

## REVIEW ARTICLE

# Forgotten fungi—the gut mycobiome in human health and disease

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## ABSTRACT

The human body is home to a complex and diverse microbial ecosystem that plays a central role in host health. This includes a diversity of fungal species that is collectively referred to as our 'mycobiome'. Although research into the mycobiome is still in its infancy, its potential role in human disease is increasingly recognised. Here we review the existing literature available on the human mycobiota with an emphasis on the gut mycobiome, including how fungi interact with the human host and other microbes. In doing so, we provide a comprehensive critique of the methodologies available to research the human mycobiota as well as highlighting the latest research findings from mycological surveys of different groups of interest including infants, obese and inflammatory bowel disease cohorts. This in turn provides new insights and directions for future studies in this burgeoning research area.

**Keywords:** gut microbiome; mycobiome; fungi; intestinal disease

## INTRODUCTION

Complex microbial ecosystems such as the human gastrointestinal (GI) tract harbour a diversity of microorganisms including bacteria, archaea, viruses and microbial eukarya (Rajilić-Stojanović, Smidt and de Vos 2007; Qin et al. 2010; Parfrey, Walters and Knight 2011; Clemente et al. 2012; Parfrey et al. 2014; Hamad, Raoult and Bittar 2016). However, the myriad of species that are potentially present are found at varying abundance levels and, thus, the presence of predominant species' may result in microorganisms that are less common being overlooked (Huber et al. 2007). This 'rare biosphere' (Sogin et al. 2006) that inhabits the human GI tract includes the fungal component or gut 'mycobiome' (Ghannoum et al. 2010).

The human gut mycobiome is receiving increased research attention (Andersen, Nielsen and Stensvold 2013; Cui, Morris and Ghedin 2013; Kumamoto 2016) due to its potential involvement in the aetiology of numerous gut-associated diseases (Ott et al. 2008; Iliev and Underhill 2013; Sokol et al. 2016). This increasing interest is largely driven by recent findings that specific fungi can modulate the host immune response and consequently may be a risk factor in immunological disorders in genetically susceptible individuals (Iliev et al. 2012). There is also an ever greater appreciation that the mycobiome may act as a reservoir for potentially opportunistic pathogens in immunocompromised hosts (Chen et al. 2011; Miceli, Díaz and Lee 2011; Polvi et al. 2015) and may play a role in many diseases that are not obviously related to, or influenced by, the gut (Chen et al. 2011;

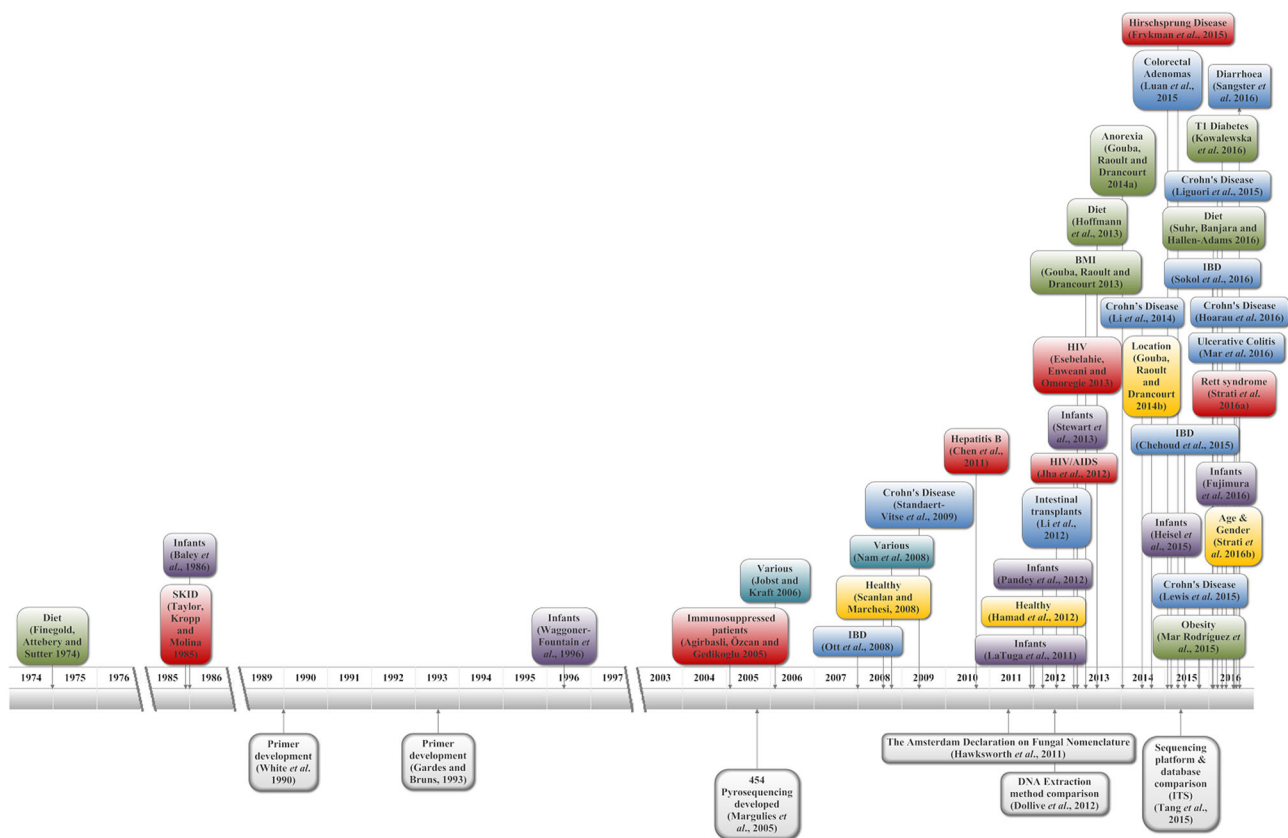


Figure 1. Timeline of studies published on the gut mycobiome and colour coded as follows: major developments (grey), GI disease (blue), immunocompromised hosts (red), healthy populations (yellow), infants (purple), diet (green) and other (teal).

Strati *et al.* 2016a). Conversely, the potential health benefits or probiotic effects of some fungal species are known but have yet to be fully explored (Hatoum, Labrie and Fliss 2012).

Historically, researchers have relied on *in vitro* culturing as the primary means of investigating fungi from the gut environment (Finegold, Attebery and Sutter 1974). However, the vast majority of microbes in the gut, including fungi, are considered refractory to cultivation which has led to large discrepancies between data obtained from culture-dependent and independent methods (Chen *et al.* 2011; Gouba, Raoult and Drancourt 2013; Browne *et al.* 2016). Although recent advances in molecular techniques that circumvent the necessity to culture microbes have been made, this research has largely focused on the non-fungal component of this ecosystem. Nonetheless, a number of important studies into the human mycobiome that use such methods are emerging. These include studies into the aetiology of various GI diseases (Ott *et al.* 2008; Iliev *et al.* 2012; Lewis *et al.* 2015; Hoarau *et al.* 2016; Mar *et al.* 2016) as well as the effect of fungal prevalence in immunocompromised hosts (Chen *et al.* 2011; Mukherjee *et al.* 2014). Mycobiome studies have also investigated the significance of subject age (Gewolb *et al.* 1999; Strati *et al.* 2016b) including children (Heisel *et al.* 2015) and adults (Scanlan and Marchesi 2008), gender (Strati *et al.* 2016b), geographical locations (Nam *et al.* 2008; Hamad *et al.* 2012), underlying conditions such as diabetes and obesity (Gouba, Raoult and Drancourt 2013; Mar Rodríguez *et al.* 2015; Kowalewska *et al.* 2016) and eating disorders (EDs) such as anorexia nervosa (Gouba, Raoult and Drancourt 2014a), together with fungal diversity associated with different body sites (Zhang *et al.* 2011; Nguyen, Viscogliosi and Delhaes 2015) (Fig. 1).

Here we review the existing literature on the human gut mycobiome in order to provide a comprehensive insight into both the methodologies available to research the gut mycobiota and also to highlight the latest research findings. We also draw on research into what is known about the human mycobiota at other body sites, in addition to detailing how fungi interact with the human host and data from other gut microbiota studies to provide both comparative insight and productive direction for future studies in this burgeoning research area.

## METHODOLOGIES TO STUDY THE HUMAN INTESTINAL MYCOBIOME

### Culture-dependent methods

As outlined, scientists have traditionally relied on culture-dependent techniques when attempting fungal surveys of complex microbial ecosystems (Horton and Bruns 2001), including the human GI tract (Finegold, Attebery and Sutter 1974; Hoog *et al.* 2005). These fungal investigations generally utilised traditional microbiological techniques such as microscopy (De Repentigny, Phaneuf and Mathieu 1992), biochemical assays (Khatib *et al.* 2001) and/or growth on selective media (Ouanes *et al.* 2013) as until relatively recently, alternatives to these methods were not developed or were too expensive for use in many laboratories.

Researchers using selective media for mycobiome research typically perform a serial dilution of faecal samples, followed by plating onto various media that have been supplemented with antibiotics (Scanlan and Marchesi 2008). These plates are

**Table 1.** Culture media used to isolate fungi from the human GI tract.

Name	Typical composition	Recommended for the culture of	Study
Sabouraud dextrose media	Mycological peptone and dextrose	Yeasts, moulds and aciduric bacteria	Finegold, Attebery and Sutter (1974) Scanlan and Marchesi (2008) Gouba, Raoult and Drancourt (2013) Hamad et al. (2012) Chen et al. (2011) Gouba, Raoult and Drancourt (2014a) Esebelahie, Enweani and Omoregie (2013) Baley et al. (1986) Waggoner-Fountain et al. (1996) Jobst and Kraft (2006) Taylor, Kropp and Molina (1985) Agırbaslı, Özcan and Gedikoğlu (2005) Kowalewska et al. (2016)
Potato dextrose media	Potato extract and dextrose	Bacteria, fungi, yeasts and moulds	Scanlan and Marchesi (2008) Gouba, Raoult and Drancourt (2013) Chen et al. (2011) Gouba, Raoult and Drancourt (2014b) Gouba, Raoult and Drancourt (2014a)
Czapek-Dox media	Sucrose, sodium nitrate, magnesium sulphate, potassium chloride, potassium phosphate dibasic and ferrous sulphate	Fungi and yeasts	Gouba, Raoult and Drancourt (2013) Gouba, Raoult and Drancourt (2014a) Taylor, Kropp and Molina (1985)
Malt extract media	Malt extract, peptone and dextrose	Yeasts and moulds	Scanlan and Marchesi (2008) Taylor, Kropp and Molina (1985)
Dixon's media	Malt extract, peptone, ox bile, tween 40, glycerol and oleic acid (Leeming and Notman 1987)	<i>Malassezia</i> species	Gouba, Raoult and Drancourt (2013) Gouba, Raoult and Drancourt (2014b) Gouba, Raoult and Drancourt (2014a)
Brain heart infusion media	Brain heart infusion (Solids), enzymatic digest of casein, dextrose, disodium phosphate, sodium chloride and enzymatic digest of animal tissue	Fastidious microorganisms	Esebelahie, Enweani and Omoregie (2013)
5% sheep blood media	Pancreatic digest of casein, papaic digest of soy meal, sodium chloride, sheep blood	Fastidious microorganisms	Baley et al. (1986)
CHROMagar Candida	Agar, chromogenic mix, chloramphenicol and peptone	<i>Candida</i> spp.	Kowalewska et al. (2016) Standaert-Vitse et al. (2009)
YPD medium	Yeast extract, bacto peptone, D-glucose and agar	Yeasts	Strati et al. (2016b)

then used to enumerate fungi and/or isolate colonies for further characterisation (Morris et al. 1996). There are a number of commercially available media types that are used in the study of the GI mycobiome, in addition to other media that can be prepared from first principles. The main media types and the fungal species they have been used to cultivate are described in Table 1. For example, commercially available media such as Sabouraud dextrose and potato dextrose are the most commonly used and have been used in the cultivation of a variety of different fungal species (see Table 2). More complex fungal media, such as Czapek-dox and Dixon's media have also been utilised in an attempt to recover more fastidious fungal species from the human gut (Gouba, Raoult and Drancourt 2014b). Although it is commonly believed that *Candida* species are the predominant species in the GI tract, the use of these media has facilitated the recovery of a wealth of other fungal species. An overview of fungi that have been recovered from culture-based gut mycobiome studies is provided in Table 2.

Conventional culture methods are, however, predisposed to a number of inherent issues and biases, many of which have been previously noted with respect to studies of the bacterial fraction of the gut community. First, culture-based studies are enormously time consuming and can require extremely long incubation times (Morris et al. 1996; Gouba, Raoult and Drancourt 2013). Furthermore, they are biased towards fast growing species and non-fastidious species that are able to utilise the nutrients contained in the culture media (Griffith et al. 2009). Such a bias is not an issue if the primary aim is to enumerate fast growing fungal species, such as *Candida albicans* which will present on plates within 48–72 h (Morris et al. 1996; Scanlan and Marchesi 2008), or where readily cultivatable clinical isolates of medical importance that require immediate attention are under investigation (Ouanes et al. 2013). However, a bias towards easy to culture species is detrimental if the aim of the study is to provide a complete survey of fungal diversity (Gouba, Raoult and Drancourt 2013). Species of fungi that do not require complex

**Table 2.** Fungal species in the gut reported in culture-dependent investigations.

Species name	Source study
<i>Aspergillus niger</i>	Taylor, Kropp and Molina (1985)
<i>Aspergillus flavipes</i>	Gouba, Raoult and Drancourt (2013)
<i>Aspergillus flavus</i>	Gouba, Raoult and Drancourt (2014b) Gouba, Raoult and Drancourt (2014a)
<i>Aspergillus glaucus</i>	Strati et al. (2016b)
<i>Aspergillus pseudoglaucus</i>	Strati et al. (2016b)
<i>Aspergillus ruber</i>	Gouba, Raoult and Drancourt (2014b) Gouba, Raoult and Drancourt (2014a)
<i>Aspergillus sydowi</i>	Taylor, Kropp and Molina (1985)
<i>Aspergillus versicolor</i>	Gouba, Raoult and Drancourt (2013) Taylor, Kropp and Molina (1985)
<i>Beauveria bassiana</i>	Gouba, Raoult and Drancourt (2013)
<i>Candida albicans</i>	Baley et al. (1986) Chen et al. (2011) Esebelahie, Enweani and Omoregie (2013) Finegold, Attebery and Sutter (1974) Gouba, Raoult and Drancourt (2014a) Heisel et al. (2015) Jha et al. (2012) Jobst and Kraft (2006) Kowalewska et al. (2016) Scanlan and Marchesi (2008) Standaert-Vitse et al. (2009) Strati et al. (2016b) Waggoner-Fountain et al. (1996)
<i>Candida deformans</i>	Strati et al. (2016b)
<i>Candida famata</i>	Agirbasli, Özcan and Gedikoğlu (2005) Jobst and Kraft (2006) Kowalewska et al. (2016)
<i>Candida fermentati</i>	Strati et al. (2016b)
<i>Candida glabrata</i>	Agirbasli, Özcan and Gedikoğlu (2005) Chen et al. (2011) Esebelahie, Enweani and Omoregie (2013) Gouba, Raoult and Drancourt (2014a) Jobst and Kraft (2006) Standaert-Vitse et al. (2009) Strati et al. (2016b)
<i>Candida guilliermondii</i>	Agirbasli, Özcan and Gedikoğlu (2005) Kowalewska et al. (2016)
<i>Candida inconspicua</i>	Jobst and Kraft (2006)
<i>Candida intermedia</i>	Strati et al. (2016b)
<i>Candida kefyr</i>	Agirbasli, Özcan and Gedikoğlu (2005) Jobst and Kraft (2006)
<i>Candida krusei</i>	Agirbasli, Özcan and Gedikoğlu (2005) Chen et al. (2011) Esebelahie, Enweani and Omoregie (2013) Hamad et al. (2012) Kowalewska et al. (2016)
<i>Candida lusitanae</i>	Agirbasli, Özcan and Gedikoğlu (2005) Kowalewska et al. (2016) Strati et al. (2016b)
<i>Candida metapsilosis</i>	Strati et al. (2016b)
<i>Candida norvogensis</i>	Agirbasli, Özcan and Gedikoğlu (2005)
<i>Candida parapsilosis</i>	Baley et al. (1986) Esebelahie, Enweani and Omoregie (2013) Heisel et al. (2015) Kowalewska et al. (2016) Scanlan and Marchesi (2008) Strati et al. (2016b) Taylor, Kropp and Molina (1985)
<i>Candida pararugosa</i>	Strati et al. (2016b)
<i>Candida rugosa</i>	Hamad et al. (2012)

**Table 2.** (Continued).

Species name	Source study
<i>Candida sphaerica</i>	Agirbasli, Özcan and Gedikoğlu (2005)
<i>Candida sp.</i>	Finegold, Attebery and Sutter (1974) Gewolb et al. (1999)
<i>Candida tropicalis</i>	Agirbasli, Özcan and Gedikoğlu (2005) Baley et al. (1986) Chen et al. (2011) Esebelahie, Enweani and Omoregie (2013) Gouba, Raoult and Drancourt (2013) Strati et al. (2016b)
<i>Candida utilis</i>	Agirbasli, Özcan and Gedikoğlu (2005)
<i>Candida zeylanoides</i>	Agirbasli, Özcan and Gedikoğlu (2005)
<i>Cladosporium sp.</i>	Gouba, Raoult and Drancourt (2013)
<i>Cladosporium sphaerospermum</i>	Taylor, Kropp and Molina (1985)
<i>Clavospora lusitanae</i>	Gouba, Raoult and Drancourt (2014a)
<i>Climacocystis sp.</i>	Gouba, Raoult and Drancourt (2013)
<i>Corticiceae sp.</i>	Gouba, Raoult and Drancourt (2014a)
<i>Cryptococcus saitoi</i>	Strati et al. (2016b)
<i>Cryptococcus sp.</i>	Agirbasli, Özcan and Gedikoğlu (2005)
<i>Cystofilobasidium capitatum</i>	Gouba, Raoult and Drancourt (2014b) Gouba, Raoult and Drancourt (2014a)
<i>Davidiella sp.</i>	Gouba, Raoult and Drancourt (2014a)
<i>Davidiella tassiana</i>	Gouba, Raoult and Drancourt (2014a)
<i>Debaryomyces hansenii</i>	Gouba, Raoult and Drancourt (2014a)
<i>Eurotium amstelodami</i>	Strati et al. (2016b)
<i>Eurotium rubrum</i>	Strati et al. (2016b)
<i>Galactomyces geotrichum</i>	Gouba, Raoult and Drancourt (2013) Hamad et al. (2012)
<i>Geotrichum sp.</i>	Agirbasli, Özcan and Gedikoğlu (2005) Kowalewska et al. (2016)
<i>Hypocrea lixii/Penicillium chrysogenum</i>	Gouba, Raoult and Drancourt (2013)
<i>Isaria farinosa</i>	Gouba, Raoult and Drancourt (2013)
<i>Lichtheimia ramosa</i>	Strati et al. (2016b)
<i>Malassezia furfur</i>	Gewolb et al. (1999)
<i>Malassezia globosa</i>	Gouba, Raoult and Drancourt (2014b) Gouba, Raoult and Drancourt (2014a) Gouba, Raoult and Drancourt (2013)
<i>Malassezia pachydermatis</i>	Gouba, Raoult and Drancourt (2014b) Gouba, Raoult and Drancourt (2014a) Gouba, Raoult and Drancourt (2013)
<i>Malassezia restricta</i>	Gouba, Raoult and Drancourt (2014b) Gouba, Raoult and Drancourt (2014a) Gouba, Raoult and Drancourt (2013)
<i>Malassezia sp.</i>	Gouba, Raoult and Drancourt (2014a)
Mould	Agirbasli, Özcan and Gedikoğlu (2005)
<i>Mucor circinelloides</i>	Strati et al. (2016b)
<i>Penicillium decumbens</i>	Taylor, Kropp and Molina (1985)
<i>Penicillium allii</i>	Gouba, Raoult and Drancourt (2013)
<i>Penicillium brevicompactum</i>	Gouba, Raoult and Drancourt (2013) Strati et al. (2016b)
<i>Penicillium citrinum</i>	Taylor, Kropp and Molina (1985)
<i>Penicillium crustosum</i>	Strati et al. (2016b)
<i>Penicillium dipodomyicola</i>	Gouba, Raoult and Drancourt (2013)
<i>Penicillium notatum</i>	Taylor, Kropp and Molina (1985)
<i>Penicillium paneum</i>	Strati et al. (2016b)
<i>Penicillium sp.</i>	Gouba, Raoult and Drancourt (2014a)
<i>Penicillium sp./P. camemberti</i>	Gouba, Raoult and Drancourt (2013)
<i>Penicillium steckii</i>	Taylor, Kropp and Molina (1985)
<i>Pichia caribbica</i>	Strati et al. (2016b)
<i>Pichia fermentans</i>	Strati et al. (2016b)
<i>Pichia kluyveri</i>	Strati et al. (2016b)

Table 2. (Continued).

Species name	Source study
<i>Pichia manshurica</i>	Strati et al. (2016b)
<i>Pleurostomophora richardsiae</i>	Strati et al. (2016b)
<i>Rhodospiridium kratochvilovae</i>	Strati et al. (2016b)
<i>Rhodotorula mucilaginoso</i>	Strati et al. (2016b)
<i>Rhodotorula rubra</i>	Waggoner-Fountain et al. (1996)
<i>Rhodotorula</i> sp.	Kowalewska et al. (2016)
<i>Saccharomyces cerevisiae</i>	Agırbaslı, Özcan and Gedikoğlu (2005) Chen et al. (2011) Jobst and Kraft (2006) Standaert-Vitse et al. (2009) Strati et al. (2016b)
<i>Saccharomyces</i> spp.	Kowalewska et al. (2016)
<i>Starmerella bacillaris</i>	Strati et al. (2016b)
<i>Torulasporea delbrueckii</i>	Strati et al. (2016b)
<i>Trichosporon asahii</i>	Gouba, Raoult and Drancourt (2014a) Hamad et al. (2012) Strati et al. (2016b)
<i>Trichosporon pullulans</i>	Baley et al. (1986)
<i>Trichosporon</i> sp.	Agırbaslı, Özcan and Gedikoğlu (2005)
<i>Yarrowia lipolytica</i>	Strati et al. (2016b)

nutrients can also mask morphologically similar, rare species on mixed primary plates and fast growing species can swamp plates, even when they are present in low levels.

As alluded to earlier, another commonly cited issue is the large discrepancies between data derived from culture-dependent and culture-independent investigations (Chen et al. 2011; Hamad et al. 2012; Gouba, Raoult and Drancourt 2013), see also Fig. 2. This problem is undoubtedly linked to inherent biases associated with culture-dependent methods as outlined and also the choice and specificity of the molecular methods used (Scanlan and Marchesi 2008). Other potential issues with culture-dependent analysis relate to the impact of storage (Ott et al. 2004a) and the freezing of faecal samples prior to their analysis. To the authors' knowledge, the effect of freezing on the ability to detect fungi from human faecal samples has only been speculated upon (Scanlan and Marchesi 2008); however, a study conducted in cattle has previously shown that the freezing and storage of faecal samples at -20°C resulted in a 2-fold reduction in the total fungal count (Griffith et al. 2009). Although the fungal species present in bovine and human faecal samples are quite different e.g. a predominance of Neocallimastigomycota in the bovine gut (Liggenstoffer et al. 2010; Nicholson et al. 2010) and Ascomycota and Basidiomycota in the human gut (Fig. 3), it is still reasonable to assume that such an effect on cultivable fungal species in human samples is likely.

Despite these difficulties and biases, the utility and importance of culturing fungi cannot be ignored and researchers have coupled this with other methods including mass spectrometry (MS) and DNA-based analysis of pure cultures in an attempt to both better identify fungi isolated from the human

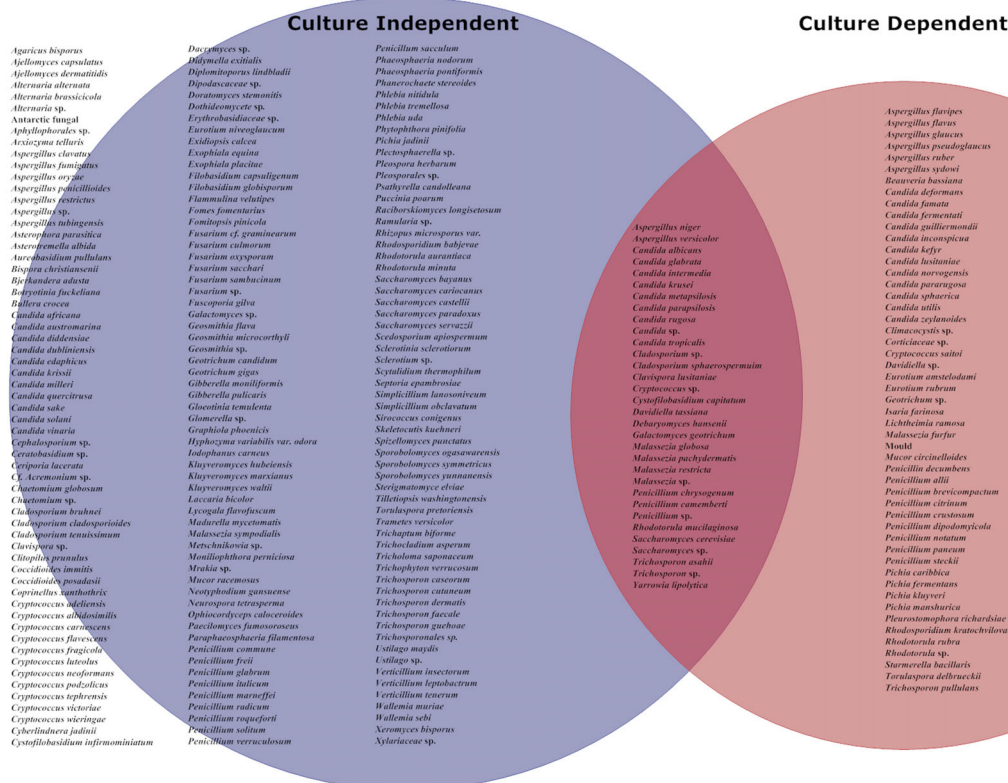
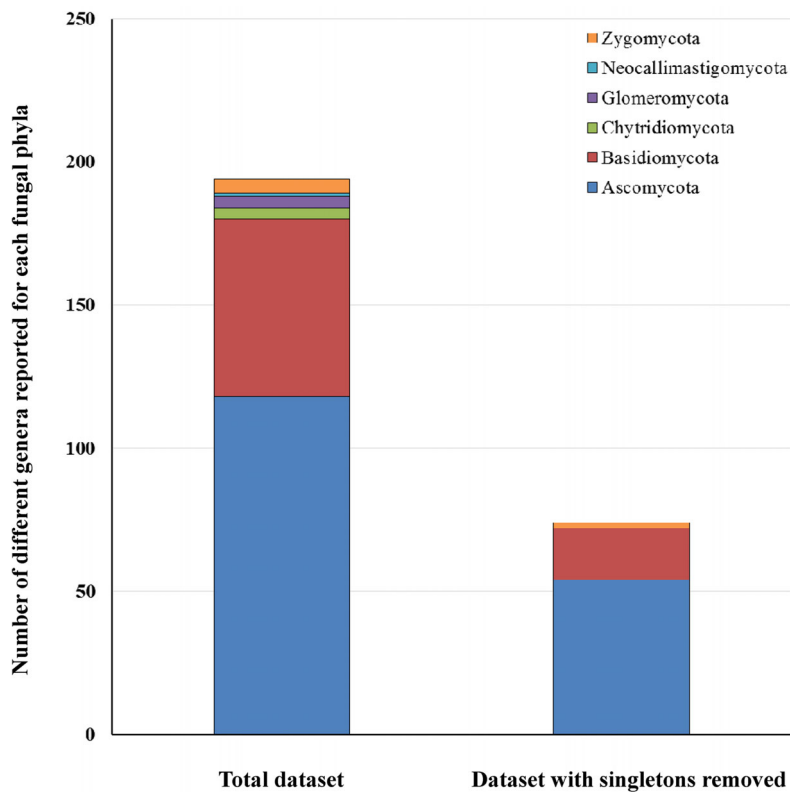


Figure 2. Commonalities and differences between fungal data (at the species level) reported in gut mycobiome studies using culture-dependent and culture-independent methodologies. This Venn diagram highlights fungal species detected by culture-independent only, culture-dependent only and species that have been detected by both methods (intersection).



**Figure 3.** Predominance of Ascomycota and Basidiomycota phyla in the human gut mycobiome. This graph illustrates the relative abundance and phylum level taxonomic affiliation of fungal genera reported in culture-independent studies of the gut mycobiome (see also Table S1). (A) Details the relative abundance of all fungal genera reported (at the phylum level) and (B) details the relative abundance of fungal genera (at the phylum level) reported following the removal of genera reported in one study only (i.e. removal of singletons).

GI and to impart a degree of high-throughput analysis (Gouba, Raoult and Drancourt 2013). Methods include the use of matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) analysis of cultured fungi in combination with internally transcribed spacer (ITS) sequence analysis (Sauer and Kliem 2010; Hamad et al. 2012; Lagier et al. 2012) which have been used to varying degrees of success. For example, in one particular study, MALDI-TOF MS failed to identify a number of cultivated fungal species (Hamad et al. 2012) which would question its overall utility when compared to the accuracy of sequence-based identification.

Recent advances in culturomics of fungi have also included the development of differential chromogenic media that allow high-throughput and semi-diagnostic culturing, which is of particular importance in clinical settings. For example, fungal infections or mycoses such as candidiasis and aspergillosis can be difficult to diagnose due to non-specific clinical presentations and a biological diagnosis may take several days. Consequently, Ouanes et al. (2013) sought to investigate the diagnostic ability of CHROMagar® *Candida* versus Sabouraud media as a primary culture media. They also assessed the ability of CHROMagar *Candida* to differentiate between the various clinically relevant species of fungi in a hospital setting. Of the samples tested, 41.6% were positive for fungi, with 50.5% of isolates identified as *C. albicans* followed by 25.1% identified as *C. glabrata*. Fungal species determination was confirmed by chlamydosporulation testing and carbohydrate assimilation tests. The specificity of CHROMagar *Candida* agar was found to positively identify *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. krusei* with the following accuracy 95.3, 96.5, 100 and 88.6%, respectively. However, this method

was found to have some limitations; the sensitivity of the CHROMagar *Candida* was 88.4% for *C. albicans* and only 66% for *C. tropicalis*. Furthermore, the media supported the growth of certain bacterial species, including *Providencia stuartii* and *Klebsiella pneumoniae*. CHROMagar® was also used to assess the presence of different *Candida* spp. present in infant faecal samples (Heisel et al. 2015). Of the 11 faecal samples tested, only two yielded fungal growth and these fungi were identified as *C. albicans* and *C. parapsilosis*. However, it is not clear if the low abundance of fungal species isolated by culture in this case was due to the choice of media or a genuine reflection of low fungal diversity in the samples tested. On this note, another important aspect of cultivation is to provide quantitative estimates of different fungal species present. Surprisingly, this data is not given in many papers; however, mean fungal counts in healthy individuals have been shown to range from  $10^2$ – $10^6$  CFU  $g^{-1}$  (Scanlan and Marchesi 2008).

Finally, the capacity to culture and isolate fungi from the human gut is also crucial to advancing our knowledge of the wealth of genetic data encoded in fungal genomes and to provide insight into their functionality within the gut ecosystem. Unfortunately, the number of publicly available sequenced fungal genomes of gut origin is considerably lower than that of bacteria (Qin et al. 2010). Even in large, multicentre studies such as the Common Fund's Human Microbiome Project program (FY2007-2015), the primary focus is on bacteria with considerably less eukaryotic reference genomes being produced (Human Microbiome Jumpstart Reference Strains Consortium et al. 2010). This lack of available fungal genomes, particularly of GI origin, leaves a major gap in our knowledge of the genetic information

and potential functionality encoded in the genomes of different fungal species inhabiting the human gut (see also Box 1).

### Box 1. Future Perspectives for Best Practice in Mycobiome Research

**Culture-independent methods:** Establish gold-standard practices for culture-independent studies to facilitate standardisation and comparative analysis of the mycobiome between different studies. Crucially this will require a consensus on DNA extraction methods, ITS-specific PCR primer choice and the adoption of best practice bioinformatics approaches using only high-quality databases. Fortunately, there is already a consensus on primer choice with the ITS recommended as the target region of choice. However, further progress will depend on the development of well curated, referenced, dedicated databases for mycological studies and the continued development of bioinformatic and computational tools that will allow us to analyse and parse large datasets.

**Culture-dependent methods:** The development of better culture-based approaches is integral to progressing mycobiome research. This is required to both isolate fungal species of interest for further experimentation, assign appropriate taxonomy and relevant phenotypic characteristics and also to generate reference genomes. A lack of available fungal genomes, particularly of GI origin, is a key gap in our knowledge of the genetic information and potential functionality encoded on fungal genomes that encompass the human mycobiome. Therefore, the requirement to increase the number of reference fungal genomes available in public databases needs to be addressed.

**Integrate disciplines:** The proper integration of all aspects of relevant research areas is needed in order to advance the study of the gut mycobiome in human health and disease. Such a multidisciplinary approach needs to include *inter alia* microbiology (including microbial ecology), traditional mycology, immunology and bioinformatics.

**Temporal data and intervention studies:** The inclusion of temporal data will allow us to monitor and analyse any changes in the mycobiome throughout the different stages of life (infant, adult, elderly), including sickness, as well as following various interventions e.g. diet and medical intervention. This is required to provide key information on a range of biotic and abiotic factors that could potentially affect the mycobiome together with providing the necessary information required to decide the best strategies for interventions to promote health and prevent the onset of disease.

### Culture-independent methods

The advent of molecular techniques has meant that scientists no longer have to rely solely on the ability to culture fungi for ecological surveys of diverse and vastly different habitats (Ott et al. 2007; Scanlan and Marchesi 2008; Buée et al. 2009; Adams et al. 2013; Lindahl et al. 2013). The application of techniques such as polymerase chain reaction (PCR), Sanger sequencing (Sanger, Nicklen and Coulson 1977) and more recently, next generation sequencing (NGS) technologies (Mardis 2013; Zoll et al. 2016) allows researchers to investigate and identify the microbes present in a habitat without the need for complex culturomics (Landlinger et al. 2009a; Shokralla et al. 2012). These

developments, together with the use of sample identifying barcode sequences, facilitate the sequencing and analysis of multiple samples at once (Binladen et al. 2007; Hoffmann et al. 2007; Parameswaran et al. 2007; Hamady et al. 2008; Berry et al. 2011). Further advances in computing capabilities have also led to the development and improvement of databases suitable for fungal analysis (Tedersoo et al. 2011). These include the UNITE and SILVA databases (Köljalg et al. 2005; Pruesse et al. 2007; Abarenkov et al. 2010), and bioinformatics pipelines such as Qiime (Caporaso et al. 2010), CloVR-ITS (White et al. 2013) and UPARSE (Edgar 2013).

This availability of relatively easy-to-use technologies means that high-throughput molecular-based studies can now be attempted with considerable ease and relatively low cost (Hamady and Knight 2009; Sboner et al. 2011). However, it is important to note that there are no gold standard approaches for the culture-independent analysis of the gut mycobiome and it is evident from published studies that a variety of different techniques have been used (Scanlan and Marchesi 2008; Iliev et al. 2012; Araujo 2014; Tang et al. 2015), including denaturing gradient gel electrophoresis (DGGE) and cloning (Scanlan and Marchesi 2008), NGS and qPCR (Heisel et al. 2015), and NGS (Tang et al. 2015). As methods vary considerably between studies and, to date, no comparative analysis of these methods has been undertaken one obvious question that arises is to what extent one can compare data obtained from different studies. This is particularly pertinent given that different methodologies are known to influence the results of culture-independent studies of the gut bacteria. Moreover, a recent comparative analysis of methods used to assess soil fungi has shown that different methodologies do influence the results and, consequently, the conclusions of the study (Liu et al. 2015). As such, when designing any sequence-based study, thorough research and prior knowledge of the various strengths and weaknesses of each technique and technology available is required (Mardis 2008). In the following sections, we review and critique the various culture-independent methodologies that have been used to study the human gut mycobiome.

### DNA extraction

The DNA extraction method used for any study is of critical importance when considering not only the design of a study (Bahl, Bergström and Licht 2012; Sartor 2015) but also when comparing data from other studies that have used different extraction methods (Henderson et al. 2013; Lozupone et al. 2013). To date, a wide range of different extraction methods have been used to recover total microbial and fungal DNA from faecal (Scanlan and Marchesi 2008; Dridi et al. 2009; Salonen et al. 2010; Wu et al. 2010; Iliev et al. 2012) and mucosal samples (Ott et al. 2004b). Considerable efforts have also been made to improve the efficacy of extraction of DNA from both digesta and faecal samples (Yu and Morrison 2004).

The structure of fungal cells (which includes a cell wall primarily composed of chitin, glucans, mannans and glycoproteins (Smits et al. 1999)) can greatly impact on both DNA yield and quality as it can be notoriously difficult to lyse (Henderson et al. 2013). One method or commercial kit may extract total DNA relatively efficiently but may fail to extract fungal DNA and thus the presence of fungi in the gut may be underestimated (Henderson et al. 2013). Consequently, a number of modifications have been made to commercially available DNA extraction kits including the addition of bead beating (Scanlan and Marchesi 2008; Chen et al. 2011) and the addition of an enzymatic, fungal-specific lysis step (Landlinger et al. 2009b; Iliev et al. 2012). Unfortunately, the

relative merits (in terms of DNA quality, quantity and species recovered) of these different modifications are not known.

Another potential problem with DNA extractions is contamination with inhibitory substances from the original starting material (Monteiro et al. 1997; Schrader et al. 2012) which can cause problems downstream when using molecular techniques such as PCR. The presence of contaminating host DNA and DNA from ingested food can also be problematic (Gouba, Raoult and Drancourt 2013; Hoffmann et al. 2013). A recent study sought to remove contaminating host DNA from extractions using an initial separation of vertebrate DNA from the microbial DNA on the basis of differences in abundance of CpG methylation (Feehery et al. 2013). This method was successful for DNA samples from human saliva, human blood, a mock malaria-infected blood sample and black molly fish. Although this study focused on removal of contaminating sequences for bacterial community analysis, research in this area could prove beneficial for future gut mycobiome analyses. Finally, the potential effect of freezing on fungal DNA recovery is another consideration (Scanlan and Marchesi 2008; Chehoud et al. 2015) as extensive work on the bacterial fraction of the gut microbiome has shown that freezing of samples prior to DNA extraction can significantly alter the community structure resulting in a higher *Firmicutes* to *Bacteroidetes* ratio (Bahl, Bergström and Licht 2012). Conversely, it has also been shown that freezing prior to extraction has no significant effect on bacterial community structure (Wu et al. 2010; Fouhy et al. 2015).

### PCR and primer choice

Subsequent to DNA extraction, a primary prerequisite of most culture-independent methods is to perform PCR on the extracted DNA. As outlined, several different culture-independent techniques have been used to investigate the gut mycobiome (Scanlan and Marchesi 2008; Chen et al. 2011; Hamad et al. 2012; Tang et al. 2015) and a range of different primers are available and have been used for fungal community analysis in environmental surveys (Anderson and Cairney 2004; Gadanho and Sampaio 2006; Lee, Lee and Young 2008; Bokulich and Mills 2013) including the human gut mycobiota, see Table 3. These primers target different regions of the fungal ribosomal DNA (rDNA) operon which includes the 26S, 5.8S, 18S and the ITS regions (Iwen, Hinrichs and Rupp 2002). Although the 18S region has been widely used in the study of fungi and other eukaryotes in the human GI tract (Scanlan and Marchesi 2008; Hamad et al. 2012; Gouba, Raoult and Drancourt 2013), 18S primers are known to amplify non-fungal species including contaminating DNA from food or from the host (Scanlan and Marchesi 2008; Hamad et al. 2012). Identification of fungi based on 18S sequencing is also difficult as it is often limited to genus or family level classification. This is due to a lack of variation in 18S rRNA sequence between fungal species that are closely related, reflecting the comparatively short period of evolution within the fungal kingdom versus the bacterial kingdom, for which the 16S rRNA gene is employed (Hugenholtz and Pace 1996).

Fortunately, a consensus regarding the preferred target or barcode for the identification of fungal species for future studies has been reached (Nilsson et al. 2009; Seifert 2009; Begerow et al. 2010; Bellemain et al. 2010; Stockinger, Krüger and Schüßler 2010; Gazis, Rehner and Chaverri 2011; Lindner and Banik 2011; Porter and Brian Golding 2011; Liu et al. 2012; Schoch et al. 2012) and it is now acknowledged that the fungal ITS region should be used (Hawksworth et al. 2011; Bates et al. 2013). The change in target region was justified in part by the presence of highly con-

served, structural regions that flank the fungal ITS region allowing for the development of primer pairs that can amplify fungal rDNA from most species. The ITS also contains sequences that are highly divergent in both sequence similarity and length between fungal species and are sufficiently different to classify fungi to species level in most cases (Hibbett et al. 2011; Yahr, Schoch and Dentinger 2016). The fungal ITS region has been targeted in environmental studies (O'Brien et al. 2005; Krüger et al. 2009; Nilsson et al. 2009; Öpik et al. 2009; Neher et al. 2013) and consequently, it is the most frequently sequenced region of the fungal genome accounting for approximately one third of the publicly available fungal sequences to date (Underhill and Iliev 2014). It consists of two regions, ITS1 and ITS2, that are separated by the conserved 5.8S region. Each region may be amplified together or separately and identifying which region has the better resolution to species level has been the focus of a number of studies (Iwen, Hinrichs and Rupp 2002; Lutzoni et al. 2004; Martin and Rygielwicz 2005; Mello et al. 2011; Bazzicalupo, Bálint and Schmitt 2013). However, there has yet to be a NGS-based investigation of human gut samples in which the merits of different ITS subregions have been explicitly tested and it is also worth noting that utilising a smaller target amplicon for bacterial community analysis results in more unique sequences, higher diversity and thus a more varied community structure (Huber et al. 2009).

An extensive diversity of fungal species have been detected in gut samples using primers that target the 18S and ITS regions (e.g. see Figs 2–4, Table 3 and Table S1, Supporting Information). The first primers to target the fungal ITS region were developed over two decades ago (White et al. 1990; Gardes and Bruns 1993) (Fig. 1), yet they are still a widely used primer set and have been utilised in a number of recent human mycobiome studies (Scanlan and Marchesi 2008; Hamad et al. 2012; Gouba, Raoult and Drancourt 2013; Hoffmann et al. 2013; Tang et al. 2015). Other primers have also been used to investigate the fungal diversity of the human gut mycobiome; however, it is difficult to fully assess the impact of primer choice for fungal studies of the gut as (outlined earlier) the experimental techniques used are not consistent across studies. Nonetheless, the effect of primer choice in determining species diversity present in the human gut mycobiome has been demonstrated by studies where the use of different primer sets on the same sample(s) has resulted in completely different data being produced from individual samples (Klingspor and Jalal 2006; Hamad et al. 2012; Toju et al. 2012; Heisel et al. 2015; Mar Rodríguez et al. 2015; Walters et al. 2015).

In studies of the human intestinal mycobiome primers targeting both the ITS1 (Hoffmann et al. 2013) and ITS2 (Heisel et al. 2015; Liguori et al. 2015) and entire ITS region (Scanlan and Marchesi 2008; Mar Rodríguez et al. 2015) have been used. Which region to target is in part largely related to the technologies available to each researcher, and reflects the fact that the entire fungal ITS region is generally considered too large to use for studies utilising NGS technology. For lower-throughput Sanger sequencing mycobiome investigations, the length of the amplicon for sequencing is generally not an issue. However, for NGS technologies, primers targeting the ITS1 region facilitated the successful utilisation of 454-pyrosequencing technology and revealed a diverse healthy human mycobiome (Hoffmann et al. 2013). Similarly, the use of the ITS2 region as a target in the study of the infant mycobiome resulted in the amplification and identification of a range of different fungi comparable to the diversity observed in bacterial studies of infants of the same age using the Illumina MiSeq sequencing platform (Heisel et al. 2015), see also Table S1. Considerable efforts have also been made

Table 3. Primers used in the investigation of fungi in the GI tract.

Primer pair	Target region	Primer sequence (forward) 5' → 3'	Primer sequence (reverse) 5' → 3'	Technology used	Sample type	Samples frozen prior to extraction Yes/No (Y/N)	Study
121F & 1147R	18S	CTG CGA ATG GCT CAT TAM AA	GAC GGT ATC TRA TCG TCT TT	Cloning & sequencing (C&S)	Faecal	Y	Hamad et al. (2012)
18S_0067a_deg & NSR 399	18S	AAG CCA TGC ATG YCT AAG TAT MA	TCT CAG GCT CCY TCT CCG G	454 pyrosequencing (454)	Faecal	Y	Dollive et al. (2012)
18SF & 5.8S1R	ITS1	GTA AAA GTC GTA ACA AGG TTT C	GTT CAA AGA YTC GAT GAT TCA C	454	Faecal	Y	Strati et al. (2016b)
AmiF & AmiR	18S	NA <sup>a</sup>	NA <sup>a</sup>	C&S	Faecal	Not stated	Strati et al. (2016a) Gouba, Raoult and Drancourt (2014a)
E528F & Univ1391	18S	CGG TAA TTC CAG CTC C	ACC TTG TTA CGR CTT	C&S	Faecal	Y	Hamad et al. (2012)
EK1F & EK-1520	18S	CTG GTT GAT CCT GCC AG	CYG CAG GTT CAC GTA C	C&S	Faecal	Y	Hamad et al. (2012)
(GC)Euk1209f & Uni1392r	18S (nested)	CAG GTC TGT GAT GCC C	ACG GGC GGT GTG TRC	DGGE, C&S	Faecal	Y	Nam et al. (2008)
Euk1a & Euk 516r	18S	CTG GTT GAT CCT GCC AG (same as EK1F)	ACC AGA CTT GCC CTC C	DGGE, C&S	Faecal	Y	Scanlan and Marchesi (2008)
EUKA & EUKB	18S	AAC CTG GTT GAT CCT GCC AGT	TGA TCC TTC TGC AGG TTC ACC TAC	C&S	Faecal	Y	Gouba, Raoult and Drancourt (2014a)
FSeq & RSeq	ITS2	ATG CCT GTT TGA GGC TC	CCT ACC TGA TTT GAG GTC	C&S Illumina Miseq	Faecal	Y	Gouba, Raoult and Drancourt (2013) Hamad et al. (2012) Pandey et al. (2012)
FunF & FunR	18S	GAT CCC TAG TCG GCA TAG TT	GTA GTC ATA TGC TTG TCT C (same as for NS1)	C&S	Faecal	Y	Hamad et al. (2012) Nam et al. (2008) Heisel et al. (2015)
				C&S	Faecal	Y	Gouba, Raoult and Drancourt (2014b) Gouba, Raoult and Drancourt (2014a)
				C&S	Faecal	Y	Hamad et al. (2012)

Table 3. (Continued).

Primer pair	Target region	Primer sequence (forward) 5' → 3'	Primer sequence (reverse) 5' → 3'	Technology used	Sample type	Samples frozen prior to extraction Yes/No (Y/N)	Study
ITS1F & TW13 and ITS1F & ITS4	ITS (nested whole ITS)	CTT GGT CAT TTA GAG GAA GTA A	TCC TCC GCT TAT TGA TAT GC (ITS4) GGT CCG TGT TTC AAG ACG (TW13)	454	Faecal	Y	Hallen-Adams et al. (2015)
ITS1F & ITS2	ITS1	CTT GGT CAT TTA GAG GAA GTA A	GCT GCG TTC TTC ATC GAT GC	Illumina HiSeq	Faecal Biopsy	Y Y	Suhr, Banjara and Hallen-Adams (2016) Luan et al. (2015)
ITS1F & ITS4	ITS (whole ITS)	CTT GGT CAT TTA GAG GAA GTA A	TCC TCC GCT TAT TGA TAT GC	Ion torrent PGM 454 454 454 Illumina MiSeq Illumina MiSeq C&S	Faecal Faecal Faecal Faecal Faecal Faecal Faecal	Y Y Y Y Y Y Y	Hoarau et al. (2016) Hoffmann et al. (2013) Dollive et al. (2012) Chehoud et al. (2015) Frykman et al. (2015) Sangster et al. (2016) Gouba, Raoult and Drancourt (2014a)
ITS2	ITS2	GTT ART CAT CGA ATC TTT	GAT ATG CTT AAG TTC AGC GGG T	C&S 454	Faecal Faecal	Y Y	Scanlan and Marchesi (2008) Gouba, Raoult and Drancourt (2013) Hamad et al. (2012) Sokol et al. (2016)
ITS3 & ITS4	ITS2	GCA TCG ATG AAG AAC GCA GCnbsp GTT GAT CCT GCC AGT ATT ATA TG	TCC TCC GCT TAT TGA TAT GC CACT ATT GGA GCT GGA ATT AC	C&S 454	Mucosa Faecal	Y Y	Liguori et al. (2015) La Tuga et al. (2011)
JVF & DSPR2	18S			C&S	Faecal	Y	Gouba, Raoult and Drancourt (2014a) Gouba, Raoult and Drancourt (2014b) Gouba, Raoult and Drancourt (2013)
MalF & MalR (also called MF & MR)	28s	TAA CAA GGA TTC CCC TAG TA	ATT AGC CCA GCA TCC TAA G	C&S Direct sequencing C&S	Faecal Faecal	Y Y	Hamad et al. (2012) Gouba, Raoult and Drancourt (2014b) Gouba, Raoult and Drancourt (2014a) Gouba, Raoult and Drancourt (2013)
				C&S	Faecal	Y	Gouba, Raoult and Drancourt (2014a)
				C&S	Faecal	Y	Gouba, Raoult and Drancourt (2013)
				C&S	Faecal	Y	Gouba, Raoult and Drancourt (2014a)
				C&S	Faecal	Y	Gouba, Raoult and Drancourt (2013)
				C&S	Faecal	Y	Hamad et al. (2012)

Table 3. (Continued).

Primer pair	Target region	Primer sequence (forward) 5' → 3'	Primer sequence (reverse) 5' → 3'	Technology used	Sample type	Samples frozen prior to extraction Yes/No (Y/N)	Study
Unnamed pair 1	<i>Candida</i> specific	TTG GTG GAG TGA TTT GTC TGC T	TCT AAG GGC ATC ACA GAC CTG	qPCR for <i>Candida</i> spp.	Faecal	Y	Gosiewski et al. (2014)
Unnamed pair 2	ITS (nested whole ITS)	AGG TCA GGA TCA ACG CTC AAG (Primer set A: H1SeqF)GTC ATT TAG AGG AAG TAA AAG TCG TAA CAA GG (Primer set B: H2SeqFb) GCA TCG ATG AAG AAC RYA GC GTA GTC ATA TCG TTG TCT C	CAT CTT GCA TGA TCC AAC CTT C (Primer set A: H1SeqRb) GCT RYG TTC TTC ATC GDT GC (Primer set B: H2SeqRb) TTC TTT TCC TCC GCT TAT TGA TAT GC	454	Faecal	Y	Mar Rodriguez et al. (2015)
NS1 & FR1	18S (nested PCR)		AIC CAT TCA ATC GGT AIT	DGGE, C&S	Faecal and mu-cosal	Y	Li et al. (2014)
NS1 & Unnamed	18S			DGGE, C&S	Faecal	Not stated	Li et al. (2012)
U1 & U2-GC	28S	GTA GTC ATA TCG TTG TCT C GTG AAA TTG TTG AAA GGG AA	GAT CCC TAG TCG GCA TAG TT (same as for FunF) GAC TCC TTG GTC CGT GTT	DGGE, C&S 454	Faecal Biopsy	Y	Hamad et al. (2012) Mukhopadhyaya et al. (2014)
fITS7 & ITS4	ITS2	GTG ART CAT CGA ATC TTT G	TCC TCC GCT TAT TGA TAT GC	DGGE, C&S	Biopsy and faecal	Y	Ott et al. (2008)
Not applicable (N/A)		TruSeq method for shotgun sequencing		RFLP, C&S	Faecal	Y	Chen et al. (2011)
				DGGE and C&S	Faecal	Y	Stewart et al. (2013)
				Illumina MiSeq	Faecal	Y	Fujimura et al. (2016)
				Illumina HiSeq	Faecal	Y	Mar et al. (2016)
					Faecal	Y	Lewis et al. (2015)

<sup>a</sup>Unable to recover information.

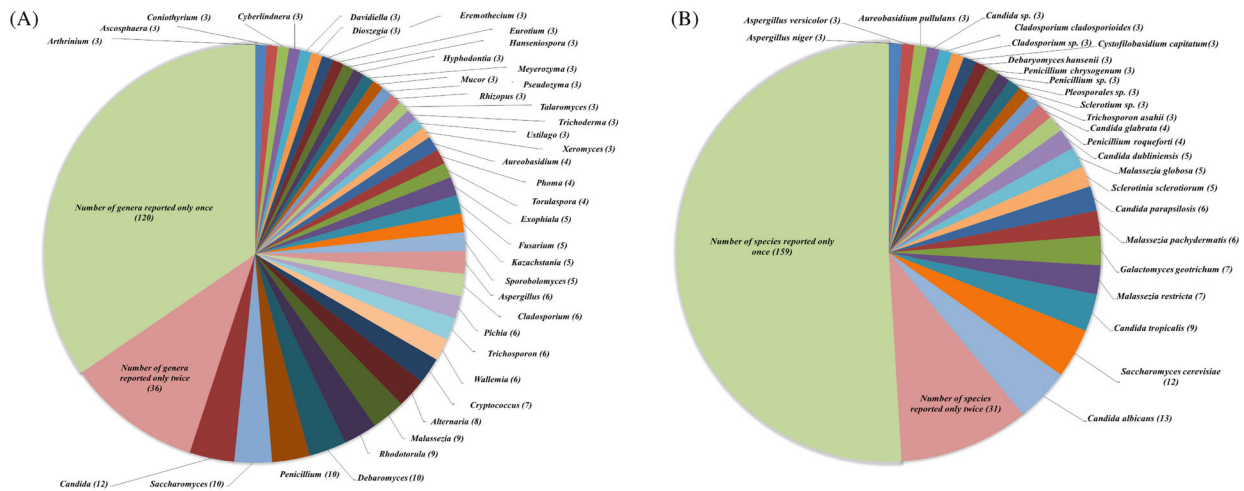


Figure 4. A diversity of different fungal genera (A) and species (B) have been reported in culture-independent studies of the gut mycobiome (see also Table S1). All genera and species that have only been reported in the literature once, and twice, have been included as single groups, respectively.

to identify primers that reduce bias and reduce errors in the sequences that are produced by PCR-based analyses of fungal populations (Borneman and Hartin 2000; Bokulich and Mills 2013; Tedersoo and Lindahl 2016). This is important as single primer mismatches to target sequences can have a significant impact on the sequence data generated (Ihrmark et al. 2012). Indeed, a recent computational analysis identified mismatches between the commonly used primers, ITS1F and ITS2, and clinically relevant fungal taxa and led the authors to advocate the use of modified sets of primers that target the ITS2 region for NGS analysis (Tedersoo and Lindahl 2016).

Issues with PCR-based analyses that have been noted for bacterial microbiome studies are also highly relevant to mycobiome studies. For example, the PCR reaction itself is prone to a certain degree of bias and error (Ishii and Fukui 2001; Engelbrektson et al. 2010; Carlsen et al. 2012) and the use of PCR can in fact result in the reporting of species that simply do not exist due to artefact formation (Kanagawa 2003; Fonseca et al. 2012). Barcoded adapters that allow the sequencing of many different samples at once can also introduce error (Jayaprakash et al. 2011; Faircloth and Glenn 2012) as can the number of cycles used in the PCR reaction. It has been shown for bacteria that, regardless of the ratio of the different templates used in a PCR reaction, after 35 PCR cycles the product ratio was biased towards an equal ratio (Kanagawa 2003). This is also relevant to human GI mycobiome studies where the starting PCR reaction mixture potentially contains many different fungal species DNA templates present at varying concentrations for amplification (Scanlan and Marchesi 2008; Heisel et al. 2015). Other errors can also arise during the PCR reaction including the formation of homoduplexes, heteroduplexes and chimeric sequences (Kanagawa 2003). These biases and PCR errors occur primarily during the later cycles in a PCR reaction and thus can be limited by reduction of the cycle number. The possibility of random errors from PCR artefacts, such as the wrong base being incorporated or primer mismatches to the template, can also be minimised by reducing the number of cycles, performing multiple PCR reactions and pooling the products prior to sequencing (Brown et al. 2013; Schmidt et al. 2013).

### Sequencing technologies

As noted, the increasing numbers of studies relating to the human mycobiome (Fig. 1) have been facilitated by the devel-

opment of high-throughput NGS technologies that have vastly improved researchers' abilities to perform in-depth analysis of complex ecosystems without the need to culture organisms (Bik et al. 2012; Hoffmann et al. 2013; Heisel et al. 2015). Previously, limitations in methods such as the laborious preparation of clone libraries limited the number of samples that could be analysed at any time resulting in potentially low coverage within samples (Ott et al. 2008; Scanlan and Marchesi 2008; Hamad et al. 2012). Despite these limitations, low-throughput molecular analyses bypassed the need to culture fungi to assess diversity and have successfully unearthed a wealth of previously unknown species (Borneman and Hartin 2000) and provided novel insight into the fungal diversity of the human gut (Ott et al. 2008; Scanlan and Marchesi 2008; Hamad et al. 2012). Although this approach is unsuitable for high-throughput analysis of DNA extracted from materials containing potentially complex mixtures of species such as in faecal samples, Sanger sequencing has been useful for the production of large reference libraries of DNA and it is anticipated that the application of newer, long read NGS technologies, such as those developed by PacBio and Oxford Nanopore, will be similarly useful in this regard.

Regardless of read length, the arrival of NGS technology (as reviewed elsewhere (Glenn 2011; Buermans and den Dunnen 2014; van Dijk et al. 2014)) affords researchers a high-throughput alternative for mycobiome studies (Heisel et al. 2015; Tang et al. 2015) due to the ability to bypass the need to use a vector-based cloning method to amplify and separate different DNA templates. A range of commercial NGS technology platforms are available to researchers planning to undertake mycological surveys, each with different capabilities and utilising different sequencing methods. These include diverse chemical methods that vary in the incorporation and also the detection of bases. Despite this, however, each NGS platform essentially shares the same workflow of an initial step of either fragmentation or amplicon library production.

The advent of NGS platforms began with the release of the Roche 454 genome sequencer in 2005 (Margulies et al. 2005), which utilises real-time sequencing-by-synthesis pyrosequencing technology. This was the first commercially available NGS platform and it has been utilised successfully in the sequencing of fungal libraries from niche environments (Jumpponen and Jones 2009; Öpik et al. 2009; Jumpponen et al. 2010; Marsh et al. 2014) and the human gut (Hoffmann et al. 2013).

However, Roche 454-pyrosequencers are no longer produced as the cost per run became considerably more than that of the subsequent generation of commercially available sequencers. A competing genome sequencer, which generated shorter reads, was released in 2007 by Illumina (formerly known as Solexa). These have since been replaced by newer Illumina sequencers, such as the HiSeq, NextSeq and MiSeq, which also rely on the same sequencing-by-synthesis approach as their predecessor but provide longer read lengths and reduced costs/Mb. The Illumina MiSeq was recently investigated for its suitability in the analysis of human mycobiome investigations of the GI tract using mock communities (Tang et al. 2015) as well as being used successfully to investigate the mycobiome of infants (Heisel et al. 2015). Ion Torrent, now owned by Life Technologies, released the Ion Personal Genome Machine (PGM) in 2010. The Ion PGM relies on the real-time detection of H<sup>+</sup> ions released as a by-product of a nucleotide attaching to the template single-stranded DNA. To the authors' knowledge, this platform has only been used once in a standalone analysis of the human gut mycobiome. It has also been used in the analysis of bacterial metagenomic sequencing (Whiteley et al. 2012) and environmental fungi (Kemler et al. 2013) and been tested for its potential for gut mycobiota analysis where it was found to be inferior to MiSeq (Tang et al. 2015). More specifically, Tang and colleagues reported a distorted read-out of their mock fungal community structure when sequenced using the PGM compared to that observed when sequenced using the MiSeq platform. Although the MiSeq did also incorporate some bias towards fungi with smaller ITS1 regions, it was considerably less than that observed on the Ion Torrent PGM. To date, other sequencers from Ion (Proton; similar to PGM but with greater outputs), PacBio (RSII) or Oxford Nanopore (MinION) have not been used for mycobiome research.

Despite the advantages of NGS technologies, they have their own particular challenges when applied to mycobiome studies. This was demonstrated in a recent non-human fungal study (Marsh et al. 2014) where variation in the length of the fungal ITS region between species required the sequencing libraries to be pooled based on size and sequenced separately in order to avoid the preferential sequencing of shorter amplicons. This variation in amplicon length within samples or 'double bands' that are seen on agarose gels and Bioanalyser traces was also reported by Tang et al. (2015). In the latter instance, all samples were sequenced together regardless of amplicon size.

### Bioinformatic analysis

There have been a variety of bioinformatics and analysis tools developed to enable the analysis of the enormous amount of data produced by large-scale studies utilising NGS technologies (Bik et al. 2012; Ursell et al. 2012). The data produced by NGS must undergo a number of important steps before it can be compared to the various databases for identification with any reliable degree of accuracy (Kunin et al. 2008). The sequences produced must be demultiplexed by sample ID, forward and reverse reads must be joined, adapter and barcode sequences removed and the sequences quality trimmed prior to further analysis (Bokulich et al. 2012). Subsequent steps include the removal of chimeric sequences (Edgar et al. 2011) using packages developed based on fungal ITS datasets (Nilsson et al. 2010, 2015). The data are then analysed to determine, as accurately as possible, the diversity and relative abundance of species present (Edgar 2004; Schloss et al. 2009; Amend, Seifert and Bruns 2010).

One of the most frequently used bioinformatic tools for the high-throughput analysis of fungal community data is the Qi-

ime pipeline, as described by Caporaso et al. (2010). This pipeline has been used to decode and quality check fungal sequences produced in a human gut mycobiome study (Hoffmann et al. 2013). Taxonomy was assigned to the sequences using BROCC (Dollive et al. 2012) before being manually curated using the BLASTn search function against the GenBank NR/NT database. Although the databases available for fungi are not as well established as those for bacterial analysis (Nilsson et al. 2006), this issue is being addressed (Vilgalys 2003; Tang et al. 2015). Several studies and reviews have focused on the merits of the databases and pipelines that are currently available (Koetschan et al. 2010, 2012; Nilsson et al. 2012; Santamaria et al. 2012; Schoch et al. 2014; Tang et al. 2015). The most recent study compared three publicly available fungal databases: the UNITE database (Kõljalg et al. 2005), the Findley database (Findley et al. 2013) and the RTL database (Schoch et al. 2014). The authors also manually created their own database (targeted host-associated fungi database) which they tailored to specifically include sequences from mouse and human samples (Tang et al. 2015). The choice of database utilised for the analysis clearly affected the outcomes of the study as evidenced by considerable differences in community compositions. This was especially apparent when the samples were also analysed by qPCR to target particular fungal species. The database used also determined the number of reported species; this is due to the relative presence or absence of particular species in different reference databases. Moreover, differences in community composition also arose from annotation issues such as those observed, for example, when using the UNITE database which identified the presence of both *Fusarium* and *Gibberella*, even though these are anamorph/telomorph names for the same fungal species. With this in mind, caution is required in the reporting of novel fungal species from sequence-based mycobiome studies alone. Although culture-independent techniques have enabled researchers to uncover a wealth of fungal species in the human gut (e.g. Table S1), a failure to respect classical mycology when undertaking mycobiome analyses has led to skewed and incorrect estimations of fungal diversity resulting in numerous revisions (Hawksworth and Rossman 1997; Hawksworth 2001, 2004; Peay, Kennedy and Bruns 2008; Blackwell 2011; Suhr and Hallen-Adams 2015). As such, it is now realised that further progress in gut mycobiome research requires the application and development of classical mycological techniques coupled with culture-independent studies in order to accurately determine the true identity, diversity and functionality of fungal species within the human gut mycobiome (Suhr and Hallen-Adams 2015; Yahr, Schoch and Dentinger 2016) (see also Box 1).

## DIVERSITY AND COMPOSITION OF THE HUMAN MYCOBIOME IN THE GENERAL POPULATION

One of the first studies that investigated the prevalence and diversity of microbial eukaryotes in the healthy human gut was published in 2008 (Scanlan and Marchesi 2008). Using a combination of culture-dependent and culture-independent methodologies, Scanlan and Marchesi detected fungi in 88% of individuals sampled (n = 17). Fungal diversity was low and colonisation was largely stable over time with the occasional transient species recovered. Of note, three species of fungi, i.e. *Galactomyces*, *Paecilomyces* and *Gloeotinia*, were detected in the faecal samples of the study participants and were stable inhabitants over time, a previously unreported occurrence in the

healthy population. Subsequently, a number of similar studies have been published and have revealed a plethora of taxa comprising the gut mycobiome (Figs 1, 2 and 4).

Hamad *et al.* (2012) also sought to investigate the diversity of total microbial eukarya in the healthy human gut; however, they only used a single faecal sample from a healthy Senegalese man. This study used 22 different primer sets (Table 3), some of which targeted specific fungi, such as *Malassezia* spp., and others that targeted all eukaryotes potentially present. Although 27 eukaryotic species were detected, fungi predominated, with 16 different fungal species observed (see Table S1). Further work by Hoffmann and colleagues also reported that the healthy human gut is host to a diverse range of fungi and further expanded upon the range of species detected in these earlier studies (Scanlan and Marchesi 2008; Hamad *et al.* 2012) (Table S1). More recently, Hallen-Adams and colleagues used NGS technology and a nested PCR approach using primers targeting the ITS region and successfully amplified products from 45 of the 49 study participants. Using this method, 72 operational taxonomic units (OTUs) were recovered and comprised human, airborne and food-associated fungi (Hallen-Adams *et al.* 2015). In contrast to the earlier study (Scanlan and Marchesi 2008), Hallen-Adams found that the gut mycobiome lacked persistent, stable or 'core' species that are typical of bacterial communities in the gut (Lozupone *et al.* 2012). This suggests that a targeted approach such as a probe-based assay might be a more rational approach given that a large proportion of fungi detected in faecal samples may be of dietary origin and therefore are not functionally relevant in the gut environment (Suhr, Banjara and Hallen-Adams 2016).

The infant gut has also been the target of mycobiota research and a relatively recent study has shown that fungi are present in the GI tract of a vaginally delivered and exclusively breast-fed infant (Koenig *et al.* 2011). This result was determined by shotgun sequencing and even though this study was performed on a healthy infant, it was observed that fungal- and viral-associated genes were enriched in a sample immediately preceding a fever, leading the researchers to suggest that this finding may be linked to the fever. Quantitative PCR (qPCR) has also been used to assess fungal prevalence in the infant gut, and researchers have shown that fungi were intermittently detectable in faecal samples at a relatively low abundance over infants' first year of life (Palmer *et al.* 2007). Another study combined NGS technology with qPCR (targeting nine clinically relevant fungi) to survey for fungi in a cohort of 11 infants. Ten of the 11 infants did not have a diagnosis of fungal infection and although only 2 infants were positive for fungi using culture-dependent methods, all infants were positive for the presence of fungi using culture-independent methods (Heisel *et al.* 2015). Species diversity varied between infants; however, all of the infants were positive for *Candida albicans* and the most abundant fungi were *C. albicans*, *Leptosphaerulina* and *C. parapsilosis*. An infant that developed mucocutaneous candidiasis was observed to have the lowest diversity of all the samples collected and, interestingly, this was also the only sample that was within the limits of detection for qPCR analysis. This further highlights the difficulties of detecting organisms such as fungi that are potentially present in human samples in low levels. Moreover, this study highlights how little is known about the fungal diversity present in infants and draws parallels between the fungal diversity shift that they observed with that seen in the bacterial microbiome of infants of the same age (Heisel *et al.* 2015). The researchers hypothesised that the relatively low diversity of fungi observed in comparison to that of adult samples was due to the age of the

study participants and the possibility that these infants may not yet have been colonised by a variety of different fungal species. Conversely, a recent study has shown that age and gender influence the composition of the gut mycobiome; here species richness of the gut mycobiome (i.e. the number of different OTUs recovered) was higher in infants and children than in adults when samples were analysed using culture-independent methods (Strati *et al.* 2016b). However, no significant age-related difference was observed from data generated using a culture-based approach. Strati and colleagues also reported the predominance of *Penicillium*, *Aspergillus* and *Candida* in their subset of healthy participants, as have also been detected in other studies (Fig. 4 and Table S1). They also investigated the effect of gender on the mycobiome and found a significant effect on the cultivable mycobiota with female subjects showing both a higher number of isolates and also species reported compared to males. Moreover, samples clustered based on gender when analysed by culture-independent analysis (Strati *et al.* 2016b).

In a study investigating the impact of the mycobiome in neonates (1 month old) on asthma development at infancy (6 months old), it was observed that bacterial  $\alpha$ -diversity was inversely correlated with fungal  $\alpha$ -diversity and both bacterial and fungal  $\beta$ -diversity were correlated with participant age. A depletion in the fungal taxa *Malassezia* and an increase in *Rhodotorula* and *Candida* correlated with a number of bacterial taxa depletions, and the overall effect was correlated with a higher risk for the development of childhood allergic asthma (Fujimura *et al.* 2016). These results led the authors to suggest an inter-kingdom co-evolution over the first year of life (Fujimura *et al.* 2016); however, further research that incorporates temporal analysis of neonates through to adulthood is required to determine if the fungal mycobiome undergoes a dramatic change equivalent to that observed for the bacterial and viral microbiomes over the first years of life (Lim *et al.* 2015; Rodríguez *et al.* 2015). Crucially, these data may also provide potentially useful information that is needed to inform us of both the timing and type of best strategies for targeted interventions to prevent the onset of disease.

## THE EFFECT OF DIET AND EATING BEHAVIOUR ON THE MYCOBIOME

The effect of diet as a key factor driving the variation observed in fungal colonisation and composition between individuals was the focus of early gut mycobiome studies (Finegold, Attebery and Sutter 1974) (Fig. 1). Research in this area looked at groups consuming a Western and a traditional Japanese diet with a view to understanding the difference in bowel cancer prevalence observed in each location (Finegold, Attebery and Sutter 1974). Little difference was observed between the fungi recovered from each group despite the differences in diet; however, the scope of the study was greatly limited by the use of a single culture medium which was incubated aerobically (Finegold, Attebery and Sutter 1974).

The relationship between diet and the mycobiome in healthy individuals has been investigated more recently using culture-independent analysis of samples from 96 individuals (Hoffmann *et al.* 2013). After strict exclusion criteria for volunteer health status and read number per sample were applied, 66 fungal genera, and 13 additional lineages which the authors were unable to classify to genus level, were detected in these samples (see Table S1). Although no conclusive evidence was provided as to whether these fungal inhabitants were permanent residents or transient species, it is notable that six of the individuals

analysed returned sequences originating from button mushrooms which can obviously be attributed to dietary contamination. The study also found that the most prevalent fungi in the gut were from the genus *Saccharomyces*, which was detected in 89% of samples. The authors did speculate that the high level of *Saccharomyces* could be due to the ingestion of yeast-containing foods such as bread and beer. Similarly, *Candida* species were found to correlate with the recent ingestion of carbohydrates. Second to *Saccharomyces*, *Candida* species were present in 57% of the samples, followed by *Cladosporium*, which was detected in 42% of samples. A more detailed analysis of the impact of diet on both bacterial and fungal diversity of the human gut partitioned dietary regimes into plant and animal-based diets and included an analysis of the food ingested by the study participants. Food items in both diets were colonised by a diversity of fungal genera including *Penicillium*, *Candida*, *Debaryomyces* and *Scopulariopsis*, and it was noted that *Penicillium* sp. and *Candida* sp., that were present in high levels within the animal and plant-based diets, showed significant ITS sequence increases in individuals sampled on these respective diets (David et al. 2013). Similarly, analysis of the mouse gut fungal mycobiome and contemporaneously ingested mouse chow revealed that a number of fungal species present in the chow survived GI transit and were detectable in ITS amplicons generated from mouse faeces (Iliev et al. 2012). Although, it is likely that a proportion of these fungi present in the gut microbiome that are of dietary origin are in fact only transient colonisers (Suhr, Banjara and Hallen-Adams 2016), emerging data do suggest that the intake of certain foods may provide an important source of inoculum of some fungal species that can colonise the human gut and that of other host species (David et al. 2013). Further work in this area is required to thoroughly assess and appreciate the contribution of diet and food-associated fungi to the diversity and functionality of the human microbiome.

In addition to diet as a source of microbial inoculum, the relationship between diet, our gut microbes and energy balance is an area of increasing interest given the current obesity epidemic (Turnbaugh and Gordon 2009). It is therefore not surprising that researchers have sought to investigate the role, if any, of the mycobiome in this regard. It was observed in two recent studies performed by Gouba and co-workers that an increased diversity of fungi were present in the gut of two respective study subjects, one of whom suffered from anorexia nervosa and the other who was classified as morbidly obese (Gouba, Raoult and Drancourt 2013, 2014a). Using a combination of culture-dependent and culture-independent methodologies, the gut microbiota of an obese Caucasian female with a BMI of 48.9 and an anorexic female with a BMI of 10.4 was determined. The results highlighted an increased fungal diversity in these individuals in comparison to studies of healthy participants reported in the literature. A variety of the fungal species detected could be attributed to food sources, including 11 of the 16 fungal species isolated in the obese individual (Gouba, Raoult and Drancourt 2013). However, it should be noted that these data should not be over-interpreted as only a single individual was analysed in each case. Fortunately, a study involving a greater number of obese individuals together with controls has been recently published (Mar Rodríguez et al. 2015). Although fungi were only classified to genus level, the researchers were able to draw several conclusions from their data. Fungi were detected in all samples (n = 52) using an ITS sequencing approach and although no differences in fungal richness were observed between the obese and control cohorts, family diversity was significantly lower in their obese cohort. In contrast to many other

studies into the human gut mycobiome, this study reported that *Penicillium*, present in 73% of samples, was the most prevalent fungal genus, followed by *Candida*, *Saccharomyces*, *Mucor* and *Aspergillus* at 55%, 55%, 38% and 35%, respectively (Mar Rodríguez et al. 2015). Although the taxonomic differences observed between the obese and control cohorts were not significant at the family level, significant differences were observed at the genus level for *Mucor*. The most abundant genera in the obese cohort were *Candida*, *Nakaseomyces* and *Penicillium*, whereas *Mucor*, *Candida* and *Penicillium* were the most abundant genera in the control cohort. Furthermore, it was observed that the genera *Mucor* and *Penicillium* negatively correlated with BMI, fat mass, android fat mass and hip circumference, whereas the genus *Aspergillus* was positively associated with adiposity. The correlation between *Mucor* spp. and leanness was investigated before and after weight loss induced by dietary change and it was observed that the relative abundance of *Mucor* spp. correlated with the degree of weight loss. However, further work is required however to determine if changes in *Mucor* spp. abundance is a contributor to or a consequence of weight loss. Moreover, further research in this area could potentially see dietary interventions that include the supplementation of host diet with foods with putative antifungal effects in order to selectively modulate the fungal profile with the ultimate aim of influencing host health (Gunsalus et al. 2016). Similarly, the use of specific microbes that can inhibit the growth of undesirable fungal species may prove another useful intervention strategy. In this regard, the negative correlation between the food-borne yeast *Debaryomyces hansenii* and *Candida* species in surveys of the gut mycobiome coupled to *in vitro* experimentation of the anti-*Candida* effects of mycotoxins produced by *D. hansenii* has led to the suggestion that *D. hansenii* could impact on *Candida* populations in the gut (Banjara et al. 2016). This is analogous to the rationale of using many species of bacteria as probiotics to mitigate the potentially harmful effects of specific gut microbes on host health.

## MYCOBIOME STUDIES BY HOST SITE

Although the primary focus of this review is to highlight the gut mycobiome and its associations with human health and disease groups, we have much to learn from the analysis of the mycobiome of various other human body sites. The methodologies and data emerging from many of the studies relating to these sites reviewed in brief below are pertinent to gut mycobiome-based investigations as they will greatly enhance our understanding of the relationships between the various fungal populations that make up the collective human mycobiome (Soll et al. 1991; Mukherjee et al. 2014). They will also better our understanding of the interactions between the fungal and non-fungal members of different microbial consortia (Krom, Kidwai and ten Cate 2014), while providing insights into the potential pathogenicity of these organisms, both in their niche environment and also when translocated to various other body sites, a phenomenon previously investigated with respect to certain bacterial species (Colucci 2015). With this in mind, we review in brief some of the significant papers from the literature relating to the oral, lung, skin, genitourinary and cardiovascular mycobiomes.

### The oral cavity

The oral cavity is an important inoculation source of fungi found elsewhere in the body, including the human gut

(Westbrook et al. 2007). Both culture-dependant and culture-independent methods, including NGS technologies, have been used to thoroughly characterise the basal oral mycobiome (Ghannoum et al. 2010; Dupuy et al. 2014). The results suggest that the distribution and profile of fungal species in the oral cavity of healthy subjects is complex and similar to that of the oral bacterial microbiome with respect to the number of species identified (Ghannoum et al. 2010). Similar to gut mycobiome studies, the portion of fungal species refractory to cultivation in the oral cavity represented a significant fraction of the total organisms identified (Ghannoum et al. 2010; Chen et al. 2011) and likewise, the oral mycobiome is dominated by *Candida* spp., which was isolated from 75% of subjects (Ghannoum et al. 2010). A related study also observed a complex and diverse basal mycobiome in the oral cavity (Dupuy et al. 2014). However, the latter found some differences in the genera present. These differences could be due to the methodologies used as results were presented at different taxonomic levels in each study or may be due to host-associated factors such as age, diet, overall health status or ethnicity. However, one interesting and perhaps surprising finding was that *Malassezia* was reported for the first time as an oral commensal (Dupuy et al. 2014). This genus has been previously associated with a variety of cutaneous disease conditions of the skin (Ashbee and Evans 2002; Sugita et al. 2002; Tajima et al. 2008).

Unsurprisingly, the human oral microbiome and mycobiome have predominantly been studied to assess their impact on oral health (Miranda et al. 2009; Wade 2013). For example, the diversity and frequency of yeasts from the dorsum of the tongue and necrotic root canals have been surveyed; however, no significant differences between colonisation of the tongue and root canal was observed. Although no direct correlation between the incidence of yeasts in the root canal and primary endodontic infection disease process was observed, a higher frequency of *Candida* spp. was noted (Miranda et al. 2009). Another relevant aspect of many studies is the role of environmental factors which can have a dramatic effect on the human mycobiome (Underhill and Iliiev 2014). These include smoking (Monteiro-da-Silva et al. 2013) and EDs (Back-Brito et al. 2012), such as anorexia nervosa and bulimia nervosa, which have all been investigated in relation to the human oral mycobiome.

The detrimental effects of smoking on the development and pathogenesis of many conditions has been described in depth (Stämpfli and Anderson 2009). A study conducted to characterise the oral mycobiome of smokers and non-smokers found that smokers have higher but not significantly different levels of both yeasts and pathogenic moulds than non-smokers. All study participants investigated in the study were colonised by fungi and subsequent analysis also showed that these fungal inhabitants were temporally stable in the oral cavity (Monteiro-da-Silva et al. 2013). Interestingly, another study noted that the incidence of *Candida* in stool is significantly correlated with smoking habits: 58% of smokers were found to have *Candida* present compared to only 29% of non-smokers (Jobst and Kraft 2006).

Given that the oral mycobiome can serve as both a reservoir of potentially pathogenic fungi and a portal for pathogens (Westbrook et al. 2007), the observation of chronic fungal colonisation by potentially pathogenic moulds such as *Penicillium*, *Cladosporium*, *Alternaria*, *Trichoderma*, *Scedosporium* and *Rhizopus* genera as well as opportunistic pathogens such as *Candida* and *Aspergillus* is of note (Monteiro-da-Silva et al. 2013). The link between alcohol and both the gut and oral mycobiome has also been investigated. Interestingly, and contrary to results observed

in the gut which do suggest a link between alcohol consumption and fungal colonisation (Hoffmann et al. 2013), analysis of the oral cavity suggest that the mycobiome is not influenced by alcohol consumption (or oral contraceptive and drug use) (Monteiro-da-Silva et al. 2013).

EDs can affect the oral mycobiome (Back-Brito et al. 2012) in a manner similar to that described for the bacterial microbiome (Lo Russo et al. 2008). ED associated factors include poor personal hygiene, the use of drugs, vomiting, modified nutritional habits, underlying psychological disturbances and nutritional deficiencies with consequent metabolic impairment (Lo Russo et al. 2008). Similarly, oral candidiasis has been linked to nutritional deficiencies in iron, zinc, vitamin K and water soluble vitamins (Lo Russo et al. 2008). Opportunistic oral infections, such as those caused by *Candida* spp., are common in those suffering from EDs; however, the mycobiome of these patients is not as well characterised as the healthy oral mycobiome (Ghannoum et al. 2010; Dupuy et al. 2014). A study seeking to rectify this disparity characterised the oral mycobiome of ED and healthy patients found that the ED group had a higher incidence of yeast colonisation, as well as a greater variability of *Candida* spp., than that observed in healthy controls (Back-Brito et al. 2012).

## The lung

Research into the lung microbiome has greatly increased in recent years and the data generated are dispelling some of long-standing views, including the perception of the deep lung as a sterile organ (Hilty et al. 2010). Correspondingly, the study of the lung mycobiome is now an emerging field of research (Nguyen, Viscogliosi and Delhaes 2015; Seed 2015; Krause et al. 2016). Research to date has been largely restricted to a small number of studies (Dynowska, Roslon and Góralaska 2006), with a strong focus on patients with cystic fibrosis (CF) (Amin 2010; Nelson et al. 2010; Delhaes et al. 2012; Harrison et al. 2012), pneumonia (Bousbia et al. 2012), lung transplants (Charlson et al. 2012) and asthma (van Woerden et al. 2013). The mycobiome of CF individuals is of particular note as sputum expectorated from the lungs can be swallowed and thus comes in contact with the digestive tract. Moreover, the link between the gut microbiome and lung was demonstrated using a murine model that showed perturbation of the mycobiome in the gut via antibiotics was instrumental in causing allergic airway disease. This was achieved through the modulation of the immune response and remote activation of macrophages causing a systemic allergic response (Kim et al. 2014).

A study investigating the microorganisms present in the lungs of transplant patients and healthy controls highlighted an inherent challenge in assessing the lung microbiome and mycobiome, as carryover of microbes from the oral cavity and upper respiratory tract invariably occurs when performing bronchoalveolar lavage (BAL) sampling (Charlson et al. 2012). It was observed that fungal level and species richness varied greatly between individuals irrespective of test or control status with respect to oropharyngeal wash (OW) samples. However, for healthy individuals, BAL samples returned very few fungal sequences even when they were present in OW samples at a high level from the same individual. Fungal species that were detected in controls were determined to be from environmental origin, whereas the BAL samples of transplant recipients were predominated by *Candida* and *Aspergillus* species. Moreover,

when present in BAL samples *Candida* was observed in OW samples from the same individual (Charlson et al. 2012).

### The skin

The skin is the largest organ of the human body and is colonised by a multitude of different microbiological species (Fierer et al. 2008; Grice et al. 2009; Findley et al. 2013). Similar to the study of the oral and gut mycobiomes, further research into the skin mycobiome is invaluable to our understanding of the basal structure and function of microbial communities as well as the potential for pathogenicity of microorganisms that inhabit a multispecies environment (Akaza et al. 2010). Such research is relevant to both healthy subjects (Gao et al. 2010) and those with skin conditions (Sugita et al. 2002; Park et al. 2012). Using the fungal ITS region and the 16S rRNA as target genes, the total fungal and bacterial populations of several different sample areas of the skin were assessed (Gao et al. 2010). *Malassezia* spp. predominated samples and accounted for 53%–80% of all fungi at the various locations sampled (Gao et al. 2010). *Malassezia* spp. have been implicated in the aetiology of dandruff, and research has been undertaken to not only determine whether this lipophilic yeast is involved in the aetiology of dandruff but also identify novel ways to prevent and treat dandruff (Park et al. 2012). The total fungal mycobiome of healthy and dandruff-afflicted human scalps was characterised using NGS, and it was shown that Ascomycota and Basidiomycota are the predominant fungal phyla found in dandruff-afflicted and healthy scalps. The predominance of these two fungal phyla has been noted in both the gut and oral mycobiome and, interestingly, the differences observed in the abundance ratio between Ascomycota and Basidiomycota in this study were dependent on the health status of the scalp. Ultimately, it was established that *Malassezia* spp. were elevated in dandruff-afflicted scalps (Park et al. 2012). Another study investigating the human skin mycobiome also implicates *Malassezia* as a compounding factor in the aetiology of atopic dermatitis (Zhang et al. 2011). Using a rRNA clone library approach to characterise the fungal community, Zhang et al. (2011) found that the populations clustered according to health status, i.e. mild/moderate, severe atopic dermatitis and healthy subjects.

### The genitourinary tract and its potential as a source of fungi for infant colonisation

The route via which humans are colonised by microbes is of fundamental interest from multiple perspectives. With respect to the human mycobiome, previous studies have sought to investigate the relationship between the gut, skin and vaginal mycobiomes of infants and their mothers (Waggoner-Fountain et al. 1996; Bliss et al. 2008; Nagata et al. 2012; Pandey et al. 2012; Song, Dominguez-Bello and Knight 2013). One approach used the various *Malassezia* species present as an indicator to determine the similarities between infant's mycobiomes and their respective mothers and found that at day 1 100% of infants surveyed ( $n = 27$ ) were colonised by *Malassezia* (Nagata et al. 2012). It was also shown that *Malassezia* profiles between mothers and their infants were concordant by day 30; however, it was not reported in this case if the babies were delivered naturally or by C-section. It has been demonstrated previously that both horizontal and vertical transmission of *Candida* spp. occur in infants (Waggoner-Fountain et al. 1996) and are influenced by mode of delivery (Bliss et al. 2008).

Fungi are well recognised members of the healthy vaginal microbiome (Drell et al. 2013). NGS technology has been used to characterise the vaginal mycobiome (Drell et al. 2013) and as outlined for other mycobiome studies, difficulties associated with bioinformatic analysis and database issues were encountered (Tang et al. 2015). Nonetheless, this study reported that the vagina was host to a diversity of fungi including 196 fungal OTUs, 16 of which were different *Candida* spp. In doing so this study highlighted a more diverse range of *Candida* spp. than had previously been described for this site. As members of these genera are typically associated with an unhealthy vaginal environment, the presence and diversity of *Candida* spp. observed were somewhat unexpected in a healthy vaginal microbiome (Drell et al. 2013). For example, a condition called vulvovaginal candidiasis affects three quarters of women at some point during their life (Sobel et al. 1998) and is characterised by *Candida* overgrowth. This condition is also linked to a number of factors, including oral contraceptive use, immunodeficiency of the host (Sobel 2000) and the use of antibiotics (Sobel et al. 1998; Fidel 2004). With respect to antibiotic usage, it is believed that this results in the depletion of protective bacteria such as *Lactobacilli* which form part of the healthy vaginal microbiome (Ravel et al. 2011).

The mechanistics of vulvovaginal candidiasis have also been characterised in some detail, and it has been shown that *Candida* spp. affect host epithelial cells in the vaginal environment by influencing cytokine and chemokine production (Steele 2002) and eliciting a proinflammatory response (Spear et al. 2008). Although the treatments available for vulvovaginal candidiasis are usually effective (Sobel et al. 1998), there is a lack of successful treatments for recurrent infections (Sobel et al. 2004). This is potentially due to the evolution of fungal resistance and therefore caution should be taken with the repeated and routine use of antifungal drugs (Sanglard and Odds 2002; Morace, Perdoni and Borghi 2014). Alternatives to antifungal drugs have been mooted, including the development of fungal vaccines (Cutler, Deepe and Klein 2007) and the use of probiotic strains (Kuhbacher 2006). With respect to the use of probiotic strains in the prevention and treatment of vulvovaginal candidiasis, the most appropriate mode of administration has been evaluated (Pirota et al. 2004; Falagas, Betsi and Athanasiou 2006) and the ability of two probiotic strains of *Lactobacillus* to reduce fungal carriage and virulence of *Candida* in the vaginal environment has been tested *in vitro* (Martinez et al. 2009). Here, the probiotic strains were capable of modulating the morphology of *C. albicans* and reducing its virulence (Martinez et al. 2009). The basis for the reduction of virulence due to changes in *Candida* morphology as well as other fungal–bacterial interactions is discussed in more detail in the section on polymicrobial interactions.

### The circulatory system

As highlighted earlier, the oral microbiota can serve as a portal of entry for pathogens to areas of the body. A number of studies have highlighted the link between the bacterial community present in oral cavities and atherosclerotic plaques (Gaetti-Jardim et al. 2009; Koren et al. 2011) and consequently these studies implicate bacteria in the chronic inflammatory process that underlies coronary atherosclerosis and cardiovascular disease. Fungal DNA signatures are also present in atherosclerotic plaques (Ott et al. 2007). Using molecular techniques to bypass the aforementioned culturing-related biases, it was demonstrated that atherosclerotic plaques harboured a diverse and variable fungal community (Ott et al. 2007). Although the study was limited to the most reliable and inexpensive molecular

techniques available at the time (DGGE, clone libraries and fluorescence *in situ* hybridisation), 19 fungal phylotypes were detected and 35 out of 38 patients were positive for fungal DNA presence by either PCR or *in situ* hybridisation analysis. This research coupled with previous analysis for bacteria in atherosclerotic plaques suggests a polymicrobial nature to cardiovascular disease (Ott *et al.* 2006).

Other diseases affecting the circulatory system such as vasculitis, for example, have also been linked to an underlying infectious agent (Pagnoux, Cohen and Guillevin 2006). Thus, diseases of unknown aetiology, such as Kawasaki disease (KD) which primarily affects the coronary arteries and is normally treated with and responds to immunoglobulin have been the focus of fungal investigations. Ishibashi and colleagues postulated that as the administration of *Candida* cell wall antigens to mice induced KD-like symptoms in mice that the response of human KD sufferers to fungi warranted further investigation. Using  $\beta$ -glucan (BG) as their preferred target, it was shown that although anti-BG antibodies were present in controls, those suffering from KD have significantly higher levels which also increased with age. As KD is often misdiagnosed when specific disease criteria are not met, this correlation with fungal antigens may serve to not only provide an insight into the disease aetiology but may also lead to better diagnostic abilities (Ishibashi *et al.* 2014).

## FUNGAL-HOST INTERACTIONS

To date, gut research has largely focused on the bacteria (Shanahan 2012, 2015) and animal models have been extensively exploited to study how members of the gut bacteria interact with the host immune system in this regard (Hooper and Gordon 2001; Hooper, Littman and Macpherson 2012; Everard *et al.* 2013). Similarly, studies on host-fungal interactions are emerging (Brown, Denning and Levitz 2012; Iliev *et al.* 2012) and in the following section, we review what is known about fungal-host interactions and their impact on disease, with a particular emphasis on diseases of the gut.

The immune system is in constant contact with a myriad of fungal species (Underhill and Pearlman 2015) and thus has a number of defence mechanisms against fungal infection (Pitman and Blumberg 2000; Cramer and Blaser 2002). One of the first lines of defence against fungi are mucosal membranes such as those that line the gut (Mowat and Viney 1997; Pitman and Blumberg 2000; Calo-Mata *et al.* 2016) and lungs (Cramer and Blaser 2002). These epithelial layers comprise part of the innate immune system and, although some fungi colonise these regions as commensal organisms (Iliev and Underhill 2013; Underhill and Iliev 2014), fungal infection or mycoses usually only becomes an issue when there is a problem with barrier function (Ford *et al.* 1991; Weindl, Wagener and Schaller 2011). These type of mycoses are important as fungal infections are difficult to treat due to the limited number of antifungal drugs available (Gonzalez *et al.* 2001), lack of antifungal vaccines (Cutler, Deepe and Klein 2007) and the prospect of antifungal resistance (Pfaller and Diekema 2004; Arendrup 2014). Fungal infections are also difficult to diagnose as clinical manifestations of disease can vary from patient to patient (Ott *et al.* 2008; Duani *et al.* 2012).

A successful immune response relies on the ability of the host to distinguish self from non-self and is mediated by the innate and adaptive immune system (Blanco and Garcia 2008; Tierney *et al.* 2012). The innate immune system plays a crucial role and facilitates the recognition of a range of microbes

through its ability to perceive various microbial signatures including lipoproteins and microbial DNA. Host cells bearing pattern recognition receptors, such as those of the innate immune system and many epithelial cells (Eriksson *et al.* 2013), are able to detect fungal cell components such as the fungal cell wall which contains a number of specific structural polysaccharide components such as chitin, mannan and BG (Smits *et al.* 1999; Poulain and Jouault 2004; Abbott *et al.* 2015). These fungal cell wall components are detected by the host's immune system and are known as pathogen-associated molecular patterns (PAMPs) (Netea *et al.* 2006). Upon recognition of the fungal PAMPs, the innate immune response is activated and switches on a variety of intracellular signalling pathways causing proinflammatory and antimicrobial responses, eventually resulting in the activation of gene expression and the synthesis of a broad range of molecules, including cytokines, chemokines, cell adhesion molecules and immune receptors that direct the adaptive immune response to invading fungal pathogen (Netea *et al.* 2006; Tang *et al.* 2012). As outlined, this recognition of microbes ultimately gives rise to adaptive immunity. However, not all microbes cause the same response or the same level of response from the host. This is largely due to the variety of PAMPs present on any microbial cell and the corresponding cocktail of innate immune recognition receptors activated by any given invading microbe. Thus, just because one fungal species elicits one response in a particular environment, that same response will not necessarily occur for all fungal species (White *et al.* 2007).

BG is a major structural component of the fungal cell wall and is known to interact with Dectin-1 (Brown and Gordon 2003), which is a type II transmembrane receptor that contains a single extracellular C-type lectin domain. Dectin-1 is thus known as a C-type lectin receptor (CLR) and acts by binding BGs and plays a role in the phagocytosis of fungal cells by macrophages and also in the production of reactive oxygen species. Dectin-1 is able to cooperate with Toll-like receptors (TLRs), such as TLR 2 (Gantner *et al.* 2003) as well as functioning independently, to elicit the production of reactive oxygen species when previously exposed to lipopolysaccharides and bacterial lipoproteins. This shows that these microbial components are capable of causing independent (Taylor *et al.* 2007; Ferwerda *et al.* 2009), yet co-operative inflammatory responses and that the recognition of these components is possible by both of these receptors independently (Gantner *et al.* 2003).

Several genetic polymorphisms have been identified which can potentially predispose the host to various GI diseases such as ulcerative colitis (UC) (McGovern *et al.* 2010). These polymorphisms have also been implicated in causing an altered immune response to specific fungal species (Glocker *et al.* 2009), the gut microbiome (Knights, Lassen and Xavier 2013) and the gut mycobiome (Iliev *et al.* 2012). It has been shown previously that mice have a diverse and abundant gut mycobiome (Scupham *et al.* 2006), and a study performed in mice showed that a polymorphism in the gene for Dectin-1 (CLE7A in humans) is linked to a severe form of UC. As outlined, Dectin-1 recognises  $\beta$ -1, 3-glucans present on the surface of almost all fungal cell walls and facilitates interactions with the indigenous mycobiome and the host response. When this response is altered, Dectin-1 can activate cytokine production and an inflammatory response against otherwise commensal fungi in the murine gut occurs (Iliev *et al.* 2012). Interestingly and converse to previous data (Iliev *et al.* 2012), it has been shown that Dectin-1 is not required in the control of *Candida albicans* in the GI tract and is only necessary in the control of systemic infection (Vautier *et al.* 2012).

In addition to Dectin-1, other CLR's have also been shown to recognise fungal cells. This includes Dectin-2 and Dectin-3 that recognise  $\alpha$ -mannan which can be found on the surface of fungal hyphae. The role of Dectin-3 and its interaction with commensal fungi in the gut was recently investigated using a mouse model (Wang et al. 2016). It was observed that mice deficient in Dectin-3 were more susceptible to chemically induced colitis than wild-type mice and that antifungal treatment could protect the Dectin-3-deficient mice from colitis (Wang et al. 2016). Although the benefits of such antifungal treatments to modulate the gut mycobiome in human inflammatory bowel disease (IBD) sufferers are currently unknown, these authors are currently recruiting human colitis patients with the aim of screening for the presence of Dectin-3 mutations. However, caution must be applied when translating results from mouse studies into human studies and treatments (Stappenbeck and Virgin 2016) as the use of antifungal drugs in another mouse model study worsened the outcome of colitis and allergic airway disease, selected for antifungal resistance and also perturbed the intestinal bacterial community structure (Wheeler et al. 2016).

### The gut mycobiome in disease

The human mycobiome has been linked to a plethora of gut-associated diseases including IBD (Ott et al. 2008; Iliev et al. 2012; Li et al. 2014; Mukhopadhyaya et al. 2014; Chehoud et al. 2015; Liguori et al. 2015; Mar et al. 2016); fungal infections (Praneenarat 2014); colorectal adenomas (Luan et al. 2015); pouchitis following ileal pouch anal anastomosis (Kuhbacher 2006), diarrhoea (Sangster et al. 2016) and intestinal allografts (Li et al. 2012), thus making it a key factor in influencing the health status of the host (Bussey 2005; Ott et al. 2008; Chen et al. 2011; Iliev et al. 2012), see also Box 2.

#### Box 2. Towards understanding the role of the human mycobiota in disease

*Separating cause from consequence:* As with much of the data generated in bacterial studies of the human microbiota, the biological significance of observed changes in the gut mycobiome between health and diseased groups is not yet fully known. This is due to the fact that differences in fungal diversity and abundance observed in disease groups compared with controls may be just incidental to the disease process and are not in fact a contributory factor. Identifying causal roles for specific fungi is undoubtedly a difficult task and will require the isolation and characterisation of suspected aetiological agents and subsequent experimentation in suitable model systems.

*Complex nature of disease:* Although specific fungal species have been implicated in intestinal disease, we also need to determine the effect of the mycobiome in disease aetiology, not just as single causative infective agents but also as potential players in polymicrobial infections. A first step towards this will require assessing any possible interactions (co-associations) between fungi and other microbes in the gut that are specific to or more prevalent or pronounced in disease processes compared to matched controls. Moreover, many diseases of the gut are multifactorial and we also need to determine and incorporate the relative importance and impact of environmental factors and host genetics on the mycobiome and the combinatory effects of the two on overall host health and disease.

### Inflammatory bowel disease

The most compelling evidence supporting a role for the gut mycobiome in human intestinal disease comes from the study of IBD (Richard et al. 2015). IBD is a collective term used to refer to a group of inflammatory disorders of the GI tract, namely Crohn's disease (CD) and UC. Although the aetiology of IBD remains unknown and the diagnosis of IBD requires a consortium of different tests, including clinical presentation, radiographic and endoscopic examinations and other histological criteria, a number of different factors have been implicated. This includes the gut microbiota, particularly the bacterial, and more recently, the fungal components of the community (Scanlan et al. 2006; Moyes and Naglik 2012; Sokol et al. 2016). Several studies have shown that the bacterial community structure is altered in individuals with IBD compared to controls with a loss of anaerobic species diversity which can have a profound effect on mucosal inflammation in IBD (Ott et al. 2004b; Korzenik 2005; Thompson-Chagoyán, Maldonado and Gil 2005; Scanlan et al. 2006; Liguori et al. 2015). More recently, studies investigating fungi in both mouse models and human cohorts, including infants, indicate that the presence of certain specific fungal species is altered in IBD when compared to controls (Ott et al. 2008; Mukhopadhyaya et al. 2014).

One of the first studies investigating the mycobiome in IBD highlighted distinct differences between IBD and control groups; however, no particular fungal species were exclusively associated with disease (Ott et al. 2008). A subsequent study analysing the bacterial and fungal communities in individuals with CD showed a reduction in bacterial diversity and an increased fungal load in patients with CD compared to healthy controls (Liguori et al. 2015). Unfortunately, this study was limited due to aforementioned issues with NGS data and publicly available fungal databases and as a result  $22\% \pm 19\%$  of sequences for healthy controls and  $10\% \pm 10\%$  in CD patients were assigned as unidentified fungi. In contrast to earlier work (Ott et al. 2008), fungal diversity was not significantly different between the two groups when assessed by qPCR; however, fungal load was significantly increased in cases of CD flare. Although genus and species level changes (which were not observed in earlier work; Ott et al. 2008) were noted (Liguori et al. 2015), this disparity between the studies is probably due to the differences in the methodologies used. However, the authors did detect one species of note, *C. glabrata*, which was positively correlated with CD (Liguori et al. 2015).

A more recent study of 235 patients with IBD, and 35 healthy controls, also found differences between the mycobiome of these two groups (Sokol et al. 2016). Although differences within the IBD group (which comprised both UC and CD patients) and between remission and relapse samples were evident, the main findings highlighted an increased Basidiomycota/Ascomycota ratio, together with increased proportion of *C. albicans* and *Malassezia sympodialis* and a decreased proportion of *Saccharomyces cerevisiae* in IBD compared with healthy controls. Significant differences were also observed between remission and relapse within the IBD cohort. For example, *C. albicans* was significantly increased in numbers during relapse compared to remission. Interestingly, fungal diversity was only decreased in UC. This finding coupled with the bacterial analysis of the samples led the authors to suggest that the specific gut environment of individuals with CD disease may favour fungi at the expense of bacteria (Sokol et al. 2016). These inter-kingdom perturbations have been also noted by others: a study investigating the microbiome in UC did not detect any overall difference in fungal  $\alpha$ -diversity or  $\beta$ -diversity between UC patients and controls

despite seeing a significant depletion in bacterial diversity in UC patients. Furthermore, no effect of ethnicity (European/South Asian) on fungal community composition in UC was noted (Mar et al. 2016). However, when patients were separated into four subgroups based on microbial co-association (as described in Lewis et al. 2015), the subgroup with the most severe UC was found to be enriched for *C. albicans* and *Debaryomyces* species as well as the bacterial genus *Bacteroides*. This increase in the Saccharomycetales fungal order has been reported in a number of earlier studies into IBD (Li et al. 2014; Chehoud et al. 2015; Lewis et al. 2015); however, it remains unclear as to the cause of this expansion. The earlier study which investigated both the mucosal and faecal mycobiome in CD by Li et al. (2014) also showed some alterations between healthy and CD patients (faecal samples) and between inflamed and non-inflamed regions of the intestinal mucosa (biopsies). This study also demonstrated correlations between the mucosal mycobiome and the host as has been observed in mice (Iliev et al. 2012).

Studies investigating inter-kingdom interactions between fungi and bacterial populations in the gut are increasing and are especially evident in studies into IBD as outlined in a number of the studies discussed earlier (Lewis et al. 2015; Hoarau et al. 2016). However, converse to many studies on IBD, a study that focused exclusively on CD patients, their healthy relatives and an unrelated healthy group found that fungal richness was increased in the unrelated healthy group. Moreover, bacterial richness was also decreased in the healthy group when compared to the CD and CD relatives groups. Taken collectively, these data highlight both the intrinsically complex relationship between bacteria and fungi irrespective of disease status (Hoarau et al. 2016) and an important caveat when using 16S and ITS metagenomic sequencing to draw conclusions on inter-kingdom interactions. This caveat relates to differences in how the 16S and ITS sequences are grouped. For example, ITS-based sequence analysis enables accurate classification to species level whereas 16S analysis is more suited to genus and family level analysis. This, coupled with difficulties in computationally dealing with differences in sequence length between fungal species and similarity thresholds which can allow the grouping of distantly related fungal species into single OTUs, can ultimately result in significant correlations being reported that are in fact just artifacts of the analysis (Peay, Kennedy and Talbot 2016).

The mycobiome of paediatric IBD patients has also been investigated (Mukhopadhyaya et al. 2014; Chehoud et al. 2015; Lewis et al. 2015). The initial failure to amplify fungal DNA from all of the samples in the study by Mukhopadhyaya and colleagues led the authors to suggest the DNA extraction method was a possible cause. Where samples were successfully amplified and analysed, the authors did note that the Basidiomycota phylum was dominant in paediatric IBD sufferers compared to Ascomycota in controls (Mukhopadhyaya et al. 2014) as is observed in adults (Sokol et al. 2016). A subsequent study into paediatric IBD observed that the species present in samples from healthy adults overlapped with both paediatric controls and paediatric IBD sufferers and also that the abundance of fungal species present significantly differed between the control and IBD paediatric groups. *Candida* OTUs were enriched in samples from IBD patients; however, the most prevalent OTU could not be assigned a single species name due to annotation issues. The second most prevalent species was *C. parapsilosis* which was also higher in paediatric IBD samples. Finally, a third OTU *Cladosporium cladosporioides* was observed more frequently in healthy samples (Chehoud et al. 2015).

Perhaps one of the most interesting studies into the gut microbiome to date involved a shotgun-metagenomic sequencing-based longitudinal analysis of paediatric CD patients and healthy control samples (Lewis et al. 2015). This approach afforded the researchers an insight into the role of the mycobiome that was free of errors and bias due to primer choice and PCR. Although more expensive than ITS sequencing, the use of shotgun-metagenomic sequencing is becoming more commonplace in surveys of the gut bacteria (Jovel et al. 2016) as shotgun sequencing removes the necessity of several individual amplicon sequencing strategies to get the complete compositional 'picture'. Using this methodology, a study by Lewis et al. (2015) found that the relative proportion of fungi present increased with both disease status and antibiotic use but was reduced by diet therapy. Here, CD participants were separated into two distinct groups based on their bacterial composition, one of which was much more similar to healthy controls than the other. The more altered group was linked to prior use of antibiotics and had increases in specific fungi and also higher concentrations of human DNA in their faeces. A total of five fungal taxa were detected in the study, all of the Saccharomycetales fungal order, i.e. *S. cerevisiae*, *Clavispora lusitaniae*, *Cyberlindnera jadinii*, *C. albicans* and *Kluyveromyces marxianus*, and all were positively enriched in CD and positively associated with a changed bacterial composition (Lewis et al. 2015). It is also worth noting that although we outlined earlier that diet can have a profound effect on the mycobiome, this particular study is the first to look at the effect of a defined formula diet on the mycobiome.

With respect to members of the Saccharomycetales, such as *S. cerevisiae*, interactions between fungi and the host are not only relevant to the complex aetiology of IBD (Main et al. 1988; Sutton et al. 2000; Zwolinska-Wcislo et al. 2009) but may also provide a potential method for diagnosis (Joossens et al. 2002). For example, the clinical presentations of UC and CD are often quite different but in ~10% of cases it is not possible to distinguish between UC and CD, and in this scenario the condition is referred to as indeterminate colitis (IC) (Joossens et al. 2002). However, given that ~60% of patients diagnosed with CD have anti-*S. cerevisiae* antibodies which are directed against oligomannosidic epitopes of the *S. cerevisiae* cell (Sutton et al. 2000; Standaert-Vitse et al. 2009), research into methods to better classify IC patients has included the use of fungal species-specific investigations targeting these serological markers. Although the use of such an approach provides a further diagnostic test for patients diagnosed with IC (Joossens et al. 2002), it also further implicates fungi in the disease aetiology of IBD and GI disease (Jawhara et al. 2008).

### Post-operative and nosocomial infections

The gut mycobiome is also implicated in infections and complications associated with surgical procedures, and consequently the mycobiome has been investigated in this regard. For example, Hirschsprung disease is a congenital condition affecting the gut in infants and children. It is often treated with surgery to remove or bypass the diseased region of the colon but is frequently followed by the complication Hirschsprung-associated enterocolitis (Frykman et al. 2015). Eighteen Hirschsprung disease patients that had corrective surgery were enrolled in a study to analyse the potential aetiology of Hirschsprung-associated enterocolitis and the effect the gut microbiota may have in the condition. The mycobiome analysis showed a reduction in fungal diversity in the enterocolitis group with increased *Candida* spp. which could be further subcategorised into high and low

*C. albicans* burden. Enterocolitis patients classified as having low *C. albicans* burden had similar *C. albicans* abundance to their Hirschsprung disease controls. The enterocolitis group also showed reduced *Malassezia* and *Saccharomyces* spp.; however, it is not known whether the overall reduced diversity and increased *Candida* spp. is as a result of the enterocolitis or if the presence of high *C. albicans* levels may play a role in the disease aetiology (Frykman et al. 2015).

Fungi are also a frequent cause of post-operative nosocomial infections following intestinal transplants (Primeggia et al. 2013), thus making mycobiome studies of relevance to both our understanding of their role in such infections associated with intestinal transplants (Li et al. 2012) and other surgical procedures, including those required for the management of IBD (Kuhbacher 2006). Similar to Hirschsprung-associated enterocolitis, pouchitis is a major post-operative complication associated with ileal pouch anal anastomosis, which is the procedure of choice in the surgical management of UC. The syndrome is clinically characterised by variable symptoms and includes increased stool frequency and fluidity, rectal bleeding, abdominal cramping, urgency and tenesmus, incontinence, fever and extra-intestinal manifestations (Gionchetti et al. 2003). As such, probiotic bacteria have been investigated for their ability to affect the colonisation of fungi in the GI tract and also for their ability to maintain remission in patients diagnosed with pouchitis (Gionchetti et al. 2003; Trojanowska et al. 2006). In an attempt to determine the mechanisms underpinning the observed protective effects, researchers characterised the bacterial and fungal microbiota in patients post-probiotic treatment (Kuhbacher 2006). The patients recruited for the study were those who had developed pouchitis after treatment with a placebo. The subsequent study found that the probiotic therapy maintained remission and most importantly was accompanied by a higher bacterial and a reduced fungal diversity. Interestingly, fungal diversity was also reduced in the placebo group; however, the level of statistical significance was not reported (Kuhbacher 2006). Ultimately, it is unclear if the observed initial high diversity could be related to the initial dose of antibiotics prior to commencement of either the probiotic therapy or the placebo.

## GUT MYCOBIOME OF IMMUNOCOMPROMISED HOSTS

It is well recognised that many fungal species are clinically relevant opportunistic pathogens in susceptible and immunocompromised patients (Gumbo et al. 1999; Campisi et al. 2002; Agırbaslı, Özcan and Gedikoğlu 2005; Jayshree et al. 2006; Kashyap, Bhalla and Uppal 2009; Geramizadeh et al. 2012; Esebelahie, Enweani and Omoregie 2013; Hammoud et al. 2013; Praneenarat 2014). Moreover, although the underlying cause of immunocompromise in individuals and patients may differ, this group are particularly susceptible to invasive fungal infection regardless of the cause of the immune suppression. In the following sections, we highlight a number of case studies that have investigated the mycobiome in a range of different groups of potentially immunocompromised individuals.

### Paediatric patients and infants

Immunocompromised paediatric patients, diagnosed with various haematologic malignancies or disease, as well as congenital immunocompromise, have been studied to assess the presence and effect of their indigenous mycobiomes (Taylor, Kropp

and Molina 1985; Agırbaslı, Özcan and Gedikoğlu 2005). The historic study of a child born with the condition severe combined immunodeficiency enabled researchers to assess fungi in an immunocompromised host in the absence of a 'disease' background (Taylor, Kropp and Molina 1985). Although limited to a single individual, researchers were able to investigate the prevalence and diversity of fungi colonising the GI tract of a child living in a controlled environment through time. In addition to low sample power, the methods were limited and should ideally be repeated with modern technologies. Nonetheless, the results were interesting and it was noted that while *Candida* was recovered in 45.5% of the faecal samples over time, colonisation did not result in invasive infection or disease over the course of the study (Taylor, Kropp and Molina 1985). Given the reduced capacity of the patient's immune system to produce immune cells such as B cells and T cells, further research into such an immunocompromised human cohort could reveal clues as to how fungi transition from commensalism to pathogenicity in immunocompromised hosts.

Although many infants are not adversely affected by fungal colonisation, neonates are considered a risk group for mycoses, with infections representing a large contributing factor to morbidity and mortality (Feja et al. 2005). Moreover, the fungal colonisation of neonates can also contribute to opportunistic invasive fungal infections (Rowen 2001; Brian Smith, Steinbach and Benjamin 2005) with the greatest threat associated with the presence of potentially invasive fungal pathogens in preterm and underweight neonates that are at risk of developing invasive diseases such as necrotising enterocolitis and sepsis. As such preterm infants are often treated with antifungal agents prophylactically (Stewart et al. 2013).

Although the importance of the mycobiome as a risk factor in premature and underweight neonates is recognised, it remains to be fully investigated. However, this topic is receiving increasing interest, especially given the fact that neonates are often treated with antibiotics which can further increase the risk of invasive disease by fungi such as *Candida*. A recent study highlighted a surprising diversity of fungi in a large proportion (64%) of extremely low birth weight infants (LaTuga et al. 2011). Of the 7 (of 11) infants positive for fungi, Saccharomycetales was the most prevalent and abundant order, constituting 38.2% of amplicons (LaTuga et al. 2011). Earlier studies of fungal colonisation of the very low birth weight infant (<1500 g) showed a fungal colonisation rate of 26.7% (Baley et al. 1986). This colonisation significantly contributed to risk factors such as cutaneous and systemic candidiasis as one third of the infants' colonised developed mucocutaneous candidiasis and 7.7% developed systemic disease despite the prophylactic use of antifungal agents. Although the study did not investigate whether colonisation was linked to the delivery method, it was noted that a greater number of infants that were colonised had been delivered vaginally compared to those delivered by caesarean section (Baley et al. 1986).

### Diabetes

Type 1 diabetes (T1D) is an increasingly prevalent autoimmune disorder that has been the focus of microbiome research including mycobiome analysis (Gosiewski et al. 2014; Soyucen et al. 2014; Kowalewska et al. 2016). In a recent study that compared the mycobiome of 53 children with T1D to 30 matched healthy controls, it was observed that children with T1D had significantly higher fungal species diversity despite there being no significant difference in the overall level of fungi in each group. A

lower incidence of *Candida albicans* was also reported in the case group (62% of all isolates identified) compared to controls (*C. albicans* accounted for 85% of strains identified). However, the recovery of the greater diversity of strains from the T1D cohort rather than a true reduction in *C. albicans* levels likely skewed the data in this manner (Kowalewska et al. 2016). Other studies have demonstrated a correlation between fungi, specifically *C. albicans*, in the gut and diabetes using qPCR (Gosiewski et al. 2014). More specifically, a higher prevalence of *C. albicans* in the guts of individuals with types 1 and 2 diabetes was observed compared with control subjects; however, no difference was observed between the diabetic cohorts (Gosiewski et al. 2014). An increased prevalence of *C. albicans* has also been observed in paediatric diabetic groups, where *C. albicans* was present in 40% of diabetic patients compared to only 14.3% of controls (Soyucen et al. 2014). Gosiewski and colleagues also observed a negative correlation between the quantity of *Candida* and the level of serum lipids in the diabetic patients. Nonetheless, the significance of a higher prevalence of *Candida* in individuals with diabetes and an understanding of the actual role of this microorganism in disease requires further work.

### Graft-versus-host disease

Another immunocompromised condition that is relevant to gut mycobiome research is graft-versus-host disease (GVHD). GVHD is a frequent complication of many transplants including haematopoietic stem cell, allogeneic stem cell and bone marrow transplants, and is caused by the transplanted cells attacking the transplant recipients' cells. GVHD frequently involves the GI tract and even though symptoms can vary, the most common include nausea, vomiting, anorexia and secretory diarrhoea and in severe cases GI bleeding, protein-losing enteropathy and ileus may also be seen (Deeg and Antin 2006). GVHD is a leading cause of morbidity and mortality and although the role of bacteria in GVHD is well established (Washington and Jagasia 2009), until recently, the role of the mycobiome in the disease had not been recognised. In the only study in this area to date, similarities between GI-GVHD and IBDs were noted (van der Velden et al. 2013). Thus, researchers sought to investigate if any relationship between fungal colonisation (specifically *Candida* colonisation) and immune receptors such as Dectin-1 function (Ariizumi et al. 2000; Iliev et al. 2012) could also be applied to the pathogenesis of GI-GVHD (van der Velden et al. 2013). Although there was no direct effect of Dectin-1 dysfunction on acute GVHD, the authors did observe that the mycobiome did have an indirect effect through the prevalence and extent of *Candida* colonisation. Moreover, a higher progression rate of grades II–IV acute GVHD and GI-GVHD was observed in patients colonised by *Candida* spp. than those that were not. However, no effect was seen in patients with isolated acute skin GVHD (van der Velden et al. 2013).

### Rett syndrome

As outlined, the aetiology of many diseases is complex and multifactorial and consequently a systems biology approach is often required to elucidate the cause and subsequent progression of many diseases. Moreover, in most cases it remains unclear if changes in the mycobiome are a consequence of the disease condition or may play a role in disease progression. It has been noted in Rett syndrome (RTT), a progressive neurological disorder, that both the mycobiome and bacterial microbiome are altered when compared to healthy controls. Although individ-

uals with RTT harbour a reduced diversity of fungi when compared to controls, this finding was not associated with the main GI symptom associated with RTT (i.e. constipation). However, a significantly higher relative abundance of *Candida* was observed in RTT subjects (Strati et al. 2016a).

### Infectious viruses—hepatitis B and HIV

Infectious viruses that affect host immunity including hepatitis B and HIV can alter the potential role of other microbes, such as fungi, in disease exacerbation and progression. As a consequence, a number of studies have sought to characterise the role of the mycobiome of individuals that have been immunocompromised by viral infections. One particular study looked at the correlation between fungi present in the GI tract and varying degrees of chronic hepatitis B infection using culture-independent techniques (Chen et al. 2011). It was found that patients with hepatitis B-associated liver cirrhosis had higher fungal species richness in their guts compared to patients with chronic hepatitis B. In contrast, little difference was observed in the enteric fungal diversity of participants positive for the hepatitis B virus and the mycobiome of healthy controls. These results indicate that the diversity of enteric fungi is positively correlated with the progression and severity of the disease in patients with varying degrees of chronic hepatitis B infection (Chen et al. 2011). It is important to note, however, that this correlation does not distinguish between cause and effect, which remains to be elucidated.

A considerable research effort has also focused on mycoses in HIV and AIDS patients given the clinical significance of fungal infections in the 37.0 million people living with HIV/AIDS worldwide (Park et al. 2009; Jha et al. 2012; Esebelahie, Enweani and Omeregie 2013; Mukherjee et al. 2014; Platt et al. 2016). Researchers have targeted the mycobiome as a source of opportunistic fungal infection (Schulze and Sonnenborn 2009) and also due to fungal-associated comorbidities in HIV/AIDS which include diarrhoea wasting syndrome (Kashyap, Bhalla and Uppal 2009). Avenues of investigation in this cohort include various studies into digestive distress to discover any potential underlying fungal causes of diarrhoea in patients with HIV/AIDS (Kashyap, Bhalla and Uppal 2009). As a number of different organisms are potentially responsible for causing diarrhoea in HIV/AIDS patients (Jha et al. 2012), researchers have surveyed the gut microbiota to assess any differences between patients that are seropositive and seronegative for HIV in order to better understand the underlying cause of the symptom and thus improve patient outcomes. The results showed that the prevalence of fungi in the gut of those seropositive for HIV was considerably higher than those seronegative for HIV (Jha et al. 2012). Differences in *Candida* carriage have also been observed in HIV patients with respect to HIV treatment (Esebelahie, Enweani and Omeregie 2013). Patients taking aggressive anti-retroviral therapy, when compared to controls and HIV patients not using the aggressive treatment, had similar *Candida* levels to the control group. Within the HIV patient group, significant differences were also observed with respect to age and gender. Although the reason for this is unclear, it was suggested that the impact of age and gender was due to decreased immune function and potential hormone imbalances, respectively (Esebelahie, Enweani and Omeregie 2013).

### *Candida* spp. and polymicrobial interactions in the gut

In this final part of the review, we first elaborate on some additional *Candida* research that is relevant to the study of the gut

mycobiome given the apparent ubiquity and considerable diversity of *Candida* spp. that have been detected in the human gut (see Fig. 2, 4, Table 2 and Table S1). Although members of this genus are generally regarded as commensal fungi in the human GI tract of healthy individuals, certain *Candida* species may also be opportunistic pathogens and affect the host, for example, during antibiotic treatment or when changes in the disease and immune status of the host occur resulting in *Candida* overgrowth. Consequently, *Candida* spp. are frequently linked with a perturbed GI environment and disease (Wingard et al. 1980; Jawhara et al. 2008; Kumamoto 2011; Dollive et al. 2013; Strati et al. 2016a). This, coupled to its ecological relevance and ease of cultivation, makes *Candida* a model organism for *in vivo* and *in vitro* analysis of fungal–host and fungal–bacterial interactions relevant to human health and disease (Reales-Calderón et al. 2013). In this regard, we also discuss what is known about *Candida* (and other fungal species) interactions with other members of the gut microbial community.

A key aspect of *Candida* virulence is cell morphology and this is also likely to be relevant to other opportunistic fungal species. For example, *C. albicans* can exhibit three different morphologies. The first is the unicellular yeast form and the second two are distinctly different filamentous forms consisting of pseudohyphae and hyphae. The ability and mechanism by which *C. albicans* switches between morphologies has been investigated with a view to understand the mechanisms involved and also as a potential means to modulate morphological variation (Vylkova et al. 2011). Nonetheless, this ability of fungi to form hyphae does not necessarily infer an ability to go undetected by the immune system. For example, Wang et al. (2001) observed that hyphal fragments from two distinct fungal pathogens were sufficient to induce cytokine production *in vitro*. One study of the interactions between Dectin-1 and the fungal species *C. albicans* has shown that *Candida* in the filamentous form does not illicit a response from Dectin-1 but that the yeast form of the fungi does (Lo et al. 1997; Gantner, Simmons and Underhill 2005). Other studies have also highlighted the link between cell morphology in *Candida* spp. and its role in pathogenicity (O'Meara et al. 2015).

As outlined earlier, the gut mycobiome has been implicated in the development of allergic asthma in children. *Candida* species have also been implicated in the development of food allergies and allergic airway disease (Noverr et al. 2004). A study utilising a murine model of chronic *Candida* GI colonisation was achieved without the use of antibiotics or immunosuppressive agents, and they found that *Candida* colonisation did in fact promote sensitisation to food allergens (Yamaguchi et al. 2006). The study sought to further investigate the mechanism by which sensitisation occurred and this was achieved by investigating the role of mast cells in the development of food allergy. It was observed that *Candida* colonisation did indeed promote the infiltration and degranulation of mast cells (Yamaguchi et al. 2006). It has also been shown that *Candida* species produce substances, such as alcohol and prostaglandin (Noverr, Erb-Downward and Huffnagle 2003), that have a proinflammatory effect on the host (Albuquerque and Casadevall 2012). Consequently, it was hypothesised that fungal colonisation may play a role in allergic airway inflammation. One study conducted concluded that *Candida* overgrowth was responsible for the promotion of macrophage polarisation resulting in altered macrophages phenotypes due to prostaglandin production in the gut by *Candida* and that this was sufficient to cause an increase in allergic airway inflammatory cell infiltration in the lung (Noverr et al. 2004).

Finally, the importance of polymicrobial interactions including those between fungi and bacteria is receiving increasing research attention. Although the majority of studies into the gut microbiome have looked at the bacterial and fungal communities independently (Peleg, Hogan and Mylonakis 2010; Oever and Netea 2014; Chehoud et al. 2015), ideally, both communities should be studied together as each population is capable of interacting with each other and the host (Gale and Sandoval 1957; Mason et al. 2012a,b). A range of fungal–bacterial interactions have been characterised and these relationships can be synergistic, antagonistic, commensal or symbiotic in nature (Kerr 1994; Klaerner et al. 1997; Hogan and Kolter 2002; Wargo and Hogan 2006; Peleg, Hogan and Mylonakis 2010; Bor et al. 2016; Kalan et al. 2016). They can influence both physical and physiological characteristics and induce morphological changes (Hogan and Kolter 2002) as well as altering the normal responses to antimicrobial agents and aid in their survival in adverse environments. The majority of studies of bacterial–fungal interactions focus on the interaction between *C. albicans* and various pathogenic bacterial species that are able to co-habit with *C. albicans* and cause mixed population persistent biofilms, and thus are an important source of inoculation, particularly for nosocomial infections (Hogan and Kolter 2002; Hogan, Vik and Kolter 2004; Nobile and Johnson 2015).

As outlined earlier, *Candida* is a polymorphic fungus and several studies have alluded to changes in the morphology being an important factor in the virulence of the species with the yeast form linked to fungal commensalism and the filamentous form implicated in opportunistic infection (Lo et al. 1997). Interactions between various fungi and bacteria in mixed populations such as those inhabiting the gut are attracting increasing interest as the ability of various strains of bacteria to modulate the morphological state of *Candida*, and potentially other fungi, is important in the combined virulence of mixed infections (Allison et al. 2016; Bor et al. 2016; Liang et al. 2016). The polymicrobial nature of many infections has highlighted the ability of different microbes, often from diverse taxonomic groupings, to form synergistic relationships. These mutualistic or synergistic relationships can provide protection from antibiotics and extracellular enzymes and also facilitate the breakdown of complex substrates. These interactions not only contribute to an organism's ability to survive in a mixed microbial community but also, potentially, within a hostile host environment (Gulati and Nobile 2016).

Studies have also shown that quorum-sensing molecules from the bacterium *Pseudomonas aeruginosa* can alter the morphology of *C. albicans* in both liquid and solid media. Here, a 3-oxo-C12 homoserine lactone *P. aeruginosa* cell-cell signalling molecule was shown to mediate the observed morphology of *Candida* without adversely affecting the growth rate of the fungi (Hogan, Vik and Kolter 2004). *Escherichia coli*, a bacterial species prevalent in the human gut (Gao, Zhao and Huang 2014), has also been shown to act synergistically with *C. albicans*. Utilising a murine model of complex polymicrobial infection, results showed that mice infected with a lethal dose of *C. albicans* combined with *E. coli* died significantly faster than mice infected with *C. albicans* alone (Ikeda et al. 1999). *Escherichia coli* also benefitted from its interaction with *C. albicans* in a polymicrobial biofilm that increased its resistance to antibiotics (De Brucker et al. 2015). As both these microbes are present in the human gut, it is interesting that *C. albicans* conferred a protective effect from antibiotic treatment in a bacterium; however, further studies in an *in vivo* model will be necessary to investigate this potential effect in the gut.

Microbial interactions are contingent on local environmental conditions and consequently, these conditions must be accounted for which can lead to practical challenges in the laboratory. Challenges such as those that have been observed in the study of bacterial–host interactions also potentially apply to bacterial–fungal interactions, the results of which suggest it is necessary to mimic the *in vivo* environment as closely as possible when investigating interactions so as not to effect the observations being made (Nordenfelt *et al.* 2012). To circumvent this problem, one possibility is to look at correlations in the co-occurrence and relative abundance of different microbes in datasets generated from sequence-based studies. For example, *Candida* and *Saccharomyces* species were positively correlated with the Archaea *Methanobrevibacter* and both fungal species were also negatively correlated with *Nitrososphaera* prevalence in one study of the gut mycobiome (Hoffmann *et al.* 2013). Syntrophic models were invoked to explain the correlation between *Candida* and *Methanobrevibacter*, and it was suggested that *Candida* may boost the breakdown of starches in carbohydrate-rich foods, freeing simpler sugars that are then fermented by bacterial species in the gut and these fermentation products are then available for *Methanobrevibacter* metabolism. Although no explanation was provided by the authors for the negative correlation between *Nitrososphaera* and *Candida* and *Saccharomyces*, it was observed that where *Nitrososphaera* was detected *Methanobrevibacter* was not, suggesting an antagonism between the two (Hoffmann *et al.* 2013). More recently, it has also been shown that the virome is also capable of influencing the mycobiome (Plotkin 2016). Here, the herpes simplex virus was shown to significantly increase the adherence of *C. albicans* to HeLa cells *in vitro* (Plotkin *et al.* 2016). These hypothesised relationships demonstrate the intrinsically complex relationships between fungi and the other inhabitants of the GI tract and highlight the need for much further research into polymicrobial and inter-kingdom interactions and their impact on host health.

## CONCLUSIONS

Here we have reviewed and highlighted key methods and research relevant to the gut mycobiome to facilitate further research into this previously neglected area, see also Box 1. Although a diverse repertoire of fungal species have been reported from gut mycobiome studies performed to date, the vast majority are affiliated with just two phyla, i.e. Basidiomycota and Ascomycota, in addition to a smaller number of Zygomycota genera. Although members of the phyla Neocallimastigomycota, Chytridiomycota and Glomeromycota have also been reported in human gut mycobiome studies, it is clear upon further interrogation that these fungal taxa are in fact extremely rare.

Currently, it remains unclear whether changes in the mycobiome observed in many diseases are a consequence of the disease process or in fact play a role in disease aetiology, see also Box 2. An example of this is the reporting of the genera *Candida*, *Aspergillus*, *Penicillium* and *Debaryomyces*, which are commonly found in the healthy gut mycobiome but are also consistently reported in cases of GI disease and perturbation. These apparently contradictory patterns may reflect species or strain-related differences in fungal composition, or contrasting susceptibilities due to host genotype or the composition of other components of the microbiome. Moreover, many of the fungal species detected in gut mycobiome studies are known to be or speculated to be of dietary origin and therefore may only represent a transient component of the community (Suhr, Banjara and Hallen-Adams 2016).

It is also evident that different methodologies can give vastly different accounts of the diversity present in any given sample. Therefore, it is clear that more studies that use comparable and all-encompassing approaches, and that employ both culture-dependent and culture-independent techniques need to be applied to large cohorts of individuals. These individuals should ideally be from different geographical regions and of different health status to enable us to define commonalities and differences between groups and individuals, assess whether a core mycobiota exists and fully appreciate the role of the mycobiome in health and disease. Moreover, bacterial community analysis is currently undergoing certain changes with researchers moving away from amplicon sequencing-based analysis and instead focusing their efforts on shotgun metagenomic sequencing (where budget allows). It is likely that a similar approach would be beneficial to the study of the human mycobiome, however, given the infancy of the field, for now, adopting this approach exclusively may be premature. Nonetheless, mycobiome analysis can benefit from the lessons and insights that have been unearthed during the study of the bacterial microbiome and thus researchers are more aware of the long-term potential consequences afforded by poor methodology choices, insufficient sample size, translation of mouse model results into human treatments, etc. Thus, a multifaceted and multidisciplinary approach is now being applied more frequently (Norman, Handley and Virgin 2014; Lewis *et al.* 2015). Consequently, we are gaining a greater appreciation of how the human gut mycobiome can serve as a reservoir of poorly characterised species of interest, invasive organisms and opportunistic pathogens and such studies offer new avenues to explore in the aetiology of disease as well as revealing the extent to which the gut mycobiome can modulate the host response. Characterising the gut mycobiome in terms of species and strain diversity both within and between groups of interest is only beginning and as with any scientific field, the accumulation of interesting findings and data will undoubtedly lead to further novel research (Huffnagle and Noverr 2013). Moreover, the functionality of the various fungal species including how they interact with the other eukaryotes and prokaryotes present in the gut and more importantly how these interactions affect us, their human hosts' remains to be elucidated. The answers have the potential to be fascinating.

## SUPPLEMENTARY DATA

Supplementary data are available at [FEMSRE](https://academic.oup.com/femsre/article/41/4/479/3738183) online.

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