Video Article

A Method to Define the Effects of Environmental Enrichment on Colon Microbiome Biodiversity in a Mouse Colon Tumor Model

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URL: https://www.jove.com/video/57182
DOI: doi:10.3791/57182

Keywords: Cancer Research, Issue 132, Mind-Body Medicine, Environmental Enrichment, Colon Cancer, Microbiota, Stool, Wound Repair

Date Published: 2/28/2018

Citation: Fuller, A.K., Bice, B.D., Venancio, A.R., Crowley, O.M., Staab, A.M., Georges, S.J., Hidalgo, J.R., Warncke, A.V., Angus-Hill, M.L. A Method to Define the Effects of Environmental Enrichment on Colon Microbiome Biodiversity in a Mouse Colon Tumor Model. *J. Vis. Exp.* (132), e57182, doi:10.3791/57182 (2018).

Abstract

Several recent studies have illustrated the beneficial effects of living in an enriched environment on improving human disease. In mice, environmental enrichment (EE) reduces tumorigenesis by activating the mouse immune system, or affects tumor bearing animal survival by stimulating the wound repair response, including improved microbiome diversity, in the tumor microenvironment. Provided here is a detailed procedure to assess the effects of environmental enrichment on the biodiversity of the microbiome in a mouse colon tumor model. Precautions regarding animal breeding and considerations for animal genotype and mouse colony integration are described, all of which ultimately affect microbial biodiversity. Heeding these precautions may allow more uniform microbiome transmission, and consequently will alleviate non-treatment dependent effects that can confound study findings. Further, in this procedure, microbiota changes are characterized using 16S rDNA sequencing of DNA isolated from stool collected from the distal colon following long-term environmental enrichment. Gut microbiota imbalance is associated with the pathogenesis of inflammatory bowel disease and colon cancer, but also of obesity and diabetes among others. Importantly, this protocol for EE and microbiome analysis can be utilized to study the role of microbiome pathogenesis across a variety of diseases where robust mouse models exist that can recapitulate human disease.

Video Link

The video component of this article can be found at https://www.jove.com/video/57182/

Introduction

Environmental enrichment (EE) studies utilize complex housing parameters to affect social stimulation (large housing cages, larger groups of animals), cognitive stimulation (huts, tunnels, nesting materials, platforms) and physical activity (running wheels). EE has been utilized by many labs to understand the effects of increased activity and improved social and cognitive interactions on disease initiation and progression using a wide array of mouse models, including barbering induced alopecia, Alzheimer's disease, Rett syndrome, and several tumor and digestive disease models[1,2,3,4,5,6].

Several mouse models have been developed to study colon tumorigenesis in mice. Perhaps the most well-defined model is the *Apc*^{Min} mouse. The *Apc*^{Min} mouse was developed in the laboratory of William Dove in 1990[7], and has been used as a mouse model of mutations in the *APC* gene that are commonly associated with human colorectal cancer. In contrast to humans harboring *APC* mutations, *Apc*^{Min} mice primarily develop small intestinal tumors, with very rare occurrence of colon tumors. However, a *Tcf4*^{Het} allele with a single knockin-knockout heterozygous mutation in *Tcf4*, vastly increases colon tumorigenesis when combined with the *Apc*^{Min} allele[8]. Recently, this mouse model of colon tumorigenesis has been used to determine the effects of EE on colon tumorigenesis[6]. In the Bice *et al*. study, the physiological and phenotypic effects of EE on males and females of four different mouse lines (*wild-type* (*WT*), *Tcf4*$^{Het/+}$ *Apc*$^{+/+}$, *Tcf4*$^{+/+}$ *Apc*$^{Min/+}$, and *Tcf4*$^{Het/+}$*Apc*$^{Min/+}$)) were defined. Perhaps the most interesting finding was that EE significantly increases the lifespan of both male and female colon tumor-bearing animals. This demonstrated that EE may reduce at least some of the symptoms associated with colon tumorigenesis, and improve animal health. Remarkably, this improved lifespan in males is not a direct result of reduced tumorigenesis, and instead was linked to the initiation of a tumor wound healing response, including improved microbiome biodiversity[6].

Several EE specific studies have been published with interesting results. However, from a technical standpoint, important results are often not translatable to other laboratories. Maintaining identical EE methodologies between different laboratories is an incredibly complex issue, not only due to enrichment devices and housing used, but also bedding, food, ventilation, breeding, genetics, activity in the room, and animal protocol requirements, among others[9,10,11]. One example is animal integration, where animals must be stably integrated into the mouse colony, therefore normalizing genetic background and diet composition, to avoid non-treatment related effects. Further, many EE studies have been completed

prior to the realization of the importance of the microbiome in disease, and the way that common mouse husbandry practices can affect the composition of the gut microbiome[10,12].

Breeding strategy and animal placement in EE can increase stress if not performed properly. Since EE studies utilize large numbers of both male and female animals and multiple genotypes, experimental setup can be difficult given the requirement for animals from several litters to be combined. Therefore, a breeding and weaning strategy was developed to allow for combining of weaned animals of the correct genotype from different litters. The primary rationale for this was to normalize the microbiota among litters and to reduce stress when animals were moved to the experimental environment. The microbiome was transmitted from the dam[10]. To provide microbial diversity to the colony, females were purchased from Jackson Labs and integrated into the colony for one month before the experiment began[9,10,12]. To further normalize microbiome biodiversity between animals, females were co-housed prior to breeding. Following breeding, communal housing during rearing and the ability to escape nursing pups improved the stress levels of maternal care[13,14], possibly furthering microbiome normalization. To prevent non-EE related effects on the microbiome, this communal housing of all experimental animals prevented fighting and additional stress that occurred when combining several males from different litters into one experimental cage. Finally, equal numbers of animals of all genotypes were included in the cages. This provided the opportunity for improved microbiota biodiversity across genotypes, and removed the contribution of coprophagia (the animal's tendency to consume stool) or possible genotype-specific behavioral differences to the overall study.

This protocol provides a strategy that expands previous EE studies to include known aspects of microbiome research, including microbiota transmission and animal colony integration for microbiota normalization, to enable more uniform microbiome populations between experimental animals. Heeding these precautions is essential due to the ability of non-treatment related microbiota differences to confound study findings. Eliminating non-EE related microbiota changes will enable researchers to specifically define the role of EE on microbiota composition during disease development and progression.

Protocol

All methods described here were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Utah.

1. Experimental Design and EE and Control Cage Setup

Note: For reference, an outline of the experimental design is illustrated (**Figure 1**).

1. **Set up control (NE) and EE cages (Figure 2).**
    1. To set up NE cages, use autoclaved conventional control cages (**Table 1**) that lack enrichment devices.
    2. For large cages, drill one hole per cage that is large enough to accommodate a grommet and tunnel (**Table of Materials**).
    3. To set up pup rearing and EE cages, connect two large autoclaved cages with a sterilized grommet secured tunnel and 2 sterilized platforms to increase floor space (**Table 1**). For EE experiments, provide sterilized running wheels, tunnels, igloos, huts, crawl balls, and nesting material within the EE cages.
    4. Place both EE and NE cages in a ventilated rack to provide equal ventilation.
    Note: Two large cages with 2 platforms (**Table 1**) allow for a maximum of 12 pregnant females per pup rearing setup[15] (see **Table of Materials**, step 3.2 in document, and **Table 2**).
    5. Feed mice *ad libitum* irradiated standard chow and autoclaved reverse osmosis water.
    6. Provide mice with sterile bedding materials.
    Note: All manipulations of empty cages and cages with animals must be done in the hood to prevent contamination. For cage manipulation of large cages, cover the hole in the cage with an adhesive film.

2. Prepare animals for breeding. Group house 15 2-month old females in a single large cage (**Table 1**) for 2 weeks prior to mating. Separately house 2-month old littermate males for 2 weeks prior to mating.
Note: The number of females for breeding is dependent on the experiment and the number of animals required. In this study, 15 females were utilized to obtain 12 plugged females, which is the maximum number allowed in the pup rearing setup described in 1.1.2 (**Table 2**).
3. For breeding, combine sire and dam animals, 1 male per 2 females. The first check in the morning should be for vaginal plugs, which may be a visual sign that the animals have mated during the night.
Note: House males and females together until each female has plugged. A vaginal plug can be identified visually, if it is external.
    1. To detect internalized plugs, a probe is inserted into the vaginal opening and if the vaginal plug is present, the probe is not easily inserted ([16]; see section 4.3.6).
    Note: Record the morning a plug is detected as ½ day, since the mating occurred in the night.
    2. Once females have vaginal plugs, transfer them to a large pup rearing cage for group housing with other mated females (Setup in step 1.1.2 without enrichment devices).
    3. Replace mated females with new unmated group housed females for mating and continue mating to obtain a maximum number of litters in a 7-day period.
    Note: All animals must have a delivery date within 7 days of each other.

4. Allow pregnant females to give birth in group housing so that all litters are raised within one large pup rearing cage (Setup in step 1.1.2 without enrichment devices).
    1. Keep track of pup numbers and birthdates, and begin genotyping pups at 7 days of age.
        1. Tattoo pups on their toes at 7 days of age with a number code to identify them[13,17].
        2. Clean the toe with 70% ethanol and gently insert a micro-tattoo device containing ink into the skin surface parallel to the toe[17] (see **Table of Materials**).
        3. Collect tissue with scissors for genotyping from the tail tips by incising a small piece of tissue from 7-day old neonates.

2. Isolate genomic DNA from tissue and perform PCR using a conventional HotSHOT method as described in [18].
3. Separate animals by sex at 14-21 days into large cages with mothers.
   Note: Ensure that the older pups are able to feed on their own, and that younger pups continue to nurse until old enough to feed on their own.
4. At 21-28 days, distribute male and female animals separately by genotype into NE or EE environments, making sure to keep equal proportions of each genotype per cage (**Figure 2A**).
   Note: Ensure that the total number of animals allowed in each NE or EE cage is based on the maximum number allowed by IACUC (**Table 2**). The NE cages have at most 5 animals (**Table 2**). In EE cages, for social stimulation, no fewer than 20, and with space restrictions, no more than 41 animals should be allowed in the EE cage (**Table 2**).

2. Stool Collection at 16 Weeks of Age

1. Begin stool collection 1 to 2 days prior to sacrifice, and separately collect stool on the day of sacrifice during dissection using sterile tools.
   Note: Collecting stool at the same time 1-2 days prior to collection may help to avoid the loss of a sample due to the possibility that no stool is present at the time of collection.
   1. To collect stool from live animals, carefully scruff the animal over a clean cage. Collect stool using sterile forceps into a sterile microfuge tube.
      Note: Animals will typically eliminate stool when immobilized, which allows for rapid stool collection directly into a sterile microfuge tube. If an animal does not immediately defecate when immobilized, place it into a clean cage and wait for the animal to defecate (typically up to 1 h).
   2. Collect stool on the day of sacrifice.
      1. For euthanizing the animal, place the animal in a bell jar containing a small container with a cotton ball soaked in isoflurane. Once cessation of breathing is observed (usually after 2 min), lay the animal on its back to allow colon dissection.
      2. Apply 70% ethanol to the mouse abdomen.
      3. Lift the skin anterior to the urethral opening with forceps, and use scissors to cut along the ventral midline until reaching the ribcage, and cut from the base of the first incision towards each leg. Fold back the skin and use scissors to cut through the peritoneal wall in the same pattern.
      4. Use forceps to grasp the distal colon at the anus to dissect and detach the distal colon from the rectum. While pulling the colon vertically with forceps, use scissors to cut through the mesentery to release the colon.
      5. Cut the colon just below the cecum and lay it on filter paper. Use forceps to lift the top of the colon tube, opening the lumen to allow one side of open scissors to be inserted. Cut longitudinally, distal to proximal, and splay open the colon lengthwise.
      6. Collect stool from the distal colon into a sterile microfuge tube using sterile forceps.

2. Store stool in a microfuge tube at -80 ˚C until time of bacterial DNA isolation.
   Note: On the day of sacrifice, in addition to the stool, collect other samples such as whole blood, serum, plasma, normal and tumor tissue from colon and small intestine, microsomes, adipose tissue, *etc*. to address defined questions in the study.

3. Genomic DNA Isolation from Stool

Note: Utilize a commercial kit to isolate microbial DNA from stool following a stool pathogen detection protocol. Remove samples directly for the -80 ˚C freezer and store on dry ice while weighing.

1. Transfer up to 220 mg of stool to a clean microfuge tube containing 1.4 mL of room temperature (RT) stool lysis buffer (see **Table of Materials**).
2. Vortex sample for 1 min to thoroughly homogenize solids (**Figure 2B**). Heat the suspension to 95 ˚C for 5 min to lyse all bacteria (including Gram-positive bacteria).
3. Vortex samples for 15 s and then centrifuge at 20,000 x g for 1 min to pellet the stool solids. Transfer supernatant to a 2-mL microfuge tube. Add one tablet to each sample to absorb PCR inhibitors, vortex until the tablet is dissolved, and incubate the sample at RT for 1 min.
4. Centrifuge the sample at 20,000 x g for 3 min and transfer the supernatant to a new microfuge tube. Centrifuge at 20,000 x g for 3 min. Aliquot 15 µL of proteinase K (20 mg/mL stock) into a new 1.5 mL microfuge tube. Pipette 200 µL of the sample into the tube containing proteinase K.
5. Add 200 µL of guanidinium chloride lysis buffer to the tube, vortex thoroughly for 15 s (see **Table of Materials**) and incubate the sample at 70 ˚C for 10 min. Add 200 µL of ethanol (96-100%) to the tubes and mix well by vortexing.
6. Place a silica based spin column in a 2-mL collection tube and apply the samples to the column. Close the lid and centrifuge for 1 min at 20,000 x g.
7. Transfer the column to a new 2 mL collection tube and add 500 µL of wash buffer 1 to the column, cap the column and centrifuge for 1 min at 20,000 x g. Transfer the column to a new 2 mL collection tube and add 500 µL of wash buffer 2 to the column, close the cap and centrifuge at 20,000 x g for 3 min.
8. With the cap closed, transfer the column to a new 2 mL collection tube, and centrifuge for an additional 1 min at 20,000 x g to remove residual wash buffer. Transfer the column to a 1.5 mL labeled microfuge tube and elute sample by adding 200 µL of elution buffer containing EDTA to the membrane (see **Table of Materials**).
9. Close the cap and incubate at RT for 1 min. Centrifuge the sample for 1 min at 20,000 x g. Discard the column.

4. DNA Concentration Determination and Sample Preparation for PCR

Note: Utilize a fluorometer and a commercially available dsDNA fluorescent assay to determine genomic DNA concentration in each sample (see **Table of Materials**). The fluorescent dye must bind double stranded DNA specifically.

1. Prepare a 1:200 dilution of each sample (1 µL of each sample in 199 µL dsDNA master mix) and a 1:50 dilution of standards. Analyze on a fluorometer using the dsDNA setting.
   Note: A high volume of DNA in PCR can be inhibitory, therefore, the volume of DNA used must not be more than 10% of the final volume of the PCR. A fluorometer enables accurate measurement of DNA in the sample, as only DNA bound to the fluorescent dye will fluoresce, eliminating the possible contribution of contaminants to the final calculated DNA concentration. This level of accurate quantitation is essential for the downstream sequencing application.
2. Prepare PCR templates diluted to 5 ng/µL with the appropriate volume of 10 mM Tris, pH 8.5 to make working template stocks of each sample.
3. Store samples at -20 °C.

5. Design Primers to the 16S Desired V Regions

1. Design primers to selectively amplify the desired V 16S rRNA regions.
2. Analyze primers with Probe Match, from the Ribosomal Database Project[19], to determine the approximate hit rate for various phyla.
   Note: For V1-V3 regions, the current study used published primers Bosshard forward[20], which bind at position 8 within the V1 region, and 533 reverse[21], which binds at position 533 within the V3 region. Primers must include overhang adapter sequences for indexing.
3. When designing primers, include adapter sequences at the 5' ends of each primer, as recommended for 16S metagenomics sequencing library preparation[22] (**Table 3**).
4. Synthesize these large primers with cartridge purification. Reconstitute desiccated primers and dilute a PCR working stock to 1 µM in 10 mM Tris, pH 8.5.

6. Amplicon PCR to Amplify the V Region(s) with Overhang Adapter Sequences Attached [22]

1. Set up the amplicon PCR reaction mix as described in **Table 4**.
2. Place an adhesive clear PCR plate seal on the plate and run the amplicon PCR using the parameters in **Table 5**.
3. (Optional) Run amplicon PCR products on an agarose gel or a high sensitivity DNA assay that enables quantitative measurement of amplicon size (see **Table of Materials**).
   Note: The amplicon size from this study is 550 bp (**Figure 3A**).

7. PCR Cleanup Using Magnetic Beads [22]

1. Centrifuge the amplicon PCR plate quickly at 1,000 x g for 1 min to collect condensation.
   Note: PCR tube strips can be used instead of PCR plates to minimize contamination. Discard tube lids and never reuse.
2. Vortex the magnetic beads to evenly disperse them, and add 20 µL of magnetic beads to each amplicon PCR well, then pipette the entire volume up and down slowly 10 times.
3. Incubate at RT for 5 min. Place the PCR plate on a magnetic stand for 2 min until magnetic beads are collected and the supernatant is clear. Remove and discard the supernatant.
4. Wash beads with 200 µL fresh 80% ethanol while the PCR plate is on the magnetic stand and incubate for 30 s at RT on the magnetic stand. Carefully remove the supernatant.
5. Repeat the wash for a second time. Now, use a fine pipette tip to remove any residual ethanol from the wells and allow air drying for 10 min.
6. Remove the PCR plate from the magnetic stand and add 52.5 µL of 10 mM Tris pH 8.5 to each well. Pipette up and down 10 times to suspend beads and incubate at room temperature for 2 min.
7. Transfer the PCR plate to the magnetic stand to collect magnetic beads and transfer 50 µL of the supernatant to a clean PCR plate. Place an adhesive clear PCR plate seal on the plate and store at -20 ˚C for up to one week.

8. Preparation of a Plate Scheme for Index PCR

Note: To generate a V1-V3 library, a second PCR was performed with an index kit (see **Table of Materials**). A default indexing scheme was used to map out unique dual index combinations for each sample (**Figure 3B** and [23]).

1. Ensure that each sample has a unique combination of 2 index primers (*i.e.*, dual indexing).

9. Perform Index PCR to Attach Barcodes to the Adaptor Sequences as Described [22] .

1. Transfer 2.5 µL of PCR amplicons (clean amplicons) to a new 96 well plate and place in an index plate fixture to aid in indexing.
2. Arrange the index 1 and index 2 primers as in the example of the prepared plate graphic (**Figure 3B**).
   Note: Visual cues are provided to avoid primer mix-ups: index 2 primer tubes should have white caps and clear solution, while index 1 primer tubes should have orange caps and yellow solution.
3. Assemble the index PCR Mix reaction as described in **Table 6**. Mix by pipetting up and down 10 times.Cover with an adhesive clear PCR plate seal and centrifuge to collect at 1,000 x g at room temperature for 1 min.

4. Run the index PCR using the parameters in **Table 7**.

10. Purify Final PCR Library

Note: This PCR clean-up is identical to step 7 above, and uses magnetic beads to perform PCR Clean-Up of the index PCR[22].

1. Centrifuge the PCR plate from step 10 quickly at 1,000 x g for 1 min to collect condensation.
2. Vortex the magnetic beads to evenly disperse them, then add 20 µL of magnetic beads to each amplicon PCR well, then pipette the entire volume up and down slowly 10 times to mix.
3. Incubate at RT for 5 min.
4. Place PCR plate on a magnetic stand for 2 min until magnetic beads are collected and supernatant is clear. Remove and discard the supernatant.
5. Wash beads with 200 µL fresh 80% ethanol while the PCR plate is on the magnetic stand and incubate for 30 s at room temperature on the magnetic stand. Carefully remove the supernatant.
6. Repeat the wash for a second time.
7. Following the second wash, use a fine pipette tip to remove any residual ethanol from the wells and allow air drying for 10 min.
8. Remove the PCR plate from the magnetic stand and add 52.5 µL of 10 mM Tris pH 8.5 to each well. Pipette up and down 10 times to suspend beads and incubate at room temperature for 2 min.
9. Transfer the PCR plate to the magnetic stand to collect magnetic beads and transfer 50 µL of the supernatant to a clean PCR plate. Place an adhesive clear PCR plate seal on the plate and store at -20 ˚C for up to one week.
10. (Optional) Run index PCR products on an agarose gel or a high sensitivity DNA assay that enables quantitative measurement of amplicon size (see **Table of Materials**).
    Note: The final indexed library size from this study was 668 bp (**Figure 3C-D**).

11. Quantify, Normalize, and Pool the Indexed Libraries for Sequencing

1. Determine the DNA concentration of each sample with a fluorometer and a dsDNA fluorescent assay kit (see Table of Materials).
    1. Prepare a 1:200 dilution of the sample (1 µL of each sample in 199 µL dsDNA master mix, which includes buffer and reagent) for each of the indexed samples and standards (190 µL dsDNA master mix and 10 µL of standard). Analyze on a fluorometer using the dsDNA setting.

2. Following the DNA concentration calculation, normalize the libraries by calculating the average library size. Do this by summing adapter lengths, index lengths, and V amplicon size from primers, and view products by agarose gel to be certain the actual size is similar to the calculated size (**Figure 3C**, see [22]).
    1. Alternatively, utilize a high sensitivity DNA assay that enables quantitative measurement of DNA integrity, amplicon size, and concentration (Table of Materials, **Figure 3D**).
       Note: In this study, the average library size was calculated based on summing adapter lengths, index lengths, and V1-V3 amplicon size from primers. The average size was 668 bp.
    2. Concentrations of samples are normalized using the formula in **Table 8**.

3. Dilute samples to 4 nM and pool 5 µL from each 4-nM sample into a single tube for sequencing.

12. Sequence the Library using a Next Generation Sequencing System and Parse the Data

1. Sequence the library.
   Note: For this study, the University of Utah High Throughput Genomics Core performed library denaturation and sample sequencing (as described in [6,22]).
2. Parse the data.
   Note: To separate data from pooled samples, index reads were identified and separated (as described in [22]).
3. Generate FASTQ files and utilize this for subsequent data analysis.

13. Analyze Sequenced Data from the 16S Amplicon Library

Note: This step is performed as described in Bice *et al*., 2017[6].

1. Install freely available data analysis tools (see **Table of Materials**;[24]).
2. Assemble demultiplexed fastq files from the sequenced data (as described in[25,26]). Discard all unassembled sequences.
3. Perform analyses following a *de novo* open taxonomic unit (OTU) picking protocol (as described in [27]).
    1. Bin sequences into a single fastq file by sampleID and group sequences with 97% or greater similarity into OTUs, as described in [28]. Align representative sequences of core set with minimum sequence length of 150 and 75% percent identity[29,30]. Assign taxonomy as described[28].
       Note: Samples can be binned and analyzed[31], followed by taxonomic assignment and OTU table construction[32].
    2. Create a mapping file that identifies descriptive names and characteristics of samples to link to sample identification and validate the mapping file[33,34].
    3. Make an OTU network that links OTUs to sample descriptions using a mapping file[35].
    4. Calculate taxonomy summaries in terms of relative abundance by summarizing taxa through plots[36].

5. Explore alpha diversity of samples at uniform sequencing depth appropriate to samples. To define the appropriate depth for alpha diversity, summarize total counts observed in each sample by using the biome summarize-table command, as described in [37].

Representative Results

Several studies have demonstrated that the practice of mind-body medicine improves health outcomes. Similarly, in mice, environmental enrichment improves outcomes including improved lifespan and tumor wound repair[6]. Therefore, an EE procedure was developed with the aim of defining the role of microbiota in this phenotype while first normalizing the microbiome prior to the initiation of the experiment (**Figure 1**). Importantly, all breeding animals are integrated into the mouse colony for at least one month prior to the commencement of breeding, and newborn pups are co-housed with mothers in one large cage to normalize microbiota transmission prior to the experiment. When animals are between 21 and 28 days old, equal numbers of each genotype are weaned into their respective housing, either EE or NE environments (**Figure 2A**). At 16 weeks, stool from all animals is collected and homogenized (**Figure 2B**), followed by bacterial DNA isolation. Finally, 16S amplicons are amplified from stool microbiome DNA and barcode indexed to allow for sequencing of all microbiome libraries simultaneously (**Figure 3**). The unique sequences identified in WT and tumor bearing animals in both NE and EE conditions are shown in **Figure 4**. Interestingly, EE does not improve biodiversity in WT animals, but vastly increases biodiversity in tumor bearing animals (**Figure 4**), demonstrating that this method allows biodiversity improvements. This increase in biodiversity can be attributed to the increased presence of the phylum Proteobacteria, with significant increases in the classes Alphaproteobacteria and Betaproteobacteria, and decreases in pathogenic Gammaproteobacteria (**Figure 5**; **Supplemental Table 1**). The largest increase is in the Betaproteobacteria class is the genus *Sutterella*, a likely commensal involved in secreted IgA degradation (**Figure 6**, also see[38]).

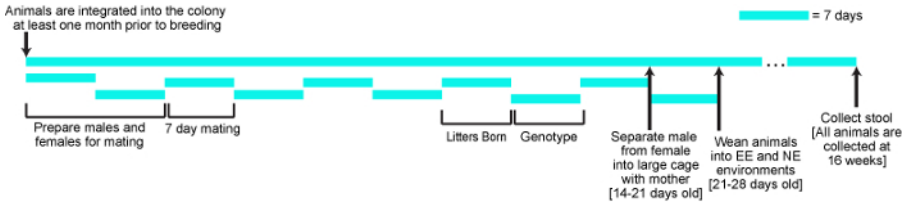

Figure 1: A representation of the experimental timeline. The short bands represent 7-day windows, as most of the protocol is accomplished in 7-day increments. This also aids in visualization of the range of pup ages across the experiment. Please click here to view a larger version of this figure.

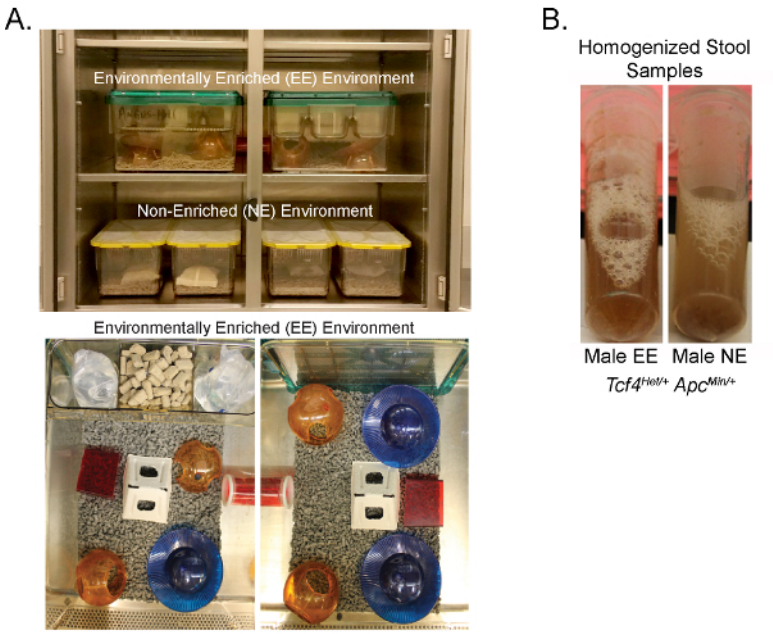

Figure 2: EE and NE housing conditions and stool homogenates (as described in the protocol). Please click here to view a larger version of this figure.

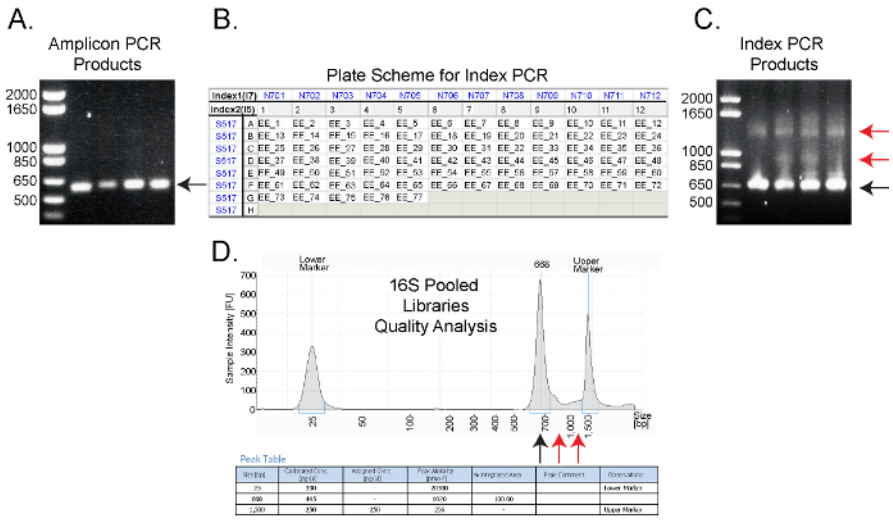

Figure 3: 16S Microbiome Library Preparation. (**A**) Unpurified PCR amplicon products derived from stool genomic DNA.(**B**) Indexing plate graphic designed as per the software used (**Table of Materials**, [23]). The dual index combinations, I7 (Index 1; Row) and I5 (Index 2; Column), are shown for each sample. Each index is 8 bp in length. The sample numbers refer to the respective mouse numbers from EE studies. (**C**) Unpurified Index PCR products. (**D**) Quality analysis of final purified and pooled 16S libraries. (**A,C,D**) Black arrows denote 550 bp amplicon and 668 bp indexed library. Red arrows denote non-specific products that are eliminated following purification as shown in **D.** Upper and Lower markers are size markers added to the sample for size reference. Please click here to view a larger version of this figure.

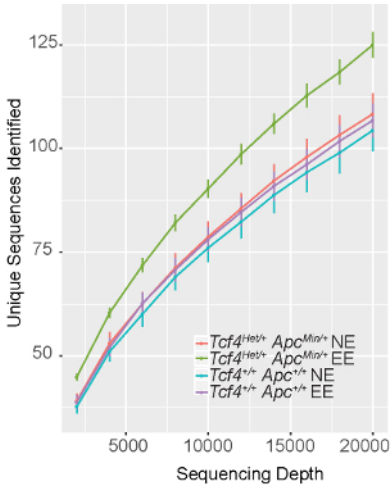

Figure 4: Changes in alpha diversity following EE of *Tcf4*$^{Het/+}$ *Apc*$^{Min/+}$ animals. Alpha diversity of WT and *Tcf4*$^{Het/+}$ *Apc*$^{Min/+}$ tumor bearing animals. At 20,000 reads, NE and EE of WT, p=0.64 and NE and EE of *Tcf4*$^{Het/+}$ *Apc*$^{Min/+}$, p=0.03 using two-sample t-test with Welch correction. Adapted from Bice *et al*., 2017[6]. Please click here to view a larger version of this figure.

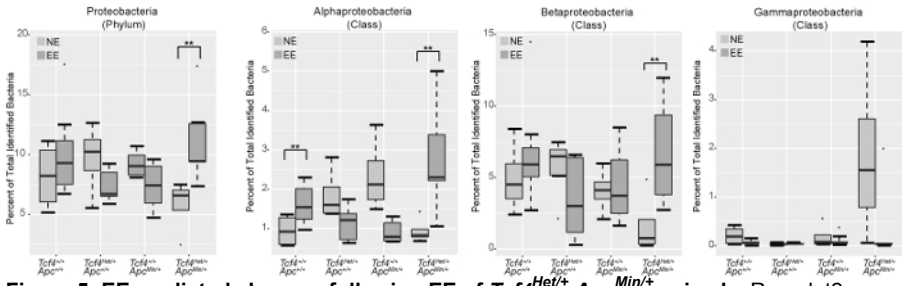

Figure 5: EE-mediated changes following EE of *Tcf4*$^{Het/+}$ *Apc*$^{Min/+}$ animals. R-ggplot2 generated box-whisker plots denoting changes in abundance of the phylum Proteobacteria and classes Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria. Outliers are noted as circles. **p=0.005 using two-sample t-tests with Welch correction. Error bars calculated using standard error of the mean (SEM). Adapted from Bice *et al*., 2017[6]. Please click here to view a larger version of this figure.

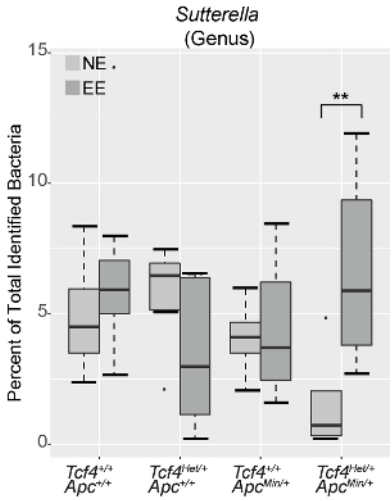

Figure 6: Changes in the relative abundance of *Sutterella* following EE of *Tcf4*$^{Het/+}$ *Apc*$^{Min/+}$ animals. Outliers are noted as circles. **p=0.005 using two-sample t-test with Welch correction. Error bars calculated using SEM. Adapted from Bice *et al*., 2017[6]. Please click here to view a larger version of this figure.

Microbe	M_WT_NE_ Mean	M_WT_NE _SEM	M_WT_N E_N	M_WT_EE_ Mean	M_WT_EE_S EM
Unassigned.Other	0.03289083	0.004809	4	0.02939822	0.00165872
k__Bacteria.Other	0.00010254	4.42E-05	4	6.80E-05	1.57E-05
k__Bacteria.p__Acidobacteria	0	0	4	0	0
k__Bacteria.p__Actinobacteria	0.00060454	0.000133	4	0.00086532	0.00029267
k__Bacteria.p__Bacteroidetes	0.6647298	0.043714	4	0.61770466	0.02522282
k__Bacteria.p__Cyanobacteria	0.00013369	4.28E-05	4	0.00225655	0.00131837
k__Bacteria.p__Deferribacteres	0.00933165	0.005574	4	0.01073413	0.00277712
k__Bacteria.p__Firmicutes	0.20133302	0.027981	4	0.22954772	0.03353101
k__Bacteria.p__OD1	2.47E-05	1.43E-05	4	1.30E-05	9.68E-06
k__Bacteria.p__Proteobacteria	0.08190355	0.014303	4	0.10123903	0.01439593
k__Bacteria.p__TM7	0.00408042	0.00174	4	0.00360984	0.0009309
k__Bacteria.p__Tenericutes	0.0048653	0.001939	4	0.00456358	0.00130957

Supplemental Table 1: Classification of Bacteria Isolated from Stool Collected from NE and EE mice. Classification across genotypes at the (**A**) Phylum, (**B**) Class, (**C**) Order, (**D**) Family, and (**E**) Genus level. Comparisons of NE and EE of the same genotype or WT to *Tcf4*$^{Het/+}$ *Apc*$^{Min/+}$. P-values are calculated using a two-sample t-test with Welch correction. Adapted from Bice *et al*., 2017[6]. Please click here to download this file.

Item	Total Area (inch^2)	Total Area (cm^2)	Cage Size (inch) L x W x H	Cage Size (cm) L x W x H
One Control Cage (NE)	68.25	440.32	10.5 x 6.5 x 5.5	26.67 x 16.51 x 13.97
One Large Cage (EE)	264.36	1706.32	13.87 x 19.06 x 7.75	35.24 x 48.42 x 19.69
Two Large Cages (EE)	528.72	3412.64	2@ 13.87 x 19.06 x 7.75	2@ 35.24 x 48.42 x 19.69
Two Platforms (EE)	93	600	2@ 11.8 x 3.94 x 2.95	2@ 30 x 10 x 7.5
Two Large Cages with Two Platforms (EE)	621.72	4013	2@ 13.87 x 19.06 x 7.75 + 2@ 11.8 x 3.94 x 2.95	2@ 35.24 x 48.42 x 19.69 + 2@ 30 x 10 x 7.5

Table 1: EE and NE Cage sizes and Floor Space.

Animals Allowed in Cage	Required Inches Squared Per Animal	EE Cage Area (Inches^2)	Total Animals allowed
Up to 25	12	622 in^2 (4013 cm^2)	up to 25
25+	15	622 in^2 (4013 cm^2)	up to 41
Female with Litter	51	622 in^2 (4013 cm^2)	up to 12

Table 2: Allowed Numbers of Animals in Cages Based on Floor Space[15].

Amplicon PCR Primers	
Forward	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**agagtttgatcMtggctcag**-3'
Reverse	5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TTACCGCGGCTGCTGGCAC** -3'
Locus specific sequences are shown in bold and the non-bold is the overhang adapter sequence.	

Table 3: **Amplicon PCR Primers.**

Amplicon PCR reaction set up	
	Volume
Microbial DNA (5ng/µl)	2.5 µl
Forward Primer (1 µM; from step 5.1.2)	5.0 µl
Reverse Primer (1 µM; from step 5.1.2)	5.0 µl
2X HotStart Ready Mix	12.5 µl
Total	25.0 µl

Table 4: **Amplicon PCR Mix.**

Amplicon PCR set up	
95 °C for 3 minutes	
25 Cycles of:	
	95 °C for 30 seconds
	55 °C for 30 seconds
	72 °C for 60 seconds
72 °C for 3 minutes	
Hold at 4 °C	

Table 5: Amplicon PCR Program Set Up.

Index PCR barcode assembly	
DNA	2.5 µl
Index Primer 1 (N7XX)	2.5 µl
Index Primer 2 (S5XX)	2.5 µl
2X HotStart Ready Mix	12.5 µl
PCR grade water	5 µl
Total	25 µl

Table 6: **Index PCR Mix.**

Index PCR Setup	
95 °C for 3 minutes	
8 cycles of:	
	95 °C for 30 seconds
	55 °C for 30 seconds
	72 °C for 30 seconds
72 °C for 5 minutes	
Hold at 4 °C	
Store at -20 °C	

Table 7: **Index PCR Program Set Up.**

Sample Concentration Formula for Pooling		
(DNA concentration in ng/µl) (660 g/mol x average library size)	x 106	= Concentration in nM
An example from this study is:		
(85.2 ng/µl) (660 g/ mol x 668 bp)	x	10^6 = 193.3 nM

Table 8: **Formula for Normalizing Before Pooling Samples**

Discussion

This procedure allows for the analysis of microbiota isolated from stool following environmental enrichment of normal or tumor bearing animals. Because these are large experiments which involve breeding to obtain many animals of different sexes and genotypes, normalizing the microbiome between animals prior to commencement of the experiment is essential to avoid non-EE related effects on microbiome biodiversity.

For consistency between NE and EE conditions, the breeding process is conducted to ensure that all mice initially have exposure to the same microbes and, therefore, are expected to have similar microbiome contents. It is possible, and likely, that mouse genotype affects microbiome composition. For this reason, mouse numbers per genotype are maintained between NE and EE conditions to be certain that any animal that is consuming stool will encounter a similar diversity of the microbiome.

Several difficulties are apparent when designing EE experiments. First, the total number of animals needed for the experiments is dependent upon the experimental details, but the total number is also limited by the number of animals allowed in the cage. For example, historical data surrounding mouse survival in a preliminary EE experiment were used to calculate the number of animals to define the mechanism underlying improved survival observed in previous experiments. From this data, a total of 17 animals in the comparison group were required for an 80% power to detect a difference in survival in a two-sided t-test where alpha=0.05. So, 4-5 animals in the control group vs. 20-24 (or up to 41) animals per cage in the experimental group must accommodate a power calculation. Therefore, several control group cages are needed along with the experimental cage. Further, with complex genotypes, it is difficult to obtain sufficient animal numbers of every genotype, which necessitates the large numbers of breeding females required. However, with other models where fewer transgenic differences are present, more animals of the same genotype can be analyzed in this system and fewer breeders are necessary. In the United States, 12 pregnant females can be housed in the 633 in^2 of space (**Table 2**). The issue with this is that as pups get older, they take up more space. Given that male pups are separated from female pups at 14-21 days, an approved exception to the space rule in certain countries may be feasible. Otherwise, male and female pups can be genotyped and selected, and then separated at a younger age with mothers to stay below the maximum mouse numbers. It is essential to gain approval for these studies and to adhere to local rules on space restrictions. Finally, with microbiota, the number of animals needed to detect even small differences in microbiota composition is difficult to calculate *a priori*. While in this study, significant differences in the microbiota were found with 4 animals per group, it is possible that increasing that number of animals would reveal microbiota that are more variable between EE mice or are only slightly altered by EE.

This method article describes the particular equipment and bedding that are used, which on the surface may not appear essential. However, several non-obvious issues that affect consistency can be encountered and need to be addressed prior to embarking on these very large, expensive, and time-consuming studies. One major issue is cage ventilation. With large numbers of animals in a cage, ventilation becomes an issue, and is an issue that most researchers do not take into account when attempting to provide consistent environments between control and experimental cages. All cages in the described setup are placed in a ventilated cabinet to equalize ventilation across the experimental and control cages. This cannot be accomplished when using cages that do not fit in a ventilated cabinet. Other means to normalize ventilation between experimental and control animals could be tested and consistently applied, but these methods are not explored in this study. Similar consistency issues arise with bedding. In the colon cancer model system used in this study, animals that have digestive disease will ingest certain types of bedding, especially corn cob bedding, leading to digestive blockages and illness. It is important to keep this in mind if the animals are known to ingest bedding when not otherwise occupied, as in the control environment. This phenomenon and the subsequent inconsistent health effects will profoundly affect all data.

The ribosomal 16S gene has been used as a means to study bacterial populations. It contains nine regions that express genetic variability, V1-V9, and interspersed conserved regions that remain relatively unchanged between bacterial species[39]. The V1-V3 region, in particular, provides the highest probability of species-level identification[39]. Similar V1-V3 studies on Colorectal Cancer (CRC) and Advanced Colorectal Adenoma found changes in three phyla of interest: Bacteroidetes, Firmicutes, and Proteobacteria[21,40,41]. It has also been reported that exercise can shift the microbial population and lead to an increase in Firmicutes[42]. For this reason, this study identified microbiome populations by using V1-V3 primers following a 16S metagenomic library prep protocol[22] to potentially define the effects of environmental enrichment on these phyla known to be altered in adenoma and CRC. This procedure can be modified to amplify and sequence other variable regions of the 16S rRNA genes. One way is to use Probe Match to understand the phylogenetic classification of microbiota present in the sample that will be identified by the probe. In this way, probes can be targeted to specifically define and phylogenetically classify microbes of interest. This allows a different characterization of the microbiota present in the stool samples, and may reveal additional EE-dependent alterations in the microbiome of tumor bearing mice that may affect disease progression.

Using this procedure, the genus *Sutterella* was identified as the most altered genus following EE of tumor bearing animals. This procedure can be adapted to accommodate studies that utilize any method meant to analyze the effects of a perturbation on microbiome composition in genetically modified models of human disease. For example, in place of EE, mice could be inoculated with *Sutterella* to define whether *Sutterella* inoculation is sufficient to increase microbial biodiversity and wound repair in 16-week-old male tumor bearing animals.

Undoubtedly, the most unique aspect of this protocol is the concern over normalizing microbiota prior to EE and maintaining microbiome diversity throughout the EE studies. Since microbiome studies are continually improving, it is likely that more robust methods for characterizing the microbiome will arise, and the microbiome characterization in this protocol will become obsolete. For example, with the current study, the probes used to amplify the 16S rRNA have bias, depending on the probes that are chosen, and do not characterize all of the bacteria present in the microbiome. While the methods used to survey and characterize the microbiome will undoubtedly improve, the basic foundation of designing and running EE experiments while keeping normalization of the microbiome in mind will remain an essential facet of EE experiments.

Disclosures

The authors declare they have no conflicts of interest.

Acknowledgements

We thank B. Dalley in the University of Utah Genomics core for library sequencing, and K. Boucher in the University of Utah Biostatistics core for statistical advice, and access to these technical cores supported by National Cancer Institute award P30 CA042014. The project described was supported by the National Cancer Institute Grants P01 CA073992 and K01 CA128891 and the Huntsman Cancer Foundation.

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