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Fungi inhabiting the healthy human gastrointestinal tract: A diverse and dynamic community

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Abstract

Fungal DNA was selectively amplified, and the ITS region sequenced, from fecal samples taken from 45 healthy human volunteers at one (21 volunteers) or two (24 volunteers) time points. Seventy-two operational taxonomic units, representing two phyla and ten classes of fungi, were recovered. *Candida* yeasts, notably *Candida tropicalis* (present in 51 samples), and yeasts in the Dipodascaceae (39 samples), dominated, while 38 OTUs were detected in a single sample each. Fungi included known human symbionts (*Candida*, *Cryptococcus*, *Malassezia* and *Trichosporon* spp.), common airborne fungi (*Cladosporium* sp.) and fungi known to be associated with food (*Debaryomyces hansenii* and high salt fermented foods; *Penicillium roqueforti* and blue cheese). In contrast with gut-associated bacteria, fungi occurred in much lower abundance and diversity, and fungal composition appeared unstable over time.

Keywords: *Candida*, CTG yeasts, Human gut microbiome, *Malassezia*

Introduction

Fungi are ubiquitous on Earth, arguably occupying more niches than any other eukaryotic kingdom. Consummate decomposers, fungi are capable of digesting materials few or no other organisms can, such as lignin and plastics. Symbiotic fungi of the rumen play a crucial role in releasing otherwise unavailable nutrients from plant matter to herbivores (Trinci et al., 1994). Some wood-boring beetles rely on the enzymes of gut fungi to digest their meals (Nguyen et al.,

2006), while others (and other groups of insects, such as termites and leaf-cutter ants) cultivate particular species and strains of fungi to predigest their food (Makonde et al., 2013, Pagnocca et al., 2008 and Popa et al., 2012).

Early studies of human gut microbiota, based on culturing organisms obtained from fecal matter, revealed representatives of all three domains of life, viz., the Bacteria, Archaea and Eukarya (Rajilic-Stojanovic et al., 2007). The characterization of the prokaryotic denizens of the healthy human gastrointestinal (GI) tract continues to yield new insights into the

role of micro-organisms in human health (Martins dos Santos et al., 2010, Peterson and Cardona, 2010 and Walter, 2008). However, prokaryotes are not the only components of the mammalian gut microbiota. The roles of gut eukaryotes in humans are just beginning to be investigated, and differing populations and/or methodologies report differing results. One paper (Raijilic-Stojanovic et al., 2007) reports the eukaryotic domain represented exclusively by fungi, while another (Scanlan and Marchesi, 2008) identifies the stramenopile *Blastocystis* as the dominant eukaryote.

In comparison with prokaryotic microorganisms, the abundance, role and diversity of fungi in the human gut have been little-studied, and the majority of studies to date have focused on the possible role of fungi in disease states (Ott et al., 2008 and Underhill and Braun, 2008), or following GI tract perturbation (Mavromanolakis et al., 2001). A majority of studies have examined *Candida* yeast species, largely on the basis of the potential for these species to serve as facultative pathogens, with Bernhardt (1998) summing up the ambivalence towards these organisms: "Fungi in the intestine – normal flora or pathogens?" (see also Schulze and Sonnenborn (2009)). With the development of a healthy human reference microbiome by the Human Microbiome Project (Human Microbiome Project Consortium, 2012), it is expected that the role of human-associated eukaryotes will come under increasing scrutiny in the next several years, and papers are just beginning to address the role of organisms other than bacteria in the healthy human (Andersen et al., 2013, Gouba et al., 2013 and Hoffmann et al., 2013).

The possibility of beneficial – or at least not overtly pathogenic – fungi living on and in humans has received very little attention. Yeast fungi are known to be symbiotic with mammals. *Saccharomyces* yeasts may occur naturally in humans where they limit inflammatory response and increase immune health; these have also been used as probiotics (Kourelis et al., 2010). Filamentous fungi, many of which decompose complex carbohydrates such as cellulose and lignin, have been studied in the digestive tracts of insects (Lichtwardt, 2008) and the rumen of cattle (Theodorou et al., 2005), and have been reported in human fecal samples (Finnegold et al., 1974).

With the potential for fungi to play an important role in human digestive health (i.e. by releasing otherwise unavailable nutrients) as well as cause harm in the growing populations of those suffering perturbation to the GI tract (immuno-compromised persons, autoimmune disorders, food allergies and intolerances), it becomes crucial to establish a baseline for the fungal composition of the healthy human gut. We report an amplicon pyrosequencing study, using fungal-specific primers, of DNA extracted from feces from 45 healthy human volunteers at two time points.

Materials and methods

Ethics statement

The human trials of this study were approved by the Institutional Review Board of the University of Nebraska (IRB Approval Numbers: 2008038840EP, 2008038840EP, and

2009019551EP), and from Kansas State University (IRB Approval number: 5298), and written informed consent was obtained from all subjects.

Sample collection and DNA extraction

As part of previous and ongoing studies on gut microbial composition (Davis et al., 2011, Martínez et al., 2010 and Martínez et al., 2013), fecal samples were provided by healthy human subjects, none of whom had been on antibiotics or a vegetarian diet within three months prior to the start of the studies or throughout their duration. DNA was extracted from the feces following the procedure of Martínez et al. (2010). In short, fecal samples were diluted 1:10 in phosphate-buffered saline (PBS; pH 7.0) upon collection and stored at -80°C until further processing. One ml aliquots of the fecal homogenates were thawed and transferred to sterile tubes containing 300 mg of 0.1 mm zirconium beads. Cell pellets were obtained by centrifugation ($8\,000 \times g$ for 5 min) and were washed three times with 1 ml ice cold PBS. Pellets were then resuspended in 100 μl lysis buffer (200 mM NaCl, 100 mM Tris, 20 mM EDTA, 20 mg ml^{-1} lysozyme, pH 8.0) and incubated at 37°C for 30 min. Next, 1.6 ml Buffer ASL from the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was added to each sample, and cells were mechanically disrupted in a MiniBeadbeater-8 (BioSpec Products, OK, USA) for 2 min at maximum speed. DNA was purified from 1.2 ml of the resulting homogenate with the QIAamp DNA Stool Mini Kit following the manufacturer's instructions. In this study, we were interested in the normal fungal flora of the unperturbed GI tract, so only baseline (collected at the beginning of the previous studies, prior to any [dietary] treatment) and washout (collected at the end of those studies, three weeks following the final treatment) samples were examined. Samples from the same individual were collected 13 or 16 weeks apart.

Isolation and sequencing of fungal DNA

Fungal DNA was selectively PCR-amplified using the fungal-specific forward primer ITS 1F (Gardes and Bruns, 1993) and the eukaryotic reverse primer TW13 (White et al., 1990). Due to the low abundance of fungal DNA (compared with bacterial DNA), a nested PCR reaction was then performed using ITS 1F and the eukaryotic ITS4 (White et al., 1990) primers, which amplify the internal transcribed spacer (ITS) 1, 5.8S, and ITS2 regions of the ribosomal RNA genes. The nested primers were modified following the Roche-454 Titanium protocol, and the A or B Titanium sequencing adapters were added (shown in *italics*). In the forward primer, the adapter was followed immediately by a unique 8-base barcode sequence (BBBBBBB), and finally the ITS1F primer, as shown:

5'-CCATCTCATCCCTGCGTGCTCCGACTCAGBBBBBBBCTTG-GTCATTAGAGGAAGTAA-3'. The reverse primer was composed of the Titanium B sequencing adapter followed by ITS4: 5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGTCCTCC-GCTTATTGATATGC-3'. Individual samples were amplified with primers containing unique barcodes, which allowed multiplexing. PCR amplification was performed on a Mastercycler Pro (Eppendorf) using Ex-Taq polymerase (TaKaRa Bio, USA) following the procedure of Martínez et al. (2010). Pyrosequencing

quencing was performed by the Core for Applied Genomics and Ecology (CAGE) at the University of Nebraska-Lincoln. Sequences were deposited in the Sequence Read Archive as BioProject PRJNA266974, BioSample accessions SRS744923–SRS744992, experiment accessions SRX760304–SRX760379.

Additionally, PCR was performed using ITS 1F in combination with ITS4NA to amplify non-ascomycete fungi (Parrent et al., 2006), and primers CM2 and 1520R were used for Cryptomycota (Lefevre et al., 2007). A nesting reaction was performed using barcoded primers with Titanium A (forward) and B (reverse) adaptors as above.

Sequence analyses

Raw ITS sequences were processed through a quality filter as described in Benson et al. (2010); sequences with an average quality score ≥ 20 were trimmed to remove adapter and primer sequences and demultiplexed based on the barcode. CD-HIT was used to generate clusters of identical sequences (Li and Godzik, 2006 and Niu et al., 2010), and clusters composed of fewer than ten sequences were discarded as probable artifacts. Clusters of ten or more sequences were subjected to a local BLAST search against GenBank's non-redundant nucleotide database (Altschul et al., 1990). One exemplar was selected from each unique combination of BLAST top hit + individual sample + time point, for a total of 412 sequences (GenBank accessions KC525444 – KC525855), and these were aligned using MUSCLE (Edgar, 2004) with default settings, as implemented in MEGA version 5 (Tamura et al., 2011). Due to the uncertainty of relying on BLAST best hits (especially for fungi for which perfect matches may not exist in the databases) coupled with high levels of sequence divergence within and between taxa, OTUs and some more inclusive clades (such as *Candida* yeasts) were subjected to more rigorous evaluation. The sequences representing each cluster composed of 100 or more hits were manually aligned in TextWrangler (Bare Bones Software, Bedford, MA), along with sequences from the public databases, prior to alignment refinement in MUSCLE. MEGA generated a single neighbor-joining tree for each analysis, using default options.

Statistical analyses

Rarefaction analysis based on observed OTU metrics was performed to assess community coverage using QIIME (v. 1.7.0) (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).

Given the binomial distribution of fungal OTUs across samples, fungal counts were transformed into binomial data where a fungal OTU was considered to be present in a sample if the observed count was greater than 10. Raw bacterial counts were normalized by dividing the total count. Raw counts were higher for fungi than for bacteria (average ~8,000 hits per sample for fungi, vs. 5,400 hits per sample for bacteria); however, this cannot be taken as a proxy for abundance, as the fungal PCR products were subject to nesting while the bacterial PCR products were not. Consequently, relative abundance within fungi was compared to relative abundance within bacteria. To test for association between bacte-

rial taxa and fungal taxa, individual logistic regressions were run with the presence/absence of a fungal taxon being the dependent variable and the log₁₀ of the normalized bacterial taxa being the independent variable. The association between the bacterial taxa and fungal taxa was quantified by the log-odds ratio of 10 fold increase in the normalized count. To control for multiple testing the false discovery rate was calculated for each comparison (Table 1).

Results

We were able to amplify fungal DNA from 45 individuals (of 49 individuals sampled): 24 from two time points and 21 from one time point (baseline or washout) for a total of 69 samples. We were unable to amplify fungal DNA from the second time point from the above-mentioned 21 individuals, while bacterial DNA was readily amplified and sequenced from the same DNA extractions.

Clustering analysis using CD-HIT yielded 10 679 clusters containing ten or more identical sequences (up to 12 695 sequences in the largest cluster; mean of 51). On the basis of sequence similarity, these clusters collapsed into 72 distinct OTUs, containing from 10 to 238 079 hits (mean = 10 499), representing two phyla and ten classes of fungi. Of the 72 OTUs, 38 were detected only once, while the most-commonly detected fungus, *Candida tropicalis*, was recovered from 51 samples. The greatest number of OTUs recovered from a single sample was 12, while seven samples contained only one fungal OTU each. Fungal OTUs, the number of samples containing each OTU, and the total number of hits to each OTU, are given in Figure 1. Supplementary Table S1 provides the fungal profiles for each individual sample in the study. Alpha diversity of the fungal fecal community measured with Shannon's (0.78 ± 0.63) and Simpson's (0.30 ± 0.24) indices (Supplementary Figure S1), revealed substantial variation in community diversity within individuals, as in some subjects only one fungal OTU was detected (null diversity), whereas in others up to 12 distinct OTUs were detected.

Forty four samples in this study, representing 39 individuals, had been previously pyrosequenced and analyzed for bacteria (Davis et al., 2011 and Martínez et al., 2010). For persons from whom both fungal and bacterial data were available, 1–11 fungal OTUs per individual were obtained (mean 3.4), while participants harbored from 31 to 54 bacterial OTUs (mean 43.2). Twenty (of 45) fungal OTUs identified in this population were only detected in a single individual, while only one bacterial OTU (of 88) was unique to a single individual.

Ascomycete yeasts in the order Saccharomycetales were the dominant OTUs detected; only two participants with detectable fungi did not carry detectable Saccharomycetalean yeasts at any time point. Within the Saccharomycetalean yeasts, two major groups could be identified: the CTG yeasts (here, *Candida* spp. and *Debaryomyces hansenii* (= *C. famata*) – a monophyletic group defined by the use of the codon CTG to encode serine rather than the canonical leucine), and the Dipodascaceae (here, species allied to *Galactomyces geotrichum* and *Geotrichum (Saprochaete) gigas*). The dominant filamentous fungus was an unidentified OTU in the ge-

Table 1. Significant associations between fungal and bacterial taxa

Fungi	Bacteria	Estimate	StdErr	WaldChiSq	ProbChiSq	fdr_p
Species	Genus					
<i>Candida albicans</i>	<i>Dorea</i>	-7.5587	3.7398	4.0852	0.0433	0.84629
<i>Candida rugosa</i>	<i>Dorea</i>	3.6407	1.6038	5.1533	0.0232	0.63964
<i>Candida rugosa</i>	<i>Faecalibacterium</i>	1.2777	0.6187	4.2648	0.0389	0.63964
<i>Candida sake</i>	<i>Anaerovorax</i>	3.3021	1.6397	4.0557	0.0440	0.83472
<i>Candida tropicalis</i>	<i>Bacteroides</i>	3.8559	1.5357	6.3046	0.0120	0.47535
<i>Candida tropicalis</i>	<i>Eubacterium</i>	-1.2160	0.6038	4.0561	0.0440	0.47535
<i>Cladosporium</i> sp.	<i>Butyricimonas</i>	-2.6424	1.1606	5.1834	0.0228	0.90404
<i>Debaryomyces hansenii</i>	<i>Asaccharobacter</i>	5.4371	2.5176	4.6639	0.0308	0.36971
<i>Debaryomyces hansenii</i>	<i>Butyricimonas</i>	-1.6462	0.7358	5.0052	0.0253	0.36971
<i>Debaryomyces hansenii</i>	<i>Collinsella</i>	1.0771	0.5455	3.8989	0.0483	0.36971
<i>Geotrichum gigas</i>	<i>Dialister</i>	-0.7198	0.3369	4.5653	0.0326	0.65094
<i>Pichia jadinii</i>	<i>Odoribacter</i>	2.0529	0.6912	8.8200	0.0030	0.12514
<i>Pichia jadinii</i>	<i>Turcibacter</i>	2.8496	1.3357	4.5516	0.0329	0.64064
Genus	Genus					
<i>Aspergillus</i>	<i>Butyricimonas</i>	-2.5294	1.2425	4.1444	0.0418	0.94299
<i>Cladosporium</i>	<i>Butyricimonas</i>	-2.6424	1.1606	5.1834	0.0228	0.90404
<i>Malassezia</i>	<i>Escherichia/Shigella</i>	-1.6267	0.7991	4.1438	0.0418	0.43876
<i>Malassezia</i>	<i>Haemophilus</i>	-2.0684	0.9510	4.7303	0.0296	0.43876
<i>Malassezia</i>	<i>Oscillibacter</i>	2.3712	1.1437	4.2986	0.0381	0.43876
<i>Malassezia</i>	<i>Ruminococcus</i>	1.5248	0.7063	4.6610	0.0309	0.43876
<i>Pichia</i>	<i>Odoribacter</i>	2.0529	0.6912	8.8200	0.0030	0.12514
<i>Pichia</i>	<i>Turcibacter</i>	2.8496	1.3357	4.5516	0.0329	0.64064
<i>Trichosporon</i>	<i>Escherichia/Shigella</i>	-1.9644	0.8612	5.2026	0.0226	0.28325
<i>Trichosporon</i>	<i>Holdemania</i>	-2.9995	1.1595	6.6924	0.0097	0.28325
<i>Trichosporon</i>	<i>Oscillibacter</i>	3.4959	1.5672	4.9756	0.0257	0.28325
<i>Trichosporon</i>	<i>Parabacteroides</i>	1.2057	0.5451	4.8923	0.0270	0.28325

Associations between fungal species and bacterial genera are at top, and between fungal genera and bacterial genera are at bottom. Estimate is the log-odds ratio for a fungal taxon being present with a 10-fold increase in a bacterial taxon. The WaldChiSq is Wald chi-square statistic, ProbChiSq is the unadjusted *p*-value, and fdr_p is the false discovery rate.

nus *Cladosporium* (Capnodiales, Dothidiomycetes, Ascomycota), detected in 14 samples with 46 193 hits. Known human symbionts such as *Candida*, *Malassezia* and *Cryptococcus* spp., and members of the Trichosporonales, were detected, with *Candida* abundant and *Malassezia* comparatively common.

Only the phyla Ascomycota and Basidiomycota (=“Dikarya”) were represented in our sequence dataset. ITS sequence diversity throughout samples is shown in Figure 2. The *Cladosporium* we detected likely represent multiple species, with top BLAST hits including GenBank accession HQ211815.1 (uncultured *Cladosporium* clone 6_j08), HQ115727.1 (*Cladosporium langeronii* isolate R1), AM410611.1 (Ascomycete sp. VTT D-041034) and GU174374.1 (uncultured fungus clone Alb-O_MayH10); these, however, appear to be closely related based on the cladogram and on sequence comparisons. This cladogram failed to distinguish *C. tropicalis* from *C. sake*, while the two can be differentiated on the basis of indels of 10 and 25 bp in the 5.8S region (Supplementary Figure S2). Within *C. tropicalis*, from 0 to 23 base pair differences were observed (within 423 aligned bp) when a given sequence was compared with GenBank EU288196.1, the closest BLAST match for the majority of our *C. tropicalis* sequences (in comparison, the closest *C. albicans* sequence differed from *C. tropicalis* EU288196.1 by 40 bases).

Several fungi that could not be satisfactorily classified on the basis of BLAST searches were detected; these are shown

in Figure 1 and Supplementary Table S1 as “unclassified ascomycete 1”, “unclassified basidiomycete 1”, “unclassified fungus 1”, etc., depending on whether sequence similarity suggested affinity with ascomycetes, basidiomycetes, or equal affinity to both. “Unclassified ascomycete” 1 and 5 were each detected in four samples; ascomycete 15 and fungus 5 in two samples, and 15 other “unclassified” taxa in one sample each. In these cases, there were no BLAST matches with sufficient sequence similarity to suggest species-level identity and/or none of the top 100 BLAST hits were identified to species (i.e. “unclassified fungus 4” shares 96 % sequence similarity to its top match, GenBank accession GQ999437, “uncultured fungus clone L042881-122-061-C01”).

In the 24 individuals from whose samples fungal DNA could be amplified from both time points, 55 OTUs were present. Detection of the same fungus at both time points from the same individual occurred less than 20 % of the time; when the two most widely-abundant fungi, *C. tropicalis* and *Geotrichum gigas*, were removed from consideration, the rate of recurrence dropped to 9.2 %.

Rarefaction analysis revealed that for the majority of samples (74.3 %) community coverage reached saturation (Supplementary Figure S3). Overall, the data indicated that 4 000 sequences per sample covered the fungal diversity of the gastrointestinal tract in humans.

Few significant associations were observed between fungi and bacteria (Table 1), and the false discovery rate was high

Figure 1. Distribution of fungi in fecal samples from healthy humans. The bar graph shows the relative abundance of the 14 taxa present in five or more samples, plus the sum of all other taxa (in blue), for all samples.

for all comparisons. This may be partly attributable to the relative paucity of common fungal OTUs (i.e. those detected in multiple samples), and to the presence/absence distribution of gut fungi, compared with the continuous distribution observed for gut bacteria. A negative association between skin-associated fungi (*Malassezia* and *Trichosporon*) and *Escherichia/Shigella* was observed at all levels above species (genus, family and order). *Candida tropicalis* showed a positive association with *Bacteroides* and a negative association with *Eubacterium*, while *G. gigas* showed a weak negative association with *Dialister*; however, the possibility that these associations are artifacts of relative OTU abundance (especially for *C. tropicalis* and *Bacteroides*) cannot be ruled out.

Discussion

The most notable results of this study were: (1) the small number of fungal OTUs detected in multiple samples, and thus the extreme limitation or absence of an observed “core” fungal gut microbiome (in contrast with bacteria); (2) the prevalence of environmental micro-organisms; and (3) the consequent instability of the fungal microbiome over time. Environmental fungi (presumably from foodborne or air-borne exposure) represent a long and unexhausted tail of

OTU diversity and, predictably, are not stable components of a microbiome, as they fail to colonize. However, even potential “core” fungal microbiota (Saccharomycetalean yeasts, and yeasts in the Dipodascaceae) were rarely detected from the same individual in two samples collected 3–4 months apart. The known human symbionts detected in this study range from commensals to opportunistic pathogens – usually within a given species. With the exception of *Candida*, fungi commonly associated with human disease (*Aspergillus fumigatus*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Penicillium marneffeii*) were not detected; unsurprisingly as (1) participants were selected from healthy individuals who reported no recent antimicrobial use, while infection with such fungi usually results in morbidity unless treated and (2) diseases caused by these fungi are not normally associated with the gut. *Candida* species are well-documented as opportunistic pathogens, causing disease ranging from vaginal yeast infections to bloodstream infections with a fatality rate approaching 49 % (Gudlaugsson et al., 2003). However, these yeasts are also commonly detected in healthy humans, with a carriage rate of from 30 to 60 % reported for the best-studied species, *C. albicans* (Moran et al., 2012). We were, thus, not surprised to detect *Candida* yeasts as the dominant taxa in this study. What was somewhat unexpected was the relative

Figure 2. Neighbor-joining tree showing diversity of fungal taxa sequenced from feces of 45 healthy volunteers. 72 distinct taxa were identified on the basis of ITS sequences; names are given for dominant and/or medically-relevant groups.

scarcity of *C. albicans* and abundance of *C. tropicalis*. *Candida albicans* is frequently reported as the most abundant *Candida* species, both in disease studies (e.g. Silva et al. (2011)) and in healthy individuals (Calderone, 2002), but was only detected in 16 % of our samples, while *C. tropicalis* was present in 73 % of the samples. *Candida albicans* may have been over-reported relative to other species in earlier literature, where identification was based solely on culture; however, current culturing methods using chromogenic substrates such as CHROMagar® *Candida*, not to mention molecular methods, can reliably distinguish between *C. albicans* and *C. tropicalis* so that does not provide a complete explanation. *Candida parapsilosis* and *C. metapsilosis* are other opportunistic pathogens that were detected in our study, but only at low levels (one sample contained both *C. parapsilosis* and *C. metapsilosis*, while another contained *C. parapsilosis* alone). *Candida dubliniensis* and *C. glabrata*, both commonly reported as pathogens, were not detected in our study.

Candida sake, the third most widely distributed fungus in our study (25 % of samples), and *D. hansenii* (= *C. fa-*

mata; 16 % of samples) both have a wide environmental distribution but are notable for their presence in food. The type strain of *C. sake* was isolated from sake, and the species has been isolated from a wide range of substrata, including grape juice, sauerkraut and frozen salmon (Lachance et al., 2011). *Debaryomyces hansenii* is a halotolerant yeast found in many cheeses; also preserved or fermented meats (Romano et al., 2006). Its appearance in the gut has been noted and attributed to the diet (particularly cheese) by Desnos-Ollivier et al. (2008). Neither *C. sake* nor *D. hansenii* grows at 37 °C, and their presence in fecal samples must, therefore, be considered allochthonous.

DNA from both pathogenic (*C. albicans*, *C. parapsilosis* and *C. tropicalis*) and food-associated (*C. quercitrusa*) *Candida* species was detected in the feces of 7 (out of 11) extremely low birth weight infants in a neonatal intensive care setting (LaTuga et al., 2011).

Malassezia species are best known from their association with mammalian skin and the human scalp, where *M. globosa* and *M. restricta* are considered primary causal agents of dandruff (Xu et al., 2007), as well as composing a part of the healthy human cutaneous microbiota (Sugita et al., 2010). Living *Malassezia* cultures have been obtained from canine feces, where they are considered cutaneous organisms that survived passage through the gastrointestinal tract following ingestion by licking colonized skin/fur (Raabe et al., 1998); from human urine, and from neonatal feces (Guého-Kellermann et al., 2011); and from an intestinal polyp of a Chinese man (culture CBS 5548, MRL 3175; <http://www.cbs.knaw.nl>). Hamad et al. (2012) detected three *Malassezia* species in fecal material from a healthy human subject in Senegal, including *M. restricta*, the primary species detected in our study. Recent work by Dupuy et al. (2014) reported *Malassezia* as a significant member of the oral microbial community, significantly broadening our understanding of *Malassezia* ecology. Presently, there is little to no data to support the supposition that *Malassezia* is a gut organism, and the likeliest origin for *Malassezia* DNA in human feces is the oral or cutaneous microbiota. However, a role for *Malassezia* in the gut cannot be ruled out and remains an intriguing possibility, especially in the light of this genus's lack of fatty acid synthase genes and consequent reliance on the host for lipid provision (Xu et al., 2007).

Scanlan and Marchesi (2008) reported *Galactomyces* – a yeast in the family Dipodascaceae – in fecal samples from 4 out of 17 individuals in their study, and found *Galactomyces* to be stable across multiple time points sampled. We detected *Galactomyces* at a lower rate – 7 out of 69 samples – but found the related *Geotrichum* aff. *gigas* in 37 samples (54 %), second only to *C. tropicalis* in frequency of detection. The Dipodascaceae in our study (top BLAST hits = *G. gigas* and *G. geotrichum*) provide comparatively poor matches to published sequences, with no more than 89 % identity to any sequences in the GenBank database. Our Dipodascaceae, therefore, cannot be assigned to a species at this time. Our samples may be classed into two distinct OTUs, one with closest sequence identity to *G. geotrichum* and deposited in GenBank with accession numbers KC525750 – KC525757, and another with closest sequence identity to *G. gigas* and with GenBank accession numbers KC525758 – KC525781.

We detected the filamentous ascomycete *Cladosporium* in 14 samples (20 %). Hoffmann et al. (2013), also using 454 sequencing of fecal samples from healthy individuals, identified *Cladosporium* in 42 % of 96 samples. *Cladosporium* was also among the most common fungal genera isolated in a study of the indigenous flora of Apollo 14 and Apollo 15 astronauts, second only to *Candida* (Taylor et al., 1973). The Apollo spacecraft study reported multiple *Cladosporium* species from bodily samples of multiple origins, including fecal, and there have since been additional sporadic reports of *Cladosporium* in fecal samples from healthy humans (Ott et al., 2008). *Cladosporium* is ubiquitous and is common in air samples; notably, in the astronauts, the same species was frequently detected in both the feces and the throat and/or gargle samples, suggesting possible inhalation followed by swallowing.

Only ascomycetes and basidiomycetes were detected in our samples, despite the use of targeted PCR primers to broaden the taxonomic diversity, such as ITS4NA, which does not amplify DNA from ascomycetes while amplifying from other fungi including basidiomycetes, zygomycetes and chytrids (Parrent et al., 2006), and CM2 and 1520R, which amplify DNA from the recently-described phylum Cryptomycota (Lefevre et al., 2007). There was no *a priori* reason for expecting to find Cryptomycota in human fecal samples – the phylum was described on the basis of isolates from pond water, soils and aquatic sediments (Jones et al., 2011) – but given the diversity of Cryptomycota habitats we considered it worth investigating. Zygomycetes and Chytridiomycota play significant roles in many insect guts and the rumen of herbivores, respectively, and the zygomycete *Rhizopus* has been implicated in invasive infections in immunocompromised patients and isolated from the human GI tract (Chen et al., 2010 and Ribes et al., 2000). The present paper used DNA isolated from feces to study the fungal component of the gut microbiome. Due to the uninvasive nature of sampling, fecal studies have been widely used to characterize the gut microbiota (Human Microbiome Project Consortium, 2012) including eukaryotes (Scanlan and Marchesi, 2008), with the understanding that fecal samples are biased towards colonic micro-organisms and may miss organisms adapted to the upper gastrointestinal tract.

In their analysis of the fungal component of the oral microbiome, Ghannoum et al. (2010) used 454 pyrosequencing of ITS PCR amplicons to detect more than 101 species from 20 healthy individuals (the 11 unculturable genera were not sequenced). Species detected both in the oral (Ghannoum et al., 2010) and gut (the present study) microbiome were *C. albicans*, *C. metapsilosis*, *C. parapsilosis*, *C. tropicalis*, *Fusarium culmorum* and *Saccharomyces cerevisiae*; both studies also detected fungi in the genera *Alternaria*, *Aspergillus*, *Cladosporium*, *Cryptococcus* and *Penicillium*, but from different species in the different studies. Many *Candida* species have been reported from multiple body sites, and *S. cerevisiae* – brewers' and bakers' yeast, and used as a nutritional supplement – could easily enter both the mouth and the gut through dietary means. In the oral cavity, 39 genera (out of 85) were detected in only one sample, comparable to the 38 (out of 72) taxa detected at a single time in our study, suggesting small-scale environmental impact (spores inhaled and/or swallowed).

The role of geographic origin and/or residence in fungal gut communities deserves further study. Scanlan and Marchesi (2008), examining 17 healthy adults in Ireland for gut eukaryotes, found the most abundant fungus to be a species identified by ITS sequence as *Gloeotinia temulenta*/*Paecilomyces fumosoroseus*, present in seven of their participants in all time points surveyed (from 3 to 6 time points); this taxon was present in 62 % of their samples but entirely absent from ours. Conversely our samples (collected in Lincoln, Nebraska and Manhattan, Kansas) contained high levels of *C. tropicalis*, which was not detected in the Irish study. Hoffmann et al. (2013), sampling in Pennsylvania, found *Saccharomyces* in 89 % of their samples, while it was only detected in 4 % of our samples, and at very low levels. As fungi may enter the gut through numerous pathways, including ingestion and possibly inhalation, locale may play a significant role.

Together with the study by Hoffmann et al. (2013) investigating both fungi and Archaea, ours is among the first to use next-generation sequencing techniques to identify gut fungi from healthy humans; 454 pyrosequencing of ITS amplicons has additionally been used to investigate oral fungi from healthy humans (Ghannoum et al., 2010), and gut microorganisms from premature infants (LaTuga et al., 2011). The utility of the technique is suggested by the number of taxa identified – 79 (Hoffmann et al., 2013) and 72 (the present study) in the feces; 101 in the oral cavity (Ghannoum et al., 2010), compared to 12 in Scanlan and Marchesi's (2008) study, which used culturing and ITS sequencing of clone libraries – although geography or other factors may also play a role in the limited diversity observed in that study. It is likely that many of the fungi detected in only a single sample play a limited role, if any, in the gut, and that these fungi are simply indicative of environmental exposure (i.e. inadvertent ingestion or inhalation of food-, air- or soilborne fungi).

The composition of gut fungal communities differs notably from that of bacteria, with healthy humans harboring thousands of bacterial OTUs in something approximating a normal distribution, while the same individuals harbor much lower fungal diversity (a dozen OTUs or less), and fungi appear to follow a binary distribution. Furthermore, the bacterial communities of healthy humans tend to remain stable over long time periods and are relatively resistant to perturbation (Costello et al., 2009 and Lozupone et al., 2012), while our data suggests that fungal communities are much less persistent and more variable over time. In addition to the observed low levels of fungal persistence in the 24 participants from whom two time points were sequenced, fungal DNA could not be amplified from a second time-point for the other 21 participants. As bacterial DNA was readily amplified from all time-points for all participants, it is highly likely that fungal DNA was genuinely below the level of detection in samples which did not amplify. It would appear that the niche for fungi in the healthy human gut is limited and that there is little scope for ecological diversification among the gut fungi.

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Supplements

Supplementary figures S1–S3 follow the References. Supplementary data, in an Excel file 45 columns x 159 rows, is attached to the repository html cover page.

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Supplementary Figure S1. Alpha diversity of the fungal fecal community. A. Shannon diversity index, and **B.** Simpson diversity index.

Supplementary Figure S2. Indels in the 5.8S rRNA region distinguishing *Candida* species. Sequence alignments for *Candida albicans*, *C. tropicalis* and *C. sake* over the 5.8S region of the rRNA genes. Bolded nucleotides are conserved between the three species.

Supplementary Figure S3. Rarefaction analysis, showing saturation at approximately 4000 sequences.

Supplementary Figure S1. Alpha diversity of the fungal fecal community.

A.

B.

A. Shannon diversity index, and B. Simpson diversity index.

Supplementary Figure S2. Indels in the 5.8S rRNA region distinguishing *Candida* species.

C. albicans   **GATTTGCTTAATTGCA**--CCACATGT**GTTTTT**CTTT-**GAA**ACAA**ACTTGCT**
C. tropicalis **GATTTGCTTAATTGCA**--CCACATGT**GTTTTT**TATT-**GAA**-CAAA-**TTTCT**
C. sake   **G-TTTGCTTAATTGCATTACACAT**--**GTTTTTT**TTAG**GAA**-----**CTTGCT**

C. albicans   **TTGGCGGTGGGCCCAGC**-----TGCCGCCAGAGGTCTAA**ACTTACAACC**-A
C. tropicalis **TTGGTGGCGGG**---A**GCAATCCTACCGCCAGAGGTTATACTA**-**GAAC**---
C. sake   **T**-----**GCAA**-----**AACCAA**

C. albicans   **ATT**
C. tropicalis **ACT**
C. sake   **ACT**

Sequence alignments for *Candida albicans*, *C. tropicalis* and *C. sake* over the 5.8S region of the rRNA genes. Bolded nucleotides are conserved between the three species.

Supplementary Figure S3. Rarefaction analysis showing saturation at approximately 4000 sequences.