November 2016

Chemopreventive Effects of Cranberry in Association with the Alteration of Gut Microbiota

xiaokun cai
University of Massachusetts Amherst

CHEMOPREVENTIVE EFFECTS OF CRANBERRY IN ASSOCIATION WITH THE ALTERATION OF GUT MICROBIOTA

A Dissertation Presented

by

XIAOKUN CAI

Submitted to the Graduate School of the

University of Massachusetts Amherst in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2016

Food Science

CHEMOPREVENTIVE EFFECTS OF CRANBERRY IN ASSOCIATION WITH THE ALTERATION OF GUT MICROBIOTA

A Dissertation Presented

by

XIAOKUN CAI

Approved as to style and content by:

_____

Hang Xiao, Chair

_____

Guodong Zhang, Member

_____

Richard J. Wood, Member

_____

Eric A. Decker, Department Head

Department of Food Science

DEDICATION

To my mom, my dad, and my families

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my advisor, Dr. Hang Xiao for offering me the opportunity to study as a Ph.D. student in his lab and his full support, expert guidance and kindly encouragement throughout my study and research. Without his patience and knowledge, my thesis work would not be completed. In addition, I would like to express my gratitude to Dr. Zhang and Dr. Wood for being my committee members for their valuable comments and feedback.

I would also like to thank to all of Xiao's lab members, past and present, Tom, Mon, Christina, Mingyue, Xian, Minqi, Fang, Zhengze, Min, Jason, Xiaoqiong, Jiazhi, Will, Tim Jingjing, Cici, Ziyuan, Fuki, Nok, Zili, Hua, Yue, Yanhui, Pan, Xuexiang, Biao, Dr.Sun, Dr.Qi, Dr.Zhang and Dr.Cao for their continuing friendship and support in the last four years. I would like to thank all of Food Science faculties, staffs and students, especially Fran, Ruth and Dave. In addition, I would like to thank all of my friends in Amherst, especially Charmaine for all their supports.

Last but not least, I would like to thank my parents and my families for the love and support they give me through my whole life. I also would like to thank my friend Xin for his kindly encouragement and support.

ABSTRACT

CHEMOPREVENTIVE EFFECTS OF CRANBERRY IN ASSOCIATION WITH THE ALTERATION OF GUT MICROBIOTA

SEPTEMTER 2016

XIAOKUN CAI,

B.S., SOUTH CHINA AGRICULTURAL UNIVERSITY, GUANGZHOU, CHINA

Ph.D., UNIVERSITY OF MASSACHUSETTS, AMHERST, MA

Directed by: Professor Hang Xiao

Accumulating evidence showed that microbiota play important roles in colonic inflammation and inflammation-associated colon carcinogenesis. Fruits and vegetable are known to have protective effects against colon carcinogenesis. Cranberry fruit contains large amount of flavonoids, phenolic acids and dietary fiber, which has been studied for their potential chemopreventive effect from in vitro model. To better understand the effect, we studied the protective effects of whole cranberry powder against colitis in mice treated with dextran sulfate sodium (DSS) and inflammation-associated colon carcinogenesis in mic treated with azoxymethane (AOM) and DSS, and its impact on gut microbiota.

In DSS-induced colitis mice model, 1.5% (w/w) whole cranberry powder mixed with regular diet was provided to mice for 32 days. The oral administration of cranberry powder significantly reduced the DAI score and inhibited the inflammation in the colon compared to the control group. Moreover, cranberry diet increase the richness and evenness of gut microbiota in mice, which played an important role on inflammation prevention. Oral intake of cranberry can

significantly inhibited the growth of *Akkermansia* and *Sutterella* while protect the growth of *Bifidobacterium* and *Lactobacillus*.

In AOM-induced colonic cancer mice model, whole cranberry powder was administered to mice through diet at 1.5% w/w for 20 weeks. Our results demonstrated that treatment with cranberry powder significantly reduced the incidence and multiplicity of colon tumors. These protective effects were associated with decreased inflammation and increased apoptosis in the colonic tumors. Using 16s rRNA amplicon sequencing, we analyzed the structure and predicted the function of fecal microbiota of the mice. Compared to that of the negative control group, fecal microbiota of AOM/DSS-treated mice showed relative abundance shifts, a decrease of the abundance of *Akkmansia*. Dietary treatment with whole cranberry powder reversed aforementioned alterations in the fecal microbiota of AOM/DSS-treated mice. Moreover, whole cranberry powder also increased the number of *Bifidobacterium* and *Lactobacillus* in the fecal microbiota. These composition alterations induced by whole cranberry powder were associated with suppressed colonic inflammation and carcinogenesis.

In conclusion, our results demonstrated that whole cranberry powder modulated the composition of gut microbiota in both DSS-treated and AOM/DSS-treated mice, which may contribute to its anti-inflammatory and anti-carcinogenic effects in the colon.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Due to the aging and the increasing population around the word, cancer burden increase dramatically. The growing adoption of cancer causing behaviors, including smoking and western diet, accelerate the growth rate of cancer incidence[1]. Colorectal cancer is one of the most commonly diagnosed cancer around the world, which caused about 608,700 deaths in 2008. North America is one of the highest colorectal cancer incidence area which can be attributed to western diet, obesity, alcohol consumption and physical inactivity [2]. Western diet, which contains excessive animal fat and red meat, and little dietary fiber and vegetables, contribute up to 80% of CRC incidence [3].

In the last decade, ample animal and epidemiological studies have revealed the role of inflammation on all steps of colon tumorigenesis. Inflammatory bowel disease(IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a chronic inflammation of all or part of digestive tract, which is a serious risk factor for one subtype of CRC, colitis-associated cancer (CAC). Comparing with other types of CRC, CAC has higher mortality since it is difficult to treat basing on current therapy method [4].

Gut microbiota is a complex communities of microorganisms which inhabiting in the gastrointestinal tract. the human gut microbiota contains approximately 500~1000 species and about 10-fold outnumber of host cells. The *Bacteroidetes* and the *Firmicutes* are the two major kind bacteria in the human while *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Fusobacteria*, and *Cyanobacteria* have small amounts [5, 6]. The composition of gut microbiota is host specific that can be affected by exogenous and endogenous factors, including gene, diet and environment. Gut microbiota can be divided into three groups according to their interaction

with the host, symbiont, commensal and pathobiont. However, some commensal bacteria can become pathobiont when their pass through the mucosa barrier [7].

Recently, a large amount of studies has indicated the role of gut microbiota on human health and disease. The gut microbiota participates in various normal host physiological activities, from immunity to host metabolism. Besides, gut microbiota can cause different aliments, including IBD and virus infection [8]. There is immunological equilibrium among different groups of the gut microbiota to maintain the health status of the host. The dysregulation of the microbiota can lead to dysfunction of the host. McFarland, L. V. have indicated that antibiotic therapy could cause diarrhea because the number of *Clostridium difficile* increased dramatically after treatment [9]. Several studies revealed that the structure of gut microbiota in CRC patients is different from the health people and the structural imbalance may contribute to the progression of CRC [10].

A large amount of studies have indicated that fruits and vegetables in diets can contribute to the improvement of health and prevention of disease, including cancers and cardiovascular diseases. Cranberry (*Vaccinium macrocarpon Ait. Ericaceae*) is a native fruit of North America that has long been consumed in various products, including juice, jam and dry fruit. Cranberry has high content of various phytochemical, including flavonoids (flavonols, anthocyanins and proanhocyanidins(PAC)), catechins and phenolic acids, which bring potential health benefits. 100 g fresh cranberry fruit contains up to 30 mg total phenol content, up to 65 mg anthocyanins. As the leading fruit sources of A-type linkage PACs, the total PACs content of cranberry on average is 180 mg per 100 g of fresh fruit weight [11]. In vitro studies demonstrate that cranberry extract can inhibit proliferation of four human colon cancer cell lines (HT-29, HCT-116, SW480,

and SW620), which may be attributed to the synergistic activities of PACs, anthocyanins and phenolic acids in cranberry [12].

Although polyphenols in cranberry exert the anti-proliferative activity, their absorption rate in vivo are very low and most of them remain intact when they reach colon where they get metabolized by gut microbiota. Researches now are interested in the interaction between polyphenols and dietary fiber and gut microbiota as new therapeutic agents to restore the microbial balance in several chronic disease [13].

Our ***long-term goal*** is to deeply understand the health benefits produced through the interaction between gut microbiota and various nutrients in whole food. To reach that goal, the ***overall objective*** of this project is to study the impact of cranberry on gut microbiota in different disease animal model. Our ***central hypothesis*** is cranberry is able to exhibit anti-inflammatory and anticancer activities. Moreover, cranberry will exert the chemopreventive effect through altering the structure and the function of gut microbiota in mice.

We will test our central hypothesis and achieve our objective by utilizing the following two ***specific aims:***

Determine the anti-inflammatory effects of cranberry and its impact on gut microbiota on colitis mouse model.

A dextran sulfate sodium (DSS)-induced colitis mice model will be used to assess the anti-inflammatory effect of cranberry *in vivo*. Histological evaluation will be performed to assess the colon adenocarcinoma formation and other parameters in DSS–treated mice. Colonic mucosa will be subjected to immunohistochemical analysis, ELISA analysis to determine the mechanism of action of cranberry on colon carcinogenesis. DNA of fecal bacteria will be extracted and undergo 16s rRNA amplicon sequencing to analysis the structure of the bacterial community.

Determine the anti-carcinogenesis effects of cranberry and its impact on gut microbiota on colon carcinogenesis mouse model.

An azoxymethane (AOM)/ dextran sulfate sodium (DSS)-induced colitis-associated colon carcinogenesis mice model will be used to assess the antitumor efficacy of cranberry *in vivo*. Histological evaluation will be performed to assess the colon adenocarcinoma formation and other parameters in AOM/DSS–treated mice. Colonic mucosa will be subjected to immunohistochemical analysis, ELISA and RT-PCR analysis to determine the mechanism of action of cranberry on colon carcinogenesis. DNA of fecal bacteria will be extracted and undergo 16s rRNA amplicon sequencing to analysis the structure of the bacterial community.

CHAPTER 2

LITERATURE REVIEW

2.1 Overiview of colorectal cancer

2.1.1 Cancer progression

Basing on the histological types, cancer can be classified into six major categories: carcinoma, sarcoma, myeloma, leukemia, lymphoma and mixed types [14]. Carcinoma, which account for up to 90% of cancer cases, is the accumulation of mutated malignant epithelial cells [15]. Carcinomas can be developed in different tissues, including head, neck, lung, breast, bladder, cervix, skin, colon and rectum [16]. Sarcoma are the cancers which develops in supportive and connective tissues. Cancer from the plasmas cells of bone marrow is classified as myeloma while leukemia refers to the cancers of bone marrow. Lymphoma are the cancers which originated in the glands of the lymphatic system.

Hanahan indicated that six principles to characterize a tumor cell: sustaining growth signals, resisting growth inhibitors, escaping from cell apoptosis, replicating infinitely, inducing angiogenesis, invading and metastasizing tissues [17]. Evasion from immune system and deregulation of cellular energetics are two emerging factors of tumor progression for last decade [18]. The tumorigenic development can be divided into three stages: initiation, promotion and progression. In the initiation stage, chemical or physical carcinogens induce the mutation of DNA in cell, which activate the oncogenes and/or inactivate of cell apoptosis genes. The second stage, promotion, is the immortally replication of mutated cell by external tumor growth promoter. During last stage, progression, the size of tumor increase continually due to the invasion and metastasis of mutated cells. Malignant neoplastic changes can be acquired, leading to the new growth of cancer [19].

2.1.2 Introduction of colorectal cancer

Colorectal carcinogenesis is a genetic mutation of single epithelial cell line resulting in the malignant proliferation of cell and dysplastic appearance in large intestine [20]. The aberrant crypt foci is first observed during the colorectal cancer progression, indicating that the abnormal proliferation of crypt cells is associated with the tumor development. Adenomatous polyposis coli (APC) gene, a potential tumor suppressor gene, is affected in the initiation of colon cancer, which promote the development polyps and invasion [21]. APC mutation initiates the Wnt pathway, in which the phosphorylation of β-catenin get inhibited, resulting in the upregulation of cellular proliferation gene, cyclin D and CD44. P53 gene mutations lead to metastasis while other additional genetic mutations lead to the growth of colon tumor size [22] [23].

Human colorectal cancer (CRC) is related advancing age, lifestyle and environmental factors. Since CRC is a complex and multifactorial disease, there is no accurate marker to screen and monitor CRC which make it difficult to detect and prevent [24].

2.1.3 Introduction of chemoprevention

The increasing amount of cancer case and death around the world throw the emphasis on the cancer therapy. Basing on the current medical therapy, it is difficult to completely cure cancer. Although it is possible to decrease the risk of the cancer by avoiding the exposure to dangerous carcinogens and the other risk factors, it is not easy to eliminate the carcinogens and risk factors thoroughly from daily life [25].

Targeting on different stages of cancer progression, cancer chemoprevention refers to the reverse, suppression and prevention of carcinogenic development by using natural, synthetic or biologic chemical agents [16]. Carcinogenic progression may be retarded when the genotypic and phenotypic mutation are prevented in different stages. Ample clinical trials suggested that

some phytochemicals from plants show benefit effect on lowering the cancer risk since they may interfere the process of cancer progression. However, the molecular mechanisms of chemoprevention effect of phytochemicals are not well studied.

2.1.4 Molecular mechanisms of chemoprevention

Basing on the affected stage of carcinogenic progression, chemopreventive phytochemicals can be classified into blocking agents and inhibiting agents [26]. Blocking agents are used to protect critical cellular molecules, like DNA, RNA and proteins, against carcinogens in initiation stage while suppressing agents can prevent or retard the stepwise development of initiated cells in promotion or the progression stage. The chemopreventive agents have influence on many cellular and molecular activities, including induction of cell-cycle, activation of cell apoptosis, detoxification of carcinogen, repair of nucleic acid, stimulation of immune system and activation of tumor inhibitor gene [27].

Mitogen-activated protein kinase (MAPK) pathway is one the common pathway which get disrupted during cancer progression, leading to the immortal cell proliferation and malignant growth. Some phytochemicals can suppress the growth signal producing by mutation gene and control the MAPK pathway to prevent the transformation from epithelia cells to adenoma [28]. The aberrant of some transcription factors have been proved to impact the cancer development. For example, overexpression of NF-κB may prevent the apoptosis and promote the growth of cancer cell and phenotypic mutation. Many chemopreventive agents from edible plants can exert the protective effect through regulating the expression of NF-κB [29]. AP1 can affect cellular differentiation and replication by modulate the expression of genes [30].

Apoptosis is a natural cellular activity to regulate cell death for eliminate the mutated cells or aged cells. In cancer progression, the process of apoptosis get suppressed which break

the equilibrium between cell growth and death. Chemopreventive phytochemical can re-active the apoptosis through two different pathways, intrinsic pathway which happen in mitochondria, exterior pathway which directly modulate the cellular death signal receptors [31]. In extrinsic pathway, the interaction between tumor-necrosis factor (TNF), or TNF-related apoptosis-inducing ligand (TRAIL) and their death receptor, TNF receptor 1(TNFR1) and death receptor 4 (DR4) result in the increasing expression of FAS-associated death domain (FADD) and the protease caspase 8. Caspase 8 continue to induce caspase 3, leading to the activation of programmed cell death [32]. In intrinsic pathway, the activation of caspase 9 by the existence of cytochrome c and apoptotic protease-activating factor 1 (APAF1) give rise to the cell death response [33].

In addition to these two pathways, several regulators can also be targeted to mediate apoptosis. p53, which can activate apoptosis and cell-cycle arrest when detect the DNA mutation, is suppressed in half of cancer types. The interaction between p53 and MDM2 inactivate p53, leading to the deregulation of cell proliferation in cancer development. Therefore, chemopreventive agents can maintain the activation of p53 to suppress the cancer progression [34]. β-Catenin is also a targeted protein for phytochemicals . β-Catenin is associated with tumorigenesis since it is a necessary protein in cell-cell adhesion machinery and WNT signaling pathway [35].

2.1.5 The role of phytochemicals in cancer prevention and therapy

Epidemiologic evidence that phytochemicals can protects against colorectal cancer is contradictory. Arthur studied a total 1905 subjects and concluded that a diet with low amount of fat and high amount of fiber, fruits and vegetables showed no positive effect on preventing the recurrence of colorectal adenomas [36]. An epidemiologic trial with 1429 subjects was

conducted by David and indicated that the wheat-bran fiber could not lower the risk of recurrence of colorectal adenomas [37]. Cascinu indicated that calcium, vitamins A and E supplement did not protect against the proliferation of colorectal cancer cell [38]. However, J.A. Baron conducted a epidemiologic trial with 930 subjects who took either calcium carbonate or placebo for four years and the results suggested that calcium can slightly lower the risk of the recurrent colorectal adenomas by binding bile and fatty acids [39].

Curcumin is widely studied for its various chempreventive effect, including anti-oxidative , anti-inflammatory and anti-cancer effects. Plummer indicated that curcumin can decrease the expression level of cyclooxygenase-2 (COX2) and NF-κB by reducing the amount of NF-κB-inducing kinase (NIK) in human colonic epithelial cells [40]. Singh reported that curcumin can prevent phosphorylation and degradation of IκB in human myeloid leukemia cell line which lead to the suppression of DNA translocation and mutation [41]. Epigallocatechin gallate (EGCG) is one kind of tea polyphenols which is extensively investigated for its anti-carcinogenic effects on different tissues, including colon, liver, pancreas, lung and skin in rodent animals. Yoshizawa and his colleagues successfully suppressed the tumor development in DMBA-initiated mouse skin with EGCG. Metz described that oral administration of green tea extract can inhibit the expression of COX2 in intestinal mucosa in azoxymethane induced colonic adenoma rat models. The studies of Gong indicated that EGCG can protect liver from malignant mutation in rats [42]. Resveratrol is a stilbenoid which can be found in the skin of grapes, blueberries and rasberries. The health benefits of red wine are mainly attributed to the presence of resveratrol, including the protective effect from cardiovascular disease and cancer. Studies showed that resveratrol can induce apoptosis and downregulation of NF-κB in both human pancreatic cancer cell lines and breast cancer MCF-7 cells [43, 44].

2.2 Overview of inflammation

2.2.1 Introduction of inflammation

Inflammation is non-specific immune response of body to injury, pathogens or harmful chemicals, which may lead to hyperemia, increasing blood flow and the molecular mediators and vasodilatation [45]. The process of inflammation manage to get rid of the stimuli which cause cellular damage, the injured or aberrant cells or tissues and activate the repairing process of body. The inflammation of body is mainly produced through two ways, innate immune response and adaptive immune response. Through pathogen-associated molecular patterns (PAMPs) and other cell products related to system defenses, the innate immunity show a quick reaction to the extrinsic chemicals which commonly exists on pathogens while the adaptive immunity only response to chemicals with specific structure [46]. When the innate immunity recognize the "invasive" agents, macrophages, a type of white blood cell, are generated to eliminate the chemicals or cells without healthy protein marker on the surface. In order to identify stimuli, macrophages can use different scavenger receptors and Toll-like receptor which can pair many pathogenic molecular like lipopolysaccharides. The interaction between Toll-like receptors and their ligands can activate the NF-κB and MAPK pathway [47]. In contrast, the adaptive immunity produce various antigen receptors like T-cell receptors which can bind with certain molecular structures to identify the specific targets. The ligations of targets and T-cell receptors can stimulate the immune system where the cytotoxic T-cells are generated to eliminate the targets and B-cells to produce antibody [48].

Despite its protective effects, a lot of chronic diseases such as arthritis, diabetes, cardiovascular disease, cancers and bowel diseases, can be attribute to inflammation when the inflammatory process get deregulated. Both innate and adaptive immune responses target on

either epithelial cells or mesenchymal cells on various organs, modulating the cellular activities of tissues, including the activation of leukocytes and the programmed cell death process, which result in the dysfunction of tissues. Through disordering the normal function of epithelial cells (i.e. endothelial cells, enterocytes), inflammatory process can initiate and promote diseases [46].

2.2.2 Introduction of inflammatory bowel disease

In United States and Europe, there are total 3.6 million people suffering from inflammatory bowel disease (IBD). Besides, the incidence and prevalence of IBD are increasing in both developed countries and developing countries which rise serious attention to this disease [49]. Inflammatory bowel disease includes two major types, ulcerative colitis (UC) and Crohn's disease (CD), which refers to the aberrant inflammation in small intestine and colon. Although UC and CD are two different diseases, the symptoms of these two diseases are similar, including abdominal pain, vomiting, diarrhea with blood [50]. Ulcerative colitis mainly happen in the colon and affect the superficial mucosal layers where severe ulceration take place and neutrophils assemble in the crypts. However, Crohn's disease can have impact on the whole gastrointestinal tract in which aggregated macrophages constitute granulomas. Crohn's disease mainly happen in ileum and the tissue damage is normally first found on Peyer's patches [51].

2.2.3 Risk factors to inflammatory bowel disease

According to the epidemiological studies, inflammatory bowel disease can be affected by different factors such as environmental factors, diet and genetic difference. The Halfvarson's study on twins with Crohn's disease indicated that genetic difference played an importance role on the disease incidence [52]. Cigarette smoking is one of the most significant environmental factors for inflammatory bowel disease even though the impact of cigarette smoking on ulcerative colitis and Crohn's disease are different. A study of 400 participants pointed out that

smoking exert protective effect against ulcerative colitis while no adverse effect on Crohn's disease [53]. Silverstein conducted a case control study on cigarette smoking and ulcerative colitis and reported that non-smoking patients is more likely to have ulcerative colitis comparing with patient who smoke [54]. Another case control study including 84 smoking patients and 84 non-smoking patients showed that smoking can lower the risk of ulcerative colitis of patients [55]. Several trials used transdermal nicotine as therapy for ulcerative colitis which suggested that the treatment of nicotine did not contribute to the remission rate but introduced other side effects to the participants [56] [57]. It is still unclear the interaction among cigarette and smoking and ulcerative colitis and the potential explanation for the unusual finding is that the multiple compounds of cigarette generate the complex effect. Despite the potential beneficial effect on ulcerative colitis, cigarette smoking has been proved to increase the risk of Crohn's disease [58].

Appendectomy is another importance environmental factor for inflammatory bowel disease. Studies reported that appendectomy can protect against ulcerative colitis while promote the Crohn's disease [59]. Koutroubakis et al. analyzed 17 studies with about 8000 participants and concluded that appendectomy dramatically decrease the risk of ulcerative colitis [60]. The epidemiological study of Andersson reported that appendectomy have adverse effect on Crohn's disease in large Swedish cohorts [61]. The possible mechanism of the different effect of appendectomy on two types of inflammatory bowel disease is the development of ulcerative colitis can be inhibited by the inflammation in appendix while Crohn's disease can be promoted through the inflammation.

Diet also plan an important role in the development of inflammatory bowel disease. The incidence rates and prevalence of inflammatory bowel disease are significantly higher in several geographic regions including northern Europe, United Kingdom and North America than others,

which is mainly affected by the different diet [62]. The consumption of sugar in diet was extensively studied for its adverse effect on inflammatory bowel disease. Russel have conducted a case control study and reported that excessive intake of food with high amount of sugar can increase the risk for the inflammatory bowel disease, especially Crohn's disease [63]. Riordan et al. have reviewed that the carbohydrate consumption was risk factors for Crohn's disease [64]. However, whether the increasing consumption of dietary fiber, fruit or vegetable can prevent inflammatory bowel diseases is still inconclusive since the intake amount may be confined due to the situation of patients.

2.2.4 The relationship between inflammatory bowel disease and colon cancer

Many studies suggested that chronic intestinal inflammation can promote the progression of colon cancer since the presence of colitis is proved to contribute to cancer development and the anti-inflammation drugs like 5-aminosalicylates can protect against the colon cancer. The sporadic colon cancer share the same pathway with colitis-associated colorectal cancer which result in the mutation of DNA and cellular damage [65]. The chromosome instability caused by inflammatory bowel disease can initiate the colorectal cancer, which can be affected by several factors, including the duration time of colitis, the affected area on colonic surface area [66]. Unlike sporadic colorectal cancer, colitis-associated colorectal cancer is initiated on multiple aberrant adenomatous polyposis coli (APC) and conquer larger surface area of mucosa.

The loss of p53 gene in the chromosome mutation is suggested to be the critical risk factor for the development of colitis associated colorectal cancer [67]. Epidemiological studies showed that the mutation of p53 in mucosa of ulcerative colitis patients was much more common than the normal people, implicating that chronic intestinal inflammation associated with the early

stage of colon cancer progression [68]. Besides, the increasing oxidative stress in inflammatory bowel disease contribute to the growth of tumor.

2.2.5 The role of phytochemicals on inflammatory bowel disease prevention and therapy

Many phytochemicals are considered to have ability to protect against inflammatory bowel disease, including flavonoids, polyphenols and dietary fibers. Flavonoids which are abundant in various fruits, vegetables and plants are widely studied for their anti-oxidative and anti-inflammatory effect in cell model and animal model [69]. Galvez et al. induced colitis in rats which received normal diet mixing with rutin, a common flavonoids in wheat, tomatoes and apricots. The study indicated that rutin can inhibit the inflammation and mucosa lesion by modulating the levels of interleukin (IL-1), IL-6, iNOS, and tumor necrosis factor α (TNF-α) [70]. Polyphenols ubiquitously present in the plant and are excessive studied for their multiple biological activity. Green tea polyphenols are the most common studied compounds for their strong anti-oxidative effect. Several studies reported that the activity of NF-κB and kinases which associated with the inflammatory process were inhibited by the green tea polyphenols and contribute to the inflammatory bowel disease prevention [71]. Catechins are one group of polyphenols which were reported to lower the risk of inflammatory bowel disease through modulating the inflammatory cytokines in both in vitro and in vivo studies [72]. Curcumin is natural phenols which widely distributed in ginger and certain spice. Studies indicated that curcumin interrupt the activation and transcription of COX-2, lipoxygenase and NF-κB, leading to the amelioration of mucosal inflammation [73]. Dietary fiber not only can modulate various inflammatory mediator such as TNF-α, IL-2 and nitric oxide, but also can interact with microorganisms which can produce short chain fatty acid to protect against the inflammatory process [74].

2.3 Overview of gut microbiota

2.3.1 Introduction of gut microbiota

Microorganisms ubiquitously distribute in the environment where human body can easily access to. Although the previous studies mainly focused on the extrinsic microorganisms which can cause human disease or their various function, more and more researches started to concern the microorganisms in/on human body [5]. The number microorganism in/on human body is about 10^{14} times more than that of human cells, which suggested that the microorganisms have a significant interaction with human body. It is estimated that more than 70% of microorganisms flourish in the colon in which microorganisms can get enough nutrients and space for their growth while the others can be founded on skin, stomach, respiratory tracts and urinary tract [75].

Gut microbiota refers to the microorganisms that live in the gastrointestinal tract, which contains approximately 66% anaerobic bacteria and 34% facultative aerobic bacteria and aerobic bacteria [76]. Human gut microbiota is composed of seven bacterial phyla, including *Bacteroidetes, Firmicutes, Proteobacteria, Verrucomicrobia, Actinobacteria*, *Fusobacteria*, and Cyanobacteria, of which *Bacteroidetes* and *Firmicutes* account for the majority. According to the multiple studies on the sequencing of gut microbiota, more than 500 species are identified in the colon, suggesting that the large diversity of microorganisms [77]. O'Hara et al. reported that amount of microorganisms are not equally distributed in the whole gastrointestinal tract. It is estimated that less than 10^4 bacteria per gram in the upper gastrointestinal tract while about 10^{12} bacteria in the lower gastrointestinal tract (Fig. 2.1A) [5] [78]. In addition to the quantity of bacteria, each part of gastrointestinal tract is occupied by different kinds of bacteria. A study analyzed the composition of bacteria in small intestine and colon and concluded that the majority

bacteria phyla in small intestine are *Firmicutes* and *Actinobacteria* while the *Fimicutes* were enriched at colon [79]. The colon is constituted by three different parts, epithelial surface, mucosa and intestinal lumen, in which the types of flourishing bacteria are different. Swidsinski et al. indicated that *Bacteroides*, *Streptococcus*, *Bifidobacterium* only present in the feces (Fig. 2.1B) [5] [80].

Human gastrointestinal tract acquires bacteria through different ways. At birth, the gut microbiota mainly come from mother's canal where the composition of gut microbiota is similar to colon. If the infant is delivered by cesarean section, both the diversity and the quantity of the gut microbiota would dramatically reduce due to the lack of exposure to mother's gut microbiota [81]. After birth, the composition of gut microbiota get shaped easily by the diet and environment but become stable after 1 year old (Fig. 2.1C) [5].

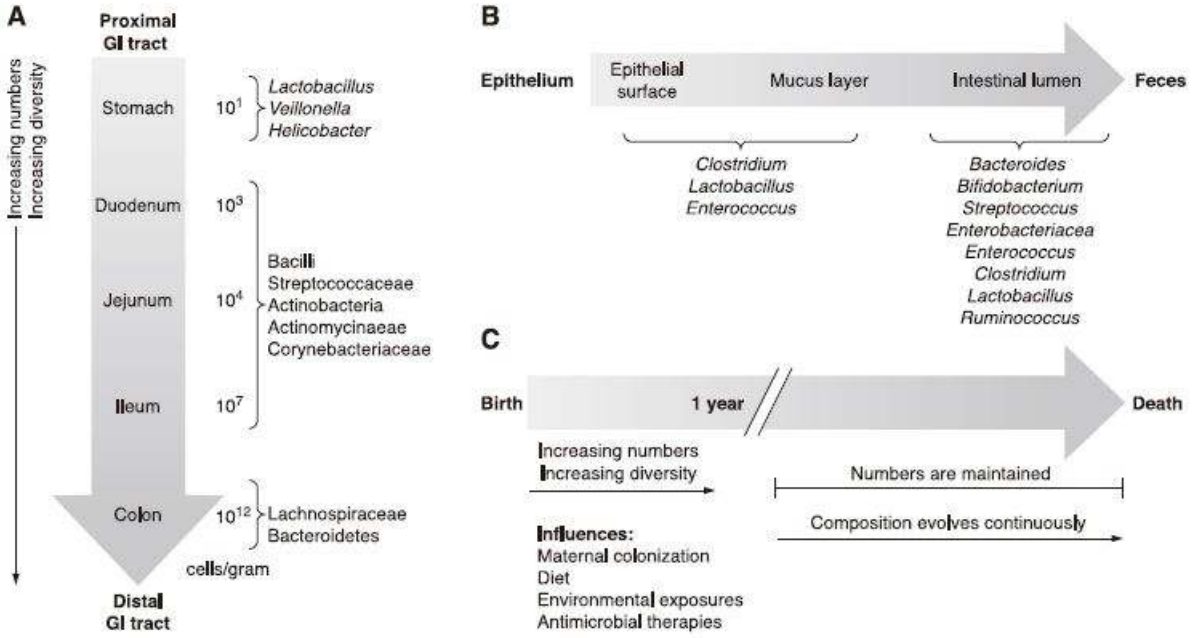


Figure 2.1 Summary of the introduction of human gut microbiota. A: the composition and quantity of gut microbiota on different sites of the gastrointestinal tract. B: the composition in the different parts of intestine. C: the factors for shaping microbial composition

2.3.2 The function of gut microbiota

The role of gut microbiota in nutrition and health of mammalian host is the result of the long time adaption and coevolution. In order to maintain the symbiosis relationship with gut microbiota, the immune system not only need to control the population and the activity area of bacteria, but also restrict the immune response to endure the growth of bacteria Gut microbiota not only can induce the immune response of the host, but also can offer nutrients through their various activities, which are the mainly function of gut microbiota [82].

The presence of gut microbiota can modulate the immune system and contribute to the immunological development. Gut microbiota is considered as the extrinsic "organ" by the immune system, which can continually trigger the immune response to protect the host. Macpherson and Harris conducted an experiment on germ free mice and reported that the immune system of germ free mice was not fully developed. For example, the germ free mice have less amount and types of immune cells comparing with the normal mice. The formation of the organs associated with immune system such as spleens and lymph nodes did not fully complete, resulting in the function deficiency [83]. Another study indicated that germ free animals were not capable to produce enough CD4+ T cells, whereas the treatment of polysaccharide of *bacteroide fragilis* can trigger the proliferation of CD4+ T cells and formation of spleen white pulp [84]. The family of Lactobacilli spp. can affect the activation of various cytokine and immune cells through modulating the differentiation of dendritic cells [85].

Gut microbiota can also protect the host against the invasive pathogens. A large number of bacteria colonize on the surface of intestinal mucosa which form a natural barrier between mucus surface and extrinsic bacteria, especially the pathogen [86]. Besides, gut microbiota can induce the immune system to generate different antimicrobial peptides, including defensins,

cathelicidins and C-type lectins, which can interact with gut microbiota to modulate their growth and composition [87]. Cash and colleagues reported that the complete composition of gut microbiota can trigger the production of antimicrobial peptides in paneth cells while the gut microbiota with low diversity may not activate the same function or induce the low intensity production [88]. Lopez et.al even pointed out that the presence of *B. thetaiotaomicron* can stimulate the production of matrilysin, leading to the increasing activation of defensins [89]. Many studies have indicated that probiotics have protective effect against various pathogens. For example, an in vitro cell cultures study reported that Lactobacillus and Bifidobacterium can inhibit the Listeria infection by the metabolites and interaction of immune response [90]. Studies also pointed out that lactic acid produced by the commensal bacteria can prevent the growth of pathogens [91].

In terms of metabolic ability, gut microbiota contribute to the host through providing nutrients and further processing xenobiotic which cannot be digested by the host. Jeremy and colleague reviewed that germ free animal gained less weight than normal animal with the same amount of food consumption [92]. The possible mechanisms is that gut microbiota can extract extra calories by utilizing indigestible compounds as well as increase the absorption rate of the nutrients by promoting the absorptive ability of intestinal epithelium. For instance, gut microbiota can digest dietary fiber, polysaccharide to short chain fatty acid, monosaccharide respectively, which can be utilize by the host (Fig 2.2) [93]. Short chain fatty acid is one of the most important metabolites of gut microbiota which is not only the energy source for the host, but also can suppress the production of toxic metabolites and the proliferation of various types of pathogen. Besides, gut microbiota can interact with lipid absorption and deposition through the augmenting the secretion of lipoprotein lipase in the gastrointestinal tract [94].

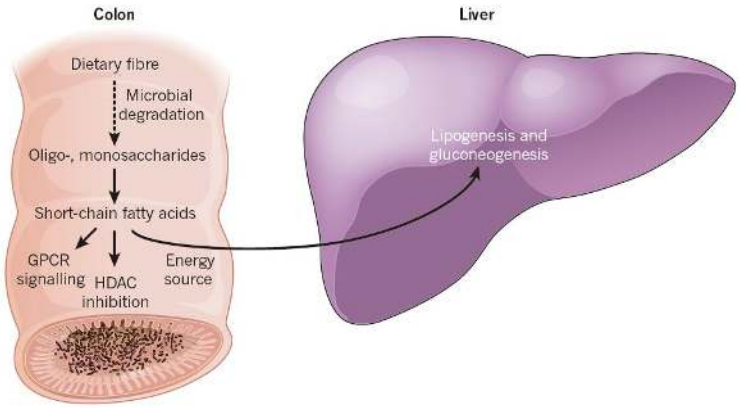


Figure 2.2 Microbial metabolic pathway of dietary fibers

2.3.3 The role of gut microbiota in colon cancer progression

Although gut microbiota bring several important benefits to the host, it also have harmful effects on human health since not all the bacteria are commensal to the host. Many studies indicated that gut microbiota is the risk factor for carcinogenesis through different mechanisms [95]. Some species of bacteria can stimulate the excessive proinflammatory signaling at the intestinal mucosa which will lead to the damage of intestinal epithelium and the initiation of colorectal carcinomas. Besides, bacteria itself is toxic to the intestinal epithelium or induce toxicity through host cells to tissue. Moreover, some groups of bacteria can transform the nutrients to the toxic product which can promote the neoplasia by damaging the intestinal mucus layer and inducing the repair process.

According to the several epidemiological studies, the composition of gut microbiota in colorectal carcinogenesis patients were significantly different from that in the health person. Moore reported that the number of family of *Bacteroides* and *Fusobacterium spp*. enriched in the patients with colon cancer, suggesting that the decreasing diversity of the gut microbiota. In

health person, the majority of gut microbiota are the family of *Lactobacillus spp.*and *Eubacterium aerofaciens*, which can produce short chain fatty acid to protect the host against pathogen [96]. The studies implicated that the presence of several species of gut microbiota may be in favor of the progression of colorectal carcinoma. In contrast, Manoj et al. indicated that probiotic (such as *bifidobacteria* and *Lactobacillus spp*) and its metabolites may plan an role on cancer prevention through the ability to eliminate the carcinogen [97].

2.3.4 The role of gut microbiota in inflammatory bowel disease development

Inflammatory bowel disease is caused by the excessive immune response in gastrointestinal tract, which can be attributed to several reasons. Gut microbiota is generally suspected to be one of the risk factors for the development of intestinal bowel disease, especially Crohn's disease. Several epidemiological studies reported that antibiotic treatment could significantly attenuate the inflammatory lesion in inflammatory bowel disease patients, suggesting that the activity of gut microbiota may stimulate the over expression of pro-inflammatory cytokines [98]. Multiple animal studies also revealed the relationship between gut microbiota and inflammatory bowel disease. Sellon et al. indicated that the development of intestinal inflammation was prevented in interleukin- 10- ($IL10^{-/-}$) mice in germ-free state while severe colitis presented in conventional environment [99]. Kent et al. described that the intestinal colitis in rats could be reversed by the usage of antibiotics [100].

The composition of gut microbiota is proved to play an important role in the development of intestinal colitis. $Tbx21^{-/-}Rag^{-/-}$ mice lost the ability to modulate and develop their immune system, which are prone to acquire severe colitis. Garrett et al. reported the health state of $Tbx21^{-/-}Rag^{-/-}$ mice can be restored after treating with antibiotic, proving that the cause of colitis in mice is gut microbiota. Moreover, the translocation of gut microbiota from $Tbx21^{-/-}Rag^{-/-}$ mice

with intestinal inflammation disease to wild-type health mice resulted in the development of colitis in wild-type mice, indicating that the certain species of bacteria are the stimuli to the intestinal inflammation [101]. Although the types of bacteria involved in the development of inflammatory bowel disease are still under investigation, several studies pointed out the presence of pathogenic *E.coli* was strongly associated with intestinal inflammation (Fig 2.3) [5]. In contrast, when severe colitis induced in mice, some bacteria in gut secreted vitamin D to protect against the inflammatory process, which may be the reaction of bacteria to the growth suppression producing by the inflammation [102]. Sokol et al. reported that *F. prausnitzii* has the ability to modulate the pro-inflammatory cytokines and restore the immunological equilibrium of the host [103].

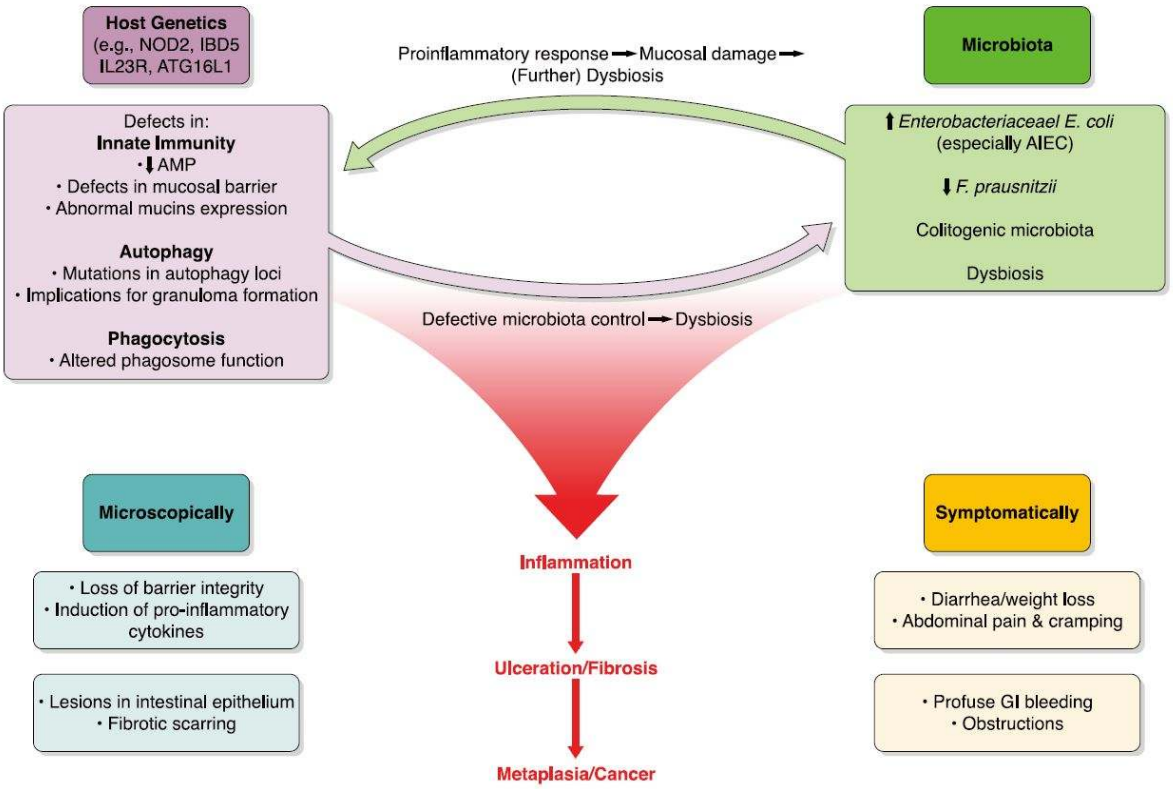


Figure 2.3 The interaction between pathogenic E.coli and the immune system

2.4 Cranberry and its phytochemicals

2.4.1 Introduction to cranberry

A large amount of scientific evidence proved that fruits and vegetables comsumption not only offer necessary nutrients to human body, but also maintain the health condition and protect body against various diseases. It has been confirmed that cardiovascular disease, colon cancer, Alzheimer's disease and obesity can be prevented through increasing intake of fruits and vegetables [104]. It is believed that the phytochemicals in fruits and vegetables such as flavonoids, phenolic compounds, stilbenes and carotenoids, plan an important role in the disease prevention [105]. Some of the phytochemical compounds are strong anti-oxidative agents which can inhibit with excessive oxidative process in the body to exert their protective effect. Others which do not possess the anti-oxidative ability may affect the disease pathway, the activity of microorganisms and the expression level of transciptor to modulate the development of disease or the immune response [106].

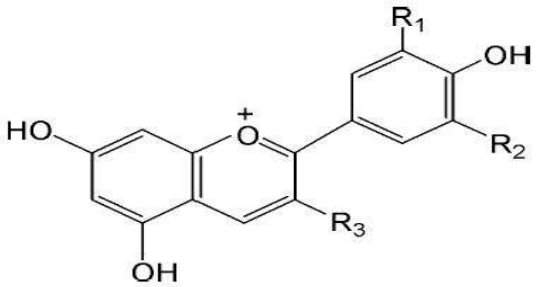
Cranberry (*Vaccinium macrocarpon*) is a kind of native American fruit, which has been consumed as food for a long time. Cranberry jam is a traditional food served in Thanksgiving meal in United States and widely consumed in north Europe [107]. In United States, the total cranberry crop was up to 8.6 million barrels, which value for 300 million dollars in 2014 [108]. The large consumption of cranberry is not only attributed to the good flavor and taste of cranberry but also to their potential health benefits. Cranberry juice has been used as the traditional treatment for urinary tract infections in woman for centuries, which believed to be the effect of one of its unique phytochemicals, proanthocyanins [109]. Recently, cranberry has been widely studied for its nutrition and strong anti-oxidative effect. It is believed that

phytochemicals of cranberry may be the natural chemopreventive agents to several diseases, such as pathogen infection, inflammation and cancer.

2.4.2 Major phytochemicals of cranberry

Cranberry is rich in various kinds of phytochemicals, including anthocyanins, proanthocyanins, phenolic acids and triterpenoids [110]. Anthocyanins is the major pigments of cranberry, which bring the red color to the ripe cranberry fruit. 100 g cranberry fruits can contian up to 140 mg anthocyanins, while the processing of cranberry juic would lost 90% of anthocyanins [111]. Wu et al. reported that thirteen types of anthocyanins (Table 2.1) were founded in freeze dried cranberry sample through LC-MS/MS, of which more than 95% are the basic backbone strutures with cyanidin and peonidin [112]. Mulleder et al pointed out that anthocyanins with glycosides would be easier to get absorbed by the body than the anthocyanins with other strutures, suggesting that the cranberry anthocyanins are the great source for anthocyanins intake [113].

Table 2.1 The strucures of anthocyanin



Structure #	Anthocyanin Name	R_1	R_2	R_3
1	cyanidin-3-*O*-galactoside	OH	H	Galactose
2	cyanidin-3-*O*-arib inoside	OH	H	Arabinose
3	cyanidin-3-*O*-glucoside	OH	H	Glucose
4	peonidin-3-*O*-galactoside	OMe	H	Galactose
5	peonidin-3-*O*-arib inoside	OMe	H	Arabinose
6	peonidin-3-*O*-glucoside	OMe	H	Glucose
7	malvidin-3-*O*-arib inoside	OMe	OMe	Arabinose
8	malvidin-3-*O*-galactoside	OMe	OMe	Galactose
9	pelargonidin-3-*O*-arib inoside	H	H	Arabinose
10	pelargonidin-3-*O*-galactoside	H	H	Galactose
11	delphinidin-3-*O*-arib inoside	OH	OH	Arabinose
12	petunidin-3-*O*-galactoside	OMe	OH	Galactose

Proanthocyanins (PACs) are a group of oligomers or polymers of flavan-3-ols, which have large molecular weight and many free hydroxyl groups. 100 g fresh cranberry fruits contain up to 418 mg PACs, which is much higher than the other commonly consuming fruits [114]. Due to their complicated structure and similarity, PACs in cranberry cannot be completely separeted and identified through current analysis method. Among the known struture of PACs (Fig 2.4) in cranberry, the majority of PACs are procyanidin A2 which has A-type linkages while procyanidin B2 with B-type linkages accounts for less than 10% [115]. Besides, A-type procyanidin is only found in cranberry fruit, indicating that cranberry is the best food source for this phytochemicals.

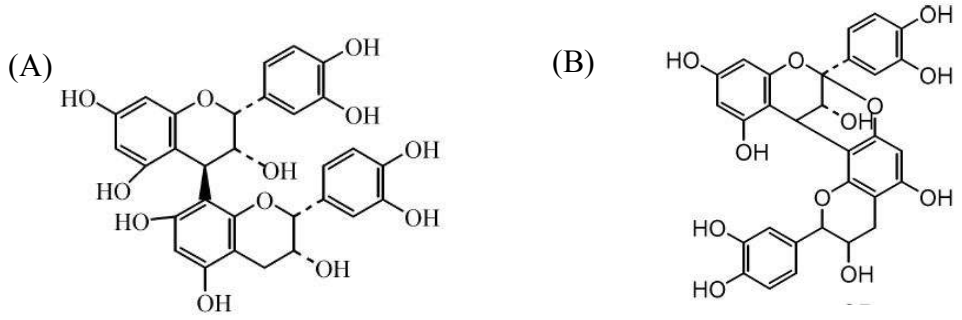


Figure 2.4 The structures of cranberry proanthocyanins: (A) procyanidin B2 (B) procyanidin A2

Ample kinds of phenolics and benzoates are found in the cranberry which contribute to the unique aroma of cranberry as well as the strong antioxidative activity. However, most of phenolics and benzoates in cranberry are binding with different compounds instead of free form. For example, Zuo et al. reported that many phenolics form a linkage with multiple polysaccharide like celluose in cranberry to increase the stability [116]. Chen et al. further quantified the amount of phenolics and conlcused that about the amount of bound phenolics in

cranberry was three times more than the free phenolics after, indicating the importance of bound phenolics in further study [117].

Terpenes and their derivatives which contribute to the odors of most of food also present in cranberry. The most recognized terpenes in cranberry is ursolic acid (Fig 2.5), a pentacyclic triterpene. The concentration of ursolic acid can be up to 110 mg in every 100 g cranberry fruits [118]. Huang et al. indicated that ursolic extracted from cranberry can mediate the expression level of pro-inflammatory cytokines, suggesting that the potent health benefits from cranberry may partly come from ursolic acid [119].

Flavonols is another group of compounds which is enriced in cranberry fruits. Quercetin, myricetin and their derivatives are the major flavonols in cranberry (Fig 2.5) [106]. Vvedenskaya et al. reported in total 22 kinds of flavonls was found in freeze dried cranberry powder, some of which have unique struture [120]. In addition to its ample diversity, the content of total flavonls in cranberry was really high, which was up tp 400 mg/kg, suggesting the potential health beneficial effect.

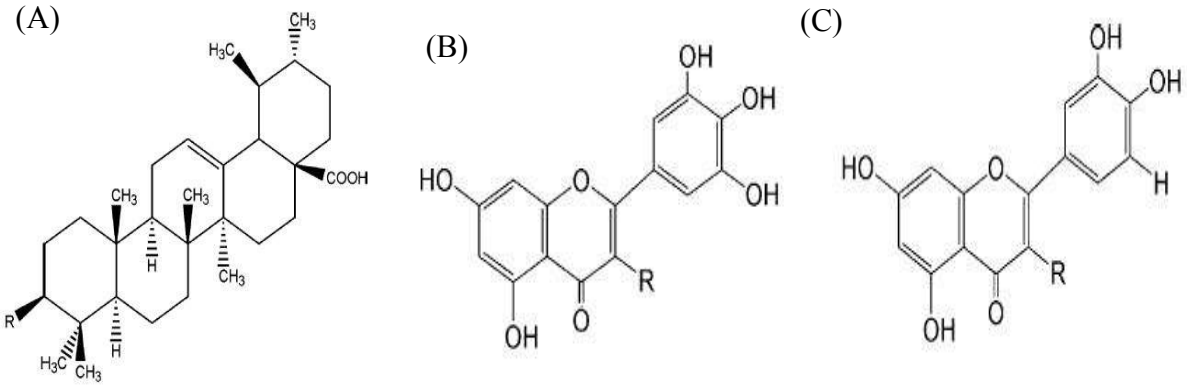


Figure 2.5 Structures of (A) ursolic acid (B) quercetin (C) myricetin in cranberry

2.4.3 The health effects of cranberry and its phytochemicals

Many studies indecated that the intake of cranberry r the phytochemicals extracted from cranberry can lead to several health beneficial effects which can be divided into two catorgries, pathogen inhibition and disease prevention.

Cranberry have long been used to treat the urinary tract infections which are mainly happen in women. It is reported that about 11 million women get the urinary tract infections every year and nearly one in five cured women re-gain the infections in 6 month [121]. The high incidence and recurring rate rise the attention to cranberry as it is a traditional folk remedy to urinary tract infections. Bailey et al. conducted a clinical study on the effect of creanberry extract on preventing urinary tract infections in women, which proved that the consumption of cranberry extract can lower the recurrent rate [122]. Stother reported the similar inhibition result by using cranberry powder, suggesting that the protective effect of cranberry agasint urinary tract infections was reliable [123]. Kontiokari et al. also pointed out that cranberry juice comsumption significantly decrease the incidence rate of urinary tract infection of 300 women [124]. Although cranberry can prevent the adult women from contracting the urinary infection, their effect on reversing the infection is barely confirmed by the clinical studies, indicating that cranberry can be used as prophylactic for urinary tract infections for women. Urinary tract infections is mainly cause by *Escherichia coli*, which is difficult to elimminate by using antibiotic since *Escherichia coli* can endure the presence of antibiotic after recurrence [125]. However, Gupta et al. indicated that cranberry prevent the adherence of *E.coli* to urinary tract instead of killing the bacteria, leading to the better way for infection prevention [126]. A-type proanthocyanins and the high acid content of cranberry are believed to be the two major phytochemicals that suppress the adhesion of *E.coli* [109].

In addition to the prevention on the urinary tract infection, cranberry also suppress the infection of other organs. Study showed that gastrointestinal disease caused by the *Helicobacter pylori* infection can be prevented by cranberry [127]. Cranberry prevent the infection through reducing the attached *h.pylori* to the epithelia surface of stomach. Burger et al. reported that cranberry extraction of compounds with high molecular weight exerted the anti-adhesion effect on *h.pylori* [128]. Accumulated evidence and studies suggested that phytochemicals in cranberry can be used to inhibit bacterial infection in the mouth, and therefore protect against dental caries and periodontal disease. Duarte et al. reported that cranberry proanthocyanins and flavonol can suppress the formation of pathogenic biofilm and reduce the infection [129]. Labrecque et al. indicated that the growth of several pathogen such as *Porphyromonas gingivalis and Treponema denticola* in mouth can be inhibited by the administration of cranberry extraction [130].

Due to their strong anti-oxidative effect, cranberry and its phytochemicals is believed to have potent anti-inflammatory activity and anti-cancer activity. Kandil et al reported that cranberry proanthocyanins can inhibited the activity of ornithine decarboxylase, which is one of the regulator of tumor suppression pathway [131]. Youdim et al. indicated that cranberry anthocyanins can mediate the expression of TNF-α and other cytokines to control the inflammation process in microvascular cells [132]. A review summarized the in vitro anti-cancer study on cranberry and conclude that phytochemicals in cranberry can be a possible chemopreventive agents for various cancer [12]. With the concentration as low as 20 μg/mL, proanthocyanins in cranberry can inhibit the growth of various types of cancer like breast and colon cancer. The concertation for ursolic acid and quercetin to show suppression effect on cancer cells were a 42 μg/mL and 20 μg/mL, respectively. Although the in vivo studies on the

beneficial effects of cranberry are contradictory, it is important to further study the potent mechanism of the health benefits of cranberry phytochemicals.

2.4.4 The bioavailability of cranberry and its phytochemicals

Bioavailability refers to the absorption rate of nutrients in the body, which can be measured by the concentration of the nutrients. The bioavailability is important for evaluating the bioactivity of nutrients in vivo study since the nutrients must be absorbed and delivered to targeted sites in order to exert their ability. The reason why in vitro studies and in vivo studies may have different result on the function evaluation of phytochemicals is the difference of the absorption rate in vitro and in vivo. Ample emerging studies focus on the bioavailability of cranberry phytochemicals (Table 2.2), indicating that the huge difference of bioavailability among various types of phytochemicals [106].

Table 2.2 Bioavailability of cranberry phytochemicals

Phytochemical Class	Peak Plasma Conc.	T_{max} (h)	Tissues Where Available	Primary Forms Detected In Vivo	Urinary Excretion*
Anthocyanins	0.001–0.2 μM	~ 0.7–4	Brain	intact, methylated	0.004—5.1%
Flavonols	0.05–7.6 μM	0.5–9.3	lungs, testes, liver, kidney, heart	intact, glucuronided, methylated, sulfated	0.07–7%
Catechins	0.03–5.9 μM	0.4–4	liver, kidney	intact, methylated	0.1–55%
PAC dimers	ND-41 nM	2	—	intact	ND-1.0%
PAC trimers and polymers	ND	ND	ND	phenolic acids, monomers (metabolism in intestine)	ND
Phenolic Acids	ND-40 μM	0.5–3	—	intact, methylated glycine conjugated	27%–39.6%
Trans-Cinnamic Acids	0.006–40 μM	0.5–3	—	intact, glucuronided, methylated, sulfated	0.3–61.7%
Stilbenes (resveratrol)	ND-32 μM	4	liver, kidney	glucuronided, sulfated	2.3%

ND = not determined

Cranberry proanthocyanins have the poorest absorption rate comparing to all the phytochemicals due to its large molecular weight and complex structure. It is suggested that the proanthocyanins have the ability to denature protein and cause potent adverse effect on body, resulting in the resistance on absorbing [133]. Deprez et al. reported that only proanthocyanins oligomers can be absorbed directly by the body while polymers go through degradation and digestion by gut microbiota in colon [134]. The metabolites of proanthocyanins with small molecular weight can be easily absorbed and exert different beneficial effects.

Anthocyanins is another poor absorbed phytochemicals in cranberry. Andres et al. indicated that small portion of anthocyanins can pass the brain barrier and get to brain, which offer a new sight for brain disease treatment [135]. Flavonols and phenolic acid have much higher bioavailability. Pedersen reported that the concentration of phenolic acid went up to 600 mg/L gallic acid equivalents in human plasma and most of them excreted through urine after consuming cranberry juice. The high content of phenolic acid in urine may contribute to the protective effect on urinary tract infection.

CHAPTER 3

INTERACTION BETWEEN CRANBERRY AND GUT MICROBIOTA AND ITS IMPLICATION ON ANTI-INFLAMMATION

3.1 Introduction

Inflammatory bowel disease (IBD), including Crohn's disease (CD) or ulcerative colitis (UC), which will lead to the epithelial dysfunction or increasing mucosal permeability, are prevalent in western countries, including western Europe and United States. It is estimated that 1.4 million patients are suffering from the IBD while $6.3 billion is used for the treatment of IBD annually [136]. Environmental factors like western diet and smoke are suspected for the major cause for the IBD, leading to alteration of the microbial structure and function in the IBD patients. In distal ileum and colon, microbiota have extraordinary interaction with the host's epithelial cells and mucosal immune system, which will affect host physiology, metabolism, nutrition and immune function. Microbiota and the immune system in the intestine form a homeostasis to maintain the health status of the intestine. However, the disruption of homeostasis will bring significant influences on both the host and the gut microbiota [137]. Abundant clinical and laboratory research has showed that intestinal inflammation responses are the protective way of body to eliminate the commensal bacterial which have pathogenic potential [138].

Whole cranberry fruit have large amount of polyphenols and dietary fiber which cannot get absorbed and digested in the body. When they reach colon nearly intact, gut microbiota plays a significant part on the depolymerization and metabolizing. Cranberry have been widely studies for its anti-microbial, anti-adhesion and antioxidant properties. In vitro researches have indicated that cranberry juice can be a promise treatment for oral infections and urinary infections [139].

Xian et al. reported that several phytochemical such as ursolic acid, quercetin and flavonol can suppress oxidative stress in the cell with EC_{50} values as low as 10μM [110]. However, due to the low bioavailability of various polyphenols in cranberry, the bioactivity of cranberry is largely influenced by the degradation process of gut microbiota. To date, the interaction between cranberry and gut microbiota have not been published.

Meanwhile, the structure and function of the gut microbiota are expected to alter in response to the digestion of cranberry. Wang et al. reported the composition of gut microbiota in mice can be mediated by dietary polysaccharides and short chain fatty acid production, resulting in the attenuation of intestinal coliis [140]. Microbial activity plays a significant role on maintaining the physiological functions of colon and regulating the immune system [141]. It is known that probiotic in the gut can protect human from the process of inflammation while a great number of pathogens or pathogens-like species can cause the diseases due to their ability to produce toxin or stimulated agents. Accumulating experimental evidence indicated that the increasing amount of pathogen in colon is associated with the development of the inflammatory bowel diseases (IBD) [142]. *Helicobacter pylori* is the example for the potentially harmful microbe, which can induce chronic gastric inflammation and cancer [143]. E.coli has been studied widely for its pro-inflammatory effect in multiple animal models. Janelle et al. indicated that the presence of *Enterobacteriaceae* in Il10-/- mice can stumulate the inflammation and further promote the tumor development [144].

Besides, using their typical enzymes, gut microbiota can metabolize a large amount of substrates, like dietary fiber which can provide various health benefits. Valcheva et.al indicated that the increasing number of *Lachnospiraceae* and *Ruminococcaceae* resulted in the increasing production of short chain fatty acid like butyrate and acetate, which protect the mediate the

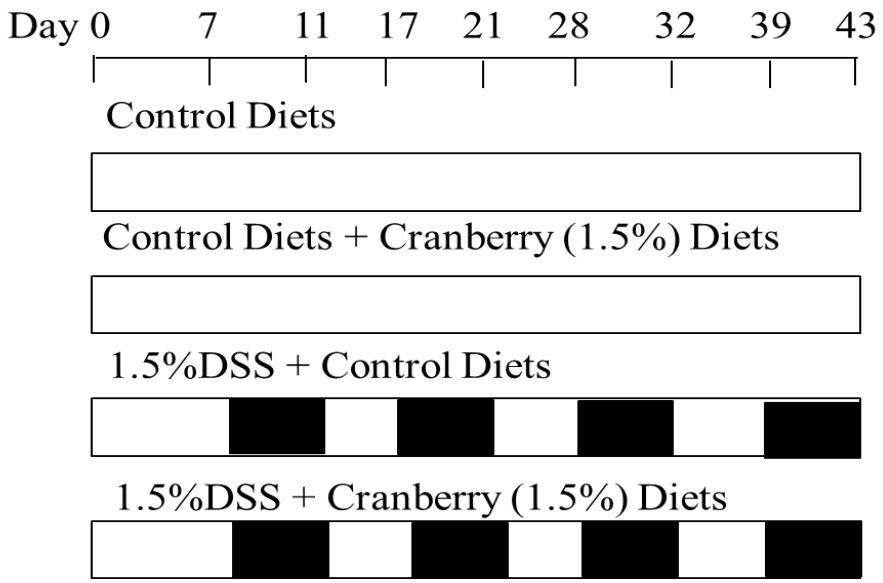
microbial translocation and inflammatory pathway [145].Therefore, modulation of the gut microbiota may be the possible way to improve the intestinal health and immune system. This an innovative work that was planned to understand the role of the cranberry and gut microbiota on anti-inflammatory effect through both mouse model. The molecular mechanism of the anti-inflammatory effect were investigated to explain the correlation between the host and them.

3.2 Materials and Method

3.2.1 Animals, diets and experimental procedure

The protocol for the animal experiment was approved by Institutional Animal Care and Use Committee of University of Massachusetts Amherst. 40 male wild-type CD-1 mice (6-8 weeks old) were obtained from Charles River Laboratories (Wilmington, MA, USA). The mice were divided into 4 groups (n=10 per each group) to receive either standard chow (AIN93G diet) or cranberry diet. Cranberry diet is made of AIN93G diet and 1.5 % (w/w) freeze dried whole cranberry powder. 5 mice were kept in a cage in animal facility where the environment maintained constant temperature (23°C) and humidity (65%). After one-week diet acclimation with respective diet, the four groups were distributed as follows: the control group, which was fed with standard chow and regular water; the DSS group, which received the standard diet and 1.5 % DSS water (wt/v, dextran sulfate sodium salt, average molecular weight 36,000–50,000) (International Lab, Chicago, IL, USA); the control-cranberry group, which received the cranberry diet and normal water; the DSS-cranberry group, which received the cranberry diet and 1.5 % DSS drinking water (wt/v). All experimental groups will be provided with respective drinking water ad libitum for 4 days and received the normal water for 7 days for total 4 cycles (Fig. 3.1). The weight of the mice were recorded every other day during the whole experiment. At the end of the third cycles of DSS water treatment, all mice were sacrificed by CO2

asphyxiation. The colons were removed from the body and got cut longitudinally to clean up the feces pellet. The weight and the length of the colon then were measured and recorded. After measurement, the whole colon were cut into two part longitudinally and kept separately by two ways for further analysis. One part of the colon were stored at -80°C for ELISA analysis while the other part were first fixed in 10% buffered formalin (pH 7.4) for 24 h and replaced by methanol for long term storage and further histopathological and immunohistochemical analysis. The feces pellet were collected from the colon and stored at -80°C for further sequencing analysis. Both the liver and the spleen were dissected from the body and weighted for evaluation of disease progression of ulcerative colitis.



Note: Black shade represents 1.5% DSS water treatment

Figure 3.1. Animal experimental design

3.2.2 Disease activity index (DAI) and histological evaluation of the colon

Disease activity index were determined basing on the scoring for weight loss, stool consistency and rectal bleeding, which were recorded every other day (Table 3.1a) [146]. The

fixed colon segments were first dehydrated by using ethanol and isopropanol, then get embedded in paraffin wax. The wax specimen were sectioned into slides which get stained by hematoxylin and eosin (H&E). Histological grading was evaluated basing on the inflammatory criteria (Table 3.1b) [147].

Table 3.1a Scores of Disease Activity Index (DAI)

Score	Body weight loss (%)	Stool consistency	Fecal blood
0	None	Normal	None
1	1~5	Soft but form	Hemoccult+
2	5~10	Soft	Blood
3	10~20	Diarrhea	Gross bleeding
4	>20		

Table 3.1b Histological scores

Score	Severity of inflammation	Extent of inflammation	Crypt damage
0	None	None	None
1	Mild	Mucosal	Basal 1/3
2	Moderate	Mucosal and submucosal	Basal 2/3
3	Severe	Transmural	Cryps lost but suface epithelium present
4			Crypts and suface epithelium lost

3.2.3 Enzyme-linked immunosorbent assay (ELISA)

Intestinal mucosa were removed and bead homogenized in phosphate buffer (PBS) solution (0.4 M NaCl, 0.05% Tween-20, 0.5% BSA, 0.1mM benzethonium, 1% protease inhibitor cocktail (Boston Bioproducts, Ashland, MA, USA)). After homogenization, the samples were centrifuged at 10,000g for 30 min at 0°C to collect the supernatant. The supernatant samples were loaded in sandwich enzyme-linked immunosorbent assay (ELISA) kits to determine the concentrations of IL-4, IL-13, IL-1β, IL-6, and TNF-α [147].

3.2.4 Isolation of DNA

Fecal pellets were collected from the mice colon to assess gut microbiota. Bacterial DNA was isolated from feces by using PowerFecal DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's protocols.

3.2.5 16S rRNA analysis

The concentration of the DNA samples was measured by NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA, US). PCR was performed to amplify the 16s rRNA gene marker using region of interest-specific primers with overhang adapters attached, which of sequences are: 16S Amplicon PCR Forward Primer = 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and (16S Amplicon PCR Reverse Primer = 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) [148]. 2x KAPA HiFi Hotstart ReadyMix (KAPA Biosystem, Wilmington, MA, US) was used as PCR reagent. Then PCR product was cleaned and purified by using AMPure XP beads (Beckman Coulter, Danvers MA, US). A second PCR was performed to attach dual indices and Illumina sequencing adapters using the Nextera XT Index Kit (Illumina, San Diego, CA, US)

followed by AMPure XP bead purification. The quantity of PCR products was determined by using Qubit dsDNA BR Assay kit (Life technology, Carlsbad, CA, US) while the size of PCR products was verified by DNA analysis ScreenTape Assay on Tape Station 2200 (Agilent Technologies, Santa Clara, CA, US). After quantification, 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, US).

3.2.6 Informatic and statistical analysis

Quantitative insights into microbial ecology (QIIME) software pipeline v1.9.1 was used to analyze the raw data files obtained from Illumina Miseq [149].

Since Miseq platform sequenced multiple samples via single sequencing run, QIIME first identified each individual samples through DNA barcode after inputting the raw data. Then QIIME removed sequence reads which have low quality to improve diversity estimates. Operational Taxonomic Units (OTUs) were clustered into represent groups and assigned to taxonomy using UCLUST. PyNAST was used in QIIME to align the sequence to constructed OTU table and phylogenetic tree basing on the public reference database. Alpha-diversity and rarefaction (number of taxa detected) was calculated and generated basing on the phylogenetic tree and OTU table. Beta-diversity and rarefaction (relative distribution of taxa in a community) was calculated and produced UniFrac distance between samples for bacterial 16S rRNA reads (evenly sampled at 600 reads per sample). UniFrac metric has two variants, weighted and unweighted. The weighted Unifrac metric consider the number of the OTUs and the relative abundance of each taxa while unweighted Unifrac metric only takes account of the number of the

OTUs [150]. The distance matrix were visualized with principal coordinate analysis (PCoA) which generated a plot with orthogonal axes explaining the maxium difference in the data [151]. The differences in the alpha-diversity were compared using a nonparametric two sample t-test with 999 Monete Carlo permutations. Results are presented as mean ± SD. The difference of the beta-diversity were test using ANOSIM with 999 permutations between every two groups.

The functional capacity of the gut microbiota were predicted using PICRUSt software. 16S rRNA sequences for taxa were input into PICRUSt to aligned KEGG gene orthologs basing on the Greengenes database [152]. STAMP software were used to calculated the difference among each group [153]. For all analyses, values of $P < 0.05$ were considered statistically significant.

3.3 Result

3.3.1 Disease activity index, colon length, and spleen enlargement

At day 32, mice in DSS-cranberry group had significantly low DAI scores compared to the mice in DSS group ($p<0.05$) (Table 3.2). The colon length in DSS group was significantly shorter than the colon length in DSS-cranberry group and control group ($p<0.05$) (Table 3.2). The spleen weight increased significantly in DSS group compared to the control group while cranberry diet reverse the weight augment ($p<0.05$) (Table 3.2).

3.3.2 Histological observation

The colonic specimens obtained from the DSS group showed serious erosive lesions with inflammatory cell infiltration and crypt shortening. However, in the DSS-cranberry group, the colonic specimens only had no erosion with just mild inflammatory cell infiltration. In both control group and control-cranberry group, no aberrant tissue was observed (Fig 3.2). Histological scores were given to each samples and the DSS group was significant higher than the DSS-cranberry groups (Table 3.2).

Table 3.2 Disease activity index (DAI), spleen weight colon length and histological score of each group

Groups	DAI	Colon length (cm)	Histological score	Spleen weight (mg)
DSS- Cranberry-	0^{c}	87.39 ± 3.85^{a}	0^{c}	125.35 ± 7.15^{b}
DSS- Cranberry+	0^{c}	94.19 ± 5.10^{a}	0^{c}	100.62 ± 4.21^{b}
DSS+ Cranberry-	3.62 ± 0.42^{a}	68.98 ± 2.01^{b}	7.11 ± 0.30^{a}	159.25 ± 18.97^{a}
DSS+ Cranberry+	1.56 ± 0.25^{b}	80.34 ± 7.42^{a}	4.55 ± 0.21^{b}	126.29 ± 8.91^{b}

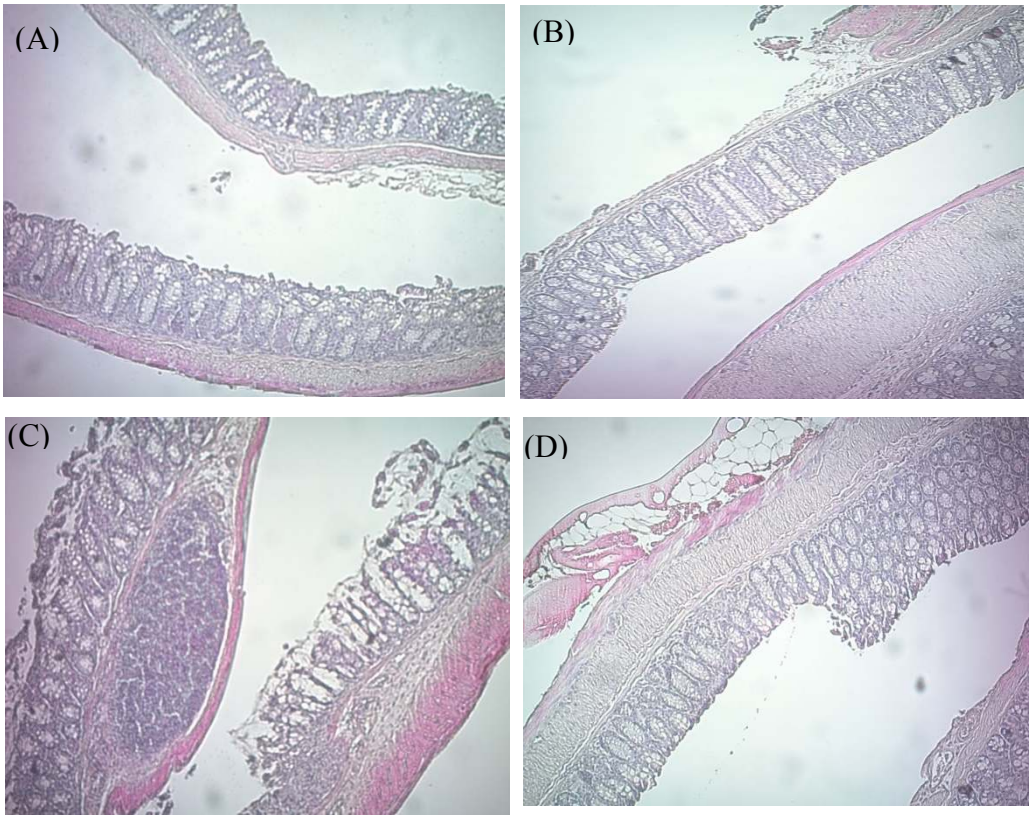


Figure 3.2 Histological characterization of the colon mucosa. Hematoxylin and eosin were used to stain the colon mucosa sample. (A) Control group (B) Control-cranberry group (C) DSS group (D) DSS-cranberry group

Figure 3.3A showed the concentration of pro-inflammatory cytokines (IL-1β, IL-10 and TNF-α) in the colonic mucosa. Compared to the control group mice, the pro-inflammatory cytokines over expressed in both DSS group mice and DSS-cranberry group mice. However, the expression of pro-inflammatory cytokines in the DSS-cranberry group mice were significantly lower than the cytokines level in the DSS group mice. The expression of IL-1β increased 80% in the DSS group while cranberry diet significantly reduced by 40% ($p<0.05$). Besides, the concentration of IL-6 elevated 95% in DSS group but the increasing rate was 65% in DSS-cranberry group, which significantly suppressed the 33% of the expression ($p<0.05$). The expression of TNF- α was enhanced by the DSS treatment by 86% while the cranberry diet significantly alleviated the expression by 37% ($p<0.05$).

The expression of cytokines in serum were different from the expression of them in colonic mucosa (Fig 3.3B). Even though there was slight reduction of the expression of TNF- α in serum in DSS-cranberry group mice, there was no significant difference between DSS-cranberry group and DSS group. Besides, the expression of IL-1β in the serum of all three groups was not high enough to identify by the Elisa kit. However, the concentration of IL-6 in serum had the similar trend as that in colonic mucosa. The DSS treatment augmented the expression of IL-6 by 78% while the cranberry diet reduced the augment by 50%. The anti-inflammatory cytokine IL-10 was significantly increased in both groups receiving DSS treatment. However, the cranberry diet enhanced the expression by 40% compared to DSS group.

The expression of IL-2, IL-12, CXCL-1and IFN-γ were measured in both colonic mucosa and serum, which were either no difference or no identified by the kit.

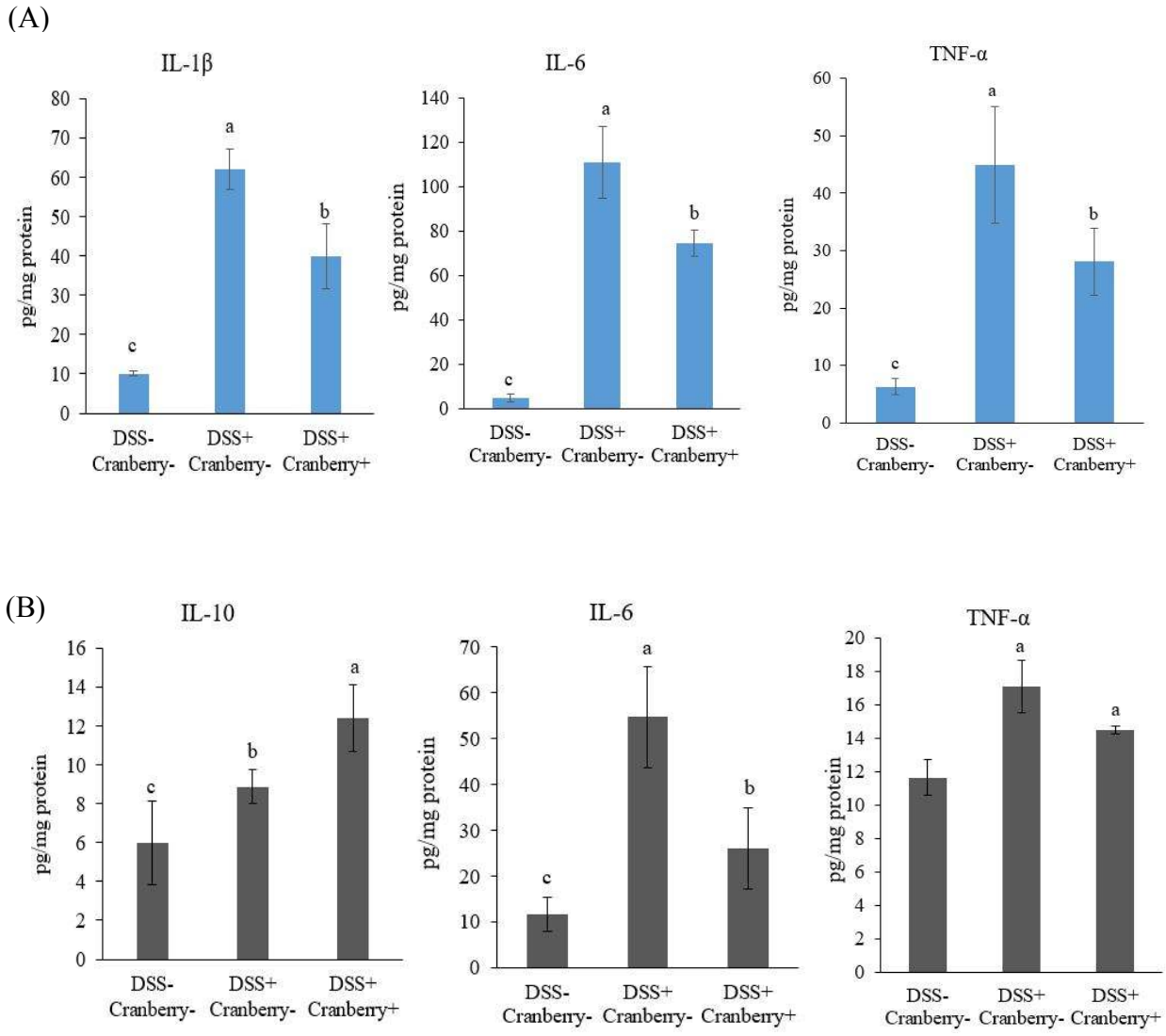


Figure 3.3 Effects of Cranberry treatment on protein levels of IL-1β, IL-6, and TNF-α in the colonic mucosa of mice (A) and serum (B). Data are shown as the mean ± SD of three independent experiments. Different letters (a, b, c) indicates statistically significant differences between groups ($p < 0.05$, n = 3).

3.3.4 Impact of cranberry on the composition of gut microbiota in mice

The result of high throughput sequencing were summarized in Table 3.3. In total, eight prokaryotic phyla were detected in the mice fecal pellet by QIIME. *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia* and *Actinobacteria* were five major phyla in all the four groups of mice (Fig. 3.4A), which was similar to other mice gut microbiota research [154]. Liner discriminant analysis (LDA) was applied to calculate the significant difference of relative abundance of phyla between different groups. LDA score of each phylum which were greater than 3.0 between every two groups indicated that the relative abundance of certain phylum in the group was significant higher than that of the other group (Fig 3.4B). Compared to control group and control-cranberry group, *Verrucomicrobia*, *TM7* and *Proteobacteria* were more abundant in DSS group while *Firmicutes* and *Actinobacteria* were less abundant. Besides, the relative abundance of *Verrucoicrobia* and *TM7* were also higher in DSS-cranberry group than the other two control groups. DSS group had the more amount of *Verrucomicrobia* and *Proteobacteria* and less amount of *Actinobacteria* than DSS-cranberry group. However, no difference was found between two control groups. The difference of these four groups indicated that cranberry treatment can reverse the alteration of the composition of gut microbiota in DSS group.

In the fourteen classes, *Bacteroidia* accounted for more than 40% in the four groups: control group 44.79%, DSS group 40.08%, control_cranberry group. 47.27% and DSS-cranberry group: 39.81%. The second dominant class were different among four groups. 28.86% and 20.32% of *Verrucomicrobiae* was identified in DSS group and DSS-cranberry group, respectively. However, 24.85% of *Bacilli* was founded in control group. In control-cranberry group, 24.05% of the bacterial class was *Clostridia*. Thirty-three families were identified in four groups of mice fecal pellet, among which, *Verrucomicrobiaceae* (28.86%) dominated in DSS

group while family *S24-7* in *Bacteroidales* order was must abundant in control group (32.64%), control-cranberry group (31.82%) and DSS-cranberry group (27.03%).

Moreover, 95 genera which belong to the eight phyla were detected in mice fecal pellet (Fig. 3.5). Among all the prokaryotic genera, *Bifidobacterium*, *Bacteroides*, *Lactobacillu*s, *Akkermansia*, *Sutterella* and two unknown genera in family *S24-7* and in order *Clostridiales* were the dominating kinds which account for about more than 50% of total bacterial cells. The relative abundance of each genera was used to calculate the difference between each group. Compared to control group, the relative abundance of 16 genera in DSS group had shifted significantly while the relative abundance of 11 genera in DSS-cranberry group had changed significantly. In control-cranberry group, there were 16 genera whose relative abundance were significantly different from that in DSS-cranberry group while the relative abundance of 13 genera were different from that in DSS group. Although no difference was detected in the phyla level between control group and control-cranberry group, 5 genera which had shifted the relative abundance was founded. Compared to the DSS group, the relative abundance of 15 genera had changed in DSS-cranberry group, in consistent with the difference in phyla level between these two groups.

Table 3.3 Summary of high throughput sequencing result

Number of observations	Total count	Minimum count	Maximum count	Mean
637	2754829	62219	272288	137741

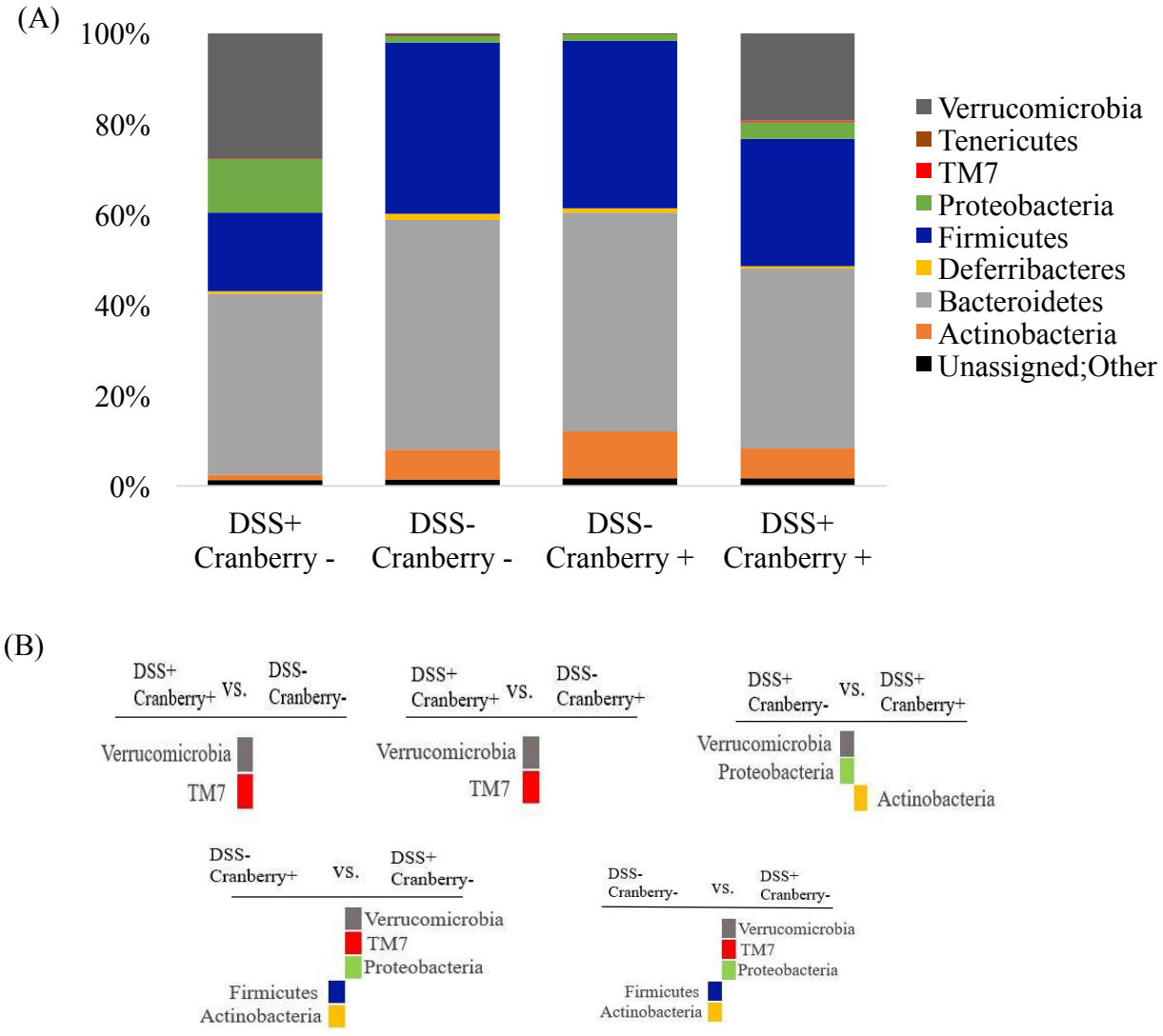


Figure 3.4 (A) Relative abundance of bacteria phyla in 4 sample groups (B) Difference in relative abundance of bacteria phyla in gut microbiota between sample groups. The LDA effect size (>3.0) was applied to identify the biomarkers.

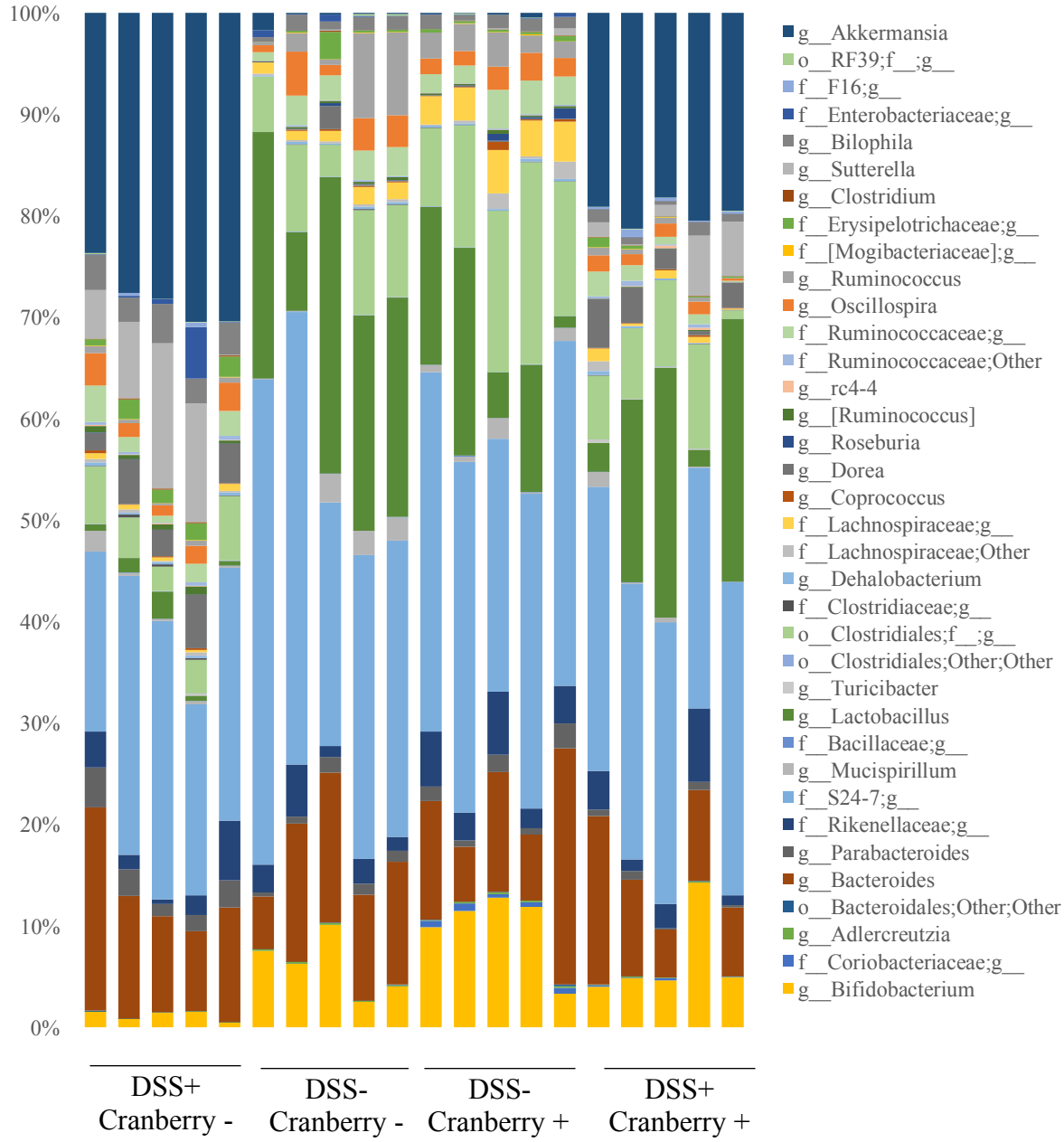


Figure 3.5 Relative abundance of bacteria genera in 4 sample groups

3.3.5 Impact of cranberry on the most abundant bacterial genera

Among all 96 genera, the relative of several most abundant bacterial genera had significantly shifted among different groups (Fig. 3.6). *Akkermansia* was the most abundant

genus in DSS group and DSS-cranberry which accounted for 28.27% and 19.32%, respectively, of the whole composition. However, less than 1% *Akkermansia* was identified in both control group and control-cranberry group. The aberrant dominance of *Akkermansia* in two DSS groups indicated that DSS treatment was in favor of the growth of *Akkermansia*, which may promote the inflammatory process in return. The cranberry diet significantly ($p<0.05$) reduced the abundance of *Akkermansia* in DSS-cranberry treatment, indicating that cranberry can prevent excessively proliferation of *Akkermansia*. In addition to *Akkermansia*, the number of *Sutterella* increased significantly ($p<0.05$) in DSS group (11.01%) and in DSS-cranberry group (2.59%), compared to control group (0.01%) and control-cranberry group (0.07%). *Bilophila* proliferated significantly ($p<0.05$) in DSS group (3.03%), which was three time more abundant than *Bilophila* in other three groups.

In contrast to the DSS group, *Lactobacillus* was the most abundant genus in the fecal pellet of mice in control group (24.50%) and control-cranberry group (19.19%). The relative abudance of *Lactobacillus* in DSS group and DSS-cranberry group significantly ($p<0.05$) dropped down to 0.51% and 14.53%, respectively. Besides, compared to DSS group (1.14%), *Bifidobacterium* genus were significantly more abundant in other three groups. The abundance of *Bifidobacterium* had 50% increasing in control-cranberry group (9.68%) compared to control group (6.51%) and DSS-cranberry group (6.42%). The abundance of Ruminococcus had decreased significantly from 2.72% (control group) to 0.46% and 0.43% in DSS group and DSS-cranberry group.

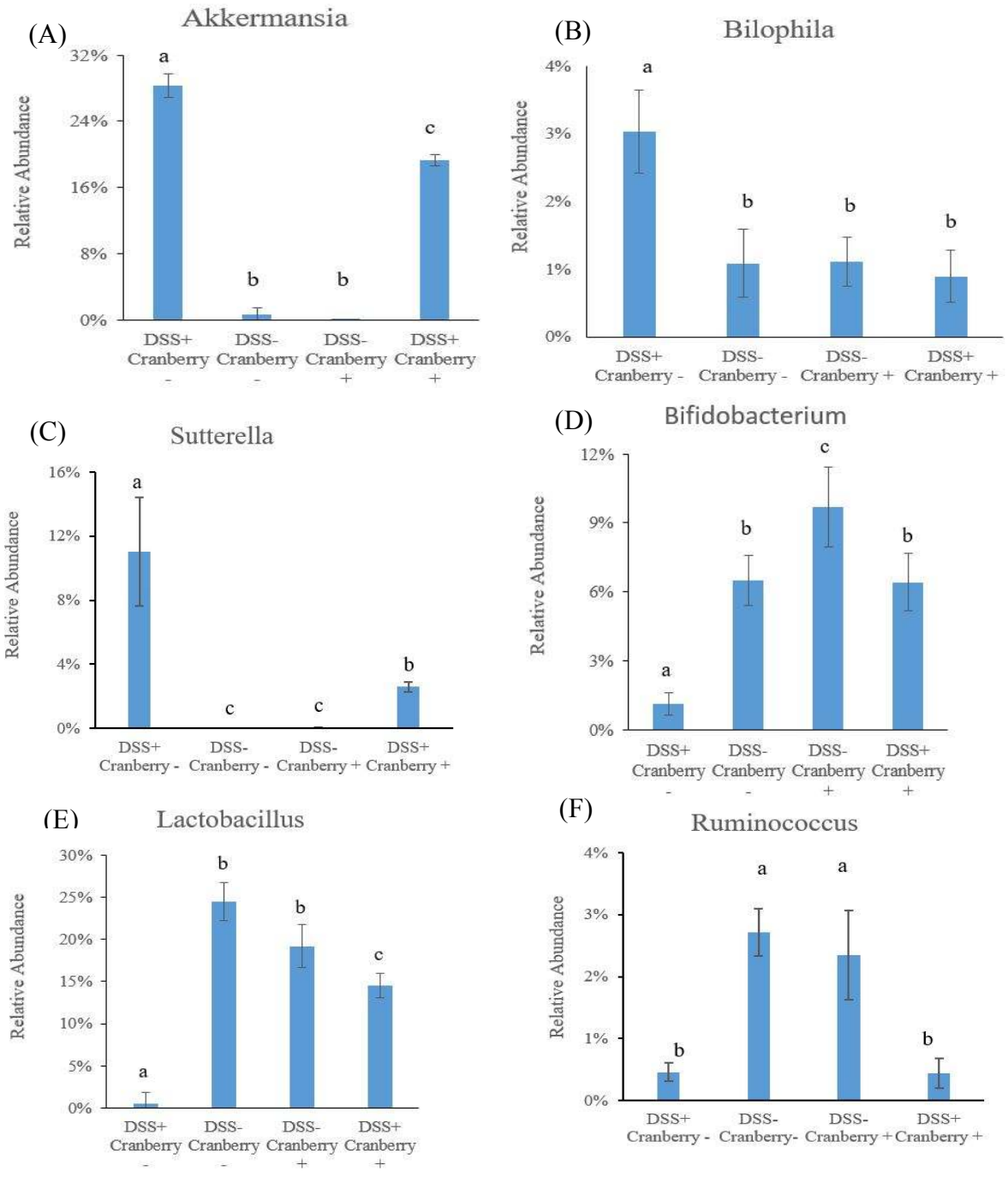


Figure 3.6 Relative abundance of genera (A) **A**kkermansia (B) Bilophila (C) Lactobacillus (D) Ruminooccus (E) Sutterella (F) Bifidobacterium. Each bar represents the mean ± SD. The bars with letters (a, b, c, d) are significantly different ($p<0.05$) from each other as determined by ANONA.

3.3.6 Impact of cranberry on the α-diversity and β-diversity of gut microbiota

Basing on the sequencing data, the bacterial α-diversity of the group receiving cranberry diet and normal water was significantly higher in subjects sampled in other three groups, which means that the most number of species were identified in control-cranberry group (Fig 3.7). Although the PD value of DSS group was the lowest, there was no significant differences in bacterial α-diversity among the control group and DSS-cranberry group. Cranberry can significantly enrich the bacterial diversity while DSS can prevent the enrichment of microbial richness by cranberry and has slightly reversing effect on the bacterial diversity in normal mice group. Although the cranberry diet can increase richness of the gut microbiota in the mice, the amount of these species did not account for enough portion to affect the total community structure. Therefore, the weighted UniFrac in cranberry diet group did not show significant difference from that of control diet group. However, the extended cranberry diet may be continually in favor of the growth of these species, leading to the shift of the community.

DSS alter the structural composition of the microbial community and cranberry attenuate the alteration (Fig 3.8). ANOSIM with 999 permutations was used to analyze statistical significance of 4 sample groups using distance matrices of weighted UniFrac. As expected, DSS significantly altered the gut microbial community of mice fed with control diet ($p< 0.05$, $R=1.0$) and cranberry diet ($p< 0.05$, $R=0.93$). Cranberry diet did not significantly shift the gut microbial community of mice in two groups receiving normal water ($p> 0.05$, $R=0.175$). However, Cranberry significantly alter the gut microbial community of mice in two groups receiving DSS water ($p< 0.05$, $R=0.75$). The community of gut microbiota in the group with DSS water and cranberry diet was also significantly different from that of the group with normal water and control diet ($p< 0.05$, $R=0.73$). However, the community of gut microbiota in the group with

normal water and control diet have more similarity with the cranberry diet and DSS water group than the community of gut microbiota in group with DSS water and control diet (R=1.0> R=0.73). This suggesting that the cranberry can alleviate the impact of DSS on the gut microbiota.

3.3.7 Impact of cranberry diet on the microbial function

Predictive metagenomics functional profile indicated that the metagenomics gene of different groups expressed in different level (Fig 3.9). The proportion of bacterial genes which related to cancers, neurodegenerative disease and xenobiotics biodegradation were higher in DSS group than in control group. Meanwhile, the expression level of genes related to cell growth, replication and repair, digestive system were lower in DSS group than in control group. Compared to DSS-cranberry group, the relative abundance of six functional genes in DSS group were higher including excretory system, immune system, cancers, lipid metabolism, neurodegenerative disease and signal transduction. However, the DSS-cranberry group mice expressed more genes related to replication and repair, enzyme families, cell growth and death.

The control-cranberry group mice had 15 different gene expression level from the DSS-cranberry group mice. In control-cranberry group, the proportion of genes related to cell motility, digestive system and nucleotide metabolism were higher while the proportion of other genes was lower such as cancers and infectious diseases. The bacterial genes in associate with cell motility, nucleotide metabolism and replication and repair were more abundant in the control group mice than in DSS-cranberry group mice. In contrast, the DSS-cranberry group mice had higher proportion of 5 kinds of genes, including excretory system, cancers, signal transduction, lipid metabolism and neurodegenerative diseases. There is no difference detected between control group mice genes expression and control-cranberry group mice.

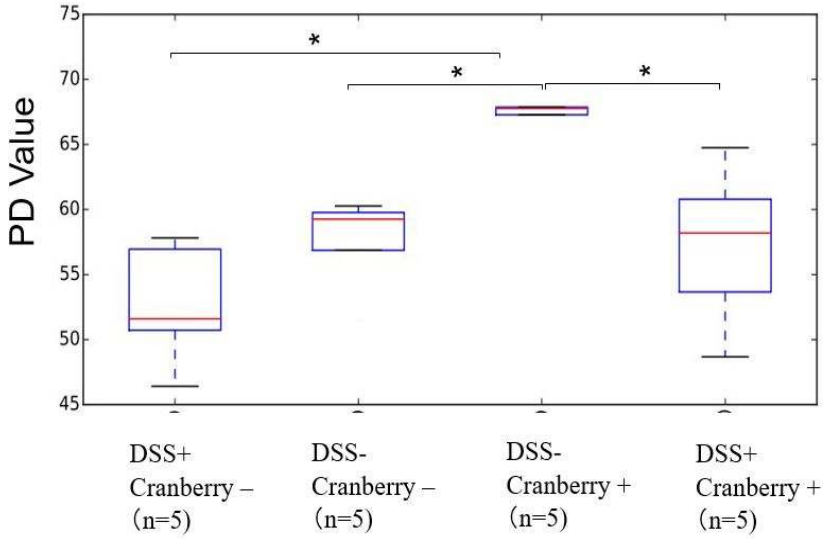


Figure 3.7 Bacterial diversity in 4 different sample groups. Box plots of alpha diversity were generated with rarefication to 60,000 reads per sample. The nonparametric P values were calculated using 999 Monte Carlo permutations. *, P value = < 0.05

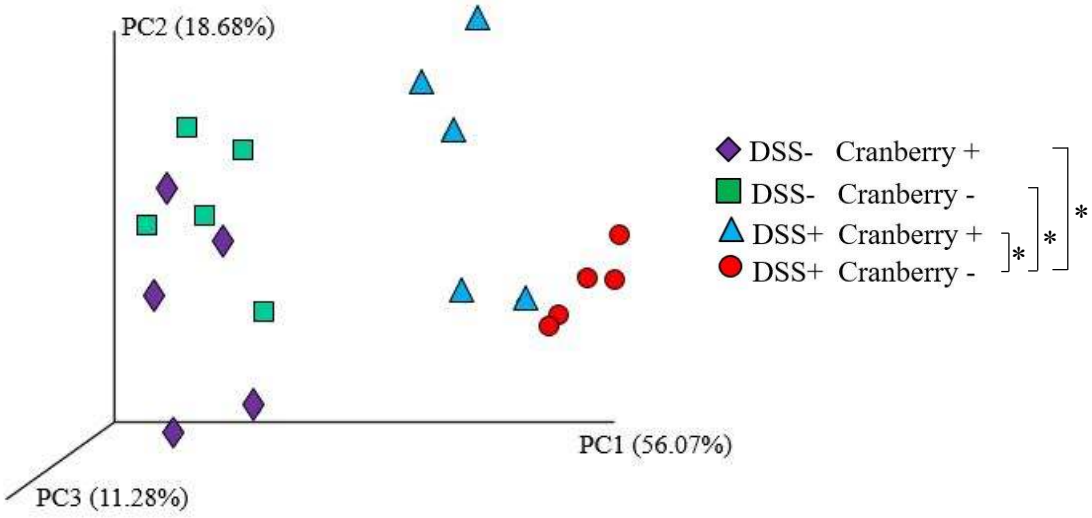
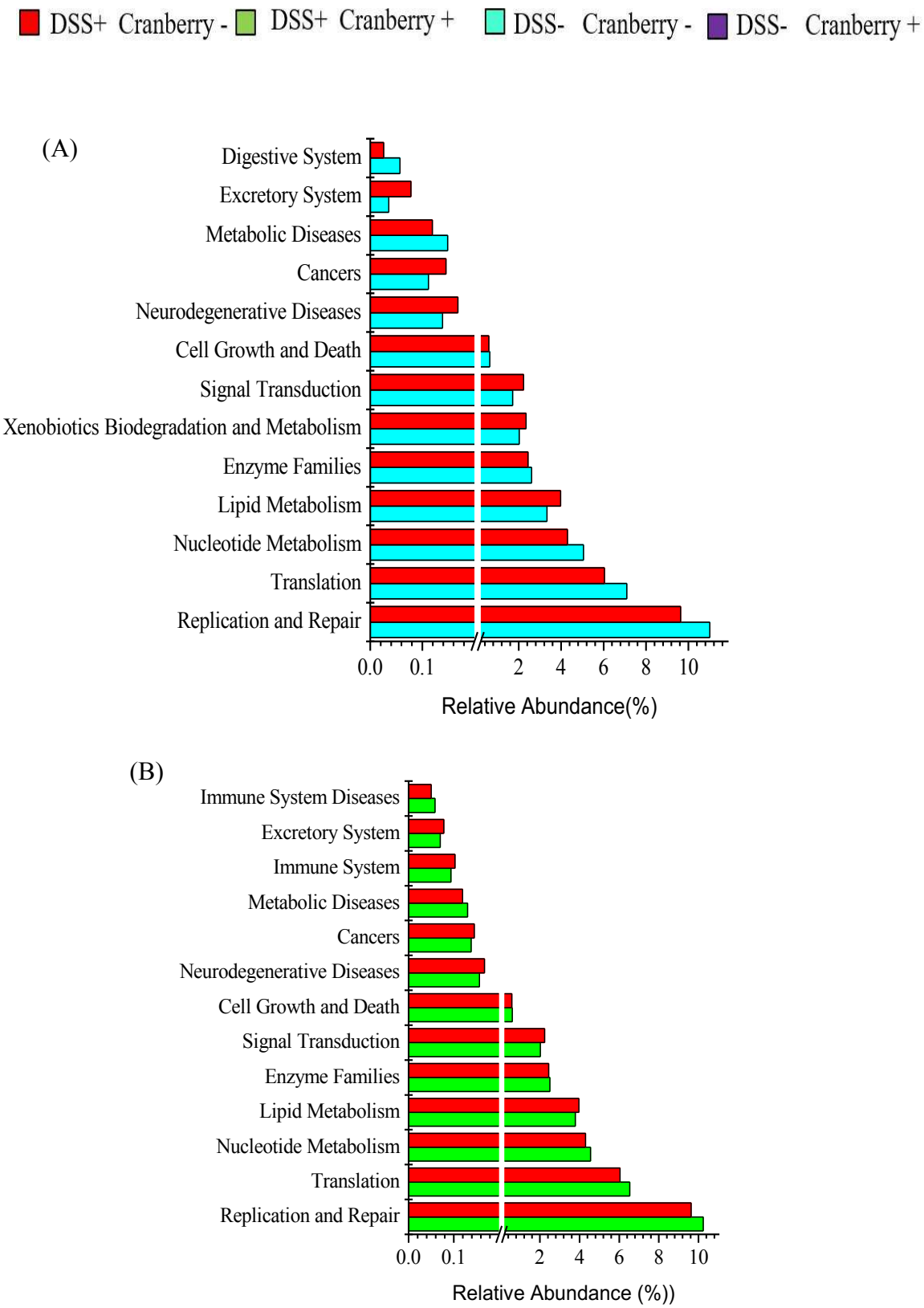


Figure 3.8 β-diversity in gut microbiota between groups. Weighted UniFrac distances PCoA graph was used to evaluate diversities between samples. *, P value <0.05.


DSS+ Cranberry -
DSS+ Cranberry +
DSS- Cranberry -
DSS- Cranberry +
(A)
Digestive System
Excretory System
Metabolic Diseases
Cancers
Neurodegenerative Diseases
Cell Growth and Death
Signal Transduction
Xenobiotics Biodegradation and Metabolism
Enzyme Families
Lipid Metabolism
Nucleotide Metabolism
Translation
Replication and Repair
0.0 0.1 2 4 6 8 10
Relative Abundance(%)
(B)
Immune System Diseases
Excretory System
Immune System
Metabolic Diseases
Cancers
Neurodegenerative Diseases
Cell Growth and Death
Signal Transduction
Enzyme Families
Lipid Metabolism
Nucleotide Metabolism
Translation
Replication and Repair
0.0 0.1 2 4 6 8 10
Relative Abundance (%))

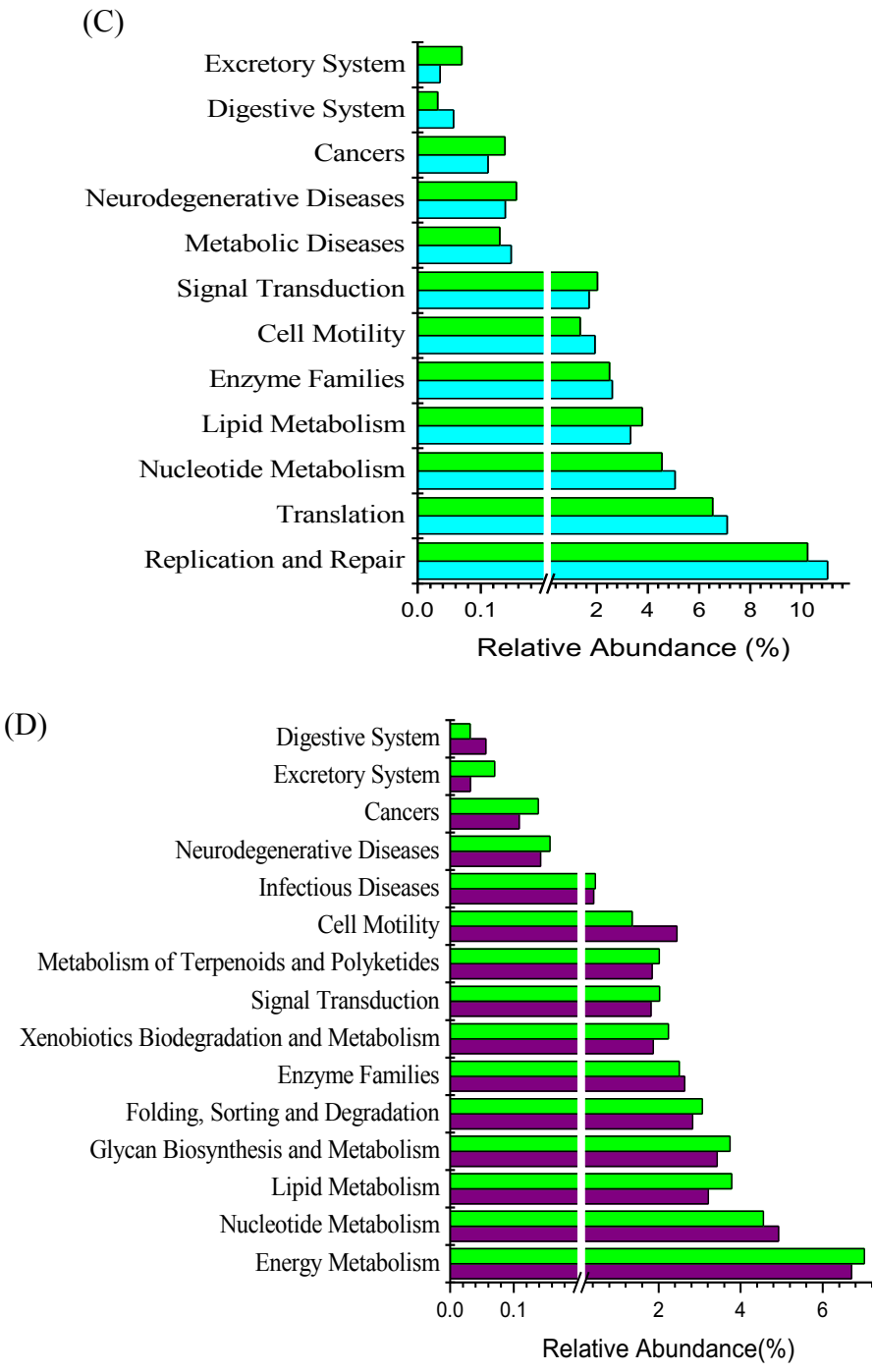


Figure 3.9 Significant differences in relative abundance of predicted metagenome function between groups. (A) DSS group and control group. (B) DSS group and DSS-cranberry group. (C) DSS-cranberry group and control group. (D) DSS-cranberry group and control-cranberry group. The STAMP was used to detect significant differences function.

3.4 Discussion

Inflammatory bowel disease (IBD) can caused several severe syndromes of the patients, including abdominal pain, rectal bleeding and diarrhea. The high incidence rate of IBD in developed countries has been attributed to the western diet with high fat, high calorie and low consumption of vegetable and fruits. Current therapy for IBD mainly suppress the immune response in the colon, resulting in the several side effects such as drug toxic and immune damage [147]. Therefore, the phytochemicals in plants which have been widely studied for their various bioactivities such as anti-oxidative and anti-inflammatory effect are thought to be a possible and safe treatment for IBD. The purpose of this study was to investigate the chemopreventive effect of cranberry on DSS-induced colitis. The results of this study showed that DSS caused severe colonic inflammatory syndromes in mice including weight loss, diarrhea and rectal bleeding (Table 3.2). The shorten colon length and the increasing spleen weight further confirmed the inflammatory process in DSS treated mice. Besides, DSS induced the damage of colonic mucosa structure, leading to the crypt lesion and inflammatory cell infiltration (Fig 3.1). However, oral administration of cranberry in diet significantly reversed the inflammatory process and damage causing by DSS. The DAI score in DSS-cranberry group was significantly lower than DSS group, indicating that cranberry diet alleviated the syndromes of colitis. No difference in the colon length and spleen weight was observed between DSS-cranberry group and control group, which further supported the anti-inflammatory effect of cranberry on the DSS induced colitis.

Studies showed that the inflammation process in the colon is modulated by the expression level of proinflammatory cytokines, such as IL-1β, IL-2, IL-6, IL-12 IFN-γ and TNF-α and anti-inflammatory cytokines like IL-4 and IL-10 [155]. This study used ELISA kit to measure the concentration of cytokines in colonic mucosa and serum. The result showed that DSS elevated

the expression of proinflammatory cytokines in colonic mucosa, including IL-1β, IL-6 and TNF-α (Fig 3.2). IL-1β modulate the inflammatory process through multiple ways. IL-1β can promote the activities of dendritic cells, macrophages and neutrophils and activate T cell and $CD4^{+}TH17$ cells [156]. TNF-α play an important role on activation of various inflammatory cells and modulation of epithelial cell permeability [147]. IL-6 is involved in the inflammatory process through modulating the proliferation of T cell. Over expression of IL-6 can activate the anti-apoptotic genes *Bcl-xl*, leading to the expansion of T-cell, which further cause the chronic intestinal inflammation [157]. The pathological studies indicated that the excessive number of T cells as well as the increasing level of tumor necrosis factor in the colonic mucosa induce Crohn's disease. High level of these three proinflammatory cytokines induced the severe inflammatory process in DSS mice group. In contrast, the intake of cranberry diet significantly suppressed the expression of these three cytokines in colonic mucosa, suggesting that cranberry can prevent the colonic inflammation through reducing the concentration of proinflammatory cytokines. Besides, the level cytokines in serum also affect the inflammatory process. The result of serum cytokines showed that DSS enhanced the expression of IL-6 and TNF-α in serum which was same in colonic mucosa (Fig 3.2). Cranberry diet significantly lower the IL-6 expression and increased the concentration of IL-10, which is an anti-inflammatory cytokines. IL-10 can mediate and suppress the expression of proinflammatory cytokines, resulting in the protective effect against the inflammation and infections [158]. Yanaba et al. also reported that IL-10 can regulate the proliferation of B10 cells, which suppressed the intestinal inflammation in a mouse model [159]. By reducing the expression of proinflammatory cytokines and promoting the secretion of anti-inflammatory cytokines, cranberry significantly ameliorate the inflammatory process in DSS-induced colitis mice.

In addition to the over expression of cytokines, accumulated evidence showed that the development of IBD is associated with the disrupted equilibrium of gut microbiota [86]. Gut microbiota play an important part in colonic immune system development, which can modulate both proinflammatory and anti-inflammatory cytokines through their presence or their activity. Ivanov et al. indicated that germ free mice lack of T_H17 cell and was abundant of $CD4^+FOXP3^+T_{reg}$ cells in colon [160]. This study used high throughput sequencing to identify the microbial community structure of the DSS-induced colitis mice and the mice with cranberry diet. The *Firmicutes/Bacterodietes* ratio was significantly lower in DSS group (0.43) than in control group (0.75), control-cranberry group (0.77) and DSS-cranberry group (0.71) (Fig3.3). The low ratio of *Firmicutes/Bacterodietes* was widely founded in IBD patients. The *Firmicutes/Bacterodietes* ratio in DSS-cranberry group was similar to control group, suggesting that the cranberry diet can protect the gut microbiota against the DSS. Moreover, the relative abundance of *Verrucomicrobia* phylum significantly increased in DSS group, which was mainly due to the excessive growth of *Akkermansia* genus (Fig 3.5). *Akkermansia* is commonly distributed in the colon of mammals, which locate within the mucus layer and is capable to degrade mucin. The aberrant number of *Akkermansia* in DSS group may due to the lesion of mucus layer caused by the DSS, which may further leaded to the over production of mucin. Dharmani et al. reported that the increasing Muc2 and Muc 3 gene expression in DSS treated animal [161]. The increasing amount of mucin can be the food source of *Akkermansia* and promoted their growth. However, one study indicated that the abundance of *Akkermansia* decresed in the IBD patients, suggesting the beneficial effect of *Akkermansia* [162]. Derrien et al. reported that the colonization of in germ free mice did not induce inflammation [163]. Therefore, the excessive proliferation of *Akkermansia* may be the result of DSS-induced colitis

rather than the cause of the inflammation. *Bifidobacterium* and *Lactobacillus* are generally regarded as probiotic, which have been proved to reduce the risk of intestinal inflammation. The reduction of these two bacterial genera in DSS group indicated that inflammatory process may inhibit the growth of them. However, the cranberry diet counteracted the reduction and the presence of probiotic and cranberry may exert the anti-inflammatory effect together. The result showed that DSS treatment reduced the microbial diversity in the gut while the cranberry diet significantly amplified the diversity in the control group and alleviate the diversity reduction in DSS group, which protected the bacterial community in colon (Fig 3.6). Moreover, DSS significantly shifted the microbial community structure while the cranberry diet reversed the alteration (Fig 3.7). The result of this study showed that DSS-induced colitis changed the composition and the function of gut microbiota in mice while cranberry ameliorate the alteration and protect the diversity of the gut microbiota, which may leading to the anti-inflammatory effect of cranberry.

These results may promote the beneficial health effect of cranberry and the importance of gut microbiota, which may improve the consumption of cranberry and probiotic product. In addition, the bioactivity of poor absorbed nutrients will be re-evaluated for their potential ability to alter the composition of gut microbiota. These results will suggest that cranberry could be the potential agent to ameliorate the IBD. The further clinical studies will need to be performed to investigate the interaction between cranberry and gut microbiota in human.

CHAPTER 4

ANTI-CANCER EFFECTS OF CRANBERRY IN ASSOCIATION WITH GUT MICROBIOTA

4.1 Introduction

Colorectal cancer (CRC) is the second most common cause of cancer death in the United States and the fourth leading cause of cancer death worldwide. More than 100,000 patients with CRC was diagnosed annually, which make it a serious health threat to human. CRC is mainly caused by the environmental factors, including environmental and food-borne carcinogens, gastrointestinal pathobionts and colonic inflammation [4]. There are two major types of CRC, including sporadic colorectal carcinoma (SCC) and colitis-asscocaited colorectal cancer (CAC). Epidemiological studies showed that patients with inflammatory bowel disease are more likely to develop colorectal cancer than the heath people, which confirmed the relation between chronic inflammation and cancer. Itzkowitz et al. further reported that colitis-associated colon cancer share several same primary molecular pathway with sporadic colon cancer, including DNA mutation and hyper-methylation [65]. Hussain et al. indicated that IBD patients inclined to have more p53 mutation causing by the oxidative damage in the colonic mucosa, which stimulated DNA mutation during cancer initiation [68].

More than 500 bacterial species live in the human colon, which play an significant role in human health and disease. Gut microbiota can offer various functions to the host, including producing vitamins and hormones, training the immune system and digesting the unused energy substrates. Therefore, gut microbiota maintain a homeostasis with the intestinal immune system and the interaction between them can affect the growth of both epithelial cells and bacteria [164].

Gut microbiota can directly metabolize intestinal mucus and dead epithelial cells, which will affect the function of the colon. One of the microbial metabolites, short chain fatty acids which are metabolizing from the indigestible carbohydrates was proved to have the ability to mediate the inflammatory process in colon. Besides, gut microbiota play an important role on detoxification of and production of carcinogen and mutagen in colon, leading to the different impact on cancer progression. An AOM-DSS induced colon cancer animal study showed the colon cancer can be prevented without gut microbiota, which further confirmed that gut microbiota could be the possible risk factor of colon cancer [165]. Some pathogen in the gut can cause infection and induce inflammation through toxins production or invasion, resulting in the promotion of CRC [166]. The dysbiosis of the community of gut microbiota

Cranberry (*Vaccinium macrocarpon*) is widely consumed in North America for its flavor and potential health benefits. Cranberry contains various phytochemicals, proanthocyanins, flavonal, anthocyanins and triterpenoids, which contributes to its anticancer, anti-adhesion and anti-inflammatory properties.

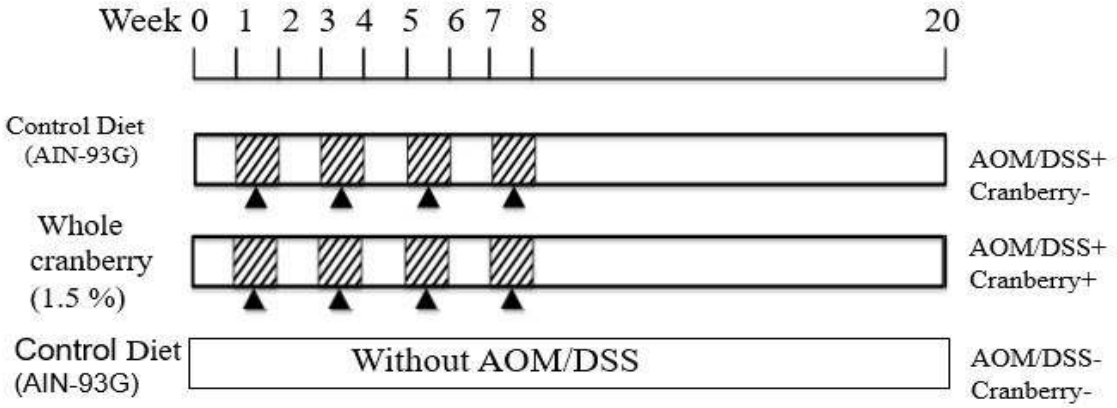
4.2 Materials and methods

4.2.1 Animals, diets and experimental design

Male CD-1 mice weighting 50-80 g were provided by Charles River Laboratories (Wilmington, MA, USA). Animals were kept in groups of five mice per cage in a temperature-controlled animal room (23 °C) with relative humidity of 65–70%, fixed lighting (12 h day/night cycle). Mice were fed with AIN93G diet for 2 weeks for acclimation. With free access to water, mice were then randomly assigned into three groups as follows: the control group, which was fed with AIN93G diet; the AOM/DSS group, which was fed with AIN93G diet; the cranberry group, which received the AIN93G diet supplemented with 1.5% w/w whole cranberry for 20 weeks.

AOM/DSS group and cranberry group were given one intraperitoneal injection of AOM (12 $mg \cdot kg^{-1}$ body weight) in saline after acclimation. After 1 week, 1.5% (w/v) DSS (molecular weight: 36,000–50,000, International Lab, Chicago, IL, USA) in drinking water was administered to the animals for 4 days, followed by 7 days of pure water, and the cycles was repeated four times. After 20^{th} weeks, CO_2 asphyxiation were used to sacrifice the animal (Fig 4.1).

Tissues were removed from the animal and weighed. The length of colon was measured then weighted after rinsing with phosphate-buffered saline (pH 7.4). Number and size of tumors of animals were inspected using dissection microscope and measured by an ocular micrometer. The sizes of tumors were determined by the following formula: tumor volume (mm^3) = $L \times W^2 / 2$, where L is the length and W is the width of the tumor. Then the colons were cut into two pieces longitudinally. Half of the colon was fixed in 10% buffered formalin for 24 h for further histopathological and immunohistochemical analysis. The other half of the colon was stored at -80°C for ELISA, RT-PCR analysis. Animal experiment was approved by Institutional Animal Care and Use Committee of the University of Massachusetts Amherst (#2014-0079).



Note: Black shade represents 1.5% DSS water treatment

Figure 4.1 Animal experimental design

4.2.2 Histological analysis

Formalin-fixed Colonic sections were stained with hematoxylin-eosin (H&E) after processing for paraffin-embedding and sectioning. The stained samples were observed under a light microscope (100×) and evaluated for surface epithelial loss and dysplasia [167].

4.2.3 Enzyme-linked immunosorbent assay (ELISA) and real-time PCR analysis

Colonic mucosa were scraped and homogenized in a phosphate buffer solution containing 0.4 M NaCl, 0.05% Tween-20, 0.5% BSA, 0.1 mM benzethonium, and 1% protease inhibitor cocktail (Boston Bioproducts, Ashland, MA, USA). The homogenates were centrifuged at 10,000g for 30 min at 0°C. The supernatant was used for quantification of cytokines, i.e. interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) by ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Real-Time qRT-PCR analysis was conducted as previously described. The primer pairs were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA) with the following primers: IL-1β F: 5′-ACCTGCTGGTGTGTGACGTT-3′, R: 5′-TCGTTGCTTGGTTCTCCTTG-3′; IL-6 F: 5′-GAGGATACCACTCCCAACAGACC-3′, R: 5′-AAGTGCATCATCGTTGTTCATACA-3′A; TNF-α F: 5′-AGCACAGAAAGCATGATCCG-3′, R: 5′-CTGATGAGAGGGAGGCCATT-3′; β-actin F: 5′-AAGAGAGGCATCCTCACCCT-3′, R: 5′-TACATGGCTGGGGTGTTGAA-3′. The copy number of each transcript was calculated with respect to the β-actin copy number, using the $2^{-\Delta\Delta Ct}$ method [168].

4.2.4 Immunoblotting

Total colonic mucosa was scraped and then lysed in 1 ml of ice-cold lysis buffer with cocktails of protease inhibitor (1:100), phosphotase inhibitor 1 (1:100), and phosphotase inhibitor 2 (1:100). Then the smaples were homogenized on ice for 15 s using ultra-sonicator

probe and centrifuged at 10,000 rpm for 10 minutes at 4 °C. Supernatant were collected and quantified to determine the protein concentration by BCA protein assay kit. 50-100 μg of total proteins were loading on SDS-PAGE and transferred on nitrocellulose membrane. The membrane was further immerged on the target antibodies followed the manufacture's recommendation and visualized by Odyssey CLx infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). Antibody for p21, p27, cyclin D, CDK 4, p-Rb, cleaved caspase-3, cleaved PARP, p53, VEGF, MMP-2 and MMP-9 were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-β actin antibody was from Sigma-Aldrich (St. Louis, MO, USA).

4.2.5 Isolation of DNA

Fecal pellets were collected from the mice colon to assess gut microbiota. Bacterial DNA was isolated from feces by using PowerFecal DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's protocols.

4.2.6 16S rRNA Analysis

The concentration of the DNA samples was measured by NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA, US). PCR was performed to amplify the 16s rRNA gene marker using region of interest-specific primers with overhang adapters attached, which of sequences are: 16S Amplicon PCR Forward Primer = 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and (16S Amplicon PCR Reverse Primer = 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) [148]. 2x KAPA HiFi Hotstart ReadyMix (KAPA Biosystem, Wilmington, MA, US) was used as PCR reagent. Then PCR product was cleaned and purified by using AMPure XP beads (Beckman Coulter, Danvers MA, US). A second PCR was performed to attach dual indices and

Illumina sequencing adapters using the Nextera XT Index Kit (Illumina, San Diego, CA, US) followed by AMPure XP bead purification. The quantity of PCR products was determined by using Qubit dsDNA BR Assay kit (Life technology, Carlsbad, CA, US) while the size of PCR products was verified by DNA analysis ScreenTape Assay on Tape Station 2200 (Agilent Technologies, Santa Clara, CA, US). After quantification, 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, US).

4.2.7 Informatic and Statistical analysis

Quantitative insights into microbial ecology (QIIME) software pipeline v1.9.1 was used to analyze the raw data files obtained from Illumina Miseq [149].

Since Miseq platform sequenced multiple samples via single sequencing run, QIIME first identified each individual samples through DNA barcode after inputting the raw data. Then QIIME removed sequence reads which have low quality to improve diversity estimates. Operational Taxonomic Units (OTUs) were clustered into represent groups and assigned to taxonomy using UCLUST. PyNAST was used in QIIME to align the sequence to constructed OTU table and phylogenetic tree basing on the public reference database. Alpha-diversity and rarefaction (number of taxa detected) was calculated and generated basing on the phylogenetic tree and OTU table. Beta-diversity and rarefaction (relative distribution of taxa in a community) was calculated and produced UniFrac distance between samples for bacterial 16S rRNA reads (evenly sampled at 600 reads per sample). UniFrac metric has two variants, weighted and unweighted. The weighted Unifrac metric consider the number of the OTUs and the relative

abundance of each taxa while unweighted Unifrac metric only takes account of the number of the OTUs [150]. The distance matrix were visualized with principal coordinate analysis (PCoA) which generated a plot with orthogonal axes explaining the maxium difference in the data [151]. The differences in the alpha-diversity were compared using a nonparametric two sample t-test with 999 Monete Carlo permutations. Results are presented as mean ± SD. The difference of the beta-diversity were test using ANOSIM with 999 permutations between every two groups.

The functional capacity of the gut microbiota were predicted using PICRUSt software. 16S rRNA sequences for taxa were input into PICRUSt to align to to Kyoto Encyclopedia of Genes and Genomes (KEGG) gene orthologs basing on the Greengenes database [152]. STAMP software were used to calculate the difference among each group [153]. For all analyses, values of $P < 0.05$ were considered statistically significant.

4.3 Results and discussion

4.3.1 Cranberry reduced the incidence and multiplicity of colonic tumors in AOM/DSS Model

Injection of a colon carcinogen AOM in combination with cyclic administration of DSS in drinking water resulted in the development of colitis, colorectal dysplasia, and cancer. Body weight was monitored twice a week as an indicator of potential toxicity of cranberry, and no difference was found between cranberry-treated group and the control during the whole experimental period (final body weights were shown in the Table 4.1). There was no difference in the weight of liver and spleen between the two groups (Table 4.1), and no apparent behavioral or appearance difference was observed either, suggesting no noticeable toxic effects caused by dietary feeding of cranberry to the mice. Colon weight/length ratio is correlated with the severity of colitis and therefore is an indicator of levels of inflammation in the colon. As shown in Table

4.1, compared to the control group, dietary treatment with cranberry significant prevented the shortening of colon length and reduced the elevated colon weight/length ratio caused by AOM/DSS. The AOM/DSS treatment resulted in 100% incidence of colon tumors and 4.97 ± 1.01 colonic tumors per mouse. Dietary administration of cranberry significantly decreased the tumor incidence by 50% and tumor multiplicity by 65%. These results demonstrated that cranberry effectively suppressed AOM/DSS-induced colon carcinogenesis in mice.

Table 4.1 Final body weight, relative organ weights, and colon assessment of mice

Group	**AOM/DSS-**	**AOM/DSS+**	**AOM/DSS+ Cranberry+**
Treatment	None	AOM/DSS	AOM/DSS/1.5% Whole cranberry
Body weight (g)	50.96± 1.47	48.33 ± 1.22	49.45 ± 1.23
Liver weight (mg)	2348.85 ± 78.36	2263.99 ± 94.74	2199.85 ± 55.20
Spleen weight (mg)	238.18± 35.58	230.67 ± 30.15	194.58 ± 16.84
Colon length (mm)	96.62 ± 2.42 [a]	87.80 ± 2.90 [b]	92.69 ± 2.99[a]
Colon weight (mm)	353.96 ± 35.47	357.84 ± 46.07	367.53 ± 56.22
Colon W/L ratio (mg/mm)	3.69± 0.29 [a]	4.00 ± 0.40 [b]	4.86± 0.84 [c]
Tumor incidence	0 [a]	80% [b]	53% [c]
Tumor multiplicity	0 [a]	5.91 ± 1.01 [b]	2.77 ± 0.88 [c]

Data are shown as the mean ± SD. *$p < 0.05$ (n = 10) versus the control group.

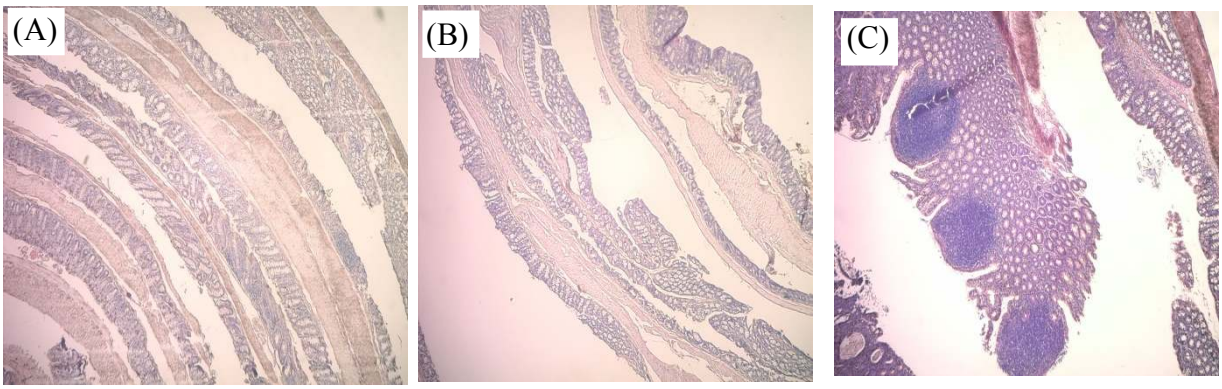


Figure 4.2 Histological characterization of the colon mucosa. Hematoxylin and eosin were used to stain the colon mucosa sample. (A) Control group (B) AOM/DSS-cranberry group (C) AOM/DSS group

4.3.2 Histological observation

The colonic specimens obtained from the AOM/DSS group showed developed invasive mucinous adenocarcinoma with inflammatory cell infiltration. However, in the AOM/DSS group, the colonic specimens had no erosion with just mild inflammatory cell infiltration. In control group, no aberrant tissue was observed (Fig 4.2).

4.3.3 Cranberry decreased the levels of pro-inflammatory cytokines in the colon of AOM/DSS Model

Accumulating studies have demonstrated that improper up-regulation of pro-inflammatory cytokines such as interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor-α (TNF-α) can accelerate the process of colon carcinogenesis. Using ELISA, we determined the effects of cranberry on the AOM/DSS-induced production of pro-inflammatory cytokines in colonic mucosa. As shown in Figure 4.3A, cranberry treatment resulted in significant decreases in the levels of IL-1β, IL-6 and TNF-α by 83%, 70% and 45%, respectively, compared to those of the control group. Next, we determined the effects of cranberry on the

mRNA expression levels of pro-inflammatory cytokines by Real-Time PCR analysis. As shown in Figure 4.2B, the mRNA expression levels of IL-1β, IL-6, and TNF-α were significantly reduced by cranberry treatment by 91%, 89%, and 95%, respectively, in comparison with those found in the AOM/DSS treatment mice. Together, our results showed that oral administration of cranberry inhibited the colon carcinogenesis at least partially by reducing cell proliferation, inducing apoptosis, and suppressing the expression levels of pro-inflammatory cytokines in the colon of AOM/DSS-treated mice.

4.3.4 Cranberry mediated the expression level of key proteins related with cell cycle progression, apoptosis, angiogenesis and metastasis in AOM/DSS-treated mice

In order to understand the molecular mechanism of the anti-cancer effect of cranberry on AOM/DSS-treated mice, this study analyzed the expression of signaling proteins involved in the apoptosis (p53, cleaved caspase-3 and cleaved PARP) in colonic mucosa. Figure 4.4A showed that cranberry diet increased the expression of all three proteins, indicating that cranberry can regulate the apoptosis. Besides, the expression of cell cycle signal protein (p21, CDK4, cyclin D and p-Rb) were also measured. Figure 4.4B showed that the expression of p21 and p27 were 3 fold and 4-fold higher while the expression of cyclin D and CDK4 were lower in cranberry group, compared to the AOM/DSS group, suggesting that cranberry could induce cell cycle arrest in G1 phase. Moreover, the expression of signal proteins involved in the angiogenesis and metastasis was significantly lower in the cranberry group than AOM/DSS group. The expression of VEGF was 2-fold lower and MMP-2 and MMP-9 were 4-fold lower in the cranberry group (Fig 4.4C). The alteration of the expression of signal protein indicated that cranberry prevented the cancer progression through multiple pathway.

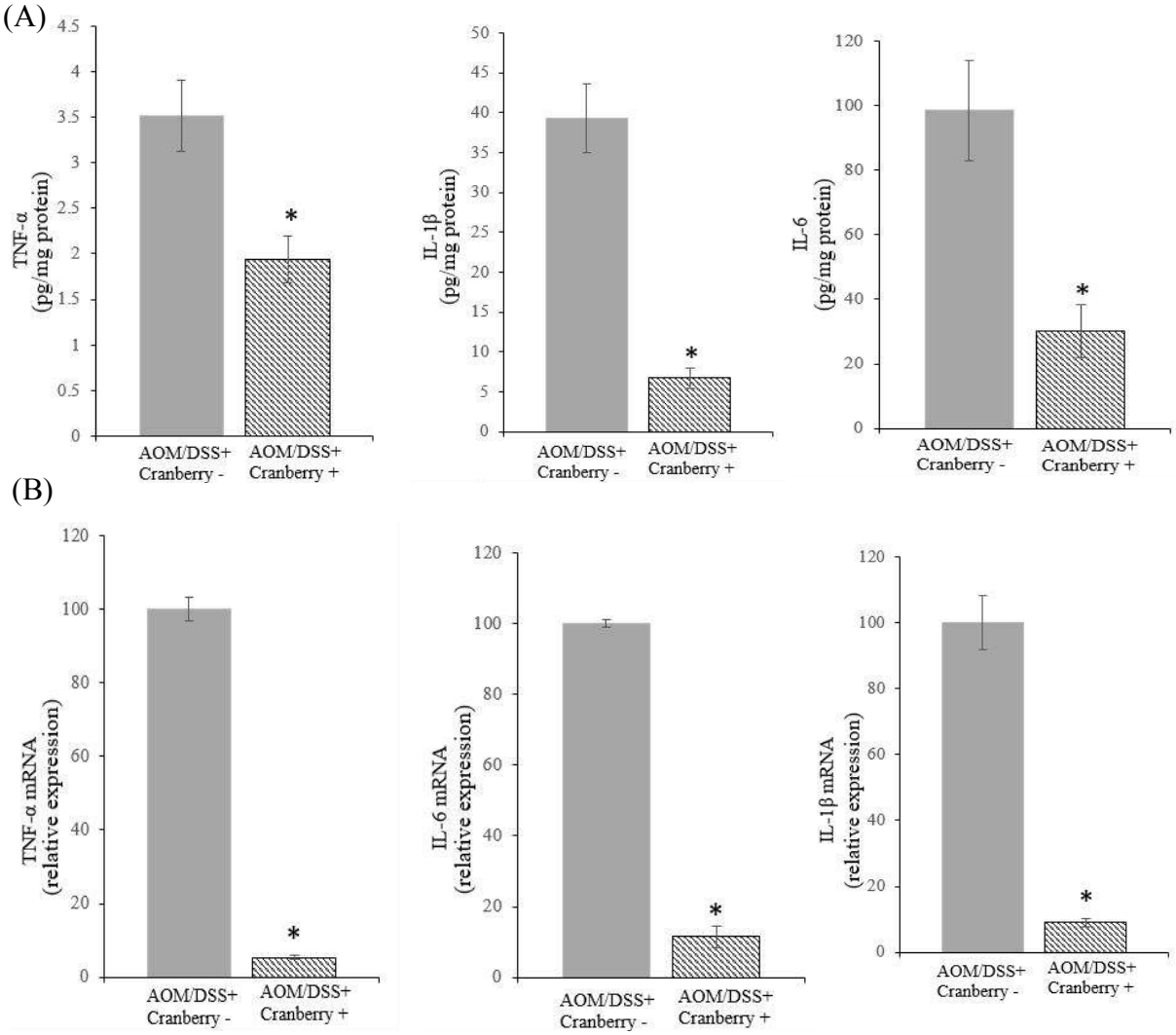


Figure 4.3. Effects of Cranberry treatment on protein levels (**A**) and mRNA levels (**B**) of IL-1β, IL-6, and TNF-α in the colonic mucosa of AOM/DSS-treated mice. Data are shown as the mean ± SD of three independent experiments. The amount of IL-1β, IL-6, TNF-α mRNA expression was normalized to that of β-actin. * indicates statistically significant differences from the control group ($p < 0.01$, n = 3).

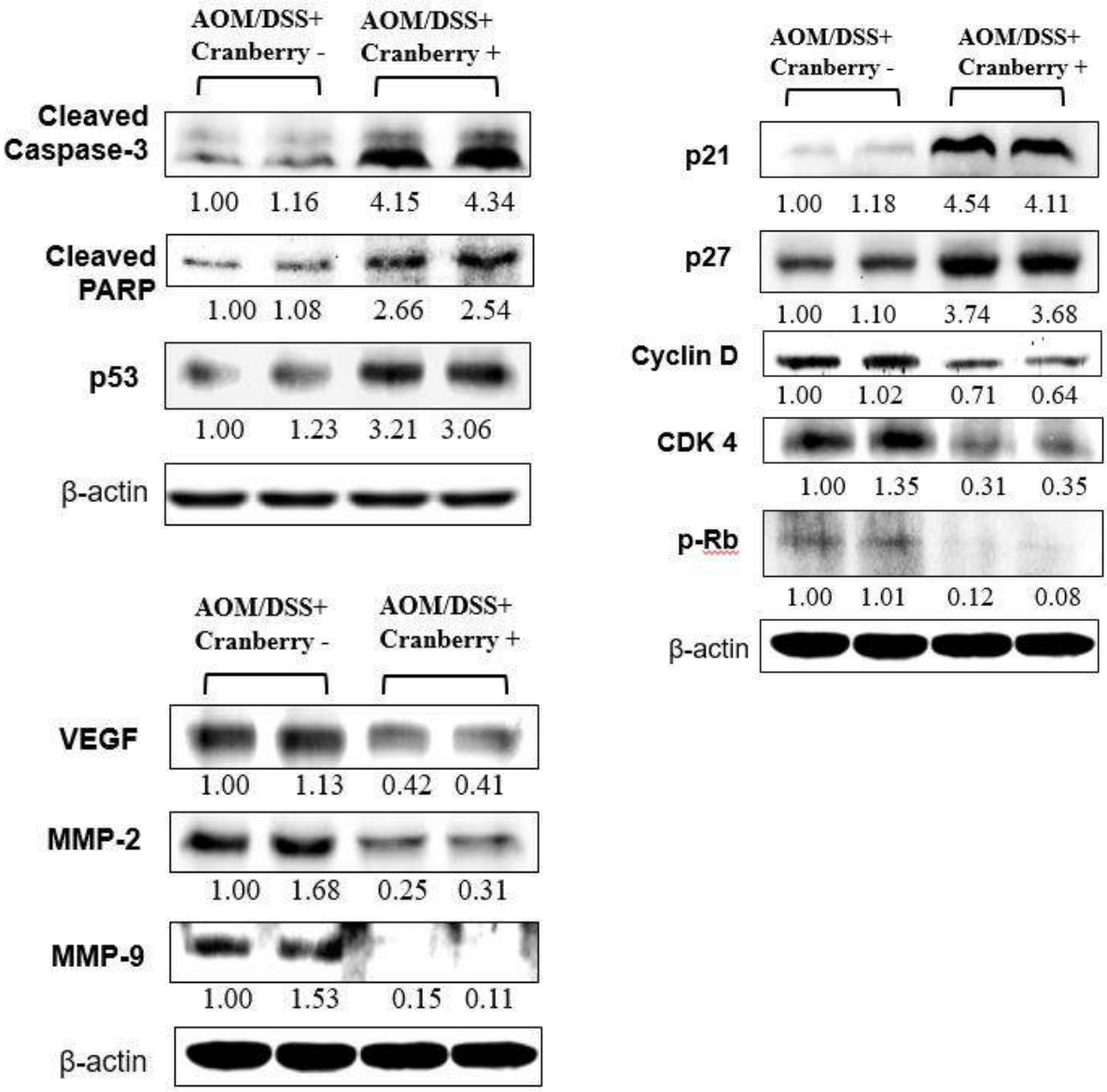


Figure 4.4. Effects of cranberry on cancer related signaling proteins in the colonic mucosa of AOM-treated mice. The numbers underneath of the blots represent band intensity (normalized to β-actin, means of three independent experiments) measured by Image J software. The SDs (all within ±15% of the means) were not shown. β-Actin was served as an equal loading control. * indicates statistical significance in comparison with the control ($p < 0.05$, n=3).

4.3.5 Impact of cranberry on the composition of gut microbiota in AOM/DSS-treated mice

Five mice were housed per cage and fecal pellets from each cage were collected and pooled after sacrificing. In order to characterize phylogenetic diversity, the 16S rRNA gene V3/V4 fragment sequencing yielded 336,876 quality reads following filtering. This provided a mean sample depth of 37,430 sequencing reads per bacterial community.

At the phylum level, *Actinobacteria*, *Bacteroidetes*, *Deferribacteres*, *Firmicutes*, *Proteobacteria*, *Verrucomicrobia* together constituted over 99% of OTUs identified in the total samples analyzed (Fig. 4.5). Compared to the control group, the relative abundances of *Firmicutes* significantly decreased while the relative abundance *Proteobacteria* significantly increased by 40% in AOM/DSS group. However, there was no significant alteration in AOM/DSS-cranberry group.

4.3.6 Impact of cranberry on the most abundant bacterial genera

At the genus level, AOM/DSS treatment and cranberry treatment exert different impact on the bacteria (Fig. 4.9). Three bacterial genus which are widely regarded as disease related increased after AOM/DSS treatment and cranberry treatment inhibited the growth of them. Compared to the control group, the relative abundance of *Clostridium*, *Escherichia* and *Staphylococcus* increased by 50%, 16%, 63%, respectively in AOM/DSS group. In contrast, no difference was founded between cranberry group and control group in terms of these genera. In addition, AOM/DSS treatment suppressed the growth of *Akkermansia*, *Bifidobacterium* and *Lactobacillus* while cranberry treatment attenuated the alteration. *Akkermansia* is a mucin degrading bacterium which proved to have ability to control inflammation. *Bifidobacterium* and lactobacillus are considered as important probiotic which may exert a range of beneficial health effects, such as the inhibition of harmful bacteria and modulation of immune responses.

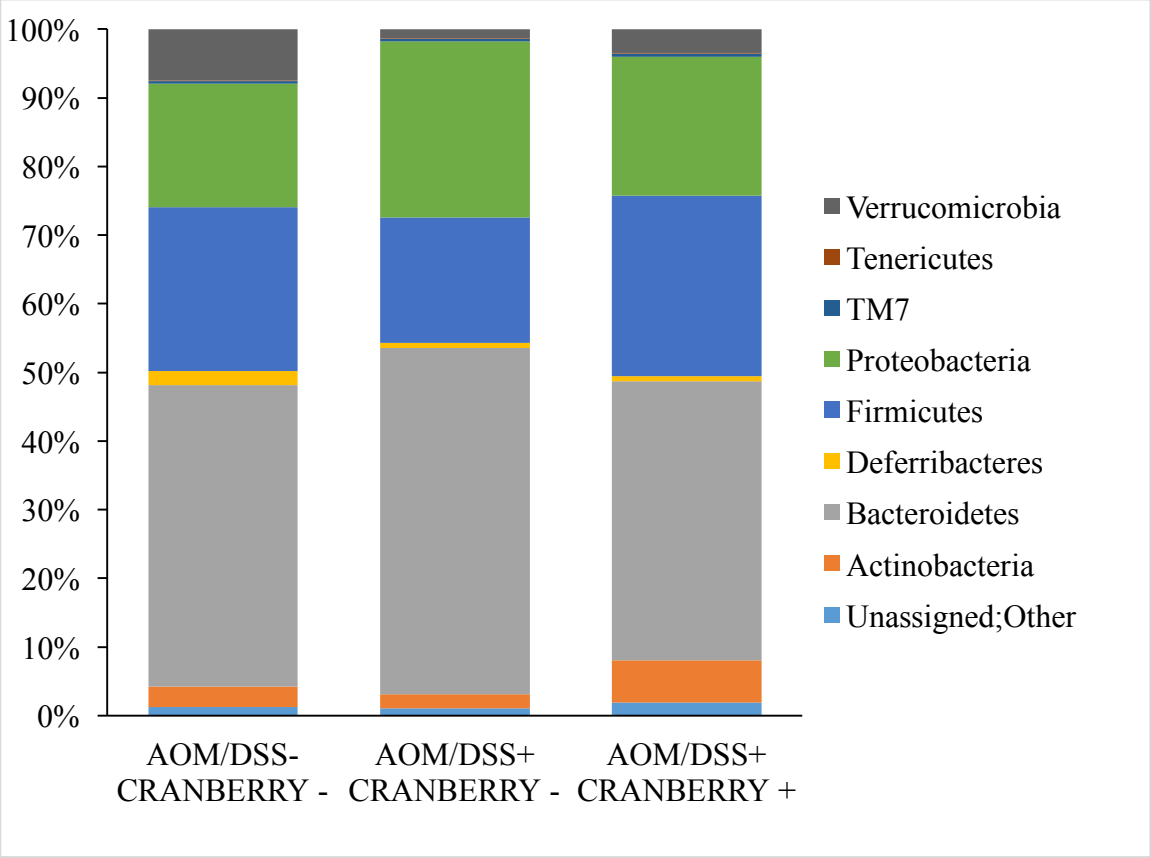


Figure 4.5 Relative abundance of bacterial phylum in response to AOM/DSS treatment and cranberry

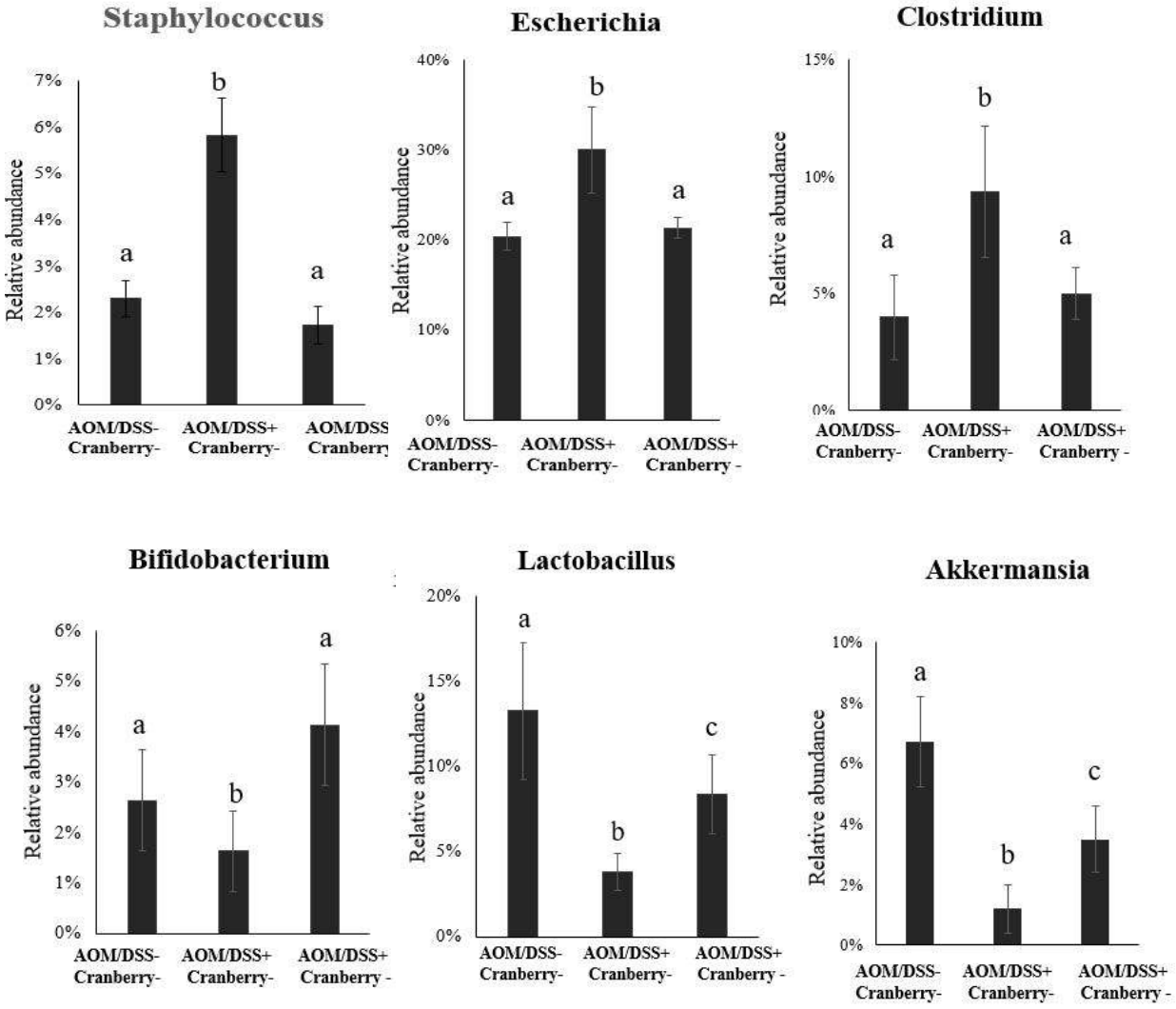


Figure 4.6 Statistical comparisons of bacteria at genus level. Data are shown as the mean ± SD. *$p < 0.05$ (n = 3).

4.3.7 Impact of cranberry on the α-diversity and β-diversity of gut microbiota

According to the high throughput sequencing data, the microbial α-diversity of cranberry groups was slightly higher than the other two groups, suggesting that the cranberry contributed to the microbial species diversity (Fig 4.7). However, there was no significant difference on the α-diversity among these three groups, which means that AOM/DSS treatment did not alter the microbial richness in the mice.

Figure 4.8 showed that AOM/DSS treatment had shifted the microbial community while cranberry alleviated the effect of AOM/DSS on gut microbiota. ANOSIM with 999 permutations was used to analyze statistical significance among three groups using distance matrices of weighted UniFrac Compared to the control group. AOM/DSS treatment significantly altered the bacterial community of gut microbiota in mice which had been consuming normal diet ($p<0.05$, $R=1.0$). Bisides, the microbial community in cranberry group was also different from that in control group ($p<0.05$, $R=0.71$) and in AOM/DSS group ($p<0.05$, $R=0.95$). However, the microbial community in cranberry group mice have more similarity with the bacterial community in the control group mice than in the DSS group ($R=1.0>R=0.73$).

β-diversity of gut microbiota is a value which evaluate the number of observed species in the sequencing samples and the quantity of each species while α-diversity only take the number of species into account. Therefore, the result of this study suggested that AOM/DSS treatment can affect the growth of gut microbiota while the cranberry can reverse or attenuate the effect and protect the microbial community, leading to the protective effect on the host.

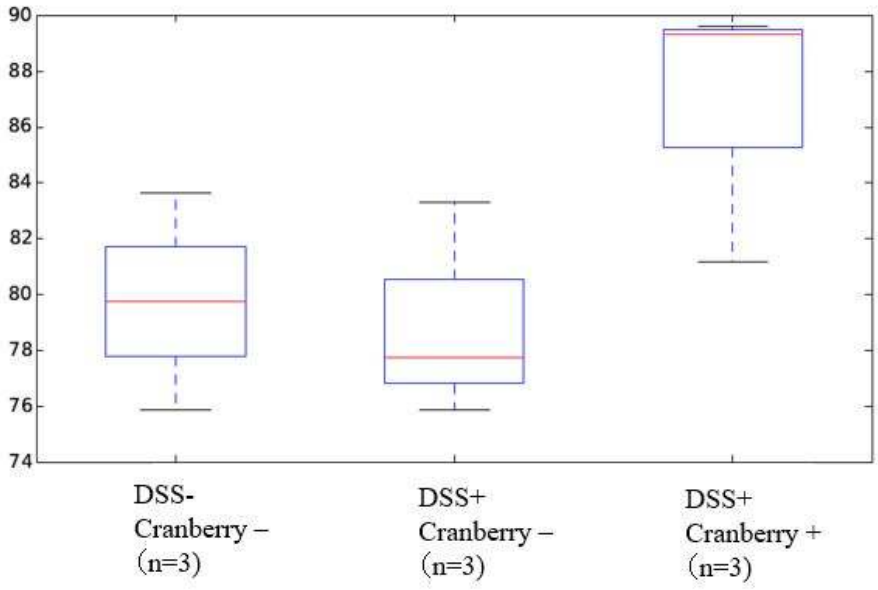


Figure 4.7 Bacterial diversity in 3 different sample groups. Box plots of alpha diversity were generated with rarefication to 30,000 reads per sample. The nonparametric P values were calculated using 999 Monte Carlo permutations.

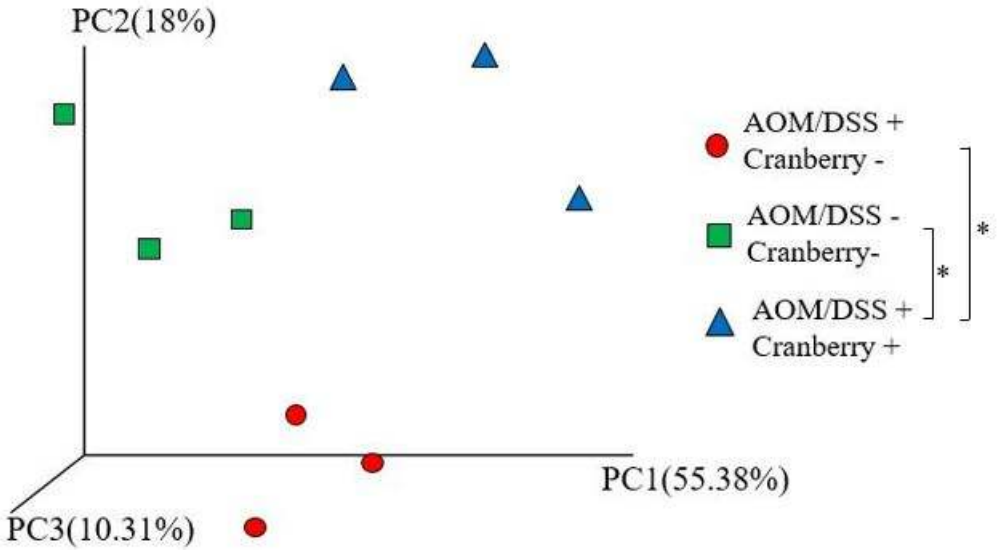


Figure 4.8 β-diversity in gut microbiota between groups. Weighted UniFrac distances PCoA graph was used to evaluate diversities between samples. *, P value <0.05.

4.3.8 Cranberry influenced predicted metagenomics function of gut microbiota of AOM/DSS Model

The metagenomics potential in the treatment groups were inferred by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) based on 16s rRNA phylogenetic data. A total of 336,876 observations were predicted across Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology groups (KO). The resultant data were categorized into 182 functional pathways encompassing the 9 samples. (Fig. 4.9).

In control group, the expression of 24 functional genes were significantly different from the AOM/DSS group. The proportion of genes in associate with cancer, which has 24 kinds of genetic function related to different kinds of cancer like colorectal cancer was significantly more abundant in of AOM/DSS group. Besides, compared to the AOM/DSS group, bacterial genes which related to the energy metabolism, metabolism of cofactors and vitamins and glycan biosynthesis were significantly higher in both control group and cranberry group, indicating that the microbial activities in these two groups are more active. In contrast, disease related genes such as immune system disease and neurodegenerative diseases were more abundant in AOM/DSS group, suggesting the potential over growth of pathogen.

In AOM/DSS group, genes related to cell growth and death and replication and repair had lower relative proportion while signal transduction genes had higher proportion, compared to other two groups, which means that the overall growth of bacterial cells may be slightly inhibited by AOM/DSS treatment while cranberry counteracted this detrimental alteration. The difference in predicted genome function further confirmed that the community of gut microbiota is associated with their function, which may affect their interaction with the host and the immunological balance.

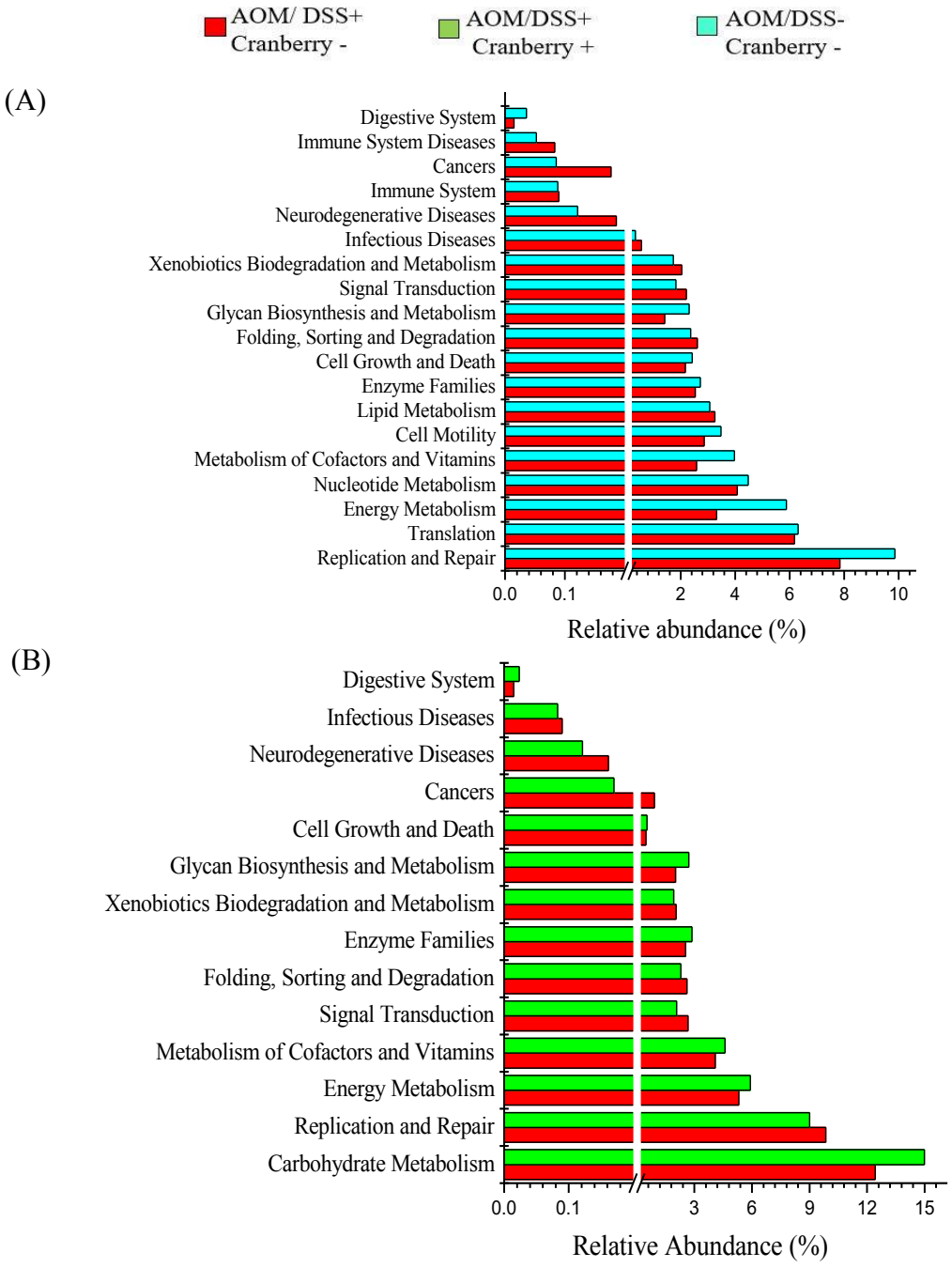


Figure 4.9 Significant differences in relative abundance of predicted metagenome function between groups. (A) AOM/DSS group and control group. (B) AOM/DSS group and AOM/DSS-cranberry group.

4.3.9 Microbiota-host networks in AOM/DSS model

Correlation networks was built among bacterial relative abundances from 16s rRNA gene amplicon sequencing, qPCR data from pro-inflammatory cytokines and tumor formation data of mice (Fig. 4.10). Data was log-normalized and the Pearson correlation coefficients r>0.6 or r<-0.6 were used to construct and visualized the correlation networks in R software environment. IL-6, IL-1β and TNF-α expression levels correlated with tumor formation and members of this cluster correlated positively with OTU 1(*Escherichia*), OTU 2 (*Clostridium*) and OTU 3 (*Staphylococcus*) while negatively with OTU 4 (*Bifidobacterium*), OTU 5 (*Lactobacillus*), OTU 6 (*Akkemansia*).

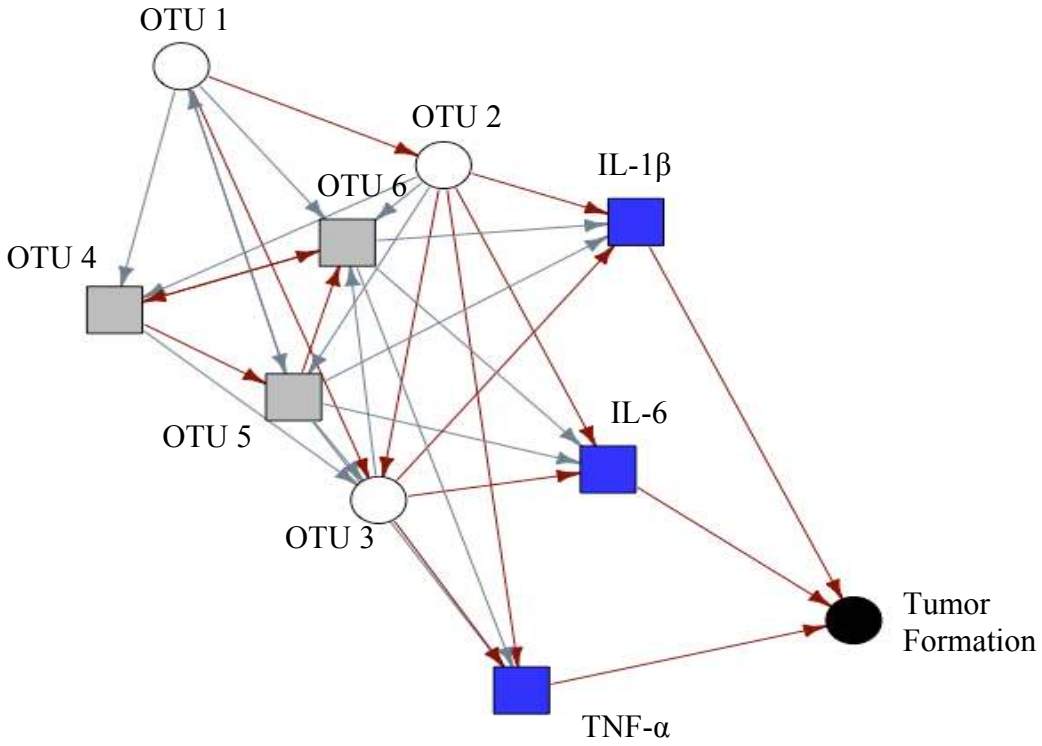


Figure 4.10 Correlation networks of 16s rRNA gene amplicon defined OTUs and host parameters

4.4 Discussion

Since cranberry and its phytochemicals were proved to have the anti-inflammatory and antioxidant effect, this study evaluated the anti-cancer properties of cranberry against colon cancer. In vitro study had been showed that phytochemicals extracted from cranberry such as ursolic acid, quercetin and proanthoanidins significantly inhibited the proliferation of HepG2 human liver cancer cells and MCF-7 breast cancer cells [110]. The result of this study were in consistence with the in vitro studies showing that oral administration of whole cranberry in diet protected the CD-1 mice from developing colitis-associated colon carcinogenesis after AOM/DSS-treatment. The result demonstrated cranberry played an important role in modulating the expression of signal proteins of the cell cycle, cell apoptosis, angiogenesis and metastasis in colonic mucosa, leading to its anti-cancer properties. Furthermore, this study proved that cranberry mediated the expression of pro-inflammatory cytokines, resulting in the prevention of cancer progression.

Moreover, as expected, AOM/DSS-treatment significantly shifted the microbial community in mice which may lead to the dysfunction of immune system and promote the development of adenoma. However, cranberry exerted protective effect on gut microbiota against the stimulation and inflammation. This effect was associated with alleviation of inflammation and the alteration of the structure of gut microbiota community. Our study further suggests that the ability of whole cranberry administration to inhibit the relative abundance of disease related bacteria while protect the health related bacteria is playing a important role in this protective effect. Moreover, the pooling of the mice feces samples bring the limitation of this study, but allowed us to focus on the alteration happening within the dominant phylum level due to the different treatment. This study provided a solid scientific basis for using whole cranberry

as a chemopreventive agent for colon cancer in human. Even though studies widely investigated the beneficial activities of pure phytochemicals in cranberry, the potent anticancer property of cranberry may be the additive and synergistic effects of all the phytochemicals in cranberry. Further study can focus on the identification and evaluation of effective phytochemicals in cranberry.

BIBLIOGRAPHY

1. Jemal, A., et al., *Global cancer statistics.* CA Cancer J Clin, 2011. **61**(2): p. 69-90.
2. Center, M.M., et al., *Worldwide variations in colorectal cancer.* CA Cancer J Clin, 2009. **59**(6): p. 366-78.
3. Buset, M., *Primary prevention of colorectal cancer.* Acta Gastroenterol Belg, 2003. **66**(1): p. 20-7.
4. Terzic, J., et al., *Inflammation and colon cancer.* Gastroenterology, 2010. **138**(6): p. 2101-2114 e5.
5. Sekirov, I., et al., *Gut microbiota in health and disease.* Physiol Rev, 2010. **90**(3): p. 859-904.
6. Savage, D.C., *Microbial ecology of the gastrointestinal tract.* Annu Rev Microbiol, 1977. **31**: p. 107-33.
7. Hooper, L.V., and Jeffrey I. Gordon, <*Commensal Host-Bacterial Relationships in the Gut.pdf*>. Science, 2001. **292.**(5519): p. 1115-1118.
8. Sommer, F. and F. Backhed, *The gut microbiota--masters of host development and physiology.* Nat Rev Microbiol, 2013. **11**(4): p. 227-38.
9. McFarland, L.V., *Antibiotic-associated diarrhea: epidemiology, trends and treatment.* Future Microbiol, 2008. **3**(5): p. 563-78.
10. Wang, T., et al., *Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers.* ISME J, 2012. **6**(2): p. 320-9.
11. Wang, S.Y. and A.W. Stretch, *Antioxidant capacity in cranberry is influenced by cultivar and storage temperature.* J Agric Food Chem, 2001. **49**(2): p. 969-74.
12. Neto, C.C., *Cranberry and blueberry: evidence for protective effects against cancer and vascular diseases.* Mol Nutr Food Res, 2007. **51**(6): p. 652-64.
13. Etxeberria, U., et al., *Impact of polyphenols and polyphenol-rich dietary sources on gut microbiota composition.* J Agric Food Chem, 2013. **61**(40): p. 9517-33.
14. Golub, T.R., et al, *Molecular Classification of Cancer Class Discovery and Class Prediction by Gene Expression Monitoring.* science, 1999. **286**(5): p. 531-537.
15. Anne S. Tsao, M.E.S.K., MD; Waun Ki Hong, MD, *Chemoprevention of Cancer.* A Cancer Journal for Clinicians, 2004. **54**(3): p. 150-180.
16. Sporn, M.B., *Approaches to prevention of epithelial.* Cancer research, 1976. **36**(7): p. 2699-2702.
17. Hanahan, D., Robert A. Weinberg, *The hallmarks of cancer.pdf.* cell, 2000. **100**(1): p. 57-70.
18. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
19. Karin, M. and F.R. Greten, *NF-kappaB: linking inflammation and immunity to cancer development and progression.* Nat Rev Immunol, 2005. **5**(10): p. 749-59.
20. Yeatman, T.J., Ann F. Chambers, *Osteopontin and colon cancer progression.* Clinical & experimental metastasis 2003. **20**(1).
21. Irby, R.B., et a, *Activating SRC mutation in a subset of advanced human colon cancers.* Nature genetics, 1999. **21**(2): p. 187-190.

22. Knudson, A.G., *Two genetic hits (more or less) to cancer.* Nature Reviews Cancer, 2001. **1**(2): p. 157-162.
23. Kinzler, K.W., Bert Vogelstein, *Lessons from Hereditary Colorectal Cancer.* Cell, 1996. **87**(2): p. 159-170.
24. Nambiar, P.R., R.R. Gupta, and V. Misra, *An "Omics" based survey of human colon cancer.* Mutat Res, 2010. **693**(1-2): p. 3-18.
25. Surh, Y.J., *Cancer chemoprevention with dietary phytochemicals.* Nat Rev Cancer, 2003. **3**(10): p. 768-80.
26. Wattenberg, L.W., *Chemoprevention of cancer.* Prev Med, 1996. **25**(1): p. 44-5.
27. Milner, J.A., et al., *Molecular targets for nutrients involved with cancer prevention.* Nutr Cancer, 2001. **41**(1-2): p. 1-16.
28. Manson, M.M., *Cancer prevention -- the potential for diet to modulate molecular signalling.* Trends Mol Med, 2003. **9**(1): p. 11-8.
29. Bharti, A.C. and B.B. Aggarwal, *Nuclear factor-kappa B and cancer: its role in prevention and therapy.* Biochem Pharmacol, 2002. **64**(5-6): p. 883-8.
30. Dong, Z., et al., *Inhibition of activator protein 1 activity and neoplastic transformation by aspirin.* J Biol Chem, 1997. **272**(15): p. 9962-70.
31. Fesik, S.W., *Promoting apoptosis as a strategy for cancer drug discovery.* Nat Rev Cancer, 2005. **5**(11): p. 876-85.
32. Nagata, S., *Apoptosis by death factor.* Cell, 1997. **88**(3): p. 355-65.
33. Prunet, C., et al., *Activation of caspase-3-dependent and -independent pathways during 7-ketocholesterol- and 7beta-hydroxycholesterol-induced cell death: a morphological and biochemical study.* J Biochem Mol Toxicol, 2005. **19**(5): p. 311-26.
34. Chene, P., *Inhibiting the p53-MDM2 interaction: an important target for cancer therapy.* Nat Rev Cancer, 2003. **3**(2): p. 102-9.
35. Kemler, R., *From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion.* Trends Genet, 1993. **9**(9): p. 317-21.
36. Schatzkin, A., et al, *Lack of Effect of a Low-Fat, High-Fiber Diet on the Recurrence of Colorectal Adenomas.* New England Journal of Medicine, 2000. **342**(16): p. 1149-1155.
37. Alberts, D.S., et al. , *Lack of effect of a high-fiber cereal supplement.* New England Journal of Medicine, 2000. **342**(16): p. 1156-1162.
38. Cascinu, S., et al., *Effects of calcium and vitamin supplementation on colon cell proliferation in colorectal cancer.* Cancer Investigation, 2000. **18**(5): p. 411-416.
39. Baron, J.A., et al., *Calcium supplements for the prevention of colorectal adenomas.* New England Journal of Medicine, 1999. **340**(2): p. 101-107.
40. Plummer, S.M., et al., *Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-kappaB activation via the NIK/IKK signalling complex.* Oncogene, 1999. **18**(44): p. 6013-20.
41. Singh, S. and B.B. Aggarwal, *Activation of transcription factor NF-kappa B is suppressed by curcumin (diferuloylmethane) [corrected].* J Biol Chem, 1995. **270**(42): p. 24995-5000.
42. Lin, J.K., *Cancer chemoprevention by tea polyphenols through modulating signal transduction pathways.* Arch Pharm Res, 2002. **25**(5): p. 561-71.
43. Banerjee, S., C. Bueso-Ramos, and B.B. Aggarwal, *Suppression of 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis in rats by resveratrol: role*

of nuclear factor-kappaB, cyclooxygenase 2, and matrix metalloprotease 9. Cancer Res, 2002. **62**(17): p. 4945-54.
44. Mouria, M., et al., *Food-derived polyphenols inhibit pancreatic cancer growth through mitochondrial cytochrome C release and apoptosis.* Int J Cancer, 2002. **98**(5): p. 761-9.
45. Ferrero-Miliani, L., et al., *Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation.* Clin Exp Immunol, 2007. **147**(2): p. 227-35.
46. Libby, P., *Inflammatory mechanisms: the molecular basis of inflammation and disease.* Nutr Rev, 2007. **65**(12 Pt 2): p. S140-6.
47. Faure, E., et al., *Bacterial lipopolysaccharide activates NF-kappaB through toll-like receptor 4 (TLR-4) in cultured human dermal endothelial cells. Differential expression of TLR-4 and TLR-2 in endothelial cells.* J Biol Chem, 2000. **275**(15): p. 11058-63.
48. Hansson, G.K., et al., *Innate and adaptive immunity in the pathogenesis of atherosclerosis.* Circ Res, 2002. **91**(4): p. 281-91.
49. Loftus, E.V., Jr. and W.J. Sandborn, *Epidemiology of inflammatory bowel disease.* Gastroenterol Clin North Am, 2002. **31**(1): p. 1-20.
50. Papadakis, K.A. and S.R. Targan, *Current theories on the causes of inflammatory bowel disease.* Gastroenterol Clin North Am, 1999. **28**(2): p. 283-96.
51. Xavier, R.J. and D.K. Podolsky, *Unravelling the pathogenesis of inflammatory bowel disease.* Nature, 2007. **448**(7152): p. 427-34.
52. Halfvarson, J., et al., *Inflammatory bowel disease in a Swedish twin cohort: a long-term follow-up of concordance and clinical characteristics.* Gastroenterology, 2003. **124**(7): p. 1767-73.
53. Odes, H.S., et al., *Effects of current cigarette smoking on clinical course of Crohn's disease and ulcerative colitis.* Dig Dis Sci, 2001. **46**(8): p. 1717-21.
54. Silverstein, M.D., B.A. Lashner, and S.B. Hanauer, *Cigarette smoking and ulcerative colitis: a case-control study.* Mayo Clin Proc, 1994. **69**(5): p. 425-9.
55. Lorusso, D., et al., *Cigarette smoking and ulcerative colitis. A case control study.* Hepatogastroenterology, 1989. **36**(4): p. 202-4.
56. Pullan, R.D., et al., *Transdermal nicotine for active ulcerative colitis.* N Engl J Med, 1994. **330**(12): p. 811-5.
57. Sandborn, W.J., et al., *Transdermal nicotine for mildly to moderately active ulcerative colitis. A randomized, double-blind, placebo-controlled trial.* Ann Intern Med, 1997. **126**(5): p. 364-71.
58. Cosnes, J., et al., *Effects of current and former cigarette smoking on the clinical course of Crohn's disease.* Aliment Pharmacol Ther, 1999. **13**(11): p. 1403-11.
59. Beaugerie, L. and H. Sokol, *Appendicitis, not appendectomy, is protective against ulcerative colitis, both in the general population and first-degree relatives of patients with IBD.* Inflamm Bowel Dis, 2010. **16**(2): p. 356-7.
60. Koutroubakis, I.E., I.G. Vlachonikolis, and E.A. Kouroumalis, *Role of appendicitis and appendectomy in the pathogenesis of ulcerative colitis: a critical review.* Inflamm Bowel Dis, 2002. **8**(4): p. 277-86.
61. Andersson, R.E., et al., *Appendectomy is followed by increased risk of Crohn's disease.* Gastroenterology, 2003. **124**(1): p. 40-6.
62. Baumgart, D.C. and S.R. Carding, *Inflammatory bowel disease: cause and immunobiology.* Lancet, 2007. **369**(9573): p. 1627-40.

63. Russel, M.G., et al., *Modern life' in the epidemiology of inflammatory bowel disease: a case-control study with special emphasis on nutritional factors.* Eur J Gastroenterol Hepatol, 1998. **10**(3): p. 243-9.
64. Riordan, A.M., C.H. Ruxton, and J.O. Hunter, *A review of associations between Crohn's disease and consumption of sugars.* Eur J Clin Nutr, 1998. **52**(4): p. 229-38.
65. Itzkowitz, S.H. and X. Yio, *Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation.* Am J Physiol Gastrointest Liver Physiol, 2004. **287**(1): p. G7-17.
66. Itzkowitz, S.H., *Cancer prevention in patients with inflammatory bowel disease.* Gastroenterol Clin North Am, 2002. **31**(4): p. 1133-44.
67. Rubin, C.E., et al., *DNA aneuploidy in colonic biopsies predicts future development of dysplasia in ulcerative colitis.* Gastroenterology, 1992. **103**(5): p. 1611-20.
68. Hussain, S.P., et al., *Increased p53 mutation load in noncancerous colon tissue from ulcerative colitis: a cancer-prone chronic inflammatory disease.* Cancer Res, 2000. **60**(13): p. 3333-7.
69. Romier-Crouzet, B., et al., *Inhibition of inflammatory mediators by polyphenolic plant extracts in human intestinal Caco-2 cells.* Food Chem Toxicol, 2009. **47**(6): p. 1221-30.
70. Galvez, J., et al., *Rutoside as mucosal protective in acetic acid-induced rat colitis.* Planta Med, 1997. **63**(5): p. 409-14.
71. Oz, H.S., et al., *Antioxidants as novel therapy in a murine model of colitis.* J Nutr Biochem, 2005. **16**(5): p. 297-304.
72. Soobrattee, M.A., et al., *Phenolics as potential antioxidant therapeutic agents: mechanism and actions.* Mutat Res, 2005. **579**(1-2): p. 200-13.
73. Bengmark, S., *Bioecological control of inflammatory bowel disease.* Clin Nutr, 2007. **26**(2): p. 169-81.
74. Hur, S.J., et al., *Review of natural products actions on cytokines in inflammatory bowel disease.* Nutr Res, 2012. **32**(11): p. 801-16.
75. Neish, A.S., *Microbes in gastrointestinal health and disease.* Gastroenterology, 2009. **136**(1): p. 65-80.
76. Gordon, J.H. and R. Dubos, *The anaerobic bacterial flora of the mouse cecum.* J Exp Med, 1970. **132**(2): p. 251-60.
77. Eckburg, P.B., et al., *Diversity of the human intestinal microbial flora.* Science, 2005. **308**(5728): p. 1635-8.
78. O'Hara, A.M. and F. Shanahan, *The gut flora as a forgotten organ.* EMBO Rep, 2006. **7**(7): p. 688-93.
79. Frank, D.N., et al., *Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases.* Proc Natl Acad Sci U S A, 2007. **104**(34): p. 13780-5.
80. Swidsinski, A., et al., *Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence in situ hybridization study in mice.* World J Gastroenterol, 2005. **11**(8): p. 1131-40.
81. Gronlund, M.M., et al., *Influence of mother's intestinal microbiota on gut colonization in the infant.* Gut Microbes, 2011. **2**(4): p. 227-33.
82. Flint, H.J., et al., *The role of the gut microbiota in nutrition and health.* Nat Rev Gastroenterol Hepatol, 2012. **9**(10): p. 577-89.

83. Macpherson, A.J. and N.L. Harris, *Interactions between commensal intestinal bacteria and the immune system.* Nat Rev Immunol, 2004. **4**(6): p. 478-85.
84. Mazmanian, S.K., et al., *An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system.* Cell, 2005. **122**(1): p. 107-18.
85. Fink, L.N., et al., *Distinct gut-derived lactic acid bacteria elicit divergent dendritic cell-mediated NK cell responses.* Int Immunol, 2007. **19**(12): p. 1319-27.
86. Round, J.L. and S.K. Mazmanian, *The gut microbiota shapes intestinal immune responses during health and disease.* Nat Rev Immunol, 2009. **9**(5): p. 313-23.
87. Salzman, N.H., M.A. Underwood, and C.L. Bevins, *Paneth cells, defensins, and the commensal microbiota: a hypothesis on intimate interplay at the intestinal mucosa.* Semin Immunol, 2007. **19**(2): p. 70-83.
88. Cash, H.L., et al., *Symbiotic bacteria direct expression of an intestinal bactericidal lectin.* Science, 2006. **313**(5790): p. 1126-30.
89. Lopez-Boado, Y.S., et al., *Bacterial exposure induces and activates matrilysin in mucosal epithelial cells.* J Cell Biol, 2000. **148**(6): p. 1305-15.
90. Corr, S.C., C.G. Gahan, and C. Hill, *Impact of selected Lactobacillus and Bifidobacterium species on Listeria monocytogenes infection and the mucosal immune response.* FEMS Immunol Med Microbiol, 2007. **50**(3): p. 380-8.
91. Sanz, Y., I. Nadal, and E. Sanchez, *Probiotics as drugs against human gastrointestinal infections.* Recent Pat Antiinfect Drug Discov, 2007. **2**(2): p. 148-56.
92. Nicholson, J.K., et al., *Host-gut microbiota metabolic interactions.* Science, 2012. **336**(6086): p. 1262-7.
93. Tremaroli, V. and F. Backhed, *Functional interactions between the gut microbiota and host metabolism.* Nature, 2012. **489**(7415): p. 242-9.
94. Backhed, F., et al., *The gut microbiota as an environmental factor that regulates fat storage.* Proc Natl Acad Sci U S A, 2004. **101**(44): p. 15718-23.
95. Zeng, H., D.L. Lazarova, and M. Bordonaro, *Mechanisms linking dietary fiber, gut microbiota and colon cancer prevention.* World J Gastrointest Oncol, 2014. **6**(2): p. 41-51.
96. Moore, W.E. and L.H. Moore, *Intestinal floras of populations that have a high risk of colon cancer.* Appl Environ Microbiol, 1995. **61**(9): p. 3202-7.
97. Kumar, M., et al., *Probiotic metabolites as epigenetic targets in the prevention of colon cancer.* Nutr Rev, 2013. **71**(1): p. 23-34.
98. Macpherson, A., et al., *Mucosal antibodies in inflammatory bowel disease are directed against intestinal bacteria.* Gut, 1996. **38**(3): p. 365-75.
99. Sellon, R.K., et al., *Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice.* Infect Immun, 1998. **66**(11): p. 5224-31.
100. Kent, T.H., et al., *Effect of antibiotics on bacterial flora of rats with intestinal blind loops.* Proc Soc Exp Biol Med, 1969. **132**(1): p. 63-7.
101. Garrett, W.S., et al., *Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system.* Cell, 2007. **131**(1): p. 33-45.
102. Froicu, M., Y. Zhu, and M.T. Cantorna, *Vitamin D receptor is required to control gastrointestinal immunity in IL-10 knockout mice.* Immunology, 2006. **117**(3): p. 310-8.

103. Sokol, H., et al., *Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients.* Proc Natl Acad Sci U S A, 2008. **105**(43): p. 16731-6.
104. Dai, Q., et al., *Fruit and vegetable juices and Alzheimer's disease: the Kame Project.* Am J Med, 2006. **119**(9): p. 751-9.
105. Vasanthi, H.R., N. ShriShriMal, and D.K. Das, *Phytochemicals from plants to combat cardiovascular disease.* Curr Med Chem, 2012. **19**(14): p. 2242-51.
106. Pappas, E. and K.M. Schaich, *Phytochemicals of cranberries and cranberry products: characterization, potential health effects, and processing stability.* Crit Rev Food Sci Nutr, 2009. **49**(9): p. 741-81.
107. Henig, Y.S. and M.M. Leahy, *Cranberry juice and urinary-tract health: science supports folklore.* Nutrition, 2000. **16**(7-8): p. 684-7.
108. DeMoranville, C., *Cranberry Nutrient Management in Southeastern Massachusetts: Balancing Crop Production Needs and Water Quality.* Horttechnology, 2015. **25**(4): p. 471-476.
109. Howell, A.B., et al., *Inhibition of the adherence of P-fimbriated Escherichia coli to uroepithelial-cell surfaces by proanthocyanidin extracts from cranberries.* N Engl J Med, 1998. **339**(15): p. 1085-6.
110. He, X. and R.H. Liu, *Cranberry phytochemicals: Isolation, structure elucidation, and their antiproliferative and antioxidant activities.* J Agric Food Chem, 2006. **54**(19): p. 7069-74.
111. Deubert, K.H., *A rapid method for the extraction and quantitation of total anthocyanin of cranberry fruit.* J Agric Food Chem, 1978. **26**(6): p. 1452-3.
112. Wu, X. and R.L. Prior, *Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: fruits and berries.* J Agric Food Chem, 2005. **53**(7): p. 2589-99.
113. Mulleder, U., M. Murkovic, and W. Pfannhauser, *Urinary excretion of cyanidin glycosides.* J Biochem Biophys Methods, 2002. **53**(1-3): p. 61-6.
114. Gu, L., et al., *Concentrations of proanthocyanidins in common foods and estimations of normal consumption.* J Nutr, 2004. **134**(3): p. 613-7.
115. Foo, L.Y., et al., *The structure of cranberry proanthocyanidins which inhibit adherence of uropathogenic P-fimbriated Escherichia coli in vitro.* Phytochemistry, 2000. **54**(2): p. 173-81.
116. Zuo, Y., C. Wang, and J. Zhan, *Separation, characterization, and quantitation of benzoic and phenolic antioxidants in American cranberry fruit by GC-MS.* J Agric Food Chem, 2002. **50**(13): p. 3789-94.
117. Chen, H., Y. Zuo, and Y. Deng, *Separation and determination of flavonoids and other phenolic compounds in cranberry juice by high-performance liquid chromatography.* J Chromatogr A, 2001. **913**(1-2): p. 387-95.
118. Neto, C.C., et al., *MALDI-TOF MS characterization of proanthocyanidins from cranberry fruit (Vaccinium macrocarpon) that inhibit tumor cell growth and matrix metalloproteinase expression in vitro.* Journal of the Science of Food and Agriculture, 2006. **86**(1): p. 18-25.
119. Huang, Y., et al., *Effects of cranberry extracts and ursolic acid derivatives on P-fimbriated Escherichia coli, COX-2 activity, pro-inflammatory cytokine release and the NF-kappabeta transcriptional response in vitro.* Pharm Biol, 2009. **47**(1): p. 18-25.

120. Vvedenskaya, I.O., et al., *Characterization of flavonols in cranberry (Vaccinium macrocarpon) powder.* J Agric Food Chem, 2004. **52**(2): p. 188-95.
121. Foxman, B., et al., *Urinary tract infection: self-reported incidence and associated costs.* Ann Epidemiol, 2000. **10**(8): p. 509-15.
122. Bailey, D.T., et al., *Can a concentrated cranberry extract prevent recurrent urinary tract infections in women? A pilot study.* Phytomedicine, 2007. **14**(4): p. 237-41.
123. Stothers, L., *A randomized trial to evaluate effectiveness and cost effectiveness of naturopathic cranberry products as prophylaxis against urinary tract infection in women.* Can J Urol, 2002. **9**(3): p. 1558-62.
124. Kontiokari, T., et al., *Dietary factors protecting women from urinary tract infection.* Am J Clin Nutr, 2003. **77**(3): p. 600-4.
125. Gupta, K., et al., *Antimicrobial resistance among uropathogens that cause community-acquired urinary tract infections in women: a nationwide analysis.* Clin Infect Dis, 2001. **33**(1): p. 89-94.
126. Gupta, K., et al., *Cranberry products inhibit adherence of p-fimbriated Escherichia coli to primary cultured bladder and vaginal epithelial cells.* J Urol, 2007. **177**(6): p. 2357-60.
127. Shmuely, H., et al., *Effect of cranberry juice on eradication of Helicobacter pylori in patients treated with antibiotics and a proton pump inhibitor.* Mol Nutr Food Res, 2007. **51**(6): p. 746-51.
128. Burger, O., et al., *A high molecular mass constituent of cranberry juice inhibits helicobacter pylori adhesion to human gastric mucus.* FEMS Immunol Med Microbiol, 2000. **29**(4): p. 295-301.
129. Duarte, S., et al., *Inhibitory effects of cranberry polyphenols on formation and acidogenicity of Streptococcus mutans biofilms.* FEMS Microbiol Lett, 2006. **257**(1): p. 50-6.
130. Labrecque, J., et al., *Effects of a high-molecular-weight cranberry fraction on growth, biofilm formation and adherence of Porphyromonas gingivalis.* J Antimicrob Chemother, 2006. **58**(2): p. 439-43.
131. Kandil, F.E., et al., *Composition of a chemopreventive proanthocyanidin-rich fraction from cranberry fruits responsible for the inhibition of 12-O-tetradecanoyl phorbol-13-acetate (TPA)-induced ornithine decarboxylase (ODC) activity.* J Agric Food Chem, 2002. **50**(5): p. 1063-9.
132. Youdim, K.A., et al., *Potential role of dietary flavonoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults (small star, filled).* J Nutr Biochem, 2002. **13**(5): p. 282-288.
133. Porter, M.L., et al., *Cranberry proanthocyanidins associate with low-density lipoprotein and inhibit in vitro Cu2+-induced oxidation.* Journal of the Science of Food and Agriculture, 2001. **81**(14): p. 1306-1313.
134. Deprez, S., et al., *Polymeric proanthocyanidins are catabolized by human colonic microflora into low-molecular-weight phenolic acids.* J Nutr, 2000. **130**(11): p. 2733-8.
135. Andres-Lacueva, C., et al., *Anthocyanins in aged blueberry-fed rats are found centrally and may enhance memory.* Nutr Neurosci, 2005. **8**(2): p. 111-20.
136. Maharshak, N., et al., *Altered enteric microbiota ecology in interleukin 10-deficient mice during development and progression of intestinal inflammation.* Gut Microbes, 2013. **4**(4): p. 316-24.

137. Guinane, C.M., and Paul D. Cotter, *<Role of the gut microbiota in health and chronic gastrointestinal disease- understanding a hidden metabolic organ.pdf>*. Therapeutic advances in gastroenterology, 2013. **6**(4): p. 295-308.
138. Mazmanian, S.K., J.L. Round, and D.L. Kasper, *A microbial symbiosis factor prevents intestinal inflammatory disease.* Nature, 2008. **453**(7195): p. 620-5.
139. Feghali, K., et al., *Cranberry proanthocyanidins: natural weapons against periodontal diseases.* J Agric Food Chem, 2012. **60**(23): p. 5728-35.
140. Wang, H., et al., *Dietary Non-digestible Polysaccharides Ameliorate Intestinal Epithelial Barrier Dysfunction in IL-10 Knockout Mice.* J Crohns Colitis, 2016.
141. Klimesova, K., et al., *Altered gut microbiota promotes colitis-associated cancer in IL-1 receptor-associated kinase M-deficient mice.* Inflamm Bowel Dis, 2013. **19**(6): p. 1266-77.
142. Gkouskou, K.K., et al., *The gut microbiota in mouse models of inflammatory bowel disease.* Front Cell Infect Microbiol, 2014. **4**: p. 28.
143. Keubler, L.M., et al., *A Multihit Model: Colitis Lessons from the Interleukin-10-deficient Mouse.* Inflamm Bowel Dis, 2015. **21**(8): p. 1967-75.
144. Arthur, J.C., et al., *Microbial genomic analysis reveals the essential role of inflammation in bacteria-induced colorectal cancer.* Nat Commun, 2014. **5**: p. 4724.
145. Valcheva, R., et al., *Soluble Dextrin Fibers Alter the Intestinal Microbiota and Reduce Proinflammatory Cytokine Secretion in Male IL-10-Deficient Mice.* J Nutr, 2015. **145**(9): p. 2060-6.
146. Cooper, H.S., et al., *Clinicopathologic study of dextran sulfate sodium experimental murine colitis.* Lab Invest, 1993. **69**(2): p. 238-49.
147. Park, M.Y., G.E. Ji, and M.K. Sung, *Dietary kaempferol suppresses inflammation of dextran sulfate sodium-induced colitis in mice.* Dig Dis Sci, 2012. **57**(2): p. 355-63.
148. Klindworth, A., et al., *Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies.* Nucleic Acids Res, 2013. **41**(1): p. e1.
149. Caporaso JG, K.J., Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI et al, *QIIME allows analysis of high-throughput community sequencing data.* Nature methods, 2010. **7**(5): p. 335-336

150. Lozupone, C.A. and R. Knight, *Species divergence and the measurement of microbial diversity.* FEMS Microbiol Rev, 2008. **32**(4): p. 557-78.
151. Navas-Molina, J.A., et al., *Advancing our understanding of the human microbiome using QIIME.* Methods Enzymol, 2013. **531**: p. 371-444.
152. Langille, M.G., et al., *Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences.* Nat Biotechnol, 2013. **31**(9): p. 814-21.
153. Parks, D.H., et al., *STAMP: statistical analysis of taxonomic and functional profiles.* Bioinformatics, 2014. **30**(21): p. 3123-4.
154. Pierre, J.F., et al., *Cranberry proanthocyanidins improve the gut mucous layer morphology and function in mice receiving elemental enteral nutrition.* JPEN J Parenter Enteral Nutr, 2013. **37**(3): p. 401-9.
155. Obermeier, F., et al., *Interferon-gamma (IFN-gamma)- and tumour necrosis factor (TNF)-induced nitric oxide as toxic effector molecule in chronic dextran sulphate sodium*

(DSS)-induced colitis in mice. Clinical and Experimental Immunology, 1999. **116**(2): p. 238-245.
156. Coccia, M., et al., *IL-1beta mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4(+) Th17 cells.* J Exp Med, 2012. **209**(9): p. 1595-609.
157. Atreya, R. and M.F. Neurath, *Involvement of IL-6 in the pathogenesis of inflammatory bowel disease and colon cancer.* Clin Rev Allergy Immunol, 2005. **28**(3): p. 187-96.
158. Ouyang, W., et al., *Regulation and functions of the IL-10 family of cytokines in inflammation and disease.* Annu Rev Immunol, 2011. **29**: p. 71-109.
159. Yanaba, K., et al., *IL-10-producing regulatory B10 cells inhibit intestinal injury in a mouse model.* Am J Pathol, 2011. **178**(2): p. 735-43.
160. Ivanov, II, et al., *Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine.* Cell Host Microbe, 2008. **4**(4): p. 337-49.
161. Dharmani, P., P. Leung, and K. Chadee, *Tumor Necrosis Factor-alpha and Muc2 Mucin Play Major Roles in Disease Onset and Progression in Dextran Sodium Sulphate-Induced Colitis.* Plos One, 2011. **6**(9).
162. Png, C.W., et al., *Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria.* Am J Gastroenterol, 2010. **105**(11): p. 2420-8.
163. Derrien, M., et al., *Modulation of Mucosal Immune Response, Tolerance, and Proliferation in Mice Colonized by the Mucin-Degrader Akkermansia muciniphila.* Front Microbiol, 2011. **2**: p. 166.
164. Othman, M., R. Aguero, and H.C. Lin, *Alterations in intestinal microbial flora and human disease.* Curr Opin Gastroenterol, 2008. **24**(1): p. 11-6.
165. Rakoff-Nahoum, S. and R. Medzhitov, *Role of toll-like receptors in tissue repair and tumorigenesis.* Biochemistry (Mosc), 2008. **73**(5): p. 555-61.
166. Chow, J., H. Tang, and S.K. Mazmanian, *Pathobionts of the gastrointestinal microbiota and inflammatory disease.* Curr Opin Immunol, 2011. **23**(4): p. 473-80.
167. Xiao, H., et al., *Green tea polyphenols inhibit colorectal aberrant crypt foci (ACF) formation and prevent oncogenic changes in dysplastic ACF in azoxymethane-treated F344 rats.* Carcinogenesis, 2008. **29**(1): p. 113-9.
168. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.