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Digestive tract mycobiota and microbiota and the effects on the immune system

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ABSTRACT

The human gastrointestinal tract exists as a complex ecosystem and contains a mycobiome and a microbiome that play central roles in host health, disease and immune system regulation. Here, we reviewed the traditional culture-dependent methods, the culturomics methods and the molecular methods used to study the gut mycobiome and microbiome. With the development of next-generation sequencing techniques, these last two methods have greatly broadened the understanding of the roles of gut bacteria in health and disease. Thus, dysbiosis of the gut microbiota and mycobiota has been found to be associated with some diseases; disruptions to the fungal and bacterial commensal communities influence the immune response and impact disease status.

1. Introduction

The human digestive tract microbiome is a complex ecosystem that harbours a diversity of microorganisms, including Bacteria, Archaea, viruses and Eukarya [1]. This microbial ecosystem plays a central role in host development, including immune system development, physiology, digestion, vitamin and nutrient production and protection against pathogen colonization [2]. The gut microbiota (bacterial community) composition is influenced by interactions among community members, the genetics of the host, dietary habits and the environment [3]. Bacteria are the most dominant group in the community and have the four following major phyla residents: *Firmicutes, Bacteroidetes, Actinobacteria* and *Proteobacteria* [1]. Therefore, the bacterial community has been well characterized and has been the focus of human microbiome research in health and disease. The mycobiota (fungal community) represents the most significant group of eukaryotes in the human gut microbiota and has been found to be associated with a wide range of diseases, such as hepatitis, inflammatory bowel diseases, and systemic mycosis [4]. In recent years, the fungal community has been gaining attention, but the field of mycobiome research is still in its infancy. The mycobiome has been traditionally studied using culture-dependent methods. To date, to investigate the role of the fungal community in both health and disease, researchers have begun to use next-generation sequencing (NGS) technology [5]. We review here the recent knowledge from the study of digestive tract mycobiota and microbiota in healthy and diseased individuals and its interaction with the host's immune system.

2. Digestive tract mycobiota and microbiota characterization

2.1. Culture-dependent methods

The preceding investigations of digestive tract fungal and bacterial diversity were based on culture methods using classic selective media to isolate fungi and bacteria [5]. Yeast and filamentous fungi are cultured in different culture media, including on Sabouraud dextrose agar, malt agar, potato dextrose agar, CZAPEK, Columbia agar [4]. A mix of broad-spectrum antibiotics are then added into the culture media to limit bacterial growth [4]. The isolated colonies are identified by classic phenotypic identification methods based on microscopic and biochemical tests. Thus, Gram, Ziehl-Neelsen, and Giemsa stains are used in bacterial microscopic identifications, while lactophenol staining is used for fungal macro- and microscopic identifications. Based on the traditional culture methods, approximately 688 bacterial species, 2

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Archaea and 88 fungal species have been isolated from the human gut [4,6]. This may reflect the approximative gut composition of bacteria at 10^{11-12} CFU per gram of faeces and of fungi at 0 up to 10^{6} CFU per gram of faeces, as reported in the literature [7,8]. The fungal species commonly isolated belong to genera of *Candida*, *Aspergillus*, and *Penicillium*, and the nine bacterial genera belong to four phyla (*Actinobacteria*, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*) [1,6]. However, traditional methods present some disadvantages, such as misidentification and time requirements. The major bias associated with traditional culture methods is the fast growth of the major population in classic nutrient growth media; thus, the minor populations, including some potential pathogenic populations, such as *Salmonella typhi* [6], are missed. A recent alternative tool, such as Matrix Assisted Laser Desorption Ionisation – Time of Flight – Mass Spectrometry (MALDI-TOF-MS), is used for both fungal and bacterial colony identifications [9,10]. The MALDI-TOF MS-based identification technique analyses the protein content from treated or intact microorganisms in the form of a spectrum that is considered to be a protein fingerprint that is specific to a microorganism. An unknown bacterial or fungal colony is identified by comparing its spectrum with the spectra in the reference database [9]. MALDI-TOF MS-based identification is simple, fast, accurate and allows for the rapid screening of large numbers of colonies isolated in culture [10]. Culturomics methods, which consist of the application of high-throughput culture conditions followed by MALDI-TOF MS and/or 16S rRNA identification, allow the known diversity of the gut microbiota to be expanded [10]. This approach has recently added 531 bacterial species to the human gut bacterial repertoire [11]. The first study that used a culturomics approach to characterize the gut mycobiota isolated 41 diverse fungal species from 14 faecal samples, of which 10 species have been reported for the first time in the human gut [12]. The advantages of culturomics are that it allows the growth of minor populations under various culture conditions and that it allows the culture of uncultured organisms that correspond to sequences not previously assigned [11]. However, there are no standard culture conditions published for mycobiome studies as there are for bacterial culturomics conditions. The bias associated with culture conditions is the lack of some 'non-cultivable' microorganisms. Additionally, the culturomics technique does not directly provide information on the enzymatic abilities of the microorganisms isolated and requires a major workload [13].

2.2. Culture-independent methods

Molecular tools have emerged since 1990 for digestive tract mycobiota and microbiota investigations. PCR-based methods used primers that targeted 16S RNA in microbiota studies and primers that targeted 18S, 5.8S, 26S, and 28S RNA and the ITS (Internal Transcribed Spacer) regions in mycobiome studies. The variability of the ITS region enables the classification of fungi into the genera and species levels. Then, computational algorithms are used to align the operational taxonomic unit (OTUs) with those from the fungal database. Other molecular techniques, such as cloning libraries as rDNA-based denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP) analysis, in situ hybridization techniques, and Sanger sequencing, have been used on stool samples or digestive mucosa biopsies to investigate the fungal and bacterial diversity [1,14,15]. All these molecular techniques helped to identify more than 30 bacterial phyla (major phyla of Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, Proteobacteria, Verrucomicrobia, and Cyanobacteria), whereas only three fungal phyla (Ascomycota, Basidiomycota, and Zygomycota) are commonly reported [1,15]. Since 2006, metagenomic studies have been used to explore gut bacterial diversity. However, this approach was not widely applied in gut mycobiota analysis. The variation and relative abundance of bacterial phyla in several studies depend significantly on the 16S hypervariable region and on the DNA extraction kit used [6].

More recently, NGS (next-generation sequencing) technologies including Roche 454, Illumina HiSeq and MiSeq, and Ion Torrent have been used to characterize the digestive tract mycobiome and microbiome in patients and healthy persons in a number of studies [12,16,17]. The high variability of reported fungi in these different studies seems to depend on host status, environment, diet and choices of primers and DNA extraction techniques. Despite the progress in technology, there are limited databases available for NGS data generated for mycobiome analyses compared to the number of bacterial databases [18].

The combination of culturomics and next-generation sequencing approaches is helpful for human digestive tract mycobiome analysis and can contribute to reducing the gap in gut mycobiota knowledge.

3. Gut mycobiota and microbiota interactions in healthy and diseased participants

3.1. In healthy participants or healthy volunteers

There are no specific fungi described in the literature that are only specific to healthy people. Several studies of the gut mycobiome in healthy participants or in healthy volunteers have reported fungal genera belonging to Ascomycota, Basidiomycota and Zygomycota [4] with the predominance of *Candida, Saccharomyces, Trichosporon* and *Cladosporium* [19]. *Candida* species are usually described as commensal yeasts in individuals with healthy gastrointestinal tracts. The first investigation combined the culture and molecular methods and reported a low diversity of fungi, approximately 1 to 3 fungal species per individual, including *Candida parapsilosis, Paecilomyces fumosoroseus,* and *Gloeotinia temulenta* [8]. Further, a large diversity and high variability of fungal species have been reported in healthy individuals in different studies [16,17,20,21]. Some fungi have been described as more prevalent in non-obese patients, such as *Mucor, Candida* and *Penicillium*, whereas *Candida, Nakaseomyces, Penicillium* and *Pichia* (telemorph of *Candida spp.*) are found predominantly in obese patients [19,22]. Most of the fungal species reported in these works were from the environment (*Cladosporium sp, Pucinia poarumn, Aspergillus restrictus*), diet (*Penicillium roqueforti*, *Saccharomyces cerevisiae*) or opportunist pathogens (*C. albicans, Candida glabrata,* and *Trichosporon asahii*) [20,23]. The mycobiome reported in healthy humans revealed a high inter-individual variability that was much less persistent in individuals and was instable over time [16]. The diversity and variability of fungal species observed in healthy individuals could be related to differences in geographical location and lifestyle, as has been observed in bacterial studies. However, further studies are needed to clarify the transmission of residential gut fungal species. Knowledges are limited on specific role of fungal species found in healthy gut compared to bacteria. Bacterial species that belong to the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria are more diverse and abundant in healthy guts. Different compositions of gut microbiota have been reported according to different locations, ages, sexes, races, and diets of the host [24]. The bacterial community has a low diversity during the first year of life and develops gradually during the period of growth. In contrast to the gut mycobiota, the bacterial community is relatively stable in adults over time [25]. Diet can impact and shape the composition of the gut microbiota. For example, the *Bacteroides* genus has been found to be associated with the consumption of animal proteins, while *Provella* are found to be associated with diets rich in carbohydrates [26]. The dominance of *Bacteroides* has been described in the microbiota from European patients, whereas *Provella* was found to be dominant in those from Africa [27]. Additionally, regarding the interaction between the bacterial and fungal communities in healthy participants, Hoffmann et al. reported a significant correlation between both Ascomycota and Basidiomycota with some bacterial lineages [17]. Indeed, this author observed a positive association between *Candida* and *Methanobrevibacter* with the *Provella* group, and each of these was found to be correlated with a diet rich in carbohydrates. Now, we know more about

some of the functions and roles of the gut microbiome, and further studies are needed to elucidate the roles of the gut mycobiome in healthy participants.

3.2. In diseased participants

Candida species have been primarily described in various diseases, such as inflammatory bowel disease (IBD), Crohn's disease (CD), ulcerative colitis (UC), and gut inflammation [4]. Opportunist pathogenic yeasts are commonly described in immunodeficient patients (HIV, cancer, and transplanted patients) [4]. The non-albicans fungal species of *Candida, Trichosporon, Rhodotorula, Cryptococcus neoformans, and Geotrichum* are increasingly emerging in the frequent diagnosis of immunodeficient patients [28]. Additionally, an increased diversity and richness of the gut mycobiome have been described in IBD, CD, UC, and hepatitis B patients compared to those in controls [14,15]. In IBD patients, Sokol et al. [29] observed a dysbiosis of the gut mycobiome with a decreased proportion of *Saccharomyces cerevisiae* and an increased proportion of *C. albicans* compared with those in the healthy controls. Fungal microbiota dysbiosis in IBD is characterized by alterations in biodiversity and composition. This observation suggested that the mycobiome might play a role in inflammatory bowel disease. Another investigation on the gut mycobiome and bacterial dysbiosis in infants reported that there was a positive association between the development of atopic wheeze, an increase in the fungal species *Pichia kudriavzevii* and a decrease in *Saccharomycetales* [30]. Similar to fungi, bacterial dysbiosis has been found to be associated with a number of diseases, including IBD, diabetes, allergy, obesity, central nervous diseases, colorectal cancer and infectious diseases [31]. Previous studies have observed a decrease in the abundance and diversity of Firmicutes in IBD patients [25]. Antibiotic treatments in infectious diseases alter the gut microbiota composition and reduce the resistance to pathogen colonization. An increase in susceptibility to *Clostridium difficile* colonization has been reported in patients with antibiotic treatment compared to that in healthy controls [25]. In diabetic patients, an alteration and a low diversity of gut microbiota have been reported [31]. A decrease in the diversity of *Bacteroides* has been reported in patients suffering from allergies. Additionally, another study observed a high abundance of some bacterial species of *Streptococcus* sp. and *Bacteroides* sp. As well as a decrease in *Ruminococcus gnavus* and *Bifidobacterium* sp. in atopic wheeze patients compared to the abundance in healthy controls [30]. The gut microbiota role is described in a number of diseases, but the mycobiome role remains unclear; therefore, further investigations are needed to elucidate the role of fungi in disease.

4. Microbiota, mycobiota and host immunity

4.1. The impact of microbiota on the immune system

Technological advances are shedding new light on the sophisticated ways in which microbes influence human health; one of the major impacts of the mammalian microbiota is its effect on the development and function of the immune system. Communities of bacteria and immune cells are closely linked, especially those residing in the intestinal tract [32]. This interaction is likely to be a key factor in the maturation of both T helper 1 (Th1) cells [33] and regulatory T cell (Treg) functions [34] and in the functional suppression of the normal neonatal propensity for T helper 2 (Th2) cell responses, which are still dominant in the perinatal period. While early hypotheses have suggested that these host-microbial interactions were largely initiated at the beginning of the postnatal period, microbes have now been detected in the placental and foetal tissues during normal pregnancies [35]. This is likely to suggest that maternal microbial transfer to the offspring begins during pregnancy. Therefore, variations in the maternal environment appear to modulate foetal immune responses, with measurable differences in both effector and Treg cell responses in the cord blood of newborns whose mothers inhabit more diverse microbial environments compared to the responses in those who do not [36]. Among mothers living on farms, Treg cells were mainly at higher levels and were more efficient in offspring after specific stimulation compared with those of nonfarming mothers. This was associated with decreased Th2 cytokine levels and less cell proliferation in the offspring of farming mothers. Exposure to several animal species also has an effect on Treg cell markers and interferon gamma (IFN-γ) secretion [37]. These well-balanced regulatory responses constrain proinflammatory Th1, Th2 and Th17 clones and curb the risk of chronic inflammatory diseases, such as allergies and autoimmune disease [38]. This has been well illustrated in rural Austria, where children in settings with rich microbial exposure have a reduced risk of allergies [37], and antenatal exposure largely accounts for this protective effect [39]. Experimental animal models also demonstrate that maternal exposure to commensal bacteria, such as *Lactobacillus rhamnoses* [40], during pregnancy can attenuate allergic sensitization and inflammation in the offspring. Thus, microbial contact during prenatal life may imprint the offspring microbiota and immune system in preparation for the much larger inoculum transferred during vaginal delivery and breastfeeding.

4.2. Fungal interactions with the human immune system

Commensal fungi are part of the intestinal microbial community in many species, such as rats, guinea pigs, rabbits, pigs, dogs, and humans, with the highest densities in the terminal colon [41]. Although few studies have suggested the presence of commensal fungi in the gut [14,41], their interaction with the immune system of the mucosa or their involvement in diseases is poorly understood.

Various specific immune mechanisms have been implicated in the clearance of fungi at mucosal surfaces, and many innate immune receptors have been shown to interact with fungal pathogens [42,43]. Among the mechanisms implicated in the clearance of fungi, C-type lectin domain family 7, member A (Dectin-1), caspase recruitment domain-containing protein 9 (CARD9), interleukin-17 (IL-17) and IL-22 have emerged as molecules that are responsible for host defence. For example, a genetic mutation in each of these molecules is associated with susceptibility to fungal infections in humans. A Dectin-1-deficient mouse model showed increased susceptibility to colitis that was characterized by increased mucosal erosion, inflammatory cell infiltration, TNF-α production and the augmented production of IFN-γ and IL-17. The more severe colitis cases in mice were accompanied by a fungal invasion of the colonic mucosa, as well as a general expansion of opportunistic fungi, such as *Candida* and *Trichosporon* species, and a decrease in the non-pathogenic *Saccharomyces* species. A Dectin-1 deficiency leads to altered immune responses by the commensal fungi in the gut; this inability to mount effective immune responses to specific intestinal fungi creates conditions that promote inflammation.

Intestinal fungi can influence immunity through both direct interactions with immune cells and the production of metabolites, such as prostaglandin E2 (PGE2), a potent immunomodulator produced by immune cells. It has been proposed that *Candida*-produced PGE2 can reach the lungs through the bloodstream, act on lung macrophages and promote allergic inflammation [44]. In addition, *Candida* can activate host cells to produce prostaglandins [45], suggesting a possibility that the overgrowth of the gut fungal microbiota may alter immune responses via PGE2. *Candida* also produces ligands for pattern recognition receptors (PRRs), including β-glucans, chitin, mannans, β-(1,2)-linked oligomannosides and fungal nucleic acids [46], which stimulate the innate immune response.

Moreover, fungi detected by the intestinal immune system can cause inflammation or tolerant immune responses and can modulate immune cell trafficking. Thus, phagocytes that digest fungi can release fungal-derived molecules into the circulation and may have immunoregulatory effects [47].

4.3. Dysbiosis and host immunity

The composition of the gut microbiota is more stable throughout adult life than the mycobiota is but can be altered as a result of bacterial infections, antibiotic treatment, lifestyle, surgery, and a long-term change in diet. Changes in the gut bacteria are initially described as "dysbiosis", a disruption of the healthy microbial communities that allows the overgrowth of fungi [48]; this indicates that, in the steady state, the bacterial communities keep fungi in check. Fungal overgrowth in the gut can induce macrophage polarization that increases allergic airway inflammatory cell infiltration [44]. In particular, *Candida* spp. infection can induce the production of inflammatory mediators by host cells. In patients with IBD, the disease has been reported to be associated with enrichment for *Candida* species (specifically *Candida dubliniensis* and *Candida parapsilosis*), suggesting that fungal dysbiosis may accompany disease. Previous results have shown that patients with diseases, such as ulcerative colitis, Crohn's disease, and gastric ulcers, were more heavily colonized with the fungus *C. albicans* [49]. However, although the study of the fungal microbiota is a rapidly emerging field, the mechanisms by which gut dysbiosis drives fungal overgrowth in the gut and affects the host immune responses remain poorly understood.

Similarly, treating animals with oral antifungal drugs has been reported to alter the bacterial microbiota. Antifungal treatment affects specific bacterial taxa with a decrease in the relative detection levels of *Bacteroides*, *Allobaculum*, *Clostridium*, *Desulfovibrio*, and *Lactobacillus* spp., while the relative detection of *Anaerostipes, Coprococcus*, and *Streptococcus* is increased [50]; this results in the alteration of the local and peripheral immune homeostasis. Matthew L. Wheeler et al. [50] showed that the treatment of mice with antifungal drugs increased the disease severity in chemically induced and T cell transfer-mediated models of experimental colitis and exacerbated the development of house dust mite-induced allergic airway disease. This provides compelling evidence to support a functional role of mycobiota in the modulation of immune function and the development of inflammatory disease.

5. Conclusion

Here, we have reviewed and highlighted the methods used to investigate the gut mycobiome and microbiome. We also reported the fungal and bacterial communities found in human health and disease and their interactions with the immune response. Compared to the roles of bacteria, the roles of the gut fungal community remain poorly understood in health and disease and require further investigation.

Declaration of Competing Interest

The authors declare no competing interests.

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