To the editor,

Thank you for taking time to read and comment on our submitted manuscript. Please find the answers to each comment below. Please let us know if you feel that any more changes need to be made or any topic addressed. Thank you again for your time.

**Response to editor’s comments:**

**Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”**

The short abstract has been edited to the following:

“Here, we present a protocol for culturing the gut microbiota of the colon *in vitro,* using a series of bioreactors that simulate the physiological conditions of the gastro-intestinal tract*.”*

**Please ensure that the protocol is made up of action steps only which consists of hard/discrete experimental steps, button clicks, knob turns etc.**

**All other details can be moved to the intro and discussion section respectively.**

**Please ensure that you write the protocol in imperative tense as if directing someone how to perform your experiment with all specific details with respect to your experiment. Do not generalize.**

**Please ensure that you answer the how question. How is this step performed.**

**Please write the steps in the order of it being performed.**

This has been fixed in the revised manuscript

**Please do not use commercial language in the manuscript. Please move this to the table of materials.**

We have removed the commercial language from most of the manuscript. However, we would like to keep a note in the materials and methods stating that we used the Twin Simulator of the Human Intestinal Microbial Ecology (TWINSHIME®) in the materials and methods section. We think this is justified for the following two reasons:

Multiple papers have been published using this system, so it is used by multiple different groups and is therefore not unique to a single company

We want to be very clear on what system we are using to cultivate the microbiota, since there are multiple types of systems available.

**Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes. Doing step 1 for your reference.**

This has been fixed in the revised manuscript

**Please remove commercial term and use generic term instead.**

This has been fixed in the revised manuscript

**This is not an action step and hence converted to a note instead.**

This has been fixed in the revised manuscript

**Please move this detail to the Table of Materials.**

This has been fixed in the revised manuscript

**We cannot have commercial language in the manuscript. Please move this part to the Table of Materials.**

This has been fixed in the revised manuscript

**We cannot have paragraph of text in the protocol section. Please consider moving some the details to intro/discussion. Protocol should only consist of action steps.**

This has been fixed in the revised manuscript

**Include a one liner note stating what is the difference between all bioreactors attached here. Why are you calling the first one as stomach and the second one as small intestine etc.**

This had been addressed in the revised manuscript using the following sentances:

“2.1.3. Label the bioreactors in the following sequence: Stomach, small intestine, ascending colon, transverse colon, descending colon. This represents the order of the gastrointestinal tract.

Note: The bioreactors can all be identical, or the stomach and small intestine bioreactors can be smaller compared to the colon bioreactors since they will hold less volume. “

**Regarding the water bath the editor asks “Where is this in the figure. “**

The water bath has been added to Figure 1

**Regarding the water bath the editor asks “What volume? “**

This depends on the type of circulating water bath used. This detail has been added to the protocol.

**Regarding the pH probes the editor asks “How? Please mark this in the figure.”**

This detail has been added to the writing and to Figure 1.

**So basically, you are differentiating on the basis of pH? This clarity needs to be brought out before the start of the experiment.**

The following sentences are added to address this comment:

Note: Setting up the bioreactors in this way means that the ascending colon region receives nutrients from the small intestine, the transverse colon region receives nutrients from the ascending colon region, and the descending colon region receives nutrients from the transverse region. In this way, the system is mimicking the sequential movement of nutrients through the gastrointestinal tract.

Note: During the initial phase, the community will differentiate based on the difference in region pH values. However, once the feeding cycles begin, the communities will further mature based on the differences in nutrient input.

**Regarding the nitrogen line the editor asks “How? Please also include a one liner note of why this is being done.”**

In response the following sentences has been added:

“Connect a nitrogen line using silicon tubing to the lid of each vessel using the designated port.”

“Note: Nitrogen is used to removed oxygen from the system and maintain anaerobiosis during the experiment.”

**Regarding the sample port the editor asks “Same as above. “**

In response the following sentences has been added:

“A metal sample tube is inserted through the lid of each bioreactor using the designated port. The metal tube extends down into the bioreactor and is used to collect fluid samples. “

“Add a small piece of silicon tubing to the top end of the sample tube and connect a luer lock. This will allow for the use of a syringe during sampling.”

**The editor asks to remove the term “luer” from luer lock.**

The term luer lock refers to a type of cap that can connect to a syringe. From my understanding the term luer lock is not trademarked or patented. Luer Lok and Luer slip are. This term can still be removed if necessary, but I don’t think it should be.

**Regarding the sample ports the editor asks “Same as above.”**

This has been added to Figure 1.

**Regarding the mucin carriers the editor asks “Please include how much is added in your case.”**

This detail has been added to the revised manuscript.

**Regarding turning on the pH probes the editor asks “How?”**

This detail has been added to the revised manuscript.

**Please describe how much? What amount is used? How do you mix in the glycerol solution? Please write all action steps.**

This has been addressed in the revised manuscript

**This is not an action step and hence can be converted to a note. Also provide ethics statement for human study. How many donors are used in tis case?**

The fecal sample that is used for our experiments is purchased from a company that harvests and processes the donated sample. Since we do not come in contact with the donors, or collect the samples, I don’t think this warrants an ethics statement regarding human studies on our end. If the editor would prefer we add some type of statement, we are willing, but I’m not sure exactly what we would say.

For each experiment we use a single donor, this has been added to the revised manuscript using the following sentences:

“For this protocol, only a single donor is used. However, this can be altered due to experimental design.”

**What is meant by manufacturer’s recommendation here? You collect the sample or the sample is commercially bought?**

We purchase the samples for our experiments from the company Openbiome. This is listed in the table of materials and addressed in the note above.

**We cannot have paragraph of text in the protocol section. Please consider moving some parts to the discussion instead.**

This has been addressed in the revised manuscript.

**Please rewrite the section as action steps providing details on how to perform each step. Please use imperative tense throughout.**

This has been corrected in the manuscript

**Notes cannot be filmed. Also we cannot have paragraph of texts in the protocol section. Please consider moving some parts to the discussion instead.**

This note was shortened to contain only pertinent information.

**We cannot have paragraph of text in the protocol section. Please consider moving some parts to the discussion.**

Paragraph will be moved to the discussion.

**Expand SCFA and use imperative tense throughout.**

This is corrected in the revised manuscript.

**Commercial term. Please move the term to the table of materials and use generic terms only.**

This has been removed in the revised manuscript.

**Please explain the result with respect to the protocol described above.**

The results provided demonstrate what type of data can be obtained by running this experiment. This was added to the first paragraph in this section. I will also tie the results in to the protocol in the discussion portion of the manuscript.

**Regarding PCoA plots the editor asks the following “Please expand”.**

The full name of PCoA was added, as was a description of what this was measuring.

**So basically, you have the same medium but different pH in different sections**

The difference in the regions is due to pH and nutrient intake. I have added the following sentences to address this in the following paragraph:

The three colon regions represented in this system are maintained at different pH values and receive different nutrient supplies. (This was mentioned in the protocol section). Based on this, it is expected that the communities in these regions will differentiate.

**Please provide explanation here for this figure.**

The following explanation has been added:

The complete system consists of the following components: A circulating water bath, nitrogen flow, a set of glass bioreactors, a set of magnetic stirrer bars and magnetic stirrers, pH probes, a computer-controlled console containing 40 peristaltic pumps, a computer monitor, and a refrigerator. The main system is composed of a set of bioreactors mimicking the stomach, small intestine, and the ascending, transverse, and descending colon regions. Two complete units are set up to run in parallel, providing for an experimental and a control group. This means that 10 bioreactors, 10 pH probes, and 10 magnetic stirrers are required for this experiment.

**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**

Based on the editor’s comments, we have completely reorganized the results and discussion section. The revised version addresses the concerns of the edits.