Novel anti-inflammatory property of polysaccharides from Lentinula edodes on colitis through necroptosis inhibition

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DOCTORAL DISSERTATION

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シイタケ由来多糖類によるネクロトーシスを介した新規腸炎抑制効果

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ABBREVIATIONS

APAF-1, apoptotic protease activating factor-1

BAK, Bcl-2 homologous antagonist killer

BAX, Bcl-2 associated X protein

BID, BH3 interacting domain

BSA, bovine serum albumin

CCL-2, c-c motif chemokine ligand 2

cDNA, complementary DNA

DAMPs, damage associated molecular patterns

DEAE, diethylaminoethyl

DNA, deoxyribonucleic acid

DSS, dextran sulfate sodium

DTT, dithiothreitol

ECL, enhanced chemiluminescence

ERK, extracellular signal-regulated kinase

FADD, fas-associated protein with death domain

FBS, fetal bovine serum

HRP, horseradish peroxidase

IFN-γ, interferon-γ

IL, interleukins

JNK, c-Jun N-terminal kinase

MAPK, mitogen-activated protein kinase

MCP-1, monocyte chemoattractant protein-1

MLKL, mixed lineage kinase domain-like pseudokinase

mRNA, messenger RNA

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Nec-1, necrostatin-1

NF-κB, nuclear factor kappa B

PCR, polymerase chain reaction

pMLKL, phosphorylated MLKL

PMSF, phenylmethylsulfonyl fluoride

PVDF, polyvinylidene fluoride

RIPA, radioimmunoprecipitation assay

RIPK1, receptor interacting protein kinase 1

RIPK3, receptor interacting protein kinase 3

RNA, ribonucleic acid

RNase, ribonuclease

ROS, reactive oxygen species

SD, standard deviation

SDS, sodium dodecyl sulfate

SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis

Syk, spleen tyrosine kinase

tBID, truncated BID

TBST, tris-buffered saline, 0.1% Tween 20

TCA, trichloroacetic acid

TLRs, toll-like receptors

TNFR1, tumor necrosis factor receptor 1

TNF-α, tumor necrosis factor-α

TRAIL, tumor necrosis factor-related apoptosis-inducing ligand

zVAD-fmk, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

CHAPTER 1

General Introduction

1.1. Intestinal epithelial cells and gut inflammation

1.1.1. ***The intestine and cellular damage***

The intestine is a specialized organ in the human body, playing an initial and crucial role in nutrient absorption and energy generation for the normal functioning of the body. Being an organ responsible for food digestion and absorption, it is constantly in contact with harmful environmental chemicals and various pathogenic and non-pathogenic microorganisms, such as fungi, bacteria, and viruses, accompanying food intake [1, 2]. Ingested toxic environmental chemicals do not only affect the activities and composition of microbiota in the lumen, they also cause serious damage to intestinal epithelial cells [3-5]. The intestinal epithelial cells (IECs) comprise three major cell types, namely the absorptive enterocytes, goblet cells, and the Paneth cells, which form both physical and chemical barrier between the immune cells in the lamina propria and the microbiota and food substance in the gut lumen. These cells are responsible for food absorption, secretion of mucin glycoproteins and mucus lining, and antimicrobial peptides, which play important roles in maintaining the intestinal epithelial homeostasis [6]. Alterations in the composition and activities of microbiota, and damage to IECs resulting from toxic environmental chemicals and food-borne microorganisms have been reported as a major cause of chronic intestinal inflammation. Damaged IECs undergo cell death and result in disruption of the mucosal barrier leading to an increased permeability to gut microbiota, and ultimately homeostatic

imbalance and systemic spread of microorganisms (sepsis) [7-10]. A compromised mucosal barrier consequently results in aberrant response of innate immune cells in lamina propria to microbiota and food substances, leading to increased secretion of proinflammatory cytokines and chronic intestinal inflammation [7, 11-13].

1.1.2. ***Inflammatory bowel disease***

Inflammatory bowel disease (IBD) is a generalized term used to describe two kinds of disorders that is characterized by a chronic, relapsing inflammation of the gastrointestinal tract, with an increasing global incidence and prevalence [14, 15]. It affects people of all age groups, and negatively impacts on every aspect of lives of the victims [16, 17]. It is subdivided into ulcerative colitis (UC) and Crohn's disease (CD). Crohn's disease could affect both the small intestine and large intestine, and it is characterized by intermittent intestinal lesions, transmural inflammation, fistula, abdominal pain, diarrhea, weight loss, loss of appetite, and perianal lesion, while ulcerative colitis affects only the colon down to the rectum, and it's characterized by continuous lesion, superficial inflammation, ulcer, severe abdominal pain, bloody diarrhea, weight loss, rectal bleeding, narrowed gut lumen, and shortened colon length [18-20]. There is currently no cure for the disease, and the pathogenesis is not completely understood [19]. However, researchers have attributed a number of mixed interacting factors ranging from, genetic predisposition, aberrant reactions of immune cells in the lamina propria to microbiota, luminal antigens and food factors, and environmental factors. The interactions of these factors result in cell death, disruption of intestinal epithelial mucosa barrier, and ultimately chronic inflammation of the gut [21]. The scheme below (Fig. 1.1) illustrates the pathophysiological mechanism of IBD, as described by Li *et al* [22], Xavier and Podolsky [23], and Pierdomenico *et al* [24].

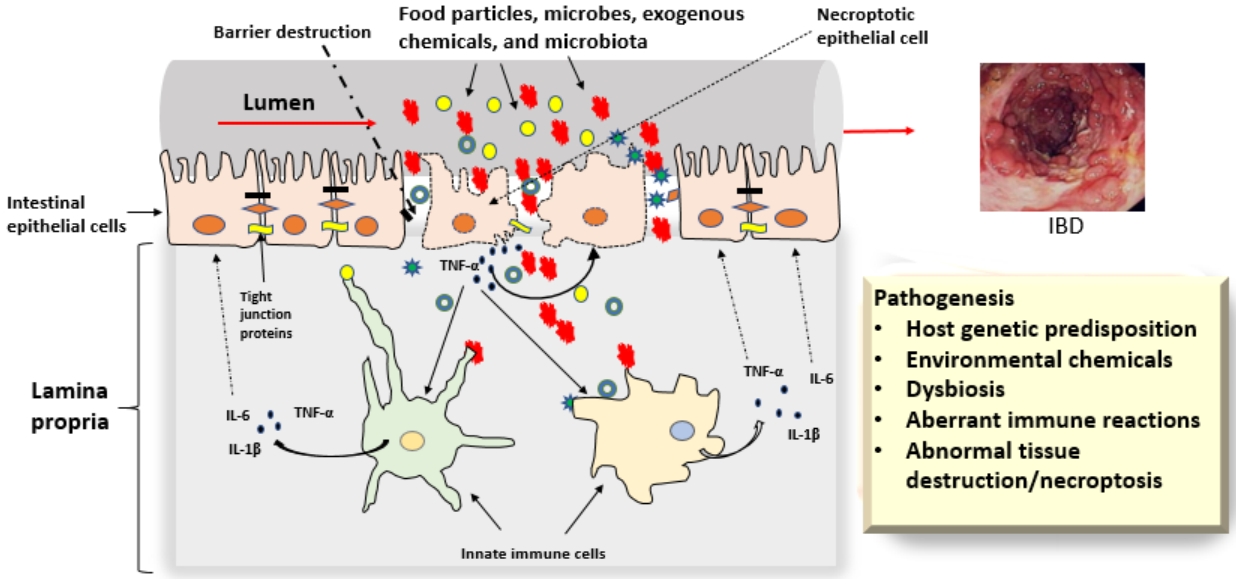


Fig. 1.1. Pathophysiological mechanism of inflammatory bowel disease

1.1.3. Necroptotic and apoptotic cell deaths

Necroptosis is a caspase-independent pro-inflammatory form of programmed cell death that is morphologically and mechanistically different from apoptosis [24]. Apoptosis could be initiated through one of two pathways, the extrinsic pathway and the intrinsic pathway. The extrinsic pathway is triggered by extracellular stimuli such as TNF-α and other ligands of tumor necrosis factor receptor superfamily, while intrinsic apoptosis is triggered by intracellular stimuli, such as DNA damage and cellular stress. The extrinsic pathway of apoptosis is mediated by the initiator caspase, caspase-8, which in turn directly activates the executor caspase, caspase-3. Caspase-8 could also activate caspase-3 and -7 through caspase-9 activation via conversion of BID to tBID and activation of BAX and BAK which translocate into the mitochondria, leading to the release of cytochrome c from mitochondria intermembrane space [25]. Release of cytochrome c into the

cytosol triggers caspase-3 and -7 (executor caspases) activation through the formation of cytochrome c/Apaf-1/caspase-9 apoptosome complex. In the same way, intrinsic apoptosis pathway is initiated through the release of cytochrome c, binding to Apaf-1, and leading to the activation of caspase-3 and -7 through apoptosome complex formation with caspase-9, hence intrinsic-mediated apoptosis is also termed cytochrome c-mediated apoptosis [25-27]. Necroptosis, on the other hand, is usually initiated by external stimuli, and it is an alternative path of cell death when apoptosis is inhibited. Necroptosis is triggered in cells by the stimulation of cell surface receptors such as TNFR1, TLR3, TLR4, Fas, and TRAIL by their respective exogenous ligands in the absence, genetic deficiency, or pharmacologic inhibition of caspase-8, or by recognition of viral or endogenous RNA by Z-DNA/RNA binding protein 1 (ZBP1) [28-31]. Being caspase-independent, it is mediated by the activities of receptor interacting protein kinases known as RIPK1, RIPK3, and MLKL (mixed lineage kinase domain-like pseudokinase). Stimulation of cell surface death receptors results in the phosphorylation and activation of RIPK1, RIPK3, and MLKL [30, 32]. Phosphorylated MLKL (pMLKL), known as the necroptosis executor, undergoes oligomerization and translocate to the plasma membrane where it binds to phosphatidylinositol phosphates to induce necroptosis [33, 34]. Unlike apoptosis, necroptosis involves cell swelling, membrane rupture, and release of DAMPs (damage associated molecular patterns), while apoptosis involves cell shrinkage, membrane blebbing, formation of apoptotic bodies, DNA fragmentation, and chromatin condensation [35]. Apoptosis has been considered an immunologically silent and anti-inflammatory cell death, while necroptosis is known as a pro-inflammatory cell death because upon membrane rupture, cellular contents are released into the microenvironment of cells which in turn trigger immune reactions. Apoptosis, on the other hand, does not involve the release of cellular contents which are held intact within the apoptotic bodies

and phagocytosed by phagocytic cells like macrophages [35]. Recent reports have suggested that necroptosis is actively involved in the etiology, pathogenesis, and progression of IBD and other inflammatory conditions [24, 36, 37], and inhibitors of necroptosis have shown promising clinical results in the treatment of inflammatory diseases. Therefore, it is important to search for natural sources of therapeutic agents against necroptotic cell death that may be of clinical benefits in the treatment of IBD.

1.1.4. ***Edible mushrooms***

Edible mushrooms have long served as a source of food and medicinal compounds in many parts of the world, ranging from America, Asia, Europe, and Africa. The health benefits attributed to mushrooms includes, anti-allergic, antiviral, antidiabetic, antioxidant activity, anti-cancer activity, anti-ageing, cholesterol-lowering activity, anti-bacteria, anti-fungal, and anti-inflammatory activity [38-41]. Perhaps, one of the prominent components of edible mushrooms which has attracted significant attention, is a group of bioactive polysaccharides known as β-glucans. Many studies have reported that β-glucans, such as lentinan (a β-1,3;1,6-glucan, derived from *Lentinula edodes*), is able to suppress intestinal inflammation by modulating the activities of microbiota and reducing the expression of pro-inflammatory cytokines, such as TNF-α, IL-8, IL-1β, IFN-γ, and IL-6 through binding to dectin-1 [42, 43].

Fig. 1.2. Shiitake mushroom (*Lentinula edodes*)

Dectin-1 is a C-type lectin-like type II membrane protein, which recognizes β-glucans found on the surface of bacteria and fungi including yeast and mushrooms. Dectin-1 is expressed on the cell surface of IECs and innate immune cells, such as macrophages, dendritic cells, and neutrophils. Ingested β-glucans are capable of inducing immune reactions or immune enhancement through binding to dectin-1 expressed on the surface of IECs [19]. However, some studies have shown that not all β-glucans are capable of activating dectin-1, and perhaps what confers glucans the ability to bind and activate dectin-1 is the presence of β-1,3- and more strongly with β-1,6 branch [42]. Researchers have not yet been able to determine the precise oligosaccharide moiety which specifically binds to dectin-1 to trigger the immune reactions [42-47]. Furthermore, the anti-inflammatory activity of lentinan has been attributed to its ability to induce internalization of TNFR1 or prevent its expression on the surface of intestinal epithelia cells through binding to dectin-1 [19, 43].

This study investigated the effect of polysaccharides from *Lentinula edodes* on ulcerative colitis and its impact on ulcerative colitis-associated necroptotic cell death. In Chapter 2, polysaccharides

from *Lentinula edodes* was orally administered to colitis mice to investigate whether the polysaccharide sample inhibited ulcerative colitis. The polysaccharide sample showed inhibitory activity against ulcerative colitis. This inhibitory activity was dependent on its carbohydrate-rich fraction. The anti-colitis activity of the polysaccharide was not abolished after deproteination, which indicated that the anti-inflammatory activity was independent of the protein content. It was also observed that the carbohydrate-rich fraction of the polysaccharides prevented ulcerative colitis in a dose-dependent manner.

Different strains of *Lentinula edodes* are known to exist, and some studies have reported that physiological activities of medicinal herbs could be strain-dependent [48]. In Chapter 3, the anti-inflammatory activity of polysaccharides from different strains of *Lentinula edodes* was investigated by oral administration of crude polysaccharide samples to colitis mice. the It was observed that polysaccharides from different strains of *Lentinula edodes* exhibited differential anti-inflammatory activity against ulcerative colitis. Chromatographic separation of crude polysaccharide samples from different strains showed differential elution profile patterns with different peaks, suggesting that the anti-inflammatory activities of the crude polysaccharides may be strain-dependent.

In Chapter 4, the effect of polysaccharides from *Lentinula edodes* against necroptosis, a regulated pro-inflammatory form of cell death, was investigated using mice and *in vitro* models of necroptosis. The results indicated that the polysaccharide sample inhibited necroptotic cell death in the colon of ulcerative colitis mice by suppressing the phosphorylation level of MLKL, pMLKL, a necroptosis executor protein. Furthermore, the results of *in vitro* necroptosis experiments indicated that the polysaccharide sample prevented necroptotic cell death in intestinal epithelial cells line, Caco-2 cells. Recent reports have shown that inhibitors of necroptosis or knockout of

the necroptosis executor, MLKL, prevented the increased expression of inflammatory cytokines, indicating that necroptotic signaling molecules induced the expression of inflammatory cytokines [49, 50]. Therefore, the results in Chapter 5 indicated that orally administered polysaccharides from *Lentinula edodes* suppressed expression of pro-inflammatory cytokines, TNF-α, IL-6, IL-1β, and IFN-γ and CCL-2 chemokine in the colon of ulcerative colitis mice. Also, the polysaccharide suppressed up-regulation of IL-8 in Caco-2 cells undergoing necroptosis, suggesting that necroptosis mediated the increase of IL-8 expression. It was also demonstrated that the anti-necroptosis activity of the polysaccharide was independent of its effects on TNFR1 expression. These results, taken together, suggested that the anti-inflammatory activity of polysaccharide from *Lentinula edodes* against ulcerative colitis is partly dependent on its ability to inhibit necroptotic cell death.

CHAPTER 2

Inhibitory Effect of Polysaccharides from *Lentinula edodes* (shiitake) against Ulcerative Colitis

2.1. Introduction

Inflammatory bowel disease (IBD) is a generalized term used to describe two kinds of disorders that is characterized by the chronic inflammation of the gastrointestinal tract, with an increasing global incidence and prevalence [14, 15]. It affects people of all age groups, and negatively impacts on every aspect of lives of the victims [16, 17]. It is subdivided into ulcerative colitis (UC) and Crohn's disease (CD). Crohn's disease could affect both the small intestine and large intestine, and it is characterized by intermittent intestinal lesions, transmural inflammation, fistula, abdominal pain, diarrhea, weight loss, and perianal lesion, while ulcerative colitis affects only the colon down to the rectum, and it is characterized by continuous lesion, superficial inflammation, ulcer, severe abdominal pain, bloody diarrhea, loss of appetite, weight loss, rectal bleeding, narrowed gut lumen, and shortened colon length [18-20]. There is currently no cure for the disease, and the pathogenesis is not completely understood [19]. However, researchers have attributed a number of mixed interacting factors ranging from, genetic predisposition, aberrant reactions of immune cells in the lamina propria to microbiota, luminal antigens and food factors, and environmental factors. The interactions of these factors result in cell death, disruption of intestinal epithelial mucosa barrier, and ultimately chronic inflammation of the gut [20].

Edible mushrooms have long served as a source of food and medicinal compounds in many parts of the world, ranging from America, Asia, Europe, and Africa. The health benefits attributed to mushrooms includes, anti-allergic, antiviral, antidiabetic, antioxidant activity, anti-cancer activity, anti-ageing, cholesterol-lowering activity, anti-bacteria, anti-fungal, and anti-inflammatory activity. Many of the beneficial health effects derived from mushrooms are triggered by their polysaccharide contents [38-46].

In this Chapter, the inhibitory effect of polysaccharides extracted from an edible mushroom, *Lentinula edodes*, on ulcerative colitis was investigated.

2.2. Materials and Methods

2.2.1. Materials

Dextran sodium sulphate (DSS, 36,000-50,000 Da) was purchased from MP Biomedicals (Canada). DEAE-sepharose CL-6B was purchased from GE Healthcare Bio-sciences AB (Sweden). *Lentinula edodes* (shiitake) was gifted by a mushrooms cultivation company in japan. Every other chemicals and reagents were standard guaranteed commercial products.

2.2.2. Extraction of polysaccharides from Lentinula edodes

Fresh fruiting bodies of *Lentinula edodes* were graciously gifted by a mushroom cultivation company in Japan (Hokuto Kinoku), and we carried out polysaccharides' extraction according to the method of Mizuno *et al* [51] with little modifications. Briefly, the fresh mushroom samples (fruiting body) were pulverized in liquid nitrogen using an Ace homogenizer (AM_7, Nihonseiki

Kaisha Ltd), and lyophilized to obtain the dried powdery sample. Twenty grams (20 g) of the dried powdery sample was extracted in 600 mL of distilled water at 100 °C for 6 h. The extracts were obtained by centrifugation at 3500 rpm, 25 ºC for 20 min, and the pellet was discarded. Ethanol (100%) in the ration of 3:1 was added to the supernatant and allowed to stand overnight at 4 °C to precipitate the polysaccharides. The supernatant and precipitate were separated by centrifugation at 3500 rpm, 4°C for 20 min, and the pellets were washed twice with 100% ethanol and centrifuged again at 3500 rpm, 4 °C for 20 min. Pellet was dissolved in distilled water and lyophilized to obtain the dried crude polysaccharides used for this study.

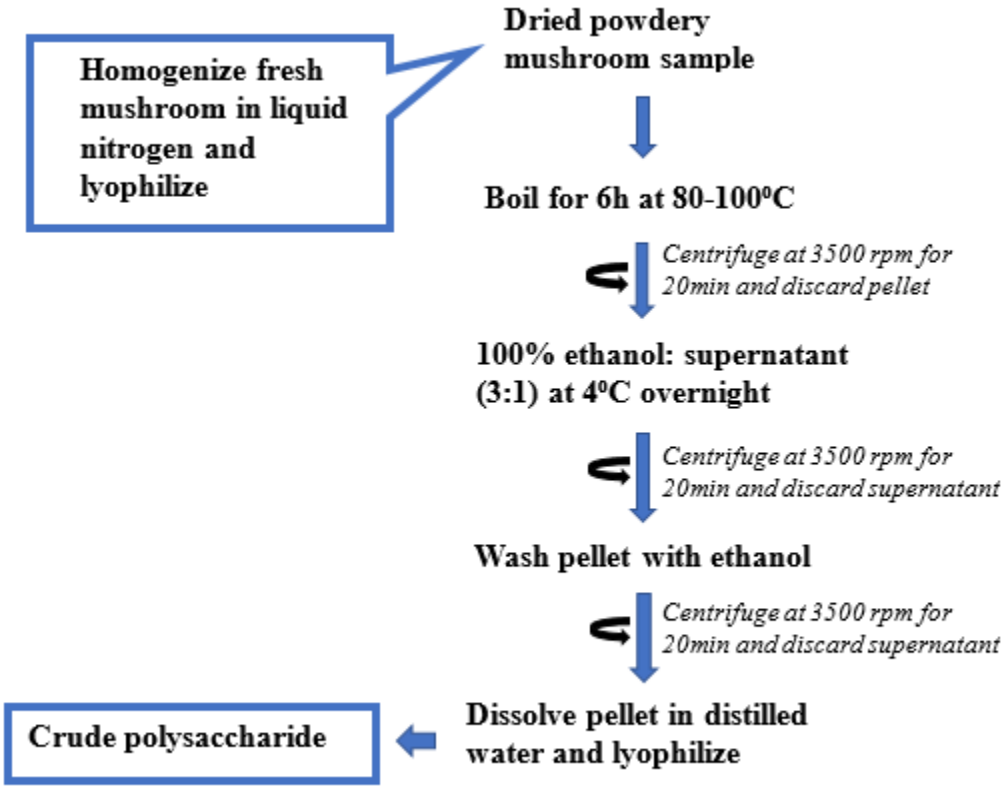


Fig. 2.1. Hot-water extraction and precipitation of crude polysaccharides from edible mushrooms

2.2.3. Chromatographic separation of polysaccharides samples

Anion exchange column chromatographic technique was used to separate the polysaccharides obtained from *Lentinula edodes*. We carried out the separation according to the method reported in Muhidinov *et al* [52]. Briefly, a slurry of DEAE-sepharose CL-6B in distilled water was packed in a column (2.4 cm × 18 cm) at a flow rate of 1 mL/min, and washed four times with distilled water to the ethanol used for preservation. The column was equilibrated with distilled water and loaded with 20 mL of 5 mg/mL of polysaccharide sample. The sample was eluted in a step wise manner with 200 mL of NaCl solution of increasing ionic strength (0.0 M, 0.5 M, and 1.0 M) at a flow rate of 1 mL/min, and eluate collected at 10ml/tube. Total carbohydrate content of the eluate in each tube was monitored at 490 nm using the phenol-sulfuric acid method [53]. The same peak fractions were pooled and dialyzed for 24 h in distilled water and lyophilized, and the two different fractions, fraction 1 (eluted with 0.0 M NaCl solution) and fraction 2 (eluted with 0.5 M NaCl solution) were labeled LeP1 and LeP2 respectively as shown in Fig. 2.3.A. The dried polysaccharides were stored at 4 ºC for subsequent use.

2.2.4. Total carbohydrate content determination

We used the phenol-sulfuric acid method [53] to determine the total carbohydrate content of the polysaccharides. Briefly, 0.4 mL of sample was mixed with 0.5 mL of 5% phenol and vortexed, followed by addition of 1 mL of concentrated sulfuric acid (H_2SO_4), vortexed, and allowed to stand for 20 min at room temperature (25 ºC). Absorbance was measured using a microplate reader (SH-9000, Corona electric, Japan) at 490 nm. D-glucose (0-100 µg/mL) was used as standard to calculate the total carbohydrate content.

2.2.5. Total protein content determination

The Lowry's method [54] was used to carry out the total protein content of the polysaccharides with some modifications. Briefly, 0.2 mL of sample was mixed with 1 mL of Lowry's solution, vortexed, and incubated at 25-30 ºC for 15 min, followed by addition of 100 µL of 1 N Folin's phenol reagent (prepared fresh), vortexed, and incubated at 25-30 ºC for 30 min. Absorbance was measured at 750 nm with a microplate reader (SH-9000, Corona electric, Japan). Bovine serum albumin (BSA) was used as standard for calculating total protein content. Lowry's solution was prepared by mixing **solution A** (4 mg/mL NaOH and 20 mg/mL Na_2CO_3 in water) and **solution B** (10 mg/mL of Potassium Sodium Tartrate and 5mg/mL $CuSO_4$ in water) in 50:1 ratio.

2.2.6. Deproteination of polysaccharides sample

Polysaccharides sample was deproteinated with trichloroacetic acid (TCA) according to the method of Trakoolpolpruek *et al* [55]. Briefly, 2mg/mL of polysaccharides was mixed with 20% TCA final concentration and stored at 4 ºC overnight. Precipitated protein was removed by centrifugation at 13,000 × g for 20 min. TCA was removed from supernatant by dialysis in distilled water for 24 h with fresh water change every 2 h. Polysaccharide was precipitated by adding three-fold of 100% ethanol overnight at 4 ºC. Precipitate was recovered by centrifugation at 3500 rpm for 20 min, washed with 100% ethanol, and lyophilized.

2.2.7. Mice

Eight-weeks old female C57BL/6 mice, purchased from Japan SLC (Shizuoka, Japan) were used for this study. Mice were acclimatized for 7 days with free access to water and food (*ad libitum*), housed in a room with controlled temperature at 23 ± 2 ºC, humidity of 50 ± 10%, and 12 h light/ dark cycle at the Life Science Laboratory, Kobe University. This study was duly approved by Kobe University Animal Care and Use Committee (approval number: 28-10-04-R1), and was carried out according to the Institution's Animal Experimentation Regulations.

2.2.8. Ulcerative colitis induction

Eight-weeks old female C57BL/6 mice were used for this experiment. DSS (dextran sulfate sodium: 36,000-50,000 Da) (2.5%, w/v), in drinking water (*ad libitum*), was used to induce ulcerative colitis in mice for 7 days according to the method described by Nishitani *et al* [19]. Mice were treated with polysaccharide samples for 7 days prior to treatment with 2.5% DSS and continued during DSS treatment for additional 10 days. Body weight of mice were recorded daily (Initial body weight at day 0 is taken as 100% (no body weight change), below 100% (body weight loss), and above 100% (body weight gain)) and mice were sacrificed by cervical dislocation at the end of treatment/administration. Colon tissues were excised and the lengths were measured as a marker for ulcerative colitis. The disease activity index (DAI), which indicate the severity of colitis, was determined according to the method shown in Jeengar *et al* [56] as shown in table 2.1.

Weight Loss		**Stool consistency**		**Hemoccult**	
Range	Score	Criteria	Score	Criteria	Score
None	0	Well-formed pellets	0	Negative	0
1-5%	1				
5-10%	2	Loose stool	2	Positive	2
10-20%	3				
>20%	4	Diarrhea	4	Gross bleeding	4

Table 2.1. Criteria for DAI scoring. The values are calculated as the sum of the scores for weight loss, stool consistency, and blood in feces.

2.2.9. Statistical analysis

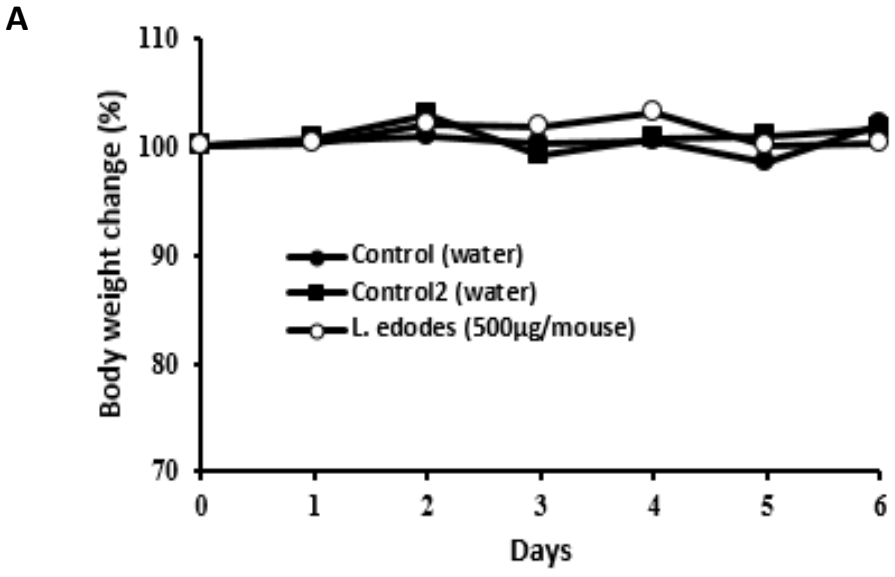
All data are expressed as means ± SD. Statistical analysis was performed by Tukey-Kramer and two-tailed standard *t* tests. Statistical significance was defined as $*p < 0.05$ and $**p < 0.01$.

2.3. Results

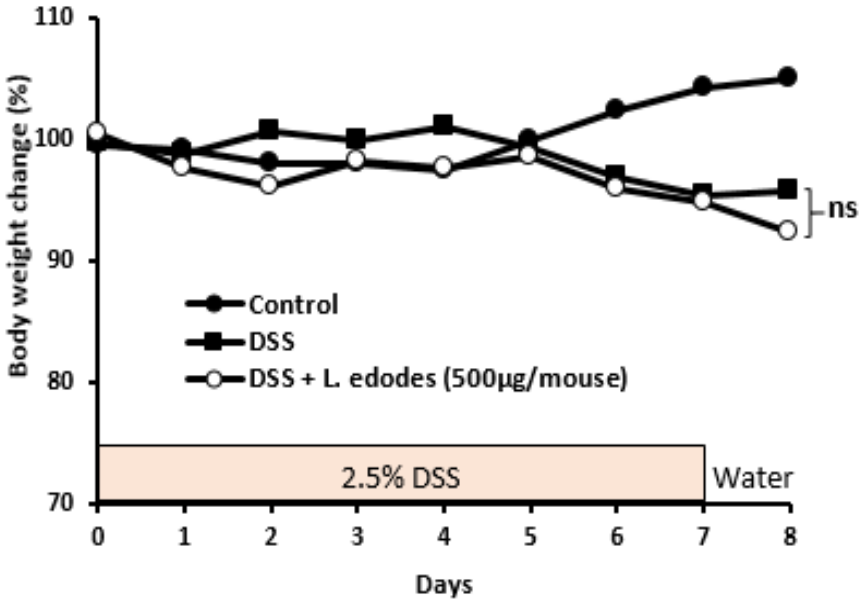
2.3.1. Polysaccharides from Lentinula edodes suppressed colitis in mice

To investigate the effect of polysaccharides extracted from *Lentinula edodes* on colitis, a DSS-induced colitis mice model was used. DSS is a relatively water soluble, negatively charged sulphated polysaccharide which is usually used as a colitogenic agent to induce acute colitis in mice. The DSS-induced colitis model is considered to be a simple model and have many similarities with human ulcerative colitis, making it a useful model for studying chronic intestinal inflammatory conditions [57] In this study, mice treated with DSS developed ulcerative colitis

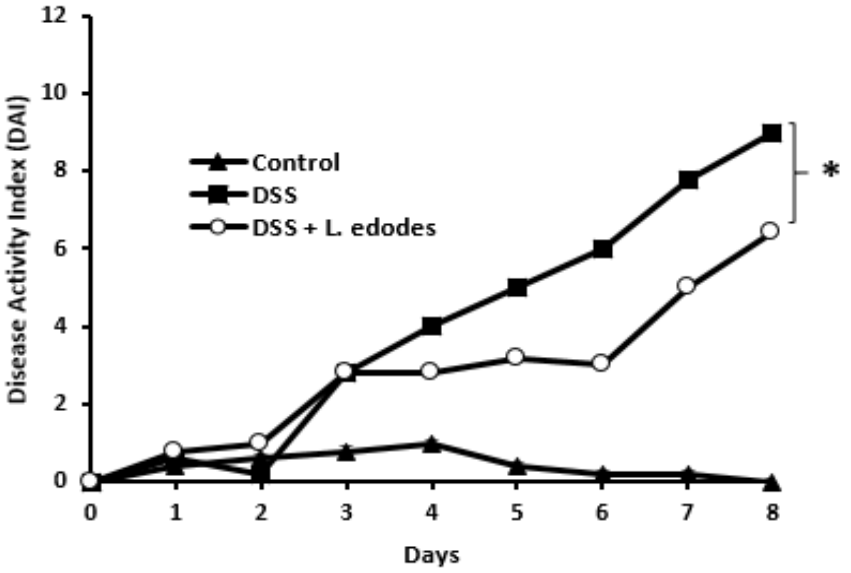
with loss in body weight, bloody diarrhea, and shortened colon length. However, in contrast to DSS-treated mice, oral administration of 500 µg/mouse polysaccharides extracts from *L. edodes* (which showed no sign of toxicity to mice, Fig. 2.2.A) significantly prevented colon length shortening (Fig. 2.2.D and E) and suppressed the severity of the disease in mice as shown by the disease activity index (DAI) (Fig. 2.2.C). DAI score indicates the severity of colitis, and was determined according to the method of Jeengar *et al* [56]. There was no significant difference in body weight of DSS-treated mice and mice orally administered with polysaccharides extracts (Fig. 2.2.B). These results suggested that polysaccharides from *Lentinula edodes* possessed inhibitory activity against ulcerative colitis in mice.



B


110
100
90
80
70
Body weight change (%)
Control
DSS
DSS + L. edodes (500µg/mouse)
ns
2.5% DSS
Water
0
1
2
3
4
5
6
7
8
Days

C


12
10
8
6
4
2
0
Disease Activity Index (DAI)
Control
DSS
DSS + L. edodes
*
0
1
2
3
4
5
6
7
8
Days

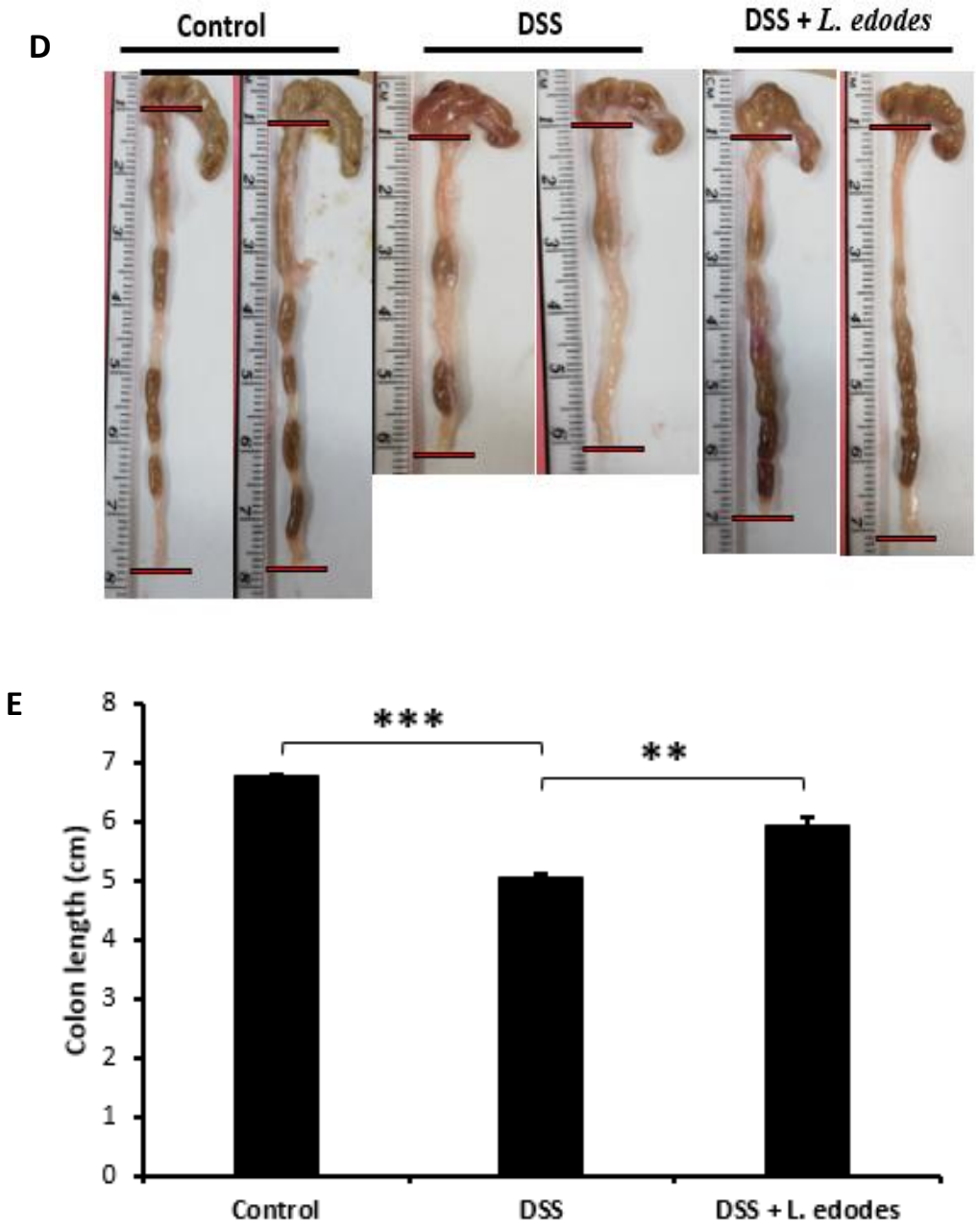
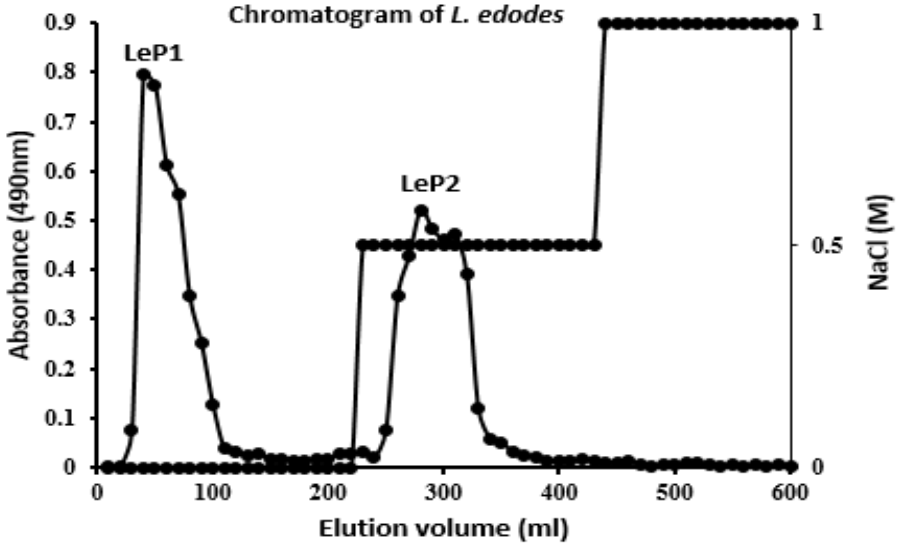


Fig. 2.2. Polysaccharides from *L. edodes* prevented colitis in mice. DSS (2.5%, w/v) was administered to mice treated with 500 μg crude polysaccharides orally for 7 days. (A) Body weight of mice administered with crude polysaccharides from *Lentinula edodes*. (B) Body weight of colitis mice treated with crude polysaccharides from *Lentinula edodes*. (C) Disease Activity index (DAI) of colitis mice treated with polysaccharides from shiitake. (D, E) Colon length of colitis mice treated with *L. edodes* polysaccharide. Values are expressed as Mean ± SD (n = 4-5). *$p < 0.05$, **$p < 0.01$, *and* ***$p < 0.001$.

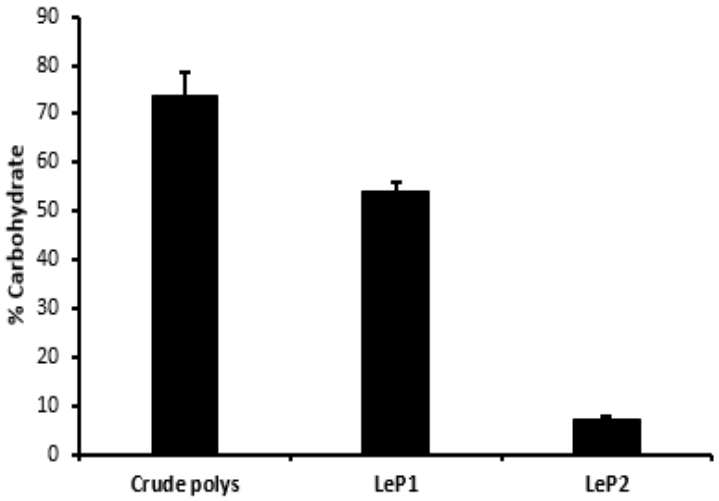
2.2.2. Anti-inflammatory activity of L. edodes polysaccharides is dependent on the carbohydrate-rich component.

The results above on polysaccharides from *L. edodes* indicated that it possessed anti-inflammatory properties against colitis in mice (Fig. 2.2 C-E), and chromatographic separation of the polysaccharides using DEAE-sepharose CL-6B suggested that it contained two different polysaccharides, LeP1 (fraction 1, eluted with 0.0 M NaCl solution) and LeP2 (fraction 2, eluted with 0.5 M NaCl solution) (Fig. 2.3.A). Carbohydrate and protein contents of LeP1 and LeP2 were carried out by phenol-sulfuric acid method and Lowry's method respectively. The result in Fig. 2.2B and C, indicated that LeP1 is a carbohydrate-rich component while LeP2 is a protein-rich component. In order to determine the component which exerted the anti-inflammatory activity above, colitis was again induced in C57BL/6 mice administered with LeP1 or LeP2 using 2.5% DSS for seven (7) days. Both LeP1and LeP2 were unable to significantly prevent body weight loss in mice (Fig. 2.3D), but their oral administration to mice resulted in a significant suppression of colon length shortening, a marker for colitis, with LeP1 showing a much stronger ability to prevent colitis in mice (Fig. 2.3E and F). Taken together, these data demonstrated that the anti-inflammatory activity of *Lentinula edodes* polysaccharides is majorly dependent on the carbohydrate-rich component.

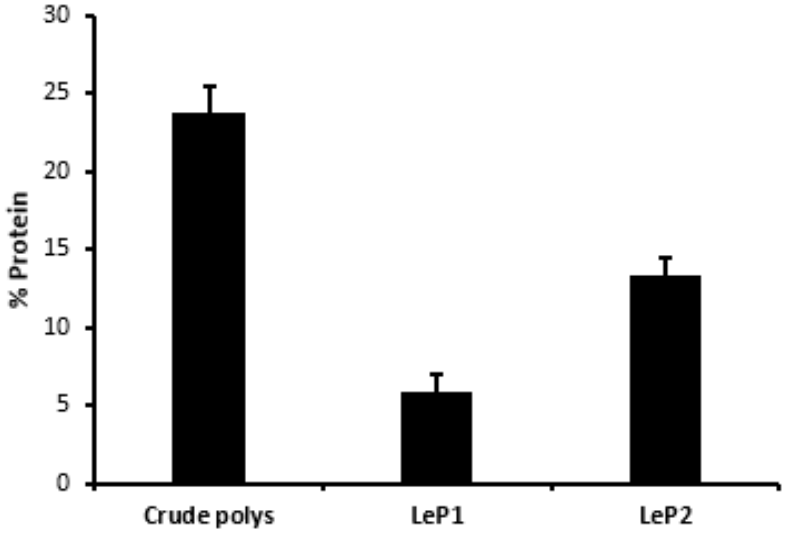
A


Chromatogram of *L. edodes*
LeP1
LeP2
Absorbance (490nm)
NaCl (M)
Elution volume (ml)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0.5
0
100
200
300
400
500
600

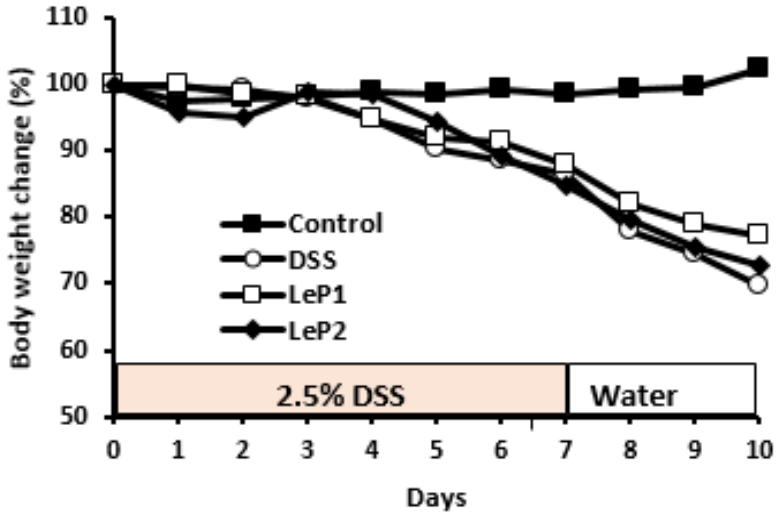
B


% Carbohydrate
0
10
20
30
40
50
60
70
80
90
Crude polys
LeP1
LeP2

C


% Protein
0
5
10
15
20
25
30
Crude polys
LeP1
LeP2

D


Body weight change (%)
110
100
90
80
70
60
50
Control
DSS
LeP1
LeP2
2.5% DSS
Water
0
1
2
3
4
5
6
7
8
9
10
Days

E


Control
DSS
DSS+LeP1
DSS+LeP2

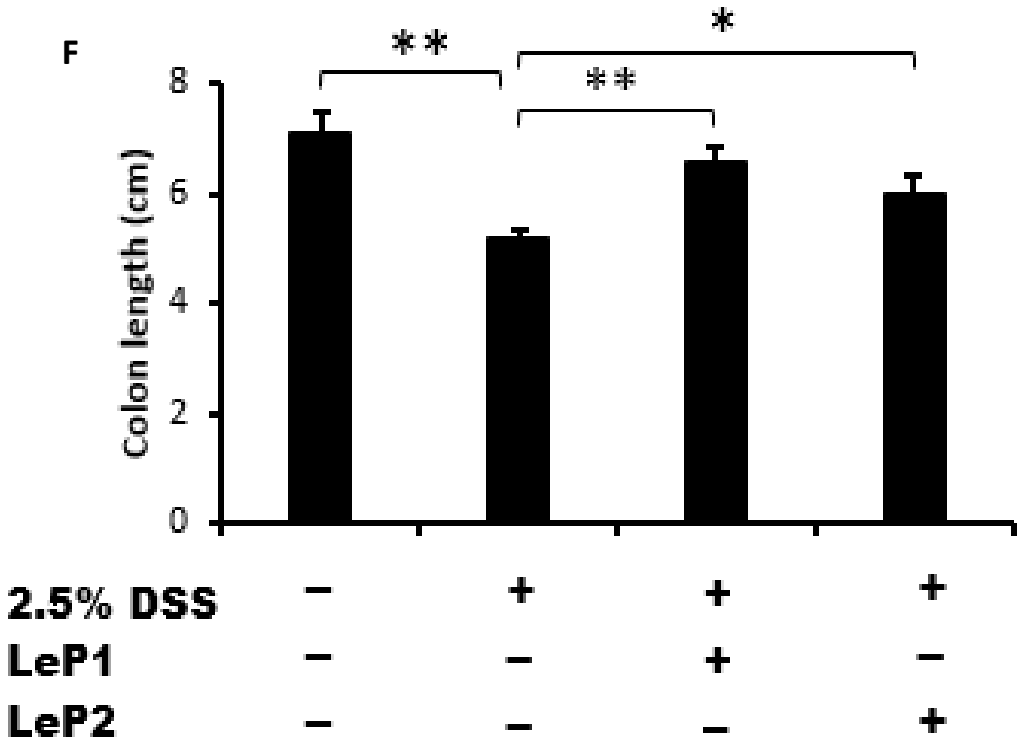
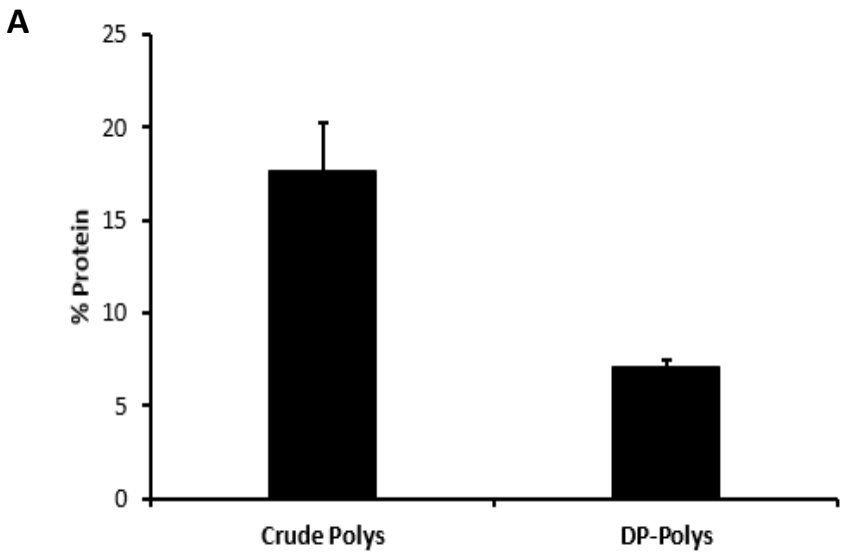


Fig. 2.3. Anti-inflammatory activity of *L. edodes* polysaccharides is dependent on the carbohydrate-rich component (LeP1). DSS (2.5%, w/v) was administered to polysaccharides (110 µg/mouse LeP1 or 365 µg/mouse LeP2)-treated mice for 7 days in drinking water. Mice were pre-treated with LeP1 or LeP2 for 7 days before colitis induction and continued for 7 days during DSS administration and an additional 3 days after DSS treatment. (A) DEAE-sepharose CL-6B column chromatographic separation of *Lentinula edodes* polysaccharides showing two peaks, LeP1 and LeP2. (B and C) Total carbohydrate and protein analysis of LeP1 and LeP2. (D) Body weight change of colitis mice treated with LeP1 or LeP2. (E and F) Colon length of colitis mice treated with LeP1 or LeP2. The different doses administered were determined from their percentage yield after chromatographic separation and lyophilization (LeP1:22% of 500 µg = 110 µg, LeP2: 73% of 500 µg = 365 µg) to represent the equivalent of 500 µg/mouse crude polysaccharides previously administered. Values are expressed as Mean ± SD (n = 4-5). *$p < 0.05$ and **$p < 0.01$.

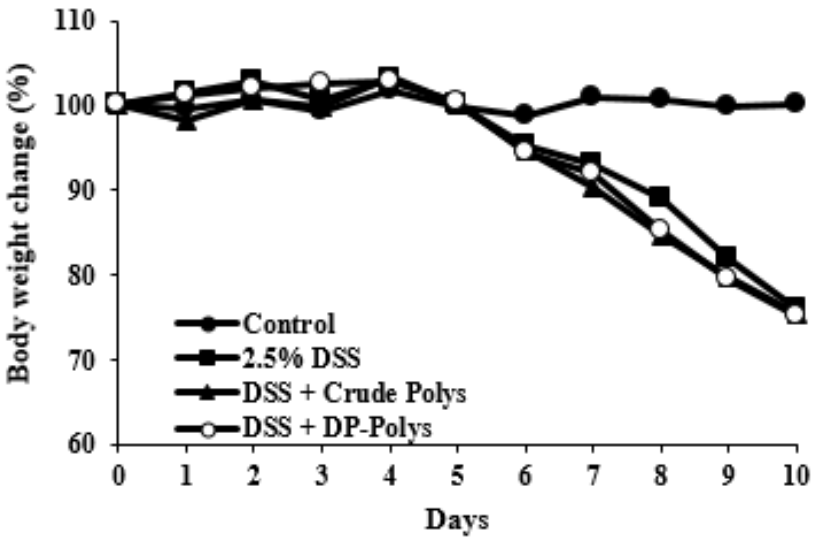
2.2.3. Deproteination did not abolish the anti-inflammatory activity of polysaccharides from Lentinula edodes.

In order to further determine whether the anti-inflammatory activity of the polysaccharide sample depended on the carbohydrate-rich component by using another method, the crude polysaccharide

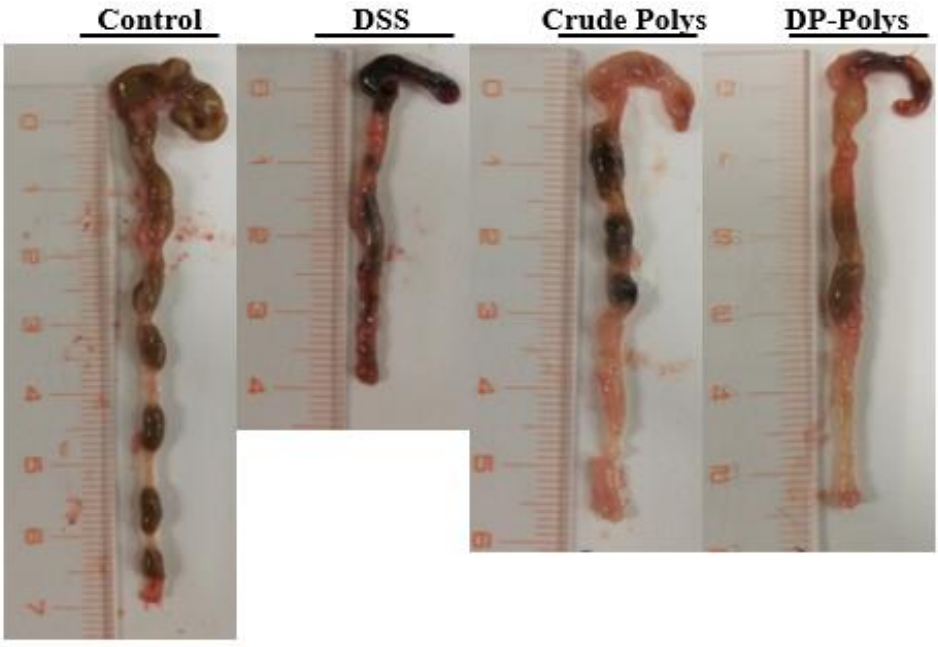
sample was deproteinated with 20% TCA final concentration overnight at 4 ºC (Fig. 2.4.A). The deproteinated polysaccharides (500 µg/mouse), named DP-Polys, was administered to DSS-induced ulcerative colitis mice for 7 days. The results obtained (Fig. 2.4.B-C) indicated that deproteinated polysaccharides suppressed ulcerative colitis in mice, as shown in the colon length, which suggested that the anti-inflammatory activity of the polysaccharide sample was independent of the protein content. Therefore, taken together with results in Fig 2.3., these data demonstrated that the carbohydrate-rich component of the polysaccharides sample was responsible for its anti-inflammatory activity.



B


Body weight change (%)
110
100
90
80
70
60
0
1
2
3
4
5
6
7
8
9
10
Days
Control
2.5% DSS
DSS + Crude Polys
DSS + DP-Polys

C


Control
DSS
Crude Polys
DP-Polys

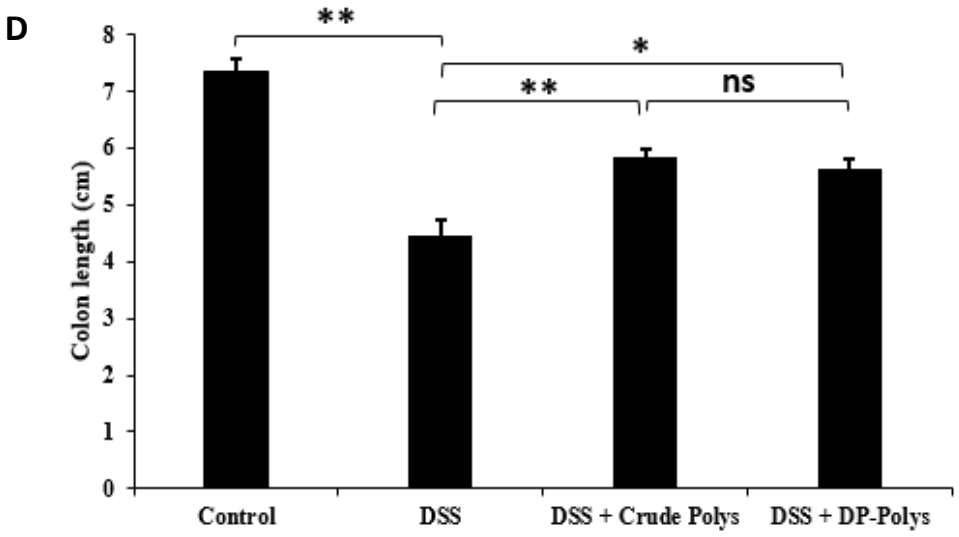
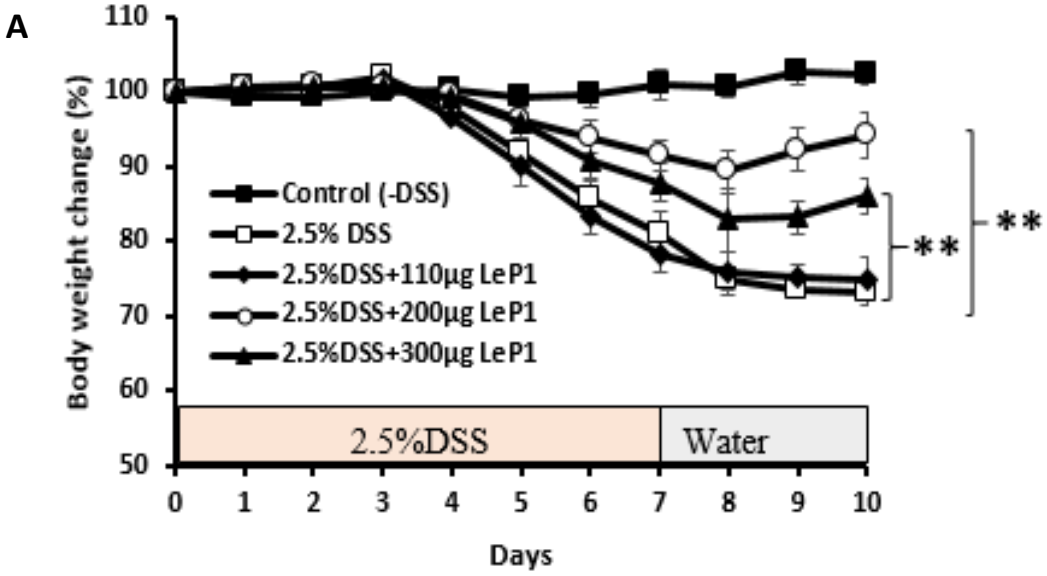


Fig. 2.4. Anti-inflammatory activity of polysaccharides from *Lentinula edodes* is independent of the protein content. DSS (2.5%, w/v) was administered to mice pre-treated with deproteinated polysaccharides (DP-Polys) (500 µg/mouse) for 7 days before colitis induction and continued for 7 days during DSS administration and an additional 3 days after DSS treatment. (A) Protein content of deproteinated polysaccharides from *L. edodes*. (B) Body weight of colitis mice treated with deproteinated polysaccharides from *L. edodes*. (C and D) Colon length of colitis mice treated with deproteinated polysaccharides from *L. edodes*. Values are expressed as Mean ± SD (n = 4-5). *$p < 0.05$ and **$p < 0.01$.

2.2.4. Anti-inflammatory activity of LeP1 from L. edodes is dose-dependent.

The results in Fig. 2.3. above, showed that LeP1 strongly suppressed colitis, but was unable to significantly prevent loss of body weight of colitis mice. To determine whether the anti-inflammatory activity of LeP1 was dependent on the administered dose. Ulcerative colitis mice were treated with different doses of LeP1 (110, 200, and 300 µg/mouse). It was observed that the ability of LeP1 to suppress body weight loss of colitis mice was dose-dependent (Fig. 2.5.A). Furthermore, shortening of colon length in colitis mice was significantly prevented, in a dose-dependent manner with the treatment of LeP1 (Fig. 2.5.B and C). These data suggested that the anti-inflammatory activity of LeP1 against ulcerative colitis is dose-dependent.


A
Body weight change (%)
110
100
90
80
70
60
50
Control (-DSS)
2.5% DSS
2.5%DSS+110μg LeP1
2.5%DSS+200μg LeP1
2.5%DSS+300μg LeP1
**
**
2.5%DSS
Water
0 1 2 3 4 5 6 7 8 9 10
Days


B
Control
DSS
110μg LeP1
200μg LeP1
300μg LeP1

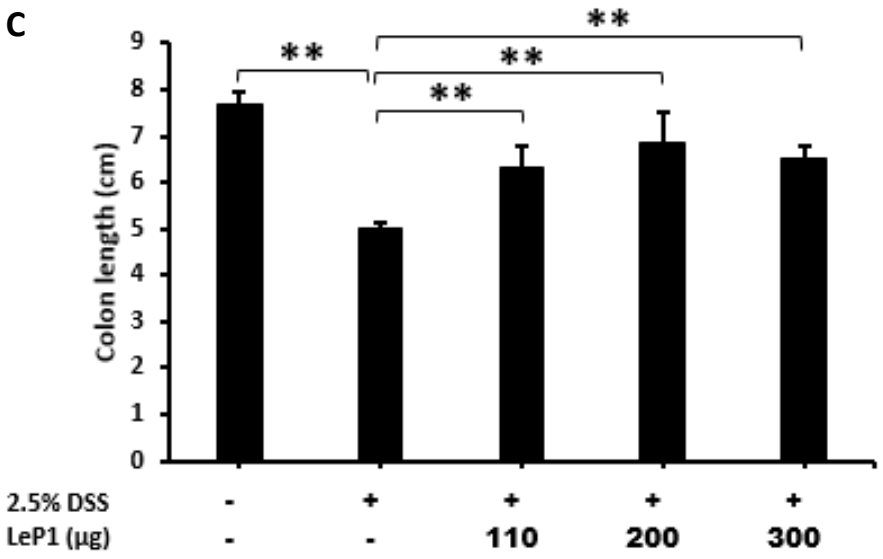


Fig. 2.5. Anti-inflammatory activity of LeP1 from *L. edodes* against colitis is dose-dependent. Mice were pre-treated with LeP1 (110, 200, or 300 µg/mouse) for 7 days before colitis induction with 2.5% (w/v) DSS and continued for 7 days during colitis induction and an additional 3 days after DSS treatment. (**A**) Body weight change of colitis mice treated with different doses of LeP1. (B and C) Colon length of ulcerative colitis mice treated with different doses of LeP1. Values are expressed as Mean ± SD (n = 4). *$p < 0.05$ and **$p < 0.01$.

2.4. Discussion

The incidence of inflammatory bowel disease has continued to increase worldwide, such that it has become a global concern. The pathogenesis is not yet fully understood, and there is no cure for the disease yet [19]. Even though some pharmaceutical drugs, such as anti-TNF-α agents (e.g. infliximab), aminosalicylates (e.g. sulfasalazine: 5-aminosalicylic acid), corticosteroids (e.g. prednisolone and budesonide), immunosuppressors (e.g. azathioprine, methotrexate, and cyclosporin), and antibiotics (e.g. metronidazole, ciprofloxacin, and rifaximin) are currently been used to manage inflammatory bowel disease, a serious adverse side effects results from their use,

which in some cases are life threatening than the disease itself [58]. Therefore, it is imperative to search for a more effective treatment with little or no adverse side effect.

Mushrooms have long served as a useful source of medicine. Medicinal compounds derived from mushrooms range from polysaccharides, proteins, polyphenols, terpenoids, flavonoids, and alkaloids. Recent reports have indicated that these compounds exerted beneficial health effects in both *in vivo* and *in vitro* studies. The results of this study are in agreement with other reports on the health effects of mushrooms [42-45].

In this study, crude polysaccharides derived from an edible mushroom, *Lentinula edodes*, was administered to ulcerative colitis mice to investigate its anti-inflammatory properties, and the data obtained indicated that the crude polysaccharide significantly prevented ulcerative colitis in mice (Fig. 2.2-2.4).

The results in Fig 2.3 and Fig. 2.4 indicated that the active component of the polysaccharides is the carbohydrate-rich fraction (LeP1). Moreover, we demonstrated that deproteination of the polysaccharides did not abolish the anti-inflammatory activity, thereby indicating that the anti-inflammatory activity was independent of the protein content (Fig. 2.4). These data are in agreement with the reports of Nishitani *et al* [19] who demonstrated that lentinan, a β-1,3:1,6-glucan, extracted from *Lentinula edodes*, suppressed ulcerative colitis in mice by inhibiting the expression of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-6, and IL-1β. Furthermore, we demonstrated that the anti-inflammatory activity of the polysaccharides was dose-dependent (Fig. 2.5), which also is in agreement with the reports of other studies [19, 43]. These data indicated that the carbohydrate-rich fraction (LeP1) of *Lentinula edodes* polysaccharides prevented ulcerative colitis in mice in a dose-dependent manner, and that these effects are independent of the

protein content. Therefore, these results suggested that dietary intake of *Lentinula edodes* by the victims of ulcerative colitis could be useful in managing the disease condition.

CHAPTER 3

Differential Activity of Polysaccharides from Strains of *Lentinula edodes* against Ulcerative Colitis

3.1. Introduction

The structure, bioactivity, and molecular weight of bioactive compounds in bacteria and fungi have been reported to be affected by their growth media composition. Studies have shown that the structures of bioactive compounds in mushrooms and bacteria could be manipulated by altering the percentage of some of the components of the growth media, or by adding some specific molecules into the growth media which in turn are incorporated into the structure their bioactive compounds during synthesis [59]. Furthermore, the report of Enman *et al* indicated that different strains of *Lentinula edodes* possessed different bioactive compounds [48], and the structural features of *Ganoderma lucidum* was reported to be influenced by growth media compositions [59]. The physiological properties of polysaccharides are dependent on the primary structure, solubility, degree of branching, molecular weight, charge of polymer, and structure in aqueous media [60]. Recent studies have shown that not all polysaccharides from mushrooms are capable of modulating the immune system [51]. Perhaps what confers glucans from mushrooms the ability to modulate the immune system by binding to dectin-1 is the presence of β-1,3- glycosidic linkages and more strongly with β-1,6 branches [61, 62]. Researchers have not yet been able to determine the precise oligosaccharide moiety which specifically binds to dectin-1 to trigger the immune reactions, and when this is eventually elucidated, it will perhaps open up a brand-new area of research.

In this chapter, the anti-inflammatory activity of polysaccharides from two other strains of *Lentinula edodes* against ulcerative colitis was investigated in order to ascertain whether polysaccharides from different strains exerted similar physiological activity.

3.2. Materials and Methods

3.2.1. Materials and reagents

Dextran sodium sulphate (DSS, 36,000-50,000 Da) was purchased from MP Biomedicals (Canada). DEAE-sepharose CL-6B was purchased from GE Healthcare Bio-sciences AB (Sweden). Two strains of *Lentinula edodes* (shiitake), named LeA and LeB, were gifted by two different mushrooms cultivation companies in japan. Every other chemicals and reagents were standard guaranteed commercial products.

3.2.2. Extraction of polysaccharides from strains of Lentinula edodes

Crude polysaccharides were extracted from the fruiting bodies of two strains of *Lentinula edodes* (LeA and LeB) according to the method outlined in chapter 2. Crude polysaccharides extract from the two strains were labeled LeAP and LeBP respectively.

3.2.3. Induction of ulcerative colitis

Mice were purchased and bred as described in chapter 2. Prior to ulcerative colitis induction with 2.5% DSS (w/v) in drinking water, mice were orally administered with 500 µg/mouse of each of

the polysaccharide samples prepared in section 3.2.2. Polysaccharide sample from previous strain of *Lentinula edodes* used in chapter 2 (Le) served as the positive control. Body weights of mice were recorded daily and colon lengths were measured after mice sacrifice.

3.2.4. Chromatographic separations of LeAP and LeBP

Crude polysaccharides samples (LeAP and LeBP) from two strains of *Lentinula edodes* (LeA and LeB) respectively, were subjected to anion column chromatographic separation using DEAE-sepharose CL-6B resin according to the method reported in Muhidinov *et al* [51] as outlined in chapter 2. Separation of LeAP resulted in two fractions named LeAP1 (fraction 1: eluted with 0.0 M NaCl solution) and LeAP2 (fraction 2: eluted with 0.5 M NaCl solution), while separation of LeBP resulted in three fractions named LeBP1 (fraction 1: eluted with 0.0 M NaCl solution), LeBP2 (fraction 2: eluted with 0.5 M NaCl solution), and LeBP3 (fraction 3: eluted with 1.0 M NaCl solution) as shown in Fig. 3.4.

3.2.5. Statistical analysis

All data are expressed as means ± SD. Statistical analysis was performed by Tukey-Kramer and two-tailed standard *t* tests. Statistical significance was defined as $*p < 0.05$ and $**p < 0.01$.

3.3. Results

3.3.1. Morphological appearance and polysaccharide yield of LeA and LeB are different

Fruiting bodies of the two strains of *Lentinula edodes* (LeA and LeB) appeared morphologically different, in color, texture, and size, which indicated some forms of genetic differences in the mushrooms which resulted in altered morphology (Fig. 3.1 A and B). Furthermore, upon hot water

extraction, the polysaccharide yield of the strains was different, with LeB having the highest yield as seen in Fig. 3.1 C.

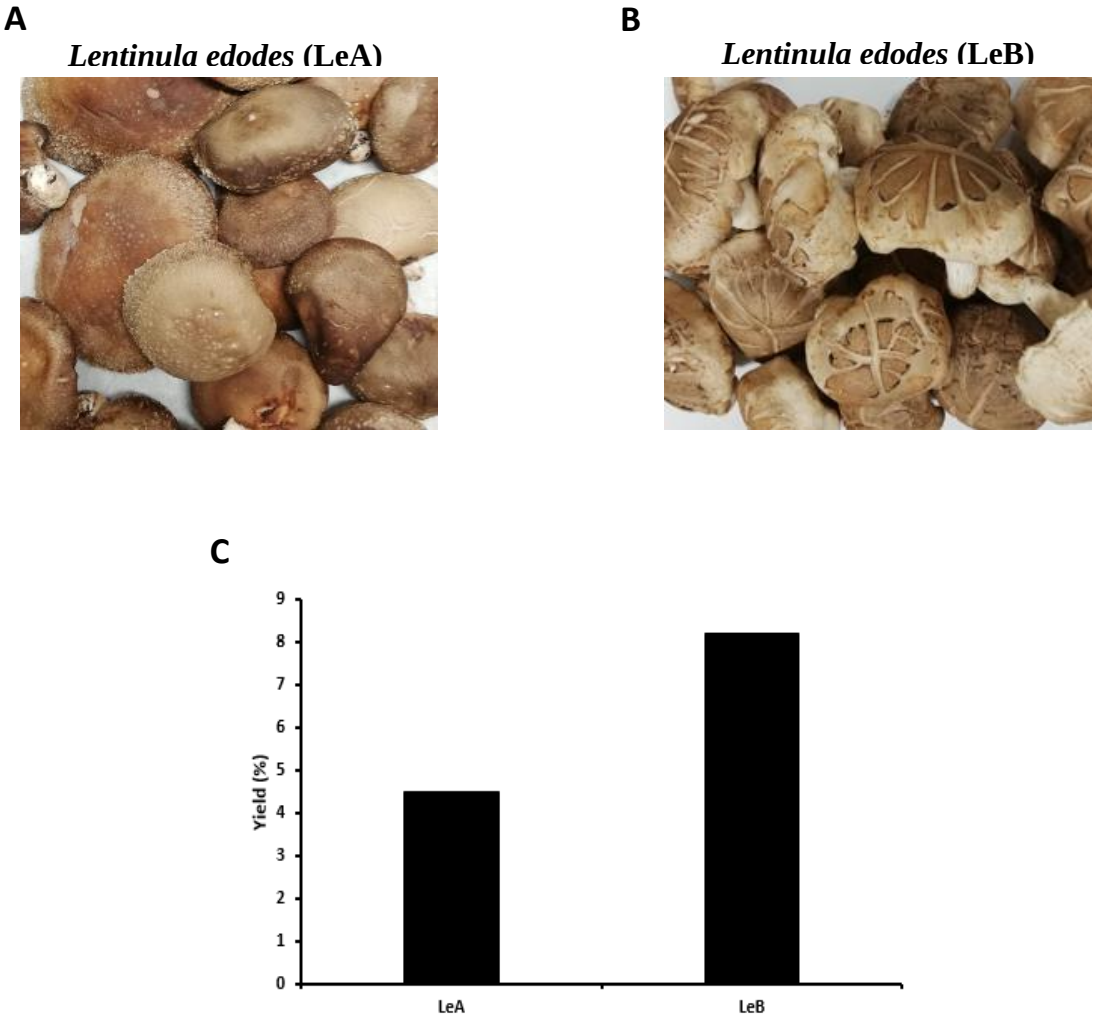


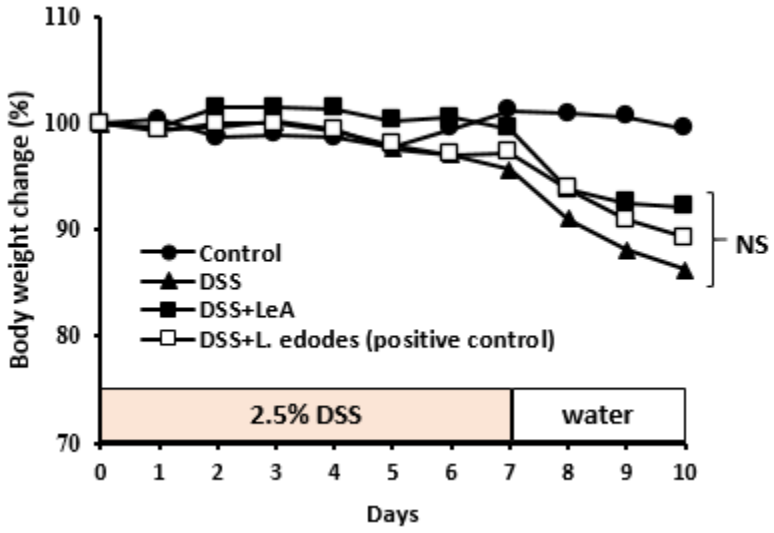
Fig. 3.1. Different appearance of strains of *Lentinula edodes*. (A) Fruit bodies of strain LeA. (B) Fruit bodies of strain LeB. (C) Crude polysaccharides yield of hot water extraction from LeA and LeB.

3.3.2. Crude polysaccharides from LeA prevented ulcerative colitis in mice

To investigate the anti-inflammatory activity of crude polysaccharides (LeAP) from LeA and compare with previous strain of *Lentinula edodes* in chapter2, LeAP (500 µg/muse) was orally

administered to ulcerative colitis mice. The results obtained indicated that LeAP significantly prevented DSS-induced colitis in mice, as seen in the colon length (Fig. 3.2. B and C) and also in the body weight (Fig. 3.2. A). These data suggested that LeAP possessed anti-inflammatory activity against ulcerative colitis comparable to the activity exerted by the crude polysaccharides from previous strain of *Lentinula edodes* (positive control) in chapter 2.

A



B

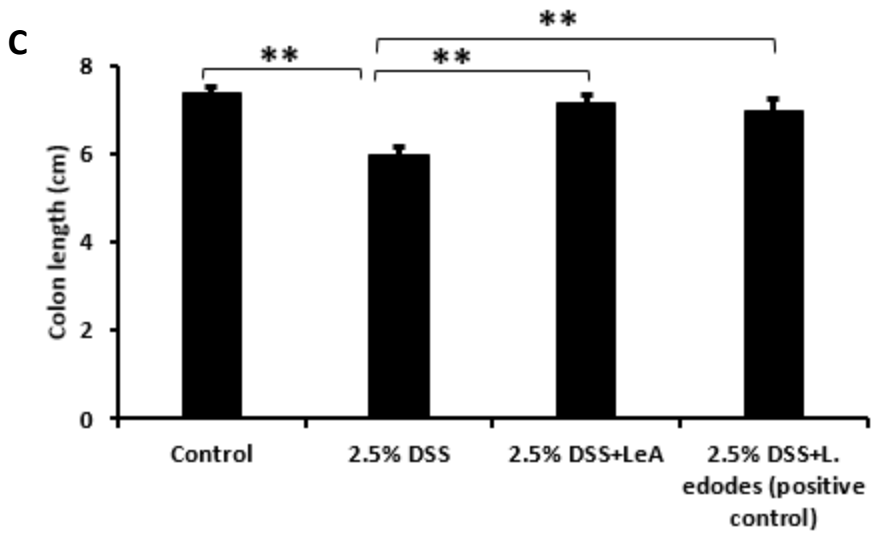


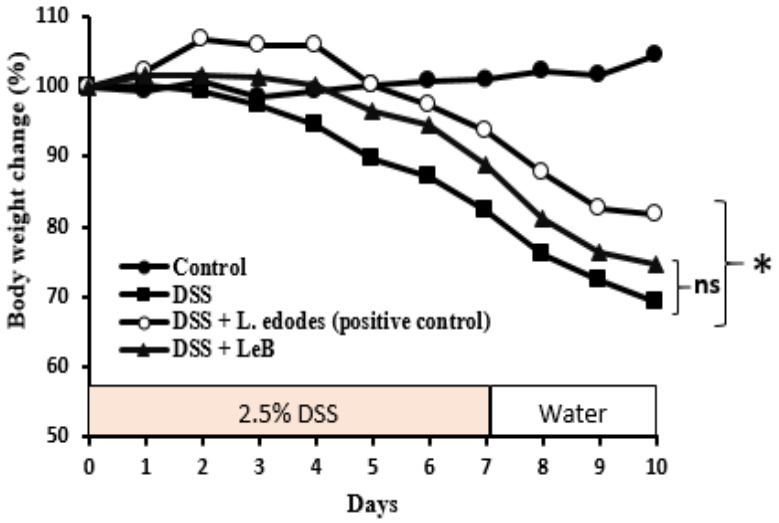
Fig. 3.2. Crude polysaccharides (LeAP) from *Lentinula edodes* strain LeA suppressed ulcerative colitis. Crude polysaccharides (LeAP: 500 µg/mouse) was administered to mice prior to colitis induction (with 2.5% DSS (w/v) in drinking water) for 7 days and during colitis induction for another 7 days, followed by additional 3 days of administration. (A) Body weight of colitis mice treated with LeAP. (B and C) Colon length of colitis mice treated with LeAP. Values are expressed as Mean ± SD (n = 5). *$p < 0.05$ and **$p < 0.01$.

3.3.3. Crude polysaccharides (LeBP) from Lentinula edodes strain LeB failed to prevent ulcerative colitis

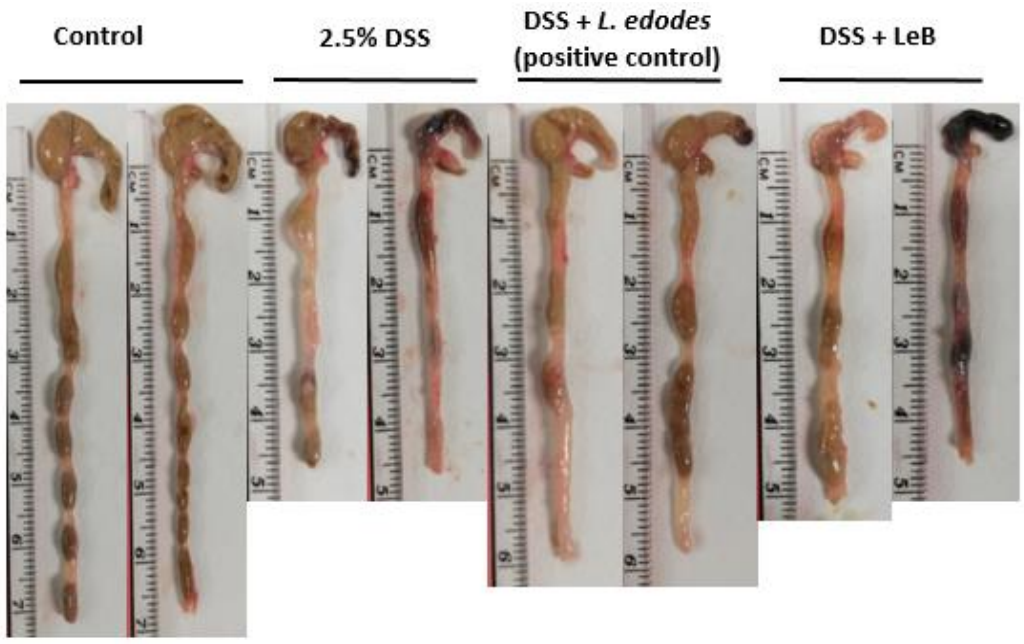
To investigate the anti-inflammatory activity of crude polysaccharides (LeBP) from strain LeB of *Lentinula edodes*, 500 µg/mouse of LeBP was orally administered to DSS-induced ulcerative colitis mice for 7 days. The results obtained indicated that LeBP did not prevent ulcerative colitis in mice. The data indicated that LeBP was unable to prevent DSS-induced body weight loss and colon shortening in mice (Fig. 3.3. A-C), which suggested that LeBP did not possess anti-inflammatory ability against ulcerative colitis. These data are different from the results obtained with crude polysaccharides extract from *Lentinula edodes* strain used in chapter 2 (LeP, positive

control) and LeA (LeAP: Fig. 3.2), which suggested that the anti-inflammatory activity may be strain-dependent.

A



B



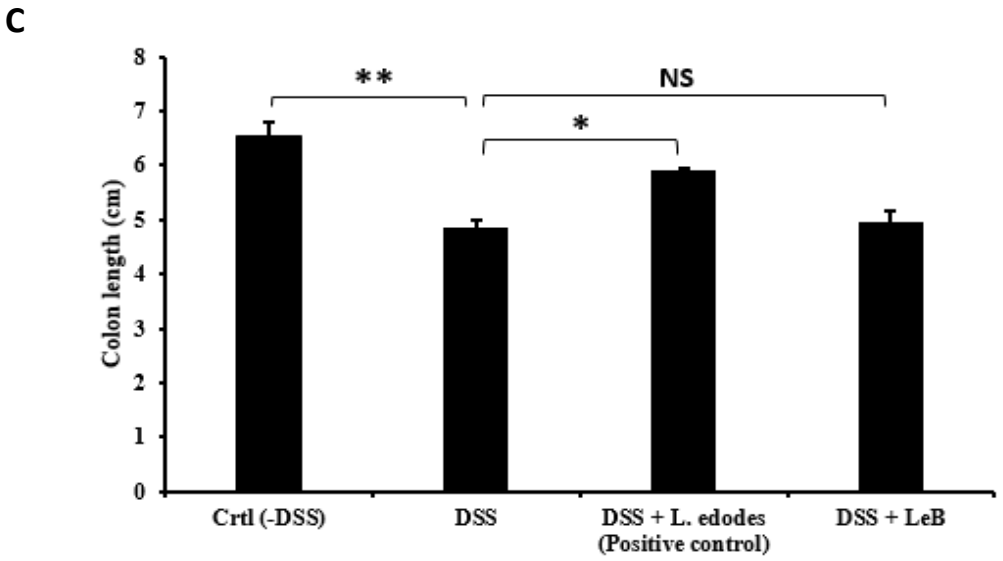


Fig. 3.3. Crude polysaccharides (LeBP) from *Lentinula edodes* strain LeB Failed to inhibit ulcerative colitis. Crude polysaccharides (LeBP: 500 µg/mouse) was administered to mice prior to ulcerative colitis induction (with 2.5% DSS (w/v) in drinking water) for 7 days and during colitis induction for another 7 days, followed by additional 3 days of administration. (A) Body weight of colitis mice treated with LeBP. (B and C) Colon length of colitis mice treated with LeBP. Crude polysaccharides from previous strain of *L. edodes* (chapter 2) that possessed anti-inflammatory activity was used as the positive control. Values are expressed as Mean ± SD (n = 4). *$p < 0.05$ and **$p < 0.01$.

3.3.4. Chromatographic separation of crude polysaccharides, LeAP and LeBP, showed different elution peaks

To further investigate the reason for the differential anti-inflammatory activity exhibited by crude polysaccharides from the different strains of *Lentinula edodes*, the polysaccharides were subjected to DEAE-sepharose CL-6B column chromatographic separation. The results obtained indicated that the elution profiles of the crude polysaccharides, LeAP and LeBP, from the two strains appeared different. The elution profile of LeAP showed two peaks (Fig. 3.4. A), while the elution

profile of LeBP showed three peaks (Fig. 3.4. B). These data suggested that the two strains of *Lentinula edodes*, LeA and LeB, may possess polysaccharides that are not similar.

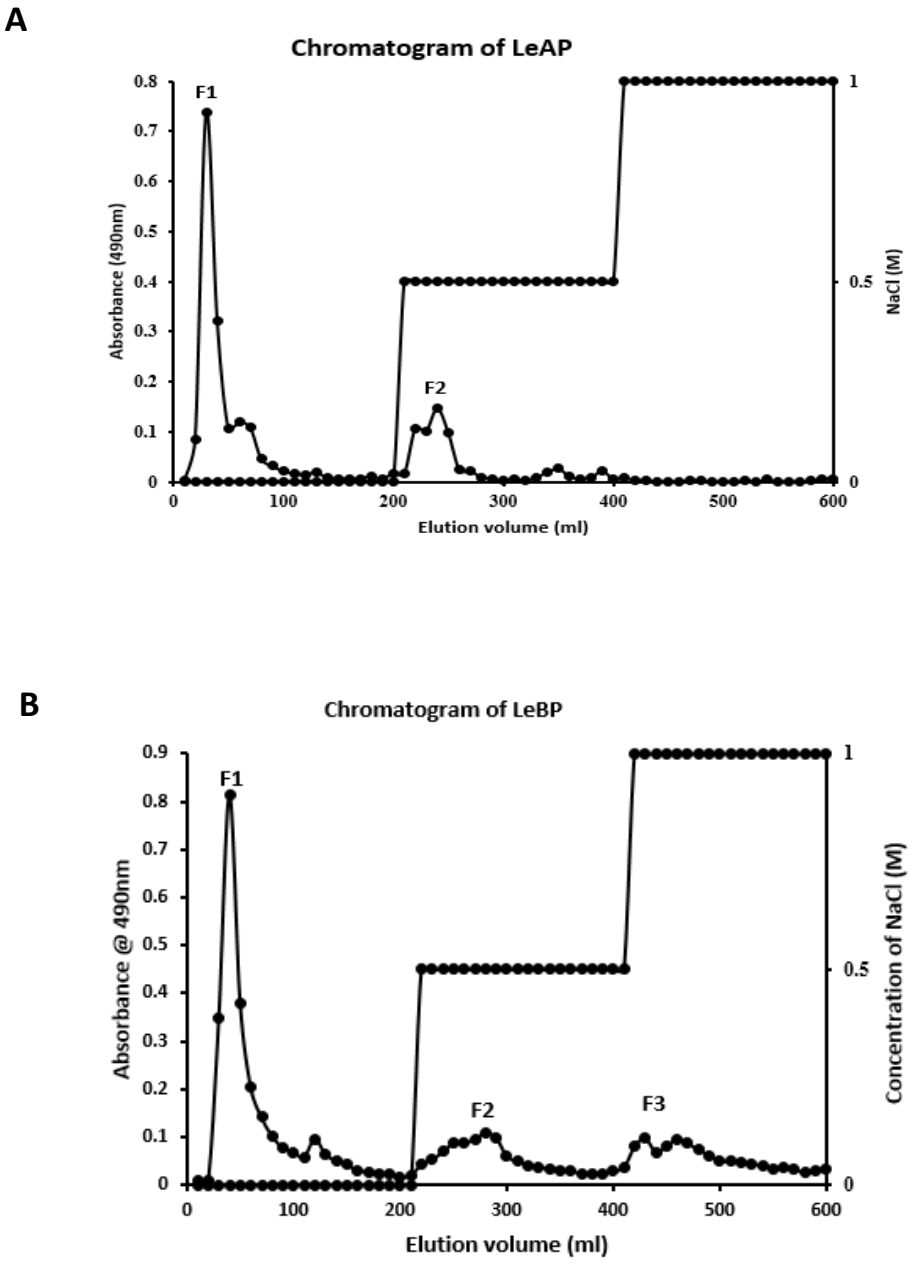


Fig. 3.4. Differential elution profiles of crude polysaccharides from strains of *Lentinula edodes*. Crude polysaccharides, LeAP and LeBP, were separated using DEAE-sepharose CL-6B column chromatography. NaCl solution of increasing ionic strength (0.0, 0.5, and 1.0 M) was used to elute the column. (A) Elution profile of LeAP showing two peaks (fractions LeAP1 (fraction 1)

and LeAP2 (fraction 2)). (B) Elution profile of LeBP showing three peaks (fractions LeBP1 (fraction 1), LeBP2 (fraction 2), and LeBP3 (fraction 3)).

3.4. Discussion

The physiological properties of polysaccharides are dependent on the primary structure, solubility, degree of branching, molecular weight, charge of polymer, and structure in aqueous media [60]. A report by Enman *et al* [48] indicated that strains of *Lentinula edodes* possessed different bioactive compounds.

Physical observation of two strains of *Lentinula edodes*, indicated that the morphological features appeared different, and the polysaccharides yield from hot water extraction of the mushrooms was equally different (Fig. 3.1). The two strains looked different in their color, texture, and size. These observations suggested that there may be some differential genetic alterations which may have accounted for their different appearances. `

To investigate whether polysaccharides from the different strains exerted similar activity against ulcerative colitis, colitis mice were orally administered with the different polysaccharides, LeAP and LeBP (Fig. 3.2 and Fig. 3.3). The result obtained indicated that LeAP prevented ulcerative colitis but LeBP did not, suggesting that the two strains may possess different kinds of polysaccharides. The results obtained with LeAP is comparable to results obtained with previous strain of *Lentinula edodes* (chapter 2). These data suggested that the anti-inflammatory activity may be dependent on the strain.

Furthermore, chromatographic separation of the crude polysaccharides showed elution profiles which appeared different with LeAP showing two peaks while LeBP showed three peaks. This is

an indication that the polysaccharides in the two strains may have some difference in their structural features, although more structural studies are needed to clarify this observation.

Therefore, this study demonstrated that the anti-inflammatory activity of polysaccharides from *Lentinula edodes* may be dependent on the strain.

CHAPTER 4

Anti-necroptosis Activity of Polysaccharide from *Lentinula edodes*

4.1. Introduction

Necroptosis is a newly described form of pro-inflammatory necrotic cell death, which has been implicated in the pathogenesis of IBD, and it is actively involved in colitis and other inflammatory disease conditions [24, 36, 37]. It is a caspase-independent immunogenic form of programmed cell death [24]. It is morphologically and mechanistically different from apoptosis, because unlike apoptosis, necroptosis involves cell swelling, membrane rupture, and release of DAMPs (damage associated molecular patterns), while apoptosis involves cell shrinkage, membrane blebbing, formation of apoptotic bodies, DNA fragmentation, and chromatin condensation [63]. Apoptosis has been considered an immunologically silent and anti-inflammatory cell death, while necroptosis is known as a pro-inflammatory cell death because upon membrane rupture, cellular contents are released into the microenvironment of cells which in turn trigger immune reactions. Apoptosis, on the other hand, does not involve the release of cellular contents which are held intact within the apoptotic bodies and phagocytosed by phagocytic cells like macrophages [37].

Necroptosis is an alternative path of cell death when apoptosis is inhibited in cells, and it is usually triggered in cells by the stimulation of cell surface receptors such as TNFR1, TLR3, TLR4, Fas, and TRAIL by their respective exogenous ligands in the absence or pharmacologic inhibition of

caspase-8, or by recognition of viral or endogenous RNA by Z-DNA/RNA binding protein 1 (ZBP1) [28-31]. Being caspase-independent, it is mediated by the activities of receptor interacting protein kinases known as RIPK1, RIPK3, and MLKL (mixed lineage kinase domain-like pseudokinase). Stimulation of cell surface death receptors results in the phosphorylation and activation of RIPK1, RIPK3, and MLKL [30, 32]. Phosphorylated MLKL (pMLKL), known as the necroptosis executor, undergoes oligomerization and translocate to the plasma membrane where it binds to phosphatidylinositol phosphates to induce necroptosis [33, 34]. Recent studies have shown that mice developed spontaneous intestinal inflammation upon conditional knockout of *caspase-8* or *FADD*, but were rescued with deletion of RIPK3 or MLKL [64-66]. The expression level of caspase-8 has been reported to be significantly reduced in the colon of IBD patients [36, 67]. These reports suggested that necroptosis played important role in the pathogenesis of IBD. Therefore, in this study, the involvement of necroptosis in ulcerative colitis was determined and the effect of polysaccharide from *Lentinula edodes* on necroptotic cell death was investigated using both *in vivo* and *in vitro models*.

4.2. Materials and Methods

4.2.1. Reagents

Dextran sodium sulphate (DSS, 36,000-50,000 Da) was purchased from MP Biomedicals (Canada). Dulbecco's Modified Eagle Medium (DMEM) containing glutamine and glucose (4.5 g/L) was purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). RPMI 1640 medium and MEM non-essential amino acids (NEAA) were purchased from Nissui Pharmaceutical (Tokyo, Japan) and Gibco (Grand Island, NY, USA) respectively. FBS (fetal

bovine serum) was purchased from Biological Industries (Beit, Israel). Recombinant human tumor necrosis factor-α (rhTNF-α) was purchased from PeproTech (Rocky Hill, NJ, USA). Necrostatin-1 (Nec-1) and zVAD-fmk (pan-caspase inhibitor) were purchased from Abcam (ab141053) and Selleck (Tokyo, Japan) respectively. MTT was purchased from Nacalai Tesque (Kyoto, Japan), mouse anti-β-actin antibody was from Santa Cruz Biotechnology (Delaware Avenue, CA), rabbit monoclonal antibody against human pMLKL (ab187091) and mouse pMLKL (ab196436) were purchased from Abcam. Mouse HRP-conjugated anti-IgG and rabbit HRP-conjugated anti-IgG were purchased from R&D Systems (Minneapolis, USA) and Cell Signaling Technology (Danvers, MA, USA) respectively.

4.2.2. *Extraction of polysaccharides from Lentinula edodes*

Crude polysaccharides used for this study was obtained from the powdery fruit bodies of *Lentinula edodes* by hot water extraction and ethanol precipitation as described in section 2.2.2 of chapter 2.

4.2.3. Chromatographic separation of crude polysaccharides

Crude polysaccharide was separated using DEAE-sepharose CL-6B column chromatography as described in section 2.2.3 of chapter 2. The two fractions obtained were named LeP1 and LeP2.

4.2.4. Colitis-associated necroptosis induction

Mice were purchased and bred as described in chapter 2. Colitis-associated necroptosis was induced in mice using 2.5% of DSS (w/v) in drinking water for 7 days. Polysaccharide samples

(Crude: 500 µg/mouse, LeP1: 110 µg/mouse, LeP2: 365 µg/mouse) were orally administered to mice prior to (7 days) and during DSS administration (7 days), followed by additional 3 days administration of the polysaccharide samples. Furthermore, increasing dose of LeP1 (110, 200, and 300 µg/mouse) were orally administered to mice to determine the dose-dependent effect of the polysaccharide sample following the same procedure as above.

4.2.5. Cell culture

Caco-2 cells (intestinal epithelial cell line) were cultured in a 75 cm^3 plastic flask containing 10 mL of DMEM (4.5 g/L glucose) supplemented with 10% FBS, 1% MEM-NEAA, 100 µg/mL streptomycin, and 100 U/mL penicillin. The cells were incubated at 37 °C in a 5% CO_2 incubator. New culture medium was changed every other two days. Cells were passaged at 80% confluence. Trypsin was used to release cells from the bottom of the flask after washing with phosphate-buffered saline (PBS), and re-plated in a new flask.

4.2.6. Cell death induction

Caco-2 cells were cultured in DMEM (4.5 g/L glucose) with fresh medium change every two days until the cells reached 80% confluence. The method of Dong *et al* [68] and Lou *et al* [69] was adopted with modifications. Briefly, the cells were harvested with trypsin and 100 µL of 2×10^5 cells/ml were seeded onto 96-well plates and incubated overnight at 37 ºC and 5% CO_2. Cell death was induced with increasing concentration of TNF-α (20, 50, and 100 ng/mL). For necroptotic cell death induction, Caco-2 cells were incubated with 50 µM zVAD, 50 µM zVAD plus 20 µM

necrostatin-1 (Nec-1), 50 µM zVAD plus 200 µg/mL or 500 µg/mL LeP1 for 2 h prior to TNF-α stimulation for an additional 24 h. Cell death was determined by MTT assay.

4.2.7. Measurement of cell death

Cell death analysis was performed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, 100 µL of Caco-2 cells (2×10^5 cells/mL) in RPMI 1640 (supplemented with 10% FBS, 100 µg/mL streptomycin, and 100 U/mL penicillin) were seeded in 96-well plate and incubated overnight at 37 ºC and 5% CO_2. Media was replaced with 100 µL of fresh media containing the different treatments, followed by additional incubation for 24 h at 37 ºC and 5% CO_2. Fresh media containing MTT at a final concentration of 0.25 mg/mL was added after removal of the old media, and incubated for 4 h at 37 ºC and 5% CO_2. The MTT-containing media was then replaced with Dimethyl Sulfoxide (DMSO, 100 µL/well) and incubated again at 37 ºC and 5% CO_2 for 20 min. Absorbance was measured at 570 nm with a microplate reader (SH-9000, Corona electric, Japan). Percentage cell survival was calculated with the formula: Cell survival (%) = $(A_a - A_o)/ (A_b - A_o) \times 100$, where A_a is absorbance of the test well, A_b is absorbance of the control well, and A_o is the absorbance of blank well.

4.2.8. Western blot analysis

Colon tissue samples (4-5 mg) were homogenized in ice-cold RIPA (radioimmunoprecipitation) buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Nonidet-P40, 0.5% Sodium deoxycholate, and 0.1% SDS) containing PMSF and a cocktail of protease and phosphatase inhibitors (aprotinin,

leupeptin, Na Fluoride, and DTT). Protein quantification was carried out by Lowry's method, and equal amounts of total protein (20 µg) were separated with SDS/PAGE (Sodium dodecyl sulfate/polyacrylamide gel electrophoresis) (10% gels) under reducing conditions (2× Laemmli buffer: 4% SDS, 10% 2-mercaptoethanol, 20% glycine, 0.004% bromophenol blue, and 0.125 M Tris-HCl (pH 6.8)). After electrophoresis, the proteins were transferred onto PVDF (polyvinylidene fluoride) membranes in running buffer (25 mM Tris base, 190 mM glycine, and 0.1% SDS (pH 8.3)) with 10% methanol. The membrane was washed four times in TBST (Tris-buffered saline containing 0.1% Tween 20) for 5 min each. Non-specific sites were blocked with 4% (w/v) BSA (bovine serum albumin) in TBST for 1 h 30 min at room temperature (25 °C). The membranes were then incubated overnight at 4 °C with anti-pMLKL (dilution of 1:1000) after washing four times with TBST for 5 min each, and anti-β-actin (dilution of 1:5000) was used as loading control. After four washes with TBST for 5 min each, the membranes were incubated with horseradish-peroxidase conjugated secondary antibodies for 2 h in TBST (dilution of 1:5000). Protein bands were visualized using enhanced chemiluminescence (ECL) Plus Western blot detection kit. ImageJ software was used to determine the relative density of the bands.

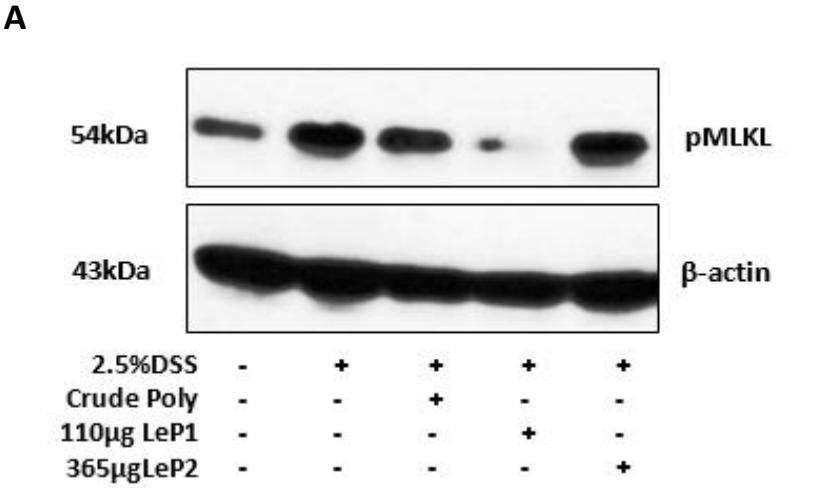
4.2.9. Statistical analysis

All data are expressed as mean ± SD. Statistical analysis was performed by Tukey-Kramer and two-tailed standard *t* tests. Statistical significance was defined as $*p < 0.05$ and $**p < 0.01$.

4.3. Results

4.3.1. LeP1 from Lentinula edodes suppressed necroptosis in mice

Recent studies on the pathophysiology of intestinal inflammation and other inflammatory disease conditions have shown that a type of cell death which is caspase-independent and morphologically different from apoptosis, known as necroptosis, is actively involved in the pathogenesis of inflammatory diseases. Necroptosis leads to the disruption of intestinal mucosal barrier which results from the death of intestinal epithelial cells (IECs) [24]. IECs death results in an increased leakage of the barrier to luminal antigens and microbiota into the lamina propria. Interactions of immune cells in the lamina propria with luminal antigens and microbiota results in the release of pro-inflammatory cytokines and consequently exacerbates intestinal inflammation [70, 71]. To determine whether necroptosis was involved in DSS-induced colitis, we analysed the level of the necroptosis executor, pMLKL (phosphorylated MLKL), which is widely used as a marker for necroptosis. Consistent with recent reports that necroptosis is actively involved in inflammatory bowel disease conditions, we found that DSS treatment to mice resulted in an increased level of pMLKL, indicating that the necroptosis pathway was activated during colitis (Fig. 4.1.A-D). However, LeP1 significantly prevented necroptosis by suppressing the phosphorylation of MLKL in a dose-dependent manner (Fig. 4.1.A-D). These data indicate that LeP1 prevented necroptosis in mice by suppressing the level of pMLKL, and this may partly account for its anti-inflammatory activity against colitis in mice.


A
54kDa
pMLKL
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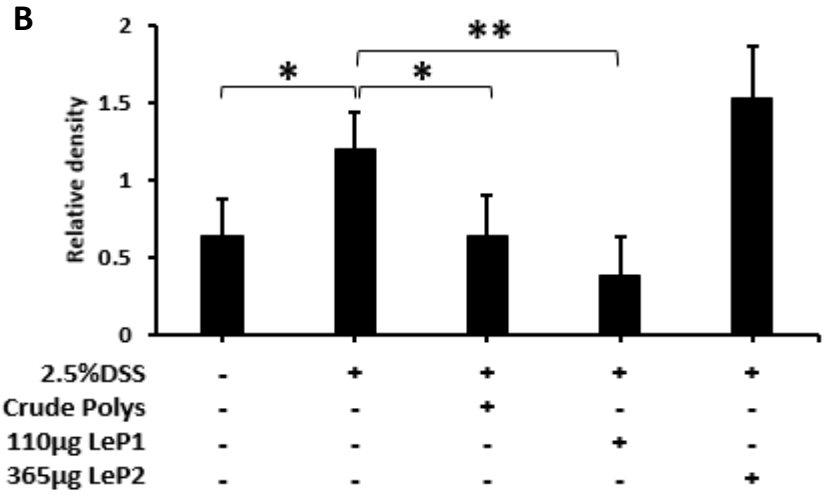
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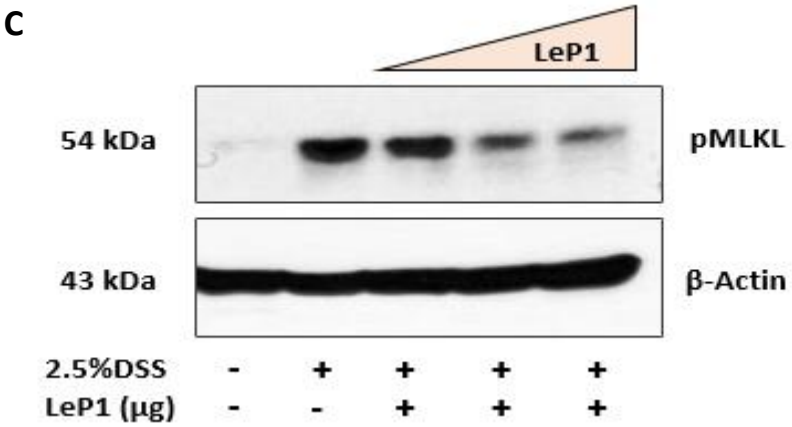

C
LeP1
54 kDa
pMLKL
43 kDa
β-Actin
2.5%DSS - + + + +
LeP1 (μg) - - + + +

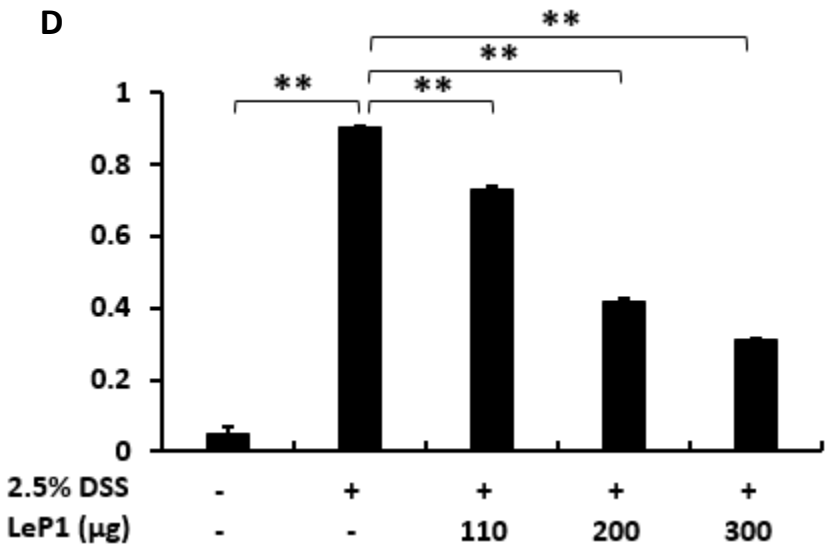


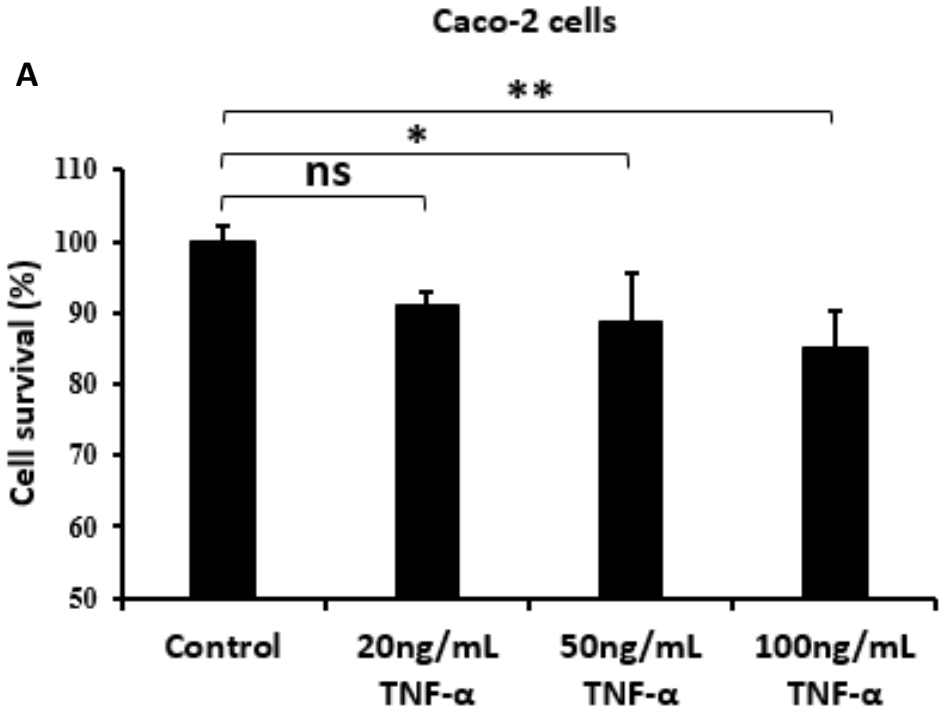
Fig. 4.1. LeP1 from *L. edodes* suppressed necroptosis in mice in a dose-dependent manner. Mice were administration with different doses of polysaccharides for 7 days prior to 2.5% DSS treatment for 7 days with a co-administration of different doses of polysaccharide for additional 10 days. (A-B) Western blot analysis of necroptosis marker, pMLKL, in the colon of mice treated with DSS + crude polysaccharides (500 µg/mouse), LeP1 (110 µg/mouse), and LeP2 (365 µg/mouse). (C-D) Representative Western blot of the necroptosis marker, pMLKL, with anti-pMLKL antibody in colon of mice treated with DSS + increasing doses of LeP1 (110, 200, and 300 µg/mouse). Values are presented as mean ± SD (n = 4). *$p < 0.05$, **$p < 0.001$.

4.3.2. LeP1 from L. edodes prevented necroptotic cell death in vitro

In order to further study the anti-necroptosis effect of polysaccharide from *Lentinula edodes*, an *in vitro* necroptosis model comprising Caco-2 cells was used. Apoptotic cell death was induced in Caco-2 cells with increasing concentration of TNF-α, and the result obtained indicate that apoptotic cell death by TNF-α is dose-dependent (Fig. 4.2.A). The reports of Nishitani *et al* [19] and Zhang *et al* [72] indicated that TNF-α was highly expressed during colitis suggesting that it played a significant role in the associated necroptotic cell death observed in our study. Therefore,

in this study, TNF-α and zVAD (A pan-caspase inhibitor) was used to induce necroptotic cell death in Caco-2 cells. We observed that treatment with zVAD-fmk, an apoptosis inhibitor, did not prevent TNF-α-induced cell death in Caco-2 cells, indicating that cell death was caspase-independent (Fig. 4.2.B). However, both 200 µg/mL and 500 µg/mL of LeP1 significantly inhibited TNF-induced necroptotic cell death in Caco-2 cells (Fig. 4.2.C). Furthermore, Nec-1, an inhibitor of necroptosis, also significantly prevented TNF-induced cell death in Caco-2 cells. Nec-1 is an allosteric inhibitor of the kinase activity of RIPK1, an important player in the RIPK1-RIPK3-MLKL necroptosis pathway (Fig. 4.2.C). These data showed that polysaccharides from *Lentinula edodes* prevented necroptotic cell death in an intestinal epithelial cell line, Caco-2 cells, *in vitro*.

RIPK1 is capable of inducing both apoptotic and necroptotic cell death through its kinase activity, and its inhibition results in blockage of its two downstream effects, apoptosis and necroptosis [73-76]. Having demonstrated that our polysaccharide sample prevented necroptotic cell death in Caco-2 cells and colon tissues of colitis mice (Fig. 4.1.A-D; Fig. 4.2.C), we next investigated whether the polysaccharide sample (LeP1) could prevent apoptotic cell death in the same manner. Surprisingly, we observed that the polysaccharide sample significantly blocked TNF-α-induced cell death in Caco-2 cells (Fig. 4.2.D). This data suggests that the polysaccharide sample may be exerting its effect on RIPK1 to suppress both necroptosis and apoptosis.


A
Caco-2 cells
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Cell survival (%)
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100
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70
60
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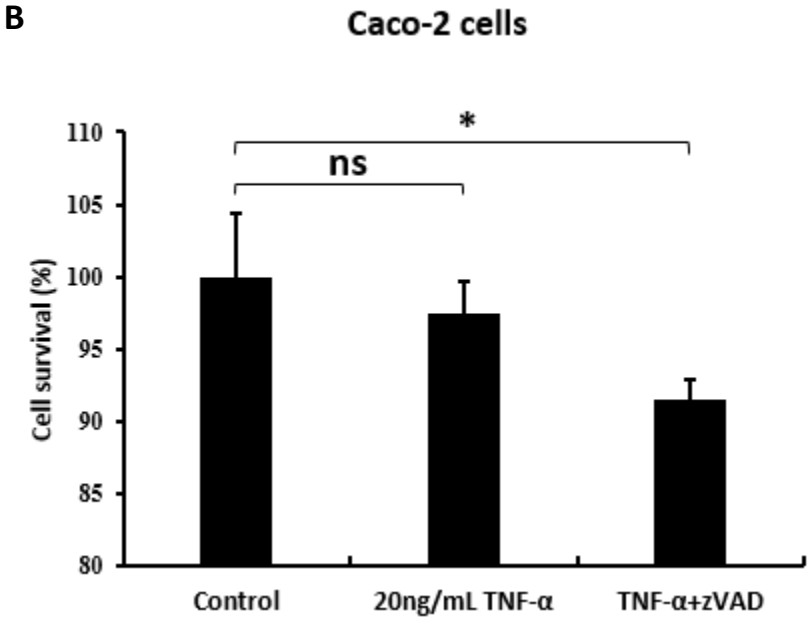
Control
20ng/mL TNF-α
50ng/mL TNF-α
100ng/mL TNF-α


B
Caco-2 cells
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Cell survival (%)
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Control
20ng/mL TNF-α
TNF-α+zVAD

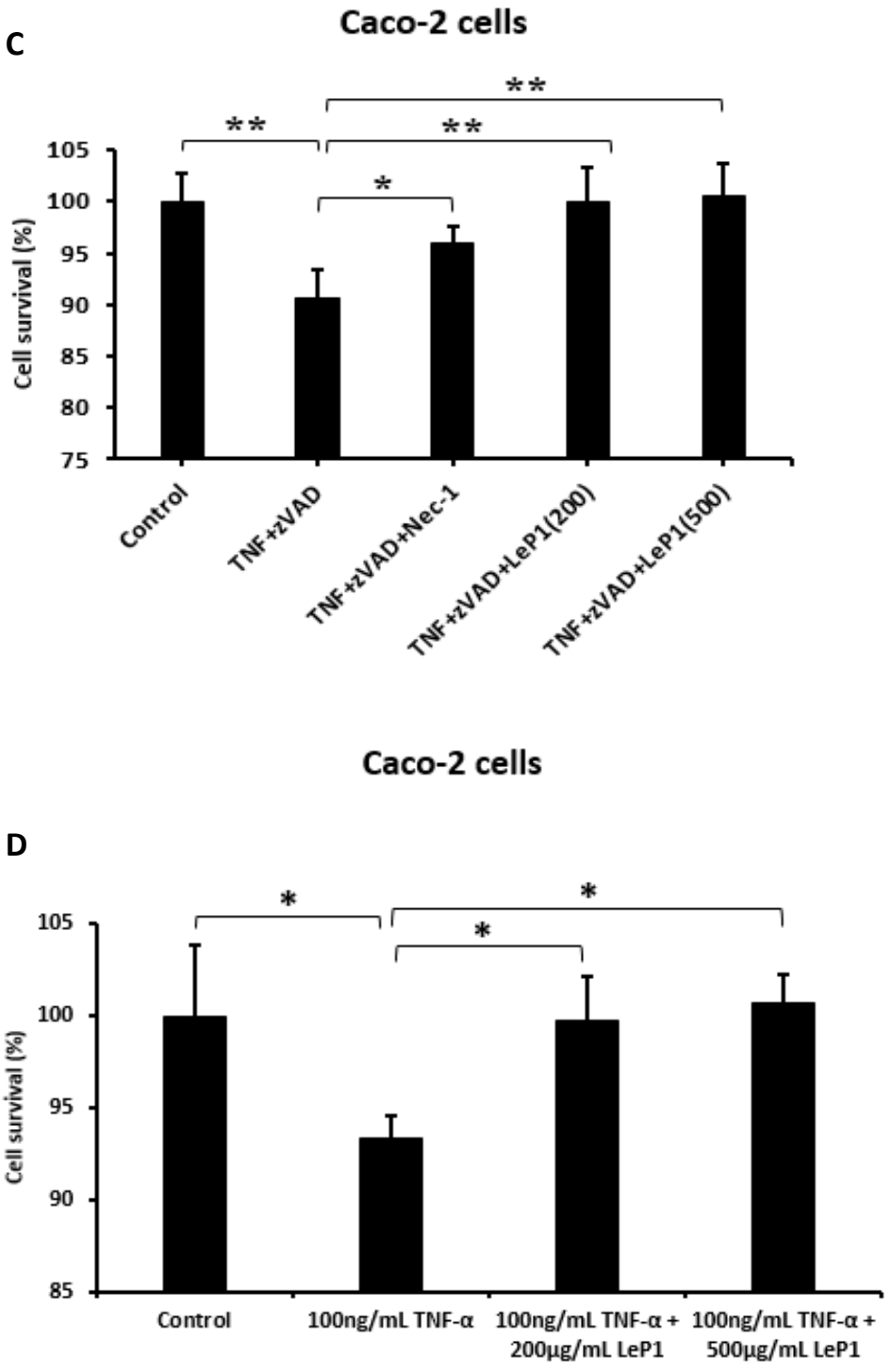


Fig. 4.2. LeP1 from *L. edodes* inhibited necroptotic cell death *in vitro*. Caco-2 cells were treated with 50 μM zVAD (caspase inhibitor), 50 μM zVAD+ 20 μM Nec-1 (necroptosis inhibitor), or 50 μM zVAD+ 200 μg/mL or 500 μg/mL of polysaccharides for 2 h prior to stimulation with 20 ng/mL rhTNF-α or not for another 24 h. (A) Induction of cell death with increasing concentration

of TNF-α (20 ng/mL, 50 ng/mL, and 100 ng/mL) for 24 h. (B) Induction of necroptotic cell death with 20 ng/mLTNF-α + 50 μM zVAD. (C) Inhibition of necroptotic cell death (induced with 20 ng/mL TNF-α+50 μM zVAD) with 20 μM Nec-1 or different doses of LeP1 (200 μg/mL or 500 μg/mL). (D) Inhibition of TNF-induced cell death by LeP1 (Caco-2 cells were treated with 200 μg/mL or 500 μg/mL polysaccharides for 2 h prior to stimulation with 100 ng/mL rhTNF-α or not for another 24 h). Cell viability was determined by MTT assay. Values are expressed as mean ± SD (n = 3-4). *$p < 0.05$, **$p < 0.001$.

4.4. Discussion

Necroptotic cell death has been reported to play important role in the pathogenesis and progression of intestinal inflammation. In this study, consistent with reports of other researchers, it was found that necroptosis was associated with DSS-induced colitis (Fig. 4.1.) [73]. However, it was demonstrated that polysaccharide derived from *Lentinula edodes* significantly suppressed colitis-associated necroptosis in a dose-dependent manner (Fig. 4.1.).

An *in vitro* necroptosis model consisting of Caco-2 cells was utilized in this study. First, an *in vitro* TNF-α-induced cell death studies using Caco-2 cells was performed, and it was found that Caco-2 cells died by necroptotic cell death in the presence of caspase inhibitor (zVAD-fmk) when stimulated with TNF-α, and also responded to TNF-α-induced cell death in a dose dependent manner (Fig. 4.2.A and B). Interestingly, it was also observed that LeP1 from *L. edodes* significantly protected Caco-2 cells from TNF-α-induced necroptotic cell death *in vitro* (Fig. 4.2. C).

RIPK1 is capable of inducing both apoptosis and necroptosis (when caspase-8 is inactivated) in cells through its kinase activity, indicating that inhibition of the kinase activity could effectively block both necroptosis and apoptosis [73-76]. Notably, it was found that the polysaccharide sample was able to prevent both TNF-α-induced necroptosis and TNF-α-induced extrinsic apoptosis in

Caco-2 cells, suggesting that it may be exerting its suppressive effect on RIPK1, which in turn resulted in the reduced level of its downstream effector, pMLKL (necroptosis executor), as observed in this study (Fig. 4.2. D). Therefore, these findings strongly suggest that the inhibition of necroptosis by polysaccharide derived from *Lentinula edodes* may partly be responsible for its activity against intestinal inflammation

CHAPTER 5

Effect of *Lentinula edodes* Polysaccharides on the Expression of Inflammatory Cytokines in Ulcerative Colitis

5.1. Introduction

Necroptosis has been shown to be actively involved in the pathogenesis and promotion of inflammatory bowel disease, as well as in the excessive expression and release of inflammatory cytokines [49, 50]. RIPK3 is an important player in the RIPK1-RIPK3-MLKL necroptosis signaling pathway. It plays dual roles upon the initiation of necroptosis, by mediating both the necroptosis-dependent DAMPs release and necroptosis-independent up-regulation of inflammatory cytokines [77]. There are various ways in which RIPK3 could up-regulate inflammatory cytokines. RIPK3 is able to stimulate the production of ROS (reactive oxygen species) and also process pro-IL-1β into active IL-1β. ROS and IL-1β, in turn, stimulate other signaling cascades which lead to up-regulation of other inflammatory cytokines [77-79]. Furthermore, studies have shown that RIPK3 directly contributes to the activation of NF-κB-mediated inflammatory cytokines expression [79-81]. RIPK3-mediated activation of the mitogen-activated protein (MAP) kinase, extracellular signal regulated kinase (ERK) and C-Jun N-terminal kinase (JNK), has also been reported [77]. DAMPs released from necroptotic cells into the extracellular environment strongly activate and induce the expression of pro-inflammatory cytokines in surrounding cells, thereby promoting the spread of inflammatory signals [82-84]. In

chapter 4, we demonstrated that polysaccharides derived from *Lentinula edodes* inhibited necroptotic cell death in the colon of colitis mice and consequently ameliorated ulcerative colitis. In this chapter 5, the effect of the polysaccharide sample on the expression level of inflammatory cytokines was investigated.

5.2. Materials and Methods

5.2.1. Reagents

Dextran sodium sulphate (DSS, 36,000-50,000 Da) was purchased from MP Biomedicals (Canada). DEAE-sepharose CL-6B was purchased from GE Healthcare Bio-sciences AB (Sweden). Dulbecco's Modified Eagle Medium (DMEM) containing glutamine and glucose (4.5 g/L) was purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). RPMI 1640 medium and MEM non-essential amino acids (NEAA) were purchased from Nissui Pharmaceutical (Tokyo, Japan) and Gibco (Grand Island, NY, USA) respectively. FBS (fetal bovine serum) was purchased from Biological Industries (Beit, Israel). Recombinant human tumor necrosis factor-α (rhTNF-α) was purchased from PeproTech (Rocky Hill, NJ, USA). zVAD-fmk (pan-caspase inhibitor) was purchased from Selleck (Tokyo, Japan). Anti-mouse β-actin antibody, anti-mouse TNFR1 antibody, anti-mouse Syk antibody, and anti-human TNFR1 antibody were from Santa Cruz Biotechnology (Delaware Avenue, CA), rabbit monoclonal anti-mouse pMLKL (ab196436) was purchased from Abcam. Mouse HRP-conjugated anti-IgG and rabbit HRP-conjugated anti-IgG were purchased from R&D Systems (Minneapolis, USA) and Cell Signaling Technology (Danvers, MA, USA) respectively. Every other chemicals and reagents were standard guaranteed commercial products.

5.2.2. Extraction of polysaccharides from Lentinula edodes

Crude polysaccharides used for this study was obtained from the powdery fruiting bodies of *Lentinula edodes* by hot water extraction and ethanol precipitation as described in section 2.2.2 of chapter 2.

5.2.3. Chromatographic separation of crude polysaccharides

Crude polysaccharide was separated using DEAE-sepharose CL-6B column chromatography as described in section 2.2.3 of chapter 2. The fraction containing the active component (fraction 1) as previously reported in chapter 2 was named LeAP1 and used for this study.

5.2.4. Ulcerative colitis induction

Mice were purchased and bred as described in chapter 2. Ulcerative colitis was induced in mice using 2.5% of DSS (w/v) in drinking water for 7 days. Polysaccharide sample (300 µg/mouse) (LeAP1) was orally administered to mice prior to (7 days) and during DSS administration (7 days), followed by additional 3 days administration of the polysaccharide sample or clean water. Body weights of mice were recorded daily, and at the end of sample administration, mice were sacrificed by cervical dislocation, colon tissues were excised and their length measured.

5.2.5. Total RNA isolation

Immediately after mice sacrifice, colon lumen was flushed with ice-cold PBS, and 4-5 mg of distal region of colon tissue was collected for total RNA isolation. Total RNA was isolated from mice colon using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) and following standard procedure for total RNA isolation. The concentration of total RNA was determined using NanoDrop 2000 spectrophotometer (Thermo Scientific, U.S.A).

5.2.6. mRNA purification using LiCl precipitation

The total RNA obtained with Sepasol extraction was subjected to purification using two-step LiCl (Lithium chloride) precipitation. LiCl precipitation removes contaminating DSS, other polysaccharides, DNA, tRNA, and nucleotides, but precipitates only mRNA, according to the method of Viennois *et al* [85, 86]. Briefly, 0.1 volume of ice-cold 8 M LiCl was added to 1 volume of total RNA sample, and was kept on ice for 2 h, followed by centrifugation at 14,000 ×g for 30 min at 4 ºC. Supernatant was discarded and pellet was redissolved in 200 µL RNase free water. Again, this step was repeated by adding 20 µL of ice-cold 8 M LiCl and kept on ice for 2 h, centrifuged at 14,000 ×g for 30 min at 4 ºC, and supernatant was discarded. Again, 200 µL of RNase free water was added to the pellet, followed by addition of 20 µL of ice-cold 3M sodium acetate (pH =5.2) and 400 µL of -20 ºC pre-chilled 100% ethanol, followed by incubation at -20 ºC for 30 min. The solution was centrifuged at 14,000 ×g for 30 min at 4 ºC**,** supernatant was discarded and pellet was washed with 100µL of -20 ºC prechilled 75% ethanol, followed by centrifugation at 14,000 ×g for 10 min at 4 ºC. The supernatant was removed and the pellet was air-dried, and redissolved in 30 µL (smaller volume than the initial starting volume of total RNA) of RNase free water. The concentration of purified mRNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, U.S.A).

5.2.7. cDNA synthesis and quantitative RT-PCR

Purified mRNA (5 µg) was used for cDNA (complementary DNA) synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) with a T100 Thermal cycler (BIO-RAD, Singapore) programmed at 25 ºC for 10 min, 37 ºC for 120 min, and 85 ºC for 5 sec. The cDNA was stored at 4 ºC for subsequent use. Quantitative real-time polymerase chain reaction was performed on a 7500 Fast-Time PCR System (Applied Biosystems) programmed at 95 ºC for 10 min, 95 ºC for 10 sec, and 60 ºC for 30 sec, using a FastStart Universal Probe Master (ROX) mix (Roche Diagnostics GmbH, Mannheim, Germany). The TaqMan probes used include Mm00607939_sl for mouse *Actb* (housekeeping gene), Hs02786624_g1 for human *GAPDH* (housekeeping gene), Mm00443258_ml for mouse *Tnfα*, Mm00446190_ml for mouse *Il6*, Mm00434228_ml for mouse *Il1b*, Mm01168134_ml for mouse *Ifng*, Mm00441242_ml for mouse *Ccl2*, Hs00174103_m1 for human *Il8*, and Mm01183349_ml for mouse *Clec7a*. Gene expressions were normalized using *β*-actin or GAPDH.

5.2.8. ***In vitro necroptosis induction***

Caco-2 cells were cultured in DMEM (4.5 g/L glucose) with fresh medium change every two days until the cells reached 80% confluence. Caco-2 cells (3.5×10^5 cells/well) were seeded onto a 12-well plate and incubated overnight at 37 ºC, 5% CO_2. The method outlined in chapter 4 was followed to induce necroptosis in Caco-2 cells and treated with LeAP1 to determine its effect on TNFR1 expression.

5.2.9. *Western blot analysis*

Colon tissue samples (4-5 mg) were homogenized in ice-cold RIPA (radioimmunoprecipitation) buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Nonidet-P40, 0.5% Sodium deoxycholate, and 0.1% SDS) containing PMSF and a cocktail of protease and phosphatase inhibitors (aprotinin, leupeptin, Na Fluoride, and DTT), while Caco-2 cells were trypsinized and washed with ice-cold PBS, followed by total protein extraction using ice-cold RIPA buffer. The remaining western blot procedure outlined in chapter 4 was followed to detect proteins of interest.

5.2.10. *Statistical analysis*

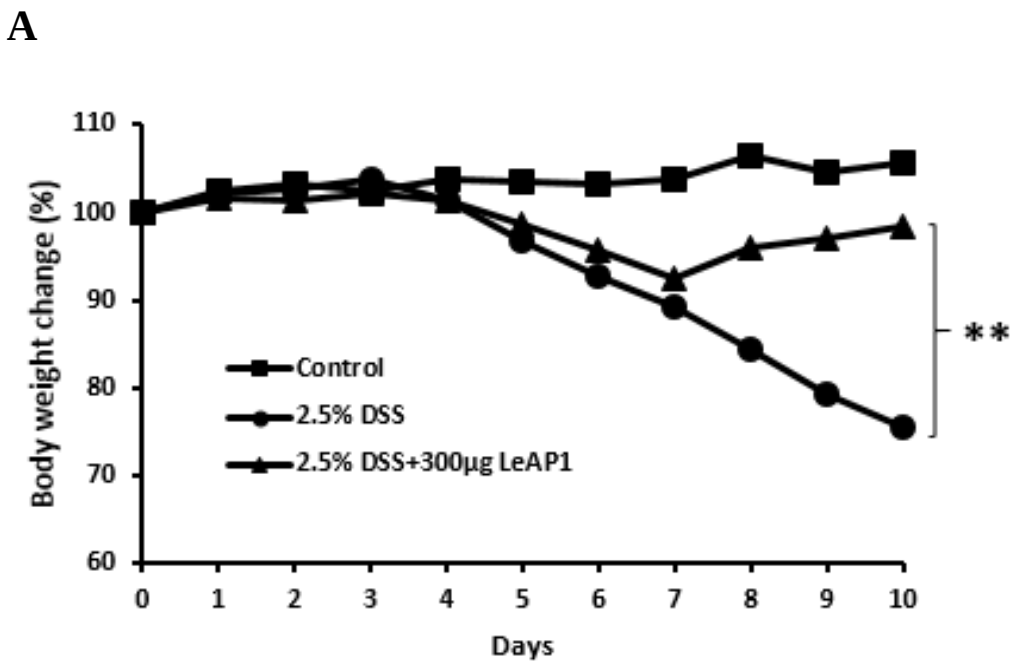
All data are expressed as mean ± SD. Statistical analysis was performed by Tukey-Kramer and two-tailed standard *t* tests. Statistical significance was defined as $*p < 0.05$ and $**p < 0.01$.

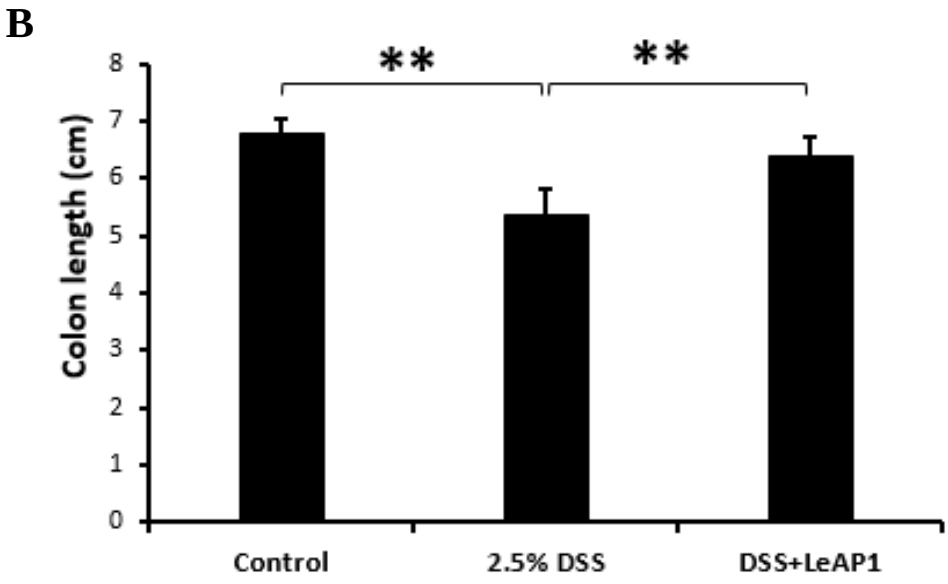
5.3. Results

5.3.1. The effect of polysaccharides (LeAP1) from Lentinula edodes on ulcerative colitis in mice

To study the effect of polysaccharides from *Lentinula edodes* on the expression level of inflammatory cytokines induced by ulcerative colitis-associated necroptosis. The polysaccharide sample (300 µg/mouse) was orally administered to ulcerative colitis mice. In chapter 4, it was demonstrated that necroptosis is actively involved in ulcerative colitis. The results obtained showed that, in consistent with previous results, the polysaccharide sample significantly prevented DSS-induced colitis in mice, as seen in both the body weight and colon length (Fig. 5.1.A-C). It was also observed that the survival rate of colitis mice which were administered with the polysaccharide sample was maintained at 100% as compared to the untreated which dropped to

80% (Fig. 5.1.D). These data suggested that, as consistent with previous results, the polysaccharide sample exerted anti-inflammatory activity.





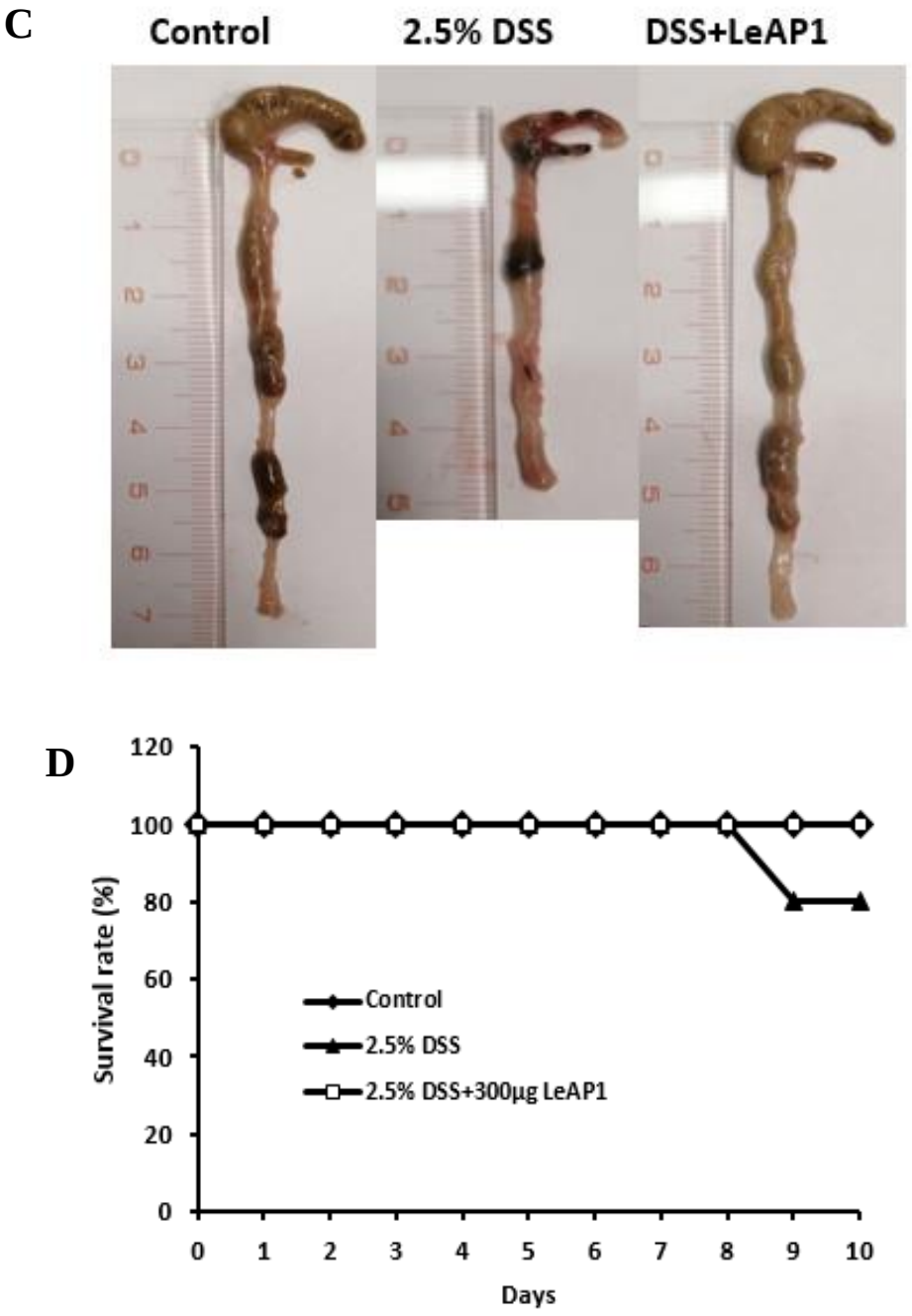
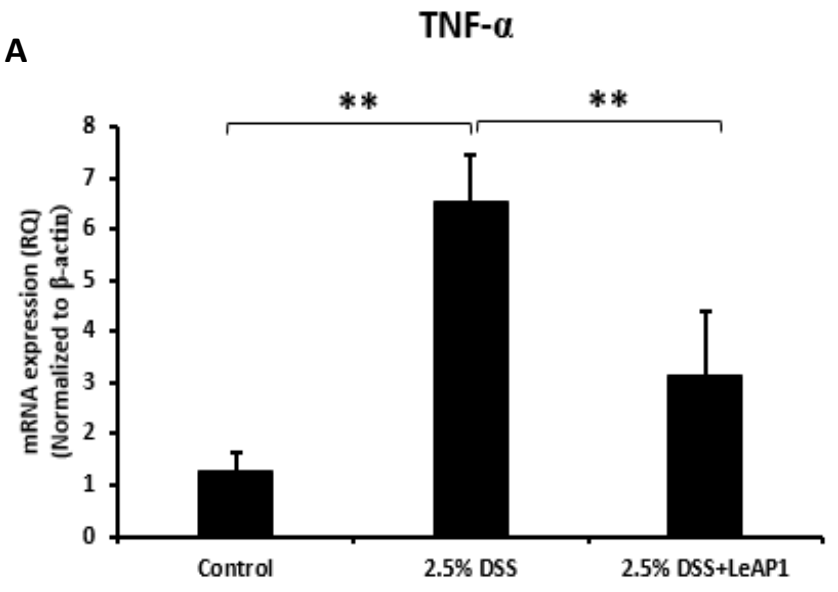


Fig. 5.1. Polysaccharides from *Lentinula edodes* (LeAP1) inhibited ulcerative colitis and maintained mice survival. Ulcerative colitis was induced in mice with 2.5% DSS (w/v) in drinking water for 7 days. Polysaccharide sample (300 µg/mouse) was orally administered to mice prior to (7 days) and during colitis induction (7 days), followed by additional 3 days of administration. (A) Body weight of mice treated with polysaccharide sample. (B and C) Colon length of mice treated with polysaccharide sample. (D) Survival rate of mice treated with polysaccharide sample. Values are expressed as mean ± SD (n = 4-5). *$p < 0.05$, **$p < 0.001$.

5.3.2. Effect of Lentinula edodes polysaccharides on the expression of inflammatory cytokines in ulcerative colitis

Necroptotic cell death is known to be associated with up-regulation of inflammatory cytokines and chemokines. In chapter 4, necroptosis was shown to be actively involved in ulcerative colitis, suggesting that there might be necroptosis-mediated up-regulation of inflammatory cytokines. To investigate this situation, ulcerative colitis was induced in mice using 2.5% DSS (w/v) in drinking water. Colitis mice were pre- and co-treated with polysaccharide sample (300 µg/mouse) from *Lentinula edodes*. The mRNA expression level of inflammatory cytokines, TNF-α, IL-6, IL-1β, IFN-γ, and CCL-2, was analysed. The results obtained indicated a significantly increased level of inflammatory cytokines in DSS-treated mice (Fig. 5.2.A-E). Furthermore, necroptosis was induced in 200 µg/mL LeAP1-treated Caco-2 cells using 20 ng/mL TNF-α + 50 µM zVAD-fmk (Pan-caspase inhibitor) for 24 h, and IL-8 mRNA expression was analysed. The data indicated that the polysaccharides sample suppressed IL-8 expression (Fig. 5.2. F and G). In addition, the data indicated that necrostatin-1 (Nec-1) (20 µM), a necroptosis inhibitor, prevented the up-regulation of IL-8, suggesting that necroptosis played an active role in mediating increased inflammatory cytokines/chemokines expression (Fig. 5.2. G). However, administered polysaccharide sample significantly prevented up-regulation of the cytokines (Fig. 5.2.A-G). These data suggested a feed-forward effect of the polysaccharide sample on necroptotic cell death in the colon of colitis mice as reported in chapter 4.


A
TNF-α
**
**
mRNA expression (RQ)
(Normalized to β-actin)
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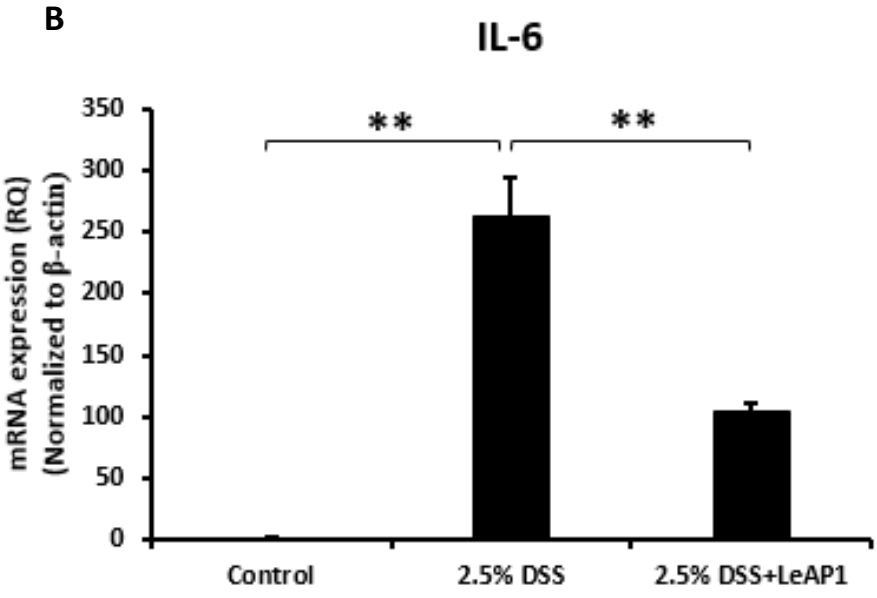
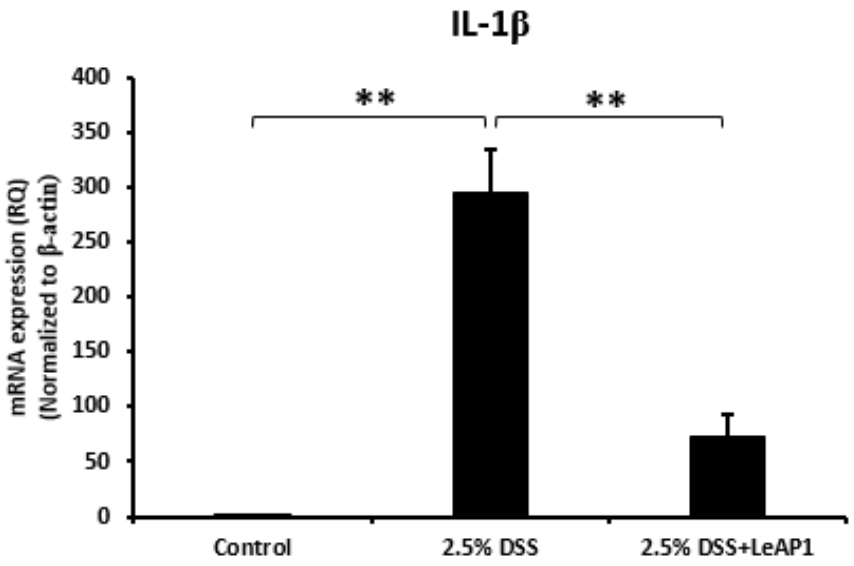
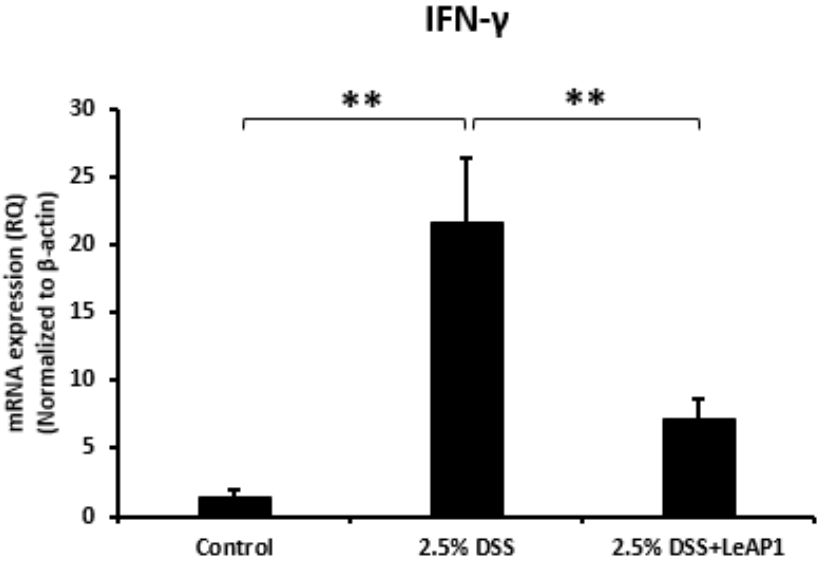
1
2
3
4
5
6
7
8
Control
2.5% DSS
2.5% DSS+LeAP1


B
IL-6
**
**
mRNA expression (RQ)
(Normalized to β-actin)
0
50
100
150
200
250
300
350
Control
2.5% DSS
2.5% DSS+LeAP1

C


IL-1β
mRNA expression (RQ)
(Normalized to β-actin)
400
350
300
250
200
150
100
50
0
**
**
Control
2.5% DSS
2.5% DSS+LeAP1

D


IFN-γ
mRNA expression (RQ)
(Normalized to β-actin)
30
25
20
15
10
5
0
**
**
Control
2.5% DSS
2.5% DSS+LeAP1

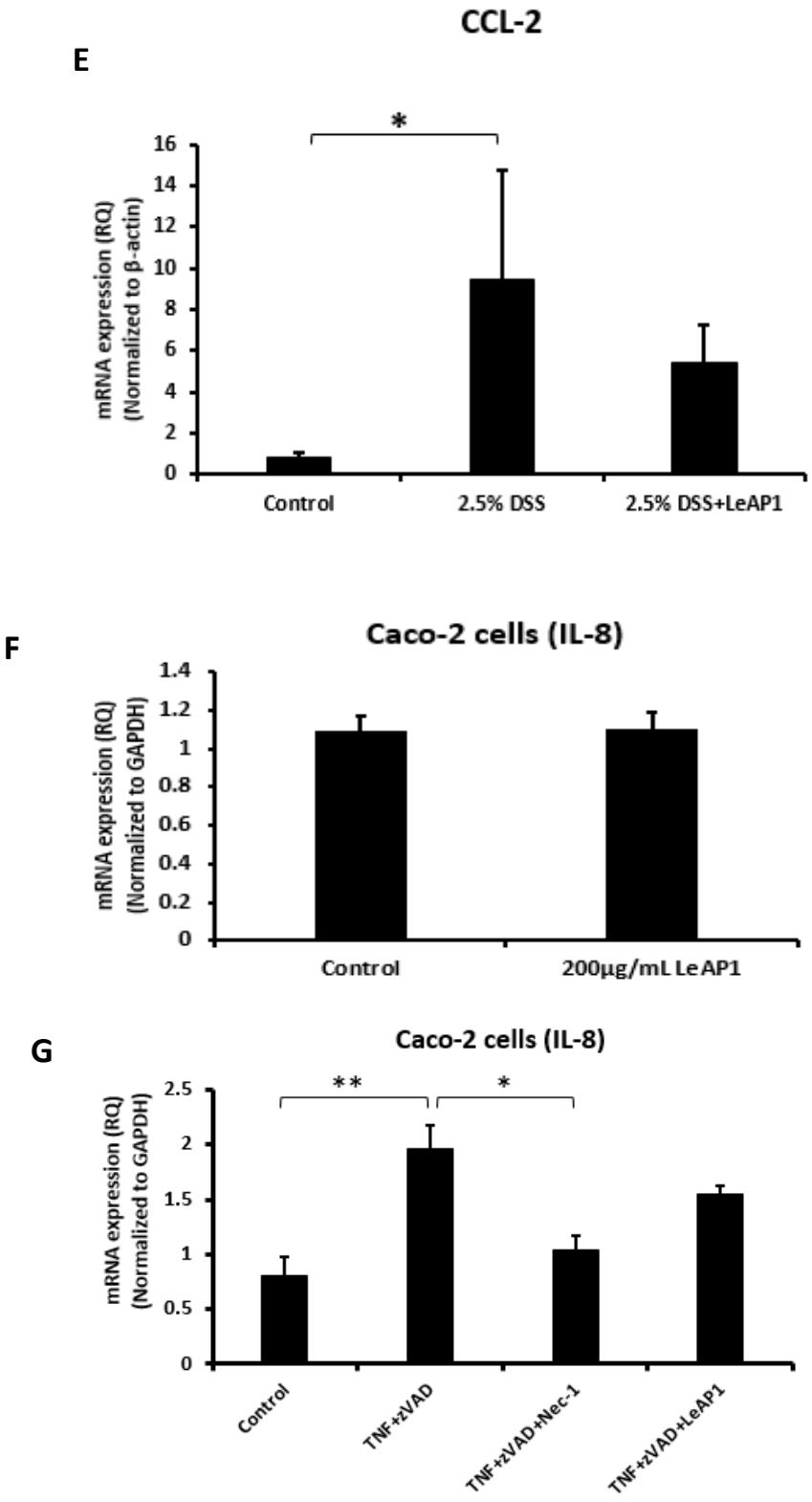
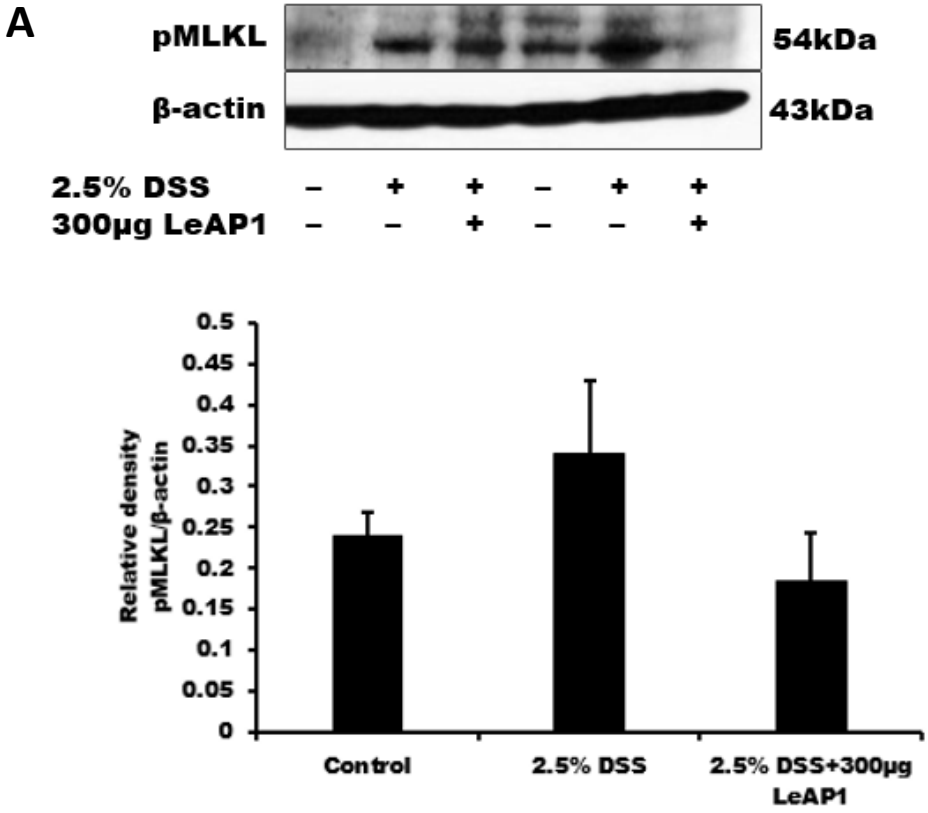


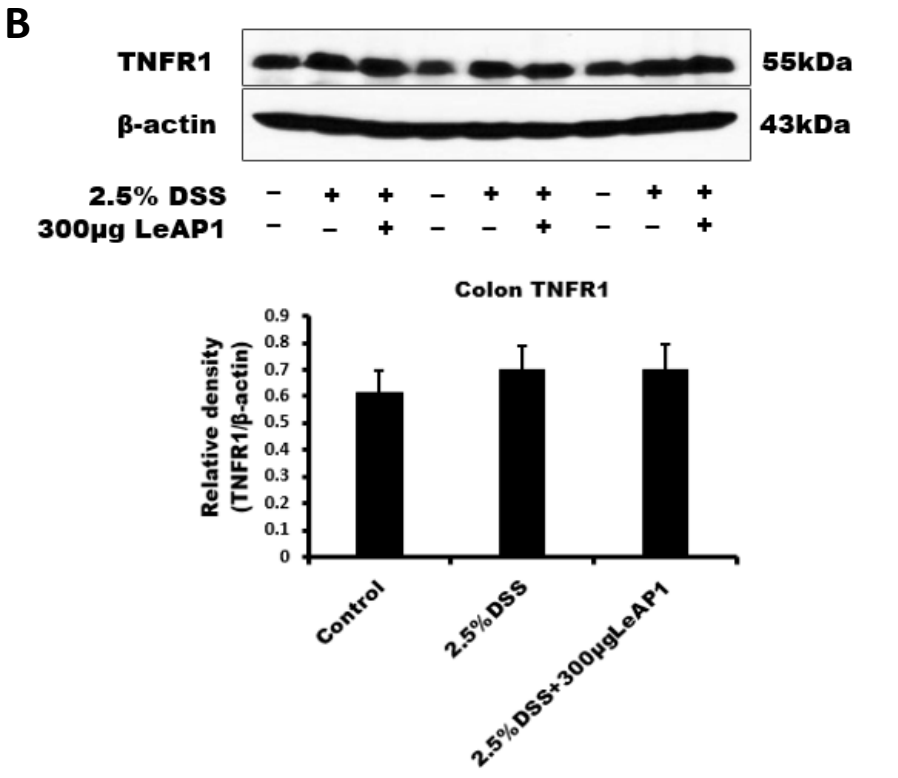
Fig. 5.2. Polysaccharides from *Lentinula edodes* inhibited up-regulation of inflammatory cytokines in ulcerative colitis mice. Polysaccharide sample (300 μg/mouse) was pre- (7 days)

and co-administered to ulcerative colitis mice for 7 days, followed by additional 3 days administration of polysaccharide sample. (A-E) mRNA expression level of inflammatory cytokines and chemokine in the colon of ulcerative colitis mice administered with polysaccharide sample. (F and G) mRNA expression of Caco-2 cells treated with 200 µg/mL LeAP1 or Nec-1 (20 µM) and necroptosis inducer (20 ng/mL TNF-α+50 µM zVAD-fmk) for 24 h. Values are expressed as mean ± SD (n = 3-4). *$p < 0.05$, **$p < 0.001$.

5.3.3 *Anti-necroptosis activity of Lentinula edodes polysaccharides is not dependent on the expression of TNFR1*

To confirm that necroptosis was associated with DSS-induced ulcerative colitis, the level of pMLKL (necroptosis executor) was determined by western blot analysis. The results in Fig. 5.3.A indicated that necroptosis actively played a role in ulcerative colitis, consistent with the data in chapter 4. However, orally administered polysaccharides (LeAP1) inhibited necroptosis and ameliorated colitis in mice. Recent reports indicated that TNF-α played important role in activating necroptosis *in vivo* through binding to its cell surface receptor, TNFR1 [24-26]. The data in Fig. 5.3.A indicated that *Lentinula edodes* polysaccharides inhibited necroptosis *in vivo* and suppressed increased expression of TNF-α (Fig. 5.2.A). The results in Fig. 5.3. B, C, and D, indicated that the polysaccharide sample did not affect the expression of TNFR1 in both *in vivo* and *in vitro* models of necroptosis, suggesting that anti-necroptosis activity of the polysaccharide is not dependent on the expression of TNFR1 on the cell surface.


A
pMLKL
54kDa
β-actin
43kDa
2.5% DSS – + + – + +
300μg LeAP1 – – + – – +
Relative density pMLKL/β-actin
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Control
2.5% DSS
2.5% DSS+300μg LeAP1


B
TNFR1
55kDa
β-actin
43kDa
2.5% DSS – + + – + + – + +
300μg LeAP1 – – + – – + – – +
Colon TNFR1
Relative density (TNFR1/β-actin)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
2.5%DSS
2.5%DSS+300μgLeAP1

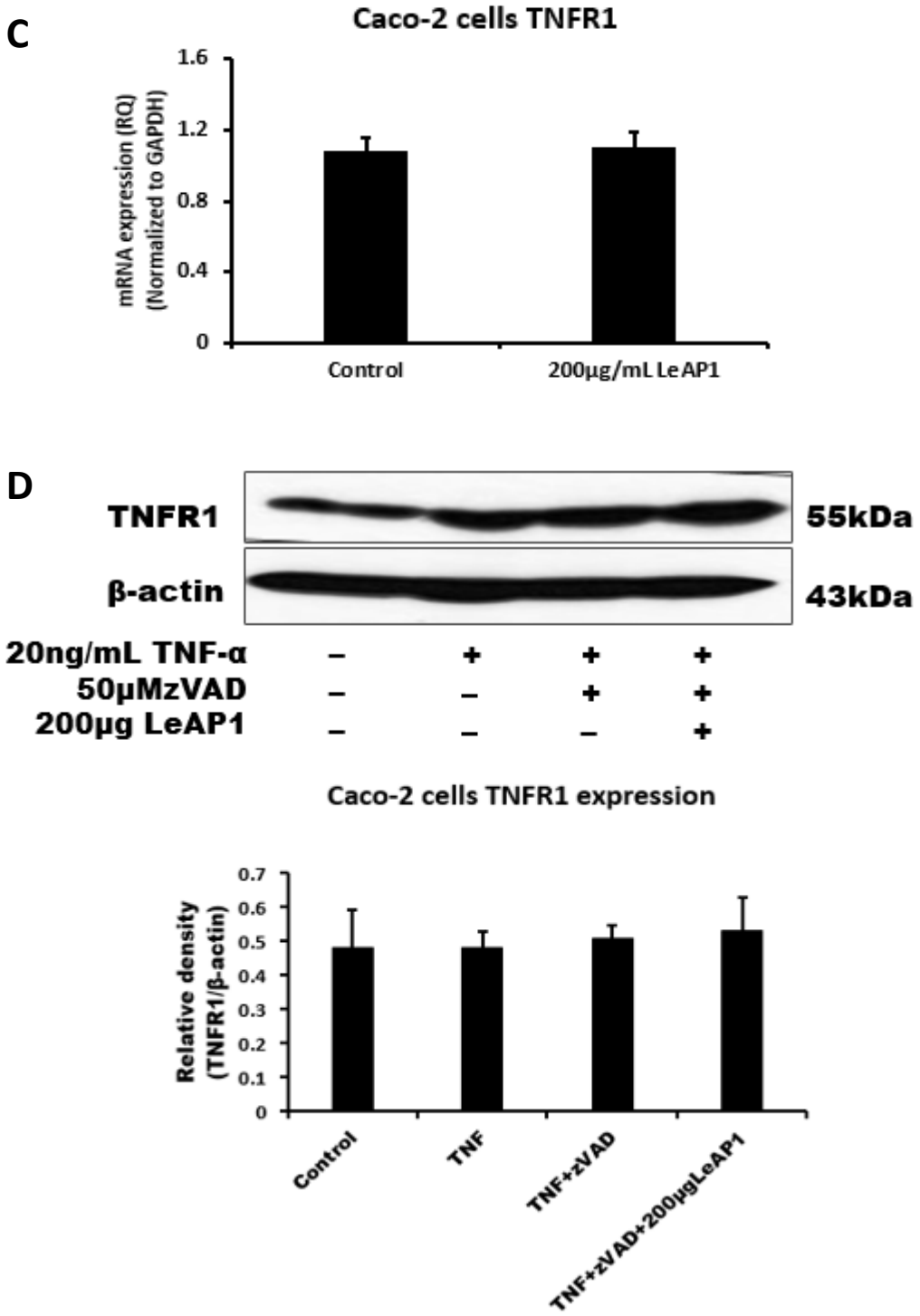


Fig. 5.3. Anti-necroptosis activity of *Lentinula edodes* polysaccharides is independent of its effect on TNFR1 expression. Polysaccharide sample (300 μg/mouse) was pre- (7 days) and co-administered to ulcerative colitis mice for 7 days, followed by additional 3 days administration of polysaccharide sample. (A) Western blot detection of necroptosis executor, pMLKL. (B) Protein

expression of colon TNFR1 of colitis mice treated with 300 µg/mouse of *Lentinula edodes* polysaccharide. To induce necroptotic cell death, Caco-2 cells were incubated with 50 µM zVAD or 50 µM zVAD+200 µg/mL LeAP1 for 2 h prior to TNF-α (20 ng/mL) stimulation for an additional 24 h. (C and D) Protein expression of TNFR1 on Caco-2 cells treated with necroptosis inducer and Lentinula edodes polysaccharide. Values are expressed as mean ±SD (n = 3). $*p < 0.05$, $**p < 0.001$.

5.4. Discussion

Studies in the last two decades on cell death have improved our understanding of cell deaths and their role in disease conditions. Necroptosis, a regulated form of pro-inflammatory cell death, has been widely shown to play active roles in the etiology and pathogenesis of IBD [24]. Recent reports indicated that inhibitors of necroptosis prevented ulcerative colitis in mice, thereby implicating necroptosis as a cause of IBD rather than a consequence of it [72]. Necroptosis signaling stimulates the expression of inflammatory cytokines which together with DAMPs are released into the extracellular environment upon cell membrane rupture, and which results in the promotion and worsening of inflammatory conditions. TNF-α is known to stimulate necroptosis upon binding to its receptor, TNFR1, in the absence or deficiency of caspase-8. In this study, increased expression of TNF-α and necroptosis were recorded in DSS-induced colitis mice model, which indicated that chemically-induced (DSS) necroptotic cell death played a role in the expression and release of inflammatory cytokines which in turn activated necroptosis in neighbouring cells and exacerbated inflammation in the form of a chain reaction.

Increased expression of inflammatory cytokines is associated with necroptotic cell death. Necroptosis is actively involved in the pathogenesis and promotion of ulcerative colitis, suggesting that necroptotic signaling molecules played crucial roles in the up-regulation of inflammatory cytokines as observed in ulcerative colitis conditions [87, 88]. In chapter 4, we demonstrated that

polysaccharides from *Lentinula edodes* prevented necroptotic cell death in the colon of ulcerative colitis mice. Therefore, the inhibition of necroptosis may have resulted to the amelioration of ulcerative colitis as reported in chapter 2 and 3. Furthermore, in this chapter 5, we observed that the expression of inflammatory cytokines (TNF-α, IL-6, IL-1β, IFN-γ, and CCL-2) was up-regulated in the colon of ulcerative colitis mice, which may have resulted from the activation of necroptotic signaling pathways in the colon. However, administered polysaccharides extract down-regulated the expression of inflammatory cytokines (Fig.5.2. A-E). Down-regulation of inflammatory cytokines may have directly resulted from the inhibition of necroptosis by the polysaccharide sample. These results are consistent with reports of other researchers who reported that necroptosis inhibitors ameliorated ulcerative colitis and prevented increased expression of inflammatory cytokines [72].

Furthermore, the effect of the polysaccharide sample on the expression of TNFR1 was investigated. In contrast to the report of Nishitani *et al* [19] who reported the endocytic effects of lentinan on TNFR1 as its anti-inflammatory mechanism, our polysaccharide sample did not show any effect on the expression of TNFR1 in both *in vivo* and *in vitro* models of necroptosis. These data indicated that the anti-necroptosis activity of the polysaccharide sample and its inhibitory effects on the expression of inflammatory cytokines were independent of the expression of TNFR1 on the cell surface, thereby suggesting a direct effect on necroptosis mediators

CHAPTER 6

General Discussion and Conclusion

Necroptosis is a caspase-independent, pro-inflammatory form of programmed necrotic cell death [28]. Unlike apoptosis, it is characterized by cell swelling, lysis, and release of intracellular contents into surrounding tissues. It is described as immunogenic because of the release of pro-inflammatory cellular contents such as interleukins and damage-associated molecular patterns (DAMPs) into extracellular medium [28]. Recent reports indicated that it is actively involved and associated with colitis and other inflammatory disease conditions [24]. Studies have suggested that Lentinan, a β-1,3-1,6-glucan, suppressed intestinal inflammation by suppressing gene expression of pro-inflammatory cytokines through binding to dectin-1 on the surface of IECs [19]. However, necroptotic cell death plays an important role in the pathogenesis and progression of intestinal inflammation, and inhibitors of necroptosis have shown promising clinical results in preventing intestinal inflammation [24, 49, 67, 68]. No study has yet reported any effect of polysaccharides from edible mushrooms on ulcerative colitis-associated necroptosis. In this study, consistent with reports of other researchers, we found that polysaccharides sample from *L. edodes* prevented ulcerative colitis in mice (chapter 2). On further study, we observed that the anti-inflammatory activity of the polysaccharides sample was exerted by its carbohydrate-rich component, and was independent of the protein content (chapter 2). Additionally, the data indicated that the carbohydrate-rich component of the polysaccharides inhibited ulcerative colitis in a dose-

dependent manner. This data was consistent with the report of Nishitani *et al* [19] who showed that lentinan, a β-glucan, suppressed colitis in mice.

A wide range of biological activities have been attributed to mushroom polysaccharides. The different structures of polysaccharides components and other bioactive compounds from different species of mushrooms are reported to be responsible for their varied activities, such as anti-inflammatory activity, anti-diabetic, immunomodulatory, hepatoprotective, anti-allergic, and anti-bacterial activities [38-41]. The difference in biological activity is attributed to the difference in their structural features such as monomeric compositions, linearity, branching points, degree of branching, chain length, molecular weight, ionic nature, and solubility. Furthermore, different strains of mushrooms have also been reported to contain similar compounds with some difference in solubility and structural features [59-61]. The structural difference in bioactive compounds found in strains of certain species of mushrooms have been reported to have some influence on their biological activities [48].

This study has demonstrated that polysaccharides derived from *Lentinula edodes* had suppressive effects against ulcerative colitis in mice. However, different strains of *Lentinula edodes* are known to exist and are cultivated in growth media with varied compositions. There is no report yet on the difference/similarity of polysaccharides from different strains of *Lentinula edodes*, and the consequent variability in their biological activities. In this study, the intestinal anti-inflammatory activities of polysaccharides from another two selected strains of *Lentinula edodes* (LeA and LeB) were investigated using animal model of ulcerative colitis. Results obtained showed that the crude polysaccharides (LeAP) from LeA suppressed ulcerative colitis in mice, suggesting that the crude polysaccharides possessed anti-inflammatory properties against colitis in mice (chapter 3). However, the crude polysaccharides (LeBP) from LeB showed no inhibitory activity against colitis

in mice as seen in both the colon length and body weight of the mice (chapter 3). These results suggest that polysaccharides from the two strains of *Lentinula edodes* exhibited differential biological activities against colitis in mice.

Furthermore, column chromatographic separation of crude polysaccharides from the strains of *Lentinula edodes* (LeAP and LeBP) using DEAE-sepharose CL-6B, showed differential elution profiles with LeAP1 having two peaks while LeBP has three peaks, which indicated that the polysaccharides may be structurally different, although further structural studies need to be done to clarify this observation. The different structural features may have accounted for their varied activities against ulcerative colitis in mice. This study is the first to demonstrate the differential anti-inflammatory activity of polysaccharides from strains of *Lentinula edodes*, thereby suggesting that the anti-inflammatory activities of *Lentinula edodes* polysaccharides may be strain-dependent (chapter 3).

Consistent with recent reports [87], it was observed in this study, that necroptosis was actively involved in DSS-induced ulcerative colitis. However, the data obtained demonstrated that the carbohydrate-rich component of the polysaccharides derived from *Lentinula edodes* significantly inhibited necroptotic cell death in the colon of ulcerative colitis mice in a dose-dependent manner, indicating that the polysaccharide sample may partly prevent ulcerative colitis through its inhibition of necroptosis.

To further explore the anti-necroptosis effect of the polysaccharides, an *in vitro* necroptosis model consisting of Caco-2 cells was utilized [37, 68]. First, an *in vitro* TNF-α-induced cell death was carried out using Caco-2 cells, and it was found that Caco-2 cells died by necroptotic cell death in the presence of caspase inhibitor (zVAD-fmk) when stimulated with TNF-α. It was also demonstrated that Caco-2 cells responded to TNF-α-induced cell death in a dose dependent

manner. Interestingly, it was observed that the polysaccharide sample from *L. edodes* significantly protected Caco-2 cells from TNF-α-induced necroptotic cell death *in vitro* (chapter 4).

RIPK1 is capable of inducing both apoptosis and necroptosis (when caspase-8 is inactivated) in cells through its kinase activity, indicating that inhibition of the kinase activity could effectively block both necroptosis and apoptosis [74-77]. Notably, it was found that the polysaccharide sample was able to prevent both TNF-α-induced necroptosis and TNF-α-induced extrinsic apoptosis in Caco-2 cells, suggesting that it may be exerting its suppressive effect on RIPK1, which in turn resulted in the reduced level of its downstream effector, pMLKL (necroptosis executor), as observed in this study (chapter 4).

Necroptotic signals result in the activation of signaling pathways leading to expression of inflammatory cytokines. The necroptosis mediator, RIPK3, has been reported to activate NF-κB and MAPK-mediated expression of inflammatory cytokines [77-80, 88]. In this study, increased expression of TNF-α and other inflammatory cytokines (IL-6, IL-1β, and IFN-γ), as well as activation of necroptosis pathway were recorded in DSS-induced colitis mice model, which indicated that chemically-induced (DSS) necroptotic cell death played a role in the expression and release of inflammatory cytokines which in turn activate necroptosis in neighbouring cells and exacerbate inflammation in the form of a chain reaction. The results in chapter 5 indicated that orally administered polysaccharides to mice prevented increased expression of inflammatory cytokines in the colon of ulcerative colitis mice. The effect of the polysaccharide sample on the expression of TNFR1 was also investigated. However, in contrast to the report of Nishitani *et al* [19] who reported the endocytic effect of lentinan on TNFR1 as its anti-inflammatory mechanism, our polysaccharide sample did not show any effect on the expression of TNFR1 in both *in vivo* and *in vitro* models of necroptosis, suggesting that the polysaccharide sample may not be similar

to lentinan. This data indicated that the anti-necroptosis activity of the polysaccharide sample and its inhibitory effects on the expression of inflammatory cytokines were independent of the expression of TNFR1 on the cell surface, thereby suggesting a direct effect on necroptosis mediators. Furthermore, the polysaccharide sample suppressed expression of monocyte chemoattractant protein-1, MCP-1 (CCL-2) and IL-8 in the colon of ulcerative colitis mice and Caco-2 cells undergoing necroptosis respectively (chapter 5), suggesting that the polysaccharides prevented infiltration of inflammatory monocytes into the inflamed colon.

Ingested toxic environmental chemicals and interactions of IECs with luminal contents, such as food antigens and microbiota, result in necroptotic cell death. Necroptotic IECs release their cytoplasmic contents, such as DAMPs, into the extracellular space, which in turn initiate necroptotic signaling cascade in neighbouring cells, and thus promote intestinal inflammation [24]. Furthermore, IECs necroptosis results in an increased permeability of mucosa barrier to luminal antigens and microbiota into the lamina propria. Interactions of immune cells in the lamina propria with luminal antigens and microbiota results in more release of pro-inflammatory cytokines and consequently exacerbates intestinal inflammation [89]. However, this study has demonstrated that orally administered polysaccharides from *Lentinula edodes* inhibited necroptotic cell death in the colon and ameliorated ulcerative colitis in mice. Therefore, these findings strongly suggest that the inhibition of necroptosis by polysaccharide extracts from *Lentinula edodes* may partly be responsible for its activity against ulcerative colitis. This study has provided evidence for the anti-necroptosis and anti-inflammatory activity of *L. edodes* polysaccharides to support its use as an alternative source of therapeutic agent against ulcerative colitis.

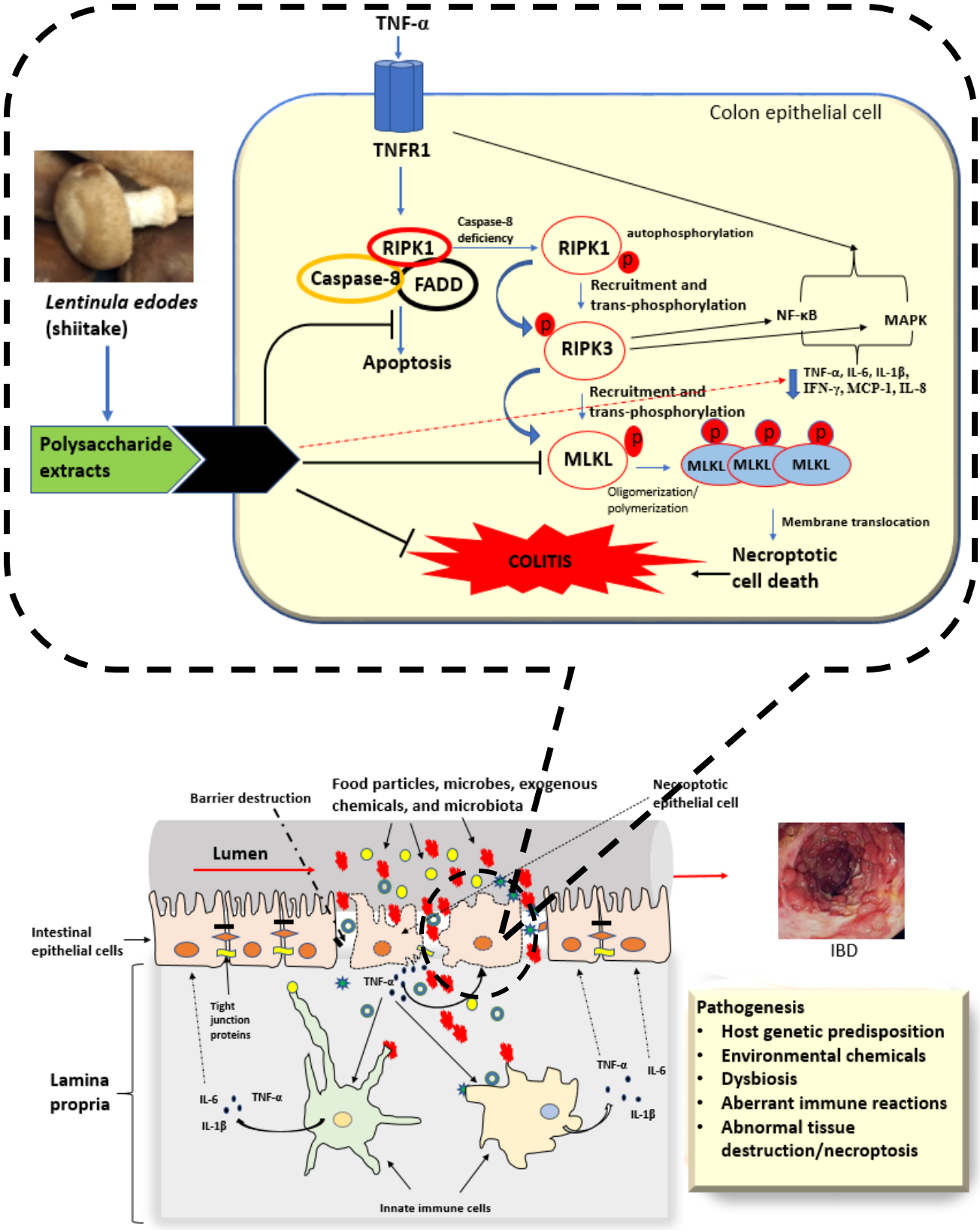


Figure 6.1. Scheme showing the mechanistic action of *Lentinula edodes* polysaccharides on IBD through inhibition of necroptosis

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LIST OF PUBLICATIONS

1. Alagbaoso C.A. and Mizuno M. Polysaccharides from Shiitake Medicinal Mushroom *Lentinula edodes* (Agaricomycetes) Suppressed pMLKL-mediated Necroptotic Cell Death and Colitis in Mice. *Int. J. Med. Mushrooms*, 2020. (**Accepted**).

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