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PROMOTION OF COLON HEALTH BY STRAWBERRY AND CRANBERRY

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PROMOTION OF COLON HEALTH BY STRAWBERRY AND CRANBERRY

A Dissertation Presented

by

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DEDICATION

I dedicate this thesis to

my beloved families and friends

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ABSTRACT

PROMOTION OF COLON HEALTH BY STRAWBERRY AND CRANBERRY

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Inflammatory bowel disease (IBD) has posed serious threats to the human health, which lead to chronic malabsorption of nutrients, abnormal pain and rectal bleeding. Both genetic predisposition and environmental factors contribute to the onset of IBD. Multiple studies suggested that dysbiosis in colon plays an important role in the development of colitis, and gut microbiota composition are different between patients with IBD and healthy individuals. Intake of whole foods, such as fruits and vegetables, may confer health benefits to the host. The beneficial effects of fruits and vegetables mainly attribute to their richness of polyphenols and microbiota-accessible carbohydrates (MACs). Components in fruits and vegetables modulate composition and associated

functions of the gut microbiota, while gut microbiota can transform components in fruits and vegetables to produce metabolites that are bioactive and important for health.

Accumulating evidence suggests that colonic inflammation may be prevented by increased consumption of certain fruits and vegetables such as berries. Cranberry and strawberry are abundant of polyphenols with various health benefits. Most studies focused on extractable polyphenols (EP), non-extractable polyphenols (NEP) were often neglected, although NEP may possess important biological functions. NEP is not significantly released from the food matrix during digestion in the stomach or small intestine, therefor they reach colon nearly intact. In the colon, the potential interaction between gut microbiota and NEP could also play an important role in colon health. NEP could be biotransformed by gut microbiota to produce bioactive metabolites that may contribute to the promotion of colon health as well. Therefore, it is important to investigate the interaction of gut microbiota and NEP in the colon in terms of production of bioactive metabolites. Meanwhile, composition and functions of gut microbiota can be altered by the presence of polyphenols in the colon, which in turn could affect colon health. The objective of this work was to characterize EP and NEP fractions from whole cranberry and strawberry and determine their potential in anti-inflammation and anti-colon cancer. EP and NEP from cranberry and strawberry showed anti-inflammatory effects in inhibiting LPS-induced production of nitric oxide in macrophages, which was accompanied by decreased expression of iNOS and increased expression of HO-1. EP

and NEP from cranberry and strawberry showed anti-cancer capacities in HCT116 cells. Flow cytometry analysis demonstrated that NEP caused a cell cycle arrest and induced a significant cellular apoptosis in colon cancer cells.

Furthermore, this study determined the protective effects of whole strawberry (WS) against dextran sulfate sodium (DSS)-induced colitis in mice. In colitic mice, dietary WS reduced the disease activity index (DAI), prevented the colon shortening and spleen enlargement, and alleviated the colonic tissue damages. The abundance of pro-inflammatory immune cells was reduced by dietary WS in the colonic mucosa, which was accompanied by the suppression of overproduction of pro-inflammatory cytokines. Western blotting and immunohistochemical analysis revealed that dietary WS decreased the expression of pro-inflammatory proteins in the colonic mucosa. Moreover, dietary WS partially reversed the alteration of gut microbiota in the colitic mice by increasing the abundance of potential beneficial bacteria, e.g. *Bifidobacterium* and *Lactobacillus,* and decreasing the abundance of potential harmful bacteria, e.g. *Dorea and Bilophila*. Dietary WS also restored the decreased production of short chain fatty acids (SCFAs) in the cecum of the colitic mice. The results revealed the anti-inflammatory effects and mechanisms of dietary WS in the colon, which is critical for the rational utilization of strawberry for the prevention of inflammation-driven diseases.

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CHAPTER 1

INTRODUCTION

Trillions of microorganisms inhabit in the large intestine. The main phyla are *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria* and *Verrucomicrobia* [1]. In recent years, studies about gut microbiota are blooming. An increasing number of diseases, such as obesity, inflammatory bowel disease, cardiovascular diseases, and metabolic syndrome, have been associated with abnormality in gut microbiota. Gut microbiota could be profoundly influenced by many factors, such as diet and health status [2]. Modulation of gut microbiota by diet is a promising and effective way to benefit the host. The effects of diet on the composition and function of gut microbiota can be beneficial or harmful. In general, dietary intake of whole fruits and vegetables has been known to beneficially affect gut microbiota by promoting the growth of beneficial bacteria and/or inhibiting the growth of the harmful bacteria (Figure 1). Fruits and vegetables are rich in microbiota-accessible carbohydrates (MACs), such as oligosaccharides, pectin, cellulose, inulin, lignans, and resistant starches, that serve as substrate for certain beneficial bacteria [3, 4]. Microflora ferments MACs to produce short-chain fatty acids (SCFAs), which in turn impact bacterial composition. On the other hand, polyphenols in fruits and vegetables can be degraded by gut bacteria to various

metabolites, and both polyphenols and their metabolites can modulate gut microbiota. This review focus on the effects of whole foods, e.g., fruits and vegetables on gut microbiota and microbiota-associated disease, e.g., obesity and colonic inflammation.

Inflammatory bowel disease (IBD) has posed serious threats to the human health [5], which lead to chronic malabsorption of nutrients, abnormal pain and rectal bleeding. Both genetic predisposition and environmental factors contribute to the onset of IBD [6]. Pathological immune response against exotic antigen and bacterial drives the progression of IBD [7]. Multiple studies suggested that dysbiosis in colon plays an important role in the development of colitis, and gut microbiota composition are different between patients with IBD and healthy individuals [8]. The incidence of colonic inflammation has also been associated with dietary patterns through the modification of gut microbiota [9]. The diet with high fat, high sugar, high protein and low fiber has been proposed to increase the risk of IBD. In contrast, dietary consumption of fruits and vegetables has been associated with lowered risk of IBD [10].

Accumulating evidence suggests that colonic inflammation may be prevented by increased consumption of certain fruits and vegetables such as berries [10, 11]. Berries (e.g. chokeberry, red raspberry, strawberry, cowberry) and their extracts have been shown to have anti-inflammatory and anticancer effects in intestine in various animal models such as azoxymethane-induced rat colon cancer model, Apc 1638+/- mouse and Muc2-/-

mouse model, and DSS-induced colitis models [12-14]. Polyphenols are a major class of dietary phytochemicals which are responsible for many health benefits. Current research on dietary polyphenols mainly focus on a fraction of polyphenols, known as extractable polyphenols (EP) [15, 16]. EP can be extracted from food with aqueous-organic solvents (e.g. water, methanol, ethanol, acetone) [17, 18], and are often considered as "total phenolic content" for various biological studies. However, another fraction of polyphenols remains in the residues after solvent extraction, and they are known as non-extractable polyphenols (NEP) [16, 19]. NEP are mainly polyphenols that bound to dietary fiber or other macromolecules through hydrophobic interaction, hydrogen bonding and covalent bonding [15, 16]. NEP is not significantly released from the food matrix during digestion in the stomach or small intestine, therefor they reach colon nearly intact [20]. In the colon, NEP may be released from food matrix by the action of microbiota and become bioavailable and bioactive. In contrast, EP tend to be absorbed and metabolized in the upper gastrointestinal tract, therefore, their biological fate is quite different from that of NEP. Only unabsorbed EP and their metabolites could reach the colon. The better colonic bioavailability of NEP than EP makes NEP promising candidates for promoting colon health.

Cranberry is a native berry fruit of North America, and it is abundant of polyphenols including flavan 3-ols, proanthocyanidins, anthocyanins and phenolic acids [21].

Majority of studies on cranberry have been focused on its antibacterial actions [22]. To date, the inhibitory effects of cranberry on colonic inflammation and colon cancer have not been adequately studied. Cranberry extracts have been shown to inhibit colon cancer cell growth and suppress pro-inflammatory enzymes expression [21]. Strawberry is one of the most popular berry fruits, which is abundant in polyphenols that have been associated with anti-oxidation, anti-inflammation and anti-cancer bioactivities. Strawberry juice exhibited anti-inflammatory effects in lipopolysaccharide-treated macrophage by decreasing the production of pro-inflammatory cytokines [23], and inhibited the cell proliferation of human colon cancer cell lines [17]. A human study showed that consumption of strawberry could increase the serum antioxidant capacity [24]. The consumption of strawberry beverage was associated with the amelioration of postprandial inflammatory response [25]. These evidences suggested the promising bioactivity of berry fruits against the colonic inflammation. Whole strawberry contains vitamins, flavonoids, phenolic acids, anthocyanins, polysaccharides and fiber [17], which may exhibit stronger anti-inflammatory effects when compared to the extracts. However, there is no detailed mechanistic information available on the potential protective effects of whole strawberry against IBD.

CHAPTER 2

LITERATURE REVIEW

Trillions of microorganisms inhabit in the large intestine. The main phyla are *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria* and *Verrucomicrobia* [1]. In recent years, studies about gut microbiota are blooming. An increasing number of diseases, such as obesity, inflammatory bowel disease, cardiovascular diseases, and metabolic syndrome, have been associated with abnormality in gut microbiota. Gut microbiota could be profoundly influenced by many factors, such as diet and health status [2]. Modulation of gut microbiota by diet is a promising and effective way to benefit the host. The effects of diet on the composition and function of gut microbiota can be beneficial or harmful. In general, dietary intake of whole fruits and vegetables has been known to beneficially affect gut microbiota by promoting the growth of beneficial bacteria and/or inhibiting the growth of the harmful bacteria (Figure 1). Fruits and vegetables are rich in microbiota-accessible carbohydrates (MACs), such as oligosaccharides, pectin, cellulose, inulin, lignans, and resistant starches, that serve as substrate for certain beneficial bacteria [3, 4]. Microflora ferments MACs to produce short-chain fatty acids (SCFAs), which in turn impact bacterial composition. On the other hand, polyphenols in fruits and vegetables can be degraded by gut bacteria to various metabolites, and both polyphenols and their metabolites can modulate gut microbiota.

This review focus on the effects of whole foods, e.g., fruits and vegetables on gut microbiota and microbiota-associated disease, e.g., obesity and colonic inflammation.

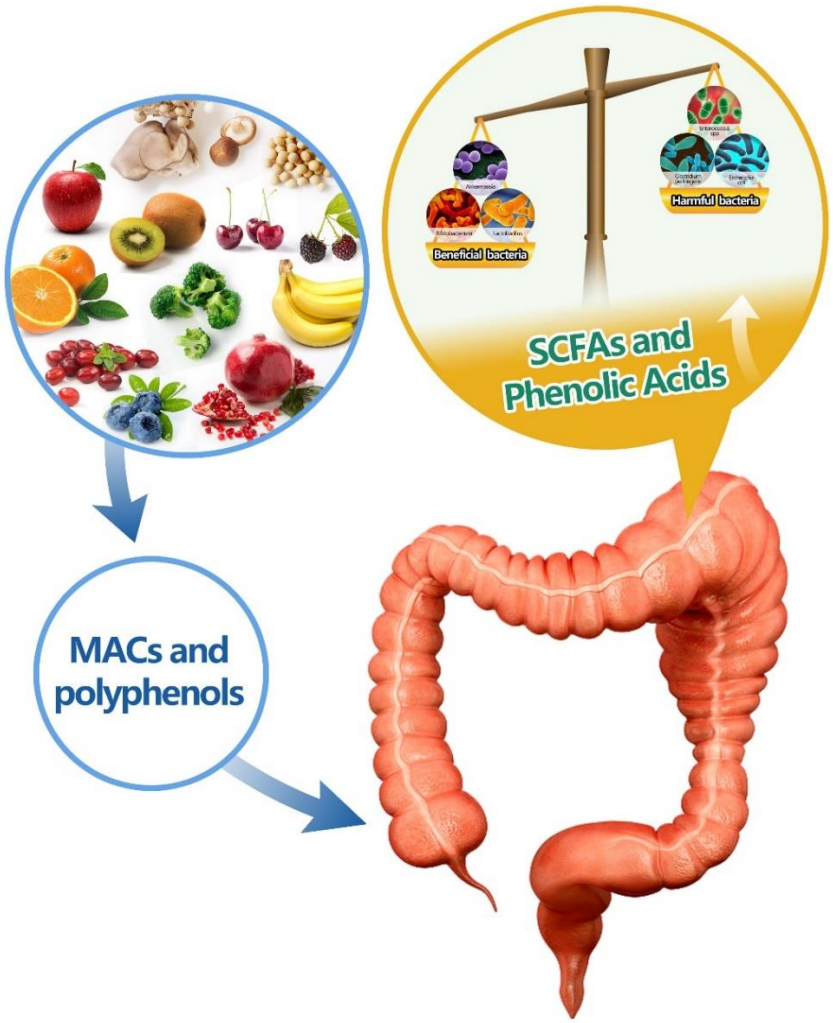

Figure. 1 The interactions between whole foods and gut microbiota. Whole foods such as fruits and vegetables are rich in microbiota-accessible carbohydrates and polyphenols. microbiota-accessible carbohydrates, such as oligosaccharides, pectin, cellulose, inulin, lignans, and resistant starches, are resistant to digestion in the upper gastrointestinal tract prior to reaching the large intestine where they interact with gut microbiota. This interaction may lead to (i) altered gut microbiota, and (ii) the production of short-chain

fatty acids from fermentation of microbiota-accessible carbohydrates by microbiota, and short-chain fatty acids may also modulate microbiota composition. After consumption of whole fruits and vegetables, a large portion of their polyphenols accumulate in the large intestine where they may interact with gut microbiota to (i) modify the structure of microbial community, and (ii) produce bioactive metabolites, such as phenolic acids, via microbiota-mediated metabolism of polyphenols. The microbial metabolites of polyphenols could modulate gut microbiota as well. The overall outcome of interactions between whole foods and gut microbiota may be an increased abundance of beneficial bacteria and a decreased abundance of harmful bacteria. Moreover, short-chain fatty acids and phenolic acids may also interact with host tissues such as colonic epithelia to impact host health.

2.1 Interactions between Whole Foods and Gut Microbiota in Disease-free Populations

Whole fruits and vegetables are known to be good food choices to maintain and promote health. Various fruits and vegetables have been studied to determine their effects on gut microbiota in healthy, disease-free populations including both humans and animals. This section provided a summary of the modulating effects of fruits and vegetables on gut microbiota, as well as the interactions between gut microbiota and the major bioactive components of fruits and vegetables. Table 1 summarized representative studies on the impacts of whole foods and their components on gut microbiota.

Table 1. Effects of whole fruits and vegetables on gut microbiota in disease-free populations.

Source	Whole foods or extracts	model	Dose	Duration	Effects on gut microbiota	Reference
apple	Fresh apple	human	Oral, 2 apples/ person/day	2 weeks	↑*Bifidobacterium,* ↑*Lactobacillus,* ↑*Streptococcus,* ↓*Enterobacteriaceae,* ↓*Pseudomonas*	[26]
	Raw whole apple	rats	Oral, 10 g/day	4 weeks	↓*Bacteroides*	[27]
	Apple pectin	rats	Oral, 7% pectin	4 weeks	↑*Anaeroplasma,* ↑*Anaerostipes,* ↑*Roseburia,* ↑*Clostridium coccoides,* ↓*Alistipes,* ↓*Parabacteroides sp,* ↓*Bacteroides*	
black raspberry	freeze-dried black raspberries powder	mice	Oral, 10% w/w	6 weeks	↓*Clostridium,* ↓*Lactobacillus,* ↑*Barnesiella,*	[28]
	freeze-dried black raspberries powder	mice	Oral, 10% w/w	7 weeks	↓*Firmicutes,* ↑*Bacteroidetes,* ↑*Verrucomicrobia,* ↑*Akkermansia muciniphila*	[29]
	freeze-dried black raspberries powder	rats	Oral, 5% w/w	6 weeks	↑*Anaerostipes,* ↑*Akkermansia,* ↑*Desulfovibrio,* ↑*Ruminococcus,* ↑*Coprpbacillus,* ↓*Acetivibrio*	[30]
	anthocyanins from black raspberry	rats	Oral, 0.2% w/w	6 weeks	↑*Anaerovorax,* ↑*Dorea,* ↓*Bifdobacterium,* ↓*Lactococcus*	
	residues fraction from black raspberry	rats	Oral, 2.25% w/w	6 weeks	↑*Anaerostipes,* ↑*Desulfovibrio,* ↑*Coprobacillus,* ↑*Victivallis,* ↑*Mucispirilum,* ↓*Streptococcus,* ↓*Turicibacter,* ↓*Acetivibrio*	

blueberry	wild blueberry drink (25 g of wild blueberry powder in 250 mL of water)	human	Oral, 25 g/day/person	6 weeks	↑*Bifidobacterium spp.*	[31]
	Lowbush wild blueberries powder (*Vaccinium angustifolium*)	rats	Oral, 8% w/w	6 weeks	↑*Bifidobacteriaceae,* ↑*Coriobacteriaceae,* ↓*Lactobacillus,* ↓*Enterococcus*	[32]
	freeze-dried blueberry powder	mice	Oral, 5% w/w	4 weeks	↑*Tenericutes,* ↓*Deferribacteres*	[33]
	water-soluble blueberry extracts	rats	Gavage, 4 ml of extracts/kg body weight/day	6 days	↑*Lactobacilli,* ↑*Bifidobacteria*	[34]
	Freeze-dried blueberry (*Vaccinium angustifolium*) pomace powder	chicken	Oral, 1% and 2% w/w	64 days	↑*Bacteroidetes,* ↑*Lactobacillus,* ↑*Bifidobacterium,* ↓*Escherichia coli,* ↓*Clostridium_Clostridiaceae,* ↓*Helicobacter,* ↓*Enterococcus*	[35]

cherry	cherry concentrate juice	human	Oral, 8 oz/day	5 days	*high-Bacteroides individuals: ↓Bacteroides, ↓Parabacteroides, ↓Alistipes, ↓Barnesiella, ↓Butyricimonas, ↓Odoribacter, ↓Porphyromonas, ↓Bifidobacterium, ↑Ruminococcus, ↑Lachnospiraceae, ↑Clostridium, ↑Clostridium XI, ↑Dialister, ↑Coprococcus, ↑Lactobacillu, ↑Streptococcus. low-Bacteroides individuals: ↓Lachnospiraceae, ↓Streptococcus, ↓Dialister, ↓Blautia, ↓Roseburia, ↑Bacteroides, ↑Prevotella, ↑Alistipes, ↑Clsotridium IV and XI, ↑Lactobacillus, ↑Bifidobacterium, ↑Ruminococcus*	[36]
citrus	citrus pectin	mice	Oral, 15% w/w	4 weeks	*↑Bacteroidetes, ↓Firmicutes*	[37]
	orange juice	human	Oral, 300 mL/day/person	2 months	*↑Lactobacillus spp., ↑Bifidobacterium spp.*	[38]
	orange juice (*Citrus sinensis*)	human	Oral, 500 mL/day/persom	7 days	*↑Mogibacteriaceae, ↑Lachnospiraceae, ↑Ruminococcaceae, ↑Veillonellaceae, ↑Enterococcaceae, ↑Coriobacteriaceae*	[39]
cranberry	freeze-dried whole cranberry powder	human	Oral, 30g/day/person	5 days	*↓Firmicutes, ↑Bacteroidetes*	[40]
	cranberry juice	human	Oral, 200mL/day/person	3 weeks	*↓Helicobacter pylori*	[41]
kiwifruit	freeze-dried green kiwifruit powder (without peel)	pigs	Oral, 25% w/w	14 days	*↑Bacteroides, ↓Enterobacteria, ↓Escherichia coli*	[42]
	freeze-dried	pigs	Oral, 6.9% w/w	14 days	*↑Bacteroides*	

	green kiwifruit fiber powder					
	freeze-dried green kiwifruit (*A. deliciosa*) powder (without peel)	rats	Oral, 10% w/w	4 weeks	↑*Lachnospiraceae*	[43]
	freeze-dried gold kiwifruit (*A. chinensis*) powder (without peel)	rats	Oral, 10% w/w	4 weeks	↑*Bacteroides–Prevotella–Porphyromonas group,* ↑*Enterococcus spp.*	
Hardy banana	freeze-dried *Musa basjoo* powder (dissolved in 20 mL ddH_2O)	mice	Gavage, 0.52, 1.04, 2.07 g/kg bw/day	7 weeks	↑*Bacteroides,* ↑*Roseburia,* ↓*Staphylococcus,* ↓*Helicobacter*	[44]
pomegranate	pomegranate extract	human	Oral, 1000 mg/day/person	4 weeks	↑*Actinobacteria,* ↑*Butyrivibrio,* ↑*Enterobacter,* ↑*Escherichia,* ↑*Lactobacillus,* ↑*Prevotella,* ↑*Serratia,* ↑*Veillonella,* ↓*Firmicutes,* ↓*Collinsella*	[45]
	pomegranate (*Punica granatum L. Mollar de Elche cv.*) juice	human	Oral, 200 mL/day/person	4 weeks	*no significant changes of microbiota*	[46]
broccoli	Cooked broccoli	human	Oral, 200 g/day/person	15 days	↑*Bacteroides,* ↑*Bacteroidetes,* ↓*Firmicutes*	[47]

	freeze-drying broccoli powder	rats	Oral, 10% w/w	4 days	↑*Akkermansia,* ↑*Oscillspira,* ↓*Clostridium,* ↓*Dorea*	[48]
mushroom	Dried *Armillariella tabescens* powder	pigs	Oral, 0.1, 0.3, and 0.9% w/w	30 days	↑*Lactobacillus spp.,* ↑*Bifidobacterium spp.,* ↓*Escherichia coli*	[49]
	freeze-dried white button mushroom powder (*Agaricus bisporous*)	mice	Oral, 1% w/w	4 weeks	↑*Bacteroidetes,* ↓*Clostridia,* ↓*Firmicutes*	[50]
	freeze-dried white button mushroom powder	pigs	Oral, 3 and 6 serving size	6 weeks	↑*Lachnospiraceae,* ↑*Ruminococcaceae,* ↑*Porphyromonadaceae,* ↓*Bifidobacteriaceae*	[51]
	freeze-dried *Pleurotus eryngii* powder	mice	Oral, 1 and 3% w/w	6 weeks	↑*Bacteroidetes,* ↑*Deferribacteres,* ↓*Firmicutes,* ↓*Verrucomicrobia*	[52]
soybean	soybean flour	rats	Oral, 41.5% w/w	14 days	↑*Prevotella,* ↑*Eubacterium*	[53]
	soy fiber	rats	Oral, 2.0% w/w	14 days	↑*Prevotella,* ↑*Bacteroidales,* ↑*Erysipelotrichaceae,* ↓*Clostridiaceae,* ↓*Adlercreuzia,*	

2.1.1 Whole Fruits and Vegetables Beneficially Impact Gut Microbiota

Among the major phyla of gut microbiota, *Firmicutes* and *Bacteroidetes* represent more than 90% of the phylogenetic types [1, 54]. Intake of whole fruits and vegetables could modulate the growth of *Firmicutes* and *Bacteroidetes*. A lowered ratio of *Firmicutes* to *Bacteroidetes* (F/B) is considered as a marker of healthier gut microbiota [28]. Daily consumption of 200 g of cooked broccoli for 18 days in human volunteers reduced the relative fecal abundance of *Firmicutes* and increase that of *Bacteroidetes*. These effects were ascribed to fiber and glucosinolates found in broccoli [47]. Human intake of cranberry (30 grams of freeze-dried powder per day for 5 days) also led to a decrease in the fecal abundance of *Firmicutes* and an increase in *Bacteroidetes*. Consistent with these human studies, dietary treatment with freeze-dried black raspberry powder (10% w/w in diet) to mice for 6 weeks lowered the F/B ratio in the fecal microbiota [28]. Feeding of cooked navy bean or black bean to mice altered fecal microbiota structure showing an increasing trend in the abundance of *Bacteroidetes*. The fecal abundance of *S24-7*, a major family of *Bacteroidetes*, was significantly increased, and the fecal abundance of *Oscillospira*, *Ruminococcus gnavus*, *Lactococcus*, *Coprococcus* and *Streptococcus*, all members from *Firmicutes*, were remarkably decreased by the intake of the beans. Moreover, concomitant with the altered fecal microbiota, dietary bean treatments enhanced multiple aspects of mucus and epithelial

barrier integrity in the mouse colon [55].

Intake of whole fruits and vegetables can promote the growth of certain beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus*. In human studies, oral intake of freeze-dried wild blueberry powder increased fecal abundance of *Bifidobacterium* and *Lactobacillus* [31, 56]. After simulated digestion, fermentation of whole apples (Renetta and Golden Delicious) with fecal bacteria from healthy donors promoted the growth of *Bifidobacterium* [57]. Whole foods also modulate gut microbiota through inhibiting potential harmful microorganisms. Cranberry intake (freeze-dried whole cranberry powder, 30 g/day for 5 days) in humans decrease the fecal abundance of bacteria that were associated with infection and antibiotic resistance, such as *Clostridia* and *Oribacterium* [40, 58]. Consumption of 2 apples per day for 2 weeks in human volunteers decreased the fecal abundance of lecithinase-positive *clostridia,* including *C. perfringens*, a potential pathogen for food poisoning [26]. Whole foods intake also showed inhibitory effects on potential harmful bacteria in animal models. Lingonberries in diet (20% w/w) caused a decrease in fecal abundance of *Lachnospiraceae, Ruminococcus* and *Oscillospira* in mice. These microorganisms were linked to obesity and type II diabetes [59]. Oral administration of bitter melon (freeze-dried powder without seeds) to rats decreased the proportion of the potential endotoxin-producing opportunistic pathogens, such as *Escherichia*, in the fecal microbiota [60]. Dietary treatment with green kiwifruit

(freeze-fried powder) decreased the fecal abundance of *Escherichia coli* [42], and feeding white mushroom (Freeze-dried powder) to mice decreased fecal abundance of *Clostridia* [50].

It is well recognized that there are considerable inter-individual variations in gut microbiota among humans. The difference in the gut microbiota composition may also render the individuals difference in responding to the same dietary intervention. For example, intake of tart cherry juice induced distinct and inverse responses in gut microbiota among human participants who had different initial levels of *Bacteroides* prior to the dietary intervention [36]. After tart cherry juice consumption, low-*Bacteroides* participants showed a decrease in *Lachnospiraceae*, *Ruminococcus* and *Collinsella*, and an increase in *Bacteroides* and *Bifidobacterium*, whereas, high-*Bacteroides* participants showed opposite changes in fecal microbiota compared to that in low-*Bacteroides* participants, i.e., an increase in *Lachnospiraceae*, *Ruminococcus* and *Collinsella*, and a decrease in *Bacteroides* and *Bifidobacterium* [36]. The mechanism underlying the interesting phenomena warrants further investigation.

The beneficial alterations of gut microbiota induced by whole fruits and vegetables were mainly attributed to microbiota-accessible carbohydrates (MACs) and polyphenols found in these whole foods [4]. The interactions between gut microbiota, MACs, and polyphenols were discussed below.

2.1.2 Interaction between Microbiota-Accessible Carbohydrates (MACs) and Gut Microbiota

MACs are indigestible by host-secreted intestinal enzymes and therefore, reach the colon largely intact, where they serve as substrates for certain gut bacteria [3]. Fruits and vegetables are generally abundant in multiple types of MACs, such as oligosaccharides, pectin, cellulose, inulin, lignans, and resistant starches [61]. Gut microbiota is dominated by phyla of *Firmicutes* and *Bacteroidetes*, both of which contain bacteria that can utilize MACs [62]. MACs interact with bacteria in the colon, resulting in favoring or inhibiting the growth of specific bacteria. For example, availability of MACs promotes the growth of certain beneficial bacteria that can readily utilize these MACs as energy sources, whereas the bacterium-derived metabolites of MACs, such as SCFAs, can inhibit the growth of certain harmful bacteria. Consequently, composition of gut microbiota can be shaped by availability or unavailability of MACs in the colon. For example, Low intake of MACs resulted in a remarkable decrease of taxa and diversity of gut bacteria relative to high intake of MACs in humanized mice [4]. High intake of MACs was associated with increased fecal richness of *Bacteroidetes*, especially the *Prevotella*, and depletion of *Firmicutes* in healthy children. Moreover, children who consumed high levels of MACs had higher levels of fecal SCFAs, which was associated with the lower abundance of fecal pathogens, such as *Escherichia* and *Shigella* [63].

Apples are abundant in pectin, a soluble fiber [64]. Consumption of 2 apples per day in human subjects increased fecal abundance of beneficial bacteria *Bifidobacterium* and *Lactobacilli*, elevated production of SCFAs, and decreased abundance of potential harmful bacteria *Enterobacteriaceae* and *Pseudomonas* [26]. *In vitro* fermentation of apple pectin with human fecal bacteria facilitated the growth of *Bifidobacterium* and *Lactobacillus*, suggesting that pectin in apples was, at least in part, responsible for the improved gut microbial ecosystem observed in the apple-eating human subjects [26]. Moreover, consumption of citrus pectin (15% w/w in the mouse diet) also led to increased fecal abundance of *Bacteroidetes* and decreased abundance of *Firmicutes* [37]. Cranberry is rich in fibers. A randomized crossover controlled trial demonstrated that daily intake of 30 grams of freeze-dried whole cranberry powder (equivalent to 2.3 cups of fresh cranberry) in humans led to a lowered F/B ratio in fecal microbiota [40]. These effects might be partially due to the action of cranberry fiber because that cranberry-derived soluble fiber xyloglucans could support the growth of *Bifidobacterium longum*, a beneficial bacterium [65]. Studies in rodents also supported the role of MACs in modulating gut microbiota. Six weeks of dietary treatment of fiber-rich white-button mushrooms increased the abundance of *Ruminococcaceae* and *Lachnospiraceae* families in mouse fecal microbiota [51]. Bacteria from these two families were known to be able to utilize plant fibers to produce SCFAs [66, 67]. Additionally, oral administration of broccoli and potato fiber significantly increased the fecal abundance of *Bacteroides*,

Prevotella and *Porphyromonas* in rats, and importantly, intake of broccoli fiber decreased the abundance of the potential pathogens, such as *Clostridium perfringens*, *Escherichia coli* and *Enterococcus spp*. [68]. Fiber-rich diet can inhibit the growth of pathogens, through the change of pH environment induced by the production of SCFAs [63, 69]. Increased microbial diversity is a marker of healthier gut microbiota [28]. Hardy banana *(Musa basjoo)* is rich in resistant starch [70]. Consumption of dried hardy banana powder (rich in resistant starch) in diet for 3 weeks led to higher α-diversity and β-diversity, as well as a decreased ratio of *Firmicutes* to *Bacteroidetes* of fecal microbiota in mice [44]. These effects could be ascribed to resistant starch in hardy banana. In support of this notion, a 17-week double-blind crossover human study demonstrated that daily intake of 33 g resistant starch (resistant starch types 4) for three weeks increased the fecal abundance of *Bacteroidetes* and lowered the fecal abundance of *Firmicutes* [71].

One of the major functions of gut microbiota is to ferment MACs to produce SCFAs primarily including acetate, propionate and butyrate. Members from *Bacteroidetes* and *Firmicutes* produce various enzymes capable of breaking down MACs to yield SCFAs, and SCFAs could positively influence gut microbiota and host gut health. The higher fecal levels of SCFAs in children consuming high amount of MACs were associated with lower abundance of pathogens [63]. In line with this study, a randomized, double-blind, placebo-controlled, parallel-group human trial showed that enema with

sodium butyrate during shigellosis led to improvement of rectal histopathology and early reduction of inflammation [72]. Furthermore, oral administration of sodium butyrate (200 mg/kg body weight) to mice remarkably increased the fecal abundance of potential beneficial bacteria, such as *Christensenellaceae*, *Blautia* and *Lactobacillus* [73]. SCFAs play important roles in the gut health. The production of SCFAs is a means of recovering energy for host body, for example, acetate can be absorbed by the liver and server as substrate for the cholesterol synthesis [74]. Importantly, SCFAs possess biological activities beneficial to the host. Dietary supplementation of SCFAs in obese rodents or humans was shown to result in promising beneficial effects. Administration of butyrate (5% w/w) to mice inhibited high-fat diet-induced body weight gain and insulin resistance, and butyrate treatment also promoted energy expenditure [75]. Oral intake of inulin-propionate ester, a means of delivery of propionate to the colon, for 24 weeks in humans prevented the weight gain in overweight humans [76].

2.1.3 Interactions between Polyphenols and Gut Microbiota

Polyphenols are abundant in whole fruits and vegetables, and they play important roles in improving the gut microbial community. Polyphenols (such as catechins) can be absorbed in the small intestine, and a significant portion of them are metabolized and excreted back to the intestinal lumen via efflux pump in the intestinal epithelium and bile secretion from liver. Conjugated and polymeric polyphenols (such as anthocyanins and

proanthocyanidins) have low absorption in the upper gastrointestinal tract and a large portion of them accumulate in the colon. In general, abundant amount of dietary polyphenols reach the colon intact or in the forms of their metabolites [77]. Polyphenols and their metabolites interact with microbiota in the colon in a reciprocal manner as bacteria can metabolize these compounds, and these compounds can modulate microbiota composition and functions. This reciprocal interaction has been linked to improved host health.

2.1.3.1 The Impact of Gut Microbiota on Polyphenols.

The biotransformation of polyphenols by gut microbiota in the colon has been extensively studied in recent years. Various metabolites have been identified, and they may have different biological functions in comparison to their parent polyphenol compounds. This is the reason why biotransformation of polyphenols by gut microbiota could play an important role in mediating health effects of dietary polyphenols. For example, flavonoid glycosides can be transformed to aglycon that are generally more bioavailable and more bioactive. Fission reactions mediated by gut bacteria lead to the breakdown of the ring structures of polyphenols and production of various phenolic acids that convey bioactivities not offered by the intact polyphenols.

Although the chemical natures of polyphenols are diverse and complex, they can

be broken down by gut microbiota to form much simpler structures such as derivatives of phenylacetic, phenylpropionic, phenylbutiric, valeric acids and urolithins [78-80]. Feeding freeze-dried powders of cranberry, blueberry or black raspberry to rats resulted in urinary excretion of about 20 different phenolic acids with hippuric, 4-hydroxyphenylacetic and 4-hydroxy-3-methoxyphenylacetic, and 4-hydroxyphenylpropioic acids being major ones [81]. The production of these phenolic acids was ascribed to the microbial breakdown of polyphenols in those berries, and the relative urinary abundance of different phenolic acids was dependent on berry types. The production of potential microbial metabolites of berry polyphenols was also investigated in humans, and the results showed similarity as well as difference between rodents and humans in response to dietary consumption of whole berries. Oral intake of wild blueberry powder (22 grams per day) in humans for 30 days significantly increased plasma levels of benzoic, 2,5-dihydroxybenzoic, vanillic, hippuric, and 3-hydroxyhipuric acids, while no significant change was observed in the urinary levels of these metabolites [46, 82]. In contrast, the urinary level of hippuric acid was increased about 6-fold while those of 2,5-dihydroxybenzoic and vanillic acids did not change after oral intake of blueberry powder in rats [81]. In another human study, consumption of cranberry powder (30 grams per day) for 5 days increased urinary levels of 3,4-dihydroxyphenylacetic and 4-hydroxy-3-methoxyphenylacetic acids [40], whereas, cranberry-fed rats showed increased urinary level of 4-hydroxy-3-methoxyphenylacetic acid, but not that of 3,4-

dihydroxyphenylacetic acid [81]. The difference in microbial metabolites of berry polyphenols between rodents and humans may be attributed to their differences in gut microbiota composition and host metabolism of polyphenols. Besides phenolic acids, many other types of microbiota-derived polyphenol metabolites have been or yet to be identified. Their contributions to human health is an important topic, which warrants further investigation.

To better understand the mechanism of microbiota-mediated biotransformation of polyphenols, it is important to identify the strains of bacteria and their genes/enzymes responsible for the production of specific metabolites of polyphenols. For example, colonic bacteria could produce multiple enzymes responsible for various deconjugation reactions of polyphenols, such as β-glucuronidase [83, 84], β-glucosidase [85], esterases [86], hydrogenases [87], dehydroxylase [85, 88] and demethylase [85, 88, 89]. *Eubacterium ramulus* and *Clostridium saccbarogumia* were able to convert cyanidin 3-glucoside, a common anthocyanin from berry fruits to 3,4-dihydroxybenzoic acid and 2,4,6-trihydroxybenzaldehyde [90]. *Streptococcus thermophiles* and *Lactobacillus plantarum* broke down mulberry anthocyanins to form chlorogenic acid, caffeic acid and ferulic acid [91]. *Bifidobacterium lactis* and *Lactobacillus gasseri* produced cinnamoyl esterase that converted chlorogenic acid to hydroxycinnamate and ferulic acid [92]. Although information on specific gut microorganism and their genes responsible for the

production of bioactive polyphenol metabolites is accumulating, the overall understanding of this subject remains preliminary, which significantly limited our ability to elucidate the complex interplay between dietary components and gut microbiota.

2.1.3.2 Modulation of gut microbiota by polyphenols from whole fruits and vegetables

As a class of major bioactive components of fruits and vegetables, polyphenols could modulate gut microbiota through stimulation of beneficial bacteria and inhibition of pathogens. The effects of polyphenol-rich extracts of fruits and vegetables on gut microbiota have been studied in humans and animals. The administration of 1% (w/w in diet) of grape seed extract in pigs for 6 days altered gut microbiota composition, i.e., increased abundance of potential beneficial bacteria, such as *Lachnospiraceae*, *Ruminococcaceae*, and *Lacobacillus* [93]. *Lachnospiraceae* and *Ruminococcaceae* are two families of bacteria in the order *Clostridiales* that could produce short-chain fatty acids by fermenting dietary fiber, thereby potentially promoting intestinal health [94]. The early-life supplementation of a grape pomace extract (200 mg/Kg·bw by daily gavage for 12 days) in mice caused an inhibition of *norank*_f_*Lachnospiraceae*, *unclassified*_f_*Lachnospiraceae*, *Mucispirillum* as well as promotion of *Akkermansia* and *Lactobacillus* in the fecal microbiota [95]. *Mucispirillum* is considered a commensal with association to the increase of inflammatory biomarkers and colitis [96]. Some members of *Lachnospiraceae* were linked to diet-induced obesity and positively correlated with

diabetes [97, 98]. Oral gavage of a bilberry anthocyanin extract (20 mg/kg bw per day for 10 weeks) to rats increased the fecal abundance of some bacteria potentially beneficial to the host, such as *Aspergillus*, *Lactobacillus*, *Bacteroides*, *Clostridiaceae*-1, the *Bacteroidales*-*S24*-*7*-group and the *Lachnospiraceae_NK4A136_*group, and at the same time reduced the abundance of potential harmful bacteria, such as *Verrucomicrobia* and *Euryarchaeota* [99]. Furthermore, thirty-nine postmenopausal women consumed 100 mg of dietary isoflavones daily for two months to characterize changes in microbial communities of the intestinal tract. The supplementation elevated the abundance of dominant microorganisms of the *Clostridium coccoides*-*Eubacterium rectale* cluster, *Lactobacillus*-*Enterococcus* group, and *Bifidobacterium* genus, and these organisms were considered beneficial to the human health in various aspects [100]. The lower ratio of *Firmicutes* to *Bacteroidetes* implicates a non-obese and healthier status. The consumption of a grape seed proanthocyanidin extract (500 mg/kg bw, daily gavage for 8 days) could increase the cecal abundance of *Bacteroidetes* and *Proteobacteria* and decreased the F/B ratio in rats [101]. In a human study, twenty healthy participants were supplemented with 1000 mg of a pomegranate extract daily for four weeks, which delivers pomegranate polyphenols in an amount equivalent to about 8 oz of pomegranate juice. It was observed that the pomegranate extract significantly decreased the fecal abundance of *Firmicutes*. [45].

The metabolites of polyphenols produced by microorganisms in the large intestine could also impact gut microbiota. *Yersinia enterocolitica* is an enteropathogen to humans and mammals and causes gastrointestinal infections after the consumption of contaminated foods [102]. Urolithins are gut microbiota-derived metabolites of ellagic acid. Urolithin-A and urolithin-B inhibited *Quorum Sensing*-mediated processes such as biofilm formation and swimming motility in *Y. enterocolitica*, which suggested that urolithins may exert antipathogenic affect against *Y. enterocolitica* in the gut *[103]*. It is important to point out that the aforementioned antipathogenic effects of urolithins were observed at the concentrations of urolithins achievable in the intestinal tract through regular diet.

2.2. Whole Foods vs. Isolated Major Components of Whole Foods in Modulating Gut Microbiota

2.3. Whole Foods and Gut Microbiota-associated Diseases

2.3.1 Obesity

Gut microbiota is a critical factor in the development of obesity. Lacking of gut microbiota in germ-free mice resulted in a resistance to the Western diet-induced obesity [108], indicating a pivotal role of gut microbiota in obesity. Microorganisms that inhabit in the large intestine can ferment MACs and produce SCFAs that are absorbed and

provide energy supply for the host [109]. Higher energy intake than expenditure could lead to obesity, and the obesity-associated microbiota were found to have higher capacity for energy harvest [54]. As the dominant phyla of gut microbiota, both *Firmicutes* and *Bacteroidetes* are capable of degrading MACs to produce SCFAs. A higher ratio of *Firmicute* to *Bacteroidetes* was observed in obese mice and humans [54]. Furthermore, production of SCFAs in the colon was found to induce obesity by enhancing lipid synthesis [110]. Dietary intake of whole fruits and vegetables was reported to impact obesity through the modulation of gut microbiota. Table 2 listed the alteration of gut microbiota in obese rodents and humans induced by dietary intervention with various whole fruits and vegetables.

The relative abundance of *Firmicutes* and *Bacteroidetes* is considered as an important marker in the development of obesity. The increase in *Firmicutes* and decrease in *Bacteroidetes* were reported in obese mice and humans with high BMIs [54, 111]. In genetically obese mice, *Bacteroidetes* abundance was 50% lower than that in lean mice, and the microbiota in obese mice was associated with more effective capacity to release calories than that in lean mice [111]. A human study also showed an inverse correlation between *Bacteroidetes* abundance and body weight [111]. Members from *Firmicutes* (such as *Ruminococcaceae*, *Lachnospiraceae*, *Clostridium*, and *Lactococcus) and Bacteroidetes* (such as *Prevotella*, *Bacteroides*, and *S24-7*) have been frequently studied

in terms of their potential contributions to the development of obesity. Consumption of wholes foods had anti-obesity effects and also resulted in decreases in the abundance of *Firmicutes* and its members and increases in the abundance of *Bacteroidetes* and its members in obese mice, for example, the decreased abundance of *Ruminococcus gnavus* by navy bean [112] and *Clostridium* by tomato and pomegranate juice [113], and the increased abundance of *Bacteroides* by lingonberry and strawberry [114-116], *Prevotella* and *S24-7* by cherry, lingonberry and navy bean [59, 112, 117].

Although the lowered *F/B* ratio has been widely used to indicate an anti-obesity profile of gut microbiota, it is worth mentioning that emerging evidence has supported the use of other microbiota-related markers to characterize obesity status. Opposite to the expected low *F/B* ratio in lean individuals, a human study showed a lowered *F/B* ratio in overweight and obese individuals in comparison to that of lean individuals [118]. Fibers can be fermented by both *Firmicutes* and *Bacteroidetes*, such as *Lactobacillus* and *Ruminococcus* from *Firmicutes*, and *Prevotella* and *S24-7* from *Bacteroidetes* [119, 120], thus both *Firmicutes* and *Bacteroidetes* could thrive at the presence of fiber-rich whole foods. For example, oral administration of mushroom powders inhibited obesity in mice, but it did not lower the *F/B* ratio [121]. This may be due to the increase of the abundance of *Firmicutes* genera *Lactobacillus*, *Lactococcus* and *Ruminococcus* at the presence of mushroom fibers [121]. Avocado and bitter melon-fed obese rats significantly lost body

weight, but they did not show a lowered *F/B* ratio in comparison with the obese rats [60, 122]. These results supported the use of markers other than the *F/B* ratio to indicate an anti-obesity microbiota profile. For example, *Prevotella*-to-*Bacteroides* ratio was used to predict the success in weight loss in humans [123]. Interestingly, the amount of fecal SCFAs has been considered as a marker of obesity in humans because that colonic production of SCFAs correlated well with the BMI and the levels of SCFAs are in accordance with the alteration of gut bacteria [118, 124].

As shown in Table 2, *Akkermansia*, a Gram-negative bacterium belonging to *Verrucomicrobia*, has attracted increasing attention in terms of its role in obesity. Calorie restriction followed by a weight-stabilizing diet improved metabolism status of overweight and obese volunteers, which was accompanied by higher fecal abundance of *Akkermansia muciniphila* [125]. The fecal abundance of *Akkermansia muciniphila* inversely correlated with body weight of rodents and humans, and the oral administration of *Akkermansia muciniphila* alleviated high-fat diet-induced metabolic disorder, such as increased fat-mass, inflammation in adipose tissue, metabolomic endotoxemia, and insulin resistance [59, 112, 117]. Importantly, obesity was associated with the reduced mucus thickness in mouse intestinal tract [126], suggesting the increased gut permeability. *Akkermansia muciniphila* administration in obese mice restored the thickness of mucus, thus enhancing the gut barrier function, as well as improving glucose

homeostasis and adipose tissue metabolism [126]. Interestingly, a protein named Amuc_1100, isolated from outer membrane of *Akkermansia muciniphila*, was capable of recapitulating the beneficial effects of *Akkermansia muciniphila* through the interaction with Toll -like receptor 2 to improve gut barrier [127].

Consumption of whole fruits and vegetables were found to increase the abundance of *Akkermansia muciniphila*. For example, oral intake of cherry [117], lingonberry [59, 115, 116], grape [128], mango [129], or navy bean [112] in obese mice decreased body weight and increased the fecal abundance of *Akkermansia muciniphila*, indicating that anti-obesity actions of these whole fruits and vegetables might be closely related to their effects in promoting the growth of *Akkermansia muciniphila*. The high levels of fibers in these whole foods might contribute to the blooming of *Akkermansia muciniphila* in the colon [130]. Furthermore, dietary polyphenols from fruits also could promote the growth of *Akkermansia muciniphila* in the colon, which was associated with their anti-obesity and anti-colonic inflammation potential [131].

Table 2. The effects of whole fruits and vegetables on gut microbiota in obesity models

source	animal model	dose	duration	biomarkers	Effects on gut microbiota	reference
avocado	overweight/obese human	Oral, 1 avocado/day/person	12 weeks	decrease in IL-1β, C-reactive protein and hepatic growth factor	↑*Dialister,* ↑*Sutterella,* ↑*Bilophila,* ↑*Holdemanella,* ↑*Herbaspirillum,* ↑*Acetivibrio;* ↓*Methanosphaera*	[122]
bitter melon (*Momordica charantia L.*)	high-fat induced obesity in rat	Gavage, 300 mg/kg body weight/day	8 weeks	decrease in fasting glucose, HOMA-IR, TNF-α, IL-6, MCP-1 and LPS concentration, increase in IL-10	↓*Proteobacteria,* ↓*Desulfovibrionaceae,* ↓*Enterobacteriaceae,* ↓*Escherichia,* ↑*Odoribacteraceae,* ↑*Allobaculum,* ↑*Butyricimonas,* ↑*Faecalibacterium,* ↑*Odoribacter*	[60]
bitter melon	high-fat induced obesity in mouse	Oral, 1.5% w/w	16 weeks	decrease in macrophages infiltration, sphingokinase1 mRNA, IL-1β, NLRP 3 inflammasome components	↑*Bacteroidetes,* ↑*Clostridiaceae,* ↑*Porphyromonadaceae,* ↑*Ruminococcus,* ↑*Lactobacillus,* ↓*Firmicutes,* ↓*Actinobacteria,* ↓*Eubacteriaceae,* ↓*Oscillibacter;* ↓*Blautia,*	[132]
bitter melon (*Momordica charantia L.*)	high-fat diet-induced obesity in rat	Gavage, 400 mg/kg body weight/day	8 weeks	decrease in LBP and HOMA-IR	↑*Verrucomicrobia,* ↑*Blautia,* ↑*Anaerotruncus,* ↑*Lactococcus,* ↑*Allobaculum,* ↑*Oceanbacillus,* ↓*Prevotella,* ↓*Anaeroplasms.*	[133]
blueberry	high-fat diet-fed rat	Oral, 10 % w/w	8 weeks	increase in ilea villus, *Mucin 2*, decrease in TNF-α, IL-1β	↑*Actinobacillus,* ↑*Aggregatibacter,* ↑*Fusobacteriaceae,*	[134]

					↑*Lactobacillales,* ↑*Porphyromonadaceae*	
broccoli	high-fat induced obesity in rat	Oral, 7.5% w/w	17 weeks	decrease in body weight gain	↑*Bifidobacterium spp.,* ↑*Lactobacillus spp.*	[135]
grape	high-fat induced obestiy in mouse	Oral, 3 and 5% w/w	11 weeks	decrease in body fat and TG, increase in ZO-1	↓*Desulfobacter spp.,* ↓*Bilophila wadsworthia,* ↑*Akkermansia muciniphila*	[128]
Lingonberry (*Vaccinium vitis-idaea L.*)	high-fat induced obesity in mouse	Oral, 20 % w/w	11 weeks	decease in plasma levels of glucose and cholesterol, serum amyloid and LBP	↑*Akkermansia,* ↑*Faecalibacterium,* ↑*S24-7,* ↑*Parabacteriodes,* ↑*Odoribacter,* ↓*Firmicutes/Bacteroidetes ratio,* ↓*Ruminococcus,* ↓*Oscillospira,* ↓*Bacteroides,* ↓*Lachnospiraceae*	[59]
Mango (*Mangifera Indica L.*)	obese individuals	Oral, 400 g/day /person	6 weeks	trend toward decreased endotoxin	*trend toward decreased Bacteroides thetaiotaomicron*	[136]
mango	high-fat induced obesity in mouse	Oral, 1 and 10 % w/w	12 weeks	increase in IL-10 and plasma insulin, no effect on glucose tolerance and body weight	↑*Bifidobacteria,* ↑*Akkermansia,* ↑*Aldercrutzia*	[129]
mushroom	high-fat induced obesity in mouse	Oral, 0.5 and 3 % w/w	4 weeks	decrease in adipocyte size and perinephric adipose tissue	*trend toward increased Allobaculum, Bifidobacterium, Ruminococcus, Lactobacillus, Lactococcus and Streptococcus, and decreased Bacteroides, Prevotella, Mucispirillum,*	[121]

					Dorea, Roseburia, Escherichia and Akkermansia	
navy bean	high-fat induced obesity in mouse	Oral, 15.7% w/w	12 weeks	increase in ZO-1, occludin and mucins; decrease in HOMA-IR, adipocyte size, NFkBp65 and STAT3	↑*Akkermansia muciniphila,* ↑*Prevotella,* ↑*S24-7,* ↓*Ruminococcus gnavus*	[112]
spinach	high-fat induced NAFLD in rat	Oral, 2.5 and 5 % w/w	5 weeks	decrease in fasting glucose, LDS-cholesterol	↑*Lactobacillus*	[137]
tomato	$BCO1^{-/-}$/$BCO2^{-/-}$ double knockout mice	Oral, 4.19 % w/w	24 weeks	decrease in pathological severity of steatosis, hepatic TG, TNF-α, IL-1β and IL-6	↓*Clostridium sp. ID4,* ↓*Clostridium*	[113]

(IL, interleukin; HOMA-IR, homeostatic model assessment for insulin resistance; TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein; NLRP, Nod-like receptor protein; TG, triglyceride; ZO-1, zonula occludens-1; LBP, LPS-binding protein; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; LDL, low-density lipoprotein; NAFLD, non-alcoholic fatty liver disease.)

2.3.2 Colonic Inflammation

In the host, homeostasis of gut microbiota is required to maintain health. When the balance of bacterial community in the colon becomes disrupted, it may trigger abnormal immune responses and induce colonic inflammation [138]. For example, certain species of bacteria were considered the stimuli to the colonic inflammation, and the increase in these bacteria, e.g. pathogenic *E. coli*, was strongly associated with colonic inflammation [139]. On the other hand, several strains of *Bifidobacterium* and *Lactobacillus* can produce anti-inflammatory effects by modulating the inflammation related cell signaling pathways [140], and the ample presence of these bacteria in the gut may protect the host against colonic inflammation. Colonic inflammation was associated with the reduction of *Firmicutes* [141], *Bacteroides* [141], *Bifidobacterium adolescentis* [142], *Faecalibacterium prausnitzii* [141], *Lactobacillus* and *Eubacterium* [143], and the increase in *Enterobacteriaceae* and *Ruminococcus gnavus* [142, 144]. Studies have supported the notion that whole fruits and vegetables inhibit colonic inflammation via modulating related bacteria. Administration of whole cranberry powder or strawberry powder in the diet of dextran sulfate sodium-treated mice for 6 weeks increased fecal abundance of *Lactobacillus* and *Bifidobacterium*, which was associated with suppression of colonic inflammation [145-147]. In a genetically induced colitis mouse model, dietary consumption of blueberry or broccoli powder (10% w/w in diet) remarkably reduced the abundance of *Clostridium perfringens* and *Escherichia coli* [148]. These bacteria were

positively associated with colonic inflammation [139, 149]. The anti-inflammatory potential of whole foods in the colon could stem from (i) the bioactive components in the whole foods, (ii) altered gut microbiota induced by the whole foods, and/or (iii) microbiota-derived metabolites of whole food components. For example, Anthocyanin-rich fractions from red raspberry showed anti-inflammatory effects against LPS-induced inflammation in macrophages, which was evidenced by the suppression of NO synthesis, as well as the downregulation of iNOS, COX-2, IL-1β and IL-6 [150]. Probiotic *Bifidobacterium* could suppress inflammation both *in vitro* and *in vivo* [151, 152]. Fermentation of MACs from whole foods by gut bacteria produces SCFAs, especially butyrate, that showed anti-inflammatory effect through inhibition of NFκB pathway and regulation of T cell functions [153, 154]. It is noteworthy that these three modes of actions often coexist in the colon and are likely to interact with each other, therefore, produce modified health outcomes in comparison with that produced by each mode of action alone. Recently, an increasing number of studies demonstrated the anti-colonic inflammation effects of whole fruits and vegetables, such as cranberry, strawberry, mushroom, and anthocyanins-containing potatoes [52, 145, 147, 155, 156]. However, the detailed mechanistic understanding of how whole food components and gut microbiota interact to produce inhibitory effects against colonic inflammation is lacking, therefore warrants further investigation.

Probiotics, such as *Bifidobacteria* and *Saccharomyces*, have been shown to offer protective effects against colonic inflammation [152]. Moreover, probiotics supplemented with food components might produce synergistic effects to confer benefits to the host. For example, a synbiotic supplementation, which contained whole plant sugar cane fiber and *Bacillus coagulans*, induced a stronger suppression on colonic inflammation than either whole plant sugar cane or *Bacillus coagulans* alone [157]. *Akkermansia*, an emerging probiotic has demonstrated anti-inflammatory effects in the colon via stimulating mucin production and improving gut barrier [126, 158, 159]. Patients with colonic inflammation showed a lower fecal abundance of *Akkermansia* [160]. Oral administration of *Akkermansia* to dextran sulfate sodium-treated mice ameliorated colonic inflammation, which was evidenced by the lower colon histological score and reduced colonic levels of pro-inflammatory cytokines [161].

Although *Akkermansia* showed consistent protective effects against obesity and diabetes, the role of *Akkermansia* in colonic inflammation remains controversy. For example, higher abundance of *Akkermansia* has been found in patients with IBD and colitic mice in comparison to the corresponding healthy counterparts [162-164]. An possible explanation is that decreased thickness of mucus layer in the inflamed colon led to the outgrowth of *Akkermansia* [165], consequently, the growth of *Akkermansia* in the lumen is elevated due to its access to ample substrates in the stool [166]. In line with

these findings, colitic mice had a high fecal abundance of *Akkermansia* than non-colitic mice, and consumption of whole strawberry or cranberry inhibited the colonic inflammation and decreased the fecal abundance of *Akkermansia* [145, 147]. It is possible that the protective effects of whole berries against colonic inflammation maintained integrity of the colonic tissue, which in turn prevented migration of *Akkermansia* from mucus to the lumen [117]. A recent study showed that oral administration of *Akkermansia muciniphila* induced colitis in germ-free $IL10^{-/-}$ mice by degrading mucus and producing LPS in the colon [167]. This finding was opposite to that of another report, where oral gavage of *Akkermansia muciniphila* inhibited colitis in mice [161]. Different cellular components of *Akkermansia* were found to exert distinct functions in colon health. For example, the outer membrane of *Akkermansia* was reported to induce inflammation by upregulating the pro-inflammatory cytokines (IL-1β and IL-6) in bone-marrow-derived macrophage [167], whereas, Amuc_1100 protein, isolated from outer membrane of *Akkermansia*, was able to improve gut barrier in high fat-fed mice [127]. These findings highlighted the complexity of gut microbiota and its role in host health.

CHAPTER 3

NON-EXTRACTABLE POLYPHENOLS FROM CRANBERRY: A POTENTIAL ANTI-INFLAMMATION AND ANTI-COLON CANCER AGENT

3.1 Introduction

Polyphenols are a major class of dietary phytochemicals which are responsible for many health benefits. Current research on dietary polyphenols mainly focus on a fraction of polyphenols, known as extractable polyphenols (EP) [15, 16]. EP can be extracted from food with aqueous-organic solvents (e.g. water, methanol, ethanol, acetone) [17, 18], and are often considered as "total phenolic content" for various biological studies. However, another fraction of polyphenols remains in the residues after solvent extraction, and they are known as non-extractable polyphenols (NEP) [16, 19]. NEP are mainly polyphenols that bound to dietary fiber or other macromolecules through hydrophobic interaction, hydrogen bonding and covalent bonding [15, 16]. NEP is not significantly released from the food matrix during digestion in the stomach or small intestine, therefor they reach colon nearly intact [20]. In the colon, NEP may be released from food matrix by the action of microbiota and become bioavailable and bioactive. In contrast, EP tend to be absorbed and metabolized in the upper gastrointestinal tract, therefore, their biological fate is quite different from that of NEP. Only unabsorbed EP and their

metabolites could reach the colon. The better colonic bioavailability of NEP than EP makes NEP promising candidates for promoting colon health.

Colon diseases such as inflammatory bowel diseases (ulcerative colitis and Crohn's disease) and colon cancer have posed serious threats to the human health. Colon cancer is one of the leading causes of death in both of male and female in the United States, and colitis is a key risk factor for colon carcinogenesis [168, 169]. The prevention of colon cancer is the most logical method to controlling it, which is suggested by accumulating evidence [170, 171]. Berries and their components have been subject of many studies for the anti-inflammatory and anticancer effects. Cell culture studies have demonstrated the inhibitory effects on human colon cancer cells by the extracts of various berries such as blackberry, black raspberry, red raspberry, blueberry, cowberry [17]. Berries (e.g. chokeberry, red raspberry, strawberry, cowberry) and their extracts have been shown to have anti-inflammatory and anticancer effects in the intestine of various animal models such as AOM-induced rat colon cancer model, Apc 1638+/- mouse and Muc2-/- mouse model, and DSS-induced colitis models [12, 14, 156].

Cranberry is a native berry fruit of North America, and it is abundant of polyphenols including flavan 3-ols, proanthocyanidins, anthocyanins and phenolic acids [21]. Majority of studies on cranberry have been focused on its antibacterial actions [22]. To date, the inhibitory effects of cranberry on colonic inflammation and colon cancer

have not been adequately studied. Cranberry extracts have been shown to inhibit colon cancer cell growth and suppress pro-inflammatory enzymes expression [21]. However, most published studies focused on EP of cranberry, and NEP has been neglected. The object of this study is to characterize EP and NEP fractions from cranberry and determine their potential in anti-inflammation and anti-colon cancer.

3.2 Materials and methods

3.2.1 Materials

Cranberry (Early Black variety) was harvested at State Bog, Wareham, MA (September 2011), and whole cranberry powder was obtained by freeze-drying from fresh cranberry fruits. The freeze-dried whole cranberry powder is deep red in color and free-flowing. procyanidin B2, procyanidin A2, cyanidin 3-arabinoside, peonidin 3-glucoside were from Shyuanye (Shanghai, China). Myricetrin, astragalin, myricetin, quercetin, ρ-coumaric acid, ferulic acid, protocatechuic acid, chlorogenic acid, caffeic acid, and benzoic acid were from Quality Phytochmicals, LLC (Ease Brunswick, NJ).

3.2.2 Extraction of EP

100g freeze-dried cranberry powder was mixed with 70% (v/v) acetone aqueous (1% acetic acid). The mixture was sonicated for 30 min at room temperature, and the supernatant was collected after centrifuging at 4000 g for 5 min. The residues were

extracted twice more, pooled the supernatants. The supernatant was concentrated and then dissolved in methanol. Hexane was used to remove fat and other highly lipophilic molecules in extracts, after which the supernatant was concentrated at 40 ℃ to dryness. EP fraction was collected and stored in -20 ℃ for further studies.

3.2.3 Extraction of NEP

The residues obtained after EP fractionation were treated with 2 M NaOH at 37 ℃ for 2 h after purging container with nitrogen to protect polyphenols from oxidation. After alkaline hydrolysis, mixture was adjusted to pH 2 with 6 M HCl, centrifuged at 4000 g for 10 min to obtain supernatant. The supernatant was extracted by ethyl acetate and diethyl ether (1:1, v/v) for three times. The Pooled supernatants were concentrated to dryness under vacuum. The NEP fraction was collected and stored in -20 ℃ for further studies.

3.2.4 Determination of total phenolic contents (TPC), flavonoid contents (FC), tannin contents (TC), and ORAC

TPC was measured using the Folin-Ciocalteu method with some modifications [172]. Sample was dissolved in 50% methanol at the final concentration 0.4 mg/mL. Twenty μL Folin-Ciocalteu reagent was added to a 96-well plate containing 20 μL sample and 20 μL distilled water. The plate was tapped gently on all sides and kept at room

temperature for 10 min, followed by addition of 140 µL 7% sodium carbonate. After 90 min at room temperature, the absorbance was measured at 760 nm (BioTek Instrument, Inc. Vermont, VT, USA). Results are expressed as microgram of gallic acid equivalents per gram.

FC was determined as previously described with some modifications [173]. Briefly, 20 µL 0.4 mg/mL sample was mixed with 100 µL distilled water and 10 µL 5% sodium nitrite in a 96-well plate. After 6 min, 20 µL 10% aluminum chloride was added in the mixture and allowed to react for 5 min. Subsequently, 50 µL 1 M sodium hydroxide was added in the mixture. Tap the plate gently on all sides for 2 min, and the absorbance was read at 510 nm (BioTek Instrument, Inc. Vermont, VT, USA). The results are expressed as microgram of catechin equivalents per gram.

TC was tested as previously described with modifications [174]. The vanillin-H_2SO_4 solution was made of 4% vanillin in methanol and 30% H_2SO_4 in methanol in the ratio of 1:1. One hundred eighty µL vanillin-H_2SO_4 solution was added in a 96-well plate containing 20 µL 0.4 mg/mL sample. The absorbance was determined at 510 nm (BioTek Instrument, Inc. Vermont, VT, USA), and results are expressed as microgram of catechin equivalents per gram.

The antioxidant capacity was determined by ORAC assay following the

previously published method with modifications using microplate reader (BioTek Instrument, Inc. Vermont, VT, USA) [175].

3.2.5 LC-MS analysis

Identification and quantification of selected flavonols, flavanols, anthocyanins and phenolic acids were carried out by LC-MS as described preciously with some modification [176]. Chromatographic separation of anthocyanins was carried out on Zorbax SB-Aq C18 column (150 mm ×4.6 mm, 5μm, Agilent Technologies, USA). Separation of flavonols, flavan 3-ols and phenolic acids were achieved on Kinetex XB-C18 column (100 mm ×4.6 mm, 2.6 μm, Phenomenex, Torrance, CA, USA). The mobile phase consisted of 0.1% formic acid in 95% water mixed with 5% ACN (solvent A), and 0.1% formic acid in ACN (solvent B). The elution program processed as follows: 0-2 min, isocratic elution with 5% B; 2-37 min, 5-95% B; 37-40 min, 95-100% B; 40-46 min, 100-5% B; 46-52 min, isocratic elution with 5% B. The flow rate was 0.4 mL/min, and injection volume was 10 μL. Proanthocyanidins B2, proanthocyanidins A2, ECG, myricetrin, astragalin, myricetin, p-coumaric acid, ferulic acid, protocatechuic acid, chlorogenic acid, caffeic acid, benzoic acid, cyanidin 3-arabinoside, peonidin 3-glucoside were used as external standards.

3.2.6 Determination of cell viability, colony formation, cell cycle and apoptosis

Assays for cell viability, colony formation, cell cycle and apoptosis were performed as we previously described [177-180]. In brief, HCT116 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). HCT116 (2,500 cells/well) were cultured in RPMI-1640 media supplemented with 5% heat-inactivated FBS, 100 units/mL of penicillin, and 0.1 mg/mL of streptomycin (Mediatech, Herndon, VA) at 37 ℃ with 5% CO_2 and 95% relative water humidity. After 24 hours of attachment, cells were treated with serial concentrations of EP and NEP for 24 h, then cell viability was determined by MTT assay [177].

HCT116 (400 cells/well) were seeded in 6-well plates. After 24 hours attachment, cells were subjected to the treatment of serial concentration of EP and NEP. The media in 6-well plate were refreshed every 2 days. After 14 days, the determination of colony formation was conducted as we described previously [180].

HCT116 (4×10^4 cells/well) were seeded in 6-well plates for cell cycle and apoptosis analysis. After 24 hours of incubation and attachment, cells were treated with 16 μg (GAE)/mL NEP for 24 h, and then cells were collected by brief trypsinization (0.25% trypsin-EDTA; Mediatech)for analysis by flow cytometry to determine cell cycle or apoptosis [177, 180].

3.2.7 Nitric oxide assay and immunoblotting

RAW 264.7 were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). RAW 264.7 cells were cultured in RPMI-1640 media supplemented with 10% heat-inactivated FBS (Medistech, Herndon, VA, USA), 100 units/mL of penicillin, and 0.1 mg/mL of streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37 ℃ with 5% CO_2 and 95% air. Griess reaction was used to determine the concentration of nitrite in the culture media, which is an indicator of NO production [181]. RAW264.7 cells (5×10^5) were seeded in 96-well plates. After 24 hours, cells were treated with different concentrations of EP or NEP for 24 hours. After 24 hours treatment, the media were mixed with an equal volume of Griess reagent mixture (reagent A: 1% sulfanilamide in 5% phosphoric acid, reagent B: 0.1% naphthylethylenediamine dihydrocholoride aqueous). Then the production of NO was determined at 540nm with a plate reader.

Whole cell lysate from macrophages was extracted as we reported previously [177-179, 182]. Briefly, RAW 264.7 cells (2.5×10^5 cells/mL) were cultured in cell culture dishes. After 24 h attachment, the media were replaced by 1μg/mL lipopolysaccharides (LPS) with or without serial concentration of NEP. After 24 h, cell lysate was collected and subject to western blot analysis as we did previously [182].

3.2.8 Data Analysis

All results were presented as mean ±SD. Analysis of variance (ANOVA) was used to compare the differences among two or more groups. $\rho < 0.05$ was considered to be statistical significance.

3.3 RESULTS AND DISCUSSION

3.3.1 EP and NEP in cranberry

Acetone and acetone aqueous are commonly used to extract polyphenols from fruits and vegetables. It was reported that acidic acetone was more effective in extracting polyphenols such as anthocyanins, flavan-3-ols and phenolic acids, than other solvents [18]. In order to increase the extraction rate of phenolic conjugates (e.g. β-glycosides), 70% acetone aqueous with 1% acetic acid was used to extract EP from cranberry (the yoeld of EP is 25,610 mg/100g dry powder) [183]. Alkaline hydrolysis was used to release NEP because it could release more NEP from plant matrix than acid hydrolysis or enzyme hydrolysis (the yield of NEP is 3,559.5 mg/100g dry powder) [184]. TPC, FC and TC of EP and NEP were shown in Figure 2. In general, EP had higher TPC, FC and TC than NEP. NEP content of cereals was found to be higher than that of EP [19, 185], while fruits had higher contents of EP than NEP [186]. The relative abundance of NEP vs. EP in cranberry was in consistence with the findings of previous studies about NEP from different fruits

including apple, orange, banana and grape [16, 187]. Both EP and NEP were more abundant in tannins than flavonoids (Figure 2).

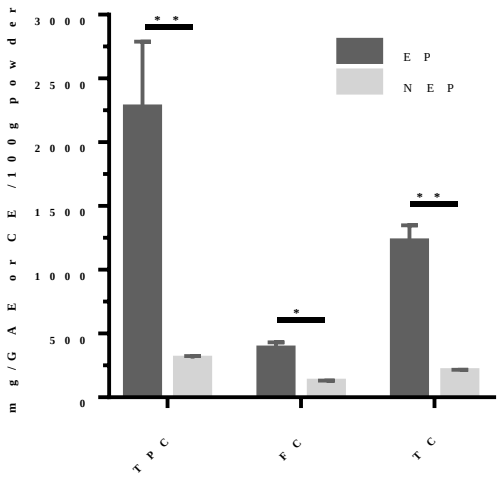

Figure. 2 Total phenolic content (TPC), flavonoid content (FC), and tannin content (TC) of EP and NEP fractions of cranberry. (GAE: gallic acid equivalents; CE: (+)-catechin equivalents). Data represent mean ± SD (n=3), * indicates $p<0.05$, ** indicates $p<0.01$.

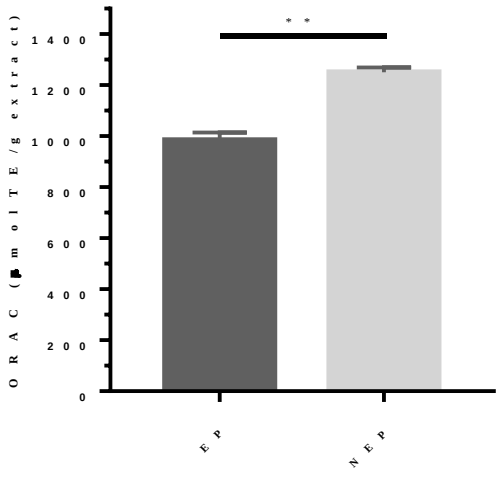

Figure. 3 Oxygen radical absorbance capacity (ORAC) of EP and NEP (TE: Trolox equivalents). Data represent mean ± SD (n=6), ** indicates $p<0.01$.

3.3.2 Chemical profiles of EP and NEP

The chemical profiles of EP and NEP were determined by HPLC-MS, results were presented in Table 3. Majority of the phenolic compounds identified by comparing their retention times and fragmentation patterns with those of standards occurred in both EP and NEP, for example procyanidin A2, myricitrin, astragalin, p-coumaric acid, protocatechuic acid, chlorogenic acid and benzoic acid. In EP fraction, the main constituents were anthocyanins, including cyanidin-3-arabinoside and peonidin-3-glucoside, whereas anthocyanins were not found in NEP fraction. The major components in NEP were phenolic acids, including chlorogenic acid, p-coumaric acid, ferulic acid, caffeic acid, protocatechuic acid and benzoic acid, and the total content of phenolic acids was more than 10 times higher than that of EP. Meanwhile, all the phenolic acids described above were found in NEP of cranberry for the first time, although some of them have been previously found in EP fraction of cranberry [188]. Previous studies reported that cranberry was abundant of proanthocyanidins that contributed to various health benefits, especially those with unusual A-type linkages [189, 190]. Importantly, proanthocyanidins were also found in NEP fraction, and their abundance was higher than that in EP. Although the major polyphenols in NEP were phenolic acids, other kinds of flavonoids were also found in NEP, such as myricitrin, myricetin, and astragalin. Although alkaline hydrolysis has been frequently used to release phenolics from fiber, it also can break the esters and glycoside linkages of phenolic components after the release,

which is a factor to be considered when interpreting the chemical profiles of NEP fraction. Proanthocyanidins or condensed tannins have been reported in EP from cranberry [191]. Interestingly, our results showed the existence of tannins in cranberry NEP and their abundance was much higher than EP, especially procyanidin A2 as shown in Table 3.

3.3.3 Antioxidant capacity of EP and NEP

ORAC was used to determine the antioxidant capacity of EP and NEP. EP and NEP showed ORAC values of 984.17 μmol TE/g(extract) and 1258.53 μmol TE/g(extract), respectively (Fig.3). The abundance of anthocyanins, flavanols, flavonols and phenolic acids in EP fraction might contribute to the antioxidant capacity, especially anthocyanins (e.g., cyanidin 3-arabinoside and peonidin 3-glucoside) which have been reported for their antioxidant capacity (Table 3) [192]. Notably, phenolic acids were the dominant phenolic constituents in NEP (Table 3), and they may responsible for the antioxidant capacity of NEP [193]. Importantly, the major compounds reported in urine after the consumption of berries were phenolic acids [81, 194], increasing numbers of studies have been focused on the phenolic acids produced from the metabolism of food components and their contribution to the human health.

Table 3 Phenolic Compounds Identified by LC-MS in EP and NEP from cranberry.

compound	Retention Time	MS (m/z)	NEP (mg/g extract)	EP (mg/g extract)
procyanidin B2	8.99	$579[M+H]^+$	N	0.09 ± 0.02
procyanidin A2	10.15	$577[M+H]^+$	1.12 ± 0.23	0.40 ± 0.00
ECG	9.98	$443[M+H]^+$	0.18 ± 0.06	0.13 ± 0.00
myricetrin	9.60	$463[M+H]^+$	1.09 ± 0.20	0.81 ± 0.02
astragalin	10.10	$447[M+H]^+$	0.58 ± 0.12	0.20 ± 0.01
myricetin	11.50	$319[M+H]^+$	0.15 ± 0.02	N
quercetin	22.72	$303[M+H]^+$	0.16 ± 0.01	0.18 ± 0.06
total flavonols and flavanols			3.28	1.81
cyanidin 3-arabinoside	26.01	$420[M+H]^+$	N	1.79 ± 0.07
cyanidin 3-galactoside	30.86	$450[M+H]^+$	N	2.10 ± 0.04
peonidin 3-galactoside	22.99	$464[M+H]^+$	N	5.43 ± 0.12
peonidin 3-glucoside	26.74	$464[M+H]^+$	N	1.95 ± 0.18
total anthocyanins				11.27
p-coumaric acid	27.02	$165[M+H]^+$	4.52 ± 0.11	0.31 ± 0.12
ferulic acid	27.30	$195[M+H]^+$	0.33 ± 0.01	N
protocatechuic acid	21.20	$153[M-H]^-$	0.46 ± 0.02	0.16 ± 0.04
chlorogenic acid	21.53	$355[M+H]^+$	10.54 ± 0.29	0.33 ± 0.13
caffeic acid	24.30	$181[M+H]^+$	0.37 ± 0.04	N
benzoic acid	7.16	$123[M+H]^+$	0.10 ± 0.03	0.66 ± 0.11
total phenolic acids			16.31	1.46

3.3.4 Anti-inflammatory effects of EP and NEP in RAW264.7 macrophages

To determine the anti-inflammatory effects of EP and NEP, their effects on the growth of RAW264.7 macrophages were firstly determined by cell viability assay. Neither EP nor NEP showed cellular toxicity at the concentration range of 2 -16 μg gallic acid equivalent (GAE)/mL (data not shown). After establishing the nontoxic concentration range, the effects of EP and NEP on nitric oxide (NO) production induced

by LPS (1μg/mL) in macrophages were determined. NO is an important inflammation mediator whose production can be induced by LPS. As shown in Figure 4, at 4 and 8 μg GAE/mL, EP treatment showed 15% inhibition of the production of NO in comparison with the LPS-treated positive control group. Although NEP caused similar suppression as EP on NO production at 4 μg GAE/mL, NEP markedly lowered the production of NO by 32% at 8 μg GAE/mL, when compared to the positive control group. At 16 μg GAE/mL, NEP caused 53% inhibition on NO production, while EP at the same concentration inhibited the production of NO by only 12%, when compared to the positive control. The difference between EP and NEP in terms of the suppression of NO production is expected to be due to their different chemical profiles. For example, higher levels of phenolic acids including chlorogenic and p-coumaric acids in NEP may contribute to the higher anti-inflammatory effects of NEP in comparison with EP [195, 196].

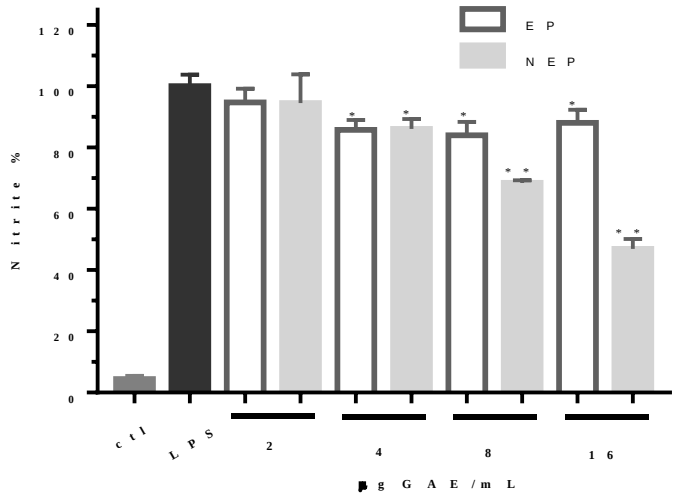

Figure. 4 Effects of EP and NEP on LPS-induced nitric oxide production in RAW 264.7 macrophages. The cells were treated with or without 1 μg/mL LPS or LPS plus different concentrations of EP and NEP fractions for 24 hours. Then the media was collected and determined the NO production as described in Materials and Methods. Results were expressed as mean ± SD (n=6). *p< 0.05 and **p<0.01 indicate statistical significance, when compared to LPS treatment group.

Since NEP had much stronger inhibitory effects than EP on NO production in RAW 264.7 macrophages, we further investigated molecular mechanism by which NEP inhibited NO production. Inducible nitric oxide synthase (iNOS) is one of the important pro-inflammatory proteins [182]. The expression of iNOS was very low without LPS treatment, while LPS treatment significantly increased iNOS expression by 22.7-fold (Figure 5A). NEP showed a dose dependent inhibition on the expression of iNOS, and NEP at 8 and 12 μg GAE/mL significantly suppressed expression levels of iNOS by 68% and 84%, respectively (Figure 4A). We examined expression levels of nuclear p50 and p-IκBα, both of which play critical role in the mediation of inflammation. p50 is the functional subunit of NF-κB pathway, and phosphorylation of IκBα results in dissociation of IκBα from NF-κB, and activated NF-κB initiates pro-inflammatory responses [182]. The results demonstrated a dose dependent inhibition on the expression of nuclear p50 by NEP, and especially, NEP at 8 and 12 μg GAE/mL decreased the levels of nuclear p50 by more than 80% when compared to that of LPS-stimulated positive control cells (Figure 5B). Also, expression levels of p-IκBα were suppressed by NEP treatment at 8 and 12 μg GAE/mL. We further determined the effects of NEP on the expression levels of HO-1, an

antioxidant enzyme, which involves in the inflammation. HO-1 expression contributes to the adaptive increase of cellular anti-oxidant capacity, and it is important targets for anti-inflammation remedy [181]. Results indicated that NEP treatment caused a dose-dependent increase in the expression of HO-1, especially, at 8 and 12 μg GAE/mL NEP increased HO-1 expression by 4.2-fold and 6.7-fold compared to the LPS-treated positive control cells, respectively (Figure 5C). The expression levels of Nrf2, a transcription factor of HO-1 gene, which involves in the induction of HO-1, was significantly increased by NEP treatment at 8 and 12 μg GAE/mL.

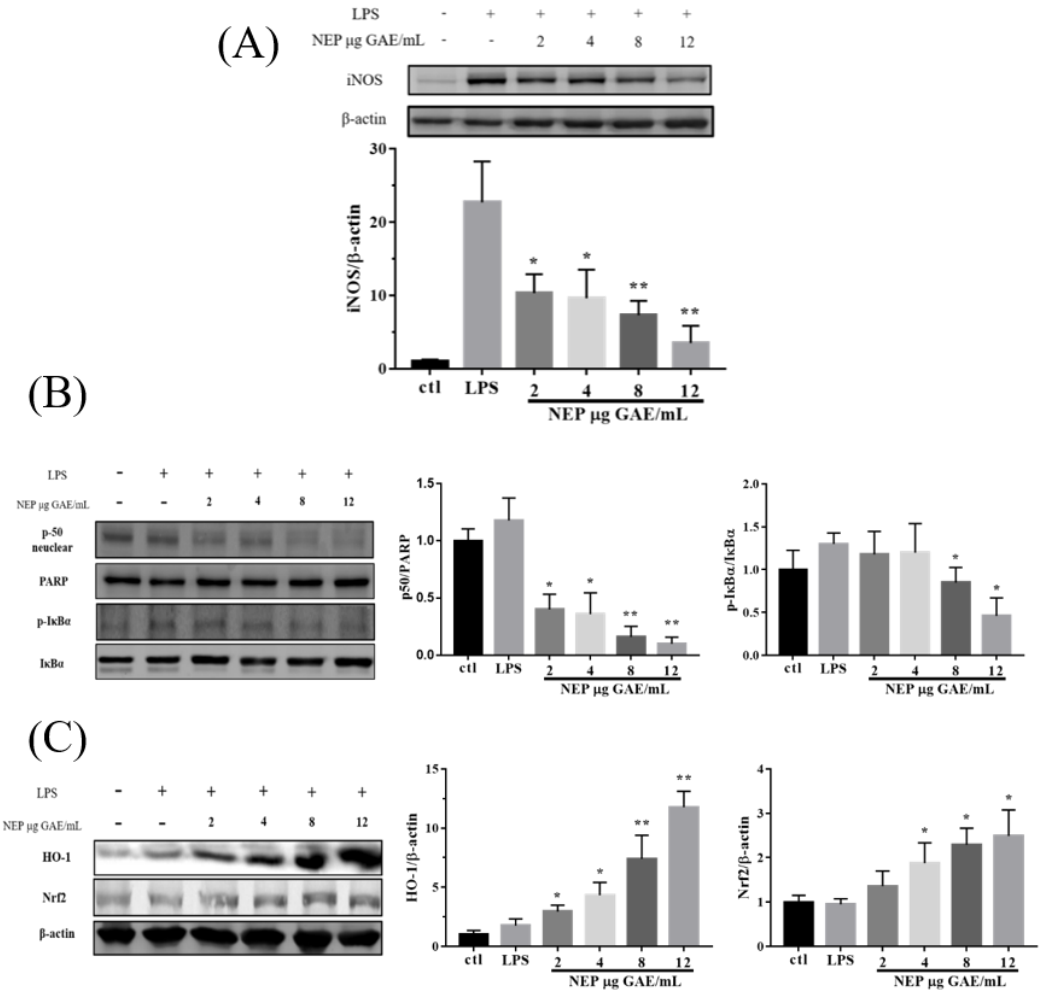

Figure. 5 Inhibitory effects of NEP on LPS-induced proteins expression of iNOS (A), p-50, p-IκBα (B), HO-1 and Nrf2 (C) in RAW 264.7 macrophages. RAW 264.7 cells were seeded in culture plates for 24 h, and then treated with different concentrations of NEP.After 24 h, cells were collected for Western blotting analysis. The numbers underneath the blots represent band intensity that was normalized by β-actin (cytosolic fraction) or PARP (nuclear fraction). *p<0.05 and **p<0.01 indicate statistical significance in comparison with LPS-treated group (n=3). The standard deviations (all within ±15% of the means) were not shown.

3.3.5 Anti-colon cancer effects of EP and NEP on human colon cancer cells

Accumulating studies suggest that various polyphenols derived from fruits and vegetables possess anticancer effects in various models. As a rich resource of

polyphenols, EP and NEP from cranberry potentially also have anticancer capacity. NEP cannot be released from the food matrix in the stomach or small intestine, they reach colon nearly intact [20], where they may show biological benefits after being released from matrix by gut microbiota-mediated fermentation. Therefore, we determined the anti-colon cancer potential of cranberry EP and NEP. Cell viability assay revealed that both EP and NEP showed inhibition on the growth of cancer cells. NEP had stronger inhibitory effects on the growth of human colon cancer HCT116 cells than EP at the concentration range of 2-16 μg GAE/mL, and NEP treatment showed a dose-dependent inhibition (Figure 6A). At the concentration of 16 μg GAE/mL, NEP and EP caused inhibition on cell viability by 20% and 60%, respectively. In consistence with the cell viability assay, colony formation assay indicated that EP and NEP showed suppression on the colony formation of colon cancer cell. And NEP had stronger inhibitory effects on colony formation of colon cancer cells, for example, at 6 μg GAE/mL, NEP and EP inhibited the colony formation of HCT116 cells by 64% and 35%, respectively (Figure 6B).

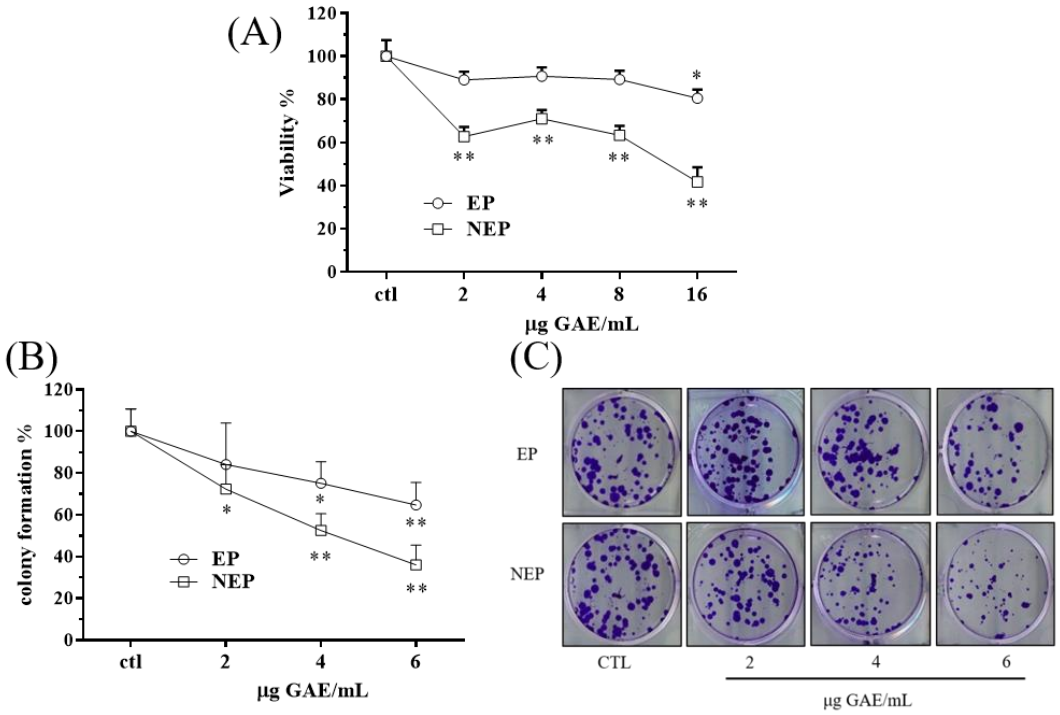

Figure. 6 (A) Inhibitory effects of EP and NEP fractions on the viability of HCT116 human colon cancer cells in MTT assay. HCT116 cells (2500 cells/well) were seeded in 96-well plates for 24 h, and then treated with serial concentrations of EP and NEP. After 24 h, cells were subjected to MTT assay. Data represent mean ± SD (n=6). (B) Inhibitory effects of NEP and EP fractions on the colony formation capacity of HCT116 cells. HCT116 (400 cells/well) were seeded in 6-well plates. After 24 hours, cells were subjected to the treatment of different concentrations of EP and NEP. The media in 6-well plate were refreshed every 2 days. After 14 days, the determination of colony formation was conducted. Data represent mean ± SD (n=3). (C)Images of colony formation ofHCT116 cells under different treatments. *$p<0.05$ and **$p<0.01$ indicate statistical significance in comparison with control group.

Since NEP showed stronger inhibitory effects on the growth of the colon cancer cells in comparison with EP, we further investigated the mechanism by which NEP inhibit the colon cancer cell viability and colony formation. We determined the effects of NEP on cell cycle progression and cellular apoptosis of HCT116 cancer cells by flow cytometry as we described previously [180]. Based on results obtained from cell viability

and colony formation assay, we selected a NEP dose of 16 µg GAE/mL to determine its effects on cell cycle progression and apoptosis. The results revealed that NEP-treatment significantly increased the cell population in G0/G1 phase and decreased the cell population in S phase in comparison with the control, suggesting a cell cycle arrest at the G0/G1 phase (Figure 7A). After treatment with NEP, early and late apoptotic cell populations were increased by 3.8-fold and 6-fold, respectively, compared to the control group (Figure 7B).

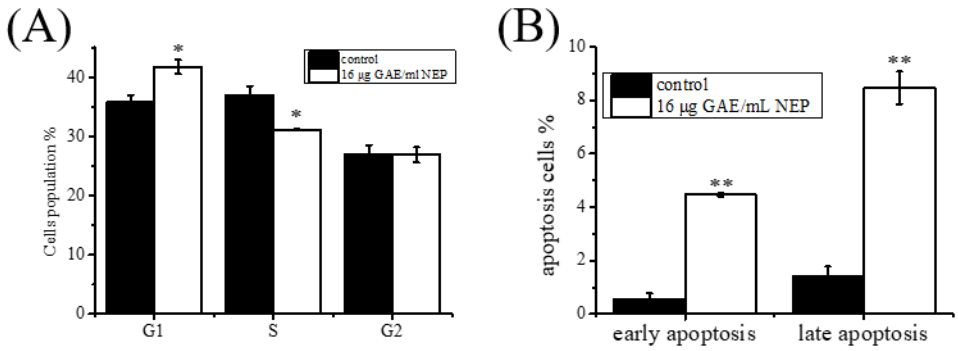

Figure. 7 Effects of NEP fraction from cranberry on cell cycle progression (A) and apoptosis (B) of HCT116 human colon cancer cells. HCT116 were seeded in 6-well plates for 24 h, then treated with or without 16 µg GAE/mL NEP. After 24 h treatment, cells were collected and subjected to cell cycle or apoptosis analysis with help of flow cytometry as described in Materials and Methods. * $p<0.05$ and ** $p<0.01$ represent statistical significance in comparison with control group (n=6).

In summary, this study provided an understanding of EP and NEP from cranberry in terms of their chemical properties and biological functions. Chemical profile of cranberry NEP was firstly determined, which showed that NEP possessed higher contents

of phenolic acids than EP. Meanwhile, NEP showed higher anti-oxidant capacity than EP (Fig.2). EP fraction in fruits have been extensively studied, such as their anti-oxidant, anti-inflammatory, anti-cancer, and antibacterial effects. However, reports about NEP have been scarce. NEP could bind to fiber and they may reach colon intact [15], so that the biological effects of NEP have been considered important for colon health. Our results indicated that both EP and NEP from cranberry had various biological effects. Importantly, NEP showed the promising anti-inflammatory and anti-cancer capacities. The high bioavailability of NEP in the colon facilitate the realization of these activities in the colnic tissues. Moreover, the potential interaction between gut microbiota and NEP could also play an important role in colon health. NEP could be biotransformed by gut microbiota to produce bioactive metabolites that may contribute to the promotion of colon health as well [197]. Therefore, it is important to investigate the interaction of gut microbiota and NEP in the colon in terms of production of bioactive metabolites. Meanwhile, composition and functions of gut microbiota can be altered by the presence of polyphenols in the colon, which in turn could affect colon health [198]. Unfortunately, the mode of interplay between NEP and gut microbiota in the colon is largely unknown, and much more efforts are needed to explore this promising research area. The knowledge obtained from our study is helpful for a better understanding of polyphenols in berries, especially the potential biological effects of NEP in the colon. Overall, this study provided the first line of evidence to support the notion that NEP could be a

promising beneficial agent for colon health. Certainly, further investigation is warranted to elucidate the detailed model of actions of NEP in the colon, especially in relationship with gut microbiota.

CHAPTER 4

NON-EXTRACTABLE PHENOLICS FROM STRAWBERRY: ANTI-INFLAMMATION AND ANTICANCER POTENTIALS

4.1. Introduction

Strawberry is an important source of polyphenolic compounds, especially phenolic acids such as ferulic, *p*-coumaric, vanillic, caffeic, syringic, and sinapic acids and flavonoids [199]. Thus, strawberry has been popularly produced and consumed because of both as its delicious taste and health beneficial properties. Phenolics are important contributors to the diverse health benefits of strawberry including anti-inflammation [200], anti-cancer [201], antioxidants and anti-diabetes [202]. In nature, phenolic compounds generally exist in the free and bound forms in plant cells. The free phenolic compounds are solvent extractable phenolics (EP). In contrast, the non-extractable phenolic compounds (NEP), which are covalently bound to the plant matrix, cannot be extracted into water or aqueous/organic solvents mixtures [203]. NEPs are high molecular weight polymeric polyphenols or individual low molecular weight phenolics mainly cross-linking with macromolecules, such as polysaccharides, dietary fiber and proteins. They can be retained in the food matrix inaccessible to solvents due to different interactions with the plant matrix [204]. It is noteworthy that most of phenolics in strawberry are NEP [15]. The total phenolic compounds with anti-inflammation [200] and anti-cancer [201] property of strawberry have been reported, which lots of bibliographic information mainly involves the biological activities of EP present in the berries [205].

Scarce information of NEPs on potential biological activities such as anti-inflammatory and anti-cancer are available [206, 207]. Although NEP is not available for extraction and absorption, they can be released under the low pH conditions of the gastrointestinal tract and upon colonic fermentation [208, 209]. Upon release, they can be absorbed in the gut lumen and exert a biological effect. Therefore, characterization of NEP and their anti-inflammation and anti-cancer activity is of paramount importance to the understanding of the health benefits of strawberries.

Colorectal cancer is the third most common cancer diagnosed in both men and women, and is one of the leading causes of death in the United States and worldwide [210]. Inflammatory bowel diseases play a key role in colon carcinogenesis. Consumption of fruits, vegetables and their bioactive constituents has been recommended to prevent colon cancer development. The health benefits of fruits and vegetables on reducing cancer risk are at least in part associated with the presence of unique polyphenolic compounds.

In this study, we firstly aimed to determine the potential anti-inflammatory effects of NEP in LPS-stimulated RAW 264.7 cells, and the underlying mechanisms of action. Secondly, we sought to investigate the inhibitory activity of NEP in HCT-116 colon cancer cells, and its effects on cell cycle progression and apoptosis.

4.2. Material and methods

4.2.1 Materials and Chemicals

Strawberry powder was obtained from Ocean Spray, Inc. (Lakeville, MA, USA). Standards of phenolics such as ferulic acid, protocatechuic acid, chlorogenic acid, caffeic acid, benzoic acid,myricetrin, astragalin, myricetin and p-coumaric acid were purchased from Quality Phytochmicals, LLC (Ease Brunswick, NJ, USA). Antibody for β-actin, HO-1, iNOS and c-FOS were purchased from Cell Signaling Technology (Beverly, MA, USA). 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT), propidium iodine (PI) and were purchased from Sigma-Aldrich (Natick, MA, USA). Annexing V/PI double staining was obtained from Bio vision (Mountain View, CA, USA). All chemical reagents used were of analytical grade.

4.2.2 Extraction of NEP

An ultrasonic-assisted extraction procedure was used for NEP [211]. Lyophilized strawberry powder (100 g) was mixed with 700 mL of 70% (v/v) acetone and then placed in an ultrasonic bath (300 Ultrasonik, Whittemore Enterprises, Inc., RanchoCucamonga, CA, USA) and sonicated at the maximum power for 30 min under refluxing conditions. After centrifugation of the resulting slurry for 5 min at 4000g (IEC Centra MP4, International Equipment Co., Needham Heights, MA, USA), the supernatant was

removed. Afterwards, the residues were subjected to extract NEP immediately [199]. Residues were hydrolyzed with 2M sodium hydroxide (NaOH) at 37 ºC for 2 h with stirring under nitrogen to protect phenolics from oxidation. The resulting slurry was acidified to pH 2 with 6M HCl and extracted three times with diethyl ether and ethyl acetate (1:1, v/v). Subsequently, supernatants were evaporated in vacuo at 4 ºC (Buchi, Flawil, Switzerland), lyophilized for 72 h at -46 ºC (Freezone, model 77530, Labconco Co., Kansas City, MO, USA) and stored at -20 ºC until further analysis. During all stages, extracts were protected from light by covering them with aluminum foil.

4.2.3 Determination of total phenolic content (TPC), flavonoid content (FC), tannin content (TC)

TPC of strawberry extracts were determined using the Folin-Ciocalteu assay with some modifications [212]. Briefly, sample was dissolved in 50% methanol to final concentration 0.4 mg/mL. 20 µL of Folin-Ciocalteu reagents were added in 96-well plate containing 20 µL of sample and 20 µL of distilled water. After incubation at room temperature for 10 min, 140 µL of 7% sodium carbonate was added. The reaction was incubated at room temperature for 90 min, followed by absorbance measurement at 760 nm using a spectrophotometer (BioTek Instrument, Inc. Winooski, VT, USA). Gallic acid was used as a standard, and TPC were expressed in milligram gallic acid equivalent (mg GAE/100g strawberry powder).

FC were determined by the aluminum colorimetric method and the catechin was used as standard [212]. 20 μL of 0.4 mg/mL sample was mixed with 100 μL distilled water and 10 μL of 5% sodium nitrite in 96-well plate. After 6-min incubation at room temperature, 20 μL of 10% aluminum chloride was added in the mixture and allowed to react for 5 min. Subsequently, 50 μL of 1 M NaOH was added in the mixture. After incubation at room temperature for 2 min, the absorbance was read at 510 nm using a spectrophotometer (BioTek Instrument, Inc.). The results are expressed as milligram of catechin equivalents per /100g strawberry powder (mg CE/100g powder).

TC was tested according to the method described by Jackson with slight modifications [213]. Vanillin- H2SO4 solution was prepared by the ratio of 4% vanillin in methanol to 30% H2SO4 in methanol of 1:1. 180 μL of vanillin-H2SO4 solution was mixed with 20 μL of 0.4 mg/mL sample in 96-well plate. The absorbance was measured at 510 nm using a spectrophotometer (BioTek Instrument, Inc.), and results are expressed as milligram of catechin equivalents (mg CE/100g strawberry powder).

4.2.4 Phenolics composition analysis

Identification and quantification of flavonols, flavanols and phenolic acids were carried out by LC-MS as described preciously with some modification [214]. Separation of flavonols, flavan 3-ols and phenolic acids were achieved on Kinetex XB-C18 column

(100 mm ×4.6 mm, 2.6 μm, Phenomenex, Torrance, CA, USA). The mobile phase consisted of 0.1% formic acid in 95% water mixed with 5% acetonitrile (solvent A), and 0.1% formic acid in acetonitrile (solvent B). The elution program processed as follows: 5% B for 2 min, changed to 95% in 35 min, raised up to100% in 5 min, decreased to 5% B in 6min, kept 5% B in 6 min, the overall analysis time was 52 min. The flow rate was 0.4 mL/min, and injection volume was 10 μL. ECG, myricetrin, astragalin, myricetin, p-coumaric acid, ferulic acid, protocatechuic acid, chlorogenic acid, caffeic acid and benzoic acid were used as external standards, and limonin was used as internal standard.

4.2.5 Cell viability, colony formation, cell cycle and apoptosis

Cell viability, colony formation, cell cycle and cellular apoptosis were performed as our previously published method [215-218]. In brief, HCT-116 (2500 cells/well) and macrophages (AML-12, CCD-18, 12500 cells/well) were seeded in 96-well plates, respectively. Serum-starved cells were treated with serial concentrations of NEP for 24 h, then cell viability was determined by MTT method [216]. For colony formation analysis, HCT-116 was plated in 6-well plate at a density of 400 cells/well. After 24 hours, cells were treated with serial concentration of NEP. The medium in 6-well plate was refreshed every 2 days. After incubation for 14 days, the determination of colony formation was conducted as previously reported [217].

For cell cycle analysis, HCT-116 was seeded in 6-well plates. After 24 hours of incubation, cells were treated with 20μg GAE/mL NEP. Cells were incubated for 24h and

subjected to analyze using flow cytometry with propidium iodide (PI) staining [218]. For apoptosis analysis, HCT-116 was treated with serial concentrations of NEP after seeded in 6-well plates. Then cells were incubated for 48 h and collected for analysis by flow cytometry [215].

4.2.6 Nitric Oxide (NO) assay

LPS-induced NO production was conducted according to the method of used in our previous publication [219]. After 24 hours of RAW 264.7 cells seeding in 96 well plate (at the concentration of 50 ×104 cells/mL with 200 µL in each well), cells were treated with LPS (1µg/mL) together with testing compound for 24 hours followed by relatively measurement of nitrite concentration which is an indicator and a more stable product of NO. Briefly, 150µL of cell culture medium was mixed with 100µL of cold Griess reagent (2% sulfanilamide and 0.2% N-(1-naphthyl) ethylenediamine dihydrochloride in phosphoric acid), the mixture was incubated at room temperature for 30 minutes, and the absorbance at 540 nm was measured in a microplate reader. Cell-free solution was used as a blank in every experiment. Percentage of nitric oxide inhibition was then relatively determined.

4.2.7 Immunoblotting

Whole cell lysate from macrophages was extracted and analyzed as previously

reported [217]. Briefly, RAW 264.7 cells were seeded in 10-cm culture dishes and incubated for 24 h to attach on dishes. Then, the fresh medium containing 1mg/mL LPS was replaced with or without serial concentrations of NEP. After 24 h, cell lysate was collected and subjected to western blot analysis as previously mentioned.

4.2.8 Data Analysis

All results were presented as mean ±SD for the indicated number of independently performed experiments. Student's t-test and analysis of variance (ANOVA) were used to compare the differences among two or more groups, respectively. $p< 0.05$ was considered statistically significantly difference.

Table 4. Phenolic profiles of NEP of strawberry fractions identified by HPLC-MS.

	Retention Time(min)	$M^{\pm}$ (*m/z*)	NEP (mg/g extract)
flavonols and flavanols			
ECG	9.98	443^{+}	0.310 ± 0.120
EGCG	9.30	459^{+}	0.140 ± 0.110
catechin	22.25	290^{+}	0.087 ± 0.008
myricetrin	9.60	463^{+}	0.074 ± 0.020
astragalin	10.10	447^{+}	3.500 ± 0.190
myricetin	11.50	319^{+}	0.077 ± 0.013
phenolic acids			
p-coumaric acid	27.02	165^{+}	23.560 ± 4.40
ferulic acid	27.30	195^{+}	1.600 ± 0.420
protocatechuic acid	21.20	153^{-}	0.890 ± 0.180
caffeic acid	24.30	181^{+}	5.100 ± 0.610
benzoic acid	7.16	123^{+}	0.840 ± 0.250

MS analysis was performed in positive and negative mode. Results are indicated as mean ± SD.

4.3 Results and discussion

4.3.1 Phenolic profiles of NEP

Regarding linkage and polymerization of NEP with protein and fiber, acidolytic method may partially release NEP. Thus, alkaline hydrolysis was conducted to release NEP by depolymerization and solubilization of cell walls [220, 221]. Figure 8 shows the total phenolic (TPC), flavonoids (FC) and tannin content (TC) of NEP fraction from 100 gram lyophilize powder. TPC of NEP fraction was 295.04±5.28 mg GAE/100g powder, FC and TC of NEP fraction was 59.28±1.62 mg CE/100g and 52.66±0.49 mg CE/100g, respectively. Phenolic acids, flavonols and flavanols of NEP were detected using accurate mass and retention data (Table 4). Six compounds of flavonols and flavanols in NEP were identified as ECG, EGCG, catechin, myricetrin, astragalin and myricetin. Moreover, five phenolic acids were identified as p-coumaric acid, ferulic acid, protocatechuic acid, caffeic acid and benzoic acid. NEP contained high levels of p-coumaric acid (23.56 ±4.4 mg/g extract), followed by caffeic acid (5.10 ±0.61 mg/g extract) and astragalin (3.50 ±0.19 mg/g extract). The phenolic profiles of NEP in the present study were partly in accordance with strawberry extractable phenolics in previous report by Aaby [222] due to ripening and growing conditions of strawberry cultivar, low stability and degradation of phenolics. Caffeic and coumaric acids have potential effects against lung and colon cancer development at adhesion and migration steps of tumor progression [223]. Because

NEP can be absorbed in the gut lumen and exert a biological effect upon their release by microbiota [224], NEP play an essential role in delivering phenolics to the colon and exhibiting biological activity. Thus, we further examined the anti-inflammation and anti-cancer efficacy of NEP.

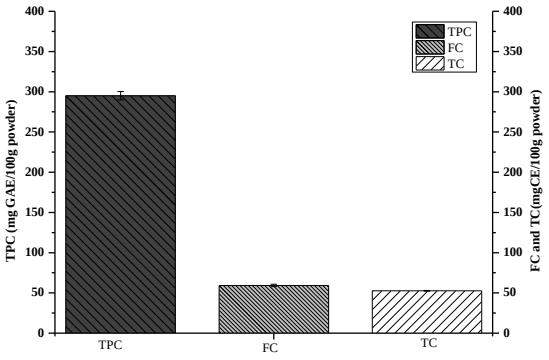

Figure. 8 TPC, flavonoids (FC) and tannin content (TC) in strawberry non-extractable phenolic fractions (NEP) from 100 gram lyophilized powder. TPC was expressed as means ± SD (n=3) of gallic acid equivalent (GAE). FC and TC were expressed as means ± SD (n=3) of catechin equivalent (CE).

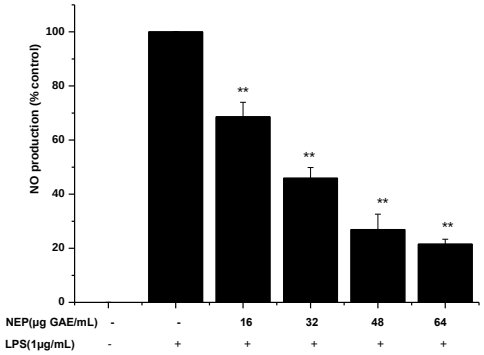

Figure. 9 Concentration-dependent inhibitory effect of NEP on LPS-induced NO production of RAW 264.7 cells. RAW 264.7 cells were exposed to NEP (16, 32, 48 and 64 μg GAE/mL) with LPS (1.0 μg/mL) treatment. After 24 h of incubation, the amount of NO in the culture supernatants was measured by Griess reaction assay. Values are expressed as mean ± SD (n=6). Comparisons of means were made using one-way

ANOVA followed by Student's t- test. ** $p < 0.01$ indicated significant differences in comparison to the LPS-treated group.

4.3.2 Inhibition of LPS-induced NO production by NEP

LPS-stimulated inflammatory models have been widely used to study the anti-inflammatory activity of natural phenolics [225]. LPS can result in the release of pro-inflammatory cytokines and activate nitric oxide (NO) cascades [200]. NO is an important fundamental component as a regulator and an effector molecule in many physiological and pathophysiological process. Using non-toxic dose range established, the effect of NEP on NO production induced by LPS (1µg/mL) in macrophages was examined. The untreated group was taken as control in RAW 264.7 macrophages. NEP treatment showed a notable reduction in the level of NO compared to control. As shown in Figure 9, NEP had dose-dependent inhibition on NO production. NO production was decreased to 68.65±5.33, 45.93±3.91, 26.95±5.68, and 21.55±1.81% at a dose range of 16-64 µgGAE/mL. The result showed that NEP exhibited the efficacy against LPS-induced damage in macrophages model.

4.3.3 Immunoblotting analysis

To investigate the molecular mechanism by which NEP inhibited inflammatory response in macrophages, we further determined the effects of NEP on the protein levels of iNOS, HO-1 and c-FOS by immunoblotting analysis. As shown in Figure 10, LPS

increased protein expression of iNOS, HO-1, and c-FOS. The protein expression of iNOS was decreased 11.8-fold by NEP treatment at 40 μg GAE/mL as compared to the LPS-treated positive control group. HO-1 is an anti-oxidative enzyme that is associated with anti-inflammatory effects [219]. Treatments with NEP at 40 μg GAE/mL significantly increased the protein levels of HO-1 by 3.03-fold compared to the LPS-treated positive control cells. AP-1 is a critical transcription factor involved in the pro-inflammatory responses [226]. The effects of NEP on the levels of c-Fos, which is the key components of AP-1 protein complex, were investigated. LPS treatment did decrease the level of c-Fos. These findings suggested that NEP might play a key role in preventing inflammation as a promising strategy by whole strawberry dietary intake.

4.3.4 Anti-cancer effects of NEP in HCT-116 colon cancer cells

To investigate anti-cancer effect of NEP, nontoxic dose range of NEP in alpha mouse liver 12 (AML-12) cells and normal colon cells (CCD-18) were firstly established. The cell viability was evaluated by MTT assay. NEP did not show significantly cellular toxicity at the concentrations from 0 to 60μg GAE/mL (Figure 11A). However, at the same concentration range, NEP dose-dependently inhibited HCT-116 cell growth, demonstrating a selective effect on cancer cells. As shown in Figure 4B, 60μg GAE/mL NEP caused 10-fold lower cell viability (10.10±2.67%) than untreated HCT-116 (control, 100±3.57%).

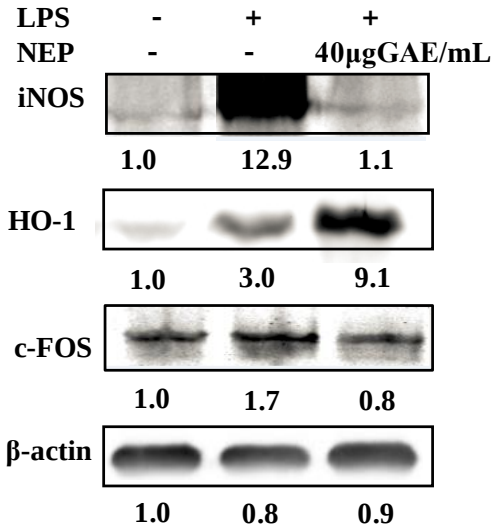

Figure. 10 Inhibitory effects of 40μg GAE/mL NEP treatment on LPS-induced protein expression of iNOS, c-FOS and HO-1 in RAW 264.7 macrophages. β-actin was served as an equal loading control.

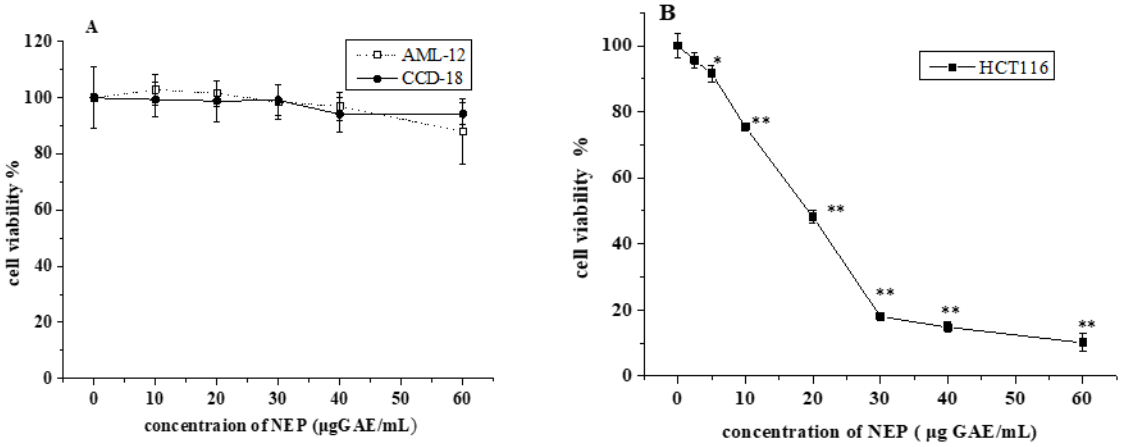

Figure. 11 (A): cell viability of alpha mouse liver 12 (AML-12) and normal colon cell (CCD-18) as normal cells treated with NEP. (B): concentration-dependent decrease in cell viability of HCT-116 colon cancer cells. Cells were treated with or without NEP under various concentrations for 24 h. Relative cell viability was assessed by MTT assay. The results were represented as percentage (mean± SD) of living cells compared to control. Comparisons of means were made using a one-way ANOVA followed by Student's t-test (n =6). *p<0.05, **p<0.01indicated the significant differences in comparison to the control group.

4.3.5 NEP suppresses colony formation of HCT-116 cells

A strong association of inflammation with colon cancer has been well recognized [227]. Investing the anti-cancer potential of NEP is of great importance for better understanding the bioactivity of strawberry. To examine long-term suppressive effects of NEP treatment against the survival of HCT116 colon cancer cells, colony formation assay was performed. HCT116 colon cancer cells were exposed to a serial concentration of NEP for 14 days, using cells without NEP treatment as a control. As shown in Figure 12 A and B, NEP showed dose-dependent inhibitory effects on the HCT-116 colony formation. The concentration of 1.25, 2.5, 5 and 10 μg GAE/mL reduced colony formation to 88.28±4.16, 79.69±6.08, 66.41±4.73 and 45.70±4.00, respectively in comparison to the control. Hence, NEP did cause long-term loss of survival of colon cancer cells [216]. The results were consistent with the cell viability that we obtained from the MTT assay. The results verified that NEP also poses significant anti-proliferation effects of colon cancer, not merely the EP as mostly previous reports [228-230].

4.3.6 NEP induced cell cycle arrest in G2/M phase in HCT-116colon cancer cells

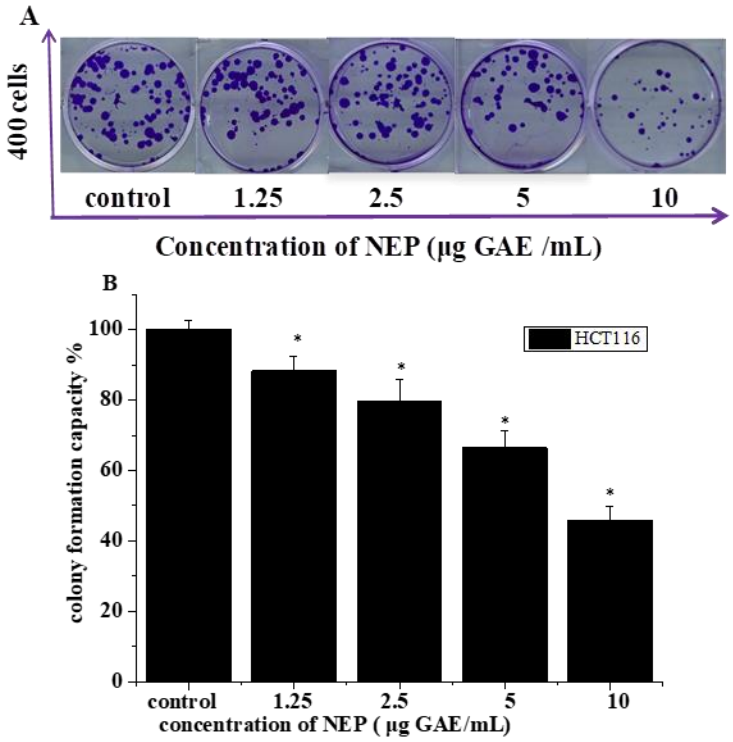

Figure. 12 Inhibitory effect of NEP on colony formation of HCT-116 human colon cancer cells. Cells were seeded 400 cells/well in six-well plates. After 24 h of incubation, cells were treated with serial concentrations of NEP. After 14 days of treatments, cells were dyed and scanned (A). The number of colonies formed was counted as described in the methods. Data represent means± SD, n=3 (B) all the treated groups showed significant difference in comparison to the control (*, $p<0.05$).

Since many dietary phytochemicals have potential to inhibit carcinogenic process by interfering with one or multiple cellular pathways and play an important role in cancer chemoprevention [216]. To further understand the mechanism by which NEP inhibited colon cancer cell proliferation, using the concentration range lower than 60 µg GAE/mL, cell cycle distribution and cellular apoptosis were detected by flow cytometry. As shown in Figure 13, the cell population in G2/M phase of HCT-116 cells treated with NEP at 20µg GAE/mL for 24 hours was significantly increased by 10% as compared to the

control cells, while the percentage of G1 phase cell population was 6% decreased and S phase was not changed in the present study (Figure 13A). It suggests DNA degradation by leading to cell death (Figure 13B). These results revealed that NEP could interrupt the cell cycle progression of colon cancer cells at the G2/M phase.

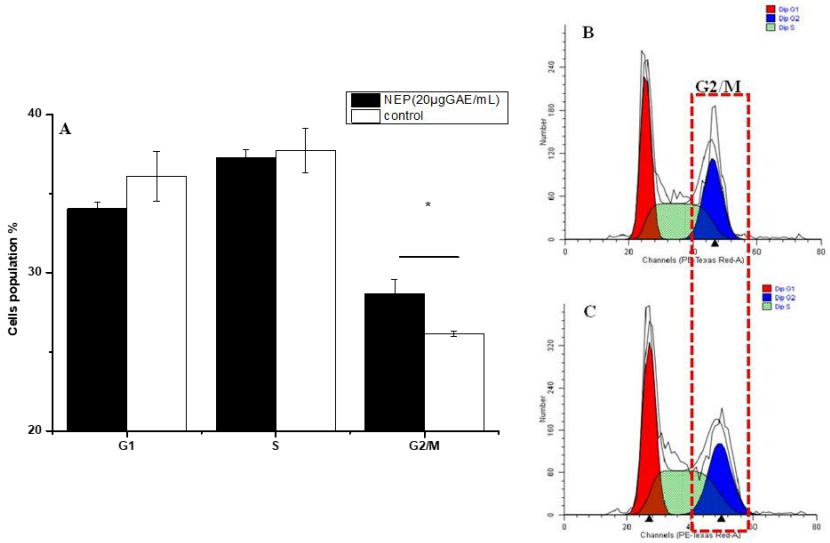

Figure. 13 NEP induced G2/M cell cycle arrest in colon cancer cell line (HCT116) after 24 hours exposure. (A): Percentage of cell population with and without 20 µg GAE/mL NEP treatment. DMSO was used in the negative control. Data represent means ± SD (n=3). *p <0.05 compared to the control. (B) and (C): PI-stained cells after NEP 20 µg GAE/mL treatment and DMSO, respectively, was analyzed by flow-cytometry. The percentage of cells in G0/G1, S and G2/M phase was calculated using Multicycle software.

4.3.7 NEP induced cellular apoptosis in HCT-116 cells

Next, we sought to determine the effect of NEP on cellular apoptosis in HCT116 cells. Early and late apoptotic cells were quantified by Annexin-V/PI double staining in HCT-116 after 48 h treatment as we described previously [216]. Early apoptotic cells

(FITC-positive), viable cells (FITC-negative and PI-negative), late apoptotic and necrotic cells (PI-positive and FITC-positive) were monitored using flow cytometry. As shown in Figure 14A-14F, NEP at the concentration range of 10-40 µg GAE/mL did significantly induce cellular apoptosis in HCT-116 cells. Compared to the control (1.17%), NEP at 10, 20, 30, 40µg GAE/mL markedly increased late apoptotic cells by 1.74- (from 1.17% to 2.03%), 5.56- (from 1.17% to 6.50%), 10.06- (from 1.17% to 11.77%) and 48.23-fold (from 1.17% to 56.43), respectively. These results showed that NEP induced extensive cellular apoptosis of HCT116 cells, which would be an effective approach to control colon cancer progression.

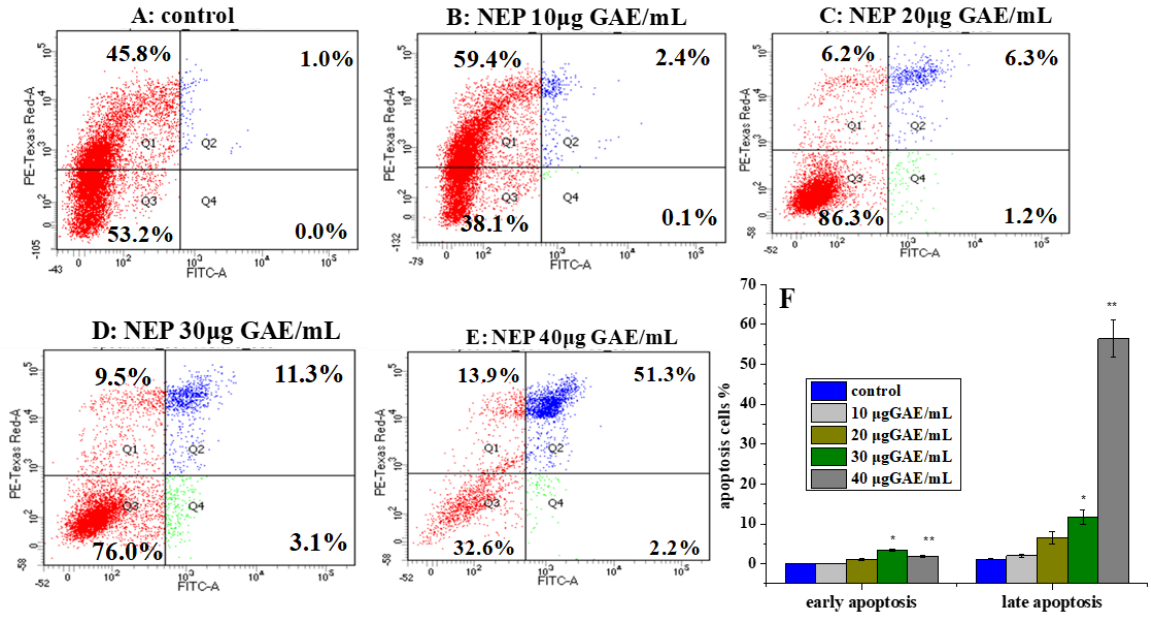

Figure. 14 Effect of NEP on HCT-116 cells apoptosis. (A-E), cells were treated with DMSO, 10, 20, 30, 40 µg GAE/mL NEP for 24 hours and subjected to flow cytometric analysis after PI and Annexin V staining. (F): percentage of cell population at different stages. All the treated groups showed significant difference in comparison to the controls (*, $p<0.05$; **, $p<0.01$).

4.4 Discussions

Phenolic compounds of strawberry have been extensively studied due to their diverse health benefits as preventing chronic inflammation and cancer [231]. These biological effects are closely associated with their non-nutritional phenolic constitutes. In nature, phenolic compounds generally occur in two forms: one form is soluble extractable phenolics (EP), another form is insoluble bound phonics (NEP). Lots of literatures have demonstrated a wide range of biological activities of EP from millet, strawberry and mulberry [211, 231-234]. The bioactivities of NEP from strawberry and other fruits were scared [235]. Since NEP bound tightly with protein and fiber through hydrogen bonding and hydrophobic interactions [236], acid, alkali and enzymatic hydrolysis methods have been used to release NEP [209]. Thus the biological effects have been underestimated. In this study, we used alkaline hydrolysis to release NEP from cell wall matrix of strawberry powder. We found that NEP of strawberry mainly contained ECG, EGCG, catechin, myricetrin, astragalin, myricetin, p-coumaric acid, ferulic acid, protocatechuic acid, caffeic acid and benzoic acid. The phenolics constitutes were distinctly different from EP, which contained plentiful anthocyanins (Table 4). The result was consistent with reports described constituents of strawberry [237].

It has been extensively elucidated that various soluble extractable phenolics (EP) inhibited to the growth of cancer cells by inducing cell cycle arrest and apoptosis, such as

EP from blackcurrant [238], blueberry and raspberry [239], purple and red corn [229]. It is well known that strawberry EP contribute greatly to the suppression of inflammation and colon cancer by strawberry [233], while the effects of NEP were unclear. NEP can be released by gut microbiota and subsequently well absorbed in vivo [240]. This prompted us to explore whether NEP exhibits the similar anti-inflammation and anti-cancer effect as EP.

NEP was extracted from strawberry by alkaline hydrolysis in this study. Our result indicated that NEP at up to 60 μg GAE/mL showed significant inhibitory effect on HCT-116 cells (Figure 11). Colony formation capability of colon cancer cells was also markedly suppressed by NEP at 10 μg GAE/mL (Figure 12). Induction of cell cycle arrest and cellular apoptosis in cancer cell is one of the effective strategies to control cancer progression. Mechanistic investigation suggested that strawberry NEP was effective in inducing colon cancer cellular apoptosis (Figure 14) and cell cycle arrest in G2/M phase (Figure 13) and thus suppressed cell proliferation of HCT116 cells (Figure 13). Our results at least partially enlarged the potent inhibitory activity of NEP on colon cancer. Further studies are planned to carry out on more cancer cells.

Apoptosis is recognized to be activate mainly through the extrinsic death receptor pathway and the intrinsic mitochondrial pathway [241]. The intrinsic mitochondrial pathway is regulated by various intracellular signals, including the mitochondrial response. The release of mitochondrial proteins, such as cytochrome C, can activate the

caspase cascade that triggers cellular apoptosis [242]. Mitochondria have an key role in pro-inflammatory signaling; in turn, pro-inflammatory mediators may also alter mitochondrial function [243], Low grade and chronic inflammatory conditions can increase the risk of malignancy from epidemiological studies and molecular studies. Chronic inflammation exhibits tumor-promoting effects by various mechanisms, including enhancing proliferation of malignant cells and promoting tumor angiogenesis [219]. NO production not only is associated with the cell apoptosis, but also is an important signaling molecule involved in regulating inflammatory response [244]. The inflammatory response is characterized by coordinating activation of both pro- and anti-inflammatory mediators [245]. LPS is the leading stimulus that initiates the macrophages host response to release pro-inflammatory cytokines [246] and in turn activate a second level of inflammatory cascades including cytokines, lipid mediators and adhesion molecules such as NO [200]. Accumulating evidence suggests that phytochemistry plays a crucial role in suppressing inflammatory response [155]. In present study, NEP treatment can produce a significant suppression on NO production (Figure 9) and iNOS expression in RAW264.7 macrophage cells. The results is in line with the reports previously, in which the expression of inflammation marker iNOS was improved by strawberry EP [200]. It demonstrated that the strong association between NO production and iNOS expression.

Recently, several phytochemistry studies have demonstrated that HO-1 plays a regulatory role in the inflammatory response by inhibiting production of pro-inflammatory cytokines [200, 219]. HO-1 has been implicated in the maintenance of

cellular homeostasis and inhibition of the inflammatory response [246]. Herein, we observed that NEP induced the increase HO-1 in the RAW 246.7 cells, which also contributed to the anti-inflammatory effect.

In conclusion, our findings suggested that NEP exerted powerful preventive ability against LPS-induced inflammation in macrophages, and inhibited the growth and proliferation of colon cancer cells via inducing G2/M phase cell cycle arrest and apoptosis. This information will provide important guidance for the chemoprevention strategies of using strawberry as nutrient fruit through dietary administration.

CHAPTER 5

DIETARY INTAKE OF WHOLE STRAWBERRY INHIBITED COLONIC INFLAMMATION IN DEXTRAN SULFATE DODIUM-TREATED MICE VIA RESTORING IMMUNE HOMEOSTASIS AND ALLEVIATING GUT MICROBIOTA DYSBIOSIS

5.1 Introduction

Inflammatory bowel disease (IBD) has posed serious threats to the human health [5], which lead to chronic malabsorption of nutrients, abnormal pain and rectal bleeding. Both genetic predisposition and environmental factors contribute to the onset of IBD [5, 6]. Pathological immune response against exotic antigen and bacterial drives the progression of IBD [247]. Multiple studies suggested that dysbiosis in colon plays an important role in the development of colitis, and gut microbiota composition are different between patients with IBD and healthy individuals [8]. The incidence of colonic inflammation has also been associated with dietary patterns through the modification of gut microbiota [248]. The diet with high fat, high sugar, high protein and low fiber has been proposed to increase the risk of IBD. In contrast, dietary consumption of fruits and vegetables has been associated with lowered risk of IBD [10].

Accumulating evidence suggests that colonic inflammation may be prevented by

increased consumption of certain fruits and vegetables such as berries [10, 249]. Berries (e.g. chokeberry, red raspberry, strawberry, cowberry) and their extracts have been shown to have anti-inflammatory and anticancer effects in intestine in various animal models such as AOM-induced rat colon cancer model, Apc 1638+/- mouse and Muc2-/- mouse model, and DSS-induced colitis models [12-14]. Strawberry is one of the most popular berry fruits, which is abundant in polyphenols that have been associated with anti-oxidation, anti-inflammation and anti-cancer bioactivities. Strawberry juice exhibited anti-inflammatory effects in LPS-treated macrophage by decreasing the production of pro-inflammatory cytokines [23], and inhibited the cell proliferation of human colon cancer cell lines [17]. A human study showed that consumption of strawberry could increase the serum antioxidant capacity [24]. The consumption of strawberry beverage was associated with the amelioration of postprandial inflammatory response [25]. These evidences suggested the promising bioactivity of berry fruits against the colonic inflammation. Whole strawberry contains vitamins, flavonoids, phenolic acids, anthocyanins, polysaccharides and fiber [17], which may exhibit stronger anti-inflammatory effects when compared to the extracts. However, there is no detailed mechanistic information available on the potential protective effects of whole strawberry against IBD. Therefore, in this study, for the first time we determined anti-inflammatory effect of whole strawberry in DSS-induced colitis in mice and elucidated the potential mechanisms involved.

5.2 Materials and methods

5.2.1 Animals, diet and experiment design

Male CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). All animal use was in compliance with regulations of Institutional Animal Care and Use Committee of University of Massachusetts Amherst. The mice were randomly divided into six groups (n=10) after one-week acclimation. Three groups were treated with 1.5% (w/v) DSS in drinking water for 4 cycles (4 days/cycle, with 7-day recovery after each of the first three DSS cycles) to induce colitis. These three colitic groups were treated with AIN93G diet containing 0%, 2.5% or 5% (w/w) free-dried whole strawberry powder, respectively, throughout the entire experiment. The other three noncolitic groups were treated with regular drinking water plus AIN93G diet containing 0%, 2.5% or 5% (w/w) WS, respectively, throughout the entire experiment.

5.2.2 LC-MS analyses of polyphenols in whole strawberry powder

Polyphenols in whole strawberry powder were extracted with 80% acetone with 0.1% acetic acid for three times. Each time, the extraction mixture was sonicated for 30 min at room temperature, and then the supernatant was collected by centrifuging at 3,500 g for 5 min. The pooled supernatant was dried under vacuum and then re-dissolved in 50% methanol aqueous before analysis by LC-MS. Identification and quantification of

polyphenols in strawberry were conducted using by LC-MS. Chromatographic separation of polyphenols was carried out on Kinetex XB-C18 column (100 mm ×4.6 mm, 2.6 μm, Phenomenex, Torrance, CA, USA). The mobile phase consisted of 0.1% formic acid in 95% water mixed with 5% CAN (solvent A), and 0.1% formic acid in ACN (solvent B). The elution program was as follows: 0-2 min, isocratic elution with 5% B; 2-37 min, 5-95% B; 37-40 min, 95-100% B; 40-46 min, 100-5% B; 46-52 min, isocratic elution with 5% B. The flow rate was 0.4 mL/min, and injection volume was 10 μL. Pelargonidin 3-glucoside, pelargonidin 3-rutinoside, proanthocyanidins B1, catechin, myricetin, kaempferol were used as external standards, and pinostilbene was used as an internal standard. All chemicals were from Sigma Aldrich.

5.2.3 Disease activity index (DAI), histological and immunohistochemistry evaluation

DAI was evaluated by the scores of bodies weight loss (the weight before each DSS treatment compared to that after the DSS treatment, scored as: 0, no change; 1, 1-5% loss; 2, 5-10% loss; 3, 10-20% loss), stool consistency (0, normal; 1, soft but firm; 2, soft; 3, diarrhea), and fecal blood (0, none; 1-2, blood; 3, gross bleeding) [250]. Once the mice were sacrificed, colons were harvested and processed for histological analysis as we previously described [176, 251, 252]. The score system was as follows: epithelium: 0, normal; 1, loss of goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts; 4, loss of crypts in large areas. Infiltration: 0, none; 1, infiltrate around crypt basis; 2,

infiltrate reaching to mucosae; 3: extensive infiltration to mucosae; 4: infiltration of submucosa [253]. Immunohistochemistry analysis of the colon was performed as we previously described [251, 254, 255]. Processed tissue sections were treated with a primary antibody against inducible nitric oxide synthase (iNOS) and visualized after the treatment with a biotin-conjugated secondary antibody (Dako, Santa Clara, CA, USA).

5.2.4 Western blot and enzyme-linked immunosorbent analysis (ELISA)

Colonic mucosa was scrapped and lysed in RIPA buffer containing a protease inhibitor cocktail, and phosphotase inhibitor. Contents of protein were determined by BCA kit. Samples were subjected to SDS-PAGE, and proteins were transferred to nitrocellulose membranes. Membranes were then processed and visualized by Odyssey CLx system (LI-COR Biosciences, Lincoln, NE, USA).

Colonic mucosa was scrapped and homogenized in MSD Tris Lysis buffer (Rockville, Maryland, USA) containing protease inhibitor cocktail and phosphotase inhibitor. The protein content was quantified by BCA kit, and then cytokines were quantified by V-PLEX ELISA kit (Meso Scale Discovery, Rockville, Maryland, USA) following the manufacture's procedure.

5.2.5 Quantification of immune cells in the colonic mucosa and GSH/GSSG in the liver

Distal colons were lysed with Hanks's balanced salt solution (1mM DTT and 5

mM EDTA) for 2 h at 4 ℃ after washing with cold PBS. Seventy μm sorters (BD Biosciences, San Jose, CS, USA) were used to obtain single-cell suspensions, followed by the staining with APC-conjugated CD11c antibody, FITC-conjugated CD45 antibody, PE/Cy7-conjugated Ly-6G/Ly-6C(Gr-1) antibody, PerCP/Cy5.5-conjugated F4/80 antibody, and isotype control antibody (Biolegend, San Diego, CA, USA). BD LSRFortessa cell analyzer (BD Biosciences, San Jose, CA, USA) was used to quantify the stained cells, and data were analyzed by FlowJo software.

Livers were rinsed in cold PBS before homogenization with PBS (1 mM EDTA), followed by centrifugation at 10,000 g for 15 min at 4 ℃. Supernatants were deproteinated with MPA reagent (Cayman Chemical, Ann Arbor, MI, USA). GSH and GSSG were quantified by a glutathione assay kit (Cayman Chemical, Ann Arbor, MI, USA).

5.2.6 Quantification of SCFAs by gas chromatography

SCFAs were extracted from cecal digesta with 0.05% phosphoric acid, followed by the vortex and centrifugation for 20 min at 14,000 rpm at 4 ℃. Supernatants were analyzed by gas chromatography with a flame ionization detector (Shimadzu GC-QP2010 SE, Tokyo, Japan). Identification of SCFAs was achieved by comparing with the chemical standards (acetic acid, propionate acid, butyric acid, isovaleric acid, and valeric

acid (Sigma-Aldrich, St. Louis, MO, USA)), and 2-ethylbutyric acid was used as the internal reference.

5.2.7 Microbial analysis

Fecal pellets were store at -80 ℃ once being collected from mouse colon until analysis. Bacterial DNA was extracted from feces using PowerFecal DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions. The concentration of DNA was measured by NanoDrop Spectrophotometer (ThermoScientific, Waltham, MA, USA). The primer sequences used to amplify V3-V4 regions of 16S rRNA gene was:16S Amplicon PCR Forward Primer = 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 16S Amplicon PCR Reverse Primer = 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. The second PCR was conducted to attach dual indices and Illumina sequencing adapters using Nextera XT Index Kit (Illumina, San Diego, CA, USA). PCR products were quantified using Qubit dsDNA BR Assay kit (Life Technology, Carlsbad, CA, USA), and the size of PCR products was verified by DNA analysis ScreenTape Assay on Tape Station 2200 (Agilent Technologies, Santa Clara, CA, USA). PCR final products were pooled in equimolar concentration and diluted to 4nM and denatured by NaOH. After combining the amplicon library and PhiX control, the samples were loaded onto the

600-cycle MiSeq Reagent kit v3 cartridge and sequenced on an Illumina MiSeq platform (Illumina Inc, San Diego, CA, USA).

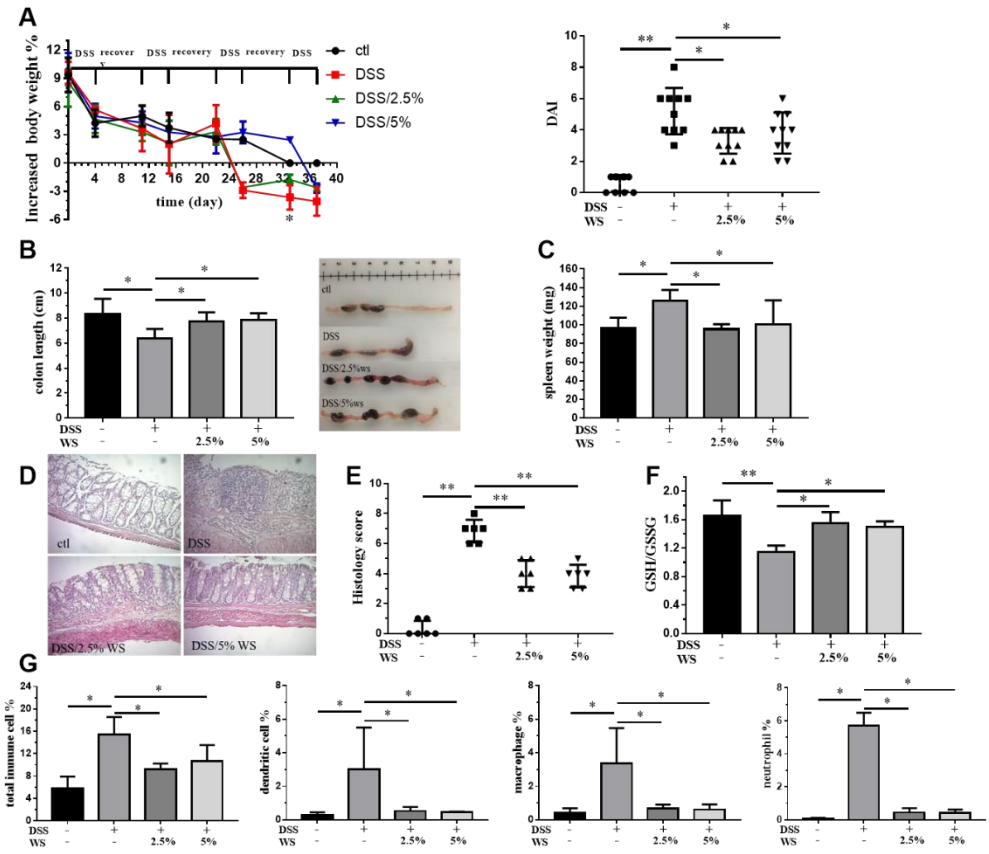

Figure. 15 Dietary WS ameliorated symptoms of DSS-induced colitis in mice. (A) Body weight change of mice, and disease activity index (DAI) (n=10 per group); (B) Colon length and representative images of the mouse colon (n=8-10 per group); (C) Spleen weights (n=8-10 per group); (D) Representative images of H&E staining of the colon tissues (magnification: 300×) (n=6 per group); (E) Histological scores of colonic abnormality (n=6 per group); (F) The ratio of GSH/GSSG in the liver (n=8-10 per group); (G) Quantification of infiltrated immune cells in the distal colonic mucosa (n=6-8 per group). Data present mean ± SD, and * indicates $p<0.05$, ** indicates $p<0.01$.

5.2.8 Data Analysis

Microbial data were analyzed by Quantitative insights into microbial ecology

(QIIME) software pipeline v1.9.1. Comparison of two or more groups was analyzed by either one-way parametric ANOVA followed by Tukey's post hoc test or one-way nonparametric ANOVA (Kruskal- Wallis test) followed by Dunn's post hoc test.

Table 5. Phenolic Compounds Identified by LC-MS in Whole Strawberry Powder (freeze-dried). Data represented mean ± standard deviation (N = 3).

compound	retention time (min)	MS(m/z)	μg/g dry powder
pelargonidin 3-glucoside	9.22	$433[M+H]^+$	1398.02 ± 38.13
pelargonidin 3-rutinoside	9.56	$580[M+H]^+$	568.49 ± 32.45
proanthocyanidins B2	5.92	$579[M+H]^+$	97.28 ± 11.48
catechin	8.29	$291[M+H]^+$	254.27 ± 18.30
kaempferol	21.028	$287[M+H]^+$	17.47 ± 0.70

5.3 Results and discussion

5.3.1 Dietary whole strawberry inhibited colitic symptoms

The doses of WS used in the experiment were 2.5% and 5% (w/w) in the mouse diet, which were equivalent to about 167 g (1.1 cup) and 334 g (2.2 cup) of fresh strawberry per day per person (60 kg), respectively. These doses are within the achievable range for humans. As shown in Figure 15A, during the experimental period, the body weight of the control mice kept increasing (at a decreasing rate) and reached plateau at the fourth DSS cycle. In contrast, DSS group (positive control) showed decreased body weight during the third DSS cycle and continued body weight loss during the third recovery period and the last DSS cycle. Dietary WS treatments slowed down the body weight loss of the DSS-treated mice, especially those fed with 5% WS. The DAI of the

DSS group was much higher than that of the control group, and WS at both 2.5% and 5.0% effectively decreased DAI (Fig. 15A). The shortening of the colon and increase of spleen weight are indicators of severity of colitis. Our results showed WS reversed the shortening of the colon and increase in spleen weight in colitic mice (Fig. 15B and C). These results demonstrated that dietary WS effectively inhibited the severity of colitis in DSS-treated mice. Strawberry is a rich source of bioactive components such as various polyphenols. Using LC-MS, we quantified the abundance of pelargonidin 3-glucoside, pelargonidin 3-rutinoside, proanthocyanidins B1, catechin, myricetin, and kaempferol in the whole strawberry powder. The results were shown in the Table 5. All of these components are potential contributors of the observed protective effects against colonic inflammation in mice. It is important to note that there might be synergistic interactions among different strawberry components, which might produce enhanced anti-inflammatory effects. The exact mechanism of actions of each component and their interactions are important focuses of future investigations.

5.3.2 Dietary whole strawberry ameliorated colonic injuries, reduced infiltration of immune cells, and improved cellular anti-oxidative status.

Hematoxylin and Eosin (H&E) staining indicated that DSS treatment caused severe colonic injuries, evidenced by the epithelium distortion, large areas of depletion of goblet cells, damage of crypts and the infiltration of inflammatory cells, resulting in

much higher histopathological scores compared to the control group (Fig. 15D and E). In contrast, WS treatments (2.5% and 5%) significantly reduced the abnormal histopathological changes in the colon of the DSS-treated mice. The preserved colon architecture suggested the protective effect of dietary WS (2.5% and 5%) against DSS-induced colonic injuries.

In response to DSS treatment, infiltration of immune cells increased in the mouse colonic mucosa, which is similar to the situation of IBD patients [256]. Increased infiltration of neutrophils was reported in DSS treated mice, along with the high oxidative stress via production of ROS in the colon [257]. Our flow cytometry results indicated that DSS treatment significantly elevated the infiltration of total immune cells, macrophage, neutrophils and dendritic cells by 2.7-folds, 8.1-folds, 6.4-folds and 10.9-folds, respectively, in the mucosa of the distal colons when compared to the control group (Fig. 15G). Dietary WS decreased the infiltration of these immune cells in the colon, especially, dietary WS almost reversed the infiltration of macrophage, neutrophils and dendritic cells. Similar to our results, polyphenol-rich apple extracts and red raspberries were shown to reduce the immune cell infiltration in a rat model of colitis [258].

Innate immunity is activated when special cells recognize pathogens, and this process triggers the production of ROS and cytokines that amplifies the host response [259]. Increased oxidative stress has been associated with extensive infiltration of

immune cells in DSS-induced colitis [260]. Antioxidants have been found effective in attenuating colitis. Glutathione is the most important intracellular ROS scavenger, and its depletion can be observed under inflammatory conditions [261]. We determined the ratio of GSH/GSSG in the liver, and the data showed that GSH/GSSG was markedly reduced in DSS-treated mice than the control mice, indicating the depletion of GSH and higher level of ROS in the DSS group than that in the control (Fig. 15F). Dietary WS (2.5% and 5%) increased the GSH/GSSG ratio to the levels similar to that of the control mice, thus suggesting the protective effects of WS against ROS in colitic mice.5.3.3 Dietary whole strawberry decreased the production of proinflammatory cytokines

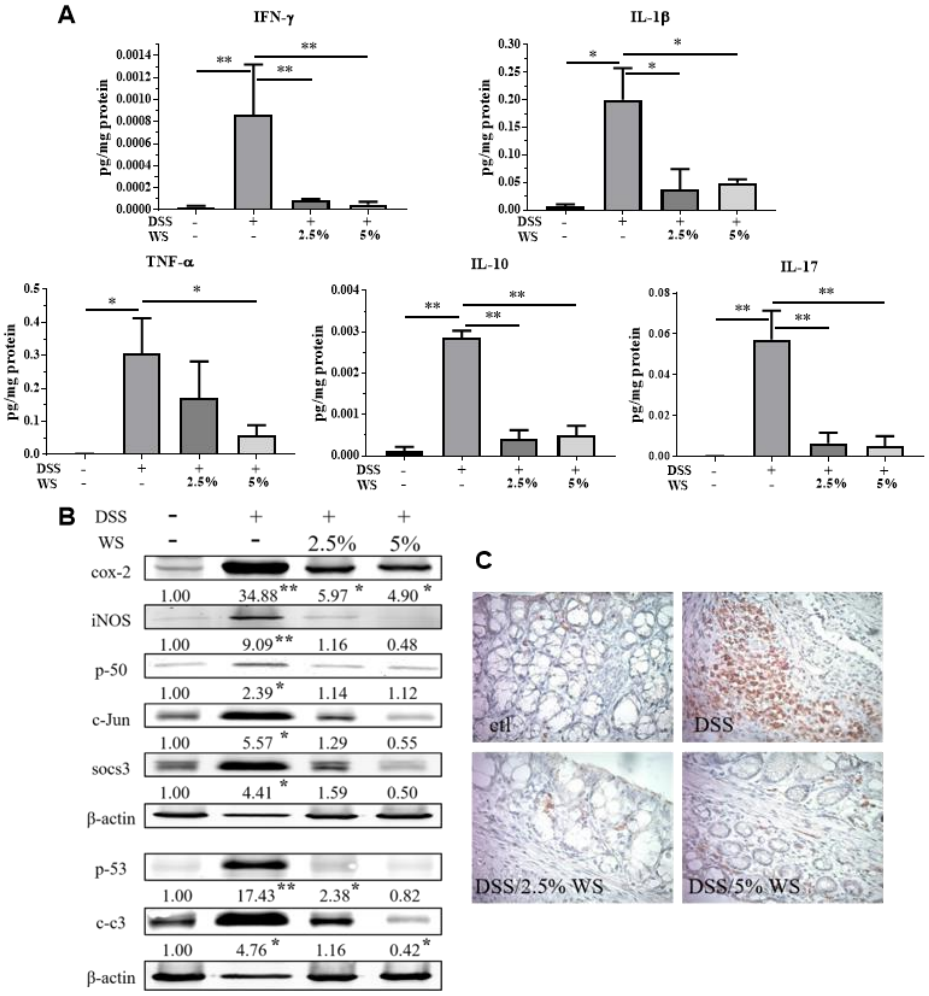

Figure. 16 (A) Quantification of TNF-α, IL-1β, IFN-γ, IL-17, and IL-10 in the colonic mucosa (n=6-8 per group); (B) Western blot analysis of key signaling proteins in the colonic mucosa (Each lane contained protein of the colonic mucosa collected from the colon of 2-3 individual animals that were pooled for immunoblot analysis. A total number of 6-8 mice from each group were analyzed. Statistical significance in comparison with the control (*$p < 0.05$, ** $p < 0.01$, $n = 3$)); (C) Representative immunohistochemistry images of iNOS in the colonic tissues (magnification: 600×). Data present mean ± SD, and * represents $p<0.05$, ** represents $p<0.01$.

Improper upregulation of proinflammatory cytokines is a marker of inflammation. IL-10 is considered an anti-inflammatory cytokine, the expression of IL-10 in colitic mice was higher than that in healthy mice (Fig. 16A), this may due to the self-defeating

response, indicating more severe colitis in DSS-treated mice. Also, as shown in Fig. 16A, DSS-treated mice showed drastically enhanced production of proinflammatory cytokines in the colonic mucosa than the control, such as TNF-α, IL-1β, IFN-γ and IL-17. Dietary WS effectively decreased the production of all five cytokines mentioned above in the DSS-treated mice. Considering the data on immune cells and cytokines, the trend of cytokine production in different groups was similar to that of the infiltration of the immune cells (Fig. 15G and Fig. 16A), which was consistent with the notion that infiltrated immune cells are the major producers of the various cytokines. These data suggest that anti-inflammatory effect of WS were at least partially due to maintenance of immune system homeostasis by reducing immune cell influx and overproduction of proinflammatory cytokines in the colon.

5.3.3 Dietary whole strawberry modulated inflammation-related signaling proteins

To investigate the mechanism by which dietary WS inhibits the production of inflammation mediators, the expression of COX-2 and iNOS were determined. We found that levels of COX-2 and iNOS were elevated in colitic mice, whereas WS successfully decreased their expressions (Fig. 16B and C). The NF-κB signaling pathway strongly influences the pathogenesis of colitis, and dictates the expression of proinflammatory proteins, such as COX-2 and iNOS, which markedly increased in the DSS-treated mice [262]. The results showed that NF-κB pathway was activated in the colon of the colitic

mice, evidenced by the elevated expression of p50, a vital subunit of NF-κB (Fig. 16B). Dietary WS effectively suppressed the activation of NF-κB signaling. Similar to these findings, polyphenol-rich fruits or extracts were found to suppress NF-κB signaling pathway in DSS-treated rodent models [11, 14]. AP-1 is another critical pathway that involved in the proinflammatory response. The DSS-induced colitis was associated with increased expression levels of c-JUN, the key component in AP-1 pathway [263]. The results showed the colonic level of c-JUN was increased by 5.6-fold in the colitic mice than the control. Whereas, dietary WS (2.5% and 5%) treatments decreased the expression of c-JUN by 73% and 91%, respectively in colitic mice.

The p53, an important tumor suppressor, can be activated and its expression level has been found increased in colitis [264], the elevated p53 expression in colitic mice is likely to be adaptive tissue response to facilitate the damaged cells. We found that the expression of p53 was increased in the colitic mice, but it was decreased by dietary WS treatments, suggesting the less severe colitis with dietary WS treatment. The activity of cleaved caspase-3 (C-C 3) was found increased in both colon cancer cells treated with LPS and the colon of the DSS-treated mice [265, 266], which may be related with tissue injuries caused by the colonic inflammation. Consistent with these findings, Figure 16B showed that DSS treatment increased expression levels of cleaved caspase 3 by 4.8-folds compared to the control. Dietary WS (2.5% and 5%) suppressed the expression of

cleaved caspase 3 by 76% and 98% compared to the DSS group, indicating that WS protected the colonic tissues against DSS-induced tissue injuries.

5.3.4 Dietary whole strawberry alleviated gut microbiota dysbiosis

Accumulating evidence suggested that gut microbiota play a vital role in colonic inflammation, and the change from “disease-free” to “disease” microbiota composition is an important characteristic of IBD [267]. In the disease-free (noncolitic) mice, i.e. the control and WS (2.5% or 5%) groups, 16S rRNA sequencing showed that 5% WS treatment significantly increased α diversity of the microbiota, which was assessed by PD whole tree (Fig. 17A), and modulated β diversity of the microbiota, which was assessed by principal coordinate analysis of the weighted UniFrac Index($P<0.05$, Fig. 17B), although 2.5% WS did not cause significant change of α or β diversity of the gut microbiota (Fig. 17A and 3B).

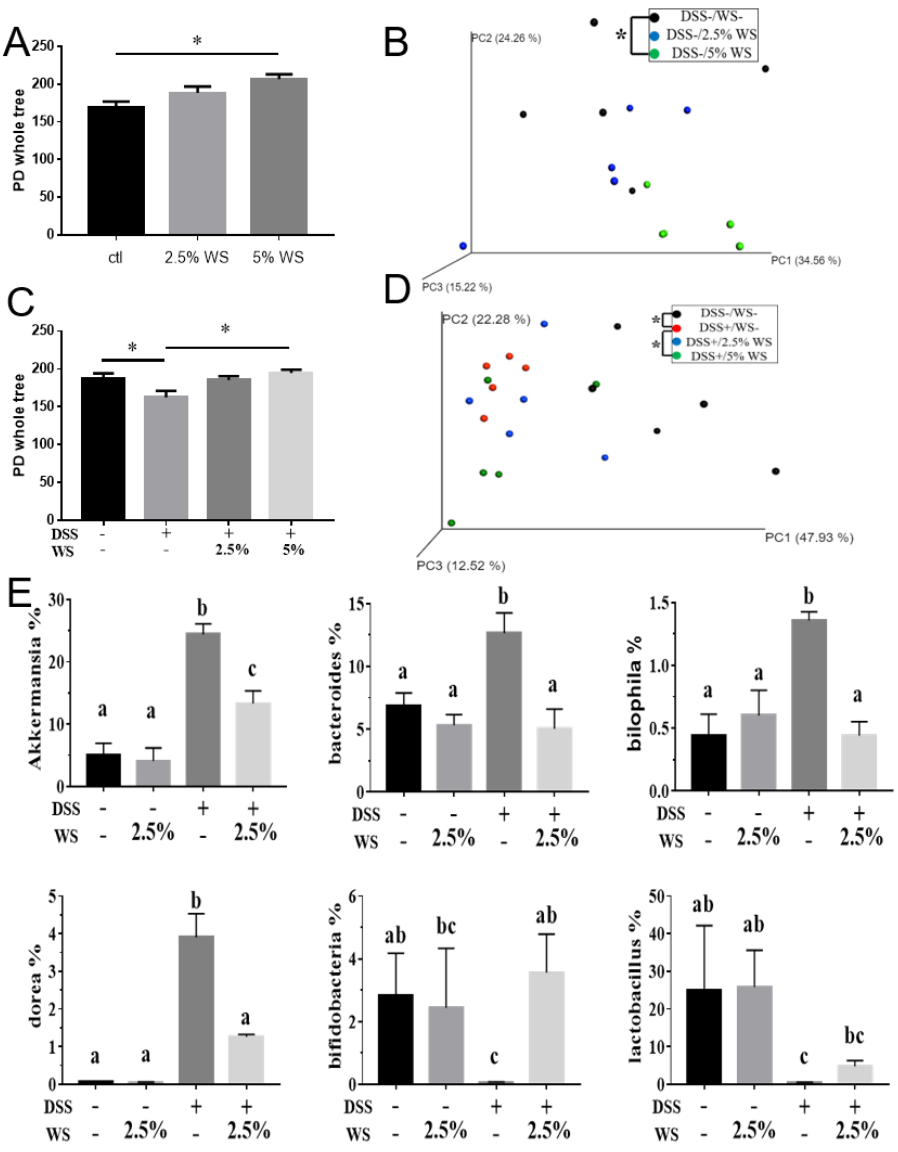

Figure. 17 The effects of WS on gut microbiota. (A) Effect of WS on α diversity of fecal bacteria in healthy mice, assessed by PD whole tree analysis; (B) Effect of WS on β diversity of fecal bacteria in healthy mice, assessed by principal coordinates analysis; (C) Effect of WS on α diversity of fecal bacteria in diseased mice, assessed by PD whole tree analysis; (D) Effect of WS on β diversity of fecal bacteria in diseased mice, assessed by principal coordinates analysis;(E) Relative abundance of bacteria taxa, different letters mean statistically differences ($p<0.05$). *$p<0.05$. Data are presented as mean ± SEM or median with 95% confidence interval (n=5).

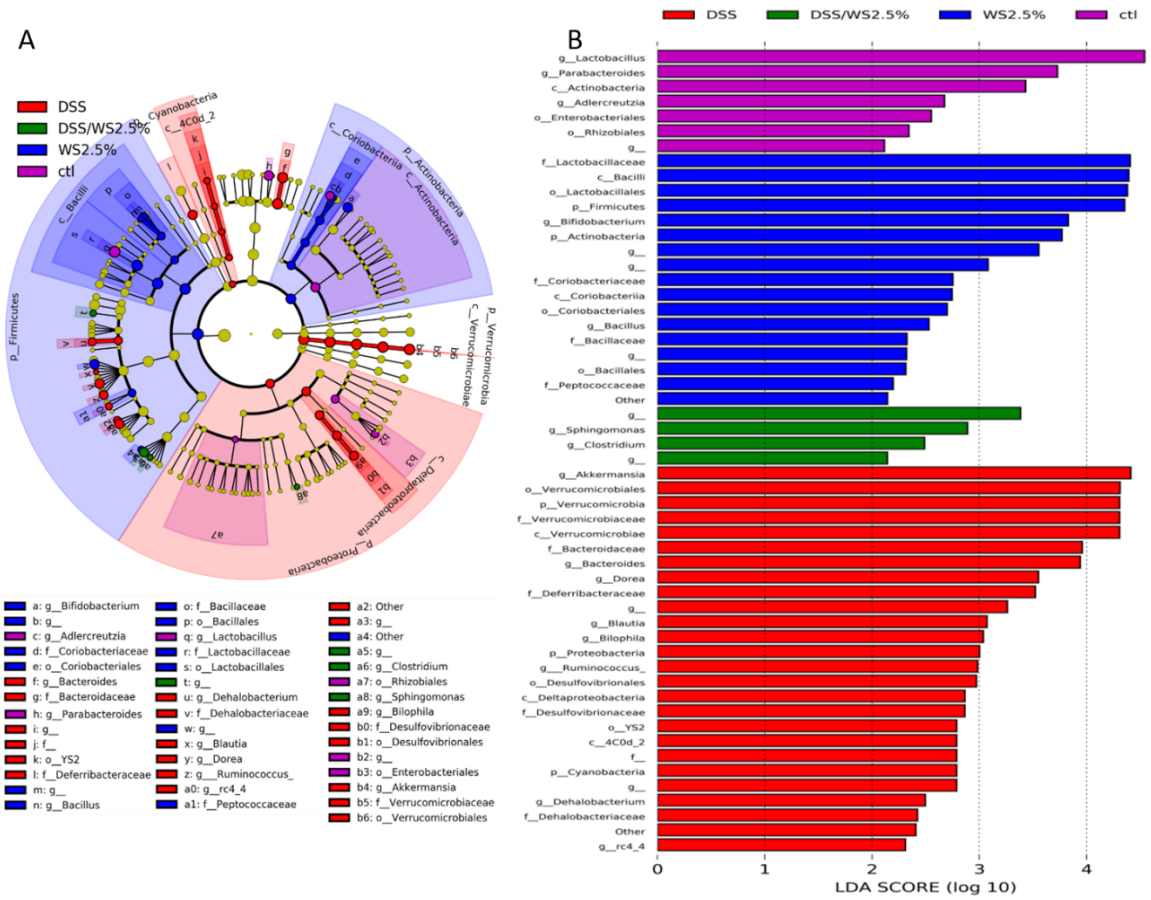

Figure. 18 LEfSe analysis of microbiota. (A) Taxonomic cladogram obtained from LEfSe analysis of 16s sequences; (B) Only taxa meeting an LDA score threshold > 2 are shown.

The α diversity was significantly decreased in colitic mice compared with noncolitic mice (Fig. 17C). 2.5% WS treatment did not significantly change α or β diversity in colitic mice. However, 5% WS treatment profoundly increased α diversity of colitic mice, which was assessed by PD whole tree (Fig. 17C). The β diversity was profoundly changed in 5% WS-treated colitic mice (Fig. 17D), as observed in the principal coordinates analysis of the weighted UniFrac Index. The three first principal coordinates explained 82.73% of the variation observed between all the groups. The change of gut microbiota between different groups was also evidenced by LEfSe analysis

(Figure 18), and Fig. 4B showed the most differentially abundant taxons of different groups. At the genus level, colitic mice showed 4.3-fold, 82.1-fold, 2.5-fold, and 2.8-fold higher abundance of Akkermansia, Dorea, Bilophila and Bacteroides and 224.1-fold and 58.1-fold lower abundance of Bifidobacterium and Lactobacillus than the noncolitic control mice, respectively (Fig. 17E). The LEfSe analysis also validated aforementioned results (Fig. 18A and 4B). Linear discriminant analysis (LDA) showed the higher abundance of Akkermansia, Dorea, Bilphila and Bacteroides in colitic mice than those in non-colitic mice (Fig. 18B).In consistent with our results, Akkermansia shows positive correlation with IBD patients [268], which may due to the Akkermansia's mucin-degrading ability and its highly immunostimulatory lipopolysaccharide activity,38 which results in high production of pro-inflammatory cytokines. Mucin is the major component of colonic mucosa, and the increased amount of Akkermansia may contribute to the mucosa damage. And dietary WS decreased both pro-inflammatory cytokines and the abundance of Akkermansia (Fig. 16A,17E and 18). The enrichment of Bilophila, as an opportunistic pathogen, is reported in colitis mice [269], which is similar to our results. Commensal Bacteroides was reported to induce colitis in mice, which is supportive to their potential role in pro-inflammation in the colitic mice that hosted higher abundance of Bacteroides than the noncolitic mice in this study [270]. Bifidobacterium and Lactobacillus have shown protective effects against colonic inflammation. Low abundance of these two bacteria in colitic mice might contribute to the development of

colitis. Intriguingly, dietary WS (2.5%) treatment at least partially or even completely reversed the alterations of gut microbiota in colitic mice by decreasing the abundance of Akkermansia, Dorea, Bilophila and Bacteroides, and increasing the abundance of Bifidobacterium and Lactobacillus. These results demonstrated dietary WS alleviated microbiota dysbiosis in DSS-induced colitis.

5.3.5 Dietary whole strawberry restored the production of SCFAs in the cecum

It has been shown that IBD was usually associated with change of gut microbiota, and concurrent decrease of SCFAs [260]. SCFAs have been found to have anti-inflammatory effects, and they were mainly produced by Bacteroidetes and Firmicutes phyla [271]. The data showed that the cecal levels of acetic acid, propionate acid, butyric acid, and valeric acid in the colitic mice 45.6%, 19.4%, 60.8% and 44.5% lower than that in the noncolitic control mice, respectively (Fig. 19A). Concomitantly, the abundance of Firmicutes was much lower in the colitic mice than the noncolitic control, which might contribute to the low levels of SCFAs.42 Dietary WS (2.5%) did not change the cecal levels of SCFAs in the noncolitic mice, but significantly increased the production of SCFAs in the colitic mice to the same levels found in the noncolitic control mice. Since WS did not increase the levels of SCFAs in noncolitic mice, it is likely that dietary WS increased SCFAs production in the colitic mice due to its effects in altering the composition of gut microbiota, such as increasing the abundance of Firmicutes.

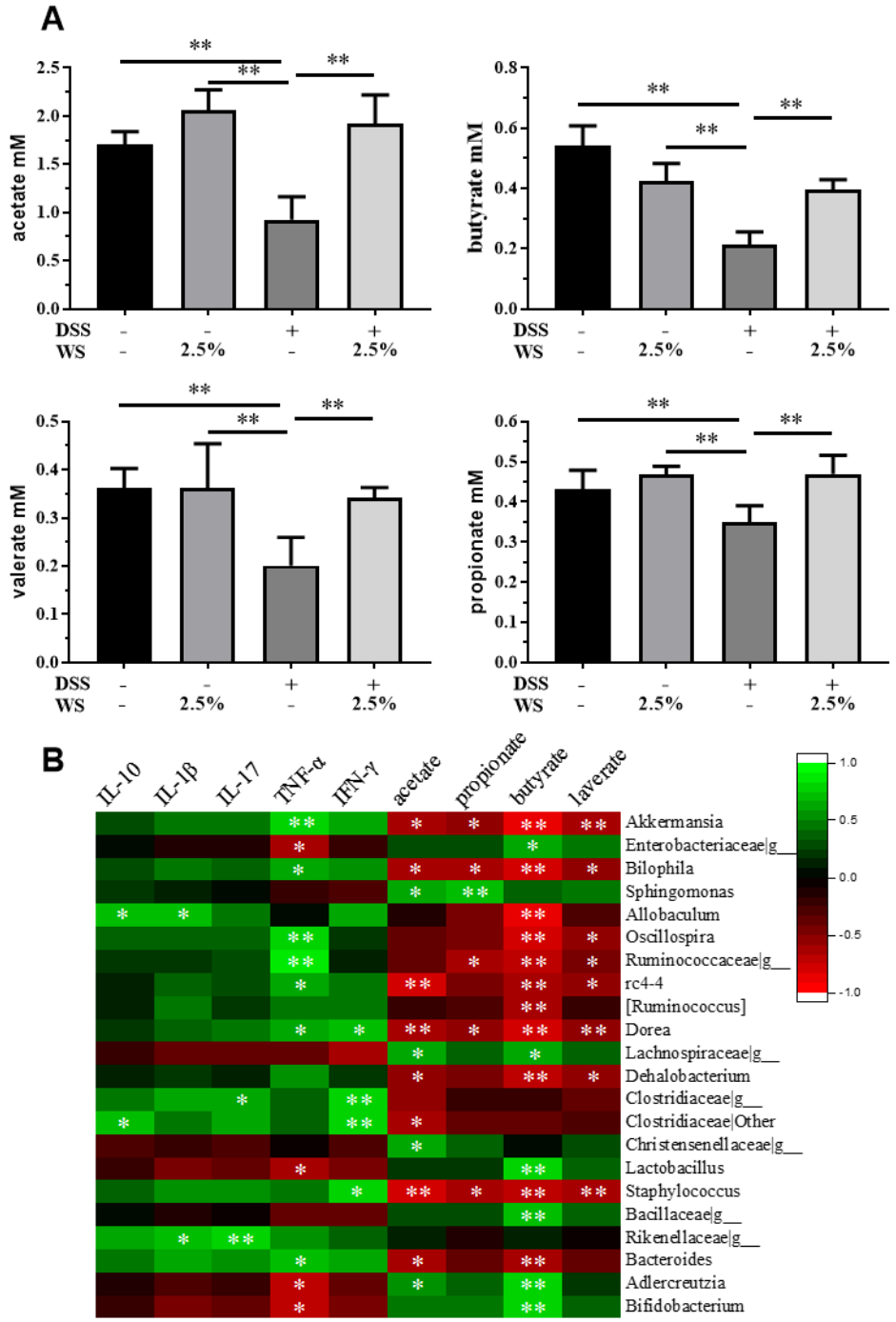

Figure. 19 (A) Quantification of SCFAs in the cecum. (B) Heatmap of the correlation between bacteria and cytokines or SCFAs. Data are presented as mean ± SD (n=5), and * represents $p<0.05$, ** represents $p<0.01$.

According to these results, colitic mice had improper upregulation of cytokines in

the colonic tissue, reduced production of SCFAs in the cecum, and increased abundance of pathogens and decreased abundance of beneficial bacterial species in the feces, when compared to the noncolitic control mice. Intriguingly, dietary WS effectively alleviated and/or reversed the aforementioned abnormality in the colitic mice. Since gut microbiota are responsible for the production of SCFAs in the colon, and can modulate the secretion of pro-inflammatory cytokines, especially when there is dysbiosis in the colon, to understand the role of gut microbiota in mediating the protective effects of dietary WS against colitis, the correlation between composition of gut microbiota and production of cytokines or SCFAs among the different experimental groups of mice was analyzed (Fig. 19B). The results showed that Akkermansia and Dorea presented a negative correlation with the production of SCFAs, especially for the butyrate that is an important anti-inflammatory component in the colon. Moreover, Akkermansia and Dorea also showed a strong positive correlation with TNF-α, a proinflammatory cytokine. Considering the relatively higher abundance of Akkermansia and Dorea in colitic mice than that in the non-colitic control mice (Fig 17E), Akkermansia and Dorea may exert proinflammatory effects in DSS-induced colitis. Consistent with our findings, the increase of Akkermansia and Dorea was previously reported in the DSS-induced colitis [272, 273]. The abundance of Bifidobacterium and Lactobacillus was negatively correlated with the production of TNF-α, and positively correlated with the production of butyrate, indicating a potential protective effect of these bacteria against colonic inflammation [274].

For the first time, these results demonstrated the protective effect of dietary WS against the development of colonic inflammation in the DSS-treated mice. This protective effect was closely associated with restoration of immune homeostasis by dietary WS, which was evidenced by that dietary WS reduced immune cell infiltration and lowered levels of proinflammatory cytokines in the colonic mucosa of colitic mice. The anti-inflammatory effects of dietary WS were also closely related with its ability to alleviate the dysbiosis of gut microbiota in the colitic mice, which was evidenced by the increased abundance of beneficial bacteria, the decreased abundance of harmful bacteria, and the increased production of SCFAs caused by dietary WS in the colon of the colitic mice. Overall, our findings provided a solid scientific basis for utilization of strawberry for the prevention of colonic inflammation.

CHAPTER 6

CONCLUDING REMARKS

Intake of whole foods, such as fruits and vegetables, profoundly modulates the composition and function of gut microbiota. These alterations of gut microbiota may (i) produce a better health status in disease-free population to lower the risks of various diseases, and (ii) alleviate the disease severity in patients with microbiota-associated diseases such as obesity and colonic inflammation. MACs and polyphenols are two major types of components responsible for the modulating effects of fruits and vegetables on gut microbiota. They have a reciprocal interaction with gut microbiota, in which MACs and polyphenols alter the structure of microbiota and microbiota transforms MACs and polyphenols to bioactive metabolites, such as SCFAs and phenolic acids, respectively. These microbiota-derived metabolites could promote host health by targeting both host tissues and gut microbiota. Furthermore, coexistence of whole food components, their metabolites and microbiota in the large intestine provides opportunities for them to interact with each other and produce certain biological functions that are otherwise impossible. The relationship among whole foods, gut microbiota, and the host is complex, which makes it challenging to study. Nevertheless, a highly interdisciplinary and dynamic approach giving special considerations of the physiological relevance of the experimental conditions is needed to elucidate the role of whole foods in modulating gut

microbiota and associated diseases.

This study provided an understanding of EP and NEP from cranberry and strawberry in terms of their chemical properties and biological functions. Chemical profile of cranberry and strawberry NEP was firstly determined, which showed that NEP possessed higher contents of phenolic acids than EP. EP fraction in fruits have been extensively studied, such as their anti-oxidant, anti-inflammatory, anti-cancer, and antibacterial effects. However, reports about NEP have been scarce. NEP could bind to fiber and they may reach colon intact [15], so that the biological effects of NEP have been considered important for colon health. Our results indicated that both EP and NEP from cranberry had various biological effects. Importantly, NEP showed the promising anti-inflammatory and anti-cancer capacities. The high bioavailability of NEP in the colon facilitate the realization of these activities in the colonic tissues. Moreover, the potential interaction between gut microbiota and NEP could also play an important role in colon health. NEP could be biotransformed by gut microbiota to produce bioactive metabolites that may contribute to the promotion of colon health as well [197]. Therefore, it is important to investigate the interaction of gut microbiota and NEP in the colon in terms of production of bioactive metabolites. Meanwhile, composition and functions of gut microbiota can be altered by the presence of polyphenols in the colon, which in turn could affect colon health [198]. Unfortunately, the mode of interplay

between NEP and gut microbiota in the colon is largely unknown, and much more efforts are needed to explore this promising research area. The knowledge obtained from our study is helpful for a better understanding of polyphenols in berries, especially the potential biological effects of NEP in the colon. Overall, this study provided the first line of evidence to support the notion that NEP could be a promising beneficial agent for colon health. Certainly, further investigation is warranted to elucidate the detailed model of actions of NEP in the colon, especially in relationship with gut microbiota.

Furthermore, this study demonstrated the protective effect of dietary WS against the development of colonic inflammation in the DSS-treated mice. This protective effect was closely associated with restoration of immune homeostasis by dietary WS, which was evidenced by that dietary WS reduced immune cell infiltration and lowered levels of proinflammatory cytokines in the colonic mucosa of colitic mice. The anti-inflammatory effects of dietary WS were also closely related with its ability to alleviate the dysbiosis of gut microbiota in the colitic mice, which was evidenced by the increased abundance of beneficial bacteria, the decreased abundance of harmful bacteria, and the increased production of SCFAs caused by dietary WS in the colon of the colitic mice. Overall, our findings provided a solid scientific basis for utilization of strawberry for the prevention of colonic inflammation.

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