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Détection Moléculaire des Eucaryotes dans les Selles de Primates : Étude Exploratoire

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Composition du Jury:

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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 nun 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.

Professeur Didier Raoult

RÉSUMÉ

Chez les mammifères, les Eucaryotes représentent une composante importante des micro-organismes peuplant le tractus digestif. Leur présence peut avoir des effets bénéfiques mais aussi néfastes, comme les parasites, chez l'hôte. Depuis la dernière décennie, de nombreux efforts ont été entrepris afin de comprendre le lien entre les composants eucaryotes du milieu intestinal et la santé de l'hôte. En effet, de nombreuses études ont été menées pour la découverte et l'évaluation de la diversité des eucaryotes peuplant ce tractus gastro-intestinal. Dans cette étude, nous nous sommes appuyés sur des techniques de culture couplées avec des méthodes moléculaires ciblant spécifiquement les gènes ribosomaux 18s et ITS dans l'objectif d'évaluer les composants eucaryotes du tube digestif de l'homme mais aussi des grands singes.

Afin de comprendre le rôle des composants eucaryotes chez des patients et des sujets sains, des échantillons fécaux, provenant d'individus sains et de personnes atteintes de tuberculose et du VIH, ont été étudiés par amplification de plusieurs couples d'amorces universelles suivies de clonage et du séquençage. Nos résultats ont montré que les champignons constituent une forte proportion de la communauté eucaryote dans le tube digestif des individus sains et malades. Ces derniers ont aussi montré que cette dominance fongique dépend de nombreux facteurs comme la situation géographique, l'état de santé de l'individu mais aussi du nombre d'échantillons analysés. Au total, 16 champignons et 2 autres micro-eucaryotes (*Entamoebahartmanni* et *Blastocystis* Sp.) ont été identifiés dans l'échantillon provenant de la personne saine. Par contre, peu d'espèces fongiques ont été identifiées via l'échantillon provenant du patient atteint de tuberculose. Cependant, un total de 78 micro-eucaryotes, comprenant 67 champignons et 2 straménopiles, ont été identifiés avec les 31 échantillons fécaux de personnes atteintes de VIH.

D'autre part, la diversité des eucaryotes qui peuplent les primates non humains tels que les grands singes demeure relativement inexploré en raison soit de la faible abondance de ces micro-organismes dans l'intestin de ces derniers, ou parce qu'ils ont reçu une attention limitée en comparaison avec l'ensemble des composants micro-eucaryotes.

Pour ces raisons, nous avons entrepris une analyse moléculaire dans le but de détecter ces micro-organismes eucaryotes, dont certains demeurent pathogènes pour l'homme, en utilisant un seul échantillon fécal prélevé chez un gorille sauvage en provenance de l'ouest du Caméroun. Ces analyses ont été suivies d'une détection moléculaire spécifique du potentiel pathogène de ces eucaryotes du tractus gastro-intestinal des gorilles sauvages. En conséquence, ils ont permis d'identifier 87 espèces eucaryotes comptant 52 champignons, 10 protozoaires, 4 nématodes et 21 espèces de plantes. Nous avons également signalé la présence de champignons pathogènes (*Candida tropicalis*), et de parasites (*Oesophagostomum bifurcum*, *Necator americanus*). Afin d'examiner d'une manière plus approfondie si ces gorilles abritaient d'autres parasites, nous avons analysé 91 échantillons fécaux à la recherche d'agents pathogènes comme la *leishmaniose*.,

Les résultats ont montré que 12 échantillons contenaient des parasites du genre *Leishmania* et 4 phlébotomes comme vecteurs. L'analyse moléculaire a été effectuée par enchaînement de 3 différentes réactions de polymérase en chaine (PCR) spécifiques aux agents de la leishmaniose. En outre, les formes promastigote et amastigote du parasite ont été visualisée dans les échantillons fécaux lorsque l'hybridation in situ par fluorescence a été réalisée. Au final, nous avons utilisé des amorces ciblant les gènes du cytochrome b et du COI avec les différents échantillons provenant des gorilles, chimpanzés, et bonobos afin d'analyser la diversité des arthropodes consumés par ces grands singes. Cinquante et 56 unités taxonomiques opérationnelles (UTO) ont été obtenues

respectivement à partir de gènes du Cyt b et COI et appartenant à 32 familles de 5 ordres (Diptera, Isoptera, lépidoptères, coléoptères et orthoptères).

ABSTRACT

Eukaryotes represent significant component of the mammalian intestinal tract. Their occurrence might have either beneficial or virulent parasitic effects on the host. Since the last decade, efforts have been made in order to understanding the link between the gut eukaryotes and host's health and thus many studies have been conducted to uncover and estimate the diversity of gut eukaryotes across intestinal digestive tract. In this thesis, we relied on the applications of both culture-dependent methods and extended molecular methods targeting the 18S rRNA and ITS and some other genes, to assess the eukaryotic components in digestive tract of both human and great apes.

In order to understand the human intestinal eukaryotic components in both health and disease states, fecal samples from healthy and patients with resistant tuberculosis and HIV infection were extensively studied using amplification with various eukaryotic universal primers followed by cloning and sequencing. Our results revealed that fungi constitute a high proportion of eukaryotic communities in gut of the healthy and sick individuals and their dominance depend on many factors including geographical location, health status and the size of samples analyzed. A total of 16 fungal species and 2 other micro-eukaryotes (*Entamoeba hartmanni* and *Blastocystis* sp.) were identified in healthy fecal sample. Contrary, a very few fungi were detected in the fecal sample from patient with resistant tuberculosis; whereas a total of 78 micro-eukaryotic species were detected (67 fungi and 2 stramenopiles) were found in 31 HIV fecal samples.

On another hand , The diversity of eukaryotes inhabiting non-human primates such as great apes remains relatively unexplored because of either the low abundance of these organisms in apes gut or because they have received limited attention from a whole-community perspective. For these reasons we undertook

an extensive molecular analysis for detecting eukaryotic microbiota including some human eukaryotic pathogens in a single fecal sample from a wild western lowland gorilla from Cameroon, and then followed by specific molecular detection of potential human eukaryotic pathogen in gastrointestinal tracts of wild population of gorillas. Our effort resulted in retrieving 87 eukaryotic species, including 52 fungi, 10 protozoa, 4 nematodes and 21 plant species. We also reported the occurrence of pathogenic fungi (*Candida tropicalis*), parasites (*Oesophagostomum bifurcum*, *Necator americanus*). To further examine whether these gorillas harbor other sever parasites, we screened 91 of their fecal samples for the presence of blood borne pathogen such as *Leishmania*. The results showed that, 12 fecal samples contained Leishmania parasites, and 4 contained phlebotomine sand fly vectors. The molecular identity was determined by running 3 different polymerase chain reaction tests for detection of *Leishmania major*. Moreover, both promastigote and amastigote forms of the parasite were visualize in fecal samples when fluorescence in situ hybridization was performed.

Finally, we used DNA-barcoding primers targeting the Cyt-b and COI genes in arthropod mitochondrial genomes to evaluate the insect-diet diversity of three African great apes gorilla, chimpanzee and bonobo by analyzing their fecal samples. A total 50 and 56 insect operational taxonomic units (OTUs) obtained from Cyt-b and COI gene libraries respectively, which belonged to 32 families from 5 orders (Diptera, Isoptera, Lepidoptera, Coleoptera, and Orthoptera).

INTRODUCTION

Eukaryotes are an important part of the primate gut. They represent a complex ecosystem and their relationship with the primate host varies from commensal or mutualistic to opportunistic or parasitic [1]. The compositions and constituents of these communities are influenced by several factors, such as the host diet, geography, physiology, and disease state [2,3].

Since the last decade, many works have been made in order to uncover the diversity of eukaryotic communities across human digestive tract [4-11]. A part of works that made on the eukaryotic community of the gut has been focused mainly on the prevalence of fungal species in the fecal samples from human through traditional culture dependent approaches [10,11]. However, culture-independent molecular techniques, comprising of direct DNA extraction from feces followed by PCR and electrophoresis or cloning, have been introduced to explore eukaryotic communities. These techniques facilitate the detection of fastidious or not-yetcultured species and opened a new avenue for understanding the eukaryotic community in gastrointestinal tracts of human.

The first culture-independent analysis for investigation the diversity of the microeukaryotes in human gut was undertook by Scanlan and his coworkers [4] and a low diversity and abundance of eukaryotes (Gloeotinia/Paecilomyces and Galactomyce) have been reported in the guts of healthy individuals. Similarly, low fungal diversity including a limited number of *Candida* species and *Saccharomyces* has been detected from the gut of Korean populations (ref). A more diverse fungal community was observed when fungal communities have been screened by Ott et al.[7] and Chen et al.[12] in patients with inflammatory bowel disease and patients with cirrhosis and chronic hepatitis B virus infection. Moreover, Li et al. also reported an increase in the size of the fungal community early after intestinal transplantation, followed by a decrease in this community

over time. Sequences of *Saccharomyces cerevisiae* and *Kluyveromyces waltii* were shown to be the dominant fungi in the gastrointestinal tract of two intestinal transplant patients [6]. The eukaryote communities have been screened in a single fecal specimen from a Senegal young healthy man using multiple sets of primers targeting almost all eukaryotic groups [8]. Hamad *et al*. reported a total of numerous fungal species belonging to both Ascomycota and Basidiomycota along with two protozoan species. Meanwhile, same PCR based-sequencing technique using 18S rRNA and ITS genes undertaken by Gouba *et al*. resulted in retrieving seven and eight fungal species in the feces of an obese individual [9] and anorexic individual, respectively.

Moreover, the diversity of the human intestinal fungi have been also assessed using next generation sequencing methods, which permit better characterization of the gut eukaryotes. La Tugaet al. and his colleagues screened intestinal fungi communities from feces in eleven extremely low birth weight infants by using deep sequencing of eukaryote-directed ITS amplicons [13]. Fungal sequences belonging to 18 orders have been retrieved from feces from these infants. ITS amplification has also revealed an abundance of Saccharomycetales and candida groups in the gut of these infants. Similarly, sequencing the Internal Transcribed Spacer region 1 (ITS1) of the rRNA locus, as performed by Hoffmann and his coworker to investigate the diversity of fungi in the gut of 96 healthy individuals who were previously characterized for their bacteria/diet relationships, resulted in the detection of 66 fungal genera and an extra 13 lineages that failed to be classified at the genus level [14].

Unfortunately not the same efforts have been applied to non-human primate as human for evaluation of eukaryotes in their gut. To date, there have been very few studies examining the whole eukaryotic community residing in the intestinal tract of the non-human primate; in fact, the majority of studies focus on the parasitological aspects of these eukaryotic communities using

coprological studies to survey the presence of intestinal parasites in wild non-human primate populations. Despite these studies, the diversity of eukaryotic communities in non-human primates, and particularly in apes, remains to be elucidated, although apes is regarded as the largest extant species of primates and share a close phylogenetic relationship with humans, and resulting in a high potential for pathogen exchange involving bacteria, viruses and gastrointestinal parasites [15-17]. The transmission of these pathogens among primates in the wild may have negative consequences for public health and wildlife conservation management [18].

The objectives of this thesis were to understand the eukaryotes components and diversity in gut of both human and non-human gut using application of culture-independent methods. However, to reach such a goal, we amplified the eukaryotes from feces with various universal primers that targeted major eukaryotic phyla.

Chapter I: Review

Article 1:

Repertory of Eukaryotes (Eukaryome) in the Human Gastrointestinal Tract: Taxonomy and Detection Methods

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Review Article

Repertory of eukaryotes (eukaryome) in the human gastrointestinal tract: taxonomy and detection methods

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SUMMARY

Eukaryotes are an important component of the human gut, and their relationship with the human host varies from parasitic to commensal. Understanding the diversity of human intestinal eukaryotes has important significance for human health. In the past few decades, most of the multitudes of techniques that are involved in the diagnosis of the eukaryotic population in the human intestinal tract were confined to pathological and parasitological aspects that mainly rely on traditionally based methods. However, development of culture-independent molecular techniques comprised of direct DNA extraction from faeces followed by sequencing, offer new opportunities to estimate the occurrence of eukaryotes in the human gut by providing data on the entire eukaryotic community, particularly not-yet-cultured or fastidious organisms. Further broad surveys of the eukaryotic communities in the gut based on high throughput tools such as next generation sequencing might lead to uncovering the real diversity of these ubiquitous organisms in the human intestinal tract and discovering the unrecognized roles of these eukaryotes in modulating the host immune system and inducing changes in host gut physiology and ecosystem.

Keywords *host species, human, parasite, tools and techniques*

INTRODUCTION OF EUKARYOTIC HUMAN GUT

Eukaryotes are an important component of the human intestinal tract (1). In addition to bacteria and viruses, they represent a complex ecosystem, and their occurrence in the gut might have either beneficial or virulent parasitic effects on the human host (2). The compositions and constituents of these communities are influenced by several factors, such as host diet, geography, physiology and disease state (3, 4).

Eukaryotic communities have been extensively studied during the few past decades across different environments and habitats, such as aquatic, terrestrial and host associated environments, using both classical means and PCR amplification, along with high-throughput sequencing of small-subunit ribosomal RNA gene (SSU rDNA) (4–9). These studies have revealed the existence of an unexpected variety of new phylotypes and have defined the major divisions in eukaryote community composition.

Since the last decade, an effort has been made to uncover the diversity of eukaryotic communities across the human digestive tract on a limited basis (7, 10–16). Studies on the eukaryotic community of the gut have focused mainly on the prevalence of both intestinal parasite infections and fungal species in the faecal samples from humans (15–18). Comprehensive analysis of the eukaryotic component of the human gut revealed the presence of more than 15 different protistan genera belonging to diverse groups such as amoebozoans, flagellates amitochondriate protozoa, ciliates, apicomplexans and stramenopiles that are known to parasitize or commensalize the intestinal tract of humans (Table 1). In addition, more than 50 helminthic genera from different groups including cestodes (tapeworms), trematodes (flukes), nematodes (roundworms) parasitize the human gut and cause infection in millions of people worldwide (Table 1). Approximately 3·5 billion people are affected by these parasites and 450 million individuals became ill due to these parasites (19). Socioeconomic status, poor sanitation, lack of access to safe drinking water and food are amongst the most common factors directly associated with luminal

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Table 1 Checklist of eukaryotes detected in the human intestinal tract

Taxa		Species	Microscope	Culture	PCR
Fungi	Ascomycota	*Acremoniumspp*			(180)
Fungi	Ascomycota	*Acremoniumfalciforme*	(181)	(181)	
Fungi	Ascomycota	*Acremonium sp.*			
Fungi	Ascomycota	*Acremoniumstrictum*		(182)	
Fungi	Ascomycota	*Ajellomycescapsulatus*			(12)
Fungi	Ascomycota	*Ajellomycesdermatitidis*			(12)
Fungi	Ascomycota	*Alternariaalternata*			(33)
Fungi	Ascomycota	*Alternaria sp.*			(180)
Fungi	Ascomycota	*Armillaria sp.*			(180)
Fungi	Ascomycota	*Arxiozymatelluris*			(7)
Fungi	Ascomycota	*Aspergilluisniger*		(183)	
Fungi	Ascomycota	*Aspergillusclavatus*			(12)
Fungi	Ascomycota	*Aspergillusflavipes*		(14, 179)	
Fungi	Ascomycota	*Aspergillusflavus*		(184)	
Fungi	Ascomycota	*Aspergillusfumigatus*		(185)	
Fungi	Ascomycota	*Aspergilluspenicillioides*			(156)
Fungi	Ascomycota	*Aspergillusrestrictus*			(33)
Fungi	Ascomycota	*Aspergillusruber*		(179)	
Fungi	Ascomycota	*Aspergillus spp.*		(157)	(180)
Fungi	Ascomycota	*Aspergillussydowii*		(183)	
Fungi	Ascomycota	*Aspergillusversicolor*		(14)	(10, 156)
Fungi	Ascomycota	*Aureobasidiumpullulans*			(13, 156)
Fungi	Ascomycota	*Beauveriabassiana*		(14)	
Fungi	Ascomycota	*Bisporachristiansenii*			(33)
Fungi	Ascomycota	*Blastoschizomycescapitatus*		(16)	
Fungi	Ascomycota	*Botryotiniafuckeliana*			(12, 13)
Fungi	Ascomycota	*Candida albicans*		(15, 16, 186, 187)	(10, 12, 13, 29, 156, 188)
Fungi	Ascomycota	*Candida austromarina*			(13, 156)
Fungi	Ascomycota	*Candida diddensiae*			(29)
Fungi	Ascomycota	*Candida dubliensis*			(13)
Fungi	Ascomycota	*Candida edaphicus*			(11)
Fungi	Ascomycota	*Candida famata*		(15)	
Fungi	Ascomycota	*Candida glabrata*		(15, 16, 189)	(13, 29, 156)
Fungi	Ascomycota	*Candida guilliermondii*		(15, 16)	
Fungi	Ascomycota	*Candida intermedia*			(156)
Fungi	Ascomycota	*Candida kefyr*		(15, 182)	
Fungi	Ascomycota	*Candida krissii*			(156)
Fungi	Ascomycota	*Candida krusei*		(7, 15, 16)	(156)
Fungi	Ascomycota	*Candida lambica*		(16)	
Fungi	Ascomycota	*Candida lusitaniae*		(15, 16)	
Fungi	Ascomycota	*Candida milleri*			(156)
Fungi	Ascomycota	*Candida norvogensis*		(15)	
Fungi	Ascomycota	*Candida parapsilosis*		(16, 187)	(10, 12, 29)
Fungi	Ascomycota	*Candida pararugosa*		(190)	
Fungi	Ascomycota	*Candida quercitrusa*			(29)
Fungi	Ascomycota	*Candida rugosa*		(7)	(7)
Fungi	Ascomycota	*Candida solani*			(156)
Fungi	Ascomycota	*Candida sp.*		(16)	(13, 156, 180, 191)
Fungi	Ascomycota	*Candida sphaerica*		(15)	
Fungi	Ascomycota	*Candida tropicalis*		(14, 16, 187)	(12, 14, 29, 156)
Fungi	Ascomycota	*Candida utilis*		(15)	
Fungi	Ascomycota	*Candida vanari*			(11)
Fungi	Ascomycota	*Candida zeynaloides*		(15)	
Fungi	Ascomycota	*Cephalosporium sp.*			(10)
Fungi	Ascomycota	*Ceratocystis spp.*			(180)

(continued)

Table 1 (*Continued*)

Taxa		Species	Microscope	Culture	PCR
Fungi	Ascomycota	*Chaetomiumglobosum*			(13, 156)
Fungi	Ascomycota	*Chaetomium sp.*			(156)
Fungi	Ascomycota	*Chaetomiumspp.*			(180)
Fungi	Ascomycota	*Cladosporidiumbruhnei*		(179)	(29)
Fungi	Ascomycota	*Cladosporiumcladosporioides*			(13, 29)
Fungi	Ascomycota	*Cladosporiumsphaerospermum*			(29)
Fungi	Ascomycota	*Cladosporium spp.*		(14)	(29, 180)
Fungi	Ascomycota	*Cladosporiumtenuissimum*			(29)
Fungi	Ascomycota	*Claviceps spp.*			(180)
Fungi	Ascomycota	*Clavispsoralusitaniae*		(33)	(33)
Fungi	Ascomycota	*Clavispsora spp.*			(180)
Fungi	Ascomycota	*Coccidioidesimmitis*			(12)
Fungi	Ascomycota	*Coccidioidesposadasii*			(12)
Fungi	Ascomycota	*Cochliobolus spp.*			(180)
Fungi	Ascomycota	*Colletotrichumspp.*			(180)
Fungi	Ascomycota	*Coniosporiumspp.*			(180)
Fungi	Ascomycota	*Davidiella sp.*		(33)	
Fungi	Ascomycota	Davidiellatassiana		(33)	
Fungi	Ascomycota	*Debaryomyceshansenii*		(33)	(33)
Fungi	Ascomycota	*Debaryomyces sp.*			(191)
Fungi	Ascomycota	*Debaryomyces spp.*			(180, 191)
Fungi	Ascomycota	*Dipodascaceae sp.*			(33)
Fungi	Ascomycota	*Doratomycesstemonitis*			(156)
Fungi	Ascomycota	*Engyodontium album*			
Fungi	Ascomycota	*Eurotium spp.*			(180)
Fungi	Ascomycota	*Eutypella spp.*			(180)
Fungi	Ascomycota	*Exophialaequina*			(33)
Fungi	Ascomycota	*Fusariumoxysporum*			(12, 13)
Fungi	Ascomycota	*Fusarium sp.*		(157)	(156, 180)
Fungi	Ascomycota	*Galactomycescandidum*		(16)	
Fungi	Ascomycota	*Galactomycesgeotrichum*		(7, 14)	(7, 10, 13, 14, 156)
Fungi	Ascomycota	*Galactomyces spp.*			(10, 180)
Fungi	Ascomycota	*Geothricum spp.*		(15)	
Fungi	Ascomycota	*Geotrichum candida*		(157)	
Fungi	Ascomycota	*Geotrichumcandidum*		(192)	
Fungi	Ascomycota	*Gloeotiniatemulenta*			(10)
Fungi	Ascomycota	*Glomerella spp.*			(180)
Fungi	Ascomycota	*Hanseniasporaspp.*			(180)
Fungi	Ascomycota	*Hansenulaanomala*		(193)	
Fungi	Ascomycota	*Histoplasmacapsulatum*	(194, 195)		
Fungi	Ascomycota	*Hyphozymavariabilis*			(156)
Fungi	Ascomycota	*Hypocrealixii*		(14)	
Fungi	Ascomycota	*Iodophanuscarneus*			(156)
Fungi	Ascomycota	*Isariafarinosa*		(14)	(156)
Fungi	Ascomycota	*Kluyveromyceshubeiensis*			(7, 188)
Fungi	Ascomycota	*Kluyveromycesmarxianus*		(196)	
Fungi	Ascomycota	*Kluyveromycesspp.*			(180)
Fungi	Ascomycota	*Kluyveromyceswaltii*			(12)
Fungi	Ascomycota	*Lasiodiplodiaspp.*			(180)
Fungi	Ascomycota	*Madurellamycetomatis*			(13)
Fungi	Ascomycota	*Melanconiumsp*			(191)
Fungi	Ascomycota	*Metschnikowiaspp.*			(180)
Fungi	Ascomycota	*Meyerozyma spp.*			(180)
Fungi	Ascomycota	*Microdochium spp.*			(180)
Fungi	Ascomycota	*Millerozymaspp.*			(180)

(continued)

Table 1 (*Continued*)

Taxa		Species	Microscope	Culture	PCR
Fungi	Ascomycota	*Miptoporusspp.*			(180)
Fungi	Ascomycota	*Mycocentrosporaspp.*			(180)
Fungi	Ascomycota	*Mycosphaerellaspp.*			(180)
Fungi	Ascomycota	*Neofusicoccumspp.*			(180)
Fungi	Ascomycota	*Neosartoryapseudofischeri*			(197)
Fungi	Ascomycota	*Ophiocordycepscaloceroides*			(156)
Fungi	Ascomycota	*Paecilomycesfumosoroseus*			(10)
Fungi	Ascomycota	*Paracoccidioidesbrasiliensis*	(194)		
Fungi	Ascomycota	*Paraphaeosphaeriafilamentosa*			(13)
Fungi	Ascomycota	*Penicilliumallii*		(14)	
Fungi	Ascomycota	*Penicilliumbrevicompactum*		(14)	
Fungi	Ascomycota	*Penicilliumcamemberti*		(14)	
Fungi	Ascomycota	*Penicilliumchrysogenum*			(13)
Fungi	Ascomycota	*Penicilliumcitrinum*		(183)	
Fungi	Ascomycota	*Penicilliumdecumbens*		(183)	
Fungi	Ascomycota	*Penicilliumdipodomyicola*		(14)	
Fungi	Ascomycota	*Penicilliumfreii*			(156)
Fungi	Ascomycota	*Penicilliumglabrum*			(13)
Fungi	Ascomycota	*Penicilliumitalicum*			(13)
Fungi	Ascomycota	*Penicilliummarneffei*	(198)	(198)	(12)
Fungi	Ascomycota	*Penicilliumnotatum*		(183)	
Fungi	Ascomycota	*Penicilliumroquefortii*			(10)
Fungi	Ascomycota	*Penicilliumsolitum*		(179)	
Fungi	Ascomycota	*Penicillium sp.*		(157)	(156, 180, 191)
Fungi	Ascomycota	*Penicilliumverruculosum*			(13)
Fungi	Ascomycota	*Penicilliumnsteckii*		(183)	
Fungi	Ascomycota	*Penicillumsacculum*			(13)
Fungi	Ascomycota	*Phaeococcomycesspp.*			(180)
Fungi	Ascomycota	*Pichiaspp.*			(180)
Fungi	Ascomycota	*Pleosporaherbarum*			(13)
Fungi	Ascomycota	*Pleosporales sp.*			(33)
Fungi	Ascomycota	*Raciborskiomyceslongisetosum*			(13)
Fungi	Ascomycota	*Saccharomyces bayanus*			(13)
Fungi	Ascomycota	*Saccharomyces cariocanus*			(13)
Fungi	Ascomycota	*Saccharomyces castellii*			(12)
Fungi	Ascomycota	*Saccharomyces cerevisiae*		(15, 182)	(7, 10–13, 29, 156, 179)
Fungi	Ascomycota	*Saccharomyces paradoxus*			(156)
Fungi	Ascomycota	*Saccharomyces servazzii*			(11)
Fungi	Ascomycota	*Saccharomyces sp.*		(16)	(156, 180, 188, 191)
Fungi	Ascomycota	*Saccharomycetales sp.*			(33)
Fungi	Ascomycota	*Sclerotiniasclerotiorum*			(12, 13)
Fungi	Ascomycota	*Septoriaepambrosiae*			(13)
Fungi	Ascomycota	*Simplicilliumlanosoniveum*			(156)
Fungi	Ascomycota	*Simplicilliumobclavatum*			(156)
Fungi	Ascomycota	*Sirococcusconigenus*			(13)
Fungi	Ascomycota	*Stemphyliumspp.*			(180)
Fungi	Ascomycota	*Sterigmatomyceselviae*			(7)
Fungi	Ascomycota	*Strelitziana spp.*			(180)
Fungi	Ascomycota	*Teratsphaeriaspp.*			(180)
Fungi	Ascomycota	*Torulasporadelbrueckii*		(196)	
Fungi	Ascomycota	*Torulasporapretoriensis*			(7)
Fungi	Ascomycota	*Torulasporaspp.*			(180)
Fungi	Ascomycota	*Toxicocladosporiumspp*			(180)
Fungi	Ascomycota	*Trichophytonverrucosum*			(12)
Fungi	Ascomycota	*UncultivatablePezizomycotina*			(156)

(continued)

Table 1 (*Continued*)

Taxa		Species	Microscope	Culture	PCR
Fungi	Ascomycota	*Uncultured ascomycete*			(13)
Fungi	Ascomycota	*Vanderwaltozymaspp.*			(180)
Fungi	Ascomycota	*Verticilliumleptobactrum*			(10)
Fungi	Ascomycota	*Westerdykellacylindrica*			(180)
Fungi	Ascomycota	*Wickerhamomycesspp.*			(180)
Fungi	Ascomycota	*Xeromycesspp.*			(180)
Fungi	Ascomycota	*Yarrowialipolytica*		(16)	(13)
Fungi	Ascomycota	*Zygosaccharomycesbisporus*		(190)	
Fungi	Ascomycota	*Zygosaccharomycesspp.*			(180)
Fungi	Basidiomycota	*Agaricusspp.*			(180)
Fungi	Basidiomycota	*Asterophoraparasitica*			(7)
Fungi	Basidiomycota	*Asterotremellaalbida*			(156)
Fungi	Basidiomycota	*Auriculariaspp.*			(180)
Fungi	Basidiomycota	*Bjerkanderaadusta*			(7)
Fungi	Basidiomycota	*Bulleracrocea*			(13)
Fungi	Basidiomycota	*Bullera sp.*			(191)
Fungi	Basidiomycota	*Ceratobasidium sp.*			(10)
Fungi	Basidiomycota	*Ceriporiaspp.*			(180)
Fungi	Basidiomycota	*Chondrostereumspp.*			(180)
Fungi	Basidiomycota	*Cinereomycesspp.*			(180)
Fungi	Basidiomycota	*Climacocystis sp.*		(14)	
Fungi	Basidiomycota	*Clitopilusprunulus*			(33)
Fungi	Basidiomycota	*Corticiaceae sp.*			(33)
Fungi	Basidiomycota	*Cryptococcus albidosimilas*			(29)
Fungi	Basidiomycota	*Cryptococcus albidus*		(16)	
Fungi	Basidiomycota	*Cryptococcus carnescens*			(13)
Fungi	Basidiomycota	*Cryptococcus fragicola*			(156)
Fungi	Basidiomycota	*Cryptococcus luteolus*		(16)	
Fungi	Basidiomycota	*Cryptococcus neoformans*			(12)
Fungi	Basidiomycota	*Cryptococcus podzolicus*			(29)
Fungi	Basidiomycota	*Cryptococcus spp.*		(15)	(29, 180, 191)
Fungi	Basidiomycota	*Cystofilobasidiumcapitatum*		(179)	(179)
Fungi	Basidiomycota	*Cystofilobasidiumspp.*			(13, 180)
Fungi	Basidiomycota	*Dacrymyces sp.*			(13)
Fungi	Basidiomycota	*Dothideomycete sp.*			(13)
Fungi	Basidiomycota	*Exidiopsiscalcea*			(13)
Fungi	Basidiomycota	*Exophialadermatitidis*		(199)	
Fungi	Basidiomycota	*Exophialaspp.*			(180)
Fungi	Basidiomycota	*Filobasidiumglobisporum*			(13)
Fungi	Basidiomycota	*Filobasidiumspp.*			(180)
Fungi	Basidiomycota	*Flammulinavelutipes*			(13)
Fungi	Basidiomycota	*Fomesfomentarius*			(33)
Fungi	Basidiomycota	*Fomitopsispinicola*			(33)
Fungi	Basidiomycota	*Graphiolaphoenicis*			(13)
Fungi	Basidiomycota	*Hymenochaetespp.*			(180)
Fungi	Basidiomycota	*Hypholomaspp.*			(180)
Fungi	Basidiomycota	*Lentinusspp.*			(180)
Fungi	Basidiomycota	*Leucosporidium sp.*			(191)
Fungi	Basidiomycota	*Lycogalaflavofuscum*			(188)
Fungi	Basidiomycota	*Malasseziaglobosa*		(14, 179)	(7, 12, 14)
Fungi	Basidiomycota	*Malasseziapachydermatis*		(14, 179)	(7, 14, 156)
Fungi	Basidiomycota	*Malasseziarestricta*		(14, 179)	(7, 14)
Fungi	Basidiomycota	*Malassezia sp.*		(33)	(33)
Fungi	Basidiomycota	*Nigrosporaspp.*			(180)
Fungi	Basidiomycota	*Ophiostomaspp.*			(180)

(continued)

Table 1 (*Continued*)

Taxa		Species	Microscope	Culture	PCR
Fungi	Basidiomycota	*Perenniporiaspp.*			(180)
Fungi	Basidiomycota	*Phanerochaetestereoides*			(7)
Fungi	Basidiomycota	*Phlebiaspp.*			(180)
Fungi	Basidiomycota	*Pityrosporum sp.*		(186)	
Fungi	Basidiomycota	*Psathyrellacandolleana*			(10)
Fungi	Basidiomycota	*Pucciniapoarum*			(33)
Fungi	Basidiomycota	*Rhodosporidiumbabjevae*			(33)
Fungi	Basidiomycota	*Rhodotorulaaurantiaca*			(13)
Fungi	Basidiomycota	*Rhodotorulaglutinis*		(16)	
Fungi	Basidiomycota	*Rhodotorulamucilaginosa*			(13)
Fungi	Basidiomycota	*Rhodotorularubra*		(16, 157)	
Fungi	Basidiomycota	*Rhodotorula sp.*		(16)	(180)
Fungi	Basidiomycota	*Sclerotium sp.*			(13, 179)
Fungi	Basidiomycota	*Skelotocutisspp.*			(180)
Fungi	Basidiomycota	*Sporobolomycesogasawarensis*			(13, 188)
Fungi	Basidiomycota	*Sporolobomycesyunnanensis*			(13)
Fungi	Basidiomycota	*Sterigmatomyceelviae*			(7)
Fungi	Basidiomycota	*Thanatephoruspp*			(180)
Fungi	Basidiomycota	*Trametesversicolor*			(13)
Fungi	Basidiomycota	*Tricholomasaponaceum*			(13)
Fungi	Basidiomycota	*Trichosporonasahii*		(7)	(7)
Fungi	Basidiomycota	*Trichosporonbeigelii*		(157)	
Fungi	Basidiomycota	*Trichosporoncaseorum*			(7)
Fungi	Basidiomycota	*Trichosporoncutaneum*			(7)
Fungi	Basidiomycota	*Trichosporondermatis*			(13)
Fungi	Basidiomycota	*Trichosporonfaecale*			(33)
Fungi	Basidiomycota	*Trichosporon spp.*		(15)	
Fungi	Basidiomycota	*Trichpatumspp.*			(180)
Fungi	Basidiomycota	*Tyromycesspp.*			(180)
Fungi	Basidiomycota	*UncultivatablePucciniomycotina*			(156)
Fungi	Basidiomycota	*Uncultured basidiomycete*			(13)
Fungi	Basidiomycota	*Uncultured ustilaginomycete*			(13)
Fungi	Basidiomycota	*Ustilagomaydis*			(13)
Fungi	Basidiomycota	*Ustilago sp.*			(13)
Fungi	Basidiomycota	*Wallemiamuriae*			(156)
Fungi	Basidiomycota	*Wallemiasebi*			(156)
Fungi	Basidiomycota	*Wallemiaspp.*			(180)
Fungi	Microsporidia	*Encephalitozoonhellem*	(200)		(200)
Fungi	Microsporidia	*Encephalitozoonintestinalis*	(200)		(200)
Fungi	Microsporidia	*Enterocytozoonbieuneusi*	(200)		(200)
Fungi	Microsporidia	*Enterocytozoonhellem*	(200)		(201)
Fungi	Microsporidia	*Microsporidium sp.*			(200)
Fungi	Unclassified	*UncultivatableAgaricomycotina*			(156)
Fungi	Unclassified	*Uncultivatable fungi*			(156)
Fungi	Unclassified	*Uncultured Banisveld*			(188)
Fungi	Unclassified	*Uncultured fungal spp.*			(13)
Fungi	Zygomycota	*Absidia sp.*			(191)
Fungi	Zygomycota	*Basidiobolusranarum*		(202, 203)	
Fungi	Zygomycota	*Mucorracemosus*	(204)	(204)	(188)
Fungi	Zygomycota	*Mucorspp.*			(180)
Fungi	Zygomycota	*Mucor spp.*		(157)	
Fungi	Zygomycota	*Rhizopusmicrosporus*			(156)
Fungi	Zygomycota	*Rhizopus spp.*		(157)	
Acanthocephala	Archiacanthocephala	*Moniliformismoniliformis*	(205)		
Helminths	Cestods	*Bertiellamucronata*	(206)		

(continued)

Table 1 (*Continued*)

Taxa		Species	Microscope	Culture	PCR
Helminths	Cestods	*Bertiella sp.*	(207)		(207)
Helminths	Cestods	*Bertiellastuderi*	(208)		
Helminths	Cestods	*Diphyllobothriumdendriticum*	(54)		(209)
Helminths	Cestods	*Diphyllobothriumlatum*	(210, 211)		(211)
Helminths	Cestods	*Diphyllobothriumnihonkaiense*	(212)		(213)
Helminths	Cestods	*Diphyllobothriumpacificum*	(214)		
Helminths	Cestods	*Diplogonoporusbalaenopterae*	(215)		
Helminths	Cestods	*Diplogonoporusgrandis*	(216)		
Helminths	Cestods	*Dipylidiumcaninum*	(217)		
Helminths	Cestods	*Echinostomailocanum*	(218)		
Helminths	Cestods	*Hymenolepisdiminutaa*	(54)		
Helminths	Cestods	*Hymenolepis nana*	(54, 56, 218)		(56)
Helminths	Cestods	*Inermicapsiferspp*	(219)		
Helminths	Cestods	*Mathevotaeninasymmetrica*	(220)		
Helminths	Cestods	*Mesocestoideslineatus*	(221)		
Helminths	Cestods	*Mesocestoidesspp*	(222)		
Helminths	Cestods	*Mesocestoidesvariabilis*	(222)		
Helminths	Cestods	*Monieziaexpansa*	(223)		
Helminths	Cestods	*Raillietinademerariensis*	(220)		
Helminths	Cestods	*Raillietina sp.*	(219)		
Helminths	Cestods	*Taeniaasiatica*	(224)		(224)
Helminths	Cestods	*Taeniasaginnata*	(224)		(121, 224)
Helminths	Cestods	*Taeniasolium*	(224)		(121, 224)
Helminths	Nematodes	*Anisakispegreffii*			(225)
Helminths	Nematodes	*Anisakis simplex*	(226)		
Helminths	Nematodes	*Ancylostomaceylanicum*	(227)		
Helminths	Nematodes	*Ancylostomacaninum*	(228)		
Helminths	Nematodes	*Ancylostomaduedonale*	(114)	(87)	(114)
Helminths	Nematodes	*Ascarislombricoides*	(54, 56, 229)		(56)
Helminths	Nematodes	*Capillariaphilippinensis*	(230, 231)		
Helminths	Nematodes	*Enterobiusvermicularis*	(54, 218)		
Helminths	Nematodes	*Heteroderasp*	(232)		
Helminths	Nematodes	*Necatoramericanus*	(114)	(87)	(114)
Helminths	Nematodes	*Oesophagostomumbifurcum*	(114)		(114)
Helminths	Nematodes	*Strongyloidesstercoralis*	(89)	(89)	(89, 233)
Helminths	Nematodes	*Ternidensdeminutus*	(234)		
Helminths	Nematodes	*Trichostrongyluscolubriformis*	(235)		(235, 236)
Helminths	Nematodes	*Trichostrongylusorientalis*	(237)		
Helminths	Nematodes	*Trichostrongylussp*	(232)		
Helminths	Nematodes	*Trichuristrichiura*	(54, 229, 238)		(238)
Helminths	Nematodes	*Trichurisvulpis*	(238, 239)		(238)
Helminths	Trematodes	*Acanthoparyphiumtyosenense*	(240)		
Helminths	Trematodes	*Amphimerusspp*	(241)		
Helminths	Trematodes	*Artyfechinostommumalayanum*	(242)		
Helminths	Trematodes	*Centrocestusarmatus*	(243)		
Helminths	Trematodes	*Dicrocoeliumdendriticum*	(244)		
Helminths	Trematodes	*Echinochasmusfujianensis*			
Helminths	Trematodes	*Echinochasmusliliputanus*	(245)		
Helminths	Trematodes	*Echinostomacinetorchis*	(243)		
Helminths	Trematodes	*Echinostomaechinatum*	(245)		
Helminths	Trematodes	*Echinostomahortense*	(243, 246)		
Helminths	Trematodes	*Echinostomailocanum*	(218)		
Helminths	Trematodes	*Echinostomamacrorchis*			
Helminths	Trematodes	*Echinochasmusjaponicus*	(242)		
Helminths	Trematodes	*Echinochasmusperfoliatus*	(245)		

(continued)

Table 1 (*Continued*)

Taxa		Species	Microscope	Culture	PCR
Helminths	Trematodes	*Echinochasmusrecurvatum*	(245)		
Helminths	Trematodes	*Echinostomarevolutum*	(247)		
Helminths	Trematodes	*Euparyphiumsp*	(242)		
Helminths	Trematodes	*Fasciola hepatica*	(54, 56, 58)		(56)
Helminths	Trematodes	*Fasciolopsisbuski*	(248)		
Helminths	Trematodes	*Fibricolaseoulensis*	(243)		
Helminths	Trematodes	*Gastrodiscoideshominis*	(249)		
Helminths	Trematodes	*Gymnophalloidesseoi*	(250)		
Helminths	Trematodes	*Gymnophalloides sp.*	(243)		
Helminths	Trematodes	*Haplorchistaichui*	(251)		(251)
Helminths	Trematodes	*Heterophyesdispar*	(243)		
Helminths	Trematodes	*Heterophyesheterophyes*	(243)		
Helminths	Trematodes	*Heterophyesnocens*	(243)		
Helminths	Trematodes	*Heterophyopsis continua*	(243)		
Helminths	Trematodes	*Himasthlamuehlensi*	(245)		
Helminths	Trematodes	*Hypoderaeumconoideum*	(245)		
Helminths	Trematodes	*Isthmiophoramelis*	(245)		
Helminths	Trematodes	*Metagonimustakahashii*	(243, 246)		
Helminths	Trematodes	*Metagonimusyokogawai*	(243)		
Helminths	Trematodes	*Opisthorchisviverrini*	(252)		(252)
Helminths	Trematodes	*Paragonimus sp.*	(253)		
Helminths	Trematodes	*Phaneropsolusbonnei*	(254)		
Helminths	Trematodes	*Plagiorchismuris*	(246)		
Helminths	Trematodes	*Plagiorchis sp.*	(243)		
Helminths	Trematodes	*Prosthodendriummolenkampi*	(255)		
Helminths	Trematodes	*Pygidiopsis summa*	(243)		
Helminths	Trematodes	*Schistosomahaematobium*	(256)		(256)
Helminths	Trematodes	*Schistosoma japonica*	(257)		(257)
Helminths	Trematodes	*Schistosomamansoni*	(256)		(256)
Helminths	Trematodes	*Schistosomamekongi*	(256)		(256)
Helminths	Trematodes	*Stellantchasmusfaloatus*	(243)		
Helminths	Trematodes	*Stictodorafuscatum*	(243, 258)		
Protozoa	Amoebozoa	*Endolimax nana*	(54, 259)	(57)	(57)
Protozoa	Amoebozoa	*Entamoebabangladeshi*			(260)
Protozoa	Amoebozoa	*Entamoebachattoni*			(261)
Protozoa	Amoebozoa	*Entamoeba coli*	(54, 56, 57, 259)	(57)	(56, 57)
Protozoa	Amoebozoa	*Entamoebadispar*	(54, 259)	(262)	(56, 262)
Protozoa	Amoebozoa	*Entamoebahartmanni*	(54, 259)		(7)
Protozoa	Amoebozoa	*Entamoebahistolytica*	(54, 56)	(262)	(56, 262)
Protozoa	Amoebozoa	*Entamoebamoshkovskii*	(99)		(99)
Protozoa	Amoebozoa	*Entamoebapolecki*	(263)		(261)
Protozoa	Amoebozoa	*Iodamoebabütschlii*	(54, 56, 57, 259)	(57)	(56, 57)
Protozoa	Apicomplexa	*Cryptosporidium andersoni*			(264)
Protozoa	Apicomplexa	*Cryptosporidium bovis*			(265)
Protozoa	Apicomplexa	*Cryptosporidium canis*	(266)		(266)
Protozoa	Apicomplexa	*Cryptosporidium cuniculus*	(267)		(267)
Protozoa	Apicomplexa	*Cryptosporidium fayeri*			(264)
Protozoa	Apicomplexa	*Cryptosporidium felis*	(268)		(268, 269)
Protozoa	Apicomplexa	*Cryptosporidium hominis*	(268, 270)		(268, 270)
Protozoa	Apicomplexa	*Cryptosporidium meleagridis*	(268)		(268)
Protozoa	Apicomplexa	*Cryptosporidium muris*	(271)		(271)
Protozoa	Apicomplexa	*Cryptosporidium parvum*	(268, 270)		(268, 270)
Protozoa	Apicomplexa	*Cryptosporidium suis*			(272)
Protozoa	Apicomplexa	*Cryptosporidium ubiquitum*	(273)		(273)
Protozoa	Apicomplexa	*Cyclosporacayetanensis*	(62)		

(continued)

Table 1 (*Continued*)

Taxa		Species	Microscope	Culture	PCR
Protozoa	Apicomplexa	*Cystoisospora belli*	(63)		(274)
Protozoa	Ciliophora	*Balantidium coli*	(51, 135)		
Protozoa	Ciliophora	*Stentorroeseli*			(33)
Protozoa	Ciliophora	*Vorticella campanula*			
Protozoa	Metamonada	*Enteromonashomini*	(58)		
Protozoa	Metamonada	*Giardia intestinalis*	(54, 259, 275)		(275)
Protozoa	Metamonada	*Giardia lamblia*	(229)		
Protozoa	Metamonada	*Retortamonasintestinalis*	(58)		
Protozoa	Metamonada	*Chilomastixmesnili*	(54)		(275)
Protozoa	Parabasalia	*Dientamoebafragilis*	(57, 58)	(57, 276)	(57, 277)
Protozoa	Parabasalia	*Pentatrichomonashominis*	(57)	(57)	(275)
Protozoa	Parabasalia	*Trichomonashominis*	(56, 58)		(56)
Protozoa	Parabasalia	*Trichomonasintestinalis*	(59)		
Protozoa	Stemenopile	*Blastocystishominis*	(54, 259, 278)	(86, 278)	(11)
Protozoa	Stemenopile	*Blastocystis sp.*	(50)		(7, 14, 50)

parasitic infections (20, 21). Intestinal parasite infection diagnosis based on microscopic examination of faecal samples for the presence of protozoan trophozoites and cysts and helminthes eggs remains the method of choice for every diagnostic parasitological lab worldwide regardless of the poor sensitivity and consistency of these methods (22, 23). However, a combination of microscopy with immunoassay or molecular tools has led to an increase both in sensitivity and specificity over the past two decades.

Recently, a range of DNA-based methods including conventional and multiplex real-time PCR assays and Loop-mediated isothermal amplification (LAMP) assays have been developed for the detection of intestinal parasites that revolutionized the research of diagnosis in field of parasitology (18).

Moreover, fungal communities have been shown to be the most abundant group of eukaryotes that reside in the human gut and play a crucial role in both the health and disease status in humans (1, 24). More than 140 fungal genera belonging to ascomycetous, basidiomycetous and zygomycetous reside as permanent or transient biota in the intestinal tract (24), and many of them do not cause harm, being either beneficial or commensal such as members of *Saccharomyces,* which are considered as probiotics and play a role in curing diarrhoea (25). In contrast, some gastrointestinal fungal species can translocate from the gut into the bloodstream and cause severe fungemia (26).

The intestinal fungi traditionally are studied by culture technique and microscopy-based approaches that rely basically on morphological and physiological criteria (27). Therefore, identification of fungi remains difficult in some cases, and the classification that considers the name of sexual states (telomorphs) over asexual vegetative growth forms (anamorphs) complicates the nomenclature (28).

However, development of culture-independent molecular techniques comprising direct DNA extraction from faeces followed by either PCR clone sequencing or next generation sequencing have been introduced to explore fungal communities in the human gut. These techniques facilitate the detection of fastidious or not-yet-cultured species and open a new avenue for understanding the fungal community in the gastrointestinal tracts of humans (7, 10, 13, 29).

For the purposes of this review, we attempt to present an overview of human gut eukarya (eukaryome), highlighting a summary of their recent taxonomies as well as the methods applied for their detection.

THE TAXONOMY OF HUMAN GUT EUKARYA

Eukaryota is one of the three domains of life (along with Bacteria and Archae) that contains organisms with cells containing a nucleus and other structures (organelles) enclosed within membranes (30). Domain Eukaryota is taxonomically divided according to recent molecular phylogenetic classification (31) into five major groups. (I) The first group, Excavata, is divided into several phyla including Metamonad, Discoba and Malawimonas (31). This group contains a variety of free-living and symbiotic forms and also includes some important parasites of humans (31). Eukaryotic species found in the human gut in this group belong to the phylum Metamonada class Parabasalids including *Dientamoeba fragilis, Pentatrichomonas hominis* and *Trichomonas* spp.

(Table 1) or classe Diplomonadida including *Giardia lamblia*. (II) The RAS group clusters three of the most diverse divisions of eukaryotes, the Alveolates, Rhizaria and Stramenopiles. The alveolates are parasites of the human intestine and are distributed along three major lineages, apicomplexans, ciliates and stramenopiles (Fig. 1, Table 1). The apicomplexans contain many known enteric parasites such as *Cryptosporidium*, *Cyclospora*, *Cystoisospora* and *Sarcocystis* (Fig. 1, Table 1), and they cause severe parasitic infection in humans (32). In contrast, the ciliate species (*Balantidium coli, Stentor roeseli* and *Vorticella campanula*) (Fig. 1, Table 1) have been reported in the human intestinal tract (33, 34). Although the stramenopiles comprise various types of algae, diatoms and other organisms that have morphological features similar to fungi, the genus *Blastocystis* (Fig. 1, Table 1) is considered to be the sole member of stramenopiles that inhabits the human gut and may cause enteritis (35). (III) Amoebozoa represent a major group of amoeboid protozoa, and they divide into two major phyla, Lobosea and Conosa (31). The genera within the phylum Conosa such as *Entamoeba*, *Iodamoeba* and *Endolimax* (Fig. 1, Table 1) inhabit the human intestinal tract and are capable of producing illness in humans (36). (IV) Opisthokonta is a grouping of all uniflagellate eukaryotes (including Metazoans and fungi) (31). Platyhelminths and nematode species belonging to the kingdom metazoa are regarded as common human gut parasitic organisms. The major Platyhelminths (Flatworms) that cause human disease fall into two classes, Cestoda (the tapeworms) and Trematoda (the flukes) (37) (Fig. 2, Table 1). Several genera of cestodes including *Bertiella*, *Diphyllobothrium*, *Echinostoma*, *Hymenolepis*, *Moniezia*, *Mesocestoides*, *Raillietina* and *Taenia* are reported to invade and infect the human gut (37); whereas, the most common human intestinal parasites belong to the *Fasciolopsis*, *Heterophyes*, *Metagonimus*, *Echinostoma*, *Opisthorchis*, *Dicrocoelium*, *Schistosoma* and *Paragonimus* genera (37) (Fig. 2, Table 1). The nematode parasite species of greatest medical importance fall into two major classes: the class Secernentea, such as *Ascaris lumbricoides*, *Ancylostoma duodenale*, *Necator americanus*, *Enterobius vermicularis, Strongyloides stercoralis* and the Enoplea class (*Trichuris trichiura*) (38) (Fig. 2, Table 1).

Fungi are a heterotrophic group of eukaryotic organisms that can occur as yeasts, moulds or as a combination of both forms. Intestinal fungi are distributed along four phyla of fungi: Zygomycota, Basidiomycota, Ascomycota and Microsporidia, which were classified recently as fungi according to several phylogenetic studies (39) (Table 1). In Zygomycota, members of the order Mucorales such as *Rhizopus*, *Mucor* and *Absidia* are reported to be found in the human gastrointestinal tract (40) (Table 1). More than 48 genera of basidiomycetes have been detected in the human digestive tube; whereas, more than 84 genera in the phylum Ascomycota have been reported in the human gut (Table 1). Finally, members of the Microsporidia phylum such as *Brachiola* spp., *Enterocytozoon* spp., *Encephalitozoon* spp., *Microsporidium* spp., *Nosema* spp., *Pleistophora* spp., *Trachipleistophora* spp. and *Vittaforma* spp. (Table 1) have been associated with human intestinal diseases (41). Finally, (V) Archaeplastida which is comprised of the Rhodophyta (red algae), the green algae and the land plants, along with a small group of unicellular freshwater algae (31). Common dietary plants such as *Triticum aestivum*, *Solanum lycopersicum*, *Allium victorialis*, *Solanum tuberosum*, *Citrus aurantium* and *Cicer arietinum* have been widely detected in the human gastrointestinal tract using extended molecular methods (7, 11, 14).

INTERACTIONS BETWEEN GUT EUKARYOTES AND HOST IMMUNE SYSTEM

The human gastrointestinal tract is home to diverse eukaryotes, which survived with the host over millions of years (8, 42). In the past, any protist or helminth species found in the human gut were viewed as a point of concern and assumed to have a negative impact on the host organism (42). In fact, besides some transient eukaryotic species that cause acute infections and stimulate the human immune system to eliminate them (the approved parasites), there are many eukaryotic organisms that colonize the human digestive tube stably and appear to provide health benefits to the host (42, 43).

Extensive research shows that certain enteric parasites have the ability to modulate the host immune system, leading to a gentler immune response and thus provide indirect protection from some immune dysregulatory diseases such as allergy, autoimmunity and colitis (44). Helminth parasitic infections mediated downmodulation of the immune system by inhibiting the host inflammatory responses through effects on both the innate and adaptive immune response (45). The suppression of human immunopathology by helminth involves $CD4^+$ regulatory T cells (Tregs, either $Foxp^{3+}$ or $Foxp^{3-}$), IL-4-responsive cells, TGF-β, IL-10 and Th2 cytokines (46). To date, scant information is available about the relationship between intestinal protists and the host immune system (42). However, the few studies performed so far suggest possible beneficial roles of *Blastocystis*, *Dientamoeba* and other common luminal protists as immune-modulators (42).

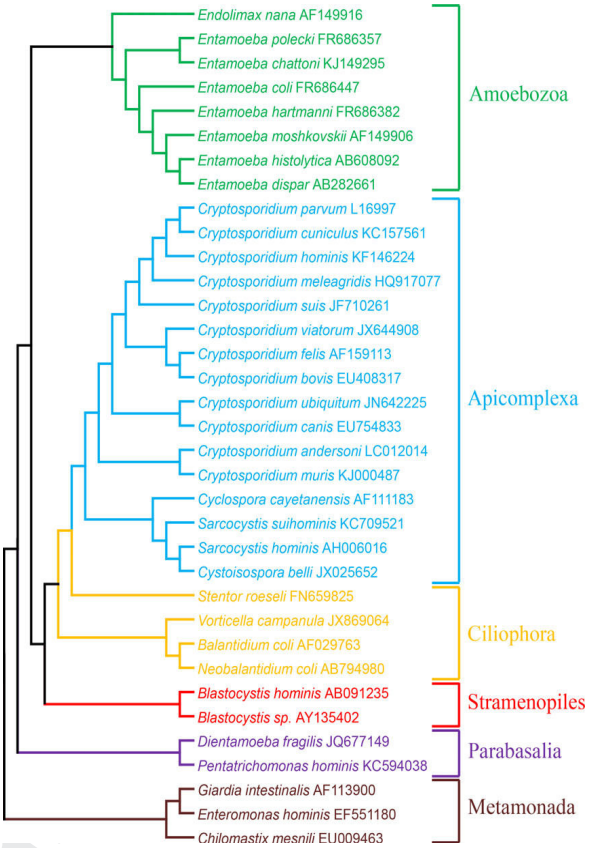

Figure 1 A phylogenetic tree of the protozoan genera detected in the human gut. Available small ribosomal subunit 18S rDNA sequences for protozoa were taken from GenBank. An alignment of eukaryotic 18s RNA sequences was made with Clustal W, and this was used to derive a phylogenetic tree with MEGA6.

THE METHODS FOR DETECTION OF HUMAN GUT EUKARYA

Human intestinal protozoa and helminths

Protozoa are microscopic unicellular eukaryotes found in almost every type of habitat; the majority of protozoa occur as free-living organisms in soil, marine and fresh water environments. In addition, they can exist as a part of the normal microbial flora in a parasitic relationship in the intestinal tracts of other organisms (47). Human intestinal protozoa exhibit a life cycle consisting of a cyst and a trophozoite stage. The cysts generally consist of a resistant wall and are expelled into human faeces where they are then transmitted by the faecal-oral route. Once the appropriate environment is established in the intestinal tract, the cysts transform into a motile, metabolically active form (trophozoite) (48). The intestinal protozoan groups such as Amoeba (*Entamoeba histolytica*), flagellates (*Giardia intestinalis*), Coccidia (*Cryptosporidium parvum*, *Cyclospora cayetanensis* and *Isospora belli*), stemenopile (*Blastocystis* sp.) and ciliates (*B. coli*) are medically important intestinal protozoans in humans (Table 1, Fig. 1), and they account for the majority of human protozoan infections in the intestinal tract (49–51).

Intestinal helminthes are complex, multicellular, eukaryotic organisms, and they represent one of the most prevalent forms of human parasitic diseases worldwide (52). It is estimated that approximately 20 major human helminth infections have been recorded (53). In general, three major groups of parasitic helminths are recognized in the human

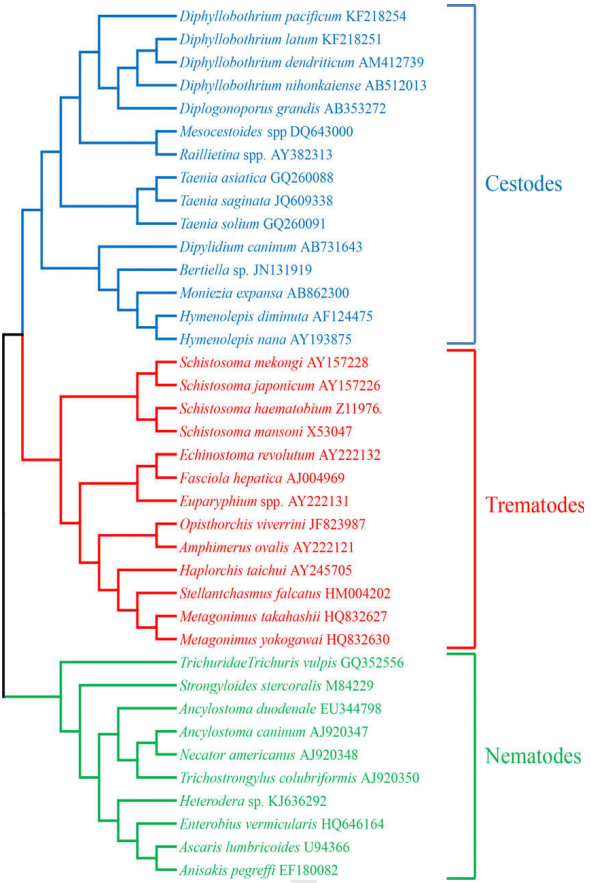

Figure 2 A phylogenetic tree of the helminthes genera detected in the human gut. Available small ribosomal subunit 18S rDNA sequences for helminthes were taken from GenBank. An alignment of eukaryotic 18s RNA sequences was made with Clustal W, and this was used to derive a phylogenetic tree with MEGA6.

intestinal tract, the nematodes (roundworms), cestoda (tapeworms) and the trematoda (flukes) (Table 1, Fig. 2).

Traditionally, intestinal protozoa and helminths have been identified through simple microscopic examination of faeces and serological methods as well as some new methods including antigen detection and nucleic acid based analysis (54–56).

TRADITIONAL APPROACHES FOR DETECTION OF LUMINAL PROTOZOA AND HELMINTHS

Microscopic techniques

For many decades, microscopic examination of faeces has been the principal tool for the detection of eukaryotes, particularly parasites for clinical purposes, despite several drawbacks associated with this technique including time needed for sample preparation and the fact that accurate diagnosis depends entirely on the skill of the laboratory microscopist (22, 23).

The microscopic technique is still used for detecting intestinal parasites in most laboratories. For example, laboratory diagnosis of intestinal amoeba (*E. histolytica Entamoeba dispar*, *Entamoeba coli*, *Entamoeba hartmanni*, *Endolimax nana* and *Iodamoeba bütschlii*) and intestinal flagellates (*G. intestinalis, D. fragilis, Chilomastix mesnili, Enteromonas hominis* and *Retortamonas intestinalis*) is made by detecting the characteristic trophozoites in a wet or permanent stained preparation or by finding their

characteristic cysts in an iodine-stained, formol-ether concentration method (54, 56–59).

Moreover, the microscopic method for identification of intestinal coccidian include finding the characteristic spherical oocysts in a faecal smear or formol-ether concentrated faecal samples using acid-fast stain or auramine-rhodamine stain (60). The oocysts of both *Cryptosporidium* species, *Cyclospora* and *Isospora,* are detected in faeces by employing modified Ziehl–Neelsen (ZN) staining and Sheather's sucrose floatation technique (61–63). Additionally, weber's chromotrope-based stain has been used for diagnosing microsporidial infections caused by *Enterocytozoon bieneusi* in faeces by light microscopy examination (64).

Microscopic identification of eggs in the faeces is the most common method for diagnosing human helminth infections. However, in some cases, macroscopic examination of faecal sample could also give information regarding the type of helminths that are present (65). Several diagnostic methods such as direct wet mount, formol-ether concentration and Kato-Katz techniques are available for detection of human intestinal helminths such as hookworm, *Dicrocoelium dendriticum, S. stercoralis, Schistosoma mansoni*, *Hymenolepis nana*, *Taenia* species and *T. trichiura* .The Kato-Katz technique is considered to be a more sensitive and reliable detection method for the diagnosis of intestinal helminths such as *S. mansoni*, *T. trichiura* and *A. lumbricoides*, rather than the other two aforementioned methods (66). Although the Kato-Katz technique is useful for the quantitative estimation of worm burdens, one major challenge that accompanies this method is the difficulty in handing out diarrhoeal faecal samples (67).

Serological based methods

Microscopic examination of faeces is the main step for diagnosis of luminal protozoan and helminth infections in human in almost all laboratories in the world (22, 23) but in certain circumstances, microscopic technique could not detect intestinal micro-eukaryotes, particularly, when density of these micro-eukaryotes in the sample is quite low. In such cases, other methods must be applied. Fortunately, over past two decades several serology-based diagnoses of intestinal protozoa such as *E. histolytica*, *G. intestinalis* and species of *cryptosporidium* have been developed to detect their corresponding antigens in faecal samples (68–70). Tests commonly used for diagnosis of protozoan parasites in the human gut include the enzyme-linked immunosorbent assay (ELISA), also called enzyme immunoassay (EIA) and the direct fluorescent antibody (DFA) assay, which is based on the reaction of antibodies with the antigenic component of protozoans, leading to formation of antigen-antibody complexes (68–73).

Some advances have been made in the use of the serological tests designed for the diagnosis of intestinal helminths, particularly acute trichinosis, strongyloidiasis and schistosomiasis infections (74–76). Moreover, several coproantigen ELISA assays have been developed during the last three decades for the diagnosis of a range of human luminal helminth infections through the detection of antigens released in human faeces. Coproantigen detection appears to be a valuable and relatively reliable approach for investigation of human taeniasis (*Taenia solium*) (77), strongyloidiasis (*S. stercoralis*) (78, 79) and fascioliasis (*Fasciola hepatica*) (80). However, this type of technique does not involve the collection of blood samples but it is subjected to common drawbacks in which cross-reactivity with other antibodies or antigens from heterologous parasites might occur (81, 82).

Co-proculture technique

Even though the cultivation of intestinal eukaryotes has a long history, not all of them are amenable to growth *in vitro*. There are many difficulties involved in the culturing of eukaryotes due to a numbers of variable factors. For example, some eukaryotes have complex life cycles as well as different morphological stages within their life cycle, and their intermediate or definitive hosts may belong to both cold- and warm-blooded animals (83, 84). Despite these challenges, many studies showed that culture methods are reliable for the detection of protozoan such as *Blastocystis hominis* and *D. fragilis* (Table 1) (85, 86) and intestinal helminths including *A. duodenale, N. americanus* and *S. stercoralis* (Table 1) (87–89). The major drawback of this approach is that the specimen to be cultured needs to be fresh and not refrigerated because refrigeration adversely affects trophozoites of certain types of protozoa (85) and the larva of helminths (90).

MOLECULAR APPROACHES

PCR-based assays

Polymerase chain reaction (PCR) is a selective target amplification assay and is considered as an attractive tool in the diagnosis of luminal parasites that are difficult to differentiate with traditional-based methods (91–93). The sensitivity and specificity of this method depends on several factors such as the DNA extraction protocol, the oligonucleotide primer sets, the size of the amplicon and the involved polymerase used in the reaction (94).

Both conventional and real-time PCR are most commonly used to diagnose a range of intestinal protozoan and helminths such as species of *Cryptosporidium* and *Entamoeba, S. mansoni, S. stercoralis* and *T. solium* (Table 1). Recently, there is an increasing trend toward the use of real-time PCR (RT-PCR) as a diagnostic tool rather than conventional PCR for detecting intestinal parasites in most laboratories (95). Indeed, RT-PCR offers numerous advantages over conventional PCR including higher specificity and sensitivity for the assay and a reduction in the likelihood of contamination. Moreover, RT-PCR has the capability of multiplexing different targets as well as quantification (96).

The first diagnostic PCR-based assay was designed to differentiate *E. histolytica* and *E. dispar* and was dependent on amplification of either the small subunit ribosomal RNA (ssrRNA) gene (97) or the gene encoding peroxiredoxin (98). In addition, several protocols have also been developed for detecting *Entamoeba moskhovskii* (99). A genus-specific PCR assay for species of *Cryptosporidium* is regarded as an appropriated diagnostic method in routine clinical laboratories (18). These assays target different regions of its genome including SSU rRNA, the oocyst wall protein (COWP) or the DnaJ-like protein, which are considered as preferential targets for diagnostic approaches (18).

Although microscopy-based *Giardia* detection is still a routine procedure in some parts of the world, some conventional and RT-PCR analyses that have been used for the detection of *Giardia* in clinical samples were based on amplification of the SSU rRNA, *gdh*, *tpi* and *bg* genes (100). More recently, a PCR-based assay has been developed to detect *Giardia duodenalis* assemblages A and B in human faeces (101). Conventional PCR assays that target SSU rRNA have previously been developed to detect and differentiate between *Blastocystis* isolates from faeces (102). Interestingly, several real-time PCR assays targeting SSU rRNA and an unknown *Blastocystis* gene have also been used for the detection of members of *Blastocystis* in human faecal samples (103, 104).

Many works have been published that employed diagnostic real-time PCR for detection of *Microsporidia* in faecal samples (105–108). A specific probe targeting the small subunit rRNA of genus *Encephalitozoon* has been designed to detect *Encephalitozoon cuniculi*, *Encephalitozoon hellem* and *Encephalitozoon intestinalis* from faecal specimens (105–107). Additionally, a multiplex real-time PCR based on small subunit rRNA amplification has been employed to simultaneously detect *E. cuniculi*, *E. hellem* and *E. intestinalis* from both fresh and formalin-fixed stool samples (108).

PCR techniques have also been applied as diagnostic tools for the detection of a variety of intestinal helminths in developed countries, although these techniques are still costly and not accessible in developing countries (93, 109). PCR-based approaches are recommended, especially in the case of differentiating two morphologically similar and practically indistinguishable species such as *A. duodenale* and *N. americanus* (110). PCR using primers derived from different genetic markers such as nuclear DNA, particularly SSU rRNA, internal transcribed spacers (ITS) and mitochondrial DNA (mtDNA) have generally been used in a wide range of applications for the identification of parasites at the species level (111–113). Several scientific works confirmed the efficacy of both the ITS-1 and ITS-2 genes as genetic markers for the specific identification of nematodes that infect humans (111, 112). A real-time PCR targeting the ITS2 gene of *N. americanus* and *A. duodenale* was developed as a screening tool for simultaneous detection of *N. americanus*, *Ancylostoma* and *Oesophagostomum* in faecal samples (114, 115). Real-time PCR based on ITS1 gene amplification enhanced the ability of this array to detect *A. lumbricoides* in faeces (116). Furthermore, in a TaqMan array, sequences from the SSU rRNA region were successfully employed to design probes for the detection and quantification *T. trichiura* along with 19 other enteropathogens (117). Additionally, several molecular techniques, including real-time PCR and multiplex PCR, have been developed for the detection of a range of human trematode infection. PCRs targeting the SSU rRNA, ITS and mitochondrial DNA genes such as cytochrome c oxidase gene (*cox1*) have been designed in many laboratories for the identification of species of *Schistosoma* (118).

Genus-specific multiplex real-time PCR targeting *cox1* to detect *S. mansoni* and *Schistosoma haematobium* DNA in faeces has been applied in faecal samples collected in northern Senegal (119).

Even though the laboratory diagnosis of cestodes mainly depends on the presence of proglottids in faeces, nested PCR and multiplex real-time PCR approaches are now available for the detection and differentiation of taeniasis. A nested-PCR assay targeting the *Tso31* gene was developed by Mayta *et al.* (120) for the specific diagnosis of *T. solium*. Additionally, a multiplex real-time PCR based on the amplification of *cox1* was developed by Yamasaki and his coworkers (121) to differentiate among *Taenia saginata*, *Taenia asiatica* and *T. solium* in faecal samples.

PCR-restriction fragment length polymorphism (RFLP)

PCR restriction fragment length polymorphism (RFLP) based analysis is a molecular technique for genetic analysis that mainly depends on differences or variations in homol-

ogous DNA sequences. These variations can be exploited for the detection and differentiation of closely related species (122). PCR-RFLP has been widely used for phylogenetic studies, but several reports considered PCR-RFLP as a powerful tool for discrimination of morphologically similar species (123–126).

Nested PCR-RFLP has successfully been applied for the identification and differentiation of morphologically related amoeba such as *E. histolytica*, *E. dispar* and *E. moshkovskii* in faecal samples (123). Furthermore, a nested PCR-RFLP analysis based on amplification of 18S ribosomal-RNA gene has been developed for identifying *Cryptosporidium* isolates including *Cryptosporidium hominis* and *C. parvum* from faeces of HIV-infected patients (124). Moreover, nested PCR-RFLP based on polymorphisms in the triose phosphate isomerase (*tpi*) gene has been employed by Amar *et al.* (125) for the identification of *G. duodenalis* assemblage A (groups I and II) and assemblage B in faecal samples. Similarly, PCR-RFLP analysis targeting the same gene demonstrated the capability of this technique to differentiate between *G. lamblia* assemblages A and B cysts isolated from human faeces (127).

PCR-RFLP has also been used for detecting and distinguishing several cestode species. For example, eggs of *T. solium* were differentiated from *T. saginata* using PCR-RFLP targeting a mitochondrial DNA (mtDNA) fragment (126). A simple PCR-RFLP analysis targeting the rDNA spanning the internal transcribed spacers is proposed by Romstad *et al.* (128) as a useful genetic marker for the identification of nematodes such as *Oesophagostomum bifurcum* and *N. americanus* to the species level (128).

Loop-mediated isothermal amplification (LAMP) Assay

Although PCR-based amplification could provide fast results, several drawbacks accompanied with this method include high cost (129) and insufficient specificity (130). Therefore, an alternate method to PCR is the loop-mediated isothermal amplification (LAMP) method in which a nucleic acid can be amplified with high specificity, sensitivity and rapidity under isothermal conditions (131, 132). This technique has proven recently to be very useful in the diagnosis of certain protozoan and helminthic infections (133, 134). It can be performed at a constant temperature (60–65°C) using a DNA polymerase called *Bst* polymerase (in which has strand displacement activity) and a set of four or six specially designed primers that recognize a total of six distinct sequences of the target DNA (132). LAMP assays based on the amplification of the *E. histolytica* hemolysin gene (HLY6) have been established by Rivera *et al.* (135) as valuable diagnostic tools for the screening of *E. histolytica* in faecal samples, whereas the first LAMP system for the detection of *Cryptosporidium oocysts* in environmental and faecal samples was developed and evaluated by Karanis *et al.* (136) using a set of primers targeting the 60-kDa glycoprotein (gp60) gene of *C. parvum.* Subsequently, Nago *et al.* (137) established a LAMP assay to detect oocysts of *C. parvum* and cysts of *G. lamblia* in clinical faecal samples.

Molecular assays based on loop-mediated isothermal amplification (LAMP) of DNA have also been developed to diagnose several helminthic infections including *S. stercoralis* in faecal specimens. More recently, a LAMP assay based on amplification of the 28S ribosomal subunit gene of *S. stercoralis* has been developed by Watts *et al.* (138) as a method for its diagnosis in faeces. Moreover, primer sets for detection and differentiation between *Taenia* species in faecal samples using LAMP were designed from the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) and cathepsin L-like cysteine peptidase (*clp*) genes (139). Interestingly, LAMP assays showed higher sensitivity in differential detection of *Taenia* species in faecal specimens than by multiplex PCR (140).

Next generation sequencing

Recently, next generation sequencing has been successfully used to identify and screen the bacterial communities in the human gut (141, 142), and this has particularly revolutionized research in the areas of the diagnosis of infectious diseases (143).

In the field of parasitology, pyrosequencing has been used for detecting and genotyping protozoan parasites, trematodes and nematodes (144–147). Pyrosequencing has been used for detecting and genotyping multiple infections of *Toxoplasma gondii* (144) and for the examination of discrete populations of *Haemonchus contortus* from small ruminants and *Plasmodium falciparum* from humans (147).

So far, this technology has not been properly employed to describe the entire eukaryotic communities in human intestinal tracts; however, some work has been conducted to evaluate the efficacy of this method for the identification of *E. moshkovskii*, *E. dispar*, *E. histolytica* and *Blastocystis* compared with other PCR-based methods (145, 146).

Intestinal fungi

Fungi are part of the Eukarya domain and include yeasts and filamentous fungi (148).

Both yeast and filamentous fungi are components of the human gut microbiota, and they play crucial roles in human health status. For example, some species including *Candida*, *Cryptococcus* and *Histoplasma* are usually considered as pathogens or opportunist pathogens under specific conditions such as immunodepression and inflammatory bowel disease (13, 149, 150).

METHODS FOR INTESTINAL FUNGAL DETECTION

Direct microscopy

Direct microscopic examination of clinical specimens is a first-line procedure to detect the presence of fungal elements mainly based on morphological criteria such as the presence of septa, hyphal diameter or ramification and yeast budding colony (151). Microscopy observation gives a presumptive diagnosis of fungi and could guide for selecting appropriate culture media for isolation.

Culture based methods

Nonselective media are generally used for the initial isolation of most fungi from clinical faecal samples and digestive tract biopsy specimens. A range of common media are generally used for the isolation of fungi including Sabouraud, dextrose agar, malt extract agar, potato dextrose agar, CZAPEK, Colombia, glycine-vancomycin-polymyxin B agar (24) and biphasic media (152, 153) supplemented with various broad spectrum antibiotic cocktail including chloramphenicol, gentamicin, streptomycin and cycloheximide to limit bacterial growth (154, 155).

A wide range of fungi such as *Candida albicans*, *Candida* non *albicans* (*Candida tropicalis*, *C. parapsilosis*, *C. rugosa*, *C. guilliermondii, C. lusitaniae*), other yeast than *Candida* (*Geotrichum, Trichosporon*, *Rhodotorula*, *Rhizopus*, *Cryptococcus, Malassezia* species) and filamentous fungi such as *Penicillium* and *Aspergillus*, have been commonly isolated from both healthy and hospitalized patients including immunosuppressed patients (14–16, 156, 157) (Table 1).

Matrix–assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS)

While microscopic identification is usually time-consuming and lacks sensitivity to distinguish between fungal species, matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS), which is a rapid, accurate, easy-to-use and inexpensive method, has been developed and played a powerful role for microorganisms identification in clinical laboratories (158). The general principle of MALDI-TOF-MS identification is based on the acquisition and analysis of protein mass and charge values from spotted isolate using mass spectrometry instrument followed by comparison with available profiles from known microorganisms stored in a database using specific software (158).

In the last decade, MALDI-TOF-MS contributed successfully to the routine identification of bacteria in clinical laboratories (158, 159) whereas less effort has been made using this technique for the identification of fungi (160). Successful identification of fungal species such as *Penicillium* spp., *Scytalidium dimidiatum, Trichophyton rubrum, Microsporidia* spp., *Candida* spp., *Cryptococcus* spp., *Saccharomyces* spp., *Trichosporon* spp., *Geotrichum* spp., *Pichia* spp., *Blastoschizomyces* spp., *Trichoderma* spp., *Rhizopus oryzae*, *Trichoderma reesei*, *Phanerochaete chrysosporium* and *Saccharomyces cerevisiae* has been performed from different specimens by MALDI-TOF-MS (28, 160–166). However, to date, no definitive study has examined this technique for human intestinal fungi except a single study conducted by Hamad *et al.* (7) who isolated and identified five fungal species including *C. albicans, Candida krusei, Galactomyces geotrichum, geotrichum, Trichosporon asahii* and *Geotrichum silvicol* from faecal samples.

Molecular detection of fungi

Molecular methods emerged over the last decade for more rapid and accurate identification of fungi when compared with traditional phenotypic methods. In addition, fungi can be identified directly from faecal specimens prior to the culturing step (12, 167).

The ribosomal operon has been mainly used as a target for fungi detection and identification (168). This operon is conserved in all fungi and contains both conserved and variable regions commonly used to design universal or specific primers for fungi identification, respectively. Fungal ribosomal operons contain the small subunit gene (18S rRNA), the large gene (28S rRNA) (D1-D2) and 5·8S rRNA separated by the internal transcribed spacers ITS1 and ITS2. Several studies described fungal species identification using 18S (169) and 28S genes (170). The conserved regions of the 18S rRNA and 28S rRNA genes are appropriate to analyse fungi at the genus level and above. The ITS and D1/D2 regions have been used for fungal identification at the level of clades and species or below in some studies (171–174). ITS has been described as a convenient universal marker for fungal identification in most mycology laboratories (175).

Species specific primers based on amplification of ITS and 28S rRNA have been designed to detect *C. albicans, C. parapsilosis, C. tropicalis, C. glabrata, C. krusei* and *S. cerevisiae* by real-time PCR in faecal samples from patients suffering from hepatitis B infection (176). Other genes have been suggested, such as the β-tubulin gene, as an alternative for a proper identification among species of *Aspergillus* and their closely related species. However, in some cases, this gene showed an inability to identify fungi at the species level (177, 178).

METAGENOMIC STUDIES OF INTESTINAL FUNGI AND OTHER EUKARYOTES

A-PCR based DNA cloning and sequencing

PCR-based methods played an important role in understanding the diversity of eukaryotes in the human gastrointestinal tract (Table 1, Fig. 3). Several studies using the 18S rRNA gene or ITS based on denaturing gradient gel electrophoresis (DGGE) clone sequencing have been conducted to explore eukaryotes, and particularly fungi, that reside in the guts of both healthy and diseased patients (10–12, 156) (Fig. 3).

Fungal communities have been screened in 57 patients with inflammatory bowel disease and 47 control individuals, a total of different 43 operational taxonomic units (OTUs) (Fig. 3) detected in the clone libraries (13). Scanlan and his coworkers reported a low diversity of fungal community in the guts of 17 healthy individuals (Fig. 3) (10). Similarly, low fungal diversity including a limited number of *Candida* species and *Saccharomyces* have been detected from the gut of Korean populations (11). Chen and his team reported higher fungal diversity in patients with cirrhosis and chronic hepatitis B virus infection when compared with HBV carriers or healthy volunteers (156). *Saccharomyces cerevisiae* and *Kluyveromyces waltii* (Fig. 3) were shown to be the dominant fungi in the gastrointestinal tract of two intestinal transplant patients (12).

Gastrointestinal fungal communities have been screened in a single faecal specimen from a young man from Senegal using multiple sets of primers targeting almost all eukaryotic groups (7). Hamad *et al.* reported a total of 16 fungal species belonging to both Ascomycota and Basidiomycota along with two protozoan species (Fig. 3). Meanwhile, a PCR based-sequencing technique using 18S rRNA and ITS genes undertaken by Gouba *et al.* resulted in retrieving seven and eight fungal species (Fig. 3) in the faeces of an obese individual (14) and anorexic individual (179), respectively.

B-Next generation sequencing methods

Recently, the metagenomic profiles of the intestinal fungal communities have been assessed using next generation sequencing methods, which permit the characterization of the entire mycobiome based on ITS sequencing (29, 180); however, one drawback of this method is the inability to identify fungi at the species level. La Tuga and his colleagues screened intestinal fungi communities from faeces in 11 extremely low birth weight infants using deep sequencing of eukaryote-directed ITS amplicons (29). Fungal sequences belonging to 18 orders have been retrieved from faeces from these infants (Fig. 3). ITS amplification has also revealed an abundance of Saccharomycetales and Candida groups in the gut of these infants. Similarly, sequencing the Internal Transcribed Spacer region 1 (ITS1) of the rRNA locus, as performed by Hoffmann and his coworker to investigate the diversity of fungi in the gut of 96 healthy individuals who were previously characterized for their bacteria/diet relationships, resulted in the detection of 66 fungal genera (Fig. 3) and an extra 13 lineages that failed to be classified at the genus level (180).

CONCLUSIONS

Despite the enormous advances in laboratory diagnosis over the last decades, the eukaryotic diversity in the human gut is still confined to those tools that are generally used for parasite detection. Laboratory diagnosis of eukaryotes, particularly the intestinal parasites, still depends mainly on traditional based methods for the identification of helminthic eggs and protozoan trophozoites and cysts in faeces specimens in many parts of world. More effective approaches, such as nucleic acid-based diagnostics, have been developed recently and could exceed the traditional methods in terms of sensitivity, specificity and reproducibility. More effort is now required to overcome the shortage of the mentioned approaches in this review and to apply new techniques such as next generation sequencing combined with bioinformatics to reveal the real diversity of these eukaryotes in the human gut as well as explaining their functions in both healthy and diseased individuals. On the other hand, more work is needed to assess the possible ways by which the eukaryome can block or modulate the host immunity to alleviate autoimmune and allergic diseases, and perhaps the development of therapy which is now well established for helminths. A positive or a negative role for other eukaryotic gut components belonging to protists and fungi should also be investigated in light of our new understanding of human eukaryome.

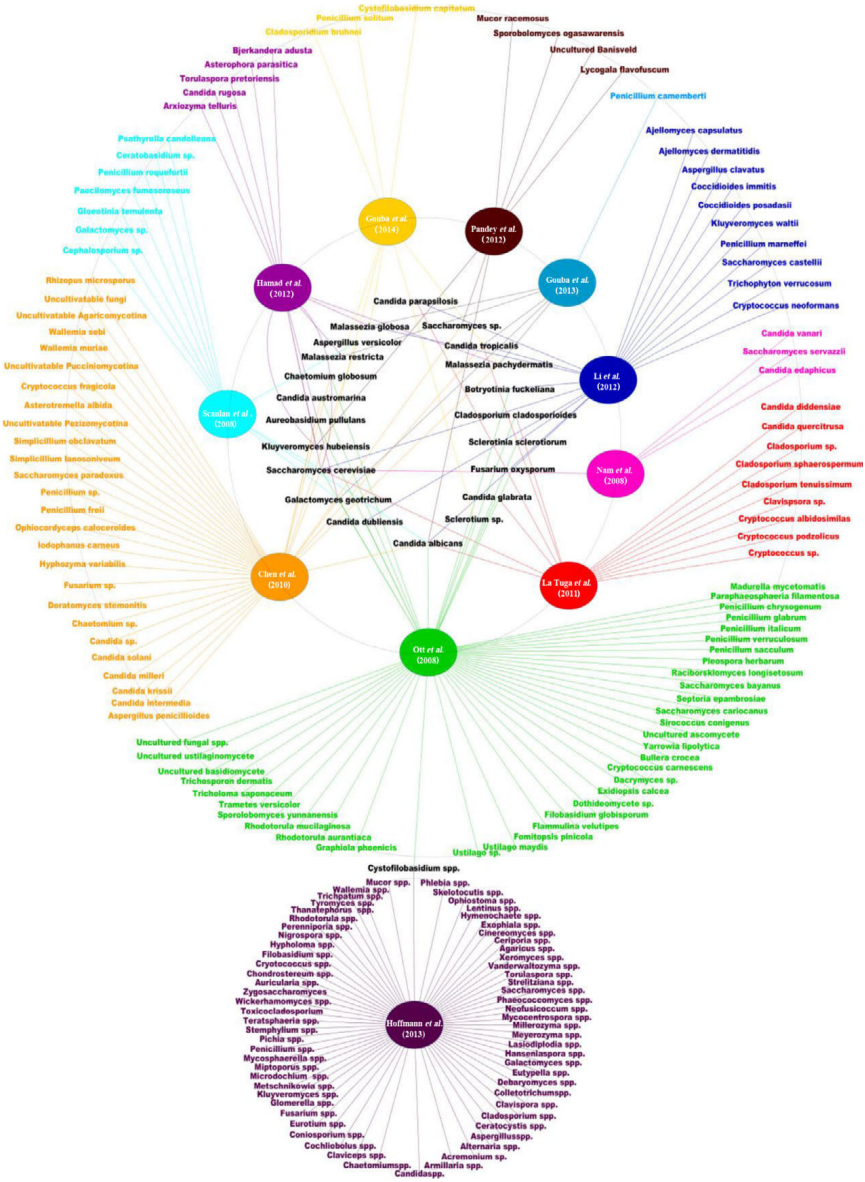

Figure 3 Metagenomic studies of intestinal fungi and other eukaryotes conducted using PCR-based DNA cloning and sequencing and Next generation sequencing methods.

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CONFLICT OF INTERESTS

The authors have no competing interests.

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Article 2:

Looking in Apes as a source of human pathogens

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Looking in apes as a source of human pathogens

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ABSTRACT

Because of the close genetic relatedness between apes and humans, apes are susceptible to many human infectious agents and can serve as carriers of these pathogens. Consequently, they present a serious health hazard to humans. Moreover, many emerging infectious diseases originate in wildlife and continue to threaten human populations, especially vector-borne diseases described in great apes, such as malaria and rickettsiosis. These wild primates may be permanent reservoirs and important sources of human pathogens. In this special issue, we report that apes, including chimpanzees (*Pan troglodytes*), bonobos (*Pan paniscus*), gorillas (*Gorilla gorilla* and *Gorilla beringei*), orangutans (*Pongo pygmaeus* and *Pongo abelii*), gibbons (*Hylobates* spp., *Hoolock* spp. and *Nomascus* spp) and siamangs (*Symphalangus syndactylus syndactylus* and *Symphalangus continentis*), have many bacterial, viral, fungal and parasitic species that are capable of infecting humans. Serious measures should be adopted in tropical forests and sub-tropical areas where habitat overlaps are frequent to survey and prevent infectious diseases from spreading from apes to people.

1. Introduction

It is well known that the majority of emerging infectious diseases are of zoonotic origin and are primarily caused by wildlife- or vector-borne pathogens [1]. The increased incidence of zoonoses highlights the critical need for real-time pathogen monitoring in wildlife animals, especially in at-risk regional "hotspots" where new emerging infectious diseases have been reported [1].

Apes (superfamily Hominoidea) include the lesser apes, known as gibbons and siamangs, that are represented by 4 genera (*Hoolock*, *Hylobates*, *Symphalangus* and *Nomascus*) and the great apes that also contain 4 genera (*Homo* (humans), *Pan* (chimpanzees and bonobos), *Gorilla* (gorillas) and *Pongo* (orangutans)) [2]. Because of their high genomic similarity and close evolutionary relationships to human beings, apes share many diseases with humans [3]. These shared infectious diseases may result from pathogens inherited from a common ancestor [4]. However, cross-species transmission between close relatives is also possible. Many factors can create opportunities for pathogen transmission between apes and humans, including their frequent contact during ecotourism, searching for food, research or simply sharing the same ecosystem (i.e., habitat overlap) [5]. The establishment of new infections in humans depends on both the pathogen and human biology (i.e., the capacity of pathogen to expand its host range and become a human pathogen) [5]. Ape pathogens most likely need very few changes, if any, to infect humans. Thus, the absence of appropriate and timely immune responses in the naive humans leads to the emergence and rapid spread of the infectious diseases [5].

Mathematical models showed that a high proportion of pathogens are shared between close relatives such as humans and apes [4–7]. Moreover, recent research has alerted the scientific communities to the emergence of human infections linked to African great apes [8]. Chimpanzees were found to be the natural reservoir of the pandemic and non-pandemic human immunodeficiency virus type 1 (HIV-1) [9], the causative agent of acquired immune deficiency syndrome (AIDS). Moreover, a new *Mycobacterium tuberculosis* strain was recovered from a wild chimpanzee [10], and gorillas were identified as the origin of the human malaria parasite *Plasmodium falciparum* [11]. *Rickettsia felis*, an emerging vector-borne pathogen that causes rickettsioses, was documented in the feces of many species of apes, including gorillas, chimpanzees and bonobos [12]. It is important to note that for AIDS and malaria, African great apes have much more variety of causative related pathogens than those in humans. This difference in pathogen variety suggests that either some specific ape pathogens have not yet been able to infect and spread widely throughout the human population or that cross-infection/adaptation of these species in human beings yet to occur [8].

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Unfortunately, the published data regarding pathogens of the apes that live in Asia remain quite poor. Large surveillance efforts in these ape populations are required to document the potential for zoonotic diseases from this continent to spread to humans.

Although the transmission of infectious diseases between apes and humans can occur in both directions, this review focuses on the importance of apes as carriers and possible source of infective organisms that have the potential to become human pathogens.

2. Apes as a reservoir and source of human pathogenic bacteria

Although bacterial and rickettsial diseases represent more than half of the emerging infectious diseases worldwide [1], the literature contains few reports regarding the pathogenic bacteria of apes and no report of transmission to human beings. Enteric bacterial agents such as *Salmonella* spp., *Shigella* spp., *Campylobacter* spp. and *Escherichia coli* can be carried by many species of gorillas [13,14]. Feces of infected animals are the most likely primary sources of these bacteria. Nizeyi et al. reported that the prevalence of isolation for *Campylobacter* spp., *Salmonella* spp., and *Shigella* spp. in mountain gorillas (*Gorilla gorilla beringei*) from Uganda is 19%, 13%, and 6%, respectively, without enteric illness in any observed gorillas [13]. *Salmonella* species and *Shigella* spp. (*Shigella sonnei*, *Shigella boydii*, and *Shigella flexneri*) were isolated principally from subadult and adult gorillas [13]. However, the prevalence of these enteropathogens may have been underestimated due to the low sensitivity of the classical methods used for their detection. The molecular survey conducted recently by Whittier et al. on *G. beringei* using real-time PCR confirmed that the prevalence of *Campylobacter* spp. can reach 85% in mountain gorilla populations [15].

Respiratory bacterial agents have also been recovered in wild great apes (chimpanzees and gorillas) [16–18]. Three bacteria including *Pasteurella multocida*, *Streptococcus pneumoniae* and *Klebsiella pneumoniae,* which are also infectious to humans, have been detected in apes that died from pneumonia. Molecular characterization of these strains indicated the presence of pathogenicity factors such as type 4 fimbriae and superoxide dismutases in *P. multocida* and pneumolysin in *S. pneumonia* that could explain their potential virulence relevance [16,17]. However, in most cases, a viral upper respiratory tract infection by metapneumovirus predisposed these great apes to bacterial infections [16–18].

Several emerging bacteria have been characterized and found to be infective in wild great apes; these bacteria include *Bacillus anthracis* [19] and *M. tuberculosis* [10]. First, two outbreaks of anthrax caused by a variant of *B. anthracis* "*B. cereus* var. *anthracis*" killed at least 6 chimpanzees in Côte d'Ivoire and 3 chimpanzees and one gorilla in Cameroon [19]. Moreover, a recently case of a wild chimpanzee infected with *M. tuberculosis* has been documented in Côte d'Ivoire [10]. The phylogenomic analyses demonstrated that this strain belongs to a new lineage of the *M. tuberculosis* complex, but it is more closely related to lineage 6 that has been described in humans and is known as *Mycobacterium africanum* (West Africa 2) [10].

Finally, *R. felis*, a fastidious intracellular pathogen transmitted to human by ectoparasites and the bites of infected mosquitoes, has also been detected in gorilla, chimpanzee and bonobo feces using molecular methods [12]. In the aforementioned study, the feces of 11% of apes living in the wild (a total of 1028 samples tested) were found to be positive for *R. felis* and *R. felis*-like organisms, thus indicating the importance of apes as potential host or reservoir for this emerging rickettsial bacterium in sub-Saharan Africa where its infection is a common public health problem [12].

3. Apes as a reservoir and source of human pathogenic viruses

There have been various studies demonstrating that apes, especially African great apes, constitute a potential reservoir and source of numerous human pathogenic viruses [20] (Fig. 1). Most species of apes, if not all, can carry retroviruses (family *Retroviridae*) including simian immunodeficiency viruses (SIVs), simian T-cell lymphotropic viruses (STLVs) and simian foamy viruses (SFVs). Thus, considerable research has been conducted to understand the prevalence, genetic diversity, geographic distribution and transmission of these viruses in ape populations [21,22]. It is now well established that the human immunodeficiency viruses HIV-1 groups M and N are very closely related to SIVcpzPtt of chimpanzees (*Pan troglodytes troglodytes*) and thus are of chimpanzee origin, while HIV-1 group P is of western gorilla (*Gorilla gorilla gorilla*) origin [22]. Despite numerous interspecies transfers of the retrovirus from apes to human, only HIV-1 group M originated from chimpanzees in Cameroon and subsequently spread worldwide to become responsible for the pandemic form of AIDS in humans [9,21,22]. Currently, no evidence has been found of SIV infections in Asian apes (orangutans and gibbons) [23]. In contrast to SIVs that are present principally in African gorillas and chimpanzees, STLVs are also retroviruses but are widely distributed among African and Asian apes including gorillas, chimpanzees, orangutans and gibbons [23,24]. In the early 2000s, a serological survey followed by molecular confirmation indicated that a wild-caught gorilla and a wild-caught chimpanzee were infected with STLV-1 strains that were closely related to HTLV-1 strains present in human inhabitants of the same region (south Cameroon); this suggests the possible transmission of STLV-1 to humans from African apes [24]. More recently, phylogenetic studies confirmed this conclusion and showed that STLVs cluster according to geographical origin rather than by host species, leading to the hypothesis that many interspecies transmissions have occurred between primates, including those from apes to humans [21,23].

Ebola virus belongs to the *Filoviridae* family and is a highly virulent virus of humans and nonhuman primates that causes severe hemorrhagic fever and death within few days. This virus has been responsible for outbreaks in several countries of Sub-Saharan Africa, such as the Democratic Republic of Congo, Gabon, Sudan, Ivory Coast, Uganda and, most recently, Guinea [25,26]. Although bats are considered the natural hosts of filoviruses, Ebola virus transmission to humans appear to be linked to direct contact with live or dead apes. Hunters that come into contact with the infected gorilla and chimpanzee carcasses are especially at risk of contracting the disease [25]. Recent surveillance of Asian apes in Indonesia for filoviruses showed that 18.4% of healthy orangutans (*Pongo pygmaeus*) are seropositive for the Ebola virus. This high seroprevalence in asymptomatic orangutans suggests that this ape may serve as carrier or host and thus could present a potential risk for humans living in this region of Asia [27].

The hepatitis B virus (family *Hepadnaviridae*) has also been characterized in apes from both Africa and Asia at high frequencies comparable to those obtained from humans in endemic zones [28]. The presence of cross-species transmission and/or recombination between human and ape hepatitis B virus variants [28] and the close genomic similarity of human and ape hepatitis B viruses [29] calls for extensive phylogenetic investigations to understand the diversity, the evolution and the worldwide spread of this virus.

Other pathogenic viruses, including adenoviruses [30] (family *Adenoviridae*), *Lymphocryptovirus* [31] and cytomegaloviruses [32] (family *Herpesviridae*), metapneumoviruses [16-18] (family *Paramyxoviridae*), polyomaviruses [33] (family *Polyomaviridae*) and enteroviruses [34] (family *Picornaviridae*), are not exclusively

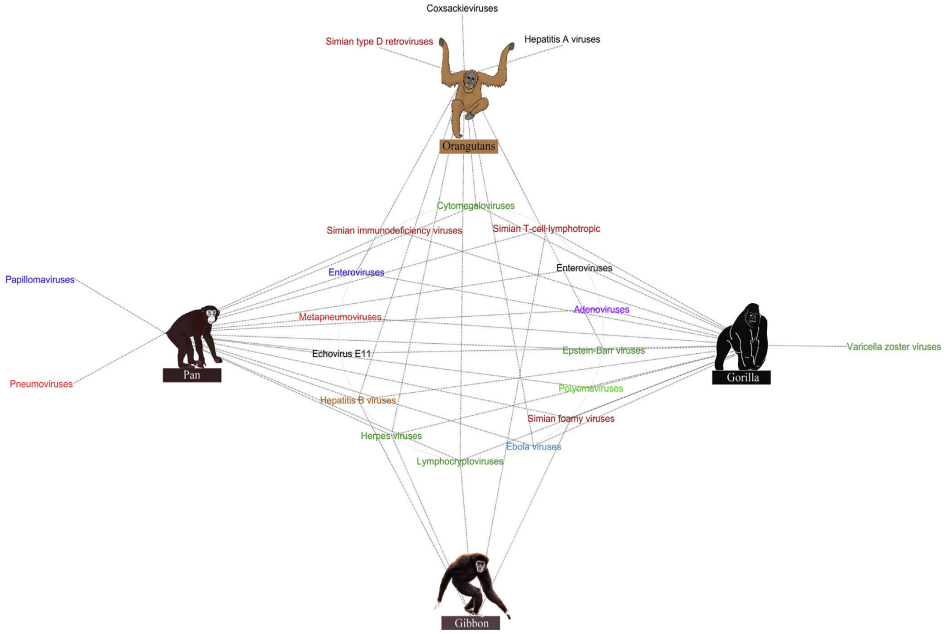

Fig. 1. Apes (chimpanzees, gibbons, gorillas and orangutans) serving as viral reservoirs. Viral genera and species are distributed into the following nine families: *Retroviridae* (dark red), *Herpesviridae* (green), *Hepadnaviridae* (orange), *Adenoviridae* (purple), *Papillomaviridae* (blue), *Filoviridae* (light blue), *Picornaviridae* (black), *Paramyxoviridae* (red) and *Polyomaviridae* (light green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

human-specific and have also been detected in apes. Moreover, based on phylogenetic analyses, some of these viruses variants found in apes are remarkably closely related to human viruses, indicating the zoonotic potential of primate viruses to spread horizontally into the local human populations [30–34].

4. Apes as a reservoir and source of human parasites

Parasitological studies from different free-ranging and captive apes have revealed a multitude of parasite species ranging from single-celled protozoa to multicellular helminths [35] (Fig. 2). The high load of these parasites in the various ape hosts may render these animals potential zoonotic reservoirs that could be responsible for an emerging parasitic zoonosis [20].

Ape parasites can be transmitted to humans through vector-mediated transmission (usually arthropods) via fecal-oral transmission or through direct or prolonged contact with apes [20]. Although little published data has demonstrated the direct transmission of parasites from apes to human, a number of zoonotic diseases have been reported, providing evidence of shared susceptibility of both apes and human to same pathogens [36]. The transmission of these pathogens among primates in the wild may have negative consequences for public health and wildlife conservation management programs [37].

4.1. Apes as reservoir for human parasitic protozoa

Regarding unicellular eukaryotic organisms, apes exhibit several blood/tissue and intestinal protozoa that can cause diseases of concern for human beings (Fig. 2). Members of the blood and tissue apicomplexan parasites of the genus *Plasmodium* have been described in gorillas, chimpanzees and bonobos [11,38–41]. Indeed, very recent data provided definitive evidence that the most virulent human malaria parasite (*P. falciparum*) originated from gorillas [11]. In addition to the occurrence of *Plasmodium* species that cause malaria (*P. falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*) in a wide range of apes, other members of the genus *Plasmodium* have also been reported to cause natural infections in apes (Fig. 2).

Furthermore, kinetoplastid protozoans, such as species of *Trypanosoma*, have been reported in gorillas, chimpanzees and orangutans [42,43], and the first natural infection cases of the hemoflagellate protozoan *Trypanosoma cruzi*, the causative agent of human Chagas disease, have been reported in chimpanzees and gibbons [44,45]. Although the transmission of this parasite requires a blood-sucking triatomine insect, direct transmission could also occur among the infected captive animals and their zookeepers [45].

The free-living amoeba *Balamuthia mandrillaris,* the etiological agent of human granulomatous amoebic encephalitis, has been identified in brain tissue from both gorillas and gibbons [46]. Although *B. mandrillaris* is regarded as an emergent threat to humans, the transmission of this pathogen from apes to human is rarely recorded. However, close contact between humans and infected non-human primates might increase the risk of transmission [46].

The gastrointestinal walls of apes are colonized by parasitic protozoan, and fecal-based studies of wild populations of gorillas, chimpanzees and orangutans revealed the presence of certain intestinal human parasites such as *Entamoeba histolytica* [6], *Giardia*

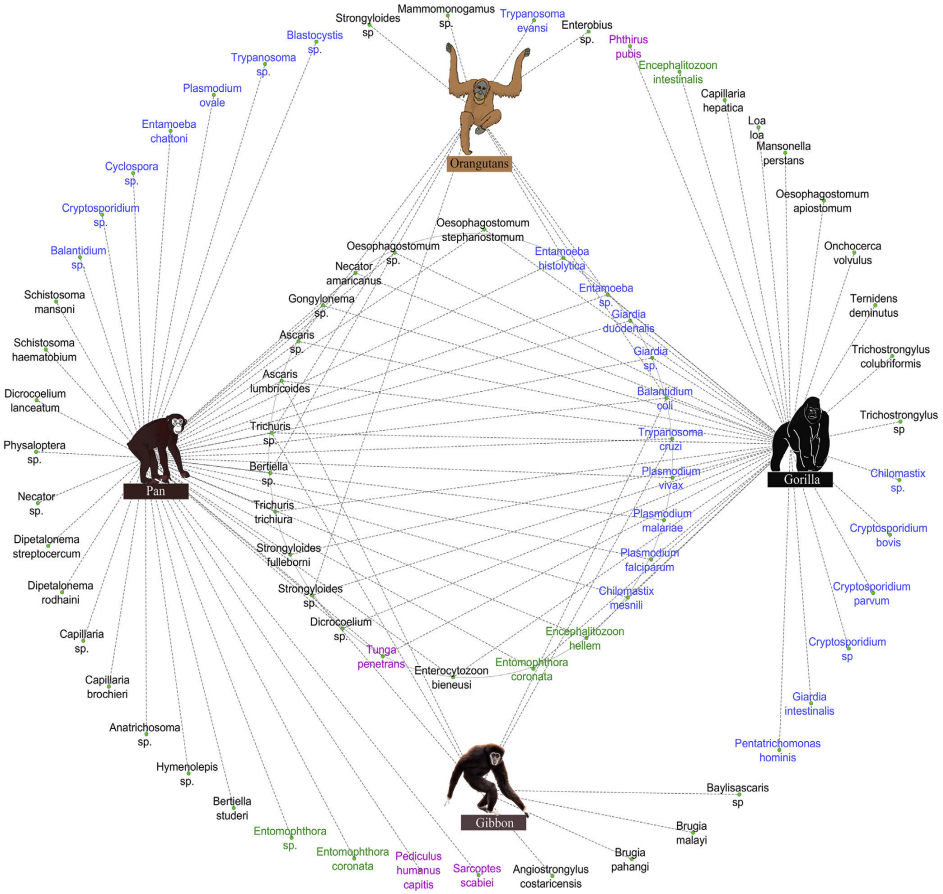

Fig. 2. Parasitic reservoir of apes (chimpanzees, gibbons, gorillas and orangutans). Helminths (black), Protozoa (blue), Fungi (green) and Arthropods (pink). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

duodenalis [6,47] and *Cryptosporidium* species [48] in the guts of these animals. Moreover, common human subtypes of *Blastocystis* sp. that are often associated with chronic infections have also been identified in the digestive tracts of both captive and free-living apes including gorillas, chimpanzees, bonobos, gibbons and orangutans [49]. Furthermore, the ciliated parasite *Balantidium coli* that cause human balantidiasis has also been detected in the stools of a wide range of gorilla, chimpanzee and orangutan populations [6]. The major source of infection with these enteric parasites is through the fecal-oral route [50]. Although there is less opportunity for the feces of wild apes to contaminate human water supplies, transmission is still possible, especially for people who are involved in caring for captive apes [51].

4.2. Apes as a reservoir of human helminths

Coprological studies revealed the presence of a variety of intestinal nematodes among gorilla, chimpanzee and orangutan populations (Fig. 2). For example, the common human enteric round worm *Ascaris lumbricoides*, the whipworm *Trichuris trichiura* and species of the pinworm *Enterobius* have been found in the gastrointestinal tracts of almost all of the great apes [6]. In addition, species of *Oesophagostomum* and *Strongyloides*, nematodes that cause oesophagostomosis and strongyloidiasis, respectively, have also been detected in the guts of African great apes [6,52]. Some reports described human infections with species of *Oesophagostomum* in northern Togo and Ghana, particularly in regions where apes live in close proximity to humans [53]. The digestive tracts of apes such as gorillas and chimpanzees can also be infected with the zoonotic parasite for *Necator americanus*, the hookworm that causes human hookworm. Molecular evidence revealed the possibility of cross-infection with *Necator* hookworms in humans and great apes that co-exist in the same area [54].

In addition to enteric nematodes, the great apes are also susceptible to a variety of filarial nematodes including *Onchocerca volvulus*, a nematode that causes human onchocerciasis in Africa

and has been observed in gorillas, *Dirofilaria immitis*, a nematode that is responsible for severe human heart problems and has been detected in orangutans, and *Loa loa*, the etiological agent of human loaiasis that has also been reported in gorillas and chimpanzees [55].

Little information regarding the diversity and transmission of cestodes and trematodes between humans and apes is available. The only cestode that is considered to be a potential zoonosis from apes is *Hymenolepsis nana*, for which chimpanzees might serve as reservoir host [56]. Regarding the parasitic trematodes, the blood flukes *Schistosoma mansoni* and *Schistosoma haematobium*, the causative agents of schistosomiasis, have been observed in chimpanzees [56,57].

4.3. Apes as reservoir for human pathogenic fungi

Little data exists concerning the occurrence and diversity of fungi in great apes. However, the primary zoonotic fungal diseases of concern for humans belong to group of microsporidia, and intracellular parasitic species belonging to the genera *Enterocytozoon* and *Encephalitozoon* have been reported in multiple species of apes [58], as shown in Fig. 2. The transmission mode of these intracellular parasites remains unclear, but it is thought that close contact between apes and humans increases the risk of transmission of these infectious diseases [58].

4.4. Apes as reservoir for arthropods causing human disease

In addition to their role as vectors of many severe parasitic diseases, arthropods such as fleas, lice, ticks, and mites also serve as intermediate biological hosts for a variety of infectious disease agents (Fig. 2). *Tunga penetrans* is a parasitic flea that cause human tungiasis and has been confirmed to use different primates as reservoirs [55]. *T. penetrans* has been observed infecting the skin of gorillas. The risk of transmission of this arthropod to humans increases in cases of direct contact, particularly in endemic areas [55].

The most prevalent sucking lice species that colonize humans are *Pthirus pubis* and *Pediculus humanus capitis*. These species have also been reported to be associated with apes, and evidence suggests that these parasites may be able to switch between human and ape hosts [55]. Interestingly, apes such as gorillas can also be parasitized by *Pthirus gorilla*, a louse that is phylogenetically a very close relative of the human parasite *P. pubis* and is regarded as a common pubic louse. It is assumed that our human ancestors contracted *P. gorilla* millions years ago by either living in close proximity to gorillas or using their bedding sites [59]. Finally, different species of mites belonging to the genus *Sarcoptes* have been documented to invade a wide range of ape hosts. The itch mite *Sarcoptes scabiei* is the causative agent of human *scabies* and has observed to also infest the skin of gorillas. The possibility of transmission of *S. scabiei* to humans increased when they came into close contact with infested animals [55].

5. Conclusion

The identification of natural or wild sources for pathogens is necessary to avoid the emergence or reemergence of infectious diseases. Apes, our closest relatives, deserve special attention because they constitute serious reservoir of many micro- and macro-pathogens for humans including viruses, bacteria, parasitic helminths and protozoans. Thus, people living close to or in direct contact with apes are at risk for inter-species transmission and infection. Non-invasive methods showed these potential interactions could be involved in many emerging infectious diseases such as HIV and malaria. However, many additional efforts are essential to detect anthropozoonoses from wild apes and to prevent transmission to humans. Future investigation of pathogens in Asian apes is required, and the large-scale monitoring of bacterial and fungal species in ape populations is needed to gain global insights into the emerging zoonotic events from wildlife.

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Chapter II:

The Eukaryotes Gut Microbiota in Human

Article 3:

Molecular Detection of Eukaryotes in a Single Human Stool Sample from Senegal

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In this study, we have compared the standard culture of eukaroytes in one stool sample from a healthy 16-year-old Senegalese man living in Dielmo, to molecular methods by the use of 25 specific and universal primers, cloning and sequencing of more than 950 clones. Our results revealed that using culture-based methods, very few fungi such as *Candida* spp., *Galactomyces* spp., and *Trichosporon asahii*, could be isolated, whereas using culture-independent methods based on various primer sets, a relatively high number of eukaryotic species could be identified in this fecal sample;A total of 27 species from one sample were found among the 977 analyzed clones. The clone libraries were dominated by fungi (716 clones/977, 73.3%), corresponding to 16 different species. In addition, 187 sequences out of 977 (19.2%) corresponded to 9 different species of plants; 59 sequences (6%) belonged to other micro-eukaryotes in the gut, including *Entamoeba hartmanni* and *Blastocystis* sp; and only 15 clones/977 (1.5%) were related to human 18S rRNA sequences. Our results revealed that the eukaryotic diversity estimated by the molecular methods was complex with numerous fungal species detected. Moreover, other eukaryotic species including plants were also found.

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Molecular Detection of Eukaryotes in a Single Human Stool Sample from Senegal

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Abstract

Background: Microbial eukaryotes represent an important component of the human gut microbiome, with different beneficial or harmful roles; some species are commensal or mutualistic, whereas others are opportunistic or parasitic. The diversity of eukaryotes inhabiting humans remains relatively unexplored because of either the low abundance of these organisms in human gut or because they have received limited attention from a whole-community perspective.

Methodology/Principal Finding: In this study, a single fecal sample from a healthy African male was studied using both culture-dependent methods and extended molecular methods targeting the 18S rRNA and ITS sequences. Our results revealed that very few fungi, including Candida spp., Galactomyces spp., and Trichosporon asahii, could be isolated using culture-based methods. In contrast, a relatively a high number of eukaryotic species could be identified in this fecal sample when culture-independent methods based on various primer sets were used. A total of 27 species from one sample were found among the 977 analyzed clones. The clone libraries were dominated by fungi (716 clones/977, 73.3%), corresponding to 16 different species. In addition, 187 sequences out of 977 (19.2%) corresponded to 9 different species of plants; 59 sequences (6%) belonged to other micro-eukaryotes in the gut, including Entamoeba hartmanni and Blastocystis sp; and only 15 clones/977 (1.5%) were related to human 18S rRNA sequences.

Conclusion: Our results revealed a complex eukaryotic community in the volunteer's gut, with fungi being the most abundant species in the stool sample. Larger investigations are needed to assess the generality of these results and to understand their roles in human health and disease.

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Introduction

The human body is home to vast and complex communities of microorganisms. It has been estimated that microbes in human bodies collectively make up approximately 100 trillion cells, ten times the number of human cells [1]. The microbial ecosystem plays important role in human metabolic activities, protection against pathogens, nutrient processing, the stimulation of angiogenesis, and the regulation of host fat storage [2,3].

The human gut is dominated by bacteria, especially species of the phyla Firmicutes and Bacteroidetes. These two phyla are spread throughout the intestinal tract and play crucial roles in human health [4,5]. In addition to bacteria, organism belonging to other domains of life, Archaea and Eukarya, are present in the human intestine [4,6].

Microbial eukaryotes represent an important component of the human gut microbiome, with different beneficial or harmful roles; some species are commensal or mutualistic, whereas others are opportunistic or parasitic [7]. This eukaryotic component of the human gut microbiome remains relatively unexplored because these organisms have a low abundance in human gut or because they received a limited attention from molecular analyses [4,8]. Thus, studying the eukaryotic diversity in the human gut can provide more complete picture of the natural communities inhabiting this niche.

The microbial eukaryote communities in the human gut have been studied primarily using selective culture techniques and microscopy-based approaches [4,9,10]. Identification was based on morphological and physiological traits. However, only a small fraction of the microorganisms present has been detected using this approach because the growth requirements for many of these organisms remain unknown [4]. Recently, molecular-based approaches, such as polymerase chain reaction (PCR) amplification of the small subunit ribosomal RNA, have been established to explore the microbial diversity in the human body [4,11,12]. In 2006, Scupham and his colleagues undertook a culture-independent analysis of fungi in mouse feces, and they identified a wide variety of fungi belonging to the phyla Ascomycota, Basidiomycota, Chytridiomycota and Zygomycota using oligonucleotide fingerprinting of rRNA genes (OFRG) [13].

More recently, in the human distal gut, culture-independent methods have shown that the diversity and abundance of eukaryotes is quite low relative to that of bacteria, and members of the genera *Gloeotinia*/*Paecilomyces* and *Galactomyces* were the most abundant [14] (Table 1). A more diverse fungal community was

observed in the study of Ott et al., 2008, in which they investigated the mucosa-associated fungal microbiota in 47 controls and 57 subjects with inflammatory bowel disease. That study showed that the majority of fungi retrieved from the fecal sample belonged to Ascomycota [15] (Table 1). Only four types of fungi (*Candida vinaria*, *Candida edaphicus*, *Saccharomyces cerevisiae* and *Saccharomyces servazzii*) and one stramenopile (*Blastocystis hominis*) were detected in fecal samples from ten Korean people using the PCR-fingerprinting method [8] (Table 1). Finally, the ileal effluent and feces from 2 intestinal transplant patients were analyzed by Li et al. [16], who reported temporal alterations in the fungal communities in these patients (Table 1). An increase in the size of the fungal community early after intestinal transplantation, followed by a decrease in this community over time, was observed. Moreover, sequence analysis of the 18S ribosomal DNA revealed that *S. cerevisiae* and *Kluyveromyces waltii* were the dominant fungal species in both patients [16].

The aim of this study is to carry out a comprehensive extended molecular analysis of the diversity of eukaryotes in one fecal sample from a young Senegalese man using amplification with various universal primers followed by cloning and sequencing.

Results

Culture-dependent Methods

Using different media, five strains of fungi were isolated from one fecal sample. The results of MALDI-TOF identification were as follows: *Candida albicans*, *Candida krusei*, *Galactomyces geotrichum*, *Trichosporon asahii* and *Geotrichum silvicola* (Table 2). Direct ITS sequencing of these strains confirmed the results of the MALDI-TOF MS analysis for *C. krusei*, *G. geotrichum* and *T. asahii*. *C. albicans* and *G. silvicola* were misidentified using MALDI-TOF MS, and the correct identities of these strains were *Candida rugosa* and *G. geotrichum*, respectively (Table 2). Finally, taking into account the ITS results, only four species of fungi were recovered through culture-based methods.

Culture-independent Methods

An important part of this study was to choose suitable PCR primers for the amplification of eukaryote sequences in the human gastrointestinal tract. One major difficulty was the tendency to amplify background bacterial, plant/animal and human DNA, which are all potentially found in DNA extracted from human feces. Twenty-five different published eukaryotic PCR primer sets were tested on fecal DNA extracts from young, healthy Senegalese man (see Table S1). For all primer sets, with the exception of the primers listed in Table 3, a negative PCR signal was obtained. The negative results with some primers could be explained by either their low sensitivities for the eukaryotic communities in the human gut or the absence of the target microorganisms in the studied sample.

Cloning was performed prior to sequencing only when direct sequencing was problematic or when the obtained sequences were not clean sequences, indicating the amplification of sequences from more than one microorganism. Finally, all PCR products were cloned and then sequenced with the exception of primers E528F/Univ1391 and FunF/FunR, which amplified *Malassezia restricta* and *G. geotrichum*, respectively (Table 3). A total of 977 clones were collected from different clone libraries that were generated using various primers and a fecal sample from a healthy male. All but 9 of the resulting sequences have sequence similarity scores of $\geq$98% when compared with existing sequences in the GenBank database. The remaining 9 sequences have similarity scores of 92% with *Malassezia pachydermatis*, and these 9 sequences may be from putative new species.

Taking the different clone libraries together, one-quarter of the obtained sequences were distributed among Viridiplantae (187 clones, 9 plant species), Stramenopile (38 clones, *Blastocystis* sp.), Amoebozoa (21 clones, *Entamoeba hartmanni*), and human 18S rDNA sequences (15 clones) (Table 3). Three-quarters of the remaining sequences (716 clones) were identified as fungi, with 16 different fungal species belonging to Ascomycota (48.4%) and Basidiomycota (24.9%) (Table 3). Seven fungal species of Ascomycete yeasts were detected: *C. rugosa*, *G. geotrichum*, *S. cerevisiae*, *Arxiozyma telluris*, *Kluyveromyces hubeiensis*, *Torulaspora pretoriensis*, and *Sterigmatomyces elviae* (Table 3). Nine basidiomycetous yeasts were also recovered from the different clone libraries, including *Trichosporon caseorum*, *T. asahii*, *Trichosporon cutaneum*, *M. restricta*, *Malassezia globosa*, *M. pachydermatis*, *Asterophora parasitica*, *Bjerkandera adusta*, and *Phanerochaete steroids* (Table 3).

Discussion

The primary objective of this study was to evaluate the broad diversity of eukaryotes in a single fecal sample from an African male using both culture-dependent and extensive culture-independent methods.

Culture-dependent versus Culture-independent Methods in this Study

Our results revealed that when using culture-dependent methods, very few fungi, including *C. rugosa*, *C. krusei*, *G. geotrichum*, and *T. asahii*, could be isolated. Among these fungi, *C. rugosa* and *C. krusei* have been previously detected by culture-based methods [12,17]. Many studies using culture-dependent approaches have found fungi as the sole eukaryotes in the human microbiome of healthy individuals and immunocompromised patients [12,14,18,19].

The culture-independent methods revealed a more vast diversity of eukaryotes, especially fungi, in our fecal sample compared with the eukaryotes that were obtained by culture-dependent methods. Among the 977 clones that were generated using different primers, 716 clones (73.3%) were belonged to fungi and corresponded to 16 species (Table 3). Among these 16 species, only 3, *C. Rugosa, G. candidum* and *T. asahii*, have been cultured. This discrepancy is due to the fact that some species require both special growth media and special conditions to be cultured. Conversely, *C. krusei*, a fungus isolated by culturing, was not detected by culture-independent methods. This result could be explained by biases in the PCR amplification [20] and/or cloning bias [21]. Interestingly, in Chen's study, molecular methods did not identify *C. krusei* from fecal samples, but this fungus was found by culturing techniques [12].

Culture-independent Methods

Different PCR primers were used to evaluate the diversity of eukaryotes in the fecal sample, and these primers were adopted from previously published studies. Some of these primers were used previously to analyze eukaryotes in the human gut (Table S1), whereas other primers were used previously to analyze eukaryotes from aquatic environments rather than the human gut (Table S1). Among the 18 micro-eukaryotic species found in our study, seven species were detected by two or more primer sets, including *T. Caseorum*, *S. cerevisiae*, *Blastocystis* sp., *G. geotrichum*, *C. rugosa*, *M. restricta*, and *M. globosa* (Table 3 and Figure 1). The remaining 11 species (Table 3 and Figure 1) were amplified using only one primer set.

Table 1. The different eukaryotes previously detected by molecular methods in the human gut using universal 18S rDNA or ITS primers.

	Taxa	Eukaryotic species	References	Eukaryotic species	References
Fungi	Ascomycota	*Acremonium* sp.	[14]	*Iodophanus carneus**	[12]
		Ajellomyces capsulatus†	[16]	*Kluyveromyces waltii*†	[16]
		Ajellomyces dermatitidis†	[16]	*Madurella mycetomatis**	[15]
		Aspergillus clavatus†	[16]	*Ophiocordyceps caloceroides**	[12]
		*Aspergillus penicillioides**	[12]	*Paraphaeosphaeria filamentosa*	[15]
		Aspergillus versicolor†	[12]	*Penicillium chrysogenum**	[14,15]
		*Aureobasidium pullulans**	[12,15]	*Penicillium freii*†	[12]
		Botryotinia fuckeliana†	[15,16]	*Penicillium glabrum**	[15]
		*Candida albicans**	[12,14–16]	*Penicillium italicum**	[15]
		*Candida austromarina**	[12,15]	*Penicillium marneffei*†	[16]
		Candida dubliensis†	[15,16]	*Penicillium* sp.*	[12]
		Candida edaphicus	[8]	*Penicillium verruculosum**	[14]
		Candida glabrata	[15]	*Penicillum sacculum**	[15]
		*Candida intermedia**	[12]	*Pleospora herbarum**	[15]
		Candida krissii†	[12]	*Raciborskiomyces longisetosum*	[15]
		*Candida milleri**	[12]	*Saccharomyces bayanus*	[15]
		Candida parapsilosis†	[16]	*Saccharomyces cariocanus*	[15]
		*Candida solani**	[12]	*Saccharomyces castellii*†	[16]
		Candida sp.	[12]	*Saccharomyces cerevisia**	[8,12,14–16]
		*Candida tropicalis**	[12,16]	*Saccharomyces paradoxus*†	[12]
		*Candida vinaria**	[8]	*Saccharomyces servazzii**	[8]
		Cephalosporium sp.	[14]	*Saccharomyces* sp.†	[12]
		*Chaetomium globosum**	[12,15]	*Sclerotinia sclerotiorum**	[15,16]
		Chaetomium sp.*	[12]	*Sclerotium* sp.†	[15]
		Cladosporium cladosporioides†	[15]	*Septoria epambrosiae*	[15]
		Coccidioides immitis†	[16]	*Simplicillium lanosoniveum**	[12]
		Coccidioides posadasii†	[16]	*Simplicillium obclavatum**	[12]
		*Doratomyces stemonitis**	[12]	*Sirococcus conigenus**	[15]
		Dothideomycete sp.†	[15]	*Trichophyton verrucosum*†	[16]
		*Fusarium oxysporum**	[15,16]	Uncultivatable Pezizomycotina†	[12]
		Fusarium sp.*	[12]	Uncultured ascomycete†	[15]
		*Galactomyces geotrichum**	[12,14–16]	*Verticillium leptobactrum*	[14]
		Gloeotinia temulenta	[14]	*Yarrowia lipolytica**	[15]
		*Hyphozyma variabilis**	[12]		
	Basidiomycota	*Asterotremella albida**	[12]	*Rhodotorula mucilaginosa*†	[15]
		Bullera crocea†	[15]	*Sporolobomyces yunnanensis**	[15]
		Cryptococcus carnescens	[15]	*Trametes versicolor**	[15]
		*Cryptococcus fragicola**	[12]	*Tricholoma saponaceum**	[15]
		Cryptococcus neoformans†	[16]	*Trichosporon dermatis**	[15]
		*Cystofilobasidium capitatum**	[15]	Uncultivatable Agaricomycotina*	[12]
		Dacrymyces sp.*	[15]	Uncultivatable Pucciniomycotina†	[12]
		Exidiopsis calcea†	[15]	Unculture basidiomycete	[15]
		*Filobasidium globisporum**	[15]	Uncultured basidiomycete†	[15]
		*Flammulina velutipes**	[15]	Uncultured ustilaginomycete†	[15]
		*Fomitopsis pinicola**	[15]	*Ustilago maydis*†	[15]
		*Graphiola phoenicis**	[15]	*Ustilago* sp.†	[15]
		Malassezia globosa†	[16]	*Wallemia muriae**	[12]
		Malassezia pachydermatis†	[12]	*Wallemia sebi**	[12]

Table 1. Cont.

	Taxa	Eukaryotic species	References	Eukaryotic species	References
		*Rhodotorula aurantiaca**	[15]		
	Zygomycota	*Rhizopus microsporus**	[12]		
	Amoebozoa	*Entamoeba coli*	[14]		
	Stramenopiles	*Blastocystis* sp.	[8,14]		
Plant	Viridiplantae	*Desmaria mutabilis*	[8]	*Physocarpus opulifolius**	[8]
		Hypseocharis pimpinellifolia†	[8]	*Rubus idaeus**	[8]
		*Parmentiera cereifera**	[8]	*Ziziphus obtusifolia**	[8]
		*Pelargonium alchemilloides**	[8]		

*Eukaryotes detected in both healthy and patient gut.
†Eukaryotes detected only in patient gut.
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Interestingly, the amplification of eukaryotic species using different sets of primers enabled us to obtain a relatively high number of species in the stool sample compared with the number of species obtained in previous molecular studies of this domain [8,12,14–16] (Figure 1). Three species identified in our sample were also found in these previous studies, including *G. geotrichum*, *S. cerevisiae*, and *Blastocystis* sp. (Figure 1). However, discordances were widespread, with many species detected only in our study and many species described previously in the human gut but not found in our work (Figure 1). These disagreements in the results may be due to many reasons. First, in our work, we studied only one fecal sample, but we used multiple universal primer sets; in contrast, in the previous studies many fecal samples were analyzed with one universal primer set [8,12,14–16]. Second, our sample was taken from a young, healthy African man, whereas the samples used in the other studies were obtained from patients with different diseases and conditions (hepatitis B virus infection, inflammatory bowel disease and post-transplantation intestine) and from patients from other geographic areas (Europe and Asia). Finally, another explanation for these discordances could be bias in the PCR and/or cloning.

Fungi are the Dominant Eukaryotes in the Human Gut

The results obtained from the sequencing of different clone libraries that were generated with various primers showed that fungi are the dominant eukaryotes in our fecal sample from Senegal. Approximately 16 fungi species were identified in the stool sample. This result is in agreement with the results of previous studies showing that fungi are widely distributed or abundant in the human gut [8,12,15].

Ascomycete Yeast in the Human Gut

Seven types of Ascomycete yeast were detected in the fecal sample using molecular methods. Among these yeasts, only *C. rugosa* and *G. geotrichum* were detected by culture-dependent methods. These species were identified using both direct ITS sequencing and analysis of the ITS clone library. The presence of these types of fungi was supported when their sequences retrieved from another 18S rRNA clone library using the JVF/DSPR2 primer. *C. rugosa* is considered to be widely distributed and abundant in the human intestine [6].

In addition, our study showed that *G. geotrichum* was widely retrieved from most of the generated clone libraries, accounting for 354 clones/977 (36.2%). This result agrees with the results of a previous study [14], in which *G. geotrichum* was most frequently found in the distal human gut using culture-independent methods. *S. cerevisiae* was also identified in the fecal sample from Senegal in the 18S rRNA clone libraries constructed using the Euk1A/Euk516r primers and the JVF/DSPR primers. This result is also in agreement with the results of previous studies [8,14–16].

To the best of our knowledge, the remaining four Ascomycete yeasts (*A. telluris*, *K. hubeiensis*, *T. pretoriensis*, and *S. elviae*) have not been described previously in the healthy human gastrointestinal tract. Thus, this report is the first these eukaryotic species in a stool sample. All of these species were found in environmental samples, including soil and leaf samples, except for *S. elviae*, which was isolated from two patients with eczematous skin lesions [22].

Basidiomycete Yeast in the Human Gut

Nine species of Basidiomycete yeast were identified from the different clone libraries generated using various primers in this study. Three species of *Trichosporon*, namely, *T. caseorum*, *T. asahii*, and *T. cutaneum*, were retrieved from the Senegalese stool sample

Table 2. Comparison of the cultured fungi identified by both MALDI-TOF MS and direct ITS sequencing.

MALDI-TOF MS	Best score of MALDI-TOF	Direct ITS	Identity %	Coverage %
Candida albicans	2.113	*Candida rugosa*	99	100
Candida krusei	2.189	*Candida krusei*	99	100
Galactomyces geotrichum	2.044	*Galactomyces geotrichum*	99	100
Trichosporon asahii	1.989	*Trichosporon asahii*	99	100
Geotrichum silvicola	2.064	*Galactomyces geotrichum*	99	100

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Table 3. Summary of resulting clone libraries in our study.

Name of primer	Fungal/Micro-eukaryotes species	No. of Clone/Total	Plant/human	No. of Clone/Total
Euk1A/Euk516r	*Trichosporon caseorum*	08/115	*Humulus lupulus*	64/115
	Saccharomyces cerevisiaei	06/115	*Artemisia annua*	23/115
	Blastocystis sp.	01/115	*Triticum aestivum*	01/115
	Trichosporon cutaneum	11/115	*Cupressus gigantea*	01/115
ITS F/ITS-4R	*Trichosporon asahii*	29/144		
	Galactomyces geotrichum	99/144		
	Candida rugosa	16/144		
E528F/Univ1391	*Malassezia restricta*	Direct sequencing		
E528F/Univ1492	*Malassezia globosa*	34/98	*Cupressus gigantea*	03/98
	Malassezia restricta	44/98	*Pinus luchuensis*	02/98
			Human 18s rRNA	15/98
JVF/DSPR2	*Saccharomyces cerevisiae*	17/132	*Humulus lupulus*	87/132
	Galactomyces geotrichum	02/132	*Solanum lycopersicum*	02/132
	Candida rugosa	03/132	*Triticum aestivum*	01/132
	Arxiozyma telluris	01/132	*Schinus molle*	01/132
	Trichosporon caseorum	14/132	*Phoenix canariensis*	01/132
	Torulaspora pretoriensis	01/132		
	Kluyveromyces hubeiensis	01/132		
	Asterophora parasitica	01/132		
NSI/FR1	*Galactomyces geotrichum*	52/96		
	Geotrichum candidum	44/96		
MF/MR	*Malassezia globosa*	79/96		
	Malassezia restricta	04/96		
	Malassezia pachydermatis	09/96		
	Sterigmatomyce elviae	02/96		
	Bjerkandera adusta	01/96		
	Phanerochaete stereoides	01/96		
EK1F/EK-1520	*Galactomyces geotrichum*	59/96		
	Blastocystis sp.	37/96		
121F/1147R	*Entamoeba hartmanni*	21/104	*Bomax ceiba*	1/104
	Galactomyces geotrichum	74/104		
	Trichosporon sp.	8/104		
FunF/FunR	*Galactomyces geotrichum*	Direct sequencing		
EUKA/EUKB	*Galactomyces geotrichum*	24/96		
	Candida rugosa	72/96		
11 primer sets	18 micro-eukaryotic species	775/977	10 species	202/977

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(Table 3). The genus *Trichosporon* is widely found in the environment, but it can occasionally be found in the gastrointestinal microbiota and can colonize human skin and the respiratory tract [23]. The three *Trichosporon* species found in our sample were not detected by previous molecular studies (Figure 1). Only *T. asahii*, which was detected by both culture-dependent and culture-independent methods in our study, has been isolated from a stool sample of 22 month-old boy with acute myeloid leukemia [24].

Malassezia, a fastidious basidiomycetous yeast, was also found in the Senegal stool sample represented by three species *M. restricta*, *M. globosa*, and *M. pachydermatis*. *Malassezia* could be found naturally on human skin but it is also able to cause cutaneous and systematic diseases [25]. Among these three species of *Malassezia*, M. *pachydermatis* and *M. globosa* have been detected previously in stool sample from health volunteers and intestinal transplant patients, respectively [12,16]. Thus, this study is the first report of molecular detection of *M. restricta* in human fecal sample (Figure 1).

The remaining three fungi belonged to *Basidiomycota* including *A. parasitica*, *B. adusta*, and *P. steroids* were not reported previously in human stool sample. Among these environmental species only *B. adusta* was previously isolated from human samples including sputa, bronchial washing and skin [26].

Other Eukaryotes in Human Gut

As well as the 16 fungal species discovered among the clone sequences, two micro-eukaryotic species were also detected (*Entamoeba hartmanni* and *Blastocystis* sp). *E. hartmanni*, which resides in the large intestine of man, is now considered to be a distinct

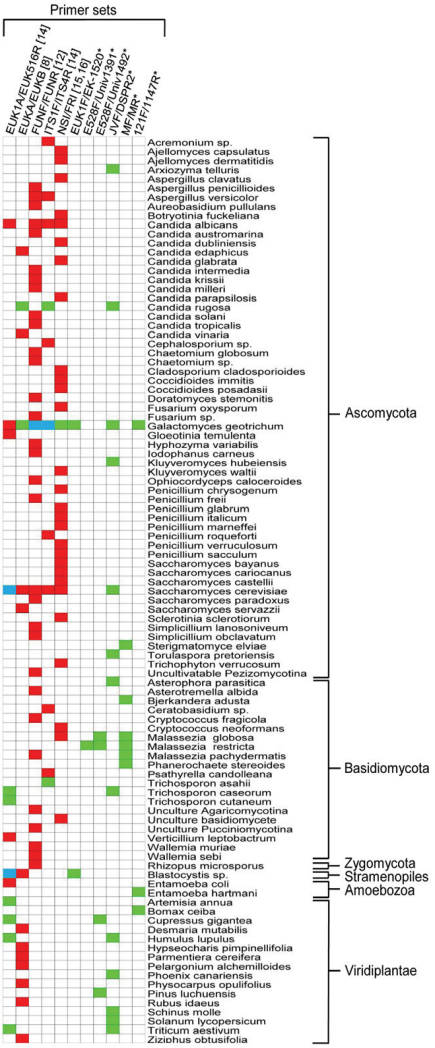

Figure 1. Comparison of our molecular results and those obtained previously for the human stool samples [8,12,14–16] using various universal primers. The universal primers used previously for the analysis of the human gut are indicated by the number of the reference. * indicates primers used for the first time to describe the human eukaryotic microbiota. Green color box = species positive in our sample and negative in the former studies; blue color box = species positive in both our sample and in the former studies; red color box = species negative in our sample and positive in the former studies.
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strain or species that is nonpathogenic and smaller than *E. histolytica* but otherwise indistinguishable from it [27]. *Blastocystis* sp also retrieved from the stool sample from Senegal, and this result concur with the study of Scanlan [14] in which *Blastocystis* was the dominant eukaryote in healthy human distal colon. Thus it may not cause disease. However, this unicellular, obligatory anaerobic protist could be common and prevalent in human with gastrointestinal illness like diarrhea and irritable bowel syndrome [28].

Finally, 9 different species of plants were detected in our sample (19.2% of the sequenced clones), with *H. lupulus* accounting for 15.5% of total sequenced clones (Table 3). The co-amplification of plant sequences with micro-eukaryote sequences has been reported previously in the literature [8] and may be due to the use of nonspecific and universal primers targeting the 18S rRNA gene. Some of these plants are consumed either as part of the diet or as a dietary supplement, including *T. aestivum*, *S. lycopersicum* and *P. canariensis*, and others plants are used as traditional medicines (e.g., *A. annua* as an anti-malarial [29], *H. lupulus* as an anxiolytic calming agent [30], and *B. ceiba* as an antioxidant [31]). The consumption of plants could explain the presence of these sequences in the stool sample.

In conclusion, we studied the eukaryotic diversity in one fecal sample from a healthy African man using extensive molecular methods with different sets of universal primers. Fungi largely dominated the clone libraries. The application of our molecular strategy in larger studies with a greater sample size, including people living in various geographic regions, is currently needed to better evaluate the occurrence/diversity of eukaryotes inhabiting the human gut and to correlate the presence of eukaryotes with human metabolism or disease. Moreover, expanded sequencing analysis using high-throughput pyrosequencing will expand the known diversity of eukaryotes in the healthy gut in the future.

Materials and Methods

Culturing and Identification of Eukaryotes

The fecal sample was obtained from a healthy 16-year-old Senegalese man living in Dielmo (a rural village in the Guinean-Sudanian zone in Senegal). Written assent was obtained from this individual; no written informed consent was needed from his guardians for this study because he was older than 15 years old (in accordance with the previous project approved by the Ministry of Health of Senegal and the assembled village population and as published elsewhere [32]). Both this study and the assent procedure were approved by the National Ethics Committee of Senegal (CNERS) and the Ethics Committee of the Institute Fédératif de Recherche IFR 48, Faculty of Medecine, Marseille, France (agreement number 09-022). The sample was serially diluted, and fivefold dilutions were spread-plated in triplicate on different media, including Sabouraud dextrose agar (BD Diagnostics, Heidelberg, Germany), Columbia culture media (BD Diagnostics, Heidelberg, Germany) and glycine-vancomycin-polymyxin B (GVPC) culture media (Biomérieux, Marcy l' Étoile, France). The plates were incubated aerobically for 48–72 h at 30 and 37°C. Colonies exhibiting different morphologies were restreaked to obtain pure cultures. The fungi were identified using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) (Microflex, Bruker Daltonics). The spectra obtained were compared with the Bruker Taxonomy database. Finally, direct internal transcribed spacer (ITS) analysis was performed for the fungal isolates as described previously to confirm the results of MALDI-TOF MS.

DNA Extraction

DNA was extracted from the frozen sample using a modified method for the Qiagen stool procedure (QIAamp DNA Tissue Kit, Qiagen Inc, Germany) [14]. Aliquots of 200 mg of fecal

sample were placed in 2 ml tubes containing a 200 mg mixture of 0.1, 0.5, and 22 m mm zirconium beads and 1.5 ml of ASL buffer (Qiagen). The sample was bead beaten at 3200 rpm for 90 seconds, followed by heating at 95°C for 10 minutes. The final pellet was suspended in 180 µl of tissue lysis buffer and incubated with proteinase K for 2 hours at 55°C. Then, the manufacturer's recommendations were followed for the purification and elution of the DNA.

Primer Selection

Twenty-two different published universal eukaryotic or fungal-specific PCR primer sets targeting the 18S rDNA and ITS sequences were used, as shown in Table S1. In addition, three specific primers for *Malassezia*, Rhodophyta, and Chlorophyta targeting the 28S rDNA, RUBISCO, and rps11-rp12 sequences, respectively, were also used (Table S1).

Genomic Amplification

Amplifications of sections of approximately 250–1,700 bp were carried out with the primers listed in Table S1. The PCR reaction mixture (final volume, 50 µl) contained 5 µl of dNTPs (2 mM of each nucleotide), 5 µl of 10x DNA polymerase buffer (QIAGEN, Courtaboeuf, France), 1 µl of MgCl2 (25 mM), 0.25 µl of HotStarTaq DNA polymerase (5 U) (QIAGEN, Courtaboeuf, France), 1 µl of each primer (Table S1) (10 pmol/µl), and 5 µl of extracted DNA. PCR was performed with a preliminary step at 95°C for 15 minutes; 40 cycles of 95°C for 45 seconds, annealing at the appropriate temperature for the primers used (see Table S1) for 30 seconds, and 72°C for 1 to 2 minutes; and a final extension step at 72°C for 5 minutes. The PCR products were analyzed using agarose gel electrophoresis and visualized by ethidium bromide staining. Then, the positive PCR products were purified using the NucleoFast® 96 PCR Kit (MACHEREY-NAGEL, Hoerdt, France) according to the manufacturer's instructions.

Cloning Procedures and Insert Amplification

Cloning of the purified PCR products was performed using the pGEM® -T Easy Vector System 2 Kit (Promega, Madison, USA) as recommended by the manufacturer. All white colonies were collected and then analyzed by PCR M13 as described previously [33].

Sequencing and Informative Data Analysis

Purified PCR-M13 inserts were sequenced in both directions using the Big Dye® Terminator V1,1 Cycle Sequencing Kit (Applied Biosystems, Courtaboeuf, France). The primers used for sequencing were M13d and M13r. The sequencing products were then run on an ABI PRISM 3130 automated sequencer (Applied Biosystems, Foster City, CA, USA). Finally, the eukaryotes were identified by comparing the obtained sequences with existing sequences in the GenBank database using the BLAST program available at the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov/, BLAST).

Nucleotide Sequence Accession Numbers

All sequences obtained in this work have been deposited in GenBank database with the accession numbers JX131688 to JX132666.

Supporting Information

Table S1 Primers used in this study.
(DOCX)

Author Contributions

Conceived and designed the experiments: DR FB. Performed the experiments: IH. Analyzed the data: IH CS DR FB. Contributed reagents/materials/analysis tools: IH CS. Wrote the paper: IH DR FB.

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Table S1: Primers used in this study.

Phyla	Primer	Sequence	Targeted Sequence	Product size	Ta	Reference	Primer used
Microsporidia	V1	CACCAGGTTGATTCTGCCTGAC	18S rRNA	250-279	55	[1]	HG
	PMP2	CCTCTCCGGAACCAAACCCTG					HG
Entamoeba	JVF	GTTGATCCTGCCAGTATTATATG	18S rRNA	600 to 650	57	[2]	HG
	DSPR2	CACTATTGGAGCTGGAATTAC					HG
Eukarya	Euk1A	CTGGTTGATCCTGCCAG	18s rRNA	570-600	55	[3]	HG
	Euk 516r-	ACCAGACTTGCCCTCC				[4]	HG
Eukarya	FUNF	GATCCCTAGTCGGCATAGTT	18s rRNA	1000	52	[5]	HG
	FUNR	GTAGTCATATGCTTGTCTC					HG
Eukarya	EUKA	AACCTGGTTGATCCTGCCAGT	18s rRNA	1800	55	[6]	HG
	EUKB	TGATCCTTCTGCAGGTTCACCTAC					HG
Eukarya	NSI	GTAGTCATATGCTTGTCTC	18s rRNA	1650	48	[7]	HG
	FR1	AICCATTCAATCGGTAIT				[8]	HG
Fungi	ITS F	CTTGGTCATTTAGAGGAAGTAA	ITS	580	50	[7]	HG
	ITS-4R	TCCTCCGCTTATTGATATGC				[9]	HG
Dinoflagellates	18ScomF1	GCTTGTCTCAAAGATTAAGCCATGC	18S rRNA	650	58	[10]	E
	Dino18SR1	GAGCCAGATRCDCACCCA					E
Trichomonads	TFR1	TGCTTCAGTTCAGCGGGTCTTCC	5,8S rRNA	338-391	60	[11]	E
	TFR2	CGGTAGGTGAACCTGCCGTTGG					E
Diplomonads	DimA	AACCTGGTTGATCTTGCCAG	18S rRNA	-	55	[12]	E
	DimB	CYGCAGGTTCACCTACGGAA					E
Kinetoplastidia	Kineto_kin1	GCGTTCAAAGATTGGGCAAT	18S rRNA	600-650	55	[13]	E
	Kineto_kin2	CGCCCGAAAGTTCACC					E
Amoeba	Ami6F1	CCAGCTCCAATAGCGTATATT	18S rRNA	830	55	[14]	E

	Ami9R	GTTGAGTCGAATTAAGCCGC					E
Acanthamoeba	JDP1	GGCCCAGATCGTTTACCGTGAA	18S rRNA	460-470	60	[15]	E
	JDP2	TCTCACAAGCTGCTAGGGAGTCA					E
Naegleria	F	GAACCTGCGTAGGGATCATTT	ITS	388 -376	55	[16]	E
	R	TTTCTTTTCCTCCCCTTATTA					E
Hartmannella	Hv1227F	TTACGAGGTCAGGACACTGT	18S rRNA	501	56	[17]	E
	Hv1728R	GACCATCCGGAGTTCTCG					E
Ciliophora	121 F	CTGCGAATGGCTCATTAMAA	18S rRNA	750	55	[18]	E
	1147R	GACGGTATCTRATCGTCTTT					E
Diatoms	18SF	GTTTCCGTAGGTGAACCTGC	18S rRNA	700-900	60	[19]	E
	28SR	GCTTATTAATATGCTTAAATTCAGCG					E
Rhodophyta	RUBI_F	CGCTGCTAAAACTTGTGGGC	RUBISCO	500	56	[20]	E
	RUBI_R	GGCGTTGTAATAAGAATCCTGG					E
Chlorophyta	UCP1_F	CAAGCWCCDGCAGAAGACC	rps11-rpl2	384	54	[21]	E
	UCP1_R	CCMAAACATAAACAAMSWCAGG					E
Euglenophyta	EAF	GTCATATGCTTYKTTCAAGGRCTAAGCC	18S rRNA	-	55	[22]	E
	EAF3	TCGACAATCTGGTTGATCCTGCCAG					E
Eukarya	E528F	CGGTAATTCCAGCTCC	18s rRNA	1000-1300	55	[23]	E
	Univ1391RE	ACCTTGTTACGRCTT				[24]	E
Eukarya	E528F	CGGTAATTCCAGCTCC	18s rRNA	1000-1300	55	[23]	E
	Univ1492RE	GGGCGGTGTGTACAARGRG				[24]	E
Eukarya	EK1F	CTGGTTGATCCTGCCAG	18s rRNA	1520	55	[25]	E
	EK-1520	CYGCAGGTTCACCTAC					E
Eukarya	EK-82F	GAAACTGCGAATGGCTC	18s rRNA	1432	55	[25]	E
	EK-1520	CYGCAGGTTCACCTAC					E
Malassezia	MF	TAACAAGGATTCCCCTAGTA	28s rRNA	580	55	[26]	E
	MR	ATTACGCCAGCATCCTAAG					E

Ta = annealing temperature

Hg = Human gut

E = Environments

RUBISCO =Ribulose-1, 5-bisphosphate carboxylase oxygenase

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Article 4:

The Gut Microbiota of a patient with Resistant Tuberculosis is more Comprehensively Studied by Culturomics than by Metagenomics

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Although the main objective of this study was to describe the composition of prokaryotic gut in patient with resistant tuberculosis, a secondary objective was also to evaluate the occurrence of eukaryotes in intestinal tract of a 63-year-old female patient with MDR tuberculosis. Culture-independent analysis for investigation the diversity of the eukaryotes in human gut was undertaken by using 36 universal primers targeting eukaryotic small ribosomal region then followed by cloning and sequencing. Our results showed only three fungi (*Candida dubliniensis, Candida albicans*, and *Galactomyces geotrichum*) in this fecal sample.

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ARTICLE

The gut microbiota of a patient with resistant tuberculosis is more comprehensively studied by culturomics than by metagenomics

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Abstract Gut microbiota consists of 10^{10} bacteria per gram of stool. Many antibiotic regimens induce a reduction in both the diversity and the abundance of the gut flora. We analyzed one stool sample collected from a patient treated for drug-resistant *Mycobacterium tuberculosis* and who ultimately died from pneumonia due to a *Streptococcus pneumoniae* 10 months later. We performed microscopic observation, used 70 culture conditions (microbial culturomics) with identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) and 16S rRNA amplification and sequencing, pyrosequencing, and 18S rRNA amplification and clone sequencing. Electron and optical microscopic observations revealed the presence of yeast, but no bacterial species were observed. By culture, only 39 bacterial species were identified, including one new species, as well as three species that have not been previously observed in the human gut. The pyrosequencing showed only 18 phylotypes, detecting a lower number of bacterial species than the culture techniques. Only two phylotypes overlapped with culturomics. In contrast, an amount of chloroplasts was found. Additionally, specific molecular eukaryote detection found three fungal species. We recovered, for the first time, more cultivable than non-cultivable bacterial species in a patient with a low bacterial load in the gut, demonstrating the depth bias of pyrosequencing. We propose that the desertification of gut microbiota in this patient is a reflection of the total body microbiota and may have contributed to the invasive infection of *S. pneumoniae*. This finding suggests that caution should be applied when treating patients with broad-spectrum antibiotics, and preventive measures should be taken in order to avoid invasive infection.

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Introduction

The composition of the gut microbiota is determined by physiological factors, such as age or geographic provenance [1, 2]. Among the external factors that influence the gut microbiota, it is well known that antibiotics, notably, vancomycin [3] and ciprofloxacin [4], tend to decrease the bacterial load in the digestive tract [5, 6]. The modifications of the gut microbiota depend on the antibiotic used and the route of administration, as well as the duration of treatment. Although the change in flora is reversible, the recovery time is variable, depending on the antibiotic regimen [6–8].

Since the 1970s, culture-based methods showed a partial overview of the gut microbiota composition and the influence that antibiotics exert on the gut flora [2, 7, 9–11]. Metagenomics and pyrosequencing have permitted a dramatic increase in knowledge, suggesting that most of the bacteria in the human gut are unable to be cultured in vitro [12, 13]. However, using only metagenomics tools induces several biases and sometimes contradictory results, suggesting that data should be carefully interpreted and there should be complementarity between pyrosequencing and exhaustive and fastidious culture-dependent methods that we have previously termed microbial culturomics [14]. This comprehensive approach had previously allowed, from three stools, to culture 341 different bacterial species, including 31 new species, with

an only 15 % overlapping with the bacteria detected by pyrosequencing performed concomitantly [14]. In addition, each genome of new bacteria cultured has been sequenced, allowing to describe approximately 10,000 ORFans [14–21]

The majority of studies examining eukaryote composition have been performed in healthy volunteers. Culture studies have found *Candida* spp. to be the most prevalent species, and metagenomic studies have found that fungi (*Candida* spp., *Galactomyces*, *Saccharomyces* spp., *Malassezia pachydermatis*) and *Blastocystis* were the most dominant protists in the gut.

Multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* are resistant to isoniazid and rifampicin, and extensively drug-resistant (XDR) mycobacteria strains are resistant to rifampicin, isoniazid, any fluoroquinolone, and one of three injectable drugs [22]. These strains are responsive to the use of broad-scale antibiotic treatment. To the best of our knowledge, no study has reported on the gut microbiota composition during XDR tuberculosis infection. In this study, we used a complementary approach with microbial culturomics, pyrosequencing, 18S rRNA amplification, and clone sequencing to determine the gut composition of a patient treated with a broad-spectrum antibiotic regimen for 4 months. The patient ultimately died of pneumonia due to *Streptococcus pneumoniae*.

Materials and methods

Stool samples

The patient was a 63-year-old female with MDR tuberculosis. Her medical history included two successive combined antibiotic treatments before her hospitalization, both of which were unsuccessful. The patient's initial weight was 60 kg, and she had lost 21 kg since the infection began. She received treatment with ethambutol, rifampicin, isoniazid, and pyrazinamide for 3 months. This antibiotic regimen was subsequently stopped, and she was treated with kanamycin for 1 month. The patient was also treated with levofloxacin for 3 weeks upon her arrival in France. At the time of stool collection, her antibiotic regimen included TMC207 (1 week), adiazine (1 month), ethambutol (4 months), linezolid (4 months), D-cycloserine (4 months), and para-aminosalicylic acid (4 months); she had also previously received trimethoprim–sulfamethoxazole (2 weeks) rifampicin (2 months), amikacin (2 months), amoxicillin–clavulanate (3 weeks), and meropenem (3 weeks). The main route of antibiotics administration was orally, with a probable high gut concentration of antibiotics, with, consequently, a direct and large gut composition disruption.

The patient did not present with any major gastrointestinal disorders, such as diarrhea or abdominal impairment, but had lost an additional 5 kg since the introduction of the new antibiotic regimen. Her progress was favorable, and she was allowed to return home. The patient was hospitalized 4 months later for fever and dyspnea, and she finally died from pneumonitis and bacteremia, probably due to *Streptococcus pneumoniae* infection documented by a positive urinary antigen test [23] and confirmed by a positive specific real-time polymerase chain reaction (PCR) assay [24] analysis on her bronchoaspiration. The patient's antibiotic regimen and weight fluctuations were collected (Fig. 1).

We collected 9 g of stool that we separated into 1-g aliquots and stored at −80 °C immediately after the collection of the sample. We obtained the patient's consent and the agreement from the local ethics committee of the IFR48 (Agreement 09-022, Marseille, France).

Microbial culturomics

Culture

Each gram of stool was diluted in 9 ml DPBS and inoculated in serial dilutions from 1/10 up to $1/10^{10}$ with different culture mediums in variable conditions. To isolate bacteria for a preliminary study of the human intestinal microbiota, we inoculated stool samples into 70 preselected culture conditions, which provided a large diversity of isolated bacteria. These culture conditions are based on a multiplication of physicochemical conditions and atmospheres, as well as passive and active filtrations, including preincubation in blood culture bottles, utilization of rumen fluid and fresh sterile stools or antibiotics, with the aim being to grow each distinct bacterial population selectively.

Each condition was observed 2 days, 1 week, 2 weeks, and 1 month after inoculation to isolate colonies that grew at different rates. In certain cases, inoculations were made up to 3 months after incubation in blood culture bottles. Additionally, we used seven supplementary culture conditions. Among these conditions, we prepared new culture media with the addition of antibiotics and cow rumen (Table S1).

Identification by mass spectrometry (MALDI-TOF)

Each deposit was covered with 2 mL of matrix solution (saturated α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile and 2.5 % trifluoroacetic acid). A Microflex spectrometer (Bruker Daltonics) was used to perform this analysis according to the manufacturer's recommendations. A maximum of 100 peaks were used for each spectrum, and these peaks were compared with the computer database at the Bruker base and base-specific lab at La Timone hospital. Our database was previously incremented with the spectra of the new bacterial species cultured during our first study. An isolate was considered as correctly identified at the

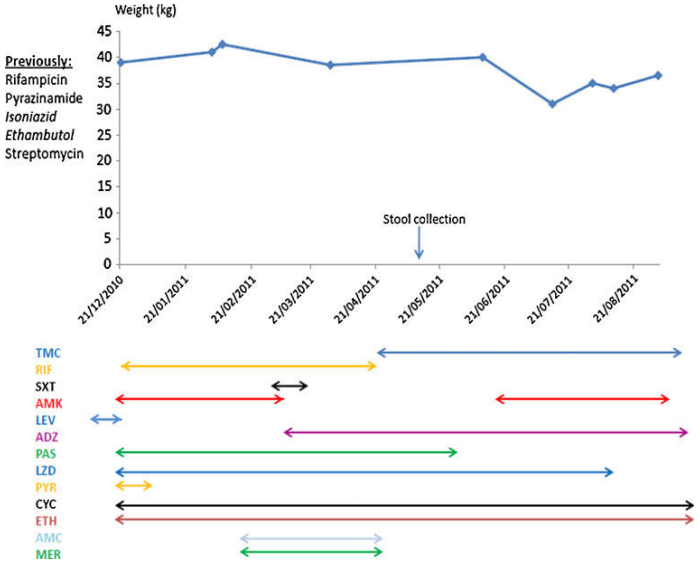

Fig. 1 Weight curve and antibiotic regimen of the patient since her hospitalization. *TMC*: TMC207, *RIF*: rifampicin, *LEV*: levofloxacin, *AMK*: amikacin, *ADZ*: adiazine, *PAS*: para-aminosalicylic acid, *PYR*: pyrazinamide, *LZD*: linezolid, *CYC*: cycloserine, *ETH*: ethambutol, *AMC*: amoxicillin–clavulanate, *MER*: meronem

species level when at least one spectrum had a score≥1.9 and a spectrum had a score≥1.7 [25]. Every non-identified colony was verified three times. When the strain remained unrecognized, the 16S rRNA gene was sequenced as previously described. All of the spectra of species identified by 16S rRNA have been added to the database. The software MALDI Biotyper 3 was used to classify the non-identified bacterial species by comparing their spectra. Only one strain per group of strains with similar spectra was sequenced, whereas the other strains were verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) after adding the spectrum to the database.

16S rRNA amplification and sequencing of the unidentified bacteria

For analyses of nucleotide sequences, bacterial DNA was extracted using the MagNA Pure LC DNA Isolation Kit III (Roche) with a MagNA Pure LC instrument. The 16S rRNA gene was amplified by PCR using the universal primer pair fd1 and rp2 and an annealing temperature of 52 °C. The primers sequences used are available in Table S2. The PCR products were purified using a NucleoFast 96 PCR kit (Macherey-Nagel). The sequence reactions were performed with the BigDye Terminator v1.1 Cycle Sequencing Kit (Perkin-Elmer), with primers 536F, 536R, 800F, 800R, 1050F, and 1050R. The products of the sequencing reaction were purified, and the sequences were analyzed using an ABI PRISM 3130x Genetic Analyzer (Applied Biosystems). The obtained sequences were compared with the GenBank database using BLAST software. A threshold similarity value of >98.7 % was chosen for identification at the species level [14]. Below this value, a new species was suspected, and the isolate was characterized in detail using phenotypic analyses and electron microscopy.

18S rRNA amplification and clone sequencing

DNA extraction

Total DNA was extracted from the frozen fecal sample using a modified method of the Qiagen stool procedure [QIAamp® DNA Stool Mini Kit (Qiagen, Courtaboeuf, France)]. An aliquot of 1.5 ml ASL buffer was added to 1 g of fecal sample and 200 mg of a mixture of 0.1-, 0.5-, and 2-mm zirconium beads. Next, the samples were mixed vigorously by agitation in a FastPrep Bio 101 agitator (Qbiogene, Strasbourg, France) at 3200 rpm for 90 s, followed by heating at 95 °C for 10 min to increase the DNA yield and proteinase K digestion. The DNA was subsequently bound to a column, washed, and eluted in TE buffer.

Genomic amplification

All universal and specific eukaryotic primers which targeted both the18S rRNA and internal transcribed spacer (ITS) gene used in this study were adopted from previously published studies (Table S3). The 50-μL PCR reaction mixture contained 5 μL dNTP (2 mM of each nucleotide), 5 μL DNA polymerase buffer (Qiagen, Courtaboeuf, France),

2 μl $MgCL_2$ (25 mM), 0.25 μL HotStarTaq DNA Polymerase (1.25 U) (Qiagen, Courtaboeuf, France), 1 μL each primer, and 5 μl DNA. The PCR cycling conditions for all of the amplifications were one cycle at 95 °C for 15 min, 40 cycles at 95 °C for 30 min, 48–60 °C for 0.5–2 min, and 72 °C for 1–2 min, followed by 72 °C for 5 min. All of the amplifications were performed in the PCR system 2720 thermal cycler (Applied Biosystems, Courtaboeuf, France). Amplification products were visualized using 1.5 % agarose gel electrophoresis, stained with ethidium bromide, and viewed under a UV light source. The PCR products were purified using the NucleoFast® 96 PCR Kit (Macherey-Nagel, Hoerdt, France), according to the manufacturer's instructions.

Cloning and sequencing

PCR products were cloned separately using the pGEM®-T Easy Vector System Kit (Promega, Madison, WI, USA). Aliquots of 150 μl of the cell suspension were plated onto LB (Luria-Bertani broth) agar plates supplemented with ampicillin (100 mg/mL), X-GAL (80 mg/mL), and IPTG (120 mg/mL), and the plates were incubated overnight at 37 °C. The positive clones were resuspended in 25 μL of distilled water and stored at −20 °C. The presence of the insert was confirmed by PCR amplification using M13 forward (5′-GTAAAACGACGGCCAG-3′) and M13 reverse primers (5′-AGGAAACAGCTATGAC-3′) (Eurogentec, Seraing, Belgium). Purified PCR products were sequenced in both directions using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing products were run on an ABI PRISM 3130 automated sequencer (Applied Biosystems). Finally, the identification of intestinal eukaryotes was performed by comparing the resulting sequences with those deposited in GenBank using the basic local alignment search (BLAST) program, available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST).

Electron microscopy

Transmitting electron microscopy of the stool samples was performed using a Morgani 268D (Philips) at an operating voltage of 60 kV. Each fecal sample was fixed using OsO_2, washed with PBS, and fixed in 2 % glutaraldehyde in 0.1 M cacodylate buffer overnight. After washing, the specimen was post-fixed in 1 % osmium dioxide in 0.1 M potassium ferricyanide for 1 h. Samples were later substituted in the Epon 815 resin type and polymerized for 3 days, and blocks of resin possessing the sample were cut using a microtome. Sections (70 nm) were stained with 3.5 % uranyl acetate and lead citrate before examination in a transmission electron microscope.

Gram staining and bacteria counting

One gram of weighted stool was dehydrated at 55 °C for 24 h and subsequently reweighted to determine the percentage of dry matter. Serial 10-fold dilutions were prepared in phosphate buffer saline with vigorous shaking between each dilution. Next, 10 μl of the 10^4 dilution was spread over a 1-cm^2 area on a slide. After heat fixation, the slides were Gram-stained with a Slide Stainer/Cytocentrifuge (Aerospray® Wescar). Observations were made in oil immersion with a 100× objective on a microscope (Leica, model DM1000) immediately following Gram staining.

Pyrosequencing

A modified version of the protocol described by Zoetendal et al. was used to extract DNA from the fecal samples [26]. The primers were designed to produce an amplicon length (480 bp) that was approximately equivalent to the average length of reads produced by the GS FLX Titanium. The primer pairs commonly used for gut microbiota were assessed in silico for sensitivity to sequences from all phyla of bacteria in the complete RDP database. We selected the bacterial primers *917 F* and *1391R* based on this assessment. The V6 region of 16S rRNA V6 was pyrosequenced with unidirectional sequencing from the forward primer with one-half of a GS FLX Titanium PicoTiterPlate Kit 70×75 per patient with the GS Titanium Sequencing Kit XLR70 after a clonal amplification with the GS FLX Titanium LV emPCR Kit (Lib-L).

We used the Mothur package and the recommended procedure for the 16S rRNA amplicon pyrosequencing analysis [27]. Briefly, raw reads were trimmed using a moving window of 50 bp and it was required that the average quality score over the region did not drop below 35. The trimmed reads were dereplicated and aligned using the SILVA reference alignment. The multiple sequence alignment was filtered based on the start position and reads with a length shorter than 200 bp were discarded. In addition, a preclustering step was performed before chimera detection by the Uchime tool implemented in the Mothur package. Finally, the phylotype identification was performed by binning reads according to their taxonomic classification using blast similarity search against the RDP database (cultivated fraction) formatted by TaxCollector [28] and with a minimum sequence identity of 98.7 %.

In addition, we analyzed the results using the operational taxonomic units (OTUs) method. We built a distance matrix and defined OTUs using a dissimilarity cutoff of 0.03. The representative sequence of each OTU was assigned at the genus level using the RDP classifier and the RDP trainset version 9. Using the Blastn algorithm, we assigned OTUs at the species level when the best blast hit was unique, with a

minimum sequence identity of 98.7 %. The multiple best blast hit cases were looked for as the most representative genus.

Mycobacterium tuberculosis real-time PCR

Extraction

The same DNA stool extract used for pyrosequencing was used for the real-time PCR.

Real-time PCR

Mycobacterium tuberculosis real-time PCR amplification and detection of IS*6110* was performed using PCR primers, with the detection probe described by El Khéchine et al. [29], and real-time PCR was performed with the Bio-Rad CFX96 real-time system (Bio-Rad).

Graphic modeling for pyrosequencing

Graphic modeling for pyrosequencing was performed using Cytoscape software [30].

Antibiotic susceptibility

Antibiotic susceptibility testing was performed according to the Antibiogram Committee of the French Microbiology Society (CASFM) and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) expert rules (http://www.eucast.org) using the Epsilon test method (bioMérieux, Marcy l'Etoile, France) on Mueller–Hinton 5 % sheep blood agar plates (Becton Dickinson, Pont-de-Claix, France).

Results

Microbial culturomics

Using this approach, we tested 73 different culture conditions, 64 of which belonged to the 70 preselected culture conditions that provided a large diversity of isolated bacteria. We tested 4,000 colonies by MALDI-TOF (MS Microflex) analysis for the rapid identification of microbial species. The four colonies not identified by MALDI-TOF were identified by 16S rRNA amplification and sequencing.

We identified 39 bacterial species from four phyla, including 20 *Firmicutes* (51 %), 15 *Actinobacteria* (38 %), three *Proteobacteria* (8 %), and one *Fusobacteria* (3 %) (Table 1). No *Bacteroidetes* were cultured from the stool sample. Among the isolated bacterial species, three had not been previously found in the human gut, including one new species, *Candidatus "Paenibacillus antibioticophila"* (GenBank accession number: KC158472). Of the 39 bacterial species that were isolated, 29 (85 %) were isolated after preincubation in anaerobic blood culture bottles with sheep rumen and sheep blood. Among the original culture conditions tested, preincubation in anaerobic culture bottles with cow rumen for 3 months led to the isolation of the new species, *Candidatus "Paenibacillus antibioticophila"*. In addition, we isolated *Candida albicans* using Sabouraud medium.

18S rRNA amplification and clone sequencing

Among the 36 primers used, four eukaryotic primers were positive (Table S3). A total of three eukaryotic species were detected, all of which were fungi (*Candida dubliniensis*, *Candida albicans*, and *Galactomyces geotrichum*), including one species that was also cultured.

Microscopic observation (Gram staining, bacterial counting, and electron microscopy)

After Gram staining, no bacteria were observed upon stool examination, despite the examination of 100 fields. However, there was yeast present, with an average of 4.2×10^7 yeast per gram of dry feces (Fig. 2a). Electron microscopy was also performed; however, no bacteria were observed during the examination of 50 fields (Fig. 2b).

Pyrosequencing

Pyrosequencing generated a total of 89,469 reads from four phyla (GenBank accession number: SRA061468). The abundance of each phyla was as follows: 74,448 reads from *Cyanobacteria/Chloroplast* (82.4 %), 8,220 from *Firmicutes* (9.1 %), 5,631 from *Proteobacteria* (6.2 %), and 1,170 from *Actinobacteria* (1.3 %). No *Bacteroidetes* were detected. Pyrosequencing identified a total of 18 phylotypes from four phyla. There were nine phylotypes from *Firmicutes* (50 %), three from *Actinobacteria* (16.7 %), two from *Cyanobacteria/Chloroplast* (11.1 %), and four from *Proteobacteria* (22.2 %) (Fig. 3). Similarly, using an OTUs-based method, the number of different OTUs was 19, with most of the reads belonging to the *Cyanobacteria/Chloroplasts* (84 %) and no OTU was identified for the *Bacteroidetes* phyla (Table S4).

Mycobacterium tuberculosis real-time PCR

To identify the *Mycobacteria* found by pyrosequencing, we performed real-time PCR on DNA extracted from the patient stool that was positive for *Mycobacterium tuberculosis*.

Table 1 Bacterial species and phylotypes identified by pyrosequencing and by culture. Species identified by both techniques are shown in bold

Culture species	Phylum	Culture species	Phylum	Pyrosequencing species	Phylum
Acinetobacter genomospecies	*Proteobacteria*	***Pantoea agglomerans***	*Proteobacteria*	***Propionibacterium acnes***	*Actinobacteria*
Actinomyces naeslundii	*Actinobacteria*	*Pediococcus acidilactici*	*Firmicutes*	*Mycobacterium*	*Actinobacteria*
Actinomyces odontolyticus	*Actinobacteria*	***Propionibacterium acnes***	*Actinobacteria*	*Streptophyta*	*Chloroplast*
Anaerococcus octavius	*Firmicutes*	*Propionibacterium granulosum*	*Actinobacteria*	*Citrus sinensis*	*Chloroplast*
Arthrobacter castelli	*Actinobacteria*	*Rothia dentocariosa*	*Actinobacteria*	*Streptococcus*	*Firmicutes*
Bacillus arsenicus	*Firmicutes*	*Rothia mucilaginosa*	*Actinobacteria*	*Lactococcus raffinolactis*	*Firmicutes*
Bacillus clausii	*Firmicutes*	*Staphylococcus capitis*	*Firmicutes*	*Blautia wexlerae*	*Firmicutes*
Brevibacillus agri	*Firmicutes*	*Staphylococcus cohnii*	*Firmicutes*	*Streptococcus parauberis*	*Firmicutes*
Brevibacterium massiliensis	*Actinobacteria*	*Staphylococcus epidermidis*	*Firmicutes*	*Enterococcus*	*Firmicutes*
Corynebacterium glucuronolyticum	*Actinobacteria*	*Staphylococcus haemolyticus*	*Firmicutes*	*Lactobacillus*	*Firmicutes*
Dermabacter hominis	*Actinobacteria*	*Staphylococcus hominis*	*Firmicutes*	*Ruminococcus torques*	*Firmicutes*
Enterococcus faecium	*Firmicutes*	*Staphylococcus pasteurii*	*Firmicutes*	*Streptococcus salivarius*	*Firmicutes*
Escherichia coli	*Proteobacteria*	*Staphylococcus vitulinus*	*Firmicutes*	*Streptococcus lutetiensis*	*Firmicutes*
Finegoldia magna	*Firmicutes*	*Staphylococcus warneri*	*Firmicutes*	*Dietzia*	*Actinobacteria*
Fusobacterium naviforme	*Fusobacteria*	*Staphylococcus intermedius*	*Firmicutes*	*Methylobacterium zatmanii*	*Proteobacteria*
Kocuria rhizophila	*Actinobacteria*	*Streptococcus mitis*	*Firmicutes*	*Rickettsia*	*Proteobacteria*
Kocuria rosea	*Actinobacteria*	*Streptococcus parasanguinis*	*Firmicutes*	***Pantoea agglomerans***	*Proteobacteria*
Microbacterium hydrocarbonoxydans	*Actinobacteria*	*Streptococcus salivarius*	*Firmicutes*	*Blastomonas natatoria*	*Proteobacteria*
Micrococcus luteus	*Firmicutes*	*Streptococcus sanguinis*	*Firmicutes*		
Moraxella osloensis	*Proteobacteria*	*Streptomyces thermovulgaris*	*Actinobacteria*		
Paenibacillus antibioticophila	*Firmicutes*	*Veillonella parvula*	*Firmicutes*		

The *Mycobacterium tuberculosis* density in the patient's stool was estimated to 700 copies per gram of wet feces using a dyeset.

Antibiotic susceptibility

Minimum inhibitory concentration (MIC) values against *Candidatus "Paenibacillus antibioticophila"* cultured from the stool sample were low for amoxicillin (MIC=0.7 mg/mL), imipenem (MIC=0.9 mg/mL), doxycycline (MIC=2 mg/mL), penicillin G (MIC=0.1 mg/mL), linezolid (0.5 mg/mL), and vancomycin (MIC=2.1 mg/mL), but high for metronidazole (MIC>12 mg/mL).

Comparing microbial culturomics and pyrosequencing

We identified significantly more bacterial species by culturomics than pyrosequencing (mid-P exact=$p<0.001$). Only two bacterial species were detected by both techniques (*Propionibacterium acnes* and *Pantoea agglomerans*). We also identified significantly more genera by culturomics ($n=24$) than pyrosequencing ($n=15$) (mid-P exact=$p<0.001$). Four bacterial genera were detected by both techniques (*Propionibacterium*, *Pantoea*, *Streptococcus*, *Enterococcus*). All of the phyla that were detected by pyrosequencing were also found in culture, except the *Chloroplast* phylum. One species from *Fusobacteria* phylum was cultured but not

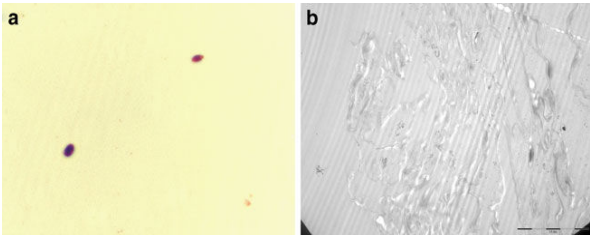

Fig. 2 Microscopic observation of the stool sample, using Gram staining (**a**) and electron microscopy (**b**) showing absence of bacteria

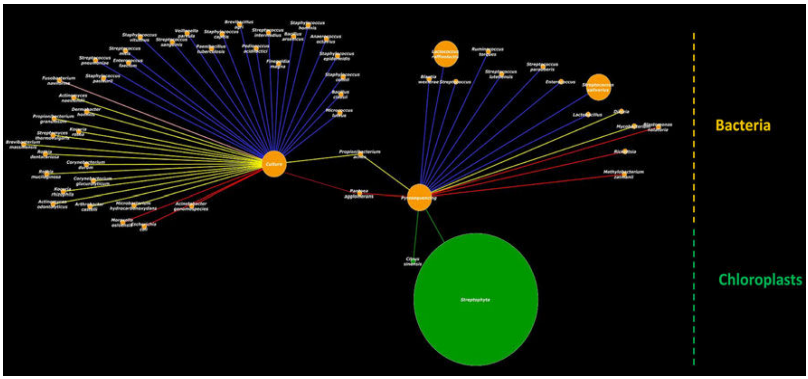

Fig. 3 Comparison of the pyrosequencing and culture results. Bacterial species isolated by culture are represented on the left and those detected by pyrosequencing on the right. The edge colors represent the phylum (*blue*: *Firmicutes*; *red*: *Proteobacteria*; *green*: *Cyanobacteria/Chloroplast;* *yellow*: *Actinobacteria*; *pink*: *Fusobacteria*). For pyrosequencing, each node represents one species or OTU assigned at the species level and the node size is proportional to the abundance of the phylotype

detected by pyrosequencing, and neither technique identified species or genera from the *Bacteroidetes* phylum.

Discussion

We are confident in our results because we used pyrosequencing of 16S rRNA amplicons targeting the V6 region, which is known to be the most variable [31], to explore the gut microbiota [32] and we also performed both phylotype and OTU assignment with similar results. Additionally, microbial culturomics was applied to this stool sample based on the most effective culture conditions that have been previously reported [14]. Both techniques have been widely used in our lab and were performed using stringent protocols. Our results are interesting because we observed a dramatic rarefaction of the gut microbiota under antibiotic pressure. Optical and electronic microscopy observations showed a complete absence of bacteria, suggesting a bacterial gut biomass of under 10^4 bacteria per gram of dry feces. There was also a partial colonization by yeast estimated at 4.2×10^7 yeast per gram of dry feces. The yeast was probably *Candida albicans*, which was found by both culture and molecular methods. In parallel, culturomics and pyrosequencing detected a total of 39 cultivated species and 18 phylotypes. Although we studied only one sample, the results are significant because the sample was provided from large gut microbiota studies [13, 14] and this was the first time that we observed such microbial poverty in a stool sample. Surprisingly, despite the critical decrease of the gut flora, microbial culturomics permitted the culture of one previously unknown new species (*Candidatus "Paenibacillus antibioticophila"*).

Indeed, this patient with a low number of bacteria (fewer than 10^4 per gram of feces) illustrates the depth bias of pyrosequencing, as shown by the significantly higher number of bacterial species identified by culturomics than phylotypes found by pyrosequencing. To the best of our knowledge, this study represents the first time that culture-dependent methods yielded more comprehensive findings than molecular methods to explore the gut flora diversity (Fig. 3). Because the most efficient metagenomic studies only allows for the detection of bacteria with a concentration greater than 10^6 bacteria per gram of feces [14], the low bacterial load of our patient sample explains the superiority of culturomics in this study. Reduced gut microbiota diversity has been previously reported in human health, particularly in infants with allergic manifestations [33, 34]. Culture-independent studies have previously shown a critical bacterial diversity reduction in patients treated with antibiotics, such as ciprofloxacin [4] or vancomycin [3]. Sterilization of the gastrointestinal tract has been previously reported in culture-dependent studies in patients treated by antibiotic regimens, including gentamicin, vancomycin, and nystatin [35], but only routine culture conditions were performed, omitting fastidious bacterial species.

Serendipitously, we found that more than 80 % of the 16S rRNA sequences were classified as *Streptophyta*, a *Viridiplantae* phylum that contains several green algae and higher plants [36]. Chloroplasts present in the human gut have been very rarely reported; however, a low number of sequences have been found in the gut of human infants [37]. Nevertheless, the use of the elastic net as a classifier [38] allowed the detection of a notably high relative abundance of *Streptophyta* from a single subject [39]. Additionally, studies examining the presence of green plant DNA in complex

microbiomes, such as the lung, did not highlight such results, despite an abundance of eukaryotes found in these environments [40]. Further, there were no previous studies exploring the eukaryotic composition of patients treated with broad-scale antibiotics. These findings suggests that the critical reduction of bacterial sequences detected, linked with the depth bias of the metagenomic studies, have permitted the amplification of DNA from green plant foods absorbed by the patient.

Unfortunately, our patient died 7 months after her hospitalization, as a result of pneumonia due to *Streptococcus pneumoniae*. The composition of the gut microbiota contributes to the development of lymphoid tissue [41], similar to how microbiome variations in early infancy induce differences in host immune responses [42]. Recent studies support the idea that the gut microbiota is able to regulate immune defense against respiratory viruses, such as influenza A [43], through the activation of lung inflammasomes. The gut flora desertification that we observed was probably a reflection of our patient's entire microbiota and has, consequently, severely compromised our patient. Thus, a global microbial desertification, particularly from the respiratory mucosa, may have contributed to the severe *Streptococcus pneumoniae* infection in our patient. Recent studies show that the airway microbiome may be linked to pneumococcal disease [44]. This finding suggests that patients treated with broad-spectrum antibiotics for a prolonged period of time should have repetitive stool sample Gram-staining and should consider protective isolation in the case of desertification of gut flora with the aim of preventing invasive infections. In addition, it may be beneficial to vaccinate this patient population systematically against pneumococcal and influenza virus, as well as meningococcal disease.

These findings highlight evidence regarding the ability of culturomics to complement metagenomic studies on the human gut. In addition, we hypothesize that the critical rarefaction of the human gut is a reflection of the entire microbiota and can facilitate invasive infections. Therefore, stringent preventive measures should be taken in order to avoid this side-effect of long-term broad-spectrum antibiotic treatment.

Conflict of interest The authors declare that they have no conflict of interest.

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LETTER TO THE EDITOR

The proof of concept that culturomics can be superior to metagenomics to study atypical stool samples

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Dear Editor,

We read with interest the comments regarding our article [1]. We are grateful to the authors for their remarks, and we agree with the biases linked to metagenomics explaining the absence of reproducibility between the different studies about the gut microbiota repertoire and have reported this elsewhere [2–4]. Nevertheless, the aim of this study was to compare the most commonly used molecular method to explore the microbiota (pyrosequencing of 16 rRNA amplicons targeting the V6 region) used as a standard with culturomics [5]. Indeed, it would be another study to compare all the pyrosequencing techniques previously reported, which was not our goal [6].

Nevertheless, we are confident in our pyrosequencing results because, as with most of the studies, we used only one hypervariable region of the 16S rRNA amplicon, and because we chose the most hypervariable V6 region as previously described for the gut microbiota exploration [6]. In addition, we pyrosequenced the amplicons using one-half of the GS Titanium Plate, generating a reasonable total of 89,469 trimmed reads for a total of 106,000 reads. These sequences have been analyzed using both phylotypes and the operational taxonomic unit method with equivalent results, and we used willingly stringent criteria to avoid erroneous assignment. These results are consistent with the microscopic analysis showing that less than 10^4 prokaryotes per gram of stools were present. It is clear that the threshold of metagenomic and 16S rDNA amplification cannot, because of inhibitors, go much lower than 10^3–10^4 bacteria per gram of stools [3].

Finally, this study is a part of the rebirth of the culture [7] that can detect a single living organism and is, therefore, indeed, more sensitive than any of the molecular techniques as reported for *Staphylococcus aureus* [5]. Nevertheless, this is the first time, because of the low number of bacteria observed in this patient, that culture yields more bacterial species than metagenomic-detected phylotypes, exemplifying the need to use a comprehensive approach based on the complementarity of the techniques rather than being blinded by the theoretical superiority of the molecular techniques.

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Article 5:

Metagenomic Analysis of Eukaryotes Microbiota in Gut of HIV-infected Patients

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Manuscript in preparation

In this work, four different extraction protocols were tested to ensure optimal recovery of representative DNAs richness in gut in the following steps. As expected, extraction methods differed in terms of their ability to extract DNA from particular species of microeukaryotes. Our results revealed that method E1 was the best among other to extract the eukaryotic DNA from feces. From the 5,328 clone sequences that collected from 31 HIV-patients and 12 control subjects, a total of 78 micro-eukaryotic species were detected (67 fungi and 2 stramenopiles found in HIV samples versus 30 fungi and 2 stramenopiles identified in feces of the healthy control individuals). Higher fungal diversity was observed in feces of both HIV infected patients and controls comparing with other eukaryotic species. Specific RT PCR for human gut parasites were also used since many parasites can't be detected by metagenomic analyses because of their low load level in human intestinal tract. Of the 39 HIV patients' fecal samples tested, 11 (28.2%) were positive for Microsporidia. Of these 11 fecal samples, 8 (20.5%) and 3 (7.7%) were PCR positive for the *Enterocytozoon bieneusi* and *Enterocytozoon intestinalis,* respectively. Moreover, specific amplification products were seen for *Giardia lamblia*, *Blastocystis* and *Hymenolepis diminuta* and acounted for 3 (7.7%), 2 (5.3) and 3 (7.7%) of 39 fecal samples from HIV infected patients. Whereas fecal samples of non HIV infected persons showed positive amplification for both *Cryptosporidium hominis* (one sample, 8.3%) and *Blastocystis* (2 samples, 16.7%) respectively.

Manuscript in preparation

Metagenomic analysis of Eukaryotes Microbiota in Gut of HIV-infected patients

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Introduction

HIV infection caused by human immunodeficiency virus (HIV) remains one of the most important infections in the world with more than 35.3 million people recorded to have HIV infection until the end of 2013 [1]. The deterioration in host immunity of patients with HIV makes them always susceptible to variety of opportunistic bacteria, fungi and parasites during their lifetime [2]. One of the major health risks in HIV-infected patients is infection by the intestinal eukaryotic population including fungi and parasites [3]. Therefore, understanding the diversity of these eukaryotes in gut of healthy individuals as well as in immunocompromised patients are needed to understand their roles in both health and disease status [4].

Until recently, the majority of works done on the eukarya of intestinal tract of HIV- infected patients have been mainly focused on known pathological and parasitological aspects of eukaryotic members of this community such as enteric parasites [2,5-10] or pathogenic fungi [3,11,12]. Indeed, many studies have used specific PCR based amplification to detect certain parasitic agent such as *Entamoeba histolytica, Cryptosporidium* spp., *Isospora belli* and members of *Microsporidia* in feces of HIV/AIDS patients [5,13-17]. However, the development of culture-independent molecular techniques, comprising of direct DNA extraction from feces followed by clone sequencing as well as high-throughput sequencing methods, offers new opportunity to estimate the diversity of eukaryotes in the human gut by providing data on the entire eukaryotic community particularly not-yet-cultured or fastidious organisms in healthy individuals [4,18-20] and patients [21-24].

The investigation using molecular approaches has been applied in patients with Crohn's disease, hepatitis B, inflammatory bowel disease and intestinal transplanted patients to report the gut fungal burden. Fungal community has been investigated in 57 patients with inflammatory bowel disease using denaturing gradient gel electrophoresis (DGGE) clone sequencing and resulted in retrieving a diverse fungal community compared with healthy controls [21]. Whereas the diversity of intestinal fungal microbiota of patients with hepatitis B was higher than that in HBV carriers and healthy volunteers [22]. Temporal alterations in the fungal communities have been observed in intestinal transplant patients when their ileal effluent and feces have been analyzed using PCR targeted 18S rRNA region [23].

No study has been conducted yet to explore the diversity of microeukaryotes and eukaryotes in the human gut of HIV- infected patients. Our study aims to investigate this diversity and the composition of human intestinal eukaryote microbiota in HIV patients. First, 4 methods

of DNA extraction were evaluated in 13 patients. Then, the best method of extraction was used in 43 individuals, including 31 patients infected with HIV, and 12 healthy and DNAs were analyzed by both conventional clone sequencing and Next-generation sequencing. Finally, as many pathogens with low load could be missed in metagenomic analyses, real-time PCRs screening important enteric parasites were applied in feces of both HIV infected patients and healthy volunteers.

Materials and Methods

Fecal Sample Collection

Forty-three fecal samples were collected in this study, 31 samples were obtained from HIV infected patients along with 12 fecal samples from healthy volunteers. Written assent were obtained from these patients as well as the healthy volunteers and these assent procedures were approved by the Ethics Committee of the Institute Fédératif de Recherche IFR 48, Faculty of Medecine, Marseille, France (agreement number 09-022). Each fecal sample was preserved as 1g-aliquots in sterile microtubes and kept at −80°C until use.

DNA extraction

In order to verify complete extraction of most eukaryotic gut representative DNAs and to minimize bias that generate from DNA extraction, four different types of DNA extraction methods were applied and tested in this study to detect the eukaryotic communities in gut of HIV infected patients. Thirteen frozen fecal samples from patients with HIV were chosen for testing the different DNA extraction methods.

Method E1: Modified Qiagen stool protocol

Total DNA was extracted from the 13 frozen fecal samples of HIV infected using a modification of the Qiagen stool procedure and the Qiamp® DNA Stool Mini Kit (Qiagen, Courtaboeuf, France) [4]. Briefly, aliquots of 200 mg of feces were added into tubes containing a 200 mg mixture of 0.1, 0.5, and 2 mm zirconium beads and 1.5 ml of ASL buffer (Qiagen). The sample was bead beaten at 3200 rpm for 90 seconds, followed by heating at 95°C for 10 minutes. The final pellet was suspended in 180 µl of tissue lysis buffer and incubated with proteinase K for 2 hours at 55°C. Then, DNA was prepared from the solution by using QIAmp spin columns (Qiagen) in an Eppendorf microcentrifuge following the manufacturer's instructions.

Method E2: Modified Qiagen stool protocol + chitinase

In this method, the procedures are the same as explained in method E1 with only one modification that 0.4 U of chitinase (Sigma) was added to proteinase K in the digestion step.

Method E3: FastDNA® kit

Aliquot of 300 mg of the 13 frozen HIV infected fecal samples were suspend in 1 ml of PBS-EDTA and centrifuged at 13,000 rpm for 5 min. The final pellet was suspended in PBS EDTA to obtain approximately 300 µl of solubilized sample. Then it was added to 1 ml of Cell Lysis solutions-Yeast (CLS-Y). Mechanical lysis was performed to the mixture by bead-beating the mixture using a FastPrep BIO 101 apparatus (Qbiogene, Strasbourg, France) for 40 seconds at a speed setting of 6.0. The samples were centrifuged for 5 minutes at 13,000 rpm. Aliquot of 600 µl supernatants were transferred to a new tube containing 600 µl of binding matrix, then the mixture was mixed gently and incubated at room temperature for 5 minutes. The samples were centrifuged again at 13,000 for 1 minute at room temperature and the pellets were resuspended thoroughly by pipetting up and down in 500 µl of prepared Salt/ethanol wash solution (SEWS-M), followed by centrifugation for 1 min. The matrix were resuspended in 100 µl of DES and incubated at 55 °C for 5 min. Finally, the samples were spinned at 13,000 rpm for 5 min, and eluted DNA were transferred to a clean microcentrifuge tube and store at -20°C for extended periods or 4°C until use.

Method E4: Protocol 5

DNA was extracted using the (protocol 5) as has been previously described (ref). Briefly, 250 mg of feces was placed in a 2-mL tube containing a mixture of 2 glass beads of 3 mm and 1.5-mL of lysis buffer (ASL) (Qiagen, Courtaboeuf, France). Mechanical lysis was performed three times by bead-beating the mixture using a FastPrep BIO 101 apparatus (Qbiogene, Strasbourg, France) at maximum speed (6.5) for 30s. Then, aliquot of 200 µL has been retrieved in new eppendorf tube and centrifuged at 12000 rpm for 10 min. Aliquot of 20 µl of 10X Glycoprotein denaturation buffer EndoHf (New England Biolabs) along with 180 µl H2O containing 1 glass bead of 2 mm were added into the retrieved supernatant and heated to 100°C for 10 min. Then, 160 µl of sterile water, 40 µl of 10X G5 buffer and 5 µL of both cellulase (SIGMA) and PNGase F (SIGMA P2619) were added to the mixture and incubated at 37°C for 13 hr. Then the manufacturer's procedure EZ1 Qiagen have been applied and the extracted DNA was stored at −20°C until use.

Primer Selection

Twenty-five different published universal eukaryotic or fungal-specific PCR primer sets as well as specific primers targeting the 18S rDNA, ITS, 28S rDNA, RUBISCO, and rps11-rp12 sequences were used, as described previously [4].

Genomic Amplification and cloning procedures and insert amplification were used as described previously [4].

Next-generation sequencing

Six primer sets targeting the 18S rDNA and ITS region to amplify the eukaryotic components in both HIV infected patients and healthy volunteers.

Real-Time PCR Assay for Detection of human intestinal parasites

Primers and probes specific to human enteric pathogens were used as described in (Tables S2). The real-time PCR reactions were conducted using 25 µL total volumes and analyzed for 44 cycles using a CFX96™ Real-Time PCR Detection System (BIO-RAD, Life Science, Marnes-la-Coquette, France) following the method recommended by the manufacturer. Amplification reactions were done as follows: 95°C for 15 min, 60°C for 0.5 min, and 72°C for 1min.

Nucleotide Sequence Accession Numbers

All sequences obtained in this work have been deposited in GenBank database with the accession numbers KP974154-KP974247.

Results

DNA extraction methods

To test the efficiency of four DNA extraction methods to retrieve the eukaryotes DNA from feces, thirteen stool samples from HIV infected patients were selected and two universal primers targeting the 18s gene for eukaryotes (Euk 1A and Euk 516r) and ITS sequence for fungi (ITS1F and ITS4R) followed by cloning and sequencing were assessed. Overall, 4,992 clones were analyzed from both PCR (1248 clones/extraction) as shown in (Table S3). The extraction method E1 resulted in the greatest yield of microeukaryotes DNA sequences (Figure 1). Thirty-seven different species of microeukaryotes were retrieved in both libraries in which 9 particular species were uniquely retrieved with this extraction (Table S4, Fig1). Whereas in extraction method E2, sequences of 31fungal species were detected using the two libraries, to which sequences of 6 fungal species were only efficiently detected in this extraction (Table S4, Fig1). Moreover, a total of 27 fungal species were obtained in extraction method E3 (Table S4, Fig1).The extraction method E4 was less powerful method

in extract the eukaryotic DNA from feces of HIV infected patients than other methods, only 20 sequences belonging to fungal species were obtained with both fungal ITS and 18s rRNA libraries (Table S4, Fig1). Consequently, the extraction method E1 was selected and used for the following analyses.

Cloning and sequencing

Among the 25 primers used, positive PCR amplifications were obtained in all the HIV patients and healthy volunteers with only Six PCR primers namely: Euk1A/Euk 516r, ITS F/ITS-4R, JVF/DSPR2 and FUNF/FUNR (positive for all the samples) and E528F/Univ1492RE and MF/MR (positive for 35 and 22 samples respectively) as shown in Table S5. Consequently, five cloning libraries were generated from the total community DNA that amplified by these general primers for each sample (Table S5). A total of 5,328 clones were subjected to sequence analysis (Table 1). After excluding plant DNA sequences that potentially represented in human fecal DNA extractions and amplified by universal primers, a total of 78 micro-eukaryotic species (67 fungi and 2 stramenopiles) were detected in 31 HIV samples, whereas 32 species (30 fungi and 2 stramenopiles) identified in feces of 12 healthy control individuals (Fig.2). Most of the species that were present in fecal samples were fungi (75 species, Table 1), while only 3 protozoan species belonging to the stramenopiles were identified. The mean number of eukaryotic species per HIV patient was 6.94 ± 1.90 [range 4-12] (taking into account the results of all PCR positive for the given patient), while the mean number of eukaryotic species per healthy sample was statistically lower (5.17 ±1.47 [range 3-8])($p = 0.006$).

The two cloning libraries, Euk1A/Euk 516r and ITS F/ITS-4R were more suitable than other libraries for analyzing eukaryotic diversity in the intestinal tract of humans. A proximately 95% of all recovered microeukaryotes in this study (total of 74 species /78) were retrieved in these two libraries (Table 1) while remaining species 5% (4/78) were retrieved with other cloning libraries (Table1).

Analysis of the clone library Euk1A/Euk 516r, allowed identification of 30 different species of microeukaryotes, 27 species (90 %) were found to be of fungal origin, whereas 3 species (10 %) were assigned to stramenopiles group. The majority of detected fungi within this library belonged to the 3 major fungal taxa: Ascomycetes (n = 15/27, 55.6 %) and Basidiomycetes (n = 11/27, 40.7%). Whereas uncultivatable chytridiomycota fungi accounted for 3.7 % of recovered fungi in this cloning library (Table S6).

Fungal species such as *Candida* spp., *Galactomyces geotrichum*, *Saccharomyces* spp., and *Trichosporon* spp. were identified in the feces of HIV infected patients and in those of

healthy volunteers. Whereas *Arxiozyma telluris*, *Candida dubliniensis*, *Candida inconspicua*, *Cryptococcus magnus*, *Filobasidium capsuligenum*, *Rhodotorula mucilaginosa*, *Penicillium marneffei* and other fungi were only retrieved in feces of HIV patients (Table 1).

The fungal clone library ITS F/ITS-4R, allowed identification of 61 different fungal species. The fungi within this library were assigned into each Ascomycetes (n = 38/61, 62.3 %), Basidiomycetes (n = 21/61, 34.4 %) and Chytridiomycota (n= 2/61, 3.3 %).

Similarly to clone library Euk1A/Euk 516r, species such as *C. magnus*, *F. capsuligenum*, and *R. mucilaginosa* and *P. marneffei* as well as member of genus *Candida* were represented only in feces of HIV patients. Moreover, the results from analyzing ITS clone library revealed that most detected fungi of genus Malassezia (*Malassezia globosa*, *Malassezia pachydermatis*, *Malassezia restricta*, *Malassezia sp.* and *Malassezia sympodialis*) were retrieved only in fecal samples of HIV patients while no Malassezia sequences were detected in feces of healthy volunteers (Table1).

Eukaryotic diversity by Next-Gen sequencing

A high throughput sequencing using Illumina machine was applied to the fecal samples after the amplification of both 18s RNA and ITS sequences. **(analyses ongoing)**

Real-Time PCR targeting the protozoan parasites and helminths

As protozoan parasites and helminths cannot be detected neither by clone-sequencing nor by metagenomic analyses because they are at low load level in human intestinal tract and only abundant eukarya can be amplified using universal primers, we used specific RT PCR for these human gut parasites.

Of the 39 HIV patients' fecal samples tested, 11 (28.2%) were positive for Microsporidia. Of these 11 fecal samples, 8 (20.5%) and 3 (7.7%) were PCR positive for the *Enterocytozoon bieneusi* and *Enterocytozoon intestinalis* respectively Table (Excel).

Moreover, specific amplification products were seen for *Giardia lamblia*, *Blastocystis* and *Hymenolepis diminuta* and acounted for 3 (7.7%), 2 (5.3) and 3 (7.7%) of 39 fecal samples from HIV infected patients.

Whereas fecal samples of non HIV infected persons showed positive amplification for both *C. hominis* 1 (8.3%) and *Blastocystis* 2 (16.7%) respectively Table (Excel).

Discussion

Human intestinal eukaryotes are studied primarily from a parasitological and pathological point of view in gut of HIV infected patients using either microscopic or culture- based

methods [2,3,5-10,12]. Since eukaryotic component of the human gut microbiome remains relatively unexplored in these immunocompromised patients with the molecular methods, therefore the investigation of the composition of the human gut eukaryote microbiota through culture independent methods is the main objective of this study.

Herein, four different extraction protocols were tested to ensure optimal recovery of representative DNAs richness in gut in the following steps. As expected extraction methods differed in terms of their ability to extract DNA from particular species of microeukaryotes (Table S3, Fig1). Our results revealed the superiority of method E1 to extract the eukaryotic DNA from feces (fig?). From the 5,328 clone sequences that collected from HIV-patients and control subjects, a total of 78 micro-eukaryotic species were detected (67 fungi and 2 stramenopiles found in HIV samples and 30 fungi and 2 stramenopiles) identified in feces of the healthy control individuals. Higher fungal diversity was observed in feces of both HIV infected patients and controls comparing with other eukaryotic species. All studies that have used culture independent approaches to investigate the micro-eukaryotes in the human gut, showed the dominance of fungi over other microeukaryotes such as enteric parasites [4,18,19,25,26]. The absence of enteric parasite in fecal samples in present study could be explained by either pretreatment of these patients with antiparasitic drug or absence/low concentration of the target parasite DNA in the studied samples.

A significant increasing in fungal diversity in gut has also been noted in certain disease such as inflammatory bowel disease and hepatitis B virus infection [21,22]. The CD4+ T-cell response is the normal gastrointestinal mucosal defense mechanism against colonization of fungi in human body [27]. Therefore, any disturbance or impairing in this immune response is making the body of patients with HIV virus to be more susceptible to fungal disease [28,29], particularly in patients with low CD4 T lymphocyte counts [3]. The colonization of some fungi such as *Candida* spp, *Aspergillus* spp, *P. marneffei* and *Geotrichum candidum* in gastrointestinal tracts of HIV infected patients, could cause many mucosal and invasive fungal infections such as mucocutaneous candidiasis [30], aspergillosis [31], penicilliosis marneffei [32] and geotrichosis [33] respectively. Our results indicated that members of the opportunistic yeasts of genus *Malassezia* were widely abundant in the feces of HIV infected patients; these fungi have been previously detected in feces of healthy individuals [4,26,34] and the colonization of *Malassezia* spp. have been reported in lower gastrointestinal tract of neonates [35].However, no reported data are available for their role in causing mucosal fungal infection. In immune-compromised patients, *Malassezia* spp were always associated with several dermal infections and systemic diseases [36].

In conclusion, Further investigations needed to find out the role of these fungi in intestinal tract of patients with HIV infected virus.

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Figue legands

Figure 1: Comparative of different microeukaryotic components extracted in 13 samples of HIV infected patients 4 methods of DNA extraction.

Figure 2: Total microeukaryotic components detected in 31 samples of HIV infected patients along with 12 fecal samples from healthy volunteers.

Table legends:

Table 1: List of microeukaryotic components detected in different cloning libraries in both HIV infected patients and healthy volunteers fecal samples.

Table S1: Primers used in this study.

Table S2: Primers and probs used for real time PCR.

Table S3:Gut microeukaryotes retrieved using four different protocol of extraction.

Table S4: Comparative of different microeukaryotic components extracted using four different protocol of extraction.

Table S5: PCR amplifications obtained through using different universal eukaryotic primer sets.

Table S6: Microeukaryotes detected using clone library Euk1A/Euk 516r

Table S7: Microeukaryotes detected using clone library ITS F/ITS-4R

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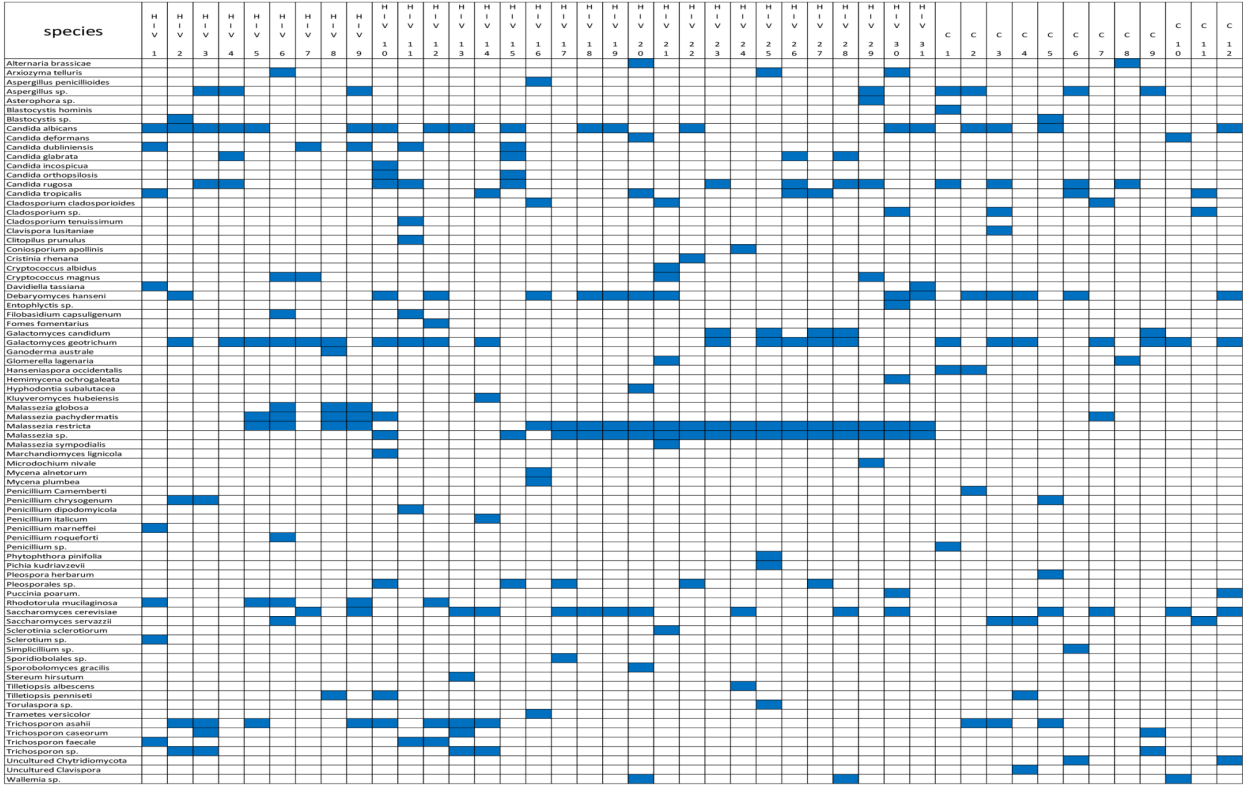
Figure 1:

Figure 2:


species
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HIV29
HIV30
HIV31
C1
C2
C3
C4
C5
C6
C7
C8
C9
C10
C11
C12
Alternaria brassicae
Arxiozyma telluris
Aspergillus penicillioides
Aspergillus sp.
Asterophora sp.
Blastocystis hominis
Blastocystis sp.
Candida albicans
Candida deformans
Candida dubliniensis
Candida glabrata
Candida incospicua
Candida orthopsilosis
Candida rugosa
Candida tropicalis
Cladosporium cladosporioides
Cladosporium sp.
Cladosporium tenuissimum
Clavispora lusitaniae
Clitopilus prunulus
Coniosporium apollinis
Cristinia rhenana
Cryptococcus albidus
Cryptococcus magnus
Davidiella tassiana
Debaryomyces hanseni
Entophlyctis sp.
Filobasidium capsuligenum
Fomes fomentarius
Galactomyces candidum
Galactomyces geotrichum
Ganoderma australe
Glomerella lagenaria
Hanseniaspora occidentalis
Hemimycena ochrogaleata
Hyphodontia subalutacea
Kluyveromyces hubeiensis
Malassezia globosa
Malassezia pachydermatis
Malassezia restricta
Malassezia sp.
Malassezia sympodialis
Marchandiomyces lignicola
Microdochium nivale
Mycena alnetorum
Mycena plumbea
Penicillium Camemberti
Penicillium chrysogenum
Penicillium dipodomyicola
Penicillium italicum
Penicillium marneffei
Penicillium roqueforti
Penicillium sp.
Phytophthora pinifolia
Pichia kudriavzevii
Pleospora herbarum
Pleosporales sp.
Puccinia poarum.
Rhodotorula mucilaginosa
Saccharomyces cerevisiae
Saccharomyces servazzii
Sclerotinia sclerotiorum
Sclerotium sp.
Simplicillium sp.
Sporidiobolales sp.
Sporobolomyces gracilis
Stereum hirsutum
Tilletiopsis albescens
Tilletiopsis pennisetі
Toruláspora sp.
Trametes versicolor
Trichosporon asahii
Trichosporon caseorum
Trichosporon faecale
Trichosporon sp.
Uncultured Chytridiomycota
Uncultured Clavispora
Wallemia sp.

Chapter III:

The Eukaryotes Gut Microbiota in Non-human Primate

Article 6:

Pathogenic Eukaryotes in Gut Microbiota of Western Lowland Gorillas as Revealed by Molecular Survey

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Because very little is known about the eukaryotic diversity in apes intestinal tract. We present as the proof-of-concept the first extensive molecular study of the occurrence of eukaryotes in fecal sample from gorilla (*Gorilla gorilla gorilla*) by the use of 35 specific and universal primers, cloning and sequencing of 1572 clones. The results revealed that eukaryotic diversity estimated by the molecular methods was complex with a total of 87 species detected in this stool sample including 52 fungi, 10 protozoa, 4 nematodes and 21 plant species, of which 52, 5, 2 and 21 species, respectively, have never before been described in gorillas. Specific real-time PCR analysis of 48 fecal samples was also conducted for screening potential human eukaryotic pathogen in gastrointestinal tracts of 21 individual western lowland gorillas (*G. g.gorilla*). The results showed the occurrence of pathogenic fungi and parasites (*Candida tropicalis* (81%), *Oesophagostomum bifurcum* (86%), *Necator americanus* (43%) and other pathogenic fungi were identified).

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Pathogenic Eukaryotes in Gut Microbiota of Western Lowland Gorillas as Revealed by Molecular Survey

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Although gorillas regarded as the largest extant species of primates and have a close phylogenetic relationship with humans, eukaryotic communities have not been previously studied in these populations. Herein, 35 eukaryotic primer sets targeting the 18S rRNA gene, internal transcribed spacer gene and other specific genes were used firstly to explore the eukaryotes in a fecal sample from a wild western lowland gorilla (*Gorilla gorilla gorilla*). Then specific real-time PCRs were achieved in additional 48 fecal samples from 21 individual gorillas to investigate the presence of human eukaryotic pathogens. In total, 1,572 clones were obtained and sequenced from the 15 cloning libraries, resulting in the retrieval of 87 eukaryotic species, including 52 fungi, 10 protozoa, 4 nematodes and 21 plant species, of which 52, 5, 2 and 21 species, respectively, have never before been described in gorillas. We also reported the occurrence of pathogenic fungi and parasites (i.e. *Oesophagostomum bifurcum* (86%), *Necator americanus* (43%), *Candida tropicalis* (81%) and other pathogenic fungi were identified). In conclusion, molecular techniques using multiple primer sets may offer an effective tool to study complex eukaryotic communities and to identify potential pathogens in the gastrointestinal tracts of primates.

The microbial communities residing in the gastrointestinal tracts of primates are complex and may play important roles in health and disease. The interactions between microbial cells and primate host cells could be either commensal or parasitic, and it is known that these interactions have an effect on the metabolic, developmental, and immunological status of the host[1,2]. The compositions and constituents of these communities are influenced by several factors, such as the host diet, geography, physiology, and disease state[3,4].

Gorillas share a close phylogenetic relationship with humans, resulting in a high potential for pathogen exchange involving bacteria, viruses and gastrointestinal parasites[5–7]. Moreover, the presence of these pathogens in wild primates may also have negative consequences for public health and wildlife conservation management[8]. To date, there have been no studies examining the entire eukaryotic community residing in the intestinal tract of the gorilla; rather, most studies focus on the parasitological aspects of these eukaryotic communities using coprological studies to survey the presence of intestinal parasites in wild gorilla populations. Several such studies have been conducted in both mountain gorillas (*Gorilla gorilla beringei*) and western lowland gorillas (*Gorilla gorilla gorilla*) in different geographical locations[9–13]. The intestinal microbiota appears to be the same among social groups and individual gorillas living in the Bwindi Impenetrable Forest in Uganda and that the flora is largely dominated by entodiniomorph ciliates and helminths, while amoebae and flagellates appear to be absent[9,10]. The coprological studies involving western gorillas also reported many species of parasites, particularly entodiniomorph ciliates and strongylates[11]. Two studies examined fecal samples from western lowland gorillas living in the Dzanga-Ndoki National Park and at Bai Hokou, Central African Republic, and found that most of individuals were infected with strongylates, whereas ascaroids and threadworms were only moderately present[12,13]. Low prevalence of *Entamoeba coli*, *Balantidium coli*, and *Iodamoeba butschlii* have also been recorded in western lowland gorillas along with trichomonads, which were the only protozoans that were present in all gorilla age-sex classes[12,13].

Despite these studies, the diversity of eukaryotic communities in primates, and particularly in gorillas, remains to be elucidated, particularly with regard to intestinal fungal diversity. In addition, the morphological descriptions of these eukaryotes are typically insufficient and thus cannot be considered in taxonomical studies. In this study, we present firstly, an extensive molecular data set of the occurrence of gastrointestinal eukaryotic microbiota including some human eukaryotic pathogens in a single fecal sample from a wild western lowland gorilla from

Cameroon, and then followed by molecular detection of potential human eukaryotic pathogen in gastrointestinal tracts of wild population of gorillas.

Results

Eukaryotes retrieved from gut microbiota of gorilla. In total, 35 existing primer sets targeting the 18S rRNA and internal transcribed spacer (ITS) genes and other specific eukaryotic genes were used to explore the diversity of the eukaryotes that were found in a fecal sample that was obtained from a wild gorilla in Cameroon (Supplementary Tables 1). Seventeen positive PCR products were obtained. Two of them (TFR1/TFR2 and 18ScomF1/Dino18SR1) were sequenced directly as obtained sequences from these two primers yield no trouble sequences, but the remaining were problematic and were thus cloned. Overall, 1,572 clones were obtained from all of the cloning libraries that were constructed in this study (Supplementary Tables 2), resulting in the detection of 87 eukaryotic species in the fecal sample (Fig. 1). Most of the species that were present were fungi (52 species, Table 1), while 10 were identified as protozoa (Table 2), 4 as nematodes (Table 3), and 21 as plants (Table 4).

Fungal diversity. Fifty-two different species of fungi were retrieved from the gorilla fecal sample following the analysis of 428 fungal clones (accounting for 59.8% of the total species detected and 27.2% of the total clones retrieved in this study). These species were detected from different cloning libraries that were generated using various primers (Table 1, Fig. 1). Most of the detected taxa belonged to the phylum Ascomycota (36 species, 69.2% of the identified taxa), followed by taxa from the Basidiomycota (15 species, 28.8% of the detected taxa). The remaining 2% of the taxa were affiliated with the phylum Zygomycota (Table 1). Only 5 fungal species were isolated through culture-dependent methods, including *Alternaria alternata*, *Cladosporium* sp., *Malassezia restricta*, *Malassezia globosa,* and *Malassezia pachydermatis*.

All of the 171 sequenced clones from the fungal ITS clone library that were generated using the ITS1-F/ITS-4R primer set were related to fungal sequences that were found in the GenBank databases with the exception of one clone belonging to the Viridiplantae (green plants) (Tables 1 and 4, Fig. 1). This amplification alone allowed for the identification of 25 different fungal species (roughly half of the fungal species detected and 28.7% of all eukaryotic species recovered in this study) (Fig. 1). The majority of the fungal sequences in this clone library were of ascomycete origin (91.2% of the total number of clones and 80.8% of the species detected); the species were assigned to different taxonomic groups, including Saccharomycetales, Pleosporales, Capnodiales, Eurotiales, Hypocreales, and Xylariales (Table 1). Only 6.4% of the sequences that were retrieved from this ITS library were related to the basidiomycetes, belonging to both Tremellales and Corticiales (Table 1). Our ITS results also showed the presence of a few zygomycetes-related sequences that were represented by *Mortierella* sp. (Table 1).

The primer set NSI/FRI, targeting the fungal 18S rRNA gene, was also used in this study, resulting in a total of 17 fungal species (16 plus one species that was retrieved from fungal ITS amplification) (Table 1, Fig. 1). In contrast to the ITS clone library, the BLAST results for the 96 clones that were obtained from this library revealed that 58 sequences (60.4%) were most closely related to the basidiomycetes, whereas 38 sequences (39.6%) were ascomycetes (Table 1).

In addition to the 41 fungal species that were recovered by the aforementioned fungal-specific primer sets, 11 were identified in other clone libraries when universal eukaryotic primers targeting 18S rDNA were used (Table 1, Fig. 1). Among these 11 species, 8 were detected using the universal eukaryotic primer set Euk1A/Euk516r.

The use of different specific and universal 18S clone libraries revealed a total of 30 fungal species in the stool sample (Fig. 1), while PCR methods using fungal ITS genes enabled the detection of 25 species (Fig. 1). Only 3 species, *A. alternata*, *Candida rugosa* and *Hanseniaspora occidentalis*, were detected in both the ITS and 18S amplifications, thus suggesting the complementarity of these approaches for the assessment of fungal communities in the gorilla intestinal tract.

Protozoal diversity. Ten different species of protozoans were detected in the gorilla fecal sample following both the sequencing of 246 protozoal-related clones (15.6% of all clones in this study) that were obtained from 8 different cloning libraries and the direct sequencing of the positive PCR product that were obtained with the TFR1/TFR2 primer set (Table 2, Fig. 1). Six species belonging to Ciliophora were detected from two of the cloning libraries, including *Blepharocorys curvigula*, *Cycloposthium bipalmatum*, *Cycloposthium ishikawai*, *Parentodinium* sp., *Triplumaria selenica* and *Troglodytella abrassarti* (Table 2, Fig. 1). Ninety-six sequences amplified by *Leishmania* specific primers were assigned to Trypanosomatidae family (Fig. 1, Table 2) as the best hit of BLAST results of these sequences was *Leishmania* sp. (with sequence coverage ranged between 69–79%). The remaining protozoans were identified as *Blastocystis* sp., *Iodamoeba* sp., and *Tetratrichomonas buttreyi*, belonging to the three taxa Stramenopiles, Amoebozoa and Parabasalia, respectively (Table 2, Fig. 1).

Helminthic diversity. Despite the use of several primers to detect the occurrences of trematodes, cestodes and nematodes in the gorilla fecal sample (Supplementary Tables 1), only the PCR amplifications using the latter primer pairs (NC1/NC2) yielded positive results; these PCR products were used to construct the nematode clone libraries (Table 3). Four nematode species, *Necator americanus*, *Libyostrongylus douglassi*, *Oesophagostomum* sp., and *Oesophagostomum stephanostomum,* were obtained from sequencing 192 positive clones in this library (Table 3, Fig. 1). The human parasitic nematode, *N. americanus*, accounted for 55.7% of this clone library (Table 3).

Plant diversity. Twenty-one plant species (Table 4) were retrieved in this study from 13 different cloning libraries that were generated using primer pairs targeting the 18S rRNA, ITS and chloroplast genes (Supplementary Tables 2). The plant-related clones constituted roughly 44.9% of all clones that were sequenced in this study. The majority of plant sequences that were detected in most of the cloning libraries belonged to *Manilkara zapota* and *Musa basjoo*, which comprised 28.6% and 16.7% of the total plant-related sequences, respectively (Table 4, Fig. 1). Only two plant species, *Schima superba* and *Davidia involucrata*, were detected from the chloroplast clone libraries that were constructed using the primer set rbcLZ1/rbcL19b; the 19 remaining plant species were retrieved from the 18S rRNA cloning libraries that were constructed from various 18S rRNA primer pairs (Table 4, Fig. 1).

Eukaryotic human pathogens in fecal samples of gorillas. Real time PCR examination (Supplementary Table 3) of 48 fecal samples from 21 individual of wild gorillas, sampled from Minton and Messok location in Cameroon, showed that 41 (18 gorillas, 86%) and 11 (9 gorillas, 43%) of gorillas' fecal samples harbor *Oesophagostomum bifurcum* and *N. americanus* respectively (Table 5). The results also showed that human pathogenic fungi such as *Candida tropicalis, Candida parapsilosis, C. rugosa, M. restricta, M. globosa*, *Trichosporon* spp. and *Trichosporon asahii* were also detected in feces of these wild animals as shown in Table 5. However, *Blastocystis sp.*, *Candida albicans*, *M. pachydermatis* and *Rhodotorula mucilaginosa* were not detected in any gorillas' feces.

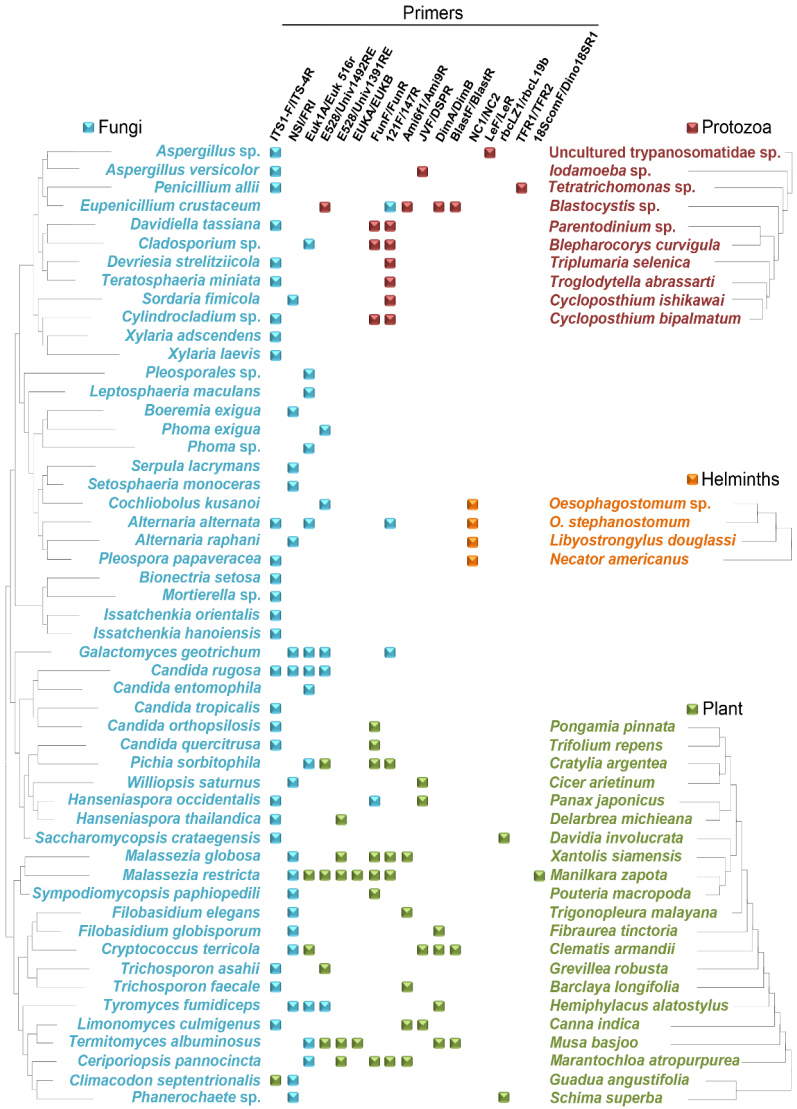

Figure 1 | The eukaryotic species that were retrieved from the gut of *Gorilla gorilla gorilla* according to the different primers used. A box indicates that the species was positive with the primer set used. Blue color = fungi; red color = protozoa; orange color = helminths; and green color = plants. The trees were constructed using the free software MEGA 5 and sequences that were retrieved from GenBank (ITS sequences for fungi and helminths and 18S rDNA for protozoa and plants).

Discussion

Previous studies focusing on the detection and identification of eukaryotic communities residing in the non-human primate gastrointestinal tract analyzed the gut microbiota using the morphological features of the flora[9–13], limiting the acquisition of knowledge regarding the real eukaryotic intestinal contents of primates. Regardless of some limitations that are associated with culture-independent methods[14], these methods have recently been used to explore the eukaryotic diversity of the human gut[15–20], and these techniques have played a crucial role in providing novel insight into the true diversity and composition of the gut microbiota. Because very little is known about both the diversity of eukaryotic organism occurring in the digestive tract of non-human primates and the presence of potential pathogens in their guts, two general approaches were used in our investigation; firstly, culture dependent and independent methods were carried out in a single stool sample from [illegible]a then additional

Table 1 | Fungal species obtained from different cloning libraries in this study

Closest relative in NCBI	Similarity%	Taxa	Taxonomic group	Targeted gene	Primer (No. of clones/total)
Alternaria alternata	99	a	Pleosporales	18s rRNA, ITS	NSI/FRI (1/96); Euk1A/Euk 516r (20/126); ITS F/ ITS-4R (15/171)
Alternaria raphani	99	a	Pleosporales	18s rRNA	NSI/FRI (3/96)
Aspergillus sp.	99	a	Eurotiales	ITS	ITS F/ITS-4R (2/171)
Aspergillus versicolor	99	a	Eurotiales	ITS	ITS F/ITS-4R (4/171)
Bionectria setosa	98	a	Hypocreales	ITS	ITS F/ITS-4R (2/171)
Boeremia exigua	99	a	Pleosporales	18s rRNA	NSI/FRI (2/96)
Candida entomophila	99	a	Saccharomycetales	18s rRNA	Euk1A/Euk 516r (1/126)
Candida orthopsilosis	99	a	Saccharomycetales	ITS	ITS F/ITS-4R (94/171)
Candida quercitrusa	99	a	Saccharomycetales	ITS	ITS F/ITS-4R (4/171)
Candida rugosa	99	a	Saccharomycetales	18s rRNA, ITS	NSI/FRI (7/96); Euk1A/Euk 516r (28/126); E528F/ Univ1492RE (17/96); ITS F/ITS-4R (13/171)
Candida tropicalis	99	a	Saccharomycetales	ITS	ITS F/ITS-4R (6/171)
Ceriporiopsis pannocincta	99	b	Polyporales	18s rRNA	Euk1A/Euk 516r (1/126)
Cladosporium sp.	99	a	Capnodiales	18s rRNA	Euk1A/Euk 516r (1/126)
Climacodon septentrionalis	99	b	Polyporales	18s rRNA	NSI/FRI (2/96)
Cochliobolus kusanoi	99	a	Pleosporales	18s rRNA	E528F/Univ1492RE (2/96)
Cryptococcus terricola	99	b	Tremellales	18s rRNA	NSI/FRI (7/96)
Cylindrocladium sp.	99	a	Hypocreales	ITS	ITS F/ITS-4R (4/171)
Davidiella tassiana	99	a	Capnodiales	ITS	ITS F/ITS-4R (6/171)
Devriesia strelitziicola	99	a	Capnodiales	ITS	ITS F/ITS-4R (3/171)
Eupenicillium crustaceum	99	a	Eurotiales	18s rRNA	121F/1147R (2/96)
Filobasidium elegans	98	b	Filobasidiales	18s rRNA	NSI/FRI (4/96)
Filobasidium globisporum	99	b	Filobasidiales	18s rRNA	NSI/FRI (5/96)
Galactomyces geotrichum	99	a	Saccharomycetales	18s rRNA	NSI/FRI (5/96); Euk1A/Euk 516r (3/126); 121F/ 1147R (1/96); E528F/Univ1492RE (27/96)
Hanseniaspora occidentalis	98	a	Saccharomycetales	18s rRNA, ITS	FunF/FunR (4/123); ITS F/ITS-4R (48/171)
Hanseniaspora thailandica	99	a	Saccharomycetales	ITS	ITS F/ITS-4R (5/171)
Issatchenkia hanoiensis	98	a	Saccharomycetales	ITS	ITS F/ITS-4R (7/171)
Issatchenkia orientalis	98	a	Saccharomycetales	ITS	ITS F/ITS-4R (2/171)
Leptosphaeria maculans	99	a	Pleosporales	18s rRNA	ITS F/ITS-4R (2/126)
Limonomyces culmigenus	99	b	Corticiales	ITS	ITS F/ITS-4R (5/171)
Malassezia globosa	99	b	Malasseziales	18s rRNA	NSI/FRI (13/96)
Malassezia restricta	99	b	Malasseziales	18s rRNA	NSI/FRI (8/96)
Mortierella sp.	99	z	Mortierellales	ITS	ITS F/ITS-4R (3/171)
Penicillium allii	99	a	Eurotiales	ITS	ITS F/ITS-4R (3/171)
Phanerochaete sp.	99	b	Polyporales	18s rRNA	NSI/FRI (2/96)
Phoma exigua	99	a	Pleosporales	18s rRNA	E528F/Univ1492RE (3/96)
Phoma sp.	98	a	Pleosporales	18s rRNA	Euk1A/Euk 516r (2/126)
Pichia sorbitophila	98	a	Saccharomycetales	18s rRNA	Euk1A/Euk 516r (20/126)
Pleospora papaveracea	99	a	Pleosporales	ITS	ITS F/ITS-4R (14/171)
Pleosporales sp.	98	a	Pleosporales	18s rRNA	Euk1A/Euk 516r (1/126)
Saccharomycopsis crataegensis	99	a	Saccharomycetales	ITS	ITS F/ITS-4R (7/171)
Serpula lacrymans	99	b	Boletales	18s rRNA	NSI/FRI (6/96)
Setosphaeria monoceras	99	a	Pleosporales	18s rRNA	NSI/FRI (12/96)
Sordaria fimicola	99	a	Sordariales	18s rRNA	NSI/FRI (7/96)
Sympodiomycopsis paphiopedili	99	b	Microstromatales	18s rRNA	NSI/FRI (5/96)
Teratosphaeria miniata	99	a	Capnodiales	ITS	ITS F/ITS-4R (4/171)
Termitomyces albuminosus	99	b	Agaricales	18s rRNA	Euk1A/Euk 516r (23/126)
Trichosporon asahii	98	b	Tremellales	ITS	ITS F/ITS-4R (4/171)
Trichosporon fecale	99	b	Tremellales	ITS	ITS F/ITS-4R (2/171)
Tyromyces fumidiceps	99	b	Polyporales	18s rRNA	Euk1A/Euk 516r (2/126); NSI/FRI (6/96); E528F/ Univ1492RE (1/96)
Williopsis saturnus	99	a	Saccharomycetales	18s rRNA	NSI/FRI (2/96)
Xylaria adscendens	99	a	Xylariales	ITS	ITS F/ITS-4R (2/171)
Xylaria laevis	99	a	Xylariales	ITS	ITS F/ITS-4R (1/171)

a, Ascomycetes; b, basidiomycetes; z, zygomycetes; ITS, internal transcribed spacer.

stool samples of wild gorillas were screened for pathogens by specific PCRs.

Fungal diversity and detection of human fungal pathogens. Culture-dependent approaches limited the number of fungi that were isolated, revealing less fungal diversity because of difficulties that were encountered in their growth using routine laboratory culture[21]. In contrast, the culture-independent methods were more effective and revealed a larger diversity of fungi[16].

Although the molecular-based methods potentially detected a wide range of fungi in this study, but it is still difficult to predict whether they are the real symbiotic fungal components of gut in these animal or potential environmental fungal contaminants. The immediate collection of feces from animal in forest is a complicate process especially with these endangered wild animals and therefore the difficulties in preventing direct contact of feces bulk with its surrounding environment during and after defecation time are a major drawback in such studies. Moreover, the natural yeast some ascomyce-

Table 2 | Protozoal species detected by various clone libraries in this study

Closest relative in NCBI	Similarity%	Taxa	Targeted gene	Primer (No. of clones/total)
Blastocystis sp.	98	Stramenopiles	18s rRNA	Ami6F1/Ami9R (4/96); BlastF/BlastR (39/48); DimA/DimB (9/96); E528F/Univ1492RE (8/96)
Blepharocorys curvigula	99	Ciliophora	18s rRNA	121F/1147R (7/96); FunF/FunR (5/123)
Cycloposthium bipalmatum	98	Ciliophora	18s rRNA	121F/1147R (8/96); FunF/FunR (6/123)
Cycloposthium ishikawai	98	Ciliophora	18s rRNA	121F/1147R (1/96)
Iodamoeba sp.	98	Amoebozoa	18s rRNA	JVF/DSPR (44/96)
Trypanosomatidae	98	Kinetoplastida	ITS1	LeF/LeR (96/96)
Parentodinium sp.	99	Ciliophora	18s rRNA	121F/1147R (2/96); FunF/FunR (2/123)
Tetratrichomonas buttreyi	99	Parabasalia	18s rRNA	TFR1/TFR2 (Direct sequencing)
Triplumaria selenica	98	Ciliophora	18s rRNA	121F/1147R (1/96)
Troglodytella abrassarti	99	Ciliophora	18s rRNA	121F/1147R (14/96)

ITS, internal transcribed spacer.

tous and basidiomycetous species that were detected in this study are considered to be saprobes and thus they possibly representing environmental fungal contaminants as they are often found in association with plants, animals and their interfaces[22]. For example several members of Saccharomycetales such as *Hanseniaspora* spp., *Saccharomycopsis crataegensis*, *Pichia* spp., *Issatchenkia* spp. and *Candida quercitrusa* regard as plant associated fungi and many of them described as common fermentative spoilage yeast[23–27]. *Candida entomophila* characterized by its ability to ferment glucose and D-xylose therefore it usually has been isolated from wood-inhabiting insects and decaying wood[28]. Furthermore, some members of orders Eurotiales, Hypocreales, Xylariales and Pleosporales that have been identified in this study are regarded as the most common environmental fungi and they abundantly occur in forest soils or on fading leaves of herbaceous and woody plants such as cosmopolitan genus of *Penicillium* (Eurotiales) which has been found to play important role as decomposers of organic materials in soil[29], several species of *Bionectria* (Hypocreales) which can be found as common saprophytes on dead broad-leaf trees in forest[30], along with members of the genus *Xylaria* (Xylariales) that usually occur as saprobes or as parasite on flowering plants in lowland forests[31] and finally some species of Pleosporales that occur as saprobic fungi on decaying leaf or animal dung[32].

Another explanation for occurrence of these fungi in feces of gorillas is the fact that these apes are herbivore in their behavior and they feed on different parts of plants in which different fungi coexist. Therefore these fungi could also represent transient contaminants in the gut of gorillas. Moreover, several of the ascomycetous yeast that were detected in this study, such as *Candida orthopsilosis*, *C. rugosa*, *C. tropicalis* and *Galactomyces geotrichum*, have been previously described in the human gut[15,19,33]. Some of the basidiomycetous yeasts that were detected in our sample, such as the *Malassezia* and *Trichosporon* species, are regarded as human pathogens[34,35]. The remaining basidiomycetous fungi that were identified in our study including the saprotrophics, wood decomposers and symbiotic fungi, such as *Termitomyces*, which is generally regarded as a nutritional source for termites[36] that are in turn regarded as a source of food, and particularly of protein and vitamins, for wild gorillas[37]. Finally, using real-time PCR, many human fungal pathogens were detected in gorilla feces including species in the genera *Candida, Malassezia* and *Trichosporon* with high prevalence of *C. tropicalis* (81%), *M. globosa* (43.5%) and *C. parapsilosis* (38%) through our survey (Table 5).

Protozoal Diversity. Numerous studies have been performed on the intestinal parasites of wild non-human primate species, especially on gorillas[9–13]; however, this study, to the best of our knowledge, is the first molecular study attempting to detect both parasitic and commensal protozoans in the gastrointestinal tract of the wild western lowland gorilla. In the present study, the majority of the intestinal protozoa that were detected in the fecal sample belonged to four different phyla: the Ciliophora, Amoebozoa, Parabasalia and Stramenopiles. The most prevalent protozoal species that were found in this study were ciliates; approximately 6 species were detected in the fecal sample, which is in agreement with results from previous studies that identified entodiniomorph ciliates in the majority of fecal samples that had been collected from wild western lowland gorillas at Bai Hokou, Dzanga-Ndoki National Park (Central African Republic) and the Lopé Reserve (Gabon)[11–13]. However, our results conflict with those from the study by Modry *et al.*[38], in which *T. abrassarti* was the sole entodiniomorph ciliate that was detected in captive western lowland gorillas at the Prague Zoo in the Czech Republic. *T. abrassarti* has also been morphologically observed in fecal specimens from wild lowland gorillas[39], where it appears to play an important role in digestion because of its ability to ferment polysaccharides in the hindguts of primates[40].

Our study described the first report of *B. curvigula*, *Parentodinium* sp. and Cycloposthiidae species, including *C. bipalmatum*, *C. ishikawa* and *T. selenica* in the gastrointestinal tract of the wild gorilla.

Members of the Amoebozoa were detected at low frequencies despite the use of many primer sets that target the 18S rRNA genes of the major groups in this phylum (Supplementary Tables 1). Only *Iodamoeba* spp. were found in the present study. The low abundance of amoebae reported here agrees with studies by Freeman *et al.*[13] and Lilly *et al.*[12], both of whom reported the low prevalence of amoebae in fecal samples of wild lowland gorillas. However, our results disagree with the study by Sleeman *et al.*[10], who reported the high prevalence of amoebae in mountain gorillas. *Blastocystis* sp. was retrieved from the intestinal tract of one gorilla in this study. Screening the 48 samples from 21 individual of gorillas has demonstrated that this protozoan is not frequent within western lowland gorillas in

Table 3 | Nematode species detected by the NC1/NC2 clone library in this study

Closest relative in NCBI	Similarity%	Taxa	Targeted gene	Primer (No. of clones/total)
Libyostrongylus douglassi	99	Nematoda	ITS	NC1/NC2 (40/192)
Necator americanus	99	Nematoda	ITS	NC1/NC2 (107/192)
Oesophagostomum sp.	99	Nematoda	ITS	NC1/NC2 (39/192)
Oesophagostomum stephanostomum	99	Nematoda	ITS	NC1/NC2 (6/192)

Table 4 | Plant species retrieved from the fecal sample of a wild gorilla

Closest relative in NCBI	Similarity%	Targeted gene	Primer (No. of clones/total)
Barclaya longifolia	98	18s rRNA	Ami6F1/Ami9R (2/96)
Canna indica	99	18s rRNA	Ami6F1/Ami9R(1/96); JVF/DSPR (3/96)
Cicer arietinum	99	18s rRNA	JVF/DSPR (2/96)
Clematis armandii	99	18s rRNA	BlastF/BlastR (4/48); DimA/DimB (13/96); Euk1A/Euk 516r (5/126); JVF/DSPR (41/96)
Cratylia argentea	99	18s rRNA	FunF/FunR (2/123); 121F/1147R (2/96); E528F/Univ1492RE (1/96)
Fibraurea tinctoria	99	18s rRNA	DimA/DimB (54/96)
Grevillea robusta	98	18s rRNA	E528F/Univ1492RE (1/96)
Guadua angustifolia	99	ITS	ITS1-F/ITS-4R (1/171)
Hemiphylacus alatostylus	99	18s rRNA	DimA/DimB (2/96)
Manilkara zapota	98	18s rRNA	FunF/FunR (79/123); 121F/1147R (36/96); Euk1A/Euk 516r (17/126); E528F/Univ1492RE (23/96); E528F/Univ1391RE (24/72); EUKA/EUKB(23/72); 18ScomF/Dino18SR1 (direct sequencing)
Marantochloa atropurpurea	99	18s rRNA	Ami6F1/Ami9R (74/96); FunF/FunR (15/123); 121F/1147R(11/96); E528F/Univ1391RE (3/72)
Musa basjoo	99	18s rRNA	BlastF/BlastR (5/48); DimA/DimB (18/96); E528F/Univ1492RE (12/96); E528F/Univ1391RE (33/72); EUKA/EUKB (49/72)
Panax japonicus	98	18s rRNA	JVF/DSPR (6/96)
Pongamia pinnata	98	18s rRNA	FunF/FunR (1/123)
Pouteria macropoda	99	18s rRNA	FunF/FunR (1/123)
Trifolium repens	99	18s rRNA	FunF/FunR (1/123)
Trigonopleura malayana	99	18s rRNA	Ami6F1/Ami9R (3/96)
Xantolis siamensis	99	18s rRNA	Ami6F1/Ami9R (12/96); FunF/FunR (7/123); 121F/1147R (10/96); E528F/Univ1391RE (11/72)
Schima superba	99	rbcLgene*	rbcLZ1/rbcL19b (72/96)
Davidia involucrata	98	rbcLgene*	rbcLZ1/rbcL19b (24/96)
Delarbrea michieana	98	18s rRNA	E528F/Univ1391RE (1/72)

*chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit.

Cameroon (Table 5). *Blastocystis* can be found in both humans and nonhuman primates[41,42]. In humans, it appears to be a causative agent of irritable bowel syndrome (IBS) in certain circumstances[41], while in non-human primates, its role remains unclear.

Either none or very few enteric flagellate protozoa have been observed in the fecal samples of wild gorillas[9–13]; additionally, only some members of the trichomonads and *Giardia* sp. have been recorded in both captive and wild gorillas, respectively[10,43]. These previous studies are in partial agreement with our study, in which only *T. buttreyi*, a trichomonad, was detected. *T. buttreyi* has also been detected in ruminant feces, and it appears to be harmless to its host[44].

Helminthic diversity and detection of human nematode pathogens. We attempted to determine the presence of trematodes, cestodes and nematodes in the fecal samples of the gorillas. Only parasites belonged to the phylum Nematoda were identified; we did not report the presence of any other groups of helminths. Previous studies that were conducted in wild lowland gorilla populations in Gabon and the Central African Republic showed also an absence of cestodes and a scarcity of trematodes in fecal samples[11–13].

The presence of nematode species, such as *O. stephanostomum,* in the intestinal tract of gorillas has been morphologically confirmed by Sleeman *et al.*[10]. *Oesophagostomum* spp. can also infect ruminants, pigs and monkeys[45]. Some reports have described human infections with *Oesophagostomum* spp., particularly in northern Togo and Ghana where they have been known to cause serious health problems[45,46]. Another nematode that was recorded in our study was *N. americanus*, which is an obligate hookworm parasite that is responsible for most common chronic infections in humans, particularly in areas of rural poverty in the tropics and subtropics[47]. This hookworm is generally transmitted through contact with contaminated soil and resides in the intestinal tract of its host[47]. The hookworm's infection with *N. americanus* has been described previously in intestinal tract of both mountain gorillas (*G. g. beringei*) inhabiting at Bwindi Impenetrable National Park, South West Uganda[48] and western lowland gorillas (*G. g. gorilla*) residing at Dzanga-Sangha Protected Areas, Southwest of Central African Republic[49]. Finally, an ostrich-specific nematode (*L. douglassi*) was also detected in our sample. As this parasite commonly infects the ostrich proventriculus and can cause libyostrongylosis, which has a high mortality rate among juvenile birds[50], the detection of this nematode in gorilla's

Table 5 | Eukaryotic Human Pathogens detected by Real Time PCR in 48 fecal samples from wild gorillas (21 individuals)

Real time PCR	Target	No. of positive fecal samples	No. of positive gorilla	Frequency
Necator americanus	ITS	11	9	43%
Oesophagostomum bifurcum	18s	41	18	86%
Candida parapsilosis	ITS	15	8	38%
Candida rugosa	ITS	4	2	9.5%
Candida tropicalis	ITS	25	17	81%
Malassezia globosa	26s	13	9	43%
Malassezia restricta	26s	14	8	31%
Trichosporon asahii	ITS	2	2	9.5%
Trichosporon spp.	ITS	13	7	33%

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feces in this study could be resulted either from environmental contamination or consumption of contaminated food items. Analyzing of more fecal samples is needed to further explaining the presence of this parasite in feces of this animal. In our survey, high percentage of both human parasitic worms; *O. bifurcum* and *N. americanus* (86% and 43% respectively) was discovered in the stool samples of western lowland gorillas from Cameroon (Table 5).

Residual plants in gut of gorilla. Gorillas are largely herbivorous and consume a wide variety of plant species (between 50 and 300)[51,52]. Studies of western gorillas have shown that fruit is an essential part of their diet[53,54], but they also eat leaves, shoots, flowers, and the woody parts of plants[51,52]. In this study, we detected 21 different plant species in the fecal sample of a wild gorilla collected around the village of Minton. Primer sets targeting both 18S rRNA and the chloroplast *rbcL* genes were used to identify the residual plant species in the gorilla feces. Unexpectedly, only two phylotypes of plants were detected when the primer targeting the chloroplast *rbcL* gene was used. These results are in agreement with those of Bradle *et al.*[55], who also detected few plants in western gorilla feces using the same primer set, which preferentially amplifies DNA from chloroplast-rich tissues, such as leaves or stems, rather than fruits, flowers and seeds[55]. Not surprisingly, roughly 19 plant species were co-amplified along with other eukaryotes in this study when universal eukaryotic primers targeting 18S rRNA were used. These plant species belong to different families that may be consumed by wild western lowland gorillas.

In conclusions, this is the first study to characterize fecal eukaryotic diversity, including fungi, in non-human primates using a comprehensive extended molecular analysis. The multiple primer set approach used herein enabled us to recover a high diversity of eukaryotes from the intestinal tract of the wild lowland gorilla, which may include human pathogens as revealed by our real-time PCR assessments in gorillas' feces. Although the detection of fungi species should be interpreted cautiously because the possibility of environmental contamination, the presence of human parasites in gorillas should be viewed as an important public health concern, particularly for surrounding rural villages where habitat overlap is frequent. Additional studies from other geographic locations and using the methodological strategy presented here are required for detailed descriptions of the occurrences and abundances of eukaryotes, including pathogens, in the guts of non-human primates, which have until now been poorly described.

Methods

Source of fecal samples. A total of 48 fecal samples were collected from 21 individual western lowland gorillas (*G. g. gorilla*) in this study (Supplementary Tables 4). One fecal sample was collected in a site near Minton village which located in south-central Cameroon and was used in this study for exploring the occurrence of gut eukaryotes in gorilla intestinal tract through using PCR-based amplification using various primers, followed by cloning and sequencing, while the rest of 47 fecal samples were collected from different sites around Messok village which located in the south-east Cameroon and were used in this study for investigating the presence of human eukaryotic pathogens in gut of gorillas. The sample collection protocol was described previously[56]. The GPS position, time and date were recorded for all samples. The fecal samples were preserved in RNA*later* (Ambion, Austin, TX) and kept at room temperature at base camps for less than 3 weeks then transported to a central laboratory and kept at −80°C.The collection of the fecal samples was approved by the Ministry of Scientific Research and Innovation of Cameroon. No other permit was required for the described field as this research was non-invasive work and the collection of the samples from soil did not disrupt the wild fauna.

DNA extraction. Total DNA was extracted from the frozen fecal samples using a modification of the Qiagen stool procedure and the Qiamp® DNA Stool Mini Kit (Qiagen, Courtaboeuf, France)[16]. The inner part of the fecal bulk was used for extraction to avoid as much as possible an eventual contamination with soil organisms and/or environmental species during collection as previously described by[54]. Aliquots of 200 mg of this part were added into tubes containing a 200 mg mixture of 0.1, 0.5, and 2 mm zirconium beads and 1.5 ml of ASL buffer (Qiagen). The samples were mixed vigorously by agitation in a FastPrep BIO 101 agitator (Qbiogene, Strasbourg, France) at 3,200 rpm for 90 seconds. Agitation was followed by heating at 95°C for 10 min to increase both the yield of DNA and proteinase K digestion before the DNA was bound to a column, washed, and eluted in TE buffer.

Genomic amplification. All universal and specific eukaryotic primers targeting both the ITS and 18S rRNA genes that were used in this study were adopted from previously published studies (Supplementary Tables 2). The 50 µL PCR reaction mixture contained 5 µL of dNTPs (2 mM of each nucleotide), 5 µL of DNA polymerase buffer (Qiagen, Courtaboeuf, France), 2 µl of $MgCl_2$ (25 mM), 0.25 µL of HotStarTaq DNA polymerase (1.25 U) (Qiagen, Courtaboeuf, France), 1 µL of each primer, and 5 µL of DNA. The PCR cycling conditions for all amplifications were as follows: 1 cycle at 95°C for 15 min, 40 cycles at 95°C for 0.5 min, 48–60°C for 0.5–2 min (Supplementary Tables 2), and 72°C for 1–2 min, followed by a final cycle at 72°C for 5 min. All amplifications were performed in a PCR system 2720 thermal cycler (Applied Biosystems, Courtaboeuf, France). Amplification products were visualized on a 1.5% agarose gel that was stained with ethidium bromide and viewed under a UV light source. The PCR products were purified using the Nucleo-Fast® 96 PCR Kit (Marcherey-Nagel, Hoerdt, France) according to the manufacturer's instructions.

Cloning, Sequencing and phylogenetic analyses. The cloning and sequencing reactions were performed as previously described[16]. The PCR products were cloned separately using the pGEM® -T Easy Vector System Kit (Promega, Madison, USA). Aliquots (150 µl) of cell suspensions were plated onto LB (Luria-Bertani Broth) agar plates that were supplemented with ampicillin (100 mg/mL), X-GAL (80 mg/mL) and IPTG (120 mg/mL), and the plates were incubated overnight at 37°C. Positive clones were suspended in 25 µL of distilled water and stored at −20°C. The presence of the insert was confirmed by PCR amplification using the M13 forward (5′-GTAAAACGACGGCCAG-3′) and M13 reverse (5′-AGGAAACAGCTATGAC-3′) primers (Eurogentec, Seraing, Belgium). The purified PCR products were sequenced in both directions using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing products were run on an ABI PRISM 3130 automated sequencer (Applied Biosystems). Finally, intestinal eukaryotes were identified by comparing the resulting sequences with those that were deposited in GenBank using the basic local alignment search tool (BLAST), which is available at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/). Phylogenetic analyses were performed using MEGA5.04 and a distance matrix neighbor-joining (NJ) approach[57].

Real-Time PCR Assay for Detection of human pathogen. Primers and probes specific to some human eukaryotic pathogens were used as described previously (Supplementary Table 3). For the primers and probes used for first time in this study, sequences corresponding to each species were collected in GenBank and aligned using multiple sequence alignment ClustalW2, and the PRIMER 3 software[58] was used to design primer sets in the conserved regions of aligned sequences. The specificity of each primer was tested using the basic local alignment search tool (BLAST), which is available at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/). The real-time PCR reactions were conducted using 25 µL total volumes and analyzed for 44 cycles using a CFX96™ Real-Time PCR Detection System (BIO-RAD, Life Science, Marnes-la-Coquette, France) following methods recommended by the manufacturer. Amplification reactions were done as follows: 95°C for 15 min, 60°C for 0.5 min, and 72°C for 1 min.

Species confirmation and Microsatellite analyses. The DNA was extracted from gorilla fecal samples in order to determine the number of individuals that carrying human eukaryotic pathogens. Total of 48 samples were genotyped at 7 polymorphic loci (D18s536, D4s243, D10s676, D9s922, D2S1326, D2S1333 and D4S1627) as described previously[56]. The gender of gorillas was determined by amplification of a region of the amelogenin gene that contains a deletion in the X, but not the Y chromosome[56]. To exclude the allelic dropout, all loci were amplified four times. Aliquot 1 µL of PCR products was mixed with 10 µL of formamide and 0.25 µL of the ladder marker (ROX GeneScan 400HD, Applied Biosystem), The resulting amplifications were analyzed by 3130xl Genetic Anlayser sequencer (Applied Biosystem, Foster City,CA). Amplification products were visualized and sized using Genemapper 3.7 software (Applied Biosystems).

Culturing and identification of fungal species. The fecal samples were serially diluted, and six-fold dilutions were spread onto potato dextrose agar (Fluka® Analytical, France), Czapek dox agar (Fluka® Analytical, France) and Dixon agar. The plates were incubated aerobically at room temperature. The colonies exhibiting different morphologies were restreaked to obtain pure cultures. The fungi were amplified using fungal primers (ITS1-F/ITS-4R) and identified as previously described[16].

Nucleotide sequence accession numbers. All sequences obtained in this work have been deposited in GenBank database with the accession numbers JX158488 to JX159965.

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Author contributions

D.R. and F.B. designed the experiments; I.H., M.K. conducted the experiments; I.H., M.K., M.P., E.D., D.R. and F.B. analyzed the results; I.H. and F.B. prepared the figure; I.H. and F.B. wrote the manuscript. All authors reviewed the manuscript.

Additional information

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Supplementary Information

Pathogenic Eukaryotes in Gut Microbiota of Western Lowland Gorillas as Revealed by Molecular Survey

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Supplementary Table 1: Primers used in this study.

Phyla	Primer	Sequence (5′ to 3′)	Targeted Sequence	Product size	Ta	Reference
Microsporidia	V1	CACCAGGTTGATTCTGCCTGAC	18S rRNA	250-279	55	[1]
	PMP2	CCTCTCCGGAACCAAACCCTG				
Entamoeba	JVF	GTTGATCCTGCCAGTATTATATG	18S rRNA	600 to 650	57	[2]
	DSPR2	CACTATTGGAGCTGGAATTAC				
Eukarya	Euk1A	CTGGTTGATCCTGCCAG	18s rRNA	570-600	55	[3]
	Euk 516r-	ACCAGACTTGCCCTCC				[4]
Eukarya	FUNF	GATCCCTAGTCGGCATAGTT	18s rRNA	1000	52	[5]
	FUNR	GTAGTCATATGCTTGTCTC				
Eukarya	EUKA	AACCTGGTTGATCCTGCCAGT	18s rRNA	1800	55	[6]
	EUKB	TGATCCTTCTGCAGGTTCACCTAC				
Eukarya	NSI	GTAGTCATATGCTTGTCTC	18s rRNA	1650	48	[7]
	FR1	AICCATTCAATCGGTAIT				[8]
Fungi	ITS F	CTTGGTCATTTAGAGGAAGTAA	ITS	580	50	[7]
	ITS-4R	TCCTCCGCTTATTGATATGC				[9]
Dinoflagellates	18ScomF1	GCTTGTCTCAAAGATTAAGCCATGC	18S rRNA	650	58	[10]
	Dino18SR1	GAGCCAGATRCDCACCCA				
Trichomonads	TFR1	TGCTTCAGTTCAGCGGGTCTTCC	5,8S rRNA	338-391	60	[11]
	TFR2	CGGTAGGTGAACCTGCCGTTGG				
Diplomonads	DimA	AACCTGGTTGATCTTGCCAG	18S rRNA	-	55	[12]
	DimB	CYGCAGGTTCACCTACGGAA				
Kinetoplastidia	Kineto_kin1	GCGTTCAAAGATTGGGCAAT	18S rRNA	600-650	55	[13]
	Kineto_kin2	CGCCCGAAAGTTCACC				
Amoeba	Ami6F1	CCAGCTCCAATAGCGTATATT	18S rRNA	830	55	[14]
	Ami9R	GTTGAGTCGAATTAAGCCGC				
Acanthamoeba	JDP1	GGCCCAGATCGTTTACCGTGAA	18S rRNA	460-470	60	[15]

	JDP2	TCTCACAAGCTGCTAGGGAGTCA				
Naegleria	F	GAACCTGCGTAGGGATCATTT	ITS	388 -376	55	[16]
	R	TTTCTTTTCCTCCCCTTATTA				
Hartmannella	Hv1227F	TTACGAGGTCAGGACACTGT	18S rRNA	501	56	[17]
	Hv1728R	GACCATCCGGAGTTCTCG				
Ciliophora	121 F	CTGCGAATGGCTCATTAMAA	18S rRNA	750	55	[18]
	1147R	GACGGTATCTRATCGTCTTT				
Diatoms	18SF	GTTTCCGTAGGTGAACCTGC	18S rRNA	700-900	60	[19]
	28SR	GCTTATTAATATGCTTAAATTCAGCG				
Rhodophyta	RUBI_F	CGCTGCTAAAACTTGTGGGC	RUBISCO	500	56	[20]
	RUBI_R	GGCGTTGTAATAAGAATCCTGG				
Chlorophyta	UCP1_F	CAAGCWCCDGCAGAAGACC	rps11-rpl2	384	54	[21]
	UCP1_R	CCMAAACATAAACAAMSWCAGG				
Euglenophyta	EAF	GTCATATGCTTYKTTCAAGGRCTAAGCC	18S rRNA	-	55	[22]
	EAF3	TCGACAATCTGGTTGATCCTGCCAG				
Eukarya	E528F	CGGTAATTCCAGCTCC	18s rRNA	1000-1300	55	[23]
	Univ1391RE	ACCTTGTTACGRCTT				[24]
Eukarya	E528F	CGGTAATTCCAGCTCC	18s rRNA	1000-1300	55	[23]
	Univ1492RE	GGGCGGTGTGTACAARGRG				[24]
Eukarya	EK1F	CTGGTTGATCCTGCCAG	18s rRNA	1520	55	[25]
	EK-1520	CYGCAGGTTCACCTAC				
Eukarya	EK-82F	GAAACTGCGAATGGCTC	18s rRNA	1432	55	[25]
	EK-1520	CYGCAGGTTCACCTAC				
Malassezia	MF	TAACAAGGATTCCCCTAGTA	28s rRNA	580	55	[26]
	MR	ATTACGCCAGCATCCTAAG				
Helminths	NC1	ACGTCTGGTTCAGGGTTGTT				[27]
	NC2	TTAGTTTCTTTTCCTCCGCT	18s rRNA	310-410	55	
Helminths	nad1T-Fw	GGKTATTCTCARTTTCGTAAGGG	18s rRNA			[28]

	nad1T-Rv	ATCAAATGGAGTACGATTAGTYTCAC		507	52	
Helminths	fashF	TATGTTTTGATTTTACCCGGG	COI			[29]
	fahR	ATGAGCAACCACAAACCATGT		400-500	56	
Helminths	Asc6	CGAACGGCTCATTACAACAG	18s rRNA		52	[30]
	Asc7	TCTAATAGATGCGCTCGTC				
Helminths	RTFlukeFa	CTTGAACGCACATTGCGGCC	ITS			[31]
	RTFlukeRa	CACGTTTGAGCCGAGGTCAG			60	
Giardia	GdhF	TCAACGTCAACCGCGGCTTCCGT	18s rRNA			[32]
	GdhR	GTTGTCCTTGCACATCTCC		500	60	
Leishmania spp	LeF	TCCGCCCGAAAGTTCACCGATA	ITS1			[33]
	LeR	CCAAGTCATCCATCGCGACACG			65	
Plasmodium spp	PLAS 1	GAGAATTATGGAGTGGATGGTG	cytochrome b	709	55	[34]
	PLAS 2	GTGGTAATTGACATCCWATCC				
Plant	rbcLZ1	ATGTCACCACAAACAGAGACTAAAGCAAGT	rbcL	158		[35]
	rbcL19b	CTTCTTCAGGTGGAACTCCAG			57	
Blastocystis spp	Blast1F	ATCTGGTTGATCCTGCCAG	18s rRNA	600	59	[36]
	Blasto1R	GAGCTTTTTAACTGCAACAAC				
Leishmania spp	rDNA-10 F	CAATACAGGTGATCGGACAGG	ITS	526	55	[37]
	rDNA-14R	CACGGGGATGACACAATAGAG				
Leishmania spp	L.cyt-S	GGTGTAGGTTTTAGTYTAGG			55	[38]
	L.cyt-R)	CTACAATAAACAAATCATAATATRCAATT	Cyt b			
Leishmania spp	LCBF	GGTGTAGGTTTTAGTTTAGG		1056	50	[39]
	LCBR2	CTACAATAAACAAATCATAATATACAATT	Cyt b			
Leishmania spp	LGITSF2	GCATGCCATATTCTCAGTGTC	ITS2	430	60	[40]
	LGITSR2	GGCCAACGCGAAGTTGAATTC				

Ta, annealing temperature; E, Environments; RUBISCO, Ribulose-1, 5-bisphosphate carboxylase oxygenase; COI, cytochrome oxidase subunit I; rbcL, ribulose-bisphosphate carboxylase gene.

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Supplementary Table 2: Cloning libraries constructed in this study.

Cloning library	No. of clones	fungi	protozoa	Helminths	plant
121F/1147R	96	3	6		
Ami6F1/Ami9R	96		1		5
BlastF/BlastR	48		1		2
DimA/DimB	96		1		4
E528F/Univ1391RE	72				4
E528F/Univ1492RE	96	5			4
Euk1A/Euk 516r	126	12			2
EUKA/EUKB	72				2
FunF/FunR	123	1	3		7
ITS1-F/ITS-4R	171	25			1
JVF/DSPR	96		1		3
LeF/LeR	96		2		
NC1/NC2	192			4	
NSI/FRI	96	17			
rbcLZ1/rbcL19b	96				2
Total clones	1572				
TFR1/TFR2	direct sequence		1		
18ScomF1/Dino18SR1	direct sequence				1

Supplementary Table 3: Primers and probes used in Real time PCR for detection of human eukaryotic pathogens in fecal samples of gorillas.

Target	Primers or probes	Sequence (5′ to 3′)	Reference
Fungi	ITS3	GCATCGATGAAGAACGCAGC	[1]
Fungi	ITS4	TCCTCCGCTTATTGATATGC	[1]
Candida albicans	CA	6FAM- ATTGCTTGCGGCGGTAACGTCC-MGB	[1]
Candida parapsilosis	CP	6FAM- ACAAACTCCAAAACTTCTTCCA-MGB	[1]
Candida rugosa	CR	6FAM- AACGCTTATTTTGCTAGTGGCC-MGB	[1]
Candida tropicalis	CT	AA CGC TTA TTT TGC TAG TGG CC	[1]
Malassezia	Mal1F	TCTTTGAACGCACCTTGC	[2]
Malassezia spp.	Mal1R	AHAGCAAATGACGTATCATG	[2]
Malassezia spp.	Mal	6FAM-ATGCCTGTTTGWGTGC-MGB	[2]
Malassezia globosa	glob	6FAM- ATAACTGCTTTTCTTCTCT-MGB	[2]
Malassezia restricta	restr	6FAM- CGCCTCCTCCCAAAC-MGB	[2]
Malassezia pachydermatis	pachy	6FAM- CCTCGCCTGACTGTTT-MGB	[2]
Blastocystis sp.	prMSJ2-F	CACACTGTGATTCTCGGG	[3]
Blastocystis sp.	prMSJ2-R	GAAATGGAAGATGGAATTGATGAC	[3]
Blastocystis sp.	Blast	6FAM-AACTCAAACTGCGTCGGATGATGCA-TAMRA	In this study
Trichosporon spp.	TrichF	GTCCGGTGGATAAAGGTAG	In this study
Trichosporon spp.	TrichR	GCTCGAACGTACCCGAAG	In this study
Trichosporon spp.	Trich	6FAM- GCCATAAAGGTGAGCTGCAGT-TAMRA	In this study
Trichosporon asahii	TaF	GTGCTTTCAGAGGCTGAGG	In this study
Trichosporon asahii	TaR	GAAGGACACACACGAGAGC	In this study
Trichosporon asahii	tasahii	6FAM- GATGTGAAGTCAGAGTTGGGAGC-TAMRA	In this study
Leishmania spp.	lishF	ACAAGTGCTTTCCCATCG	[4]
Leishmania spp.	lishR	CCTAGAGGCCGTGAGTTG	[4]
Leishmania spp.	liesh	6FAM- CGGTTCGGTGTGTGGCGCC-TAMRA	[4]
Rhodotorula mucilaginosa	RhodF	GTGCACTTGTTTGGGATAG	In this study
Rhodotorula mucilaginosa	RhodF	CTTCATCGATGCGAGAGC	In this study
Rhodotorula mucilaginosa	Rhod	6FAM- CTCTCGCAAGAGAGCGAACTCCT-TAMRA	In this study
Necator americanus	Na58F	CTGTTTGTCGAACGGTACTTGC	[5]
Necator americanus	Na158R	ATAACAGCGTGCACATGTTGC	[5]

Necator americanus	Na81MGB	6FAM- CTGTACTACGCATTGTATAC	[5]
Oesophagostomum bifurcum	oeso31F	GTTTGTCGAACGATGCTTACATTT	[5]
Oesophagostomum bifurcum	oeso136R	CGAGGTCACGACATCCGTG	[5]
Oesophagostomum bifurcum	Obif63TMGB	6FAM- CCTCGTTCTAGATAAGAAAT-MGB	[5]

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Supplementary Table 4: Microsatellite analyses of gorillas' fecal samples used in this study.

Fecal sample*	Individual ID	Date of collection (day/mo/yr)	Sex†	D18S536‡	D4S243‡	D10S676‡	D9S922‡	D2S1326‡	D2S1333‡	D4S1627‡
MS7209	MSg-ID1	21/10/2007	M	150/150	181/189	188/196	260/276	251/251	318/334	230/238
MS7204	MSg-ID1	21/10/2007	M	150/150	181/189	196/196	260/276	251/251	318/334	230/238
MS7211	MSg-ID1	21/10/2007	M	138/150	181/181	196/196	260/276	251/251	318/334	230/238
MS7193	MSg-ID1	21/10/2007	M	150/150	181/189	196/196	260/276	251/251	318/334	230/238
MS7202	MSg-ID1	21/10/2007	M	150/150	181/189	196/196	260/276	251/251	318/334	230/238
MS7166	MSg-ID1	21/10/2007	M	150/150	189/189	188/196	260/260	251/251	318/318	230/230
MS7178	MSg-ID1	21/10/2007	M	150/150	181/189	196/196	260/276	251/251	318/334	230/238
MS7172	MSg-ID2	21/10/2007	F	146/146	189/201	196/196	268/276	275/275	298/298	234/238
MS7175	MSg-ID3	21/10/2007	M	146/146	177/181	180/196	258/272	255/259	286/314	230/238
MS7187	MSg-ID3	21/10/2007	M	146/146	177/181	180/196	258/272	255/259	286/314	230/238
MS7190	MSg-ID3	21/10/2007	M	146/146	177/181	180/196	258/272	255/259	286/314	230/238
MS7212	MSg-ID4	21/10/2007	M	146/150	189/189	180/196	264/276	263/281	314/334	234/238
MS7179	MSg-ID5	21/10/2007	M	146/146	189/189	196/196	274/277	263/275	318/334	230/238
MS7180	MSg-ID5	21/10/2007	M	146/146	189/189	196/196	274/277	263/275	318/334	230/238
MS7200	MSg-ID5	21/10/2007	M	146/146	189/189	196/196	274/277	263/275	318/334	230/238
MS7158	MSg-ID6	19/10/2007	M	150/154	185/185	200/204	274/280	251/255	322/326	234/234
MS7159	MSg-ID7	19/10/2007	M	146/150	189/197	200/204	276/284	247/251	326/334	-
MS7161	MSg-ID8	19/10/2007	M	142/150	189/189	200/200	266/270	251/255	322/322	278/278
MS7176	MSg-ID8?	21/10/2007	M	142/150	189/189	200/200	266/270	251/255	322/322	242/242
MS7152	MSg-ID9?	18/10/2007	M	150/154	177/193	196/200	266/270	279/279	298/334	-
MS7154	MSg-ID9?	18/10/2007	M	150/154	177/193	196/200	266/270	279/279	298/334	-
MS7153	MSg-ID9	18/10/2007	M	150/154	177/193	196/200	266/270	279/279	298/334	234/238
MS7155	MSg-ID9	18/10/2007	M	150/154	177/193	196/200	266/270	279/279	298/334	234/238
MS7156	MSg-ID9	18/10/2007	M	150/154	177/193	196/200	266/270	279/279	298/334	234/238
MS7157	MSg-ID9	18/10/2007	M	150/154	177/193	196/200	266/270	279/279	298/334	234/238

MS7162	MSg-ID10	19/10/2007	F	150/154	185/189	196/196	270/274	271/275	294/294	246/262
MS7188	MSg-ID11	21/10/2007	M	142/146	177/197	196/200	262/280	267/267	298/314	238/242
MS7168	MSg-ID12	21/10/2007	M	146/154	181/197	196/200	270/274	251/255	298/328	234/238
MS7169	MSg-ID12	21/10/2007	M	146/154	181/197	196/200	270/274	255/255	298/328	234/238
MS7170	MSg-ID12	21/10/2007	M	146/154	181/197	196/200	270/274	255/255	298/328	234/238
MS7171	MSg-ID13	21/10/2007	M	146/150	181/189	196/200	270/274	259/283	298/330	334/338
MS7173	MSg-ID14	21/10/2007	M	146/150	193/197	180/200	276/280	255/267	306/306	230/242
MS7174	MSg-ID14	21/10/2007	M	146/150	193/197	180/200	276/280	255/267	306/306	230/242
MS7197	MSg-ID14	21/10/2007	M	146/150	193/197	180/200	276/280	255/267	306/306	230/242
MS7177	MSg-ID15	21/10/2007	M	142/146	181/189	200/200	262/284	251/251	314/330	230/242
MS7181	MSg-ID15	21/10/2007	M	142/146	181/189	188/200	262/284	251/251	314/330	230/242
MS7182	MSg-ID15	21/10/2007	M	142/146	181/189	200/200	262/284	251/251	314/330	230/242
MS7183	MSg-ID16?	21/10/2007	M	146/150	189/189	196/204	266/284	271/275	298/318	234/234
MS7192	MSg-ID16	21/10/2007	M	146/150	189/189	196/204	266/284	271/275	298/318	234/234
MS7195	MSg-ID16	21/10/2007	M	146/150	189/189	196/204	266/284	271/275	298/318	234/234
MS7184	MSg-ID17	21/10/2007	M	146/150	181/189	196/200	266/276	259/283	298/330	234/238
MS7185	MSg-ID17	21/10/2007	M	146/150	181/189	196/200	266/276	259/283	298/330	234/238
MS7186	MSg-ID17	21/10/2007	M	146/150	181/189	196/200	266/276	259/283	298/330	234/238
MS7191	MSg-ID18	21/10/2007	M	150/150	189/193	196/200	264/268	267/245	298/314	234/238
MS7160	MSg-ID19	19/10/2007	M	146/150	189/197	200/204	276/284	247/247	334/334	-
MS7163	MSg-ID20	21/10/2007	M	146/150	193/197	180/200	274/280	255/267	306/306	230/242
MS7201	MSg-ID20	21/10/2007	M	146/150	193/197	180/200	274/280	255/267	306/306	230/242
MIN6877	MINg-ID21	08/07/2007	M	146/150	173/189	192/196	260/276	275/287	322/322	242/274

*Fecal sample collected from Messok (MS) and Minton (MIN).

†M, male; F, female.

‡Seven Short tandem repeat (STR) loci were amplified from fecal DNA. Two alleles per locus are shown. -, repetitively negative.

Article 7:

Wild Gorillas as a Potential Reservoir of *Leishmania major*

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Over the past several years, it has been found that blood-borne pathogens such as *Plasmodium falciparum*, a causative agent of malaria, and the human immunodeficiency virus (HIV) arose from parasites originating in wild apes, suggesting that these animals constitute the initial reservoir and the source of infections that affect humans. Herein, in order to show that wild western lowland gorillas in southern Cameroon may also serve as reservoir for *Leishmania*, we screened 91 fecal samples from wild western lowland gorillas (*Gorilla gorilla gorilla*) in Cameroon for the presence of these pathogens, the results revealed that of 91 wild gorilla feces samples, 12 contained *Leishmania* parasites and 4 contained phlebotomine sand fly vectors. The molecular identity of *L. major* was determined using *Leishmania*-specific primers targeting three different genes. Moreover, fluorescence *in situ* hybridisation was performed to visualise these parasites in the faeces of these animals. We detected the presence of both the promastigote and amastigote forms *of L. major* in fecal samples of wild western lowland gorillas in Cameroon. Our work creates a paradigm for future studies and highlights the potential of this primate to serve as a host for *L. major* and other emerging pathogens in this region, which has implications for containment efforts.

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MAJOR ARTICLE

Wild Gorillas as a Potential Reservoir of *Leishmania major*

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Vector-borne parasites of the genus *Leishmania* are responsible for severe human diseases. Cutaneous leishmaniasis, a common form of the disease, is most often caused by the transmission of *Leishmania major* to humans by female phlebotomine sand flies. Apes are increasingly being seen as a source of zoonotic diseases, including malaria and rickettsiosis. To examine whether gorillas harbor *Leishmania* species, we screened fecal samples from wild western lowland gorillas (*Gorilla gorilla gorilla*) in Cameroon for the presence of these pathogens. Of 91 wild gorilla fecal samples, 12 contained *Leishmania* parasites, and 4 contained phlebotomine sand fly vectors. The molecular identity was determined by running 3 different polymerase chain reaction tests for detection of *L. major*. Next, fluorescence in situ hybridization was performed to visualize *L. major* parasites in fecal samples from the gorillas. Both promastigote and amastigote forms of the parasite were found. This work strongly suggests that wild gorillas carry pathogenic *Leishmania* parasites.

Keywords. *Leishmania major*; gorilla; detection; feces; PCR; FISH.

Vector-borne parasites of the genus *Leishmania* are responsible for severe human diseases collectively termed "leishmaniasis." This neglected disease is among the most significant tropical infectious diseases; according to the World Health Organization, leishmaniasis is a major public health problem, with an estimated 350 million people at risk of infection each year [1] in 88 countries worldwide. Cutaneous leishmaniasis, a common form of the disease in many countries in Africa, is most often caused by the transmission of *Leishmania major* to humans by female phlebotomine sand flies [2, 3]. In Cameroon, the disease has been reported in the northern and eastern regions, where the insect *Phlebotomus duboscqi* has been identified as a potential vector [2, 4], but no cases have been recorded in the southern region. To date, there is a paucity of data about the reservoir of these particular protozoan parasites in Central Africa; this is a major obstacle in our understanding of the epidemiology of leishmaniasis in these regions [4].

The transmission of cutaneous leishmaniasis caused by *L. major* is principally zoonotic [5], with many infected mammals, particularly wild rodents and domestic animals, regarded as potential reservoirs of *Leishmania* parasites [5, 6]. Humans are considered accidental hosts of the disease. Recently, wild great apes have been shown to be potential reservoirs for human pathogens [7–9], including bacteria, viruses, and parasites [7, 10–12]. There has been increasing evidence that several human pathogenic agents have their origin in our closest relatives, the nonhuman primates [7]. New findings concerning severe human infectious diseases have fuelled this concept. For example, the parasite *Plasmodium falciparum*, the agent of the most severe form of malaria in humans, likely originated from *Plasmodium* parasites of gorilla origin [10]; the precursor of the pandemic form of human immunodeficiency virus type 1 (HIV-1) is of chimpanzee origin [13]; and *Rickettsia felis,* the agent of flea-borne spotted fever, has been found to be carried by apes [11]. These recent insights were obtained through noninvasive techniques, using fecal samples from great apes, which are easily found in the wild; this method circumvents the difficulties involved in obtaining blood

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samples from wild apes. Thus, collection and analysis of fecal samples proved to be an alternative to investigating pathogens that circulate in these endangered animals [12].

Herein, we test the presence of *Leishmania* species in fecal samples from western lowland gorillas in Cameroon. We initially used real-time polymerase chain reaction to detect the presence of *Leishmania* species and subsequently identify the *Leishmania* species present as *Leishmania* major. Then, the organisms in the stool samples were visualized directly, using fluorescence in situ hybridization.

METHODS

Sample Collection

Fecal samples from wild western lowland gorillas (*Gorilla gorilla gorilla*) were collected in 2011 at 1 site located in south central Cameroon. Overall, fecal samples were primarily collected around night nests or feeding sites. For almost all samples, the global positioning system position and estimated time of deposition were recorded, and the species origin was defined in the field according to nesting sites, prints, vocalizations, and morphological and physical aspects of the samples. About 20 mg of dung was collected in a 50-mL tube that contained 20 mL of RNAlater (Ambion, Austin, TX). These tubes were kept at base camps at ambient temperature for a maximum of 3 weeks and were subsequently transported to a central laboratory for storage at −80°C. The collection of the fecal samples from the soil was approved by the Ministry of Scientific Research and Innovation of Cameroon.

DNA Extraction

DNA was extracted from frozen samples using a Qiagen stool protocol (QIAamp DNA Tissue Kit, Qiagen, Germany) with minor modifications [14]. Aliquots (200 µL of each fecal sample /RNAlater) were placed in 2-mL tubes containing a 200-mg mixture of 0.1, 0.5, and 2 mm zirconium beads and 1.5 mL of Buffer ASL (Qiagen). The samples were disrupted with a bead beater at 3200 rpm for 90 seconds, followed by heating at 95°C for 10 minutes. The final pellet was resuspended in 180 µL of tissue lysis buffer and incubated with proteinase K for 2 hours at 55°C. DNA purification and elution were then performed according to the manufacturer's recommendations.

Species Confirmation and Microsatellite Analyses

For all fecal samples, the species origin was also determined by amplifying a 386-bp fragment spanning the 12S gene using primers 12S-L1091 (5′-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT) and 12S-H1478 (5′-TGACTGCAGAGGGTGACGGGCGGTGTGT) as previously described [15]. For microsatellite analysis, to determine the number of infected individuals, samples were genotyped at 8 loci (D18s536, D4s243, D10s676, D9s922, D2S1326, D2S1333, D4S1627, and D9S905) as previously described [16].

Real-Time PCR

Leishmania parasites and their insect vectors were screened by specific real-time PCR, using the following primers: leishF (5′-ACAAGTGCTTTCCCATCG) and leishR (5′-CCTAGAGGCCGTGAGTTG), SF18sF (5′-CGTCGCTACTACTGATGA) and SF18sR (5′-TCGGTCAACTTCTGTGTA), and the probes leishP (6FAM-CGGTTCGGTGTGTGGCGCC-TAMRA) and SF18sP (6FAM-GTTTAGTGAGGTCTCTGGACGTGTGC –MGBNFQ). Real-time PCR was performed using a CFX96 Real-time System (Bio-Rad, Marnes la Coquette, France). The final volume of the PCR mixture was 25 µL, and each mixture contained 12.5 µL of the Probe Master kit (Qiagen, Courtaboeuf, France), 0.5 µL of each primer and probe, 6 µL of distilled water, and 5 µL of extracted DNA. The amplification conditions were as follows: an initial denaturation step at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds and annealing and elongation at 60°C for 60 seconds.

Genomic Amplification and Sequencing

Amplification of 500-, 430- and 820-bp segments of 18S ribosomal DNA (rDNA), the internal transcribed spacer (ITS) region, and the cytochrome b (*Cytb*) gene, respectively, of the *Leishmania* kinetoplast was performed with the primers described previously [17–19]. Each PCR reaction mixture (final volume, 50 µL) contained 5 µL of dNTPs (2 mM of each nucleotide), 5 µL of 10× DNA polymerase buffer (Qiagen, Courtaboeuf, France), 1 µL of $MgCl_2$ (25 mM), 0.25 µL of HotStarTaq DNA polymerase (5 U; Qiagen, Courtaboeuf, France), 1 µL of each primer (10 pmol/µL), and 5 µL of extracted DNA. PCR was performed with a preliminary step at 95°C for 15 minutes followed by 40 cycles of 95°C for 45 seconds, an annealing temperature based on the primers (55°C–60°C) for 30 seconds, and 72°C for 1–2 minutes, with a final extension step at 72°C for 5 minutes. The PCR products were analyzed using agarose gel electrophoresis and visualized by ethidium bromide staining. Positive PCR products were subsequently purified using the NucleoFast 96 PCR Kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer's instructions.

Purified PCR products were sequenced in both directions, using the Big Dye Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing products were run on an ABI PRISM 3130 automated sequencer (Applied Biosystems, Foster City, CA). Finally, *Leishmania* species were identified by comparing the sequences obtained with existing sequences in the GenBank database, using the BLAST program, accessible online at the National Center for Biotechnology Information website (available at: http://www.ncbi.nlm.nih.gov).

Construction of a Phylogenetic Tree

A multiple sequence alignment of the sequences obtained for the 18S ribosomal RNA (rRNA), ITS, and *cytB* genes was used as an input to examine phylogenetic relationships. An

NJ tree was constructed using MEGA 4 [20] and was supported by 1000 bootstrap replicates.

Fluorescence In Situ Hybridization (FISH)

A 10-μL aliquot of each fecal sample was spotted onto poly-L-lysine glass slides and air dried, and the samples were then fixed in 100% methanol. The slides were rinsed with washing buffer (20 mM Tris-HCl [pH 7.6], 0.01% sodium dodecyl sulfate, and 112 mM NaCl) for 10–15 minutes. Fixed samples were hybridized by the application of 10 μL of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.6], 0.01% sodium dodecyl sulfate, and 30% formamide) containing 5 ng of a specific oligonucleotide probe (Leish_18S_840) [21]. The slides were then incubated at 48° C for 2 hours. The hybridized slides were washed in washing buffer preheated to 48°C. Finally, the slides were rinsed with distilled water, air dried, and mounted with solution containing DAPI (ProLong Gold Antifade Reagent; Molecular Probes, Cergy-Pontoise, France). The hybridized slides were viewed with a Leica fluorescence microscope (Leica Microsystems, Germany). For positive control, *L. major* promastigotes (MHOM/SU/73/5-ASKH) were cultured in vitro in M199 and 10% fetal calf serum and processed for double FISH staining as described above.

Nucleotide Sequence Accession Numbers

All sequences obtained in this work have been deposited in GenBank database, with the accession numbers KF981802 to KF981817.

RESULTS

Molecular Detection and Identification of *L. major* in Gorillas' Feces

In this study, 91 gorilla fecal samples collected at 1 site located at the southeastern border of the Dja National Reserve in south-central Cameroon were investigated for the presence of *Leishmania* parasites and their insect vectors, using specific real-time PCR probes targeting 18S rRNA genes for *Leishmania* species and sand flies. Interestingly, 12 of these fecal samples (13.2%) showed positive results, revealing the presence of *Leishmania* parasites in these stool samples, whereas only 4 samples were positive for the vector (Figure 1). To confirm the molecular identity of the *Leishmania* parasites in these samples, 3 primer sets targeting 18S rDNA [17] (500 bp), the ITS region (430 bp) [18], and the *Cytb* gene (820 bp) [19] of *Leishmania* kinetoplasts were used. Good-quality sequences for each gene were obtained from 8 wild gorilla fecal samples. Phylogenetic analysis revealed that all of the new sequences clustered together and matched the sequences of *L. major*, which causes cutaneous leishmaniasis in humans (Figure 2*A*–2*C*). Microsatellite analyses [16] showed that 6 of these fecal samples belonged to 4 different gorillas (Figure 1). Analysis of the remaining 2 samples was not completed because the DNA from these samples was of poor quality.

FISH Staining of Gorillas' Feces

To further examine *L. major* in the feces of wild gorillas in southern Cameroon, the PCR-positive fecal samples were

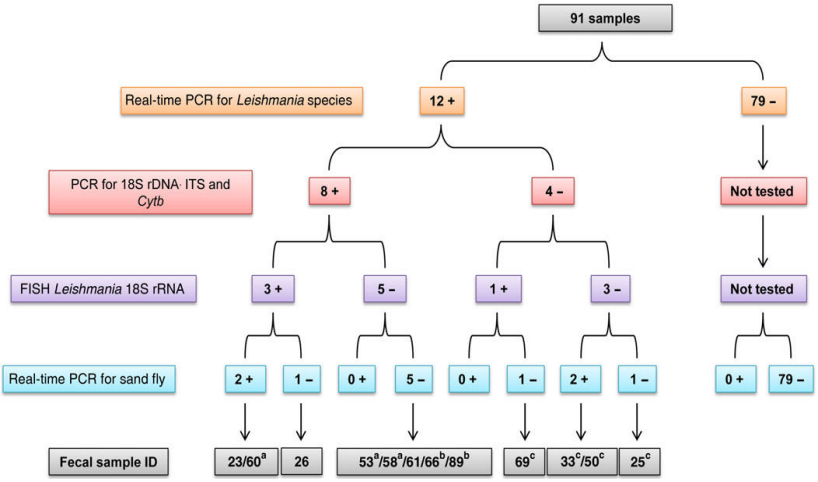

Figure 1. Overview of the analysis of the 91 gorilla stool samples tested for the presence of *Leishmania* species by real-time polymerase chain reaction (PCR); sequencing of 18S ribosomal RNA, internal transcribed spacer (ITS), and *Cytb* PCR products; fluorescence in situ hybridization; and real-time PCR to detect the sand fly vector. [a]Samples collected from the same gorilla. [b]No reliable results were obtained from the microsatellite analyses. [c]Microsatellite analyses were not performed in these samples. Abbreviation: rDNA, ribosomal DNA.

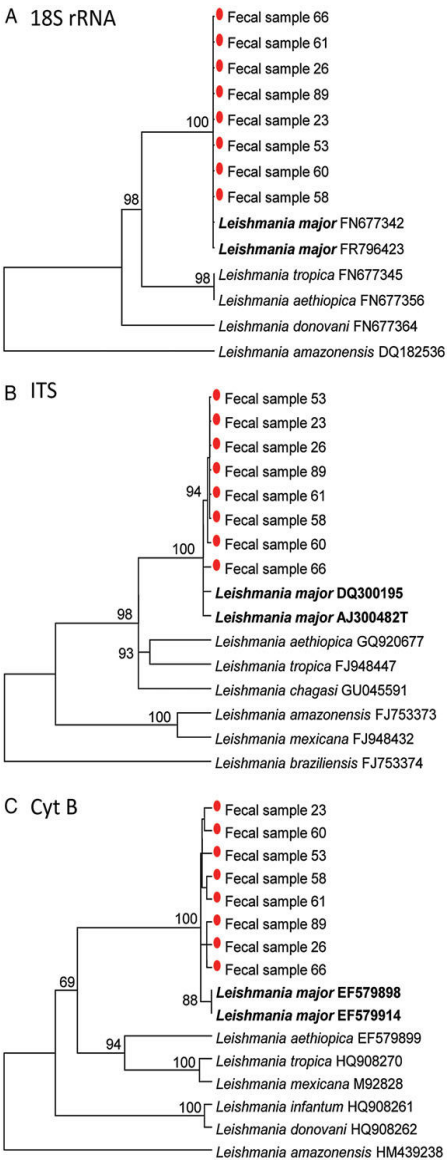

Figure 2. Phylogeny of the *Leishmania* species sequences amplified from wild western lowland gorillas stool samples, based on the 18S ribosomal RNA (rRNA) gene (500 bp; *A*), the internal transcribed spacer (ITS) region (430 bp; *B*), and *Cytb* (820 bp; *C*).

analyzed by double FISH staining with DAPI and the 18S-FAM probes, a technique [21] that has been successfully used to identify *Leishmania* parasites in human patients with cutaneous leishmaniasis. Here, we revealed the presence of both lifecycle stages of *L. major*: the promastigote form, typically present in the sand fly, and the amastigote form, typically detected in

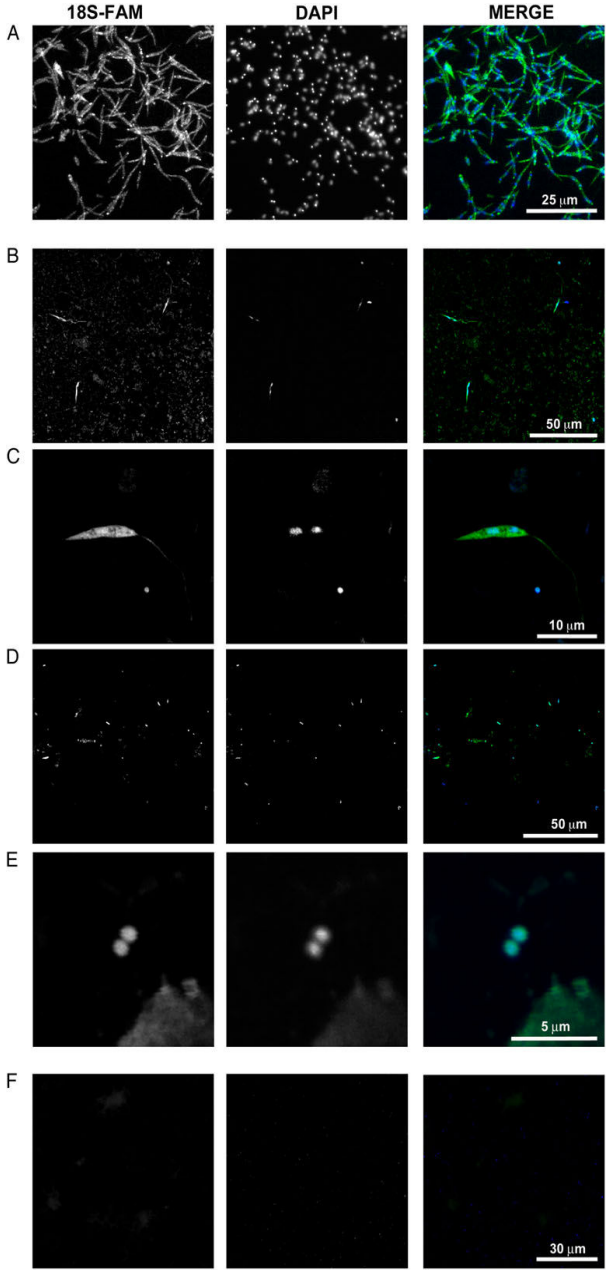

Figure 3. Confocal microscopy images of stool samples stained with the FAM-labeled 18S ribosomal RNA *Leishmania*-specific probe and the nuclear dye DAPI. *A*, Images of axenic *Leishmania major* promastigotes double stained by fluorescence in situ hybridization. *B–E*, Images of stool sample number 23 (40× original magnification [*B*, *D*] and 100× original magnification [*C*, *E*]), showing the presence of *L. major* promastigote parasites (*B*, *C*) and *L. major* amastigote forms (*D*, *E*). *F*, Images of stool sample number 31 (*Leishmania*-free sample; 100× original magnification).

Table 1. Number of *Leishmania* Promastigote and Amastigote Forms and Other Microorganisms Present in Gorilla Fecal Samples

Fecal Sample ID	DAPI Positive/18S rRNA Positive/ Promastigote Forms	DAPI Positive/ 18S rRNA Positive/ Amastigote Forms	DAPI Positive/ 18S rRNA Negative/Other Organisms
23	12	34	4
26	2	41	7
60	6	35	9
69	0	37	13

Stool samples were processed for fluorescence in situ hybridization, using DAPI and *Leishmania*-specific 18S-FAM probes. Several random fields were selected, and 50 DAPI-positive nuclei were randomly picked and analyzed for 18S ribosomal RNA (rRNA) staining. Double-fluorescent DAPI- and 18S rRNA–positive *Leishmania* were categorized as promastigotes or amastigotes according to their form and size (promastigote, elongated and flagellated; amastigote, round and aflagellated). DAPI-positive and 18S rRNA–negative cells were considered as unknown microorganisms.

infected vertebrate host cells, in 4 of 12 PCR-positive gorilla stool samples (Figures 1 and 3). In addition, by counting the number of *Leishmania* promastigotes, *Leishmania* amastigotes, and other microorganisms based on the DAPI-18S staining, we provided evidence of a high prevalence of the parasite amastigote forms in the 4 PCR-positive stool samples. As expected, we also detected other unknown microorganisms, as judged by the presence of numerous DAPI-positive, 18S-negative cells (Figure 3 and Table 1).

DISCUSSION

Cutaneous leishmaniasis caused by *L. major* is transmitted to various mammalian hosts via approximately 30 species/subspecies of sand flies belonging to the genera *Phlebotomus* (in Europe, North Africa, the Middle East, and Asia) and *Lutzomyia* (in the United States) [22]. An additional 40 sand fly species have also been implicated in this transmission [22]. Several studies documented that insects can be part of the diet for African great apes because they supply nutrients that may be absent in plants, including protein, particularly certain types of amino acids; vitamins, such as B12; and mineral elements, such as iron and manganese. The consumption of insects by apes has previously been recorded on the basis of direct observations and/or trace signs in the feces [23]. However, DNA-based diet analyses revealed recently trophic links for these animals and an unexpectedly diverse insect repertoire on their menu [24].

The presence of *L. major* in these stool samples may reflect infection by *L. major*, consumption of *L. major*–infected sand flies [24], or agglutination of infected insects into the feces before collection. Although the detection of the intact promastigote form of *L. major* in feces after the consumption of infected flies by gorillas seems unlikely because of the mechanical digestion of the digestive tract and because the parasites that were detected cannot be released from vectors that have been swallowed by the apes, we cannot exclude any of these 3 hypotheses because both the amastigote and promastigote forms of *L. major* were detected in these fecal samples. As amastigotes should normally be absent in sand flies, they have been present in the gorilla independent of the sand fly vector. Thus, a mix of these hypotheses is very likely possible. Therefore, we can speculate that the gorillas were infected with *L. major* due to the presence of relatively large amounts of amastigote and promastigote forms of *L. major* in fecal samples (Table 1). The presence of the promastigote form is unusual in cutaneous leishmaniasis; however, a promastigote-like form has been detected in human lesions of cutaneous leishmaniasis [25, 26].

Apes have been identified as a reservoir for vector-borne diseases, including malaria [10] and rickettsiosis [11], and stool specimens from wild apes have been considered reliable samples for determining the presence of pathogens. Owing to the nature of the samples analyzed, this study could not directly assess whether the wild gorilla population of Cameroon examined here also developed symptomatic cutaneous leishmaniasis. However, our results clearly indicate that the gorilla stool specimens contained substantial amounts of *L. major* parasites, in both the promastigote and the amastigote forms. Thus, this study strongly suggests that great apes might play a role as reservoir hosts for *L. major* parasites.

The presence of *L. major* in gorilla feces opens a new avenue of thinking about the role of primates as potential reservoir hosts for vector-borne diseases. As described in humans [27–29], this finding most likely suggests that great apes may be asymptomatic carriers for *Leishmania* parasites. Indeed, the vectors can have a similar affinity for both humans and our closest relatives, the nonhuman primates [30]. As such, the potential presence of leishmaniasis in great apes also suggests that most biting insects feeding on gorillas are likely to become infected by *Leishmania* species, and this vector/parasite and host cycle appears to be extremely difficult to terminate. Moreover, this finding could explain why a large number of diseases appear to emerge from the intertropical zone: many nonhuman primates are continuously exposed to microorganisms, particularly microorganisms vectored by biting insects, and great apes probably represent permanent natural reservoirs of human pathogens [12].

In conclusion, the presence of *Leishmania* parasites and other emerging human pathogens in gorilla feces should be viewed as a point of concern for public health in the region, particularly in rural villages where habitat overlap is frequent. Additional studies are needed to determine whether the presence of *Leishmania* in gorillas is widespread and whether sympatric chimpanzee populations also harbor *Leishmania* parasites. Finally, studies on human populations living in these rural forest areas have to be performed for the presence of asymptomatic or

paucisymptomatic *Leishmania* infections, to elucidate the paradigm of the apparent absence of clinical cases in this region.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Reply to Bastien et al

To the Editor—We thank Bastien et al for their comments on our recent article [1]. However, we completely disagree with them since our findings concerning the presence of *Leishmania* in the feces of wild gorillas represent a major point of concern for public health in Cameroon. The application of modern diagnostic tools can determine information about emerging pathogens and can document spatial changes in the distribution of these agents and theirs hosts and vectors.

Before responding scientifically to the comments from Bastien et al, we need to highlight some points. First, our work underwent extensive peer review before it was accepted and published as an article containing original research findings. Moreover, our laboratory is considered one of the pioneering laboratories in the world, providing advanced diagnostic laboratory services (with >400 000 polymerase chain reaction [PCR] analyses/year). Additionally, it is a reference laboratory for many of the world's emerging and neglected infectious diseases.

We are confident in the PCR results from our article [1] because we performed 3 different PCR analyses on 3 different sequences of *Leishmania* organisms (18S ribosomal DNA, internal transcribed spacer region, and Cytb gene), in addition to the first screening of the stool samples via real-time PCR. Furthermore, we excluded the possibility of contamination of these fecal samples by *Leishmania* DNA from our laboratory, as we did not use any positive control during the PCR procedure, and until recently, we had never performed PCR on this organism. Moreover, feces from gorillas have previously been shown to contain other blood-borne pathogens, such as *Plasmodium falciparum* [2], *Rickettsia felis* [3], and human immunodeficiency virus [4]. For these reasons, it is also possible that these great apes may be reservoirs for *Leishmania* organisms, which is how other neglected or emerging human pathogens spread in Africa. Finally, both *Leishmania*-positive and *Leishmania*-negative stool samples from gorillas, as well as DNA, were sent to Switzerland for external, blind molecular validation by Dr G. Greub (a coauthor on this reply). His laboratory results are in agreement with our findings. Indeed, in his laboratory, using their accredited *Leishmania*-specific PCR used for diagnostic purposes (ie, a PCR adapted from Mary et al [5]), he amplified all 5 native samples blindly submitted, with high parasite load of about 500–18 000 copies/5 μL. These positive results are unlikely due to PCR contamination, since in the setting of the diagnostic laboratory of Lausanne's university hospital, which processes >25 000

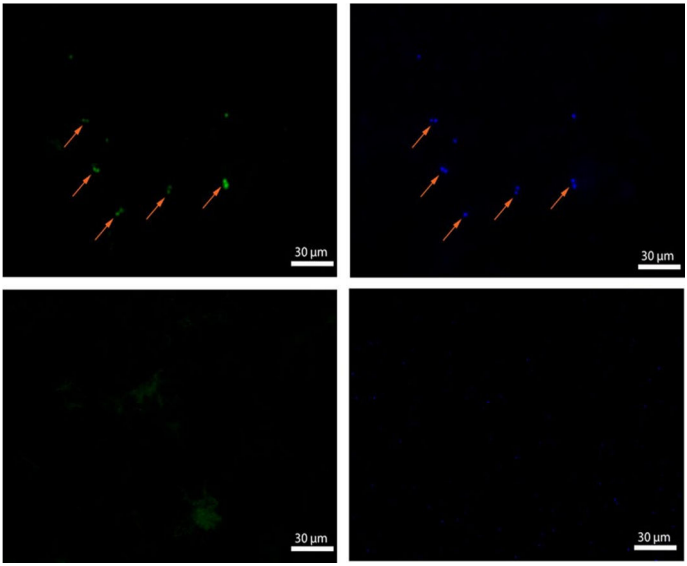

Figure 1. Confocal microscopy images of a stool sample stained with the FAM-labeled 18S ribosomal RNA *Leishmania*-specific probe Leish_18S_651 (left panels) and the nuclear dye DAPI (right panels). The top panels show a fecal sample positive for *Leishmania* organisms by polymerase chain reaction (PCR) analysis. The bottom panels show a sample negative for *Leishmania* species by PCR.

PCR analyses/year, false-positive results due to PCR contamination are rarely experienced (<1 case/10 000 PCR analyses), and when they occurs, only very low numbers of DNA copies are amplified, a situation contrasting with the high parasite load observed here in all 5 positive samples received. Please note that the primers used here (primer 1, CTTTTCT GGTCCTCCGGGTAGG; primer 2, CCA CCCGGCCCTATTTTACACCAA; and probe, TTTTCGCAGAACGCCCCTACC CGC [Fam-MGB]) are relatively specific since they do not amplify all *Leishmania* species, but they have a sequence 100% similar to that of the kinetoplast of *Leishmania major*. Finally, the minor-groove format of the probe further increases the specificity of this PCR.

We also disagree with Bastien et al in their contention that the ingestion of sandflies by primates seems unlikely and has never been reported. Recent studies showed that insect consumption by apes could be achieved either directly (through insect predation) or indirectly (incidentally, when apes feed on different plant parts containing insects that burrow into it or feed on them) [6, 7]. Indeed, DNA sequences that belong to small insects, such as sandflies and mosquitoes, have been detected in the feces of nonhuman primates through extensive molecular analysis [6, 7]. For this reason, we speculated that the detected promastigote form of *Leishmania* organisms in the fecal samples might result from the fact that the vectors are part of the diets of these wild animals. However, we agree that sand flies do not feed on feces; nevertheless, we do not exclude the possibility that these flies may be trapped in feces. A very recent study showed that the presence of animal dung is considered a factor that is associated with *Phlebotomus argentipes* density [8].

In their letter, Bastien et al comment on the experimental results obtained via fluorescence in situ hybridization (FISH) and their doubts concerning the amastigote form of *Leishmania* species. The FISH staining of the stool samples performed in our work used the specific Leish_18S_651 probe in combination with DAPI staining. This *Leishmania*-specific probe was adapted from Frickmann et al [9] and correctly identified all tested *Leishmania* species and excluded closely related species (*Trypanosoma* species). In parallel with this probe, DNA staining with DAPI allowed the authors to reliably differentiate specific probe binding from autofluorescing cells. We used the same strategy (ie, DAPI counterstaining with a *Leishmania*-specific probe) to perform this discrimination, and we are confident in our results. We have also used FISH to analyze stool samples from gorillas that were *Leishmania* negative by PCR. Figure 1 shows no cells that are double positive by means of the Leish_18S_651 probe and DAPI staining

in these samples, which confirms the specificity of the amastigote staining observed in Figure 3*D* and 3*E* from our earlier article [1]. This pattern was similar in all 3 PCR-negative stool samples tested, and in each sample, 3 different areas were analyzed. Moreover, we could further confirm the presence of the amastigote form of *L. major* in gorilla stool samples from the sand fly PCR results obtained in this study (Table 1 from our earlier article [1]). FISH staining indicated an absence of the promastigote form of *L. major* in stool sample number 69 (Table 1 from our earlier article [1]), while results of sand fly PCR on the same sample also indicated the absence of sand flies in the stool sample. This result confirms that the positive signal from the FISH staining of sample 69 belongs to the amastigote form, rather than the promastigote form, because the amastigote form is not found in sand fly hosts (Table 1 from our earlier article [1] and Figure 1).

We agree that a serological survey would be much more convincing and might answer many questions: Are these wild gorillas really infected with *Leishmania* species or are they only carriers? If they are infected, is it only a local infection (cutaneous leishmaniasis) or a systemic infection (due to the presence of *Leishmania* amastigotes in gorilla feces)? In addition, how was *L. major* introduced to the stool samples? Unfortunately, it is impossible to collect blood specimens from theses endangered animals, and even the observation of these protected animals requires difficult-to-obtain authorization.

Finally, many factors, such as global climate change, urbanization, and immigration, have effects on different microorganisms, their vectors, and their reservoir. These interactions lead to changes in the incidence and natural distribution of infectious diseases far from their areas of endemicity [10, 11]. Furthermore, Dr B. Dondji, who is among the authors who commented on our article, referred to 326 cutaneous leishmaniasis cases; most of these cases were seen in immigrants from the southern forest of Cameroon [12]. He has also commented on the necessity of identifying new probable foci in the southern forest and determining the repertory of this parasite in that area [12]. Thus, we think that application of new tools, such as molecular biology, in the field of ecological parasitology could shed light on previously unrecognized zoonotic risks for the local population and pave the way to new discoveries regarding parasite life cycles, epidemiological data, and the detection and identification of parasites, their transmission, and colonization.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Article 8:

Detection of Termites and Other Insects Consumed by African Great Apes using Molecular Fecal Analysis

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Investigating the diets of apes has previously been reported based on direct macroscopic inspection of scats; however some insect food-items may have not identified using this method. In this article we have analysed for the first time the insects eaten by African great apes through amplification and cloning of insect mitochondrial DNA from regions of the Cyt-b and COI genes in their fecal samples. A total of 1056 clones were sequenced for Cyt-b and COI gene libraries, which contained 50 and 56 operational taxonomic units (OTUs), respectively. BLAST research revealed that the OTUs belonged to 32 families from 5 orders (Diptera, Isoptera, Lepidoptera, Coleoptera, and Orthoptera). While ants were not detected by this method, the consumption of flies, beetles, moths, mosquitoes and termites was evident in these samples. Such work highlights the potential of non-invasive methods to expand the known dietary repertoire of primate populations, in particular those that remain unobservable. We think that our study can present a seminal work for studying and analyzing, by molecular methods, the diet of wild primates in future

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Detection of Termites and Other Insects Consumed by African Great Apes using Molecular Fecal Analysis

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The consumption of insects by apes has previously been reported based on direct observations and/or trail signs in feces. However, DNA-based diet analyses may have the potential to reveal trophic links for these wild species. Herein, we analyzed the insect-diet diversity of 9 feces obtained from three species of African great apes, gorilla (*Gorilla gorilla gorilla*), chimpanzee (*Pan troglodytes*) and bonobo (*Pan paniscus*), using two mitochondrial amplifications for arthropods. A total of 1056 clones were sequenced for *Cyt-b* and *COI* gene libraries, which contained 50 and 56 operational taxonomic units (OTUs), respectively. BLAST research revealed that the OTUs belonged to 32 families from 5 orders (Diptera, Isoptera, Lepidoptera, Coleoptera, and Orthoptera). While ants were not detected by this method, the consumption of flies, beetles, moths, mosquitoes and termites was evident in these samples. Our findings indicate that molecular techniques can be used to analyze insect food items in wild animals.

Investigating the diets of primate populations elucidates their behavioral ecology and evolution and clarifies dietary differences among the same species in different habitats and among different species in shared habitats[1]. The consumption of insects is widespread among nonhuman primates, and their entomophagy depends on their body size. Small primates such as galagos (*Galago crassicaudatus* and *Galago senegalensis*), pottos (*Perodicticus potto*), and tarsiers (*Tarsius spectrum*) are obligate insect eaters; their diet is composed mainly of insects belonging to the orders Lepidoptera, Orthoptera and Hymenoptera[2–4]. The diets of medium-bodied primates such as red colobus monkeys (*Procolobus tephrosceles*) and blue monkey (*Cercopithecus mitis*) consist mainly of young leaves, flowers, and unripe fruit; they are also known to eat insects less frequently than small primates[5,6]. Although the large-bodied great apes have a high frugivory[7–11] and folivory component to their diet[12,13], there is also an insectivorous component in the diet of gorilla, chimpanzee, bonobo, orangutan and gibbon populations[14–20].

Insects comprise 0.1–1% of the fresh weight of the daily food ingested by both gorillas and chimpanzees[21], but they may provide disproportionate nutritional benefits due to their role as protein sources. Insects are particularly valuable because they provide certain amino acids, vitamins (such as B12) and minerals (including iron (Fe) and manganese) that may be absent in plant foods[17,22–24].

The insect prey items vary among gorilla species living in different habitats. In mountain gorillas (*Gorilla beringei beringei*), insectivory is uncommon[18,25,26], but they feed on ants (*Dorillus* spp.) to a variable degree[18,26]. In contrast, Grauer's gorillas (*Gorilla beringei graueri*) consume ants more regularly than mountain gorillas, perhaps because the insects are more prevalent[27]. Furthermore, western gorillas (*Gorilla gorilla gorilla*) feed on insects, indicating that insects are an important aspect of the diet of these gorillas[10,14,16,17,28,29].

There is considerable temporal variation in insect consumption among different chimpanzee populations. Both forest-inhabiting chimpanzees in central Africa and savannah-dwelling chimpanzees in western Africa consume insects, such as termites, throughout the year[21,30–32]. In East Africa and at Mt. Assirik in Senegal, chimpanzees feed on termites only seasonally[33]. There is not much variation in insect consumption by different chimpanzee groups. Generally, chimpanzees eat ants, termites, bees, wasps, caterpillars and beetle grubs[29,34,35]. In contrast, arthropods comprise a small fraction of the diet of wild bonobos. The most important non-plant food sources for bonobos are invertebrates, including larvae, termites, ants, bees, earthworms and millipedes[36–39]. Insectivory is also reported for orangutans; especially during times of fruit scarcity[40], orangutans start to prey on insects including termites, ants, bees, gall wasps, crickets, caterpillars and bush crickets[19,41,42]. Wild Sumatran orangutans (*Pongo pygmaeus abelii*) make and use tools (twigs, sticks and branches) for extractive insect

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foraging[41]. Very little data is available on the diet of gibbons, although fruit is their main food source, which is supplemented with some arthropods including caterpillars and termites[20,43].

The studies cited above are based either on direct observations of feeding apes or morphological classification of insect remains in ape feces. Another avenue for investigation of insectivory is the study of feeding remains left by apes (e.g. to identify insect species eaten from a recently disturbed ant nest or encountered termite mound). Although these classical methods can provide direct records for both qualitative and quantitative behavior (i.e. insect prey choice and frequency of insect consumption) and they are inexpensive. However, direct observations of insects consumed by apes are limited in each individual trial by difficulties in tracking apes in their native habitat; this approach can be time-consuming and therefore impractical for studying insectivory in non-human primates[44]. Furthermore, the damage to insect bodies and distortion of their morphology by the mechanical action of the digestive tract creates major challenges for direct analyses of insects in animal feces[44]. Alternative molecular methods may provide better identification and global detection of insects in primates feces compared with these classical methods and may also elucidate the feeding behavior of primates[45,46].

The first PCR-based method for detecting animal prey in primate feces was used by Hofreiter et al.[46], who detected fragments of vertebrate mitochondrial DNA in fecal samples of gorillas and bonobos. In a metagenomic study with primers targeting the arthropod cytochrome b (*Cyt-b*) gene, Pickett et al.[45] investigated the insect diets of six sympatric New World monkeys living in a western Amazon rainforest. To date, molecular analyses of great ape insectivores using high throughput sequencing have not been reported. However, using this technique to target the cytochrome c oxidase I (*COI*) fragment is effective for studying the arthropod diet of bats and, thus, could be applicable to other insectivores[47,48].

In this study, we evaluated the insect-diet diversity of three African great apes gorilla, chimpanzee and bonobo by analyzing fecal samples (N = 9) with DNA-barcoding primers targeting the *Cyt-b*[49] and *COI*[50] genes in arthropod mitochondrial genomes.

Results

A total of 1056 clones were collected from the cloning libraries generated by using both primer sets. First, 24 clones per sample and per amplification were sequenced and analyzed. No additional operational taxonomic units (OTUs)[51] were found when we sequenced 24 additional clones from each sample. Moreover, in one sample (G1), sequencing 144 clones did not increase the number of OTUs that were obtained from the first 24 sequenced clones. Finally, after examining the 1056 clones, 1006 clones presented good quality sequences that did not contain termination codons within the translated sequences. Analyses of these 1006 clones resulted in the detection of 106 different insect OTUs in the 9 fecal samples from bonobos (B1, B2 and B3), chimpanzees (C1, C2 and C3) and gorillas (G1, G2 and G3) with both *Cyt-b* and *COI* genes (Fig. 1 and 2).

Analysis of the *Cyt-b* gene revealed 18 OTUs from insects belonging to 12 families in 4 orders (Isoptera, Diptera, Lepidoptera and Coleoptera) in the fecal samples from bonobos (Fig. 1 and Table 1). Nineteen OTUs belonging to 10 families from Diptera and Coleoptera and 16 OTUs belonging to 11 families from 5 orders (Diptera, Isoptera, Lepidoptera, Coleoptera and Orthoptera) were identified in the feces of chimpanzees and gorillas, respectively, with the same primer set (Fig. 1 and Table 1).

The *COI* bonobo clone libraries yielded 23 OTUs belonging to 12 families within the orders Diptera and Lepidoptera (Fig. 2 and Table 2). Similarly, 19 and 21 OTUs from 12 and 10 families within the orders Diptera, Lepidoptera and Coleoptera were retrieved from the *COI* chimpanzee and gorilla clone libraries, respectively (Fig. 2 and Table 2).

Taking the PCR results together, we detected a variety of arthropod OTUs in the fecal samples from these three African great apes, including fruit flies, moths, beetles, butterflies, mosquitoes and termites (Fig. 1 and 2 and Tables 1 and 2). A total of 32 families from 5 orders were present in at least one of the African great apes studied (Fig. 3). Eight of these families (Carabidae, Chrysomelidae and Staphylinidae from the order Coleoptera and Cecidomyiidae, Muscidae, Psychodidae, Sciaridae and Sepsidae from the order Diptera) were commonly detected in feces from the 3 African great apes species (Fig. 3). Three types of OTUs belonging to the order Isoptera (termites) were only identified in gorilla and bonobo feces (G1, G2 and B2), as shown in Fig. 1 and 3. However, no OTUs assigned to the families Formicidae, Apidae and Tabanidae were identified in our samples (Fig. 3).

Discussion

Investigating the diets of animals by applying molecular methods to fecal samples is useful for the study of wild animals that are difficult to observe[52–54]. Very few studies have examined the insect-diet diversity eaten by primates through molecular approaches. Hofreiter et al.[46] investigated the presence of vertebrate prey DNA in bonobo and gorilla feces through PCR-based methods, and more recently, Pickett et al.[45] evaluated insect diversity in fecal samples from new world monkeys using a single arthropod primer set. Thus, our study is the first to analyze insect diversity in fecal samples from African great apes using two primers (targeting the *Cyt-b* and *COI* genes in the mitochondrial genome of arthropods). These primers have been used to successfully analyze the arthropod prey of tiger beetles and bats[49,50]. In this study, we detected 106 insect OTUs (41, 38 and 37 for bonobos, chimpanzees and gorillas, respectively) using these primers (Fig. 1 and 2, Tables 1 and 2). Compared with behavioral observations and/or analysis of trail signs in ape feces, we found many previously unknown insect families that are consumed by African great apes (Fig. 3). Many insects, such as species in the orders Coleoptera and Lepidoptera or caterpillars detected in this study (Tables 1 and 2) have strong associations with plants[55,56]. Some of these insect species, such as member of family Chrysomelidae, feed on different plant parts[57]. Consequently, they could be eaten incidentally (secondary predation) when African great apes feed on plants. Thus, one advantage of using a molecular approach to examine insects consumed is the inclusion of those consumed via secondary predation. These species may not have been otherwise detected through classical approaches, but they are still components of the diet that have nutritional value. These indirectly eaten insects may also contribute to an understanding of the feeding ecology and foraging strategy of a species. Interestingly, different OTUs from termites were identified in both gorilla and bonobo feces (Fig. 1 and Table 1). Termites are commonly preyed on by African great apes[21,33] (Fig. 3). *Macrotermes* species, which are the largest termites in Africa and live in different habitat types, are an important insect meal for both western gorillas and chimpanzees residing in southeastern Cameroon[21] (Fig. 3). Despite the wide global distribution of some of the insect species found in our work, such as those belonging to Drosophilidae, no data on the occurrence of the remaining species in Cameroon and DRC are available.

We were unable to amplify any of Formicidae species in this study (Fig. 3 and Tables 1 and 2) using both arthropod primers, and the macroscopic examination of fecal samples did not detect any visible hard parts (e.g. chitinous exoskeleton or jointed appendages) of these insects. Their absence could be due to degradation of insect DNA during its journey through the digestive tube, the small sample size or insufficient mitochondrial DNA caused by bias in the DNA extraction, amplification and cloning process.

While DNA-based fecal analyses are useful for evaluating the diets of wild animals, several challenges and limitations should be noted when employing this method. Although, no hard insect parts were

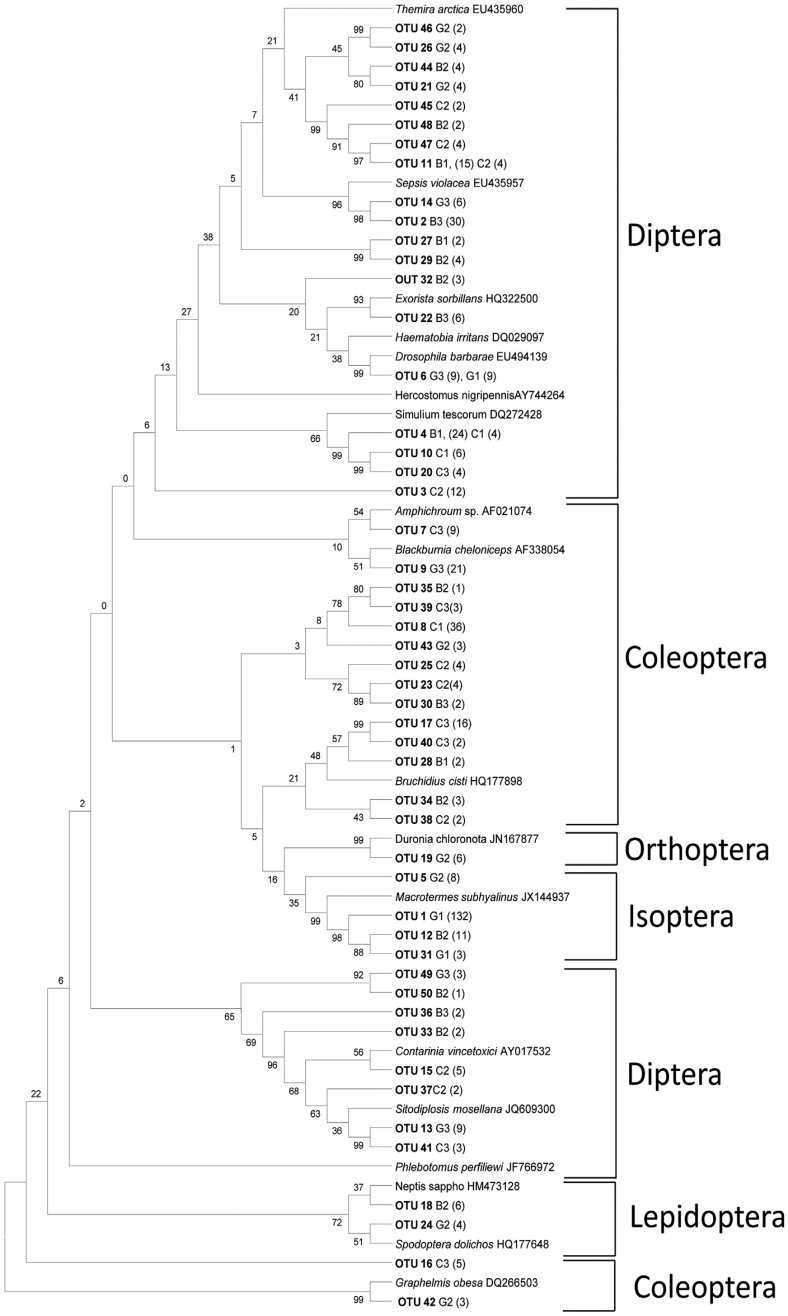

Figure 1 | Insect OTUs detected in African great ape fecal samples using CB3/CB4 primers targeting the *Cyt-b* gene. The neighbor-joining tree was constructed using MEGA 5 and supported by 500 bootstrap replicates. The insect species listed with accession numbers are the closest matching sequences from GenBank. G1, G2 and G3: fecal samples from gorillas; C1, C2 and C3: chimpanzees; B1, B2 and B3: bonobos.

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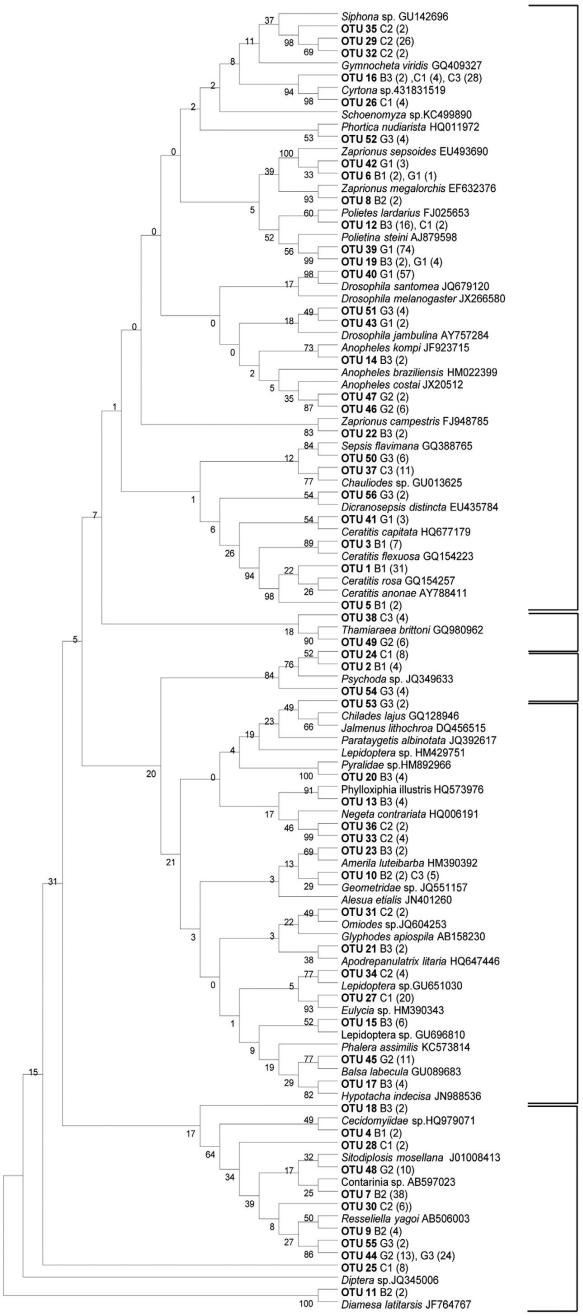

Figure 2 | Insect OTUs detected in African great fecal samples using ZBJ-ArtF1c/ZBJ-ArtR2c primers targeting the *COI* gene. The neighbor-joining tree was constructed using MEGA 5 and supported by 500 bootstrap replicates. The insect species listed with accession numbers are the closest matching sequences from GenBank. G1, G2 and G3: the fecal samples from gorillas; C1, C2 and C3: chimpanzees; B1, B2 and B3: bonobos.

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Table 1 | Insect OTUs detected in 9 fecal samples from wild African great apes using *Cyt-b* targeting primers

Ape	Sample	OTU (No. of Clones)	Order	Family	Common name
Bonobo	B1	4 (24)	Diptera	Simuliidae	Flies (Black flies)
Bonobo	B1	11 (15)	Diptera	Sepsidae	Flies (Ensign flies)
Bonobo	B1	27 (2)	Diptera	Tephritidae	Flies (Fruit flies)
Bonobo	B1	28 (2)	Coleoptera	Chrysomelidae	Beetles (Leaf beetles)
Bonobo	B2	12 (11)	Isoptera	Termitidae	Termite
Bonobo	B2	18 (11)	Lepidoptera	Nymphalidae	Butterflies (Brush-footed butterflies)
Bonobo	B2	29 (4)	Diptera	Tephritidae	Flies (Fruit flies)
Bonobo	B2	32 (3)	Diptera	Drosophilidae	Flies (Fruit flies)
Bonobo	B2	44 (4)	Diptera	Muscidae	Flies (House flies)
Bonobo	B2	48 (2)	Diptera	Sepsidae	Flies (Ensign flies)
Bonobo	B2	50 (1)	Diptera	Fanniidae	Flies (True Flies)
Bonobo	B2	33 (2)	Diptera	Cecidomyiidae	Flies (Gall gnats)
Bonobo	B2	34 (3)	Coleoptera	Chrysomelidae	Beetles (Leaf beetles)
Bonobo	B2	35 (1)	Coleoptera	Carabidae	Beetles
Bonobo	B3	2 (30)	Diptera	Sepsidae	Flies (Ensign flies)
Bonobo	B3	22 (6)	Diptera	Muscidae	Flies (House flies)
Bonobo	B3	36 (2)	Diptera	Cecidomyiidae	Flies (Gall gnats)
Bonobo	B3	30 (2)	Coleoptera	Staphylinidae	Beetles (Rove beetles)
Chimpanzee	C1	4 (4)	Diptera	Simuliidae	Flies (Black flies)
Chimpanzee	C1	10 (6)	Diptera	Chironomidae	Flies (nonbiting midges)
Chimpanzee	C1	8 (36)	Coleoptera	Scarabaeidae	Beetles (Scarab beetles)
Chimpanzee	C2	3 (12)	Diptera	Tachinidae	Flies (True flies)
Chimpanzee	C2	11 (4)	Diptera	Sepsidae	Flies (Ensign flies)
Chimpanzee	C2	15 (5)	Diptera	Cecidomyiidae	Flies (Gall gnats)
Chimpanzee	C2	37 (2)	Diptera	Cecidomyiidae	Flies (Gall gnats)
Chimpanzee	C2	45 (2)	Diptera	Anthomyiidae	Flies
Chimpanzee	C2	47 (4)	Diptera	Anthomyiidae	Flies (House flies)
Chimpanzee	C2	23 (4)	Coleoptera	Scarabaeidae	Beetles (Scarab beetles)
Chimpanzee	C2	25 (4)	Coleoptera	Chrysomelidae	Beetles (Leaf beetles)
Chimpanzee	C2	38 (2)	Coleoptera	Chrysomelidae	Beetles (Leaf beetles)
Chimpanzee	C3	7 (9)	Coleoptera	Staphylinidae	Beetles (Rove beetles)
Chimpanzee	C3	16 (5)	Coleoptera	Scarabaeidae	Beetles (Scarab beetles)
Chimpanzee	C3	17 (16)	Coleoptera	Dytiscidae	Beetles (Water beetles)
Chimpanzee	C3	39 (3)	Coleoptera	Scarabaeidae	Beetles (Scarab beetles)
Chimpanzee	C3	40 (2)	Coleoptera	Dytiscidae	Beetles (Water beetles)
Chimpanzee	C3	20 (4)	Diptera	Chironomidae	Flies (nonbiting midges)
Chimpanzee	C3	41 (3)	Diptera	Cecidomyiidae	Flies (Gall gnats)
Gorilla	G1	1 (132)	Isoptera	Termitidae	Termite
Gorilla	G1	31 (3)	Isoptera	Termitidae	Termite
Gorilla	G1	6 (9)	Diptera	Drosophilidae	Flies (Fruit flies)
Gorilla	G2	42 (3)	Coleoptera	Carabidae	Beetles
Gorilla	G2	43 (3)	Coleoptera	Chrysomelidae	Beetles (Leaf beetles)
Gorilla	G2	19 (6)	Orthoptera	Acrididae	Grasshoppers
Gorilla	G2	21 (4)	Diptera	Sepsidae	Flies (Ensign flies)
Gorilla	G2	26 (4)	Diptera	Muscidae	Flies (House flies)
Gorilla	G2	46 (2)	Diptera	Muscidae	Flies (House flies)
Gorilla	G2	24 (4)	Lepidoptera	Arctiidae	Moths (tiger moths)
Gorilla	G2	5 (8)	Isoptera	Rhinotermitidae	Termite
Gorilla	G3	6 (9)	Diptera	Drosophilidae	Flies (Fruit flies)
Gorilla	G3	13 (9)	Diptera	Cecidomyiidae	Flies (Gall gnats)
Gorilla	G3	14 (6)	Diptera	Sepsidae	Flies (Ensign flies)
Gorilla	G3	49 (3)	Diptera	Fanniidae	Flies (True flies)
Gorilla	G3	9 (21)	Coleoptera	Carabidae	Beetles

G1, G2 and G3: fecal samples from gorillas; C1, C2 and C3: chimpanzees; B1, B2 and B3: bonobos.

visible in feces prior to analysis, fecal sample contamination is possible and, unfortunately, difficult to investigate especially for insects that burrow into feces. However, we minimized this limitation by using the inner part of the fecal mass for DNA extraction, which reduced contamination with soil organisms, flies and their eggs deposition.

DNA extraction, PCR amplification and cloning biases were also limitations in this study. The amount of feces used for DNA extractions represented only a small fraction of the total feces bulk. Low DNA concentration, species with low frequency in samples and the limited number of tested clones also may have prevented the detection of certain prey items. Moreover, the primers used may not have amplified the mitochondrial sequences of some insect species, so these sequences were lost. Another challenge with molecular diet analyses using DNA from feces is that one cannot determine whether the insects present in the guts of African great apes were the result of primary or secondary predation or contamination from the edible parts of plants. Although the primer sets we used amplified a wide range of arthropods, the incompleteness of insect sequence data in GenBank prevented us from assigning the recovered OTUs at genus or species level. For this reason, the taxonomic identifications were done at family level according to the E-values of BLAST results. Exhaustive studies using molecular techniques along with conventional morphological taxonomy are needed to enrich the public

Table 2 | Insect OTUs detected in 9 fecal samples from wild African great apes using *COI* targeting primers

Ape	Sample	OTU (No. of clones)	Order	Family	Common name
Bonobo	B1	1 (31)	Diptera	Tephritidae	Flies (Fruit flies)
Bonobo	B1	2 (4)	Diptera	Psychodidae	Flies (drain flies)
Bonobo	B1	3 (7)	Diptera	Tephritidae	Flies (Fruit flies)
Bonobo	B1	4 (2)	Diptera	Cecidomyiidae	Flies (Gall gnats)
Bonobo	B1	5 (2)	Diptera	Tephritidae	Flies (Fruit flies)
Bonobo	B1	6 (2)	Diptera	Drosophilidae	Flies (Fruit flies)
Bonobo	B2	7 (38)	Diptera	Cecidomyiidae	Flies (Gall gnats)
Bonobo	B2	8 (2)	Diptera	Drosophilidae	Flies (Fruit flies)
Bonobo	B2	9 (4)	Diptera	Cecidomyiidae	Flies (Gall gnats)
Bonobo	B2	11 (2)	Diptera	Chironomidae	Flies (nonbiting midges)
Bonobo	B2	10 (2)	Lepidoptera	Noctuidae	Moths (Owlet moths)
Bonobo	B3	12 (16)	Diptera	Muscidae	Flies (House flies)
Bonobo	B3	14 (2)	Diptera	Culicidae	Mosquitoes
Bonobo	B3	16 (2)	Diptera	Curtonotidae	Flies (Quasimodo flies)
Bonobo	B3	18 (2)	Diptera	Cecidomyiidae	Flies (Gall gnats)
Bonobo	B3	19 (2)	Diptera	Muscidae	Flies (House flies)
Bonobo	B3	22 (2)	Diptera	Drosophilidae	Flies (Fruit flies)
Bonobo	B3	13 (4)	Lepidoptera	Sphingidae	Moths (Hawk moths)
Bonobo	B3	15 (6)	Lepidoptera	Nymphalidae	Butterflies (Brush-footed butterflies)
Bonobo	B3	17 (4)	Lepidoptera	Noctuidae	Moths (Owlet moths)
Bonobo	B3	20 (4)	Lepidoptera	Pyralidae	Moths (snout moths)
Bonobo	B3	21 (2)	Lepidoptera	Noctuidae	Moths (Owlet moths)
Bonobo	B3	23 (2)	Lepidoptera	Noctuidae	Moths (Owlet moths)
Chimpanzee	C1	12 (2)	Diptera	Muscidae	Flies (House flies)
Chimpanzee	C1	16 (4)	Diptera	Curtonotidae	Flies (Quasimodo flies)
Chimpanzee	C1	24 (8)	Diptera	Psychodidae	Flies (drain flies)
Chimpanzee	C1	25 (8)	Diptera	Sciaridae	Flies (Dark-winged fungus gnats)
Chimpanzee	C1	26 (4)	Diptera	Curtonotidae	Flies (Quasimodo flies)
Chimpanzee	C1	28 (2)	Diptera	Cecidomyiidae	Flies (Gall gnats)
Chimpanzee	C1	27 (20)	Lepidoptera	Geometridae	Moths (geometer moths)
Chimpanzee	C2	29 (26)	Diptera	Tachinidae	Flies (True flies)
Chimpanzee	C2	30 (6)	Diptera	Cecidomyiidae	Flies (Gall gnats)
Chimpanzee	C2	32 (2)	Diptera	Tachinidae	Flies (True flies)
Chimpanzee	C2	35 (2)	Diptera	Tachinidae	Flies (True flies)
Chimpanzee	C2	31 (2)	Lepidoptera	Geometridae	Moths (geometer moths)
Chimpanzee	C2	33 (4)	Lepidoptera	Cosmopterigidae	Moths (Cosmet moths)
Chimpanzee	C2	34 (4)	Lepidoptera	Erebidae	Moths
Chimpanzee	C2	36 (2)	Lepidoptera	Cosmopterigidae	Moths (Cosmet moths)
Chimpanzee	C3	10 (5)	Lepidoptera	Noctuidae	Moths (Owlet moths)
Chimpanzee	C3	16 (28)	Diptera	Curtonotidae	Flies (Quasimodo flies)
Chimpanzee	C3	37 (11)	Diptera	Simuliidae	Flies (Black flies)
Chimpanzee	C3	38 (4)	Coleoptera	Carabidae	Beetles
Gorilla	G1	6 (1)	Diptera	Muscidae	Flies (House flies)
Gorilla	G1	19 (4)	Diptera	Muscidae	Flies (House flies)
Gorilla	G1	39 (74)	Diptera	Muscidae	Flies (House flies)
Gorilla	G1	40 (57)	Diptera	Drosophilidae	Flies (Fruit flies)
Gorilla	G1	41 (3)	Diptera	Drosophilidae	Flies (Fruit flies)
Gorilla	G1	42 (3)	Diptera	Drosophilidae	Flies (Fruit flies)
Gorilla	G1	43 (2)	Diptera	Drosophilidae	Flies (Fruit flies)
Gorilla	G2	44 (13)	Diptera	Cecidomyiidae	Flies (Gall gnats)
Gorilla	G2	46 (6)	Diptera	Drosophilidae	Flies (Fruit flies)
Gorilla	G2	47 (2)	Diptera	Drosophilidae	Flies (Fruit flies)
Gorilla	G2	48 (10)	Diptera	Cecidomyiidae	Flies (Gall gnats)
Gorilla	G2	49 (6)	Coleoptera	Staphylinidae	Beetles (Rove beetles)
Gorilla	G2	45 (11)	Lepidoptera	Noctuidae	Moths (Owlet moths)
Gorilla	G3	44 (24)	Diptera	Cecidomyiidae	Flies (Gall gnats)
Gorilla	G3	50 (6)	Diptera	Sepsidae	Flies (Ensign flies)
Gorilla	G3	51 (4)	Diptera	Drosophilidae	Flies (Fruit flies)
Gorilla	G3	52 (4)	Diptera	Syrphidae	Flies (Hoverflies)
Gorilla	G3	54 (4)	Diptera	Psychodidae	Flies (drain flies)
Gorilla	G3	55 (2),	Diptera	Cecidomyiidae	Flies (Gall gnats)
Gorilla	G3	56 (2)	Diptera	Tephritidae	Flies (Fruit flies)
Gorilla	G3	53 (2)	Lepidoptera	Riodinidae	Butterflies (metalmarks)

G1, G2 and G3: fecal samples from gorillas; C1, C2 and C3: chimpanzees; B1, B2 and B3: bonobos.

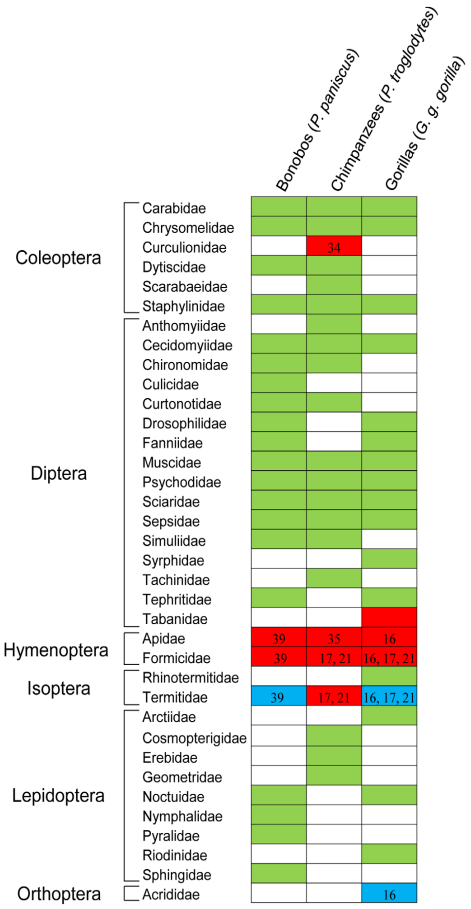

Figure 3 | Summary of insect consumption by three species of African great apes. Orders and families of known insect prey identified through DNA analysis, direct observation or morphological analysis of prey remains in feces. Arthropod families obtained from *Cyt-b* and *COI* sequences and from different individuals of the same species were pooled together. Green cells indicate insect families that were identified as diet items in this study. Red cells indicate insect families that were not detected in this study. Blue cells indicate insect families that were detected in this study by DNA and in other studies through classical methods.

databases with complete arthropod mitochondrial genome sequences, which will identify insects at the species level. Finally, prey sequence counts recovered using traditional cloning/sequencing or pyrosequencing[58] cannot quantify insect consumption or provide insights to insect choice by African great apes. Thus, combining molecular tools with classical observation methods provides a more complete picture of the complex diet of wild animals, including primates.

In conclusion, molecular fecal analysis is an emerging method that can provide valuable insight into the insectivory aspect of primate diet and can expand the list of insect foods ingested by a study population. The high number of insect OTUs detected in fecal samples using this strategy confirms the importance of molecular applications for analyzing direct and indirect insect consumption in African great apes. For detailed descriptions of the occurrence and abundance of insects in African great ape feces using next generation sequencing, additional studies using larger numbers of samples collected in different seasons are needed. This approach will clarify intra- and inter-species dietary variation in wild primates.

Methods

Source of the fecal samples. A total of 9 fecal samples were used in this study. Three fecal samples from wild gorillas (*G. g. gorilla*) were collected at a forest site near Messok (sample G1) and at a forest site near Mambele (samples G2, G3) (Messok and Mambele are two towns located southeast and east of Cameroon, respectively). Three fecal samples from chimpanzees (*P. troglodytes*) were collected at a forest site near Mambele (samples C1, C2) and at a forest site near Messok (sample C3), and three fecal samples (B1, B2 and B3) from bonobos (*P. paniscus*) were collected at the Lomako-Yokokala faunal reserve in the Democratic Republic of Congo (DRC). The sample collection protocol was described previously[59]. Briefly, samples were collected near night nests or feeding sites. For all samples, the time, date and GPS position were recorded, and the species of origin was determined according to prints, collection sites and morphological aspects of the samples as well as by amplifying a 386 bp mtDNA fragment spanning the 12SrRNA gene (using primers 12S-L1091 5′-AAAAAGCT-TCAAACTGGGATTAGATACCCCACTAT-3′ and 12S-H1478 5′-TGACTGC-AGAGGGTGACGGGCGGTGTGT-3′). The time between defection and collection was estimated at <24 h, according to the physical texture of the samples. Collected samples were saved at base camps at ambient temperature in RNA*later* (Ambion, Austin, TX) for less than 3 weeks and transported to a central laboratory for storage at −80°C. No experimentation was conducted on these animals. The collection of fecal samples from the soil was approved by the Ministry of Scientific Research and Innovation of Cameroon and DRC. No other permit was required, as this research was non-invasive work, and the collection of the samples did not disrupt wild fauna.

DNA extraction. The outer layer of fecal bulk was peeled carefully with a sterile scalpel and polystyrene tweezers (approximately 1–2 mm was removed). The inner part of the fecal bulk was used for extraction to avoid a possible contamination with soil organisms and/or the risk of egg deposition by some flies, which would result in the amplification of non-prey organisms. DNA was extracted using a modified version of the Qiagen fecal procedure (QIAamp DNA Tissue Kit, Qiagen Inc., Germany)[60]. A 200-mg aliquot of each fecal sample was placed in a 2-ml tube containing 200 mg of a mixture of 0.1-, 0.5-, and 2-mm zirconium beads and 1.5 ml of ASL buffer (Qiagen). The sample was bead-beaten at 3200 rpm for 90 seconds, followed by heating at 95°C for 10 minutes. The final pellet was suspended in 180 µl of tissue lysis buffer and incubated with proteinase K for 2 hours at 55°C. The manufacturer's recommendations were followed for the purification and elution of DNA.

Primer selection and genomic amplification. To minimize primer bias, two different primers targeting *Cyt-b* and *COI* genes in the mitochondrial genome of arthropods were used[49,50]. The first PCR was previously used to study the prey of tiger beetles after whole DNA extraction from predatory specimens, which provided lower identity sequences compared with the GenBank database but sufficiently high scores to identify the order, family and occasionally the genus[49]. The second PCR was used to analyze arthropod prey in bat feces, enabling the authors to identify 37 prey taxa from 15 fecal samples (identification at the species level in 72% of the analyzed clones)[50]. The primer sets ZBJ-ArtF1c (AGATATTGGAACWTTATATTTTATTTTTGG)/ ZBJ-ArtR2c (WACTAATCAATTWCCAAATCCTCC)[49] and CB3 (GAGGAGCAA-CTGTAATTACTAA)/CB4 (AAAAGAAARTATCATTCAGGTTGAAT)[50] amplified 157 and 410 bp segments of mitochondrial DNA, respectively. The PCR reaction mixture (final volume 50 µl) contained 5 µl of dNTPs (2 mM of each nucleotide), 5 µl of 10× DNA polymerase buffer (QIAGEN, Courtaboeuf, France), 1 µl of $MgCl_2$ (25 mM), 0.25 µl of HotStarTaq DNA polymerase (5 U) (QIAGEN, Courtaboeuf, France), 1 µl of each primer (10 pmol/µl), and 5 µl of extracted DNA. The PCR was performed with an initial denaturation at 95°C for 15 minutes, followed by 40 cycles of 95°C for 45 seconds, an annealing temperature specific for the primers used (57°C for ZBJ-ArtF1c/ZBJ-ArtR2c and 46°C for CB3/CB4) for 30 seconds, 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The PCR products were analyzed using agarose gel electrophoresis (1.5%) and visualized with ethidium bromide staining. Positive PCR products were subsequently purified using the NucleoFast® 96 PCR Kit (MACHEREY-NAGEL, Hoerdt, France) according to the manufacturer's instructions.

Cloning procedures and insert amplification. The purified PCR products were cloned with the pGEM®-T Easy Vector System 2 Kit (Promega, Madison, USA), as recommended by the manufacturer. All white colonies were analyzed by PCR with the M13d (5′-GTAAAACGACGGCCAG) and M13r (5′-CAGGAAACAGCTA-TGAC) primers, as described previously[60].

Sequencing and data analysis. Correct sizes of purified PCR-M13 inserts were sequenced in both directions using the Big Dye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Courtaboeuf, France). The M13d and M13r primers were

used for sequencing. The sequencing products were run on an ABI PRISM 3130 automated sequencer (Applied Biosystems, Foster city, CA, USA).

The sequences were corrected with the CodonCode Aligner software (4.0.1). Sequence similarity was determined through multiple alignment software using ClustalW2. Sequences with base-pair mismatches, insertions or deletions leading to stop codons in every possible reading frame were excluded from further analysis. A cloned sequence was designated an "operational taxonomic unit" (OTU)[51] if it had more than 2% sequence divergence from all other recovered sequences across approximately 400 bp and 157 bp of *Cyt-B* and *COI*, respectively[45]. Phylogenetic analyses (neighbor-joining tree) were performed using MEGA 5, supported by 500 bootstrap replicates.

To make taxonomy assignments, the OTUs were compared with a BLAST database of pre-assigned sequences in GenBank (available at the National Center for Biotechnology Information website: http://www.ncbi.nlm.nih.gov/), and the closest available matching hit with the lowest E-value was assigned as the taxonomic identification for each OTU. As many arthropod species and genera shared or had very close E-values, OTUs were assigned to the family level.

Nucleotide sequence accession numbers. All sequences generated from the primer set CB3/CB4 were deposited in the GenBank database with the accession numbers JX500757 to JX501234. The sequences obtained from the primer set ZBJ-ArtF1c/ZBJ-ArtR2c were not deposited because sequences shorter than 200 bp are not accepted in the GenBank database.

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Author contributions

D.R. and F.B. designed the experiments; I.H. conducted the experiments; I.H., E.D., D.R. and F.B. analyzed the results; I.H. and F.B. prepared the figures; I.H. and F.B. wrote the manuscript. All authors reviewed the manuscript.

Additional information

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Conclusions and Perspectives

Conclusions and Perspectives

One approach for gaining insights into gut eukaryotes composition is through analysis of eukaryotic microbial 18S rRNA genes followed by cloning and sequencing. Generally one or two eukaryotic universal primers have used in previous published studies to estimate the diversity of gut eukaryotes. Our study is the first study that has used a large number of universal primers to analyze the eukaryotes in fecal samples. Using this strategy (i.e. multiple sets of primer) enabled us to increase the number of eukaryotes detected in fecal samples or in another way the combination of different primer sets can increase the eukaryotic diversity detected. According to our data, non-significant difference in eukaryotic gut diversity was observed in intestinal tract of healthy and sick individuals. In both cases fungi constitute a high proportion of the total gut eukaryotes detected in our studies. Moreover, the retrieved eukaryotic communities in gut varied from individual to another depending on many factors including geographical location, individual health status and the size of fecal samples analyzed.

Despite a relative dearth of information on the diversity of eukaryotic in non-human primate, the scientific results in our published articles outlined above represent the evolution of our view of the gut eukaryotes in great apes particularly in wild gorilla. This is the first study to characterize fecal eukaryotic diversity, including fungi, in great apes using a comprehensive extended molecular analysis. The multiple primer set approach used herein enabled us to recover a high diversity of eukaryotes from the intestinal tract of the wild lowland gorilla including human pathogens such as pathogenic fungi (*Candida tropicalis*), parasites (*Oesophagostomum bifurcum*, *Necator americanus*) and blood borne pathogen such as *Leishmania*. The presence of such human pathogens in gorillas should be viewed to be a more important public health

issue as they may be particularly susceptible to exchanging pathogens with apes because they range widely into human habitats.

In this thesis, we also accomplished an evaluation of the insect-diet diversity in African great apes (gorilla, chimpanzee and bonobo) via molecular analysis of their fecal samples with DNA-barcoding primers targeting the arthropod mitochondrial genomes. The large insect OTUs retrieved in their fecal samples confirm the necessary of using this molecular array to get valuable insight into the insectivory aspect of primate diet and to expand the list of insect foods ingested by great apes.

Finally, we think that more effort are now required to overcome the limitation of this method and apply new techniques such as next generation sequencing combined with bioinformatics to reveal the actual diversity of these eukaryotes in gut as well as explaining their impact in both healthy and diseased status as well as additional studies from other geographic locations are required for detailed descriptions of the occurrences and abundances of eukaryotes, including pathogens, in the guts of non-human primates, which have until now been poorly described.

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