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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.

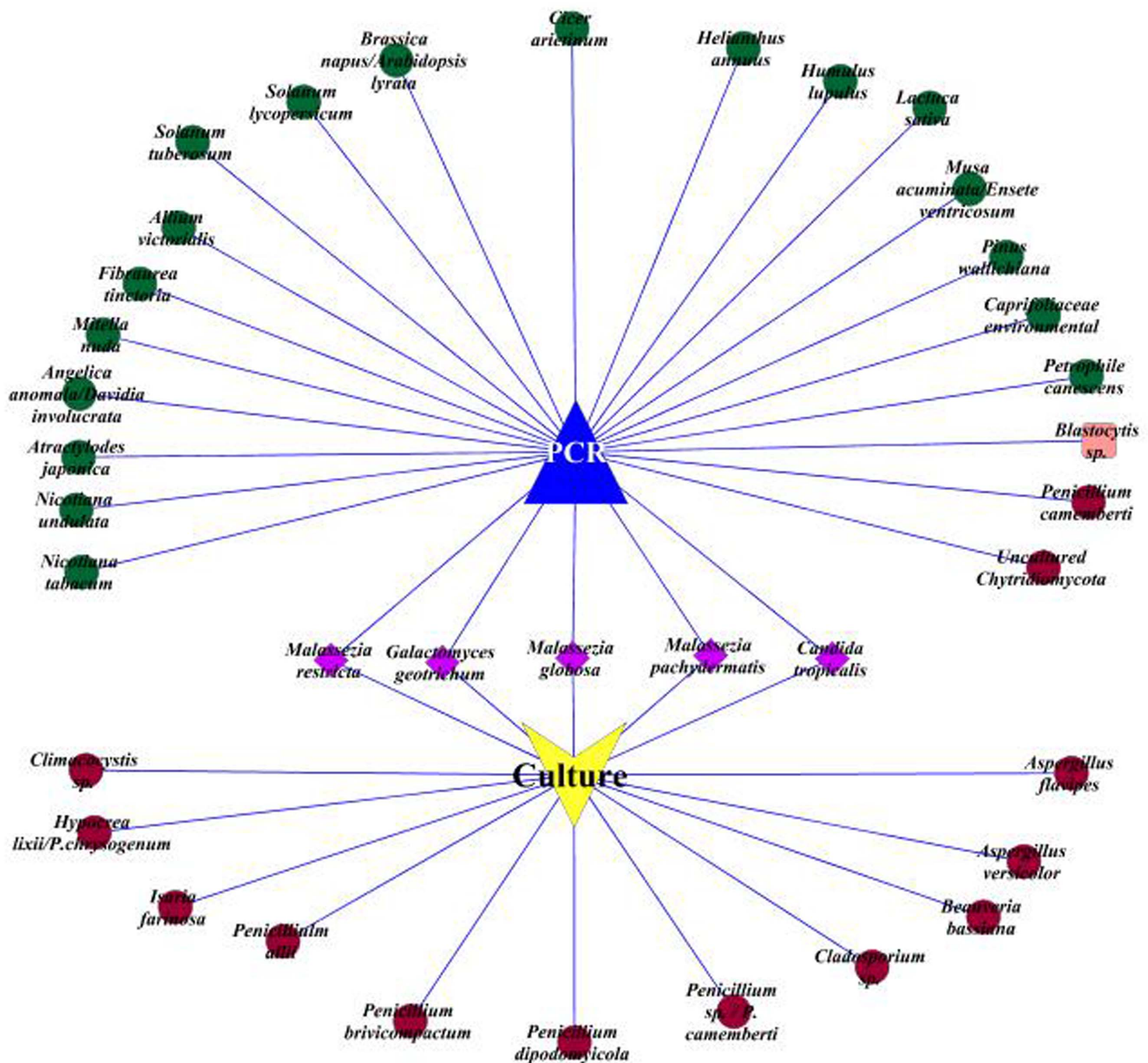

Figure 1. Eukaryotes detected by PCR and culture. Lines connect species found by the two methods. (green color represents plant, red are fungi, pink color are protozoan, purple color are fungi identified by two methods).
doi:10.1371/journal.pone.0059474.g001

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 micro-eukaryotes 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

doi:10.1371/journal.pone.0059474.t004

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

doi:10.1371/journal.pone.0059474.t005

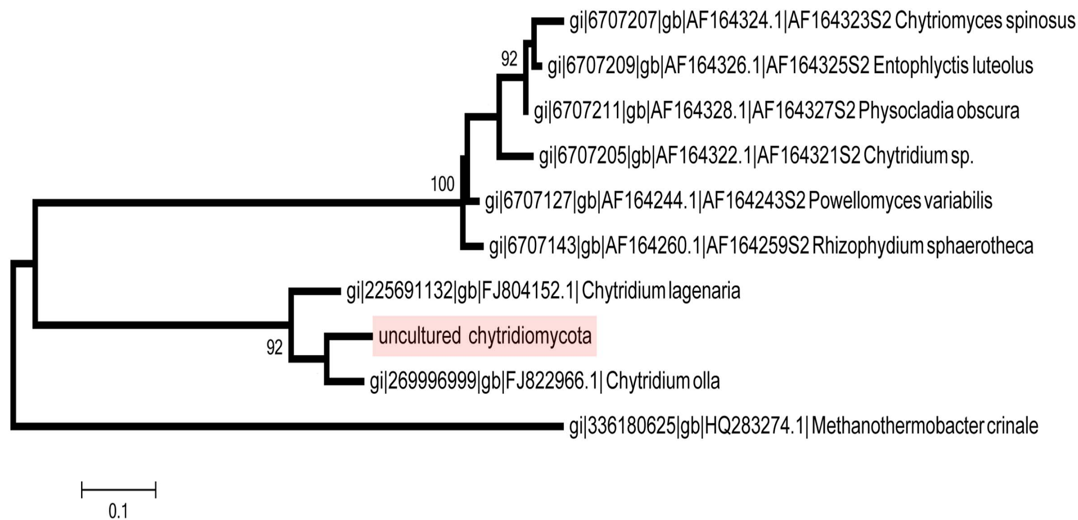

Figure 2. Phylogenetic tree of 18S rRNA gene sequences of uncultured Chytricomycota.
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(ASL) (Qiagen). Mechanical lysis was performed by bead-beating the mixture using a FastPrep BIO 101 apparatus (Qbiogene, Strasbourg, France) at level 4.5 (full speed) 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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