The Effects of Cooked Whole Asparagus (*Asparagus officinalis* L.) and its Purified Bioactive, Rutin, on Symptoms of DSS-induced Acute Colitis and Recovery in C57BL/6 Mice

By

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The Effects of Cooked Whole Asparagus (Asparagus officinalis L.) and Its Purified Bioactive, Rutin, on Symptoms of DSS-Induced Acute Colitis and Recovery in C57BL/6 Mice

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University of Guelph, 2013

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This thesis explored the effects of cooked whole asparagus and its purified bioactive, rutin, on colitis symptoms and disease progression in mice using a chemically-induced model of colitis. This model mimics active colitis and recovery states of ulcerative colitis. C57BL/6 mice were fed a basal diet supplemented with 2% asparagus or 0.025% rutin for 3 weeks. Colitis was induced by 2% dextran sodium sulfate in drinking water for 7 days. Asparagus diet was determined to contain higher antioxidant capacities than rutin diet through antioxidant assays. During active colitis, consumption of asparagus alleviated some clinical symptoms (stool consistency, stool blood, and spleen hypertrophy) of colitis. In recovery, asparagus-fed mice were improving in terms of regenerating crypts, surface epithelial, and goblet cells, potentially due to its rutin content. Overall, these findings advocate that asparagus can be therapeutic in treating symptoms during active colitis and recovery phases of ulcerative colitis.
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# Table of Contents

Chapter 1 : Literature Review .................................................................................................................. 1
1.1 Inflammatory Bowel Disease and Ulcerative Colitis ........................................................................ 2
   1.1.1 Overview ................................................................................................................................. 2
   1.1.2 Epidemiology .......................................................................................................................... 2
1.2 Risk Factors of IBD ........................................................................................................................... 3
   1.2.1 Genetic Susceptibility .............................................................................................................. 4
   1.2.2 Immune System and Microflora ............................................................................................... 5
   1.2.3 Environmental Factors ........................................................................................................... 6
1.3 Animal Models of IBD ......................................................................................................................... 7
   1.3.1 Overview ................................................................................................................................. 7
   1.3.2 Dextran Sodium Sulfate Model of Colitis ............................................................................... 8
1.4 Etiology ................................................................................................................................................ 14
   1.4.1 Inflammatory Mediators in Intestinal Immunity ....................................................................... 14
   1.4.2 Reactive Oxygen Species and Oxidative Stress ...................................................................... 18
1.5 Complications with Ulcerative Colitis ............................................................................................. 19
1.6 Therapy ............................................................................................................................................... 20
   1.6.1 Conventional Treatments ........................................................................................................ 20
   1.6.2 Complementary and Alternative Treatments .......................................................................... 21
   1.6.3 Natural Dietary Interventions ................................................................................................ 23
1.7 Phenolic Compounds and Health ....................................................................................................... 24
   1.7.1 Nomenclature and Overview .................................................................................................. 24
   1.7.2 Flavonoids ................................................................................................................................ 25
   1.7.3 Pure Phenolic Compounds in Experimental IBD ..................................................................... 26
1.8 Rutin and Quercetin ............................................................................................................................ 26
   1.8.1 Overview ................................................................................................................................. 26
   1.8.2 Rutin and Quercetin in vivo and in vitro ................................................................................ 28
1.9 Asparagus (*Asparagus officinalis* L.) ............................................................................................... 35
   1.9.1 Overview ................................................................................................................................. 35
   1.9.2 Phenolic Compounds in Asparagus ......................................................................................... 36
Chapter 2: Study Rationale, Objectives, and Hypotheses

2.1 Asparagus (Asparagus officinalis L.)

2.2 Hypothesis

2.3 Objectives

Chapter 3: Chemical Characterization of Asparagus Flour and Assessing the Suitability of Supplementing Asparagus into C57BL/6 Mice Diets

3.1 Introduction

3.2 Experiment 1: Asparagus Flour Preparation and Rutin Analysis

3.2.1 Materials and Methods

3.2.1.2 Analysis of Rutin Concentration in Asparagus Flour

3.2.1.3 Macronutrients and Soluble Fibre in Asparagus Flour

3.3 Experiment 2: Determining the Suitability of Supplementing Asparagus into C57BL/6 Mice Diets

3.3.1 Materials and Methods

3.3.2 Results and Discussion

3.4 Summary

Chapter 4: The Effects of Rutin on Acute Colitis Symptoms and Disease Progression in DSS exposed C57BL/6 Mice

4.1 Introduction

4.2 Materials and Methods

4.2.1 Diet Preparation

4.2.2 Mice

4.2.3 Study Design

4.2.4 Colonic MPO Assay

4.2.5 Colon Histopathology

4.2.6 Statistical Analyses

4.3 Results

4.3.1 Determination of Rutin in Diets

4.3.2 Pre-DSS Food Intake and BW

4.3.3 Food and Water Intake During Acute Colitis

4.3.4 DAI During Acute Colitis
List of Tables

TABLE 1-1: A SUMMARY OF SOME COMMONLY STUDIED TH1 CYTOKINES .......................................................... 17
TABLE 1-2: QUERCETIN AND RUTIN IN EXPERIMENTAL COLITIS IN VIVO AND IN VITRO ................................. 31
TABLE 1-3: PROXIMATE ANALYSIS OF ONE SERVING (1 CUP) OF RAW ASPARAGUS [222] .............................. 35
TABLE 3-1: MACRONUTRIENT COMPOSITION OF ASPARAGUS FLOUR .......................................................... 48
TABLE 3-2: COMPOSITION OF EXPERIMENTAL DIETS (BD AND 12% ASPARAGUS) ........................................ 50
TABLE 4-1: COMPOSITION OF EXPERIMENTAL DIETS (BD AND 0.1% RUTIN) ..................................................... 60
TABLE 4-2: DAI SCORING SYSTEM .................................................................................................................. 63
TABLE 4-3: HISTOLOGICAL GRADING OF COLON EROSION DURING DSS-INDUCED ACUTE COLITIS ............ 66
TABLE 4-4: HISTOLOGICAL GRADING OF GOBLET CELL DEPLETION DURING DSS-INDUCED COLITIS ......... 67
TABLE 5-1: MACRONUTRIENT COMPOSITION OF ASPARAGUS FLOUR .......................................................... 88
TABLE 5-2: COMPOSITION OF EXPERIMENTAL DIETS (BD, 2% ASPARAGUS, AND 0.025% RUTIN) ............... 90
List of Figures

Figure 1-1: Chemical structures of phenolic compounds ................................................. 25
Figure 3-1: Asparagus preparation .................................................................................. 45
Figure 3-2: Rutin concentration in asparagus flour ......................................................... 47
Figure 3-3: Determination of rutin in basal diet (BD) and 12% asparagus diet .......... 52
Figure 3-4: Food intake and body weight (BW) measured over 21 days ....................... 54
Figure 3-5: Determination of rutin in 3, 6, and 12% asparagus diets ......................... 55
Figure 4-1: Experimental Design .................................................................................. 63
Figure 4-2: Method of collecting distal colon sections .................................................. 64
Figure 4-3: Histological grading of colon erosion in DSS-induced acute colitis .......... 66
Figure 4-4: Histological grading of goblet cell depletion in DSS-induced acute colitis...... 67
Figure 4-5: Determination of rutin in basal diet (BD), and 0.025, 0.05, and 0.1% rutin diets .................................................................................................................. 69
Figure 4-6: Pre-DSS food intake and BW ....................................................................... 70
Figure 4-7: Food and water intake during DSS-induced acute colitis .............................. 71
Figure 4-8: Effects of rutin diets (0.025%, 0.05%, and 0.1%) on DAI clinical symptoms of DSS-induced acute colitis ........................................................................................................ 73
Figure 4-9: Effects of rutin diets on organ size parameters in DSS-induced acute colitis... 75
Figure 4-10: Effects of rutin diets on distal colon myeloperoxidase (MPO) during DSS-induced acute colitis ............................................................................................................ 76
Figure 4-11: Effects of rutin diets on distal colon histopathology .................................... 77
Figure 5-1: Analysis of rutin concentration in asparagus flour ....................................... 87
Figure 5-2: Experimental design .................................................................................... 93
Figure 5-3: Histological grading of colon erosion during recovery ............................... 94
Figure 5-4: Histological grading of goblet cell depletion during recovery.................... 95
Figure 5-5: Determination of rutin in BD, 2% asparagus, and 0.025% rutin diets ........... 97
Figure 5-6: Antioxidant activity of BD, 2% asparagus, and 0.025% rutin diets ............. 98
Figure 5-7: Pre-DSS food intake and BW ....................................................................... 99
Figure 5-8: Food and water intake during DSS-induced acute colitis .............................. 100
Figure 5-9: Effects of 2% asparagus and 0.025% rutin diets on DAI during DSS-induced acute colitis .................................................................................................................. 101
Figure 5-10: Effects of 2% asparagus and 0.025% rutin diets on organ size parameters during DSS-induced acute colitis ............................................................................................. 102
Figure 5-11: Effects of 2% asparagus and 0.025% rutin diets on myeloperoxidase (MPO) activity during acute colitis ................................................................................................. 103
Figure 5-12: Histopathology of distal colon erosion and goblet cell depletion during acute colitis ............................................................................................................................. 104
Figure 5-13: Food and water intake during colitis recovery ........................................... 105
Figure 5-14: Impact of 2% asparagus and 0.025% rutin diets on survival during colitis recovery in mice ................................................................. 106
Figure 5-15: Effects of diets on DAI during colitis recovery ................................................................. 107
Figure 5-16: Effects of 2% asparagus and 0.025% rutin diets on organ size parameters during colitis recovery ............................................................................. 108
Figure 5-17: Effects of 2% asparagus and 0.025% rutin diets on myeloperoxidase (MPO) activity during colitis recovery ............................................................................. 109
Figure 5-18: Histopathology of distal colon erosion during colitis recovery ........................................ 110
Figure 5-19: Histopathology of distal colon goblet cell depletion during colitis recovery ........................................................................................................................................ 111
Figure 5-20: Serum T_h1 cytokine multiplex analysis in colitis recovery .............................................. 112
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>5-ASA</td>
<td>5-aminosalicylic acid</td>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>AAE</td>
<td>Acetic acid equivalent</td>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cells</td>
<td>mdr1a⁻/⁻</td>
<td>Multidrug resistance gene-deficient</td>
</tr>
<tr>
<td>BD</td>
<td>Basal diet</td>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow-derived macrophages</td>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>c.i.</td>
<td>Colitis induction</td>
<td>MZ</td>
<td>Monozygotic twins</td>
</tr>
<tr>
<td>CAC</td>
<td>Colitis-associated colorectal cancer</td>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>CAM</td>
<td>Complementary and alternative medicine</td>
<td>NFDL</td>
<td>NF-κB-dependent luciferase</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>DAI</td>
<td>Disease activity index</td>
<td>OMAFRA</td>
<td>Ontario Ministry of Agriculture, Food and Rural Affairs</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
<td>ORAC</td>
<td>Oxygen radical absorbance capacity</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran sodium sulfate</td>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>DW</td>
<td>Dry weight</td>
<td>PUFAs</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>DZ</td>
<td>Dizygotic twins</td>
<td>QOL</td>
<td>Quality of life</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
<td>RCT</td>
<td>Randomized controlled trials</td>
</tr>
<tr>
<td>FOS</td>
<td>Fructo-oligosaccharides</td>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>FRAP</td>
<td>Ferric reducing/antioxidant power assay</td>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>GBF</td>
<td>Germinated barley foodstuff</td>
<td>SA-PE</td>
<td>Streptavidin-phycoerythrin</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
<td>SCFA</td>
<td>Short-chain fatty acids</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>GPX</td>
<td>Glutathione peroxidase</td>
<td>SPF</td>
<td>Specific-pathogen-free</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
<td>TE</td>
<td>Trolox equivalent</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human umbilical vein endothelial cells</td>
<td>TH</td>
<td>T helper cell</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
<td>TJ</td>
<td>Tight junctions</td>
</tr>
<tr>
<td>IECs</td>
<td>Intestinal epithelial cells</td>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
<td>TNBS</td>
<td>2,4,6-trinitrobenzene sulfonic acid</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
<td>UC</td>
<td>Ulcerative colitis</td>
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Chapter 1 : Literature Review
1.1 Inflammatory Bowel Disease and Ulcerative Colitis

1.1.1 Overview

Inflammatory bowel disease (IBD) is a spontaneous chronic, relapsing, and remitting disorder of the gastrointestinal tract (GIT) that comprises two major subtypes: ulcerative colitis (UC) and Crohn’s disease (CD) [1]. Both conditions have significant impacts on the quality of life (QOL) of patients, producing similar disease symptoms such as abdominal pain, bleeding, diarrhea, and weight loss. However, UC and CD are dramatically distinct with regards to disease location, morphology, and histopathology. In UC, intestinal inflammation is restricted to the large intestine (colon), whereas CD can encompass any part of GIT, but involves mostly the distal small intestine (ileum) and the proximal colon [2, 3]. Morphologically, inflammation in UC affects only the mucosal layer of the intestinal wall, but with CD, it can involve all layers of the intestinal wall. UC and CD do share many inflammatory similarities such as epithelial barrier dysfunction, but histologically, UC usually consists of more neutrophil infiltration, goblet cell mucin depletion, and prominent crypt abscesses, while CD consists of skip lesions, non-necrotizing granulomas, and pseudopolyps [4]. Although most patients with IBD can lead productive lives with the use of medications and surgery, IBD is considered a lifetime disease that has substantial personal burden with increased morbidity [3].

1.1.2 Epidemiology

IBD has been traditionally considered a disease of developed Western countries [5]. However, IBD is now emerging as a global disease as the incidence and prevalence of the
disorder is increasing with time and in various regions around the world [6, 7]. In Europe and North America, the annual incidence rates for UC ranges from 0.6 to 24.3 cases, and from 2.2 to 19.2 cases per 100,000, respectively [5, 6], while values are markedly decreased in Asia and the Middle East with 0.1 to 6.3 cases per 100,000 [5, 6]. With regards to prevalence rates for UC, North America has the highest with 7,000 to 46,000 newly diagnosed cases each year [5]. Although the incidence and prevalence rates in Western countries such as North America and Europe are still quite high, the rates are starting to stabilize in those high-incidence populations, while continuing to increase in low-incidence nations such as those in southern and eastern Europe [8-10] and Asia [11-15]. For instance, time trend statistics from four Asian studies [14] demonstrated that there was: a 5-fold increase in UC incidence amongst Japanese patients within 20 years [16]; a 7-fold increase within 10 years in Seoul, Korea [11]; and a 6-fold increase in Hong Kong Chinese within 20 years [17]. Overall, the burden of UC appears to be increasing over time as developing nations become increasingly industrialized [5, 18], which stresses the need for improved therapeutic and preventative treatments.

1.2 Risk Factors of IBD

As a multifactorial disorder, the development of UC is influenced by several factors including genetic susceptibility of the host, the host immune system, and other environmental factors [2, 19-21].
1.2.1 Genetic Susceptibility

Population-based studies suggest that genetic susceptibility plays a role in the pathogenesis of IBD, where both CD and UC are clustered as related polygenic disorders [22]. About 20% of people with UC have a close relative with IBD [23], and the risk of getting IBD increases 2 to 13 times for offspring of patients who have IBD. For patients who have UC, their offspring would have an occurrence of 6.26%, and for CD patients, the occurrence would be 9.2% [24]. However, the cause of IBD cannot be attributed to only genetics as monozygotic (MZ; genetically identical) and dizygotic (DZ) twin studies have shown low concordance rates. Combined data from a Swedish [25], British [26], and a Danish [27] twin study indicated that the concordance rates for UC in MZ and DZ twins were 15% and 4%, respectively [28].

There has also been substantial progress in the last 2 years in characterizing the susceptibility genes involved in IBD using genome-wide association scanning in large studies of cases and controls [29-31]. CD and UC have been confirmed in 99 susceptibility loci/genes, 71 associated with CD and 47 with UC [32, 33]. There are multiple genes involved in the interleukin (IL)-23/Th17 signalling pathway, including STAT3 and IL-12B, which have been noted to be important in regulating intestinal immune homeostasis [34]. Another signaling pathway that appears to be playing a major role in IBD pathogenesis is the IL-10 pathway [35, 36]. IL-10 is a key anti-inflammatory cytokine that helps induce and maintain tolerance toward commensal bacteria [36]. Case-control studies have provided evidence that IL-10 polymorphisms are significantly associated with IBD occurrence [37-39]. Gene targeting animal studies using IL-10 gene-deficient mice have discovered that unless these mice were kept in germ-free conditions, they would develop spontaneous
enterocolitis with mucosal hyperplasia, increased numbers of crypt cells, and inflammatory cell response, to just name a few [40, 41].

1.2.2 Immune System and Microflora

It has been speculated that IBD arises from an inappropriate activation of the immune system to the colonic microflora in part due to immunoregulatory defects of the colonic mucosa [42]. Human GIT is colonized with an immense community of symbionts and commensals that have vital roles in immune functioning, nutrient processing, and other host activities [43]. In a healthy, non-diseased gut, these symbiotic and commensal bacteria live peacefully with their host, but in a diseased state or in a genetically susceptible individual, the relationship between indigenous gut microbes and their hosts can shift from commensalism toward pathogenicity [43]. One genetic variant that has been identified in CD patients, NOD2, has been implicated in altering the immune system so that it initiates an amplified response to bacteria, causing inflammation [44]. A recent study showed that NOD2 mutation in CD aggravated nuclear factor kappa B (NF-κB) activity and IL-1β processing, suggesting initiation and/or promotion of mucosal inflammation [45]. Conversely, UC has been associated with a mutation in the Toll-like receptor (TLR)-4 gene in humans [46], which is needed to detect lipopolysaccharide (LPS) contained in the cell walls of Gram-negative bacteria in order to activate the innate immune system [47]. Thus, carriers with this TLR-4 mutation would be at an increased risk for Gram-negative infections and an activated mucosal immune system.

A continuous stimulation of the mucosal immune system can lead to uncontrolled inflammation and subsequent defects to the intestinal epithelial barrier function,
particularly an increase in permeability of the intestinal epithelial cells (IECs) [48, 49]. This barrier is needed to protect against luminal antigens and microorganisms from entering the body [50]. Without this, commensal and pathogenic bacteria can cross over and illicit a perpetual cycle of inflammation with innate and adaptive immune cells [51].

1.2.3 Environmental Factors

The significant growth in the incidence of UC during the last half century, particularly with developing countries with a history of low prevalence, may indicate that environmental risk factors are playing a major role in disease development [5, 19]. Emigration [52-54], industrialization, and urbanization may explain the increase in UC incidence as changes to occupational status, diet, and lifestyle habits have all been implicated as potential environmental risk factors for UC [5, 18]. For example, UC is more prevalent in higher socioeconomic groups, affecting more white than blue-collar workers. Workers who have occupations outdoors are less prone to developing UC, whereas sedentary indoor workers have an increased risk [55].

The current role of dietary components and habits on the development and progression of UC is not conclusive. The most consistent association noted in dietary studies has been the connection between an increased risk of UC with high intakes of total fats, omega-6 fatty acids, and red meat, and a decreased risk of UC with high vegetable and fruit intake [56]. A recent case-control study also reported that a low consumption of fibre with a high consumption of refined sugar were significantly associated with the development of UC [57].

Interestingly, it has been widely publicized that there is an inverse relationship between UC and cigarette smoking [58-60]; however, a meta-analysis showed that former
cigarette smokers had a 70% greater risk of developing UC compared to those who never smoked [61]. The mechanisms underlying this protective effect are currently unknown, but smoking can alter a wide range of both innate and adaptive immune functions [62]. For instance, in clinical trials, smoking has reduced mucosal cytokine production [63], decreased distal bowel permeability [64], and increased colonic mucus production [65].

Environmental factors are undoubtedly involved with the pathogenesis of UC, and it is probable that the complex interactions between each factor may be responsible for the rising incidence worldwide.

1.3 Animal Models of IBD

To fully understand the molecular events that may occur during colonic inflammation, reliable and reproducible models of colitis are necessary. To test various hypotheses regarding the etiology of IBD, numerous experimental models of colitis have become available in the last decade [66]. Progress in understanding the mechanisms involved in IBD has increased due to these models. Presently, experimental models of colitis can be characterized as genetic [gene knockout (KO), transgenic], immunological (adoptive cell transfer), or chemical-based [67].

1.3.1 Overview

Gene KO models involve ‘knocking-out’ or making a specific gene or DNA region inoperative, in order to understand the roles and functions of that specific deleted gene or DNA compared to a wildtype control. Some well-studied gene KO models of IBD include IL-10 KO and IL-2 KO/IL-2 receptor KO mice, which have been found to develop spontaneous
colitis after 3 months of age [67, 68]. Transgenic models are also used to investigate the role and functions of specific genes or DNA. Yet, transgenic models usually use mice that are genetically engineered to overexpress a certain gene, such as with the IL-7 transgenic mice; mice overexpressing IL-7 were discovered to develop spontaneous acute colitis within 1-3 weeks of age [68]. Immunological models comprise of transferring specific host cell populations, often T cells, to severe combined immunodeficiency (SCID) mice to illicit colonic inflammation. These models allow for the study of immunopathology and immune regulation in the intestine [69]. Lastly, the most common chemicals used to induce colitis in rodents include dextran sodium sulfate (DSS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS), which are administered orally and rectally, respectively [67, 68]. All of these experimental models can produce diverse ulceration patterns at different locations in the GIT. For instance, the DSS model closely resembles UC since ulcerations occur in the colon, whereas TNBS mimics CD since ulcerations occur in the upper intestinal tract [70]. While none of these experimental models can fully replicate human IBD, they are still quite useful in developing novel therapeutic strategies and elucidating the etiology of IBD. For the purpose of this thesis, the DSS-induced colitis model will only be used and discussed.

1.3.2 Dextran Sodium Sulfate Model of Colitis

To date, DSS is one of the most common chemical compounds to induce colitis in animals. In the DSS model, colitis is induced by adding DSS into the animals’ drinking water [71, 72]. Acute or chronic colitis can be induced depending on the concentration, duration, and frequency of DSS administration. To induce acute colitis, the amount of DSS that is added to drinking water is usually between 2 and 5%, and it is given to the animals daily
for short periods (4-14 days) [71, 73]. Meanwhile, to induce chronic colitis, a low concentration of DSS, such as 1%, is given continuously for long periods (e.g. 100 days [74] or even up to 6 months [75]), or through cycles of relatively high doses of DSS and normal drinking water [71, 73]. The following sections will only focus on acute colitis induced by DSS.

1.3.2.1 DSS Overview

DSS is a polyanionic derivative of dextran, a complex polymer of straight and branched chains of glucose. Dextran is synthesized from sucrose by specific lactic-acid bacteria, such as *Leuconostoc* spp and *Streptococcus* spp [76]. When dextran is esterified with chlorosulphonic acid, it produces DSS, where the sulphur content is about 17%, which is equivalent to approximately two sulfate groups per glucosyl residue of the dextran molecule [71].

IBD is known to be a complex multifactorial disease, which is not reproducible in any cell culture system. Therefore experimental models of IBD are most crucially needed to investigate the pathophysiologies. Since 1985, when the first model of DSS was used to induce colitis in hamsters [77], the model has been extensively used over the years with other animals. Now the DSS model is widely accepted as a highly reproducible and simple-to-induce model that is applicable to various animals (e.g. mice, rats, hamsters) [71, 78]. The model is also inexpensive, may either induce acute or chronic inflammation, and is perceived as a representative model of experimental colitis as it mimics symptoms of UC, clinically and histologically [70, 71, 79, 80]. However, the severity of DSS-induced inflammation is dependent on the animal strain, DSS molecular weight (MW), and degree of
sulfation, and as previously mentioned, the concentration or dose, and frequency and duration of exposure of DSS [78].

Mice can show different susceptibilities and responsiveness to DSS-induced colitis, and this may be due to the genetics (strain and gender) and microbiological aspects of the animal. A study analyzing DSS-induced colitis (3.5% DSS of 36-45 kDa MW for 5 days) in nine strains of mice discovered major differences in DSS responsiveness among inbred strains [81]. C3H/HeJ, NOD/Ltj, NOD-scid inbred strains were very susceptible to DSS-induced lesions, while 129/SvPas and DBA/2J inbred mice showed less susceptibility [81]. Other studies have found that BALB/c mice, which are often used in DSS-colitis studies, were also less susceptible to DSS compared to IQL/Jic [82] or C57BL/6 mice [83]. A significant gender bias had also been found within the nine mouse strains exposed to DSS; male mice had increased histological colon scores of severity and hyperplasia compared to female mice [81]. There are also differences in the recovery process from inflammatory damage, as Melgar et al. (2005) have shown that after DSS withdrawal (3% DSS for C57BL/6 and 5% DSS for BALB/C for 5 days), BALB/c mice were able to partly recover from the colitis insult within one week, while C57BL/6 mice progressed into chronic colitis [83]. This indicates that genetic factors are contributing to DSS susceptibility and are involved in the regulation of the mucosa in either limiting the inflammatory response and/or resisting inflammatory damage.

The MW of DSS is also important in determining the severity of colitis that will be induced [84]. DSS is highly variable in terms of MW, ranging from 5 kDa to up to 1,400 kDa [78]. Administration of DSS at three different MW (i.e. 5, 40, and 500 kDa) produced various degrees of colitis severity that was also location-dependent in BALB/c mice [84].
DSS of 5 kDa produced only sporadic lesions in the cecum and upper colon. Meanwhile, DSS of 40 kDa produced more severe colitis than 5 kDa, affecting the middle and distal third of the large bowel. And lastly, 500 kDa produced no lesions in the large bowel of BALB/c mice; it was determined that this MW was too large, thereby preventing its passage through the mucosal membrane to exert its effects [84].

1.3.2.2 Clinical, Histological, and Molecular Features of DSS-induced Colitis

The clinical and histological symptoms of DSS-induced colitis are most reflective of UC. In the acute phase of DSS-induced colitis, weight loss, increase in stool consistency (diarrhea), presence of bloody stool, reduced physical activity, and a decrease in food and water intake are initially observed [71, 78]. These clinical symptoms can be seen within 2 days post-colitis induction [78], and the inflammation is entirely established within 7-10 days [81].

Histologically, DSS-induced acute colitis affects mainly the mucosa of the colon, but can spread to the submucosa and muscularis mucosa [71]. The typical histological characteristics include: crypt distortion; epithelial cell loss; increased mucosal edema and permeability; immune cell infiltration (e.g. neutrophils, macrophages, and lymphocytes) into the lamina propria and submucosa; goblet cell loss and decreased mucin production; presence of ulcers; cryptitis; and crypt abscesses [71, 78, 83, 85]. Cryptitis and crypt abscesses are common features of human IBD, and they involve transepithelial migration of neutrophils into mucosal epithelial lining and crypt lumen, respectively [83]. Crypt loss and subsequent crypt shortening occur around day 2 or 3 of DSS intake, and are thus considered the earliest histological signs of colitis, even before the development of inflammation [71, 85]. And by day 5 post-administration of DSS, erosion and inflammation
are observed [85]. It has been found that the removal of DSS results in recovery, including a restitution of normal colonic architecture [81, 86], yet the extent of recovery is dependent on mouse strain [83].

Molecularly, there have been various biomarkers detected with DSS-induced colitis, which are also associated in human UC. For instance, myeloperoxidase (MPO) is a mammalian enzyme that is stored in neutrophils and macrophages, which are recruited during inflammatory processes. Thus, MPO activity is an indicator of the degree of neutrophil infiltration and has been positively correlated with worsened disease state in IBD patients [87], and also positively correlated with an increased exposure to DSS [88]. Furthermore, the expression of proinflammatory cytokines (e.g. IL-10, IL-12, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and IL-1β) are also significantly increased in the colon of IBD patients [89], and with DSS induction in mice [88].

Overall, the DSS model of acute colitis is an effective model for UC as it reliably produces clinical, histological, and molecular features that are representatively observed in UC patients. Therefore, this model is deemed suitable for investigating the pathogenesis and therapeutic options for colitis.

1.3.2.3 Pathogenesis of DSS-induced Colitis

The mechanisms through which DSS initiates colitis are presently unknown, but there have been several proposed hypotheses involving an increase in gut permeability (e.g. mucus layer, tight junctions (TJ), epithelial cells). The increase in gut permeability would allow the permeation of luminal bacteria and large molecules, such as DSS.

First, due to the structure of DSS, it is likely that DSS can directly alter the mucus structure within the large intestine. There are two mucus layers, made up of a gel-forming
mucin called MUC2, which are found firmly attached to the epithelium surface [90]. These layers are there to prevent luminal bacteria from crossing over into our bodies. It was found through an in vitro study that DSS can cause quick changes to the mucus layers, increasing permeability to bacteria [91]. The glycans on MUC2 mucin from human colon carries multiple negative charges on sulfate residues [92]. Mucin sulfation is deemed important for mucus function as mice lacking the Nas1 sulfotransporter that is needed for sulfation of mucins, have increased susceptibility to colitis [93]. So it is probable that sulfated DSS can be readily soluble in the sulfated MUC2 mucin network, thereby allowing the sulfates in the DSS to destabilize the interactions that maintain the organization of the mucin network.

Second, DSS can directly alter gut permeability by interacting with TJ proteins. Studies indicate that TJ proteins, such as zona occludens-1, were reduced by DSS as early as day 1 and increased gut permeability by day 3, which were both observed before colonic inflammation [85, 94].

Lastly, DSS may be changing gut permeability by directly acting as a toxin to colonic epithelial cells. In vivo studies have reported an increase in epithelial cell apoptosis with DSS-induced colitis [95, 96]. Vetuschi et al. (2002) found that a 7 day colitis induction with DSS in Sprague-Dawley rats increased epithelial apoptotic index by 20-fold compared to healthy controls. Araki et al. (2010) also reported a 5-fold increase in epithelial apoptosis in DSS-induced BALB/c mice compared to controls [95]. They also found that there was a significant reduction (about 2-fold) in cell proliferation, thereby causing defects in the integrity of the epithelial barrier [95]. Therefore, it appears that DSS can directly affect the balance between apoptosis and proliferation of colonic epithelial cells.
In light of the above, changes in gut permeability from DSS interfering and causing injury to the mucus layers, TJs, and epithelial cells, would allow the permeation of luminal bacteria and content into colonic mucosa. An activated inflammatory response, further damage to epithelial cells, and basal crypts would all likely ensue due to this permeation. Similar to other experimental models of IBD, the DSS-induced colitis model does not fully reflect human UC, but the proposed hypotheses of DSS pathogenesis is well correlated with what is known with human IBD. Research has found that UC patients also had reduced adherent mucus [97], changes in TJ protein expression [49], and increased epithelial cell apoptosis [98]. Overall, this demonstrates that the DSS-induced colitis model is not only reliable, reproducible, and simple to use, but it is also the most current representative experimental model of human UC, clinically, histologically, molecularly, and pathogenically.

1.4 Etiology

The exact etiology of IBD remains unknown; however, with the vast amount of experimental models that are currently available, as previously discussed, it is possible to test various hypotheses [66]. Progress in understanding the mechanisms involved in IBD have risen due to these models. For the purpose of this thesis, the role of inflammatory mediators and oxidative stress in IBD pathogenesis will only be discussed.

1.4.1 Inflammatory Mediators in Intestinal Immunity

The epithelium, containing a dense network of innate and adaptive immune cells, is central in monitoring the integrity and health of the mucosa by eliciting appropriate responses to invading bacteria and antigens. Amongst the antigen-presenting cells (APCs),
dendritic cells (DC) are the most commonly found subtype in the intestinal lamina propria [99]. Once activated by interaction with an antigen or injury, they initiate the adaptive immune response by migrating to lymph nodes where they interact with T and B cells. T helper cell (T\(_h\)) gets activated by activated DC and they differentiate into either T\(_h\)1 or T\(_h\)2 cells. T\(_h\)1 or T\(_h\)2 cells then migrate to the site of injury to secrete specific small signalling proteins called cytokines, which help mediate the inflammatory pathway [100].

Cytokines can be categorized into either pro- or anti-inflammatory (immunosuppressor), or both (immunomodulatory), depending on the way they influence inflammation [101] (Table 1-1). T\(_h\)1 cells are involved with macrophages and are marked by an upregulation of TNF-\(\alpha\), IL-1\(\beta\), IFN-\(\gamma\), IL-6, and IL-17, while T\(_h\)2 cells are mainly involved with B cells and increases in IL-4, IL-5, and IL-13 secretions [102]. These increases in cytokine release can initiate the recruitment of other immune cells, such as natural killer (NK) cells, neutrophils, and macrophages. These immune cells in turn increase the levels of cytokines, amplifying the process of inflammatory defense. For instance, NK cells can secrete IFN-\(\gamma\) and TNF-\(\alpha\) [103], and neutrophils can release IL-1\(\beta\), IL-10, IL-6, and TNF-\(\alpha\) [104]. Macrophages, in particular, participate in an autoregulatory loop, where they become activated by certain cytokines, such as IFN-\(\gamma\) and TNF-\(\alpha\), and then become deactivated by producing IL-10 [105].

Research over the past decade has proposed that dysfunctions of the intestinal immune system and cross-reactivity against host epithelial cells may be one of the etiologies of IBD. A failure to control normally protective cell-mediated responses, as described above, can result in sustained activation of the mucosal immune system and uncontrolled overproduction of pro-inflammatory cytokines and mediators. As previously
mentioned, patients with IBD have been found to have increased polymorphisms, such as with IL-10 [37-39] and the TLR-4 gene [46], compared to healthy controls. Data from different genetically engineered or immune-manipulated animal models of colitis have also supported this hypothesis. For instance, studies involving mice with targeted deletions of IL-2 and IL-10 have resulted in spontaneous and chronic colitis [106]. These findings have resulted in the emergence of novel drug and dietary therapies for IBD which target specific cytokines or receptors in the inflammatory cascade, including TNF-α and IFN-γ [102, 107].
<table>
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<th>Cytokine (Secreted by)</th>
<th>Activities</th>
<th>References</th>
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| **IL-10** (neutrophils, T cells, B cells, NK cells) | Anti-inflammatory/immunosuppressor:  
- Inhibits antigen presentation and suppresses release of pro-inflammatory cytokines (e.g. IFN-γ, TNF-α) | [104, 108, 109] |
| **IL-1β** (neutrophils, resident macrophages, DC, monocytes) | Pro-inflammatory:  
- Immune defense against infection, ↑ transmigration of leukocytes (e.g. neutrophils)  
- Resets hypothalamus thermoregulatory center, leading to ↑ in body temperature | [104, 110] |
| **TNF-α** (neutrophils, APCs, NK cells) | Pro-inflammatory:  
- Stimulates IL-1β and IL-6 secretion, inhibits apoptosis, ↑ fibroblast and procoagulant proliferation  
- Activates neutrophils, macrophages, B cells, ↑ IFN-γ by T cells | [104, 111] |
| **IL-6** (neutrophils, T cells, resident macrophages) | Pro- and anti-inflammatory (immunomodulatory):  
- Induces B cell differentiation, T cell proliferation and differentiation, ↑ IL-2  
- Mediator in fever and acute phase responses  
- Can control pro-inflammatory cytokine levels (e.g. TNF-α) | [104, 112, 113] |
| **IL-17A** (T<sub>H</sub> cells) | Pro- and anti-inflammatory (immunomodulatory):  
- Recruits immune cells to peripheral tissues (needs NF-κB activation)  
- Induces TNF-α, IL-6, and IL-1β | [114-116] |
| **IFN-γ** (NK cells) | Pro- and anti-inflammatory (immunomodulatory):  
- Antiviral and antiproliferative  
- Activates neutrophils, NK cells, and vascular endothelial cells, promotes T and B cell differentiation | [117] |
1.4.2 Reactive Oxygen Species and Oxidative Stress

Another proposed mechanism of IBD pathogenesis is through oxidative stress caused by an imbalance of prooxidant and antioxidant pathways involving reactive oxygen species (ROS) [118]. ROS are chemically reactive molecules containing oxygen that are normally produced as byproducts of normal metabolism [119]. Direct measurements of ROS are problematic due to their short half-lives; however, present techniques estimate oxidative status by observing key antioxidant enzymes, such as glutathione (GSH), superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX) [119]. Furthermore, assays for antioxidant capacity, such as the oxygen radical absorbance capacity (ORAC) and ferric reducing/antioxidant power assay (FRAP), can provide a general assessment of antioxidant status [120].

It has been noted that there is increased free radical DNA damage and decreased plasma antioxidant defences in both UC and CD [121]. Rodent models have been used to evaluate the status of ROS during colitis. Typical observations included an increased upregulation of ROS-inducing enzymes, such as lipid peroxidase, inducible nitric oxide synthase (iNOS), MPO, and GPX; and a downregulation of antioxidant enzymes, such as SOD, GSH, catalase, and glutathione S-transferase (GST) [122-124]. Esworthy et al. (2001) has also provided a causal link between ROS in UC pathogenesis; they showed that mice lacking the antioxidant GPX developed symptoms similar to UC even in the absence of any chemical insults [124]. Recent studies have proposed that oxidative stress may be a component by which cytokine gene expression is upregulated through the activation of NF-κB [125].
Aside from having imbalances in the prooxidant and antioxidant pathways, an imbalance with inflammatory mediators in intestinal immunity, as previously discussed, may also contribute to the increased production of ROS in UC. One known characteristic of UC is the infiltration of neutrophils and immune cells into inflamed mucosa [4]. These immune cells are not only secreting cytokines, chemokines, and adhesion molecules to help mediate the inflammatory process, but they also secrete ROS that increases epithelial permeability [126]. Activated neutrophils are also known to generate superoxide radicals, hydrogen peroxide, and MPO, which all have been involved with tissue damage [127].

Currently, it is unknown whether oxidative stress occurs prior to immune cell recruitment or the infiltration of immune cells into inflamed tissue causes an increase oxidative stress. Yet, it is recognized that there are homeostatic imbalances in either or both of the pathways involved in oxidative stress and intestinal immunity. Overall, this highlights the need for additional research to investigate potential treatments in targeting both ROS and inflammatory mediators.

1.5 Complications with Ulcerative Colitis

Aside from a decreased QOL and personal and financial burdens [3], there are additional health complications for UC patients due to the relapsing and remitting nature of the disease. Through an epidemiological study, Langholz et al. (1994) found that 25% of UC patients underwent remission within the first 3-7 years after diagnosis; 57% had recurring relapses; and that the cumulative probability of having a relapse was 90% after 25 years of follow-up [128].
The first complication with UC is the need for a coloproctectomy caused by the acute severe colitis, which occurs in 20-25% of patients after 10-25 years [128, 129]. Long-term problems after surgery include mortality, with an average annual rate of 0.7%, and morbidity, such as reoperations and disturbance of sexual function [130].

The second and most fatal complication is the onset of colitis-associated colorectal cancer (CAC) due to persistent, chronic inflammation [131]. Within 30 years of IBD onset, more than 20% of patients will develop CAC and more than 50% will die from it because the disease is very difficult to treat [132].

The final complication of UC is the development of extra-intestinal manifestations. Patients with active UC can acquire disorders involving the joints (23%), skin (15%), mouth and eye (each 4%) [133, 134]. Other complications include osteoporosis (3%), liver disease (5%), and peptic ulcers (10%) [134-136].

1.6 Therapy

In the absence of a cure, therapy is directed towards alleviating disease symptoms and/or maintaining remission (i.e. free from symptoms) to achieve an improved QOL. Although disease-related mortality is low, morbidity is high since most UC patients are continuously on medications, but when those fail, surgery (colectomy) is often required.

1.6.1 Conventional Treatments

Currently, conventional medications for UC include aminosalicylates, corticosteroids, antibiotics, and immunomodulators. Sulfasalazine, and its derivative, mesalazine or 5-aminosalicylic acid (5-ASA; mesalamine) are the most commonly used forms of aminosalicylates. These have been used as first-line therapies for many years
during active phases and for the induction and maintenance of remission [137]. They have been proposed to act upon epithelial cells by various mechanisms to regulate the release of cytokines [138] and ROS [139]. Corticosteroids, such as prednisone and hydrocortisone, are usually used for acute episodes of UC. Their mechanisms of action include inhibiting certain cytokines, and inflammatory pathways such as the arachidonic acid cascade [140]. Unlike aminosalicylates and corticosteroids, antibiotics such as metronidazole and vancomycin have been less effective in UC patients [141]. Immunomodulators are a new class of drugs that are presently being investigated for UC patients to avoid long-term usage of corticosteroids. Immunosuppressive agents, azathioprine and 6-mercaptopurine, inhibits the proliferation of T and B lymphocytes by causing chromosomal breaks [142, 143].

Although most of these drugs are deemed safe to use, there are potential adverse side effects with certain individuals. For instance, side effects from using 5-ASA can occur in 10-45% of people, depending on the dose used, and symptoms may range from diarrhea to pancreatitis [144]. Moreover, a prolonged use of steroids can increase the risk of osteoporosis [145], impair wound healing, and cause hyperglycemia [146]. Adverse effects of metronidazole include peripheral neuropathy, nausea, and neutropenia [142], while immunomodulators can cause bone marrow suppression, pancreatitis, and/or minor allergies characterized by abdominal pain, rash, and fever [143].

1.6.2 Complementary and Alternative Treatments

UC patients are increasingly using complementary and alternative medicine (CAM), which include traditional practices such as acupuncture, as they believe they are less toxic
than conventional drugs, Chinese herbal medicine, aromatherapy, and/or reflexology [147]. CAM overall appears to be more popular in Western populations than European countries with currently up to 50% using some form of CAM [148]. Yet, the usage of CAM is still quite high with European and Asian patients as herbal therapy seems to be the most commonly reported therapy for IBD [149, 150].

Traditional Chinese medicine is a broad range of practices that has been a tradition for more than 2,000 years. There have been various randomized controlled trials (RCT) regarding certain herbal medicines in the treatment of UC, such as Kui jie qing [151], aloe vera gel [152], wheat grass juice [153], and germinated barley food (GBF) stuff [154]. All of these RCT reported positive results; for instance, 72% of patients treated with Kui jie qing (alum, Halloysite, Calamine, Indigo naturalis, and plum-blossom tongue-pointing pills) enemas were considered cured compared to 9% of controls who took sulfasalazine, oral prednisolone, and prednisolone enemas [147]. It also appears that there are greater effects when CAM and conventional therapy are given together than when either of the treatments is administered separately [155]. For example, patients treated with only GBF, which consisted of dietary fibre and glutamine-rich protein, had worse clinical disease activity compared to conventional therapy alone, but when GBF and 5-ASA were combined, rectal bleeding and diarrhea were reduced significantly in UC patients [154].

Although CAMs may work for some or many individuals over conventional means, it is still worth noting that they are not always safe as most herbal remedies are unregulated [156]. Unpurified herbal extracts contain a vast array of biologically active compounds, which may have either beneficial or adverse effects. There have been some reports of significant poisoning effects of some herbs and even severe side effects involving herb-drug
interactions [157-159]. Adulterations have also been discovered in herbal remedies, such as the deliberate inclusion of prescription medicines (e.g. corticosteroids) [160], and/or other toxic contaminants like lead, mercury, and arsenic to induce a desired effect [161]. Therefore, a licensing authority similar in conventional medicine is urgently needed for herbal preparations in order to enforce quality control and regulate the production of safe CAM products for UC therapy.

1.6.3 Natural Dietary Interventions

Over the past decade, the use of dietary interventions in achieving UC remission has increasingly become an area of great interest as they have been found to provide effective therapy without the adverse effects as seen with conventional drugs and CAM [162-165]. Some natural dietary components that have been studied extensively are prebiotics, polyphenols, and essential polyunsaturated fatty acids (PUFAs). Prebiotics are non-digestible dietary fibre (e.g. psyllium, inulin, germinated barley) that are consumed by endogenous protective bacteria to stimulate their growth. The beneficial effects of prebiotics are due to the metabolites or short-chain fatty acids (SCFAs) produced from fermentation by colonic bacteria [166]. SCFAs are important in upholding colonic barrier integrity and function by serving as an energy source for enterocytes [167]. Moreover, SCFAs, such as butyrate, have been found to reduce colitis and anti-inflammatory cytokines, IL-1β, TNF-α, and IL-17 [168-171]. Conversely, phenolic compounds, such as curcumin, quercetin, rutin, and resveratrol, can exert anti-inflammatory effects by modulating NF-κB [172-174] and mitogen-activated protein kinases (MAPKs) [175] signalling pathways and levels of pro-inflammatory markers [174, 176] in animals with induced colitis. The role of
phenolics in UC will be discussed further in the succeeding sections. Lastly, omega-3 PUFAs such as eicosapentaenoic acid and docosahexaenoic acid have also been found to alleviate a number of inflammatory diseases including UC [177-180]. Proposed mechanisms of PUFAs may also be through the reduction of NF-κB activity and expression of pro-inflammatory cytokines [178, 181].

Overall, the current usage of conventional medical treatments by UC patients in the form of anti-inflammatory (e.g. 5-ASA) and immunosuppressive (e.g. corticosteroids) drugs [182], and the unconventional therapies involving CAM, all derive adverse side effects. Nutrition is now proposed to be a potential candidate in IBD management due to the evidence purported in the last decade with anti-inflammation and certain food components, such as PUFAs and prebiotics. Therefore, diet-based approaches may play a pivotal role in the clinical care of all patients with UC and hold promise for both preventing and treating the disease.

1.7 Phenolic Compounds and Health

1.7.1 Nomenclature and Overview

As previously mentioned, phenolic compounds are one class of natural dietary interventions in alleviating symptoms of UC. Phenolic compounds are naturally occurring plant molecules found abundantly in fruits and vegetables, with currently more than 8,000 phenolic structures still to be discovered [183]. The base structure of a phenolic compound comprises an aromatic or benzene ring with a carboxyl group attached (Figure 1-1A) [184]. There are many classes of phenolics and they are all defined by the nature of their carbon
backbone and the functional derivatives (esters, methyl esters, glycosides, etc.) that are attached [185].

![Chemical structures of phenolic compounds](image)

**Figure 1-1: Chemical structures of phenolic compounds**
The above shows the chemical base structure of (A) a phenolic compound, and chemical structures of (B) rutin, and (C) quercetin. Modified from [184, 186, 187].

### 1.7.2 Flavonoids

One of the most common phenolic classes is the flavonoid family; almost all plant tissues are able to synthesize flavonoids. Presently, there are more than 4,000 naturally occurring flavonoids that have been identified. Flavonoids are most often found in nature as glycosides (a compound bound to a sugar or carbohydrate moiety), but can occur as aglycones (non-sugar compound) in woody tissues and particularly from food processing.
effects [188, 189]. Their base structure consists of two aromatic rings linked by three carbons that are usually in an oxygenated C ring [188].

Flavonoids can be additionally classified into six groups depending on their heterocycle C ring: flavones, flavanones, flavonols, isoflavones, flavanols (catechins), and anthocyanidins [188]. Human consumption of all flavonoids is about a few hundred milligrams [190] to 650 mg/day [191]. The most commonly consumed flavonoids are the flavonols and flavones. Some common flavonols include quercetin, kaempferol, and myricetin found in onions, apples, broccoli, and tea, to name a few, whereas the most commonly consumed flavones are luteolin and apigenin found in celery, spinach, and green pepper [192].

1.7.3 Pure Phenolic Compounds in Experimental IBD

From a vast amount of in vitro and in vivo evidence, phenolic compounds have been acknowledged to be antioxidant and anti-inflammatory, and therefore have been proposed to be an alternative approach to preventing or treating inflammatory diseases, including IBD [193]. Quercetin, and its glycoside, rutin, have been the most investigated phenolic compounds in relation to IBD (Table 1-2), and therefore will be discussed in this thesis.

1.8 Rutin and Quercetin

1.8.1 Overview

Rutin (C_{27}H_{30}O_{16}; 610.517 g/mol; Figure 1-1B), also called rutoside, quercetin-3-rutinoside, and sophorin, is a flavonoid glycoside found in asparagus, buckwheat, fruit rinds, and the leaves and petioles of Rheum species [194-196]. Rutin has been found to be
chemically stable in both phosphate buffer and hydrochloric acid conditions (mimicking the small intestine and stomach environments, respectively) [197]. When ingested, rutin travels intact to the large intestine, where it is then rapidly hydrolyzed to its aglycone quercetin ($C_{15}H_{10}O_7$; 302.236 g/mol; Figure 1-1C) by β-glucosidase activity of colonic microflora, such as Bacteroides distasonis, B. uniformis, and B. ovatus [198, 199].

Quercetin itself is a flavonol and is also widely distributed in nature and can be found in foods such as apples, onions, and red grapes [200-203]. The total average intake of flavonols (quercetin, myricetin, kaempferol) and flavones (luteolin, apigenin) was observed to be 23 mg/day, with about 70% from quercetin [204].

In terms of bioavailability, rutin has been found to be absorbed more slowly than quercetin in Wistar rats according to observed concentrations of quercetin metabolites in plasma [205]. Carbonaro and Grant (2005) also found the same results but in rats of the Hooded Lister strain; although uptake rates were similar for quercetin and rutin during the first 5 minutes of exposure to the compounds, the maximum percentage of flavonoid that was taken up by the gut over the following 35 minutes was significantly higher for quercetin (60%) than for rutin (45%) [206]. It has been speculated that the absorption of rutin by colonic epithelial cells is delayed in comparison with quercetin because the sugar moiety on the C ring of rutin must be hydrolyzed by microflora in the large intestine prior to absorption [207]. Moreover, in two studies observing the pharmacokinetics of quercetin and rutin in healthy volunteers, it was found that quercetin was actively absorbed from the small intestine and possibly even in the stomach, while the majority of rutin can travel intact to the distal parts of the small intestine or the colon to be deglycosylated and then absorbed [208, 209]. This has also been confirmed in animal studies where they did not see
any deglycosylation of rutin in the upper intestine, but rapid deglycosylation in the large intestine [205, 210]. Thus, it may be hypothesized that quercetin and rutin would have different anti-inflammatory effects in diseases where the occurrence of inflammation is location-dependent along the GIT, such as in UC and CD.

1.8.2 Rutin and Quercetin in vivo and in vitro

1.8.2.1 Rutin

Rutin is proposed to be anti-inflammatory and anti-oxidative as it has been shown to significantly reduce colitis severity in several chemically-induced colitis rodent models (Table 1-2). One example is a study by Kwon et al. (2005), who used a DSS-induced colitis model with female ICR mice and fed them a diet containing either 0.001, 0.01 or 0.1% rutin or quercetin for 2 weeks. After 2 weeks, they found that dietary rutin, even at a low dose (0.01%) attenuated colorectum shortening and IL-1β production. In addition, oral administration of 0.1% rutin 3 days after DSS treatment significantly reversed colitis, as shown by suppression of both colorectum shortening and IL-1β production by 43 and 52%, respectively, compared to the control [176]. Another notable finding was from the study by Kim et al. (2005), who found that an oral administration of rutin [10 mg/Kg body weight (BW)] ameliorated TNBS-induced colitis in rats, reduced MPO activity to about 55% of the control and was just as effective as sulfasalazine (30 mg/Kg BW), a common colon-specific drug for the treatment of IBD [210].

The effects of rutin have been reported to act through suppressing pro-inflammatory cytokine production in mice, such as IL-1β and IL-6 [176], reducing MPO activity [210-212], and alleviating oxidative stress in the colon by counteracting GSH.
depletion [212, 213], which is an essential antioxidant; a depletion of GSH has been seen to cause a cascade of events resulting in cell death [211]. Moreover, pre-treatment of rutin (25-100 µM) can dose-dependently inhibit the expression of NF-κB and TNF-α production in LPS-stimulated human umbilical vein endothelial cells (HUVECs) [214].

1.8.2.2 Quercetin

Compared to rutin, there have only been three animal experimental colitis studies concerning quercetin, in which only one study found beneficial results (Table 1-2). In the first study, a rectal administration of 25 µM (300 µL) of quercetin was found to be just as effective as 20 mM (300 µL) 5-ASA, a common IBD-treating drug, in healing the damaged colon [210]. However, the other two studies found no significant effects with DSS-induced colitis severity after giving quercetin orally [172, 176]. The conflicting results may be due to a bioaccessibility issue; most of the quercetin may not have reached the colon as they could have been absorbed in the small intestine [176]. As discussed previously, quercetin has been found to be actively absorbed from the small intestine and possibly even in the stomach, while the majority of rutin can travel intact to the distal parts of the small intestine or the colon to be deglycosylated and then absorbed [208, 209]. This may explain why quercetin has not been seen to ameliorate DSS-induced experimental UC in mice when quercetin was ingested orally [172, 176], but intracolonic administration of quercetin was able to heal the colonic damage, as well as lower MPO activity, dose-dependently in rats [210].

In vitro models suggest that quercetin may be exerting its effects through inhibiting TNF-α and NF-κB-mediated pathways, including suppressing pro-inflammatory cytokines, such as IL-6, IL-8, and IL-1β [172, 210, 215, 216].
In summary, with the vast amount of evidence purporting the anti-inflammatory and anti-oxidative activities of rutin and quercetin \textit{in vivo} and \textit{in vitro}, the roles of these phenolic compounds as therapeutic tools in managing colonic inflammation is very promising. The ability of rutin and quercetin to improve symptoms of colitis, reduce MPO activity, and modulate TNF-\(\alpha\) and NF-\(\kappa B\) signalling and levels of pro-inflammatory cytokines, encourages the use of these natural compounds clinically. These results also promote future research to explore the effects of consuming whole foods rich in rutin or quercetin, such as asparagus, in experimental colitis.
Table 1-2: Quercetin and rutin in experimental colitis *in vivo* and *in vitro*

<table>
<thead>
<tr>
<th>Phenolic Compound</th>
<th>Study Design</th>
<th>Animal Model</th>
<th>Results</th>
<th>Reference</th>
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<tr>
<td><strong>Quercetin</strong></td>
<td>300 µL (10, 25, 50, and 100 µM) in phosphate buffered saline (PBS) rectally, 1 day after colitis induction (c.i.), 6 d</td>
<td>Male Sprague-Dawley rats, 15 mg/0.3 mL/rat TNBS (rectally)</td>
<td>Reduced colitis severity (colonic damage with regards to inflammation and ulceration severity) in a dose-dependent manner, lower MPO activity.</td>
<td>[210]</td>
</tr>
<tr>
<td></td>
<td>1 mg/Kg/d, 5 d after 5% DSS c.i.</td>
<td>Female Wistar rats, 5% DSS, 5 d, then 2% DSS for 10 d</td>
<td>No significant reduction in colitis severity.</td>
<td>[172]</td>
</tr>
<tr>
<td></td>
<td>0.001 (60 µg/d), 0.01 (0.6 mg/d), or 0.1% (6 mg/d), 14 d prior or 3 d after c.i.</td>
<td>Female ICR mice, 5% DSS, 1 wk</td>
<td>No significant effect on histological signs of colitis severity.</td>
<td>[176]</td>
</tr>
<tr>
<td><strong>Rutin</strong></td>
<td>10 mg/Kg/d solution rectally or orally, or 10 mg/Kg/d pellets coated with caffeic acid/HPMC/algic acid; sodium alginate/HPMC/zinc acetate; or sodium alginate/chitosan, 5 d</td>
<td>Male Wistar rats, 42 mg/Kg TNBS (rectally)</td>
<td>Pellets coated with alginate/chitosan and rectal rutin solution showed the best efficacy in colonic healing clinically and histologically (i.e. erosion of mucosa), and decreased colon weight/BW ratio and MPO activity.</td>
<td>[211]</td>
</tr>
<tr>
<td>Dose/Method</td>
<td>Treatment</td>
<td>Effect</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>0.1% rutin in AIN-76A diet, 15 or 18 wk</td>
<td>Male multidrug resistance gene-deficient (mdr1a⁻/⁻) mice</td>
<td>No significant effect on histological signs of colitis severity.</td>
<td>[217]</td>
<td></td>
</tr>
<tr>
<td>10 mg/Kg/d, 1 day after c.i., 6 d</td>
<td>Male Sprague-Dawley rats, 15 mg/0.3 mL/rat TNBS (rectally)</td>
<td>Significantly healed the damaged colon and reduced MPO activity to about 55% of control.</td>
<td>[210]</td>
<td></td>
</tr>
<tr>
<td>0.001 (60 µg/d), 0.01 (0.6 mg/d), or 0.1% (6 mg/d), 14 d prior or 3 d after c.i.</td>
<td>Female ICR mice, 5% DSS, 1 wk</td>
<td>Reduced colitis severity (BW loss and histologically), attenuated DSS-induced colon shortening, decreased IL-1β mRNA and protein expression, and IL-6 mRNA expression in a dose-dependent manner.</td>
<td>[176]</td>
<td></td>
</tr>
<tr>
<td>Acute colitis: 5, 10, 25, 50, 100 mg/Kg in dH₂O, 48, 24, and 1 hr prior c.i., and 24 hr after c.i.</td>
<td>Female Wistar rats, 10 mg TNBS (rectally)</td>
<td>Acute colitis: 10 and 25 mg/Kg increased mean food intake, reduced macroscopic colonic damage (i.e. colonic mucosa) after 48 hr; 10 mg/Kg significantly had a lower loss of BW; GSH contents were increased by 66.6%, 46.5% and 53.4% at doses of 5, 10, and 25 mg/Kg; 25 mg/Kg reduced MPO and alkaline phosphatase (AP) activity.</td>
<td>[212]</td>
<td></td>
</tr>
<tr>
<td>Chronic colitis: 10 and 25 mg/Kg/d in dH₂O, 24 hr after c.i. for 1 wk or 2 wks</td>
<td></td>
<td>Chronic colitis: After 1 or 2 wks, 10 and 25 mg/Kg reduced colonic damage score (i.e. ulcer lesions, hyperemia, thickening of bowel wall), MPO activity and increased GSH content; 2 wks</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
treatment reduced AP activity and inhibition of LTB₄ synthesis.

| 10, 25, 100 mg/Kg, 48, 24, and 1 hr prior c.i. | Female Wistar rats, 4% acetic acid (v/v) (rectally) | 25 and 100 mg/Kg reduced histologic injury and prevented increase in AP activity, and 25 mg/Kg counteracted GSH depletion. | [213] |

**In vitro**

<table>
<thead>
<tr>
<th>Phenolic Compound</th>
<th>Study Design</th>
<th>Cell Model</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>Mode-K cells stimulated with TNF in absence or presence of 100 µmol/L quercetin</td>
<td>Primary IEC were isolated from the ileum of TNFαRE/WT mice (develop spontaneous experimental ileitis) to grow Mode-K cells</td>
<td>100 µmol/L inhibited TNF-induced IFN-γ-inducible protein IP-10 and macrophage MIP-2 gene expressions at both 40 and 44 µmol/L. Molecularly, quercetin inhibited akt phosphorylation and NF-κB binding to the IP-10 and MIP-2 gene promoters, therefore further blocking cofactor recruitment and HAT activity at the chromatin of these promoters.</td>
<td>[215]</td>
</tr>
<tr>
<td>Quercetin</td>
<td>TNF-α with presence or absence of 5, 25, 50, or 100 µM, 16 h post-transfection</td>
<td>Human colon epithelial cell lines HT-29, HCT116, and SW620, transfected with NF-κB-dependent luciferase (NFDL) plasmid</td>
<td>Significantly inhibited TNF-α-mediated NFDL expression by attenuating IL-8 upregulation, dose-dependently.</td>
<td>[210]</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>Neutrophils incubated with or without LPS (100 ng/mL) for 6 hr. Some neutrophils pretreated with quercetin (40 µM) for 30 min and then 100 ng/mL LPS for 3 hr.</td>
<td>Blood from healthy volunteers; neutrophils isolated using 3% dextran sedimentation and density gradient centrifugation</td>
<td>LPS induced IL-6 expression of neutrophils, but after pre-treatment of neutrophils with quercetin for 30 min, the effects of LPS on increasing IL-6 (mRNA and level) were eliminated.</td>
<td>[216]</td>
</tr>
<tr>
<td>---</td>
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<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Pretreatment with 0, 2.5, 5, 25, 50, or 100 µM for 6 hr, then stimulated with LPS (100 ng/mL) for 3 hr</td>
<td>HUVECs</td>
<td>Significantly decreased NF-κB activation, and TNF-α production, dose-dependently (25-100 µM).</td>
<td>[214]</td>
<td></td>
</tr>
<tr>
<td><strong>Rutin</strong></td>
<td>TNF-α with presence or absence of 5, 25, 50, or 100 µM, 16 h post-transfection</td>
<td>Human colon epithelial cell lines HT-29, HCT116, and SW620, transfected with NF-κB-dependent luciferase (NFDL) plasmid</td>
<td>No significant inhibition of TNF-α-mediated NFDL expression.</td>
<td>[210]</td>
</tr>
</tbody>
</table>

Presence or absence of 1, 10, or 50 µM for 1 hr, then stimulated with LPS (50 ng/mL) for 12 and 24 hr

Bone marrow-derived macrophages (BMDM)

Pre-treatment dose-dependently inhibited secretion of TNF-α and IL-1β, and the phosphorylation of the IκB-α protein (hence inhibiting activation of NF-κB pathway). | [172] |
1.9 Asparagus (*Asparagus officinalis* L.)

1.9.1 Overview

One vegetable that has recently attracted attention for its potential health benefits is asparagus (*Asparagus officinalis* L.). Asparagus is a member of the Asparagales family and is related to onions, leeks, and garlic [218]. The harvestable portions of asparagus are the tops (also known as shoots), while the bottoms (also known as butts) are discarded during processing as they are not marketable [219].

The consumption of asparagus in Canada has been steadily increasing over the years. Specifically, the intake has nearly doubled over the past 20 years to about 0.2 Kg asparagus per person according to Statistics Canada [220]. Asparagus is rich in numerous macro- and micronutrients (Table 1-3), including being a good source of folate [221].

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Units</th>
<th>1 cup (134 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proximates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>g</td>
<td>124.91</td>
</tr>
<tr>
<td>Energy</td>
<td>kcal</td>
<td>27</td>
</tr>
<tr>
<td>Protein</td>
<td>g</td>
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</tr>
<tr>
<td>Total lipid (fat)</td>
<td>g</td>
<td>0.16</td>
</tr>
<tr>
<td>Ash</td>
<td>g</td>
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</tr>
<tr>
<td>Carbohydrate, by difference</td>
<td>g</td>
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</tr>
<tr>
<td>Fibre, total dietary</td>
<td>g</td>
<td>2.8</td>
</tr>
<tr>
<td>Sugars, total</td>
<td>g</td>
<td>2.52</td>
</tr>
<tr>
<td>Sucrose</td>
<td>g</td>
<td>0.31</td>
</tr>
<tr>
<td>Glucose (dextrose)</td>
<td>g</td>
<td>0.87</td>
</tr>
<tr>
<td>Fructose</td>
<td>g</td>
<td>1.34</td>
</tr>
</tbody>
</table>

Table 1-3: Proximate analysis of one serving (1 cup) of raw asparagus [222]
One serving (1 cup) of asparagus provides approximately 70 µg/DFE of folate, which is about one fifth of the RDA in adult males and non-pregnant and -lactating females [222, 223]. Folate has been linked with modulating the risk of developing cancers, most notably in the colorectum [224, 225]. It has been noted that IBD patients are at a high risk for developing folate deficiency and CAC due to the ablation of two receptor/carrier-mediated pathways for folate transport [226]. Asparagus is also a good source of dietary fibre (soluble and insoluble), providing 2.8 g per serving, or about 7 and 9% of the daily adequate intake needs for adult men and women, respectively [227]. Dietary fibre from germinated barley foodstuff (GBF) has been found to reduce colitis severity during active or relapsing states of colitis in mouse and clinical studies [228-230]. Aside from being rich in folate and dietary fibre, asparagus has also been found to have high antioxidant capacity in part from the phenolic compounds embedded in its food matrix [231].

1.9.2 Phenolic Compounds in Asparagus

Among 23 commonly consumed vegetables, the highest antioxidant activity based on dry weight was seen in asparagus [232]. In recent pharmacological studies, the main bioactive compounds in asparagus that are responsible for antitumoral and antioxidant activities are the phytochemicals: saponins, flavonoids, and hydroxycinnamates [233, 234].

The most abundant known flavonoid in asparagus is rutin, which has been shown to exert antioxidant properties [235]. Maeda et al. (2005) has reported that rutin represents 60-80% of the total phenolic content of purple and green asparagus extracts [231]. It was observed that rutin in green spears of 12 asparagus hybrid lines yielded levels ranging from 0.015 to 0.45% of fresh weight. The level of rutin varies with asparagus genotype as
well as the tissue location. For instance, rutin has been found to be more abundant at the upper portions of the spears, while the bottoms of asparagus contain a very small quantity of rutin (less than 0.01% of fresh weight from three lines of sampled asparagus) [236].

As previously discussed, phenolic compounds and phenolic-rich foods can be prospective natural dietary interventions for managing and treating IBD because of their anti-inflammatory properties (Table 1-2). Therefore, rutin-rich asparagus can be a potential candidate for alleviating active symptoms and maintaining remission in IBD.
Chapter 2: Study Rationale, Objectives, and Hypotheses
2.1 **Asparagus (Asparagus officinalis L.)**

As discussed in Chapter 1, UC, characterized by relapsing and remitting inflammation of the colon, is becoming quite prevalent worldwide. The disease not only reduces the QOL of UC patients, but it also results in additional health complications, including an increase in CAC risk. Current conventional therapies, such as aminosalicylates, immunomodulators, and antibiotics, are effective in controlling disease symptoms, but are also associated with severe side effects. Therefore, the use and research of natural dietary interventions in relieving symptoms and achieving UC remission has garnered a great deal of attention in the last decade. A body of evidence has advocated that natural dietary treatments, such as prebiotics, SCFAs, and phenolic compounds, can effectively reduce intestinal inflammation in UC.

Asparagus is a potential natural dietary treatment for UC patients as it is rich in micronutrients and macronutrients, dietary fibre, and phenolic compounds. Dietary fibres are known as prebiotics, and the SCFAs that are produced as a result of bacterial fermentation, have been shown to reduce colitis and anti-inflammatory cytokines. SCFAs also have a vital role in maintaining colonic barrier integrity and function by serving as an energy source for enterocytes. Asparagus is also known for its phenolic content, including high concentrations of rutin. Many pre-clinical *in vitro* and *in vivo* models of experimental IBD have demonstrated that pure phenolic compounds have anti-inflammatory and anti-oxidative properties. Therefore, with asparagus containing high amounts of phenolic compounds, it is a prospective treatment for alleviating symptoms and/or delaying UC progression.
Although there have been numerous \textit{in vitro} and \textit{in vivo} studies purporting the anti-inflammatory and antioxidant effects of rutin, and its respective aglycone quercetin, there have been no studies observing the association between rutin-rich asparagus intake and intestinal inflammation \textit{in vivo}. In fact, there have been no studies conducted using asparagus in diets of healthy or diseased rodents. Additionally, no studies observing the effects of rutin in DSS-induced colitis in the C57BL/6 mouse strain have been conducted. From previous research, the strain of animal used is a significant factor in influencing the severity of DSS-induced colitis. Thus, this thesis will involve three studies: a pilot feeding trial to determine if mice will consume diet supplemented with asparagus; a rutin-dose response study to determine if rutin effectively reduces colitis in the C57BL/6 mouse strain; and finally, a DSS-induced colitis study comparing the effectiveness of asparagus and purified rutin during acute colitis and recovery in reducing colitis symptoms and disease progression.

\subsection*{2.2 Hypothesis}

The overall thesis hypothesis is that asparagus, as a rich source of rutin, will reduce UC symptoms and progression in mice through anti-inflammatory and anti-oxidative mechanisms.

Specifically, it is hypothesized that:

1) C57BL/6 mice will consume a diet supplemented with cooked asparagus flour

2) Rutin will reduce colitis symptoms and disease progression in the C57BL/6 mouse strain exposed to DSS

3) Dietary asparagus will reduce colitis symptoms and disease progression similar to rutin in C57BL/6 mice exposed to DSS during acute colitis and recovery.
2.3 Objectives

The thesis objectives are to:

**Study 1:**

1. Characterize macronutrients, including dietary fibre, and concentration of rutin in cooked asparagus flour.
2. Determine if C57BL/6 mice will consume diet supplemented with cooked asparagus flour.

**Study 2:**

Determine if rutin will alleviate DSS-induced acute colitis symptoms and disease progression (mimicking active colitis/relapse in UC) in C57BL/6 mice.

**Study 3:**

1. Determine if dietary cooked asparagus will reduce DSS-induced acute colitis symptoms and disease progression in C57BL/6 mice.
2. Investigate the effects of dietary cooked asparagus and equivalent levels of purified rutin during colitis recovery in C57BL/6 mice.
Chapter 3: Chemical Characterization of Asparagus Flour and Assessing the Suitability of Supplementing Asparagus into C57BL/6 Mice Diets
3.1 Introduction

One vegetable that has attracted attention for its prospective health benefits is asparagus (*Asparagus officinalis* L.), a member of the Asparagales family. The consumption of asparagus in Canada has been steadily increasing over the last few years. Specifically, the consumption nearly doubled over the past 20 years to about 0.2 Kg asparagus per person according to Statistics Canada in 2008 [220]. Asparagus is rich in numerous macro- and micronutrients, including being a good source of folate [221].

Among 23 commonly consumed vegetables, including beans, broccoli and cabbage, the highest antioxidant activity based on dry weight was observed in asparagus [232]. The main bioactive compounds in asparagus that are responsible for antitumoral and antioxidant activities are the phytochemicals: saponins, flavonoids, and hydroxycinnamates [233, 234]. Asparagus particularly contains a high concentration of rutin, a flavonoid, which attributes to about 60-80% of its total phenolic content [231]. Due to the anti-inflammatory properties of rutin and quercetin in various *in vivo* [176, 210-213] and *in vitro* [172, 210, 214-216] colitis models, rutin-rich asparagus may also exert anti-inflammatory and anti-oxidative activities in IBD. Yet, no study has reported the use of asparagus in rodent diets. Therefore, this first study was conducted as a pilot trial to characterize asparagus flour in terms of rutin and macronutrient content and determine the palatability of diets supplemented with asparagus flour fed to C57BL/6 mice.
3.2 Experiment 1: Asparagus Flour Preparation and Rutin Analysis

3.2.1 Materials and Methods

3.2.1.1 Preparation of Asparagus Flour

A batch (51 Kg) of Guelph Millennium asparagus spears (8 mm diameter, about 22 cm long) was provided by the University of Guelph/Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) Research Program. They were harvested at Dalton White farms in Otterville, Ontario, and collected during the last week of May 2011. The asparagus was washed in cold distilled water, patted dry with paper towels, and then the upper portions were collected by cutting the top 7 cm of the spear. Only the asparagus tops were used in the experiment as they contain the highest concentration of rutin compared to the bottom portions of the spear [196]. The asparagus spear tops were steamed on cooking trays for 5 minutes (Figure 3-1A and B), and then freeze-dried (-50°C; vacuum 0.5 mmHG; 48 hours) in a VirTis freeze dryer at the Guelph Food and Technology Centre pilot plant. The freeze-dried asparagus was then ground into flour first with a Leeson Explosion Proof Industrial blender for 1 minute, sieved (1,680 µM), and then further ground with a Black&Decker® SmartGrind™ CBG100S stainless steel coffee grinder for 1 minute, followed by a final sieving (600 µM). The final asparagus flour was stored in aluminum foil bags at -80°C until further use (Figure 3-1C).
Figure 3-1: Asparagus preparation
Washed Guelph Millennium asparagus tops were (A) placed on stainless steel trays and then (B) placed in a steamer for 5 minutes. (C) Freeze-dried cooked asparagus tops was ground into flour and stored in aluminum foil bags at -80°C until further use.

3.2.1.2 Analysis of Rutin Concentration in Asparagus Flour

3.2.1.2.1 Phenolic Compounds from Asparagus Extraction Conditions

Phenolic compounds were extracted from 0.5 g of freeze-dried asparagus flour with 80% methanol (MeOH). Each sample in 15 mL sterile polypropylene tubes was combined with 10 mL of 80% MeOH and then put on a Roto-Shake Genie rotator (Scientific Industries, Inc., USA) overnight at a rotating speed of 7. Tubes were centrifuged at 24°C in IEC Centra CL3R Refrigerated centrifuge (Thermo Electron Corporation, USA) at 3,000 rpm (24,451 g) for 5 minutes, and supernatants were transferred to their respective labelled 50 mL sterile polypropylene tube. The asparagus pellets were resuspended in 6 mL of 80% MeOH, and then put on the rotator for 1 hour. Tubes were then centrifuged again at 3,000 rpm (24,451 g) for 5 minutes, and supernatants were transferred to their respective labelled 50 mL sterile polypropylene tube. Resuspension in 80% MeOH was repeated once more, and then the volume of each supernatant in its 50 mL tube was topped-up to 25 mL with 80% MeOH. From each 50 mL tube, 3 mL was obtained and filtered (0.45 µM) with a syringe into a brown vial for high-performance liquid chromatography (HPLC) analysis.
3.2.1.2.2 Detection and Quantification of Rutin and Quercetin by HPLC

The HPLC system (Agilent Technology 1100 Series, Palo Alto, Ca, 1999) used to analyze the asparagus flour phenolic extract was equipped with a quaternary pump, an inline degasser, a thermostatic autosampler, and a DAD detector (detects signals from 190 nm to 600 nm). A Phenomenex Phenosphere 5 µ ODS (2) 80 A column (150 X 4.60 mm) was used for the separation. The binary mobile phase consisted of 0.2% acetic acid (Solvent A) and MeOH (Solvent B). The column was equilibrated with 30% Solvent B, and a gradient program was used as follows: 30 to 70% Solvent B in 10 minutes, and 70 to 100% Solvent B in 0.5 minutes. Maintenance of 100% Solvent B occurred for 3 minutes and then brought back to 30% Solvent B for 4 minutes. The flow rate was 1.0 mL/min. The injection volume was 10 µL for all samples and standards. The detector was set at 360 nm for the chromatogram output; and the absorption spectra of each peak were also monitored and stored. Rutin and quercetin in the samples were identified by comparing their retention times and UV absorbance spectra with the standards. Quantification was carried out by integration of the peak areas at 360 nm. Response was linear between the tested range from 0.00625 to 0.3 mg/mL, and the linear regression $R^2$ was greater than 0.9999. Rutin and quercetin standards were purchased from Sigma (Sigma Chemicals, USA), and all samples were analyzed in triplicate.

3.2.1.3 Macronutrients and Soluble Fibre in Asparagus Flour

Asparagus flour macronutrients (carbohydrate, fat, protein), total dietary and soluble fibre, ash, and moisture content were analyzed by Maxxam Analytics (Mississauga, ON).
3.2.2 Results and Discussion

The concentration of rutin in asparagus flour was determined by HPLC to be 8.36 mg rutin/g asparagus dry weight (DW). Quercetin was not detected, indicating that rutin was not metabolized during steaming, freeze-drying, and grinding of the asparagus (Figure 3-2).

![Graph showing rutin and quercetin concentrations](image)

**Figure 3-2: Rutin concentration in asparagus flour**
Rutin was extracted from 0.5 g of asparagus flour (in triplicate) through a series of MeOH washes. A final 10 µL volume of sample and standards (rutin and quercetin) were used to detect rutin by HPLC (360 nm). All peaks were identified by comparison of retention times and UV absorbance spectra with the commercial standards.

Proximate analysis of asparagus flour showed that asparagus is high in protein and fibre, with more than one third being soluble fibre (Table 3-1). Asparagus flour is also low in dietary fat and available carbohydrates, as well as rich in minerals, as indicated by its high ash content.
Table 3-1: Macronutrient composition of asparagus flour

<table>
<thead>
<tr>
<th></th>
<th>g/100 g DW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>5.244</td>
</tr>
<tr>
<td>Protein</td>
<td>49.03</td>
</tr>
<tr>
<td>Available carbohydrates</td>
<td>10.5</td>
</tr>
<tr>
<td>Total Fibre</td>
<td>23.2</td>
</tr>
<tr>
<td>Soluble fibre</td>
<td>7.6</td>
</tr>
<tr>
<td>Insoluble fibre</td>
<td>15.6</td>
</tr>
<tr>
<td>Ash</td>
<td>8.8</td>
</tr>
<tr>
<td>Moisture</td>
<td>3.3</td>
</tr>
</tbody>
</table>

3.3 Experiment 2: Determining the Suitability of Supplementing Asparagus into C57BL/6 Mice Diets

3.3.1 Materials and Methods

3.3.1.1 Preparation of Asparagus Diet for Mice

Since asparagus had not previously been supplemented into animal diets it was not known what level in the diet would be palatable for mice. Therefore, the asparagus concentration in diet was initially chosen to be equivalent to the level of purified rutin known to be effective in reducing colitis in mice. Kwon et al. (2005) previously fed ICR mice a diet containing 0.001, 0.01 or 0.1% rutin for 2 weeks, and found that 0.1% rutin was the most effective in reducing colitis symptoms and disease progression [176]. Therefore, supplementing a mouse diet with a level of asparagus resulting in 0.1% rutin was chosen for this first feeding trial. Since the asparagus flour contained 8.36 mg rutin/g asparagus...
DW as found in Experiment 1, 0.1% rutin diet would be achieved by supplementing rodent diet with 12% asparagus flour.

Experimental diets, AIN-93G basal diet (BD) and BD supplemented with 12% asparagus flour (120 g asparagus flour/Kg BD), were prepared by Harlan Laboratories, Inc. (Madison, WI). The AIN-93G diet formulation was modified to contain 5% corn oil instead of soybean oil, and the total fibre content (cellulose) was increased to 7% from 5%. AIN-93G diet levels of protein, carbohydrate, fat, and fibre were adjusted in the 12% asparagus diet to account for these nutrients found in asparagus flour (Table 3-1). Therefore, both diets had equivalent macronutrients and were isocaloric (Table 3-2). The diets were packaged in plastic sandwich bags and stored in a walk-in refrigerator (about 4°C) throughout the feeding trial.
Table 3-2: Composition of experimental diets (BD and 12% asparagus)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>AIN-93G BD</th>
<th>12% asparagus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asparagus flour</td>
<td>0</td>
<td>120</td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>132</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Corn starch</td>
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<td>359</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>132</td>
<td>132</td>
</tr>
<tr>
<td>Corn oil</td>
<td>70.0</td>
<td>64.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>70.0</td>
<td>42.2</td>
</tr>
<tr>
<td>Mineral mix</td>
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<td>35.0</td>
</tr>
<tr>
<td>Vitamin mix</td>
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<td>10.0</td>
</tr>
<tr>
<td>Choline bitartrate</td>
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<table>
<thead>
<tr>
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</thead>
<tbody>
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<td>19.3</td>
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<tr>
<td>Carbohydrate</td>
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</tr>
<tr>
<td>Fat</td>
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</tr>
</tbody>
</table>

3.3.1.2 Study Design

All protocols and animal procedures used in this study were approved by the Animal Care and Use Committee (University of Guelph). Five week old male C57BL/6 mice (n=39) were obtained from Charles River (Portage, MI) and were acclimatized for 1 week ad libitum on water and BD. Mice were grouped into 3 or 4 per cage and maintained in controlled room conditions: 23±2°C room temperature (RT), 50±10% humidity, and 12-hour light-dark cycle. Mice were fed BD (n=13) or asparagus diet (n=26) ad libitum for 3 weeks and BW and diet intake were measured a minimum of twice weekly.
3.3.1.3 Rutin and Quercetin Analysis of BD and 12% Asparagus Animal Diets

A sample of approximately 10 g of each animal diet was collected from cages after 3 days (representing the longest duration of RT storage). A sample of 0.5 g of each diet was then analyzed by HPLC for rutin levels to verify that the BD had no levels of rutin and 12% asparagus diet contained 0.1% rutin. Quercetin levels were also measured to ensure that rutin was stable in the 12% asparagus diet after preparation and during storage conditions. The rutin extraction and HPLC analyses procedures are as outlined above in section 3.2.1.2.

3.3.1.4 Statistical Analyses

All values are expressed as means ± SEM. Week 1 food intake and BW were analyzed by unpaired t-tests (***=p<0.0001), and week 2-3 food intake and BW were analyzed by one-way ANOVA on each day (SNK and Dunn’s post-hoc test, respectively). A difference with p<0.05 was considered significant. All statistical analyses were performed using Sigma Plot 12.0 (Systat Software Inc, USA).

3.3.2 Results and Discussion

As indicated by the HPLC profile, BD did not contain detectable levels of rutin, and rutin was detected in the 12% asparagus diet (Figure 3-3). Rutin was also not metabolized during preparation or storage of the 12% asparagus diet as quercetin was not detected.
Rutin was extracted from 0.5 g of BD and 12% asparagus diet through a series of MeOH washes. A final 10 µL volume of each sample and standards (rutin and quercetin) were used to detect rutin and quercetin by HPLC (360 nm). All peaks were identified by comparison of retention times and UV absorbance spectra with the commercial standards. Each diet was run in triplicate.

During the first week of feeding, 12% asparagus diet was not well tolerated by the mice as indicated by significant decreases in diet intake and BW compared to the BD group (Figure 3-4A and B). An increase in stress and aggression in the asparagus-fed mice was also observed, therefore it was concluded that the 12% level of asparagus was not palatable by the mice, and thus reduced levels of asparagus diets were prepared to determine if the palatability could be improved. This was achieved by preparing 3 and 6% asparagus diets by grinding 12% asparagus diet pellets, and mixing with appropriate amounts of BD. BD was also ground and fed to mice in the BD group to ensure equal diet texture and consistency across groups. Mice in the 12% asparagus group were then divided into three groups: 1) 3% asparagus (n=9); 2) 6% asparagus (n=9); and 3) 12% asparagus
(n=8), such that the mean initial BW of each group was not significantly different from each other (p>0.05).

After dividing mice into their new treatment groups, diet intake and BW began to increase (Figure 3-4C and D). By day 21, the average daily intake of mice in the 3% and 6% asparagus groups were comparable to the BD group, while the 12% asparagus diet group were not able to improve to BD levels. Body weight gain was not significantly different between diet groups, but amongst the asparagus groups, although not significant (p=0.156), mice fed 3% asparagus appeared to gain more body weight compared to 6 and 12% asparagus-fed mice.
Figure 3-4: Food intake and body weight (BW) measured over 21 days

During week 1, the (A) average food intake per mouse (n=8-9) and (B) %BW change were collected at days 3 and 6 or 7 (n=22-26), and during weeks 2 to 3, the (C) average food intake per mouse (n=4-9), and (D) %BW change (n=8-13) were collected daily. Data points are expressed as the mean ± SEM. Means with different letters are significantly different from each other (p<0.05). Statistical analyses were performed using (A and B) unpaired t-test (***=p<0.0001), (C) one-way ANOVA (SNK post-hoc test), and (D) one-way ANOVA on each day (Dunn's post-hoc). BD=basal diet.

To ensure that the mixing of the diets was correct, we analyzed the rutin levels in 3 and 6% asparagus diets as described in section 3.2.1.2. As shown by the HPLC profile, all diets contained detectable levels of rutin (Figure 3-5). Through using the standard curve, 12% asparagus indeed contained 0.1% rutin (1.04 mg rutin/g pelleted diet), while rutin levels in 6% asparagus was 0.05% rutin (0.503 mg rutin/g pelleted diet), and 3%
asparagus contained about 0.025% rutin (0.232 mg rutin/g pelleted diet). There was also no breakdown of rutin during mixing since quercetin was not detected.

Figure 3-5: Determination of rutin in 3, 6, and 12% asparagus diets
Rutin was extracted from 0.5 g of 3, 6, and 12% asparagus diets through a series of MeOH washes. A final 10 µL volume of each sample and standards (rutin and quercetin) were used to detect rutin and quercetin by HPLC (360 nm). All peaks were identified by comparison of retention times and UV absorbance spectra with the commercial standards. Rutin concentration rutin of each diet was interpolated from the standard curve. Each diet was run in triplicate.

3.4 Summary

This was the first study to characterize dietary fibre, macronutrient levels by proximate analysis, and the concentration of rutin by HPLC in cooked Guelph Millennium asparagus tops. Proximate analysis showed that Guelph Millennium asparagus contained high levels of minerals protein and low levels of dietary fat and available carbohydrates. Moreover, there were high amounts of dietary fibre, with more than one third from soluble fibre. Rutin concentration was also determined to be 8.36 mg/g asparagus DW. The rutin in the asparagus diets was also not broken down during steaming, freeze-drying, grinding,
and storage conditions. This study was also the first to execute a feeding trial with asparagus-supplemented diet in C57BL/6 mice. The results showed that a dose of 12% asparagus was not palatable to C57BL/6 mice, but 3% asparagus diet was. Initially, 12% asparagus (equivalent to 0.1% rutin) was selected because Kwon et al. (2005) discovered that ICR mice fed a diet containing 0.1% rutin had the greatest impact in reversing colitis [176]. However, since 12% asparagus was not well tolerated by C57BL/6 mice, the next step is to confirm if 0.025% rutin (equal to 3% asparagus) can effectively reduce symptoms of DSS-induced acute colitis in C57BL/6 mice.
Chapter 4: The Effects of Rutin on Acute Colitis Symptoms and Disease Progression in DSS exposed C57BL/6 Mice
4.1 Introduction

Fruits and vegetables are a major source of phenolic compounds in human diets [183]. Due to their structure, phenolics are known to have anti-oxidative and anti-inflammatory activities that have been associated with human health benefits [237, 238]. One of the most common classes of phenolics is the flavonoid family since they are synthesized by almost all plant tissues [188, 189]. Out of the flavonoids, the most frequently consumed compound is rutin (C_{27}H_{30}O_{16}; 610.517 g/mol), also called rutoside; quercetin-3-rutinoside; and sophorin. Rutin is found in asparagus, buckwheat, fruit rinds, and the leaves and petioles of Rheum species [194-196]. Upon ingestion, rutin is metabolized by gut microflora to its aglycone, quercetin (C_{15}H_{10}O_{7}; 302.236 g/mol) [198, 199]. Quercetin itself is a flavonol and is also widely distributed in nature and can be found in foods such as apples, onions, and red grapes [200-203].

IBD, a remitting and relapsing inflammatory condition, is now emerging as a global disease as the incidence and prevalence is increasing with time, geographically [5-7, 18]. Currently, the annual incidence rates of UC in North America is 2.2 to 19.2 cases per 100,000 [5, 6], and prevalence of IBD is known to be the highest in North America, with about 7,000 to 46,000 newly diagnosed cases each year [5]. Existing conventional therapies, such as aminosalicylates, corticosteroids, antibiotics, and immunomodulators, can produce adverse side effects that can range from hyperglycemia to pancreatitis [142-145]. Rutin and quercetin have demonstrated anti-inflammatory properties in various in vivo [176, 210-213] and in vitro [172, 210, 214-216] models of colitis, and thus may prove effective in alleviating inflammation during relapse in IBD patients.
It has been found in a colitis model by Kwon et al. (2005) that ICR mice fed a diet containing 0.001, 0.01 or 0.1% rutin for 2 weeks dose-dependently improved colitis symptoms [176]. Mice fed 0.1% rutin had the greatest impact, preventing colitis by the end of the study [176]. Evidence has also shown that there are differences in susceptibility to DSS in various mouse strains [81]. For instance, BALB/c mice, which are often used in DSS-induced colitis studies, are less susceptible to DSS compared to IQL/Jic [82] or C57BL/6 mice [83]. Therefore, it is questionable whether 0.1% rutin would also be effective in alleviating DSS-induced colitis symptoms and disease progression in C57BL/6 mice as it was found to be with ICR mice [176].

In Chapter 3, it was discovered that 3% asparagus was the most palatable to C57BL/6 mice as diet intake was comparable to BD. When looking at these three diets in terms of rutin content, 3% asparagus would be equivalent to 0.025% rutin, while 6 and 12% asparagus would equal to 0.05 and 0.1% rutin, respectively. However, from Chapter 3, it can be seen that 12% asparagus (0.1% rutin) was not well-tolerated by C57BL/6 mice, meaning it would not be practical to proceed with a colitis intervention using this dose of asparagus in C57BL/6 mice.

To date, there has not been a study examining the effects of purified rutin in C57BL/6 mice using the DSS-induced colitis model. Therefore, the objectives of this study are to determine if rutin can be effective in reducing acute colitis symptoms and progression in the C57BL/6 mouse strain, and establish whether a low rutin dose (that is achievable through an asparagus supplemented diet) is also effective.
4.2 Materials and Methods

4.2.1 Diet Preparation

Four experimental diets were prepared similar section 3.3.1.1. The same AIN-93G BD as in Chapter 3 and BD supplemented with 0.1% rutin diet were used in this study (Table 4-1). The 0.025 and 0.05% rutin diets were made by grinding 0.1% rutin diet pellets and mixing with appropriate amounts of BD. BD was also ground and fed to mice in the BD group to guarantee equal diet texture and consistency across groups. The rutin trihydrate used was purchased from Sigma (Sigma Chemicals, USA) with 97% purity.

<table>
<thead>
<tr>
<th>Ingredients (g/Kg diet)</th>
<th>AIN-93G BD</th>
<th>0.1% rutin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin trihydrate</td>
<td>0</td>
<td>1.10</td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Corn starch</td>
<td>377</td>
<td>376</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>132</td>
<td>132</td>
</tr>
<tr>
<td>Corn oil</td>
<td>70.0</td>
<td>70.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>70.0</td>
<td>70.0</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.50</td>
<td>2.50</td>
</tr>
<tr>
<td>TBHQ antioxidant</td>
<td>0.014</td>
<td>0.014</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nutrients (% kcal from)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>19.2</td>
<td>19.2</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>63.2</td>
<td>63.2</td>
</tr>
<tr>
<td>Fat</td>
<td>17.6</td>
<td>17.6</td>
</tr>
</tbody>
</table>
4.2.1.1 Determination of Rutin in Diets

After the rutin diets were made, rutin concentration was verified using procedures described in section 3.2.1.2 to ensure correct mixing.

4.2.2 Mice

All protocols and animal procedures used in this study were approved by the Animal Care and Use Committee (University of Guelph). Five week old male C57BL/6 mice \((n=62)\) were obtained from Charles River (Portage, MI) and were acclimatized for 1 week \textit{ad libitum} on water and BD. Mice were housed into 3 or 4 per cage under controlled room conditions: \(23\pm2^\circ\text{C}\) RT, \(50\pm10\%\) humidity, and 12-hour light-dark cycle.

4.2.3 Study Design

The experimental protocol is summarized in Figure 4-1. Six week old C57BL/6 mice \((n=62)\) were divided into four treatment groups: 1) BD \((n=26)\); 2) \(0.025\%\) rutin \((n=12)\); 3) \(0.05\%\) rutin \((n=12)\); and 4) \(0.1\%\) rutin \((n=12)\), such that the mean BW of each group was not significantly different from each other \((p>0.05)\). Then each group was fed their respective diet for 2 weeks, and during this period, diet was provided fresh daily, and food intake and BW were recorded daily. After 2 weeks, experimental colitis was induced by adding \(2\%\) (w/v) DSS (MP Biomedicals, USA, MW 36,000-50,000) in the drinking water for 7 days. Half of the BD group received DSS-free drinking water and served as healthy controls. During this time the mice continued on their respective diet \textit{ad libitum}.

During the DSS cycle, food and water intake were measured daily. Furthermore, mice were monitored daily for disease activity index (DAI), which is a research tool to quantify clinical symptoms of colitis. The DAI is an average of three scores, which includes
measuring BW loss, stool consistency, and stool blood. BW loss was calculated as the % difference compared to BW at the start of the DSS cycle. Stool consistency and stool blood scores were determined by smearing fresh fecal samples onto Hemoccult SENSA test slides (Beckman Coulter, USA). The three scores were on a scale from 0 to 3 with 0 representing the normal/healthy condition, and 3 representing the worst clinical outcome (Table 4-2).

Mice were euthanized prior to study completion if malocclusion was evident, BW loss exceeded 20% of initial BW, or if a DAI score of 3 was achieved.

At the end of the DSS cycle, all mice were sacrificed by cervical dislocation from a trained lab technician. Blood collection by cardiac puncture was also completed by the lab technician. The entire colon and cecum were excised for macro and micro analyses of colonic inflammation. Colon length was measured from the ceco-colonic junction to the rectum with a ruler. The colon was cleaned of fecal material, weighed, and washed with 1X PBS. Then 1 cm colon sections of the distal colon were collected and fixed in 10% buffered formalin solution for later histological analysis (Figure 4-2). The remaining colon segments were snap frozen in liquid nitrogen. Organs (spleen and cecum, with and without its digesta) were excised, weighed, and snap-frozen in liquid nitrogen. All snap-frozen tissues were stored in -80°C for later analyses.
**Figure 4-1: Experimental Design**

C57BL/6 mice were acclimatized on BD for 7 days and then switched over to experimental diets for the remainder of the study. After 14 days of experimental diets, a 7-day DSS cycle started where all mice received 2% DSS in their drinking water, except for half of the BD group who received DSS-free water. During the 7 days, collection of diet and water intake, and DAI and body condition of mice were monitored daily. At the end of the 7 days, all mice were sacrificed and tissue parameters and samples were collected.

**Table 4-2: DAI scoring system**

<table>
<thead>
<tr>
<th>Score</th>
<th>% BW loss score</th>
<th>Stool consistency score</th>
<th>Stool blood score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0-1%</td>
<td>Normal</td>
<td>Normal (- Hemoccult)</td>
</tr>
<tr>
<td>1</td>
<td>1-5%</td>
<td>Soft, but formed</td>
<td>+ Hemoccult</td>
</tr>
<tr>
<td>1.5</td>
<td>---</td>
<td>---</td>
<td>Trace of blood</td>
</tr>
<tr>
<td>2</td>
<td>5-10%</td>
<td>Very soft</td>
<td>Visible blood</td>
</tr>
<tr>
<td>3</td>
<td>10-20%</td>
<td>Diarrhea</td>
<td>Rectal bleeding</td>
</tr>
</tbody>
</table>
Figure 4-2: Method of collecting distal colon sections
Colons from (A) Basal diet-fed and (B) DSS-treated mice were excised, cut once (as indicated by the red line), and 1 cm sections of the distal colon were fixed in 10% buffered formalin solution for later histological analyses.

4.2.4 Colonic MPO Assay

Colonic MPO levels were measured using a MPO ELISA kit (Cederlane Laboratories Ltd, Canada) according to the manufacturer’s instructions. Frozen distal colons were weighed then transferred into homogenization tubes containing 5 ceramic beads (2.8 mm, MO BIO Laboratories Inc., CA) and lysis buffer (200 μL/10 mg colon). The lysis buffer comprised of 28 μg aprotinin/mL, 1 μg leupeptin/mL, and 1 mM Phenylmethanesulfonyl fluoride in a Tris buffer with 10% glycerin and 5 mM EDTA. Samples were homogenized using a Powerlyzer TM (MO Bio laboratories Inc, USA) for 20 seconds at 3500 rpm. Powerlyzer TM is a tissue homogenizer incorporated with bead-beating technology. The homogenized samples were centrifuged at 1500 rcf (g) for 15 minutes at 4°C. Supernatants were collected and then centrifuged twice more to ensure the purity of the sample.
Supernatants were collected and stored in -80°C until MPO analysis. Diluted colon protein lysates were analyzed for MPO levels as per kit instructions. MPO concentration (ng/mL) was read with a plate reader (BioTek PowerWave XS2, USA) at 450 nm. Final MPO concentrations were corrected for dilution factor and wet tissue colon weight, and then log transformed [log units (u)/g].

4.2.5 Colon Histopathology

At sacrifice, 1 cm distal colon was excised and fixed in 10% formalin for 24 hours, and then transferred to 70% ethanol (EtOH) until paraffin embedding. Tissues were embedded in paraffin, sectioned (5 μm), and stained with Haematoxylin & Eosin or Alcian Blue for histological analyses of crypt erosion and goblet cell depletion, respectively. The histological scoring system for colon erosion consisted of 5 scores with 1 showing normal crypts and intact surface epithelial cells; scores 2 to 4 representing an increase in crypt erosion and surface epithelial cells; and 5 indicating a total loss of crypt architecture and surface epithelial cells (Table 4-3 and Figure 4-3). The scoring system for goblet cell depletion also consisted of 5 scores with 1 displaying normal intact goblet cells; scores 2 to 4 showing a marked increase in goblet cell loss; and 5 with a total loss of goblet cells (Table 4-4 and Figure 4-4). Each cross section received a score from 1 to 5. All slides were scored blindly by three different individuals, and the final scores were averaged.
Table 4-3: Histological grading of colon erosion during DSS-induced acute colitis

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal with intact crypts and surface epithelial cells</td>
</tr>
<tr>
<td>2</td>
<td>Loss of &lt;1/3 of crypts, but surface epithelial cells are still visible</td>
</tr>
<tr>
<td>3</td>
<td>Loss of 2/3 of crypts, but some crypt structures and most surface epithelial cells are still visible</td>
</tr>
<tr>
<td>4</td>
<td>Loss of &gt;2/3 of crypts, but most surface epithelial cells are still visible</td>
</tr>
<tr>
<td>5</td>
<td>Loss of entire crypts and surface epithelial cells</td>
</tr>
</tbody>
</table>

Figure 4-3: Histological grading of colon erosion in DSS-induced acute colitis

Haematoxylin & Eosin-stained distal colon cross-sections were scored for erosion based on five scores: (1) normal with intact crypts and surface epithelial cells; (2) loss of <1/3 of crypts, but surface epithelial cells are still visible; (3) loss of 2/3 of crypts, but some crypt structures and most surface epithelial cells are still visible; (4) loss of >2/3 of crypts, but surface epithelial cells are still visible as indicated by the black arrow; and (5) loss of entire crypt and surface epithelial cells (20X magnification).
### Table 4-4: Histological grading of goblet cell depletion during DSS-induced colitis

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal with intact goblet cells</td>
</tr>
<tr>
<td>2</td>
<td>Loss of &lt;1/3 of goblet cells; increase in number/size of goblet cells</td>
</tr>
<tr>
<td>3</td>
<td>Loss of 2/3 of goblet cells</td>
</tr>
<tr>
<td>4</td>
<td>Loss of &gt;2/3 of goblet cells</td>
</tr>
<tr>
<td>5</td>
<td>Complete loss of goblet cells</td>
</tr>
</tbody>
</table>

Figure 4-4: Histological grading of goblet cell depletion in DSS-induced acute colitis
Alcian blue-stained distal colon cross-sections were scored for goblet cell depletion based on five scores: (1) normal with intact goblet cells; (2) loss of <1/3 of goblet cells; increase in number/size of goblet cells; (3) loss of 2/3 of goblet cells; (4) loss of >2/3 of goblet cells; and (5) complete loss of goblet cells (20X magnification).

#### 4.2.6 Statistical Analyses

All values are expressed as means ± SEM. Food and water intake, and DAI were analyzed by one-way ANOVA on each day (SNK post-hoc test). For organ size parameters,
one-way ANOVA (SNK post-hoc test) was used for colon weight-to-length ratio and cecum weight, while Dunn’s post-hoc test was used for spleen weight. MPO activity (with log_{10} transformed data), and histopathology scores (colon erosion and goblet cell depletion) were analyzed by one-way ANOVA (Dunn’s post-hoc test). A difference with p<0.05 was considered significant. Statistical analyses were performed using Sigma Plot 12.0 (Systat Software Inc., USA).

4.3 Results

4.3.1 Determination of Rutin in Diets

As indicated by the HPLC profile, rutin was detected in all diets, except the BD (Figure 4-5). Through using the standard curve, the 0.1% rutin diet contained 0.926 mg rutin/g pelleted diet, the 0.05% rutin diet contained 0.434 mg rutin/g pelleted diet, and 0.025% rutin diet had 0.221 mg rutin/g pelleted diet. This indicates that rutin in 0.05% rutin diet was indeed half of 0.1% rutin diet; and the rutin in 0.025% rutin diet was also half of 0.05% rutin diet. Moreover, rutin was not metabolized during preparation or storage of the rutin diets as quercetin was not detected.
Figure 4-5: Determination of rutin in basal diet (BD), and 0.025, 0.05, and 0.1% rutin diets

Rutin was extracted from 0.5 g of 0.025, 0.05, and 0.1% rutin diets through a series of MeOH washes. A final 10 µL volume of each sample and standards (rutin and quercetin) were used to detect rutin and quercetin by HPLC (360 nm). All peaks were identified by comparison of retention times and UV absorbance spectra with the commercial standards. Rutin concentration of each diet was determined by standard curve. Each diet was run in triplicate.

4.3.2 Pre-DSS Food Intake and BW

After 14 days of feeding, there were no significant differences between groups with regards to food intake and BW gain (Figure 4-6).
Figure 4-6: Pre-DSS food intake and BW
The (A) average daily intake per mouse (n=3-9) and (B) % change in BW (n=12-26) was measured over 14 days. Data points are expressed as the mean ± SEM.

4.3.3 Food and Water Intake During Acute Colitis

During the 7 day DSS cycle, DSS caused a significant reduction in diet and water intake. By the end of DSS cycle, there were no significant differences among DSS-treated groups with diet (Figure 4-7A) and water intake (Figure 4-7B).
Figure 4-7: Food and water intake during DSS-induced acute colitis

The (A) average daily diet intake per mouse \( (n=3-5) \) and (B) daily water intake per mouse \( (n=3-5) \) was measured over 7 days during 2% DSS-induced acute colitis. Data points are expressed as the mean ± SEM. Means with different letters within the same day are significantly different from each other \( (p<0.05) \). Within each day, some letters are representing more than one treatment group. Statistical analyses were performed using one-way ANOVA (SNK post-hoc test) on each day. BD=basal diet, DSS=dextran sodium sulfate.

4.3.4 DAI During Acute Colitis

The clinical symptoms of DSS-induced colitis are most reflective of UC. In the acute phase of DSS-induced colitis, weight loss, diarrhea, and the presence of bloody stool are initially observed [71, 78]. These clinical symptoms can be seen within 2 days post-colitis induction [78].

DSS-induced weight loss is usually a result of reduced diet intake and an indicator of increased inflammation [71, 78]. We observed that mice started to experience BW loss starting from days 4 and 5 of DSS exposure in the BD+DSS and rutin-treated groups, respectively (Figure 4-8A). By day 7, BW loss was significantly different between mice fed 0.025% rutin compared to the remaining DSS-treated groups.
Increase in stool consistency or diarrhea is also a common phenomenon seen in DSS-induced colitis, similar in UC, which is caused by an increased permeability of the intestinal cells [239] or hyperosmolarity in the lumen induced by DSS [240]. As observed in our study, significant stool consistency changes occurred in all DSS-exposed mice by day 1, which then worsened over time (Figure 4-8B). Collectively, rutin-treated mice attenuated the onset of diarrhea at the end of the DSS cycle; however, this effect was not dose-dependent.

Increased stool blood, leading to rectal bleeding, is also often observed in DSS-induced colitis, which is usually a result of damaged crypts and surface epithelium, and ulcer formation [71, 78]. Hemoccult positive stools were observed in all DSS-treated groups within 1 day of DSS exposure, and throughout the cycle, DSS-treated groups were not significantly different from each other (Figure 4-8C).

When all scores were combined (DAI), 0.1% rutin-fed mice had a significantly lower DAI score than mice in the BD+DSS group at the end of the 7-day acute colitis cycle (Figure 4-8D). Overall, rutin-fed mice had significantly lower DAI scores than the BD+DSS group, though this effect was not dose-dependent.
Figure 4-8: Effects of rutin diets (0.025%, 0.05%, and 0.1%) on DAI clinical symptoms of DSS-induced acute colitis

During the 7-day 2% DSS cycle, mice (n=12-14) were scored daily for (A) BW loss, (B) stool consistency, and (C) stool blood. The average of all three scores was expressed as (D) DAI. All data are expressed as the mean ± SEM. Means with different letters within the same day are significantly different from each other (p<0.05). Within each day, some letters are representing more than one treatment group. Statistical analyses were performed using one-way ANOVA (SNK post-hoc test) on each day. BD=basal diet, DSS=dextran sodium sulfate.

4.3.5 Organ Size Parameters During Acute Colitis

Not only does DSS induce colitis symptoms (DAI), but it also affects the colon, and extraintestinal organs, such as the cecum and spleen. It is believed that DSS shortens the colon by damaging crypts [71, 78] and increasing colon weight by inducing a thickening of the colon via edema and muscular hypertrophy [86]. As observed in this study, DSS
induced an increase in colon weight to length ratio; however, none of the rutin diets were able to significantly attenuate this parameter (Figure 4-9A).

Cecum weight, usually known to be an indicator of dietary fibre fermentation [241], has also been affected by DSS. DSS has been shown to inhibit fermentation [242], thereby reducing the production of anti-inflammatory SCFAs, such as butyrate [168-171, 243]. DSS can also increase cecum weight by directly causing inflammation to caecal tissue, which would result in focal mucosal edema and infiltration of mononuclear or mixed inflammatory cells [244]. In this study, there was not a significant attenuation of DSS-induced cecum weight with rutin-fed mice (Figure 4-9B).

Lastly, DSS can induce an increase in spleen weight (i.e. hypertrophy) as observed by several rodent studies as a response to increased colonic inflammation [83, 245-248]. In this study, it was found that mice fed the 0.025% rutin diet had significantly reduced spleen hypertrophy, having similar spleen weights as the mice in the BD group (Figure 4-9C).
Figure 4-9: Effects of rutin diets on organ size parameters in DSS-induced acute colitis

Data shows the effects of rutin diets on (A) colon weight-to-length ratio (n=11-12), and extraintestinal organs: (B) cecum (n=11-13) and (C) spleen (corrected for BW; n=11-14) during 7 days of 2% DSS-induced colitis. All data are expressed as the mean ± SEM. Means with different letters are significantly different from each other (p<0.05). Statistical analyses were performed using (A and B) one-way ANOVA (SNK post-hoc test) and (C) one-way ANOVA (Dunn’s post-hoc test). BD=basal diet, DSS=dextran sodium sulfate.
4.3.6 Colonic MPO Activity During Acute Colitis

MPO activity is an indicator of the degree of neutrophil infiltration that has been positively correlated with a worsened disease state in IBD patients [87], and is also positively correlated with an increased exposure to DSS [88]. In this study, colonic MPO activity was increased by DSS, but none of the rutin groups were able to attenuate this increase (Figure 4-10). This may be attributed to the large variations seen between all treatment groups before the data was transformed (data not shown).

![Figure 4-10: Effects of rutin diets on distal colon myeloperoxidase (MPO) during DSS-induced acute colitis](image)

After 7 days of 2% DSS-induced colitis, distal colon protein lysates \((n=12-14,\text{ in duplicate})\) were used in a commercial ELISA kit to detect MPO activity. All data are expressed as the mean ± SEM. Means with different letters are significantly different from each other \((p<0.05)\). Statistical analysis was performed using one-way ANOVA (Dunn’s post-hoc) on log\(_{10}\) transformed data. BD=basal diet, DSS=dextran sodium sulfate.
4.3.7 Colon Histopathology During Acute Colitis

4.3.7.1 Colon Erosion and Goblet Cell Depletion

The typical histological characteristics of DSS-induced colitis include: crypt distortion; luminal epithelial cell depletion; and loss of mucin-producing goblet cells within crypts [71, 78, 83, 85]. It is believed to be caused by the ability of DSS to alter the mucus layer lining the luminal epithelium [91]. DSS would then be able to act as a direct toxin to surface epithelial cells to increase cell apoptosis [95, 96] and gut permeability [71, 85, 94], leading to the permeation of luminal microflora and antigens to activate an inflammatory response that can result in damaged basal crypts. As observed in our study, there was a significant increase in colon erosion and goblet cell depletion scores with DSS, yet rutin was not able to significantly affect this change (Figure 4-11).

![Colon Erosion](image1)

![Goblet Cell Depletion](image2)

**Figure 4-11: Effects of rutin diets on distal colon histopathology**
Distal colon cross-sections were scored for (A) colon erosion (n=10-13) and (B) goblet cell depletion (n=11-14) after 7 days of 2% DSS-induced colitis. All data are expressed as the mean ± SEM. Means with different letters are significantly different from each other (p<0.05). Statistical analyses were performed using one-way ANOVA (Dunn’s post-hoc). BD=basal diet, DSS=dextran sodium sulfate.
4.4 Discussion

The first objective of this study was to determine if rutin was effective in reducing DSS-induced acute colitis symptoms and disease progression in C57BL/6 mice. After feeding C57BL/6 mice three doses of rutin (0.025, 0.05, and 0.1%) in diet for 2 weeks prior to and during DSS colitis induction, there were mild effects from all rutin doses in suppressing clinical symptoms, such as stool consistency and DSS-induced spleen hypertrophy. The second objective was to ascertain if rutin at its lowest dose (that is achievable through an asparagus diet as observed in Chapter 3), could also decrease DSS-induced colitis symptoms in C57BL/6 mice. It was found that 0.025% rutin was effective, especially with significantly attenuating spleen hypertrophy. Although the beneficial effects observed from the rutin diets were not dose-dependent, the results still established that the beneficial effects could be attained from a low rutin dose, which can be acquired through diet.

However, unlike Kwon et al. (2005) [176], our findings showed that consumption of 0.025, 0.05, and 0.1% rutin in diet by C57BL/6 mice did not dose-dependently attenuate clinical symptoms. There may be mouse strain differences in terms of BW gain and diet intake, and microflora responsible for metabolism of rutin and eliciting and/or promoting immune-mediated colonic inflammation.

A notable difference between the C57BL/6 mice used in this study and the ICR mice in the study by Kwon et al. (2005) was their BW prior to colitis induction and the amount of diet intake they consumed each day. The ICR mice were on average 30 g and consumed about 6 g of pelleted diet each day (200 g/Kg BW) [176], while the C57BL/6 mice in our study were on average less than 20 g before colitis induction and consumed about 3 g of
powder diet each day (150 g/Kg BW). A study by Bachmanov et al. (2002) has verified that there are substantial strain variations in food and water intakes independent from BW from 28 inbred strains [249]. Moreover, the ICR mice were fed rutin in MF pellets, which is a non-purified diet, while the C57BL/6 mice in this study were fed rutin supplemented to a purified diet, AIN-93G. Goto et al. (2010) has found different effects of feeding mice fructooligosaccharides (FOS) in purified and non-purified diets in DSS-induced colitis, with the non-purified diet having more of a protective effect. The authors speculated that this may have been due to an increased production of SCFAs and water holding capacity in the intestinal contents that were observed in mice fed the non-purified diet [250]. These differences would ultimately produce varying end results, depending on how much rutin was attained by the animal prior to and during DSS exposure.

There was also an observed aggravated BW loss within the 0.025% rutin group; there were two mice in the same cage that lost considerable BW starting at day 5, which explains for the large variation seen with BW loss in this group. When these two mice are removed from the DAI data analyses, 0.025% rutin is comparable with 0.05 and 0.1% rutin (data not shown). These two mice were not excluded because they did not have malocclusion, but it is possible that they were not eating 0.025% rutin diet but in its place ate cage bedding and/or exhibited coprophagia. It has been found in A/J mice that failed to consume a synthetic low-fat diet, began to eat their own cage bedding and displayed coprophagia in order to prevent starvation [251]. Since we monitored diet intake by cage (n=3 or 4), and estimated the diet intake per mouse, there is no way of knowing the exact individual intake of each mouse.
Another strain difference could be in microbial ecology and the ability of C57BL/6 and ICR mice to metabolize rutin. It is known that before absorption can occur, rutin has to be hydrolyzed to its aglycone, quercetin, by β-glucosidase activity of colonic microflora, such as *Bacteroides distasonis*, *B. uniformis*, and *B. ovatus* [198, 199]. Therefore, the transformation of native phenolics, such as rutin, into their respective metabolites is dependent on the individuals and their intestinal microflora. In humans, both metabolite “producers” and “non-producers” have been reported [252, 253]. For instance, only one-third to one-half of the population contains specific microbiota (that is currently unidentified), who are able to metabolize daidzein, a soy isoflavone, to equol that has been claimed as a potent estrogenic metabolite with great antioxidant activity [254]. In mice, gut microbiota is related to both genetic and environmental factors. Genetically, the gut microbiota of inbred mice, such as C57BL/6 mice [255], is more similar between animals than those of outbred mice, such as ICR mice [256, 257]. Environmentally, when scientists compared C57BL/6 mice bred from two different locations, they revealed significant differences in microbial profile, but when they compared C57BL/6Sca mice raised in separate rooms within the same breeding center, they found the profiles were not significantly different from each other [256]. Therefore, the degree of rutin metabolism is dependent on individual variation in the composition of the colonic microbiota.

Moreover, the composition of the colonic microflora in mice is also vital in determining the inflammatory response involved in colitis. One of the theories of IBD etiology is an inappropriate activation of the immune system by colonic microflora, which could occur before or after immunoregulatory defects of the colonic mucosa [42]. It has been reported that: 1) luminal bacteria are needed for the development of chronic
immune-mediated intestinal inflammation; 2) commensal bacterial species have dissimilar pro-inflammatory capabilities, with some being more destructive than others; and 3) each species has a different role in the inflammatory process [258]. For example, *B. vulgatus* has been associated with increased active colitis. Germfree B27 TG rats colonized with six different obligate and facultative intestinal anaerobic bacteria, including *B. vulgatus*, developed much more active colitis and gastritis compared to littermates colonized with the same selected bacteria without *B. vulgatus* [259]. Moreover, treatment of metronidazole (an antibiotic that suppresses *Bacteroides* spp) in B27 TG rats raised under specific-pathogen-free (SPF) environment was able to treat and prevent the onset of colitis [258]. Thus, the severity of colonic inflammation would be dependent on colonic microflora populations.

The most prominent result observed in this study was a reduction in DSS-induced spleen hypertrophy with 0.025% rutin-fed mice. This increase in DSS-induced spleen hypertrophy has also been observed in other rodent studies [83, 245-248]. The spleen is known as a functionally diverse organ with active roles in immunosurveillance, filtering blood, storing monocytes, and hematopoiesis [260]. It is possible that spleen enlargement occurs when there is increased inflammation within the body, such as in the colon. Thus, an increase in spleen weight could mean that it is performing its normal functions at an amplified rate to: clear microorganisms and antigens from the blood stream; initiate immune reactions; synthesize IgG, an antibody isotype that controls infections of body tissues; and remove abnormal red blood cells [261]. The attenuation by 0.025% rutin-fed mice could be attributed to the anti-oxidative nature of rutin and quercetin [212, 213, 262, 263]. ROS are highly reactive molecules produced by normal metabolism that can cause
damage to tissues [119]. During inflammation, activated immune cells are recruited to manage inflammation, but sequentially they increase ROS production [126, 127]. Therefore, rutin and quercetin may be directly acting as a free radical scavenger, eliminating ROS, and/or maintaining a balance in the antioxidant defense system [212, 213, 262, 263].

In previous studies, rutin was able to attenuate the increase in DSS-induced colonic MPO levels (indicator of neutrophil infiltration) [210, 211] and colon histopathology scores [176, 210, 212], but in this study, there were no significant attenuations seen from rutin diets. These dissimilar results could, again, be attributed to strain and study differences. Cecum weight has not been measured in any rodent studies observing rutin. Cecum weight, typically known to be an indicator of dietary fibre fermentation [241], has also been affected by DSS. DSS has been shown to inhibit fermentation [242], thereby reducing the production of anti-inflammatory SCFAs, such as butyrate [168-171, 243]. Thus, for dietary-fibre containing diets to counteract the inhibition of fermentation caused by DSS, there must be an adequate level of dietary fibre to produce SCFAs. But in this study, the level of dietary fibre in all rutin diets was the same as the BD group. Therefore, we did not see an attenuation of DSS-induced cecum weight by any rutin groups.

In summary, this study demonstrated that rutin was effective in reducing some markers of colitis, such as DSS-induced stool consistency, and spleen hypertrophy in C57BL/6 mice. Yet, the outcomes were weaker compared to previously reported rutin effects in other mouse strains, which may be accounted by strain differences in BW gain and diet intake as well as microflora status. Although the beneficial effects of rutin in this
study were not dose-dependent, the lowest rutin dose (0.025%) was still seen to be effective in attenuating the onset of diarrhea and reducing spleen hypertrophy.
Chapter 5: The Effects of Asparagus and its Purified Bioactive, Rutin, on DSS-induced Acute Colitis and Recovery in C57BL/6 Mice
5.1 Introduction

IBD, comprising of UC, is a lifetime disease of remitting and relapsing inflammation of the large intestine [1]. Since UC patients are often interchanging between a relapse or remissive state, potential therapies should aim to target either of these states, or both, to reduce the time in relapse and accelerate towards remission. Foods that are rich in natural dietary compounds with anti-inflammatory and anti-oxidative activities are thus proposed as potential treatments for IBD patients. For instance, the juice from Cydonia oblonga Miller (Quince), a fruit tree cultivated in many countries, contains high amounts of flavonoids, namely rutin, quercetin, and kaempferol. Quince juice can significantly diminish inflammation and ulcer indices in TNBS-induced colitis in rats [264]. There are currently limited studies observing the effects of certain whole foods or dietary components during recovery from DSS-induced colitis. Yet, from the limited studies that have been completed, positive results have been reported. For instance, FOS, a prebiotic that stimulates the colonic growth of bifidobacteria to promote intestinal health, was found to produce a faster recovery from DSS-induced colonic damage with increased crypt depth and crypt area compared to the control in C57BL/6 mice [265].

As observed in Chapter 4, and in various rodent models, rutin can significantly reduce experimentally-induced colitis symptoms, possibly owing to its anti-inflammatory [176, 210-213] and anti-oxidative nature [212, 213, 262, 263]. A recent review has established that quercetin, the aglycone of rutin, can exhibit protective effects on intestinal tight junction (TJ) barrier function via the assembly and expression of TJ proteins [266], such as claudin-4 [267]. Hence, it is probable that 0.025% rutin could also offer benefits during recovery in repairing the damaged colon.
To date, there has not been a study examining the effects of phenolics in recovery from DSS-induced colitis nor cooked whole asparagus in acute colitis or remissive states of experimental UC. With asparagus containing high concentrations of rutin and additional anti-inflammatory bioactives (e.g. soluble fibre), asparagus is a potential candidate for alleviating symptoms during colitis and remission, possibly more so than just purified rutin. Asparagus may also be harbouring other phenolic compounds with antioxidant potential. Therefore, in order to expand the therapeutic potential of rutin and rutin-rich asparagus, the objective of this study is to evaluate the effects of cooked asparagus (equivalent to 0.025% rutin) and purified 0.025% rutin in diet using both a DSS-induced acute colitis (i.e. relapse) and recovery model in C57BL/6 mice.

5.2 Experiment 1: Asparagus Flour Preparation and Rutin Analysis

5.2.1 Materials and Methods

5.2.1.1 Preparation of Asparagus Flour

A batch (25 Kg) of Guelph Millennium asparagus spears (8 mm diameter, about 22 cm long) was provided by the University of Guelph/OMAFRA Research Program. They were harvested at Dalton White farms in Otterville, Ontario, and collected during the first week of May 2012. The procedures in preparing the asparagus flour is the same as described in section 3.2.1.1.

5.2.1.2 Analysis of Rutin Concentration in Asparagus Flour

The procedures used in extracting rutin from asparagus flour and measuring rutin concentrations by HPLC are described in section 3.2.1.2.
5.2.1.3 Macronutrients and Soluble Fibre in Asparagus Flour

Asparagus flour macronutrients (carbohydrate, fat, protein), total dietary and soluble fibre, ash, and moisture content were analyzed by Maxxam Analytics (Mississauga, ON).

5.2.2 Results and Discussion

As indicated by the HPLC profile, rutin was detected in asparagus flour (Figure 5-1). Moreover, quercetin was not detected, indicating that rutin was not broken down during steaming, freeze-drying, and grinding of the asparagus. Using a rutin standard curve, the concentration of rutin in asparagus flour determined to be 12.92 mg rutin/g asparagus DW.

Figure 5-1: Analysis of rutin concentration in asparagus flour
Rutin was extracted from 0.5 g of asparagus flour through a series of MeOH washes. A final 10 µL volume of sample and standards (rutin and quercetin) were used to detect rutin by HPLC (360 nm). All peaks were identified by comparison of retention times and UV absorbance spectra with the commercial standards. Concentration of rutin was interpolated from the standard curve. Each diet was run in triplicate.
Proximate analysis of asparagus flour revealed that asparagus is high in protein, and low in dietary fat and available carbohydrates, as well as rich in minerals, as indicated by its high ash content (Table 5-1). Asparagus flour is also rich in dietary fibre, with more than one third being soluble fibre. The macronutrient composition of the asparagus flour in this study is also comparable with the asparagus flour used in Chapter 3.

Table 5-1: Macronutrient composition of asparagus flour

<table>
<thead>
<tr>
<th></th>
<th>g/100 g DW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>5.486</td>
</tr>
<tr>
<td>Protein</td>
<td>45.58</td>
</tr>
<tr>
<td>Available carbohydrates</td>
<td>15.8</td>
</tr>
<tr>
<td>Total Fibre</td>
<td>21.7</td>
</tr>
<tr>
<td>Soluble fibre</td>
<td>7.9</td>
</tr>
<tr>
<td>Insoluble fibre</td>
<td>13.8</td>
</tr>
<tr>
<td>Ash</td>
<td>8.5</td>
</tr>
<tr>
<td>Moisture</td>
<td>3</td>
</tr>
</tbody>
</table>

5.3 Experiment 2: Determining the Effects of Asparagus and Purified Rutin on DSS-induced Colitis and Recovery in C57BL/6 Mice

5.3.1 Materials and Methods

5.3.1.1 Preparation of Diets for Mice

It was found in Chapter 4 that 0.025% rutin can significantly reduce some parameters of colitis in C57BL/6 mice. Therefore, in this study, a level of asparagus that was equivalent to 0.025% rutin was added to rodent AIN-93G BD. Since the asparagus flour
was found to contain 12.92 mg rutin/g asparagus DW in Experiment 1, supplementing rodent diet with 2% asparagus flour achieved 0.025% rutin in the diet.

Experimental diets: AIN-93G BD; BD supplemented with 2% asparagus flour (20 g asparagus flour/Kg BD); and BD supplemented with 0.025% rutin (0.28 g rutin/Kg BD) were prepared by Harlan Laboratories, Inc. (Madison, WI). The rutin trihydrate used was purchased from Sigma (Sigma Chemicals, USA) with 97% purity. The AIN-93G diet formulation was modified to contain 5% corn oil instead of soybean oil, and the total fibre content (cellulose) was increased from 5% to 7%. AIN-93G diet levels of protein, carbohydrate, fat, and fibre were adjusted in the 2% asparagus diet to account for the nutrients determined in asparagus flour (Table 5-1). Therefore, all diets had equivalent macronutrients and were isocaloric (Table 5-2). The diets were packaged in plastic sandwich bags and stored in a walk-in refrigerator (about 4°C) throughout the intervention.
Table 5-2: Composition of experimental diets (BD, 2% asparagus, and 0.025% rutin)

<table>
<thead>
<tr>
<th>Ingredients (g/Kg diet)</th>
<th>AIN-93G BD</th>
<th>2% asparagus</th>
<th>0.025% rutin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin trihydrate</td>
<td>0</td>
<td>0</td>
<td>0.280</td>
</tr>
<tr>
<td>Asparagus</td>
<td>0</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>189</td>
<td>200</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3.00</td>
<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Corn starch</td>
<td>377</td>
<td>373</td>
<td>377</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>132</td>
<td>132</td>
<td>132</td>
</tr>
<tr>
<td>Corn oil</td>
<td>70.0</td>
<td>68.9</td>
<td>70.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>70.0</td>
<td>65.7</td>
<td>70.0</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>35.0</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.50</td>
<td>2.50</td>
<td>2.50</td>
</tr>
<tr>
<td>TBHQ antioxidant</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
</tr>
<tr>
<td>Nutrients (% kcal from)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>19.2</td>
<td>19.2</td>
<td>19.2</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>63.2</td>
<td>63.2</td>
<td>63.2</td>
</tr>
<tr>
<td>Fat</td>
<td>17.6</td>
<td>17.6</td>
<td>17.6</td>
</tr>
</tbody>
</table>

5.3.1.2 Determination of Rutin in Diets

After the diets were made, rutin concentration was confirmed using procedures described in section 3.2.1.2 to ensure proper diet preparation.
5.3.1.3  Antioxidant Activity of Diets

The remaining soluble phenolic extracts from analyzing rutin by HPLC were used in measuring antioxidant activity of diets by ORAC and FRAP.

5.3.1.3.1  ORAC

Antioxidant activity of diet sample extracts was determined by the ORAC assay according to procedures described by Li et al. (2011) with modifications [268]. A stock solution of fluorescein (Sigma Chemicals, USA; \(144.65 \times 10^{-3}\) mM) was prepared by dissolving 4.8 mg of fluorescein in 100 mL phosphate buffer (75 mM, pH7.4). The working fluorescein solution (\(8.68 \times 10^{-6}\) mM) was prepared daily by mixing 9 μL of the stock solution with 14.991 mL of 75 mM phosphate buffer. The AAPH (Sigma-Aldrich, USA) radical solution (153 mM) was also prepared daily by dissolving 414 mg of AAPH in 10 mL of 75 mM phosphate buffer. Serial dilutions of Trolox (0-100 μM) were used to generate a standard curve from which the ORAC values of the samples were generated. As the ORAC assay is extremely sensitive, the samples were diluted appropriately before analysis to avoid interference. In this study, soluble phenolic extracts were diluted 25X and AHBP and BHBP were diluted 200X with phosphate buffer.

In a 96-well plate, 150 μL of fluorescein working solution and 25 μL of sample, blank (phosphate buffer), or standards were loaded to each well. The plate was heated for 30 seconds at 37°C prior to the addition of AAPH. The fluorescence was read immediately after the addition of AAPH and continuously every minute for 1 hour. Each sample was analyzed in triplicate using a microplate reader (BioTek Fluorescent, USA) with fluorescence filters containing an excitation wavelength of 485 nm and an emission
wavelength of 528 nm. The ORAC values, expressed as μM of Trolox equivalent (TE)/g DW of diet, were calculated from the Trolox standard curve.

5.3.1.3.2 FRAP

The FRAP assay used for determining antioxidant activity in diet sample extracts was based on the procedures described by Li et al. (2011) [268]. Standards (0-1,000 μM ascorbic acid) and samples at 10 μL were added to a 96-well microplate in triplicate. In each well, 300 μL of working FRAP regent was added. The FRAP reagent was made daily and consisted of acetate buffer (300 mM, pH 3.6), 2,4,6-tripyridyl-s-triazine (Sigma-Aldrich, USA; 10 mM), and FeCl₃·6H₂O (20 mM) at a ratio of 10:1:1 (v/v/v). After 30 minutes of incubation at RT, the absorbances were recorded at 593 nm using a microplate reader (BioTek, PowerWave XS2, USA). The FRAP values, expressed as μM of acetic acid equivalent (AAE)/g DW of diet, were calculated from the ascorbic acid standard curve.

5.3.1.4 Mice

All protocols and animal procedures used in this study were approved by the Animal Care and Use Committee (University of Guelph). Five week old male C57BL/6 mice (n=99) were obtained from Charles River (Portage, MI) and were acclimatized for 1 week ad libitum on water and BD. Mice were grouped into 3 or 4 per individual stainless steel cage under controlled room conditions: 23±2°C RT, 50±10% humidity, and 12-hour light-dark cycle.

5.3.1.5 Study Design

The experimental protocol is summarized in Figure 5-2. Mice (n=99, 6 week old) were divided into three treatment groups: 1) BD (n=35); 2) 2% asparagus (n=33); and 3)
0.025% rutin \((n=30)\), such that the mean BW of each group was not significantly different from each other \((p>0.05)\). Then each group of mice was fed their respective diet for 2 weeks, and during this period, food intake and BW of each group were recorded. After 2 weeks, the mice were given 2\% (w/v) DSS (MP Biomedicals, USA, MW 36,000-50,000) in the drinking water for 7 days to induce colitis. Half of the BD group received DSS-free drinking water and served as healthy controls. During this time the mice continued on their respective diet \textit{ad libitum}. At the end of the 7 days, mice (BD+DSS and 0.025\% rutin+DSS: each \(n=11\); and 2\% asparagus+DSS: \(n=12\)) were sacrificed to evaluate the effects of asparagus on symptoms and progression of acute colitis. The remaining animals were switched back to normal drinking water in order to induce recovery.

During the DSS cycle, food and water intake were measured daily. Furthermore, mice were monitored daily for DAI according to the procedures as described in section 4.2.3. Sacrifice methods as well as organ measurements were also the same as detailed in section 4.2.3.

\textbf{Figure 5-2: Experimental design}

C57BL/6 mice were acclimatized on BD for 7 days and then switched over to experimental diets for the remainder of the study. After 14 days of experimental diets, a 7-day DSS cycle started and all mice received 2\% DSS in their drinking water, except for half of the BD group who received DSS-free water. At the end of the 7 days, a subset of mice was sacrificed and the remaining mice started on a 5-day recovery cycle with normal drinking water. Sacrifice occurred again at the end of the recovery cycle. During DSS-induced acute colitis and recovery, DAI was monitored and diet and water intake were collected daily.
5.3.1.6  Colonic MPO Assay

Colonic MPO activity was measured by using the method as outlined in section 4.2.4.

5.3.1.7  Colon Histopathology

Colon cross sections were fixed, embedded, cut, and scored according to the procedures described in section 4.2.5. The scoring system and representative sections for colon erosion and goblet cell depletion during acute colitis are described in section 4.2.5 (Table 4-3 and Table 4-4, and Figure 4-3 and Figure 4-4). The scoring system and representative histological sections for colon erosion and goblet cell depletion during recovery are shown in Table 4-3 and Figure 5-3, and Table 4-4 and Figure 5-4, respectively.

Figure 5-3: Histological grading of colon erosion during recovery
Distal colon cross-sections were scored for erosion based on five scores: (1) normal with intact crypts and surface epithelial cells; (2) loss of <1/3 of crypts, but surface epithelial cells are still visible; (3) loss of 2/3 of crypts, but some crypt structures and most surface epithelial cells are still visible; (4) loss of >2/3 of crypts, but surface epithelial cells are still visible; and (5) loss of entire crypt and surface epithelial cells (20X magnification).
Figure 5-4: Histological grading of goblet cell depletion during recovery
Distal colon cross-sections were scored for goblet cell depletion based on five scores: (1) normal with intact goblet cells; (2) loss of <1/3 of goblet cells; increase in number/size of goblet cells; (3) loss of 2/3 of goblet cells; (4) loss of >2/3 of goblet cells; and (5) complete loss of goblet cells (20X magnification).

5.3.1.8 Serum Cytokine Multiplex Analysis

The Th1 analytes that were measured included IL-10, IL-1β, IL-6, IL-17, TNF-α, and IFN-γ. A TH17 magnetic bead-based multiplex sandwich immunoassay from the Bio-plex Pro™ Assays (Bio-Rad Inc., CAN) was used. This assay contained dyed microspheres (i.e. beads) conjugated with a monoclonal capture antibody that were specific for a target protein. Serum samples were thawed and run in duplicates. According to the manufacturer's instructions, antibody-coupled beads were incubated with the serum samples (1:3 dilution), and then incubated with biotinylated detection antibody to create a sandwich complex. The final step was incubation with streptavidin-phycoerythrin (SA-PE) conjugate to serve as a fluorescent reporter. The standards used consisted of 0.54 to 11,900 pg/mL. Bound molecules were read using a Bio-Plex 200 System (Bio-Rad Inc.,
CAN), which is based on Luminex fluorescent-bead-based technology with a flow-based dual laser detector and high-speed digital processor.

5.3.1.9 Statistical Analyses

All values are expressed as means ± SEM. ORAC and FRAP antioxidant capacities were analyzed by one-way ANOVA (SNK post-hoc test). Food and water intake were analyzed by two-way ANOVA (SNK post-hoc test). DAI were analyzed by one-way ANOVA on each day (SNK post-hoc test). For organ size parameters, one-way ANOVA (SNK post-hoc test) was used for colon weight-to-length ratio and cecum weight, while Dunn’s post-hoc test was used for spleen weight. MPO activity (with log_{10} transformed data), and histopathology scores (colon erosion and goblet cell depletion) were analyzed by one-way ANOVA (SNK post-hoc test). A difference with p<0.05 was considered significant. Serum cytokines were analyzed by one-way ANOVA (Dunn’s post-hoc test for IL-1β, IL-6, and IL-10, and SNK post-hoc for IL-17, IFN-γ, and TNF-α). Statistical analyses were performed using Sigma Plot 12.0 (Systat Software Inc., USA).

Kaplan-Meier survival curves were generated using GraphPad Prism 5.03 (GraphPad Software Inc., USA) and analyzed by Log-Rank (Mantel Cox) χ² test. Survival curves of the DSS exposed treatment groups were considered significantly different from healthy controls if the p value was less than the Bonferroni-corrected threshold which was set at p<0.017.
5.3.2 Results

5.3.2.1 Determination of Rutin in Diets

As indicated by the HPLC profile, rutin was not detected in BD, while 2\% asparagus and 0.025\% rutin diets indeed contained equal detectable levels of the compound (Figure 5-5). Furthermore, there was no rutin breakdown during preparation or storage of the rutin diets as quercetin was not detected. By using the standard curve, both 2\% asparagus and 0.025\% rutin diets were validated to contain 0.025\% rutin.

![HPLC profile showing rutin and quercetin peaks](image)

**Figure 5-5: Determination of rutin in BD, 2\% asparagus, and 0.025\% rutin diets**
Rutin was extracted from 0.5 g of BD, 2\% asparagus, and 0.025\% rutin diets through a series of MeOH washes. A final 10 \µL volume of each sample and standards (rutin and quercetin) were used to detect rutin and quercetin by HPLC (360 nm). All peaks were identified by comparison of retention times and UV absorbance spectra with the commercial standards. Concentration of rutin was interpolated from the standard curve. Each diet was run in triplicate.

5.3.2.2 Antioxidant Activity of Diets

The experimental diets, 2\% asparagus and 0.025\% rutin, were assessed for antioxidant activity by two well-established assays, ORAC and FRAP. In both the assays, 2\%
asparagus and 0.025% rutin had significantly higher antioxidant activities compared to BD, yet 2% asparagus diet exhibited the most antioxidant activity (Figure 5-6).

Figure 5-6: Antioxidant activity of BD, 2% asparagus, and 0.025% rutin diets
Antioxidant activities of BD, 2% asparagus, and 0.025% rutin diets were assessed in triplicate by (A) ORAC and (B) FRAP assays. All data are expressed as the mean ± SEM. Bars with different letters are significantly different from each other (p<0.05). Statistical analysis was performed using one-way ANOVA (SNK post-hoc test).

5.3.2.3 Pre-DSS Food Intake and BW

Both 2% asparagus and 0.025% rutin diets were well tolerated by the mice as diet intake and BW gain was comparable to mice in the BD group (Figure 5-7).
**Figure 5-7: Pre-DSS food intake and BW**
The (A) average daily intake per mouse \((n=8-10)\) and (B) % change in BW \((n=30-35)\) was measured every 3 days. Data points are expressed as the mean ± SEM.

### 5.3.3 Part 1: Acute Colitis Results

#### 5.3.3.1 Food and Water Intake During DSS-induced Acute Colitis

During the 7 day DSS cycle, DSS caused a significant decrease in diet intake (Figure 5-8). By the end of the cycle, all DSS-treated groups were not significantly different from each other in diet and water intake.
5.3.3.2 DAI in Acute Colitis

During acute colitis, DSS induced BW loss (Figure 5-9A), and an increase in stool consistency (i.e. diarrhea) (Figure 5-9B) and stool blood (i.e. rectal bleeding) (Figure 5-9C), which corresponds with previous studies [71, 78]. By the end of the DSS cycle, there were no significant differences among DSS-treated groups with BW loss; however, 2% asparagus+DSS significantly attenuated the onset of diarrhea and rectal bleeding. Through taking the average of all three scores, the total DAI score was not significantly different between DSS-treated groups (Figure 5-9D).
Figure 5-9: Effects of 2% asparagus and 0.025% rutin diets on DAI during DSS-induced acute colitis

During the 7-day 2% DSS cycle, mice (n=6-27) were monitored daily for (A) % BW loss, (B) stool consistency, and (C) stool blood. The average of all three scores was expressed as (D) DAI. Means with different letters are significantly different from each other (p<0.05). Within each day, some letters are representing more than one treatment group. Statistical analyses were performed using one-way ANOVA (SNK post-hoc test) on each day. BD=basal diet, DSS=dextran sodium sulfate.

5.3.3.3 Organ Size Parameters During Acute Colitis

During colitis, DSS increased colon weight to length ratio, and cecum and spleen weights. There were no significant differences between DSS-treated groups with cecum weight and colon weight to length ratio (Figure 5-10A and B); however, DSS-induced spleen weight enlargement was significantly attenuated by 2% asparagus+DSS (Figure 5-10C).
Figure 5-10: Effects of 2% asparagus and 0.025% rutin diets on organ size parameters during DSS-induced acute colitis

The effects of diets on (A) colon weight-to-length ratio (n=6-12) and extraintestinal organs: (B) cecum (n=6-11) and (C) spleen weights (n=6-11) were measured during 7 days of 2% DSS-induced acute colitis. All data are expressed as the mean ± SEM. Means with different letters are significantly different from each other (p<0.05). Statistical analyses were performed using (A and B) one-way ANOVA (SNK post-hoc test), and (C) one-way ANOVA (Dunn's post-hoc test). BD=basal diet, DSS=dextran sodium sulfate.

5.3.3.4 Colonic MPO Activity During Acute Colitis

In acute colitis, MPO activity was increased by DSS, but there was not a significant difference amongst DSS-treated groups (Figure 5-11).
Figure 5-11: Effects of 2% asparagus and 0.025% rutin diets on myeloperoxidase (MPO) activity during acute colitis

The effects of diets on distal colon MPO (n=5-12, in duplicate) after 7 days of 2% DSS-induced colitis. All data are expressed as the mean ± SEM. Means with different letters are significantly different from each other (p<0.05). Statistical analyses were performed using one-way ANOVA (SNK post-hoc test) on log_{10} transformed data. BD=basal diet, DSS=dextran sodium sulfate.

5.3.3.5 Acute Colitis Colon Histopathology: Colon Erosion and Goblet Cell

Some of the earliest histological signs of DSS-induced colitis are: increases in crypt distortion; epithelial cell loss; mucin-producing goblet cell loss; presence of ulcers; cryptitis; and crypt abscesses [71, 78, 83, 85]. The epithelium, containing a dense network of innate and adaptive immune cells, is central in monitoring the integrity and health of the colonic mucosa by eliciting appropriate responses to invading bacteria and antigens [99]. It has been found that the removal of DSS results in recovery, including a restitution of normal colonic architecture and an increase in crypt cell proliferation as an integral part of the repair process [81, 86].
In this study, there were no effects observed between DSS-treated groups during colitis with regards to colon erosion (Figure 5-12A) and goblet cell depletion (Figure 5-12B).

![Colon Erosion](image)

![Goblet Cell Depletion](image)

**Figure 5-12: Histopathology of distal colon erosion and goblet cell depletion during acute colitis**
The effects of 2% asparagus and 0.025% rutin diets on (A) colon erosion \((n=11-12)\) and (B) goblet cell depletion \((n=6-12)\) during 7 days of 2% DSS-induced acute colitis. Data are expressed as the mean ± SEM. Means with different letters are significantly different from each other \((p<0.05)\). Statistical analyses were performed using one-way ANOVA (SNK post-hoc test). BD=basal diet, DSS=dextran sodium sulfate.

### 5.3.4 Part 2: Colitis Recovery Results

#### 5.3.4.1 Food and Water Intake During Colitis Recovery

Once DSS was removed and replaced with normal drinking water again during the 5 day recovery cycle, the DSS-treated mice began to increase their diet and water intake by day 3 (Figure 5-13). By the end of the 5 day recovery cycle, all DSS-treated groups were normally consuming diet and water comparable to the BD group.
Figure 5-13: Food and water intake during colitis recovery
The (A) average daily diet intake per mouse (n=2-5) and (B) daily water intake per mouse (n=2-5) was measured over 5 days of colitis recovery with water. Data points are expressed as the mean ± SEM. Within in each day, means with different letters are significantly different from each other (p<0.05). Within each day, some letters are representing more than one treatment group. Statistical analyses were performed using two-way ANOVA (SNK post-hoc test). BD=basal diet, DSS=dextran sodium sulfate.

5.3.4.2 Kaplan-Meier Survival Curve in Colitis Recovery

During the recovery period, the survival rate was notably different between groups (Figure 5-14). As mentioned in the methods section 5.3.1.5, mice were removed from the study and terminated if their BW loss exceeded 20% of their initial body weight and if they experienced excessive diarrhea and stool blood loss. Based on these parameters, 50% (6 of 12) of the mice in the BD+DSS group remained in the study throughout the 5 day recovery period, while 67% (10 of 15) and 85% (11 of 13), remained in the study from the 2% asparagus+DSS and 0.025% rutin+DSS groups, respectively. This resulted in a significant difference in survival between the DSS group (p=0.0057) and the healthy controls (BD), while the survival of the asparagus (p=0.031) and rutin (p=0.17) groups did not differ from healthy controls. These results indicate that asparagus and rutin supplemented diets improved colitis recovery, with rutin being more effective than asparagus.
Figure 5-14: Impact of 2% asparagus and 0.025% rutin diets on survival during colitis recovery in mice
Kaplan-Meier survival curve demonstrating the % of mice \((n=12-15)\) which survived within different treatment groups during the 5 day recovery period with water following a 7-day 2% DSS-induced acute colitis. Curve was analyzed by Log-Rank (Mantel Cox) \(\chi^2\) test and treatment groups were considered significantly different from healthy controls if the p value was less than the Bonferroni-corrected threshold which was set at \(p<0.017\). BD=basal diet, DSS=dextran sodium sulfate.

5.3.4.3 DAI in Colitis Recovery

At the start of the recovery cycle, BD-fed mice who previously received DSS continued to lose BW until day 4, while mice fed 0.025% rutin+DSS significantly started to gain BW compared to those fed BD+DSS and 2% asparagus+DSS (Figure 5-15A). At day 4, 0.025% rutin+DSS significantly improved colitis recovery in terms of BW gain compared to the BD+DSS group, followed by the 2% asparagus group, though not significantly different from mice in the BD+DSS. With stool consistency and stool blood, there were no significant differences amongst DSS-treated groups by the end of the DSS cycle (Figure 5-15B). There was a decrease in stool blood by all DSS-treated groups throughout the recovery period, which was diminished to healthy control levels by day 5 (Figure 5-15C). Lastly, the total DAI score decreased in all treatments groups by day 7, but 0.025% rutin+DSS group
significantly decreased this score, followed by mice in the 2% asparagus+DSS group (Figure 5-15D).

**Figure 5-15: Effects of diets on DAI during colitis recovery**
During the 5-day recovery cycle, mice (n=6-15) were monitored daily for (A) % BW loss, (B) stool consistency, and (C) stool blood. The average of all three scores was expressed as (D) DAI. Data are expressed as the mean ± SEM. Means with different letters are significantly different from each other (p<0.05). Within each day, some letters are representing more than one treatment group. Statistical analyses were performed using one-way ANOVA (SNK post-hoc test) on each day. BD=basal diet, DSS=dextran sodium sulfate.
5.3.4.4 Organ Size Parameters During Colitis Recovery

During recovery, colon weight-to-length ratio was also not significantly different between DSS-treated groups (Figure 5-16A), and there was no DSS effect observed with cecum weight (Figure 5-16B).

![Figure 5-16: Effects of 2% asparagus and 0.025% rutin diets on organ size parameters during colitis recovery](image)

The effects of asparagus and rutin diets on (A) colon weight-to-length ratio and (B) cecum weight were measured during colitis recovery. All data are expressed as the mean ± SEM. Means with different letters are significantly different from each other (p<0.05). Statistical analyses were performed using one-way ANOVA (SNK post-hoc test). BD=basal diet, DSS=dextran sodium sulfate.

5.3.4.5 Colonic MPO Activity During Colitis Recovery

Similar to what was observed in acute colitis, at the end of the 5 day recovery cycle, MPO activity was increased by DSS, but there was not a significant difference amongst DSS-treated groups (Figure 5-17).
**Figure 5-17: Effects of 2% asparagus and 0.025% rutin diets on myeloperoxidase (MPO) activity during colitis recovery**

The effects of diets on distal colon MPO \( (n=5-9, \text{ in duplicate}) \) after 5 days of colitis recovery with water. All data are expressed as the mean ± SEM. Means with different letters are significantly different from each other \((p<0.05)\). Statistical analyses were performed using one-way ANOVA (SNK post-hoc test) on \( \log_{10} \) transformed data. BD=basal diet, DSS=dextran sodium sulfate.

5.3.4.6 Colitis Recovery Colon Histopathology: Colon Erosion and Goblet Cell Depletion

Unlike what was observed in acute colitis, during recovery, significant effects were observed between treatment groups for colon erosion (Figure 5-18) and goblet cell depletion (Figure 5-19). The BD+DSS group was the slowest in improving colon erosion, while 2% asparagus and 0.025% rutin significantly regenerated crypts and surface epithelial cells compared to BD+DSS, with 0.025% rutin having the greatest impact on colon erosion recovery (Figure 5-18). These patterns were also seen with goblet cell depletion. The 0.025% rutin-fed mice had the greatest impact in increasing the regeneration of mucin-producing goblet cells, followed by 2% asparagus (Figure 5-19).
Thus, the decreased survival rate in mice fed BD+DSS during colitis recovery may potentially be attributed to slow improvements in colon architecture and goblet cell regeneration.

![Colon Erosion Diagram](image)

**Figure 5-18: Histopathology of distal colon erosion during colitis recovery**
The effects of 2% asparagus and 0.025% rutin diets on (A) colon erosion (n=9-13) during 5 days of colitis recovery with water. (B) Representative sections are shown (10X magnification). Data are expressed as the mean ± SEM. Means with different letters are significantly different from each other (p<0.05). Statistical analyses were performed using one-way ANOVA (SNK post-hoc test). BD=basal diet, DSS=dextran sodium sulfate.
Figure 5-19: Histopathology of distal colon goblet cell depletion during colitis recovery
The effects of 2% asparagus and 0.025% rutin diets on (A) goblet cell depletion (n=6-13) during 5 days of recovery with water. (B) Representative sections from recovery are shown (10X magnification). All data are expressed as the mean ± SEM. Means with different letters are significantly different from each other (p<0.05). Statistical analyses were performed using one-way ANOVA (SNK post-hoc test). BD=basal diet, DSS=dextran sodium sulfate.
5.3.4.7 Serum Cytokine Multiplex Analysis in Colitis Recovery

Mouse serum from the recovery phase showed that DSS significantly increased IL-1β and IL-10, but mice fed 0.025% rutin attenuated these increases (Figure 5-20A and C). DSS also was shown to increase IL-6, IL-17A, and IFN-γ, and TNF-α during recovery (Figure 5-20B, D-F), but there were no significant differences amongst DSS-treated groups, which may be due to the large variances.

Figure 5-20: Serum Th1 cytokine multiplex analysis in colitis recovery
Serum from mice fed 2% asparagus and 0.025% rutin diets during the 5 day recovery were analyzed for Th1 cytokines by multiplex analysis: (A) IL-1β (n=8), (B) IL-6 (n=8), (C) IL-10 (n=8-9), (D) IL-17A (n=7-8), (E) IFN-γ (n=8-9), and (F) TNF-α (n=8-9). All data are expressed as the mean ± SEM. Means with different letters are significantly different from each other (p<0.05). Statistical analyses were performed using (A-C) one-way ANOVA (Dunn’s post-hoc test), and (D-F) one-way ANOVA (SNK post-hoc test). BD=basal diet, DSS=dextran sodium sulfate.
5.3.5 Discussion

The objectives of this study were to determine the effects of whole cooked asparagus and 0.025% rutin-supplemented diets during DSS-induced acute colitis and recovery in C57BL/6 mice. After feeding the mice 2% asparagus or 0.025% rutin in their diet for 2 weeks prior to and during DSS colitis induction, the 2% asparagus diet reduced some clinical symptoms of colitis (e.g. stool consistency, stool blood, and spleen enlargement). Though, these improvements were not reflected in histological scores with asparagus diet. During recovery, both 2% asparagus and 0.025% fed mice had significantly greater survival than the DSS group as they did not differ from healthy controls. Furthermore, 0.025% rutin was more effective than 2% asparagus in recovery in terms of increased diet and water intake, BW gain, total DAI score, improvement in histological scores, and reductions in IL-1β and IL-10 levels in serum. Overall, this was the first study to show the effects of rutin in recovery and whole asparagus during acute colitis and recovery.

In Chapter 4, rutin was observed in DSS-induced colitis, and it was found that mice fed 0.025% rutin significantly reduced spleen enlargement. When cross-comparing between Chapter 4 and this study, 0.025% rutin was analogous between studies, producing similar clinical, molecular, and histological data in colitis. During colitis, mice fed 2% asparagus significantly improved some clinical symptoms compared to those fed 0.025% rutin fed (e.g. stool consistency and stool blood). It is likely that the matrix of the asparagus is contributing anti-inflammatory and/or anti-oxidative effects. Prior to starting the intervention, an analysis of the antioxidant capacities of all diets was completed by FRAP and ORAC assays. It was found that the 2% asparagus diet had significantly higher antioxidant capacity compared to
the 0.025% rutin diet. This indicates that 2% asparagus contains additional anti-oxidative compounds, including phenolics other than rutin. The attenuation in clinical colitis symptoms could be attributed to the anti-oxidative nature of rutin and quercetin [212, 213, 262, 263], as well as other bioactives (e.g. vitamins, minerals) and unidentified phenolic compounds within asparagus [238, 269, 270]. It is probable that prior to colitis, the bioactives in asparagus matrix prepared the colon and/or increased the endogenous antioxidant defense system for potential insults. Therefore, after colitis is initiated, asparagus bioactives could then directly eliminate ROS and/or maintain a balance in the antioxidant defense system [212, 213, 238, 262, 263, 269]. It is not likely that the anti-inflammatory effects of asparagus during active colitis could be from mainly dietary fibre as there were no significant differences seen with the cecum weight (usually known to be an indicator of dietary fibre fermentation [241]). It is very probable that there was not enough dietary fibre in the 2% asparagus diet. Although asparagus itself is a good source of dietary fibre, a level of 2% asparagus in diet is minimal. Vitamin K, which is well known for its role in blood clotting, is found abundantly in asparagus. This vitamin could have aided in reducing the onset of rectal bleeding. Vitamin K injections have been used to treat infants who have had potentially fatal rectal bleeding in the first month of life [271].

The most prominent effects were observed in the recovery phase when DSS was removed and C57BL/6 mice were given normal drinking water for 5 days. Mice fed 0.025% rutin significantly consumed more diet and water starting on days 1 and 2, respectively, compared to 2% asparagus and DSS groups, which is an indication of increased recovery. This may explain why 0.025% rutin-fed mice recovered quicker than BD+DSS and 2% asparagus groups in terms of BW gain and total DAI score, thereby improving in other
parameters (e.g. histopathology scores). Mice fed 2% asparagus also significantly improved in these measures, but not as much as 0.025% rutin-fed mice. One reason could be because there was overall reduced diet intake (including asparagus diet intake) as a result of DSS-induced acute colitis, thus there would be a reduction in obtaining anti-oxidative and anti-inflammatory rutin and other bioactives in asparagus matrix during active colitis. Another reason could be due to a bioavailability issue; there could be delays in releasing rutin from certain components of the asparagus matrix, such as soluble fibre [272], whereas rutin would be readily free in the 0.025% rutin diet to exert effects during recovery.

Moreover, there were significant differences seen with serum cytokine levels during recovery. The expression of IL-1β, TNF-α, IL-6, and IL-10 has been observed to increase as early as 1 day post-DSS treatment [88]. After 5 days of DSS followed by 7 days of water treatment in C57BL/6] mice, the expressions of TNF-α, IL-1β, IL-6, IL-10, and IFN-γ [88] were increased, and by 28 days of water, there were high levels of IL-1β, IL-6, and IL-17 [83]. In this study, high levels of TNF-α, IL-1β, IL-6, IL-10, IL-17, and IFN-γ induced by DSS were also observed. However, mice fed 0.025% rutin diets had mildly reduced IL-1β and IL-10 levels in their serum compared to BD+DSS and 2% asparagus groups. These results coincide with previous studies regarding rutin and quercetin with IL-1β. Quercetin, released from rutin, has been found to significantly inhibit IL-1β levels in BMDM [172]. Furthermore, a recent study discovered that the dose-dependent inhibition of IL-1β by quercetin leads to an inhibition of LPS-induced DC activation, which is the most potent APC [273]. There has not been in vitro studies looking at rutin in regards to IL-1β, but one study has observed that rutin can reduce colitis severity in vivo by inhibiting IL-1β mRNA and protein expression [176]. There is also a lack of studies observing quercetin and rutin with regards to IL-10 levels, but it is likely that
these two compounds are either directly or indirectly reducing levels of neutrophils, T cells, B cells, and NK cells that are producing IL-10 [104, 108, 109]. In this study 0.025% rutin was more effective in reducing IL-1β and IL-10 levels during recovery than 2% asparagus. This may be because 0.025% rutin-fed mice consumed more diet compared to 2% asparagus-fed mice during the 5-day recovery, thereby obtaining more rutin and its respective aglycone, quercetin. There could also be a bioavailability issue occurring, resulting in a delay of rutin release from specific components of the asparagus food matrix, such as soluble fibre [272], to exert effects.

There may be two reasons why rutin was not found to be beneficial in colitis, but was during recovery, and these include: 1) the dose of DSS and 2) distinct mediators and mechanisms involved in colitis and recovery. The dose of DSS to initiate colitis is very vital, especially with the specific mouse strain used as evidence has suggested that there are differences in susceptibility to DSS in various mouse strains [81]. For instance, BALB/c mice, which are often used in DSS-induced colitis studies, are less susceptible to DSS compared to IQI/Jic [82] or C57BL/6 mice [83]. Although the DSS dose used (2%) in C57BL/6 mice is comparable with other studies using C57BL/6 [274-276], it is still possible that this DSS dose was too strong, thereby masking any protective effects of rutin during colitis. Secondly, a relapsing and a recovering state are two distinct phases, producing dissimilar mediators and mechanisms of inflammation and repair. This can be supported by the evidence that there are different levels of cytokines secreted and detected during colitis and recovery. Levels of IL-1β, TNF-α, IL-6, and IL-10 are usually seen 1 day post-DSS colitis induction [88]. And after 5 days of DSS treatment followed by 7 days of water, the same levels of cytokines are present but there is an addition of IFN-γ [88], and by 28 days of water, elevated cytokine levels are
reduced to only IL-1β, IL-6, and IL-17 [83]. Therefore, it is possible that rutin and asparagus may be interacting differently with certain mediators of inflammation during colitis and recovery.

Overall, this study demonstrated for the first time that during acute colitis, C57BL/6 mice fed 2% asparagus had the greatest reductions in colitis severity as indicated by the decrease in DSS-induced diarrhea onset and rectal bleeding, and attenuation in DSS-induced spleen hypertrophy. Yet, during recovery, 0.025% rutin-fed mice recovered the quickest in terms of BW gain and total DAI, reductions in IL-1β and IL-10, and crypt, epithelial and goblet cell regeneration. Mice fed 2% asparagus also recovered quicker than the control group. It is likely that 0.025% rutin-fed mice recovered quicker than the 2% asparagus group because they consumed more diet than mice fed 2% asparagus, thereby obtaining an increased level of rutin. However, there has been research suggesting that food matrices, including dietary fibre, can interfere or delay the release of antioxidants [272]. Therefore, to further understand the role of cooked asparagus in gut health (diseased and healthy states), future bioavailability and bioaccessibility studies are warranted.
Chapter 6: Thesis Overview, Future Directions, and Implications
6.1 Thesis Overview

This thesis was comprised of three studies using C57BL/6 mice: 1) chemical and proximate characterization of asparagus flour and an assessment of the suitability of supplementing asparagus in C57BL/6 mice diets; 2) an investigation of the effects of rutin on symptoms and disease progression in DSS-induced acute colitis in C57BL/6 mice; and 3) an assessment of whole cooked asparagus and purified rutin in DSS-induced acute colitis and recovery. Asparagus is known to be a good source of micro- and macronutrients, dietary fibre, and phenolic compounds, yet, for this thesis, the phenolic compounds of asparagus, particularly rutin and quercetin were emphasized.

In Chapter 3, the objectives were to first characterize the macronutrients and rutin concentration in cooked asparagus, and then the second objective was to test if asparagus would be palatable to C57BL/6 mice. Cooked asparagus was found to contain high mounts of rutin, protein, and fibre, particularly soluble fibre, low in dietary fat and available carbohydrates, and rich in minerals. For the second objective, 12% asparagus was supplemented in mouse diet, because it was equivalent to 0.1% rutin, which was the level that has seen to be the most effective in reducing colitis in ICR mice by Kwon et al. (2005) [176]. One week into the feeding, it was discovered that the mice were not tolerating the diet well compared to the BD group as indicated by the decrease in diet intake and BW gain. Thus, the 12% asparagus was modified with BD to create two more doses, 3 and 6% asparagus (0.025 and 0.05% rutin, respectively) in powder form. It was concluded that 12% asparagus (0.1% rutin) was not tolerable with C57BL/6 mice, yet, 3% asparagus (0.025% rutin) was.
In Chapter 4, the effects of rutin (0.025, 0.05, and 0.1%) on DSS-induced acute colitis in C57BL/6 mice were examined. As seen with ICR mice [176], we wanted to verify if 0.1% rutin would also have the greatest reductions in colitis severity in C57BL/6 mice. Yet, after giving the respective diets for 3 weeks, we did not see significant differences amongst rutin doses for most of the measured parameters, except that rutin was able to attenuate stool consistency changes leading to diarrhea compared to BD+DSS mice. Also, the most significant difference observed was an attenuation of DSS-induced spleen hypertrophy by 0.025% rutin. Therefore, based on these results, rutin can alleviate DSS-induced acute colitis in C57BL/6 mice, but not dose-dependently.

In Chapter 5, I investigated the effects of consuming whole cooked asparagus and rutin in C57BL/6 mice during acute colitis and recovery, mimicking the relapse and recovery states of UC. Based on Chapters 3 and 4, we used an asparagus dose of 2%, containing 0.025% rutin. Before the intervention began, an analysis of the diets for antioxidant activity by FRAP and ORAC assays was completed. The 2% asparagus diet had significantly higher antioxidant activity compared to 0.025% rutin, which denotes that 2% asparagus must contain other anti-oxidative bioactives and phenolic compounds. Yet, it must be kept in mind that human digestive systems may be more efficient in releasing phenolics from food matrices than lab chemical extraction methods, so the antioxidant activity of 2% asparagus diet may be greater than what was reflected by FRAP and ORAC assays. One of the limitations of current chemical extraction methods of phenolic compounds is that they cannot fully represent human digestive system. During acute colitis, mice fed 2% asparagus significantly attenuated DSS-induced colitis symptoms. Though in recovery, 2% asparagus fed mice had significant improvements in colitis symptoms.
symptoms, mice fed 0.025% rutin had greater improvements in disease symptoms compared to the 2% asparagus group. It is unknown at this point whether these effects are because 0.025% rutin-fed mice ate more at the beginning, or if it is a bioavailability issue. If it is the latter, then there could have been a delay in releasing rutin from certain components of the asparagus food matrix, such as soluble fibre [272], whereas rutin is readily free in the 0.025% rutin diet. However, from past research, these beneficial effects can be attributed to the anti-oxidative and anti-inflammatory properties of rutin and quercetin [172, 176, 210-213, 216, 248, 261, 262, 268, 269, 277].

Overall, this thesis has determined: the amounts of macronutrient, fibre, and rutin, and the suitability of using cooked asparagus in animal diet (Chapter 3); the effect of rutin in alleviating DSS-induced acute colitis symptoms and disease progression (Chapter 4); and the effects of whole cooked asparagus and purified rutin in DSS-induced acute colitis and recovery (Chapter 5), all with C57BL/6 mice. This is the first study to demonstrate that whole cooked asparagus is beneficial to colon health during relapse (inflammatory) and recovery states of experimental UC.

6.2 Future Directions

6.2.1 Asparagus Characterization

Currently, a full characterization of asparagus bioactives has not been completed, including phenolics. From the FRAP and ORAC analyses, it appears that the asparagus diet contains other phenolic compounds aside from rutin. A full characterization of all the bioactives and phenolic compounds in asparagus would be helpful in understanding how asparagus can affect colon health in terms of the individual bioactive it contains, as well as
determine if there are any interactions (synergism or antagonism) between bioactives. Mass-spectrometry combined with liquid chromatography is an efficient and highly accurate method that can be used to identify unknown bioactives in asparagus, including determining the structure of those compounds [278-280].

6.2.2 Bioavailability and Bioaccessibility

Bioaccessibility studies are needed to understand how accessible rutin or other bioactives from asparagus is to the colon in order to then elucidate the mechanisms involved with asparagus bioactives in colon health. Direct interactions between phenolics and components of food, such as dietary fibre, proteins, and polysaccharides, can occur, which can affect absorption [281]. For instance, with regard to flavonols, much higher plasma concentrations were obtained when quercetin glucosides were given to fasted volunteers in the form of a water-alcohol solution [282] than when an equivalent quantity was ingested in foods like onions, apples, or a complex meal [283, 284]. A recent review has also established that dietary fibre can limit the bioavailability of phenolic compounds in fruits and vegetables. The authors concluded that the main reasons were because: 1) the phenolic compounds are not well released from fruit and vegetable matrices; 2) dietary fibre entraps the phenolic compounds during digestion in the upper intestine, and 3) some antioxidants may be bound to polysaccharides and thus require enzymatic hydrolysis to be absorbed, but this is restricted by dietary fibre matrices formed in the chyme [272]. Therefore, to assess the bioavailability of rutin from asparagus, a feeding study with cooked asparagus or purified rutin in healthy subjects is needed. The amount of rutin and
quercetin metabolites can be measured in either blood serum or plasma, fecal, or urine samples [285].

6.2.3 Mechanisms of Action of Asparagus on Colon Health

6.2.3.1 Gut Barrier Integrity

The preservation of gut barrier integrity, including: mucosal barrier; luminal epithelial cell and tight junction interactions; and a balance between elimination of damaged cells and generation of new cells in intestinal crypts; is very important in preventing antigen penetration, injury, and stress of the colon [286]. There has been evidence to suggest that certain food components can increase intestinal gut barrier integrity. For instance, substantial amounts of dietary fibre, both soluble and insoluble, in the diet can increase colonic mucosal production in rats [287]. Fermentable fibre can also provide SCFAs that are an important energy source for colonocytes [288]. Feeding asparagus to healthy subjects and assessing their colons could indicate whether or not asparagus can increase gut barrier integrity and prepare the colon for potential impending insults from toxins and pathogens, for instance. Assessments could be through histological analyses (Hematoxylin & Eosin for colon morphology and Alcian Blue for mucus-producing goblet cells) and gene expressions of MUC2 [289, 290]. MUC2 is a gel-forming mucin that makes up the mucosal barrier and is known to be the first line of defense in protecting against damaging agents within the lumen [90]. It is possible that quercetin may have effects on MUC2 and other MUC genes within the colon. It has been found that quercetin can inhibit human neutrophil elastase-induced MUC5AC expression in human airway epithelial cells [291], so it may be possible that phenolics can affect MUC genes involved in
the colon. This could further elucidate the mechanisms of action of asparagus with regards to colon health.

6.2.3.2 Antioxidant Status

Aside from potentially enhancing gut barrier integrity, asparagus may also be able to increase the endogenous antioxidant defense system in a healthy state prior to colitis or in remission prior to relapse. There has been research demonstrating that the antioxidant capacities of serum or plasma from healthy subjects are increased after the ingestion of antioxidant-rich foods [292-294]. From FRAP and ORAC analyses, it was determined that asparagus diet contained the highest antioxidant capacities compared to BD and rutin diets. However, it is unknown whether asparagus diet increased antioxidant levels systemically in the blood and/or locally at the colon tissue. Therefore, analyses of the antioxidant capacity in serum and/or colon tissue are warranted through FRAP, ORAC, or GSH assays [120, 295].

6.2.3.3 Direct Effects on NF-κB Inflammatory Pathway

Though serum cytokines were measured after the 5 day recovery cycle, an analysis of serum cytokines during acute colitis still needs to be completed. Since it is vital to understand which cytokines are elevated and/or attenuated during active colitis by the treatments, I will be completing this analysis sometime in the near future using the same multiplex kit as described in section 5.3.1.8. It is also essential to complete to be able to compare with the recovery serum cytokine data.

One main pathway that may be playing a major role in the inflammatory process of DSS-induced colitis is the NF-κB pathway. NF-κB exists in the cytoplasm of cells in an
inactive form associated with inhibitory proteins [296]. When NF-κB is exposed to activation signals such as TNF-α or ROS binding to cell surface receptors, the inhibitory proteins associated with NF-κB are broken down. An increased activation of NF-κB leads to increased expression of proinflammatory cytokines and immunoregulatory mediators [296]. Certain components of the diet (e.g. butyrate, polyphenols, omega-3 PUFAs) have been demonstrated to be effective inhibitors of NF-κB activation [297-299]. Hence, asparagus, which is rich in polyphenols and other anti-inflammatory bioactives, may be interacting with the NF-κB pathway. In order to confirm this, NF-κB assessments from colon tissue using an ELISA kit should be completed [300].

6.2.3.4 Colonic Microflora Profile

The human intestinal tract consists of a complex community of microbiota (10^{11} to 10^{12} cells/gram) that is critical in influencing bodily health [301]. The intestinal microflora varies between individuals and these variations are dependent on non-dietary (e.g. geographic location, occupation) and dietary factors [301]. A diet consisting of prebiotics (e.g. fructo-oligosaccharides, inulin) can significantly increase fecal concentrations of bifidobacteria, which are considered key players in health and well-being, including reducing disease risk in humans [288, 302]. Thus, asparagus matrix may also have the ability to enhance health-promoting bacteria prior to colitis.

During colitis, however, evidence has suggested that there is a shift in bacteria populations in UC patients [303] and in rodents of DSS-induced colitis to pro-inflammatory Gram-negative intestinal bacteria [304]. Most phenolics, such as rutin, in food are in the form of esters, glycosides, or polymers that cannot be absorbed in their native form. They must be hydrolyzed or cleaved by intestinal enzymes (e.g. β-glucosidase) or by colonic
microflora, respectively, before they can be absorbed. Therefore, it is vital to verify if microflora (e.g. Bacteroides population producing β-glucosidase) is decreased during colitis in C57BL/6 mice, and whether or not this affected rutin metabolism. To assess microflora populations prior to and during colitis, rutin metabolites can be identified in fecal or colon tissue samples via culturing techniques and/or polymerase chain reaction-denaturing gradient gel electrophoresis [301, 305].

6.3 Implications

Overall, the findings of this thesis are influential to managing symptoms of UC, a prevalent condition of remitting and relapsing inflammation of the large intestine [1]. As previously discussed, current conventional therapies for UC have several limitations, including severe side effects, drug dependence, and antibiotic resistance [142-146]. Thus, these limitations encourage the use of natural dietary interventions among UC patients, such as PUFAs, prebiotics, and phenolic compounds. Currently, the link between diet, etiology, and signs and symptoms of UC is unclear. Yet, it has been noted by UC patients that they often experience food sensitivities, particularly with cereals, milk, eggs, vegetables, and citrus fruits [306]. These food sensitivities could be dependent on the time they are eaten (i.e. during relapse or remissive states) [307]. Certain dietary foods, such as high meat or alcoholic beverage intake, have also been identified to increase the likelihood of relapse in UC patients [308].

In the end, the aim for IBD therapy is to downregulate inflammation, reduce the incidence of relapse, increase the healing time, and/or maintain remission without the reliance of pharmacotherapy and surgical intervention. Nutrition currently offers a
prospective role in meeting these aims, but without additional research, it remains uncertain whether the modification of diets would have a role in IBD management. As demonstrated in this thesis, cooked whole Guelph Millennium asparagus can be beneficial during relapse and recovery of experimental UC. This advocates for increased public awareness of the health benefits of asparagus. An increased rate of asparagus consumption could also be economically profitable for farmers and for Ontario as Southern Ontario grows most of the Guelph Millennium asparagus.
Chapter 7 : References


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132. *!!! INVALID CITATION !!!*


