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Reduction in Mucosal Barrier Markers with Soy Protein Diet but not Lactobacillus rhamnosus GG in DSS-Treated Mice

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ABSTRACT

Inflammatory bowel disease (IBD) is a common ailment affecting people of all ages. Even though the pathogenesis of IBD has not been fully elucidated, there is evidence that it involves interactions between genetic susceptibility, aberrant activation of the immune system, and the environment. Inflammation of the colon (colitis) results in pain, diarrhea, hemorrhage, and weight loss. Within the colon, the family of mucins (MUC) and trefoil factors (TFF) facilitate mucosal protection. Recent studies found that probiotic administration could influence the colonic mucin layer. Additionally, food components may affect gut microflora or have influence on the mucin barrier. Our aims were to explore the role of probiotic administration in modulating *MUC* and *TFF* gene expression in the dextran sodium sulfate (DSS) induced colitis mouse model, and to examine interactions with casein, whey, or soy protein diets. C57BL/6 mice were assigned to three diet groups. Diets were formulated to substitute casein, whey or soy protein on an equivalent basis. On day 6, each diet group was divided into 2 groups, with or without *Lactobacillus rhamnosus* GG (LGG) added daily to the diet. Starting on day 12 the mice were given 2% DSS in drinking water for 4 days and then euthanized 1 day later. A casein diet group with neither LGG nor DSS treatment served as a control. Colon tissues and cecal samples were collected and analyzed by quantitative RT-PCR. LGG-specific gene expression was found in cecum contents of all mice given LGG but none of the untreated mice. Mice fed whey protein had significantly higher LGG quantity compared to mice fed casein diets. Colonic *MUC1* and *TFF3* gene expression were increased by DSS treatment. In mice fed soy protein, induction of *MUC1* and *TFF3* expression after DSS was suppressed. Mice fed whey protein had intermediate

TFF3 gene expression compared to the other diet groups. There was no overall probiotic effect on any of the parameters. As a marker of inflammation, *TNF-α* gene expression was increased after DSS treatment. Mice fed soy protein, however, had suppressed *TNF-α* expression compared to the other treatment groups, which was consistent with the colon length change and colonic inflammation score. In conclusion, soy protein suppressed the DSS-induced inflammatory stimulation of *MUC*, *TFF* and *TNF-α* gene expression independently of the probiotic. Whey protein may act as a prebiotic in the colon to increase microbial survival, but these factors failed to prevent the inflammatory response. Future studies are needed to identify the bioactive components in the soy protein that modulate the barrier function in the colon and have protective effects against colitis.

CHAPTER 1

LITERATURE REVIEW

1. Physiological and cellular characterization of the colon mucosa

The colon is the distal region of the digestive system in vertebrates. The major functions of the colon are to absorb water and electrolytes, absorb vitamins synthesized by the bacteria in the lumen, and compact and store fecal matter until it can be expelled. The colon wall consists of four layers, from outer surface inward, including adventitia, muscularis externa, submucosa and mucosa. The adventitia is the outmost connective tissue covering the colon. It consists of several layers of epithelia. The muscularis externa consists of an inner circular layer and a longitudinal outer muscular layer, which are responsible for gut motility. The submucosa is the layer of dense irregular connective tissue with blood vessels, lymphatics, and nerves between the smooth muscle and the mucosa. The most inner layer of the gastrointestinal (GI) tract is the mucosa, which surrounds the lumen (1). This layer comes in direct contact with food, and is responsible for absorption of nutrients, secretion of mucus, and the mucosal barrier it forms is an important component of the immune system. Lymphoid follicles consisting of B lymphocytes, T lymphocytes, dendritic cells and macrophages are present in colonic mucosa and may extend through the muscularis mucosa into the submucosa (2). Going from inside the lumen radially outwards, the mucosa can be divided into epithelium, lamina propria, and muscularis mucosa.

The epithelium is composed of absorptive epithelium, goblet, and endocrine cells. The epithelium contains no enzyme-secreting cells. Instead, it consists mainly of goblet cells that secrete mucus. There are some endocrine cells in the colon secreting small amounts of

somatostatin that regulates digestion. Paneth cells are not normally present in the mucosa of the colon. The presence of Paneth cells more distally indicates metaplastic changes seen in chronic infection such as in patients with ulcerative colitis (3). Paneth cells secrete antimicrobial substances such as defenses, lysozyme, and phospholipase A2 indicating a role for Paneth cells in host defense (4). The lamina propria is a thin layer of loose connective tissue with capillaries and lymphoid cells which lies under the epithelium. It contains several cell types, including plasma cells, T cells, NK cells, eosinophils, mast cells, and macrophages, most of which are responsible for local, immunologically mediated host defense against harmful agents from the lumen. In inflammatory conditions, there is inflammatory cell infiltration by neutrophils, macrophages and lymphocytes. The muscularis mucosa is the thin layer of smooth muscle separating the lamina propria from the submucosa (1).

Crypts of Lieberkuhn are straight tubular glands that are found in the epithelial lining of the colonic mucosa. There are millions of crypts in a normal colon. Crypts contain about 2000 cells, which are maintained by stem cells (undifferentiated progenitor cells) located near or at the bottom of each crypt (5). Large numbers of mucus-secreting goblet cells fill the crypts of the colon. Branching of the crypts, increasing of plasma cells and lymphocytes between the bases of the crypts and the muscularis mucosa, and neutrophil infiltration in crypt epithelium are histopathological characteristics of chronic inflammation and inflammatory bowel disease (IBD) (6).

Aberrant crypt foci (ACF) are clusters of abnormal tube-like glands in the lining of the colon and rectum, which can be found before the early stage of colorectal polyps. The appearance

of ACF is one of the earliest changes that might lead to colon cancer. They can be detected under a microscope with methylene blue staining (7).

There is a large population of microbes in the gut. It is reported that the bacteria density in the human colon may be as abundant as 10^{10}-10^{12} colony-forming units per gram of colonic contents (8). The commensal bacteria collectively make up the intestinal microbiota. The human host and its microbiota constitute a complex ecosystem whose equilibrium serves as a remarkable example of reciprocal adaptation (9). Most microbes belong to the genera *Bacteroides, Clostridium, Fusobacterium, Eubacterium, Ruminococcus, Peptococcus, Peptostreptococcus,* and *Bifidobacterium*. Other genera, such as *Escherichia* and *Lactobacillus,* are present to a lesser extent (10). The commensal bacteria are essential to human health. They form resistance to pathogen colonization via producing antimicrobial substances, changing luminal pH and directly competing against pathogens for nutrients, thereby obstructing and inhibiting pathogen invasion (11, 12). Commensal bacteria also facilitate the development and optimal functioning of the host immune system (13).

The mucous layer is the first defense barrier between bacteria and the epithelial cells in the gut (14-18). The mucous layer lining the surface epithelium of the intestine acts as a lubricant and protective physical barrier between the mucosal surface and the luminal contents. Particles, bacteria, and viruses are trapped in the mucous layer and eliminated by the peristaltic movement of the gut. In the presence of this barrier, pathogens and antigens cannot gain access to the underlying epithelium and are blocked from invading the body (2). The mucous layer consists of mucus glycoprotein (mucins) and trefoil factors that are secreted by goblet cells. Mucin is a family of high molecular weight, heavily glycosylated proteins

produced by goblet cells. The mucins can form gels. Trefoil factors are stable secretory proteins by goblet cells expressed in GI mucosa, which may protect the mucosa from insults, stabilize the mucus layer and affect healing of the epithelium (19, 20).

Mucins that form the protective barrier to the mucosa are divided to three main types, including secretory (gel forming) mucins, membrane bound mucins, and a group of nonclassified mucins. The secretory mucins include MUC2, MUC5AC, MUC5B, and MUC6. Secretory mucins are produced in goblet cells of the GI tract. Membrane mucins are MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12 and MUC17 (21). The membrane bound mucins are produced in nonspecialized serous or epithelial cells, for instance, the enterocytes (22). MUC1, MUC2, MUC3 and MUC4 are the major mucins expressed in the large intestine in humans. In particular, MUC2 is the main secreted colonic mucin (23). The mucin genes in mice are similar to those found in human. It has been found in BALB/c mice that MUC3 is the most commonly expressed mucin in the large intestine, followed by MUC2. In contrast, MUC1 and MUC4 are poorly expressed (24). The mucin barrier is critical in limiting contact between bacteria and epithelial cells. Dysfunction of this protective barrier may lead to IBD. For example, MUC2 knockout mice spontaneously developed colitis, indicating that MUC2 is important for colonic protection (25).

The trefoil factor (TFF) peptides TFF1, TFF2, and TFF3 are expressed by mucus-secreting cells, mainly in the GI tract, with specific cellular localization of each peptide. TFF1 is expressed by surface epithelial cells of the stomach, and TFF2 in mucous neck cells, pyloric glands, and Brunner’s glands, whereas TFF3 is mainly located in goblet cells of the small and large intestine (26). TFF3 is a key peptide in mucosal protection and repair. It cooperates

at the epithelial barrier with mucin glycoproteins to enhance the protective barrier. Knockout of the TFF3 gene led to an increased susceptibility to dextran sulfate sodium (DSS)-induced colitis, and intraluminal infusion of TFF3 rescued the TFF3 defect through coordinated promotion of intestinal cell migration and inhibition of intestinal cell apoptosis (27). On the other hand, overproduction of TFF3 is found in a variety of GI inflammatory conditions, such as necrotizing enterocolitis and IBD, which indicates that dysregulation of TFF3 occurs during inflammatory conditions (28).

2. Overview of inflammatory bowel diseases

Inflammatory bowel diseases are a group of chronic inflammatory conditions in the colon and small intestine. According to data published in 2004, there were as many as 1.4 million persons in the United States and 2.2 million persons in Europe suffering from IBD (29). The incidence of IBD has increased during recent decades, not only in Western countries, but also in some regions where IBD had been less common such as Japan (30).

IBD includes the two major diseases, Crohn's disease and ulcerative colitis (31). There are several differences between these conditions. The main difference is the location of the inflammatory changes. Crohn's disease can affect any part of the alimentary tract, from mouth to anus, even though most of the cases start in the terminal ileum. In contrast, ulcerative colitis is restricted to the colon and the rectum. Crohn's disease typically involves transmural inflammation, and the lesions may be patchy and segmental, whereas ulcerative colitis develops diffuse mucosal inflammation. The histological feature of Crohn's disease is

the aggregation of macrophages which usually form non-caseating granulomas, while ulcerative colitis is distinguish by the neutrophils aggregation within lamina propria and crypts, where they form microabscesses. Depletion of goblet cell mucin is also common in ulcerative colitis (32). The major symptoms of IBD include diarrhea, hemorrhage, abdominal pain, and weight loss, which subsequently affect the quality of life for patients (33). There is evidence that patients with ulcerative colitis and Crohn's disease had a higher risk of developing intestinal cancer, even though the mechanisms have not been fully understood (34-37). O'Connor et al. (38) explored these mechanisms, and stated that IBD patients were exposed to constant activation of nuclear factor-kappaB (NF-κB) and cyclooxygenase-2/ prostaglandin pathways, release of proinflammatory cytokines, and increased local levels of reactive oxygen and nitrogen species. Collectively, the chronic presence of these inflammatory signals can have carcinogenic effects through the following major processes: 1) by enhancing oxidative stress, which promotes DNA mutagenesis thereafter contributing to tumor initiation; 2) by activating prosurvival and antiapoptotic pathways in epithelial cells, thus contributing to tumor promotion; and 3) by creating an environment that supports sustained growth, angiogenesis, migration, and invasion of tumor cells, thereby supporting tumor progression and metastasis (38, 39). Due to the rising incidence and significant association with human health, IBD has been extensively studied during the last several decades.

3. Etiology of IBD

Even though the pathogenesis of IBD has not been fully understood, there is evidence that it

involves interactions between genetic susceptibility, immune system, and the environment, most notably the colonic microflora (40).

The importance of genetic factors in IBD was originally suggested by epidemiological studies showing familial aggregation of IBD and by twin studies. The *NOD2* (*CARD15*) gene, which is located on chromosome 16, was the first susceptibility gene characterized in Crohn's disease (41-45). In 1996, Hugot et al. (45) performed a genome-wide search on two consecutive and independent panels of families with multiple Crohn's disease patients and found that a susceptibility locus for Crohn's disease, named *IBD1*, was located near the pericentromeric region of chromosome 16. Further study was conducted to identify the specific gene in this region. Subsequently, the *NOD2* gene has been sequenced in 50 Crohn's disease patients (41). There were 13 polymorphisms identified. Two amino acid changes involving single nucleotide polymorphisms (Arg702Trp and Gly908Arg) and one frameshift mutation (3020insC) that leads to a premature stop codon was also linked to Crohn's disease. Bacterial peptidoglycan may activate NOD2 and thereafter activates the NF-κB and mitogen-activated protein (MAP) kinase signaling pathways, which results in the production of proinflammatory cytokines (46, 47). Recently, the use of genome-wide association studies (GWAS) has provided new insights into primary pathogenetic mechanisms. Besides *NOD2*, there are more genes identified, including *NOD1, DLG5, TLR4, OCTN1* and *OCTN2*. The GWAS also facilitated the recognition of additional genes such as *ATG16L1*, *IL23R*, *IRGM* and *PTGER4* (48, 49). For instance, *ATG16L1* is the autophagy gene that was found to regulate secretion of interleukin-1β, interleukin-18 and suppress intestinal inflammation.

Mice lacking *ATG16L1* in hematopoietic cells were extremely susceptible to DSS induced acute colitis (50).

Innate immune response is the first line of defense against microbial infection and provides a rapid response to pathogens. Recent evidence supports that innate immune responses play an essential role in initiating the inflammatory cascade and help to shape the subsequent and characteristic pathological adaptive immune responses associated with IBD (51). Several types of components from bacteria and viruses such as lipopolysacharide (52), peptidoglycan, flagellin, and lipoproteins, can be detected by pattern recognition receptors (PRRs) of the host. Interaction of these ligands with their cognate PRRs activate intracellular signaling cascades that induce the expression of a variety of overlapping and unique genes involved in inflammatory and immune responses (52). The toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD) receptor families of PRRs are found to play important roles in mucosal homeostasis and relate to the pathogenesis of IBD (53). It has been described that the expression of TLR3 and TLR4 were modulated in IBD. TLR3 was downregulated in intestinal epithelial cells during active Crohn's disease, whereas TLR4 was increased in both Crohn's disease and ulcerative colitis (54). The activation of the NOD2 by bacterial peptidoglycan leads to the production of proinflammatory cytokines as mentioned above (46, 47).

Several environmental risk factors for IBD have been investigated, including smoking, appendectomy, oral contraceptives, diet, breastfeeding, infections, antibiotics, and childhood hygiene (55). Migrant studies have clearly demonstrated that lifestyle and environmental influences are more important to IBD etiology than regional, ethnic, and racial differences

because individuals moving from low-occurrence areas to high-occurrence areas show an increased incidence of disease (29). For instance, a 3-year prospective study of ulcerative colitis in a city in the United Kingdom found that extensive colitis was more frequent in the second-generation migrants than in the first. The incidence was comparable to the European community (56). It was found that consumption of fast foods conferred a high risk for IBD (57), while raw vegetable and fruits, whole grain, and coffee were suggested to have protective effects (57, 58).

Bacteria have long been considered essential to the development of IBD (59). It was observed that in the different parts of the intestine, the locations with more bacteria were those where there were higher incidence of inflammation (60). An increasing number of clinical studies in patients with IBD and laboratory studies in animal models point to the important role of the microbiota in the initiation and development of inflammation (60, 61). Giaffer et al. (62) accessed the microbiota of IBD patients and observed an increase of *Escherichia coli* and a decrease of *Bifidobacteria* in patients with Crohn's disease. In another similar study, the fecal microbiota of patients with IBD was specified via fluorescent in situ hybridization adapted to flow cytometry. It was found that there was a significantly higher percentage of bacteria that could not be recognized by the probes in patients with IBD compared to healthy subjects, which indicated there were more unusual bacteria in the fecal samples of IBD patients. They also observed a decrease of *Clostridium*, a commensal bacteria genera, in IBD patients (61). The necessity of bacteria in the establishment of experimental colitis models also supports the importance of bacteria in IBD etiology. Early in 1998, Sellon et al. (59) demonstrated that resident enteric bacteria were necessary for

developing spontaneous colitis. Transgenic mice kept under germ free conditions did not develop colitis until they were administered bacterial strains. Consistently, it was shown that if the animals were treated with antibiotic first, experimental colitis could not be induced (63). Bacteria which can adhere to and invade the intestinal mucosa may be especially critical, for instance, *E. coli* (64). *E. coli* are the major facultative anaerobic, Gram-negative species of the normal intestinal microbiota. In normal human intestine, commensal *E. coli* cannot adhere to the apical face of intestinal epithelial cells. However, the pathogenic *E. coli*, which hereditably obtain virulence genes encoding adhesive factors, are able to adhere to the apical face of intestinal epithelial cells and cause pathogenic reactions (65). For instance, the enterotoxigenic *E. coli* (65), enteropathogenic *E. coli* (66) and enterohemorrhagic *E. coli* (67) were found to have the ability to attach to the epithelium and cause gastroenteritis. *E. coli* strains are suspected to be associated with the pathogenesis of Crohn's disease. A microflora examination found that *E. coli* were found abnormally predominant in early as well as chronic lesions of Crohn's disease patients. They consisted 50% to 100% of the total amount of aerobes and anaerobes (68).

4. IBD animal models: classification, DSS model

Dependable, reproducible, and clinically relevant experimental colitis models are needed to study the molecular events related to the disease progression and to explore the prevention strategies for IBD and subsequent colon cancer (69). There are several categories of experimental colitis models, including genetically engineered models, spontaneous colitis models, inducible colitis models, and adoptive transfer models (70). Genetically engineered

models consist of gene knockout mouse models and transgenic mouse/rat models. Mice with knockout of several genes, such as interleukin-2 (*IL-2*) (71), *IL-10* (72), *STAT3* (73), tumor necrosis factor (TNF)-3′ untranslated region (74), intestinal trefoil factor (27), and T cell receptor (75), have been shown to develop colitis when harboring a conventional microbiota. *IL-17* transgenic mice (70), *STAT4* transgenic mice (76), *HLA B27* transgenic rats (77) are transgenic models that have been reported. Models of spontaneous colitis include C3H/HeJBir mice (78) and SAMP/Yit mice (79). Several chemicals have been utilized to induce colitis in rodents, such oxazolone (80), DSS (81), 2,4,6-trinitrobenzene sulfonic acid (TNBS) (82), and carrageenan (83). CD4+/CD45RB^{high} T cells transfer colitis (84) and colitis induced by transfer of hsp60-specific CD8 T-cells (85) are the two adoptive transfer models that have been established.

The DSS-induced colitis model originally reported by Okayasu et al. (81) has been widely used to investigate colitis. This model is easy to develop, and the characteristics of this model are close to those of ulcerative patients. In addition, DSS can be used to induce both acute colitis and chronic inflammation (86). DSS interferes with the intestinal epithelial barrier first, which is a physical barrier against excessive entry of bacteria from gut lumen into the lamina propria, and then stimulates local inflammation as the secondary phenomenon (87). Acute colitis could be induced by 4-day treatment of 5% DSS (88), while a cycle of 5% DSS administration, followed by a 2-5 week recovery period resulted in chronic colitis in Swiss Webster mice, which was characterized by slower epithelial regeneration and an adaptive immune response characterized by both Th1 as well as Th2 cytokine profiles, including IFN-γ and IL-4, respectively (86). In general, DSS treatment causes weight loss, diarrhea with

blood and/or mucus, shortening of the colon, erosion of the mucosal epithelium, and acute neutrophilic infiltration all of which are similar to the clinical and histological features of ulcerative colitis (81, 87).

Widely used inflammatory markers for DSS induced colitis include myeloperoxidase (MPO) activity, proinflammatory cytokines such as IL-1β, IL-6, IL-12, TNF-α, IFN-γ, and chemokines KC and MIP-2 (87). These markers are usually measured in the colon samples or the plasma by several techniques such as western blotting, enzyme-linked immunosorbent assay (ELISA), or polymerase chain reaction (PCR). The transcription factor NF-κB signal pathway is an important pathway associated with colitis. Experimental colitis models are characterized by NF-κB activation and relative proinflammatory cytokines upregulation (87, 89, 90). For instance, in the study by Reed et al. (89), rats were treated with 5% DSS in drinking water and sacrificed daily for 6 days. NF-κB activation increased on day 1 and again on days 4 through 6. The colonic mRNA levels for IL-1β and TNF-α rose significantly between 2 and 4 days. Increased iNOS mRNA levels, MPO activity, and mucosal damage occurred on day 6. In another study, C57BL/6 mice were treated with 3.5% DSS for various times. The proinflammatory cytokines of colonic mucosa such as IL-1β, IL-6, IL-12, TNF-α, and IFN-γ were increased after DSS treatment (87). The histological examination has been widely used as another inflammatory parameter. The histological scores are usually based on the severity of mucosal epithelial damage, glandular alterations, and the character of lamina propria cellular infiltration. Mucosa height, mucosa erosion, inflammatory score, edema score, and inflammatory cell infiltration are evaluated in the tissue sections stained with hematoxylin and eosin (91).

5. MUC genes and DSS induced colitis

MUC genes are altered in IBD. In a study that examined colon biopsies for *MUC1* expression by immunochemistry using MUC1-specific antibody and analyzed MUC1 serum concentrations by ELISA in IBD patients, overexpression of MUC1 was found (92). The increased MUC1 levels were correlated with clinical disease severity. This study suggested that MUC1 could be used as an inflammatory biomarker and assisted monitoring the disease activity of IBD (92).

Alterations of *MUC* gene expression are also observed in DSS-induced colitis models (24, 93, 94). Hoebler et al. (24) treated BALB/c mice with 1% DSS for 5 days to induce acute inflammation, followed by 0.5% DSS for 28 days to induce chronic inflammation. It was found that *MUC2* gene expression was not modified by DSS treatment, while *MUC3-specific* mRNA expression was increased after acute colitis only in the cecum and distal colon. *MUC1* and *MUC4* gene expression were enhanced significantly by colitis. This suggested that mucin production helps to maintain and to recover the protective capacities of the mucus layer, which were changed during experimental colitis (24). In another study, Sprague-Dawley rats were administered 5% DSS for 9 days followed by 2% DSS for 18 days to induce chronic colitis (93). It was observed that DSS-treated rats had higher mRNA levels of colonic *MUC2* and *MUC3* compared with pair-fed controls (93). In the study by Renes et al (94), Wistar rats were given 7% DSS in the drinking water for 7 days, followed by a 3 week recovery period. Immunohistochemistry observation showed that MUC2 was produced by goblet cells in the proximal and distal colon from crypt base to the surface epithelium. Alterations in the numbers and localization of goblet cells were observed during and after

DSS treatment. On day 2, MUC2 positive goblets accumulated in the surface epithelium along the length of the colon. As DSS administration proceeded (day 5 and 7), there were more MUC2 positive goblet cells located along the surface epithelium. Biochemical analysis of MUC2 protein levels showed a progressive increase in MUC2 in the proximal colon during DSS treatment, and MUC2 levels rose four-fold over that of the control levels on day 7. Thereafter, the MUC2 levels decreased but were still higher compared to the control levels. In the distal colon, MUC2 tended to decrease during the active inflammatory phase, and returned to control levels during the regenerative phase (94).

In brief, *MUC* gene expression was altered in IBD patients and experimental colitis models. The *MUC* genes might be used as targets of IBD prevention and therapy.

6. TFF genes and DSS induced colitis

Trefoil factor play an important role in mucosal protection in the intestine. There is increasing evidence indicating the association of TFF dysregulation with IBD. A clinical study found that serum concentrations of TFF1, TFF2, and TFF3 were significantly increased in IBD patients compared with healthy controls (95). The presence of increased TFF3 levels correlated with clinical and biochemical parameters of disease activity in ulcerative colitis patients. After medical therapy, TFF levels tended to reduce, which was consistent with clinical and biochemical remission (95).

Upregulation of TFF3 peptides and/or *TFF3-specific* mRNA in colonic samples was observed in DSS-induced colitis of mice or rats (94, 96, 97). In the study by Renes et al. (94),

Wistar rats were given 7% DSS in the drinking water for 7 days, followed by a 3 week recovery period. Immunohistochemistry observation showed that, TFF3 was expressed in the upper crypts in the proximal colon and upper two-thirds of the crypts in the distal colon. On day 2, TFF3 positive goblet cells accumulated in the surface epithelium in the whole colon. As DSS administration proceeded (day 5 and 7), there were more TFF3 positive goblet cells found in the surface epithelium. Protein dot blotting showed an increase of TFF3 peptide in the colon samples (94). Consistently, it was observed in the DSS-induced colitis using a BALB/c mouse model that both TFF3 peptide and mRNA were increased significantly (24, 98). As opposed to the finding in IBD patients, *TFF1* and *TFF2* mRNA were not changed significantly in DSS-induced colitis of mice, which indicates that DSS induced colitis might have an acute pathology, while IBD lesions exhibit a more chronic nature (98).

How TFF3 mediates its protective and healing effects on the epithelium is not fully understood. It has been proposed that these effects are mediated by cellular responses which might involve receptor-ligand interactions. In vitro experiments demonstrated that TFF3 enhanced the expression of decay-accelerating factor (DAF), which is another important mucosal defensive protein interrupting the complement activation pathways, and thus protects the tissues from autologous complement-mediated injury (99, 100). Andoh et al. (100) found that TFF3 induced an increase in DAF protein and mRNA expression in human and rat intestinal epithelial cells in normal conditions. It has been shown that TFF3 blocks complement C3 deposition. In HT-29 cells, TFF3 increased *DAF* mRNA stability and enhanced DAF promoter activity via NF-κB motif and induced activation of NF-κB-DNA binding activity. In summary, TFF3 promoted protection of epithelial cells from

complement-mediated damage via up-regulation of DAF expression contributing to a robust mucosal defense (100).

Due to the association between TFF dysregulation and IBD, the functions of TFF genes in colitis should be further investigated. Animal studies can be conducted to explore how the modulation of TFF genes would affect the incidence and development of colitis.

7. TNF-α as an inflammatory marker in DSS induced colitis

It is well documented that TNF-α is an inflammatory marker associated with DSS-induced colitis (87, 89, 90). It is a 17 kDa polypeptide produced by many cell types including activated monocytes and macrophages, neutrophils, T and B lymphocytes, mast cells, basophils, eosinophils, NK cells, endothelial cells, smooth muscle cells, and astrocytes (101). When macrophages or other cells are stimulated with LPS in the cell wall of Gram-negative bacteria, TNF-α is secreted as one of the earliest proinflammatory cytokines and enter the systematic circulation (102). It functions as a chemoattractant for neutrophils, and facilitates them to stick to the endothelial cells for migration. It enhances phagocytosis and stimulates production of IL-1 and prostaglandin E2 in macrophages. In the hypothalamus, as an endogenous pyrogen, it increases the thermoregulatory set-point in the hypothalamus and causes fever (103, 104). In the liver, TNF-α stimulates the acute phase response and increases protein synthesis, leading to an increase in C-reactive protein and a number of other mediators. The synthesis of TNF-α is induced by the activation of the transcription factor NF-κB. After activation, NF-κB translocates to the nucleus and induces gene transcription of

several cytokines, such as IL-1β, IL-2, IL-6, IL-8, IL-12, TNF-α, IFN-γ, and COX-2 (89, 102).

Reed et al. (89) investigated the temporal relationship between NF-κB activation and the expression of proinflammatory cytokines and other peptides in the rat DSS colitis model. Rats were given 5% DSS in their water and sacrificed daily for 6 days. NF-κB activation, TNF-α and other cytokines protein levels and gene expression were measured by electrophoretic mobility shift assay and relative RT-PCR, respectively. It was observed that NF-κB activation increased on day 1 of the DSS administration and again 3 days later. The level of *TNF*-α-specific mRNA rose significantly on day 5.

An study by Yan et al. (87) observed the dynamic changes of inflammatory parameters including TNF-α in the DSS-induced colitis mouse model. They found that the production of TNF-α increased progressively during the DSS treatment, and reached the maximum amount on the 5^{th} day. After withdrawing the DSS, TNF-α maintained a high level in the colon tissues until 2 weeks after the treatment, when the animals were sacrificed (87).

Egger et al. (105) demonstrated that the induction of TNF-α in acute murine DSS colitis was dose-dependent. In their study, four groups of BALB/c mice were given different concentrations of DSS (0, 2.5, 5 and 7.5%) in drinking water for 7 days ad libitum. Cytokine-specific mRNA levels in the colon tissues were determined by competitive quantitative RT-PCR. It was observed that gene expression for *TNF*-α was progressively upregulated with increasing dosage of DSS (105).

8. Probiotics and barrier function

Probiotics were first described by Lilly and Stillwell in 1965 (106). They are selective nonpathogenic living microorganisms, including some commensal bacterial species, that protect host health and may help prevent or/and treat some diseases (106). The World Health Organization (FAO/WHO, 2002) has defined probiotics as "living microorganisms which, when consumed in adequate amounts as part of food, confer a health benefit on the host." Certain *Lactobacillus, Bifidobacterium,* and *Saccharomyces* species are commonly utilized probiotics. *Lactobacillus* and *Bifidobacterium,* especially *Lactobacillus rhamnosus, L. acidophilus, Bifidobacterium longum,* and *B. bifidum* are the most commercially used species of lactic acid bacteria in products such as probiotic yogurts and probiotic pills (31, 107). Among these lactic acid bacteria, *L. rhamnosus* has been thoroughly investigated and has been shown to survive gastric, bile and intestinal secretion, adhere to the intestinal epithelium and colonize the colon after oral consumption in a 12-day human study (108). Probiotics have been found to enhance the health state of the GI tract and have been applied to prevent and treat several intestinal inflammatory disorders, such as necrotizing enterocolitis and IBD (31, 109-112). There are several studies about the beneficial effects of probiotics on IBD. Gupta et al. (113) observed that *L. rhamnosus* GG (LGG) was beneficial in children with Crohn's disease. In this study, four children with mildly to moderately active Crohn's disease were given 10^{10} colony-forming units of LGG in enterocoated tablets twice daily for 6 months. The results showed that LGG decreased the disease activity 1 week after the initiation of the administration and the protective effect was maintained during the study period. The double sugar permeability test suggested that the probiotic administration

improved intestinal permeability, which was consistent with the changes of the disease activity. Consistent with these observations, a meta-analysis study showed that LGG was effective in treating acute diarrhea in children (114). An animal study found that LGG administration decreased MPO activity in the TNBS model of colitis (115). Three distinct cellular and molecular mechanisms for probiotic regulation of IBD were demonstrated by Vanderpool et al. (31) in a recent review paper: i) Probiotics produce antibacterial substances and compete with pathogens and toxins for adherence to the intestinal epithelium and thus block pathogenic bacterial effects; ii) Probiotics regulate immune responses by modulating innate immunity and pathogen-induced inflammation via toll-like receptors mediated signaling pathways; iii) Probiotics regulate intestinal epithelial homeostasis by enhancing intestinal epithelium survival, promoting barrier function and stimulating protective responses.

Intestinal mucins and TFF are components of a complex regulatory network that acts as the first line of host defense against enteric pathogens. Abnormal regulation of this network might lead to inflammatory conditions of the intestine. There is evidence supporting that probiotics have certain effects on the modulation of intestinal barrier function. In healthy Wistar rats, it was observed that oral administration of the probiotics mixture VSL#3 containing 3 *Bifidobacterium* strains (*B. breve, B. longum,* and *B. infantis*), 4 *Lactobacillus* strains (*L. acidophilus, L. plantarum, L. paracasei,* and *L. bulgaricus*) and *Streptococcus thermophilus* on a daily basis for 7 days increased basal luminal mucin content by 60%. The isolated rat colonic loops that exposed to the probiotic formula had increased amount of mucin secretion and a higher gene expression of *MUC2*. Meanwhile, *MUC1* and *MUC3* gene

expression were slightly increased (116). Consistently, another study showed that LGG enhanced gastric mucosal integrity in Sprague-Dawley rats by upregulating *MUC6*-specific mRNA expression and increasing the mucus layer thickness (117). On the other hand, under inflammatory conditions, probiotic administration could normalize the abnormal stimulation of *MUC- or TFF-specific* mRNA expression. Khailova et al. (111) found that *B. bifidum* normalized the increased levels of MUC3 and TFF3 in a rat model of necrotizing enterocolitis. Another study also showed that oral administration of LGG reduced mucin gene expressions in the DSS induced colitis mouse model (118).

In summary, use of probiotics can inhibit the growth of pathogens, modulate the immune response, and enhance the barrier function of the gut, thereby promoting gut health. Thus, probiotic is beneficial in preventing and/or treating intestinal inflammation.

9. Dairy proteins and barrier function

Casein was originally defined in 1956 as those phosphoproteins that precipitate from raw skim milk by acidification to pH 4.6 at 20°C (119). According to the homology of their primary structures, casein was divided into four families, including α_{S1}-casein, α_{S2}-casein, β-casein and κ-casein (119). Casein accounts for nearly 80% of protein in cow's milk (120). It is used as the basic protein source in the AIN 93 rodent diet formulation (121).

Whey is obtained from the cheese-making process. It is the liquid remaining after milk has been curdled and strained to remove the casein (120). It contains water, proteins, lactose, vitamins, minerals, and traces of fat. Whey protein represents 20% of the total protein

content of cow's milk. It is used commercially as a nutritional supplement. Whey protein contains 5 major proteins, including 35% β-lactoglobulin, 12% α-lactalbumin, 12% glycomacropeptide, 12% proteose peptone, and 8% immunoglobulins, which together make up over 80% of the total whey protein. It also contains 5% serum albumin, 1% lactoferrin, 0.5% lactoperoxidase, and some minor proteins (120).

Whey protein contains bioactive components such as glycomacropeptide and lactoferrin, which may be beneficial in preventing colitis (122, 123). Glycomacropeptide and lactoferrin also have prebiotic properties that have been shown to support the growth of bifidobacteria and lactobacilli and thereafter promote gut health (124). An in vivo study found that adding 16% cheese whey protein in the diet significantly increased the amount of lactobacilli and bifidobacteria in fecal samples of the rats after 3 weeks (125). In addition, whey protein is a rich source of threonine, cysteine, serine and a certain amount of proline, which are the major amino acid constituents of GI secretory mucins (126).

Faure et al. (127) found that the supplementation of threonine, cysteine, serine and a certain amount of proline increased the production of mucins and ameliorated histological changes in DSS-induced colitis in rats. From 8 days before to 28 days after induction of colitis, male Sprague-Dawley rats were fed a control diet or diets supplemented with 2 different doses of an amino acid mixture containing L-threonine, L-serine, L-proline, and L-cysteine. It was found that the higher dose of amino acids increased the number of MUC2-containing goblet cells in the surface epithelium of the ulcerated area, enhanced mucin production in the colon by 95% and improved histological scores. Recently, the protective effects of whey protein against DSS-induced colitis were explored with respect to how whey protein changed the

microbiota and mucin (125). During this study, colitis was induced by 3% DSS treatment for 7 days. Rats were divided into three diet groups containing casein, cheese whey protein, or casein supplemented with threonine and cysteine. Whey protein reduced DSS-induced mucosal gene expression of inflammatory markers such as IL-1β, calprotectin, and inducible nitric oxide synthase and relieved the clinical signs of diarrhea and hematochezia. Fecal mucin secretion was increased by whey protein without altering MUC2 gene expression. In addition, whey protein increased the number of lactobacilli and bifidobacteria in fecal samples. The rats fed the casein supplemented with threonine and cysteine diet exhibited similar effects. This study suggested that whey protein provided protection against colitis via enhancing mucin synthesis and stimulating the survival of the probiotics (125).

10. Soy protein and barrier function

Soybean has been considered a good source of protein throughout history. There is approximately 50% protein in the soybean. Soybean protein provides all of the essential amino acids for human nutrition, thus, it is called a "complete protein". Edible soy protein is derived from defatted soy flour and has a high solubility in water (128). Several studies have shown that soy protein isolate with isoflavones is protective against colon cancer, although the mechanisms are not well defined (129, 130). Vis et al. (131) observed that soy protein influenced several colon cancer risk parameters such as cell proliferation, fecal fat, fecal bile acid, and alkaline phosphatase in a rat model. Cai et al. (132) examined the effects of soy protein on the intestinal barrier function. Acute enteritis was induced by radiation in Sprague-Dawley rats. Then the rats were assigned to two groups fed either soy protein or casein

protein. Bacterial translocation rates, lipopolysacharide (LPS) concentrations in serum, and mucosal thickness were analyzed. It was found that soy protein decreased bacterial translocation, LPS levels in the circulation, and improved mucosa repair suggesting that soybean protein alleviated radiation damage and protected the bowel barrier function in rats.

Many of the health benefits of soy protein attribute to its isoflavones, which are a family of phytoestrogens found mainly in soybeans. Soy contains many types of isoflavones, but the most beneficial are genistein and daidzein (133). In vitro studies found that genistein inhibited the proliferation of colon cancer cells and promoted the apoptosis of these cells (134, 135). An animal study investigated the effect of genistein on IBD with the TNBS induced chronic colitis rat model (136). They found that 14-day oral administration of 100 mg/kg genistein reduced colonic expression of COX-2 mRNA and protein and decreased MPO activity compared to the control group. These results suggested that oral administration of genistein exerted anti-inflammatory effects in experimental colitis animals (136).

Recently, a bioactive peptide named lunasin in soy protein has been isolated. Lunasin is composed of 43 amino acids residues and a predicted helix with structural homology to a conserved region of chromatin binding protein (137). Lunasin has been found to be bioavailable in humans. A human study showed that amino acid sequences from lunasin were present in plasma samples after soy intake for 30 min and 1 h, whereas no peptides from lunasin were detected in plasma samples without soy intake (138).

It has been found that lunasin had anti-inflammation effects via inhibiting the NF-κB pathway in the macrophages (139, 140). In vitro study by De Mejia et al. (139) found that lunasin and lunasin-like peptide purified from defatted soybean flour inhibited the pro-

inflammatory markers such as IL-1β, IL-6, NF-κB transactivation, COX-2 expression, and nitric oxide synthase expression in RAW 264.7 macrophages. Another in vitro experiment by Hernández-Ledesma et al. (140) used LPS-stimulated RAW 264.7 macrophages to study the anti-inflammatory effects of lunasin. They observed that lunasin reduced the production of reactive oxygen species (ROS) by LPS-induced macrophages in a significant dose-dependent manner and inhibited the release of pro-inflammatory cytokines TNF-α and IL-6. These results supported the conclusion that lunasin had anti-inflammation properties and may explain some of the GI health benefits of soy protein.

It has also been proposed that soy protein may influence the growth of probiotics. Soy products have been given much attention lately as dairy replacers and carriers for probiotics, without the lactose intolerance factors. For instance, soy yogurt is made by fermenting soymilk with probiotics, mainly *L. bulgaricus* and *S. thermophilus* (141, 142). Tsinberg et al. (143) studied the growth features of 3 strains of *Bifidobacterium* and 3 strains of *Lactobacillus* in hydrolysate-milk or hydrolysate-soybean media (HMM and HSM, respectively) comparatively. The bacterial cells were investigated morphometrically with atomic force microscopy. They found that HSM promoted a higher growth rate of the strains than HMM, and this effect was more obvious for the bifidobacteria as compared to the lactobacilli (143). However, a later study that determined the utilization of lactose and production of organic acid in reconstituted skim milk (RSM) and RSM supplemented with soy protein isolate (RSMS) had different findings (144). Six probiotic organisms were grown in the two media, including 4 strains of *Lactobacillus* and 2 strains of *Bifidobacteria*. The results were that the viable microbial populations in RSMS were lower than those in RSM

due to lower pH of the former, even though all 6 probiotic organisms produced significantly more acetic acid in RSMS than RSM. This study suggested that the addition of soy protein enhanced lactose utilization and acetic acid production but did not increase the growth of probiotic microorganisms (144).

11. Hypothesis

Intestinal mucins and TFF compose a network that acts as the first line of host defense against enteric pathogens. Dysregulation of *MUC* and *TFF* genes were found in IBD patients and DSS-induced colitis models. Previous studies supported the concept that probiotics can modulate the mucin secretion and mucin gene expression in healthy subjects or subjects with active inflammatory conditions. Whey protein and soy protein have the potential to stimulate the growth of probiotic organisms and/or regulate the *MUC* and *TFF* gene expression in vitro and in vivo. Hence, we hypothesized that probiotic administration would enhance colonic barrier function via modulating mucin and *TFF3* gene expression in the DSS-induced colitis mouse model. Casein, whey, or soy diets would differentially affect the colonic barrier function directly by modulating mucin gene expression and TFF3 and/or by enhancing the growth of the probiotic organism.

CHAPTER 2

Reduction in mucosal barrier markers with soy protein diet but not *Lactobacillus rhamnosus* GG in DSS-treated mice

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Abstract

The incidence of inflammatory bowel diseases (IBD) has increased during recent decades. Within the colon, the family of mucins and trefoil factors (TFF) facilitate mucosal protection. Probiotic administration influences the intestinal mucin layer. Additionally, food components may affect gut microflora or have direct effects on the mucin barrier. Our objective was to determine if diet and/or administration of *Lactobacillus rhamnosus* GG (LGG) would attenuate dextran sulfate sodium (DSS)-induced colitis by altering expression of the *MUC* and *TFF* genes. C57BL/6 mice were fed diets containing casein, soy, or whey protein along with or without LGG for 12 days. Seven days after starting LGG diets, the mice were given 2% DSS for 4 days. One day after the DSS treatment the colonic and cecal tissues and fecal samples were collected and analyzed by quantitative RT-PCR for MUC, TFF genes and LGG specific gene. Whey protein significantly increased cecal LGG content compared to the other diets. *MUC1* gene expression was induced by DSS and diets containing soy protein

dramatically suppressed the induction. Colonic TFF3-specific mRNA was upregulated by DSS and decreased by soy and whey protein. Soy protein also reduced the inflammatory scores, TNF-α expression and prevented colon shortening. There was no overall effect of LGG on these parameters. In conclusion, soy protein suppressed the DSS-induced inflammatory stimulation of *MUC*, *TFF* and *TNF*-α gene expression independently of the probiotic. Whey protein may act as prebiotic to increase microbial survival, but failed to prevent the inflammatory response. Hence diet but not probiotic affected the colonic mucosal barrier in DSS-treated mice.

Key words: IBD, MUC, TFF, probiotic, soy protein, whey protein

Introduction

Inflammatory bowel diseases (IBD) are a group of chronic inflammatory conditions of the small and large intestine, and includes two major diseases, Crohn's disease and ulcerative colitis (31). In 2004, as many as 1.4 million persons in the United States and 2.2 million persons in Europe suffered from IBD (29). The incidence of IBD has continued to increase during recent decades, not only in the Western countries, but also in some regions where IBD had been less common such as Japan (30). There is evidence that patients with ulcerative colitis and Crohn's disease have a higher risk of developing intestinal cancer, even though the mechanisms have not been fully understood (34-37). O'Connor et al. (38) demonstrated that IBD patients were exposed to constant activation of nuclear factor-kappaB (NF-κB) and cyclooxygenase-2/prostaglandin pathways, release of proinflammatory cytokines, and increased local levels of reactive oxygen and nitrogen species. Collectively, the chronic presence of these inflammatory signals may have carcinogenic effects (38).

Even though the pathogenesis of IBD is not fully elucidated, there is evidence that it involves interactions between genetic susceptibility, aberrant activation of the immune system, and the environment (40). A number of environmental risk factors for IBD have been explored, including smoking, appendectomy, oral contraceptives, diet, breastfeeding, infections, antibiotics, and degree of childhood hygiene (55). Migrant studies have clearly demonstrated that lifestyle and environmental influences are more important to IBD etiology than regional, ethnic and racial differences as evidenced by the increased risk of IBD for individuals moving from low-occurrence areas to high-occurrence areas (29).

The DSS-induced colitis model originally reported by Okayasu et al. (81) has been used to investigate colitis. This model is easy to develop, and the characteristics are very similar to those of ulcerative colitis patients. Inflammatory parameters for DSS-induced colitis include increased myeloperoxidase (MPO) activity, proinflammatory cytokines such as IL-1β, IL-6, TNF-α, IFN-γ, and histological scores based on the severity of mucosal epithelial damage, glandular alterations, and the character of lamina propria cellular infiltration (87, 91).

The mucous layer overlying colonic epithelial cells consists of mucus glycoproteins (mucins or MUC) and trefoil factors (TFF) that are secreted by goblet cells is a first defense barrier between bacteria and the mucosal cells in the gut (14, 15, 17, 18). In the presence of a functional barrier, pathogens and antigens cannot gain access to the underlying epithelium and are thereby blocked from invading the body (2).

The MUC1, MUC2, MUC3 and MUC4 glycoproteins are the major mucins expressed in the large intestine in humans. In particular, MUC2 is the main secreted colonic mucin (23). Loss of this protective barrier may predispose to IBD. For instance, *MUC2* knockout mice

spontaneously developed colitis, indicating that the presences of *MUC2* was important for colonic protection (25). Overexpression of *MUC1*-specific mRNA was found in IBD patients (92) and *MUC1* and *MUC4* gene expression were enhanced significantly in the mouse model of DSS-induced colitis (24).

The TFF1, TFF2, and TFF3 proteins are produced by mucus-secreting cells, mainly in the gastrointestinal (GI) tract, with specific cellular localization of each peptide. TFF3 is mainly located in goblet cells of the small and large intestine (26). TFF3 is a key peptide in mucosal protection and repair. It interacts with mucin glycoproteins to enhance the protective epithelial barrier function. Knockout of the *TFF3* gene led to an increased susceptibility to colitis after exposure to DSS, and intraluminal infusion of TFF3 rescued the TFF3 deficiency through coordinated promotion of intestinal cell migration and inhibition of intestinal cell apoptosis (27). In contrast, overproduction of TFF3 is found in a variety of GI inflammatory conditions, such as necrotizing enterocolitis and IBD, which suggests that upregulation of *TFF3*-specific mRNA is a feature of GI inflammation (28).

Probiotics have certain effects on the modulation of intestinal barrier function. Gupta et al. (113) observed that *L. rhamnosus* GG (LGG) was beneficial in children with Crohn's disease. In healthy Wistar rats, oral administration of the probiotics mixture VSL#3 on a daily basis for 7 days increased the amount of mucin secretion and a higher gene expression of *MUC1, MUC2,* and *MUC3* in the colon (116). However, under inflammatory conditions, probiotic administration normalized the abnormal regulation of *MUC*- or *TFF*-specific mRNA (111, 145).

Whey protein contains bioactive components such as glycomacropeptide and lactoferrin, which have prebiotic properties to support the growth of bifidobacteria and lactobacilli and subsequently promote gut health (124). Soy products have been given much attention lately as dairy replacers and carriers for probiotics, without the lactose intolerance factors (142). It has been suggested that soy protein may promote the growth of *Bifidobacteria* and *Lactobacillus* species (143). In addition, an animal study demonstrated the ability of soy proteins to enhance intestinal barrier function (132).

Based on the interactions between the mucus barrier, use of probiotics, and different protein supplements, we hypothesized that probiotic administration would enhance colonic barrier function via modulating *MUC* and *TFF* gene expression in the murine model of DSS induced colitis. Furthermore, we hypothesize that casein, whey, or soy protein supplemented diets would differentially affect the colonic barrier function by modulating *MUC* gene expression and *TFF* and/or by enhancing the growth of the probiotic organism(s).

Materials and Methods

Animals and diet

All animal protocols were approved by the Iowa State University Animal Care and Use Committee. Female C57BL/6NHsd *Helicobacter* negative mice, aged 6-7 weeks (Harlan, Haslett, MI) were housed individually, exposed to 12 h light and dark cycles and given free access to tap water. Casein and whey protein isolates will be purchased from commercial sources and the soy protein, depleted of isoflavones will be obtained from the Archer Daniels Midland Company. Powdered diets were prepared using a modification to the standard AIN-

93G formula (121) as shown in **Table 1** and were stored frozen. Immediately prior to feeding each day, the diets were quantitatively mixed with water to form a thick paste. This was done to assure survivability of the probiotic. Body weight and food intake were monitored daily.

Table 1 Diet compositions

	Source	casein[1]	soy	whey
			g/kg	
Casein	MP Biomedicals	200	0	0
Whey protein	Leprino Foods, Inc.	0	0	250
Soy protein concentrate	Archer Daniels Midland	0	275	0
DL-methionine[2]	MP Biomedicals	3	3	3
Sucrose	General Stores	100	100	100
Corn Starch	MP Biomedicals	397.5	322.5	347.5
Dyetrose	Dyets, Inc.	132	132	132
Cellulose[3]	General Stores	50	50	50
Safflower oil[4]	MP Biomedicals	20	20	20
Corn oil[4]	General Stores	50	50	50
AIN-93VX vitamin mix	MP Biomedicals	10	10	10
AIN-93G mineral mix	MP Biomedicals	35	35	35
Choline bitartrate	MP Biomedicals	2.5	2.5	2.5
Energy content, kcal/kg		3868	3746	3933
Nitrogen content, g/kg		30.4	30.4	30.4

[1]same as for AIN-93G with the exceptions noted in superscripts below and that tert-butyl-hydroquinone was omitted (diets stored at -15ºC).

[2]used instead of L-cystine

[3]used instead of Solka-Floc® fiber

[4]used instead of 70 g/kg soybean oil

Bacterial growth conditions

Lactobacillus rhamnosus GG ATCC 53103 was acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA) as a lyophilized sample and maintained as frozen stocks at −75°C in deMan-Rogosa-Sharpe medium (MRS, #288130, BD Diagnostic Systems, Sparks, MD) containing 20% (v/v) glycerol. Working cultures of *L. rhamnosus* ATCC 53103 were maintained as slants on MRS at 4°C, and sub-cultured in MRS broth at 37°C under stationary culture conditions. During each day of the feeding trial, 100 mL of the bacterial cell suspension containing 3 x 10^{10} CFU/mL was prepared by inoculating 100 mL of MRS in a 250 mL Erlenmeyer flask and incubating overnight at 37°C under stationary conditions.

The culture broth was centrifuged (12,000 x g, at 4°C for 10 min) and cell pellet was washed twice with phosphate buffered saline (PBS) to remove traces of nutrient medium. One gram of bacterial cell pellet was re-suspended aseptically into 5 mL of PBS. From this bacterial suspension, 100 µL (20 mg of bacterial pellet) was pipetted onto the surface of each moistened diet placed in a plastic disposable 10 mL beaker and then fed the mice. Cell counts of the bacterial suspension were taken half-way through the bacterial feeding period and on the last day of the bacterial treatment showed 6 x 10^{11} CFU/mL and 3 x 10^{11} CFU/mL, respectively.

Treatments

Mice were acclimated for 5 d to meal-fed training with casein, soy, or whey protein diets. On day 6, each diet group was divided into two groups placed in separate rooms: one for those given *L. rhamnosus* GG (LGG) and the other room for a saline control added daily to the surface of diets freshly moistened by mixing 0.4 g, 1.1 g, or 0.4 g tap water per gram of diet

to casein, soy, or whey powdered diets, respectively for 12 d. Diets were given during the first 5-6 h of the dark cycle. To prevent bacterial contamination of the control group, lab coats, gloves, and animal caretakers were dedicated to each room. Seven days after starting the LGG diets, mice were given drinking water without or with 2% dextran sodium sulfate (DSS, #160110, MP Biomedicals, Solon, OH) that was prepared fresh daily. After the 4-day DSS treatment period, the DSS solution was replaced with water for 24 h and then mice were euthanized by carbon dioxide asphyxiation. The colon and cecum were removed and the colon length was measured. The colon was flushed with PBS, weighed and prepared for histology or RNA extraction and stored at -80ºC. The cecum was weighed and the cecal contents were removed and both were stored at -80ºC for RNA or DNA extraction.

Histological staining and goblet cell counts

Half of the longitudinal distal colon was fixed in 10% neutral buffered formalin overnight. Specimens were placed in 70% ethanol until embedded in paraffin, sectioned at 7µm and stained with alcian blue as previously described (146). Briefly, deparaffinized and hydrated slides were soaked in 1% alcian blue with 3% acetic acid for 30 min then rinsed in 3% acetic acid, washed in slow-running water and counterstained for 5 min in Kernechtrot stain (0.1% nuclear fast red in 5% aluminum sulfate) before dehydrating and mounting with acrytol mounting media (Surgipath, Richmond, IL). To determine the number of goblet cells per colonic crypt, the numbers of alcian blue-stained cells of 4-6 crypts for each of 2-4 regions of the colon slide were counted under light microscopy at 400x magnification and the results were expressed as the mean value per crypt.

Colon histological measurements and colitis scoring

Colon tissue showed the typical 3-layer morphology with the crypt layer (from the luminal end of the crypt to the surface of the thin basal muscle layer) bordered on the basal part with a thin muscle layer then a "space" sometimes filled with immune cells defined as the inflammatory layer (from the surface of the thin basal muscle layer under the crypts to the surface of the lower thicker muscle layer) and lastly, a thicker muscle layer. The thicknesses of these layers in 2-4 regions of the colon slide for each mouse were measured at 400x magnification, using AxioVision 4.6.3 software and expressed as the mean ± SE per experimental group.

Colitis was scored over the whole colon slide by an observer blinded to the treatment groups according the morphological criteria described by Suzuki et al. (147): 0 = normal colon mucosa; 1 = shortening the basal 1/3 of crypts with slight inflammation and edema in the lamina propria; 2 = loss of the basal two-thirds of the crypts with moderate inflammation in the lamina propria; 3 = loss of all the crypts with severe inflammation in the lamina propria, but with the surface epithelium still remaining; 4 = a loss of all the crypts and surface epithelium with severe inflammation in the mucosa, muscularis propria and submucosa.

RNA isolation

Distal pieces (0.5 cm) of colon and cecum were placed in 1 mL RNALater® (Applied Biosystems, Foster City, CA) at 4ºC overnight, removed from RNALater® and stored at -80ºC. Total RNA was isolated using the Trizol method with RNAse-free reagents. Briefly, tissue was homogenized in 1 mL Trizol (Invitrogen, Carlsbad, CA) with an Omni homogenizer (#TH115, Omni International, Kennesaw, GA), mixed with 0.2 mL chloroform, centrifuged 12,000 x g for 15 min and the RNA was precipitated from the aqueous layer

using 0.5 mL isopropanol followed by washing twice with 1 mL iced 75% ethanol. The resulting RNA was dissolved in 50 µL nuclease-free water, heated for 5 min to 65ºC, treated with a TURBO DNA free kit (# AM1907, Applied Biosystems, Foster City, CA) according to the manufacturer's instructions, diluted 1:5 with nuclease-free water and RNaseOUT® (Invitrogen, Carlsbad, CA) and stored at 4ºC. The concentration of RNA was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

DNA extraction from cecal contents

DNA was extracted from cecal contents using the UltraClean® Fecal DNA Isolation Kit (#12811-100, MoBio Laboratories, Inc. Carlsbad, CA) with the following modifications to the manufacturer's instructions. Before step 5, proteinase K (MoBio #1222-2) was added and heated to 55ºC for 1 hour and, in step 12, Solution CB3 (MoBio #12240-50-3) was used instead of solution S3. The concentration of extracted DNA (stored at 4ºC) was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Quantitative real-time polymerase chain reaction

Primers mixed with TaqMan® probes in a kit for real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) for the following mouse genes were obtained commercially (TaqMan® Gene Assay, Applied Biosystems, Foster City, CA): trefoil factor-2 (TFF2, # Mm00447491_m1), intestinal trefoil factor-3 (TFF3, # Mm00495590_m1), transmembrane mucin-1 (Muc1, # Mm00449604_m1), mucin-2 (Muc2, # Mm00458299_m1), intestinal mucin-3 (Muc3, # Mm01207064_m1), mucin-4 (Muc4, # Mm00466886_m1), keratin 19 (Krt19, # Mm00492980_m1), tumor necrosis factor-alpha (TNF-α, #

Mm99999068_m1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, # Mm99999915g1). Each 20 µL PCR reaction was performed in duplicate and consisted of 1.766 ng RNA, 125 nM probe, 450 nM forward and 450 nM reverse primers and used qScript One-Step Fast MGB qRT-PCR master mix (low ROX) kit (# 95084, Quanta Biosciences, Gaithersburg, MD) according to manufacturer's instructions. Real time RT-qPCR was performed using a Stratagene Mx3005P™ QPCR system (Agilent Technologies, Inc, Santa Clara, CA) and the following cycling conditions: cDNA synthesis for 5 min at 50ºC, denaturation for 30 s at 95°C, amplification for 50 cycles at 95°C for 3 s and at 60°C for 30 s. To determine the optimal RNA concentration for RT-qPCR reactions, a preliminary dilution study was performed from 1:21 to 1:50,000 on a mixture of all mouse RNA samples to determine the best range for each target standard curve. Calculations for the optimal dilution of each RNA sample and each final target standard curve was accomplished using PREXCEL-Q software (148). To normalize expression to epithelial cells (Larouche-2010), amplification of target mRNA was expressed relative to amplification of the keratin-19 (Krt19) gene using the $2^{-\Delta Ct}$ method.

Quantitative bacterial PCR

The choice of probe and primers specific to the LGG is based on Ahlroos and Tynkkynen (149), who isolated and sequenced a unique random amplified polymorphic DNA band from LGG. This band is identified from a NCBI BLAST search as LGG insertion sequence ISLrh1 transposase gene (GenBank: EU709012.1). To distinguish this gene as from the GG and not the 705 *Lea rhamnosus* strain and to choose an MGB probe, Primer Express 2.0 software (Applied Biosystems, Foster City, CA) was used over the area where there was a difference

in the SNP sequence between the two strains. The real time qPCR of LGG was as above, except that 0.0776 ng DNA was used in each sample PCR reaction with the following probes and primers specific to this strain and synthesized commercially (Applied Biosystems, Foster City, CA): probe (6FAM-ATCGCGCTTGAACG-MGB), forward primer (5'-AAGTTCGCTTTTATGGGTAATGGA-3') and reverse primer (5'-GAAGGACTGCACGGTGTCACT-3'). Amplification of the target gene was expressed as relative DNA expression using the $2^{-\Delta Ct}$ method.

Statistical analysis

All data are expressed as means ± SE. Data was analyzed by individual F-tests for linear contrasts of means to assess the effects of diet, treatment, bacteria and the interactions of these factors on gene expression using the SAS GLM procedure and significant individual posthoc comparisons ($P < 0.05$) are indicated by groups with different letters.

Results

Mice were trained to meal feed in order to ensure complete intake of viable probiotic bacteria. The mice tolerated the experimental diets well and there was no significant difference in food intake among experimental groups (data not shown). Without LGG, mice fed the casein diet and treated with DSS had lower final body weight compared to those not treated with DSS (**Figure 1**). However, this effect was ameliorated with the addition of the probiotic to the diet. Comparing the final body weights of mice treated with DSS to the casein group not treated with DSS, casein and whey protein diets resulted in lower body weights while soy protein diets did not. Addition of the probiotic to these diets also prevented

the decrease in final body weight.

Colon length was reduced in mice fed the casein diet and treated with DSS compared to those not treated with DSS (**Figure 2**). Addition of the probiotic to the casein diet did not prevent the shortening of the colon in these animals. Within mice treated with DSS, colon length was greater in mice fed the soy protein diet compared to either casein or whey. The colon length of the mice fed the soy protein diet was not different from the casein fed mice without DSS treatment. Addition of probiotic had no effect on colon length for any treatment group.

Quantification of LGG in fecal samples

LGG was detected in the cecal contents of all mice fed LGG but none of those not given the probiotic (**Table 2**). Mice fed the whey protein diet had significantly higher LGG quantity compared to mice fed the casein or soy protein diets, but there was no significant difference between mice fed soy and those fed casein. DSS treatment had no effect on expression of LGG in the mice.

Colonic histological study

In colons of mice fed casein, DSS treatment produced an increase in most of the inflammatory parameters and addition of probiotic to the diet did not affect this response (**Table 3**). Among mice treated with DSS, casein and whey diets tended to produce similar changes in the colon with no differences due to probiotic feeding. One exception to this observation was that mice fed whey protein had significantly lower mucus layer thickness compared to the casein fed animals when both were fed the probiotic. In contrast, mice fed soy protein and treated with DSS tended to have colon inflammatory measures similar to the

casein fed mice without DSS, and this was similar with or without probiotic (**Figure 3**). Also, mice fed the soy protein diet without probiotic had significantly higher goblet cells per crypt compared to the other treatment groups (without probiotic). This effect of soy protein was not present when comparing within animals fed the probiotic.

MUC gene expression in colon and cecum

In mice fed casein, *MUC1* gene expression was increased by DSS treatment (**Figure 4A**). Among the mice treated with DSS, casein and whey protein diets resulted in similar levels of MUC1-specific mRNA expression while those fed soy protein had significantly ($p < 0.05$) lower levels. Addition of probiotic to the diets did not influence *MUC1* gene expression. In the cecum, DSS also induced *MUC1* gene expression in mice fed casein (**Figure 4B**). After DSS treatment, *MUC1* gene expression was similar in mice fed the three dietary proteins, although the soy fed animals had levels that were not statistically different from the casein fed animals without DSS. Among the *MUC2, MUC3* and *MUC4* genes, expression was not significantly altered by DSS treatment in mice fed casein in either colon or cecum (**Table 4**). Overall, diet and probiotic treatment had little effect on *MUC2*, 3 or 4 gene expression in the colon or cecum. However, *MUC2* and *MUC4* expression were significantly higher in colon of mice fed soy protein compared to casein when neither group received the probiotic. And in the cecum, *MUC3* expression was higher in mice fed soy protein compared to whey when no probiotic was fed. When the mice were given the probiotic, diet had no effect on *MUC2*, 3 or 4 expression in the colon, but in the cecum, expression of *MUC3* was less in mice fed casein compared to soy or whey.

TFF gene expression in colon and cecum

In mice fed casein, adding the probiotic to the diet significantly increased expression of *TFF3* in the colon (**Figure 5A**). DSS treatment also increased *TFF3* in casein fed animals, and the increase was greater when the mice consumed the probiotic. However, after DSS treatment *TFF3* expression in the colon was lower in mice fed soy or whey protein compared to casein, and there was no effect of the probiotic in these animals. The expression of *TFF3* in the animals fed soy or whey protein and treated with DSS was not different from that in mice fed casein without DSS treatment. A different pattern in *TFF3* expression was observed in the cecum with lower expression following DSS treatment in the casein fed animals and a further decrease in mice fed the probiotic with DSS (**Figure 5B**). In DSS treated mice fed the soy or whey protein diets, *TFF3* expression in the cecum was not affected by the probiotic and levels were not different from the casein fed animals without DSS. *TFF2* gene expression was not affected by DSS, diet or probiotic in the colon, however, in the cecum it was increased by probiotic feeding in all diet groups (data not shown).

TNF-α gene expression in colon and cecum

TNF-α gene expression was significantly higher in casein fed animals following DSS treatment in both the colon (**Figure 6A**) and the cecum (**Figure 6B**). In mice treated with DSS and fed soy protein however, *TNF-α* expression was lower than in mice fed casein or whey. Addition of the probiotic to the diet only affected expression of *TNF-α* in the cecum of mice fed whey protein.

Comparisons of MUC, TFF genes in control and DSS groups in colon and cecum

The relative gene expression of the inflammatory markers in colonic (**Figure 7A**) and cecal tissue (**Figure 7B**) in response to DSS were compared. In colon of mice not treated with DSS, *MUC3* was the most abundant, followed by *MUC2*, *MUC4*, and *MUC1*. *TFF2* was poorly expressed, whereas *TFF3* was abundant. *CK19* and *GAPDH* were similarly expressed in colon and cecum. With DSS treatment, colonic expression of *MUC1*, *TFF3*, and *TNF-α* were significantly increased ($p < 0.05$), while *MUC3* was suppressed. In the cecum, only *MUC1* and *TNF-α* were increased with DSS while *TFF3* and *CK19* were suppressed.

Discussion

Given the current increase in IBD in the United States, finding dietary interventions to reduce or alleviate symptoms is urgently needed. It is becoming increasingly evident that dietary factors and colonic microbiota play significant roles in modulating symptoms of IBD and that there are complex interactions between dietary components and microbial populations. Hence, it is of value to carry out research that combines studies of microbial and dietary interventions in models of IBD. To clarify these interactions, we quantified the probiotic population in the lower bowel following oral supplementation in animals fed different sources of protein. Whey protein resulted in a four-fold higher expression of LGG in the cecum compared to casein, which was consistent with previous findings by Sprong et al. (125). They found that cheese whey protein administration increased the amount of *Lactobacillus* and *Bifidobacteria* in fecal samples of rats compared to casein. This effect may be attributed to certain components in the whey protein, such as glycomacropeptide or lactoferrin, which are not present in casein. A previous study found that they supported the growth of *Bifidobacteria* and *Lactobacillus* and suggested support of gut health (124). In

addition, threonine and cysteine that are abundant in whey protein were proved to promote the growth of *Bifidobacteria* and *Lactobacillus* in rats (125). Soy protein did not support retention of LGG in the present study. Recent reports about the prebiotic potential of soy protein are controversial. Tsinberg et al. (143) found that hydrolysate-soybean media promoted a higher growth rate of the *Bifidobacteria* and *Lactobacillus*, whereas Pham et al. (144) observed that the addition of soy protein did not increase the growth of probiotic microorganisms. It is clear that soy protein are not uniform in their ability to support the growth of probiotics, hence a careful, systematic assessment of soy protein and probiotic interactions is needed and illustrates the importance of quantifying probiotic survival.

The DSS induced colitis model applied in the present study is a well-established and generally accepted model for human colitis. In general, DSS treatment causes weight loss, diarrhea, shortening of the colon, erosion of the mucosal epithelium, and acute neutrophilic infiltration (81, 87). TNF-α is also a well-documented inflammatory marker that is increased in DSS-induced colitis models (87, 89, 90). In our study, the animals were euthanized within 24 hours after ending DSS treatment, hence we examined very early changes in intestinal response. Even at this early phase, colon length was significantly reduced by DSS in mice fed casein or whey. Additionally, the proinflammatory cytokine TNF-α was induced in both colon and cecum in mice fed casein or whey, which further confirmed colitis in these DSS treated mice. The soy fed mice, in contrast, did not experience these changes, suggesting an anti-inflammatory effect of soy protein, which is in concordance with previous studies (139, 140). The isoflavones in soybean were found to exert anti-inflammatory effects, including reducing colonic expression of COX-2 mRNA and protein and decreasing MPO activity in

TNBS induced colitis animal model (136). However, the soy protein we used was depleted of isoflavones, therefore we may exclude the contribution of isoflavones to these anti-inflammatory effects. De Mejia et al. (139) found that a bioactive peptide named lunasin, purified from defatted soybean flour, inhibited the pro-inflammatory markers such as IL-1β, IL-6, NF-κB transactivation, COX-2 expression, and nitric oxide synthase expression in RAW 264.7 macrophages. Similarly, Hernández-Ledesma et al. (140) used LPS-stimulated RAW 264.7 macrophages to study the anti-inflammatory effects of lunasin. They observed that lunasin reduced the production of reactive oxygen species (ROS) by LPS-induced macrophages in a significant dose-dependent manner and inhibited the release of pro-inflammatory cytokines TNF-α and IL-6.

Our working hypothesis was that diet and/or probiotic bacterium feeding could generate a protective effect in the IBD model through regulation of the mucin and trefoil factor genes. These factors have been implicated previously to be critical in the progression and severity of IBD. Mucins are a family of high molecular weight, heavily glycosylated proteins produced by goblet cells. The mucous layer lining the surface epithelium of the intestine acts as a lubricant and protective physical barrier between the mucosal surface and the luminal contents (2). *MUC1*, *MUC2*, *MUC3* and *MUC4* are the major mucins expressed in the large intestine in humans (23). The mucin genes in mice are similar to human. It has been found in the BALB/C mice that *MUC3* is the most commonly expressed mucin in the large intestine, followed by *MUC2*. In contrast, *MUC1* and *MUC4* are poorly expressed (24). The trefoil factor (TFF) peptides are a family of small protease-resistant proteins characterized by one or more trefoil motifs (150). *TFF1*, *TFF2*, and *TFF3* are expressed in mucus-secreting cells,

mainly in the GI tract, with specific cellular localization of each peptide. *TFF1* is expressed in surface epithelial cells of the stomach, and *TFF2* in mucous neck cells, pyloric glands, and Brunner's glands, whereas *TFF3* is mainly located in goblet cells of the small and large intestine (26). As is well known, the mucous layer that consists of mucins and trefoil factors secreted by goblet cells forms the first barrier against pathogens in the gut (19, 20). MUC and TFF genes are critical for protecting the intestine from inflammation. In addition, *MUC1* plays a role in regulating the cell growth and the differentiation via the mitogen-activated protein kinase pathway (151). In vitro experiments demonstrated that TFF3 enhanced the expression of decay-accelerating factor (DAF), which is another important mucosal defensive protein interrupting the complement activation pathways, and thus protected the tissues from autologous complement injury (99, 100). The upregulation of these genes in the early stage of acute colitis indicates the role of MUC1 and TFF3 in the response to injury and maintaining the mucosal barrier integrity (24).

Among the MUC genes, *MUC1* is most associated with IBD. Overexpression of *MUC1* was found in the serum and colon biopsies of IBD patients and increased MUC1 levels correlated with clinical disease severity (92). Additionally, in BALB/C mice, *MUC1* gene expression was enhanced significantly by DSS treatment (24). In these animals, alteration of *MUC1* suggested that mucin production was adapted to maintain and recover the protective capacities of the mucus layer, which were changed during experimental colitis (24). In agreement with these findings, we found DSS increased *MUC1* gene expression in both the colon and the cecum. This suggested that in the inflammatory condition caused by DSS treatment, a compensation of *MUC1* gene expression was stimulated in order to maintain the

protective capacities of the mucus layer and prevent the colon from further damage.

In the casein fed mice, *MUC2*, *MUC3* and *MUC4* gene expression was not altered by DSS treatment in either colon or cecum in the present study. This is in contrast to previous findings. *MUC2* in the colon was increased following 5% DSS treatment for 9 days in Sprague Dawley rats (93). In Wistar rats treated with 7% DSS for 7 days, colonic *MUC2* started to increase 2 days after the DSS treatment, and the increase was maintained during the regenerative phase only in the proximal colon (94). However, in BALB/C mice *MUC2* was not changed following a 5 day treatment with 1% DSS when the mice were euthanized right after the DSS treatment (24). *MUC3* and *MUC4* were increased the day after a 5-day 1% DSS treatment in BALB/C mice and 9-day 5% DSS treatment in Sprague Dawley rats (24, 93). These discrepancies in *MUC2*, *MUC3* and *MUC4* response to DSS may be a result of differences in the animal models and or severity of the inflammatory response. *MUC2* seemed to be induced immediately after a longer period of high dose (5% and 9%) DSS in rats, but not after 5-day treatment with a low dose (1%) in BALB/C mice, which might suggest that *MUC2* induction occurs in more severe inflammation. Both *MUC3* and *MUC4* were induced immediately after 5-day 1% DSS treatment in BALB/C mice and 9-day 5% DSS treatment in Sprague Dawley rats, which suggested that *MUC3* and *MUC4* might be induced early after moderate inflammation in some animal stains.

We observed in casein fed mice that *TFF3* expression was induced by DSS in the colon, as was previously reported. Renes et al. (94) treated Wistar rats with 7% DSS in the drinking water for 7 days, followed by a 3 week recovery period. By Immunohistochemistry, TFF3 was expressed in the upper crypts in the proximal colon and upper two-thirds of the crypts in

the distal colon. On day 2 of DSS treatment, TFF3 positive goblet cells accumulated in the surface epithelium in the entire colon. As DSS administration progressed (day 5 and 7), there were more TFF3 positive goblets cells found in the surface epithelium. Protein dot blotting showed an increase of TFF3 peptide in the colon samples (94). Consistently, it was observed in the DSS-induced colitis BALB/C mice model that both TFF3 peptide and mRNA were increased (24, 98). *TFF2* was not induced in either the colon or cecum by DSS in the present study, which is in agreement with previous findings (98). This contrasts with findings in IBD patients where TFF2 mRNA was not changed significantly in DSS-induced colitis of mice, which indicates that DSS-induced colitis might have an acute pathology, while IBD lesions exhibit a more chronic nature (98). A significant finding of our study was that, soy protein significantly suppressed the increase in *MUC1*, *TFF3* and *TNF-α* caused by DSS in the colon, while neither casein nor whey had this effect. A primary component of soy that has been well studied for bioactivity is the estrogenic isoflavone. However, the soy protein we used was processed to remove the majority of the isoflavones, hence these compounds cannot explain the current findings. However, other potentially bioactive compounds in soy have also been identified. De Mejia et al. (139) and Hernández-Ledesma et al. (140) found that the soy-specific compound lunasin had anti-inflammation effects via inhibiting the NF-κB pathway in macrophages. In their studies, NF-κB transactivation, IL-1β, IL-6, TNF-α, COX-2 expression and nitric oxide synthase expression were suppressed by lunasin (139, 140). We did not quantify lunasin in our soy protein; however, information on lunasin concentration of soybean cultivars and commercial soy proteins is available in another publication. De Mejia et al. (152) used a developed ELISA method to identify lunasin and quantify the variations in concentration in 144 selected, diverse soybean accessions. They found that soy protein

concentrate, isolate, and hydrolyzate contained 2.8, 3.75, and 4.4 g lunasin/100 g flour, respectively, while soy flour and soy flakes contained 1.24 g lunasin/100 g flour. There was very little or no lunasin in isoflavone-enriched products (152). In our study, the mice ate about 4g of the diet each day, which contained 1.1g soy protein concentrate. The lunasin in soy protein concentrate was approximately 2.8 g/100g, thus the daily intake of lunasin for the mice was about 30.8 mg/day. There is no available data about the dose of lunasin in an animal study yet, thus we could not compare the dose in our study with other publications and confidently decide if the amount of lunasin consumed by the mice would be significant. Given the finding that soy protein affected the mucosal barrier markers in our study, further study of the potential mechanisms, and/or bioactive components seems warranted.

Neither casein nor whey protein alleviated colitis symptoms, histological parameters, or expression of *TNF-α*, indicating that dairy proteins did not have protective effects against DSS-induced colitis. Whey protein did not change the *MUC* gene expression in the present study, which was in accordance with the observation by Sprong et al. (125). They found that feeding 16% cheese whey protein increased the mucin production in the feces of DSS-treated rats without altering *MUC* gene expression (125). They also found that animals fed casein supplemented with threonine and cysteine exhibited a similar response, suggesting that these amino acids, which are the major amino acid constituents of GI secretory mucins (126), may provide the protective effect.

Gupta et al. (113) observed that administration of 10^{10} colony-forming units of LGG twice daily for 6 months was beneficial to children with mildly to moderately active Crohn's disease. LGG feeding decreased disease activity within one week and the protective effect

was maintained throughout the study period. Similarly in rats, LGG administration decreased myeloperoxidase activity following TNBS treatment (115). In contrast to these previous studies, we found no effect of the probiotic on the inflammatory parameters in the colon or cecum. In fact, the soy diet which retained the lowest level of LGG in the cecal contents was the most effective in ameliorating the effects of DSS, while the whey protein diet that generated the highest cecal load of LGG was largely ineffective. Hence, our hypothesis that a diet which supports optimal probiotic survival would generate the largest protective effect was not supported for LGG specifically in the current study.

In summary, we demonstrated that *MUC1* and *TFF3* gene expression were increased by DSS treatment. In mice fed soy protein, induction of *MUC1* and *TFF3* was suppressed. Whey and casein protein diets and LGG at the levels selected for this study were ineffective in altering the inflammatory or mucosal barrier parameters. As a marker of inflammation, *TNF-α* gene expression was increased after DSS treatment. Mice fed soy protein, however, had suppressed *TNF-α* expression compared to the other treatment groups, which was consistent with the colon length and histological measures. In conclusion, soy protein suppressed the DSS-induced inflammatory stimulation of *MUC*, *TFF* and *TNF-α* gene expression independently of the probiotic. Whey protein may act as prebiotic in the colon to increase microbial survival, but these factors failed to prevent the inflammatory response in the DSS induced colitis mouse model.

ACKNOWLEDGEMENTS

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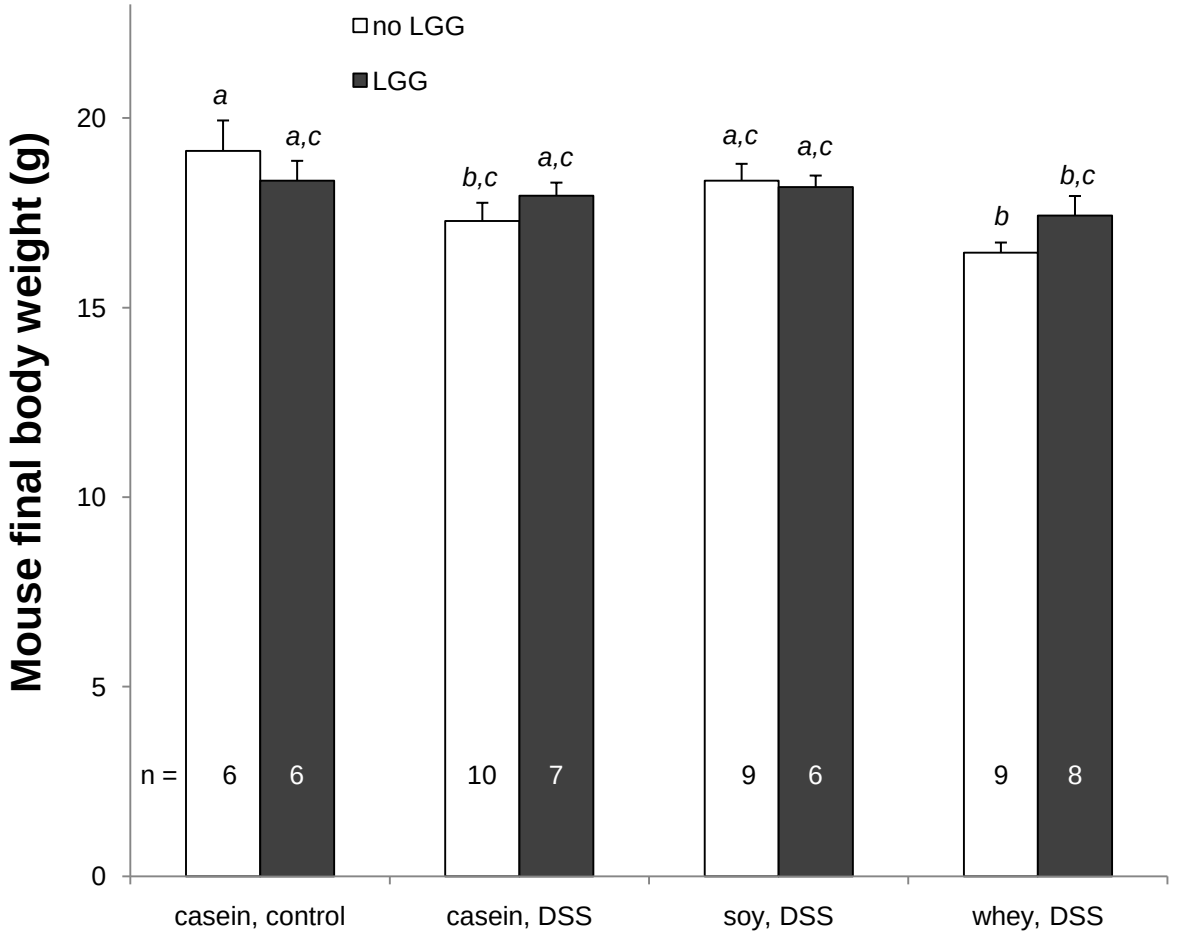

Figure 1 Final body weight of mice fed casein, soy or whey protein diets with or without LGG. Values are mean ± SE, n = 6-10. Means without a common letter differ, $P < 0.05$.

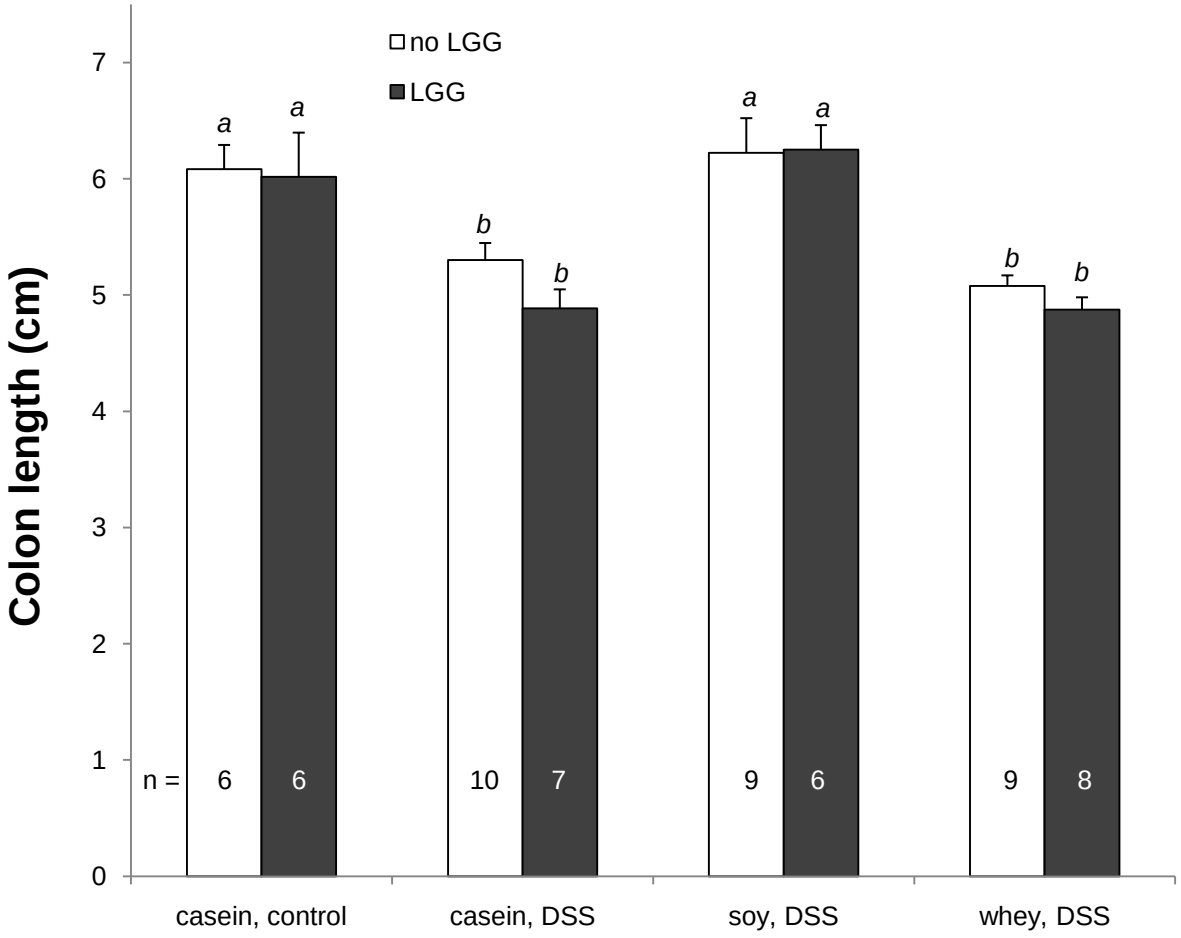

Figure 2 Length of colon from mice fed casein, soy or whey protein diets with or without LGG. Values are mean ± SE, n = 6-10. Means without a common letter differ, $P < 0.05$.

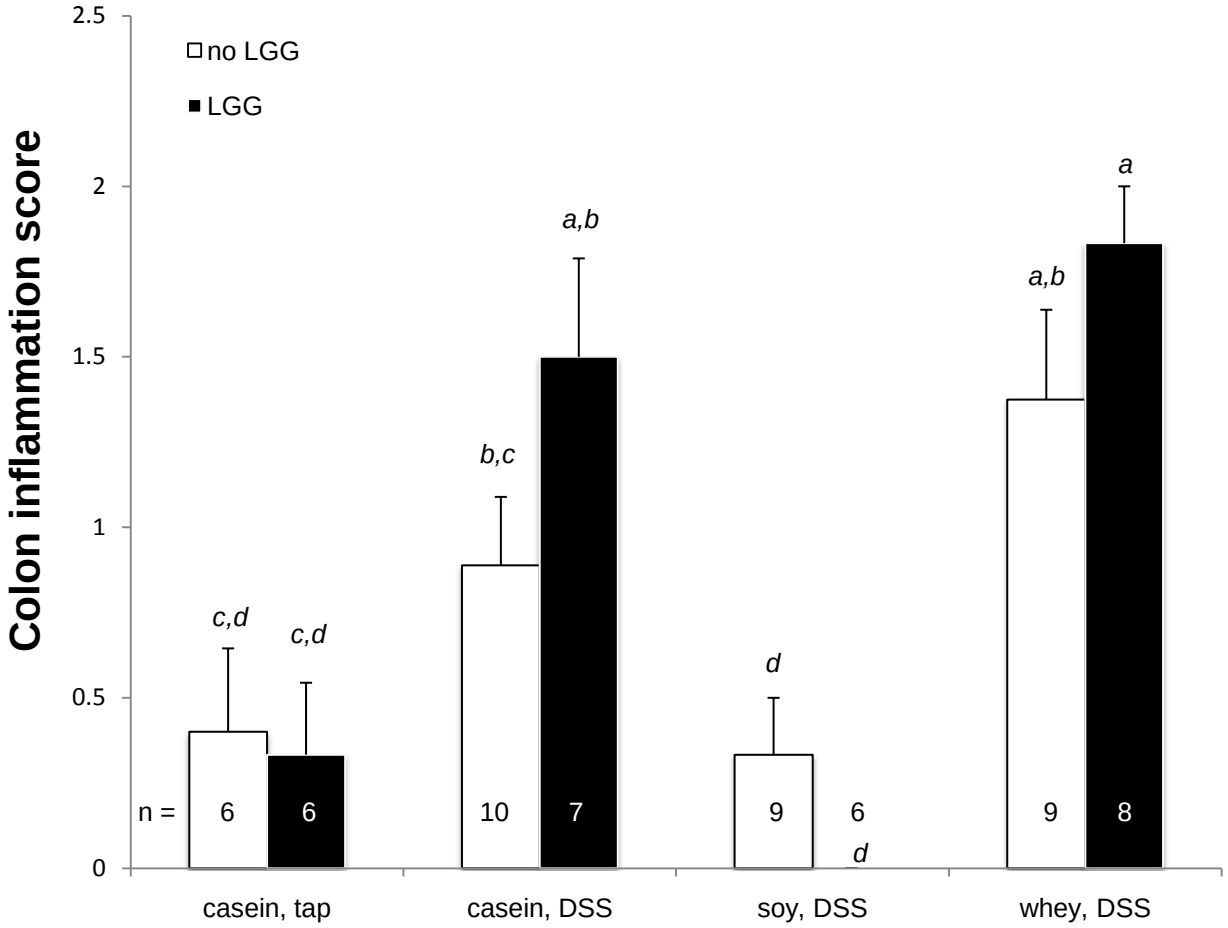

Figure 3 Colon inflammation score from mice fed casein, soy or whey protein diets with or without LGG. Values are mean ± SE, n = 6-10. Means without a common letter differ, $P < 0.05$.

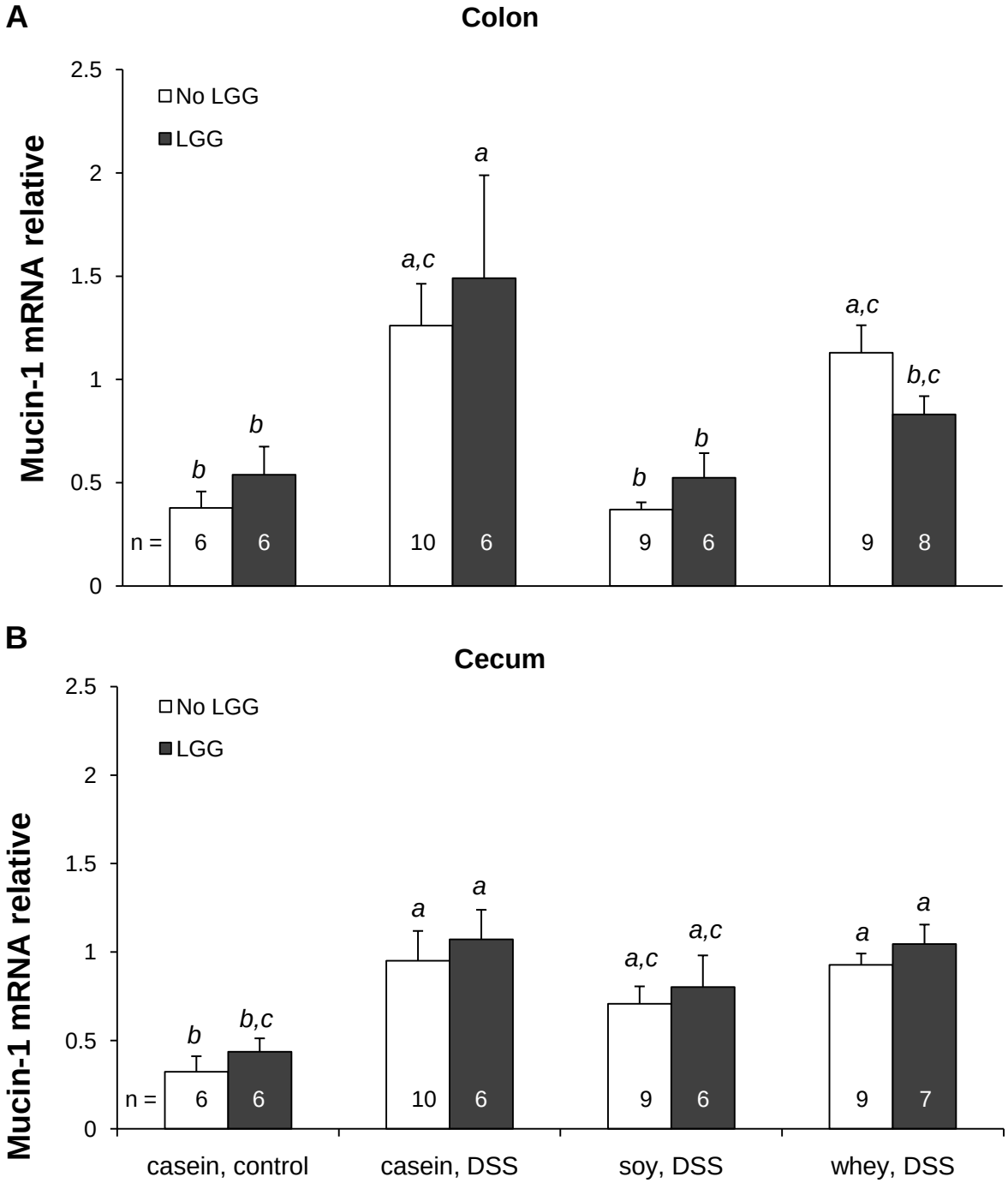

Figure 4 mRNA expression of *MUC1* gene in mouse colon (A) and cecum (B) in mice fed casein, soy or whey protein diets with or without LGG. *MUC1* gene expression was quantified by quantitative real-time PCR and data were normalized to keratin 19 expression. Values are mean ± SE, n = 6-10. Means without a common letter differ, $P < 0.05$.

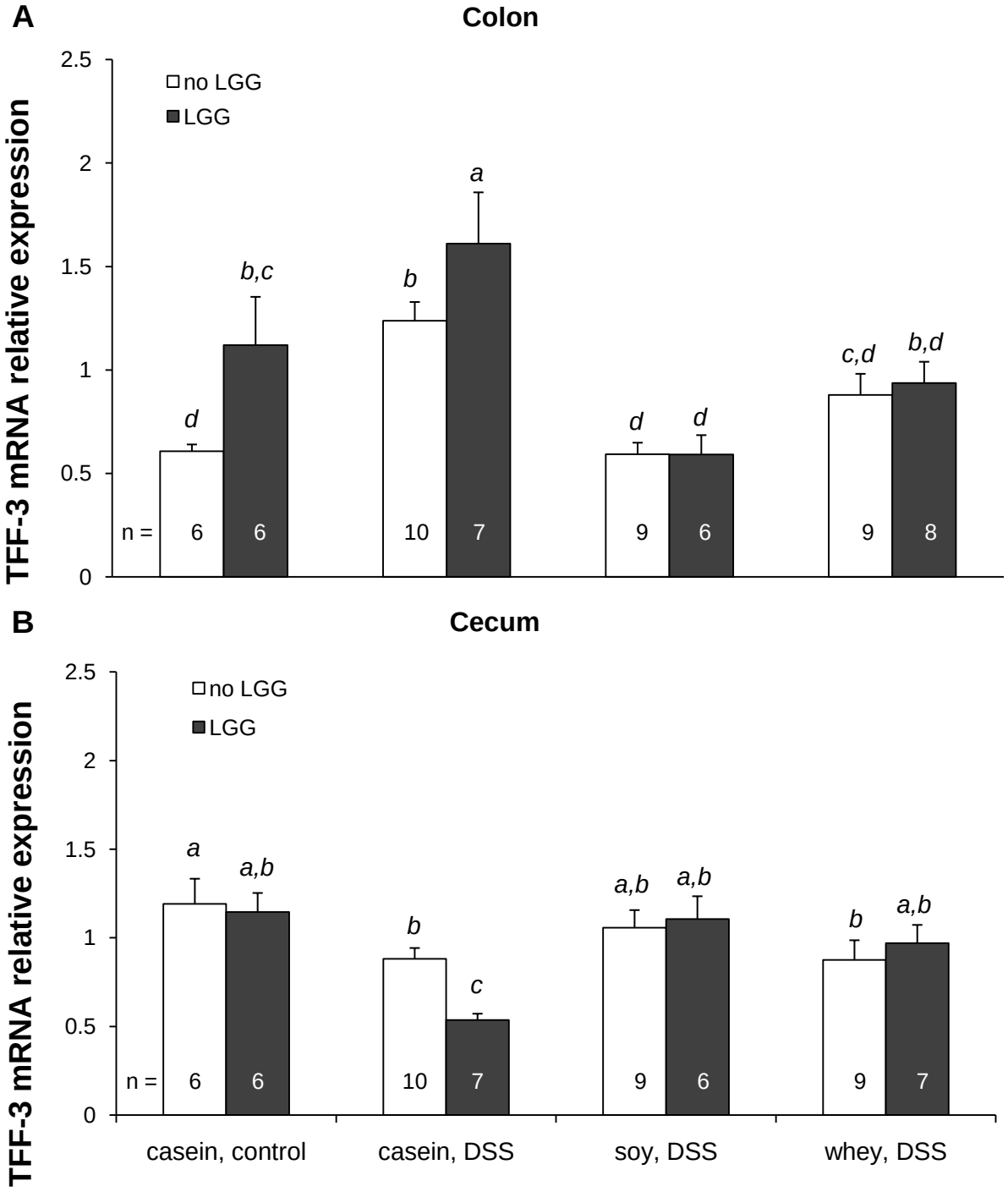

Figure 5 mRNA expression of *TFF3* gene in mouse colon (A) and cecum (B) in mice fed casein, soy or whey protein diets with or without LGG. *TFF3* gene expression was quantified by quantitative real-time PCR and data were normalized to keratin 19 expression. Values are mean ± SE, n = 6-10. Means without a common letter differ, $P < 0.05$.

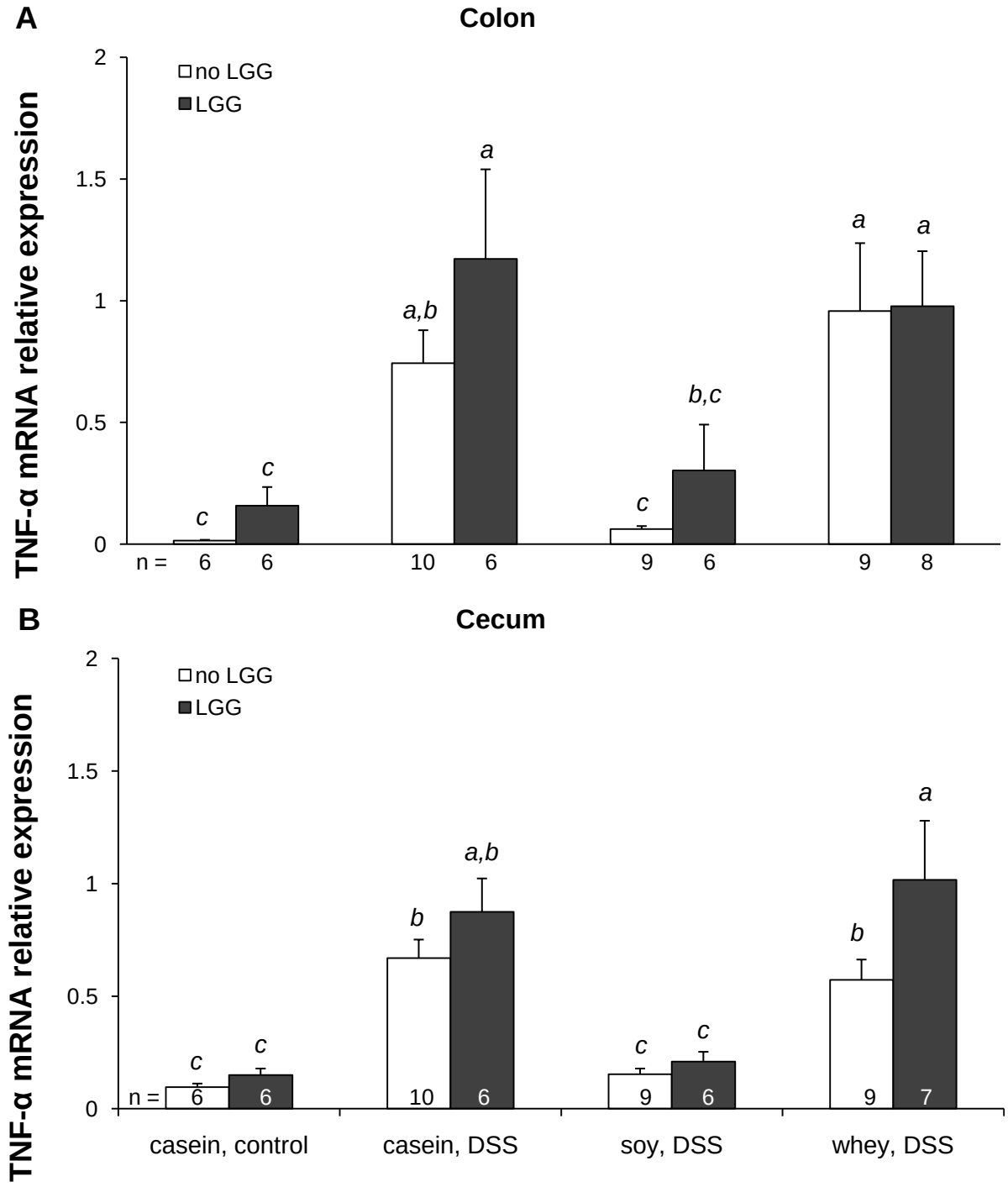

Figure 6 mRNA expression of *TNF-α* gene in mouse colon (A) and cecum (B) in mice fed casein, soy or whey protein diets with or without LGG. *TNF-α* gene expression was quantified by quantitative real-time PCR and data were normalized to keratin 19 expression. Values are mean ± SE, n = 6-10. Means without a common letter differ, $P < 0.05$.

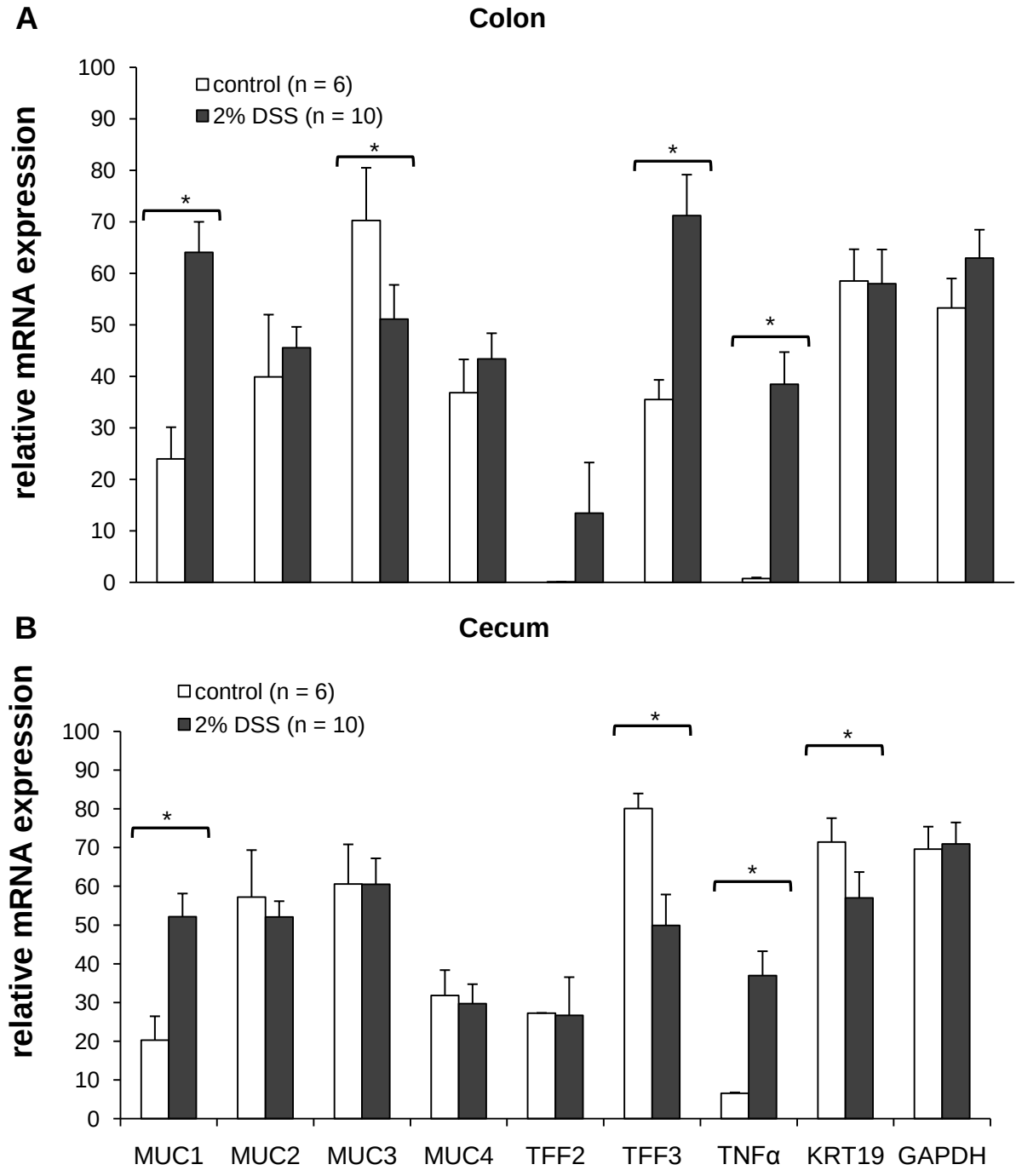

Figure 7 Comparisons of colonic (A) and cecal (B) *MUC*, *TFF*, keratin 19 and *GAPDH* gene expression in control or DSS treated mice. All diet and probiotic treatments combined. Gene expression was quantified by quantitative real-time PCR. The data was not normalized to internal control gene. Values are mean ± SE, n = 6-10. A significant DSS effect ($P < 0.05$) between casein groups indicated with black brackets.

Table 2 Effect of diets on relative expression of LGG transposase gene in mouse cecal contents[1]

	Diets without LGG				Diets with LGG			
	Casein		Soy	Whey	Casein		Soy	Whey
	control	DSS	DSS	DSS	control	DSS	DSS	DSS
	n = 6	n = 10	n = 9	n = 9	n = 6	n = 7	n = 6	n = 8
LGG transposase[2]	ND[3]	ND	ND	ND	16.35 ± 7.62[b]	12.78 ± 5.18[bc]	3.90 ± 1.66[bc]	52.07 ± 13.1[a]

[1] values are means ± SE. Means without a common letter differ, $P < 0.05$.

[2] probe and primers were used that are specific to LGG.

[3] ND: not detectable.

Table 3 Effects of 2% DSS and diet on mouse colon histology[1]

Colon histological measurements	Diets without LGG				Diets with LGG			
	Casein		Soy	Whey	Casein		Soy	Whey
	control n = 4-5	DSS n = 9	DSS n = 9	DSS n = 7	control N = 6	DSS n = 4	DSS n = 6	DSS n = 6
inflammation score[2]	0.40 ± 0.2^{cd}	0.90 ± 0.2^{bc}	0.30 ± 0.2^{d}(9)	1.40 ± 0.3^{ab}(8)	0.30 ± 0.2^{cd}	1.50 ± 0.3^{ab}	0.00 ± 0.0^{d}	1.80 ± 0.2^{a}
crypt layer thickness[3]	1.48 ± 0.08^{d}	2.00 ± 0.18^{bc}	1.80 ± 0.07^{cd}	2.08 ± 0.14^{bc}	1.91 ± 0.11^{bd}	2.35 ± 0.35^{ab}	1.89 ± 0.12^{bd}	2.69 ± 0.18^{a}
Inflammatory layer thickness	0.47 ± 0.08^{d}	0.92 ± 0.09^{c}	0.50 ± 0.06^{d}	1.09 ± 0.12^{c}	0.50 ± 0.15^{d}	1.12 ± 0.25^{bc}	0.31 ± 0.03^{d}	1.40 ± 0.10^{ab}
muscle layer thickness	1.14 ± 0.14^{b}	1.56 ± 0.16^{ab}	1.84 ± 0.18^{a}	1.57 ± 0.13^{ab}	1.73 ± 0.20^{ab}	2.04 ± 0.32^{a}	1.91 ± 0.43^{a}	2.06 ± 0.14^{a}
total thickness	3.09 ± 0.27^{d}	4.49 ± 0.36^{bc}	4.14 ± 0.14^{cd}	4.74 ± 0.29^{bc}	4.13 ± 0.42^{cd}	5.51 ± 0.86^{ab}	4.11 ± 0.48^{cd}	6.15 ± 0.29^{a}
mucus layer thickness	0.58 ± 0.14^{b}	0.48 ± 0.04^{b}	0.47 ± 0.07^{b}	0.49 ± 0.05^{b}	0.57 ± 0.14^{b}	1.31 ± 0.44^{a}	0.60 ± 0.08^{b}	0.53 ± 0.07^{b}
goblet cells per crypt	7.10 ± 1.0^{b}	8.90 ± 0.9^{b}	13.90 ± 1.2^{a}	7.90 ± 0.5^{b}	9.10 ± 0.7^{b}	8.00 ± 0.6^{b}	9.20 ± 0.8^{b}	7.20 ± 0.6^{b}

[1] values are means ± SE for the number of mice indicated (exceptions in parenthesis) averaged for 2-4 regions per cross section of alcian blue-stained colon slide. Means in a row with superscripts without a common letter differ, $P < 0.05$.

[2] inflammation scoring is as in Materials and Methods.

[3] Thicknesses are measured in microns (μm).

Table 4 Effects of 2% DSS and diet on relative mRNA expression in mouse colon and cecum[1]

mRNA	Diets without LGG				Diets with LGG			
	Casein		Soy	Whey	Casein		Soy	Whey
	Control	DSS	DSS	DSS	control	DSS	DSS	DSS
	n = 6	n = 10	n = 9	n = 9	n = 6	n = 6	n = 6	n = 7
colon								
Mucin-2	0.813 ± 0.301^{ab}	0.826 ± 0.072^{b}	1.335 ± 0.159^{a}	0.891 ± 0.125^{ab}	1.014 ± 0.084^{ab}	1.354 ± 0.423^{a}(7)	1.094 ± 0.150^{ab}	0.972 ± 0.073^{ab}(8)
Mucin-3	1.175 ± 0.079^{b}	0.900 ± 0.085^{bc}	0.910 ± 0.088^{bc}	0.776 ± 0.066^{c}	1.627 ± 0.325^{a}	0.908 ± 0.116^{bc}	0.931 ± 0.165^{bc}	0.789 ± 0.074^{c}(8)
Mucin-4	0.718 ± 0.186^{b}	0.807 ± 0.109^{b}	1.323 ± 0.213^{a}	1.077 ± 0.205^{ab}	0.751 ± 0.108^{b}	0.844 ± 0.280^{ab}	1.015 ± 0.109^{ab}	0.890 ± 0.129^{ab}(8)
cecum								
Mucin-2	0.846 ± 0.088^{ab}	0.918 ± 0.047^{b}	0.694 ± 0.067^{b}	1.054 ± 0.059^{a}	1.014 ± 0.133^{a}	0.894 ± 0.081^{ab}(7)	1.010 ± 0.130^{a}	1.039 ± 0.038^{a}
Mucin-3	0.874 ± 0.068^{c}	1.075 ± 0.072^{bc}	1.305 ± 0.083^{ab}	1.019 ± 0.066^{c}	1.009 ± 0.063^{c}	0.837 ± 0.055^{c}	1.510 ± 0.135^{a}	1.407 ± 0.201^{a}
Mucin-4	0.478 ± 0.075^{d}	0.535 ± 0.064^{d}	0.719 ± 0.110^{ac}	0.535 ± 0.100^{bc}	0.598 ± 0.108^{bc}	0.521 ± 0.062^{bc}	0.850 ± 0.152^{ab}	0.808 ± 0.173^{bc}

[1] values are means ± SE for the n values indicated (exceptions in parenthesis). Means without a common letter differ, $P < 0.05$.

CHAPTER 3

DISCUSSION

Probiotic bacteria have been utilized for health benefits for several decades (106). Probiotics have been found to promote a healthy state of the GI tract and have been applied to prevent and treat several intestinal inflammatory disorders, such as necrotizing enterocolitis and inflammatory bowel disease (IBD) (31, 109-112). Evidence suggests that probiotics have certain effects on the modulation of intestinal barrier function in both normal and inflammatory conditions (112, 116-118).

Recently, soy and whey proteins have gained popularity as nutrition supplements in the market. They both have various potential modes of action for protecting human health. Soy protein has been shown to have protective effects on colon cancer, although the mechanisms are not well defined (129, 130). Animal studies have found that soy protein improved the intestinal barrier function and reduced bacteria translocation in the gut (132). In addition, it has been found that soy protein had anti-inflammation effects via inhibiting the NF-κB pathway in macrophages (139, 140). Whey protein contains bioactive components such as glycomacropeptide and lactoferrin, which may be beneficial in preventing colitis (122, 123). Glycomacropeptide and lactoferrin also have prebiotic properties that have been shown to support the growth of *Bifidobacteria* and *Lactobacillus* and thereby promoting gut health (124, 125). In addition, threonine and cysteine that are abundant in whey protein were proved to promote the growth of *Bifidobacteria* and *Lactobacillus* in rats (125).

Therefore, we designed the present study to investigate whether a probiotic bacterium, in

combination with casein, soy or whey protein would have protective effects on colitis, with special focus on modulation of the mucous barrier. Since casein is used as the basic protein source in the AIN 93 rodent diet formulation (121), we used it as the control diet in our study.

Both casein and whey protein are derived from cow milk and they are easily digestible proteins. The digestibility rates of casein and whey protein in rats were 99% and 100%, respectively (153). Soy protein is somewhat less digestible. The apparent ileal digestibility of soy protein concentrate was 75.4% in growing pigs (154). The casein used in our study was 95% purity, whereas the soy protein and whey protein included some other components (**Table 7**). The soy protein contained 69% protein, 19% fiber and 3% fat and 7% ash. The whey protein contained 81.5% protein, 5.5% fat, 6% lactose and 3% ash. Not only casein, but also whey protein and soy protein were good sources of essential amino acids (EAAs). They provided sufficient EAAs for the growing mice (**Table 8**). We proposed that the specific bioactive components or amino acids in the soy protein and whey protein could affect the gut microbiota and barrier function, thereby modulating colitis.

We chose the DSS animal model based on our previous experiments. Our lab had previously tested different doses of DSS (1%, 2% and 3%) in C57BL/6 female mice. It was found that treating the C56BL/6 mice with 2% DSS for 4 days resulted in moderate colitis, defined by the MPO activity and the changes of several proinflammatory cytokines such as IL-1β, IL-6, and TNF-α. The dose and administration period for LGG were based on previous publications by others (116, 153). The diet was prepared according to AIN 93 rodent diet formulation, with relative changes in the protein sources. Because we wanted to investigate the protective effects of the probiotic/diet in this study, we euthanized the animal just one day

after finishing the DSS treatment without giving the mice a recovery period. To investigate how these conditions would influence colitis recovery, another study using a different end point would need to be done.

The chronic inflammation can be induced by 5-9 day high dose DSS treatment followed by 2-4 week low dose DSS treatment. Hoebler et al. (24) treated BALB/c mice with 1% DSS for 5 days to induce acute inflammation, followed by 0.5% DSS for 28 days to induce chronic inflammation. It was found that *MUC3* mRNA level was increased after acute colitis. *MUC1* and *MUC4* gene expression were enhanced significantly by colitis (24). In another study, Sprague-Dawley rats were administered 5% DSS for 9 days followed by 2% DSS for 18 days to induce chronic colitis (93). It was observed that DSS-treated rats had higher mRNA levels of colonic *MUC2* and *MUC3* compared with pair-fed controls (93). In the study by Renes et al. (94), Wistar rats were given 7% DSS in the drinking water for 7 days, followed by a 3 week recovery period. Biochemical analysis of MUC2 protein levels showed a progressive increase in MUC2 in the proximal colon during DSS treatment, and MUC2 levels rose to fourfold of the control levels on day 7. If we extend the DSS treatment in our study, for instance, continue 1% DSS treatment for 3 weeks after the 4-day 2% DSS treatment, we may develop a chronic inflammation model. According to the chronic inflammation models mentioned above, we may see the induction of *MUC2*, *MUC3* and *MUC4* by DSS. We expect that soy protein would suppress these MUC genes. Because the administration time of the LGG and whey protein is extended, we might expect that the probiotic and whey protein show a protective effect against colitis.

A limitation about the experimental design of the present study was that we did not include groups of mice fed soy or whey protein without DSS treatment. This was partially attributed to budget limitations. If we included these groups, there would be four more groups, thus 12 groups in total. Another consideration was that, the DSS effects on the parameters could be observed within the casein groups which did have with or without DSS. But our primary aim was to compare the differences of the *MUC*, *TFF* gene expression and inflammatory markers in colitis animals among different diet groups, which was achieved with the present experimental design.

In our study, we only tested one specific strain of probiotic, the LGG, which has been found beneficial in Crohn's diseases (113). LGG did not show a protective effect at the level selected for this study. Besides LGG, other strains of *Lactobacillus* or *Bifidobacteria* may be used. For example, the commercial probiotics mixture VSL#3 containing 3 *Bifidobacterium* stains (*breve*, *longum*, and *infantis*), 4 *Lactobacillus* stains (*acidophilus*, *plantarum*, *paracasei*, and *bulgaricus*) and *Streptococcus thermophilus* has been utilized widely in publications exploring how probiotics affect IBD (116, 153, 154). In healthy Wistar rats, it was observed that oral administration of VSL#3 on a daily basis for 7 days increased basal luminal mucin content by 60%. The isolated rat colonic loops that exposed to the probiotic formula increased the amount of mucin secretion and a higher gene expression of *MUC2*. Meanwhile, *MUC1* and *MUC3* gene expression were slightly increased (116). Another animal study by Fitzpatrick et al. (155) found that the probiotic formulation VSL#3 improved DSS-induced colitis in weaning rats. Colonic IL-1β, MPO activity were decreased by the probiotic application. Sood et al. (156) found the patients with mild-to-moderately active

ulcerative colitis had significantly greater decreases in disease scores and individual symptoms after 6-week VSL#3 administration, compared with the placebo group.

There are other diet factors that may influence the mucus barrier. Butyrate, an end-product of carbohydrate fermentation, was found to modulate the secretion of mucins (157, 158). Gaudier et al. (158) treated mice daily for 7 days by rectal enemas of butyrate (100 mM) versus saline. They demonstrated that butyrate stimulated the gene expression of both secreted (*MUC2*) and membrane-linked (*MUC1, MUC3, MUC4*) mucins in the colon without changing the number of MUC2-positive epithelial cells. However, butyrate caused a 2-fold decrease in the thickness of adherent mucus layer, which needed further studies to explore the mechanisms. Since dietary fibers could be fermented by the microbes in the colon and produce butyrate (159), we may also test different fibers and determine how they would modulate the mucus barrier.

Discussion about the methods in the present study

1. New method for probiotic administration

In the present study, we developed a new method to administer LGG. Instead of using orogastric gavage to introduce the probiotic into the stomach of the mice as previously reported (116, 160), we added the LGG, resuspended in PBS, on the top of wet diets and then meal-fed the mice. The mice had been adjusted to the meal-fed method before we started the experimental period and they consumed most of the food during the allowed feeding time, thus we could ensure the LGG intake. There was no diet effect, probiotic effect on the food

consumption. During the DSS treatment period, the food consumption in the DSS groups was decreased compared the control groups, but the food consumption decrease seemed less obvious in the soy protein groups (**Table 5A and 5B**). The mice body weight decreased during the acclimation time, but increased when we started the experimental period (**Figure 8**). Over the 4-day DSS treatment, most of mice did not lose weight, but the average body weight gain was reduced in the DSS groups compared to the groups without DSS treatment. There was no significant difference between the diet groups, and LGG did not show significant effect on the body weight change (**Figure 9**). There was no significant difference in body weight gain among the casein, soy protein and whey protein groups. In addition, we conducted an experiment to test the viability of LGG after being left in the diet for 2 hours and the result confirmed that LGG remained viable. After 1h in the soy diet (mixed with 110% water), the concentration of LGG changed from $3x10^{10}$CFU/ml to $1x10^{10}$CFU/ml. The concentration after 2h was $1.5x10^{10}$CFU/ml. In the casein diet (mixed with 40% water), the concentrations of LGG after 1h and 2h were $2x10^{8}$CFU/ml and $1x10^{8}$CFU/ml, respectively. Given that the amount of LGG rarely declined within the test time, we presumed that the LGG would stay viable in the diet within the feeding time (5h). Therefore, our method to administer LGG was feasible. An obvious advantage of this method was that it did not produce stress in the animals as would have occurred with gavage administration. De Meijer et al. (161) observed that orogastric gavage increased stress and affected food intake when trying to develop a diet-induced obesity murine model. They suggested that the laboratory routines, such as orogastric gavage, may intervene in the results of the experiments and should be taken into account when designing animal studies.

2. Goblet cell staining

In the current study, the histological sections were stained with 1% alcian blue for goblet cell quantification (146). The alcian blue stain is a popular staining method in histology. At a pH of 2.5, the alcian blue will stain for both sulfated (sulphomucins) and carboxylated (sialomucins) mucopolysaccharides which are found in the goblet cells located in the intestine. The alcian blue dye is a positively charged, very large molecule that will bind to the negatively charged low density mucin found in the goblet cells, staining it blue in color (162). In general, the goblets cells were stained well in our study, however, the boundary of some goblet cells were not clear, and there was some non-specific staining, which made it difficult to count the numbers of the cells in each crypt. An alternative to measuring goblet cell density would be immunohistochemical staining using an anti-mucosal epithelial membrane mucin antibody (163). The specific antibody-antigen binding can stain the goblet cells in a more specific manner and may improve the accuracy of the quantification.

3. Mucin synthesis measurement

Mucin synthesis rates would be a direct measure of the mucin expression. Other researchers have quantified mucin synthesis (125, 127). Sprong et al. (125) analyzed fecal mucin in rats treated with DSS. Briefly, fecal mucin was extracted from freeze-dried feces and quantified fluorometrically as previously described by Bovee-Oudenhoven et al. (164). Mucin synthesis rates can also be measured in vivo. Faure et al. (93) determined mucin synthesis rates in the rats using gas chromatography-combustion-isotope ratio mass spectrometry with purified mucins from the intestine.

4. Histological study

The parameters we used in the present study included the inflammation score, thickness of crypt layer (from the luminal end of the crypt to the surface of the thin basal muscle layer), the thickness of the inflammatory layer (from the surface of the thin basal muscle layer under the crypts to the surface of the lower thicker muscle layer) and thickness of the whole colon, which have been widely used by others (160, 165). The inflammation score was based on the morphological criteria described in by Suzuki et al. (147): 0 = normal colon mucosa; 1 = shortening the basal 1/3 of crypts with slight inflammation and edema in the lamina propria; 2 = loss of the basal two-thirds of the crypts with moderate inflammation in the lamina propria; 3 = loss of all the crypts with severe inflammation in the lamina propria, but with the surface epithelium still remaining; 4 = a loss of all the crypts and surface epithelium with severe inflammation in the mucosa, muscularis propria and submucosa.

An additional parameter that could be used in the future is the counting of polymorphonuclear neutrophil granulocytes (PMN) in the colon sections. PMN is the most abundant cell type in intestinal lesions in IBD (166). It was found that DSS-induced colitis in mice was characterized by PMN infiltration into the colonic mucosa. Thus, the quantification of PMN within the lamina propria could be used as an inflammatory marker in DSS-induced colitis (167).

5. LGG quantification in fecal samples

In our study, we used quantitative RT-PCR to quantify the amount of LGG in the fecal

samples. The 16S rDNA gene sequence is widely used as a target for genus- and species-level identification. We extracted the fecal DNA and amplified the specific gene (*L. rhamnosus* transposase) and calculated the relative amount of LGG. LGG-specific gene expression was found in cecum contents of all mice given LGG but none of the untreated mice. We housed the mice in separate rooms and made specific effort to prevent cross contamination, which was very successful. Mice fed whey protein had significantly higher LGG expression than the other diet groups; therefore we concluded that the quantitative RT-PCR was a reliable method. Another way to quantify probiotic recovery from the intestine would be the bacteria culture method. As described by Nanda Kumar et al. (160), feces samples are collected from cecum and diluted. The bacteria can be cultured in MRS medium in an anaerobic incubator for 24 hours, and the number of the LGG colonies counted.

Future work

1. Identify the bioactive components in the soy protein that modulate the barrier function.

We found that soy protein mediated the abnormal expression of MUC and TFF genes in DSS treated animals, which indicated the potential of soy protein in preventing or treating IBD or other intestinal diseases. However, further studies are needed to identify the bioactive components in the soy protein.

The protective effects of soy protein could attribute to the three aspects. Firstly, the soy protein concentrate we used in our study is not pure protein. It is a mixture of 69% protein and 19% fiber and a small quantity of other undefined compositions. These compositions

other than the protein may contribute to the benefits of soy protein. As well known, short-chain fatty acids (SCFA) produced during fermentation of plant fibers in the colon are beneficial for preventing colon inflammation and cancer (146). The second aspect is the bioactive peptides in the soy protein. At last, specific amino acid compositions may contribute to the protective properties of soy protein.

One property of any potential bioactive component is that they survive digestion in the stomach and in the small intestine, so that they can reach to the colon and exert their functions. A newly isolated bioactive peptide, lunasin, may be a candidate. Lunasin has been found to be bioavailable in humans. A human study showed that amino acid sequences from lunasin were present in plasma samples after soy intake for 30 min and 1 h, whereas no peptides from lunasin were detected in plasma samples without soy intake (138). It has been measured that the lunasin in soy protein concentrate was 2.8 g/100g (152). In our study, the mice ate about 4g of the diet each day, which contained 1.1g soy protein concentrate, thus the daily intake of lunasin for the mice was about 30.8 mg/day. In the in vitro study by De Mejia et al. (139), they treated the macrophages with 50μM lunasin and found that the lunasin showed a significant anti-inflammatory effect. There is no available data about the dose of lunasin in animal study yet, thus we could not compare the dose in our study with other publications and confidently decide if the amount of lunasin consumed by the mice would be significant.

Further in vitro or in vivo studies may be conducted to test if lunasin is the bioactive component that provides the protective effect observed. Lunasin is commercially available (140) or can be purified from defatted soybean flour (139). To examine its effects, we may

treat cell lines with lunasin and measure the gene expression of *MUC* and *TFF* by PCR or western blotting. For instance, the human intestinal epithelial goblet cells HT29-Cl.16E (168), either in their basal state or treated with interferon-gamma could serve as a model of inflammation (169). Animal colitis models can also be used to investigate the effects of lunasin on the mucous barrier. In the animal models, we may also test the digestibility of lunasin by quantifying the amount in fecal samples and in the serum to confirm the bioavailability of lunasin.

Both soy and whey are good sources of essential amino acids. The differences of essential amino acid profile of casein, soy protein concentrate and whey protein are shown in **Table 8**. The major differences are: soy protein contains significantly more arginine, while whey protein has more leucine, lysine and threonine. Whether these differences would be associated with their different effects on colon inflammation, or whether specific amino acid(s) exhibit anti-inflammatory potential is unknown and further investigations are needed.

2. Investigate the pathways involved in the MUC, TFF gene expression.

After identifying the bioactive components in the soy protein, further studies may be conducted to investigate the pathways that may be involved in the modulation of *MUC*, *TFF* genes. *MUC* gene expression has been found to be associated with the MAPK pathway (151). We could explore how soy protein affects the factors in this pathway and how these changes are eventually related the colon inflammation. There are multiple regulatory pathways for *TFF*, including PI3K/AKT pathway, the Rho-ROCK cascade, COX-2/TXA2-R/Gαq

signaling, PLC/PKC, MAP kinase and EGFR signaling (170). We may study how soy protein would regulate these pathways and affect the incidence and development of colon inflammation.

3. Human feeding study

Based on the results that soy protein showed protective effects on DSS-induced colitis in the present study, we may propose to conduct a human feeding trial to further explore this response. Human subjects with IBD could be recruited and randomly assigned to the control diet group or soy protein diet group. The protein supplements can be given in a powder form and consumed once daily with milk or other beverages (171). The composition of the diets would be the same only except for the proteins. Casein would be used as the control protein. MUC1-4, TFF1-3, TNF-α concentrations in the serum can be measured in the beginning and at the end of the experimental period. Disease activity based on presence of diarrhea, bloody stools, abdominal pain and fever would be recorded during the study (172). These parameters should be compared between the control group and the soy protein group. The diet effects can be analyzed.

In conclusion, our study investigated the protective mechanisms of diets and LGG in a mouse model of IBD, with an emphasis on the barrier function. We found that soy protein suppressed the DSS-induced inflammatory stimulation of MUC, TFF and TNF-α gene expression independently of the probiotic. Whey protein may act as prebiotic to increase

microbial survival, but these factors failed to prevent the inflammatory response. Our hypothesis that LGG would enhance barrier function was not supported by the data, however, we proved that diets differentially affected the barrier function and showed protect effects against colitis. Further studies are needed to identify the bioactive components in the soy protein that exert the protective effects. The pathways involved in the *MUC*, *TFF* gene expression should be investigated. In addition, a soy protein human feeding trial would be helpful for better understanding the potential of soy protein in IBD prevention and treatment. In the present study, we developed a new method for probiotic administration. The methods for colonic histological study and quantitative RT-PCR for *MUC*, *TFF* genes and LGG-specific gene were reliable. As alternative, mucin synthesis measurement could be conducted to evaluate mucin expression. Fecal bacteria culture method would be another way to quantify probiotic recovery from the colon.

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APPENDIX

Abbreviations:

DSS: dextran sodium sulfate

LGG: *Lactobacillus rhamnosus* GG

Figure 8 Body weight of mice fed experimental diets

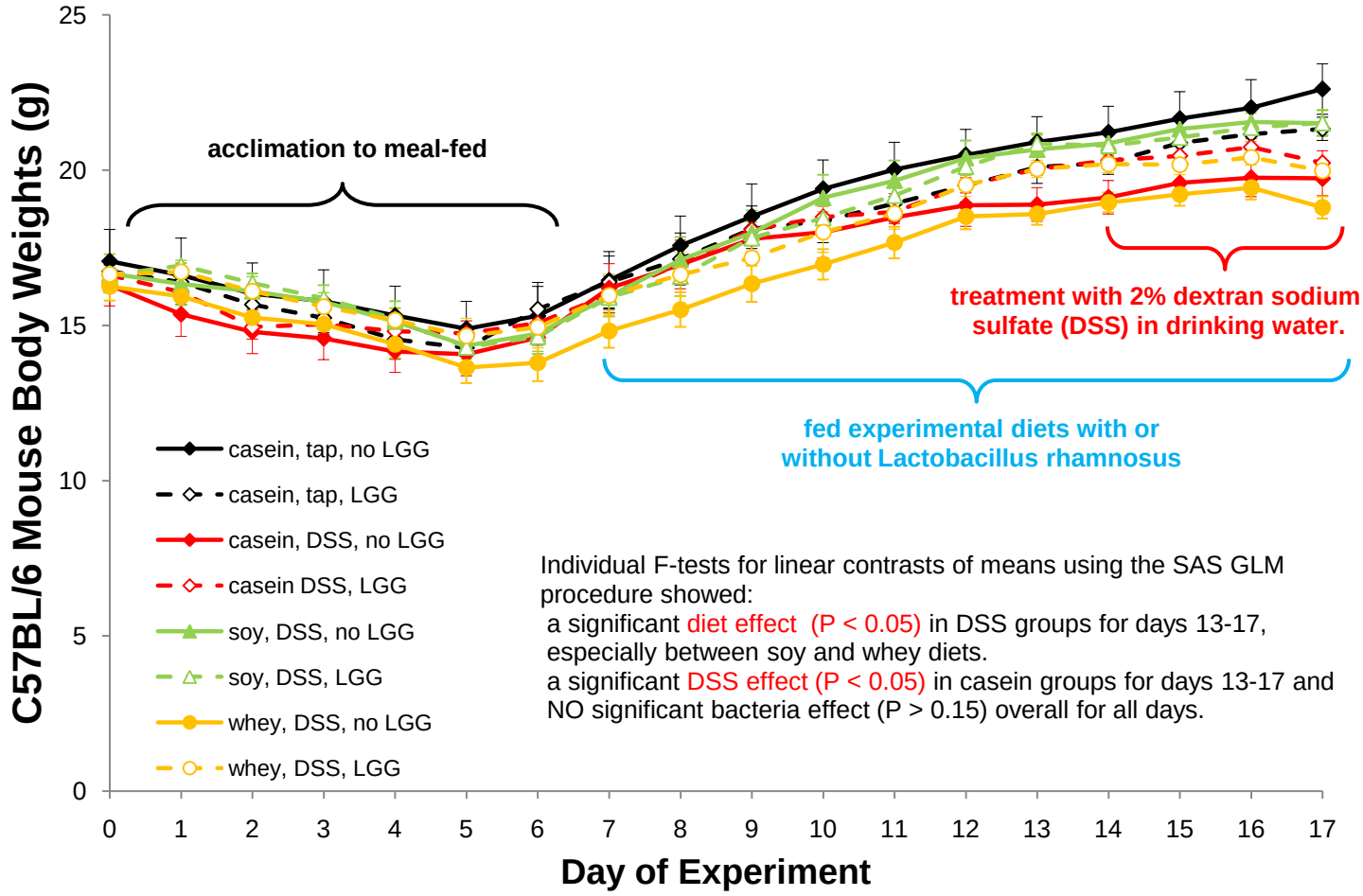


Figure 9 Change in body weight over 4-day DSS treatment

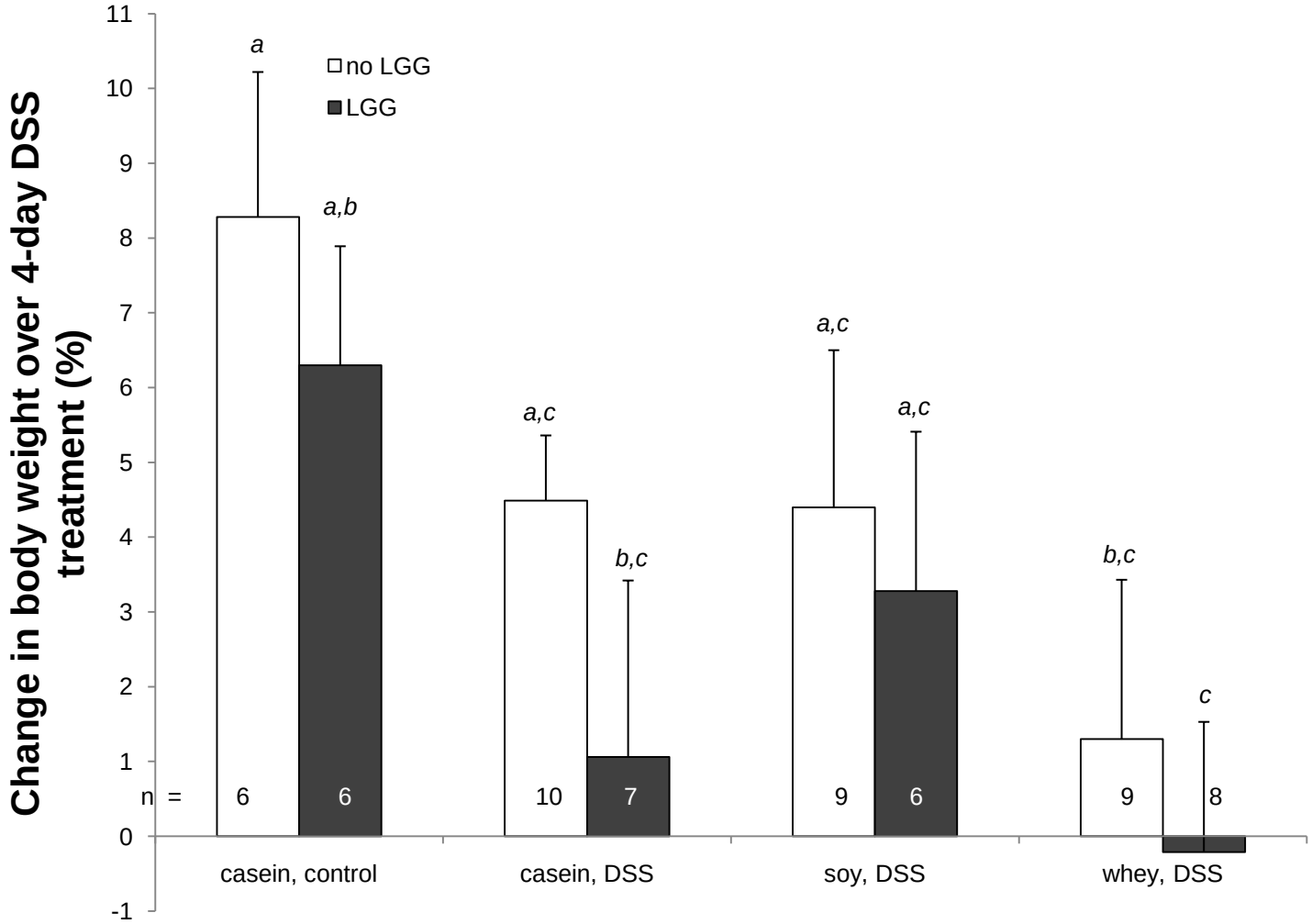


Table 5A Effects of diet and bacteria on food consumption (dry weight) before DSS treatment*

Food Consumption gram/day	Diets without LGG			Diets with LGG		
	Casein *n = 16*	Soy *n = 9*	Whey *n = 9*	Casein *n = 13*	Soy *n = 6*	Whey *n = 7*
Day 1	2.99 ± 0.09	2.57 ± 0.16	2.42 ± 0.10	2.73 ± 0.36	2.52 ± 0.20	2.82 ± 0.10
Day 2	3.10 ± 0.08^{a}	2.87 ± 0.17^{ab}	2.58 ± 0.14^{b}	3.05 ± 0.12^{ab}	2.82 ± 0.17^{ab}	2.88 ± 0.11^{ab}
Day 3	3.20 ± 0.10	3.14 ± 0.15	2.80 ± 0.11	3.18 ± 0.09	3.29 ± 0.17	2.92 ± 0.05
Day 4	2.94 ± 0.14	3.21 ± 0.15	2.76 ± 0.09	2.88 ± 0.10	3.11 ± 0.07	3.03 ± 0.17
Day 5	2.91 ± 0.10^{ab}	3.21 ± 0.09^{a}	2.88 ± 0.07^{ab}	2.78 ± 0.10^{b}	3.21 ± 0.09^{ab}	2.97 ± 0.13^{ab}
Day 6	2.77 ± 0.09	3.21 ± 0.09	2.84 ± 0.14	2.92 ± 0.11	3.07 ± 0.20	3.10 ± 0.12
Day 7	2.66 ± 0.11^{b}	3.13 ± 0.12^{ab}	2.78 ± 0.15^{ab}	2.86 ± 0.12^{ab}	3.36 ± 0.13^{a}	2.96 ± 0.18^{ab}

Table 5B Effects of DSS, diet and LGG on food consumption (dry weight) during DSS treatment*

Food Consumption gram/day	Diets without LGG				Diets with LGG			
	Casein		Soy	Whey	Casein		Soy	Whey
	Control *n = 6*	DSS *n = 10*	DSS *n = 9*	DSS *n = 9*	Control *n = 6*	DSS *n = 7*	DSS *n = 6*	DSS *n = 7*
Day 0, no DSS	2.80 ± 0.18^{a}	2.21 ± 0.09^{b}	3.04 ± 0.14^{a}	2.65 ± 0.18^{a}	2.62 ± 0.11^{a}	2.66 ± 0.13^{a}	3.00 ± 0.28^{a}	2.85 ± 0.14^{a}
Day 1, DSS	2.65 ± 0.22^{ab}	2.23 ± 0.08^{bc}	2.51 ± 0.21^{ab}	1.98 ± 0.31^{c}	2.77 ± 0.10^{ac}	2.52 ± 0.22^{bc}	2.91 ± 0.06^{a}	2.44 ± 0.13^{bc}
Day 2, DSS	2.62 ± 0.20^{ac}	2.17 ± 0.15^{b}	2.74 ± 0.14^{ac}	2.16 ± 0.14^{b}	2.78 ± 0.09^{ac}	2.49 ± 0.09^{bc}	3.01 ± 0.18^{a}	2.54 ± 0.07^{bc}
Day 3, DSS	2.63 ± 0.24^{ad}	2.07 ± 0.12^{bc}	2.57 ± 0.15^{ad}	1.61 ± 0.21^{c}	2.60 ± 0.11^{ad}	1.98 ± 0.14^{bc}	2.80 ± 0.15^{a}	2.21 ± 0.11^{bd}

*values are means ± SE for the n values indicated.

Means without a common letter differ, $P < 0.05$.

Table 6 Effects of 2% DSS and Diet on selected mouse organ weights and parameters

mouse parameter	Diets without LGG				Diets with LGG			
	Casein		Soy	Whey	Casein		Soy	Whey
	Control n = 6	DSS n = 10	DSS n = 9	DSS n = 9	Control n = 6	DSS n = 7	DSS n = 6	DSS n = 8
final body weight	19.13 ± 0.80^{a}	17.28 ± 0.48^{bc}	18.35 ± 0.44^{ab}	16.45 ± 0.26^{c}	18.34 ± 0.53^{ab}	17.95 ± 0.35^{ab}	18.18 ± 0.30^{ab}	17.43 ± 0.52^{bc}
liver/body weight ratio*	56.1 ± 4.1^{a}	50.8 ± 1.9^{ab}	46.7 ± 1.2^{bc}	50.4 ± 1.4^{ab}	47.3 ± 1.7^{bc}	49.7 ± 2.2^{abd}	43.2 ± 1.8^{cd}	52.6 ± 2.9^{ab}
spleen/body weight ratio*	3.05 ± 0.21^{ac}	3.22 ± 0.25^{ac}	2.85 ± 0.16^{ac}	3.63 ± 0.49^{ab}	2.71 ± 0.23^{c}	3.33 ± 0.26^{ac}	2.48 ± 0.19^{c}	3.64 ± 0.31^{ab}
cecum/body weight ratio*	9.33 ± 0.74^{bc}	8.61 ± 0.82^{c}	12.4 ± 0.37^{a}	10.8 ± 0.95^{ab}(8)	8.50 ± 0.81^{c}	8.05 ± 0.76^{c}	12.2 ± 0.60^{a}	9.01 ± 0.64^{bc}(7)
colon/body weight ratio*	8.86 ± 1.09^{d}(5)	10.1 ± 0.36^{bcd}	9.76 ± 0.27^{bcd}	11.6 ± 0.57^{a}	9.18 ± 0.28^{cd}	10.3 ± 0.42^{ad}	10.7 ± 0.61^{ac}	11.0 ± 0.64^{ab}
colon length	6.1 ± 0.2^{a}	5.3 ± 0.1^{b}	6.2 ± 0.3^{a}	5.1 ± 0.1^{b}	6.0 ± 0.4^{a}	4.9 ± 0.2^{b}	6.2 ± 0.2^{a}	4.9 ± 0.1^{b}
colon weight/length ratio*	27.3 ± 3.0^{c}	33.4 ± 2.0^{ac}	29.6 ± 2.4^{c}	37.5 ± 2.0^{ab}	28.7 ± 2.7^{c}	38.0 ± 1.9^{ab}	31.5 ± 2.5^{ab}	39.3 ± 2.2^{a}

* values for ratios are shown as (x 1000)

± standard error, Significant individual posthoc comparisons are indicated by different italic superscripted letters.

Table 7 Protein analysis

	Casein	Soy protein concentrate	Whey protein
%Protein	95	69	81.5
% Fat	1.5	3	5.5
% Lactose	0.2	–	6
% Ash	2.1	7	3.0
% Fiber	–	19	–
Calories (kcal/100g)	360	290	394

Sources: product data sheet from the companies.

Table 8 Contents of essential amino acids (EAA) in the casein, soy protein and whey protein

Amino Acid	Casein[1]	Soy protein[2]	Whey protein[3]	Mice requirement[4]
	g EAA/100g of Protein			g EAA/100g diet
Isoleucine (BCAA)	5.9	4.7	6.0	0.5
Leucine (BCAA)	9	7.7	10.4	0.7
Valine (BCAA)	6.9	5	3.6	0.9
Lysine	7.3	6.5	9.5	0.5
Methionine	2.9	1.3	2.3	0.1
Phenylalanine	5.2	5	3.6	0.4
Threonine	4	3.8	7.5	0.9
Tryptophan	1.3	1.2	1.1	0.1
Histidine	2.7	2.6	2.0	0.2
Arginine	3.7	7.2	2.7	1.5

BCAA: branch chain amino acid.

Sources: [1]MP Biomedicals; [2]Archer Daniels Midland; [3]Leprino Foods Dairy Products. [4]John AM, Bell JM. Amino acid requirements of the growing mouse. J. Nutr. 106: 1361-1367, 1976.

Probiotic work protocol

By Debjani Mitra, FSHN

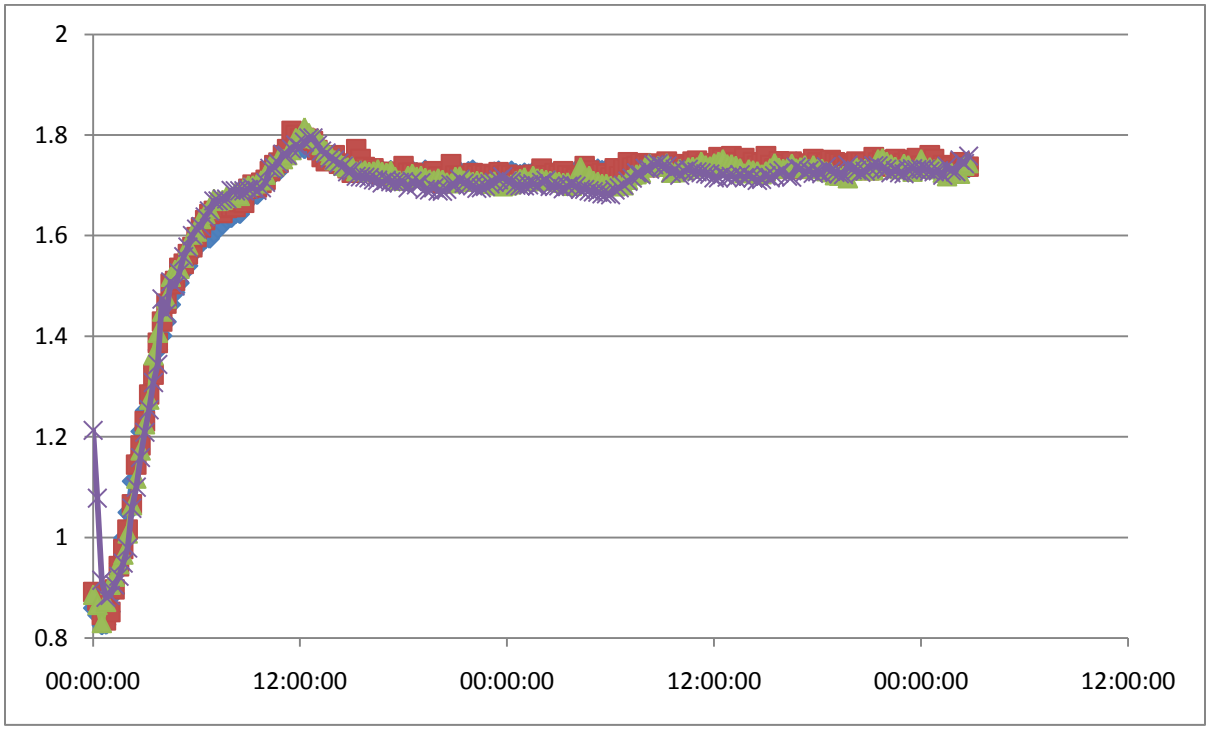
Strain: *Lactobacillus rhamnosus* GG (LGG) ATCC 53103

Revival of lyophilized culture

1) Prepare MRS broth tubes and agar plates.
2) Inoculate 2 MRS broth tubes with lyophilized LGG powder.
3) Incubate overnight at 37°C.
4) Streak out on MRS agar plates for purity check and incubate overnight at 37°C.
5) Pick out single isolated colony and inoculate a broth tube. Incubate overnight at 37°C.
6) Subculture into second broth tube. Incubate overnight at 37°C.
   Result: Pure and isolated colonies of LGG were seen which were used to prepare pure culture suspensions each time.

Growth curve plot

7) Start Bioscreen run with 50µL culture broth +150 µL MRS broth, incubate for 24h.
8) Note time taken to reach maximum cell concentration ("T") from growth curve.



Result: Culture reaches stationary phase by 14h.

Estimation of cell concentration. By track plating

9) Subculture into 300mL MRS broth in flask. Incubate at 37°C for 24h for cell count study.
10) Centrifuge cell suspension at time, dissolve pellet in 1X PBS (10mL).
Result: 600mL=11.8g of pellet.
11) Prepare 8 dilutions of culture broth in PBS (10^2, 10^4, 10^6, 10^7, 10^8, 10^9& 10^10)
12) Perform track plating of dilutions from 10^4, 10^6 to 10^10 and find cell concentration (cfu's/ml) .

Result: Cell concentration= 3*10^10cfu's/mL or
1.5*10^12cfu's/g

Viability testing of L. rhamnosus in mice feed

13) Dissolved cell pellet from step 11 will be used here.
14) Prepare mice feed in water in beakers: 40%for casein, 110% for soy.
15) Add 1mL of cell suspension.
16) Leave beaker at RT for 1-2 hour.
17) Collect total of 1g of sample from each of the 3 beakers. Samples will be collected from at least 3 random locations in the feed.
18) Sample is dissolved in 100mL PBS and vortexed to mix well (10^2). Then diluted serially in PBS (10^4, 10^6, 10^8, 10^10).
19) Perform track plating of dilutions from 10^2 to 10^10 and find cell concentration (cfu's/g of feed).

Result:

	1h	2h
Soy	1*10^10cfu's/mL	1.5*10^10cfu's/mL
Casein	2*10^8cfu's/mL	1*10^8cfu's/mL

Sample preparation every morning for the probiotic feed

20) 24h culture broth is centrifuged, pellet collected.
21) 1g of pellet is transferred aseptically to a tube and 5mL PBS is added to it to get a suspension.
22) Cell counts were taken of the 1g Lactobacillus pellet dissolved in 5mL PBS on 4/2/10 (mid-way of feeding trial) and showed 6*10^11 cfu's/ml.
23) Cell counts taken on 4/8/10 (end of feeding trial) showed 3*10^11 cfu's/ml.