We thank all referees for their constructive criticism. We have now substantially revised our manuscript in line with the referee's comments.

Editorial comments:
Changes to be made by the author(s):
1. 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.

We have examined the manuscript with great care to correct the spelling and grammar mistakes.

2. Please revise lines 190-193, 250-252, 283-286, 294-295, 346-348, and 350-351 to avoid textual overlap with previously published work.

We have revised these lines to avoid overlap with previous publications.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Sigma- Aldrich, Sutter Instruments Inc., Harvard Apparatus, Adaptive Science Tools, ThermoFisher, BacLight, etc.

We have deleted all commercial language and brand names as the editor referred, BacLight, Leica SP8, Sigma-Aldrich, Sutter Instruments Inc., Harvard Apparatus, Adaptive Science Tool. The reagents and instruments are listed in the table of materials.

4. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

We have revised line 94 to avoid personal pronouns and line 107, 111, 197 to avoid colloquial phrases.

5. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

We have revised line 105, 184 to use imperative tense.

6. For each protocol step, 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. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We have gone through protocol to make sure each step has no more than 3 actions.

7. 1.1: What volume of Danieau medium is used?

We have added that 10ml of Danieau medium is used in line 91.

8. 2.4.1: When are the cultures harvested? What volume of PBS is used to wash?

The cultures were harvested at 16 hours after inoculation. Bacteria were washed with 1 mL PBS.

9. 3.1: Please define dfp. How many larvae are used? How large is the Petri dish?

We have defined dpf as days post-fertilization, and added the number of larvae and the size of the Petri dish in line 143 and line 146.

10. 3.4: Please specify the size of the needle.

We have added the size of the microinjection needle at step 1.2.

11. 4.1: Please use a superscripted number for the reference (Pham et al., 2008).

We have used a superscripted number at line 173.

12. 4.2: Please specify the gnotobioc conditions.

We apologized that a typo caused the misunderstanding and we have corrected it as gnotobiotic conditions.

13. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

We have now highlighted the protocol text that should be covered by the video in yellow.

14. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

We carefully went through the highlighted text to make sure a logical flow and at least one action per step is presented.

15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We also highlighted the sub-steps containing the necessary details.

16. Please reference the macrophage phagocytosis movie in the manuscript.

We have added one sentence to refer the movie 1 in line 275.

17. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.

We have added the two softwares that were used, Fiji and Leica X, also the *C. difficile* strain, and the zebrafish stains were added.

18. References: Please do not abbreviate journal titles; use full journal name.

We have changed the abbreviated journal titles into their full names.

We thank all reviewers for having read our manuscript with great care, and we are thankful for the helpful advice. Below we will provide answers to all concerns raised by the reviewers in a point by point manner. We hope to clarify the raised questions satisfactorily and point out the individual changes that we incorporated into the manuscript, which we believe has significantly improved with the reviewer's help.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript by Li et al. describes a protocol to inoculate larval zebrafish with C. difficile by injection into the GI tract or oral microgavage, followed by observation of bacterial interactions with phagocytes by fluorescence microscopy, or determination of gut colonization by C. diff culturing from homogenized gut samples or 16S rRNA amplification.
Although protocols describing infection of zebrafish, including with intestinal pathogens have been published, there has been debate about whether the larval gut would be sufficiently low in oxygen to support the growth of strictly anaerobic species. Therefore, a protocol describing infection of zebrafish larvae with C. diff. which would demonstrate the feasibility of colonization with an obligate anaerobe, as well as provide a way to study this clinically relevant pathogen and its interaction with phagocytes in vivo, would be of great interest.

Major Concerns:
The manuscript needs some substantial improvements to unambiguously show that C. diff indeed colonizes the gut, at least in germfree larvae, rather than just transiently passing through the GI tract. Below are a list of suggestions for additional representative results to include that would demonstrate that colonization is indeed the outcome of this protocol (see comment 18).
Additionally, there is a list of suggestions to make the protocol more specific and therefore easier to reproduce, since in its current form some critical details are omitted.

We thank the reviewer for the deailed response and the many helpful advice, which we have attempted to integrate into the manuscript. Regarding the colonization of *C. diff* of the intestine, we share the view of the other reviewers that inoculating the gut directly with actively growing cells and recovering *C. diff* colonies from spores isolated from the GI tract three days later clearly argues against a transient passage of *C. diff* through the gut.

Nevertheless, the purpose of JoVE manuscripts is foremost to present and teach new methodological approaches, and we are convinced that the several reoutes and analytical protocols for working with *C. diff* in zebrafish are new and helpful for the community.

Minor Concerns:
1. Larval (5dpf) zebrafish are used as a model, therefore the title should be expanded to "Development of a larval zebrafish infection model for Clostridioides difficile".

We have changed our title accordingly.

2. Introduction: It should be made clear that C. diff exists in two forms - vegetative spores and actively growing cells, and that infections are done with cells rather than spores.

We have now pointed out that *C. difficile* form spores, and that we have used actively growing *C. difficile* cells, not spores, for this study.

3. Infection in hospital settings often happens by ingestion of spores that persist in the environment. Have the authors tried to gavage larvae with spores to see if they will germinate in the gut and colonize? This should be mentioned in the discussion section.

We indeed tried to infect gnotobiotic larvae with *C. difficile* spores. However, we obtained the same result as for active *C. difficile* cells, in which infected zebrafish did not show symptoms of CDI but *C. difficile* spores were detected.

4. Infection routes described are microinjection into the intestine and microgavage into the mouth. Have water immersion or food-borne infection been tested as alternative routes of infection? If so, what was the outcome? If not, why not? It would be good to include mention of this in the discussion. In particular, another group has achieved C. diff colonization of larval fish by immersion. This work should be referenced (Valenzuela et al., 2018).

We initially tested immersion of zebrafish in *C. difficile* cells, but these tests did not seem to colonize the zebrafish intestinal tract and did not elicit an innate immune response. This is in contrast to the findings reported in Valenzuela et al., 2018, which we now cite in our protocol. The only explanation that we currently have for this discrepancy is that E3 medium used by Valenzuela et al. may favor infection by immersion compared to Danieau medium used in our study. In addition, we did not further follow immersion protocols, as such incubation does not allow to precisely determine the time of infection, which is why we aimed to achieve a better temporal control over pathogen administration.

5. l.89: What is the composition of 30% Danieau medium?

The composition of 30% Danieau is 0.12 mM $MgSO_4$, 0.18 mM Ca $(NO_3)_2$, 0.21 mM KCl, 1.5 mM HEPES pH 7.2, 17.4 mM NaCl. We have now added the receipt in line 91-93.

6. L.116f.: What C. diff. strain was used by the authors? What temperature was used for growth in BHIS liquid medium? How many hours was the broth culture grown for over night?

C. difficile R20291 stain was used, which is now mentioned in line 124. Bacteria were cultured at 37 °C under anaerobic conditions and the culture was grown for 16 h.

7. L.125: What OD600 was typically achieved in the BHIS culture prior to PBS wash and BacLight staining? Was the OD600 adjusted to a specific density prior to BacLight staining, or only after? It seems the initial density prior to staining would affect the intensity of staining.

Typically, ODs of 1-1.2 were achieved by culturing using this method. The OD was adjusted prior to the staining. This is now mentioned in the protocol in line 138 and 139.

8. L.134: What zebrafish line was used for the experiments?

Wild-type, Brass and two stable transgenic lines, *Tg(mpeg1.1: KalTA4)*^{bz16}/Tg(*4xUAS-E1b:EGFP)*^{hzm3} and *Tg(lyZ: KalTA4)*^{bz17}/Tg(*4xUAS-E1b:EGFP)*^{hzm3} were used for the experiments. This is mentioned in lines 281 and 304 respectively.

9. L.134: How as the tricaine solution prepared? Was it buffered and how?

Tricaine powder is dissolved in double-distilled water and adjusted pH to 7 with 1 M Tris-HCL solution, then store at 4 °C. This is now mentioned in line 143-145.

10. Step 3.4. should be highlighted in yellow and included in the video.

We have now highlighted step 3.4. in yellow.

11. L.146. Freshly pulled microinjection needles are usually calibrated with mineral oil prior to use to ensure injection of the correct volume. Is this not done or just not described as part of the protocol?

We have now added more details about how to calibrate the injection needle in step 1.2.

12. Step 3.6. should be highlighted in yellow and should be shown as part of the video, or a corresponding cartoon/animation should be included in the video to demonstrate the location of the injection site in relation to the larval anatomy.

We have now highlighted step 3.6. in yellow to include it in the video presentation.

13. L.190: Step 5.8. should be highlighted in yellow and be included in the video to demonstrate correct microgavage.

We have now highlighted step 5.8. in yellow to include it in the video presentation.

14. L.214: The section about dissection of zebrafish larval intestines should start with a one-sentence introduction to describe that this procedure is used to recover viable bacteria to determine bacterial burden or profile bacterial communities in the zebrafish intestine following C. diff. colonization. Is it necessary to dissect out the intestine, or are similar results obtained with whole-larvae homogenates? Please include this in the discussion.

We revised the title of step 7 as 'Dissection of Zebrafish Larval Intestine to Recover Viable *C. difficile*'. We have also added one introductory sentence in line 232 to emphasize that the intestinal dissection is necessary for the recovery of the viable *C. difficile*. This explanation is repeated in the discussion in lines 371-372.

15. L.249 should read 5dpf.

We have now corrected this sentence.

16. L.304: please give the absolute CFUs microgavaged into the larvae, rather than CFUs/ml.

We have now included the volume of the *C. difficile* that were injected. With a concentration of the inoculum of 10^8cfu/mL (line 139) the absolute CFUs can be approximately determined.

17. L.311: describe in the legend what the 800bp band corresponds to. Details of primers used for PCR amplification should be included in the protocol.

The 800 bp band corresponds to the *C. difficile* specific 16S rDNA band that is produced using the primers:

Cdiff16Sfw: 5' GTG AGC CAG TAC AGG 3'
Cdiff16Srev: 5' TTA AGG AGA TGT CAT TGG 3'
We have added the sequence of primers in step 7.5.

18. It would be useful to include quantitative data of how many CFU of C. diff could typically be recovered from larval guts at various time points post infection.

We agree with this point, but injection of inoculum varies with slight differences in capillary openings, and variability in OD within a certain range. Also slight difference in larval age could affect the outcome, thus at best an approximation could be performed. As the aim of this manuscript is not to report studies about *C. diff* infection dynamics in zebrafish, but to demonstrate the general feasibility of a method and to provide possible technological approaches we think that we have covered the scope of the journal by showing that *C. diff* can be applied to the zebrafish intestine by microgavage and subsequently successfully recovered over several days. These approaches therefore lay the technical ground for a later thorough analysis of infection dynamics.

19. Is the issue of C. diff imaging at later time points really because of bleaching? In our experience, backlight stained bacteria in the gut of zebrafish can be imaged over at least 24 hours without bleaching. The issue may rather be that the bacteria grow and the dye is diluted as a result of cell division. Quantitation of C. diff burden over time (as suggested in 18) will also shed more light on this issue.

We were surprised to learn that Baclight stained bacteria in the gut of zebrafish can be imaged over 24 hours. However, if the dye is not bleached, dilution of the bacteria could be one explanation. Given our results that we could not recover actively growing cells later that 24 hours post-infection, but that spores could be isolated on the following days, we think that spore formation may be responsible for the loss of staining. The sporulation process is divided into several stages, during which physiological land marks change, for example, the assembly of the coat possible affecting labeling intensity. We now mention this possible explanation in the discussion in line 291.

20. L.348: It would be useful to include an image of fixed fish with bacteria stained post-fixation, to unambiguously visualize the presence of both C. diff cells and spores in the gut.

We intended to perform such whole mount immunostaining using an antibody against *C. difficile*. But no appropriate antibody was available for this purpose.

21. L.358: Which are the two anaerobic species the authors are referring to here?

They are Lactobacillus *paracasei and Eubacterium limosum* and we have included them in line 389.

Reviewer #2:

Manuscript Summary:

The authors describe two alternative injection routes to infect the zebrafish intestinal tract with C. difficile, an important anaerobic human pathogen commonly associated with gastrointestinal infections in hospitalised settings. Very few reports have previously adopted microgavage techniques in zebrafish to directly deliver bacteria to the intestine. Additionally, the work described here is particularly challenging because of the bacteria being anaerobic (which also makes it impossible to conventionally trace them with fluorescence reporters which require oxygen to fluoresce). The manuscript is therefore very valuable because it details how to deliver pathogens to the intestine of larval zebrafish and how to track them in vivo (although only for a short period).

Notably, applying this model, the authors suggest sporulation of C. difficile in vivo in the zebrafish. They also have data suggesting active invasion. As the interaction with the microbiome is very important in C. difficile pathogenesis in humans, it is also a very important finding that the natural zebrafish microbiome also can affect the optimal establishment of C. difficile infection in the zebrafish intestine. In turn, the relative ease to rear zebrafish larvae in gnotobiotic conditions until the delivery of the infection (compared to e.g. mice) holds great promise to exploit this model for further research in C. difficile pathogenesis.

Broadly, the injection method described here can be useful to a large spectrum of researchers interested in gastrointestinal pathogens and to other researchers attempting to track anaerobic bacteria in vivo.

We appreciate this positive view about our manuscript and hope to clarify all remaining questions.

I have only minor concerns, mostly related to textual adaptations to clarify protocols and interpretation of the representative results (see comments below).

Major Concerns:

No major concerns to address

Minor Concerns:

- L 62-64 Establishing a transparent zebrafish model (...) "is required" --> The sentence feels like an overstatement. I'd suggest rephrasing.

We have now rephrased the sentence to avoid overstatement. The sentence reads now: ”Therefore, establishing a transparent zebrafish model to reveal the innate immune cell response to *C. difficile* directly inside a living vertebrate organism would facilitate such innate immune cell studies.”

- L 99-100 Can the authors write more precisely how they measure the diameter of the tip of the needle to be 10-20 um?

We now include more details of the measurement of the tip of the needle, which ranges within 30-40 µm. This information can be found in step 5.2.

- L 129 Can the authors also include the volume of PBS in which to wash the C. diff (as this may affect the intensity of final staining)?

The bacteria were washed in 1 mL PBS.

- L 134 It should probably be "0.02-0.04 % Tricaine (Tricaine, Sigma-Aldrich) in 30% Danieau medium" and not Tricaine alone.

Thank you for pointing it out. We have now corrected this point.

- L 143 Where is the 0.5 % phenol red solution from and is it made in PBS?

Yes, in PBS. We have now added it to the protocol.

- L 150 Can the author include how they determine the volume of the drop? Eg. Do they inject in a drop of mineral oil and measure the diameter of the drop using a graticule to then determine the volume?

Yes, we have now included the details about drop size measurement in step 1.2.

- L 153 The fish are embedded in agar. Is the agar scraped off the injection area before injection, or is the needle pierced through the agar too? Doesn't this lead to needle clogging?

The needle pierced through the agar and it didn't cause needle clogging. We have now revised the text to be more precise.

- L 162 Are the fish maintained into gnotobiotic conditions just until the injection procedure and then transferred into standard medium and standard maintenance protocols? If so, it might be useful to state it clearly here.

We have now modified the text to precisely indicate how we maintained the fish and mention under point 4.2:

4.2 All gnotobiotic zebrafish larvae are maintained under gnotobiotic conditions until 5 dpf or before the gavage. After gavage, zebrafish larvae are transferred into a standard incubator, but with sterile 30% Danieau medium.

- L 169 Can the authors include needle diameter here too as they do for the protocol above?

We have now added that the needle diameter is 30-40 µm.

- L 187 As mentioned for the protocol above, can the author include how they measure the volume of inoculum?

We have now added more details in step 1.2.

- L 190 As mentioned for the protocol above, is needle operated also through the layer of agar or is it necessary to remove this from the area?

The needle passed through the agar. We have revised the text in this part.

- L 196 How are the larvae removed from agarose?

The larvae were released from agarose with a flexible microloader tip. We have now added it into the text, which now reads:

5.9 Following gavage, rescue the infected zebrafish larvae from the agarose with a flexible microloader tip by first cutting the agarose away and then by lifting the larvae, transfer these larvae into sterile 30 % Danieau medium.

- L 271 "Therefore, another approach to detect C. difficile was established." I am not sure what this is referred to, where is this other approach described?

We have now revised the sentence as "an intestinal dissection method was established to detect *C. difficile*."

- L 283-289 This sentence is unclear. Can C. difficile be harvested from the intestine >24hpi and can it not be harvested before this time point? If so, it is unclear to me how C. difficile is not detectable before 24h but it is detectable later on. Can the authors write more on how exactly they think that the indigenous microbial communities prevent C. difficile invasion and make it undetectable early upon injection, but not in later stages? Is it because the microbiome delays colonisation (but this can still happen later on) and collection/washing of intestines before 24h results in loss of all the delivered bacteria before they could invade?

We have revised this sentence to be more precise: "However, *C. difficile* was only detected before 24 h post-infection both with and without TCA".

- L 291 It is not clear to me why the bacteria grow at 24h in TCA- medium and then stop growing later on. This should be clarified. Is this because TCA is necessary for spore germination and after 24h all residual C. difficile is only in the form of spores?

This suggestion is what we think happens based on our findings that cells can only be isolated until around 24 hours post-infection, while afterwards only spore-derived bacterial colonies could be obtained. We have added a sentence "which suggested that total *C. difficile* in the gut had formed spores" in line 319-320 to clarify this conclusion.

- L 338-339 Following from the point above, do the authors have evidence that C. difficile proliferates in the intestine of zebrafish? Is it possible that the staining is lost because of proliferation/dilution too? Does the staining used here remain on C. difficile spores?

One of the reviewers made a similar suggestion (Reviewer #1, Minor Concerns 19) and we agree that spore formation including changes in the membrane could be the cause for the loss of fluorescence. Currently we have no indication for pronounced proliferation of *C. diff*.

- Figures: Can the authors include labels for colours and timepoints in all the images? Also, the legend for Figure 1 should inform of the injection route, and weather 1A is bacteria already within the fish or in culture. Figure 3 should state clearly that these fish were gnotobiotic at the time of the gavage. Also, I feel it would have been very valuable to have a more complete figure showing also the result for conventionalised (non-gnotobiotic) zebrafish, as these are discussed in the text (but not shown).

We have now included labels for colors and timepoints in Figure 1 and Figure 2. We have also revised the legend of Figure 1 and 3 to indicate the *C. difficile* used in zebrafish and gnotobiotic zebrafish larvae. Given that we found that conventional zebrafish could not maintain *C. difficile* in the gut for longer than 24h, we did not follow this further, which is why we would not like to show these results here.

- Table of material: it could be handy to have the C. difficile strain information here too.

We have added the information of *C. difficile* strain in Table of material.

Typos:
- Throughout, generally percentages and temperature notations do not require spaces eg. 30 % --> 30%; 4 °C --> 4°C.

We have corrected the space between numbers and percentages. However, the Instruction for Authors says:" Include a space between all numbers and the corresponding unit", so we kept the space for temperature notations.

- Some minor inconsistencies in style for the references throughout. eg. compare L55 and L65 with L53 and L60 (presence/absence of spaces)
- L 120 inconsistent spacing between digits and units throughout, eg. 1 g/L vs 15g/L.
- L 119 "filtratino" --> filtration
- L 196 steril --> sterile
- L 249, L 279 dfp --> dpf

Thank you for pointing out the typos. We have now carefully corrected all of them.

Reviewer #3:
Manuscript Summary:
The authors injected fluorescently labeled C.difficile into 5 dpf zebrafish gut and observed infiltration of neutrophils and macrophages with phagocytosis. To mimic a natural course of infection, the authors also used microgavage to deliver the labeled bacteria in the gut. However the bacteria lose the fluoresence label after 5 hpi and there are minimal immune response. The authors then generated gnotobiotic zebrafish and performed microgavage again. This time, no imaging was performed and bacteria can be recovered from dissected gut up to 120 hpi. However, whether the bacteria can replicate and cause disease, tissue damage or innate activation is not reported. Overall, the work is valuable providing a new way of labeling the bacteria and attempts in establishing the zebrafish infection model.

We are grateful for this positive judgment about our manuscript.

Major Concerns:
The major concern is that the bacteria detection method can not test for CFU or whether the bacteria can establish productive infection after inoculation.
Figure 2 will be more informative if the experiment is performed with the gnotobiotic zebrafish where infection is more likely to be successful.
lack of the evidence that this bacteria can establish productive infection in zebrafish despite various attempts is a major concern.

We agree with the reviewer that a better way to detect *C. difficile* would be helpful to directly follow the behavior of these pathogenic cells and allow for quantitative analysis. We have attempted for quite some time to generate florescent protein expressing *C. difficile*, but we could not obtain convincing fluorescent labeling yet. We will maintain our efforts and hope to be able to provide further conclusive data about *C. difficile* behavior soon.

Minor Concerns:
line 90, receipt of 30% Danieau medium missing. The volume and how the dissolved solution is stored is missing.

We have added the recipe for 30% Danieau and the storing conditions in line 91-93.

a catalog number of normal agarose will be helpful.

We have added the catalog number behind of normal agarose at line 111.

line 119, typo, filtratino

Thank you for pointing out these typos, which we have corrected now.

line 125, how to monitor the initial amount of bacteria before labeling is missing. what is the volume of PBS used for washing?

The bacterial concentration was adjusted using optical density. Bacteria were stained at an OD600 of 1. One mL of PBS was used. We have added this information to the manuscript now.

line 130, how many CFU/ml is equivalent to OD600=1?

OD600 is approximately equivalent to 10^8 cfu/mL. We included this in the manuscript in line 139

illustration or video of dissection of larval intestine will be informative

We have highlighted step 7.3 to demonstrate the dissection during the video recording.

methods of 16S rRNA PCR is missing.

We now provide the following information:

For the 16S-PCR a colony was resuspended in 50 µL of H_2O and boiled at 95 °C for 15 min. Lysed debris as pelleted by centrifugation (14000 rpm, 2 min, RT) and 2 µL of the supernatant were used as template in a 25 µL PCR-reaction using *C. difficile*-specific primers (s.a.). When bacteria from liquid culture were used, 1 mL of culture was harvested, and once washed with 1 mL PBS (14000 rpm, 2 min, RT). The pellet was resuspended in 100 µL of H_2O_{dd} and treated as above.

Reviewer #4:
Manuscript Summary:
In general, this is a well-conducted research that can be consider for publication in JOVE. This protocol aims to established a non-invasive microgavage protocol to deliver C. difficile directly into the intestine of zebrafish larvae by intubation through the open mouth. This novel infection method closely mimics the natural infection route of C. difficile in humans. Therefore, using zebrafish-C difficile ineraction can help in decipher those virulence and host factors important for pathogenesis of this important pathogen. The use of gnotobiotic zebrafish larvae to predispose the zebrafish to be infected is a great idea that can be follow in other enteropathogens infections in this host model.

We are thankful for this positive judgment about our work.

Major Concerns:
My major concern is related with the absent of details regarding the production of gnotobiotic zebrafish larvae. Despite the authors refer this section to a well-established method, in a journal like JOVE all methodological details are of a great importance. I suggest that a summary of the published protocols by Pham et al., 2008 should be included.

We have inserted a summary of the protocol in the Protocol section to highlight the key steps.

This part can now be found in a new chapter 4.

Minor Concerns:
Include more references that compare injections with other immersion methods in zebrafish larvae in other bacterial pathogens is highly desirable in the discussion section. For example BMC Immunol. doi: 10.1186/1471-2172-12-58; Front Cell Infect Microbiol. doi: 10.3389/fcimb.2017.00334

We have extended the discussion on this point and added these literatures as references.