Immunomodulatory effects of aronia berries in inflammatory bowel disease

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Immunomodulatory effects of aronia berries in inflammatory bowel disease

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Inflammatory bowel disease (IBD) is a growing public health burden in the United States and many other industrialized nations. Dietary, genetic, and environmental factors contribute to IBD pathogenesis. T helper (Th) cell dysfunction, particularly the Th17/T regulatory (Treg) cell axis, is considered to be a major mechanism for the initiation and persistence of IBD. Fruit and vegetable consumption is associated with a lower risk of developing IBD. The overall hypothesis of this work was that aronia berry modulates intestinal Th17/Treg homeostasis to inhibit colitis development. Mice were fed a diet with 4.5% aronia berry in the adoptive transfer model of colitis for up to 54 days or fed 4.5% aronia berry concurrent with dextran sulfate sodium (DSS) administration in the drinking water for 7 days. Aronia berry consumption inhibited wasting associated with T cell adoptive transfer and DSS-induced colitis. Aronia extracts and microbial polyphenol catabolites were incubated along with stimulated Jurkat T cells. Aronia extracts, neutral phenols, and the polyphenol catabolites 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylpropionic acid inhibited TNF-α production in Jurkat T cells. Mice were fed diets with 4.5% aronia berry, 0.16% aronia polyphenol extract, or 2.12% polyphenol depleted aronia berry for 7 days and then another 7 days concurrent with DSS administration in the drinking water. No diet inhibited DSS induced weight loss, improved intestinal barrier function, or improved histopathological damage. The 4.5% aronia berry diet decreased colonic tumor necrosis factor (TNF)-α. Finally, the 4.5% aronia berry diet was fed to mice in the adoptive

transfer model of colitis. At 5 weeks post transfer, the aronia-fed group had greater proportions of Treg in the lamina propria (LP), mesenteric lymph nodes (MLN), and spleen than the control group. Th17 IL-10^{+} and IL-22^{+} subpopulations in LP and spleen also increased in the aronia-fed group. Induction of Treg in MLN and reductions in pro-inflammatory cytokines in the colon were dependent on T cell IL-10 at 3 weeks post-transfer. Aronia consumption improved histopathological scores in the absence of T cell IL-10, although overall histological score was higher than in mice having T cell IL-10. Increased Treg and nonpathogenic Th17 by aronia consumption and overall anti-colitic activity are promising steps towards establishing dietary recommendations for IBD prevention. Future experiments should determine components in fruits and vegetables conferring inhibition of IBD, the role of the microbiota, and the metabolic, molecular, and cell-mediated mechanisms of action.

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Chapter 1

Introduction

1.1 Introduction

Increased consumption of dietary polyphenols is associated with reduced chronic disease risk (1). Aronia berry is among the most concentrated sources of dietary polyphenols (2). Aronia berry is widely consumed in Eastern Europe and Russia; cultivation has increased in the U.S. Midwest over the past decade. Relative to other berries, aronia is rich in cyanidin-type anthocyanins, hydroxycinnamic acids, and tannins, many of which are common to other berries. Because of its increasing cultivation, polyphenol content, and polyphenol profile, aronia berry is a compelling fruit to utilize for dietary interventions for chronic disease risk.

Inflammatory diseases are pervasive in the U.S. Among them, inflammatory bowel disease (IBD) is a growing public health burden, affecting more than 3 million individuals in industrialized nations (3). Dietary, genetic, and environmental factors contribute IBD pathogenesis. T helper (Th) cell dysfunction, particularly the Th17/regulatory T (Treg) cell axis is considered to be a major mechanism for the initiation and persistence of IBD (4). Less is known about dietary factors that influence IBD development or dietary approaches to manage existing IBD. Population based studies indicate that fruit and vegetable intake inhibits IBD (5–7). Preclinical studies (which are reviewed in Chapter 2) have identified that polyphenols from fruits and vegetables inhibit IBD (8). These studies are limited in their translatability by supraphysiologic doses, usage of models of IBD that are chemically induced, and a lack of attention to cell-mediated inflammation.

Because of these limitations, further studies are needed to identify the mechanisms by which fruit polyphenols modulate intestinal immune function at the cellular level. We previously demonstrated that aronia extract modulated key cytokines involved in the development of colitis from murine splenocytes (9). Aronia extract inhibited interleukin (IL)-6, the determinant

cytokine for differentiation of naïve T helper cells to Th17 cells (10) and increased the anti-inflammatory cytokine IL-10 (9). Furthermore, grape seed proanthocyanidins were shown to promote a less pathogenic Th17/Treg profile in a murine model of arthritis (11). The overall hypothesis of this work was that aronia berry modulates intestinal Th17/Treg homeostasis to inhibit colitis development. The approach utilized animal models of inflammatory bowel disease as well as an in vitro approach to determine the effects of aronia polyphenols on inflammatory cytokine production by T cells.

The first research chapter (Ch. 3) describes how aronia berry polyphenols modulate T cell cytokines and inhibit colitis-associated wasting in rodent models. The second research chapter (Ch. 4) describes how the food matrix affects the anti-colitic activity of aronia berry consumption in the dextran sulfate sodium (DSS) model of colitis. The third research chapter (Ch. 5) describes how aronia berry affects the Th17/Treg axis and the importance of T cell derived IL-10 to immune homeostasis in the adoptive transfer model of colitis.

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Chapter 2

Literature review

A review of the efficacy of dietary polyphenols in experimental models of inflammatory bowel disease

The basis of this chapter was published as:

Martin DA, Bolling BW. A review of the efficacy of dietary polyphenols in experimental models of inflammatory bowel diseases. Food Funct. 2015;6:1773–86.

2.1 Abstract

Crohn's disease and ulcerative colitis presently have no cure and are treated with anti-inflammatory drugs, monoclonal antibodies targeting pro-inflammatory cytokines, and/or other pharmaceuticals. A variety of rodent models have been used to model chronic and acute colitis. Dietary polyphenols in foods and botanicals are of considerable interest for prevention and treatment of colitis. Many dietary polyphenols have been utilized for prevention of colitis in rodent models. Berries, green tea polyphenols, curcumin, and stilbenes have been the most extensively tested polyphenols in rodent models of colitis. The majority of polyphenols tested have inhibited colitis in rodents but increasing doses of epigallocatechin-3-gallate (EGCG), green tea extract, isoflavones, flaxseed, and α-mangostin have exacerbated colitis. Few studies have examined combinations of polyphenols or other bioactives for inhibition of colitis. Translating polyphenol doses used in rodent models of colitis to human equivalent doses shows that many studies have utilized doses higher than could be attained in foods. This may imply that supplemental doses are required to inhibit colitis. The ability to translate polyphenol treatments in rodent models is likely to be limited by species differences in xenobiotic metabolism and microbiota. Given these limitations, data from polyphenols in rodent models suggests merit for pursuing additional clinical studies for prevention of colitis.

2.2 Introduction

Inflammatory bowel diseases (IBD) include both ulcerative colitis (UC) and Crohn's disease (CD). UC may affect the rectum and entire colon in an uninterrupted pattern of inflammation that is generally confined to the mucosa (1). In CD, inflammation is typically located at the ileum and the colon, but it can affect any region of the colon and often occurs in an interrupted pattern. CD may also involve strictures and fistulas, but these are not typical for UC (1). Pharmacotherapy for IBD includes anti-inflammatory drugs and anti-tumor necrosis factor-α (TNF-α) monoclonal antibodies but does not cure the disease (2). Thus, colectomy may be needed when pharmacotherapy is inadequate to control inflammation. A significant number of studies have investigated the role of diet on IBD prevention and treatment, as previously reviewed (3). The objective of this paper is to review recent data from rodent models about polyphenols for the prevention and treatment of IBD.

Polyphenols and other phenolic compounds are secondary plant metabolites characterized by an aromatic or phenol ring structure. Polyphenols can be further divided into classes according to the number of phenolic rings and the bonds that join the rings. These classes include stilbenes such as resveratrol, flavonoids such as epigallocatechin gallate (EGCG), and phenolic acids such as caffeic acid, among others. Polyphenols have antioxidant activity, modulate cell signaling pathways, and have anti-inflammatory properties (4). Dietary sources of polyphenols include berries, grapes and wine, tea, chocolate, coffee, and a variety of fruits and vegetables, and their content is indexed in nutrient databanks (5,6). Polyphenol intake can reach gram amounts, especially for individuals that consume multiple servings of fruits, vegetables, tea, or coffee each day (5). For example, habitual coffee consumption can supply 500-800 mg hydroxycinnamic acids (5).

Human intervention trials for polyphenol-based IBD treatment are limited. However, pilot studies of curcumin for UC or CD (7–9) bilberries for UC (10), or ECGC for UC (11) have had promising results. Other dietary and botanical interventions for colitis have been recently reviewed elsewhere (3,12,13). While polyphenol intake and IBD risk have not been studied using epidemiological approaches, IBD risk has been inversely associated with childhood fruit and vegetable consumption (14,15). These promising clinical and epidemiological studies have increased interest in the therapeutic and prophylactic use of polyphenols for IBD. Pre-clinical evidence is needed to design effective clinical studies of polyphenols for IBD. Therefore, the purpose of this review is to summarize and analyze the use of polyphenols in animal models of IBD.

2.3 Experimental models of IBD

A number of rodent models have been used for investigating dietary treatments for IBD (16). Inflammation in rodent-based IBD models can be induced by chemicals, immune cell transfer, or target gene manipulations that reflect defects in epithelial integrity, innate immunity, or adaptive immunity. Inflammation characteristic of IBD can be chemically-induced by administration of dextran sulfate sodium (DSS), 2,4,6-trinitrobenzenesulfonic acid (TNBS), or acetic acid. Typically, colitis is induced in rodents by administration of DSS in drinking water with various cycles and doses, leading to highly reproducible acute or chronic colitis (16). DSS disrupts epithelial barrier integrity and leads to an inflammatory immune response to gut microbiota. Consequently, DSS treatment induces inflammation through T helper (Th)1, Th2, and Th17 responses (17). TNBS induces colitis in susceptible rodent strains and alters colonic or microbiota proteins, rendering them immunogenic to the host (16). TNBS colitis induction has

been useful in studying T helper cell immune responses (primarily Th1), cytokine secretion patterns, cell adhesion and immunotherapy (16). Transfer of $CD45RB^{HI}$ T cells isolated from donor mice and transferred to severe combined immunodeficiency (SCID) or Recombinase Activating Gene (*Rag*) $1/2^{-/-}$ mice causes intestinal inflammation (18). Lack of functional proteins in mice can also lead to colitis. These include interleukin (IL)-10 and IL-10 receptor knockouts, T-cell receptor (TCR)-α chain knockouts, and manipulations causing the overexpression of signal transducer and activator of transcription (STAT)4 (16). To our knowledge the IL-10 receptor$^{-/-}$, TCRα chain knockouts, and STAT4 overexpression models have not been used to evaluate the effects of polyphenols or other plant metabolites in colitis development. In contrast, most studies of polyphenols in IBD models have used chemically-induced colonic inflammation or *Il10*$^{-/-}$ mice that spontaneously develop colitis (**Table 2.1**).

2.4 Polyphenols and polyphenol-rich foods and extracts used in animal models of IBD

Polyphenol-rich fruits, vegetables, and other sources are composed of multiple polyphenol classes and other bioactives. For example, anthocyanin-rich berries also contain significant quantities of proanthocyanidins and dietary fiber (19,20). For the purposes of this review, preparations or unpurified sources of polyphenols are grouped with the most-abundant class of polyphenol identified in its composition. A number of polyphenols have been administered in animal models of colitis, which will be examined next (**Figure 2.1**, **Table 2.1**).

2.4.1 Anthocyanins

Berries, grapes, and other pigmented plants can be rich sources of anthocyanins and have been applied to experimental models of IBD. The cost of isolation and purification of anthocyanins has precluded treatment with individual compounds.

2.4.1.1 Grape juice

Grape juice (GJ) concentrate was administered in drinking water to rats with TNBS-induced colitis 24 h or 7 d post-induction and continued until d 16 post-induction (21). GJ (1%) treatment initiated at d 7 reduced TNBS-induced macroscopic and histological damage scores, which was accompanied by reduced colonic TNF-α mRNA and inducible nitric oxide synthase (iNOS) expression independent of nuclear factor-κB (NF-κB) or intercellular adhesion molecule 1 (ICAM-1) (21). The 24 h treatment with GJ or higher 2% GJ dose at 7 d did not reduce macroscopic and histological damage (21). Another study of GJ examined multiple dosing regimens in TNBS-induced colitis in rats (22). Administration of 1% GJ at 7 d after TNBS-administration reduced macroscopic and histological damage scores and reduced colonic cyclooxygenase-2 (COX-2), TNF-α and iNOS proteins (22). However, treatment with 1% or 2% GJ at 24 h after TNBS-induction did not prevent macroscopic or histological markers of inflammation (22).

2.4.1.2 Blueberry, bilberry, raspberry, hawthorn berry, aronia berry, and mulberry

Anthocyanin-rich blueberry extract was orally administered to mice at 10, 20, or 40 mg/kg bw for 6 d after TNBS-induced colitis (23). Blueberry extract dose-dependently protected against TNBS-induced weight loss, diarrhea, and mortality. The 20 and 40 mg/kg doses reduced

colon shortening, macroscopic and histological damage, and decreased myeloperoxidase (MPO) activity. All blueberry extract doses decreased colonic IL-12, TNF-α, and interferon (IFN)-γ mRNA and increased colonic IL-10 mRNA (23).

Wistar rats were given 10 mg/kg of an anthocyanin-rich fraction from blueberries by oral gavage for 8 d after TNBS administration (24). The treatment improved weight gain, colonic macroscopic damage score, colonic weight/length ratio, and reduced MPO expression (24).

Dried bilberry (20% w/w) and anthocyanin-rich bilberry extract (10 or 1% w/w) were added to chow diet and fed to mice 2 wk prior to and concurrent with acute (7 d) and chronic (4 × 7 d cycles) DSS treatment (25). Bilberry extract, but not whole bilberry, reduced acute-DSS colonic histological inflammation score, but both extracts and whole bilberry prevented the decrease in colon length. Bilberries, but not the 10% anthocyanin extract decreased mesenteric lymph node (MLN) TNF-α excretion, while both forms reduced MLN IFN-γ excretion. After chronic DSS-induced colitis, bilberry and bilberry extract reduced colon shortening, but only bilberry extract reduced colonic histological inflammation scores and excretion of IFN-γ and IL-6 from MLNs (25).

A 5% black raspberry diet was fed to spontaneously-colitic *Il10*$^{-/-}$ mice for 8 wk (26). Black raspberry feeding reduced colonic ulceration in the mucosa and submucosa in mice (26). Black raspberry powder was also administered at 5 or 10% of the diet to C57BL/6J mice treated with 3% DSS for 7 d (27). Both black raspberry doses prevented DSS-induced weight loss, colon shortening, and mucosal ulceration. The 10% black raspberry-treated mice had less colonic *Il1β* and TNF-α mRNA, and decreased COX-2 and prostaglandin E2 (27).

Wistar rats were given 100 mg/kg hawthorn berry extract by oral gavage 3 d before and 7 d after induction of colitis by intrarectal 4% acetic acid (28). Hawthorn berry extract prevented

colitis-induced body weight loss and inhibited increases in colonic MPO activity and nitric oxide (28). Hawthorne berry extract treatment prevented colonic lipid peroxidation and macroscopic and histopathological inflammatory damage scores in rats (28).

BALB/c or C57BL/6 mice were given 10 or 100mg/kg aronia berry extract orally for 10 d initiated at the start of a 7 d 5% DSS administration (29). Both aronia doses suppressed weight loss and colon shortening and 100 mg/kg suppressed the disease activity index, decreased serum IL-6 and TNF-α, and suppressed epithelial cell and crypt damage (29).

An anthocyanin rich red raspberry fraction was given by intraperitoneal injection at a dose of 20 mg/kg to BALB/c mice daily starting 1 d prior to a 10 d administration of 2% DSS (30). The treatment reduced weight loss, colon shortening, and the histological damage score (30).

BALB/c mice were supplemented with 5 or 10% mulberry fruit powder for 10 d, followed by 3% DSS for 9 d. Mulberry fruit supplementation protected against DSS-induced body weight loss, spleen weight increase, and colon shortening (31). Colon histopathology was likewise improved (31).

2.4.2 Flavan-3-ols and green tea

Flavan-3-ols, particularly (+)-catechin and (-)-epicatechin are widely distributed in plants, including nuts, berries, chocolate, and wine (6). Green tea and green tea extract are rich in flavan-3-ols, including EGCG, epigallocatechin, epicatechin gallate, and epicatechin (32).

2.4.2.1 EGCG

EGCG has been administered in a wide range of doses in rodent models of colitis. In mice with TNBS-induced colitis, EGCG was administered twice per day by intraperitoneal (IP) injections at a 10 mg/kg dose (33). EGCG injections reduced diarrheal severity, weight loss, and macroscopic and histologic indices of inflammation. Despite inhibition of colitis, plasma TNF-α, IL-6, and IL-10 were not modulated by EGCG treatment. EGCG decreased DNA binding of NF-κB and activator protein-1 in the colon (33). In a DSS mouse model of colitis, diets were supplemented with 0.01% or 0.05% ECGC for one week prior and subsequent to DSS administration to male ICR mice (34). EGCG feeding attenuated DSS-induced weight loss and colon shortening, infiltration of inflammatory cells, disruption of crypt structure, and other histological characteristics (34). EGCG also inhibited acetic acid-induced colitis in rats (35). Oral gavage of 50 mg EGCG/kg/d 24 h after induction of colitis reduced body weight loss, bloody stools, and diarrhea after 3-4 d. At 8 d, EGCG treatment markedly improved macroscopic and microscopic signs of colon damage and inflammation, as well as inhibited serum TNF-α and IFN-γ (35). Similarly, a daily oral gavage of only 6.9 mg EGCG/kg/d with 2.9 mg piperine/kg/d as a bioavailability enhancer inhibited DSS-initiated chronic colitis in mice (36). In contrast to these studies, higher doses of ECGC exacerbated inflammation in an acute DSS-induced colitis in mice (37). Mice fed 0.1% and 0.5% EGCG experienced more aggressive weight loss than the control group (37). Consumption of 0.1% EGCG prevented colon shortening, but higher EGCG doses did not (37). Mice on the 0.5% EGCG diet experienced more severe rectal bleeding compared to DSS-control mice (37). The 0.1% EGCG diet is equivalent to approximately 0.5 g EGCG/d for a human consuming 2,000 kcal/d (37). The EGCG content of green tea brewed at 80 °C for 3 minutes ranged from ~100-350 mg/L (38). Achieving 0.5 g EGCG/d is thus nearly

impossible without supplementation. The evidence indicates that EGCG at high doses exacerbates colitis in rodents, but at lower doses it is protective (**Figure 2.2a**). It may be prudent for individuals with IBD to avoid excessive doses of supplemental EGCG until further evidence of its safety and efficacy is demonstrated in human intervention studies.

2.4.2.2 Green tea extract

Dietary green tea polyphenols (GTP) have been used as a treatment in DSS-induced colitis models (39–41) and the *Il10*$^{-/-}$ (42) and multidrug-resistance transporter (*Mdr1)a*$^{-/-}$ (43) spontaneous colitis models. A transcriptomic and metabolomics approach to characterizing GTP protection in spontaneously colitic *Mdr1a*$^{-/-}$ mice identified 1,343 genes in the colon differentially expressed in the GTP-fed group (43). Pathways related to the immune and inflammatory response were decreased, and genes associated with xenobiotic metabolism were increased. At the proteomic level, the negative acute phase response, endoplasmic reticulum stress response, inflammation and inflammatory response were downregulated by GTP consumption, while nuclear factor (erythroid-derived 2)-like 2 (Nrf2), proteins involved in tight junction signaling, and oxidative stress response proteins were upregulated (43). Like EGCG, doses < 0.5% GTP inhibited DSS-induced colitis, while doses of 0.5% to 1% GTP fortified in mouse chow increased colitis symptoms (**Figure 2.2b**). Furthermore, doses of 1% GTP had increased mortality in mice with DSS-induced colitis (39,40) and *Il10*$^{-/-}$ mice had increased weight loss (42). GTP-induced exacerbation of colitis may be related to increased colonic IL-1β rather than IL-6 or TNF-α (41).

2.4.3 Proanthocyanidins and cocoa

Proanthocyanidins are polymeric flavan-3-ols with 2 to >100 constituent units. Proanthocyanidin polymers are predominately “B-type”, having a single interflavan bond, although “A-type” proanthocyanidins are most abundant in cranberries. While cocoa, grapeseed extract, and berries are rich in proanthocyanidins, it should be noted that crude extracts are likely to contain other bioactive components, such as catechins, anthocyanins, and other flavonoids.

Isolated apple proanthocyanidins, having degree of polymerization of 2-15, inhibited oxazolone and DSS-induced colitis (44). C57BL/6 mice were provided 0.1, 0.3, or 1.0% apple proanthocyanidins in the drinking water for 14 d prior to a 4 d 2.5% DSS treatment (44). DSS-treated mice consuming 1% apple proanthocyanidins lost significantly less weight, had less colon shortening and all survived to 20 d, compared to 40% survival in the control group (44). DSS-treated mice showed multifocal inflammatory cell infiltration, erosion of the epithelium and gland destruction, while consumption of 1% apple proanthocyanidins yielded almost normal histologic morphology (44).

Intragastric administration of a grape-seed proanthocyanidin extract (95% proanthocyanidins) reduced recurrent TNBS-induced colitis in Wistar rats (45). Rats were given 100, 200, or 400 mg proanthocyanidins/kg 24 h after a second TNBS administration and continuing for 7 d (45). All doses of grape seed proanthocyanidins inhibited weight loss and macroscopic damage scores (45). The medium and high doses of proanthocyanidins preserved colonic antioxidant function by inhibiting reductions in superoxide dismutase and glutathione peroxidase activities (45). All proanthocyanidin doses decreased colonic TNF-α, the ratio of phosphorylated to unphosphorylated inhibitor of κB kinase, and NF-κB levels (45).

Oligonol, an enzymatically-prepared low-molecular weight proanthocyanidin mixture also inhibited DSS-induced colitis in ICR mice (46). Mice consumed 0.5 or 5 mg/kg/d oligonol in water for 7 d prior to and during a 7 d 3% DSS exposure (46). Oligonol attenuated body weight loss, rectal bleeding, colon shortening, and diarrhea during the DSS treatment (46). Oligonol dose-dependently decreased the colonic inflammatory histological grade (46). Furthermore, oligonol inhibited DSS-induced inhibitor of κB (IκB)α degradation, NF-κB p65 phosphorylation, and STAT3 phosphorylation in the colon (46).

Cocoa and cocoa extract consumption inhibited DSS-induced colitis in mice (47,48). A catechin and proanthocyanidin cocoa extract was administered at 500 mg/kg to mice on d 1 and 4 during a 7 d 5% DSS administration (47). Mice treated with cocoa polyphenols were protected from colon shortening and weight loss (47). Cocoa polyphenols improved stool consistency, reduced colonic histological scores, MPO activity, and reduced colonic phosphorylated STAT3 and phosphorylated STAT1α (47). In another study, consumption of a 50 g/kg cocoa-enriched diet for 14 d before DSS treatment prevented colonic mononuclear cell infiltration and goblet cell loss and reduced serum TNF-α in colitic Wistar rats (48).

2.4.4 Isoflavones and soy

Dietary isoflavones are present in soy foods, including soymilk, tofu, miso, and soy protein. Dietary isoflavones are predominately malonyl-glycosides, but heat treatment and fermentation liberate genistein and daidzein, isoflavone aglycones (49). Equol and *O*-desmethylangolensin are unique microbial metabolites of daidzein (50).

Mixed soy isoflavones enriched in daidzein inhibited DSS-induced colitis in C57BL/6 female mice (51). Oral gavage of 80 mg isoflavones/kg/d (daidzein, genistein, and glycitein,

7:1:2) was provided to mice for one week prior to and concurrent with a 4 d 2% DSS dose (51). Isoflavone-treated mice were protected from weight loss, had decreased severity and extent of inflammation and crypt damage (51). MLN cells from isoflavone-treated mice produced less IFN-γ, IL-12p40, and IL-6 and produced more IL-10 when stimulated *ex vivo.* The percentage of $CD80^+$-and $CD86^+$ -$CD11b^+$ cells in the MLN was also decreased by isoflavone treatment, indicating decreased activation in antigen presenting cells (51). In contrast, lower doses of individual isoflavones exacerbated a 4% DSS-induced colitis in female BALB/c mice (52). Gavage of 20 mg/kg bw of genistein, daidzein, or equol for a week prior to 4 d of 4% DSS treatment reduced survival relative to the non-treated DSS-control group (52). In contrast, lower equol concentrations (2 and 10 mg/kg bw) did not increase weight loss (52). MLN cells from 20 mg/kg equol-treated mice stimulated *ex vivo* with CD3 monoclonal antibody produced less IL-10 than control mice, but did not differ in TNF-α, IFN-γ, or IL-4 (52). One hundred mg genistein/kg bw for 14 d prior to TNBS treatment in male Wistar rats inhibited colonic MPO activity and COX-2 protein (53). Exposure to ~0.05% genistein and daidzein in prenatal and post-natal diets increased TNBS-induced colitis in Wistar rats, evidenced by colonic weights and MPO activity (54).

2.4.5 Flavanones

Flavanones include compounds such as hesperetin, naringenin, and eriodictyol. Citrus polyphenols are predominately flavanone glycosides. Citrus juices contain hesperidin (hesperetin 7-*O*-rutinoside) from ~1 to 50 mg/100 mL (6).

Hesperidin inhibited DSS-induced colitis in BALB/c mice (55). An 80 mg/kg hesperidin oral dose inhibited a 7 d, 5% DSS-induced colitis to a similar extent as a 500 mg /kg

sulfasalazine treatment, based on an observed disease activity index (55). Similarly, the aglycone naringenin inhibited a 9 d, 2% DSS-induced colitis (56). Consumption of diets containing 0.3% naringenin protected mice from DSS-induced body weight loss, colon shortening, and an increase in the disease activity index score (56). Naringenin fed mice had improved intestinal barrier function determined by reduced colonic permeability and plasma lipopolysaccharide-binding protein, and higher concentrations of occludin, junctional adhesion molecule-A, and claudin-3 compared to the DSS treatment alone (56). Additionally, naringenin feeding inhibited the colonic cytokines IFN-γ, IL-6, and IL-17A (56). Another study of naringenin in a DSS-induced model of colitis in C57BL/6 mice found that a 50 mg/kg bw oral dose reduced colitis and decreased colonic toll-like receptor 4, phospho-NFκB p65, TNF-α, and IL-6 protein (57).

2.4.6 Flavonols

Quercetin (58–60), quercetin 3-*O*-rutinoside (rutin) (61–63), kaempferol (64), morin (65), and myricitrin (66) have been tested in rodent models of colitis. Delivery and slow-release of quercetin to the colon through pectin and casein-based microcapsules improved the efficacy of quercetin to inhibit acetic acid-induced colitis in Swiss mice (59). Doses of 100 mg quercetin/kg free or in microcapsules were provided 2 h prior to and 10 h after administration of acetic acid. At 18 h, quercetin microcapsules, but not free quercetin reduced macroscopic colonic damage scores, MPO activity, IL-1β, and IL-33, and increased colonic IL-10 (59). Consumption of a 2% rutin-supplemented diet by BALB/c mice inhibited markers of inflammation in 5% DSS-induced colitis models (61,67). Rutin decreased colonic IL-1β, IL-6, and TNF-α, and reduced colon shortening, but did not prevent colitis-induced weight loss (61). A lower dose of rutin, ~0.05%

in diets of C3H/HeOuJ mice for 7 d prior to and during a 6 d 1.25% DSS treatment improved body weight, reduced colonic MPO activity, and inhibited colonic IL-17 and iNOS mRNA (63). However, this dose of rutin did not significantly reduce colon histopathology scores or protect from colon shortening (63).

The effect of pre-feeding kaempferol prior to DSS-treatment relative to concurrent treatment was examined by Park et al. (64). C57BL/6J mice were fed diets enriched with 0.1% or 0.3% kaempferol starting 2 wk before or concurrent to a 4 d 2% DSS exposure (64). Kaempferol consumption continued until 1 wk after the start of DSS exposure (64). The experimental diets significantly lowered disease activity index scores, with the greatest inhibition occurring in the mice fed 0.3% kaempferol prior to DSS exposure (64). All kaempferol treatments improved histological scores of colitis with the greatest effect in 0.3% kaempferol pre-fed group (64). Thus, kaempferol prefeeding appears to confer greater protection in DSS-induced colitis than treatment post-initiation.

2.4.7 Other purified flavonoids

Gavage of luteolin protected against colitis in 2% DSS-treated C57BL6/6CrSlc mice (68). IP injection of astilbin to C57BL/6 mice prevented colonic inflammation and increased IL-10 and TGF-β in splenic dendritic cells after treatment of 2.5% DSS for 7 d (69). Consumption of 5 mg/kg apigenin reduced acetic acid-induced colitis in mice (70). Intragastric administration of 2 g baicalin/kg/d to Sprague Dawley rats prevented TNBS-induced colitic damage evaluated histologically (71).

2.4.8 Stilbenes and resveratrol

Stilbenes are present in microgram to milligram quantities in grapes, wine, peanuts, tree nuts, and berries (6). Dietary stilbenes exist as glycosylated forms, e.g. resveratrol 3-O-glucoside, or as aglycones, e.g. resveratrol, and more than 40 types of stilbenes have been identified in foods (72).

The anti-colitic properties of supplemental resveratrol have been examined in DSS-treated mice (73–77) and spontaneously-colitic *Il10*$^{-/-}$ mice (78). Supplemental piceatannol has also been administered to DSS-treated mice (77,79). Supplemental resveratrol consumption (20 mg/kg diet) prevented 5% DSS-induced weight loss and colitis-induced mortality (76). In the same study, resveratrol consumption increased colonic IL-10 protein and inhibited colonic TNF-α protein after DSS treatment (76). Gavage of 100 mg resveratrol/kg bw to C57BL/6 mice during a 7 d 3% DSS exposure decreased serum IFN-γ, TNF-α, IL-6, and IL-1β and reduced lamina propria CD4^{+} T cells expressing IFN-γ and TNF-α (75). Lamina propria cells from resveratrol-treated mice also had increased sirtuin 1 expression and decreased p-IκBα expression following DSS treatment (75). In *Il10*$^{-/-}$ mice, 10, 50, or 100 mg resveratrol/kg bw was administered every other day after development of colitis (78). Treatment of 100 mg resveratrol/kg bw reduced colitic symptoms and weight loss, while lower doses did not (78). Immunosuppressive CD11b^{+}Gr-1^{+} myeloid derived suppressor cells were increased in the spleen and lamina propria of mice given the highest dose of resveratrol (78). Resveratrol treatment also decreased colonic excretion of IFN-γ, TNF-α, IL-6, IL-12, and IL-1β (78).

2.4.9 Curcuminoids and turmeric

Turmeric contains the curcuminoids curcumin, demethoxycurcumin, and bis-demethoxycurcumin (80). Curcumin treatment has been evaluated in Mdr1a$^{-/-}$ spontaneously colitic mice (81), DSS-treated BALB/c and C57BL/6 mice (82–84), Wistar rats with acetic acid-induced colitis (85), and Sprague-Dawley rats with TNBS-induced colitis (86). In these models, consumption of 0.2% to 0.6% curcumin-supplemented chow, or oral gavage of 50 to 200 mg curcumin/kg bw/d inhibited colitis. In a 52 d chronic DSS model of colitis, a 200 mg turmeric extract/kg bw dose inhibited reduced histological scores of inflammation and lowered the disease activity index (82). Consumption of 0.6% curcumin reduced DSS-induced colonic TNF-α, IFN-γ, COX-2, and iNOS proteins (84).

Notably, curcumin has poor oral bioavailability, so a number of studies have successfully improved delivery through emulsions, liposomes, and nanotechnology-based approaches (87–89). Strategies to increase curcumin solubility and delivery to the colon have increased the therapeutic efficacy of curcumin in rodent colitis models. Equivalent doses of curcumin loaded in solid lipid microparticles or hydroxypropyl- β-cyclodextrin increased recovery from DSS-induced colitis than curcumin alone (100 mg curcumin per kg bw) (90,91). Similarly, curcumin-Zn(II) complexation improved curcumin water solubility and a 20 mg per kg bw dose improved histopathological scores more than curcumin alone in mice with acetic acid-induced colitis (92). Thus, solubility and bioavailability should be considered in formulating curcumin interventions for colitis.

2.4.10 Gingerols and ginger

Ginger contains the polyphenolic gingerols, shoagols, paradols, and zingerone (93). Consumption of ginger extract inhibited acetic acid-induced colitis in Wistar rats (94). Oral gavage of 200 or 400 mg ginger extract/kg bw inhibited colonic lesions to a similar extent as a 500 mg sulfasalazine/kg bw. Ginger extract treatment improved colonic antioxidant function and reduced colonic MPO activity and TNF-α (94). Ginger extract or zingerone was co-administered with TNBS to BALB/c mice (95). Treatment with ginger extract from 0.1 to 100 mg/kg bw or zingerone from 1 to 100 mg/kg bw dose-dependently decreased macroscopic colonic inflammation in TNBS-treated mice (95). Microarray analysis of colonic genes indicated IL-17, IL-1β, IL-6, IFN-γ, and TNF-α pathways were modulated by ginger extract and zingerone (95).

2.4.11 Ellagitannins and pomegranate

Ellagitannins are hydrolysable tannins containing a hexahydroxydiphenoyl moiety which is metabolized to ellagic acid upon digestion. Ellagic acid is extensively metabolized by gut microbiota and exists as urolithins or urolithin conjugates in circulation (96). A wide variety of ellagitannins have been identified in fruits such as pomegranates (punicalagin, corilagin), raspberries (sanguiin H-6, lambertianin C), and walnuts (glansrin A, B, and C, casuarictin) (97). Ellagic acid (98,99) pomegranate extract, corilagin (100), and urolithin A (101) have been administered in rodent models of colitis.

Ellagic acid alone (0.5% diet) prevented chronic 1% DSS-induced disease activity index and histological markers of colitis in C57BL/6 mice (98). However, 2% ellagic acid supplemented diet did not prevent weight loss or disease activity index induced by a 7 d

treatment of 5% DSS to BALB/C mice (98). Ellagic acid reduced DSS-induced colonic COX-2 and iNOS expression, p38 mitogen activated protein kinase (MAPK) phosphorylation, NF-κB IκBα phosphorylation, NF-κB p65 nuclear translocation, and STAT3 phosphorylation in mice (98). IP injection of corilagin at 7.5, 15, or 30 mg/kg bw for 7 d to C57BL/6 mice inhibited colitis induced by a 5 d-2% DSS treatment (100). Corilagin treatment dose-dependently improved colon length, histological score and MPO activity (100). The colonic cytokines TNF-α, IL-1β, and IL-6, but not IL-10 were modulated by corilagin treatment (100). Since ellagitannins are extensively metabolized, it is plausible that dietary intake of corilagin would lead to a different outcome than IP injection in a model of colitis. Urolithin A-supplemented diets, providing 2.2 mg/kg bw/d for 25 d concurrent and following a 5 d, 5% DSS exposure, inhibited colitis as graded histologically (101). Urolithin A consumption also increased fecal bifidobacteria and lactobacilli (101). Consumption of 250 or 500 mg pomegranate extract/kg chow by Wistar rats prior to and following TNBS-induced colitis prevented weight loss and reduced colonic TNF-α and macroscopic and histological markers of colonic inflammation (99).

2.4.12 Hydroxycinnamic acids

Chlorogenic acid (102), caffeic acid (63,102), hydrocaffeic acid (103), and sodium ferulate (104) have been administered in rodent models of colitis. Chlorogenic acid is hydrolyzed to caffeic acid prior to absorption in the small intestine (105). Caffeic acid and chlorogenic acid were supplemented at 1 mM in the water of C57BL/6 mice 7 d prior to and concurrent with an 8 d, 3% DSS treatment (102). Chlorogenic acid, but not caffeic acid treatment prevented DSS-induced weight loss in mice, although both caffeic and chlorogenic acids improved diarrhea scores and histological grading of colitis (102). Hydrocaffeic acid is a colonic catabolite of

caffeic acid and other polyphenols. Hydrocaffeic acid prevented weight loss and reduced colonic TNF-α, IL-1β, and IL-8 mRNA in Fischer 344 rats treated with 4% DSS (103). Thus, hydroxycinnamic acid catabolites appear to retain anti-inflammatory activity.

2.4.13 Other purified phenolics

A number of other polyphenols have been solely administered in rodent models of colitis. These include apocynin (acetovanillone) (67), canolol (4-vinyl-2,6-dimethoxypehnol) (106), oleuropein, a phenolic secoiridoid from olive (61,107), chrysin, a flavone (108,109), silymarin (110), icariin (prenylated kaempferol diglucoside) (111), esculetin and 4-methylesculetin (coumarin derivatives) (112), hydroxytyrosol and tyrosol (113,114), and α-mangostin (a xanthone) (115). Notably, C57BL/6 mice consuming 112 mg α-mangostin/kg bw with DSS-induced colitis had worsened symptoms and greater colonic injury (115).

2.4.14 Other dietary sources of polyphenols

Other extracts or polyphenol-rich foods and ingredients have been used for treatment in rodent models of colitis. These extracts have a mixed composition that do not have a dominant polyphenol class or bioactive class. Foods that have been tested include cranberry (116), propolis (117), olive oil (113,114), almond skin powder (118), apples and apple polyphenols (119,120), flaxseed and its hulls and kernels (containing lignans) (121), lemon verbena (122), and oat bran and blueberry fiber (123). While other polyphenol diets conferred protection against colitis in rodents, it should be noted that 10% flaxseed diets increased disease activity index in 2% DDS-treated C57BL/6 mice (121).

2.4.15 Use of nutritional interventions in the adoptive transfer model

There is a limited record in the use of nutritional interventions in the adoptive transfer model of colitis. Female SCID mice were fed a control diet or a diet enriched with rice fiber for two weeks prior to adoptive transfer and were sacrificed 7 weeks after the transfer (124). Rice fiber supplementation attenuated weight loss, MLN T cell activation, mucosal damage, and decreased serum IL-6 (124). A barley derived prebiotic attenuated body weight loss, reduced IFN-γ and IL-6 mRNA, and attenuated colonic mucosal damage in the same experimental design (125). The adoptive transfer model has also been used to study the effects of dietary fish oil (126,127).

2.5 Dose relevance of polyphenols in rodent models of IBD

It is critical to consider the dose of polyphenols when interpreting results of published studies or designing a new experiment. It may be possible to demonstrate a polyphenol or extract inhibits colitis in rodents, but if the dose is not relevant to human consumption the findings may not be translatable. Allometric scaling or body surface area approaches can be used to predict a "human equivalent dose" from rodent models (**Table 2.2**) (128,129).

As an example, treatment with a human equivalent of 30 mg resveratrol/d inhibited DSS-induced colitis in mice (76). Wine has a maximum of ~14.3 mg resveratrol/L, with most red wines having 2-5 mg resveratrol/L (130). Other dietary sources of resveratrol have mg to sub-mg quantities (6). While consumption of 30 mg of resveratrol from the diet is not feasible, gram-quantities of supplemental resveratrol could be used to provide this dose (131). In contrast, another study treated colitic mice with a human dose equivalent to 8.4 g apple polyphenols/d, which may not be possible to consume (119). Another study used a human equivalent of 121.6

mg morin/d, but to our knowledge, a supplemental morin is not on the market, and sub-milligram quantities are present in food (6,65). Thus, to optimize translation of research using rodent models of colitis, doses and treatment regimens should be carefully selected. This is particularly necessary as over-supplementation of polyphenols could exacerbate colitis (39,40,115,121).

2.6 Experimental limitations of studying molecular targets with respect to polyphenol metabolism

Upon consumption polyphenols can be subjected to metabolism by the host, extensively catabolized by gut microbiota, or be poorly absorbed. This complicates *in vitro* and animal models investigating the molecular targets of polyphenols as they may not reflect the metabolite(s) presented to cells or tissue in humans. Although many polyphenol metabolites are bioactive, they may not be active in the same manner as the parent compounds and generally have reduced bioactivity relative to parent compounds (132).

Unabsorbed and catabolized polyphenols may be active in the gastrointestinal system (132). This makes polyphenols an attractive target in the treatment of inflammatory bowel diseases. Phytochemicals must still be bioaccessible to be bioactive in the gut. Thus, they must be liberated from the food matrix to exert effects on the cells in the gut. Indeed, both polyphenols and other phenolic compounds exist in the fecal water but exhibit high interindividual variation (133). Unabsorbed polyphenols may interact directly with microbiota, mucosal cells, and dendritic cell projections in the intestinal lumen. There is a greater need to understand how targeted polyphenol delivery systems could be used to optimize treatment for colitis.

For example, chlorogenic acid undergoes extensive transformation before absorption. Chlorogenic acids are hydroxycinnamic acids such as caffeic acid or ferulic acid linked to quinic acid through an ester bond (134). Before reaching the colon, chlorogenic acid can be cleaved to its phenolic acid and quinic acid portions, glucuronidated, sulfated, and methylated (105). It may enter circulation and re-enter the intestinal lumen via enterohepatic recirculation. Therefore, the compounds reaching the colon tissue and gut microbiota could be the parent compounds or one of the many metabolites which arise from its biotransformation of hydroxycinnamic acids. The bioactivity would then be dependent upon the metabolites' interactions, not just the parent compound.

It should also be noted some aspects of rodent microbiota and xenobiotic metabolism are dissimilar to humans (135,136). Furthermore, colitis is associated with changes in gut microbiota and xenobiotic metabolism (137–139). A number of studies in rodents and humans have demonstrated that polyphenol consumption modifies microbiota composition (140–142). The extent that these species differences affect the ability to translate polyphenol treatment rodent models to humans is presently unknown.

The efficacious agents for anti-IBD activity are not known. It is not known whether it is the parent polyphenols themselves, the microbial catabolites, phase I and II-enzyme derivatives (such as methylated or glucuronidated polyphenols), or a combination of these that generate biological effects in vivo. Parent polyphenols exhibit low plasma C_{max} and $t_{1/2}$ values, while bacterial catabolites exhibit higher C_{max} and $t_{1/2}$ values (143). Because of increased systemic exposure, it is highly plausible that health effects of polyphenols may be due to the bacterial catabolites. On the other hand, substantial evidence indicates that polyphenols improve intestinal barrier function (56,144,145) which in turn could ameliorate IBD.

2.7 Conclusions and future directions

A significant number of isolated polyphenols and polyphenol-rich extracts reduce colitis in rodent models. The doses of polyphenols applied in rodent IBD models vary widely and should be given careful consideration in experimental design. Using infeasible human equivalent doses may not be warranted, as it may induce harmful effects and makes translation to humans more difficult. Future consideration of targeted delivery of polyphenols may improve their ability to inhibit colitis.

Polyphenols modulate a large number of targets relevant to inflammation and colitis. Polyphenols consistently modulate NF-κB pathway and a number of polyphenols inhibit the MAPK cascade and induce Nrf2. These targets enable polyphenols to inhibit inflammatory responses in a variety of ways. Polyphenols reduce proinflammatory cytokines, induce anti-inflammatory cytokines, inhibit nitric oxide (NO) production, induce immunosuppressive properties in immune cells, and inhibit COX-2 activity.

In contrast to drugs, which generally have well-defined and specific targets, the broad actions of polyphenols on inflammation mechanisms could be an advantage to IBD therapy. However, caution should be exercised when designing adjuvant polyphenol treatments. Polyphenols can exert pharmacokinetic and pharmacodynamic interactions, leading to enhanced or antagonistic actions of more traditional pharmacotherapy. More work is needed to test the safety of using polyphenols as adjuvants to current IBD drugs.

The extant literature on this subject is highly redundant; there has been little novelty or innovation. These papers utilize the same outcome measures, only differing in the specific time courses, dietary intervention and duration. It is well established that polyphenols (with some exceptions) and polyphenol rich foods reduce the intestinal inflammation and weight loss

associated with colitis. While less reliable and robust, they generally decrease at least some inflammatory cytokines and other mediators such as COX-2 and iNOS (which may be confounded by Type 1 error when many measurements are taken). The field has not moved forward with the advances in immunology and intestinal physiology.

There are many facets of the biology and physiology of IBD that have not been explored in polyphenol research. Chiefly, we do not know how dietary polyphenols affect the innate and adaptive arms of the immune system. Polyphenols or their metabolites may act on antigen presenting cells, other innate immune cells such as neutrophils, or the B and T cells of the adaptive immune system. Th1, Th2, and Th17 cells are known drivers of IBD in both humans and rodents (1), but the effect of dietary interventions on these cells in IBD is non-existent, although grape seed proanthocyanidin extract decreased the frequency of Th17 cells and increased Treg cells in an arthritis model (146). Some papers have focused on the impact of polyphenols on microbiota composition, but this is mostly exploratory and causation is not being addressed. How polyphenols work in these models may be a key question to answer in the quest to translate these findings to dietary interventions in humans.

Intestinal barrier function is compromised in IBD (147), individuals with quiescent IBD (148), and in relatives of IBD who themselves do not have IBD (149). Improving intestinal barrier function is an important mechanism by which polyphenols may modulate susceptibility to colitis, especially since improvements in barrier function may not be dependent on the bioavailability of the polyphenols. Numerous in vitro studies point to improved intestinal barrier function, but very few have addressed this aspect in vivo. Barnett et al. (43) found an increase in proteins involved in tight junction signaling with green tea polyphenol supplementation but did not assess the tight junction proteins themselves. Azuma et al. (56) found an attenuation of DSS-

induced changes in tight junction protein expression, as well as occludin and claudin-3 localization with naringenin supplementation. They also found that plasma FITC-dextran and LPS-binding protein were lower in naringenin fed mice (56). Others have investigated demonstrated improvements in intestinal permeability and/or tight junction protein restoration with grape seed proanthocyanidins (150) and chlorogenic acid (144), but neither of these were models of colitis.

Resveratrol, curcumin, quercetin and anthocyanin-rich foods appear to have the most evidence for efficacy and safety in rodent colitis models. Despite the considerable number of positive studies on polyphenols for IBD treatment in rodents, few polyphenols have been examined in human intervention studies of colitis. Although evidence for the effectiveness of polyphenols for IBD in humans remains very limited, results from these pre-clinical studies indicate an opportunity for developing anti-colitic polyphenol-based treatments.

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2.9 Figures and Tables

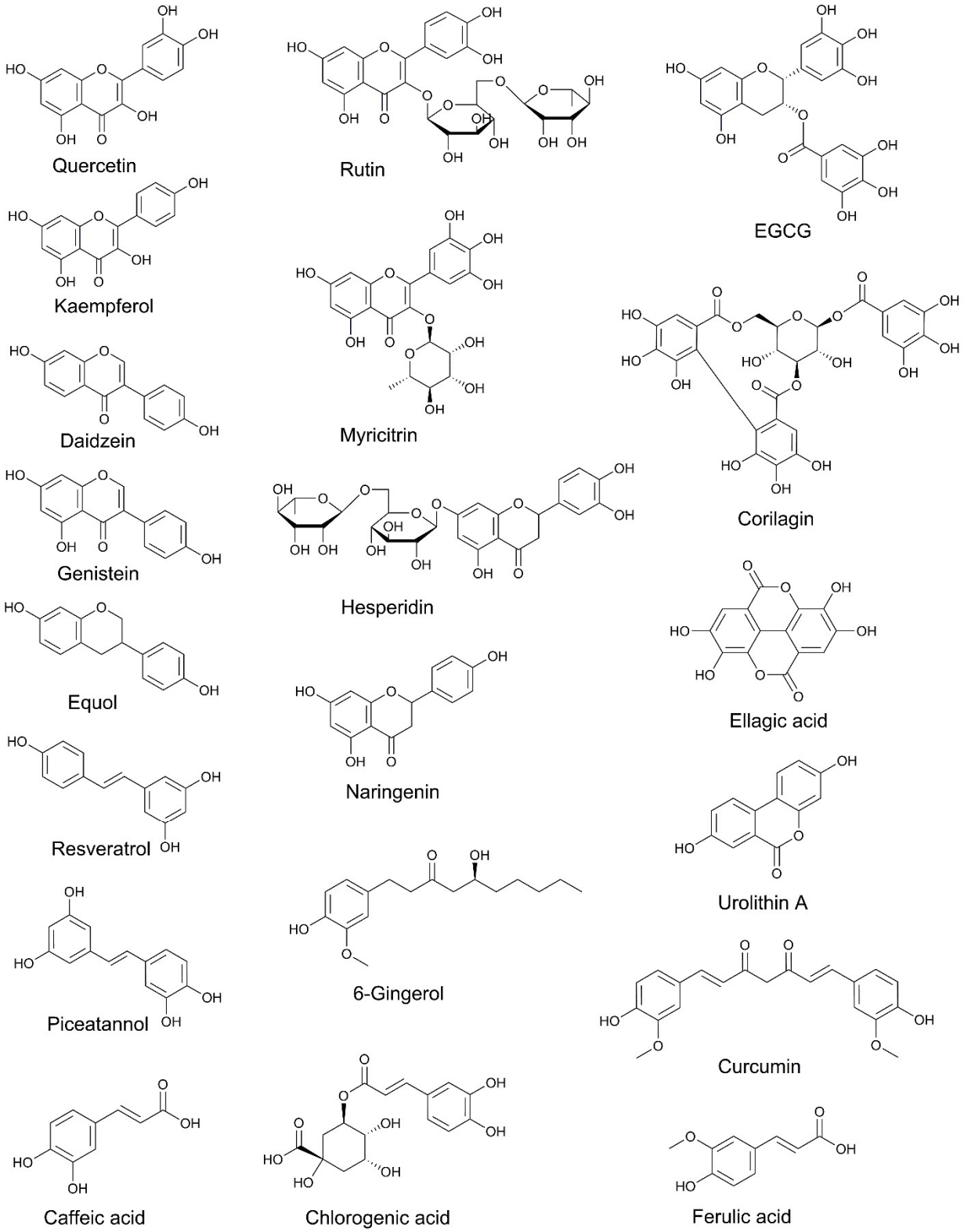

Figure 2.1 Structures of polyphenols used singularly as treatment in rodent models of colitis.

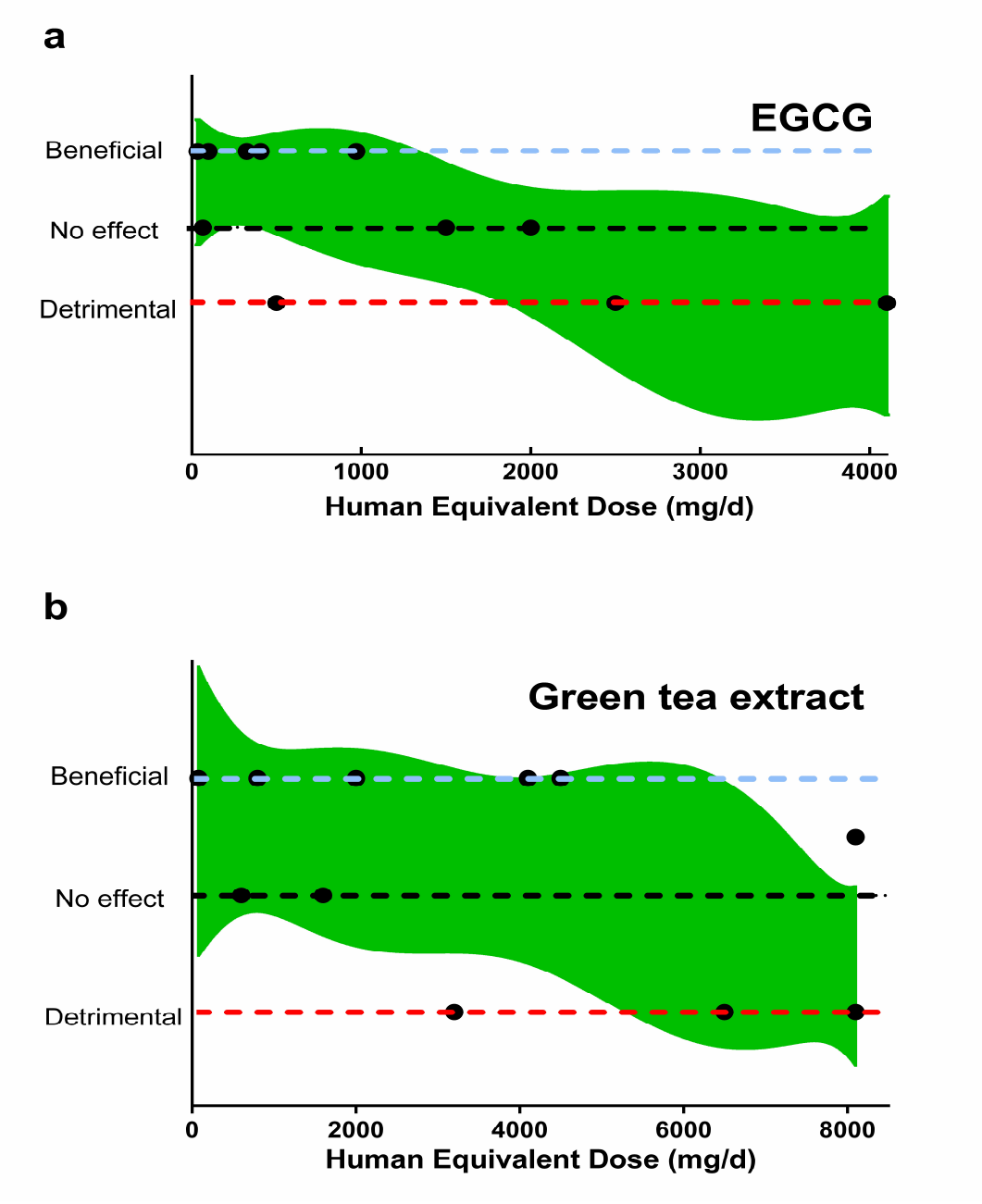

Figure 2.2 Results and human equivalent doses of **(a)** EGCG and **(b)** green tea polyphenols in rodent models of colitis, based on data from Table 2.2. A 90% confidence band of a best-fit quadratic non-linear regression is plotted for reference.

1

Table 2.1 Polyphenol classes used as treatments in rodent IBD models.

IBD Model	Polyphenol class	Intervention (Reference)
DSS-induced colitis	Anthocyanin	Bilberries and bilberry anthocyanin extract (25), black raspberry powder (27), aronia berry extract (29), goji berry (151), mulberry fruit (31), anthocyanin rich red raspberry fraction (30)
	Flavan-3-ol	EGCG (34,37), green tea polyphenols (36,39–41)
	Isoflavone	Genistein, daidzein and equol (52), daidzein rich isoflavones extract (51)
	Proanthocyanidin	Oligonol (46), apple proanthocyanidins (44), cocoa extract (47), cocoa (48)
	Flavanone	Hesperidin (55), naringenin (56,57)
	Flavonol	Quercetin (58,60), rutin (61–63) kaempferol (64), myricitrin (66)
	Other flavonoids	Luteolin (68), astilbin (69)
	Stilbene	Resveratrol (73–77), piceatannol (77,79)
	Hydrolysable tannin	Ellagic acid (98), corilagin (100), pomegranate extract and urolithin A (101)
	Curcuminoid	Curcumin (83,84), *Curcuma longa* extract (82)
	Hydroxycinnamic acid	Caffeic acid and chlorogenic acid (102), hydrocaffeic acid (103)

TNBS/DNBS-induced colitis	Anthocyanin	Grape juice concentrate (21,22), blueberry extract (23), blueberry anthocyanin fraction (24)
	Flavan-3-ol	EGCG (33)
	Isoflavone	Genistein and daidzein (53,54)
	Proanthocyanidin	Grape seed proanthocyanidins (45)
	Other flavonoid	Baicalin (71)
	Curcuminoid	Curcumin (86)
	Gingerol	Ginger extract, zingerone (95)
	Hydrolysable tannin	Pomegranate extract (99,101)
Il10$^{-/-}$ spontaneous colitis	Anthocyanin	Black raspberry (26)
	Flavan-3-ol	Green tea polyphenols (42)
	Stilbene	Resveratrol (78)
Mdr1a$^{-/-}$ or	Flavan-3-ol	Green tea polyphenols (43)

HLA-B27 transgenic rats, spontaneous colitis	Flavonol	Rutin (81)
	Curcuminoids	Curcumin (81)
Acetic acid-induced colitis	Anthocyanins	Hawthorn berry extract (28)
	Flavonol	Quercetin (59)
	Other flavonoids	Apigenin (70)
	Curcuminoid	Curcumin (85)
	Gingerol	Ginger extract (94)
	Hydroxycinnamic acid	Ferulate (104)
Adoptive transfer	Flavonol	Rutin (62)

Table 2.2 Human equivalent doses and treatment regimens previously utilized in rodent models of IBD

Treatment and Citation	Human Equivalent Dose[A]	Experimental Dose	Time Course	Outcome
Resveratrol (74)	8.6 mg/d	1 mg/kg/d, F344 rats	20 d prior to and concurrent with a 5 d 5% DSS treatment	inhibition of colitis, modified microbiota
Resveratrol (76)	30 mg/d	20 mg/kg diet, C57BL/6 mice	concurrent with a 5 d, 3% DSS treatment, and 21 d post-DSS.	improve mortality, inhibition of colitis
Resveratrol (77)	48.6 mg/d	10 mg/kg bw, ICR mice	concurrent with a 7 d, 2.5% DSS treatment	inhibition of colitis
Resveratrol (75)	48.6, 243, 486 mg/d	10, 50, 100 mg/kg bw, C57BL/6 mice	concurrent with a 7 d, 3% DSS treatment, and for 7 d following DSS	highest dose protective against colitis
Resveratrol (73)	58, 116, 232 mg/d	75, 150, 300 mg/kg diet, C57BL/6 mice	7 d prior to, concurrent with a 7 d, 1% DSS treatment, and 7 d following DSS	inhibition of colitis at two highest doses

Curcumin (83)	243 mg/d	50 mg/kg bw, BALB/c mice	7 d after a 7 d, 5% DSS treatment	inhibition of colitis
curcumin extract (82)	973 mg/d	200 mg/kg/d, BALB/c mice	7 to 21 d following induction of colitis by 52 d, 2.5% DSS; For 7 d after a 7 d, 5% DSS treatment	inhibition of chronic colitis at d 20, inhibition of acute colitis
Curcumin (81)	~1.6 – 2.0 g/d	0.2% diet, *Mdr1a*$^{-/-}$ mice	from 6 to 24 wk	inhibition of spontaneous colitis
Curcumin (85)	1.9 g/d	100 mg/d, Wistar albino rats	10 d prior to and 2 d following acetic acid treatment	inhibition of colitis
Curcumin (86)	1.9 g/d	100 mg/kg bw, Sprague-Dawley rats	7 d after induction of colitis by TNBS	inhibition of colitis
Curcumin (84)	~4.4 g/d[B]	0.6% diet, 18 mg/d C57BL/6 mice	2 wk prior to and concurrent with 0.7% DSS treatment for 15 cycles of 7 d DSS, 10 d no DSS (total 37 wk)	inhibition of colitis at cycle 11-15

EGCG (36)	34 mg/d	6.9 mg/kg bw, C57BL/6 mice	1 wk prior to and concurrent with 60 d, 2% DSS alternating treatment	inhibition of weight loss, some inhibition of colitis
EGCG (34)	65, 324 mg/d[C]	0.01, 0.05% diet, ICR mice	7 d prior to and concurrent with 7 d, 1.5% DSS treatment	some inhibition of colitis at highest dose
EGCG (33)	97 mg/d (IP)	20 mg/kg bw (IP), C57BL/6 mice	1 to 7 d after induction of colitis by TNBS	inhibition of colitis
EGCG (35)	405 mg/d	50 mg/kg bw, Sprague-Dawley rats	7 d after induction of colitis by acetic acid	inhibition of colitis
EGCG (37)	0.5, 1.5, 2.5 g/2,000 kcal	0.1, 0.3, 0.5% EGCG diet, C57BL/6, CD-1 mice	1 wk prior, concurrent with 1.5% DSS treatment, and 3 d after DSS treatment	highest dose increased rectal bleeding, lowest and highest doses increased weight loss
EGCG (42)	0.97, 2, 4.1 g/d[D]	0.12%, 0.25%, 0.5% diet, BALB/c	10 d, concurrent and after 7 d, 3% DSS	highest dose increased weight loss, lowest dose reduced colitis

GTE (40)	0.08, 0.8, 8.1 g/d[D]	0.01, 0.1, 1% GTE diet, ICR mice	concurrent with 6 d, 5% DSS treatment	highest dose induced mortality, lower doses inhibited colitis
GTE (41)	0.6, 1.6, 3.2, 6.5 g/d[C]	0.1, 0.25, 0.5, 1% GTE in diet, ICR mice	concurrent with 6 d, 25% DSS treatment	increased colitis at two highest doses
GTE (42)	2, 4.1, 8.1 g/d[D]	0.25, 0.5, 1% GTE diet, *Il10*$^{-/-}$ mice; 1% GTE diet, BALB/c mice	*Il10*$^{-/-}$: from 4 wk age; BALB/c: For 10 d, concurrent and after 7 d, 3% DSS	*Il10*$^{-/-}$: highest dose exacerbated colitis, lower doses improved colitis; BALB/c: prevented weight loss, improved colitis somewhat
GTE (43)	4.5 g/d	0.6% GTE diet, *Mdr1a*$^{-/-}$ mice	10 wk	inhibition of colitis
GTE (39)	8.1g/d[D]	1% GTE in diet, ICR mice	concurrent with 6 d, 5% DSS treatment	disrupted kidney function

Abbreviations: EGCG: epigallocatechin gallate; GTE: green tea extract; DSS: dextran sulfate sodium;

[A]Human equivalent dose based on 60 kg adult using body-surface area calculation (128). 60 kg was used in the reference and is between the 58 kg “reference woman” and the 70 kg “reference man.”

[B]Assuming 20 g bw for female C57BL/6 mice

[C]Assuming 30 g bw male ICR mice, consuming 4 g diet/d

[D]Based on 3 g diet/18 g mouse

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Chapter 3

Aronia berry (*Aronia mitschurinii* 'Viking') inhibits colitis in mice and inhibits T cell tumor necrosis factor-α secretion

The basis of this chapter was published as:

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3.1 Abstract

Aronia berries are rich in polyphenols with anti-inflammatory activity. We hypothesized that aronia berry consumption modulates intestinal immune function and prevents colitis by modulating T-cells. The aims of the present work were to assess the immunomodulatory potential of 'Viking' aronia berry (black chokeberry, *Aronia mitschurinii*) in vivo and to determine the extent aronia berry polyphenols or known microbial polyphenol catabolites inhibit T cell tumor necrosis factor (TNF)-α production in vitro. Aronia berry consumption increased colonic IL-10 secretion in healthy mice, but did not inhibit ex vivo cytokine secretion of lipopolysaccharide-stimulated spleen and colon tissue. Aronia berry consumption inhibited wasting associated with T cell adoptive transfer and dextran sulfate sodium-induced colitis. Aronia extracts, neutral phenols fraction, and the polyphenol catabolites 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylpropionic acid inhibited TNF-α production in Jurkat T cells. Therefore, T cells and microbial catabolism may mediate anti-inflammatory effects of aronia consumption in the colon.

3.2 Introduction

Increased fruit and vegetable consumption is associated with reduced risk of inflammatory bowel disease (IBD) (1). IBD includes Crohn's disease and ulcerative colitis and develops as an aberrant immune response to the intestinal microbiota (2). Pilot trials in humans of polyphenol-rich foods for IBD treatment including bilberry (3) and curcumin (4–6) have been promising. Similarly, a variety of polyphenol sources inhibit colitis in rodent models (7). Despite work in this area, little is known about how plant components affect the basal state of the immune system or specific immune cells to confer a reduced risk of IBD.

IBD immunology involves numerous innate and adaptive immune cells and both regulatory and inflammatory cytokines (2). Of these cytokines, interleukin (IL)-10 is an important immunoregulatory cytokine produced mainly by T cells (8,9). In contrast, IL-6 is a pleiotropic, but often proinflammatory cytokine and is responsible for the initial differentiation of naïve T cells into potentially pathogenic T-helper (Th)-17 cells (10) and inhibits regulatory T cell (Treg) development (11). Tumor necrosis factor (TNF)-α is also proinflammatory and signals through TNF receptors to initiate angiogenesis, Paneth cell death, activation of macrophages and effector T cells, and intestinal barrier dysfunction (9). TNF-α from macrophages, adipocytes, fibroblasts and T cells is markedly augmented in patients with IBD (9). TNF-α inhibitors are important pharmacological agents for managing IBD (12).

Polyphenols appear to modulate immune function in a cell- and cytokine-specific manner. For example, aronia berry extracts inhibited IL-6 production in lipopolysaccharide (LPS)-stimulated primary murine splenocytes and increased IL-10 excretion only in unstimulated splenocytes (13). Furthermore, aronia berry polyphenols are extensively catabolized by gut microbiota and metabolized by host tissue (14). Because of this, a combination of in vivo and in

vitro models considering the metabolic fate of polyphenols are needed to clarify mechanisms by which aronia consumption can modulate IBD risk or have therapeutic effects. We hypothesized that aronia berry consumption modulates intestinal immune function and prevents colitis through a T-cell based mechanism. The aims of the present work were to assess the immunomodulatory potential of 'Viking' aronia berry (black chokeberry, *Aronia mitschurinii*) in the colon and to determine the potential for aronia berry polyphenols or bacterial catabolites of polyphenols (**Figure 3.1**) to inhibit T cell TNF-α in vitro.

3.3 Materials and methods

3.3.1 Chemicals and reagents

Gentamycin, 2-mercaptoethanol, ionomycin, LPS, dibutyryl-cAMP, DMSO, 3,4-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylpropionic acid, ferulic acid, chlorogenic acid, neochlorogenic acid, quercetin-3-galactoside, quercetin-3-glucoside, and quercetin-3-rutinoside were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's phosphate buffered saline (DPBS), RPMI 1640 with 2 mM L-glutamine, fetal bovine serum (FBS), sodium pyruvate, minimum essential medium (MEM), and 10% buffered formalin were purchased from Thermo Fisher Scientific (Waltham, MA). Dextran sulfate sodium (DSS, MW: 36,000-50,000) was purchased from MP Biomedicals (Santa Ana, CA). Aronia extract powder for in vitro studies was a gift from Naturex-DBS LLC (South Hackensack, NJ). Cyanidin-3-galactoside and cyanidin-3-glucoside standards were purchased from Chromadex (Irvine, CA). Antibiotic/antimycotic solution and FBS for Jurkat T cell experiments were purchased from Gibco (Waltham, MA). RPMI 1640 used for Jurkat T cell experiments was

purchased from Corning Cellgro (Corning, NY). Other reagents, including HPLC grade solvents were purchased from either Thermo Fisher Scientific or Sigma-Aldrich.

3.3.2 Experimental diets

Diets were formulated to provide a dose equivalent to ~1 cup of fresh, whole Viking berries for a 70 kg human. This was based on a 25 g mouse consuming ~3.5 g diet providing 6.3 g lyophilized aronia berry per kg body weight. Using the human equivalent dose (HED) approach as described by Reagan-Shaw, Nihal, and Ahmad (15), a 4.5% berry-fortified diet has a HED of 35.8 g aronia berry powder or 149 g fresh aronia berries for a 70 kg human (**Supplemental Method 1**).

Lyophilized aronia berries were prepared from fresh 'Viking' aronia berries grown by Dr. Mark Brand at a test plot located in Storrs, CT. Berries were harvested at apparent ripeness. Stems, debris, and apparently inedible berries were removed prior to freezing at -20 °C. The berries were then lyophilized, ground to a powder using an IKAA11 Basic Grinder (St. Louis, MO). Berry powder was stored at -80 °C until formulation of the diets. Diets were purchased from Harlan Teklad (Madison, WI) and pelleted in-house for basal and adoptive transfer experiments or by Harlan Teklad for the DSS experiment. Aronia berry powders were substituted for corn starch at 4.5% weight basis in the AIN-93M rodent diet. The control diet consisted of 91% AIN93M-Mod, 4.5% sucrose, and 4.5% corn starch. The intervention diet consisted of 91% AIN93M-Mod, 4.5% sucrose, and 4.5% Viking aronia powder.

In-house pelleting was done by mixing the diet powders and adding water until a homogeneous mass was obtained. Pellets with 1.3 cm diameter and 2.5-4 cm length were placed

on a tray and frozen at -80 °C. Frozen pieces were lyophilized and the dried diet "pellets" were stored at 4 °C until being placed in cage feeder baskets.

3.3.3 Effects of aronia consumption on healthy C57BL/6J mice

Male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 4 weeks old and were fed standard chow diets upon arrival to the animal facility. The aronia-supplemented diet or standard AIN-93M diets were fed to C57BL/6J mice for 4 weeks starting at 12 weeks of age (n = 8 per group). Mice were anesthetized under isoflurane and sacrificed by cervical dislocation. Experiments were approved by the University of Connecticut Institutional Animal Care and Use Committee (IACUC #A12-016). Cytokines relevant to IBD development were assessed ex vivo in primary colon tissue and splenocytes.

3.3.4 Incubation of colon tissue for cytokine analysis

Colonic cytokine production was determined ex vivo as previously described by Sellon et al. (16). Upon sacrifice, the mouse colon was excised, and the intestinal contents were removed. The colon was then opened longitudinally and shaken in a tube of ice-cold DPBS to wash out remaining intestinal content. The colon tissue was then cut into ~1 cm segments with a scalpel and transferred to a 50 mL conical tube containing 20 mL RPMI 1640 with 2 mM L-glutamine with 50 μg gentamycin/mL. The conical tubes were then gently shaken using an orbital shaker for 30 min at 23 °C. Subsequently, media was decanted, and the tissue segments were blotted dry and weighed. Colon tissue segments were selected randomly for each mouse and placed in 24-well plates, with ~50 mg tissue/well and 1 mL RPMI 1640 with 2 mM L-glutamine, gentamicin (50 μg/mL), 1x antibiotic/antimycotic, 5% fetal bovine serum, 1 mM pyruvate and 0.5 μM 2-

mercaptoethanol. For stimulation experiments, media contained 2 μg LPS/mL. For ex vivo inhibition experiments, 2 mg gallic acid equivalents aronia extract/mL was also applied to colons from mice fed control diets (**Supplemental Method 2**). Tissue was then cut into smaller pieces within the plate wells and incubated for 18.5 h in a cell culture incubator at 37 °C and 5% CO_2. Colon tissue culture media were then collected and stored at -80 °C until further analysis.

3.3.5 Splenocyte stimulation and incubation

Splenocyte cytokine production was determined ex vivo as previously described (17), with modifications. Mouse spleens were dissected and strained through a 70 μm cell strainer (Becton Dickinson, Franklin Lakes, NJ). Cells were then pelleted in MEM by centrifuging at 400 × g for 5 min at 4 °C. Red blood cells were then lysed by adding 5 mL 0.15 M ammonium chloride with 10 mM potassium carbonate. After 5 min, 30 mL DPBS was added, cells were pelleted, and washed twice with MEM and resuspended in MEM. Cells were counted in a Bio-Rad (Hercules, CA) TC-10 cell counter. Cells were then plated at 10^6 cells/well with 250 μL of MEM containing 10% FBS and supplemented with amino acids, dextrose, sodium pyruvate, antibiotics, antimycotic, and 2-mercaptoethanol (17). LPS-stimulation experiments contained 1 or 10 μg LPS/mL. Cells were incubated for 12-26.5 h at 37 °C with 5% CO_2. Following incubation, the cells were pelleted as described above, and the supernatants were stored at -80 °C until analysis.

3.3.6 Measurement of colon tissue and splenocyte supernatant cytokines

Cytokines from colon tissue and splenocyte incubations were determined in frozen supernatants. Upon thawing media, TNF-α, IL-6, IL-10, and IL-17 were determined by

commercial enzyme-linked immunosorbent assay (ELISA) kits according to manufacturer's instructions (Platinum ELISA kits, eBioscience, San Diego, CA). The limits of quantification were 31.6, 31.3, 15.6, and 7.8 pg/mL, respectively. Intra-assay variation averages were 3.7%, 16.7%, 5.1%, and 3.5%, respectively.

3.3.7 Effects of aronia consumption on adoptive transfer colitis

Colitis was induced in recombinase activating gene-1 (*Rag1*)$^{-/-}$ mice by adoptive transfer of $CD4^{+}CD45RB^{HI}$ cells from C57BL/6J mice (18). Male *Rag1*$^{-/-}$ mice were purchased from the Jackson Laboratory at 3 weeks old and given free access to chow diets. $CD4^{+}CD45RB^{HI}$ cells from splenocytes of male C57BL/6J mice (Jackson Laboratory) were isolated by magnetic separation and flow assisted cell sorting (18). Briefly, splenocytes were isolated as above, $CD4^{+}$ T cells were magnetically separated (Invitrogen, Carlsbad, CA) and stained with anti-CD4-FITC (eBioscience) and anti-CD45RB-APC (eBioscience) and flow sorted on a Becton Dickinson FACSAriaII. $CD4^{+}CD45RB^{HI}$ cells or sterile phosphate buffered saline (PBS) were injected intraperitoneally into *Rag1*$^{-/-}$ mice at 9 weeks of age. Upon injection, mice were divided into the control diet, or the 4.5% aronia berry diet (n = 6/group). A colitis symptom score (19) was used to evaluate mouse behavior and appearance at the end of the experiment. Mice were sacrificed by cervical dislocation after isoflurane anesthesia when 25% of original body weight was lost or after 54 days.

The colon was harvested and cleared of fecal content by manual expulsion, opened longitudinally, and then washed with PBS. It was laid flat and open onto chromatography paper and secured with parafilm. Colons were fixed in 10% neutral buffered formalin for 2-3 h. The fixed colons were "swiss rolled" and placed into histological cassettes and stored in 70% ethanol

at 4 °C. Fixed colon tissues were processed to paraffin by standard methods and 4-5 μm sections were stained with hematoxylin and eosin by the Connecticut Veterinary Medical Diagnostic Laboratory at the University of Connecticut. Colitis severity was determined by a veterinary pathologist in a blinded manner. Experiments were approved by the University of Connecticut (IACUC #A12-016).

3.3.8 Effects of aronia consumption on DSS-induced colitis

Colitis was induced in C57BL/6J mice by consumption of DSS in drinking water (20,21). Male C57BL/6J mice were purchased at 4 weeks old from The Jackson Laboratory and were fed standard chow diet upon arrival to the animal facility. At 6-15 weeks old, mice were given 3% DSS in the drinking water for 1 wk. Concurrent with DSS administration, mice were fed either an AIN93M control diet or a 4.5% aronia supplemented diet (n=9/group). A group without DSS supplementation and fed the control diet was utilized as a non-colitic control (n=9). One week after initiating DSS administration and test diets, mice were sacrificed by CO_2 asphyxiation followed by cervical dislocation. Experiments were approved by the University of Wisconsin-Madison IACUC (#A01583).

3.3.9 Determination of colitis severity in DSS-induced colitis

Colitis symptom scores were evaluated daily upon initiation of DSS administration. Colon length was measured to the nearest mm, cleared of fecal content by manual expulsion and flushing with PBS. Colon tissues were then blotted dry and weighed. After weighing, the entire colon was placed into histological cassettes and fixed for 3 hr in 10% neutral buffered formalin and stored in 70% ethanol at 4 °C. Fixed colon tissues were processed to paraffin and then

stained with hematoxylin and eosin by the University of Wisconsin Comparative Pathology Laboratory. Colitis severity was determined by a certified clinical pathologist in a blinded manner.

Colonic tissue cytokines were assessed in DSS-supplemented and non-colitic control mice. Colon tissue was homogenized with a handheld motorized mortar and pestle system (Thermo Fisher Scientific) in a buffer containing 150 mM NaCl, 20 mM Tris, 1mM EDTA, 1mM EGTA, 1% Triton X-100, phosphatase inhibitor II and protease inhibitor solution (Sigma-Aldrich). The resulting homogenate was centrifuged at 12,000 × g for 30 min. The supernatant was transferred to a new tube and stored at -80 °C. Protein concentration was determined by Pierce BCA protein assay kit (Thermo Fisher Scientific). The concentrations of IL-10, interferon (IFN)-γ, IL-1β, IL-2, IL-6, and TNF-α were determined by electrochemiluminescence immunoassay using the Meso Scale Diagnostics multiplex assay (Rockville, MD). The limits of quantification were 1.39, 0.176, 0.792, 0.663, 0.979, and 0.376 pg/mL, respectively. Intra-assay variations were 6.9, 17.2, 11.7, 12.6, 18.7, and 10.0 %, respectively.

3.3.10 Effects of aronia polyphenols and catabolites on T cell TNF-α production in vitro

The ability of aronia extract, fractions, and known colonic catabolites to modulate TNF-α excretion was evaluated in vitro using human T cell cultures. Jurkat T cells (Clone E6-1 (ATCC® TIB-152™)) were purchased from American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 with 10% FBS and were used between passages 4 and 21. For stimulation of cytokine production, 0.2 mL aliquots of a 2×10^6 cells/mL suspension were seeded in U-bottom microplates (VWR, Radnor, PA) and stimulated with phorbol 12-myristate 13-acetate (PMA) (Calbiochem, La Jolla, CA) and ionomycin (50 ng/mL and 1 μg/mL,

respectively, "PI") for 4 h at 37 °C and 5% CO_2. Cells were treated with 50 to 250 µg/mL aronia berry extract, 1.25 to 446 µM aronia polyphenol fractions, 0.5-10 µg/mL proanthocyanidin fraction, 0.1 to 100 µM polyphenol catabolite standards, 100 µM dibutyrl-cAMP as a positive control, or a vehicle control containing 0.6% DMSO for 4 h. The plate was then centrifuged at 600 × g for 10 min at 4 °C and the supernatant was stored at -80 °C until analysis. Supernatant TNF-α was determined by an ELISA kit according to the manufacturer's instructions (Human TNF-α ELISA MAX™ Deluxe, BioLegend, San Diego, CA). The limit of quantification for TNF-α was 7.8 pg/mL, and the mean intra-assay variation was 5.0%.

3.3.11 Aronia polyphenol fractionation

Aronia berry extract was fractionated to isolate anthocyanins, neutral phenols, and proanthocyanidins. Anthocyanins and neutral phenols were isolated by sequential elution from C18 solid phase extraction (SPE) cartridges (Thermo Fisher Scientific) (22). Aronia extract containing 12.4% anthocyanins was loaded onto preconditioned 500 mg C18 SPE cartridges (Thermo Fisher Scientific). Organic acids and sugars were eluted with 0.01% HCl in water and discarded. Neutral phenols were eluted with 20 mL ethyl acetate, and anthocyanins with 4 mL methanol having 0.01% HCl (22). The fractions were aliquoted and dried under nitrogen gas. For isolation of proanthocyanidins, frozen 'Viking' aronia berries were freeze dried, ground to powder and extracted with acetone/water/acetic acid (70:29.5:0.5, v/v/v) by sonication and agitation as previously described (23). The acetone/water/acetic acid extract was applied to a Sephadex LH-20 column (GE Healthcare, Waukesha, WI), eluted with 30% methanol in water (v/v) to remove low molecular weight compounds, and then 70% acetone in water (v/v) to elute

proanthocyanidins (23). The proanthocyanidin-containing fraction was dried under nitrogen gas at 40 °C and stored at -20 °C until analysis.

3.3.12 Analysis of aronia extract and polyphenol fractions

Aronia polyphenols were quantitated using a Dionex Ultimate 3000 UHPLC (Sunnyvale, CA) equipped with an autosampler, diode-array-detector, fluorescence detector, and column oven. Anthocyanins, hydroxycinnamic acids, and flavonols were analyzed by reverse-phase chromatography, using an Agela Technologies (Wilmington, DE) Venusil XBP Phenyl column (250 mm × 4.6 mm, 5 μm) with a column oven temperature of 30 °C. Separation was with mobile phase A consisting of 5% formic acid in water and mobile phase B consisting of 100% methanol. Using a flow speed of 1 mL/min, the gradient was initially 5% B, then increased from 5 to 35% B over 40 min, from 35 to 95% B over 40 to 45 min, and held at 95% from 45 to 50 min. The gradient was returned to initial conditions over 55 to 57 min and was equilibrated until 65 min. Absorbance was monitored at 254, 280, 370, and 520 nm. The major aronia polyphenols cyanidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-arabinoside, cyanidin-3-xyloside, protocatechuic acid, chlorogenic acid, neochlorogenic acid, quercetin-3-galactoside, quercetin-3-glucoside, and quercetin-3-rutinoside (rutin) were identified based on known elution order and comparison to authentic standards (23). Anthocyanins were quantitated as cyanidin-3-glucoside (Chromadex, Irvine, CA) equivalents, due to lack of a commercial high-purity cyanidin-3-galactoside standard. All other phenolics were quantitated on the basis of authentic external standards.

Proanthocyanidins were quantitated by hydrophilic interaction chromatography (HILIC) chromatography using the UHPLC system described above. A ThermoFisher Hypersil Silica

column (250 mm × 4.6mm, 5 μm) was equipped and the column oven was set to 35°C. A gradient of mobile phase A (98% acetonitrile, 2% acetic acid, v/v) and mobile phase B (95% methanol, 2% acetic acid, 3% water, v/v/v) was applied at a 1 mL/min flow rate. Mobile phase B was initially 7%, then increased from 7 to 37.6% over 60 min, then increased to 100% B over 3 min and held for an additional 7 min. The solvent proportion was then returned to 7% B over 6 min, and then held at 7% B for 10 min. Proanthocyanidin monomers, oligomers (degree of polymerization (DP) < 11), and polymers (DP > 11) were quantitated as (+)-catechin equivalents by fluorescence detection with 230 nm excitation and 321 nm emission.

3.3.13 Statistical analyses

Statistical significances for ex vivo cytokine production by colon tissue and splenocytes, colitis symptom score in the adoptive transfer experiment, and tissue cytokines in the DSS experiment were determined by two-tailed student's T-tests. Survival was tested by the Log-rank (Mantel-Cox) test. Animal weight across time was subjected to two-way repeated measures ANOVA followed by Tukey's test. Colon length, weight, weight/length ratio, spleen weight, and histological summary score in the DSS experiment were tested by one-way ANOVA followed by Tukey's test. TNF-α production by Jurkat T cells was determined by one-way ANOVA with Dunnett's multiple comparisons test. P values < 0.05 were considered significant.

3.4 Results

3.4.1 Effects of aronia consumption on cytokine production in healthy mice

Consumption of 4.5% aronia berry did not inhibit ex vivo stimulated cytokine production by colon and spleen tissue from healthy C57BL/6J mice. Ex vivo splenocyte secretion of IL-6 following LPS stimulation was not different between the aronia or control groups (**Figure 3.2a,b**). Similarly, ex vivo colon tissue secretion of IL-6, IL-17A, TNF-α, and IL-10 after LPS stimulation were not different between dietary treatments (**Figure 3.2c-f**). In contrast, unstimulated ex vivo colon tissue secretion of IL-10 was increased by 100% in the aronia fed mice relative to the control group ($P = 0.0348$) (**Figure 3.2g**). IL-17 and IL-6 were not significantly different in the aronia-fed mice ($P = 0.0535$ and $P = 0.0850$, respectively) (**Figure 3.2h,i**). Ex vivo application of aronia extract to colon tissue of control mice did not modulate cytokine expression (data not shown).

3.4.2 Effects of aronia consumption on adoptive transfer colitis

Aronia consumption prevented T cell adoptive transfer colitis-associated weight loss ($P < 0.05$) (**Figure 3.3a**) and inhibited the symptoms of colitis ($P = 0.0308$) (**Figure3.3b**). Additionally, aronia feeding enhanced survival ($P = 0.0195$ vs. noncolitic control), as mice were euthanized at a body weight loss of at least 25% from the weight at T cell transfer to minimize unnecessary distress (**Figure 3.3c**).

The colons of aronia-fed mice exhibited mild to severe inflammation (**Figure 3d**). Notably, animals on the control diet who developed early weight loss (day 37-48) had zero to very few foci of very mild inflammation. In summary, although aronia consumption appeared to

delay initiation of colitis as evidenced by weight loss and mortality, it did not completely inhibit colitis development after adoptive transfer.

3.4.3 Effects of aronia consumption on DSS-induced colitis

Aronia consumption also inhibited weight loss in mice with DSS-induced colitis ($P < 0.05$) (**Figure 3.4a**) and reduced clinical symptom scores at days 7-8 during DSS treatment ($P < 0.05$) (**Figure 3.4b**). DSS treatment reduced colon lengths of the control mice, relative to the non-colitic control ($P < 0.05$), but aronia consumption inhibited this shortening (**Figure 3.4c**). In contrast, colon weight-length ratios were increased in both DSS-treated groups, relative to the non-colitic control ($P < 0.05$) (**Figure 3.4d**). DSS treatment increased spleen weights relative to the non-colitic control ($P < 0.05$), but this indicator of inflammation was also inhibited by aronia consumption (**Figure 3.4e**). Similar to adoptive transfer, aronia consumption did not significantly reduce histological scores of colonic inflammation relative to control-fed mice (**Figure 3.4f**). DSS treatment also increased colonic tissue IL-10, IFN-γ, IL-1β, IL-2, IL-6, and TNF-α (**Figure 3.5a-f**). Among these, IL-1β was increased by aronia feeding ($P < 0.05$), but no other cytokines were modulated.

3.4.4 Effects of aronia polyphenols and catabolites on T cell TNF-α production in vitro

Given that aronia inhibited the colitic weight loss and other indicators of colitis, and the significant role of T cell TNF-α in mediating colitis, we further evaluated the ability of aronia polyphenols and known colonic catabolites to inhibit TNF-α secretion in cultured Jurkat T cells. Aronia extract inhibited PI stimulated TNF-α excretion from Jurkat cells at 250 μg extract/mL ($P < 0.05$) (**Figure 3.6a**), without a significant loss of viability (data not shown). Fractionation of

aronia yielded isolates enriched in anthocyanins, neutral phenols, and proanthocyanidins (**Supplemental Figures A.3.S1-A.3.S3**). In the anthocyanin fraction, anthocyanins were mainly cyandin-3-galactoside (53%) and cyanidin-3-arabinoside (23%) (**Supplemental Table A.3.S1**). The neutral phenols fraction consisted mainly of chlorogenic acid (46%), neochlorogenic acid (44%), protocatechuic acid (6%) and quercetin-3-galactoside (3%). Proanthocyanidins were mainly DP >10 (**Supplemental Table A.3.S2**). Polyphenol fractions were assessed for inhibition of TNF-α in Jurkat cells. The anthocyanin fraction (1-10 μM) and proanthocyanidin fraction (0.5-4 μg/mL) did not inhibit PI-stimulated TNF-α (**Figure 3.6b,c**). In contrast, the neutral phenols (111.5-445.9 μM) inhibited PI-stimulated TNF-α dose-dependently ($P < 0.05$) (**Figure 3.6d**).

Aronia polyphenols are extensively catabolized, resulting in a series of hydroxyphenyl microbial catabolites. Ferulic acid is also a major product formed as a consequence of methylation of caffeic acid in tissues. The ability of these catabolites to inhibit PI-induced TNF-α was variable (**Table 3.1**). 3,4-Dihydroxyphenyl-propionic acid inhibited TNF-α production at 100 and 0.1 μM and 3,4-dihydroxyphenyl-acetic acid inhibited TNF-α production at 0.1 μM ($P < 0.05$). However, 3,4-dihydroxy-benzoic acid (protocatechuic acid) and ferulic acid did not inhibit TNF-α production by Jurkat cells at any tested concentration.

3.5 Discussion

Aronia berry consumption inhibited the onset of weight loss in DSS- and T cell transfer-induced colitis and increased colonic IL-10 in healthy mice. However, aronia consumption did not significantly inhibit the ex vivo response to LPS stimulation in healthy mice. In our prior work using ex vivo primary mouse splenocytes, incubation with aronia extract also increased the

production of anti-inflammatory IL-10 (13). Therefore, it appears that aronia polyphenols can directly stimulate immunocyte IL-10 production, which may explain the higher levels of this cytokine in the mouse colon. In contrast, aronia extract and isolated polyphenols inhibited IL-6 ex vivo in PI-stimulated $CD4^-$ and $CD4^+$ lymphocyte populations (13). However, aronia consumption did not inhibit ex vivo stimulation of IL-6 by LPS in the present study. Thus, it appears that the concurrent presence of high levels of aronia polyphenols are necessary to inhibit immunocyte proinflammatory cytokines.

T cells and macrophages function in the adaptive and innate immune responses, so it is important to consider the function of dietary polyphenols on both of these cell types. In the present study, we report that aronia polyphenols decrease inflammatory mediators in Jurkat T cells. In contrast, most prior research on the anti-inflammatory mechanisms of aronia has utilized cultured macrophages. Aronia juice concentrate reduced pro-inflammatory cytokines in primary human monocytes and decreased LPS-stimulated activation of NF-κB in RAW 264.7 macrophages (24). A crude aronia extract inhibited LPS-stimulated inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 expression in RAW 264.7 cells, at a dose as low as 1 μg/mL (25). In the present work, we did not observe anti-inflammatory effects in Jurkat T cells until aronia extract reached 250 μg/mL. Intestinal lumen concentrations of polyphenols and catabolites are generally going to be higher than plasma concentrations, and therefore intestinal resident T cells would plausibly be exposed to relatively high concentrations of the compounds. Consumption of 100 g fresh aronia berries would yield about 330 mg of extractable anthocyanins (23), making lumen concentrations of total anthocyanins in the low millimolar range if berries were consumed with a limited amount of other foods. Thus, the anticipated luminal concentrations of berry phytochemicals are in-range of the in vitro concentrations employed in

the present study. Assuming continuous consumption and cecal volume in mice, the cecal anthocyanin concentration would be within a mid-micromolar range (i.e. somewhat higher than 250 μg anthocyanins/mL); therefore, polyphenol concentrations between the in vitro and in vivo experiments in the present study are relatively similar. At physiologic plasma concentrations, parent polyphenols did not inhibit T cell TNF-α. However, it is notable that certain microbiota-derived catabolites did inhibit TNF-α production in Jurkat T cells at physiologically relevant plasma levels (26) and may thus contribute to the health benefits of polyphenol consumption.

Aronia has inhibited the inflammatory response in other pre-clinical models of chronic disease. Topical administration of aronia concentrate reduced the expression of TNF-α, IL-1β, and IL-6 in a model of mouse ear edema (27). In rats fed a fructose rich diet, plasma TNF-α and IL-6 protein levels, and adipose tissue IL-1β, IL-6, and TNF-α gene expression levels were reduced by 6 wk aronia extract supplementation (28).

Our studies utilizing in vitro and in vivo approaches are in general agreement with human trials in that aronia treatment decreases inflammatory mediators associated with chronic diseases. Serum TNF-α was decreased in adults with mild hypertension that consumed 300 mL fresh aronia juice and aronia powder for 16 wk (29). Monocyte chemotactic protein-1 (MCP-1) and high-sensitivity C-reactive protein (hs-CRP) was reduced in adults taking statins post myocardial infarction after consuming 255 mg aronia extract/d for 6 weeks (30). However, consumption of 500 mg aronia extract/d in healthy adult former smokers did not improve markers of chronic inflammation but did reduce total and LDL cholesterol levels (31).

Limited data are available to inform our understanding of how effective aronia or other berries may be in preventing or managing IBD. Other berries may have similar effects on inhibiting colitis, given their rich polyphenol content. For example, bilberry consumption inhibits

DSS-induced colitis in BALB/c mice (32). Bilberry consumption also improved disease activity of patients with ulcerative colitis (3). Dietary administration of components of the aronia neutral phenols fraction, rutin and chlorogenic acid, inhibit DSS-induced colitis in mice (33,34). Furthermore, dietary administration of 3,4-dihydroxyphenylpropionoic acid inhibited acute DSS-induced colitic wasting (35).

The present study suggests that increased basal levels of colonic IL-10 may be important to the anti-colitic mechanism of aronia consumption. IL-10 is an important immunoregulatory cytokine, and *Il10*$^{-/-}$ mice spontaneously develop colitis when colonized by microbiota (16). IL-10 deficiencies in T cells (36) and Tregs (37) also lead to spontaneous colitis. IL-10 functions to inhibit T cell activation and also suppresses the development of pathogenic Th17 cells in colitis (38).

It is notable that aronia consumption prevented weight loss in both the DSS and adoptive transfer models of colitis. The DSS model of colitis histologically resembles human ulcerative colitis but is driven by a Th1/Th17 type response, especially in C57BL/6 mice (20); ulcerative colitis is a Th2 driven disease in humans (2). The DSS model and the adoptive transfer model require the presence of the microbiota, but for different reasons. The adoptive transfer model more closely mimics colitis as an aberrant immune response to the gut microbiota and colitis does not develop in germ free animals (39). On the other hand, the DSS model requires microbiota so that the immune system will initiate an inflammatory response to the mucosal injury caused by DSS (40).

Aronia consumption delayed the onset of colitic weight loss in both models but did not strongly inhibit colitis histopathology. Furthermore, in the DSS model, colonic IL-1β was increased in the aronia-fed mice. Given the similar degree of colitis, the significance of increased

IL-1β is unclear. Evaluating cytokines and cellular events at earlier stages of these models may provide insight about the mechanism(s) associated with reduced wasting. Although aronia conferred protection in these models of severe colitis, the doses and treatment length needed to inhibit a more moderate colitis also warrant further investigation.

Aronia or metabolite inhibition of T cell TNF-α, may partly contribute to the anti-inflammatory effect. TNF-α inhibitors are an important tool in the clinical management of IBD (12). Anti-TNF-α biologics have varying degrees of efficacy and many patients experience an eventual failure of the drug due to production of antibodies against the TNF antagonists (41). Inhibiting immunocyte TNF-α by polyphenols may be an important biological feature of the anti-colitic activity of aronia consumption, but the lack of inhibition at later stages in the DSS model of colitis suggests that this mechanism may be more important at earlier stages in the model.

Aronia berry consumption increased colonic IL-10 in healthy mice but did not otherwise broadly modulate immune function. Aronia berry consumption also inhibited the onset of T cell transfer and DSS-induced colitis in mice. Aronia extract, non-anthocyanin polyphenols, and metabolites inhibited Jurkat T cell TNF-α in vitro. This combined with the inhibition of wasting by aronia consumption in the T cell transfer colitis model, suggests that T cells are at least partly involved in the anti-colitic mechanism. Certain catabolites of anthocyanins appear to be more active than parent compounds in inhibiting TNF-α in cultured T cells. Thus, metabolic fate of polyphenols is an important consideration for the putative anti-colitic activity of aronia berry. These findings warrant further investigation into the cell-mediated mechanisms of colitis inhibition by aronia or other berries.

3.6 Acknowledgments

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3.7 Figures and Tables

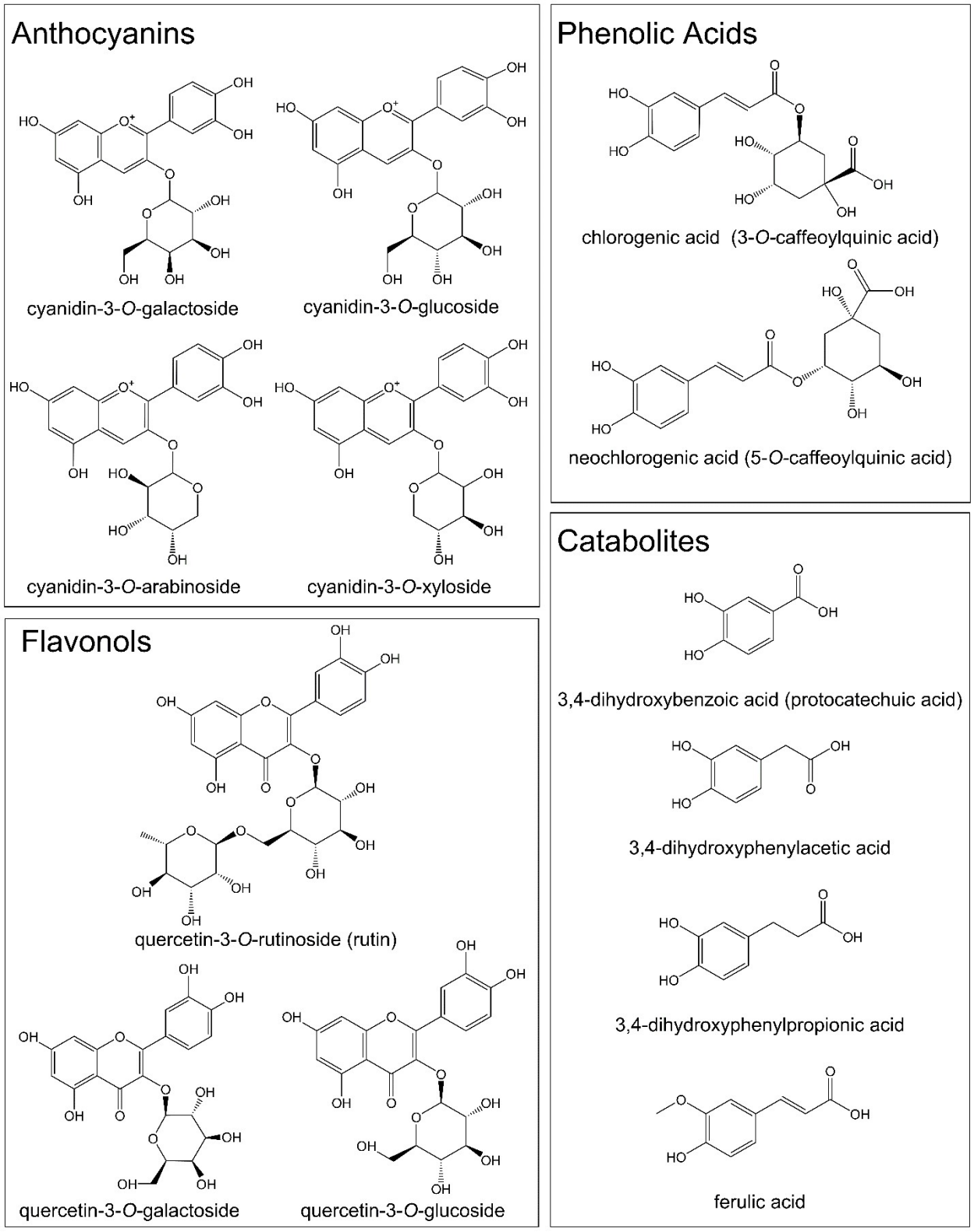

Figure 3.1. Major polyphenols reported in aronia berry and polyphenol catabolites utilized in the present study.

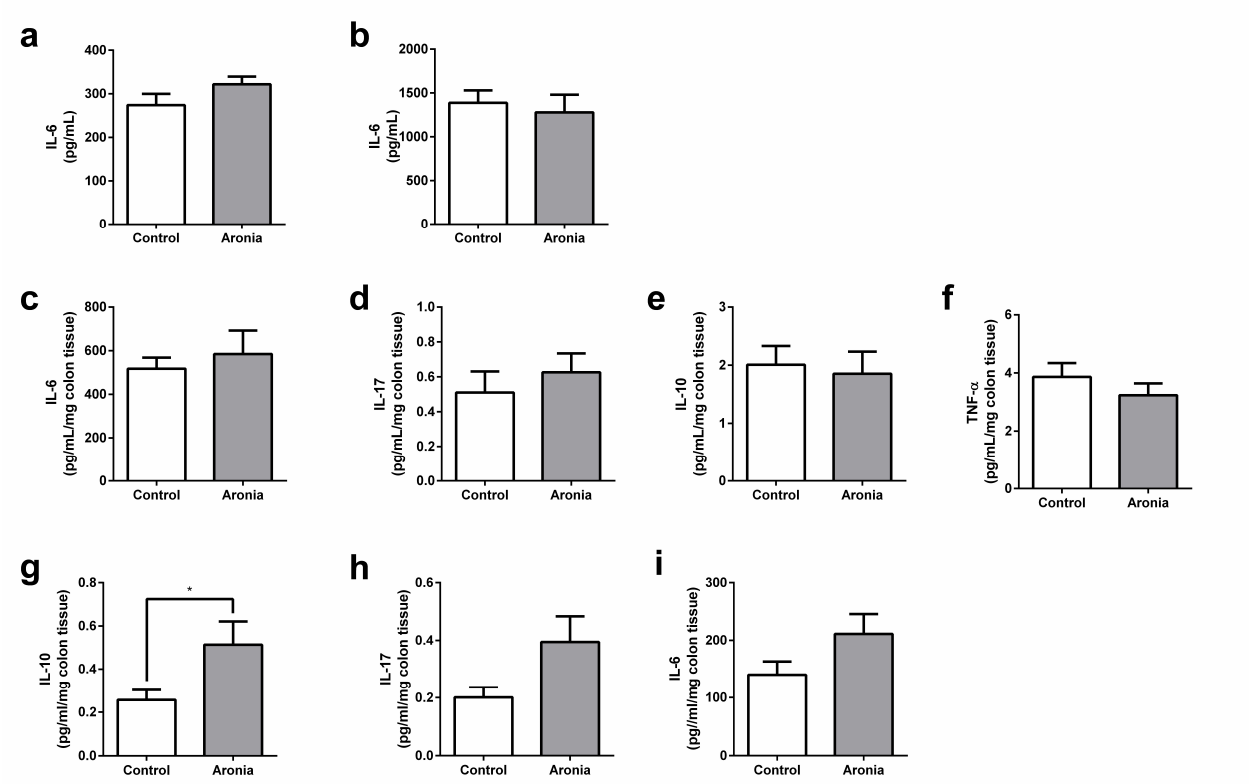

Figure 3.2. Ex vivo cytokine production by splenocytes and colon tissues of C57BL/6J mice fed control or 4.5% aronia supplemented diets for 4 weeks (n = 8 per group). **(a,b)** Interleukin (IL)-6 excretion by splenocytes after incubation with **(a)** 10 µg lipopolysaccharides (LPS)/mL for 12 h, or **(b)** 1 µg LPS/mL for 17 h. **(c-f)** Cytokine excretion from colon tissue after stimulation with 2 µg LPS/mL for 18.5 h, **(c)** IL-6, **(d)** IL-17, **(e)** IL-10, **(f)** Tumor necrosis factor (TNF)-α. **(g-i)** Cytokine excretion from colon tissue without stimulation **(g)** IL-10, **(h)** IL-17, **(i)** IL-6. Data represent means ± SEMs, n = 8/group. Statistical significance was determined by two-tailed student's T-tests. *Different between treatments, $P < 0.05$.

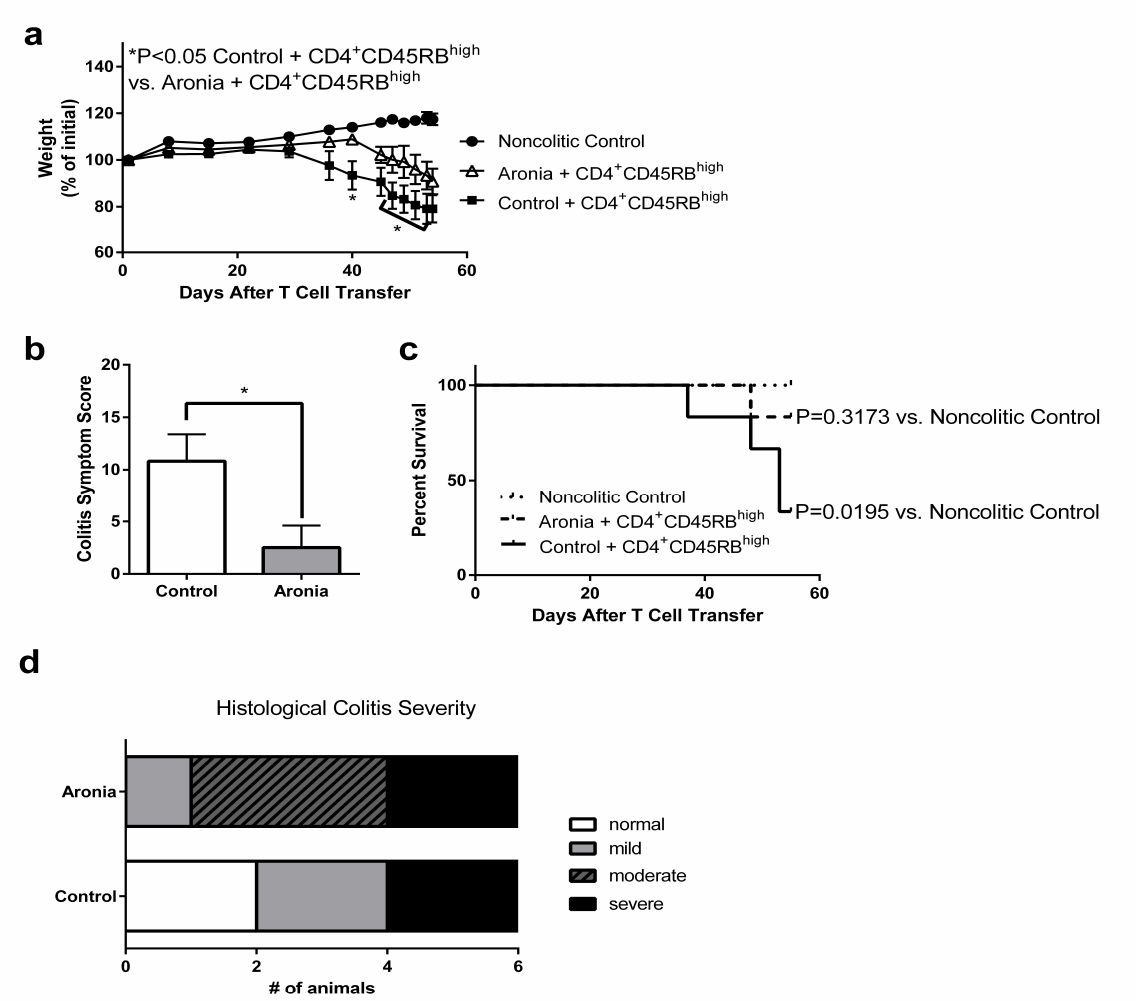

Figure 3.3. Effect of aronia berry feeding on weight loss and survival in the adoptive transfer model of colitis. $CD4^{+}CD45RB^{HI}$ cells from C57BL/6J mice were transferred by intraperitoneal injection into recombinase activating gene 1 $(Rag1)^{-/-}$ mice and mice were placed on control or 4.5% whole aronia berry diet. **(a)** Weight. Statistical significance determined by two-way repeated measures ANOVA followed by Tukey's test. Data represent means ± SEMs. n = 6 per group. *Different from colitic control, $P < 0.05$. **(b)** Colitis symptom score. Statistical significance determined by two-tailed student's T test. *Different between treatments, $P < 0.05$. **(c)** Survival curve. Statistical significance determined by Log-rank (Mantel-Cox) test, n = 6 per group. **(d)** Histological grading of colitis severity.

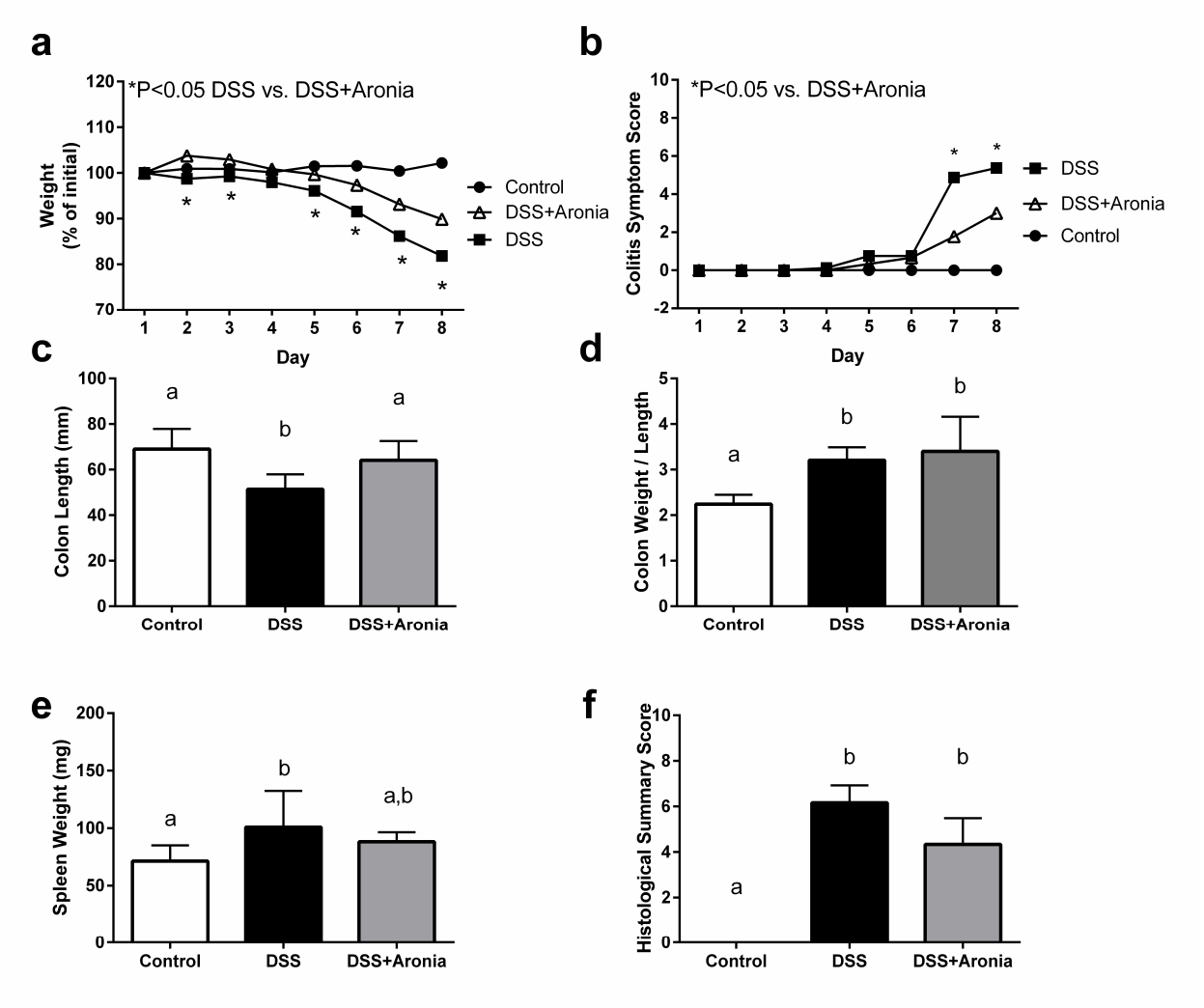

Figure 3.4. Effect of aronia feeding on indicators of colitis in dextran sulfate sodium (DSS)-induced colitis. Mice were fed either control diet or 4.5% whole aronia berry diet concurrent with 3% DSS in the drinking water for 1 week. **(a)** Weight. Statistical significance assessed by two-way repeated measures ANOVA followed by Tukey's test, n = 9 per group. *Different between DSS + aronia group, $P < 0.05$. **(b)** Colitis symptom score. Statistical significance assessed by two-way repeated measures ANOVA followed by Tukey's test, n = 9 per group. *Different from DSS + aronia group, $P < 0.05$. **(c)** Colon Length. n = 8-9 per group. **(d)** Colon weight/length ratio. n = 8-9 per group. **(e)** Spleen weight. n = 8-9 per group. **(f)** Histological Summary Score. n = 3 per group. For panels **c-f**: Data represent means ± SEMs. Statistical

significance determined by one-way ANOVA followed by Tukey's test. Bars bearing different letters are significantly different, $P < 0.05$.

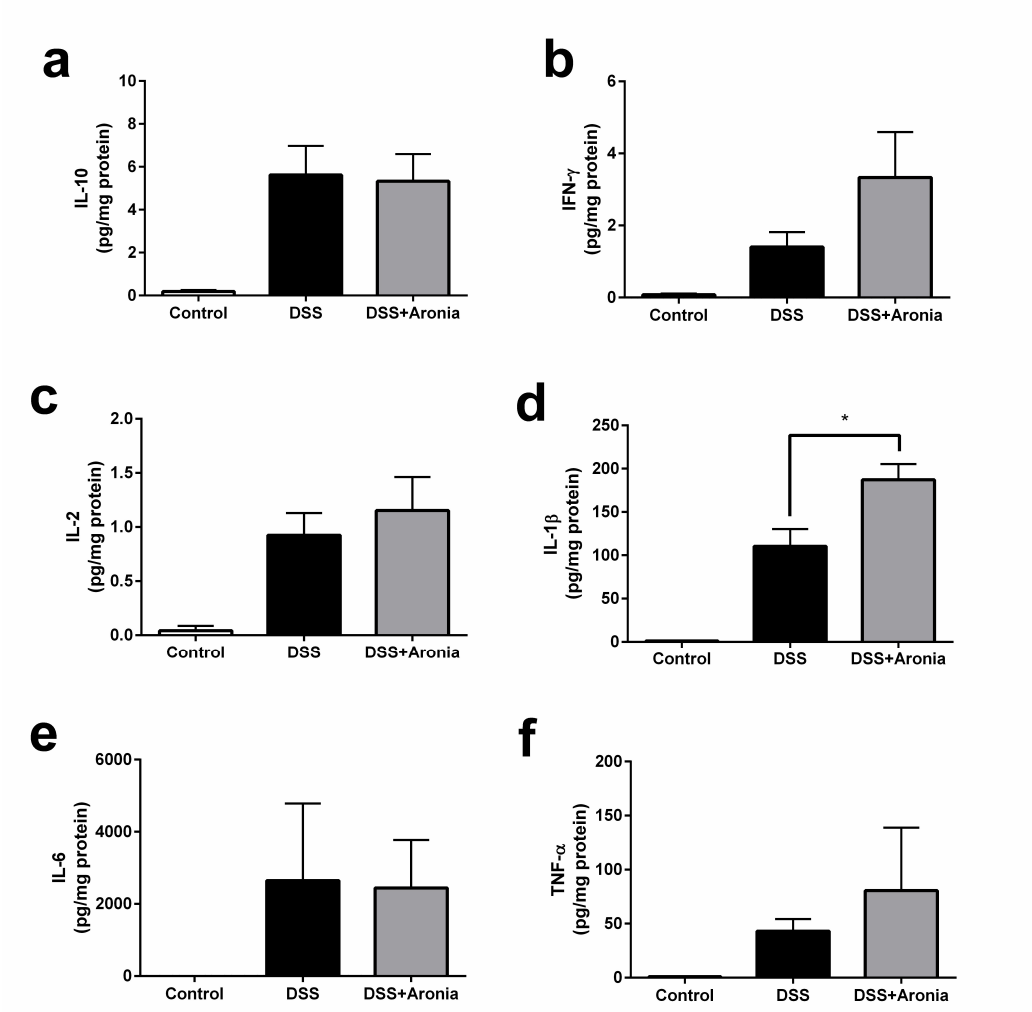

Figure 3.5. Effect of aronia feeding on colon tissue cytokines in dextran sulfate sodium (DSS)-induced colitis. Mice were fed either control diet or 4.5% whole aronia berry diet concurrent with 3% DSS in the drinking water for 1 week. Colon tissue cytokines were determined by Meso Scale Diagnostics multiplex immunoassay. **(a)** Interleukin (IL)-10, **(b)** Interferon, (IFN)-γ, **(c)** IL-1β, **(d)** IL-2, **(e)** IL-6, **(f)** Tumor necrosis factor (TNF)-α. Data represent means ± SEMs. Student's t-tests were used to determine statistical differences between DSS treatment groups. n = 3/group, *Different between treatments, $P < 0.05$.

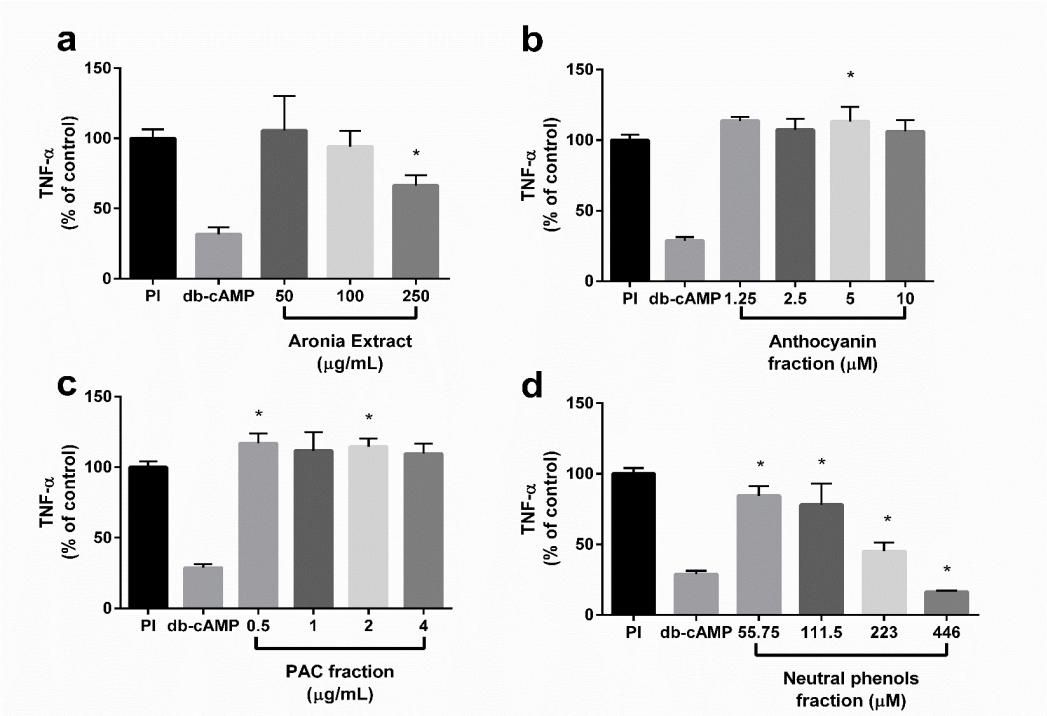

Figure 3.6. Inhibition of Jurkat T cell tumor necrosis factor (TNF)-α secretion by **(a)** Aronia extract, **(b)** Aronia anthocyanin fraction, **(c)** Proanthocyanidin (PAC) fraction, and **(d)** Aronia neutral phenols fraction, Jurkat T cells were simultaneously stimulated with phorbol 12-myristate 13-acetate and ionomycin (PI) and treated with 100μM dibutyryl-cAMP (db-cAMP), Aronia extracts, or aronia fractions for 4 hr. TNF-α production was determined by enzyme-linked immunosorbent assay (ELISA) and is expressed as a percentage of the control (PI), and the control response varied from 400-455 pg TNF-α/mL among experiments. Statistical significance determined by one-way ANOVA with Dunnett's multiple comparisons test, *Different from PI control, $P < 0.05$. Data represent means ± SEMs, n = 3-6. db-cAMP not included in statistical analyses, shown for comparison only.

Table 3.1

Effects of microbial polyphenol catabolites on Jurkat T cell tumor necrosis factor (TNF)-α production.[a]

Polyphenol	TNF-α, % of Control			
	0.1 μM	1 μM	10 μM	100 μM
3,4-dihydroxybenzoic acid	91.9 ± 1.7	90.7 ± 3.6	98.7 ± 1.8	100 ± 4
3,4-dihydroxyphenylacetic acid	79.4 ± 4.1*	88.9 ± 2.3	89.0 ± 2.3	95.1 ± 1.7
3,4-dihydroxyphenylpropionic acid	84.9 ± 1.6*	90.8 ± 1.0	93.6 ± 8.3	85.9 ± 2.8*
ferulic acid	87.7 ± 1.7	92.3 ± 0.7	102 ± 2	96.6 ± 2.5

[a]Jurkat T cells were simultaneously stimulated with phorbol 12-myristate 13-acetate and ionomycin (PI) and treated as indicated for 4 hr. TNF-α production was determined by enzyme-linked immunosorbent assay (ELISA) and is expressed as a percentage of the PI control. Statistical significance determined by one-way ANOVA with Dunnett's multiple comparisons test, *Different from PI control, $P < 0.05$. Data represent means ± SEMs of n = 3-6 biological replicates.

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Chapter 4

The anti-colitic activity of aronia polyphenols and fiber

4.1 Abstract

Aronia berries have polyphenols and fiber, which are both putatively anti-colitic. The objective of this study was to study the effects of aronia berry polyphenols, fiber, and whole aronia berries in dextran sulfate sodium (DSS)-induced colitis. Mice were prefed diets supplemented with aronia extract (AE), polyphenol depleted aronia powder (D), or whole aronia berries (AB) for 1 week. DSS was administered at 3.5% in the drinking water while the diets continued for another 7 days. Colonic cytokines were determined by electrochemiluminescence and intestinal permeability was determined by translocation of FITC-dextran to the plasma after oral gavage. Polyphenol content of the diet was determined by HPLC. Diets differed in both fiber and polyphenol content. No diet inhibited DSS-induced weight loss. There was transient improvement in colitis symptoms with AB, AE, and D. Histological damage induced by DSS was not improved by test diets. AB inhibited colonic tumor necrosis factor (TNF)-α, but no other cytokines were affected. DSS appeared to increase intestinal permeability, but no aronia based diet provided measurable improvement. The null results were unexpected, based upon the literature and the previous chapter, but may be related to a diminished response of the mice to DSS administration and/or source of the materials for the diet. Further work is needed to determine which component(s) of aronia berry confer anti-colitic activities.

4.2 Introduction

The Western diet is characterized by low intakes of fruits, vegetables, and fiber, which increases the risk of inflammatory bowel diseases (IBD) (1). Whole berries, fibers, berry extracts, and isolated polyphenols inhibit the severity of colitis in rodent models (2,3). However, the specific contributions of polyphenols, fibers, and non-extractable polyphenols (NEPs) to dietary inhibition of colitis have not been systematically investigated.

In many fruits and vegetables, polyphenols are distributed as extractable or non-extractable. NEPs are those polyphenols that are not extractable intact by organic solvents. NEPs are delivered to the colon intact because of their covalent or non-covalent associations with food matrix fibers and proteins (4). In contrast, extractable polyphenols are partially absorbed in the upper gastrointestinal tract and the remaining fraction reaches the colon and catabolized by the microbiota. For example, following anthocyanin-rich aronia berry extract consumption, ~96% of the increase in plasma polyphenols was from microbial polyphenol catabolites (5). Thus, the majority of ingested extractable aronia berry polyphenols are delivered to the colonic microbiota and degraded. The differing metabolic fate of extractable and non-extractable polyphenols suggests that anti-colitic activity may vary between these fractions. Furthermore, NEPs are closely associated with dietary fiber which is also anti-colitic (reviewed in (3)).

The immunology of IBD is complex and involves a number of cell types and cytokines that mediate inflammation (6). Furthermore, intestinal barrier function is compromised in inflammatory bowel disease (IBD) (7). Poor intestinal barrier function increases pro-inflammatory signaling in the gut and increases risk of IBD and other chronic diseases. Whole fruit and polyphenols improve barrier function in vitro (8) and in vivo (9–11). Improving

intestinal barrier function may be an important mechanism by which berries and other polyphenol rich foods contribute to colitis reduction in animal models and may point to why diets high in fruits and vegetables are protective against colitis.

The objective of this study was to evaluate anti-colitic activity of whole aronia berry, extractable aronia polyphenols, and NEPs and fiber using the DSS model of colitis. It was hypothesized that the anti-colitic effects of aronia would be mediated by improvement in intestinal barrier function. Identifying the aronia components that are most effective at inhibit IBD pathology could help aid the design of medical foods or dietary recommendations targeted at reducing IBD risk.

4.3 Materials and methods

4.3.1 Solvents and reagents

Whole aronia berry powder (Full Spectrum, Lot EK004095 K287/002/A14) and ethanolic aronia extract (Lot EK004255 C203/046/A14) was obtained from Naturex (South Hackensack, NJ). Cyanidin-3-galactoside and cyanidin-3-glucoside standards were purchased from Chromadex (Irvine, CA). Ethanol meeting USP specifications for polyphenol depletion of diet was obtained from Decon Labs (King of Prussia, PA). Emprove® acetone for polyphenol depletion of diet was obtained from EMD Millipore (Billerica, MA). FITC-Dextran was purchased from Millipore Sigma (FD4, MW 3000-5000, St. Louis, MO). Folin-Ciocalteu reagent and 4-dimethylaminocinnamaldehyde were purchased from Sigma Aldrich (St. Louis, MO). Gallic acid was purchased from Acros Organics (Morris Plains, NJ). (+)catechin was purchased from Cayman Chemical (Ann Arbor, MI). HPLC grade solvents and other reagents or chemicals

were purchased from Sigma-Alrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA).

4.3.2 Preparation of diets

For this study, diets consisted of the control diet (AIN-93M, Envigo Teklad, Madison, WI), whole aronia berry-supplemented diet (AB), aronia extract-supplemented diet (AE), and a diet depleted of extractable polyphenols from aronia berry diet (D) prepared in-house. For D, aronia berry powder extracted with acetone/water (70:30, v/v) 3 times for 24 h, and with ethanol/water (95:5, v/v) 5 times for 24 h. Depletion of extractable polyphenols was confirmed by the Folin assay for total polyphenols, the pH differential assay for anthocyanins, and the 4-dimethylaminocinnamaldehyde (DMAC) assay for proanthocyanidins (see below). AB and AE were calibrated to deliver equivalent extractable polyphenols, whereas D was calibrated to deliver non-extractable material equivalent to AB (**Supplemental Method 1**). At the expense of corn starch, aronia berry powder, depleted aronia powder, and aronia extract were incorporated at 4.5, 0.16, and 2.12 %, respectively to AB, AE, and D by Envigo Teklad. Fiber testing was conducted by Eurofins Analytical Laboratories (New Orleans, LA) using standard methods (AOAC Official Method 950.02, AOCS Official Method Ba 6-84) that determine crude fiber as the loss on incineration of the dried residue remaining after digestion of the sample with dilute sulfuric acid and dilute sodium hydroxide. Consumption of 4.5% whole aronia berries approximates a 70 kg human consuming ~1 cup of fresh aronia berries/d according to the method by Reagan-Shaw (12), as described in the previous chapter.

Depletion of total phenols in the whole berry powder serial extracts was determined by the Folin method. Briefly, 10 μL of serial extracts or a gallic acid in water standards were

incubated with 15 µL Folin-Ciocalteu reagent, 45 µL 20% Na_2CO_3, and 230 µL ultrapure water in a clear plastic 96-well flat-bottomed plate. Absorbance was measured at 765 nm in a spectrophotometer.

Depletion of extractable anthocyanins in the whole berry powder serial extracts was determined by the pH differential assay. Briefly, extracts were diluted in pH 1 0.025 M KCl or pH 4.5 0.4 M sodium acetate and pipetted into a clear plastic 96-well flat-bottomed plate. Absorbance was read at 520 and 700 nm. Anthocyanins were calculated by the following formula:

$$\frac{[(A_{520\ nm} - A_{700\ nm})pH\ 1 - (A_{520\ nm} - A_{700\ nm})pH\ 4.5]449.2\ x\ DF\ x\ 10^3}{26{,}900\ (molar\ extinction\ coefficient,\ L\ x\ mol^{-1})}$$

DF = dilution factor

Depletion of extractable proanthocyanidins in the whole berry powder serial extracts was determined by the DMAC assay. Briefly, extracts were diluted with a 72.8% ethanol solution in water. A (+)catechin standard curve was constructed with 91% ethanol. Seventy µL diluted extracts or catechins standards were pipetted into a clear plastic 96-well flat-bottomed plate. Then, 210 µL of 0.1% 4-dimethylaminocinnamaldehyde in acidified ethanol (91% ethanol, water, 37% HCl, 75:12.5:12.5 v/v/v) (DMAC) was pipetted into the wells. Absorbance at 640 nm was read in a 25 °C spectrophotometer 25 minutes after addition of 0.1% DMAC.

4.3.3 Mice and housing

C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were bred by the University of Wisconsin-Madison Research Animal Resource Center. Mice were transferred to the University of Wisconsin-Madison Department of Animal Sciences housing facility and acclimatized on chow diet for at least 1 week. At ~10 weeks of age, mice were fed

the four test diets (n = 14/group). After 1 week on the test diets, half of the animals were sacrificed as described below (n = 7/group, “prefeeding”). The remaining half were administered 3.5% DSS (13,14) (MP Biomedicals # 0216011001, MW: 36,000-50,000, Santa Ana, California) for 7 days and sacrificed as described below. Mice were weighed and monitored for external colitis symptoms daily according to a standardized scoring system (15).

4.3.4 Determination of polyphenols in test diets

Polyphenols were extracted from diets according to previously described methods (16). Briefly, powdered diets (40 g) were diluted in 160 mL of 70% acetone, 29.5% ultrapure water, and 0.5% acetic acid (v/v/v), sonicated for 5 min, and centrifuged at 950 × g for 10 min. The pellet was re-extracted twice, and the supernatant extracts were combined and dried at 40 °C in a rotary evaporator.

Fractions enriched in anthocyanins and proanthocyanidins were isolated from the dried extracts by Sephadex LH-20 chromatography (16). Briefly, dried extracts were reconstituted in 30% methanol in water and applied to a 2.5 cm diameter column with ~6 g of Sephadex LH-20 equilibrated in 30% methanol. Columns were first eluted with 50 mL each of 30% methanol in water to obtain an anthocyanin-rich fraction and proanthocyanidins were then eluted with 100 mL of 90% acetone in water. Both fractions were condensed by the rotary evaporator and stored at -20 °C prior to analysis.

Additionally, 0.5 g powdered diets were hydrolysed in 40 mL of 62.5% aqueous methanol with 2 mg/mL of tert-butylhydroquinone (TBHQ) and 10 ml of 6 M HCl at 90 °C for 2 h to release bound polyphenols (17). The suspensions were sonicated for 5 minutes and then centrifuged at 1,400 × g at 25 °C for 5 minutes. The supernatant was stored at -20 °C.

For HPLC analysis of polyphenols, reconstituted fractions or extracts were resolved using a Dionex Ultimate 3000 UHPLC (Thermo Scientific, Sunnyvale, CA) equipped with a temperature-controlled autosampler, a diode array detector, and a fluorescence detector as described in the previous chapter. For anthocyanin and flavonoid analysis, a Kinetex EVO C18 column (250 mm ×4.6 mm, 5 μm; Phenomenex, CA) and a gradient solvent system consisting of 5% formic acid in water (A) and methanol (B) were used for anthocyanin fractions (0 min, 5% B; 1 min, 55% B; 40 min, 35% B; 50 min, 95% B; 55 min, 95% B, 57 min, 5% B; flow rate 1 mL/ min). For proanthocyanidin analysis, the column was Lichrosorb Diol-5 (250 mm × 4 mm, 5 μm; Supelco, PA) and the gradient solvents were 2% acetic in acetonitrile (A) and 2% acetic and 3% water in MeOH (B) at a flow rate of 1 mL/min (0 min, 7% B; 3 min, 7% B; 60 min, 37.6% B; 63 min, 100% B; 70 min, 100% B, 76 min, 7% B). Proanthocyanidins were quantitated as (+)catechin equivalents by degree of polymerization (DP).

4.3.5 Assessment of intestinal permeability

Intestinal permeability was assessed by translocation of FITC-dextran in mice that were prefed test diets and after 1 week of DSS treatment. On the day of sacrifice, mice were deprived of food and water for 4 h and then gavaged with 60 mg/kg FITC-Dextran dissolved in phosphate buffered saline (PBS) (13). After 4 h, mice were anesthetized under isoflurane and blood was obtained by an axillary bleed. Blood was immediately transferred to heparinized blood collection tubes (Becton Dickinson, Franklin Lakes, NJ) and plasma was obtained by centrifugation at 1,500 × g for 15 min at 4 °C. Plasma was snap frozen in liquid nitrogen and stored at -80 °C.

Plasma samples were diluted with PBS and 100 μL was placed in a 96 well plate in duplicate. Fluorescence was measured with excitation at 490 nm and emission monitored at 520

nm in a Varioskan Flash (Thermo Fisher Scientific). A standard curve of 1.56 to 100 µg/mLwas constructed with FITC-Dextran dissolved in PBS.

4.3.6 Colon tissue histology and cytokines

Upon sacrifice, the large intestine was excised and feces were collected by manual expulsion. Colon tissue was weighed and then cut into thirds. The proximal third was Swiss rolled and placed into 10% neutral buffered formalin for 24 h. Colon tissues were then transferred to ethanol/water (70:30, v/v) and stored at 4 °C. Fixed colon tissues were processed to paraffin and then stained with hematoxylin and eosin by the University of Wisconsin Research Animal Resource Center Comparative Pathology Laboratory. Colitis severity was determined by a certified clinical pathologist.

The distal third of the colon was snap frozen in liquid nitrogen and stored at -80 °C. Colon tissue was weighed into bead (ceramic, 1.4 mm) homogenization tubes (Thermo Fisher Scientific) and homogenized in buffer (150 mM NaCl, 20mM Tris-pH 7.5, 1mM ethylenediaminetetraacetic acid (EDTA), 1mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 1% Triton-X-100, phosphatase inhibitors II and III and protease inhibitor (Sigma, St. Louis, MO). Homogenate was centrifuged at 12,000 × g for 15 minutes at 4 °C. The supernatant was transferred to a new tube and stored at -80 °C. Protein content of colon tissue homogenate was determined by the Pierce BCA assay (Thermo Fisher Scientific). Concentrations of interferon (IFN)-γ, interleukin (IL)-10, IL-17, IL-1β, tumor necrosis factor (TNF)-α, IL-23, and IL-6 in the supernatant were determined by electrochemiluminescence immunoassay using the Meso Scale Diagnostics multiplex assay (Rockville, MD).

4.3.7 Statistical analysis

Data are expressed as means ± SEMs. Body weights were expressed as a percentage of the animals' weight at the start of DSS administration. Time course data were analysed by repeated measures two-way ANOVA with Dunnett's multiple comparisons test, with $P < 0.05$ considered significant. Cross-sectional data (e.g. colon lengths, colon weight to length ratios, spleen weights, plasma FITC-dextran, histological scores, and colon cytokines) were analyzed by one-way ANOVA with Dunnett's multiple comparisons test, with $P < 0.05$ considered significant.

4.4 Results

4.4.1 Polyphenol composition of the test diets

Sequential extraction with acetone/water and aqueous ethanol depleted nearly all extractable polyphenols from whole aronia berry powder **(Figure 4.1**). A small amount of cyanidin (3.96%) recovered after acid hydrolysis and some polymeric proanthocyanidins were detected as NEPs in diet D (**Tables 4.1-4.3**). AE contained a total of ~625 nmol/g non-proanthocyanidin polyphenols, while AB contained ~733 nmol/g. AB contained 258% more anthocyanins and 46.5% less hydroxycinnamic acids than AE (**Table 4.2**). Considering proanthocyanidins, AB contained 71% more trimers, 424% more tetramers, 2570% more pentamers, 5211% more heptamers, and 806% more polymers than AE, with only dimers being similar between AB and AE (**Table 4.3**). Heptamers through 11-mers were not detected in AE. Only polymers were detected in D, having only 7.5% of AB and 68% of AE. Aronia berry

powder contained 19.4% fiber while polyphenol depleted berry powder contained 24.9% fiber (data not shown), which would yield 0.873% fiber in AB and 0.528% fiber in D.

4.4.2 Effect of aronia diets on DSS-induced colitis

None of the aronia-based diets protected from DSS-induced weight loss relative to the control (**Figure 4.2a**). Colitis symptoms were inhibited 83% by D after 4 days of DSS administration and by AB, AE, and D after 5 days of DSS administration. However, after 6 days, only AB and D inhibited symptoms, and at day 7 only AB inhibited symptoms (**Figure 4.2b**). At day 7, colon lengths, colon weight to length ratios, and spleen weights were similar among all DSS treated groups (**Figure 4.3a-c**). Although administration of DSS-induced histological signs of inflammation, there were no differences in total histological score between groups given DSS (**Figure 4.3d**). DSS administration significantly increased colonic IL-10 (5.5-fold), IL-17A (14-fold), IL-1β (3.5-fold), IL-23 (18%), and IL-6 (9.5-fold), but not IFN-γ (-55%, $P > 0.05$) (**Figure 4.4, Supplemental Table A.4.S7).** Consistent with day 7 symptom score, colonic TNF-α was inhibited by AB supplemented diet, but IFN-γ, IL-10, IL-17A, IL-1β, IL-23, and IL-6 were not affected by any diet (**Figure 4.4**).

4.4.3 Intestinal barrier function in healthy and DSS-treated mice

Translocation of FITC-dextran to the plasma was not different between dietary treatments in either the prefeeding portion or the DSS portion (**Figure 4.5**), although DSS nonsignificantly increased plasma FITC-dextran by about 20% across all diets.

4.5 Discussion

Consumption of a commercially-available whole aronia berry powder marginally improved external colitis symptoms and reduced colonic TNF-α after 7 d of 3.5% DSS treatment. In contrast, AE and D reduced external colitis symptoms marginally only at days 4 and 5 during DSS treatment. Aronia-based diets did not significantly affect other markers of colitis in this study. However, in a previous study by another group, cranberry, cranberry polyphenols or 0.7% cranberry fiber conferred protection against 1% DSS (18). Also, the present study contrasts the results of Chapter 3, where freeze-dried aronia powder consumption inhibited DSS colitis to a greater extent than AB. The magnitude of DSS-induced weight loss was also less than reported in Chapter 3. Some variables were different, such as less concentrated DSS (3%), and test diets were provided concurrently with DSS treatment. In the present study, DSS induced a significant change in histological findings and overall scores, indicating the induction of inflammation, but none of the diets affected the histological findings. The lot of DSS, the dietary change, and the source of the mice may have contributed to a diminished response to DSS treatment.

The reduction in colonic IFN-γ with the administration of DSS in the present study contrasts with the previous chapter where colonic IFN-γ increased. IFN-γ is a characteristic Th1 cytokine (6). Distinct microbial communities generate different susceptibilities and immune responses in DSS-induced colitis (19). The microbial community differences between the mice in Chapter 3 and this chapter could explain the variation in the response to DSS and the skewing of the immune system away from a Th1 response.

Other anthocyanin rich extracts have been efficacious in experimental IBD; thus it is not clear why this intervention was not. Oral administration of 100 mg/kg aronia extract in a DSS

mouse model protected against weight loss, colon shortening, and histological score (20). Food consumption peaked in this study at about 3g/mouse/day. Mice consuming AE would be consuming approximately 190 mg/kg/d aronia extract at this level of consumption, although food consumption drops during the course of DSS administration. In the aforementioned study (20), 10 mg/kg/d aronia extract inhibited colon shortening, but other measures were not affected. The effectiveness of aronia extract therefore appears to be dose-dependent and possibly administration route dependent.

TNF-α is an important cytokine driving the pathology of IBD (6) and is the target of a number of biologics used to treat IBD as well as other inflammatory disorders (21). The reduction in colonic TNF-α by AB suggests a modest efficacy by this diet. This marker should not be over-interpreted as there were no differences in other cytokines or colonic histopathology. Many other dietary interventions have been shown to reduce colonic and plasma TNF-α (reviewed in Chapter 2 and (2)). This mechanism may contribute to the prevention of IBD and plausibly the alleviation of IBD by diets high in fruits and vegetables.

Due to sample availability and analytical constraints, it was not possible to collect all of the FITC-dextran measurements at the same time, so the prefeeding group and the DSS treated group were analyzed separately. Plasma FITC-dextran in the prefeeding group was similar to levels reported previously for C57BL/6J mice (22). DSS administration increased intestinal permeability, but there were no differences in plasma FITC-dextran between any of the diets in either the prefeeding or the DSS portions of the experiment. This suggests that components of aronia had no effect on intestinal barrier function in the present study.

The differences in the anti-colitic activity of aronia between this study and those described in Chapter 3, are possibly due to compositional differences between the test material.

In our prior studies, we utilized fresh, whole aronia berries that were freeze dried, ground to powder, and incorporated into the diet. In the present study, we used commercially available whole aronia powder and a commercially available aronia extract. The aronia berry powder was obtained by drum drying, which may contribute to some changes in the composition of the berries due to thermal treatment (23). Indeed, the diets containing freeze-dried aronia berries used in the following chapter had nearly 3 times the amount (by moles) of total extractable non-proanthocyanindin polyphenols and ~1.4 times the total proanthocyanindins than the commercial whole aronia berry diet (**Tables 4.1-4.3, Supplemental Tables A.4.S4-A.4.S6)**. Anthocyanins in the commercial whole berry diet were almost 71 percent lower than the diet prepared from freeze dried berries, which is consistent with a previous study which showed that Saskatoon berries dried at 75 °C had an 85% reduction in anthocyanin content (24). Additionally, the variety/cultivar of the aronia berry used in the present study was unknown. Polyphenol content, especially anthocyanin content can vary widely between different varieties of aronia (16). Although the fiber content of the freeze dried berries was not determined, it is unlikely to be substantially different than the drum dried berry powder. The reduced dose of polyphenols in the berry powder may account for the minimal efficacy in this model. It is also plausible that aronia may effect a certain type of immune response, such as that generated in Chapter 3, as opposed to the immune response in the present study which appeared to be skewed away from Th1.

In conclusion, consumption of a commercial aronia berry conferred minimal protection in an acute DSS colitis mouse model. Aronia extract and polyphenol-depleted aronia fiber likewise conferred minimal protection against external colitis symptoms and no protection against colonic inflammation. Furthermore, test diets did not improve intestinal barrier function diets after 1 week prefeeding or treatment with DSS. Given these results, it is apparent that the anti-colitic

activity of aronia berry depends on test material and colitis model. Therefore, further studies should carefully evaluate doses and sources of intervention diets, timing, and method of colitis induction.

4.6 Acknowledgments

Beth Gray at the University of Wisconsin-Madison Research Animal Research Center (RARC) did histology slide preparation. Annette Gendron-Fitzpatrick of the RARC did histology interpretation and scoring. Jiyuan Liu obtained the polyphenol composition data of the aronia powders, extracts, and diets. Jinchao Li from Zhenhua Liu's lab at the University of Massachusetts-Amherst performed the MSD analysis of colon cytokines.

4.7 Figures and Tables

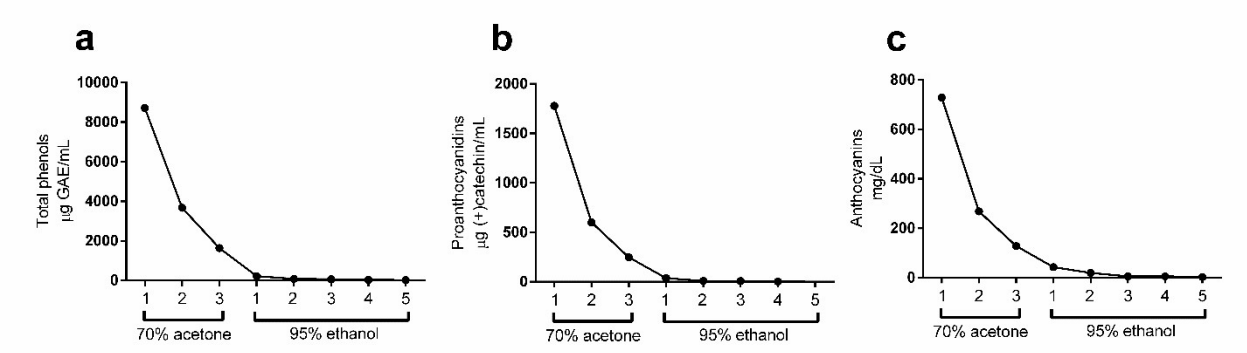

Figure 4.1. Whole aronia berry powder was serially extracted with acetone/water (70:30, v/v) 3 times for 24 h, and with ethanol/water (95:5, v/v) 5 times for 24 h. **(a)** Determination of total phenols in extracts by the Folin assay **(b)** Determination of proanthocyanidins in extracts by DMAC assay **(c)** Determination of anthocyanins by the pH differential method

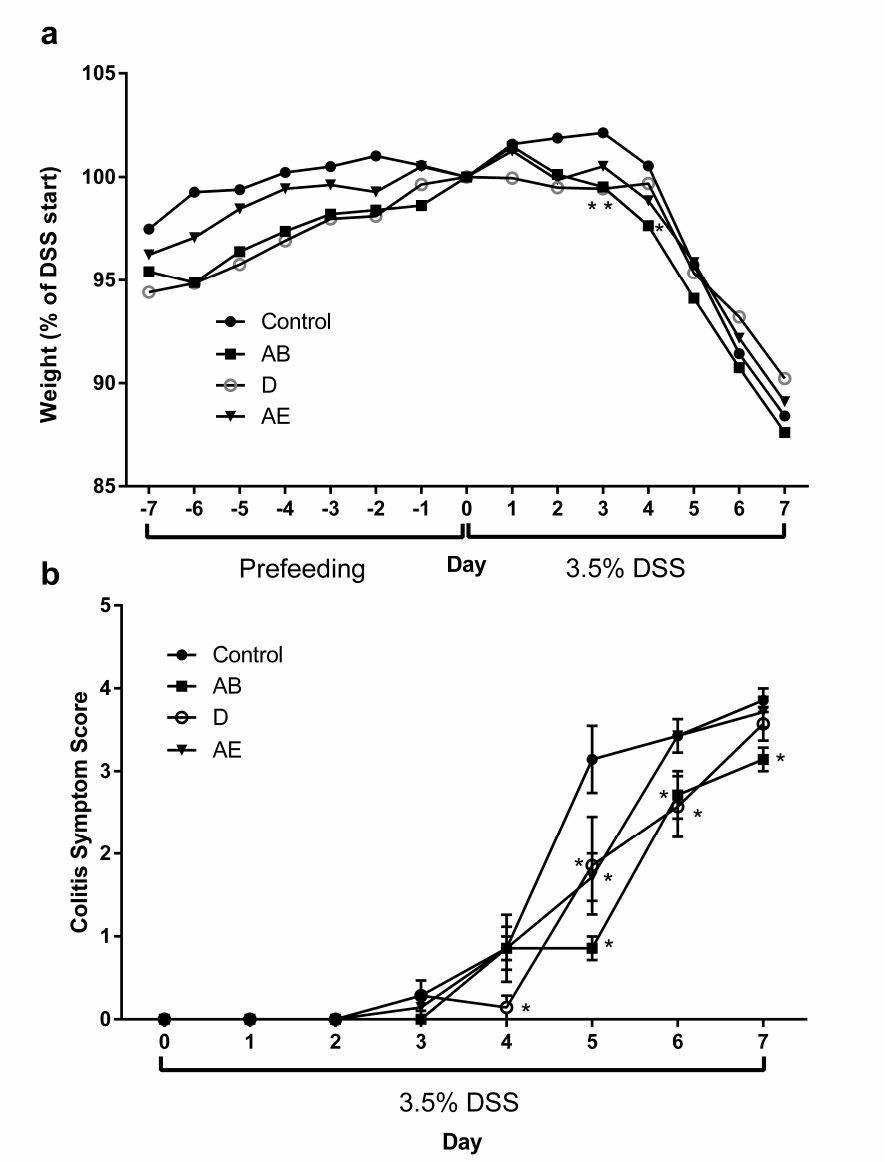

Figure 4.2. C57BL/6J mice were fed a control diet, 4.5% whole aronia berry powder diet (AB), 2.12% polyphenol depleted aronia berry powder diet (D), or 0.16% aronia extract diet (AE). Mice were given 3.5% dextran sulfate sodium (DSS) in the drinking water for 1 week, 1 week after the start of the diet **(a)** Weight. Analyzed by repeated measures two-way ANOVA with Dunnett's multiple comparisons test (n = 7/group) **(b)** Colitis symptoms were observed on a daily basis. Analyzed by repeated measures two-way ANOVA with Dunnett's multiple comparisons test. Data are expressed as means ± SEMs

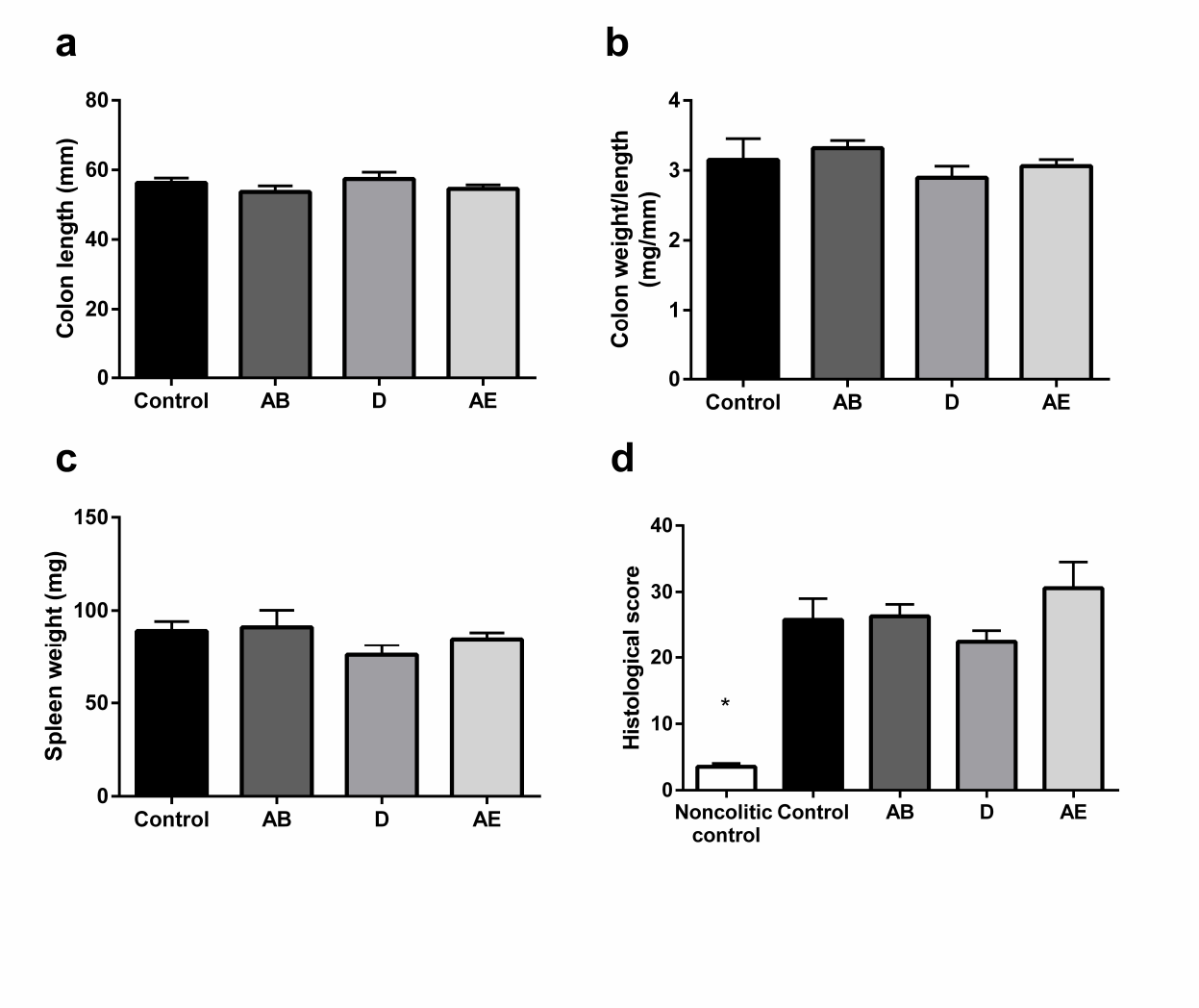

Figure 4.3. C57BL/6J mice were fed a control diet, 4.5% whole aronia berry powder diet (AB), 2.12% polyphenol depleted aronia berry powder diet (D), or 0.16% aronia extract diet (AE). Mice were given 3.5% dextran sulfate sodium (DSS) in the drinking water for 1 week, 1 week after the start of the diet. **(a)** Colon length (n = 7/group) **(b)** Colon weight:length ratio(n = 7/group) **(c)** Spleen weight (n = 7/group) **(d)** Fixed colon tissues were stained with hematoxylin and eosin and were assessed by a pathologist. (n = 7/group) **a-d** analyzed by one-way ANOVA with Dunnett's multiple comparisons test. *P < 0.05 vs. Control. Data are expressed as means ± SEMs

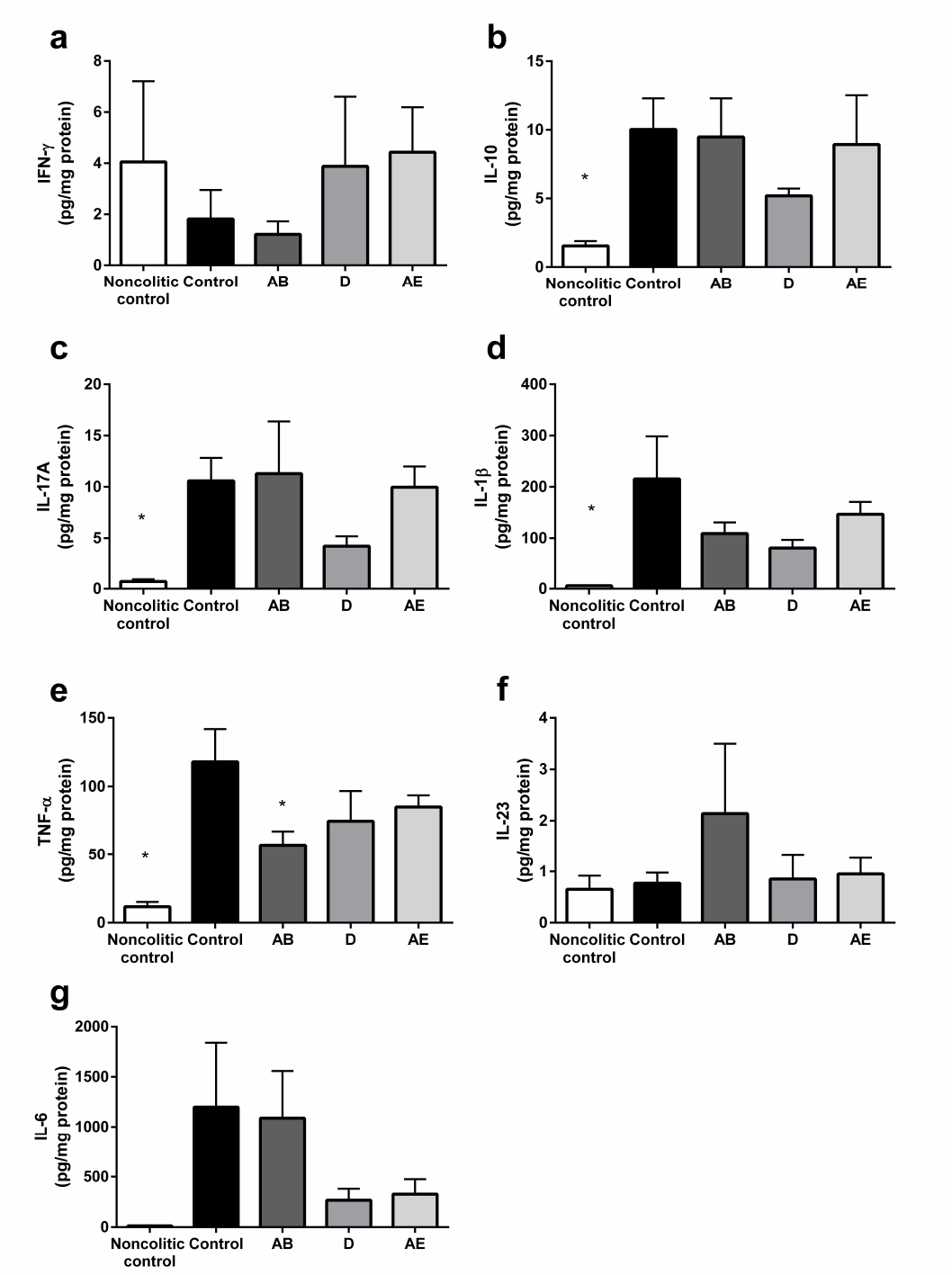

Figure 4.4. C57BL/6J mice were fed a control diet, 4.5% whole aronia berry powder diet (AB), 2.12% polyphenol depleted aronia berry powder diet (D), or 0.16% aronia extract diet (AE). Mice were given 3.5% dextran sulfate sodium (DSS) in the drinking water for 1 week, 1 week after the start of the diet. Cytokine concentration in the colon tissue was determined by

electrochemiluminescence immunoassay using the Meso Scale Diagnostics multiplex assay. Protein content of colon tissue was determined by the Pierce BCA assay **(a)** interferon (IFN)-γ **(b)** interleukin (IL)-10 **(c)** IL-17A **(d)** IL-1β **(e)** tumor necrosis factor (TNF)-α **(f)** IL-23 **(g)** IL-6. Statistical differences assessed by one-way ANOVA followed with Dunnett's multiple comparisons test. n = 7/group. *$P < 0.05$ vs. Control. Data are expressed as means ± SEMs.

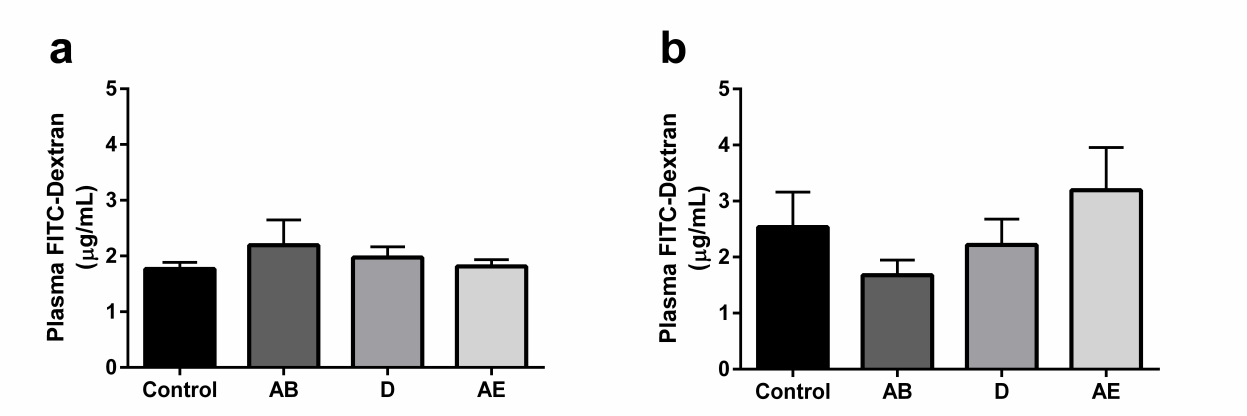

Figure 4.5. C57BL/6J mice were fed a control diet, 4.5% whole aronia berry powder diet (AB), 2.12% polyphenol depleted aronia berry powder diet (D), or 0.16% aronia extract diet (AE). Mice were given 3.5% dextran sulfate sodium (DSS) in the drinking water for 1 week, 1 week after the start of the diet. Mice were starved for 4 h, administered 60 mg/kg FITC-dextran by oral gavage, and then sacrificed 4 h later. Fluorescence of FITC-dextran was measured in plasma with excitation at 490 nm and emission monitored at 520 nm at the end of the prefeeding portion (n = 7/group) **(a)** and at the end of DSS administration (n = 6-7/group) **(b)**. Data are expressed as means ± SEMs

Table 4.1. Polyphenol content of diets (nmol/g) determined after acid hydrolysis in methanol.

Polyphenol	4.5% Full Spectrum aronia berry (AB)	2.2 % Polyphenol-depleted diet (D)	0.16% Aronia extract (AE)
Caffeic acid	<LOD	<LOD	<LOD
Cyanidin-3-O-galactoside	<LOD	<LOD	<LOD
Cyanidin	356 ± 87	14.1 ± 7.7	100 ± 33
Quercetin	21.3 ± 3.2	<LOD	16.1 ± 1.1

Data are means ± SD, n = 3, LOD = limit of detection

Table 4.2. Polyphenol content of diets (nmol/g) determined after extraction with acetone/water/acetic acid (70:29.5:0.5, v/v/v).

Polyphenol	**4.5% Full Spectrum aronia berry (AB)**	**2.2 % Polyphenol-depleted diet (D)**	**0.16% Aronia extract (AE)**
Protocatechuic acid	109 ± 0.9	<LOD	118 ± 3
Chlorogenic acid	88 ± 0.5	<LOD	163 ± 4
Caffeic acid	119 ± 4	<LOD	182 ± 2
Cy3Gal	305 ± 10	<LOD	116 ± 2
Cy3Glu	10.5 ± 0.7	<LOD	4.77 ± 0.16
Cy3A	90.9 ± 12.5	<LOD	37.8 ± 7.8
Cy3X	7.38 ± 3.06	<LOD	2.06 ± 0.26
Q3Glu	0.57 ± 0.18	<LOD	<LOD
Rutin	2.84 ± 0.74	<LOD	1.66
Q3Gal	<LOD	<LOD	<LOD
Quercetin	<LOD	<LOD	<LOD

Data are means ± SD, n = 3, LOD = limit of detection

Table 4.3. Proanthocyanidin content of diets (nmol catechin equivalents/g) determined after extraction with acetone/water/acetic acid (70:29.5:0.5, v/v/v).

Proanthocyanidin	**4.5% Full Spectrum aronia berry (AB)**	**2.2 % Polyphenol-depleted diet (D)**	**0.16% Aronia extract (AE)**
Monomers	ND	<LOD	<LOD
Dimers	7.79 ± 0.23	<LOD	7.59 ± 2.35
Trimers	5.38 ± 0.04	<LOD	3.14 ± 0.03
Tetramers	4.35 ± 0.11	<LOD	0.83 ± 0.10
Pentamers	4.54 ± 1.17	<LOD	0.17 ± 0.03
Hexamers	4.78 ± 0.09	<LOD	0.09 ± 0.003
Heptamers	3.92 ± 0.13	<LOD	<LOD
Octamers	3.65 ± 0.16	<LOD	<LOD
Nonamers	2.82 ± 0.09	<LOD	<LOD
Decamers	2.06 ± 0.04	<LOD	<LOD
Undecamers	1.45 ± 0.08	<LOD	<LOD
Polymers	174 ± 7	13.1 ± 3.8	19.2 ± 3.5

Data are means ± SD, n = 3, LOD = limit of detection

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Chapter 5

Aronia berry modulates cytokines and the Th17/Treg axis in a T cell IL-10 dependent manner in adoptive transfer colitis

5.1 Abstract

Increased fruit consumption is associated with reduced risk of colitis. However, little is known about how fruit polyphenols affect T cell homeostasis. We hypothesized the anti-colitic effects of the polyphenol-rich aronia berry (*Aronia mitschurinii* 'Viking') were mediated through Th17 and Treg. Colitis was induced in recombinase activating gene-1 deficient mice injected with syngeneic $CD4^+CD62L^+$ naïve T cells. Mice consumed either a 4.5% w/w aronia berry-supplemented or a control diet concurrent with T cell transfer. Aronia consumption prevented colitic weight loss induced by naïve T cells and reduced colon weight/length ratios and colon myeloperoxidase activity relative to the control diet. Compared to the control, aronia-fed mice had increased proportions of mesenteric lymph node (MLN) Tregs from 3-7 weeks after transfer. Similarly, at 5 weeks after transfer lamina propria and spleen Th17 IL-10^+ and IL-22^+ were also increased proportionally in the aronia-fed group. 3 weeks after transfer, increased MLN Treg and inhibition of multiple cytokines was dependent on T cell IL-10. The immunomodulatory effect of aronia consumption was concurrent with increased diversity of microbiota. Thus, aronia berry consumption modulates immunity by increasing proportions of Th17 and Treg in a T cell IL-10 dependent manner.

5.2 Introduction

The increasing global prevalence of inflammatory bowel disease (IBD) has been partly attributed to poor diet quality (1). Inadequate consumption of fruits and vegetables increases the risk of many chronic diseases. Similarly, epidemiological evidence suggests that increased fruit, vegetable, or polyphenol consumption may reduce the risk of IBD (2,3). The genetic and environmental risk factors that contribute to IBD risk are complex, but T cell dysfunction appears to be a significant mechanism responsible for initiation and pathology of IBD (4).

Fruits, vegetables, and certain isolated plant constituents inhibit colitis in preclinical models through a variety of mechanisms. These include maintenance of intestinal barrier function, inhibition of cytokines, modulation of gut microbiota, and inhibition of pro-inflammatory transcription factors, as reviewed elsewhere (5–7). In an open-label trial, bilberry consumption inhibited disease activity scores and modulated colonic cytokine expression in individuals with moderate ulcerative colitis (8,9).

Less is known about how consumption of fruits, vegetables, and their components affect T cell function in colitis. T cells are required for the anti-colitic activity of apple polyphenols in mice (10). Regulatory T (Treg) cells could be a potential target for dietary modulation of colitis, as green tea polyphenol epigallocatechin gallate (EGCG) induced forkhead box P3 (FoxP3) expression and Treg fequency in vitro (11). Dietary fiber intake reduced symptoms of dextran sulfate sodium (DSS)-induced colitis in mice and increased T helper (Th)17/Treg ratios in lymphocytes from mesenteric lymph node (MLN) tissue (12).

The aronia berry is rich in cyanidin-type anthocyanin polyphenols (13). Cyanidin and cyandin-glycosides inhibit Th17 signaling by binding directly to interleukin-17 receptor A (IL-17RA) (14). Aronia polyphenols inhibited T cell interleukin-6 (IL-6) and tumor necrosis factor-

alpha (TNF-α) release in vitro (15,16). Furthermore, consumption of whole aronia berry increased colonic excretion of interleukin-10 (IL-10) in healthy mice and inhibited wasting associated with DSS and T cell-transfer colitis (16). However, the mechanisms responsible for this inhibition are not known. In this study, we present evidence that aronia berry consumption reduces inflammatory Th17 differentiation and increases Treg in the adoptive transfer model of colitis, and these effects were associated with increased cecal microbiota diversity and dependent on T cell IL-10 3 weeks after T cell transfer.

5.3 Materials and Methods

5.3.1 Reagents amd Antibodies

Ethylenediaminetetraacetic acid (EDTA), phosphate buffered saline (PBS), ammonium chloride, potassium carbonate, acetone, acetic acid, methanol, acetonitrile, and were purchased from Fisher Scientific (Waltham, MA). Collagenase D, DNase I, dispase II, phosphatase inhibitor I, phosphatase inhibitor II, and protease inhibitor were purchased from Sigma-Aldrich (St. Louis, MO). DL-Dithiothreitol (DTT) was purchased from Dot Scientific (Burton, MI). Dulbecco's phosphate buffered saline (DPBS), minimum essential medium (MEM), and fetal calf serum (FCS) were purchased from Thermo Scientific Hyclone (Waltham, MA). Antibiotic/antimycotic, L-glutamine, amino acids, and sodium pyruvate were purchased from Gibco (Life Technologies, Carlsbad, CA). Phorbol-12-myristate-13-acetate (PMA) was from Calbiochem (Gibbstown, NJ). Ionomycin was from Invitrogen (Carlsbad, CA). Brefeldin A (Golgi Plug^{TM}) and anti-CD16/CD32 were both from BD Biosciences (San Jose, CA). Ghost

Dye™ Red 780 was purchased from Tonbo Biosciences (San Diego, CA). FoxP3/transcription factor staining buffer set was purchased from eBioscience (San Diego, CA).

Monoclonal antibodies were used for flow cytometry. PE-CF594 conjugated anti-CD3 (clone: 145-2C11), BUV737 conjugated anti-CD4 (clone: GK1.5), BB 515 conjugated anti-CD25 (clone: PC61), and Brilliant Violet 510 conjugated anti-TNF-α (clone: MP6-XT22) were purchased from BD Biosciences (San Jose, CA). Brilliant Violet 785 conjugated anti-interferon (IFN)-γ (clone: XMG1.2), APC conjugated anti-IL-17A (clone: TC11-18H10.1), PerCP-Cy5.5 conjugated anti-IL-10 (clone: JES5-16E3), and PE conjugated anti-IL-22 (clone: Poly5164) were purchased from BioLegend (San Diego, CA). PE-Cy7 conjugated anti-FoxP3 (clone: FJK-16s) was purchased from eBioscience (San Diego, CA).

5.3.2 Induction of adoptive transfer colitis and experimental design

Mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and the University of Wisconsin-Madison Research Animal Resource Center maintained colonies. Mice were housed under controlled environmental conditions with a 12-h light-dark cycle at the University of Wisconsin-Madison. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison.

Colitis was induced by adoptive transfer as previously described (17). C57BL/6J mice were donors for recipient C57BL/6J-background recombinase activating gene (*Rag)1*$^{-/-}$ mice. Briefly, donor mice were euthanized at 6-8 wk old and spleens were dissected. The $CD4^+CD62L^+$ naïve T cells were isolated from splenocytes using the $CD4^+CD62L^+$ naïve T cell isolation kit (Cat # 130-106-643, Miltenyi Biotec Inc., San Diego, CA) following the manufacturer's protocol. Then, colitis was induced in *Rag1*$^{-/-}$ mice by transferring 5×10^5

purified $CD4^+CD62L^+$ naïve T cells intraperitoneally from gender and age-matched donor mice. Another group of *Rag1*$^{-/-}$ mice was administered sterile PBS, serving as the non-colitic control (sham, n = 21). *Rag1*$^{-/-}$ mice reconstituted with $CD4^+$ T cells ($CD4^+$ T Cell Isolation Kit, Miltenyi Biotec) from C57BL/6J mice were included as a non-colitic control (18) at the 3 wk timepoint. Also at the 3 wk time point, both C57BL/6J and *Il10*$^{-/-}$ mice served as donors of purified $CD4^+CD62L^+$ naïve T cells. After the transfer, the mice were randomly assigned to either the AIN-93M diets (control, n = 21) or a modified AIN-93M diet supplemented with 4.5% lyophilized "Viking" aronia berry powder (Bellbrook Berry Farm, Brooklyn, WI) at the expense of corn starch (aronia, n = 21) (Envigo Teklad, Madison, WI). This dose is equivalent to a 70 kg adult consuming ~ 1 cup of fresh aronia berries per day (19). The aronia-supplemented diet contained 847.4 ± 53.9 nmol phenolic acids, 1439.7 ± 250.0 nmol anthocyanins and 233.3 ± 17.4 nmol proanthocyanidins per gram of diet (**Supplemental Tables A.4.S4-A.4.S6)** Food intake and body weights of mice were recorded for the duration of the experiment. In addition, mice were periodically inspected for outward colitis symptoms based on a previously developed instrument (20). Mice were euthanized 3, 5, or 7 weeks after the transfer.

5.3.3 Cell isolation from splenocytes, mesenteric lymph nodes (MLN), and lamina propria (LP)

Splenocytes were obtained as previously described (15). Briefly, spleens were removed and passed through a 70 μm cell strainer. Cells were pelleted in MEM by centrifuging at 400 × g for 5 min at 4 ºC and resuspended in 2 mL MEM. Then, 5 mL 0.15 M ammonium chloride with 10 mM potassium carbonate were added to lyse the red blood cells. After 5 min at room temperature, 30 mL DPBS was added. Then, splenocytes were washed twice with PBS and

pelleted. MLNs were extracted and dissected mechanically, and a single-cell suspension was prepared like the isolation of splenocytes.

LP lymphocytes were isolated as previously described (21). Briefly, the colon was flushed with ice-cold PBS, opened longitudinally, and cut into small pieces. The pieces were incubated with calcium- and magnesium-free HBSS supplemented with 1 mM DTT and 5 mM EDTA at 37 ºC for 20 min under slow rotation to remove epithelial cells. This process was repeated twice. After passing through a 100 μm cell strainer, the remaining tissues were then digested with PBS containing 0.5 mg/mL collagenase D, 0.5 mg/mL DNase I, and 3 mg/mL dispase for 20 min at 37 ºC under slow rotation. The released cells were passed through a 40 μm cell strainer. The digestion was repeated twice. The isolated cells were pooled together for flow cytometry. Isolated cells were counted by a Scepter™ 2.0 cell counter (EMD Millipore, Billerica, MA).

5.3.4 Flow cytometry

Splenocytes, MLN, and LP were cultured with PMA (50 ng/mL)/ ionomycin (1 μg/mL) (PI) and 5 μg/mL Brefeldin A. Cells were incubated at 37 ºC w/ 5% CO_2 for 5 h and washed twice with MEM following incubation. Nonspecific binding was blocked using anti-CD16/CD32 and cells were surface stained with Ghost Dye™ Red 780. Then, cell surfaces were stained with anti-CD3, anti-CD4, and anti-CD25. Cell permeabilization and fixation were achieved by using the FoxP3/transcription factor staining buffer set according to manufacturer's instructions. Then cells were stained intracellularly with anti-TNF-α, anti-IFN-γ, anti-IL-17A, anti-IL-10, anti-IL-22, and anti-FoxP3. Data were acquired on a BD LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA), with data analyzed with FlowJo v10.1 software (Tree Star, Ashland, OR).

5.3.5 Histopathology and immunohistochemistry (IHC)

Histopathology and IHC were used to assess colonic inflammation. Harvested colons were flushed with ice-cold PBS and blotted dry. Then, colons were Swiss-rolled, fixed in formalin, paraffin embedded, sectioned, and stained at the UW-Madison Comparative Pathology Lab. The hematoxylin and eosin stained slides were graded by an expert pathologist in a blinded manner. Based on the severity of inflammation, samples were graded 0 for unaffected, 1 for mildly affected, 2 for moderately affected, and 3 for severely affected. Then the grades were multiplied by the percent area of the colon affected and then added together for a final score.

For IHC staining, each slide was firstly double-stained with either rabbit monoclonal anti-mouse CD4 (Cat # ab183685, Abcam, Cambridge, MA) and rat monoclonal anti-mouse FoxP3 (Cat # 14-5773-82, Invitrogen, Carlsbad, CA), or rabbit monoclonal anti-mouse CD4 (Cat # ab183685, Abcam, Cambridge, MA) and rat monoclonal anti-mouse retinoic acid receptor-related orphan receptor (ROR)γt antibody (Cat # 14-6981-80, Thermo Fisher Scientific, Waltham, MA). Then, each slide was stained by secondary antibodies of goat anti-rabbit Alexa Fluor 488 (Cat # A-11008, Invitrogen, Carlsbad, CA) and goat anti-rat Alexa Fluor 594 (Cat # A-11007, Invitrogen, Carlsbad, CA). Finally, each slide was counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Cat # D1306, Invitrogen, Carlsbad, CA). Visualization was performed on a fluorescent microscope (Nikon, Eclipse Ti, Melville, NY). Images were captured at the proximal, middle, and distal section of colon at 200× magnification. The image format was TIFF with a resolution of 1,600 × 1,200 pixels. Cell count was performed on ImageJ V1.5 which was a Java image processing and analysis program based on NIH Image (http://imagej.nih.gov/ij/docs/index.html). Briefly, the cell counting consisted of binary image conversion, rolling ball background subtraction, threshold adjustment, watershed segmentation,

and the analyze particles tool. A macro was created by recording the commands to enable continuous and automated analysis (22).

5.3.6 Myeloperoxidase (MPO) activity and colon weight/length ratio

MPO activity and colon weight/length ratio were determined as secondary markers of inflammation. MPO activity was measured by a commercial colorimetric assay kit (Cat # MAK068, Sigma-Aldrich). Briefly, ~ 50 mg of colon tissue was homogenized with 400 μL of the provided buffer and the supernatant was collected. The supernatant was aliquoted into two vials, with one for MPO activity measurement according the manufacturer's instruction, and the other for protein concentration measurement by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Colon length and weight were recorded after flushing colons with ice-cold PBS and blotting dry.

5.3.7 Multiplex cytokine analysis

After cleanup, 10-20 mg of colon tissues were mixed with 250 μL of Tris lysis buffer in the presence of phosphatase inhibitor I, phosphatase inhibitor II, and protease inhibitor. The mixture was homogenized with 1.4 mm ceramic beads in a Fisher Scientific™ Bead Mill 4 homogenizer. After incubation on ice for 30 min, the homogenized samples were centrifuged at 12,000 × g for 30 min at 4 ºC. The supernatant was collected for measuring cytokine and protein concentration. Colonic cytokines were measured with a customized multiplex cytokine panel that included IL-17A, IL-23, IFN-γ, TNF-α, IL-1β, IL-6, IL-10, IL-2, and IL-22 on the QuickPlex SQ 120 imager (Meso Scale Discovery, Rockville, MD). Total protein concentrations were

determined using a commercially available Pierce™ BCA Protein Assay Kit on a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA).

5.3.8 Cecal material collection and bacterial 16S rRNA analysis

Cecal material was collected from the mice shortly after sacrifice at wk 3, transferred to sterile microcentrifuge tubes, and flash frozen in liquid nitrogen. The samples were then stored at -80 °C until processing. DNA was then extracted from the cecal material using a slightly modified method than previously described (23). The Illumina MiSeq platform (paired end, 2x250-bp) was used to sequence the extracted genomic material. Sequence processing and clean-up was performed using mothur v1.39.5 (24) and a previously defined protocol current as of February 16, 2018 (25). Briefly, contigs were formed from paired-end duplex sequence reads. Sequences with ambiguous bases, longer than 300-bp, and homopolymers greater than 8-bp were removed prior to aligning them to the SILVA 16S rRNA gene reference alignment database. Chimeric sequences were then removed using the UChime algorithm (26). Any sequences classified as Archaea, Eukaryota or that did not classify to the Kingdom level were culled from the data set. Taxonomic classification was assigned by clustering sequences into operational taxonomic units (OTUs) using the opticlust algorithm and a 3% dissimilarity level. These OTUs were assigned the lowest possible taxonomic classification from the GreenGenes reference database (27) using a naïve Bayesian classification based on the RDP classifier, requiring an 80% pseudo bootstrap confidence score (24,28).

All differential abundance statistical analysis calculations were performed on data normalized to the lowest number of reads in a sample, which was 21,408. Richness (Chao1, ACE) and alpha diversity (Shannon, Inverse Simpson) of the samples were calculated in mothur

by performing 1000 iterations at a 97% confidence level. Normalcy of the frequency in each of the indices was determined using the Shapiro-Wilk test. ANOVA analysis with Tukey HSD (normal distribution) or Kruskal-Wallis and Wilcoxon rank sum tests (non-normal distribution) were used to determine the significance of the differences. Beta diversity was calculated in R v3.4.3 utilizing the *vegan, ape, and phyloseq* packages. Estimates were made using Bray-Curtis dissimilarity and UNIFRAC (weighted and unweighted). Statistical significance was confirmed via permutational multivariate analysis of variance (PERMANOVA) in the *vegan* package in R. The relative abundance of phyla occurring in the sequences was calculated in R using the *phyloseq* package. Low abundance taxa were removed from the data set by excluding phyla occurring in less than 2% of sequences. Individual OTUs were identified for further analysis using similarity percentages (SIMPER) analysis in R, identifying the species that contribute most to Bray-Curtis dissimilarity between groups, totaling to 70% of the variation. Significance was determined using the Kruskal-Wallis rank sum test, taking into account false discovery rate. In OTUs with significant ($P < 0.05$) differences in relative abundance, post hoc significance was determined using the Wilcoxon rank sum test.

5.3.9 Statistical analyses

All results were expressed as means ± SEMs. Statistical analysis was conducted on SAS 9.4 software (Cary, NC). The significance level was set at $\alpha = 0.05$ for all tests. Body weight changes were analyzed by two-factor repeated measures (RM) ANOVA (PROC MIXED) with time and treatment group as independent variables. Multiple comparisons were conducted between groups at different weeks with Tukey's test. For histological analysis at 3 wk, significance testing in wild type recipients was by ANOVA with Tukey's test for multiple

comparisons, significance testing in *Il10*$^{-/-}$ recipients was by Student's T test. For data that followed normal distribution, group difference was determined by ANOVA with Tukey's test for multiple comparisons (PROC GLM). For nonparametric data, the Mann–Whitney U-test and Kruskal–Wallis test were used to compare two groups and more than two groups, respectively (PROC NPAR1WAY). Comparisons between two means were performed using GraphPad Prism software (La Jolla, CA). Colonic cytokine differences were determined by T tests between diets within recipient cell type; when variances between the groups were different, Welch's correction was used.

5.4 Results

5.4.1 Aronia supplementation inhibits adoptive transfer colitis

Adoptive transfer of naïve T cells to immunocompromised mice induces colitis through expansion of pro-inflammatory Th17 in the absence of Treg. We utilized T cell transfer to evaluate the extent aronia berry consumption inhibited colitis. Adoptive transfer of $CD4^+CD62L^+$ cells to *Rag1*$^{-/-}$ mice fed the control diet induced weight loss, relative to the sham group by week 4 after transfer (**Figure 5.1a**). Consumption of lyophilized aronia powder concurrent with T cell transfer inhibited colitic weight loss at weeks 5 through 7 (**Figure 5.1a**). The colitis symptom scores of aronia-fed mice were less than the control group at weeks 5 and 7 (**Supplementary Figure A.5.S1a,b**). Aronia consumption inhibited colonic myeloperoxidase activity, indicating a reduction of neutrophil infiltration **(Supplementary Figure A.5.S1c**). T cell transfer increased colon weight/length ratios at weeks 5 and 7 relative to the sham, but the aronia-fed group had mean colon weight/length ratios less than the control (**Figure 5.1b,c**).

Histopathological analysis indicated induction of mild to severe colitis by T cell transfer, but both the aronia and control-fed groups had similar levels of colonic inflammation at weeks 5 and 7 (**Figure 5.1d,e**). Thus, despite the induction of severe colitis by T cell transfer, aronia berry consumption inhibited wasting and colonic edema.

5.4.2 Aronia increases MLN Treg populations concurrent with weight loss inhibition

Because aronia inhibited wasting induced by T cell transfer colitis, we investigated the T cell populations associated with these effects at week 7. Given the ability of aronia to inhibit IL-6 and TNF-α in vitro and induce IL-10 in vivo in healthy mouse colons, we hypothesized aronia consumption would increase Treg and inhibit Th17 after transfer of naïve T cells. At week 7 after transfer, LP and splenic Treg and Th17 proportions were similar between the aronia and control groups (**Figure 5.2a, Supplementary Figure A.5.S2a**). However, at week 7, MLN Treg were increased by aronia consumption, but no differences were observed in MLN Th17 (**Figure 5.2a**). Consistent with the observation that MLN Treg were increased by aronia consumption, aronia but not the control diet had increased colonic IL-10 relative to the sham (**Figure 5.2b**). T cell transfer induced colonic TNF-α, IL-6, IL-17A, IFN-γ, IL-1β, IL-2, IL-22, and IL-23, but these cytokines were not affected by aronia consumption (**Figure 5.2b**, **Supplementary Figure A.5.S2b**). Thus, at the later stage of adoptive transfer colitis, aronia consumption increased the proportion of MLN Treg which was associated with increased colonic IL-10.

5.4.3 Consumption of aronia increases differentiation of anti-inflammatory Th17 populations in LP 5 weeks after T cell transfer

Given the dynamic nature colitic wasting, we next evaluated our hypothesis at 5 weeks after T cell transfer. Utilizing IHC analysis, the colitic control group showed pervasive infiltration of $CD4^+$ cells at week 5 relative to the sham (**Figure 5.3a**). The number of colonic $CD4^+$ cells was reduced by aronia consumption (**Figure 5.3b**). The proportion of Treg cells ($FoxP3^+$) in $CD4^+$ cells in the aronia group was more than double than the control (**Figure 5.3c**). Contrary to our hypothesis, aronia consumption also increased the proportion of colonic Th17 cells ($ROR\gamma t^+$) in $CD4^+$ relative to the control diet (**Figure 5.3d,e**). The colonic cytokines TNF-α, IL-6, IL-17A, IFN-γ, IL-1β, IL-2, IL-22, and IL-23 were significantly increased in the colitic control compared to the sham group ($P < 0.05$, **Figure 5.3f, Supplementary Figure A.5.S2**). Consumption of the aronia-supplemented diet attenuated colonic cytokines without affecting TNF-α, IL-17, IL-1β, or IL-10. Notably, aronia supplementation suppressed colonic IFN-γ by 75.2% ($P < 0.05$) and tended to reduce IL-23 and IL-6 relative to the control group (**Figure 5.3f, Supplementary Figure A.5.S2**). Flow cytometry analysis was utilized to determine Treg and Th17 in LP, MLN, and spleen. Consistent with IHC analysis, the percentage of Treg cells ($CD4^+FoxP3^+$) in the LP of the aronia-fed group was more than double than the colitic control group (**Figure 5.4**). The aronia-fed group also had proportionally more MLN and splenic Treg cells than the control group (**Figure 5.4**). In addition, the proportion of Th17 in the LP was increased in the aronia group, but not at the MLN or spleen (**Figure 5.4**). Given the increased proportion of Th17 in the LP, Th17 subpopulations were further assessed in the LP, MLN, and spleen. Compared with the control group, the aronia group had increased proportion of Th17 ($CD3^+CD4^+IL\text{-}17A^+$) $IL\text{-}10^+$ and $IL\text{-}22^+$ cells in the LP and spleen but not in the MLN (**Figure**

5.5a,b,c; Supplementary Figure A.5.S4). However aronia consumption did not alter the proportion of Th17 IFN-γ^+ or Th17 TNF-α^+ in the LP, MLN, or spleen (**Figure 5.5a,b,c**). Likewise, the proportions of $CD3^+CD4^+$IL-$17A^-$IFN-γ^+ or $CD3^+CD4^+$IL-$17A^-$TNF-α^+ were not different between diets (**Supplementary Figure A.5.S5**). Aronia consumption increased the proportions of IL-10^+ Treg ($CD3^+CD4^+FoxP3^+$IL-10^+) in LP, MLN and spleen (**Figure 5.5d**), although colonic IL-10 was similar between the aronia and control groups (**Figure 5.3f**). Therefore, aronia consumption induced both Treg and anti-inflammatory Th17 in the LP, but only Treg were induced in the spleen and MLN.

5.4.4 T cell IL-10 is essential for colonic cytokine modulation upon aronia consumption

Because IL-10 is central to the Treg and Th17 populations induced by aronia consumption, we further determined if T cell IL-10 was essential to the immunomodulatory activity of aronia berry consumption. To test this, we transferred $CD4^+CD62L^+$ cells isolated from spleens of wild type or *Il10*-deficient mice to *Rag1*$^{-/-}$ mice that were fed the control or aronia diets concurrent with injection. As a non-colitic control, *Rag1*$^{-/-}$ mice received $CD4^+$ splenocytes from wild type mice. Groups injected with $CD4^+CD62L^+$ cells were fed aronia or control diets concurrent with injection. We evaluated cell populations, colonic cytokines, and markers of colitis at 3 weeks after transfer because we hypothesized differences would be evident prior to colitic weight loss. At this time, aronia and control diets had similar body weights, regardless of donor type (**Figure 5.6a**). Aronia-fed mice that received *Il10*$^{-/-}$ $CD4^+CD62L^+$ cells also had similar body weight relative to the control (**Figure 5.6a**). Aronia berry-fed mice had lower colon weight/length ratios than controls, regardless of donor type (**Figure 5.6b**). Histopathological scores of colitis were not different between the WT recipients

and non-colitic controls (**Figure 5.6c**). In contrast, histopathological scores in *Il10*$^{-/-}$ recipients were increased from WT donors, and the aronia diet had less inflammation than the control (**Figure 5.6c**). Colonic TNF-α, IL-6, IFN-γ, IL-22, IL-17A, IL-10, and IL-4 were significantly reduced in aronia fed mice that received cells from wild type donors (**Figure 5.6d**). Aronia consumption did not inhibit colonic cytokines in mice reconstituted with *Il10*$^{-/-}$ naïve T cells (**Figure 5.6d**). IL-23 was not affected by diet or T cell donor type (**Figure 5.6d**). Aronia consumption did not modulate proportions of Th17 in the LP, MLN, or spleen among donor types (**Supplementary Figure A.5.S6a,b,c**). Treg populations in the LP and spleen were not different among donor types or diets (**Figure 5.6e**). However, functional T cell IL-10 was required for an increased proportion of Treg in the MLN (**Figure 5.6e**). Therefore, prior to the onset of colitic wasting, aronia consumption increases MLN Treg and robustly inhibits colonic cytokines. These dietary immunomodulatory effects are dependent on functional T cell IL-10.

5.4.5 Aronia consumption induces cecal microbial diversity prior to colitic weight loss

Upon adoptive transfer to immunocompromised mice, naïve T cells proliferate and differentiate in response to the gut microbiota. Therefore, we sought to characterize the extent aronia consumption affected the composition of the gut microbiota prior to the onset of colitic wasting. Diet and colitis significantly affected the cecal microbial community at 3 weeks after transfer of WT naïve T cells determined by 16S rRNA gene sequencing. Aronia consumption increased α-diversity, measured by Shannon ($F = 47.1$, $P < 0.001$) and Inverse Simpson indices ($P < 0.001$), without affecting richness assessed by ACE ($P = 0.93$) or Chao1 ($P = 0.30$) (**Figure 5.7a,b, Supplementary Figure A.5.S7**). Aronia consumption also impacted beta diversity by increasing clustering by UNIFRAC analysis (weighted and unweighted) (**Figure 5.7c,**

Supplementary Figure A.5.S7). The differential clustering of the colitic aronia and colitic control groups were significant by PERMANOVA (UNIFRAC (weighted) F=12.11, P < 0.001; UNIFRAC (unweighted) F = 2.934, P < 0.001; Bray-Curtis F = 11.152, P < 0.001). Notably, aronia consumption impacted phylum composition in colitic mice by increasing relative abundance of Bacteroidetes, Firmicutes, and Proteobacteria, and decreasing Verrucomicrobia. At the species level, colitic aronia-fed mice had lower *Akkermansia muciniphila*, *Bacteroides acidifans,* and increased unclassified order Bacteroidales, an unclassified species belonging to the genus *Dorea*, and *Ruminococcus gnavus* relative to the control, among others (**Supplementary Table A.5.S1**). Thus, aronia consumption concurrent with naïve T cell transfer distinctly altered the cecal microbiota relative to the control diet.

5.5 Discussion

Consumption of polyphenol-rich aronia berry inhibited colitis-associated markers and modulated the Th17/Treg axis in the T cell adoptive transfer model. Aronia consumption resulted in early changes in the gut microbiota and increased MLN Treg cell differentiation and later increased proportions of anti-inflammatory Th17 cells, therefore inhibiting the proliferation and infiltration of effector T cells in colon tissue. In the adoptive transfer model of colitis, induction of Treg inhibits expansion of effector cells (29). Reduction of IL12/23 by an IL-12/23p40 monoclonal antibody increased induced regulatory T-cells (iTregs) in the adoptive transfer model (29). Notably, colonic IL-23 was reduced at later stages of colitis, which may partly explain induction of Tregs. Also, IL-6 inhibition may partly explain increased Treg and IL-10^{+} cells, as blockade of IL-6 increased IL-$10^{+}CD4^{+}$ cells in the adoptive transfer model (30). IL-6 blockade during adoptive transfer colitis also inhibited TNF-α and IL-17 (30), which was

observed only in the early model stages after aronia consumption. Aronia berry consumption also inhibited colonic IFN-γ, but IL-17A^{+}IFN-γ^{+} differentiation was similar between dietary treatments. Based on in vitro studies, aronia polyphenols may directly inhibit T cell cytokine excretion (15). These extra-intestinal effects were mediated through T cell IL-10. Notably, recombinant IL-10 therapy is not effective for Crohn's disease treatment (31). Since IL-17^{+} IL-10^{+} are non-pathogenic and promote host defense (32) and Treg IL-10 is critical in IBD (33), the cellular source(s) of IL-10 may be essential for functional inhibition of IBD. Notably, aronia berry consumption inhibits colitis in the absence of T cell IL-10, apart from modulation of colonic cytokines and adaptive immunity at 3 wk, indicating that aronia may affect innate immunity, barrier function, and/or other mechanisms to contribute to its anti-colitic activity.

Aronia supplementation modulated T cell populations early in the adoptive transfer model, but mice still developed severe inflammation at later stages of the model. The inability of aronia berry consumption to inhibit weight loss and progressive late-stage inflammation was similar to CD4^{+}CD45RB^{HI} cells to *Rag1*$^{-/-}$ mice (16). Other reports of dietary treatments affecting colitis progression in adoptive transfer are sparse, but *L. salivarius* Ls-33 could reduce but not ablate weight loss during late progression of adoptive transfer colitis (34). Thus, it is unclear if any dietary treatment could prevent the progression of colitis at later stages in the adoptive transfer model.

The inhibitory actions of aronia berry consumption on adoptive transfer colitis appear unique from prior dietary interventions in the adoptive transfer model. Oral gavage of 57 mg/kg/day of rutin inhibited CD4^{+}CD62L^{+} transfer colitis, and reduced colonic expression of TNF-α, IFN-γ, IL-6, and IL-17 (35). Capsaicin injections inhibited total CD4^{+} cells in MLN, as

well as ex vivo MLN TNF-α, IL-17, and IL-1β production (36). Intake of fish oil did not modulate Treg or Th17 cells at 4 weeks after adoptive transfer to *Rag2*$^{-/-}$ mice (37).

Anthocyanin-rich fruits may also inhibit colitis by mechanisms independent of T cell function. Blueberry consumption reduced colonic cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), and modulated antioxidant function in 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in rats (38). Black raspberry consumption also inhibited colonic COX-2 expression in DSS-colitis in mice (39). Anthocyanins inhibited TNF-α-induced COX-2 expression and activity via nuclear factor-kappa B (NF-κB) and nuclear factor erythroid 2-related factor 2 (Nrf2) in cultured colonic epithelial cells (40). Similarly, red raspberry anthocyanins inhibited COX-2 and reduced NF-κB signaling in macrophages (41). Blueberry also improved colonic glutathione (GSH)/glutathione disulfide (GSSG) ratios in rats with TNBS-induced colitis (38). Aronia polyphenol consumption also modulates colonic antioxidant function in mice (42,43). It is plausible that the aronia berry consumption also has anti-inflammatory mechanisms independent of T cell differentiation.

Given the varying chemical composition of different berries and anthocyanin-rich fruits, it is unlikely that all fruits share a common anti-colitic mechanism. Anthocyanin hydroxylation, glycosylation, and methylation affect IL-17RA receptor binding affinity, as well as ex vivo anti-inflammatory activity after lipopolysaccharides (LPS)-stimulation (14,15). Blueberry, bilberry, grape juice, and black raspberry consumption inhibited colonic TNF-α in DSS- or TNBS-induced colitis, whereas aronia berry did not (16,39,44–46). Also, the form of polyphenols consumed during colitis is an important consideration, as bilberry anthocyanins had distinct differences in cytokine modulation relative to consumption of whole bilberries (45). Bilberry anthocyanins were more effective at inhibiting colonic IFN-γ than whole bilberry in chronic DSS

colitis, but bilberry more effectively inhibited colonic IFN-γ during acute DSS colitis (45). Bilberry contains both cyanidin- and delphinin-type anthocyanins, while aronia contains primarily cyanidin-type (47). The importance of this compositional difference to IBD is not known. Taken together, the methods to induce colitis, degree of inflammation, and intervention composition affect the outcome of dietary approaches for colitis prevention in rodents.

Aronia berry consumption inhibited a pathogenic T helper cell profile in adoptive transfer colitis in *Rag1*$^{-/-}$ mice by increasing Treg and anti-inflammatory Th17 populations. The earliest events included increased proportion of MLN Tregs and altered cecal microbiota. Several phyla or species modulated by aronia consumption have been associated with colitis. *Dorea longicatena* was positively associated with Crohn's disease remission in patients who had undergone ileocolonic resection (48). *Akkermansia muciniphilia* was increased in IBD patients and induces colitis in *Il10*$^{-/-}$ mice (49,50). *R. gnavus* has been positively associated IBD, but regionally associated with uninflamed tissue (49,51). Aronia broadly affected cecal microbiota, and these changes may mediate host-microbiota interactions and alter naïve T cell differentiation. Changes in microbial antigens and/or bacterial metabolites may alter host immune homeostasis.

In conclusion, this study reveals the dynamic nature of polyphenol-rich berry consumption on T cell differentiation during colitis. The immunomodulatory effects of aronia berry consumption included early induction of MLN Tregs dependent upon functional T cell IL-10. Immunomodulatory events prior to extensive differentiation of pro-inflammatory Th17 mediate dietary inhibition of colitis by aronia berry consumption. Notably, the intermediate protective mechanism(s) were independent of colonic TNF-α, a major target of biologics for IBD

and instead may relate to inhibition of IFN-γ and IL-6. Dietary strategies to increase Tregs and IL-10^{+} cells may be viable and distinct mechanism from existing therapeutic approaches.

5.6 Acknowledgments

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5.7 Figures

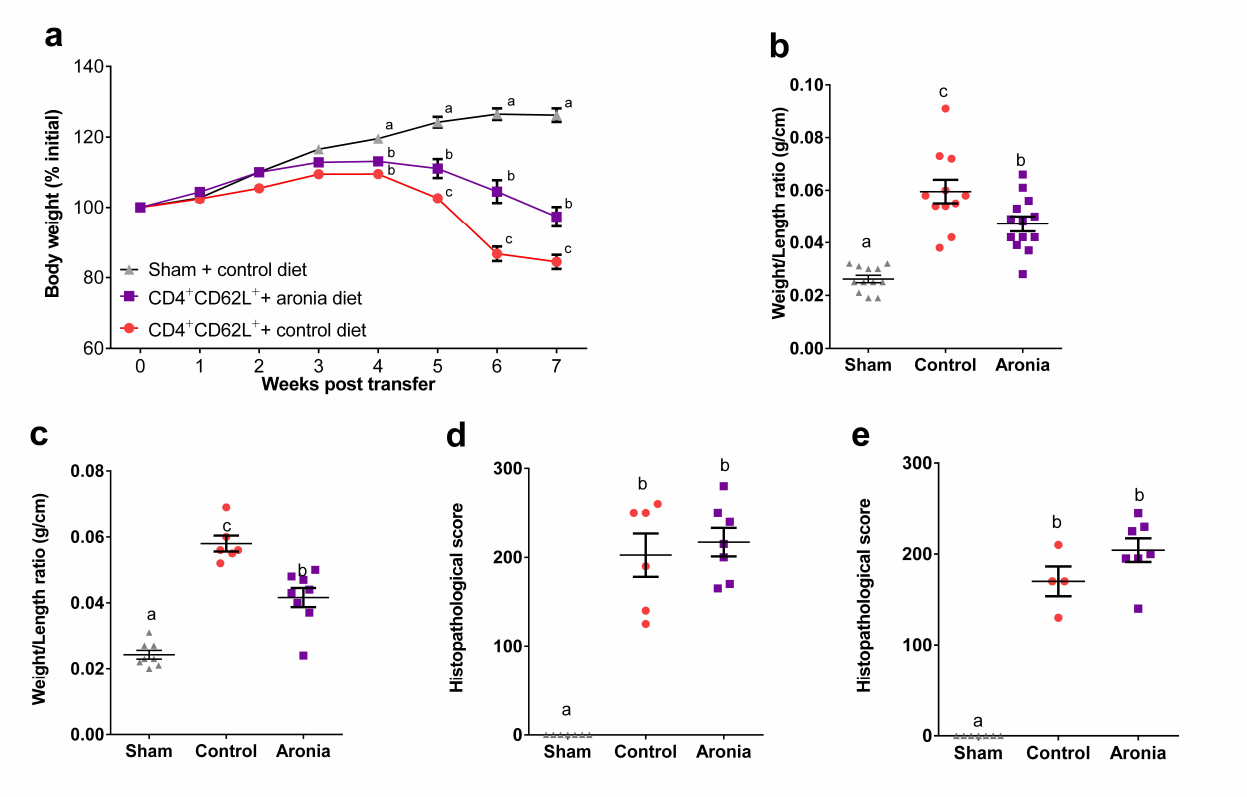

Figure 5.1. Consumption of 4.5% aronia berry-supplemented diet inhibits markers of T cell transfer colitis in mice. Splenic $CD4^+CD62L^+$ cells from C57BL/6J mice or vehicle (sham) were transferred to *Rag1*$^{-/-}$ mice. Mice consumed the control (AIN-93M) or aronia-supplemented diets. **(a)** Body weight after T cell transfer, as percentage of initial body weight at transfer (n = 21/group). Colon weight/length ratio of mice at **(b)** week 5 (n = 11-14/group) and **(c)** week 7 (n = 6-8/group). Histopathological scores of colons from mice sacrificed at **(d)** week 5 (n = 6-7/group) and **(e)** week 7 (n = 4-8/group). Data are means ± SEMs. Body weight changes within each group were determined by two-way repeated measures ANOVA (PROC MIXED) with time and treatment group as independent variables. Multiple comparisons were conducted between groups at different weeks with Tukey's test. Data bearing different letters indicate significant within-week differences ($P < 0.05$). For colon weight/length and histopathological scores,

significance testing was by ANOVA with Tukey's test for multiple comparisons (PROC GLM). Groups bearing different letters indicate significant differences between treatments ($P < 0.05$).

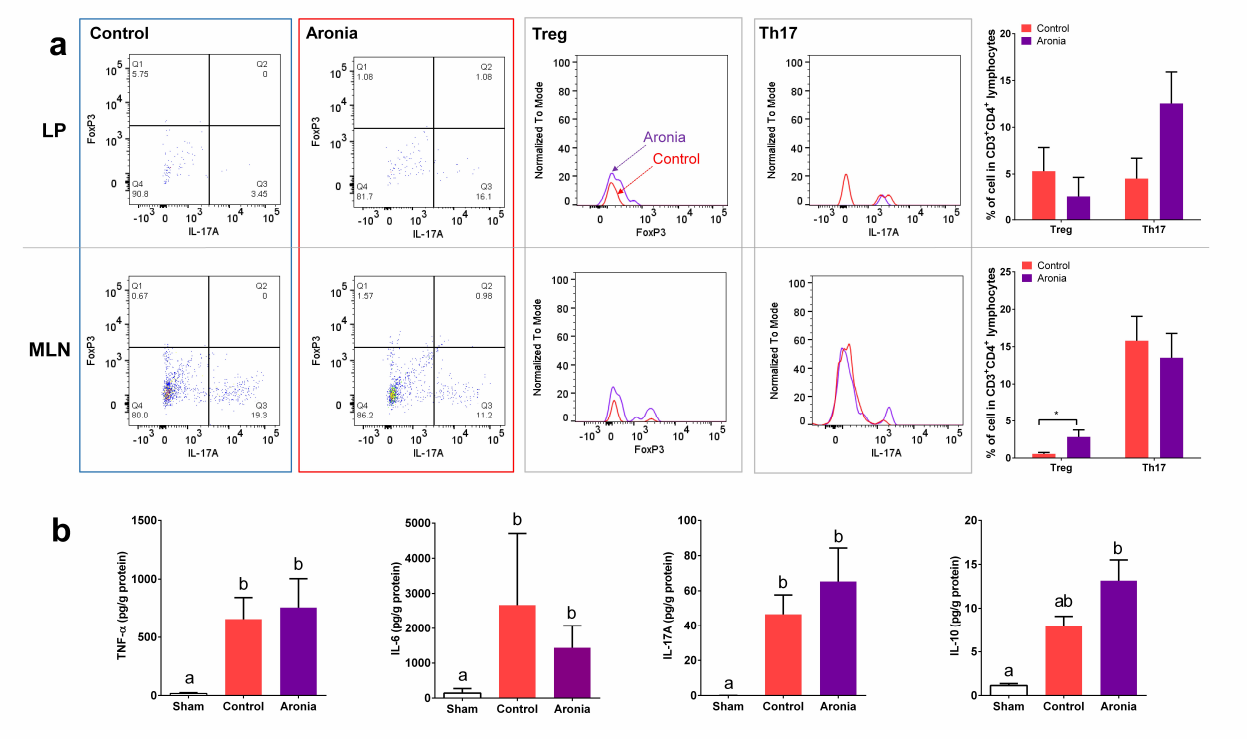

Figure 5.2. Aronia consumption increases proportion of Tregs in MLN but not LP 7 wk after adoptive transfer of $CD4^+CD62L^+$ cells to *Rag1*$^{-/-}$ mice. Flow cytometry analysis of $CD4^+CD3^+$ populations in **(a)** LP and MLN with representative plots, and **(b)** colonic tissue cytokines (TNF-α, IL-6, IL-17A, and IL-10). Bar graph data are means ± SEMs, n = 6-7/group, cell populations were compared by Mann–Whitney U-test (PROC NPAR1WAY). *, control vs. aronia, $P < 0.05$. Cytokines were assessed Kruskal–Wallis test with Dunn's test for multiple comparison (PROC NPAR1WAY). Bars bearing different letters indicate significant differences between treatments ($P < 0.05$).

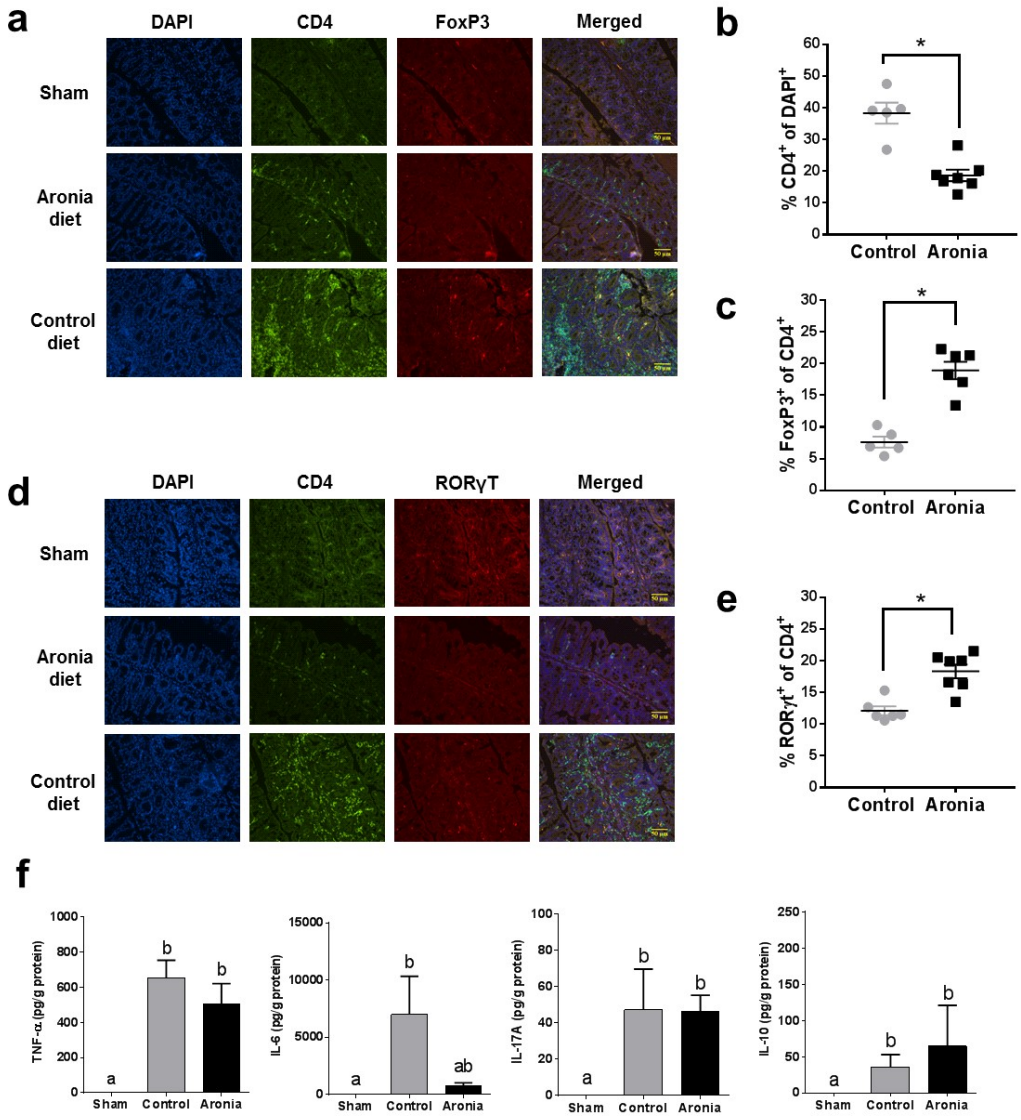

Figure 5.3. Aronia consumption inhibits $CD4^+$ cells and IL-6, while increasing proportion of FoxP3 and RORγT in $CD4^+$ cells in colon in *Rag1*$^{-/-}$ mice at 5 wk after vehicle injection (sham) or transfer of splenic $CD4^+CD62L^+$ cells from C57BL/6J mice (n = 5-7/group). **(a)** Representative IHC staining for nuclei (DAPI, blue), CD4 (Alexa Fluor 488, green) and FoxP3 (Alexa Fluor 594, red) in colon sections (200 ×). **(b)** Relative expression of $CD4^+$ cells of $DAPI^+$ cells. **(c)** Relative expression of $CD4^+$ and $FoxP3^+$ cells in colons. **(d)** Representative IHC staining of CD4 and RORγt in colon sections (200 ×). **(e)** Relative expression of $ROR\gamma t^+$ cells in

colonic $CD4^+$ cells. **(f)** Cytokines (TNF-α, IL-6, IL-17A and IL-10) in colon tissue at wk 5. The percentage of cell populations was assessed for significance by the Mann–Whitney U-test (PROC NPAR1WAY). *, control vs. aronia, $P < 0.05$. Cytokines were assessed Kruskal–Wallis test with Dunn's test for multiple comparisons (PROC NPAR1WAY). Bars bearing different letters indicate significant differences between treatments ($P < 0.05$).

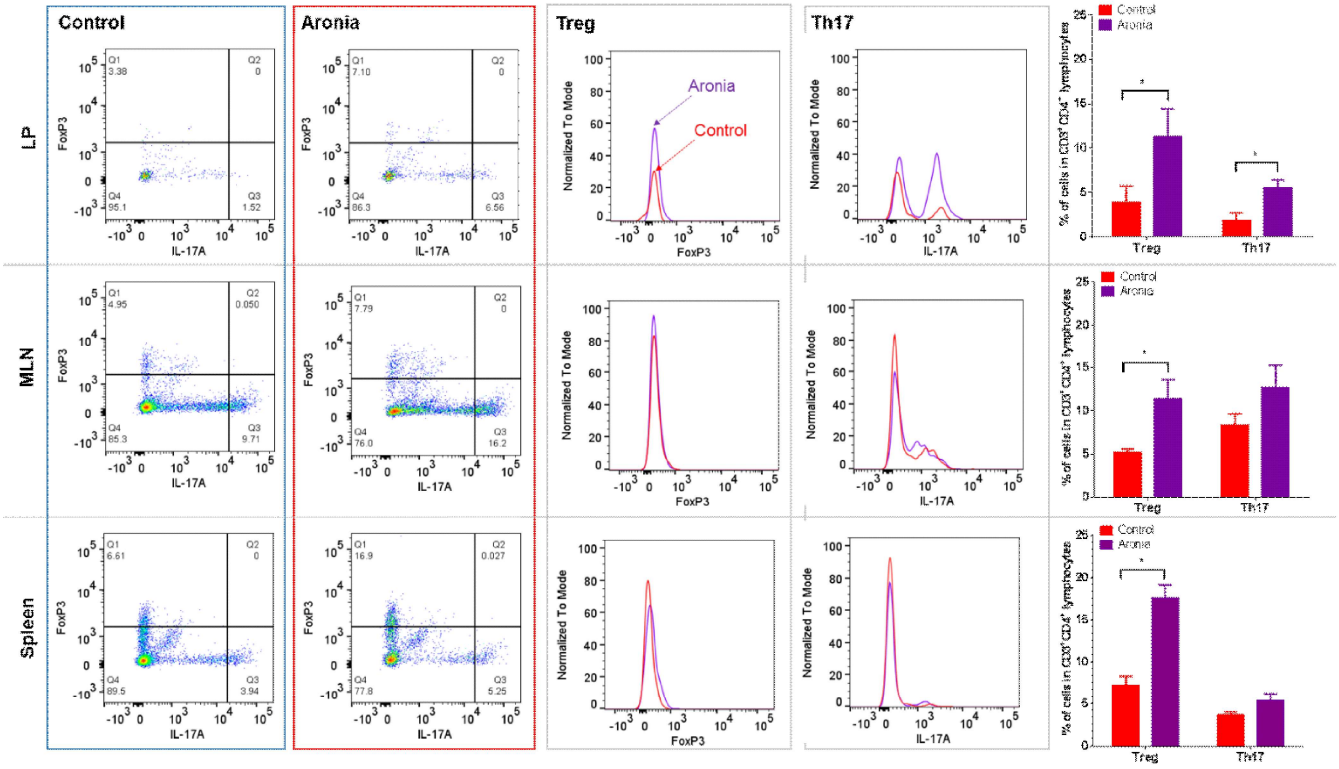

Figure 5.4. Flow cytometry analysis of $CD3^{+}CD4^{+}$ lymphocyte populations from *Rag1*$^{-/-}$ mice 5 wk after T cell transfer fed aronia berry-supplemented (4.5%) or control diets (n = 6-7/group). Representative dot plots and aggregate data from LP, MLN, and spleen. Cell populations were compared by Mann–Whitney U-test (PROC NPAR1WAY). *, control vs. aronia, $P < 0.05$.

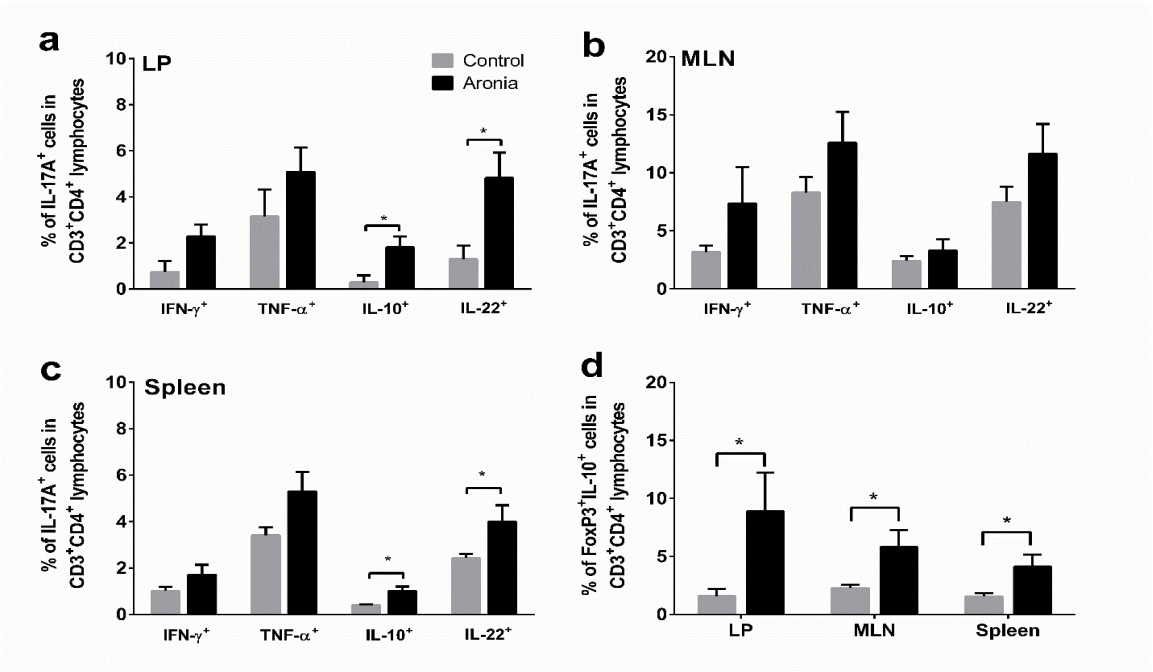

Figure 5.5. Lymphocyte subpopulations in LP, MLN and spleen from *Rag1*$^{-/-}$ mice at 5 wk after T cell transfer and fed control or aronia-supplemented diets (n = 6-7/group). Cells were surface stained with anti-CD3 and anti-CD4, and intracellularly stained with anti-IL-17A, anti-FoxP3, anti-IFN-γ, anti-TNF-α, anti-IL-10, and anti-IL-22. Gating was based on Fluorescence-Minus-One (FMO) staining. Relative expression of Th17 sub-populations in **(a)** LP, **(b)** MLN, and **(c)** spleen, and **(d)** Treg ($CD3^+CD4^+FoxP3^+$)$IL\text{-}10^+$ cells in LP, MLN, and spleen. Cell populations were assessed for significance by Mann–Whitney U-test (PROC NPAR1WAY). *, control vs. aronia, $P < 0.05$.

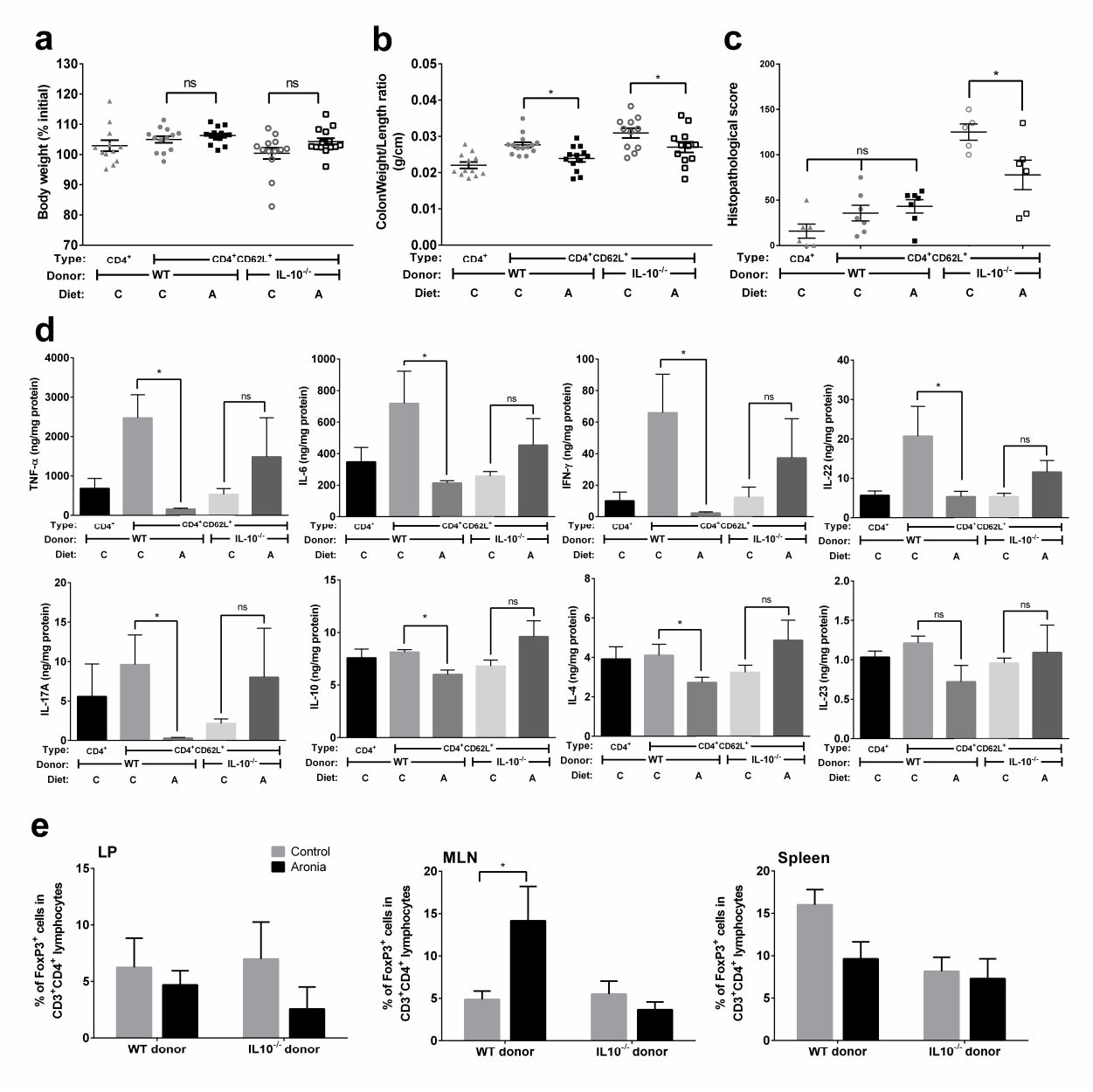

Figure 5.6. Immunomodulation by aronia consumption is dependent on T cell IL-10. Wild type or *Il10*$^{-/-}$ CD4^{+}CD62L^{+} cells or wild type (WT) CD4^{+} cells (non-colitic control) were transferred to to *Rag1*$^{-/-}$ mice consuming aronia-supplemented (A) or control diets (C). After 3 weeks: **(a)** Body weight. Significance testing was by one-way ANOVA with Sidak's multiple comparisons test, n = 12-14/group. **(b)** Colon weight-length ratios; significance testing was by ANOVA with Tukey's test for multiple comparisons, n = 11-14/group. **(c)** Histopathological analysis;

significance testing in WT recipients was by one-way ANOVA with Tukey's test for multiple comparisons, significance testing in *Il10*$^{-/-}$ recipients was by Student's T test. **(d)** Colonic cytokines (TNF-α, IL-6, IFN-γ, IL-22, IL-17A, IL-10, IL-4, and IL-23). Differences were determined by T tests between diets within recipient cell type; when variances between the groups were different, Welch's correction was used, n = 3-7/group. **(e)** Flow cytometry analysis of Treg ($CD3^+CD4^+FoxP3^+$) in lymphocytes from LP, MLN, and spleen. Significance testing was by two-way ANOVA with Sidak's multiple comparisons test, n = 4-7/group. Data represent means ± SEMs . *, control vs. aronia, $P < 0.05$.

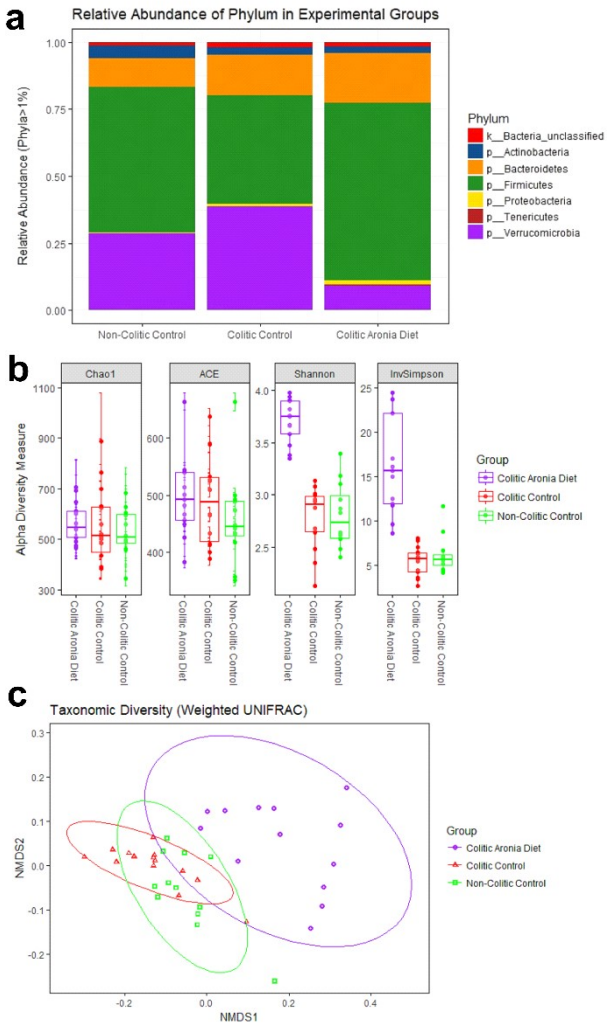

Figure 5.7. Consumption of aronia berry induces changes in the gut microbiome composition of colitic mice. At 3 wk after adoptive transfer of $CD4^+$ cells (non-colitic control) and naïve T cells to *Rag1*$^{-/-}$ mice consuming the control diet or aronia-supplemented diet, cecal contents were analyzed by 16S rRNA sequencing. Comparing the groups, the **(a)** mean relative abundance of identified phyla, **(b)** alpha diversity measures (Chao1, ACE, Shannon, Inverse Simpson), and **(c)** beta diversity by weighted UNIFRAC analysis plotted in two dimensions using NMDS.

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Chapter 6

Conclusions

6.1 Re-evaluation of hypothesis

The overall hypothesis of this work was that aronia berry modulates intestinal Th17/Treg homeostasis to inhibit colitis development. Consistent with the hypothesis, aronia polyphenols and microbial catabolites directly inhibited T cell cytokines *in vitro*. Freeze dried aronia berry, but not other preparations or an ethanolic extract inhibited dextran sulfate sodium (DSS) colitis-associated wasting. Thus, anti-colitic effects appear to depend on the berry preparation or the colitis model. Freeze dried aronia berry powder consistently inhibited wasting in the adoptive transfer model. Therefore, these data support that the anti-colitic mechanisms of aronia consumption are in part mediated through T cells.

Consistent with our overall hypothesis, Treg were induced with aronia feeding in the adoptive transfer model of colitis. However, the site of Treg induction was time-dependent. The earliest site of Treg induction was the MLN. Unexpectedly, aronia berry consumption increased Th17 populations in the adoptive transfer model. However, subpopulation analysis suggested that these cells were non-pathogenic Th17. Consistent with the hypothesis, T cell IL-10, a major effector cytokine of Treg, contributed to the modulation of colonic cytokines and induction of Treg in the MLN. Unexpectedly, colon weight/length ratios and histopathological scores were improved by aronia berry in the absence of T cell IL-10. This indicates that aronia berry exhibits inhibitory mechanisms outside of the adaptive immune system and could be due to modulation of gut microbiota, intestinal barrier function, and/or the innate immune system. Notably, aronia berry did not improve histopathological scores in the DSS model, and only improved the score in the adoptive transfer model in the absence of T cell IL-10. The inability of aronia to improve histological scores while promoting a more anti-inflammatory Th17/Treg balance is a paradox yet to be resolved.

6.2 Advancement of field

This work increases the knowledge of cell mediated effects of dietary interventions in inflammatory models. Only a few reports (1–3) have examined the effects of dietary interventions on the cellular mediators of inflammation. To the best of our knowledge, the work described here is the first use of whole fruits in the adoptive transfer model of colitis. To the best of our knowledge, Chapter 5 is the most comprehensive characterization of T cell homeostasis in a dietary study of colitis.

This work provides new insight about the effects of microbiota-derived polyphenol catabolites. This work has demonstrated that bioavailable microbial catabolites of polyphenols can directly suppress T cell inflammatory cytokine production. Given that, in general, dietary polyphenols have low bioavailability, rapid metabolism, and rapid excretion, it would be difficult to infer that polyphenols within the food matrix are immediate effectors of anti-inflammatory activity. Microbial catabolites of anthocyanins have higher C_{max} and $t_{1/2}$ values than the parent polyphenols (4,5). To the best of our knowledge, we have presented the first evidence that microbial polyphenol catabolites can directly affect T cell inflammatory cytokine production. This data joins a small, but growing body of literature showing direct effects of microbial catabolites on different types of cells (6–8).

These studies also utilized physiologically relevant dose of whole food, as opposed to pharmaceutical doses of extracts/single polyphenols. As discussed in Chapter 2, many studies utilize supraphysiologic doses of polyphenols or extracts, which makes more difficult and distant the translation to dietary recommendations. While this does not preclude supplemental polyphenols for IBD in future research in this area, these preclinical studies suggest that anti-colitic activity of aronia berry occurs at feasible dietary intake of a whole food.

6.3 Importance

This work is an important step in defining dietary components that affect colitis development and the immune system. Preclinical studies are needed to design human interventions studies that can yield more precise dietary recommendations for IBD. IBD patients often use diet or supplements beyond traditional therapy (9), but there is little guidance for dietary approaches to manage IBD (10). It is incumbent on the research community to identify and evaluate potential treatment approaches, including diet, which is generally low risk especially when compared to some immunosuppressive drugs. Dietary management may also help improve quality of life and the many complications that are experienced in patients with IBD.

Among pre-clinical colitis models, the adoptive transfer model is among the most physiologically relevant IBD models (11). This provides greater plausibility to the hypothesis that berries or other polyphenol-rich foods can inhibit IBD in humans. Furthermore, the protective effects of aronia berry consumption was associated with favorable changes in Th17/Treg homeostasis, which is implicated in both types of human IBD. Dietary approaches have been shown to be well tolerated in human trials (12–14); if effective, this would be a simple recommendation for a generally highly motivated patient population. Until more research is done, the best dietary advice to offer IBD patients is, “to eat a well-balanced diet, such as the Mediterranean-style diet, avoiding processed foods or foods that they self-identify as worsening their symptoms” (15).

Additionally, early dietary intervention may be an important risk modifier, especially for those at higher risk of developing IBD. There are a number of environmental and genetic factors that are risk factors of IBD (16), and many people who have relatives with IBD exhibit gut

dysbiosis (17) and increased gut permeability (18). Early intervention may prevent development of what may be considered a “predisease state” (17) to overt IBD.

6.4 Future directions

The work described in this dissertation enables several new research directions. The contribution of components in aronia berry responsible for colitis inhibition should be evaluated in the adoptive transfer model of colitis. Only one other instance of this approach is known to us (19) evaluated components of cranberries in a DSS model. Many fruits and vegetables contain polyphenols, so other fruits and vegetables may also affect T cell homeostasis in IBD. If polyphenols alone are efficacious, it could provide a unique opportunity to manage active IBD. The current practices in medical nutrition therapy for active IBD (although not necessarily supported by a wealth of evidence) are to follow a low fiber diet or utilize enteral nutrition (20,21). The dose response should also be investigated to determine effective doses as well as possible toxicity issues; numerous case reports of toxicity are reported for green tea based dietary supplements (22,23).

The role of the microbiota should be clarified with regard to polyphenol-rich dietary interventions for IBD. A number of factors could be important to the benefits of berries. The microbiota may be responsible for generating the main bioactive components-whether they are short chain fatty acids or polyphenol catabolites. The presence of polyphenols may change the microbial community or microbial metabolism which may mediate the effects on the immune system. Dietary intervention efficacy could be dependent on microbiota composition or activity, which would be important for enabling precise dietary interventions on the basis of microbiota.

The mechanisms of Treg induction may be related to cellular glucose availability and metabolism (24), the availability of short chain fatty acids (SCFAs), *Clostridia* abundance (25), or polyphenol metabolites and catabolites. Studies utilizing genetic manipulations of glucose transporters and G protein coupled receptors (which serve as receptors for SCFAs) could help elucidate the contribution of macronutrient availability changes resulting from aronia feeding. Defined microbial communities with known metabolic capacities would be useful in determining the contribution of the gut microbiota to protection against colitis and Treg induction.

This work further enables future investigations on cell-based mechanisms by which aronia berry modulates T cell homeostasis. Investigating how the antigen presenting cells, particularly dendritic cells, respond to aronia berry consumption would help understand the mechanism and site-specificity of Treg induction. In mice, the current consensus suggests that Treg induction for the small intestine occurs in the mesenteric lymph nodes, but it is less clear for the large intestine (25). First, work is needed to determine if aronia berry induces Tregs in the small intestine. Since the luminal contents would be quite different in the small intestine and large intestine, there may also be differences in mechanism of action. Further, the impact of T cell IL-10 should also be investigated at later stages of the adoptive transfer model of colitis. It is possible that various stages of colitis have different dependency on this cytokine. In summary, IBD inhibition by fruits and vegetables would be better understood by determining components of IBD inhibition in fruits and vegetables, determining the role of the microbiota, and determining the metabolic, molecular, and cell mediated mechanisms of action.

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Appendix

A.3 Appendix materials for Chapter 3

A.3.1 Supplemental Method 1

Determination of human equivalent dose (HED) of aronia berry. The HED of the present study was determined using **Equation 1**. The animal dose of 0.1575 g/d was determined using the assumption that mice consumed 3.5 g/d of 4.5% aronia berry diets (**Equation 2**). Thus, a 6.3 g/kg body mass (bm) animal dose for a 0.025 kg bm mouse was obtained (**Equation 3**). Using Equation 1, the HED was determined as 0.5108 g of freeze-dried aronia powder per kg (**Equation 4**), that is equivalent to 35.75 g freeze-dried berries for a 70 kg human (**Equation 5**). Given the fraction of dried matter in fresh aronia berries, the HED is equivalent to consumption of 149 g of fresh aronia berries (**Equation 6**).

$$HED\ \left(\frac{g}{kg}\right) = Animal\ dose\ \left(\frac{g}{kg}\right) \times \frac{Animal\ Km}{Human\ Km} \quad \textbf{(Equation 1)}$$

$$Animal\ dose\ \left(\frac{g}{d}\right) = 0.045g \times \frac{3.5g}{day} = \frac{0.1575\ g}{day} \quad \textbf{(Equation 2)}$$

$$Animal\ dose\ \left(\frac{g}{kg\ bm}\right) = \frac{0.1575g}{0.025\ kg\ bm} = \frac{6.3g}{kg\ bm} \quad \textbf{(Equation 3)}$$

$$HED\ \left(\frac{g}{kg}\right) = \frac{6.3g}{kg} \times \left(\frac{3}{37}\right) = 0.5108\,\frac{g}{kg} \quad \textbf{(Equation 4)}$$

$$HED = 0.5108\ g \times 70\ kg = 35.75\ g\ dried\ berries\ per\ 70\ kg\ individual \quad \textbf{(Equation 5)}$$

$$\frac{35.75\ g\ dried\ berries}{0.24\ g\ dried\ berries\ per\ 1\ g\ fresh\ berry} = 149\ g\ fresh\ aronia\ berries \quad \textbf{(Equation 6)}$$

A.3.2 Supplemental Method 2

Folin assay of aronia extracts used in ex vivo colon incubations. Total phenols in aronia berry extract were determined by the Folin method. Briefly, 10 µL of aronia extract or a gallic acid in water standards were incubated with 15 µL Folin-Ciocalteu reagent, 45 µL 20% Na_2CO_3, and 230 µL ultrapure water in a clear plastic 96-well flat-bottomed plate. Absorbance was measured at 765 nm in a spectrophotometer.

A.3.3 Supplemental Figures and Tables

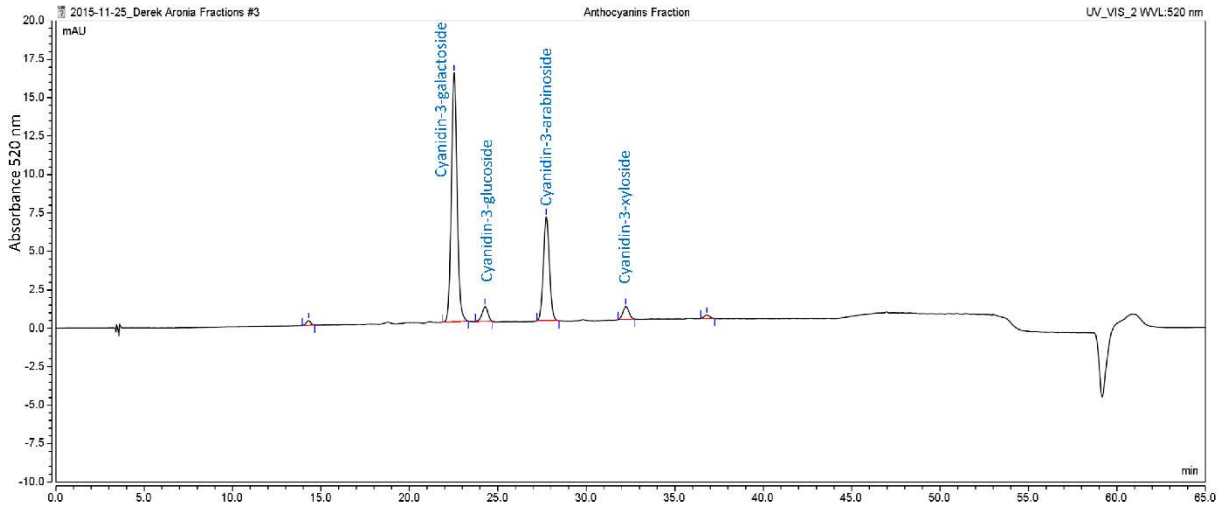

Figure A.3.S1. HPLC chromatogram of aronia berry extract anthocyanin fraction.

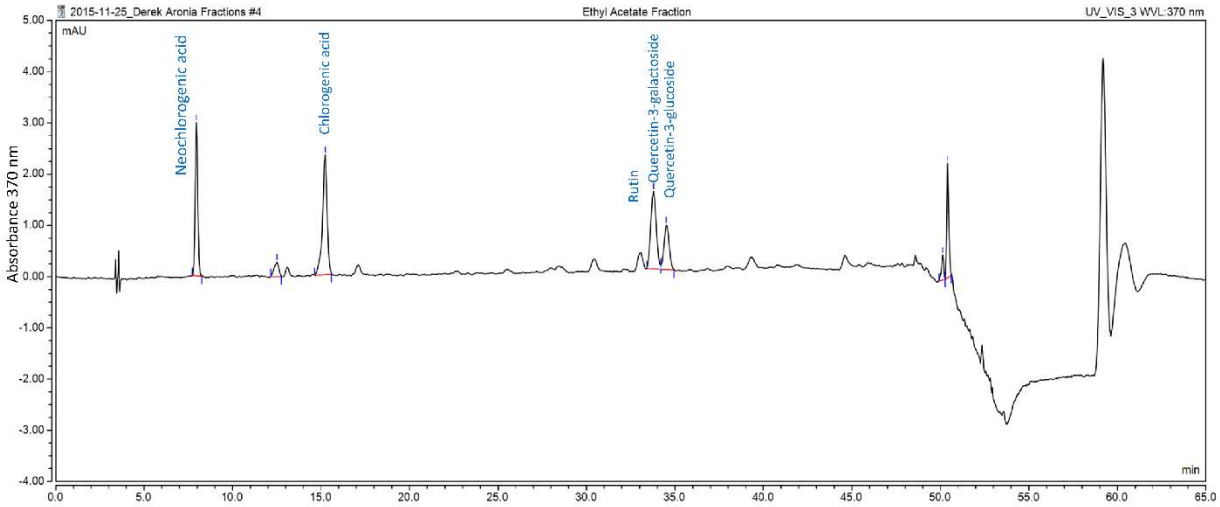

Figure A.3.S2. HPLC chromatogram of aronia berry extract neutral phenols fraction.

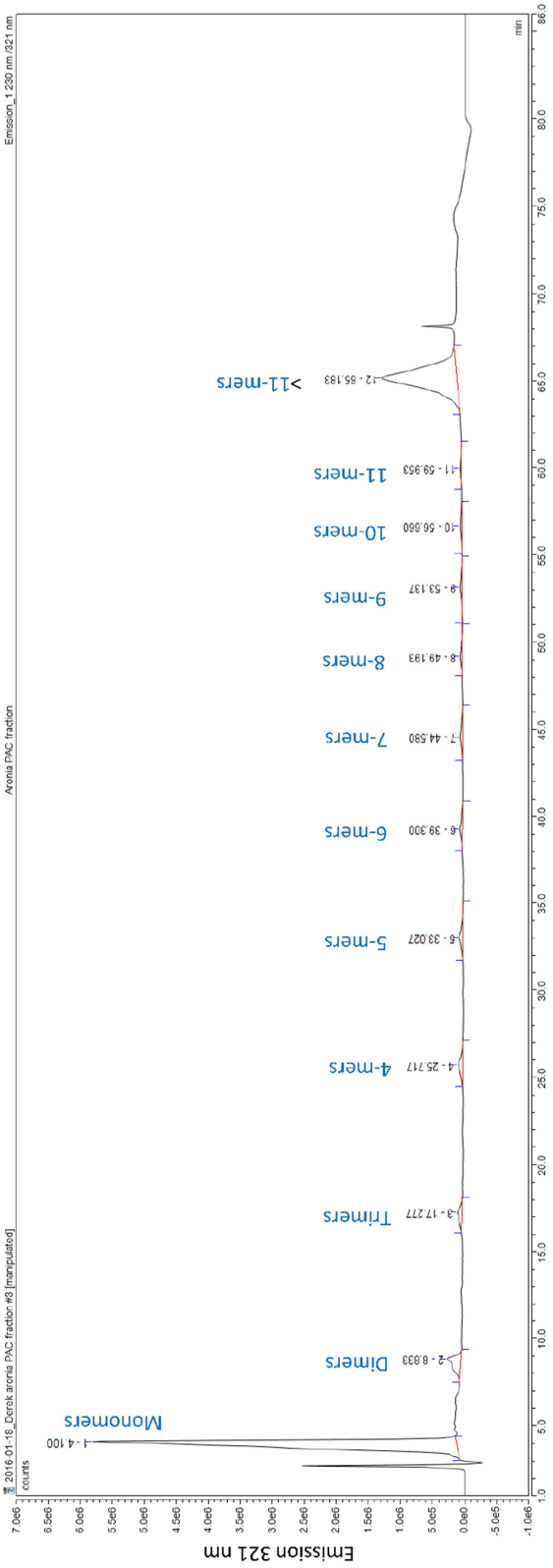

Figure A.3.S3. Hydrophilic interaction chromatography (HILIC) resolution of aronia berry proanthocyanidin fraction.

Supplemental Tables

Supplemental Table A.3.S1. Content of phenolics in aronia extracts and fractions. Concentrations of polyphenols under the anthocyanin fraction and neutral phenols fraction columns refer to the highest concentration used in the in vitro assay.

Polyphenol	Research material content			
	Extract (mg/g)	Extract (μmol/g)	Anthocyanin fraction (μM)	Neutral phenols fraction (μM)
Cyanidin-3-galactoside	79 ± 1	180 ± 1	5.73	1.32
Cyanidin-3-glucoside	5.6 ± 0.1	12 ± 0	0.354	-[b]
Cyanidin-3-arabinoside	35 ± 0	83 ± 1	2.54	1.07
Cyanidin-3-xyloside	4.8 ± 0.0	11 ± 0	0.326	-
Protocatechuic acid	10 ± 0	67 ± 0	-	26.7
Neochlorogenic acid	12 ± 0	16 ± 0	-	196
Chlorogenic acid	130 ± 2	1500 ± 20	1.81	205
Quercetin-3-galactoside	2.9 ± 0.0	23 ± 0	-	15.2
Quercetin-3-glucoside	0.86 ± 0.01	1.3 ± 0.5	-	-
Quercetin-3-rutinoside (rutin)	5.2 ± 0.3	8.6 ± 0.5	-	-

[a]Data are means ± SD
[b]-, below limit of detection.

Supplemental Table A.3.S2. Concentration of proanthocyanidins in cell culture experiments when tested at 4 μg catechin equivalents/mL cell culture media.

Degree of polymerization	μg catechin equivalents/mL
1	2.20
2	0.14
3	0.06
4	0.06
5	0.06
6	0.04
7	0.04
8	0.03
9	0.03
10	0.02
11	0.01
>11	1.31
Sum	4.00

A.4 Appendix materials for Chapter 4

A.4.1 Supplemental Method 1. Determination of incorporation rates of polyphenol depleted aronia berry powder and aronia extract

Determination of incorporation percentage of polyphenol depleted aronia berry in diets. 200 g aronia berry powder serially extracted with acetone/water (70:30, v/v) 3 times for 24 h, and with ethanol/water (95:5, v/v) 5 times for 24 h yielded 94.4 g depleted aronia powder.

$\frac{94.4\ g}{200g} = 0.472$ **(Equation 1)**

$0.472\ \times 4.5\% = 2.12\%$ **(Equation 2)**

Determination of incorporation percentage of aronia extract in diets. HPLC analysis determined that there were 4.437 mg anthocyanins/g berry powder and 122.725 mg anthocyanins/g aronia extract.

$0.045\ \times 4.437 = 0.1996\ mg$ anthocyanins/g diet **(Equation 3)**

$\frac{199.6\ mg/kg\ anthocyanins}{122.725\ mg/g\ anthocyanins} = 1.626$ g extract/kg diet **(Equation 4)**

A.4.2 Supplemental Tables

Supplemental Table A.4.S1. Polyphenol content of berry powders (nmol/g) determined after acid hydrolysis in methanol.

Polyphenol	Full-spectrum Aronia powder	Freeze-dried Aronia powder
Caffeic acid	737 ± 71	702 ± 23
Cyanidin-3-O-galactoside	249 ± 0.1	2,391 ± 212
Cyanidin	21,697 ± 3,158	69,104 ± 6,553
Quercetin	1,278 ± 152	1,922 ± 145

Data are means ± SD, n = 3

Supplemental Table A.4.S2. Polyphenol content of berry products (nmol/g) determined after extraction with acetone/water/acetic acid (70:29.5:0.5, v/v/v).

Polyphenol	Aronia Extract	Full-spectrum Aronia powder	Freeze-dried Aronia powder
Protocatechuic acid	51,937	7,292 ± 175	2,288 ± 128
Chlorogenic acid	55,663	4,475 ± 114	7,591 ± 158
Caffeic acid	86,112	5,871 ± 227	9,458 ± 28
Cy3Gal	89,175	10,765 ± 212	32,716 ± 815
Cy3Glu	49,068	575 ± 38	965 ± 18
Cy3A	34,375	4,286 ± 82	9,515 ± 174
Cy3X	3,760	282 ± 183	998 ± 3
Q3Glu	45.0	9.66 ± 6.41	62.6 ± 0.2
Rutin	2,573	57.1 ± 27.8	302 ± 4
Q3Gal	<LOD	<LOD	<LOD
Quercetin	<LOD	<LOD	<LOD

Data are means ± SD, n = 3, LOD = limit of detection

Supplemental Table A.4.S3. Proanthocyanidin content of aronia berry products (nmol catechin equivalentsl/g) determined after extraction with acetone/water/acetic acid (70:29.5:0.5, v/v/v).

Proanthocyanidin	Aronia Extract	Full-spectrum Aronia powder	Freeze-dried Aronia powder
Monomers	3,656	<LOD	<LOD
Dimers	12,971	224 ± 14	24.7 ± 7.8
Trimers	6237	84.8 ± 0.1	136 ± 20
Tetramers	1845	116 ± 1	184 ± 11
Pentamers	<LOD	121 ± 2	183 ± 3
Hexamers	<LOD	151 ± 2	240 ± 5
Heptamers	<LOD	128 ± 4	180 ± 3
Octamers	<LOD	113 ± 4	165 ± 1
Nonamers	<LOD	88.3 ± 2.6	129 ±2
Decamers	<LOD	65.2 ± 0.9	103 ± 4
Undecamers	<LOD	49.4 ± 0.4	81.2 ± 1.4
Polymers	384669	8,885 ± 1508	12,302 ± 2,889

Data are means ± SD, n = 3, LOD = limit of detection

Supplemental Table A.4.S4. Polyphenol content of diet (nmol/g) determined after acid hydrolysis in methanol.

Polyphenol	Freeze-dried aronia (4.5%)
Caffeic acid	<LOD
Cyanidin-3-O-galactoside	<LOD
Cyanidin	1,307 ± 69
Quercetin	9.2 ± 2.8

Data are means ± SD, n = 3, LOD = limit of detection

Supplemental Table A.4.S5. Polyphenol content of diet (nmol/g) determined after extraction with acetone/water/acetic acid (70:29.5:0.5, v/v/v).

Polyphenol	Freeze-dried aronia (4.5%)
Protocatechuic acid	170 ± 3
Chlorogenic acid	263 ± 0.3
Caffeic acid	309 ± 23
Cy3Gal	1,066 ± 19
Cy3Glu	28.8 ± 0.7
Cy3A	290 ± 18
Cy3X	22.1 ± 4.2
Q3Glu	1.29 ± 0.13
Rutin	6.54 ± 1.16
Q3Gal	<LOD
Quercetin	<LOD

Data are means ± SD, n = 3, LOD = limit of detection

Supplemental Table A.4.S6. Proanthocyanidin content of diet (nmol catechin equivalentsl/g) determined after extraction with acetone/water/acetic acid (70:29.5:0.5, v/v/v).

Proanthocyanidin	**Freeze-dried aronia (4.5%)**
Monomers	<LOD
Dimers	8.71 ± 0.99
Trimers	1.65 ± 0.12
Tetramers	5.93 ± 0.28
Pentamers	5.87 ± 0.11
Hexamers	6.31 ± 0.20
Heptamers	4.50 ± 0.05
Octamers	3.88 ± 0.13
Nonamers	2.95 ± 0.10
Decamers	2.19 ± 0.11
Undecamers	1.61 ± 0.13
Polymers	258 ± 11

Data are means ± SD, n = 3, LOD = limit of detection

Supplemental Table A.4.S7. Cytokine concentrations (pg/mg protein) in colon tissue homogenate

Cytokine	Noncolitic Control	Control	AB	D	AE
IFN-γ	4.047 ± 3.166	1.818 ± 1.131	1.218 ± 0.5085	3.874 ± 2.727	4.432 ± 1.758
IL-10	1.541 ± 0.3521	10.03 ± 2.259	9.478 ± 2.815	5.196 ± 0.5191	8.929 ± 3.585
IL-17A	0.7070 ± 0.2198	10.60 ± 2.219	11.29 ± 5.088	4.218 ± 0.9678	9.976 ± 2.023
IL-1β	5.920 ± 1.054	215.4 ± 83.27	109.1 ± 21.85	80.99 ± 15.87	146.8 ± 23.98
TNF-α	11.70 ± 3.453	117.9 ± 23.94	57.23 ± 9.906	74.63 ± 22.08	85.07 ± 8.529
IL-23	0.6530 ± 0.2656	0.7714 ± 0.2070	2.143 ± 1.355	0.8501 ± 0.4702	0.9517 ± 0.3172
IL-6	12.40 ± 2.384	1,199 ± 641.0	1,089 ± 469.9	267.7 ± 112.9	327.0 ± 148.4

Data are presented as mean ± SEM. Interferon (IFN)-γ, n = 7/group; interleukin (IL)-10, n = 6-7/group; IL-17A, n = 7/group; IL-1 β, n = 7/group; tumor necrosis factor (TNF)-α, n = 7/group; IL-23, n = 2-6/group; IL-6, n = 7/group.

A.5 Appendix materials for Chapter 5

A.5.1 Supplemental Figures and Tables

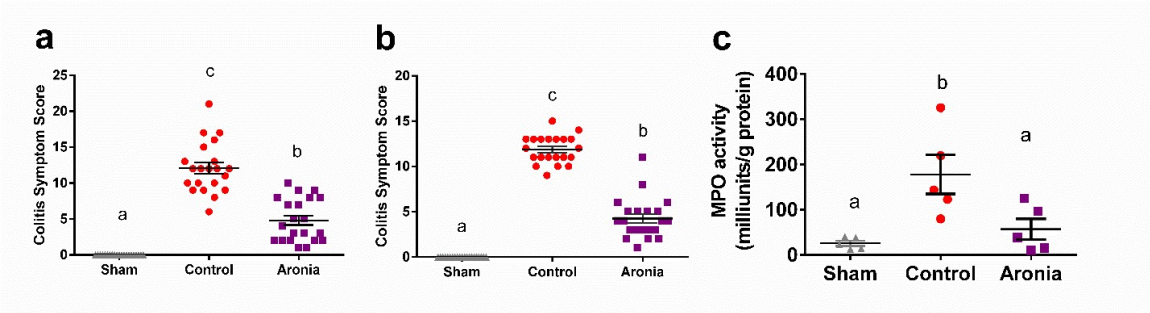

Figure A.5.S1. Consumption of 4.5% aronia berry-supplemented diet inhibits markers of T cell transfer colitis in mice. Splenic $CD4^{+}CD62L^{+}$ cells from C57BL/6J mice or vehicle (sham) were transferred to *Rag1-/-* mice. Mice consumed the control (AIN-93M) or aronia-supplemented diets. Colitis symptom score at (**a**) 5 wk and (**b**) 7 wk after transfer, and (**c**) colonic myeloperoxidase activity (MPO) at 5 wk after transfer. Data are means ± SEMs. Significance testing was by ANOVA with Tukey's test for multiple comparison (PROC GLM). Groups bearing different letters indicate significant differences between treatments ($P < 0.05$).

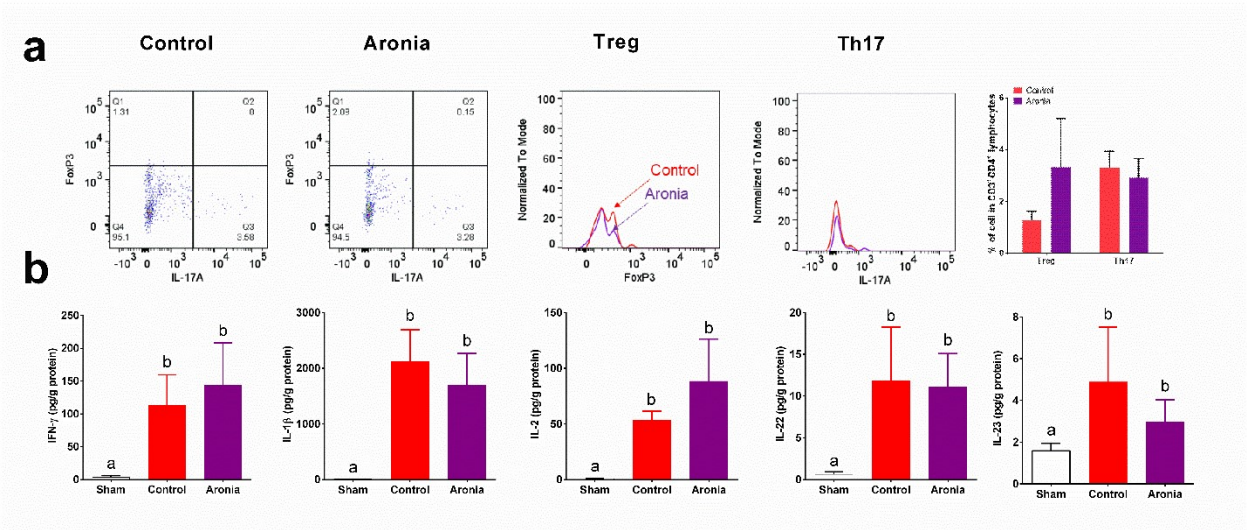

Figure A.5.S2. At 7 wk after T cell transfer, aronia consumption does not alter (**a**) Treg or Th17 in spleen determined by flow cytometry, or (**b**) colonic interferon (IFN)-γ, interleukin (IL)-1β, IL-2, IL-22, or IL-23 in *Rag1*$^{-/-}$ mice. Bar graph data are means ± SEMs, n = 6-7/group, cell populations were compared by Mann–Whitney U-test (PROC NPAR1WAY). *, control vs. aronia, $P < 0.05$. Cytokines were assessed Kruskal–Wallis test with Dunn's test for multiple comparisons (PROC NPAR1WAY). Bars bearing different letters indicate significant differences between treatments ($P < 0.05$).

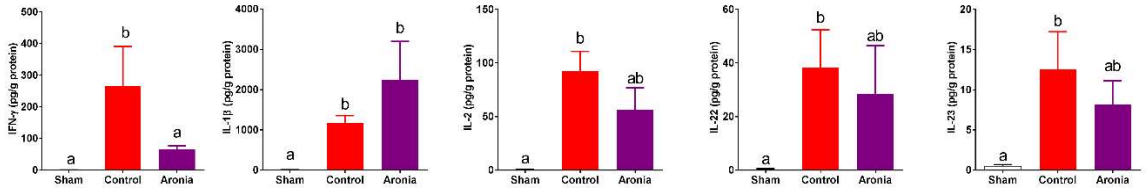

Figure A.5.S3. Aronia consumption modulates colonic cytokines at 5 wk after naïve T cell transfer in *Rag1*$^{-/-}$ mice. Data are means ± SEMs, n = 6-7/group, cell populations were compared by Mann–Whitney U-test (PROC NPAR1WAY). *, control vs. aronia, $P < 0.05$. Cytokines were assessed Kruskal–Wallis test with Dunn's test for multiple comparisons (PROC NPAR1WAY). Bars bearing different letters indicate significant differences between treatments ($P < 0.05$).

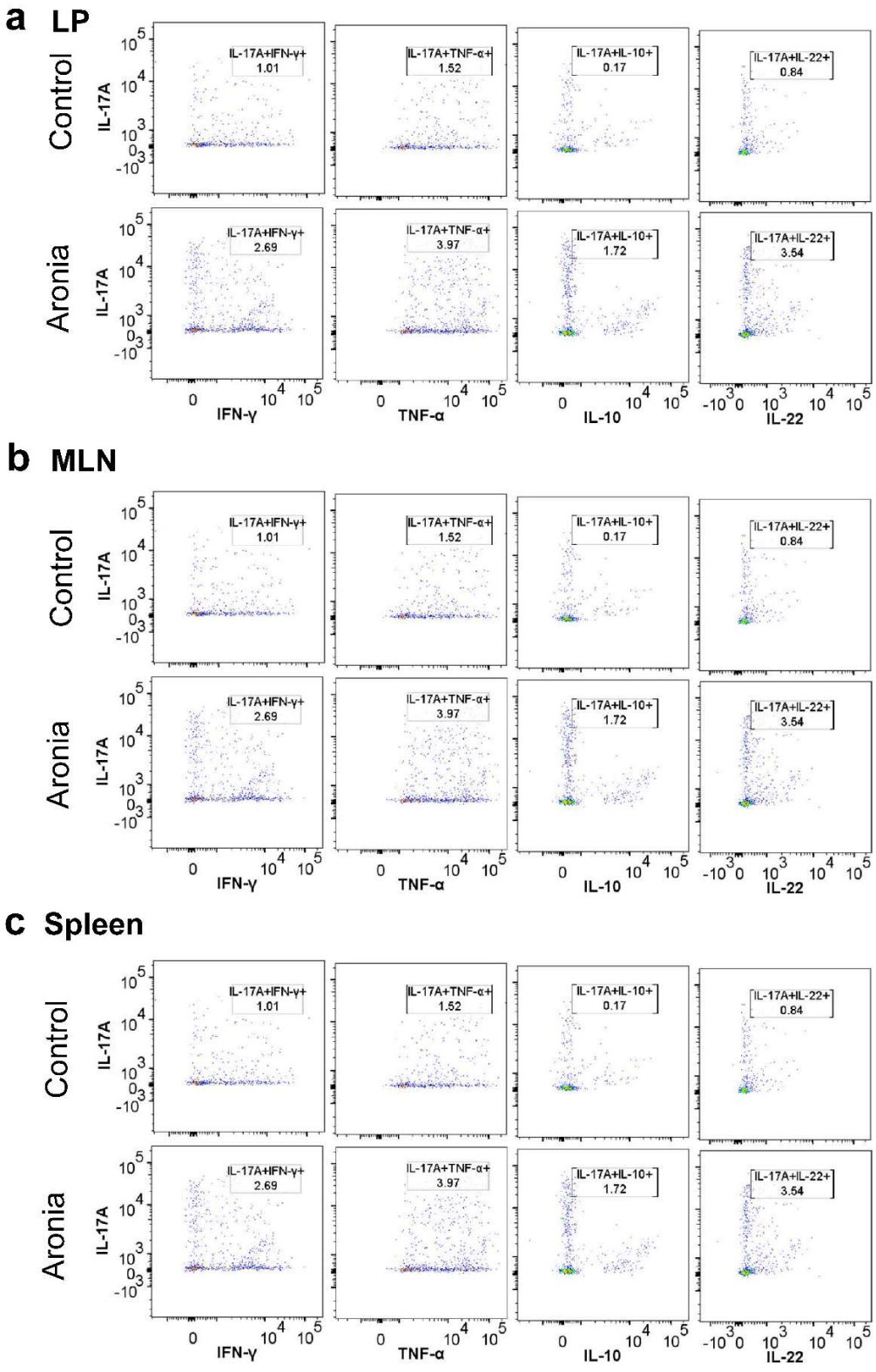
a LP
Control
Aronia
IL-17A
IL-17A+IFN-γ+
1.01
IL-17A+TNF-α+
1.52
IL-17A+IL-10+
0.17
IL-17A+IL-22+
0.84
IL-17A+IFN-γ+
2.69
IL-17A+TNF-α+
3.97
IL-17A+IL-10+
1.72
IL-17A+IL-22+
3.54
IFN-γ
TNF-α
IL-10
IL-22
b MLN
c Spleen

Figure A.5.S4. Representative flow cytometry analysis of Th17 subpopulations isolated from (**a**) lamina propria (LP), (**b**) mesenteric lymph nodes (MLN), and (**c**) spleen at 5 wk after naïve T cell transfer to *Rag1*$^{-/-}$ mice.

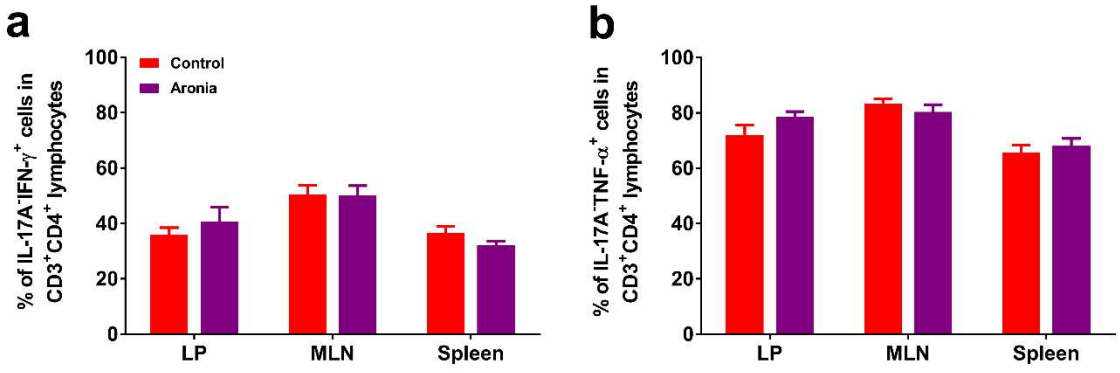

Figure A.5.S5. Aronia supplementation does not affect interleukin (IL)-17A^{-} CD3^{+}CD4^{+} (**a**) interferon (IFN)-γ^{+} or (**b**) TNF-α^{+} proportions in lamina propria (LP), mesenteric lymph nodes (MLN), or spleen 5 wk after adoptive transfer of naïve T cells to *Rag1*$^{-/-}$ mice. Cell populations were compared by Mann–Whitney U-test (PROC NPAR1WAY).

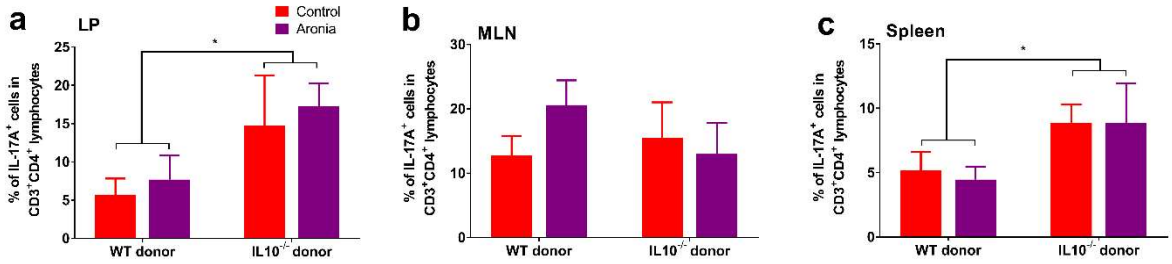

Figure A.5.S6. Aronia consumption does not alter Th17 in *Rag1*$^{-/-}$ mice at 3 wk after adoptive transfer of naïve T cells from wild type or *Il10*$^{-/-}$ donors. Flow cytometry analysis of Th17 ($CD3^+CD4^+IL\text{-}17A^+$) in (**a**) lamina propria (LP), (**b**) mesenteric lymph nodes (MLN), and (**c**) spleen. Data are means ± SEMs of n = 5-7/group. Comparisons were by 2-way ANOVA with the diet and donor type as factors. *, $P < 0.05$ between donor types.

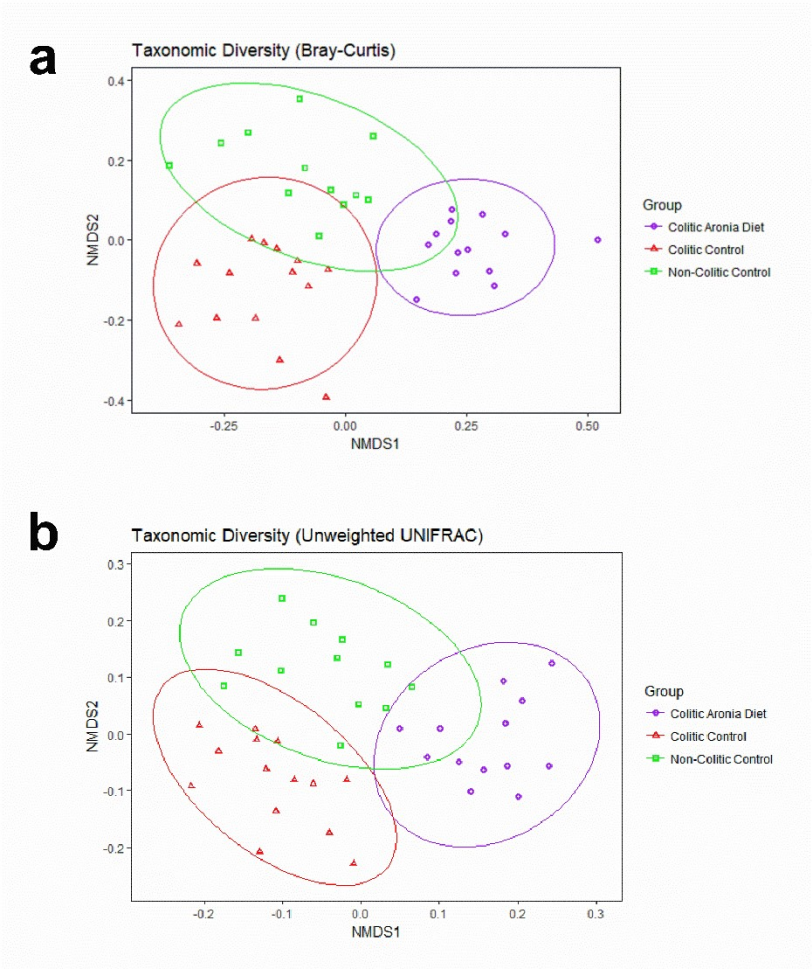

Figure A.5.S7. Consumption of aronia berry induces changes in the gut microbiome composition of colitic mice. At 3 wk after adoptive transfer of $CD4^+$ cells (non-colitic control) and naïve T cells to *Rag1*$^{-/-}$ mice consuming the control diet or aronia-supplemented diet, cecal contents were analyzed by 16S rRNA sequencing. Using the (**a**) Bray-Curtis analysis and (**b**) unweighted UNIFRAC analysis, plotted in two dimensions using NMDS.

Supplemental Table A.5.S1. Mean relative abundance of 16S rRNA reads from cecal microbiota of *Rag1*$^{-/-}$ reconstituted with CD4^{+} and fed AIN-93M diets (non-colitic control) or reconstituted with naïve T cells and fed AIN-93M (control) or the control diet fortified with 4.5% aronia berry powder (aronia).

OTU number	Identification	Mean relative abundance (%)			P-value
		non-colitic control	control	aronia	
1	*Akkermansia muciniphila*	26.3 ± 9.8	41.6 ± 12.1#	9.30 ± 10.1*	0.00001
2	*Allobaculum* (Unclassified)	24.1 ± 10.0	17.2 ± 6.6#	16.5 ± 5.3	0.00497
4	*Bacteroides acidifaciens*	1.49 ± 0.59	3.25 ± 1.05#	1.96 ± 0.91*	0.00170
5	*Family Lachnospiraseae (Unclassified)*	0.650 ± 1.17	2.31 ± 1.03#	3.04 ± 2.85	0.00263
6	*Order Bacteroidales* (Unclassified)	0.882 ± .650	0.538 ± 5.41#	3.35 ± 2.18*	0.00003
7	*Olsenella (Unclassified)*	2.37 ± 1.46	1.72 ± 0.59#	1.26 ± 0.46	0.04946
8	*Bacteroides (Unclassified)*	1.39 ± 0.54	0.973 ± 0.58#	1.83 ± 1.22	0.01985
9	Family *Lachnospiraceae* (Unclassified)	0.017 ± 0.041	0.139 ± 0.422	2.90 ± 2.60*	0.00011
10	Oscillospira (Unclassified)	0.778 ± 0.793	2.46 ± 1.06#	0.479 ± .782*	0.00003
11	*Lactococcus* (Unclassified)	1.36 ± 0.51	0.817 ± 0.256#	1.13 ± 0.32	0.00040
12	Oscillospira (Unclassified)	0.674 ± 0.327	0.394 ± 0.159	1.83 ± 0.67*	0.00001
13	*Family Lachnospiraceae (Unclassified)*	1.07 ± 0.78	0.541 ± 0.439 #	1.40 ± 1.06	0.01669
17	*Lactobacillus* (Unclassified)	1.44 ± 2.48	0.220 ± 0.273	1.27 ± 0.964*	0.00954
19	*Family S24-7* (Unclassified)	0.0011 ± .0041	1.22 ± 0.74	1.27 ± 0.99	< 0.00001
20	*Dorea* (Unclassified)	0.140 ± 0.070	0.117 ± 0.074	2.34 ± 1.29*	< 0.00001
21	*Family Lachnospiraceae (Unclassified)*	0.0025 ± 0.0047	0.0011 ± 0.0027	1.78 ± 5.64*	0.00959
23	*Family S24-7* (Unclassified)	0.0025 ± 0.0032	1.61 ± 1.04#	0.808 ± 1.24	0.00049
24	*Phylum Bacteroidetes* (Unclassified)	0.609 ± 1.036	1.92 ± 1.22	0.0003 ± 0.0014*	0.00031
25	*Kingdom Bacteria* (Unclassified)	0.511 ± 0.904	1.33 ± 0.61#	0.303 ± 0.435	0.00114
27	Family *Lachnospiraceae* (Unclassified)	0.0412 ± 0.0407	0.161 ± 0.116	1.85 ± 1.70*	0.00063
29	*Clostridium* (Unclassified)	0.111 ± 0.083	0.165 ± 0.137	1.69 ± 1.68*	< 0.00001
31	*Ruminococcus gnavus*	0.0365 ± 0.0360	0.0253 ± 0.0171	1.77 ± 1.37*	< 0.00001
37	Oscillospira (Unclassified)	0.0318 ± 0.0191	0.0332 ± 0.0142	1.11 ± 1.13*	< 0.00001
39	*Ruminococcus bromii*	0.884 ± 1.185	0.0015 ± 0.0022#	0.626 ± 1.019	0.02538

Data are means ± SDs, n = 12-14/group. OTU's were identified using similarity percentages calculated using Bray-Curtis Dissimilarity distances. Significance was determined using Kruskal-Wallis rank sum test, with post hoc analysis via the Wilcoxon rank sum test. $P < 0.05$ *, different than control; # different than non-colitic control.