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Identifying Host Associated Bacteria Involved in Systemic DNA Damage and Diet-Induced Obesity

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by

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Chapter One: Identifying Host-Associated Intestinal Bacteria Involved in Systemic DNA Damage in a Mouse Model of Intestinal Inflammation.

ABSTRACT

Bacteria have been implicated as contributing factors in colorectal cancer (CRC), yet identifying the causal bacteria has remained challenging. To identify such organisms, we examined the host-associated bacteria in an inflammatory bowel disease (IBD) mouse model (*IL-10*$^{-/-}$) that exhibits increased levels of CRC and systemic DNA damage in the peripheral blood. High-throughput sequence analysis of 16S rRNA genes identified several intestinal bacteria that were differentially abundant between the *IL-10*$^{-/-}$ and control mice, including *Lactobacillus johnsonii*, *Lactobacillus animalis*, and *Bacteroides vulgatus*. These bacteria were tested for their ability to affect DNA damage in cell culture and animal experiments. Oral administration of the bacterium that was more abundant in the control mice (*L. johnsonii*) decreased systemic DNA damage in mice treated with dextran sodium sulfate (DSS) while the bacteria that were more abundant in the *IL-10*$^{-/-}$ mice increased DNA damage in cell culture (*L. animalis*, *B. vulgatus*) and wild type mice (*L. animalis*). We posit that examining the host-associated intestinal bacteria allowed us to identify these functionally important microorganisms, and we anticipate that this approach may be useful for identifying bacteria involved in other diseases where bacteria play a causal role.

INTRODUCTION

Intestinal bacteria have been implicated in cancer from as early as the 1960s. An estimated 18% of cancers are cause by infectious agents, but the majority of them are viruses (1). The most studied bacterium involved in cancer is *Helicobacter pylori* in gastric cancer. It is the only bacteria to be classified as a carcinogen by the International Agency for Research on Cancer (2).

Strong evidence supporting a role for intestinal bacteria in cancer comes from animal models where these microorganisms are altered or removed. There have been several studies in rats using different chemicals to induce CRC that have shown fewer tumors in animals that were germ free compared to the conventionally colonized controls (3-6). A similar result was shown with an azoxymethane (AOM)-dextran sodium sulfate (DSS) model of CRC, where tumors were reduced when an antibiotic was orally administered (7). In addition, similar results were obtained in genetically susceptible mouse models including $Apc^{Min/+}$ germ free mice (8) and $Nod1^{-/-}$ mice given antibiotics (9), which both developed fewer tumors than the control mice.

Bacterial involvement is not just associated with CRC. In a chemically induced liver cancer model, germ free mice or mice treated with antibiotics produced fewer tumors than the control mice (10). Similar results were obtained in another liver cancer model in which mice were chemically induced and fed a high fat diet, resulting in fewer liver tumors when the mice were treated with antibiotics compared to the control mice

(11). Breast cancer has also been shown to be affected by the intestinal microbiota, as germ free animals in a chemically induced rat model had fewer mammary tumors than the conventionally colonized control rats (12). Similar results were obtained with a chemically induced lung cancer and germ free rats (13).

In animal models, several individual bacteria have been shown to increase cancer risk or tumors in cancer prone mice and in chemically induced cancers. In the *Apc*$^{Min/+}$ mouse model, *Fusobacterium* (14) and an enterotoxigenic *Bacteroides fragilis* (15) were able to promote tumor formation compared to vehicle controls and a non-enterotoxigenic strain of *B. fragilis*. In a germ free *IL-10*$^{-/-}$ model, *Enterococcus faecalis* was able to induce DNA damage and cancer compared to a vehicle control due to superoxide production, which was shown through a mutant unable to produce superoxide (16). In an AOM *IL-10*$^{-/-}$ model, a genotoxic *E. coli* strain containing a polyketide synthase (pks) island was able to increase DNA damage and the number of tumors versus a pks deficient mutant and the vehicle control (17).

While some progress has been made in finding bacteria involved in carcinogenesis in animal models, in humans the data is mostly correlative. *Chlamydia* strains have been thought to be a risk factor for lung (18) and cervical (19) cancers because of epidemiological data showing previous infection leads to a higher risk of developing cancer. *Streptococcus bovis* has been correlated with CRC and found in tumors (20), but no causal link has been shown. *Fusobacterium* (21) and *E. coli* (22) have

also been associated with cancer as they are both found in higher levels in tumors than in healthy tissues in the same subjects.

Inflammatory bowel disease (IBD) is associated with higher risk for CRC, which appears to involve a bacterial and immune component (23). In IBD, there is a defective epithelial barrier allowing more contact between microbes and the host epithelium, which makes it likely that intestinal bacteria play a role in disease progression. In *IL-10*$^{-/-}$ mice, a model for IBD, spontaneous colitis develops and AOM treatment produces higher numbers of tumors compared to control mice (24). However, when these mice are in germ free conditions, colitis does not develop (25) and treatment with AOM does not produce tumors (24). In addition, also in the *IL-10*$^{-/-}$ model, systemic DNA damage was shown to occur due to inflammation (26), and bacteria likely play a role in this model because germ free animals do not develop colitis (25).

Identifying causal microorganisms in diseases with unknown etiologies, including IBD and CRC, has been challenging. We posit that greater success will be obtained by examining the bacteria closely associated with the host, because these organisms are more likely to influence host phenotypes given their proximity to the host. To test this hypothesis, we examined the host-associated bacteria in the small intestine and colon of *IL10*$^{-/-}$ and control mice using an Illumina sequence analysis.

METHODS

Mouse Experiments

Eight to ten week-old male C57BL/6 (B6), C3H/HeJ (wild type control mice), and C3Bir.129P2 (B6)-Il10^{tm1Cgn}/Lt (*IL-10*$^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All experiments were in accordance with the guidelines set by the University of California Riverside Institution Animal Care and Use Committee.

Host Associated Sampling

Host associated samples from the small intestine and colon of the mice were obtained as previously described (27). Briefly, small intestines and colons were longitudinally opened, washed three times with PBS, incubated with dithiothreitol (DTT) (MP Biomedicals, Santa Ana, CA) at 37^{o}C, and then the DTT-liberated host cells including epithelial cells and intraepithelial lymphocytes were separated from the tissue and collected using a 70-um filter. Finally, the intestinal epithelial cells were isolated by density gradient centrifugation, and these cells comprised our host-associated samples. A portion of these samples were frozen for DNA extraction for the culture-independent analysis of the host-associated bacteria while another portion was applied to Brucella Agar (Becton Dickinson, Franklin Lakes, NJ) supplemented with 5% defibrinated sheep blood (Colorado Serum Company, Denver, CO) (BBA) and incubated anaerobically at 37^{o}C for four days. The resulting bacterial growth was analyzed for a culture-based depiction of the bacteria from these host-associated samples.

DNA Extraction and Library Construction

DNA was extracted from the host-associated samples and the bacterial culture samples using the Power-Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) as described by the manufacturer with a 30-second beat-beating step using a Mini-Beadbeater-16 (BioSpec Products, Bartlesville, OK). Bacterial rRNA genes were amplified as previously described (Ruegger et al. 2014). Briefly, twenty-five microliter PCR amplification reactions were conducted in an MJ Research PTC-200 thermal cycler (Bio-Rad Inc., Hercules, CA). PCR primers targeted a region of the hypervariable V6 region of the 16S rRNA gene and contained a 14-bp barcode on the reverse primers. PCRs contained: 50 mM Tris (pH 8.3), 500 µg/mL bovine serum albumin, 2.5 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate (dNTP), 400 nM of forward PCR primer, 200 nM of each reverse PCR primer, 2.5 µL of DNA template, and 0.25 units JumpStart *Taq* DNA polymerase (Sigma-Aldrich, St. Louis, MO). The PCR reaction parameters were 94°C for 5 minutes; 35 cycles of 94°C for 20 seconds, 50°C for 20 seconds, and 72°C for 30 seconds, and followed by 72°C for 5 minutes. The PCR products were then purified with a MinElute 96 UF PCR Purification Kit (Qiagen, Valencia, CA) and quantified using a Nanodrop 2000c (Thermo Scientific, Valencia, CA). Libraries were constructed by combining an equal amount of the amplified rRNA genes from each sample. An Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA) was used for DNA sequencing.

Data Analysis

Sequence data was demultiplexed and poor quality reads removed using QIIME (28) as previously described (29). ANOVAs were also performed using QIIME.

Bacterial Isolation

Lactobacillus johnsonii and *Lactobacillus animalis* were isolated from stored frozen bacterial cultures obtained from the host-associated samples using Lactobacilli MRS Agar (Becton Dickinson, Franklin Lakes, NJ) at 37° under anaerobic conditions. *Bacteroides vulgatus* was isolated in the same manner using Brucella Agar (Becton Dickinson, Franklin Lakes, NJ) supplemented with 5% defibrinated sheep blood (Colorado Serum Company, Denver, CO) (BBA). The frozen cultures were streaked on plates and single colonies were isolated, re-streaked for single colonies, and the isolates were verified by sequence analysis of the 16S rRNA gene.

***In vitro* Bacterial Infection**

Human intestinal epithelial cells (C2BBe1) (ATCC, Manassas, VA) were seeded at $5x10^5$ per well in 6-well tissue culture plates, and allowed to grow for 3 days. Cells were then inoculated with PBS, *L. johnsonii*, *L. animalis* or *B. vulgatus* at a multiplicity of infection (MOI) of 80:1 determined by OD_{600} and incubated for 20 hours at 37°C with 5% CO_2. Cells were then washed twice with PBS and removed with 0.25% Trypsin-EDTA (Life Technologies, Grand Island, NY). Cells were then washed with PBS and centrifuged at 600g for 6 minutes at room temperature. Cells were fixed with 2.5%

paraformaldehyde (Electron Microscopy Services, Hatfield, PA) in PBS for 15 minutes at room temperature, washed with PBS, centrifuged at 600g for 6 minutes at room temperature and placed on ice for γ-H2AX staining.

In vivo Bacterial Infection

Ten to twelve week-old wild type B6 mice (Jackson Laboratories, Bar Harbor, ME) were administered 4% dextran sodium sulfate (DSS) (MP Biomedicals, Santa Ana, CA) in their drinking water for 5 days. Each day of the DSS-treatment, mice were also orally inoculated with *L. johnsonii, L. animalis* or PBS control at 10^9-10^{10} CFU bacteria per mouse. On the sixth day, the mice were euthanized using CO_2 and peripheral blood was collected by cardiac puncture into a tube containing 10-ul of 0.5 EDTA. Blood was then diluted with an equal volume of PBS and treated for 8 minutes in 10 volumes of ACK lysis buffer (0.85% NH_4CL, 10mM $KHCO_3$, 0.1mM Na_2EDTA) at room temperature followed by diluting with 10 volumes of PBS. The blood was then centrifuged at 500g for 10 minutes at room temperature and the pellet, containing the peripheral blood leukocytes (PBLs), was washed with PBS then incubated on ice for 10 minutes in PBS-1% BSA (Sigma-Aldrich, St. Louis, MO) containing a 1:10 dilution of Fc Block (BD Biosciences, San Jose, CA) before adding a 1:50 dilution of CD45-APC-Cy7 (BD Biosciences, San Jose, CA) and incubating on ice for 30 minutes. Cells were then washed with PBS-1% BSA and centrifuged at 600g for 6 minutes at 4° C. The pellet was then fixed in 2.5% paraformaldehyde in PBS for 15 minutes at room temperature,

washed with PBS-1% BSA, and then centrifuged at 600g for 6 minutes at 4° C and placed on ice for γ-H2AX staining.

γ-H2AX Detection

Cells were resuspended in PBS-1% BSA-0.4% saponin (Sigma-Aldrich, St. Louis, MO) for 10 minutes on ice, centrifuged at 600g for 6 minutes at 4°C and incubated with a 1:50 dilution of rabbit anti-γ-H2AX antibody (Millipore, Billerica, MA) in PBS-1% BSA-0.4% saponin for 60 minutes on ice. The cells were then washed once with PBS-1 % BSA and centrifuged at 600g for 6 minutes at 4° C and resuspended in a 1:200 dilution of Alexa 488 donkey anti-rabbit (Life Technologies, Grand Island, NY) in PBS-1% BSA-0.4% saponin for 30 minutes on ice. Cells were analyzed using a BD FACSCanto II Flow Cytometer (BD Biosciences, San Jose, CA) and FlowJo analysis software v.8.7.3 (TreeStar Software, Ashland, OR). Cell populations were determined by gating on CD45+ for PBLs and forward scatter vs. side scatter to identify lymphocytes or live cell gate for the Caco cells and measured for DNA damage using an anti-γ-H2AX antibody (Millipore, Billerica, MA).

RESULTS AND DISCUSSION

A bacterial rRNA gene analysis was used to examine the host-associated intestinal bacteria from a mouse model (*IL-10*$^{-/-}$) of inflammatory bowel disease, CRC and systemic DNA damage. This study focused on host-associated bacteria because we

posit that they are more likely to influence host phenotypes given their proximity to the host.

To identify individual bacteria that may contribute to the differential phenotypes exhibited by this model, we used a high throughput sequence analysis to identify bacterial 16S rRNA gene sequence types (phylotypes) whose population densities were both abundant (greater than 8% of the total sequencing reads) and statistically different between the *IL-10*$^{-/-}$ and wild type control mice in the small intestine and colon (Figures 1.1A and B and Table 1.1). Differences in specific bacteria between the two genotypes were observed in samples where the DNA was directly extracted from the host-associated samples (culture-independent), and from bacteria that were grown on BBA culture media that was inoculated with the host-associated samples (culture). In the small intestine, the most differentially abundant phylotype in the control mice had high sequence identity to *Mycoplasma wenyonii* (culture-independent) and *Lactobacillus johnsonii* (culture). In the small intestine, the most differentially abundant phylotype in the *IL-10*$^{-/-}$ mice had high sequence identity to *Lactobacillus animalis* (culture-independent and culture). In the colon, the most differentially abundant phylotype in the control mice had high sequence identity to *M. wenyonii* (culture-independent) and *L. johnsonii* (culture). In the colon, the most differentially abundant phylotype in the *IL-10*$^{-/-}$ mice had high sequence identity to *Bacteroides vulgatus* (culture-independent and culture). We were able to obtain pure cultures of all of these bacteria except for the *M. wenyonii* phylotype, which was therefore not studied in the following experiments. We predict that the intestinal bacteria

that are higher abundance in the *IL-10*$^{-/-}$ mice compared to the control mice play a causative role in the intestinal inflammation, CRC and systemic DNA damage exhibited by these mice and that the intestinal bacteria that are higher abundance in the control mice compared to the *IL-10*$^{-/-}$ mice are inhibiting these phenotypes. We focused our studies on the most abundant bacteria, because we posit that they are the most likely to influence the host.

To determine whether these bacteria may be contributing to the differing CRC phenotype exhibited by the *IL-10*$^{-/-}$ and control mice, we measured the abilities of these bacteria to damage DNA, an intermediate marker for cancer, in human colonic epithelial cells *in vitro* (C2BBe1) (Figure 1.2). These experiments used an anti-γ-H2AX antibody, which binds to a specific phosphorylation event associated with DNA double-strand breaks (30). *Lactobacillus johnsonii*, which was the most differentially abundant phylotype in the culture-based analysis of the small intestine and colon from the control mice, did not produce DNA damage compared to the vehicle control (PBS) when it was incubated with the C2BBe1 cells. Conversely, the most differentially abundant bacteria in the small intestine and colon of the *IL-10*$^{-/-}$ mice, *L. animalis* and one of the *B. vulgatus* isolates (BV12), caused significant DNA damage, while another *B. vulgatus* isolate (BV8) did not cause DNA damage. Both *B. vulgatus* isolates had 100% sequence identity to the 16S rRNA gene obtained by the high throughput rRNA sequence analysis. However, they were identified as being different strains based on their growth characteristics and rRNA-ITS gene sequences. Overall, these *in vitro* cell culture

experiments supported our predictions – that the differentially abundant intestinal bacteria in the *IL-10*$^{-/-}$ mice are contributing to the CRC phenotype while those in the control mice are inhibiting it.

To test the latter prediction, we performed an experiment with wild type B6 mice that were administered 4% DSS in their drinking water for five days and orally administered a bacterium once per day for the same five days. In this experiment, we examined *L. johnsonii*, which was the most differentially abundant cultured bacterium in the control mice. We then analyzed the lymphocytes from peripheral blood because DNA damage has been previously shown to be high in this cell type when mice are treated with DSS (26). Administration of *L. johnsonii* reduced the levels of DNA damage in the lymphocytes compared to the PBS vehicle control (Figure 1.3), which was consistent with our prediction.

Next, we tested whether the differentially abundant bacteria in the *IL-10*$^{-/-}$ mice can cause DNA damage in mice. In this experiment, we orally administered *L. animalis* to wild type B6 mice for two days, and then measured the amount of DNA damage in the peripheral blood leukocytes on the third day. Compared to the PBS vehicle control, *L. animalis* caused increased DNA damage, which was also consistent with our prediction (Figure 1.4).

A review of the scientific literature identified several studies that either corroborated or contradicted our results. In a study that used similar methods, and that came from the Borneman lab, *L. johnsonii* was shown to be in higher abundance in cancer resistant mice than in cancer prone mice, where isogenic *Atm*$^{-/-}$ mice harboring different intestinal bacteria caused this differential phenotype (31). When this bacterium was orally administered to the cancer-prone mice, it was able to reduce systemic DNA damage, which is consistent with the results from this current study. Conversely, two studies showed that *B. vulgatus* was more abundant in healthy human subjects than those with CRC (24, 32). We did not find any studies linking *L. animalis* or *M. wenyonii* with DNA damage (genotoxicity) or CRC.

Summary and Future Directions

We have demonstrated that our approach for identifying functionally important bacteria involved in systemic DNA damage shows considerable promise, because intestinal bacteria that were differentially abundant between the *IL-10*$^{-/-}$ and control mice were able to either increase or decrease DNA damage in cell culture and mouse experiments in a predictable manner. We posit that this successful outcome is a result of examining host-associated intestinal samples, which has two important attributes. First, it focuses the study on those microorganisms that are in closest proximity to the host, which is a feature that should enable them to have the biggest impact on the host. Second, by examining such a narrow habitat, it reduces the amount of abundant and statistically

different organisms to a very small number, which makes it easier to identify and validate the causal organisms.

Future work will include testing all of the bacteria that were differentially abundant between the *IL-10*$^{-/-}$ and control mice in the *in vivo* mouse experiments, which will include testing the *B. vulgatus* isolates. To examine the *M. wenyonii* phylotype, we will endeavor to culture this organism. We also plan on increasing the number of replicates in the experiments assessing DNA damage (Figures 1.2-4). To attempt to identify molecules that cause systemic DNA damage, we will perform a series of comparative "omics" experiments on the two *B. vulgatus* isolates, which are phylogenetically very similar but which exhibit differing abilities to affect DNA damage. We also plan on inoculating germ-free mice with our putatively pro- and anti-DNA damaging bacteria, and monitoring DNA damage and colitis over time. Finally, thus far, we have only examined the influence of our bacteria on an intermediate marker of cancer. We plan on determining how oral administration of our bacteria influence tumor formation in the AOM-DSS model, because recent results have shown that administration of antibiotics to mice that are exposed to three cycles of AOM-DSS dramatically reduces the number of colorectal tumors compared to a non-antibiotic control (33). If successful, we will be encouraged to use our method to identify relevant bacteria in animals or humans with diseases where bacteria appear to be contributing to the etiology including obesity (34), asthma (35), Alzheimer's (36), diabetes (37) and many more.

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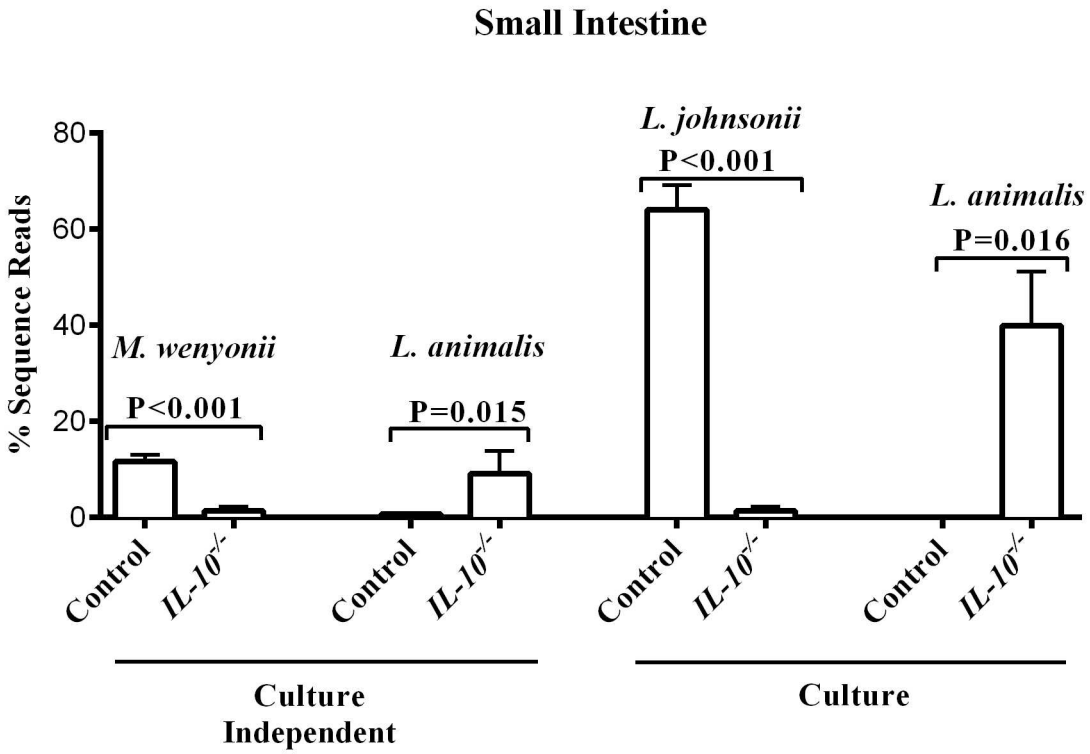
I thank Dr. Emma Wilson for her training and guidance in flow cytometry, and for generously allowing me to use her flow cytometer to conduct these studies. I also thank Drs. Moorthy Krishnan and Declan McCole for their assistance with the cell culture experiments.

A.


Small Intestine
% Sequence Reads
80
60
40
20
0
M. wenyonii
P<0.001
L. animalis
P=0.015
L. johnsonii
P<0.001
L. animalis
P=0.016
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IL-10-/-
Control
IL-10-/-
Control
IL-10-/-
Control
IL-10-/-
Culture Independent
Culture

B.

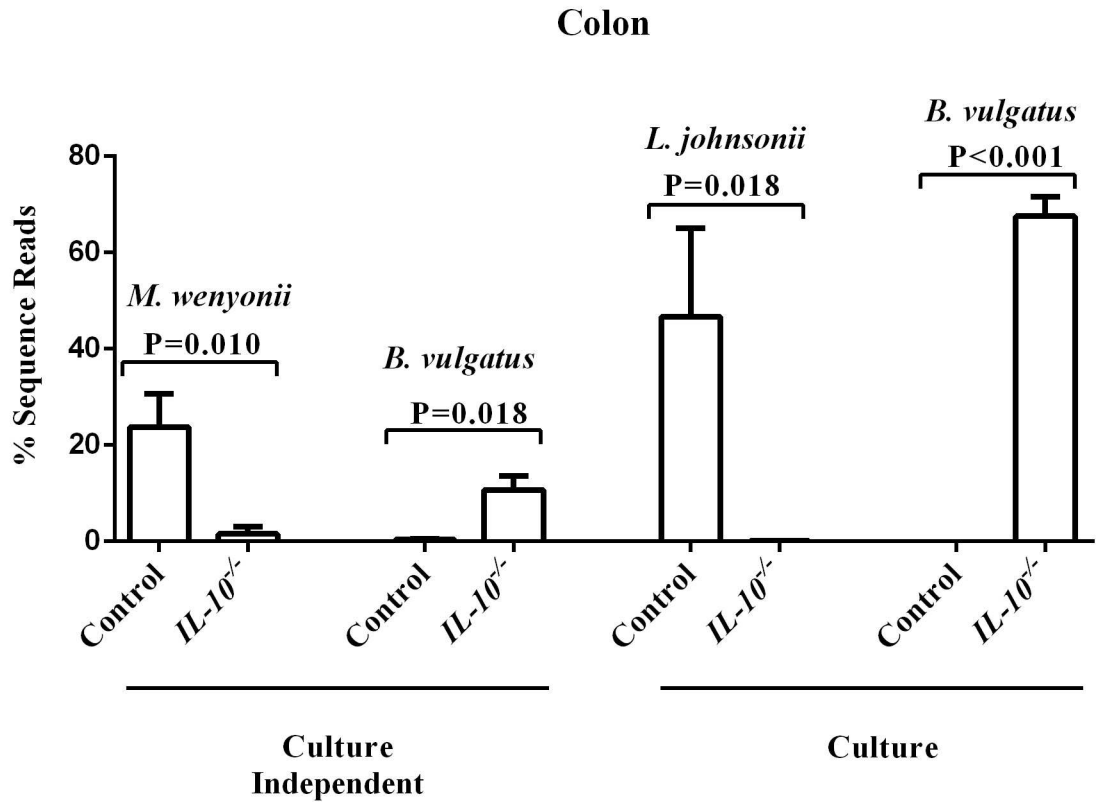

Figure 1.1. High Throughput Bacterial rRNA Sequence Analysis of Intestinal Bacteria in *IL-10*$^{-/-}$ and Control Mice. These graphs show the percentage of sequence reads of the most abundant (greater than 8% of the total sequencing reads) intestinal bacterial phylotypes that were statistically different bacteria between the *IL-10*$^{-/-}$ and control mice from culture based and culture independent analyses of the small intestine (A) and colon (B). Probability values are from two-sided Mann Whitney U-Tests (n=4-9). Experiments were performed at least two times. Standard error is indicated for each column.

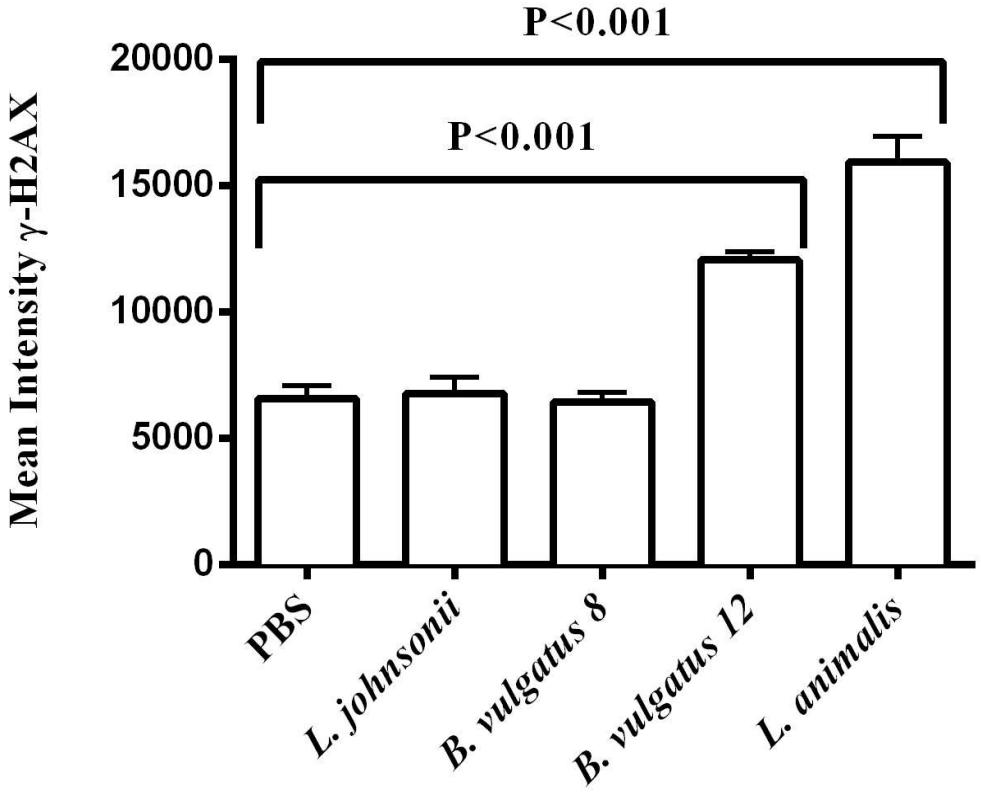

Figure 1.2. DNA Damage Caused by Bacteria in Cell Culture Experiments. Bacteria were incubated with human colonic epithelial cells (C2BBe1) for 20 hours and then DNA damage was measured by flow cytometry using an anti-γ-H2AX antibody. Probability values are from Kruskal-Wallis tests (n=2-3). Standard error is indicated for each column.

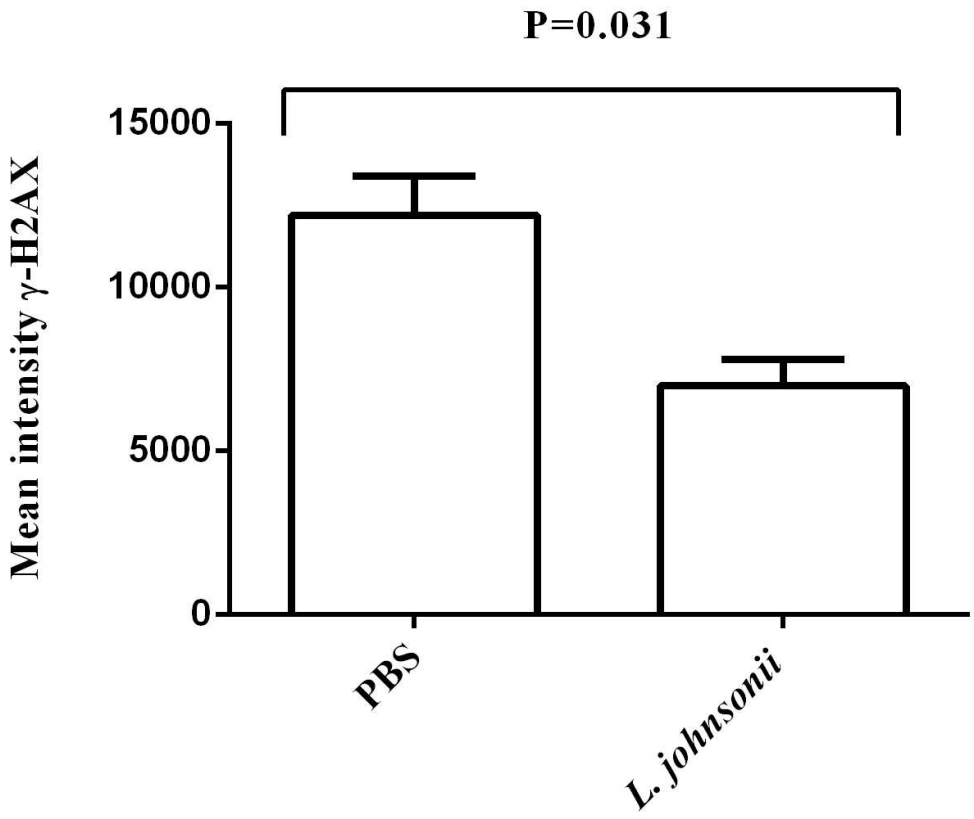

Figure 1.3. Attenuation of DNA Damage By *L. johnsonii* in Wild Type B6 Mice Treated with DSS. Wild type B6 mice were administered 4% DSS in their drinking water for 5 days and orally administered 10^{10} CFU of *L. johnsonii* or the PBS vehicle control for each of the same 5 days. On the sixth day, DNA damage of lymphocytes from peripheral blood was measured by flow cytometry using an anti-γ-H2AX antibody. The probability value was obtained by using a two-tailed Student's t-test (n=2-3). Standard error is indicated for each column.

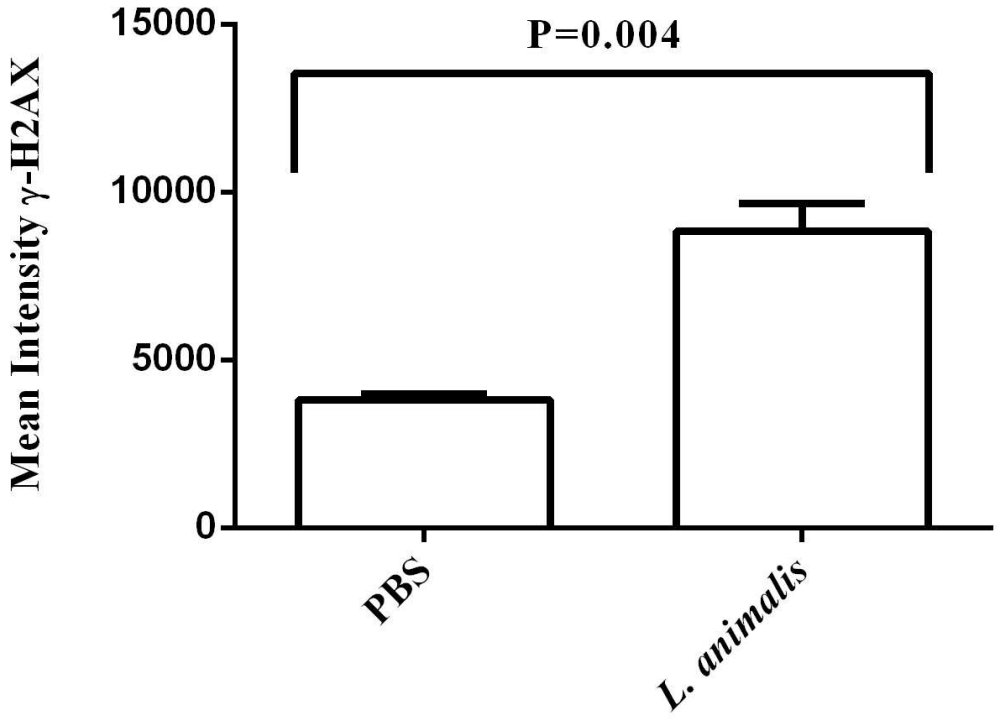

Figure 1.4. DNA Damage Caused by *L. animalis* in Wild Type B6 Mice. Wild type B6 mice were orally administered with 10^{10} CFU of *L. animalis* or the PBS vehicle control for 2 days. On the third day, DNA damage of peripheral blood leukocytes was measured by flow cytometry using an anti-γ-H2AX antibody. The probability value was obtained by using a two-tailed Student's t-test (n=2-3). Standard error is indicated for each column.

Table 1.1. Host associated bacteria that were differentially abundant between *IL-10*$^{-/-}$ and control mice.

Nearest Cultured Relative (accession) (% identity)*	**Intestinal Region**	**Method****	**Control (% reads)**	***IL-10*$^{-/-}$ (% reads)**	**P*****
Mycoplasma wenyonii (NR_074477) (100%)	Small Intestine	Culture-Independent	11.4%	1.4%	0.016
Lactobacillus animalis (KJ412485) (100%)	Small Intestine	Culture-Independent	0.7%	9.0%	0.016
Lactobacillus johnsonii (CP006811) (100%)	Small Intestine	Culture	64.0%	1.4%	0.016
Lactobacillus animalis (KJ412485) (100%)	Small Intestine	Culture	0.0%	39.6%	0.016
Mycoplasma wenyonii (NR_074477) (100%)	Colon	Culture-Independent	23.5%	9.4%	0.021
Bacteroides vulgatus (NR_074515) (100%)	Colon	Culture-Independent	0.9%	7.6%	0.011

Lactobacillus johnsonii (CP006811) (100%)	Colon	Culture	46.4%	0.0%	0.001
Bacteroides vulgatus (NR_074515) (100%)	Colon	Culture	0.0%	67.2%	0.001

*Some of the sequences had 100% identity to more than one bacterial species, but for simplicity, only one is listed.
**Small subunit rRNA gene sequences came from directly extracting DNA from the host-associated samples (Culture-Independent) or from the bacteria that grew on Brucella blood agar after inoculating the media with the host-associated samples (Culture).
***Probability values from two-sided Mann Whitney U-Tests. Experiments were performed at least two times. Numbers of mice ranged from 4 to 9.
Only those bacteria that were statistically different between the *IL-10*$^{-/-}$ and control mice and were comprised of more than 8% of the total sequence reads are shown.

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Chapter 2: Identifying Host-Associated Intestinal Bacteria Involved in Diet-Induced Obesity.

ABSTRACT

Obesity is becoming an epidemic that affects one-third of adults in the USA and which has multiple causes including genes, diet, activity level and intestinal bacteria. Using a diet-induced obesity model, we compared the host-associated intestinal bacteria of mice fed four different diets: a standard vivarium chow and three high-fat diets. Mice fed the vivarium chow gained significantly less weight over the course of the experiment compared to mice fed the high fat diets. By using a high-throughput sequence analysis of the rRNA-ITS gene region, we identified several bacteria, including *E. faecium*, *L. animalis*, SFB and *C. innocuum*, whose abundance varied by diet type. We predict that the intestinal bacteria that are higher in abundance in mice fed the high fat diets compared to mice fed the vivarium chow play a causative role in the diet-induced obesity observed in this experiment, and that the bacteria that are higher in abundance in mice fed the vivarium chow compared to mice fed the high fat diets inhibit obesity. Future Koch's postulates like experimentation using germ-free mice will be used to test these predictions.

INTRODUCTION

Obesity is a disease defined by an excess of body weight, which carries a risk of developing diabetes, heart failure, numerous cancers and other chronic health issues (1).

Obesity has become an epidemic over the last couple of decades, affecting one-third of adults and 17% of children in the USA (2). Obesity appears to be a complex disease with multiple components including genes, diet, activity as well as the intestinal microbiota, which can all influence each other (3, 4).

The human gut is a complex environment. Estimates suggest that the number of bacteria in the gut is about 10 times the total number of human cells in an individual (5). The bacteria in the gut play a role in a variety of host functions and are necessary for normal immune development, digestion and production of nutrients that humans are unable to make (6).

The link between microbes and obesity was demonstrated in a study using a diet-induced obesity model, which showed that germ free mice do not become obese (7). In addition, transfer of an entire microbiome by oral administration of feces to germ-free mice produced obese mice (8). Due to the large number of bacterial species in the intestines, it has been difficult to find a direct link between individual bacteria and diseases such as obesity.

We posit that examining intestinal bacteria closely associated with the host will allow us to identify bacteria that influence obesity. To test this hypothesis, we used high throughput sequencing of the rRNA-ITS region to examine host-associated intestinal bacteria of a diet-induced mouse model of obesity.

METHODS

Animals

Male C57/Bl6 mice were weaned at 3 weeks of age and assigned randomly to one of the four diets used in the study. The animals were maintained on a 12:12 hour light-dark cycle in a non-specific pathogen free vivarium at the University of California, Riverside (UCR). Three to four animals were housed per cage. Individual mouse weights were recorded once per week. Care and treatment of animals was in accordance with guidelines from the University of California Riverside Institutional Animal Care and Use Committee.

Diet Study

Three isocaloric diets were formulated in conjunction with Research Diets, Inc. (New Brunswick, NJ) and provided 5.56 kcal energy/gm. Our high fat diet (HFD) had 40% total fat with 36% of the fat calories from coconut oil and 4% from soybean oil. The oils used in diet formulation were standard commodity oils. In the high soybean oil diet (LAHFD), 15% of the calories from coconut oil were replaced with those from soybean oil to give a final concentration of 21% fat calories from coconut oil and 19% from soybean oil of which 10% were from linoleic acid (LA), the major polyunsaturated fatty acid (PUFA) present in soybean oil. Regular soybean oil used in LAHFD was replaced by an equal amount of genetically modified (GMO) high oleic soybean oil to formulate our Plenish high fat diet (Plen). The total amount of carbohydrates was constant across all the diets. Regular vivarium (Viv) chow (Purina Test Diet 5001, Newco Distributors,

Rancho Cucamonga, CA) was used as a low fat control. Diets were provided in pellet form, twice weekly for up to 24 weeks and the amount of food consumed was monitored.

Host Associated Sampling

Host associated samples from the small intestine and colon of the mice were obtained as previously described (9). Briefly, small intestines and colons were longitudinally opened, washed three times with PBS, incubated with dithiothreitol (DTT) (MP Biomedicals, Santa Ana, CA) at 37^{o}C, and then the DTT-liberated host cells including epithelial cells and intraepithelial lymphocytes were separated from the tissue and collected using a 70-um filter. Finally, the intestinal epithelial cells were isolated by density gradient centrifugation, and these cells comprised our host-associated samples. A portion of these samples were frozen for DNA extraction for the culture-independent analysis of the host-associated bacteria while another portion was applied to Brucella Agar (Becton Dickinson, Franklin Lakes, NJ) supplemented with 5% defibrinated sheep blood (Colorado Serum Company, Denver, CO) (BBA) and incubated anaerobically at 37^{o}C for four days. The resulting bacterial growth was analyzed for a culture-based depiction of the bacteria from these host-associated samples.

DNA Extraction and Library Construction

DNA was extracted from the host-associated samples and the frozen bacterial culture samples using the Power-Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) as described by the manufacturer with a 30-second beat-beating step using

a Mini-Beadbeater-16 (BioSpec Products, Bartlesville, OK). Bacterial rRNA-ITS regions were amplified as previously described (10). Briefly, twenty-five microliter PCR amplification reactions were conducted in an MJ Research PTC-200 thermal cycler (Bio-Rad Inc., Hercules, CA). PCR primers targeted the rRNA-ITS region and contained a 14-bp barcode on the reverse primers. PCRs contained: 50 mM Tris (pH 8.3), 500 µg/mL bovine serum albumin, 2.5 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate (dNTP), 400 nM of forward PCR primer, 200 nM of each reverse PCR primer, 2.5 µL of DNA template, and 0.25 units JumpStart *Taq* DNA polymerase (Sigma-Aldrich, St. Louis, MO). The PCR reaction parameters were 94°C for 5 minutes; 35 cycles of 94°C for 20 seconds, 50°C for 20 seconds, and 72°C for 30 seconds, and followed by 72°C for 5 minutes. The PCR products were then purified with a MinElute 96 UF PCR Purification Kit (Qiagen, Valencia, CA) and quantified using a Nanodrop 2000c (Thermo Scientific, Valencia, CA). Libraries were constructed by combining an equal amount of amplified rRNA genes from each sample. An Illumina MiSeq (Illumina, Inc., San Diego, CA) was used for DNA sequencing.

Data Analysis

Sequence data was demultiplexed and poor quality reads removed using QIIME (11) as previously described (10). ANOVAs were also performed using QIIME.

RESULTS AND DISCUSSION

A bacterial rRNA-ITS gene analysis was used to examine the host-associated intestinal bacteria of a diet-induced mouse model of obesity. We focused our study on the host-associated bacteria, because we posit that their proximity to the host enables them to have the greatest impact on the host.

To identify individual bacteria that may contribute to the differential phenotypes exhibited by this model, we used a high throughput sequence analyses to identify bacterial rRNA-ITS sequence types (phylotypes) whose population densities were both abundant (greater than 5% of the total sequencing reads) and statistically different between the diets (Figure 2.1A-D, Table 2.1). Mice fed any of the three high fat diets (LAHFD, HFD and Plen) gained significantly more weight over the 23-week study than mice fed the standard vivarium chow (Viv) with no difference in calorie consumption (Figure 2.2). Differences in specific bacteria between the diets were observed in samples where the DNA was directly extracted from the host-associated small intestine and colon samples (culture-independent), and from bacteria that were grown on BBA culture media that was inoculated with the host-associated samples (culture). In the small intestine, the most differentially abundant phylotype in the Viv-fed mice had high sequence identity to *Candidatus Arthromitus* Segmented Filamentous Bacteria (SFB) (culture-independent) and *Lactobacillus animalis* (culture). In the small intestine, the most differentially abundant phylotype in mice fed any of the three high fat diets had high sequence identity to a *Ureaplasma* sp. (culture-independent) and *Enterococcus faecium* (culture). In the

colon, the most differentially abundant phylotype in the Viv-fed mice had high sequence identity to *Barnesiella viscericola* (culture-independent) and *L. animalis* (culture). In the colon, the most differentially abundant phylotype in mice fed any of the three high fat diets had high sequence identity to *Pseudomonas putida* (culture-independent) and *Parabacteroides goldsteinii* (culture). Several other differentially abundant bacteria associated with the high fat diets included *Clostridium innocuum*, *Escherichia coli*, *Propionibacterium acnes*, and *Lactobacillus johnsonii* (Table 2.1). One other differentially abundant bacterium that was associated with vivarium diet was *Eubacterium rectale* (Table 2.1).

Several of the intestinal bacteria that we identified in this study have been previously associated with obesity. Close relatives of our putative pro-obesity bacterium, *C. innocuum*, which is a member of the family Erysipelotrichaceae, have been associated with or causally linked to obesity. A study examining different diets showed that obese animals exhibited a large increase in members of the Erysipelotrichaceae, most closely related to *Eubacterium dolichum, E. cylindroides, and E. biforme* (12). In a germ-free study, another member of the Erysipelotrichaceae, *Clostridium ramosum*, was shown to increase mouse weight compared to inoculation with eight common intestinal bacteria, when both bacterial treatments were combined with a high fat diet (13). In the same study, these authors also showed that mice gained more weight when *C. ramosum* was co-inoculated with the eight common intestinal bacteria compared to the eight bacteria without *C. ramosum*.

Other investigations that identified bacteria with similar trends as our study included a lymphotoxin knockout mouse model, which was resistant to diet-induced obesity and which led to an increase in segmented filamentous bacteria (14). In addition, *E. coli* was found to be higher in abundance in overweight preschool children than their leaner counterparts (15).

Investigations that identified bacteria with opposite trends of our study included one that showed that a high protein-low carbohydrate diet, which promoted weight loss, was associated with a decrease in *Eubacterium rectale* (16). One other study showed that *E. faecium* in combination with other bacteria reduced adipocyte circumference in rats (17).

We did not find prior studies relating weight gain/loss to *L. animalis, B. viscericola, P. putida, P. goldsteinii, L. johnsonii, P. acnes or Ureaplasma.*

Summary and Future Directions

We used a high throughput sequence analysis of host-associated intestinal samples from mice that were fed different diets to identify bacteria that were associated with varying levels of body weight. We predict that the intestinal bacteria that are higher abundance in mice fed any of the high fat diets compared to mice fed the vivarium chow play a causative role in the diet-induced obesity observed in this experiment, and that the

bacteria that are higher in abundance in mice fed the vivarium chow compared to mice fed any of the high fat diets inhibit obesity.

Future research will include performing Koch's postulates like experiments in germ-free mice to test the aforementioned predictions. Toward this goal, thus far, we have been able to obtain pure cultures of *E. faecium*, *L. animalis*, and *C. innocuum*.

ACKNOWLEDGEMENTS

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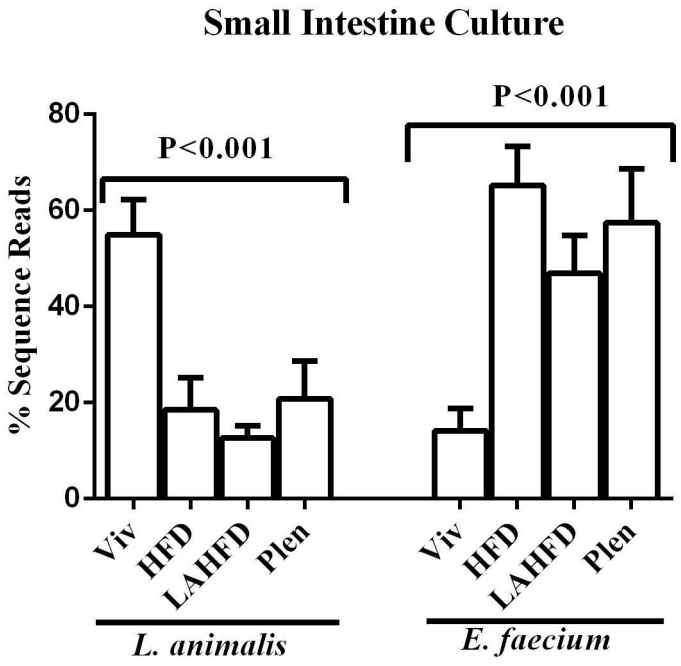
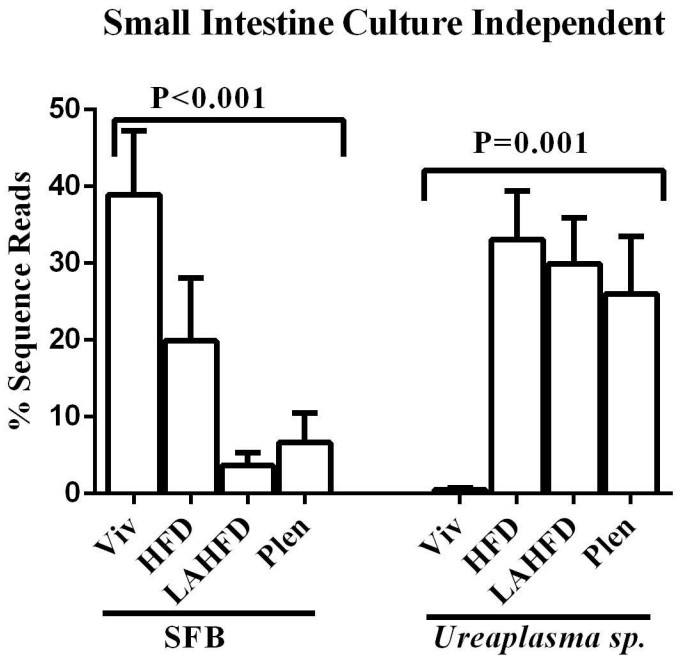
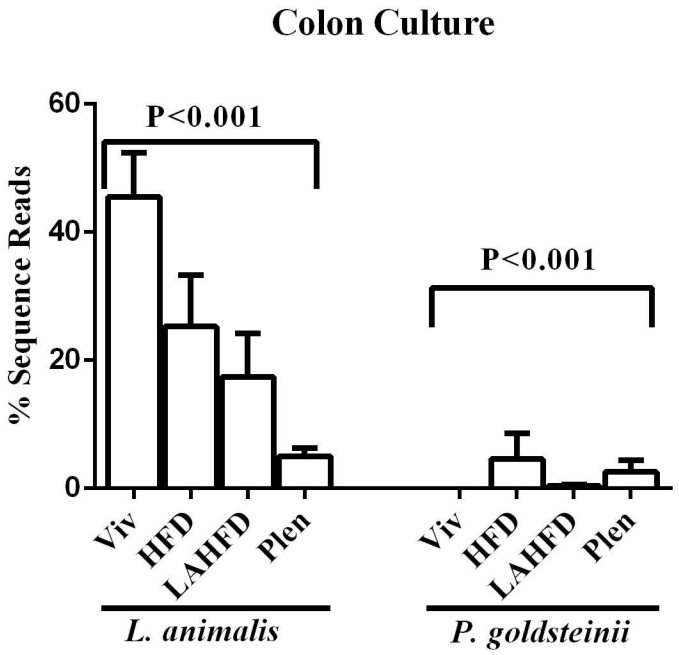
A.


Small Intestine Culture
P<0.001
P<0.001
% Sequence Reads
80
60
40
20
0
Viv
HFD
LAHFD
Plen
Viv
HFD
LAHFD
Plen
L. animalis
E. faecium

B.


Small Intestine Culture Independent
P<0.001
P=0.001
% Sequence Reads
50
40
30
20
10
0
Viv
HFD
LAHFD
Plen
Viv
HFD
LAHFD
Plen
SFB
Ureaplasma sp.

C.


Colon Culture
% Sequence Reads
60
40
20
0
P<0.001
P<0.001
Viv
HFD
LAHFD
Plen
Viv
HFD
LAHFD
Plen
L. animalis
P. goldsteinii

D.

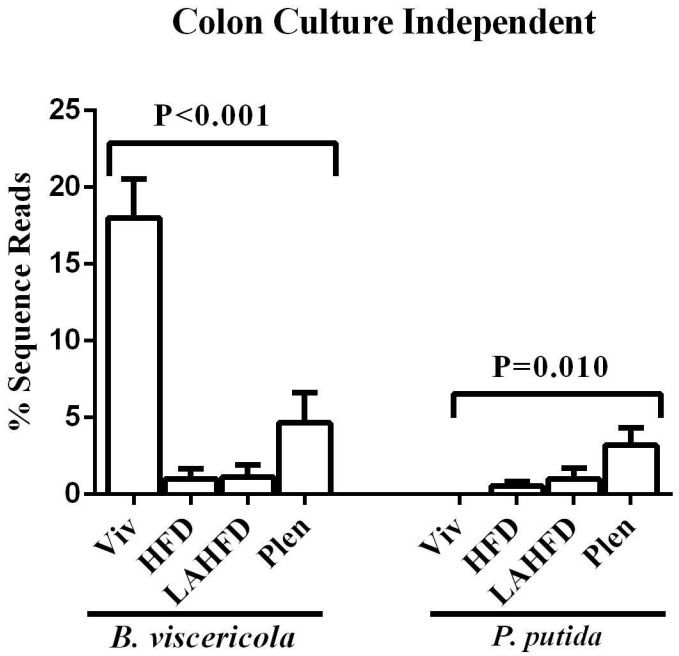

Colon Culture Independent
% Sequence Reads
25
20
15
10
5
0
P<0.001
P=0.010
Viv
HFD
LAHFD
Plen
Viv
HFD
LAHFD
Plen
B. viscericola
P. putida

Figure 2.1. High Throughput Bacterial rRNA-ITS Sequence Analysis of Intestinal Bacteria from Mice Fed Four Different Diets. These graphs show the percentage of sequence reads of the most abundant intestinal bacterial phylotypes that were statistically different ($P<0.05$) between mice fed four different diets: standard vivarium chow (Viv), high coconut fat diet (HFD), high soy bean oil diet (LAHFD), and high oleic soybean oil diet (Plen). These values were compared by the Kruskal-Wallis test (nonparametric ANOVA) using Dunn's test for multiple comparison. n = 11-12 mice for each of the four diets. Standard error is indicated for each column. The intestinal habitats examined and sampling methods used were: A (small intestine, culture), B (small intestine, culture-independent), C (colon, culture), and D (colon, culture-independent).

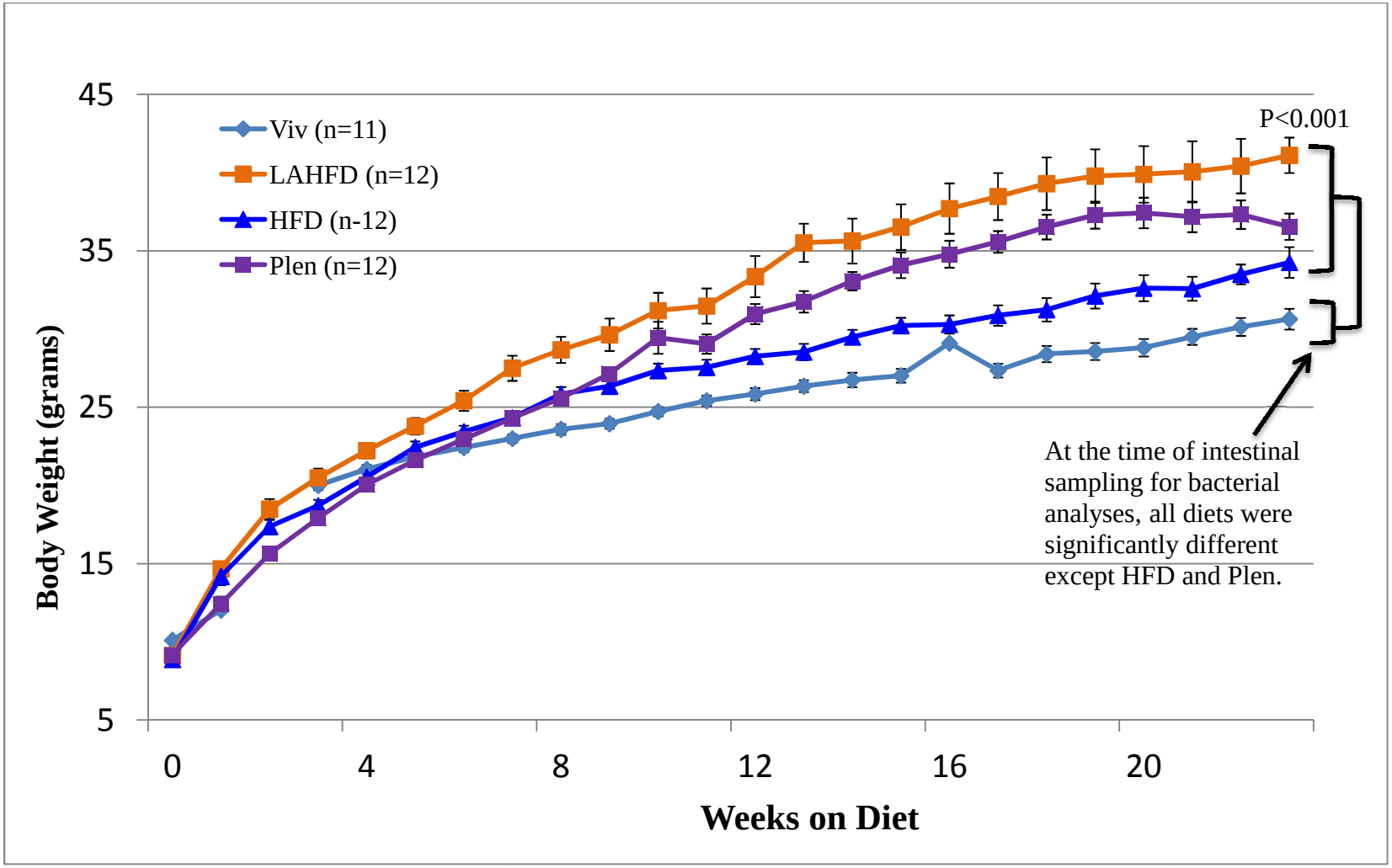

45
35
25
15
5
Body Weight (grams)
0
4
8
12
16
20
Weeks on Diet
Viv (n=11)
LAHFD (n=12)
HFD (n-12)
Plen (n=12)
P<0.001
At the time of intestinal sampling for bacterial analyses, all diets were significantly different except HFD and Plen.

Figure 2.2. Diet-Induced Changes in Mouse Weight Over Time. C57/BL6 mice were fed one of four different diets for 23 weeks and weight was measured once per week throughout the experiment. The weight values at the end of the experiment were compared by the Kruskal-Wallis test (nonparametric ANOVA) using Dunn's test for multiple comparison. n = 11-12 mice for each of the four diets. Diets were: standard vivarium chow (Viv), high coconut fat diet (HFD), high soybean oil diet (LAHFD), and high Plenish oil diet (Plen).

Table 2.1. Bacteria differentially abundant in the small intestine and colon of mice fed four different diets.

Nearest Cultured Relative (accession) (% identity/coverage)	**Intestinal Region**	**Method**	**LAHFD**	**Plen**	**VIV**	**HFD**	**P**
Enterococcus faecium (2558860813.AISM01000028) (100%/100%)	Small Intestine	Culture	46.8ab	57.4ac	14.2	65.2bc	0.000
Lactobacillus animalis (AY526615) (98%/100%)	Small Intestine	Culture	12.6ab	20.8ac	54.9	18.5bc	0.003
Clostridium innocuum (2534682210.AGYV01000003) (85%/70%)	Small Intestine	Culture	1.8ab	3.7	0.0ac	0.2bc	0.001
Lactobacillus animalis (AY526615) (97%/100%)	Colon	Culture	17.4abc	5.0ad	45.5be	25.3cde	0.001
Parabacteroides goldsteinii (EU136697) (99%/100%)	Colon	Culture	0.4ab	2.6ac	0.0	4.6bc	0.000
Ureaplasma sp. (JF731007) (100%/78%)	Small Intestine	Culture-Independent	29.9a	26.0a	0.5	33.1a	0.000
Candidatus Arthromitus sp. SFB (AP012209) (100%/100%)	Small Intestine	Culture-Independent	3.6ab	6.7ac	38.9d	19.9bcd	0.001
Escherichia coli (CP008801) (100%/100%)	Small Intestine	Culture-Independent	3.0a	3.8a	0.6	3.7a	0.001
Escherichia coli (CP008957) (100%/100%)	Small Intestine	Culture-Independent	3.0ab	3.3ac	0.4d	2.1bcd	0.000
Propionibacterium acnes (CP003195) (100%/100%)	Small Intestine	Culture-Independent	2.7ab	2.1ac	0.4d	1.5bcd	0.000
Lactobacillus johnsonii (CP006811) (100%/100%)	Small Intestine	Culture-Independent	2.5abc	3.7ad	0.1be	1.2cde	0.011
Eubacterium rectale (CP001107) (84%/100%)	Small Intestine	Culture-Independent	0.7ab	2.5acd	6.2c	0.5bd	0.001

Barnesiella viscericola (CP007034) (94%/35%)	Small Intestine	Culture-Independent	0.6a	0.7a	7.5	0.7a	0.001
Eubacterium rectale (CP001107) (84%/100%)	Small Intestine	Culture-Independent	0.7ab	2.5acd	6.2c	0.5bd	0.001
Barnesiella viscericola (CP007034) (94%/35%)	Colon	Culture-Independent	1.1a	4.7a	18.0	1.0a	0.000
Barnesiella viscericola (CP007034) (94%/35%)	Colon	Culture-Independent	0.2a	0.1a	10.2	0.2a	0.000
Barnesiella viscericola (CP007034) (94%/35%)	Colon	Culture-Independent	0.1a	2.6a	3.3	0.1a	0.000

Diets were: standard vivarium chow (Viv), high coconut fat diet (HFD), high soybean oil diet (LAHFD), and high oleic soybean oil diet (Plen). Values in diet columns are mean-percent bacterial rRNA-ITS reads from an Illumina sequence analysis. These values were compared by the Kruskal-Wallis test (nonparametric ANOVA), using Dunn's test for multiple comparison; mean % sequencing reads with the same letter in each row are not significantly different ($P > 0.05$). n = 11-12 mice for each of the four diets. Bacteria with the same name have different rRNA-ITS sequences. Only those bacteria that were statistically different between at least two of the diets and that were comprised of more than 5% of the total sequence reads are shown.

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