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Adam Irvin
West Virginia University

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Diet Induced Alteration of the Murine Intestinal Microbiome After Antibiotic Ablation

Adam Irvin

**Thesis submitted
to the School of Medicine
at West Virginia University**

in partial fulfillment of the requirements for the degree of

**Master of Science in
Biomedical Sciences**

**Christopher F. Cuff, Ph.D, Chair
Rosana Schafer, Ph.D
P. Roco LaSala, M.D.**

Department of Microbiology, Immunology and Cell Biology

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ABSTRACT

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Adam Irvin

Background and Objective

We detected a dominant non-cultivable gram-positive coccobacillus associated with cultivable yeast in feces of mice treated with a cocktail of broad-spectrum antibiotics while being fed a partially defined mouse chow that is commonly used for nutritional studies. The goal of this study was to determine the identity of the non-cultivable gram-positive coccobacillus.

Methods

Fecal samples were analyzed by Gram stain, quantitative flow cytometry, next generation sequencing of the V3 or V4 regions of the 16S rRNA gene, fluorescence microscopy, and sequence analysis of the *tuf* gene, which can distinguish among *Streptococcus* and *Staphylococcus* genera.

Results

Fecal samples from antibiotic-treated mice displayed a ten-fold decline in bacterial cell number. High-throughput sequencing of the variable regions of the 16S rRNA gene and *tuf* gene sequencing identified the major phylotype as *Lactococcus*. *Lactococcus* did not grow from fecal cultures, although *Enterococcus casseliflavus* did grow, which was a confounding finding. The mouse chow was heavily contaminated with non-viable *Lactococcus*. Appearance of intestinal yeast was dependent on the specific chow, although yeast was not detected in the chow.

Conclusion

Dead bacteria in food can modulate the intestinal microbiome. Whether and how food-derived dead bacteria alter intestinal physiology needs to be determined.

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GENERAL INTRODUCTION

From birth until death, microorganisms, especially bacteria, coexist with humans in a mutualistic relationship. The host provides nourishment and a suitable environment for the bacteria, and the bacteria play a role in establishing and maintaining a healthy immune system^[1], prevent colonization of pathogens^[2] and contribute to host nutrition by synthesizing vitamins (i.e. vitamin K and biotin) and metabolizing non-digestible starches^[3-5]. There are an estimated 10^{14} bacterial cells (at least an order of magnitude more than the total number of our own cells)^[6-7] that colonize the human body, with the majority residing in areas that include the skin surface, oral cavity, nasal cavity, genitourinary system and the gut^[8]. The compilation of microorganisms and their collective genome within a niche is referred to as a microbiome^[9-11], which can consist of bacteria, viruses, fungi and Archaea^[12-14].

The intestinal microbiome has been identified as a key player in several processes that include; metabolic, nutritional, physiologic and immunologic^[15]. The gut is sterile until the moment of birth, at which

point the intestinal microbiome becomes established. The microbiome expands and increases in diversity through adolescence and into adulthood, at which point the population and diversity begins to decline^[16].

At birth, the intestinal microbiome can be influenced by means of delivery. A study found that babies delivered by vaginal birth had a significantly higher population of *Actinobacteria* than children delivered by caesarean section^[17]. However, the microbiome quickly changes with the introduction of nutrients.

Intestinal microbiomes of newborns are greatly influenced by the source of nourishment they receive. Infants that are breast-fed have a larger population of *Actinobacteria* when compared to formula-fed infants, and have nearly a two-fold increase in total number of bacteria^[18]. With the introduction of solid foods the microbiome takes a dramatic shift, and *Actinobacteria* percentages drop significantly while *Bacteroidetes* and *Firmicutes* become the dominant phyla^[19].

The trend of a microbiome that is primarily dominated by an equal ratio of *Bacteroidetes* and *Firmicutes* continues through adolescence and into adulthood. In adulthood, *Firmicutes* become the primary phylum in average, healthy adults microbiome. This trend continues throughout life as the number of bacteria and diversity decline^[20].

The human gut normally possesses one of the densest populations of bacteria found on Earth^[21-22]. Populations of bacteria within the gut have been enumerated at levels reaching 10^{12} cells per ml of intestinal content with over 10,000 different phylotypes identified^[22]. Molecular approaches with Next-gen sequencing have detected that approximately only 1% of bacteria residing in the gut can be recovered by culture-based approaches^[23].

A variety of factors play a role in establishing and maintaining the gut microbiome to include: age, diet, health, geographic location and genetics^[24-30]. Dysbiosis within microbiomes have been linked to certain diseases: cancer, obesity, diabetes, atherosclerosis and inflammatory bowel disease^[31-32].

Antibiotics introduced into the intestinal tract disrupt the microbiome by suppressing the total bacterial population and diversity in a variety of ways. Broad-spectrum antibiotics are often prescribed to treat infections to ensure eradication of the pathogenic bacteria. In the past little regard was taken to the effects that these antibiotics have on the normal microbiota, and how they could promote colonization of pathogens, such as *Clostridium difficile*. One study has shown that antibiotics introduced to infants are capable of modulating the microbiome even 8 wks after terminating antibiotic use^[33].

Antibiotic use became the standard of care for treating infections in the 1940s and has been widely used since. They have played a large role in extending human longevity; however antibiotic-resistant pathogens have wreaked havoc on health care and patients for at least the past two decades^[34]. However, within the last decades with the improvements to high-throughput sequencing, the true significance of the microbiota within the gastrointestinal tract suppressing the colonizing of pathogens is being realized.

Murine models of intestinal microbiome disruption are often used to demonstrate the effects of antibiotics. Antibiotics are typically administered either through drinking water or by oral gavages, and are used to selectively target groups of bacteria. Numerous cocktails of antibiotics have been used to either ablate the gut flora of mice to present a sterile gut environment or to target certain genera of bacteria^[35-41]. One recipe of antibiotics that is commonly used in studies is a combination of vancomycin, ampicillin, neomycin and metronidazole^[36, 39, 42]. The metronidazole targets anaerobic bacteria, neomycin is effective against Gram-negative bacteria, vancomycin is highly effective against Gram-positive organisms, and the ampicillin targets the remaining bacteria and has broad spectrum capabilities. In these studies, it is evident that the antibiotics suppress either the selected commensal bacteria or ablate nearly the entire microbiome. An alternative approach to studying the microbial ecology of the small intestine is through the use of germ-free mice. One such study showed colonizing germ-free mice with known commensal bacteria was capable of preventing introduced pathogens from colonizing^[43].

Prebiotics and probiotics have been studied for beneficial effects as supplements for the commensal bacteria in the gut. Prebiotics, such as cellulose and tagatose, are non-digestible food ingredients that modulate the gastrointestinal environment to promote the growth of select bacteria. A prebiotic mixture of galactooligosaccharides, brand name Bimuno, has demonstrated potential relief to patients suffering from inflammatory bowel disease. Probiotics are commensal bacteria, such as *Lactobacillus* and *Bifidobacterium*, which are commonly used to help augment existing commensal bacteria within the gut. There are conflicting reports on the clinical efficacy of probiotic use; however a ground-breaking study focused on fecal transplantations from healthy donors to treat patients with *Clostridium difficile* infections^[44]. The results were remarkable, with greater than 95% of patients cleared of infection without a subsequent infection. Fecal transplants are currently being researched as potential treatments.

Apart from bacteria, yeast and Archaea play a role as commensal organisms within the human intestinal microbiome. It has been documented that yeast can propagate in response to suppressing bacterial growth through antibiotic-treatment. Interestingly, the

lineage of the yeast changes under long-term antibiotic treatment^[36].

Archaea, such as *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*, are considered normal microbiota of the human intestines and have an essential role.^[45] As anaerobic bacteria metabolize pectin and other complex sugars, hydrogen and methanol are released as byproducts. Both hydrogen and methanol reduce the efficiency of fermentation, and these Archaea methanogens use both hydrogen and methanol as a source of energy. Although Archaea has not been identified to exist within the murine gut, given the similarities of composition and environment between the human and murine gut, it is reasonable to believe that Archaea could exist within the murine gut as a commensal organism.

The present study was undertaken to examine the role of diet in affecting the intestinal microbiome; particularly in the context of antibiotic-induced perturbations in an intestinal ecosystem. We initially wanted to add quantitative analysis to previously reported studies that relied on culture methods^[46] or qPCR^[36] to quantify perturbation of the 16S gene in studies of the microbiome. We also wanted to explore the existence of Archaea within the murine intestinal microbiome.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice, aged 6 weeks old were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were randomly separated into two groups and housed in the Health Sciences Center Animal Care Facility 5 mice per cage under approved protocol number 12-0506.3. Upon arrival the mice were placed on either a controlled diet (referred to as Open Source chow), D12450B from Research Diets Inc (New Brunswick, New Jersey) or on 2018 Teklad Global 18% Protein Rodent Diet chow ad libitum, dependent upon the experiment. The mice were allowed to acclimate for 5 days prior to use.

Antibiotics

The antibiotics vancomycin (0.5 mg/mL), ampicillin (1.0 mg/mL), metronidazole (1.0 mg/mL) and neomycin (1.0 mg/mL) were dissolved into filtered (0.22 μ m), sterile water and delivered to mice in light-protected water bottles ⁽³⁵⁾. Aspartame (3.75mg/mL) was also added to the antibiotic containing water to make the water palatable for the mice.

All antibiotics were purchased through Sigma Aldrich (St Louis, MO). The vancomycin, ampicillin and neomycin were United States Pharmacopeia (USP) certified, and metronidazole was traceable to USP. The antibiotic water was made fresh every 48 hours, due to the loss of efficacy of the ampicillin in solution. Based on the minimum inhibitory concentration (MIC) against a laboratory strain of *E. coli*, it was determined that ampicillin lost 90% of its efficacy within 24 hours in solution. Due to the nature of the prolonged study and to recapitulate the results from Hildebrandt et al. [22], the decision to change the water at 48 hour intervals was made.

Sample Collection and Processing

To ensure that fresh fecal pellets were collected, mice were placed into sterile, empty pipette tip boxes for up to 3 minutes. The mice were under direct observation the entire time to ensure that once a fecal pellet was expelled, mice were placed back into their home cages and the pellets placed into 1.5 mL Eppendorf tubes. If the fecal pellets were contaminated by urine, the samples were discarded. The samples were weighed and then emulsified in 1 mL of sterile water using a Pellet Pestle Motor from Kimble Chase Kontes (Vineland, NJ). The samples

were then centrifuged at 800 x g for 1 minute to remove fecal debris. Five hundred uL of the supernatants were diluted in 500 uL of sterile water in a fresh 1.5 mL Eppendorf tubes.

DNA Isolation

DNA was isolated from murine fecal pellets by processing fecal pellets as described above and purifying the DNA using QIAamp DNA Stool Mini Kit from Qiagen (Valencia, CA, USA) following the manufacturer's instructions. Genomic DNA was stored at - 20°C.

PCR and Fragment Purification

The PCR primer sequences, the conditions for amplification of the V3 region of the 16S ribosomal RNA gene, and multiplexed DNA sequencing strategy were as described in Bartram et al.^[47] unless otherwise indicated. The PCR primers for the V4 region of the 16S ribosomal RNA gene were as described in Caparoso et al.^[48]. High-pressure liquid chromatography purified PCR primers for the V3 region were obtained from Integrated DNA Technologies (Coralville, IA, USA) and the V4 region primers were obtained from Invitrogen (Grand Island, NY, USA). Bacterial DNA was amplified using an AccuPrime PCR Kit (Invitrogen

Life Technologies, Grand Island, NY, USA) on a Techne Genius Model FGEN02TP Thermal Cycler using the following conditions: 95°C for 6 minutes denature; 95°C for 2 minutes, 50°C for 2 minutes, 72°C for 2 minutes 30 cycles; 72°C for 4 minutes extend. Each reaction contained 1 U TAQ polymerase, 5 µl 10x buffer 1(600 mM Tris-SO₄ (pH 8.9), 180 mM (NH₄)₂SO₄, 20 mM MgSO₄, 2 mM dGTP, 2 mM dATP, 2 mM dTTP, 2 mM dCTP, thermostable AccuPrime™ protein, 10% glycerol), 20 µM forward primer, 20 µM reverse primer, and up to 60 ng DNA in a total volume of 50 µl. PCR reactions were performed in triplicate and reaction products were pooled prior to purification. Because there was a low concentration of DNA in the samples from the treated mice, it was necessary to perform 30 cycles of amplification to obtain sufficient material to view on gels. Pooled PCR products were purified by electrophoresis through 2% agarose in Tris/acetate/EDTA gels and the bands corresponding to approximately 300 base pairs for V3 and bands corresponding to approximately 400 base pairs for V4 were excised and purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia,CA, USA) according to the manufacturer's directions. The V3 and V4 regions both vary in length by about 30 base pairs among different species of bacteria, and the sequences obtained and analyzed in this study showed

similar size variability. The amplicon of the V3 region ranged from 296 to 327 base pairs, of which 160 base pairs were the primer. The amplicon of the V4 region ranged from 373 to 402 base pairs, of which 172 base pairs were the primer.

PCR Amplification with Genus Specific Primers

Bacterial DNA was amplified with either on a Techne Genius Model FGEN02TP Thermal Cycler using the following conditions: 95°C for 6 minutes denature; 95°C for 2 minutes, 50°C for 2 minutes, 72°C for 2 minutes 20 cycles; 72°C for 4 minutes extend. Each reaction contained 1 U TAQ polymerase, 5 µl 10x buffer 1(600 mM Tris-SO₄ (pH 8.9), 180 mM (NH₄)₂SO₄, 20 mM MgSO₄, 2 mM dGTP, 2 mM dATP, 2 mM dTTP, 2 mM dCTP, thermostable AccuPrime™ protein, 10% glycerol), 20 µM forward primer, 20 µM reverse primer, and up to 100 ng DNA in a total volume of 50 µl. The *Lactococcus*^[61] and *Enterococcus*^[62] specific primers are listed in Table 3.

Illumina HiSeq High Throughput (HT) Sequencing

Libraries were sequenced in a 2 x 125 bp paired-end strategy on an

Illumina HiSeq1000, so that the forward and reverse reads could be assembled into a single contig. Indexed libraries were pooled so that 12 libraries were sequenced in each lane of the flow cell. Eight pmols of the pooled libraries were clustered onto an Illumina v2 sequencing flow cell using an Illumina cBOT. Reads were converted from Illumina bcl format to fastq format and separated into bins based on exact match to the index using CASAVA 1.8.2 (Illumina, San Diego CA, USA). The Illumina HiSeq was performed at Marshall University (Huntington, West Virginia.) Sanger sequencing was performed by the Molecular Medicine core facility at West Virginia University and MiSeq sequencing was performed by the West Virginia University Genomics core facility.

HiSeq Data Processing

Sequence files were initially processed by removing sequences corresponding to linkers and primers by automated batch processing using scripts written in-house. In an effort to reduce artifacts generated by sequencing errors, a strict quality filtering protocol was employed that reduced the number of analyzed sequences to approximately 35% of the total number of sequences generated. Nevertheless, an average of more than two million high quality reads was obtained from each

sample. Quality filtering of DNA sequences was performed using the following steps: 1) Sequences were first filtered by the Illumina software to eliminate the poorest reads (Q score ≥ 30) and imperfect primer matches. 2) The forward and reverse sequences were matched to construct a sequence that spanned the entire region between the primers with a program written in-house. The original Illumina sequences were all 125 bases in length, which is where the run ended. The pairing strategy overlaid the two 3' ends starting with an overlap of 58 bases for the V3 region. The overlap window was extended one base at a time to 89 bases until a perfect match was obtained in the overlap region. Any pair of sequences that did not match at 100% identity in any of the size windows was discarded. This step eliminated 56% of the sequences, which overwhelmingly had the lowest quality scores. In general, the sequence quality was better in the middle than at the end, so this preferentially eliminated sequences with sequencing artifacts. 3) Paired sequences with a Phred quality score of less than five were discarded. This removed a few remaining low quality sequences, especially any that had low quality in the regions between the primers and the overlap. 4) The sequences were clustered by matching against the Greengenes database, which is a curated collection of known

bacterial 16S sequences. Sequences that did not match any of the known bacterial sequences with 97% identity were discarded. This removed chimeras and most major PCR artifacts and represented approximately 5% of the total remaining sequences. Matching at 97% identity meant that any single base PCR artifacts would be combined with the corresponding authentic sequence (since the region is about 100 bases long, up to three single base changes will be ignored). 5) The resulting table of operational taxonomic units (OTUs) was filtered to remove any sequences that appeared less than 150 times. Because of the length of the V4 region, we were unable to perform the paired end strategy. Scripts written in-house in biopython were used to convert the filtered Illumina data to the FASTA format for analysis by QIIME for taxonomic assignment and measurements of microbial diversity, but scripts to do this are now part of QIIME. To process Illumina generated files in QIIME, the file headers were changed to begin with '`>sample_number`' where '`sample`' is the sample number and '`number`' is the number of the sequence in the file. All of the sequences were then combined into one file for analysis with QIIME.

QIIME

All QIIME analyses were performed on a virtual server hosted by Amazon Web Services using an existing QIIME image. The server had the following specification: QIIME 1.8.0 EBS East XLARGE (ami-438d5b2a). The following QIIME scripts were used during analysis and default parameters were used unless otherwise noted: 1) ‘pick_reference_otus_through_otu_table.py’ matched sequences at 97% sequence identity with OTUs associated with specific bacterial phylotypes in the Greengenes database (4Feb2014); 2) ‘summarize_taxa_through_plots.py’ generated bar graphs of the relative abundance of different taxa in each sample; 3) ‘alpha_rarefaction.py’ generated alpha rarefaction plots; 4) ‘pick_rep_set.py’, ‘align_seqs.py’, ‘filter_alignment.py’, and ‘make_phylogeny.py’ were chained to generate a phylogenetic tree of the OTUs.

***Tuf* Gene Analysis**

The *tuf* gene encoding elongation factor Tu was amplified with the *tuf* forward primer: (5'-CCAATGCCACAACTCGT-3') and the *tuf* reverse primer (5'-CCTGAACCAACAGTACGT-3') as described in Li et al. [49].

Cloning of the *tuf* gene was carried out using a TOPO® TA for sequencing kit from Invitrogen (Grand Island, NY, USA) according to the manufacturer's instructions. The 830 bp *tuf* gene was amplified by PCR using AccuPrime PCR Kit (Invitrogen Life Technologies, Grand Island, NY, USA) on a Techne Genius Model FGEN02TP Thermal Cycler using the following conditions: 95°C for 6 minutes denature; 95°C for 2 minutes, 50°C for 2 minutes, 72°C for 2 minutes 30 cycles; 72°C for 4 minutes extend. Each reaction contained 0.5 µl TAQ polymerase, 5 µl 10x buffer 1 (600 mM Tris-SO₄ (pH 8.9), 180 mM (NH₄)₂SO₄, 20 mM MgSO₄, 2 mM dGTP, 2 mM dATP, 2 mM dTTP, 2 mM dCTP, thermostable AccuPrime™ protein, 10% glycerol), 20 µM forward primer, 20 µM reverse primer, and up to 60 ng DNA in a total volume of 50 µl. For cloning, the amplified fragment was inserted into the pCR4-TOPO plasmid (4.0 Kb). Electrically competent cells (*E. coli* TOP10) were transformed with the insert-containing plasmids. The recombinant clones were selected by growth on LB agar with ampicillin (100 µg/ml) following incubation for 18 hrs at 37°C with 5% CO₂. The presence of the insert in *E. coli* TOP10 transformants was verified by colony PCR using the *tuf* primers. Plasmids from transformants were extracted using the Mini-Prep 96 from Macconnell Research [San Diego, CA, USA]

according to the manufacturer's instructions. Five mL cultures grown in Magnificent Broth with ampicillin were incubated overnight. 2 mL of the cultures were placed in each well and applicator combs inserted. Lysis tablets were then added and allowed to incubate at room temp for 15 mins. Purified DNA was then removed from the corresponding wells on the back of the cartridge and the samples were sequenced by WVU Genomics Core facility by capillary (Sanger) sequencing.

Flow Cytometric Detection and Quantification of Fecal Bacteria

Fifty uL of the suspensions from fecal pellets were diluted in 950 uL of sterile 0.5% NaCl for a final dilution of 1/400. Utilizing Invitrogen's (Grand Island, NY, USA) Bacteria Counting Kit for flow cytometry, 1 µL of SYTO BC stain was added to the diluent as well as 10 µL of 1×10^8 /mL of microspheres, per manufacturer's instructions. Samples were analyzed with a Becton Dickinson FACSCalibur using the following settings: FSC E00 gain; SSC 350 voltage; FL1 412 voltage; FL2 550 voltage and FL3 650 voltage. A region was placed around the beads to capture a quantity of 10,000 beads; another region was placed to capture the number of bacteria that were counted in comparison to the

10,000 beads. WINMDI 2.8 software, (Joe Trotter, Scripps Research Institute, La Jolla, CA) was used to analyze the flow cytometer data.

Fluorescent Microscopy

Fifty μL of the suspensions from fecal pellets were diluted in 950 μL sterile 0.85% NaCl, either 1 μL of *BacLight* Red bacterial stain (Invitrogen, Grand Island, NY, USA), 1 μL of SYTO 9 stain (Invitrogen, Grand Island, NY, USA) , or 1 μL of both were added and samples incubated for 15 minutes at room temperature in the dark. Ten μL of sample was then placed on glass microscope slides, 1 drop of ProLong Gold (Invitrogen, Grand Island, NY, USA) was added and a cover slip applied. The slides incubated for 24 hours at room temperature in the dark to allow the coverslip to seal in accordance with manufacturer's instructions, and were examined by confocal microscopy.

Metagenomics analysis

Predictive functional profiling of the microbiome was performed using the 16S rRNA sequencing data and then running the following scripts through the PICRUSt database:^[50] 1) `normalize_by_copy_number.py`; 2)

predict_metagenomes.py; 3) categorize_by_function.py. QIIME commands were then executed on the file: 1) summarize_taxa.py (with –md_identifier=KEGG_Pathways flag); 2) plot_taxa_summary.py.

RESULTS

Gram stains of suspensions from fecal pellets from control mice and antibiotic-treated mice

Suspensions of fecal pellets from both control and antibiotic-treated mice were collected after two weeks of treatment and analyzed by Gram stain. Samples from control mice displayed a very diverse population of bacteria, which consisted of both Gram-positive and Gram-negative rods and cocci and was consistent with normal gastrointestinal microbiota (Fig 1A). Gram stains of samples from the antibiotic-treated mice showed distinctly homogeneous Gram-positive coccobaccilli, as well as some yeast (Fig 1B), suggesting that antibiotic treatment altered the normal intestinal microbiota.

Quantitative flow cytometry performed on fecal pellets from control and antibiotic-treated mice

Quantitative cell counts were performed on suspensions of fecal bacteria by flow cytometry. Ten samples from each group were analyzed and an approximate 1- $\log_{10}$ reduction in the number of bacteria was found in the antibiotic-treated samples when compared to

the control samples (Fig 2A). As was observed on the Gram stains, the control mice had a diverse population (Fig 2B) of bacteria as determined by forward scatter, while the antibiotic-treated mice had a homogeneous population (Fig 2C).

Fluorescent microscopy performed on fecal suspension to determine cellular composition

To confirm that the Gram stain and flow cytometric assays were detecting bacteria, samples were stained with dual fluorescent stains (Fig 3A and B) and examined using confocal microscopy. A merged view (Fig 3C) displays that the putative bacteria contain nucleic acid and their cell walls are comprised of peptidoglycan.

Sequencing of V3 region of 16S rRNA from DNA extracted from antibiotic-treated and control mice feces

Paired-end sequencing of the V3 region of the 16S rRNA gene was performed to identify the Gram-positive coccobaccilli in the antibiotic-treated mice, and characterize the fecal microbiomes of the treated and non-treated mice. Figure 4 represents a graphic display of the Organizational Taxonomic Units (OTU) table generated from QIIME, and

shows substantial increase in *Lactococcus* species in the antibiotic-treated group when compared to the control group. Within the antibiotic-treated group, there is a decrease in *Bacteroidetes* and non-*Lactococcus*, *Firmicutes*. Although the *Lactococcus* is present in the control group as well, it is in much smaller quantities when compared to the antibiotic-treated samples.

Sequencing of V4 region of 16S rRNA from DNA extracted from antibiotic-treated and control mice feces

Due to the length of the V4 region of the 16S rRNA gene, contigs could not be generated as were with the V3 region because of only 1 or 2 base overlaps. The V4 region sequencing (Fig 5) displayed a loss of *Bacteroidetes* and an increase in *Lactococcus* in the antibiotic-treated samples when compared to the control samples, albeit the changes were not as dramatic in the V4 results as displayed in the V3 results. A small percentage of *Archaea* was also detected in antibiotic-treated samples.

Culturing

Numerous attempts at culturing the coccobacilli that were observed by Gram stain in the antibiotic-treated samples yielded predominantly

overgrowth of yeast (Fig 6A). Upon closer examination, there were also scant pinpoint, α -hemolytic colonies. These colonies were isolated (Fig 6B), DNA was extracted, and the entire 16S rRNA gene was PCR-amplified and sequenced. Sequence analysis identified the alpha hemolytic colonies as *Enterococcus casseliflavus*, not *Lactococcus*. The inconsistency between the HT sequencing and culture results required additional analysis.

Cloning of *Tuf* gene

Sequence variation in the *tuf* gene, a prokaryotic elongation factor, has been previously used to differentiate species of Gram-positive cocci (Li et al.^[39]) Therefore fecal DNA from antibiotic-treated mice was amplified using degenerate *tuf*-specific primers, cloned, and sequenced.

14 of 14 samples yielded a 99% match with *Lactococcus* through BLAST. These results confirmed the results from the V3 sequencing data.

Growth time point assay

After numerous unsuccessful attempts to grow *Lactococcus* from the fecal samples from antibiotic-treated mice on a variety of bacterial media, fecal samples from the antibiotic-treated mice were cultured in

evaporated milk. Aliquots were removed at time 0, and every 8 hours of incubation for a 24-hour period. DNA samples from each time point were amplified using *Lactococcus* or *Enterococcus* specific primers. The reactions resulted in an initial positive reaction for the *Lactococcus* with no increase in the levels of PCR product through the 24-hour time period, indicating that the *Lactococcus* was not replicating. However, although the initial sample failed to amplify with *Enterococcus* specific primers, weak positive results were detected at 16 hours and strong positive observed at 24-hours (Fig 7A). Gram stains were performed on the sample at 24 hours and demonstrated Gram-positive coccobaccilli detected and yeast in the culture (Fig 7B).

Examination of the chow

To identify the source of the *Lactococcus*, a 20 mg sample of the Open Source Chow was processed in the same manner as the fecal samples and Gram stained (Fig 8A). The Gram stain results of the Open Source chow demonstrated that the chow was heavily contaminated with Gram-positive coccobaccilli, with no yeast observed. A sample of the 2018 Teklad Global 18% Protein Rodent Diet chow normally used in HSC vivarium was then examined in the same manner as the Open

Source chow and Gram stained (Fig 8B). No bacteria were visualized on the Gram stain of the 2018 Teklad Global 18% Protein Rodent Diet chow. To determine if the bacteria from the Open Source chow was *Lactococcus*, PCR reactions were performed on both the Open Source chow and the 2018 Teklad Global 18% Protein Rodent Diet chow with both specific and universal primers for 16S rRNA (Fig 9). The results demonstrated that both the 2018 Teklad Global 18% Protein Rodent Diet chow and the Open Source chow contained DNA from bacteria, however the Open Source Chow contained *Lactococcus* DNA.

Flow cytometric analysis of the chow

The Open Source and 2018 Teklad Global 18% Protein Rodent Diet chows were analyzed by flow cytometry to compare the forward scatter characteristics of bacteria in the chow with bacteria in the fecal samples of antibiotic-treated mice. The Open Source chow had a distinct bacterial population that resembled the fecal samples from the antibiotic-treated mice (Fig 10A), while the 2018 Teklad Global 18% Protein Rodent Diet chow lacked a distinct population (Fig 10B). A pure culture of *Lactococcus* was analyzed for comparison (Fig 10C).

Quantitative analysis revealed the bacterial cell count for the Open Source chow to be 3×10^{12} per gram.

Live/Dead Analysis

To determine the viability of the *Lactococcus* present within the Open Source chow, a Live/Dead analysis was performed using Invitrogen's LIVE/DEAD BacLight Bacterial Viability kit according to manufacturer's instructions. The analysis showed that the *Lactococcus* from the Open Source chow was non-viable (Fig 11).

Effects of 2018 Teklad Global 18% Protein Rodent Diet chow

To determine the differences of gastrointestinal microbiomes when the mice were placed on different chow, 15 additional C57BL/6 mice were acquired from Jackson Laboratory. Upon receipt, the mice were handled in the exact manner as before with the exception of placing the mice on 2018 Teklad Global 18% Protein Rodent Diet chow rather than the Open Source Diet. DNA extracted from fecal pellets from both antibiotic-treated and control mice were amplified using V3 16S rRNA primers and sequenced (Fig 12) using an Illumina MiSeq. The antibiotic-treated mice produced an overwhelming dominance- >95%

in all 5 mice- of a lactose fermenting *Enterobacteriaceae*. The short V3 regions of the V3 region of the 16S rRNA were unable to differentiate between species of *Enterobacteriaceae* due to homology of the genus within the V3 region. With the removal of the Open Source Diet no *Lactococcus* was detected.

Metagenomic evaluation

PICRUSt Bioinformatics software was utilized to predict metagenomic functions from the 16S rRNA sequencing data (Fig 13.) The most notable change was a reduction in the content of genes predicted to play a role in motility, which correlates with *Lactococcus* being a non-motile organism.

Detection of yeast in feces is dependent on chow

Ingestion of the 2018 Teklad Global 18% Protein Rodent Diet chow not only produced a dominant *Enterobacteriaceae*, but also failed to produce yeast in the feces of antibiotic-treated mice. Yeast was not detected in the feces of the mice that were fed 2018 Teklad Global 18% Protein Rodent Diet chow and antibiotic treatment for 10 weeks by either culture or Gram stain. Another group of mice that were on

antibiotic treatment and fed the 2018 Teklad Global 18% Protein Rodent Diet chow for 3 weeks and then converted to Open Source chow for the remaining 7 weeks also produced the *Enterobacteriaceae* with the absence of yeast. In comparison, the mice that were on antibiotic treatment and the Open Source chow displayed yeast by culture and Gram stain after 2 weeks. (Data not shown)

Table 1. Yeast in feces of antibiotic-treated mice dependent on chow	
Chow	Yeast
Open Source	+
Teklad Global	-
Teklad Global/converted to Open Source *	-

*Mice were fed Teklad Global chow for 3 wks and then converted to Open Source chow for remaining 7 weeks

Table 2. Sequences of primers used in the construction of libraries for Illumina sequencing. Lowercase letters denote adapter sequences necessary for binding to the flow cell, underlined lowercase are binding sites for the Illumina sequencing primers, bold uppercase highlight the index sequences and regular uppercase are the V3 or V4 region primers.^[37]

Forward Primers Sequence (5' to 3')	
V3_Fmod	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctNNNNCTACGGGAGGCAGCAG
V4_Fmod	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctNNNNGTGCCAGCGCCGCGGTAA
Reverse Primers	
V3_1R	caagcagaagacggcatacagagat CGTGAT gtgactggagttcagacgtgtgctcttccgatctATTACCGCGGCTGCTGG
V3_2R	caagcagaagacggcatacagagat ACATCG gtgactggagttcagacgtgtgctcttccgatctATTACCGCGGCTGCTGG
V3_3R	caagcagaagacggcatacagagat GCCTAA gtgactggagttcagacgtgtgctcttccgatctATTACCGCGGCTGCTGG
V3_4R	caagcagaagacggcatacagagat TGGTCA gtgactggagttcagacgtgtgctcttccgatctATTACCGCGGCTGCTGG
V3_5R	caagcagaagacggcatacagagat CACTGT gtgactggagttcagacgtgtgctcttccgatctATTACCGCGGCTGCTGG
V3_6R	caagcagaagacggcatacagagat ATTGGC gtgactggagttcagacgtgtgctcttccgatctATTACCGCGGCTGCTGG
V3_7R	caagcagaagacggcatacagagat GATCTG gtgactggagttcagacgtgtgctcttccgatctATTACCGCGGCTGCTGG
V3_8R	caagcagaagacggcatacagagat TCAAGT gtgactggagttcagacgtgtgctcttccgatctATTACCGCGGCTGCTGG
V3_9R	caagcagaagacggcatacagagat CTGATC gtgactggagttcagacgtgtgctcttccgatctATTACCGCGGCTGCTGG
V3_10R	caagcagaagacggcatacagagat AAGCTA gtgactggagttcagacgtgtgctcttccgatctATTACCGCGGCTGCTGG
V3_11R	caagcagaagacggcatacagagat GTAGCC gtgactggagttcagacgtgtgctcttccgatctATTACCGCGGCTGCTGG
V3_12R	caagcagaagacggcatacagagat TACAAG gtgactggagttcagacgtgtgctcttccgatctATTACCGCGGCTGCTGG
V4_13R	caagcagaagacggcatacagagat CGTACT gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_14R	caagcagaagacggcatacagagat GACTGA gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_15R	caagcagaagacggcatacagagat GCTCAA gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_16R	caagcagaagacggcatacagagat TCGCTT gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_17R	caagcagaagacggcatacagagat TGAGGA gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_18R	caagcagaagacggcatacagagat ACAACC gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_19R	caagcagaagacggcatacagagat ACCTCA gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_20R	caagcagaagacggcatacagagat ACGGTA gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_21R	caagcagaagacggcatacagagat AGTTGG gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_22R	caagcagaagacggcatacagagat CTCTCT gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_23R	caagcagaagacggcatacagagat CAAGTG gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT
V4_24R	caagcagaagacggcatacagagat CCTTGA gtgactggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT

Table 3. Genus specific primers used to identify *Lactococcus* and *Enterococcus*.

<i>Lactococcus</i> specific primers (5' to 3')	
LacF	GTACTTGTACCGACTGGAT
LacreR	GGGATCATCTTTGAGTGAT
<i>Enterococcus</i> specific primers (5' to 3')	
Ent1	TACTGACAAACCATTCATGATG
Ent2	AACTTCGTCACCAACGCGAAC

A **B**

Figure 1. Gram stains from fecal pellets from control mice (A) and antibiotic-treated mice (B). In (A) there is a very diverse population of both Gram-positive and Gram-negative organisms both rods and cocci. Samples from antibiotic-treated mice (B) showed distinct homogeneous Gram-positive coccobaccilli, as well as yeast. Photos are taken at 630X.

Figure 2. Quantitative flow cytometry performed on suspension from fecal pellets from control and antibiotic-treated mice on Open Source diet. Samples from 10 mice in each group were analyzed and displayed an average of a 1- log_{10} difference in bacterial load (A). A heterogeneous population of cells is displayed in the control sample (B), when compared to the antibiotic-treated sample (C), which displays a distinct homogenous population of bacteria. (i) bacterial cells population, (ii) microspheres population, (iii) debris.

Figure 3. Fluorescent microscopy of fecal suspensions stained for bacterial identification. Utilizing a dual stain; Syto 9, which stains the nucleic acid green (A), a peptidoglycan stain that fluoresces red (B) and then merging (C), confirmed that the cells contained DNA and peptidoglycan. Photos are taken at 630X.

Figure 4. Taxonomic assignment of phylotypes found in feces from control and antibiotic-treated mice at the class level from V3 region 16S rRNA sequencing analysis. The total height of the bar represents 100% of the assigned sequences after quality filtering, and the height of the individually colored bars represents the percentage of the class of organisms. The expansion of *Lactococcus* within the antibiotic-treated group is shown, while they also lose a large percentage of other *Firmicutes* and *Bacteroidetes*. The control mice had a small percentage of *Lactococcus*, but displayed a larger heterogeneity of bacterial population.

Figure 5. Taxonomic assignment of phylotypes found in feces from control and antibiotic-treated mice at the class level from V4 region 16S rRNA sequencing analysis . The total height of the bar represents 100% of the assigned sequences after quality filtering and the height of the individually colored bars represents the percentage of the class of organisms. The expansion of *Lactococcus* within the antibiotic-treated group is still present, but not near the percentages as seen with the V3 data (Fig 4.) A loss of *Bacteroidetes* was still observed, however a decrease in non-*Lactococcus Firmicutes* was not detected with the V4 sequencing data.

Figure 6. Cultures of feces from antibiotic-treated mice. Aerobic cultures of the fecal pellets from the antibiotic-treated mice yielded an abundant growth of yeast (A). Upon closer examination, there were pinpoint α -hemolytic colonies that were subcultured for isolation on new 5% sheep blood agar plate (B).

Figure 7. Culturing of mouse fecal pellets and growth time point assay. A 24-hour growth rate, time point assay was performed and the growth of *Lactococcus* and *Enterococcus* were compared in culturing in condensed milk. PCR amplification (A) was performed with bacteria-specific primers at four separate time points; 0, 8 hours, 16 hours and 24 hour time points. Results indicate that the *Lactococcus* is present initially but never propagates, and *Enterococcus* is detected at 16 hours with an increase of PCR product at 24 hours. A Gram stain of the milk at 24 hours (B) displays Gram-positive cocci and yeast.

Figure 8. Gram stain analysis (A) of Open Source and 2018 Teklad Global 18% Protein Rodent Diet chow (B). The Gram stain displays homogenous, Gram-positive coccobacilli in the Open Source diet that resembles the organism from the fecal samples, whereas no bacterial cells were observed in the 2018 Teklad Global chow.

Figure 9. PCR analysis demonstrates *Lactococcus* DNA present in the Open Source Diet chow. The Open Source Diet chow and the 2018 Teklad Global chow were emulsified in sterile, distilled water and PCR reactions were performed for 16S rRNA as well as *Lactococcus* specific primers.

Figure 10. Flow cytometric analysis of chow. Open Source Diet (A) has a distinct bacterial population that resembles the fecal samples of the antibiotic-treated mice (see Fig 2B) and this population is absent in 2018 Teklad Global chow (B). An analysis on a purified culture of *Lactococcus* is shown in (C) for comparison.

Figure 11. Viability analysis of chow via flow cytometry.

Invitrogen's Live/Dead kit for bacteria utilizes Syto9 and propidium iodide (PI) to elicit different staining characteristics between cells that have an uncompromised cellular wall versus cells that are non-viable and have perforations within the cell wall. If the cell wall has been compromised the PI will quench the Syto9 and present a homologous population, as seen in the *E. coli* cells fixed with 4% paraformaldehyde (A). A dual population will be seen in viable cells that maintain their cell walls, as seen in the sample of cultured *Lactococcus* (B). When the Open Source chow is examined (C) there is a loss of the dual population, identifying that the bacterial cell wall has been compromised and the cells are not viable.

Figure 12. Taxonomic assignment of the GI microbiome after sequencing V3 region of 16S rRNA sequencing from antibiotic-treated and control mice fed 2018 Teklad Global chow. The total height of the bar represents 100% of the assigned sequences and the height of the individually colored bars represents the percentage of the order of organisms.

Figure 13. Metagenomic analysis. Predictive metagenomic functions were applied through PICRUST bioinformatics software. The most notable change between the antibiotic-treated and control group was in motility, which correlates with *Lactococcus* being a non-motile organism.

Figure 14. Quantitative flow cytometry performed on suspension from fecal pellets from control and antibiotic-treated mice on Teklad global chow. Samples from 3 mice in each group were analyzed and displayed an average of a 2-log_{10} difference in bacterial load. The average cell count of the control group was 5.3×10^6 and the antibiotic-treated group was 5.9×10^4 respectively. A heterogeneous population of cells is displayed in the control sample (A), when compared to the antibiotic-treated sample (B). Of note, most of the cells counted in the antibiotic-treated sample (B) appear to be debris.

DISCUSSION

Preliminary studies in this project were undertaken to examine the role of diet in affecting the intestinal microbiome; particularly in the context of antibiotic-induced perturbations in an intestinal ecosystem. We initially wanted to add quantitative analysis to previously reported studies that relied on culture methods^[41] or qPCR^[34] to quantify perturbation of the 16S gene in studies of the microbiome.

Quantitative flow cytometry revealed an average of a 1- $\log_{10}$ decrease in bacteria in the fecal samples from antibiotic-treated mice when compared to the control fecal samples. This result conflicts with previously reported 2 to 4- $\log_{10}$ decreases with qPCR^[36, 42]. A possible explanation for this disparity is variation in the number of 16S rRNA gene copies that each bacterial organism possesses, the number of copies can vary from 1 to 15 with an average of 7.^[43] Thus, if remaining bacteria have a disproportionately low copy number of 16S gene then this could account for the discrepancy.

Flow cytometric analysis of fecal suspensions from antibiotic-treated mice showed a homogenous population that was staining with the DNA stain SytoBC, and by forward scatter measurement were within the same parameters of known bacterial cells. Further, Gram stains of fecal suspensions from the antibiotic-treated mice yielded a morphologic picture dominated by Gram-positive coccobaccilli. When fecal suspensions from both antibiotic-treated and control samples were diluted to equal concentrations based on the flow cytometry data, the bacterial populations appeared equivalent in number by Gram stain in all observed fields. This result conflicted with the 2 to 4 log₁₀ difference that was previously reported. This was an unexpected finding given the type of antibiotics used in the system; the metronidazole should eliminate the anaerobic bacteria, neomycin is effective against Gram-negative bacteria, vancomycin is highly effective against Gram-positive organisms, and the ampicillin should eradicate the remaining bacteria due to its broad spectrum capabilities. Multiple attempts at culturing the organism aerobically, anaerobically and microaerophilically were essentially negative, with the exception of the growth of yeast (that was present in low numbers in the Gram stains) and very low numbers of other colonies, which conflicts with the Gram stain and flow cytometry

data. The potential explanations included: 1) the Gram-positive coccobaccillus is a fastidious bacteria that was highly antibiotic resistant; 2) the organism was Archaea; 3) material identified as a microorganism by flow cytometer and Gram stain was actually debris; 4) the Gram-positive organism was dead.

The idea that the organism could be Archaea was provocative since *Methanobrevibacter smithii* is known to colonize the human gut^[44], but to our knowledge it is unknown if they reside within the murine gut. However, PCR analysis of the fecal suspensions of the antibiotic-treated mice failed to amplify with Archaea specific primers (data not shown), and fluorescent staining for both peptidoglycan and DNA provided further evidence that the putative cells were bacteria.

PCR amplification was carried out at 30 cycles to ensure adequate concentrations could be recovered to generate the libraries. PCR reactions were performed with equivalent amounts of DNA quantified by nanodrop, however it is impossible to distinguish between mouse DNA and bacterial DNA in the measurement.

High-throughput sequencing was performed on the fecal suspension to compare the gut microbiome from the antibiotic-treated mice to the control mice fed Open Source chow. Sequence analysis of both the V3 and V4 regions of the 16s rRNA gene indicated an expansion of *Lactococcus*. The increase in *Lactococcus* was more evident in analysis of the V3 region; however there was still an increase when the V4 region was analyzed from the antibiotic-treated samples as well. The presence of *Lactococcus* was confirmed by PCR amplification of fecal DNA with *Lactococcus* specific primers. Therefore, based on morphological determinants, high-throughput sequencing, and PCR performed with specific primers, we concluded that the organism present in the feces of the antibiotic-treated mice was *Lactococcus*. This result is consistent with the findings presented by Dollive et al.^[36] However, *Lactococcus* is not a fastidious organism so it should have been readily cultivated, and although previous studies have identified vancomycin resistant strains of *Lactococcus*^[53] an ampicillin resistant strain has not yet been identified.

The differences in the sequencing analysis between the V3 and V4 (Fig 4 & 5) regions can be explained by the length of product, the length of the V3 product allowed us to overlap reads and create contigs, where the V4 region was too long and the sequence analysis was created from single strand reads. The numbers at the bottom of the graphs correlate with the mouse number and NT refers to the control group, no antibiotic treatment given and the ABX is the antibiotic-treated group. For both the V3 and V4 sequence runs the same number of samples were processed, however some of the samples failed to produce adequate reads due to various reasons.

Repeat culturing of the feces of the antibiotic-treated mice revealed scant, pinpoint α -hemolytic colonies mixed in with the yeast, which we initially surmised was *Lactococcus*. Significant efforts to enrich this bacterium in *Lactococcus*-specific media (M17 agar supplemented with 10% sucrose and 0.5% glucose) were fruitless, and Sanger sequencing of the 16S gene amplified from purified colonies identified the organism as *Enterococcus casseliflavus*, not *Lactococcus*. Reexamination of the sequences from the high-throughput sequencing data confirmed that a very small percentage of *Enterococcus* within the fecal suspensions of

the antibiotic-treated mice was detected. Nevertheless, comparison of the previous V3 and V4 sequences in the high-throughput data that were identified as *Lactococcus* did not match the V3 or V4 region of the 16S gene from the *Enterococcus isolates*. Previous studies have reported that some species of *Enterococcus* have been shown to be resistant to vancomycin and ampicillin^[53-55], and follow-up testing on the isolates from the fecal samples from the antibiotic-treated mice found the *Enterococcus* strain to be resistant to vancomycin and intermediate to ampicillin (data not shown). Thus we were forced to resolve the seemingly incongruous dilemma of whether the organism that is observed by Gram-stain and flow cytometry is *Lactococcus* or *Enterococcus*, and if it was *Lactococcus* why did it not grow in culture.

A previously published study identified a region within the bacterial elongation factor *tuf* gene that was capable of differentiating between members of *Enterococcus* and *Lactococcus*.^[49] Therefore, *tuf* gene sequencing of DNA from fecal suspensions from antibiotic-treated mice was performed, and 100% yielded a match for *Lactococcus*. Now convinced that the organism in question was *Lactococcus*, we were still perplexed that it would not grow. Studies have shown that both

Lactococcus and *Enterococcus* will propagate in milk^[55, 58], so a growth assay was established using the milk as a medium and feces from antibiotic-treated mice as the inoculum. It was determined that the *Enterococcus* was propagating in the milk, however *Lactococcus* DNA that was detected initially failed to produce an increase in intensity by PCR throughout 24 hours of culture. Thus, we concluded that the *Lactococcus* present in the feces was non-viable, but the source was still unknown.

The possibility that the *Lactococcus* was a contaminant in the Open Source chow prompted the examination of the chow. Gram stains revealed that the chow was contaminated with Gram-positive coccobaccilli, and PCR analysis confirmed the organism to be *Lactococcus*. Cultures of the food yielded no growth, so it was concluded that the bacteria were non-viable. A quantitative flow cytometric evaluation of the Open Source chow determined a bacterial count of 3×10^{12} bacteria/g of chow. With an average daily consumption of 4.5 g of chow/per mouse, the mice were consuming an average of 1.4×10^{13} non-viable *Lactococcus* per day. An average of 1.1×10^{10} *Lactococcus* cells/g of feces was recovered from the antibiotic-treated mice, with an

average of ~100 mg of feces occupying the intestines at any given time that would account for 10^{12} *Lactococcus* cells residing within the intestines. Our bacterial counts conflict with Dollive et al.^[36] previously reported 5×10^4 bacteria/per fecal pellet; one potential explanation is that the DNA within the dead *Lactococcus* is degrading and lacks the stability to amplify by qPCR, however the SytoBC stain is still capable of binding to the DNA making detection by the flow cytometer possible. Given this scenario, the dead *Lactococcus* could be thought of as an empty peanut shell that facilitates transport of its degrading DNA throughout the intestinal tract. Interestingly, we discovered proportional levels of *Lactococcus* present throughout the intestinal tract, to include the small intestines just above the ileocecal junction, which is normally has a much lower concentration of microorganisms at an average of 10^4 bacterial cells /g^[57].

Lactococcus is widely used in the dairy industry because of its homofermentor capabilities^[58]; it solely produces lactic acid as it metabolizes glucose. *Lactococcus* acidifies milk rapidly and prevents the overgrowth of other bacteria that would cause spoilage^[58]. One of the main sources of protein in the Open Source chow is casein, which is

derived from mammalian milk. It is quite possible that the *Lactococcus* in the chow is from the acidified milk that is used to introduce casein into the finalized pelletized chow. Casein is also present in Open Source Hi-fat diets that are commonly used in nutritional studies, which explains results from earlier studies with the presence of *Lactococcus*-like bacteria in fecal samples from antibiotic-treated mice fed a high-fat diet (Cuff et al. unpublished).

After the discovery of the *Lactococcus* in the Open Source chow we wanted to repeat the microbiome analysis of the V3 region with the mice on another chow. Teklad Global 2018 is the normal chow used in the WVU Health Sciences vivarium, and was confirmed to be negative for *Lactococcus*. *Firmicutes* and *Bacteroidetes* represented the majority of the bacterial population in the control mice samples that were fed Open Source chow; however with the Teklad Global chow there was a larger percentage of *Bacteroidetes* and a smaller percent of *Firmicutes* than with the Open Source chow. These data reflect the removal of the *Lactococcus* (which is in the *Firmicute* order) from the chow. The antibiotic-treated samples had a >95% population of a lactose fermenting *Enterobacteraceae* that was identified as *Escherichia coli* by

Vitek analysis performed in the laboratory at Ruby Memorial hospital, Morgantown, WV. Our findings coincide with Hill et al.^[42], in respect to an increase in *Enterobacteraceae* in mice that are treated with antibiotics administered through drinking water, and no *Lactococcus* discovered when diet did not contain casein.

The antibiotic-treated fecal samples also showed a loss of bacterial motility genes when predictive metagenomic analysis was performed through PICRUSt, which is consistent with an increase in *Lactococcus*, which is non-motile. Although in minuscule percentages, Archaea was identified by sequencing the V4 region of 16S rRNA in the antibiotic-treated mice.

Interestingly, there was no yeast recovered from the feces of the antibiotic-treated mice that were fed the Teklad Global even after 10 wks of treatment. Another group of antibiotic-treated mice that were fed the Teklad Global for 3 wks and were then converted to the Open Source chow for the remaining 7 wks also failed to produce yeast in the feces. However, yeast was recovered at day 7 of antibiotic treatment in the feces of mice that were fed only the Open Source. Speculation as to

why the mice that were fed the Teklad Global chow failed to grow yeast from the feces is that the Teklad Global lacked the sucrose supplementation that was included in the Open Source chow. The Open Source chow consists of 35% sucrose, which yeast utilize as a source of nutrients, especially *Candida spp.*^[52] In the mice that were fed solely the Open Source chow, we speculate that the addition of the sucrose supported the growth of *Candida* and outcompeted antibiotic-resistant bacteria from colonizing. We further speculate that the intestines within the mice fed the Teklad Global chow from the beginning supported the antibiotic resistant bacteria the opportunity to colonize and flourish to outcompete the yeast once the Open Source chow was introduced. An experimental approach to check if the dead *Lactococcus* was promoting the growth of the yeast would be to fortify the Teklad Global chow with dead *Lactococcus* and feed it to the mice.

Our results demonstrate that ingestion of chow contaminated with non-viable *Lactococcus* is capable of passage through the intestinal tract and be displayed as the dominant bacteria within the feces in this model of antibiotic-ablation of the intestinal microbiome. It is possible for bacteria present in food to modulate intestinal microbiome studies. The

results of our study have paved the way for potential future studies of whether and/or to what extent food-derived, non-viable bacteria can alter intestinal physiology.

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