



# Dietary broccoli impacts microbial community structure and attenuates chemically induced colitis in mice in an Ah receptor dependent manner



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3071)

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114764)

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## ABSTRACT

Consumption of broccoli mediates numerous chemo-protective benefits through the intake of phytochemicals, some of which modulate aryl hydrocarbon receptor (AHR) activity. Whether AHR activation is a critical aspect of the therapeutic potential of dietary broccoli is not known. Here we administered isocaloric diets, with or without supplementation of whole broccoli (15% w/w), to congenic mice expressing the high-affinity *Ahr*<sup>b/b</sup> or low-affinity *Ahr*<sup>d/d</sup> alleles, for 24 days and examined the effects on AHR activity, intestinal microbial community structure, inflammatory status, and response to chemically induced colitis. Cecal microbial community structure and metabolic potential were segregated according to host dietary and AHR status. Dietary broccoli associated with heightened intestinal AHR activity, decreased microbial abundance of the family *Erysipelotrichaceae*, and attenuation of colitis. In summary, broccoli consumption elicited an enhanced response in ligand-sensitive *Ahr*<sup>b/b</sup> mice, demonstrating that in part the beneficial aspects of dietary broccoli upon intestinal health are associated with heightened AHR activity.

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## 1. Introduction

Plants of the genus *Brassica*, often referred to as cruciferous vegetables (e.g. broccoli, cabbage, Brussels sprouts, etc.), provide critical components of a healthy diet, such as vitamins, minerals, and fiber. Consumption of these plants correlates with decreased

incidence of cancer (Latte, Appel, & Lampen, 2011). While individual chemical constituents found in broccoli have been reported in isolation to exhibit beneficial properties, the relative importance of each chemical within the context of dietary broccoli in conferring these beneficial effects is poorly understood (Bradlow, Sepkovic, Telang, & Osborne, 1999; Higdon, Delage, Williams, & Dashwood, 2007; Zhang, Talalay, Cho, & Posner, 1992).

Broccoli (*Brassica oleracea*) has been extensively studied for its content of various nutritional phytochemicals, such as glucosinolates (Kushad et al., 1999). Enzymatic hydrolysis of glucosinolates by plant myrosinases generates numerous metabolites including isothiocyanates, thiocyanates, and epithionitriles (Fenwick, Heaney, & Mullin, 1983) which exhibit diverse biological activities. Perhaps one of the most studied components of this class of glucosides are indole-3-carbinol (I3C), indole-3-acetonitrile (I3ACN) and 3,3'-diindolylmethane (DIM), breakdown products of

**Abbreviations:** AHR, aryl hydrocarbon receptor; I3C, indole-3-carbinol; DIM, 3,3'-diindolylmethane; ICZ, indolo[3,2-b]carbazole; I3ACN, indole-3-acetonitrile; Ltr-1, 2-(indol-3-ylmethyl)-3,3'-diindolylmethane; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; OTU, operational taxonomic unit; DSS, dextran sodium sulphate; EAE, experimental autoimmune encephalitis; T<sub>reg</sub>, regulatory T-cell; T<sub>H</sub>17, T-Helper-17 cell; IBD, irritable bowel disease.

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the glucosinolate, glucobrassicin. I3C and its oligomeric products exhibit multiple biological activities, including induction of apoptosis, decreased DNA-adduct formation, and reduced estrogen signaling, but have been characterized and extensively investigated as ligands and modulators of aryl hydrocarbon receptor (AHR) activity (Aggarwal & Ichikawa, 2005). Additionally, gastric acid-mediated condensation of I3C in the stomach yields 2-(indol-3-yl methyl)-3,3'-diindolylmethane (Itr-1) and indolo[3,2-b]carbazole (ICZ), a potent ligand and activator of the AHR (Bjeldanes, Kim, Grose, Bartholomew, & Bradfield, 1991). As such, broccoli represents a rich source of dietary AHR ligands (Hubbard, Murray, & Perdue, 2015).

The AHR is a ligand activated transcription factor, of the basic region helix-loop-helix-PER/ARNT/SIM homology super-family, first identified as the mediator of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxicity (Poland & Knutson, 1982). The receptor possesses a promiscuous ligand binding domain that is responsive to an array of xenobiotic compounds, such as polycyclic aromatic hydrocarbons (PAH's) and polychlorinated biphenyls (PCBs) (Poland & Knutson, 1982). The AHR has been historically characterized for its capacity to induce metabolism of exogenous compounds through transcriptional regulation of the cytochrome P450 gene battery (Denison, Fisher, & Whitlock, 1988; Rowlands & Gustafsson, 1997). The physiological roles of the AHR have expanded beyond xenobiotic metabolism, to include an array of endogenous functions. This concept is supported by the generation and phenotypic characterization of AHR null mice, which exhibit numerous physiological anomalies, including immune dysfunction, decreased hepatic vasculature development, and reduced fertility (Baba et al., 2005; McMillan & Bradfield, 2007).

Additional studies provide evidence that ligand-activated AHR participates in the maintenance of intestinal homeostasis. AHR null mice, relative to their wild-type counterparts, are more susceptible to various modes of intestinal challenge, such as chemically-induced colitis or *C. rodentium* infection (Li et al., 2011; Qiu et al., 2012). Protection is also conferred in ligand-responsive mice by administration of AHR agonists, such as TCDD and I3C (Furumatsu et al., 2011; Takamura et al., 2010). In the absence of exogenous agonists, AHR activity is likely mediated by ligands produced endogenously or provided via dietary sources, such as cruciferous vegetables. Administration of whole broccoli has been previously shown to alter the resident microflora in mice and to decrease colonic inflammation (Paturi et al., 2012). The mechanism(s) by which broccoli confers these activities and their dependency upon AHR-activation have not been fully investigated.

Here, the impact of broccoli consumption upon AHR activation, intestinal microbial community structure, and intestinal homeostasis is examined *in vivo*. To delineate the physiological impacts of broccoli consumption that associate with AHR ligand-responsiveness we utilized congenic mice expressing either the *Ahr*<sup>b/b</sup> (high-affinity) or *Ahr*<sup>d/d</sup> alleles (low-affinity) (Nebert & Bausserman, 1970). Mice harboring the *Ahr*<sup>d/d</sup> allele exhibit decreased sensitivity to many prototypical AHR ligands (Poland & Glover, 1975). Data presented here demonstrate that a significant component of the beneficial effects of broccoli can be attributed to activation of the AHR signaling pathway, resulting in an altered microbiome and protection from chemically induced colitis.

## 2. Materials and Methods (additional methodology can be found in the supplement)

### 2.1. Animals and husbandry

All animal studies were performed with approval and under the support of the Institutional Animal Care and Use Committee

(IACUC Protocol #45967, The Pennsylvania State University). C57BL6/J mice were originally purchased from Jackson Laboratories (Bar Harbor, ME). C57BL6/J-*Ahr*<sup>b/b</sup> and derived C57BL6/J-*Ahr*<sup>d/d</sup> were subsequently bred in-house. Animals were housed in autoclaved polypropylene cages with corn cob bedding in a specific pathogen-free environment with *ad libitum* access to indicated diets and water. Germ-free 129S6/SvEv-*Il10*<sup>-/-</sup> mice were purchased from the National Gnotobiotic Resource Center at the University of North Carolina School of Medicine. Germ-free C57BL6/J mice were bred in-house and maintained by The Pennsylvania State University Gnotobiotic Animal Research Facility.

### 2.2. Preparation of broccoli diet

Broccoli of the Lieutenant cultivar was used for generation of a custom broccoli diet. Broccoli was thoroughly rinsed, chopped finely, freeze-dried, and stored at -80°C prior to manufacturing of custom diet. Purified AIN-93G and the custom broccoli rodent diets were manufactured by Dyets Inc. (Bethlehem, PA). The nutritional components of the 15% (w/w) broccoli diet were adjusted to ensure similar nutritional and caloric content profiles relative to that of defined AIN-93G diet. The diet composition was adjusted based upon previously established nutritional component concentrations in broccoli (Salunkhe & Kadam, 1998). The compositions of both the control AIN-93G diet and customized 15% broccoli diet are listed in [Supplementary Table S1](#).

### 2.3. Feeding studies

C57BL6/J-*Ahr*<sup>b/b</sup> and *Ahr*<sup>d/d</sup> mice were weaned onto a standard animal chow diet. Mice (8–10-week-old) were acclimatized to AIN-93G purified rodent chow for seven days. This was followed by continuation of the AIN-93G diet in control groups or administration of the 15% broccoli diet for 24 days, unless otherwise indicated.

### 2.4. 16S rDNA gene Illumina MiSeq analyses

Bacterial DNA was amplified across the V4/V4 region of the 16S rRNA gene (Primers available in [Supplementary Table S2](#)) with FastStart high-fidelity amplification kit (Roche, Indianapolis, IN) and the following cycling conditions (94 °C, 3 min; 94 °C, 15 s, 55 °C, 45 s; 72 °C, 1 min for 30 cycles; 72 °C, 8 min). Product amplification was confirmed by agarose gel electrophoresis and the observation of a single product of 359 bp. The amplified V4 16S rRNA gene products were transferred to the Genomics Core Facility (The Pennsylvania State University) for 16S sequencing using the Illumina MiSeq platform (150 × 150 paired end). Sequence files were then obtained and analyzed using the Mothur software pipeline (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). Reads were trimmed at 320 bp and aligned to the SILVA database. Chimaeras were removed using UChime (Edgar, Haas, Clemente, Quince, & Knight, 2011) and reads were classified at a 75% cutoff using the ribosomal database project's (RDP) training set. The summary file was used for further taxonomic analysis. Further sequence analysis involved converting the trimmed fasta file into a distance file and aligning it to a phylogenetic tree. This tree file was compared to an operational taxonomic unit (OTU) file for Generalized Unifrac (GUnifrac) analysis (Chen et al., 2012; Chen, Liu, Ling, Tong, and Xiang, 2012).

### 2.5. Metagenomic analysis of the microbiome

A repeated analysis of the raw sequence data was completed again using the Mothur software pipeline with the reads aligned to the GreenGenes database (Kozich et al., 2013). This alignment

is needed to create a biom file for **Phylogenetic Investigation of Communities by Reconstruction of Unobserved States** (PICRUSt) analysis (Langille et al., 2013). Significantly different predicted pathways were discovered using **LDA effect size** (LEfSe). LEfSe combines the Kruskal-Wallis and the Wilcoxon statistical tests to show biologically relevant and statistically significant pathways.

## 2.6. RNA isolation and quantitative PCR expression analyses

Mice were euthanized by carbon dioxide asphyxiation and tissues were excised and immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Tissues were homogenized in 1 mL Tri Reagent (Sigma, St. Louis, MO) together with 10–20 zirconia/silica beads (1 mm diameter) (BioSpec Products, Bartlesville, OK) using a Bertin Precellys 24 homogenizer (VWR, Radnor, PA). Total RNA was isolated according to manufacturer's protocol. A total of 1.5  $\mu\text{g}$  RNA per sample was utilized as template for cDNA synthesis using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. cDNA was diluted 1:10 in nuclease-free water. Quantitative PCR reactions totaling 20  $\mu\text{L}$  were comprised of 6  $\mu\text{L}$  dilute cDNA template, 10  $\mu\text{L}$  PerfeCTa SYBR mastermix (Quanta Biosciences, Gaithersburg, MD), 2  $\mu\text{L}$  forward primer (3 nM), and 2  $\mu\text{L}$  reverse primer (3 nM). Reactions were performed using the cycling conditions ( $95^{\circ}\text{C}$ , 3 min;  $95^{\circ}\text{C}$ , 30 s, target-specific annealing  $^{\circ}\text{C}$ , 72  $^{\circ}\text{C}$ , 45 s for 40 cycles; 72  $^{\circ}\text{C}$ , 5 min) on a CFX Connect platform (Biorad). Primers can be found in [Supplementary Table S2](#).

## 2.7. Illumina Hiseq colonic RNA expression profiles

C57BL6/J *Ahr*<sup>b/b</sup> male mice were fed control ( $n = 3$ ) or broccoli ( $n = 3$ ) supplemented diet as described above. Animals were euthanized by carbon dioxide asphyxiation and placed into an anaerobic chamber (Coy Lab Products, Grass Lake, MI) containing an atmosphere comprising 3.3% hydrogen and <15 ppm oxygen. Tissues were excised under anaerobic conditions, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . RNA was extracted as described above and subjected to further purification using the RNeasy purification kit (Qiagen, Hilden, Germany). The RNA samples were transferred to the Genomics Core Facility (The Pennsylvania State University) for Poly-A selection and sequencing on the Illumina HiSeq 2500 (150 bp single end reads). RNAseq reads were aligned to the *Mus musculus* genome (mm10, RefSeq genes) using TopHat version 2.0.13 with default parameters (Trapnell, Pachter, & Salzberg, 2009). Alignment results are given in [Supplementary Table S3](#). Reads mapping to genes were counted using HTSeq-count version 0.5.4p3 with parameters '-s no -a 10' (Anders & Huber, 2010).

## 2.8. Ingenuity pathway analysis

To eliminate read bias, mapped reads were normalized to transcript length to generate normalized RPKM reads (Reads per kilobase per million) for further analyses. Reads were filtered by those whose expression varied significantly between groups, according to a Student's two-tailed *t*-test. Significantly altered genes that exhibited at least a 1.5-fold change in expression were analyzed using Ingenuity Pathway Analysis software (Qiagen, Hilden, Germany) according to manufacturer's instructions.

## 2.9. Statistical analysis

Where indicated, two-tailed unpaired parametric Student's *t*-test was performed. Correlation analyses were performed using Pearson's correlation coefficient (*r*) with a confidence interval of

95% and a two-tailed significance test. Significance thresholds of  $*p < 0.05$ ,  $**p < 0.01$  and  $***p < 0.001$  were applied. Statistical analyses and graphing was performed using Graphpad Prism v5.

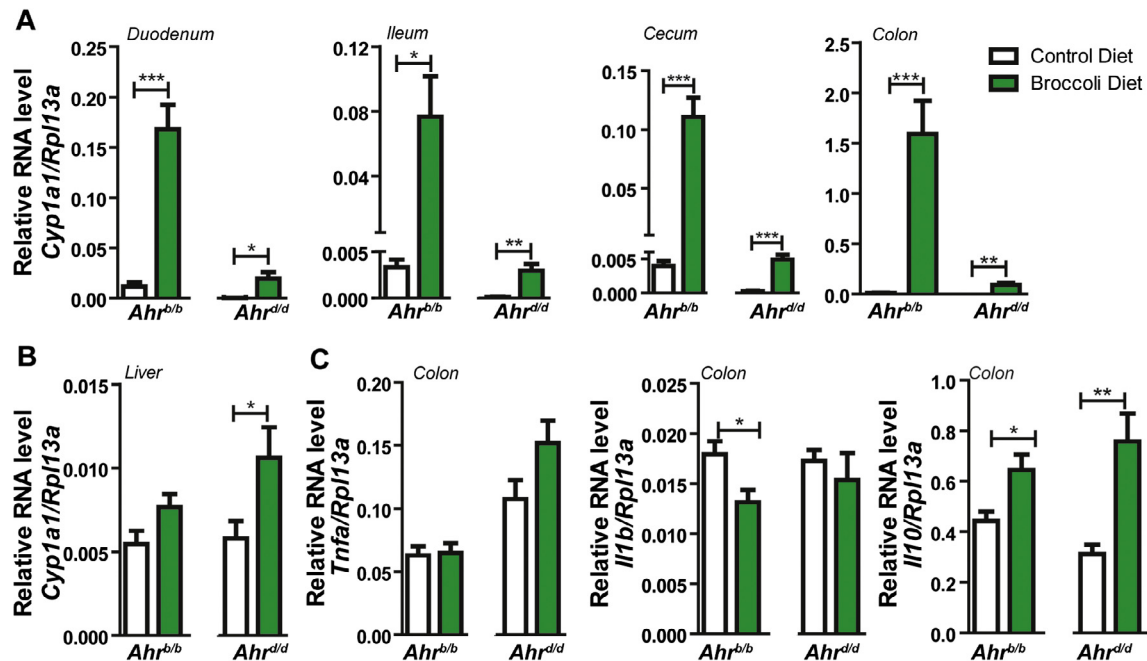
## 3. Results

### 3.1. Dietary broccoli induces the prototypic AHR target Gene, *Cyp1a1*

Activation of the AHR in response to dietary administration of whole broccoli was initially assessed in C57BL6/J mice that express the high affinity *Ahr*<sup>b/b</sup> allele. Mice were acclimatized to a defined pellet diet of AIN-93G (Low in AHR ligands) for seven days, and then administered finely ground diets containing concentrations of 0% or 10% (w/w) freeze-dried broccoli for an additional seven days. Broccoli at 10% in the diet promoted a significant 3-fold elevation in duodenal *Cyp1a1* expression ([Supplementary Fig. S1](#)). In our judgment, this represented a modest level of AHR mediated activity and thus 15% broccoli was tested and a significant 15-fold induction of *Cyp1a1* was observed ([Fig. 1A](#)). Thus, all further studies utilized a 15% (w/w) broccoli diet on an AIN-93G background in which the nutritional composition has been controlled to ensure similar caloric content relative to the AIN-93G control diet. Importantly, these data indicate that AHR-mediated intestinal *Cyp1a1* expression is dose-dependently sensitive to dietary broccoli. Glucosinolate levels vary between different broccoli cultivars and storage conditions. Extractions of the Lieutenant cultivar we used and subsequent quantification via HPLC-MS revealed glucobrassicin content to be 1.6  $\mu\text{moles/g}$  (dry weight). This value is within previously established ranges of glucobrassicin levels detected in different varieties of broccoli cultivars (Kushad et al., 1999). Analysis of 15% broccoli diet consumption rates revealed a mean intake of 3.34 g per day, which equates to 354  $\mu\text{g}$  of glucobrassicin per day, which is equivalent to a mean intake of 14 mg/kg/day.

### 3.2. Broccoli supplemented diet facilitates differential activation of the AHR in congenic *Ahr*<sup>d/d</sup> and *Ahr*<sup>b/b</sup> mice

The physiological impacts of broccoli consumption associated with activation of the AHR were evaluated using congenic male C57BL6/J mice possessing either the ligand-sensitive *Ahr*<sup>b/b</sup> or less-sensitive *Ahr*<sup>d/d</sup> alleles. Mice were acclimatized to AIN-93G diet for seven days, followed by a 24-day dietary intervention of modified AIN-93G rodent diet supplemented with 15% broccoli ( $n = 16$  per genotype, 8 per diet). Examination of the percentage increase in body weight over 24 days revealed no significant contribution of either diet or genotype. ([Supplementary Fig. S2](#)). Epididymal adipose tissue weight was found to be unaltered by dietary broccoli in *Ahr*<sup>b/b</sup> mice, however, a decrease in mean epididymal adipose mass of 22.5% occurred in broccoli fed *Ahr*<sup>d/d</sup> mice relative to control-fed mice ([Supplementary Fig. S2](#)). Gastrointestinal activation of AHR by dietary broccoli was assessed by quantification of *Cyp1a1* expression in the duodenum, ileum, cecum, and colon ([Fig. 1A](#)). Duodenal *Cyp1a1* expression was enhanced significantly relative to controls in both *Ahr*<sup>b/b</sup> and *Ahr*<sup>d/d</sup> animals, 15-fold and 85-fold, respectively. However, upon comparison of genotypes, *Ahr*<sup>b/b</sup> mice were found to express 9-fold higher levels of *Cyp1a1* in the duodenum than *Ahr*<sup>d/d</sup> mice, in response to dietary broccoli. Similar dietary and genotypic trends of AHR transcriptional activity were observed throughout the intestinal tract, i.e. the ileum, cecum, and colon ([Fig. 1A](#)). Systemic penetrance of broccoli derived AHR ligands and resultant AHR activation, were evaluated by quantification of hepatic *Cyp1a1* expression ([Fig. 1B](#)). Contrary to observations in intestinal tissues, broccoli diet did not mediate a significant increase in AHR target gene expression within the



**Fig. 1.** *Ahr* allele status dictates responsiveness and systemic penetration of broccoli-derived agonists. (A) Real time PCR quantification of (A) intestinal *Cyp1a1* expression, (B) Hepatic *Cyp1a1* expression, and (C) Colonic inflammatory gene expression (*Tnfa*, *Il1b*, *Il10*) normalized to eukaryotic *Rpl13a* from *Ahr<sup>b/b</sup>* and *Ahr<sup>d/d</sup>* mice fed AIN-93G (control) or isocaloric broccoli diet (15%) for 24 days. Data represent the mean gene expression ( $n = 8$  per genotype/diet group)  $\pm$  standard error of mean (SEM).

*Ahr<sup>b/b</sup>* genotype. However, within the *Ahr<sup>d/d</sup>* mice, a significant two-fold increase in hepatic *Cyp1a1* expression was observed. In addition, it was previously observed that administration of AHR ligands can alter intestinal inflammatory status (Furumatsu et al., 2011). Colonic expression of the cytokines *Tnfa*, *Il1b*, and *Il10* associated differentially with dietary or genotypic status (Fig. 1C). Expression of *Tnfa* was elevated in *Ahr<sup>d/d</sup>* relative to *Ahr<sup>b/b</sup>* mice and increased further following broccoli consumption. Expression of proinflammatory *Il1b* was reduced by 25% by consumption of broccoli, but this effect was only observed in *Ahr<sup>b/b</sup>* mice. Broccoli consumption also mediated a 1.5–2-fold increase in anti-inflammatory cytokine *Il10* expression, irrespective of *Ahr* genotype.

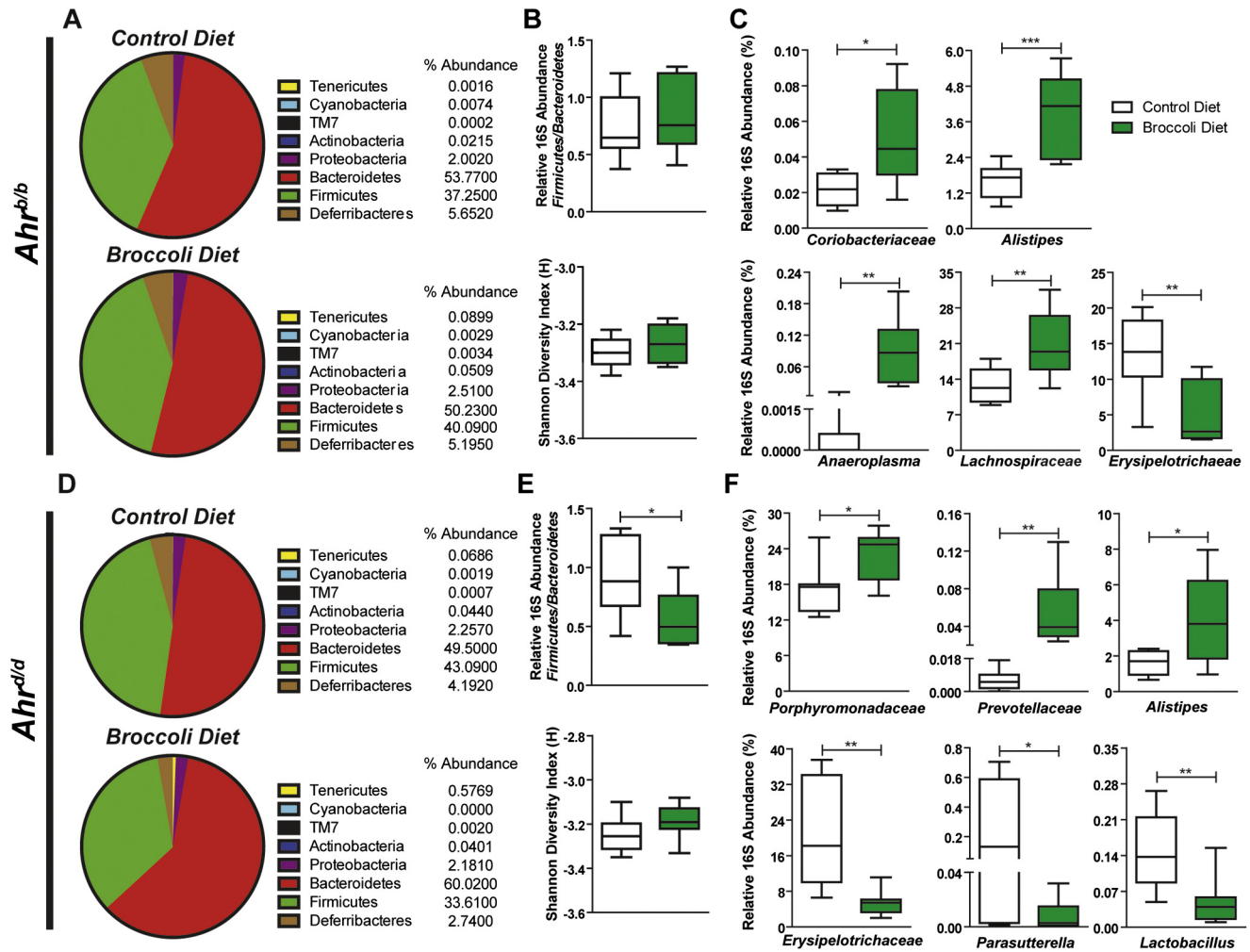
### 3.3. Dietary broccoli and genotype alter microbial community structure

AHR status is reported to influence intestinal microbial community structure (Murray, Nichols, Zhang, Patterson, & Perdew, 2016). To assess the combinatorial impact of AHR status and consumption of broccoli upon microbial community structure, we performed bacterial 16S rRNA gene profiling of DNA isolated from cecal luminal contents of *Ahr<sup>b/b</sup>* and *Ahr<sup>d/d</sup>* mice following 24 days of broccoli consumption. Cecal microbial population profiles were obtained from 16S rRNA gene sequencing (mean 155,000 reads/sample,  $n = 8$  per group). Comparative analyses of phylogenetic differences between genotypes and dietary broccoli supplementation were conducted using g-unifrac. Differences in bacterial populations are calculated as distances based upon the presence of abundant (weighted unifrac) and rare (unweighted unifrac) bacterial populations, where a shorter distance between points indicates similarities in overall microbial composition. Analyses of microbial composition by g-unifrac indicate that individuals segregate into distinct groups dependent upon genotype and diet (Supplementary Fig. S3).

Phyla level analyses of 16S rRNA gene sequencing derived from *Ahr<sup>b/b</sup>* mice fed a control or broccoli diet identify significant

differences in the percentage abundance of 16S reads associated with *Tenericutes* (57-fold increase), *Cyanobacteria* (3-fold decrease), *TM7* (15-fold increase), and *Actinobacteria* (2-fold increase) (Fig. 2A). Consumption of broccoli diet by *Ahr<sup>b/b</sup>* mice did not significantly influence 16S reads associated with *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, or *Deferibacteria*, which together constitute over 90% of the total reads for all samples analyzed. As such, there was no significant change in the *Firmicutes/Bacteroidetes* ratio or the overall microbial community diversity as measured by the Shannon Diversity Index score associated with dietary broccoli (Fig. 2B). In total, 16S reads aligned with approximately 224 different taxonomic classifications per individual (Supplementary Figs. S4–S8). Significant variations in the relative abundances of 35 taxa associated with broccoli consumption in *Ahr<sup>b/b</sup>* mice (Fig. 2C). The increased abundance of 16S reads associated with *Actinobacteria* in broccoli fed *Ahr<sup>b/b</sup>* mice correlated with a 2-fold enrichment of the family *Coriobacteriaceae* ( $r = 0.9999$ ,  $p < 0.0001$ ). Within the *Bacteroidetes* phylum, a significant increase (2-fold) within the family *Rikenellaceae* was observed in broccoli fed *Ahr<sup>b/b</sup>* mice, correlating with an increase of the genus *Alistipes* ( $r = 1.0$ ,  $p < 0.0001$ ), which accounted for a 2% increase relative to total 16S reads. Significant enrichment of 16S reads associated with the class *Mollicutes* (57-fold) correlated with a corresponding increase in the genus *Anaeroplasm* ( $r = 1.0$ ,  $p < 0.0001$ ). Increased abundance of 16S reads associated with the class *Clostridia* was found to strongly correlate with the family member *Lachnospiraceae* ( $r = 0.9485$ ,  $p < 0.0001$ ). Increased 16S reads associated with *Clostridia* accounted for an average 11% increase relative to total 16S reads. Conversely, a trend towards depletion of 16S reads associated with the class *Erysipelotrichia*, which accounted for an average decrease of 8% relative to total reads, was observed in broccoli fed *Ahr<sup>b/b</sup>* mice. Decreased *Erysipelotrichia* was found to strongly correlate with a decrease in the family member *Erysipelotrichaceae* ( $r = 1.0$ ,  $p < 0.0001$ ). Notably, 16S reads associated with four genera of microbes, *Asaccharobacter*, *Syntrophococcus*, *Anaerostipes*, and *Ruminococcus*, are solely present in *Ahr<sup>b/b</sup>* mice fed a broccoli diet.





**Fig. 2.** Dietary broccoli mediates divergence of cecal microbial community structure in *Ahr<sup>b/b</sup>* and *Ahr<sup>d/d</sup>* mice. Pie chart representation and relative abundance of bacterial phyla from control or broccoli-fed (A) *Ahr<sup>b/b</sup>* and (D) *Ahr<sup>d/d</sup>* mice. Broccoli impacts *Firmicutes/Bacteroidetes* ratio and overall microbial diversity as determined by the Shannon Diversity Index found in (B) *Ahr<sup>b/b</sup>* and (E) *Ahr<sup>d/d</sup>* mice. Relative 16S rDNA abundances of microbial taxa that are significantly altered by administration of broccoli diet in (C) *Ahr<sup>b/b</sup>* and (F) *Ahr<sup>d/d</sup>* mice. Data represent the mean relative 16S rDNA abundance (%) ( $n = 8$  per genotype/diet group)  $\pm$  SEM.

Phyla level analyses of 16S rRNA gene sequencing between *Ahr<sup>d/d</sup>* mice fed a control or broccoli diet identify significant differences in the percentage abundance of 16S reads associated with *Bacteroidetes* (1.2-fold increase) and *TM7* (3-fold increase) (Fig. 2D). Consumption of broccoli diet by *Ahr<sup>d/d</sup>* mice did not coincide with significant phyla variations in 16S reads associated with *Proteobacteria*, *Tenericutes*, *Actinobacteria*, *Cyanobacteria*, *Firmicutes*, or *Deferibacteria*. The increase in reads associated with the phyla *Bacteroidetes* accounted for an average 11% increase relative to total 16S reads and facilitated a significant decrease in the relative *Firmicutes/Bacteroidetes* ratio in broccoli-fed *Ahr<sup>d/d</sup>* mice (Fig. 2E). Analysis of microbial diversity in *Ahr<sup>d/d</sup>* mice identified no significant change in Shannon Diversity Index associated with broccoli consumption (Fig. 2E). In total, relative 16S reads associated with 224 different taxonomic classifications were quantified per individual (Supplementary Figs. S4–S8). Expanded 16S sequence analyses identified 29 significant changes in relative taxa abundance associated with dietary broccoli in *Ahr<sup>d/d</sup>* mice (Fig. 2F). The increased abundance of 16S reads associated with *Bacteroidetes* in broccoli-fed *Ahr<sup>d/d</sup>* mice correlated with an enrichment of three families *Porphyromonadaceae* ( $r = 0.6108$ ,  $p < 0.05$ ), *Prevotellaceae* ( $r = 0.6479$ ,  $p < 0.01$ ), and *Rikenellaceae* ( $r = 0.5768$ ,  $p < 0.05$ ). The significant increase in 16S reads associated with the family *Rikenellaceae* correlated with an increase of the genus *Alistipes* ( $r = 1.0$ ,

$p < 0.0001$ ). This increase in 16S reads associated with *Alistipes* accounted for a 2% increase relative to total 16S reads. A decrease in 16S reads associated with the order *Burkholderiales* (27-fold) correlated with a corresponding decrease in the genus *Parasutterella* ( $r = 0.9576$ ,  $p < 0.0001$ ). A reduction in 16S reads associated with the class *Bacilli* (3-fold) was found to strongly correlate with the family member *Lactobacillus* ( $r = 0.9858$ ,  $p < 0.001$ ). A trend towards depletion of 16S reads associated with the class *Erysipelotrichia*, which accounted for an average decrease of 15% relative to total reads, was observed in broccoli fed *Ahr<sup>d/d</sup>* mice. Decreased *Erysipelotrichia* strongly correlated with a decrease in the family member *Erysipelotrichaceae* ( $r = 1.0$ ,  $p < 0.0001$ ). Within *Ahr<sup>d/d</sup>* mice, 16S reads associated with the family *Staphylococcaceae* are only observed in broccoli-fed *Ahr<sup>d/d</sup>* mice. Enrichment of 16S reads associated with the increase in *Staphylococcaceae* correlate with a corresponding increase in the genus *Staphylococcus* ( $r = 0.7660$ ,  $p < 0.001$ ).

Alterations in bacterial community composition between *Ahr<sup>b/b</sup>* and *Ahr<sup>d/d</sup>* mediated by broccoli were compared to identify changes associated with increased sensitivity to AHR ligands (Fig. 3). Unique changes in 16S read abundance of three phyla, four classes, three orders, four families, and 12 genera were observed in broccoli-fed *Ahr<sup>b/b</sup>* mice, relative to controls. In contrast, 16S reads associated with one phylum, two classes, four orders, five families,

and eight genera were found to be uniquely altered in *Ahr*<sup>d/d</sup> mice fed broccoli. Shared changes within the bacterial community compositions, independent of genotype and AHR activation status, identified significant variations within one phylum, one class, one order, two families, and four genera. More specifically, broccoli consumption was associated with increased 16S read abundance corresponding to the phylum TM7, family Rikenellaceae, and genera *Alistipes* and *Anaerostipes*. Decreased 16S read abundance associated with the class Erysipelotrichia, order Erysipelotrichales, family Erysipelotrichaceae, and the genus *Dorea* was observed following broccoli administration regardless of *Ahr* genotype. Notably, the relative abundance of bacteria within the genus *Flavonifractor* was significantly decreased in *Ahr*<sup>b/b</sup> mice, but significantly elevated in *Ahr*<sup>d/d</sup> mice upon broccoli consumption. Overall these data demonstrate significant differences in intestinal microbe community structure that segregate according to the host's diet and *Ahr* status.

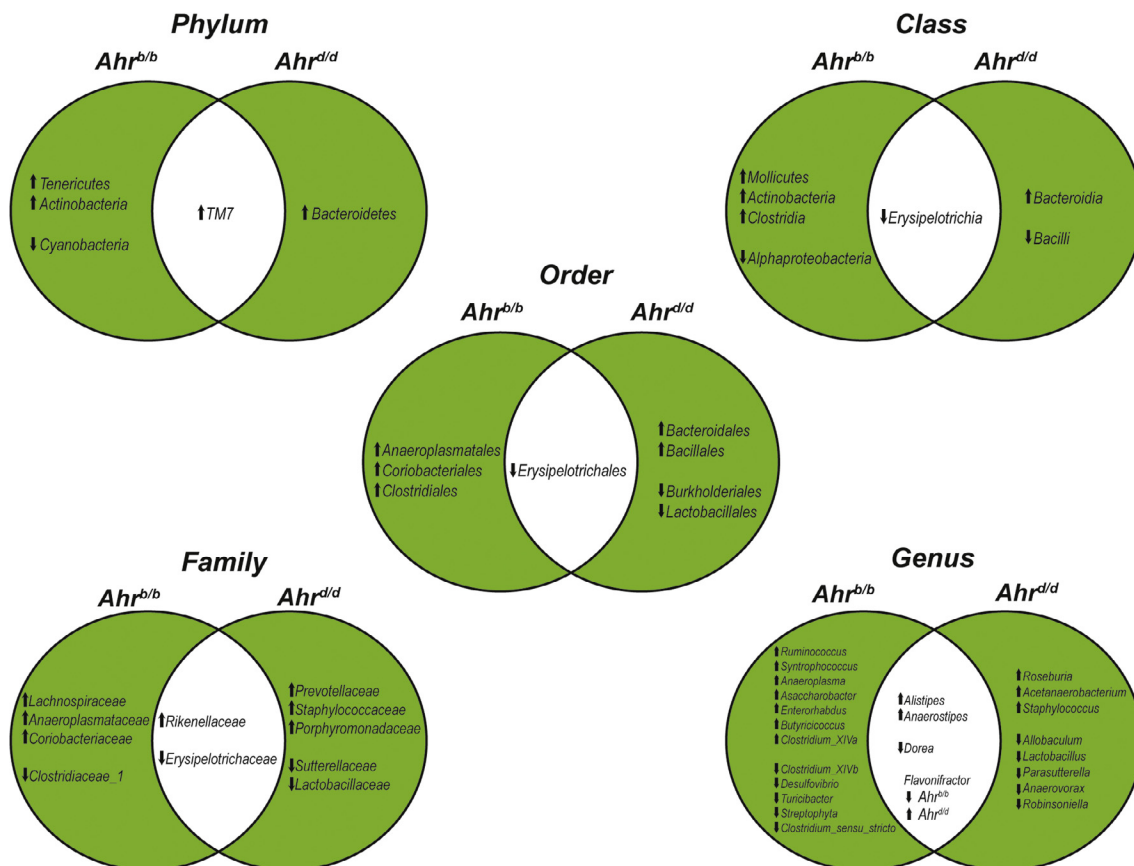
#### 3.4. Microbial status does not associate with an enhanced capacity to produce AHR ligand from indole-3-carbinol

It is well documented that acid condensation reactions of I3C yield potent AHR ligands (Bradfield & Bjeldanes, 1987). Microbial metabolism of I3C may contribute to endogenous formation of AHR ligands, and could impact the observed physiological effects of dietary broccoli by increasing AHR ligand bioavailability in the distal colon (Kwon, Grose, Riby, Chen, & Bjeldanes, 1994). To address this hypothesis, we performed parallel dietary feeding studies in which conventional and germ-free C57BL6/J mice were fed a purified diet with or without supplementation of I3C (125 mg/kg) for seven days. Intestinal AHR activation was compared

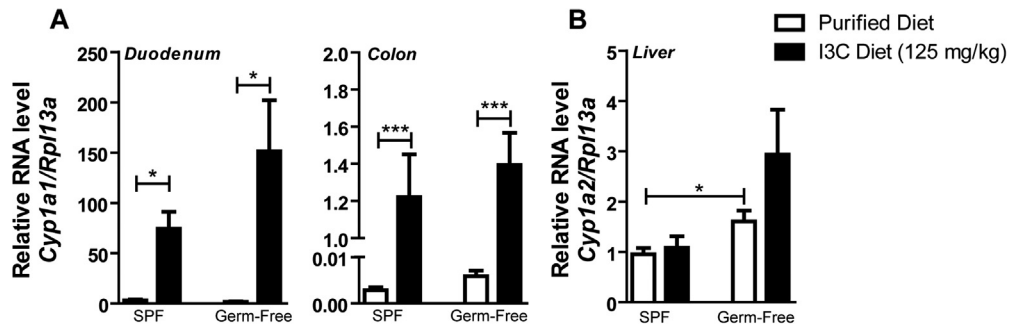
between animals by quantitative PCR analysis of *Cyp1a1* expression (Fig. 4A). Similar duodenal expression profiles of *Cyp1a1* were observed in conventional and germ-free mice. In contrast to previous findings, we observed no significant increase in colonic AHR activity following I3C administration attributed to alteration of microbial status. Equivalent levels of basal AHR activity were observed in conventional and germ-free animals, suggesting minimal microbial contribution to intestinal generation of I3C-derived AHR ligands. Hepatic AHR activity, as measured by *Cyp1a2* induction, was not elevated in conventional mice by consumption of I3C (Fig. 4B). However, basal hepatic AHR activity increased in germ-free relative to conventional mice.

#### 3.5. Metagenomic analyses of microbial communities identify differential metabolic potential associated with dietary broccoli and AHR status

Having established that dietary broccoli and *Ahr* status can mediate divergence of overall cecal microbial community composition in mice, we utilized whole genome metagenomic pathway analyses to assess differences in microbial metabolic potential. Numerous alterations in microbial metabolic capacity were found to be mediated by host consumption of broccoli (Fig. 5). In total, 49 and 46 microbial metabolic pathways were significantly altered by broccoli ingestion in *Ahr*<sup>b/b</sup> or *Ahr*<sup>d/d</sup> mice, respectively. Broccoli consumption associated with a decreased representation of ten metabolic pathways: riboflavin metabolism, lysine degradation, nicotinate and nicotinamide metabolism, D-glutamine/D-glutamate metabolism, terpenoid backbone biosynthesis, nitro-toluene degradation, photosynthesis, amino-sugar/nucleotide-sugar metabolism, thiamine metabolism, and seleno-compound



**Fig. 3.** *Ahr* status contributes to alteration of cecal microbe populations by broccoli. Venn diagram plots depict significant ( $p < 0.05$ ) changes in microbial taxa that occur dependently (green) or independently (white) of *Ahr* status, based upon relative 16S rDNA abundances.



**Fig. 4.** Bacterial status does not influence formation of broccoli-derived AHR ligands. Specific-pathogen free (SPF) and Germ-free (GF) C57BL6/J-*Ahr*<sup>b/b</sup> mice were fed control or indole-3-carbinol (I3C) (125 mg/kg) diets for a duration of 7 days, followed by real time PCR quantification of (A) Duodenal/colonic expression of prototypic target gene *Cyp1a1* and (B) Hepatic expression of *Cyp1a2*, both normalized to eukaryotic *Rpl13a*. Data represent the mean gene expression (SPF + control diet n = 5, SPF + I3C diet n = 6, GF + control diet n = 4, GF + I3C diet n = 6) ± SEM.

metabolism, independent of *Ahr* status. In *Ahr*<sup>b/b</sup> mice, dietary broccoli associated with decreased abundance of 18 pathways involved in amino acid metabolism (22%), genetic information processing (44%), metabolism of other amino acids (6%), and miscellaneous pathways (28%). In *Ahr*<sup>d/d</sup> mice, broccoli associated with decreased abundance of 15 pathways involved in amino acid metabolism (13%), carbohydrate metabolism (13%), genetic information processing (13%), glycan biosynthesis and metabolism (7%), lipid metabolism (13%), metabolism of other amino acids (13%), and miscellaneous pathways (27%).

Increased representation of nine metabolic pathways: methane metabolism, phosphonate/phosphinate metabolism, valine/leucine/isoleucine biosynthesis, starch/sucrose metabolism, drug metabolism, arginine/proline metabolism, C5-branched dibasic amino acid metabolism, sphingolipid metabolism, and vitamin B6 metabolism all were observed in both *Ahr*<sup>b/b</sup> and *Ahr*<sup>d/d</sup> broccoli-fed mice. In *Ahr*<sup>b/b</sup> mice, broccoli associated with increased abundance of 12 pathways involved in carbohydrate metabolism (42%), lipid metabolism (25%), metabolism of cofactors and vitamins (8%), and miscellaneous pathways (25%). In *Ahr*<sup>d/d</sup> mice, broccoli associated with increased abundance of 12 pathways involved in amino acid metabolism (17%), genetic information processing (8%), glycan biosynthesis and metabolism (17%), lipid metabolism (13%), metabolism of cofactors and vitamins (33%), and miscellaneous pathways (25%). This data provides further support to the concept that *Ahr* status and consumption of broccoli can influence microbial community structure and their metabolic contribution to overall host physiology.

### 3.6. <sup>1</sup>H-NMR analysis of cecal metabolite profiles from broccoli-fed *Ahr*<sup>b/b</sup> and *Ahr*<sup>d/d</sup> mice

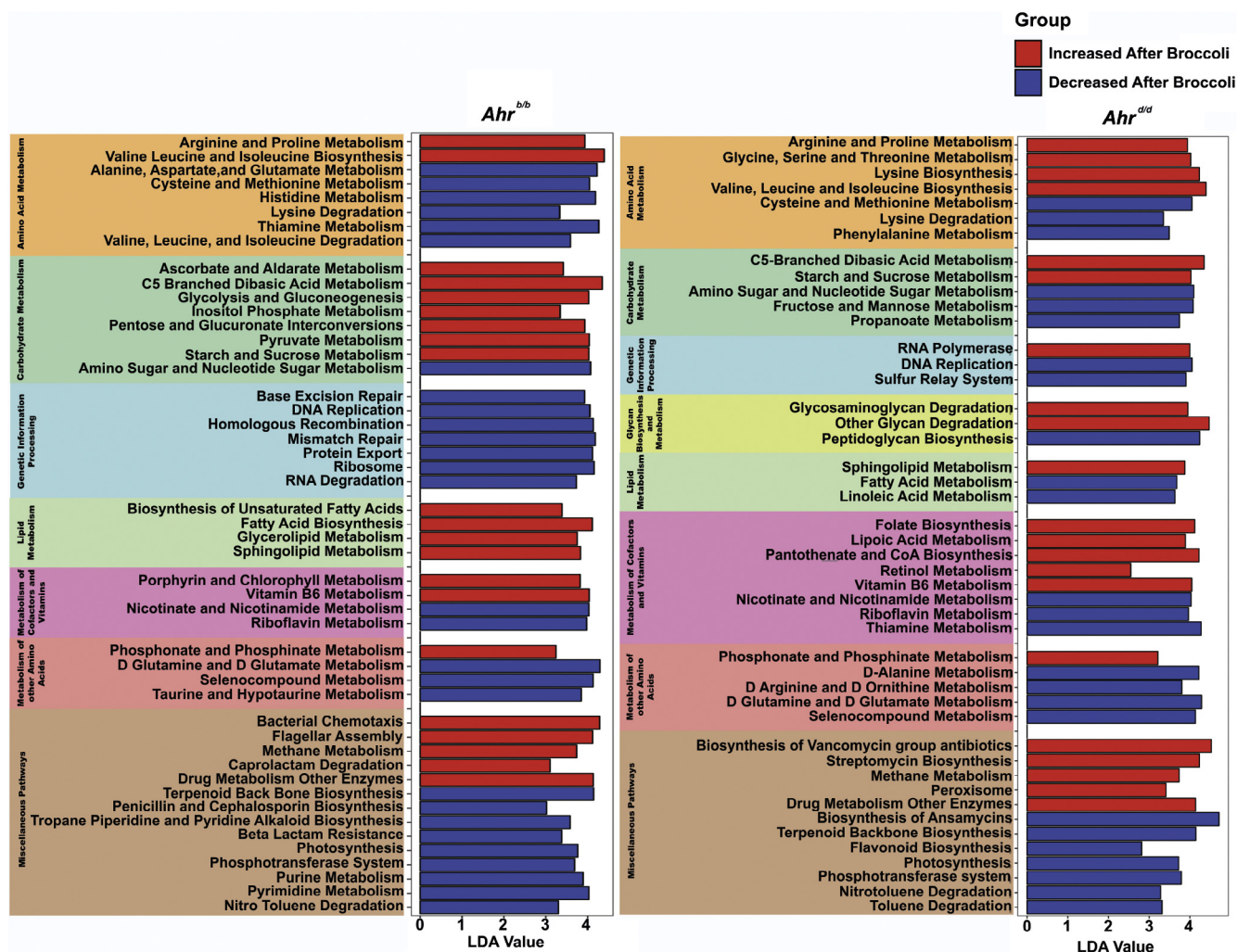
The observed variance in the metabolic potential of intestinal microbes associated with broccoli consumption and *Ahr* status suggest possible changes in microbial metabolic output and subsequent alteration of metabolite profiles in the cecal luminal contents. Relative quantification of short chain fatty acids (SCFAs), glucose, lactate, bile acids, and amino acids levels within cecal content extracts were measured by <sup>1</sup>H-NMR targeted peak integration analyses (Supplementary Fig. S9). Of the metabolites measured, only the SCFA propionate and lactate were found to be significantly decreased in *Ahr*<sup>b/b</sup> mice following administration of broccoli. In contrast, broccoli consumption associated with significant changes in the luminal concentration of numerous metabolites in *Ahr*<sup>d/d</sup> mice. Concentrations of acetate, a SCFA that is a principle energy source for host epithelial cells, and bile acids, host-derived emulsifiers and bioactive signaling factors, were elevated in the cecal contents of *Ahr*<sup>d/d</sup> broccoli-fed mice. In contrast, glucose, tyrosine, and

phenylalanine metabolite concentrations were significantly decreased upon broccoli administration. These data provide support that the host genotype, dietary status, and resultant alteration of microbial populations can combinatorially influence intestinal metabolite availability, which may further influence host signaling and gene expression.

### 3.7. Dietary broccoli attenuates symptoms of chemically induced colitis

The AHR has been previously investigated for its role in reducing disease severity associated with chemically induced inflammatory models such as dextran sodium sulfate (DSS) colitis (Takamura et al., 2010). Utilizing a DSS-colitis model, we investigated the capacity for dietary broccoli to remediate intestinal disease severity in conjunction with differential AHR activation potential through use of *Ahr*<sup>b/b</sup> and *Ahr*<sup>d/d</sup> mice (Fig. 6A). Mice were given access to control or broccoli diets for 14 days prior to administration of 3.5% DSS in drinking water. Mice were maintained on these diets for the remainder of the study. DSS-supplemented water was administered for six consecutive days, at which point the mice were switched to tap water for 48 h prior to euthanasia. Animals were monitored daily for weight loss, and clinical symptoms of disease severity, i.e. stool consistency, blood in stool, rectal bleeding, and lethargy. As stated previously, broccoli consumption alone does not cause significant change in weight relative to control mice (Supplementary Fig. S2). *Ahr*<sup>b/b</sup> mice exposed to DSS and fed a control diet exhibited marked decreases in body weight following day 4 of administration. The onset of weight-loss in *Ahr*<sup>b/b</sup> mice was delayed by administration of broccoli diet, which led to significant differences in the percent of weight lost that was maintained throughout the duration of the study time course. *Ahr*<sup>d/d</sup> broccoli-fed mice also exhibited attenuated weight loss, however this effect was only observed during days four and five of DSS administration. By day six, the last day of DSS exposure, significant attenuation of overall weight-loss is singularly observed in *Ahr*<sup>b/b</sup> broccoli-fed mice, which exhibit only a 5% decrease in total body weight, compared to an average 12% decrease in all other experimental groups (Fig. 6B). Colitis disease activity was quantified for the initial four days of the study when fecal pellets were produced by all observed individuals. Disease activity scores indicate significant attenuation of assessed clinical parameters associated with broccoli-fed *Ahr*<sup>b/b</sup> mice relative to *Ahr*<sup>b/b</sup> control diet and *Ahr*<sup>d/d</sup> broccoli diet groups (Fig. 6C). An enlarged spleen is indicative of a heightened inflammatory status or infection and is known to occur in both acute and chronic models of DSS-colitis. Both *Ahr*<sup>b/b</sup> and *Ahr*<sup>d/d</sup> mice displayed significant attenuation of the clinical manifestation of splenomegaly as evidenced by a respective 31%





**Fig. 5.** Metagenomic analysis of differential microbial metabolic potential. Significantly different predicted pathways mediated by broccoli were discovered using LDA effect size (LEfSe). LEfSe combines the Kruskal-Wallis and the Wilcoxon statistical tests to show biologically relevant and statistically significant pathways. Metabolic pathways were grouped by established KEGG classifications. Data represent linear discriminate analysis (LDA) score for indicated pathways that significantly increase (red) or decrease (blue) due to broccoli consumption in *Ahr<sup>b/b</sup>* and *Ahr<sup>d/d</sup>* mice.

and 33% lowered mean spleen weight relative to control fed mice (Fig. 6D).

Our previous data indicates broccoli is protective against DSS-chemical challenge. However, this phenotype may be a consequence of broccoli mediated host adaptation prior to or an active response during intestinal challenge. Histological analyses of mucosal damage by assessment of severity/extent of inflammatory cell infiltration and loss of intestinal crypt morphology displayed no difference between control and broccoli-fed *Ahr<sup>b/b</sup>* mice (Supplementary Fig. S10). Previous studies found that ligand-activation of the AHR facilitates decreased symptom severity in chemically-induced inflammatory models by enhancement of anti-inflammatory regulatory T-cell ( $T_{reg}$ ) differentiation (Quintana et al., 2008). In the absence of an inflammatory challenge broccoli had no effect upon  $T_{reg}$  differentiation in *Ahr<sup>b/b</sup>* or *Ahr<sup>d/d</sup>* mice. Although increased splenic T-Helper 17 ( $T_{H17}$ ) cell numbers (1.6-fold) were observed in *Ahr<sup>d/d</sup>* mice (Supplementary Fig. S11). Upon challenge with DSS, broccoli mediated a significant reduction (3-fold) in splenic pro-inflammatory  $T_{H17}$  cells, but no increase in  $T_{reg}$  cell populations in *Ahr<sup>b/b</sup>* mice (Fig. 6E). To examine possible timeline differences in the onset of intestinal inflammation, a non-invasive measurement of fecal lipocalin 2 (Lcn2) was utilized. The lack of fecal Lcn2 production at day 4 in broccoli-fed

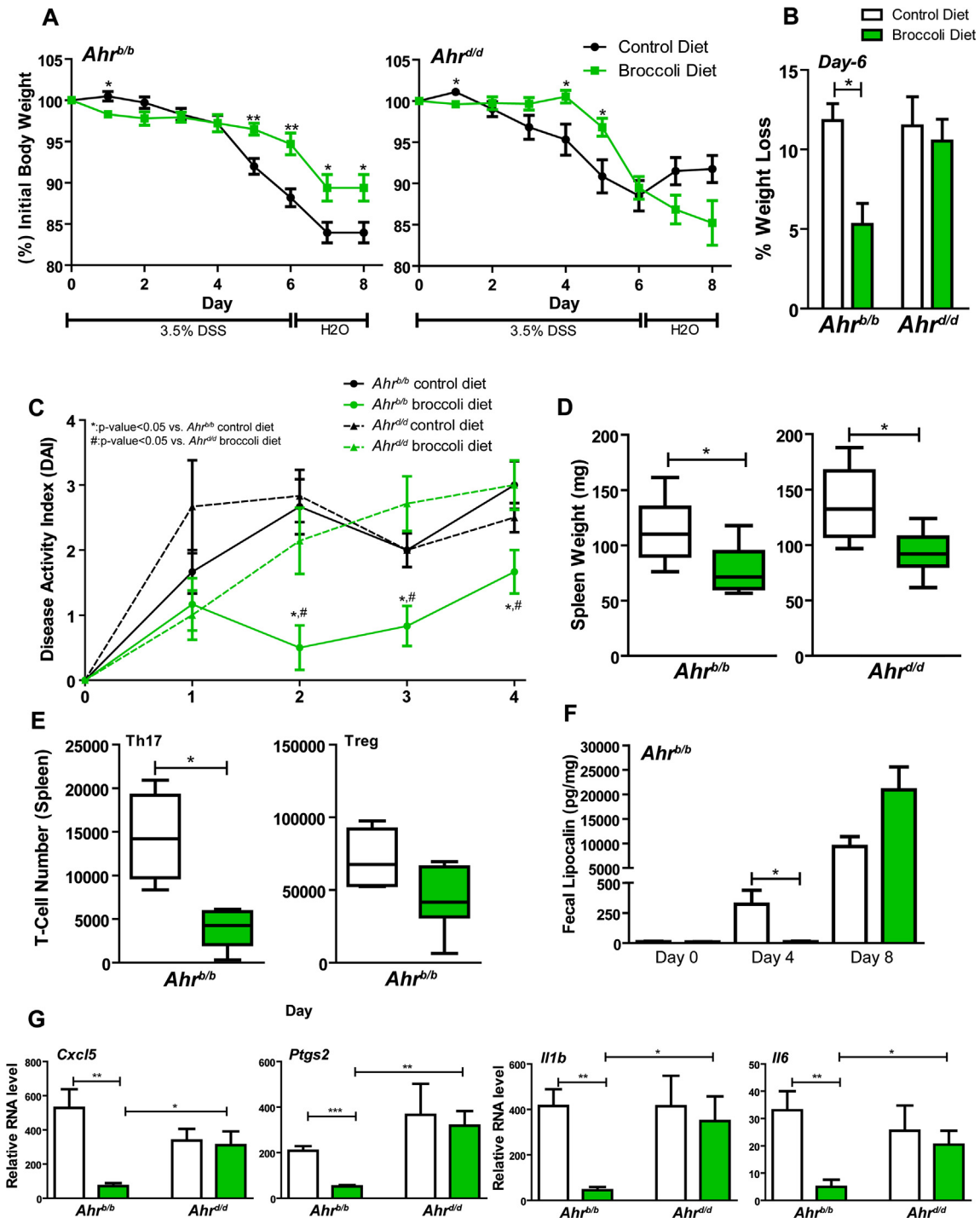
*Ahr<sup>b/b</sup>* mice indicate delayed onset of DSS-induced intestinal stress (Fig. 6F). Taken together, these data suggest that broccoli consumption may prime the host to better adapt to instances of intestinal challenge.

To assess whether broccoli fed mice exhibited an attenuated constitutive inflammatory status Nanostring™ hybridization was performed. Quantification of colonic inflammatory gene expression identified four genes [*Cxcl5* (4-fold), *Ptgs2* (6-fold), *Il1b* (8-fold), and *Il6* (4-fold)] that exhibited significantly decreased expression in *Ahr<sup>b/b</sup>* mice fed broccoli, in contrast to broccoli-fed *Ahr<sup>d/d</sup>* mice (Fig. 6G).

### 3.8. Microbial reconstitution of germ-free *Il10<sup>-/-</sup>* mice

Administration of DSS induces colitis by disruption of intestinal epithelial barrier integrity, which increases microbial translocation out of the intestinal lumen. Microbial composition, the presence of opportunistic pathogens, and host genetic risk factors all concomitantly contribute to susceptibility to intestinal inflammatory disease. A lack of AHR signaling promotes colonization of segmented filamentous bacteria and an increased intestinal inflammatory tone (Murray et al., 2016). This finding suggests that AHR activity and dietary broccoli may promote differential





**Fig. 6.** Sensitivity to broccoli derived AHR ligands contributes to attenuation of DSS-colitis disease severity. Attenuation of DSS-mediated body weight loss in response to consumption of broccoli in (A) *Ahr<sup>b/b</sup>* and *Ahr<sup>d/d</sup>* mice. (B) Influence of broccoli consumption on day 6 of DSS exposure. (C) Disease activity index (DAI) and (D) spleen weight were assessed in treated mice. (E) Box and whisker plot depicting *Ahr<sup>b/b</sup>* splenic  $T_{H17}$  and  $T_{reg}$  numbers following broccoli administration and DSS challenge. (F) ELISA quantification of fecal lipocalin (pg/mg) at days 0, 4, and 8 of DSS challenge in *Ahr<sup>b/b</sup>* fed control or broccoli diet. Data are shown as means  $\pm$  SEM (n = 6 per genotype/diet). (G) Nanostring hybridization analysis of colonic inflammatory cytokines expression (*Cxcl5*, *Ptgs2*, *Il1b*, *Il6*) in broccoli-fed *Ahr<sup>b/b</sup>* and *Ahr<sup>d/d</sup>* mice.

colonization of microbial communities that vary in their intrinsic capacity to induce intestinal inflammation. To compare the contribution of microbial communities upon intestinal inflammatory status we utilized a germ-free *Il10<sup>-/-</sup>* mouse reconstitution model, in which cecal microbes from broccoli and control fed donors were gavaged into *Il10<sup>-/-</sup>* mice. Their innate lack of *Il10* expression predisposes them to onset of spontaneous colitis following reconstitution of enteric bacteria (Sellon et al., 1998). Microbial

restitutions from *Ahr<sup>b/b</sup>* mice fed control or broccoli diets mediated similar colonic expression of pro-inflammatory cytokines (*Tnfa*, *Il1b*, *Il6*, *Il17a*, and *Lcn2*), suggesting that broccoli-dependent microbial differences are unable to induce a heightened intestinal inflammatory status (Supplementary Fig. S12). Therefore, the observed benefit of broccoli upon *Ahr<sup>b/b</sup>* mice in DSS-colitis models is likely attributed to effects upon host gene expression. In contrast, microbial communities derived from

broccoli-fed *Ahr<sup>d/d</sup>* mice mediate a heightened colonic inflammatory tone in recipient mice, characterized by significantly increased expression of *Tnfa* (3-fold), *Il1b* (4-fold), *Il6* (2-fold), and *Il17a* (2-fold) (Supplementary Fig. S13).

### 3.9. RNA-Seq analysis of dietary broccoli impact upon colonic gene expression

RNA-sequencing was utilized as a non-targeted high-throughput method to further investigate the impact of dietary broccoli upon colonic gene expression in *Ahr<sup>b/b</sup>* mice on day 8 of the DSS exposure study. Reads were mapped to 10,163 known transcripts (Fig. 7A). Transcripts that displayed significantly altered expression ( $p < 0.05$ , by student's *t*-test) between control and broccoli diet groups were compared via Bland-Altman plot. In total 608 targets were found to exhibit at least a 1.5-fold change in expression (212 upregulated, 396 down-regulated) (Fig. 7B). The top 20 increased and decreased transcripts are listed in the Supplementary Table S4. Additionally, a number of genes were found to be induced by dietary broccoli that have roles in the maintenance of intestinal epithelial homeostasis *Reg1*, *Reg2*, *Tff2*. In addition, dietary broccoli enhanced *Ccl28* expression, which regulates mucosal homing of T and B lymphocytes and possesses potent antimicrobial activity against *Candida albicans*, Gram-negative bacteria, and Gram-positive bacteria (Hieshima et al., 2003; Kunkel & Butcher, 2003). To confirm the inducibility of these genes by broccoli consumption, real-time PCR was performed and the data obtained confirmed enhanced colonic expression in *Ahr<sup>b/b</sup>* mice of *Reg1* (6-fold), *Reg2* (5-fold), *Tff2* (30-fold), and *Ccl28* (6-fold) (Fig. 7C). Ingenuity pathway analysis (IPA) predicted alteration of six canonical pathways associated with consumption of dietary broccoli; triacylglycerol degradation, retinol biosynthesis, DNA damage-induced 14-3-3 $\sigma$  signaling, role of CHK proteins in cell cycle checkpoint control, cyclins and cell cyclin regulation, and tight junction signaling (Fig. 7D). Of these pathways, cyclins and cell cycle regulation was predicted to be significantly elevated by broccoli consumption, in contrast, the role of CHK proteins in cell cycle checkpoint control were suggested to be significantly decreased. Overall these data suggest that dietary broccoli can significantly alter host colonic gene expression and such alterations may enhance the proliferative capacity of the intestinal mucosa.

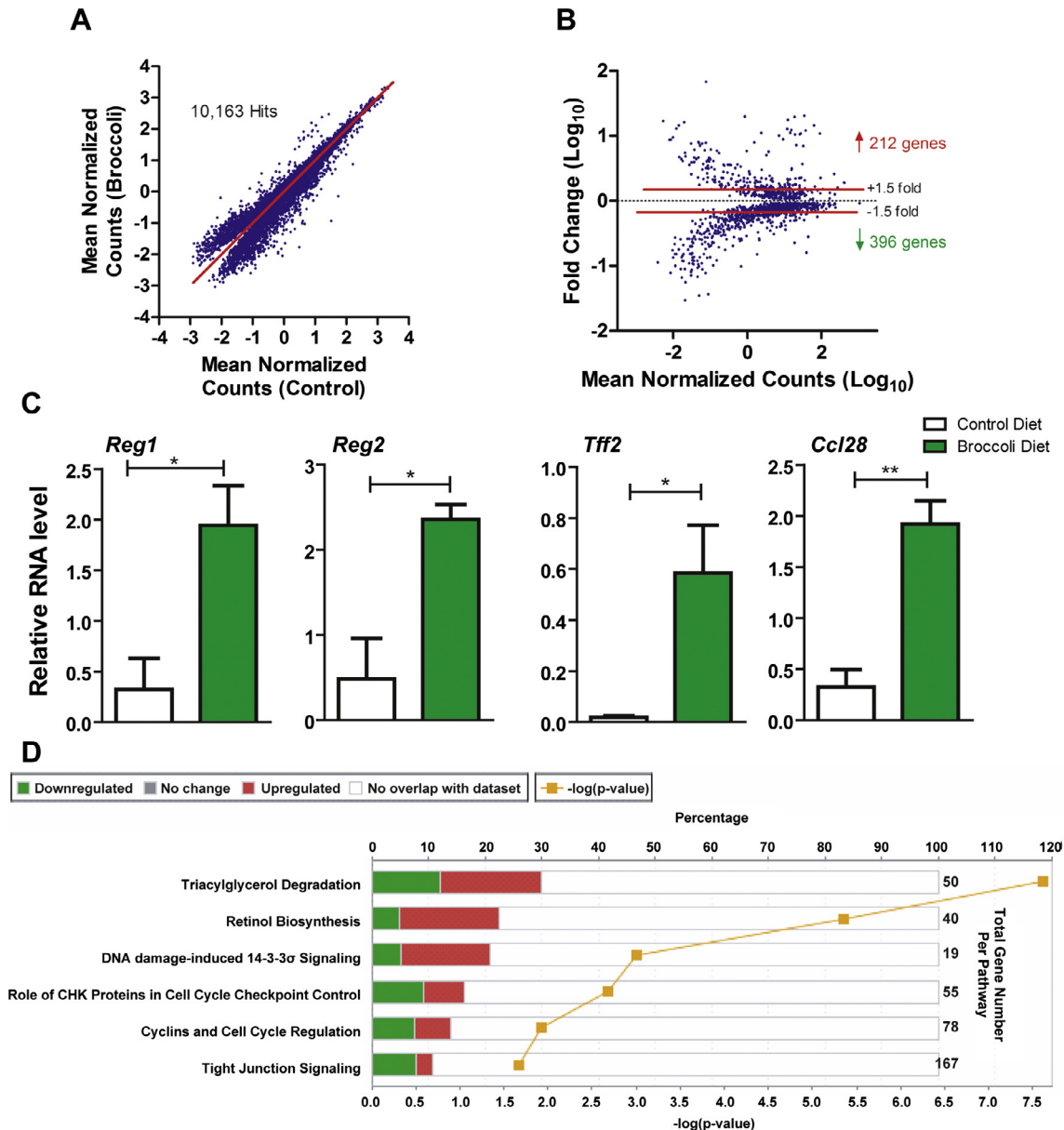
## 4. Discussion

In the present study, we observed that broccoli mediated a greater level of intestinal AHR activation in *Ahr<sup>b/b</sup>* relative to *Ahr<sup>d/d</sup>* mice. This is likely an indication of a reduced capacity for the *Ahr<sup>d/d</sup>* isoform to bind and respond to broccoli-derived ligands. Previous studies suggest administration of broccoli will activate the AHR; however, these studies fail to consider the array of chemical components present in broccoli that could impact uptake and metabolism of I3C or antagonize the AHR. We found that the administration of the chemical mixture within broccoli does not mitigate AHR activation. Notably we observe the occurrence of broccoli mediated benefits and AHR activation at relatively low daily doses of I3C (4.64 mg/kg), which is 15–60-fold lower than that used in previous studies (Julliard et al., 2016; Stoner et al., 2002). While heightened doses of I3C may promote intestinal health, they will lead to systemic circulation of AHR ligands that are associated with development of hepatocarcinoma (NTP, 2014; Stoner et al., 2002). We found that broccoli consumption did not mediate upregulation of hepatic AHR activity in *Ahr<sup>b/b</sup>* mice. This was in direct contrast to significantly increased AHR activity in the liver of *Ahr<sup>d/d</sup>* mice. This elevation of hepatic AHR activity is an indicator of systemic circulation of broccoli derived AHR

ligands. The strain difference observed is likely a consequence of differential induction of drug metabolism enzymes by the intestinal epithelium, which has recently been identified as the “gate keeper” to systemic circulation of AHR ligands (Schiering et al., 2017). Elevated hepatic AHR activity, as a result of xenobiotic treatment, is associated with increased risk of hepatic steatosis and is likely mediated in part by AHR induced expression of the fatty acid transporter, CD36 (Angrish, Mets, Jones, & Zacharewski, 2012; Kawano et al., 2010). These results suggest that the effective dose of AHR ligands present within broccoli can promote local intestinal AHR activation and its associated benefits, while avoiding systemic exposure.

The microbiome is now widely considered an additional organ within an organism through its influence upon host immune system development, displacement of pathogenic organisms, energy utilization and metabolism. Broccoli consumption is associated with altered cecal microbe community composition and metabolism, as well as decreased prevalence of bacterial species associated with Crohn's Disease (Paturi et al., 2012). We find that consumption of broccoli and AHR responsiveness combinatorially contributes to the divergence of numerous taxa. Broccoli consumption was found to associate with a marked expansion of the genus *Alistipes* and decreased prevalence of the *Erysipelotrichaceae* family, independent of *Ahr* status. *Alistipes* bacteria that have been found to be significantly more abundant in the gut microbiota of healthy subjects, compared to patients diagnosed with non-alcoholic fatty liver disease (Jiang et al., 2015). Several reports have documented potential roles for *Erysipelotrichaceae* in host physiology and disease. Enriched abundance of *Erysipelotrichaceae* is associated with the prevalence of colorectal cancer in human epidemiological studies (Chen et al., 2012; Chen, Liu, Ling, Tong, and Xiang, 2012) and mouse models of 1,2-dimethylhydrazine-induced colon cancer (Zhu et al., 2014). Additionally, increases in *Erysipelotrichaceae* correlate with the development of TNF-driven Crohn's disease-like transmural inflammation (Schaubeck et al., 2015). Decreased abundance of *Erysipelotrichaceae* has been found to associate with increased consumption of flavonoids, such as quercetin (Ettxeberria et al., 2015), and may suggest a mechanism for broccoli to elicit a similar effect. Therefore, broccoli mediated depletion of *Erysipelotrichaceae* may contribute to an improved host intestinal inflammatory status. Relative 16S rRNA gene abundance of *Erysipelotrichaceae* represent on average 14–20% of total assigned reads in both *Ahr<sup>b/b</sup>* and *Ahr<sup>d/d</sup>* control fed mice, which are depleted 8–15% following broccoli consumption. However, differential displacement of *Erysipelotrichaceae*, a member of the *Firmicutes* phylum, occurs in *Ahr<sup>b/b</sup>* relative to *Ahr<sup>d/d</sup>* mice. Such that in *Ahr<sup>b/b</sup>* mice, 16S read abundance associated with *Firmicutes* is maintained following the loss of *Erysipelotrichia* due to a corresponding increase in *Clostridia* associated 16S reads. In contrast, *Erysipelotrichia* are displaced by the microbiota taxonomically classified within the *Bacteroidetes* phylum in *Ahr<sup>d/d</sup>* mice, facilitating a significant decrease in the *Firmicutes*:*Bacteroidetes* ratio. The relative proportion of *Firmicutes*:*Bacteroidetes* has been demonstrated to correlate to host adiposity. Specifically, a lower ratio, or increased abundance of *Bacteroidetes*, corresponds to decreased incidence of obesity in *ob/ob* genetic mouse models through altered caloric utilization by the host conferred by the resident microflora (Ley et al., 2005; Turnbaugh et al., 2006). We find that alteration of the *Firmicutes*:*Bacteroidetes* ratio by broccoli in *Ahr<sup>d/d</sup>* mice corresponded to decreased epididymal adiposity, not observed in *Ahr<sup>b/b</sup>* mice. In summary, the data indicates that broccoli is able to significantly alter cecal microbial composition and metabolism through mechanisms that associate with or are independent of host AHR status.

Our studies utilizing congenic mice under specific pathogen free or germ-free conditions fed purified diets supplemented with I3C indicate that intestinal microbes are unlikely to contribute to



**Fig. 7.** RNA-sequencing identified differential colonic gene expression profiles mediated by dietary broccoli in *Ahr<sup>h/h</sup>* mice after DSS exposure on day 8. (A) Dot Plot displays distribution of reads that were mapped to known transcripts, deviation from the diagonal indicate differential expression due to broccoli consumption, relative to controls. (B) Bland-Altman plot of  $\log_{10}$  mean normalized (RPKM) counts vs. fold-change in expression. Ingenuity Pathway Analysis identified elevation of 4 genes associated with intestinal health altered by broccoli consumption (*Reg1*, *Reg2*, *Tff2*, and *Ccl28*). (C) Real time PCR quantification of *Reg1*, *Reg2*, *Tff2*, and *Ccl28* normalized to eukaryotic *Rpl13a*. Data represent the mean gene expression ( $n = 3$  per diet group)  $\pm$  standard error of mean (SEM). (D) Ingenuity Pathway Analysis identified top canonical pathways altered by broccoli consumption in the colon of *Ahr<sup>h/h</sup>* mice. For a given pathway, green bars indicate the percentage of down-regulated genes, and red bars indicate the percentage of up-regulated genes.

increased production of AHR ligands from cruciferous vegetables. In contrast, the enhanced sensitivity of germ-free mice, relative to conventional controls, to I3C with regard to hepatic *Cyp1a2* expression suggests that the intestinal microbiota may metabolically eliminate I3C within the intestine, thus limiting systemic AHR activation. Upon further examination, control fed animals were found to display no significant differences in intestinal AHR activation as measured by *Cyp1a1* expression. This suggests that intestinal AHR activity in mice is not increased by the metabolic activity of resident microflora. In contrast, human AHR is known to respond to numerous microbial-derived molecules, such as indole derivatives (Hubbard et al., 2015; Hubbard, Murray, & Perdew, 2015). Therefore, microbial derived ligands may heighten human intestinal AHR activity prior to consumption of dietary

ligands. This effect could lower the amount of dietary agonist required to mediate a physiological impact upon the intestinal tract. Also the gastric pH of humans is lower than that of mice (1.5 to 3.5 versus 3.1 to 4.5) (Kararli, 1995), which could further enhance the efficiency of I3C conversion to potent AHR agonists and decrease the amount of broccoli consumption required to be beneficial.

The capacity for AHR ligands to mitigate disease severity in models of autoimmune (EAE), pathogenic (*C. rodentium*), and chemical challenge (i.e. chemically-induced colitis) are well documented (Rouse, Singh, Nagarkatti, & Nagarkatti, 2013; Schiering et al., 2017). Administration of the broccoli-derived phytochemical I3C and its principle gastric-condensation products (DIM, and ICZ) has also been shown to promote similar effects (Rouse et al., 2013).



In our study, we find that DSS-induced colitis severity is significantly attenuated by prior and concurrent administration of dietary broccoli. Notably, mitigation of DSS-mediated weight-loss was found to initially occur in both *Ahr<sup>b/b</sup>* and *Ahr<sup>d/d</sup>* phenotypes, but maintained in *Ahr<sup>b/b</sup>* mice following day six of the experimental time course. It is likely that the benefit of broccoli consumption is not wholly dependent upon glucobrassicin, but other bioactive constituent phytochemicals such as sulfurophane and phenylethyl isothiocyanate (Stoewsand, 1995). However, the concomitant decreases in severity of weight loss, disease activity, and inflammatory cytokine expression (*Cxcl5*, *Ptgs2*, *Il1b*, *Il6*) observed in *Ahr<sup>b/b</sup>* mice fed broccoli, but not *Ahr<sup>d/d</sup>* mice, suggest that AHR responsiveness to dietary agonists may be a significant contributor to broccoli-associated maintenance of intestinal homeostasis. Previous studies suggest that AHR activation promotes a decreased inflammatory tone by enhanced differentiation of naïve CD4<sup>+</sup> T-cells to favor formation of anti-inflammatory T<sub>reg</sub> cells (Funatake, Marshall, & Kerkvliet, 2008; Rouse et al., 2013). In contrast, we find that broccoli consumption and associated activation of the receptor does not promote elevated differentiation of T<sub>reg</sub> cells, but does reduce the number of resident T<sub>H17</sub> cells. Also, reconstitution experiments using colitis-prone *Il10<sup>-/-</sup>* mice indicate that the therapeutic effect of broccoli in *Ahr<sup>b/b</sup>* mice is not likely a consequence of altered intestinal inflammatory signaling as a result of increased virulence or pathogenicity of resident microbes. Therefore, the broccoli-associated benefit observed in the models of intestinal challenge likely originates from alterations of host gene expression.

To investigate additional mechanisms of the therapeutic effect of broccoli consumption upon the host, we utilized high throughput RNA sequencing to evaluate colonic gene expression profiles in mice after dietary intervention. These data suggest that broccoli consumption is able to significantly modulate expression of hundreds of genes. Ingenuity pathway analyses suggest that broccoli is able to significantly upregulate expression of cyclin and cell cycle regulation genes and down-regulate others involved in cell cycle checkpoint control. Elevated colonic expression of *Reg1*, *Reg2*, and *Tff2* genes was observed in broccoli-fed *Ahr<sup>b/b</sup>* mice. Both *Reg1* and *Reg2* have been found to be upregulated in cases of intestinal amebiasis (Peterson et al., 2012) or irritable bowel disease (IBD) (Dieckgraefe et al., 2002) and thought to maintain intestinal epithelial barrier function by acting as a mitogenic or anti-apoptotic signaling factor. Also *Tff2* signaling is known to promote gastric mucosal healing through actions that likely involve both inhibition of acid secretion and stimulation of mucosal proliferation (Farrell et al., 2002). In summary these data would suggest that enhanced intestinal proliferative potential could be an effect of broccoli consumption and likely would enhance epithelial barrier function and protection against intestinal challenge.

Currently, over 1–2 million people in the United States and Canada have been diagnosed with irritable bowel disease (IBD), including ulcerative colitis and Crohn's disease (Loftus, 2004). The pathogenesis of IBD is characterized by a dysregulation of host proinflammatory signaling pathways in response to luminal microbes (Kaser, Zeissig, & Blumberg, 2010). The Crohn's and Colitis Foundation of America suggest that patients avoid cruciferous vegetables due to their fibrous nature and capacity to increase local gas production and irritation. However, our results would suggest that broccoli may be of preventative benefit in cases of heightened intestinal inflammation, such as Crohn's disease and colitis. Consumption of puréed broccoli or dietary supplementation of low doses of I3C may provide a heightened therapeutic effect, while minimizing risks for patients. Notably, the scale of anti-carcinogenic and anti-inflammatory effect of cruciferous vegetable consumption has been found to directly associate with glucosinolate levels (Lippmann et al., 2014). Allometric calculations indicate

humans (65 kg) would need to consume 900 g (5 cups) of raw broccoli daily to receive the scaled effective dose used in this study (Hu & Hayton, 2001). Selection of broccoli cultivars that yield higher levels of glucosinolates may further enhance observed chemo-protective effects and enhanced maintenance of intestinal homeostasis, while requiring lower levels of consumption. Alternatively, other subspecies of *Brassica oleracea* could be substituted in place of broccoli, such as Brussels sprouts, which can contain up to 4.3-fold higher glucobrassicin levels than the utilized broccoli lieutenant cultivar (Kushad et al., 1999).

## 5. Conclusions

Broccoli consumption alters the host microbiome and improves intestinal resistance to chemical challenge, which suggests there is a therapeutic effect on the maintenance of intestinal homeostasis. Most importantly, this latter effect of broccoli consumption is mediated through AHR activation. Therefore, the selection of broccoli cultivars with increased levels of glucosinolates, especially those that lead to the formation of AHR ligands, may be of increased health benefit. Furthermore, numerous other foodstuffs contain AHR ligands that may also exhibit similar health effects (Jeuken et al., 2003).

## Acknowledgements

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## Disclosure/Conflict of interest

The authors declare no conflict of interest.

## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jff.2017.08.038>.

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