Journal of Visualized Experiments

An Ex Vivo Assay to Study Candida albicans Hyphal Morphogenesis in the Gastrointestinal Tract

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61488R1
Full Title:	An Ex Vivo Assay to Study Candida albicans Hyphal Morphogenesis in the Gastrointestinal Tract
Section/Category:	JoVE Immunology and Infection
Keywords:	Candida albicans, Hyphal Morphogenesis, Ex vivo assay and GI tract
Corresponding Author:	Shankar Thangamani Midwestern University Glendale, AZ UNITED STATES
Corresponding Author's Institution:	Midwestern University
Corresponding Author E-Mail:	sthang@midwestern.edu
Order of Authors:	Ross Monasky
	Sonia Villa
	Shankar Thangamani
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Glendale, Arizona and USA

TITLE:

An Ex vivo Assay to Study *Candida albicans* Hyphal Morphogenesis in the Gastrointestinal Tract

AUTHORS AND AFFILIATIONS:

Ross Monasky[1], Sonia Villa[2] and Shankar Thangamani[3*]

[1]College of Veterinary Medicine, Midwestern University, 19555 N. 59th Ave. Glendale, AZ 85308, USA

[2]Masters in Biomedical Science Program, College of Graduate Studies, Midwestern University, 19555 N. 59th Ave. Glendale, AZ 85308, USA

[3]Department of Pathology and Population Medicine, College of Veterinary Medicine, Midwestern University, 19555 N. 59th Ave. Glendale, AZ 85308, USA

Corresponding Author:

Shankar Thangamani (sthang@midwestern.edu)

Email Addresses of Co-Authors:

Ross Monasky (rmonas@midwestern.edu)

Sonia Villa (svilla63@midwestern.edu)

SUMMARY:

The ex vivo assay described in this study using gut homogenate extracts and immunofluorescence staining represents a novel method to examine the hyphal morphogenesis of *Candida albicans* in the GI tract. This method can be utilized to investigate the environmental signals regulating morphogenetic transition in the gut.

ABSTRACT:

Candida albicans hyphal morphogenesis in the gastrointestinal (GI) tract is tightly controlled by various environmental signals, and plays an important role in the dissemination and pathogenesis of this opportunistic fungal pathogen. However, methods to visualize fungal hyphae in the GI tract in vivo are challenging which limits the understanding of environmental signals in controlling this morphogenesis process. The protocol described here demonstrates a novel ex vivo method for visualization of hyphal morphogenesis in gut homogenate extracts. Using an ex vivo assay, this study demonstrates that cecal contents from antibiotic treated mice, but not from untreated control mice, promote *C. albicans* hyphal morphogenesis in the gut content. Further, adding back specific groups of gut metabolites to the cecal contents from antibiotic-treated mice differentially regulates hyphal morphogenesis ex vivo. Taken together, this protocol represents a novel method to identify and investigate the environmental signals that control *C. albicans* hyphal morphogenesis in the GI tract.

Keywords:

Candida albicans, hyphal morphogenesis, ex vivo assay, glucose, secondary bile acids, and GI tract

INTRODUCTION:

Candida albicans is an opportunistic, polymorphic fungal pathogen that is normally commensal, but can undergo a morphological change into a virulent form capable of causing life-threatening infections in immunocompromised individuals[1-13]. *C. albicans* is a leading cause of systemic nosocomial infections, with a 40–60% mortality rate even with antifungal treatment[2,14,15]. Though *C. albicans* resides in different host niches including the female reproductive system[16,17], the oral cavity of healthy individuals[18] and the gastrointestinal (GI) tract[19,20], the majority of the systemic infections originate from the GI tract and furthermore, the source of systemic infection is often confirmed to be the GI tract[21-34]. *C. albicans* pathogenicity in the GI tract is influenced by a wide range of factors; however, a major characteristic necessary for virulence is the transition from a yeast cell morphology into a virulent hyphal cell morphology[35-44]. *C. albicans* attachment and dissemination from the GI tract during infection is highly associated with its capacity to transition from a commensal yeast into virulent hyphae, allowing the fungi to cause invasive disease[44-53].

A variety of factors in the gut, including n-acetylglucosamine, regulate hyphal formation by *C. albicans*. Therefore, it is crucial to narrow the gap in knowledge regarding the hyphal morphogenesis of this fungal pathogen in the GI tract[54-56]. Recent evidence indicates that various gut metabolites differentially control the hyphal morphogenesis of *C. albicans* in vitro[57-60]. However, technical constraints present issues when attempting to study *C. albicans* hyphae formation in in vivo gut samples, especially staining yeast and hyphae cells and quantitative analysis of hyphal development. To understand *C. albicans* hyphal morphogenesis in the GI tract, an ex vivo method was developed using soluble extracts of homogenized gut content from mice to study the effect of metabolites on fungal hyphal morphogenesis. Utilizing gut samples from mice that are resistant and susceptible to *C. albicans* GI infection, this method will help to identify and study the effect of metabolites, antibiotics and xenobiotics on fungal hyphal morphogenesis in the GI tract.

PROTOCOL:

All animal protocols were approved by Midwestern University Institutional Animal Care and Use Committee (IACUC) as described before[57]. The Institutional Animal Care and Use Committee at Midwestern University approved this study under MWU IACUC Protocol #2894. The MWU animal care policies follow the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals and the policies laid out in the Animal Welfare Act (AWA).

1. Mice study standard protocol

1.1. Use male and female C57BL/6J mice at least six weeks old. Supplement them with sterile water with or without cefoperazone (0.5 mg/mL).

1.1.1. Co-house mice in groups of 5, with each cage containing either all male or all female mice. Provide the mice standard mouse chow and water (via a 400 mL bottle) at all times.

1.1.2. Check cages daily to ensure food and water levels are enough, and to examine mice for signs of distress.

1.2. Replace the water with cefoperazone every 48 h to ensure fresh antibiotic is being provided regardless of remaining water in the cage feeding bottles.

1.3. After 5–7 days of cefoperazone treatment, euthanize mice via CO_2 asphyxiation observing established IACUC protocol. Confirm death via cervical dislocation.

1.4. Dissect mice using autoclave-sterilized sharp ended scissors and autoclave-sterilized forceps.

1.4.1. After euthanasia, secure the animal was secured to a dissection surface by pinning all limbs such that the abdomen is exposed.

1.4.2. Spray the abdominal region with 70% ethanol to prevent fur from sticking to forceps, scissors, or gut sections during dissection.

1.4.3. Use forceps to pinch and lift a section of skin at the base of the abdomen and create a small incision through the skin and underlying fascia using scissors. Take great care when making this incision to avoid puncturing the cecum or intestinal wall.

1.4.4. Extend this cut to the rib cage, partially exposing the peritoneal cavity. Make a cut starting at the point of the initial incision on either side extending upward and laterally.

1.4.5. Pull these flaps laterally and pin to the dissecting surface to fully expose the peritoneal cavity.

1.5. Extract the GI tract using forceps, while using scissors to make cuts superior to the stomach and at the distal region of the large intestine to ensure collection of the greatest amount of gut content from each section.

1.6. When removing the GI tract, take care to avoid rupturing the individual components. Separate the stomach, small intestine, cecum, and large intestine individually using scissors at their proximal and distal ends.

1.7. For collection of each gut contents from each section, make a single incision at the distal end of each section using scissors, followed by manually expelling the gut content into a 1.5 mL microcentrifuge tube using forceps.

1.8. Store gut contents at -80 °C for ex vivo assays.

2. Preparation of yeast extract-peptone-dextrose (YPD) agar plates

2.1. To a 1 L glass bottle add 25 g of yeast extract peptone-dextrose broth powder, 10 g of agar, and ultrapure water to a final volume of 500 mL.

2.2. Autoclave at 121 °C for 30 min on a liquid cycle to sterilize the media.

2.3. Under a laminar flow hood, pour approximately 20 mL of agar media into a sterile Petri plate. 500 mL of agar media should yield approximately 25 plates.

2.4. Store plates at 4 °C until ready for use.

3. Ex vivo prep for hyphal morphogenesis assay

3.1. Streak a fresh culture of *C. albicans* SC5314 onto a YPD agar plate and incubate overnight at 30 °C.

3.2. Pick two to three medium-sized individual colonies from overnight grown *C. albicans* SC5314 culture and re-suspend in 1 mL of phosphate buffered saline (PBS).

3.3. Retrieve frozen gut contents from the -80 °C freezer and thaw at 25 °C.

3.4. Weigh about 150 mg of gut contents into a new 1.5 mL tube.

3.5. Re-suspend the gut contents with 150 µL of PBS (gut content and PBS at a 1:1 weight to volume ratio).

3.6. Vortex at high speed for 30 s to homogenize the gut contents and allow to sit at room temperature for about a minute.

3.7. Centrifuge the homogenates at 1000 x *g* for 3 min.

3.8. Transfer the supernatant to a new 1.5 mL tube.

3.9. Repeat steps 3.7 and 3.8 to remove all debris in the supernatant.

3.10. Add 10 µL of the *C. albicans* SC5314 inoculum prepared above to this supernatant

3.11. Mix well and incubate at 37 °C for 4 to 5 h.

4. Exogenous addition of metabolites to the gut homogenate extracts for the hyphal morphogenesis assay

4.1. Retrieve frozen gut contents from the -80 °C freezer and re-suspended in PBS at 1:1 ratio (weight: volume).

4.2. Add desired concentration of gut metabolites to the gut content and PBS mixture.

4.3. Vortex at high speed for 30 s to homogenize the gut contents containing metabolites and allow to sit at room temperature for about 10 min.

4.4. Centrifuge the homogenates at 1000 x *g* for 3 min.

4.5. Transfer the supernatant to a new 1.5 mL tube. Repeat steps 4.4 and 4.5 to remove all debris in the supernatant.

4.6. Add 10 µL of the *C. albicans* SC5314 inoculum prepared above to this supernatant. Mix well and incubate at 37 °C for 4 to 5 h.

5. *C. albicans* morphogenesis assay (immunostaining and imaging)

5.1. Centrifuge the samples at 1000 x *g* for 2 min and discard the supernatant via pipetting.

5.2. Fix the samples in 100 µL of 2% paraformaldehyde (PFA) for 15 min.

5.3. Centrifuge at 1000 x *g* for 2 min and discard supernatant via pipetting.

5.4. Wash the samples twice with 1 mL of PBS. To wash samples, re-suspend the pellet in PBS by pipetting gently. Do not vortex the sample as this can damage hyphal structures. After re-suspension, centrifuge at 1000 x *g* for 2 min and discard the supernatant via pipetting.

5.5. Incubate the samples at room temperature in 100 µL of PBS containing polyclonal *C. albicans* antibody (1:100 dilution) for 30 min.

5.6. Wash the samples three times with 1 mL of PBS.

NOTE: When using a fluorescent antibody, it is recommended that all dilution and wash steps be performed in dim light to avoid photo bleaching and improve sample longevity.

5.7. Incubate the samples at room temperature for 15 min in 100 µL of PBS containing anti-Rabbit IgG Alexafluor 488 antibody at 1:500 dilution. Perform incubation in a dark drawer or room to avoid photo bleaching.

5.8. Wash the samples three times with 1 mL of PBS.

5.9. Re-suspend the samples in 100 µL of PBS and transfer to a 96-well plate for imaging.

NOTE: When not being imaged, it is recommended that the 96-well plate be wrapped in aluminum foil to avoid photo bleaching.

5.10. Image fungal cells using 20x and 40x objective lenses using a fluorescence imaging microscope. Use a green fluorescent protein (GFP) filter (excitation wavelength 470/40 and emission wavelength 525/50) to detect fluorescence.

REPRESENTATIVE RESULTS:

These results along with previous findings from the Thangamani laboratory[60] indicate that when *C. albicans* is grown ex vivo in gut homogenate extracts taken from the stomach, small intestines and large intestines of untreated control and antibiotic-treated mice, *C. albicans* generally develops with a yeast morphology (**Figure 1B**). However, when grown in the cecal extract from antibiotic-treated, *C. albicans* readily undergoes morphogenesis, resulting in samples containing yeast and hyphae forms (Figure 1B); this does not occur in control mice. This supports previous results, which showed a significant increase in hyphae forms in samples grown in antibiotic-treated cecal extracts, but not in any other antibiotic-treated gut extracts[60]. These results suggest that antibiotic treatment causes changes in the cecal environment, which induce hyphal morphogenesis of *C. albicans.* Additionally, the specific localization of this phenotype noticed only in the cecum also suggests that these hyphae-promoting conditions may not necessarily present throughout the GI tract, but instead are restricted to specific segments of the GI tract depending on the availability of nutrients, metabolites and other unknown molecules.

Since the cecal extract of antibiotic-treated mice promotes the morphogenesis of *C. albicans* [57-60], we examined whether exogenous addition of a selected group of gut metabolites (identified from previous in vitro studies to the cecal content of cef-treated mice) will affect the morphogenesis of *C. albicans* ex vivo. Previous work performed by the Thangamani laboratory has characterized the metabolomics profile of cecal content homogenate extracted from untreated and antibiotic-treated mice, revealing significant changes in the abundance of various metabolites as a result of antibiotic-treatment—specifically, decreased abundance of secondary bile acids and increased abundance of carbohydrates[60]. Further, this study identified that secondary bile acids and carboxylic acids inhibit hyphae development, whereas carbohydrates including glucose, promote the hyphal morphogenesis of *C. albicans* in vitro[60]. Results indicate that adding back a pool of inhibitory gut metabolites containing deoxycholic acid (DCA, 0.5 mg/mL), lithocholic acid (LCA, 0.1 mg/mL), palmitic acid (0.1 mg/mL), p-tolylacetic acid (0.1 mg/mL), sebacic acid (0.5 mg/mL), 2-methylbutyric acid (0.5 mg/mL), and lactic acid (5 mg/mL) to the cecal homogenate of cef-treated mice completely inhibited hyphal morphogenesis ex vivo. On the other hand, exogenous addition of glucose (1 mg/mL) to the cecal homogenate of cef-treated mice showed a massive hyphal development ex vivo (**Figure 2B**). Collectively, these findings indicate that addition of gut metabolites back to the cecal homogenate of the cef-treated mice differentially regulates the morphogenesis of *C. albicans,* thus confirming previous in vitro findings. These results indicate that gut metabolites play a critical role in hyphal morphogenesis of *C. albicans* and understanding the gene targets and signaling pathways modulated by these

metabolites will aid in the development of new therapeutic approaches to prevent and treat *C. albicans* infections.

FIGURE LEGENDS:

Figure 1 : Ex vivo assay to determine the effect of cefoperazone treatment on *C. albicans* hyphal morphogenesis in the gut contents. (**A**) Protocol schematic outline. (**B**) Antibiotic-treated (top panels) and non-treated (bottom panels) gut contents were taken from the stomachs, small intestines, cecums, and large intestines of C57BL/6J mice. Gut contents inoculated with *C. albicans* SC5314 were incubated at 37 °C for 4–5 h and stained with *C. albicans* antibody. Cells were imaged at 40x magnification. Representative images are shown here.

Figure 2: Exogenous addition of gut metabolites to the cecal contents from cef-treated mice on hyphae formation of *C. albicans* ex vivo. (**A**) Protocol schematic outline. (**B**) Inhibitory gut metabolites pool containing DCA (0.5 mg/mL), LCA (0.1 mg/mL), palmitic acid (0.1 mg/mL), p-tolylacetic acid (0.1 mg/mL), sebacic acid (0.5 mg/mL); 2-methylbutyric acid (0.5 mg/mL), and lactic acid (5 mg/mL) or glucose (1 mg/mL) were added back to the cecal content of cef-treated mice, mixed thoroughly and incubated at 37 °C for 15 min to carry out the ex vivo hyphae assay. Cecal contents inoculated with *C. albicans* SC5314 were incubated at 37 °C for 4–5 h and stained with *C. albicans* antibody. Cells were imaged at 40x magnification. Representative images are shown here.

DISCUSSION:

The method described here presents a novel way to investigate the effect of antibiotic, dietary, xenobiotic and therapeutic impacts on *C. albicans* hyphal morphogenesis in the GI tract. Since the majority of systemic infections originate from the GI tract[21-34] and hyphae formation is a critical virulence factor that promotes the dissemination of *C. albicans* from the GI tract, understanding the factors that controls this morphogenesis in the GI tract will expand the knowledge about pathogenesis mechanisms and identify novel treatment options.

While the method presented here is relatively straightforward, certain steps discussed below were identified as critical and important. (i) The initial inoculum of *C. albicans* should be optimal to allow for both growth and hyphal morphogenesis of fungi. With the limited availability of nutrients in the gut homogenate extracts, higher volume of inoculum may significantly reduce the fungal growth and morphogenesis process. However, the growth of different clinical isolates and strains are likely to be variable, thus optimizing the inoculum and incubation time for specific *C. albicans* isolates is essential. (ii) Multiple centrifugation steps when preparing the gut homogenate extract were found to be crucial to remove the debris in gut contents as much as possible. (iii) Due to the relatively low speed of centrifugation (to avoid damaging hyphal structures), care must be taken to avoid cell loss during immunostaining steps in this protocol.

Alternative methods to visualize fungal hyphae in the GI tract have been used in the past, with certain advantages and limitations associated with each method. One relatively notable method

using fluorescent in situ hybridization (FISH) to visualize fungal hyphae in the GI tract has been recently demonstrated by the Witchley et al.[61,62]. This is a promising in vivo method currently available to detect *C. albicans* hyphae directly in the GI tract, however the complexity of this protocol makes it difficult to adapt it to rapid, large scale initial screening studies. Traditional histopathology methods have also been used in the past to vitalize *C. albicans* yeast and hyphae forms in the GI tract. However, observation and imaging of fungal cells with basic histopathology, and Hematoxylin and Eosin (H/E) stains remains challenging, as many standard fixation methods have the potential to disrupt the mucosal layer of GI tract samples, often damaging hyphal structures in the process and leading to contradictory reports over the relative abundance of hyphal cell morphology during infection[63-66] . This method was developed to avoid damage to hyphae during processing to address this issue. In addition, tissue explants have been used as a way to examine biological conditions ex vivo, however these methods are generally focused and useful for examining the adherence or invasion potential of *C. albicans*[67], but also they generally exclude the majority of metabolomics and microbiome components that contribute to in vivo pathogenesis. Although the ex vivo protocol described here does not completely mimic in vivo GI environment as described previously[61,62], it provides the closest possible conditions that *C. albicans* encounters in the gut environment compared to in vitro methods using artificial growth conditions.

This protocol can be used for basic screening assays to identify the impact of environmental signals in the GI tract on *C. albicans* hyphal morphogenesis. This method allows for large groups of compounds including small molecule inhibitors, novel antimycotics, and metabolites to be screened rapidly for hyphal development, and could be used in screening therapeutic treatments or identifying risk factors for systemic disease. Since *C. albicans* colonizes throughout the GI tract, this protocol will further aid identifying the environmental signals present in the specific segments of GI tract that control hyphal morphogenesis in individuals taking antibiotics, chemotherapeutic agents, and in patients with metabolic disorders including diabetes mellitus. Ultimately the method described here allows for quick characterization of hyphal morphogenesis in *C. albicans* over a wide range of environmental factors in a manner that is more biologically relevant than current in vitro methods and is substantially faster and more resource efficient than current in vivo methods.

ACKNOWLEDGEMENTS:

The authors acknowledge resources and support from Midwestern University Cellular and Molecular Core Research facility.

DISCLOSURES:

The authors have no competing financial interests or other conflicts of interest.

REFERENCES:

1 Huffnagle, G. B., Noverr, M. C. The emerging world of the fungal microbiome. *Trends in Microbiology.* **21** (7), 334-341, doi:10.1016/j.tim.2013.04.002, (2013).

2 Wisplinghoff, H. et al. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clinical Infectious Diseases.* **39** (3), 309-317, doi:10.1086/421946, (2004).
3 Hajjeh, R. A. et al. Incidence of Bloodstream Infections Due to Candida Species and In Vitro Susceptibilities of Isolates Collected from 1998 to 2000 in a Population-Based Active Surveillance Program. *Journal of Clinical Microbiology.* **42** (4), 1519-1527, doi:10.1128/JCM.42.4.1519-1527.2004, (2004).
4 Lockhart, S. R. et al. Species Identification and Antifungal Susceptibility Testing of Candida Bloodstream Isolates from Population-Based Surveillance Studies in Two U.S. Cities from 2008 to 2011. *Journal of Clinical Microbiology.* **50** (11), 3435-3442, doi:10.1128/JCM.01283-12, (2012).
5 Pfaller, M. et al. Epidemiology and outcomes of candidemia in 3648 patients: data from the Prospective Antifungal Therapy (PATH Alliance(R)) registry, 2004-2008. *Diagnostic Microbiology and Infectious Disease.* **74** (4), 323-331, doi:10.1016/j.diagmicrobio.2012.10.003, (2012).
6 Angarone, M. Fungal infections in cancer patients. *Cancer Treatment and Research.* **161** 129-155, doi:10.1007/978-3-319-04220-6_4, (2014).
7 Brown, G. D. et al. Hidden killers: human fungal infections. *Science Translational Medicine.* **4** (165), 165rv113, doi:10.1126/scitranslmed.3004404, (2012).
8 Calton, E. A. et al. Invasive bacterial and fungal infections in paediatric patients with cancer: incidence, risk factors, aetiology and outcomes in a UK regional cohort 2009-2011. *Pediatric Blood & Cancer.* **61** (7), 1239-1245, doi:10.1002/pbc.24995, (2014).
9 Carter, J. H. et al. Medical management of invasive fungal infections of the central nervous system in pediatric cancer patients. *Pediatric Blood & Cancer.* **62** (6), 1095-1098, doi:10.1002/pbc.25331, (2015).
10 Low, C. Y., Rotstein, C. Emerging fungal infections in immunocompromised patients. *F1000 Medicine Reports.* **3** 14, doi:10.3410/M3-14, (2011).
11 Mousset, S. et al. Treatment of invasive fungal infections in cancer patients-updated recommendations of the Infectious Diseases Working Party (AGIHO) of the German Society of Hematology and Oncology (DGHO). *Annals of Hematology.* **93** (1), 13-32, doi:10.1007/s00277-013-1867-1, (2014).
12 Perfect, J. R., Hachem, R., Wingard, J. R. Update on epidemiology of and preventive strategies for invasive fungal infections in cancer patients. *Clinical Infectious Diseases.* **59 Suppl 5** S352-355, doi:10.1093/cid/ciu639, (2014).
13 Sipsas, N. V., Kontoyiannis, D. P. Invasive fungal infections in patients with cancer in the Intensive Care Unit. *International Journal of Antimicrobial Agents.* **39** (6), 464-471, doi:10.1016/j.ijantimicag.2011.11.017, (2012).
14 Falagas, M. E., Apostolou, K. E., Pappas, V. D. Attributable mortality of candidemia: a systematic review of matched cohort and case-control studies. *European Journal of Clinical Microbiology and Infectious Diseases.* **25** (7), 419-425, doi:10.1007/s10096-006-0159-2, (2006).
15 Chi, H. W. et al. Candida albicans versus non-albicans bloodstream infections: the comparison of risk factors and outcome. *Journal of Microbiology, Immunology and Infection.* **44** (5), 369-375, doi:10.1016/j.jmii.2010.08.010, (2011).

16 Drell, T. et al. Characterization of the vaginal micro- and mycobiome in asymptomatic reproductive-age Estonian women. *PLoS One.* **8** (1), e54379, doi:10.1371/journal.pone.0054379, (2013).
17 Merenstein, D. et al. Colonization by Candida species of the oral and vaginal mucosa in HIV-infected and noninfected women. *AIDS Research and Human Retroviruses.* **29** (1), 30-34, doi:10.1089/aid.2012.0269, (2013).
18 Ghannoum, M. A. et al. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS Pathogens.* **6** (1), e1000713, doi:10.1371/journal.ppat.1000713, (2010).
19 Hoffmann, C. et al. Archaea and fungi of the human gut microbiome: correlations with diet and bacterial residents. *PLoS One.* **8** (6), e66019, doi:10.1371/journal.pone.0066019, (2013).
20 Noble, S. M., Gianetti, B. A., Witchley, J. N. Candida albicans cell-type switching and functional plasticity in the mammalian host. *Nature Reviews Microbiology.* **15** (2), 96-108, doi:10.1038/nrmicro.2016.157, (2017).
21 Samonis, G. et al. Prospective evaluation of effects of broad-spectrum antibiotics on gastrointestinal yeast colonization of humans. *Antimicrobial Agents and Chemotherapy.* **37** (1), 51-53 (1993).
22 Sahni, V. et al. Candidemia--an under-recognized nosocomial infection in Indian hospitals. *The Journal of the Association of Physicians of India.* **53** 607-611 (2005).
23 Meijer-Severs, G. J., Joshi, J. H. The effect of new broad-spectrum antibiotics on faecal flora of cancer patients. *Journal of Antimicrobial Chemotherapy.* **24** (4), 605-613 (1989).
24 Kennedy, M. J., Volz, P. A., Edwards, C. A., Yancey, R. J. Mechanisms of association of Candida albicans with intestinal mucosa. *Journal of Medical Microbiology.* **24** (4), 333-341, doi:10.1099/00222615-24-4-333, (1987).
25 Miranda, L. N. et al. Candida colonisation as a source for candidaemia. *Journal of Hospital Infections.* **72** (1), 9-16, doi:10.1016/j.jhin.2009.02.009, (2009).
26 Nucci, M., Anaissie, E. Revisiting the source of candidemia: skin or gut? *Clinical Infectious Diseases.* **33** (12), 1959-1967, doi:10.1086/323759, (2001).
27 Raponi, G., Visconti, V., Brunetti, G., Ghezzi, M. C. Clostridium difficile infection and Candida colonization of the gut: is there a correlation? *Clinical Infectious Diseases.* **59** (11), 1648-1649, doi:10.1093/cid/ciu637, (2014).
28 Guastalegname, M., Russo, A., Falcone, M., Giuliano, S., Venditti, M. Candidemia subsequent to severe infection due to Clostridium difficile: is there a link? *Clinical Infectious Diseases.* **57** (5), 772-774, doi:10.1093/cid/cit362, (2013).
29 Nerandzic, M. M., Mullane, K., Miller, M. A., Babakhani, F., Donskey, C. J. Reduced acquisition and overgrowth of vancomycin-resistant enterococci and Candida species in patients treated with fidaxomicin versus vancomycin for Clostridium difficile infection. *Clinical Infectious Diseases.* **55 Suppl 2** S121-126, doi:10.1093/cid/cis440, (2012).
30 Krause, R., Krejs, G. J., Wenisch, C., Reisinger, E. C. Elevated fecal Candida counts in patients with antibiotic-associated diarrhea: role of soluble fecal substances. *Clinical and Diagnostic Laboratory Immunology.* **10** (1), 167-168 (2003).
31 Krause, R. et al. Role of Candida in antibiotic-associated diarrhea. *The Journal of Infectious Diseases.* **184** (8), 1065-1069, doi:10.1086/323550, (2001).

32 Zuo, T. et al. Gut fungal dysbiosis correlates with reduced efficacy of fecal microbiota transplantation in Clostridium difficile infection. *Nature Communications.* **9** (1), 3663, doi:10.1038/s41467-018-06103-6, (2018).
33 Delaloye, J., Calandra, T. Invasive candidiasis as a cause of sepsis in the critically ill patient. *Virulence.* **5** (1), 161-169, doi:10.4161/viru.26187, (2014).
34 Cole, G. T., Halawa, A. A., Anaissie, E. J. The role of the gastrointestinal tract in hematogenous candidiasis: from the laboratory to the bedside. *Clinical Infectious Diseases.* **22 Suppl 2** S73-88 (1996).
35 Lo, H.-J. et al. Nonfilamentous C. albicans mutants are avirulent. *Cell.* **90** (5), 939-949 (1997).
36 Gale, C. A. et al. Linkage of adhesion, filamentous growth, and virulence in Candida albicans to a single gene, INT1. *Science.* **279** (5355), 1355-1358 (1998).
37 Bendel, C. M. et al. Systemic infection following intravenous inoculation of mice with Candida albicans int1 mutant strains. *Molecular genetics and metabolism.* **67** (4), 343-351 (1999).
38 Toenjes, K. A. et al. Small-molecule inhibitors of the budded-to-hyphal-form transition in the pathogenic yeast Candida albicans. *Antimicrobial agents and chemotherapy.* **49** (3), 963-972 (2005).
39 Carlisle, P. L. et al. Expression levels of a filament-specific transcriptional regulator are sufficient to determine Candida albicans morphology and virulence. *Proceedings of the National Academy of Sciences.* **106** (2), 599-604 (2009).
40 Fazly, A. et al. Chemical screening identifies filastatin, a small molecule inhibitor of Candida albicans adhesion, morphogenesis, and pathogenesis. *Proceedings of the National Academy of Sciences.* **110** (33), 13594-13599 (2013).
41 Pande, K., Chen, C., Noble, S. M. Passage through the mammalian gut triggers a phenotypic switch that promotes Candida albicans commensalism. *Nature genetics.* **45** (9), 1088 (2013).
42 Bar-Yosef, H., Gonzalez, N. V., Ben-Aroya, S., Kron, S. J., Kornitzer, D. Chemical inhibitors of Candida albicans hyphal morphogenesis target endocytosis. *Scientific reports.* **7** (1), 5692 (2017).
43 Mendelsohn, S., Pinsky, M., Weissman, Z., Kornitzer, D. Regulation of the Candida albicans hypha-inducing transcription factor Ume6 by the CDK1 cyclins Cln3 and Hgc1. *mSphere.* **2** (2), e00248-00216 (2017).
44 Vila, T. et al. Targeting Candida albicans filamentation for antifungal drug development. *Virulence.* **8** (2), 150-158 (2017).
45 Pande, K., Chen, C., Noble, S. M. Passage through the mammalian gut triggers a phenotypic switch that promotes Candida albicans commensalism. *Nature Genetics.* **45** (9), 1088-1091, doi:10.1038/ng.2710, (2013).
46 Lo, H. J. et al. Nonfilamentous C. albicans mutants are avirulent. *Cell.* **90** (5), 939-949 (1997).
47 Bar-Yosef, H., Vivanco Gonzalez, N., Ben-Aroya, S., Kron, S. J., Kornitzer, D. Chemical inhibitors of Candida albicans hyphal morphogenesis target endocytosis. *Scientific Reports.* **7** (1), 5692, doi:10.1038/s41598-017-05741-y, (2017).

48 Carlisle, P. L. et al. Expression levels of a filament-specific transcriptional regulator are sufficient to determine Candida albicans morphology and virulence. *Proceedings of the National Academy of Sciences of the United States of America.* **106** (2), 599-604, doi:10.1073/pnas.0804061106, (2009).
49 Mendelsohn, S., Pinsky, M., Weissman, Z., Kornitzer, D. Regulation of the Candida albicans Hypha-Inducing Transcription Factor Ume6 by the CDK1 Cyclins Cln3 and Hgc1. *mSphere.* **2** (2), doi:10.1128/mSphere.00248-16, (2017).
50 Bendel, C. M. et al. Effects of Alteration of the Candida albicans Gene INT1 on Cecal Colonization in Orally Innoculated Mice. *Pediatric Research.* **45** 156A, doi:10.1203/00006450-199904020-00929, (1999).
51 Gale, C. A. et al. Linkage of adhesion, filamentous growth, and virulence in Candida albicans to a single gene, INT1. *Science.* **279** (5355), 1355-1358 (1998).
52 Toenjes, K. A. et al. Small-molecule inhibitors of the budded-to-hyphal-form transition in the pathogenic yeast Candida albicans. *Antimicrobial Agents and Chemotherapy.* **49** (3), 963-972, doi:10.1128/AAC.49.3.963-972.2005, (2005).
53 Fazly, A. et al. Chemical screening identifies filastatin, a small molecule inhibitor of Candida albicans adhesion, morphogenesis, and pathogenesis. *Proceedings of the National Academy of Sciences of the United States of America.* **110** (33), 13594-13599, doi:10.1073/pnas.1305982110, (2013).
54 Naseem, S., Gunasekera, A., Araya, E., Konopka, J. B. N-acetylglucosamine (GlcNAc) induction of hyphal morphogenesis and transcriptional responses in Candida albicans are not dependent on its metabolism. *Journal of Biological Chemistry.* **286** (33), 28671-28680, doi:10.1074/jbc.M111.249854, (2011).
55 Piispanen, A. E., Hogan, D. A. PEPped up: induction of Candida albicans virulence by bacterial cell wall fragments. *Cell Host & Microbe.* **4** (1), 1-2, doi:10.1016/j.chom.2008.06.005, (2008).
56 Xu, X. L. et al. Bacterial peptidoglycan triggers Candida albicans hyphal growth by directly activating the adenylyl cyclase Cyr1p. *Cell Host & Microbe.* **4** (1), 28-39, doi:10.1016/j.chom.2008.05.014, (2008).
57 Guinan, J., Thangamani, S. Antibiotic-induced alterations in taurocholic acid levels promote gastrointestinal colonization of Candida albicans. *FEMS microbiology letters.* **365** (18), fny196 (2018).
58 Guinan, J., Villa, P., Thangamani, S. Secondary bile acids inhibit Candida albicans growth and morphogenesis. *Pathogens and disease.* **76** (3), fty038 (2018).
59 Guinan, J., Wang, S., Hazbun, T. R., Yadav, H., Thangamani, S. Antibiotic-induced decreases in the levels of microbial-derived short-chain fatty acids correlate with increased gastrointestinal colonization of Candida albicans. *Scientific Reports.* **9** (1), 1-11 (2019).
60 Gutierrez, D. et al. Antibiotic-induced gut metabolome and microbiome alterations increase the susceptibility to Candida albicans colonization in the gastrointestinal tract. *FEMS microbiology ecology.* **96** (1), fiz187 (2020).
61 Witchley, J. N. et al. Candida albicans morphogenesis programs control the balance between gut commensalism and invasive infection. *Cell Host & Microbe.* **25** (3), 432-443. e436 (2019).

62 Witchley, J. N., Penumetcha, P. M., Noble, S. M. Visualization of Candida albicans in the Murine Gastrointestinal Tract Using Fluorescent In Situ Hybridization. *JoVE (Journal of Visualized Experiments).* (153), e60283 (2019).
63 Johansson, M. E., Hansson, G. C. Preservation of mucus in histological sections, immunostaining of mucins in fixed tissue, and localization of bacteria with FISH. *Mucins. pp* 229-235. Springer. (2012).
64 Lossinsky, A. S. et al. The histopathology of Candida albicans invasion in neonatal rat tissues and in the human blood-brain barrier in culture revealed by light, scanning, transmission and immunoelectron microscopy scanning. *Histology and histopathology*. (2006).
65 Rosenbach, A., Dignard, D., Pierce, J. V., Whiteway, M., Kumamoto, C. A. Adaptations of Candida albicans for growth in the mammalian intestinal tract. *Eukaryotic Cell* **9**, 1075-1086. (2010).
66 Vautier, S., et al. C andida albicans colonization and dissemination from the murine gastrointestinal tract: the influence of morphology and T h17 immunity. *Cellular Microbiology* **17**, 445-450, (2015).
67 Lyman, C., Navarro, E., Garrett, K., Roberts, D., Pizzo, P., Walsh T. Adherence of Candida albicans to bladder mucosa: development and application of a tissue explant assay. *Mycoses.* **42**, 255-259. (1999).

Figure 1

Click here to access/download;Figure;Figure 1.tif

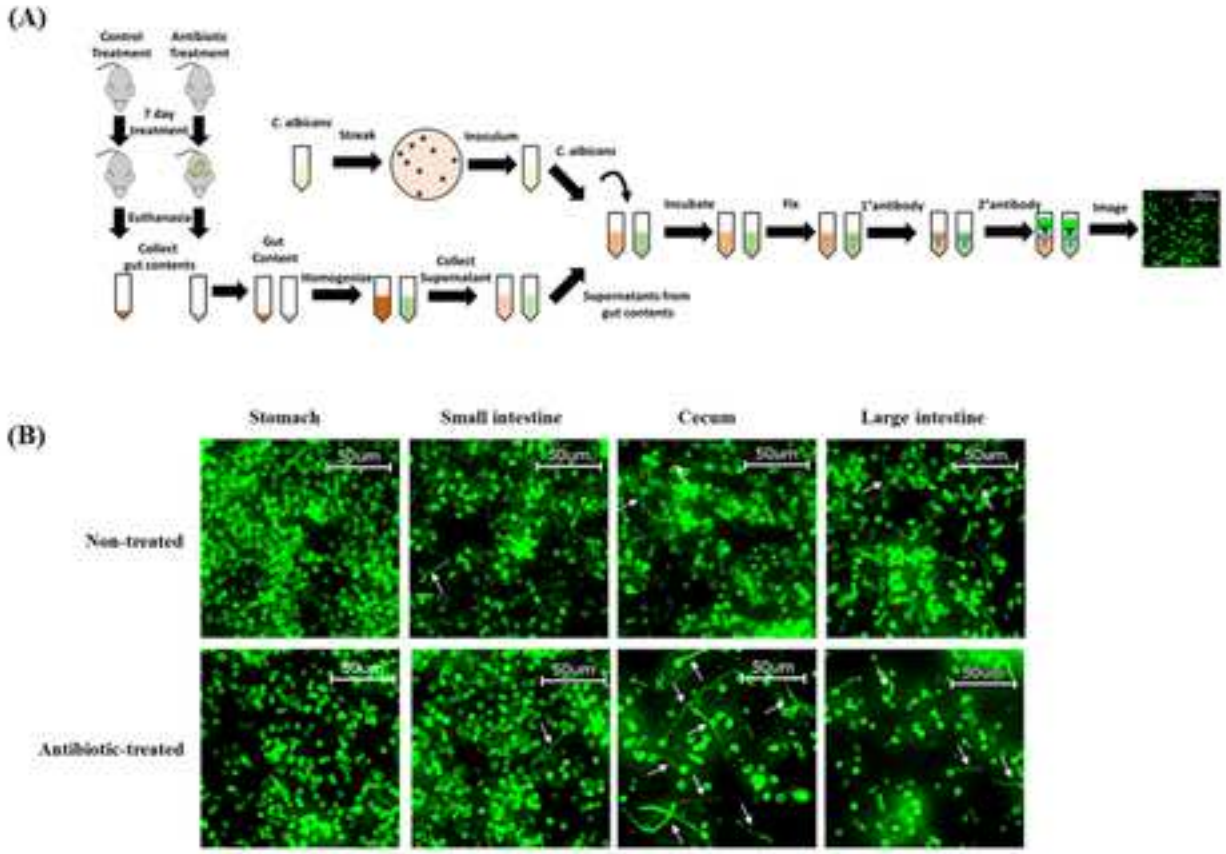
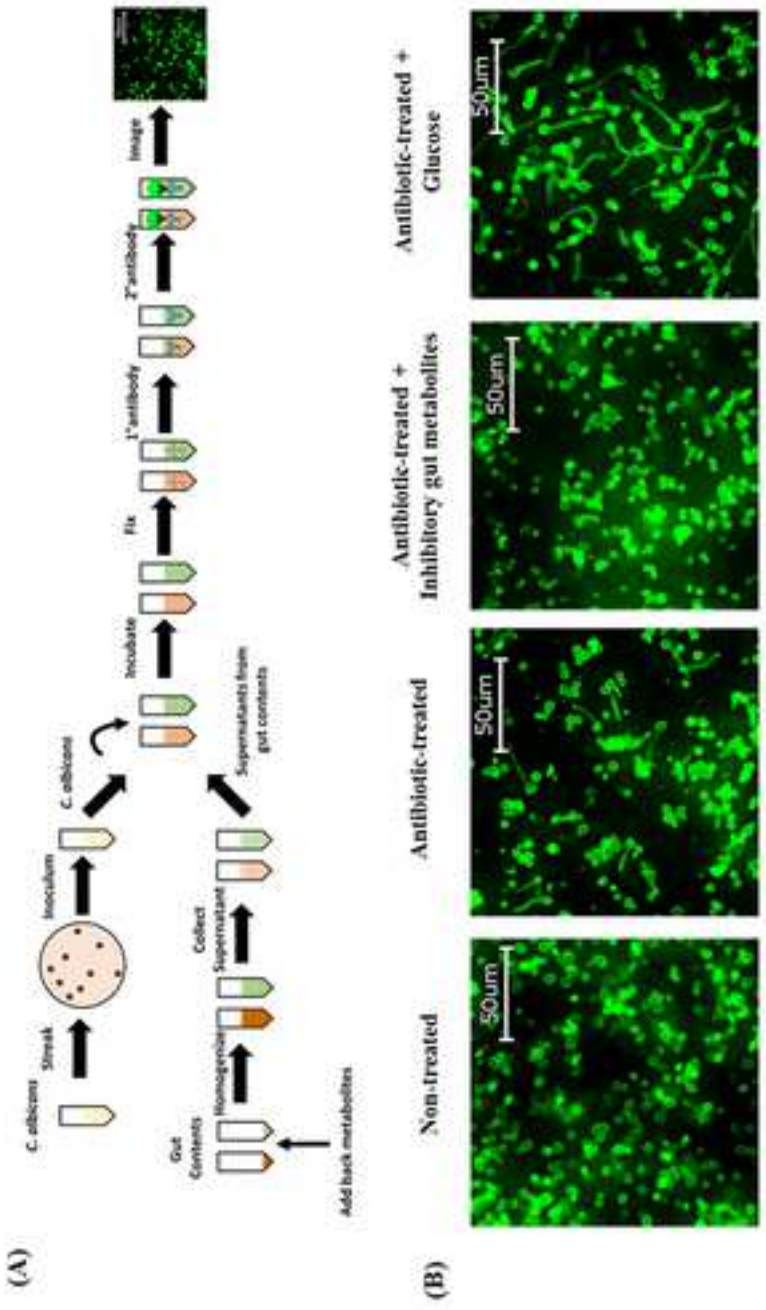


Figure 2

Click here to access/download;Figure;Figure 2.tif



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1 - 10 µL Pipet Tips	Fisher Scientific	02-707-454	Misc
100 - 1000 µL Pipet Tips	Fisher Scientific	02-707-400	Misc
20 - 200 µL Pipet Tips	Fisher Scientific	02-707-451	Misc
2-methylbutyric acid	Sigma	193070-25G	hyphal-inhibitory compound
488 goat anti-rabbit IgG	Invitrogen (Fisher)	A11008	IF Staining secondary ab
Agar	Fisher	BP1423-500	YPD agar component
Automated Imaging Microscope	Keyence	BZX700	
Candida Albicans Antibody	Invitrogen (Fisher)	PA1-27158	IF Staining primary ab
cefoperazone	Cayman	16113	antibiotic
deoxycholic acid	Sigma	30960	hyphal-inhibitory compound
D-Glucose	Fisher	D16-500	hyphal-promoting compound
forceps	Fisher	08-885	
lactic acid	Alfa Aesar	AAAL13242-06	hyphal-inhibitory compound
lithocholic acid	Sigma	L6250-10G	hyphal-inhibitory compound
palmitic acid	Sigma	P5585-10G	hyphal-inhibitory compound
Paraformaldehyde	Alfa Aesar	A11313	IF Staining fixative
Phosphate-buffered saline (PBS), 10x	Alfa Aesar	J62692	PBS component
p-tolylacetic acid	SCBT	sc-257959	hyphal-inhibitory compound
sebacic acid	Sigma	283258-250G	hyphal-inhibitory compound
sharp ended scissors	Fisher	28301	
sterile Milli-Q water	N/A	N/A	Misc
YPD Broth	BD Biosciences	242810	YPD agar component

MIDWESTERN UNIVERSITY

COLLEGE OF VETERINARY MEDICINE

April 22, 2020

Dear Editor:

Please find enclosed a revised manuscript entitled "**An *Ex Vivo* Assay to Study *Candida albicans* Hyphal Morphogenesis in the Gastrointestinal Tract"** by Monasky et al. for consideration in the *Journal of Visualized Experiments*. We thank the reviewers for their careful review of the manuscript and recommendations. We have revised the manuscript and addressed the reviewers' recommendations and provided clarifying comments below. We highlighted all the major changes in the manuscript.

Editorial Comments

Q1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: Thank you for the suggestion. The manuscript has been edited for spelling and grammar.

Q2. Unfortunately, there are a few sections of the manuscript that show overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 56-57, 65-67, 140-146,

Shankar Thangamani, DVM, PhD | Cactus Wren 336-N | 19555 N. 59th Avenue | Glendale | Arizona 85308
Email: sthang@midwestern.edu | Phone: 623.537.6378 | Fax: 623.537.6399

Response: Thank you for your feedback. These sections as well as other sections with similarity to our previous published work have been edited where possible.

Q3. Please sort the Materials Table alphabetically by the name of the material.

Response: The Materials Table has now been sorted alphabetically as requested.

Q4. Please include 2-3 more keyword phrases.

Response: The manuscript has been edited to include additional keywords.

Q5. Please include a Summary that clearly describes the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

Response: The manuscript now includes a Summary section detailing the method and its potential applications.

Q6. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Shankar Thangamani, DVM, PhD | Cactus Wren 336-N | 19555 N. 59th Avenue | Glendale | Arizona 85308
Email: sthang@midwestern.edu | Phone: 623.537.6378 | Fax: 623.537.6399

Response: The manuscript has been updated to include more detailed methods and explanations of specific steps.

Q7. How were the mice housed/fed?

Response: The manuscript includes updated methods for how the mouse strains were maintained during the study.

Q8. How were the mice euthanized? Any anesthesia?

Response: The manuscript now details euthanasia methods used during the study.

Q9. 1.2: How were the gut contents collected?

Response: The manuscript now details the specific dissection and gut content collection methods used during this study.

Q10. 2.1: How was the YPD plate prepared?

Response: The manuscript now details the preparation of the YPD agar media.

Q11. 2.6: Vortex/homogenize for how long?

Response: The manuscript now details the vortex speed and duration.

Shankar Thangamani, DVM, PhD | Cactus Wren 336-N | 19555 N. 59th Avenue | Glendale | Arizona 85308
Email: sthang@midwestern.edu | Phone: 623.537.6378 | Fax: 623.537.6399

Q12. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Response: The manuscript now details all centrifuge speeds as centrifugal force units.

Q13. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: The manuscript has been edited to remove personal pronouns.

Q14. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Response: Thank you for this feedback. We have revised the Discussion section as suggested.

Q15. Please include a Disclosures section, providing information regarding the authors'

Shankar Thangamani, DVM, PhD | Cactus Wren 336-N | 19555 N. 59th Avenue | Glendale | Arizona 85308
Email: sthang@midwestern.edu | Phone: 623.537.6378 | Fax: 623.537.6399

competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

Response: A Disclosures section has been added to the manuscript.

Q16. Please do not abbreviate journal titles.

Response: All journal titles have been written in full.

Reviewer #1

Manuscript Summary:

In this text portion of the JoVE manuscript, Monasky et al. describe methodology and results for differential morphological aspects of C. albicans in ex vivo murine gut contents depending on the specific area of the gut and the presence/absence of antibiotics and metabolites. Overall the methodology could be useful for studying aspects of C. albicans morphological transition and regulation in the gut. There are several suggestions, however, to strengthen the manuscript.

Major Concerns:

Q1. The authors refer the gut preparation as 'gut contents'. However, it is more 'gut homogenate extracts'. The authors should be clear throughout the manuscript that the tissues collected are homogenized with the soluble fraction used for the assays.

Shankar Thangamani, DVM, PhD | Cactus Wren 336-N | 19555 N. 59th Avenue | Glendale | Arizona 85308
Email: sthang@midwestern.edu | Phone: 623.537.6378 | Fax: 623.537.6399

Response: Thank you for this feedback. The manuscript has been updated to clarify that the soluble fraction of the homogenized sample is being used in these experiments.

Q2. Do the authors have any information on the general contents of the soluble extracts used? Are microbes present? There is no indication of filtering. Hence, some information as to the general properties of the extract would be useful.

Response: Thank you for this feedback. The Thangamani lab has previously examined cecal gut homogenate extracts for metabolomic and microbiome profiles for both antibiotic and non-antibiotic treated mice (Gutierrez, *et al.*, 2019) providing an overall view on the metabolites and specific phyla and families present in the cecal homogenate extracts. This previous data primarily shows a decrease in Bacteroidetes and an increase in Firmicutes during antibiotic treatment, among other changes. Similarly, there were substantial changes in metabolomics profiles during antibiotic treatment, including antibiotic-induced decreases in secondary bile acids and increases in carbohydrates. A general overview of this information has been added to the manuscript.

Q3. By the initial read of the abstract this reviewer expected the authors to show the morphological aspects of Candida on actual ex vivo explant tissues more or less laid out in a humidified manner. Have the authors attempted such a design? If similar results could be shown on explant tissues rather than homogenate extracts, data would overall be stronger. Perhaps there

Shankar Thangamani, DVM, PhD | Cactus Wren 336-N | 19555 N. 59th Avenue | Glendale | Arizona 85308
Email: sthang@midwestern.edu | Phone: 623.537.6378 | Fax: 623.537.6399

are issues related to microbial contamination with such a design, although similar issues likely apply to homogenate extracts as well. Some discussion of the justification for homogenate extracts as opposed to tissue explants would be in order.

Response: Thank you for this suggestion. Currently, we have not attempted this sort of explant design. We agree that integration of the host gut tissue would make for a stronger argument, and for future studies, it may be valuable to incorporate this into the overall design. However, we believe that the *ex vivo* assay using gut homogenate extracts is a worthwhile model to investigate the impact of changes in metabolites and microbiome on fungal hyphal morphogenesis. The protocol described here is relatively inexpensive, fast and applicable for large-scale initial screening and identification of environmental signals present in the gut content and their effects on fungal hyphal morphogenesis. We have briefly discussed the existing methods in the discussion section of the revised manuscript.

Minor Concerns:

Q1. The cecum and large intestine show somewhat similar results based on the representative images shown. Perhaps the large intestine should be included in the discussion of contents that will support hyphal growth under antibiotic conditions. If the general consensus though is that cecum is much more permissive overall then perhaps better representative images should be shown.

Shankar Thangamani, DVM, PhD | Cactus Wren 336-N | 19555 N. 59th Avenue | Glendale | Arizona 85308
Email: sthang@midwestern.edu | Phone: 623.537.6378 | Fax: 623.537.6399

Response: Overall the cecum and large intestine do show similar percent hyphae development in untreated samples, but only the cecal samples show a significant increase in the antibiotic-treated samples. A better representative image for these conditions have been added to the manuscript and further we have cited our previous publication where we have quantified the hyphae cells in cecum and large intestine.

Q2. The headings in the Methods need modification. #2 should be 'Ex vivo prep for hyphal morphogenesis assay'. #3 should be 'Exogenous addition of metabolites to the gut homogenate extracts for the hyphal morphogenesis assay'. #4 should be 'C. albicans morphogenesis assay (immunostaining and imaging)'

Response: Thank you for this feedback. These suggestions have been incorporated into the manuscript.

Reviewer # 2

The manuscript by Monasky et. al demonstrates an ex vivo method to study Candida albicans hyphal morphogenesis in the gastrointestinal tract. This method provides the closest possible condition in contrast to artificial growth media and can be useful to the scientific community to study fungal pathogenesis in the gut. I have some minor comments and suggestions, which would improve the manuscript quality to the readers.

Shankar Thangamani, DVM, PhD | Cactus Wren 336-N | 19555 N. 59th Avenue | Glendale | Arizona 85308
Email: sthang@midwestern.edu | Phone: 623.537.6378 | Fax: 623.537.6399

Q1. Why cecum but not stomach, small and large intestine was selected to study the effect of inhibitory and promoting metabolites on hyphal morphogenesis in Figure 2?

Response: Thank you for this feedback. We chose to focus on the cecal content specifically for Figure 2 because only cecal content from antibiotic-treated mice promotes hyphal morphogenesis as documented in our recent publication (Gutierrez, *et al.*, 2019) and in Figure 1.

Q2. Provide some more details about the rationale behind choosing specific concentration of metabolites in Figure 2.

Response: The concentration of metabolites were chosen based on the *in vivo* levels of each metabolites present in the cecum as previously determined through mass spectroscopy (Guinan & Thangamani, 2018, Gutierrez, *et al.*, 2019).

Q3. Can histopathology staining methods can be used to stain in vivo hyphae?

Response: Previous work has used histopathology methods to stain *C. albicans in vivo*, however differentiation of fungal cells from host cells with basic histopathology stains are challenging. We have briefly discussed this in the revised version of the manuscript.

Q4. Authors briefly mentioned about In Situ Hybridization methods reported in reference 62. Explaining briefly about in vivo staining methods available to date in the discussion section would be valuable to the readers.

Shankar Thangamani, DVM, PhD | Cactus Wren 336-N | 19555 N. 59th Avenue | Glendale | Arizona 85308
Email: sthang@midwestern.edu | Phone: 623.537.6378 | Fax: 623.537.6399

MIDWESTERN UNIVERSITY

COLLEGE OF VETERINARY MEDICINE

Response: Thank you for this feedback. The Discussion section has been updated to include an overview of existing *in vivo* and *ex vivo* methods and some of their benefits and drawbacks in comparison to our method.

We would like to have this revised manuscript considered for publication in ***The Journal of Visualized Experiments*.**

Thank you very much.

Sincerely,

Shankar Thangamani, DVM, PhD

Assistant Professor of Microbiology

Department of Pathology and Population Medicine

College of Veterinary Medicine, Midwestern University

19555 N. 59th Ave. | Glendale, AZ 85308, USA