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Characterization and Investigation of Fungi Inhabiting the Gastrointestinal Tract of Healthy and Diseased Humans

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CHARACTERIZATION AND INVESTIGATION OF FUNGI INHABITING THE GASTROINTESTINAL TRACT OF HEALTHY AND DISEASED HUMANS

by

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Gastrointestinal microbiome studies have failed to include fungi in total community analyses. As a result, their diversity and function in the gut is poorly understood. Recent work has begun to uncover the role intestinal fungi play in diet, immune system development, interactions with other microorganisms in the gut, and pathogenesis of diseases. Advances in sequencing technologies allow for the ability to profile the fungal gut microbiome ("mycobiome") in healthy and diseased states. This thesis explores the mycobiome in 1) healthy humans with a vegetarian diet and 2) pediatric small bowel transplant recipients that develop fungal bloodstream infections.

The gut mycobiome from healthy adult humans with a vegetarian diet was determined using two sequencing technologies and a commercially available probe-based method. The use of three culture-independent methods demonstrated that the gut mycobiome is best characterized using a combination of methods, as each method has strengths, weakness, and biases. In addition, this study provides insight into the allochthonous nature of fungal inhabitants in the gut and demonstrates that fungal diversity in the gastrointestinal tract is diet related. It is apparent that a wide variety of fungi can be identified from the human gastrointestinal tract.

Further, the intestinal mycobiome of seven pediatric small bowel transplant recipients was characterized. Transplant patients are subject to multiple risk factors and have a correspondingly high incidence of candidemia. Molecular typing methods were used to assess whether candidemia in small bowel transplant recipients was acquired endogenously (from their own gut microbiota) or exogenously. Our results suggest that small bowel transplant patients are infected both by *Candida* colonizing their gastrointestinal tract and from exogenous sources. Ultimately, these results underline the importance of *Candida* surveillance in small bowel transplant institutes.

"Never, never, never give up."

— Winston Churchill

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PREFACE

This thesis is comprised of four chapters characterizing the fungal communities in the gastrointestinal tract of healthy and diseased humans. A primary goal of this research was to raise awareness of gut fungi and discuss methods to characterize this understudied group of microorganisms. **Chapter 1** provides a review of the current understanding of the mycobiome of the human gastrointestinal tract. **Chapter 2** provides a profile of the gut mycobiome from healthy adults with a vegetarian diet using two culture-independent sequencing methods and a commercially available fungal detection assay. In **Chapter 3**, our efforts were aimed at surveying gut fungi populations in small bowel transplant patients over time and determining the source(s) of yeasts causing life-threatening bloodstream infections. Finally, **Chapter 4** summarizes the impact of this work and discusses the continual efforts needed to explore the role of the mycobiome in human health and disease.

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CHAPTER 1

FUNGI OF THE HUMAN GASTROINTESTINAL TRACT: A REVIEW

1.1 THE GASTROINTESTINAL MICROBIOTA

All surfaces of the human body exposed to the environment are colonized with complex and diverse populations of microorganisms from all three domains of life [33]. A majority of the microbes colonizing the human host are harbored within the gastrointestinal (GI) tract, making the GI tract home to one of the densest microbial communities on Earth. Approximately 100 trillion microorganisms inhabit the human gut and outnumber human host cells by a factor of 10 to 1 [214]. Sequencing an estimated 3.3 million human intestinal bacterial genes demonstrated the gut microbial genome is approximately 150 times greater than the human genome [163]. This population of microbes, collectively termed the gastrointestinal microbiota, has received much attention and has been at the forefront of biological research for the last decade.

The acquisition of - and colonization by - microbes residing in the GI tract of newborn infants is an emerging field of study. Traditionally, it was believed that the gastrointestinal tract of neonates is sterile *in utero* and immediately becomes colonized by microbes from their surroundings upon birth. However, recent culture-independent research shows the placenta harbors a unique collection of nonpathogenic bacterial commensals from the phyla Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, and Fusobacteria [1]. Previous analyses also show bacteria and fungi are present in the amniotic fluid, placenta, and umbilical cord [46, 91, 168]. In addition, bacteria such as *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Lactobacillus* have been cultured from the meconium, the newborns' first passed stool [135]. Collectively, support for microbial transmission from mother to fetus *in utero* has led to the 'early programming hypothesis'

stating that factors acting *in utero* program the risks for adverse health conditions later in life [62].

The development of the infant's intestinal microbiota begins early in life and colonization order is greatly influenced by a number of lifestyle factors. Newborns are first exposed to their mother's microbiota, and depending of the mode of delivery, vaginal or cesarean, their microbiota resembles the vaginal or skin microbiota of their mother [5, 49]. In the subsequent days following birth, newborns are continuously faced with a number of environmental factors that become major determinants of the composition of the gut microbiota. Feeding mode is claimed to substantially affect the gut microbiota. Breastfed infants (as compared to formula-fed infants) develop and establish a microbiota rich in beneficial microbes such as *Bifidobacterium* by the end of the first week [16, 76, 156, 226]. In addition, human breast milk contains secretory IgA and free oligosaccharides that help develop the immune system and protect infants from colonization by pathogens [123, 170]. Other extrinsic factors orchestrating the development of the gut microbiota include the use of antimicrobial agents, hygienic conditions, family structure, and home environment [4, 44]. Throughout the first year of life, the infant intestinal tract evolves from a nearly sterile environment to a diverse assemblage of microorganisms, where it will slowly begin to resemble the microbiota of an adult and remain relatively stable.

1.2 ROLE OF THE GASTROINTESINAL MICROBIOTA IN HEALTH AND DISEASE

The gastrointestinal microbiota plays a central role in the well-being of the human host and is therefore often termed ‘the forgotten organ’ [151]. The microbiota has a profound functional role in energy harvest and digestion. Complex polysaccharides and proteins that escape human digestion in the small intestine serve as the main substrates to resident colonic microbes [39]. The microbiota breaks down these undigested nutrients into the short-chain fatty acids (SCFA) acetate, butyrate, and propionate. SCFAs become readily available to the human host and provide energy that would otherwise be lost. Production of SCFAs has been shown to confer health benefits and decrease the risk of colon cancer [73, 218]. In addition, the microbiota synthesizes vitamins essential to the host [7].

The GI tract is the primary site of interaction between the human immune system and microbiota and, therefore, is of substantial importance for immunological development. Studies show the gut microbiota is responsible for the development of immune cells such as secretory IgA and provides signaling molecules that aid in maturation of the host immune system [128, 140]. In conjunction with the immune system providing critical disease resistance and a line of defense against pathogens, the gastrointestinal microbiota aids in limiting colonization by pathogens via a phenomenon known as colonization resistance [206].

While commensal microbial communities in the gut carry out a series of advantageous and essential functions, perturbation of the system creates a dysbiosis. Dysbiosis refers to a microbial imbalance in the gut with increased levels of harmful

microbes and reduced levels of beneficial microbes. Imbalances in the composition of the microbiota have been associated with a number of digestive diseases such as ulcerative colitis, Crohn's disease, inflammatory bowel disease, irritable bowel syndrome, and colon cancer [90, 120, 144, 160, 220]. In addition, recent research has indicated that disruptions in the gut microbiome and relative abundances of certain members are associated with complex, non-digestive diseases such as obesity, type 2 diabetes, chronic fatigue syndrome, arthritis, liver disease, and autism [36, 54, 106, 115, 183, 189]. Determining what constitutes a healthy microbiota is essential for evaluating deviations associated with disease.

1.3 DIVERSITY OF THE GASTROINTESTINAL MICROBIOTA

The gastrointestinal tract extends from the oral cavity to the anus with well-defined anatomical sections within. Each individual component of the GI tract has a specialized function for the digestion and absorption of food. Microorganisms within the digestive tract are not randomly distributed but instead are preferentially localized to specific regions in different numbers and taxonomic distributions [117]. Properties of each section in the digestive tract such as pH, rapid luminal flow, bile salts, and oxygen availability are driving factors that shape microbial diversity by supporting or discouraging growth [95, 210]. A majority of the gut microbiota is anaerobic, with a lower proportion of facultative anaerobes and aerobes [64, 77].

Microbial numbers increase in a gradient from the stomach to the colon (Table 1.1). The upper gastrointestinal tract (stomach, duodenum, jejunum, proximal ileum) contains a relatively low number of microorganisms. Very few organisms ($<10^2$

cells/mL), consisting of acid-tolerant lactobacilli and streptococci, are present in the stomach and upper small intestine due to harsh pH conditions [117]. Cell densities in the proximal small intestine range from 10^4-10^5 cells/mL of intestinal content [210]. The lower gastrointestinal tract (distal ileum, colon) contains a much higher population and diversity of microorganisms—10^8 cells/gram in the distal ileum and approaching 10^{10}-10^{12} cells/gram in the colon. Less acidic pH, low concentration of bile acids, and longer retention due to slower peristalsis are several properties that distinguish the colon from the rest of the GI tract and allow it to be the primary site of colonization [210]. Fecal samples are, therefore, commonly used as a snapshot of the gut microbiome.

Table 1.1 Microorganism groups and counts in different sections of the gastrointestinal tract [186].

Microorganisms	Stomach	Jejunum	Ileum	Colon
Aerobic and facultatively anaerobic microorganism groups				
Enterobacteria	0-10^2	0-10^3	10^2-10^6	10^4-10^{10}
Enterococci	0-10^3	0-10^4	10^2-10^6	10^5-10^{10}
Staphylococci	0-10^2	0-10^3	$10^{2-}10^5$	10^4-10^7
Lactobacilli	0-10^3	0-10^4	10^2-10^5	10^6-10^{10}
Fungi	0-10^2	0-10^2	10^2-10^3	10^2-10^6
Anaerobic microorganism groups				
Bacteroides spp.	Rare	0-10^2	10^3-10^7	10^{10}-10^{12}
Bifidobacteria	Rare	0-10^3	10^3-10^5	10^8-10^{12}
Anaerobic streptococci	Rare	0-10^3	10^2-10^4	10^8-10^{11}
Clostridia	Rare	Rare	10^2-10^4	10^6-10^{11}
Eubacteria	Rare	Rare	Rare	10^9-10^{12}

Note: Figures in colony forming units (CFU) per mL or per g intestinal content.

Lastly, the diversity of the microbiota is divided into two categories: indigenous (autochthonous) and transient (allochthonous) microorganisms. Autochthonous microorganisms are ubiquitous in the GI tract, readily colonize available niches, and are stable over time, whereas allochthonous microorganisms cannot readily colonize the GI tract [53]. Allochthonous species are merely transient members that are unable to establish themselves in a foreign community. These species mainly originate from consumption or ingestion of air and food or water. The distinction between autochthonous and allochthonous microorganisms in the gut is crucial to understand how organisms interact with their host in terms of whether they effect colonization and succession [117].

1.4 COMPONENTS OF THE HUMAN GUT MICROBIOME

Bacteria are the most abundant members in the gut and, therefore, have overshadowed the presence of other members of the gut microbiome. In fact, this complex community of intestinal microbes spans all three domains of life - Bacteria, Archaea, and Eukarya - and includes bacteria, protists, fungi, and viruses. Few studies have investigated the contribution of microbial residents other than bacteria to function of the human host.

1.4.1 PROKARYOTIC DIVERSITY

Over 99% of the genes in the human gut microbiome are of bacterial origin and the number of bacterial species reported from the gut is generally believed to be between 500-1,000 [163, 221]. Fifty-five bacterial phyla have been described from the human gut;

however, the two most dominant phyla are Bacteroidetes and Firmicutes [12, 55, 184]. Five other common phyla, present in lower proportions, are Proteobacteria, Fusobacteria, Verrucomicrobia, Cyanobacteria, and Actinobacteria. One study involving fecal samples from 124 Europeans showed each individual harbored at least 160 species of bacteria and a majority of the species was shared among individuals [163].

Prokaryotic, single-celled members of the Kingdom Archaea reside alongside bacteria in the gut [163]. The archaea, once considered bacteria, consist of halophiles and thermophiles, found in extreme environments; and methanogens, found in a broad range of habitats. Only a few methanogens are found in the human gut. *Methanobrevibacter smithii* is considered the predominant archaeon; *Methanosphaera stadtmaniae* and *Methanobrevibacter oralis* are also present in the gut [51, 131, 133, 181]. The carriage rate of methanogens in the human gut ranges from 25-95% [51, 194]. Intestinal methanogens play a role in the pathogenesis of obesity [132, 176, 225].

1.4.2 VIRAL DIVERSITY

One aspect of the human gut microbiome that has received little attention is the viral component, referred to as the "human virome." Human feces contain at least 10^9 eukaryotic and prokaryotic viruses per gram [167]. The adult virome consists of DNA and RNA viruses, the majority being bacteriophages and plant viruses, respectively [25, 224]. Bacteriophages influence human health by contributing genes to their host bacteria that affect overall community and structure of the microbiota [31]. Bacteriophage repertoires vary widely between individuals and are likely the reason for inter-individual variation in gut bacterial communities [134]. Eukaryotic viruses also have significant

effects on human health, causing gastroenteritis and a host of acute, chronic, and fatal diseases [32].

1.4.3 EUKARYOTIC DIVERSITY

Eukaryotes in the human gut are less extensively studied than prokaryotes, yet remain an important component of the microbiome. The Domain Eukarya encompasses a vast diversity of organisms spanning from animals, plants, and fungi to protozoa, slime molds, and algae. A bulk of the eukaryotes found in the human gut, as expected, are microeukaryotes and ignorance of these organisms becomes a major limitation of gut microbiome studies.

Protista (now split into several kingdoms) comprises a collection of single-celled, eukaryotic organisms including protozoa, unicellular algae, slime molds, and water molds. Several protists are known to cause disease, however, few have been described as asymptomatic commensals in the gut [22, 162]. Studies show that the protist *Blastocystis* and fungi are the dominant eukaryotes in the gut [142, 180]. Also, the protists *Blastocystis* and *Dientamoeba* have been observed in 50% of some Danish cohorts, and are nearly absent in those with inflammatory bowel disease [8]. Although protists make up a small component of the microbiome, further research is necessary to understand their role in health and disease.

Fungi are eukaryotic organisms that can occur as single-celled yeasts, multicellular filamentous molds, or macroscopic organisms such as mushrooms, and range from pathogens to commensals and parasites to mutualists. Populations of fungi colonize the human host at multiple different body sites—gastrointestinal tract, oral

cavity, skin, vaginal tract, and lungs (Figure 1.1) [50, 58, 63, 80, 207]. Most human-associated fungi are opportunistic pathogens in that disease is caused when the host immune system becomes weakened or natural barriers are disrupted. Well-known fungal infections range from skin mycoses such as athlete's foot, ringworm, and yeast infections, to more life-threatening systemic mycoses caused by *Cryptococcus*, *Candida*, and *Aspergillus.* An estimated 75% of women will encounter a yeast infection during their lifetime and roughly 20-25% of the world's population has skin mycoses [79, 190]. Fungi represent the majority of eukaryotes in the human gut microbiome and the remaining sections of this review detail and depict what is currently known about the relatively new field of human gut mycology.

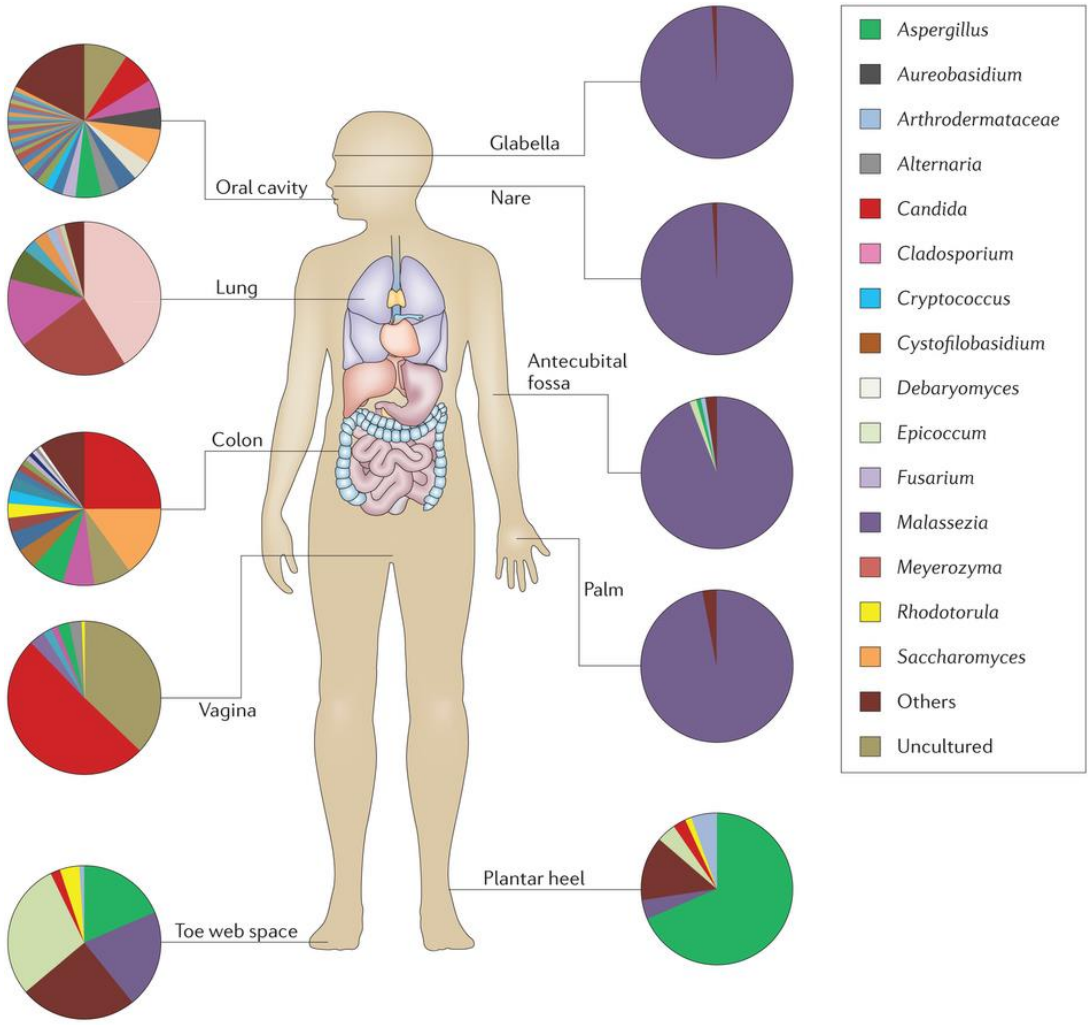


Figure 1.1 The human mycobiota [204].

1.5 THE HUMAN GASTROINTESTINAL MYCOBIOME: A NEW FRONTIER

Gut bacteria are the most abundant microorganisms in the gastrointestinal tract, and therefore, have been the focus of much human microbiome research over the past decade. The extent of biodiversity of the "mycobiome" (fungal biota) in the human gastrointestinal tract remains to be defined. The term mycobiome was coined in 2010 to differentiate the fungal biota from the bacterial biota, as the word "microbiota" is

frequently treated as synonymous with bacteria [63]. On July 6, 2013, using a PubMed search, the word “mycobiome” appeared in the literature 10 times [38]. As of March 6, 2015 a PubMed search showed at least 32 publications using the word “mycobiome.” The field is clearly advancing and increasing awareness allows gut microbiologists to address the same questions to gut fungi as have been studied in bacteria.

1.5.1 HISTORY

Yeasts were first identified in the human GI tract in the early nineteenth century [104]. However, at the time no effort was made to identify and characterize these yeasts. In the early 1900s, the presence of yeasts in feces was sporadically documented in the medical literature and studies tried to link their presence to gastrointestinal diseases, such as sprue (a digestive disease characterized by malabsorption and commonly reported in tropical regions) [13, 23, 109]. Years later, just before World War II, isolates from human clinical samples were identified as yeasts of the genus *Monilia*, which were later reclassified into the genus *Candida* [105, 121, 122]. Culturing *Candida* from the intestines of healthy humans and those with gastrointestinal disturbances became a popular area of research during the mid-1900s. *Candida* was the most commonly isolated yeast from fecal samples, yet its significance and occurrence in stools had yet to be elucidated due to the fact that heavy loads of *Candida* species were collected from healthy humans [23].

The long known history of yeasts in the gastrointestinal tract and their peculiar presence led physicians in the late 1980s to propose hypotheses linking unhealthy lifestyles to *Candida* overgrowth in the intestine [186, 202]. Mycophobia was also

spreading due to falsely interpreting yeasts isolated from the mouth or stool as fungal infections [187]. Gut mycology has therefore become a field in need of attention and advancement.

1.5.2 GASTROINTESTINAL COLONIZATION BY FUNGI

As stated previously, microbial colonization starts at birth. The vaginal tract is home to a large number of yeasts and, not surprisingly, neonates born vaginally are colonized by yeasts from their mother's vaginal microbiota [21]. Infant colonization by *Candida* spp. is therefore transmitted vertically from the maternal microbiota and also horizontally from the environment and hands of health care workers [21, 116]. The prevalence of *Candida* spp. gut colonization in newborns on the day of birth is roughly 23%, increasing to 50% by four months [56, 174]. Yeast carriage rates in infants are approximately 10^3-10^5 CFU/g feces [16, 56]. Additionally, 96% of neonates have detectable *Candida* species in the oral cavity by the first month of life [171].

Every human being is colonized with fungi [85]. Fungi are detectable in all sections of the GI tract of about 70% of healthy adults, normally at 0-10^3 fungal cells per ml or g of intestinal contents [186]. Table 1.1 (above) shows the relative abundance of fungi in each section of the gut and outlines the disparity that exists between fungal and bacterial residents of the GI tract. Culture-independent analyses show fungal genes constitute less than 0.1% of the human gut microbiome [163]. The low abundance of fungi in the gut microbiome undoubtedly relegates them to part of the 'rare biosphere' [191]. The rare biosphere is of significance as it potentially serves as a reservoir for

pathogens or keystone species that have a critical role in maintaining the structure and function of the human gut microbiome [85].

The oral cavity is asymptomatically colonized by *Candida* and harbors a wide diversity of fungi, likely from food and airborne spores [11, 63]. The rapid luminal flow and low pH of gastric acid allows few microorganisms to colonize the stomach and functions as a barrier to prevent ingested pathogens from reaching the intestine [124]. A number of acid-resistant bacteria exist in the stomach as well as a few acid-tolerant fungi such as *Candida* and *Phialemonium* [19, 208]. The diversity of fungi in the small intestine is largely unexplored due to the difficulty in collecting samples [211]. Ileal effluent collected from intestinal transplant recipients showed colonization by *Saccharomyces cerevisiae*, *Candida* spp., *Cryptococcus neoformans*, and *Aspergillus clavatus* [112]. The properties of the large intestine facilitate extensive microbial colonization and proliferation, and a wide diversity of fungal genes is detected in the lower gastrointestinal tract.

1.5.3 FUNGAL DIVERSITY OF THE INTESTINAL MYCOBIOME

Two large-scale projects, the Human Microbiome Project and MetaHit, provide an initial compilation of the gut microbiome ecosystem from 242 and 124 humans, respectively [86, 158, 163]. As revealing as the results have been, publications to date from these projects focused exclusively on the bacterial component and the fungal diversity was not considered. Smaller-scaled, fungal-specific studies using culture-dependent and/or culture-independent methods have thus been conducted but are limited.

Previous culture-dependent studies identified *Candida* spp. as the most common fungi in the GI tract. With the development of advanced culturing methodologies and PCR, the modern era of mycobiome studies has exposed a more in-depth survey of both culturable and unculturable fungi in the gut. At present only a handful of mycobiome characterization studies exist. To the best of our knowledge, we compiled a comprehensive list of fungal species reported from the GI tract of humans by culture-dependent and culture-independent studies (Table 1.2; includes: healthy and diseased adults and infants, fungi from stomach, small intestine, large intestine, differing diets and varying geographic locations). Data was compiled December 2014 from articles spanning 1917-2014. Also, included in the list are fungi detected in our lab from healthy humans on a vegetarian diet reported in Chapter 2 of this thesis (denoted S in Table 1.2). If a more current name for a fungus exists than that used in the initial publication, the current name is also listed. Fungi reported from the literature but not specified to the species level are excluded from the list if a species from that genus, family or class is listed. Taxa reported in the literature that are indistinguishable from one another at the level of the target gene used are listed in the same row.

Table 1.2 Comprehensive catalog of fungi reported in the gastrointestinal tract of humans by culture-dependent and -independent methods.

Phylum	Fungal Taxa
Ascomycota	*Ajellomyces capsulatus* [112], Current name: *Histoplasma capsulatum*
	Ajellomyces dermatitidis [112]
	Alternaria alternata [68, S], *A. brassicicola* [113], *A. arborescens, A. citri, A. mali, A. tenuissima* [S]
	Amphisphaeriaceae sp. [48]
	Arxiozyma telluris [75], Current name: *Kazachstania telluris*

Ascocoryne cylichnium [S]
Ascocoryne sarcoides [S]
Aspergillus clavatus [112, 113]
Aspergillus flavipes[1] [66]
Aspergillus flavus[1] [67], *Aspergillus oryzae* [S]
Aspergillus fumigatus [203, S]
Aspergillus microviridicitrinus[1] [34]
Aspergillus niger[2] [34, 74, 199, S]
Aspergillus penicillioides [30]
Aspergillus restrictus [68]
Aspergillus sydowii[1] [199], *Aspergillus versicolor*[2] [30, 34, 66, 180, 199]
Aureobasidium pullulans[2] [30, 152, 198]
Beauveria bassiana[1] [66]
Bispora christiansenii [68], Current name: *Intralichen christiansenii*
Blastoschizomyces capitatus[1] [97] (also reported as *Dipodascus capitatus*, [203]) Current name: *Geotrichum capitatum*
Botryotinia fuckeliana [112, 152], Current name: *Botrytis cinerea*
Candida albicans[2] [6, 10, 18, 30, 34, 46, 59, 60, 68, 74, 92, 97, 108, 112, 113, 118, 152, 153, 180, 192, 195, 198, 208, S] (also reported as *Endomyces albicans*[1] [9], *Oidium albicans*[1] [9], *Parasaccharomyces ashfordii*[1] [9])
Candida colliculosa[1] [10, 18]
Candida diddensiae [108]
Candida dubliniensis [68, 112, 113, 152]
Candida glabrata[2] [6, 18, 30, 68, 92, 97, 108, 118, 152, 195] (also reported as *Cryptococcus glabratus*[1] [9], *Torulopsis glabrata*[1] [34])
Candida guilliermondii[1] [6, 10, 34, 97], Current name: *Blastodendrion artzii*
Candida inconspicua[1] [10]
Candida intermedia [10, 30]
Candida kefyr[1] [6, 18, 92], Current name: *Atelosaccharomyces pseudotropicalis*
Candida krusei[1] [6, 10, 18, 30, 34, 75, 97, 118, 198] (also reported as *Mycoderma monosa*[1] [9]), Current name: *Candida acidothermophilum*
Candida lambica[1] [97], Current name: *Candida fimentaria*
Candida lusitaniae[2] [6, 10, 18, 28, 97] (also reported as *Clavispora lusitaniae*[2] [68])
Candida metapsilosis [74]
Candida milleri [30], Current name: *Candida humilis*

	Candida norvegensis[1] [6], Current name: *Candida mycoderma* var. *annulata*
	Candida quercitrusa [108]
	Candida orthopsilosis[2] [10, 28]
	Candida parapsilosis[2] [10, 18, 28, 34, 74, 97, 108, 112, 118, 180, 198, 199, 208]
	Candida pelliculosa[1] [10], Current name: *Candida beverwijkiae*
	Candida rugosa[2] [74, 75, 97) (also reported as *Mycoderma rugosa*[1] [9])
	Candida sake [74, 203] (also reported as *Candida austromarina* [30, 152])
	Candida solani [30]
	Candida sphaerica[1] [6, 10]
	Candida stellata[1] (reported as *Torulopsis stellata* [34])
	Candida temnochilae[1] [10]
	Candida tropicalis[2] [6, 10, 18, 28, 30, 34, 66, 74, 97, 108, 112, 113, 198, 203, S] (also reported as *Monilia candida* Bon. [9, 208])
	Candida utilis[1] [6, 10], Current name: *Candida guilliermondi* var. *niratophila*
	Candida valida[1] [10], Current name: *Candida krusei* var. *vanlaeriana*
	Candida vinaria [142], Current name: *Trigonopsis vinaria*
	Candida zeynaloides[1] [6] (also reported as *Candida krissii* [30])
	Ceratocystis sp. [80]
	Chaetomium globosum [30, 42, 152]
	Cladosporium cladosporioides[2] [28, 34, 108, 152, S], *C. bruhnei* [67], *C. herbarum*[1] (= *Davidiella tassiana* [68, 74, 198]), *C. macrocarpum*[1] [198], *C. sphaerospermum*[2] [108, 198, 199], *C. tenuissimum* [108]
	Claviceps purpurea [48]
	Coccidioides immitis [112]
	Coccidioides posadasii [112]
	Cochliobolus sp. [80]
	Colletotrichum sp. [80]
	Coniosporium sp. [80]
	Cryptococcus aggregatus[1] [9], Current name: *Candida pararugosa*
	Curvularia lunata[1] [34]
	Cyberlindnera jadinii [S] (also reported as *Pichia jadinii* [74])
	Debaryomyces carsonii[1] [10], Current name: *Priceomyces carsonii*
	Debaryomyces fabryi [28]
	Debaryomyces hansenii[2] [28, 48, 68, 74, S] (also reported as *Candida famata*[1] [6, 92]

Dendrostilbella sp.[1] [110]
Diaporthales sp. [108]
Didymella exitialis [74]
Diplodia mutila [S]
Doratomyces stemonitis [30]
Epicoccum nigrum [S]
Eurotium niveoglaucum [S]
Eurotium rubrum [S] (also reported as *Aspergillus ruber*[1] [67])
Eutypella sp. [80]
Exophiala equina [68]
Exophiala heteromorpha [S]
Fusarium graminearum [S], *F. culmorum* [74]
Fusarium oxysporum [112, 152]
Fusarium sambucinum [74]
Galactomyces geotrichum[2] [30, 66, 68, 74, 75, 152, 180]
Geosmithia flava [74]
Geosmithia microcorthyli [74]
Geotrichum candidum[2] [75, 97] (also reported as *Galactomyces candidum* [68])
Geotrichum gigas [74], Current name: *Saprochaete gigas*
Gibberella moniliformis [113]
Gloeotinia temulenta/Paecilomyces fumosoroseus [180]
Glomerella sp. [74, 80]
Hanseniaspora sp. [80]
Hortaea werneckii [28]
Hyphozyma variabilis var. *odora* [30]
Iodophanus carneus [30]
Isaria farinosa[1] [66]
Kluyveromyces hubeiensis [75, 153]
Kluyveromyces lactis [42]
Kluyveromyces waltii [112], Current name: *Lachancea waltii*
Lasiodiplodia sp. [80]
Lecythophora sp. [S]
Leptosphaerulina chartarum [S]
Lodderomyces elongisporus[1] [10]

Madurella mycetomatis [152]
Magnaporthe grisea [42]
Metschnikowia sp. [74, 80]
Meyerozyma sp. [80]
Microdochium sp. [80]
Millerozyma sp. [80]
Mycocentrospora sp. [80]
Nectria haematococca [42], Current name: *Haematonectria haematococca*
Neofusicoccum sp. [80]
Neosartorya fischeri [42, S]
Neotyphodium gansuense [113]
Neurospora tetrasperma [113]
Nigrospora sp. [80]
Ochrocladosporium elatum[1] (reported as *Cladosporium elatum* [198])
Ophiocordyceps caloceroides [30], Current name: *Cordyceps caloceroides*
Ophiocordyceps sinensis [S]
Ophiostoma sp. [80]
Orbiliales sp. [108]
Paecilomyces variotii[1] [34]
Paraphaeosphaeria filamentosa [152], Current name: *Neophaeosphaeria filamentosa*
Parasaccharomyces thomasii[1] [9]
Penicillium allii[1] [66], *P. camemberti*[2] [66, S], *P. chrysogenum*[2] (= *Penicillium notatum*) [34, 68, 113, 152, 199, S], *P. commune* [203], *P. dipodomyicola*[1] [66], *P. freii* [30], *P. italicum* [152], *P. solitum* [67]
Penicillium brevicompactum[1] [66]
Penicillium chermesinum[1] [34]
Penicillium citrinum[1] [199]
Penicillium decumbens[1] [199]
Penicillium glabrum [152]
Penicillium marneffei [112]
Penicillium ochrochloron[1] [34]
Penicillium roqueforti [74, 180, 203, S]
Penicillium sacculum [152]
Penicillium steckii[1] [199]
Penicillium turbatum [S]

Penicillium verruculosum [152]
Phaeococcomyces sp. [80]
Phaeosphaeria nodorum [113]
Phaeosphaeria pontiformis [74]
Phialemonium sp. [208]
Phialocephala lagerbergii [S]
Phoma sp.[1] [110]
Pichia kudriavzevii [S]
Pichia manshurica[1] [10]
Plenozythia sp.[1] [110]
Pleospora herbarum [152]
Podosphaera xanthii [S]
Pseudosaccharomyces stevensii[1] [9]
Raciborskiomyces longisetosus [152], Current name: *Epipolaeum longisetosum*
Ramularia sp. [74]
Saccharomyces bayanus [28, 152]
Saccharomyces cariocanus [152]
Saccharomyces castellii [112, 113], Current name: *Naumovia castellii*
Saccharomyces cerevisiae[2] [6, 10, 28, 30, 42, 67, 68, 74, 75, 92, 108, 112, 113, 118, 142, 152, 180, 203, S]
Saccharomyces paradoxus [30]
Saccharomyces servazzii [142], Current name: *Kazachstania servazzii*
Scedosporium apiospermum [74]
Scheffersomyces stipites [42]
Sclerotinia sclerotiorum [68, 112, 113, 152]
Sclerotium sp. [67, 152]
Scopulariopsis sp. [42]
Scytalidium thermophilum [74]
Septoria epambrosiae [152]
Simplicillium lanosoniveum [30]
Simplicillium obclavatum [30]
Sirococcus conigenus [152]
Stemphylium sp. [80]
Sterigmatomyces elviae [75]
Strelitziana sp. [80]

	Talaromyces stipitatus [42]
	Teratosphaeria sp. [80]
	Torula nigra[1] [110]
	Torulaspora pretoriensis [75]
	Toxicocladosporium sp. [80]
	Trichocladium asperum [74]
	Trichoderma lignorum[1] [34], Current name: *Trichoderma viride*
	Trichoderma longibrachiatum [S]
	Trichophyton verrucosum [112]
	Verticillium leptobactrum/*Verticillium insectorum* [180]
	Westerdykella cylindrical [68]
	Xeromyces sp. [80]
	Xylariales sp. [108]
	Yarrowia lipolytica[2] [42, 68, 97, 152]
	Zygosaccharomyces bisporus [9]
	Zygosaccharomyces rouxii [28]
Basidiomycota	*Agaricus bisporus* [48, S]
	Armillaria sp. [80]
	Asterophora parasitica [75]
	Asterotremella albida (nom. inval.) [30], Current name: *Sporobolomyces albidus*
	Auricularia sp. [80]
	Bjerkandera adusta [75]
	Bullera crocea [152], Current name: *Dioszegia crocea*
	Ceriporia lacerate [74]
	Chondrostereum sp. [80]
	Cinereomyces sp. [80]
	Climacocystis sp.[1] [66]
	Clitopilus prunulus [68]
	Cryptococcus albidosimilis [108]
	Cryptococcus albidus[1] [97]
	Cryptococcus amylolyticus [S]
	Cryptococcus carnescens [152]
	Cryptococcus fragicola [30]
	Cryptococcus humicola (reported as *Candida humicola* [28])

	Cryptococcus laurentii[1] [34]
	Cryptococcus luteolus[1] [97], Current name: *Hannaella luteola*
	Cryptococcus neoformans [112, 113]
	Cryptococcus ovoidea[1] [9]
	Cryptococcus podzolicus [108]
	Cryptococcus tephrensis [74, S]
	Cystofilobasidium capitatum[2] [67, 152]
	Dacrymyces sp. [152]
	Erythrobasidiaceae sp. [74]
	Exidiopsis calcea [152]
	Exobasidiomycetes sp. [80]
	Filobasidium capsuligenum [68]
	Filobasidium globisporum [68, 152]
	Flammulina velutipes [152]
	Fomes fomentarius [68]
	Fomitopsis pinicola [68, 152]
	Fuscoporia gilva [74]
	Graphiola phoenicis [152]
	Hymenochaete sp. [80]
	Hypholoma sp. [80]
	Laccaria bicolor [113]
	Lentinus sp. [80]
	Malassezia globosa[2] [28, 42, 66, 67, 68, 75, 112, S]
	Malassezia pachydermatis[2] [30, 66, 67, 68, 75, S]
	Malassezia restricta[2] [28, 66, 67, 68, 74, 75, S]
	Malassezia slooffiae [S]
	Malassezia sympodialis [74, S]
	Moniliophthora perniciosa [42, 113], Current name: *Crinipellis perniciosa*
	Mrakia sp. [74, S]
	Oidium lactis[1] [9], Current name: *Oospora lactis*
	Phanerochaete stereoides [75]
	Phlebia nitidula [74]
	Phlebia uda [74], Current name: *Mycoacia uda*
	Pholiota sp. [S]
	Piptoporus sp. [80]

	Postia placenta [42], Current name: *Rhodonia placenta*
	Psathyrella candolleana [180]
	Puccinia poarum [68]
	Rhodosporidium babjevae [68]
	Rhodotorula aurantiaca [152]
	Rhodotorula glutinis[1] [97] (also reported as *Saccharomyces glutinous*[1] [9])
	Rhodotorula minuta[1] [34]
	Rhodotorula mucilaginosa[2] [34, 68, 152]
	Sporobolomyces ogasawarensis [153]
	Sporobolomyces yunnanensis [152]
	Thanatephorus sp. [80]
	Tilletiopsis washingtonensis [74]
	Torula rubra[1] [9], Current name: *Rhodotorula rubra*
	Trametes versicolor [152]
	Trichaptum sp. [80]
	Tricholoma saponaceum [152]
	Trichosporon asahii[2] [10, 68, 75]
	Trichosporon caseorum [75]
	Trichosporon cutaneum [75]
	Trichosporon dermatis [28, 152]
	Trichosporon faecale [68]
	Trichosporon guehoae [74]
	Tyromyces sp. [80]
	Ustilago maydis [42, 152]
	Wallemia muriae [30, S]
	Wallemia sebi [30, 48]
Chytridiomycota	*Spizellomyces punctatus* [113]
Microsporidia	*Enterocytozoon bieneusi [42]*
Zygomycota	*Entomophthora* sp. [48]
	Mucor racemosus [153]
	Rhizopus microsporus [28]
	Syncephalastrum racemosum[1] [34]

Note: No superscript indicates fungi were detected by culture-independent method.
[1]Fungi were detected by culture-dependent method.
[2]Fungi were detected by both culture-dependent and -independent methods.

The most commonly detected fungi in the GI tract of humans among the 36 studies listed in Table 1.2 are *C. albicans* (25 studies), *Saccharomyces cerevisiae* (19), *C. tropicalis* (17), *C. parapsilosis* (13), *C. glabrata* (12), *C. krusei* (10), *Malassezia globosa* (8), *M. restricta* (7), and *Debaryomyces hansenii* (7). *Candida* species are known to dominate the GI tract of humans and their presence is not surprising. To the best of our knowledge, at least 268 fungal taxa have been reported in gut, which is considerably lower than the estimated 500-1,000 bacteria residing in the gut [163, 221]. Additionally, a majority of the fungi were only reported in one study.

1.5.4 STABILITY OF THE MYCOBIOME

Fungi residing in the gastrointestinal tract can originate from a number of sources. Fungi are ubiquitous in the environment and are used as food and in food production, making their presence in the gut a reflection of the individual's diet and environmental encounters [180]. DNA extracted from food and fecal samples show that foodborne fungi from the diet transiently colonize the gut [42]. Species of fungi found in the gut known to colonize the skin and respiratory tract are also thought to be transient members [98]. Due to the transient and allochthonous nature of numerous fungal members in the gut, stability of the gut mycobiome over time is low [74]. Table 1.2 also illustrates the dynamic and variable nature of the mycobiome in the number of fungi identified in a single study. Fungal populations in the murine gut vary substantially over time and are influenced by the environment, while bacterial populations remain relatively stable [47]. There are both more bacterial species and more individual bacteria in the gut than fungi and thus, bacterial communities may be more robust [204].

1.6 MEDICALLY RELEVANT FUNGI: PATHOGENIC YEASTS

Fungal diseases, especially those caused by opportunistic pathogens, have become increasingly important over the past few decades. The success of modern medicine and the explosion of new antibacterial agents have led to greater survival of immunocompromised patients while creating populations highly susceptible to fungal infections. The most common yeasts found associated with disease are *Candida, Histoplasma, Blastomyces,* and *Cryptococcus* and to a lesser extent species of *Geotrichum, Malassezia, Pichia, Rhodotorula, Saccharomyces,* and *Trichosporon*. With the exception of *Blastomyces,* each of these fungal genera has been reported in the GI tract. The most common filamentous fungi associated with disease found in the gut are *Aspergillus* spp. Additionally; other fungi found in the gut and causing infections include *Alternaria, Scedosporium, Paecilomyces,* and *Trichoderma*.

1.6.1 *CANDIDA* SPECIES

Candida species are normal, harmless commensals on many human body sites and have been consistently reported in gut fungi studies. *C. albicans* is the predominant commensal yeast of humans. *Candida* spp. are also opportunistic pathogens of humans and cause disease when mucosal barriers are disrupted and defects in the immune system occur. They can invade and cause infections ranging from superficial infections of the skin to life-threatening systemic infections. At least 17 *Candida* species, out of the approximately 150 known species, cause disease in humans, the most common being *Candida albicans, C. dubliniensis, C. glabrata, C. guilliermondii, C. krusei, C. lusitaniae, C. parapsilosis,* and *C. tropicalis* [139]. Recent studies show that *Candida*

spp. are the fourth most-common cause of nosocomial bloodstream infections [216, 217]. An estimated 60,000 cases of disseminated candidiasis occur each year in the U.S, with an estimated cost of 2-4 billion dollars [157, 215].

In the years prior to 1990, the predominant species causing invasive *Candida* infections was *C. albicans* [157]. *C. albicans* lacks an environmental reservoir and is almost exclusively associated with humans and possibly other warm-blooded animals and avian species [26, 89, 119, 169]. *C. albicans* is considered the most virulent species of the genus *Candida* because it contains an arsenal of virulence factors and fitness attributes that contribute to pathogenesis. The most widely studied virulence factors include adhesins, secreted hydrolytic enzymes, and morphogenesis.

Adherence to the host cell is an essential first step of pathogenesis. *C. albicans* expresses adhesins on the surface of the cell that allow it interact will host cells and ultimately colonize host tissues. The agglutinin-like sequence (ALS) protein family contains the most recognized *C. albicans* adhesins and consists of eight large cell-surface glycoproteins for adherence to mammalian cells [83]. In addition to host cell adherence, *Candida* species produce three-dimensional structured microbial community biofilms surrounded by a matrix of exopolymeric material on biotic and abiotic surfaces [102, 165]. The formation of *Candida* biofilms becomes important clinically because of biofilms' increased resistance to antifungals and protection from host immune defenses [14]. Biofilms forming on implanted medical devices such as catheters, shunts, prosthetic devices, and dentures serve as a reservoir for infection and direct access to the body [164].

Extracellular proteolytic activity is another *C. albicans* pathogenicity mechanism. The best-characterized *Candida* proteases are the secreted aspartyl proteinases (Sap) [136, 137]. Sap proteins help digest molecules for nutrient uptake, distort host cells to enable adhesion and tissue invasion, and digest components of the host immune system to evade attack [141]. *In vitro* models using *sap* deletion mutants show attenuated virulence and demonstrate Sap proteins contribute to the overall virulence and are required for disseminated infection [84, 177, 182].

Morphological switching is a virulence strategy used by *C. albicans* to aid in its pathogenic potential. When *C. albicans* comes into contact with a surface, yeast cells transition into hyphal forms [103]. Both morphological forms are important for pathogenicity; the hyphal form aids in cellular invasion and the yeast form is primarily involved in dissemination [17, 179]. Quorum sensing regulates the yeast-to-filamentous transition in *C. albicans* by molecules by farnesol [82]. *C. albicans* also has a range of fitness attributes that allow the yeast to adapt to pH changes, stress, and nutrient starvation [127].

In recent years, there has been an increased prevalence of infections caused by species of *Candida* other than *C. albicans*. The introduction and widespread use of antifungals has led to an increased number of infections caused by *C. glabrata,* due to its resistance to fluconazole, itraconazole, and moderate (dose-dependent) resistance to other antifungal agents [201]. Distribution of *Candida* infections varies among countries and institutions; *C. glabrata* is considered the second most common cause of *Candida* infections in the U.S. and Europe, yet ranks behind *C. parapsilosis* and *C. tropicalis* in South America and Asia, respectively [35, 107, 159, 197]. Reasons for geographical

differences are of interest and outline the complexity of epidemiology. Multiple risk factors including clinical practice, antifungal therapies, and colonization of health care workers likely support the emergence of non-*albicans* species as predominant pathogens [150].

1.6.2 *ASPERGILLUS* SPECIES

Invasive aspergillosis (IA) has increased significantly in recent decades and is the most common filamentous fungal lung infection in immunocompromised patients [69, 130]. *Aspergillus* species have a worldwide distribution and their ubiquity in the environment exposes humans to *Aspergillus* spores daily. *Aspergillus fumigatus* is the most prevalent species of the 200 aspergilli described and is the main cause of human IA [40]. *A. flavus, A. glaucus, A. niger, A. nidulans, A. parasiticus,* and *A. terreus* are also commonly associated with aspergillosis [138]. *Aspergillus* species are also well known for their ability to produce carcinogenic mycotoxins, aflatoxin, and ochratoxin, and show resistance to some antifungals [45, 94]. Numerous species of *Aspergillus* have been reported in the GI tract of humans (Table 1.2).

1.7 CHARACTERIZATION OF THE GASTROINTESTINAL FUNGAL COMMUNITY

The correct identification of fungi is of great importance in clinical settings, and especially in novel studies attempting to characterize microbial communities from different ecological niches, such as the gastrointestinal tract. Methods for exploring and characterizing the microbiota from the GI tract have greatly evolved over time. Wide

ranges of techniques are available to identify fungi, all differing in specificity, reproducibility, time, and cost. While each method has its own advantages and limitations, advances over the years have improved our understanding of the human GI tract ecosystem.

1.7.1 Culture-dependent methods

The first studies identifying the fungi of the gastrointestinal tract date back to the early 1900s [9, 99]. The ability to culture fungi was critical for discovering and identifying their presence. At the time it was known that feces contain a large number of bacteria compared to fungi and ordinary culture medium and plating methods would not be sufficient for the isolation of fungi from the gut. Therefore media inhibiting bacterial growth, such as Sabouraud's agar, were commonly used [9, 13]. Historically, identification and classification of yeasts was based on readily observable gross colony features (color, size, shape), microscopic examination, and biochemical properties. Over time, culture-dependent methods including selective media, nutrient utilization and fermentation, susceptibilities to yeast killer toxins, susceptibilities to chemicals and antifungal drugs, secondary metabolites, fatty acid composition, cell wall composition, and protein composition have been indispensable for increasing our understanding and classifying fungi [70].

Although advancements have been made in culture-dependent methods, these methods present several disadvantages and study limitations that restrict the ability for mycologists to accurately characterize fungi in fecal samples. In culture-based studies, dominant populations (e.g. *Candida* spp.) can mask the detection and diversity of low-

abundance organisms. In addition, current fungal culture techniques may be inadequate to optimally cultivate organisms that require microbe–microbe interactions, since culture fails to reproduce ecological niches and symbiotic relationships encountered in the natural environment [15, 29]. The culturable fraction is also distorted because replication times vary among microorganisms and fast-growing species efficiently outcompete others [143].

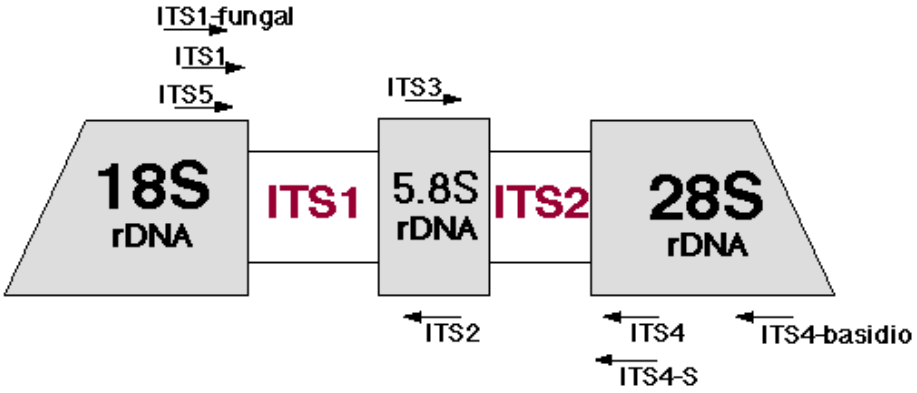
1.7.2 Culture-independent methods

Considering the above limitations of culture-dependent methods, the advent of culture-independent methods has allowed researchers to more efficiently characterize the mycobiome. In particular, the development of polymerase chain reaction (PCR) in the 1980s revolutionized culture-independent fungal detection methods [173]. In the ensuing years, the introduction of multiple culture-independent DNA-based methods for characterizing the gut microbiota became available—denaturing gel gradient electrophoresis (DGGE), oligonucleotide fingerprinting of ribosomal RNA genes (OFRG) and restriction fragment length polymorphism (RFLP). While these methods have improved the ability to study the mycobiota, they still have a reduced ability to identify fungi at the species level and quantify taxa [38].

The advent of high-throughput DNA sequencing technologies has become the preferred method for mycobiome studies [48, 185]. Sanger sequencing and, more recently, next-generation amplicon sequencing are capable of distinguishing species using genes within the fungal ribosomal RNA gene cluster—18S small subunit rDNA, 28S large subunit rDNA, and the internal transcribed spacer (ITS1 or ITS2) (Figure 1.2).

rRNA genes are appealing targets for fungal identification because they are highly multicopy in each genome. Fungal rRNA genes have highly conserved regions serving as primer binding sites to determine the sequences of adjacent variable regions by PCR amplification. In fungi, 28S provides much greater resolution than 18S, and continues to be used for within-genus (i.e., species-level) identification and phylogenetic studies, while 18S is rarely adequate to distinguish species [41] (in contrast with the analogous bacterial 16S gene, which is widely used for identification). ITS genes are less conserved and, therefore, are considered the best target for fungal species and subspecies identification [161]. Also, researchers must select which ITS gene (ITS1, ITS2 or ITS1-5.8s-ITS2) to amplify for identification (Figure 1.2). Next-generation sequencing of these genes has provided the most accurate account of the fungal composition of the human gut microbiome.

A)



B)

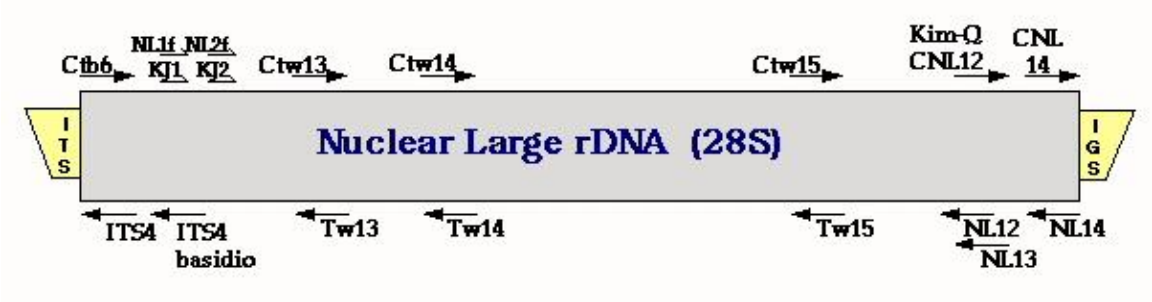


Figure 1.2 Schematic representation of the A) fungal ribosomal gene cluster and B) 28s nuclear large rDNA with binding locations of PCR primers
(http://nature.berkeley.edu/brunslab/; accessed February 2015).

1.7.3 Culture-dependent vs. culture-independent methods

Few studies have used a combination of culture-dependent and -independent methods for the identification of gut fungi and compared the results. One study exploring the diversity of gut eukaryotes in a Senegalese man identified 16 fungal species by molecular methods targeting the ITS, 18s, and 28s genes and only four fungal species by culturing, using three culture media [75]. Three of the four fungi isolated by culture were also detected by sequencing; the exception was *C. krusei*. An earlier gut fungi study using

culture-dependent and -independent methods also highlighted a culturing bias. *Candida* spp. were identified as the predominant species by cultivation methods and *Gloeotinia*/*Paecilomyces* and *Galactomyces* were found to be the predominant species using clone libraries [180]. Chen and colleagues found 37 fungal OTUs using clone libraries while only five species, from two genera (*Candida* and *Saccharomyces*), were isolated by two culture media [30]. The culture-independent method in this study failed to detect *C. krusei* and *C. glabrata*.

1.8 THE GUT MYCOBIOME IN HEALTHY AND DISEASED STATES

1.8.1 Benefits of intestinal fungi

Compared to the vast amount of literature on the benefits of bacteria in the human gut, the beneficial role of gut fungi – if any – remains largely unexplored. No strong evidence exists for a mutualistic or beneficial relationship with the gut mycobiome [85]. The only fungus studied in any detail with indications of treating gastroenteritis is the probiotic yeast *Saccharomyces cerevisiae* var. *boulardii* ("*S. boulardii*") [222]. Clinical trials using *S. boulardii* as a therapeutic strategy have shown significant efficacy in treatment and prevention for various types of diarrhea including traveler's diarrhea, antibiotic associated diarrhea, and inflammatory bowel disease [24, 71, 72, 129]. In addition to the presumed health benefits, it is proposed that probiotic yeasts may positively interact with probiotic bacteria by enhancing their survival, and overall display a synergistic effect [20, 196]. Another potential medical application of probiotic yeasts involves their ability to express disease-fighting proteins known as killer toxins, or mycocins, against pathogenic yeasts, such as *Candida* spp. [212].

1.8.2 Mycobiome and disease

Since the renowned Greek physician Hippocrates first described oral candidiasis in 400 BC, researchers and clinicians have aimed to explore the roles of commensal and pathogenic fungi in human health and disease [3]. In particular, cataloging the gut mycobiome in health and disease has been a biological hot spot in recent years. Defining fungi present and/or absent as well as alterations in the fungal community in the gut during different disease states may inform knowledge of cause and effect. Do these fungi contribute to or protect from disease? Do they cause disease directly or indirectly?

Gastrointestinal colonization by fungi such as *C. albicans* is suggested as the major reservoir and source of invasive fungal infections such as invasive candidiasis [148]. *C. albicans*, the predominant gut fungus, colonizes the GI tract but has the ability to invade tissues and disseminate in the body when the gut microbiome is disrupted, intestinal mucosal permeability is increased, or the host is immune-suppressed. *Candida* gut colonization has been associated with a number of diseases such as diabetes, hematologic malignancies, Crohn's disease, and graft-versus-host disease; and patient populations such as hospital inpatients and extremely low birth weight infants (Table 1.3). Patients with gastrointestinal disorders such as ulcerative colitis and Crohn's disease are more frequently and heavily colonized by *C. albicans* than are control groups [100, 193].

In the gut, the disease states studied thus far exhibit associations between fungal diversity and disease. This has been demonstrated in patients with Crohn's disease, hepatitis B, and pouchitis [30, 101, 113, 152]. In many of these cases, fungal diversity is positively correlated with disease progression and severity. However, this is not the case

for the mycobiome at other diseased body sites. The human lung, previously thought to be sterile, harbors a lower diversity of fungi in patients with cystic fibrosis than healthy controls [78]. This is also true in the nasal mycobiome of allergic patients [188]. Drawing broad conclusions about fungal diversity, colonization, and disease is difficult due to the low number of studies, sample numbers, and uncertainty across body sites. Gut fungi data indicates a role for the mycobiome in the pathogenesis of a number of diseases and suggests that altering the mycobiome may aid in preventing or ameliorating disease. The question yet remains whether or not disease causes changes in the mycobiome or vice versa. As the field advances, mycobiome composition and alterations may be a predictor of disease and more research will continue to define the association between fungi and disease.

Table 1.3 Changes in the gut mycobiota associated with disease.

Disease status	Sample type and size	Methodology	Primers	Findings	Reference
Type 1 diabetes	Feces, n=35	Culture	n/a	*C. albicans* identified in 40% of subjects; significant difference from 35 controls	[192]
Inflammatory bowel disease	Colonic biopsies, Crohn's disease n=31 Ulcerative colitis n=26	18s rDNA, DGGE, cloning, Sanger sequencing	NS0/EF3 NS1/FR1 BF2/TR1	Higher mean fungal diversity in patients with CD; significant difference from 47 controls	[152]
Crohn's disease	Ileal mucosa & feces, n=19	18s rRNA, DGGE, cloning, Sanger sequencing	NS1/FR1, EF390/GC-FR-1	Fungal richness and diversity significantly increased in patients, *C. albicans* & *C. tropicalis* abundant in patients and absent in 7 controls	[113]
Familial Crohn's disease	Feces & mouth swabs, n= 129 CD patients n=113 healthy relatives	Culture, biochemical tests, latex agglutination	n/a	*C. albicans* significantly more frequent in CD patients and their healthy relatives than from 14 control families	[193]
Preterm infants (<32 weeks)	Feces, n=32, (7 NEC, 13 sepsis)	28s rDNA, DGGE, cloning, Sanger sequencing	U1/U2-GC	Fungal colonization in half of infants; No fungal species were observed in infants who developed necrotizing enterocolitis	[195]
Intestinal transplant	Ileal effluent & feces, n=2	18s rDNA, DGGE, cloning, Sanger sequencing	NS1/FR1 EF390/GC-FR1	High fungal diversity soon after transplant; less diversity >16-20 weeks post-transplant	[112]
Hepatitis B	Feces, cirrhosis n=38, chronic hepatitis B n=35	Culture 18s, cloning, RFLP, Sanger sequencing	Unspecified	Diversity of enteric fungi positively correlated with disease progression	[30]

Table 1.3 Continued.

Disease status	Sample type and size	Methodology	Primers	Findings	Reference
Hematologic malignancy or disorder	Feces, n=80	Culture, germ tube test, biochemical test	n/a	Non-*albicans Candida* spp. significantly higher in patients, *C. glabrata* more prevalent in patients than 61 controls	[6]
Hospital inpatients	Feces, n=34	Culture, germ tube test, biochemical test	n/a	Inpatients higher prevalence of yeast, *C. glabrata* more prevalent in inpatients	[97]
Extremely low birth weight infants	Feces, n=11	ITS, 454 pyrosequencing, metagenomic shotgun sequencing	ITS3/ITS4	*Candida* spp. and *Clavispora* sp. dominated, *Candida quercitrusa* most abundant	[108]
Diabetes	Feces, type 1 n=27, type 2=17	Quantitative real-time PCR	18s, FAM *Candida* probe	*Candida* spp. significantly greater in type 1 & type 2 patients than 17 controls	[65]
Graft-versus-host disease	Feces & oral, GVHD n=59 GI-GVHD n=37	Culture	n/a	Patients colonized with *Candida* spp. had a significantly higher rate of grade II-IV acute GVHD	[205]
Pouchitis	Biopsy & feces, n=36	18s, DGGE	NS1/FR1-GC	Patients who develop pouchitis have higher fungal diversity and lower bacterial diversity	[101]
Varying symptoms & complaints	Feces, n=308	Culture	n/a	Smoking habits highly associated to *Candida* colonization; also associated with *Candida*-vaginitis	[92]
Gastric ulcers	Gastric juice, n=293	Culture, biochemical test	n/a	Significant fungal colonization $>10^4$ CFU/ml in patients with gastric ulcers	[227]

1.9 DIET AND THE GASTROINTESTINAL MYCOBIOME

Diet is an environmental factor that influences the structure and diversity of microorganisms in the gut [42]. Resident bacterial populations are associated with diet and long-term dietary patterns [42]. However, few studies have investigated the relationship between diet and the human intestinal mycobiome. One recent human study demonstrated that consumption of high amounts of carbohydrates was positively associated with *Candida* spp. colonization, while a diet high in amino acids, fatty acids, and protein was negatively associated with *Candida* spp. [80]. Fungal abundance in the gastrointestinal tract was also strongly associated with the composition of short-term diet. Animal-based diets significantly increase *Penicillium* spp. [42], while Ukhanova et al. showed pistachio and almond consumption does not significantly increase gut fungal OTUs [203].

Intestinal populations of microorganisms are suspected of contributing to obesity by way of diet [111]. The role of gut fungi in obesity is unknown. Only one study has addressed this, focusing on the fungal communities in the human gut of a single obese person and detecting 18 fungal species [66]. Conversely, a study identifying the fungal community of a single anorexic human reported a “restricted” diversity of 10 fungal species [67]. However, this number is not unusual for healthy individuals on a conventional diet [74].

1.10 THE INTESTINAL MYCOBIOTA AND THE HOST IMMUE SYSTEM

The GI tract is home to the majority of both the human body’s commensal microorganisms and immune cells. Little is known about the interaction between

commensal intestinal fungi and the host immune system. Several players of the immune system have been reported to play a critical role in fungal recognition and host defense against disseminated fungal infections. These include Dectin-1, IL-17, and IL-22 [88]. Dectin-1 is a C-type lectin that plays a role in the innate immune response by functioning as a pattern-recognition receptor and recognizing beta-glucans found in the cell walls of fungi. In mice, Dectin-1 is of critical importance to defend against pathogenic fungi such as *Pneumocystis carinii* and *C. albicans* [172, 200]. Humans deficient in Dectin-1 suffer recurrent mucocutaneous candidiasis infections [57]. Genetic polymorphisms of Dectin-1 have also been associated with increased severity of ulcerative colitis [87, 204].

The cytokines IL-17 and IL-22 have been implicated in mucosal and antifungal immunity. Mice deficient in components of the IL-17 pathway show increased susceptibility to oral candidiasis and skin infections [37, 93]. Furthermore, IL-22 serves as a first-line of defense against candidiasis in mice because IL-22 deficient mice are more susceptible to GI candidiasis [43]. Mucosal immune responses to fungi are different across human body sites. A complete understanding of the interaction between commensal fungi and the immune system has yet to be unveiled.

1.11 CROSS-KINGDOM INTERACTIONS; POST-ANTIBIOTIC INTESTINAL COMMUNITY REASSEMBLY

Interactions among microorganisms within the GI tract have long been recognized as important determinants of community function [178]. Inhabitants are unlikely act in isolation in the gut and, therefore, live in complex associations with one another. Residents of the gastrointestinal microbiota are constantly interacting with one another,

competing for nutrients and space and producing compounds that directly inhibit growth or kill potential competitors [114]. Microbes in the gut may also form mutualistic and/or commensal interactions with one another cooperating via syntrophy - using metabolic waste products of another organism as nutrients - and constructing interspecies antibiotic-resistant biofilms [166, 219]. Hoffman et al. has best described the correlations between fungi-bacteria and fungi-archaea [80]. The fungal phyla, Ascomycota and Basidiomycota, significantly correlate with bacterial lineages. *Candida* and *Saccharomyces* commonly co-occur with bacterial taxa *Faecalibacterium*, *Bacteroides*, Lachnospiraceae, and Ruminococcaceae and these fungal genera are also positively associated with the archaeon *Methanobrevibacter* and negatively associated with the archaeon *Nitrososphaera*. Further analysis showed that *Candida* was negatively associated with *Bacteroides*.

Bacteria and fungi interact with one another within an ecosystem physically and chemically [154]. These interactions influence overall survival, colonization, and virulence. Perhaps the most important bacterial-fungal interaction led to the discovery of penicillin [61]. Physical interactions between fungi and bacteria include forming mixed biofilms with one another and the ability of bacteria to attach to fungal hyphae, inhibiting filamentation and biofilm formation [2, 155]. Bacteria also produce antifungal molecules that affect morphology and virulence of *Candida* [81]. Bacteria have the ability to modify the environment by lowering the pH and preventing hyphae formation by *C. albicans* [27].

Bacteria and fungi are known to inhabit, and interact with one another at, multiple human body sites. The species composition of the two may become beneficial or

detrimental to the human host. Within the gastrointestinal microbiota, colonization resistance is a known interaction that prevents overgrowth by endogenous organisms. The bacterial microbiota reduces *C. albicans* colonization by excluding and out-competing *Candida* for adhesion sites and producing inhibitory molecules [96]. Controlling fungal colonization, especially by opportunists, with the use of probiotic bacteria is appealing. One study showed the probiotic bacteria, *Lactobacillus acidophilus* and *Lactobacillus casei,* induce protection against systemic candidiasis in mice [209]. Short chain fatty acids (butyric acid) produced by lactic acid bacteria also inhibit filamentation and may prevent *C. albicans* from causing disease [146].

1.11.1 Post-antibiotic recolonization

The ecological balance in the human gastrointestinal tract is important and modulated, in part, by intestinal bacteria preventing fungal colonization and overgrowth. However, when broad-spectrum antibiotics are administered they have the potential to wipe out the entire bacterial community in the GI tract, perturb the equilibrium and allow the host to become susceptible to infections by normally benign, commensal fungi such as *C. albicans* [175]. Antibiotics are, therefore, a major risk factor for candidemia in hospitalized patients [149, 213, 223].

Conversely, post-antibiotic recolonization of the bacterial gut microbiota is shaped and altered by the presence of *Candida*. In the stomach of microbiome-disturbed mice, *C. albicans* prevents *Lactobacillus* regrowth after antibiotic treatment and promotes colonization of *Enterococcus* [125]. Presence of *C. albicans* in the murine cecum after antibiotic treatment promotes Bacteroidetes populations, *Enterococcus faecalis*

persistence, and inhibits *Lactobacillus johnsonii* [126]. Another recent study demonstrated that the exogenous addition of *C. albicans* during treatment with the cefoperazone in mice led to yeast overgrowth and substantially altered reassembly of the bacterial community after treatment [52]. The combination of antibiotics and fungal microbiota has been shown to promote the development of allergic airway disease [145, 147].

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CHAPTER 2

CHARACTERIZATION OF THE HUMAN GUT MYCOBIOME: VEGETARIAN DIET AS AN ILLUSTRATION

2.1 ABSTRACT

The fungal inhabitants of the gastrointestinal tract have been previously overlooked in human microbiome studies. Here we present a survey of the fungal microbiota in 16 fecal samples from healthy humans with a vegetarian diet. Fungi were identified using molecular cloning and 454/Roche amplicon pyrosequencing of ITS gene tags; additionally, samples were queried with a Luminex xTAG analyte specific reagent (ASR) assay for fungal pathogens. Two fungal phyla were detected, with Ascomycota present in all samples and Basidiomycota present in 81% of samples. Both sequencing methodologies detected fungi in each fecal sample and in combination identified at least 57 fungal OTUs. The number of OTUs in each sample ranged from 2-25. A total of 31 fungal genera were found overall; 14 genera were present in only one sample and four genera were present in only two samples. *Malassezia* was the most abundant genus, followed by *Aspergillus* and *Fusarium.* Other abundant genera included *Penicillium, Alternaria*, 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. We also detected 17 fungal species not previously reported from the gut. It is apparent that a wide variety of fungi can be identified from the human gastrointestinal tract.

2.2 INTRODUCTION

Over the last few decades, the composition and role of the bacterial gut microbiota in humans has been studied extensively. The bacterial microbiota play an integral part in the digestion of food, colonization resistance to invading pathogens, and development of the immune system [8, 14, 25]. Until recently, microbiome studies have failed to include eukaryotes in total community analyses, and eukaryotic diversity and function in the gut is poorly understood. However, fungi are consistently identified as the dominant human gut eukaryotes [44]. Knowledge of fungi in the gut opens a novel area of research, where the same questions once asked about the role of gut bacteria in health and disease are now being directed at the fungal microbiota ("mycobiota").

Fungi are ubiquitous in the environment, and it is not surprising that humans have co-evolved with fungi on many surfaces of the body [27]. Fungi are successful commensals and pathogens of humans, despite the relative paucity of specialized mammalian fungal pathogens [39]. Yeasts were first recognized to inhabit the gastrointestinal (GI) tract in the early 1800s [5, 30]. However, only recently have we started to appreciate and question their role. Cultivation studies show fungi to be detectable in all sections of the GI tract of 70% of healthy adults, while the abundance – 10^6 fungal cells per gram of feces – is orders of magnitude smaller than that of gut bacteria [4, 48, 49]. Alterations of the gut mycobiome have been associated with hepatitis B and inflammatory bowel disease and increased fungal colonization has been shown to impair mucosal barrier defense allowing translocation of gram-negative bacteria [7, 11, 41]. Mycobiome research also represents an important field of study due to the increasing

number of invasive fungal infections in immunocompromised populations such as AIDS, cancer, and organ transplant patients [35].

Culture-dependent methods used to characterize the intestinal mycobiome are unable to optimally cultivate organisms of low abundance or those that require microbe–microbe interactions [3]; additionally, many gut microbes require a modified atmosphere for optimal growth. Thus, culture-independent methods, such as PCR-based cloning and high-throughput DNA sequencing of fungal rRNA and ITS genes have become the preferred method for mycobiome studies [12, 47]. No method has been unanimously used and difficulties in defining the complete mycobiome are attributed to low sample numbers, individual variation, limited attention, and a lack of consistent identification methods and gene targets. In addition, several recent intestinal fungi investigations described the diversity of the mycobiome based on a single fecal sample [18, 19, 22].

Factors such as diet, genetics, and environment play an influential role in composition of the gut microbiota and help explain why differences in microbial communities exist between human hosts [36]. Few studies have attempted to explore the association between enteric fungi and diet [9, 23]. One previous study examining the association between diet and intestinal fungi showed fungal abundance of *Candida* spp. were positively correlated with recent consumption of a high carbohydrate diet, while *Candida* spp. were negatively associated with diets high in amino acids, fatty acids, and protein [23]. In an effort to deepen the understanding of dietary factors, we investigated the intestinal mycobiome of healthy adults with a vegetarian diet.

It is evident that the present view of the intestinal mycobiome is incomplete. In this study, we used molecular cloning and high-throughput ITS gene pyrosequencing to

obtain a comprehensive survey of the human gastrointestinal mycobiome. We additionally used a commercially available assay using multiplex-PCR with labeled primers and subsequent sample hybridization to fluorescent microspheres (Luminex xTAG analyte specific reagents [ASRs], Luminex Corporation, Austin, TX). This assay was developed to target clinically relevant fungi [2], which include species of *Candida* and *Aspergillus* that may be detected in the gut. Here we provide preliminary characterization of the fungal communities in 16 fecal samples from the gastrointestinal tract of healthy adult humans with a vegetarian diet. Furthermore, we compare three culture-independent methods, and provide the first evaluation of Luminex fungal ASRs on fecal DNA from healthy humans.

2.3 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 subjects to participate in this study. Fecal samples were obtained from 15 healthy human adults (6 male, 9 female; one male subject provided two fecal samples two months apart, n=16). Study subjects were self-identified vegetarians (may or may not eat eggs and/or dairy; no meat, fish or poultry consumption) 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

DNA was extracted using a modified method of the QIAamp® DNA stool mini kit (QIAGEN) [38]. Briefly, feces were thawed on ice, and approximately 200 mg of sample was diluted ten-fold in ice-cold phosphate buffered saline (PBS). Fecal homogenates were transferred into a 2-ml sterile bead-beating tube containing 300 mg of 0.1-mm zirconium beads, and fungal cells were collected by centrifugation at room temperature at 8,000 x *g* for 5 minutes. The cell pellets were washed three times with ice-cold PBS. The pellets were then resuspended in 100 µl lysis buffer (200 mM NaCl, 100 mM Tris pH 8.0, 20 mM EDTA) containing 20 mg/ml of lysing enzymes and incubated at 37°C for 30 minutes. Using the kit reagents, 1.7 ml of Buffer ASL was added to each tube and vortexed until the stool sample was thoroughly homogenized. Mechanical cell lysis was performed by bead-beating each sample for two minutes at maximum speed (MiniBeadbeater-8, BioSpec Products, USA). Isolation and purification of DNA from fecal samples was continued following the manufacturer's recommendations.

PCR amplification

PCR was performed using primers targeting the ITS1-5.8S-ITS2 region. Semi-nested PCR was used to detect fungal DNA amongst the predominant non-fungal DNA present in fecal samples. The first round of amplification utilized the fungal-specific ITS1-F forward primer [15] and the universal eukaryotic reverse primer TW13 [54] (see Table 2.1 for primer sequences). Each PCR reaction mixture included 12.5 µl of REDTaq ready mix (Sigma-Aldrich, St. Louis, MO), 2 µl (10 µM) of each oligonucleotide primer, 7.5 µl molecular biology-grade water, and 1 µl DNA template for a final volume of 25 µl.

PCR reactions were performed on an Eppendorf Mastercycler and cycling conditions were as follows: initial denaturation for 94°C for 4 min and 30 cycles at 94°C for 30 sec, 52°C for 1 min, 72°C for 1.5 min, followed by a final extension step of 72°C for 10 min. After the initial reaction, a second reaction was performed using the primers ITS1-F and ITS4 [54] and 1 µl of the template DNA from the first reaction. PCR amplicons were separated using a 0.7% agarose gel in 1 x TAE buffer, stained with 0.5 µg/ml ethidium bromide and visualized. PCR products were confirmed with bands around 500-600 bp. Negative controls were used throughout the reactions to ensure contamination did not occur.

Fusarium-specific PCR was performed with the following primer combinations: GOFW and GORV, which specifically amplify a 435 bp fragment from the galactose oxidase gene of *F. graminearum* [10] (see Table 2.1 for primer sequences); $OPT18F_{470}$ and $OPT18R_{470}$, which specifically amplify a 470 bp fragment from *F. culmorum* [46]; and 3CON, 3D3A, 3D15A, and 3NA, used in a multiplexing reaction to specifically amplify 243, 610 and 840 fragments from the *Tri3* gene in *Fusarium* producing 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, and nivalenol, respectively [50]. *F. graminearum* PH-1 was included as a positive control, and 25 µl reactions followed the protocols from the respective papers. Additionally, nested reactions were prepared using the same primers and protocols as above, with 1 µl of the initial PCR reaction as template, to account for the potentially low copy number in DNA extracted from fecal samples.

Table 2.1 PCR primers used in this study.

Primer	Sequence	Reference
ITS1-F	5'-CTTGGTCATTTAGAGGAAGTAA-3'	[15]
TW13	5'-GGTCCGTGTTTCAAGACG-3'	[54]
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	[54]
GOFW	5'-ACCTCTGTTGTTCTTCCAGACGG-3'	[10]
GORV	5'-CTGGTCAGTATTAACCGTGTGTG-3'	[10]
$OPT18F_{470}$	5'-GATGCCAGACCAAGACGAAG-3'	[46]
$OPT18R_{470}$	5'-GATGCCAGACGCACTAAGAT-3'	[46]
3CON	5'-TGGCAAAGACTGGTTCAC-3'	[50]
3D3A	5'-CGCATTGGCTAACACATG-3'	[50]
3D15A	5'-ACTGACCCAAGCTGCCATC-3'	[50]
3NA	5'-GTGCACAGAATATACGAGC-3'	[50]

Molecular cloning

PCR products were purified using the Wizard Genomic DNA purification kit (Promega, Madison, WI). Purified products were ligated into pGEM®-T Easy vectors and transformed into JM109 High Efficiency Competent cells (Promega, Lyon, France) following the manufacturer's instructions. Subsequently, 10 colonies negative for β-galactosidase activity were chosen and sequenced by dideoxy (Sanger) 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 [29] and the curated FUNCBS database, as accessed through the Centraalbureau voor Schimmelcultures (www.cbs.knaw.nl/Collections/BioloMICSSequences.aspx?file=all; accessed July 2014). Sequences have been deposited in GenBank as accession # KP196567-KP196602.

Pyrosequencing of the fungal ITS genes

Extracted DNA from fecal samples was selectively amplified by PCR using primers targeting the ITS1-5.8S-ITS2 genes. PCR reaction mixtures consisted of 25 µl REDTaq ready mix (Sigma-Aldrich, St. Louis, MO), 4 µl ITS1-F (10 pmol), 4 µl TW13 (10 pmol), 15 µl molecular biology-grade water, and 2 µl DNA template. PCR was performed on an Eppendorf Mastercycler using the following conditions: 95°C for 15 min and 30 cycles of 94°C for 30 sec, 52°C for 30 sec and 72°C for 1 min, followed by a final elongation for 72°C for 10 min. PCR products were purified using the Wizard SV Gel and PCR Clean-up Kit (Promega, Madison, WI). Following cleanup, amplicon products from all samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corp., MA, USA). Samples were sequenced at MR DNA (Shallowater, TX) using tag-encoded FLX amplicon pyrosequencing on 454/Roche GS FLX Sequencer technology [13, 51]. Data processing and analysis followed Hume and colleagues [26]. Rarefaction analysis based on observed OTU metrics was performed to assess community coverage using QIIME (v. 1.8.0) [6] with default settings. Alpha diversity of the samples was measured based on Simpson and Shannon's coefficients in QIIME [6]. One sample with null diversity (only one OTU detected) was removed from analysis. Raw sequences have been deposited in GenBank under BioProject PRNJA268649.

Luminex Fungal xTAG ASR Assay

A multiplex xTAG fungal analyte-specific reagent (ASR) assay consisting of 23 ASRs developed by Luminex Molecular Diagnostics was used for the simultaneous

detection and identification of multiple fungal pathogens in the fecal samples. The following ASRs were used: xTAG *Aspergillus flavus*, xTAG *Aspergillus fumigatus*, xTAG *Aspergillus niger*, xTAG *Aspergillus terreus*, xTAG *Blastomyces dermatitidis*, xTAG *Candida albicans*, xTAG *Candida glabrata*, xTAG *Candida guilliermondii*, xTAG *Candida krusei*, xTAG *Candida lusitaniae*, xTAG *Candida parapsilosis*, xTAG *Candida tropicalis*, xTAG *Coccidioides immitis*, xTAG *Cryptococcus neoformans*, xTAG *Fusarium*, xTAG *Histoplasma capsulatum*, xTAG *Mucor indicus*, xTAG *Pneumocystis jirovecii*, xTAG *Rhizopus microsporus*, xTAG *Rhizopus arrhizus*, xTAG *Scedosporium apiospermum*, xTAG *Scedosporium prolificans*, and xTAG *Tremella*. Multiplex PCR, bead hybridization, and detection were performed on DNA extracted from fecal samples above following the protocol of Babady and colleagues [2]. The samples were analyzed on the Luminex MAGPIX system (Luminex Corp., Austin, TX) and median fluorescence intensity (MFI) values were calculated by the xPONENT 4.2 software. A threshold value of 225 MFI was set for positive targets, values below 200 MFI were negative, and 200-224 MFI were equivocal.

2.4 RESULTS

Sequencing methods

The gut mycobiota of 16 fecal samples from humans with a vegetarian diet was characterized using molecular cloning and 454/Roche amplicon pyrosequencing. Each method was able to identify fungi in the samples provided after amplification of the fungal ITS genes. The gut mycobiota was first identified using a traditional Sanger sequencing-based method via molecular cloning and secondly by 454/Roche

pyrosequencing of the ITS1-ITS2 genes. After removing singletons from pyrosequencing data, which account for a major source of biases [52], the total number of sequences obtained from 16 samples was 186,495 (mean 11,656; median 7,802). The predominant fungal taxa identified differed for the two methods. Molecular cloning identified *Aspergillus* as the most abundant genus and 454 pyrosequencing identified *Fusarium*, *Malassezia*, and *Penicillium* as the most abundant genera. Pyrosequencing identified 41 fungal operational taxonomic units (OTUs) that cloning failed to detect. In contrast, *Leptosphaerulina chartarum* was only detected by molecular cloning.

Identification of fungi in fecal samples

Combining both sequencing methods, two fungal phyla were observed in the samples: Ascomycota and Basidiomycota. Ascomycetes were present in all 16 samples and basidiomycetes were found in 13 samples. A total of 31 fungal genera were detected (mean 7; median 7; range 2-14 per sample). Fourteen genera were present in only one sample and four genera were present in only two samples. *Malassezia* was the most commonly detected genus, in 81% of samples (Figure 2.1). *Aspergillus* and *Fusarium* were the second most commonly detected genera, present in 75% of samples, followed by *Penicillium* (69%), *Alternaria* (56%), and *Candida* (50%). Other fungal genera found in 25% or more of samples included *Agaricus*, *Cladosporium*, and *Debaryomyces.*

At the lowest discernible taxonomic level, pyrosequencing and molecular cloning identified at least 57 fungal operational taxonomic units (OTUs). The number of OTUs in an individual sample ranged from 2-25 (mean 12; median 10.5). Twenty-seven fungal OTUs were present in only one sample and 6 OTUs were present in only two samples.

The most abundant OTUs included: *Malassezia restricta* (81%), *Fusarium* cf. *graminearum* (75%), *Aspergillus* cf. *niger* (69%), *Penicillium* cf. *roqueforti* (69%), *Penicillium* cf. *commune* (63%), *Alternaria* spp. (56%), and *Debaryomyces hansenii* (44%). Other ascomycetes identified included *Aspergillus* cf. *oryzae*, *Candida albicans*, *Candida tropicalis*, *Galactomyces* sp., *Ophiocordyceps sinensis*, and *Saccharomyces cerevisiae*. Across all the samples analyzed, no species were common to all individuals. The overall distribution of fungi in 16 fecal samples obtained from healthy human vegetarians by 454 pyrosequencing is shown in Figure 2.2. Alpha diversity of the fungal fecal community was measured with Shannon's (1.70 +/- 0.86) and Simpson's (0.53 +/- 0.25) indices (Figure 2.3). Both indices 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 24 distinct OTUs were detected by pyrosequencing. Rarefaction analysis revealed that for the majority of samples community coverage reached saturation (Figure 2.4). For 75% of the samples, 600 sequences per sample covered the fungal diversity of the gastrointestinal tract in humans.

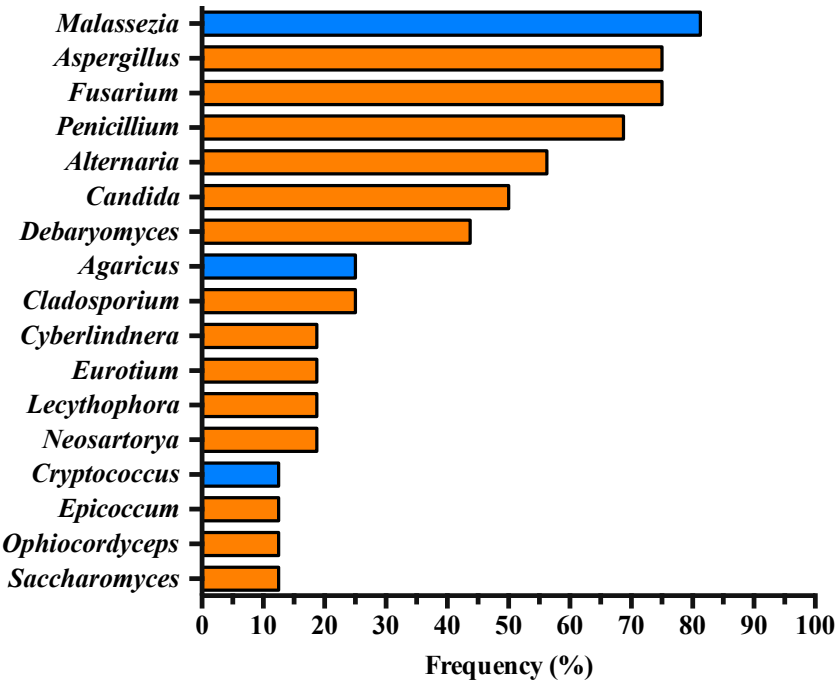

Figure 2.1 Detection frequency of fungal genera in 16 fecal samples using molecular cloning and 454 pyrosequencing. Only genera present in greater than ten percent of samples are shown. Phylum affiliation for each genus is indicated: Ascomycota (orange), Basidiomycota (blue).

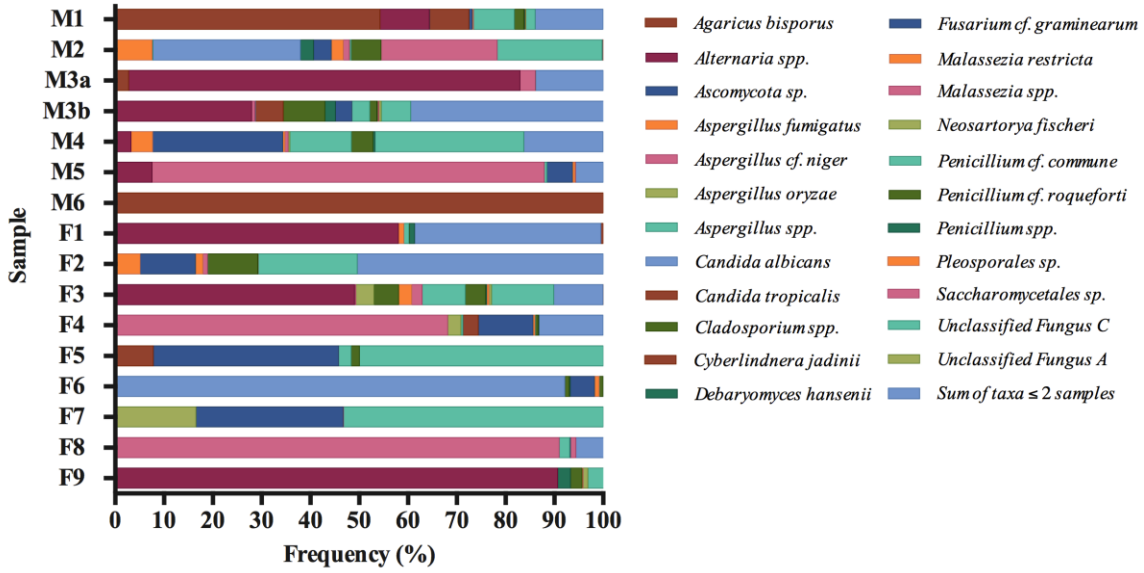

Figure 2.2 Overall distribution of gut fungi. Fungi in more than fifteen percent of fecal samples from healthy individuals with a vegetarian diet obtained by 454 pyrosequencing are shown.

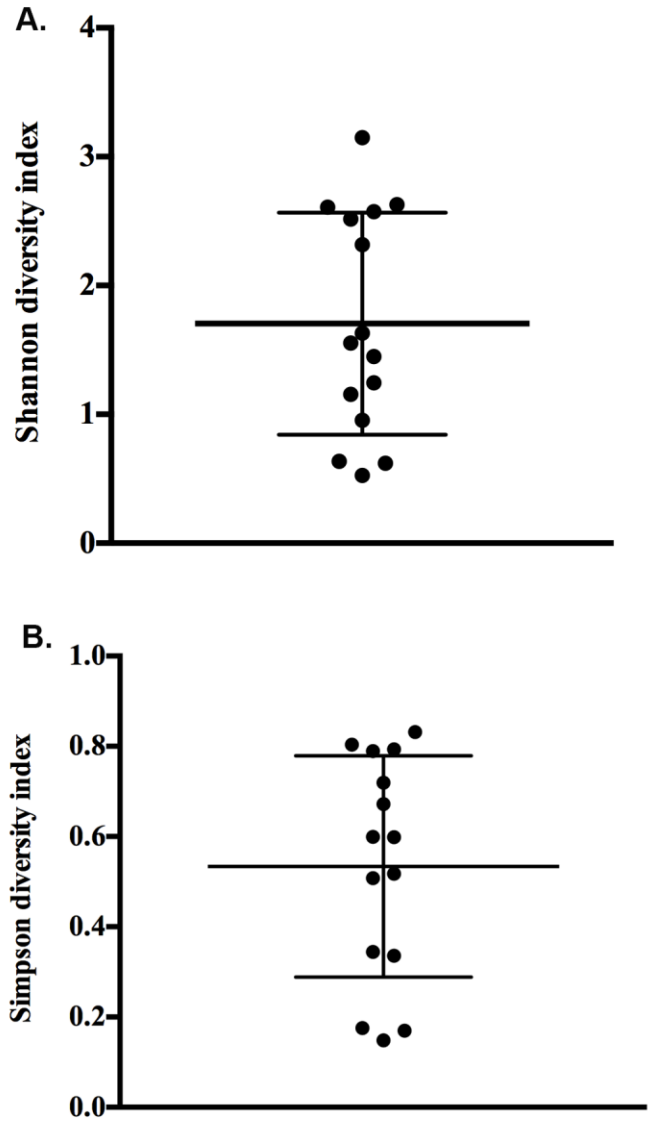

Figure 2.3 Alpha diversity of the fungal fecal community. A) Shannon's diversity index, and B) Simpson's diversity index.

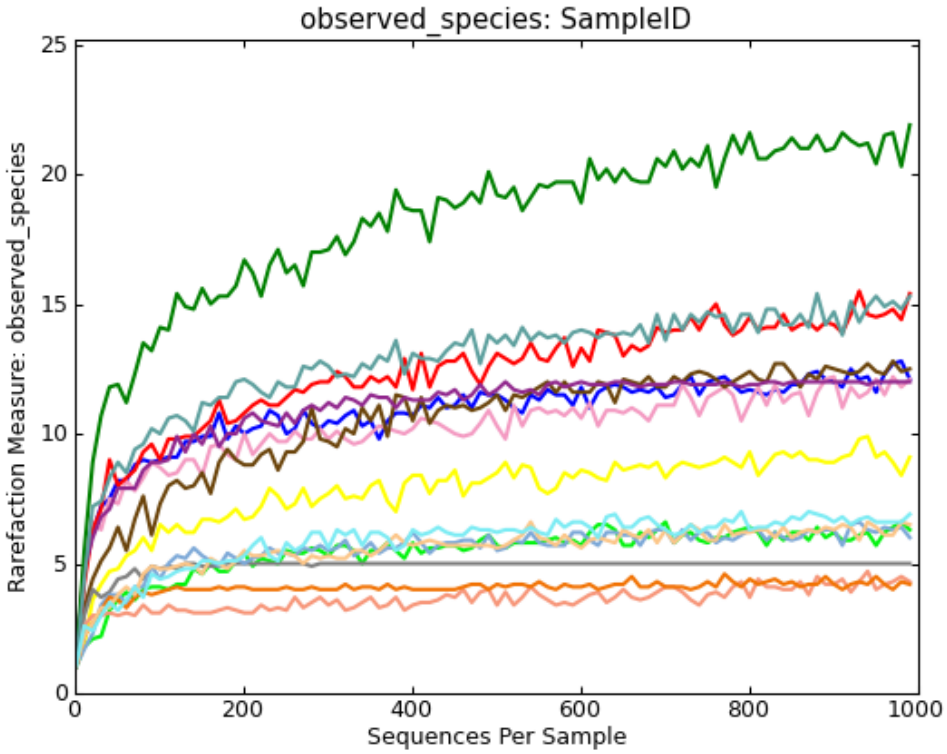

Figure 2.4 Rarefaction analysis of ITS pyrosequencing tags in fecal samples from adults with a vegetarian diet, showing saturation at approximately 600 sequences.

Fusarium PCR

One or more *Fusarium* species affiliated with *F. graminearum* was detected in many samples. Our lab does ongoing research with *Fusarium graminearum* Michigan field isolate PH-1 (FGSC 9075, NRRL 31084). To determine whether the *Fusarium* detected in the samples was due to lab contamination, several *Fusarium*-specific PCR reactions were performed. Galactose oxidase PCR amplifies a 435 bp fragment of *F. graminearum* while not amplifying related species [10], while the randomly-selected OPT18 primers specifically amplify a 470 bp fragment from *F. culmorum* [46]. Only one of the study samples yielded PCR results consistent with strain PH-1, though multiple

bands of other sizes amplified (Figure 2.5), suggesting 1) the presence of *Fusarium* other than *F. graminearum*; 2) that the high level of *Fusarium* detected by all detection methods was not due to contamination with the lab organism; and 3) amplification of non-*Fusarium* species (also present in the DNA) by the PCR primers.

Fungal xTAG ASR Assay

A commercially available fungal ASR panel allows for the detection of 23 species of fungi: 22 human pathogens and *Tremella*, which is included as an internal control. Six of the fungi detected by the sequencing methods have ASRs available: *Aspergillus flavus, A. fumigatus, A. niger, Candida albicans, C. tropicalis,* and *Fusarium*. The ASR assay detected *Fusarium* in 10 samples, *C. albicans* in three, and *C. tropicalis* in two (Table 2.2). Despite *Aspergillus* species being detected in 10 samples by pyrosequencing and cloning, the ASR assay only detected *A. flavus* (= *A. oryzae*) in a single sample. While we performed all 23 ASRs on all samples, the ASR assay did not detect any species that had not also been detected by sequencing.

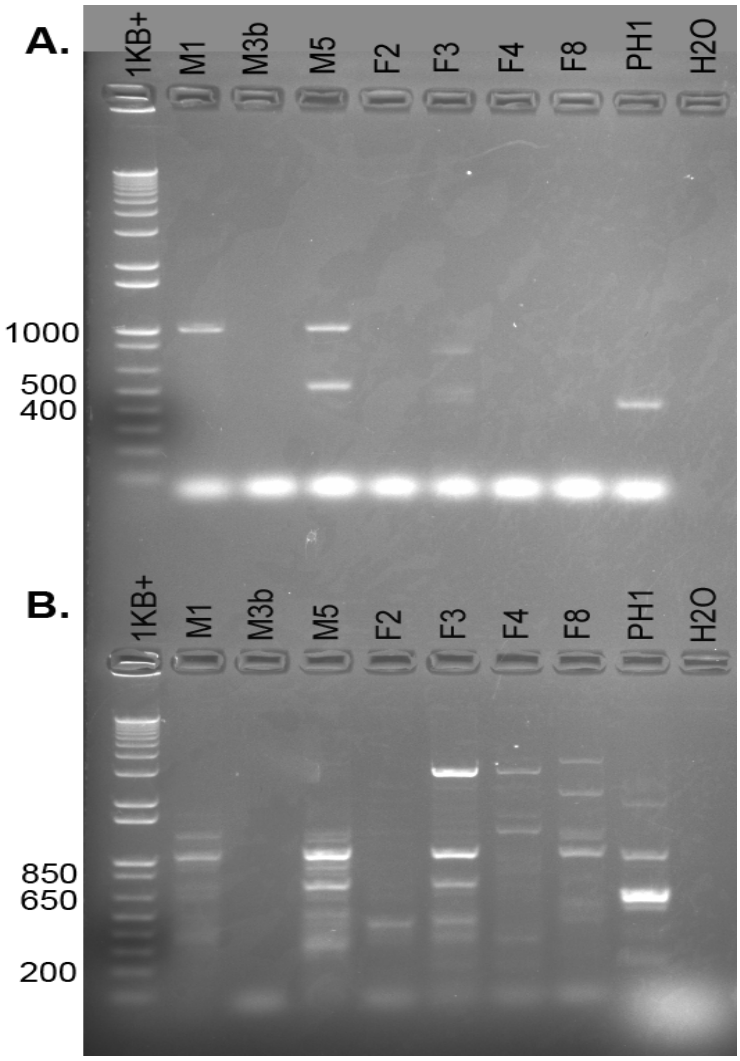

Figure 2.5 A) Galactose oxidase and B) Tri3 PCR products from DNA samples identified as containing *Fusarium*. 1KB+: 1 KB Plus ladder (Invitrogen), PH1: *Fusarium* graminearum strain PH-1 (NRRL 31084). The galactose oxidase primers yield a product of 435 bp for *Fusarium graminearum*, present in PH1 and study samples M5 and (possibly) F3. The multiplexing Tri3 primers yield bands of 243, 610 and 840 bp for 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, and nivalenol, chemotypes, respectively. A band of the appropriate size for 15-acetyldeoxynivalenol is present in PH1, M5 and F3; other bands are visible, but do not correspond with validated trichothecene mycotoxin chemotypes. Clear differences are visible between PH1 and study samples.

Table 2.2 Fungi detected in fecal samples from healthy adult vegetarians, by 454 pyrosequencing, cloning, and Luminex ASR assays.

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.	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
Ascocoryne sarcoides	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
Ascomycota sp.	4	2	0	0	0	0	0	0	3	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. *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 sp.	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0
Candida tropicalis	562	0	0	292	0	0	5	0	0	0	284	181	2	0	0	0
Capnodiales sp.	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

Table 2.2 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 niveoglaucum	0	0	0	0	0	0	0	0	2	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
Mrakia sp.	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0
Mycosphaerellaceae sp.	0	0	0	2	0	0	0	0	0	4	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

Table 2.2 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 spp.	12	0	0	4	7	0	0	628	2	34	14	0	2	0	0	0
Penicillium turbatum	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0
Phialocephala lagerbergii	0	0	0	6	0	0	0	0	0	0	0	0	0	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
Sphaerotheca fusca	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
Torulaspora sp.	0	0	0	13	0	0	0	0	0	0	0	0	0	0	0	0
Trichocomaceae spp.	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 "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
Wallemia muriae	0	0	0	0	0	7	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

Numbers = number of 454 sequences. Clones are indicated by a box around the corresponding cell. ASR data is color-coded: green for present, red for absent, and yellow for equivocal.

Temporal analysis of fungal diversity

The subject who provided fecal samples at two different time points showed little similarity and stability over time (Figure 2.2; M3a & M3b). The fecal sample from the first time point yielded 15,959 hits from five OTUs and the second fecal sample yielded 5,099 hits from 24 OTUs. Only three OTUs were common between both time points (*Agaricus bisporus*, *Alternaria* spp., and *Aspergillus* cf. *niger*). Four genera were detected in the first time point and 14 genera were detected in the second time point.

2.5 DISCUSSION

The gut mycobiome of adult vegetarians

We have demonstrated that the composition of the human gut microbiome is diverse among healthy adult humans with a vegetarian diet, identifying a total of at least 57 different OTUs from two fungal phyla, with a mean of approximately 12 OTUs per subject. On average, the vegetarian gut mycobiome does not appear diminished in diversity compared to the mycobiome of healthy individuals on a conventional diet and is more diverse compared to the mycobiome of an individual with anorexia nervosa [18, 23]. This study shows that the detectable fungal diversity is not limited to only a few fungal genera or species; however, it may be argued that use of the term “mycobiome” for all of these species is misleading, as a majority are allochthonous. In addition, we have also discovered fungal species previously undescribed in the human gut (Table 2.3).

Here we report the most commonly detected gut fungus among vegetarians to be *M. restricta*. Other gut fungi studies detect *M. restricta* and *Malassezia* species in the gut [19, 22]. There is considerable variation among human gut fungal culture-independent

studies regarding which fungal species is the most dominant. *Gloeotinia*/*Paecilomyces*, *Galactomyces*, *Candida*, *Saccharomyces*, and *Penicillium* have all been documented as the most abundant fungi in the GI tract of the studied cohort [7, 23, 33, 41, 45]. Abundance discrepancies among studies may be due to differences in in the individuals' diet, environment, host genetics, age, gender, ethnicity, health status or ultimately the chosen methodology for detection.

Table 2.3 Unique fungal species from the gastrointestinal tract of vegetarians not previously detected in human gut mycobiome studies.

Phylum	Class	Order	Taxa	Ecological Niche
Ascomycota	Pezizomycotina	Botryosphaeriales	*Diplodia mutila*	Causes tree branch cankers; pathogen of apples, pears, grapes
		Chaetothyriales	*Exophiala heteromorpha*	Environmental fungus; causes phaeohyphomycosis infections
		Erysiphales	*Sphaerotheca fusca*	Powdery mildew fungus
		Eurotiales	*Eurotium niveoglaucum*	Widespread environmental osmophile
			Penicillium turbatum	Rotten wood
		Helotiales	*Ascocoryne cylichnium*	Saprobic fungus of hardwoods and conifers
			Ascocoryne sarcoides	Saprobic jelly fungus
			Phialocephala lagerbergii	Wood-inhabiting fungus
		Hypocreales	*Ophiocordyceps sinensis*	Caterpillar fungus; used as herbal supplement
			Trichoderma longibrachiatum	Common house mold; causes allergic fungal sinusitis
		Pleosporales	*Epicoccum nigrum*	Common in environment; plant pathogen cereals and nuts, spoilage
			Leptosphaerulina chartarum	Leaf blight; high-level xylanolytic enzyme production
		Sordariales	*Lecythophora* sp.	Dematiaceous mold; isolated from soil and from plant debris
	Saccharomycotina	Saccharomycetales	*Pichia kudriavzevii*	Spoilage yeast; found in soil, fruits; surface biofilms
Basidiomycota	Agaricomycotina	Agaricales	*Pholiota* sp.	Wood-rotting saprobic mushroom
		Tremellales	*Cryptococcus amylolyticus*	Soil organism
	Ustilaginomycotina	Malasseziales	*Malassezia slooffiae*	Human skin commensal-external ear canal; dermatitis in goats

A previous study in our lab, employing the same DNA extraction, amplification, and 454 pyrosequencing methods on fecal samples from adults approximately from the same geographical location (upper Midwest) on a conventional diet, identified *C. tropicalis* as the most abundant gut fungus, present in 57% of 69 samples, followed by *Galactomyces* in 49%. [21]. *Malassezia, Aspergillus, Fusarium,* and *Penicillium* species were identified, but from a much smaller proportion of samples than in this study; conversely, only one vegetarian sample contained *Galactomyces*. *Candida tropicalis* was detected in 25% of the vegetarian samples, and the genus *Candida* in 50%.

The relationship between diet and the gut mycobiome remains poorly understood. Bacterial microbiome studies have shown that the microbiota of an individual with a vegetarian diet is substantially different and shifted in composition in comparison with a conventional diet [28, 55]. This suggests that fungal abundance may also be a reflection of diet and shifts towards certain populations may occur depending on the chosen dietary habits of the individual. Further research is necessary to determine how diet can induce significant compositional changes in the gut microbiota.

Identification of fungi using ITS

The internal transcribed spacers between the nuclear ribosomal RNA genes have rightly been selected as fungal barcoding sequences, due to their ease of amplification, multicopy nature, and extreme sequence divergence allowing for species-level identification in a majority of cases [47]. However, fungal identification is only as good as the available databases, and not all species have been sequenced. Additionally, some species are so closely related that ITS cannot accurately distinguish between them. In this

study, indistinguishable species include *Alternaria alternata/A. arborescens/A. brassicicola/A. citri/A. mali/A. tenuissima*, *Aspergillus flavus/A. oryzae*, *Aspergillus niger/A. carbonarius/A. foetidus/A. tubingensis*, *Cladosporium cladosporioides/C. bruhnei/C. colocasiae/C. herbarum/C. macrocarpum/C. oxysporum/C. pseudocladosporioides/C. sphaerospermum/C. tenuissimum, Fusarium graminearum/F. culmorum/F. asiaticum*; *Penicillium camemberti/P. allii/P. aurantiogriseum/P. chrysogenum/P. commune/P. dipodomyicola/P. freii/P. glandicola/P. italicum/P. solitum,* and *P. roqueforti/P. carneum/P. paneum*. We refer to these as *Aspergillus* cf. *niger*, *Fusarium* cf. *graminearum,* etc.; it should be understood that these are not precise species identifications.

OTUs of interest

Among the ascomycetes, members of the genus *Aspergillus* were commonly detected among the samples. *Aspergillus* species play important roles in industry, the environment, and human health. *Aspergillus* cf. *oryzae,* found in three samples, encompasses *A. oryzae*, used in soy fermentations, and its wild progenitor, the toxigenic *A. flavus*. *Aspergillus fumigatus,* detected in three samples, is one of the most ubiquitous airborne fungi, and humans will inhale hundreds of *Aspergillus* conidia per day [17, 24]. Consequently, *A. fumigatus* has become the leading cause of aspergillosis and causes fatal invasive infections, resulting in death in 50% of all cases [32]. In addition, our study showed 11 samples of *Aspergillus* cf. *niger*. *A. niger* and related species are capable of causing human pulmonary infections and contaminating a wide range of food commodities [39, 42, 43].

Penicillium species were also commonly detected in the GI tract of our study cohort. *Penicillium* is a large, diverse genus of fungi capable of growing in almost any environment [43]. *Penicillium* species are among the main causes of food spoilage because of their psychotropic characteristic, allowing them to tolerate refrigeration temperatures. Many *Penicillium* species detected in this study might be a result of consuming contaminated foods. In addition, *P. camemberti* and *P. roqueforti* are widely used in the production of mold-ripened cheeses.

The frequency of *Candida* species detected in this study was 50%. *Candida* species, including *C. albicans* and *C. tropicalis*, are commensals of the gastrointestinal, reproductive, and respiratory tracts and skin. *Candida* species are of substantial clinical significance because of their ability to become opportunistic pathogens responsible for a spectrum of diseases, ranging from mucosal, cutaneous, invasive infections when host resistance is decreased [27]. *Candida* species are considered to be the most widely distributed and dominant fungi in the human gut mycobiome. In healthy individuals, carriage of *Candida albicans* is estimated to be 30-60% [40].

Five species of *Malassezia* were identified from the gut of our cohort: *M. globosa*, *M. pachydermatis*, *M. restricta*, *M. slooffiae*, and *M. sympodialis*. *Malassezia* species are lipophilic yeasts found naturally on the skin and are associated with a number of skin diseases and dandruff [20]. *Malassezia* has been commonly detected in fecal samples [7, 18, 22, 33], however, here we report for the first time detection of *M. slooffiae*. The detection of *Malassezia* species across gut mycobiome studies may suggest a role for its presence in the gut due to the favorable lipid-rich environment provided by the host's

diet. Thus, more research is needed to determine if *Malassezia* species are true gut commensals or merely contaminants.

The introduction of unequivocal food-associated fungi, including dietary mushrooms, is not uncommon in the human distal gut [9, 12]. The basidiomycete, *Agaricus bisporus* (white button mushroom, portabello, crimini) was detected in four subjects. *A. bisporus* is the most commonly cultivated edible mushroom worldwide [31] and the most widely consumed mushroom in Western countries; its detection in feces is almost certainly due to consumption. The yeasts *Saccharomyces cerevisiae* and *Debaryomyces hansenii,* are used in the production of fermented foods. *Ophiocordyceps,* detected in two individuals, is used as a dietary herbal supplement for a variety of therapeutic practices.

Other fungi detected in this study, such as *Epicoccum nigrum, Diplodia mutila, Alternaria* spp., *Podosphaera xanthii,* and *Fusarium* cf. *graminearum,* are known plant pathogens. These fungi may be present in the intestinal tract due to the consumption of contaminated foods or consumables requiring the use of fungi for production. Fungi for which this study provides the first report in the human gut are shown in Table 2.3, together with probable sources.

Transient nature of intestinal fungi

Our results demonstrate that many fungi present in the gut are of allochthonous origin due to their known presence in the environment or foodstuffs. We cannot conclude that all of the fungi reported in this study are autochthonous organisms and will remain in the gut over time; indeed, the ecology (when known) of the organisms (Table 2.3)

suggests a plausible environmental source for most, while several grow poorly if at all at human body temperatures. Bacterial gut microbiome studies show bacteria to be temporally stable over time [36, 53]. One study documented the transient nature of foodborne fungal microbes in the distal gut by sequencing the ITS genes from food and fecal samples [9]. A previous study of the micro-eukaryotic diversity in the gut demonstrated a temporally stable fungal gut community using a fungal internal transcribed spacer-based analysis [45]; conversely, Hallen-Adams et al. [21] observed high variability and low persistence of gut fungi from 24 individuals' samples at two time points. The subject in the current study who was sampled at two different time points showed little similarity over time. The paucity of data from multiple study subjects limits the ability to draw broad conclusions of fungal stability in the gut over time.

Comparison of methods

Fungi are increasingly being recognized as an important component of the gut microbiota. Over the last two decades, cloning and Sanger sequencing have been the foundation for microbial sequencing [16]. 454 pyrosequencing represents a method for rapidly characterizing microbial communities in a high-throughput, efficient, relatively rapid and cost-effective manner [37]. In the current study, we used a combination of two different sequencing methods to identify the fungal composition in the gut of humans with a vegetarian diet. In some instances one method and not the other detected fungal sequences. 454 pyrosequencing identified 41 OTUs that cloning failed to detect. That pyrosequencing should detect more OTUs than cloning was not unanticipated; pyrosequencing generated several thousands of sequences per sample, while 10 clones

from each cloning reaction were selected for further analysis, and only those with distinct restriction patterns were sequenced. Tedersoo et al. [52] reported that by combining Sanger sequencing and 454 pyrosequencing they were able to identify 141 species of ectomycorrhizal fungi in root samples. Approximately 66% of them were common between the two methodologies, and they concluded considerable methodology biases towards certain lineages existed. These two methods, especially when used in conjunction with one another, represent potentially useful methods to characterize the mycobiome due to the low abundance and diversity of gut fungi.

Of more interest is the correlation between the two methods; 55% of the OTUs identified by cloning were the same OTUs identified by pyrosequencing, and in 25% of the samples, the OTUs pyrosequencing identified as highest abundance (Figure 2.2, Table 2.2) were present in the clone sequences. Several species detected by cloning were not among the tops hits identified by pyrosequencing in their respective sample. For example, cloning was able to identify *Cyberlindnera jadinii* in a sample that only had six pyrosequencing hits. Cloning was able to identify six OTUs that had less than 20 hits in pyrosequencing, while 16 OTUs identified by cloning never appeared in the pyrosequencing data of that sample.

Of the six taxa found in our samples for which ASRs were available, the ASRs showed the highest detection of *Fusarium* (10 samples), which also represented the most commonly detected OTU in the pyrosequencing (11 samples) data. In 3 samples *Fusarium* was detected by all three methods; in 5 samples by ASRs and pyrosequencing; in 3 samples by cloning and pyrosequencing; and in 1 sample by cloning alone. In 1 sample, *C. albicans* was detected by all three methods, in 2 samples by ASRs alone, and

in 2 samples by pyrosequencing alone. The ASR assay detected *C. tropicalis* from 2 out of 4 samples in which it was detected by pyrosequencing.

The *Aspergillus fumigatus* and *A. niger* ASRs failed to detect anything, despite high representation of these OTUs in the sequencing data (3 samples with *A. fumigatus* and 6 with *A.* cf. *niger* by pyrosequencing, and 10 samples with *A.* cf. *niger* clones). 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 and colleagues [2], evaluating the ASR assay on clinical samples, report that certain isolates they had identified as *A. niger* were not detected by the *A. niger* ASR and were subsequently determined to be *A. tubingensis*; additionally, Babady and colleagues [2] report suboptimal detection of *A. fumigatus* and *A. flavus.*

All methods discussed 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 [52]. A serious limitation in microbiome studies in general is the difficulty in obtaining reliable measures of OTU abundance within sample, let alone making between-sample comparisons. It is tempting to conclude that a higher number of 454 sequences or clones, or higher median fluorescence in the case of ASRs, equates to greater abundance, but in truth the pool may be influenced by PCR biases; the limitations of each technology; and the multicopy nature of the nuclear ribosomal region, which differs substantially in copy number between organisms (180 copies in the sequenced strain of *Fusarium graminearum* vs. 55 copies in *Candida albicans*; Liti and colleagues [34] report copy numbers ranging from 54-511 in different strains of *Saccharomyces cerevisiae*). Amend, Seifert and Bruns [1]

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 quantitative data; however, alone and in combination, a snapshot of gut fungal diversity is produced.

In conclusion, the human gut mycobiome is diverse and dynamic. Many fungi are allochthonous due to the high level of exposure to environment or food-associated fungi. One must be cautious in drawing conclusions about the role of fungi, as a majority are "passing through," and their contribution to the gut ecology is likely to be minimal. The different methodologies displayed differing strengths, weaknesses and biases: notably, the ASR assay is limited to detecting species for which there are primers, cloning is limited by the number of clones selected for sequencing, and all three methods are subject to PCR biases. As gut mycologists continue to expand gut mycobiome studies we will begin to understand their beneficial and/or detrimental roles in human health.

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CHAPTER 3

EPIDEMIOLOGICAL INVESTIGATION OF *CANDIDA* SPECIES CAUSING BLOODSTREAM INFECTION IN PEDIATRIC SMALL BOWEL TRANSPLANT RECIPIENTS

3.1 ABSTRACT

Small bowel transplantation (SBT) is a life-saving medical procedure for patients with short bowel syndrome; however, these patients remain at high risk for bloodstream infections. Gastrointestinal carriage and exogenous acquisition of *Candida* are potential sources of infection. This research aims to characterize the SBT recipient gut microbiota over time following transplant and investigate the epidemiology of candidemia in seven pediatric SBT recipients at the University of Nebraska Medical Center. *Candida* species from the recipient ileum and bloodstream were identified by ITS sequence and identified to strain by multilocus sequence typing and randomly amplified polymorphic DNA. Twenty-one ileostomy samples harbored at least one *Candida* species (*C. albicans*, *C. glabrata*, *C. lusitaniae*, *C. parapsilosis*, *C. tropicalis*). Bloodstream infections were caused by *C. parapsilosis* (3), *C. albicans* (2), *C. glabrata* (1), *C. orthopsilosis* (1), and *Wickerhamomyces anomalus* (1). Two transplant patients were confirmed to harbor the same strain of *Candida* in the ileum and bloodstream while a third patient had distinct ileal and bloodstream *Candida* strains. Results are congruent with at least two infections arising from the recipient GI tract (*C. albicans*, *C. glabrata*) and one infection arising from *Candida* other than that colonizing the recipient GI tract (*C. parapsilosis*). Results also suggest two patients were infected by the same strain of *C. parapsilosis*. Antifungal susceptibility testing revealed that all isolates, with the exception of three, showed susceptibility to all the antifungals tested. One *C. glabrata* isolate showed multidrug resistance to itraconazole, amphotericin B, and posaconazole and was classified as susceptible dose-dependent to fluconazole and voriconazole.

3.2 INTRODUCTION

Short bowel syndrome (SBS) is a fatal gastrointestinal disorder characterized by the inability to absorb enough nutrients and fluids to sustain life [86]. The most common cause of pediatric SBS is extensive loss of the small bowel due to congenital birth defects (e.g. midgut volvulus, gastroschisis, intestinal atresia) or surgical removal to treat intestinal diseases (e.g. necrotizing enterocolitis). Pediatric SBS is defined as the loss of ≥70% of small intestinal length (either congenital or acquired), equating to no more than 75 cm of the small intestine remaining in a full-term neonate [69]. One population-based estimate of neonatal SBS reports 24.5 cases per 100,000 live births [91] and mortality rates of SBS are consistently reported at 20-40% [78, 85, 91].

Despite high mortality rates, major advances have been made to increase the lifespan of those suffering from SBS. Introduction of parenteral nutrition (PN) is important for survival and has transformed the management of pediatric SBS [75]. PN bypasses gastric digestion and provides a complete source of nutrients, vitamins, and electrolytes intravenously. PN support allows time for intestinal adaptation of the residual bowel to occur [66]. However, the process of intestinal adaptation is unpredictable and pediatric patients receiving long-term PN often face life-threatening complications, including PN-associated liver disease and catheter-related sepsis.

Small bowel transplantation (SBT) is considered a last-resort treatment option for pediatric patients with extreme SBS or those suffering life-threatening complications from PN [42]. Since 1990, when the first successful intestinal transplant was documented [30], intestinal transplants have been successfully employed and survival rates have steadily increased [86]. Small bowel transplants have a one-year graft and recipient

survival rate of 62-86% in pediatric patients [58]. Additionally, SBT is a cost-effective treatment option. Beyond the first year, average costs associated with SBT can be less than the average costs of prolonged PN [33, 75, 80].

However, two important factors exist when patients are selected to receive a SBT: 1) increased immunosuppressive therapy to preclude immune rejection of the transplanted tissue and 2) microbial colonization of the recipient and/or donor allograft [86]. SBT recipients have a higher incidence of allograft rejection than other solid-organ transplants, due to the high immunogenicity of the bowel, and therefore require more potent immunosuppression therapy [64]. Breakdown and loss of the mucosal barrier during episodes of allograft rejection or breach of the gastrointestinal tract during surgery can translocate enteric organisms from the recipient or donor bowel into circulation, consequently causing bloodstream infections. Bloodstream infections are common complications after intestinal transplant and represent a major cause of morbidity and mortality among SBT recipients. In one study, 70% of pediatric SBT recipients developed a bloodstream infection within one year after transplantation [24]. *Enterococcus*, *Klebsiella*, and *Candida* were the main causes of infection. Prevention of translocation-associated infections remains a significant challenge, despite improvements in antirejection and antimicrobial therapy.

Candida species are the fourth most common cause of hospital-acquired bloodstream infections in the United States [95]. *Candida* species including *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. metapsilosis*, *C. parapsilosis*, and *C. tropicalis* are normal, asymptomatic residents of the gastrointestinal and reproductive tract and skin of most individuals. In healthy individuals, carriage of *C. albicans* is estimated to be 30-

60% [52]. However, *Candida* species can also act as opportunistic pathogens that are capable of infecting a broad range of body sites when host resistance is decreased. *Candida* species are responsible for a spectrum of diseases, including mucosal, cutaneous, and invasive infections.

Of particular concern is when *Candida* crosses the epithelial barrier and gains access to the bloodstream, causing candidemia and potentially spreading throughout the body, causing infections of other organs termed invasive candidiasis (IC). The overall incidence of IC is estimated at 63,000 cases/year in the United States [63]. This life-threatening infection is responsible for mortality rates reaching 49% [32] and medical care costs of an estimated $1.7 billion per year [94]. IC continues to be a persistent public health problem and infections due to *Candida* are the most common and fatal fungal bloodstream infections among pediatric SBT recipients [23].

Patients receiving solid organ transplantations present a unique combination of risk factors that predispose them to develop fungal bloodstream infections with *Candida*. In particular, pediatric SBT recipients constitute a population with an increased incidence for IC, with up to 25% of recipients developing invasive candidiasis [23]. Risk factors for candidemia among SBT recipients include immunosuppressive therapies, broad-spectrum antibiotics, use of central venous catheters, invasive abdominal surgery, environmental hospital exposures, and repeated endoscopy surveillance [25, 71, 76]. In addition, these patients experience disruption of the gastrointestinal barrier during surgery and the introduction of a foreign microbiota with the donor allograft.

The *Candida* implicated in candidemia may be of endogenous or exogenous origin [31]. Candidemia is often believed to derive from an endogenous source, as many

individuals harbor *Candida* species in their gastrointestinal tract. The carriage of *Candida* in the donor or remnant recipient organ may act as a reservoir for pathogenic fungi and an endogenous source of IC [76]. Donor organs can also carry commensal *Candida* or may become infected during hospitalization of the donor or during procurement and preservation of the organ [31, 39]. Transplantation of an infected organ can serve as another possible source of infection.

There is now increasing evidence for exogenous sources of *Candida* originating from the hospital environment, contaminated implanted medical devices, or from the skin. Infection with *C. parapsilosis* is often linked to an exogenous source because of its ubiquity in the environment and its presence on the skin of healthy humans. Carriage of *C. parapsilosis* on the hands of health care workers has long been recognized [38] and plays an important role in the transmission of opportunistic yeasts to hospitalized patients. Horizontal spread of *C. parapsilosis* infections from nurses to infants has been documented [37, 47]. In addition, nosocomial infections with *C. parapsilosis* are related to the contamination of indwelling central venous catheters used for the administration of PN [55]. The ability of *C. parapsilosis* to produce biofilms on catheter instruments is a common cause of IC because it gives the yeast direct access into the bloodstream upon insertion [13, 45].

Molecular typing methods have proven to be valuable to analyze strain relatedness in order to perform epidemiological assessment of candidiasis outbreaks. Two classes of typing methods exist for molecular characterization of *Candida* species—non-DNA-based and DNA-based [73]. The first class, non-DNA-based, includes strain typing that indirectly assays the genotype by multilocus enzyme electrophoresis (MLEE).

MLEE is a method that assesses and compares multiple core metabolic enzyme fingerprints by analyzing their electrophoretic mobilities on a gel. While MLEE has shown to be highly discriminatory and reproducible, this method assays the genome indirectly, may take several days to complete, and is difficult to master [10, 15, 73].

The second class of molecular typing methods for *Candida* species is DNA-based. Conventional DNA-based techniques used to genotype *Candida* in epidemiological studies include electrophoretic karyotyping with pulsed-field gel electrophoresis (PFGE), restriction enzyme analysis (REA) with Southern blotting, random amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP) [3, 5, 9, 49, 65]. These methods have the potential to discriminate at the strain level, but potential drawbacks associated with these methods include low reproducibility, low discriminatory power, and the need for trained personnel [73].

The conventional DNA-based techniques described above allow for easy strain differentiation within a laboratory; however, comparison of data between laboratories is nearly impossible. Therefore, exact DNA-based methods that are more unambiguous and reproducible, such as multilocus sequence typing (MLST), offer the best way at present to strain-type *Candida* [11]. MLST has been successfully applied to several *Candida* species including *C. albicans*, *C. glabrata*, *C. krusei*, and *C. tropicalis*, but not *C. parapsilosis* [12, 22, 40, 81, 82]. MLST protocols are based on the analysis of nucleotide sequence polymorphisms in 400- to 500-bp internal fragments of 6-8 independent housekeeping genes. MLST is a robust typing technique because of the highly reproducible nature of the data generated and allows for laboratories to easily compare and exchange data. Internet databases have been constructed for a number of organisms,

including multiple *Candida* species, and allow for the sharing of global epidemiological data (http://www.mlst.net/databases/). Many *Candida* epidemiological studies using MLST have been reported and show the method to be highly discriminating and stable [70, 90]. Ultimately, it is recommended to employ at least two independent techniques to verify the results for a set of given isolates [87].

The prevention and treatment of candidemia among SBT recipients is controversial [31]. Antifungal prophylaxis is appealing for recipients of organ transplants due to the high mortality rate of invasive fungal bloodstream infections post-transplant. However, extensive use of antifungal prophylaxis selects for resistant *Candida* species. The recent emergence of azole-resistant species (*C. glabrata* and *C. krusei*) is likely due to widespread use of fluconazole and other antifungal agents [77]. Additionally, a shift towards non-*albicans Candida* species causing fungemia has been observed [79].

Fluconazole is routinely used for antifungal prophylaxis; however, choice of drug, who should receive it, and when to initiate/discontinue prophylaxis is not well understood, therefore, universal use cannot be justified to all transplant recipients [77]. Antifungal prophylaxis should ideally be based on institutional trends. Established candidiasis infections are routinely treated with amphotericin B, azoles, and echinocandins [59]. Problems associated with antifungal treatments include potential toxicity, drug interactions with immunosuppressant agents, high costs, and patient compliance. Also, poor compliance with current antifungal management guidelines by transplant physicians has been documented [53].

Small bowel transplantation is a life-preserving treatment for pediatric short bowel syndrome patients facing intestinal failure or life-threatening complications from

parenteral nutrition. Recipients of SBT represent a population of patients with a combination of unique risk factors, making them highly susceptible to fungal bloodstream infections, mainly caused by *Candida* species. Thus, it is essential to identify exogenous or endogenous *Candida* yeasts implicated in infection and understand their origin in order to improve survival rates and prevent fungal infections for recipients of small bowel transplants. Clinicians must understand the epidemiology of *Candida* in their institution and know which *Candida* species are implicated in infection in order to select appropriate treatment options for their patients.

To our knowledge, no studies have investigated the intestinal microbiota or origin of *Candida* causing candidemia in SBT recipients. In the present work, we defined the gut mycobiota of SBT recipients and genotyped ileum and bloodstream isolates to infer origin of infection. We also assessed isolates to determine if patients shared the same strains of *Candida*. The data obtained in this study will contribute to the knowledge base of *Candida* epidemiology and infections acquired by SBT recipients at the University of Nebraska Medical Center.

3.3 MATERIALS AND METHODS

Sample collection

A total of seven pediatric short bowel syndrome patients who received small bowel transplantations and later developed candidemia at the University of Nebraska Medical Center were included in this study. The patient cohort comprised 3 females and 4 males with a mean age of 23.1 months (median: 20 months; range: 16-37 months). The University of Nebraska Medical Center is a premier facility for SBT, conducting ~20

transplants per year. Ileostomy and blood samples were collected from recipients under UNMC Institutional Review Board protocol #417-02, and informed consent was received from the caregivers of all patients. Candidemia was diagnosed on the basis of symptoms and a positive blood culture identified using the API 20C test (BioMérieux, Marcy l'Etoile, France). Ileostomy samples prior to and concurrent with infection (up to nine) were collected post-transplantation during hospitalization and stored at -80°C until processing (Table 3.1). Culture positive bloodstream isolates from each recipient following infection were isolated and maintained on Sabouraud dextrose agar.

Extraction of DNA from Ileostomy Samples

DNA was extracted from ileostomy samples following the protocol of Oh and colleagues [57]. Briefly, 300 µl of ileal effluent was centrifuged at 10,000 x g for 5 minutes. Cell pellets were resuspended with 750 µl lysis buffer (200 mM NaCl, 100 mM Tris [pH=8.0], 20 mM EDTA, 20 mg/mL lysozyme) and transferred to a 2 ml tube containing 300 mg of 0.1-mm zirconium beads (BioSpec Products). Cell suspensions were incubated at 37°C for 45 minutes and 85 µl of 10% sodium dodecyl sulfate and 40 µl of proteinase K (15 mg/ml) was added. Samples were then incubated for 45 minutes at 60°C. After the second incubation, 500 µl of phenol-chloroform-isoamyl alcohol (25:24:1) was added, and the samples were bead beaten at maximum speed for 2 minutes and immediately cooled on ice. Samples were centrifuged at 13,500 x *g* for 5 minutes and the top aqueous layer was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and twice with chloroform-isoamyl alcohol. DNA was precipitated by the addition of 2.5 volumes of 100% ethanol, 1/10 volume of 3M sodium acetate (pH 5.2)

and 50 µg/ml GlycoBlue (Ambion) at -20°C overnight. The samples were centrifuged at full speed for 25 minutes and the resulting DNA pellets were washed with 70% ethanol, air-dried, and dissolved in 50 µl sterile water.

Isolation of yeasts from ileostomy samples

To obtain pure culture isolates from ileostomy samples, approximately 250 µl of ileal effluent was grown overnight at 37°C with shaking (200 rpm) in yeast nitrogen broth (YNB; 6.7 g/L yeast nitrogen base, 10 g/L glucose, 0.76 g L-asparagine, 50 mg/L chloramphenicol, 20 mg/L gentamicin sulfate, sterilized by filtration) and yeast extract peptone dextrose broth (YPD; 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 50 mg/L chloramphenicol, pH 5.6). Volumes of 100 µl were plated after 24, 48, and 72 hours on YPD (supplemented with 50 mg/L chloramphenicol), CHROMagar Candida (Paris, France), potato dextrose agar (PDA, Oxoid; supplemented with 50 mg/L chloramphenicol), and dichloran rose bengal chloramphenicol (DRBC; 10 g/L glucose, 5 g/L peptone, 1 g/L monopotassium phosphate, 0.5 g/L magnesium sulfate, rose bengal 0.025 g/L, dichloran 0.002 g/L chloramphenicol 0.10 g/L). Plates were incubated at 37°C and monitored daily for 2 weeks. Multiple colonies from each plate were further isolated on the basis of color and morphology and maintained at 4°C.

Table 3.1 Outline of patients and sample collection.

Patient	Age	Sex	Sample Name	Date of Collection	Source of Sample
P32	28 mo.	F	32_I_1	18 Mar 2005	Ileostomy
			32_B_1	28 Mar 2005	Blood
P33	37 mo.	F	33_I_1	15 Aug 2005	Ileostomy
			33_I_2	29 Sept 2005	Ileostomy
			33_B_1	7 Oct 2005	Blood
			33_I_3	24 Mar 2006	Ileostomy
P39	23 mo.	M	39_I_1	7 June 2004	Ileostomy
			39_B_1	8 Oct 2004	Blood
			39_I_2	22 Oct 2004	Ileostomy
P47	20 mo.	F	47_I_1	13 Sept 2004	Ileostomy
			47_I_2	24 Sept 2004	Ileostomy
			47_I_3	28 Sept 2004	Ileostomy
			47_B_1	30 Sept 2004	Blood
			47_I_4	1 Oct 2004	Ileostomy
			47_I_5	5 Oct 2004	Ileostomy
			47_B_2	6 Oct 2004	Blood
			47_I_6	12 Oct 2004	Ileostomy
			47_I_7	19 Oct 2004	Ileostomy
			47_I_8	27 Oct 2004	Ileostomy
			47_I_9	1 Nov 2004	Ileostomy
P62	16 mo.	M	62_I_1	14 July 2005	Ileostomy
			62_I_2	22 July 2005	Ileostomy
			62_B_1	30 July 2005	Blood
			62_I_3	2 Aug 2005	Ileostomy
			62_I_4	2 Sept 2005	Ileostomy
			62_I_5	7 Oct 2005	Ileostomy
P74	20 mo.	M	74_I_1	22 June 2006	Ileostomy
			74_I_2	12 July 2006	Ileostomy
			74_B_1	20 July 2006	Blood
			74_I_3	3 Oct 2006	Ileostomy
			74_I_4	27 Oct 2006	Ileostomy
			74_B_2	7 Nov 2006	Blood
			74_I_5	15 Nov 2006	Ileostomy
			74_I_6	17 Nov 2006	Ileostomy
P88	18 mo.	M	88_B_1	3 Jan 2007	Blood
			88_I_1	4 Jan 2007	Ileostomy
			88_I_2	16 Jan 2007	Ileostomy

Extraction of DNA from pure culture bloodstream and ileal Isolates

DNA was extracted from ileal and bloodstream isolates following the protocol of Harju et al. [34]. Yeast colonies were grown overnight at 37°C in YPD medium (1% yeast extract, 2% peptone, 2% glucose) with 200 rpm shaking and pelleted by centrifugation at 20,000 x *g* for 2 minutes. Pellets were resuspended in 200 µl of Harju lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]). The samples were frozen at -80°C for 10 minutes and then immersed into a 95°C water bath for 1 minute. The freeze/thaw process was repeated. Sterile pestles were used to disrupt cell walls, and then samples were vortexed vigorously for 30 seconds. After the addition of 200 µl of chloroform and 2 minutes of vortexing, samples were centrifuged at 20,000 x *g* for 3 minutes. The upper aqueous layer was transferred into a microcentrifuge tube containing 400 µl of ice-cold 100% ethanol, mixed with inversion, and incubated at -20°C for at least 10 minutes. The samples were centrifuged again at 20,000 x *g* for 5 minutes. DNA pellets were washed with 500 µl ice-cold 70% ethanol followed by air-drying at room temperature. DNA was dissolved in 50 µl sterile water.

Genomic amplification

The extracted DNA from each ileostomy sample and isolate was selectively amplified by PCR using fungal-specific primers ITS1-F (5'-CTTGGTCATTTAGA GGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [27, 93]. PCR reaction mixtures consisted of 1.25 U of TaKaRa Ex Taq (Shiga, Japan), 5 µl of 10X Ex Taq buffer, 4 µl of dNTP mixture (2.5 mM each), 20 pmol of each primer, and 1 µl of

genomic DNA. Amplifications were performed with the following PCR cycling conditions: initial denaturation at 94°C for 4 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 1 minute, elongation at 72°C for 1.5 minutes and a final extension step at 72°C for 10 minutes. PCR amplicons were separated using a 0.7% agarose gel in 1 x TAE buffer and visualized with 0.5 μg/ml ethidium bromide staining.

Cloning and sequencing

PCR products from ileostomy samples were cloned using the pGEM® -T Easy Vector System Kit (Promega, Lyon, France) following the manufacturers instructions to obtain a profile of the intestinal fungi. For each sample, 25 colonies negative for β-galactosidase activity were chosen and those with distinct *Eco*R1 patterns were sequenced on an ABI 3730xl platform at Michigan State University's Research Technology Support Facility (East Lansing, MI). Fungal sequences were identified by sequence homology using nucleotide BLAST against the UNITE curated fungal ITS sequence database [44] and the curated FUNCBS database, as accessed through the Centraalbureau voor Schimmelcultures (www.cbs.knaw.nl/Collections/BioloMICSSequences.aspx?file=all; accessed September 2014).

Multilocus sequence typing

All *C. albicans* and *C. glabrata* ileostomy and bloodstream isolates were typed by MLST as described previously [12, 22] (Table 3.2). MLST protocols are not currently

available for *C. parapsilosis.* For *C. albicans*, seven housekeeping gene fragments were sequenced (*AAT1a*, *ACC1*, *ADP1*, *MPIb*, *SYA1*, *VPS13*, *ZWF1b*). For *C. glabrata*, six housekeeping gene fragments were sequenced (*FKS*, *LEU2*, *NMT1*, *TRP1*, *UGP1*, *URA3*). Each gene fragment was amplified using 50 µl PCR reactions containing 100 ng of genomic DNA, 1.25 U of TaKaRa Ex Taq (Shiga, Japan), 5 µl of 10X Ex Taq buffer, 4 µl of dNTP mixture (2.5 mM each), and 25 pmol of each primer (Table 3.2). PCR conditions for *C. albicans* MLST reactions were as follows: 94°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute, relevant annealing temperature (Table 3.2) for 1 minute, and 72°C for 1 minute, with a final extension period of 10 minutes at 72°C. PCR conditions for *C. glabrata* MLST reactions were as follows: 94°C for 7 minutes, followed by 30 cycles of 94°C for 1 minute, relevant annealing temperature (Table 3.2), and 74°C for 1 minute, with a final extension period of 10 minutes at 74°C.

All PCR products were purified using the Wizard SV Gel and PCR Clean-up Kit (Promega, Madison, WI). Following cleanup, amplicons were sequenced in both directions using the same primers used for PCR. Sequences were aligned and assembled using ClustalW implemented in BioEdit Sequence Alignment Editor version 7.3.2. For *C. albicans*, heterozygosities were assessed by visual inspection of the chromatograms and designated using the International Union of Pure and Applied Chemistry nomenclature [12]. Sequences were entered into the MLST databases (http://pubmlst.org/calbicans/ & http://cglabrata.mlst.net/) and assigned an allele profile for each locus and an overall sequence type (ST).

Table 3.2 *C. albicans* and *C. glabrata* MLST schemes.

Locus	Gene product	Primer sequence (5'-3')	Annealing Temp (°C)	No. bp sequenced
C. albicans				
AAT1a	Asparate aminotransferase	F: ACTCAAGCTAGATTTTTGGC	52	373
		R: CAGCAACATGATTAGCCC		
ACC1	Acetyl-coenzyme A carboxylase	F: GCAAGAGAAATTTTAATTCAATG	55	407
		R: TTCATCAACATCATCCAAGTG		
ADP1	ATP-dependent permease	F: GAGCCAAGTATGAATGATTTG	55	443
		R: TTGATCAACAAACCCGATAAT		
MPIb	Mannose phosphate isomerase	F: ACCAGAAATGGCCATTGC	52	375
		R: GCAGCCATGCATTCAATTAT		
SYA1	Alanyl-RNA synthetase	F: AGAAGAATTGTTGCTGTTACTG	55	391
		R: GTTACCTTTACCACCAGCTTT		
VPS13	Vacuolar protein sorting protein	F: TCGTTGAGAGATATTCGACTT	55	403
		R: ACGGATGGATCTCCAGTCC		
ZWF1b	Glucose-6-phosphate dehydrogenase	F: GTTTCATTTGATCCTGAAGC	52	491
		R: GCCATTGATAAGTACCTGGAT		
C. glabrata				
FKS	1,3-Beta-glucan synthase	F: GTCAAATGCCACAACAACAACCT	55	589
		R: GCACTTCAGCAGCGTCTTCAG		
LEU2	3-Isoprpylmalate dehydrogenase	F: TTTCTTGTATCCTCCCATTGTTCA	54	512
		R: ATAGGTAAAGGTGGGTTGTGTTGC		
NMT1	Myristoyl-CoA, protein *N*-myristoyltransferase CoA	F: GCCGGTGTGGTGTTGCCTGCTC	59	607
		R: CGTTACTGCGGTGCTCGGTGTCG		
TRP1	Phosphoribosyl-anthranilate isomerase	F: AATTGTTCCAGCGTTTTTGT	50	419
		R: GACCAGTCCAGCTCTTTCAC		
UGP1	UTP-glucose-1-phosphate uridylyltransferase	F: TTTCAACACCGACAAGGACACAGA	57	616
		R: TCGGACTTCACTAGCAGCAAATCA		
URA3	Orotidine-5'phosphate decarboxylase	F: AGCGAATTGTTGAAGTTGGTTGA	53	602
		R: AATTCGGTTGTAAGATGATGTTGC		

Random amplified polymorphic DNA

C. albicans, *C. glabrata*, and *C. parapsilosis* isolates were also typed by RAPD using the primer Oligo 2 (5'-TCACGATGCA-3') as described previously [9]. *C. parapsilosis* isolates were further typed with the primer OPE-04 (5'-GTGACATG-3') [8]. Additional control *Candida* strains were added to each RAPD reaction to test discriminatory power. Amplification reactions were performed as described above for PCR with 10 ng of genomic DNA. PCR was performed as follows: 94°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 36°C for 30 seconds, and 72°C for 2 minutes, with a final extension period of 10 minutes at 72°C. PCR amplicons were separated using a 1.5% agarose gel in 1x TAE buffer at 80 V for 90 min and visualized with 0.5 μg/ml ethidium bromide staining. Isolates were considered distinct if banding patterns differed.

Antifungal susceptibility test

Antifungal susceptibility was assessed using a commercially available colorimetric microdilution panel Sensititre YeastOne YO-9 (TREK Diagnostic Systems). The Sensititre YeastOne system provides antifungal susceptibility testing and minimum inhibitory concentration (MIC) determination against nine antifungals: amphotericin B (0.12 – 8 μg/ml), 5-flucytosine (0.06 – 64 μg/ml), anidulafungin (0.015 – 8 μg/ml), caspofungin (0.008 – 8 μg/ml), micafungin (0.008 – 8 μg/ml), fluconazole (0.12 – 256 μg/ml), itraconazole (0.015 – 16 μg/ml), posaconazole (0.008 – 8 μg/ml), and voriconazole (0.008 – 8 μg/ml). Testing was carried out following the manufacturers instructions. After 24-48 hours of incubation, panels were observed and MIC end points

were determined as evidenced by a color change from blue to red. Isolates were categorized according to the CLSI breakpoints (CLSI) reported by the manufacturer. Breakpoints were not available for posaconazole and amphotericin B, therefore, isolates inhibited by >1 mg/ml of amphotericin B and >1 mg/ml of posaconazole were considered resistant [26, 61].

3.4 RESULTS

Prevalence of *Candida* in ileal effluent from SBT recipients

Profiling fungi of the ileum revealed that SBT recipients harbored several *Candida* and non-*Candida* species (Table 3.3). *C. albicans* was the most predominant species, detected in the ileum of four patients (57%) and 11 out of 28 ileostomy samples (40%). *C. glabrata* was identified in three patients and 10 samples (35%). *C. parapsilosis* was obtained from two patients and six samples (21%). *C. tropicalis* was detected in two samples (7%) from two patients (28%) and *C. lusitaniae* (also known as *Clavispora lusitaniae*) was detected in one patient (14%), one sample (3.5%). Two patients (P33 and P62) harbored three species of *Candida* in the ileum and two patients (P47 and P74) harbored 2 species of *Candida* in the ileum. P32 and P39 each harbored one species of *Candida*. No *Candida* was detected in the ileum of P88.

Yeasts causing bloodstream infection in SBT recipients

Species taxonomic classification was determined for each blood culture and the results are shown in Table 3.3. With the exception of P62, all bloodstream infections were caused by *Candida*. *C. parapsilosis* was the most predominant species isolated from

the bloodstream of the patient group (3 patients). *C. albicans* was the second most-isolated species from the bloodstream, in two patients, while *C. glabrata, C. orthopsilosis*, and *Wickerhamomyces anomalus* (formerly *Pichia anomala*) were each isolated from one patient. P74 showed positive blood cultures of two different yeasts (*C. parapsilosis* and *C. albicans*).

Table 3.3 Identification of fungi in ileostomy and blood samples.

Sample	***Candida* ileal species**	**Non-*Candida* ileal species**	**Blood isolate**
32_I_1	*C. albicans*	*Epicoccum nigrum, Penicillium* cf. *roqueforti*, *Acremonium* sp.	
32_B_1			*C. parapsilosis**
33_I_1	*C. albicans*	*Penicillium* cf. *roqueforti*, *Cladosporium* sp., *Pichia kudriavzevii*	
33_I_2	*C. albicans**	*Pichia kudriavzevii*	
33_B_1			*C. albicans**
33_I_3	*C. albicans, C. glabrata. C. tropicalis**	*Pichia kudriavzevii **	
39_I_1	*C. glabrata**		
39_B_1			*C. glabrata**
39_I_2	*C. glabrata**		
47_I_1		*Epicoccum nigrum, Penicillium* cf. *roqueforti*	
47_I_2	*C. parapsilosis*	*Debaryomyces* sp., *Trichoderma* sp., *Penicillium* cf. *roqueforti*	
47_I_3	*C. parapsilosis, C. glabrata**	*C. glabrata*	
47_B_1			*C. parapsilosis**
47_I_4	*C. parapsilosis** *C. glabrata**	*Penicillium* cf. *roqueforti*	
47_I_5	*C. parapsilosis, C. glabrata**	*Penicillium* cf. *roqueforti*	
47_B_2			*C. parapsilosis**
47_I_6	*C. glabrata*		

Table 3.3 Continued.

Sample	*Candida* ileal species	Non-*Candida* ileal species	Blood isolate
47_I_7	*C. glabrata**		
47_I_8	*C. glabrata*		
47_I_9	*C. parapsilosis*, *C. glabrata**		
62_I_1		*Penicillium* cf. *roqueforti*	
62_I_2	*C. albicans*	*Fusarium* sp., *Penicillium* cf. *roqueforti*	
62_B_1			*W. anomalus**
62_I_3	*C. albicans*, *C. tropicalis*	*Penicillium* cf. *roqueforti*, *Fusarium* sp., *Debaryomyces hansenii*, *Pichia fermentens*	
62_I_4	*C. albicans*, *C. parapsilosis**		
62_I_5	*C. albicans*	*Penicillium* cf. *roqueforti*	
74_I_1	*C. albicans*	*Penicillium* cf. *roqueforti*	
74_I_2		*Penicillium* cf. *roqueforti*	
74_B_1			*C. parapsilosis**
74_I_3	*C. albicans*, *C. lusitaniae*		
74_I_4	*C. albicans*	*Epicoccum nigrum*, *Penicillium* cf. *roqueforti*	
74_B_2			*C. albicans**
74_I_5		*Aureobasidium pullulans*	
74_I_6		*Saccharomyces cerevisiae*	
88_B_1			*C. orthopsilosis**
88_I_1		*Penicillium* cf. *roqueforti*	
88_I_2		*Epicoccum nigrum*, *Penicillium* cf. *roqueforti*, *Saccharomyces cerevisiae*, *Pichia kudriavzevii*	

*Obtained in culture

MLST analysis of isolates

All *C. albicans* (n=3) and *C. glabrata* (n=8) isolates were subjected to MLST and results are shown in Table 3.4 and Table 3.5. Two sequence types were identified for each species. Twelve alleles were identified among the seven loci of *C. albicans* isolates, including two new alleles, which generated a new, unique diploid sequence type. Fourteen polymorphic nucleotide sites were found between the two *C. albicans* sequence types. Both *C. albicans* sequence types detected in this study (ST2854 & ST2855) are novel isolates and have been deposited in the MLST database. Additionally, two sequence types (ST2 & ST3), consisting of 12 alleles and 23 variable nucleotide sites were identified among the six loci of *C. glabrata* isolates.

For P33, ileostomy and blood *C. albicans* isolates yielded one sequence type (ST2854). *C. albicans* causing bloodstream infection in P74 was sequence type 2855, distinct from P33. *C. glabrata* ileostomy and bloodstream isolates from P39 shared sequence type 3. P47, who developed candidemia by *C. parapsilosis*, had gastrointestinal colonization by *C. glabrata*. However, the *C. glabrata* isolated from this individual had a sequence type 2, which was a distinct genotype from P39. In both species for which MLST was possible, sequence type and thus strain was consistent within a patient over time, but differed between patients.

Table 3.4 MLST genotypes of *C. albicans* isolates studied.

Sample	Allele number							MLST Genotype
	AAT1a	*ACC1*	*ADP1*	*MPIb*	*SYA1*	*VPS13*	*ZWF1b*	
33_I_2	156	4	4	4	204	4	4	2854
33_B_1	156	4	4	4	204	4	4	2854
74_B_2	35	7	6	4	4	4	239	2855

Table 3.5 MLST genotypes of *C. glabrata* isolates studied.

Sample	Allele number						MLST Genotype
	FKS	*LEU2*	*NMT1*	*TRP1*	*UGP1*	*URA3*	
39_I_1	5	7	8	7	3	6	3
39_B_1	5	7	8	7	3	6	3
39_I_2	5	7	8	7	3	6	3
47_I_3	1	2	2	1	1	1	2
47_I_4	1	2	2	1	1	1	2
47_I_5	1	2	2	1	1	1	2
47_I_7	1	2	2	1	1	1	2
47_I_9	1	2	2	1	1	1	2

RAPD analysis of isolates

RAPD fingerprinting with primer Oligo 2 was performed on all cultured *C. albicans* and *C. glabrata* isolates (Figure 3.1 and Figure 3.2). Four *C. albicans* isolates generated three different RAPD profiles. *C. albicans* isolates recovered from the ileostomy and bloodstream of P33 had the same RAPD profile, A. The bloodstream isolate from P74 gave a distinct RAPD profile, B (*C. albicans* was detected in ileostomy samples from P74 but could not be cultured). The common laboratory strain *C. albicans* SC5314 also gave a distinct pattern, C. Nine *C. glabrata* isolates generated three different RAPD profiles. *C. glabrata* isolates obtained from the ileostomy and bloodstream of P39

all had same RAPD profile D. *C. glabrata* ileostomy isolates from five consecutive ileostomy samples over a one month period in P47 generated RAPD profile E, while control strain *C. glabrata* NRRL Y-65 gave a distinct RAPD pattern F. The RAPD results obtained from *C. albicans* and *C. glabrata* isolates are congruent with strain typing data by MLST (Table 3.6). No RAPD patterns for *C. albicans* or *C. glabrata* were shared across individuals.

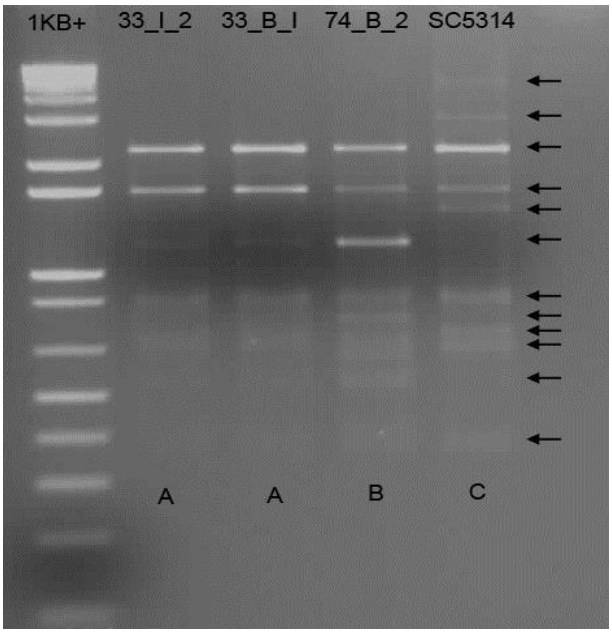

Figure 3.1 RAPD fingerprint patterns of *C. albicans* isolates obtained with primer Oligo 2. Fingerprints indicated with the same letter have equal genotypes. Arrows indicate fragments found in any profiles of the strains. First lane 1 KB+ molecular size marker. Laboratory strain *C. albicans* SC5314 included with isolates.

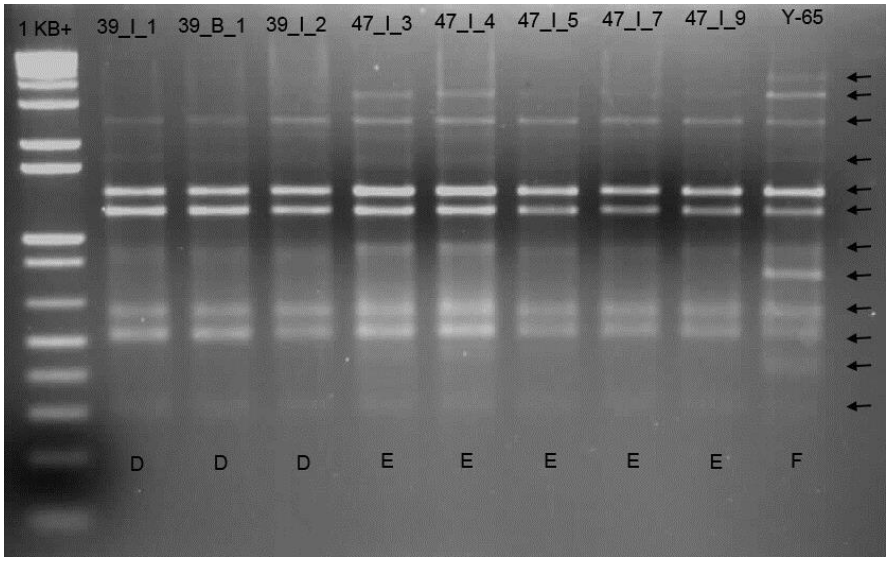

Figure 3.2 RAPD fingerprint patterns of *C. glabrata* isolates obtained with primer Oligo 2. Fingerprints indicated with the same letter have equal genotypes. Arrows indicate fragments found in any profiles of the strains. First lane 1 KB+ molecular size marker. *C. glabrata* NRRL Y-65 included with isolates.

In addition, RAPD was performed on *C. parapsilosis* isolates with primer Oligo 2 (Figure 3.3). Seven *C. parapsilosis* isolates generated two different RAPD profiles. P47 *C. parapsilosis* isolates showed two distinct RAPD patterns between ileostomy and bloodstream. The two *C. parapsilosis* blood cultures were collected from this patient before and after the ileostomy sample. Both blood cultures generated the same RAPD pattern H. However, the ileostomy isolate showed RAPD pattern G. Interestingly, the two ileostomy samples prior to the first bloodstream collection harbored *C. parapsilosis*, but viable organisms could not be cultured. Three *C. parapsilosis* isolates, collected from the ileostomy of P62 and the blood of P32 and P74, gave the same RAPD pattern G. Control *C. parapsilosis* 232, isolated from cheese, also gave the RAPD pattern G. Since a *C. parapsilosis* MLST scheme does not exist, primer OPE-04 was also used for RAPD to

confirm or contradict results obtained by primer Oligo 2. Primer OPE-04 likewise yielded two different patterns among the seven isolates, in agreement with primer Oligo 2 (Figure 3.4).

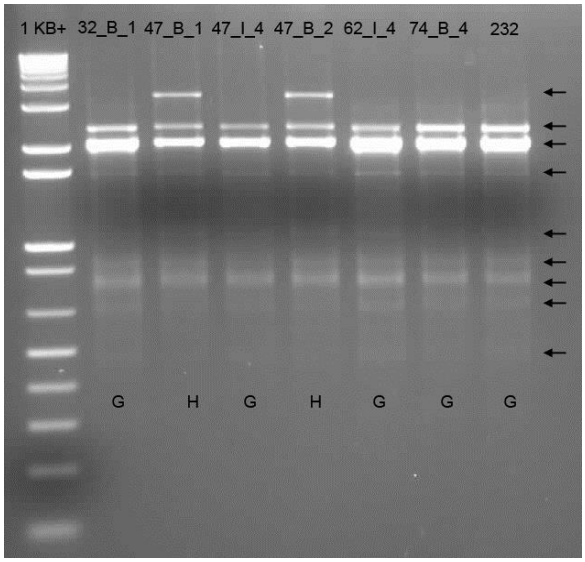

Figure 3.3 RAPD fingerprint patterns of *C. parapsilosis* isolates obtained with primer Oligo 2. Fingerprints indicated with the same letter have equal genotypes. Arrows indicate fragments found in any profiles of the strains. First lane 1 KB+ molecular size marker. *C. parapsilosis* 232 isolated from cheese included with isolates.

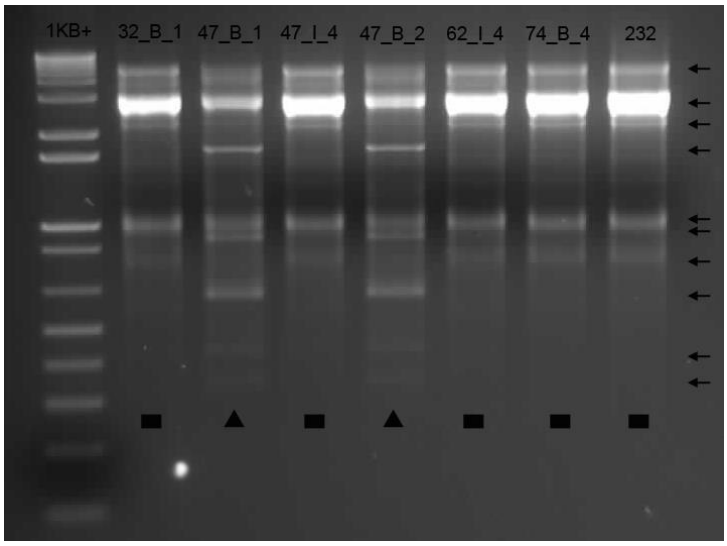

Figure 3.4 RAPD fingerprint patterns of *C. parapsilosis* isolates obtained with primer OPE-04. Fingerprints indicated with the same shape have equal genotypes. Arrows indicate fragments found in any profiles of the strains. First lane 1 KB+ molecular size marker. *C. parapsilosis* 232 isolated from cheese included with isolates.

Table 3.6 Genotype of cultured ileal (I) and bloodstream (B) isolates.

Sample	Culture	RAPD genotype	MLST genotype
32_B_1	*C. parapsilosis*	G	n/a
33_I_2	*C. albicans*	A	2854
33_B_1	*C. albicans*	A	2854
39_I_1	*C. glabrata*	D	3
39_B_1	*C. glabrata*	D	3
39_I_2	*C. glabrata*	D	3
47_I_3	*C. glabrata*	E	2
47_B_1	*C. parapsilosis*	H	n/a
47_I_4	*C. parapsilosis*	G	n/a
	C. glabrata	E	2
47_I_5	*C. glabrata*	E	2
47_I_7	*C. glabrata*	E	2
47_B_2	*C. parapsilosis*	H	n/a
47_I_9	*C. glabrata*	E	2
62_I_4	*C. parapsilosis*	G	n/a
74_B_1	*C. parapsilosis*	G	n/a
74_B_2	*C. albicans*	B	2855
Control	*C. albicans*	C	n/a
Control	*C. glabrata*	F	n/a
Control	*C. parapsilosis*	G	n/a

The chronological carriage of *Candida* genotyping patterns is reported in Figure 3.5. At two points, samples were collected from multiple patients during the same time period. In both cases, patients carried distinct strains from one another. *C. parapsilosis* isolates of RAPD profile G were detected consistently over time in four patients. Isolates of all other MLST and RAPD profiles were each restricted to a single patient, in our sampling. No one genotype dominated the patient population.

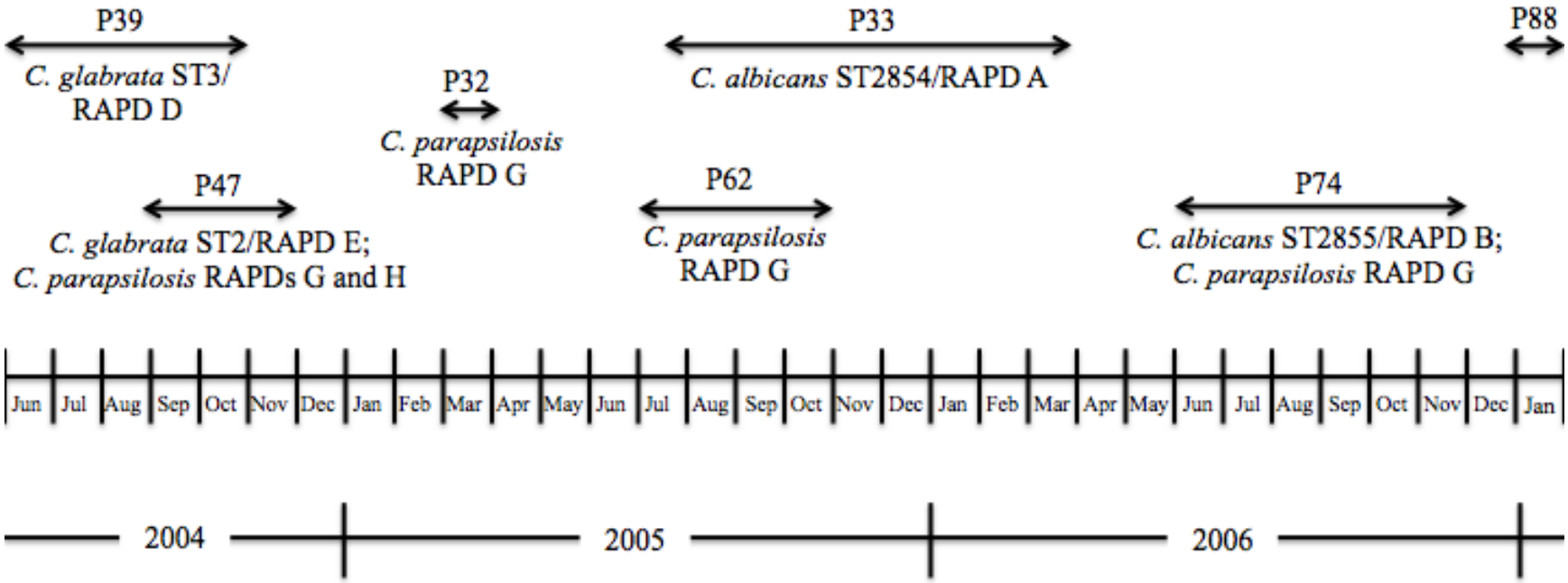

Figure 3.5 Chronological outline of patient sample collection. The MLST sequence types and RAPD profiles are indicated for each patient.

Antifungal susceptibility

The antifungal susceptibilities and MICs for the yeast isolates against nine antifungals using the YeastOne system are shown in Table 3.7. *C. glabrata* isolate 39_B_1 was classified as susceptible dose-dependent to fluconazole and voriconazole based on MIC interpretative criteria from the manufacturer. This isolate showed multidrug resistance against amphotericin B, itraconazole, and posaconazole. *C. parapsilosis* isolates 47_B_1 and 47_B_2 were resistant to amphotericin B. *C. albicans*, *C. orthopsilosis*, and *W. anomalus* isolates were uniformly susceptible to all antifungals. For all isolates, the range of MICs were: amphotericin B (0.25-2 μg/ml), itraconazole (0.06-2 μg/ml), posaconazole (0.03-2 μg/ml), and voriconazole (0.03-2 μg/ml). Fluconazole showed a wide range of MICs among the isolates (0.5-32 μg/ml), as well as micafungin (<0.008-2 μg/ml), anidulafungin (0.015-2 μg/ml), and caspofungin (0.015-2 μg/ml). 5-flucytosine showed a narrow range of MICs among the isolates (<0.06-0.25 μg/ml).

Table 3.7 Summary of MIC values and antifungal susceptibility of bloodstream isolates determined by YeastOne.

Isolate	MIC[1] (μg/ml) & Susceptibility[2]																	
	AB		AND		CAS		FC		FZ		IZ		MF		PZ		VOR	
	MIC	S	MIC	S	MIC	S	MIC	S	MIC	S	MIC	S	MIC	S	MIC	S	MIC	S
C. albicans																		
33_B_1	1	S	0.03	S	0.03	S	0.12	S	0.5	S	0.06	S	0.008	S	0.03	S	0.03	S
74_B_2	1	S	0.03	S	0.03	S	0.12	S	4	S	0.12	S	<0.008	S	0.06	S	0.12	S
C. glabrata																		
39_B_1	2	R	0.06	S	0.25	S	<0.06	S	32	S-DD	2	R	0.015	S	2	R	2	S-DD
C. orthopsilosis																		
88_B_1	1	S	1	S	0.5	S	0.12	S	1	S	0.12	S	0.5	S	0.12	S	0.12	S
C. parapsilosis																		
32_B_1	1	S	2	S	0.25	S	0.25	S	1	S	0.12	S	1	S	0.06	S	0.03	S
47_B_1	2	R	2	S	2	S	0.12	S	2	S	0.06	S	2	S	0.06	S	0.06	S
47_B_2	2	R	2	S	2	S	0.12	S	2	S	0.06	S	2	S	0.06	S	0.06	S
74_B_1	0.25	S	1	S	0.25	S	0.06	S	1	S	0.06	S	1	S	0.03	S	0.15	S
W. anomalus																		
62_B_1	0.5	S	0.015	S	0.015	S	<0.06	S	2	S	0.06	S	0.015	S	0.12	S	0.06	S

[1] AMB, amphotericin B; AND, anidulafungin; CSP, caspofungin; FC, 5-flucytosine; FZ, fluconazole; ITC, itraconazole; MF, micafungin; PSC, posaconazole; VOC, voriconazole.
[2] S, susceptible; S-DD, susceptible dose-dependent; I, intermediate; R, resistant.

3.5 DISCUSSION

Small bowel transplantation is a life-preserving treatment for pediatric short bowel syndrome patients facing intestinal failure or life-threatening complications from parenteral nutrition. The increasing incidence of *Candida* infections and the high mortality rates post-transplant warrant further research into the epidemiology of nosocomial fungal infections. The present study describes a cohort of small bowel transplant patients who developed bloodstream infection post-transplant at the University of Nebraska Medical Center from 2004-2007. We employed two molecular typing methods to determine the potential source of nosocomial candidemia infections in SBT recipients.

Bloodstream infections in this study were mainly caused by *C. parapsilosis* (43%). Prior to 1990, the predominant species causing *Candida* infections was *C. albicans* [62]. In recent years there has been an emergence of infections caused by non-*albicans* species [1, 6, 36]. *C. albicans* was isolated from the bloodstream in only two out of the seven patients. Non-*albicans* species caused the majority of infections. *C. orthopsilosis*, recently separated from the *C. parapsilosis* complex with *C. metapsilosis* [81], was identified in the bloodstream of one patient. *C. orthopsilosis* is known to be responsible for bloodstream infections, however, documented cases are limited [28, 83, 84].

One bloodstream infection was caused by *Wickerhamomyces anomalus* (formerly *Hansenula anomala* and *Pichia anomala*; teleomorph *Candida pelliculosa*, current teleomorph name *Candida beverwijkiae*). *W. anomalus* is a ubiquitous yeast found naturally in the environment and various fermentations. This yeast transiently colonizes

the oropharyngeal and gastrointestinal tract of humans [16, 54]. *W. anomalus* is rarely found as a cause of infection; however, it has been implicated in a few hospital outbreaks in neonatal and pediatric intensive care units [2, 16, 41, 60]. *W. anomalus* was not identified in the intestine of P62 from whom it was isolated from the blood. Therefore, it is possible that this patient acquired *W. anomalus* from an exogenous source. Exogenous acquisition of *W. anomalus* infection is the suggested route of transmission [2].

This study shows that various species of *Candida* are present in the gastrointestinal tract of SBT patients. This is not surprising, as the human gut is known to harbor multiple *Candida* species [35]. At least one fungal taxon was found in every ileostomy sample. Ileostomy samples contained an average of two fungal species and up to six species in one sample. This is the first study characterizing the fungal intestinal microbiota of small bowel transplant recipients. It is possible that the cloning method used to characterize the intestinal mycobiota did not obtain a complete comprehensive survey of fungi in the ileostomy samples. The number of clones selected for sequencing and the failure to sequence every clone is a limitation of cloning that may result in defining a limited diversity. Fungi of low abundance may have not been detected in ileostomy samples. Moreover, of interest is the fact that all cultured organisms were detected by molecular cloning, but not vice versa. While next-generation sequencing methods may describe the complete mycobiota in full, cloning may be the most cost-efficient and practical way to determine the mycobiota in these patients due to the limited diversity of gut fungi expected.

We detected previous digestive tract colonization with *Candida* in six of the patients with candidemia. Our findings suggest that at least two bloodstream infections

(P33 and P39) originated from *Candida* colonizing the gastrointestinal tract. This is in keeping with the findings of other studies that identified the same strain in the intestine and blood [19, 43, 51, 67]. Gastrointestinal colonization is an important source of candidemia, although only a few studies using molecular typing methods exist and sample numbers are small [55]. Many studies only report an association between anatomic site of *Candida* colonization as a risk factor for subsequent candidemia and invasive candidiasis in hospitalized and critically ill patients [17, 48]. The epidemiology of candidemia in small bowel transplant recipients has not been previously investigated. However, it is not surprising that patients in our study were infected by endogenous *Candida* due to the invasive nature of this procedure that disrupts the integrity of the gastrointestinal barrier and allows translocation of microorganisms into the bloodstream.

P47 had different strains of *C. parapsilosis* colonizing the intestine and infecting the bloodstream, suggesting that candidemia derived from an exogenous source of *C. parapsilosis*. While the possibility of hospital transmission cannot be discounted, other possibilities exist and include mutation within the patient and increase in prevalence of a previously-undetectable strain. Microvariation, small detectable changes in DNA sequence among serial isolates or as separate clones from individual patients, is a known phenomenon for strains of *C. albicans* [46, 56]. The majority of documented *C. parapsilosis* infections are of exogenous origin. *C. parapsilosis* is the most common fungus recovered from the hands and studies have implicated health care workers' hands as the source of nosocomial candidemia [50, 88]. Using PFGE and RAPD, Lupetti et al. [47] demonstrated horizontal transmission of *C. parapsilosis* infection from the hands of a health care worker to a newborn. Another study showed dissemination of *C.*

parapsilosis between patients and hospital staff, but was unable to determine whether the initial infection originated in patient or staff [45]. Another possible route of *C. parapsilosis* transmission could be contaminated parenteral nutrition and intravascular devices [18, 21, 92].

C. parapsilosis was not detected in ileostomy samples from P32 and P74, who developed bloodstream infection with *C. parapsilosis*. It is possible that these two individuals exogenously acquired *C. parapsilosis*. Endogenous *C. parapsilosis* infections are rarely seen [51]. P74 was co-infected by two *Candida* species. *C. parapsilosis* was the first organism recovered from the blood, but ileostomy samples show an absence of *C. parapsilosis* in the gut prior to infection. Interestingly, *C. albicans* was present in three ileostomy samples prior to *C. albicans* detection in the blood. It is possible that this patient was infected exogenously by *C. parapsilosis* and endogenously by the patient's own intestinal *C. albicans*. The inability to culture *C. albicans* from the patient's ileum precluded strain typing and thus confirmation of a possible endogenous source. Additionally P88, who had no gastrointestinal colonization with *C. orthopsilosis*, may have acquired candidemia exogenously.

Studies have documented the ability of *Candida* isolates to persist in hospital settings and cause temporally-associated infections [4, 90]. In our study, temporal analysis revealed multiple species were responsible for causing bloodstream infections during this time period. At two different time points, samples collected from two patients overlapped, but the patients did not carry the same genotype. However, the same *C. parapsilosis* genotype G in four patients was documented at multiple time points. Therefore, there is potential evidence that *C. parapsilosis* strains had a common source.

Dissemination could have been due to health care workers, although they are required to wash hands before and after patient contact as well as wear gloves. Samples from health care workers or the environment were not taken; however, of potential significance is that *C. parapsilosis* isolates of genotype G were detected throughout this study.

Methods used to strain-type *Candida* species have greatly advanced our knowledge of fungal epidemiology. MLST and RAPD were applied in this study to help elucidate the origin of candidemia. MLST has been shown to be more discriminating than RAPD for *C. albicans* [70]. In our study, MLST identified distinct isolates between patients, but did not distinguish between isolates within a patient, whether ileostomy or bloodstream. This suggests independent, endogenous acquisition of candidemia. Strain-typing with RAPD was used as a complement to MLST. RAPD is known to have low reproducibility but is useful in strain differentiation due to its speed and cost-efficiency. The fingerprints of the RAPD patterns yielded the same conclusions as MLST for *C. albicans* and *C. glabrata* isolates. Primer Oligo 2 was sufficient to strain-type *C. albicans* and *C. glabrata*.

Since a *C. parapsilosis* MLST scheme does not exist [81], RAPD was necessary in order to obtain epidemiological information. RAPD fingerprints generated by both primer Oligo 2 and OPE-4 gave the same results: one RAPD fingerprint detected in samples from four patients (as well as a control isolate from cheese), and a distinct fingerprint in two bloodstream samples from P47, whose ileostomy *C. parapsilosis* yielded the other fingerprint. It is possible that other RAPD primers would further differentiate between our isolates; conversely, genetic variation in *C. parapsilosis* may be minimal.

There is increasing concern regarding antifungal resistance in *Candida,* especially *C. parapsilosis*. A study of a neonatal intensive care unit during a 12-year period showed that an outbreak of candidemia was caused by a *C. parapsilosis* strain with fluconazole resistance due to the long-term use of fluconazole prophylaxis in this institute [74]. In our study, two *C. parapsilosis* isolates from the same patient showed resistance to amphotericin B. MICs reported for *C. parapsilosis* isolates were within the MIC ranges in other studies [14, 29]. Usually, *C. albicans* is susceptible to azoles, echinocandins, and polyenes [68]; our *C. albicans* isolates were susceptible to all antifungal agents. *Wickerhamomyces anomalus* was susceptible to all antifungals in this study. *W. anomalus* does not show intrinsic resistance to any antifungals; however, susceptibility to itraconazole has been reported to be poor [20].

One of the limitations of this study was the lack of environmental isolates. It was not possible to determine the exact exogenous source of candidemia in any of the patients. Also, this study is unable to determine whether the donor bowel was colonized by *Candida* and consequently, the source of infection. Donor-derived *Candida* infections have been documented in kidney transplant recipients [7, 89]. To date, no efforts have been made to identify *Candida* species and/or strains from the donor small bowel to investigate the origin of candidemia in SBT recipients.

In this study, *C. parapsilosis* isolates were only typed using RAPD. RAPD is known to have low reproducibility and may not be the best method for epidemiological studies. The isolates could not be typed by MLST because attempts to construct a *C. parapsilosis* MLST scheme have been unsuccessful thus far due to the lack of sequence

variation [81]. Other methods such as PFGE-based typing and microsatellite markers may be more discriminatory for epidemiological investigations of *C. parapsilosis* [45, 72].

Another limitation of this study was the inability to culture viable organisms from several ileostomy samples. In some instances a species could be identified by PCR and sequencing from an ileostomy sample but a subsequent isolate could not be obtained. Techniques to detect species in mixed matrices are possible with the use of universal or species-specific primers. Conversely, strain typing aims to differentiate specific strains and requires pure culture of each isolate [87]. Thus, while we know an ileostomy sample harbored a specific species, determining intestinal origin could not be elucidated because of the inability to strain type. However, it must be noted that the samples we used were collected nearly a decade ago and no special steps had been taken to ensure long-term survival of microorganisms. The fact that we were able to culture as many isolates as we were bodes well for future studies with newly-collected material.

In conclusion, these findings highlight the possibility of hospitalized SBT patients acquiring candidemia both endogenously and exogenously. Although the exact route of infection in these transplant patients is not clear, our results suggest that cross infection with *C. parapsilosis* may have occurred and *C. parapsilosis* strains of profile G are frequent and may represent an endemic strain. Attempts need to be aimed at reducing *Candida* gut colonization with the use of antimicrobials and antifungals. Also, focused efforts should be made to ensure proper sterilization of equipment and intravenous devices. Implementing surveillance programs to ensure correct use of gloves and proper hand washing is important to prevent spread of infection. Ultimately, these results underline the importance of *Candida* surveillance in small bowel transplant institutes and

knowing the source of *Candida* implicated in infection (recipient, donor, or hospital acquired) to inform pre-transplant interventions and future treatment will improve outcomes for SBT patients.

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CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 OUTLOOK ON THE ROLE OF THE GUT MYCOBIOME

Fungi undoubtedly reside in the human gastrointestinal tract and form a small but persistent part of the gastrointestinal microbiota. So why is it important to understand their contribution to the gut microbiota as a whole and the human host? Gut mycologists have only begun to answer this question and have conducted preliminary studies on many aspects of fungal existence in the gut. Like the bacterial microbiota, there is now increasing evidence that fungi play a role in the human gut ecology and health. Efforts need to continue focusing on the function of fungi in the human gastrointestinal tract, as a myriad of questions remain and the field lags immensely behind bacterial gut microbiota research. It is gratifying that studies are beginning to question the interactions of fungi with diet, immune responses, other microbial communities, and diseased states. As the field advances, studies will unveil the importance of the mycobiome and specific fungal members as a factor of human health.

4.2 CONCLUSIONS

In Chapter 2, our goal was to help advance and stimulate the relatively new field of gut mycobiome research. To date, most research has focused on characterizing the bacterial members of the gastrointestinal tract and the human mycobiome is years behind the state of the art represented by bacterial microbiome studies. The human gut mycobiome is diverse and relatively unstable, and the search for a “core mycobiome” (with the exception of a very few species of human-associated yeasts) may prove futile due to high level of exposure to environment or food-associated fungi. To date, next-generation sequencing platforms show the greatest diversity in gut fungi; however, as so

many fungi are allochthonous, this high diversity may be misleading and targeted approaches to identify specific organisms from a limited pool of species of interest may prove more meaningful. As gut mycologists continue to expand gut mycobiome studies using larger sample sizes, geographic distribution sampling, dietary influences, and diseased states we will begin to understand their beneficial and/or detrimental roles in human health.

In Chapter 3, we surveyed the full gut fungal microbiota of small bowel transplant patients to determine the source of *Candida* implicated in candidemia. Using genotyping methods, we were able to determine endogenous and exogenous sources of *Candida* causing candidemia. Although this study has only a limited number of isolates, we were able to hypothesize that *C. albicans* and *C. glabrata* infections were caused by endogenous sources of *Candida* and infection with *C. parapsilosis* was caused from an exogenous source. The proposed work highlights the wide spread nature of *Candida* causing endogenous and exogenous candidemia infections in hospital settings.

4.3 FUTURE DIRECTIONS

Chapter 2 provides a framework for future studies interested in characterizing the fungi of the gastrointestinal tract. Future studies will need to use larger sample sizes in order to understand variation of fungal communities between individuals. In addition, serial samples from individuals need to be collected over time to gain a better understanding of the stability of gut fungi over time. Culture-dependent and -independent methods need to be performed in conjunction with one another due to the methodology biases. Encompassing samples from multiple individuals with different lifestyles, dietary

habits, and geographic locations will allow researchers to better understand gut mycology around the world.

Chapter 3 provides baseline data on the gut ecology of *Candida* yeasts after small bowel transplantation. In this study it was possible to determine if the gastrointestinal tract was a potential source of infection. However, due to the limited samples available, we were unable to determine if the *Candida* causing infection was from donor or recipient origin. Donor organs could serve as a source of infection. Collecting, identifying, and strain-typing *Candida* yeasts from the donor small bowel at the time of transplant and the recipient small bowel prior to transplant would allow us to differentiate between a donor or recipient endogenous source of candidemia.

Collecting donor-recipient ileostomy sample pairs, tracking *Candida* strain presence or absence over time for each donor-recipient pair would provide data on which strains seem to have competitive advantages, and information on the *Candida* strains likely to cause candidemia. It would be possible to determine whether strains of *Candida* were present in the patient's microbiota at the time of transplant or were donor-derived. Also, use of sequencing and strain-typing would: 1) show whether single or multiple *Candida* species/strains are present at a given time point, 2) track *Candida* populations and prevalence over time and observe whether donor and recipient strains coexist or if one predominates.

Small bowel transplantation presents a unique opportunity to directly study the merging of two distinct microbial populations in humans. The use of next generation sequencing to conduct a comprehensive survey of the gastrointestinal microbiota (bacteria and fungi) would allow us to evaluate the interactions of two gut microbial

populations after merging donor and recipient gut microbiomes via transplant. Collecting samples from both the donor and recipient at the time of transplant would be necessary to study this. Observing gut microbiota populations over time would allow us to view ecological processes, such as competition or synergy. Studying the merging of two microbial communities will allow us to study if donor microbes establish themselves or if the recipient microbiota is resilient to disturbance. Also, are particular microbes consistently dominant, regardless of source? And does the abundance of particular bacterial taxa correlate with *Candida* abundance or infection?