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MODULATION OF THE INTESTINAL MICROBIOTA BY 15KDA SELENOPROTEIN EXPRESSION IN INFLAMMATORY COLON CANCER

by

Jessica A. Canter

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THESIS APPROVAL PAGE

This is to certify that the thesis prepared by **Jessica Canter** entitled **Modulation of the Intestinal Microbiota by 15 kDa Selenoprotien Expression and Inflammatory Colon Cancer**, has been approved by her committee as satisfactory completion of the requirement for the degree of Master of Science.

Chair, Thesis Committee — Date: 04/27/2015

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Committee Member — Date: 4/27/15

Janet DeLany, DEd — Date: 5-8-15
Dean, College of Graduate Education and Research

ABSTRACT

MODULATION OF THE INTESTINAL MICROBIOTA BY 15KDA SELENOPROTEIN EXPRESSION IN INFLAMMATORY COLON CANCER

JESSICA A. CANTER

The intestinal microbiota is sensitive to inflammation that may induce dysbiosis leading to colon cancer. Selenium, when incorporated into the 15 kDa selenoprotein (Sep15) has a tissue-specific role in carcinogenesis. $Sep15^{-/-}$ mice are protected from pre-neoplastic colonic lesions, aberrant crypt foci (ACF). These mice were used to investigate relationships among the intestinal microbiota, Sep15, and tumorigenesis in inflammatory colon cancer using azoxymethane (AOM) and dextran sulfate salt (DSS). $Sep15^{-/-}$ and $Sep15^{+/+}$ mice were given adequate selenium for 20 weeks alongside AOM/DSS or control treatment. Colonic ACF, tumor formation, and pro-inflammatory serum cytokines were analyzed, and murine fecal samples were utilized to categorize the intestinal microbiota. My results demonstrate that in this model, $Sep15^{-/-}$ mice are protected from ACF but not colonic tumorigenesis, and $Sep15^{-/-}$ mice may resist changes in microbiota content. Sep15 has a complex role in colon cancer initiation but may not control cancer promotion or impact microbiota composition.

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INTRODUCTION

Trillions of microorganisms reside within the digestive tracts of higher living organisms and have established a rather efficient commensal relationship [1]. Bacteria are the most prevalent of these microorganisms, and of these bacteria 99% are anaerobic, and 60% of these organisms belong to the phylum Firmicutes [2]. The composition of this microbial community is not uniform among individuals or within one individual's lifetime [3]. These microorganisms, referred to as the intestinal microbiota, have essential roles in many physiological processes in mammalian hosts. These processes include signaling the mucosal immune response, defense against bacterial pathogens, the maturation and proliferation of intestinal epithelial cells, and the digestion of food [2]. Bacteria residing in the gut ferment carbohydrates and other compounds that mammals cannot digest independently. This results in the formation of metabolites, such as butyrate, which protects human colon cells from DNA damage and inhibits the progression of colonic carcinoma cells [3]. When the microbiota becomes unbalanced, diseases such as inflammatory bowel disease, Crohn's disease, colitis, and colon cancer become a possible threat to human health [4, 5].

Colon cancer remains the second leading cause of cancer related deaths in the United States with an estimated 93,090 new cases and 39,610 deaths in 2015 [6]. Risk factors for the development of this cancer include a diet high in red meats, cigarette smoking, family history of colon cancer, age 50 years or older, and chronic intestinal inflammation [6]. One of the earliest indicators of colorectal cancer development is the formation of aberrant crypt foci (ACF), which are pre-neoplastic lesions in the form of abnormal tube-like glands in the colorectal lining tissue that form before adenomas in the

progression of colon cancer. A previous study demonstrated that the number of ACF has a strong relationship with the number of tumors formed in the colon [7].

Intestinal inflammation is thought to promote colon cancer through a variety of different mechanisms. Inflammatory cytokines such as IL-6, IL-23, and TNF-α have been shown to form a microenvironment in the gut that stimulates colitis-associated colorectal cancer [1]. This was also seen in mice deficient in IL-10 that are sensitive to colitis. The IL-10-deficient mice also had reduced species richness of the intestinal microbiota, but an increased presence of *Verrucomicrobia, Bacteroidetes,* and *Proteobacteria* when compared to control mice with adequate IL-10. Certain strains of these bacterial organisms, such as *Escherichia coli,* have genotoxic capabilities that induce DNA damage and promote carcinogenesis [1]. On the other hand, species such as *Lactobacillus acidophilus* and *Bifidobacterium longum* have been shown to have an inhibitory effect on carcinogen-induced colonic DNA damage [3]. Interestingly, such a change in species diversity remained unchanged in a study whereby mice were treated with the colon-specific carcinogen azoxymethane (AOM) to induce tumorigenesis [1]. Intestinal inflammation, not tumorigenesis, was thought to be the cause for diminished microbial diversity. Although tumorigenesis was not the cause of change in diversity, the remaining *Enterobacter* species in these mice were correlated with severe carcinoma in the IL-10-deficient mice [1]. This strongly suggests a pattern of inflammation selecting for certain bacterial species and that these bacterial-specific factors significantly contribute to the development of colon cancer.

Trace elements in the mammalian diet are known to alter the efficacy of colonization of the gastrointestinal tract and diversity of the microbiota, because some

trace elements are highly toxic to certain bacterial species but may be essential to others [8]. Selenium is an essential trace element found in many foods commonly consumed in the U.S. diet. Soil contains inorganic forms of selenium that are converted by plants and other organisms to organic forms, such as selenocysteine and selenomethionine [9]. The recommended daily allowance (RDA) for selenium is 55 μg/day in adults and increases to 60 μg/day for pregnant women, which is easily attained in the average American diet [10]. Common adequate sources of selenium in the US American diet include red meats, fish, broccoli, poultry, eggs, and whole grain foods [11, 12].

The essential nature of selenium appears to be primarily due to its incorporation into selenoproteins, which are encoded by 25 different genes in humans. These proteins are found in prokaryotes, Archaea, and eukaryotes with the exception of yeasts and higher plants [13]. Selenoproteins are built upon the 21st amino acid, selenocysteine, encoded by the codon UGA [14]. This codon also serves as a stop codon; however, in this case a selenocysteine-specific tRNA is recruited by a selenocysteine insertion sequence in the 3' un-translated region incorporating selenocysteine to the polypeptide chain instead of stopping translation [14].

Selenoproteins play crucial roles in cellular processes such as DNA synthesis, apoptosis, and protection from oxidative damage. Previous studies have shown an inverse relationship between dietary selenium levels and the risk of colon cancer. Increased levels of dietary selenium in mice caused an increase in diversity of the intestinal microbiota when compared to mice fed a diet adequate in selenium, which is commonly associated with a healthier state [8]. The intestinal microbiota also compete with the host

cells for selenium and therefore are capable of altering the host's selenium level and its subsequent selenoprotein expression [15].

Among the many selenoproteins, the 15 kDa selenoprotein (Sep15) has a strong tissue-specificity and is found in all mammals. In human and mice, it is expressed in high levels in liver, prostate, kidney, testis, and brain. Sep15's primary function appears to be in oxidative protein folding in the endoplasmic reticulum, yet its entire function has yet to be determined [14, 16]. The expression of Sep15 is regulated by dietary selenium and signaling in the cellular misfolded protein response [17]. In the human genome, Sep15 is found at chromosome locus 1p31, which is often mutated or deleted in cancer cases [14, 18]. It has also been shown that Sep15 expression is lower in cells derived from malignant mesothelioma, prostate, and hepatocarcinoma tissues [18, 19]. However, in stark contrast to this presumably protective function of Sep15 in these previous studies, the role of Sep15 in colon cancer seems to follow an opposing model: lack of Sep15 appeared to result in a reversal of the cancer phenotype in mouse and human colon cancer cells [20, 21].

To further elucidate the role of Sep15 in cancer, a Sep15 knockout ($Sep15^{-/-}$) model was created using C57-Black 6 mice. Sep15 was systemically knocked out in these mice by the targeted removal of one protein-coding region of exon 2 of the Sep15 gene [22]. To create littermate controls for comparison with these mice, heterozygous $Sep15^{+/-}$ mice were backcrossed to create a pseudo-wild type mouse group as well as a $Sep15^{-/-}$ mouse group from the same set of parents. This preserved any genetic background as well as environmental factors that may influence the development of the animals [23]. These $Sep15^{-/-}$ mice have a typical morphology with no visible phenotypic abnormalities.

Sep15$^{-/-}$ mice do, however, appear to have increased levels of serum inflammation in the form of interferon gamma (IFN-γ) expression [24] and develop cataracts early in life [22]. Despite the apparent increase in inflammation it has also been shown in a previous study that these Sep15$^{-/-}$ mice produce significantly fewer ACF when exposed to azoxymethane (AOM) than littermate control mice [FIGURE I, [24]].

As part of my thesis, I investigated possible reasons why mice lacking Sep15 seemed to be protected against chemically induced colon carcinogenesis, and had demonstrated an altered expression of IFNγ. Because the intestinal microbiota has such influence on the development of colitis, and certain microbes may further escalate this inflammatory response, there may be a yet unseen interaction between the microbiota and this unique *sep15* expression environment. This interaction may be pivotal in describing colon carcinogenesis in this model, not only through the formation of ACF but also tumorigenesis. Our lab hypothesized that mice lacking Sep15 retain a more diverse intestinal microbial community than littermate controls, and that this diversity ameliorates the cancer phenotype caused by inflammatory and carcinogen treatment. This could occur through a balance between beneficial and genotoxic intestinal bacteria or how this unique *in vivo* environment of *sep15* expression and inflammation interacts with the intestinal diversity of these organisms.

To investigate this, the role of inflammation on the intestinal microbiota and chemically-induced colon tumorigenesis was simulated and analyzed using this Sep15 knockout mouse model. The intestinal microbiota of Sep15 knockout and litter mate control mice injected with the colon-specific carcinogen AOM and an inflammatory agent (in drinking water), dextran sulfate salt (DSS), were categorized and compared to

Sep15 knockout and litter mate control mice treated with only saline and water. Pro-inflammatory serum cytokines were analyzed to examine inflammatory responses in mice from each group. The tumorigenesis observed in each mouse genotype and treatment was quantified for correlation with the species richness of the intestinal microbiota. The results of this study contributes to the understanding the roles of Sep15 expression and intestinal microbial diversity on the development of inflammatory colon cancer. This knowledge may be useful in further investigation into personalized cancer prevention and treatment.

MATERIALS AND METHODS

Animal Care Disclosure and Study Organization

All mice used in this experiment were handled and sacrificed in a humane manner in accordance with the Institutional Guidelines at the National Cancer Institute at the National Institutes of Health (NIH) in Bethesda, Maryland. The Animal Ethics Committee at the NIH previously approved these experiments with proper permit documentation obtained from the Institutional Animal Care and Use Committee, and documents are on file both at the NIH and at Towson University.

Only male Sep15 knockout ($Sep15^{-/-}$) and littermate control mice ($Sep15^{+/+}$) were used to eliminate sex as a variable in the experiment. Weanling mice of both genotypes were fed standard chow with 0.1 ppm of dietary selenium for 3 weeks. Mice of each genotype ($Sep15^{-/-}$ N= 13, $Sep15^{+/+}$ N= 18) were then subcutaneously injected with AOM (10 mg/kg in ~100uL saline) or saline as a control ($Sep15^{-/-}$ N= 12, $Sep15^{+/+}$ N= 9). At 7 weeks of age, these mice were given two one-week treatments with 2% DSS or

water as a control, with one week of water treatment for each group between DSS or control treatments. At 10 weeks, all mice were maintained on regular drinking water alongside the 0.1 ppm selenium diet until sacrifice upon the end of the study at 20 weeks. Unequal sample sizes among groups are due to the unfortunate premature death of some mice, or the early termination of unhealthy mice to prevent unnecessary suffering.

Bacterial DNA Isolation and Barcoded Amplification

Fecal matter samples were collected from $Sep15^{-/-}$ and littermate $Sep15^{+/+}$ mice at the time of sacrifice and frozen in TE buffer (pH 8) at -20°C. Bacterial DNA from these fecal matter samples was extracted using the QIAmp DNA Stool Mini Kit (Qiagen) with the protocol for Isolation of DNA from Stool for Pathogen Detection and frozen at -20°C for storage. This eluted DNA was then purified using the Genomic DNA Clean & Concentrator Kit (Zymo). The purity and concentration of DNA in each sample was quantified using the Nanodrop 2000 (Fisher Scientific) and all template DNA was normalized by nanograms per microliter of nucleic acid content for further use.

PCR amplification of the bacterial genomic DNA using the GoTaq Green Master Mix (Promega) and all reactions were synthesized at a volume of 50 µl with approximately 100 ng of template DNA in each reaction. Primers for this reaction were selected from the 16s Metagenomic Library Preparation protocol (Illumina) and were designed to flank the V3 and V4 region of the bacterial 16s rRNA gene [25]. These primers were synthesized by IDTechnologies with an overhang adapter sequence specific to the Illumina system to flank the 16s rRNA primers (Forward Primer: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCGTCGGCAGCGT

CAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') (Reverse Primer: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). The reactions were run on the Perkin Gene Amp PCR System 2400 at an initial 94° C for 2 minutes, followed by 29 cycles of 94° C for 20 seconds, 48° C for 30 seconds, and 72° C for 1 minute with a final extension step of 72° C for 10 minutes. To visualize successful amplification, amplicons were electrophoresed on a 1% agarose gel.

After successful PCR synthesis, amplicons were cleaned following the Illumina MiSeq 16s Metagenomic Library Preparation protocol using Ampure XP Beads and 80% ethanol. Nextera XT v2 indices, sets A, B, and C, were then ligated to the cleaned amplicons using PCR facilitated by reagents from the KAPA HiFi HotStart ReadyMix to allow for multiplexing of library samples during sequencing. These final indexed amplicons were cleaned with Ampure XP Beads using the same protocol mentioned in the previous step. To prepare for sequencing, all indexed amplicons were quantified for nucleic acid content using a Qubit fluorometer and then normalized for sequence library pooling.

Amplicon Sequencing and Bioinformatics

The pooled amplicon library was prepared using an Illumina MiSeq V2 reagent kit for 151 base pairs, paired end sequencing for 500 cycles using diluted PhiX DNA as an internal control. After sequencing, the Illumina MiSeq Reporter Software Metagenomics Workflow taxonomically classified these 16S region amplicons using the Greengenes Database. These data were sorted by the percent phyla represented within each fecal sample for calculations of total diversity within the sample. Using the average

of percent phyla diversity, the top 5 most abundant phyla were compared among treatment and genotype groups using a 2-way ANOVA to determine any significant differences. Similar analysis was performed to determine differences in bacterial phyla composition between groups of mice that did or did not develop tumors throughout the study.

Tumor Analyses

After $Sep15^{-/-}$ and $Sep15^{+/+}$ mice were sacrificed, colons were excised from anus to caecum and rinsed with phosphate-buffered saline (PBS). These tissues were opened longitudinally and stored in 70% ethanol or 10% formalin for subsequent analysis. Each of these colons was measured for length in centimeters with a ruler, accurate to 1 millimeter. Tumor formation was measured by counting the total number of tumors formed in each colon using a dissecting microscope as well as determining the mass of each tumor in milligrams using a digital scale accurate to 10^{-4} grams. To analyze for ACF formation, colonic tissues were stained with methylene blue (1 g/L in PBS) and examined using a dissecting microscope by a blinded examiner to avoid any detection bias. The mean was calculated for tumor number, mass, and number of ACF formed in each genotype and treatment group. These data were used in correlation analyses with the bacterial species richness of the microbiota in these groups to observe any association between tumor burden and microbial content.

Serum Cytokine Analysis

Upon sacrifice, blood was taken from all mice by cardiac puncture, and centrifuged in heparinized tubes at 6000 rpm for 5 minutes. Serum was then frozen and stored at -80° C for later analysis. Using the mouse TH1/TH2 7-Plex assay kit, protein

levels of interferon-γ, interleukin-12p70, interleukin-6, tumor necrosis factor-α, KC/GRO (CXCL1, GROα,), interleukin-1β, and interleukin-10 were measured in a sandwich immunoassay format using a SECTOR Imager 2400 per manufacturer's protocol (MesoScale Discovery). An eight-point standard curve was used to calculate the concentration of cytokines in each murine serum sample and all samples and standards were analyzed in duplicate (technical replicates).

RESULTS

General Growth Data

Upon sacrifice, the mass of mice from both genotypes and treatments were measured to determine the mean weight gain in each group. A 1-Way ANOVA with Bonferroni's Multiple Comparison Test was performed to determine significant differences among groups. $Sep15^{+/+}$ mice (mean = 14.588 g) gained significantly less weight than $Sep15^{-/-}$ mice (mean = 20.6 g) under control (no chemical treatment) conditions ($p < 0.05$). Control $Sep15^{-/-}$ mice gained significantly more weight than $Sep15^{-/-}$ mice treated with AOM/DSS (mean = 11.859 g, $p < 0.0001$) [Figure II].

Colons from all mice were excised and measured for total length. A 1-Way ANOVA followed by Bonferroni's Multiple Comparison Test did not indicate any significant differences in length of colons among mice of either genotype or treatment groups [Figure III].

Aberrant Crypt Foci Formation and Tumorigenesis

When exposed to AOM/DSS treatment and maintained on a diet adequate in Se (0.1ppm), $Sep15^{-/-}$ mice formed significantly fewer (mean = 0.455 ACF +/- 0.282 SEM)

aberrant crypt foci (ACF) when compared to $Sep15^{+/+}$ mice (mean = 3.389 +/- 0.797 SEM) (p = 0.0094, T-Test) [FIGURE IV]. Although $Sep15^{-/-}$ mice are protected from the formation of pre-neoplastic lesions in the form of ACF, these mice did however form tumors in the duration of the study. The percent of mice within each genotype group that formed tumors under AOM/DSS treatment remains fairly equal at approximately 60% of total mice ($Sep15^{-/-}$ mice = 63.64%, $Sep15^{+/+}$ mice = 61.11%) [FIGURE V]. In concordance with this, no significant differences were found among the raw number of tumors formed in either AOM/DSS-treated genotype (T-Test, mean $Sep15^{-/-}$ mice = 3.3 tumors +/- 1.1 SEM, $Sep15^{+/+}$ mice = 2.2 tumors +/- 0.4 SEM) [FIGURE VI]. The mass of tumors formed in $Sep15^{-/-}$ mice (mean = 0.0193g +/- 0.0085 SEM) at an adequate Se diet, although not statistically significant, are slightly higher than those found in $Sep15^{+/+}$ mice (mean = 0.0162 g +/- 0.0084 SEM) [FIGURE VII].

Intestinal Microbiota Composition

Among all combinations of treatments and genotypes in this study, the same 5 bacterial phyla were most abundant in the total sequence reads within groups. These phyla were Bacteroidetes, Firmicutes, Proteobacteria, Verrucomicrobia, and a group labeled as Unclassified due to limits in sequence identity matching. No significant differences were found between intestinal micrbiota in untreated $Sep15^{+/+}$ and $Sep15^{-/-}$ mice in the percent of total sequence reads attributed to the 5 most abundant phyla; however slight variations in abundance in the phyla Firmicutes and Verrucomicrobia were observed [FIGURE VIII]. This suggests that the absence of Sep15 alone may result in gradual shifts in microbial composition within the intestinal tract. Mice from both the $Sep15^{-/-}$ and $Sep15^{+/+}$ genotype, when treated with AOM/DSS, appear to become more

similar in microbiota diversity with a slight variation between genotypes only seen in the phylum Verrucomicrobia. Although this trend is intriguing, again no significant differences were determined among groups [FIGURE IX].

To further explore this phenomenon, the microbiota composition was compared among $Sep15^{+/+}$ mice treated with AOM/DSS that either did or did not form tumors, as well as $Sep15^{+/+}$ mice given control treatment of saline/water only. Significant differences were found between $Sep15^{+/+}$ mice treated with AOM/DSS with tumor formation and $Sep15^{+/+}$ mice given control treatment in both the phylum Firmicutes ($p < 0.05$) and Verrucomicrobia ($p < 0.001$) [FIGURE X]. In $Sep15^{-/-}$ mice in these same categories, AOM/DSS treatment with and without tumor formation and control treatment, no significant differences were found [FIGURE XI].

Pro-Inflammatory Serum Cytokines

Serum interferon-γ, interleukin-12p70, interleukin-6, tumor necrosis factor-α, KC/GRO, interleukin-1β, and interleukin-10 levels were determined. No significant differences were found between genotype or treatment groups [FIGURE XII].

DISCUSSION

The previous study by Tsuji *et al*. in 2012 demonstrated that $Sep15^{-/-}$ mice formed significantly fewer pre-neoplastic lesions, ACF, than $Sep15^{+/+}$ mice under AOM/DSS treatment [FIGURE I]. Although only few ACF develop into tumors over time, it is widely accepted that ACF formation is an indicator of colonic tumorigenesis [7]. Thus, for this thesis, we hypothesized that $Sep15^{-/-}$ mice would therefore develop fewer, if any,

colonic tumors when exposed to AOM/DSS treatment in an extended study with observation over 20 weeks.

As the data in this study clearly demonstrate, the formation of ACF in $Sep15^{-/-}$ mice was again significantly reduced ($p = 0.0094$) [FIGURE IV]. However, much to our surprise, $Sep15^{-/-}$ mice did not form fewer tumors than their $Sep15^{+/+}$ counterparts [FIGURE VI]. This is in stark contrast to the hypothesis formed by Tsuji *et al*. in 2012 and indicates that there are possibly far more complex interactions between Sep15 expression and chemically-induced colon cancer involved. Similar disparities between ACF formation and colon tumorigenesis in studies using rats had been described in the past, and more recently, investigators are debating the usage of either β-catenin-accumulated crypts or mucin-depleted foci in rat models utilizing AOM/DSS treatment as more accurate predictors of colon tumorigenesis than ACF [26, 27].

The causation of the contrast in ACF formation and tumorigenesis in the Sep15 knockout mouse model presented in this thesis is yet unknown. The abundance of pro-inflammatory serum cytokines was measured in an attempt to clarify this matter. Although no statistically significant differences were found between $Sep15^{+/+}$ and $Sep15^{-/-}$ mice in the expression of these cytokines, there are observable trends that show AOM/DSS treatment increases expression in some cases (IFNγ, IL-10, IL12-p70, IL-1β, KC/GRO, and TNF-α) [FIGURE XII]. Variability in cytokine levels among mice within each group may account for a lack of consensus in mean serum concentrations, which is to be expected at times when using a live animal model. Further investigation is required to determine the meaning of these data and their involvement in carcinogenesis in this model.

Yet another variable at play is the intestinal microbiota. In $Sep15^{-/-}$ and $Sep15^{+/+}$ mice under control and AOM/DSS treatment, the most abundant bacterial phylum recorded in fecal extracts was Firmicutes [FIGURE VIII, FIGURE IX]. These data are consistent with other C57BL/J6 mouse studies in models of both dietary selenium modulation and colitis [8, 28]. Firmicutes are also implicated in human pathology, so the significance in this phylum's abundance may translate to human health. Firmicutes undergo short chain fatty acid metabolism and produce metabolites such as butyrate, which may have cancer preventative functions [29]. This phylum has also been tied to inhibiting growth of pathogens in the gut, decreasing the pH within the colon, water and sodium absorption in colonic tissues, and the synthesis of cholesterol [29].

In humans, colon cancer has been associated with a loss in intestinal microbial diversity with particular significance in a loss of Gram-positive fiber fermenting organisms, such as *Clostridia* within Firmicutes, with an increase in the presence of Gram-negative pro-inflammatory bacteria, such as *Fusobacterium* and *Porphyromonas* [30]. When comparing healthy and cancerous human colon lumen tissue, significant differences can be found within colonization of Firmicutes, Bacteroidetes, and Proteobacteria [31]. It has been posited that inflammation is the key to the reduction of microbial diversity, particularly the loss of beneficial butyrate metabolizing organisms and an increase in pro-inflammatory organisms such as *Bacteroides* to induce colon tumorigenesis [32]. These inflammation-enriched bacterial genera may have direct connections to tumorigenesis through their virulence factors, tumor-inducing gene products, metabolites, and antigens [32]. The question remains to determine if colon

cancer is tied more directly to a gain of pro-inflammatory organisms in the microbiota, or a loss of healthy anti-inflammatory organisms [32].

Dietary selenium and selenoproteins are involved in cancer prevention and promotion, so examining their role in colon cancer as well as the diversity of the microbiota is important. In this study, $Sep15^{-/-}$ mice appear to be more resistant to changes in microbiota composition among groups of mice treated with AOM/DSS that did and did not form tumors when compared to mice given control treatment [FIGURE XI]. The mechanism behind this is unknown but small, unequal sample sizes among groups could also account for differences seen in $Sep15^{+/+}$ mice between these same groups [FIGURE X]. Because other investigators have seen modulations in major phyla, such as Bacteroidetes and Firmicutes, with changes in dietary selenium as well as an increase in total microbial diversity with increases in dietary selenium [8], and because a selenium-deficient diet may exacerbate colitis and tumor formation in mice treated with AOM/DSS [33], our future analyses will take into account varying levels of dietary selenium, such as selenium-deficiency and supra-nutritional selenium status, and their effects on intestinal microbiota composition and associated ACF formation and tumorigenesis.

To fully understand the role of the intestinal microbiota in a $Sep15^{-/-}$ and $Sep15^{+/+}$ environment, the data presented should be expansively analyzed in the future. For instance, a comparison of beta-diversity among groups of $Sep15^{-/-}$ and $Sep15^{+/+}$ mice in both AOM/DSS and control treatment might be helpful to determine relationships between microbial diversity and tumor pathology in this model. This relationship could be calculated using principal coordinate analysis, as well as relationships with ACF

formation, dietary selenium and serum cytokine levels. Ultimately, there may be more accurate pathological biomarkers for colon cancer [26, 27], or there may be a network of cellular relationships involving Sep15 and other selenoproteins in the process of cancer initiation and promotion that could explain the unexpected results.

Future experiments are required to determine regulatory mechanisms of Sep15 in healthy and cancerous colon tissue, as well as the effects on other relevant genes, such as the WNT/beta-catenin pathway, in these tissues when Sep15 is absent. This could offer a possible explanation to the observed phenomenon. It would also be informative to determine the effects of inflammation alone in the $Sep15^{-/-}$ mouse model to determine any possible interplay with other networks of genes or the intestinal microbiota. Finally, modulating dietary selenium above and below the adequate status used in these experiments may induce differences in the expression of other selenoproteins, and therefore cause differences in the other investigated factors contributing to colon cancer.

In summary, mice lacking Sep15 in an AOM/DSS model of chemically-induced colon carcinogenesis are protected from ACF formation but ultimately not from tumorigenesis. Even though $Sep15^{-/-}$ mice appear to have an altered pro-inflammatory cytokine status compared to littermate controls, serum cytokine levels were not statistically significantly different, regardless of treatment. $Sep15^{-/-}$ mice did, however, cultivate differences in intestinal microbiota structure from their $Sep15^{+/+}$ counterparts. The full function of Sep15 in a model of colon carcinogenesis as well as in inflammatory colitis continues to be elucidated.

APPENDIX - FIGURES

FIGURE I

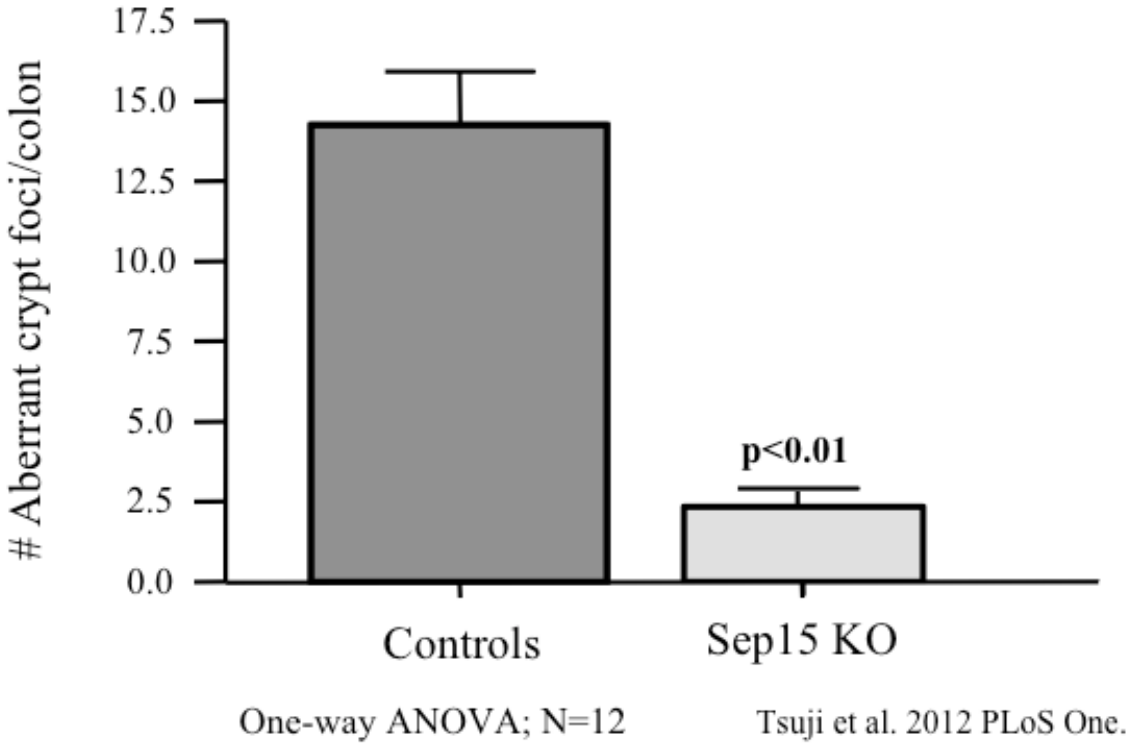

Figure I – Aberrant crypt foci (ACF) formation in $Sep15^{+/+}$ (Control) and $Sep15^{-/-}$ (Sep15 KO) mice from Tsuji *et al*., 2012. $Sep15^{-/-}$ mice form significantly fewer ACF when exposed to azoxymethane than $Sep15^{+/+}$ mice. (One-way ANOVA, p , 0.01).

Figure II

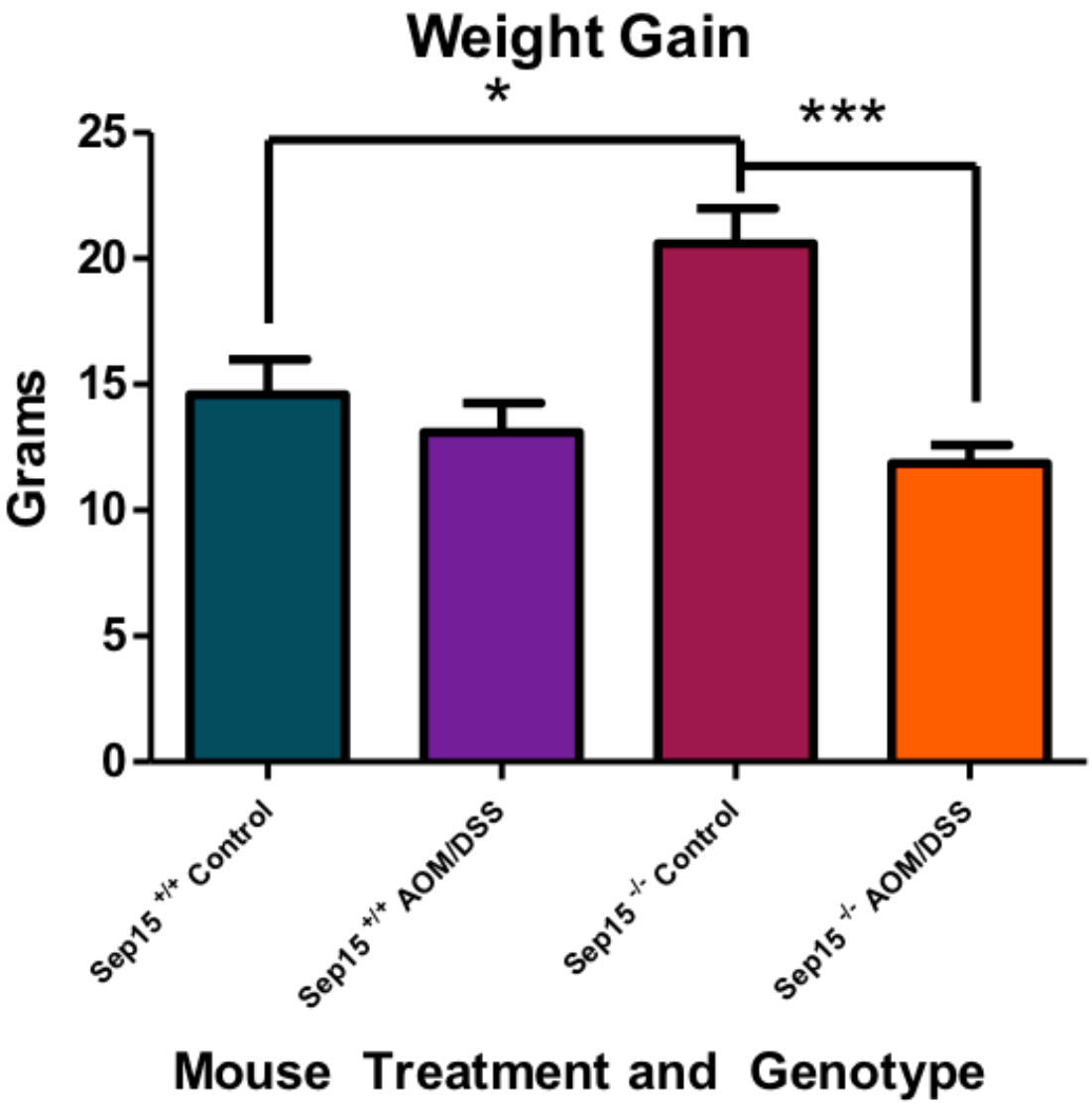

Figure II – Weight gain (grams) in $Sep15^{+/+}$ and $Sep15^{-/-}$ mice under both saline/water (control) and AOM/DSS treatment upon sacrifice after 20 weeks. (One-way ANOVA,* = $p < 0.05$, *** = $p < 0.0001$).

Figure III

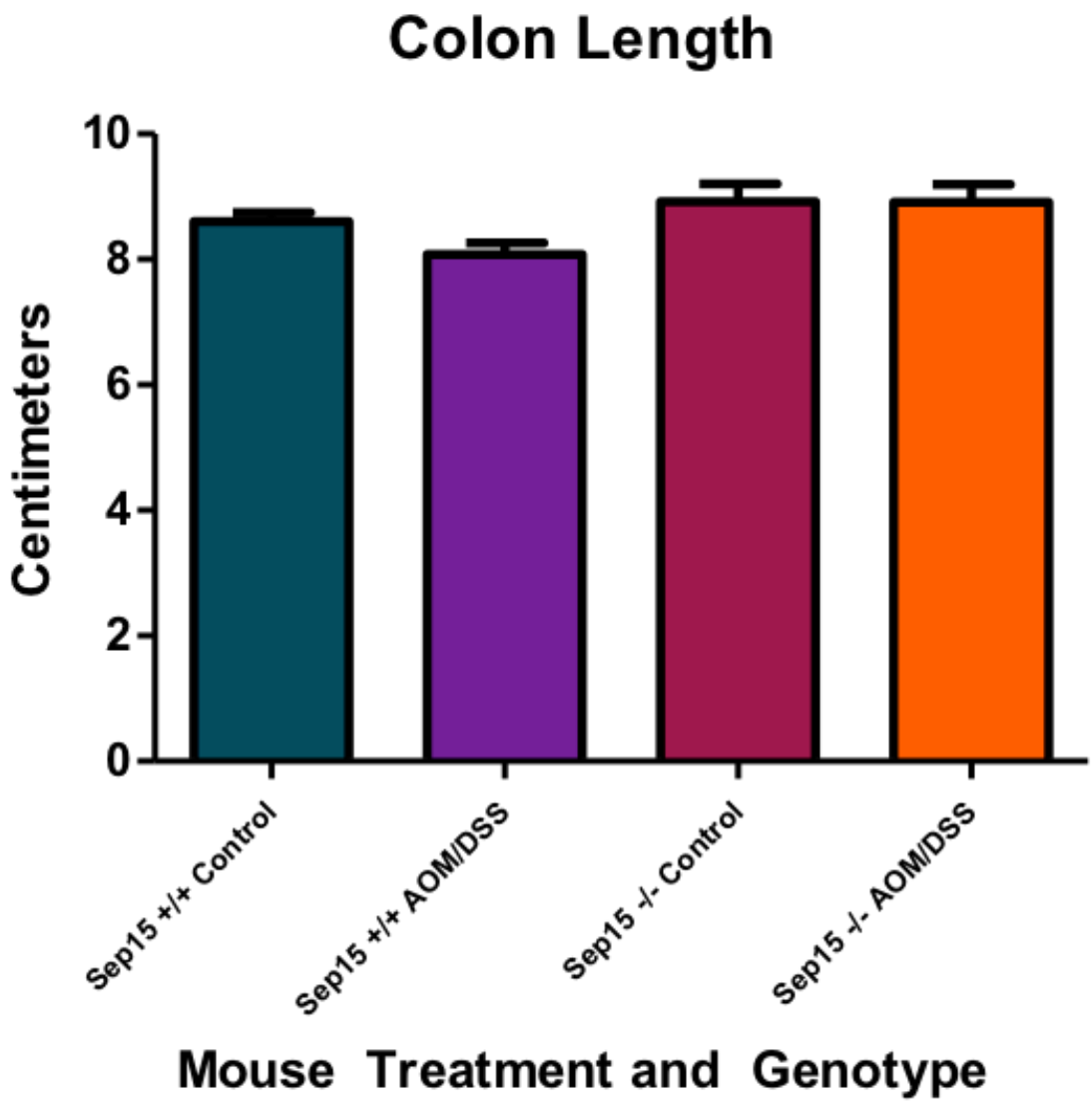

Figure III – Colon length (centimeters) in $Sep15^{+/+}$ and $Sep15^{-/-}$ mice under both saline/water (control) and AOM/DSS treatment upon sacrifice after 20 weeks. (One-way ANOVA).

Figure IV

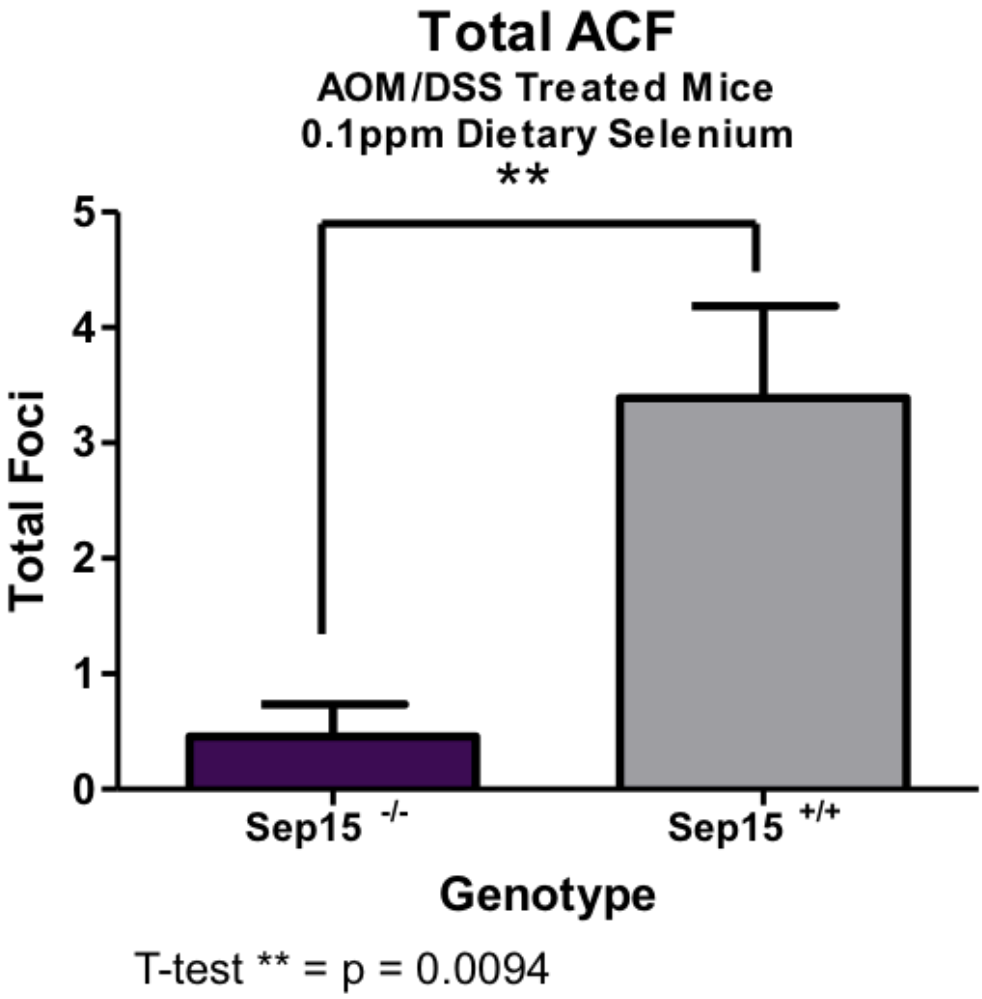

Figure IV – Aberrant crypt foci (ACF) formation in $Sep15^{+/+}$ and $Sep15^{-/-}$ mice under AOM/DSS treatment upon sacrifice after 20 weeks. $Sep15^{-/-}$ mice formed significantly fewer ACF than $Sep15^{+/+}$ mice (T-test ** = p = 0.0094).

Figure V

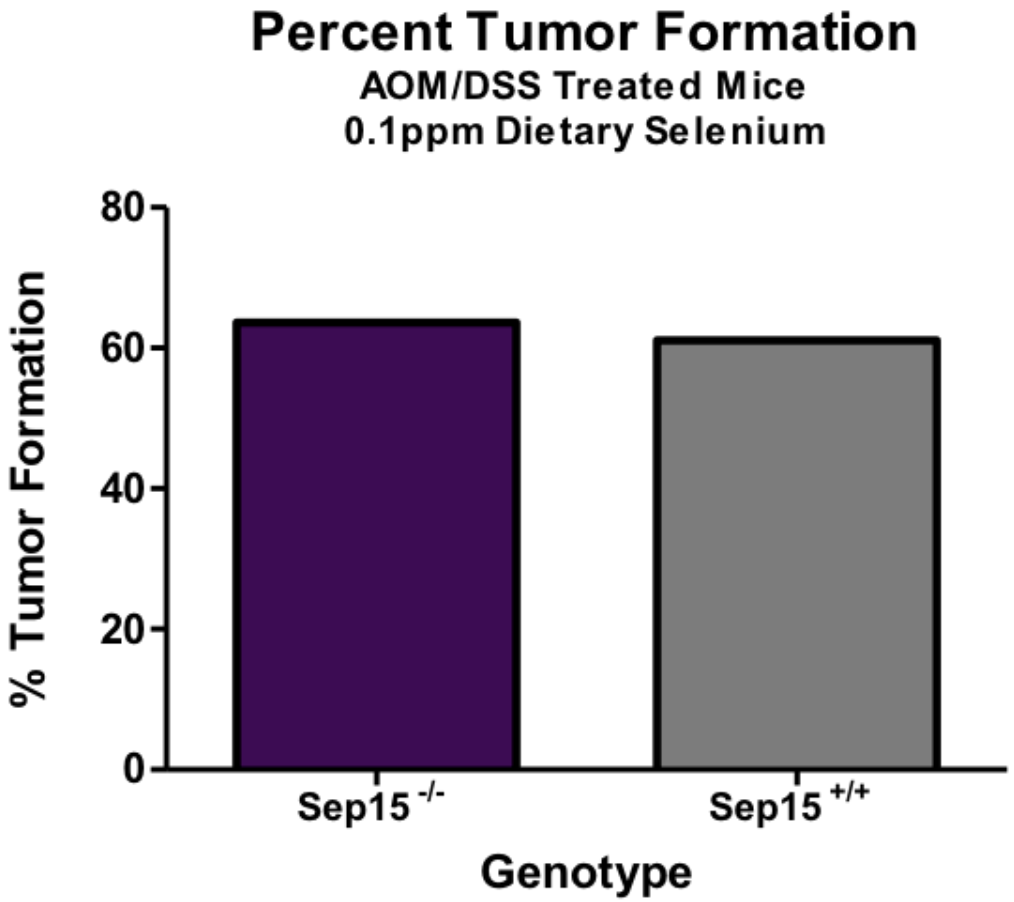

Figure V – Percent of all $Sep15^{+/+}$ and $Sep15^{-/-}$ mice under AOM/DSS treatment that formed tumors upon sacrifice after 20 weeks.

Figure VI

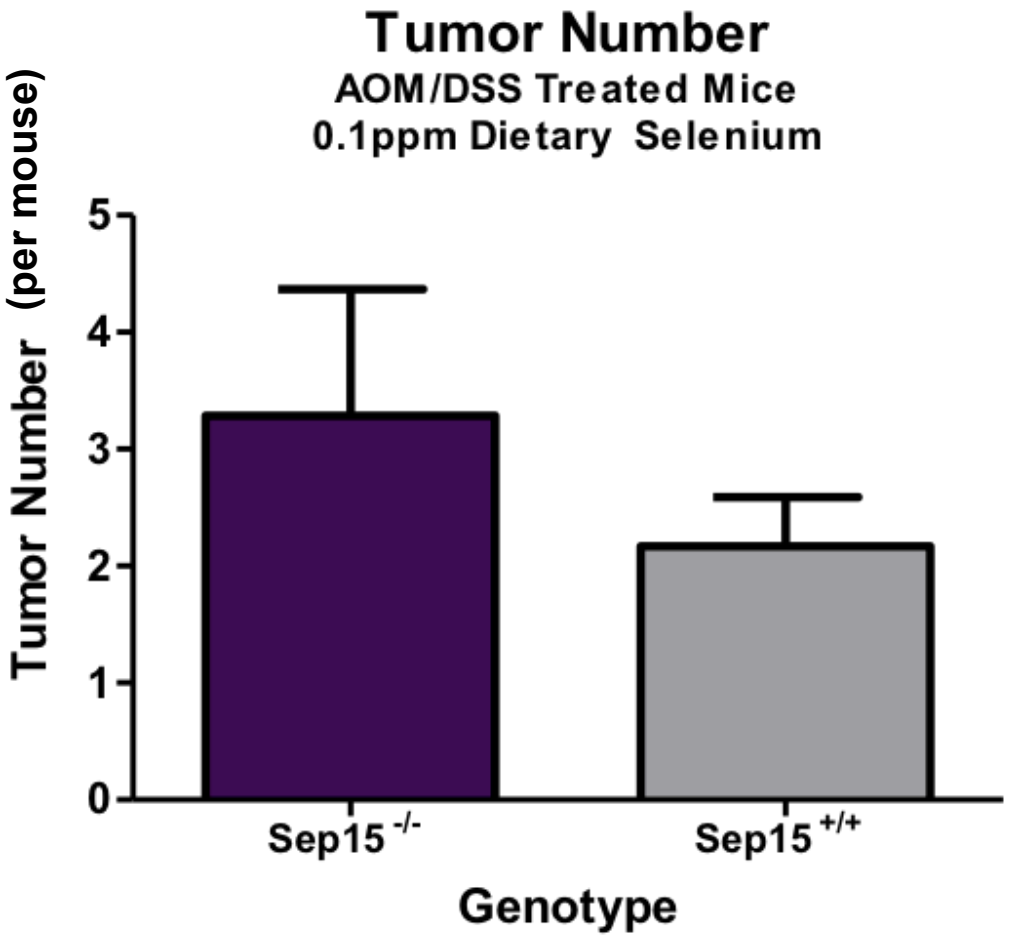

Figure VI – The number of tumors formed per mouse in $Sep15^{+/+}$ and $Sep15^{-/-}$ mice with AOM/DSS treatment upon sacrifice after 20 weeks. (T-test).

Figure VII

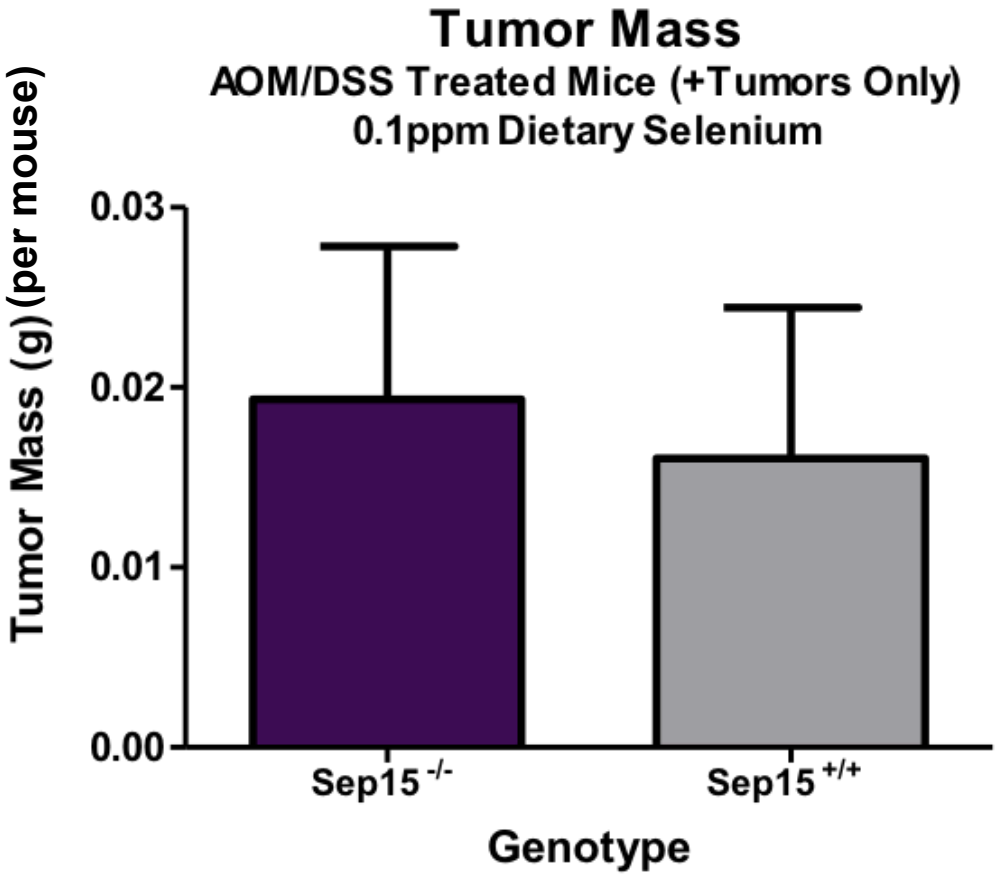

Figure VII – The mass of tumors (grams) formed per mouse in $Sep15^{+/+}$ and $Sep15^{-/-}$ mice with AOM/DSS treatment upon sacrifice after 20 weeks. (T-test).

Figure VIII

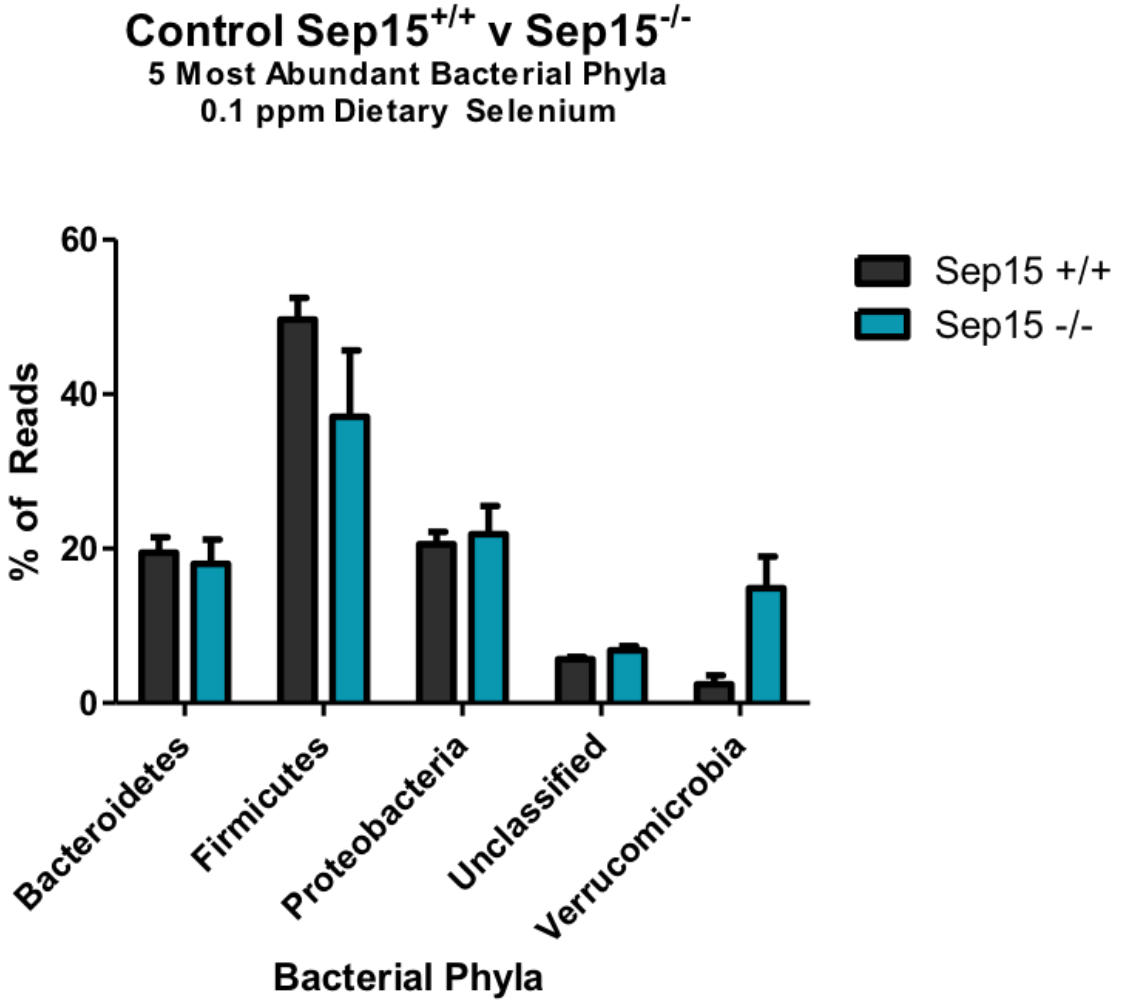

Figure VIII – The percent of sequencing reads assigned to the 5 most abundant bacterial phyla present in fecal samples from $Sep15^{+/+}$ and $Sep15^{-/-}$ mice given saline/water (control) treatment. (Two-way ANOVA).

Figure IX

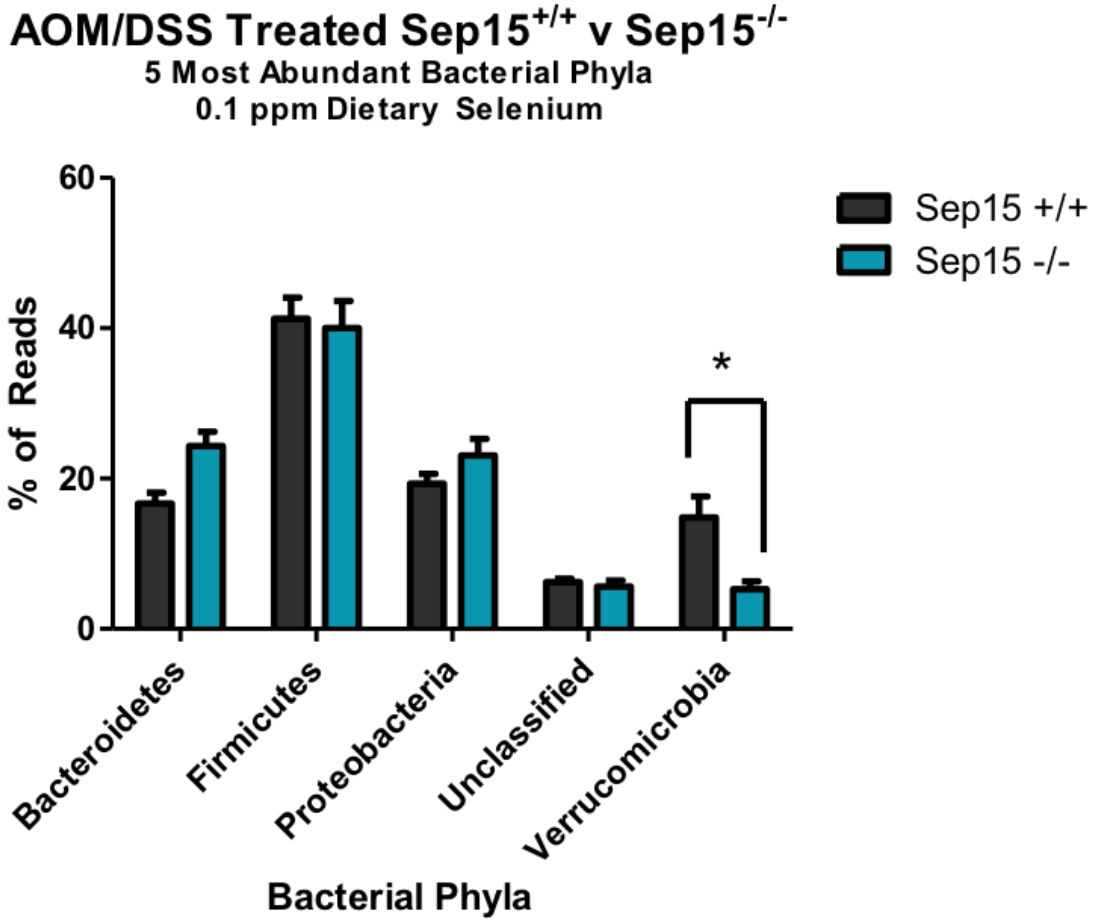

Figure IX – The percent of sequencing reads assigned to the 5 most abundant bacterial phyla present in fecal samples from $Sep15^{+/+}$ and $Sep15^{-/-}$ mice treated with AOM/DSS. (Two-way ANOVA, * = $p < 0.05$).

Figure X

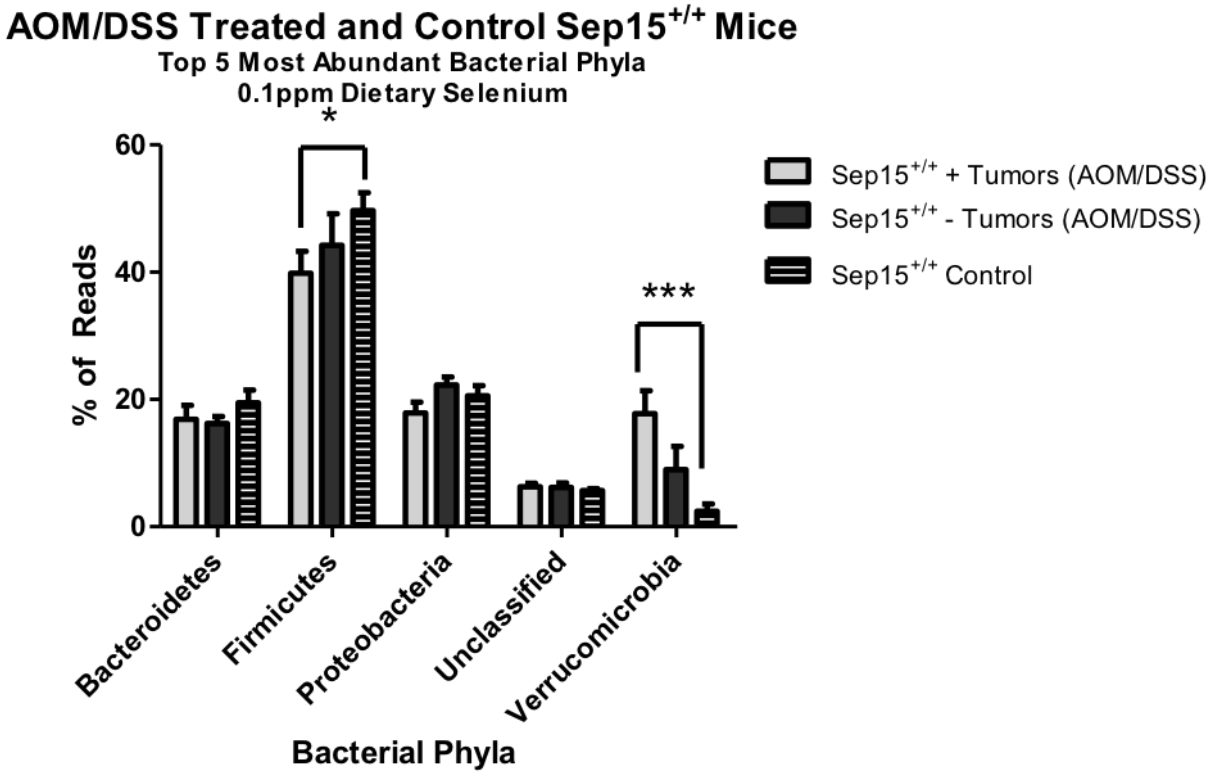

Figure X – The percent of sequencing reads assigned to the 5 most abundant bacterial phyla present in fecal samples from $Sep15^{+/+}$ mice treated with AOM/DSS that were positive or negative for tumor formation compared to mice given saline/water (control) treatment. (Two-way ANOVA, * = $p < 0.05$, *** = $p < 0.001$).

Figure XI

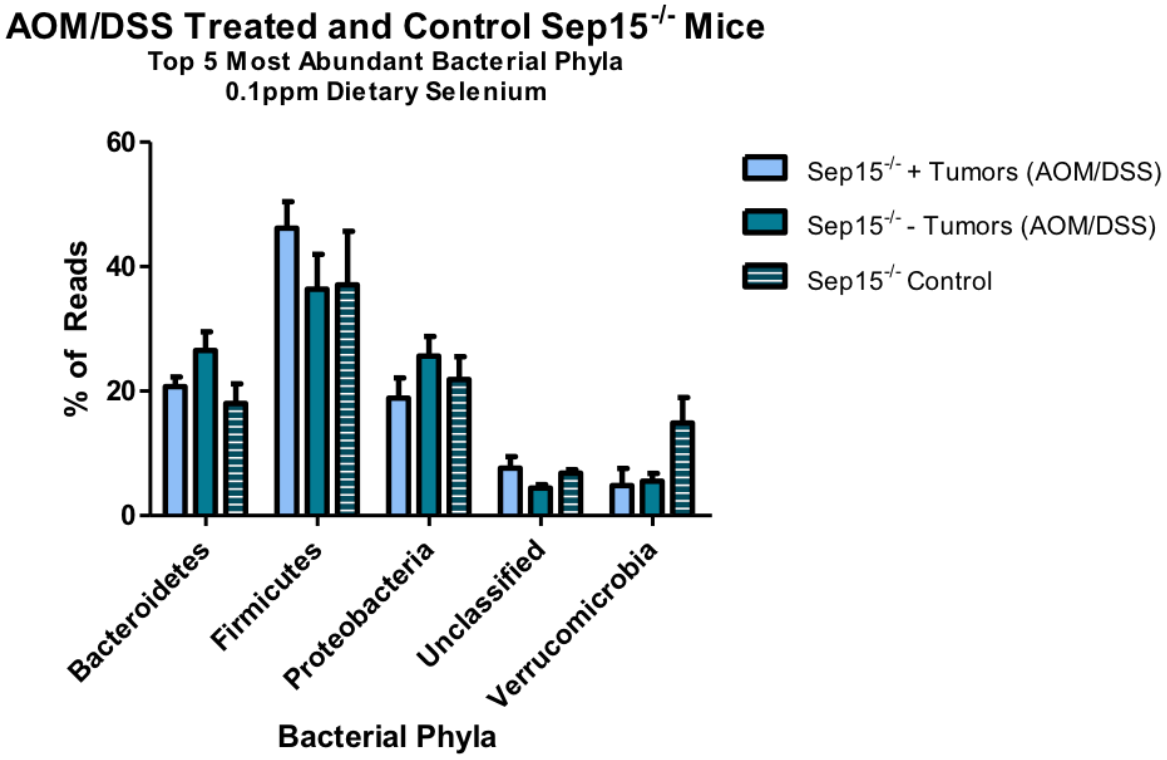

Figure XI – The percent of sequencing reads assigned to the 5 most abundant bacterial phyla present in fecal samples from Sep15$^{-/-}$ mice treated with AOM/DSS that were positive or negative for tumor formation compared to mice given saline/water (control) treatment. (Two-way ANOVA).

Figure XII

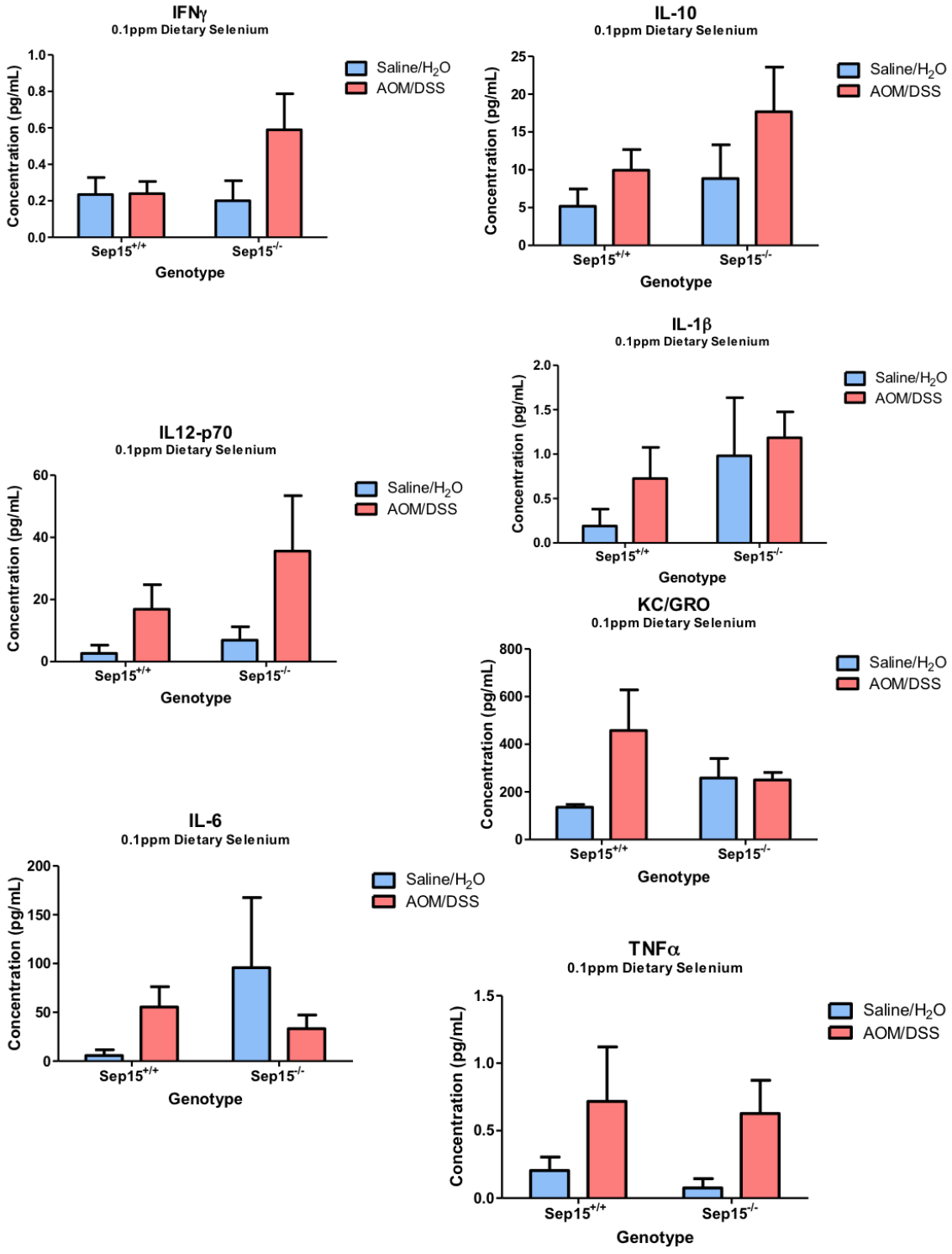

Figure XII – Serum pro-inflammatory cytokines in $Sep15^{+/+}$ and $Sep15^{-/-}$ mice treated with AOM/DSS or saline/water (control). (Two-way ANOVA).

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Jessica A. Canter

[redacted]

Education

M.S. Biology - Expected Completion May 2015
Towson University – Towson, MD
Thesis Title: *Modulation of the Intestinal Microbiota by 15 kDa Selenoprotein Expression in Inflammatory Colon Cancer*

B.S. Biology (Cell & Molecular Biology) – December 2012
Towson University – Towson, MD

Research Experience

Master's Thesis Research, Graduate Research Assistant (August, 2013 – Present)
Towson University – Towson, MD
- Examine the effects of selenoprotein expression and dietary selenium interacting with the intestinal microbial community in the development of inflammatory colon cancer in a murine model.

Research Associate (January, 2013 – August, 2013)
Towson University – Towson, MD
- Investigated the use of animal manure extracts for the preparation of a renewable cell culture media for both bacterial and algal cultures.

Undergraduate Research Assistant (January, 2011 – January, 2013)
Towson University – Towson, MD
- Analyzed virulence factors of *Mycoplasma gallisepticum* in an attempt to correlate measured virulence with clinical pathogenicity observed in songbirds.

Teaching Experience

Graduate Teaching Assistant (August, 2013 – Present)
Towson University – Towson, MD
- Medical Microbiology/Essentials of Microbiology
  - Lecture students on microbiology laboratory concepts and skills as they relate to a clinical environment
  - Grade student laboratory practical exams and projects

Professional Service, Leadership and Awards

Leadership Experience
- Towson University Fisher College of Science and Mathematics College Council – Graduate Student Representative (2014-2015)
- Beta Beta Beta, Upsilon Eta Chapter – Webmaster (2014-2015)
- Towson Biology Graduate Student Association – Founding Member (2013-2015)
- Kappa Kappa Psi, Iota Delta Chapter – Recording and Corresponding Secretary (2009-2010), Vice President of Membership (2011)

- Towson University Marching Band – Section Leader, Field Set-Up Committee Chair (2010)

Affiliation with Professional Societies

- American Society for Biochemistry and Molecular Biology (2014)
- United States Mycoplasmology Consortium (2013)
- American Society for Microbiology: Division G (2012)

Awards

Towson University – Towson, MD

- Graduate Student Association Travel Award (June, 2014)
- Undergraduate Research Committee Travel Award (June, 2012)
- Fischer College of Science and Mathematics Travel Award (June, 2012)

Publications and Presentations

Textbook Chapters

Canter J.A., L.E. Rosso, and P.A. Tsuji. Trace Minerals. (Invited book chapter, in preparation) In *Encyclopedia of Food and Health*. Caballero, Finglas, Toldrá, Editors. Elsevier, Oxford, UK.

May, M., Arjoon A.V., **Canter J.A.**, Dunne D.W., and Terrell B. "Mycoplasmology", in the Encyclopedia of Life Support Systems. United Nations Educational, Scientific, and Cultural Organization (UNESCO)-EOLSS Publishers, Ltd, Oxford, OXF United Kingdom. Online 2012. [http://www.unesco.org/en/services/projects/eolss/]

Oral Presentations

Canter J.A., Sheckells S.E., Saylor C.V., Carlson B.A., Gladyshev V.N., May M., Davis C.D., Hatfield D.L., and Tsuji P.A. Effects of Dietary Selenium and 15 kDa Selenoprotein Expression on Intestinal Microbiota and Inflammatory Colon Cancer. International Symposium on Trace Elements in Man and Animals; 2014 June; Orlando, FL.

Canter J.A,, May M., and Ley D. *Mycoplasma gallisepticum* Infection in House Finches. Towson University MB3 Club Seminar; 2011 May; Towson, MD.

Poster Presentations

Canter J.A., Sheckells S. E., Saylor C.V., Patterson A., Carlson B.A., Gldyshev V.N., Yu Y., Cao L., May M., Davis C.D., Hatfield D.L., and Tsuji P.A. Modulation of Intestinal Microbiota by Dietary Selenium and 15 kDa Selenoprotein Expression in Inflammatory Colon Cancer. Experimental Biology: 2015 March: Boston, MA.

Canter J.A., Sheckells S.E., Saylor C.V., Carlson B.A., Gladyshev V.N., May M., Davis C.D., Hatfield D.L., and Tsuji P.A. Effects of Dietary Selenium and 15 kDa Selenoprotein Expression on Intestinal Microbiota and Inflammatory Colon Cancer. American Institute for Cancer Research Conference on Food, Nutrition, Physical Activity, and Cancer; 2014 October; Washington, DC.

Hartig N.A., **Canter J.A**., Gough A., May M., and Casey R. Analysis of Manure Extracts as Cell Culture Growth Media. Towson University Student Research and Scholarship Expo; 2013 April; Towson, MD.

Canter J.A., May M., and Ley D. Sialidase Activity and Hemolysis in Songbird Isolates of *Mycoplasma gallisepticum*. Colonial Academic Alliance Undergraduate Research Conference [2012 April; Norfolk, VA], Towson University Student Research and Scholarship Expo [2012 April; Towson, MD], and American Society for Microbiology General Meeting [2012 June; San Francisco, CA], International Organization for Mycoplasmology 19th congress [2012 July; Toulouse, Midi-Pyrénées, France. Presented by M. May].