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TITLE:

Applying Advanced *In Vitro* Culturing Technology to Study the Human Gut Microbiota

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Gut microbiota, *in vitro* bacterial culturing, ascending colon, transverse colon, descending colon, short chain fatty acids, bacterial community, bioinformatics

SHORT ABSTRACT:

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.

LONG ABSTRACT:

The human gut microbiota plays a vital role in both human health and disease. Studying the gut microbiota using an *in vivo* model, is difficult due to its complex nature, and its diverse association with mammalian components. The goal of this protocol is to culture the gut microbiota *in vitro*, which allows for the study of the gut microbiota dynamics, without having to consider the contribution of the mammalian milieu. Using *in vitro* culturing technology, the physiological conditions of the gastro intestinal tract are simulated, including parameters such as pH, temperature, anaerobiosis, and transit time. The intestinal surface of the colon is simulated by adding mucin-coated carriers, creating a mucosal phase, and adding further dimension. The gut microbiota is introduced by inoculating with the human fecal material. Upon inoculation with this complex mixture of bacteria, specific microbes are enriched in the different longitudinal

(ascending, transverse and descending colons) and transversal (luminal and mucosal) environments of the *in vitro* model. It is crucial to allow the system to reach a steady state, in which the community and the metabolites produced remain stable. The experimental results in this manuscript demonstrate how the inoculated gut microbiota community develops into a stable community over time. Once steady state is achieved, the system can be used to analyze bacterial interactions and community functions or to test the effects of any additives on the gut microbiota, such as food, food components, or pharmaceuticals.

INTRODUCTION:

The gut microbiota is a community of micro-organisms that reside in the human gastrointestinal tract (GIT). This community reaches maximum concentration in the colon, which is estimated to hold 10^{13} - 10^{14} bacteria, from 500-1000 species, that live in symbiosis with the colon milieu^{1,2}. The composition and functionality of the gut microbiota change spatially along the GIT, forming region specific communities, with the most diversity found distally²⁻⁵. For each anatomical region, separate microbial communities reside in the lumen and on the mucosal lining⁶. The lumen community has more direct access to nutrients as substrates move through the luminal compartment⁷. Despite this, some bacteria reside preferentially in the mucus layer, utilizing mucin produced by the colon cells as an energy source^{1,5,8}. The difference in microenvironments between the luminal and mucosal phases results in divergence and the development of phase specific communities. Together, these communities provide metabolic functions, such as nutrient metabolism and the production of vitamins, and immunological functions, such as preventing the colonization of human pathogens^{1,3,9}. The gut microbiota also works functionally in conjugation with the human colon cells³.

As an important part of the human GIT, it is not surprising that the gut microbiota is known to contribute to both host health and disease status^{3,9-12}. A shift in the gut microbial population has been associated with multiple human diseases, including GIT disorders like intestinal bowel disease (IBD) and intestinal bowel syndrome (IBS), but also other diseases, such as obesity, circulatory disease, and autism^{3,9-12}. Metabolites produced from the gut microbiota have a global effect, reaching locations far from the gut¹²⁻¹³. For example, the gut-brain axis is associated with mental disorders like anxiety and depression¹⁴. Therefore, studying the gut microbiota is important to multiple fields of research, and is applicable to many diseases, even those not often associated with the GIT.

While it is widely acknowledged that studying the gut microbiota is important, it is a complicated endeavor. Multiple animal models are available, from small animals like zebrafish, rats, and mice, to larger ones like monkeys and pigs¹⁵⁻¹⁹. However, the application of these animals in terms of the human gut microbiota is not straightforward, since these animals have a unique bacterial community that has evolved based on environment and diet, and they are anatomically distinct from humans²⁰⁻²¹. The use of human subjects removes the question of relevance yet introduces another set of challenges. Human studies are expensive, time consuming, and are ethically constrained¹¹. Moreover, confounding factors influence the gut microbiota in human studies, including age or developmental stage, environment, diet, medication, and genetic factors^{2,4,22}. There are also restrictions on what can be tested in humans, and which type of samples can be

harvested at what times⁴.

One critical disadvantage of using an *in vivo* system to study the gut microbiota is the presence of mammalian components. The gut microbiota and human cells interact with one another, and in an *in vivo* setting, it is impossible to distinguish the two. The metabolites produced by the gut microbiota are taken in by the colon cells, so measurements cannot be calculated with precision. Therefore, any mechanistic study must be limited to end-point measurements¹¹. Another major disadvantage for *in vivo* studies is the inability to harvest samples from the different regions of the GIT longitudinally²³. This does not allow for the assessment of changes that may occur in the microenvironments of the colon over time¹². Many *in vivo* studies, including human studies, rely on analysis of fecal samples to detect changes to the gut microbiota¹². While this is informative, it does not provide data on the gut microbiota across the GIT and does not differentiate between the luminal and mucosal communities⁵⁻⁸.

For the gut microbiota, the application of an *in vitro* method is required to study the dynamics of the bacterial community, without interference from the mammalian components. Using an *in vitro* method allows for the tight control of environmental conditions¹⁰, testing of multiple parameters simultaneously, and the ability to sample longitudinally, and in large volumes¹¹. Since an *in vitro* method utilizes a mechanical device and not a host, no considerations are needed for age, environment, diet, or genetic background. These systems can be used to test either the entire gut microbiota community, only selected organisms, or even single strains. Importantly, *in vitro* results are reproducible, yet retain a level of diversity comparable to *in vivo* studies^{11,22}.

Depending on the hypothesis in question and the desired results, *in vitro* studies can be performed in numerous ways. They can utilize single-vessel systems and simple methods, such as incubating samples with fecal homogenate²⁴ or performing single batch cultures over the course of 24-48 h²⁵. They can also be accomplished using single-vessel systems and more complex methods, such as using a chemostat system to produce a stable gut microbial community¹¹. However, the use of a single reactor can over-simplify the microbiota¹² since it only represents one section of the colon, even though the colon is composed of the ascending, transverse, and descending regions.

In order to study the gut microbiota community that develops in the different regions of the colon (the ascending, transverse, and descending regions), a complex, multi-stage system can be employed. In these systems, multiple vessels are set up to mimic the different regions of the colon, so the gut microbiota of the ascending, transverse, and descending regions are cultivated independently. These vessels are connected, using pumps to move substrates in sequence, from the ascending to the transverse to the descending colon regions, mimicking the flow of nutrients through the GIT.

The objective of this study was to demonstrate how a 5-stage *in vitro* culture system (see **Table of Materials**) can be used to cultivate the gut microbiota community, and to demonstrate community dynamics in terms of stability and composition. In this system, one vessel represents the stomach and one represent the small intestine. The colon is divided into three regions

(ascending, transverse, descending), with one vessel representing each region²⁶. In this experimental setup, two complete systems were run in parallel, with Unit 1 containing mucin carriers to represent the mucosal surface and Unit 2 containing no mucin carriers. The communities that developed in the luminal and mucosal phases of each region were compared to each other, and to the fecal inoculum over time using 16S rRNA gene sequencing and SCFA analysis. The results presented demonstrate the type of community, both in terms of composition and functionality, which can be produced from this type of *in vitro* system.

PROTOCOL:

1. Materials and Preparations

Note: The defined medium is purchased as a powder (see **Table of Materials**). The composition of the defined medium in g/L is the following: Arabinogalactan (1.2), Pectin (2.0), Xylan (0.5), Glucose (0.4), Yeast extract (3.0), Special peptone (1.0), Mucin (2.0), L-cysteine-HCl (0.2).

1.1. Prepare the defined medium

1.1.1. Fill a 4 L flask with 2 L of double distilled, deionized water.

1.1.2. Add 29.2 g of defined medium powder to the water and mix until completely homogenized.

1.1.3. Add a magnetic stirrer and loosely screw on the lid.

1.1.4. Autoclave at 121°C for 30 min.

1.1.5. After cooling, store the prepared defined medium in a refrigerator at 4 °C with constant agitation (magnetic stirrer).

1.2. Prepare pancreatic juice

1.2.1. Autoclave 2L of distilled, deionized water in a 5L vessel with a stirrer bar added, at 121°C for 30 min.

1.2.2. After autoclaving, allow the water to cool to room temperature.

1.2.3. Add 12.5 g NaHCO₃, 6 g bile, and 0.9 g pancreatin. Place on magnetic stirrer to mix.

Note: The pancreatic juice should be prepared fresh every 2-3 days and refrigerated with agitation after it is made.

1.3. Phosphate buffer

1.3.1. Place a 1L flask containing a magnetic stirrer bar on a magnetic stirrer. Add 0.5 L of distilled,

deionized water and turn on agitation.

1.3.2. Next, add 6.8 g KH_2PO_4 , 8.8 g K_2HPO_4 , and 0.1 g sodium thioglycolate. Top off with water to a final volume of 1 L.

1.3.3. After the components have completely dissolved, adjust the pH to 7.0 using NaOH.

1.3.4. Pour the buffer into a 2 L vessel with a screw cap lid and autoclave at 121°C for 30 min. Ensure that the lid is screwed on loosely and not closed tightly.

1.3.5. After removing from the autoclave, allow the solution to cool to the room temperature. Store the buffer at room temperature for up to 2 weeks.

1.4. Prepare Mucin agar carriers

1.4.1. Autoclave plastic mucin carriers (see **Table of Materials**), forceps, plastic mesh (tubular shape), and zip ties at 131°C for 30 mins.

1.4.2. Autoclave 300 mL of double distilled, de-ionized water at 131 °C for 30 min. Store at room temperature until use.

1.4.3. In a biosafety cabinet with the laminar flow, fill sterile Petri dishes with the plastic mucin carriers using sterile forceps.

Note: The mucin carriers are hollow plastic circles that are filled with mucin agar. Once filled, the lid can be placed on top and these can be set aside.

1.4.4. To prepare mucin agar, add 3 g of bacterial agar and 15 g of porcine mucin to the 300 mL of autoclaved water.

1.4.5. In a fume hood, boil this solution, then remove from the heat source and allow to cool for 1 min. Repeat the boiling and cooling for a total of 3 times.

1.4.6. Once the glass vessel containing the mucin agar solution is cool enough to touch, move it into the biosafety cabinet with laminar flow.

1.4.7. In the biosafety cabinet with the laminar flow, pour the liquid mucin agar over the plastic mucin carriers that were placed in the Petri dish. Ensure that all the carriers are filled completely with mucin agar.

1.4.8. Place the lid back on the Petri dish and allow it to cool under laminar flow.

1.4.9. To assemble the mucin carriers, take the autoclaved plastic netting and insert mucin carriers containing the solidified mucin agar from the Petri dish using sterile forceps.

1.4.10. Use zip ties to close the ends of the mesh. In this way the net functions to contain the mucin carriers filled with mucin agar. Store at 4 °C until use.

2. Set up, Inoculation, and Running of the System

2.1. Set up of the culturing system

Note: The Twin Simulator of the Human Intestinal Microbial Ecosystem was used for this study.

2.1.1. Place 10 bioreactors containing stirrer bars on magnetic stirrers and turn on agitation.

Note: Each Unit will consist of 5 bioreactors, so 2 units will require 10 bioreactors.

2.1.2. Connect 5 bioreactors to each other using silicon tubing in sequence by way of the peristaltic pump (**Figure 1**). Use the pumps to transverse the fluid contents from one bioreactor to the next.

2.1.3. Label the bioreactors in the following sequence: Stomach, small intestine, ascending colon, transverse colon, descending colon to represent 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. 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.

2.1.4. Place the defined medium and pancreatic juice in a refrigerator at 4 °C with constant agitation using magnetic stirrers. Using silicon tubing, connect the defined medium to the stomach bioreactor via a peristaltic pump and connect the pancreatic juice to the small intestine via a peristaltic pump.

2.1.5. Add solidifying agent to a urine drainage bag. Connect the descending colon to the urine drainage bag by way of the peristaltic pump. Dispose of the solidified as biological waste during the experiment.

2.1.6. Using silicone tubing, connect the water jacket of each vessel in order, and connect each end to the circulating water bath. Fill the circulating water bath with distilled water (this amount will vary depending on the type of circulating water bath used) and set to 37 °C.

2.1.7. Using silicon tubing, connect the acid and the base to the lid of each vessel by way of peristaltic pumps. The recommended concentrations are 0.5M HCl and 0.5M NaOH.

2.1.8. Insert the pH probes to each vessel through the designated port in the lid. This port is designed to hold the pH probe and screw into the lid (**Figure 1**). Set the pH for each region as follows: Stomach, pH=2; Small intestine, pH=6.7-6.9; Ascending colon, pH = 5.6-5.9; Transverse colon, pH=6.15-6.4; Descending colon, pH=6.6-6.9.

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.

2.1.9. Connect a nitrogen line using silicon tubing to the lid of each vessel using the designated port. The nitrogen line should flow in the following sequence: stomach, small intestine, ascending colon, transverse colon, descending colon. Ensure each unit has its own flush to avoid cross contamination.

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

2.1.10. Insert a metal sample tube 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.

2.1.11. Add a small piece of silicon tubing to the top end of the sample tube and connect a Luer Lock to allow for the use of a syringe during sampling.

2.2. Inoculation of the system

2.2.1. Fill the colon regions with the defined medium using the following volumes: 500 mL in the ascending colon, 800 mL in the transverse colon, and 600 mL in the descending colon.

2.2.2. Add mucin agar carriers to the colon regions, in an amount proportional to the volume of the reactor. For this experiment, 60 carriers per reactor were used.

2.2.3. Remove any excess defined medium using the sample tube and a syringe since adding the carriers raises the level of fluid in the bioreactor.

2.2.4. Turn on the pH probes to adjust the pH in each reactor using the computer.

2.2.5. Turn on the nitrogen flush for 20 mins.

Note: The fecal homogenate used for inoculation is purchased as 10% feces homogenized in 10% glycerol solution (see **Table of Materials**). The fecal sample is randomly selected from a pool of donors with the following criteria: An American consuming a typical western diet (not vegan or vegetarian), between 21-45 years of age, antibiotic free for at least 1 year, with an average Body Mass Index (18.5-24.9). For this protocol, only a single donor is used. However, this can be altered due to experimental design.

2.2.6.2. Prior to inoculation, thaw the fecal homogenate following the manufacturer's guidelines.

2.2.7. Inoculate each colon reactor by adding the fecal homogenate in an amount equal to 5% of the reactor volume through the sample port, using a 60 mL syringe (25 mL for the ascending colon, 40 mL for the transverse colon, 30 mL for the descending colon).

2.2.8. Grow the cultures in the bioreactors overnight with pH control.

2.2.9. After the overnight growth, harvest samples of the luminal fluid from each colon region as detailed below in section 3. After sampling is completed, turn on the computer program to begin feeding.

2.3. Daily feeding cycles

Note: The daily feeding cycles are computer automated.

2.3.1. Three times a day, pump 140 mL of the defined medium into the stomach bioreactor and allowed to incubate for 1 h.

2.3.2. After 1 h incubation, pump the contents of the stomach into the small intestine. At the same time, pump 60 mL of pancreatic juice into the small intestine.

2.3.3. In the small intestine, turn on pH adjustment to pH 6.7-6.9, and allow mixture to incubate for 90 mins. During incubation, turn on the nitrogen flush for each system for a total of 10 min each.

2.3.4. After the 90 min incubation, turn on the pumps from the small intestine to the ascending colon, the ascending colon to the transverse colon, the transverse colon to the descending colon, and the descending colon to the waste simultaneously.

2.4.4. Experimental timeline

Note: The *in vitro* system used here is operated in a continuous fashion for approximately 8 weeks in length. Below is a recommended experimental timeline, however, this can be modified depending on the requirements and objective of the experiment.

2.4.1. Set up system following steps listed in step 2.1.

2.4.2. Harvest samples from overnight growth following the protocol below. Begin the daily cycles, feeding three times a day following the protocol above.

2.4.3. Run the experiment for a total of 2 weeks to allow the bacteria to stabilize.

Note: Running the system means that the pH is maintained, the daily feeding cycles are followed three times a day, and the mucosal carriers are changed 2-3 times each week.

2.4.4. After stabilization, run the experiment for 2 weeks as the control period.

2.4.5. After the control period, use the system to test different components or variables, known as the treatment period.

2.4.6. After the treatment period, stop adding the components or variables and return the conditions to normal. This is considered the wash-out period and is used to determine whether or not changes to the community are permanent.

3. Harvesting Samples from the System

Note: During the experiment, samples can be harvested from the luminal or mucosal phase of any region at any time, following the below guidelines.

3.1 Harvesting luminal samples

3.1.1. Harvest luminal fluid samples through the sample port that extends into the middle of the culture liquid. Begin by cleaning the Luer Lock on the sample port with either 70% ethanol or the small alcohol pad and allow to dry.

3.1.2. Ensure that all air is removed from a 30 mL syringe and connect it to the sample port. Draw up and down 3-5 times to ensure proper mixing of the vessel components. After, draw a total of 30 mL of culture.

3.1.3. Aliquot the luminal samples as needed into sterile falcon tubes and store on ice. After sampling is completed transfer these to a -80 °C freezer.

3.1.4. For SCFA analysis, 15 mL of the luminal sample is spun at 4 °C, 5,000 x *g* for 10 min. The supernatant is syringe filtered using a PES 0.2 µM filter. Store the filtered supernatant at -80 °C.

3.1.4.1. For DNA analysis, spin 1 mL of luminal fluid at 4 °C, 5,000 x *g* for 10 min. Discard the supernatant and store the pellet at -80 °C.

3.1.4.2. As a backup, store 10 mL of luminal fluid at – 80 °C for most experiments.

3.2 Harvesting mucosal samples

Note: Change mucosal carriers every 2-3 days.

3.2.1. Remove and bring out the prepared mucin carriers from the refrigerator.

3.2.2. Turn on the nitrogen flush and open the reactor lid quickly. Remove 50% of mucin carriers and add new ones.

3.2.3. Seal the lid quickly and keep the nitrogen flush on for another 20 min.

3.2.4. For DNA extraction, aliquot 0.25- 0.5 g of agar into a 2 mL tube and stored at -80 °C until needed.

4. DNA Extraction, Sequencing, and Analysis

Note: DNA is extracted using the CTAB DNA extraction method with physical homogenization in a fume hood²⁹. Following extraction, a spectrophotometer is used to quantify the amount of DNA in each sample.

4.1 Prepare CTAB buffer

4.1.1. Dissolve 4.2 g K_2HPO_4 and 4.09 g NaCl in 200 mL of deionized, distilled water, then autoclave at 121 °C for 30 mins.

4.1.2. Allow the solution to cool to room temperature.

4.1.3. Add 10 g of Hexadecyltrimethylammonium bromide (CTAB) and heat to 60 °C with agitation.

4.1.4. After all the CTAB is dissolved, remove the solution from heat and cool to room temperature.

4.2 Prepare PEG-6000 solution

4.2.1. Dissolve 300 g of polyethylene glycol 6000 and 93.5 g NaCl in 1 L of deionized, distilled water.

4.2.2. After all particles are dissolved, autoclave the solution at 121 °C for 30 min.

4.2.3. After autoclaving, cool the solution to room temperature before use.

4.3 Perform DNA extraction

4.3.1. Add 500 μ L of CTAB buffer and 500 μ L of Phenol-Chloroform-Isoamyl alcohol to the 2 mL sample tube and vortex to homogenize.

4.3.2. Transfer the entire contents of the sample tube to a 0.1 mm physical homogenizing tube (see **Table of Materials**).

4.3.3. Homogenize the tubes for 20 s at the highest setting two times, with a 20 s pause in between, using a physical homogenizer (see **Table of Materials**).

4.3.4. Centrifuge the homogenized tubes at 3000 x *g* for 5 mins.

4.3.5. Transfer 300 µL of the supernatant to a clean, 1.7 mL tube and add 500 µL of CTAB buffer to the original tube.

4.3.6. Homogenize this tube again using the methods in steps 4.3.2-4.3.3 and centrifuge at 3000 x *g* for 5 min.

4.3.7. After centrifugation, transfer another 300 µL of supernatant to the new tube which now contains 600 µL.

4.3.8. Add a total of 600 µL Chloroform-Isoamyl alcohol to the tube containing 600 µL of the supernatant.

4.3.9. Invert the tubes to mix, and then centrifuge briefly. Transfer 500 µL of the upper phase to a clean 1.7 mL tube. Ensure only to take the top phase.

4.3.10. Next, add 1000 µL of Peg-6000 solution, invert the tubes to mix, and then incubate at room temperature for 2 h.

4.3.11. After incubation, centrifuge the tubes at 18,200 x *g*, 4 °C, for 10 min.

4.3.12. Discard the supernatant and clean the pellet with ice cold 70% ethanol.

4.3.13. Centrifuge the tubes again at 18,200 x *g*, 4 °C, for 10 min. Discard the supernatant and allow the pellet to dry in the biosafety cabinet under laminar flow.

4.3.14. After the pellet is dry, add 75 µL of RNase free, DNase free, sterile water to each tube.

4.3.15. Quantify the resuspended DNA by a spectrophotometer and then send it out for 16S rRNA gene sequencing.

5. Short Chain Fatty Acid (SCFA) Detection and Analysis

5.1. Thaw the frozen samples at 40°C for 30 min and extract short chain fatty acids using diethyl ether in a 2:1 (v:v) ratio.

5.2. Transfer the organic phase to a GC/MS (see **Table of Materials**) equipped with a column, 30 m, 0.25 mm ID, 0.25 µm, (see **Table of Materials**) where 10 µL is injected.

5.3. Set the injection port temperature and initial column temperature to 260 °C and 125 °C

respectively. Hold the initial temperature for 1 min, and then increase this to 250 °C, over 11.5 min with a column flow rate of 1 mL/min.

Note: The MS temperature for the ion source is 220°C and 250°C for the interface.

5.4. Quantify the amount of each SCFA using standard curves and 2-methylhexanoic acid as the internal standard.

REPRESENTATIVE RESULTS:

The above protocol describes set up, inoculation, and running of a 5-stage *in vitro* system to study the gut microbiota of the colon. To generate the data presented below, following DNA extraction, 16S rRNA marker gene DNA sequencing of the V1V2 region was performed using the high throughput sequencing (*e.g.*, MiSeq Illumina platform) by the Microbiome Center at the Children's Hospital of Philadelphia²⁷. QIIME (Quantitative Insight into Microbial Ecology) version 1.9²⁸ was used to process the sequencing data and statistical analysis was performed on the R environment for statistical computing²⁹. Taxonomic assignments were generated using the Greengenes 16S reference database^{30,31}. OTU relative abundance values were calculated by dividing the OTU read count by the total number of reads in the sample. SCFA levels were quantified using the protocol described.

The *in vitro* communities achieve a steady state equilibrium

The ability to study the gut microbiota and short chain fatty acid (SCFA) production *in vitro* with a multi-stage system requires that the microbial communities reach a steady state. This occurs after inoculation when the taxa have become established in their niches, and the composition of the community and their metabolites are no longer fluctuating. In a steady state, the community and its products remain the same over time. For *in vitro* gut microbiota studies, stability is a key requirement; without stability, it is impossible to determine whether or not observed changes are occurring due to the experimental conditions, or if they are due to variation. According to what has been proposed in the literature, a community can be considered stable when there is 80% similarity between time points³².

Using the results of 16S rRNA marker gene sequencing, Principal Coordinates Analysis (PCoA) plots based on unweighted and weighted UniFrac distances were generated for the communities that developed in each region of the system over time (**Figure 2**). An unweighted analysis compares the communities in terms of the presence/absence of species. The weighted analysis compares them based not only on the presence/absence of species but also considers their abundance. For Unit 1, this included both the luminal and mucosal phases, and for Unit 2 only the luminal phase. Based on this analysis, it was observed that the community changed appreciably between days 1 and 7. However, from day 11 post inoculation until the end of the experiment, the samples occupied a small region of the PCoA space. This was true for sample-sample distance scores based on OTU presence-absence (**Figure 2A**) or on OTU abundance (**Figure 2B**). Thus, it was observed that, after 11 days, the abundance and the types of bacteria were stable from one sample point to the next. This pattern was observed for both the luminal and mucosal phases of all three colon regions of Unit 1 and the luminal phase of Unit 2. These

results illustrated that both the luminal and mucosal phases reach stability and that this occurred at the same time.

System stability was also probed by analyzing the production of short chain fatty acids (SCFAs) over time. The three most prominent SCFAs (propionic acid, butanoic acid, and acetic acid)³⁸ were measured in each sample over the course of the experiment using gas chromatography GC/MS. These measurements revealed that propionic acid, butanoic acid, and acetic acid fluctuated from the start of the experiment until day 15 post inoculation (**Figure 3A-C**). After day 15, the amounts of these SCFAs produced in each colon region remained constant, with only minimal changes occurring until the end of the experiment (**Figure 3A-C**). The difference between time points was an average of 6.8% for propionic acid, 7.2% for acetic acid, and 8.02% for butanoic acid. This suggests that similar to the community composition, the metabolic properties of the community entered a steady state, as indicated by the production of stable amounts of SCFAs over time. Both Unit 1 and Unit 2 produced similar amounts of SCFAs, with no significant differences between the two ($p > 0.05$). This indicated that the production of SCFAs was not affected by the presence or absence of the mucosal community. It should be noted that the point of stability determined in this experiment is similar to that reported previously, in which it was stated that community stabilization in a 5-stage *in vitro* system occurred approximately 2 weeks post inoculation³³⁻³⁵.

The communities developed in the *in vitro* system are similar to the inoculum

The second required element for an *in vitro* gut microbiota experiment is that the community developed in the model preserves the microbial diversity of the fecal inoculum. Since the system used in this experiment provides for three distinct colon regions it cannot be expected that any one region be exactly the same as the fecal inoculum. However, it is expected that the members of each community are derived from the fecal inoculum, and all together maintain a similar level of diversity as the inoculum.

Based on the results of 16S rRNA marker gene sequencing, the average, stable community for the luminal and mucosal phase of each colon region was determined (Days 15-28 post inoculation) and compared to the community of the fecal inoculum (**Figure 4A**). These results demonstrate that the communities which developed in the individual colon regions of the *in vitro* system were similar to the fecal inoculum in composition. The two most prominent orders in the reactor, *Clostridiales* and *Bacteroidales*, matched those observed in the inoculum. However, several low-abundance bacterial orders in the colon regions were different from the inoculum, with the most prominent differences between orders *Burkholderiales* and *Synergistales* (**Figure 4A**).

The alpha diversity for *in vitro* system was compared to the inoculum, as another measure of community structure after stabilization. The Shannon index, calculated for each region over time, reached a similar level of diversity as the inoculum for all regions, except for luminal samples from the ascending region (**Figure. 4B**). Taken together, these results demonstrate that the *in vitro* culture system was able to produce a community comparable to the fecal inoculum, both in terms of composition and diversity (**Figure 4A-B**).

Using an *in vitro* system allows for the development of the region and the phase specific communities

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 differentiate³³. The stable (Days 15-28) luminal communities in both Unit 1 and Unit 2 were determined at the family level and plotted according to relative abundance (**Figure 5**). The divergence between the three colon regions in terms of the abundance of specific taxa, demonstrates that each region develops a unique community. This is also supported by the results of **Figure 2** and **Figure 4A**. In **Figure 2**, the mature communities that developed in each region cluster at different locations in the PCoA chart. In **Figure 4A**, the communities in each colon region differed in the percentages of the dominant order members.

For this experiment, Unit 1 was provided with mucosal carriers, while Unit 2 had no mucin carriers. Based on this experimental design, the contribution of the mucosal surface could be examined. For comparison purposes, the stable (Days 15-28) luminal and mucosal communities for Unit 1, at the family level, were plotted together according to relative abundance (**Figure 6**). It is clear that the composition of the luminal and mucosal communities for all three colons differ in the abundance of some taxa, the most prominent being *Lachnospiraceae* and *Bacteroidaceae*. These results also demonstrate that the mucosal communities in the three regions are different from each other. For example, *Clostridiaceae* is enriched in the mucosal communities of the descending and transverse regions, and *Veillonellaceae* is higher in the mucosal phase of the ascending colon, but not in the transverse or descending colon regions. Taken together, the results presented in these figures show that there is a clear difference between the communities in each phase and each region for some taxa, illustrating that the composition between the regions is similar and that there is a difference in abundance.

Although analysis of DNA sequencing reveals the development of a region-specific community, there is no apparent difference in the production of SCFAs between regions. The average ratio of Acetic Acid: Propionic Acid: Butanoic Acid for the stable community (Day 15-28) was calculated for each region (**Figure 7A**). While the ratios of the different SCFA remained fairly similar, there is an increase in the total amounts of SCFAs produced between the ascending, transverse and descending regions, with the highest levels found in the descending region (**Figure 7B**). This was true for both Unit 1 and Unit 2.

FIGURE AND TABLE LEGENDS:

Figure 1. Illustration of the 5-stage, *in vitro* experimental design. 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.

Figure 2. The *in vitro* community stabilizes by day 11 post inoculation. PCoA analysis based on (A) Unweighted and (B) Weighted Unifrac distances for the luminal and mucosal phase of each region over time for Unit 1 and for the luminal phase of Unit 2. The plot is faceted into the components after calculating the PCoA axes.

Figure 3. The production of SCFAs by the *in vitro* system stabilize by day 15 post inoculation. Measurements of Propionic Acid, Butanoic Acid, and Acetic Acid over time for the (A) Ascending colon (B) Transverse colon and (C) Descending colon. The experiment was performed in triplicate. The results represent an average of three independent measurements, with error bars depicting the standard deviation.

Figure 4. Comparison of the stable communities from the *in vitro* system to the fecal inoculum. (A) The stable communities (D15-28), at the order level were averaged and formatted in pie charts for both the mucosal and luminal phase of each colon region, and for the fecal inoculum. (B) The Shannon diversity for the mucosal and luminal phase of each colon region compared to the fecal inoculum (black dotted line).

Figure 5. The luminal and mucosal phase in each colon region promote the growth of distinct communities. The average relative abundance, at the family level, for the stable communities (D15-28) for the luminal and mucosal phase, and the inoculum, were calculated and plotted together for each colon region. Error bars represent the standard deviation between timepoints. AC1= Ascending colon Unit 1; TC1= Transverse colon Unit 1; DC1= Descending colon Unit 1; AC2= Ascending colon Unit 2; TC2= Transverse colon Unit 2; DC2= Descending colon Unit 2.

Figure 6. Each colon region of the *in vitro* system develops a unique community. The average relative abundance, at the family level, for the stable communities (D15-28) in each colon region, and the fecal inoculum, were calculated and plotted together. The experiment was performed in triplicate. The results represent an average of three independent measurements, with error bars depicting the standard deviation.

Figure 7. The ratio of Acetic Acid: Propionic Acid: Butanoic Acid is similar for each colon region. (A) The amounts of acetic acid, propionic acid, and butanoic acid for the stable communities (D15-28) of each colon region were calculated and converted to a ratio. (B) The ratios were plotted as percentages for each colon region. Error bars represent the standard deviation between timepoints. AC1= Ascending colon Unit 1; TC1= Transverse colon Unit 1; DC1= Descending colon Unit 1; AC2= Ascending colon Unit 2; TC2= Transverse colon Unit 2; DC2= Descending colon Unit 2.

DISCUSSION:

In vitro culturing systems have been developed to study the gut microbiota of the large intestine. They use apparatuses designed to simulate the physiological conditions of the gastro intestinal

tract, promoting the growth of a mature gut microbial community for each region of the colon³³. While the concept is logical and comprehensible, the actual running of *in vitro* culturing systems to study the gut microbiota requires precision and an understanding of what is required and expected to produce reliable results.

The required elements for an *in vitro* gut microbiota experiment are that the community must reach stability after inoculation and that it must maintain the microbial diversity of the fecal inoculum. If the experiment is run properly, these two required elements are achieved readily and predictably. Critical steps in the protocol that will affect system stability and diversity are the following: The preparation of defined medium and pancreatic juice must be accurate, and delivery of these substrates must be consistent. Anaerobic conditions must be maintained during the experiment. The pH of each intestinal region must be accurately maintained over time. Changes in these three parameters during the experiment may result in population fluctuations and variable metabolite production.

Using this type of multi-stage *in vitro* system has several advantages. Notably, they simulate the different regions of the colon. They can also be designed to simulate *in vitro* digestion of food in the upper GIT, including the enzymatic components from saliva, and the addition of pancreatin and bile. A mucosal phase can be incorporated into each colon reactor, adding further complexity⁸. This results in the development of the region and the phase specific microbial communities that can be individually analyzed and compared. These types of systems can be readily manipulated, depending on experimental design through physical reconfiguration, alterations of the physiological parameters such as pH, temperature, or transit time, or inoculation with specific fecal samples²⁶. Importantly, research has illustrated that the communities developed in these *in vitro* systems represent the gut microbial community^{11,26,34}.

While the use of multi-stage *in vitro* systems can be used to mechanistically study the gut microbiota, they do have limitations. First, while the physiological conditions are programmable, they are fixed using specific average values, thus representing the average person. This is a simplification since there is considerable inter-individual variability^{12,23}. Second, although the lack of mammalian components is one reason to use an *in vitro* system, it also must be considered a limitation. The lack of certain mammalian components, like the immune system, can result in differences between the *in vitro* model and the corresponding *in vivo* microenvironment^{12,23}. However, these limitations do not detract from the value and insight that can be gained from using *in vitro* systems to mechanistically study the gut microbiota. Finally, the cultures are grown in glass bioreactors with no absorption of water or metabolites. This is an important difference, and the effect can be seen in the measurement of SCFAs where the total amounts of SCFAs are increasing from the ascending to transverse to descending colon regions (**Figure 7**). This is opposite of what is observed *in vivo*, where the highest concentrations of SCFAs are found proximally, in the ascending region, and the lowest concentrations found distally³⁶. However, when the net production of SCFAs is considered for each region, then it can be determined that the most SCFA production is occurring in the ascending colon. It should also be mentioned here that bacterial load was not measured in this experiment. Differences in bacterial load between the regions in this system may have an effect on the total amounts of SCFAs produced.

In conclusion, it is well known that the gut microbiota plays a role in both human health and disease, and it is considered to function as a mediator between diet and metabolic health^{37,38}. Here, the application of an *in vitro* system to study the gut microbiota was discussed, and the results from an experiment demonstrating the development of a stable community were presented. An *in vitro* system has many advantages and promotes the development of a complex and dynamic community, which is illustrated in the described experiment. This type of system is best used to study the interactions and changes within the gut microbiota community in response to external factors, such as food components and medicines. The results of these *in vitro* studies can then be supplemented with *in vivo* studies, to gain a deep understanding of the function and contribution of the gut microbiota to both health and disease.

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DISCLOSURES:

The authors have no competing financial interests. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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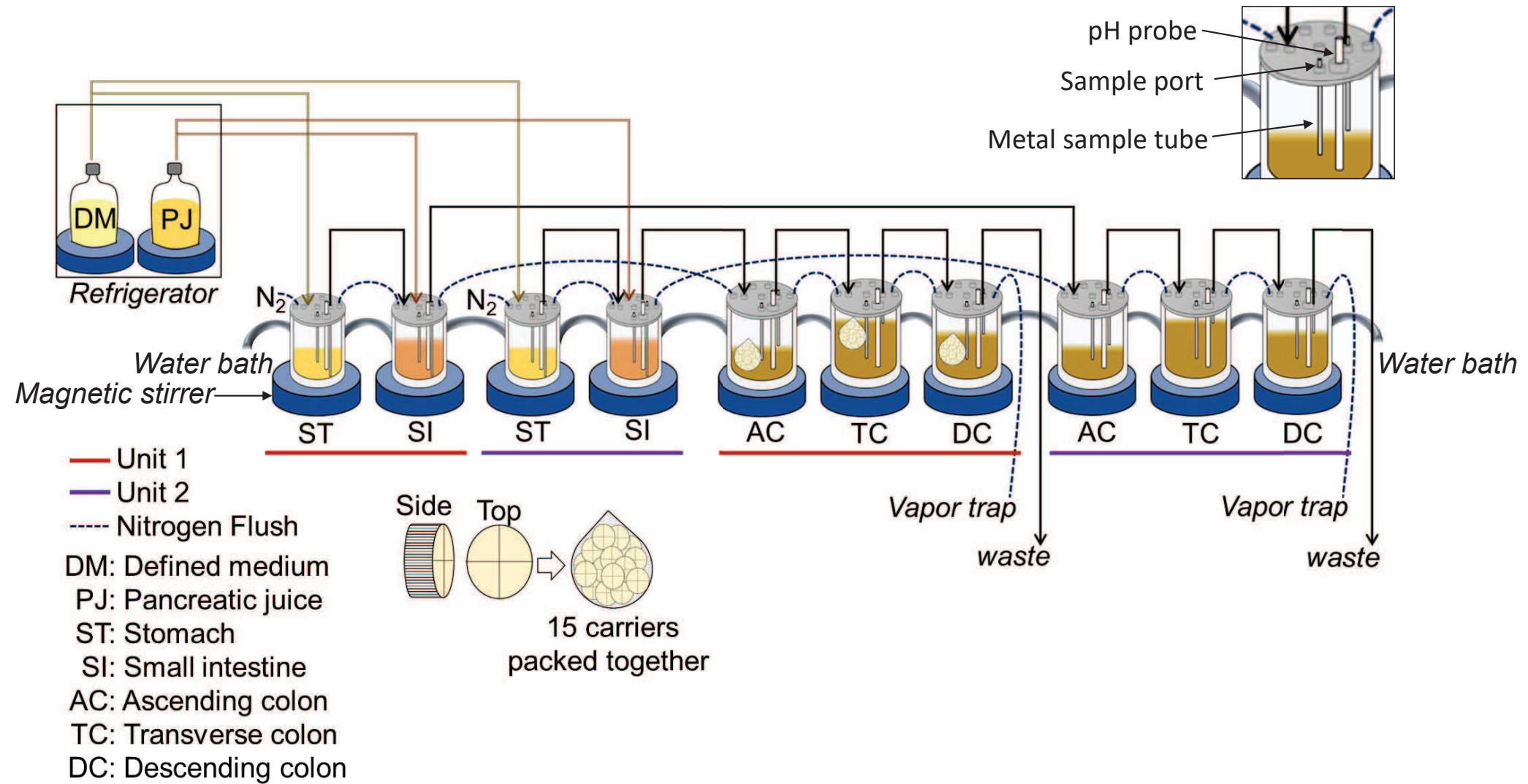


Figure 2

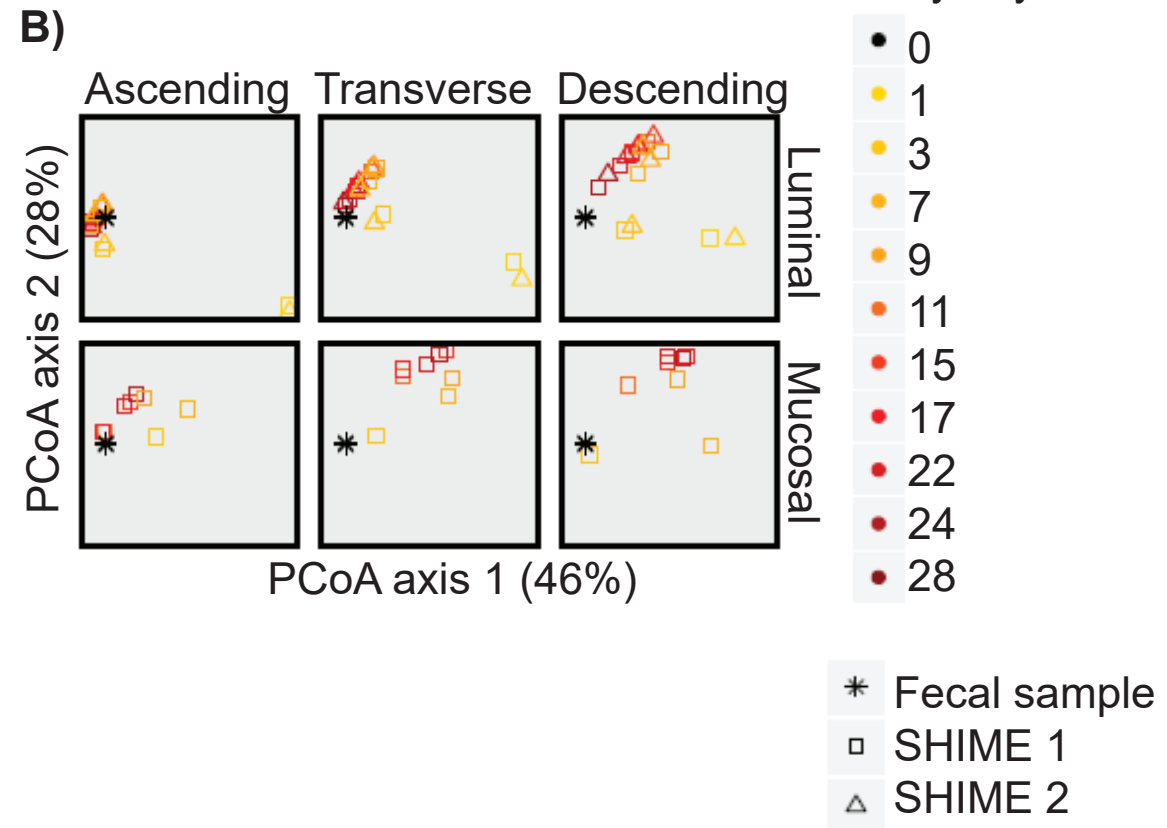
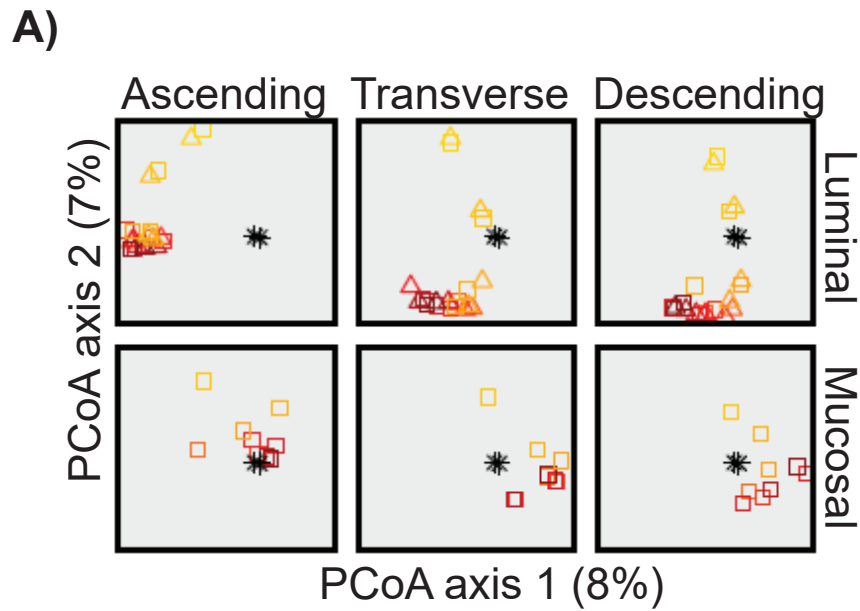


Figure 3

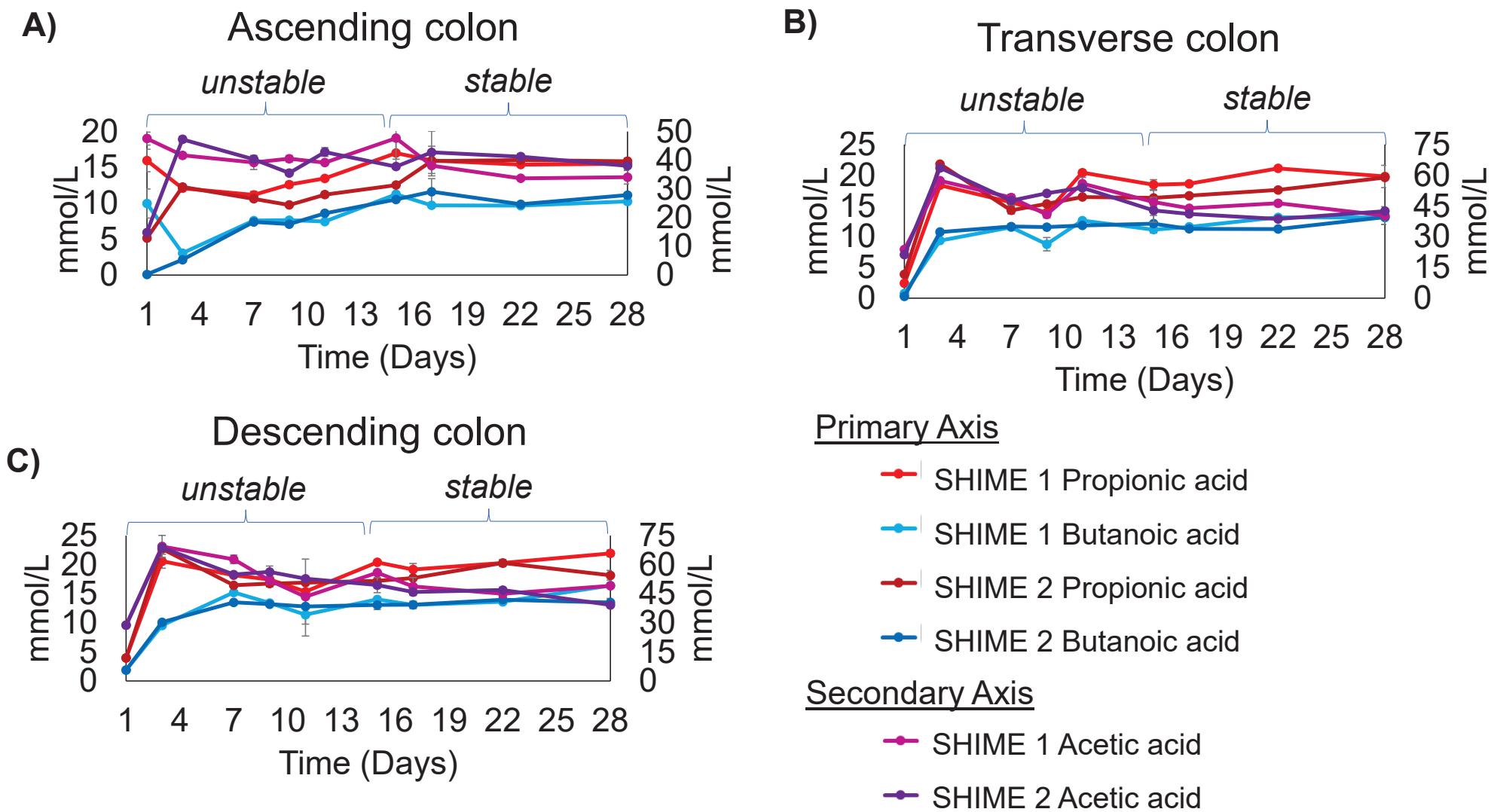
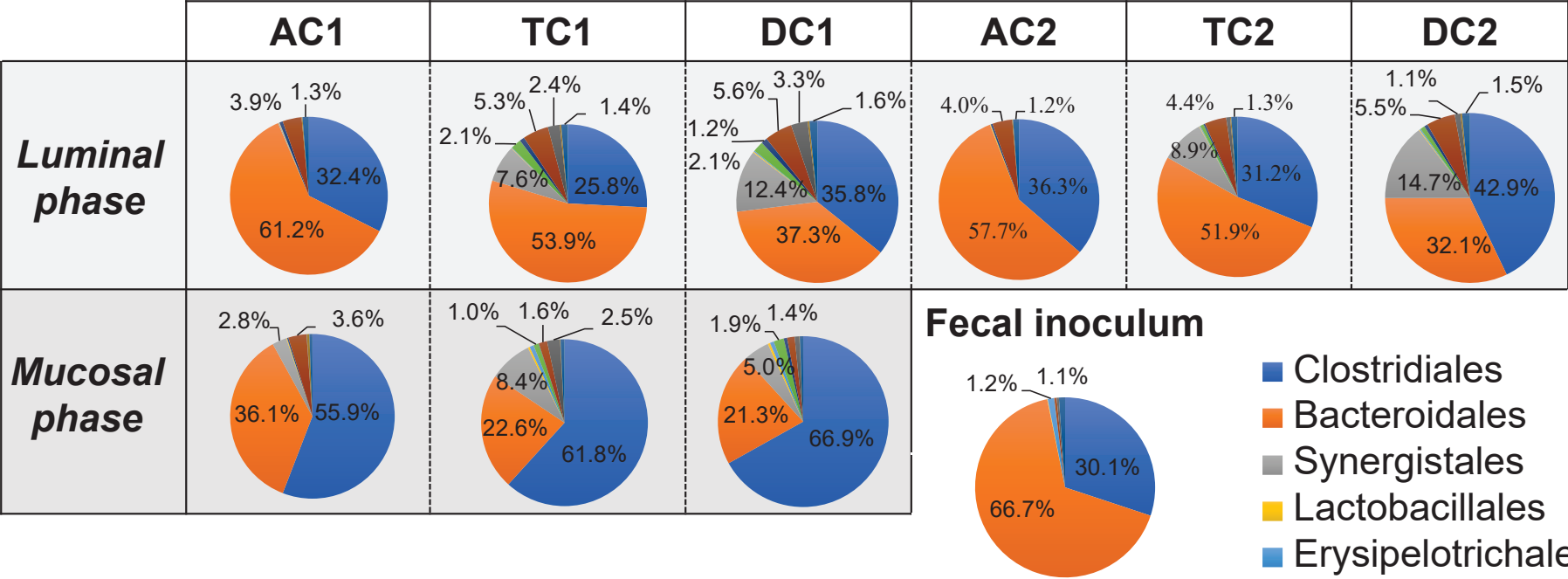


Figure 4

A)



B)

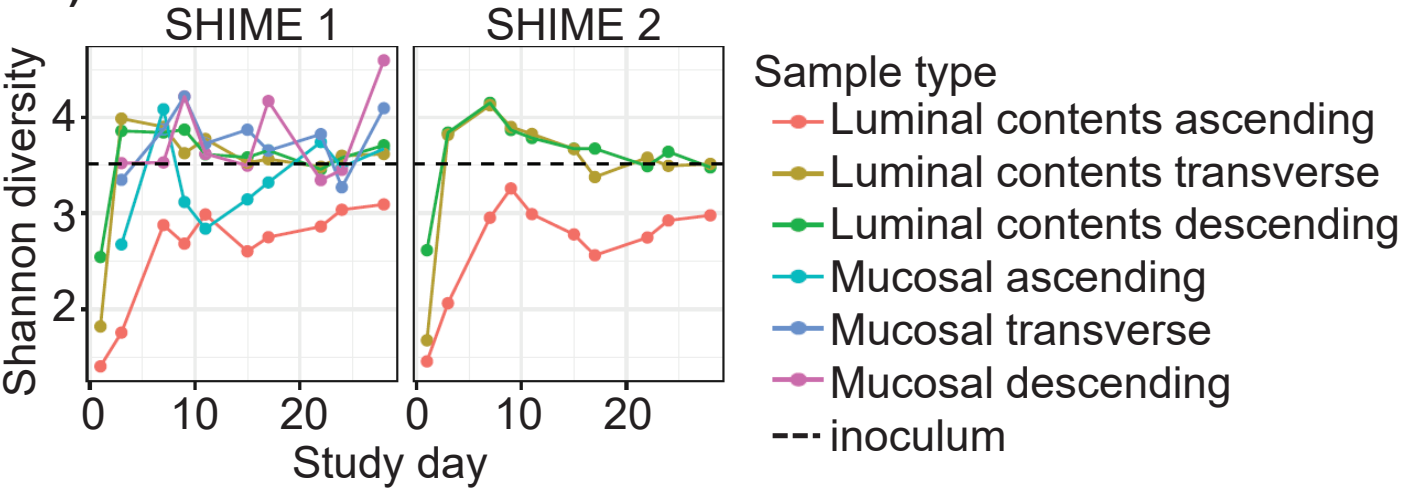


Figure 5

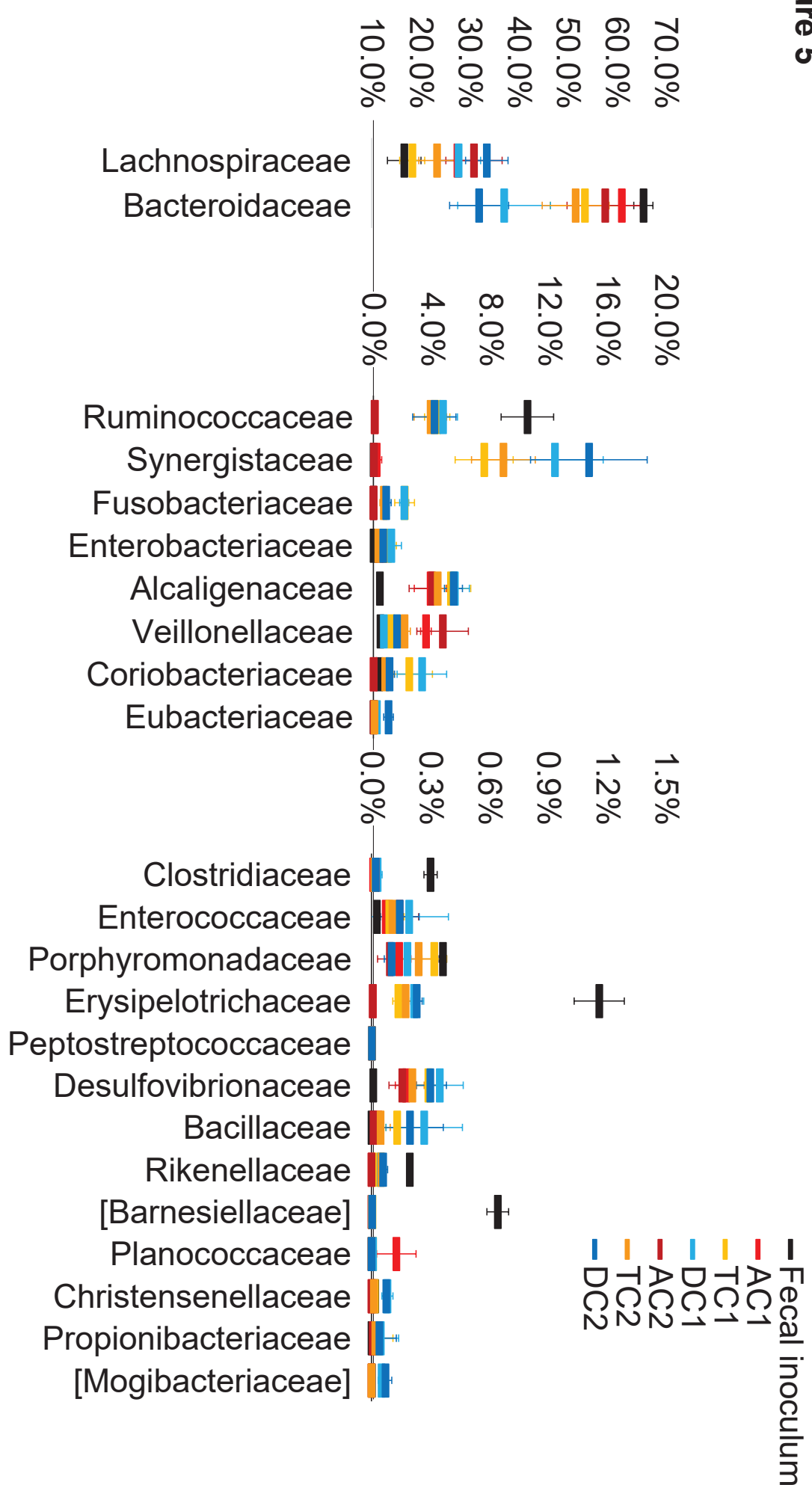


Figure 5

Figure 6

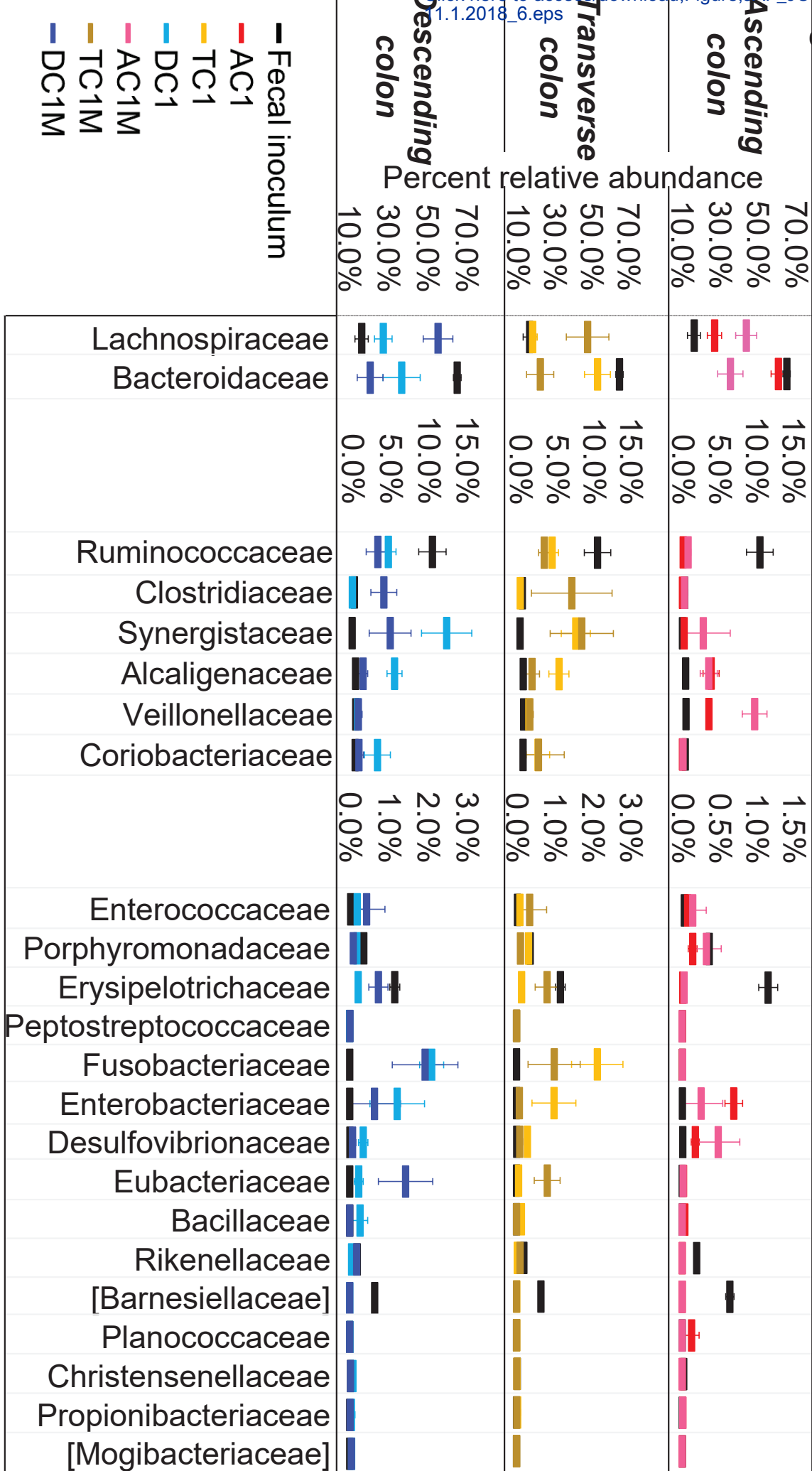
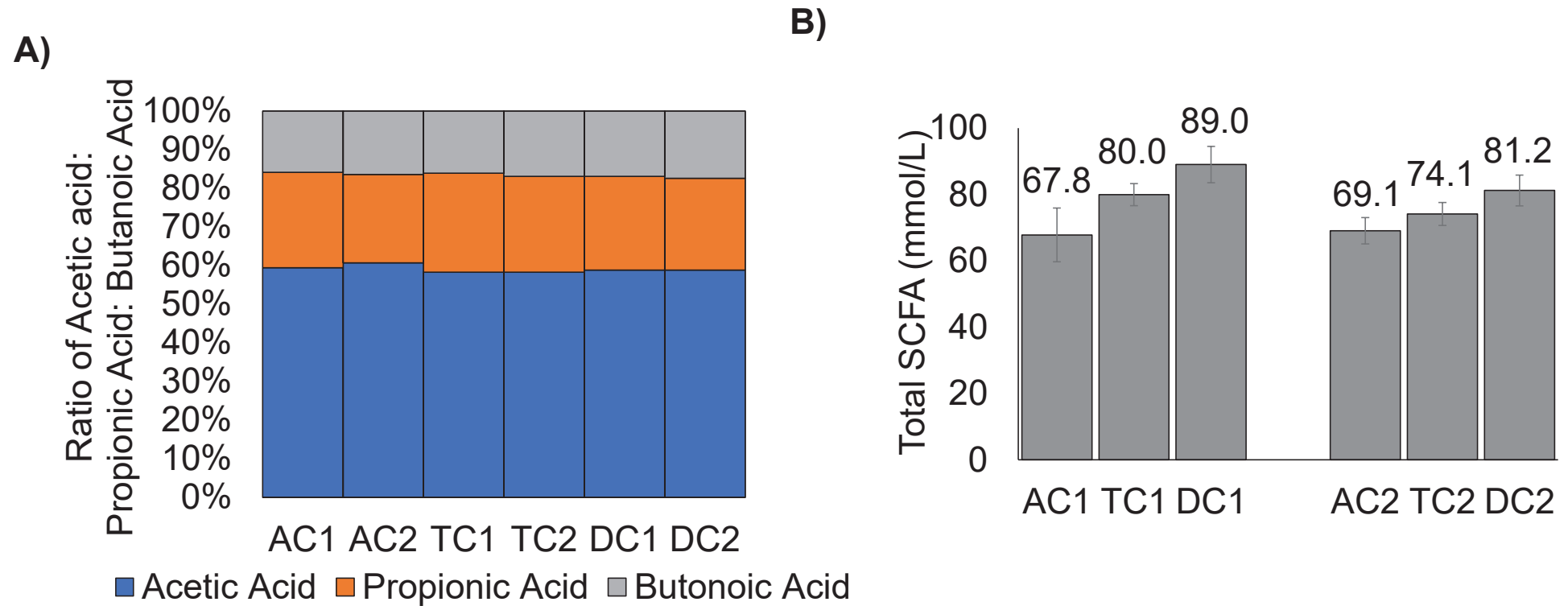


Figure 6

Figure 7

Name of Material/ Equipment	Company	Catalog Number
TWINSHIME	Prodigest	NA
defined medium (Adult M-SHIME growth medium with starch)	Prodigest	NA
Masterflex tubing	cole Parmer	NA
Urine Drainage bag	Bard	NA
Labsorb	Sigma-Aldrich	NA
Fecal sample	Openbiome	NA
Syringes	Becton Dickson	NA
Defined medium	Prodigest	NA
Oxgall Bile	Becton Dickson	NA
Pancreatin	Sigma-Aldrich	NA
Glass ware	Ace Glass	NA
Porcine mucin	Sigma-Aldrich	NA
Bacteriological agar	Sigma-Aldrich	NA
Sterilization pouches	VWR	NA
BeadBug	Benchmark Scientific	NA
Triple-Pure High Impact Zirconium 0.1mm Bead beater tube	Benchmark Scientific	NA
RNAse free, DNAse free, sterile water	Roche	NA
Shimadzu QP2010 Ultra GC/MS	Shimadzu	NA
Stabilwax-DA column, 30m, 0.25mm ID, 0.25µm	Restek	NA
plastic mucin carriers	Prodigest	NA

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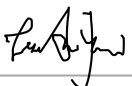
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To the editor,

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:

1. 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
2. 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 sentences:

“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.