Studies on biological effects of mulberry fruits against microbial composition and intestinal inflammation

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CONTENTS

LIST OF ABBREVIATIONS

In thesis

ASC: apoptosis-associated speck-like protein containing a caspase recruitment domain

Casp-1: Caspase-1

CD: Crohn's disease

COX-2: cyclooxygenase-2

C. perfringens: *Clostridium perfringens*

DAI: disease activity index

DGGE: denaturing gradient gel electrophoresis

DSS: dextran sodium sulfate

E. coil*: *Escherichia coli

GSH-Px: glutathione peroxidase

HE: hematoxylin-eosin

IL: interleukin

IBD: Inflammatory bowel disease

L. plantarum*: *Lactobacillus plantarum

M. alba: morus alba

M. nigra: morus nigra

M. rubra: morus rubra

MFP: mulberry juice freeze-dried powder

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

NLRPs: binding oligomerization domain protein-like receptors

PAMP: pathogen-associated molecule pattern

PPRs: pattern recognition receptors

PCR: polymerase Chain Reaction

qPCR: quantitative real-time polymerase Chain Reaction

RT-qPCR: reverse transcription quantitative polymerase Chain Reaction

RNS: reactive nitrogen species

ROS: reactive oxygen species

SCFAs: short-chain fatty acids

SD: standard deviation

SE: standard error

TMAO: Trimethylamine N-oxide

TNBS: 2,4,6-trinitrobenzene sulphonic acid

TNF-*α*: tumor necrosis factor alpha

TLRs: Toll-like receptors

UC: ulcerative colitis

In Tables

COX-2: cyclooxygenase-2

EGCG: (-)-epigallocatechin-3-gallate

GSH: glutathione

GSH-Px: glutathione peroxidase

iNOS: inducible nitric oxide synthase

LPO: lipid peroxides

MMP-2: matrix matalloprotinase-2

MPO: myeloperoxidase

MSE: Moringa (*Moringa oleifera Lam.*) seed extract

NO: nitric oxide

PAI: plasminogen activator inhibitor

SOD: superoxide dismutase

Th: T helper

TIMP-2: tissue inhibitor of matrix matalloprotinase-2

u-PA: urokinase-plasminogen activator

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ABSTRACT

Inflammatory bowel disease (IBD) has attracted widespread attention because the incidence of it shows an increasing trend from 1990. This disease is characterized as chronic and relapsing intestinal inflammation. The factors such as diet, smoking, mucosal immunity, and gut microbiota are considered as the risk factor of this disease. The integrity of epithelial tissue is an important guarantee for intestinal health and gut microbiota plays a crucial role in intestinal homeostasis. There are reported that the composition of intestinal microbiota associates with the pathogenesis of ulcerative colitis (UC) and Crohn's disease (CD). The gut microbiota has been shown to influence homeostasis of the intestinal mucosa by enhancing barrier function. As mucosal barrier integrity is essential for blocking the access of microorganisms to underlying tissues. Studies in rodent model have linked tissue damage and disruption of the epithelial barrier in the gut to cytokine imbalances. UC is generally recognized as an immune-mediated disorder resulting from abnormal interaction between intestinal microbiota and mucosal immune cells.

The differential compositions of intestinal microbiota have been also reported in the patients with IBD. As a kind of strictly anaerobic bacteria, *Bacteroides* spp. played a vital role in the induction of colitis. The results of bacterial contents analysis have showed that *Bacteroidetes* spp. has highly representation in UC patients, but contained lower in healthy adults, respectively. There have been also reported that two bacteria species, *Bifidobacterium* spp. and *Clostridium perfringens* group, are known as beneficial factors to UC. However, the etiology of this disease is unclear.

Current therapies to IBD patients have limited efficacy because of their potentially serious side-effects in long-term use. Hence, we have considered that the development of new drugs and the effective useful way of supplements in combination are the important approaches. Recently, natural plants containing polyphenol have attracted wide attention because polyphenol have many biological functions such as anti-inflammation. For example, there was reported that grape pomace extracts depressed the colonic inflammation by preventing the expressions of pro-inflammatory genes and regulating oxidative stress in colitis. We have focused on the mulberry which has already used as a traditional medicine and contains rich beneficial ingredients such as polyphenols especially anthocyanin.

The aims of this study were as follows: (1) to evaluate the microbial compositions after the mulberry juice freeze-dried powder (MFP) supplementation. (2) to evaluate the inhibitory effect of mulberry fruits against the acute colitis and its mode of action which focused on both microbial compositions and gene expression related to inflammation.

In Chapter 2, to evaluate the compositional changes, the microbial DNA from the feces was analyzed by denaturing gradient gel electrophoresis (DGGE) and DNA sequencing for bacterial species. C57BL/6 mice (male, 5 weeks old) were randomly divided into 2 groups (n＝5). These mice fed the diet with or without MFP for 12 weeks. At the end of the experiment, the feces were collected, and the change of intestinal microbiota was analyzed. The results of DGGE showed that the number of bands in MFP diet group was decreased compared with normal diet group. Moreover, the results of DNA sequence showed that MFP diet decreased the bands of *Lactobacillus plantarum,* which possibly led to the disruption of the cell and contributed to promoting cellular lysis. Additionally, the band of *Bifidobacterium pseudolongum* was shallower in the MFP group compared with the normal diet group. Those results proved that mulberry diet modified the composition of intestinal microbiota.

In Chapter 3 and 4, the mitigative effects of MFP against intestinal inflammation were evaluated using DSS-induced acute colitis mouse model. BALB/c mice (male, 7 weeks old) were randomly divided into 6 groups (n＝3). In the first three weeks, mice were fed with diet supplied with (Group 1 to 4) or without (Group 5 and 6) MFP. In the fourth week, mice received water containing 5% (w/v) DSS (Group 1, 3, and 5) or not (Group 2, 4, and 6). Meanwhile, the mice in Group 1 and 2 continued to be fed with MFP diet while the mice in other groups (Group 3 to 6) were fed with normal diet. The body weight and fecal condition of mice were daily checked. The disease activity index (DAI) score was calculated according to body weight loss and symptoms of this disease. At the end of this experiment, the mice were sacrificed, their colonic tissues and feces were collected. The proximal colon was immediately frozen at -80˚C until use. The mRNA expressions of IL-1β and TNF-α were evaluated by RT-qPCR using the proximal colon tissue. The middle and distal colons were fixed with 10% formaldehyde for histopathological observation and the microscopic score was evaluated according to crypt destruction, extent of disease, and the condition of immunocyte infiltration. Furthermore, the bacterial compositions of feces were determined by qPCR. The results

showed that severe body weight loss and tissue damages were not observed in Group 1 mice. DAI score in mice fed MFP was significantly decreased compared with the mice not fed MFP. At the same time, the mRNA expression of IL-1β and TNF-α in Group 1 mice were lower than the expression of those in Group 3 mice. A remarkable decrease was observed for *Bifidobacterium* spp. and *Clostridium perfringens* subgroup, whereas a significant increase was observed for the *Bacteroides* spp. in mice fed without MFP. Those results indicated that MFP inhibited the colonic inflammation by modifying the intestinal microbiota in DSS-induced acute colitis mice model. The mRNA expressions of both NLRP3 and NLRP6 were determined by RT-qPCR using the proximal colon tissue. Additionally, the middle and distal colons were fixed with 10% formaldehyde for counting the number of goblet cell. The results showed that an increase was observed for the number of goblet cell and NLRP6 expression in mice fed MFP compared with the mice not fed MFP. NLRP6 is highly expressed in goblet cells and this inflammasome has been considered an essential factor for mucosal self-renewal, cell proliferation, and regulation of intestinal microbiota through mucus secretion and anti-microbial peptide production from goblet cells. These results indicated that MFP maintained the number of goblet cell by modifying the expression of NLRP6 in DSS-induced acute colitis. MFP suppressed body weight loss, colon shorting, and inflammatory level in the colon. Meanwhile, MFP increased the contents of *Bifidobacterium* spp. and *C. perfringens* subgroup, whereas decreased the *Bacteroides* spp. in feces. Moreover, MFP increased the expression of NLRP6 inflammasome and maintained the number of goblet cell.

In conclusion, the beneficial bacteria are dominant in the gut by MFP supplementation. Furthermore, MFP supplementation leads to protect mucus condition by the expression of NLRP6 inflammasome and the maintenance of goblet cells. As those results, MFP supplementation mitigates the intestinal inflammations.

Keywords: DSS-induced acute colitis, Goblet cells, Inflammasome, Microbiota, Mulberry, NLRP6, Polyphenol.

CHAPTER 1

General Introduction

1.1 Mulberry fruits have some bioactivities that depend on special components

Mulberry is used as a traditional medicine which depends on some special components such as polyphenols. This plant belongs to the genus *Morus* and the family *Moraceae* and is cultivated in India, Africa, America, Asia and Europe (Khan et al., 2013). Mulberry approximately contains 24 species, and the functions of white mulberry (*Morus alba*), red mulberry (*Morus rubra*), and black mulberry (*Morus nigra*) are deeply investigated in functional food field (Khalifa, et al., 2018). The nutrients of those including sugar, ascorbic acid, fatty acid, phenols, mineral elements. Whereas, the contents of nutrients are different for mulberry species showed in Table 1.1 (Ercisli and Orhan, 2007; Imran, et al., 2010). According to this table, the content of total fat in *Morus nigra* (*M. nigra*) indicates in the middle between that in *Morus alba* (*M. alba*) and that in *Morus rubra* (*M. rubra*). Moreover, the contents of total phenolics and total flavonoids in *M. nigra* are significantly higher than the contents of those in *M. alba* and *M. rubra*, and the taste of *M. nigra* is better than others because of lower PH value (nearly 3) (Yang, et al., 2010). The major polyphenol components of mulberry fruit including anthocyanins, flavonols, benzoic acids, flavonoids, and hydroxycinnamic acids (Zhang, et al., 2018). Among those components, the anthocyanins are considered as a key factor and contain cyanidin-3-glucoside and cyanidin-3-rutinoside. Those two ingredients relate with the control of fruit color (Gerasopoulos and Stavroulakis, 1997; Liu, et al., 2004). Recently, accumulative scientific results have indicated that mulberry fruits had some bioactivities such as antioxidant, anti-obesity, and anticancer (Table 1.2).

1.1.1 Antioxidant effect

As far as we know, anaerobes microorganisms can survive under anaerobic environment, however, their growth is inhibited or stopped under a high oxygen environment (more than 21% oxygen). However, there are also some anaerobic bacteria and facultative anaerobes in intestinal tract of human being. Therefore, the content of

oxygen plays a key role in our health. There are two methods to produce oxygen in human body. On the one hand, it is an unavoidable method that the body makes oxygen by accident of chemistry, meanwhile, there are some molecular reactions with oxygen to maintain the normal function of human being. Of course, the oxygen which makes from this method is useful and necessary for health. On the other hand, a large amount of superoxide is generated from some phagocytes to inhibit tissue injury which is caused by any pathogenic factors (Holland, 2010). Under this condition, some diseases can be induced by excessive phagocyte activation such as IBD (Halliwell, 2012). During this procedure, reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a key role and control the development of disease (Sheng, et al., 2014; Fridovich, 1998). ROS levels significantly increase under some stresses such as heat stress and contribute to oxidant damages. RNS and ROS result in cell damages. In order to maintain cell homeostasis, some endogenous antioxidant factors such as active superoxide dismutases and H_2O_2-removing enzymes help to inhibit the oxidation reaction. Unfortunately, it is not adequate just to depend on those factors against oxidant damages (Halliwell, 1996). Therefore, it is necessary to research other ways to reduce oxidant damages. Recently, some researches have proved that mulberry fruit had excellent antioxidant properties (Table 1.2).

It has been proved that various extract methods and store methods affect the contents of polyphenols (Yu, et al., 2014; Ojha, et al., 2017; Pehluvan, Kaya, et al., 2015). Moreover, the antioxidant capacities of mulberry fruit also relate to the purification methods (Qian, et al., 2015; Jiang, et al., 2013). The total polyphenolic content of *M. nigra* is higher than that in *M. alba* (Arfan, et al., 2012; Hosseini, et al., 2018). The antioxidant mechanism of mulberry fruit remains unclear. However, some scientific results suggested that this mechanism relates to (1) mulberry fruit can inhibit α-glucosidase activity, increase antioxidant enzymatic activities such as catalase and glutathione peroxidase (GSH-Px) (Wang, et al, 2013). (2) mulberry fruit can inhibit intracellular glutathione depletion and restore the mitochondrial membrane function (Chen, et al., 2017). (3) mulberry fruits can decrease the atherogenic index and lipid peroxidation, increase serum high-density lipoprotein cholesterol and superoxide dismutase (Yang, et al., 2010; Natić, et al., 2015). Those mechanisms are proved in mice model and cells culture (Bao, et al., 2016).

The effective component against oxidant is polyphenol extracted from mulberry fruit (Kamiloglu, et al., 2013). The anthocyanin is considered the most effective ingredient of polyphenol against oxidant (Jin, et al., 2015). The anthocyanin is water-soluble vacuolar pigments. The contents of anthocyanins are different for the three important species of mulberry fruit and the content of anthocyanins in *M. nigra* is higher than those in *M. alba* and *M. rubra* (Aramwit, et al., 2010). In order to determine the function of anthocyanins, they were purified from mulberry fruit and the components of anthocyanins including cyanidin-3-glucoside, cyanidin-3-rutinoside and pelargonidin-3-glucoside (Wu, et al., 2013a). The results suggested that the effective part against oxidant is 3,4-dihydroxy substituent in the B ring from anthocyanins (Du, et al., 2008). However, it needs to be further investigated for the mechanism of antioxidant in polyphenols from mulberry fruit using different cells and animal models.

1.1.2 Anti-obesity

Recently, obesity is growing into a global problem and it typically results from over-eating (especially an unhealthy diet) and lack of exercise. It has been proved that obesity is related to some diseases such as a migraine in women (Pavlovic, et al., 2017), renovascular disease (Zhang and Lerman, 2015), diabetes (Coleman, 1978), cardiovascular disease (Caleyachetty, et al., 2017), non-alcoholic fatty liver disease (Tarantino, 2008), and cancer (Kim, et al., 2006). Current therapies to treat obesity are increasing exercise and adjusting diet. However, the prevention of obesity is more important than treatment. Recently, accumulative scientific results have indicated that polyphenols had the function of anti-obesity. The polyphenol can reduce the body weight gain by stimulating the cellular energy expenditure and suppressing the expression of fatty acid synthase (Ikarashi, et al., 2011; Lin, & Lin-Shiau, 2006; Sergent, et al., 2012). As a natural fruit, mulberry fruit is a good candidate to lose weight because it contains rich polyphenols. The mechanism of anti-obesity in mulberry fruit was investigated using animal model and cells showed in Table 1.2.

The mulberry fruit can reduce the level of obesity by decreasing the body weight, fasting plasma glucose and insulin, and improving glucose control during intraperitoneal glucose tolerance test; moreover, reducing protein levels of oxidative stress markers (manganese superoxide dismutase) and inflammatory markers (monocyte chemoattractant protein-1, inducible nitric oxide synthase, C-reactive protein, tumor necrosis factor-α and interleukin-1) in liver and adipose tissue (Lim, et

al., 2013a; Lim, et al., 2013b). Furthermore, a publication reported that anthocyanins extracted form mulberry fruit inhibited obesity by improving the function of mitochondrion via p38-AMPKPGC1α pathway (You, et al., 2015). The cyanidin-3-glucoside and cyanidin3-rutinoside are major components against obesity (Huang, et al., 2011; Liu, et al., 2008; Liu, et al., 2009).

The effect of them for the anti-obesity function is dose-dependent, and the higher content, the more effective (Ghadimi, et al., 2017). Furthermore, they also improved the impaired hepatic function and significantly decreased the leptin secretion (Wu, et al., 2013). Leptin is a product of the obese gene and secretes in white adipose tissue, it plays an important role in regulating energy homeostasis and lipid metabolism. The obesity animal had a high concentration of leptin has been reported. Moreover, cyanidin 3-glucoside and cyanidin 3-rutinoside also increased the number of mitochondria during brown adipogenesis (You, et al., 2017), which has a relationship with the function of anti-obesity (Wu, et al., 2013; Yoneshiro, et al., 2013).

1.1.3 Anticancer

Cancer is a serious disease and features by the uncontrolled cell proliferation and propagation, meanwhile, lung cancer and breast cancer are common types of cancer (Danaei, et al., 2005; El-Sheekh, & El-Kassas, 2014). There are a lot of people die from cancer every year, moreover, there are also 14.1 million new cancer cases occur has been reported (Torre, et al., 2015). Long-term anti-cancer drugs and chemotherapy have caused cancer patients great pain. Recently, it has been indicated that the polyphenols of mulberry fruit affected the cellar differentiation, proliferation, and apoptosis using cancer cell lines or animal tumor models (Jeong, et al., 2010). The researchers have been devoting themselves to the development of new drugs or methods and the effective way of supplements in combination. Recently, it has been reported that mulberry fruit has the potential to become an excellent candidate for cancer prevention because of their bioactivities including antioxidant, anti-obesity, detoxification activity, induction of apoptosis, antiproliferation and antiangiogenic activity (Chon, et al., 2009).

Chen et al. found that the anthocyanins extracted from mulberry fruit decreased the expressions of matrix metalloproteinase-2 and urokinase-plasminogen activator, enhanced the expression of tissue inhibitor of matrix metalloproteinase-2 and plasminogen activator inhibitor using highly metastatic A549 human lung carcinoma

cells (Chen, et al., 2006). Likewise, anthocyanin-rich mulberry inhibited cancer cell growth by regulating some genes signals such as p38/p53 (Huang, et al., 2011). The cyanidin-3-rutinoside and cyanidin-3-glucoside extracted from anthocyanin were considered as effective components of anticancer. Except for anthocyanin, hydroxycinnamic acid derivatives of mulberry fruits can kill the cancer cells by increasing ROS production (Trivellini, et al., 2016). It needs further research about the mechanism of mulberry fruit in anticancer function.

1.1.4 Other effects

The polyphenols from mulberry fruit are reported to have others bioactivities, such as antidiabetic and hypolipidemic. The main mechanism includes the polyphenol from mulberry fruit (1) prevented the progressively declining of insulin secretion through protecting β-cell and enhanced hepatic tissues glucose uptake (Sarikaphuti, et al., 2013); (2) increased levels of phosphorylated AMPK, which suppressed hepatic glucose production via an AMPK-mediated reduction in the protein level of genes encoding G6Pase and PEPCK (Choi, et al., 2016). The effective components are also cyanidin-3-glucoside and cyanidin-3-rutinoside. Moreover, they can increase the levels of phosphorylated AMPK, AS160, and GLUT4 levels in the plasma membrane (Choi, et al., 2016).

Hyo Geun Kim *et al.* reported that mulberry fruit has the functions of anti-apoptotic by regulating ROS, NO generation, Bcl-2, Bax proteins, mitochondrial membrane depolarization, and caspase-3 activation (Kim, et al., 2010). Furthermore, mulberry fruit also has the functions of anti-stress, anti-HIV and vitamin C-synergized radical scavenging activity (Sakagami, et al., 2007). On the other hand, mulberry fruit contains the functions of antimicrobial activity and anti-fatigue by inhibiting the growth of some bacteria and fungi strains (Minhas, et al., 2016; Jiang, et al., 2013).

1.2 Inflammatory bowel disease

1.2.1 Epidemiology

IBD, which includes UC and CD, is characterized by chronic or relapsing uncontrolled immune activation and inflammation in the intestinal tract. CD is limited to the mucosa, and it was first described by Dr. Burrill Crohn, et al. The crypt distortion, crypt distortion, and goblet cells loss were observed in the mucosa. UC was first

described by Dr. Samuel Wilks. The deep fissuring ulcers, fistulas, patchy changes, and granulomas usually were observed in submucosal (Lawley, 1994). Since 1990, the disease has spread rapidly from Europe to Asia, Africa, and other regions. The incidence of IBD has been reported from 24.3 million persons per year to 396 million persons per year and the symptoms including diarrhea, fever, bloody feces and body weight loss (Lovasz, et al., 2013). This disease becomes a global disease and leads to a serious decline in patients' life quality. Unfortunately, this disease also increases the risk of intestinal cancer. However, the etiology of IBD still remains unclear. It is reported that some factors such as environmental factors, diet, intestinal microbiota, and mucosal immunity have relation to this disease. In addition, IBD tends to have a genetic predisposition and CD is higher than UC (Langholz, et al., 1994; Sands, 2007; Halme, et al., 2006).

The integrity of intestinal structure is essential to maintain intestinal homeostasis. Recently, intestinal microbiota has been considered as an "organ" in the body because it plays an important role in intestinal immune response (Possemiers, et al., 2011). It is widely accepted that the disruption of the epithelial barrier triggers an invasion of the bacteria into the mucosal layer, resulting in activation of the inflammatory process that contains immune response. *Bacteroides* and *E. coli* are regarded as two important pathogenic factors for the induction of colitis, furthermore, *Bacteroides* alone is more active than *E. coli* alone (Penders, 2005). However, the mechanism of this induction is incompletely understood. It is reported that the intestinal microbiota has effect on the expression of some genes such as transforming growth factor beta 1 (TGF-β) (McGovern, et al., 2010; Franke, et al., 2010). The model is useful to study the contribution of innate immune systems in intestinal inflammation and presumably due to the toxic effects of DSS on the epithelial barrier dysfunction.

The inflammatory process is accompanied by immunological cell infiltration, the unregulated production of proinflammatory cytokines and ROS in the lamina propria of the colon. ROS are also generated from mitochondria during adenosine triphosphate synthesis, and excessive amounts of ROS can trigger a large amount of damage to DNA and protein. It is suggested that highly oxidative stress by ROS has a strong correlation with UC development (Keshavarzian, et al., 1992). High level of ROS is generated by immune cells such as infiltrated active macrophages and leukocytes in the colon as a result in a series of responses that prolong inflammation.

1.2.2 Research progress for therapies of IBD

The therapies of IBD including anti-inflammatory drugs, immune system suppressors, antibiotics, and surgery. The anti-inflammatory drugs (for instance, mesalamine and balsalazide) are used as the first step to treat this disease. However, when infection occurs, it is necessary to use antibiotics such as ciprofloxacin and metronidazole. However, it is not enough for some patients who only depend on some anti-inflammatory drugs or antibiotics. A combination of these drugs works better than one drug alone, moreover, some gene expression inhibitor is used to treat this disease such as tumor necrosis factor (TNF)-alpha inhibitors (Murch, et al., 1993). However, adverse side effects of drugs should be considered in long-term use (Cosnes, 2009). On the other hand, physical training has been suggested to be protective against the onset of IBD. During this process, muscles release biologically active myokines which exert the direct anti-inflammatory effects and inhibit the release of proinflammatory mediators from visceral fat (Bilski, et al., 2014).

It is widely accepted that immune cells secrete products involved in the initiation and preservation of inflammatory responses. Those inflammatory responses contribute to the injury of intestinal tissue. It has been proved that several pro-inflammatory cytokines are involved in the progression of IBD. For instance, interleukin (IL) - 1β releases from monocytes and macrophages. It can induce the inflammation and it is a high expression in the intestinal tract of UC patients (Li, et al., 2014). IL-18 originates from mucosal T cells, it can increase the T helper (Th)1 response and inhibit the expression of IL-10 (Kanai, et al., 2001). The expressions of IL-18 and IL-1β are significantly different between the healthy adults and IBD patients. Mucus, which secrets from goblet cells, plays an important role in resisting the invasion of foreign bacteria and pathogens. Goblet cell hyperplasia is influenced by the expression of IL-33 which also up-regulates the expression of IL-5 and IL-13 (Schmitz, et al., 2005; Liu, et al., 2009). Besides, the expression of IL-6 also increases in the IBD patients compared with the healthy person. IL-6 increases the inflammatory levels by activating signal transducer and activator of transcription 3 (Mitsuyama, et al., 1995). Finally, the expression of TNF-α correlates with disease severity of IBD because TNF-α can increase the expression of IL-1β, IL-6, and IL-33 (Sanchez-Muñoz, et al., 2008; Murch, et al.,1993). Base on the functions of cytokines in the pathogenesis of UC and CD, inhibiting the expression of pro-inflammatory cytokines was considered as an effective

therapy against IBD (Fischer, et al., 2016; Feagan, et al., 2013; Wyant, et al., 2015). Depending on those mechanisms, cyclosporine-A and methotrexate are used to treat IBD because they can inhibit the secretion of pro-inflammatory cytokines and induce apoptosis (Terdiman, et al., 2013; Steiner, et al., 2014; Matsuda, & Koyasu, 2000; Nielsen, et al., 2007; Wessels, et al., 2008). Currently, anti-TNF therapy is widely used to treat IBD. Unfortunately, there are some patients do not respond to this therapy. Ozanimod can suppress experimental colitis by inducing lymphopenia (Sandborn, et al., 2016). In addition, Suzuki *et al* reported that Carbohydrate sulphotransferase 15 (CHST15) reduced inflammation and fibrosis in CD clinical trials by small-interfering RNAs mediated silencing of the CHST15 base on mucosal healing of epithelial cell (Suzuki, et al., 2017).

Some dietary therapies have been shown in Table 3. IBD related to diet, it was an available therapy to modulate diet to treat CD and UC. For instance, protein, which is a common component, exists in many kinds of foods including meat, eggs, and milk. High protein diet provides abundant amino acids which benefit mucosal healing during some diseases (Liu, et al., 2013). It has been proved that high protein diet was benefical for repairing the colonic epithelium during colitis in the early phase, while this diet increased colonic inflammation in post-induction phase (Lan, et al., 2016). Moreover, Vitamin D also can reduce colonic inflammation during 2,4,6-trinitrobenzene sulphonic acid (TNBS)- induced colitis. Interestingly, Vitamin D cannot reduce the inflammatory levels of the colon in oxazolone-induced colitis (Liu, et al., 2016). Compared with traditional medicine (Vinod Prabhu, & Guruvayoorappan, 2014; Mao, et al., 2017; Kim, et al., 2017; Kanauchi, et al., 1998), calcium propionate and lecithinized-superoxide dismutase can ameliorate colitis by regulating the expression of cytokines (Hori, et al., 1997; Ghadimi, et al., 2017).

Recently, some scientific results indicated that polyphenols have the function of anti-inflammation. Therefore, some plants or fruits rich polyphenols usually were considered as good candidates because they contained rich polyphenols (Oz, et al., 2013; Choi, et al, 2011). The anti-inflammatory mechanism of polyphenol remains unclear; however, this mechanism may relate to the regulation of pro-inflammatory cytokines. For instance, the polyphenols can decrease the expression of proinflammatory molecules (COX-2, IL-1β, and IL-6) and inhibit inflammatory response by blocking some pathways (NF-kB/p65 and pERK/MAPK) (Sánchez-Fidalgo, et al., 2012;

Boussenna, et al., 2016; Qian, et al., 2015). NF-kB is an especially key regulator implicated in the control of genes involved in the inflammatory cascade. In addition, hydroxytyrosol acetate and curcumin, which were purified from plants or fruits, can decrease inflammatory levels of colon tissues by regulating the expression of proinflammatory genes (Sánchez-Fidalgo, et al., 2015; Deguchi, et al., 2007).

1.3 Intestinal flora affects human health

Food is processed by our gastrointestinal system primarily to supply energy and key functional elements to the body. Dietary components, which contain carbohydrates and protein, are served as fuel to maintain organ function and promote cellular growth and recycling. As the major location to absorb nutrition from the diet, the human intestinal tract is the major surface for microbial colonization. The intestinal habitat of an individual contains 300–500 different species of bacteria (Savage, 1977). Among them, the four dominant bacterial phyla are *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* in the human gut (Khanna, & Tosh, 2014; Guarner, & Malagelada, 2003). Ilya Metchnikov, who is called as "the father of modern probiotics", suggested that regular consumption of lactic acid bacteria in fermented dairy products was associated with enhanced health including promoting longevity (van de Guchte, et al., 2006). He also forecasted that any changes in intestinal microbiota could increase susceptibility to some diseases. After that, the function of intestinal flora was deeply investigated. Some ideas were reported that critical functions of the commensal flora including protection against epithelial cell injury (Rakoff-Nahoum, et al., 2015), regulation of host fat storage (Bäckhed, et al., 2004), and stimulation of intestinal angiogenesis (Stappenbeck, et al., 2002).

As Metchnikov forecast, the occurrence of some diseases relates to the imbalance of intestinal flora (Qin, et al, 2010). For instance, the imbalance of intestinal flora is associated with the occurrence of diabetes, cardiovascular and cerebrovascular diseases, obesity, inflammatory enteritis, gastrointestinal cancer, and autoimmune diseases (Zackular, et al., 2013). Recently, diabetes has posed a great threat to human health because the incidence of the disease is increasing rapidly around the world. The content of some butyrate-producing bacteria significantly decreased in patients compared with the healthy people, while the content of pathogenic bacteria (*Bacteroides*, *Proteobacteria*) rapidly increased in patients' intestinal tract (Qin, et al., 2012; Larsen,

et al., 2010). The imbalance of intestinal flora may contribute to this disease. Cardiocerebrovascular disease, which has led to a dramatic increase in mortality and morbidity, is a common disease especially occurs in middle-aged and elderly people (over 50 years old). Trimethylamine N-oxide (TMAO) can promote atherosclerosis by upregulation of multiple macrophage scavenger receptors linked to atherosclerosis (Koeth, et al., 2013). The intestinal microbiota plays a key role in TMAO production (Miele, et al., 2015; Wang, et al., 2011). More recently, with the continuous improvement of living standards, obesity has become a problem plaguing human beings. The scientific results have proved that the contents of *Bacteroidetes* and *Firmicutes* significantly increased in obese mice compared with slim mice, and obese microbiome has an increased capacity to harvest energy from the diet (Ley, et al., 2006; Turnbaugh, et al., 2006). Furthermore, colonization of germ-free mice with an 'obese microbiota' results in a significantly greater increase in total body fat than colonization with a 'lean microbiota'. Those results suggest that modulation of intestinal flora is a new trend against obesity.

However, some bacteria have protective effects on the intestine, which is called probiotics. The probiotics mainly contain *Bifidobacterium* spp., *Enterococcus* spp., *Propionibacterium* spp., and *Lactobacillus* spp. (SÁnchez, et al., 2009). Probiotics can inhibit colonization of pathogens through competition for common receptors of adhesion, improve the barrier function of the epithelial lining, and modulate the immune responses (Adlerberth, et al., 1996). Interestingly, those functions are significantly impacted by dietary factors. Moreover, the consumption of probiotics relates to the function of anti-inflammation, gut-modulating properties, and anti-oxidant (Resta-Lenert, & Barrett, 2006; Kobatake, et al., 2017; Thomas, et al., 2015). The fecal transplant of probiotic was used as a treatment (Scott, et al., 2015).

According to those backgrounds, the functions of MFP was evaluated in this research. In chapter 2, the effect of MFP on the intestinal microbiota was determined by DGGE and DNA sequencing. After that, the mice were induced acute colitis by DSS and the change in intestinal flora was determined by qPCR in chapter 3. Finally, the number of goblet cell and mRNA expression of NLRPs (NLRP3, ASC, NLRP6) was evaluated by qRT-PCR in chapter 4. The aims of this research were to determine the effect of MFP supplementation against DSS-induced acute colitis and understand the mechanism of its focus on the intestinal microbiota and inflammasomes.

Table 1. The contents of mulberry fruit. Fruit weight, moisture, pH, TAc (total acidity), TSS (total soluble solids), TDW (total dry weight), total fat, total phenolics, and total flavonoids contents of mulberry species (originated from Ercisli & Orhan, 2007).

	Black (*Morus nigra*)	White (*Morus alba*)	Red (*Morus rubra*)
Fruit weight (g)	4.37^{a}	3.49^{b}	2.14^{c}
Moisture (%)	72.6	71.5	74.6
pH	3.52^{c}	5.60^{a}	4.04^{b}
TAc (%)	1.40^{a}	0.25^{b}	1.37^{a}
TSS (%)	16.7^{b}	20.4^{a}	15.9^{b}
TDW (%)	27.4^{ab}	29.5^{a}	24.4^{b}
Total fat (%)	0.95^{ab}	1.1^{a}	0.85^{b}
Total phenolics (mg GAE/100g fresh mass)	1422^{a}	181^{c}	1035^{b}
Total flavonoids (mg QE/100g fresh mass)	276^{a}	219^{b}	29^{c}

※ Values in the same column with different lower-case letters were significant as $p<0.05$.

Table 2. The effects of polyphenols from mulberry in antioxidant, anti-obesity, and anti-cancer in vivo and vitro researches.

Effects	Specie of mulberry	Condition of mulberry fruit	Type of study	Results	Reference
Antioxidant	Morus alba	mulberry juice purification; mulberry marc purification	Mice model	Antioxidant capacity: mulberry juice purification<mulberry marc purification	Jiang et al, 2013
	Morus alba	Ethyl acetate-soluble extract	Mice model	α-glucosidase activity↓; fasting blood glucose↓; glycosylated serum protein ↓ ;antioxidant enzymatic activities ↑	Wang et al, 2013
	Morus nigra and Morus alba	Sugar-free extracts	Mulberry extract	Total polyphenolic contents, antioxidant activities of Morus nigra > those in Morus alba	Arfan et al, 2012
	Morus nigra; Morus rubra; Morus alba	Crude extract	Mulberry extract	Phenolics in Morus nigra were the highest compared with others.	Dimitrova et al, 2014
	Morus alba	Fruit powder by drying	HepG2 cells	intracellular GSH depletion ↑; restored the mitochondrial membrane function	Chen et al, 2017
Anti-obesity	Morus alba	Frozen fruit	Frozen fruit	mulberry polyphenolics act as potent superoxide anion radical scavengers and reducing agents	You et al, 2015
	Morus alba	Freeze-dried powder	Rat model	Atherogenic index ↓; serum high-density lipoprotein cholesterol↑; serum and liver content of thiobarbituric acid related substances↓; SOD↑; GSH-Px ↑; lipid peroxidation↓	Yang et al, 2010b
		Fruit powder	Mice model	Body weight gain↓; fasting plasma glucose and insulin↓; protein levels of oxidative stress markers ↓; inflammatory markers↓ .	Lim et al, 2013a
		Fruit powder	Mice model	suppress hepatic fat accumulation; epididymal adipocyte size↓;protein levels of oxidative stress markers ↓; inflammatory markers↓ .	Lim et al, 2013b
	Morus alba	Fruit extract	BAT-cMyc cells and C3H10T1/2 cells	Expression of the transcription factor A and the nuclear respiratory factor-1 ↑;Cyanidin-3-glucoside was the most abundant anthocyanins	You et al, 2015
		Anthocyanins from mulberry fruit	Mice model	Improve impaired hepatic function; insulin resistance ↓; the size of adipocytes ↓; leptin secretion ↓	Wu et al, 2013a
		Mulberry fruit juice	Mice model	body weight gain↓; serum cholesterol↓; insulin resistance↓; lipid accumulation↓; leptin secretin↓	Wu et al, 2013b
Anti-cancer		Extract anthocyanins from mulberry	AGS cell line; mice model	distribution of hypodiploid phase (apoptotic peak)↑; suppressed cell survival and tumorigenesis; induced apoptotic death in AGS cells.	Huang et al, 2011
	Morus alba	Extract anthocyanins from mulberry	A549 human lung carcinoma cells	Expressions of MMP-2 and u-PA ↓;enhance the expression of TIMP-2 and PAI; inhibition on the activation of c-Jun and NF-kB.	Chen et al, 2006

Table 3. The functional foods or components were used to prevent or treat IBD.

Food or component	Type of study	Dosis	Results	Reference
Cheongilppong	mice model cell culture	Cheongilppong fruits 10 μl /g Cheongilppong branches 1mg /g	Reduced body weight loss DAI ↑ Maintained length of colon length NO production ↓	Choi et al, 2011
Green tea polyphenols	mice model	Green tea polyphenols High 1% sulfasalazine 50 mg/kg	Colonic damage ↓ Histological scores ↓ TNF-α, IL-6, and serum amyloid A ↓ Restored antioxidants levels in hepatic and colon EGCG reduced leptin levels	Boussenna et al, 2016b
Vitamin D	mice model	0.5 μg /kg body weight	Preserve the local histology alleviate inflammation suppress apoptosis maintain tight junction function decrease permeability	Liu et al, 2016
Moringa seed extract (MSE)	mice model	150 mg/kg Moringa seed extract 50 mg/kg 5-aminosalicyic acid	MSE: DAI scores ↓, colon length ↑, colonic inflammation/damages↓, NO↓ MSE: pro-inflammatory KC ↓, TNF-α，MPO， fecallipocalin-2, IL-1, and IL-6↓, MSE: tight -junction , pro-inflammatory biomarkers ↓	Kim et al, 2017

Continued to **Table 3**.

Food or component	Type of study	Dosis	Results	Reference
Mulberry fruit powder	mice model cell culture	5% or 10% mulberry fruit powder	Expression of proinflammatory molecules (iNOS, COX-2, IL-1β，IL-6) ↓ Inhibition on inflammatory response by blocking NF-kB/p65 and pERK/MAPK pathway Ameliorated symptoms of colitis in mice model	Qian et al, 2015
Rhizophora apiculata	mice model	10 mg/kg body weight	Macroscopic score and wet weight of damaged colon ↓ Anti-oxidant enzymes including SOD and GSH ↑ LPO and NO↓, inflammatory mediators including MPO, LDH,iNOS,COX-2 and TNF-α↓ Inhibits the translocation of NF-kB p65 and p50 subunits	Rezayat et al, 2018
Calcium propionate	mice model	186.22 g/mol	Ameliorated DSS-induced colitis and attenuated the sum of histologic colitis scores Plasma IFN- and calprotectin ↓, enhance PGlyRP3 in response to DSS treatment	Ghadimi et al, 2017
Hydroxytyrosyl acetate	mice model	0.1 %	DAI ↓, improvement of histological damage; expression of iNOS, COX-2, and MPO ↓ Down-regulated JNK phosphorylation and prevented nuclear translocation level of p65	Sanchez et al, 2015a
Curcumin	mice model	2%	Inhibited the body weight loss; DAI, histological colitis score, and MPO activity ↓ Microscopically, mucosal edema, cellular infiltration, and epithelial disruption ↓ blocked the NF-kB activation in the mucosa	Deguchi et al, 2007

Continued to **Table 3**.

Food or component	Type of study	Dosis	Results	Reference
Extra virgin olive oil	mice model	40 mg/kg	Attenuated the clinical and histological signs of damage Improving results from DAI and reducing about 50% mortality Extra virgin olive oil added hydroxytyrosol showed better results. COX-2 and iNOS were downregulated; activation of p38 MAPK ↓	Mao et al, 2016
Qingchang wenzhong decoction	rat model	low-dose group 0.3g/kg medium-dose group 0.6g/kg high-dose group 1.2g/kg	Alleviated colitis-associated inflammation; upregulated serum MSP and RON expression pAkt levels ↓; depressed claudin-2 expression	Oz et al, 2013
Grape pomace extracts (GPEs)	rat model	Alicante-S extract 5g/kg Alicante-P extract 8.2g/kg Pinot-S 2.9g/kg	Clinical signs and colon shortening↓; myeloperoxidase activity ↑ GPEs modulated antioxidant enzyme activities GPEs prevented the increase in pro-inflammatory cytokines levels GPEs prevented the up-regulation of ICAM-1 and MMP-9	Sanche et al, 2012
High-protein diet	mice model	normoproteic (NP) 140g/kg whole milk protein high-protein (HP) 530g/kg whole milk protein	during colitis induction phase, the symptoms were similar in DSS-NP and DSS-HP group during colitis resolution phase, inflammation intensity: DSS-HP group > DSS-ND group colonic crypt height in DSS-HP > DSS-NP group	Lan et al, 2016
Germinated barely foodstuff	rat model	100g/kg	prevented bloody diarrhea and mucosal damage mucosal protein and RNA content↑	Kanauchi et al, 1998

CHAPTER 2

Effects of Mulberry Fruit on Gut Flora in Feces from Mice

2.1 Introduction

The main function of intestinal tract is to obtain energy and nutrients from food, intestinal flora plays an important role in this process. Intestinal flora, which relates to nutrition absorption, energy metabolism, and mucosal immunity, is an important "organ" in the body (Luo, et al., 2018). The imbalance of intestinal flora or their metabolite changes will affect the normal function of the host (Kim, et al., 2018). Food has a great influence on the composition and function of intestinal bacteria (Llewellyn, et al., 2018). Long-term dietary habits play an important role in determining the composition of intestinal flora (Sonnenburg, & Bäckhed, 2016; Choi, et al., 2011; Kanauchi, et al., 1998; Lan, et al., 2016). Mulberry is a fruit, which is often used as medicine and it is widely studied because of its rich polyphenols. It has been reported that polyphenols have many biological functions including anti-oxidation, anti-inflammation, and immune regulation (Rahman, et al., 2018). Therefore, intestinal flora can be influenced by mulberry fruit and this result relates to the health of human.

Polyphenols in food are collectively called "dietary polyphenols", which are important functional components of plant foods (Laparra, & Sanz, 2010). Polyphenol is a generic term for plant constituents with several phenolic hydroxyl groups in the molecular structure (Sánchez-Fidalgo, et al., 2015; Deguchi, et al., 2007). Flavonoids (anthocyanins, flavonols, and flavanones) and non-flavonoids (caffeic acid, ferulic acid, and chlorogenic acid) are classified according to their structures. The main absorption site of polyphenol is small intestine which contains a large number of bacteria (Walle, 2004). The bioavailability of polyphenols was influenced by intestinal flora (Cardona, et al., 2013). Polyphenol inhibited some disease especially IBD by modifying the intestinal flora (Boussenna, et al., 2016; Mao, et al., 2016; Sánchez-Fidalgo, et al., 2012; Oz, et al., 2013; Kim, et al., 2017; Liu, et al., 2016). The polyphenols could selectively inhibit the growth of some pathogenic bacteria (*Enterobacter* spp.), whereas they also could promote the growth of some probiotics (*Lactobacillus* spp. and *Bifidobacterium* spp.) (Yamakoshi, et al., 2001; Tzounis, et al., 2008). There are reported that the function of polyphenol relates to the permeability of cell membranes (Smith, & Mackie,

2004). On the contrary, some researchers believed that the metabolites of polyphenols can regulate intestinal flora to protect the intestinal mucosa, rather than polyphenols (Parkar, et al., 2008). Therefore, it needs to be further researched.

In the present research, the aim was to investigate the effect of MFP on intestinal flora in mice model. Male C57BL/6 mice were randomly divided into two groups. The mice in Group 1 were supplied with MFP diet, whereas the mice in Group 2 were supplied with normal diet. After 12 weeks, the mice were sacrificed and the feces were collected. The change of intestinal flora was determined by DGGE method and DNA sequence.

2.2 Materials and Methods

2.2.1 Diet preparation and reagents

Mulberry fruit used in this study was harvested in the Shiraishi Island of Okayama prefecture. The contents of anthocyanins, as major polyphenols in this fruit, were 80 mg/g of cyanidin-3-glucoside and 24 mg/g of cyanidin-3-rutinoside. MFP from the 5 x concentrated mulberry fruit juice kindly given by Okayamaken Seikabutsu Hanbai Co. Ltd (Okayama, Japan) was prepared in the freeze dryer (TD-81TA, Tokyo Rikakikai *Co., Ltd.,* Tokyo, Japan). The anthocyanin content was 7 mg/g MFP. MFP was stored at -20˚C until use. MFP-containing diet was prepared as a mix with the normal diet (AIN93G: Oriental Yeast *Co., Ltd*., Tokyo, Japan). MFP diet contains 0.136 mg MFP per day for each mouse.

Normal diet was mice AIN93G powder which was bought from Oriental Yeast co., Ltd. (Tokyo, Japan). The major components including 39.75% cornstarch, 20.0% milk casein, 13.2% pregelatinized cornstarch, 10.0% granulated sugar, 7.0% purified soybean oil, and 5% cellulose powder. All the other chemicals were purchased from Sigma (Tokyo, Japan).

2.2.2 Animals and experimental design

C57BL/6 (male, 5 weeks old) were purchased from Charles River Laboratories Japan, Inc (Osaka, Japan). The animals were housed at a constant temperature (22 ± 1 ˚C) with a 12-h dark/light cycle and had access to tap water and food. All procedures were approved by the Animal Care and Use Committee, Okayama University and were

conducted in accordance with the Policy on the Care and Use of the Laboratory Animals, Okayama University.

The experiment lasted for 12 weeks. The mice (5 weeks old) were randomly divided into 2 groups (n = 5) and were maintained on different diets. The mice in MFP group were supplied with MFP diet, whereas the mice in the control group were fed with normal diet. At the end of the experiment, all the mice were sacrificed. The feces were collected and immediately frozen at -80˚C for further analysis.

2.2.3 DGGE

The bacterial DNA in feces was extracted and purified using the QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. A variable (V6-V8) region of the bacterial 16S rDNA gene was amplified by PCR using OneTaq® DNA Polymerase (BioLabs, MA, USA) with the 20 pmol/µl forward primer GC-16S rDNA V6/8f (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCTTAC-3′) and the 20 pmol/µl reverse primer 16S rDNA V6/8r (5′-CGGTGTGTACAAGACC-3′). The total volume was 50µl which contained 10 mM/µl dNTP, 10 µl 5 x PCR buffer, 1µl forward and reverse primer, and 0.25µl polymerase. The PCR protocol involved an initial denaturation step at 95°C for 1 min and then subjected to 40 cycles of denaturation at 95˚C for 30 sec, annealing for 30 sec at 58 ˚C, and 72 ˚C for 50 sec. Finally, the extension step was performed at 72 ˚C for 7 min.

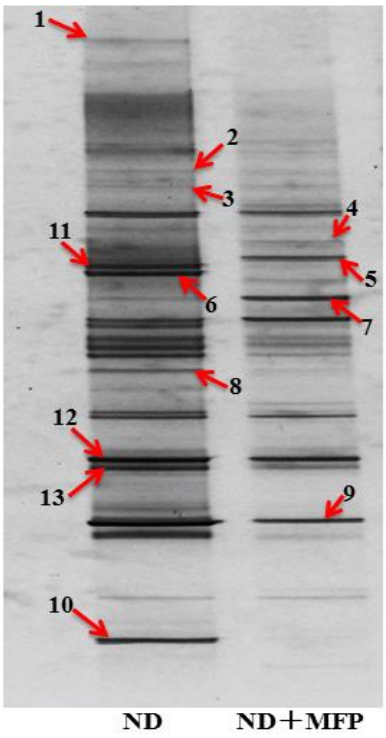
The DGGE was performed using a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Inc., Tokyo, Japan). The PCR products were added directly into 80 g/L polyacrylamide gels which were prepared in a denaturing gradient from 25% to 60% using 7 M urea and 400 mL/L formamide as 100% denaturants. Electrophoresis was performed at 150 V for 8 h at 60°C. After electrophoresis, the gels were stained with SYBR Green (Cambrex Bio Science Inc., Rockland, ME, USA) and photographed under ultraviolet illumination.

Choosing the special band by comparing the difference between MFP Group and control Group. After that, the selected bands were accurately removed from the DGGE gels, and the DNA was amplified by PCR using 20 pmol/µl forward primer 16S rDNA V6/8f (without the GC-clamp) and 20 pmol/µl reverse primer 16S rDNA V6/8r primers.

The total volume was 50µl which contains 10 mM/µl dNTP, 10 µl 5 x PCR buffer, 1µl forward and reverse primer, and 0.25µl polymerase. Samples were heated at 95˚C for 1 min and then subjected to 40 cycles of denaturation at 95˚C for 30 sec and annealing for 30 sec at 58˚C, and 72 ˚C for 50 sec. Finally, the extension step was performed at 72 ˚C for 7 min. Then, the PCR products were purified using Wizard® SV gel and PCR clean-up system (Promega, Madison, WI) according to the manufacturer's protocol. The purified PCR products were cloned into the pGEM®-T vector, and the resulting plasmids were transformed into *Escherichia coli* (*E. coli*) DH5α competent cells (Dyna Express TA cloning kit; BioDynamics Laboratory Inc., Tokyo, Japan). After that, the samples were sent to company to finish BigDye and DNA sequences. Finally, the bacterial species were determined using the basic local alignment search tool (BLAST) in NCBI.

2.3 Results

DGGE results showed that the number of bacterial bands decreased after feeding with MFP diet compared with the control group (Fig.1). The bacterial bands were numbered according to the different locations in Fig.1. Bacterial bands of NO. 1, 6, 8, 10, and 11 were clearly observed in the control group compared with MFP group. Contrarily, the bacterial band of NO.5 and 7 were more significant in MFP group than those in the control group. Those results indicated that MFP increased some bacteria, whereas also decreased bacteria. DNA sequence results showed that band NO. 2 to 8, and 11 were uncultured bacteria, and band NO.1 was *Lactobacillus plantarum.* Band NO.10 was *Atopobiaceae bacterium*. Band NO. 9, 12, 13 were same species and belonged to *Bifidobacterium pseudolongum*.



1.*Lactobacillus plantarum*
2.Uncultured bacterium
3.Uncultured bacterium
4.Uncultured bacterium
5.Uncultured bacterium
6.Uncultured bacterium
7.Uncultured bacterium
8.Uncultured bacterium
9.*Bifidobacterium pseudolongum*
10.Atopobiaceae bacterium
11.Uncultured bacterium
12.*Bifidobaterium pseudolongum*
13.*Bifidobaterium pseudolongum*

Figure 1. The fecal DGGE and BLAST results in feces from the MFP diet group and normal diet group. The fecal DGGE and BLAST results in feces from the MFP diet group and normal diet group. The C57BL/6 (male, 5 weeks old) mice were randomly divided into two groups, and the mice fed with or without MFP diet for 12 weeks. At the end of the experiment, the feces were collected. The fecal DGGE results were showed in right, and the special bands were picked up to further determine using DNA sequence. The BLAST results were showed in left.

2.4 Discussion

As we all know, diet has an important effect on intestinal flora. The gut microbiota relates to the mucosal immune system and plays a key role in the stability of intestinal environment, absorption of nutrition, and preventing the colonization of pathogenic bacteria (Gome, & Malcata, 2005; Cunningham-Rundles, et al., 2002). There are reported that the normal bacterial flora is a prerequisite for normal physiological function proved by some gene-knock mouse models (Chu, et al., 2014; Kado, et al., 2001; Balish, & Warner, 2002). In this experiment, mulberry, as a food rich in polyphenols, modified the composition of intestinal flora, which was consistent with previous research results (Özkan, et al., 2004; Requena, et al., 2010; Yamakoshi, et al., 2001). On the one hand, the biological function of polyphenols may depend on their influence on gut microbiota and modulation to certain metabolic pathways such as KEGG pathways of ATP-binding cassette transporters (Cheng, et al., 2018). On the other hand, polyphenol also modulated the gut microecology to protect the health of the host by improving the proportion of beneficial bacteria in intestinal flora (Parkar, et al., 2008). Moreover, metabolites from dietary polyphenols also benefited the host (Pasinetti, et al., 2018).

Epithelial tissue integrity is an important guarantee for intestinal homeostasis. Epithelia are an important component of intestinal epithelial tissue and are closely connected to each other by tight junctions (Turner, 2006). Tight junctions are made up of complex lipoprotein structures that form fibrils that traverse the lateral plasma membrane to interact with proteins from the adjacent cell. As far as we know, *Bifidobacterium* spp., which belong to probiotics, can maintain intestinal homeostasis by enhancing epithelial cell barrier function (Ulluwishewa, et al., 2011; Ewaschuk, et al., 2008). This bacterium was first isolated from the feces of lactating infants by Henry Tissier in 1899 (Gomes, & Malcata, 1999). After that, *Bifidobacterium* spp. was also found in fermented food and digestive tract (Ventura, et al., 2007; Klijn, et al., 2005). The content of this bacteria in infancy accounted for 80% of intestinal flora and played a dominant role in the function of intestinal flora (Turroni, et al., 2012). During human development, the content of *Bifidobacterium* spp. gradually decreases, and finally tends to be stable. This bacterium is an important member of human intestinal flora and one of the indicators of intestinal health. There are reported that *Bifidobacterium* spp. can

protect intestinal epithelia from harmful factors by modulating the expression of cytokines, maintaining the integrity of tight junction, and promoting the mucin secretion (Krumbeck, et al., 2018; Engevik, et al., 2018; Paveljšek, et al., 2018). In this research, band NO. 9, 12, and 13 represent *Bifidobacterium pseudolongum*, however, only band NO.13 is shallower in MFP group compared with the control group. This result is not enough to prove that MFP diet reduces the content of *Bifidobacterium pseudolongum*, and it needs further proof in future experiments such as qPCR.

L. plantarum, which is a widespread member of the genus *Lactobacillus*, belongs to lactic acid bacteria which plays a key role in intestinal food fermentation (Leal-sánchez, et al., 2003; Ruiz-Barba, et al., 1994). Interestingly, the content of *L. plantarum* in fresh products is low (Ercolini, et al., 2006; Durán, et al., 1993). The size of *L. plantarum* strain WCFS1 is 3.3Mb which belongs to the largest size of lactic acid bacterium which relates to the diversity of environmental niches (Kleerebezem, et al., 2003; Makarova, & Koonin, 2007). *L. plantarum* possibly leads to the disruption of the cell and contributes to the promotion of cellular lysis (Ruiz-Barba, et al., 1990). The DGGE result showed that MFP diet significantly decreased the contents of *L. plantarum* in feces compared with the control group. This result is consistent with former researches (Ruiz-Barba, et al., 1993; Landete, et al., 2008; Durán, et al., 1993). The reduction of *L. plantarum* may benefit the biological function of polyphenols.

2.5 Conclusion

In the present research, the influence of MFP on intestinal flora was investigated using mice model. DGGE results showed that the bacterial bands in MFP group and control group were clearly different. By the DNA sequence, the special bands were determined. According to blasting results, the band of *L. plantarum*, which can lead to the disruption of the cell and contribute to the promotion of cellular lysis, was changed. In conclusion, these results indicated the intestinal microbiota was modified by mulberry fruit.

CHAPTER 3

Mulberry Diet Modifies Intestinal Flora in DSS-Induced Acute Colitis

3.1 Introduction

Intestinal flora mainly concentrates in the colon and small intestine. Aerobic bacteria and facultative anaerobic bacteria are predominating bacteria in the small intestine, however, anaerobic bacteria mainly locate in the colon, such as *Bacteroides* spp. and *Bifidobacterium* spp. (Kanauchi, et al., 2003). The dominant bacteria in the feces of healthy adults including *Bacteroides* spp., *Bifidobacterium* spp., *Lactobacillus* spp., *C. leptum*, *C. coccoides*, and *Enterobacter* spp. (Sghir, et al., 2000). The intestinal flora is stable for the healthy person, furthermore, the pathogenesis of some diseases relates to disturbance of intestinal homeostasis (Zhang, et al., 2015; Palmela, et al., 2018). Recently, IBD becomes one of the global diseases to threaten the health of human. This disease contains Crohn's disease and ulcerative colitis. The former can affect any part of the digestive tract, whereas the latter mainly damage in the colon. The inflammation is usually confined to the mucosa by histological observation.

It has been reported that differential compositions of intestinal microbiota in patients with IBD (Bamola, et al., 2017). The population of *Enterobacter* spp. and *Enterococcus* spp. in the feces of UC patients were significantly higher than those in the feces of healthy persons, meanwhile, the content of *Bifidobacterium* spp. was significantly lower (Schirmer, et al., 2018). Moreover, the content of facultative anaerobes in feces also increased (Linskens, et al., 2001). *Escherichia coli* with adhesion and invasion characteristics was isolated from the patient's feces, furthermore, the pathogenic factors including adhesin, hemolysin and necrotizing toxin (Dickinson, et al., 1980). The content of *Bifidobacterium* spp. was significantly decreased in feces of CD patients, and the concentration of bacteria in patients' mucosa was significantly higher than that in healthy persons (Swidsinski, et al., 2005; Seksik, et al., 2003). After the occurrence of IBD, the proliferation of pathogenic bacteria produced more bacterial metabolites to induce inflammation, such as lipopolysaccharide and glycoprotein-polysaccharide peptidoglycan-polysaccharide (Schmiedlin-Ren, et al., 2016; Bamias,

et al., 2017; Qian, et al., 2015). The scientific results have been proved that some *Clostridium* species are considered as the pathogenic factors, such as *C. difficile* and *E. coil*, by promoting the accumulation of immune cells (Sun, et al., 2018). On the other hand, some bacteria, such as *C. butyricum and Bifidobacterium* spp., are probiotic bacteria able to reduce intestinal inflammation by improving the mucosal barrier function and modulating immune responses (Banaszkiewicz, et al., 2015). Contrarily, short-chain fatty acids (SCFAs), which are produced by anaerobic bacteria during the fermentation of indigestible food ingredients, have a certain anti-inflammatory effect. It has been reported that the content of SCFAs was significantly decreased in intestinal tract of IBD patients compared with healthy human (Ross, & Cohen, 2004). Therefore, SCFAs are considered to use for the treatment of IBD, among those SCFAs, butyrate was widely studied. The symptoms of IBD were alleviated by butyrate enema therapy for the patients (Yan, et al., 2018). However, not all SCFAs can be used as medicine for colitis, because some SCFAs are quickly absorbed by upper intestine after oral administration. This makes it difficult for these SCFAs to reach the colon such as 5-aminosalicylic acid (Dooley, et al., 2004; Yokoe, et al., 2003).

Moreover, some bacteria were observed in the intestinal mucosal surface of IBD patients (Kleessen, et al., 2002; Schultsz, et al., 1999). The gut microbiota influences the homeostasis of the intestinal mucosa via its barrier function (Thursby, & Juge, 2017). Mucosal barrier integrity is essential to block the access of microorganisms to underlying tissues. Studies in a rodent model have linked tissue damage and the disruption of the epithelial barrier in the gut to cytokine imbalances (Schleimer, & Berdnikovs, 2017). It led to inflammation if the pathogenic bacteria and their metabolites invaded into epitheliums. Exceptionally, *Listeria* antigens were found in the mucosal lamina propria and inside of macrophages in the mesenteric lymph nodes of CD patients (Liu, et al., 1995). However, *Listeria* was considered to be associated with secondary infection after enteritis, not pathogenic bacteria (Yokoe, et al., 2003).

In addition, studies have shown that impairment of intestinal barrier integrity in IBD patients can lead to increased permeability, which is also one of the pathogenic factors of colitis (Hollander, 1988). Some pathogenic bacteria can invade into intestinal epitheliums during the impaired intestinal barrier or higher permeability, which leads to local inflammation or involvement of the entire mucosa. It has been proved that pathological T cells were activated during this process (Nenci, et al., 2007). The

intestinal mucosal barrier is maintained by tight junctions between intestinal epithelium and mucus. The claudins 5 and 6 are important components of tight connection, and the expression of them are down-regulated in IBD patients (Heller, et al., 2005; Prasad, et al., 2005; Katza, et al., 1989). N-cadherin transgenic mice with defects in tight junction, which leads to local intestinal inflammation (Hermiston, & Gordon, 1995). Therefore, protecting the integrity of intestinal epithelial cells is also a vital method to prevent colitis.

UC is generally recognized as an immune-mediated disorder resulting from the abnormal interaction between intestinal microbiota and mucosal immune cells. The reduction of colitis relates to some immune deficiencies has been reported (Khor, et al., 2006; Meisel, et al., 2017). IL-10 can express on many kinds of cells including macrophages, dendritic cells, moreover, it also regulates the function of many kinds of immune cells including T cells, B cells, and natural killer cells (Asadullah, et al., 2003; Moore, et al., 2001). IL-10 gene-deficiency mice spontaneously develop intestinal inflammation; however, this kind of mice do not cause colitis under sterile conditions (Davidson, et al., 2000; Murai, et al., 2009). It proved that common gut flora was necessary for the induction of colitis. Moreover, the expression of IL-10 plays a key role in inflammatory response especially in IBD (Abdel-Motal, et al., 2019; Biswas, et al., 2018). Moreover, IL-2-deficient mice spontaneously induced colitis, especially the pathological damages of intestine was similar to that in human (Sadlack, et al., 1993). IL-7 maintained the survival of $CD4^{+}$ cells and memory T cell and then induces IBD (Chetoui, et al., 2010).

Toll-like receptors (TLRs), which are distributed in a variety of immune-related cells (such as neutrophils, macrophages, T cells, and B cells), play a key role in innate and adaptive immune responses (Lancaster, et al., 2005). TLRs are important pattern recognition receptors (PPRs) for pathogen-associated molecule pattern (PAMP). TLRs mediate the secretion of host-related cytokines and the production of natural immune responses by recognizing the lipid-like structure of pathogenic microorganisms and their cell walls. TLR-deficient mice spontaneously developed colitis, furthermore, the expression of pro-inflammatory cytokines was increased, which associated with the development of colitis (Kutikhin, 2011; Vlantis, 2016).

The imbalance of intestinal flora leads to the destruction of intestinal homeostasis, which contributes to the damage of intestinal mucosa, which then induces the

inflammatory response. Probiotics are beneficial bacterial to the host, and they are also helpful in maintaining the homeostasis of intestinal flora (Schrezenmeir, & Vrese, 2001). Probiotics can protect intestinal mucosa from pathogenic factors by improving the expression of anti-inflammatory cytokines, promoting the secretion of IgA, increasing the phagocytic function of macrophages, and competing the location of adherent epithelium with pathogenic bacteria (Perdigon, et al., 1995; Dotan, & Rachmilewitz, 2005; Coconnier, et al., 1993; Bernet, et al., 1994). At present, probiotics, such as *Bifidobacterium* spp. and *Lactobacillus* spp., have been extensively studied and are used as a treatment of IBD (Mays, & Nair, 2018; Okada, et al., 2018; Rong, et al., 2018; Mendes, et al., 2018).

The mulberry fruit contains rich polyphenols which have the function of anti-inflammation (Rezayat, et al., 2018). In the present research, the aim was to evaluate the inhibitory function of mulberry fruit against DSS-induced colitis and understand the mechanism focus on the change of gut microbiota. BALB/c mice were randomly divided into 6 groups. In the first three weeks, mice were fed with diet supplied with or without MFP. In the fourth week, mice received water containing 5% (w/v) DSS or not. Meanwhile, the mice in Group 1 and 2 continued to be fed with MFP diet while the mice in other groups were fed with normal diet. At the end of experiment, all the mice were sacrificed. The mRNA expressions of both cytokines and NLRPs were evaluated by RT-qPCR using the proximal colon tissue. The middle and distal colons were fixed with 10% formaldehyde for histopathological observation. Furthermore, the compositions of feces bacteria were determined by qPCR.

3.2 Materials and Methods

3.2.1 Diet preparation and reagents

The MFP diet, normal diet, and reagents the same as 2.2.1.

DSS 5000 was purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). The hematoxylin was bought from Pharmaceutical co., Ltd. (Osaka, Japan). All other chemicals were purchased from Sigma (Tokyo, Japan).

3.2.2 Animals and experimental design

BALB/c mice (male, 7 weeks old) were purchased from Charles River Laboratories Japan, Inc (Osaka, Japan). The animals were housed at a constant

temperature (22 ± 1˚C) with a 12-h dark/light cycle and had access to tap water and food. All procedures were approved by the Animal Care and Use Committee, Okayama University (OKU-2017096) and were conducted in accordance with the Policy on the Care and Use of the Laboratory Animals, Okayama University.

The experimental periods were 28 days in total. In the first 3 weeks, mice (7 weeks old) were randomly divided into 6 groups (n = 3) and were maintained on different diets or drinking water, as shown in Table 4. Briefly, the diet with (Group 1 to 4) or without (Group 5 and 6) MFP (final anthocyanin concentration: 50 mg/kg) was supplied daily for the first 3 weeks. Then, the mice in Groups 1, 3, and 5 were supplied drinking water containing 5% (w/v) DSS for 1 week; and body weight and fecal condition in mice were monitored daily. The mice in Groups 2, 4, and 6 were supplied with normal water. At the same time, the mice (in Groups 1 and 2) received a fourth week supply of the diet with MFP. At the end of the experiment, all the mice were sacrificed, and their colonic tissues were collected, and lengths measured. The DAI scores were calculated according to the fecal condition and body weight loss (Table 5) as described elsewhere (Li, et al., 2014). Then, the colon was divided into three parts after tissue collection. The proximal part of colon was immediately frozen at -80˚C for gene expression analysis. Both middle and distal colon were fixed with 10% formaldehyde for histopathological observation.

Table 4. The grouping of mice.

n = 3	Week 0-3	Week 4	
	MFP	MFP	5% DSS
Group 1	+	+	+
Group 2	+	+	-
Group 3	+	-	+
Group 4	+	-	-
Group 5	-	-	+
Group 6	-	-	-

Table 5. The scoring system for Disease activity index.

Score	Weight loss	Stool consistency	Bloody stool
0	None	Normal	None
1	1% - 5%	Loose stool	Occult bleeding
2	6% - 10%		
3	11% - 20%		
4	＞20%	Diarrhea	Gross bleeding

3.2.3 Histopathological examination

The middle and distal regions were collected from mice and immediately fixed with 10% formaldehyde for 2 days. Then the samples were washed 24 hours using rung water. After dehydration and ethanol elimination, the samples were embedded in paraffin, sectioned at 5-μm thickness, and de-paraffinized. Sectioned specimens were stained using hematoxylin eosin (HE) solution. The tissues were stained in hematoxylin (Pharmaceutical co., Ltd. Osaka, Japan) at 10 minutes, and the nucleus was dyed in blue after the running tap water washed 17 minutes. After that, samples were stained in eosin (Sigma, Tokyo, Japan) at 2 minutes, and the cytoplasm was dyed in pink after the tap water washed 30 seconds. HE specimens were observed under the light microscope (Olympus FSX100, Olympus, Tokyo, Japan) to evaluate the tissue damage (magnification, x200 and x400). The microscopic score was calculated according to the extent of disease, the destruction of crypts, and the level of inflammation in colonic tissues (Table 6) (Dieleman, et al., 1998).

3.2.4 Gene expression analysis

Total RNAs were extracted from colonic tissues in each mouse using the SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer's instructions. The concentration of RNA was quantified using SmartSpec plus Spectrophotometer (Bio-Rad Laboratories Inc., Tokyo, Japan). One microgram of total RNA was subjected to reverse transcription with oligo (dT18) primers using the First Strand cDNA synthesis kit (Takara Biotechnology, Shiga, Japan) according to manufacturer's instructions. All the cDNA preparations were stored at -20˚C until further use.

The quantitative real-time polymerase chain reaction (qRT-PCR) was conducted in the Mini Opticon Real-Time PCR System (Bio-Rad Laboratories Inc.) using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix Kit (Agilent Technologies, West Cedar Creek, TX). Expression values were normalized to GAPDH in the same sample and then normalized to the control. The sequences of the primer pairs used for qRT-PCR amplification are listed in Table 8 (located in Chapter 3). Samples were heated at 95˚C for 5 min and then subjected to 40 cycles of denaturation at 95˚C for 5 sec and annealing/elongation for 10 sec at annealing/elongation temperatures described in Table 4. The amplifications were performed on three independent samples, with

triplicate reactions carried out for each sample. The relative mRNA level was calculated using the $2^{-\Delta\Delta CT}$ method.

3.2.5 Assessment of bacterial contents using qPCR

Fecal DNA samples from mice in all the 6 groups were extracted using the QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. qPCR amplification and detection were performed in the Mini Opticon Real-Time PCR System (Bio-Rad Laboratories Inc.) using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix Kit (Agilent Technologies).

To determine of the number of *Bacteroides* spp., *Bifidobacterium* spp., and *C. perfringens* subgroup present in each sample, fluorescent signals detected from serial dilutions in the linear range of the assay were averaged and compared to a standard curve generated with standard plasmid DNA in the same experiment. The primer pairs and qPCR conditions used were those described by Rinttilä et al. (Table 7) (Rinttilä, et al., 2004). The resulting levels of total bacteria were assessed using a 16S specific primer pair (357f: 5'-CTCCTACGGGAGGCAGCAG-3' and (517r: 5'-ATTACCGCGGCTGC TGG-3'). Samples were heated at 95˚C for 5 min and then subjected to 35 cycles of denaturation at 95˚C for 15 sec and annealing at 60˚C for 20 sec, extending at 72 ˚C for 30 sec. Finally, samples were heated at 80 ˚C to 85 ˚C for 30 sec. The 16S rRNA sequences from each bacterial strain [*Bacteroides fragilis* (JCM 11019T), *Bifidobacterium longum* (JCM 1217T), *C. perfringens* (JCM 1290 T), and *E. coli* (JCM 1649T)] were cloned. The plasmid inserted with the 16S rRNA sequence from each bacterial strain was used to define the standard curve.

3.2.6 Statistical analysis

Data were expressed as the mean ± standard error of the mean (SE) or the mean ± standard deviation (SD) of three independent experiments. Data were statistically evaluated by one-way analysis of variance followed by Tukey's HSD using IBM SPSS Statistics software. Values of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered statistically significant.

Table 6. The scoring system for Histological damages.

Score	Extent of disease	Crypt Destruction	Degree of inflammation
0	None	None	No evidence
1	< 25%	1/3 destruction	Scattered infiltrating mononuclear cells (1 – 2 foci)
2	26 – 50%	2/3 destruction	Moderate inflammation with multiple loci
3	51 – 75%	Only epithelium intact	High level of inflammation with vascular density
4	76% <	Epithelium and Mucous layers are destructed	Maximal severity of inflammation and loss of goblet cell

Table 7. Group-or Species-specific 16s-targeted primers.

	Primer sequence (5'→3')	Amplicon size (bp)	Annealing temperature (°C)
Total bacteria	357F: 5'-ACTCCTACGGGAGGCAGCAGG-3'	200	60
	517R: 5'-ATTACCGCGGCTGCTGGG-3'		
***Bacteroides-Prevotella-Porphyromonas* group**	F: 5'-GGTGTCGGCTTAAGTGCCATG-3'	140	68
	R: 5'- CGGA(C/T) GTAAGGGCCGTGC-3'		
Bifidobacterium spp.	F: 5'-TCGCGTC(C/T) GGTGTGAAAGG-3'	243	58
	R: 5'-CCACATCCAGC(A/G) TCCACG-3'		
***Clostridium perfringens* group**	F: 5'-ATGCAAGTCGAGCGA(G/T) G-3'	120	55
	R: 5'-TATGCGGTATTAATCT(C/T) CCTTT-3'		

3.3 Results

3.3.1 Body weight.

Prior to DSS administration, body weight did not differ among mice groups (data not shown). The body weight after DSS administration is shown in Fig. 2A. The body weight in mice of the DSS treatment groups (Groups 1, 3, and 5) was decreased after DSS administration compared with the mice in normal water groups (Groups 2, 4, and 6). In Group 5 mice compared with Group 6 mice, the body weight gain was decreased by about 25%. After DSS treatment 1 day, the body weight loss in Group 3 and 5 mice started to increase, while the reduction of body weight in Group 1 mice started after DSS administration 3 days. At the same time, the weight decreases in Group 3 mice indicated in the middle between that in Group 1 and 5 mice. Additionally, the body weight loss in Group 1 was significantly inhibited compared with that in Group 5 mice after DSS administration 2 days and 3 days, meanwhile, that in Group 3 occurred in the intermediate (Fig. 2B).

3.3.2 Colon length.

The colon length was calculated (Fig. 3A) and the colon length was significantly shortened in the DSS administration group without MFP (Group 5, Fig. 3B). However, the colon length in Group 1 was similar to the normal water groups (Group 2, 4, and 6) and significantly longer than that in Group 5 mice. The colonic length in Group 3 mice indicated in the middle between that in Group 1 mice and that in Group 5 mice.

3.3.3 Disease activity index

Compared with normal water group mice (Group 6), the DAI score in DSS administration groups mice significantly increased (Fig. 4A). This score in Group 1 was immediately increased after DSS treatment 1 day, and higher than other two DSS treatment groups (Group 1 and 3). The Group 3 mice score indicated in the middle between Group 1 mice score and Group 5 mice score. The DAI score increased gradually over the last 3 days in the DSS administration group (Fig. 4B). However, disease signs appeared later in the MFP-administered group (Group 1). The DAI score in Group 1 mice was significantly lower than those in Group 3 and Group 5 mice. Thus, MFP significantly attenuated the clinical symptoms of colitis.

3.3.4 Histopathological observation.

Both middle and distal colon regions were observed for histopathological examination using HE staining. In mice administered DSS, the colons showed destruction of the epithelial layer and the infiltration of inflammatory cells, such as neutrophils, extending through the mucosa and sub-mucosa (Fig. 5A). MFP administration attenuated the extent and severity of lesions and improved the epithelium architecture. The infiltration of inflammatory cells in Group 1 mice was significantly less than that in Group 5 mice, and that in Group 3 mice were located in the middle between in Group 1 mice and in Group 5 mice. The microscopic scores were calculated according to crypt destruction, the extent of disease, and the condition of immunocyte infiltration. The score in Group 1 mice was similar to normal water groups (Group 2, 4, and 6) and lower than that in Group 3 and 5 mice (Fig. 5B). In Group 1, MFP administration allowed only a moderate inflammatory infiltration, indicating an important reduction of colon damage.

3.3.5 Gene expression analysis

The expression of proinflammatory cytokines was down-regulated by MFP administration (Fig. 6). The mRNA expression levels of IL-1β, TNF-α in Group 3 were higher than those in Group 1. In another word, MFP inhibited the inflammatory levels in colonic tissues. This result was consistent with the previous results (body weight loss, colon length, DAI score, and microscopic score).

3.3.6 Bacterial content analysis

qPCR analyses were performed to quantity *Bacteroides* spp., *Bifidobacterium* spp., and *C. perfringens* subgroup in fecal samples (Fig. 7). The total bacterial population count was $10^{9\text{-}10}$ copies/g in control fecal samples, and a significant decrease was observed in the total bacterial contents in Group 3 mice fecal samples. The decrease of the *Bifidobacterium* spp. copy number in both Group 3 and 5 mice was significant when compared with Group 1 mice ($p < 0.05$). A significant increase was observed for the *Bacteroides* spp. population in group 5 compared with Group 3 mice ($p < 0.05$). The *C. perfringens* subgroup population in Group 1 mice was much higher than that in both Groups 3 and 5 ($p < 0.05$).

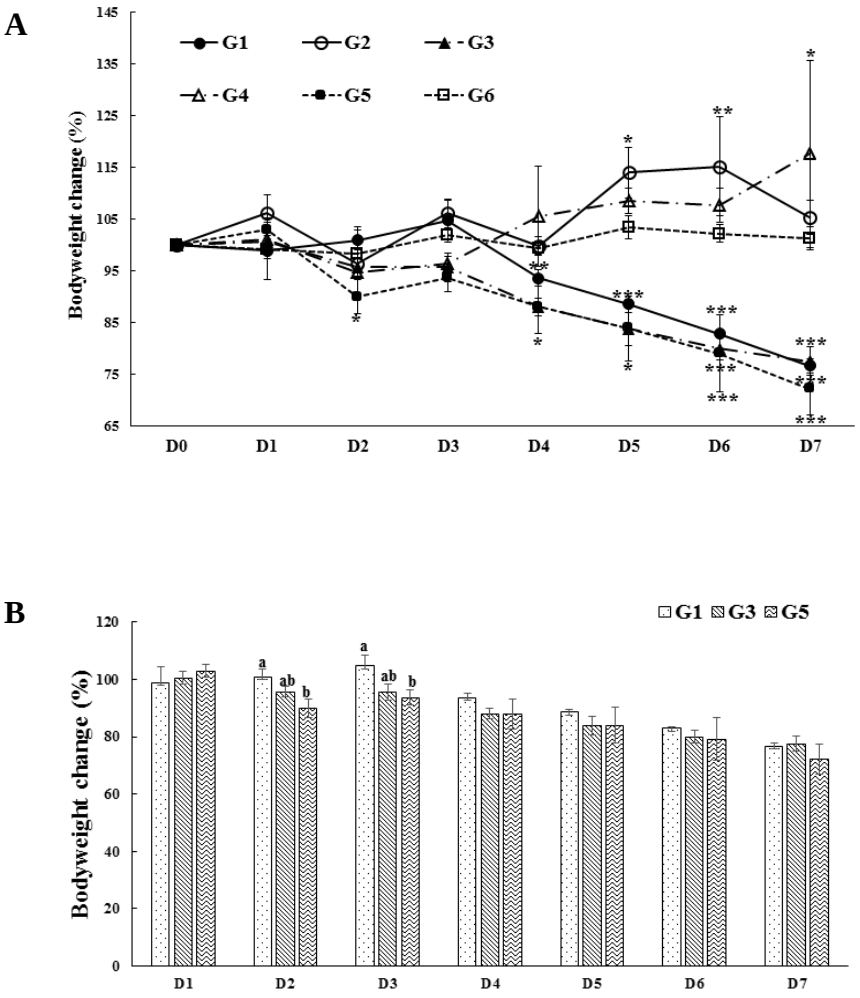


Figure 2. The influence of MFP on the body weight loss in DSS-induced acute colitis. During DSS treatment week, the body weight was daily checked. (A) The results of statistical significance in six groups. (B) The comparative results between G1, G3, and G5. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Different letters (a, b, c) indicate significant differences between groups at $p < 0.05$. All data are represented as the mean ± SD. G: group. D: day.

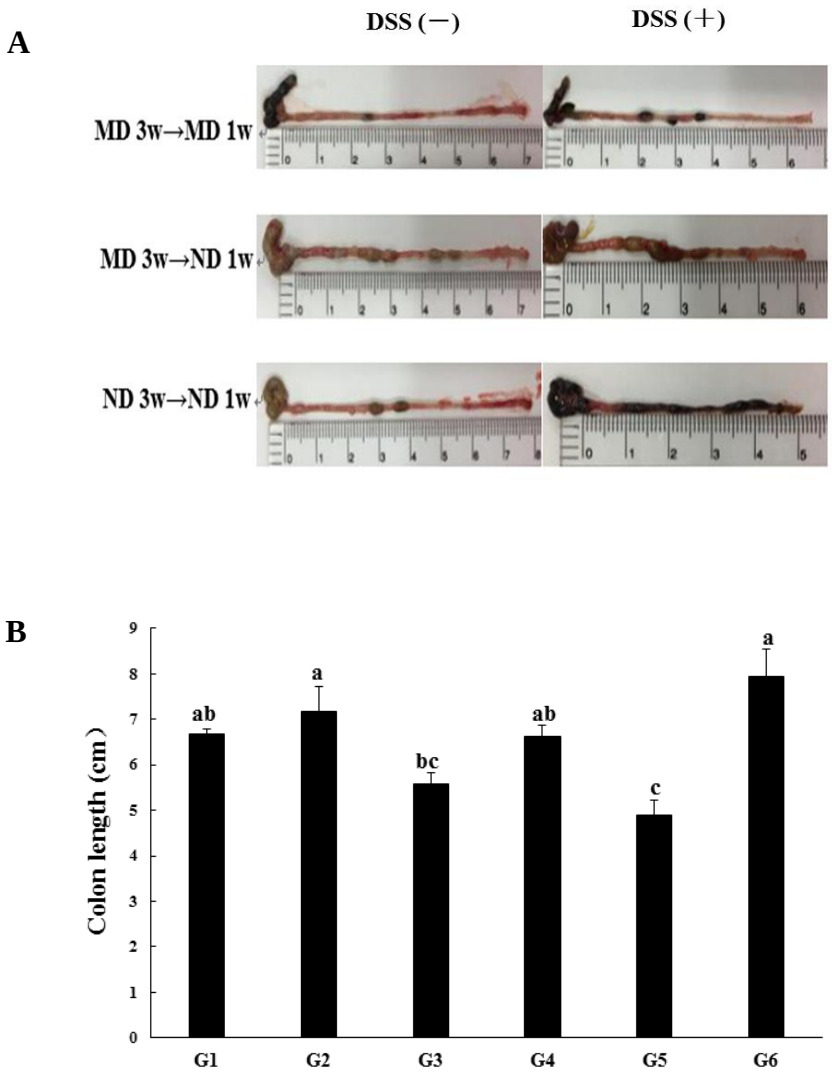


Figure 3. The influence of MFP on colon length in DSS-induced colitis. At the end of experiment, the mice were sacrificed and the colonic tissues were collected. (A) The length of colon was calculated and (B) the statistical analysis of the colon length among the mice groups. Different letters (a, b, c) indicate significant differences between groups at $p < 0.05$. All data are represented as the mean ± SD.

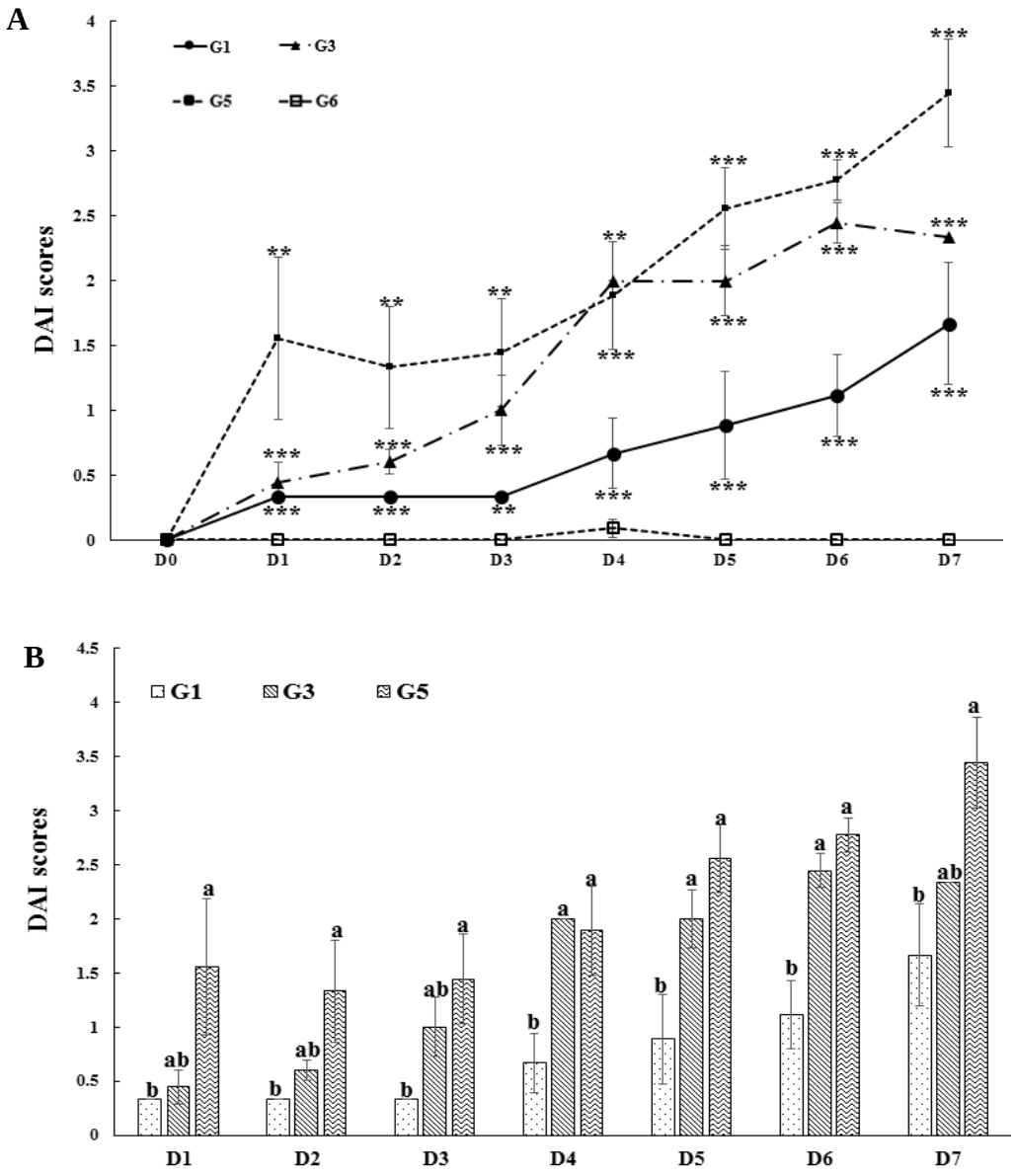


Figure 4. The DAI scores were influenced by MFP in DSS-induced colitis. During the 5% DSS-induced week, the body weight and stool condition were monitored daily. DAI scores were measured according to the fecal condition and body weight loss. (A) The results of statistical significance among G1, G3, G5, and G6. (B) The comparative results between G1, G3, and G5. *p < 0.05, **p < 0.01, ***p < 0.001. Different letters (a, b, c) indicate significant differences between groups at p < 0.05. All data are represented as the mean ± SD.

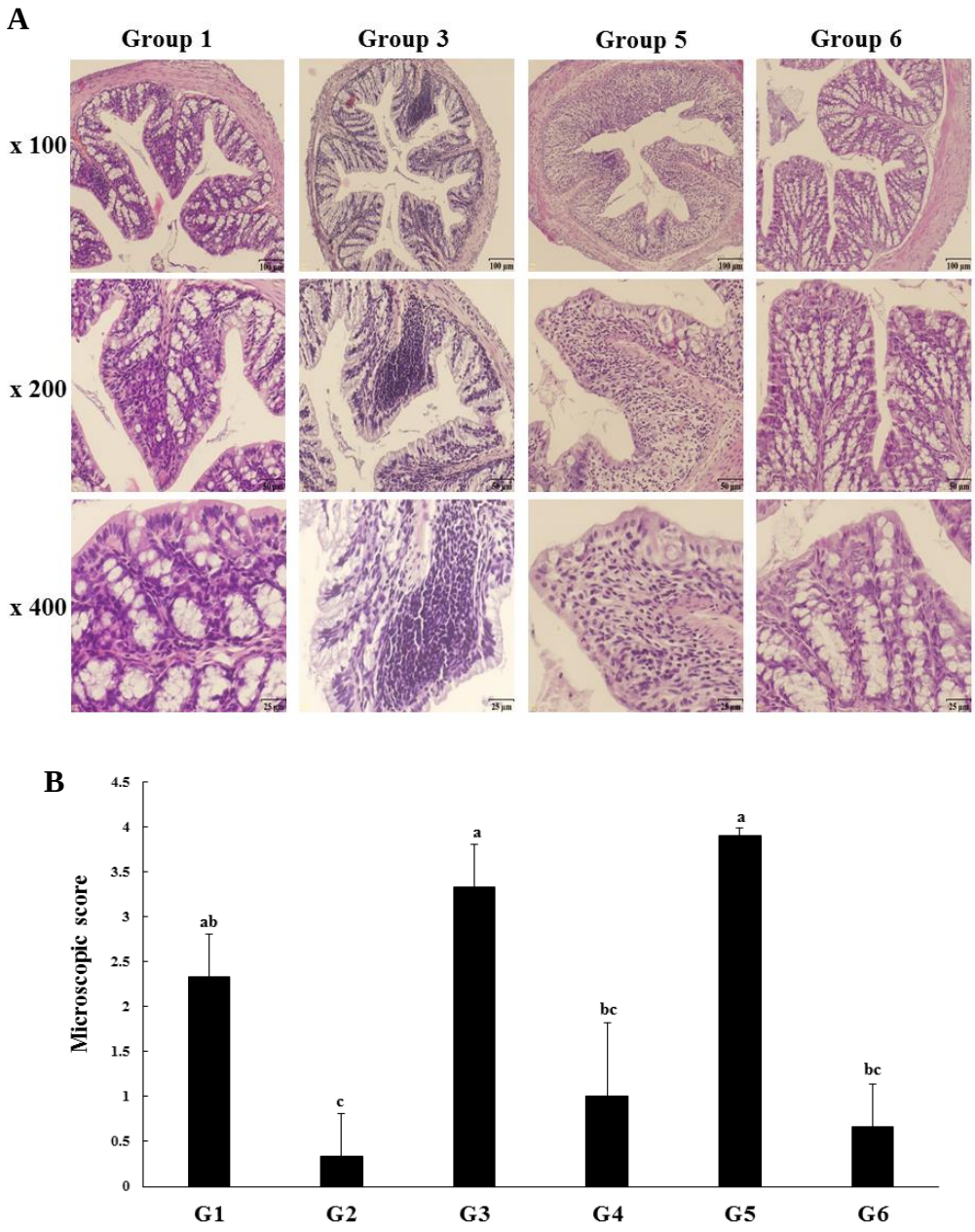


Figure 5. Histological observation of colon in DSS-induced colitis. At the end of the experiment, all the mice were sacrificed, and their colonic tissues were collected. Histological analysis of colon sections obtained from DSS-induced colitis mice with/without mulberry using HE staining. (A) HE-stained specimens observed under light microscopy. The arrowheads indicate the infiltration of inflammatory cells. (B) The microscopic scores are indicated. The microscopic score was calculated according to the scoring system. Different letters (a, b, c) indicate significant differences between groups at $p < 0.05$.

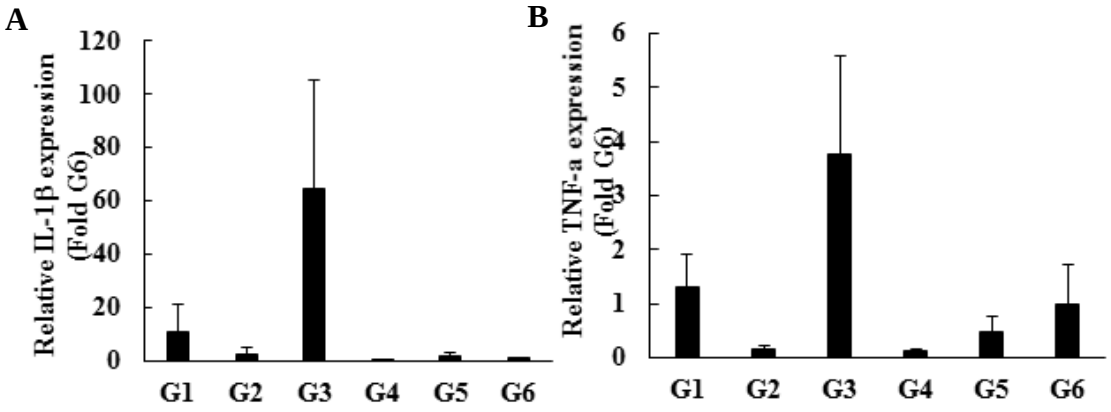


Figure 6. mRNA expression levels of cytokines in colonic tissues. At the end of the experiment, all the mice were sacrificed, and their colonic tissues were collected. mRNA expression levels of cytokines (IL-1β and TNF-α) in colonic tissues from the mice with/without MFP and DSS. All data are represented as the mean ± SE of three separate experiments and analyzed by a one-way ANOVA followed by Tukey's HSD using IBM SPSS Statistics software.

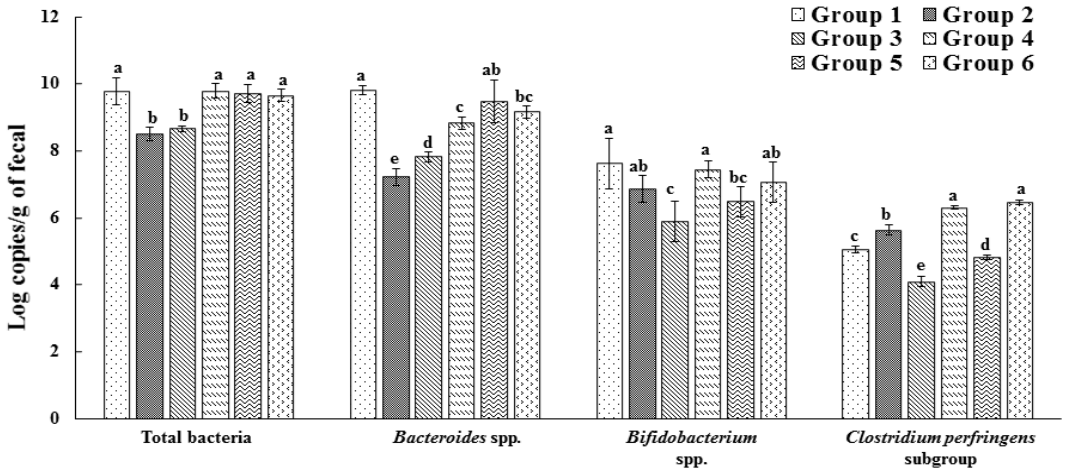


Figure 7. qPCR detection of intestinal bacterial composition. The fecal samples for qPCR detection of bacteria were collected at the end of experiments. Fecal DNA samples from mice in all the 6 groups were extracted, and qPCR amplification and detection were performed. *Bacteroides* spp., *Bifidobacterium* spp., and *C. perfringens* subgroup contents are presented as the bacterial copy number in fecal microbiota. Different letters (a, b, c, d, and e) indicate significant differences between groups at $p < 0.05$. All data are represented as the mean ± SD of three separate experiments and analyzed by a one-way ANOVA followed by Tukey's HSD using IBM SPSS Statistics software.

3.4 Discussion

IBD has attracted widespread attention because the incidence of it shows an increasing trend from 1990. Current therapies to IBD patients have limited efficacy because of their potentially serious side-effects in long-term use (Bernstein, 2018; Bernstein, 2015). This disease is considered as a continuous or chronic intestinal inflammation which caused the imbalance between immune responses and gut microbiota. This imbalance contributes to mucosal disruption and ulceration. Under this condition, the pathogenic bacteria easier invade into the mucosal layer, which leads to the activation of immune cells. the infiltration of a large number of immune cells is observed in the mucosa during active colitis (Stevceva, et al., 2001). In particular, macrophages play a key role in this disease pathogenesis because they release proinflammatory cytokines and ROS, which is an important factor in the mechanism of IBD pathogenesis (Martini, et al., 2017). In the context of limited therapy options for patients with IBD, biological and clinical benefits, such as inflammation modulation, was recently reported for fruits, such as berries, containing anthocyanin. Anthocyanins are non-toxic water-soluble pigments with great anti-oxidative capacity. Anthocyanins from blueberry and grape have proven to mitigate IBD symptoms (Yu, et al., 2011). In this study, I have focused on the mulberry fruit richer in anthocyanin content than other berries. Anthocyanins are major functional polyphenols in this fruit, probably explaining their use in China and Japan in traditional medicines against various diseases (Sugimoto, et al., 2009; Jin, et al., 2005; Yang, et al., 2010; Naowaboot, et al., 2009; Enkhmaa, et al., 2005).

Some methods are used into induced colitis in mice model, such as chemical reagents, gene-deficient mice (Nan, et al., 2018; Dong, et al., 2015; Rudolph, et al., 1995; Yassin, et al., 2018; Fitzpatrick, et al., 2018). The UC model established by chemical method mainly utilizes the direct stimulation of chemical substances to damage the mucosal barrier of colon and increase permeability. Then inflammatory responses are induced. This method is widely used in experiments because of its short time, good repeatability, and obvious symptoms. The chemical substances mainly contain DSS, 2,4-dinitrochlorobenzene (DNCB), 2,4,6-trinitrobenzenesulfonic acid sol (TNBS), acetic acid, and oxazone (Heller, et al., 2002; Thippeswamy, et al., 2011; Bai, et al., 2015; Lee, et al., 2015; Wang, et al., 2011; Yunusi, et al., 2015). Among those

chemical substances, we used the DSS to induce the colitis. DSS mouse colitis model was first established by Japanese scholar Ohkusa in 1985, and it is the most ideal colitis model at present. The clinical manifestation and mucosal damage degree are DSS concentration-dependent, and the difference of total DSS intake does not affect the degree of colonic inflammation (Egger, et al., 2000).

The aim of this study was to determine the effect of mulberry fruit against UC and the mechanism of its focus on the change of intestinal microbiota in DSS-induced colitis. Our results indicate that MFP reduces the major symptoms of DSS-induced acute colitis, including body weight loss, colon shortening, and colonic inflammation (Fig. 1 and 2). Feeding the mice, the MFP mixed diet modified the colonic microenvironment, which may play an important role in reducing DSS-induced colonic injury and inflammation and/or maintaining the gut barrier, as evidenced by the reduced DAI score and histological damage. Moreover, few infiltrated immune cells were observed in mice fed with MFP suggesting that MFP can protect the mucosal barrier and suppress the activation of macrophages. Thus, the oral administration of MFP significantly mitigates the disease severity in DSS-induced acute colitis, suggesting that a daily supplement of MFP has health benefits.

IL-1β is a multifunctional cytokine secreted by monocytes, mast cells, smooth muscle cells, and endothelial cells. It plays a key role in the initiation and development of host response to the pathogenic invasion and is an important regulator of immune and inflammatory initiation (LaRock, et al., 2016). IL-1β participates in the inflammation response, activating lymphocytes, and promoting immune cell infiltration at the site of injury and inflammation (Jain, et al., 2018). It has been proved that the expression of IL-1β was higher in UC patient compared with the healthy adults, and this process may relate to the release of hydrogen peroxide (Leal, et al., 2008). TNF-α has a wide range of biological activities, and it combines with TNF receptors on the cell surface to perform a variety of biological functions and promote the occurrence and development of inflammatory responses. This cytokine can improve the phagocytic function of immune cells including neutrophils and macrophages, and leads to secretion of IL-1、IL-2、IL-4、IL-6、IL-8. Moreover, it also induces the expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 and mediates the inflammatory cell adhesion (Thakkar, et al., 2018; Kim, et al., 2001). It is important

that TNF-α effects on the expression of NF-kB pathway which plays a central role in inflammation through its ability to induce transcription of proinflammatory genes especially in IBD (Evans, et al., 2018; Subramanian, et al., 2018; Tak, & Firestein, 2001; Rezayat, 2018). In this study, the results showed that MFP reduces the mRNA expression of IL-1β and TNF-α in mice fed MFP, suggesting that MFP acts as a powerful antioxidant and cancels the ROS influence in the colonic microenvironment.

Several studies have reported that the intestinal flora plays a key role in the pathogenesis of intestinal inflammation and that intestinal microbial dysbiosis is closely associated with the pathogenesis of DSS-induced UC (Matsuoka, & Kanai, 2015; Cao, 2017; Sasaki, & Klapproth, 2012; Knights, et al., 2013). Animal models indicate that intestinal bacteria have different proinflammatory activities in relation to the induction of colitis. For instance, *Bifidobacterium* spp., a kind of probiotic, has an inhibitory effect on DSS-induced colitis (Venturi, et al., 1999; Nanda Kumar, et al., 2008). *Bacteroides* spp. and *E. coli* are considered as two pathogenic bacteria for colitis (Bloom, et al., 2011; Rath, et al., 1999). *Clostridium* spp. promote the accumulation of regulatory T cells, which play a key role in mucosal immunity (Atarashi, et al., 2001). Some *Clostridium* species are considered as the pathogenic factors, such as *C. difficile* (Sun, et al., 2018). On the other hand, *C. butyricum*, which belongs to *C. perfringens* group, is probiotic bacteria able to reduce intestinal inflammation by improving the mucosal barrier function and modulating immune responses (Banaszkiewicz, et al., 2015). In this study, the contents of *Bifidobacterium* spp. and *C. perfringens* group were determined; the results indicate that those two bacterial groups were significantly increased in mice fed MFP of Group 1 compared with Group 3 and Group 5 mice that were not fed MFP (Fig 4). As these probiotic bacteria reduce mucosal inflammation by modulating cytokine production, decreasing oxidative stress, and enhancing the mucosal barrier (McCarthy, et al., 2003; Amaretti, et al., 2013; Madsen, et al., 1999), our results suggest that MFP increases immunomodulation in the colon by the increased contents of these bacteria.

3.5 Conclusion

In the present research, MFP reduced the body weight loss, colon shortening, and inflammatory level in colonic tissue. Additionally, the content of beneficial bacteria (*Bifidobacterium* spp. and *C. perfringens* group) was significantly increased, while *Bacteroides* spp. population was significantly decreased in MFP diet groups mice compared with the that in normal diet groups mice. In conclusion, MFP attenuates disease severity by modifying the bacterial content in DSS-induced acute colitis. MFP contains the anthocyanins, cyanidin-3-glucoside and cyanidin-3-rutinoside as functional antioxidative ingredients. These findings suggest that MFP has beneficial health effects.

CHAPTER 4

Mulberry Diet Regulates the Expression of NLRPs in DSS-Induced Colitis

4.1 Introduction

The intestinal immune cells are the first line of protection for intestinal homeostasis because there is no immune organ in the intestine. The innate and adaptive immunity plays a key role in resistance to pathogenic factors invasion. On the one hand, adaptive immunity is mediated by B lymphocytes and T lymphocytes, while excessive immune response can lead to tissue damage. On the other hand, innate immunity plays a major role in the early phase of infection because it is a quick response to pathogenic factors. The antigen-presenting cells such as dendritic cells and macrophages mediate this process. During the process of IBD, the bacteria invaded the mucosa layer to induce the injury of intestinal tissues. Several families of innate receptors are involved in the recognition of microbe-associated molecular patterns, such as TLRs and nucleotide-binding oligomerization domain protein-like receptors (NLRs) (Jang, et al., 2015). Inflammasomes are innate sensors in which apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) connects the sensing of microbial molecular patterns or cellular stress by NLRs to Caspase-1 (Casp-1)-mediated maturation of IL-1β and IL-18 (Vanaja, et al., 2015). The results in the study using Casp-1 knockout mice have strongly suggested that Casp-1 plays a key role in DSS-induced colitis (Błażejewski, et al., 2017).

The NLRP3 inflammasome plays a key role in inflammation because disease-associated NLRP3 mutations enhance Casp-1 activation and IL-1β production (Abderrazak, et al., 2015). Furthermore, a decreased NLRP3 level and decreased IL-1β secretion were recently reported to be linked with increased susceptibility to CD in humans (Zaki, et al., 2010). On the other hand, ASC-dependent inflammasomes, such as NLRP6, were identified as a key immune axis regulating the intestinal microbiota (Elinav, et al., 2013). The NLRP6 inflammasome has been shown to play critical roles in defense against infection, autoinflammation, and tumorigenesis. As NLRP6 is highly expressed in epithelial cells, especially goblet cells, this inflammasome has been

considered an essential factor for mucosal self-renewal, cell proliferation, and regulation of intestinal flora through mucus secretion and anti-microbial peptide production from goblet cells and the epithelium (Levy, et al., 2017; Wlodarska, et al., 2014). Intestinal mucus layer, which mainly composed of MUC2 mucin secreted by goblet cells, is the protective layer of intestinal tract (Tawiah, et al., 2018). It provides guarantees for intestinal defense against endogenous or exogenous stimulation and microbial invasion. And it also contributes to maintaining the balance of intestinal symbiotic flora (Arike, et al., 2016).

In the present research, the colonic tissues were the same as Chapter 3. The aim was to evaluate the influence of MFP on the function of inflammasomes including NLRP3 and NLRP6 in DSS-induced colitis. The mRNA expressions of NLRPs were evaluated by RT-qPCR using the proximal colon tissue. The number of goblet cell was calculated according to the HE staining results using the middle and distal colons.

4.2 Materials and Methods

The mice grouping was the same as Chapter 3 (Table 4).

4.2.1 Gene expression analysis

Total RNAs were extracted from colonic tissues in each mouse using the SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer's instructions. The concentration of RNA was quantified using SmartSpec plus Spectrophotometer (Bio-Rad Laboratories Inc., Tokyo, Japan). One microgram of total RNA was subjected to reverse transcription with oligo (dT18) primers using the First Strand cDNA synthesis kit (Takara Biotechnology, Shiga, Japan) according to manufacturer's instructions. All the cDNA preparations were stored at -20˚C until further use.

The qRT-PCR was conducted in the Mini Opticon Real-Time PCR System (Bio-Rad Laboratories Inc.) using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix Kit (Agilent Technologies, West Cedar Creek, TX). Expression values were normalized to GAPDH in the same sample and then normalized to the control. The sequences of the primer pairs used for qRT-PCR amplification are listed in Table 8. Samples were heated at 95˚C for 5 min and then subjected to 40 cycles of denaturation at 95˚C for 5 sec and annealing/elongation for 10 sec at annealing/elongation

temperatures described in Table 4. The amplifications were performed on three independent samples, with triplicate reactions carried out for each sample. The relative mRNA level was calculated using the $2^{-\Delta\Delta CT}$ method.

4.2.2 Histopathological examination

The middle and distal regions were embedded in paraffin, sectioned at 5-μm thickness, and de-paraffinized. Sectioned specimens were stained using a HE solution. HE specimens were observed under the light microscope (Olympus FSX100, Olympus, Tokyo, Japan) to count the number of goblet cells (magnification, x200 and x400). HE specimens (3 specimens/each region/mouse) were made, and then the number of goblet cells were counted in three fields of each specimen under the light microscope (magnification, x 400).

4.2.3 Statistical analysis

Data were expressed as the mean ± SE of the mean or the mean ±SD of three independent experiments. Data were statistically evaluated by one-way analysis of variance followed by Tukey's HSD using IBM SPSS Statistics software.

4.3 Results

4.3.1 Gene expression analysis.

The mRNA expression of NLRP3 in Group 3 was higher than it in Group 1, however, the difference was not statistically significant (Fig. 8). However, both NLRP6 and ASC in Group 1 seemed to be expressed at higher levels than those in Groups 3 or 5, but the differences were not statistically significant (G1 vs G3: $p = 0.347$; G1 vs G5: $p = 0.163$).

4.3.2 The number of goblet cell.

The number of goblet cell in Group 1 epithelia was significantly higher than those in Groups 3 or 5 ($p<0.05$, Fig. 9). The number of goblet cell in Group 5 was significantly lowest compared with Group 1 and Group 3 ($p<0.05$). The number of goblet cell in Group 3 indicated in the middle between Group 1 and 5.

Table 8. The primer sets for cytokines and NLRPs.

Primer set 1	Primer sequence (5′- -3′)	Annealing temperature (° C)
IL-1β	F: 5'- TCGGACCCATATGAGCTGA-3' R: 5'- CCACAGGTATTTTGTCGTTGC-3'	52
NLRP3	F: 5'-ACCTCCAAGACCACTACGG -3' R: 5'-AAAACAACAGGCTAAGGA -3'	52
GAPDH	F: 5'-GTTCCTACCCCCAATGTGTCC -3' R: 5'- TAGCCCAAGATACCCTTCAGT-3'	52
Primer sets 2	**Primer sequence (5′- -3′)**	**Annealing temperature (° C)**
TNF-α	F: 5'-CATCTTCTCAAAATTCGAGTGACAA -3' R: 5'-TGGGAGTAGACAAGGTACAACCC -3'	60
ASC	F: 5'-ACAGAAGTGGACGGAGTGCT-3' R: 5'-CTCCAGGTCATCACCAAGT -3'	60
NLRP6	F: 5'-TGACCAGAGCTTCCAGGAGT -3' R: 5'-TTTAGCAGGCCAAAGAGGAA -3'	60
GAPDH	F: 5'-AGGTCGGTGTGAACGGATTTG -3' R: 5'-TGTACACCATGTAGTTGAGGTCA -3'	60

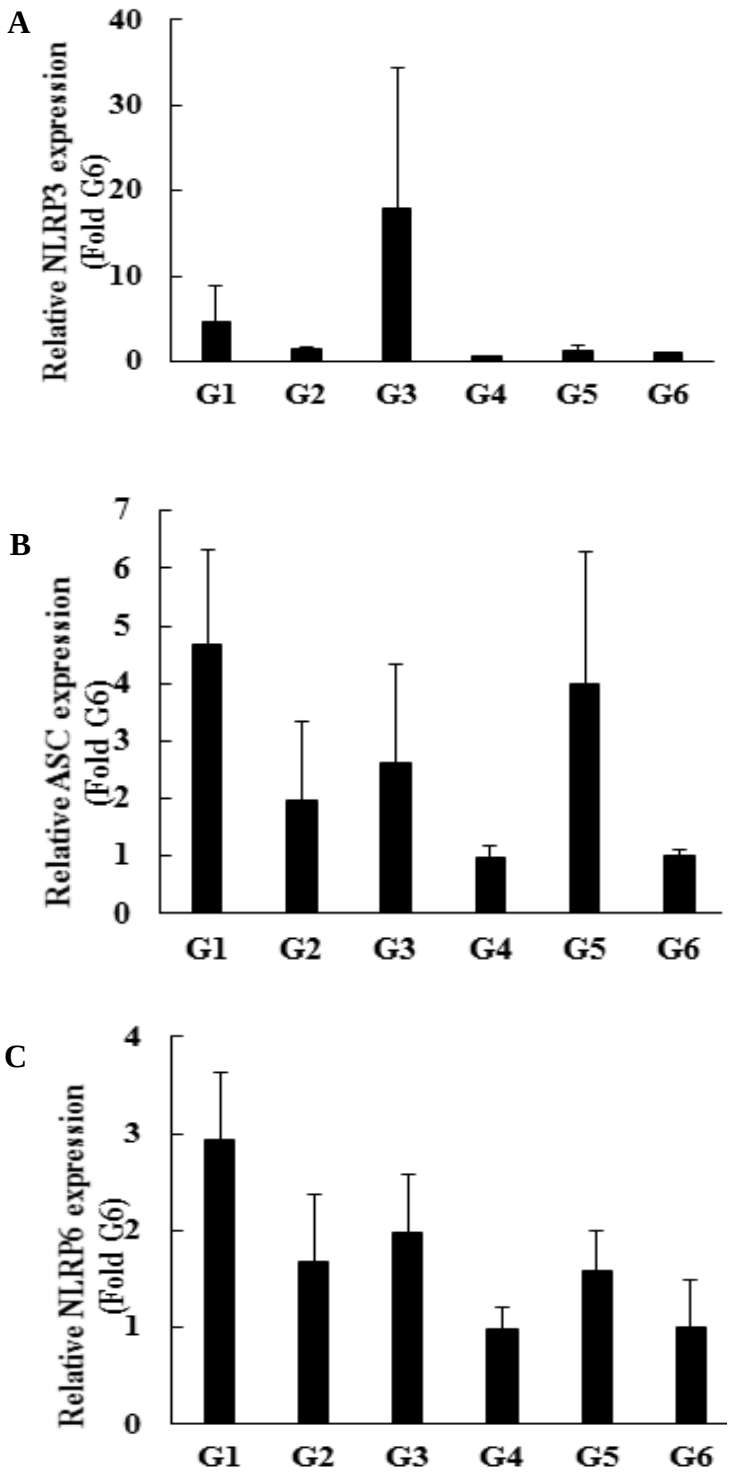


Figure 8. mRNA expression levels of NLRPs in colonic tissues. At the end of the experiment, all the mice were sacrificed, and their colonic tissues were collected. mRNA expression levels of NLRPs (NLRP3, ASC, and NLRP6) in colonic tissues from the mice with/without MFP and DSS. All data are represented as the mean ± SE of three separate experiments and analyzed by a one-way ANOVA followed by Tukey's HSD using IBM SPSS Statistics software.

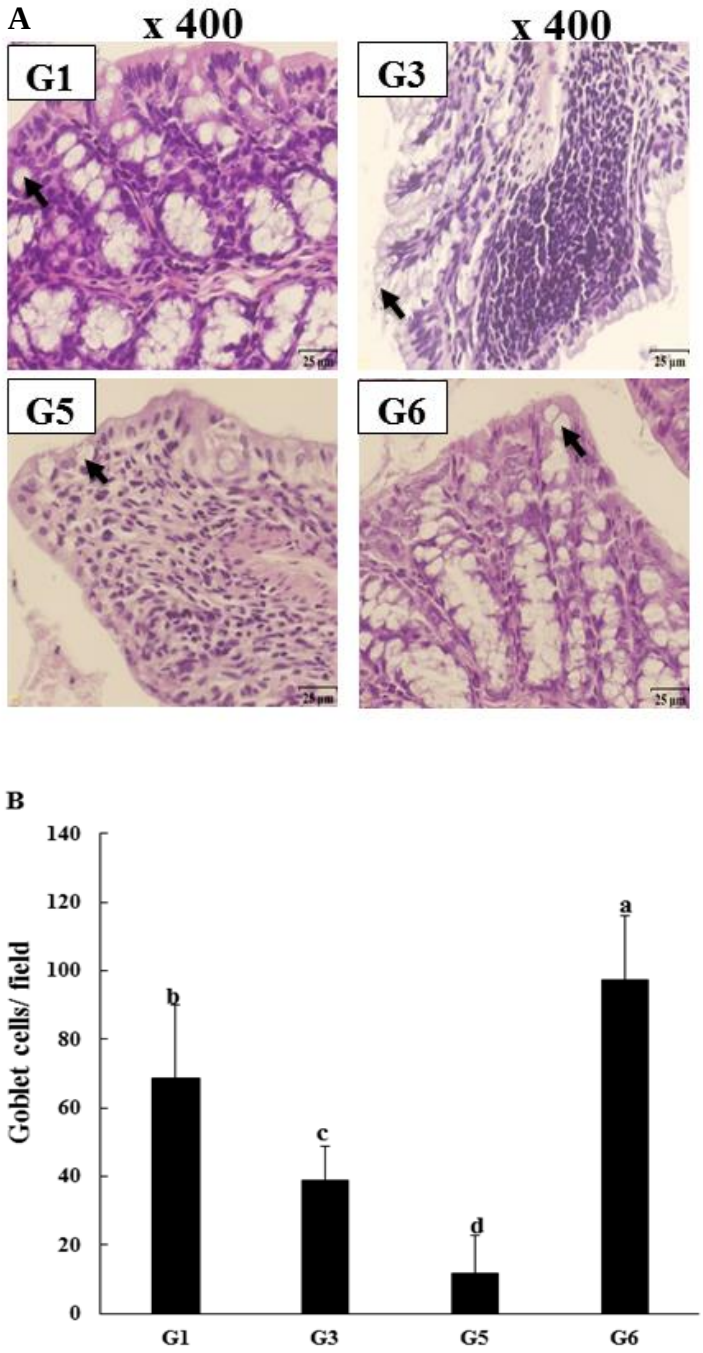


Figure 9. The number of goblet cell in colonic tissue. At the end of the experiment, all the mice were sacrificed, and their colonic tissues were collected. HE-stained specimens observed under light microscopy. (A) The goblet cells were indicated using arrows. (B) The number of goblet cell was counted in three fields of each specimen under the light microscope (magnification, x 400). Different letters (a, b, c, d, and e) indicate significant differences between groups at $p < 0.05$. All data are represented as the mean ± SD of three separate experiments and analyzed by a one-way ANOVA followed by Tukey's HSD using IBM SPSS Statistics software.

4.4 Discussion

The cases of IBD which characterized chronic inflammation and the ulcerative colitis increase worldwide. This disease not only reduces the patients' quality of life but also increase the risk of colon cancer. Currently, the major treatments are depending on the anti-inflammation and anti-oxidant drugs, which have side-effects in long-time use. Recently, researchers have reported that some inflammasomes play a key role in maintaining homeostasis of intestinal environment (Ratsimandresey, et al., 2017; Rauch, et al., 2017; Zhu, et al., 2017). The inhibitory function of mulberry fruit was proved in previous results (Chapter 3), while the influence of MFP on the inflammasomes especially NLRP6 and NLRP3 inflammasome need further investigate.

The NLRP3 inflammasome activation by ROS and/or danger signals, including pathogen-associated molecular patterns, results in the Casp-1-dependent processing of IL-1β and IL-18 (de Zoete, et al., 2014; Anand, et al., 2012). IL-1β participates in the inflammation response, activating lymphocytes, and promoting immune cell infiltration at the site of injury and inflammation. Our data show that MFP reduces the mRNA expression of IL-1β and NLRP3 inflammasomes in mice fed MFP, suggesting that MFP acts as a powerful antioxidant and cancels the ROS influence in the colonic microenvironment.

Our result of the increased expression of NLRP6 inflammasomes in mice fed MFP suggest a key role of NLRP6 in intestinal homeostasis by regulating IL-18 expression, secreting mucin, and producing anti-microbial peptides from goblet cells. The mucus overlying the epithelial cells is secreted mainly by goblet cells, and the secretion is regulated by NLRP6 inflammasomes (Wlodarska, et al., 2014). Our results showed that goblet cells are maintained in Group 1 mice compared with Group 3 and 5 mice, suggesting that MFP promotes the formation of NLRP6 inflammasomes and preserves the goblet cell number. This might lead to a protective mucus barrier in the colon, resulting in the inhibition of DSS-induced acute colitis. Moreover, NLRP6 inflammasomes can negatively regulate the NF-kB and MAPK signaling pathways to control the expression of proinflammatory cytokines (Anand, et al., 2012). NLRP6 inflammasome-deficient mice are characterized by the infiltration of inflammatory cells in the lamina propria and the exacerbation of colitis (Elinav, et al., 2011).

4.5 Conclusion

In the present research, the mRNA expression of NLRP6 and ASC in Group 1 was higher than that in Group 3. At the same time, the goblet cells in Group significantly increased compared with that in Group 3 and 5. It has been reported that the activity of goblet cells, which secrete the mucin and anti-microbial peptides, was regulated by the expression of NLRP6. In conclusion, those results indicated that MFP maintained the intestinal mucosal by increasing the expression of NLRP6 and maintaining the goblet cells.

In conclusion, the beneficial bacteria are dominant in the gut by MFP supplementation. Furthermore, MFP supplementation leads to protect mucus condition by the expression of NLRP6 inflammasome and the maintenance of goblet cells. As those results, MFP supplementation mitigates the intestinal inflammations. These findings suggest that MFP has beneficial health effects.

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