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Le mycobiome digestif humain : étude exploratoire

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AVANT PROPOS

Le format de présentation de cette thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille. Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette thèse. Par ailleurs, la thèse est présentée sur article publié, accepté ou soumis, associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

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Psaume 103:2

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Sommaire

Résumé

Le mycobiome digestif comprend l'ensemble des espèces de champignons contenu dans le tube digestif. La variation et la composition du mycobiome peuvent être influencées par des facteurs tels que l'alimentation, la physiologie, l'environnement et le style de vie de l'hôte. Les études initiales sur les champignons du tube digestif basées sur l'approche culture et la microscopie se sont focalisées sur la recherche spécifique des champignons pathogènes de l'homme tels que *Candida albicans*, *Candida glabrata*, *Penicillium marneffei*, *Histoplasma capsulatum*, *Cryptoccocus neoformans* et *Basidiobolus ranarum*. L'investigation des communautés de microorganismes dans les différentes parties du corps humain par métagénomique, dans le cadre du « Human Microbiome Project » et en particulier « Metagenomics of the human intestinal tract » a suscité un intérêt nouveau dans la recherche pour le mycobiome digestif. En effet, les outils de biologie moléculaire, de séquençage des clones et plus récemment le séquençage à haut débit permettent d'explorer la diversité mycobiome digestif. Cependant, très peu de travaux ont été spécifiquement consacrés à l'étude des micro-eucaryotes dont le mycobiome, compris comme l'ensemble des espèces de champignons microscopiques.

Au cours de cette thèse, nous avons réalisé dans un premier temps une revue de la littérature sur le mycobiome digestif afin d'établir le répertoire des espèces de champignons décrites dans le tube digestif et leur implication dans les infections

digestives et systémiques. Nous avons répertorié les espèces de champignons décrites chez les sujets sains et avons comparé ce répertoire à celui retrouvé dans les différentes pathologies.

Puis dans un second temps, notre travail expérimental a combiné l'approche culture et l'approche moléculaire basée sur le séquençage des clones pour explorer la diversité du mycobiome digestif. Nous avons répertorié une variété d'amorces PCR dans la littérature ciblant le gène 18S rRNA et ITS rRNA. En appliquant ces outils moléculaires et la culture à l'analyse d'une selle chez un sujet obèse nous avons détecté 16 espèces de champignons dont 11 espèces sont associées à l'alimentation avec 8 espèces de champignons observées pour la première fois dans les selles.

Ensuite, la même approche appliquée à une selle collectée chez un sujet anorexique a permis de détecter 8 espèces de champignons dont 5 espèces associées au régime alimentaire du sujet et 3 espèces retrouvées pour la première fois dans les selles humaines. Il ressort de ce travail que la diversité des champignons est liée en partie au régime alimentaire.

Dans un dernier temps en utilisant la même méthodologie, nous nous sommes intéressés à la diversité du mycobiome en fonction de l'origine géographique des sujets. Pour cela, nous avons exploré la communauté du mycobiome dans les selles provenant des quatre continents Afrique, Asie, Amérique et l'Europe. Dans ce travail, nous avons détecté 40 espèces de champignons provenant de

l’environnement dont certains pathogènes opportunistes. Il ressort de ce travail que certaines espèces comme *Candida et Malassezia* sont ubiquitaires, *Galactomyces, Saccharomyces* et *Trichosporon* sont retrouvés en commun avec différentes régions alors que la majorité des espèces restent spécifiques à chaque région. Ce travail a permis d’identifier 16 espèces de champignons qui n’avaient jamais été retrouvées dans le tube digestif chez l’homme.

Pour conclure, les travaux effectués dans le cadre de cette thèse ont permis de détecter 27 espèces de champignons pour la première fois dans les selles humaines, notre travail à donc contribuer à élargir la connaissance du répertoire des champignons dans le tube digestif humain.

Mots clés : selle, obèse, anorexique, 18SrRNA, ITS rRNA, clone séquençage PCR, culture.

Abstract

The human gut mycobiome, comprising of all fungal species detected in the gut. Several factors can affect the gut mycobiota composition and variation such as host diet, physiology, envrironment and life style. Previous studies on gut mycobiome based on culture and microscopy focused on specific pathogens in gut as *Candida albicans*, *Candida glabrata*, *Penicillium marneffei*, *Histoplasma capsulatum*, *Cryptococcus neoformans* et *Basidiobolus ranarum.* The Human Microbiome Project and the Metagenomics of the Human intestinal tract projects has led to new interest in the study of the human gut mycobiome. Recently, culture-independent approaches including PCR-based molecular clone libraries sequencing and high-throughput sequencing allowed to explore the diversity of gut mycobiota.

In this thesis, firstly, we reviewed fungal species described in the human gut and their implication in systemic infections. We reported from literature fungal species described in healthy individuals compared to repertoire described in diseased individuals.

Secondly, in our experimental work we used molecular and culture approaches to explore gut mycobiota diversity related to host physiology. We selected various set of PCR primers from literature targeting 18S rRNA gene and ITS rRNA gene.

Combining molecular and culture tools in stool specimen from an obese individual we detected 16 fungal species, 11 were linked to food and 8 species were found for first in the human stools. Using the same approaches in an anorexic individual stool we identified 8 fungal species, five were associated to subjected diet collected and three fungal species were observed for the first time in the human stools. From these two studies, we observed that the gut mycobiome diversity is part associated to diet.

Using the same methodology, we to explored gut mycobiota diversity according to different geographical locations. For this, fungal diversity was screened in stools samples from four continents Africa, America, Asia and Europe. We identified 40 different fungal species from environement and human pathogens. *Malassezia* spp., *Candida* spp. are ubiquitous, *Galactomyces* spp., *Saccharomyces cerevisiae* and *Trichosporon asahii* are found common in some areas and most of species are observed to be specific to each area. From this work we detected 16 fungal species never described in the human gut.

To conclude, in this thesis we detected for the first time in the human gut a total of 27 fungal species, our work contributed to increase the knowledge of repertoire of the human gut mycobiome.

Keywords: Stools, obese, anorexic, 18S rRNA, ITSr RNA, clone sequencing, PCR, Culture.

Introduction

Dans le cadre du projet « the Human Microbiome Project » aux Etats Unis et « Metagenomics of the human intestinal tract» en Europe des travaux ont montré l'importance du microbiote intestinal pour la santé et sa contribution dans le métabolisme et le système immunitaire de l'hôte [1–3]. Cependant la majorité de ces travaux sont focalisés sur les bactéries. Les études sur le mycobiome digestif (ensemble des espèces de champignons microscopiques) sont très récentes et il y'a très peu de données comparé aux données disponible sur le microbiote bactérien [4].

Les travaux initiaux sur les champignons de la flore fécale humaine basés sur la culture et la microscopie ont évalué la prévalence des champignons chez des patients hospitalisés, immunodéprimés, des patients souffrant d'une maladie inflammatoire de l'intestin et des sujets sains ont rapporté une ou deux espèce par individu [5,6]. Ces travaux méthodes ont rapporté une très faible diversité limitée à une ou deux espèces appartenant aux genres *Candida, Sacchaormyces, Galactomyces Trichosporon* et *Rhodoturula* [5,6]. *Candida albicans* est une espèce commensale pouvant être pathogène dans des conditions spécifiques (chimiothérapie, immunodépression) et associée à l'inflammation de l'intestin [7,8]. Par ailleurs, une augmentation significative ($P<0.001$) de la prévalence *C. albicans* et *Candida glabrata* a été observée chez les patients hospitalisé comparés au sujets témoins [5,6].

Les outils développés ces dernières années, à savoir l'approche moléculaire basée sur la PCR, le séquençage des clones et plus récemment le séquençage à haut débit permettent une exploration de la diversité du mycobiome dans les différentes parties du corps y compris la peau, le vagin, la cavité orale et l'intestin. Les marqueurs moléculaires des gènes 18S rRNA 28S et ITS rRNA sont couramment utilisés pour analyser la diversité de la communauté des champignons dans le tube digestif à partir des selles et des biopsies du tube digestif.

Des études antérieures basées sur la PCR et le séquençage des fragments par électrophorèse sur un gel en gradient dénaturant à partir des selles des individus sains ont observé une faible diversité limitée à *Candida, Galactomyces* et *Saccharomyces* [9,10]. Un travail similaire basé sur la PCR et le séquençage des clones à partir des selles d'un individu sénégalais a détecté 16 différentes espèces de champignons [11]. Cependant, un travail récent basé sur le pyrosequençage a détecté une large diversité comprenant 66 genres de champignons dans les selles de 98 individus sains [12]. Cette étude a montré une corrélation positive entre certaines espèces de champignons et des phyla bacteriens. Une correlation positive a été observée entre *Fusarium, Bryantella* et *Anaerostipes*, entre *Candida* et *Bacteroides*, et entre *Pichia* et *Syntrophococcus.* Une corrélation positive significative a aussi été trouvée entre *Candida* et la prise récente d'un repas riche en carbohydrates [12].

D’autres travaux ont rapporté une augmentation significative de la diversité des champignons chez des patients infectés par le virus de l’hépatite B et des patients ayant une maladie inflammatoire de l’intestin comparés au sujets témoins [13,14]. Une large diversité des champignons a été observée dans les selles et les effluents intestinaux de l’ iléostomie chez des patients ayant subi une transplantation de l’intestin [15].

L’exploration du mycobiome dans les selles de 11 nouveaux-nés de faible poids a permis de détecter 13 espèces chez sept nouveaux-nés, les espèces dominantes étaient *Candida* spp. et *Clasvispora* spp. [16]. La revue de ces différents travaux de la littérature a fait l’objet d’une revue soumise dans Journal of Medical Mycology. Au vue des travaux rapportés dans la littérature, portant sur la variation du mycobiome en fonction de l’état de santé de l’hôte et de l’âge, nous nous sommes intéressés également à l’exploration de la diversité du mycobiome digestif en fonction du poids de l’hôte mesuré par son « body mass index » et de son origine géographique.

Plus précisément les objectifs de cette thèse étaient de combiner l’approche moléculaire basée sur la PCR et le séquençage des clones avec la culture pour :

- analyser la diversité du mycobiome dans les selles d’un individu obèse
- analyser la diversité du mycobiome dans les selles d’un individu anorèxique

- explorer la diversité géographique du mycobiome à partir des selles d’individus venant des quatre continents Afrique, Amérique, Asie et Europe.

Partie I: Revue de la littérature

The human gut mycobiome as a source of systemic infection

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The human gut mycobiome as a source of infections

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Culture-based, molecular analyses and metagenomics studies indicate that the human gut mycobiome is comprising of a total of 247 species from 126 genera of yeasts and filamentous fungi members of Ascomycota, Basidiomycota and Zygomycota phyla. Fourty species have been uniquely found by culture only, 188 species by culture-independent methods and 19 species were found by the two approaches. Fungal diversity does not significant differ according to sex but *Basidiobolus ranarum* is significantly more prevalent in male and *Paecilomyces fumosoroseus* in female. Fungal diversity is significantly higher in adults than in babies. Whereas 118 fungal species are found in healthy individuals, there is only 42 species in inflammatory bowel disease patients, 29 in hepatitis-B patients and 11 in HIV-infected patients. Also, 27 species are uniquely found in inflammatory bowel disease patients, 17 species in hepatitis-B patients and *H. capsulatum* is uniquely found in HIV-infected patients. Gut mycobiota is a source of systemic infection with *Candida albicans* and *Candida glabrata*. This review gives an update into the repertoire of fungi in the human gut in healthy

and patients reported by culture and molecular approaches. Fungal culturomics must be enforced in order to further get insights in the human gut mycobiota.

Keywords human, gut, mycobiome, culture-dependant, Culture-independant, infections.

Introduction

The human mycobiome, comprising of all fungal species detected among the human microbiota is emerging as an essential component of such microbiota [1]. Over the last 20 years, culture-independent methods including high-throughput sequencing yielded new data on the mycobiome in the different sites of the human body including gut, vaginal, skin and oral mycobiota from healthy and diseased individuals [2–5]. Culture approaches focused on the prevalence of fungal species in population studies and yielded only one or two species per individual [6–9]. Ascomycota, Basidiomycota and Zygomycota are the only phyla being currently detected in the human gut [10]. Here, we review the repertoire of fungal species forming the human gut mycobiome, its variations in healthy and diseased individuals and the gut mycobiota as a source for systemic infections. We reviewed 61 articles published in English from 1998 to 2013 using mycobiome, human intestinal fungi, human gastrointestinal fungi, fecal fungal, fungemia, candidemia as key words to search in PubMed database.

Methods for investigating human gut mycobiota

Clinical specimens available for fungal culture include stools, gastric biopsies and colonic biopsies. These specimens could be inoculated on seven different solid media including Sabouraud dextrose agar, malt agar, inhibitory mould agar, Potato dextrose agar, CZAPEK, Columbia culture medium and glycine-vancomycin-polymyxin B culture medium for up to two weeks and examined two or three time per week (**Figure 1a**). A broad-spectrum antibiotic cocktail including chloramphenicol, gentamicine, streptomycine and cycloheximide is added in order to limit bacterial overgrowth [11,12]. Inoculated media are incubated at 25 °C or room temperature to isolate filamentous fungi and at 35-37°C four up to five days for yeast [6,8,13–15]. *Candida albicans, Candida glabrata, Candida rugosa, Candida parapsilosis, Candida tropicalis*, *Candida dubliniensis, Candida krusei* and *Candida lusitniae*

are commonly isolated from stools [6,8,10]. Recently, matrix–assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) revolutionized the identification of such fungi as their identification is readily achieved within a few minutes after minimal preparation of colonies [16]. Interestingly, 29 species from stools, colonics biopsies already cultured are identifiable by using MALDI-TOF-MS and commercially available databases which contain over 150 fungal species (**Table 1**). MALDI-TOF-MS allows for high-throughput identification of cultured fungi, thus it could enabling the rebirth of culture. However, culture-based studies usually incorporated only one medium or media specific to yeast for isolation that limited the growth of other fungal species [6,8,17,18]. This contrasts with the concept of culturomics, recently applied to the study of gut bacterial using several culture conditions for isolation followed by MALDI-TOF-MS identification to tremendously extend the repertoire of bacterial species and recover new species [19,20].

In addition to culture, gut mycobiome is studied by culture-independent molecular tools. Accordingly, the specific detection and identification of fungi in digestive tract specimens basically relied on culture-independent techniques as recently reviewed [1]. Most of fungal diversity was revealed in the human gut using culture-independent methods, compared to culture methods which detected only 40 fungal species, 188 species found by molecular approaches and 19 species have been detected by the two approaches (Figure 1a). Altogether, a total of 247 species and 126 genera form the human gut mycobiota comprising of members of Ascomycota (63%), Basidiomycota (32%), Zygomycota (3%) and unclassified fungi (2%). *Penicillium* spp., *Saccharomyce*s spp. and *Candida glabrata* were reported uniquely in stools [10], whereas *Basidiobolus ranarum*, *Histoplasma capsulatum* and *Paracoccidioides brasiliensis* were found only in colonic and gastric biopsy specimens [21–23].

Age and sex-related variations

A total of 53 different fungal species have been reported in 71 male individuals and 38 species in 35 female individuals, a difference which is not statistically significant (p=0.0645). However, *Basidiobolus ranarum* was significantly more prevalent in male (85% prevalence P=0.023) and *Paecilomyces fumosoroseus* in female (70% prevalence, P= 0.035). After age stratification, a total of 14 species have been reported in eight babies, 18 species from 13 young individuals 1-17-year-old and 38 species from 80 adult individuals 18-80-year -old. The diversity observed in adults is significantly higher than that in babies (P=0.037). Fungi are detected in gastrointestinal tract in about 70% of healthy adults and *Candida* are detected in 96% of neonates in the first month of life [24]. Another study observed a higher prevalence of fungi in adults compared to children (up to 3 years) although the difference did not reach statically significance: *C. lusitaniea* and *C. parapsilosis* were found uniquely in children whereas *C. glabrata* and *C. krusei* were found uniquely in adults [8]. Pyrosequencing analysis of stools collected from 11 low-weight neonates detected 13 species in seven dominated by *Candida* spp. and *Clasvispora* spp [25]. However, another study using clone libraries sequencing based on 18S rRNAgene screening micro-eukaryotes in neonates (0 to 30 days) did not detected any fungi species whereas seven fungal species were detected in their mothers [26]. The contract observed in these two studies could be from the gene targeted and the methods of sequencing.

Geographical variation

The different studies reported gut fungal species in 25 countries from four continents (Africa, America, Asia and Europe) (**Figure 1b**). A total of 101 different fungal species have been reported in America, 89 species in Europe, 84 species in Asia and 24 species in Africa (**Table S1 supplementary data)**. *Basidiobolus ranarum, Candida albicans, Candida glabrata, Candida krusei, Candida parapsilosis, Candida tropicalis, Galactomyces geotrichum* and *Histoplasma capsulatum* are ubiquitous whereas 68 were reported uniquely in America, 64

species in Europe, 54 species in Asia and 10 species in Africa (**Table S1 supplementary data** [8,14,16,17,22]. Also, restrictive diversity of one to four species per individual was observed in two studies in stool specimens in healthy individuals from Korea, Ireland and UK whereas a large diversity of 16 species was found in one healthy individual from Senegal [15,16,27].

Later observation is meaningful as number of fungi species reported by regions positively correlates with the number of studies which describe fungi. Most of fungal species were reported by studies from America (18 articles), Asia (19 articles) and Europe (21articles).

Gut mycobiota and diseases

Gut mycobiota has been analyzed in the course of 13 diseases including inflammatory bowel disease, hepatitis B, intestinal transplant, kidney transplant, ulcer gastric, gastritis, leukemia, a patient with haematological disorder, HIV infection, other immunodeficiencies, paracoccidioidomycosis, basidiobolomycosis, anorexia, and obesity by comparing in hospitalized patients, and healthy individuals **(Figure 1b).** *C. albicans* is found in (63%) healthy individuals, (70%) in hospitalized patients and patients with inflammatory bowel disease (91%), HIV infection (87%), hepatitis B, diarrhea, gastric ulcer, gastritis, other immunodeficiency, (low weight infant, and intestinal transplant) [6,8,28,29]. However, there is a restrictive diversity of fungal species in diseased individuals with 42 different fungal species in patients with inflammatory bowel disease, 29 species in hepatitis B patients and 11 species in HIV-infected patients (figure 1b); 27 species are uniquely found in inflammatory bowel disease patients, 17 species uniquely in hepatitis B patient and *H. capsulatum* in HIV-infected patients (**Table S2 supplementary data**) [10,29–33]. Culture-based studies exploring fecal fungal flora focused on prevalence of fungi in healthy, hospitalized and immunosuppressive patients [6,8,17,18]. Significant high prevalence ($P<0.001$) of *C. glabrata* was observed in hospitalized patients compared to healthy controls [6,8]. *Candida* species

were to be significantly more prevalent in patients with HIV infection and recent antibiotic use in diarrhea than in healthy controls [28, 34-35]. Intestinal colonization by *Candida* species was significantly found associated to inflammatory bowel disease and intestinal graft-versus-host-disease [29,36]. An increased significant fungal diversity was detected using molecular methods in patients with inflammatory bowel disease and hepatitis B compared with healthy controls [10,30]. Sixteen different fungal species have been detected in two intestinal transplant patients from stools and intestinal effluents [37]. We observed previously in an obese and anorexic individual 16 and 10 fungal species related to subject diet [14]. Additional, a large diversity including 66 genera of fungi were detected in 98 healthy with different diet compositions [38]. *Saccharomyces* (89%, *Candida* (57%) and *Cladosporidium* were the most prevalent genus and *Candida* was found positively correlated with diets in high carbohydrates. Some strict pathogens including opportunist pathogens *Aspergillus flavus, Basidibolus ranarum, Cryptococcus noeformans, Exophilia dermatidis, Histoplasma capsulatum, Paracoccidioides brasiliensis* and *Penicillium marneffei* were isolated in the diarrhea and case of compromised [21,22,30–42].

Gut mycobiota as a source of infections

Gastrointestinal fungal species can translocate from gut into bloodstream [43]. Molecular typing is used to trace the source of fungemia [44]. Molecular typing methods including pulsed-field gel electrophoresis karyotyping, PFGE on fragments generated by restriction endonucleases and PCR fingerprinting by random amplified polymorphic DNA (RAPD) allow to show the similar fungal species isolated from blood and gastrointestinal sites in patient. Only five studies used molecular typing to evaluate the gut fungal as a source of candidemia. Using molecular typing based on PFGE the same strain of identical DNA of *C. albicans* has been detected from stool and blood in one HIVinfected patient and in five patients with candidemia [45,46]. The same profile of *C albicans* has been detected from

blood and stool in 13 of 26 patients with candidemia using molecular typing [43]. The source of candidemia has been evaluated in 35 of 2087 neonate patients and molecular typing detected identical DNA pattern of *C. albicans* from stool and blood of 15 patients with candidemia [47]. Finally, similar studies performed in six patients with candidemia detected the same strain of *C. glabrata* from stool and blood in one patient [49].

Conclusion

A total of 247 fungal species and 126 genera form the gut mycobiota detected by culture and molecular approaches from stool and gastrointestinal biopsy specimens. Culture approaches detected limited 40 species compared to molecular methods which detected 188 species and adding 19 species were found by the two methods. The culture culturomics concept developed to investigate bacteria [50] could be adopted to study gut mycobiome in order to increase the repertoire of gut fungi and precise its role in intestinal and systemic infection.

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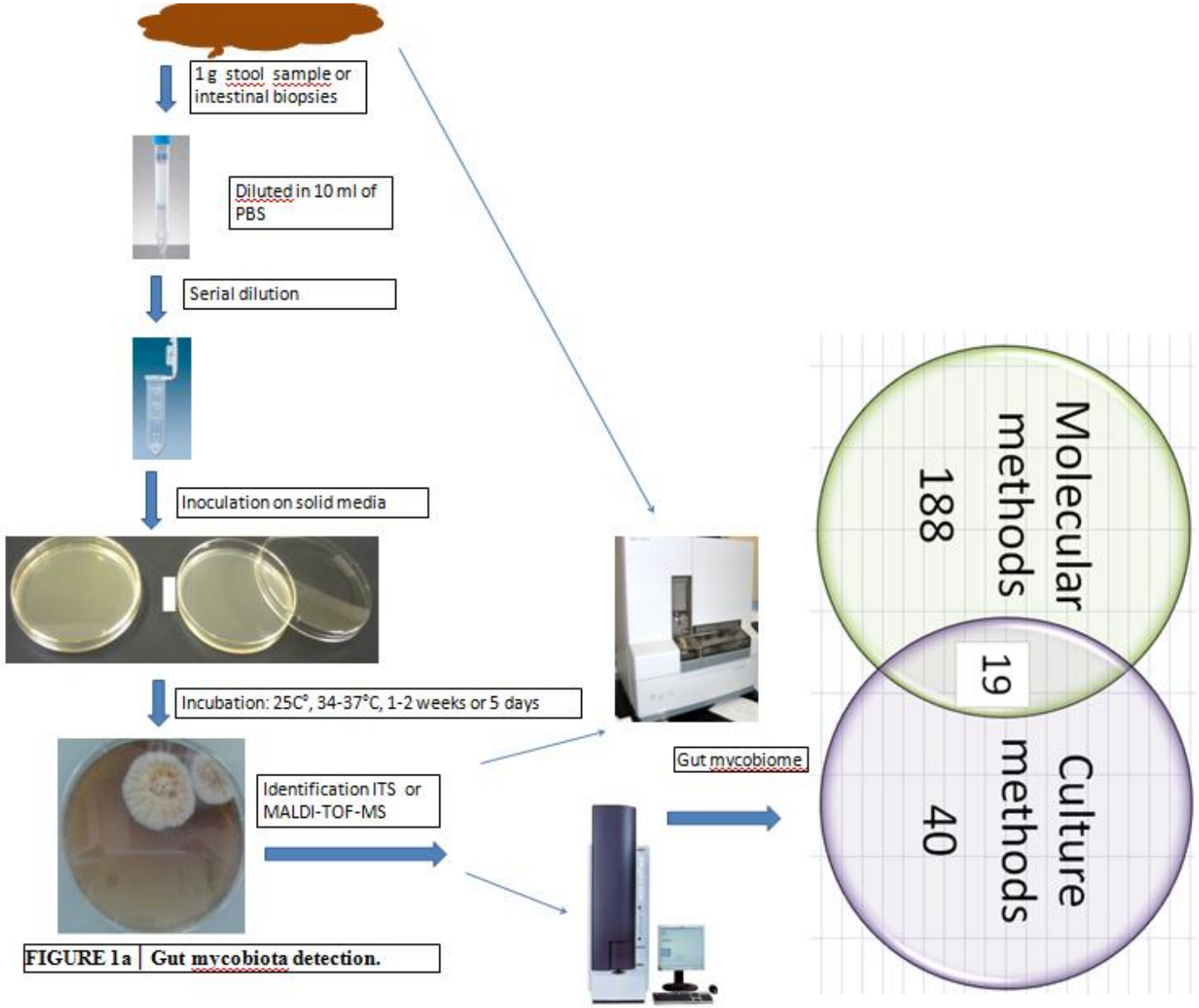

FIGURE 1a | Gut mycobiota detection.

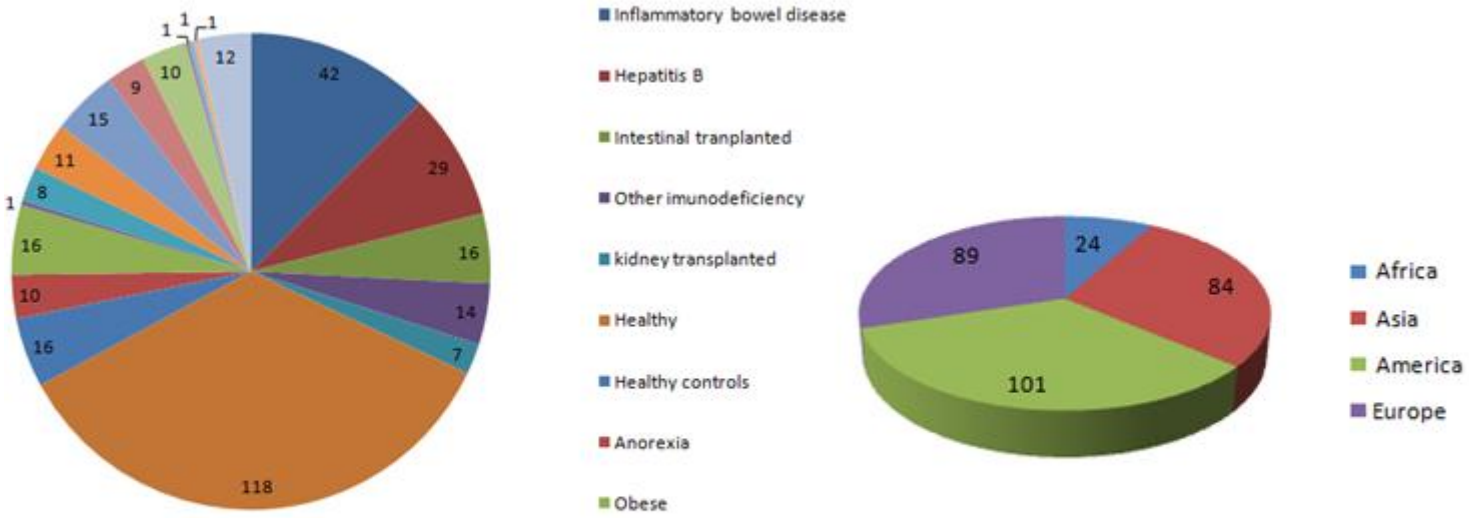

FIGURE 1b | Gut mycobiota variation with diseases and four continents.

Table 1 | Gut fungal species isolated by culture and identify by MALDI-TOF-MS.

Gut mycobiota isolated by culture	MALDI TOF-MS identification
Aspergillus flavipes	Yes
Aspergillus flavus	Yes
Aspergillus ruber	
Aspergillus spp.	
Aspergillus versicolor	Yes
Basidiobolus ranarum	
Beauveria bassiana	yes
Blastoschizomyces capitatus	
Candida albicans	Yes
Candida dubliniensis	Yes
Candida famata	
Candida glabrata	Yes
Candida guilliermondii	Yes
Candida incospicua	Yes
Candida kefyr	Yes
Candida krusei	yes
Candida lambica	yes
Candida lusitaniae	Yes
Candida norvogensis	Yes
Candida parapsilosis	Yes
Candida sphaerica	Yes
Candida spp.	

Candida tropicalis	Yes
Candida utilis	Yes
Candida zeynaloides	Yes
Climacocystis sp.	
Cryptococcus albidus	
Cryptococcus luteolus	
Cryptococcus neoformans	Yes
Cryptococcus spp.	
Exophiala dermatidis	Yes
Fusarium spp.	
Galactomyces candidum	Yes
Galactomyces geotrichum	Yes
Geothricum spp.	
Geotrichum candida	
Geotrichum candidum (Goetrichum silvoca)	yes
Histoplasma capsulatum	
Hypocrea lixii	
Isaria farinosa	
Malassezia globosa	
Malassezia pachydermatis	Yes
Malassezia restricta	
Mucor spp.	
Paracoccidioides brasiliensis	
Penicillium allii	
Penicillium brevicompactum	
Penicillium camemberti	Yes

Penicillium dipodomyicola	
Penicillium solitum	
Penicillium spp.	
Rhodotorula glutinis	Yes
Rhodotorula rubra	
Rhodotorula sp.	
Saccharomyces cerevisiae	Yes
Saccharomyces sp.	
Trichosporon asahii	Yes
Trichosporon beigelii	
Yarrowia lipolytica (Candida lipolytica)	Yes

Table S1 | Supplementary data fungal species distribution by continent. (* means species found uniquely in the corresponding country).

Africa	Asia	America	Europe
*Arxiozyma telluris**	*Ajellomyces capsulatus**	*Absidia sp.**	*Acremonium sp.**
*Asterophora parasitica**	*Ajellomyces dermatitidis**	*Acremonium*	*Aspergillus flavipes**
Basidiobolus ranarum	*Aspergillus clavatus**	*Agaricus**	*Aspergillus flavus*
*Bjerkandera adusta**	*Aspergillus flavus*	*Alternaria**	*Aspergillus ruber**
Candida albicans	*Aspergillus penicillioides**	*Armillaria**	*Aspergillus versicolor*
Candida glabrata	*Aspergillus spp.**	*Aspergillus*	*Aureobasidium pullulans*
Candida krusei	*Aspergillus versicolor*	*Auricularia**	*Basidiobolus ranarum*
Candida parapsilosis	*Asterotremella albida**	*Basidiobolus ranarum*	*Beauveria bassiana**
*Candida rugosa**	*Aureobasidium pullulans*	*Blastoschizomyces capitatus**	*Botryotinia fuckeliana*
Candida spp.	*Basidiobolus ranarum*	*Bullera sp.**	*Bullera crocea**
Candida tropicalis	*Botryotinia fuckeliana*	*Candida*	*Candida albicans*
Galactomyces geotrichum	*Candida albicans*	*Candida albicans*	*Candida austromarina**
Histoplasma capsulatum	*Candida dubliniensis*	*Candida glabrata*	*Candida diddensiae**
Kluyveromyces hubeiensis	*Candida edaphicus**	*Candida guilliermondii*	*Candida dubliensis**
Malassezia globosa	*Candida famata **	*Candida krusei*	*Candida dubliniensis*
Malassezia pachydermatis	*Candida glabrata*	*Candida lambica**	*Candida glabrata*
Malassezia restricta	*Candida guilliermondii*	*Candida lusitaniae*	*Candida guilliermondii*
*Phanerochaete stereoides**	*Candida intermedia**	*Candida parapsilosis*	*Candida krusei*
Saccharomyces cerevisiae	*Candida kefyr**	*Candida quercitrusa**	*Candida parapsilosis*
*Sterigmatomyce elviae**	*Candida krissii**	*Candida sp.*	*Candida spp.*
*Torulaspora pretoriensis**	*Candida krusei*	*Candida tropicalis*	*Candida tropicalis*
*Trichosporon asahii**	*Candida lusitaniae*	*Ceratocystis**	*Candida zeynaloides*
*Trichosporon caseorum**	*Candida milleri**	*Ceriporia**	*Cephalosporium sp.**
*Trichosporon cutaneum**	*Candida norvogensis**	*Chaetomium**	*Ceratobasidium sp.**
	Candida parapsilosis	*Chondrostereum**	*Chaetomium globosum*
	*Candida solani**	*Cinereomyces**	*Cladosporidium bruhnei**
	Candida sp.	*Cladosporium*	*Cladosporium cladosporioides**
	*Candida sphaerica**	*Cladosporium sp.**	*Climacocystis sp.**
	Candida spp.	*Cladosporium sphaerospermum**	*Cryptococcus carnescens**
	Candida tropicalis	*Cladosporium tenuissimum**	*Cryptococcus neoformans*

Candida utilis	*Claviceps**	*Cystofilobasidium capitatum**
*Candida vanaria**	*Clavispora**	*Dacrymyces sp. **
Candida zeynaloides	*Clavispsora sp.**	*Debaryomyces*
Chaetomium globosum	*Cochliobolus**	*Dothideomycete sp.**
*Chaetomium sp. **	*Colletotrichum**	*Exidiopsis calcea**
*Coccidioides immitis**	*Coniosporium**	*Exophiala dermatitidis**
*Coccidioides posadasii**	*Cryotococcus*	*Filobasidium globisporum**
*Cryptococcus fragicola**	*Cryptococcus albidus**	*Flammulina velutipes**
Cryptococcus neoformans	*Cryptococcus luteolus**	*Fomitopsis pinicola**
*Cryptococcus spp.**	*Cryptococcus neoformans*	*Fusarium oxysporum*
*Doratomyces stemonitis**	*Cryptococcus podzolicus**	*Galactomyces candidum**
Fusarium oxysporum	*Cryptococcus sp.**	*Galactomyces geotrichum*
*Fusarium sp. **	*Cryptococcus albidosimilas**	*Galactomyces sp.**
Galactomyces geotrichum	*Cystofilobasidium*	*Gloeotinia temulenta**
*Geothricum spp.**	*Debaryomyces sp.**	*Graphiola phoenicis**
*Geotrichum candida**	*Dictyoglomus sp.**	*Histoplasma capsulatum*
*Geotrichum candidum**	*Filobasidium*	*Hypocrea lixii **
Histoplasma capsulatum	*Fusarium*	*Isaria farinosa**
*Hyphozyma variabilis**	*Galactomyces*	*Madurella mycetomatis**
*Iodophanus carneus**	*Galactomyces geotrichum*	*Malassezia globosa*
Kluyveromyces hubeiensis	*Glomerella**	*Malassezia pachydermatis*
*Kluyveromyces waltii**	*Hanseniaspora**	*Malassezia restricta*
*Lycogala flavofuscum**	*Histoplasma capsulatum*	*Neosartorya pseudofischeri**
Malassezia globosa	*Hymenochaete**	*Paecilomyces fumosoroseus**
Malassezia pachydermatis	*Hypholoma**	*Paraphaeosphaeria filamentosa**
Mucor racemosus	*Kluyveromyces*	*Penicillium allii**
*Mucor spp.**	*Lasiodiplodia**	*Penicillium brevicompactum**
*Ophiocordyceps caloceroides**	*Lentinus**	*Penicillium camemberti**
*Penicillium freii**	*Leucosporidium sp. **	*Penicillium chrysogenum**
*Penicillium marneffei **	*Melanconium sp**	*Penicillium dipodomyicola**
Penicillium sp.	*Metschnikowia**	*Penicillium glabrum**
*Penicillium spp.**	*Meyerozyma **	*Penicillium italicum**
*Rhizopus microsporus**	*Microdochium**	*Penicillium roquefortii **
*Rhizopus spp.**	*Millerozyma**	*Penicillium sp.*
Rhodotorula rubra	*Miptoporus**	*Penicillium verruculosum**

*Saccharomyces castellii**
Saccharomyces cerevisiae
*Saccharomyces paradoxus**
*Saccharomyces servazzii **
Saccharomyces sp.
*Sclerotinia sclerotiorum**
*Simplicillium lanosoniveum **
*Simplicillium obclavatum**
*Sporobolomyces ogasawarensis**
*Trichophyton verrucosum**
*Trichosporon beigelii**
*Trichosporon spp.**
*Uncultivatable Agaricomycotina**
*Uncultivatable fungi**
*Uncultivatable Pezizomycotina**
*Uncultivatable Pucciniomycotina**
*Uncultured Banisveld**
*Wallemia muriae**
*Wallemia sebi**

Mucor
*Mycocentrospora**
*Mycosphaerella**
*Neafusicoccum**
*Nigrospora**
*Ophiostoma**
Paracoccidioides brasiliensis
Penicillium
Penicillium sp.
*Perenniporia**
*Phaeococcomyces**
*Phlebia**
*Pichia**
Rhodotorula
Rhodotorula rubra
Rhodotorula sp.
Saccharomyces
Saccharomyces sp.
*Skelotocutis**
*Stemphylium**
*Strelitziana **
*Teratsphaeria**
*Thanatephorus**
Torulaspora
*Toxicocladosporium**
*Trichpatum**
*Tyromyces**
*Wallemia**
*Wickerhamomyces**
*Xeromyces**
Yarrowia lipolytica
*Zygosaccharomyces**
*Eurotium**
*Eutypella**
Exophiala

*Penicillum sacculum**
*Pleospora herbarum**
*Psathyrella candolleana **
*Raciborskiomyces longisetosum**
*Rhodotorula aurantiaca**
*Rhodotorula glutinis**
*Rhodotorula mucilaginosa **
*Saccharomyces bayanus**
*Saccharomyces cariocanus**
Saccharomyces cerevisiae
Sclerotinia sclerotiorum
*Sclerotium sp.**
*Septoria epambrosiae**
*Sirococcus conigenus**
*Sporolobomyces yunnanensis**
*Trametes versicolor**
*Tricholoma saponaceum**
*Trichosporon dermatis**
*Uncultured ascomycete **
*Uncultured basidiomycete**
*Uncultured fungal spp.**
*Uncultured ustilaginomycete **
*Ustilago maydis**
Ustilago sp. *

Table S2 | supplementary data fungal species distribution in diseases (* means species uniquely found in the corresponding disease).

Healthy	Inflammatory bowel disease	Hepatitis B	HIV
Acremonium	*Aureobasidium pullulans*	*Aspergillus penicillioides**	*Candida albicans*
*Acremonium sp.**	*Bjerkandera adusta*	*Aspergillus versicolor*	*Candida glabrata*
Agaricus	*Bullera crocea**	*Aureobasidium pullulans*	*Candida krusei*
*Alternaria**	*Candida albicans*	*Candida albicans*	*Candida parapsilosis*
*Armillaria**	*Candida austromarina*	*Candida austromarina*	*Candida spp.*
*Arxiozyma telluris**	*Candida dubliensis**	*Candida intermedia*	*Candida tropicalis*
Aspergillus	*Candida incospicua**	*Candida krissii*	*Cryptococcus neoformans*
Aspergillus ruber	*Candida* spp.	*Candida milleri**	*Geotrichum candida*
Aspergillus versicolor	*Chaetomium globosum*	*Candida solani**	*Histoplasma capsulatum**
*Asterophora parasitica**	*Cladosporium cladosporioides*	*Candida tropicalis*	*Penicillium marneffei*
*Auricularia**	*Cryptococcus carnescens**	*Chaetomium globosum*	*Saccharomyces cerevisiae*
Bjerkandera adusta	*Cystofilobasidium capitatum*	*Chaetomium sp. **	
Blastoschizomyces capitatus	*Dacrymyces* sp. *	*Cryptococcus fragicola**	
Candida	*Dothideomycete* sp. *	*Doratomyces stemonitis**	
Candida albicans	*Exidiopsis calcea**	*Fusarium sp. **	
*Candida edaphicus**	*Filobasidium globisporum**	*Galactomyces geotrichum*	
Candida glabrata	*Flammulina velutipes**	*Hyphozyma variabilis **	
Candida guilliermondii	*Fomitopsis pinicola**	*Iodophanus carneus**	
Candida kefyr	*Fusarium oxysporum*	*Ophiocordyceps caloceroides**	
Candida krissii	*Galactomyces geotrichum*	*Penicillium sp.**	
Candida krusei	*Graphiola phoenicis**	*Rhizopus microsporus**	
Candida lusitaniae	*Madurella mycetomatis**	*Saccharomyces cerevisiae*	
Candida parapsilosis	*Penicillium chrysogenum**	*Saccharomyces paradoxus*	

*Candida rugosa**	*Penicillium italicum**	*Saccharomyces sp.*
Candida spp.	*Penicillum sacculum**	*Simplicillium lanosoniveum **
Candida tropicalis	*Pleospora herbarum**	*Simplicillium obclavatum **
Candida zeynaloides	*Raciborskiomyces longisetosum**	*Uncultivatable Pezizomycotina **
*Cephalosporium sp.**	*Rhodotorula aurantiaca**	*Wallemia muriae**
*Ceratobasidium sp.**	*Rhodotorula mucilaginosa **	*Wallemia sebi**
*Ceratocystis**	*Saccharomyces cerevisiae*	
*Ceriporia**	*Sclerotinia sclerotiorum*	
Chaetomium	*Sclerotium sp.*	
*Chondrostereum**	*Sirococcus conigenus**	
*Cinereomyces**	*Sporobolomyces ogasawarensis*	
Cladosporium	*Trametes versicolor**	
*Claviceps**	*Tricholoma saponaceum**	
Clavispora	*Trichosporon dermatis**	
*Cochliobolus**	*Uncultured ascomycete **	
*Colletotrichum**	*Uncultured basidiomycete**	
*Coniosporium**	*Uncultured ustilaginomycete **	
Cryotococcus	*Ustilago maydis**	
*Cryptococcus albidus**	*Ustilago sp. **	
*Cryptococcus luteolus**	*Yarrowia lipolytica*	
*Debaryomyces**		
*Eurotium**		
*Eutypella**		
Exophiala		
Filobasidium		
Fusarium		
Fusarium spp.		
Galactomyces		

Galactomyces candidum
Galactomyces geotrichum
Galactomyces sp.
Geothricum spp.
Geotrichum candida
Gloeotinia temulenta
Glomerella
Hanseniaspora
Hymenochaete
Hypholoma
Kluyveromyces
Kluyveromyces hubeiensis
Lasiodiplodia
Lentinus
Lycogala flavofuscum
Malassezia globosa
Malassezia pachydermatis
Malassezia restricta
Metschnikowia
Meyerozyma
Microdochium
Millerozyma
Miptoporus
Mucor
Mucor racemosus
Mucor spp.
Mycocentrospora
Mycosphaerella

Neafusicoccum
Nigrospora
Ophiostoma
Paecilomyces fumosoroseus
Penicillium
Penicillium freii
Penicillium glabrum
Penicillium roquefortii
Penicillium spp.
Perenniporia
Phaeococcomyces
Phanerochaete stereoides
Phlebia
Pichia
Psathyrella candolleana
Rhodotorula
Rhodotorula glutinis
Rhodotorula rubra
Rhodotorula sp.
Saccharomyces
Saccharomyces bayanus
Saccharomyces cerevisiae
Saccharomyces paradoxus
Saccharomyces sp.
Skelotocutis
Sporobolomyces ogasawarensis
Stemphylium
Sterigmatomyce elviae

Strelitziana
Teratsphaeria
Thanatephorus
Torulaspora
*Torulaspora pretoriensis**
*Trichosporon asahii**
Trichosporon beigelii
*Trichosporon caseorum**
*Trichosporon cutaneum**
Trichosporon spp.
*Trichpatum**
*Tyromyces**
*Uncultured Banisveld**
*Uncultured fungal spp.**
Wallemia
*Xeromyces**
Yarrowia lipolytica
*Zygosaccharomyces**

Table 1 | Gut fungal species isolated by culture and identify by MALDI-TOF-MS.

Gut mycobiota isolated by culture	MALDI TOF-MS identification
Aspergillus flavipes	Yes
Aspergillus flavus	Yes
Aspergillus ruber	
Aspergillus spp.	
Aspergillus versicolor	Yes
Basidiobolus ranarum	
Beauveria bassiana	yes
Blastoschizomyces capitatus	
Candida albicans	Yes
Candida dubliniensis	Yes
Candida famata	
Candida glabrata	Yes
Candida guilliermondii	Yes
Candida incospicua	Yes
Candida kefyr	Yes
Candida krusei	yes
Candida lambica	yes
Candida lusitaniae	Yes
Candida norvogensis	Yes
Candida parapsilosis	Yes
Candida sphaerica	Yes
Candida spp.	
Candida tropicalis	Yes
Candida utilis	Yes

Candida zeynaloides	Yes
Climacocystis sp.	
Cryptococcus albidus	
Cryptococcus luteolus	
Cryptococcus neoformans	Yes
Cryptococcus spp.	
Exophiala dermatidis	Yes
Fusarium spp.	
Galactomyces candidum	Yes
Galactomyces geotrichum	Yes
Geothricum spp.	
Geotrichum candida	
Geotrichum candidum (Goetrichum silvoca)	yes
Histoplasma capsulatum	
Hypocrea lixii	
Isaria farinosa	
Malassezia globosa	
Malassezia pachydermatis	Yes
Malassezia restricta	
Mucor spp.	
Paracoccidioides brasiliensis	
Penicillium allii	
Penicillium brevicompactum	
Penicillium camemberti	Yes
Penicillium dipodomyicola	
Penicillium solitum	

Penicillium spp.	
Rhodotorula glutinis	Yes
Rhodotorula rubra	
Rhodotorula sp.	
Saccharomyces cerevisiae	Yes
Saccharomyces sp.	
Trichosporon asahii	Yes
Trichosporon beigelii	
Yarrowia lipolytica (Candida lipolytica)	Yes

Commentaire: Review

Le mycobiome apparait comme une composante importante du microbiote humain. Cependant, il est peu étudié comparé à la diversité des travaux disponibles sur les bactéries. Les travaux antérieurs sur le mycobiome digestif basés sur la méthode de culture, évaluant la prévalence des champignons chez les patients et les sujets sains, ont révélé une très faible diversité limitée à une ou deux espèces par individu [5,6]. Le développement récent des outils moléculaires à savoir la PCR suivie du séquençage des clones et le séquençage à haut débit ont permis d'explorer la diversité du mycobiome dans les différentes parties du corps dont la peau, le vagin, la cavité orale et l'intestin [1]. Ainsi, ces travaux ont rapporté une variation du mycobiome digestif en fonction de l'état de santé de l'hôte et de l'âge dans différents pays du monde. Les champignons couramment décrits dans le tractus digestif sont membre des phyla Ascomycota, Basidiomycota et Zygomycota.

Dans cette revue, nous avons répertorié un total de 247 espèces de champignons décrites dans le tractus digestif de champignons appartenant à 126 genres. L'approche moléculaire a détecté 188 espèces, la culture 40 espèces et 19 espèces ont été rapportées par les deux approches. Deux études ont rapporté une augmentation significative de la diversité du mycobiome chez les patients ayant une inflammation de l'intestin et les patients infectés par le virus de l'hépatite B comparés aux sujets témoins. Un total de 33 espèces ont été décrites uniquement

chez les sujets sains, 27 espèces chez les patients ayant une inflammation de l'intestin, 17 espèces chez les patients infectés par le virus de l'hépatite B et *Histoplasma capsulatum* chez les patients infectés par le VIH. Une augmentation significative de la prévalence de *C. glabrata* a été observée les patients hospitalisés comparés aux sujets témoins.

Une variation non-significative du mycobiome en fonction de l'âge a été observée : *Candida lusitaniea* et *C. parapsilosis* ont été trouvés chez des enfants alors que *C. glabrata* and *C. krusei* ont été observés chez des adultes [6]. *Basidiobolus ranarum* est retrouvé plus prévalent chez les individus de sexe masculin (85% prévalence P=0.023) et *Paecilomyces fumosoroseus* chez les individus de sexe féminin (70% prévalence, P= 0.035).

Les champignons du digestif ont été rapportés dans des études menées dans 25 pays. *B. ranarum, C. albicans, C. glabrata, C. krusei, Candida parapsilosis, Candida tropicalis, Galactomyces geotrichum* and *Histoplasma capsulatum* sont ubiquitaires alors que 68 différentes espèces sont reportés uniquement en Amérique, 64 espèces en Europe, 54 espèces en Asie et 10 espèces en Afrique [11,17–19].

Le typage moléculaire (avec l'électrophorèse sur champs expulsé (PFGE)) de *C. glabrata* et *C. albicans* dans les selles et le sang des patients a permi d'établir que les souches de fungémie étaient identiques à celles du mycobiome intestinal [20–23].

Cette revue a fourni un répertoire des champignons décrits dans le tube digestif des patients et des sujets sains rapportés par l'approche molécaire et la culture. Le concept de la culturomic devrait être adopté pour élargir la vision sur le mycobiome digestif.

Partie II: Article 1

Plant and Fungal Diversity in Gut Microbiota as Revealed by Molecular and Culture Investigations

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Plant and Fungal Diversity in Gut Microbiota as Revealed by Molecular and Culture Investigations

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Abstract

Background: Few studies describing eukaryotic communities in the human gut microbiota have been published. The objective of this study was to investigate comprehensively the repertoire of plant and fungal species in the gut microbiota of an obese patient.

Methodology/Principal Findings: A stool specimen was collected from a 27-year-old Caucasian woman with a body mass index of 48.9 who was living in Marseille, France. Plant and fungal species were identified using a PCR-based method incorporating 25 primer pairs specific for each eukaryotic phylum and universal eukaryotic primers targeting 18S rRNA, internal transcribed spacer (ITS) and a chloroplast gene. The PCR products amplified using these primers were cloned and sequenced. Three different culture media were used to isolate fungi, and these cultured fungi were further identified by ITS sequencing. A total of 37 eukaryotic species were identified, including a Diatoms (*Blastocystis* sp.) species, 18 plant species from the *Streptophyta* phylum and 18 fungal species from the *Ascomycota*, *Basidiomycota* and *Chytridiocomycota* phyla. Cultures yielded 16 fungal species, while PCR-sequencing identified 7 fungal species. Of these 7 species of fungi, 5 were also identified by culture. Twenty-one eukaryotic species were discovered for the first time in human gut microbiota, including 8 fungi *(Aspergillus flavipes, Beauveria bassiana, Isaria farinosa, Penicillium brevicompactum, Penicillium dipodomyicola, Penicillium camemberti, Climacocystis* sp. and *Malassezia restricta)*. Many fungal species apparently originated from food, as did 11 plant species. However, four plant species (*Atractylodes japonica*, *Fibraurea tinctoria*, *Angelica anomala*, *Mitella nuda*) are used as medicinal plants.

Conclusions/Significance: Investigating the eukaryotic components of gut microbiota may help us to understand their role in human health.

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Introduction

The human gut contains a wide variety of microorganisms known as the microbiota [1]. At birth, the human gut is sterile and is then colonized by bacteria originating from the mother, environment and diet [2,3]. Several studies have revealed the importance of gut microbiota in host health and the contribution of these microbes to diverse functions, including metabolism, immune function and gene expression [4]. Gut microbes produce a large arsenal of enzymes that are naturally absent from humans, which contribute to food digestion, energy harvesting and storage [5,6]. Two bacterial phyla, *Firmicutes* and *Bacteroidetes*, dominate in the gut microbiota. Some studies have shown a reduction in the relative proportion of *Bacteroidetes* in obese individuals compared to lean individuals [5,7]. Additionally, it has been observed that the microbiota of obese individuals extract more energy from the diet than the microbiota of lean individuals [1].

The gut microbiota is comprised of Viruses, Bacteria, Archaea and Eukaryotes [8]. Accordingly, there are much data available about the bacterial community. However, few studies have investigated eukaryotic communities in the human gut, resulting in a dearth of information about these communities. Previous studies that have used molecular methods to explore the eukaryotic community in the guts of healthy individuals detected only *Galactomyces* and *Candida* fungi and *Blastocystis hominis* as prevalent species [9,10]. Additional studies have reported increased fungal diversity in ill patients compared to healthy individuals [11–13].

Thus, our study aimed to examine the repertoire of plants and fungi in the gut of an obese human using both PCR-sequencing and culturing techniques.

Results

Molecular Detection

Mixing *Acanthamoeba castellanii* DNA and stool DNA yielded a positive amplification using specific primer pair for *Acanthamoeaba* (JPD1/JDP2). Among the 25 primers pairs, 17 yielded an exact sequence with an appropriate positive control, whereas no positive control was available for 8 primer pairs (Table 1 & Table 2). Only 5 of these 25 eukaryotic PCRs yielded amplification product with the stool specimen, while the negative controls exhibited no amplification. The analysis of a total of 408 clones identified 7

fungal species, 18 plant species and one Diatoms (*Blastocystis* sp.) species (Table 3). GenBank reference number of the best hit similarly to our sequences for each organism were: *Galactomyces geotrichum* (JN903644.1); *Penicillium camemberti* (GQ458039.1), *Malassezia globosa* (AY743604.1), *Malassezia pachydermatis*(AB118940.1), *Malassezia restricta* (AY743607.1), uncultured *Chytridiomycota* (GQ995333.1) *Candida tropicalis* (DQ515959.1).

Fungi Isolated Using Culture Media

In all experiments, the negative control plates remained sterile. A total 16 different fungal species were isolated (Table 4). Nine species of fungi (*M. globosa*, *M. restricta*, *M. pachydermatis*, *Penicillium allii*, *Penicillium dipodomyicola*, *G. geotrichum*, *Cladosporidium* sp., *Climacocystis* sp. and *C. tropicalis*) were cultured on Dixon agar medium. Three species of fungi (*Penicillium* sp./*P. commune*/*P. camemberti*, *Aspergillus versicolor*, *Beauveria bassiana*) were cultured on Potato Dextrose media. Two species of fungi (*Aspergillus flavipes*, *Isaria farinosa*) were cultured on CZAPEK medium. Two species (*Hypocrea lixii*/*Penicillium chrysogenum*, *Penicillium brevicompactum*) were cultured on both PDA and CZAPEK media, and *C. tropicalis* was cultured on both Dixon agar and PDA media. Five of the cultured species of fungi (*G. geotrichum*, *C. tropicalis*, *M. pachydermatis*, *M. globosa*, and *M. restricta)* were also identified by clone sequencing, while 11 fungi were detected only by culture (Figure 1). *Penicillium*, *Aspergillus*, *Galactomyces*, *Beauveria*, *Candida*, *Cladosporidium*, *and Isaria* are members of the Ascomycota phylum and *Malassezia* and *Climacocystis* are members of the Basidiomycota phylum.

Discussion

The PCR-based and culture-based results obtained here are validated by the fact that all the negative controls remained negative, precluding the possibility of cross contamination from the laboratory. Also, we ensured the absence of potential PCR inhibitors in the stool specimen. At last, the PCR systems yielded expected result with appropriate positive controls including Fungi which have been shown to be diffult to lyse [14]. Accordingly, we combined mechanical and enzymatic lysis to optimize recovery of DNA from Fungi as previously reported [9,14–15]. These data allowed to interpret negative results as true negatives. The 18S rRNA, ITS and chloroplast genes amplified in this study are molecular markers commonly used for eukaryotic screening [11,16–22]. These genes are conserved in all eukaryotes and contain variable regions suitable for primer design.

However, this is the first study to use a multiple set of primers for molecular approach to screen eukaryotic communities in a stool sample from an obese person. The combination of culture-dependent and culture-independent cloning and sequencing revealed a previously unsuspected diversity of eukaryotes among the human intestinal microbiota. Indeed, we detected a total of 37 eukaryotic species; only 16 of these species had been previously

Table 1. Eukaryotic and fungi primers selected in this study.

Taxon	Primer	Target	PCR product size (bp)	Annealing temperature and number of cycles	Reference
Amoeba	AmiF1/Ami9R	18S rRNA	670	55°C 30 s 40cyles	[47]
Acanthamoeba	JDP1/JDP2	18S rRNA	460–470	60°C 60 s 40cylces	[48]
Entamoeba	JVF/DSPR2	18S rRNA	662–667	55°C 60 s 40cycles	[49]
Hartmanella	HV1227F/HV1728R	18S rRNA	502	56°C 30 s 40cycles	[50]
Naegleria	F/R	ITS	376–388	55°C 30 s 35cycles	[51]
Ciliophora	121F/1147R	18S	750–1000	55°C 60 s 30cycles	[52]
Chlorophyta	UCP1F/UCP1R	Rsp11-rpl2	384	54°C 60 s 35cyles	[53]
	UCP2F/UCP2R	Rsp11-rpl2	391	56°C 60 S 35cycles	
Diatoms	18S/28R	18s-28srRNA	700–900	60°C 30 s 35cycles	[54]
Dinoflagellate	18ScomF1/Dino18SR1	18S rRNA	650	58°C 60 s 40cycles	[55]
Diplomonads	DimA/DimB	18S rRNA			[56]
Euglenophyta	EAF/EAF3	18S rRNA	1000	62°C 90 s 25cycles	[57]
Kinetoplastidia	Kinetokin1/kinetokin2	18S rRNA	600–650	56°C 60 s 35cycles	[58]
	KinSSUF1/KinSSUR1	18S rRNA	427–600	60°C 60 s 35cycles	[59]
Microsporidia	V1/PMP2	18S rRNA	250–279	55°C 30 s 35cycles	[60]
Rodhophyta	URP1_F/URP1_R	rps10-dnaK	464	52°C 60 s 35cyles	[53]
	URP2_F/URP2_R	rps10-dnaK	1772	52°C 60 s 35cyles	
Trichomonads	TFR1/TFR2	5,8SrNRA, ITS	338–391	60°C 30 s 35cycles	[61]
Fungi	MalF/MALR	26S	580	55°C 45 s 40cycles	[62]
Fungi	NS1/FR1	18S rRNA	1650	48°C 45 s 35cycles	[63]
	ITS1F/ITS4R	ITS	Variable	50°C45 s 40cycles	[9]
Fungi	FunF/funR	18S	1000	52°C30 s 40cycles	[12]
Universal	Euk1A/EUK516r	18S	560	50°C 30 s 35cycles	[9]
eukaryote	EUK528/1391R	18S	1000–1300	55°C 60 s 30cylces	[64]
Plant	rbcLZ1/rbcL19b	Chloroplast	157	40°C30 s 40 cycles	[16]

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Table 2. Results of PCR testing with positive control. NA non available.

Taxon	Primers	Positive control	PCR	Blast coverage%	Blast identity %	GenBank refence number
Amoeba	AmiF1/Ami9R	*Acanthamoeba castellanii*	Positive	100	99	*A.castellani* (GU001160.1)
		Hartmannella vermiformis	Positive	100	99	*H. vermiformi* (DQ123623.2)
Acanthamoeba	JDP1/JDP2	*Acanthamoeba castellanii*	Positive	100	99	*A. castellanii* (GU001160.1)
Entamoeba	JVF/DSPR2	NA				
Hartmannella	Hv1227F/Hv1728R	*Hartmannella vermiformis*	Positive	100	99	*H. vermiformis* (HM363627)
Naegleria	F/R	NA				
Ciliophora	121 F/1147R	*Colpoda steinii*	Positive	100	99	*C. steineii* (DQ388599.1)
Chlorophyta	UCP1F/UCP1R	*Chlorella vulgaris*	Positive	95	93	*C. vulagaris* (AB001684.1)
Chlorophyta	UCP2F/UCP2R	*Chlorella vulgaris*	Positive	95	93	*C. vulagaris* (AB001684.1)
Diatoms	18S/28R	NA				
Dinoflagellates	DinocomF1/Dino18SR1	*Poterioochromonas malhamensis*	Positive	100	98	*P. malhamensis* (FN662745.1)
Diplomonads	DimA/DimB	NA				
Euglenophyta	EAF/EAF3	NA				
Kinetoplastidia	Kinetokin1/kinetokin2	*Leshmania major*	Positive	99	99	*L. major* (FN677342.1)
Kinetoplastidia	KinSSUF1/KinSSUR1	*Leshmania major*	Positive	99	99	*L. major* (FN677342.1)
Microsporidia	V1/PMP2	*Encephalitozoon hellem*	Positive	100	99	*E. hellem* (AF039229.1)
Rhodophyta	URP1F/URP1R	NA				
Rhodophyta	URP2F/URP2R	NA				
Trichomonads	TFR1/TFR2	NA				
Fungi	MalF/MalR	*Malassezia restricta*	Posisitve	100	98	*M. restricta* (JN980105)
Fungi	ITS1F/ITS4R	*Candida albicans*	Posisitve	100	99	*C. albicans* (L28817.1)
Fungi	NSR1/FR1	*Candida albicans*	Positive	100	99	*C. albicans* (JN940588.1)
Fungi	FunF/FunR	*Candida albicans*	Positive	100	99	*C. albicans* (JN940588.1)
Universal Eukaryotes	euk528F/1391R	*Acanthamoeba castellanii*	Positive	98	99	*A. castellanii* (GU001160.1)
	Euk1A/Euk516r	*Acanthamoeba castellanii*	Positive	100	99	*A. castellanii* (GU001160.1)
Chloroplast Plant	rbcLZ1/rbcL19b	*Solanum* sp.	Positive	98	94	*S. physalifolium* (HQ23562)

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reported to be present in the gut microbiota. Interestingly, the culturing of the sample in using only three different culture media identified more than twice the fungal species than did the different PCR-based molecular methods (Table 5). Accordingly, culturing yielded *A. flavipes, P. brevicompactum, B. bassiana, P. dipodomyicola, M. restricta, Climacocystis* sp. and *I. farisona*, which have not been previously detected in human stool samples. This result differs from previous studies that cultured only one or two *Candida* spp. and *Saccharomyces* spp. from healthy individuals [9–12]. Our culture conditions were different from those used by Scanlan and Chen [9,12], as we incubated our cultures at 25°C for two weeks. We also did not use the same medium as Khatib [23]. Our use of Dixon medium allowed us to isolate a wide variety of fungi (9 species). Our results can be explained by our subject's obese status; it is possible that obese individuals harbor more fungi. Most of the fungi (11 species) identified in our study are known to be associated with dietary sources. In particular, *G. geotrichum* and *P. camemberti* are used as starters for the production of many cheeses [24–25]. Accordingly, *G. geotrichum* has been commonly reported in human stool samples [9–12]. *P. brevicompactum*, which was also identified in our study, has been previously reported to be part of the oral microbiome in healthy individuals, but it has not been identified among the gut microbiota [26]. *P. brevicompactum* is frequently isolated from smoked dry-cured hams [27]. The *P. dipodomyicola* species that was identified in this study has also been reported in food [28]. The *A. flavipes* and *P. allii* species are usually found to be associated with cereal grains [29–31]. To the best of our knowledge, we are the first to report the presence of this species in a stool sample from an obese individual using a culture-dependent method. The *A. versicolor* species found in this stool sample is an environmental airborne fungal species [32]. *A. versicolor* and *P. chrysogenum* have also been previously isolated from dry cured meat products [33]. Accordingly, previous studies have detected these species in human stool samples [11,12]. The *Cladosporidium* sp. isolated from our subject's stool sample is often found on fruit, such as grapes [34], and has been previously reported in stool samples [11].

The *B. bassiana* and *I. farisona* detected in this study are entomopathogenic fungi that are used as biocontrol agents in agriculture [35], which can explain their presence in the human gut. *C. tropicalis*, which was also isolated from our subject's stool sample, has commonly been reported in human stool [23], in the intestine of normal individuals (up to 30%) and in the oral microbiome of healthy individuals [36]. The *Climacocystis* sp. detected here is an edible fungus, which explains the detection of this fungus in this stool sample. This fungus was not found to be present in stool in previous studies.

The *Malassezia* species isolated from our subject's stool sample are normal flora found on the skin of 77–80% of healthy adults [37]. These species were also found in scalp skin from healthy

Table 3. Sequencing results on PCR products from clones.

Primers	clones	Sequences of Species	Blast Identity% and coverage%	Kingdom
ITS1F/ITS4R	75	96% *Galactomyces geotrichum*	99 and 99	Fungi
		4% *Penicillium camemberti*	99 and 99	Fungi
MalFMalR	57	*28.07% Malassezia pachydermatis*	92 and 100	Fungi
		17.54% Malassezia restricta	100 and 99	Fungi
		54.4% Malassezia globosa	99 and 99	Fungi
EUK1A/EUK516r	104	20.4% *Blastocyctis* sp.	99 and 99	Protist
		0.96% *Uncultured Chytridiomycota*	95 and 99	Fungi
		0.96% *Fibraurea tinctoria*	98 and 100	Plant
		1.9% *Allium victorialis*	98 and 100	Plant
		3% *Nicotiana tabacum*	99 and 99	Plant
		0.96% *Helianthus annuus*	96 and 100	Plant
		0.96% *Caprifoliaceae environmental*	98 and 99	Plant
		0.96% *Petrophile canescens*	98 and 99	Plant
		60% *Solanum lycopersicum*	99 and 99	Plant
		5% *Humulus lupulus*	98 and 100	Plant
		3% *Cicer arietinum*	99 and 98	Plant
		0.96% *Pinus wallichiana*	100 and 98	Plant
		0.96% *Mitella nuda*	100 and 98	Plant
JVF/DSPR2	*141*	94.32% *Galactomyces geotrichum*	98 and 99	Fungi
		0.71% *Candida tropicalis*	98 and 99	Fungi
		0.71% *Citrus aurantium*	99 and 100	plant
		4.25% *Atractylodes Japonica*	98 and 99	Plant
		0.71% *Pinus wallichiana*	99 and 100	Plant
		78% *Nicotiana undulate*	98 and 99	Plant
rbcLZ1/rbcL19b	*31*	*3% Musa acuminata/Ensete ventricosum*	99 and 99	Plant
		6.25% Lactuca sativa	99 and 99	Plant
		3% Solanum tuberosum	100 and 99	Plant
		3% Brassica napus/Arabidopsis lyrata	100 and 99	Plant
		6.25% Angelica anomala/Davidia involucrata/Aucuba japonica	100 and 99	Plant

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volunteers [38]. However, *M. pachydermatis* and *M. globosa* were previously found in stool from healthy and ill subjects [12,13] by culture-independent methods. We report for the first time the detection of *M. restricta* in stool by molecular methods. The *Malassezia* species that were detected by culture-independent methods in this study were confirmed by culture. The presence of these fungi in our subject's stool sample could be either a contaminant from the subject's skin or a part of human gut flora, so more investigation is needed to confirm these results. The uncultured *Chytridiomycota* detected in this stool sample is a member of the *Chytridiomycota* family (Figure 2). Some *Chytridiomycota* species infect potatoes and tomatoes [39], which could explain the incidence of these fungi in the human gut. To the best of our knowledge, we are the first to report this species in a stool sample from an obese subject.

In addition to fungi, we detected 11 plant species, all of which are known to be associated with human food and traditional medicines. We identified the dietary plants *Solanum lycopersicum* (tomato), *Allium victorialis* (onion family), *Solanum tuberosum* (potato), *Citrus aurantium* (orange), *Cicer arietinum*, *Musa acuminata/Ensete ventricosum* (banana), *Lactuca sativa*, *Humulus lupulus* (hops), *Pinus wallichiana*. *Helianthus annuus* (sunflowers) and *Brassica napus*. The sequences of *Nicotiana tabacum* and *Nicotiana undulate* that we identified might be linked to the consumption of cigarettes by the patient. A previous study has also reported the presence of *N. tabacum* and *C. arietinum* in human stool [40].

The diversity of the plant species found in the stool sample can be explained by the patient's diet. Because of her obesity, she may have a diet rich in plants. Some of the plant sequences found in this stool sample, such as *Atractylodes japonica*, *Fibraurea tinctoria*, *Angelica anomala* and *Mitella nuda*, are used as medicinal plants [41]. The genus *Atractylodes* has been found in the oral microbiome of healthy individuals [26]. The plants that we identified in this study are similar to those found in Nam's study, which detected different plants from 10 Korean individuals [10]. We did not find the same plant species as those identified from Korean subjects because our obese subject did not have the same diet and lived in a different environment.

Finally, the *Blastocytis* sp. that we detected is commonly found in healthy microbiota [9,10] and is associated with irritable bowel syndrome.

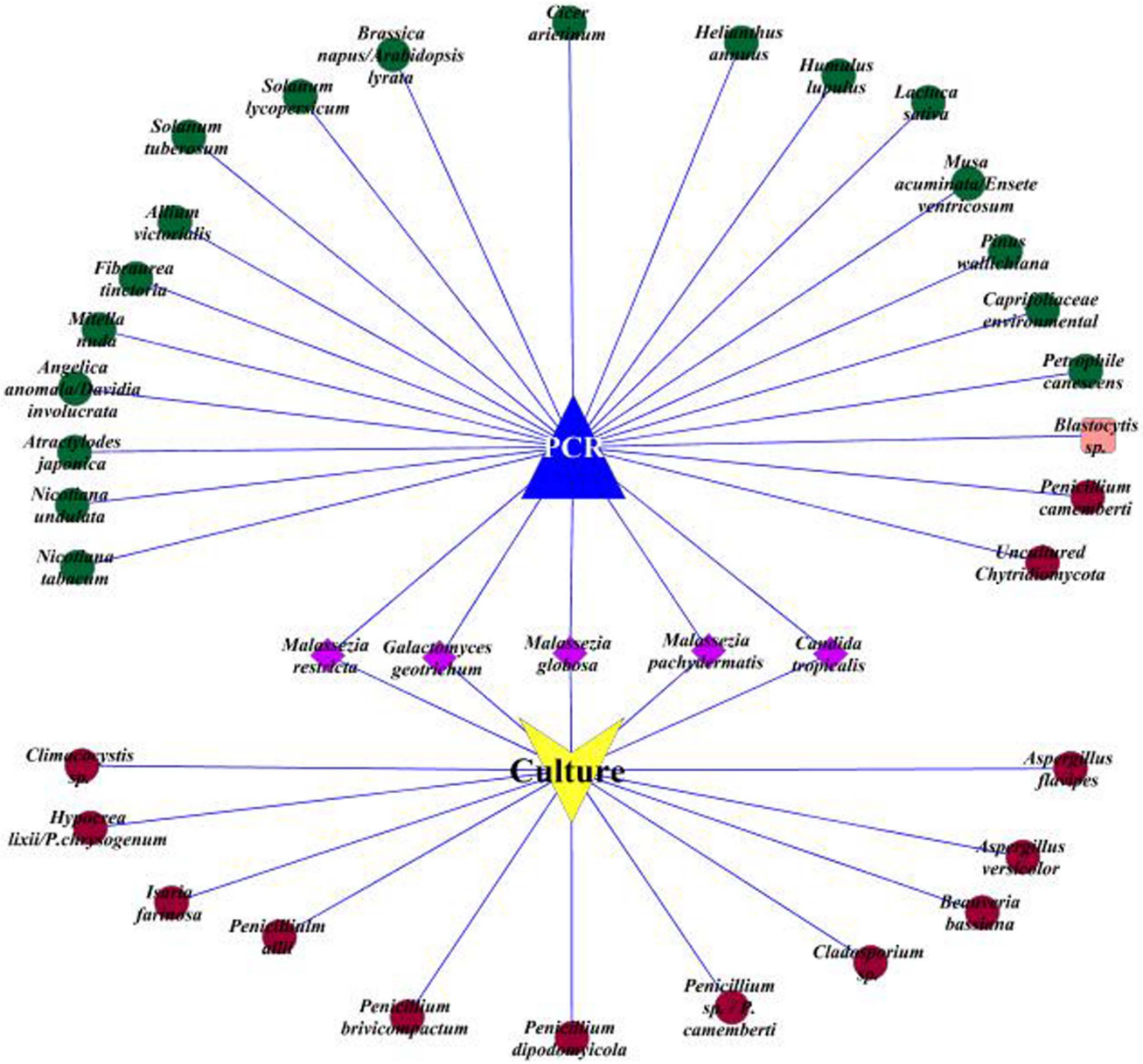

Figure 1. Eukaryotes detected by PCR and culture. Lines connect species found by the two methods. (green color represents plant, red are fungi, pink color are protozoan, purple color are fungi identified by two methods).
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Conclusions

Of 40 phyla of protists described in literature, eight phyla (Diatoms, Apicomplexa, Ciliate, Parabasalids, Fornicata, Amoebozoa, Microsporidia, Fungi) have been previouslsy detected in human gut [42]. However, most species including *Gardia intestinalis* (Parabasalids), *Blastocystis hominis* (Diatoms), *Cryptosporidium parvum* (Apicomplexa), *Balantidium coli* (ciliates), *Dientamoeba fragilis* (Fornicata), *Entameba histolytica* (Archamoeba), *Encephalitozoon intestinalis* (Microsporidia) and *Candida tropicalis* (Fungi) have been reported in patients with digestive tract disease [42–44]. Here, we showed that representatives of two of these eight phyla (Fungi and Blastocystis) can be also detected in one individual without digestive tract disease. Among 19 microeukaryotes found in this individual, five fungal species were detected using PCR-based and culture approaches, 16 fungal species were detected by culture and eight species including seven different fungi and one *Blastocystis* were detected by molecular methods. Accordingly, a total of 13 plants species and eight fungi including *Aspergillus flavipes*, *Beauveria bassiana*, *Isaria farinosa*, *Penicillium brevicompactum*, *Penicillium dipodomyicola*, *Penicillium camemberti*, *Climacocystis* sp. and *Malassezia restricta* were detected for the first time in the human gut microbiota. These data illustrate that eukaryotes have to be searched in the digestive tract using a combined approach and that culture must be kept as a key approach. As a a single stool sample was used herein, results here reported constitute a baseline for futher studies to assess eukaryotic diversity in healthy and diseased individuals from various geographical origins.

Table 4. Fungi cultured using different culture media.

PCR ITS from cultured fungi	% Coverage and % Identity	Media for culture
Penicillium sp./*P. camemberti*	99 and 100	PDA
Hypocrea lixii/*Penicillium chrysogenum*	99 and 98	PDA/CZAPEK
Penicillium brevicompactum	95 and 97	PDA/CZAPEK
Penicillium allii	99 and 99	Dixon agar
Penicillium dipodomyicola	99 and 100	Dixon agar
Aspergillus flavipes	100 and 99	CZAPEK
Aspergillus versicolor	100 and 99	PDA
Beauveria bassiana	99 and 99	PDA
Isaria farinosa	97 and 98	CZAPEK
Galactomyces geotrichum	100 and 100	Dixon agar
Malassezia globosa	100 and 99	Dixon agar
Malassezia restricta	100 and 99	Dixon agar
Malassezia pachydermatis	100 and 93	Dixon agar
Candida tropicalis	99 and 100	Dixon agar/PDA
Cladosporium sp.	100 and 99	Dixon agar
Climacocystis sp.	98 and 96	Dixon agar

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Materials and Methods

Fecal Sample Collection

One stool specimen was collected in a sterile plastic container from a 27-year-old Caucasian woman, who weighed 120 kg with a body mass index (BMI) of 48. 9 and lived in Marseille, France. After collecting the stool sample, 1 g aliquots were preserved in sterile microtubes stored at −80°C until use. The patient provided her written consent to participate in the study, and the agreement of the local ethics committee of the IFR48 was obtained (agreement number 09-022, Marseille, France). The subject did not take antibiotic or antifungal treatments in the month prior to the stool collection, but we were not given information about her diet.

DNA Extraction

DNA was extracted using the Qiamp® stool mini kit (Qiagen, Courtaboeuf, France) as has been previously described [9]. Briefly, 200 mg of stool was placed in a 2-mL tube containing a 200 mg mixture of 0.1–0.5 mm glass beads and 1.5-mL of lysis buffer

Table 5. Cultured fungi compared to fungi detected by PCR and sequencing.

Cultured fungi	PCR cloning sequencing-detected fungi
Galactomyces geotrichum	*Galactomyces geotrichum*
Malassezia globosa	*Malassezia globosa*
Malassezia restricta	*Malassezia restricta*
Malassezia pachydermatis	*Malassezia pachydermatis*
Candida tropicalis	*Candida tropicalis*
Cladosporium sp.	
Climacocystis sp.	
Penicillium sp./*P. camemberti*	*P. camemberti*
Hypocrea lixii/*Penicillium chrysogenum*	
Penicillium brevicompactum	
Penicillium allii	
Penicillium dipodomyicola	
Aspergillus flavipes	
Aspergillus versicolor	
Beauveria bassiana	
Isaria farinosa	
	Uncultured Chytridiomycota

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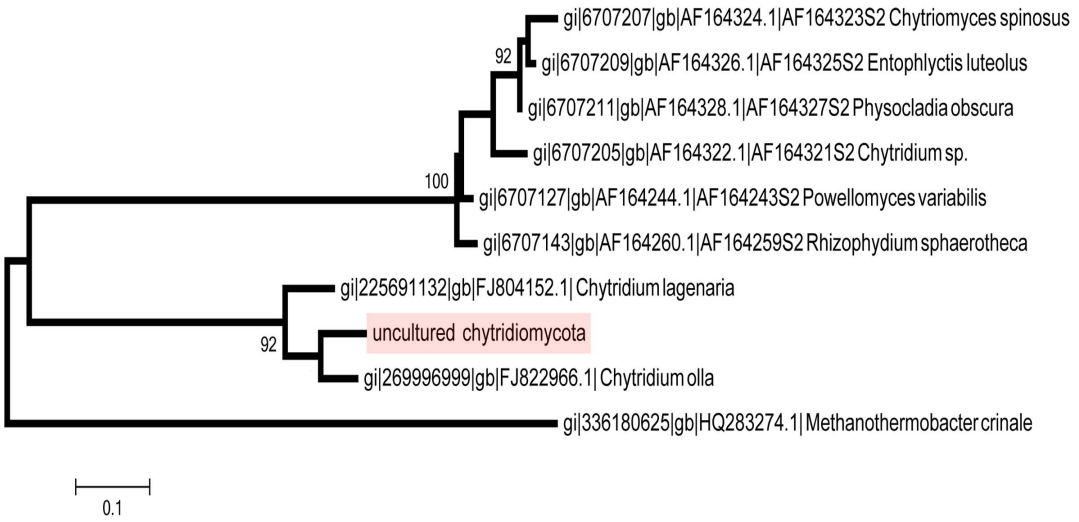

Figure 2. Phylogenetic tree of 18S rRNA gene sequences of uncultured Chytricomycota.
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(ASL) (Qiagen). Mechanical lysis was performed by bead-beating the mixture using a FastPrep BIO 101 apparatus (Qbiogene, Strasbourg, France) at level 4.5 (full speed) for 90 s. A minor modification was made to the manufacturer's procedure by increasing the proteinase K incubation time to 2 h at 70°C. For all DNA extractions, 200 µL of distilled water was used as a negative control. The extracted DNA was stored at −20°C until use.

PCR Amplification

A total of 25 eukaryotic primer pairs for PCR were selected from the literature and used to amplify the 18S rRNA gene, internal transcribed spacer (ITS) and a chloroplast gene (Table 1). Each set of primers was blasted against corresponding taxa of each phylum in nucleotide BLAST program from the National Center for Biotechnology Information (NCBI) to test its ability to amplify the corresponding phylum. The sets of primers for were selected on the basis of a 100% coverage and a 100% identity shown by at least one of the primer from a set. Primers which yielded negative PCR were tested using positive controls specific for each phylum (Table 2). For each eukaryotic primer pair, the 50 µL PCR reaction mixture contained 5 µL of dNTPs (2 mM of each nucleotide), 5 µL of DNA polymerase buffer (Qiagen) 2 µl of $MgCL_2$ (25 mM), 0.25 µL HotStarTaq DNA polymerase (1.25 U) (Qiagen), 1 µL of each primer (Eurogentec, Liège, Belgium) and 5 µl of DNA. PCR was performed with a 15 min initial denaturation at 95°C followed by cycles of 95°C for 30 sec. The initial extension was performed at 72°C for 1 min, and the 5 min final extension was performed at 72°C. The annealing temperature and the number of cycles used for each primer are presented in Table 1. All PCRs were performed using the 2720 thermal cycler (Applied Biosystems, Saint Aubin, France). A reaction made up of buffer without DNA was used as a negative control for each PCR run. Amplified products were visualized with ethidium bromide staining after electrophoresis using a 1.5% agarose gel. The PCR products were purified using the Nucleo- Fast® 96 PCR Kit (Marcherey-Nagel, Hoerdt, France) according to the manufacturer's instructions. To test for potential PCR inhibitors, 1 µl of *A. castellanii* was added to 4 µl of stool DNA prior to PCR amplification.

Cloning and Sequencing

PCR products were cloned separately using the pGEM® -T Easy Vector System Kit (Promega, Lyon, France) as described by the manufacturer. The presence of the insert was confirmed by PCR amplification using M13 forward (5′-GTAAAACGACGGC-CAG-3′) and M13 reverse (5′-AGGAAACAGCTATGAC-3′) primers (Eurogenetec) and an annealing temperature of 58°C. PCRs were performed as described above. Purified PCR products were sequenced in both directions using the Big Dye® Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems, Villebon-sur-Yvette, France) with the M13 forward and M13 reverse primers. These products were run on an ABI PRISM 3130 automated sequencer (Applied Biosystems). Eukaryotes were identified by comparing our obtained sequences with the sequences in the GenBank database using BLAST. The sequence alignments were performed using the clustalw algorithm for multiple sequence alignments (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page = /NPSA/npsa_clustalwan.html). Phylogenetic trees were constructed using the Mega version 5 bootstrap kimura2-parameter model [45].

Fungi Culture and Identification

One gram of stool was diluted in 9 mL of sterile phosphate-buffered saline (PBS), and a six-fold serial dilution from 10^{-1} to 10^{-6} was prepared in PBS. Each dilution was spread in duplicate on potato dextrose agar (PDA) (Sigma-Aldrich, Saint-Quentin Fallavier, France), Czapeck dox agar (Sigma-Aldrich) supplemented with chloramphenicol (0.05 g/l) and gentamycin (0.1 g/l), and Dixon agar [46] supplemented with chloramphenicol (0.05 mg/mL) and cycloheximide (0.2 mg/mL). Dixon agar medium was prepared by adding 1 L of distilled water to a mixture of 36 g of malt extract, 6 g of peptone, 20 g of ox bile, 10 mL of Tween 40, 2 mL of glycerol, 2 mL of oleic acid and 12 g of agar (Sigma-Aldrich). The mixture was heated to boiling to dissolve all components, autoclaved (20 min at 121°C) and cooled to approximately 50°C. Agar plates made from this media were placed in plastic bags with humid gas to prevent desiccation and incubated aerobically at room temperature (~25°C) in the dark. The Dixon Agar medium plates were incubated aerobically at 30°C. Growth was observed for two weeks. The solution used for dilution of the sample was spread on the same media and

incubated in the same conditions as a negative control. DNA extracted from colonies as described above was amplified with the fungal primers ITS 1F/ITS 4R and MalF/Mal R. The purified PCR products were submitted to direct sequencing using the ITS1R/ITS4 and MalF/Mal R primers with the Big Dye® Terminator V1,1 Cycle Sequencing Kit (Applied Biosystems) as described above. When the peaks of the sequence overlapped, the amplicons were cloned as described above.

All sequences superior to 200 base pairs are available in GenBank with reference number (KC143356–KC143757).

Author Contributions

Conceived and designed the experiments: MD DR. Performed the experiments: NG. Analyzed the data: MD. Contributed reagents/materials/analysis tools: DR. Wrote the paper: NG MD DR.

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Commentaire 1: article 1

Dans ce travail, le répertoire des micro-eucaryotes (champignons et des plantes) a été investigué dans une selle d'un sujet caucasien âgé de 27 ans, de sexe féminin avec un indice de masse corporelle de 48.9, en utilisant une approche moléculaire en parallèle de la culture. Pour ce qui concerne l'approche moléculaire, 25 couples d'amorces sélectionnés à partir de la littérature et ciblant le gène 18SrRNA et ITS rRNA ont été utilisés et les produits d'amplification ont été clonés et séquencés.

Cinq couples d'amorces ont généré des résultats positifs. L'analyse de 408 clones a donné 267 (65.4%) séquences correspondant à des séquences de champignons, 116 (28.4%) de plantes et seulement 25 (6.1%) correspondaient à un protiste. L'approche moléculaire a identifié 18 plantes, sept champignons et un diatome (*Blastocystis* sp.

Pour ce qui est de la culture, trois milieux différents de culture de champignon (PDA, CEZAPEK, Dixon agar) ont été utilisés pour l'isolement des champignons dont l'identification a été réalisée par séquençage de l'ITS rRNA. La culture a permis d'identifier 16 espèces de champignons dont cinq espèces avaient été identifiée par l'approche moléculaire.

La combinaison des deux méthodes a permis d'identifier au total 37 espèces d'eucaryotes comprenant un diatome (*Blastocystis* sp.), 18 plantes appartenant au phylum Streptophyta et 18 espèces de champignons appartenant aux phyla

Ascomycota, Basidiomycota et Chydridiomycota. Huit espèces de champignons (*Aspergillus flavipes, Beauveria bassiana, Isaria farinosa, Penicillium brevicompactum, Penicillium dipodomyicola, Penicillium camemberti, Climacocystis* sp. et *Malassezia restricta*) ont été détectées pour la première fois dans le tractus intestinal. Ces espèces avaient décrites dans des aliments à l'exception de *M. restricta* qui était retrouvé sur la peau [24–27]. Les séquences de plantes identifiaient des plantes de tomate, d'oignons, de pomme de terre, de laitue, de fruit et des plantes médicinales. Parmi les espèces identifiées, 11espèces de champignons et de 11 plantes provenaient vraisemblablement d'une source alimentaire.

La culture a identifié un nombre de champignons plus élevé que l'approche moléculaire. Le milieu Dixon agar qui est décrit comme étant spécifique au genre *Malassezia* a favorisé l'isolement de 9 champignons dont 3 du genre *Malassezia*. Des champignons différents ont été isolés par les trois milieux d'où l'importance d'utiliser une variété de milieu pour augmenter la diversité des espèces (Table 4, article 1). Un travail similaire utilisant trois milieu (PDA, Sabouraud dextrose agar et malt agar) n'a isolé que deux espèces de champignons. Nos résultats diffèrent de ce travail par le fait que nous avons effectué une incubation à température ambiante alors que le travail que ce travail précédant à fait une incubation à 37°C.

La diversité du microbiote des eucaryotes répertoriée dans ce travail a montré l’importance de la combinaison des deux approches.

Partie II: article 2

Gut microeukaryotes during anorexia nervosa: one case report.

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Gut microeukaryotes during anorexia nervosa: one case report.

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Abstract

Background

Few studies focused on eukaryote community in the human gut. Here, the diversity of microeukaryotes in the gut microbiota of an anorexic patient was investigated using molecular and culture approaches.

Case presentation

A 21-year-old Caucasian woman was admitted in an intensive care unit for severe malnutrition in anorexia nervosa. One stool specimen was collected from an anorexic patient, culture and PCR-based explorations yielded restricted diversity of fungi but four microeukaryotes *Tetratrichomonas* sp., *Aspergillus ruber Penicillium solitum* and *Cladosporium bruhnei* previously undescribed in human gut.

Conclusions

Establishing of microeukaryotes repertoire in gut microbiota contributes to understand its role in human health.

Keywords

Anorexia nervosa, gut, microeukaryotes, PCR, culture

Background

Few studies focused on the diversity of microeukaryotes and eukaryotes in the human gut of standard weight and obese individuals. The investigation using molecular methods approaches reported an increased fungal burden patients with Crohn's disease, hepatitis B, inflammatory bowel disease and intestinal transplanted patients compared to healthy individuals [1, 2]. The

aim of this study was to make a comprehensive analysis of eukaryote communities in the gut of an anorexic human using both PCR-sequencing and culture techniques.

Case presentation

A 21-year-old Caucasian woman living in Marseille, France was admitted in an intensive care unit weighing 27.7 kg and measuring 1.63 m with a 10.4 kg/m^2 body mass index (BMI). The patient suffered a severe form of anorexia nervosa with severe malnutrition. The investigation of patient dietary habits indicated that she usually drank tea**,** nonfat milk, fruit juice, cereals, a piece of bread and a few fruits for breakfast. For lunch or dinner, she ate vegetables zucchini, carrots, beans, few noodles, rice or grilled bread, fish, turkey, vegetarian steak, nuts, milk products and regularly drank tea and fruit juices. She flavored meals with cinnamon, fennel seeds, curry and brewer's yeast. The patient provided her written consent to participate to the present study and the agreement of the local ethics committee of the IFR48 was obtained for this study (agreement number 09-022, Marseille, France). One stool sample collected aseptically the first day of hospitalization was preserved as 1g aliquots in sterile microtubes stored at -80°C until use. The patient had no antibiotic or antifungal treatment in the month prior to the stool collection.

DNA extracted using the Qiamp® stool mini kit (Qiagen, Courtaboeuf, France) as previously described was stored at -20°C until use [3]. A set of 35 eukaryotic PCR primer pairs retrieved from the literature were used to amplify the 18S rRNA gene and the internal transcribed spacer (ITS rRNA) of fungi, protozoa, helminthes, arthropods and plants (Table 1) (4). Potential stool PCR inhibitors were tested by mixing *Acanthamoeba castellanii* DNA with DNA from stool specimen prior to PCR, as previously described [4]. Distilled water was used as negative control in all PCR reactions. PCRs were performed using the 2720 thermal cycler (Applied Biosystems, Saint Aubin, France). PCR products purified using the Nucleo-Fast® 96 PCR Kit (Marcherey-Nagel, Hoerdt, France) were cloned separately using the

pGEM® -T Easy Vector System Kit (Promega, Lyon, France). PCR amplification using M13 forward (5'-GTAAAACGACGGCCAG-3') and M13 reverse (5'-AGGAAACAGCTATGAC-3') primers (Eurogentec, Seraing, Belgium) were performed on white colonies to confirm the presence of insert. Purified PCR products were sequenced using M13 primers and the Big Dye® Terminator V1.1 Cycle Sequencing Kit on ABI PRISM 3130 automated sequencer (Applied Biosystems). Sequences were compared with those available in GenBank database using BLAST. Seven of 35 (20%) pairs yielded a PCR product with the stool specimen as did control *A. castellanii* DNA. The analysis of 348 clones identified 28 eukaryotic species comprising of 17 (61%) Viridiplantae species, eight (29%) fungi *Saccharomyces cerevisiae, Penicillium solitum, Cladosporium bruhnei, Cystofilobasidium capitatum, Sclerotium* sp., *Malassezia pachydermatis, Malassezia restricta* and *Malassezia globosa*; two metazoa *Mytilus trossulus* and *Mytilus galloprovincialis* and one protozoan *Tetratrichomonas* sp. (Table 2). Original sequences here reported have been deposited in GenBank database with accession numbers JX132667 to JX133078.

Furthermore, one gram of stool was diluted in to 9 mL sterile phosphate-buffered saline spread in duplicate on potato dextrose agar (PDA) (Sigma-Aldrich, Saint-Quentin Fallavier, France), Czapeck dox agar (Sigma-Aldrich) supplemented with 0.05 g/L chloramphenicol and 0.1 g/L gentamycin and Dixon agar [5] supplemented with 0.05 mg/mL chloramphenicol and 0.2 mg/mL cycloheximide [4]. Plates were incubated aerobically at room temperature (~25° C) in the dark, excepted for Dixon agar plates which were incubated aerobically at 30°C. The phosphate-buffered saline solution was spread on the same media and incubated in the same conditions as negative controls. Growth was observed for two weeks. DNA extracted from colonies as described above was amplified with the fungal primers ITS 1F / ITS 4R. Purified PCR products were sequenced as described above. While negative control plates remained

sterile, six fungi including *Aspergillus ruber, Aspergilllus flavus, C. capitatum, M. globosa, M. restricta* and *M. pachydermatis* grew in the two media (Table 3).

Discussion

Here, mycological data were certified since negative controls introduced in both PCR-based and culture-based observations remained negative. Moreover, four fungi were detected by culture as well as by PCR-sequencing. Combining the two methods, a total of ten different fungal species were detected, including *S. cerevisiae, A. flavus, M. pachydermatis, M. globosa* and *M. restricta* previously detected in stools of healthy individuals and patients; and *C. capitatum* and *Sclerotium* sp. previously detected in intestinal biopsy from inflammatory bowel disease [3, 4, 6-9]. Moreover, *A. ruber, P. solitum, C. bruhnei* and *Tetratrichomonas* sp. have not been previously detected in the human although *Tetratrichomonas* sp. has been previously found in the oral cavity and respiratory tract [10].

The diversity of fungal species here observed (ten fungal species) is low compared to that observed in a previous study in an obese patient where sixteen fungal species have been detected [4]. This contrasts with previous observations that the repertoire of bacterial species was comparable in anorexic and obese individuals [11]. Other studies showed a more diverse fungal repertoire in patients than in healthy individuals [1, 2].

Most of eukaryotic species here identified in stools were associated with foods consumed by the patient. *A. ruber* and *A. flavus* have been described in cereals and in human oral mycobiome [12, 13]. *P. solitum* and *C. bruhnei* were previously described on the surface of fruit [14, 15] and *S. cerevisiae* is used in brewing beer. Also, *M. trossulus* and *M. galloprovincial* are seafood here reported for the first time in the human gut. Moreover, 15/17 (88%) plants that were detected from the patient's gut could be linked to the food being

consumed by the patient including edible nuts *(Juglans nigra, Juglans regia, Carya glabra*), herbal teas (*Angelica gigas, Dryas octopetala, Panax notoginseng, Convolvulus arvensis*) or infusions (*Lycium barbarum*), vegetables (*Trigonella foenum-graecum, Foeniculum vulgare, Fallopia japonica, Laurus nobilis* and *Phaseolea environmental*) and fruits (*Prunus persica*). *Humulus lupulus* is hops, which are used in the brewing industry. Similar link between dietary habits and gut microbiota has been made in Malawian twins with kwashiorkor and children in Burkina Faso [16, 17]. Also, *Candida* and *Sacharromyces* have been previously found to be associated to diet [18].

Conclusions

Here, exploration of microeukaryotes in one stool specimen in patient with severe anorexia, correlated with dietary and found restrictive diversity in fungi despite the detection of four species previously unreported in human gut. Establishing the repertoire of microeukaryotes in gut microbiota is necessary to then understand its role in the human health.

Consent

Written informed consent was obtained from the patient for publication of this case report. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

Competing interests

The authors declare that they have no competing interests.

Authors'contributions

NG analyzed the stool sample and prepared the manuscript. MD and DR evaluated the draft and suggested revisions. All authors reviiewed and approved the final manuscript.

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142 **Table 1** Ten Eukaryotic primers used in complement with those reported in a previous study (2)

143

Taxon	Primer	Target	PCR product size (bp)	Annealing temperature (°C)	Reference
Helminthes	NC1/NC2	18s rRNA gene	310-410	55	[19]
	TDR5/TDR3		1700	57	[19]
Blastocystis spp.	SF/SR	18s rRNA gene	600	55	[20]
Plasmodium spp.	PLAS1/PLAS2	cytochrome b gene	709	55	[21]
	PLAS3/PLAS4		709	55	[21]
Plant	rD5-ITS2/rb1-ITS2f	ITS-2 gene	350	59	[22]
Arthropoda	ZBJ-ArtF1c/ZBJ-ArtR2c	mitochondrial coxI gene	157	56-57	[23]
	CB3/CB4	cytochrome b gene	410	46	[24]
Leishmania spp.	LeF/LeR	18S rRNA gene	330	65	[25]
	LEI70R/LEI70L			65	[25]

144

145 **Table 2** PCR results and clone sequencing

Primers	Total number of clones	Number of clones per species	Species	Blast coverage%	Blast coverage identity %	Phylum
EUK1A/EUK516r	43	6/43	*Mytilus trossulus* (seefood)	100	99	*Metazoan*
		12/43	*Lycium barbarum* (tea)	100	98	*Viridiplantae*
		6/43	*Juglans nigra* (nut)	96	99	*Viridiplantae*
		8/43	*Phaseoleae environmental*	100	99	*Viridiplantae*
		2/43	*Panax notoginseng* (ginger)	100	99	*Viridiplantae*
		2/43	*Fallopia japonica* (infusion)	100	99	*Viridiplantae*
		3/43	*Angelica gigas* (infusion)	99	99	*Viridiplantae*
		2/43	*Humulus lupulus*	100	98	*Viridiplantae*
		1/43	*grevillea robusta*	99	98	*Viridiplantae*
		1/43	*Atractylodes japonica*	100	99	*Viridiplantae*
JVF/DSPR2	60	24/60	*Lycium barbarum*	99	98	*Viridiplantae*
		9/60	*Prunus persica* (fruit)	96	99	*Viridiplantae*

		14/60	*Phaseoleae environmental*	99	99	*Viridiplantae*
		5/60	*Carya glabra* (nut)	96	99	*Viridiplantae*
		2/60	*Saccharomyces cerevisiae*	100	99	*Fungi*
		1/60	*Laurus nobilis culinaire*	99	99	*Viridiplantae*
		5/60	*Mytilus galloprovincialis* (seefood)	99	99	*Metazoan*
TFR1/TFR2	25	15/25	*Tetratrichomonas* sp.	100	100	*Protozoan*
		10/25	*Humulus lupulus*	100	98	*Viridiplantae*
MalF/MalR	56	34/56	*Malassezia pachydermatis*	100	93	*Fungi*
		12/56	*Malassezia restricta*	100	99	*Fungi*
		6/56	*Malassezia globosa*	100	99	*Fungi*
		4/56	*Cystofilobasidium capitatum*	100	99	*Fungi*
CuF/CUR	52	20/52	*Trigonella foenum-graecummecinal* (vegetables)	100	99	*Viridiplantae*
		8/52	*Juglans regia* (nut)	100	99	*Viridiplantae*
		19/52	*Foeniculum vulgare* (vegetables)	100	99	*Viridiplantae*

		5/52	*Convolvulus arvensis* (tea)	100	99	*Viridiplantae*
FunF/FunR	42	9/42	*Dryas octopetala* (tea)	99	99	*Viridiplantae*
		5/42	*Penicillium solitum*	99	99	*Fungi*
		4/42	*Cladosporium bruhnei*	100	99	*Fungi*
		2/42	*Prunus persica* (fruit)	99	98	*Viridiplantae*
		22/42	*Sclerotium* sp.	94	99	*Fungi*
rD5-ITS2/rb1-ITS2	70	70/70	*Palaquium formosanum*	100	93	*Viridiplantae*

Table 3 Fungi cultured from stool collected in patient with severe malnutrition and anorexia nervosa.

Species	ITS sequences Blast coverage %	ITS sequences Blast identity %	Culture media
Aspergillus ruber	99	100	Potato dextrose agar
Aspergillus flavus	100	100	Potato dextrose agar
Cystofilobasidium capitatum	100	99	Dixon agar
Malassezia globosa	100	99	Dixon agar
Malassezia restricta	100	99	Dixon agar
Malassezia pachydermatis	100	94	Dixon agar
			Dixon agar

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Commentaire 2: article 2

Peu de travaux ont porté sur le répertoire des micro-eucaryotes détectés dans le tube digestif de personnes ayant un poids standard comparé à celui de personnes obèses et aucun travail de ce type n'avait été réalisé chez des personnes anorexiques. L'objectif de ce travail était d'investiguer pour la première fois le répertoire des micro-eucaryotes dans la selle d'un sujet anorexique âgé de 21 ans, avec un indice de masse corporelle de 10.4, en utilisant une approche moléculaire combinée à la culture. Les informations sur le régime alimentaire du sujet ont été également collectées. Ce régime était composé de lait écrémé, de jus de fruit, de fruits, de légumes, de pain, de céréales, de noix, d'infusions de thé et de levure de bière.

Dans ce travail nous avons augmenté le nombre de couple d'amorces (35 couples d'amorce) pour la détection moléculaire des eucaryotes cependant, 7 couples d'amorces ont généré une amplification positive (Table 1, Table 2 article 2). Un total de 348 clones ont donné 231 (66.4%) séquences de plantes, 91 (26.1%) séquences de champignons, 15 (4.3%) séquences de protistes et 11(3.2%) séquences de metazoan. L'analyse des clones a permis d'identifier 28 espèces d'eucaryotes dont 17 (61%) plantes, 8 (29%) champignons (*Saccharomyces cerevisiae, Penicillium solitum, Cladosporium bruhnei, Cystofilobasidium capitatum, Sclerotium* sp., *Malassezia pachydermatis,*

Malassezia restricta et *Malassezia globosa*), deux metazoa *Mytilus trossulus* et *Mytilus galloprovincialis* et un protiste (*Tetratrichomonas* sp.).

Six espèces ont été identifiées par la culture (*Aspergillus ruber, Aspergilllus flavus, C. capitatum, M. globosa, M. restricta et M. pachydermatis)* dont quatre avaient été identifiées par l'approche moléculaire.

La combinaison des deux approches a permis de détecter un total de 10 espèces de champignons.

Parmi les espèces identifiées, cinq espèces de champignons (*A. ruber, A. flavus*, *P. solitum, C. bruhnei* et *S. cerevisiae)* et 15 plantes ont été associées au régime alimentaire particulier chez cette personne. *A. ruber, A. flavus, P. solitum* et *C. bruhnei* avaient été décrits dans les fruits et les céréales et *S. cerevisiae* est utilisée comme levure de bière [28–30].

Les séquences de plantes correspondaient à des fruits, des noix, de légumes, d'infusion de thé (Table 2, article 2). De travaux similaires sur des coprolithes ont permis d'identifier des séquences de plantes, de poisson et de viande permettant de re-composer en partie, le régime alimentaire de nos encêtres [31–33]. L'analyse des selles contemporaines chez des sujets coréens a également permis de reconstituer le régime alimentaire de ces personnes [34].

Nos travaux vus en perspective de la littérature, indiquent qu'il est possible d'avoir une indication sur le contenu du bol alimentaire par la détection des

micro-eucaryotes et cette approche aurait un intérêt en médecine légale pour déterminer le bol alimentaire d'un défunt. Ce travail nous a également permis d'identifier pour la première fois dans les selles humaines trois champignons *A. ruber P. solitum* et *C. bruhnei* trouvés dans les fruits et un protiste *Tetratrichomonas* sp. qui avait été décrit uniquement dans le tractus respiratoire [35].

Partie III: article 3

Exploration of gut microbiota yields eighteen new micro-eukaryotes species

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En préparation.

Exploring the human gut microbiota in tropical countries yields fifteen new microeukaryotes species

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Abstract

The repertoire of microeukaryotes in the human gut remains poorly explored mainly in individuals living in northern hemisphere countries. We further explored this repertoire using PCR-sequencing and culture in seven individuals living in four tropical countries. A total of 41 microeukaryotes including 38 different fungal species and three protists were detected. Four fungal species *Davidiella tassiana*, *Davidiella* sp., *Corticiaceae* sp. and *Penicillium* sp. were uniquely detected by culture; 27 fungal species were uniquely detected using PCR-sequencing and seven species *Candida albicans, Candida glabrata, Trichosporon asahii, Clavispora lusitaniae*, *Debaryomyces hansenii, Malassezia restricta* and *Malassezia* sp. were detected using both molecular and culture methods. The diversity indice was of 2 in Amazonia, 1.8 in India, 1.7 in Segenal and 1.5 in Polynesia. The pooled diversity was of 0.22 in southern hemisphere (Polynesia and Amazonia) inhabitants compared to the diversity published 0.94 in northern hemisphere inhabitants (P-value=10^{-4}). Fourteen microeukaryotes were shared by the seven individuals whereas 27 species were found in only one individual including 11 species in Amazonia, nine species in Polynesia, five species in India and two species in Senegal. Here, 13 fungal species and two protists *Stentor roeseli* and *Vorticella campanula* were observed for first time in the human gut. This study revealed a previously unsuspected diversity in the repertoire of human gut microeukaryotes, suggesting spots for further exploring this repertoire.

Keywords: Microeukaryotes, gut, stools, tropical countries, PCR-sequencing, culture.

Introduction

The human gut microbiota is a complex ecosystem comprising of bacteria, archaea, virus and eukaryotes referred to as the gut microbiota[1] . It has been observed that the composition of gut microbiota depends on environmental factors [2,3]. Numerous studies focused on gut bacteria, but the repertoire of gut microeukaryotes has been poorly explored [4–9]. Previous study demonstrated the interaction between gut microbiota community and the virulence of some eukaryotes are linked the presence of some bacteria [10]. Recently, high-throughput sequencing and clone library sequencing of gut microeukaryote community indicated that fungi and *Blastocystis* were the two dominant components of gut microeukaryote community [5–7,9,11]. Interestingly, fungal abundance was found to be significantly associated with recently consumed foods: in particular *Candida* spp. abundance significantly correlated with recent consumption of diet rich in high carbohydrates [12]. Likewise, our previous study on eukaryote community in an obese individual revealed fungi diversity related to diet [5]. A diversity of eukaryotic fungi was detected in healthy individuals and infants with low weight [6,7,9]. Despite evidence for the gut microeukaryote community being influenced by the environment, a few studies have been reported from a limited number of individuals, mainly living in the northern hemisphere countries. Indeed, twelve studies three issued from Europe [5,8,9], three from the USA [7,12,13], two from China [4,14], one from India [15], one from Turkey [16], one from Korea [11] and one from Senegal [6] **(Figure 1)**. Therefore, the current corpus of knowledge may not be representative of the actual diversity of this repertoire, as no data issued from among southern hemisphere countries such as Polynesia and Amazonia. Here, in an effort to broaden knowledge on gut microeukaryotes, we investigated microeukaryotes in seven individuals living in four tropical countries.

Materials and methods

Fecal sample collection

The study was approved by the local ethics committee of the Institut Fédératif de Recherche 48 (IFR 48, Marseille, France; agreement number 09-022). After the participants written consent was were obtained. Total of seven stool samples were collected in three individuals from Polynesia (Iles Raietea, rural area), two individuals from Amazonia (Manaus, urban area, forest area), one individual from Senegal (Dielmo, rural area) and India (New Dheli). No specific pathology was reported in any of these individuals. Each stool sample was preserved as 1-g aliquots in sterile microtubes stored at -80°C until use.

DNA-based analyses.

Total DNA was extracted using the Qiamp® stool mini kit (Qiagen, Courtaboeuf, France) as previously described using mechanic and enzymatic lyses [5]. Potential PCR inhibitors were tested by mixing *Acanthamoeba castellanii* DNA with DNA extracted from stool specimen prior to PCR, as previously described [5]. A set of 35 eukaryotic PCR primer pairs obtained from the literature were used to target the 18S rRNA gene and the internal transcribed spacer (ITS). The PCR reaction (50 µL final volume) contained 5 µL of dNTPs (2 mM of each nucleotide), 5 µL of DNA polymerase buffer (Qiagen), 2 µL of $MgCL_2$ (25 mM), 0.25 µL HotStarTaq DNA polymerase (1.25 U) (Qiagen), 1 µL of each primer (Eurogentec, Seraing, Belgium) and 5 µL of DNA. PCR included a 15-min initial denaturation at 95°C followed by 40 cycles including denaturation at 95°C for 30-sec and extension at 72°C for 1 min. A 5-min final extension was performed at 72°C. All PCRs were performed using the 2720 thermal cycler (Applied Biosystems, Saint Aubin, France). PCR buffer without DNA was used as a negative control for each PCR run. PCR products were visualized by electrophoresis using a 1.5% agarose gel. The PCR products were purified using the Nucleo- Fast® 96 PCR Kit (Marcherey-Nagel, Hoerdt, France) according to the manufacturer's instructions. PCR products were cloned separately using the pGEM® -T Easy Vector System Kit as described by the manufacturer (Promega, Lyon, France). Forty-eight white colonies were collected from

each PCR product and sequenced using the Big Dye® Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems, Villebon-sur-Yvette, France) and M13 primers on ABI PRISM 3130 automated sequencer (Applied Biosystems). Sequences were compared with sequences available in the GenBank database using BLAST. All the sequences obtained in this work have been deposited in GenBank database with accession number KF768259-KF768340.

Culture and identification of fungi. Stool samples were diluted in sterile phosphate-buffered saline (PBS) and cultured on potato dextrose agar (PDA) (Sigma-Aldrich, Saint-Quentin Fallavier, France) from potato infusion and dextrose, Czapeck dox agar (Sigma-Aldrich), semi-synthetic solid medium containing sucrose as C-source and sodium nitrate as the sole source of nitrogen supplemented with 0.05 g/l chloramphenicol and 0.1 g/l gentamycin, Sabouraud dextrose agar (BD diagnostic system) and Dixon agar (16) supplemented with 0.05 mg/mL chloramphenicol and 0.2 mg/mL cycloheximide. Dixon agar medium was prepared as previously described [5]. Agar plates were kept in plastic bags with humid gas to prevent desiccation and incubated aerobically at room temperature (~25° C) in the dark. Dixon agar medium plates were incubated aerobically at 30°C. Growth was observed for two weeks. The dilution solution of the sample was spread on the same media and incubated in the same conditions as a negative control. Fungi were identified with ITS 1F / ITS 4R and MalF/Mal R. Purified PCR were sequenced using the ITS1R/ ITS4 and MalF/Mal R primers with the Big Dye® Terminator V1,1 Cycle Sequencing Kit (Applied Biosystems). When the peaks of the sequence overlapped, the amplification products were cloned.

Statistical analysis

The species diversity from stool in each tropical country was compared using Sahannon – Wiener index (H) [17]. The Shannon-Wienner indice diversity was calculated using R software (R version 3.0.1 (2013-05-16).

We collected data from studies previously reporting in northern hemisphere, tropical regions and temperate regions's inhabitants to calculate the diversity using Mann-Whitney/Wilcoxon and Bartlett's tests with PASW softward.

Results

Culture-independent methods.

In all PCR-based experimental the negative controls remained negative. Among the primers tested, four set of primers yielded positive amplifications **(Table 1)**. A. *castellanii* DNA mixed with stool sample yielded positive amplification. A total of 1,056 clones were sequenced and 528 sequences identified 37 microeukaryotes including 34 fungal species and three protists *Stentor roeseli, Vorticella campanula* and *Blastocystis* sp. (**Table1**). Plant and human DNA sequences were excluded for analysis. Species distribution in stools from geographical locations is shown in **Figure 2.** *Malassezia* spp. and *Candida* spp. were detected in all stools from different locations.

Culture dependant-methods.

While the negative control plates remained sterile, *Candia albicans, Candida glabrata, Trichosporon asahii, Clavispora lusitaniae, Debaryomyces hansenii, Malassezia restricta* and *Malassezia* sp. were cultured from Amazonia, Senegal, Polynesia and India. Four fungal species *Corticiaceae* sp. *Davidiella tassiana, Davidiella* sp., and *Penicillium* sp. were detected uniquely by culture-dependant methods in stool samples from (India, Amazonia and Polynesia) (**Table 2**).

Overall results. Combining of the two approaches yielded a total of 38 different fungal species and three protists including *S. roeseli, V. campanula and Blastocystis* sp. Thirteen fungal species and two protists were observed for the first time in the human gut (**Table 3**). Fourteen fungal species including *C. albicans, C. glabrata, C. lusitaniae, D. hansenii,*

142 *Galactomyces candidum, Galactomyces geotrcichum, Malassezia globosa, M. restricta*
143 *Malassezia* sp*. Penicillium* sp., *Rhodotorula mucilaginosa, Saccharomyces cerevisiae, T.*
144 *asahii* and *Westerdykella cylindrica* were shared by the 7 individuals whereas 27
145 microeukeuryotes were found in only one individula including 11 species in Amazonia, nine
146 species in Polynesia, five species in India and 2 species in Senegal were found specific in
147 each tropical country (**Table 4).**

148 **Fungal diversity in stools.**The Shannon-Wiener indice showed high fungal diversity in stools
149 from Amazonia (diversity indice, 2), India (diversity indice, 1.8), Polynesia (diversity, 1.414)
150 and Senegal (diversity indice, 1.7) **(Table 5).** The pooled diversity was of 0.12 in southern
151 hemisphere regions Amazonia and Polynesia and 0.94 in stool specimens reported in northern
152 hemisphere regions in USA, Europe, India, Turkey, Korea and Senegal (P-value=10^{-4}). The
153 diversity in tropical regions in Amazonia, Polynesia, Korea and Senegal was of 0.22 vesus
154 0.84 in temperate regions from USA, Europe and China (P-value=10^{-4}).

155 **Discussion**

156 Here, further exploration of gut microeukaryotes in stool specimens collected from in seven
157 individuals living in four tropical countries yielded new data about this poorly explored
158 component of the gut microbiota. The data were certified since negative controls introduced
159 in both PCR-based and culture-based observations remained negative. Indeed, fifteen species
160 were observed for the first time in the human gut. *S. roeseli* detected in stool from India and
161 *V. campanula* in stool from Amazonia are ciliates previously described from environment in
162 particular in freshwater [18,19]. Moreover, environmental fungal detected in stool from
163 Amazonia including *Puccinia poarum, Rhosporidium babjevae, Phytophthora pinifolia,*
164 *Alternaria alternate, Aspergillius restrictus, Bispora christiansenii, D. tassiana, Davidiella*
165 sp. and *W. cylindrical* were previously described as plant pathogen or from fresh water [20–

26]. *F. capsuligenum* was previously found in fruit, brewery and in soil [27]. These fungal species have not been previously reported in the human gut.

Some opportunist pathogens fungi including *C. albicans, C. glabrata, Filobasidium globisporum, T. asahii, C. lusitaniae, Rhodotorula mucilaginosa, M. restricta, M. globosa* and *Yarrowia lipolytica* were previously described in the human gut [4,5,8,14]. *Geotrichum candidum* and *Saccharomyces cerevisiae* were encountered in the human gut and associated with the consumption of cheese and brewery [15,28,29]. Similar study found a correlation between diet and fungi detected in gut [12]. *Candida nivariensis* is rarely encountered as responsible for vulvovaginal candidiasis [30]. *Exophiala equina* is an environmental fungi previously reported in dialysis water and subscutaneous abcesses [31,32].

Previous studies on eukaryotes diversity in people from Korea, the United Kingdom and Senegal detected some fungal species different from our study and this could be related of individuals location or diet. Here, the high diversity of micro-eukaryotes observed in Amazonia, India and Africa could be related to individual environment. Similar study on gut bacteria microbiota found the bacteria related to host environment and diet [2,3,33]. Our findings are in the same line with previous observation that found that fungal species and protists are dominant components of gut micro-eukaryotes [9].

The 0.94 diversity observed in stools specimens collected in northern hemisphere regions was higher compared to 0.12 diversity reported in southern hemisphere (P-value=10^{-4}). The diversity observed in temperate regions 0.84 was high compared to .022 in tropical regions (P-value=10-4). However, the fact that most of studies have been performed in temperate regions including in 694 individuals may bias this observation.

Conclusion

A diversity of 41 micro-eukayotes species including 38 fungal species and three protist was detected in stool samples collected from four different tropical locations. The diversity was observed in stool from Amazonia, India and Segenal. Some fungal species (14 species) are share with different geographical locations but we detected 27 microeukaryotes species specifics according to geographical location. A total of 13 fungal species and two protists including *Stentor roeseli* and *Vorticella campanula* were observed in the human gut for the first time. Diversity of gut microeukaryotes appear to depend in host environment and diet. These data plea for more extensive studies being performed in specimens collected from various geographical regions to further establish the human gut microeukaryote repertoire.

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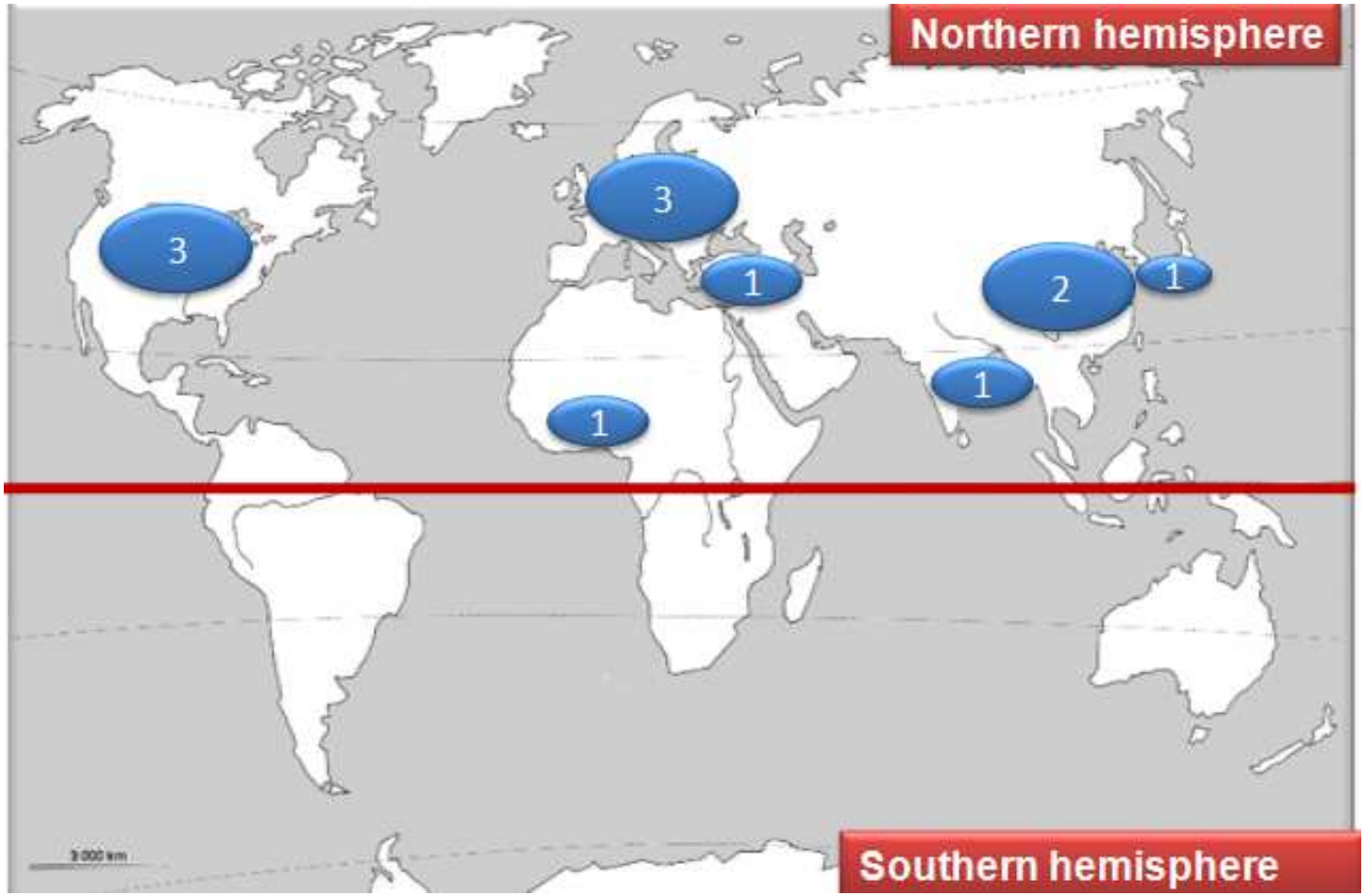

Fig1:Microeukaryotes studies in northen hemisphere. Blue circles represente the number of studies.

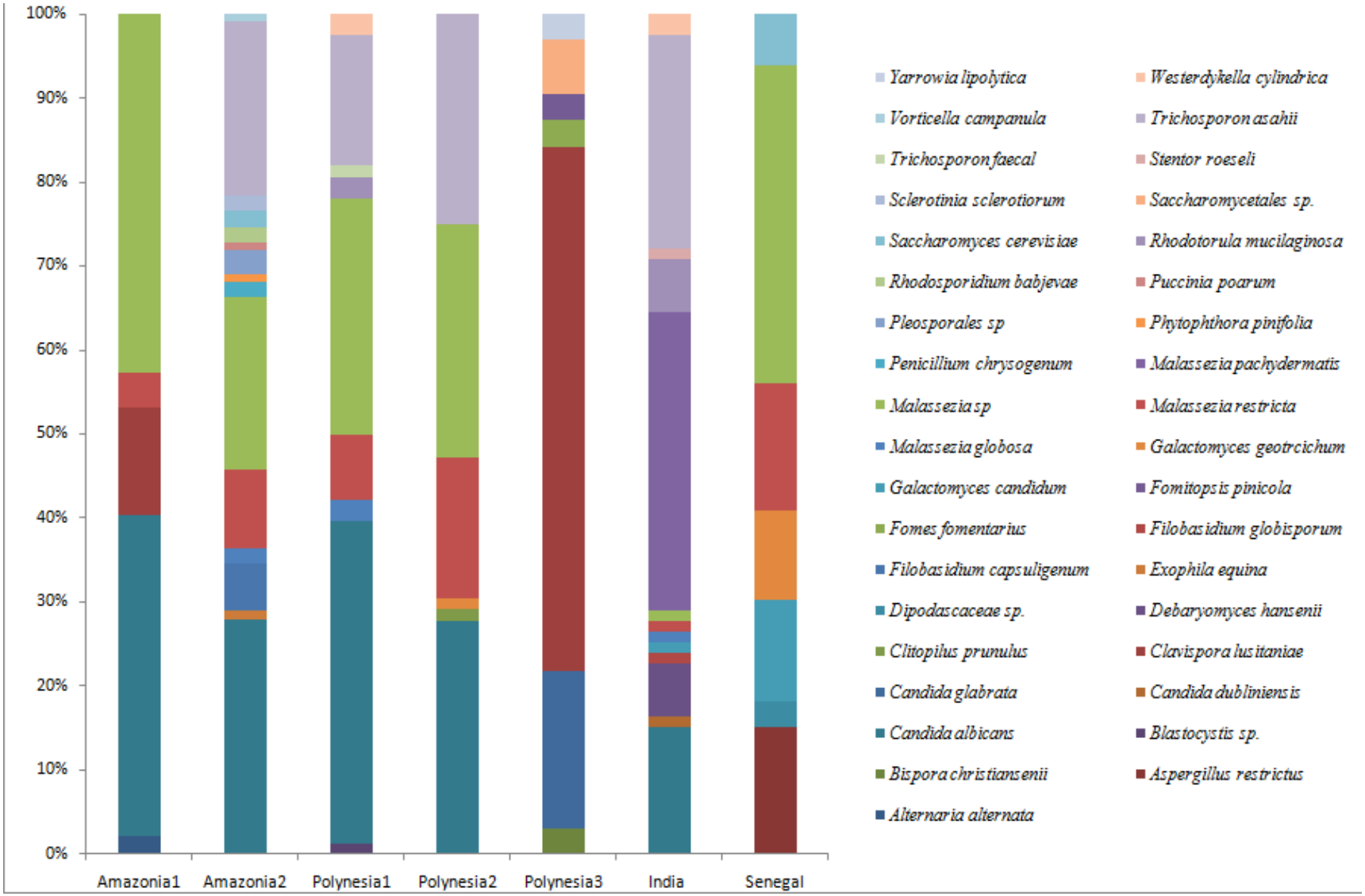

Fig 2. Microeukaryotes species distributions in 7 stool samples from different geographical locations detected by PCR-based methods.

337

338 **Table 1**. Clone library of micro-eukaryotesin stool from different locations using three primers

Stool samples locations	ITS1F/ITS4R	Euk1A/Euk516r	AmiF/AmiR	MAlF/MalR
Amazonia 1	*Candida albicans* (36/48) *Clavispora lusitaniae* (12/48)		*Alternaria alternata* (2/48)	*Malassezia* sp. (40/48 *Malassezia restricta* (4/48)
Amazonia 2	*Candida albicans* (30/48) *Trichosporon asahii* (12/48)	*Vorticella campanula* (1/48) *Saccharomyces cerevisiae* (2/48) *Phytophthora pinifolia* (1/48) *Trichosporon asahii* (10/48) *Pleosporales* sp.(3/48) *Puccinia poarum* (1/48) *Sclerotinia sclerotiorum* (2/48) *Rhodosporidium babjevae (2/48)*	*Filobasidium capsuligenum (*6/48) *Penicillium chrysogenum* (2/48) *Vorticella campanula* (1/48) *Exophiala equina*(1/48)	*Malassezia* sp.(22/48) *Malassezia restricta* (10/48) *Malassezia globosa* (2/48)
Polynesia1	*Trichosporon asahii* (12/48) *Candida albicans* (30/48) ***Trichosporon faecale1/48***	*Westerdykella cylindrica* (2/48) *Rhodotorula*		*Malassezia* sp.(22/48) *Malassezia restricta* (6/48) *Malassezia globosa* (2/48)

		mucilaginosa (2/48) *Blastocystis* sp. (1/48)		
Polynesia 2	*Trichosporon asahii* (18/48) *Candida albicans* (20/48) *Galactomyces geotrcichum* (1/48)	*Clitopilus prunulus* (1/48)		*Malassezia* sp.(20/48) *Malassezia restricta* (12/48)
Polynesia 3	*Saccharomycetales* sp. (2/48) *Clavispora lusitaniae* (20/48) *Fomitopsis pinicola* (1/48) *Fomes fomentarius* (1/48) ,	*Candida glabrata* (6/48) *Bispora christiansenii* (1/48) *Yarrowia lipolytica* (1/48)		
India	*Candida albicans* (12/48) *Trichosporon asahii* (20/48 *Geotrichum candidum (*1/48)	*Debaryomyces hansenii* (5/48) *Filobasidium globisporum* (1/48) *Candida dubliniensis* (1/48) *Stentor roeseli* (1/48) *Rhodotorula mucilaginosa* (5/48) *Westerdykella cylindrica* (2/48)		*Malassezia* pachydermatis (28/48) *Malassezia restricta* (10/48) *Malassezia globosa* (5/48)

Senegal	*Galactomyces geotrichum* (7/48) *Dipodascaceae* sp. (2/48) *Galactomyces candidum* (8/48)		*Saccharomyces cerevisiae(4/48)* *Aspergillus restrictus (*10/48)	*Malassezia restricta* (10/48) *Malassezia* sp.(25/48)

339

340 **Table 2**. Fungal species isolated from four culture media and identified by ITS1/ITS4R primers.

Geographical location	PDA	SDA	CZPEK	DA
Amazonia1	*Candida glabrata* *Candida albicans*	*Candida glabrata* *Candida albicans*	*Candida glabrata* *Candida albicans*	*Candida albicans* *Candida glabrata* *Davidiella tassiana*
Polynesya1		*Trichosporon asahii* *Penicillium* sp.		
Polynesya2		*Candida albicans*		*Candia albicans* *Malassezia restricta* *Trichosporon asahii* *Davidiella* sp.

Polynesya3	*Trichosporon asahii* *candida albicans*	*Trichosporon asahii* *candida albicans*		*Debaryomyces hansenii* *Trichosporon asahii*
India	*Candida albican* *Trichosporon asahii* *Clavispora lusitaniae*	*Trichosporon asahii* *Clavispora lusitaniae* *Corticiaceae* sp.	*Penicillium sp.*	*Malassezia restricta* *Candida albicans* *Malassezia sp*

341

342 **Table 3.** Micro-eukaryotes described for the first time in human gut.

Micro-eukaryotess first description in the human gut	Affiliation
Aspergillus restrictus	Fungi
Alternata alternaria	Fungi
Bispora christiansenii	Fungi
Corticiaceae sp.	Fungi
Davidiella sp.	Fungi
Davidiella tassiana	Fungi
Exophiala equina	Fungi
Filobasidium capsuligenum	Fungi
Fomes fomentarius	Fungi
Phytophthora pinifolia	Fungi
Pleosporales sp.	Fungi
Puccinia poarum	Fungi
Rhodosporidium babjevae	Fungi
Stentor roeseli	Protist
Vorticella campanula	Protist

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346 **Table 4**. Micro-eukaryotes species found specifics by geographical locations

Amazonia	Polynesia	India	West africa(Senegal)
			Aspergillus
Filobasidium capsuligenum	*Blastocystis* sp.	*Candida dubliniensis*	*restrictus*
Penicillium chrysogenum	*Trichosporon faecale*	*Corticiaceae* sp	*Dipodascaceae sp.*
Phytophthora pinifolia	*Clitopilus prunulus*	*Filobasidium globisporum*	
Pleosporales sp	*Davidiella* sp.	*Malassezia pachydermatis*	
Puccinia poarum	*Bispora christiansenii*	*Stentor roeseli*	
Rhodosporidium babjevae	*Fomes fomentarius*		
Sclerotinia sclerotiorum	*Fomitopsis pinicola*		
Vorticella campanula	*Saccharomycetales sp*		
Exophiala equina	*Yarrowia lipolytica*		
Alternaria alternata			
Davidiella tassiana			

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Table 5. Shannon indice for micro-eukaryotes diversity in stools from different locations

Stool samples geographical locations	Shannon Indice
Amazonia1	1,210
Amazonia2	2,003
Polynesia1	1,554
Polynesia2	1,475
Polynesia3	1,214
India	1,83
Senegal	1,709

Commentaire 3: article 3

En combinant l'approche moléculaire et la culture, nous avons exploré la diversité du mycobiome et des micro-eucaryotes dans des échantillons de selles d'individus habitant différentes régions géographiques du monde, Amazonie, Polynésie, Inde, Sénégal et France.

Sur 35 couples d'amorces quatre couples d'amorce ont généré des résultats positifs. Nous avons obtenu 528 clones dont 3 séquences identifiaient des protistes et 525 (99%) séquences identifiaient des champignons. L'approche moléculaire a détecté 34 espèces de champignons dont 27 spécifiques à la culture, la culture seule quatre champignons et 7 champignons ont été détecté par la combinaison des deux méthodes. Nous avons trouvé un total de 41 micro-eucaryotes. Nous avons observé que 14 espèces champignons sont retrouvés partagés entres les différentes régions alors que la majorité 27 espèces restent détectées une seule fois dans une région donnée. Les champignons identifiés dans ce travail proviennent de l'environnement ou de l'alimentation et des espèces pathogènes comme *Candida nivariensis* et *Exophilia equina* ont été également retrouvées dans les selles.

Des travaux similaires sur le microbiote bactériens dans les selles des individus habitant différentes régions du monde ont trouvé une corrélation entre la composition du microbiote et l'origine géographique des sujets [36,37]. Nos

résultats sont similaires à ces travaux du fait que nous avons trouvé des espèces de champignons spécifiques à une région donnée (Table 4) article 3).

Ce travail a permis aussi de détecter pour la première fois dans les selles humaines13 espèces de champignons et deux protistes *Stentor roeseli* en Inde et *Vorticella campanula* en Amazonie qui n'avaient jamais été décrits dans le tractus digestif de l'homme. Ces deux protistes ont été décrits dans des eaux douces [38,39].

Conclusions et perspectives

Notre travail expérimental a commencé par la mise au point et la standardisation de l'extraction de l'ADN, de la PCR et de la culture pour la détection des micro-eucaryotes dans les selles. Les eucaryotes sont connus pour avoir des parois cellulaires difficiles à lyser pour cela nous avons effectué une lyse mécanique avec de la poudre de verre suivie des étapes de fastprep pour une lyse des parois en combinant avec une lyse enzymatique fournie dans le kit QiampH stool mini kit (Qiagen,Courtaboeuf, France) [40]. Les selles peuvent contenir également des inhibiteur de la PCR tels que les lipides, les selles biliaires, l'hémoglobine, les polysaccharides [40]. Pour cela, avons mélangé l'ADN de *Acanthamoeba castellanii* à l'échantillon de selle pour tester de potentiels inhibiteurs de la PCR à chaque extraction d'ADN. Nous avons également testé 35 couples d'amorce avec 13 contrôles positifs appropriés disponibles dans notre laboratoire pour évaluer l'efficacité des amorces sélectionnés et seulement 18 paires ont généré des résultats positifs. Nous n'avons pas eu de contrôles positifs pour 17 amorces couples d'amorces. Les résultats négatifs obtenus avec les couples d'amorces pourraient s'expliques par l'absence d'organismes ciblés dans les échantillons ou simplement par le faite que ces amorces ne fonctionnent pas.

Seulement 7 couples d'amorces ITSF1/ITS4R, EUK1A /EUK516r, AmiF/AmiR, JVF/DSPR2, rbcl1/rbclz, FunF/FunR, Mal/MalR ont donné des résultats positifs sur les échantillons de selle; parmi eux, deux couples (ITSF1/ITS4R, EUK1A /EUK516r) ont toujours donnés des amplifications

positives sur toutes les selles analysées. Nous suggérons l'utilisation de ces derniers dans les études futures sur les microeucaryotes. Dans la littérature il été a été rapporté que IITS1R/ITS4R favorise l'amplification du phylum des Ascomycota pour cela nous suggérons l'association ITS1F/ITS4B décrit pour amplifier les Basidiomycota [41].

La suite de l'expérimentation a porté sur l'incubation des champignons à différentes températures dans des milieux appropriés dans le souci de déterminer celle favorable à la croissance des champignons. Les résultats obtenus montre que l'incubation à température ambiante favorisait la croissance des champignons sur les milieux PDA et CZAPEK.

Dans un deuxième temps, nous avons appliqué les protocoles standardisés pour explorer le mycobiote digestif dans les selles de deux individus présentant un indice de masse corporelle différent. La comparaison de l'analyse de ces deux selles a permis d'observer des espèces de champignons spécifiques à chaque individu et correspondant au régime alimentaire. Ces espèces pourraient être considérées comme une flore transitoire apportée par les aliments. *Candida tropicalis* détecté chez le sujet obèse et *Sclerotium* sp. chez le sujet anorexique sont couramment décrits dans les selles humaines et leur détection n'est donc pas spécifique de la situation de la personne. Aussi, des espèces communes retrouvées chez ces individus telles que *Malassezia restricta, Malassezia globosa et Malassezia pachydermatis* pourraient correspondre à une flore

commensale. Nous avons également observé une prédominance de séquences de champignons chez la personne obèse alors que chez la personne anorexique les séquences de plantes étaient prédominantes. Les séquences de protistes étaient minoritaires dans les deux cas.

Ensuite, nous avons utilisé les mêmes protocoles dans sept échantillons pour explorer la diversité des micro-eucaryotes en fonctions des origines géographiques. Nous avons identifié 14 espèces partagées entre les différentes régions géographiques et 27 espèces spécifiques à chaque région. 13 espèces de champignons et deux protistes ont été trouvés pour la première fois dans les selles humaines.

Ces résultats, bien qu'intéressants, restent encore préliminaires. Il est important de mener des recherches approfondies particulièrement sur un nombre élevé avant de tirer d'avantage de conclusions.

Avant d'entreprendre nos travaux, un total de 236 espèces de champignons avait été rapporté dans le tube digestif humain. Notre recherche a permis d'identifier un total de 24 espèces de champignons et trois protistes qui n'avaient jamais été observés dans le tube digestif. Nos travaux ont contribué à un apport de 10% du répertoire mycobiome digestif par rapport à ce qui était connu dans la littérature.

Notre étude a permis de révéler que le répertoire des micro-eucaryotes dépend de l'environnement des individus. Les protocoles standardisés que nous avons

mis en place pourront être utilisés pour explorer les micro-eucaryotes dans des environnements différents.

L'accent devrait être mis sur la culturomic dans les études futures en variant les conditions et les milieux de culture pour champignons et en utilisant le MALDI-TOF-MS pour une identification rapide des espèces.

Nous suggérons également une approche métagénomique à haut débit avec une variété d'amorce pour analyser un grand nombre de séquences afin d'augmenter la diversité des espèces.

Pour l'instant, il est difficile de parler de la physiologie et de la pathologie des micro-eucaryotes chez les patients et les personnes en bonne santé. On n'a pas assez de connaissance sur le répertoire des micro-eucaryotes qui apparemment dépend de l'environnement. Le développement des outils d'exploration du mycobiome digestif permettra une meilleure connaissance du répertoire qui permettra dans une perspective lointaine d'envisager la corrélation entre le mycobiome digestif et les maladies.

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