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Artemisia supplementation differentially affects the mucosal and luminal ileal microbiota of diet-induced obese mice

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Abstract

Objective—The gut microbiome has been implicated in obesity and metabolic syndrome; however, most studies have focused on fecal or colonic samples. Several species of *Artemisia* have been reported to ameliorate insulin signaling both in vitro and in vivo. The aim of this study was to characterize the mucosal and luminal bacterial populations in the terminal ileum with or without supplementation with *Artemisia* extracts.

Materials/Methods—Following 4 weeks of supplementation with different *Artemisia* extracts (PMI 5011, Santa or Scopa), diet-induced obese mice were sacrificed and luminal and mucosal samples of terminal ileum were used to evaluate microbial community composition by pyrosequencing of 16S rDNA hypervariable regions.

Results—Significant differences in community structure and membership were observed between luminal and mucosal samples, irrespective of diet group. All *Artemisia* extracts increased

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the Bacteroidetes:Firmicutes ratio in mucosal samples. This effect was not observed in the luminal compartment. There was high inter-individual variability in the phylogenetic assessments of the ileal microbiota, limiting the statistical power of this pilot investigation.

Conclusions—Marked differences in bacterial communities exist dependent upon the biogeographic compartment in the terminal ileum. Future studies testing the effects of *Artemisia* or other botanical supplements require larger sample sizes for adequate statistical power.

Keywords

microbiota; PMI 5011; obesity; insulin resistance

1. Introduction

Humans host 10^{13} - 10^{14} microorganisms in the gastrointestinal tract, collectively containing 100 fold more genes than the mammalian cells [1]. Experiments in germ-free mice suggest a key role of the gut microbiota in regulating fat mass and insulin resistance [2].

Transplantation of the microbiota from obese *ob/ob* mice into lean mice induces greater dietary energy harvesting and more fat gain than transplantation from lean donor mice [3]. Studies in both humans and mice have identified a correlation between obesity and the Bacteroidetes:Firmicutes ratio, with higher proportions of Firmicutes associated with obesity [3-5].

The biogeography of the gastrointestinal microbiota directly impacts host responses. Commonly, microbiota studies have either used stool samples or cecal contents; however, the small intestine is the primary site of fat absorption. It contains fewer bacteria than the large intestine, but is the site of the greatest concentration of immune cells in the body. The ileum may thus play a key role in maintenance of systemic homeostasis through the mucosal associated immune system [6]. Further, a survey of host transcriptional modulation by the digestive tract microbiota identified the ileum as the region with the most significant microbiota-induced gene expression [7].

The gut microbiota resides within both the intestinal lumen and the mucosal layer of the intestinal wall; however, there has been little study of the difference between luminal and mucosal bacterial populations. Mucosal-associated bacterial communities in murine studies have higher *Mucispirillum* and *Lactobacillus* genera counts and lower frequencies of the *Alistipes* relative to the luminal compartment [8]. The close spatial association of mucosal bacteria and the epithelial cells of the intestine suggests an important role in intestinal homeostasis and health [9-11].

We report here our preliminary characterization of the luminal and mucosal populations of the terminal ileum in mice fed a high fat diet, with or without supplementation by different *Artemisia* extracts. The extracts, PMI 5011, Santa and Scopa, have been shown to improve metabolic parameters in vivo [12-16]. We found pronounced differences in luminal and mucosal populations, accompanied by substantial inter-individual variability. In order to probe the role of *Artemisia* in future studies, it will be necessary to use large sample sizes.

2. Methods

2.1 Animal Studies and Botanical Extracts

Animal studies were conducted at Pennington Biomedical Research Center's AALAC-approved facility, and were approved by the Institutional Animal Care and Use Committee. C57BL/6J-60% animals (18 weeks old, 5 per group) were randomized into 4 groups (1-Control, 2-PMI-5011, 3-Santa, 4-Scopa), single housed and fed assigned diets (60% fat Research Diet #D12492, +/- botanical extract) ad libitum for 28 days. PMI 5011, Santa and Scopa extracts were prepared at Rutgers University, provided by the Botanical Research Center of Rutgers/PBRC, and mixed in diet as detailed in [14-16].

2.2 Sampling of Microbiota in the Terminal Ileum

The luminal microbiota was collected by expressing the contents from the transected 4-5cm portion of the terminal ileum. The ileum was then opened longitudinally and overt residual fecal material removed. The mucosal compartment was sampled by scraping the ileal mucosa with a DNA-free glass slide. Samples were immediately snap frozen in liquid nitrogen and stored at -80C.

2.3 DNA Isolation and 16S rDNA hypervariable region PCR Amplification

DNA was isolated from luminal and mucosal samples using the QIAamp DNA stool mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The 16S rDNA hypervariable regions V3-V5 were PCR amplified using barcoded, gene-specific primers (357F 5'-CCTACGGGAGGCAGCAG-3' and 926R 5'-CCGTCAATTCMTTTRAGT-3') adapted for Roche GS FLX Titanium sequencing using Taq DNA Polymerase High Fidelity kits (Invitrogen) followed by library preparation (GS FLX Titanium LV emPCR Kit; GS Titanium Sequencing Kit XLR70; Roche).

2.4 Pyrosequencing and Primary Data Analysis

Pyrosequencing was performed on a Roche 454 GS FLX+ by the LSU Microbial Genomics Core Facility. After on-machine amplicon processing, 759,563 reads were obtained and processed using the QIIME 1.6 pipeline (<http://qiime.org>) [17]. Reads < 250 bp or > 600bp, those with > 6 ambiguous bases, homopolymer runs of more than 6 bases, mean quality score < 25 or a non-matching primer sequence were discarded. Denoising, Operational Taxonomic Unit (OTU) picking at 97% identity, chimera removal and taxonomic assignment were performed using QIIME scripts and workflows. Taxonomic classification was performed down to the species level (where available) using the RDP (Ribosomal Database Project) tool [18].

2.5 Taxa Composition and Species Diversity Analysis

OTUs were summarized by experimental group (Control, PMI 5011, Santa, Scopa), and/or site of collection (luminal or mucosal). The relative abundance of each OTU was examined at the Phylum, Class, Order, Family, Genus and Species levels. Alpha (within a community) and beta (between communities) diversity metrics as well as taxonomic community assessments were produced using QIIME scripts.

For alpha diversity, rarefaction curves were produced by plotting Shannon's diversity metric against the number of sequences considered from a sample. Subsequent analysis of diversity was performed at a depth of 2600 sequences per sample. Statistical significance was compared using a non-parametric permutation test for a pairwise comparison of categories. All *p*-values were Bonferroni-corrected for multiple testing.

For beta diversity, principle coordinates analysis (PCoA) plots were produced, using both weighted (considers abundance of each species) and unweighted (considers presence or absence of each species) UniFrac metrics [19]. Statistical significance was assessed with a non-parametric permutation test to compare a chosen category. Beta diversity was further assessed by hierarchical clustering using Unweighted Pair Group Method with Arithmetic mean (UPGMA). Results were viewed using FigTree software (<http://tree.bio.ed.ac.uk/software/figtree/>).

ANOVA was used to test for differences in relative abundance of specific OTUs for each group using the QIIME pipeline. We calculated *p*-values, Bonferroni corrected *p*-values, and False Discovery Rate corrected values. An unweighted *g* test was used to analyze the presence/absence of OTUs across categories.

3. Results

Individual OTU tables for each sample show substantial variability between samples (Fig. 1A); however, consistent with other reports, the primary phylum are *Bacteroidetes* and *Firmicutes* in all samples. *Actinobacteria* and *Proteobacteria* are also well-represented in mucosal samples.

The ratio of the phyla *Bacteroidetes*:*Firmicutes* has been used as an indicator of microbial imbalance associated with a high fat diet [3, 20, 21]. All three *Artemisia* extracts increased the mean mucosal, but not luminal, *Bacteroidetes*:*Firmicutes* ratio (Fig. 1B); however, results were not significant due to small sample size (*n*=5) and interindividual variability.

The diversity of the gut microbiome within each sample was examined using the Shannon alpha diversity metric. No botanical-mediated difference in alpha diversity was observed (data not shown). However, significantly greater diversity was found within the mucosal compartment relative to the luminal microbiota (*p*=0.002) (data not shown).

Similarly, the unweighted UniFrac metric showed significant differences in beta diversity (*p*=0.00001) and community structure by hierarchical clustering between the luminal and mucosal compartments (Fig. 1C,D). No significant differences in beta diversity were identified based upon botanical supplementation.

There are substantial differences in both abundance (data not shown) and presence of specific bacterial groups between the mucosal and luminal microbiota. Unweighted *g* tests reveal that there are numerous OTUs that occupy only one of these two niches (Table 1, Bonferroni-corrected *p* values less than 0.05 shown only).

Discussion

In summary, this pilot study did not detect significant differences in microbial populations as a result of *Artemisia* supplementation. However, promising trends, such as a lower Bacteroidetes:Firmicutes ratio, were present in mucosal samples that may prove interesting in future studies with larger cohorts. We found significant differences between luminal and mucosal microbial populations within the terminal ileum. The mucosal population was more diverse, and many OTUs were present only in luminal or mucosal niches. Further, for the OTUs common to both compartments, abundance varied dramatically between compartments. This grouping probably reflects different metabolic niches and substrate availability at luminal and mucosal sites.

The role of the microbiota in the terminal ileum remains largely undescribed, with most work focusing on cecal microbiota. However, gene transcription related to innate immunity and lipid and fatty acid absorption is highly responsive to the microbiota within the ileum [7]. Our results suggest that the mucosal microbiota of the terminal ileum may be a worthwhile target in efforts to modulate metabolism. Mucosal bacteria are in closer contact to epithelial cells of the ileum than luminal bacteria. It has also been suggested that, by forming adherent biofilm populations, mucosal populations may have distinctly different capacities for substrate oxidation, and may be able to modulate inflammation [11].

The primary limitation of this pilot study is the substantial interindividual variability. Due to this unexpectedly high variability, our group size was insufficient to detect statistically significant effects of *Artemisia* supplementation. This variability may be partially explained by single housing the mice; co-housed mice are coprophagic and, thus, maintain a more similar microbiota. However, one of the few studies to directly compare microbiota in different intestinal segments also found marked variability in duodenum, jejunum and ileum, but tight clustering in cecum and colon, potentially as a result of different biomass [7]. Overall, the high diversity of mucosal samples suggests that it may be a productive target for modulation by *Artemisia* extracts; however, larger sample sizes will be essential for future experiments.

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Abbreviations

Santa	<i>Artemisia santafolia</i> extract
Scopa	<i>Artemisia scoparia</i> extract
PMI 5011	<i>Artemisia dracunculus</i> extract

OTU	Operational Taxonomic Unit
RDP	Ribosomal Database Project
PCoA	principal coordinates analysis
UPGMA	Unweighted Pair Group Method with Arithmetic mean

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Figure 1.

Community structure varies significantly in luminal and mucosal subcompartments. A) Class level taxonomic groupings for each sample. C- Control, P- PMI 5011, Sa- Santa, Sc- Scopa. B) Mean ratio of Bacteroidetes:Firmicutes for each botanical grouping in mucosal samples. C) Principle coordinates analysis of beta-diversity in luminal and mucosal samples. D) Hierarchical clustering using the UPGMA method for luminal and mucosal samples.

Table 1Unweighted G-test comparing presence/absence of OTUs in luminal or mucosal compartments¹

OTU	G value	G probability	Bonferroni corrected	Site	Consensus Lineage
836	52.9	1.92E-11	4.79E-09	Lumen	None
1005	35.8	8.46E-08	2.11E-05	Lumen	None
1157	35.8	8.46E-08	2.11E-05	Lumen	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__S24-7; g__; s__
992	32.1	4.98E-07	0.000125	Lumen	None
1265	32.1	4.98E-07	0.000125	Lumen	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__
809	32.1	4.98E-07	0.000125	Lumen	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__
4	28.9	2.40E-06	0.000599	Lumen	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales
1053	28.9	2.40E-06	0.000599	Lumen	None
1158	25.9	9.86E-06	0.002464	Lumen	None
1285	25.9	9.86E-06	0.002464	Lumen	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__; s__
795	25.9	9.86E-06	0.002464	Lumen	None
131	25.9	9.86E-06	0.002464	Lumen	None
812	25.9	9.86E-06	0.002464	Lumen	k__Bacteria
110	23.3	3.58E-05	0.00894	Lumen	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Ruminococcus; s__
581	23.3	3.58E-05	0.00894	Lumen	None
905	23.3	3.58E-05	0.00894	Lumen	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae
764	20.8	0.000117	0.029233	Lumen	None
325	20.8	0.000117	0.029233	Lumen	None
921	52.9	1.92E-11	4.79E-09	Mucosal	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Faecalibacterium
395	52.9	1.92E-11	4.79E-09	Mucosal	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Roseburia; s__
991	52.9	1.92E-11	4.79E-09	Mucosal	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Lactococcus; s__
1233	45.0	9.09E-10	2.27E-07	Mucosal	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Dialister
821	45.0	9.09E-10	2.27E-07	Mucosal	None
504	39.8	1.18E-08	2.96E-06	Mucosal	None
673	39.8	1.18E-08	2.96E-06	Mucosal	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides; s__plebeius
1125	39.8	1.18E-08	2.96E-06	Mucosal	None
1161	39.8	1.18E-08	2.96E-06	Mucosal	None
5	35.5	9.69E-08	2.42E-05	Mucosal	None
534	35.5	9.69E-08	2.42E-05	Mucosal	None
758	35.5	9.69E-08	2.42E-05	Mucosal	None

OTU	G value	G probability	Bonferroni corrected	Site	Consensus Lineage
1070	31.7	5.95E-07	0.000149	Mucosal	None
556	31.7	5.95E-07	0.000149	Mucosal	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Carnobacteriaceae
1162	31.7	5.95E-07	0.000149	Mucosal	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales
939	31.7	5.95E-07	0.000149	Mucosal	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Faecalibacterium; s__
1286	31.7	5.95E-07	0.000149	Mucosal	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Veillonella; s__parvula
1176	28.4	2.98E-06	0.000744	Mucosal	None
1136	28.4	2.98E-06	0.000744	Mucosal	None
532	28.4	2.98E-06	0.000744	Mucosal	None
620	25.4	1.27E-05	0.003174	Mucosal	None
989	25.4	1.27E-05	0.003174	Mucosal	None
496	25.4	1.27E-05	0.003174	Mucosal	None
71	25.4	1.27E-05	0.003174	Mucosal	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Dialister; s__invisus
1139	25.4	1.27E-05	0.003174	Mucosal	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Leuconostocaceae; g__Leuconostoc
151	22.7	4.77E-05	0.011927	Mucosal	None
861	22.7	4.77E-05	0.011927	Mucosal	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__S24-7; g__s__
144	22.7	4.77E-05	0.011927	Mucosal	None
752	22.7	4.77E-05	0.011927	Mucosal	None
161	22.7	4.77E-05	0.011927	Mucosal	None
882	22.7	4.77E-05	0.011927	Mucosal	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae
473	22.7	4.77E-05	0.011927	Mucosal	None
954	22.7	4.77E-05	0.011927	Mucosal	None
468	22.7	4.77E-05	0.011927	Mucosal	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae
210	22.7	4.77E-05	0.011927	Mucosal	None
911	22.7	4.77E-05	0.011927	Mucosal	None
892	22.7	4.77E-05	0.011927	Mucosal	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides; s__ovatus
706	22.7	4.77E-05	0.011927	Mucosal	None
15	20.8	0.000117	0.029233	Mucosal	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pasteurellales; f__Pasteurellaceae; g__Haemophilus; s__parainfluenzae
1270	20.6	0.000128	0.031993	Mucosal	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__s__
675	20.1	0.00016	0.039935	Mucosal	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae
361	20.1	0.000161	0.040321	Mucosal	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Faecalibacterium
942	20.1	0.000161	0.040321	Mucosal	None
84	20.1	0.000161	0.040321	Mucosal	None

OTU	G value	G probability	Bonferroni corrected	Site	Consensus Lineage
568	20.1	0.000161	0.040321	Mucosal	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Streptococcus
936	20.1	0.000161	0.040321	Mucosal	None
301	20.1	0.000161	0.040321	Mucosal	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Lachnospira; s__

¹ OTUs (Operational Taxonomic Units) shown for $p > 0.05$ after Bonferroni correction for multiple comparisons only. A G-test was used to perform an unweighted comparison of OTU presence/absence in luminal and mucosal compartments. The Site column lists the compartment in which the OTU is present.