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,

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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.

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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.

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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'

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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.

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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

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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.

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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.

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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.

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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,

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