BENEFICIAL EFFECTS OF DIETARY RED RASPBERRY AND PURPLE POTATO ON COLITIS IN EXPERIMENTAL MICE MODELS

By

SHIMA BIBI

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of SHIMA BIBI find it satisfactory and recommend that it be accepted.

Meijun Zhu, Ph.D., Chair

Barbara Rasco, Ph.D.

Michelle McGuire, Ph.D.

Min Du, Ph.D.
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Abstract

by Shima Bibi, Ph.D.
Washington State University
December 2017

Chair: Meijun Zhu

Inflammatory bowel diseases (IBD), Crohn's disease, and ulcerative colitis are chronic relapsing disorders of the gastrointestinal-tract characterized by intestinal-inflammation. Persistent intestinal-inflammation severely impairs intestinal-integrity and activates immune responses increasing the likelihood of colorectal cancer (CRC)-development. Diets rich in polyphenolic and fiber have an inverse association with IBD-development. Red raspberries (RB) and purple potato (PP) are rich sources of bioactive compounds including polyphenols and fiber. The overall aim of this dissertation was to evaluate the effect of dietary RB and PP supplementation on the intestinal barrier function and health in ulcerative colitis, using dextran sulfate sodium (DSS)-induced colitis, a common colitis murine model for IBD research.

In the first study, six-week-old-male mice were fed a control or RB (5% w/w, \( n=20 \)/group) diet for 6-weeks. At 4th-week, approximately half of mice in each-group (\( n=12 \)/group) were subjected to 2.5% DSS-induction for 6-days, followed by 6-days of recovery. RB decreased DSS-induced colitis symptoms, colonic structure distortion, IL-1\( \beta \), IL-6, IL-17,
COX-2, TNF-α, NF-κB signaling, neutrophil infiltration, and xanthene oxidase, while upregulated catalase content in DSS-mice suggesting anti-inflammatory and anti-oxidant activity. RB increased barrier strengthening by reducing claudin-2, and enhancing claudin-3, ZO-1, mucin-2, and activated AMP-activated protein kinase in DSS-mice.

In the second study, the role of RB on prevention of CRC-risk during chronic colitis was examined. Mice were fed with control or RB (5% w/w, n=13/group) diet for 10-weeks. At 4th-week, mice were subjected to two-repeated-cycles of 1% DSS (7-days-DSS-treatment, and 14-days-recovery). RB reduced the colitis-symptoms, inflammation, and immune responses, and facilitated epithelium repair associated with suppressed β-catenin and STAT3 signaling. RB enhanced the tumor suppressor p53 stability and reduced oncogenes expression.

In summary, RB delayed colitis onset, reduced inflammatory and immune responses, improved intestinal epithelium repair and barrier function, and facilitated anti-cancerous effects.

In the third study, mice were fed with a control AIN-93G basal-diet or the diet supplemented with PP (10% w/w, n=20/group) for 7-weeks. At 5th-week, colitis was induced in half of the mice (n=12/group) by 2.5% DSS-induction for 7-days followed by 7-days of recovery. PP improved colitis symptoms, colonic structure distortion, and inflammation in the DSS-mice, suggesting protective effect.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ iii
ABSTRACT .............................................................................................................................. iv
LIST OF TABLES ...................................................................................................................... x
LIST OF FIGURES ..................................................................................................................... xi

CHAPTER ONE: INTRODUCTION ......................................................................................... 1

CHAPTER TWO: LITERATURE REVIEW ................................................................................. 6

1.1 Introduction - the gastrointestinal tract (GIT) ................................................................. 6
1.2 Mucosa .............................................................................................................................. 6
1.3 The intestinal epithelium ................................................................................................. 8
1.4 Intestinal epithelial cells ................................................................................................. 8
1.4.1 Epithelial cells proliferation and differentiation ......................................................... 9
1.4.2 Epithelial cells in regulation of gut barrier function .................................................. 11
1.5 Tight junctions ............................................................................................................... 12
1.6 Gut microbiome ............................................................................................................. 13

2. The intestine as an immune organ .................................................................................... 14
2.1 The immune system in intestine ................................................................................... 14
2.2 Induction of immune responses and inflammation in intestine .................................... 15
2.3 Epithelial cells and gut immune system ......................................................................... 17
2.4 Gut microbiota and immune system of intestine ............................................................ 19
3. IBD........................................................................................................................................20
3.1 Pathogeneses...........................................................................................................20
3.2 Therapies of IBD..................................................................................................................21
3.3 Alternative strategies ........................................................................................................22
3.4 Experimental mouse models of IBD..................................................................................23
4. Fruits and vegetables promote gut health ........................................................................24
4.1 Raspberry and potato bioactive components .................................................................25
4.2 Mechanisms linking beneficial effects of RB and PP on intestinal health ...................26
4.2.1 Regulation of inflammatory NF-κB cascades by RB and PP bioactive constituents ....26
4.2.2 Down-regulation of oxidative stress by RB and PP bioactive constituents...............28
4.3 Regulation of pathways linked to proliferation and CRC development by RB and PP bioactive constituents..................................................................................................................29
4.3.1 Wnt/β-catenin..................................................................................................................30
4.3.2 STAT3..................................................................................................................................31
4.3.3 p53......................................................................................................................................32
4.4 RB and PP bioactive constituents promote differentiation............................................33
4.5 RB and PP bioactive constituents regulate TJs assembly...............................................34
5. Effect of potato on gut microbiome and intestinal epithelial health..............................35
5.1 Gut microbiome and intestinal health: a brief overview...............................................36
5.2 Potato and gut microbiome .............................................................................................37
5.2.1 Potato resistant starch and microbiome .................................................................38
5.2.2 Potato fiber and microbiome.....................................................................................39
5.2.3 Potato polyphenols and microbiome ................................................................................................................. 40
5.3 Potato polyphenols and gut microbial metabolites in intestinal epithelium barrier function .......................................................................................................................................................................................... 41
5.3.1 Potato polyphenols reduces proliferation and improves differentiation and barrier function...................................................................................................................................................................................................................... 42
5.3.2 Gut microbial metabolites promote intestinal barrier function ................................................................................................................................................................................................................................................................................. 43
5.4 Concluding remarks ........................................................................................................................................................................................................................................................................................................................................................................... 45
6. Summary and perspective ........................................................................................................................................................................................................................................................................................................................................................................... 46
7. Figures and legends ......................................................................................................................................................................................................................................................................................................................................................................................................................... 47
8. References ........................................................................................................................................................................................................................................................................................................................................................................................................................................... 49

CHAPTER 3: DIETARY RED RASPBERRIES ATTENUATE DEXTRAN SULFATE SODIUM-INDUCED ACUTE COLITIS ........................................................................................................................................................................................................................................................................................................................................................................................................................................................................................................... 85
1. Abstract ......................................................................................................................................................................................................................................................................................................................................................................................................................................................................................... 85
1. Introduction ......................................................................................................................................................................................................................................................................................................................................................................................................................................................................................... 86
4. Results ......................................................................................................................................................................................................................................................................................................................................................................................................................................................................................... 92
5. Discussion ......................................................................................................................................................................................................................................................................................................................................................................................................................................................................................... 94
6. Conclusion ......................................................................................................................................................................................................................................................................................................................................................................................................................................................................................... 97
7. References ......................................................................................................................................................................................................................................................................................................................................................................................................................................................................................... 99
8. Figures and legends ......................................................................................................................................................................................................................................................................................................................................................................................................................................................................................... 105
9. Supplementary tables ......................................................................................................................................................................................................................................................................................................................................................................................................................................................................................... 109

CHAPTER 4: DIETARY RED RASPBERRY REDUCES COLORECTAL INFLAMMATION AND CARCINOGENIC RISK IN DSS-INDUCED CHRONIC COLITIS IN MICE ........................................................................................................................................................................................................................................................................................................................................................................................................................................................................................................... 113
1. Abstract ......................................................................................................................................................................................................................................................................................................................................................................................................................................................................................... 113
## Table of Contents

2. Introduction................................................................................................................. 114

3. Material and Methods .............................................................................................. 115

4. Results......................................................................................................................... 118

5. Discussion.................................................................................................................... 121

6. Conclusion ................................................................................................................. 125

7. References ............................................................................................................... 126

8. Figures and legends ................................................................................................. 132

9. Supplemental material ............................................................................................ 137

CHAPTER 5: DIETARY PURPLE POTATO SUPPLEMENTATION ATTENUATES COLITIC SYMPTOMS IN DSS-INDUCED COLITIS IN MICE .................................................. 144

1. Abstract ...................................................................................................................... 144

2. Introduction ................................................................................................................ 145

3. Material and Methods ............................................................................................. 146

4. Results ...................................................................................................................... 149

5. Discussion ............................................................................................................... 150

6. Conclusion .............................................................................................................. 152

7. References ............................................................................................................. 153

8. Tables ...................................................................................................................... 157

9. Figures and legends .............................................................................................. 159

CHAPTER 6: CONCLUSION AND FUTURE WORK ......................................................... 163
LIST OF TABLES

Table S1.1: Composition of the experimental diets ................................................................. 106-107

Table S1.2: Primer sequences used in qRT-PCR assays in colonic tissue ..............................108

Table S2.1: Composition of the CON and 5% RB experimental diets given to male (C57BL/6J)
mice for 10-weeks.........................................................................................................................135

Table S2.2: Mineral Mix used at 10 g/kg of diets......................................................................136

Table S2.3: Disease activity index (DAI) scoring criteria .........................................................137

Table S2.4: Primer sequences used in qRT-PCR assays of colonic tissue ...............................138

Table 3.1: Composition of the experimental diets ....................................................................154

Table 3.2: Disease activity index (DAI) scoring criteria ............................................................155
LIST OF FIGURES

Figure 1.1 RB supplementation improves symptoms of DSS-induced colitis ........................................... 102

Figure 1.2 RB supplementation ameliorates gut histopathobiological score and inflammation in DSS-induced colitis ........................................................................................................... 103

Figure 1.3 RB supplementation attenuates neutrophil infiltration and related oxidative stress in the colonic tissues of mice with DSS-induced colitis ............................................................................ 104

Figure 1.4 RB supplementation enhances gut barrier and AMPK phosphorylation in colonic tissues of mice with DSS-induced colitis ......................................................................................................... 105

Figure 2.1 Symptoms of DSS induced chronic colitis in CON (□) or RB (■) fed mice ................. 129

Figure 2.2 Inflammatory mediators, immune cells and related adhesion signaling in DSS induced mice fed with CON (□) or RB (■) supplemented diets ................................................................. 130

Figure 2.3 Epithelial cell differentiation and proliferation markers in DSS induced mice fed with CON (□) or RB (■) supplemented diets ..................................................................................................... 131

Figure 2.4 β-catenin and signal transducer and activator of transcription (STAT) 3 signaling in DSS induced mice fed with CON (□) or RB diets (■) ......................................................................................... 132

Figure 2.5 p53 and its downstream signaling in the colon of DSS induced mice fed with CON (□) or RB (■) diets ........................................................................................................................................ 133

Figure S 2.1 Weekly body weight in male (C57BL/6J) mice fed with CON (□) or RB (■) supplemented diets for 10-weeks ............................................................................................................. 139

Figure 3.1 Feed intake and body weight before DSS treatment ........................................................... 156

Figure 3.2 Symptoms of DSS-induced colitis in mice ............................................................................ 157
Figure 3.3 PP supplementation improves DSS-induced colon shortening ........................................158

Figure 3.4 PP supplementation improves gut histopathobiological score and inflammation in DSS-induced colitis ........................................................................................................................................159
CHAPTER ONE: INTRODUCTION

The human body interacts with the external environment through different sites. The sites include skin and mucosa of nostrils, oral cavity, urinary tract, and gastrointestinal tract (GIT). The GIT is the largest mucosal surface in the body that is directly facing a diverse external environment. This environment consists of diverse food, commensal, and other microbes, and their derived antigens that constantly stimulate the intestinal immune system (Kuhn et al. 1993). Persistent activation of the intestinal immune system both due to pathogenic microbes or food toxic antigens greatly affects the GIT health and can lead to chronic disease of the bowel.

Inflammatory bowel disease (IBD) is a complex, chronic, and relapsing disease of the GIT encompassing Crohn's disease (CD) and ulcerative colitis (UC), both of which are immunologically mediated. In CD inflammation can occur at any site of the GIT from mouth to anus, while in UC the colon is affected. Recurring intestinal mucosal injury and sustained inflammation, increase the likelihood of colorectal cancer (CRC). Though the exact etiology of IBD is unknown, the following factors are associated with common pathogenesis: genetics, persistent activation of immune system by pathogenic microbes, chronic inflammation, unbalanced intestinal microbiota (dysbiosis), and defective mucosal barrier function (Sartor 2006).

IBD creates huge health expenses in the USA and around the world (Molodecky et al. 2012). The incidence of IBD is parallel with the development of populations and adaptation to a western lifestyle, which mainly include changes in dietary habits, less physical activity, and more stress (Loftus 2004). The possible pharmacological therapies for IBD include long-term usage of anti-inflammatory drugs, while other could be surgery or combination of both (Camilleri et al.
However, the long-term drug treatment is not reliable because it can cause serious side effects. Therefore, alternative approaches with low risk effects are needed. Dietary interventions such as intake of fruits and vegetables rich in polyphenolics and fiber will be good alternatives for reducing the risk of and potentially preventing IBD.

Berries are one of the nutritional fruits that contain high amounts of plant phenolics, fiber and other important macro and micronutrients. The different colors of berries such as blue, black, purple and red are mostly due to the presence of anthocyanins which have antioxidant content (Tulio et al. 2008). Among the berries, red raspberries (RB) are rich sources of essential nutrients and polyphenolics namely anthocyanins and ellagitannins. The chemical compositions and health beneficial aspects and biological activity of the red raspberries have been reviewed in detail (Rao and Snyder 2010). The whole raspberries have anti-oxidative activity (de Souza et al. 2014), and black raspberries have shown anti-inflammatory effects following dextran sodium sulfate (DSS)-induced injuries in mice (Montrose et al. 2011). However, the beneficial effects of whole RB on the colitis models have not been studied.

Potato is one of the main staple foods used by people throughout the world. The potato is a rich source of health beneficial nutrients which includes ascorbic acid, potassium, dietary fiber (when skins are eaten), magnesium, phosphorus, B-vitamins, and phenolic compounds (Camire et al. 2009). The polyphenol anthocyanin is a major antioxidant in red and purple cultivars of potato (Reyes et al. 2005). The anthocyanins in potato are more stable than other food sources due to acetylation, which make potatoes unique among other anthocyanin containing food sources since anthocyanins commonly are relatively unstable (Eichhorn and Winterhalter 2005; Xiao and Hogger 2015). In recent studies, purple potato (PP) anthocyanins have induced
apoptosis in human stomach cancer cell lines, and steamed cooked purple potato were shown to suppress the growth of mouse stomach cancer (Hayashi et al. 2006). Similarly, potato extract has induced apoptosis in LNCaP and PC-3 prostate cancer cell lines showing anti-cancer properties (Reddivari et al. 2007). However, the potential health benefits of consuming whole PPs on intestinal function and especially in the active IBD have not been investigated.

To better explore the potential health beneficial aspects of whole freeze-dried RB and PP on prevention of UC, the overall aim of this study was to evaluate the effect of dietary RB and PP supplementation on the intestinal barrier function and intestinal health in active IBD using DSS-induced experimental colitis mice models. The specific objectives of this study were:

1: To determine the effects of whole RB supplementation on acute colitis using DSS-induced colitis mouse model. We hypothesized that pre-feeding of whole RB in mice had a protective effect against the severity of symptoms of DSS-induced experimental colitis possibly through its anti-inflammatory activity.

2: To examine the effect of dietary RB supplementation on inflammation, epithelium repair and oncogenic signaling in DSS-induced chronic colitis in mice. We hypothesized that dietary RB facilitates epithelium repair and reduces the risk of CRC due to its anti-inflammatory property.

3: To determine the effect of PP supplementation on the DSS-induced acute colitis in mice. We hypothesized that PP being rich source of anti-inflammatory anthocyanin and fiber would improve DSS-induced colitis symptoms in mice.
1. References


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CHAPTER TWO: LITERATURE REVIEW

1.1 Introduction - the gastrointestinal tract (GIT)

The GIT is a long contentious tube starting from mouth, running through the body, and ending at the anus. The GIT has specialized compartments that digest the material put in at the mouth, extract useful components from it, and then expel the waste material at the end. This whole process is under the control of complex signaling including the release of different hormone with the presence of food in the mouth. The anatomical parts with their physiology include, mouth that chews the food, esophagus that propels the food from mouth to stomach, stomach that both chemically and mechanically breakdowns the food, small intestine that digests and absorbs the nutrients, pancreas that secrets enzymes for digestion into the small intestine, and large intestine that digests and absorbs the remaining nutrients, and expels the waste end products from the body through the rectum. The small intestine is further divided into the duodenum, the jejunum and the ileum, and the large intestine is divided into the cecum and colon (Ganong and Ganong 1995).

The intestine inner surface is irregular due to several projections to enhance the surface area (200 m² for an adult human) for digestion and absorption. Every organ of the GIT has distinct differentiated cell types that helps digestion, absorption of nutrients, and metabolic homeostasis. The intestine inner layer, called mucosa, consists of intestinal epithelium, lamina propria (LP) and muscularis mucosae (layers of smooth muscle fibers) forms the largest mucosal surface in the body that is directly facing a diverse external environment (Rescigno 2011).

1.2 Mucosa

The mucosa is a contiguous layer covering the intestine that forms a barrier between the
luminal content and the remainder of the host, making mucus as the first line of defense in intestine. It limits the entry of lumen microorganisms and toxic food substances into the epithelium (Linden et al. 2008). The GIT contiguous mucosal gel lining can be divided into two layers. An outer loosely adherent layer, which is easy to remove by suction, and an inner firmly adherent layer attached to the mucosa (Atuma et al. 2001). The outer layer habitats microbiome colonization, while the inner layer is almost void of microbes, providing a barrier against bacterial adhesion and invasion (Johansson et al. 2008).

The hydrated gel of mucosal lining is mainly composed of mucins (MUCs) secreted by goblet cells. MUCs are heterogeneous family of large complex O-glycosylated proteins. This family contains at least 16 human MUCs with a varying profile of expression between different tissues and the most divers profile of expression in the GIT. MUCs family contains 3 sub-families, the gel-forming secreted MUCs, the non-gel-forming secreted MUCs (MUC-7 secreted in saliva), and the cell surface MUCs. The gel-forming MUCs include MUC-2, MUC-5AC, MUC-5B, MUC-6, and MUC-19, forming a major portion of the mucus. MUC-2 is the major mucin in intestine. The cell surface MUCs include MUC-1, MUC-3A/B, MUC-4, MUC-12, MUC-13, MUC-15, MUC-16, MUC-17, and MUC-20 (Linden et al. 2008). MUC-1 is studies most extensively, having a prominent role in cancer development rather than defense (Byrd and Bresalier 2004; Furr et al. 2010). MUC-1 interacts with β-catenin, linking MUC-1 with the Wingless and Int (Wnt) signaling involved in epithelial cell proliferation (Li et al. 2001; Ren et al. 2002).

The mucosa provides a significant barrier, and aberrant mucin production has been reported in immune-mediated diseases such as IBD. Mice lacking genes for MUC-2 develop
spontaneous colitis (Heazlewood et al. 2008; Van der Sluis et al. 2006). While mice deficient in Muc1(-/-) gene, show enhanced mucus, and protects against DSS-induced colitis through less recruitment of T cells to the inflamed colon (Petersson et al. 2011).

1.3 The intestinal epithelium

Underlying the mucus, intestinal epithelium consists of a single layer of tightly connected epithelial cells, which forms a barrier to allow nutrients and restrict bacteria, viruses and toxic substance. The intestinal epithelium is derived from the endoderm layer. The pseudostratified endoderm consists of undifferentiated cells goes-through a columnar transformation forming structures termed villi. The small intestine epithelium consists of long and thin villi, while colonic epithelium contains wide and flat villi. The villi are separated by a downward proliferating inter-villus epithelium called crypts (Heath 2010). The epithelium of small intestine is specialized for digestion and absorption of nutrients with some highly specialized and metabolically active epithelial cells.

The epithelial cells are generated at the bottom of the crypts and upon differentiation slowly move up to the villus. Each cell on the villus is lined by many microvilli, which form a brush border and have many enzymes that help in digestion and absorption. The colonic epithelium does not have villi and its main function is water absorption (Heath 2010).

1.4 Intestinal epithelial cells

Intestinal epithelial cells, lined up in the epithelium, include the absorptive cells called enterocytes, the secretory cells that produce MUCs called goblet cells, the hormones producing cells called enteroendocrine cells, and the antimicrobial peptides (AMPs) producing cells called Paneth cells (Flier and Clevers 2009). Besides these cells, other specialized epithelial cells are
present in the part of follicle-associated epithelium. They are called membranous or microfold cell (M cell) that take up and transport antigens from the lumen to the mucosal immune system (Miller et al. 2007), and brush cells (BCs) that helps in the chemosensory process (Sbarbati and Osculati 2005).

1.4.1 Epithelial cells proliferation and differentiation

The intestinal epithelium undergoes self renewal process every 3-4 days, which is maintained by a small population of progenitor cells, called stem cells, located at the base of the crypts that give rise to the four differentiated cell lineages (enterocytes, goblet cells, enteroendocrine cells and Paneth cell) (Cheng and Leblond 1974). Usually, under homeostatic conditions, a stem cell divides asymmetrically forming 2 daughter cells, one daughter stem cell and one daughter committed cell. The committed cell differentiates to become one of the 4 types of mature epithelial cells. During intestinal injuries, the stem cell can divide symmetrically forming 2 daughter stem cells to replenish the damaged stem cells (Booth and Potten 2000).

After committing to the specific lineage, the enterocytes, goblet cells, and the enteroendocrine cells partially commit to the transit-amplifying cells at the bottom of the crypt while they are migrating to the top of crypt. The transit amplifying cells divide 4-5 times and usually stay there for 2 days until full differentiation. Upon reaching the villi they are fully differentiated, and finally go through spontaneous apoptosis at the top of the villi and then shed into the lumen. The Paneth cells, unlike the other 3 differentiated epithelial cells, stay at the bottom of the crypt, produce AMPs, and are absent in the colon (van der Flier and Clevers 2009). Alteration in the turnover rate of epithelial cell proliferation and differentiation can lead to improper barrier function (Yang et al. 2015), and hyperplasia due hyperproliferation in colitis.
increasing the risk of malignancies (Babyatsky et al. 1996).

The Wnt/β-catenin pathway is the main force behind epithelial cell proliferation during normal condition and in the diseased state. β-catenin is the main component of this pathway. In the absence of Wnt stimuli, β-catenin is targeted for degradation through a complex consists of adenomatous polyposis coli (APC), casein kinase I, glycogen synthase kinase 3 (GSK3), and axin. This complex degrades β-catenin through its phosphorylation and keeps its levels low. In the presence of Wnt stimulus, the degradation complex does not degrade the β-catenin resulting in its higher levels, which lead to β-catenin translocation in to the nucleus. Inside the nucleus β-catenin form complex with Groucho on T cell factor (TCF) transcription factors, activating transcription of the of Wnt target genes responsible for differentiation and proliferation (Behrens 1999; MacDonald et al. 2009; Moon et al. 2004).

The Notch pathway plays a critical role in the cell lineage specification (Stanger et al. 2005). Active Notch signaling leads to the transcription of Hairy/Enhancer of Split (Hes). Hes-1 represses transcription of the bHLH transcription factor Math-1 to regulate the enterocytes differentiation in intestine (Jensen et al. 2000). Inactivation of Notch pathway via Hes1 deletion in mouse results in the excessive formation of Paneth, goblet, and enteroendocrine cells (Jensen et al. 2000; Suzuki et al. 2005). The terminal differentiation of goblet cells is carried out by a zinc-finger transcription factor called Kruppel-like factor 4 (Klf-4). Loss of Klf-4 results in the loss of goblet cells and MUC-2. Loss of MUC-2 results in the absence of Alcain blue staining, which is used as a marker of goblet cells differentiation (Katz et al. 2002). Klf-4 also plays an important role in the differentiation of enterocytes. Intestinal alkaline phosphatase (ALPi), a differentiation marker of enterocytes, is the target gene of Klf-4 (Hinnebusch et al. 2004). Hes-1,
Klf-4, and ALPi play a regulatory role in the differentiation of epithelial cells and are used as differentiation markers in different studies.

1.4.2 Epithelial cells in regulation of intestinal barrier function

The goblet cells secret MUCs and thus create the first line of defense against microbes. The importance of goblet cells MUCs can be emphasized by the development of spontaneous colitis and susceptibility to inflammation-induced colon cancer in Muc-2 knocked out mice (Van der Sluis et al. 2006; Velcich et al. 2002). Furthermore, goblet cells derived products including trefoil factor 3 (TFF3) and resistin-like molecule-β (RELMβ) help to promote the intestinal barrier function. TFF3 crosslinks with mucin glycoproteins providing mucosal structural integrity and acts as a signal that enhances epithelial repair, and epithelial cells migration (Dignass et al. 1994; Taupin et al. 2000). RELMβ facilitates MUC-2 production, mediates macrophage and adaptive CD4+ T cell responses following infection, and inhibits parasite chemotaxis during GIT nematode infection (Artis et al. 2004; Nair et al. 2008).

Enterocytes can produce some AMPs, for example the C-type lectin, and regenerating islet-derived protein IIIγ (REGIIIγ) both in small intestine and in colon. Additionally, Paneth cells secrete many AMPs such as defensins, cathelicidins and lysozyme in the small intestine (Bevins and Salzman 2011; Gallo and Hooper 2012). The C-type lectins disrupt the Gram-positive cell wall peptidoglycans, and the defensins and cathelicidins target surface membranes of bacteria (Gallo and Hooper 2012; Mukherjee et al. 2014) regulating both commensal and pathogenic bacteria and limiting bacterial resistance to antimicrobial responses. REGIIIγ in the small intestine limits bacteria entry into epithelium, and it is produced when epithelial cells recognize signals of commensal microbes (Vaishnava et al. 2011). Both AMPs and mucins...
interaction results in concentrated antimicrobial activity at the epithelium (Meyer-Hoffert et al. 2008) limiting the bacterial quantity and diversity, which can reach or cross the epithelium and interact with the underlying immune system.

1.5 Tight junctions

Tight junctions (TJs) are complexes of trans-membrane proteins that seal the adjacent epithelial cells near the apical surface to regulate permeability and physiologically active intestinal barrier function. A large number of proteins are involved in the formation of TJs complexes, which include intracellular proteins: zonula occludens (ZO-1, ZO-2, and ZO-3), cingulin, 7H6 and ZA-1, the integral or trans-membrane protein occludin, claudin, junctional adhesion molecules and tricellulin (Laukoetter et al. 2006; Suzuki 2013; Ulluwishewa et al. 2011).

The ZO family proteins are membrane associated proteins in which the intracellular domains of scaffold proteins interact with extracellular loops of proteins in occludin and claudins, and anchor them to cytoskeletal actin thus tightly sealing the epithelium (Fanning et al. 1998; Itoh et al. 1999). ZO-1 knocked out cells show delayed assembly of occludin and claudins into the TJs, indicating ZO-1 importance in the regulation of TJs assembly (Umeda et al. 2004).

The TJs claudins consist of around 24 proteins that express differently in different tissues. The pore-forming claudins that increase the epithelial permeability include claudin-2, claudin-7, claudin-12, and claudin-15. The barrier forming claudins include claudin-1, claudin-3, claudin-4, claudin-5, claudin-8, claudin-9, claudin-11, and claudin-14 (Suzuki 2013).

The role of TJs occludin remained debatable. Occludin is the constituent of filaments in the TJs complexes (Furuse et al. 1996). Though mutation or overexpression of occludin affects
trans-epithelial resistant (Balda et al. 1996; McCarthy et al. 1996), knocked-downing of occludin does not affect the viability of mice and demonstrates normal TJs morphology (Saitou et al. 2000).

The TJs are constantly remodeled due to the dynamic environment of the intestine, and delocalization of TJs with enhanced permeability is reported in active IBD (Nalle and Turner 2015; Suzuki 2013). Studies suggest that several food nutrients (glutamine, polyphenols, and probiotics) enhance and protect TJs barrier integrity (Suzuki 2013), and hence could be potential therapeutic tools for IBD.

1.6 Intestinal microbiome

Intestinal microbiome is a complex community of millions to trillions microbes residing in the human intestine, which co-evolved with its host over the life span and helps its host in many ways such as by protecting against enteropathogens (Candela et al. 2008; Fukuda et al. 2011; Lozupone et al. 2012), extracting nutrients and energy from diets (Macfarlane and Englyst 1986; Sonnenburg et al. 2005), and maintaining the immune function (Kau et al. 2011; Olszak et al. 2012; Roeselers et al. 2013). The imbalanced intestinal microbiota, i.e. dysbiosis, is linked to malnutrition (Kau et al. 2011), obesity (Ley et al. 2006; Turnbaugh et al. 2008), IBD (Dicksved et al. 2008; Frank et al. 2007), and colon cancer (Lupton 2004), suggesting critical effects of intestinal microbe on human health.

Intestinal microbial-host interaction promotes both the functional and structural development of GIT. Intestine formation has compromised in germ free (GF) mice showing reduced capillaries network at the villous and potentially less absorption of nutrients (Stappenbeck et al. 2002), and impaired peristalsis of the GIT (Husebye et al. 1994). Further,
microbiota protects TJs, the epithelium barrier function, and epithelium repair following injuries (Cario et al. 2007; Hooper et al. 2001; Rakoff-Nahoum et al. 2004), while the role of microbial interaction in counteracting stress-induced intestinal damage has been reviewed (Lutgendorff et al. 2008).

Diet reshapes the intestinal microbiota (David et al. 2014), and dietary bioactive components promote the growth and activity of beneficial microbiota (Davenport et al. 2014; Ridaura et al. 2013; Walker et al. 2011). Intestinal bacteria produce short chain fatty acids (SCFAs), which also decrease in IBD patients (Viladomiu et al. 2013). SCFAs acetate, propionate, and butyrate are mostly produced via bacterial fermentation of dietary fiber, resistant starch, and soluble oligosaccharides of fruits and vegetables in the colon, which become energy sources for colonic epithelial cells, and regulate immune responses effectively (Fung et al. 2012; Viladomiu et al. 2013).

2. The intestine as an immune organ

The lumen of the intestine harbors commensal and potentially pathogenic microbes and diverse food antigens. The intestine epithelium is challenged to eliminate the pathogenic microbes, limit the localization and growth of commensal microbes, and remove or inactivate toxic food antigens. For this purpose, the intestine mucosa, epithelium, and the underlying LP are equipped with secondary lymphoid tissues and immune cells that provide adaptive immune responses at the site of infection. Therefore, aside from the digestive function, the GIT is a lymphoid organ, with lymphoid tissues collectively called gut-associated lymphoid tissue or GALT (Nagler-Anderson 2001).

2.1 The immune system in intestine
The GALT can be divided into two immunological important sites; the inductive site and the effector site. The inductive site includes mesenteric lymph nodes (MLNs), and Peyer’s patches, and the effector site includes intraepithelial compartment and LP (Mowat 2003).

The Peyer’s patches are the lymphoid aggregates which are located in the mucosa of small intestine. They contain B cells, T cells, macrophages, dendritic cells (DCs), and M cells present at the follicle associated epithelium (Kerneis et al. 1997; Mowat 2003). The MLNs are the largest lymph nodes in the body. The lymphocytes accumulation in the MLNs requires L-selectin and adhesion molecules α4β7 integrin. L-selectin direct lymphocytes to the peripheral, and α4β7 integrin direct lymphocytes to the mucosal tissues, making MLNs as a cross road for the recirculation of lymphocytes (Wagner et al. 1998).

The LP is a layer of connective tissues underlying the epithelium, and the intraepithelial compartment is located above the basement membrane and between the columnar epithelial cells (Lefrancois and Lycke 2001). The normal mucosa and epithelium is heavily populated with immune cells, the effector T cells (CD4+ T cells, CD8+ T cells), with a greater proportion of CD4+ T cells in the intraepithelial compartment of the colon (Cheroutre et al. 2011).

2.2 Induction of immune responses and inflammation in intestine

Under healthy conditions, the epithelium routinely modulates the luminal contents and assesses intestinal microbe activity to regulate the immune system thus maintaining homeostasis via activation of immune cells, and anti-inflammatory cytokine responses. In response to epithelium leak or microbial assault, the epithelial cells secrete an array of chemokines, which prompt chemotaxis of immune cells into the mucosa to mediate innate and adaptive immunity (Eckmann and Kagnoff 2005).
The pro-inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 stimulate the epithelial cells to activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. In unstimulated cells, NF-κB is sequestered in the cytoplasm as an inactive non-DNA-binding complex by partnering with inhibitor κB protein (IκB) (Caamano and Hunter 2002; Santangelo et al. 2007; Senftleben and Karin 2002). Once stimulated by cytokines, IκB is phosphorylated by IκB kinase which degrades IκB. The free NF-κB enters the nucleus to function as transcription factors and initiate the expression of inflammatory genes (Tak and Firestein 2001). The process eliminates pathogens and promotes tissue healing, however, uncontrolled signaling leads to persistent inflammation that damages the epithelium causing disease (Dongarra et al. 2013).

When the inflammatory response is not controlled, the adaptive immune responses are activated. DCs present in the LP, and GALT of the intestine, migrate to the MLNs, present antigens and activate naive CD4+ T cells (Coombes and Powrie 2008). Upon activation, the CD4+ T cells proliferate and differentiate into subsets, type 1 helper T (Th1) cells and IL-17-producing helper T (Th17) cells. Effective trafficking and diffusion of CD4+ T cells from blood to the intestinal is carried out by the CD4+ T cells interaction with epithelial and endothelial cells through integrins and the corresponding ligands cellular adhesion molecules, fibronectin, chemokine receptors, and chemokines (Thomas and Baumgart 2012). The integrin α4β7 present on the CD4+ T cells (Schweighoffer et al. 1993) interacts with mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1), intercellular adhesion molecule-1 (ICAM-1) (Hubbard and Rothlein 2000), and vascular cell adhesion protein 1 (VCAM-1) expressed on endothelial cells (Elices et al. 1990) to diffuse from blood to intestine immunological sites, where they execute
their function (Gorfu et al. 2009).

The rapid diffusion and inappropriate retention of T cells are hallmarks of chronic inflammation associated with aggravated colitis (Adams and Eksteen 2006). In inflamed intestine, the circulation routes of the T cells alter due to aggravated changes in the adhesion molecules and activated endothelial cells (Salmi and Jalkanen 2005). Anti-inflammatory drugs used in the treatment of IBD inhibit the expression ICAM-1, and VCAM-1 (Mori et al. 1999; Salmi et al. 1994), and treatment with antibodies against MAdCAM-1 and α4β7 improved intestinal inflammation, and colitis in the animal models (Picarella et al. 1997). Similarly humanized anti-α4β7 antibody alleviates inflammation in UC (Feagan et al. 2000; Feagan et al. 2003). In the DSS-induced colitis, the adaptive immune responses are evoked after day 6 of DSS treatment (Kozlowski et al. 2013), and the persistent activation of T cells and aberrant gut homing leads to provoked colitis (Adams and Eksteen 2006; Perse and Cerar 2012). Most of the studies performed on gut homing of T cells in IBD, have investigated CD8+ T cells.

2.3 Epithelial cells and intestinal immune system

Epithelial cells produce frequent immunoregulatory signals which are essential to tolerate immune cells, limit inflammation, and direct proper innate and adaptive immune cell responses against pathogenic and commensal microbes. Epithelial cells produce cytokines IL-25, transforming growth factor β (TGFβ), and thymic stromal lymphopoietin (TSLP) in response to bacteria through pattern recognition signaling. IL-25 results in the basophil progenitors, and TSLP results in the multipotent progenitor type 2 cells expansion and differentiation into DCs and macrophages (Rimoldi et al. 2015; Zaph et al. 2008; Zeuthen et al. 2008). The two distinct populations of mononuclear phagocytes are the pre DC-derived CD11c+CD103+ DCs and
monocyte-derived CD11clowF4/80+CX3CR1hi intestine resident macrophages (Varol et al. 2009; Zigmond and Jung 2013). The CD103+ DCs are migratory antigen-presenting cells in nature, and they move to MLNs and Peyer’s patches upon activation presenting live bacteria and antigen to the adaptive immune cells (Macpherson and Uhr 2004; Schulz et al. 2009). Thus promoting immune tolerance via the differentiation of forkhead box P3 (FOXP3+) regulatory T cells in TGFβ/retinoic acid dependent mechanism (Coombes et al. 2007; Schulz et al. 2009). The CX3CR1hi intestine resident macrophages stay in close contact with epithelial cells and get rid of the pathogens and commensal bacteria that cross the epithelium (Niess et al. 2005; Schulz et al. 2009). These macrophages are promoting tolerance in the intestinal LP via IL-10 production that suppresses production of inflammatory cytokine from colitic T cells and enhances regulatory T cell function (Kayama et al. 2012; Murai et al. 2009).

Epithelial cells help in the direct transport of secretory immunoglobulins across the epithelium. Immunoglobulins, IgA, produced by plasma cells in the LP, get attached to the polymeric immunoglobulin receptor on the basolateral membrane of epithelial cells and actively transcytoses into the lumen (Johansen and Kaetzel 2011). The collaborative association between IgA secreting B cells and epithelial cells aids on an adaptive immune component to the epithelium, which regulates commensal bacterial populations in order to maintain epithelial and immune cell homeostasis (Johansen et al. 1999; Shulzhenko et al. 2011; Suzuki et al. 2004).

The M cell takes up both specific receptor-mediated microbes and nonspecific antigens from the lumen and process it to the immune system of intestine. The small intestine goblet cells contribute to this process by delivering soluble luminal antigens to DCs (McDole et al. 2012). In addition, sub-epithelial mononuclear phagocytes interact with epithelial cells and samples lumen
contents via trans-epithelial DCs (Chieppa et al. 2006; Rescigno et al. 2001). All these suggest that the epithelial cells sampling of luminal contents limits and controls bacterial/antigen translocation to assist appropriate tolerogenic and anti-pathogenic responses (Shan et al. 2013).

2.4 Intestinal microbiota and immune system

The GF animals have shown reduced number of DCs, and colonization with *Escherichia coli* recruited sufficient DCs to intestine (Haverson et al. 2007; Williams et al. 2006), suggesting an important role of the intestinal microbiota in regulating the immune system. Further, the microbial-derived ATP can induce a subset of DCs that stimulate the differentiation of Th17 cells (Atarashi et al. 2008). The number of systemic macrophages is also low in GF pigs (Zhang et al. 2008). In GF mice, the functions of peritoneal macrophages is compromised (Mitsuyama et al. 1986; Morland and Midtvedt 1984), lacking the macrophage activation markers (Mikkelsen et al. 2004). Further, GF rats have low number of systemic neutrophils with an impaired phagocytic killing, and superoxide production, and a higher myeloperoxidase (MPO) activity when compared to conventional rats (Ohkubo et al. 1990). In fact, the microbiota peptidoglycan primes the innate immune system, enhances killing of the *Streptococcus pneumoniae* and *Staphylococcus aureus* via bone marrow-derived neutrophils (Clarke et al. 2010), showing systemic immunomodulation by intestinal microbiota. Moreover, SCFAs interact with G-protein-coupled receptor 43 expressed on immune cells, and reduce inflammation in the DSS-induced colitis model (Maslowski et al. 2009). All together these studies suggest a profound role of intestinal microbiota in the host immune system regulation and thus can have an effect on immune modulated diseases such IBD.
3. IBD

IBD is a complex chronic and relapsing disease of the GIT, which is immunologically mediated. The two main types of IBD are Crohn’s disease (CD) and ulcerative colitis (UC) (Kaser et al. 2010). In CD, the inflammation (transmural in nature) can occur at any sight of the intestine from mouth to anus, in other words it can affect the entire intestinal wall. In UC, the inflammation (non-transmural) is restricted to colon, which affects mucosa and distort crypt architecture. Common features of both the conditions include diarrhea, blood in stool, weight loss, abdominal discomfort and pain, fever, and fatigue (Baumgart and Sandborn 2007).

3.1 Pathogeneses

Research has shed light on the pathogenesis of this complex disease by using animal models, and studying molecular genetics in human in a combination with clinical trials; however, the etiology of IBD remains an enigma. The most common hypothesis to explain the pathogenesis of the IBD is that the persistent activation of immune responses to the infection caused by intestinal microbes, or to environmental factors, lead to chronic intestinal inflammation, and impaired epithelial barrier function in addition to genetic susceptibility (Hill and Artis 2010; Kaser et al. 2010; Maloy and Powrie 2011).

IBD affects around 1.5 million people in the USA and 2.2 million people in the Europe (Loftus 2004). The incidences of IBD have rapidly increased in the USA and Europe in the last decades of 20th century, and is becoming common in the areas of the world where people are adopting a Western lifestyle (Loftus 2004), which resulted in huge health cost in the USA and around the world (Molodecky et al. 2012). Further, chronic recurring inflammation in IBD increases the risk of colorectal cancer (CRC) development (Itzkowitz and Yio 2004). CRC is the
third most common cancer in the USA and Europe, and is becoming common around the world (Ferlay et al. 2015; Siegel et al. 2014).

Epidemiologically it has been revealed that both genetic and environmental factors are associated with IBD induction. The first CD related gene, nucleotide-binding oligomerization domain-containing protein (NOD2), was recognized in the 2001 (Hugot et al. 2001; Ogura et al. 2001), and other studies identified 163 different risk alleles in white populations (Ogura et al. 2001), showing the familial nature of these diseases. In addition to the genetic risk factors, environmental risk factors are associated with the development of IBD (Ananthakrishnan 2015). The environmental risk factor includes the internal environmental risk factor “intestinal microbiota” and the external environmental risk factors such as exposure to breastfeeding and antibiotic during infancy, and exposure to smoking, life stressors, life style, and diet in adulthood (Ananthakrishnan 2015). Among which, high dietary saturated fats, enhanced depression, improper sleep, and low levels of vitamin D and dietary fiber, and dysbiosis have been associated with IBD incidence (Ananthakrishnan 2015). The environmental factors are modifiable and studies evaluating these risk factors are very important to reduce the risk of IBD development.

3.2 Therapies of IBD

Traditionally, therapies of IBD aim to modulate the immune responses and reduce inflammation to induce remission and delay relapse. The course of IBD treatment can be medical, or surgical in nature, or it can be a combination of both. Drugs used for IBD treatment include anti-inflammatory, 5-aminosalicylic acid (5-ASA), corticosteroids, mesalazine, immunosuppressive drugs such as prednisone and azathioprine (Baumgart and Sandborn 2007).
The TNF-α inhibitors, antibodies against IL-6 and IL-12/23, small molecules including Janus kinase inhibitors, and inhibitors of leukocyte trafficking to intestinal sites of inflammation are also reported (Coskun et al. 2016). Further, the surgical treatment includes resection (removing portions of the intestines), colectomy (removing the colon), or proctocolectomy (removing the colon and rectum), and surgery for abscesses (pus filled mass), and fistulas (abnormal tract) (Connelly and Koltun 2013; Ferrari et al. 2016; Hwang and Varma 2008). Though these medical and surgical therapies are effective, they can have unbearable side effects and health risks, which can limit their long term usage (Crowe et al. 2017; Rogler 2010; Xu et al. 2004). Therefore, it is important to find out the alternative strategies using alone or in combination with medication/surgery to minimize the side effects in patients and reduce the risk of disease development in susceptible population.

3.3 Alternative strategies

Diet is one of the modifiable environmental risk factor of IBD. Diets high in saturated fats and low in fruits and vegetables have been associated with IBD development (Ananthakrishnan et al. 2014). The Western high-fat diet (HFD) aggravates DSS-induced colitis in mice (Kim et al. 2010), further exacerbated by the intake of heame (van der Logt et al. 2013), and even maternal HFD consumption during gestation and lactation predisposes offspring to a higher susceptibility to DSS-induced colitis through increased inflammatory responses (Bibi et al. 2017b). On the contrary, bioactive components of fruits and vegetable, polyphenolics and dietary fibers, reduce the colitis symptoms and decrease the risk of IBD development in mice due to their putative anti-inflammatory and anti-oxidative properties (Bibi et al. 2016; Bibi et al. 2017a; Yang et al. 2015). Therefore, consumption of diet rich in fruits and vegetables or their
bioactive constituents can be a good alternative in reducing IBD development. Some of the pilot studies using curcumin for UC or CD treatment (Hanai et al. 2006; Holt et al. 2005; Suskind et al. 2013), and bilberries or green tea polyphenolic (−)-epigallocatechin-3-gallate (EGCG) for UC patients treatment have shown effective results (Biedermann et al. 2013; Dryden et al. 2013), increasing interest in the therapeutic use of polyphenolic rich food for IBD management.

3.4 Experimental mouse models of IBD

Different experimental mouse models have been used for examining mechanism and dietary treatments for IBD (Wirtz and Neurath 2007). For IBD induction, using chemicals, immune cell transfer, and deletion of some target gens (such as IL-10) can induce inflammation in rodents (mouse or rat), which causes impairment in epithelial barrier integrity, and manipulates innate/adaptive immune response. The IL-10 knocked out mouse model is a genetically engineered model, which is extensively used to understand etiology of IBD (Kuhn et al. 1993). Histological characteristics of colitis such as epithelial hyperplasia, mucin depletion, crypt abscesses, and ulcers with inflammatory cell infiltration into the LP and submucosa, are similar to those of human IBD (Berg et al. 1996; Bleich et al. 2004; Kuhn et al. 1993)

The chemically induced IBD models include DSS, 2,4,6-trinitrobenzenesulfonic acid (TNBS), and acetic acid induced models. The DSS-induced colitis model is the most widely used model. DSS is administered in drinking water, using different doses and cycles with a high reproducibility of both acute and chronic colitis (Wirtz and Neurath 2007). DSS impairs the barrier integrity due to cytotoxic effect of DSS on epithelial cells disrupting TJs (Cooper et al. 1993; Poritz et al. 2007) and a quick alterations in the colonic mucosal layer making it permeable to bacteria (Johansson et al. 2010) proceeding inflammation, which is induced through Th1, Th2,
and Th17 responses (Perše and Cerar 2012; Wen et al. 2015).

4. Fruits and vegetables promote intestinal health

Fruits and vegetables contain nutrients including dietary fiber, vitamins, minerals, and polyphenols that all play important role in health. These bioactive constituents regulate immune responses, modulate inflammatory pathways and alter intestinal microbiota to exert beneficial effect on intestine (Montrose et al. 2011; Sanchez-Fidalgo et al. 2015; Yang et al. 2014; Zheng et al. 2014). Polyphenols are bioactive components having anti-oxidative, anti-inflammatory and anti-cancerous activities. The most common division of polyphenols includes five main classes; phenolic acids, flavonoids, stilbenes, lignans, and anthocyanins. In addition, polyphenols may be conjugated to other nutrients such as carbohydrates, or to themselves or to organic acids in fruits and vegetables. After digestion, the mixture of polyphenols cannot be absorbed directly, and most of them are metabolized and absorbed at lower intestine by the microbes present there (Manach et al. 2004), indicating their profound interaction directly with the intestinal. Dietary fiber passes through the digestive system without digestion. Dietary fiber absorbs water and expands, which helps the bowel movement and reliefs symptoms of irritable bowel syndrome (IBS) and constipation (Lembo and Camilleri 2003). The action of water absorption by fiber reduces pressure inside the intestinal tract and can help prevent diverticulosis (Aldoori et al. 1998). Dietary fiber becomes a substrate of the intestinal microbiota which upon degradation produce SCFAs, providing nutrients for colonic epithelial cells (Lin et al. 2014). Degradation of polyphenols by intestinal microbes results in bacterial-derived-metabolites of polyphenols, which demonstrate anti-inflammatory activity that further strengthen the polyphenolic effects on intestinal health. For example, equol is bacterial-derived metabolite of daidzein, which possess
strong anti-inflammatory activity than daidzein (Blay et al. 2010), bacterial-derived metabolites of quercitrin reduce inflammatory cascades via NF-κB pathway both in vivo and in vitro more efficiently than quercitrin (Comalada et al. 2005), and bacterial-derived metabolites of quercetin suppress cyclooxygenase 2 (COX-2) in human adenoma cells LT97 (Miene et al. 2011). All these suggest that fiber and polyphenols rich fruits and vegetables can exert beneficial effect on intestinal health both directly or indirectly through microbial conversions.

4.1 Raspberry and potato bioactive components

Berry fruits are usually consumed in fresh and or processed forms. The fruit is small fleshy and very perishable. Different types of berries include blackberries (Rubus spp.), blueberries (Vaccinium corymbosum), strawberries (Fragaria ananassa), black raspberries (Rubus occidentalis), and red raspberries (Rubus idaeus), which all are cultivated commercially (Seeram 2008).

RB as a whole fruit contains substantial amount of health beneficial components including ellagitannins, flavonoids, vitamin C, fiber, and other macro and micro nutrients (Mullen et al. 2002; Noratto et al. 2017). There are 50 different polyphenolic compounds in RB (Carvalho et al. 2013) with anthocyanins and the ellagitannins, sanguin H-6, and lambertianin C as major polyphenols (Borges et al. 2010; Gasperotti et al. 2010), aiding to a high radical scavenging capacity of RB (de Ancos et al. 2000). In addition, RB contains substantial amount of fiber. According to USDA Nutrient Database (NDB no. 09518), there is 6.5% dietary fiber in frozen RB that upon freeze-drying RB, increases to 35.1%. In this dietary fiber 1.6 ± 0.2% is soluble and 33.5 ± 0.1% is insoluble fiber (Noratto et al. 2017), which further add up to the health beneficial effects of freeze dried RB.
Potato (*Solanum tuberosum* L.) is one of the main staple foods used by people throughout the world. Potato is a rich source of health beneficial nutrients which includes carbohydrates (resistant and non-resistant starches), dietary fiber (when skin is eaten), potassium, magnesium, phosphorus, B-vitamins, vitamin C, and polyphenols (Camire et al. 2009). Potato especially pigmented potato contains a high level of polyphenols, such as chlorogenic acid and anthocyanin. Chlorogenic acid is abundant in white potato (Mattila and Hellström 2007), while anthocyanin is a major antioxidant in colored flesh potatoes (Ezekiel et al. 2013; Reyes et al. 2005). Other reported phenolics in potato include catechin, caffeic acid, ferulic acid, gallic acid, and malvidin (Leo et al. 2008; Nara et al. 2006; Reddivari et al. 2007). The anthocyanins in potato are more stable due to acetylation, which makes potatoes unique among other anthocyanins containing food sources since anthocyanins commonly are relatively unstable (Eichhorn and Winterhalter 2005; Xiao and Hogger 2015). Due to the high contents of anthocyanins in colored flesh potatoes, the consumption of colored potatoes has been increasing in the USA (NPC 2015). Moreover, people with gluten sensitivities can consume potato as a gluten-free alternative to wheat and other grains (Vazquez–Roque et al. 2013).

**4.2 Mechanisms linking beneficial effects of RB and PP on intestinal health**

The polyphenolics and fiber present in RB and PP can regulate different signaling pathways that promote intestinal epithelial health. In this section, we review studies on pathways that link these bioactive constituents to the intestine health and reduce the risk of IBD and CRC development.

**4.2.1 Regulation of inflammatory NF-κB cascades by RB and PP bioactive constituents**

Inflammation is a very basic phenomenon of the host defense in response to harmful
stimuli, pathogens, and tissue injuries. However, persistent chronic inflammation is the root cause of IBD, and is strongly associated with CRC development (Bernstein et al. 2001; Eaden et al. 2001; Itzkowitz and Yio 2004; Ullman and Itzkowitz 2011). The NF-κB protein is the main inflammatory transcription factor, which upon activation, promotes expression of the inflammatory cytokines, TNF-α, inducible nitric oxide synthase (iNOS), COX-2, ICAMs, and VCAMs, and induction of lymphocytes, leucocytes, monocyte and macrophages (Karin et al. 2004).

Polyphenols play a pivotal role in inhibiting the NF-κB pathway at multiple points (Biasi et al. 2011; Bibi et al. 2016; de la Lastra and Villegas 2005; Izzi et al. 2012; Song et al. 2011b; Velmurugan et al. 2010; Wang et al. 2013a). Polyphenols such as anthocyanins from different berries attenuates lipopolysaccharide (LPS)-induced NF-κB(p65) translocation to the nucleus in macrophages (Lee et al. 2014). Similarly, the anthocyanin rich extract of RB, and polyphenols from black raspberry root suppressed IL-1β, IL-6, iNOS, COX-2 and NF-κB inflammatory cascade in LPS/IFN-γ-stimulated RAW264.7 macrophages (Kim et al. 2013; Li et al. 2014). Further, blueberry extract (rich in malvidin and chlorogenic acid), dietary black raspberry, dietary caffeic acid, and dietary ellagic acid administration reduced the expression of IL-6, IL-17, iNOS, IFN-γ, COX-2, TNF-α, and phospho-IκBα of the NF-κB pathway in DSS-induced colitis in mice (Marin et al. 2013; Montrose et al. 2011; Pervin et al. 2016; Rosillo et al. 2011; Ye et al. 2009), while dietary goji berry, a source of polyphenolics and dietary fiber, reduced the expression of IL-6, COX-2, IFN-γ, ICAM-1, VCAM-1, recruitment of leukocytes (neutrophils), and monocyte chemoattractant protein-1 (MCP-1) in the colon of DSS-colitis mice (Kang et al. 2016). Reduction in inflammatory responses due to polyphenolics has improved the IBD.
symptoms in these experimental models of colitis (Kang et al. 2016; Li et al. 2014; Montrose et al. 2011; Pervin et al. 2016; Ye et al. 2009). In addition, dietary RB reduced the plasma IL-6 in obese diabetic mice (Noratto et al. 2017) and dietary PP suppressed the HFD-induced IL-6 in colonic mucosa of the pigs (Sido et al. 2017). Anthocyanin rich extract of bilberry reduced the IFN-γ-induced expression of MCP-1, IL-6, TNF-α, and ICAM-1 in human monocytic THP-1 cells (Roth et al. 2014), and anthocyanins from black soybean seed coats reduced the expression of ICAM-1 and VCAM-1 in IFN-γ-induced endothelial cells (Nizamutdinova et al. 2009).

4.2.2 Down regulation of oxidative stress by RB and PP bioactive constituents

Oxidative stress is caused by the impairment between pro-oxidative and anti-oxidative system. Reactive oxygen species (ROS) produced due to cellular metabolism are eliminated by the anti-oxidant mechanism in the body, and the maintenance of this redox homeostats balance is critical for proper normal function of the tissues and organs (Kanninen et al. 2011; Pi et al. 2010; Reuter et al. 2010). The anti-oxidant defense enzymes that counteract with excess ROS directly include superoxide dismutases (SODs) that convert superoxide to hydrogen peroxide ($\text{H}_2\text{O}_2$); catalases and peroxidases that catalyze $\text{H}_2\text{O}_2$ to water; and the glutathione and thioredoxin that reduce thiol groups of oxidized proteins (Berndt et al. 2007).

The intestinal epithelium is in constant contact with oxidative stress due to the presence of oxidants produced from food material and pathogens. Further, ROS are released by the neutrophils, macrophages, and immune cells upon epithelium damage in response to microbial antigens (Bhattacharyya et al. 2014). The massive activation and infiltration of neutrophils into the mucosa of GIT during chronic inflammation result in huge amount of ROS, aggravating IBD status (Alzoghaibi 2013; Bhardwaj 2008; Pravda 2005). ROS production is thus directly related
to inflammation (Morgan and Liu 2011; Park et al. 2012).

Polyphenols possess anti-oxidative property. The presence of multiple hydroxyl groups and phenyl rings linked to aromatic rings make polyphenols efficient scavengers of the free radicals, and alternative therapeutic agents in IBD management (Biasi et al. 2011). Polyphenols from different plant sources exhibited anti-inflammatory and anti-oxidative effect on several disease models (Cao et al. 2008; Chis et al. 2009; Cuevas et al. 2011; Edgecombe et al. 2000; Nadour et al. 2012; Periasamy and Alshatwi 2013; Rajamurugan et al. 2012; Tsuda et al. 2000). The anthocyanin rich fraction of blueberry inhibited ROS production by elevating SODs activity in liver of acrylamide-induced oxidative stress mouse model (Zhao et al. 2015). Similarly, anthocyanin from red-fleshed apples reduced the ROS production by increasing the expression of SOD1, catalase, and glutathione peroxidase in Rosup-induced oxidative stressed porcine granulosa cells (Xiang et al. 2017). Dietary caffeic acid reduced the production MPO, inflammatory cells infiltrate and ulceration of colon in DSS-induced colitis in mice (Ye et al. 2009).

4.3 Regulation of pathways linked to proliferation and CRC development by RB and PP bioactive constituents

Due to exposure to a divers external environment, intestinal epithelium undergoes a self-renewal every 3-4 days which requires a homeostatic epithelial cells proliferation and differentiation (Booth and Potten 2000; Cheng and Leblond 1974). Impairment in the cell proliferation and differentiation can negatively affect the renewal of epithelium, increasing the possibilities of IBD, and cancer development (Camilleri et al. 2012; Groschwitz and Hogan 2009; Hilsden et al. 1999; Vaarala 2012; Yu 2009). Intestinal epithelium wound healing is
dependent on the homeostasis of three cellular process including restitution, proliferation, and
differentiation of epithelial cells into functional cells in the injured epithelium (Dignass 2001;
Paclik et al. 2008), which requires intricate regulatory mechanisms to prevent uncontrolled
proliferation that eventually can lead to hyperplasia and CRC. Under chronic inflammation,
Wnt/β-catenin and signal transducer and activator of transcript (STAT) 3 signaling pathways are
activated and promote epithelial cell proliferation (Ibrahem et al. 2014). On the contrary, the key
tumor suppressor protein p53 (Bates and Vousden 1996; Yu et al. 2003) is reduced under chronic
inflammation and is lost in about 50% of malignant tumors (Agarwal et al. 1998). Polyphenols
can regulate these signaling reducing IBD symptoms and the risk of CRC development.

4.3.1 Wnt/β-catenin

Intestinal inflammation is known to enhance Wnt/β-catenin signaling, which further
stimulates cell proliferation and CRC development (Claessen et al. 2010). Under normal
condition β-catenin protein is degraded from its multiprotein complex in the cytoplasm and the
remaining binds to E-cadherin and supports cell–cell adhesion in adherens junctions (Rubinfeld
et al. 1993; Su et al. 1993). However, mutation, alteration or the loss of function in APC causes
disruption of the multiprotein complex, which omits degradation and leads to overexpression of
β-catenin (Sieber et al. 2000). Similarly, in CRC, the translocation of β-catenin from the cell
membrane (E-cadherin-bound β-catenin) to either cytoplasm or nucleus, can result in aberrant
transcription signaling (Brabletz et al. 2002). In addition, in carcinoma, overexpressed MUC-1
competes with E-cadherin for binding to β-catenin, and forms MUC-1-β-catenin complex, which
enters to nucleus. Entrance of β-catenin to the nucleus activates expression of oncogene protein
Cyclin D1 (Cnd1) and myelocytomatosis (Myc) (Baldus et al. 2004; Wang et al. 2011a; Xue et
Myc is the first identified downstream target gene of Wnt pathway in CRC cells, which is expressed in all cycling cells of the intestine (He et al. 1998).

Dietary polyphenols down regulate the Wnt/β-catenin signaling in colonic cancer cells (Wang et al. 2012), and related proliferation with reduced expression of Ccdn1, and Myc in the colon of IL-10 knocked out mice (Yang et al. 2015). PP polyphenols possess anti-proliferative activity in early and advanced colon cancer cell lines (Madiwale et al. 2011; Madiwale et al. 2012), reduce proliferation, levels of β-catenin and its downstream Ccdn1 and Myc in CRC cells, and related proliferation with reduced expression of Ccdn1, and Myc in the colon of IL-10 knocked out mice (Yang et al. 2015). PP polyphenols possess anti-proliferative activity in early and advanced colon cancer cell lines (Madiwale et al. 2011; Madiwale et al. 2012), reduce proliferation, levels of β-catenin and its downstream Ccdn1 and Myc in vitro and in vivo (Charepalli et al. 2015). Similarly, purple and red potatoes repress the proliferation of stomach cancer cells both in vitro and in vivo (Hayashi et al. 2006). Furthermore, oral administration of anthocyanins from mirtocyan (2g, for one week) decrease the proliferation of CRC cells in patients (Thomasset et al. 2009). Dietary black raspberries (60g/day) supplementation decreased promoter methylation of Wnt pathway negative regulators in tumors samples of CRC patients (Wang et al. 2011b), and reduced the β-catenin level and colonic ulceration in mice (Wang et al. 2013b). Polyphenolic rich nordic berries reduced the β-catenin and Ccdn1 in multiple intestinal neoplasia/+mice (Misikangas et al. 2007). All these suggest that polyphenols can reduce proliferation by down-regulating Wnt signaling and thus can reduce the risk of CRC.

**4.3.2 STAT3**

STAT3 pathway is involved in the transcription of genes related to cell proliferation and survival (Buettner et al. 2002), and is activated in 50-60% of CRC (Morikawa et al. 2011). STAT3 is also activated by IL-6 with the proliferation of pre-malignant cells during CRC (Grivennikov et al. 2009). Further, STAT3 is associated with β-catenin regulation. In CRC cells,
knocked-down-STAT3 reduced β-catenin mRNA levels, while IL-6 stimulation increased both STAT3 and β-catenin mRNA (Ibrahem et al. 2014), and STAT3 inhibition suppressed β-catenin with significant inhibition of cell proliferation (Kawada et al. 2006). In addition, STAT3 binding site in the promoter region of MUC-1 is involved in the IL-6 induced overexpression of MUC-1 gene in human mammary ductal carcinoma cells (Gaemers et al. 2001). All these indicated that these pathways can crosstalk with each other during IBD, and can enhance proliferation and tumor formation during chronic inflammatory conditions such as IBD.

Dietary polyphenolics targeting STAT3 suggests a therapeutic role of polyphenols in melanomas (Momtaz et al. 2017). Anthocyanin, cyanidin-3-glicoside, reduces the TNF-α-induced proliferation and STAT3 activation in vascular smooth muscle cells (Luo et al. 2012). Bioactive compounds in black raspberry inhibited proliferation of CD3/CD28-activated human CD4+ and CD8+ T lymphocytes, and activation of STAT3 (Mace et al. 2014). Further, IFN-γ-induced activation of STAT3 was reduced by anthocyanins from black soybean seed coats in endothelial cells (Nizamutdinova et al. 2009), and anthocyanin rich extract from bilberry in THP-1 cells (Roth et al. 2014).

4.3.3 p53

Protein p53 is one of the main core tumor suppressor protein that inhibits cell growth, arrests cell cycle, and can activate apoptotic genes causing death of the affected cells (Bates and Vousden 1996). The p53 activity is governed by controlling the stability of the p53 protein which is lost in about 50% of all human cancers (Agarwal et al. 1998). Stability of the p53 protein is regulated by many pathways including its phosphorylation, inhibition of murine double minute 2 (Mdm2) synthesis, and expression of p14ARF. Mdm2 degrades p53 protein (Kubbutat et al. 2006).

4.4 RB and PP bioactive constituents promote differentiation

RB and PP can enhance the differentiation of intestinal epithelial cells, due to polyphenols, fiber, and their microbial-fermented products. In fact, potato fiber and resistant starch increased the number of goblet cells, in the colon of rats fed red meat (Paturi et al. 2012), possibly by the production of SCFAs. SCFA butyrate promotes mucosal restitution and differentiation, and reduces inflammation and tumor proliferation (Awad et al. 1995; D'Argenio and Mazzacca 1999). Further, polyphenolic PP extract increases epithelial differentiation in Caco-2 cells, which is also associated with the activation of AMP-activated protein kinase (AMPK) (Sun et al. 2017). Depletion of goblet cells and MUC-2 in colon is one of the features of IBD (Van der Sluis et al. 2006; Velcich et al. 2002; Yang et al. 2015). We found that RB increased MUC-2 expression and attenuated the DSS-induced colitis in mice (Bibi et al. 2017c).
Further, GSE supplementation has promoted the differentiation indicated by enhanced expression of differentiation markers Klf-4, Hes-1, ALPi, and goblet cells, and increased villus length in IL-10 knocked out mice (Bibi et al. 2016; Yang et al. 2015).

### 4.5 RB and PP bioactive constituents regulate TJs assembly

Dietary bioactive components can modulate the expression of intestinal TJs. PP extract, and SCFA butyrate enhance TJ assembly in Caco-2 cells (Peng et al. 2009; Sun et al. 2017), and vitamin D enhances TJ formation and prevents dextran sodium sulfate (DSS)-induced epithelial disruption (Kong et al. 2008). Chlorogenic acid reduces intestinal permeability and increases intestinal TJ ZO-1 expression in the weaned rats after lipopolysaccharide inflammatory challenge (Ruan et al. 2014). Naringenin, polyphenol present in citrus fruits, enhances TJs formation in Caco-2 cells (Noda et al. 2012), and attenuates DSS-induced colitis in BALB/c mice partially via TJs improvement (Azuma et al. 2013). Polyphenolic-rich GSE increases ZO-1 expression in the intestinal epithelium of rats (Goodrich et al. 2012; Song et al. 2011a), and reduces the expression of pore forming TJ protein claudin-2, in mice (Wang et al. 2013a). Polyphenols regulate the TJ assembly through cross-talk with other signaling (Yang et al. 2017). For example the increased expression of claudin-1 in IL-10 knocked out mice is due to the down-regulation of NF-κB signaling (Wang et al. 2013a), and anthocyanin rich extract of raspberries attenuates DSS-induced histological damage of the colon architecture by inhibiting mitogen-activated protein kinases (MAPKs) (Li et al. 2014). Theaflavins-3'-O-gallate increases the expression of TJs claudin-1, and ZO-1 associated with phosphorylation of AMPK (Park et al. 2015), and 6-Gingerol protects the DSS-induced intestinal inflammatory disorder via activation of AMPK (Chang and Kuo 2015). All these suggest that RB and PP polyphenols can exert their
beneficial effects in TJs regulation through multiple pathways.

5. Effect of potato on intestinal microbiome and intestinal epithelial health

Potato is a rich source of beneficial nutrients including carbohydrates (resistant and non-resistant starches), dietary fibers, polyphenols, vitamins and minerals (Camire et al. 2009). Potato especially pigmented potato contains a high level of polyphenols, such as chlorogenic acid and anthocyanin. Chlorogenic acid is abundant in white potato (Mattila and Hellström 2007), while anthocyanin is a major antioxidant in colored flesh potatoes (Reyes et al. 2005). Polyphenols are bioactive components in plant foods that are known for their anti-oxidative, anti-inflammatory and anti-cancerous activities. Polyphenols are conjugated to other nutrients such as carbohydrates, organic acids or themselves. The majority of them are not able to absorb directly, and are metabolized and absorbed at lower intestine by the microbes present there (Manach et al. 2004), indicating a direct interaction of polyphenols with the intestinal microbes. Degradation of polyphenols by intestinal microbes results in bacterial-derived-metabolites, which may increase polyphenol bioavailability and further strengthen their effects on intestinal health (Blay et al. 2010; Comalada et al. 2005; Miene et al. 2011). Resistant starches and dietary fibers also pass the small intestine and reach to the lower intestine, where they were degraded by intestinal microbiota and produce SCFAs, providing nutrients for colonic epithelial cells (Lin et al. 2014) and exerting beneficial effects on intestine. For example, butyrate promotes cell differentiation, cell-cycle arrest and apoptosis of the colonic epithelial cells exerting protective effect in prevention of colon cancer (Wong et al. 2006). These indicated that the potato phytonutrients associated with potato consumption can have a profound effect on the intestinal microbiome and intestine health due to the synergistic effect of resistant starches, fiber, and polyphenols in
potatoes, which will be discussed in this review.

5.1 Intestinal microbiome and intestinal health: a brief overview

Intestinal microbiome has been considered as a “separate organ” in the body due to its metabolic activities and interaction with the host. They exert beneficial effects on human health by protecting against enteropathogens (Candela et al. 2008; Fukuda et al. 2011; Lozupone et al. 2012), extracting nutrients and energy from diets (Macfarlane and Englyst 1986; Sonnenburg et al. 2005), and maintaining the immune function (Kau et al. 2011; Olszak et al. 2012; Roeselers et al. 2013). The imbalanced intestinal microbiota, i.e dysbiosis, is linked to malnutrition (Kau et al. 2011), obesity (Ley et al. 2006; Turnbaugh et al. 2008), inflammatory bowel diseases (IBD) (Dicksved et al. 2008; Frank et al. 2007), and colon cancer (Lupton 2004), suggesting critical effects of intestinal microbe on human health (Figure 1).

Classification, functional diversity, and the role of intestinal microbiome in health and diseases have been extensively reviewed elsewhere (Lozupone et al. 2012; Sekirov et al. 2010b), and here we provide a brief overview to contextualize their relationship with potato consumption and intestinal health. The intestinal microbiome contains around 35,000 different species (Xenoulis et al. 2008), and is dominated by Bacteroidetes and Firmicutes phyla whereas Actinobacteria (*Bifidobacterium* spp.), Proteobacteria (*Escherichia coli*), Verrucomicrobia (*Akkermansia muciniphila*), Fusobacteria, and Cyanobacteria are present in minor proportions (Eckburg et al. 2005). The intestinal microbiota carryout a number of biochemical functions. They produced SCFAs, *Bacteroides and Prevotella* genera produce propionate (El Kaoutari et al. 2013; Reichardt et al. 2014) whereas Firmicutes phylum dominantly generates butyrate (Louis et
al. 2010), and metabolize resistant starches (Ze et al. 2012). *Bacteroides, Eubacterium, Propionibacterium, Fusobacterium, Bifidobacterium, Lactobacillus, Clostridium, Enterobacterium, Veillonella, Enterococcus, Enterobacteria,* and *Streptococcus* produce vitamin K (Hill 1997), and vitamin K deficiency causes death of germ free (GF) mice (Hirayama et al. 2007). The intestine formation is compromised in GF mice showing reduced capillaries network at the villous and potentially less absorption of nutrients (Stappenbeck et al. 2002), and impaired peristalsis of the gastrointestinal track (GIT) (Husebye et al. 1994). Colonization of the GF mice with *Bacteroides thetaiotaomicron* increased level of sodium-glucose cotransporter, pancreatic lipase-related protein-2, colipase, and apolipoprotein A-IV, improving host nutrients absorption (Hooper et al. 2001). Furthermore, *B. thetaiotaomicron* colonization in GF mice strengthened the epithelial barrier function by increased levels of immunoglobulin A, decay-accelerating factor, and small proline-rich protein-2 (Hooper et al. 2001). Detailed key roles of intestinal microbes in metabolism of food/drugs, development and modulation of the intestinal epithelium and immune system, has been revived in (Sekirov et al. 2010a) and protection in counteracting stress-induced intestinal damage has been reviewed in (Lutgendorff et al. 2008).

### 5.2 Potato and intestinal microbiome

The microbial ecosystem in the intestine is dynamic and influenced by numerous factors especially dietary factors. Diet is one of the key mediators in shaping intestinal microbiome composition and their metabolites (Graf et al. 2015). Potato as an important staple food provides a rich source of phytonutrients including resistant starches, dietary fibers and polyphenols, which are expected to improve intestinal microbial ecology.
5.2.1 Potato resistant starch and microbiome

Starch constitutes about 18% of fresh weight in potato, which is packed in granules composed of amylose and amylopectin in a ratio of 1:3 (Jansen et al. 2001). Amylose to amylopectin ratio and the phosphorylation of starch can impact the digestibility of starch. The amylopectin structure is more digestible than the amylose. Higher-amylose starches become more crystalline upon cooling following cooking, resulting in physiologically resistant starch showing resistance to the digestive enzymes (Camire et al. 2009). The amylose starch accounts for about 31% of the total starch in potato (Jansen et al. 2001), making potato as a good choice for resistant starch (Englyst et al. 1992). Resistant starches are not digested in the small intestine, pass through and reach to the colon, where they are fermented by intestinal microbiota (Macfarlane and Englyst 1986). In the distal intestine, resistant starches enhance probiotic abundance in intestinal microbiome (Sun et al. 2016). Resistant starch from raw potato increases the abundance of *Coprococcus, Ruminococcus*, and *Turicibacter* genera, while decreases *Clostridium, Dorea*, and *Sarcina* genera in the cecum and colon microbiota of pigs, associated with changes in microbial metabolites (Sun et al. 2016). In addition, resistance starches stimulate the butyrate-producing *Faecalibacterium prausnitzii*, while reducing the abundance of potentially pathogenic members of the *Gammaproteobacteria*, such as *Escherichia coli* and *Pseudomonas* spp. in intestinal microbiota of pig (Haenen et al. 2013), thereby improving colonic health (Wong et al. 2006). In addition, MSPrebiotic® (commercialized *Solanum tuberosum* extract containing 70% resistant starch) 12-weeks supplementation reduced Proteobacteria (*Escherichia coli/Shigella*) abundance in older-aged-people that was observed at base line of the study. Further, MSPrebiotic® increased endogenous *Bifidobacteria* and the level
of fecal butyrate in the older-aged-people with a significant reduction in the usage of stool softener medication (Alfa et al. 2017). Colored (red and purple) potato flake diets enrich cecal *Lactobacillus* and *Bifidobacterium* (Han et al. 2008). These findings suggest that potato as a potential natural source of resistant starches can modulates intestinal microbiota and microbiota-derived metabolites, further boosting intestinal health.

**5.2.2 Potato fiber and microbiome**

Potato is a good source of dietary fibers, which mostly locate in the thickened cell walls of the periderm constituting about 1–2% of the tuber (Lazarov and Werman 1996). Potato is one of the most popular fiber sources in the American diet (Slavin 2008). Dietary potato fibers lowers the abundance of colonic *Bacteroides-Prevotella-Porphyromonas* group and increases *Bifidobacterium* spp. and/or *Lactobacillus* spp., as well as the concentration of SCFAs including acetate, butyrate, and propionate (Pastuszewska et al. 2010; Paturi et al. 2012). Fiber fractions from industrial potato pulp, fermented *in vitro* by microbes derived from fecal samples of healthy human volunteers, increase the density of *Bifidobacterium* (Thomassen et al. 2011). Potato fibers increase the abundance of *Faecalibacterium* and *Lachnospira* genera of phylum Firmicutes, while decrease abundance of *Fusobacterium* genus of phylum Fusobacteria in the feces of healthy adult dogs (Panasevich et al. 2015b). Dietary fiber modified from potato, dextrin, stimulates the abundance of the members of the phyla Bacteroidetes (*Prevotella, Bacteroidetes*), and Actinobacteria (*Bifidobacterium*), and reduces the abundance of phylum Firmicutes (*Clostridium, Lactobacillus*), after nine weeks in the intestinal microbiota of rats fed a high-fat diet (Barczynska et al. 2017). In addition, potato fermentable fibers attenuate dextran sulfate sodium (DSS)-induced colitis in mice due to the enhanced SCFAs production.
Collectively, these studies indicated that potato consumption can modulate intestinal microbiome both at phylum and genus levels due to the presence of fiber, and can help support intestinal health.

5.2.3 Potato polyphenols and microbiome

Potato especially pigmented potato can contain a high level of polyphenols. Pigmented potato enriches anthocyanins (Ezekiel et al. 2013), while chlorogenic acid is abundant polyphenolic compound in all potato verities (Mattila and Hellström 2007). In addition, contents of other polyphenols present in potato such as catechin, caffeic acid, ferulic acid, gallic acid, and malvidin have also been reported in different verities (Leo et al. 2008; Nara et al. 2006; Reddivari et al. 2007). Polyphenols, transformed and metabolised by intestinal microbiota (Manach et al. 2004), modulate intestinal microbiota, which was first reviewed in (Selma et al. 2009) to understand the two way phenolic and microbiome interaction on human health. Tea polyphenols including epicatechin, catechin, 3-O-methylgallic acid, gallic acid, and caffeic acid repressed the growth of *Clostridium perfringens*, *Clostridium difficile*, and *Bacteroides* spp. in fecal cultures (Lee et al. 2006). We previously showed that polyphenol-rich grape seed extract (GSE) augmented the abundance of beneficial bacteria, specifically *Lactobacilli* and *Bacteriodes*, in intestinal microbiota of interleukin (IL)-10 deficient mice (Wang et al. 2013a). In a human volunteer study, regular intake of a high-cocoa flavonol drink increased *Lactobacilli* and *Bifidobacteria* in feces (Tzounis et al. 2011). Similarly, purple-fleshed potato diet consumption increased fecal *Clostridia* and *Lachnospiraceae* of pigs, with an increased *Bacteroidetes* to *Firmicutes* ratio, leading to the suppression of colon-systemic oxidative stress and inflammation in high fat diet fed pigs (Reddivari et al. 2013). Furthermore, the intestinal
microbial transformation of polyphenols increases the bioavailability and biological activity of polyphenols in diets via the hydrolytic breaking down from complex polyphenols into smaller bioactive components, which can be easily absorbed across the intestinal epithelium to exert beneficial effects on the host (Crozier et al. 2010; Del Rio et al. 2010). Indeed, microbial-derived metabolite of quercetin (3,4-dihydroxyphenylacetic acid), and that of chlorogenic/caffeic acid (3-(3,4-dihydroxyphenyl)-propionic acid) up-regulate glutathione S-transferase T2 and downregulate cyclooxygenase-2 in human colon adenoma LT97 cell lines (Miene et al. 2011) increasing potential of polyphenols after metabolism in the intestinal. Furthermore, the microbial-derived metabolites of chlorogenic acids, such as dihydrocaffeic acid, dihydroferulic acid, and feruloylglycine improve cognitive function and protect against neuronal degeneration (Verzelloni et al. 2011). All these suggest that polyphenol-rich potato can exert beneficial effect on intestinal health both directly or indirectly through microbial conversions. Further studies are required to enrich understanding of the complex interactions between potato phytochemicals, their metabolites and intestinal microbiome.

5.3 Potato polyphenols and intestinal microbial metabolites in intestinal epithelium barrier function

Intestinal epithelium forms the largest, most critical and highly selective barrier in our body (Maloy and Powrie 2011). Perturbation of intestinal epithelial barrier function is a central pathogenic factor for food allergy and a number of other health problems of intestinal origin (Camilleri et al. 2012; Groschwitz and Hogan 2009; Hilsden et al. 1999; Vaarala 2012; Yu 2009). The intestinal epithelium consists of a single layer of tightly connected epithelial cells (Heath 2010), which undergoes self-renewal every 3-4 days, a process requiring delicate balance

Being absorbed at the lower intestine, polyphenols can affect the epithelium renewal and barrier function due to their putative anti-inflammatory and anti-proliferative actives directly, or indirectly by modulating intestinal microbiota. Prominent among many factors, intestinal microbiota modulates intestinal inflammation and barrier function through their metabolites (Arpaia and Rudensky 2014; Camilleri et al. 2012). Through fermentation, intestinal microbiota produce SCFAs including butyrate, acetate and propionate (Wong et al. 2006), which suppress intestinal inflammation and enhance epithelial integrity.

5.3.1 Potato polyphenols reduces proliferation and improves differentiation and barrier function

Polyphenols are known to prevent hyperproliferation directly or by suppressing signaling pathways stimulating cell proliferation (Wang et al. 2012; Yang et al. 2015). Purple potato polyphenols possess anti-proliferative activity to early and advanced colon cancer cell lines (Madiwale et al. 2011; Madiwale et al. 2012), which is associated with reduced Wingless and Int (Wnt)/β-catenin signaling in vitro and in vivo (Charepalli et al. 2015). Similarly, purple and red potatoes repress the proliferation of stomach cancer cells both in vitro and in vivo (Hayashi et al. 2006).

The intestinal epithelium is covered by lubricating gel called mucus, which is mostly composed of mucin 2 (MUC-2) secreted by the fully differentiated goblet cells. Depletion of
goblet cells and MUC-2 in colon is one of major features of IBD (Van der Sluis et al. 2006; Velcich et al. 2002; Yang et al. 2015). In addition to their inhibitory effects against proliferation, polyphenolics promote the differentiation of epithelial cells. Polyphonic-rich GSE promotes epithelial differentiation as evidenced by enhanced expression of differentiation markers, and increased goblet cell density and villus length of the epithelium in IL-10 knock out mice (Bibi et al. 2016; Yang et al. 2015). In addition, potato fiber and resistant starch diet increase the number of goblet cells, in the colon of rats fed red meat (Paturi et al. 2012). Polyphenolic rich purple potato extract enhances epithelial differentiation in both Caco-2 cells and ex vivo intestinal as indicated by increased alkaline phosphatase (AP) activity and contents of villin and E-cadherin (Sun et al. 2017). Further, the strengthening ability of purple potato extract is associated with the activation of AMP-activated protein kinase (AMPK) concomitant with the elevated expression of the transcriptional factor CDX2, which is critical in regulating intestinal epithelial differentiation (Sun et al. 2017). Tight junction (TJ) proteins seal the adjacent epithelial cells near the apical surface to regulate permeability and maintain intestinal barrier function. Further, purple potato extract enhances the TJ assembly in Caco-2 cells by activating AMPK which reduces permeability and strengthens barrier function (Sun et al. 2017). Polyphenols regulate the TJ assembly through multiple signaling which has been reviewed elsewhere (Yang et al. 2017).

### 5.3.2 Intestinal microbial metabolites promote intestinal barrier function

Evident from the above literature, intestinal microbiome is necessary for digestion of fiber, resistant starches, polyphenols, and acquisition of vitamin. Intestinal microbiome communicates with the host intestinal immune system directly or through their metabolites, SCFAs, to regulate the barrier function. SCFAs reduction reported in IBD patients (Viladomiu et
al. 2013) further pointing out the importance of these metabolites’ functions in intestinal barrier. SCFAs facilitate mucosal development (Tamate et al. 1962), and epithelial cell division (Galfi et al. 1986; Sakata and Tamate 1978) in the rumen, and stimulate epithelial proliferation in the colon of rats (Sakata and Engelhardt 1983). SCFA, butyrate, can promote barrier integrity by regulating inflammatory responses of intestinal macrophages (Chang et al. 2014) and the differentiation of colonic regulatory T cells (Furusawa et al. 2013). Butyrate inhibited the proliferation and increased differentiation and apoptosis in non-differentiated and high-proliferative adenocarcinoma HT-29 cells (Comalada et al. 2006). Oral administration of SCFAs (acetate, propionate, and butyrate) restores the turnover rate of epithelial cells in antibiotic treated specific pathogen free mice, and promotes the intestinal organoids development in vitro (Park et al. 2016). SCFAs stimulate MUC-2 expression in intestinal epithelial monolayers of T84 and LS174T cells co-cultured with myofibroblasts CCD-18Co, suggesting muco-protective effect of bacterial-derived metabolites (Willemsen et al. 2003). Microbial-derived butyrate promotes barrier function via IL10 receptor dependent repression of pore forming TJ claudin-2 (Zheng et al. 2017), and enhances the assembly of TJ in Caco-2 cells by activating AMPK (Peng et al. 2009). Moreover, bacterial-derived butyrate regulates epithelial barrier function via stabilization of hypoxia-inducible factor, which is a transcription factor coordinating barrier protection (Kelly et al. 2015). SCFAs improve barrier function by protection and repair of Caco-2 cell monolayer against disrupting agents (LPS/TNF-α) (Chen et al. 2017). Indole, secreted by intestinal bacteria and detected in human feces, increases mucin production and transepithelial resistance, and reduces inflammation in HCT-8 cells (Bansal et al. 2010). Further, colonization of the mice with single segmented filamentous bacteria induce the Th17 subset of CD4+ T cells that produce IL-
17/IL-22, promoting the production of antimicrobial peptides and tissue repair, and resistance to intestinal *Citrobacter rodentium* infection (Ivanov et al. 2009). Pregnane X receptor (PXR)-deficient (Nr1i2−/−) mice have “leaky” intestine physiology associated with up-regulated Toll-like receptor signaling (Venkatesh et al. 2014), showing bacterial metabolites regulation of the barrier function, as PXR can accommodate diverse small bacterial metabolites (Watkins et al. 2001).

Collectively, potato consumption may have beneficial effect on intestinal health by promoting barrier integrity likely due to its polyphenol and, fiber contents, and intestinal bacterial fermented products.

### 5.4 Concluding remarks

Potato as a main staple food can provide a rich source of phytonutrient including resistant starches, dietary fibers, and polyphenols, which become a substrate for intestinal microbiota. These bioactive compounds and their microbial-derived metabolites contribute to intestinal health by direct interaction with intestinal epithelium, and mostly by modulation of intestinal microbiome compositions. Consumption of whole potato (red, yellow, white, or purple) might promote the abundance of commensal beneficial bacteria and inhibit the growth of pathogenic bacteria exerting a prebiotic effect. Polyphenol-microbial-derived metabolites and SCFAs can further strengthen the intestinal barrier function by nourishing epithelial cells, enhancing their differentiation, mucus production and TJ expression (Figure 2). Therefore, potato consumption may beneficially balance intestinal microbiota and help prevent intestinal chronic diseases of in the host. Further studies are required to investigate the relationship between potato consumption and intestinal microbiota in human and their metabolic output in animal models and clinical
trials.

6. Summary and perspective

Mucosal lining of the GIT is the largest mucosal surface that is facing a diverse external environment. This direct contact makes it susceptible to damage and inflammation which compromise the intestinal barrier function and result in IBD. Presently, IBD does not have cure and mostly it is treated with anti-inflammatory drugs, or antibodies targeting specific cytokines. The food we consume has great effect on the intestinal health, and diet rich in polyphenols and fiber is one of the main focus for treatment of IBD. Dietary polyphenols modulate multiple pathways linked to IBD. Polyphenols target the NF-κB inflammatory signaling at multiple points, reduces oxidative stress, and suppress immune responses. Polyphenols and fiber induce differentiation, reduce the proliferation by targeting Wnt/B-catenin and STAT3 signaling, and inducing p53 stabilization. Dietary polyphenols and fiber improves TJ assembly, modulate intestinal microbiome and increase SCFAs. All together, these pathways crosstalk with each other. Most of the studies conducted on polyphenols for IBD treatment are in animal models, either using a single polyphenol or whole food approach. Few studies have been conducted in humans, however, the promising results from animal studies can give a hope for developing new anti-IBD polyphenols treatments. Further the whole food approach can give more opportunities to use diet as medicine, therefore, future research on the consumption of fruits and vegetables rich in polyphenols (RB and PP) in clinical trials is suggested.
7. Figures and legends

Figure 1. Intestinal microbiota and our health. Genetics and environmental factors such as life style, hygiene conditions, and diet influence the intestinal microbial composition. Dysbiosis affects immune system, and causes intestinal disorders, such as IBD, IBS, and CRC, as well as metabolic disorders, such as obesity, diabetes and CVD. Dysbiosis causes chronic inflammation which is one of the main cause of intestinal and metabolic disorder and is also associated with the immune system dysregulation. CRC: colorectal cancer, CVD: cardiovascular disease, IBD: inflammatory bowel disease, IBS: irritable bowel syndrome.
Figure 2. Effect of potato diet on intestinal microbiota and barrier function. Potato bioactive constituents including resistant starch, fiber and polyphenolics reach to the colon where they become a substrate for intestinal microbiota. Microbial conversion of these constituents results in promotion of beneficial microbe’s growth and the production of SCFAs. The growth of beneficial microbes and SCFAs strengthens intestinal barrier function by inducing tight junction assembly, epithelial cell differentiation, and mucus thickness. SCFAs: Short chain fatty acids.
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73
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79


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CHAPTER 3: DIETARY RED RASPBERRIES ATTENUATE DEXTRAN SULFATE SODIUM-INDUCED ACUTE COLITIS

1. Abstract

Persistent intestinal inflammation severely impairs intestinal integrity resulting in inflammatory bowel disease. Red raspberries (RB) are a rich source of bioactive compounds; their beneficial effect on the protection against the development of colitis was evaluated in the current study using a dextran sulfate sodium (DSS)-induced acute colitis mouse model. Six-week-old mice were fed a standard rodent research diet supplemented with RB (0 or 5% w/w, n=20 each group) for 6 weeks. At the 4th week of dietary treatment, approximately half of the mice in each dietary group (n=12 each group) were subjected to 2.5% DSS induction for 6 days, followed by 6 days of recovery, to induce colitis. RB supplementation decreased body weight loss (P ≤ 0.01), disease activity index (P ≤ 0.01), and colon shortening (P ≤ 0.05) in the DSS-treated mice. In addition, RB supplementation protected the colonic structure (P ≤ 0.01), associated with suppressed NF-κB signaling and reduced expression of inflammatory interleukin (IL)-1β, IL-6, IL-17, cyclooxygenase-2, and tumor necrosis factor-α in DSS-treated mice. RB supplementation reduced neutrophil infiltration, monocyte chemoattractant protein-1 mRNA expression, and xanthine oxidase content, but enhanced catalase content in DSS-treated mice. Consistently, RB supplementation reduced pore forming tight junction protein claudin-2, increased barrier strengthening claudin-3, zonula occluden-1 protein content and mucin (MUC)-2 mRNA level, and activated AMP-activated protein kinase (AMPK) in DSS-treated mice. In conclusion, dietary RB protected against inflammation and colitis symptoms induced by DSS, providing a promising dietary approach for the management of colitis.
1. Introduction

Inflammatory bowel disease (IBD), encompassing Crohn's disease (CD) and ulcerative colitis (UC), is a chronic and relapsing disorder of the intestine. Around 1.5 million people are suffering from IBD in the United States, and is becoming common in the developing countries (Loftus 2004; Vanamala et al. 2008). The common etiological factors of IBD include genetics, dysregulated immune response, chronic inflammation, unbalanced gut microbiota (dysbiosis), and defective mucosal barrier function (Sartor 2006). Pathologically, colitis is characterized by prominent infiltration of neutrophils into colonic lesions, epithelial cell necrosis, and ulceration of the mucosal and submucosal layers (DeRoche et al. 2014). Infiltrated neutrophils release reactive oxygen species (ROS) inducing local inflammation (Nishida et al. 1997), and impairing barrier function (Natsui et al. 1997). The proper intestinal epithelial barrier function depends on the integrity of mucus layer, and the expression and assembly of tight junction (TJ) proteins. AMP-activated protein kinase (AMPK), the main regulator of cellular energy balance, is important for TJ assembly and proper barrier function of the gut epithelium (Park et al. 2015; Zhang et al. 2006). Disrupted mucus layer and TJ complexes further exacerbate inflammatory response aggravating colitis (Poritz et al. 2007; Renes et al. 2002).

Evident from the epidemiological studies, intake of diet high in fruits and vegetables, and low in fats has been associated with lower risk of IBD (Ananthakrishnan 2015; Hou et al. 2011). Berry fruits contain high amounts of polyphenolics (Shahidi and Ambigaipalan 2015; Tulio et al. 2008), which are known for their anti-oxidative (de Souza et al. 2014), anti-inflammatory (Montrose et al. 2011), and anti-carcinogenic activities (Stoner et al. 2007). Red raspberries (RB) reduced oxidative stress in obese diabetic (db/db) mice (Noratto et al. 2017), and its anthocyanin
rich extract (RB-ARF) reduced inflammatory cascades in lipopolysaccharide (LPS)/IFN-γ-stimulated RAW264.7 macrophages (Li et al. 2014). The RB-ARF intraperitoneal injections suppressed histological indices of damage of colonic tissue in dextran sulfate sodium (DSS)-induced BALB/c mouse (Li et al. 2014), a commonly used experimental colitis model resembling to human UC (Elson et al. 1995). RB as a whole fruit contains substantial amount of health beneficial components including ellagitannins, flavonoids, vitamin C, fiber, and others macro and micro nutrients (Mullen et al. 2002; Noratto et al. 2017), suggesting that whole RB supplementation might have a better protective effect than extracts (Seeram 2008) against DSS-induced colitis, which has not been tested yet. Therefore, we hypothesized that pre-feeding of whole RB in mice would have a protective effect against the severity of symptoms of DSS-induced experimental colitis possibly through the anti-inflammatory activity of RB.

3. Material and Methods

3.1 Raspberry powder preparation and supplemental dose justification

Organic frozen RB (Lucerne Foods, Inc. Boise, ID, USA) were purchased from a local Safeway store in Pullman Washington. From the retail food label of frozen RB, the percent daily value (%DV) for total carbohydrates is 7 %, dietary fiber is 20%, vitamin C is 15%, calcium is 2%, and iron is 6%. RB were freeze-dried in VirTis freeze drier (Vertis Co. Gardiner, NY, USA), and were powdered using cyclone mill (Model 3010-060, UDY Corp. Fort Collins, CO, USA). The total polyphenolics in RB is ~11 g gallic acid equivalent (GAE)/kg of dry weight. The RB powder contains 4.24 ± 0.12% protein, 1.91 ± 0.03% fat, 0.81 ± 0.02% ash, 16.14 ± 0.45% moisture. Per a recent publication, freeze-dried RB contains 35.1% dietary fiber in which 1.6 ± 0.2% is soluble and 33.5 ± 0.1% is insoluble fiber (Noratto et al. 2017). The powder was shipped
overnight to the Research Diets, Inc. (New Brunswick, NJ, USA) for making customized rodent research diets containing 0% and 5% of RB on dry weight basis. The dose of RB (5%) supplement was 50 g/kg of the diet. The average daily feed consumption of mouse was 2.40 g/mouse, which equals to 120 mg RB per day for an adult mouse of 20 g (i.e., 6 g RB/day/kg body mass). This converts to about 29.0 g of RB daily consumption for a 60 kg human per the published formula (Reagan-Shaw et al. 2008). The similar supplementation level of RB was used in a recent mice study (Noratto et al. 2017).

3.2 Experimental design and animal diet

Six-week-old wild-type C57BL/6J male mice (Jackson Laboratory, Bar Harbor, ME, USA) were randomized into 2 groups, receiving a standard rodent diet (CON, Research Diets Inc.), or a CON supplemented with RB (5% of dry feed weight, Research Diets Inc.) for 6 weeks. Detailed dietary information was listed in the supplementary Table S1.1. Only male mice were used in the study to avoid the confounding effect of sex. There were 20 mice in each dietary group.

At 4th week of dietary treatment, mice at each dietary group were further randomly divided into 2 sub-groups, receiving a regular tap water with 0 or 2.5% DSS (MP Biomedicals, Santa Ana, CA, USA). This resulted in 4 dietary groups: neither DSS nor RB (CON, n=8), DSS without RB (DSSC, n=12), RB only (RB, n=8), and DSS plus RB (DSSRB, n=12). The CON and RB groups were given normal drinking water while DSSC and DSSRB were given drinking water containing 2.5% DSS for 6 days, followed by 6 days of recovery period with normal drinking water. The DSS-induction and recovery period was used to mimic human UC symptoms (Elson et al. 1995; Kang et al. 2016). Mice were monitored on daily basis for water consumption, body weight, fecal consistency, and blood in the stool throughout the DSS-
treatment and recovery period. All mice were housed in a temperature-controlled room with a
12h light and 12h dark cycle and had free access to diet and drinking water. No difference was
observed in the average amount of water consumption and feed intake among treatment groups.
All animal procedures were approved by the Washington State University Animal Care and Use
Committee (BAF#04316-010).

3.3 Assessment of colitis symptoms and disease activity index

The disease activity index (DAI) score was assessed by the combined score of weight loss
compared to initial weight (scored as 0-4), stool consistency (scored as 0-4), and blood in the
stool (scored as 0-4). The scores were recorded daily during the DSS- induction and recovery
period, according to the previously described method (Kang et al. 2016).

3.4 Tissues collection and processing

Mice were anesthetized with CO₂ inhalation and followed by cervical dislocation. The
colon section was dissected, and a 5 mm segment of distal colon was fixed in freshly prepared
4% (w/v) paraformaldehyde (pH 7.0), processed and embedded in paraffin. The remaining colon
tissue was opened by a longitudinal cut, rinsed in PBS, frozen in liquid nitrogen, and stored at
-80 °C for later biochemical analyses.

3.5 Histological evaluation of colonic ulceration

Paraffin embedded distal colonic tissues were sectioned at 5μm thickness, deparaffinized
and subjected to haematoxylin and eosin (H&E) staining. Histological examination and imaging
were done under Lecia DM2000 LED light microscope (200x, Leica Microsystems Inc.,
Chicago, IL, USA). For pathobiological scoring, each colonic section was scored blindly using a
previously published score criteria (Kang et al. 2016), and 9 sections per animal at constant
interval were used. The scores of crypt damage (0-4 scale), severity of inflammation (0-3 scale), and depth of injury (0-3 scale) were recorded individually. The summation of the scores resulted in the total pathobiological score ranging from 0 to a maximum of 10 per distal colonic section.

3.6 Immunoblotting analyses

Immunoblotting analyses were conducted as previously described (Bibi et al. 2016; Zhu et al. 2007). Briefly, protein extracts from colonic tissues were separated by sodium dodecyl sulfate polyacrylamide (10%) gradient gels and transferred to nitrocellulose membranes. After blocking with 5% w/v nonfat dried skimmed milk, membranes were overnight incubated with the selected primary antibodies at 4°C. Then the blot membranes were subsequently subjected to three times rinse of PBS with 0.5% Tween 20 (PBST), incubation with either IRDye 680 goat anti-mouse or IRDye 800CW goat anti-rabbit secondary antibodies (Li-Cor Biosciences, Lincoln, NE, USA) and then three times rinse of PBST. Finally, the bands were visualized and quantified using the Odyssey Infrared Imaging System and Image Studio™ Lite software (Li-Cor Biosciences, Lincoln, NE, USA). Bands density was normalized to the β-actin content. Antibodies against catalase, cyclooxygenase-2 (COX-2), interleukin (IL)-1β, IL-6, 90ccludin-/total AMP-activated protein kinase (AMPK), and 90ccludin-/total p65 were from Cell Signaling Technology (Beverly, MA, USA). Antibodies against claudin-2, claudin-3, 90ccludin and zonula occluden-1 (ZO-1) were from Invitrogen (Rockford, IL, USA), and the antibody against xanthine oxidase (XO) was from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Anti-β-actin antibody was from the Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA, USA).

3.7 Neutrophil immunohistochemical analyses
The immunohistochemical staining, scoring and analyses of neutrophil were carried out as described previously (Zhu et al. 2007). Briefly, paraffin embedded colonic tissues sections were deparaffinized, hydrated and antigen retrieved. Sections were blocked in goat serum, overnight incubated with anti-Ly-6B.2 antibody (Bio-Rad Laboratories Inc., Hercules, CA, USA), and then incubated with a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 30 min. Signals were visualized using the Vectastain ABC and DAB peroxidase (HRP) substrate kits (Vector Laboratories Inc., Burlingame, CA, USA) and haematoxylin counterstaining. Leica DM2000 LED light microscope (200x, Leica Microsystems Inc. Chicago, IL, USA) was used for images.

Neutrophil infiltration scores were assessed blindly by two researchers per our established method (Bibi et al. 2017). The scores for depth of neutrophil infiltration (scored as 0–3) and staining intensity (scored as 0–4), which was the percent area positive (0, none; 1, <25%; 2, 25–50%; 3, 50–75%; 4, >75%), were recorded individually. The summation of both scores result in 0–7 per distal colonic section. Nine sections per animal at constant interval were used for microscopic examination and score assessment.

3.8 Quantitative Reverse Transcriptase (qRT)-PCR analyses

Total RNA was extracted from the powdered colonic tissue using Dynabeads® mRNA DIRECT™ Purification Kit (Invitrogen, Carlsbad, CA, USA) followed the manufacturer protocol. cDNA was synthesized with the iScript™ cDNA synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). qRT-PCR was performed on a Bio-Rad CFX384 real-time thermocycler. 18S was used as the reference gene (Wang et al. 2013). Primer sequences used in the study were provided in supplementary Table S1.2.
3.9 Statistical analysis

Data were analyzed as a complete randomized design using General Linear Model of Statistical Analysis System (2000), and were expressed as mean ± standard error of mean (SEM). Two-tailed Student’s T-test was used for calculating statistical significance between sample means. A significant difference was considered as $P \leq 0.05$.

4. Results

4.1 RB supplementation ameliorates symptoms of DSS-induced colitis

Mice without DSS-treatment did not show any colitis symptom regardless of dietary groups (Figure 1.1). A 6-day of 2.5% DSS-treatment successfully induced the clinical symptoms of colitis in mice (Figure 1), as shown by body weight loss (Figure 1.1A) and vast increase in DAI scores (Figure 1.1B). All mice survived during the DSS-induction and recovery period. RB supplementation significantly reduced the body weight loss (Figure 1.1A) and the DAI scores (Figure 1.1B) during both the DSS-induction and recovery stages, indicating that RB had a protective role in both those stages. Moreover, an obvious swelling along with shortening in colon length was observed in DSS-treated mice, which was again ameliorated by RB supplementation (Figure 1.1C, $P \leq 0.05$).

4.2 RB supplementation protects histological architecture and reduces inflammation in DSS-induced colitis

The colonic tissues of mice without DSS-treatment showed a typical histological architecture as expected (Figure 1.2A). The DSS-treatment caused injuries to the colon. Most of the epithelial cells were disappeared along with the loss of mucosa and crypts, indicating ulceration in the distal colon of DSS-treated mice (Figure 1.2A). The histopathobiological score
induced by DSS-treatment was reduced by RB supplementation (Figure 2A, $P \leq 0.01$).

Along with severe mucosal damage and elevated histopathobiological score, DSS-treatment enhanced both the protein and mRNA expression of COX-2, IL-1β, and IL-6 in the colonic tissues of DSSC-mice, which were recovered by RB supplementation (Figure 1.2B-F). The mRNA expression of IL-17 and TNF-α was very low in both CON and RB groups without DSS treatment, but their expression was markedly elevated in DSS-treated mice, which was further attenuated by RB supplementation (Figure 1.2G). Consistently, DSS-treatment dramatically activated NF-κB inflammatory signaling as shown by enhanced p65 phosphorylation, which was again attenuated by RB supplementation (Figure 1.2B&H). These data collectively suggest that RB supplementation protected the colon against the DSS-induced colitis and associated mucosal injuries possibly due to its anti-inflammatory activity.

4.3 RB supplementation reduces neutrophil recruitment and related oxidative stress in DSS-induced colitis

DSS-induction led to a prominent and extensive neutrophil infiltration in the distal colon of mice, which was ameliorated due to RB supplementation (Figure 1.3A, $P \leq 0.05$). In agreement, mRNA level of monocyte chemoattractant protein (MCP)-1, a potent chemoattractant of neutrophils, was dramatically increased in DSS-treated group, while RB supplementation decreased MCP-1 expression in DSS-treated mice (Figure 1.3B, $P \leq 0.05$). Xanthine oxidase (XO) is a source of ROS and neutrophil recruitment in mucosal injuries (Nishida et al. 1997). Consistent with the observed neutrophil infiltration, XO content drastically increased in DSSC-mice ($P \leq 0.01$), which was prevented by RB supplementation ($P \leq 0.01$) (Figure 1.3C). Furthermore, RB supplementation restored catalase content, which was decreased in DSSC-mice.
4.4 RB supplementation protects tight junction proteins and activates AMPK in DSS-induced colitis

Consistent with enhanced neutrophil infiltration and deteriorated mucosal structure, the content of pore-forming TJ protein claudin-2 was increased \((P \leq 0.01)\) in the colonic tissues of DSSC-mice (Figure 1.4A), which was restored due to RB supplementation (Figure 1.4A). RB supplementation also improved the barrier strengthening TJ protein claudin-3 \((P \leq 0.01)\), and ZO-1 \((P \leq 0.10)\) in the colonic tissues of DSS-treated mice (Figure 1.4A). In addition, the mRNA expression of MUC-2 was significantly reduced by DSS-treatment \((P \leq 0.05)\), again elevated by RB supplementation (Figure 1.4B, \(P \leq 0.05)\). RB supplementation also enhanced AMPK activation (Figure 1.4C, \(P \leq 0.05)\), consistent with elevated contents of barrier forming TJ protein and MUC-2.

5. Discussion

Raspberry contains high amounts of dietary fiber and polyphenolic compounds, which are well known for their anti-oxidative and anti-inflammatory effects (de Souza et al. 2014; Rao and Snyder 2010). However, few studies related to the beneficial effects of RB and its phytochemicals on intestinal health are available in the literature.

DSS-induced colitis shows clinical, histological, and microscopic resemblances to human UC, with a well characterized onset of colonic injury and epithelial barrier disruption (Elson et al. 1995), making the model suitable for screening of potential therapeutics of IBD (Egger et al. 2000; Valatas et al. 2013). We showed that dietary whole RB had a protective role in the DSS-induced colitis as indicated by attenuated colitis symptoms, inflammation, and mucosal damage,
indicating that the whole RB can be used as a complementary dietary supplement for UC patients. In alignment with our finding, intraperitoneal injections of RB-ARF, started one day prior of DSS-treatment, reduced DSS-induced body weight loss and histological damage (Li et al. 2014), and blueberry extract rich in malvidin and chlorogenic acid lowered the DAI scores, and improved the histology of the colonic tissues of DSS-treated mice (Pervin et al. 2016).

Inflammation plays a crucial role in the pathogenesis of colitis, and NF-κB (p65) is a major pathway in the inflammatory cascades. In this study, dietary RB down-regulated the NF-κB signaling and reduced the production of inflammatory markers in DSS-treated mice. Similar to our findings, the RB-ARF suppressed inflammation in LPS/IFN-γ-stimulated RAW264.7 macrophages in vitro (Li et al. 2014), and blueberry extract down-regulated NF-κB inflammatory cascade in colonic tissues of DSS-mice (Pervin et al. 2016). Inhibition of inflammatory enzyme COX-2 by non-steroidal anti-inflammatory drugs (NSAIDs) (Paiotti et al. 2012), and blocking of IL-6 by anti-IL-6 antibody alleviated inflammation in UC patients (Bernardo et al. 2012). We found that proinflammatory enzyme COX-2 was markedly increased in DSS-treated group, consistent with previous reports (Kang et al. 2016; Pervin et al. 2016), which was suppressed by RB supplementation. In addition, dietary RB supplementation mitigated IL-6 mRNA expression and protein content in DSS-treated mice, highlighting the effectiveness of dietary RB in protection against colitis development.

DSS-treatment led to severe infiltration of neutrophils into colonic lesion. Dietary RB supplementation largely prevented the neutrophil infiltration in the colon, partially explained by the reduced expression of IL-6, IL-1β, IL-17, and TNF-α in DSSRB-mice. Concomitantly, RB also reduced MCP-1, the main chemokine mediating neutrophil migration and infiltration, in the
colon. Neutrophils enhance oxidative stress via production of ROS, and enhanced XO activity generates oxygen free radicals that enhance mucosal lesions (Nishida et al. 1997). Conversely, catalase converts ROS into water, thus reduces oxidative stress in DSS-induced colitis (Pervin et al. 2016). RB was capable of reducing the oxidative stress by reducing XO and enhancing catalase content in the colonic tissues of DSS-treated mice, which further contributes to its protective role in colitis. Supporting our findings, blueberry extract supplementation reduced the oxidative stress and enhanced the serum catalase activity in the DSS-treated mice (Pervin et al. 2016), while black raspberry supplementation reduced COX-2 expression and colonic ulceration in DSS-treated mice, though no change in inflammatory cell infiltration and oxidative stress was noted (Montrose et al. 2011), which could be due to the compositional differences in phytochemicals among blueberry, black raspberry and RB, and the doses of DSS used for colitis induction and the duration of RB supplementation.

Exact mechanisms by which DSS causes colitis and JT loss remain poorly defined. The possibilities include cytotoxic effect of DSS on epithelial cells disrupting TJ (Cooper et al. 1993; Poritz et al. 2007) and a quick alterations in the colonic mucosal layer making it permeable to bacteria (Johansson et al. 2010) proceeding inflammation. Important JT proteins include claudins, occludin and ZO-1. ZO-1 anchors occludin and claudins in cytoskeletal actin tightly sealing the epithelium (Fanning et al. 1998; Itoh et al. 1999). Miss-localization of occludin and claudins is involved in active IBD, with an upregulated pore-forming claudin-2 in CD patients (Zeissig et al. 2007). Disrupted TJ causes extra-junctional localization of occludin forming a complex with the extrinsic death receptor (Beeman et al. 2009) suggesting its involvement in apoptosis (Beeman et al. 2012). Reduced mRNA expression of MUC-2 has been associated with
the development of UC in experimental colitis (Renes et al. 2002), and DSS-treatment decreased thickness of the inner mucus layer and enhanced gut permeability to bacteria (Johansson et al. 2010). Consistent with the previous findings and improved colitis symptoms, RB supplementation mitigated the elevation of claudin-2 protein content, enhanced claudin-3 protein content and MUC2 mRNA expression, and has tendency to enhance the ZO-1 content in DSS-induced colitis. Interestingly, occludin was increased in DSS-treated mice, which might be due to redistribution of TJ after disruption by DSS-injury, providing a protective mechanism of cell death in regulating barrier function of epithelium (Beeman et al. 2012), which warrants further investigation. AMPK suppresses the inflammatory responses (Sag et al. 2008), which improves epithelial barrier function (Sun et al. 2017). Associated with improved colitis symptoms and intestinal barrier, AMPK activity was enhanced in RB supplemented DSS-treated mice. In support of our finding, theaflavins-3’-O-gallate enhanced the phosphorylation of AMPK as well as expression of ZO-1 (Park et al. 2015), and 6-gingerol protected the DSS-induced intestinal inflammatory disorder associated with AMPK activation (Chang and Kuo 2015).

RB as whole fruit contains around 50 different polyphenolic compounds (Carvalho et al. 2013) with anthocyanins and the ellagitannins, sanguin H-6 and lambertianin C as major polyphenols (Borges et al. 2010; Gasperotti et al. 2010). Using the whole fruit approach, we were not able to identify the specific bioactive component in RB responsible for protection against DSS-induced damages. The beneficial effect of RB can be attributed to the synergistic effects of high polyphenolic and fiber content inherent in RB.

6. Conclusion

In summary, dietary RB protected mice against the DSS-induced colitis possibly due to
its anti-inflammatory activity, by reducing cytokine expression, neutrophil recruitment and oxidative stress, and ultimately reducing the colonic mucosal and epithelial damages in the colon. Thus, RB supplementation has preventive roles in IBD symptoms and related gut disease, which can make it a good dietary choice in IBD management.
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8. Figures and legends

**Figure 1.1** RB supplementation improves symptoms of DSS-induced colitis. (A) Body weight loss (percent of initial body weight). (B) Disease activity index (DAI). A higher score represents more severe symptoms. (C) Representative colon images at necropsy and colon length. CON: Control, RB: Red raspberry, DSSC: DSS-treated control mice, DSSRB: DSS-treated mice with RB supplementation. Means ± SEM, n = 12 for DSSC and DSSRB, and 8 for CON and RB. *: $P \leq 0.05$, and **: $P \leq 0.01$
Figure 1.2 RB supplementation ameliorates gut histopathobiological score and inflammation in DSS-induced colitis. (A) Representative distal colonic images of hematoxylin and eosin (H&E) staining (200x), and pathological quantification score. (B) Representative immunoblots. (C&D) Relative protein and mRNA expression of COX-2. (E&F) Relative protein and mRNA expression of IL-1β and IL-6. (G) mRNA expression of IL-17 and TNF-α. (H) Relative protein expression of p65 and phosphorylated p65. CON: Control, RB: Red raspberry, DSSC: DSS-treated control mice, DSSRB: DSS-treated mice with RB supplementation. 18s rRNA mRNA was used as the reference gene. Means ± SEM, n = 12 for DSSC and DSSRB, and 8 for CON and RB. #: P ≤ 0.10, *: P ≤ 0.05, **: P ≤ 0.01.
Figure 1.3 RB supplementation attenuates neutrophil infiltration and related oxidative stress in the colonic tissues of mice with DSS-induced colitis. (A) Representative images of neutrophil immunohistochemical staining and quantification scores of neutrophil infiltration. (B) mRNA expression of MCP-1. 18s rRNA mRNA was used as the reference gene. (C) Catalase and xanthine oxidase (XO) content. CON: Control, RB: Red raspberry, DSSC: DSS-treated control mice, DSSRB: DSS-treated mice with RB supplementation. Means ± SEM, n = 12 for DSSC and DSSRB, and 8 for CON and RB. #: $P \leq 0.10$, *: $P \leq 0.05$, **: $P \leq 0.01$. 
Figure 1.4 RB supplementation enhances gut barrier and AMPK phosphorylation in colonic tissues of mice with DSS-induced colitis. (A) Representative immunoblots and relative contents of tight junction proteins. (B) mRNA expression of MUC-2. 18s rRNA mRNA was used to the reference gene. (C) Representative immunoblots and relative protein content of total and phosphorylated AMPK. CON: Control, RB: Red raspberry, DSSC: DSS-treated control mice, DSSRB: DSS-treated mice with RB supplementation. Means ± SEM, n = 12 for DSSC and DSSRB, and 8 for CON and RB. #: $P \leq 0.10$, *: $P \leq 0.05$, and **: $P \leq 0.01$. 
9. Supplementary tables

<table>
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<th>Ingredient</th>
<th>CON (D12450K)</th>
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<td>179 (716)</td>
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1Diets were purchased from Research Diets Inc. (New Brunswick, NJ, USA) and information of diet composition was provided by the company.  
2Freez-dried RB powder contains 77% carbohydrates were adjusted accordingly.
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10. References


CHAPTER 4: DIETARY RED RASPBERRY REDUCES COLORECTAL INFLAMMATION AND CARCINOGENIC RISK IN DSS-INDUCED CHRONIC COLITIS IN MICE

1. Abstract

**Background:** Ulcerative colitis (UC) causes recurring intestinal mucosal injury and sustained inflammation, increasing the likelihood of colorectal cancer (CRC)-development. Dietary red-raspberry (RB) is a rich source of phytonutrients known to have anti-inflammatory activity. However, the role of RB on CRC-prevention in chronic colitis has not been examined.

**Objective:** The objective of current study was to examine the effect of dietary RB-supplementation on inflammation, epithelium repair and oncogenic signaling in DSS-induced chronic colitis in mice. We hypothesized that RB facilitates epithelium repair and reduces CRC-risk due to its anti-inflammatory property.

**Methods:** Six-week-old-male (C57BL/6J) mice were fed a control or RB (5% of dry feed weight, n=12/group) diet for 10-weeks. Starting the 4th-week, mice were subjected to 2-repeated cycles of 1% DSS (7-day-DSS-treatment plus 14-day-recovery) and were monitored daily for disease activity index (DAI). Colonic tissues were collected at the end of study for histochemical/immunohistochemical and biochemical analysis.

**Results:** RB-supplementation reduced the DAI and histological damage \((P \leq 0.01)\), expression of inflammatory mediators \((P \leq 0.01)\), infiltration of CD4 T cells \((P \leq 0.05)\), and \(\alpha 4\beta 7\) integrin and related adhesion molecules \((P \leq 0.01)\). Furthermore, RB-supplementation facilitated epithelium repair evident by enhanced goblet cell density, expression of transcription factors including Kruppel-like factor-4 \((Klf-4)\) and Hairy and enhancer of split 1 \((Hes-1)\), terminal differentiation markers, \(Muc-2\) and intestinal alkaline phosphatase \((P \leq 0.01)\). Conversely,
proliferating cell nuclear antigen (PCNA, \( P \leq 0.01 \)), as well as \( \beta \)-catenin and signal transducer and activator of transcription3 (STAT3) signaling were reduced \( (P \leq 0.05) \) by RB-supplementation. In addition, RB-supplementation enhanced p53 stability and reduced oncogenic gene expression.

**Conclusion:** RB-supplementation reduced DAI and risk of CRC-development during recurring colitis in mice, suggesting that RB is a good dietary supplement for patients with UC and related gut inflammatory diseases.

2. Introduction

Ulcerative colitis (UC), a common form of inflammatory bowel disease (IBD), is categorized by a chronic recurring inflammation of the colon, which is known to increase the risk of colorectal cancer (CRC) development (Itzkowitz and Yio 2004). CRC is the third most common cancer in the USA and Europe, and is becoming common around the world (Ferlay et al. 2015; Siegel et al. 2014). Repeated mucosal injuries of the intestinal epithelium result in aberrant activation of inflammatory signaling, and accumulation of immune cells further amplifying the disease severity (Itzkowitz and Yio 2004; Naito et al. 1998). Epithelium repair or mucosal healing is a process of coordinated interplay between inflammation, cell proliferation, migration, and differentiation of epithelial cells in the intestinal epithelium (Dignass 2001; Paclik et al. 2008), which requires intricate regulatory mechanisms to prevent uncontrolled proliferation that eventually can lead to hyperplasia and CRC. Under chronic inflammation, Wingless and Int (Wnt)/\( \beta \)-catenin and signal transducer and activator of transcript (STAT) 3 signaling pathways are activated and promote epithelial cell proliferation (Ibrahim et al. 2014). On the contrary, the key tumor suppressor protein p53 (Bates and Vousden 1996; Yu et al. 2003) is reduced under
chronic inflammation and is lost in about 50% of malignant tumors (Agarwal et al. 1998).

Among cancers, CRC etiology is unique and highly affected by diets. Western high calorie diets with low dietary fibers and polyphenolic compounds are known to increase CRC incidences. Thus, increasing fruit consumption can reduce the risk of CRC and enhance the efficacy of anti-cancerous drugs and or therapies (Martin et al. 2013; Stoner and Mukhtar 1995), primarily mediated by their bioactive components. Red-raspberry (RB) contains around 50 different bioactive compounds (Carvalho et al. 2013b) including anthocyanins, ellagitannins, sanguin H-6, and lambertianin C (Borges et al. 2010; Gasperotti et al. 2010). RB extract containing bioactive components exerts anti-oxidative (de Souza et al. 2014) and anti-inflammatory activities (Li et al. 2014). Dietary RB reduces the severity of dextran sulfate sodium (DSS) induced colitis in mice (Bibi et al. 2017). Additionally, in vitro studies showed that RB extract inhibits proliferation and induces apoptosis (Coates et al. 2007; God et al. 2010), but the effects of dietary RB on CRC prevention has not been tested. We hypothesized that dietary whole RB supplement can reduce the risk of CRC development in mice experienced chronic colitis likely associated with its anti-inflammatory and anti-proliferative properties.

3. Material and Methods

3.1 Animal care and experimental designs

Six-week-old wild-type male mice (C57BL/6J) were randomized into two groups (n = 12 each group), receiving a standard rodent diet (CON, Research Diets Inc., New Brunswick, NJ, USA), or a CON supplemented with RB (5% of dry feed weight, Research Diets Inc., New Brunswick, NJ, USA) for 10-weeks. The pelleted diets with or without raspberry were stored at -20°C under vacuum package in dark, and fresh diets were provided to mice weekly. The
composition of RB powder have been published previously (Bibi et al. 2017), which contains ~11 g gallic acid equivalent (GAE)/kg of dry weight. The freeze-dried RB was powdered then shipped to Research Diets, Inc. (New Brunswick, NJ, USA) overnight for customized rodent research diets preparation. The detailed diet information was listed in supplemental Table S2.1&2.2. The dose of RB (5%) supplement equals to about 29.0 g of RB daily consumption for a 60 kg human per the published formula (Reagan-Shaw et al. 2008). The detailed calculation can be found in our recent publication (Bibi et al. 2017), where the same supplemental level of RB was used.

For chronic colitis induction, after four weeks of the dietary treatments, mice were treated with 1% DSS (MW = 36,000-50,000, MP Biomedicals, Santa Ana, CA, USA) in drinking water for 2-repeated cycles. Each cycle consisted of 7-day of 1% DSS treatment in water, followed by 14-day of recovery by providing normal drinking water. Repeated cycles of DSS exposure were used to mimic the recurring nature of colitis in human (Elson et al. 1995). Mice without DSS induction were not included in the present study, because our previous study has shown that RB-supplementation has barely an effect on mice without DSS treatment (Bibi et al. 2017).

After receiving DSS treatment, mice were monitored daily for colitis symptoms during both DSS cycles. At the end of the study mice were sacrificed and colonic tissues were collected for histological and biochemical analyses. Mice were housed in a temperature-controlled room with a 12 h light and 12 h dark cycle, and had a free access to diet and drinking water. There was no difference in feed intake and water consumption. All animal procedures were approved (BAF # 04316-001) by the Washington State University Animal Care and Use Committee. Tissues were collected and processed according to the previously published process (Bibi et al. 2017).
3.2 Colitis symptom assessment and disease activity index

During DSS induction and the recovery stages, mice under treatment were monitored for body weight loss, fecal consistency, and blood in the stool using the previously published scoring criteria (Hamamoto et al. 1999; Kang et al. 2016). The disease activity index (DAI) score was calculated as the sum of the above three scores as listed in Supplemental Table S2.3.

3.4 Histological assessment of colonic ulceration and goblet cells

The fixed distal colonic tissue sections were deparaffinized and subjected to haematoxylin and eosin (H&E) staining. The pathological scores of the distal colon were evaluated and recorded blindly using previously published scoring criteria (Hamamoto et al. 1999; Kang et al. 2016). For goblet cell staining, the colonic sections were subjected to Alcian blue (pH 2.5) staining, examined and quantified using the Image J 1.30v software (split color channels) as described previously (Wang et al. 2013a).

3.5 Immunoblotting analysis

Immunoblotting analyses were conducted per our published method (Zhu et al. 2007). Band density was quantified using the Odyssey Infrared Imaging System and Image Studio™ Lite software (Li-Cor Biosciences, Lincoln, NE, USA), and normalized to the β-actin content. Antibodies used in the study were listed in the supplemental material.

3.6 Immunohistochemical analysis

Immunohistochemical analyses were carried out as previously described (Zhu et al. 2007). CD4 antibody was purchased from eBioscience, Inc. (San Diego, CA, USA) and integrin α4β7 antibody was from Abcam (Cambridge, MA, USA). The CD4 T cell infiltration scores were assessed blindly according to the distribution and degree of CD4 positive cells staining per crypt.
ranging from 0 to a maximum of 4 (0 = no cells staining at all, 1 = 0-25% of crypt, 2 = 25-50% of crypt, 3 = 50-75% of crypt, 4 = 75-100% of crypt). The α4β7 integrin filtration score was assessed blindly using the distribution of α4β7 integrin staining per crypt ranged from 0 to 4, plus the intensity of the stain signal per whole section ranging from 0 to 4 (0 = no staining signal no intensity, 1 = 0-25% of whole section, 2 = 25-50% of whole section, 3 = 50-75% of whole section, 4 = 75-100% of whole section). This results in the total quantified score ranging from 0 to a maximum of 8 per distal colonic section. Nine sections per animal at constant interval were used for microscopic examination and score assessment.

3.7 Quantitative reverse transcriptase (qRT)-PCR analysis

Total RNA extraction, cDNA synthesis and qRT-PCR were performed as previously described (Bibi et al. 2017; Wang et al. 2013a). 18s rRNA was used as the reference gene. Primer sequences used in the study were listed in Supplemental Table S2.4.

3.8 Statistical analysis

Data were analyzed using one-way ANOVA and two-tailed Student’s t-test. P ≤ 0.05 was considered to be statistically significant. Each mouse is considered as an experimental unit. Data were expressed as mean ± standard error of mean (SEM).

4. Results

4.1 Dietary red-raspberry ameliorates colitis symptoms and histological ulceration

There was no difference in the weekly body weight between the treatment groups before DSS treatment (Supplemental Figure 1). RB-supplementation decreased DAI scores during both DSS treatment cycles (Figure 2.1A), and the difference became progressively larger during the second DSS cycle (Figure 2.1A). Morphologically, RB supplemented mice had improved colonic
architecture with less inflammation, and less crypt distortion compared to that of CON mice (Figure 2.1B), which was confirmed by histopathological scores (Figure 2.1B).

4.2 Dietary red-raspberry reduces inflammation and associated signaling pathways

Accompanying with lower DAI and histopathological scores, RB-supplementation reduced both mRNA and protein levels of IL-6 and COX-2 in colonic tissues (Figure 2.2A). Consistently, the mRNA levels of \( \text{Il-17} \) and interferon gamma (\( \text{Ifng} \)) in colonic tissues were also reduced by RB-supplementation (Figure 2.2B). Chronic induction of inflammatory cytokines evokes the adaptive immune responses that further amplify the disease symptoms (Adams and Eksteen 2006; Cheroutre et al. 2011). To further investigate the effect of RB-supplementation on the adaptive immune signaling, we evaluated the infiltration of CD4 T cells. Concomitantly, RB-supplementation reduced the CD4 T cell infiltration (Figure 2C) and \( \alpha4\beta7 \) integrin staining (Figure 2.2F); mRNA levels of inflammatory markers, intercellular adhesion molecule 1 (\( \text{Icam-1} \)), vascular cell adhesion protein 1 (\( \text{Vcam-1} \)), mucosal vascular addressin cell adhesion molecule 1 (\( \text{Madcam-1} \)) and chemokine (C-X-C motif) ligand 1 (\( \text{Cxcl-1} \)) were all reduced in the colonic tissues of DSS treated mice with RB supplementation (Figure 2.2D-E). Collectively, these data show that RB-supplementation reduced DSS-induced chronic inflammation and resultant adaptive immune responses.

4.3 Dietary red-raspberry supplementation improves epithelium repair as indicated by enhancing differentiation of the epithelial cells in chronic colitis

Reduction in inflammation leads to subsequent epithelial repair or mucosal healing, which requires epithelial cell differentiation into functional cells in the epithelium (Dignass
Consistently, RB-supplementation enhanced goblet cell density in the colonic tissues of chronic colitis (Figure 2.3A). Concomitant with enhanced goblet cells, RB-supplementation elevated the mRNA levels of *Muc-2* and goblet cell differentiation marker Kruppel-like factor 4 (*Klf-4*), as well as intestinal alkaline phosphatase (*Alpi*), a differentiation marker of enterocytes, in colonic tissues (Figure 2.3B). Further, both mRNA and protein levels of HES-1 were enhanced, while that of proliferating cell nuclear antigen (PCNA), a marker of cell proliferation, was reduced by RB-supplementation (Figure 2.3C).

### 4.4 Dietary red-raspberry supplementation suppresses β-catenin and STAT3 signaling in chronic colitis

Cytosolic β-catenin has an important role in cell adhesion and intracellular signaling including cell proliferation (Ilyas and Tomlinson 1997). Intestinal inflammation is known to enhance Wnt/β-catenin signaling (Liu et al. 2012). RB supplementation reduced the protein content of phosphorylated β-catenin (Ser 552), β-catenin, and mucin 1, while enhanced E-cadherin in the colonic tissues (Figure 2.4A-C). Furthermore, phosphorylation of STAT3, involved in proliferation and survival of tumor cells (Ibrahim et al. 2014), was suppressed by RB supplementation in the colonic tissues of mice (Figure 2.4D).

### 4.5 Dietary red-raspberry supplementation suppresses oncogenic signaling via enhancing tumor suppressor p53 expression

Activation and stabilization of tumor suppressor p53 results in cell cycle arrest and apoptosis. RB supplementation enhanced p53 and its phosphorylation at Ser 20 and Ser 15 in the colonic tissues of mice (Figure 2.5A). In addition, p19ARF, a small tumor suppressor protein that stabilizes p53, and mRNA of *p21*, a cyclin-dependent kinase inhibitor that is downstream target
of p53, were expressed highly in the RB supplemented colonic tissues (Figure 2.5B-C). Consistently, the mRNA levels of the p53 downstream targets, anti-apoptotic B-cell lymphoma 2 (Bcl-2), myeloid cell leukemia 1 (Mcl-1), proliferative Cyclin D1 (Ccd1) and oncogenic myelocytomatosis (Myc) were reduced in RB supplemented colonic tissues of mice (Figure 2.5D-E).

5. Discussion

Chronic colitis is characterized by active inflammation during relapse separated by remissions, frequently with a progressive increase in wound region and duration of the disease, which might eventually lead to CRC occurrence (Itzkowitz and Yio 2004). Consumption of fresh fruits, vegetables and dietary fiber is associated with reduced risk of CRC-development (Watson and Collins 2011); phytonutrients in plant foods potentially reduce the risk of cancer via multiple mechanisms (Martin et al. 2013; Stoner and Mukhtar 1995). RB is a rich source of polyphenolic compounds, vitamin B and C, folate and dietary fiber (Borges et al. 2010; Carvalho et al. 2013a; Gasperotti et al. 2010; Probst 2015). Dietary RB reduced the severity of DSS-induced acute colitis (Bibi et al. 2017). In the current study, we examined the protective effects of dietary RB against chronic colitis, which causes colonic mucosal inflammation and associated epithelial dysplasia predisposing to CRC (Elson et al. 1995). We found that RB reduced the severity of chronic colitis and inflammation, modulated immune responses, enhanced epithelium repair, and reduced oncogenic signaling suggesting that dietary RB can reduce the risk of CRC-development in subjects with chronic colitis.

Chronic colitis is associated with increased production of inflammatory cytokine IL-6 (Grivennikov et al. 2009) and other inflammatory mediators including COX-2, IFNG, and
CXCL-1 (Cheng et al. 2011; Paiotti et al. 2012). IL-17-expressing CD4 T cells present in the intestinal lamina propria produce IL-17 (Atarashi et al. 2008). IL-17 stimulates IL-6, which activates STAT3, mediating CD4 T cells maturation, proliferation and perpetuation into inflammatory intestinal tissues (Atarashi et al. 2008; Atreya et al. 2000). As a result, CD4 T cells are aberrantly activated in colitis (Adams and Eksteen 2006; Cheroutre et al. 2011), which express α4β7 integrin to further attract leucocytes (Behm and Bickston 2009). Vedolizumab, an integrin antagonist antibody, significantly induces mucosal healing and enhances remission in UC patients (Parikh et al. 2012). In agreement, RB-supplementation reduced the expression of inflammatory mediators, CD4 T cell density, the content of α4β7 integrin, and adhesion molecules in chronic colitis, clearly showing the protective effect of RB on inflammation and pathological changes of DSS-induced colitis.

Intestinal epithelium wound healing is dependent on the homeostasis of three cellular process including restitution, proliferation, and differentiation of epithelial cells into functional cells in the injured epithelium (Dignass 2001). Goblet cell is one of the differentiated functional lineages of epithelial cells that secrete MUC-2, the major mucin of the mucosal layer shielding the underlying epithelium from pathogenic microbes and toxic luminal content (Birchenough et al. 2015). Depletion of goblet cells and MUC-2 is a characteristic of intestinal inflammation in IBD (Bibi et al. 2016; Gersemann et al. 2009; Yang et al. 2015). Goblet cell staining and mRNA expression of Muc-2 and the lineage specific differentiation factors such as Klf-4 and Hes-1, along with Alpi were heightened in RB supplemented mice, indicating enhanced epithelial cell differentiation. RB-supplementation also reduced cell proliferation, thus decreasing chance of CRC-development. The total polyphenolics could be the main contributor for these protective
effects. Indeed, the polyphenolic rich grape seed extract (GSE) protects epithelial integrity by reducing proliferation and enhancing differentiation in the intestine of Il-10 knocked out mice (Bibi et al. 2016; Yang et al. 2015); berry extracts reduce the proliferation of LNCaP prostate cancer cells, and HT-29 and HCT116 colon cancer cells (Seeram et al. 2006).

Several signaling pathways regulate cell proliferation and differentiation, of which Wingless and Int (Wnt)/β-catenin pathway dominates. Intestinal inflammation is known to enhance Wnt/β-catenin signaling, which further stimulates cell proliferation and CRC development (Claessen et al. 2010). In the current study, we found that RB-supplementation reduced the β-catenin content, consistent with a previous report in which black raspberry supplementation reduced the β-catenin level and colonic ulceration (Wang et al. 2013b). In addition, GSE supplementation down regulated the Wnt/β-catenin pathway and related proliferation in the colon of Il-10 knocked out mice (Yang et al. 2015). STAT3 transcriptionally regulates β-catenin expression (Ibrahim et al. 2014), and epithelial cell proliferation and survival (Grivennikov et al. 2009); STAT3 is activated in 50-60% of CRC (Morikawa et al. 2011). RB-supplementation reduced STAT3 activation, which could be partially explained by reduced expression of IL-6, an upstream activator of STAT3 signaling (Grivennikov et al. 2009). The suppression of STAT3 and β-catenin signaling pathways reduces the risk of CRC. In agreement with our results, curcumin derivative small compound FLLL32 inhibited STAT3 activity, which reduces CRC development (Lin et al. 2010). Triptolide (a diterpenoid triepoxide extracted from the traditional Chinese medicinal herb inhibited CRC progression associated with STAT3 suppression (Wang et al. 2009).

Protein p53 is a tumor suppressor protein (Bates and Vousden 1996), which is reduced or lost in cancer cells (Agarwal et al. 1998). Phosphorylation of p53 at Ser 15 and Ser 20 stabilizes
p53 by reducing its interaction with mouse double minute 2 (MDM2), which mediates p53
degradation (Hastak et al. 2003). On the other hand, p14<sup>ARF</sup> (p19<sup>ARF</sup> in mouse) stabilizes p53 by
acting as an inhibitor of MDM2 (Hastak et al. 2003). We found that RB supplementation
stabilized the p53 protein correlated with increased phosphorylation at Ser 15, Ser 20, and an
upregulated level of p19<sup>ARF</sup>. These changes could be due to the biological effects of polyphenols.
Green tea polyphenols, epigallocatechin-3-gallate (EGCG), stabilized p53 by inducing its
phosphorylation at Ser 15 and Ser 20, and p14ARF-mediated downregulation of MDM2 in
human prostate carcinoma LNCaP cells (Hastak et al. 2003). p21 (CIP1/WAF1) is a cyclin-
dependent kinase inhibitor and one of the p53 downstream mediators, which initiates cell cycle
arrest, and interacts with PCNA to inhibit DNA replication (Warbrick et al. 1997). The p53
stabilization increases transcriptional activity of p21/WAF1, the expression of pro-apoptotic Bax
while reducing the anti-apoptotic BCL-2 (Hastak et al. 2003; Yu et al. 2003). Consistently,
dietary RB enhanced <i>p21</i> expression and reduced <i>Bcl-2</i>, <i>Mcl-1</i>, <i>Ccdn1</i>, and <i>Myc</i>, showing that
RB suppressed epithelial cell proliferation and oncogenic signal activation in chronic colitis.

Bioactive components in RB whole fruit include vitamins, minerals, fiber, antioxidants,
and polyphenols (Noratto et al. 2017). RB freeze-dried powder contains ~11 g gallic acid
equivalent (GAE)/kg of dry weight (Bibi et al. 2017) with anthocyanins and the ellagitannins,
sanguin H-6 and lambertianin C as major polyphenols (Borges et al. 2010; Gasperotti et al.
2010). RB anthocyanins supplementation to mice reduced production of <i>IL-6</i>, <i>IL-1β</i>, COX-2, and
inducible nitric oxide synthase, and suppressed nuclear factor kappa-B signaling (Li et al. 2014).
RB ellagic acid demonstrates immune regulatory functions in various animal studies (Burton-
Freeman et al. 2016), and anti-proliferative and apoptotic activities in Caco-2 cells (Larrosa et al.
In addition, RB contains 1.6% soluble and 33.5% insoluble fiber (Noratto et al. 2017), which provide substrates for gut fermentation to produce short chain fatty acids and is expected to generate additional beneficial effects (Jakobsdottir et al. 2014). Using the whole fruit approach, beneficial effects of RB could be attributed to the combined effects of high polyphenolic and fiber contents in RB.

6. Conclusion

In summary, dietary RB reduced the severity of chronic colitis, colonic ulceration, inflammation and associated signaling. RB facilitated the epithelium repair by enhancing epithelial cell differentiation, Muc-2 production, and reducing proliferation, which were associated with reduced β-catenin and STAT3 signaling. Moreover, dietary RB-supplementation stabilized the tumor suppressor p53 and reduced oncogenic signaling, suggesting RB is a good dietary choice to reduce the risk of CRC-development in subjects with chronic colitis.
7. References


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8. Figures and legends

**Figure 2.1** Symptoms of DSS induced chronic colitis in CON (□) or RB (■) fed mice. (A) Disease activity index scores of mice subjected to two cycles of DSS treatment, each cycle consists of seven days of 1% DSS treatment and 14 days of recovery process without 1% DSS treatment; a higher score correlates with severer symptoms. (B) Pathological quantification scores in distal colonic tissues and representative images of hematoxylin and eosin (H&E) staining (200×). CON; control, RB; red-raspberry. Means ± SEM, n = 12, **: $P \leq 0.01$. 

132
Figure 2.2 Inflammatory mediators, immune cells and related adhesion signaling in DSS induced mice fed with CON (□) or RB (■) supplemented diets. (A) Relative protein and mRNA expression of IL-6 and COX-2. (B) mRNA expression of Il-17 and Ifng. (C) Representative images and quantification scores of CD4 T cell immunohistochemical staining in distal colonic tissue. (D-E) mRNA expression of adhesion molecules. (F) Representative images and quantification score of α4β7 integrin immunohistochemical staining (200×). CON; control, RB; red-raspberry. Means ± SEM, n = 12, *: P ≤ 0.05, and **: P ≤ 0.01.
Figure 2.3 Epithelial cell differentiation and proliferation markers in DSS induced mice fed with CON (□) or RB (■) supplemented diets. (A) Alcain blue staining of goblet cells (200×) and quantification score. (B) mRNA expression of Muc-2, Klf-4 and Alpi. (C) mRNA and protein expression of HES-1 and PCNA. CON; control, RB; red-raspberry. Means ± SEM, n = 12, *: P ≤ 0.05, and **: P ≤ 0.01.
Figure 2.4 β-catenin and signal transducer and activator of transcription (STAT) 3 signaling in DSS induced mice fed with CON (□) or RB diets (■). (A) Relative protein contents of β-catenin and p-β-catenin (Ser 552). (B) Relative protein contents of mucin 1 and E-cadherin. (C) mRNA expression of Muc-1. (D) Relative protein contents of total, phosphorylated (Tyr 705) and acetylated (Lys 685) STAT3. CON; control, RB; red-raspberry. Means ± SEM, n = 12, #: $P \leq 0.10$, *: $P \leq 0.05$, and **: $P \leq 0.01$. 
Figure 2.5 p53 and its downstream signaling in the colon of DSS induced mice fed with CON (□) or RB (■) diets. (A) Relative protein expression of total and phosphorylated p53 (B) Relative protein content of p19ARF. (C) mRNA expression of p21. (D-E) mRNA expression of anti-apoptotic and oncogenic genes. CON; control, RB; red-raspberry. Means ± SEM, n = 12, #: $P \leq 0.10$, *: $P \leq 0.05$, and **: $P \leq 0.01$. 
9. Supplemental material

Antibodies and chemicals:

Antibodies against β-catenin, phospho β-catenin (Ser 552), cyclooxygenase (COX)-2, E-cadherin, HES-1, IL-6, MUC-1, phospho-/total STAT3, acetylated STAT3 (Lys 685), phospho-p53 at Ser 15 and Ser 20, were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against p53, p19ARF and PCNA were from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Anti-β-actin antibody was from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA). IRDye 680 goat anti-mouse and IRDye 800CW goat anti-rabbit secondary antibodies were purchased from Li-Cor Biosciences (Lincoln, NE, USA). Anti-mouse CD4 antibody was from eBioscience, Inc. (San Diego, CA, USA) and anti-integrin alpha 4+beta 7 antibody was from Abcam (Cambridge, MA, USA). The Vectastain ABC and DAB kits were purchased from Vector Laboratories Inc. (Burlingame, CA, USA).
Table S2. 1 Composition of the CON and 5% RB experimental diets\(^1\) given to male (C57BL/6J) mice for 10-weeks.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>CON (D12450K)</th>
<th>5% RB (D14072001)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/kg diet</td>
<td>g/kg diet</td>
</tr>
<tr>
<td>Casein, 30 Mesh</td>
<td>189.6</td>
<td>189.6</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>521.3</td>
<td>471.3</td>
</tr>
<tr>
<td>Maltodextrin 10</td>
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<td>142.2</td>
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<tr>
<td>Sucrose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cellulose, BW200</td>
<td>47.4</td>
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<tr>
<td>Soybean Oil</td>
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<td>23.7</td>
</tr>
<tr>
<td>Lard</td>
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<tr>
<td>Mineral Mix S10026</td>
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<td>9.5</td>
</tr>
<tr>
<td>DiCalcium Phosphate</td>
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<td>12.3</td>
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<tr>
<td>Calcium Carbonate</td>
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<td>5.2</td>
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<tr>
<td>Potassium Citrate, 1 H(_2)O</td>
<td>15.6</td>
<td>15.6</td>
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<tr>
<td>Vitamin Mix V10001(^2)</td>
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<td>9.5</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>1.9</td>
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</tr>
<tr>
<td>Freez-dried RB powder(^3)</td>
<td>0</td>
<td>50.0</td>
</tr>
<tr>
<td>FD&amp;C yellow dye 5</td>
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<tr>
<td>FD&amp;C red dye 40</td>
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</tr>
<tr>
<td>FD&amp;C blue dye 1</td>
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</tr>
<tr>
<td>Total</td>
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<td>1000.0</td>
</tr>
<tr>
<td>Protein (g)</td>
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<tr>
<td>Carbohydrates (g)</td>
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<td>Fiber (g)</td>
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<td>Fat (g%)</td>
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<tr>
<td>Raspberry (g%)</td>
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<td>5</td>
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<td>Protein (kcal%)</td>
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<td>18</td>
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<tr>
<td>Carbohydrates (kcal%)</td>
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<td>71</td>
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<tr>
<td>Fat (kcal%)</td>
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<td>10</td>
</tr>
</tbody>
</table>

\(^1\)Diets were purchased from Research Diets Inc. (New Brunswick, NJ, USA) and information of diet composition was provided by the company.


\(^3\)Freez-dried RB powder contains 77% carbohydrates, which were adjusted accordingly. Detailed composition of RB powder can be found at (Bibi et al. 2017c)
Table S2.2 Mineral Mix\(^1\) used at 10 g/ kg of diets

<table>
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<tr>
<th>Ingredient</th>
<th>g</th>
<th>Amount in 10 g</th>
</tr>
</thead>
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<tr>
<td>Sodium Chloride (39.3% Na, 60.7% Cl)</td>
<td>259</td>
<td>1.0 g Na</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.6 g Cl</td>
</tr>
<tr>
<td>Magnesium Oxide, Heavy, DC USP (60.3% Mg)</td>
<td>41.9</td>
<td>0.5 g Mg</td>
</tr>
<tr>
<td>Magnesium Sulfate, Heptahydrate (9.87% Mg, 13.0% S)</td>
<td>257.6</td>
<td>0.33 g S</td>
</tr>
<tr>
<td>Ammonium Molybdate Tetrahydrate</td>
<td>0.3</td>
<td>1.6 g Mo</td>
</tr>
<tr>
<td>Chromium Potassium Sulfate (10.4% Cr)</td>
<td>1.925</td>
<td>2.0 g Cr</td>
</tr>
<tr>
<td>Copper Carbonate (57.5% Cu)</td>
<td>1.05</td>
<td>6.0 g Cu</td>
</tr>
<tr>
<td>Ferric Citrate (17.4% Fe)</td>
<td>21</td>
<td>37 g Fe</td>
</tr>
<tr>
<td>Manganese Carbonate Hydrate (47.8% Mn)</td>
<td>12.25</td>
<td>59 g Mn</td>
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<tr>
<td>Potassium Iodate (59.3% I)</td>
<td>0.035</td>
<td>0.2 g I</td>
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<td>Sodium Fluoride (45.2% Fl)</td>
<td>0.2</td>
<td>0.9 g Fl</td>
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<tr>
<td>Sodium Selenite (45.7% Se)</td>
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<td>0.16 g Se</td>
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<tr>
<td>Zinc Carbonate (52.1% Zn)</td>
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<td>29 g Zn</td>
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<td>Sucrose</td>
<td>399.105</td>
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<td><strong>Total</strong></td>
<td>1000</td>
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\(^1\)Research Diets Inc. (New Brunswick, NJ, USA) information of Mineral Mix composition was provided by the company
### Table S2.3 Disease activity index (DAI) scoring criteria modified from the published criteria (Hamamoto et al. 1999)

<table>
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<th>Score</th>
<th>Body weight loss (%)</th>
<th>Stool consistency</th>
<th>Bleeding in the stool</th>
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<tbody>
<tr>
<td>0</td>
<td>&lt;=1%</td>
<td>Firm</td>
<td>No blood</td>
</tr>
<tr>
<td>1</td>
<td>1&lt;=5%</td>
<td>Soft but can pick up</td>
<td>Stool color changed</td>
</tr>
<tr>
<td>2</td>
<td>5&lt;=10%</td>
<td>Soft can’t pick up</td>
<td>Visible blood, small area</td>
</tr>
<tr>
<td>3</td>
<td>10&lt;=15%</td>
<td>Can’t pick up, no shape</td>
<td>Visible blood, large area, not on all the stool</td>
</tr>
<tr>
<td>4</td>
<td>&gt;15%</td>
<td>Watery</td>
<td>Gross blood on most of the stool</td>
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</table>

The DAI score is the sum of the score of weight loss, stool consistency and bleeding.
<table>
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<tr>
<th>Gene Name</th>
<th>Accession No.</th>
<th>Product Size</th>
<th>Direction</th>
<th>Sequence (5'-3')</th>
<th>Reference</th>
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<tr>
<td>Alpi</td>
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<td>271 bp</td>
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<td>GCTTAGAGCCCTACACCGAC</td>
<td>(Bibi et al. 2016)</td>
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<tr>
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<td>Reverse</td>
<td>GAAAGTAACCAGCGGTTGGA</td>
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<td>Bel-2</td>
<td>NM_009741.5</td>
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<td>GAGGCTGGGATGCTTTTTGT</td>
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<td>GCAGGTTTGTCAGCCTACT</td>
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<tr>
<td>Ccdn1</td>
<td>NM_007631.2</td>
<td>166 bp</td>
<td>Forward</td>
<td>TCAAGTGGTCGAGAAGGAGATT</td>
<td>(Yang et al. 2015)</td>
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<td>Cox-2</td>
<td>NM_011198.3</td>
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<td>Cxcl-1</td>
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<td>Icam-1</td>
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<td>(Yang et al. 2015)</td>
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</table>
Supplemental Figure 1

**Figure S1.** Weekly body weight in male (C57BL/6J) mice fed with CON (□) or RB (■) supplemented diets for 10-weeks. Values are means ± SEMs, n = 12. CON, control; RB, red-raspberry.
10. References

Bibi, S., et al. (2016), 'Grape seed extract improves small intestinal health through suppressing inflammation and regulating alkaline phosphatase in IL-10-deficient mice', *J Fun Foods*, 20, 245-52.

Bibi, S., et al. (2017a), 'Dietary green pea protects against DSS-induced colitis in mice challenged with high-fat diet', *Nutrients*, 9 (5), 509.

Bibi, S., et al. (2017b), 'Maternal high-fat diet consumption enhances offspring susceptibility to DSS-induced colitis in mice', *Obesity (Silver Spring)*, 25 (5), 901-08.


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1. Abstract

**Background:** Polyphenols anthocyanins have been shown anti-inflammatory, anti-proliferative and anti-cancerous properties with beneficial effect on gut health. However, the role of anthocyanin rich purple potatoes (PP) on modulating symptoms of inflammatory bowel disease (IBD) in vivo has not been investigated yet.

**Objective:** The objective of the current study was to determine the beneficial effect of PP supplementation on IBD using a DSS-induced colitis mice model. We hypothesized that PP being rich source of anti-inflammatory anthocyanins and fiber can improve DSS-induced colitis symptoms in mice.

**Methods:** Six-week-old C57BL/6J male mice were fed with a standard AIN-93G basal diet or diet supplemented with PP (0 or 10% w/w, n=20 each group) for 7 weeks. At the 5th week of dietary treatment, for colitis induction, approximately half of mice in each dietary group (n=12 each group) were subjected to 2.5% DSS in drinking for 7 days, followed by 7 days of recovery using normal drinking water.

**Results:** In healthy mice with no DSS induction, PP supplementation had no effect on the body weight gain. PP supplementation improved DSS induced body weight loss, diarrhea and gross bleeding, resulting in a significantly lower disease activity index. Further, PP supplementation reduced the colon shortening induced by DSS treatment. Moreover, histological examination of the distal colonic tissue of mice showed that PP supplementation reduced the colonic mucosal damage, crypt distortion, and inflammation in the intestinal tissue in response to DSS damage.
**Conclusion:** PP supplementation possesses protective effect against DSS-induced colitis symptoms, which is associated with improved colonic histology and inflammation, suggesting PP as a good dietary choice in IBD patients.

**2. Introduction**

Inflammatory bowel disease (IBD), encompassing Crohn’s disease (CD) and ulcerative colitis (UC), is a chronic disease of the intestinal track with unknown etiology. IBD is characterized by episodes of relapse and remission, with symptoms of loose stool/diarrhea, abdominal pain and discomfort, and rectal bleeding during the relapse (Alrubaiy et al. 2015). The incidences of IBD are increasing rapidly in the developing countries, and is still a burden in the USA and Europe causing huge health costs (Loftus 2004). Possible treatment of IBD include long term usage of anti-inflammatory or immunosuppressive drugs with or without surgery, which have profound side effects (Baumgart and Sandborn 2007), pointing out to the need for searching alternative therapeutic strategies. Epidemiological studies have revealed an inverse association for consumption of whole grains, vegetables and fruits and the risk of IBD development (Ananthakrishnan 2015), suggesting diet as one of the modulators for IBD management (Durchschein et al. 2016). In fact, diet rich in bioactive compounds such as black raspberry (Montrose et al. 2011), blueberry (Pervin et al. 2016), red raspberry (Bibi et al. 2017) and goji berry (Kang et al. 2016) exhibited protective effects in dextran sulfate sodium (DSS)-induced colitis.

Purple potato (PP) contains health beneficial nutrients including B-vitamins, ascorbic acid, minerals, dietary fiber and polyphenolics especially anthocyanin as a major antioxidant (Camire et al. 2009; Eichhorn and Winterhalter 2005; Reyes et al. 2005; Xiao and Hogger 2015).
PP anthocyanin suppressed the growth of colon cancer cell lines (Madiwale et al. 2012), mouse stomach cancer (Hayashi et al. 2006b), and prostate cancer cell lines (Reddivari et al. 2007), while PP (20% w/w) supplementation reduced azoxymethane-induced (AOM) colon tumorigenesis in A/J male mice (Charepalli et al. 2015). Further, potato fermentable fiber supplementation attenuated DSS-induced colitis symptoms due to the short chain fatty acids (SCFAs) production (Panasevich et al. 2015). However, the potential health benefits of consuming whole PP on the active IBD have not been investigated. The aim of current was to determine the role of PP in improving IBD symptoms using DSS-induced colitis mice model.

3. Material and Methods

3.1 PP powder and experimental diets

PP (Cultivar Purple Pelisse) were provided by Prof. Roy Navarre from Irrigated Agriculture Research and Extension Center, Prosser, WA, USA. PP were washed, dried, cut to small pieces and were freeze dried in VirTis freeze drier (Vertis Comp. Gardiner, NY, USA) and ground into powder at School of Food Science Washington State University Pullman, WA.

The diet was composed of standard AIN-93G basal mouse chow supplemented with PP (0, and 10%) powder in agar. A 4% (w/v) agar solution was prepared by heating 10 g of agar in 250 ml ddH2O (microwaved on high for 4 min), stirred, and microwaved again for 1 min. The AIN-93G basal diet was combined with PP powder (0, or 10%) (Table 1), added to the 4% agar solution at 50 ºC, and mixed with a hand mixer (Hamilton Beach Brands, Southern Pines, NC, USA). The diets were allowed for 4 h, at 4 ºC to solidify, then cut into 2 cm³ chunks and frozen at -80 ºC.

3.2 Experimental design and animal care
Six-week-old wild-type C57BL/6J male mice (Jackson Laboratory, Bar Harbor, ME, USA) were randomized into 2 groups having 20 mice in each group. One group received a standard AIN-93G basal agar based control diet (CON), and other group received a CON diet supplemented with 10% PP powder (substituted for corn starch) for 7 weeks. At 5th week of dietary treatment, mice at each dietary group were further randomly divided into 2 sub-groups, receiving a regular tap water with 0 or 2.5% DSS (MP Biomedicals, Santa Ana, CA, USA). This resulted in a total 4 dietary groups: no DSS no PP (CON, n=8), DSS without PP (DSSC, n=12), PP only (PP, n=8), and DSS with PP (DSSPP, n=12). The CON and PP groups were under normal drinking water while DSSC and DSSPP were exposed to a 7 days treatment of 2.5% DSS dissolved in drinking water, followed by 7 days of recovery treatment with normal drinking water.

Mice were monitored daily for water consumption, body weight, fecal consistency, and blood in the stool throughout the DSS treatment and recovery stage. All mice were housed in a temperature controlled room with a 12h light and 12h dark cycle and had free access to diet and drinking water. No difference was observed in the average amount of water consumption among treatment groups. All animal procedures were approved by the Washington State University Animal Care and Use Committee (BAF#04316-010).

3.3 Monitoring colitis symptoms and disease activity index (DAI) assessment

The scores for body weight loss compared to initial weight (scored as 0-4), stool consistency (scored as 0-4), and blood in the stool (scored as 0-4) were recorded daily during the DSS-induction and recovery period, according to the previously described method (Kang et al. 2016). The DAI score was assessed by the combined score of weight loss, fecal consistency and blood
in the stool (Table 2).

3.4 Colon tissue length, collection and fixation

Mice were anesthetized with CO₂ inhalation and followed by cervical dislocation. The whole colon section having cecum was removed, placed straight on the scale without stretching and colon length was measured from cecum to anus. A 5 mm segment of distal colon was dissected and was fixed in freshly prepared 4% (w/v) paraformaldehyde (pH 7.0), processed and embedded in paraffin. The remaining colon tissue was opened by a longitudinal cut, rinsed in PBS, frozen in liquid nitrogen, and stored at -80 °C for later biochemical analyses.

3.5 Histological evaluation of DSS-induced colonic inflammation

The paraffin embedded distal colonic tissues were sectioned at 5µm thickness, deparaffinized and subjected to haematoxylin and eosin (H&E) staining. Histological examination and imaging were done under Lecia DM2000 LED light microscope (200x, Leica Microsystems Inc., Chicago, IL, USA). For pathobiological scoring, each colonic section was scored blindly using a previously published score criteria (Kang et al. 2016), and 9 sections per animal at constant interval were used. The scores of crypt damage (0-4 scale), severity of inflammation (0-3 scale), and depth of injury (0-3 scale) were recorded individually. The summation of the scores resulted in the total pathobiological score ranging from 0 to a maximum of 10 per distal colonic section.

3.6 Statistical Analysis

Data were analyzed as previously described (Zhu et al. 2010). A complete randomized design using General Linear Model of Statistical Analysis System (General Linear Model of Statistical Analysis System, SAS, 2000). Data were expressed as mean ± standard error of mean.
A significant difference was considered as $P \leq 0.05$.

4. Results

4.1 Dietary PP supplementation attenuates DSS-induced colitis symptoms

PP supplementation had no effects on the average feed intake and body weight gain of the mice before DSS-treatment (Figure 1A&B). In the DSS-induced groups, all mice on both CON and PP diet survived during DSS treatment and recovery period. Exposure to DSS treatment induced watery diarrhea, and gross bleeding in the stool, which were significantly alleviated by PP supplementation in DSS-mice (Figure 2A&B). Consistently, PP supplementation reduced the DSS-induced body weight loss (Figure 2C). The DAI scores increased till day 7 of DSS-treatment and then started decreasing during the recovery process. PP supplementation significantly ameliorated the DAI scores throughout the treatment and recovery phases when compared to the DSSC-mice (Figure 2D) indicating that PP attenuated the disease symptoms.

4.2 Dietary PP supplementation protects DSS-induced colonic shortening

Colon length is considered as an indirect marker of inflammation (Peng et al. 2010). Mice under CON and PP dietary treatment with no DSS treatment had morphologically normal colons with no swelling (Figure 3A). DSS treatment resulted in swelling and shortening of the colon, which was protected PP supplementation evident from the measured colon length (Figure 3B).

4.3 PP supplementation reduces DSS-induced mucosal damage improving histological architecture of the colon

The colonic tissues from the mice on CON and PP diets with no DDS treatment showed normal histology with no mucosal or crypt damages (Figure 4A). The DSS treatment induced
injuries to the colon epithelium as shown (Figure 4B) most of the epithelial cells were
disappeared along with the loss of mucosa and crypts, indicating inflammation in the distal
colon. PP supplementation protected the colon against DSS-induced injuries, which was
confirmand by the low histopathobiological score in DSSPP-mice (Figure 4C).

5. Discussion

Diet rich in polyphenols and fiber can potentially modulate IBD symptom. PP is a rich
source of anthocyanins. Anthocyanin might play an important role in PP due to the anti-
proliferative, pro-apoptotic, and anti-cancerous properties (Charepalli et al. 2015; Hayashi et al.
2006b; Hayashi et al. 2006a; Madiwale et al. 2012; Reddivari et al. 2007). Recently, our
colleagues found that PP extract promotes gut barrier function by increasing trans-epithelial
electrical resistance in vitro (Sun et al. 2017). In the present study, we investigated the role of PP
as a whole food approach in reducing the symptoms of active IBD, and a protective effect was
observed.

Symptoms of IBD can be mild to severe during the relapses and can decrease or even
disappear during remissions. Generally, symptoms associated with the inflammatory damage of
the intestine include diarrhea, rectal bleeding in the stool, and weight loss (Bernstein et al. 2010).
DSS induces symptoms of IBD in mice with histological and microscopic resemblances to
human IBD (Elson et al. 1995). PP supplementation reduced the DSS-induced symptoms of
colitis in mice showing a protective role of PP during the diseases active stage and recovery
period. This protective effect of PP can be due to its bioactive constituents especially
anthocyanin and fiber. In fact, dietary supplementation of red raspberry, rich in anthocyanin and
fiber (Bibi et al. 2017), and dietary potato fermentable fiber supplementation attenuated DSS-
induced colitis symptoms in mice (Panasevich et al. 2015).

Due to chronic intestinal inflammation, the accumulation of extracellular cellular matrix in mucosal and submucosal layers contribute to shortening of the colon in IBD (Rieder and Fiocchi 2008), making colon length as an indicator of DSS-induced inflammation (Peng et al. 2010). DSS causes mucosal damages of the intestine due to direct cytotoxic effect on the intestinal epithelium (Ni et al. 1996), resulting in mucus reduction, crypt distortion, epithelial necrosis, and infiltration of inflammatory cells (Cooper et al. 1993; Elson et al. 1995; Hamilton et al. 2011; Kolaczkowska and Kubes 2013). Accompanying with alleviated disease symptoms, PP supplementation protected the colon shortening showing an anti-inflammatory effect on the colon, which was further confirmed by the microscopic evaluation of colonic tissue showing less mucosal damage, crypt distortion and inflammation, and a lower pathological score.

PP contains resistant and non-resistant starches, proteins, and vitamins, anthocyanin and dietary fiber (Camire et al. 2009). Dietary fiber, resistant starches, and polyphenolics in legumes have shown beneficial effect on intestinal health (Monk et al. 2015; Monk et al. 2016; Monk et al. 2017). Using the whole food approach, we were not able to conclude that which bioactive component in PP was responsible for protection against DSS-induced damages. However, based on the previous studies, the beneficial effect of PP can be possible to the synergistic effect of anthocyanin, resistant starches and dietary fiber in PP (Haenen et al. 2013; Han et al. 2008; Paturi et al. 2012; Reddivari et al. 2013; Wong et al. 2006). Dietary fiber and resistant starches can modulate the gut microbiota and enhance SCFAs production, attenuating DSS-colitis in mice (Panasevich et al. 2015). Further, upon digestion dietary fiber absorbs water and expands, which helps the bowel movement and reliefs symptoms of an irritable bowel syndrome and constipation.
(Lembo and Camilleri 2003). All these suggest that PP can exert beneficial effect on gut health.

6. Conclusion

Dietary PP reduced the symptoms of DSS-induced colitis in mice indicated by low DAI scores, which were associated with reduced inflammation as indicated by improved colon length, and proven by the histological examination of colonic tissues, clearly showing the effectiveness of PP supplementation in improving IBD symptoms. Thus, PP being rich in bioactive constituents may be useful in the management of IBD.
7. References


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### 8. Tables

Table 3.1 Composition of the experimental diet

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>% of AIN-93G purified rodent diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON diet (%)</td>
</tr>
<tr>
<td>Casein</td>
<td>20</td>
</tr>
<tr>
<td>Dyetrose</td>
<td>13.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5</td>
</tr>
<tr>
<td>Salt/mineral mix</td>
<td>3.5</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>1</td>
</tr>
<tr>
<td>L-cystine</td>
<td>0.3</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.25</td>
</tr>
<tr>
<td>t-Butylhydroquinone</td>
<td>0.0014</td>
</tr>
<tr>
<td>Corn starch</td>
<td>39.7</td>
</tr>
<tr>
<td>PP powder</td>
<td>0</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>7</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>
Table 3.2 Disease activity index (DAI) scoring criteria modified from Hamamoto et al.1999

<table>
<thead>
<tr>
<th>Score</th>
<th>Body weight loss (%)</th>
<th>Stool consistency</th>
<th>Bleeding in the stool</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;=1%</td>
<td>Firm</td>
<td>No blood</td>
</tr>
<tr>
<td>1</td>
<td>1&lt;=5%</td>
<td>Soft but can pick up</td>
<td>Stool color changed</td>
</tr>
<tr>
<td>2</td>
<td>5&lt;=10%</td>
<td>Soft can’t pick up</td>
<td>Visible blood, small area</td>
</tr>
<tr>
<td>3</td>
<td>10&lt;=15%</td>
<td>Can’t pick up, no shape</td>
<td>Visible blood, large area, not on all the stool</td>
</tr>
<tr>
<td>4</td>
<td>&gt;15%</td>
<td>Watery</td>
<td>Gross blood on most of the stool</td>
</tr>
</tbody>
</table>

The DAI= Sum of the score of weight loss, stool consistency, and bleeding
9. Figures and legends

**Figure 3.1** Feed intake and body weight before DSS treatment. (A) Feed intake. (B) Body weight. CON: Control, PP: Purple potato. Means ± SEM, n = 20
Figure 3.2 Symptoms of DSS-induced colitis in mice. (A) Fecal consistency score. (B) Fecal blood score. (C) Body weight loss (percent of initial body weight). (D) Disease activity index (DAI) score during DSS treatment and recovery process; a higher score correlates with severer symptoms. CON: Control, PP: Purple potato, DSSC: DSS-treated control mice, DSSPP: DSS-treated mice with PP supplementation. Means ± SEM, n = 12 for DSSC and DSSPP, and 8 for CON and PP. *: $P \leq 0.05$, and **: $P \leq 0.01$
Figure 3.3 PP supplementation improves DSS-induced colon shortening. (A) Representative colon images at necropsy. (B) Mean colon length (cm). CON: Control, PP: Purple potato, DSSC: DSS-treated control mice, DSSPP: DSS-treated mice with PP supplementation. Means ± SEM, n = 12 for DSSC and DSSPP, and 8 for CON and PP. **: $P \leq 0.01$
Figure 3.4 PP supplementation improves gut histopathobiological score and inflammation in DSS-induced colitis. (A-B) Representative distal colonic images of hematoxylin and eosin (H&E) staining (200x). (C) Quantified pathological score. CON: Control, PP: Purple potato, DSSC: DSS-treated control mice, DSSPP: DSS-treated mice with PP supplementation. Means ± SEM, n = 12 for DSSC and DSSPP, and 8 for CON and PP. **: P ≤ 0.01
IBD is a chronic relapsing disease of the gastrointestinal track characterized by chronic intestinal inflammation. The etiology of IBD is unknown with no cure. Furthermore, the persistent and recurring intestinal inflammation during the episodes of remission and relapse, increasing the risk of CRC development in subjects of IBD. The traditional therapies for IBD include anti-inflammatory and immune modulatory drugs and surgery, which causes severe side effect due to long term treatment of the diseases. Recently, research is focusing on the use of dietary bioactive compounds as an alternative either alone or supplemented with the therapeutic drugs. RB and PP are rich sources of bioactive compounds that possess anti-inflammatory, anti-oxidative and anti-cancerous properties. In the present study, the beneficial effects of dietary RB and PP supplementation was evaluated on the gut barrier function and intestinal health in active IBD using DSS-induced experimental colitis mice models.

In the first study, the beneficial effects of RB supplementation on DSS-induced acute colitis were examined. RB supplementation decreased body weight loss, DAI scores, and colon shortening, and protected the colonic structure, associated with suppressed NF-κB signaling and reduced expression of IL-1β, IL-6, IL-17, COX-2, and TNF-α in DSS-induced colitis mice. RB supplementation reduced neutrophil recruitment, associated MCP-1 mRNA expression, and oxidative stress enzyme, XO, protein content, and enhanced the anti-oxidative catalase protein content in DSS-induced colitis mice. Furthermore, RB supplementation reduced the expression of pore forming tight junction protein claudin-2, while increased the expression of barrier strengthening claudin-3, ZO-1 protein content and MUC-2 mRNA level, and activated AMPK in DSS-induced colitis mice. Thus, dietary RB protected mice against the DSS-induced acute colitis.
possibly due to its anti-inflammatory activity, by reducing cytokine expression, neutrophil recruitment and oxidative stress, and ultimately reducing the colonic mucosal and epithelial damages in the colon, suggesting preventive roles of dietary RB in IBD symptoms and related gut disease.

In the second study, the effects of dietary RB supplementation on inflammation, epithelium repair and oncogenic signaling associated with the CRC were examined using a DSS-induced chronic colitis mice model. RB supplementation reduced the DAI scores and expression of the inflammatory markers. Further, RB supplementation suppressed the infiltration of CD4+ T cells, expression of α4β7 integrin and related adhesion molecules signaling of the adaptive immune system. Consistently, RB supplementation facilitated epithelium repair, as indicated by enhanced goblet cell density and expression of transcription factors including Klf-4 and Hes-1, as well as terminal differentiation markers, MUC-2 and ALPi, and reduced expression of PCNA. These results were associated with reduced expression of β-catenin and STAT3 signaling in the RB supplemented mice. Moreover, RB supplementation enhanced the tumor suppressor p53 stability and reduced its downstream anti-apoptotic Bcl-2 and oncogenic Myc and Ccdn1 gene expression. These findings suggested that RB supplementation reduced disease indices and risk of CRC development during recurring colitis in mice.

In the third study, the beneficial effect of PP supplementation on DSS-induced colitis symptoms was evaluated. In the healthy mice with no DSS, PP supplementation had no effect on the body weight gain. PP supplementation improved body weight loss, stool consistency and gross bleeding in the stool, with a significantly decreased resultant DAI scores in DSS-treated mice. Further, PP supplementation reduced the colon shortening, and reduced the histological
colonic mucosal damage, crypt distortion, and inflammatory cell infiltrate in the DSS-treated mice. Thus, we demonstrated that PP supplementation possesses protective effect against DSS-induced colitis symptoms.

The future studies should determine the bioactive components in RB and PP, should determine the effectiveness of RB and PP at low doses in animal colitis models, and should explore the role of RB and PP in gut microbial modulation and underlying in depth mechanisms of their protection.