10. Mycobiome-Specific Protocols

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10.1. Purpose

The following protocols are a set of methods used at the Quadram Institute (QI) to study and characterise the fungal community that resides within the human gastrointestinal (GI) tract, namely the gut mycobiome. The culture-based methods have been developed to isolate and culture, where possible, viable enteric fungi from fresh or refrigerated human faecal samples. The non-culture-based protocols provide a workflow for isolating fungal DNA from human faecal samples that can be used as a template to prepare amplicon libraries for fungal community profiling using the Illumina MiSeq sequencing platform.

10.2. Introduction

The human gut harbours a diverse variety of yeasts and other fungal species [1], which are typically present in far lower abundance than their enteric bacterial counterparts. Indeed, in a recent metagenomic study, fungi were estimated to make up just 0.1% of the total microbiota in the healthy adult gut [2]. However, given that fungi are generally much larger in volume than bacteria, they are still likely to constitute a sizeable portion of the intestinal microbiota biomass [3]. This means they can have a significant impact, either beneficial or deleterious, on human health [4].

In view of their low abundance in faecal samples, it is essential when isolating enteric fungi or extracting fungal DNA, to include steps that take this bias into consideration. Fungal isolation and cultivation requires selective growth media (e.g. yeast extract-malt extract [YM]; Sabouraud dextrose [SD]) supplemented with antibiotics to suppress/inhibit the growth of bacteria, which are likely to be present in large numbers. In addition, fungi have tough outer cell walls, which can make then ddifficult to lyse. This means that when extracting fungal DNA from faecal samples it is advisable to use a combination of chemical and

mechanical lysis approaches to maximise DNA recovery. Using an amplicon-based sequencing approach can also help overcome the low abundance issue, as fungi typically have far more copies of the ribosomal rRNA genes that collectively make up the ribosomal DNA (rDNA) array, than bacteria. For instance, *Saccharomyces cerevisiae*, a food-associated yeast often found in the human gut, can have between 50 and 100 copies of the rDNA array per genome depending on the individual strain [5]. Currently, the non-coding ITS1 and ITS2 regions of the rDNA array are widely used as the biomarkers of choice for next generation sequencing (NGS) of amplicons and for determining enteric fungal community composition [6-8]. Here at the Quadram Institute, we use ITS1 amplicon sequencing to profile the mycobiota of the human gut.

10.3. Materials (including products, kits, solutions etc.)

10.3.1 Culture-based (fungal cultivation)

- Sterile 1x PBS buffer (used as a diluent)
- SD broth (40 g/L dextrose and 10 g/L peptone) supplemented with chloramphenicol (0.05 g/L) and kanamycin (0.05 g/L).
- SD agar (40 g/L dextrose, 10 g/L peptone and 20 g/L agar) supplemented with chloramphenicol (0.05 g/L) and kanamycin (0.05 g/L)
- YM broth (10 g/l glucose, 3 g/L malt extract, 5 g/l peptone and 3 g/L yeast extract) supplemented with penicillin (25 U/mL) and streptomycin (25 U/mL)
- YM agar (10 g/l glucose, 3 g/L malt extract, 5 g/l peptone, 3 g/L yeast extract and 20 g/l agar) supplemented with penicillin (25 U/mL) and streptomycin (25 U/mL)
- Petri dishes (Triple vent)
- 20 mL Glass bottles (Plastic capped)
- 15 mL Corning® centrifuge tube
- Sterile spatulas
- Benchtop vortex (e.g., Vortex-Genie®)
- Sterile, aerosol-resistant pipette tips (of varying volume capacities: 1 µl to 1 ml)
- Plastic pellet pestles (sterile)
- Plastic L-shaped spreaders (sterile)
- Parafilm
- Incubator (set to 30ºC)
- Sterile 20% glycerol

10.3.2. Non-culture-based

- Benchtop microcentrifuge (up to 16,000x g)
- 1.5 ml LoBind microcentrifuge tubes
- QIAamp PowerFecal Pro DNA Kit (Qiagen)
- FastPrep-24 benchtop homogeniser (MP Biomedicals)
- Qubit 3.0 Fluorometer (Invitrogen)
- Qubit dsDNA Broad-Range (BR) assay kit (Invitrogen)
- NanoDrop spectrophotometer
- 0.6 ml microcentrifuge tubes
- 0.2 ml PCR tube strips & lids
- KAPA2G Robust PCR kit (Roche)
- Mini-fuge with 8-place rotor for 0.2 ml tubes
- Thermal cycler, 0.2 ml tube compatible (e.g., Biometra TRIO instrument)
- Midori Green Direct DNA stain
- Blue/Green LED Transilluminator
- KAPA Pure beads (Roche)
- Elution buffer (EB) (10 mM Tris-HCl)

10.4. Methods (step by step procedure)

10.4.1 Culture-based

When culturing fungi from human faecal samples, it is important to use either fresh or refrigerated samples, as freezing samples prior to culturing can significantly reduce the overall recovery of viable fungi. Indeed, a single freeze/thaw cycle results in a 10-fold reduction in fungal abundance post freezing compared with fresh samples [9]. Furthermore, fungal growth can vary considerably depending upon growth conditions (e.g., aerobic vs semi-anaerobic). Thus, to maximise fungal recovery we recommend that culturing should, whenever possible, be carried out in liquid media (semi-aerobic) as well as on solid media (aerobic). The latter method, also known as spread plating, can also be used if required to determine faecal fungal load (i.e., fungal colony forming units (CFUs) g-1 of faeces).

In view of their general low abundance within the human gut, selective media should be used for the recovery of viable fungi from human faecal samples. YM and SD media are the recommended growth media of choice, as both are used routinely for the cultivation of yeast and other fungi from a variety of sources, including human faecal samples. In addition, each growth medium should be supplemented with antibiotics to suppress/inhibit bacterial overgrowth. In our experience, the addition of two antibiotics, rather than just one, works more effectively to inhibit bacterial growth.

Prior to culturing, either aerobically or semi-aerobically, a faecal homogenate should first be prepared from the faecal sample, subsequent to use as the inoculum. Details of how to do this are provided in the next section (10.4.1.1).

Note: to avoid the risk of possible culture contamination, inoculation of all media should be carried out aseptically either in a safety cabinet or next to a lit Bunsen flame.

10.4.1.1. Faecal homogenate preparation

When handling human faecal samples, all processing steps should be carried out in a Class 2 Safety Cabinet. Ensure the cabinet is sterilised prior to use, by thoroughly wiping down all surfaces with 70% ethanol.

1. Using a sterile spatula, weigh out between 0.1 and 1.0 g of fresh faecal material from the sample into a sterile 15 mL Corning® centrifuge tube or similar.

Note: the quantity of faeces used is dependent upon the original amount provided by the study participant (e.g. young infant vs adult).

2. Add sterile 1x PBS buffer to the centrifuge tube to produce a 10x diluted suspension (e.g., for 0.5 g of faeces, add 5.0 mL 1x PBS).

3. Mix thoroughly by vortexing (at maximum speed), for 30 to 60 s to produce a uniform homogenate.

Note: the length of vortexing is dependent upon faecal consistency. If necessary, a sterile plastic pellet pestle or 1 mL pipette tip can be used to break up any large particulate matter that may remain in suspension after initial vortexing. An additional round of vortexing is then required.

4. Once a uniform faecal homogenate has been produced, it is ready to use for inoculating liquid media (i.e., semi-aerobic cultivation) and for spread plating onto solid agar media (i.e., aerobic cultivation).

10.4.1.2. Semi-aerobic liquid fungal culturing

1. For semi-aerobic cultivation (i.e., liquid culturing), add 200 µL of faecal homogenate to 10 mL of SD broth (supplemented with chloramphenicol and kanamycin) pre-dispensed in a

sterile 20 mL glass bottle and vortex thoroughly to mix. A duplicate culture should be set up if there is sufficient homogenate (i.e., at least 1.0 mL).

2. Repeat step 1, but add 200 µL of homogenate to 10 mL of YM broth (supplemented with penicillin and streptomycin). Again, set up in duplicate if sufficient homogenate is available.

3. Incubate all broth cultures at 30ºC without agitation.

4. Broth cultures should be monitored daily for signs of microbial growth. This may manifest itself as an increase in turbidity, appearance of gas bubbles (due to microbial fermentation) or a combination of both.

5. Standard light microscopy, if available, is recommended as an additional means to monitor for signs of fungal growth in liquid cultures. Using aseptic technique throughout, to avoid possible culture contamination, a small volume of the broth culture (e.g. a 3-4 µL aliquot) should be removed and examined microscopically. At 200x and 400x magnification, yeast and other fungal cells can be readily distinguished from bacterial cells based on their larger size. For instance, cells of *Saccharomyces cerevisiae*, a food-associated yeast often present in the human gut [1], measure between 5 and 10 µm in length [10]. In contrast, cells of *Bacteroides thetaiotamicron*, one of the most common bacterial species found in the human gut flora, typically measure just 1 µm.

6. Once fungal growth is detected, the liquid culture should be serially diluted in sterile 1x PBS buffer (see Figure 2 below) and spread plated onto agar plates of the same selective medium (e.g., YM broth onto YM agar). If more than one fungal species is present in the broth, then spread plating is an effective method to isolate and purify each individual species.

Note: a separate triple-vented agar plate should be used for each individual dilution.

7. Firstly, using the undiluted broth culture, pipette out a 50 ul aliquot onto the centre of an antibiotic supplemented agar plate of the same selective medium (i.e., pipette YM broth culture samples onto YM agar). Using a sterile plastic L-shaped spreader, spread the sample evenly over the entire surface of the agar, carefully rotating the Petri dish at the same time. Once completed, allow the plate to air dry for 5 minutes.

8. Repeat the process for each 10-fold dilution, using a fresh agar plate and new spreader for each separate dilution.

9. A sample of diluent (i.e., PBS) should also be spread plated onto a separate agar plate. This acts as a negative control, and is used to monitor for contamination.

Note: a positive growth control should also be included and for this, we use a strain of *Candida albicans* (NCYC 3115, a human isolate), as this fungal species is a common human gut commensal [1].

10. Once all spread plates have been inoculated, transfer to a 30ºC incubator, and incubate upside down (i.e., lid down) in darkness.

11. After 2 days incubation, check plates for signs of fungal growth (i.e., appearance of fungal colonies). Check again after 1 week's incubation for the appearance of potential slow growing species. Figure 1 below shows an isolate of *Candida parapsilosis* growing on an antibiotic supplemented SD agar spread plate. This fungal pathobiont was cultured from a faecal sample from a young infant.

12. Depending on overall fungal colony number, pick at least two well-separated colonies of each different colony type (i.e., morphotype) and re-plate onto fresh agar medium of the same type (e.g., YM). The aim is to capture as much of the (viable) fungal diversity as possible, given the fact that differing colony morphology (e.g. colour, texture, elevation and margin type) can be used as a reliable means of identifying and isolating different fungal species. At this stage, there should be no need to use antibiotic supplemented medium, but it can be used if bacterial growth persists.

13. Incubate plates at 30ºC.

14. Once good growth has been achieved, a sample of each colony type can be picked and stored in sterile 20% glycerol stock at -80ºC, prior to future characterisation (e.g., species identification, phenotyping, genome sequencing etc.).

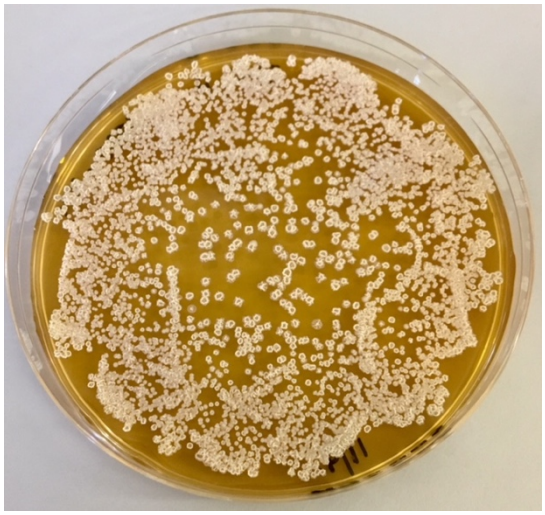

Figure 1. An isolate of *Candida parapsilosis* recovered from a faecal sample from an infant (shown here growing on antibiotic-supplemented SD agar)

10.4.1.3. Aerobic fungal culturing

1. For aerobic cultivation by spread plating, first set up a series of 10-fold dilutions from the faecal homogenate (dilution range: 10^{0} to 10^{-4}).

2. To set up the first ten-fold dilution (i.e., 10^{-1} or 10x dilution), add 100 uL of the faecal homogenate to a sterile 1.5 mL centrifuge tube containing 900 uL sterile 1x PBS (diluent). Mix thoroughly by vortexing.

3. Carry out a second ten-fold dilution by transferring 100 uL of the first ten-fold dilution (Step 2) into a new sterile 1.5 mL centrifuge tube containing 900 uL sterile 1x PBS, and mix thoroughly by vortexing. This represents the 10^{-2} or 100x dilution.

4. Repeat the process twice more to produce a total of 4 ten-fold dilutions, from 10^{-1} to 10^{-4} (i.e., from 10x to 10,000x diluted, respectively). Figure 2 below shows a schematic of how to set up a dilution series.

Figure 2. Setting up a 10-fold dilution series

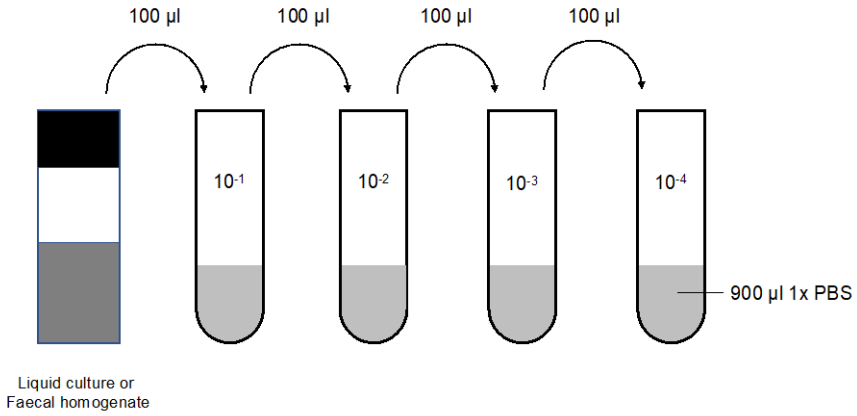

Figure 2. Setting up a 10-fold dilution series

5. For cultivation on solid medium, pipette a 50 µl aliquot of each dilution of faecal homogenate (i.e., 10^0 to 10^{-4}) onto a separate agar plate. Duplicate spread plates should be set up for each separate dilution.

6. Use a sterile disposable plastic L-shaped spreader to spread each diluted homogenate evenly across the entire surface of each antibiotic supplemented SD agar plate. Allow each plate to air dry. This represents Plate Set 1.

7. Repeat the procedure, using antibiotic supplemented YM agar plates. This represents Plate Set 2.

8. Transfer both sets of agar plates (Sets 1 & 2) to a 30°C incubator.

9. After 2 days incubation, inspect all plates for signs of fungal growth, notably for fast growing species (e.g., *Candida albicans*).

10. Continue incubating all plates for a further 5 days. This additional incubation time allows for the detection and cultivation of any slower growing fungal species that may also be present.

11. Some bacterial species can acquire antibiotic resistance, and so may still grow on these supplemented plates. In view of this, we strongly recommend that representative colonies should be examined using a standard light microscope if available. As already discussed,

yeast and filamentous fungi can readily be distinguished from bacteria based on their larger cell size

10.4.2 Non-culture based

10.4.2.1 DNA extraction

The following protocol, which uses the commercial Qiagen QIAamp PowerFecal Pro DNA Kit coupled with high-speed bead beating, has been developed as an effective manual method for maximising the recovery of low abundance fungal DNA from human faecal samples.

Please note: all centrifugation steps should be performed at room temperature.

1. Using a sterile spatula, add 200-250 mg of faecal sample to a clean 1.5 ml microcentrifuge tube.
2. Add 800 µl of Solution CD1 and vortex vigorously to mix.

Note: a plastic pellet pestle can be used to break up any large particulate matter that may remain after vortexing. At his stage, a second round of vortexing may be necessary to produce a uniform homogenate.

3. Before transferral to a PowerBead Pro tube, spin the tube briefly to ensure all beads have settled at the bottom. Once done, transfer the faecal homogenate to the tube using a 1 ml pipette tip.

Note: use of a wide-bore tip may help at this stage, especially if the homogenate still contains particulate matter after vortexing.

4. Homogenize samples using a FastPrep-24 benchtop instrument (MP Biomedicals) at 6.0 m/s for 1 minute**.**

Note: if larger sized DNA of higher molecular weight is required (e.g., for Nanopore long read sequencing), then sample homogenization should be done by vortexing using a benchtop vortex (e.g., Vortex-Genie®). Vortex at maximum speed for 10 minutes. When vortexing, tubes should be secured horizontally on a vortex adapter for 1.5-2 ml tubes.

5. After homogenization, centrifuge the PowerBead Pro tube at 15,000 **x *g*** for 1 minute.

6. Transfer the supernatant to a clean 2 mL microcentrifuge tube.

7. Add 200 µl of solution CD2 and vortex for 5 seconds.

8. Centrifuge at 15,000 **x *g*** for 1 minute. Without disturbing the pellet, transfer the supernatant (up to 700 µl) to a clean 2 ml microcentrifuge tube.

9. Add 600 µl of solution CD3 and vortex for 5 seconds.

10. Load 650 µl of the lysate onto an MB Spin Column (supplied in kit) and centrifuge at 15,000 **x *g*** for 1 minute.

11. Discard the flow-through and repeat step 10 to ensure all lysate has passed through the spin column.

12. Carefully place the spin column into a clean 2 ml microcentrifuge collection tube.

Note: avoid transferring any flow-through onto the spin column.

13. Add 500 µl of solution EA to the spin column and centrifuge at 15,000 **x *g*** for 1 minute.

14. Discard the flow-through and place the spin column back into the same collection tube.

15. Add 500 µl of solution C5 to the spin column and centrifuge at 15,000 **x *g*** for 1 minute.

16. Discard the flow-through and place the spin column into a new 2 ml collection tube and centrifuge at 16,000 **x *g*** for 2 minutes.

17. Carefully place the spin column into a new 1.5 ml elution tube.

Note: to maximise DNA recovery, we use 1.5 mL LoBind microcentrifuge tubes in place of the elution tubes supplied.

18. Add 50 µl of solution C6 (10 mM Tris) to the centre of the filter membrane.

Note: do not disturb the (white) filter membrane when pipetting.

19. Centrifuge at 15,000 x ***g*** for 1 minute. Discard the spin column. The extracted DNA is now ready to be quantified and quality checked. For short-term storage, the DNA should be stored frozen at -15 to -30ºC, while for long term storage, we recommend that it is kept frozen at -70ºC or below.

Important note: an empty bead-beating tube should be included as an extraction control, and treated exactly the same as for the tubes containing faecal samples. This control represents the 'kitome' and is used to identify DNA contaminants that may be introduced during DNA extraction (e.g., from reagents etc.).

10.4.2.2. Quantification and quality checking

For DNA quantification and quality checking, at the Quadram Institute we use the Qubit 3.0 Fluorometer and associated Qubit dsDNA Broad-Range (BR) assay kit, following the manufacturer's instructions. The BR assay kit is highly selective for double-stranded DNA (dsDNA) and has a quantitation range of 1 to 1000 ng/ µl. In our experience, typical DNA yields from human faecal samples using the above extraction protocol can often exceed 100 ng/ µl, and so the BR assay kit is ideally suited for accurate quantification. To minimise overall sample loss, we recommend that no more than 2 µl of extracted DNA should be used for quantification purposes. As an additional quality control (QC) check, DNA purity can be determined, if required (e.g., for commercial DNA sequencing submission), by spectrophotometry. At the Quadram Institute we use a benchtop NanoDrop spectrophotometer for determining A_{260}/A_{280} and A_{260}/A_{230} ratios. Again, sample loss is kept to a minimum as only a single 1-ul aliquot of sample is required. Further details on general DNA QC are provided in Section 4: DNA Extraction and Quality Control.

10.4.2.3. Fungal ITS1 amplification

The following protocol is used at the Quadram Institute to routinely prepare fungal ITS1 amplicon libraries for sequencing using the Illumina MiSeq platform. It is an amplicon-based approach that capitalises on the fact that the ribosomal DNA (rDNA) array, which includes the non-coding ITS1 and ITS2 regions, is present in multiple copies in fungi. Indeed, in *Saccharomyces cerevisiae*, a food-associated yeast often present in the human GI tract, the number of rDNA copies can range from 50 to 100 copies per strain [5]. For amplification of the ITS1 region we use the 'universal' fungal primers ITS1F and ITS2 [11, 12], modified to include the Illumina sequencing adapters, thus allowing a one-step amplification prior to indexing PCR. The Illumina-modified ITS1 primer sequences are shown below in Table 1.

Table 1. Fungal ITS1 primer sequences. Illumina adapter sequences are shown underlined.

Primer:	**Sequence (5' to 3'):**
ITS1F	CTTGGTCATTTAGAGGAAGTAA
ITS1F_MiSeq	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTTGGTCATTTAGAGGAAGTAA
ITS2	GCTGCGTTCTTCATCGATGC
ITS2_MiSeq	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTGCGTTCTTCATCGATGC

The ITS1 amplicon library PCR that we use is detailed below.

Note: duplicate amplification reactions are set up for each faecal DNA sample, and positive and negative controls are included in each separate PCR run.

All amplifications are carried out in a 25 µl reaction volume in 0.2 ml PCR tube strips.

Note: All reagents, as well as template DNA, once thawed should be kept on ice during PCR set up. Set up time should be kept as short as conveniently possible, to prevent reagents and DNA from warming to room temperature.

1. PCR master mix preparation: add the following reagents to a 0.6 ml microcentrifuge tube in the order shown below:

PCR-grade H_2O	8.9 µl
5x GC buffer (included in kit)*	5.0 µl
Primer mix (10 pmol/ul)	0.5 µl
dNTP mix (10 mM each)	0.5 µl
KAPA2G DNA polymerase (5U/µl)	0.1 µl

* contains 1.5 mM $MgCl_2$ at 1x concentration

All volumes shown above are for a single reaction and do not include the template.

Note: when preparing the PCR master mix, prepare for n+1 samples, to allow for possible pipetting error.

2. Mix gently by vortexing and briefly centrifuge to ensure all contents collect at the bottom of the tube.

3. Place the PCR master mix on ice.

4. For human faecal DNA samples, we routinely use 100 ng of DNA as template, diluted to a final working concentration of 10 ng/µl.

Note: in our experience, 100 ng is usually sufficient for successful fungal DNA amplification from human faecal DNA samples.

5. Add 10 µl of template DNA to each individual 0.2 ml tube.

Note: while the same pipette tip can be used to dispense the replicate aliquots of the same sample, a new tip must be used for separate samples to avoid cross-contamination between samples.

6. Positive and negative controls should be included in each separate PCR run. As a positive control, we use 0.1 ng of *Candida albicans* DNA, and PCR-grade water for a negative control.

7. Once all DNA templates and controls have been added, briefly centrifuge the PCR tube strips to ensure all contents collect at the bottom of the tubes.

Note: a mini-fuge with an 8-place rotor for 0.2 ml tubes can be used for this purpose.

8. Add 15 µl of PCR master mix to the top of each tube.

Note: to reduce overall pipette tip usage, a single pipette tip can be used to dispense the master mix to all tubes. However, at this stage, care must be taken to ensure template and master mix do not mix between tubes to avoid cross contamination.

9. Add lid strips, ensuring that each individual lid is firmly pressed into place.

10. Briefly centrifuge the PCR tube strips to ensure all contents collect at the bottom of the tubes.

11. Load the PCR tube strips onto a 0.2 ml tube compatible thermal cycler with a heated lid.

Note: at the Quadram Institute we use a Biometra TRIO, a triple block thermal cycler with a fast ramp rate.

12. For fungal ITS1 amplification, when using either standard or modified ITS1F and ITS2 primers, we use the following cycling parameters:

1 cycle	95ºC, 5 mins
35 cycle	95ºC, 30 s; 55ºC, 30 s; 72ºC, 30 s
1 cycle	72ºC, 5 mins

Note: The temperature of the heated lid should be set to 99ºC (to prevent sample evaporation); the typical PCR run time on a Biometra TRIO thermal cycler is approx. 80 mins.

13. The size of the ITS1 region varies quite markedly between different fungal species and genera [13], and can typically range from 100 to 400 bp in length. This means that standard 1% agarose gel electrophoresis is an effective method to evaluate overall PCR success as well as provide an estimate of ITS1 amplicon size. For agarose gel analysis, we typically run a 5 ul aliquot of each sample replicate to which 0.5 µl of Midori Green Direct DNA stain has been added. This is a safe stain, developed to replace toxic ethidium bromide, and allows DNA to be visualised under blue light using a blue/green LED transilluminator.

10.4.2.4. Library preparation and sequencing

Following agarose gel analysis, sample replicates are combined and a 0.7x SPRI purification done using KAPA Pure Beads with the purified DNA eluted in 20 ul of EB buffer (10 mM Tris-HCl). The purified DNA is then ready for the second PCR (i.e., the Indexing PCR), where the sequencing barcodes are added to each sample. Full details of the bead clean-up procedure, how the library indexing PCR is set up, and the amplification conditions used are provided in Section 5: 16S Illumina Library Preparation (5.4.4, Barcoding, PCR 2). After a second SPRI clean-up, samples are quantified, normalised and pooled in equal quantities. ITS1 library pools are run, at a final concentration of 8 pM, on an Illumina MiSeq instrument using the Illumina MiSeq Reagent Kit v3 (600-cycle) with 2x 300 bp output. All sequencing is

done in-house at Quadram Institute. Full details of the sequencing protocol used are provided in Section 5: 16S Illumina Library Preparation (5.4.9, Final dilutions; 5.4.10, Loading the run on the sequencer), with the ITS1 raw data analysed locally on the MiSeq instrument using MiSeq reporter.

10.4.2.5. Bioinformatics Analysis

A comprehensive description of the current mycobiome analysis pipeline developed at the Quadram Institute and used for characterising the human gut mycobiome is provided in Section 7: Microbiome Informatics (7.4.2; 16S/ITS1 amplicon sequencing [metabarcoding]). Briefly, after the initial pre-processing step, where adapters, barcodes and reads with ambiguous reads are removed using fastp [14], the primary analysis is done using DADA2 [15], with taxonomic annotation achieved using the UNITE Fungal ITS database [16]. The main output files from this analysis include representative sequences (amplicon sequence variants, ASVs), feature table and associated metadata. Secondary analysis is done using MicrobiomeAnalyst [17], an interactive web-based platform for downstream visualisation (e.g., relative abundance plots) and statistical analyses (e.g. alpha diversity).

10.5. Tips and Troubleshooting

SD and YM can be used for culturing a wide variety of different fungi, including *Candida* and *Saccharomyces* species. However, lipophilic fungi (e.g. *Malassezia*) do not grow well, if at all, on these and require specialised media (e.g. *Malassezia* medium).

For identification and cultivation of true gut fungal commensals (e.g. *Candida albicans*), incubation can be done at the higher temperature of 37ºC, rather than 30ºC as used in our culture-based protocols.

It is good practice to use both positive and negative controls in all PCR runs and DNA extractions. The inclusion of a negative control can help identify any contaminants that may be introduced during processing, from PCR reagents and from the DNA extraction kit.

When doing database searches (e.g., to confirm species identity) it is essential to ensure any comparisons are made against authenticated reference strains (i.e., species type strains). For instance, when using BLASTN for such purposes to search rRNA/ITS databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the ‘Sequences from type material’ option should be selected.

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