Doctoral Thesis

EFFECTS OF LEMON PEEL POWDER ON INTESTINAL BARRIER AND INFLAMMATION

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March, 2021

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ABBREVIATIONS

ANOVA, analysis of variance

CD, Crohn's disease

Ccl-2, C-C motif chemokine ligand 2

CXCL-2, chemokine C-X-C motif ligand-2

DSS, dextran sodium sulfate

IBD, inflammation bowel disease

IL, interleukin

IFNγ, interferon-γ

LC/MS/MS, liquid chromatography tandem mass spectrometry

LP, lemon peel

MetOH, methanol

qRT-PCR, quantitative reverse transcription-polymerase chain reaction

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SCFA, short chain fatty acids

SEM, standard error of the mean

TGFβ, transforming growth factor-β

TNF-α, tumor necrosis factor-α

UC, ulcerative colitis

ZO, zonula occludens

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ACKNOWLEDGEMENTS

First and foremost I offer my sincerest gratitude to my supervisor, Professor Takuya Suzuki who has supported me throughout my thesis with his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. In many ways I have learnt much from him.

Besides my advisor, I greatly appreciate my committee members: Prof. Shimamoto Tadashi, and Prof. Obitsu Taketo, for their insightful comments and the hard question which incented me to widen my research from various perspectives.

I gratefully acknowledge the funding sources that made my Ph.D. studying possible. I was funded by the scholarship of the Government of Viet Nam fellowship for 3 years. My study was also supported by Hiroshima University that opened up to the horizon of my new knowledge.

I also would like to acknowledge to the Teachers and Laboratories of Graduate School of Biosphere Science in Hiroshima University that has provided the support and equipment I have needed to produce and complete my research. My sincere thanks also goes to the staff members of the Student support office who has supported me greatly.

I thank my fellow lab mates for being fantastic colleagues and friends, for sharing their great knowledge and research experience. They made the lab a fun and uniquely vibrant place with happy time of cherry bloom party, welcome party and so on. My time in Japan was made enjoyable in large part thanks to many friends who has become a special part of my life. Thanks so much for all of my international friends who has always met me with warmth and friendship, and helped me along the way in both study and spirit.

Last but not least, I would like to thank my family. I cannot thank my mother enough for her loving, and encouraging me spiritually and especially to my husband who is always beside me to share with me the happy times as well as the difficult times, together trying and passing the challenge in life and achieved the result today.

Obtaining the educational opportunity in Hiroshima University is the foundation for my success in the future. I wish my teachers and my friends all the happiness and good health.

Chapter 1

INTRODUCTION

1.1 Citrus limon

Citrus genus is the most important fruit tree crop in the world with a production estimated at 12246 tons (FAO, Statistical bulletin, 2016). According to the morphological system established by Tanaka (1969), lemon is classified as *Citrus limon*. Lemon is a yellow or pale yellow fruit with 5-10 seeds, known throughout the world (Jideani, 2012). Lemon is popularly used in beverages, ice creams, desserts, salad dressings, and many meat and vegetable dishes (Xi *et al.*, 2017).

Lemon is the third most important citrus species after orange and mandarin (González-Molina *et al.*, 2010) and well-known for nutritional and health-promotion values (Dong *et al.*, 2019). Lemon has been investigated for a broad spectrum of biological activities, including anti-microbial, anti-cancer property, anti-inflammatory and can serve as an adsorbent for removing pollutants such as metal ions (Bhatnagare et al., 2010). These activities are strictly related with many important natural components, such as phenolic compounds, vitamin C, dietary fibers, essential oils and carotenoids (Del Rio et al. 2004), alkaloids and flavonoids, which also play a key role as nutraceuticals (John *et al.*, 2017). Therefore, the lemon fruit is more and more becoming a popular health-promoting fruit.

Lemon fruits have a strong commercial value in the fresh products market and food industry. However, excepting the used parts in processing industry, other inedible parts were wasted (Xi *et al.*, 2017). Thus, the industrial consumption of lemon generates high amounts of wastes and by-products such as peels, seeds and pulps matrix that constitutes an important source of bioactive compounds with potentials for animal feed, manufactured food, and health care (González-Molina *et al.*, 2010).

1.1.1 Structure of lemon fruit

The anatomy of lemon is similar to other citrus fruits. The flesh is covered by the pericarp which is like a leathery rind. The pericarp is made up of three distinct layers, including exocarp, mesocarp, and endocarp (Jideani, 2012).

Exocarp (flavedo) is the outermost layer of the pericarp and forms the tough outer skin of the fruit, which bears oil glands and pigments. Flavedo is mostly composed of cellulosic material, but also contains other components, such as essential oils, paraffin waxes, steroids, triterpenoids, fatty acids, pigments (carotenoids, chlorophylls, flavanoids), bitter principles (limonene), and enzymes (Jideani, 2012).

Mesocarp (albedo) is the middle layer of the pericarp situated between the exocarp and then endocarp. It is a part of the peel, which is commonly removed by hand. The albedo contains celluloses, soluble carbohydrates, pectin and proto-pectin, flavonoids, amino acids, and vitamins. The albedo also contains higher flavanone compared to juice vesicles or flavedo. The albedo and flavedo contain higher concentration of bitter components and pectin than other parts of the fruit (Jideani, 2012).

Endocarp is the inside layer of the pericarp which directly surrounds the seeds and is the edible portion, divided into 10-14 segments (carpels) separated by thin septa. The walls of the vesicles are composed of cellulose, hemicellulose, pectin, proto-pectin, sugars, flavonoids, and other minor components such as amino acids and vitamin C (Jideani, 2012).

1.1.2 Composition of lemon peel by-product

Lemon has been valued as a fundamental part for a healthy diet. It is well established that the lemon fruit and its by-products constitute an interesting source of nutrients and non-nutrient compounds which are beneficial for the normal growth of human and the improvement of the human physiological systems. The representative of nutritional composition of fresh lemon peel was presented at **Table 1.1**. The by-products obtained from the lemon transformation are represented by peels, pulps, seeds, and waste water.

Table 1.1 Variation of proximate chemical composition of raw lemon peel (Source: USDA, 2009) (Muhammad Siddiq *et al.*, 2012)

Component	Unit	Raw lemon peel
Water	g	81.6
Energy	Kcal	47
Protein	g	1.5
Lipid	g	0.3
Carbohydrate	g	16
Fiber	g	10.6
Sugars	g	4.17

The lemon peels, which is the main residue generated by lemon juice industry, accounts for 50 – 60 % of the whole fruit weight. These by-products obtained from the lemon transformation are represented by peels, pulps, seeds and waste water. These are known to be rich in bioactive molecules (Russo *et al.*, 2014). However, the comprehensive information about their nutrition is still scarce. It is composed of external part (flavedo), and the internal spongy part (albedo) (Amarowicz *et al.*, 2009). Lemon peels were presented with high total phenolic contents as well as rich in flavonoids. Caffeic acid and chlorogenic acid are the major phenolic acids in lemon, followed by gallic and ferulic acids (González-Molina *et al.*, 2010; Xi *et al.*, 2017). Besides, flavanones are the major flavonoids of the lemon fruits, and hesperidin is the predominant flavanone, followed by hespertin and eriocitrin (Russo *et al.*, 2014; Nogata *et al.*, 2006). Naringin is also present as a major flavonoid in lemon peels (Jasleen and Gurpreet, 2015). The lemon peel also contains polymethoxylated flavone such as sinensetin, and tangeretin. Lemon peels highly contain the three flavones such as: diosmetin 6,8-di-C-glucoside, vicenin-2, and diosmin (González-Molina *et al.*, 2010).

It has been shown that the major component of albedo is the dietary fiber (soluble and insoluble). A previous study showed that the total dietary fiber in the lemon peel was 53 % (Russo *et al.*, 2014). Although the lemon peel includes different types of dietary

fibers, pectin is one of the most abundant fiber. The albedo portion of spent lemon peel contains about 35 – 40 % pectin on a dry weight basis and is an important raw material for commercial pectin production. Pectin is a functional ingredient used in many processed food products. It is used extensively as a gelling agent and mouth-feel enhancer. The major processing steps in refining citrus pectin is the extraction from the peel through an acid treatment. The pectin is recovered by precipitation with alcohol. The lemon has a higher level of pectin than that of orange or tangerine, neither of which is typically used for commercial pectin production (González-Molina *et al.*, 2010).

To conclude, since the several studies show beneficial effects of bioactive compounds present in the lemon byproducts, the lemon peel powder can be successfully re-used as a source of functional foods.

1.2 Dietary fiber

Fiber comprises cellulose, noncellulosic polysaccharides such as hemicellulose, pectic substances, and a noncarbohydrate component lignin. These are mainly the structural components of the plant cell wall, and function like a skeleton for the plants to maintain their shape and structure (Ötles and Ozgoz, 2014). Traditionally, the dietary fiber was defined as the portions of plant foods that were resistant to digestion by human digestive enzyme; this included polysaccharides and lignin. More recently, the definition has been expanded to "dietary fiber is the remnants of the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the human large intestine (Dhingra *et al.*, 2012). Dietary fiber supplement is the popular ingredient in meals of people all over the world. High-fiber foods potentially play an adjunctive role in offering the health benefits including of the reduction of chronic constipation, the attenuation of blood glucose response, less cardiovascular disease, and hypocholesterolemic effect (Slavin, 2013; Fuller *et al.*, 2016).

Pectin has a linear polymer of galacturonic acid connected with α (1-4) bonds. Some regions of this backbone are substituted with α (1-2) rhamnopyranose units from which side-chains of neutral sugars such as galactose, mannose, glucose, and xylose exist. Citrus

fruits contain the pectin from 0.5 to 3.5 % pectin with a large concentration located in the peel. (Lattimer and Haub, 2010). Several studies demonstrated that intake of dietary pectin in the prevention of diseases such as high glucose level, high cholesterol level, myocardial injury, inflammation, endotoxemia, and heavy metal contamination (Roth *et al.*, 1995; Kim, 2005; Sánchez *et al.*, 2008; Zhang et al., 2015). Recently, many peoples prefer "natural", "clean", and "healthy" ingredients in foods. Pectin is strongly positioned in the context of these current trends due to its positive functionality and existence in nature. The versatility as a highly effective stabilizer and texturizer also helps the frequency in use. It is expected that pectin will continue to deliver the significant value and enable manufactures to meet consumers' evolving expectations (CP Kelco - A Huber company).

In the human intestines, pectins are fermented by the resident microbiota in the large intestine, mainly in the colon. The presence of pectins affects the microbial composition and activity, and thereby often increases the production of short chain fatty acids (SCFAs) by the microbes in the colon. The SCFAs production as a result of the pectin consumption are often related to the health benefits. In the *in vitro* fermentation using human fecal microbiota, pectin from sugar beet and soybean significantly stimulated the growth and activity of the genera *Bifidobacterium* and *Lactobacillus*, which have been shown to protect enterocytes from an acute inflammatory response (Olano-Martin et al., 2002). In rats, citrus pectin tended to increase not only the total SCFAs concentration, but also the proportion of propionate and butyrate (Dongowski et al., 2002). Propionate has the potential to reduce blood cholesterol concentrations, while butyrate is an important energy source for intestinal epithelial cells and plays a role in the maintenance of colonic homeostasis (Tian *et al.*, 2016).

1.3 Intestinal barrier function of intestinal tight junction

Intestinal barrier integrity is a prerequisite for homeostasis of mucosal function, which is balanced to maximize absorptive capacity, while maintaining efficient defensive reactions against chemical and microbial challenges. Mounting evidence demonstrates that disruption of epithelial barrier integrity resulting in the increased the mucosal permeability

are recognized to play a role in the pathophysiology of a variety of disorders, such as inflammatory bowel disease (IBD), irritable bowel syndrome, obesity, diabetes, metabolic syndrome, and necrotizing enterocolitis (Bron *et al.*, 2017). The intestinal barrier is organized by different barrier components and structures, but the tight junction structure expressed in intestinal epithelial cells is one of the major determinants of intestinal barrier (Suzuki, 2013).

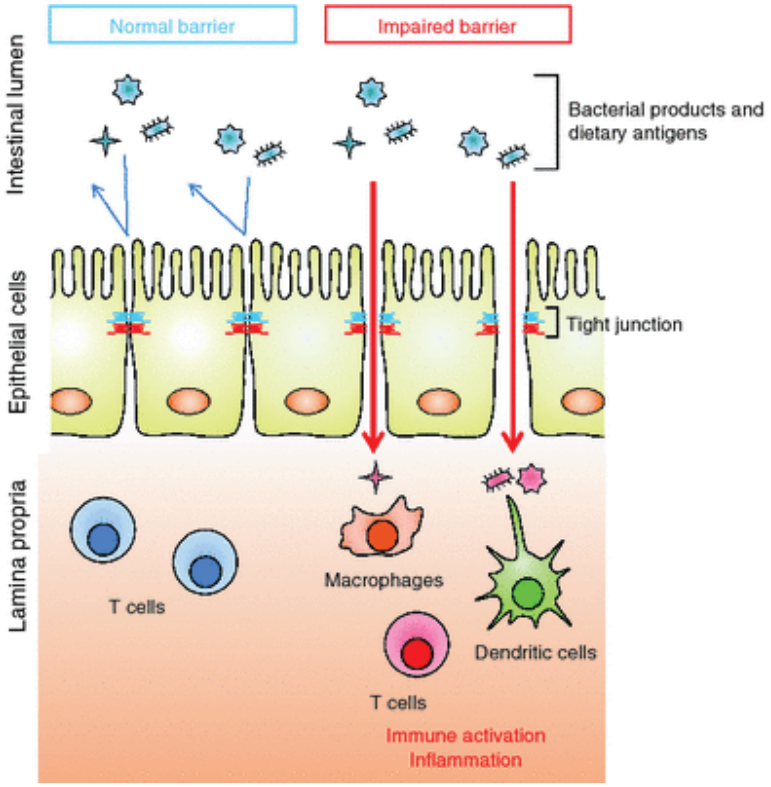


Figure 1.1 Barrier function of intestinal tight junctions (source: Suzuki, 2013).

An important component of the intestinal barrier is the intercellular junctional complex, which is crucial for the maintenance of barrier integrity. The tight junction constitutes the barrier both to the passage of ions and molecules through the paracellular pathway and to the movement of proteins and lipids between the apical and the basolateral domains of the plasma membrane (González-Mariscal *et al.*, 2003). Tight junctions are a multifunctional complex that forms a seal between adjacent epithelial cells near the apical

surface. They seal the paracellular space between epithelial cells, thus preventing paracellular diffusion of microorganisms and other antigens across the epithelium. Tight junction are not static barriers but highly dynamic structures that are constantly being remodeled due to interactions with external stimuli, such as food residues and pathogenic and commensal bacteria. They can regulate the entry of nutrients, ions, and water while restricting pathogen entry and thus regulating the barrier function of the epithelium (Fanning et al., 1999; Ulluwishewa *et al.*, 2011). The Tight junction structure is a multiple protein complex, consisting of transmembrane and cytosolic plaque proteins, including the transmembrane proteins, occludin, and claudins, whose extracellular loops directly interact with adjacent cells to create a barrier against luminal noxious molecules (Furuse *et al.*, 1993, 2002).

Occludin is an ~65 kDa teraspan protein with two extracellular loops. Occludin has been linked to the regulation of intermembrane diffusion and paracellular diffusion of small and large molecules (Fanning et al., 1999; Hartsock and Nelson, 2008). Many researches have shown intestinal tissue expression of occludin to be markedly decreased in patients with intestinal permeability disorders, including crohn's disease (CD), ulcerative colitis (UC), and celiac disease, and in animal model of IBD. It has been proposed that the decreased occludin expression may be an important mechanism leading to the increase in intestinal epithelial tight junction permeability (Fries W *et al.*, 1999; Gassler N *et al.*, 2001; Al-Sadi *et al.*, 2011).

The claudins family consists of at least 24 members ranging from 20 to 27 kDa. Claudins directly regulate the gate function as paracellular tight junction channels that have biophysical properties similar to those of traditional ion channel including ion charge selectivity, permeability dependent on ion concentration, and competition for movement of permeable molecules (Chiba *et al.*, 2008; Hartsock and Nelson, 2008).

The intracellular region of the transmembrane protein is bound to cytosolic plaque proteins, such as zonula occludens (ZO), which anchors the tight junction complex to the actin cytoskeleton (Rao *et al.*, 2002). Although the tight junction barrier is regulated by endogenous factors, such as growth factors, cytokines, and hormones (Suzuki et al., 2008;

Al-Sadi *et al.*, 2010; Suzuki et al., 2011), dietary factors, such as polyphenolic compounds and dietary fibers also have a role in its regulation (Suzuki and Hara, 2009; Noda et al., 2013; Mayangsari and Suzuki, 2018). Thus, plant-derived food material rich in polyphenols and fibers could be developed as a novel tool against intestinal damage and inflammation.

1.4 Inflammatory bowel disease (IBD)

IBD comprises a group of idiopathic chronic inflammotry intestinal conditions of which CD and UC are the two main categories. UC is usually confined to the colon, while CD affects any part of the gastrointestinal tract. The prevalence of IBD are reportedly highest in the United States and the Northern Europe. However, the incidence of IBD is now also increasing in other regions including Asia, where economic developmemnt and industrialization quickly occur. Other factors such as gender, age, and ethnicity also influence the incidence rate of the IBD (Cosnes *et al.*, 2011). Although the IBD pathogenesis is complicated, genetic susceptibility coupled with environmental risk factors such as dietary habits, smoking, stress and lack of exercise, as well as medications and surgery are thought to be associated with the IBD development (Biasi *et al.*, 2013).

IBD is a chronic and recurrent disease of the digestive tracts, which is characterized by an abnormal immune response in the mucosal tissues. The intestinal mucosa has the dual purposes of providing a barrier to prevent bacteria and toxins in the intestines into the circulatory system, while simultaneously absorbing the nutritional components (Bischoff *et al.*, 2014; Wallace *et al.*, 2014). The IBD arises possibly from an impaired epithelial barrier leading to an exacerbated immune response to the resident microflora. Down-regulation of tight junction proteins has been observed in inflamed tissues which assessed by the enhancement of pro-inflammatory cytokines include interleukins (IL) 1, 2, 6, 7, and TNF (tumor necrosis factor). The increased permeability in patients with IBD is related to their disease activity and is predictive of relapse after pharmacological and surgical relief from inflammation (Yu *et al.*, 2015; Fukui, 2016). To maintain the intestinal homeostasis, the innate immune system in the mucosa must be able to distinguish between commensal

bacteria and pathogenic microorganisms. Thus, the improving the intestinal barrier and microflora has a play important role in our health. Intestinal integrity is a hallmark of intestinal health and appropriate microflora, which can maintain the maximized absorptive capacity with appropriate defense system against noxious substances (Toumi *et al.*, 2014; Yue *et al.*, 2019). Current strategies for the treatment of IBD firstly aim the induction of remission, followed by maintaining the remission. Patients are usually treated with corticosteroids, immune-modulators, and anti-TNF-α agents although immunosuppressive therapies and anti-TNF-α agents are sometimes associated with a higher risk of infections and the patients eventually require surgical intervention, indicating that current therapeutic options are insufficient. Moreover, the high cost of biological therapies contributes to the increasing financial burden of health care. The disadvantages of pharmacological therapies in IBD emphasize the need for non-pharmacological options (Bron *et al.*, 2017).

In this regard, low intake of the dietary fibers has been associated with the incidence of IBD, since the prebiotic activity of fiber can stimulate the selective growth of the intestinal *Lactobacilli* and *Bifidobacteria*, which are thought to be beneficial for the intestinal health. These bacteria also produce SCFAs. That could improve mucosal barrier functions and modulate the immune system. Our research group also demonstrated that the fermentable fibers reduce the intestinal inflammation and barrier defect in mice (Hung and Suzuki, 2016). However, the roles of prebiotics in the prevention and suppression of IBD are still unclear (Bron *et al.*, 2017). Chronic inflammation in IBD is characterized by massive leukocytes infiltration of the mucosal tissues. The activated leukocytes produce a wide spectrum of pro-inflammatory cytokines. With regard to the likely combination of genetic and environmental factors in IBD pathogenesis, variants of multiple genes involved in the microbe recognition, lymphocyte activation, cytokine signaling, and intestinal epithelial defense could make a given population more susceptible to environmental attack (Nunes *et al.*, 2011).

In IBDs, both the structure and the function of the intestinal barrier are compromised, with a loss of tolerance to normal dietary components and/or excessive response to pathogens, which all contribute to amplify the overal inflammatory process.

Under normal conditions, inflammatory reactions within the intestinal mucosa are quantitatively and temporally controlled by a delicate balance between pro-inflammatory (TNF-α, IL-1, IL-6, IL-8, IL-17, and IL-23) and anti-inflammatory (IL-4, IL-10, IL-11, and TGF-β) cytokines (Biasi *et al.*, 2013).

The main hypothesis on the develoment and progression of IBD is based on impairment of immune tolerance to the gut commensal microbiota, thought to be due to a genetic predisposition of the host, which leads to chronic intestinal inflammation and mucosal damage (Biasi *et al.*, 2013).

1.5 Aims and outline of the thesis

It is evident that intestinal epithelial barrier dysfunction and inflammation are closely involved in IBD. Recently, there are more research-focuses on herbal medicines lemon peels against diseases. Therefore, the purpose of this study to evaluate the effect of lemon peel containing diets in the murine model of experiment colitis. After that, the lemon peel powder was extracted by methanol to separate into polyphenol- and dietary fiber-rich fractions, to determine the active components in the lemon peel powder.

This dissertation includes 4 chapter:

+ Chapter 1: Introduction including background information of this research is described. Especially, some keywords such as IBD, intestinal epithelial barrier, dietary fiber, and polyphenols are briefly explained.

+ Chapter 2: The preventive effect of lemon-peel containing diet on the intestinal barrier and inflammation in the murine model of colitis was examined.

+ Chapter 3: The lemon peel powder is rich in polyphenols and dietary fibers. We aimed to reveal the potential ingredients in the lemon peel powder, especially with focusing on the dietary fibers and polyphenolic compounds (hesperidin, eriocitrin, diosmin, narirutin, and coumarin) to ameliorate the experimental colitis in mice.

+ Chapter 4: The general conclusion and outcome of this research are described. The future scope is also included.

Chapter 2

THE EFFECT OF LEMON PEEL POWDER IN THE MURINE MODEL OF COLITIS

2.1 Introduction

IBD is characterized by inflammation of the intestinal tract that can ultimately lead to decreased epithelial barrier function. It commonly refers to UC, and CD, the two chronic inflammatory conditions (Perše and Cerar, 2012; M'Koma, 2013). Over 1 million residents in the USA and 2.5 million in Europe are estimated to have the IBD with substantial costs for health care. Moreover, the IBD has emerged in newly industrialized countries in Asia, South America, and the Middle East and has evolved into a global disease with rising prevalence in every continent (Kaplan, 2015). Currently, aminosalicylates, glucocorticoids, immunosuppressive agents, and biological drugs are used to treat IBD patient. However, it occasionally does not work sufficiently (non-responders) and also could induce serious side-effects (Mahadevan, 2004). Therefore, the development of novel preventive or therapeutic approaches using natural herbal medicines is desired. In this study, we examined the effect of the lemon-peel powder which is rich in dietary fiber as well as flavonoid, phenolic acid, and essential oil, in a murine model of experimental colitis.

2.2 Materials and method

2.2.1 Chemicals

Dextran sulfate sodium (DSS; molecular weight: 36,000-50,000) was purchased from MP Biomedicals (Santa Ana, CA, USA). Rabbit anti-ZO-1 (61-7300), occludin (71-1500), claudin-3 (34-1700), claudin-4 (36-4800), claudin-7 (34-9100), and goat Alexa Fluor 488-conjugated anti-rabbit IgG antibodies (A11034) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Rabbit anti-ZO-2 antibody was purchased from Santa Cruz Biotechnology (sc-1148, Dallas, TX, USA). Horseradish peroxidase-conjugated anti-rabbit IgG antibody was purchased from Sera Care (074-1506, Milford,

MA, USA). All other chemicals were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

2.2.2 Preparation of lemon peel

Lemon peel (LP; the peel of lemon after squeezing lemon juice) was purchased from Pokka Sapporo Food & Beverage Co., Ltd (Tokyo, Japan). The lemon fruits were originally grown and cropped in Setouchi area. The diced peel was freeze-dried and milled converted to a fine powder with a mill mixer (IFM-66ODG; Iwatani, Osaka, Japan). The resultant powder was passed through a mesh sieve and designated as whole LP powder.

2.2.3 Nutritional analysis

The nutritional components, including protein, lipid, ash, and moisture in the whole LP powder were analyzed using the Kjeldahl (J M Lynch and Barbano, 1999), Soxhlet extraction (Rosenblum, Garris and Morgan, 2002), dry ashing (Association of Official Agricultural Chemists [AOAC] 942.05) (Thiex, Novotny and Crawford, 2012), and air-oven drying methods (AOAC 930.15) (Thiex, 2009), respectively. The results of the composition was shown at **Table 2.1**.

Table 2.1 Nutrition profile of Lemon peel powder (%)

Moisture	7.0
Dry matter	93
Crude protein	0.7
Crude lipid	4.3
Ash	3.9

2.2.4 Animals

All study protocols were preapproved by the Animal Use Committee of Hiroshima University (authorization No. C19-19). The animal experiment was performed in accordance with the regulations and standard guides for laboratory animals. Male Balb/c

mice (7 week-olds) were purchased from Charles River Inc. (Yokohama, Japan). The mice were allowed to acclimate for 7 days with feeding AIN-93G (Reeves, Nielsen and Fahey, 1993) control diet and water *ad libitum*. All mice were housed under conditions with controlled room temperature (20 – 24 ºC), relative humidity (40 – 60 %) and lighting from 8:00 to 22:00 throughout experiment.

2.2.5 Experimental design

Mice (n=28) were randomly divided into four groups: the control, DSS, DSS + 2.5% LP, DSS + 5% LP (seven mice/group). The control and DSS groups were fed the control diet for the 16-days experimental period. The DSS + 2.5% LP, DSS + 5% LP groups were fed the diets containing 2.5% LP, 5% LP by weight, respectively, through the experimental period. Seven days after the start of feeding the experimental diets, 3 DSS treatment groups were administered 2% (w/v) DSS solution through their drinking water for 9 d to induce experimental colitis, whereas the control group only received distilled water. After DSS administration for 9 days, mice were euthanized by exsanguination under isoflurane anesthesia. The colon was quickly dissected and colon length was measured. The colon was isolated for histological, immunoblot, quantitative reverse transcription-polymerase chain reaction (qRT-PCR), and immunostaining analyses, as described below.

2.2.6 Macroscopic indicators of colitis

The body weight of mice was monitored daily throughout the experiment period. The percentage of body weight gain during 7 days pre-feeding treatment diet was calculated using this method: [(Body weight on day X – Body weight on initial day)/ Body weight on initial day] x 100. After the start of DSS administration the severity of colitis was assessed daily by Disease Activity Index (DAI) (Cooper *et al.*, 1993; Chassaing *et al.*, 2015) the DAI was evaluated, by the presence of gross blood in the feces, the stool consistency, and body weight loss (**Table 2.2**). The percentage of body weight loss was calculated using the following method: [(Body weight on day X – Body weight on initial day)/ Body weight on initial day] x 100.

Table 2.2 The clinical score of murine colitis experiment

Score	Diarrhea stool	Bloody stool	Weight loss (% of initial)
0	Normal	Normal color	<1
1	Mildly soft	Brown color	1-5
2	Very soft	Reddish color	6-10
3	Watery	Bloody stool	11-20
4	More watery	More bloody	>21

2.2.7 Immunoblot analysis

The segment of mouse colonic tissue (appx. 1.5 cm in length) was washed with saline, frozen in nitrogen liquid, and preserved at -80 ^{o}C until analysis. Samples were homogenized in lysis buffer (1 % [w/v] sodium dodecyl sulfate, 1 % [v/v] Triton X-100, 1 % [w/v] sodium deoxycholate, and 30 mmol/L 2-amino-2-(hydroxymethyl)-1,3-propanediol trimethylolaminomethane) with protease and phosphatase inhibitors by Kinematica polytron homogenizer PT 2500 E (Switzerland) to extract the protein. The lysate was centrifuged at 13000 rpm for 10 min at 4 ^{0}C and the supernatant were mixed with Laemmli sample buffer and subjected to immunoblot analyses of tight junction proteins. Protein (20 μg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. Membranes were bloted for ZO-1, ZO-2, occludin, claudin-3, claudin-4, and claudin-7 using specific antibodies in combination with HRP-conjugated anti-rabbit IgG antibody as previously described (Hung and Suzuki, 2016; Ogata *et al.*, 2017). The blots were developed using enhanced ECL chemiluminescence detection reagents (Perkin Elmer Life Sciences, Waltham, MA, USA). Quantification was performed by densitometric analysis of specific bands on the immunoblots with the use of Image J software (National Institutes of Health, Bethesda, MD, USA).

2.2.8 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The colonic expression of cytokines and chemokines, such as interleukin 6 (*Il6*), *Il17A*, chemokine (C-X-C motif) ligand 2 (*Cxcl2*), and C-C motif chemokine ligand 2 (*Ccl2*) was determined by qRT-PCR. The primers sequences of these genes were shown in (**Table 2.3**). Total RNA from mouse colonic tissues was isolated using a NucleoSpin® RNA kit (Macherey-Nagel, Duren, Germany) and reverse-transcribed into cDNA using the ReverTra Ace qPCR RT Master Mix kit (Toyobo, Osaka, Japan), according to the manufacturers' instructions. The PCR reaction was performed in a StepOne Real-Time PCR System (Thermo Fisher Scientific) with 2x Brilliant III Ultra-Fast SYRB Green qPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) in accordance with the manufacturer's protocol. The expression of the target genes in each sample set was calculated by the delta delta Ct method. Change in the mRNA expression levels were determined after normalized to ribosomal protein L13 (*Rpl13*) gene expression which served as an internal control.

Table 2.3 Primer sequences for qRT-PCR

	Forward	Reverse
mIl6	TCCATCCAGTTGCCTTCTTG	CATTTCCACGATTTCCCAGAG
mIl17a	AGCTGGACCACCACATGAAT	ACACCCACCAGCATCTTCTC
mCxcl-2	AGTGAACTGCGCTGTCAATG	ACTTTTTGACCGCCCTTGAG
m Ccl2	GGAATGGGTCCAGACATACATTA	TAGCTTCAGATTTACGGGTCAAC
mRpl13	TGGTTGTCACTGCCTGGTACTT	CCTGCTGCTCTCAAGGTTGTT

2.2.9 Fecal organic acid analysis

Fresh fecal samples were collected 4 days after the DSS administration to determine organic acid, with minor modifications (Han *et al.*, 2015). Briefly, fecal samples were diluted and homogenized with a 9 times volume of distilled water. The supernatant obtained after centrifugation was deproteinized with 62.5 % acetonitrile. Organic acids were chemically derivatized by 3-nitrophenylhydrazine to their 3-nitrophenyhydrazones.

Crotonic acid was used as an internal standard. Derivatives of the organic acids were determined by liquid chromatography tandem mass spectrometry (Waters, Germany).

2.2.10 Histopathology

Mouse colon tissues were embedded in optimal cutting temperature (OCT) compounds (Sakura Finetek Japan, Tokyo, Japan) and frozen tissue sections (8 µm) were prepared on glass slides using LEICA CM1850 microtome (Germany). Sections were fixed in 4 % (w/v) paraformaldehyde and were stained with Mayer's Hematoxylin and Eosin, as previously described (Mayangsari and Suzuki, 2018). Sections were dehydrated in gradient alcohol and preserved with Eukitt Cover (Sigma). Tissue sections were visualized on microscope (Leica, Wetzlar, Germany).

2.2.11 Immunofluorescence analysis in mouse colon tissues

The tight junction proteins, claudin-3 and claudin-7, in mouse colonic tissues were analyzed by immunostainning. Briefly, the mouse colon was embedded in OCT compound and frozen tissue sections (8 µm) were prepared on glass slides using LEICA CM1850 microtome (Germany). Colon sections were fixed with 4 % paraformaldehyde for 10 min at room temperature. The sections were blocked with 5 % normal goat serum in 4 % skimmed milk for 30 min. The specimens were incubated with corresponding primary antibodies at 4 ^{o}C for overnight in a humidifying box. The specimens were incubated with goat-anti-rabbit-IgG conjugated with AlexaFluor488 and DAPI (4′,6-diamidino-2-phenylindole) for 1 hr in a humidifying box at dark place. The specimens were preserved in a mounting fluid, and the fluorescence was visualized with LCM 700 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) (Kawabata *et al.*, 2018).

2.2.12 Statistical analysis

All data are expressed as mean with standard error of mean. Statistical analyses were performed using Predictive Analytics Software (PASW) Statistics 18. Statistical differences among groups were determined by one-way analysis of variance (ANOVA)

followed by a Tukey-Kramer test. Differences were considered significant at a $p < 0.05$. The sample size was calculated using the POWER procedure for one-way ANOVA, considering $p < 0.05$ with a power of 0.80 (SAS Institute, Cary, NC, USA) and using the results of our previous studies.

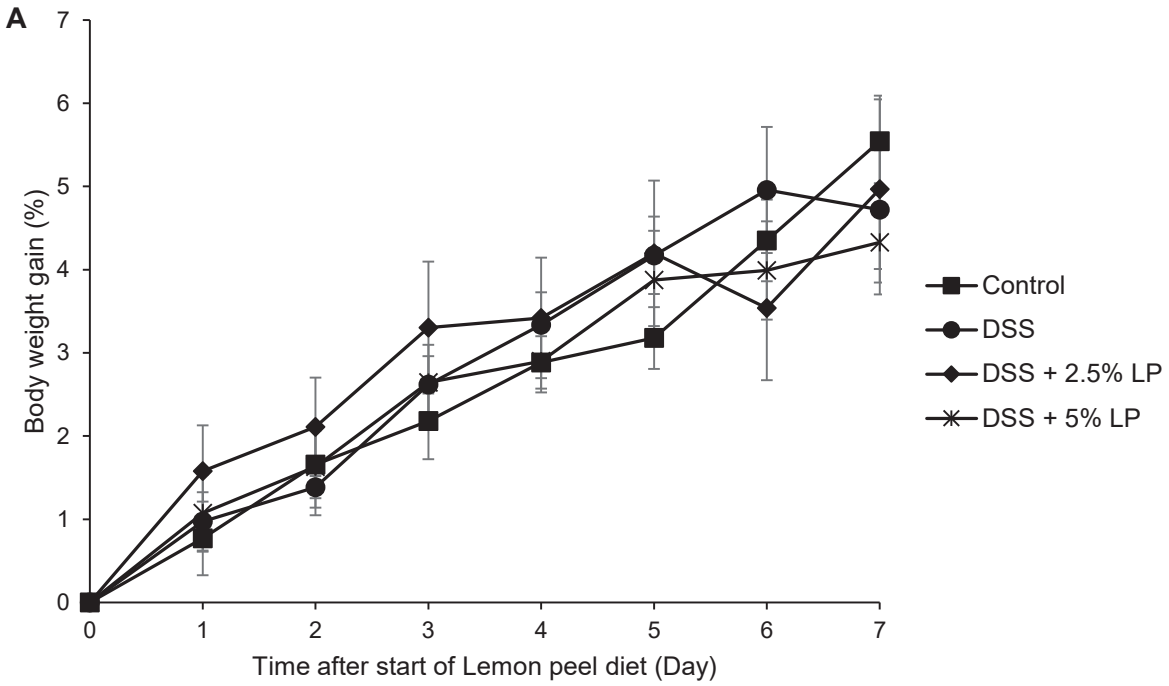
2.3 Resutls

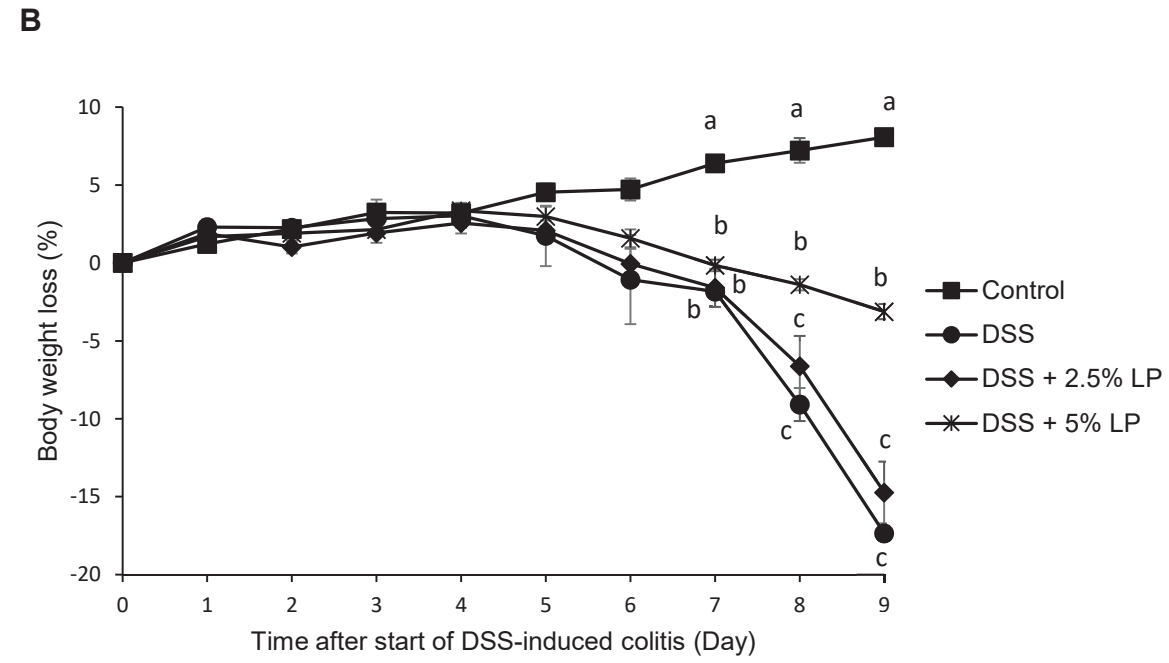
2.3.1 Effect of LP diet on body weight and colitis clinical score

Mice in the two groups were pre-fed with the LP diets containing LP powder at 2.5% and 5%, respectively for 7 days before induction of colitis. The LP diets did not influence the percentage of body weight gains in the 7 days (**Figure 2.1A**), indicating the safety for mice. At and after day 7, the body weight loss was evident in the DSS group compared to the control group, suggesting that the DSS administration successfully induced the colitis (**Figure 2.1B**).The body weight in the DSS + 5% LP group were higher than those in the DSS group at days 8 and 9.

To further assess the severity of colitis, the DAI score based on stool consistency, stool bleeding, and body weight loss were examined (**Figure 2.1C**). The DAI score in the control group remained at 0 throughout the experiment. The DAI scores in the DSS + 5% LP group at and after day 4 were lower or tended to be lower than those in the DSS group. The DAI score in the DSS + 2.5% LP groups at day 8 was lower than that of the DSS group.

The colon length is known to be another indicator of colitis. As shown in **Figure 2.1D**, the colon length was shortened by 29% by DSS administration. Feeding the 5% LP diet prevented the shortening of the colon.


A
Body weight gain (%)
Time after start of Lemon peel diet (Day)
Control
DSS
DSS + 2.5% LP
DSS + 5% LP


B
Body weight loss (%)
Time after start of DSS-induced colitis (Day)
Control
DSS
DSS + 2.5% LP
DSS + 5% LP

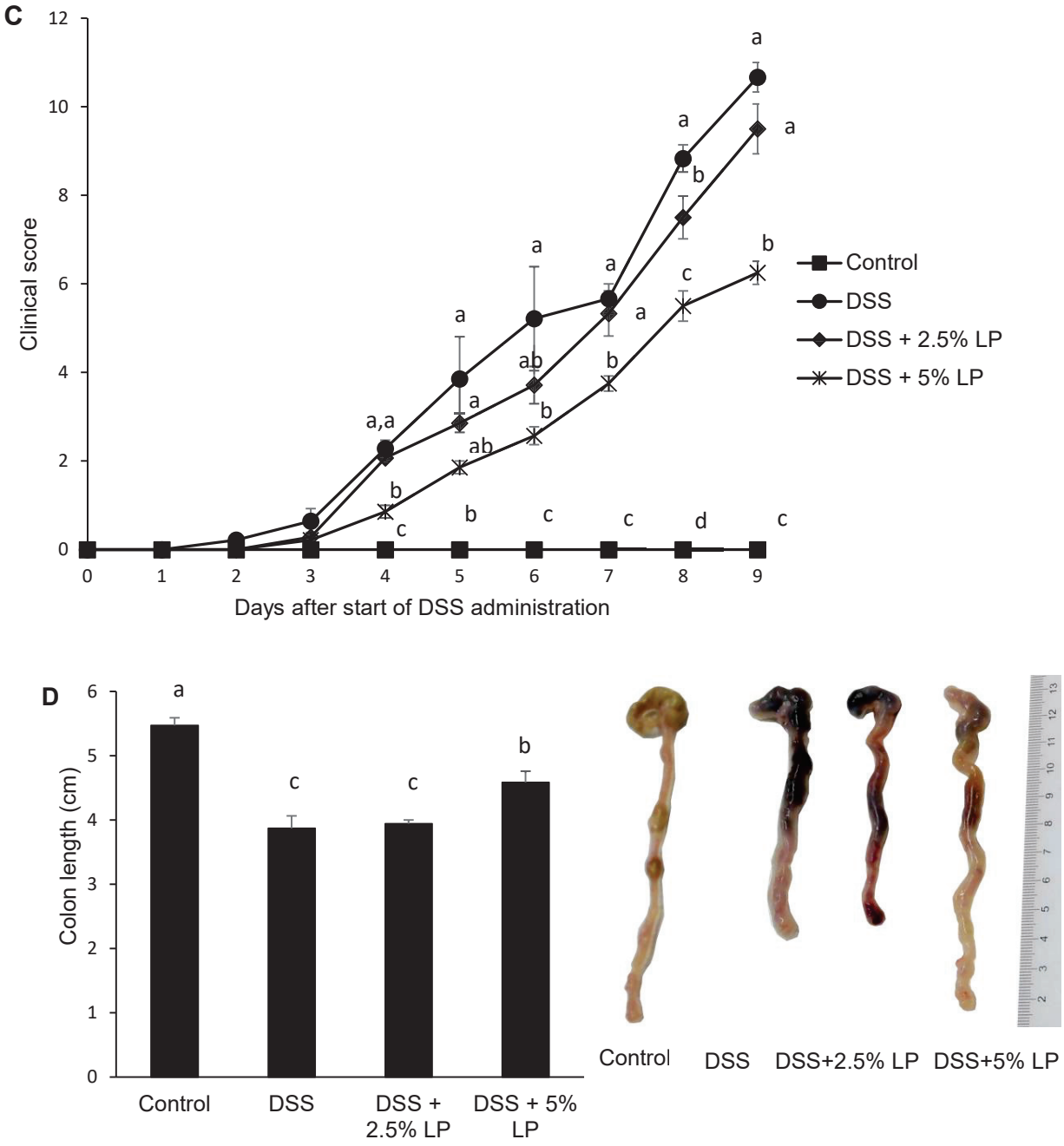


Figure 2.1 Effects of LP powder on body weight gain and clinical score in dextran sodium sulfate (DSS)-induced colitis mice. Body weight changes before (A) and after (B) DSS administration, clinical score (C), and colon length (D) of mice fed diets with and without LP powder, with or without DSS administration. Values are the mean ± SEM (n=7). Means without a common letter differ, $p < 0.05$

2.3.2 Effect of LP diet on improvement epithelial barrier function

To investigate the mechanisms underlying the LP-mediated enhanced of intestinal epithelial barrier function, we performed the immunoblot analysis of occludin, ZO-1, ZO-2, claudin-3, claudin-4, and claudin-7. Tight junction protein plays a crucial role in maintaining intestinal barrier integrity. The results demonstrated that the colonic expression of tight junction protein decreased by DSS administration (**Figure 2.2**). Remarkably, the LP powder diet attenuated the decreased expression of some tight junction proteins examined. Among them, claudin-3, claudin-4, claudin-7, occludin, and ZO-2 protein expression levels in the DSS + 5 % LP group were higher compared to the DSS group. Although the 2.5 % LP diet tended to restore the claudin-3 and claudin-7 expressions, the restoration was not statistically significant.

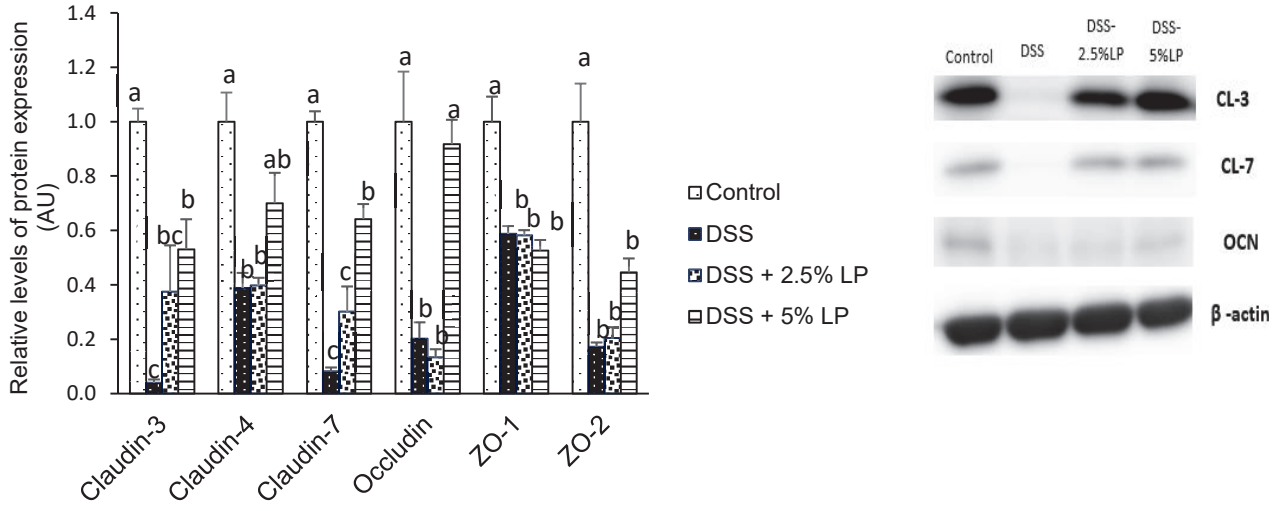


Figure 2.2 Effects of LP powder on tight junction protein expression in the colon of dextran sodium sulfate (DSS)-induced colitis mice. Protein expression of zonula occludens (ZO)-1, ZO-2, occludin, claudin-3, claudin-4, and claudin-7 in the colon of mice fed diets with and without lemon peel powder, with or without DSS administration, as determined by immunoblot analysis. Values are the mean ± SEM (n=7). Means without a common letter differ, $p < 0.05$. AU, arbitrary unit.

The intercellular localization and expression of the tight junction protein in the colon were visualized by immunofluorescence (**Figure 2.3**). Tight junction proteins were observed in the epithelial cells of the colons with different patterns of expression and localization throughout the crypts in control mice. Claudin-3 were highly expressed in the intercellular junction of the epithelial cells located on the luminal surface and in the upper crypts. Claudin-7 were also observed in the basolateral membrane of the epithelial cells. The DSS administration severely impaired the expression and localization of these tight junction proteins. The colons of mice in the DSS + 5 % LP group showed relatively intact expression and localization of the tight junction proteins in comparison with those in the DSS group. Accordingly, the lemon peel supplement partially restored the tight junction structure in the colon. These results were consistent with those of the immunoblot analysis of tight junction protein shown in **Figure 2.3**.

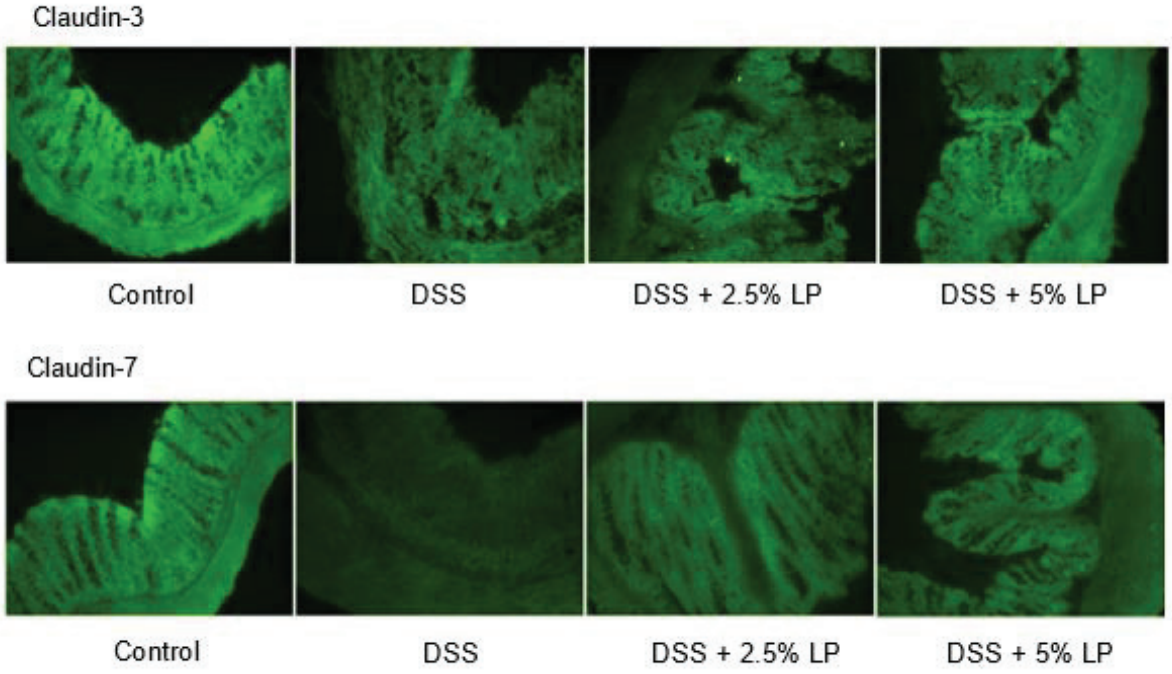


Figure 2.3 Effects of LP powder on claudin-3, and claudin-7 expression in the colon of dextran sodium sulfate (DSS)-induced colitis mice. Immunolocalization of claudin-3 and claudin-7 in the colon of mice fed diets with and without LP powder, with or without DSS administration, as analyzed by immunofluorescence microscopy. Representative images of seven mice in each group are shown.

2.3.3 Effect of LP powder on mRNA expression of inflammatory mediators

In IBD, the altered levels of inflammatory cytokines often correlate with the severity of disease symptoms. The qRT-PCR analysis was employed to examine the expression of inflammatory mediators in the colon of mice. As shown in **Figure 2.4**, the DSS administration significantly increased the gene expression of the inflammatory mediators the *Ccl2*, *Il6*, and *Cxcl2* in comparison to those in the control group, up 250-, 500-, and 40-fold, respectively. Interestingly, the 5 % LP supplement significantly reduced the *Il6* and *Cxcl-2* levels (**Figure 2.4A and 2.4B**). Similar to *Il6* and *Cxcl-2*, the gene expression level of *Il17a* and *Ccl2* were downregulated by 5 % LP supplement although the reduction were not significant (**Figure 2.4C and 2.4D**).

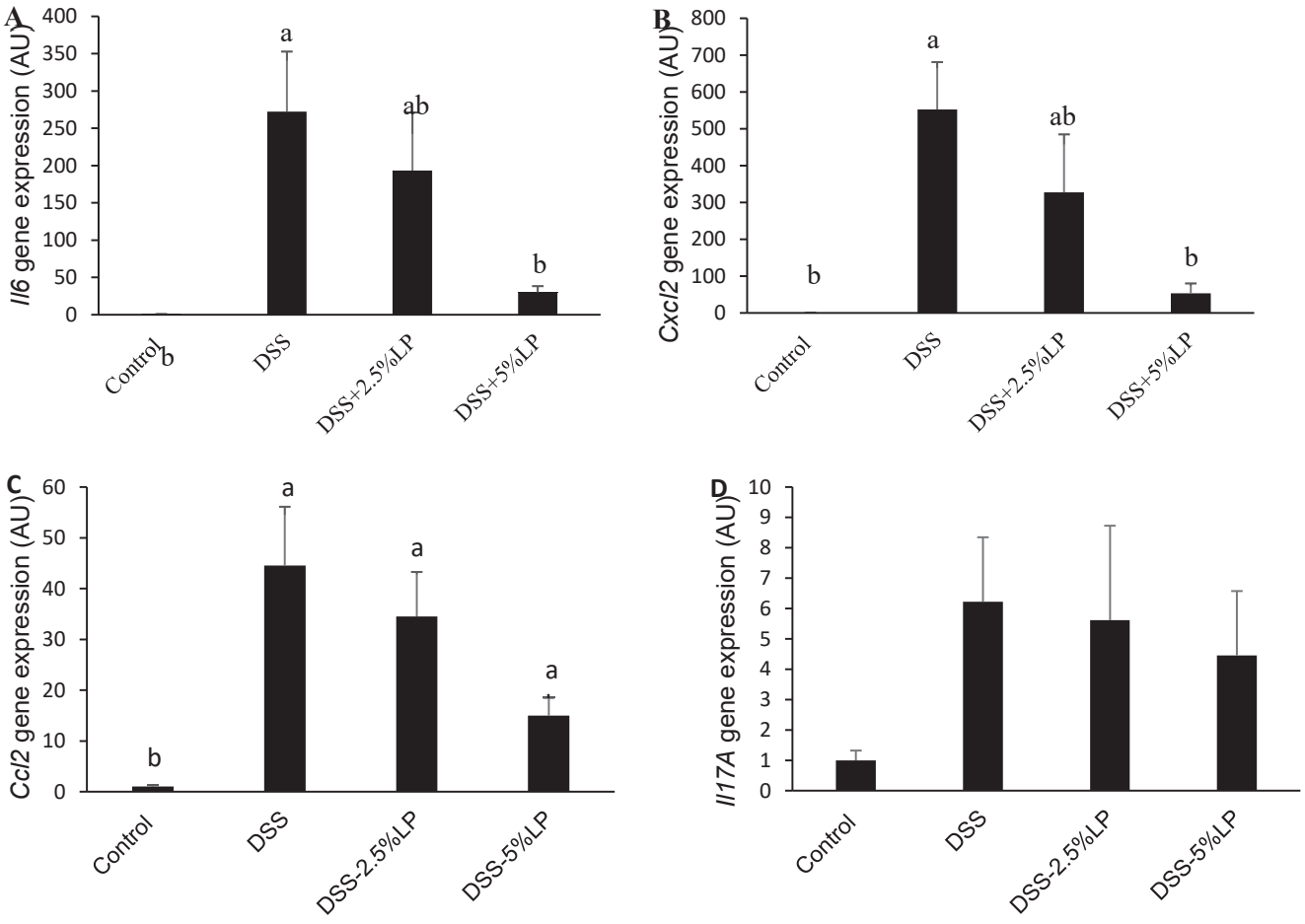


Figure 2.4 Effects of LP powder on inflammatory cytokines expression in the colon of dextran sodium sulfate (DSS)-induced colitis mice. Gene expression of *Il6* (A), *Cxcl-2* (B), *Ccl2* (C), and *Il17A* (D) in the colon mice fed diets with and without LP powder, with or without DSS administration, as determined by quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis. Values are the mean ± SEM (n=7). Means without a common letter differ, $p < 0.05$. AU, arbitrary unit.

2.3.4 Histopathological analysis of DSS-induced colitis

Histological examination of colons in DSS-induced colitis mice showed mucosal thickening, loss of crypts, and an increase in infiltrating lymphocytes (**Figure 2.5**). These abnormalities were absent in the control group. The administration of LP powder at 2.5 % and 5 % improved the mucosal structure, indicated by the decreased lymphocytes and

mucosal erosion. However, the ameliorative effects were more evident in the DSS + 5 % LP than in the DSS + 2.5 % LP. These findings suggested the potential of LP powder to reduce the IBD.

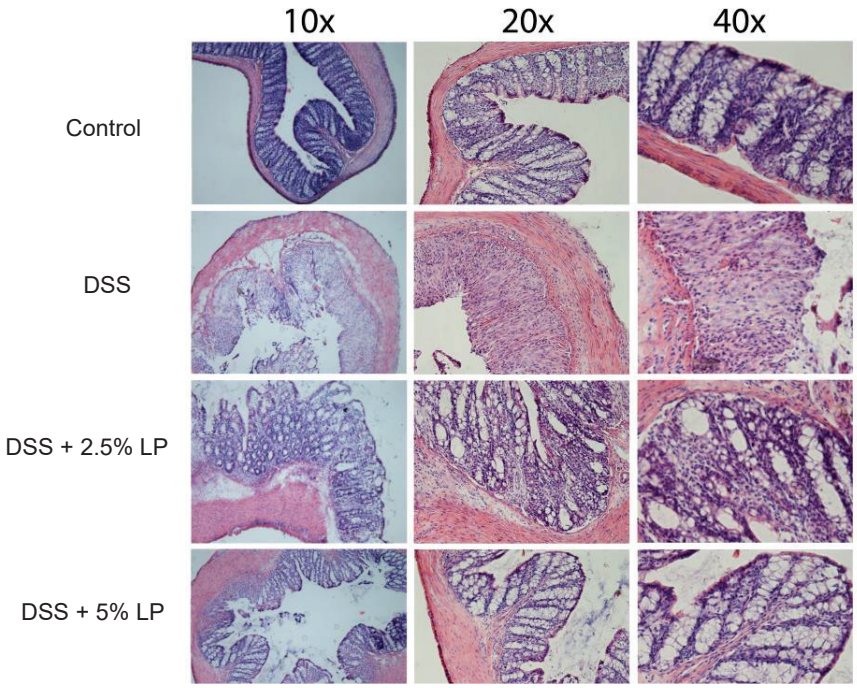


Figure 2.5 Effects of LP powder on mucosal structure in dextran sodium sulfate (DSS)-induced colitis mice. Mucosal structure of mice fed diets with and without LP powder, with or without DSS administration. Colonic sections of mice were stained with hematoxylin and eosin. Representative images of seven mice in each group are shown. Images were taken using a digital inverted microscope with a 10X, 20X, and 40X objective, respectively.

2.3.5 Effect of LP powder on fecal organic acids

SCFAs are major metabolites of undigested carbohydrates including dietary fibers by intestinal microorganisms. These acids have crucial functions in colonic homeostasis. The acetic, propionic and iso-butyric acids were lower in the DSS group compared to those in control group although the reductions were not significant because of big deviations.

The LP powder at 2.5 % and 5 % restored or tended to restore the decreased acetic acid and iso-butyric acid.

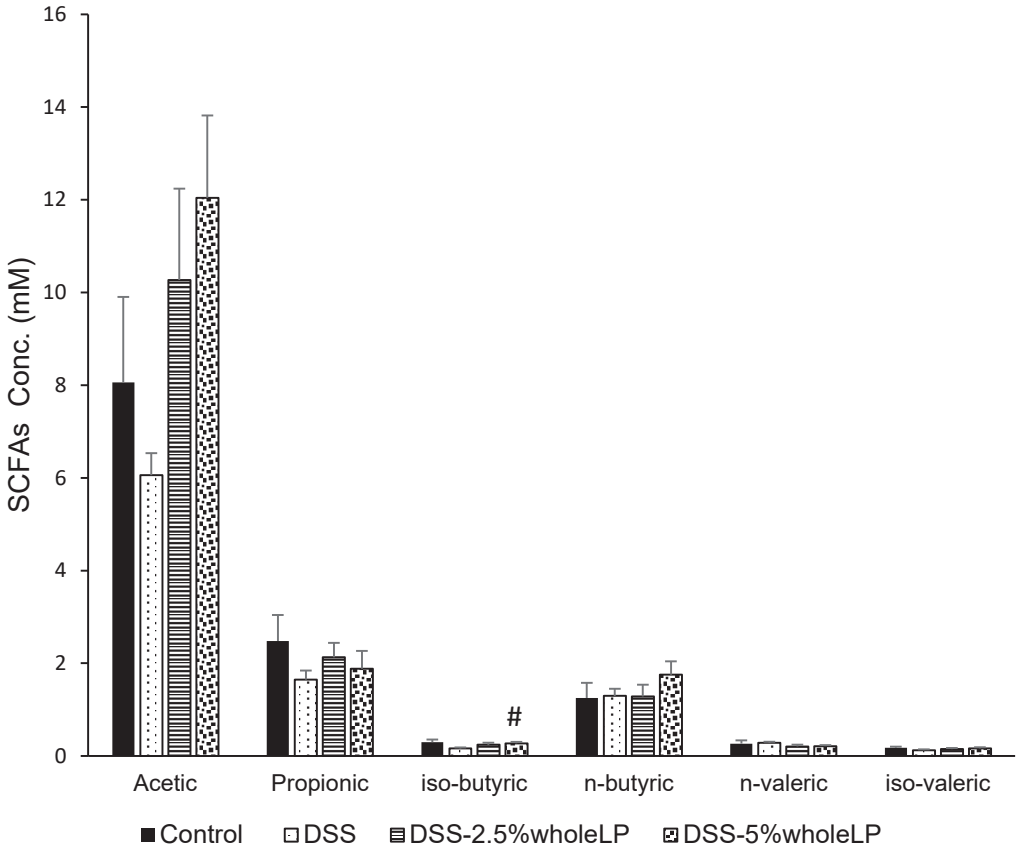


Figure 2.6 Effects of LP powder on SCFA acids concentrations in feces of dextran sodium sulfate (DSS)-induced colitis mice. Fecal concentrations of acetate, propionate, n-butyrate, iso-butyrate, n-valerate, and iso-valerate in mice fed diets with and without LP powder, with or without DSS administration, as determined by liquid chromatograph/tandem mass spectrometry (LC/MS/MS) analysis. Values are the mean ± SEM (n=7). Means without a common letter differ, $p < 0.05$.

2.4 Discussion

IBD is a chronic and debilitating condition of the intestines that is becoming more common in the world (Isidro *et al.*, 2017). The increasing global burden of IBD during the next decade will require a two-pronged solution that involves the basic research into the

clinical interventions to prevent IBD and innovations in the delivery of care to patients with IBD (Langholz, 2010). The DSS-induced colitis mouse model is often used in the IBD research, because the mice exhibit the disease symptoms similar to human IBD. The precise mechanisms by which DSS induces intestinal inflammation is still unclear, but it is likely that the result of damage to the epithelial barrier of the large intestines allowing the permeation of luminal pro-inflammatory molecules into the mucosa trigger the immune activation (Chassaing *et al.*, 2015). To examine the potential of LP powder against the IBD, we have characterized the effects of LP powder in the DSS-colitis mice. The DSS administration in mice clinically induced the bloody stool, diarrhea stool, as well as body weight loss and colon shortening. These results are similar to previous studies from other research groups as well as in our laboratory (Cho *et al.*, 2011; Hung and Suzuki, 2016; Ogata *et al.*, 2017; Kawabata *et al.*, 2018; Li *et al.*, 2018; Mayangsari and Suzuki, 2018; Huynh *et al.*, 2019). The histological examination indicated the marked mucosal destruction and infiltration of inflammatory cells in the involved colonic segment indicating robust activation of immune response, resulting in the injured colon.

Tight junction structure provides a physical barrier against pathogens and has an important role in maintaining intestinal homeostasis. The tight junction barrier disruption is closely associated with the pathogenesis of various intestinal and systemic inflammatory diseases including the IBD. Previous studies demonstrate that altered intestinal tight junction protein expression and redistribution such as occludin and claudin family are observed in the involved segment of IBD (Vetrano *et al.*, 2008; Suzuki, 2013). In the present study, we investigated whether the dietary LP powder suppresses the intestinal inflammation and tight junction disruption in the DSS-induced colitis mice. The results suggested that the reduction of colitis by LP powder is involved in the restored tight junction protein including occludin, claudin-3, and claudin-7.

Inflammatory cytokines are indispensable signals in the mucosa-associated immune system for maintaining normal intestinal homeostasis (Műzes *et al.*, 2012). Some studies reported that the roles of pro-inflammatory cytokines such as IL-6, -8, -17, and -1β, and IFN-γ in the DSS-induced colitis as well as IBD such as UC and CD (Pullman *et al.*, 1992;

Ardozzone and Porro, 2005). We determined the colonic pro-inflammatory cytokines gene expression such as *Il6*, *Cxcl-2*, *Ccl2*, *Il17a* (Fig.4), and *Il10* (the result is not shown). The administration of LP powder at 5 % reduced these cytokines expression (but the reduction of *Ccl2* and *Il17A* were not significant). Our results suggest that the protection of tight junction barrier by LP powder is involved in the reductions of cytokines expression.

The next question is which components in the LP powder are the mediators in the reduction of colitis. The LP powder is known to be rich in dietary fiber and polyphenolic compound. Then, we determined the fecal SCFAs which are the metabolites of dietary fiber by intestinal microorganisms. It has been demonstrated that SCFAs reinforce the tight junction integrity and suppress the inflammatory reaction in the intestines (Venegas *et al.*, 2019). Feeding the LP powder increased or tended to increase some SCFAs including acetate, n-butyrate and iso-butyrate. On the other hand, some polyphenol such as nariagin and hesperitin, which present in the LP powder, have been reported to reduce the experiment of inflammation and colitis (Xu *et al.*, 2009; Salaritabar *et al.*, 2017; Polat *et al.*, 2018). Further studies are needed to reveal the active components in the LP powder.

In summary, the results in this study show that the LP powder reduces the DSS-induced colitis in mice. This reduction is possibly involved in the restored tight junction barrier and suppressed cytokines in the colon. The SCFAs are possibly ones of the mediators in the effect of LP powder, but further studies should be required to understand the underlying mechanism. Therefore, I planned to examine effects of different fractions of LP powder in the next experiment.

Chapter 3
BIOACTIVE COMPONENT RESPONSIBLE FOR THE LEMON PEEL-MEDIATED REDUCTION OF COLITIS

3.1 Introduction

In chapter 2, our study demonstrated that feeding LP powder reduced the intestinal inflammation and barrier defect in a murine model of colitis. Next question is which components in the LP powder responsible for the effect. Structurally, the LP consists of the outer layer, called flavedo, and the inner layer, called albedo. The flavedo contains high amounts of polyphenolic compounds, such as hesperidin, diosmin, eriocitrin, and narirutin whereas the albedo is rich in dietary fibers, particularly pectin. Many studies demonstrated the beneficial effect of polyphenols in different diseases including inflammatory diseases. Whereas, dietary fibers such as pectin are also known to exhibit the beneficial effect on the intestinal health. In addition, the SCFAs produced from the intestinal fermentation of dietary fibers have essential roles in the intestinal homeostasis. Accordingly, we hypothesized that either or both polyphenols or dietary fibers is responsible for the LP-mediated reduction of colitis.

The objective of this study was to reveal which components are the active components in LP powder, polyphenols or dietary fibers. We separated the LP powder into two fractions, which were rich in polyphenolic compounds and dietary fibers. The effects of the two LP fractions in the experimental colitis mice was examined.

3.2 Materials and methods

3.2.1 Chemicals

Dextran sulfate sodium (DSS; molecular weight: 36,000-50,000) was purchased from MP Biomedicals (Santa Ana, CA, USA). Rabbit anti-ZO-1 (61-73000), occludin (71-1500), claudin-3 (34-1700), claudin-4 (36-4800), claudin-7 (34-9100), and goat Alexa Fluor 488-conjugated anti-rabbit IgG antibodies (A11034) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Rabbit anti-ZO-2 antibody was purchased from

Santa Cruz Biotechnology (sc-11448, Dallas, TX, USA). Horseradish peroxidase-conjugated anti-rabbit IgG antibody was purchased from SeraCare (074-1506, Milford, MA, USA). All other chemicals were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

3.2.2 Preparing the fraction of methanolic extract of *Citrus limon* peel powder

Fresh *Citrus limon* peel was purchased from Pokka Sapporo Food & Beverage (Tokyo, Japan). The whole dry LP powder was prepared as shown in 2.2.2.

Whole LP powder contained polyphenols and dietary as major bioactive components at high concentrations. To examine which component have a role in the ameliorative effect on colitis, whole LP powder was separated into two fractions rich in polyphenols and fibers, respectively, using a methanol (MetOH) extraction method. Whole LP powder (45 g) was vigorously mixed with 300 mL MetOH, sonicated using an ultrasonic bath (Bransonic 1210; Emerson Electric, St. Louis, MO, USA) for 60 min, and allowed to stand for 16 hr. The mixture was centrifuged at 10,000 x g at 15 ºC for 20 min to allow the separation into supernatant and pellet. The pellet was immersed in 300 mL MetOH, vigorously mixed, and centrifuged again. Two additional extractions were performed in the same manner. The pellet obtained through the repeated extractions was air-dried, milled to a fine powder, and designated as the MetOH extraction residue (MetOH residue) of LP powder. The supernatant obtained through the repeated extractions was filtered and evaporated under vacuum at 50 ºC. The resultant concentrate was dispersed in 45 g of maize starch powder for a faithful handling, air-dried, and designated as MetOH extract of LP powder.

3.2.3 Nutrient components analysis

The nutritional components, including protein, lipid, ash, and moisture in the whole LP powder and the MetOH residue were analyzed as described in 2.2.3. The fiber content in whole LP powder and MetOH residue was determined using the enzymatic-gravimetric method (AOAC 985.29 and 991.43) (McCleary *et al.*, 2012). Because LP powder is rich in

polyphenols, such as hesperidin, eriocitrin, diosmin, and narirutin (Del Río *et al.*, 2004; Xi *et al.*, 2017), the polyphenols in whole LP powder and MetOH extract were determined using liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis (Acquity UPLC-TQD; Water, Milford, MA, USA). Confirmatory analyses were equipped with the collision energy voltages in the range 2 – 100 eV and were applied the collision energy range 20 – 80 as well as the instrument was operated in negative electrospray ionization mode. Nitrogen was used in both for desolvation gas (650 L/hr) and cone gas (35 L/hr). Desolvation and source temperature were set at 450 ºC and 150 ºC, respectively. Data processing were performed by the MassLynx software ver 4.1.

3.2.4 Animals

All study protocols were preapproved by the Animal Use Committee of Hiroshima University (authorization No. C19-19), and animal experiments were performed in accordance with Hiroshima University guidelines for the care and use of laboratory animals. Male 7-week old Balb/c mice were purchased from Charles River Japan (Yokohama, Japan). All mice were housed under conditions with a controlled room temperature (20 - 24 ºC), relative humidity (40 - 60 %), and lighting from 8:00 to 20:00 throughout the experimental period. The mice were allowed to acclimate for 7 days with the AIN-93G-based control diet (Reeves, Nielsen and Fahey, 1993) and water *ad libitum*, prior to the start of the experiment.

3.2.5 Diet preparation

Whole LP powder, MetOH extract, and MetOH residue were added to the AIN-93G-based control diet at 5, 6.5, and 4.4% by weight, respectively. LC/MS/MS analysis demonstrated that the recovery ratio of hesperidin, eriocitrin, and diosmin, major polyphenols in the whole LP powder, in the MetOH extract was approximately 97, 77, and 89 %, respectively, of those from the original whole LP powder **(Table 3.2)**. To ensure that these three polyphenols were present at concentration at least equivalent to those in the 5 % whole LP powder diet, the MetOH extract was added to the control diet at 6.5 %.

Additionally, the fiber content in the MetOH residue increased to 1.13-fold that in the original powder; thus, to ensure that concentration was approximately equivalent to that in the 5 % whole LP powder diet, the MetOH residue was added to the control diet at 4.4 %.

3.2.6 Experimental design

Mice (n=35) were randomly divided into five groups: the control, DSS, DSS + whole LP, DSS + MetOH extract, and DSS + MetOH residue (seven mice/group). The control and DSS groups were fed the control diet for the 16 days experimental period. The DSS + whole LP, DSS + MetOH extract, and DS + MetOH residue groups were fed the diets containing 5 % whole LP powder, 6.5 % MetOH extract, and 4.4 % MetOH residue by weight, respectively, through the experimental period. Seven days after the start of feeding the experimental diets, four DSS treatment groups were administered 2% (w/v) DSS solution through their drinking water for 9 days to induce experimental colitis, whereas the control group only received distilled water. After DSS administration for 9 days, mice were euthanized by exsanguination under isoflurane anesthesia. The colon was quickly dissected and colon length was measured. The colon was isolated for histological, immunoblot, quantitative reverse transcription-polymerase chain reaction (qRT-PCR), and immunostaining analyses, as described below.

3.2.7 Macroscopic indicators

Disease Activity Index (DAI) were evaluated daily as described in 2.2.6.

3.2.8 Immunoblot analysis

Tight junction protein were evaluated by Western bloting as described in 2.2.7

3.2.9 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Cytokines gene expression were evaluated by qRT-PCR as described in 2.2.8

3.2.10 Fecal organic acid analysis

Fecal organic acids were evaluated by LC/MS/MS as described in 2.2.9

3.2.11 Histopathology

Histological examination of colons were stained with Mayer's Hematoxylin and Eosin as described in 2.2.10

3.2.12 Immunofluorescence analysis in mouse colon tissues

Cellular localization of claudin-3 was examined by immunofluorescent staining as described in 2.2.11

3.2.13. Statistical analysis

All data are expressed as mean with standard error of mean. Statistical analyses were performed using Predictive Analytics Software (PASW) Statistics 18. Statistical differences among groups were determined by one-way analysis of variance (ANOVA) followed by a Tukey-Kramer test. Differences were considered significant at a $p < 0.05$. The sample size was calculated using the POWER procedure for one-way ANOVA, considering $p < 0.05$ with a power of 0.80 (SAS Institute, Cary, NC, USA) and using the results of our previous studies.

3.3 Resutls

3.3.1 Phytochemical characterization of LP powder and its fractions

We analyzed the nutrition profile of whole LP powder and two fractions that were reported in (**Table 3.1)**. Some studies reported that LP was abundant in dietary fiber which was the major material to produce industrial pectin (CP Kelco A Huber Company, 2005; Russo *et al.*, 2014b) as well as phenolic compounds such as hesperidin, eriocitrin, diosmin, and narirutin (Nogata *et al.*, 2006; Russo *et al.*, 2014b; Xi *et al.*, 2017). The sum of protein, lipid, ash, and moisture was 15.9% by weight in whole LP powder. The amount of total dietary fiber in whole LP powder was 47.1%, which included water-soluble fiber at 12.3% and water-insoluble fiber at 34.8%. In agreement with the previous studies (Del Río *et al.*,

2004; Xi *et al.*, 2017), hesperidin, eriocitrin, and diosmin were found at high amounts in whole LP powder. Protein, lipid, and ash were mostly retained in the MetOH residue of LP powder, and the values were roughly similar to those in whole LP powder. The total dietary fiber amount in the MetOH residue was 53.1%, which was 1.13 times higher than that of the original whole LP powder. Protein, ash, and fiber were not analyzed in the MetOH extract, because these nutrients were not extracted by MetOH. The hesperidin amount in the MetOH extract was equivalent to that in the whole LP powder, but the amounts of eriocitrin, diosmin, and narirutin were 77, 89, and 51%, respectively, of those in whole LP powder. Our analytical results were used to design the experimental diets.

Table 3.1 Nutritional composition of whole lemon peel (LP) powder, MetOH extract, and MetOH residue

	Whole LP	MetOH extract	MetOH residue
Protein (g/100g powder)	0.7	N.A.	07
Lipid (g/100g powder)	4.3	N.A.	5.8
Ash (g/100g powder)	3.9	N.A.	4.0
Dietary fiber (g/100g powder)	47.1	N.A.	53.1
Water soluble	12.3	N.A.	17.8
Water insoluble	34.8	N.A.	35.3
Moisture	7.0	N.A.	9.2
Polyphenols (mg/100g powder)			
Hesperidin	427	423	N.D.
Eriocitrin	174	134	N.D.
Diosmin	146	130	N.D.
Narirutin	26.5	13.5	N.D.

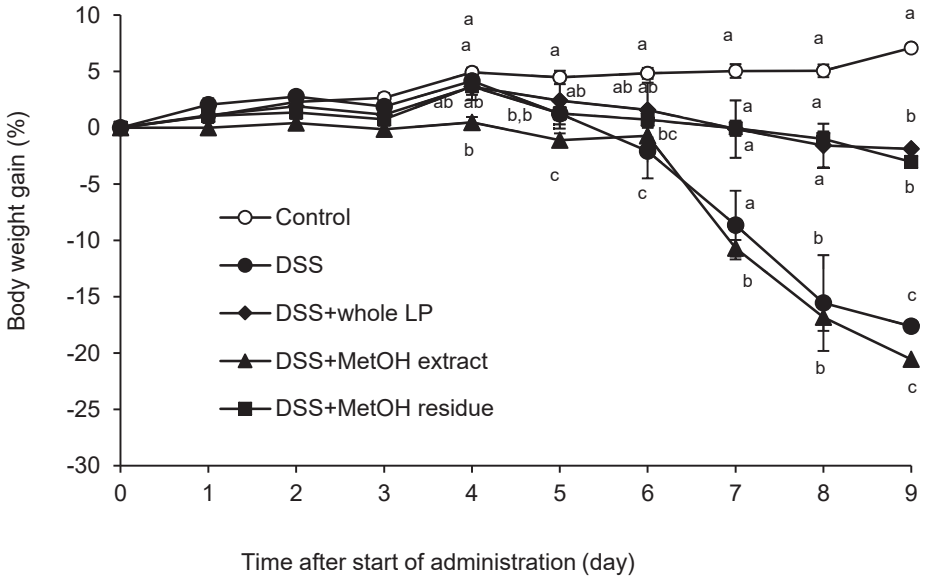
(N.A: not analyzed, N.D: not detected)

3.3.2 Effects of whole LP powder and its fractions on body weight, colitis clinical score and colon length

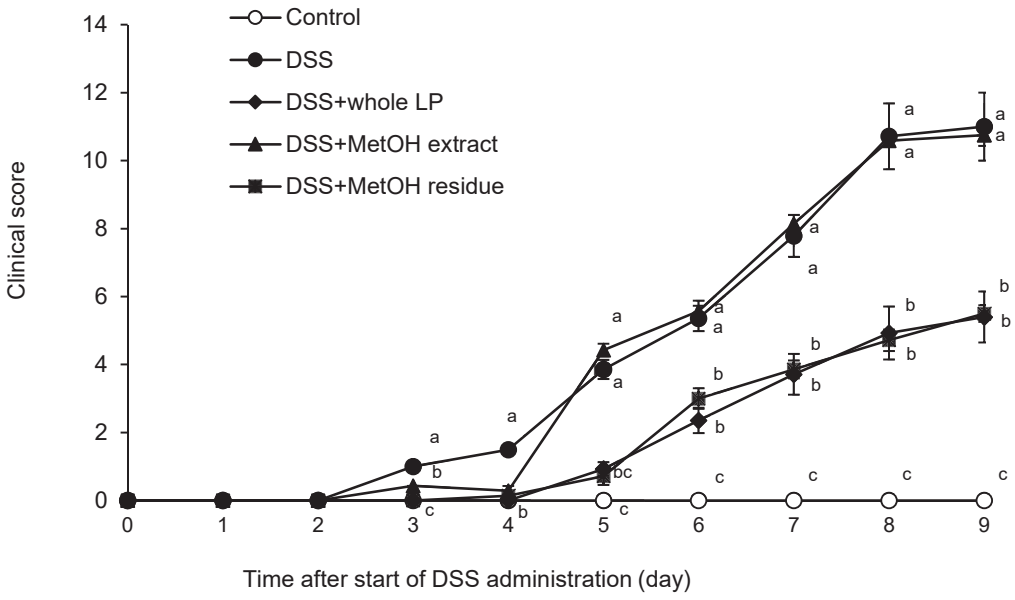
To reveal which components were responsible for the effect of LP powder, the DSS-induced colitis mice were fed with the MetOH extract (rich in polyphenols) and MetOH extraction residues (rich in dietary fibers) as well as the whole LP poweder. The DSS administration reduced the body weight and increased the DAI in a manner similar to the previous results in Chapter 2 (**Figure 3.1A, B**). Body weight gain in the DSS group was lower than those in the control group at, and after, day 4. Feeding mice whole LP powder and MetOH residue attenuated body weight loss at, and after, day 6. The clinical score in the DSS group was higher than that in the control group at, and after, day 3, and the increments were partially attenuated by feeding mice whole LP powder and MetOH residue. The clinical score in the MetOH extract group was lower than that in the DSS group at days 3 and 4; however, the ameliorative effects on body weight gain and clinical score were not observed.

Colon length is inversely associated with the severity of DSS-induced colitis. Shortening of colon was observed in the DSS group as compared with control group (**Figure. 3.1C**). Feeding mice with whole LP powder and MetOH residue attenuated this colon shortening.

A


Body weight gain (%)
Time after start of administration (day)
Control
DSS
DSS+whole LP
DSS+MetOH extract
DSS+MetOH residue

B


Clinical score
Time after start of DSS administration (day)
Control
DSS
DSS+whole LP
DSS+MetOH extract
DSS+MetOH residue

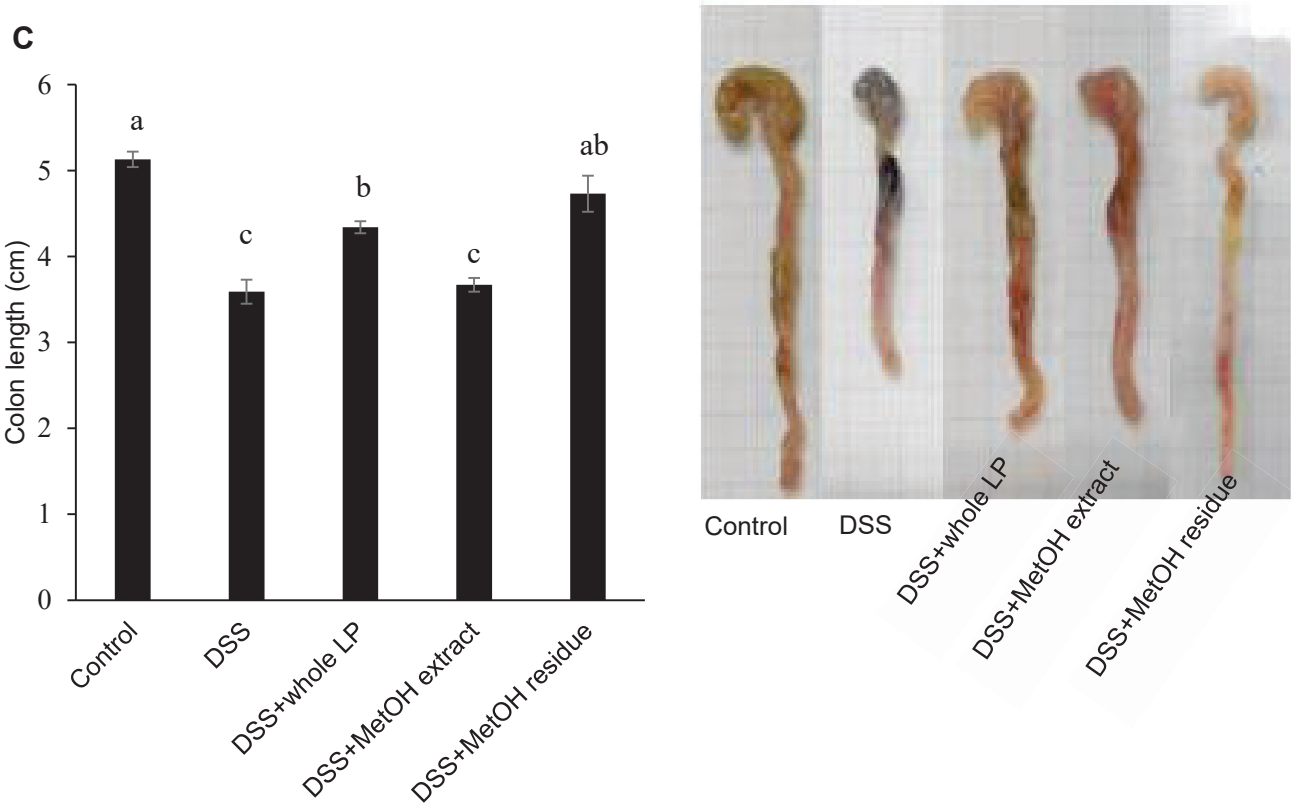


Figure 3.1 Effects of whole LP powder, methanol (MetOH) extract, and MetOH extraction residue (MetOH residue) on body weight gain and clinical score in dextran sodium sulfate (DSS)-induced colitis mice. Body weight gain (A) and clinical score (B), colon length (C) of mice fed diets with and without whole LP powder, MetOH extract, and MetOH residue, with or without DSS administration. Values are the mean ± SEM (n=7). Means without a common letter differ, $p < 0.05$.

3.3.3 Effect of whole LP powder and its fractions on the colonic tight junction barrier

An impaired intestinal tight junction barrier has a role in the pathogenesis of IBDs (Farhadi *et al.*, 2003). The expression of colonic tight junction protein was shown in **Figure. 3.2**. The colonic expression of ZO-1, ZO-2, claudin-3, and claudin-7 in the DSS group was lower than those in the control group. DSS administration reduced occludin expression, but the reduction was not statistically significant. Feeding mice whole LP

powder restored the decreased expression of these tight junction proteins, although the restoration in claudin-7 expression was not significant.

Feeding mice MetOH residue also restored ZO-2, claudin-3, and claudin-7 expression. Meanwhile, any tight junction proteins examined in the DSS + MetOH extract group did not differ from those in the DSS group. Because claudin-3 is one of the major isoform expressed in the colonic epithelium and whole LP powder and MetOH residue significantly restored its expression, its cellular localization was examined by immunofluorescence analysis (**Figure 3.3**). In the control group, claudin-3 was observed at basolateral, as well as in the junctional regions of epithelial cells. DSS administration impaired expression and localization, while feeding mice whole LP powder and MetOH residue, but not the MetOH extract, partially, but clearly, restored them. These data suggest that the restoration of the colonic tight junction barrier by whole LP powder and MetOH residue was involved in the reduction of colitis.

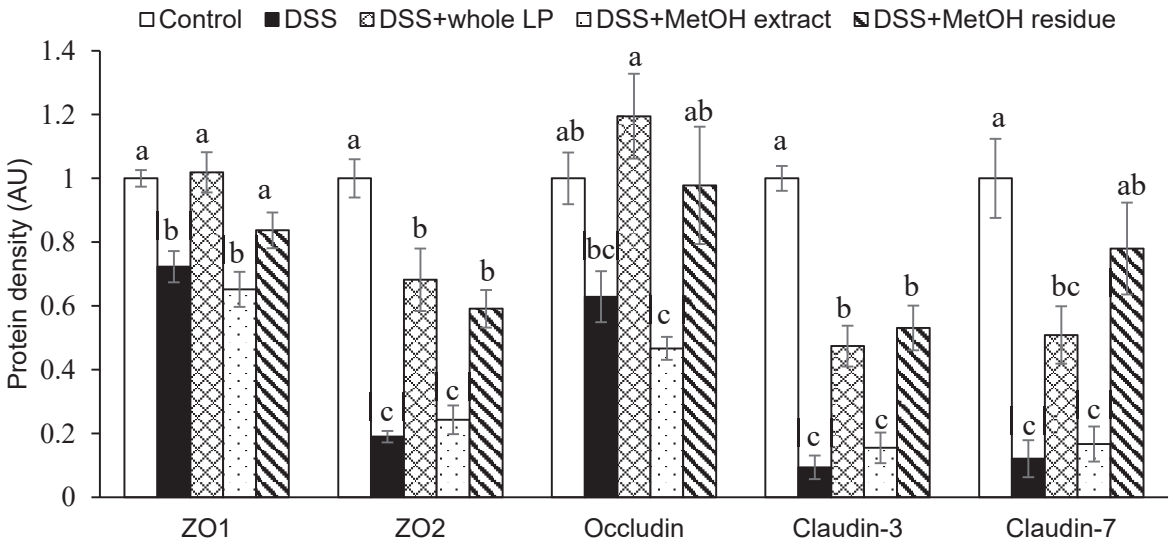


Figure 3.2 Effects of whole LP powder, methanol (MetOH) extract, and MetOH extraction residue (MetOH residue) on tight junction protein expression in the colon of dextran sodium sulfate (DSS)-induced colitis mice. Protein expression of zonula occludens (ZO)-1, ZO-2, occludin, claudin-3, and claudin-7 in the colon of mice fed diets with and without whole LP powder, MetOH extract, and MetOH residue, with or without DSS

administration, as determined by immunoblot analysis. Values are the mean ± SEM (n=7). Means without a common letter differ, $p < 0.05$.

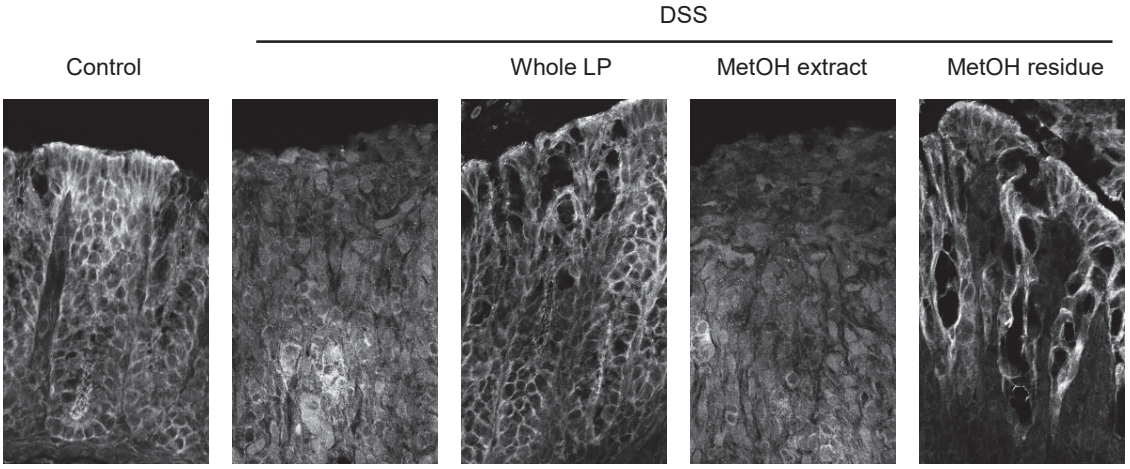


Figure 3.3 Effects of whole LP powder, methanol (MetOH) extract, and MetOH extraction residue (MetOH residue) on claudin-3 expression in the colon of dextran sodium sulfate (DSS)-induced colitis mice. Immunolocalization of claudin-3 in the colon of mice fed diets with and without whole LP powder, MetOH extract, and MetOH residue, with or without DSS administration, as analyzed by immunofluorescence microscopy. Represenatitve images of seven mice in each group are shown.

3.3.4 Effect of whole LP powder and its fractions on colonic gene expression

IBD refers to a group of disorders characterized by chronic inflammation of the gastrointestinal tract. In patients with IBD, the dysregulation of the innate and adaptive immune pathways possibly contributes to the abnormal intestinal inflammatory response. The inflammatory gene expression in the colonic tissue of mice after the DSS administration was shown in **Figure. 3.4**. DSS administration upregulated expression of *Il6*, *Il17A*, *Cxcl2*, and *Ccl2* in colonic tissues, whereas feeding mice whole LP powder and MetOH residue suppressed expression of *Il6*, *Cxcl2*, and *Ccl2*, but not *Il17A*. These results suggest that the suppression of the inflammatory reaction was another mechanism underlying the reduction of colitis. The MetOH extract modestly suppressed *Cxcl2* expression, but did not influence the other genes.

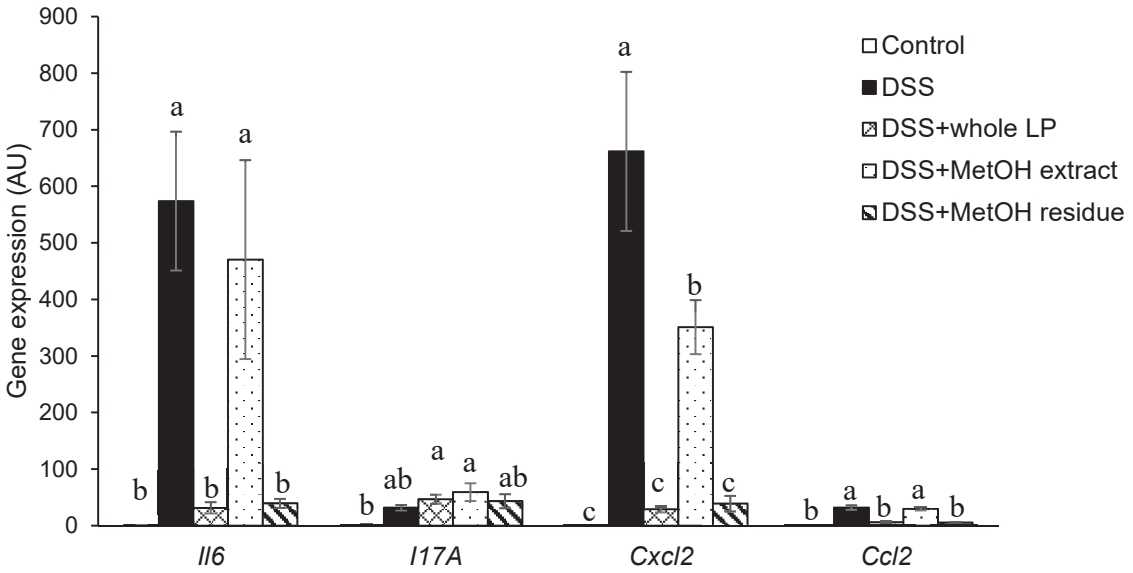


Figure. 3.4 Effects of whole LP powder, methanol (MetOH) extract, and MetOH extraction residue (MetOH residue) on inflammatory cytokines expression in the colon of dextran sodium sulfate (DSS)-induced colitis mice. Gene expression of *Il6*, *Il17A*, *Cxcl2*, and *Ccl2* in the colon mice fed diets with and without whole LP powder, MetOH extract, and MetOH residue, with or without DSS administration, as determined by quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis. Values are the mean ± SEM (n=7). Means without a common letter differ, $p < 0.05$.

3.3.5 Effect of whole LP powder and its fractions on colonic histopathology

Histopathological analyses were performed to investigate morphological changes of the colon. Colonic sections stained by Mayer's Hematoxylin and Eosin are shown in **Figure. 3.5**. The results showed that the colon of the DSS group exhibited distortion of crypts, loss of goblet cells, severe epithelial injury, and inflammatory cell infiltration in the mucosa and submucosa in a similar manner to the previous study in Chapter 2. The mucosal structures were well-preserved in the DSS + MetOH residue group as well as DSS + whole LP group. The ameliorative effect of the MetOH extract looked relatively lower than the other 2 preparation.

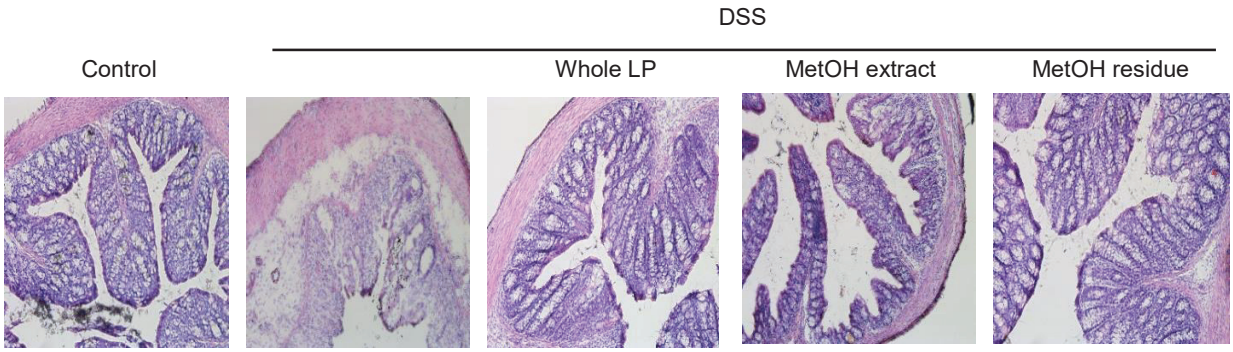


Figure. 3.5 Effects of whole LP powder, methanol (MetOH) extract, and MetOH extraction residue (MetOH residue) on mucosal structure in dextran sodium sulfate (DSS)-induced colitis mice. Mucosal structure of mice fed diets with and without whole LP, MetOH extract, and MetOH residue, with or without DSS administration. Colonic sections of mice were stained with hematoxylin and eosin. Representative images of seven mice in each group are shown.

3.3.6 Effect of whole LP powder and its fractions on fecal organic acids

Organic acids, including short-chain fatty acids (SCFAs), are major metabolites of undigested carbohydrates from intestinal microorganisms (Cook and Sellin, 1998). Because the MetOH residue, rich in dietary fibers, exhibited ameliorative effects in colitis mice in a manner partially similar to that of whole LP powder, we speculated that the microbial metabolism of dietary fibers in whole LP powder was involved in the reduction of colitis. Acetate, n-butyrate, iso-butyrate, and lactate in the DSS + whole LP group and acetate and n-butyrate in the DSS + MetOH residue group were higher than those in the DSS group. There was no difference in propionate, n-valerate, and iso-valerate among the groups.

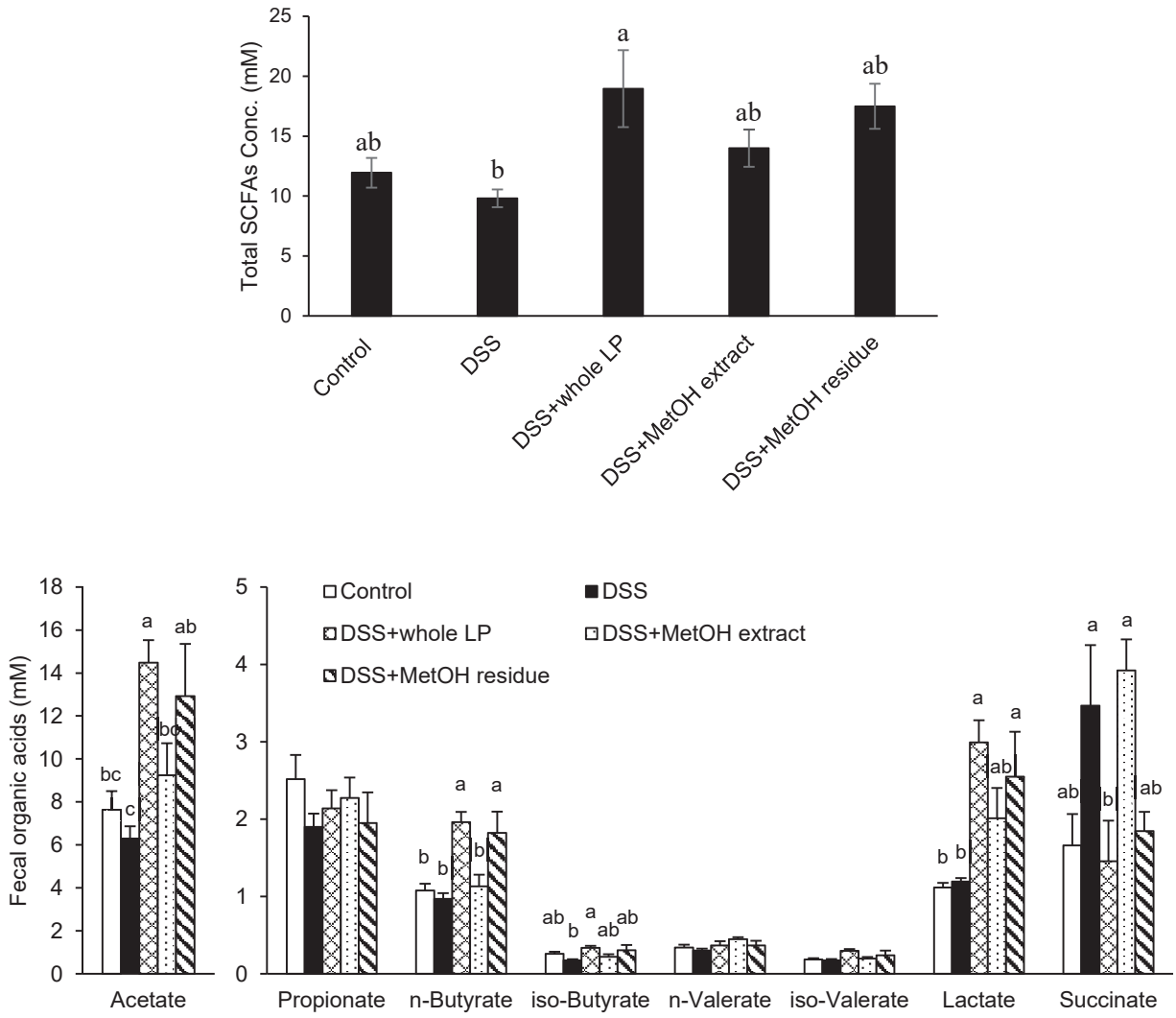


Figure 3.6 Effects of whole LP powder, methanol extract, and MetOH extraction residue on organic acid concentrations in feces of dextran sodium sulfate (DSS)-induced colitis mice. Fecal concentrations of acetate, propionate, n-butyrate, iso-butyrate, n-valerate, iso-valerate, lactate, and succinate in mice fed diets with and without whole LP powder, MetOH extract, and MetOH residue, with or without DSS administration, as determined by liquid chromatograph/tandem mass spectrometry (LC/MS/MS) analysis. Values are the mean ± SEM (n=7). Means without a common letter differ, $p < 0.05$.

3.4 Discussion

In chapter 2, we demonstrated that the supplemental whole LP powder reduced the intestinal inflammation and barrier defect in DSS-colitis mice. This suggest that the whole LP powder could be develop to a novel functional food against intestinal diseases. This experiment shown in chapter 3 aimed to reveal the active components in the whole LP powder. One of the most important findings of the present study was that the MetOH residue, rich in dietary fibers, exhibited ameliorative effects on colitis in a manner similar to that of whole LP powder. In addition, feeding mice the MetOH residue, as well as whole LP powder, increased SCFAs in feces, such as acetate and n-butyrate, major microbial metabolites of undigested carbohydrates. These data suggest that these SCFAs, at least in part, contributed to whole LP powder-mediated reduction of colitis. Feeding mice fermentable fibers leading to increased luminal SCFAs reduces intestinal inflammation and barrier defects in colitis mice (Hung and Suzuki, 2016). Clinical studies have shown lower fecal SCFAs levels in patients with IBDs than those in healthy subjects (Huda-Faujan *et al.*, 2010; Machiels *et al.*, 2014).

At least two mechanisms are involved in the acetate- and n-butyrate-mediated reduction of colitis: the regulation of the colonic tight junction barrier and inflammatory cytokine production. The intestinal tight junction barrier limits the permeation of luminal noxious molecules into mucosal tissues and prevents uncontrolled activation of immune cells (Turner, 2009). Feeding mice whole LP powder and MetOH residue restored the structure of the colonic tight junction barrier. SCFAs, including acetate and n-butyrate, enhance and protect the integrity and structure of the intestinal tight junction barrier (Suzuki et al., 2008; Kelly *et al.*, 2015; Miao *et al.*, 2016; Yan and Ajuwon, 2017). Acetate and n-butyrate enhance tight junction barrier integrity in rat cecum and intestinal Caco-2 cells (Suzuki et al., 2008). Additionally, feeding mice whole LP powder and MetOH residue suppressed inflammatory cytokine expression, including *Il6* and *Cxcl2* (a murine homologue of Il8). SCFAs, including acetate and n-butyrate, suppress the inflammatory response of intestinal epithelial cells (Hung and Suzuki, 2018). Stimulation of intestinal Caco-2 cells and mouse colonic tissues with tumor necrosis factor-α induces inflammatory

cytokine production, including IL-6 and IL-8, but treatment with acetate and n-butyrate reduces cytokine production (Hung and Suzuki, 2018). IL-8, secreted by epithelial cells, recruits neutrophils into inflamed colonic tissues, where neutrophils develop mucosal inflammation through intercellular interactions with other cells, such as macrophages and T cells (Mitsuyama *et al.*, 1994). In clinical studies, various immune cells, including neutrophils, accumulate in inflamed tissues of patients with ulcerative colitis (Holcombe *et al.*, 1994). Administration of a neutralizing antibody against the IL-8 receptor and C-X-C motif chemokine receptor 2 attenuates disease symptoms in DSS-induced colitis mice (Farooq *et al.*, 2009). Thus, the SCFA-mediated reduction of inflammatory cytokines, such as IL-6 and IL-8, seems to be another mechanism underlying the reduction of colitis.

Although we hypothesized that dietary fiber has an important role in whole LP powder-mediated reduction of colitis, other bioactive components, such as polyphenols also marginally contribute. This was because the restoration of tight junction proteins such as ZO-1 and occludin by the MetOH residue was not as effective as that by whole LP powder. In addition, feeding mice MetOH extract partly, but significantly, reduced DSS-induced *Cxcl2* expression in colonic tissues. Oral administration of hesperidin (10-40 mg/kg body weight) and eriocitrin (20 mg/kg body weight) reduces disease symptoms in DSS-induced colitis in mice (Guo *et al.*, 2019a; Guo *et al.*, 2019b). The doses of hesperidin and eriocitrin achieved through feeding mice whole LP powder and MetOH extract were approximately equivalent to 17 and 7 mg/kg body weight, respectively, under the feeding conditions of this study. At least, hesperidin in whole LP powder and MetOH extract seems to exhibit ameliorative effects on colitic mice (Guo *et al.*, 2019b). The reason for the weaker ameliorative effect of the MetOH extract than that was expected might be due to minor differences in experimental conditions. Previously, hesperidin was administered to C57BL/6 mice by oral gavage (Guo *et al.*, 2019b), whereas, in this study, whole LP powder was mixed in the diet and provided to Balb/c mice.

Although these results suggest that SCFAs target the tight junction barrier and inflammatory reaction in the colonic epithelium and mediate the protective effect of whole LP powder in colitic mice, additional mechanisms may also exist. N-butyrate promotes the

expansion of regulatory T cells, which suppress an inflammatory reaction (Furusawa *et al.*, 2013). Acetate also induces the intestinal production of immunoglobulin A, which protects intestinal tissue from inflammation (Shumer, 2017). Possible involvement of these mechanisms should be investigated in further studies.

In conclusion, dietary fibers largely contributed to LP powder-mediated reduction of intestinal inflammation and damage in colitis mice. Although the underlying mechanisms were not fully understood, these results suggest that SCFAs produced from colonic fermentation of dietary fibers in LP powder protected the colonic tight junction barrier and suppressed an inflammatory response. Further studies will investigate the possible application of LP powder to treat intestinal damage and inflammation.

Chapter 4

GENERAL CONCLUSION

IBD has emerged in newly industrialized countries as well as the developed countries and evolved into a global disease with rising prevalence in every continent. Thus, the IBD has affected the health of millions people and has posed a considerable global burden of disease. Nutritional and health-promoting values of *Citrus limon* have been traditionally known. Especially, LP contains many bioactive components carrying anti-oxidative, anti-inflammatory, anti-allergic, anti-viral, anti-mutagenic, and anti-carcinogenic effects. Recently, more researches focus on the lemon peel as herbal medicines to remedy different diseases. The purpose of this study to examine the protective effect of LP powder against the intestinal inflammation in mice. We also examined the active components in the LP powder.

In the first experiment (in chapter 2), we examined the effect of whole LP powder at different doses 2.5 % and 5 % (w/w) on the DSS-induced colitis in mice. The results demonstrated that whole LP powder attenuated the inflammation and damage of the colon in mice. The effect of whole LP powder seems to occur through the up-regulation the tight junction protein such as occludin, claudin-3 and claudin -7, as well as the down-regulation of pro-inflammatory cytokines such as *Il6* and *Cxcl-2*. Our findings imply the novel application of the whole lemon peel as food supplements against the intestinal diseases.

In second experiment (in chapter 3), we examined the bioactive components responsible for the effect of whole LP powder in DSS-induced colitis mice. Mice were fed with the polyphenol-rich MetOH extract and the dietary fiber-rich MetOH extraction residues. The results showed that the dietary fibers rather than polyphenols were responsible for the LP-mediated reduction of colitis. Although the underlying mechanisms for the effect of whole LP powder are still unclear, the SCFAs produced through the bacterial metabolism of dietary fiber at least in part contribute to the protection of the colonic tight junction barrier and the reduction of inflammatory cytokine expression.

In conclusion, our findings have established the foundation for the future application of whole LP powder against the intestinal damage and inflammation. Although the safety of whole LP powder for humans are protected based on the eating experience, the efficacy in humans should be required in the further studies.

REFERENCES

Al-Sadi, R. *et al.* (2010) 'IL-1beta-induced increase in intestinal epithelial tight junction permeability is mediated by MEKK-1 activation of canonical NF-kappaB pathway.', *The American Journal of Pathology*, 177(5), pp. 2310–2322.

Al-Sadi, R. *et al.* (2011) 'Occludin regulates macromolecule flux across the intestinal epithelial tight junction barrier', *American Journal of Physiology - Gastrointestinal and Liver Physiology*, 300(6), pp. 1054–1064.

Amarowicz, R. *et al.* (2009) 'Influence of postharvest processing and storage on the content of phenolic acids and flavonoids in foods', *Molecular Nutrition and Food Research*, 53, pp. 151–183.

Ardozzone and Porro, B. (2005) 'Biologic therapy for inflammatory bowel diseases', *Drugs*, 65(16), pp. 2253–2286.

Bhatnagar, A., Minocha, A. K. and Sillanpää, M. (2010) 'Adsorptive removal of cobalt from aqueous solution by utilizing lemon peel as biosorbent', *Biochemical Engineering Journal*, 48(2), pp. 181–186.

Biasi, F. *et al.* (2013) 'Inflammatory bowel disease: Mechanisms, redox considerations, and therapeutic targets', *Antioxidants & Redox Signaling*, 19(14), pp. 1711–1747.

Bischoff, S. C. *et al.* (2014) 'Intestinal permeability - a new target for disease prevention and therapy', *BMC Gastroenterology*, 14(1), pp. 1–25.

Bron, P. A. *et al.* (2017) 'Can probiotics modulate human disease by impacting intestinal barrier function ?', *British journal of nutrition*, 117, pp. 93–107.

Chassaing *et al.* (2015) 'Dextran sulfate sodium (DSS)-induced colitis in mice benoit', *Current Protocols in Immunology*, 104(1), pp. 1-14.

Chiba, H. *et al.* (2008) 'Transmembrane proteins of tight junctions', *Biochimica et Biophysica Acta*, 1778, pp. 588–600.

Cho, E. J. *et al.* (2011) 'Anti-inflammatory effects of methanol extract of Patrinia scabiosaefolia in mice with ulcerative colitis', *Journal of Ethnopharmacology*, 136(3), pp. 428–435.

Cook, S. I. and Sellin, J. H. (1998) ‘Review article: Short chain fatty acids in health and disease’, *Alimentary Pharmacology and Therapeutics*, 12(6), pp. 499–507.

Cosnes, J. *et al.* (2011) ‘Epidemiology and natural history of inflammatory bowel diseases’, *Gastroenterology*, 140(6), pp. 1785-1794.

Daniel E Shumer (2017) ‘Microbiota metabolite short chain fatty acid acetate promotes intestinal IgA response to microbiota which is mediated by GPR43’, *Physiology & behavior*, 176(12), pp. 139–148.

Dhingra, D. *et al.* (2012) ‘Dietary fibre in foods: A review’, *Journal of Food Science and Technology*, 49(3), pp. 255–266.

Dong, X. *et al.* (2019) ‘The maturity degree, phenolic compounds and antioxidant activity of Eureka lemon [Citrus limon (L.) Burm. f.]: A negative correlation between total phenolic content, antioxidant capacity and soluble solid content’, *Scientia Horticulturae*, pp. 281–289.

Dongowski, G., Lorenz, A. and Proll, J. (2002) ‘The degree of methylation influences the degradation of pectin in the intestinal tract of rats and in vitro’, *Journal of Nutrition*, 132(7), pp. 1935–1944.

Fanning, A. S., Mitic, L. L. and Anderson, J. M. (1999) ‘Transmembrane proteins in the tight junction barrier.’, *Journal of the American Society of Nephrology : JASN*, 10(6), pp. 1337–45.

Farhadi, A. *et al.* (2003) ‘Intestinal barrier : An interface between health and disease basement epithelium membrane coat secretory IgA mucosal M cell unstirred water layer immunoglobulin macrophage endothelium dandrite cell basophil mast cell lymphocyte’, *Journal of Gastroenterology and Hepatology*, 18, pp. 479–497.

Farooq, S. M. *et al.* (2009) ‘Therapeutic effect of blocking CXCR2 on neutrophil recruitment and dextran sodium sulfate-induced colitis’, *Journal of Pharmacology and Experimental Therapeutics*, 329(1), pp. 123–129.

Fries W *et al.* (1999) ‘Experimental colitis increases small intestine permeability in the rat’, *Lab Invest*, 79, pp. 49–57.

Fukui, H. (2016) ‘Increased intestinal permeability and decreased barrier function:

does it really influence the risk of inflammation?', *Inflammatory Intestinal Diseases*, 1(3), pp. 135–145.

Fuller, S. *et al.* (2016) 'New horizons for the study of dietary fiber and health: A review', *Plant Foods for Human Nutrition*, 71(1), pp. 1–12.

Furusawa, Y. *et al.* (2013) 'Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells.', *Nature*, 504(7480), pp. 446–450.

Furuse, M. *et al.* (1993) 'Occludin: A novel integral membrane protein localizing at tight junctions', *Journal of Cell Biology*, 123(6 II), pp. 1777–1788.

Furuse, M. *et al.* (2002) 'Claudin-based tight junctions are crucial for the mammalian epidermal barrier: A lesson from claudin-1-deficient mice', *Journal of Cell Biology*, 156(6), pp. 1099–1111.

Gassler N *et al.* (2001) 'Inflammatory bowel disease is associated with changes of enterocytic junctions', *Am J Physiol Gastrointest Liver Physiol*, 281, pp. 216–228.

González-Mariscal, L. *et al.* (2003) 'Tight junction proteins.', *Progress in Biophysics and Molecular Biology*, 81(1), pp. 1–44.

González-Molina, E. *et al.* (2010) 'Natural bioactive compounds of Citrus limon for food and health', *Journal of Pharmaceutical and Biomedical Analysis*, 51(2), pp. 327–345.

Guo, G. *et al.* (2019a) 'Anti-inflammatory effects of eriocitrin against the dextran sulfate sodium–induced experimental colitis in murine model', *Journal of Biochemical and Molecular Toxicology*, 33(11), pp. 1-8.

Guo, K. *et al.* (2019b) 'Hesperidin protects against intestinal inflammation by restoring intestinal barrier function and up-regulating treg cells.', *Molecular Nutrition & Food Research*, 63(11), p. e1800975.

Han, J. *et al.* (2015) 'An isotope-labeled chemical derivatization method for the quantitation of short-chain fatty acids in human feces by liquid chromatography – tandem mass spectrometry', *Analytica Chimica Acta*, 854, pp. 86–94.

Cooper *et al.* (1993) 'Clinicopathologic study of dextran sulfate sodium experimental murine colitis', *Laboratory Investigation*, 69, pp. 238–249.

Hartsock, A. and Nelson, W. J (2008) 'Adherens and tight junctions : Structure ,

function and connections to the actin cytoskeleton', *Biochimica et Biophysica Acta*, 1778, pp. 660–669.

Hartsock, A. and Nelson, W. J (2008) 'Adherens and tight junctions: Structure, function and connections to the actin cytoskeleton', *Biochimica et Biophysica Acta - Biomembranes*, 1778(3), pp. 660–669.

Holcombe, R. F. *et al.* (1994) 'Correlation of serum interleukin-8 and cell surface lysosome-associated membrane protein expression with clinical disease activity in systemic lupus erythematosus.', *Lupus*, 3(2), pp. 97–102.

Hung, T. V and Suzuki, T. (2016) 'Dietary fermentable fiber reduces intestinal barrier defects and inflammation in colitic mice', *The Journal of Nutrition*, 146(10), pp. 1970–1979.

Hung, T. V and Suzuki, T. (2018) Short-chain fatty acids suppress inflammatory reactions in Caco-2 cells and mouse colons, *Journal of Agricultural and Food Chemistry,* pp. 108-117.

Huynh, E. *et al.* (2019) 'Protective effects of protegrin in dextran sodium sulfate-induced Mmurine colitis'. *Front Pharmacol.*, 10, pp. 1–12.

Isidro, R. A. *et al.* (2017) 'The Probiotic VSL#3 modulates colonic macrophages, inflammation, and microflora in acute trinitrobenzene sulfonic acid colitis', *Journal of Histochemistry and Cytochemistry*, 65(8), pp. 445–461.

Lynch J M and Barbano, D. M. (1999) 'Kjeldahl nitrogen analysis as a reference method for protein determination in dairy products', *J AOAC Int.*, 82(6), pp. 1389–1398.

Jasleen and Gurpreet (2015) 'An insight into the role of citrus bioactives in modulation of colon cancer', *Journal of Functional Foods*, 13, pp. 239–261.

Jideani, V. A. J. and Jideani I. A. (2012) *Tropical and subtropical fruits: Postharvest Physiology, Processing and Packaging - Chapter 20*, *Wiley-Blackwell*, pp. 377-399.

John, S. *et al.* (2017) 'Investigation on phytochemical profile of *Citrus limonum* peel extract', *International Journal of Food Science and Nutrition*, 2(1), pp. 65–67.

Kaplan, G. G. (2015) 'The global burden of IBD: From 2015 to 2025', *Nature*

Reviews Gastroenterology and Hepatology, 12(12), pp. 720–727.

Kawabata, A. *et al.* (2018) Citrus kawachiensis peel powder reduces intestinal barrier defects and inflammation in colitic mice, *Journal of Agricultural and Food Chemistry*, 66, 422, pp. 10991-10999.

Kelly, C. J. *et al.* (2015) 'Crosstalk between microbiota-derived short-chain fatty acids and intestinal epithelial HIF augments tissue barrier function', *Cell Host Microbe*, 17(5), pp. 662–671.

Kim, M. (2005) 'High-methoxyl pectin has greater enhancing effect on glucose uptake in intestinal perfused rats', *Nutrition*, 21(3), pp. 372–377.

Langholz, E. (2010) 'Current trends in inflammatory bowel disease: The natural history', *Therapeutic Advances in Gastroenterology*, 3(2), pp. 77–86.

Lattimer, J. M. and Haub, M. D. (2010) 'Effects of dietary fiber and its components on metabolic health', *Nutrients*, 2(12), pp. 1266–1289.

Li, Y. H *et al.* (2018) 'A characterization of pro-inflammatory cytokines in dextran sulfate sodium-induced chronic relapsing colitis mice model', *International Immunopharmacology*, 60, pp. 194–201.

M'Koma, A. E. (2013) 'Inflammatory bowel disease: An expanding global health problem', *Clinical Medicine Insights: Gastroenterology*, 6, pp. 33–47.

Machiels, K. *et al.* (2014) 'A decrease of the butyrate-producing species roseburia hominis and faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis', *Gut*, 63(8), pp. 1275–1283.

Mahadevan, U. (2004) 'Medical treatment of ulcerative colitis', *Clinics in Colon and Rectal Surgery*, 17(1), pp. 7–19.

Mayangsari, Y. and Suzuki, T. (2018) 'Resveratrol ameliorates intestinal barrier defects and inflammation in colitic mice and intestinal cells', *Journal of Agricultural and Food Chemistry*. American Chemical Society, 66(48), pp. 12666–12674.

McCleary, B. V *et al.* (2012) 'Determination of insoluble, soluble, and total dietary fiber (CODEX definition) by enzymatic-gravimetric method and liquid chromatography: collaborative study', *J AOAC Int.*, 95(3), pp. 824–844.

Miao, W. *et al.* (2016) 'Sodium butyrate promotes reassembly of tight junctions in Caco-2 monolayers involving inhibition of MLCK/MLC2 pathway and phosphorylation of PKCβ2', *International Journal of Molecular Sciences*, 17(10), pp. 1–12.

Mitsuyama, K. *et al.* (1994) 'IL-8 as an important chemoattractant for neutrophils in ulcerative colitis and Crohn's disease', *Clinical and Experimental Immunology*, 96(3), pp. 432–436.

Siddiq M. *et al.* (2012) *Tropical and Subtropical Fruits: Postharvest Physiology, Processing and Packaging*. ISBN: 978-0-813-81142-0, *Wiley-Blackwell,* pp. 1-656.

Műzes, G. *et al.* (2012) 'Changes of the cytokine profile in inflammatory bowel diseases', *World J Gastroenterol 2012*, 18(41), pp. 5848–5861.

N. Huda-Faujan *et al.* (2010) 'The impact of the level of the intestinal short chain fatty acids in inflammatory bowel disease patients versus healthy subjects', *The Open Biochemistry Journal*, 4, pp. 53–58.

Noda, S., Tanabe, S. and Suzuki, T. (2013) 'Naringenin enhances intestinal barrier function through the expression and cytoskeletal association of tight junction proteins in Caco-2 cells', *Mol Nutr Food Res*, 57(11), pp. 2019–2028.

Nogata, Y., Sakamoto, K., Shiratsuchi, H., Ishi, T., *et al.* (2006) 'Flavonoid composition of fruit tissues of citrus species', *Biosci. Biotechnol. Biochem*, 70(1), pp. 178–192.

Nunes, T. *et al.* (2011) 'Familial aggregation in inflammatory bowel disease: Is it genes or environment?', *World Journal of Gastroenterology*, 17(22), pp. 2715–2722.

Ogata, M. *et al.* (2017) 'Supplemental psyllium fibre regulates the intestinal barrier and inflammation in normal and colitic mice', *British Journal of Nutrition*, 118(9), pp. 661–672.

Olano-Martin, E., Gibson, G. R. and Rastall, R. A. (2002) 'Comparison of the in vitro bifidogenic properties of pectins and pectic-oligosaccharides', *Journal of Applied Microbiology*, 93(3), pp. 505–511.

Ötles, S. and Ozgoz, S. (2014) 'Health effects of dietary fiber', *Acta Scientiarum Polonorum, Technologia Alimentaria*, 13(2), pp. 191–202.

Perše, M. and Cerar, A. (2012) 'Dextran sodium sulphate colitis mouse model: Traps and tricks', *Journal of Biomedicine and Biotechnology*, 31(4), pp. 1-13.

Polat, F. R. *et al.* (2018) 'Effect of hesperetin on inflammatory and oxidative status in trinitrobenzene sulfonic acid-induced experimental colitis model.', *Cellular and Molecular Biology (Noisy-le-Grand, France)*, 64(11), pp. 58–65.

Pullman, W. E. *et al.* (1992) 'Enhanced mucosal cytokine production in inflammatory bowel disease', *Gastroenterology*, 102(2), pp. 529–537.

Rao, R. K. *et al.* (2002) 'Tyrosine phosphorylation and dissociation of occludin-ZO-1 and E-cadherin-β-catenin complexes from the cytoskeleton by oxidative stress', *Biochemical Journal*, 368(2), pp. 471–481..

Reeves, P. G., Nielsen, F. H. and Fahey, G. C. (1993) 'AIN-93 purified diets for laboratory rodents: Final Report of the American Institute of Nutrition Ad Hoc Writing Committee on the reformulation of the AIN-76A rodent diet', *The Journal of Nutrition*, 123(11), pp. 1939–1951.

Del Río, J. A. *et al.* (2004) '*Citrus limon*: A source of flavonoids of pharmaceutical interest', *Food Chemistry*, 84(3), pp. 457–461.

Rosenblum, L., Garris, S. T. and Morgan, J. N. (2002) 'Comparison of five extraction methods for determination of incurred and added pesticides in dietary composites', *J AOAC Int*, 85(5), pp. 1167–1176.

Roth, J. A. *et al.* (1995) 'Pectin improves colonic function in rat short bowel syndrome', *Journal of Surgical Research*, pp. 240–246.

Russo, M. *et al.* (2014a) 'Underestimated sources of flavonoids, limonoids and dietary fiber: Availability in lemon's by-products', *Journal of Functional Foods*, 9, pp. 18–26.

Russo, M. *et al.* (2014b) 'Underestimated sources of flavonoids, limonoids and dietary fiber: Availability in orange's by-products', *Journal of Functional Foods*, 9, pp. 150–157.

Salaritabar, A. *et al.* (2017) 'Therapeutic potential of flavonoids in inflammatory bowel disease: A comprehensive review.', *World Journal of Gastroenterology*, 23(28), pp.

5097–5114.

Sánchez, D. *et al.* (2008) ‘Highly methoxylated pectin improves insulin resistance and other cardiometabolic risk factors in Zucker fatty rats’, *Journal of Agricultural and Food Chemistry*, 56(10), pp. 3574–3581.

Slavin, J. (2013) ‘Fiber and prebiotics: Mechanisms and health benefits’, *Nutrients*, 5(4), pp. 1417–1435.

Suzuki, T. (2013) ‘Regulation of intestinal epithelial permeability by tight junctions’, *Cellular and Molecular Life Sciences*, 70(4), pp. 631–659.

Suzuki, T. and Hara, H. (2009) ‘Quercetin enhances intestinal barrier function through the assembly of zonnula occludens-2, occludin, and claudin-1 and the expression of claudin-4 in caco-2 cells’, *Journal of Nutrition*, 139(5), pp. 965–974.

Suzuki, T., Seth, A. and Rao, R. (2008) ‘Role of phospholipase Cgamma-induced activation of protein kinase Cepsilon (PKCepsilon) and PKCbetaI in epidermal growth factor-mediated protection of tight junctions from acetaldehyde in Caco-2 cell monolayers.’, *The Journal of biological chemistry*, 283(6), pp. 3574–83.

Suzuki, T., Yoshida, S. and Hara, H. (2008) ‘Physiological concentrations of short-chain fatty acids immediately suppress colonic epithelial permeability’, *British Journal of Nutrition*, 100(2), pp. 297–305.

Suzuki, T., Yoshinaga, N. and Tanabe, S. (2011) ‘Interleukin-6 (IL-6) regulates claudin-2 expression and tight junction permeability in intestinal epithelium.’, *The Journal of Biological Chemistry*, 286 (36), pp. 31263–71.

Thiex, N. (2009) ‘Evaluation of analytical methods for the determination of moisture, crude protein, crude fat, and crude fiber in distillers dried grains with solubles’, *J AOAC Int*, 92(1), pp. 61–73.

Thiex, N., Novotny, L. and Crawford, A. (2012) ‘Determination of ash in animal feed: AOAC official method 942.05 revisited’, *J AOAC Int.*, 95(5), pp. 1392–1397.

Tian, L. *et al.* (2016) ‘Effects of pectin supplementation on the fermentation patterns of different structural carbohydrates in rats’, *Molecular Nutrition & Food Research*, 60(10), pp. 2256–2266.

Toumi, R. *et al.* (2014) 'Probiotic bacteria lactobacillus and bifidobacterium attenuate inflammation in dextran sulfate sodium-induced experimental colitis in mice', *International Journal of Immunopathology and Pharmacology*, 27(4), pp. 615–627.

Turner, J. R. (2009) 'Intestinal mucosal barrier function in health and disease', *Nature Reviews Immunology*, 9(11), pp. 799–809.

Ulluwishewa, D. *et al.* (2011) 'Regulation of tight junction permeability by intestinal bacteria and dietary components 1 , 2', *The Journal of Nutrition*, 141, pp. 769–776.

Venegas, D. P. *et al.* (2019) 'Short chain fatty acids (SCFAs)mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases', *Frontiers in Immunology*, 10, pp. 1–16.

Vetrano, S. *et al.* (2008) 'Unique role of junctional adhesion molecule-A in maintaining mucosal homeostasis in inflammatory bowel disease', *Gastroenterology*,, 135, pp. 173–184.

Wallace, K. L. *et al.* (2014) 'Immunopathology of inflammatory bowel disease', *World Journal of Gastroenterology*, 20(1), pp. 6–21.

Xi, W. *et al.* (2017) 'Characterization of phenolic profile and antioxidant capacity of different fruit part from lemon (Citrus limon Burm.) cultivars', *Journal of Food Science and Technology*, pp. 1108–1118.

Xu, L. *et al.* (2009) 'Modulating effect of Hesperidin on experimental murine colitis induced by dextran sulfate sodium.', *Phytomedicine : international journal of phytotherapy and phytopharmacology*, 16(10), pp. 989–95.

Yan, H. and Ajuwon, K. M. (2017) 'Butyrate modifies intestinal barrier function in IPEC-J2 cells through a selective upregulation of tight junction proteins and activation of the Akt signaling pathway', *PLos One*, 12(6), pp. 1–20.

Yu, Q. *et al.* (2015) '*Lactobacillus* protects the integrity of intestinal epithelial barrier damaged by pathogenic bacteria', *Frontiers in Cellular and Infection Microbiology*, 5, pp. 1–7.

Yue, B. *et al.* (2019) 'Inflammatory bowel disease: A potential result from the

collusion between gut microbiota and mucosal immune system', *Microorganisms*, 7(10), pp. 1-21.

Zhang, W., Xu, P. and Zhang, H. (2015) 'Pectin in cancer therapy: A review', *Trends in Food Science and Technology*, 44(2), pp. 258–271.