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Corresponding Author:	Benjamin Dreskin VA Greater Los Angeles Healthcare System Los Angeles, California UNITED STATES
Corresponding Author's Institution:	VA Greater Los Angeles Healthcare System
Corresponding Author E-Mail:	benjamin.dreskin@va.gov
Order of Authors:	Benjamin Dreskin
	Kayti Luu
	Tien Dong
	Jihane Benhammou
	Venu Lagishetty
	John Vu
	Daniel Sanford
	Francisco Durazo
	Vatche Agopian
	Jonathan Jacobs
	Joseph Pisegna
	Shehnaz Hussain
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TITLE:

Specimen Collection and Analysis of the Duodenal Microbiome

AUTHORS AND AFFILIATIONS:

Benjamin W. Dreskin[1], Kayti Luu[1], Tien S. Dong[1,2,3], Jihane Benhammou[1,2,4], Venu Langishetty[2,3], John Vu[1], Daniel Sanford[1], Francisco Durazo[2,4], Vatche G. Agopian[4], Jonathan P. Jacobs[1,2,3], Joseph R. Pisegna[1,5], Shehnaz K. Hussain[6]

[1]Division of Gastroenterology, Hepatology and Parenteral Nutrition, Veterans Administration Greater Los Angeles Healthcare System, Los Angeles, CA, USA

[2]The Vatche and Tamar Manoukian Division of Digestive Diseases, Department of Medicine, David Geffen School of Medicine at University of California, Los Angeles, CA, USA

[3]UCLA Microbiome Center, David Geffen School of Medicine at University of California, Los Angeles, CA, USA

[4]Department of Surgery, University of California, Los Angeles, CA, USA

[5]Department of Medicine and Human Genetics, David Geffen School of Medicine at University of California, Los Angeles, CA, USA

[6]Cedars-Sinai Cancer and Department of Medicine, Cedars-Sinai Medical Center, Los Angeles, CA, USA

benjamin.dreskin@va.gov
kayti.luu@va.gov
shehnaz.hussain@cshs.org
tsdong@mednet.ucla.edu
JBenhammou@mednet.ucla.edu
VLagishetty@mednet.ucla.edu
john.vu@va.gov
daniel.sanford@va.gov
Fdurazo@mcw.edu
VAgopian@mednet.ucla.edu
JJacobs@mednet.ucla.edu
joseph.pisegna@va.gov

Corresponding authors:
Benjamin W. Dreskin
Kayti Luu
Shenaz K. Hussain

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SUMMARY:

In this manuscript, we discuss a novel method to sample and analyze the duodenal microbiome. This method provides an accurate depiction of microbial diversity and composition in the duodenum and could be useful for further investigation of the duodenal microbiome.

ABSTRACT:

Shifts in the microbiome have been correlated with the physiology and pathophysiology of many organ systems both in humans and in mouse models. The gut microbiome has been typically studied through fecal specimen collections. The ease of obtaining fecal samples has resulted in many studies that have revealed information concerning the distal luminal gastrointestinal tract. However, few studies have addressed the importance of the microbiome in the proximal gut. Given that the duodenum is a major site for digestion and absorption, its microbiome is relevant to nutrition and liver disease and warrants further investigation. Here we detail a novel method for sampling the proximal luminal and mucosal gut microbiome in human subjects undergoing upper endoscopy by obtaining duodenal aspirate and biopsies. Specimen procurement is facile and unaffected by artifacts such as patient preparatory adherence, as might be the case in obtaining colonic samples during colonoscopy. The preliminary results show that the luminal and mucosal microbiomes differ significantly, which is likely related to environmental conditions and barrier functions. Therefore, a combination of duodenal aspirate and biopsies reveal a more comprehensive picture of the microbiome in the duodenum. Biopsies are obtained from the descending and horizontal segments of the duodenum, which are anatomically close to the liver and biliary tree. This is important in studying the role of bile acid biology and the gut-liver axis in liver disease. Biopsies and aspirate can be used for 16S ribosomal RNA sequencing, metabolomics, and other similar applications.

INTRODUCTION:

The intestinal microbiome has become an area of increased interest in recent years. It is now understood that the diverse bacterial population in the gut can differ based on a variety of factors, including genetics, diet, medication, and environmental influences[1]. Studies have also identified unique microbial profiles linked to varying gastrointestinal diseases, such as obesity, inflammatory bowel disease, and liver disease[2,3]. The majority of studies focus on profiling the microbiome of the large intestine through the analysis of fecal and distal mucosal samples[4]. Although the highest concentration of intestinal bacteria resides in the colon (10^{12} bacteria/gram), there nevertheless is a complex community of microbes residing in the duodenum (10^3/g), jejunum (10^4/g), and ileum (10^7/g) that plays a key role in digestive metabolism and absorption[5].

The small intestine serves as the primary site of nutrient breakdown and absorption in the gastrointestinal tract. Commensal bacteria lining the small intestine play a fundamental role in aiding in the chemical breakdown of food substrates and in the release of bioactive compounds that aid in nutrient absorption[6]. These interactions contribute to a complex environment of microbe-microbe and host-microbe activity in the small intestine[7]. A study observing the small intestine microbiota in murine models found that germ-free mice fed a high fat diet had impaired lipid absorption but, when colonized with jejunal microbiota, had a direct increase in lipid absorption[6]. A human pilot study profiling the duodenal microbiota of obese and healthy

individuals found that the duodenal microbiota of obese individuals had alterations in fatty acid and sucrose breakdown pathways, likely induced in a diet-dependent relationship[8]. Furthermore, dysbiosis in the small intestine microbiota has been identified in several diseases including small intestinal bacterial overgrowth, short-bowel syndrome, pouchitis, environmental enteric dysfunction, and irritable bowel syndrome[7].

We are interested in the relationship between the microbiome and different stages of chronic liver disease. Specifically, the duodenum serves as the first site of chemical breakdown and nutritional absorption in the small intestine. Additionally, portal hepatic circulation brings nutrients and metabolites to the liver, where they are processed and regulated into the bloodstream. The anatomical proximity between the gut and the liver creates an environment susceptible to pro-inflammatory responses that can arise due to failure in the gut barrier or alterations in the gut microbiome[9]. Studies investigating the microbiome and liver disease progression have identified microbial dysbiosis in patients with non-alcoholic fatty liver disease (NAFLD), steatohepatitis (NASH), alcoholic liver disease, and cirrhosis[10,11]. While the majority of studies characterize the microbiome of the colon, we were interested in investigating the small intestine microbiome in relation to liver disease. By utilizing the novel method presented here, we have identified unique duodenal microbial profiles in patients with liver cirrhosis in relation to diet[12].

As characterization of the small intestine microbiome continues to become an area of increased interest, it is necessary to develop uniform techniques for obtaining samples that accurately represent the small intestine microbiota. However, there are challenges associated with specimen procurement that have complicated the study of the small intestine microbiome environment. Current sampling methods require invasive procedures that are often subject to contamination, as outlined by Kastl et al[7]. Here we detail a novel method for obtaining duodenal aspirate and biopsies for microbial analysis from patients with liver disease undergoing esophagogastroduodenoscopy.

PROTOCOL:

Duodenal samples were obtained at the Veteran Affairs Greater Los Angeles Healthcare System, Cedars-Sinai Medical Center, and the Ronald Reagan UCLA Medical Center after the clinical protocol for the Microbiome, Microbial Markers and Liver Disease (M3LD) study was accepted by the institutional review board of the local ethics review committee. Written informed consent was obtained from all participating patients.

1. Consent of participants

1.1. Approach subjects with a diagnosis of liver cirrhosis of any etiology with endoscopies scheduled for variceal screening or portal hypertension for recruitment. Exclude patients with hepatocellular carcinoma (HCC).

1.2. Introduce the study, carefully ensure participant understanding, and ask the participant if they would like to volunteer in the study. Complete an informed consent form if they agree.

1.3. Ensure that a properly trained and credentialed medical professional performs the endoscopy.

2. Specimen collection

2.1. Anesthetize the patient with 50 mg of fentanyl and 2 mg of midazolam. Increase the dosage as needed until the patient is moderately anesthetized and can tolerate the endoscope.

2.2. Insert an endoscope (e.g., Pentax EG29-i10) into the patient's esophagus and progress toward the pylorus.

NOTE: The endoscope size will vary based on the patient's physiology. Do not use the endoscope for any aspiration prior to sample collection to avoid contaminating the endoscope channel with gastric or oropharyngeal secretions.

2.3. Obtain a microscope slide and blunt 18 Gauge syringe needle to prepare for specimen procurement. Prepare a sterile pad which will be used to transfer biopsies from the microscope slide to cryovials.

2.4. When the scope arrives at the second portion of the duodenum distal to the ampulla of Vater, aspirate any fluid present through a sterile disposable aspiration catheter passed through the working channel of the upper endoscope and collect it into a 40 mL disposable specimen container. Flush up to 30 mL of additional sterile water by syringe into the duodenum and aspirate the fluid into a fluid specimen trap to collect a total of 15 mL.

2.5. Immediately remove and cap the aspirate container attached to the suction component of the catheter. Place the container into a thermos over ice and into a specimen transport bag for transfer to the processing lab.

2.6. Insert a 2.8 mm single-use biopsy forceps into the port on the side of the endoscope to remove tissue specimens for research. Perform 2 random passes of the forceps within the second portion of the duodenum, collecting two bites of tissue in each pass.

2.7. Use a blunt 18 Gauge syringe needle to extract tissue samples from the forceps and onto the microscope slide before transferring the samples to four separate empty 2 mL cryovials. Tightly screw on the caps.

2.8. Insert all 4 tightly capped cryovials into an empty container, such as a urine cup, and create a bath, consisting of 20 mL of ethanol and dry ice, to flash freeze the biopsies.

2.9. Place the biopsy cryovials in an insulated Styrofoam container on a bed of dry ice and transfer the specimens to a -80 °C freezer for long term storage to preserve the specimens.

3. Aspirate processing

3.1. Following biospecimen collection, immediately transfer the biospecimens to a BSL2 or higher laboratory for processing.

3.2. Gently invert the container of aspirate fluid four times, and then aliquot approximately 1.5 mL of the fluid into 2 mL cryovials.

3.3. Store specimens at -80 °C until batch analysis can be performed.

4. Questionnaire administration and collection of clinical data

4.1. Ask participants to complete questionnaires to elicit information on diet, demographics, smoking /alcohol habits, medical conditions/medications, and quality of life[13].

4.2. Review medical charts to capture clinical data including clinical labs, imaging data, medications, and medical complications.

5. DNA extraction

5.1. Perform DNA extraction immediately upon removing the duodenal biopsies and aspirate from storage at -80 °C. No preparatory steps are required to extract DNA from duodenal biopsy specimens.

5.2. Conduct DNA extraction and sequencing for all of the samples on the same day to decrease the risk of a batch effect in analysis. Set up a library specific to the collected specimens before the samples can be sequenced.

5.3. Use a DNA microprep kit to extract DNA from duodenal biopsy specimens, per the manufacturer's protocol[14].

5.4. Add the maximum amount of sample per protocol guidelines to a lysis tube containing 750 µL of lysis solution and a 0.7 mL dry volume of 2 mm glass beads.

5.5. Secure the lysis tubes in a bead beater with a tube holder and run the beater at maximum speed for at least five minutes. The time will vary based on the bead beater and the sample being analyzed.

5.6. Centrifuge the lysis tubes in a microcentrifuge at 10,000 x g for 1 min.

5.7. Transfer up to 400 µL of supernatant through a filtered collection tube and centrifuge again at 8000 x g for 1 min.

5.8. Add 1200 µL of binding buffer to the filtrate in a microcentrifuge tube and mix thoroughly until homogenous.

5.9. Transfer 800 µL of the mixture to a spin column and tube assembly. Centrifuge at 10,000 x g for 1 min. Discard the flow-through.

5.10. Place the spin column on a new centrifuge tube with 400 µL of DNA wash buffer and mix thoroughly until homogenous. Centrifuge at 10,000 x g for 1 min. Discard the flow-through.

5.11. Repeat step 5.10 two times, with 700 µL and then 200 µL DNA wash buffer per wash.

5.12. Add 20 µL of elution buffer to the spin column in a new centrifuge tube. Incubate for one minute, then centrifuge at 10,000 x g for 1 min to elute the DNA. Discard the column.

5.13. Filter the DNA again. The DNA is now ready for PCR amplification.

6. DNA amplification

6.1. Perform PCR amplification of the V4 region of the 16S ribosomal RNA gene by 250 × 2 paired-end sequencing on aMiSeq, HiSeq or NovaSeq sequencer[15].

6.2. Create a 96-well plate primer plate. Create a master mix of 165 µL of ILHS_515f conserved forward primer (100 µM stock) and 2970 µL of molecular biology grade water. Add 28.5 µL of this mix to each well.

6.3. Add 1.5 µL of corresponding reverse primer from the 96 well plate of barcoded IL_806r unique primers (100 µM stock). These barcodes will identify unique patient samples.

6.4. Create a master mix of water (23.1 µL x 3.3 x number of samples), PCR buffer (3 µL x 3.3 x number of samples), dNTPs (0.6 µL x 3.3 x number of samples), and JumpStart Taq DNA polymerase (0.3 µL x 3.3 x number of samples).

NOTE: 16S DNA amplification should be performed in triplicate.

6.5. Add 81 µL of master mix to each well in the first 96 well plate. Add 6 µL of DNA and 3 µL of primer mix to each well. Use a P200 multichannel pipette to mix the wells, then pipette 30 µL into the corresponding well of each of the other two PCR plates. Seal the plate.

6.6. Use the following thermal cycler settings: 94 °C x 3 min; 35 cycles: 94 °C x 45 s, 50 °C x 1 min, 72 °C x 1.5 min. After 35 cycles: 72 °C x 10 min, 94 °C x 5 min, 4 °C indefinitely.

7. Cleanup and library setup

7.1. Employ a PCR or DNA cleanup kit to purify the PCR products. Elute the DNA with 30 μL of PCR grade (DEPC-treated) water.

7.2. Use a UV-visible spectrum spectrophotometer that has the capability to quantify PCR samples.

7.3. Open the spectrophotometer software on computer desktop. Click on **Nucleic Acid**.

7.4. Wipe the port with distilled water. Add 1 μL of 1x PCR buffer/water to port and calibrate.

7.5. Enter the sample ID and pipette 1 μL of sample onto the port.

7.6. Close the port and hit **Measure**.

NOTE: Avoid air bubbles when adding samples onto the port.

7.7. Wipe off the sample with lab wipes and distilled water, and then prepare the next sample. Record values with each sample. The computer should also auto-save.

7.8. Combine 250 ng of each amplified PCR product in 1 tube. This is the DNA library.

7.9. Deliver the library, along with reads 1 and 2 and the index sequencing primers to a sequencing lab for sequencing.

NOTE: If a local laboratory is not available, a commercial sequencing lab can be utilized for this step.

8. Data formatting

8.1. Sequencing data provides results in forward and reverse .fastq files. Convert this data into a data sheet and clean so that the information can be analyzed for downstream applications.

8.2. Open the R programming software. Follow the instructions and code listed in the supplemental coding file to proceed through the DADA2 pipeline and clean the DNA sequencing data[16]. Download the SILVA reference database for the taxonomy assignment after the sequences have been passed through the pipeline[17].

8.3. Briefly, load the DADA2 package in R. Define a path in which all of the .fastq files are saved in the same directory and extract the files into R.

8.4. Read the names of the fastq files and match the lists of forward and reverse fastq files.

8.5. Inspect the quality of the forward reads and reverse reads.

NOTE: Nucleotide sequences can be truncated to maintain high quality sequences.

8.6. Filter and trim the fastq files. Use the learnErrors method to learn the parametric error model that DADA2 uses. This will show the observed and expected error rates for each possible nucleotide substitution (A → C, A →T, A→G, etc.).

8.7. Apply the sample inference algorithm to determine the number of unique sequences in each sample and further inspect the dada-class object to arrive at the number of true unique variants per sample.

8.8. Merge the forward and reverse reads to obtain complete and denoised sequences.

8.9. Construct an amplicon sequence variant (ASV) table.

8.10. Remove chimeras from the ASV table. Chimeric sequences are a single sequence resulting from two more abundant parent sequences.

8.11. Track the total number of reads that have passed through the pipeline. DADA2 will use SILVA to assign taxonomy to each unique sequence. The accuracy can be analyzed by including a community of known bacteria sequences in the DADA2 pipeline and comparing DADA2 sequence variants to the expected composition of the community.

NOTE: The DNA sequencing data is now cleaned and ready for analysis based on the specific scope and goals of the research project.

REPRESENTATIVE RESULTS

Population differences between mucosal and luminal microbiome of proximal gut

Previous studies have found differences in the microbial populations of luminal and mucosal colon specimens[4-5, 18]. The preliminary results show that duodenal aspirate and biopsy specimens can measure for both luminal and mucosal microbiota in the proximal gut. Furthermore, we have found that these microbiome populations are distinct from one another (**Figure 1**). Additionally, bile acids in the aspirate correlate strongly with microbiome composition of the aspirate (**Figure 2**).

FIGURE LEGENDS

Figure 1: Microbial differences were identified based on sampling type and location in the proximal gut. (A) Principal coordinates (PC) analysis plot of microbial composition from aspirate samples representative of the luminal microbiome (red) compared to duodenal biopsy samples representative of the mucosal microbiome (blue). **(B)** Phylum summary for luminal versus mucosal populations. **(C)** Log2 fold changes (FC) show differential abundance by genus between

duodenal aspirate (red) and biopsy (blue) specimens at $q < 0.05$. Dot size corresponds to genus abundance and color corresponds to phylum. **(D)** Genus level taxonomic summary by sampling type.

Figure 2: Correlation Matrix of bile acids and microbiota from duodenal aspirate samples in which color represents correlation. The matrix was created with the corrplot and Hmisc packages in R which use Spearman correlation and adjust for false discovery rate.

Supplemental File: DADA2 code

DISCUSSION

Studies of the microbiome are incredibly important, as this complex ecosystem has a critical role in energy homeostasis, immunologic responses, and metabolism[19]. Regional microbiome differences exist that may reflect the distinct physiological functions of various regions of the gastrointestinal tract, which may affect different disease states[20]. The fecal microbiome is most commonly studied but more recently the small intestine microbiome has come under investigation[6,21]. There is a need to develop a uniform protocol for further study of the proximal gut microbiome and its effects on gastrointestinal health and disease states.

This method allows the investigation of both the luminal and mucosal components of the microbiome of the proximal gut. This has advantages over a nasogastric tube method that cannot consistently procure post-pyloric samples and risks contamination by the oropharyngeal tract. Similarly, collecting biopsies via ileostomy risks exposure to the skin and external environment[7]. There are populations requiring routine endoscopies for screening and surveillance, such as cirrhotic patients or patients with gastric or esophageal cancer[22]. Obtaining samples from patients who are already undergoing screening removes the challenge of consenting patients to voluntarily tolerate an invasive sampling procurement, as described by Angelakis et al.[8]. However, this may create a potential limitation for studies aiming to investigate gastrointestinal physiology that does not require regular screening. Collecting samples by esophagogastroduodenoscopy allows the investigator to precisely analyze microbiome composition from the second and third portions of the duodenum, depending on the area of interest. Forceps are fed through the endoscope port and therefore avoid contamination by the oral cavity microbiome. Additionally, biopsies are associated with very minimal risk and aspiration is routinely used during endoscopy to clear and better visualize the mucosa. This method is valuable in that it is easy to simultaneously collect duodenal aspirates and biopsies for luminal and mucosal proximal gut microbiome analysis.

Modifications can be made to the specimen procurement protocol based on reagent availability and ease of use. In this protocol, a blunt 18 Gauge syringe needle was used to remove biopsies from the forceps but this could easily be substituted with a smaller or larger blunt syringe needle or dressing forceps. Using forceps may allow the sample procurer to transport specimens directly from endoscope forceps to cryovials and to allow the specimens to be frozen more quickly. Biopsies can be placed into cryovials with freezing medium and placed into a freezing container with isopropanol to freeze the tissue samples at a constant, gradual rate until they reach -80 °C.

Liquid nitrogen tissue storage could also be valuable depending on the anticipated time from tissue collection to DNA extraction and analysis. Biopsies should not be fixed in formalin, which can disrupt duodenal microbiota and cause issues with DNA extraction and amplification. If the patient is hesitant to donate tissue biopsies, aspirate alone can provide useful information, however the mucosal microbiome cannot be evaluated in this case. Aspirates can also be analyzed by a metabolomics approach or by correlating luminal samples with bile acid concentrations. Further metabolomics analysis can be added by including a blood sample collection from a patient's pre-procedure.

There are several bioinformatics pipelines that can be used in place of DADA2 that have tradeoffs in sensitivity, specificity, and degree of consensus between outputs[23]. Once samples have been sequenced and denoised, the possibilities for analysis are very broad. Briefly, sequencing data can be analyzed for alpha and beta diversity, coupled with a metabolomics approach to identify microbial byproducts. QIIME 2 is a useful microbiome analysis package that can generate a variety of different data manipulations from raw DNA sequencing inputs[24]. This allows flexibility in comparison of the microbial profiles in different disease states. The DNA extraction and sequencing protocols detailed in this manuscript can also be used to study oral, fecal and tissue-based microbiomes.

There are potential limitations to this method that should be considered when devising a protocol to study the duodenal microbiome. There is little correlation between duodenal and fecal microbiota, so results may not be generalizable to other regions of the gastrointestinal tract[25]. Studies comparing duodenal to oral and fecal microbiota in several disease states have found the communities to be unique as well[26,27]. Concomitant medications should be reviewed at the time of endoscopy. It is critical to ensure that participants have not used antibiotics in the past month, which are known to affect microbial diversity in the gut[28,29]. Cholestyramine or proton pump inhibitor usage could potentially affect microbial diversity and confound results[30,31]. Additionally, flexible endoscopes cannot be heat sterilized and it is possible for biofilms to form, potentially confounding microbial analysis of duodenal biopsies. Microbiological inspection of endoscopes should be considered when reviewing facility endoscope sterilization protocols.

This manuscript details a duodenal microbiome sampling protocol from initial biopsy and aspirate specimen procurement during endoscopy to DNA extraction, sequencing and data formatting to prepare for downstream applications. This method has direct applications specifically to research regarding liver disease. Absorbed macromolecules, microbiota, and their metabolites are carried from the gastrointestinal tract to the liver by portal circulation[12]. Cirrhosis impairs gastrointestinal epithelial barrier function, which can increase exposure of the liver to these potentially immunogenic features and has also been associated with microbial dysbiosis[25]. Dysbiosis of the gut microbiome has been linked to the pathogenesis of autoimmune diseases and other changes in immune function[32,33]. Following this reasoning, the proximal gut microbiota may also be important for the outcomes of liver and other gastrointestinal organ transplantation outcomes due to effects on the anti-graft immune response. Obtaining duodenal aspirate and mucosa samples may be useful for identifying and further investigating the specific roles of small bowel microbiota in gastrointestinal physiology and disease.

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DISCLOSURES:

The authors have no competing financial conflicts of interest to report.

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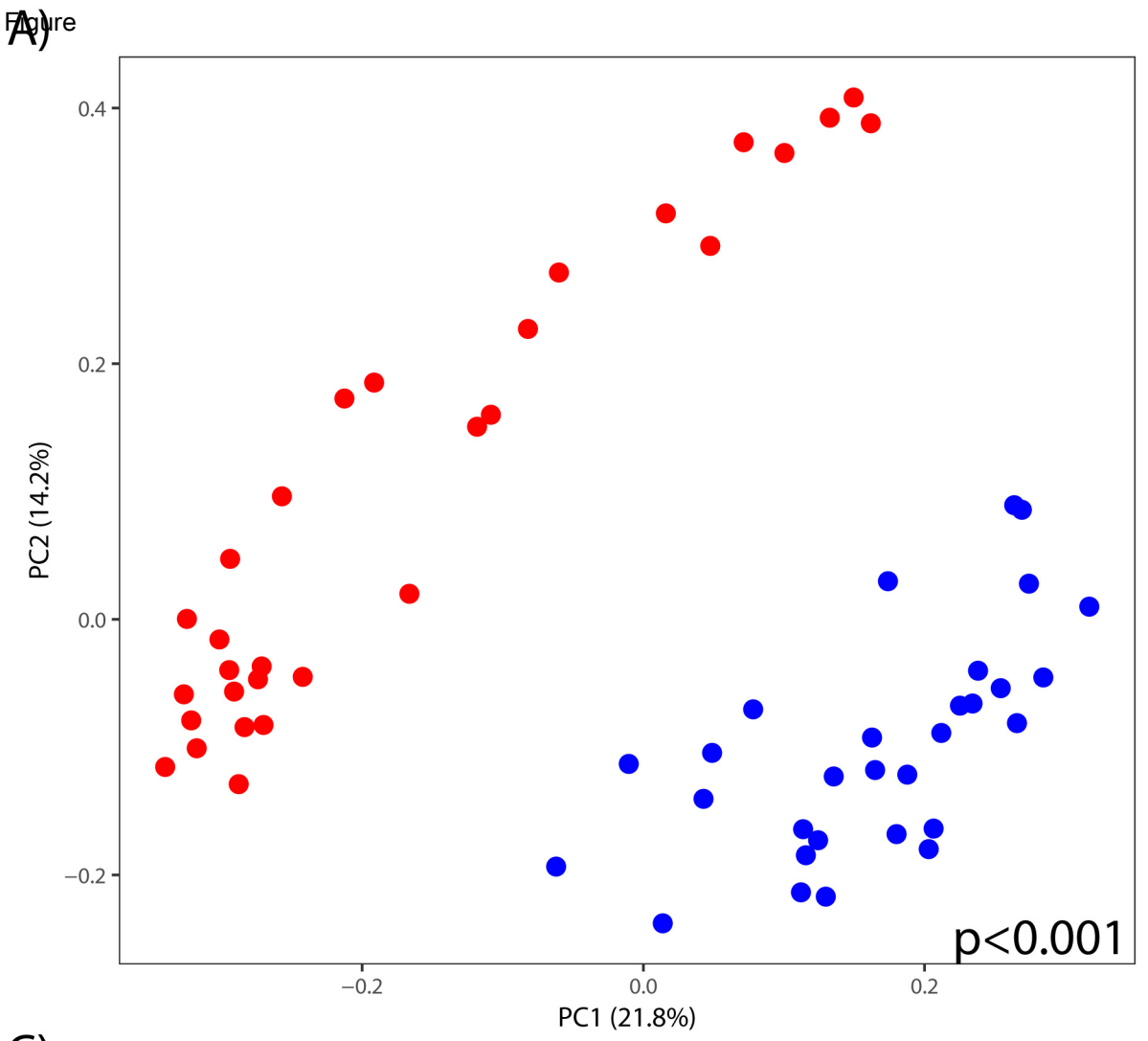
Figure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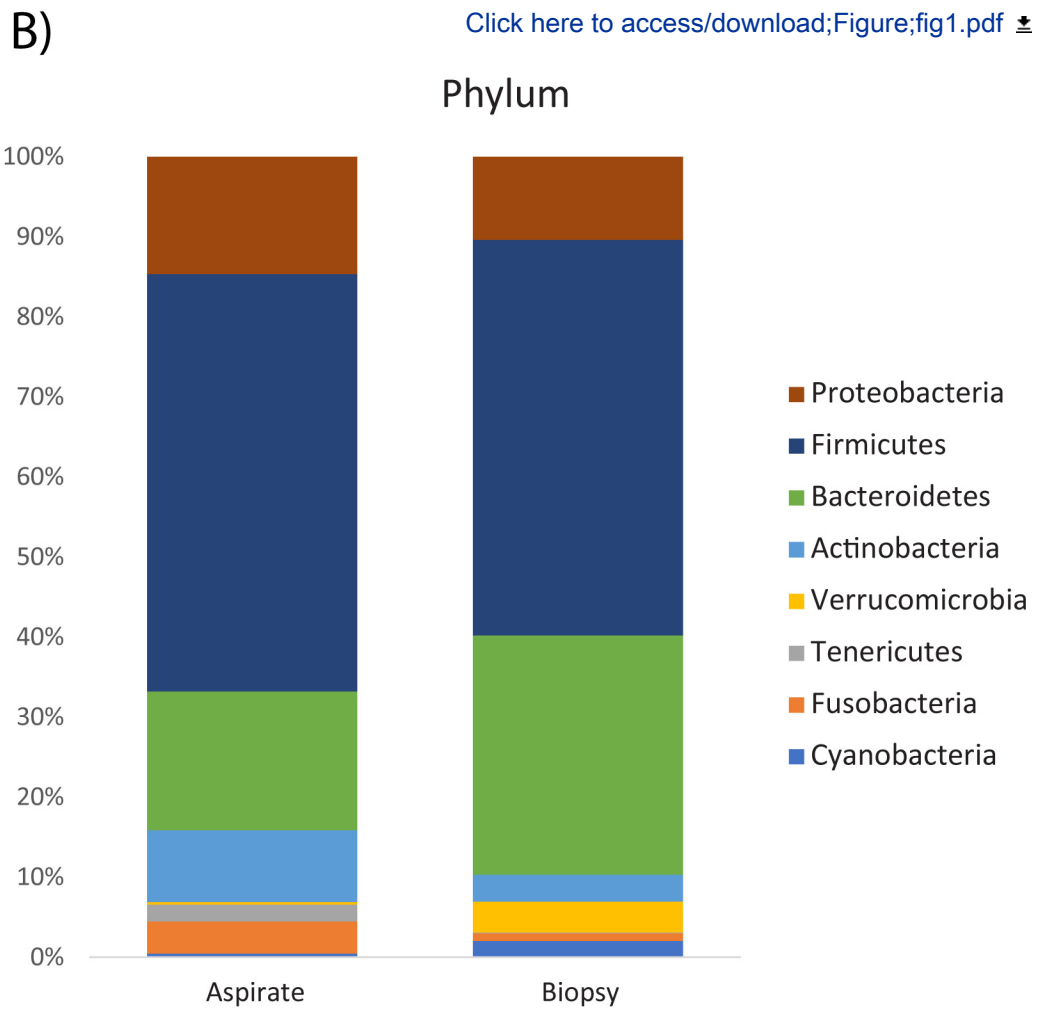
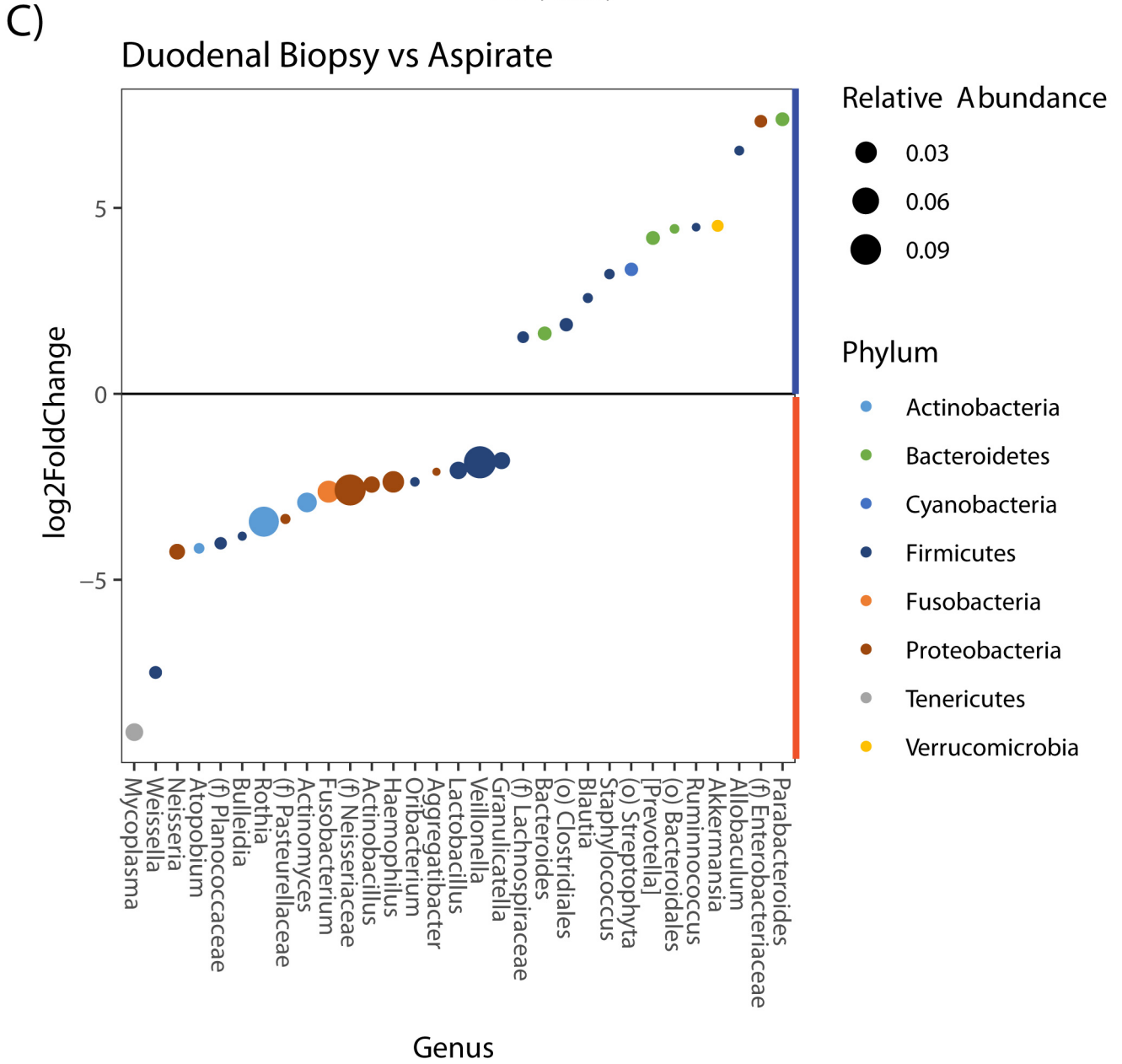
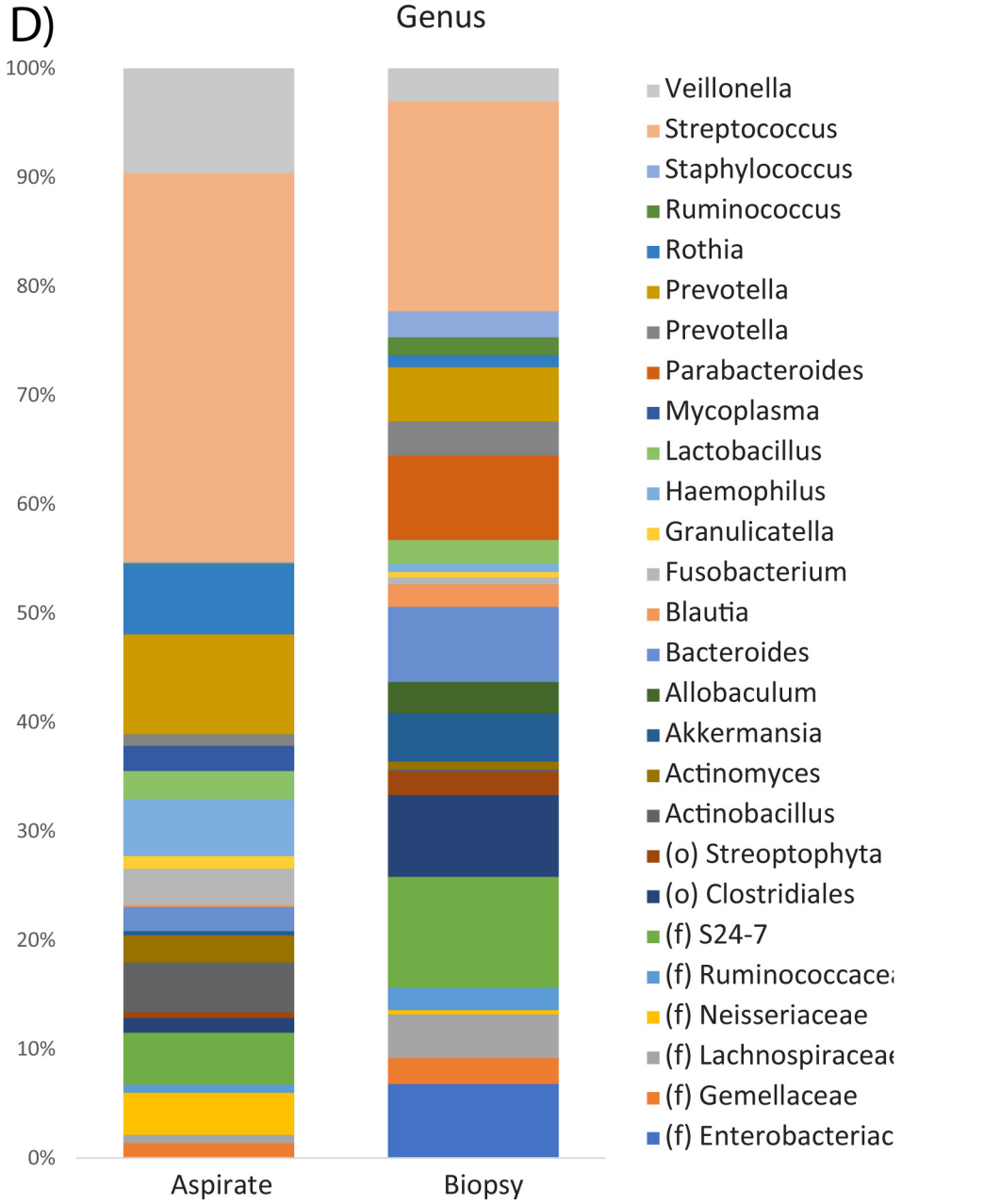
Aspirate
Biopsy


B)
Phylum
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Aspirate
Biopsy
Proteobacteria
Firmicutes
Bacteroidetes
Actinobacteria
Verrucomicrobia
Tenericutes
Fusobacteria
Cyanobacteria


C)
Duodenal Biopsy vs Aspirate
log2FoldChange
5
0
−5
Mycoplasma
Weissella
Neisseria
Atopobium
(f) Planococcaceae
Bulleidia
Rothia
(f) Pasteurellaceae
Actinomyces
Fusobacterium
(f) Neisseriaceae
Actinobacillus
Haemophilus
Oribacterium
Aggregatibacter
Lactobacillus
Veillonella
Granulicatella
(f) Lachnospiraceae
Bacteroides
(o) Clostridiales
Blautia
Staphylococcus
(o) Streptophyta
[Prevotella]
(o) Bacteroidales
Ruminococcus
Akkermansia
Allobaculum
(f) Enterobacteriaceae
Parabacteroides
Genus
Relative Abundance
0.03
0.06
0.09
Phylum
Actinobacteria
Bacteroidetes
Cyanobacteria
Firmicutes
Fusobacteria
Proteobacteria
Tenericutes
Verrucomicrobia


D)
Genus
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Aspirate
Biopsy
Veillonella
Streptococcus
Staphylococcus
Ruminococcus
Rothia
Prevotella
Prevotella
Parabacteroides
Mycoplasma
Lactobacillus
Haemophilus
Granulicatella
Fusobacterium
Blautia
Bacteroides
Allobaculum
Akkermansia
Actinomyces
Actinobacillus
(o) Streoptophyta
(o) Clostridiales
(f) S24-7
(f) Ruminococcace
(f) Neisseriaceae
(f) Lachnospiracea
(f) Gemellaceae
(f) Enterobacteriac

Figure

Click here to access/download;Figure;fig2.pdf

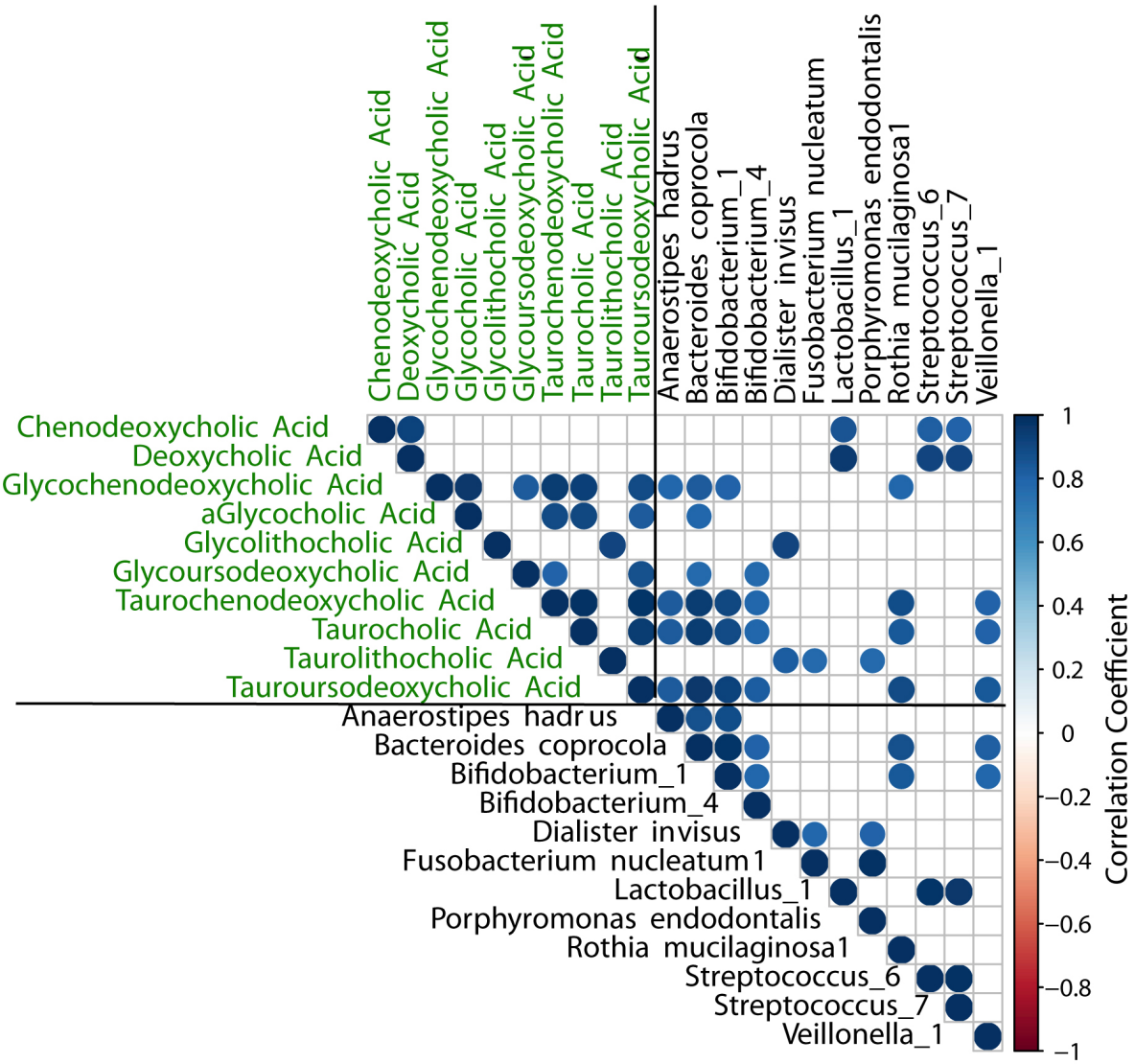


Table of Materials

Name of Material/ Equipment	Company
2 mL cryovials	Corning
96-well plates	Applied Biosystems
dNTPs	Sigma
Dry ice	
EG29-i10 endoscope	Pentax
Epoch microplate spectrophotometer	Biotek
Ethanol	Sigma Aldrich
HiSeq 2500	Illumina
IL_806r reverse primer	IDT DNA technologies
ILHS_515f forward primer	IDT DNA technologies
JumpStart Taq DNA	Sigma
Mucus specimen trap	Busse Hospital
Nanodrop Gen5 software	ThermoFisher Scientific
PCR buffer	Sigma
PCR cleanup kit	Zymo Research
Radial Jaw 4 Jumbo Forceps	Boston Scientific
Vioscreen dietary questionnaire	VioCare
ZymoBIOMICS DNA Microprep Kit	Zymo Research

Catalog Number	Comments/Description
430659	
4306737	
D7295	
	Provided by institution
N/A	Endoscope size may vary depending on patient physiology
N/A	
676829	
N/A	
custom	custom primers
custom	custom primers
D4184	
405	40 cc specimen trap with transport cap
P2192	
D4204	
M00513343	2.8mm Jaw OD
N/A	
D4300	25 ug binding capacity

Rebuttal Letter

Jaydev Upponi, Ph.D.
A Novel Method for Specimen Collection and Analysis of the Duodenal Microbiome
Journal of Visualized Experiments

Dear Dr. Upponi,

We would like to thank our reviewers and editor for the time they spent providing us with valuable feedback on our manuscript entitled, "A Novel Method for Specimen Collection and Analysis of the Duodenal Microbiome." We have gone through the comments carefully and adapted the manuscript to address your comments and concerns. Please see below an item-by-item response to the comments from our reviewers and editor:

1. Please revise the highlighting of the protocol to be under 3 pages. This is a hard production limit to ensure that videography can occur in a single day. I would suggest not highlighting step 8. If step 8 is to be filmed, we need explicit user input commands on how to perform these steps: File | Save | etc. or run xxx -x, etc. We have revised the highlighted sections to focus on specimen procurement, and the initial DNA extraction portion of the protocol.
2. References 31 and 32 and not cited in the written manuscript but are included in the References. Please revise. References 31 and 32 have been added as citations in the final paragraph of the discussion.
3. The Food Questionaire: Is this file required in the submission or will a citation suffice? If the file is included, please provide copyright permissions from Viocare. We have reviewed and believe the citation will suffice. We have removed the file from the supplemental materials.
4. Please remove the Zymo Research product manual as a citation would suffice in the written manuscript. We have removed the Zymo Research product manual from the supplemental materials and cited this protocol within the written manuscript.

We hope you find our manuscript suitable for publication and look forward to hearing from you.

Sincerely,

Shehnaz K. Hussain, PhD, ScM
Department of Medicine, Cedars-Sinai Medical Center
700 N. San Vicente Blvd, Pacific Design Center G599

West Hollywood, CA 90069
Tel./Fax: 310-429-9859
Email: shehnaz.hussain@cshs.org

Rebuttal Letter

Jaydev Upponi, Ph.D.

A Novel Method for Specimen Collection and Analysis of the Duodenal Microbiome

Journal of Visualized Experiments

Dear Dr. Upponi,

We would like to thank our reviewers and editor for the time they spent providing us with valuable feedback on our manuscript entitled, "A Novel Method for Specimen Collection and Analysis of the Duodenal Microbiome." We have gone through the comments carefully and adapted the manuscript to address your comments and concerns. Please see below an item-by-item response to the comments from our reviewers and editor:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. We have carefully proofread the manuscript to correct any spelling or grammar issues and made every effort to address your comments.

2. Please provide an email address for each author. The email address of each corresponding author has been included in the manuscript. The email addresses of the remaining authors have been updated in the JoVE editorial manager.

3. Please provide 6-12 keywords/key phrases.
We have updated our keywords to include the terms "Duodenal microbiota; liver cirrhosis; diet; upper endoscopy; bile acid; microbiome" as keyword/key phrases.

4. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. We have reviewed the protocol and expanded on areas that were previously vague to ensure that the written protocol will be easy to follow in conjunction with the video publication.

5. Given that you are performing endoscopy and collecting tissue specimens, please specify how you anesthetized your patients. Patients are anesthetized with midazolam and fentanyl. Dosage typically starts at 2mg and 50mg respectively, and is up-titrated based on the patient's tolerance of the endoscope.

7. 2.2 and 2.6: What is the gauge of the syringe needle? The blunt syringe needle used in this protocol is 18-gauge. This is not a specific requirement, as other needle sizes would perform equitably in removing the biopsy from the forceps.

8. 4.1: Please consider providing the questionnaire as supplementary material. The dietary questionnaire is an adaptive branching website, so it is impossible to provide the questionnaire as supplementary material. We have attached a PDF of the list of foods covered by this questionnaire, as well as a citation to the publication evaluating this food frequency questionnaire.

9. Please ensure that all text (e.g., 4.1 and 4.2) in the protocol section is written in the imperative tense as if telling someone how to do the technique (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." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc. We have examined the protocol and changed all verbiage to the imperative tense.

10. 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. 5.1 actually describes aspirate analysis and 6.2 elaborates points in 6.1. Please consider renumbering 5.2, 5.3, 5.4, and 5.5 as 5.1.1, 5.1.2, 5.1.3, and 5.1.4; and renumbering 6.2 as 6.1.1; and renumbering 6.4-6.8 as 6.3.1-6.3.5. We have adjusted the numbering throughout the Protocol to match this more detailed framework.

11. Given that you have highlighted 5.2 and 5.3, please provide sufficient details for filming. If you will only show the results in the video, please cite a reference for osmolality determination. We have determined that this section of the protocol is slightly outside the scope of this manuscript. Osmolality, protein and visual color grade normalization are not directly relevant to analyzing duodenal microbiota. These steps could be relevant in a future analysis of the data prepared from this protocol. We have mentioned aspirate analysis for bile acid concentrations in the discussion section as an option to consider for future investigators.

12. 5.5: To what values are the protein concentration, osmolality, and visual color grade being normalized, and how much do you dilute the samples? Upon further review by our authors, we have decided that normalization of aspirate samples is outside of the scope of this manuscript. Normalization was done to account for differences in bile acid concentrations but is unrelated to DNA extraction. Therefore, we have removed this section from the protocol.

13. 6.2: If you plan to film the DNA extraction, please provide more details. If you will only show the pellet or the DNA solution obtained by extraction, please cite a reference. A detailed protocol for DNA extraction was added.

14. 7.1: As you have highlighted this step, please provide more details for DNA cleanup to facilitate filming. We have expanded upon the DNA cleanup step to facilitate filming and better elucidate why the step is performed.

15. 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 and Reagents.
For example: NanoDrop software
We have removed all commercial language from the protocol and updated the Table of Materials and Reagents.

16. 8: Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. We have gone into more detail throughout the protocol on how to use software and perform each step of the protocol to go along with the video.

17. Fig. 2's legend mentions corrplot and Hmisc packages in R. Is this included in step 8?
The plots shown in figure 2 are meant to serve as representative figures that can result after the sample sequences are cleaned and formatted. The purpose of this protocol is to describe sample acquisition and preparation for analysis, and therefore a description of specific analyses is outside the scope of this paper.

18. As we are a methods journal, please revise the Discussion (total 3-6 paragraphs) to add the following in detail with citations:
a) Any modifications and troubleshooting of the technique

We have revised the Discussion section to include modifications for specimen procurement and storage, as well as coding packages to use to analyze sequencing data.

b) Any limitations of the technique
We have revised the Discussion section to include potential limitations to this technique and method of study, including comments from our reviewers regarding concomitant medication confounders and endoscope contamination.

19. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.
A Disclosures section has been added to the manuscript. The authors have no competing financial conflicts of interest to report.

20. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate journal names. We have corrected the reference formatting to follow the sample formatting provided.

21. Please sort the Materials Table alphabetically by the name of the material.
The Materials table has been updated and sorted alphabetically by material.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Thank you for well design study. It will contribute to make a new approach small bowel associated disorders

Major Concerns:
N/A

Minor Concerns:

In my opinion, it is also need to be cited about patients history of using bile acid sequestrane in article discussion

Thank you for pointing out this concomitant medication that could affect study results. In the case of our study, all medications for each patient in this study were reviewed and no patients were taking cholestyramine. Your point is well received, however, and we have added this as a potential confounding factor mentioned in the discussion section.

Reviewer #2:

Thank you for the opportunity to review this interesting manuscript. In this article, Dreskin RW et al. aimed to discuss a novel method to sample and analyze the duodenal microbiome. They detail a novel method to sample the proximal luminal and mucosal gut microbiome in human subjects undergoing upper GI endoscopy by obtaining duodenal aspirate and biopsies.
The article is interesting, well-written and assessment of gut microbiota is topical. It would be interesting to know:

- How the biofilm formation in Endoscopes can alter your results? Please see related publications (PMID: 23554415, PMID: 32169614, PMID: 31413166)

No issues with biofilm or contaminations of endoscopes have been detected through routine testing at our facility. We believe our facility's decontamination protocol is sufficient and adequate to remove biofilms, and do not believe this is a confounding factor. We will address this in the discussion for future investigators to consider based on the decontamination protocols at their study site.

- **How these results correlate with fecal or oral microbiota in this population?**

Literature has shown that the duodenal microbiome is more closely related to the oral microbiome than the fecal microbiome. However, these microbial communities are nevertheless significantly different, and difficult to correlate. We have added mention of this in the Discussion section for readers to consider as they analyze duodenal microbiome results.

We hope you find our manuscript suitable for publication and look forward to hearing from you.

Sincerely,

Shehnaz K. Hussain, PhD, ScM
Department of Medicine, Cedars-Sinai Medical Center
700 N. San Vicente Blvd, Pacific Design Center G599
West Hollywood, CA 90069
Tel./Fax: 310-429-9859
Email: shehnaz.hussain@cshs.org