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Sequence-based Methods for Detecting and Evaluating the Human Gut Mycobiome

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Sequence-based methods for detecting and evaluating the human gut mycobiome

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Significance and Impact of the Study: Fungi play a role in human gut ecology and health. The field lags immensely behind bacterial gut microbiota research, and studies continue to identify new fungi in fecal samples from healthy humans. However, many of these 'new' species are incapable of growth in the human GI tract, let alone making a meaningful contribution to the gut microbial community. Fungi actually inhabiting and impacting the gut likely constitute a small set of species, and an optimized, targeted, probe-based assay may prove to be the most sensible way of quantifying their abundances.

Abstract
We surveyed the fungal microbiota in 16 fecal samples from healthy humans with a vegetarian diet. Fungi were identified using molecular cloning, 454 pyrosequencing and a Luminex analyte-specific reagent (ASR) assay, all targeting the ITS region of the rRNA genes. Fungi were detected in each fecal sample and at least 46 distinct fungal operational taxonomic units (OTUs) were detected, from two phyla — Ascomycota and Basidiomycota. *Fusarium* was the most abundant genus, followed by *Malassezia, Penicillium, Aspergillus* and *Candida*. Commonly detected fungi such as *Aspergillus* and *Penicillium*, as well as known dietary fungi *Agaricus bisporus* and *Ophiocordyceps sinensis*, are presumed to be transient, allochthonous members due to their abundance in the environment or dietary associations. No single method identified the full diversity of fungi in all samples; pyrosequencing detected more distinct OTUs than the other methods, but failed to detect OTUs in some samples that were detected by cloning and/or ASR assays. ASRs were limited by the commercially available assays, but the potential to design new, optimized assays, coupled with speed and cost, makes the ASR method worthy of further study.

Keywords: environmental mycology, fungi, microbiome, molds, PCR, yeasts

Introduction

Recently, the composition and role of the gut microbiota in humans has become a focal point of study. Bacteria are the most abundant micro-organisms in the gastrointestinal tract, and therefore, have been the focus of much human microbiome research. The extent of biodiversity of the "mycobiome" (fungal biota) in the human gastrointestinal tract remains to be defined and large-scale human microbiome projects have mostly excluded fungal components (Qin *et al.* 2010; Human Microbiome Project Consortium 2012). Smaller-scale, fungal-specific studies exist but are limited in making accurate estimates of diversity.

Gut fungi are commonly reported as difficult to cultivate, thus culture-independent methods relying on DNA sequencing have become preferred methods for mycobiome studies (Schoch *et al.* 2012). No method has been universally adopted and difficulties in defining the complete mycobiome are attributed to low sample numbers, individual variation, limited research attention and a lack of consistent identification methods and gene targets. Furthermore, several recent intestinal fungi investigations are based on a single fecal sample (Hamad *et al.* 2012; Gouba *et al.* 2013, 2014).

In this study, we use internal transcribed spacer (ITS) DNA sequence to characterize the fungal communities in 16 fecal samples from the gastrointestinal tract of healthy

adult humans with a vegetarian diet. Additionally, we compare three culture-independent methods, and provide the first evaluation of Luminex fungal ASRs on fecal DNA from healthy humans. As with previous studies (David *et al.* 2014; Hallen-Adams *et al.* 2015), we find few common fungal species, and the majority of species that are detected can be attributed to the environment. Although our present view of the intestinal mycobiome is incomplete, and further sampling from diverse populations would add to our knowledge, the sparseness of fungi in fecal samples implies that efforts to comprehensively characterize fungi in the gut are likely to require very deep metagenomic sequencing and that targeted approaches would yield the most meaningful information.

Results and discussion

Detection and identification of gut fungi

Two fungal phyla were detected in the samples — Ascomycota (all 16 samples) and Basidiomycota (13 samples). Using all methods combined, a total of 28 genera were detected (mean and median 8; range 2–17 per sample). Ten genera were present in only one sample and five genera were present in only two samples. *Fusarium* was the most commonly detected genus, in 88% of samples (Fig. 1). *Malassezia* was present in 81% of samples, followed by *Penicillium* (75%), *Aspergillus* (68%) and *Candida* (63%).

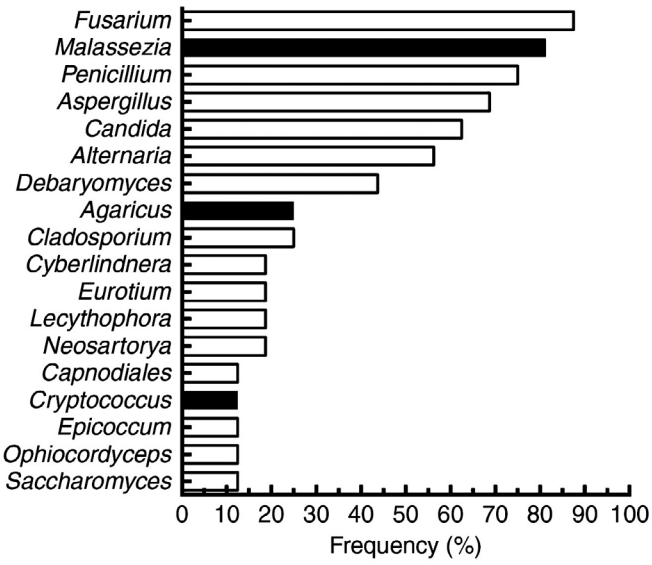


Figure 1. Detection frequency of fungal genera in 16 fecal samples. Only genera present in >10% of samples are shown. Phylum affiliation for each genus is indicated: Ascomycota (white), Basidiomycota (black).

At the lowest discernible taxonomic level, 46 operational taxonomic units (OTUs) were detected. The number of OTUs in an individual sample ranged from 2 to 22 (mean 11; median 10.5). Eighteen OTUs were detected in only one sample and five OTUs were detected in only two samples. The most abundant OTUs included: *Fusarium* cf. *graminearum* (88%), *Malassezia restricta* (81%), *Aspergillus* cf. *niger* and *Penicillium* cf. *roqueforti* (69%) and *Penicillium* cf. *commune* (63%). No species was common to all samples. The two fecal samples taken from the same individual showed little similarity (Fig. 2; M3a & M3b). The fecal sample from the first time point yielded seven OTUs and the second fecal sample yielded 22, with only three detected at both time points (*Agaricus bisporus*, *Alternaria* sp. and *A.* cf. *niger*). Male and female participants did not differ significantly in number of OTUs, while most OTUs were detected from both male and female participants.

The most commonly detected gut fungus among our study participants was *F.* cf. *graminearum.* Among studies published thus far, a variety of different taxa have been observed to dominate different individuals, including *Gloeotinia/Paecilomyces*, *Galactomyces*, *Candida*, *Saccharomyces* and *Penicillium* (Ott *et al.* 2008; Scanlan and Marchesi 2008; Chen *et al.* 2011; Li *et al.* 2012; Hoffman *et al.* 2013). A previous study in our lab, employing the same amplification methods and 454 pyrosequencing on fecal samples from adults approximately from the same geographical location on a conventional diet, identified *Candida tropicalis* as the most abundant gut fungus, present in 57% of 69 samples (Hallen-Adams *et al.* 2015). Given the lack of consensus regarding the dominant species in different studies, it seems reasonable to conclude that there may be few truly autochthonous species and that dominant species are attributable to differences in the individuals' diet and/or other environmental factors, including interactions with other members of the microbiota.

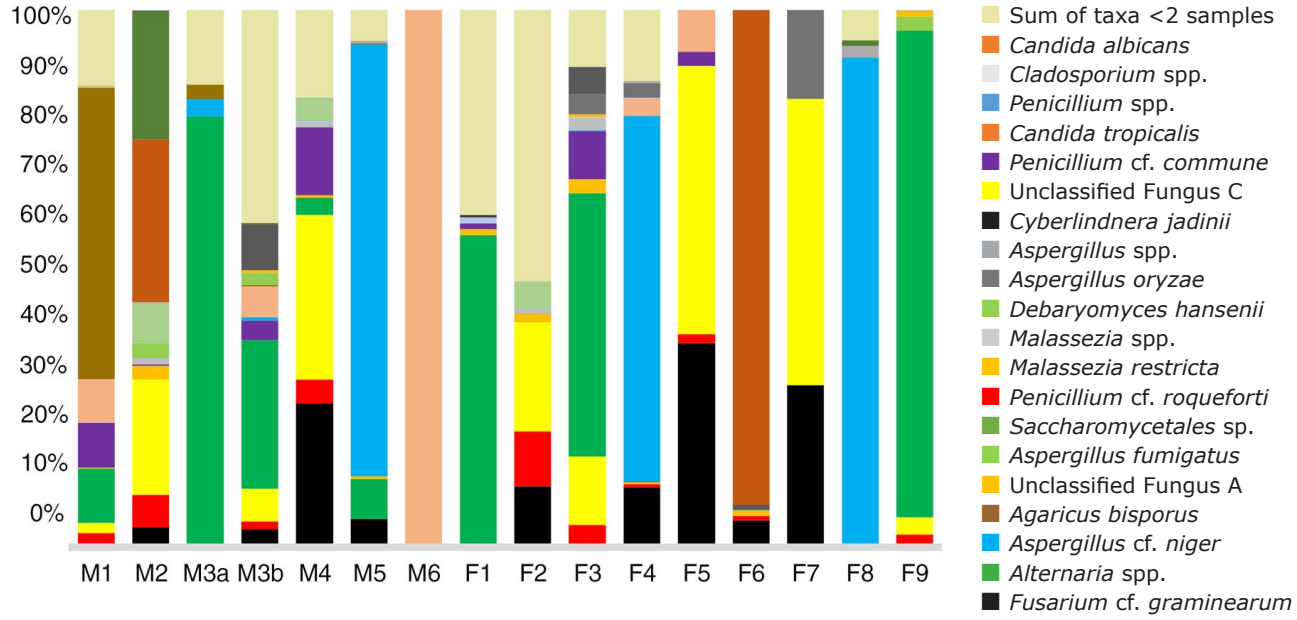
OTUs of interest

Commensals and pathogens

Five species of *Malassezia* were identified from the gut of our cohort. *Malassezia* species are lipophilic yeasts found naturally on the skin, and are associated with a number of skin diseases and dandruff (Findley *et al.* 2013), but this genus is also commonly detected in fecal samples (Chen *et al.* 2011; Hamad *et al.* 2012; Li *et al.* 2012; Gouba *et al.* 2013). The dependence of *Malassezia* species on host lipids does not preclude a niche in the gut; more research is needed to determine if *Malassezia* species are true gut commensals or merely contaminants.

Candida species were detected in 63% of our study samples. *Candida* species are considered to be the most widely distributed and dominant fungi in the human gut mycobiome, but are notorious for their ability to become

Figure 2. Overall distribution of gut fungi. Fungi in >15% of fecal samples from healthy individuals with a vegetarian diet obtained by 454 pyrosequencing are shown.



opportunistic pathogens (Iliev and Underhill 2013). In healthy individuals, carriage of *Candida albicans* is estimated at 30–60% (Moran *et al.* 2012).

Environmental and dietary fungi

Aspergillus and/or *Penicillium* species were detected in all but one sample. *Aspergillus* cf. *oryzae* (three samples) encompasses *A. oryzae*, used in soy fermentations, and its wild progenitor, the toxigenic *Aspergillus flavus*. *Aspergillus fumigatus*, detected in three samples, is among the most ubiquitous airborne fungi, and humans inhale hundreds of *Aspergillus* conidia per day (Goodley *et al.* 1994). In addition, our study showed 11 samples containing *A.* cf. *niger,* a species group contaminating a wide range of food commodities (Pitt and Hocking 2009). *Penicillium* species are among the main causes of food spoilage and many *Penicillium* species detected in this study might be a result of consuming contaminated foods. In addition, *Penicillium camemberti* (indistinguishable by ITS from *P. commune*) and *P. roqueforti* are widely used in the production of mold-ripened cheeses.

Species indistinguishable from *F. graminearum* by ITS sequencing were abundant, detected in seven samples by cloning, ten by ASR and eleven by pyrosequencing (fourteen samples overall). A previous study found *F.* cf. *graminearum* in one sample out of 69 (Hallen- Adams *et al.* 2015). These species are widespread in the environment and in plant materials, while growing poorly if at all at 37°C.

Agaricus bisporus (white button mushroom, portabello, crimini) was detected in four subjects; its detection in feces is almost certainly due to consumption, as are the yeasts *Saccharomyces cerevisiae* and *Debaryomyces hansenii* which were found in six subjects and are used extensively in the production of fermented foods. The detection of foodborne fungi in the distal gut by ITS sequencing of food and fecal samples has been documented by David *et al.* (2014), and it may therefore be common to detect these species transiently in individuals consuming such foods. *Ophiocordyceps*, detected in two individuals, is used as a dietary supplement for a variety of therapeutic practices. Other fungi detected in this study, such as *Epicoccum nigrum* and *Alternaria* spp., are known plant pathogens and could also be present in the gut due to the consumption of contaminated foods. In comparing vegetarians with persons on a conventional western diet (Hallen-Adams *et al.* 2015), we observe little overlap in the gut mycobiota. Notably, the vegetarian mycobiota is dominated by what we would consider dietary or environmental fungi (including a high diversity of plant pathogens), while samples from conventional diet participants are dominated by *Candida* and Dipodascaceae yeasts.

When investigating microbial diversity in any ecosystem, it is important to recognize that not all organisms detected contribute equally to ecosystem function. This concept is illustrated by the fact that many fungi detected in the fecal samples, in our study and in others, can be ruled out as gut residents due to their inability to grow at 37°C, e.g. most *Penicillium* species, *D. hansenii* (Pitt and Hocking 2009), *Agaricus bisporus, Ophiocordyceps sinensis* and others. Furthermore, the ecology of some species (e.g. *O. sinensis*, an obligate pathogen of insect larvae) strongly suggests allochthonous origin. Few taxa (Saccharomycetalean yeasts, *Malassezia*) are consistently detected in fecal samples, either within or between studies; these are the species likeliest to be truly "gut fungi," although their ecological roles and niches within this complex ecosystem are yet unknown.

Comparison of methods

The distribution of fungi detected by pyrosequencing is shown in Fig. 2. The total number of sequences obtained from 16 samples was 186,495 (mean 11,656; median 7802). Alpha diversity of the fungal fecal community was measured with Shannon's (1.70 ± 0.86) and Simpson's (0.53 ± 0.25) indices (Fig. S1). Both indices revealed substantial variation in community diversity within individuals (from one to 24 OTUs). Rarefaction analysis revealed that for the majority of samples community coverage reached saturation (Fig. S2). For 75% of the samples, 600 sequences per sample covered the fungal diversity of the gastrointestinal tract in humans.

ASR assays are available for six of the fungi detected by the sequencing methods: *A. flavus, A. fumigatus*, *A. niger, C. albicans, Candida tropicalis* and *Fusarium*. The ASR assay detected *Fusarium* in 10 samples, *C. albicans* in three and *C. tropicalis* in two (Table S1). While we performed all 23 available ASRs on all samples, the assays did not detect any further species. ASR assays showed the highest rate of detection of *Fusarium* (10 samples), which also represented the most commonly detected OTU in the pyrosequencing data (11 samples).

In three samples *Fusarium* was detected by all three methods; in five samples by ASRs and pyrosequencing; in three samples by cloning and pyrosequencing; and in one sample by cloning alone. In one sample, *C. albicans* was detected by all three methods, in two samples by ASRs alone, and in two samples by pyrosequencing alone. The *A. fumigatus* and *A. niger* ASRs failed to detect anything, despite high representation of these OTUs in the sequencing data (Table S1). As our *A.* cf. *niger* is imperfectly identified, it is possible that this ASR shows high fidelity to *A. niger sensu stricto*, and is not detecting the OTU(s) in our samples. Babady *et al.* (2011) report suboptimal detection of *A. niger, A. fumigatus* and *A. flavus* using this assay. Methods differed in probability of detection at $P < 0.05$ for *A.* cf. *niger* and *C. tropicalis*.

Molecular cloning identified *Aspergillus* as the most abundant genus and pyrosequencing identified *Fusarium*, *Malassezia* and *Penicillium* as the most abundant genera. Pyrosequencing identified 41 OTUs that cloning failed to detect. In contrast, *Leptosphaerulina chartarum* was only detected by molecular cloning. Tedersoo *et al.* (2010) reported that by combining Sanger sequencing and 454 pyrosequencing 66% of the species were common between the two methodologies, and reported considerable methodological biases towards certain lineages.

Despite the relatively low level of fungal diversity detected in our samples (compared with bacterial diversity) it was somewhat surprising that only 25% of the samples had sequences in the clone libraries that were also abundant OTUs in the pyrosequencing data (Fig. 2, Table S1). Indeed, several species detected by cloning were not among the top hits identified by pyrosequencing in their respective sample, and 16 OTUs identified by cloning never appeared in the pyrosequencing data of that sample. Different methods may show preferential amplification and detection of different sequences.

All three methods used are limited by dependence on PCR during sample preparation, the results of which are influenced by primer choice and template characteristics as well as initial template abundance (Tedersoo *et al.* 2010). The complexity of the community is also a contributing factor; in 454-based pyrosequencing, bacterial taxa that are less than 0.1% of the community are highly subject to sample error. Beyond the known effects of sample error and PCR bias, the multicopy nature of the nuclear ribosomal region in fungi also confounds quantification. The nuclear rRNA genes differ substantially in copy number between organisms (180 copies in the sequenced strain of *F. graminearum vs* 55 copies in *C. albicans*). Amend *et al.* (2010) report a tenfold difference in 454 read counts between species from samples prepared with the same spore counts of each species, and conclude that while read abundance may be quantitative within species, between-species comparisons are unreliable. We are not confident that any method used in this study provides highly quantitative data; however, alone and in combination, a snapshot of gut fungal diversity is produced.

No single method identified the full fungal diversity in every sample; however, we must ask whether identifying the full fungal diversity is a meaningful goal if a majority of gut fungi are 'passing through', and their contribution to the gut ecology is likely to be minimal. The gut mycologists' first priority should be the development of a probable 'core mycobiome', likely including various Saccharomycetalean yeasts such as *Candida, Saccharomyces* (Hoffman *et al.* 2013) and *Galactomyces* (Hallen-Adams *et al.* 2015) and *Malassezia* (Dupey *et al.* 2014). Common environmental fungi that are also commonly detected in fecal samples (*Cladosporium* [Hoffmann *et al.* 2013], *Aspergillus, Penicillium* and *Fusarium*) need further investigation to determine which species may actually persist to play any role in gut ecology and which can be discounted based on physiological constraints (Suhr and Hallen-Adams 2015). Ultimately, a targeted assay with ASR-like probes optimized for known gut fungi and knowable (or easily validated) quantitative performance is likely to be more rapid and cost-effective than continuing use of shotgun approaches to capture an illusory species richness.

Materials and methods

Collection of fecal samples

The Institutional Review Board of the University of Nebraska approved all study protocols (IRB Approval Number: 20111112037EP). Written informed consent was obtained from all participants. Fecal samples were obtained from 15 human adults (six male, nine female; one subject provided two samples on dates 2 months apart; $n = 16$) who self-identified as 'healthy', and had not taken antifungal medications within the previous 6 months. Study subjects were self-identified vegetarians and the participant ages ranged from 19 to 48, with a mean of 23 and a median of 20. All participants were from the Midwestern United States. After collection, each fecal sample was immediately preserved at –80°C until the time of processing.

DNA isolation and PCR amplification

A ~200 mg subsample was suspended in phosphate-buffered saline, with the addition of 300 mg 0.1 mm zirconium beads, and pelleted by centrifugation at 8000 g for 5 min and DNA was extracted using a modified method of the QIAamp® DNA stool mini kit (Qiagen, Hilden, Germany) (Martínez *et al.* 2010). A nested PCR amplification was as described in Hallen-Adams *et al.* (2015). Products of the first PCR reaction were used for both molecular cloning and pyrosequencing; primers for molecular cloning did not contain pyrosequencing adaptor sequences or barcodes. Negative controls were used throughout to ensure contamination did not occur. PCR products were purified using the Wizard Genomic DNA purification kit (Promega, Madison, WI) following the manufacturer's instructions.

Molecular cloning

PCR products were cloned using pGEM T-Easy (Promega) following the manufacturer's instructions. Ten colonies negative for β-galactosidase activity were evaluated by *Hae*III and *Eco*RI restriction digest, and those with differing profiles were sequenced by dideoxy sequencing at Michigan State University's Research Technology Support Facility (East Lansing, MI). Samples were identified by sequence homology using nucleotide BLAST against the UNITE curated fungal ITS sequence database (Kõljalg *et al.* 2013) and the curated FUNCBS database (http://www.cbs.knaw.nl/Collections/BioloMICSSequences.aspx?file=all ; accessed July 2014). Sequences were deposited in GenBank as accessions KP196567– KP196602.

Pyrosequencing of the fungal ITS genes

ITS PCR reactions were performed as described above and samples were sequenced by MR DNA (Shallowater, TX) using tag-encoded FLX amplicon pyrosequencing on 454/Roche GS FLX Sequencer technology (Dowd *et al.* 2008; Sun *et al.* 2011). Data processing and analysis followed Hume *et al.* (2012). MrDNA clustered denoised sequences into OTUs at 97% similarity. We further combined sequences differing by more than 3% but BLASTing to the same species in the UNITE and/or FUNCBS databases. Rarefaction analysis based on observed OTU metrics was performed to assess community coverage using QIIME (ver. 1.8.0) (Caporaso *et al.* 2010) with default settings. Alpha diversity of the samples was measured based on Simpson and Shannon's coefficients in QIIME (Caporaso *et al.* 2010). One sample with null diversity (only one OTU detected by pyrosequencing) was removed from analysis. OTUs detected <10 times were discarded as probable artifacts. Raw sequences have been deposited in GenBank under BioProject PRNJA268649.

Luminex fungal xTAG ASR assay

A multiplex fungal analyte-specific reagent (ASR) assay developed by Luminex Molecular Diagnostics (Austin, TX) was used for the simultaneous detection and identification of 23 species in the fecal samples. Multiplex PCR, bead hybridization and detection were performed on DNA extracted from fecal samples above following the protocol of Babady *et al.* (2011). The samples were analyzed on the MAGPIX system (Luminex Corp., Austin, TX) and the median fluorescence intensity (MFI) was calculated by XPONENT 4.2 (Luminex Corp., Austin, TX). A threshold value of 225 MFI was set for positive targets, values below 200 MFI were negative and 200–224 MFI were equivocal. Cochran's Q test, as implemented using RVAideMemoire in R (Hervé 2015), was used to compare each method using the six taxa for which ASRs were available.

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Conflict of Interest — The authors declare no conflict of interest associated with this research.

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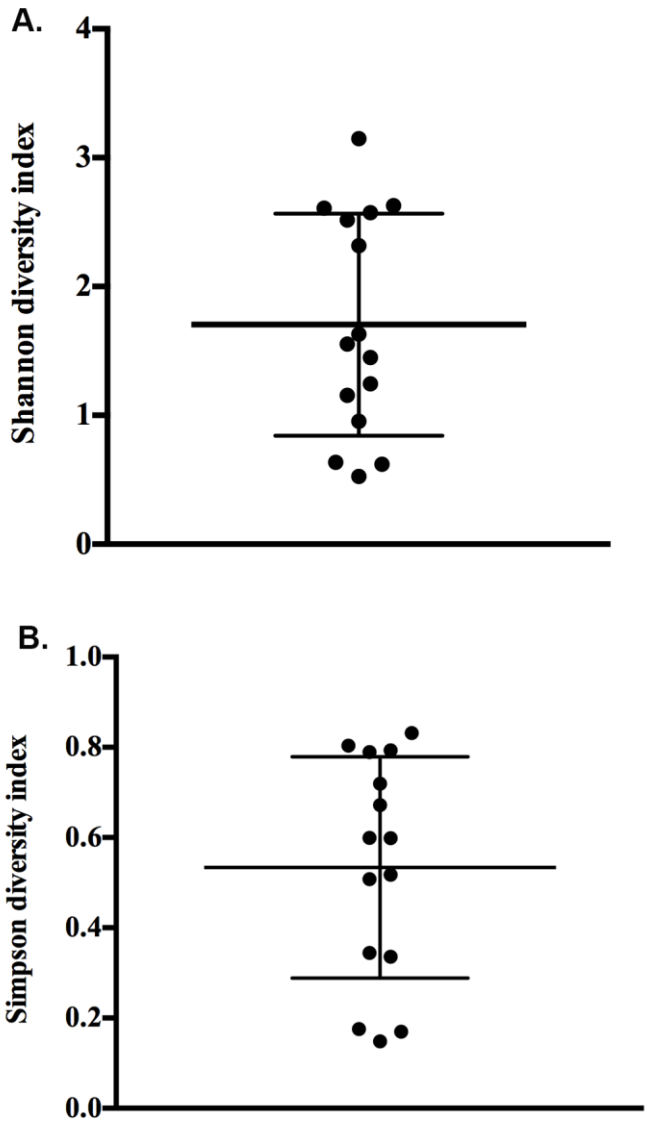
Supporting Information (following)

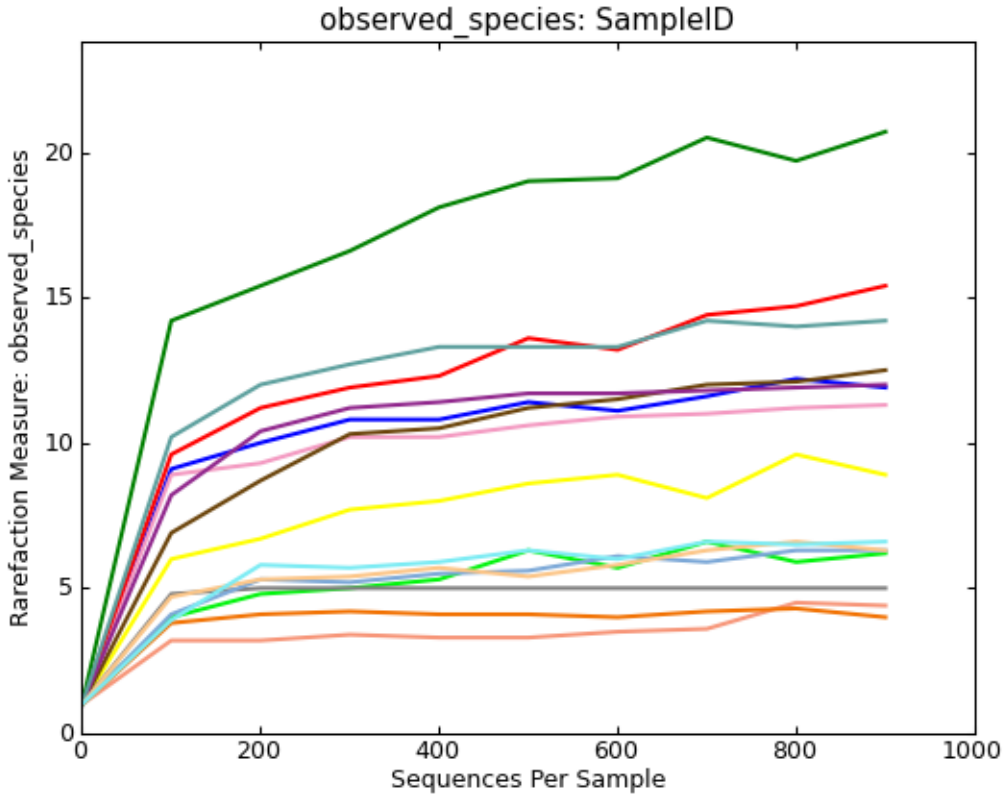
Figure S1. Alpha diversity of the fungal fecal community.

Figure S2. Rarefaction analysis of ITS pyrosequencing tags in fecal samples from adults with a vegetarian diet.

Table S1. Fungi detected in fecal samples from healthy adult vegetarians, by 454 pyrosequencing, cloning and Luminex ASR assays.



Supplementary Figure 1. Alpha diversity of the fungal fecal community. A) Shannon's diversity index, and B) Simpson's diversity index.



Supplementary Figure 2. Rarefaction analysis of ITS pyrosequencing tags in fecal samples from adults with a vegetarian diet.

Supplementary Table 1. Fungi detected in fecal samples from healthy adult vegetarians, by 454 pyrosequencing, cloning, and Luminex ASR assays. Numbers representing sequencing hits in 454 pyrosequencing. Outline boxes indicate detection by cloning. For the six OTUs for which ASR assays were available, green indicates detection, pink indicates no detection, and yellow indicates equivocal detection. OTUs given in bold are reported for the first time in fecal samples from healthy humans.

OTU	M1	M2	M3a	M3b	M4	M5	M6	F1	F2	F3	F4	F5	F6	F7	F8	F9
Agaricus bisporus	3781	0	438	12	0	15	0	0	0	0	0	0	0	0	0	0
Alternaria spp.[1]	704	0	12800	1413	54	1008	0	34497	0	7761	0	0	0	5	0	2433
Ascocoryne cylichnium	0	0	0	0	0	0	0	0	1340	0	0	0	0	0	0	0
Aspergillus fumigatus	0	548	0	0	74	0	0	0	396	0	0	0	0	0	0	0
Aspergillus cf.[1] *niger*	0	0	506	33	0	10862	0	0	0	39	5290	0	0	0	10226	0
Aspergillus cf. *oryzae*	0	0	0	0	0	0	0	0	0	590	211	0	0	1373	0	0
Aspergillus spp.	0	8	4	0	0	74	0	0	0	0	29	0	0	0	240	0
Candida albicans	2	2197	0	5	0	0	0	0	0	0	0	0	18933	0	371	0
Candida tropicalis	562	0	0	292	0	0	5	0	0	0	284	181	2	0	0	0
Capnodiales sp.[1]	0	0	0	18	0	0	0	0	0	37	0	0	0	0	0	0
Cladosporium spp.	0	0	0	436	0	0	0	0	0	802	0	0	184	0	0	0
Cryptococcus amylolyticus	0	0	0	1336	0	0	0	0	0	0	0	0	0	0	0	0
Cryptococcus tephrensis	0	0	0	0	0	0	0	0	0	1551	0	0	0	0	0	0
Cyberlindnera jadinii	0	14	0	0	0	0	0	279	0	6	0	0	0	0	0	0
Debaryomyces hansenii	0	196	0	111	0	0	0	0	0	0	0	0	37	0	2	70
Diplodia mutila	0	0	0	0	0	295	0	0	0	0	0	0	0	0	0	0

Supplementary Table 1 Continued.

OTU	M1	M2	M3a	M3b	M4	M5	M6	F1	F2	F3	F4	F5	F6	F7	F8	F9
Epicoccum nigrum	0	0	0	216	0	0	0	0	0	0	0	0	0	0	0	0
Eurotium rubrum	0	0	0	0	0	0	0	0	2475	0	0	0	0	0	0	0
Eurotium sp.	0	0	0	0	0	0	0	0	0	0	890	0	0	0	257	0
Exophiala heteromorpha	0	0	0	246	0	0	0	0	0	0	0	0	0	0	0	0
Fusarium cf. *graminearum*	47	264	0	170	455	710	0	0	890	0	868	883	1026	2513	18	0
Galactomyces sp.	0	0	2208	0	0	0	0	0	0	0	0	0	0	0	0	0
***Lecythophora* spp.**	576	0	0	0	250	0	0	0	0	0	0	0	0	0	0	0
Leptosphaerulina chartarum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Malassezia globosa	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0
Malassezia pachydermatis	0	0	0	0	0	14	0	0	0	0	0	0	0	0	0	0
Malassezia restricta	13	179	0	0	8	73	0	671	120	422	30	0	195	2	0	0
Malassezia slooffiae	0	0	0	171	0	0	0	0	0	0	0	0	0	0	0	0
Malassezia sp.	5	89	0	0	11	10	0	0	73	345	0	0	0	5	0	0
Malassezia sympodialis	0	0	0	0	0	450	0	0	0	0	0	0	0	0	0	0
Neosartorya fischeri	0	4	0	0	6	0	0	0	3	0	0	0	0	0	0	0
Ophiocordyceps sinensis	0	0	0	0	0	0	0	0	120	0	40	0	0	0	0	0
Penicillium cf. *commune*	582	22	0	187	210	0	0	627	4	1398	9	60	4	0	0	0

Supplementary Table 1 Continued.

OTU	M1	M2	M3a	M3b	M4	M5	M6	F1	F2	F3	F4	F5	F6	F7	F8	F9
Penicillium cf. *roqueforti*	136	445	0	75	74	0	0	56	802	656	42	39	161	0	0	64
Penicillium sp.	12	0	0	4	7	0	0	628	2	34	14	0	2	0	0	0
***Pholiota* sp.**	0	0	0	0	0	0	0	0	0	0	36	0	0	0	0	0
Pichia kudriavzevii	0	0	0	0	0	0	0	22692	0	0	0	0	0	0	0	0
Pleosporales sp.	6	0	0	9	0	0	0	0	4	73	0	0	0	0	0	3
Rhodotorula sp.	332	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Saccharomycetales sp.	0	1729	0	8	0	0	0	0	0	0	0	0	0	0	119	2
Saccharomyces cerevisiae	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Torulaspora sp.	0	0	0	13	0	0	0	0	0	0	0	0	0	0	0	0
Trichocomaceae sp.	0	0	0	0	0	0	0	0	18	5	0	0	0	0	0	0
Trichoderma longibrachiatum	0	0	0	0	0	0	0	0	0	0	20	0	0	0	0	0
Unclassified[2] "Fungus A"	10	0	0	24	0	0	0	0	0	89	0	0	0	0	0	27
Unclassified "Fungus C"	134	1558	0	308	510	0	0	0	1590	2015	0	1161	0	4438	4	84
Unclassified "Fungus D"	61	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sum from 454	6970	7255	15956	5099	1670	13518	5	59456	7844	15830	7763	2324	20546	8336	11237	2683

[1]Where “spp.” or “cf.” are used, this indicates that closely related species cannot be distinguished by ITS sequence, and more than one species may consequently be included. The use of “sp.” indicates a single OTU (based on 97% sequence similarity) that does not show a sufficient match to database entries to be placed to species.

[2]Unclassified OTUs BLAST with moderate sequence homology to both ascomycete and basidiomycete fungi, but with no conclusive hits to any named organism.