (E). Data in (B, C, E) are the mean of six or more independent experiments, each from different donors. Error bars represent SEM. One-way ANOVA was used to compare different experimental conditions (*=p-value ≤ 0.05; **=p-value ≤ 0.01; ***=p-value ≤ 0.001; ****=p-value ≤ 0.0001). The percentages of P1 subpopulation, obtained from all experimental conditions of 4 independent experiments, were correlated to their IFN-a levels using Pearson correlation (F). The R value indicates the correlation coefficient.

Fungal Nucleic Acids Activate Human pDCs

In order to investigate the mechanism inducing blood DC activation by S. cerevisiae, we firstly analyzed the involvement of TLR7 and TLR8, able to recognize microbial RNA, in SK-1- mediated DC activation. We performed a set of experiments in the presence of a synthetic antagonist inhibitor of TLR7 in pDCs and TLR8 in cDCs. First, human-purified DCs were pre-treated for 30 min with the inhibitor and then stimulated for 48 h with SK-1 at MOI 5. We analyzed the IFN-a production in pDCs and IL-6 production in cDCs, and we compared the results with those obtained in SK-1-stimulated DCs without inhibitor. Interestingly, IFN-a production in pDCs, not IL-6 in cDCs, is significantly reduced in the presence of TLR7-8 inhibitor (Figure 3A). Importantly, we tested different doses of TLR7-8 inhibitor, and the results confirmed that TLR8 is not involved in IL-6 production by cDCs stimulated with SK-1 (Supplementary Figure S4A), while it is involved in those stimulated with R848, which is the ligand of TLR7-8 (Supplementary Figure S4B). Since pDCs also express TLR9, which recognizes microbial DNA, we cultured SK-1-stimulated pDCs in the presence of TLR7-9 inhibitor. We observed a reduction of IFN-a levels in pDCs stimulated with SK-1 and pre-treated with TLR7-9 inhibitor (Figure 3B). However, we could not appreciate an additive effect due to the inhibition of both TLR7 and TLR9 signaling compared with the inhibition of TLR7 alone (Figure 3B). In contrast, the expression of CD80, CD86, and PD-L1 seems to be not mediated by fungal nucleic acids. In fact, PD-L1 expression in SK-1- stimulated pDCs and cDCs was not affected by the presence of TLR7-8-9 inhibitors (Figures 3C–F), and the CD80 and CD86 expression, respectively, were weakly increased in pDCs in the presence of TLR7-8 inhibitor (Figures 3C, D). These results suggest that IFN-a production in pDCs could be mediated by the interaction between yeast nucleic acids and TLR, while the upregulation of surface markers on the pDC surface depends on other pathways, which are further activated in response to TLR7 inhibition. On the other hand, recognition of whole SK-1 by cDCs occurs through other pathways independent of TLR8.

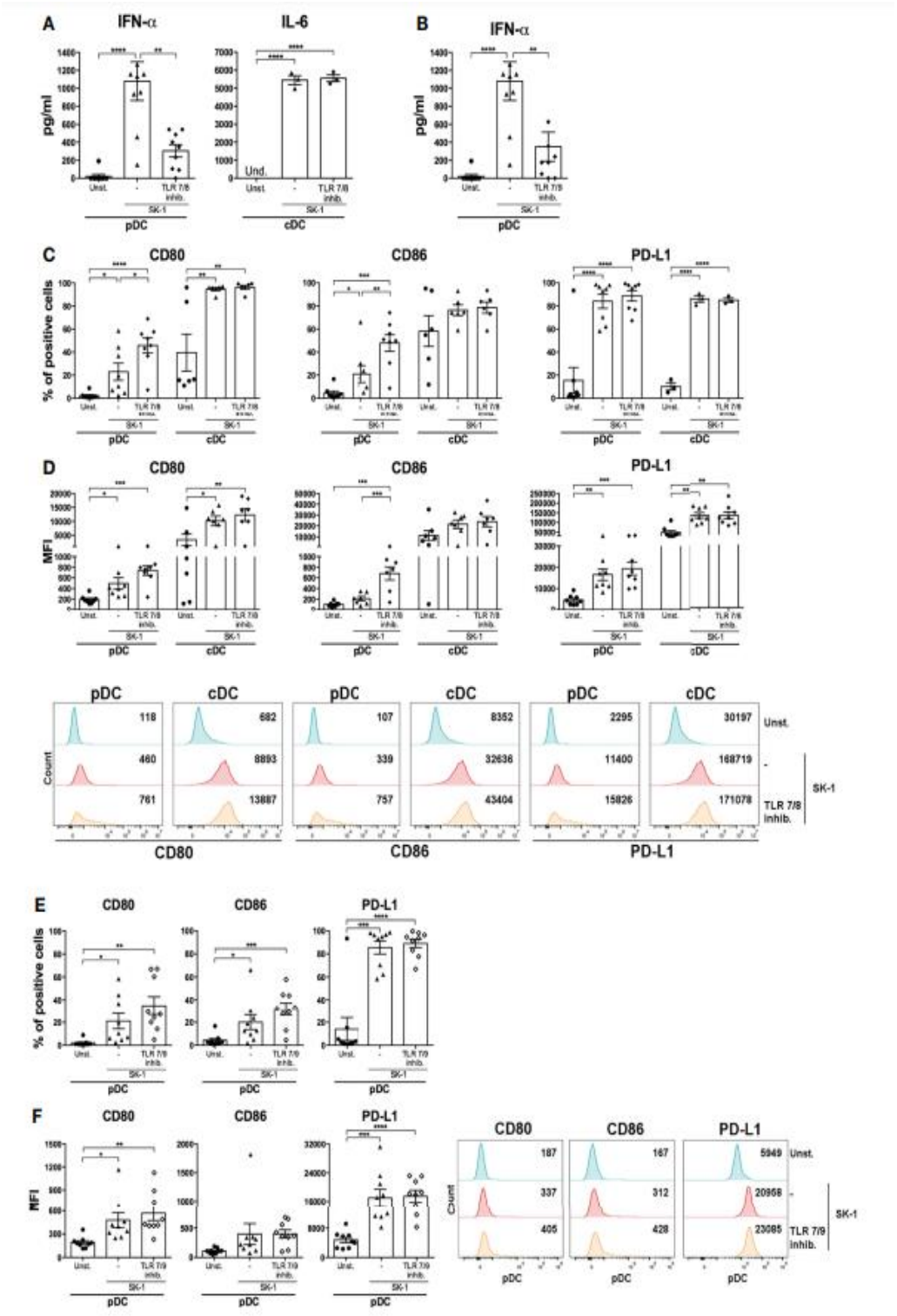

Figure 3. | IFN-a production by SK-1-stimulated plasmacytoid dendritic cells (pDCs) is mediated by sensors of nucleic acids. Human pDCs and conventional dendritic cells (cDCs) purified from the peripheral blood of healthy donors were pre-treated for 30 min with TLR 7/8 (1 mM) or with TLR 7/9 (1 mM) and cultured for 48 h without stimulation (Unst.) or with the laboratory strain of Saccharomyces cerevisiae SK-1 at multiplicity of infection = 5 (SK-1/DC). The levels of IFN-a or IL-6 were measured in the culture supernatants by ELISA (A, B). The expression of molecules CD80, CD86, and PD-L1 was analyzed by flow cytometry and reported as the percentage of positive cells and median fluorescence intensity (C–F). The graphs show the mean ± SEM of three or more independent experiments, each from different donors. Twoway ANOVA was used to compare different experimental conditions (*=p-value ≤ 0.05; **=p-value ≤ 0.01; ***=p-value ≤ 0.001; ****=p-value ≤ 0.0001).

In order to directly assess whether *S. cerevisiae* nucleic acids induce IFN-a production by pDCs, we stimulated blood pDCs with RNA and DNA extracted from SK-1. We found that both nucleic acids induce cell cluster formation typical of pDC activation (Figure 4A) and IFN-a production (Figure 4B). To further characterize this response, we performed experiments in the presence of nucleases specifically degrading single-strand (ss) or double-strand (ds) yeast nucleic acids. We found that degradation of ssRNA, ssDNA, and dsDNA of SK-1 nucleic acids inhibits IFN-a production by human pDCs (Figure 4C). Interestingly, nucleic acids from SK-1 do not induce the production of pro-inflammatory cytokine IL-6 by cDCs (Supplementary Figure S5). Moreover, RNA and DNA from SK-1 induce P1-pDC differentiation (Figure 4D), upregulation of the activation markers (CD80 and CD86), and the inhibitory marker PD-L1 at a similar extent to whole SK-1 (Figures 4E, F). These data collectively indicate that yeast nucleic acids specifically activate human pDCs, leading to IFN-a, CD80, CD86, and PD-L1 overexpression. In order to investigate the role of TLRs in recognizing SK-1 nucleic acid in pDCs, we used TLR inhibitors in yeast DNA- and yeast RNA-treated cells, and we found that TLR7 and TLR7/9 inhibitors partially reduce IFNa production by SK-1 RNA and DNA, respectively (Figure 5A). The percentage of P1-pDC subpopulation (Figures 5B, C) and the expression of CD80, CD86, and PD-L1 are not affected by the presence of TLR7 and TLR9 inhibitors (Figures 5D, E).

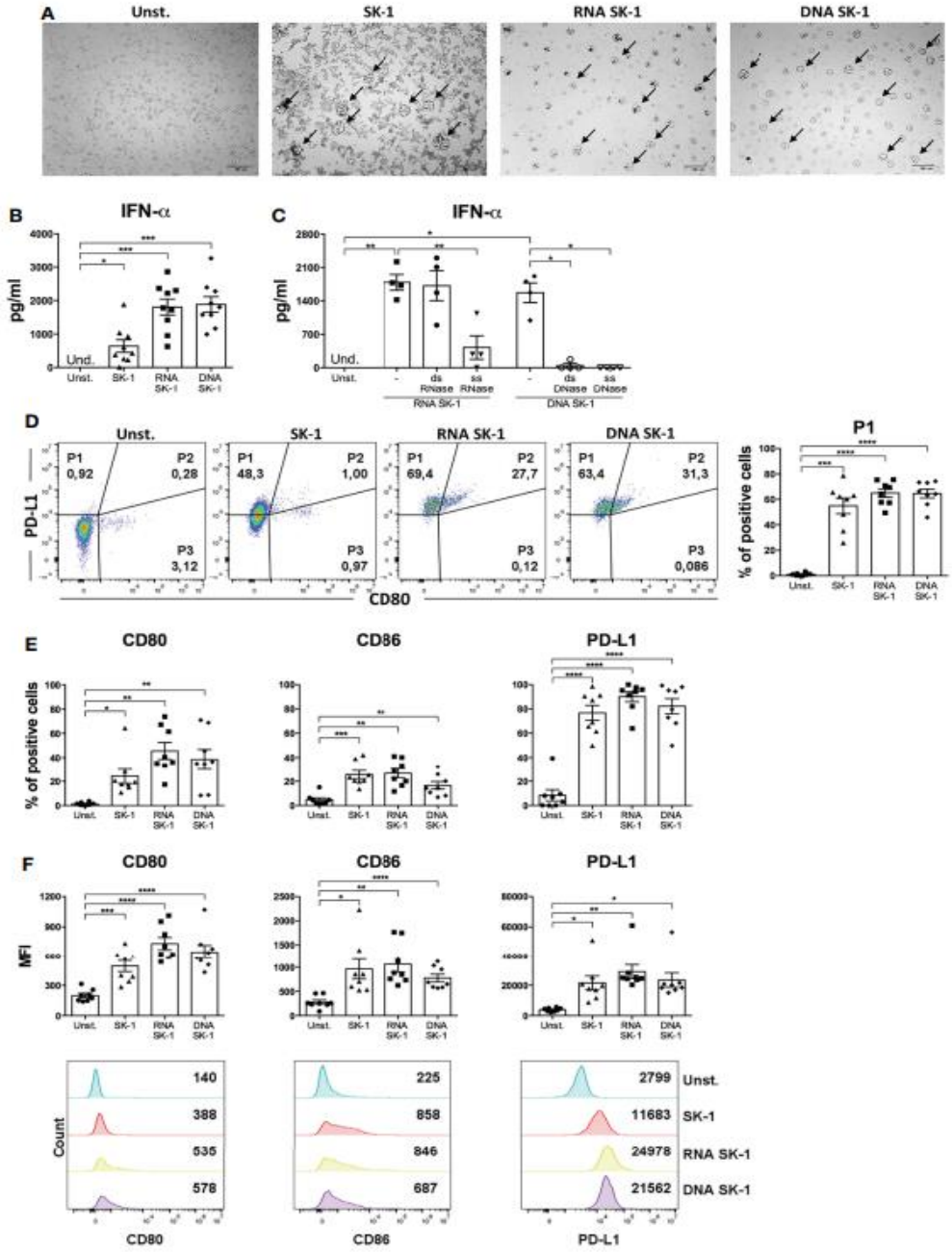

Figure 4. RNA and DNA of SK-1 induce activation of human plasmacytoid dendritic cells (pDCs). Human pDCs purified from the peripheral blood of healthy donors were cultured for 48 h with RNA or DNA (0.2 mg) extracted from Saccharomyces cerevisiae SK-1 and pre-treated with Dotap (10 ml/mg of nucleic acids) for 30 min at 37° C without stimulation (Unst.) and with the laboratory strain of Saccharomyces cerevisiae SK-1 at multiplicity of infection = 5 (SK-1/pDC). Photos taken with an optical microscope show the cell clusters formed by pDCs upon activation highlighted by arrows (A). The pictures are representative of four experiments, each from a different donor. DNA and RNA were pre-treated with nucleases where indicated. The levels of IFN-a were measured in the culture supernatants by ELISA (B, C). The percentage of the P1-pDC subpopulation was evaluated by flow cytometry (D). The percentages and median fluorescence intensity of costimulatory molecules CD80, CD86, and PD-L1 were analyzed by flow cytometry (E, F). The graphs show the mean ± SEM of eight independent experiments, each from different donors. One-way ANOVA was used to compare different experimental conditions (*=p-value ≤ 0.05; **=p-value ≤ 0.01; ***=p-value ≤ 0.001; ****=p-value ≤ 0.0001)

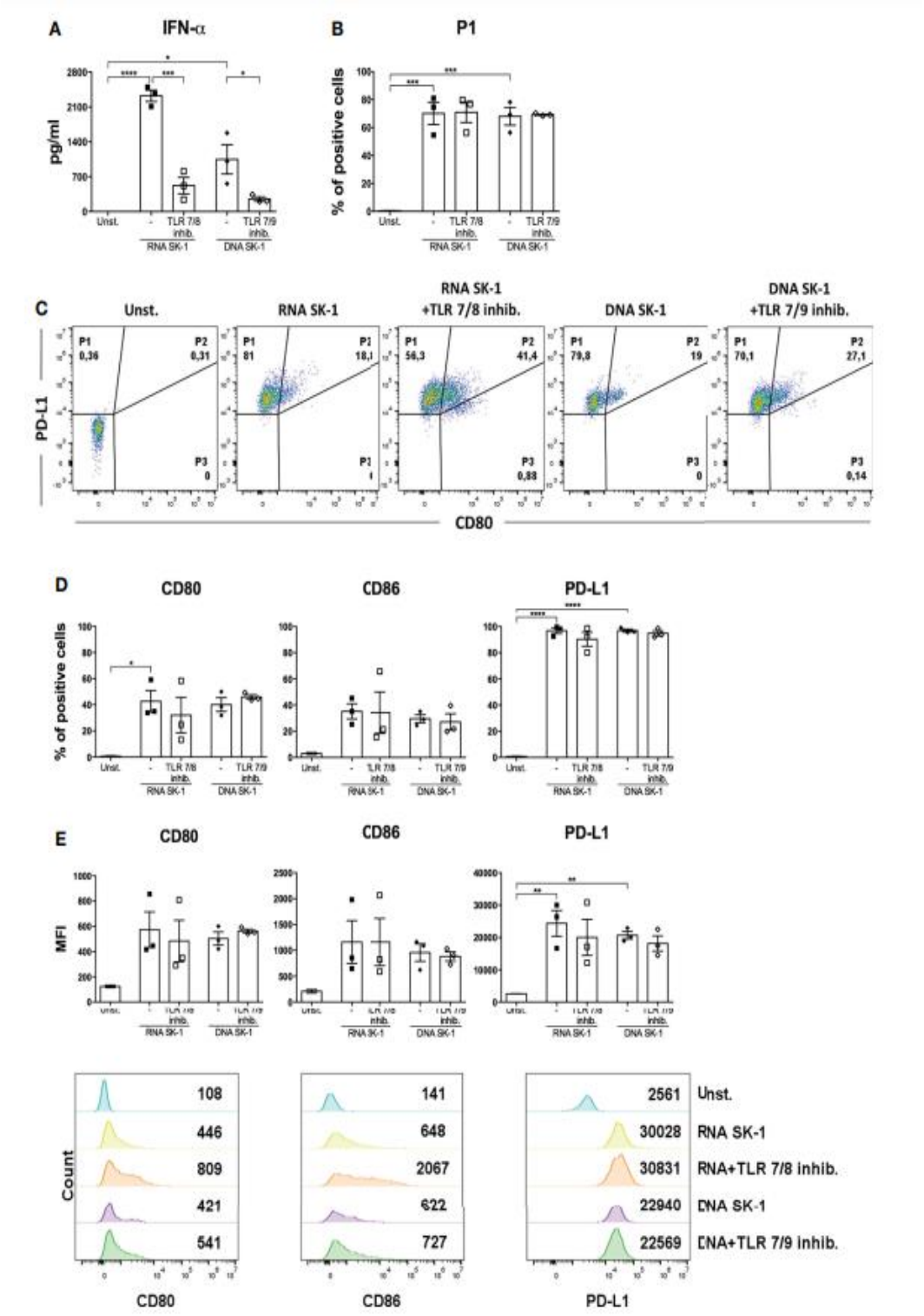

Figure 5. IFN-a production by human plasmacytoid dendritic cells (pDCs) stimulated with SK-1 nucleic acids is partially mediated by TLR7 and TLR9. Human pDCs purified from the peripheral blood of healthy donors were pre-treated for 30 min with TLR7/8 (1 mM) or with TLR7/9 (1 mM) and cultured for 48 h with RNA or DNA (0.2 mg) after incubation with Dotap (10 ml/mg of nucleic acids) for 30 min at 37°C without stimulation (Unst.). The levels of IFN-a were measured in the culture supernatants by ELISA (A). The percentage of the P1-pDC subpopulation was evaluated by flow cytometry (B, C). The percentages and median fluorescence intensity of costimulatory molecules CD80, CD86, and PD-L1 were analyzed by flow cytometry (D, E). The graphs show the mean ± SEM of three independent experiments, each from different donors. Two-way ANOVA was used to compare different experimental conditions (*=p-value ≤ 0.05; **=p-value ≤ 0.01; ***=p-value ≤ 0.001; ****=p-value ≤ 0.0001).

SK-1-Primed pDCs and cDCs Induce IL10- and IL-17-Producing CD4 T Cells

Activated DCs perform important adaptive functions in naïve CD4 T cell priming and polarization. Given the differential response of pDCs and cDCs to S. cerevisiae in terms of cytokine production and costimulatory molecule expression, we hypothesized a functional specialization towards distinct T helper (Th) profiles. We stimulated naïve CD4 T cells with antiCD3 to simulate antigen recognition and with SK-1-primed DCs to study the impact of the upregulation of their cytokines and costimulatory receptors on Th polarization. We analyzed the production of typical Th cytokines (IFN-g for Th1, IL-4 for Th2, IL-17 for Th17, and IL-10 for T regulatory cells) in T cells co-cultured with SK-1-primed DCs compared with T cells cocultured with unstimulated DCs and T cells stimulated with antiCD3 and anti-CD28. We found that IL-4 is not significantly modulated in the different experimental conditions (Figures 6A, B), while SK-1-primed cDCs induce significant IL-17 production compared with unstimulated cDCs (Figure 6B) and SK-1- primed pDCs induce significant IL-10 production compared with unstimulated pDCs (Figure 6A). Concomitantly to IL-17 production, we observed a significant reduction of IFN-g by naïve CD4 T cells co-cultured with SK-1-primed cDCs (Figure 6B).

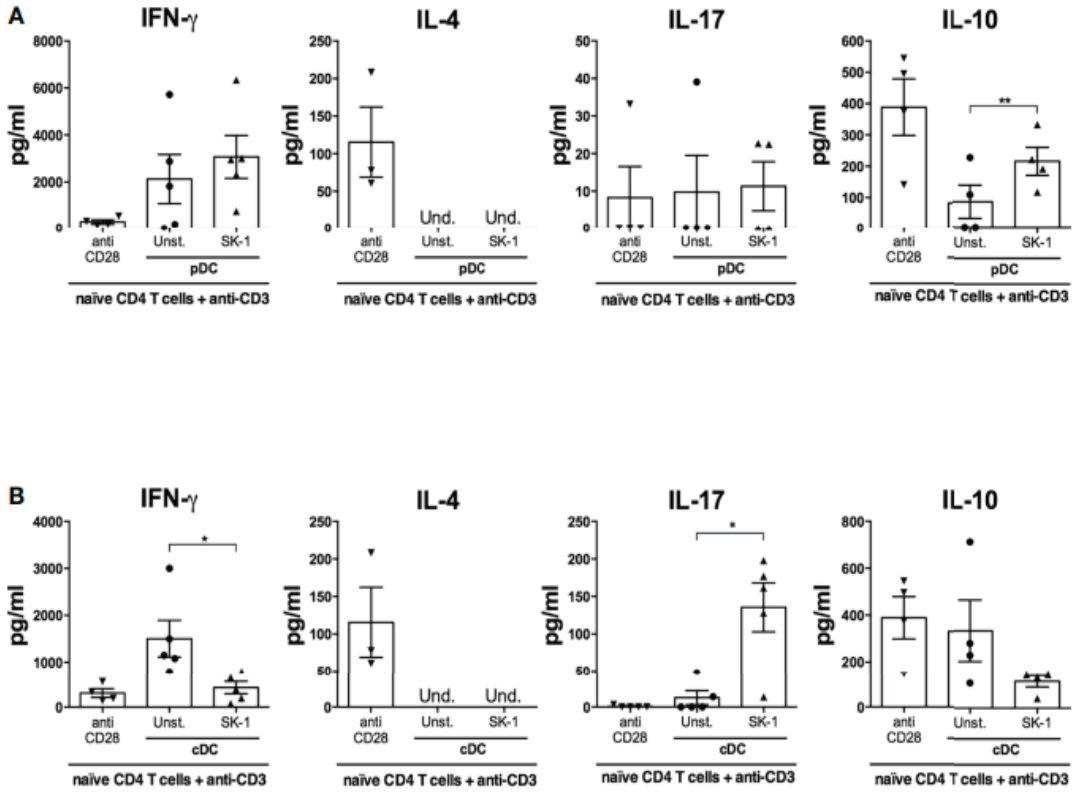

Figure 6. Plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs) primed with SK-1 induce IL-10- and IL-17-producing CD4 T cells, respectively. Human naïve CD4 T cells were cultured in the presence of anti-CD3/CD28 beads or placed in a co-culture for 5 days with unstimulated or SK-1-primed pDCs or cDCs in the presence of anti-CD3. After 5 days, the cells were re-stimulated with anti-CD3 (co-culture) or anti-CD3/CD28 beads (CD4 T cells alone). Supernatants were collected after 24 h of re-stimulation, and the levels of IFN-g, IL-4, IL-17, and IL-10 in co-cultures with pDCs (A) and cDCs (B) were measured by ELISA. The graphs show the mean ± SEM of four or five independent experiments, each from different donors. Paired Student's t-test was used to compare unstimulated and stimulated DCs (*=p-value ≤ 0.05; **=p-value ≤ 0.01).

MATERIALS AND METHODS

Purification of DC Subpopulations (cDCs and pDCs) From Adult Blood

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation (GE Healthcare) from the whole blood of healthy donors (Santa Lucia Foundation, Rome, Italy). Approval by the ethics committee of the Santa Lucia Foundation, Rome (Italy), and written informed consent in accordance with the Declaration of Helsinki from all participants were obtained before the study was initiated. PBMCs (400 × 106) were enriched using the Human Pan-DC Pre-Enrichment Kit (Stemcell Technologies), specific for the purification of all DC type, by negative selection. After isolation, the cells were stained with the following antibodies: anti-human CD11c PE-Dazzle594 (BD Biosciences), anti-human CD4 PC7 (Beckman Coulter), anti-human CD3 PE (Beckman Coulter), anti-human CD14 PE (Immunological Science), antihuman CD16 PE (Miltenyi Biotec), anti-human CD56 PE (Beckman Coulter), anti-human CD19 PE (Miltenyi Biotec), and anti-human CD235a (eBioscience). Highly purified pDC and cDC cells were selected and sorted by 6-way sorter MoFlo Astrios (Beckman Coulter) by using the following analysis as previously described (18): lineage (CD3, CD14, CD16, CD56, CD19, CD235a) - , CD4+ , CD11c- for pDCs and lineage (CD3, CD14, CD16, CD56, CD19, CD235a) - , CD4+ , CD11c+ for cDCs. The sorted cells had a purity of over 95 and 94%, respectively, for pDCs and cDCs, as confirmed by flow cytometry analysis (Supplementary Figures S1A, B). This gating strategy includes only pDCs CD123+ and cDCs HLA-DR+ cells (Supplementary Figure S2).

In Vitro Stimulation of pDCs and cDCs

pDCs and cDCs were cultured separately in 96-well flat-bottom half-area plates (Corning) at a density of 5 × 104 per well in RPMI 1640 with 5% of human serum for 48 h at 37°C with 5% of CO2, in the absence of stimuli or in the presence of R848 (InvivoGen; 100 ng/ml), SK-1 (Saccharomyces cerevisiae; MOI: 0.1–1–5–10 SK-1 cells/cDC or pDC). For the SK-1 nucleic acid stimulation experiments, the RNA and DNA (0.2 mg) were extracted from SK-1 as previously described (16), and their quality and purity were verified by agarose gel electrophoresis and using a Nanodrop 2000 spectrophotometer (Thermofisher; Supplementary Figure S6). SK1 RNA and SK-1 DNA samples were used for pDC stimulation pretreated with Dotap (Merck; 10 ml/mg of nucleic acids). Pre-treatment of nucleic acids with ezDNase or RNase III (Thermofisher) was performed to degrade dsDNA and dsRNA, respectively, while S1 nuclease (Thermofisher) was used to degrade ssDNA and ssRNA. For the TLR inhibition experiments, cDCs and pDCs were pretreated for 30 min with TLR7/8 (Miltenyi Biotec ODN 2087) or TLR7/9 (Miltenyi Biotec ODN 2088) inhibitors (1 mM) and then stimulated with SK-1 (MOI 5).

Naïve CD4 T Cells and DC Subset Coculture

pDC and cDC subsets from healthy donors were cultured separately in 96-well flat-bottom half-area plates (Corning) at a density of 5 × 104 per well in RPMI 1640 with 5% of human serum in the absence of stimuli or in the presence of SK-1 (MOI 5). CD4 T lymphocytes were purified from the PBMCs of the same healthy donors by immunomagnetic selection using antiMouse IgG MicroBeads (Miltenyi Biotec). After isolation, the cells were stained with anti-human CD4 PC7 (Beckman Coulter), anti-human CD45RA BV421 (BD Biosciences), antihuman CD45RO PE (BD Biosciences), and anti-human

CD27 APC (Beckman Coulter), and CD4 naïve T cells were sorted by Astrios high-speed cell sorter (Beckman Coulter) as CD4high, CD45RAhigh, CD45RO- , and CD27+ . The sorted cells had a purity of over 97%, as shown by flow cytometry (Supplementary Figure S7). After 24 h, the stimulated DCs were collected and cocultured with naive CD4 T cells at a density of 5 × 104 DCs and 5 × 104 ymphocytes (ratio 1:1) per well in X-VIVO 15 serum-free medium (Lonza) in 96-well round-bottom plates (Falcon) coated with anti-CD3 (BD Biosciences; 10 mg/ml). Naïve CD4 T cells stimulated with Dynabeads CD3/CD28 T cell expander (1 bead per cell; Life Technologies) were used as control. After 5 days, the cells were harvested and washed, and viability was determined by Trypan Blue exclusion. Then, the cells were resuspended in X-VIVO 15 serum-free medium (Lonza) at a concentration of 1 × 106 cells/ml and restimulated with anti-CD3 for 24 h in 96-well flat-bottom plates (Falcon). The conditions of incubation were stable (temperature at 37°C with 5% CO2).

Flow Cytometry Analysis

pDCs and cDCs were harvested after 48 h of culture, resuspended in an EDTA-containing medium, and then stained for 15 min at 4°C with the following antibodies: antihuman CD4 PC7 (Beckman Coulter), anti-human PD-L1 PE (Biolegend), anti-human CD86 APC (Miltenyi Biotec), and antihuman CD80 BV650 (BD Bioscience). The samples were washed in EDTA-containing medium, acquired using CytoFLEX cytometer (Beckman Coulter) and analyzed by FlowJo-10 software (version 10.3.0).

Analysis of Cytokine Production

Cytokines were measured in supernatants from pDC and cDC cultures, respectively, using IFN-a ELISA (Invitrogen, Human IFN alpha Antibody Pair Kit), IL-6 ELISA (R&D Systems, Human IL-6), IL-17 ELISA (R&D Systems, Human IL-17), IL4 ELISA (R&D Systems, Human IL-4), IFN-g ELISA (R&D Systems, Human IFN-g), and IL-10 ELISA (R&D Systems, Human IL-10) according to the manufacturer's instructions.

Statistical Analysis

Statistical analyses were performed using one-way ANOVA, twoway ANOVA, or Student's t-test, depending on the number of experimental conditions and independent variables. We used GraphPad Prism software (version 6.01, GraphPad Software). Data were presented as mean ± standard error (SEM). The pvalues of 0.05 or less were considered statistically significant.

DISCUSSION

SK-1 is a fungus with phylogenetic similarity to yeasts isolated from fermentation in the African area (19) and is most likely one of the constituents of the human microbiota derived from the ingestion of fermentation products. However, S. cerevisiae has also been found in the bloodstream of immunocompromised patients (20–22), thus considering this fungus an emerging opportunistic pathogen (23). Therefore, our study is useful for understanding the interaction of mucosal DCs with intestinal microbiota and for the analysis of the anti-fungal immunity of blood DCs against pathogenic S. cerevisiae. The results from this study revealed that SK-1 is able to activate human blood pDCs and cDCs, indicating that both DC subsets have an important role in modulating the immune response following a fungal stimulus. Importantly, we observed a differential activation of

pDCs and cDCs by SK-1, suggesting a distinct role of each DC subset in the anti-fungal immunity and in the interaction with intestinal microbiota. Specifically, SK-1 induces a high induction of IFN-a and expansion of a subpopulation of pDCs, called P1, characterized by the expression of PD-L1 molecule on the cell surface. However, SK-1 does not stimulate a strong maturation process in these cells; in fact, the co-stimulatory molecules CD80 and CD86 are only weakly induced, unlike what happens as a consequence of their activation mediated by viruses (24), which, in our system, is simulated by R848. A robust IFN-a production associated to a weak induction of costimulatory molecules at the pDC surface is a typical response of CpG-A (25). Thus, similarly to CpG-A, fungal nucleic acids could form a large multimeric complex upon internalization that is retained in early endosomes and signals through MyD88 and IRF-7 (26). We observed that SK-1 promotes the expansion of P1-pDC subpopulation (PD-L1+ CD80-), which is specialized in the production of IFN-a and in the induction of the antiinflammatory cytokine IL-10 by T cell lymphocytes (17). Consistently, our data showed that pDCs stimulated with SK-1 induce IL-10-producing T cells. It is known that IL-10 and IFNa synergistically promote the differentiation of a particular population of regulatory T lymphocytes known as Tr1 (IL-10+ , IFN-g+, IL-2- / low, and IL4-), which is capable of suppressing the proliferation of T lymphocytes (27). These results altogether suggest that SK-1, by inducing pDCs to produce IFN-a and the consequent generation of IL-10-producing T cells, may contribute to the generation of immunoregulatory T cells. The activation of a regulatory response by pDCs has been described in previous papers (7, 28–30), suggesting that this is a typical property of pDCs regardless of the nature of the activating stimulus. Moreover, the immunosuppressive activity of T regulatory cells has been described in fungal infections (31–34). Specifically, the anti-inflammatory role of DCs in response to fungi is mediated by the enzyme indoleamine 2,3-dioxygenase (IDO) (35), which is associated with the induction of IL-10- producing T regulatory cells (33). Thus, the metabolic pathway involving tryptophan catabolism could be involved in pDC response to SK-1 and local tolerogenic responses. Given the importance of the anti-inflammatory responses in chronic autoimmune diseases, such as multiple sclerosis and Crohn's disease, pDC response to SK-1 could play a protective role in these diseases. In contrast to pDCs, cDCs express high levels of CD80 and CD86 in response to SK-1, produce high levels of the inflammatory cytokine IL-6, and promote a Th17 response, suggesting that the interaction between S. cerevisiae and cDCs in the gut contributes to mucosal inflammation and to mucosal host defense against fungal infection (36). Importantly, cDCs promote a simultaneous reduction of IFN-g production by CD4 T cells that suggests a prominent role of S. cerevisiae and cDCs in regulating the balance between IL-17-producing (Th17) and IL-17/IFNg-producing (Th1/17) cells. It is already known that the yeast cell wall, characterized by galactose and glucosamine (chitin moieties), glucose (betaglucan), and mannose (mannans), induces the inflammatory response in monocyte-derived DCs (14). Our data suggest that the components of the yeast cell wall are also involved in the activation of blood cDCs, cells which share numerous similarities with monocyte-derived DCs. However, here, for the first time, we reported that the nucleic acids of S. cerevisiae specifically induce cell activation and IFN-a production in pDCs. In particular, ssRNA, ssDNA, and dsDNA derived from SK-1 are involved in pDC activation. Importantly, the induction of IFN-a is partially mediated by TLR7 and TLR9, while the upregulation of co-stimulatory molecules in pDCs is mediated by other pathways. Among the potential receptors of yeast PAMPs, we suppose that galectin-3 and Fc-g receptor, recognizing yeast mannans, and DNA-

PK, cGAS (CGAS), MRE11, DHX36, DHX9, DDX41, and DDX60, sensing yeast nuclei acids, may have a role in SK-1-mediated activation because they are expressed by human pDCs (Supplementary Figures S8A, B). We do not observe an additive effect in IFN-a blocking due to the inhibition of both TLR7 and TLR9 signaling compared with the inhibition of TLR7 alone that could be due to a different effectiveness exerted by TLR7/8 and by TLR7/9 inhibitors on TLR7 or to the involvement of other DNA sensors in pDCs. In this context, it has been reported that pDCs express DHX36, DHX9 (37), and cGAS (38) (Supplementary Figure S8B), which are TLR9-independent DNA sensors. Previous studies reported that nucleic acids induce the maturation of DCs and generate an anti-fungal immune response (39, 40). Specifically, DNA is recognized by TLR9 in C. albicans (41, 42), A. fumigatus (43), and C. neoformans (44) infection, while S. cerevisiae and C. albicans RNA are recognized by TLR7 (40, 45) and C. albicans and A. fumigatus RNA by MDA5 (39, 40, 46, 47). Interestingly, our data indicate that TLR8 inhibitor does not reduce IL-6 production nor the upregulation of co-stimulatory molecules in cDCs, indicating that the SK-1 cell wall components are the main stimuli for cDCs and that the response to SK-1 nucleic acids is a typical feature of pDCs. These results altogether indicate that the exposure to S. cerevisiae triggers pro- or anti-inflammatory responses depending on the interaction with cDCs or pDCs, respectively. Importantly, the nucleic acids of S. cerevisiae are a specific anti-inflammatory trigger for pDCs. This information indicates that the opposite role of pDCs and cDCs in response to S. cerevisiae could mediate the equilibrium between the proinflammatory and the anti-inflammatory immune responses in the gut, which is important either for gut homeostasis or during a S. cerevisiae opportunistic infection. The immune dysregulation, characterized by an imbalanced prevalence of cDCs or pDCs, could alter this equilibrium and could lead to the development of autoimmune diseases or exacerbation of fungal infections.

Future studies aimed to investigate the molecular mechanisms leading to IFN-a production by pDCs, such as the identification of the activating RNA or DNA sequence of S. cerevisiae, could open new perspectives towards therapeutic approaches for dysbiosis-related diseases, such as probiotic intervention. On the other side, the identification of the molecular mechanisms leading to the pro-inflammatory activity of cDCs by S. cerevisiae could be useful for the therapeutic targeting of chronic inflammatory diseases and for a better understanding of the mechanisms underlying the immune response during fungal infections in immunocompromised patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the IRCCS Fondazione Santa Lucia. The patients/ participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS and GG performed the research and analyzed the data. MC and GR performed some experiments. MDB performed sorting experiments. SR cultured and provided S. cerevisiae and

nucleic acids of SK-1. DC and LB contributed to the research design. DFA designed the research and analyzed the data. EV designed the research, analyzed the data, and wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.850404/ full#supplementary-material

Supplementary Figure 1 | Gating strategy for the purification of blood dendritic cells (DCs). Human peripheral blood mononuclear cells purified from the peripheral blood of healthy donors were enriched using the Human Pan-DC Pre-Enrichment Kit (EasySep) and then labeled with specific antibodies conjugated with a fluorochrome. Using a cell sorter, the plasmacytoid dendritic cells (pDCs) were isolated as lineage (CD3, CD14, CD16, CD19, CD56, CD235a) - CD4+ , CD11c- , while conventional dendritic cells (cDCs) were isolated as lineage (CD3, CD14, CD16, CD19, CD56, CD235a) - CD4+ , CD11c+ (A). The purity of the isolated pDCs and cDCs, evaluated by flow cytometry after purification, is more than 94% (B).

Supplementary Figure 2 | The human blood plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs) used in this study are CD123+ and HLA-DR+ cells, respectively. Human peripheral blood mononuclear cells purified from the peripheral blood of healthy donors were labeled with specific antibodies conjugated with a fluorochrome. pDCs were identified as lineage (CD3, CD14, CD16, CD19, CD56, CD235a) - CD4+ , CD11c- , while cDCs were identified as lineage (CD3, CD14, CD16, CD19, CD56, CD235a) - CD4+ , CD11c+ . Staining of CD123 and HLA-DR before (A) and after sorting (B) reveals that pDCs are CD123+ cells, while cDCs are HLA-DR+ cells. The plots show the data of a representative of more experiments.

Supplementary Figure 3 | Fluorescence-minus-one control (FMO) for PD-L1 staining. Human plasmacytoid dendritic cells (pDCs) purified from the peripheral blood of healthy donors were cultured for 48 h without stimulation (Unst.), with R848 (1 mg/ml), or with the laboratory strain of Saccharomyces cerevisiae SK-1 at multiplicity of infection = 5 (colony-forming unit SK-1/pDC). Cells were stained with anti-CD80 BV650 (FMO control) or anti-PD-L1 PE and anti-CD80 BV650 (PD-L1 staining). Percentage of PD-L1+ cells (A), MFI of PD-L1 (B), and percentage of P1, P2, P3 pDC subpopulations (C) were represented. Plots and histograms are from one representative of more experiments.

Supplementary Figure 4 | Human conventional dendritic cell (cDC) activation by SK-1 is not affected by TLR8. Human cDCs purified from the peripheral blood of healthy donors were cultured for 48 h

without stimulation (Unst.) and pre-treated for 30 min with different doses of TLR inhibitor, as indicated, with the laboratory strain of Saccharomyces cerevisiae SK-1 at multiplicity of infection = 5 (SK-1/conventional dendritic cells) (A), or with 5 mg/ml TLR8 inhibitor and R848 (0.1 mg/ml) (B). The levels of IL-6 were measured in the culture supernatants by ELISA. The graphs show the mean ± SEM of 3 independent experiments, each from different donors. Two-way ANOVA was used to compare different experimental conditions (*p-value ≤ 0.05).

Supplementary Figure 5 | Nucleic acids from Saccharomyces cerevisiae SK-1 do not induce the production of IL-6 by conventional dendritic cells (cDCs). Human cDCs purified from the peripheral blood of healthy donors were cultured for 48 h with RNA or DNA (0.2 mg) extracted from S. cerevisiae SK-1 and pre-treated with Dotap (10 ml/mg of nucleic acids) for 30 min at 37°C without stimulation (Unst.) and with the laboratory strain of S. cerevisiae SK-1 at multiplicity of infection = 5 (SK-1/ cDC). The levels of IL-6 were measured in the culture supernatants by ELISA. The graphs show the mean ± SEM of three independent experiments, each from different donors. One-way ANOVA was used to compare different experimental conditions (*p-value ≤ 0.05).

Supplementary Figure 6 | The quality and purity of DNA and RNA extracted from SK-1. The integrity of DNA and RNA samples isolated from SK-1 was analyzed by 1% agarose gel electrophoresis (A). The measurements of absorbance 260/280 ratio of DNA and RNA samples were performed by Nanodrop spectrophotometer (B).

Supplementary Figure 7 | Gating strategy for the purification of human blood naïve CD4 T cells. Human peripheral blood mononuclear cells purified from the peripheral blood of healthy donors were labeled with specific antibodies conjugated with a fluorochrome and, by using a cell sorter, naïve CD4 T cells were isolated as CD4high, CD45RAhigh, CD45RO- , and CD27+ (A). The purity of the isolated naïve CD4 T cells, evaluated by flow cytometry after purification, is more than 97% (B). The plots show the data of a representative of all experiments performed with dendritic cells or naïve CD4 T cells.

Supplementary Figure 8 | Expression of the yeast receptor in human plasmacytoid dendritic cells (pDCs). The expression values of yeast pattern recognition receptors, cell wall (A) and nucleic acid sensors (B), on pDCs freshly isolated from the blood of healthy donors were extracted from Affymetrix data (Human Genome U133 Plus 2.0 arrays) (48). The dashed line is the threshold of signal detection. White bars represent the mRNA levels of the receptors expressed on pDCs; gray bars indicate that the receptors were not detectable. The data are the mean of seven independent experiments, each from different donors. The error bars represent standard deviation.

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Chapter 3

Transitional mycobiomes-rural urban transition leads to reduction of diversity and loss of fungal species in the gut mycobiome of african families

Submitted to *Microbiome*

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Abstract

Urbanization probably represents the global change with the highest impact on human lifestyle. Rural-to-urban transition produced drastic changes in social, food and human health care dynamics. These changes also reflect a remodeling of the intestinal microbial communities with a loss of bacterial richness and diversity and the related encoded functions. As consequence to dietary and lifestyle changes following rising of social-economic status, disease epidemiology is also radically changing with an increase in the incidence of non-communicable diseases. The bacterial fraction of the transitional microbiomes was investigated, but the shaping of intestinal fungal communities following this human transition are almost unexplored. Here, we show changes of gut mycobiota composition and lifestyle of families from Burkina Faso living in areas at different levels of urbanization (rural, semi-urbanized and urban areas), thus following a Western-related gradient. We also compared these changes with that of Italian families, as representative of Western world. These variations were also studied within family members (i.e., mother, father, and children). We observed that families living in rural areas showed a greater intestinal mycobiota complexity, in terms of diversity and composition, compared to the urbanized families of Burkina Faso and Italy. We also found that the mycobionts differed within family members only in rural and in semi-urbanized areas. Furthermore, a differential abundance gradient of the 33 detected fungal ASVs was associated to rural- to-urban transition. We identified 12 fungal species affected by urbanization gradient. These species are known for their immunogenic activity and overall, the impact on human health. Our results shed light on the not yet well-explored transitional mycobiota suggesting a legitimate role of fungal community in the hypothesis of hygiene and implication on human health.

Introduction

The human gut microbiome represents a complex ecosystem teeming with different types of microorganisms such as bacteria, fungi, archaea, and viruses which influence host metabolism, cognitive activities and overall, the human well-being (Gilbert et al., 2018; Kho et al., 2018). This ecosystem is constantly affected by environmental factors which can switch the microbial community imbalance. Among these, globalization has produced substantial changes in the human lifestyle, these changes in the nutritional field are resumed by the term "globesity" due to the radical diet upheaval and the harmful effects on human health (Costa-Font & Mas, 2016). However, diet is only one of the factors on which globalization acts. Globalization changes the local and global dynamics which in turn cause macro- and micro-urban changes imposing a different level of urbanization.

Urbanization has determined, over time, a series of habits focused on minimizing the exposure to pathogenic microorganisms, increasing the use of antibiotics and hygiene precautions, as well as the development of food sterilization procedures. Consequently, urbanization has produced undesirable effects on the human gut microbial ecosystem, eradicating potentially beneficial microorganisms and their related encoded functions (Zuo et al., 2018). This microbial depletion in a wide time scale has been exemplified by the hygiene hypothesis described by David Strachan (Strachan, 1989), subsequently conceptually enriched by Blaser and Falkow introducing the concept

of disappearing microbiota that represent the loss of specific ancestral microorganisms within the human core microbiota due to the Western-related habits (Blaser & Falkow, 2009). Recently, the impoverishment of intestinal bacterial communities was also connected to the change of habits determined by the recent COVID-19 pandemic. In this case, the hygiene hypothesis was linked with the weakness in the structure of the microbiota and a significantly higher incidence to the adverse effects produced by COVID-19 infection (Finlay et al., 2021). African populations living without any influence of urbanization, in terms of socio-economic and health care conditions, represent the ideal settings to observe the effects of urbanization on the human microbiota. The Interaction with environmental fungal communities is essential for the development of immunocompetence from the first years of life (Rook, 2013). The ability of fungi to interact and modulate immune activity through cell wall structures underlies several chronic inflammatory disorders (Gow & Netea, 2016). Therefore, the knowledge concerning the communication between fungi and the human immune system has been central for the development of therapeutic solutions against infections and vaccines (Rappuoli et al. 2014).

The effect of the non-Western diet in Burkina Faso populations has been linked to beneficial effects such as the increase in the levels of short-chain fatty acids (SCFAs) produced by the gut bacterial community (De Filippo et al., 2010). The effects of urbanization on gut microbiome composition were also investigated in a follow up study in Burkina (De Filippo et al., 2017) and in two distinct rural populations of Papua New Guinea (Martinez et al., 2015) and in rural and urban individuals in South Africa (Kabwe et al., 2020). All the studies highlight a trend of reduced bacterial diversity, depletion of Prevotellaceae and SCFA as associated with urbanization. Overall research focused on the gut microbiome's response to urbanization has connected contemporary lifestyles to a loss of bacterial diversity and a propensity to develop typical Western conditions such as obesity and autoimmune diseases (Zuo et al., 2018; Blaser, 2017; Vangay et al., 2018). The fungal communities closely adapted to the human intestine (Strati et al., 2016b) undergo the impact of the environmental factors and their disequilibrium has strong implications on human health (Strati et al., 2016a; Strati et al., 2017). Thus, to date, the influence of urbanization on bacterial microbiome has significantly increased, however, the knowledge related to the fungal microbiota, also referred as mycobiota, remains virtually untapped. To investigate the role of urbanization on the fungal communities of the human gut we compared the mycobiomes within family members stratified for age and gender from Italy and Burkina Faso areas. Urbanization rate in Sub-Saharan Africa is the highest globally; while the Westernization of lifestyle is already pervasive in metropolitan areas, as people in rural areas are still close to a traditional lifestyle but starting to be exposed to the increasing contamination of Western-like factors. This trend highlights socio-economic developments as well as a transition from subsistence farming to more specialized occupations. The epidemiology of diseases is also drastically shifting. Numerous studies have suggested that by 2030, the prevalence of non-communicable diseases (NCDs) such cancer, chronic renal and respiratory illnesses, and dietary-related disorders may surpass incidences of infectious diseases in sub-Saharan Africa due to changes in urban lifestyle and nutrition (Kiawi et al., 2006; Frank et al., 2014; Dake et al., 2016).

The demographic transition in Burkina Faso, a nation in West Africa, is still in its early stages. The primary urban area comprises Ouagadougou's capital city and its surrounding area. Here, the lifestyle changes typical of urban populations have already occurred (Bosu, 2015). On the other hand, Burkina Faso's rural areas still exhibit a traditional rural lifestyle that is mostly based on Neolithic-like subsistence farming and with minimal contact with the rest of the world. Few studies have previously characterized the microbiota and dietary habits of rural and urban populations (Becquey et a., 2010; De Filippo et al., 2017; Sié et al., 2018; Casari et al., 2022), but a thorough investigation of the gut mycobiota of the entire family unit, i.e., father, mother, and children, who live in environments with different degrees of urbanization, among different geographic areas, is still lacking. It is critical to assess the impact of environmental factors on the gut mycobiome across a larger sample of participants in order to determine the implications on host-microbiota dynamics and health. Currently, few studies have focused on the characterization of the eukaryotic components of the human gut of African individuals (Hamad et al., 2016; Kabwe et al., 2020; Nel Van Zyl et al., 2022). Therefore, it is well known the importance of studying the mycobiome variations according to different life stages given the different impact exerted by fungi on human health during the lifespan (Collado et al., 2015; Koren & Rautava, 2020).

The aim of the present work is to understand how the effect of urbanization, expressed by the transition of human populations from rural to urban environment, influence the structure of the intestinal mycobiota. For this purpose, we decided to expand the previous surveys on the individuals of Burkina Faso (De Filippo et al 2010 and 2017) to a wider cohort, considering the effect of diet, geography and social status, in parallel to the differences related to age, sex and family, by sampling feces from families and households from three different environments, representing three phases of the demographic transition process from rural to urban environment, and comparing them with an Italian cohort of healthy families as Western controls. We demonstrated that fungal diversity was significantly influenced by the degree of urbanization and this varied within family members only as a function of the area of origin. In fact, the fungal community changed within the family members in semi-urban and rural areas only. The taxonomic variants that significantly changed within the dataset followed the same pattern, highlighting the presence of abundance gradients depending on the degree of urbanization.

Material and Methods

Participant enrolment criteria for urban and rural areas

In Burkina Faso thirty healthy families (adults and children) have been selected from the same ethnic group, the Mossi ethnicity, based on different socio-economic conditions, lifestyle, environments (rural and urban areas), diets, hygiene habits and sanitation. These parameters were collected by questionnaire for each individual subject and summarized in Supplementary Table 1. The enrolled cohorts are the followings: (i) 10 families (composed of at least 2 parents and up to 4 children) living in rural villages located around Nanoro town, in environments resembling that of Neolithic subsistence farmers and eating a traditional vegetarian diet (based on millet, sorghum, Niebè legumes and Nerè vegetable), as representative of a traditional and rural population far from Western influence on diet and lifestyle; (ii) 10 families (composed of at least 2 parents and up to 4

children) from the small town of Nanoro, corresponding to an initial urbanization status. These families are still used to eating cereals and legumes, similarly to the rural population but, depending on their socio-economic status, their diet also contains once-a-week meat or dried fish. (iii) 10 families (composed of 2 parents and up to 4 children) in mid-high economic conditions living in the capital city Ouagadougou with exposure to western food and living in a clean environment. As representatives of the Western lifestyle and diet, 46 Italian families (composed of 2 parents and up to 4 children) living in urban areas have been enrolled. By selecting families from the same ethnic background, and by enrolling families with children, we avoid bias related to ethnic differences, age or sex.

Inclusion and exclusion criteria

Inclusion criteria for the families were as follows: (i) Households composed of a biological father and mother(s) living in the same household with at least one child, in apparently good health; (ii) belonging to the Mossi ethnic group (for the African cohort). To exclude acute illnesses at the time of enrolment, individuals who had fever (>38.5 ◦C) in the previous 72 h were excluded. Furthermore, thanks to the questionnaire, the presence of diseases associated with the gastrointestinal tract (IBD) was investigated thus considering for computational analyses only healthy subjects (see 'Condition' in supplementary table 1).

Ethics

In August 2018, the National Ethics Committee of Burkina Faso granted ethical clearance (reference number 2018-8-104[1]). Adults who participated gave their informed consent. Primary caregivers signed off on children under the age of 18. By assigning each participant an identifying code, confidentiality was maintained.

*Sample collection and DNA extractio*n

For each participant a stool sample was collected by a commercial sterile collection tube supplied with RNAlater (Thermo Fisher Scientific), then stored at -80°C to preserve nucleic acids. The DNeasy PowerSoil Pro Kit (Qiagen) was used to extract total DNA from 250 mg (wet weight) of each fecal sample according to the manufacturer's instructions. The Qubit 4 Fluorometer (Thermo Fisher Scientific) and the 1x dsDNA High Sensitivity kit were used to assess DNA concentration before the downstream analyses.

ITS1 amplification, library preparation and sequencing

For each DNA sample the fungal internal transcribed spacer 1 (ITS1) was amplified using a specific primer set for the ITS1 rDNA region (ITS1f: 5'- CTTGGTCATTTAGAGGAAGTAA-3' and ITS2r: 5'-GCTGCGTTCTTCATCGATGC-3')(White et al., 1990), with overhang Illumina adapters. Library preparation was performed according to the Fungal Metagenomic Sequencing Demonstrated Protocol (Document # 1000000064940 v01). Sequencing was performed on the Illumina MiSeq platform (Illumina) with the V3 chemistry 600 cycle, paired-end 300 protocol, at the Department of Biology of the University of Florence, Italy.

Amplicon sequence variance assemblage

Both primer sequences were removed by using cutadapt version 1.15 (Martin, 2011) in paired-end mode. If a primer sequence was not found, the entire sequence was discarded together with its pair to reduce possible contamination. The raw sequences were processed using DADA2 pipeline version 1.14.1 (Callahan et al., 2016) to infer amplicon sequence variants (ASVs). The low-quality reads were filtered using the "filterAndTrim" function with a maximum number of expected error thresholds of 2 for forward and reverse read pairs and a minimum cut-off length of 70bp. Error rate estimation ("learnErrors" function) and denoising ("dada" function) with default parameters were performed. Denoised reads were merged using "mergePairs" function discarding those with any mismatches and/or an overlap length shorter than 20bp. Chimeric sequences were also removed ("removeBimeraDenovo" function) and taxonomic classification was produced by using DECIPHER package version 2.14.0 against the Warcup database for fungal ITS1 (Deshpande et al., 2016). All sequence variants not classified as Fungi and/or with no fungal counts were removed from the dataset to properly perform the downstream analyses.

Statistical analysis

Statistical analyses were performed in R environment version 4.1.2 (R Core Team, 2021).

Sequencing depth was depicted by rarefaction curves generated by using the "ggrare" function of "ranacapa" package version 0.1.0 (Kandlikar, 2022). Mean relative abundance was calculated by using "microbiomeutilities" package version 1.0.16 (Shetty & L. Lahti, 2022). Variations in fungal diversity (beta diversity analysis) were inspected using "vegan" package version 2.6.2 (Oksanen et al., 2022). In detail, samples distribution was displayed by principal coordinate analysis (PCoA) using the "cmdscale" function of the "stat" package performed on distance matrices based on Bray-Curtis diversity index and Jaccard similarity coefficient to infer quantitative and qualitative differences respectively. The data were normalized before multidimensional analysis removing counts present less than 5 times within the sample dataset when the analysis was performed on the entire dataset and removing counts present less than 1 times within the sample dataset when the analysis was performed on the datasets divided according to the four geographical areas, in both cases then sample counts were transformed in relative abundances to reduce coverage bias among samples. Permutational multivariate analysis of variance using distance matrices (adonis PERMANOVA) was performed to inspect differences between sample groups by using the "adonis2" function of "vegan" package version 2.6.2. (Oksanen et al., 2022). Adonis PERMANOVA was tested on multiple factors formula (formula = ~Area * Family degree + Age range + Sex) considering the interaction between area and family degree due to the crossed design experiment. Pairwise comparisons on variance among geographical areas were assessed using pairwise adonis PERMANOVA by using the "pairwise.adonis" function from "pairwiseAdonis" package version 0.4 (Arbizu, 2017) adjusting resulting p-values with Holm correction method. The same model formula was used to inspect differences in alpha diversity metrics, first fitted using linear model ("lm" function of "stats" R package) then inspected by analysis of variance Anova type II from "car" package version 3.1.0 (Fox & Weisberg, 2019). Significant effects were inspected by pairwise Wilcoxon test by using the "wilcox_test" function from the "rstatix" package version 0.7.0 adjusting resulting p-values with Benjamini-Hochberg correction method (Kassambara, 2021). Correlation between alpha metrics and age was produced by the "stat_cor" function of "ggpubr" package version 0.4.0 (Kassambara,

2020). To assess differences on variance related to the family degree only, thus excluding the geographic area effect, the adonis PERMANOVA was also performed on multiple factors formula (formula = ∼Family degree + Age range + Sex) after separating the dataset according to four geographical areas. All multivariate analyses were conducted setting a number of permutations of 1000 and the r-squared values were used to inspect the amount of variance explained. The dispersion among sample group centroids was computed using the ”betadisper” function of “vegan” package version 2.6.2. (Oksanen et al., 2022) and the differences in dispersion were tested using the analysis of variance (anova) and Tukey post-hoc test (TukeyHSD function from “stats” R package) to inspect significant pairwise contrasts. After singleton removal the differential abundance analysis was performed using the likelihood-ratio test (LRT) method by the “DESeq” function from the “DESeq2” package version 1.34.0 (Love et al., 2014). LRT was used to test significance of change in deviance between a full model (Area + Family degree) and reduced model (Family degree) provided in the model formula. Size factor estimation was performed using the “poscount” method and dispersions to the mean intensity were fitted by using “local” method. Statistically significant counts (alpha = 0.05) were scaled using variance stabilizing transformation (VST) then clustered using “pheatmap” package version 1.0.12 (Kolde, 2019). Figures were produced using “ggplot2” package version 3.3.6 (Wickham, 2016) and edited using open-source graphics editor Inkscape (http://inkscape.org/).

Results

DADA2 pipeline produced a total of 13'594'609 reads inferred from internal transcribed spacer 1 region (ITS1) and clustered in a total of 10’526 amplicon sequence variants (ASVs). After quality filtering (see materials and methods for additional details), two samples that showed zero fungal counts (ITIBD21F1 and F304N) were removed from the dataset to properly perform the downstream statistical analyses, thus a total of 8’364’411 reads clustered in a total of 1’655 different fungal ASVs were obtained. The reads distribution within sample groups shows the presence of ASVs with average reading levels higher in African areas than Italy area, with an increasing trend towards the level of ruralization compared to the average number of reads within the dataset (median number of reads of 13’334.5 counts, represented by the dashed red line in figure S1a). The rarefaction curves showed that the number of ASVs (referred as Species Richness in figure 1b) detected as a function of sequencing depth reached a *plateau* condition indicating that our sampling effort was effective in assessing the natural fungal diversity within sample groups (Figure S1b). The dataset was mainly represented by the taxa belonging to the Class Saccharomycetes which showed different compositional transformed mean abundance distribution according to the geographical areas (hereafter areas) (Italy: 44%, Ouagadougou: 57%, Nanoro: 48%, Rural villages: 52%) (Table S2 and figure S2). Furthermore, taxa belonging to the Class Eurotiomycetes and Dothideomycetes were widely represented within the dataset showed differential distribution in the four different areas (Eurotiomycetes; Italy: 25%, Ouagadougou: 13%, Nanoro: 12%, Rural villages: 6.5%. Dothideomycetes; Italy: 9.7%, Ouagadougou: 9%, Nanoro: 14%, Rural villages: 21%) (Table S2 and figure S2). Interestingly, regardless the sequencing depth, the rate of unknown sequence variants was on average higher in the African datasets, particularly in the rural villages area. Class-level

unknown rates were 3.14%, 9.2%, 10.8%, and 7.4% for Italy, Ouagadougou, Nanoro, and rural villages areas, respectively. Accordingly to this trend, genus-level rates were 5.2%, 13.9%, 16.2% and 18.2% for Italy, Ouagadougou, Nanoro and rural villages areas, respectively.

Changes in fungal diversity depending on geographical area and family degree

The samples distribution was explored by Principal Coordinates Analysis (PCoA) performed on distance matrices achieved by quantitative (Bray-Curtis) and qualitative (Jaccard) indexes to fully describe similarity and dissimilarity between sample groups (PCoA in figure 2 and figure S3 respectively). Both multidimensional analyses showed a sharp separation among sample groups according to the area, in particular a clear separation between the Italy area and the three African areas together was evident (Figure 2a figure S3a). Differences in beta diversity were then tested by multivariate permutational analysis of variance (adonis PERMANOVA) by using multilevel factors formula (see material and methods for additional details). The sample groups separation highlighted in the ordination analysis were statistically assessed by adonis PERMANOVA, which showed that the area and the interaction between area and family degree were the only statistically significant factor affecting the gut fungal assemblage (Table S3). The impact of the tested factors in explaining variance were proved using a multivariate analysis approach for both Bray-Curtis and Jaccard diversity indexes (Figure 2b, figure S3b and table S3). The dispersion tested for area, family degree and their interaction produced significant results for area and interaction only (Anova, $p<0.001$), however pairwise test on all 66 pair combinations within the area and family degree interaction tested by using the Tukey test showed that this significance was driven by solely 13 and 9 significant contrasts for the Bray-curtis and Jaccard distances, respectively (Table S4). Pairwise adonis PERMANOVA was performed to highlight beta diversity variation according to the urbanization degree. The only non-significant contrast was Ouagaodugou vs Nanoro, two African areas with contiguous urbanization levels while all the other combinations showed significant results (Figure 2c, figure S3c and tableS5). This was confirmed by the levels of variance explained which showed the highest value for the two opposite poles in the level of urbanization, i.e., the Italy and the African rural villages groups (colored gradient cells in figure 2c and figure S3c). As mentioned previously, the family degree was not significant when tested individually but only in interaction with the area, therefore the microbiota differed within family members only as a function of the area. Thus, the dataset was divided according to the four different areas in order to better explore the diversity related to the family degree only.

The areas with a greater degree of urbanization (Italy and Ouagadougou) did not show significant differences in fungal diversity within the family members as depicted by the adonis PERMANOVA (Table S6), while rural areas and areas with an intermediate level of urbanization (Rural villages and Nanoro) showed significant variations in fungal diversity within the family members (table S6). The sample separation between the family members was shown by multidimensional analysis (PCoA based on Bray-Curtis distance in figure 2d). Therefore, the variance explained was higher in the groups with the highest level of urbanization, i.e., Nanoro and rural villages (figure 2e and table S6). The adonis pairwise PERMANOVA analysis identified the group pairs that significantly influenced the multivariate analysis, i.e., Children vs Mother (R2: 0.05, Holm p-adj: 0.009) in the Nanoro group and Father vs Mother (R2: 0.07, Holm p-adj: 0.04) in the Rural villages group.

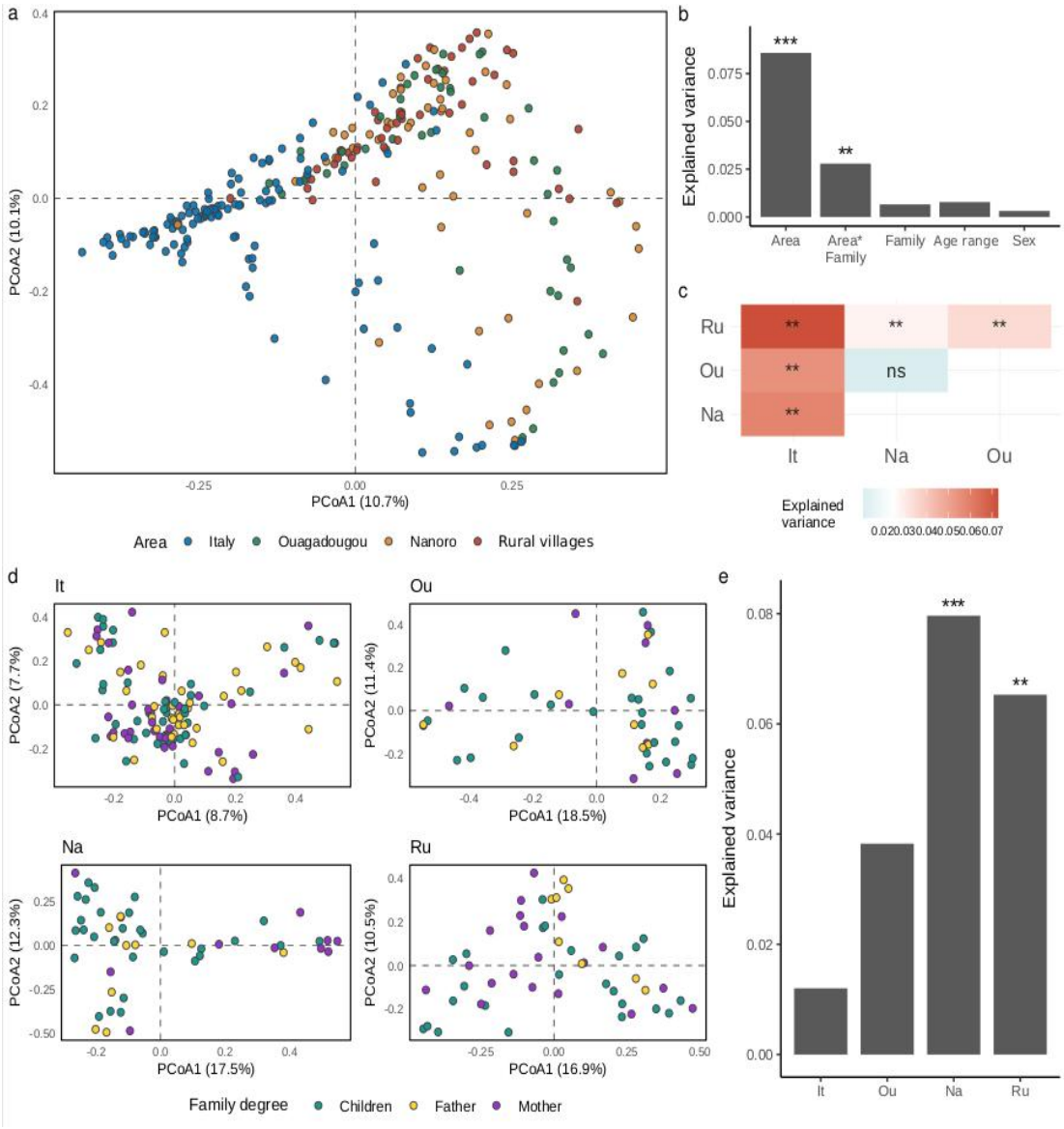

Figure 2. Fungal distribution related to the geographical areas and family degree. Principal Coordinates Analysis (PCA) based on Bray-Curtis distance matrix depicted the four different geographical areas represented following the color scheme shown in the legend (Area) (a). Statistically significant factors and variance explained value from adonis PERMANOVA performed on Bray-Curtis distance matrix reported using barplot (b). Statistically significant contrasts and variance explained from pairwise adonis PERMANOVA (Holm correction method) performed on Bray-Curtis distance matrix displayed for each group comparison were reported in the heatmap using color gradient, from lower value (blue scale) to higher value (red scale) (c). PCA based on Bray-Curtis distance matrix depicted the family degree represented following the color scheme shown in the legend (Family) (d). Statistically significant factors and variance explained values from adonis PERMANOVA performed on Bray-Curtis distance matrix reported using barplot (e). The four different geographical areas compared in the adonis PERMANOVA and pairwise adonis PERMANOVA (c, e) were reported by using the following label code, It: Italy, Ou: Ouagadougou, Na: Nanoro, Ru: Rural villages. Statistically significant results were represented using asterisks (ns, not significant; *, p-value <0.05; **, p-value <0.01; ***, p-value <0.001).

The degree of ruralization affects fungal richness and diversity

To assess differences in alpha diversity measures among different areas and family members we first carried out the type II Anova on fitted linear model formula (see material and methods section for additional details). The analysis of variance highlighted the area as the only significant term for each index considered (Table S7). According to the analysis of variance results we better explored significant contrasts within area groups only by pairwise Wilcoxon test (Benjamini-Hochberg

correction method). The alpha diversity metrics were higher in areas with the lower level of urbanization as highlighted by the significant contrasts produced by Wilcoxon test as depicted in figure 3 and supplementary table 8. In particular, all area groups showed significant differences in the total number of ASVs except for the Ouagadougou vs Nanoro pair (Figure 3a and Observed in table S8). Furthermore, we observed that total ASVs decreased as a function of increasing age regardless of the area (Pearson corr, R=-0.18, p<0.001 in figure 3b). Wilcoxon test performed on Shannon and Inverse simpson indices showed a smaller number of significant contrasts compared to the total observed ASVs. All group pairs tested for Shannon index metric showed significant results except for areas with similar levels of urbanization, that is Ouagadougou vs Nanoro and Nanoro vs Rural villages (figure 3 and table S8). Therefore, the only groups showing significant differences in the Inverse Simpson index values were represented by the areas with opposite levels of urbanization, i.e., Italy vs Nanoro and Italy vs Rural villages (figure 3 and table S8).

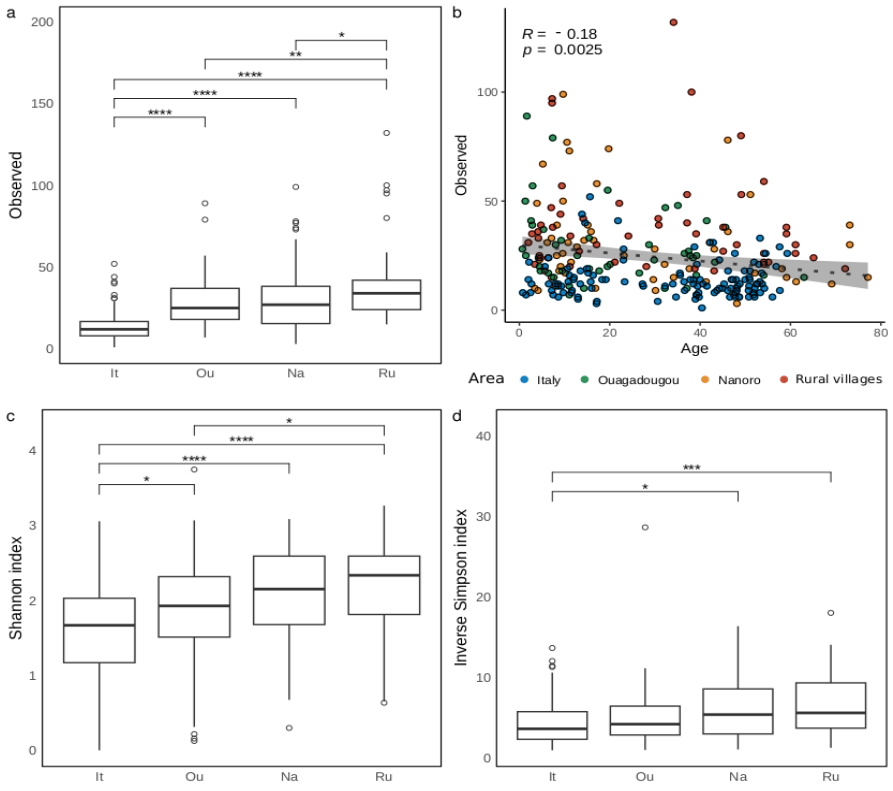

Figure 3. Fungal diversity differences among geographical areas. Differences in alpha diversity measure across different areas were represented using boxplot and significant contrast after pairwise Wilcoxon test (Benjamini-Hochberg correction method) were reported using asterisks (*, p-value <0.05; **, p-value <0.01; ***, p-value <0.001; ****, p <0.0001) (a,c,d). Alpha diversity measures were reported on y-axis while sample groups were reported on x-axis. Correlation between the total number of ASVs and the age (years) of subjects was depicted by using scatterplot and correlation results from Pearson correlation methods displayed on top-left corner (b). Samples in the scatterplot were depicted according to the Area by using the color palette reported in the legend. Local regression line with the 95% confidence interval were reported. The four different geographical areas compared were reported by using the following label code, It: Italy, Ou: Ouagadougou, Na: Nanoro, Ru: Rural villages.

To inspect fungal distribution within sample groups we performed log-likelihood ratio test (LRT) using DESeq2. The analysis produced a total of 33 different ASVs reporting a significant distribution across different areas and family members (Figure 4 and Table S9). Significant ASVs corresponded

to 1.99% of the ASVs profiled in the whole fungal communities (33 on 1655) however representing 59.9% of the total fungal abundance with mean abundance within sample dataset of 50.5% and standard error of 1.83%. Sequence variant clustering reported in figure 4 (Complete linkage method) showed an ASVs abundance distribution according to the area. Within the two main branches, were depicted four different clusters reporting a pattern of distribution mainly referred to the Italy area and the three African areas together (Figure 4). In particular, 8 differential abundant ASVs were mainly referred to the Italy area, their taxonomy annotation enclosed 3 fungal species (*Rhodotorula mucilaginosa*, *Candida sake*, *Verticillium leptobactrum*) and 4 fungal genera (*Eupenicillum*, *Debaryomyces*, *Penicillium*, *Eurotium*). The 3 clusters referred to the African areas depicted 25 different ASVs which included 9 fungal species (*Epicoccum sorghi*, *Candida mesorugosa*, *Candida tropicalis*, *Aspergillus flavus*, *Kluyveromyces marxianus*, *Cryptococcus flavus*, *Aspergillus penicillioides*, *Malassezia restricta*, *Cyberlindnera fabianii*) and 10 fungal genera (*Pichia*, *Candida*, *Epicoccum*, *Eurotium*, *Trichosporon*, *Davidiella*, *Aspergillus*, *Gibberella*, *Rhizopus*, *Plectosphaerella*). All significant ASVs and the related taxonomic annotation were reported in supplementary table 9.

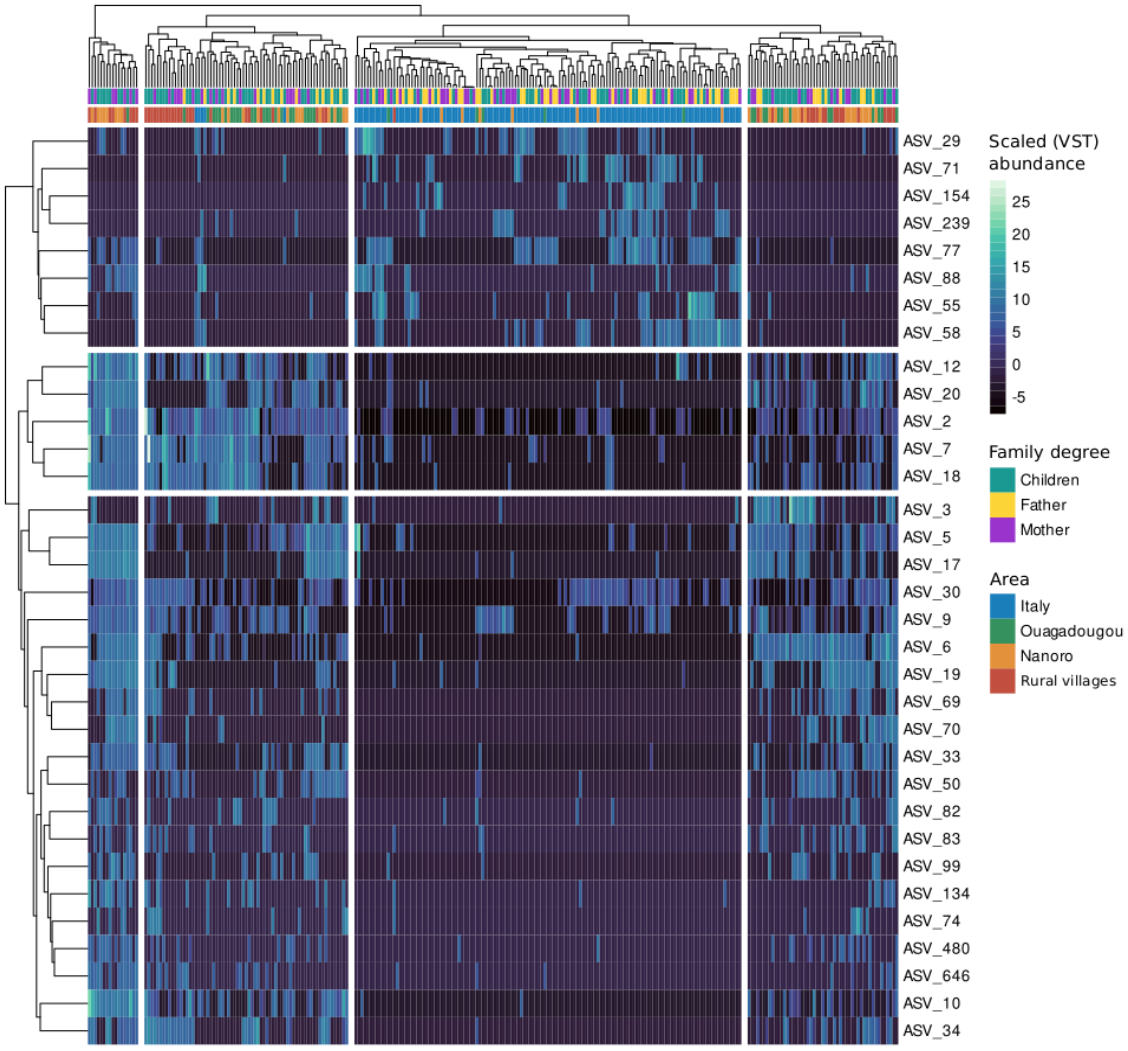

Figure 4. Sequence variants clustering based on scaled abundance across geographical areas and family degree. Amplicon sequence variants whose abundances significantly differed within the dataset (loglikelihood ratio test of DESeq2) were clustered according to their mean variance-stabilized abundance. Euclidean distance was used as a measure in the cluster dendrogram displayed in the rows and columns. Complete linkage was used as a method to produce the hierarchical clusters. Significant VST scaled variants were reported on the heatmap rows while samples were reported on the columns. Samples were displayed according to the factors present in the DESeq model (Area and Family degree) following color pattern legend.

To widely explore the differences in relative abundances of significant ASVs (Significantly enriched ASVs from LRT analysis) within different areas a pairwise comparison by using Wilcoxon test (Benjamini-Hochberg correction method) was performed. We produced significant contrast considering the deepest taxonomic rank obtained (Species level) (figure 5 and table S10) and for the restant part of significant ASVs produced (Figure S4 and table S11). The results from the Species-level pairwise test showed that the abundance of the fungal species detected follows the urbanization/ruralization degree criterion. The abundance of fungal species *Candida mesorugosa*, *Cryptococcus flavus*, *Cyberlindnera fabianii*, *Epicoccum sorghi*, *Kluyveromyces marxianus*, and *Malassezia restricta*, associated with African areas, follow an abundance gradient inversely related to the level of urbanization (Figure 5). ASVs related to the genus *Pichia* (ASV_2, ASV_7, ASV_18 in figure S4 and table S11) significantly more associated with rural areas, when collapsed to genus level they also showed significant variations in abundance within family members, in particular mothers showed significantly higher levels of fathers (Wilcoxon test - Benjamini-Hochberg adjusted: p-adj=0.007). No significant variations within family members were found in the other areas.

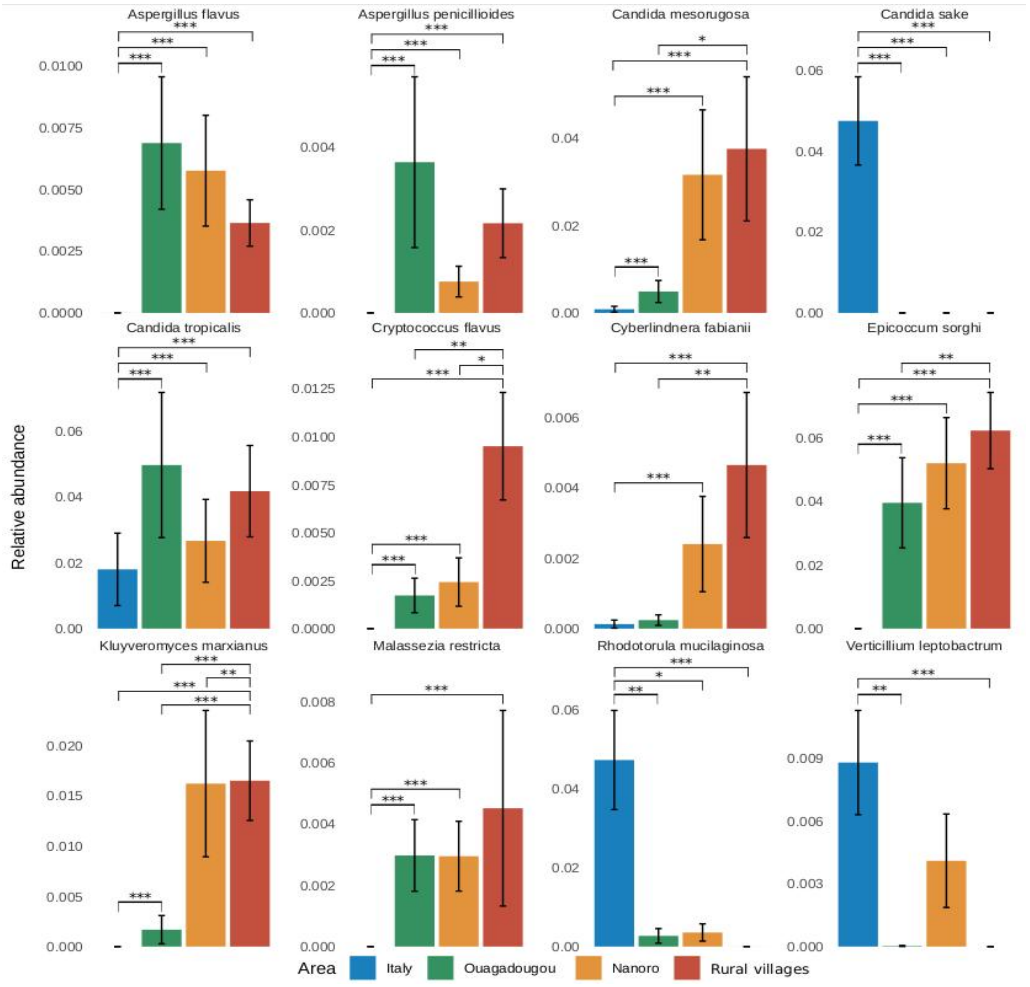

Figure 5. Pairwise comparisons of significant fungal species abundance. Relative abundance of significant Species-level ASVs (loglikelihood ratio test of DESeq2) displayed according to the four different areas. Differences in the abundance values were tested by using Wilcoxon test (Benjamini-Hochberg correction method). Different areas were depicted following the color pattern in the legend. Significant contrasts were displayed using asterisks (*, p-value <0.05; **, p-value <0.01; ***, p-value <0.001).

Discussion

Globalization of foods, medical care, social dynamics, and sanitation is drastically changing human microbiomes. Urbanization is the first step towards globalization, and represents a set of social, cultural, and economic variations that have a strong impact on the human condition. Urbanization plays a crucial role in modulating human's health and for this reason several studies that have emerged in recent years have focused on the effect of urbanization on the human microbiome. The increasing level of urbanization is one of the factors associated with the increasing incidence of several autoimmune diseases, including IBD and cardiovascular disease. It has been shown that increasing levels of urbanization and IBD both produce a decrease in the diversity levels of the gut bacterial microbiota thus predisposing to the onset of IBD conditions (Zuo et al., 2018). Immune training and shaping of the immune responses occur early in life, thus understanding the variations in the fungal diversity and composition of adults and children in different degrees of urbanization holds the key to understanding which fungal species could be associated with the Hygiene Hypothesis therefore with the predisposition towards possible IBD conditions. Our results demonstrate that the level of urbanization plays a crucial role in the diversity, composition, and assembly of intestinal fungal communities, producing effects also within family members. We investigated the effect of urbanization within family members, considering three African areas at different levels of urbanization and a western country (Italy) represented the maximum expression of the level of urbanization (western pattern conditions). The complexity of the experimental design made it possible to highlight how the fungal microbiota within the family members did not show variations in diversity in the two groups with the highest level of urbanization, namely Ouagadougou (capital of Burkina Faso) and Italy. Conversely, the microbiome differed between family members in the mid-urbanization area (Nanoro) and in rural villages which are the areas with the highest degree of ruralization. Thus, we described the dependence of the fungal microbiota structure on the degree of urbanization, furthermore we also demonstrated that the development of the fungal microbiota within family members is also related to the degree of urbanization. The fact that differences between family members can be observed only in the rural settings, with a very uniform lifestyle within the family, indicates that greater exposure to environmental yeasts and fungi, rather than dietary habits or lifestyle is likely the main driver of the differences observed. The alpha diversity measures increased (see Results section for the significance) depending on the level of urbanization, i.e., showing minimum values in the Italy group and maximum values in the rural villages and this occurred for all the indices tested. In this case, unlike the beta diversity analysis, the interaction between geographical area and family degree was not significant, highlighting that only the geographical area acted as a driver for changing the alpha diversity measures. Differential abundance analysis sorted 33 different sequence variants that significantly change within sample groups. Among these, we highlighted 12 ASVs classified at the deepest level of taxonomic assignment (Species level) whose abundances varied following a gradient of urbanization. In particular we identified several fungal species affected by urbanization, relevant for their interactions with the immune system and overall for the implication on human health. These results likely underestimate the extent of this diversity, since a large number of reads from the African microbiomes, especially from the rural ones could not be properly assigned. This strongly highlights

the importance of further investigations on the genomic sequence of African yeasts and fungi, to allow a more refined and improved assignment of the reads (Huffnagle et al., 2013) (Galagan et al., 2005). These species are known to be associated with humans and others have rarely been found on humans, some are known to induce differential immune responses in human populations and some are well known opportunistic pathogens in transition from commensalism. *C. mesorugosa* was described as a pathogen causing several infections and was isolated from clinical samples in Ghana (Adjapong et al., 2015) and Brasil (Chaves et al., 2013). *A. flavus*, *A. penicillioides* and ASVs related to the *Aspergillus* genus were all significantly enriched within the African groups. The decrease of *Aspergillus* abundance in *Clostridium difficile* infections (CDI) has suggested it's beneficial role in decreasing the risk of CDI (Cao et al., 2021). Therefore, the association of *A. penicillioides* with healthy individuals rather than CDI was also corroborated because its abundance increased following fecal transplantation in CDI subjects (Zuo et al., 2018). Furthermore, *M. restricta* was proven to exert a regulatory role on the immune system, even Kesavan et al. described that *Malassezia* was related to a significant decrease in pro-inflammatory cytokines (Kesavan et al., 1998). The increased abundance of *K. marxianus* according to the rural gradient can be explained by the high presence of this yeast in several African fermented products (Motey et al., 2020). *K. marxianus* is also known for its regulatory activity on the immune system, in particular, *K. marxianus* exert the activation of Foxp3+ Treg cells, thus reducing the levels of inflammation and potentially, in the long-term scale, preventing the appearance of chronic inflammatory disease (Smith et al., 2016). The co-presence of fungal species with potentially immunogenic profiles in non-immunocompromised subjects (healthy donors) could trigger a combination of protective effects able, over time, of reducing the onset of chronic inflammatory diseases. Again, the fact that several of the discriminating species is not associated to food production and fermentations suggest that the environment is the main driver of the differences observed. Overall, we hypothesize that the urbanization and consequently the decrease of specific fungal species represent one of the key factors towards chronic inflammatory conditions correlated to the western-related condition. We also consider that this difference in fungal species composition among family members, evident in the rural villages but absent in the cities, could be the result of contamination with environmental fungal species that are very likely passengers and did not underwent transition from colonizers to invaders, typical of the pathogens, and that could potentially be driver of the activation of innate immune responses. Since the occurrence of non-communicable diseases follows the trend of fungal reduction, our results suggest changes in yeast communities as a legitimate explanation for the hygiene hypothesis. We can conclude that this study strongly stresses the importance of further investigations on the influence of environmental fungi on human health, exploring the immunomodulatory properties of African yeasts, evaluating their potential role in self sustainability of health.

Availability of data and materials

The ITS1 raw sequences data has been deposited to the European Nucleotide Archive (ENA) under the accession code PRJEB59322. All results from statistical analyses were mentioned by writing in the main text, represented as figures in the main text or reported as supplementary tables and figures.

Author contributions

DC and PL conceived the work, SR and NM wrote the manuscript SR and BC produced the metagenomic libraries and sequencing, NM analyzed the data. All authors revised and approved the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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Chapter 4

Changing Dietary Habits: The Impact of Urbanization and Rising Socio-Economic Status in Families from Burkina Faso in Sub-Saharan Africa

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Abstract

(1) Background: Sub-Saharan Africa is experiencing the fastest urbanization worldwide. People in rural areas still have a traditional and rural lifestyle, whereas the Westernization of diet and lifestyle is already evident in urban areas. This study describes dietary habits of families in Burkina Faso living at different levels of urbanization. (2) Methods: Data on lifestyle, socioeconomic conditions, health status and anthropometry were collected from 30 families living in rural villages, a small town, and the capital city. A food frequency questionnaire and a 24 h recall diary were used to estimate dietary habits and macronutrients intake. (3) Results: The urban cohort showed a more diversified diet, with a higher intake of animal protein and, especially in children, a higher consumption of simple sugars. Fiber intake was significantly higher in the rural and semi-urbanized cohorts. As expected, overweight and obesity gradually increased with the level of urbanization. In semi-urbanized and urban families, we observed coexistence of under- and over-nutrition, whereas in rural families, a portion of children were wasted and stunted, and adults were underweight. (4) Conclusions: These three cohorts represent a model of the effect on diet of rural-to-urban migration. Rural diet and traditional habits are replaced by a Western-oriented diet when families move to urbanized areas. This dietary transition and increased socio-economic status in newly developing urban areas have a major impact on disease epidemiology, resembling the past evolution in Western countries.

1. Introduction

Industrialization in high-income countries has historically led to rural-to-urban migration, alongside a change in nutrition from mostly plant-based foods (vegetables, fruit, wholegrains, legumes) to hyper-caloric diets rich in total and saturated fats, cholesterol, animal protein, added salt and sugar and poor in fiber [1–3]. Currently, several regions in the world, including sub-Saharan Africa (sSA), are experiencing rapid urbanization, with a constant increase of people moving from rural to urban areas. This transition reflects socio-economic changes and a shift from subsistence farming to more specialized jobs. As a consequence, diet changes from a traditional towards a Western-like diet [4], with easier access to a greater food variety [5], including highly caloric and processed foods. Disease epidemiology is also radically changing. While the burden of infectious diseases remains high in many rural low-income areas, obesity and non-communicable diseases (NCDs) related to diet—once typical of the Western world—are rapidly increasing in the newly developing urban areas [6–9], accounting for almost half of all deaths and disability in low-to-middle-income countries [10]. In sSA, due to urban lifestyle and nutrition transition [11–14], many studies have described that NCDs, such as cardiovascular diseases, diet-related diseases, chronic kidney and respiratory diseases and cancers, will overtake infectious disease cases by 2030 [15–18]. These changes promote the adoption of behaviors that favor unhealthy diets and lifestyles, such as inadequate fruit and vegetable consumption and increased energy intake, harmful alcohol consumption, smoking and physical inactivity [19,20].

Burkina Faso, a country in West Africa, is in an early stage of demographic transition. The capital city of Ouagadougou and its surroundings represent the main urban area. Here, changes in lifestyle and dietary habits characteristic of urbanized populations have already taken place [21]. On the other hand, non-urban regions of Burkina Faso still present a traditional rural lifestyle mainly based on

subsistence agriculture, with marginal contacts with the globalized world. A few studies have previously described dietary patterns of rural and urban children [22–24] and urban adults in Burkina Faso [25], but a detailed study of the current dietary habits of people living in environments at different levels of urbanization is missing. Here, we describe the dietary and lifestyle habits of families of the same ethnicity living in areas at different phases of the rural-to-urban transition in Burkina Faso: rural villages, a semi-urbanized town (Nanoro) and the capital city Ouagadougou.
The aim of the study was to investigate the effect of the rural-to-urban transition, focusing on households and family units as the center of a shared lifestyle and diet.

2. Materials and Methods

2.1. Characteristic of the Region, Setting and Study Population

The current study was conducted in Burkina Faso from February 2019 to January 2020. The population of Burkina Faso is rapidly growing (20.9 million with a growth rate of 2.8% in 2019) [26] in particular with an annual urban growth rate remaining constantly high over recent years (4.9–6.7% per year in the decade 2009–2019) [27]. All individuals were enrolled during the dry season, which in Burkina Faso runs approximately from October to April, with variations depending on the region. In particular, the rural and the semi-urbanized cohorts were enrolled during February 2019, and the urban cohort in January 2020. Thirty families were enrolled from three areas at different levels of urbanization: (i) Three rural villages from Boulkiemde province: Boulpon (geographic coordinates 12◦390000 N 2◦4 00000 W), Godo (12◦0100000 N−2 ◦3400000 W), and Poessi (12◦20014.5500 N 2 ◦13025.2000 W); (ii) the small town of Nanoro (Boulkiemde province, 12◦410 N 2◦120 W), with a population of 7542 inhabitants; (iii) the Capital city, Ouagadougou (12◦2102600 N 1 ◦3207 00 W), with 2453 million inhabitants (Figure 1).

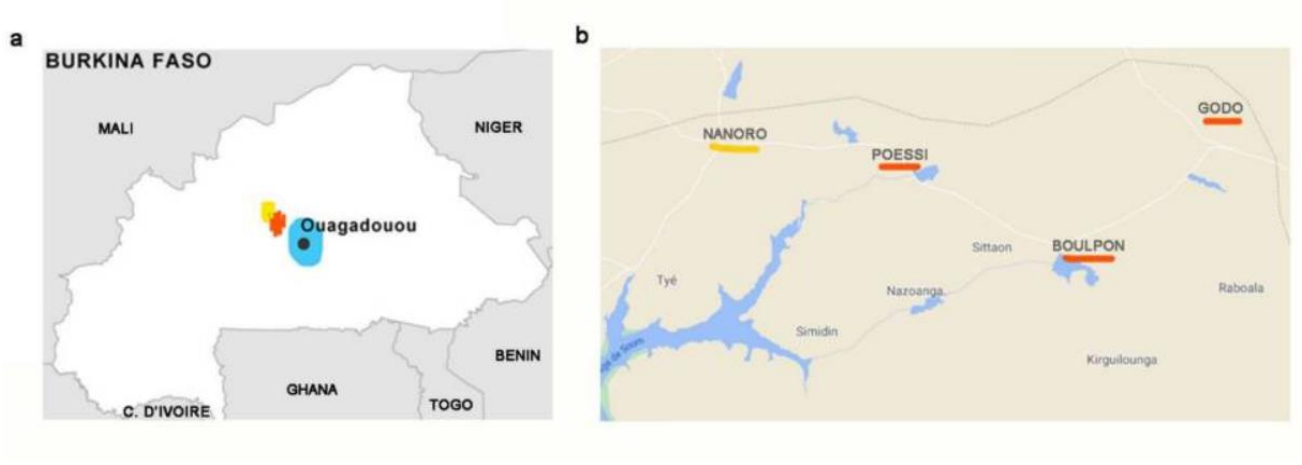


Figure 1. Map of Burkina Faso and areas of the three enrolled cohorts. (**a**) In blue: Urban area of the capital city Ouagadougou; in yellow: The small town of Nanoro (initial level of urbanization); in red: Rural area. (**b**) Detailed map. In red, the three rural villages of Boulpon, Godo and Poessi; in yellow, the town of Nanoro.

The Nanoro Health and Demographic Surveillance System (HDSS) [28], a surveillance organ established by the Clinical Research Unit of Nanoro (CRUN), randomly selected the households and

families living in the three areas (rural, semi-urbanized and urban) based on different socio-economic conditions, lifestyle, diet, hygiene and sanitation. In rural Burkina Faso, most people live from subsistence agriculture. Rural villages are composed of clusters of huts built using soil, wood and straw (Figure 2). Rural families are on average poor; children are at high risk of infectious diseases and malnutrition, especially during the lean season between harvests (usually between May and September). Electricity is unavailable and the population gets water from wells or dams. Daily foods include cereals (millet, sorghum, maize), legumes (cowpeas, locally called Niébé, Vigna unguiculata) and vegetables (baobab leaves, wild herbs, tomatoes, peppers or Nerè, the fruit of Parkia biglobosa tree). Families usually share a single main meal made of tô, a thick porridge obtained from ground millet, sorghum or corn served with a sauce made from local vegetables, herbs, soumbalà (a condiment made of Néré seeds) and peanuts. Sometimes tô is supplemented with a small piece of meat (mainly goat, sheep and chicken, less frequently beef and pork) or dried fish. Young children are usually fed bouille, a liquid porridge made with millet, soy, peanuts and sugar.

Figure 2. Life in Burkina Faso. (**a**) Rural village (Boulpon), (**b**) semi-urbanized area (Nanoro town), (**c**) the capital city Ouagadougou. Personal photographs taken by Paolo Lionetti and Silene Casari.

In the town of Nanoro (Figure 2), there are urban groupings of small brick houses. In this semi-urbanized area, a few families have access to private sources of water, but all of them use public wells. Electricity is commonly absent in the houses, and all households host livestock in their courtyards. In this area, children are at risk of infectious disease and at low–medium risk of

malnutrition. Diets are still predominantly plant-based but different products, such as cereal flour, rice, legumes, fruit, meat and dried fish can be easily found at the bi-weekly local market, together with foods from neighboring regions. People occasionally consume highly processed and Western-like food. In the capital city of Ouagadougou (Figure 2), select wealthy families live in concrete or brick buildings with access to a private water source and electricity. With regard to diet, these families have a typical Burkinabe diet, rich in whole grain cereals, such as millet, sorghum and maize, legumes and local fruit and vegetables. The rapid urbanization of this area is bringing with it an increased wealth, with greater food availability and variety of processed food (such as fruit juices, snacks, sweets and bakery products) from supermarkets.

A total of 159 individuals (parents and children) were enrolled from three cohorts as follows:

• Rural cohort: 10 households (n = 54 individuals) living in the rural villages of Boulpon (n = 4 households), Godo (n = 3) and Poessi (n = 3).

• Semi-Urbanized cohort: 10 households (n = 55 individuals) from Nanoro.

• Urban cohort: 10 households (n = 50 individuals) living in the capital city of Ouagadougou selected among wealthy households.

Inclusion criteria for the families were as follows: (i) Households composed of a biological father and mother(s) living in the same household with at least one child, aged between 2 and 18 years, in apparently good health; (ii) belonging to the Mossi ethnic group, the main ethnic group in Burkina Faso. In order to exclude acute illnesses at the time of enrolment, individuals who had had fever (>38.5 ◦C) in the previous 72 h were excluded.

2.2. Ethics

Ethical clearance was obtained from the National Ethics Committee of Burkina Faso in August 2018 (reference number 2018-8-104). Enrolled adults gave informed consent. For children under 18, consent was given by primary caregivers. Confidentiality was maintained by allocating an identification code for each participant used on all questionnaires.

2.3. Data Collection and Nutritional Analyses

A questionnaire on lifestyle, health status and dietary habits was administered on site to each recruited individual. In cases of illiteracy, a member of local trained staff conducted a direct interview. The questionnaire was written in French and translated into Moorè (local language of Mossi ethnicity). Support of the local staff was ensured. Collected data were entered into a database. The dietary section of the questionnaire was tailored to the study population and included a semi-quantitative food frequency questionnaire (FFQ) and a 24-h diet recall, from which daily energy and macronutrient intakes were estimated. Food frequency referred to the week prior to the interview and regarded all the main local foods, as well as processed and refined foods. For every food item, pictures of the portion size of dishes (small–medium–large) were shown to estimate the amount of food consumed by each individual. In Supplementary Material Table S1, the estimated quantities for each portion size, divided for age groups, are reported. For analytical food composition, FAO/INFOODS Food Composition Databases were used [29]. 2.4. Dietary Diversity Score Data from the 24-h diet recall and from the questionnaire of weekly frequency consumption for each individual were used to calculate a dietary diversity score (DDS), a qualitative measure of

the variability of food consumption, based on FAO guidelines for dietary diversity [30]. It is considered an indicator of macro- and micronutrient adequacy for all age groups [30–34]. Food items consumed during the seven days prior to the interview were categorized into 12 food groups (cereals, white tubers and roots, vegetables, fruits, meat, eggs, fish and seafood, legumes, nuts and seeds, milk and milk products, oils and fats, sweets, spices and condiments). A score equal to 1 was assigned for every food group included in the individual's diet without considering a minimum intake for the food group. The sum of the scores represents each individual's diet diversity score (minimum 0 and maximum 12).

2.5. Anthropometry and Classification of Nutritional Status

Trained fieldworkers collected anthropometry measurements. Individuals <18 years of age were considered children. Weight and height were measured for all enrolled individuals, with participants wearing light clothing and no shoes. Data on the nutritional status of children (<18 years) were derived from WHO databases [35]. These data include measurements of stunting (height-for-age, Z score < −2 SD), wasting (weight-for-height, Z score < −2 SD, if under 5 years; BMI < −2 SD, if 5–17 years, for moderate acute malnutrition, weight-for-height, Z score < −3 SD, if under 5 years; BMI < −3 SD, if 5–17 years for severe acute malnutrition) and overweight (weight-for-age Z-score > +2 SD, if under 5 years; BMI > +2 SD, if 5–17 years) [36]. For adult nutritional status, BMI was calculated and classified according to BMI cut points for underweight, overweight and obesity (<18.0 kg/m2 , ≥25.0 kg/m2 , and ≥30.0 kg/m2 , respectively), based on the standard classifications of nutritional status as reported in the WHO website [37].

2.6. Statistical Analysis

Data were managed and analyzed using Microsoft Office Excel 2016 and IBM SPSS Statistic 20 software (IBM Corp., Armonk, NY, USA). Categorical variables were described as frequency and percentages. Continuous variables were evaluated for normal distribution using Kolmogorov–Smirnov goodness-of-fit test. Normally distributed continuous variables were presented as mean +/− standard deviations (SDs). Non-normally distributed variables were presented as medians (interquartile ranges (IQRs)). Univariate analyses were carried out on questionnaire results about lifestyle, health, anthropometric measures and dietary habits. Analyses of statistical differences among categorical variables were carried out using Chi-square or Fisher Exact test, where appropriate. We performed unpaired Student t test and Wilcoxon–Mann–Whitney tests to assess disparities among quantitative continuous variables. All statistical tests were two sided and $p < 0.05$ was considered as the statistically significant threshold.

3. Results

3.1. Characteristics of the Three Burkinabè Cohorts

In this study, 159 individuals (children and adults) took part belonging to households and families living in three areas at different level of urbanization (rural villages, semi-urbanized area around Nanoro town and the capital city Ouagadougou). The age range was from 0.7 to 77.1 years (mean

age 24.7 years ± 20.4 standard deviation). Demography and socio-cultural status of the study populations are shown in Table 1. Living conditions of the households enrolled in the three different environments, including water source, cooking energy, light source, and coexistence with livestock are reported in Supplementary Material Table S2. Parents in all three cohorts were aged > 18 years. Enrolled families in the rural and semi-urbanized areas were large, composed of a man with one or more wives and their respective children, all sharing the same household. Polygamy is widely practiced (100% in rural and 60% in semi-urbanized households; Supplementary Material Table S2). In the capital city, the selected wealthy families were all monogamous (Supplementary Material Table S2). Christian and Muslim religions are widespread in the three areas, especially in the urban cohort. However, in the rural villages, traditional animism is still the most practiced religion (Table 1). As for education, in the rural cohort, men and women are mostly illiterate, and children usually do not attend school, often because of the long walking distance from home. In the semi-urbanized cohort, education of adults is low, especially among women (77.8% and 81.8% of male and female caregivers, respectively, did not receive any education). All children of the semi-urbanized cohort attend school, also due to the proximity of school from their home. In the urban cohort, education and schooling rates are high (88.9% and 80% of male and female adults, respectively, completed at least a primary cycle education, and 85.7% of enrolled children attended school (Table 1)). With regard to occupations, agriculture is the main activity in the rural and semi-urbanized cohorts, especially for women (100% of women are housewives and farmers in both cohorts), while different manual jobs can be found among men. In the capital city of Ouagadougou, adults work mostly in professional or specialized jobs (44.4% of males and 40% of females). Concerning habitual alcoholic beverage consumption, fermented beverages are very popular in Burkina Faso. “Dolo”, a low alcoholic grade beverage (about 4–6% *v*/*v*) produced by fermentation of sorghum, is commonly consumed in rural areas [38]. Adults of the rural cohort reported habitual alcohol consumption (62%, 18/29), mostly based on dolo (55.2%, 16/29; Table 1). In the semi-urbanized and urban cohorts, dolo consumption was lower (17.4%, 4/23, for semi-urbanized and 12.5%, 3/24, for urban adults). In the capital city adults consume mainly commercial alcoholic drinks, such as beer, wine or spirits (72.7%, 8/24). Regarding smoking, a small proportion of adults of the semi-urbanized and urban cohorts declared they usually smoke cigarettes (Table 1). In urban areas, transport and mobility are mainly via motorized means (66.7% of the adult individuals declared they use scooters and cars; Table 1) and lifestyle is predominantly sedentary. In rural and semi-urbanized areas, people move mainly on foot or by bike (89.7% and 69.6% of the adults, respectively; Table 1), they have an active lifestyle, also due to heavy physical work.

Table 1. Demographic and socio-cultural characteristics of study participants.

	Rural (R) *n.* (%)	Semi-Urbanized (SU) *n.* (%)	Urban (U) *n.* (%)
Participants (n = 159)	54 (34.0)	55 (34.6)	50 (31.4)
Fathers/mothers (n = 26/40)	8 (30.8)/19 (47.5)	9 (34.6)/11 (27.5)	9 (34.6)/10 (25.0)
Offspring (n = 93)	27 (29.0)	35 (37.6)	31 (33.4)
Children Males/females (n = 44/49)	14 (31.8)/13 (26.5)	16 (36.4)/19 (38.8)	14 (31.8)/17 (34.7)
Age distribution (years)			
<5 (n = 27)	11 (40.7)	6 (22.3)	10 (37.0)
5–13 (n = 43)	12 (27.9)	19 (44.2)	12 (27.9)
14–18 (n = 13)	2 (15.4)	7 (53.8)	4 (30.8)
>18–77 (n = 76)	29 (38.1)	23 (30.3)	24 (31.6)
Parents/caregivers (n = 66)	27 (40.9)	20 (30.3)	19 (28.8)
Religion of Parents/caregiver			
Muslim	6 (22.2)	7 (35)	8 (42.1)
Christian Catholic	7 (25.9)	5 (25)	9 (47.4)
Christian Protestant	2 (7.5)	6 (30)	2 (10.5)
Traditional (animist)	12 (44.4)	2 (10)	0 (0)
Education level of parents/caregivers			
Males			
No school education	8 (100)	7 (77.8)	1 (11.1)
Primary school	0 (0)	0 (0)	3 (33.3)
Intermediate	0 (0)	1 (11.1)	3 (33.3)
High or more	0 (0)	1 (11.1)	2 (22.2)
Females			
No school education	18 (94.7)	9 (81.8)	0 (0)
Primary school	1 (5.3)	2 (18.2)	2 (20)
Intermediate	0 (0)	0 (0)	6 (60)
High or more	0 (0)	0 (0)	2 (20)
Occupation of parents/caregivers			
Males			
Manual worker	8 (100)	8 (88.9)	4 (44.4)
Professional	0 (0)	1 (11.1)	4 (44.4)
Retired	0 (0)	0 (0)	1 (11.2)
Females			
Housewife/farmer	19 (100)	11 (100)	4 (40)
Student	0 (0)	0 (0)	1 (10)
Manual worker	0 (0)	0 (0)	1 (10)
Professional	0 (0)	0 (0)	4 (40)
School attendance among children at school age (6–16 y)			
Attending	0 (0)	21 (100)	12 (85.7)

	Rural (R) *n.* (%)	Semi-Urbanized (SU) *n.* (%)	Urban (U) *n.* (%)
Habitual alcohol consumption (parents/caregivers)			
Alcoholic fermented products (e.g., dolo)	16 (55.2)	4 (17.4)	3 (12.5)
Alcoholic beverages (beer, wine, spirits)	2 (6.9)	0 (0)	8 (33.3)
Smoking (parents/caregivers)			
Smoking habit	0 (0)	1 (4.3)	1 (4.2)
Means of transport of adults (>18 y)			
Walk and/or bike	26 (89.7)	16 (69.6)	8 (33.3)
Scooter and/or car	3 (10.3)	7 (30.4)	16 (66.7)

3.2. Energy and Macronutrient Intake Estimation Per Area

Energy and macronutrient intake for adults and children are reported in Supplementary Material Tables S3 and S4, respectively. Calories, carbohydrates and fat intake did not differ significantly among the families in the three areas. In contrast, the intake of protein, fibers and simple sugars

differed significantly (Figure 3). Total protein intake was significantly higher among the urban participants compared to the other two groups. Almost half of the total protein amount was of animal origin in the urban cohort, both in adults and children. Among adults, the consumption of simple sugars did not differ significantly between urban and semi-urbanized participants, but it was significantly lower in rural participants. Among children, those in the urban setting had a significantly higher intake of simple sugars compared to children from other areas. Fiber intake in adults was highest in those from the rural area, followed by those in the semi-urbanized area and the capital. Sixty-nine percent of rural adults reached the recommended fiber intake (12.6–16.7 g/1000 kcal for adults according to EFSA [39]), 26% in the semi-urbanized group and 0% in the urban adults. In children, fiber intake was similarly high in both rural and semi-urbanized cohorts (100% and 97% of children reached the recommended intake of 8.4 g/1000 kcal [39]), but significantly lower in the urban children (50% of children were not reaching the recommended fiber intake; Supplementary Material Figure S1).

3.3. Food Frequency Consumption and Estimation of Dietary Diversity

Weekly frequency of consumption of major food items (such as meat, fish, eggs, cheese, dairy, main carbohydrate sources, fruit and vegetables, sweet foods) is reported in Figure 4. Food items that contribute to protein intake are consumed daily by 19% of rural participants, 31% of semi-urbanized participants and 92% of urban participants. In rural families, protein sources are mainly legumes and, sometimes, small quantities of meat or fish, whereas eggs or dairy are not consumed. In contrast, urban participants showed a more diversified diet with more frequent consumption of fish, meat, eggs and dairy during the week compared to the rural cohort. In addition, fruits and vegetables are consumed more often among the urban participants than the rural ones. Among carbohydrate sources, to is the main rural meal, while bread, rice and pasta are frequent alternatives in the urban cohort. The semi-urbanized participants showed intermediate frequency of consumption for almost all food groups. Interestingly, 10% of urban participants reported daily consumption of sweet items (including industrial cookies, confectioneries and sweets), while the rural and semi-urbanized cohorts rarely consume them. None of the rural participants consumed meals at restaurants and only 2% of semi-urbanized participants had consumed more than two restaurant meals in the week preceding the interview. In contrast, 22% of urban participants consumed restaurant meals ranging from twice to more than seven times per week. A small proportion of rural individuals (8%) declared having consumed meals at a local market (Supplementary Material Table S5). Food items purchased from supermarkets were consumed only by urban participants. In order to assess differences in food variety consumed among the three cohorts, the dietary diversity score (DDS) was calculated. The DDS was similar across the rural and semi-urban families, but significantly higher in urban families (Figure 5).

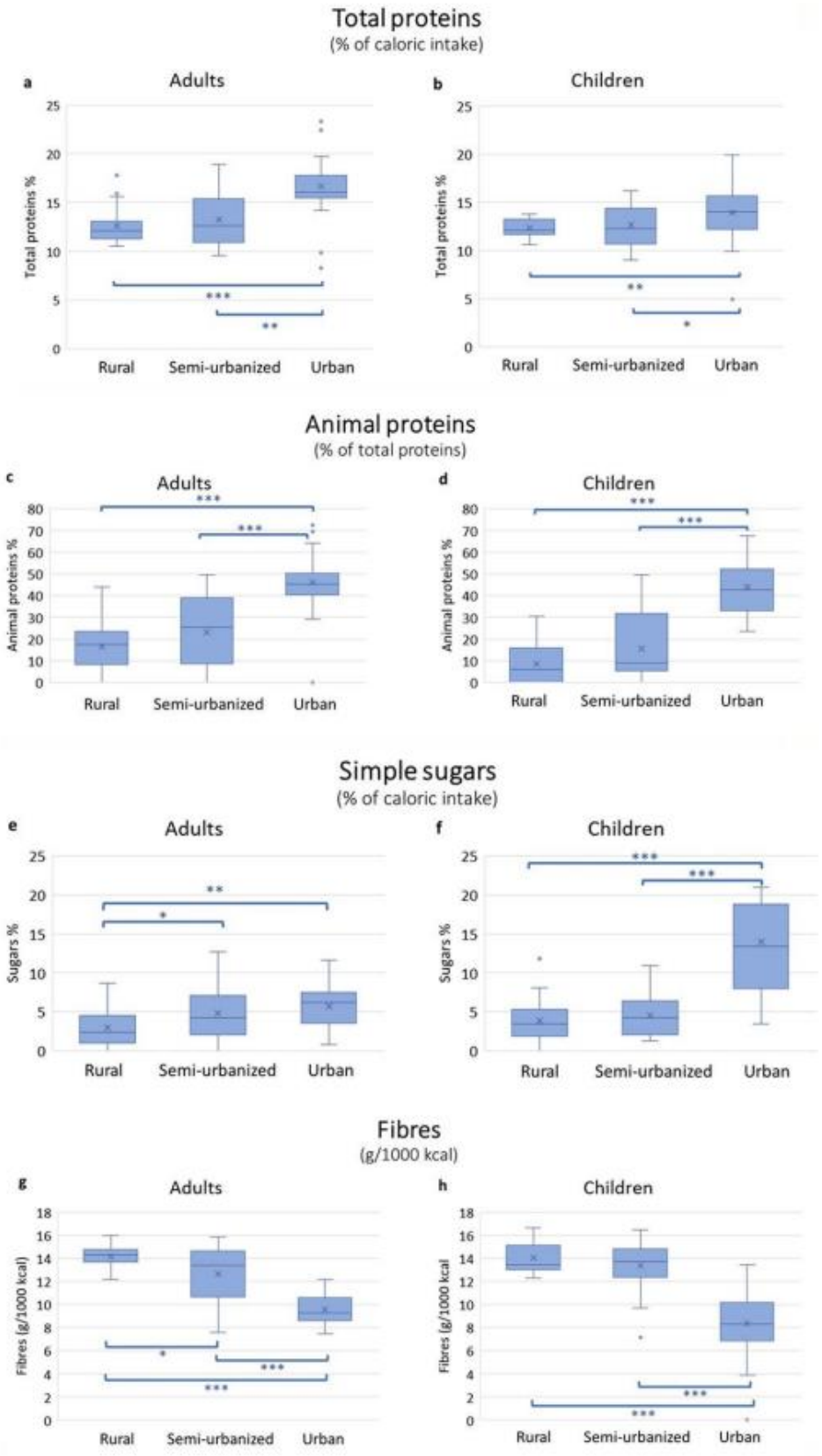
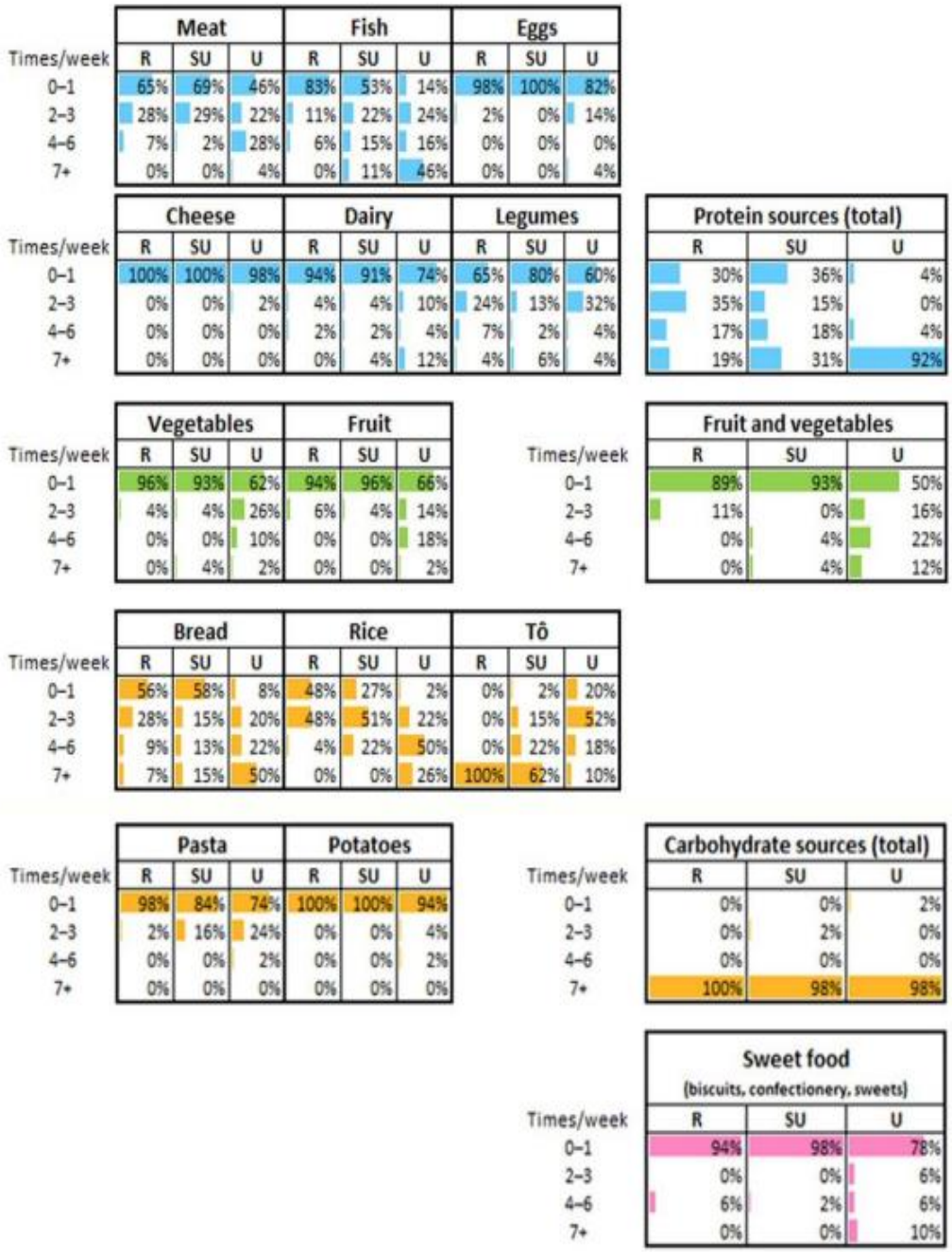


Figure 3. Macronutrient intake in adults and children in the rural, semi-urban and urban areas. The box plots represent median with interquartile range. Total proteins expressed as percentage of total caloric intake in adults (a) and children (b). Animal proteins as percentage of total proteins in adults (c) and children (d). Simple sugars expressed as percentage of total caloric intake in adults (e) and children (f). Fiber intake expressed as grams per 1000 kcal in adults (g) and children (h). Statistically significant by Mann–Whitney test *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.



Meat				Fish			Eggs		
Times/week	R	SU	U	R	SU	U	R	SU	U
0–1	65%	69%	46%	83%	53%	14%	98%	100%	82%
2–3	28%	29%	22%	11%	22%	24%	2%	0%	14%
4–6	7%	2%	28%	6%	15%	16%	0%	0%	0%
7+	0%	0%	4%	0%	11%	46%	0%	0%	4%

Cheese			Dairy			Legumes		
Times/week	R	SU	U	R	SU	U	R	SU	U
0–1	100%	100%	98%	94%	91%	74%	65%	80%	60%
2–3	0%	0%	2%	4%	4%	10%	24%	13%	32%
4–6	0%	0%	0%	2%	2%	4%	7%	2%	4%
7+	0%	0%	0%	0%	4%	12%	4%	6%	4%

Protein sources (total)		
R	SU	U
30%	36%	4%
35%	15%	0%
17%	18%	4%
19%	31%	92%

Vegetables			Fruit		
Times/week	R	SU	U	R	SU	U
0–1	96%	93%	62%	94%	96%	66%
2–3	4%	4%	26%	6%	4%	14%
4–6	0%	0%	10%	0%	0%	18%
7+	0%	4%	2%	0%	0%	2%

Fruit and vegetables		
Times/week	R	SU	U
0–1	89%	93%	50%
2–3	11%	0%	16%
4–6	0%	4%	22%
7+	0%	4%	12%

Bread			Rice			Tô		
Times/week	R	SU	U	R	SU	U	R	SU	U
0–1	56%	58%	8%	48%	27%	2%	0%	2%	20%
2–3	28%	15%	20%	48%	51%	22%	0%	15%	52%
4–6	9%	13%	22%	4%	22%	50%	0%	22%	18%
7+	7%	15%	50%	0%	0%	26%	100%	62%	10%

Pasta			Potatoes		
Times/week	R	SU	U	R	SU	U
0–1	98%	84%	74%	100%	100%	94%
2–3	2%	16%	24%	0%	0%	4%
4–6	0%	0%	2%	0%	0%	2%
7+	0%	0%	0%	0%	0%	0%

Carbohydrate sources (total)		
Times/week	R	SU	U
0–1	0%	0%	2%
2–3	0%	2%	0%
4–6	0%	0%	0%
7+	100%	98%	98%

Sweet food (biscuits, confectionery, sweets)		
Times/week	R	SU	U
0–1	94%	98%	78%
2–3	0%	0%	6%
4–6	6%	2%	6%
7+	0%	0%	10%

Figure 4. Weekly frequency of food consumption. The frequency of consumption of the categorized food items was referred to the seven days prior to the interview. Data are referred to all the enrolled individuals, without distinction of age. R = rural; SU = semi-urbanized; U = urban cohorts. Percentages may not add up to exactly 100% due to rounding. Right panel: Table of the total protein sources showing the proportion of individuals from the three cohorts that consume food rich in proteins (such as meat, fish, egg, cheese, dairy and legumes). Similarly, table of fruit and vegetables showing the proportion of individuals consuming weekly both fruits and vegetables; table of total carbohydrate sources showing the proportion of individuals consuming weekly foods rich in carbohydrates (such as bread, rice, to, pasta and potatoes); table of sweet food showing the proportion of individuals consuming weekly foods rich in simple sugars.

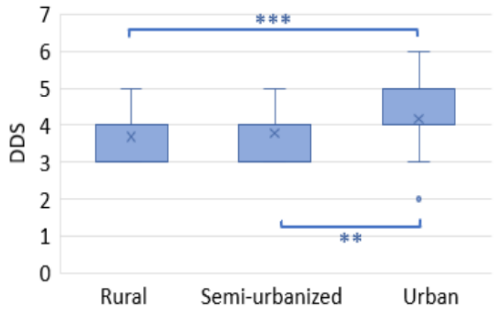


Figure 5. Dietary diversity score (DDS). The box plots show the median and interquartile range (IQR). "x" is the mean and blue dots are outliers. *** $p < 0.001$ ** $p < 0.01$ (Mann–Whitney test).

3.4. Nutritional Status Changes across the Cohorts

The nutritional statuses of adults and children from the different areas are shown in Figures 6 and 7, respectively. The BMI in adults showed a gradient with rural adults having the lowest BMI and urban adults the highest (Figure 6a,b). In Supplementary Material Table S6, adults' BMI score divided by gender is reported. As expected, overweight and obesity are absent in the rural cohort, whereas an increasing proportion of adults from the semi-urbanized or urban area are overweight (13% and 25%, respectively) or obese (9% and 17%, respectively). In contrast, in the rural cohort, 28% of adults are underweight. Among children, standard deviations of BMI-for-age (5–18 years) and weight-for-height (under 5 years) were calculated to obtain the proportion of children who were underweight or suffered acute malnutrition (Figure 7a,b). Thirty-six percent of rural children were malnourished (16% with moderate acute malnutrition, MAM, and 20% with severe acute malnutrition, SAM). This proportion was lower in the semi-urbanized (15.6% MAM, 9.4% SAM) and in the urban (7.7% MAM, 0% SAM; Figure 7a) children. With regard to chronic malnutrition, there were no differences in the prevalence of stunting (defined as a height-for-age below −2 SD) between the rural and semi-urbanized children (20% and 22%, respectively). In contrast, the proportion of stunted children was reduced (8%) in urban children (Figure 7c).

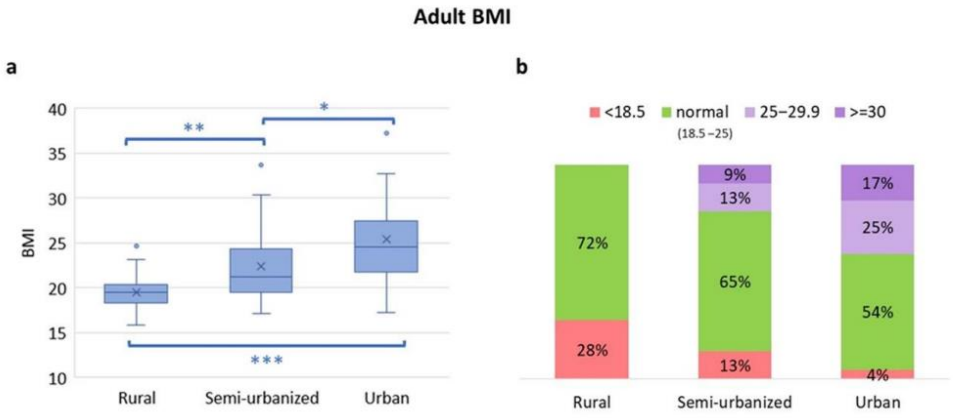


Figure 6. Comparison of the BMI categories among adults from rural, semi-urban and urban areas. (**a**) BMI distribution. Boxplots show median and interquartile range. "x" is the mean and blue dots are outliers. Statistically significant by Mann–Whitney test *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$. (**b**) Proportion of adults with underweight (BMI < 18.5 kg/m2), normal weight (BMI 18.5–25 kg/m2), overweight (BMI 25–29.9 kg/m2) and obesity (>30 kg/m2) across the areas.

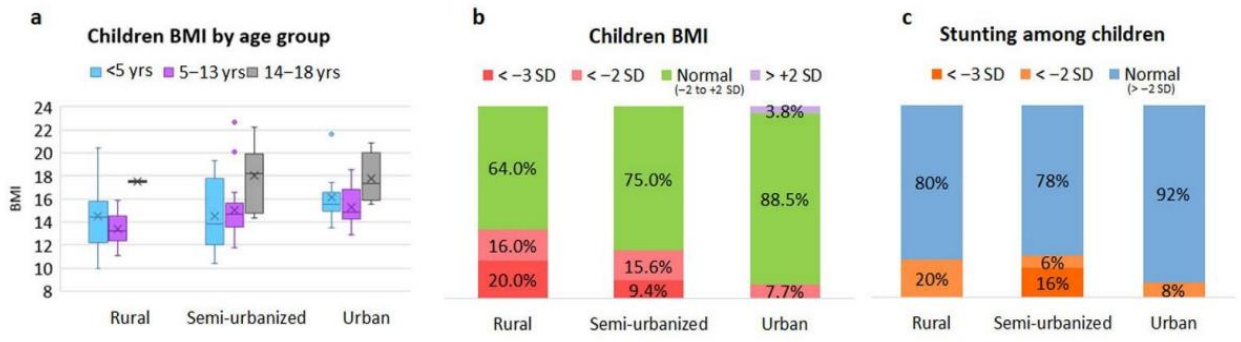


Figure 7. Comparison of BMI categories among children from the rural, semi-urban and urban areas. (**a**) BMI by age group, boxplots show median and interquartile range (IQR). "x" is the mean and blue dots are outliers (**b**) BMI distribution. BMI < −2 SD defines acute malnutrition; <−3 SD severe acute malnutrition. For children < 5 years, SD of weight-for-height was considered instead of BMI. (**c**) Prevalence of stunting among children; <−2 SD as moderate stunting, <−3 SD as severe stunting. yrs, years old.

Finally, in the semi-urbanized and urban families we observed a co-presence of family members affected by either under- or over-nutrition (three out of 10 households and in two out of 10, respectively; Figure 8). On the other hand, in the rural and semi-urbanized households, no adult or child was overweight, but, generally, at least one family member (child or adult) was underweight/wasted or stunted, when compared to urban families (Figure 8).

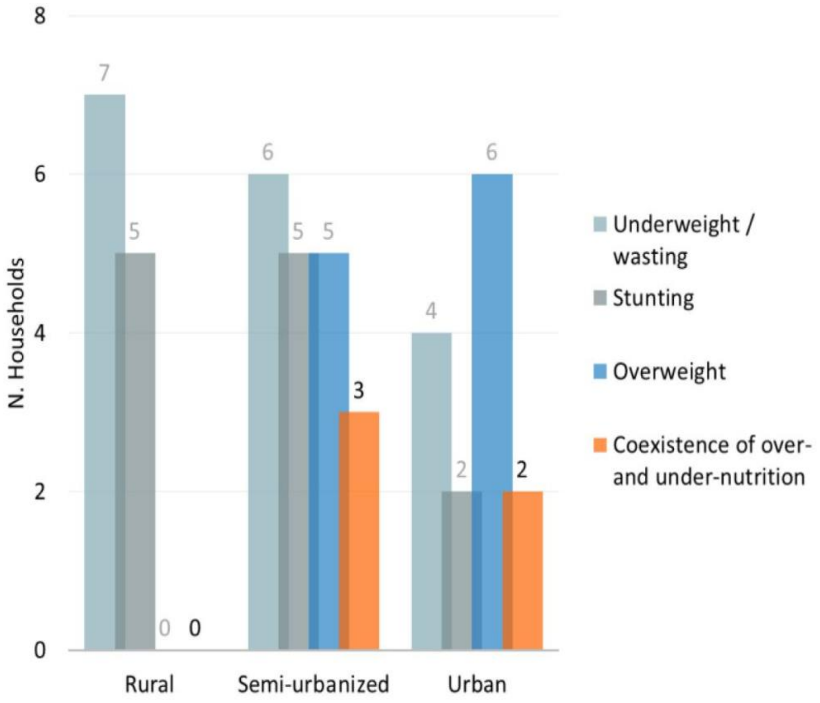


Figure 8. Double burden of malnutrition. Distribution of different forms of malnutrition (under- or overweight) within families. The number of households with at least one family member affected by underweight, stunting or overweight was reported.

4. Discussion

This study describes dietary habits and lifestyle of families belonging to the same ethnic group and living at different stages of urbanization in Burkina Faso. In accordance with previous reports [40,41,42,43], we observed pronounced socio-cultural and economic changes, associated to different dietary patterns, across families living in rural areas, in semi-urban areas and in the capital city. The main changes observed were in (i) family composition: Polygamy in the rural and semi-urbanized cohorts versus monogamy in the urban area; (ii) education: High illiteracy in rural areas, especially among women; (iii) employment: Manual and field workers in the rural and semi-urbanized areas, more specialized jobs in the city; (iv) religion: Traditional animism in rural villages versus prevalence of monotheistic religion in the urban area; (v) dwelling typology: Traditional huts built with bundles of straw stored against their circular walls made of mud bricks without electricity and water versus brick houses with electricity and water supplies in the cities. The families in the selected areas represent a model that allows us to observe the effect of rapid urbanization and rural-to-urban transition, as well as the consequent socio-economic changes in dietary habits, lifestyle and health. It is worth noting that we selected households in the capital city with a medium–high income to observe the effect of this transition, similarly to what has happened in the past in the Western countries [27,44]. Therefore, dietary habits of low-income urbanized individuals were not investigated. As observed in our previous studies in children from Burkina Faso [22,23] and as described in other reports [1,2,3,4,11,14,19,20,21,45], the fast urbanization is bringing along a transition towards Western diets and lifestyles, increasing the distance from traditional rural areas where access to food is limited to local products and follows seasonal availability. In urban settings, supermarkets and restaurants provide access to a great variety of food items. Ultra-processed food, soft drinks and foods rich in additives, salt, sugar and saturated fats are easily accessible, determining a nutrition transition towards a Western-style diet. In our study, to collect information on dietary habits of the three cohorts, trained field staff administered both 24 h recall diary and a seven-day food frequency questionnaire (FFQ). The 24-h dietary recall and the FFQ are useful tools for dietary assessment and epidemiological purposes, and FFQ is often used in epidemiological studies to estimate long-term dietary exposure. These methods are mainly used for their applicability in large samples and their capability to categorize foods based on their intake [46,47]. Dietary assessment methods have potential limitations and they are hugely argued over in literature in order to keep attention on them and to ensure an adequate evaluation of dietary pattern and macronutrients intake in population surveys. A potential limitation of such dietary assessment methods is due to the risk of bias and under- or overestimation (mainly due to memory and portion size estimation) or caused by missing information when assessing habitual diets. A recent study in adolescents from Burkina Faso [48] validated the 24-h dietary recall with the support of the observed weighed food records for estimating nutrient intakes. In our study, a potential limitation of the dietary assessment methods could be the discrepancies in data collection between the 24-h dietary recall and the FFQ. Similarly, to support the compilation of both the diary and the questionnaire, pictures of size portions were shown to participants. Moreover, to limit as much as possible biases on the collected dietary information and to make sure that collected data were reliable, results of both 24-h dietary recall and of FFQ were compared in order to more precisely

assess macronutrient intakes. In general, in the three cohorts of this study we observed that the intake of proteins, especially animal proteins, and simple sugars increases with the level of urbanization, while fiber intake showed an opposite trend. Similarly, others countries, such as Ghana in West Africa [49], or Tanzania in East Africa [45], or other world regions, such as rural China [50], have experienced fast urbanization and changes of dietary patterns across the rural-to urban transition. These studies described mainly a reduction of carbohydrates and fiber consumption and increased intake of animal protein and fat in urban populations. Despite a low availability of fruits and vegetables outside the urban settings during the dry season (when the interview was conducted), Burkinabè people acquired dietary fiber from tô and/or bouille, both made with whole ground grains, and from plant-based ingredients used in the added sauce. Rural and semi-urbanized individuals showed higher fiber consumption compared to urbanites. These observations confirm in whole families what we previously reported in small cohorts of Burkinabè children living at different levels of urbanization compared to European children [22,23]. We previously showed that a fiber-rich diet, typical of the rural population, enriches the gut microbiota of bacterial species that are able to ferment dietary indigestible polysaccharides, producing short-chain fatty acids (SCFAs). These metabolites are well known for their anti-inflammatory role and for their contribution to the maintenance of health [51]. In contrast, a Western-style diet of urban people, rich in protein, simple sugars and fat and poor in fiber, has the potential to deeply influence gut microbiota composition, as well as the host's health and development [52,53,54,55]. In recent years, a general increase in non-communicable conditions typical of Western industrialized countries, such as inflammatory bowel diseases (IBDs), type 2 diabetes, obesity, allergies and colorectal cancer [56] has been observed also in low-income countries, in association with improvement of socio-economic conditions and Western-like dietary habits. In the present study, the nutritional and anthropometric status changed significantly across the rural, semi-urban and urban areas. Overweight and obesity in children and young adults are rapidly increasing all over the world, albeit at different rates, depending on geographic location and following different development models [57]. Similarly, rural areas in China have experienced the co-existence of overweight and obesity, undernutrition and micronutrient deficiencies associated to changes of dietary habits [50,58]. In the past, childhood overweight was considered an exclusive problem in high-income countries but we are currently witnessing an increase even in low- and middle-income countries (LMICs), and especially in urban areas [59]. The World Health Organization (WHO) estimated that 39 million children under the age of 5 and 340 million children and adolescents between 5 and 19 years are overweight or obese [56]. Overweight and obesity in these age groups deserve particular attention, as they represent a particular risk factor linked to the onset of numerous chronic diseases, such as diabetes, cardiovascular diseases and some types of cancer [60]. Furthermore, pre-adolescent and adolescent obesity is considered a strong predictor of obesity in adulthood [61]. It has been calculated that more than a third of children and about half of adolescents who are overweight maintain this condition when they become adults [62], with all the consequences that this entails in terms of health and social costs. On the other hand, malnutrition is also still a major public health problem in LMICs. Across the areas in our study, the prevalence of underweight and acute malnutrition (wasting) decreased with the increase in urbanization level. The prevalence of child stunting was similar in the non-urban children, but much lower in the capital city. In addition to diet, the high

prevalence of recurrent infections, including malaria, in the rural environments compared to urban areas is an important factor [63]. Infections can cause rapid weight loss in children and, in the long term, threaten the achievement of full development. Prolonged undernutrition leads to stunting, a severe condition with long-term consequences such as impaired neurocognitive development. The last nutritional survey in communities and host sites conducted in Burkina Faso in 2019 [64] reported a prevalence of severe wasting and wasting among children under 5 years of age of 1.0% and 8.1%, respectively, 23.8% of stunting and 1.6% of overweight. In our randomly selected families, even though children under 5 years represent a small proportion among the overall child group, we observed that the prevalence of severe wasting, moderate wasting and stunting were significantly higher in the rural children compared to urban children. In contrast, in the urban cohort, wasting and stunting were not detected in children under 5 years of age, whereas one child out of ten was obese. The "double burden of malnutrition" refers to "the coexistence of undernutrition along with overweight, obesity or diet-related NCDs, within individuals, households and populations, and across the life-course" [65]. This condition is often observed as a side-effect of urbanization, demographic transition, changes in occupational structure and shifts in patterns of diet and physical activity [65,66,67]. As previously described in literature [68], in some urban families enrolled in this study, we found the coexistence of under- and over-nutrition. This condition was not observed in rural families where we reported various forms of undernutrition. However, it is interesting to highlight how the nutrition transition starts well before full-fledged urbanization. In fact, families from the semi-urbanized cohort showed a higher presence of the double burden within the same household, emphasizing the risk of areas at an early stage of urbanization paying the double price of the consequences of under- and over-nutrition. Nonetheless, nutritional interventions in these areas are still focused almost uniquely on undernutrition.

5. Conclusions

Burkina Faso is a low-income sub-Sahelian country at an early stage of urbanization. Rapidly urbanizing areas, like the capital city of Ouagadougou, are quickly showing the effects of the globalization of food systems and the westernization of both diet and lifestyle. This phenomenon is consequently associated with the increase in conditions typical of the globalized world, including overweight, obesity and other NCDs. We are aware that the health outcomes of urbanized populations and tailored interventions to break the emergent epidemic may not be fully identified in a short period of time. In the future, the complex interactions among urbanization, environment, diet, socio-economic and cultural status requires longitudinal population studies to deeply describe dietary transition and its evolution over time, as well as health determinants and outcomes in African urban areas. On the other hand, rural areas of sub-Saharan Africa continue to lag behind, especially in terms of education and health, with high levels of illiteracy and high prevalence of infections and different forms of under-nutrition like child wasting and stunting or adult underweight.

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Supplementary Materials
The following are available online at https://www.mdpi.com/article/10.3390/nu14091782/s1, Figure S1: Proportion of individuals in the three cohorts, adults (upper panel) and children (bottom panel), who consume an adequate quantity of fibre based on the recommended standards. Table S1: Food portion sizes estimated for the main food items. An average standard composition of tô sauce was included in our calculation. For bouillie, an average composition was calculated (for 100 g of dry product: millet 60 g, soy 20 g, peanuts 10 g, sugar 9 g). Table S2: Living conditions of the households enrolled in the three different environments. Table S3: Estimation of macronutrients intake in adults of the three cohorts. Data are reported as median and interquartile range (IQR). Table S4: Estimation of macronutrients intake in children of the three cohorts. Data are reported as median and Interquartile range (IQR). For fibre, absolute amount in grams is not reported due to differences in age and weight of children. Table S5: Comparison of the weekly frequency of meals not prepared at home among the three cohorts, in particular food consumed at restaurant and food from a local market. Table S6: Adult BMI divided by gender. Median and IQR are reported.

Author Contributions
Conceptualization, P.L.; methodology, S.C., E.B., S.D., K.D., B.K. and H.T.; formal analysis, S.C., E.B., L.S., A.G. and S.R. (Sonia Renzi); investigation, S.C., S.D., K.D., B.K., P.L. and H.T.; writing—original draft preparation, S.C., M.D.P. and P.L.; writing—review and editing, S.C., M.D.P., L.S., S.R. (Sara Renzo), M.P., Q.d.M., T.P., H.T., D.C. and P.L.; funding acquisition, P.L. and D.C. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement
The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of the National Ethics Committee of Burkina Faso (protocol code 2018-8-104).

Informed Consent Statement
Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

Conflicts of Interest

The authors declare no conflict of interest.

Footnotes

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Conclusions

Fungi have accompanied mammalian, and specifically human, evolution by establishing various type of relationships. Despite fungi being the most abundant and ubiquitous eukaryotes, it has been suggested that only a very small fraction of their diversity is currently known, leaving room for major investigations. Understanding and deepening the processes underpinning fungi-host interaction is becoming feasible thanks to technical advances and well-established ecological and evolutionary methods.

Historically, human eating habits have repeatedly changed due to climate change, migration to new environments and for cultural and lifestyle changes. With the Neolithic revolution, a wide-scale human transition towards agriculture and settlements, the introduction of fermentation has become a standard practice for naturally preserving food sources. This ancient technology has made fungi part of our diet and of our gut microbial ecosystem. Beside diet, human microbiota's dynamics are strongly affected by environment and general lifestyle. The rural environment has characterized our development for the last 10,000 years, but in the last century, the Western world has undergone a drastic demographic transition from rural to urban with a consequent change in lifestyle, eating habits and socio-economic conditions.

The gut microbiota has co-evolved with the changes in human physiology over time, also following the change in exposure to microorganisms, determined by the improvement of sanitation standards, to the possibility of access to clean water and the reduction of direct contact with animals. This transition has undoubtedly also had a big impact on disease epidemiology: while infectious diseases are still the leading cause of death in many poor rural areas of the world, particularly in third world countries, noncommunicable diseases have increased exponentially in newly developed urban areas, with particular emphasis on non-communicable inflammatory and autoimmune diseases, such as allergies and chronic inflammatory bowel diseases (IBD).

Globalization of foods, medical care, social dynamics, and sanitation is drastically changing human microbiomes; recent studies have linked contemporary habits to bacterial diversity loss and a proclivity to develop typical Western conditions such as obesity and autoimmune conditions. However, its impact on the mycobiota has not been clarified yet.

Urbanization is the first step towards globalization, and represents a set of social, cultural and economic variations that have a strong impact on the human condition. Urbanization plays a crucial role in modulating human's health and for this reason several recent studies have focused on the effect of urbanization on the human microbiome. At present, urbanization rate in Sub-Saharan Africa (SSA) is the highest globally; while the Westernization of lifestyle is already pervasive in metropolitan areas, people in rural areas are still close to a traditional lifestyle but starting to be exposed to the increasing contamination of Western-like factors. Thus, SSA represent the the ideal context in which investigating the effect of urbanization on the human microbiota in real time.

The results of the in-depth assessment of the gut mycobiota of whole families/households here discussed, demonstrate that the level of urbanization plays a crucial role in the diversity, composition, and assembly of intestinal fungal communities, producing effects also within family

members. Immune training and shaping of the immune responses occur early in life, thus enrolling adults and children coming from different degrees of urbanized environments held the key to understanding which fungal species could be associated with the Hygiene Hypothesis therefore with the predisposition towards possible IBD conditions. The extensive scope of the study's setup enabled the researchers to bring out how the fungal microbiota within family members remained comparable in diversity in the two groups with the highest rates of urbanization, namely Ouagadougou (Burkina Faso's municipality) and Italy. Conversely, the microbiome differed between family members in the mid-urbanization area (Nanoro) and in rural villages which are the areas with the highest degree of ruralization. Thus, we described the dependence of the fungal microbiota structure on the degree of urbanization, furthermore we also demonstrated that the development of the fungal microbiota within family members is also related to the degree of urbanization. The alpha diversity measures increased depending on the level of urbanization, i.e., showing minimum values in the Italy group and maximum values in the rural villages and this occurred for all the indices tested. In this case, unlike the beta diversity analysis, the interaction between geographical area and family degree was not significant, highlighting that only the geographical area acted as a driver for changing the alpha diversity measures. Differential abundance analysis sorted 33 different sequence variants that significantly change within sample groups. Among these, we highlighted 12 ASVs classified at the deepest level of taxonomic assignment (Species level assignment) whose abundances varied following a gradient of urbanization. These species are known to be associated with humans and others have rarely been found on humans, some are known to induce differential immune responses in human populations, and some are well known opportunistic pathogens in transition from commensalism.

The co-presence of fungal species with potentially immunogenic profiles in non-immunocompromised subjects (healthy donors) could trigger a combination of protective effects able, over time, of reducing the onset of chronic inflammatory diseases. So has been hypothesize that the urbanization and consequently the decrease of specific fungal species represent one of the key factors towards chronic inflammatory conditions correlated to the western-related condition. Moreover, has been consider that this difference in fungal species composition among family members, evident in the rural villages but absent in the cities, could be an interesting novel target to understand the hygiene hypothesis.

The findings here discussed show the importance of investigating the interactions between the yeast component of the microbiota and their host. Only using evolution as a magnifying lens, together with modern analyses, we will understand the ecological transition of fungi from passengers to colonisers and invaders and unravel the subtle networks discriminating the role of the mycobiota in health from disease.