ANTHOCYANINS AND POLYSACCHARIDES INTERACTION ON GUT BARRIER FUNCTION

by

Wenyi Fu

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STATEMENT OF COMMITTEE APPROVAL

Dr. Lavanya Reddivari, Chair
Department of Food Science

Dr. Bruce R. Hamaker
Department of Food Science

Dr. Tzu-Wen Cross
Department of Nutritional Science

Approved by:
Dr. Arun K. Bhunia

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LIST OF ABBREVIATIONS

PP	Purple potato
ACNs	Anthocyanins
APC	Anthocyanin-pectin complex
AIC	Anthocyanin-inulin complex
ASC	Anthocyanin-starch complex
ACC	Anthocyanins-cellulose complex
AMC	Anthocyanins-mixture complex
APM	Anthocyanins-pectin mixture
AIM	Anthocyanins-inulin mixture
ASM	Anthocyanins-starch mixture
ACM	Anthocyanins-cellulose mixture
AMM	Anthocyanins-mixture mixture
SEM	Scanning electron microscope
SCFA	Short-chain fatty acids
IBD	Inflammatory bowel disease
UC	Ulcerative colitis
CD	Crohn’s disease
TJ	Tight junctions
ZO	Zonula occludens
MUC	Mucins
FW	Fresh weight
DW	Dry weight
IL	Interleukin
GI	gastrointestinal
NFκB	Nuclear factor-kappa B
TNF	Tumor necrosis factor
DFs	Dietary fibers
TLR2	Toll-like receptor

DSS	Dextran sulfate sodium
DE	degree of esterification
RS	Resistant starch
β-CD	β-cyclodextrin
DLS	Dynamic light scattering
FTIR	Fourier Transforms Infrared Spectroscopy
TPC	Total phenolic content
MAC	Monomeric anthocyanin content
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
FBS	Fetal bovine serum
GAC	Gallic acid equivalents
C3G	Cyanidin-3-glucoside
DMEM	Dulbecco's Modification of Eagle's Media
LPS	Lipopolysaccharide
AST	Aspartate aminotransferase

ABSTRACT

Anthocyanin-containing foods are beneficial against chronic diseases. Anthocyanins exist as acylated and complexed with other macronutrients such as carbohydrates and proteins in the food matrix. Isolated anthocyanins are unstable and can be easily destabilized by environmental factors (pH, light, temperature). Furthermore, the colonic concentration of isolated anthocyanins is low compared to when in a food matrix. Complexation is a technique used to stabilize anthocyanins. This study aims to investigate which polysaccharides can stabilize the isolated anthocyanins from purple potatoes and enhance the colonic concentration of anthocyanins via complexation. The health benefits of anthocyanin-polysaccharide complexes were also evaluated *in vivo*. The different polysaccharides used were pectin, inulin, starch, cellulose, and their mixture in equal proportions. Pectin exhibited the best binding capacity with anthocyanins (anthocyanin-pectin complex; APC).

APC retained more anthocyanins compared to other complexes after the simulated upper GI digestion. The electrostatic interaction of APC quantified by zeta potential showed that anthocyanins were positively charged, and pectin was slightly negatively charged under pH 2.0 condition. The increased particle size of APC was observed indicating the agglomeration between particles. The physical surface structure of APC was changed after complexation compared to APM (anthocyanin-pectin mixture) as observed by SEM. The stability of anthocyanins in APC was better at pH 3, 5 and 7 and showed higher temperature tolerance than isolated anthocyanins. Furthermore, APC significantly decreased LPS-induced increase in gut permeability in Caco-2 cell model. APC restored the gut barrier function *in vivo* by reducing gut permeability and increasing mucus thickness in a DSS-induced colitis mouse model. APC also increased the total short-chain fatty acids especially butyrate concentration. In conclusion, APC enhanced the stability of isolated anthocyanins and improved the gut barrier function compared to isolated anthocyanins.

CHAPTER 1. INTRODUCTION

1.1 IBD

Inflammatory bowel diseases (IBD) are characterized by disorders in the human digestive tract. The United Kingdom, the United States and Northern Europe have the highest prevalence of IBD [1]. The IBD cases are increasing in both developed and developing countries [2]. Globally, about 6.8 million people are suffering from IBD [3]. Whites have the highest prevalence of IBD, followed by Africans and Hispanics [4]. Asia has the least prevalence compared to Western countries [5]. Hospital records revealed that 15-29-year-old age group had the highest IBD incidence [6]. IBD patients encounter a lot of difficulties, such as career interruptions, social stigma and quality of life reduction [7]. Many studies have emerged to understand the IBD pathogenesis due to the severity of IBD. Furthermore, the development of preventive and therapeutic strategies for IBD has attracted more and more attention in the last decade.

1.1.1 IBD treatments

Although many therapeutic options are developed to target IBD symptoms, numerous side effects have weakened the potential of IBD treatments. Induction and maintenance of remission are two strategies to treat IBD. Exogenous glucocorticoids are widely used to prolong the remission stage and reduce inflammation, which is considered as first-line therapy to reduce the disease activity of IBD [8]. Corticosteroids are effective in the short term, but they have severe negative side effects with long-term intakes, such as swelling, weight gain, acne, hair growth, and mental health issues. Aminosalicylates are applied for inflamed gut and flare-ups of ulcerative colitis while the 5-aminosalicylic acid from aminosalicylates might lead to headaches, nausea, cramping, rash or fever. Immunomodulators (immunosuppressants and immunostimulants) are widely used as a long-term treatment for IBD. The common side effects of immunomodulators are headache, vomiting, diarrhea, nausea and low blood cell count. Cyclosporine and infliximab are alternatives in steroid-refractory IBD, which are developed from living organisms. Long-term steroid use can cause osteoporosis; however, a sudden stop of steroid medication causes weakness, body aches, joint pain, and a decrease in appetite. Furthermore, the use of antibiotics might lead to infections, abscesses and pouchitis. In addition, antibiotics not only eliminate pathogenic

bacteria but also beneficial bacteria from IBD patients. Overall, the negative side effects of IBD therapy and disruption of life quality of the IBD patients necessitate alternate therapies.

1.1.2 IBD pathogenesis

IBD includes ulcerative colitis (UC) and Crohn's Disease (CD). UC is characterized by a non-infectious chronic inflammation limited to the colonic mucosa, but CD targets at any segment of the gastrointestinal (GI) tract [3]. IBD causes impaired intestinal barrier function, gut bacterial dysbiosis and colonic inflammation [9]. High gut permeability is associated with impaired gut barrier function, which represents the reduction of mucus thickness, and a low abundance of tight junctions (TJ) within the epithelial cells. In addition, the compromised barrier function allows the pathogenic bacteria or toxins to pass through the gut and enter the bloodstream. Gut bacterial dysbiosis is another IBD symptom implicated in several chronic diseases, and various digestive disturbances including bloating, constipation and stomach cramps. Furthermore, the critical manifestation of IBD is colonic inflammation. Chronic colonic inflammation causes weight loss, rectal bleeding, abdominal pain, diarrhea and fatigue. IBD also increases the risk of other comorbidities, such as colon cancer and cardiovascular diseases.

The pathogenesis of IBD is not fully understood, but the environmental and genetic factors are widely known to trigger IBD. Diet, drugs, stress, environment and lifestyle are environmental factors. Diet can either aggravate or ameliorate IBD. Sasson *et al.* reported that an unhealthy diet is related to intestinal inflammation and diet can dysregulate the immune system, alter intestinal permeability and microbial dysbiosis [10]. A diet high in fat and protein but low in fruits and vegetables might also explain the high incidence of IBD in Western countries [11]. On the other hand, the intake of dietary fibers, prebiotics, probiotics and avoidance of lactose and processed complex carbohydrates can help to ameliorate symptoms in IBD patients [12]. Unlike medication, a plant-based diet with anti-inflammatory activity can serve as an effective, safe and low-cost preventive strategy against the rising IBD incidence [13]. Therefore, it is no exaggeration to say that the global efforts in the etiology and treatment of IBD have reached the culmination for hundreds of years [14].

1.1.3 Impaired intestinal barrier function

The improvement of gut barrier function is key in IBD prevention and treatment. Impaired epithelial barrier function is closely related to the pathogenesis of other diseases, such as food allergies, type I diabetes, and many systemic diseases [15]. The intestinal epithelium is a semipermeable layer, which selectively absorbs nutrients. The intestinal mucosal barrier can be classified as chemical and physical barriers. The antimicrobial peptides work as chemical agents to prevent pathogenic microorganisms, while the mucus layer and TJ provide physical effects. The intestinal epithelium consists of a layer of epithelial cells, which are connected by TJ. Enterocyte and goblet cells are two differentiated cell types to produce colon epithelium. The intestinal epithelium separates the host's internal milieu from the external environment and is considered as a significant mechanical barrier [16]. Furthermore, the intestinal epithelium cells can mediate the crosstalk between gut microbes and host immunity and interact with host immune cells via various gut environmental stimulations [17]. Other than the intestinal epithelial cell layer, a thin brush border is another important component for intestinal epithelium. To sum up, impaired epithelial barrier increases gut permeability, triggers an immune reaction and inflammatory response. Therefore, a well-defined intestinal epithelium plays an important role in defining the integrity of the intestinal barrier and function.

Many *in vitro* and *in vivo* studies have investigated the epithelial barrier function. Caco-2 cells are human colon carcinoma cells and serve as an intestinal model to test the permeability of the cell monolayers *in vitro*. Caco-2 cells spontaneously differentiate into a monolayer of cells with the typical characteristics of absorptive enterocytes with brush border layer [18]. *In vivo*, the rodent studies measure serum FITC-dextran concentrations after oral gavage to determine the gut permeability or gut barrier integrity [19].

1.1.4 Tight junctions (TJs)

TJs are multiprotein junctional complexes that play an important role in many gut-related diseases. The transmembrane proteins are involved in TJ formation. They include claudin, occludin, junctional adhesion molecules and tricellulin, and intracellular proteins (zonula occludens (ZOs), cingulin, 7H6 and ZA-1) [20]. TJs mainly contribute to intestinal barrier function. Occludin was the first integral membrane TJ protein [21]. Otani and Furuse reported that the ZO

family is indispensable in TJ assembly, especially ZO-1 and ZO-2. This is because ZO-1 and ZO-2 are scaffolding proteins that can form oligomers and interact with other TJ-associated membrane proteins [22]. Chemical-induced colitis models are characterized with decreased expression of TJ proteins, mucus layer thinning and increased gut permeability.

1.1.5 Mucus layer

The mucus layer is considered as a passive, host-designed chemical complex barrier, which is largely composed of glycoproteins called mucins. The human mucin (MUC) family consists of 17 genes. Mucins contain a large number of high-density clusters of *O*-linked glycans and are able to form gels for lubrication and serve for cell signaling [23]. The mucin-type glycans of MUC gene-encoded proteins frequently form a cross-linked connection in an aqueous solution, resulting in a high viscosity gel (mucus). Mucin 2 (MUC2) is encoded by the MUC2 gene acting as gel-forming mucin in the lumen of the mammalian small intestine and colon [24]. As a secreted mucin, MUC2 is secreted from goblet cells, which protects epithelial cells and lines the colon. Malin *et al.* revealed that the inner mucus layer contains no bacteria in the colon because this firm mucus layer is rich in the high concentration of MUC2, and small pore size blocking the bacteria [25].

1.2 Whole foods

Whole foods are minimally processed and refined plant foods including fruits (berries, orange and apple), vegetables (tomato, carrot and onion), whole grains, legumes and nuts. Whole foods retain to the greatest extent the micronutrients and macronutrients, vitamins, beneficial phytochemicals and dietary fibers compared to processed foods. Accumulated evidence supports the role of whole foods in lowering the rates of heart disease, cancer, type 2 diabetes and colonic diseases [26]. Furthermore, a plant-based diet is gaining more attention against IBD because it can reduce the medication burden. Numerous whole foods are naturally rich in phytochemicals, which can offer many beneficial effects on colon health (Table 1).

Table 1. Beneficial effects of whole foods on colon health.

Whole foods	Effects on colon health	Reference
Purple potato (*Solanum tuberosum* spp.)	Reduced high-calorie diet-induced inflammation, lowered the risk of colon cancer.	[27]
	Ameliorated DSS-induced colitis in a mice study.	[28]
Wild blueberry	The presence of polyphenols and fibers in wild blueberry resulted in the increased relative abundance of intestinal bifidobacteria	[29]
Juçara pulp (*Euterpe edulis*)	The *in vitro* fermentation revealed that the microbial population was changed and SCFA production was shifted due to the combination of fibers and polyphenol.	[30]
Navy and black bean	Both navy and black bean supplementation (phenolic compounds, resistant starch, and galactooligosaccharides) altered gut microbial community activity and promoted gut barrier integrity.	[31]
Strawberry	The whole strawberry decreased the production of inflammation mediators (cyclooxygenase-2 and iNOS) and suppressed the activation of NF-κB signaling (influences the pathogenesis of colitis) in DSS-consuming mice compared to the mice consuming the control diet.	[32]

Potato, as the main food crop after rice and wheat, is one of the most consumed vegetables by over one billion people. Potato is rich in resistant starch, vitamin C, potassium, magnesium, phosphorus, B-vitamins, protein, dietary fiber and lipids (only a tiny fraction) [33]. Purple potato (PP) is gaining more interest because of the diversity of polyphenols (anthocyanins and phenolic acids). For instance, Han *et al*. reported that PP contains various anthocyanidins, including malvidin, peonidin, pelargonidin and petunidin [34]. The anthocyanin content of PP is in the range of 5.5 to 51 mg/100 g FW (fresh weight) [35]. Based on Charepalli *et al*., PP might function in the reduction of cancer stem cells and tumor incidence by suppressing Wnt/β-catenin signaling and increasing mitochondria-mediated apoptosis [36]. Moreover, PP can improve the diversity of gut microbiota, gut barrier function, prevent and reverse chronic colonic and systemic inflammation [28]. The mRNA expression of colonic inflammatory cytokines IL-6, IL-9 and IL-17A were reduced in the mice consuming cooked navy and black bean diets for 2 weeks in a DSS-induced colitis model [68]. Although many studies have shown the beneficial effects of whole foods that are mainly attributed to the richness of polyphenols and microbiota-accessible carbohydrates, it is very difficult to dissect the major contribution of any single component in a matrix.

1.3 Anthocyanins

Dietary anthocyanins are natural phytochemicals belonging to the group of flavonoids (Fig. 1). Anthocyanidins are cyanidin, delphinidin, pelargonidin, peonidin, malvidin and petunidin (Fig. 2). They are unstable and rarely found in nature [37]. The presence of flavylium ion and their peculiar electron distribution contributes to the instability of anthocyanidins [38]. More than 635 anthocyanins have been identified and derived from these six anthocyanidins [39]. Anthocyanins mainly exist in the form of glycosides in fruits and vegetables, majorly providing red, purple and blue colors, attracting pollinators and seed dispersers, and resisting abiotic/biotic stresses [40]. Anthocyanins can act as pH indicators due to their colors and are changed depending upon pH, for example, a red-pink color in acidic solutions (pH 1-6), a reddish-purple color in neutral conditions (pH 7) and green color in alkaline or basic environment (pH 8-14) [41]. Additionally, anthocyanins are located within the vacuole of plant cells, enclosed by tonoplast and cytoplasmic lipid membranes, and then encapsulated by the plant cell wall [42]. In flowers, pectin forms large non-dialyzable and co-pigment complexes with anthocyanins [43]. The key functions of anthocyanins in plants include protection from excess light, UV-B radiation attenuation and antioxidant activity [44]. Moreover, anthocyanins have been shown anti-oxidant, anti-microbial, anti-inflammatory, anti-cancer, anti-obesity and anti-diabetes properties [45].

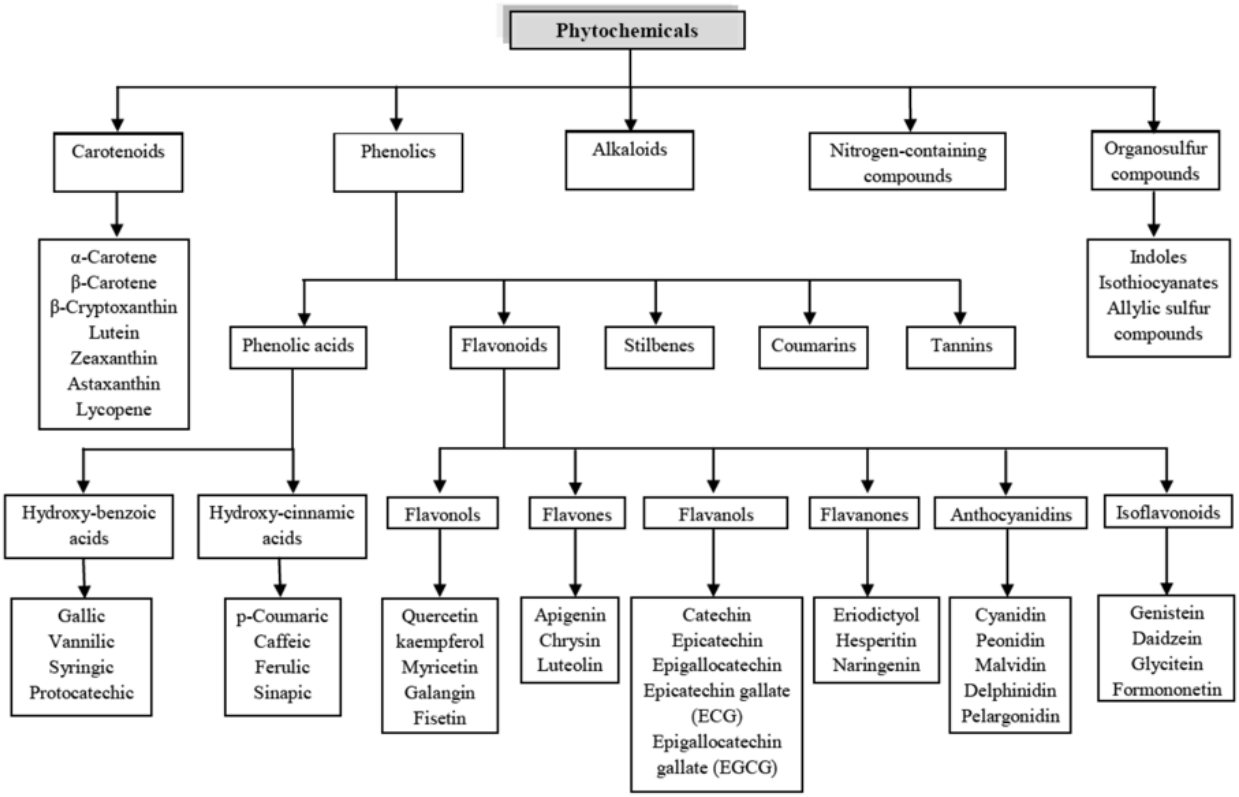


Figure 1. Classification of plant phytochemicals [46].

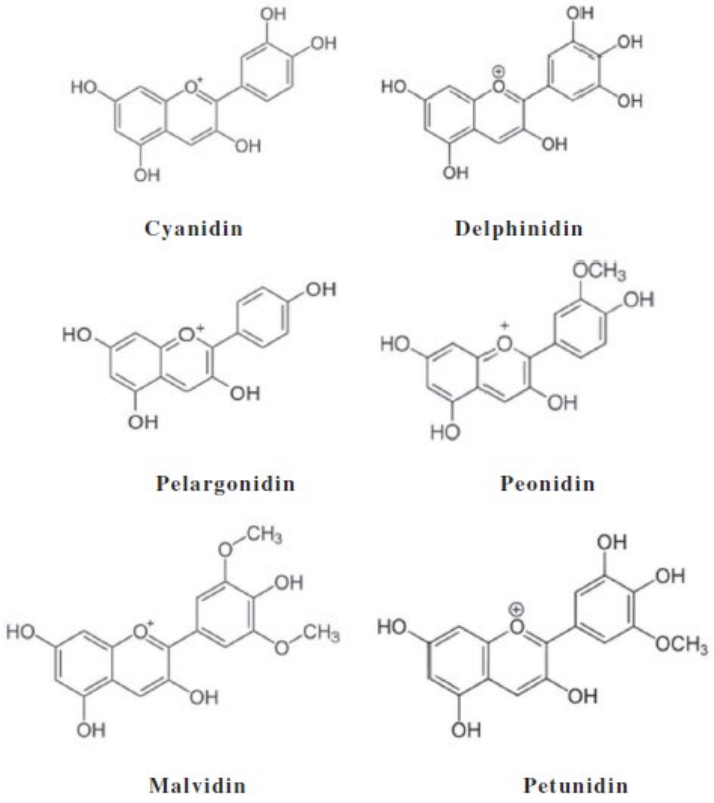


Figure 2. Structures of six major anthocyanidins [45].

1.3.1 Anthocyanin metabolism:

During human digestion, anthocyanins are released from the food matrix and then go through biotransformation via the human GI digestive process by endogenous enzymes [47] and metabolized in the liver and intestines. Schmitt *et al.* revealed that the liver enzymes sulfated and glucuronidated cyanidin [48]. Anthocyanins are more stable in the stomach due to the lower pH. In the small intestine, anthocyanins experience degradation because the pH is around 7.5. Anthocyanin glycosides are absorbed rapidly and efficiently in the small intestine by the intestinal epithelial cells through glucose transporter 2 and possibly Na^+/glucose cotransporter 1 [49]. Unabsorbed anthocyanins will reach the colon, serve as substrates for gut microbiota fermentation, and undergo decomposed catalyzation. The microbial metabolism of anthocyanins involves the breaking of C-ring leading to hydroxylated aromatic compounds derived from the A-ring and releasing the B-ring in many phenolic acids [50]. Hydrolysis, fission and demethylation are three pathways for biotransformation when anthocyanins are exposed to gut microbiota [45].

Anthocyanin glycosides are hydrolyzed to aglycones and degraded into simple phenolic acids by colonic microbiota [49]. Furthermore, the most abundant anthocyanin metabolites are glucuronidated and methylated compounds [51].

1.3.2 Anthocyanins and gut health

Anthocyanin extracts from different sources were studied and shown to have a strong correlation to gut health. Cremonini *et al.* reported that microbiota composition and MUC2 levels could be restored by anthocyanins supplementation in high-fat diet-induced obesity in mice [52]. Anthocyanins can interfere with the cell cycle, induce anti-proliferation and apoptosis which inhibit colon cancer [53]. In addition, anthocyanins can inhibit the transcription factor nuclear factor-kappa B (NFκB) that activates the inflammatory signaling pathway [54]. Anthocyanins suppressed the secretion of tumor necrosis factor (TNF), another essential mediator of inflammation in IBD [12]. Furthermore, other IBD-associated pro-inflammatory mediators were all significantly inhibited by bilberry extract and cyanidin-3-*O*-glucoside under cell culture conditions [55]. An *in vitro* fermentation experiment conducted by Yan *et al*. showed that the anthocyanins from *L. ruthenicum* regulated the intestinal microbiota composition and maintained intestinal health [56]. In conclusion, anthocyanins can suppress colonic inflammation and majorly impact gut health, which has been proved by *in vitro* and *in vivo* studies.

Many studies showed that intestinal permeability can result in paracellular transport of gut bacterial endotoxins and toxins from foods and trigger systemic inflammatory response [52]. Anthocyanins might be considered as an important medium for extenuating gut permeability. Anthocyanins are limited to absorption by passive diffusion due to their highly water-soluble nature. However, anthocyanidins are more hydrophobic and can diffuse across the mucosal epithelium passively [57]. Anthocyanins can be metabolized by phase II enzymes after absorption [58]. The consumption of anthocyanins-rich foods stimulates the secretion of MUC1 and MUC3, which are membrane-associated mucins, related to the epithelial injury repair process [59]. Moreover, a growing number of studies on anthocyanin-rich extracts exhibited therapeutic potential against IBD, especially enhancing the gut barrier function [58].

1.3.3 The stability of isolated anthocyanins

Many environmental factors, such as light, temperature, and pH [40] can destabilize the anthocyanin molecules. When the temperature rises to 60 °C, anthocyanins have poor stability and become colorless [60]. Besides, the structural transformation of anthocyanins is pH-dependent, and there are four major anthocyanin forms found in equilibria: red flavylium cation, blue quinoidal base, colorless carbinol pseudo base and colorless chalcone (Fig. 3) [61]. Anthocyanins are electron donors, can form flavylium cation and become highly soluble in water at lower pH conditions [62]. The core flavylium cation causes anthocyanins to be highly sensitive to pH [63]. Additionally, the bioavailability of anthocyanins is influenced by their chemical structures and forms in the food matrix. Yi *et al.* reported that the glucose-based anthocyanins have higher bioavailability than the galactose-based anthocyanins [64]. Furthermore, the increasing number of studies on the stability of anthocyanins points out encapsulation [65][66] and complexation [67][68] with fibers to improve the stability. In this study, anthocyanins from PP were complexed with different polysaccharides for the increment of anthocyanins' stability.

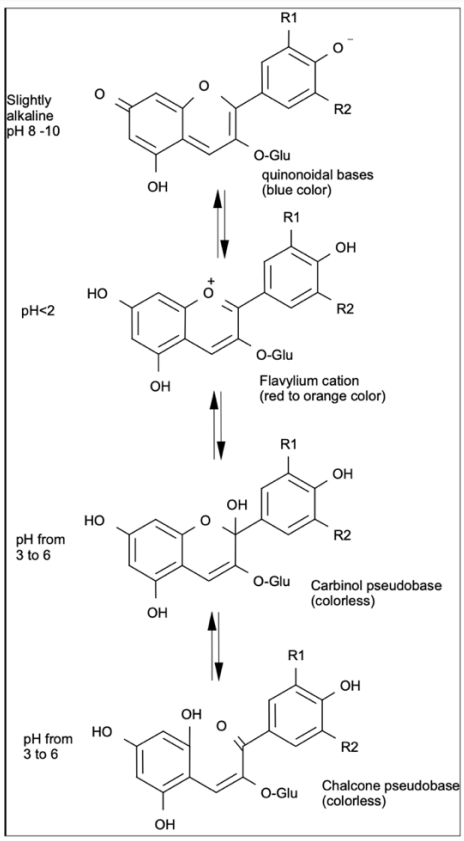


Figure 3. Effect of pH conditions on different anthocyanin forms [61].

1.4 Polysaccharides

Polysaccharides comprise dietary fibers (DFs) and starches. DFs are resistant to human endogenous enzymes [69] and are considered as prime substrates for microbial fermentation in the colon. The metabolic pathways of fermentation mainly release the SCFAs, which are essential for colonic mucosal health. DFs are indispensable components of the human diet because they provide a variety of health benefits. For example, people who had the highest level of fiber intake showed about 29% lower risk of coronary heart disease compared to those who had the lowest fiber consumption [70]. Weickert *et al.* reported that the diet with high insoluble DF improved insulin sensitivity, which contributed to reducing the risk of diabetes [71]. Also, high dietary fiber intake can lower the risk of coronary heart disease, stroke, hypertension, diabetes and obesity development [72].

The production of pro-inflammatory cytokines, such as IL-6, IL-8, IL-1β and TNFα are affected by SCFA via NF-*κ*B activation [73]. A mice study done by Desai *et al*. reported an increase in mucus-degrading bacteria, mucus layer thinness, and pathogen susceptibility in DF deficient diet [74]. Another mice study revealed that the mice with a diet lacking microbiota-accessible carbohydrates, typically DFs, resulted in reducing mucus layer thickness, increasing proximity of microbes to the epithelium and the expression of inflammatory markers [75]. Furthermore, piglets fed with 10% wheat bran fiber for 30 days could up-regulate the gene expression of ZO-1 and Toll-like receptor 2 (TLR2) and improve the intestinal barrier function [76].

Recently, prebiotic supplements are becoming more popular due to their ability to promote the growth of the commensal bacteria in the human GI tract. The prebiotic dietary fibers have positive effects on the modulation of gut microbial composition in a structure-dependent manner. Prebiotics can utilize the substrates by fermentation and construct a beneficial colonic environment. The commensal microbes have a profound impact on intestinal physiology and mucosal immune functions because they are in contact with the epithelial cells solely on the apical membrane [77]. Inulin and oligofructose have been proven to be effective prebiotics and used to develop new food products for enhancing the gut microbiota to resist acute infections [78]. Burkitt stated that fiber is an important factor in maintaining normal bowel behavior [79]. Rats exposed to Trinitrobenzenesulfonic acid after two weeks of a fiber-supplemented diet (5% *Plantago ovata*

seeds) showed attenuation of the inflammatory response [80]. In addition, the rats with DSS-induced colitis reduced the mucosal lesion scores, tissue myeloperoxidase activity and inflammation after inulin supplementation compared to the control rats [81]. Another rat experiment with the same DSS-induced colitis with resistant starch as diet showed improvement in caecal and distal macroscopic and histological observations compared with other treatment groups [82].

1.4.1 Pectin

Pectin is a polysaccharide of galacturonic acid. The derivatives of pectin are connected by α-1,4-linkages (Figure 4). Pectin can be found naturally in fruits and vegetables' cell walls and intercellular layers. In plant cell walls, pectin is the most complex polysaccharide both structurally and functionally [83]. The degree of esterification (DE) includes high ester, low ester conventional and low ester amidated [84]. Citrus peels and apple pomace are two main sources of commercial pectin [85]. Pectin is widely applied in food, pharmaceutical and other industries. For instance, pectin is a functional ingredient in jellies, jams, frozen foods, fruit drink concentrates, fruit juice and many desserts. In the pharmaceutical industry, pectin showed various biological activities, such as reduction of GI disorders, blood cholesterol via restricting the bile acid reabsorption, serum glucose and insulin content as well as delayed gastric emptying and anti-cancer activities [86][87][88]. Recently, many studies revealed the synergistic effects of pectin and anthocyanins on gut health. For example, a study reported that the pectin extracted from blueberry powder bonded with anthocyanins can contribute to the stability of anthocyanins under GI simulation [89].

1.4.2 Inulin

Inulin is a fructan-type soluble polysaccharide, with β-(2→1)-glycosidic bonds of fructose moieties. Inulin is commonly extracted from vegetables, including onion, asparagus, leek, garlic and chicory root [90], and mainly functions as carbohydrate storage in plants. Moreover, inulin can be used as a vehicle for drug delivery in the pharmaceutical industry, and have the potential to be the source of fructose in the biotechnology industry [91]. The consumption of inulin increases the frequency of bowel movements because it adds bulk to stool, and functions in the inhibition of pathogenic microorganisms. Studies have proved that inulin is a type of prebiotic with the ability

to promote the growth of specific beneficial gut bacteria [92]. More specifically, Uerlings *et al*. stated that inulin was correlated with higher production of SCFA and can upregulate the gene expression of TJ in IPEC-J2 cells [93]. Another *in vitro* study showed that the dried chicory root (containing 75% inulin) provided protective effects by reducing the gut permeability that was induced by an apical stressor [94].

1.4.3 Resistant starch

Resistant starch (RS) is another insoluble DF. It also enters the colon for microbial fermentation to promote SCFA production, optimize physiological function and inhibit pathologies [95]. RS occurs naturally in oats, grains (sorghum and barley), beans, legumes, green bananas, etc. Their residues are predominantly α-1,4-linked glucans [96]. There are four types of resistant starch (Figure 5), and have different descriptions: RS 1 is found in whole or partly milled grains and considered as a physically protected form; RS 2 is present as raw granules; RS 3 is retrograded starch and RS 4 is chemically modified starch [97].

Furthermore, RS has shown promising effects for improving human health. The decrease of fasting cholesterol and triglyceride levels was ascribed to the long-term consumption of RS in a diet because RS increased the viscosity of intestinal digesta, which retarded the interactions between sugars and fatty acids [98]. Resistant starches play a role in preventing and/or controlling chronic human diseases, such as diabetes, colon cancer, and obesity [99]. The investigation using rodent models showed that RS intake was correlated to colonic pH, SCFA composition, and enzymatic activity and bacterial taxa abundance [99].

1.4.4 Cellulose

Cellulose is a linear glucose polysaccharide linked by β-1,4 glycosidic linkages (Figure 4) and is considered an insoluble DF with a rigid structure. In nature, cellulose is the most abundant polysaccharide and it is a major component of cell walls that impacts strength [100]. Cellulose supplementation is beneficial to human health. As a hydrophilic bulking agent, cellulose has the potential to aid in defecation. Furthermore, cellulose can increase satiety in hypocaloric diets. Also, the cellulose nanofiber consumption and exercise together inhibited the rise of body weight and fat mass and enhanced glucose tolerance in mice [101]. Kim *et al*. conducted a mice study and

reported that the high-cellulose diet maintained gut homeostasis by alternating gut microbiota and metabolites [102]. Another study revealed the importance of dietary cellulose in protecting mammalian large bowel and ameliorating intestinal inflammation in a DSS-induced colitis mouse model [103].

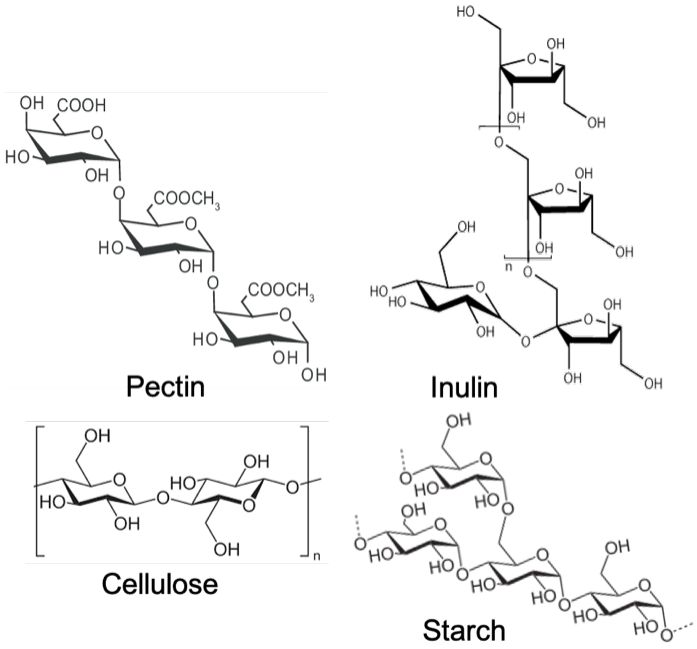


Figure 4. Chemical structures of pectin, inulin, cellulose and starch.

Type of Resistant Starch	Description	Examples
RS1	Physically inaccessible, non-digestible matrix	Whole or partly milled grains and seeds
RS2	Tightly packed, ungelatinized starch granules	Raw potato starch Green bananas High-amylose cornstarch
RS3	Retrograded starch (cooled gelatinized starch)	Cooked and cooled potato, bread and pudding
RS4	Chemically modified starch	Etherized, esterified or cross-bonded starches (used in processed foods)

Figure 5. Types of resistant starch.

1.5 Anthocyanins complexation with polysaccharides

Due to the low stability of anthocyanins, encapsulation and copigmentation are two methods used to stabilize anthocyanins. Encapsulation includes spray/freeze-drying, gelation, emulsification and polyelectrolyte complexation. Biopolymers, phenolic compounds and metal ions are the common copigments in the copigmentation technique with anthocyanins [104]. There are many strategies to determine the stability of anthocyanins, such as color stability against pH changes, anthocyanin concentration remaining after *in vitro* digestion and colonic anthocyanins measurement.

Zeta potential analyzer is a tool to measure the electrostatic potential from the particle surface (zeta potential) and the particle size called dynamic light scattering (DLS). Zeta potential analysis is related to electrophoretic mobility and is used to estimate the electrostatic interaction between particles. As Guan and Zhong revealed, the complexation of anthocyanins and gum Arabic was a hydrophobic attraction based on the Zeta-potential data [105]. Scanning Electron Microscope (SEM) is a type of electron microscope for capturing the images of the sample surface by a focused beam of high-energy electrons. Surface morphology can characterize the structure of samples. For instance, Patel *et al.* illustrated SEM for the morphological characterization of anthocyanins encapsulated with soy protein isolated, jackfruit seed starch and emulsifier (NBRE-15) [106]. Fourier Transforms Infrared Spectroscopy (FTIR) is another chemical analytical technique, which measures the infrared absorption spectrum to identify the chemical bonds of a molecule. The determination of anthocyanins in red wines was done by FTIR because FTIR has good repeatability and reproducibility [107]. In addition, FTIR was used to measure the infrared spectroscopy of the malvidin-3-*O*-glucoside-β-lactoglobulin complex for the determination of secondary structure changes of proteins [108].

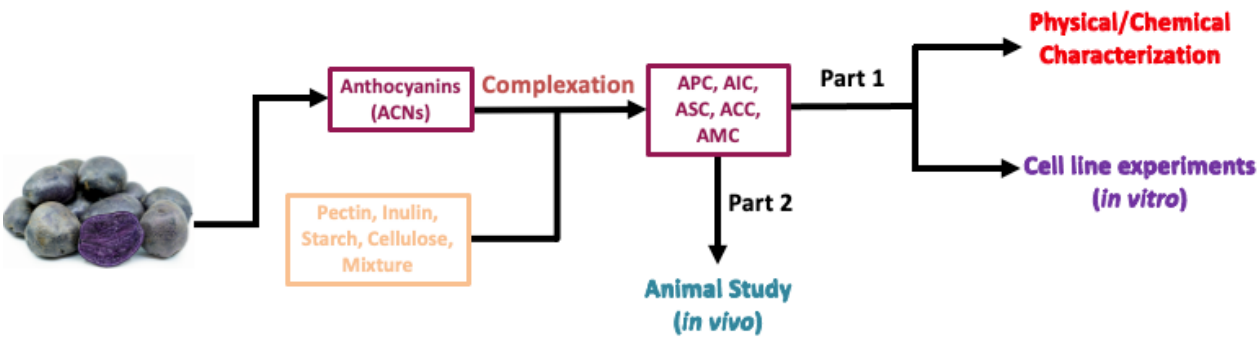
Zeta-potential analyzer, FTIR and SEM were applied in many studies of anthocyanins. According to Jiang *et al.*, Fe_3O_4/anthocyanin magnetic bio-composite was produced through physical intermolecular adsorption or covalent cross-linking then analyzed via SEM and FTIR for bio-composite characterization while zeta potential analyzer for determining the sizes and the zeta potentials of nanoparticles that are dispersed in water [109]. Another study involved microencapsulation of anthocyanin extract (from red raspberry) with soy protein isolate, gum Arabic also applied FTIR, zeta potential, and SEM (morphology) to analyze the properties [110].

Moreover, DLS, FTIR and SEM were utilized to determine the particle sizes, structural and physical properties, respectively of rutin, which was encapsulated with different starch nanoparticles [111].

1.6 Hypothesis

The isolated anthocyanins are not stable and may not confer the same health benefits compared to whole foods. Therefore, in this study, we hypothesized that the complexation with polysaccharides imitates the food matrix which can improve the stability of plant-based anthocyanins and function in colon health.

1.7 Experimental design and objectives



Objective 1: Determine the role of polysaccharides in purple potato anthocyanin stability against changes in pH and temperature and intestinal availability after upper GI digestion.

Objective 2: Role of anthocyanin-polysaccharide complexes in the restoration of gut barrier function in DSS-induced colitis mouse model.

CHAPTER 2. COMPLEXATION WITH POLYSACCHARIDES ENHANCE THE STABILITY OF ISOLATED ANTHOCYANINS

Abstract

Plant foods containing anthocyanins are beneficial against chronic diseases, but the stability and bioavailability of isolated anthocyanins are low compared to when in whole foods. Polysaccharides in plant foods can complex with anthocyanins to improve stability. However, polysaccharides differ in their complexation efficiency with anthocyanins. Furthermore, the ability of polysaccharides to improve the stability and bioactivity of anthocyanins is still unknown. In this study, we complexed anthocyanins with four different polysaccharides (pectin, inulin, starch, cellulose, and their mixture in equal proportions) and characterized and monitored them by zeta potential, dynamic light scattering, scanning electron microscopy and Fourier-transform infrared spectroscopy. The stability of anthocyanins in the complex was measured both in terms of the amount that was bound to the polysaccharide after digestion and the structural stability under different pH and temperature conditions. The role of complexes in improving barrier function was determined using *in vitro* Caco-2 cell model. The electrostatic interaction of complexes quantified by zeta potential showed that anthocyanins were positively charged while the polysaccharides were slightly negatively charged up to pH 2.0. Dynamic light scattering data reported an increased particle size of anthocyanin-pectin complex (APC) due to agglomeration. Pectin increased the concentration of bound anthocyanins compared to other polysaccharides after the simulated digestion. In addition, anthocyanins in APC were more stable at pH 3, 5 and 7 for a longer duration at a higher temperature compared to isolated anthocyanins. APC reduced the LPS-induced increase in cell permeability compared to isolated anthocyanins. In conclusion, the isolated anthocyanins from purple potatoes showed distinct binding capacity with different polysaccharides. Pectin showed the best complexation efficiency with anthocyanins and enhanced anthocyanin stability and bioactivity *in vitro*.

2.1 Introduction

Natural phytochemicals such as anthocyanins are well known for their anti-carcinogenic, antioxidant and anti-inflammatory effects. Anthocyanins are stored in the vacuole [42], and mainly

exist in the form of glycosides and impart red, purple and blue colors to fruits, vegetables and flowers [112][113]. Isolated anthocyanins are less stable, and more susceptible to changes in environmental factors (pH, temperature and light) compared to the anthocyanins in a whole food matrix. Anthocyanins will release from the food matrix in the human GI tract as the food is digested by endogenous digestive enzymes [47]. Unabsorbed anthocyanins reach the colon and serve as substrates for gut microbiota metabolism. Moreover, anthocyanins have exhibited the ability to modulate the gut microbiota. For example, an *in vitro* experiment conducted by Yan *et al*. showed that the anthocyanins from *L. ruthenicum* upregulated the abundances of *Bifidobacterium* and *Allisonella* [56]. Furthermore, the gut bacterial metabolites of anthocyanins are shown to be more effective compared to parent anthocyanins in reducing inflammation [114]. The IBD (inflammatory bowel disease)-associated pro-inflammatory mediators (IP-10, I-TAC, sICAM-1 and GRO-α) were significantly inhibited by bilberry anthocyanin extracts [55].

Anthocyanins that are present within a whole food matrix are more stable because of their interaction with the polysaccharides compared to isolated anthocyanins [115]. A simulated GI digestion study revealed that the stability of anthocyanins was significantly higher in red cabbage than in extracts [116]. Various delivery techniques such as encapsulation, emulsification, gelation, and complexation have been used to stabilize the isolated anthocyanins [117]. A decreased degradation rate of cyanidin-3-*O*-glucoside under simulated GI conditions was observed after complexation with β-cyclodextrin [67]. Biopolymers, phenolic compounds and metal ions are used to stabilize anthocyanins [117]. As Koh *et al.* mentioned, pectin extracted from the blueberry powder when bonded with three different anthocyanin standards increased the stability of isolated anthocyanins under GI simulation [89]. In addition, anthocyanins from strawberries encapsulated with inulin were stable under high temperatures [118]. Although many polysaccharides, especially dietary fibers, have been reported to ameliorate gut health by reducing gut bacterial dysbiosis [119], inflammation [120], and improving gut barrier function [74], some of the starch hydrolysis products might aggravate gut inflammation and reduce MUC2 production [121]. Thus, it is not known which polysaccharides play a role in improving the stability of anthocyanins.

Our study focused on the complexation of extracted anthocyanins from purple potatoes (PP) with different polysaccharides include pectin, inulin, starch, cellulose, and their mixture in equal proportions. PP are important source of anthocyanins. Out of 16 anthocyanins identified, petunidin

3-caffeoyl-rutinoside-5-glucoside and malvidin 3-*p*-coumaroyl-rutinoside-5-glucoside are the predominant anthocyanins in PP [28]. Importantly, PP exhibited anti-inflammatory [35], anti-colitic [28], anti-hypertensive [122] and anti-diabetic effects [123] as a whole food matrix. However, as of yet, no studies have assessed the role of complexation in improving the stability of isolated PP anthocyanins. The objectives of this study were (1) to identify the polysaccharide and anthocyanin complex that improves the stability of anthocyanins both in terms of concentration of free and bound anthocyanins after simulated digestion; (2) to characterize the structure of complexes.

In this study, all the complexes were evaluated through simulated *in vitro* GI digestion and changes in pH. Zeta potential, dynamic light scattering, scanning electron microscopy and Fourier-transform infrared spectroscopy were used to characterize the complexes. The bioavailability and bioactivity were measured *in vitro* using a Caco-2 cell permeability assay.

2.2 Materials and Methods

2.2.1 Materials

Purple potatoes (*Solanum tuberosum* L. var. Purple Majesty; PP) were harvested from San Luis Valley Experiment Station (Center, Colorado, USA). They were washed, baked (oven; 180 °C, 50 min), freeze-dried (VirTis Ultra 35L Pilot Lyophilizer; Warminster, PA, USA), and stored at -80 °C for further use.

2.2.2 Polyphenol extraction and anthocyanin fractionation

The free extraction of free polyphenols from freeze-dried PP was done by 80% methanol (pH = 3.0), followed by vortexing for 10 min, and incubation on ice. After 30 min, solutions were vortexed for another 5 min before the centrifugation (10000 rpm, 10 min, 4°C). For bound polyphenol extraction, sample residues after free extraction were subjected to acid hydrolysis using 2% formic acid. The concentrated extracts were fractionated on a Hypersep C18 cartridge (2000 mg, Thermo Scientific) that had already been activated by 80% methanol and equilibrated with acidified water. The elution of extracts was performed with three different solutions: (1) 0.001% HCl-acidified water for eluting sugars, (2) ethyl acetate to elute phenolic acids and (3) 80%

methanol (pH = 3.0) to elute anthocyanins. The anthocyanin fractions were collected, evaporated, and stored at -80 °C for further analyses.

2.3 Determination of total phenolic content

Total phenolic content (TPC) was determined by using a Folin-Ciocalteu reagent (Sigma-Aldrich) [124]. The reagent (0.2 M) and Na_2CO_3 (7.5%) were allowed to react with samples and the intensity of blue color due to product molybdenum–tungsten blue [125] was measured at 765 nm by a UV-Visible spectrophotometer with gallic acid as a standard.

2.4 Determination of total anthocyanin content

The pH-differential method [126] was applied for the determination of monomeric anthocyanin content (MAC). Potassium chloride buffer (0.025 M; pH=1) and 0.4 M sodium acetate (pH=4.5) were added to samples separately under an appropriate dilution factor (DF). The absorbances were measured at 525 nm and 700 nm by a plate reader. MAC was calculated using the following formula.

$$MAC\ \left(\frac{mg}{L}\right) = \frac{A \times MW \times DF \times 1000}{\varepsilon \times 1}$$

Where;

$$A = Absorbance = (A_{525} - A_{700})_{pH1.0} - (A_{525} - A_{700})_{pH4.5}$$

$$\varepsilon = Molar\ absorptivity = 26900\ \frac{L}{mol \cdot cm}$$

The results were expressed as cyanidin-3-glucoside equivalents (Molecular weight; MW=449.2).

2.4.1 Determination of ABTS radical scavenging activity

The 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma-Aldrich) radical-scavenging activity assay is also known as Trolox (Sigma-Aldrich) equivalent antioxidant capacity assay [127] was used in this study. The green-blue stable radical cationic chromophore (ABTS•+) was produced by the reaction of ABTS solution (95.2 mg in 25 mL 2.45 mM potassium persulfate) and standards/samples. Then, the absorbances were measured at 734 nm by UV-Visible

spectrophotometer after incubation (10 min at 30 ℃) and the antioxidant activity was calculated using Trolox as a standard.

2.4.2 Complexation

The complexation of anthocyanins with polysaccharides (apple pectin, chicory inulin, potato starch and colloidal microcrystalline cellulose from Sigma-Aldrich, St. Louis, MO, USA) was performed according to the method of Li *et al*. [128]. Different ratios of anthocyanins to polysaccharides (1:5, 1:20, 1:50 and 1:100) were tried, and it was found that the best complexation occurred at 1:20. Complexation was conducted by dispersing samples into HCl-acidified water (pH = 2.0) at 1:20 (anthocyanins: polysaccharide) ratio and shaken overnight at 4°C. Complexes were freeze-dried and stored at -80°C for further experiments.

2.4.3 Characterization of complexes

ZETA potential and dynamic light scattering (DLS) analyses

The zeta potential and DLS measurements were performed using a Zetasizer Nano ZS (Zetasizer Nano ZS, Malvern Instruments Inc., Malvern, U.K.). All the zeta potential and DLS measurements were carried out at 25 ℃. Zeta potential was read from pH 12.00 to 1.00 in 1.00-unit increments with a 0.20 pH unit tolerance and three readings per pH value using an MPT-2 Autotitrator (Malvern Instruments Inc., Malvern, U.K.). The samples (polysaccharides: 5 mg/mL; anthocyanins: 0.25 mg/mL) were suspended in water zeta potential and DLS readings. All measurements for zeta potential and DLS were conducted in triplicate and the averages were reported.

Scanning electron microscopy (SEM)

Morphological characterization of complexes obtained under optimized conditions was done by coating with gold/palladium using a sputter coater followed by SEM analysis (FEI Nova NanoSEM).

FTIR spectroscopy

Fourier-transform infrared spectroscopy (FTIR) was specifically performed for the identification of bonds. The experiments were done by FTIR (Nicolet Nexus 670 FTIR, Thermo Fisher Scientific, Waltham MA), with 34 scans at a resolution of 4 cm^{-1}.

2.4.4 Stability of anthocyanins

Concentration of anthocyanins after in vitro digestion

Simulated digestion was performed following the method described by Brodkorb *et al.* [129] with some modifications. This digestion model was composed of three fractions: oral, gastric, and intestinal fractions, which imitate the human upper GI tract. For the oral phase, simulated salivary fluid (SSF) and salivary amylase (75 U/mL; Sigma-Aldrich) were added into the dispersions and gently shaken at 37 °C for 2 min. The samples were then adjusted to pH 2.0 and added to simulated gastric fluid (SGF) and pepsin (4 mg/mL; Sigma-Aldrich) and incubated at 37 °C for 120 min in the gastric phase. The mixtures were then neutralized in the intestinal phase with simulated intestinal fluid (SIF), bile salts (10 mM; Sigma-Aldrich) and trypsin (10 mg/mL; Sigma-Aldrich). Following incubation at 37 °C for 120 min, HCl was applied for enzyme inhibition followed by dialysis (Spectra/Por®3 Dialysis Membrane; MWCO: 3.5 kD; Fisher Scientific) for 2 days in water. Samples that remained in the dialysis bag were freeze-dried and quantified for total phenolics and anthocyanins.

Complexation efficiency

The complexation efficiency was calculated using the loss of the TPC and MAC before and after upper GI digestion. The equation was expressed as:

$$TPC\ complexation\ efficiency\ (\%) = \frac{(TPC_{before} - TPC_{after})}{TPC_{before}} \times 100$$

$$MAC\ complexation\ efficiency\ (\%) = \frac{(MAC_{before} - MAC_{after})}{TMAC_{before}} \times 100$$

Effect of pH on the stability of anthocyanins

Isolated anthocyanins and APC (anthocyanin-pectin complex) were dissolved in water with different pH adjusted by HCl (pH 2, 3, 5, 7). All the samples had the same anthocyanin content (0.3 mg/mL), they were transferred into glass vials to observe the initial color at room temperature (RT). After 4 h at RT, pictures were captured to exhibit the color change. Then, samples were heated by setting the plate heater to 95 ℃ for 6 h and the color differences were recorded. The temperature was lowered to 40 ℃ for color observation at 24 h and 96 h.

2.4.5 Cell culture

Human colon epithelial cells, Caco-2 (ATCC®HTB-37) with 10-30 passages were used. The cultivation of the cells was performed in Dulbecco's Modification of Eagle's Media (DMEM) with 4.5 g/L glucose, L-glutamine and sodium pyruvate, supplemented with 10% (*v*/*v*) fetal bovine serum (FBS), 1% (*v*/*v*) MEM non-essential amino acids solutions (100×), and 1% (*v*/*v*) penicillin-streptomycin (10,000 units penicillin and 10 mg streptomycin/mL) under a humidified atmosphere of 5% CO_2 at 37 °C. All chemicals were obtained from Sigma-Aldrich and Fisher Scientific.

Colonic concentration of anthocyanins in vitro

Caco-2 cells in 12-well filter Transwell inserts were differentiated for 14 days. On day 15, serum media was washed, and anthocyanins, pectin and APC were dissolved in serum-free DMEM for applying to the upper compartment (0.5 mL; 10 μg/mL). After 24/48 h, media from apical and basolateral were collected, evaporated and reconstituted (80 % methanol, pH = 2.0) for anthocyanin content measurement.

Colonic permeability assay in vitro

The Caco-2 cells were seeded onto a 12-well filter Transwell insert at a density of about 1×10^6 cells/cm^2/insert in DMEM cell culture medium (0.5 mL apical and 1.5 mL basolateral). The culture medium was changed every other day for 14 days. On day 15, the differentiated Caco-2 cell monolayer was washed with PBS, serum-free DMEM, and pre-treated with lipopolysaccharide (10 μg/mL; LPS; Sigma-Aldrich) and FITC-dextran (5 mg/mL; Sigma-Aldrich) for 72 h. Anthocyanins, pectin and APC were applied for a total of 48 h. The medium was collected from

the lower compartment on day 18 and the FITC levels were measured to calculate the cell permeability.

2.4.6 Statistical analysis

Values were expressed as mean ± SEM from 4, 6 or 8 independent measurements. Statistical analyses were performed by GraphPad 8, using One/Two-way ANOVA and Tukey's multiple comparisons. When $p < 0.05$, the difference was considered significant.

2.5 Results and discussion

2.5.1 Total phenolic and anthocyanin content of purple potato, purple potato extracts and polysaccharides.

Table 2 details the TPC, MAC and antioxidant activity of PP, isolated anthocyanins and polysaccharides. The bound and free anthocyanin content of purple potatoes were 6.10 ± 0.1 mg GAE/g DW (dry weight) and 1.51 ± 0.02 mg GAE/g DW, respectively. The total bound phenolics and anthocyanins were 2-fold compared to the free phenolics and anthocyanins in PP. The antioxidant activity of purple potato extracts was about 23.4% higher for bound polyphenols, which is because of the increased phenolic and anthocyanin contents. As Yang and Ge *et al.* reported, the phenolic and anthocyanin content is positively correlated to the antioxidant activity [130][131]. Polysaccharides were also analyzed for phenolic and anthocyanin content. Except for pectin, none of the dietary fibers showed any presence of phenolics and anthocyanins after free extraction. The use of pectin in this study was from apple and it is a rich source of polyphenols. A study reported that the total phenolics ranged from 0.73 – 2.17 mg GAE/g FW (fresh weight) in apples [132], and the apple pomace is the main by-product in the apple juice industry that is normally used to extract abundant pectin [133]. The mixture had 0.25 ± 0.03 mg GAE/g DW bound phenolics due to the presence of pectin.

The *in vitro* simulated digestion imitates human upper GI digestion. After the simulated digestion, the increase of free phenolics (65.2%) and anthocyanins (68.8%) were observed in PP. This is because enzymatic hydrolysis facilitates the cleavage of bonds and can help to increase the extraction efficiency [134][135]. Anthocyanin fractions were obtained by the fractionation of concentrated crude extracts into sugars, phenolic acids and anthocyanins using C18 cartridges. The

isolated anthocyanins showed 99.48% loss in anthocyanins after simulated digestion. Unlike the whole PP, the concentrations of phenolics and anthocyanins were increased after upper GI digestion while the isolated anthocyanins showed a high loss indicating low stability. In the case of polysaccharides, after simulated digestion, about 97% of phenolics were lost in pectin. About 0.44 ± 0.03 mg GAE/g DW of free and 0.31 ± 0.01 mg GAE/g DW of bound phenolics were detected in starch. This may be due to starch digestion by the enzymes allowing phenolics to be released [136]. After digestion, the bound phenolics measured in the mixture might come from pectin and remaining undigested starch. Overall, PP as a whole food matrix retained more phenolics and anthocyanins than isolated anthocyanins. Also, polysaccharides had low phenolic content after GI digestion, which will not affect the complexation efficiency.

Table 2. Free and bound TPC, MAC and antioxidant activity (ABTS) of PP, isolated anthocyanins from PP and polysaccharides.

	Samples	Free			Bound		
		Phenolics[1]	Anthocyanins[2]	Antioxidant activity (ABTS[3])	Phenolics	Anthocyanins	Antioxidant activity (ABTS)
Before digestion	Purple potato (mg/g PP DW)	2.07 ± 0.1$^{*\#}$	0.55 ± 0.008*	4.62 ± 0.1$^{\bullet}$	4.03 ± 0.06$^{*\#}$	0.97 ± 0.02$^{*\#}$	6.03 ± 0.3$^{\bullet}$
	Isolated anthocyanins (mg/mL)	2.49 ± 0.02	1.47 ± 0.05	0.75 ± 0.003	NA	NA	NA
	Pectin (mg/g)	0.24 ± 0.008*	ND	0.33 ± 0.2	1.48 ± 0.1*	ND	0.59 ± 0.09
	Mixture (mg/g)	ND	ND	ND	0.25 ± 0.03	ND	ND
After digestion	Purple potatoes (mg/g PP DW)	5.95 ± 0.3$^{*\#}$	1.76 ± 0.1$^{*\#}$	ND	3.80 ± 0.2$^{*\#}$	0.50 ± 0.04$^{*\#}$	ND
	Isolated anthocyanins (mg/mL)	0.083 ± 0.008$^{\#}$	0.021 ± 0.005$^{\#}$	ND	NA	NA	NA
	Pectin (mg/g)	ND	ND	ND	0.061 ± 0.01	NA	ND
	Starch (mg/g)	0.44 ± 0.03	ND	ND	0.31 ± 0.01	NA	ND
	Mixture (mg/g)	ND	ND	ND	0.072 ± 0.02	NA	ND

[1]Expressed as gallic acid equivalents (GAE); [2]expressed as cyanidin-3-glucoside (C3G) equivalents; [3]expressed as Trolox equivalents. Values are shown as means ± SEM (n = 4). ND means not detected; NA means not applicable. Means with *represents significance between free and bound phenolics and anthocyanins. $^{\bullet}$represents significance between free and bound antioxidant activity at $p < 0.01$; $^{\#}$ represents significance between before and after suggestion at $p < 0.01$.

2.5.2 Characterization of complexes

Zeta potential

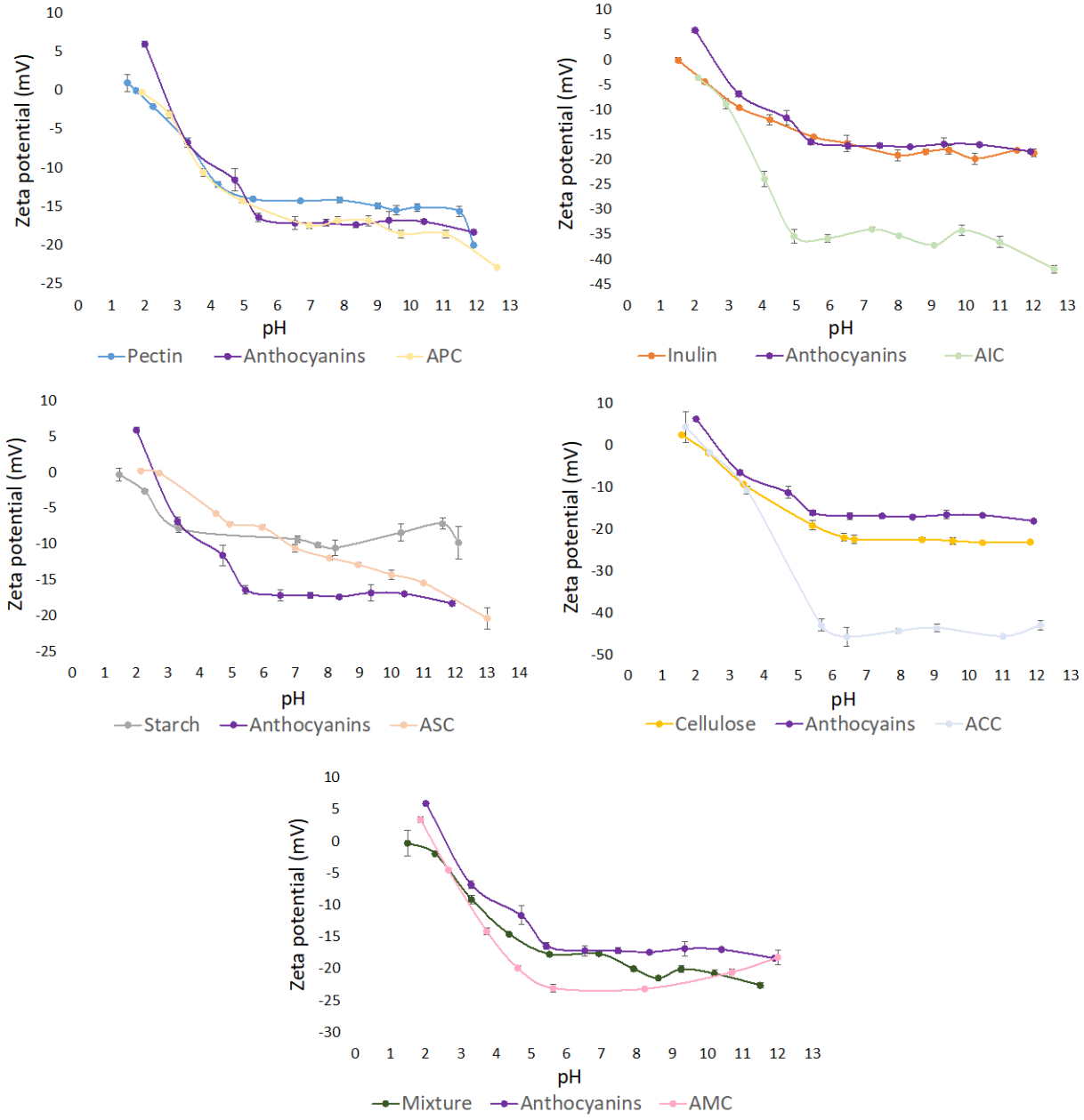


Figure 6. Zeta potential (mV) at different pH levels of polysaccharides, isolated anthocyanins from PP and complexes. Values are shown by means ± SEM (n = 3). APC, anthocyanin-pectin complex; AIC, anthocyanin-inulin complex; ASC, anthocyanin-starch complex; ACC, anthocyanin-cellulose complex; AMC, anthocyanin mixture of polysaccharides complex.

Four polysaccharides and a mixture of these polysaccharides were complexed with anthocyanins. Fig. 6 presents the zeta potential of isolated anthocyanins, polysaccharides, and

respective complexes from pH 1.5 – 12.5. As expected, all the particles were positively charged at a low pH and became significantly more negative at a high pH. This is due to the intrinsic ionization constant of the hydroxyl groups in the polysaccharides. At high pH conditions, samples were able to accept an H+ ion from the acidified solution, increasing their observed surface charge consistent with previously reported work in similar polymers [137][138]. The magnitude of the electrostatic repulsion or attraction between anthocyanins and polysaccharides particles can affect their complexation efficiency. When one particle is positively charged and the other one is negatively charged, the electrostatic attraction can lead to agglomeration [139]. Both structure and electric charge of anthocyanins are pH-dependent. Anthocyanins act as cations in acidic conditions while as anions in alkaline conditions. Anthocyanin zeta potential decreased from 6.03 ± 0.2 to -6.74 ± 0.3 mV as the pH was increased from 2.0 to 3.3. Anthocyanins are positively charged due to the stable flavylium cation at an acidic pH condition [140]. Pectin (-1.06 ± 0.3 mV), inulin (-2.71 ± 0.2 mV), starch (-1.78 ± 0.2 mV), cellulose (-0.0928 ± 0.4 mV) and mixture (-1.33 ± 0.4 mV) showed slightly negative zeta potential at pH = 2.0.

Particle size and polydispersity index through dynamic light scattering

Table 3. Particle size and polydispersity index of polysaccharides, anthocyanins and complexes.

Samples	Z-average (nm)	Polydispersity index
Pectin	1299 ± 14^{a}	0.44 ± 0.002^{a}
Inulin	685.0 ± 24^{b}	0.49 ± 0.06^{a}
Starch	724.8 ± 23^{b}	0.50 ± 0.02^{a}
Cellulose	21080 ± 1200^{c}	1^{b}
Mixture	556.2 ± 20^{d}	0.52 ± 0.04^{a}
Anthocyanins	296.6 ± 7^{e}	0.45 ± 0.03^{a}
APC	1327 ± 12^{a}	0.39 ± 0.03^{a}
AIC	469.2 ± 29^{f}	0.67 ± 0.06^{c}
ASC	434.8 ± 29^{f}	0.50 ± 0.02^{a}
ACC	1545 ± 119^{a}	0.99 ± 0.005^{b}
AMC	658.6 ± 40^{bd}	0.44 ± 0.04^{a}

Values are means ± SEM (n = 6). Similar letters superscript indicated differences at $p < 0.05$ using Tukey's test. APC, anthocyanin-pectin complex; AIC, anthocyanin-inulin complex; ASC, anthocyanin-starch complex; ACC, anthocyanin-cellulose complex; AMC, anthocyanin mixture of polysaccharides complex.

The particle size (Z-average) and polydispersity index (PDI) of samples were measured by Zetasizer (Table 2). DLS measures the light scattering intensity of particles in solution and then observes the correlation of the initial scattering pattern to the change over time. The size of the particle is related to Brownian motion, where small particles move more than the larger particles. In this way, particles that are 1/10 the wavelength of the laser being used can be quantified [141]. Isolated anthocyanins had the expected smallest particle size (296.6 ± 7 nm) which is intuitive when observing its relatively small chemical structure. Among the polysaccharides, cellulose had the largest particle size (21080 ± 1200 nm) while inulin had the smallest particle size (685.0 ± 24 nm). After complexation, only APC and AMC exhibited increases in particle size. Increased particle size represents the possible complexation success. Padayachee *et al.* suggested that ionic

interactions were found between anthocyanins and pectin [42]. The particle size of AIC, ASC and ACC were significantly decreased after complexation, which might signify the failure in bond formation. A few studies have investigated the ability of inulin in the encapsulation with different sources of anthocyanins [142][143]. The complexation illustrated in this study occurred at 4 ℃, while the complexation (i.e. encapsulation) of inulin with anthocyanins required a much higher temperature [118]. Therefore, the lower particle size found in AIC might be ascribed to the low-temperature condition during complexation. PDI describes the width of the particle size distribution, and larger particles also tend to have higher PDI values [141]. The range of PDI is from 0 to 1, and a higher PDI indicates a less homogeneous particle size distribution [144]. Most of the samples apart from cellulose, AIC and ACC showed relatively homogeneous particle size distributions.

Scanning electron microscopy (SEM)

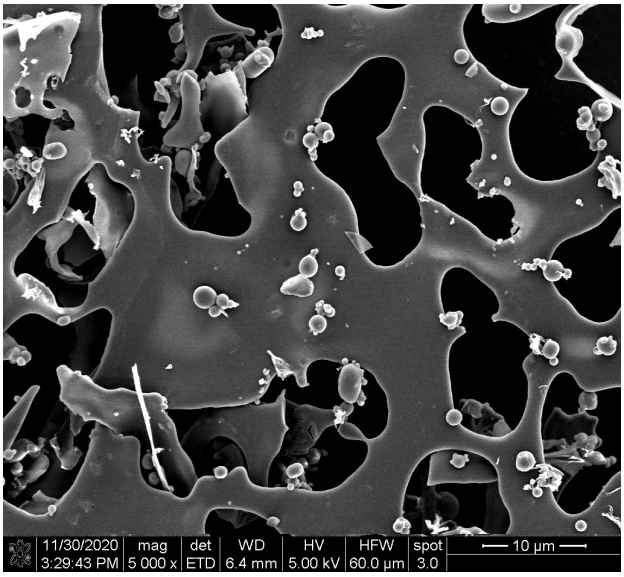


Figure 7. Scanning electron microphotograph of freeze-dried isolated anthocyanins from purple potatoes.

SEM was used to better understand the surface morphology of the polysaccharides and complexes. SEM micrographs revealed a smooth spherical structure of freeze-dried anthocyanin extracts from purple potatoes in Fig. 7. Particles with smooth surfaces have a smaller contact area than rough surface particles, which are less susceptible to degradation reactions [145]. The particle size of anthocyanins was 296.6 nm (Table 3), which is in line with the dimension in SEM

observation. Fig. 8 shows the surface morphology of different polysaccharides and complexes. Additionally, the mixtures (APM, AIM, ASM, ACM and AMM) were made without complexation and subjected to SEM for comparison with complexes. Pectin (Fig. 8A) had a flake and filament structure while starch (Fig. 8G) exhibited smooth spherical and oval shapes. Also, cellulose (Fig. 8J) and pectin appeared to contain additional features (irregular and rough surfaces). Ruffled structures (Fig. 8D) were observed in inulin, which was similar to the SEM micrographs of commercial inulin powder reported by Zarroug *et al.* [146].

APC (Fig. 8C) exhibited a smooth and glossy sheet structure. APC processed complexation led to a smoother surface than APM (Fig. 8B). Anthocyanins as hydrophilic colorants are compatible with water-based gel polysaccharides like pectin [147]. The interaction between pectin and anthocyanins can be attributed to the carboxylic groups of the pectin backbone [148]. Particle agglomeration was seen in APM, which might be due to the formation of a suspension with anthocyanins instead of complexation.

Anthocyanins were dispersed within the layers of inulin in Fig. 3E while AIC (Fig. 8F) displayed a sheet structure that was similar to the surface morphology of APC, but the sheet surface of AIC was rough. The complexation process involved shaking, which might explain the sheet structures that were observed in APC and AIC. The surface morphology of ASM (Fig. 8H) and ASC (Fig. 8I) did not exhibit significant structural differences suggesting no complex formation occurred. In addition, ACM (Fig. 8K) displayed the attachment of anthocyanin particles to the cellulose surface. Aggregation of cellulose particles was found in ACC (Fig. 8L). The interaction of anthocyanins with cellulose was associated with a weak hypochromic effect [149]. Anthocyanins were scattered on the surface of four polysaccharides (Fig. 8N).

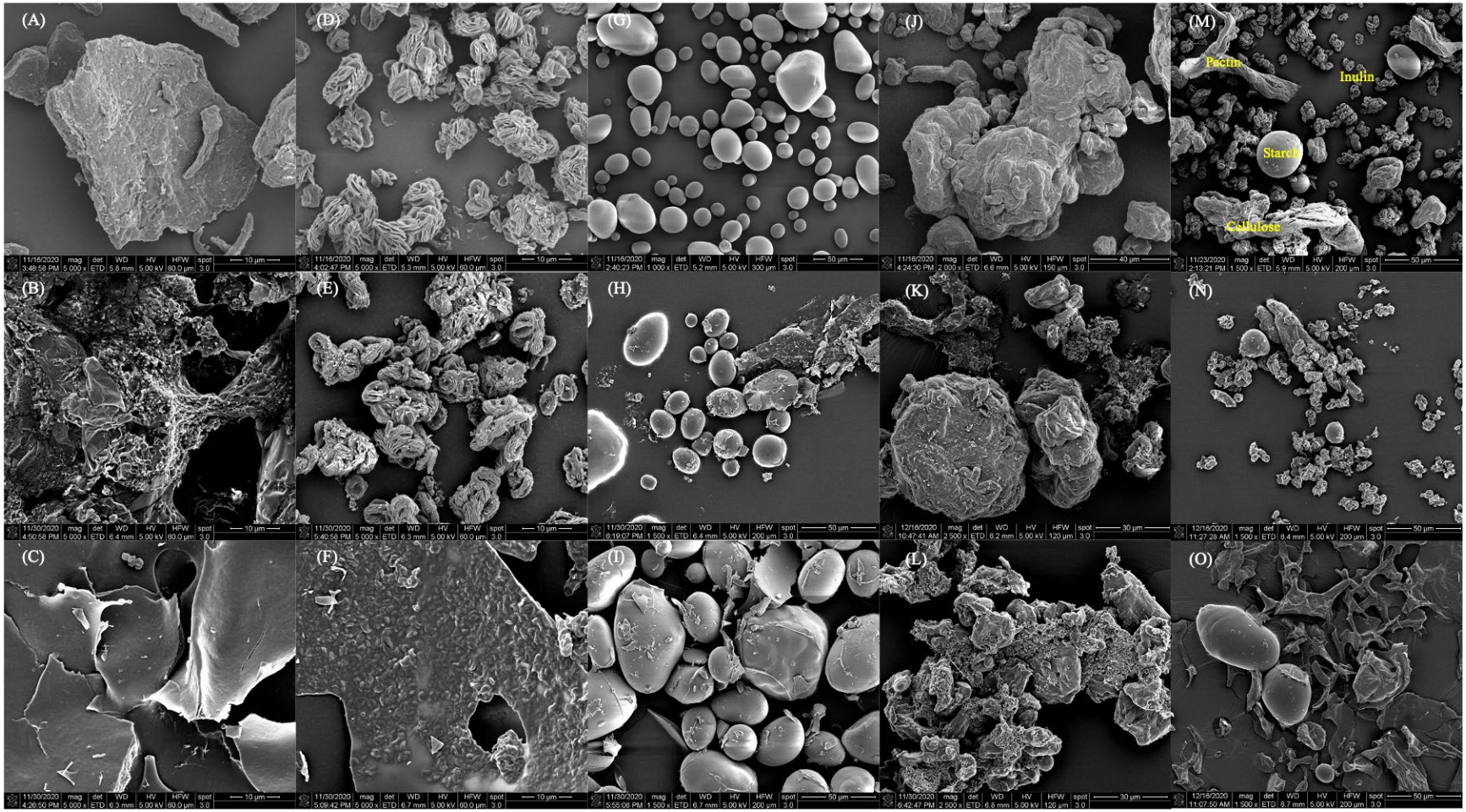


Figure 8. Scanning electron microphotographs of (A) pectin; (B) APM; (C) APC; (D) inulin; (E) AIM; (F) AIC; (G) starch; (H) ASM; (I) ASC; (J) cellulose; (K) ACM; (L) ACC; (M) mixture of four polysaccharides; (N) AMM; (O) AMC. APM, anthocyanin-pectin mixture; APC, anthocyanin-pectin complex; AIM, anthocyanin-inulin mixture; AIC, anthocyanin-inulin complex; ASM, anthocyanin-starch mixture; ASC, anthocyanin-starch complex; ACM, anthocyanin-cellulose mixture; ACC, anthocyanin-cellulose complex; AMM, anthocyanin mixture of polysaccharides mixture; AMC, anthocyanin mixture of polysaccharides complex.

Structural analysis using FTIR

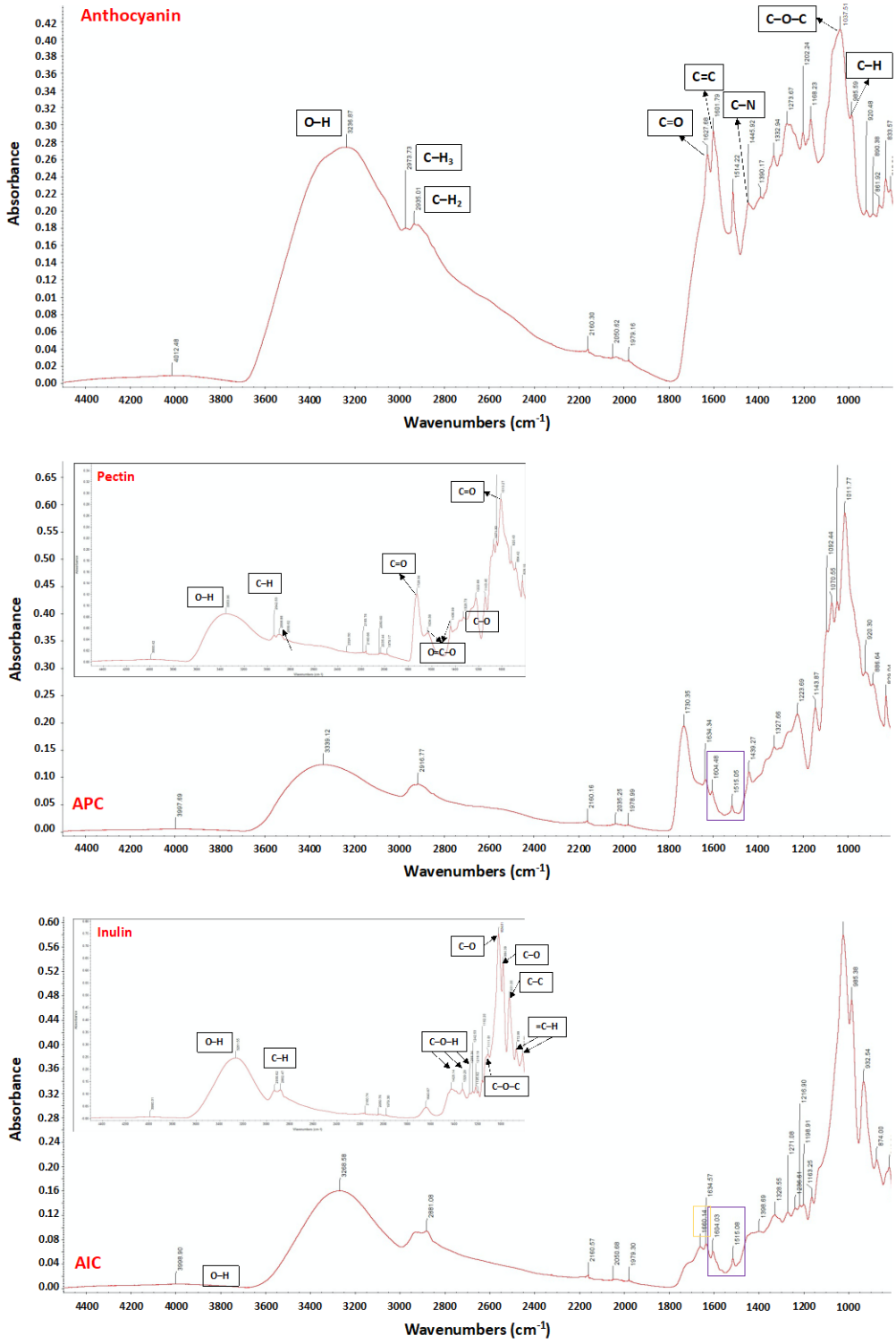
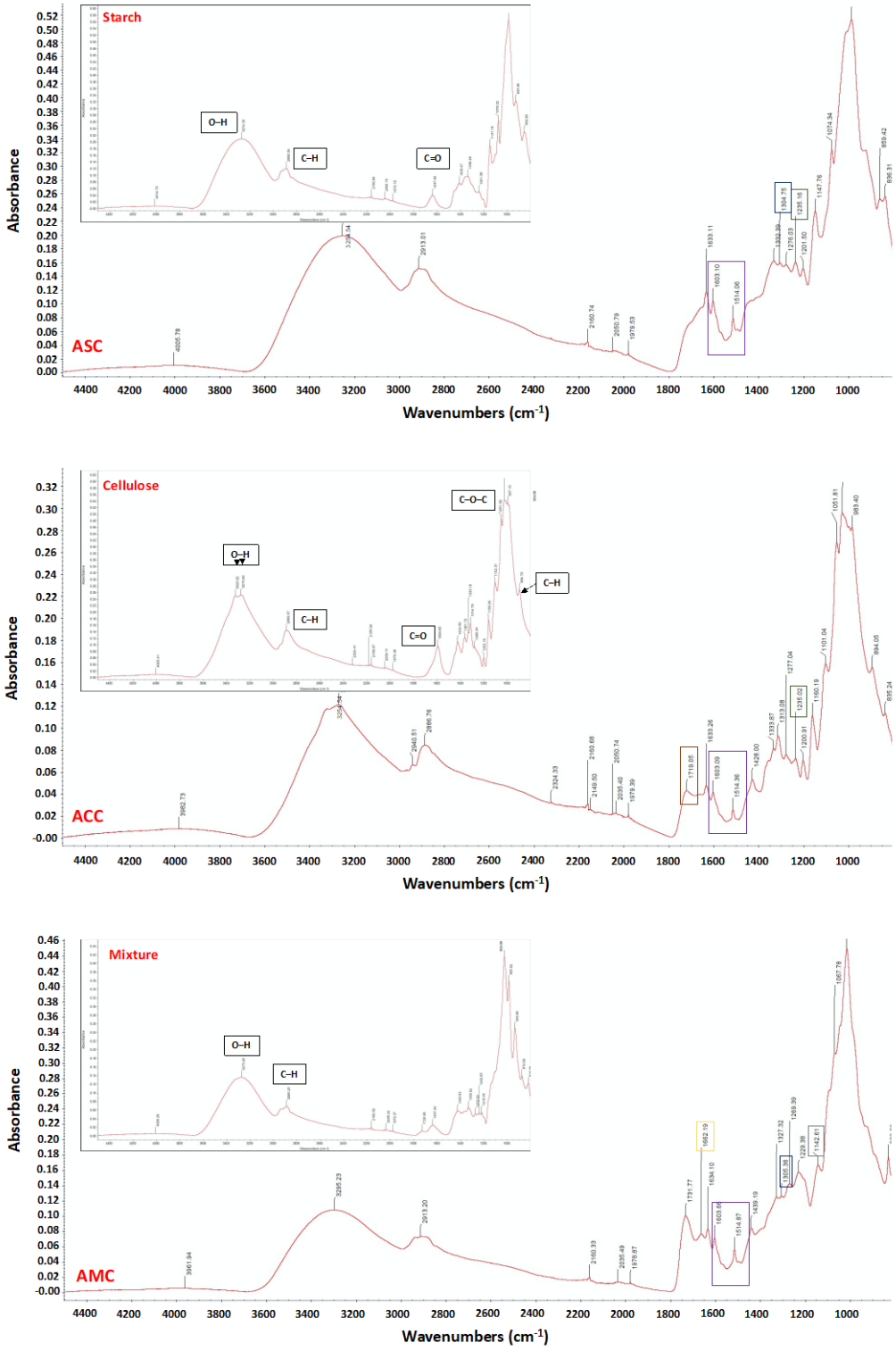


Figure 9. FTIR spectra of anthocyanins, APC, pectin, AIC, inulin, ASC, starch, ACC, cellulose, AMC, mixture. APC, anthocyanin-pectin complex; AIC, anthocyanin-inulin complex; ASC, anthocyanin-starch complex; ACC, anthocyanin-cellulose complex; AMC, anthocyanin mixture of polysaccharides complex.

Figure 9 continued



FTIR was employed to characterize anthocyanins, polysaccharides and complexes (Fig. 9). FTIR spectroscopy is a powerful characterization technique to detect organic molecules'

functional groups including hydroxyls, carbonyls, esters, amides, etc. The FTIR spectra of polysaccharides are ascribed to stretching vibrations of free, inter- and intra-molecular hydroxyl groups at similar broad bands peaking around 3350-3200 cm^{-1}. Additionally, the wavelength ranges from 3000 to 2800 cm^{-1} are the characteristic bands of C-H bonding. Anthocyanins from purple potatoes exhibited bands belonging to the hydroxyl group (3236 cm^{-1}), saturated hydrocarbon groups (2973 cm^{-1}: methyl group; 2935 cm^{-1}: methylene group), and the band at 1445 cm^{-1} was attributed to aromatic ring (C-N) vibration [150]. The other absorbance bands were visible at wavelengths of 1627, 1601, 1514, 1445, 1390, 1332, 1273, 1202, 1168, 1037 and 985 cm^{-1} in anthocyanins. The C=C stretching (1604 cm^{-1}) and N-O stretching (1515 cm^{-1}) found in anthocyanins were observed in APC, AIC, ASC, ACC and AMC.

The infrared spectra in pectin has important bands found at 1728, 1634, 1438, 1328, 1222, 1143, 1071, 1010, and 828 cm^{-1} [174][152]. The peaks at 1728 and 1634 cm^{-1} indicated ester carbonyl (C=H) and carboxylate ion stretching, respectively [153]. The stretching bands of APC highly resemble pectin due to the high ratio of pectin in the complex. The infrared spectra of inulin had bands at 3261, 2882, 1640, 1426, 1329, 1266, 1111, 1024, 984, 931 and 872 cm^{-1}, which are similar to the spectra reported by El-Kholy *et al*. [154]. The strong peak at around 1024 cm^{-1} might be the inulin pyranose ring due to the C-O and C-C stretching vibrations [155]. There are two peaks at 1660 and 1634 cm^{-1} (C=C stretching) that emerged in AIC. The 1147 and 1076 cm^{-1} bands found in starch are attributed to bending and asymmetric stretching of C-O, C-C, O-H and C-O-C glycosidic bonds [156]. Two new FTIR spectral peaks at 1304 cm^{-1} and 1235 cm^{-1} are found in ASC compared to starch and anthocyanins. The absorbance bands at 1304 cm^{-1} and 1235 cm^{-1} can be corroborated with a C-C/C=C vibration and antisymmetric stretching of PO^{2-}, respectively [157][158]. In ACC, the bands at 1719 cm^{-1} correspond to ester carbonyl groups (C=O stretch) which were not found in cellulose and anthocyanins. FTIR proved the complexation of polysaccharides and anthocyanins.

2.5.3 Stability of anthocyanins in complexes

Concentration of anthocyanins bound to polysaccharides after GI digestion

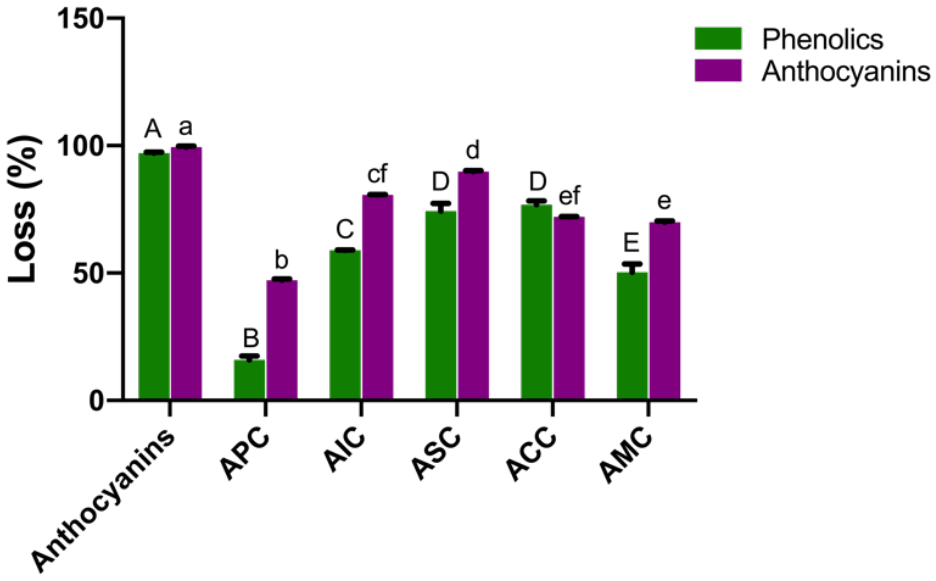


Figure 10. Loss in the percentage of phenolics and anthocyanins in isolated anthocyanins and complexes after simulated digestion. Values are shown by means ± SEM (n = 4), different letters on the bars indicate differences between different polysaccharides at $p < 0.05$. APC, anthocyanin-pectin complex; AIC, anthocyanin-inulin complex; ASC, anthocyanin-starch complex; ACC, anthocyanin-cellulose complex; AMC, anthocyanin mixture of polysaccharides complex.

The complexes were subjected to simulated digestion and the percent loss of phenolics and anthocyanins after digestion are presented in Fig. 10. The isolated anthocyanins were more susceptible to upper GI digestion. This agrees with work that found a reduction in the stability of anthocyanins from commercial black currant juice in the intestinal fluid with pancreatin [159]. In addition, Yang *et al.* revealed that there was a dramatic decrease in the concentration of anthocyanins from red wine in the simulated intestinal digestion [160]. Furthermore, Podsędek *et al.* reported that the loss of anthocyanins of anthocyanin-rich extract from red cabbage was about 87% and only 32% loss was observed in whole cabbage [116]. Therefore, complexation as a stabilizing method is necessary to help anthocyanins resist the loss in the upper GI tract to enter the colon.

The concentration of bound anthocyanins after digestion is highly dependent on complexation efficiency. Pectin had the best binding capacity with anthocyanins compared to other polysaccharides. Although all the polysaccharides significantly improved the anthocyanin

concentration, APC exhibited the lowest loss percentage in phenolics (16.00%) and anthocyanins (47.13%), suggesting a higher concentration of bound anthocyanins. In plant cells, pectin had the highest affinity for phenolic acids and proanthocyanins [161]. Apple pectin is a type of soluble fiber with a high level of methyl esterification [162]. Liu *et al.* revealed strong hydrophobic interactions between methyl groups of pectin and the dihydropyran heterocycles (C-ring) of procyanidins [163]. However, soluble blueberry pectin (high methoxyl) showed the least binding ability with anthocyanins as reported by Lin *et al* [43]. This suggests that the interactions may likely be driven through electrostatic interactions of oppositely charged particles, confirmed through FTIR and zeta potential.

Stability of anthocyanins with changes in pH and temperature

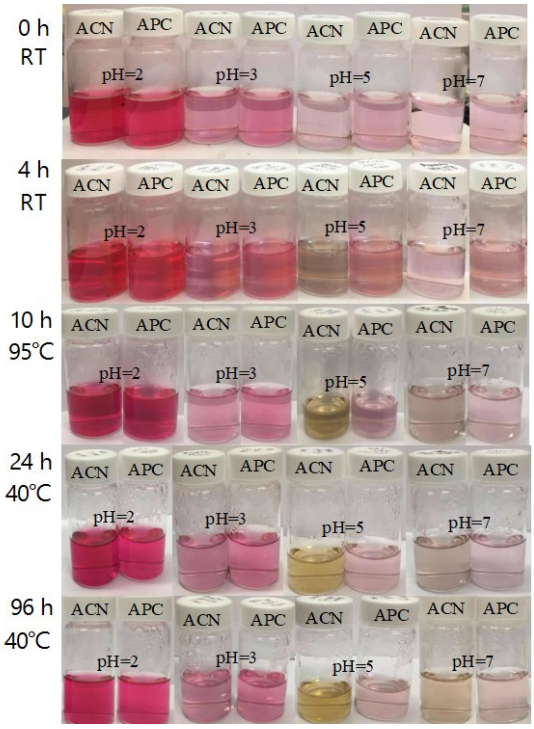


Figure 11. The intensity of anthocyanins in extract and APC at different pH and temperature; ACN, fractionated anthocyanins; APC, anthocyanin-pectin complex.

Isolated anthocyanins were unstable, and their structural stability was altered by the changes in pH and temperature in Fig. 11. At neutral pH, anthocyanins normally show a purple hue and then turn to blue at an increasing pH [62]. In Fig 6, isolated anthocyanins and APC showed rose-red color and good stability at pH = 2.0 and exhibited the highest stability even when the

temperature was increased. A study reported that the stability of anthocyanins at acidic conditions is higher [164]. In addition, APC showed significantly better stability than isolated anthocyanins with the increased pH and temperatures. As Buchweitz *et al.* reported, the stability of anthocyanins was significantly improved by pectin compared to the anthocyanin extracts under 20 ℃ conditions for 18 weeks [165]. At pH = 5.0 and 7.0, the color fading was noticeable after 4 h. At pH = 5.0, the increased temperature reduced the intensity of the color of isolated anthocyanins than APC. This shows that APC can stabilize anthocyanins at high pH and temperature conditions.

Permeability of anthocyanins in vitro

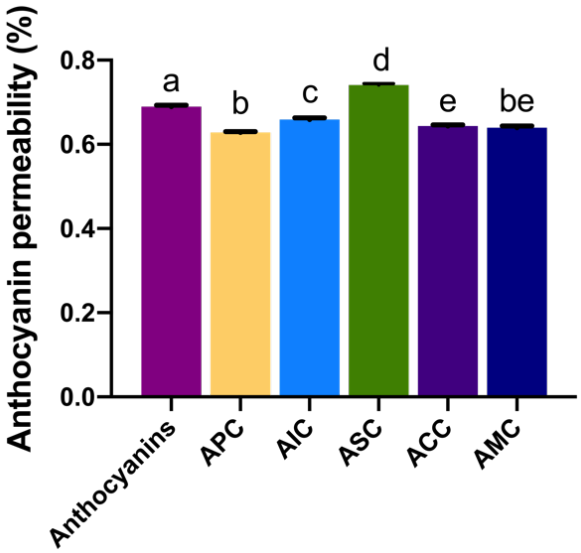


Figure 12. Anthocyanin permeability (%) of isolated anthocyanins and complexes. Values were shown as means ± SEM (n = 8), different letters on the bars indicate differences between the means of complexes at $p < 0.05$. APC, anthocyanin-pectin complex; AIC, anthocyanin-inulin complex; ASC, anthocyanin-starch complex; ACC, anthocyanin-cellulose complex; AMC, anthocyanin mixture of polysaccharides complex.

The colonic permeability of anthocyanins after the simulated *in vitro* upper GI digestion was measured using Caco-2 monolayer. After 24-hour exposure (Fig. 12), the concentration of anthocyanins that are not absorbed and are available for gut bacteria from the complexes was measured. Many studies used the Caco-2 cell monolayer to investigate anthocyanin absorption [166][167]. Cheng *et al.* reported increased intestinal absorption of anthocyanins with anthocyanin-phospholipid complex [168]. A higher anthocyanin concentration in the basolateral chamber indicates higher permeability and less complexation. Thus, the disruption of the complex

was quantified by a high permeability percentage. It is expected that the isolated extracts had a high anthocyanin permeability indicating a lower abundance in the colon. APC exhibited significantly lower permeability of anthocyanins compared to AIC, ASC, and ACC. Pectin might be considered a good candidate to improve colonic concentrations of isolated anthocyanins.

2.5.4 Colonic permeability in vitro

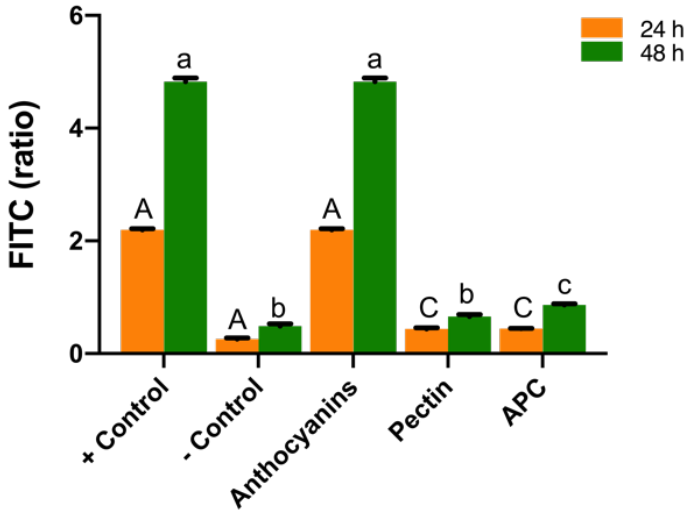


Figure 13. The relative FITC-dextran ratio. Caco-2 permeability at 24 h was calculated: 24 h/0 h, and at 48 h: (24 h + 48 h)/0 h. Values are shown as means ± SEM, different letters on the bars indicate differences at $p < 0.05$. APC, anthocyanin-pectin complex.

Differentiated Caco-2 cells were used to determine the role of isolated and complexed anthocyanins in reducing colonic cell permeability. The FITC-dextran permeability assay in the Caco-2 cell model is presented in Fig. 13. The differentiated cells were treated with LPS (10 μg/mL) for three days to induce damage and increase permeability in this study. The permeability of the gut barrier was monitored by the FITC-dextran concentration in the basolateral chamber. Higher the FITC concentrations, the higher the permeability and barrier dysfunction [169]. On day four, LPS was washed, and treatments (anthocyanins and APC; 20 μg/mL; pectin: 10 μg/mL after upper GI digestion) were applied in the basolateral chamber for 24 h and 48 h. High FITC-dextran measured in anthocyanin treated wells, which explained that the isolated anthocyanins failed to restore the barrier function. Although studies have reported that the isolated anthocyanins from plant foods inhibit the disruption of Caco-2 intestinal barrier function [170][171], researchers

suggested that the protective effects of anthocyanin-rich extracts in barrier integrity are positively correlated to cyanidin and delphinidin but not malvidin and peonidin [172]. Petunidin and malvidin are two major anthocyanidins in purple potatoes [28], which might explain the anthocyanins did not restore the intestinal permeability in the LPS-induced Caco-2 monolayer model. Moreover, we used the extracts after *in vitro* digestion, where the anthocyanins were subjected to pH changes rendering them ineffective. The barrier function was restored by pectin and APC treatment at 24 and 48 h. The effect of APC in reducing the LPS-induced permeability is due to pectin in the complex rather than the anthocyanins [173]

2.6 Conclusion

In this work, we have shown that pectin complexed with isolated anthocyanins from purple potatoes at pH 2.0, through electrostatic interaction and hydrogen bonds, enhanced the stability of anthocyanins and their colonic concentrations. In conclusion, pectin is the best candidate for the complexation with anthocyanins to improve GI stability and barrier function.

CHAPTER 3. ANTI-COLITIC EFFECTS OF ANTHOCYANIN-PECTIN COMPLEX *IN VIVO*

Abstract

Ulcerative colitis (UC) is characterized by chronic colonic inflammation, impaired barrier function and gut bacterial dysbiosis. Though anthocyanin-containing potatoes have been shown to reduce inflammation and colonic permeability in colitis mice, isolated anthocyanins are not stable. We and others have shown that anthocyanin complexation with polysaccharides improved the stability of anthocyanins against varying pH and temperature and simulated upper gastrointestinal (GI) digestion *in vitro*. However, it is not clear whether the complexation of anthocyanins with pectin (APC) improves the anti-colitic activity *in vivo*. This study evaluated the role of APC in improving gut barrier integrity, reducing gut permeability and inflammation using a DSS-induced murine model of colitis. Age- and gender-matched C57/BL6 mice were assigned to six treatments (negative control, DSS, isolated anthocyanins, pectin, APC and purple potatoes) for 2 or 4 weeks. On week four all animals excepted the negative control group were exposed to DSS in drinking water for four days. The concentration of anthocyanins remaining after upper GI digestion was high in APC compared to isolated anthocyanins. Mice in APC group showed a significant reduction in the gut permeability and increase in colonic mucus thickness and fecal butyric acid levels compared to DSS mice. These parameters indicate improved gut barrier function. APC supplementation showed a reduction in liver weight and serum aspartate aminotransferase, a marker of liver damage compared to anthocyanins and pectin. However, APC did not reduce the expression of IL-6 and IL-1β, while the PP group exhibited a better anti-inflammatory effect than other treatment groups. Isolated anthocyanins, pectin, APC and purple potatoes significantly reduced the IL-17 levels compared to the DSS group. In conclusion, APC resisted the DSS-induced increase in gut barrier dysfunction by reducing gut permeability, increasing mucus thickness and butyric acid levels.

3.1 Introduction

Inflammatory bowel disease (IBD) is a global disease, encompasses ulcerative colitis (UC) and Crohn's disease (CD). Impaired intestinal barrier, gut bacterial dysbiosis and colonic

inflammation are three main characteristics of IBD [9]. The risk factors of IBD include lifestyle, genetic background, immunity, and environmental factors. Over 6.8 million people were suffering from IBD globally in 2017 [3], and the worldwide IBD incidence continues to grow. Corticosteroids, amino-salicylates, immunomodulators and antibiotics are the pharmacotherapy options and commonly used to treat IBD, but with many negative side effects [174]. Thus, the attention of functional foods as substitutes for IBD treatment is growing worldwide.

Dietary anthocyanins (ACNs) are natural phytochemicals in fruits and vegetables that belong to the group of flavonoids. Isolated anthocyanins are not stable, and can be degraded by many environmental factors, such as temperature, pH and light. In the whole food matrixes, anthocyanins are strongly stabilized because of their complexation with macronutrients, and their colonic anthocyanin concentration is higher than the isolated anthocyanins. A study done by Podsędek *et al.* reported that the loss of anthocyanins in anthocyanin-rich extracts was significantly higher than the red cabbage after simulated digestion [116]. To enhance the colonic anthocyanin concentration of isolated anthocyanins, many stabilizing methods are developed. Tan *et al.* reported that encapsulation can be done by spray/freeze-drying, emulsification, gelation and polyelectrolyte complexation to stabilize isolated anthocyanins. In addition, many different biopolymers such as protein and pectin are materials used in anthocyanins stabilization [104].

Anthocyanins (ACNs) have shown anti-microbial, anti-cancer, anti-obesity, anti-diabetes, and anti-inflammatory properties [45]. Many *in vitro* and *in vivo* studies have revealed the beneficial effects of isolated anthocyanins in the colon. In a TNFα-induced Caco-2 cell monolayers model, cyanidin-*O*-glucoside and delphinidin-*O*-glucoside increased TEER (transepithelial electrical resistance) and decreased FITC-dextran permeability [172]. Besides, anthocyanins extracted from *L. ruthenicum* regulated the intestinal microbiota composition and maintained intestinal health in an *in vitro* fermentation [56]. Black rice anthocyanin-rich extract administration decreased the histological score and downregulated the TNF-α gene expression levels in an *in vivo* dextran sulfate sodium (DSS)-induced colitis mouse model [175]. However, the colonic concentration of isolated anthocyanins is quite low due to their low stability.

Pectin is a polysaccharide of galacturonic acid and found naturally in the cell walls and intercellular layers of fruits and vegetables. As a functional ingredient, pectin is commonly added

to jellies, jams, juice and many desserts. Furthermore, pectin showed various biological activities including the modulation of gastrointestinal disorders, blood cholesterol via restricting the bile acid reabsorption, serum glucose and insulin content and anti-cancer activities in the pharmaceutical industry [86][87][88]. Additionally, in a chemical-induced colitis mouse model pectin supplementation ameliorated the colitis symptoms and tissue damage [176]. Pectin can also be utilized by gut bacteria in enhancing gut immunity and improving intestinal integrity [177].

The aim of the present study was to investigate the anti-colitic effects of APC in a well-defined DSS-induced colitis mouse model. Isolated anthocyanins, pectin and purple potato were also used in this study to compare the beneficial effects.

3.2 Materials and Methods

3.2.1 Animals

Five-week-old male C57BL/6 mice (n = 60) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and randomly assigned to 6 groups: Control, Control + DSS, ACN + DSS, Pectin + DSS, APC + DSS and purple potato diet (PP) + DSS groups. All animal procedures were approved by the Purdue University Animal Care and Use Committee (PACAUC approval No. 1810001817) who adheres to the recommendations of the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

3.2.2 Diet and experimental design

Purple potatoes (*Solanum tuberosum* L. var. Purple Majesty) were harvested from San Luis Valley Experiment Station (Center, Colorado, USA). They were washed, baked (oven; 180°C, 50 min), freeze-dried (VirTis Ultra 35L Pilot Lyophilizer; Warminster, PA, USA), ground and stored at -80°C. The purple potato diet (PP) included 20% baked and freeze-dried purple potato powder. ACN + DSS group was gavaged with the anthocyanins extracted from freeze-dried purple potato powders and fractionated on a Hypersep C18 cartridge (2000 mg, Thermo Scientific). The standard apple pectin was purchased from Sigma-Aldrich, St. Louis, MO, USA and used in the Pectin + DSS group. APC + DSS group was gavaged with anthocyanin extracts that were complexed with pectin. AIN-93G diet (ENVIGO Teklad, Madison, WI, USA) was the control diet. Table 4 shows the nutritional information of the AIN-93G diet and purple potato diet. The treatment groups, ACN

+ DSS, Pectin + DSS and APC + DSS, were administrated by oral gavage. The dosage of anthocyanins was 7.6 mg anthocyanin extracts/100 g body weight, pectin was 38 mg pectin/100 g body weight. APC + DSS group was exposed to 7.6 mg anthocyanins and 38 mg pectin in complexation/100 g body weight. Treatment groups and Control + DSS group induced colitis by DSS (dextran sulfate sodium; M. W. = 40 kDa; MP Biomedicals (Solon, OH, USA)) administration in water.

Table 4. Composition of Diets [g/kg] and Selected Nutrient Information.

Formula	Control	PP
Potato Powder	-	200.000
Corn Starch	427.492	252.322
Casein	200.000	180.230
L-Cystine	3.000	3.000
Maltodextrin	132.000	132.000
Sucrose	100.000	100.000
Soybean Oil	40.000	40.000
Cellulose	50.000	44.940
Mineral Mix, AIN-93G-MX [94046]	35.000	35.000
Vitamin Mix, AIN-93-VX [94047]	10.000	10.000
Choline Bitartrate	2.500	2.500
TBHQ [antioxidant]	0.008	0.008
Protein [% by weight]	17.700	17.700
Carbohydrate [% by weight]	62.800	61.700
Fat [% by weight]	4.200	4.200
Kcal/g	3.600	3.600

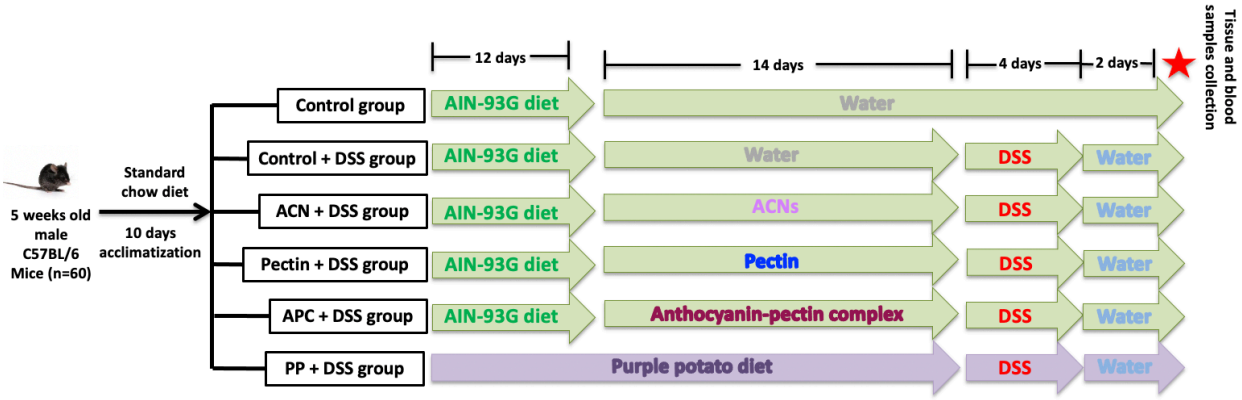


Figure 14. Experimental design. Mice were maintained on standard chow for 10 days and then assigned to 6 groups randomly. Control and Control + DSS groups were gavaged with drinking water. The treatment groups included ACN + DSS, Pectin + DSS, APC + DSS and PP + DSS. In the last week, colitis was induced by 3% DSS treatment in mice drinking water for four days. On day 5, mice received normal drinking water without DSS. The intestinal permeability was measured, and mice were sacrificed for sample collection on day 6.

3.2.3 Sample collection and measurement

Fecal samples were collected every two days through the whole experiment period. Mice were euthanized under CO_2 asphyxiation after DSS treatment. Blood collection was done by cardiac puncture, and cecum, cecal digesta, colon, colon digesta, spleen, liver and kidney were harvested and weighed immediately before storage.

3.2.4 Determination of total phenolics and anthocyanins

APC, purple potatoes and mice cecal digesta samples were measured and extracted with 50% ethanol at pH 3.0. Mixtures were centrifuged (10000 rpm, 10 min, 4°C) after 10 min vortexing and 30 min incubation on ice. Supernatants were collected into a new tube, then 2% formic acid was added to the residue for acid hydrolysis at 60°C for 15min. After centrifugation, supernatants were combined for the measurement of TPC and MAC. Total phenolic content (TPC) was measured by the reaction of samples with Folin-Ciocalteu reagent (Sigma-Aldrich; 0.2 M) and Na_2CO_3 (7.5%). After 30 min incubation at 37°C, absorbance was measured at 765 nm using a microplate reader. Monomeric anthocyanin content (MAC) was measured by the pH-differential method [178]. Buffers with pH 1.0 and 4.5 were added into samples separately. The absorbance was measured at 525 and 700 and the MAC was obtained by the following equation below:

$$MAC\ \left(\frac{mg}{L}\right) = \frac{A \times MW \times DF \times 1000}{\varepsilon \times 1}$$

Where;

$$A = Absorbance = (A_{525} - A_{700})_{pH1.0} - (A_{525} - A_{700})_{pH4.5}$$

$$\varepsilon = Molar\ absorptivity = 26900\ \frac{L}{mol \cdot cm}$$

The results were expressed as cyanidin-3-glucoside equivalents (Molecular weight; MW=449.2).

3.2.5 Histopathology

10% neutral formalin was used to fix the colon tissue at room temperature for 24 hours. Next, tissues were embedded in the paraffin blocks, sectioned (4 μm), stained with hematoxylin and eosin (H&E) and then observed under the microscope. A board-certified veterinary pathologist scored the distal colon sections based on the standard histopathological morphology shown in Table 5.

Table 5. Histomorphological scale.

Criterion	Score
Mucosal hyperplasia	3 = Marked 2 = Moderate 1 = Mild 0 = Normal
Epithelial cell death	3 = Ulcerations 2 = Erosions 1 = Superficial epithelial sloughing, single cell necrosis 0 = Normal
Mononuclear infiltrate	3 = Markedly increased 2 = Moderately increased 1 = Slightly increased 0 = Normal
Polymorphonuclear leukocyte infiltrate + eosinophils	3 = Markedly increased 2 = Moderately increased 1 = Slightly increased 0 = Normal
Eosinophils	3 = Markedly increased 2 = Moderately increased 1 = Slightly increased 0 = Normal
Crypt architectural distortion/death	3 = 50% or greater of the crypts in the mucosa 2 = 10%–50% 1 = <10% 0 = Normal
Involvement of the submucosa	3 = 50% or greater of the submucosal diameter 2 = 10%–50% 1 = <10% 0 = Normal

3.2.6 Intestinal permeability assay

Mice were gavaged with 150 µL/mouse FITC-dextran (100 mg/mL), and then blood was collected into BD Microtainer® tubes (BD, Franklin Lakes, NJ) via the submandibular vein, and centrifuge (2500 g, 2 min) for serum. The BioTek™ Cytation™3 Cell Imaging Reader (Thermo Fisher Scientific Inc., Winooski, VT, USA) was used to measure the fluorescence intensity (λex = 490

nm; λem = 520 nm) of FITC-dextran. The intestinal permeability was calculated by the FITC-dextran standard curve (1000000, 10000, 5000, 2500, 1250, 625, 312.5, 0 ng/mL).

3.2.7 Colonic mucus thickness

Carnoy solution [methanol, chloroform and glacial acetic acid (6:3:10, v/v/v)] and Alcian blue solution (Electron Microscopy Sciences, Hatfield, PA, USA) were used to fix and stain the fresh colon tissues that have embedded in paraffin. The staining process followed the protocol done by Desai *et al.* [179].

3.2.8 RNA extraction and RT-PCR analysis

The total RNA from colon tissues was extracted by the PureLink™ Mini Kit (Invitrogen; Carlsbad, CA, USA), and reverse-transcription was done by qScriptTM cDNA SuperMix (Quantabio, Beverly, MA) synthesis kit. The Biosystems™ PowerUp™ SYBR™ Green Master Mix (Waltham, MA, USA) and QuantStudio 3 Real-Time PCR System (Waltham, MA, USA) were applied for real-time PCR. Initially, denaturation to generate single-stranded DNA templates was achieved by heating up to 95°C for 10 min, and then followed by 45 cycles of annealing at 95°C for 10 s. In the end, the extension step was held for 60 s at 55°C. As an endogenous reference for colonic inflammatory cytokines gene expression analysis, the expression level of β-actin was used. The primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA) and listed in Table 6.

Table 6. Real-time PCR primers.

Gene	Primers
IL-6	Forward: 5′-AGTTGCCTTCTTGGGACTGA-3′
	Reverse: 5′-CAGAATTGCCATTGCACAAC-3′
IL-17	Forward: 5′-TCAGACTACCTCAACCGTTCC-3′
	Reverse: 5′-ATGTGGTGGTCCAGCTTTCC-3′
IL1-β	Forward: 5′-GCCCATCCTCTGTGACTCAT-3′
	Reverse: 5′-AGGCCACAGGTATTTTGTCG-3′
ZO-1	Forward: 5′-AGGACACCAAAGCATGTGAG-3′
(zonula occludens-1)	Reverse: 5′-GGCATTCCTGCTGGTTACA-3′
Claudin-1	Forward: 5′-TCTACGAGGGACTGTGGATG-3′
	Reverse: 5′-TCAGATTCAGCAAGGAGTCG-3′
MUC2	Forward: 5′-GCTGACGAGTGGTTGGTGAATG-3′
	Reverse: 5′-GATGAGGTGGCAGACAGGAGAC-3′
β-Actin	Forward: 5′-AGCCATGTACGTAGCCATCC-3′
	Reverse: 5′-CTCTCAGCTGTGGTGGTGAA-3′

3.2.9 MUC2 protein quantification

About 100 mg frozen distal colons were measured, homogenized (5 min at high speed) by bullet blender in the reinforced tube with 1 mL PBS, stored overnight at -20℃, then subjected to two freeze-thaw cycles. Centrifuged (5,000g; 5 min) samples were quantified for the total protein content by a Pierce BCA Protein Assay Kit [Fisher Scientific, Hanover Park, IL]. The MUC2 protein content was quantified by a custom MUC2 ELISA kit (AVIVA SYSTEMS BIOLOGY, San Diego, CA).

3.2.10 Examination of aspartate aminotransferase (AST) activities

The aspartate aminotransferase (AST) test is a blood test that quantified liver injury. This test is done by following the manufacturer's instructions.

3.2.11 Short-chain fatty acid extraction and GC analysis

Mice cecal digesta (50 mg) were homogenized in 0.5% phosphoric acid (600 µL) using the bullet blender. Before centrifugation (17000 g; 10 min; 4°C), fecal samples were vortexed under a medium-high speed for about 1 min. Ethyl acetate with 0.014% heptanoic acid (about 300 ~ 400 µL) was added to the supernatant with a thorough vortex in a 2 mL screw-thread autosampler vial and stored at -80°C. The identification and quantification of SCFAs were done by an Agilent 7890A GC (GC-FID 7890A, Santa Clara, CA, USA) with a fused silica capillary column (Nukson SUPELCO No: 40369-03A, Bellefonte, PA, USA). Standards (acetic, butyric, isobutyric, propionic, valeric, isovaleric and caproic acids) and the internal standard (heptanoic acid) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and used to calculate the SCFAs concentration of fecal samples.

3.2.12 Statistical analysis

All values were expressed as mean ± SEM. Statistical analysis was performed by GraphPad 8, using T-test, One/Two-way ANOVA, Tukey's and Fisher's LSD multiple comparison tests. When $p < 0.05$, the difference is considered significant.

3.3 Results

3.3.1 Concentration of anthocyanins retained after simulated GI digestion

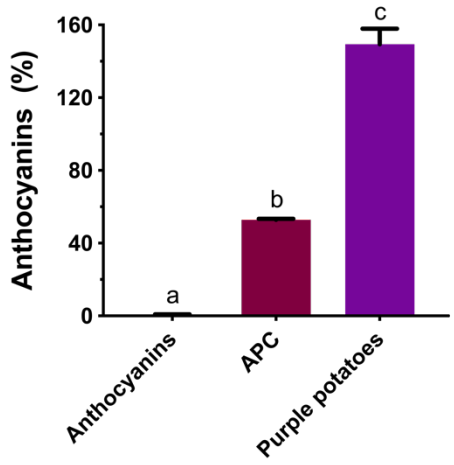


Figure 15. Anthocyanins (%) retained after simulated digestion. Values are shown by means ± SEM (n = 4), different letters on the bars indicate differences between different treatment groups at $p < 0.01$. APC, anthocyanin-pectin complex.

The *in vitro* simulated digestion includes oral, gastric and intestinal phases with different enzymes to imitate the human upper gastrointestinal (GI) tract digestion. The concentration of anthocyanins retained after the simulated digestion of anthocyanin-rich extracts, APC and purple potatoes are shown in Fig 15. Isolated anthocyanins showed the highest loss (99.48%) and the lowest retention (0.52%) after simulated digestion. However, only 47.13% of anthocyanins were lost when they are complexed with pectin in APC, and about 52.87% of anthocyanins can enter the small intestine. Purple potatoes significantly retained anthocyanins. About 149.41%

anthocyanins were measured after upper GI digestion, and the enzymatic hydrolysis facilitated the cleavage of bonds and increased the extraction yield of anthocyanins.

3.3.2 Cecal phenolics and anthocyanins

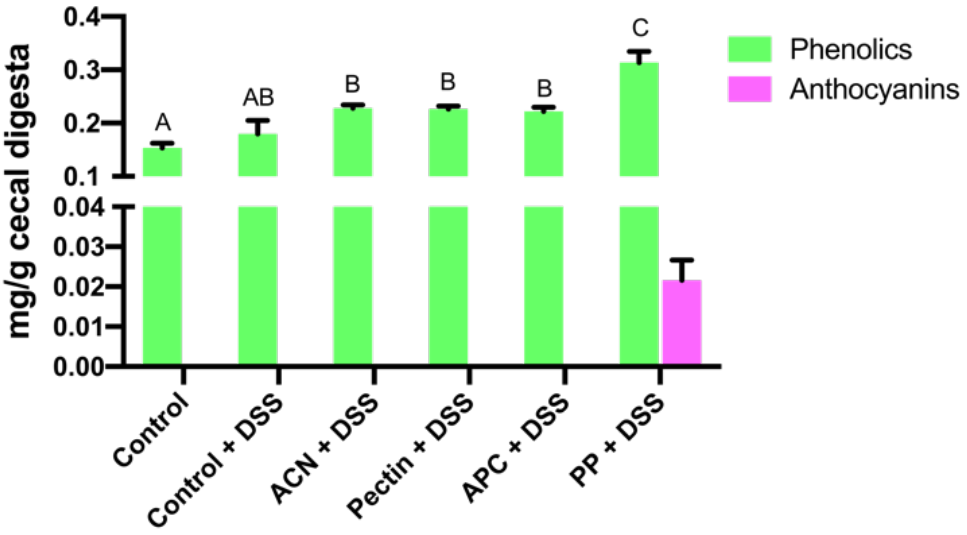


Figure 16. Total phenolics and anthocyanins measured in cecal digesta. Values are shown by means ± SEM (n = 6), different letters on the bars indicate differences between different treatments $p < 0.05$. ACN + DSS, anthocyanin group; Pectin + DSS, pectin group; APC + DSS, anthocyanin-pectin complex group; PP + DSS, purple potato diet group.

Food intake and weight gain during the four weeks before colitis induction did not differ among treatment groups apart from the anthocyanin diet group, indicating that pectin and APC were well tolerated by mice. Phenolics and anthocyanins were measured in cecal digesta (Fig. 16) to determine the concentration of anthocyanins entering the colon. PP + DSS group significantly increased the total phenolic content (0.313 ± 0.02 mg/g cecal digesta), and about 0.022 ± 0.005 mg/g anthocyanins were detected. Although the total phenolic content measured in the ACN + DSS group (0.23 ± 0.006 mg/g), Pectin + DSS (0.23 ± 0.005 mg/g) and APC + DSS group (0.22 ± 0.008 mg/g) were significantly increased compared to the Control group (0.15 ± 0.009), no significant differences were observed compared to the Control + DSS group (0.18 ± 0.03).

3.3.3 Gut barrier function

Serum levels of FITC dextran were measured after oral administration. Mice exposed to DSS showed the highest FITC-dextran serum concentration indicating increased gut permeability. Mice with APC and purple potato administration significantly decreased the FITC concentrations than the Control + DSS group (Fig 17A). Anthocyanins complexed with pectin could resist the DSS-induced increase in gut permeability. About 34.95% and 14.32% lower FITC-dextran concentrations were observed in ACN + DSS group and Pectin + DSS group, respectively compared to the Control + DSS group although the difference was not significant. Fig. 17B shows the adherent mucus layer thickness of different treatment groups. The Control group had the thickest (0.78 ± 0.04 mm) mucus layer and was observed by Alcian blue staining (Fig. 17J). A significant reduction in the mucus layer thickness (0.035 ± 0.004 mm) was observed in mice exposed to DSS in control + DSS group. APC, anthocyanins, pectin and purple potato supplementation increased the mucus layer thickness compared to the DSS group.

The shortening of colon length is one of the main biological markers in the DSS-induced colitis mouse model [180]. The length of the colon is shown in Fig. 17C and 17J. In this study, Mice exposed to DSS showed shortened colons and was not significantly increased in ACN + DSS group (5.40 ± 0.2 cm), Pectin + DSS group (5.48 ± 0.2 cm), APC + DSS group (5.90 ± 0.1 cm) and PP + DSS group (5.93 ± 0.1 cm). Histology score (Fig. 17D) was calculated based on the parameters presented in Table 5, to assess the severity of DSS-induced colitis. Control + DSS group had significantly higher colonic histological damage than the Control Group. None of the treatment groups exhibited a significant reduction in the histology score compared to the Control + DSS group.

To further determine the effect of anthocyanins, pectin, APC and purple potatoes in colonic mucosal barrier function, the gene expression levels of MUC2 (Fig. 17E), ZO-1 (Fig. 17G), Claudin-1 (Fig. 17H) and MUC2 protein content (Fig. 17F) were quantified. DSS exposure increased the gene expression of MUC2, ZO-1 and Claudin-1 and reduced the MUC2 protein levels in mice compared to the control mice. Mice supplemented with APC did not differ significantly from control and DSS mice in MUC2 gene expression, but had significantly higher claudin-1 gene expression compared to control mice and lower ZO-1 gene expression compared to Control + DSS group. Anthocyanin supplemented mice gene expression levels of MUC2, ZO-

1 and Claudin-1 were similar to that of control mice and significantly lower compared to control + DSS mice. Pectin + DSS group and PP + DSS had significantly higher MUC2 protein content compared to the Control + DSS group. In the PP + DSS group, both gene expression level and protein content of MUC2 were higher than in other treatment groups. Pectin + DSS group followed a similar trend.

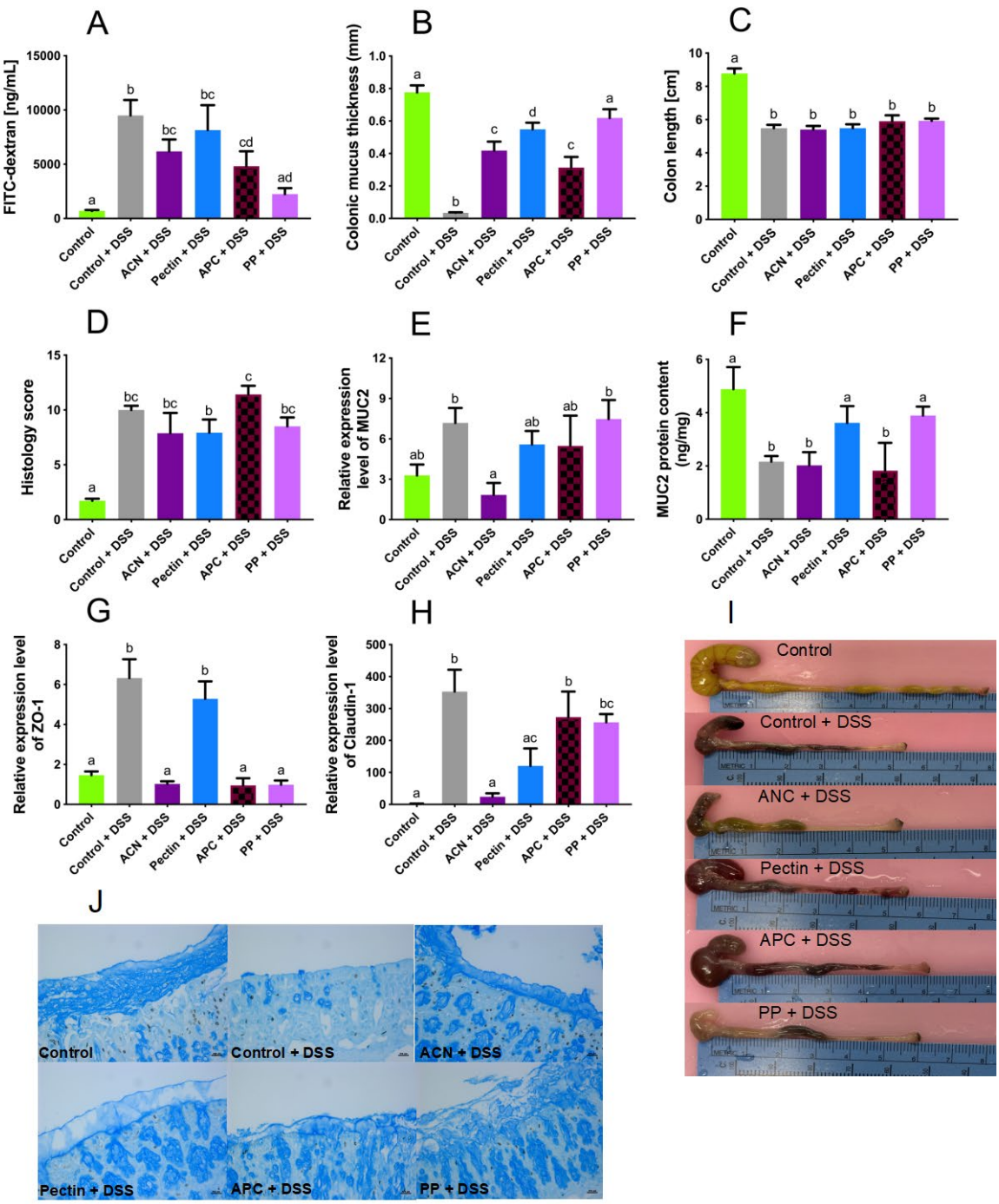


Figure 17. Gut barrier parameters. Gut permeability was measured by FITC-dextran concentration in serum (A); gut barrier function was evaluated by mucus thickness (B) colon length (C), histology score (D), the expression of MUC2 (E), ZO-1 (G) and Claudin-1 (H) and MUC2 protein (F); Alcian blue-stained colon sections represented the (I) (magnification, 10x, scale bar: 200 μm), and images of cecum and colon (J). Values are shown by means ± SEM (n = 10 to 20), different letters on the bars indicate differences between different treatments $p < 0.05$. ACN + DSS, anthocyanin group; Pectin + DSS, pectin group; APC + DSS, anthocyanin-pectin complex group; PP + DSS, purple potato diet group.

3.3.4 Colonic inflammation

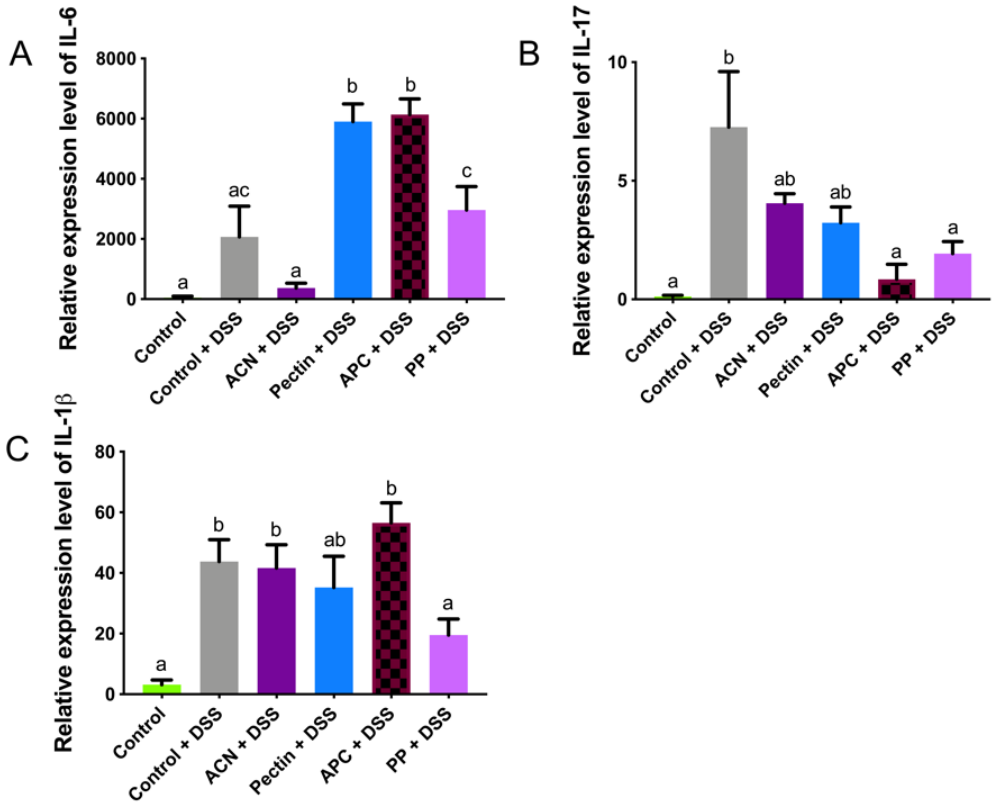


Figure 18. Effect of anthocyanins, pectin, APC and purple potatoes on colonic inflammatory markers. The relative mRNA expression levels of IL-6 (A), IL -17(B) and IL-1β (C). Values are shown by means ± SEM (n = 4 to 8), different letters on the bars indicate differences between different treatments $p < 0.05$. ACN + DSS, anthocyanin group; Pectin + DSS, pectin group; APC + DSS, anthocyanin-pectin complex group; PP + DSS, purple potato diet group.

Along with impaired barrier function, colonic inflammation is another main characteristic of colitis [181]. Thus, the gene expression levels of three pro-inflammatory cytokines including IL-6 (Fig. 18A), IL -17 (Fig. 18B) and IL-1β (Fig. 18C) were determined. APC and pectin administration showed significantly higher gene expression of IL-6 than the Control + DSS group. Studies have shown that IL-6 acts as both pro-inflammatory and anti-inflammatory cytokine involved in several immune pathways [182]. PP + DSS group did not differ from control + DSS group in mRNA expression levels of IL-6, while ACN + DSS group mice had a lower expression level compared to control + DSS. However, the gene expression of IL-17 was significantly reduced in APC and purple potato supplemented mice compared to Control + DSS group. Furthermore, the gene expression of IL-1β was significantly reduced only in purple potato supplemented group, but

not in ACN, pectin and APC groups compared to control + DSS group. In conclusion, APC group showed a similar anti-inflammatory effect as of anthocyanins and pectin groups but was less effective compared to purple potato diet group in DSS-induced colitis mice.

3.3.5 Effect of anthocyanins, pectin, APC and purple potatoes on liver injury parameters

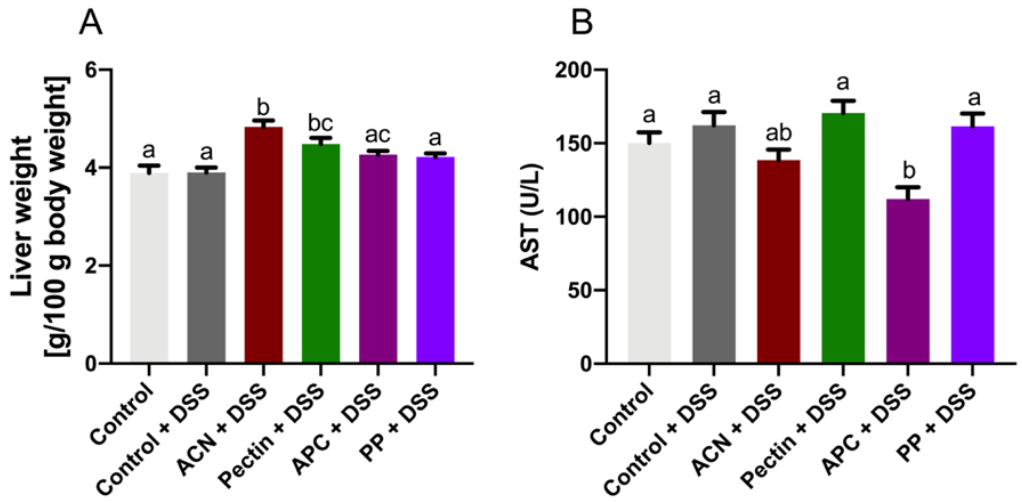


Figure 19. Liver weight to body weight ratio (A) and serum aspartate aminotransferase (AST) levels (B). Values are shown as means ± SEM (n = 6), different letters on the bars indicate differences between different treatments $p < 0.05$. ACN + DSS, anthocyanin group; Pectin + DSS, pectin group; APC + DSS, anthocyanin-pectin complex group; PP + DSS, purple potato diet group.

Liver weights and serum AST levels were used to evaluate the hepatic damage in mice (Fig. 19). A significant increase in liver weight was observed in the ACN + DSS group and Pectin + DSS group. Surprisingly, no changes were observed in the liver weight in either the Control group or Control + DSS group. Mice in APC + DSS and PP + DSS groups showed lower liver weights compared to ACN + DSS and pectin + DSS groups. DSS exposure did not increase the AST level compared to the control mice. Mice in APC + DSS showed a significant reduction in AST level compared to controls.

3.3.6 Short-chain fatty acids

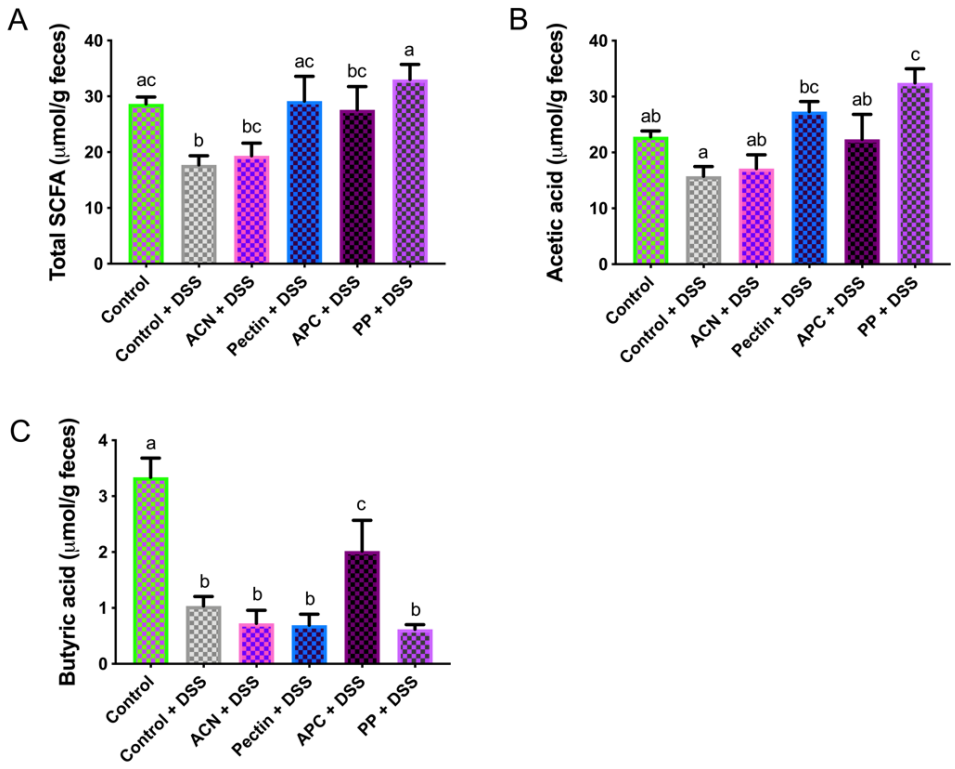


Figure 20. SCFA levels in cecal digesta of mice. A, B and C: Total SCFAs, acetic acid and butyric acid, respectively. Values are shown as means ± SEM (n = 6), different letters on the bars indicate differences between different treatments $p < 0.05$. ACN + DSS, anthocyanin group; Pectin + DSS, pectin group; APC + DSS, anthocyanin-pectin complex group; PP + DSS, purple potato diet group.

To investigate the effects of anthocyanins, pectin, APC and purple potatoes on gut microbiota-derived metabolites, the content of cecal SCFAs in mice was measured using GC. SCFAs are produced by the fermentation of dietary fibers and play an important role in colonic health. The Control + DSS group had the lowest total SCFAs abundance (17.70 ± 1.6 μmol/g feces) (Fig. 20A). The total SCFAs measured in PP + DSS group (33.02 ± 2.7 μmol/g feces) were the highest among all the treatment groups. The total SCFAs measured in the APC + DSS group (27.59 ± 1.6 μmol/g feces) as well as Pectin + DSS group (29.16 ± 4.4 μmol/g feces) were higher than ACN + DSS group (19.35 ± 2.3 μmol/g feces). APC diet group exhibited increased total SCFAs might be due to the presence of pectin. Moreover, APC administration had slightly higher acetic acids (22.33 ± 1.7 μmol/g feces) compared to ACN + DSS group (17.11 ± 2.5 μmol/g feces) and lower than Pectin + DSS group (27.32 ± 1.8 μmol/g feces) (Fig. 20B). APC + DSS group showed significantly higher butyric acid content (2.02 ± 0.2 μmol/g feces) than the other three treatment

groups and Control + DSS group (Fig. 20C). Butyrate plays an important role in the promotion of intestinal barrier function.

3.4 Discussion

With the growing prevalence of IBD, many natural substrates were studied to ameliorate the IBD symptoms. Purple potatoes are rich in anthocyanins and exhibited anti-inflammatory effects in colitis mice [28]. Anthocyanins are more stable in the whole food matrix than in isolated forms. Complexation is a technique to stabilize the isolated anthocyanins, and many biopolymers such as proteins, dietary fibers have been proved as stabilizing agents [104]. Previously, our lab studied the complexation efficiency of different polysaccharides complexed with the isolated anthocyanins from purple potatoes. Pectin exhibited the best binding capacity with anthocyanins compared to inulin, starch and cellulose. Pectin is a soluble dietary fiber that is fermented by gut microbiota to produce beneficial metabolites in the colon, and improve gut barrier function [183]. In this study, the isolated anthocyanins, pectin, APC and purple potatoes were used to evaluate their beneficial effects in gut barrier function, anti-inflammation and SCFAs production in a DSS-induced colitis mouse model. Our results reported that APC might exhibit the beneficial effects in gut health.

A high serum FITC-dextran levels represent high gut permeability. FITC-dextran is a fluorescent probe and is normally used for permeability assay [184]. As expected, DSS exposure increased the serum FITC-dextran concentrations indicating increased permeability. APC and purple potato administration showed significantly reduced FITC-dextran compared to Control + DSS group. The protective role of purple potatoes in gut permeability was also reported by Li *et al.* [28]. Isolated anthocyanins were not as effective as PP and APC in reducing the permeability. This may be because the isolated anthocyanins are susceptible to degradation in the upper GI tract digestion [116].

The mucus layer is composed largely of glycoproteins called mucins. Mucin 2 (MUC2) acts as gel-forming mucin in the lumen of the small intestine and colon [24] and is secreted by goblet cells and considered as the building blocks of colonic mucus. ZO-1 and Claudin-1 are genes encoding tight junction proteins. [185]. The thinning of the mucus layer was observed in the DSS-induced colitis mouse model [186]. Mucus thinning was also observed in the Control + DSS group.

In this study, the APC significantly increased the colonic mucus thickness. However, APC administration did not reverse the shortened colon length induced by the DSS challenge, which might be due to the short treatment period and high DSS exposure. DSS exposure increased relative gene expression levels of MUC2, ZO-1 and Claudin-1. This may be a compensatory increase due to DSS-induced colonic epithelial damage. ACN + DSS significantly downregulated the gene expression levels of MUC2, ZO-1 and Claudin-1 compared to the DSS group, which were not significantly different from control mice. Pectin + DSS group showed a thick colonic mucus layer and high MUC2 protein content, which indicated that pectin could promote the integrity of the mucus layer as a dietary fiber. Similar results have been revealed by Beukema *et al.* [187]. Different sources of pectin have specific structural characteristics. The high methoxyl pectin derived from apple was used in this study. This conclusion was also confirmed by a study that reported that pectin increased the abundance of specific cecal bacteria and metabolites that mainly function in the production of MUC2 [188].

The gene expression levels of IL-6, IL-17 and IL-1β were used to evaluate the anti-inflammatory effects of different treatment groups. IL-6 functions in immune response and preparation for host defense when tissue damage and pathogen invasion occurred [189]. IL-17 is produced by T helper 17 cells [190], and is mainly mediated by the innate immune system and inflammation [191]. IL-1β plays a role in the pathogenesis of inflammation. In this study, acute colitis was induced by DSS administration in mice which was accompanied by the infiltration of pro-inflammatory cytokines in the colon. APC-treated mice had reduced expression levels of IL-17; however, APC treatment failed to downregulate the gene expression of IL-6 and IL-1β. Anthocyanin and pectin administration also did not downregulate the gene expression levels of IL-6 and IL-1β but reduced IL-17 expression levels. Numerous studies have stated the protective effects of pectin and anthocyanins in suppressing IBD [176][175], but their anti-inflammatory effects may not be significant in this study. PP + DSS group exhibited the best ability in reducing colonic inflammation compared to other treatment groups. This may be due to the greater stability of anthocyanins in the food matrix against upper GI digestion and increased colonic concentrations.

Although DSS did not induce any hepatic damage in mice, the liver weight in anthocyanin- and pectin-treated groups was significantly higher than control and DSS groups. Therefore, the AST levels were determined to further discuss whether the liver injury has occurred. Growing

evidence suggests that the gut-liver-axis can indirectly and directly affect liver function by SCFAs [192]. However, no significant differences in AST levels were observed among all the groups except APC + DSS. Normally, the level of AST in mice is 50-100 U/L [193], the high AST levels measured in treatment groups might be due to the stimulation from gavage. The low AST levels observed in APC group suggest the protective role of APC against liver injury.

The undigested fibers that enter the colon are colonic fuel for bacterial fermentation and SCFAs production, which contributes to many health benefits. As Tan *et al.* mentioned, SCFAs have anti-inflammatory, anti-microbial effects, and the ability to alter gut integrity and regulate cell proliferation [194]. Acetic acid, butyric acid and propionic acid are three major SCFAs from the fermentation of dietary fibers and resistant starch [195]. In this study, acetic acid accounted for the largest proportion of the total SCFAs and is commonly produced by the bacterial groups of phylum Bacteroidetes [196]. Butyrate can maintain gut hemostasis by its anti-inflammatory effects and is a prime substrate for colonocytes [197]. DSS challenge significantly decreased the production of SCFAs compared to the control group. ACN + DSS group exhibited similar total SCFAs to the Control + DSS group. This phenomenon was also found in acetic acid and butyric acid contents. A significant increase in total SCFAs was observed in the APC- and pectin-treated groups compared to Control + DSS group. A study reported that the increase of total SCFAs was observed in the high methoxyl pectin fermentation [177]. APC + DSS facilitated the production of butyric acid, which may be due to the synergistic effects of anthocyanins and pectin. Anthocyanins and dietary fibers have been proved to modulate gut microbiota [198]. The regulation of microorganisms can improve the gut ecological environment and increase SCFAs. Moreover, Kilua *et al.* reported that the fermentation of purple sweet potato polyphenols and two dietary fibers positively modulated the microbial composition [199]. In conclusion, APC enhanced the production of SCFAs, especially butyric acid.

3.5 Conclusion

The results of the present study demonstrated that APC restored the gut barrier function and increased the production of SCFAs and downregulate the gene expression of IL-17 in a DSS-induced colitis mouse model.

CHAPTER 4. CONCULSIONS AND RECOMMENDATIONS FOR FUTURE WORK

This study has investigated the interaction between anthocyanins and polysaccharides in gut barrier function. The complexation of isolated anthocyanins with polysaccharides improved the stability of the purple potato anthocyanins. Pectin showed the best binding capacity with anthocyanins compared to inulin, starch and cellulose. Complexation caused significant changes in the surface morphology that was observed using a scanning electron microscope. FTIR was used to obtain the chemical bonds of complexes. C=C and N-O are two specific chemical bonds from purple potato anthocyanins that were detected in the anthocyanin-pectin complex (APC). Zeta potential measured the net charges on the anthocyanins and pectin and showed the potential electrostatic interaction in APC at lower pH. Furthermore, APC enhanced the anthocyanin stability against the changes in pH and temperature and increased the colonic concentration of anthocyanins after the simulated upper GI digestion. The Caco-2 cell line model revealed that APC could increase the colonic availability of anthocyanins. APC restored the LPS-induced high permeability *in vitro*. In mice models of colitis, APC reduced the high gut permeability and enhanced the colonic mucus layer thickness compared to DSS control. APC increased the MUC2 gene expression levels. Although APC did not suppress the gene expression levels of IL-6 and IL-1β, it reduced IL-17 expression levels significantly. AST levels were measured in mice serum to determine hepatic damage. Anthocyanins and pectin administration increased the hepatic damage while only APC group maintained the AST levels within the normal range. Moreover, APC administration prevented the DSS-induced bacterial dysbiosis and increased total SCFAs and butyric acid. In summary, APC enhanced the colonic concentration of anthocyanins *in vitro*, and it was considered as a protective strategy to restore the gut barrier function in a chemically induced colitis mouse model.

Further studies related to this project may include the use of different polysaccharides. Polysaccharides have different patterns which can influence the complexation efficacy with anthocyanins. In this study, although pectin exhibited the best binding ability among other polysaccharides, high methoxyl pectin and low methoxyl pectin still have different capacities to complex with anthocyanins. Thus, a more detailed selection of polysaccharides for complexation

still needs more studies. Furthermore, the complex was gavaged for only two weeks because of the stress that was induced during gavage. Therefore, the fabrication of APC, anthocyanins and pectin into diet not only can increase the treatment period but also can reduce the extra stress for mice which might be a better way to determine the anti-colitic effects. In addition, bacterial dysbiosis is strongly related to constipation and diarrhea in colitis patients. Thus, the determination of bacterial composition and bacterial metabolites can better explain the anti-colitic effects of APC, anthocyanins, pectin and purple potato.

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