Doctoral Thesis

Regulation of suppressor of cytokine signaling 1 in the intestine by dietary fibers

GERTRUDE CYNTHIA SITOLO

Graduate School of Biosphere Science
Hiroshima University

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GERTRUDE CYNTHIA SITOLO

Department of Biofunctional Science and Technology
Graduate School of Biosphere Science
Hiroshima University

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CERTIFICATE OF APPROVAL

We hereby certify that the doctoral dissertation of Ms. Gertrude Cynthia Sitolo, entitled "**Regulation of suppressor of cytokine signaling 1 in the intestine by dietary fibers**" was accepted in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Food Science and Nutrition**.

Takuya SUZUKI, Ph.D.
Academic supervisor
Professor in Food Functional Science
Food Science and Biofunctions Division
Graduate School of Biosphere Science
Hiroshima University

Asakawa MANABU, Ph.D.
Committee member on Final Examination
Professor in Marine Bioresource Chemistry
Food Science and Biofunctions Division
Graduate School of Biosphere Science
Hiroshima University

Hiroyuchi NAKANO, Ph.D.
Committee member on Final Examination
Professor in Food Microbiology and Hygiene
Food Science and Biofunctions Division
Graduate School of Biosphere Science
Hiroshima University

Yoshihiro SAMBONGI, Ph.D.
Dean
Professor in Microbial Biochemistry
Molecular and Applied Sciences Division
Graduate School of Biosphere Science
Hiroshima University

ABSTRACT

Fermentable dietary fibers, such as guar gum (GG) and its hydrolysate, partially hydrolyzed guar gum (PHGG) are known for their various health benefits particularly through their fermented microbial metabolites, short chain fatty acids (SCFAs). This study examined the effects of fermentable GG and PHGG on suppressor of cytokine signaling 1 (SOCS1), a negative regulator of inflammatory signaling on intestinal epithelial cells of mouse colon and human intestinal Caco-2 cells, focusing on the role of SCFAs. The results showed that GG fiber uses different mechanisms in regulating intestinal SOCS1. In the small intestine the fiber appears to upregulate SOCS1 in its intact form through TLR-2 and Dectin-1 pathways whereas in the colon, GG-mediated SOCS1 upregulation was influenced by microbial activity since antibiotic administration reduced SCFAs production and suppressed SOCS1 expression. However, specific structure of intact GG may be responsible for induction of SOCS1 protein expression in the small intestine since the hydrolysate could not upregulate SOCS1. Butyrate upregulated SOCS1 in Caco-2 cells and could be the possible candidate responsible for upregulation of SOCS1 *in vivo*. This study demonstrated that dietary interventions such as the use of PHGG/GG fiber increase intracellular levels of SOCS1 protein and may be an alternative approach in the fight against inflammatory disease.

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LIST OF ABBREVIATIONS

AU	Arbitrary unit
DF	Dietary fiber
GG	Guar gum
GPR	G protein-coupled receptor
IEC	Intestinal epithelial cell
IL	Interleukin
INF	Interferon
JAK	Janus kinase
NF- κB	Nuclear factor-κB
PHGG	Partially hydrolyzed guar gum
SCFA	Short chain fatty acids
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activators of transcription
TLR	Toll-like receptor
Tregs	Regulatory T cells

CHAPTER 1

INTRODUCTION

1.1 Background

The intestinal epithelium is an effective and dynamic compartmentalized organ that maintains a distinct physical and biochemical barrier, pertinent for a constant state of homeostasis. The epithelium selectively permits entry of nutrients while maintaining an effective barrier against microbes, antigens and toxins. Because intestinal epithelium continuously faces the luminal noxious molecules, it may lead to loss of barrier function and innate immunity thereby triggering inflammatory responses. Initiation and progression of intestinal inflammation involve several activities but recruitment of neutrophils to the site of inflammation is the basis of the inflammatory response. In the process, pathogens stimulate a proinflammatory cascade producing different inflammatory cytokines which leads to further inflammatory pathways culminating into mucosal inflammation (Suzuki, 2013; Coleman and Haller, 2018). Thus, suppression of proinflammatory cytokines are indispensable for intestinal homeostasis.

Suppressor of cytokine signaling1 (SOCS1) is a member of a large superfamily of intracellular proteins that regulate cytokine signal transduction as negative feedback molecules. SOCS1 exerts its effect on Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signaling pathway by directly inhibiting catalytic activity of JAK tyrosine kinase. Cytokines mediate many physiological processes including proliferation, differentiation, innate and adaptive responses through intracellular signaling cascades involving JAK/STAT pathways (Krebs and Hilton, 2000). Accumulating evidence suggest that these pathways are tightly regulated such that deficiency of SOCS1 leads to aberrant

signaling transduction which provoke excessive proliferation of cytokines often resulting into inflammatory and tumorigenic diseases (Alexander et.al., 1999; Horino et.al., 2008). It is therefore self-evident that SOCS1 is crucial for normal functioning of cytokine signaling which eventually contributes to regulation of inflammation and maintenance of intestinal immune homeostasis.

Non-digestible carbohydrates such as dietary fibers (DFs) confer several health benefits to humans, including establishments and maintenance of intestinal health. DFs include remnants of edible plant cells, polysaccharides, lignin and associated substances and are resistant to digestion in the small intestine but available as substrates in the colon. Consumption of DFs influences various physiological effects on human health, subject to their physiochemical properties such as solubility, viscosity and fermentability. The physiological effects of the fiber-rich diets are often associated with the increased production of short chain fatty acids (SCFAs) through microbial fermentation by gut microbiota. Several studies have demonstrated the effect of SCFAs on health as they modify cellular processes, such as gene expression, differentiation, proliferation, chemotaxis and apoptosis through different mechanisms (Dai and Chau, 2017; Deehan et. al., 2017).

Guar gum (GG) fiber, a water-soluble dietary fiber obtained from guar seeds is one of the most studied dietary fiber with various applications in the food industry. GG fiber is composed of galactomannan and characterized by high viscosity and high fermentability (Yoon et. al., 2008). Previous study from our laboratory, established that GG and partially hydrolyzed guar gum (PHGG) increased the luminal SCFAs production which in part may have contributed to suppression of inflammatory immune responses and reduced barrier defects in the colon of colitic mice (Hung and Suzuki, 2016). Furthermore, it was demonstrated that intact GG directly upregulated SOCS1 expression, regulating

inflammatory responses in the small intestine through activation of toll-like receptor (TLR)-2 and dectin-1 signaling pathways (Van Hung and Suzuki, 2017). However, the regulation of the intestinal SOCS1 expression by GG was not validated *in vivo*. In addition, the effects of GG and the underlying mechanisms could differ between small and large intestines, because GG passes the small intestine with its intact structure but is easily metabolized by intestinal microorganisms in the colon.

1.2 Purpose

The purpose of this study was to investigate the role of dietary fibers particularly GG on regulation of intestinal SOCS1 and examine microbial activity *in vivo. In vitro* study used human intestinal Caco-2 cells to verify the impact of SCFAs on SOCS1 expression. An outline of of the content of this dissertation is presented as follows:

Chapter 1: This chapter presents background information and the purpose of this study.

Chapter 2: In this chapter, all the fundamentals and literature review are laid-out. The chapter carries information about the intestinal epithelium, maintenance of intestinal homeostasis, regulation of chronic information through cytokine signaling, dietary fibers and their health benefits.

Chapter 3: Guar gum fiber uses different mechanisms to upregulate SOCS1 in the small intestine and colon in mice. This chapter aimed at validating upregulation of SOCS1 expression in the small intestine and investigate the effect of the fiber on SOCS1 expression in the colon.

Chapter 4: Fermentable fibers increase SCFAs and upregulate SOCS1 in mice colon. This chapter aimed at comparing the influence of GG and PHGG on intestinal SOCS1 expression and SCFAs production.

Chapter 5: Antibiotic administration suppresses guar gum fiber-mediated SOCS1

expression in the colon. This chapter examined the role of microbial activity with associated SCFAs production on SOCS1 expression.

Chapter 6: Butyrate influences SOCS1 upregulation in intestinal Caco-2 cells. This chapter assessed the role of SCFAs on SOCS1 expression using human intestinal Caco-2 cells.

Chapter 7: This chapter carries the summary of the study and future studies to be undertaken.

CHAPTER 2

FUNDAMENTALS AND LITERATURE REVIEW

2.1 The intestinal epithelium

The gastrointestinal epithelium is a single layer of intestinal epithelial cells (IECs) lining the small and large intestines which functions as a barrier surface protecting mammalian host from the external environment. IECs form a physical and biochemical barrier to keep away commensals and pathogenic microorganisms. The presence of pattern-recognition receptors on the cell surface aid in sensing microbial stimuli triggering the cells to participate in a series of coordinated immune responses, appropriate for tolerance and anti-pathogenic responses. This ensures reinforcement and maintenance of immunomodulatory function which is influential for development and homeostasis of mucosal immune cells (Peterson and Artis, 2014).

The intestinal epithelium is dynamic and plays a crucial role in reinforcing barrier function, establishing and maintaining intestinal homeostasis by segregating pathogenic microbes while regulating commensals and host immune system. However, it is a teamwork achieved in conjunction with additional specialized IEC lineages. Adjacent IECs are joined by specific intercellular tight junction proteins which provide selective permeable barrier, limiting entry of luminal deleterious molecules while permitting absorption of nutrients and water. The proteins include zonula occludens, occludin, the claudin family and joint adhesion molecules (Suzuki, 2013; Van Itallie and Anderson, 2014).

Overlaying the IECs is the mucus layer secreted by goblet cells. The outer proteoglycan gel mucus layer is habitat for intestinal microbiota while the inner layer is devoid of microbiota and houses molecules such as antimicrobial peptides (AMPs), secretary immunoglobulin A (sIgA), microbial-associated molecular patterns and others. AMPs come from Paneth cells and are responsible for establishing and maintaining intestinal microbiota in addition to function in

host defense. sIgA block microbial translocation across the epithelial barrier and are produced by Lipopeptide plasma cells (Peterson and Artis, 2014), propagating teamwork in promoting exclusion of bacteria from epithelial surface. T cells and antigen presenting cells, dendritic cells (DCs) and macrophages are found beneath the epithelial lining in the lamina propria. These cells come into play when Microfold and goblet cells facilitate transport of luminal antigens across epithelial barrier to DCs and microphages sample through transepithelial dendrites (Coleman and Haller, 2018). The collective functions of all these molecules and cells provide a dynamic barrier, protecting the host from infection and endless exposure to potentially inflammatory stimuli. Figure 2.1 depicts the interplay of various cells and molecules to maintain intestinal epithelial barrier integrity.

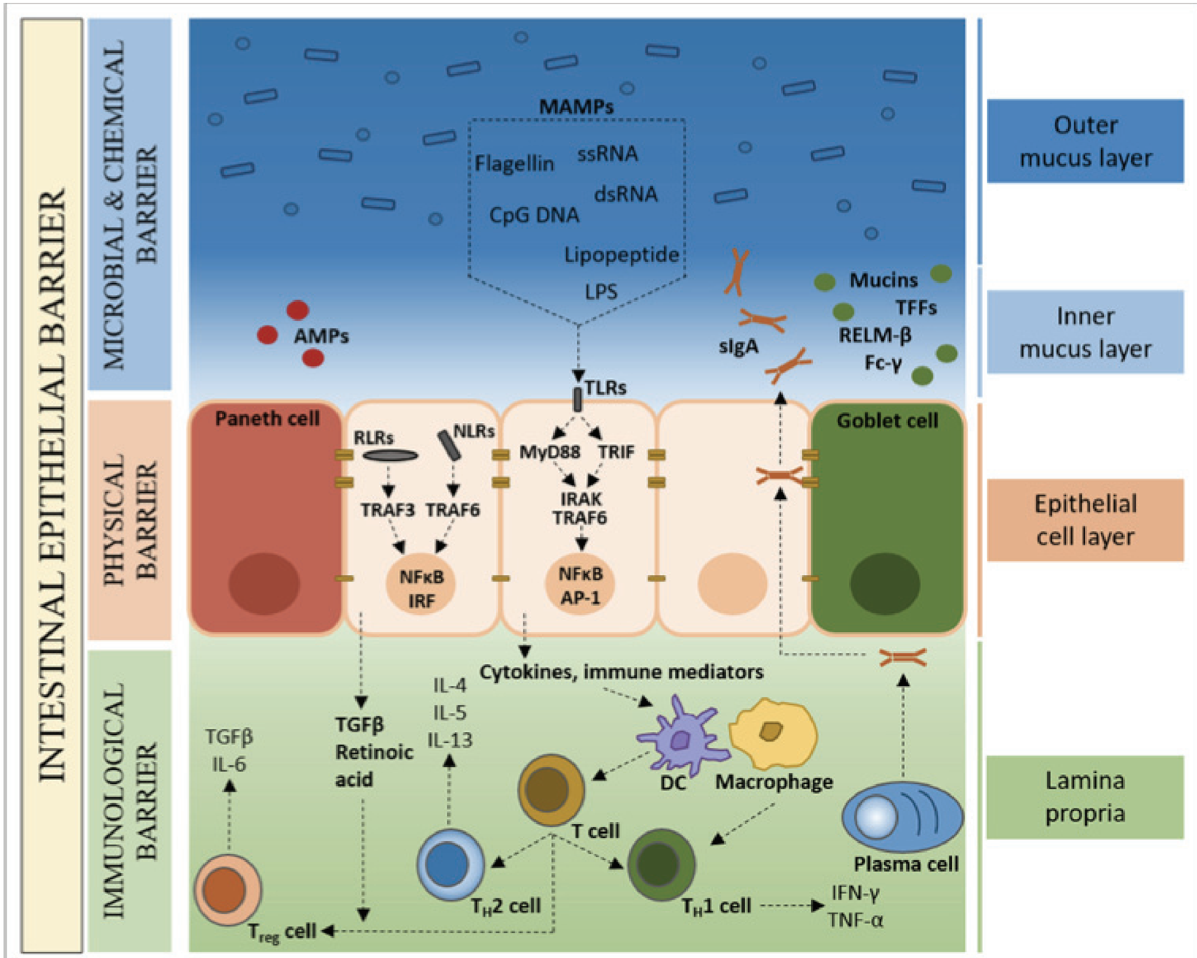


Figure. 2.1 Colonic intestinal epithelium and associated cells (source: Coleman and Haller, 2018)

2.1.2 **Intestinal homeostasis**

The intestinal mucosa works under intense conditions and relies on tight cellular and molecular control of mechanisms as means of adaptation to constant antigen pressure. Although a well-established intestinal barrier is impermeable to commensals and other intestinal microorganisms, there is a likelihood that a certain number of pathogens and toxin may transverse the epithelium thereby triggering immune response. The collective efforts of both immune and non-immune cells (epithelial, mesenchymal, endothelial and nerve cells) exchange regulatory signals through production of mediators such as cytokines and initiation of cell event i.e. proliferation and apoptosis, which eventually facilitate and amplify cell interaction as well as inflammation (Peterson and Artis, 2014).

Under normal physiological state, inflammation is a regulated process characterized by infiltration of mucosal tissue by leukocytes, due to presence of foreign antigen and the process terminates once the antigen is eliminated. Cytokines, as mediators, initiate a crosstalk between cells through their cell surface receptors and regulate numerous events including trafficking of leucocytes and apoptosis. Once the antigen is eliminated intestinal immune response is suppressed. The normalization involves immunosuppressive cytokines such as Interleukin (IL)-10 and transforming growth factor (TGF-β) produced by regulatory T cells (Treg) which limit proliferation of lymphocytes and control of activated macrophages and other immune cells (Coleman and Haller, 2018). Together, the epithelial barrier, supportive cells and mucosal immune system participate in intestinal immune response and maintain intestinal homeostasis.

2.1.3 Chronic intestinal inflammation.

Dysregulation of intestinal homeostasis result in chronic inflammation when the host loses tolerance towards normal enteric microflora. Studies have linked chronic intestinal inflammation to disrupted barrier integrity and increase in mucosal permeability (Hung and

Suzuki, 2016; Pastorelli et. al., 2013). Furthermore, increased production of cytokines in favor of proinflammatory cytokines, defects in toll-like receptor (TLR) signaling and immunosuppressive mechanisms are evident in the inflammatory diseases (Neurath, 2014). During these intestinal inflammatory responses, nuclear factor-kappa B (NF-κB) and mitogen activated protein kinase pathways are of particular interest as they respond to extracellular signaling and alter gene expression. Specifically, NF-κB, as a cytoplasmic transcription factor is a key regulator of inflammatory gene expression. Cellular stimulation by signals related to pathogens and stress activates NF-κB which translocate into the nucleus to regulate expression of various genes including those of proinflammatory cytokines and chemokines i.e. tumor necrosis factor α (TNF-α), interleukin (IL) IL-1β, IL6, IL-8, cell surface receptors, adhesion molecules and inflammatory enzymes (i.e. Phospholipase A2, inducible nitric oxide synthase [iNOS], cyclooxygenase-2 [CoX-2]) among others (Dinarello, 2000, Tak and Firestein, 2001).

2.2 **Regulation of chronic inflammation through cytokine signaling.**

Progression of inflammation is dependent on action of pro-inflammatory cytokines. Treatment strategies mostly focus on inhibiting production of proinflammatory mediators as well as suppressing initiation of inflammatory responses. One of the most practical ways is to suppress positive signaling pathways of proinflammatory cytokines, such as Janus kinase (JAK)/ signal transducers and activators of transcription (STAT) pathway (Hanada and Yoshimura, 2002).

2.2.1 **Suppressor of cytokine signaling 1**

Cytokine signal transduction by JAK/STAT pathway is regulated by a family of proteins referred to as suppressor of cytokine signaling (SOCS) proteins. These intracellular proteins are an eight-member large superfamily ranging from SOCS 1 to SOCS7 and cytokine-induced STAT inhibitor (CIS) with a central Scr-homology 2(SH2) domain and C-terminal

SOCS box. Expression of these proteins in cell lines, is a response to stimulation by various cytokines and their overexpression inhibit cytokine signaling thus negatively regulating cytokine signaling (Yoshimura et. al., 2012).

Among the SOCS family members, SOCS1 has been investigated extensively. SOCS1 exerts its effect on JAK/STAT signaling pathway by directly inhibiting catalytic activity of JAK tyrosine kinase. SOCS1 proteins possess a kinase inhibitory region which function as a pseudo-substrate and is essential for suppression of cytokine signaling (Krebs and Hilton, 2000). Studies have reported the significance of these proteins in maintaining homeostasis. Mice lacking SOCS1 developed a complex fatal neonatal disease and were hyperresponsive to viral infection typically induced by interferon γ (IFN γ) (Alexander et. al. 1999). Similarly, SOCS1 deficiency led to inflammation and dysbiosis of gut microbiota consequently inducing a pro-colitogenic environment (Gendo et. al., 2018). Kimura et. al., (2005) showed that SOCS1 suppressed LPS-induced IL-6 production by regulating the JAK/STAT pathway. In mouse colon, SOCS1 is suggested to prevent DSS-induced colitis by inhibiting IFNγ/STAT1 signaling and mediates in intestinal tolerance involving a mechanism distinct from IL-10 and Tregs (Horino et. al., 2008; Chinen et. al., 2011). Moreover, in a clinical study, low levels of SOCS1 expression was considered a poor prognostic indicator in gastric cancer (Li et. al., 2015).

2.3 Dietary fibers

Dietary fibers (DFs) is a broad term for a category of non-digestible food ingredients comprising of oligosaccharides, non-starch polysaccharides, analogous polysaccharides and lignin with associated health benefits. They are resistant to digestion by endogenous enzymes in the small intestine but are subjected to fermentation by gut microbiota in the colon. To qualify as a dietary fiber, edible carbohydrate polymer should (a) occur naturally in plant based foods such as fruits, vegetable, cereals and legumes; (b) be obtained from food raw materials

by means of physical, enzymatic or chemical methods and have physiological benefits; (c) or can be synthetic carbohydrate polymers also with physiological benefits which has been demonstrated by generally accepted scientific evidence (Makki et. al., 2018). DFs are heterogenous in nature, subdivided into non-starch polysaccharides (NSPs), resistant starch (RS) and resistant oligosaccharides (RO) mostly from plant based, but may also come from bacteria or fungi (figure 2.2). Further classification is based on origin, chemical composition, physiochemical characteristics and to a lesser extent, degree of polymerization i.e. chain length (Deehan et. al., 2017).

DFs vary in their physiochemical properties to the extent that a slight difference can influence the physiological effect since the physiological effect depends on their physiochemical properties. Among others, the properties include solubility, viscosity and fermentability. Based on solubility, dietary fibers are either soluble i.e pectin, gums and polysaccharides or insoluble i.e cellulose, hemicellulose and lignin. This property is responsible for technological functionality and physiological effect. Viscosity is mainly associated with most soluble fibers such as gums, pectins, psyllium and β-glucans. In the gastrointestinal tract, the viscous nature of these soluble fibers allows them to respond more like solids than liquids giving them an edge in delayed gastric emptying as evidenced with ingestion of fiber-rich diet. Although solubility and viscosity influence functionality of DFs, fermentability is of particular importance for the purpose of modulating gut microbiota and is also a major determinant of bacterial metabolites which are associated with various health benefits to the host. On the contrary, insoluble fiber are less fermentable and mostly increase fecal bulk and water holding capacity. (Dai and Chau, 2017; Gill et. al., 2018; Mudgil and Barak, 2013).

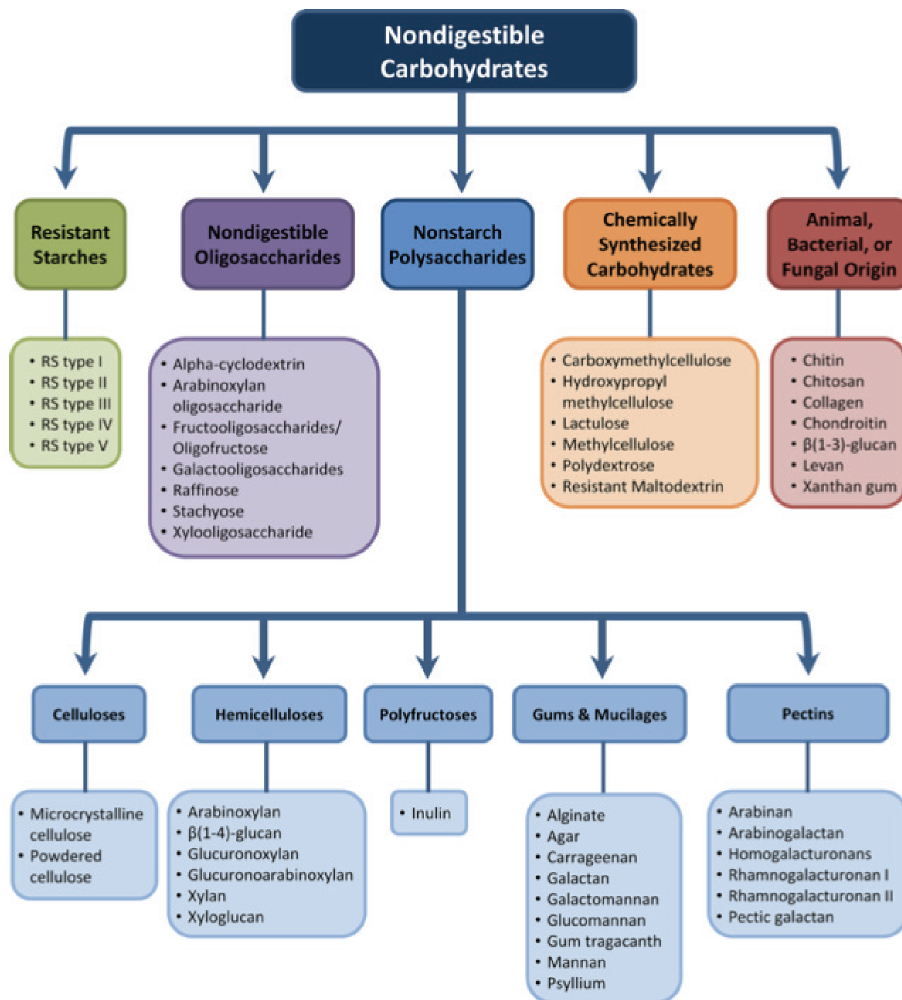


Figure 2.2 Classification of non-digestible carbohydrates (Source: Deehan et. al., 2017)

2.3.1 Healthy benefits of dietary fibers.

Dietary fibers have been studies extensively in recent years because of their beneficial physiological effects. Fermentable fibers change composition and function of microbial communities residing in the gut. The symbiotic relationship between gut microbiota and the host has a pivotal role in physiological functions to do with energy metabolism, barrier integrity and the immune system (Kaczmarczyk et. al., 2012; Wang et. al., 2018).

Fiber-rich diets have been reported to be effective in treatment of postprandial glycemia and diabetes. Recent studies showed that DF intake improved fasting blood glucose concentration and glycated hemoglobin A1c levels, enhancing overall glucose homeostasis in participants with type 2 diabetes (Gibb et. al., 2015; Zhao et. al., 2018). Likewise, in a comparative study between European and rural African children, DF intake increased microbial richness and biodiversity towards SCFAs producing bacterial communities including *Firmicutes* and *Bacteroides* species (De Fillipo et. al., 2010). David et. al., (2014) shared similar sentiments with an increase in production of SCFAs which are believed to be at the center of the immunomodulatory effects.

SCFAs are main end products of intestinal microbial fermentation of non-digestible carbohydrates. Most common ones are acetate, propionate and butyrate which are found in abundance in the caecum and the proximal colon where bacteria are densely populated. Their uptake by intestinal epithelial cells is either passive (in non-ionized form) or active transport, in ionized form, which involves monocarboxylate transporter 1 (MCT-1) or sodium-dependent monocarboxylate transporter 1 (SMCT-1) (Cumming et. al., 1987; den Besten et. al., 2013) Production of SCFAs reduce luminal pH thereby inhibiting pathogenic microorganisms while increasing absorption of minerals such as calcium, magnesium (Coudray et. al., 2003; Rios-Covian et. al., 2016; Shigematsu et. al., 2004). These SCFAs modify several cellular processes including gene expression, differentiation, proliferation, chemotaxis and apoptosis through

inhibition of histone deacetylase (HDAC), activation of G-protein coupled receptors (GPRs), stabilization of hypoxia-inducible factor and can also serve as energy substrates. Butyrate is preferred energy source for colonocytes and is crucial for maintenance of colonic epithelium as it affects proliferation, differentiation and apoptosis of these cells, whereas acetate and propionate are transported to peripheral tissues and the liver via the portal vein where they act as signaling molecules and regulate various biological processes (Correa-Oliveira et, al., 2016).

Studies have demonstrated the influence of SCFAs in homeostatic regulation. Macia et. al. (2015), demonstrated that SCFAs through GPR43 and GPR109a activated inflammasome which promotes conversion of pro-IL-8 to IL-18 thereby regulating inflammation. Butyrate, through GPR109a- signaling facilitated anti-inflammation properties in macrophages and dendritic cells in mice colon epithelium and enabled them to induce differentiation of Treg cells and IL-10 producing T cells (Singh et. al., 2014). In another study, oral supplementation of butyrate exhibited potency in attenuating colitis *in vivo* whereas *in vitro,* butyrate decreased proinflammatory cytokines TNF-a, IL-6 and IL-12 while upregulating IL-10 through reversal of histone acetylation and blocking NF-kB pathway (Lee et.al. 2017). Similarly, SCFAs are capable of stabilizing hypoxia-inducible factor-1 in intestinal epithelial cells by increasing oxygen consumption in the cells which leads to depletion of intracellular oxygen, triggering HIF-1 stabilization (Kelly et. al., 2015).

2.3.2 Guar Gum

Guar gum (GG), *Cyamopsis tetragonolobus*, is a drought-resistant annual leguminous plant, originally from India and Pakistan. It is a galactomannan polysaccharide of the endosperm of guar seeds with approximately 10,000 polymeric molecules. The structural backbone of GG fiber is made up of (1→4)-linked-β-D-manopyranose and (1→6)-linked-α-D-galactose side chains attached at O-6 (figure 2.3). The molar ration of galactose to mannose depends on origin but falls in the range of 1:1.5-1.8. Galactomannans are found in a variety of

natural sources and some plants which contain them include locust bean gum, soybeans, coffee beans, coconut meat, alfalfa seeds, sugar beets, and even pineapple (Ellis et. al., 2001; Yoon et. al., 2008)

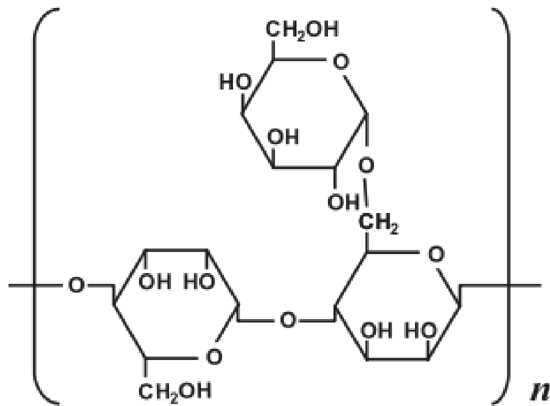


Figure. 2.3 Structure of guar galactomannan (source: Yoon et. al., 2008)

The fiber is highly soluble in water and forms viscous solutions with less than 1% of food weight, earning its commercial use by the food industry as a thickener and stabilizer (Theuwissen and Mensink, 2008). However, due to high viscosity of GG, its utilization as a dietary fiber supplement is limited, especially liquid food products. As such GG is enzymatically hydrolyzed to yield a partially hydrolyzed guar gum fiber (PHGG) (Fig 2.4). PHGG is produced through an endo-β-mannanase hydrolysis and carries same structural features as GG. Its molecular size is one-tenth the original length of GG. As a white powder, PHGG is soluble, colorless, tasteless and transparent in water solutions. Reducing $\beta 1 \rightarrow 4$ mannosidic linkages in PHGG reduces its viscosity and is advantageous because the fiber can be used in various food products such as juices, shakes, cereals, soups, yoghurt, meal replacements, and baked products. It can also be used in enteral nutrition products, if need be, by clinical settings (Mudgil and Barak, 2014; Slavin and Greenberg, 2003; Yoon et. al., 2008).

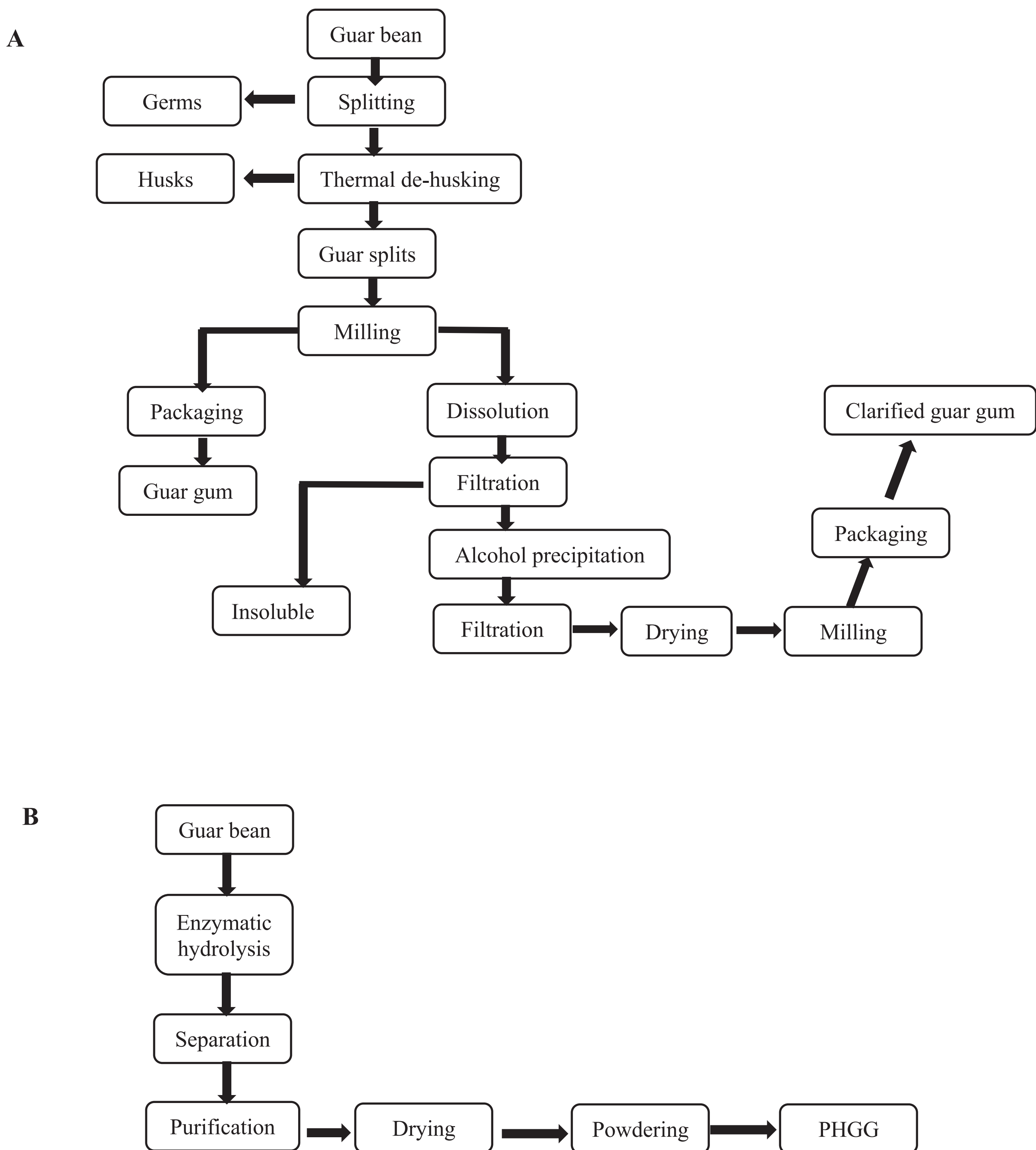


Fig. 2.4 Flow diagram for industrial manufacturing of (A) GG and (B) PHGG (adapted from Mudgil et. al., 2014; Yoon et. al., 2008)

Both clinical and animal studies have documented the physiological effect of PHGG and GG fibers such as cholesterol lowering, control of diabetes and digestive system improvement. For example, Dalla'Ahba and others, (2013), showed that supplemental PHGG improved cardiovascular and metabolic profile as evidenced in reduced waist circumference, glycated hemoglobin A1c, urinary albumin excretion, and serum *trans*-fatty acids. Apart from that, supplemental PHGG had a prebiotic effect in healthy subjects when it improved the growth and activity of beneficial intestinal bacteria (Ohashi et. al., 2015). Prebiotics are non-digestible food ingredients that stimulate growth or activity of protective endogenous microflora and enhance host's defence system, however, not all dietary carbohydrates have prebiotic activity. A food substance is prebiotic if it can resist gastric acidity, gastrointestinal absorption, hydrolysis by brush-border enzymes; can be fermented by specific intestinal microflora and is capable of selectively stimulating growth and activity of the gut microbiota (Panesar and Bali, 2016).

In animal models, GG and its hydrolysate have been shown to be effective in amending intestinal barrier defects, attenuating inflammation, modulating luminal microbiota and increasing SCFAs production (Hung and Suzuki, 2016; Hung and Suzuki, 2018a; Naito et. al., 2006; Takagi et. al., 2016). In addition, Van Hung and Suzuki (2017) unveiled another effect of GG fiber in upregulation of a cytokine signalling suppressor, SOCS1, in the small intestine via activation of TLR2 and dectin-1 pathways with a remarkable anti-inflammatory activity.

CHAPTER 3

GUAR GUM FIBER USES DIFFERENT MECHANISMS TO UPREGULATE SOCS1 IN THE SMALL INTESTINE AND COLON IN MICE

3.1 **Introduction**

Consumption of dietary fiber modulate the composition and metabolic functions of gut microbiota, which provide a link between diet and different physiological states through generated microbial products, such as SCFAs. These compounds influence the intestinal immune system to constantly maintain a delicate balance between immunity to pathogens and tolerance to commensals, in a state of intestinal homeostasis (Kumar et. al., 2012; Holscher, 2017). The intestinal mucosa relies on tight cellular and molecular control of mechanisms as means of adaptation to constant antigen pressure. Thus, aberrant signaling in control mechanisms that control cellular processes attracts production of proinflammatory cytokines which lead to intestinal inflammation and then to inflammatory bowel diseases (Neurath, 2014).

SOCS1 regulates cytokine signal transduction along the JAK/STAT pathway. Cytokines mediate various physiological processes including proliferation, differentiation, through their cognate surface receptors which initiate intracellular signal transduction cascades changing the transcriptional profile of a cell when JAK/STAT pathway is activated (Krebs and Hilton, 2000, Linossi et. al., 2013). Studies have shown that SOCS1 is a critical regulator of signal transduction. Mice developed a complex fatal neonatal disease and were hyperresponsive to viral infection typically induced by IFNγ due to SOCS1 deficiency (Alexander et. al. 1999). In a different study, SOCS1 is suggested to prevent DSS-induced colitis in mouse colon, by inhibiting IFNγ/STAT1 signaling and mediating in intestinal

tolerance without relying on IL-10 and Tregs (Horino et. al., 2008; Chinen et. al., 2011). Thus, SOCS1 has anti-inflammatory properties.

Accumulating evidence suggests that supplemental non-digestible carbohydrates such as dietary fibers have various beneficial effects on our health. Guar gum (GG) fiber, a water-soluble dietary fiber, is obtained from guar seeds, mainly composed of galactomannan, and characterized by high viscosity and high fermentability (Yoon et. al., 2008). Previous study by a research group in our laboratory established that GG and PHGG increased the luminal SCFAs production which in part may have contributed to suppression of inflammatory immune responses and reduced barrier defects in the colon of colitic mice (Hung and Suzuki, 2016). Another study demonstrated that intact GG directly upregulated SOCS1 expression, regulating inflammatory responses in the small intestine through activation of TLR-2 and dectin-1 signaling pathways (Van Hung and Suzuki, 2017) However, the regulation of the intestinal SOCS1 expression by GG was not validated *in vivo* yet. In addition, the effects of GG and the underlying mechanisms could differ between small and large intestines, because GG passes the small intestine with its intact structure but is easily metabolized by intestinal microorganisms in the colon.

The study in this chapter aimed at validating GG fiber influence on SOCS1 expression in the small intestine and investigating its effect on colonic SOCS1 expression in healthy mice.

3.2 Materials and methods

3.2.1 Materials

Mouse monoclonal anti-SOCS1 antibody was purchased from MERCK (Darmstadt, Germany). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG was purchased from SeraCare (Milford, MA, USA). GG was provided by Taiyo Kagaku (Yokkaichi, Japan). All other chemicals were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

3.2.2. Mice and diets

Animal study protocols were pre-approved by the Animal Use Committee of Hiroshima University, and all mice were maintained in accordance with the Hiroshima University guidelines for the care and use of laboratory animals (authorization no. C15-10-3). The experiment used male BALB/c mice (7-week-old, ~21 g) obtained from Charles River Japan (Yokohama, Japan). Mice were housed in cages (3 mice/cage) under controlled temperature of 22 ± 2°C; relative humidity of 40–60%; and light exposure from 08:00 to 20:00. They were allowed to acclimatize to the laboratory environment with free access to an AIN-93G-formula control diet (Table 3.1) and distilled water *ad libitum* for a week prior to the start of the experiments.

3.2.3 Experimental design

Mice (n = 18) were randomly divided into three groups: control, 5% GG, 10% GG (n = 6 per group). The 5% and 10% GG groups were fed a diet containing 5% and 10% (w/w) GG fiber, respectively, for 14 d of the experimental period. GG was added to the control diet by substitution for an equal amount of starch. The control group was fed the control diet during the experimental period. Mouse body weight was evaluated on a daily basis throughout the

experiment. At the end of experiments, mice were killed by exsanguination under isoflurane anesthesia. The small intestine and colon were collected, and the intestinal epithelial cells were isolated as described below. Ceca were collected for tissue examination and organic acid analysis of cecal contents.

Table 3.1. Composition of test diets

Ingredient	Control diet[a]
	g/kg diet
Casein[b]	200
α-Corn starch[c]	529.5
Sucrose	100
Soybean oil	70
Choline bitartrate	2.5
L-Cystine	3
Mineral mixture[d]	35
Vitamin mixture[d]	10
Cellulose[e]	50

[a] GG and PHGG-containing diets were prepared by substitution of GG and PHGG for equal amounts of starch.

[b] Casein (ALACID; New Zealand Daily Board, Wellington, New Zealand).

[c] α-Corn starch (Amylalpha CL; Chuo-Shokuryou Co. Ltd, Aichi, Japan).

[d] Mineral and vitamin mixtures were prepared according to the AIN-93G formulation.

[e] Powdered cellulose (Just fiber; International Fiber Corporation, New York, USA).

3.2.4 Isolation of IECs

IECs were isolated according to Hung and Suzuki, (2018a). Briefly, small intestine and colonic tissues, about 3 cm long, were washed with saline and cut into small segments. The pieces were transferred into isolation buffer (3 mL) comprising of Ca^{2+}- and Mg^{2+}-free HBSS supplemented with 0.1μM DDT, 5 μM EDTA, protease and phosphatase inhibitors. To maximize release of the IECs, the mixture was incubated for 1 hour with gentle agitation and then vigorous shaking. The supernatants were collected and centrifuged at 5, 000 x *g* for 2 min at 4°C to sediments the pellets. The pellets were prepared for immunoblot analysis.

3.2.5 Immunoblotting

Isolated IECs were homogenized in appropriate volume of lysis buffer containing 1% (w/v) sodium dodecyl sulfate (SDS), 1% (v/v) Triton X-100, and 1% (w/v) sodium deoxycholate in 30 mM Tris with protease and phosphatase inhibitors (pH 7.4), by using a Polytron®-type homogenizer (KINEMATICA AG, Lucerne, Switzerland). Immunoblotting analysis of SOCS1 was performed as previously described by Azuma et. al., (2013). Protein concentration was measured using BCA method. IEC pellets were mixed with Laemmli sample buffer (3X concentrated) containing 6% (wt:v) SDS, 30% glycerol (v:v), 155 (v:v) 2-mercaptoethanol and 0.02% (wt:v) bromophenol blue in 188 mmol/L Tris (pH6.8) and subjected to heat (95°C) for 5 minutes. 20 μg proteins were separated by SDS-PAGE and transferred on polyvinylidene difluoride membrane. The blots were developed using the ECL chemiluminescence method (Amersham Imager 680, Uppsala, Sweden). The SOCS1 protein expression was normalized to total protein measurements on polyvinylidene difluoride membranes by Ponceau S (Gilda and Gomes, 2013). Quantification of protein expression was done by densitometric analysis of specific bands on the immunoblots using ImageJ software (NIH, Bethesda, MD, USA).

3.2.6 SCFAs analysis

SCFAs in cecal contents were determined by ultra-performance liquid chromatography–mass spectrometry. Cecal contents were homogenized 9X the volume of distilled water and centrifuged at 13, 000 x *g* for 10 min at 4 °C. The resulting supernatant was deproteinized with 50 % (v/v) acetonitrile and incubated with 50 mM 3-nitrophenylhydrazine, 30 mM water-soluble carbodiimide, and 6% pyridine. The chemical derivatives of SCFAs were quantified by ultra-performance liquid chromatography–mass spectrometry (Waters, Milford, MA, USA).

3.2.7 Calculations and Statistical analysis

Body weight gains of mice were calculated using the following formula: body weight gain (mg/d) = [final body weight – initial body weight] (mg)/experimental period (d). The cecal tissue weights were shown as values standardized by body weight. All data are presented as the means and standard error of the means (SEM). Statistical analyses were performed by one-way analysis of variance followed by the Tukey-Kramer post-hoc test. Pearson correlation coefficients were calculated to assess the relationship between cecal SCFAs and colonic SOCS1 expression. All statistical analyses were conducted with a significance level of $\alpha = 0.05$ ($P < 0.05$) using Statcel 3 program (OMS Publishing, Saitama, Japan).

3.3 Results

3.3.1 Body parameters

Feeding mice 5% and 10% GG diets showed that diets had no impact on body weight gain of mice (Fig. 3A). However, the weights of cecal tissue and contents in the 5% and 10% GG groups increased significantly compared to the control group (Fig. 3B-C).

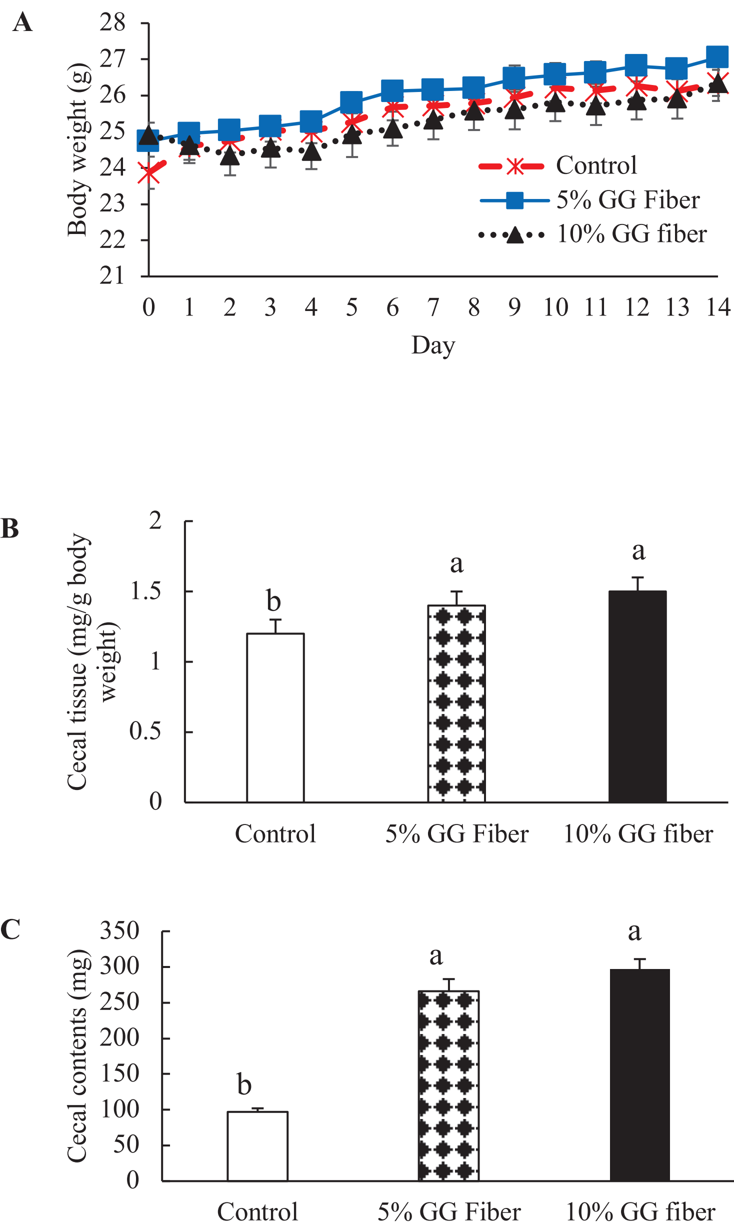


Figure 3.1 Effect of GG fiber on body parameters. (A), body weight. (B), cecal tissue weight. (C), cecal contents. The values are means ± standard error measurement (SEM) n = 6. Means without a common letter differ (Tukey-Kramer post-hoc test, $P < 0.05$).

3.3.2 Intestinal expression of SOCS1 and cecal SCFAs profile.

Previous study in our laboratory demonstrated that GG fiber increases SOCS1 protein expression in the small intestine (Hung and Suzuki, 2017) but the regulation was not validated *in vivo*. Besides, GG is fermented by gut microbiota to yielding SCFAs. In this experiment, feeding GG increased SOCS1 protein expression in epithelial cells of both the small intestine and colon (Fig. 3.2). In the small intestine, SOCS1 expression of 10% GG group was higher than that in the control group. In the colon, the SOCS1 expression of 5% and 10% GG groups significantly increased compared to the control group.

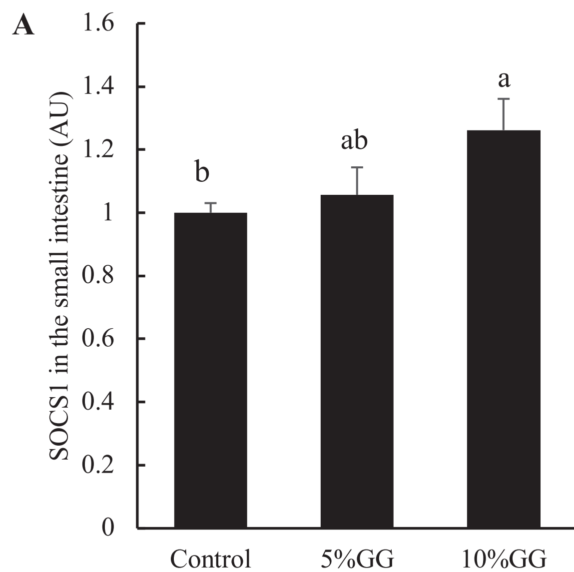
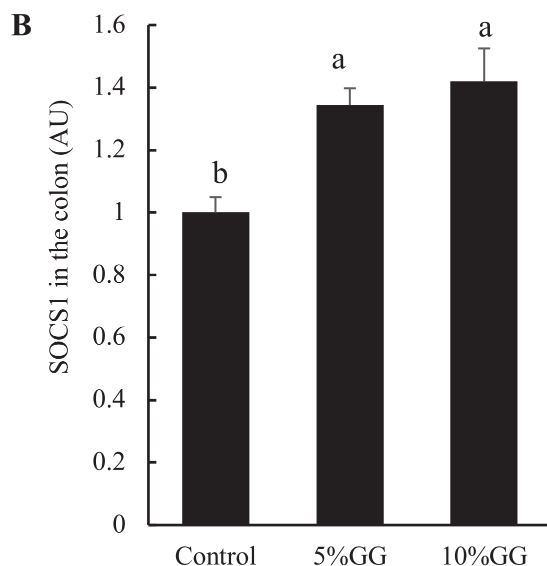


Figure. 3.2 SOCS1 expression in the small intestine and colon of mice fed the control, 5% GG, and 10% GG. Protein expression of SOCS1 levels in the epithelial cells of small intestine (A) and colon (B) was determined by immunoblot analysis. The values are means ± SEM (n = 6). Means without a common letter differ (Tukey-Kramer post-hoc test, $P < 0.05$).



SCFAs are major metabolites of undigested foods including GG by intestinal microorganisms. Cecal pool of acetate, propionate, *n*-butyrate, and *iso*-valerate in the 5% and 10% GG groups were significantly higher than those in the control group (Fig. 3.3). The changes were also observed in the 5% GG group for *iso*-butyrate in comparison to the control group.

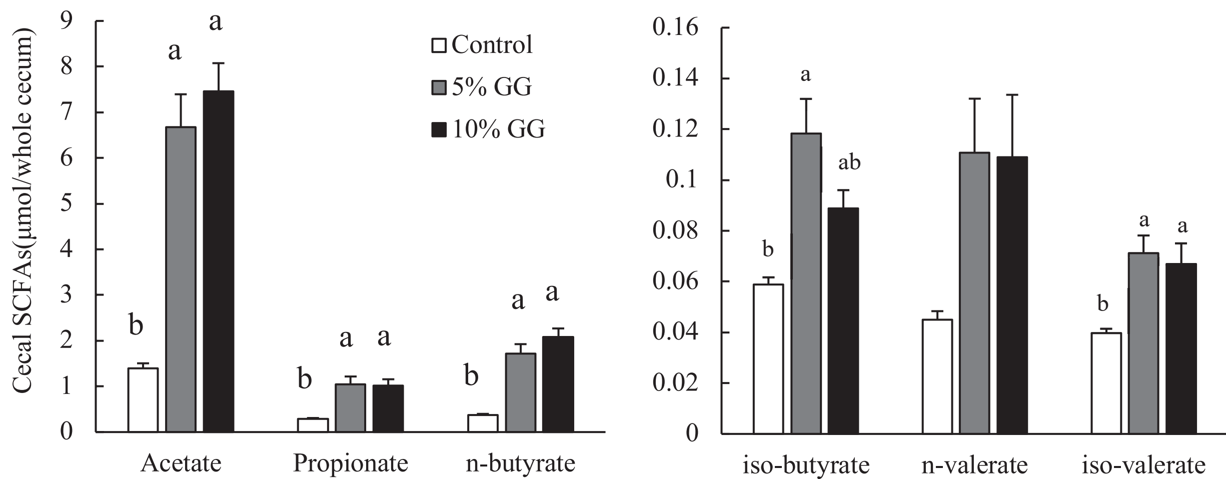


Figure. 3.3 SCFAs pool in the cecum of mice fed the control, 5% GG, and 10 % GG diets for 14 days. The cecal SCFA was determined by ultra-performance liquid chromatography–mass spectrometry. The values are means ±SEM (n = 6). Means without a common letter differ (Tukey-Kramer post-hoc test, $P < 0.05$).

Both major SCFAs and isoforms correlated positively with SOCS1 expression and was much stronger with acetate, propionate, n-butyrate and iso-valerate (Table 3.2).

Table 3.2. Correlation between colonic SOCS1 expression and cecal SCFAs pool in mice

SCFAs	Correlation coefficient value
Acetate	0.71**
Propionate	0.88**
n-butyrate	0.67**
iso-butyrate	0.57*
n-valerate	0.53*
iso-valerate	0.81**

*$P < 0.05$ and **$P < 0.01$ according to Pearson's correlation analysis.

3.4 Discussion and conclusion

Dietary fibers affect gut function and overall health of the host. While the upper GI tract largely support absorption of nutrients, digestion process mainly depends on exocrine secretion of the pancreas and liver. In the small intestine DFs are not digested nor absorbed and the microbial activity in the upper part of the GI tract are relatively low (Charter et. al., 2015). However, research has shown that some DFs and oligosaccharides are recognizable by toll-like receptors and lectin receptors in intestinal epithelial cells (Brown et. al., 2003; Sahasrabudhe et. al., 2016; Tsoni and Brown 2008). Moreover, the research group in our laboratory have reported that the intestinal Caco-2 cells can directly recognize the specific structure of GG, through TLR-2 and dectin-1 and induce SOCS1 expression (Van Hung and Suzuki, 2017). In the current study, only 10% GG diet increased the expression of SOCS1 in the small intestine. It is likely that the GG-mediated SOCS1 expression in the small intestine of mice occurs through such mechanism. However, the luminal concentration of the intact GG may matter, hence no effect on SOCS1 expression was evident in mice fed 5% GG diet. On the contrary, both 5 and 10% GG diets increased the SOCS1 expression in a similar manner in the colon. This evidence may suggest that supplemental GG can induce SOCS1 expression more effectively in the colon than the small intestine even at low dietary levels.

Microbial conversation of dietary DF yields SCFAs, acetate, propionate and butyrate in particular, which are known for their immunomodulatory effects. Regardless of GG fiber concentration in the colon, SCFAs profiles were similar between 5% and 10% GG diets. Furthermore, using Pearson correlation coefficient analysis, SOCS1 expression correlated well with SCFAs production in the colon, suggesting that these microbial metabolites may have, in part, contributed to the upregulation of the protein, a mechanism different from SOCS1 upregulation in the small intestine.

Intestinal epithelium continuously faces the luminal noxious molecules, including microbes and antigens that could trigger inflammatory responses (Suzuki, 2013). For instance, lipopolysaccharides stimulate the TLR4-mediated proinflammatory cascade which activates a transcription factor, NF-κB, leading to the mucosal inflammation orchestrated by cytokines (Manco et. al., 2010). SOCS1 functions as the negative regulator of cellular inflammatory reaction and its deficiency leads to hyperactivation of transcriptional factors such as STAT1, STAT3 and NF-κB (Takahashi et. al., 2011). In a murine model of colitis, the SOCS1 heterozygous mice had more severe colitis compared to wild-type mice (Horino et. al., 2008), indicating the preventive roles of SOCS1 in the intestinal inflammation. Accordingly, GG-mediated SOCS1 expression in this study may promote anti-inflammatory regulation.

The study in this chapter showed that GG fiber upregulate intestinal SOCS1 by different mechanisms, however other isoforms of SOCSs such as SOCS2 and SOCS3 were not examined. Although only a few studies provide a direct insight into roles of other SOCS isoforms in the intestinal inflammation, SOCS3 seems to function in intestinal epithelial cells. Yong et. al., (2019) demonstrated that SOCS3, but not SOCS4, negatively regulated the activity of NF-κB via degradation of MyD88-adapter-like protein in porcine intestinal cells. SOCS3 also influenced the proliferation of intestinal epithelial cells (Thagia et. al., 2015). Further studies are required to understand the roles of SCFAs in the regulation of other SOCS isoforms.

CHAPTER 4

FERMENTABLE FIBERS INCREASE SCFAs AND UPREGULATE SOCS1 IN MICE COLON

4.1 Introduction

Dietary fibers have various beneficial physiological effects on our health, often linked to physiochemical properties they possess. Although fibers differ in their chemical composition as well as physiochemical properties, technological treatment permits modification of the physiochemical properties to optimize the functional and physiological properties of these fibers (Guillon and Champ, 2000).

Partially hydrolyzed GG (PHGG) is a product of controlled endo-β-mannanase hydrolysis of GG that has same chemical structure as unhydrolyzed GG but less in molecular size by one-tenth. Because of reduced size, it is lower in viscosity compared to GG and is preferred as a dietary fiber supplement (Yoon et. al., 2008). Both GG and its hydrolysate are fermentable and impart physiological benefits on the host through modulation of colonic microbiota and stimulating SCFAs production. For example, in healthy subjects, supplemental PHGG fiber increased *Bifidobacterium* and butyrate-producing bacterial species in human colon (Ohashi et. al., 2015). In a randomized clinical trial study, it was demonstrated that dietary supplementation with PHGG improved cardiovascular and metabolic profiles in patients with type 2 diabetes. It was observed that waist circumference, glycated Hb, urinary albumin excretion and serum *trans*-fatty acids were reduced while only weight reduction was evident in the control group (Dall'alba et. al., 2013). In animal studies the fibers have been reported to reduce colonic mucosal damage while enhancing barrier integrity in colitic mice (Hung and Suzuki, 2016; Naito et. al., 2006; Takagi et. al., 2016) and are capable of ameliorating chronic kidney disease in mice (Hung and Suzuki, 2018a).

The previous study demonstrated that intact GG is capable of increasing SOCS1 in the small intestine possibly via TLR-2 and dectin-1(Van Hung and Suzuki, 2017) whereas chapter 3 confirmed the results with GG affecting SOCS1 expression in the colon as well. However, mechanism of GG-mediated SOCS1 expression is not fully understood. Furthermore, PHGG effect on intestinal SOCS1 expression is not known despite being structurally similar to GG. Thus, the study in this chapter aimed to understand the GG-mediated intestinal SOCS1 expression in comparison with PHGG.

4.2 Materials and methods

4.2.1 Materials

Mouse monoclonal anti-SOCS1 antibody was purchased from MERCK (Darmstadt, Germany). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG was purchased from SeraCare (Milford, MA, USA). GG and PHGG (Sunfiber®) were provided by Taiyo Kagaku (Yokkaichi, Japan). PHGG (average molecular mass, 20 kDa) was prepared by treating GG fiber (average molecular mass, 300 kDa) with β-endo-galactomannase from a strain of *Aspergillus niger*. All other chemicals were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

4.2.2. Mice and diets

Animal study protocols were pre-approved by the Animal Use Committee of Hiroshima University, and all mice were maintained in accordance with the Hiroshima University guidelines for the care and use of laboratory animals (authorization no. C15-10-3). Male BALB/c mice (7-week-old, ~21 g) obtained from Charles River Japan (Yokohama, Japan) were used and other experimental conditions were consistent as described in Chapter 3.

4.2.3 Experimental design

Mice (n = 18) were randomly divided into three groups: control, 10% PHGG, 10% GG (n = 6 per group). The treatment groups were fed a diet containing 10% PHGG and 10% GG fiber (w/w), for 14 d of the experimental period. PHGG and GG was added to the control diet by substitution for an equal amount of starch. The control group was fed the control diet for the entire experimental period. The rest of the procedure were as described in Chapter 3.

4.2.4 Isolation of IEC and immunoblot analysis

IECs were isolated and protein expression of SOCS1 was determined as previously described in Chapter 3.

4.2.5 SCFAs analysis

SCFAs determination in cecal contents followed the protocol explained in Chapter 3 and the chemical derivatives of SCFAs were quantified by ultra-performance liquid chromatography–mass spectrometry (Waters, Milford, MA, USA).

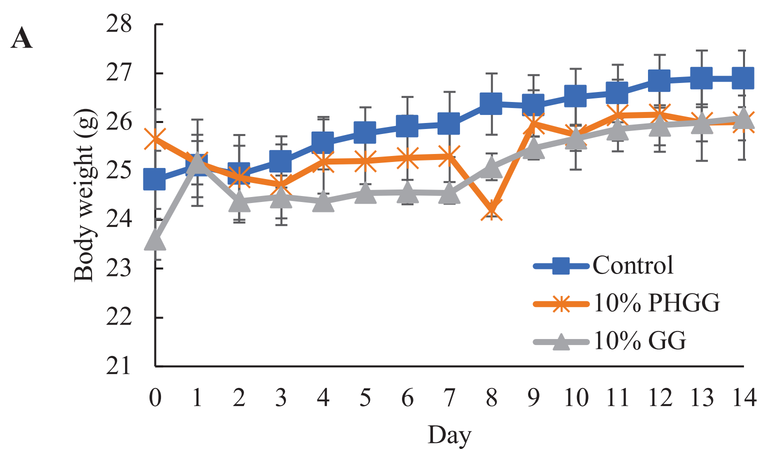
4.2.6 Statistical analysis

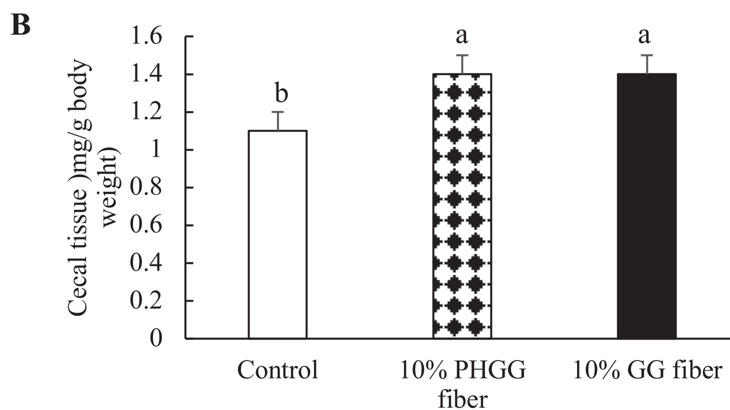
All data are presented as the means and standard error of the means (SEM). Statistical analyses were performed, and analysis was conducted as described in Chapter 3.

4.3 Results

4.3.1 Body parameters

PHGG is a product of controlled partial enzymatic hydrolysis of GG and is highly fermentable by microbiota. Feeding the PHGG and GG diets did not have any impact on body weight gain. However, the weights of cecal tissue and contents for fiber diet groups (Fig. 4.1) were higher than those in the control group. The weight of cecal contents in fiber diet groups tripled in comparison with the control group.





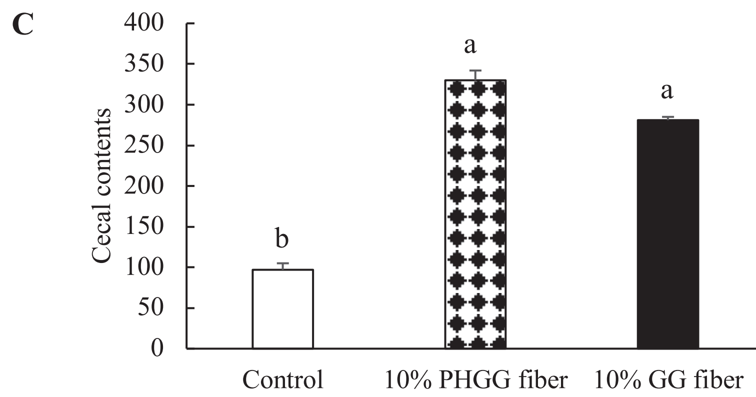


Figure. 4.1 Effect of GG and PHGG fiber on body parameters. (A), body weight. (B), cecal tissue weight. (C), cecal contents. The values are means ± standard error measurement (SEM) n = 6. Means without a common letter differ (Tukey-Kramer post-hoc test, $P < 0.05$).

4.3.2 SOCS1 expression and cecal SCFAs profile.

In the small intestine, SOCS1 expression in the GG group, but not PHGG group, was higher than that in the control group (Fig. 4.2A), whereas, in the colon, the SOCS1 expression in both GG and PHGG groups was higher than that in the control group (Fig. 4.2B). Feeding PHGG as well as GG increased cecal pool of major SCFAs, acetate, propionate, and *n*-butyrate (Fig. 4.3), and the values were significantly higher than those of the control group. No effect of fiber diets was observed on concentration of *iso*-butyrate, *n*-valerate and *iso*-valerate.

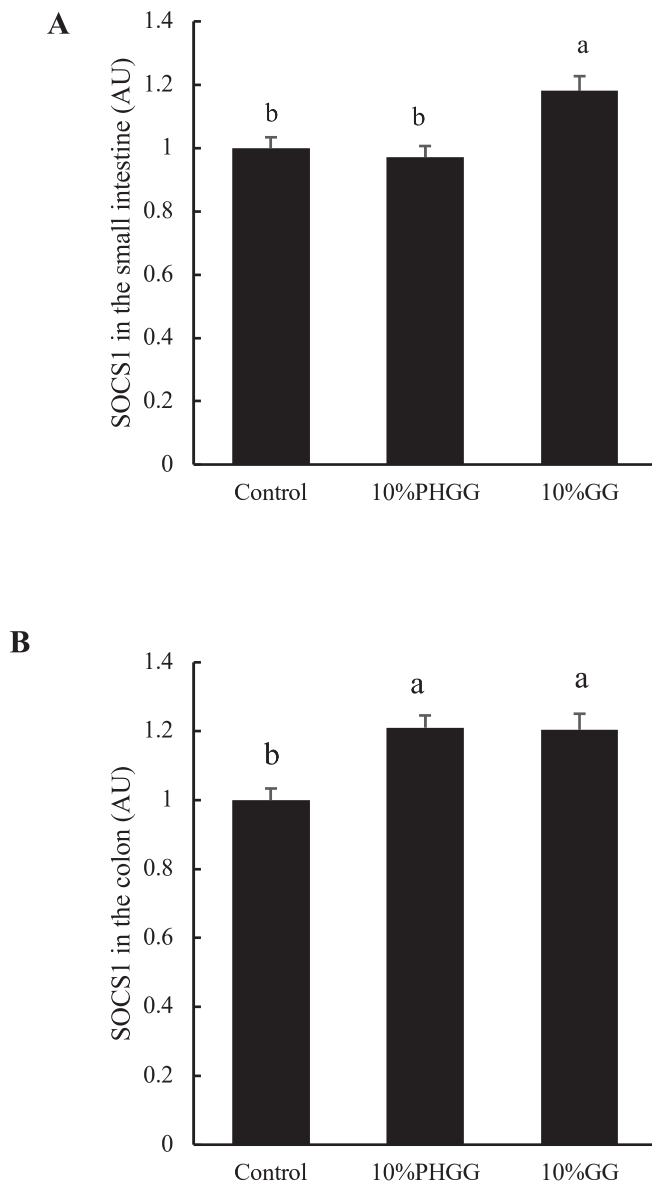


Figure. 4.2 SOCS1 expression in the small intestine and colon of mice fed the control, 10% PHGG, and 10% GG diets for 14 days. Protein expression of SOCS1 levels in the epithelial cells of small intestine (A) and colon (B) was determined by immunoblot analysis. The values are means ± SEM (n = 6). Means without a common letter differ (Tukey-Kramer post-hoc test, $P < 0.05$).

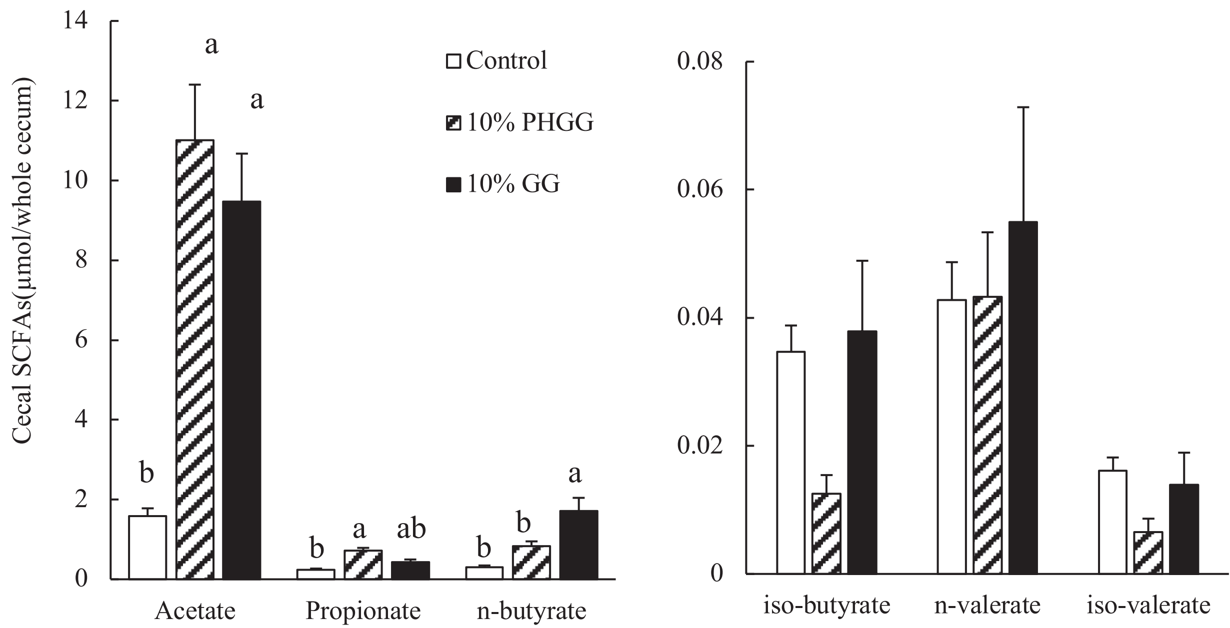


Figure. 4.3 SCFAs pool in the cecum of mice fed the control, 10% PHGG, and 10% GG diets for 14 days. The cecal SCFA was determined by ultra-performance liquid chromatography–mass spectrometry. The values are means ±SEM (n = 6). Means without a common letter differ (Tukey-Kramer post-hoc test, $P < 0.05$).

To consider the role of SCFAs production in the regulation of the colonic SOCS1 expression, Pearson correlation coefficients between cecal SCFAs pool and colonic SOCS1 expression were also calculated in this study. Except for the propionate (Table 4.1), the two major SCFAs, acetate, *n*-butyrate positively correlated with the colonic SOCS1 expression while the minor SCFAs had correlatedly negatively with colonic SOCS1 expression.

Table 4.1. Correlation between colonic SOCS1 expression and cecal SCFAs pool in mice

SCFA	Correlation coefficient value
Acetate	0.55*
Propionate	0.45
n-butyrate	0.51*
iso-butyrate	-0.38
n-valerate	-0.11
iso-valerate	-0.59**

*$P < 0.05$ and **$P < 0.01$ according to Pearson's correlation analysis.

4.4 Discussion and conclusion

A growing body of evidence from both clinical and animal studies suggests that consumption of dietary fibers improve metabolic syndrome profile and promote intestinal health (Dall'alba et. al. 2013; Hung and Suzuki, 2016; Naito et. al., 2006; Ohashi et. al., 2015; Takagi et. al., 2016). The study in this chapter demonstrates that GG and its hydrolysate may contribute to anti-inflammatory responses through upregulation of SOCS1.

In agreement with the previous study using intestinal Caco-2 cells, supplemental GG, but not its hydrolysate, PHGG, induced SOCS1 expression in the small intestine of mice. PHGG is a product of controlled endo-β-mannanase hydrolysis of GG and its molecular weight is reduced in size by one-tenth, thereby reducing the viscosity. Although they are structurally similar, the intestinal cells can directly recognize the specific structure of GG, and not PHGG, through TLR-2 and dectin-1 and induce SOCS1 expression. Hence, the expression of SOCS1 by GG in the small intestine is mostly likely through such mechanism.

Fibers escape digestion in the upper gastrointestinal tract but are fermented in the colon producing organic acids such as SCFAs. These acids are known to promote cell proliferation, differentiation, apoptosis among others. In turn, these activities promote intestinal homeostasis (Deehan et. al., 2017; Hung and Suzuki, 2016; Takagi et. al., 2016). In this study both PHGG and GG influenced SOCS1 expression in the colon. In addition, cecal SCFAs pool particularly acetate, propionate and *n*-butyrate increased in fiber diet groups. Similar to results in Chapter 3, a positive correlation, especially for acetate and butyrate, and the colonic SOCS1 expression was also evident, indicating that SCFAs may be responsible for PHGG/GG-mediated SOS1 expression in the colon.

The results of the study in this chapter showed that supplemental GG, but not its hydrolysate, PHGG, induced SOCS1 expression in the small intestine of mice possibly due to specific structure of intact GG that are recognized by TLR-2 and dectin-1 pathways, as reported

by previous study. In the colon, both fibers are influential in inducing SOCS1 expression, probably with involvement of microbial metabolites.

CHAPTER 5

ANTIBIOTIC ADMINISTRATION SUPPRESSES GUAR GUM FIBER-MEDIATED SOCS1 EXPRESSION IN MICE COLON

5.1 Introduction

In chapter 4 of this study, results showed that PHGG failed to influence SOCS1 expression in the small intestine. However, both fibers influenced SOCS1 expression in the colon in a similar manner, suggesting that microbial fermentation to produce SCFAs may have played a role. This chapter describes the effect of microbial activity on SOCS1 expression in the colon.

The gastrointestinal tract is a habitat for a diverse cluster of microorganisms and provides a platform for microbiota-host interaction. A growing body of evidence has highlighted the effectiveness of gut microbiome in providing metabolic, immunologic and protective functions which are critical for host's health. However, their population is influenced by a several factors including host physiology (age, stress, diseases), genetics and environmental factors such as use of antibiotics and other living conditions (Holscher, 2017; Sonnenburg and Backhed, 2016). Increasingly, diet is being recognized as the strategic environmental factor that facilitates the composition and metabolic function of the GI microbiota. The gut microbiota produces specific enzymes that enable them to depolymerize and ferment DFs into absorbable SCFAs. Reduced dietary fiber intake alters microbial diversity and production of microbial metabolites, thereby increasing chances of intestinal inflammatory response (Desai et. al., 2016). It has already been reported that GG undergoes fermentation in the colon to produce SCFAs which impart various physiological benefits to the host including regulation of inflammation (Hung and Suzuki, 2016; 2018a) suggesting that altering the microbial niche environment has serious consequences on intestinal homeostasis.

The aim of this chapter was to examine the role of intestinal microbial activity with associated SCFAs production in the GG-mediated expression of SOCS1 in the colon.

5.2 Materials and methods

5.2.1 Materials

Mouse monoclonal anti-SOCS1 antibody was purchased from MERCK (Darmstadt, Germany). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG was purchased from SeraCare (Milford, MA, USA). GG and PHGG (Sunfiber®) were provided by Taiyo Kagaku (Yokkaichi, Japan). PHGG was prepared as explained in Chapter 4. All other chemicals were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

5.2.2. Mice and diets

Animal study protocols were pre-approved by the Animal Use Committee of Hiroshima University, and all mice were maintained in accordance with the Hiroshima University guidelines for the care and use of laboratory animals (authorization no. C15-10-3). Male BALB/c mice (7-week-old, ~21 g) obtained from Charles River Japan (Yokohama, Japan) were used and other experimental conditions were consistent as described in Chapter 3.

5.2.3 Experimental design

Mice (n = 24) were randomly divided into four groups: control+water, control+antibiotics, 10% GG+water, and 10% GG+antibiotics (n = 6 per group). The control+antibiotics and 10% GG+antibiotics groups were administered with the antibiotic cocktail (vancomycin 500 mg/L, polymyxin B 200 mg/L and neomycin 500 mg/L) via drinking water for 35 d of the experimental period. The control+water and 10% GG+water groups received distilled water alone during the experimental period. After twenty-one days of antibiotic administration, the 10% GG+water and 10% GG+antibiotics groups were fed the diet with 10% GG for 14 d, whereas, the control+water and control+antibiotics groups were

fed the control diet. At the end of the experiment, the rest of the procedure followed as described in Chapter 3.

5.2.4 Isolation of IEC and immunoblot analysis

IECs were isolated and protein expression of SOCS1 was determined as previously described in Chapter 3

5.2.5 SCFAs analysis

SCFAs determination in cecal contents followed the protocol explained in Chapter 3 and the chemical derivatives of SCFAs were quantified by ultra-performance liquid chromatography–mass spectrometry (Waters, Milford, MA, USA).

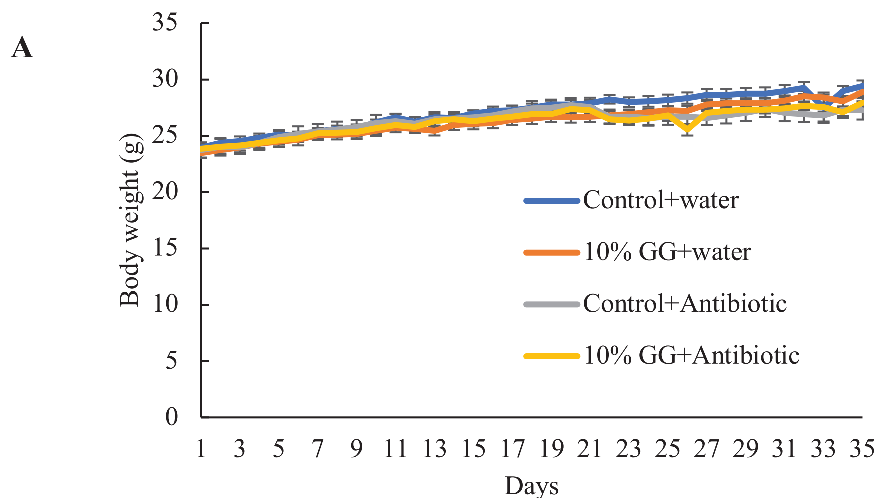
5.2.6 Statistical analysis

All data are presented as the means and standard error of the means (SEM). Statistical analyses were performed by two-way Analysis of variance, and analysis was conducted as described in Chapter 3.

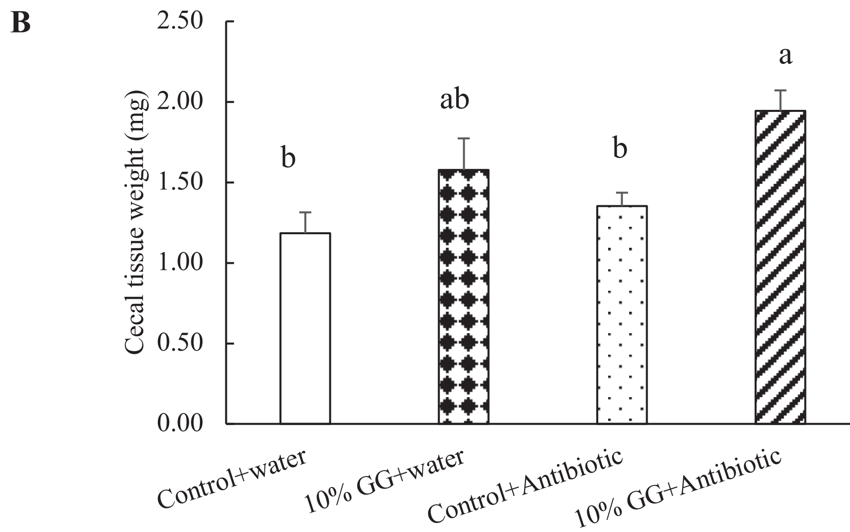
5.3 Results

5.3.1 Body parameters

Administration of antibiotics negatively influenced the body weight gain of mice (Fig. 5.1). However, no pathological sign was observed in these mice throughout the experimental period. Both antibiotic administration and GG feeding increased the weights of cecal tissues and contents although the effect of GG was not significant (*P*=0.07, 2-way ANOVA). There was no interaction effect between the GG feeding and antibiotic administration in these parameters.



	2-way ANOVA		
	Body weight	Cecal tissue	Cecal content
Antibiotics (A)	0..91	< 0.01	< 0.01
Diet (D)	0.54	0.07	< 0.01
A X D	0.47	0.49	0.39



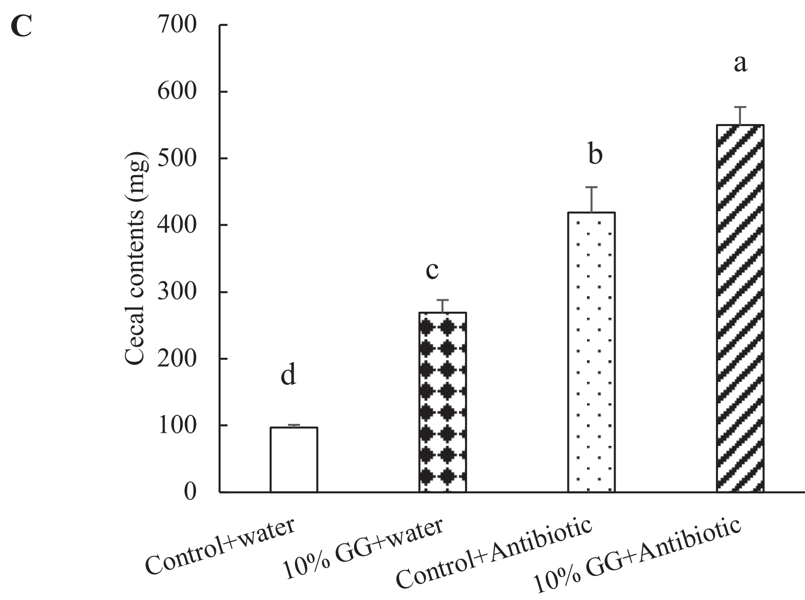


Figure. 5.1 Effect of antibiotic on body parameters. (A), body weight. (B), cecal tissue weight. (C), cecal contents. The values are means ± standard error measurement (SEM) n = 6. Means without a common letter differ (Tukey-Kramer post-hoc test, $P < 0.05$).

5.3.2 Colonic SOCS1 expression and cecal SCFAs profile.

The antibiotic administration and GG feeding negatively and positively influenced the colonic SOCS1 expression, respectively, based on the 2-way ANOVA (Fig. 5.1). Upregulation of the colonic SOCS1 expression by GG was observed in mice without antibiotic administration in a similar manner to results in previous chapters.

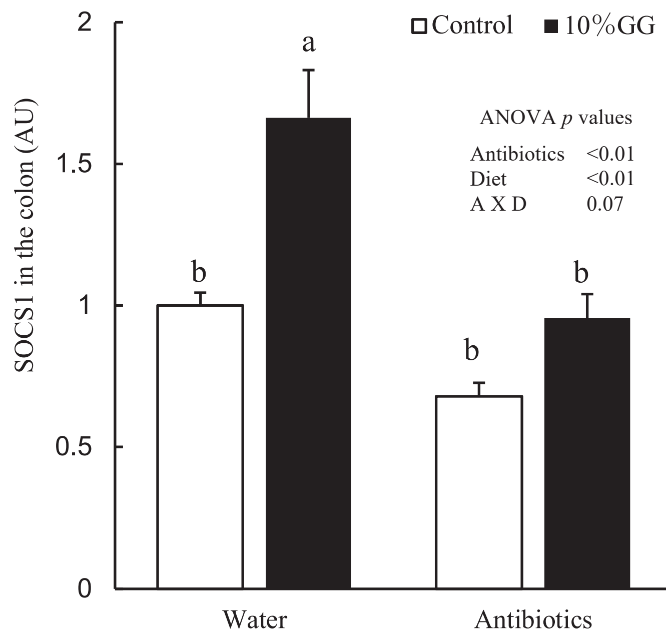
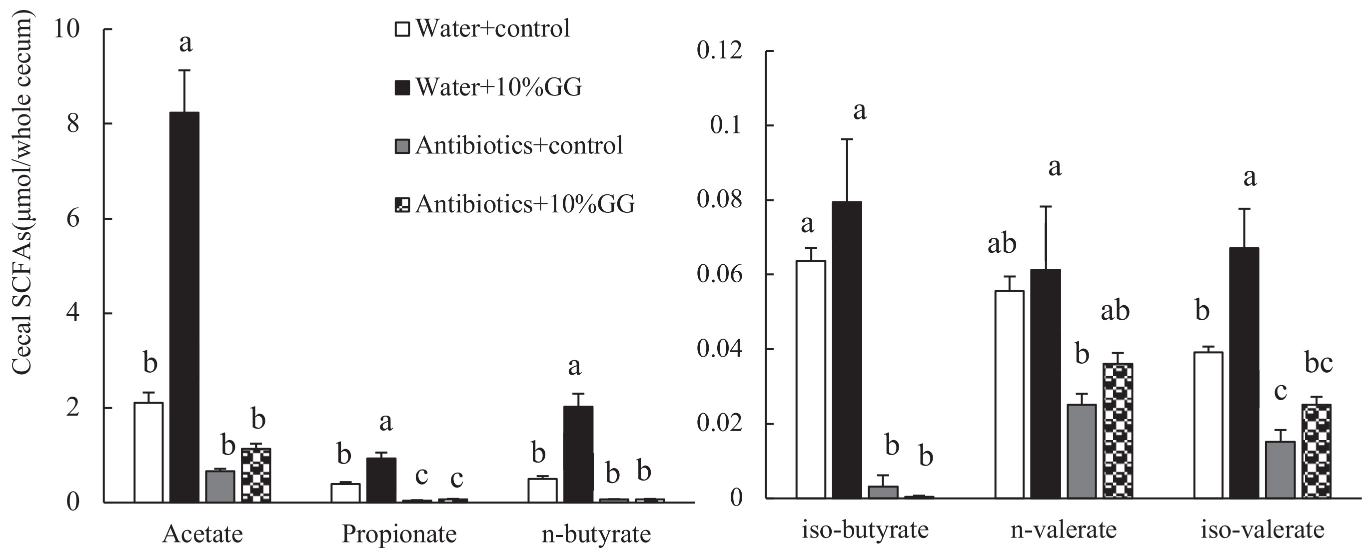


Figure. 5.1 SOCS1 expression in the colon of mice administered water or antibiotics and supplemented with or without GG for 14 days. Expression of SOCS1 levels in the colon was determined by immunoblot analysis. The values are means ± SEM (n = 6). Means without a common letter differ (Tukey-Kramer post-hoc test, $P < 0.05$).

The antibiotic administration completely abolished the upregulation by GG and the SOCS1 expression in the antibiotics+GG group was lower than that in the water+GG group. In the absence of antibiotic administration, feeding GG increased the cecal pool of SCFAs in a manner similar to results in previous chapters. Acetate, propionate, *n*-butyrate, and *iso*-valerate in the water+GG group were higher than those in the water+control group. The 2-way ANOVA detected the significant effect of antibiotics on the pool of all SCFAs, indicating that the antibiotic administration suppressed the cecal microbial activity and SCFA production in mice fed both the control and GG diets. The significant interactions between the antibiotic administration and GG feeding were observed in acetate, propionate and *n*-butyrate, because the antibiotic administration depleted these SCFAs irrespective of diets provided. Furthermore, Pearson's correlation analysis showed that all SCFAs except *n*-valerate correlatedly positively with SOCS1 expression in the colon.



ANOVA *p* values

	Acetate	Propionate	n-butyrate	iso-butyrate	n-valerate	iso-valerate
Antibiotics	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Diet	< 0.01	< 0.01	< 0.01	0.46	0.36	< 0.01
A x D	< 0.01	< 0.01	< 0.01	0.31	0.77	0.13

Figure. 5.2 SCFAs pool in the cecum of mice administered water or antibiotics and supplemented with or without GG for 14 days. The cecal SCFA was determined by ultra-performance liquid chromatography–mass spectrometry. The values are means ±SEM (n = 6). Means without a common letter differ (Tukey-Kramer post-hoc test, $P < 0.05$).

Table 5.2 Correlation between colonic SOCS-1 expression and cecal SCFA pools in mice

SCFA	Correlation coefficient value
Acetate	0.75**
Propionate	0.76**
n-butyrate	0.90**
iso-butyrate	0.58**
n-valerate	0.31
iso-valerate	0.69**

*$P < 0.05$ and **$P < 0.01$ according to Pearson's correlation analysis.

5.4 Discussion and conclusion.

Dietary fiber intake modifies gut microbiota composition and metabolism by stimulating the growth and activity of beneficial protective bacteria and subsequently host physiology. The physiological effects of the fiber-rich diets are often associated with the increased production of SCFAs through microbial fermentation in which acetate, propionate and butyrate dominate the SCFAs pool in the colon (Koh et. al., 2016). GG is highly fermentable by intestinal microorganisms and similar to previous results in Chapter 3 and 4 of this study, the fiber diet consistently upregulated SOCS1 expression in the colon. However, the antibiotic administration abolished the GG-mediated SOCS1 expression in the colon, suggesting that microbial fermentation is involved in the increased colonic SOCS1 expression by GG.

A healthy gut microbiota is prime for maturation and development of the immune system (Smith and Garrett, 2011). Desai and others (2016) showed that a low-fiber diet reduced microbial diversity and SCFAs yield as microbial metabolism shifted towards utilization of dietary and endogenously supplied proteins and mucins thereby damaging colonic mucus barrier and increasing pathogen susceptibility. Although microbial composition and production of SCFAs rely on diet as a major determinant, host genetic background and other environmental factors influence microbial community (Holscher, 2017). This study showed that the antibiotic administration suppressed the SOCS1 expression in the colon of mice fed the control diet as well as the GG diet. However, a substantial SOCS1 expression in the colon was still observed when the antibiotic administration depleted the cecal SCFAs pool, although the increment was not statistically significant according to the Tukey-Kramer post-hoc test. It can only be speculated that the increment occurred by intact GG, because the antibiotics reduced the microbial activity, resulting in the increased intact GG in the colon. This may imply that colonic SOCS1 expression can be regulated by endogenous mechanisms as well as

intestinal microbial activity. Furthermore, the significant increase in cecal content in the group fed 10% GG + antibiotic could be attributed to intact GG that reached the colon and was not fermented.

In conclusion, microbial activity appears to facilitate the GG-mediated upregulation of SOCS1 in the colon of mice. Antibiotic administration reduced microbial activity, consequently affecting SOCS1 expression in the colon.

CHAPTER 6

BUTYRATE INFLUENCE SOCS1 UPREGULATION IN INTESTINAL CACO-2 CELLS

6.1 Introduction

Chapter 5 of this study demonstrated that microbial activity is involved in GG-mediated SOCS1 expression in mice colon. Moreover, Chapters 3-5 showed a correlation between expression of SOCS1 and SCFAs production. This chapter explored on the role of SCFAs on SOCS1 expression using human intestinal Caco-2 cells.

Short chain fatty acids (SCFAs) are main end products of intestinal microbial fermentation of non-digestible carbohydrates that escape enzymatic digestion in the small intestine. They are found in abundance in the large intestine where bacteria are densely populated. These SCFAs modify several cellular processes, such as gene expression, differentiation, proliferation, chemotaxis and apoptosis through different mechanisms including inhibition of histone deacetylase and activation of G protein-coupled receptors (GPRs). Butyrate is the major energy source for colonocytes and is very important for maintenance of colonic epithelium through proliferation, apoptosis and differentiation of the cells while acetate and propionate are mostly transported to the liver and peripheral tissues via the portal vein where they act as signaling molecules and regulate various biological processes (Corrêa-Oliveira et. al., 2016)

Several studies have documented the influence of SCFAs in intestinal homeostatic regulation. Macia et. al. (2015) demonstrated that SCFAs through GPR43 and GPR109a activated an epithelial inflammasome which promoted conversion of pro-interleukin (IL)-18 to IL-18 thereby regulating inflammation. SCFAs have also been reporte to reduced TNFα-induced inflammatory signaling in intestinal cells (Hung and Suzuki, 2018b). Similarly,

Butyrate facilitated anti-inflammation properties in macrophages and dendritic cells of the mouse colon and enabled them to induce differentiation of regulatory T cells and IL-10-producing T cells (Singh et. al, 2014). In another study, oral supplementation of butyrate exhibited potency in attenuating colitis *in vivo* and proinflammatory cytokines TNF- α, IL-6 and IL-12 decreased with butyrate treatment *in vitro,* (Lee et. al., 2017). This chapter then, investigated the potential of SCFAs in upregulation of SOCS1 in Caco-2 cells.

6.2 Materials and methods

6.2.1 Materials

Mouse anti-SOCS1 antibody was purchased from MERCK (Darmstadt, Germany). Horseradish peroxidase-conjugated anti-mouse IgG was purchased from SeraCare (Milford, MA, USA). Cell culture reagents and supplies were from Thermo Fisher Scientific Inc. (Waltham, MA, USA). All other chemicals were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

6.2.2 Cell culture

Human intestinal Caco-2 cells (American Type Culture Collection, Manassas, VA, USA) were used. The Caco-2 cells were propagated and maintained under standard cell culture conditions in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum. The cells were seeded in 24-well plates at the density of 4.4×10^4 cells/cm^2 and cultured for 14 d prior to experiments. Passage for cultures used, ranged from 48 to 68 and the medium was refreshed every 3 days.

6.2.3 Treatment of Caco-2 cells with SCFAs

The cells were incubated with or without acetate (20 and 40 mM), propionate (2.5 and 5 mM), n-butyrate (2.5 and 5 mM), iso-butyrate (0.15 and 0.3 mM), n-valerate (0.2 and 0.4 mM), and, iso-valerate (0.25 and 0.5 mM) for 24 h and subjected to the immunoblot analysis of SOCS1 as described below. The SCFA concentrations used were based on the physiological concentrations in the colon of mice and humans (Cummings et. al., 1987; Hu et. al., 2012).

6.2.4 Immunoblot analysis

The Caco-2 cells were lysed in the appropriate volume of the lysis buffer. Protein expression of SOCS1 was determined as previously described in Chapter 3.

6.2.5 Statistical analysis

All data are presented as the means and standard error of the means (SEM). Statistical analyses were performed by one-way Analysis of variance, and analysis was conducted as described in Chapter 3

6.3 Results

6.3.1 SOCS1 expression in Caco-2 cells

Results from previous chapters showed a correlation between colonic SOCS1 expression and some SCFAs production. Because of this, the effect of individual SCFAs on SOCS1 expression in intestinal Caco-2 cells was examined. The expression of SOCS1 in cells incubated with 5 mmol/L n-butyrate was higher than that in the control cells, whereas, the effect of other SCFAs was not evident as far as SOCS1 expression was concerned.

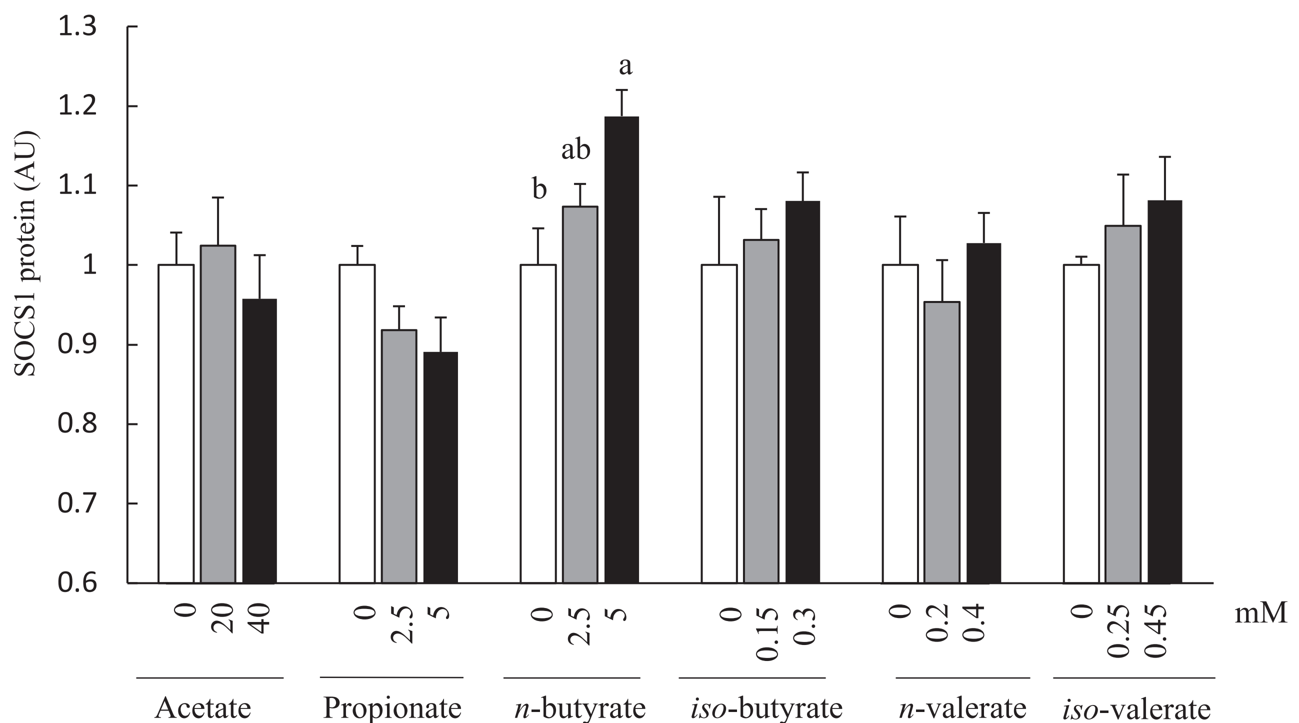


Figure. 6.1 SOCS1 expression of in Caco-2 cells treated with individual SCFAs for 24 hrs. Expression of SOCS1 levels were determined by immunoblot analysis. The values are means ± SEM ($n = 4$). Means without a common letter differ (Tukey-Kramer post-hoc test, $P < 0.05$).

6.4 Discussion and conclusion

A growing body of evidence has highlighted immunomodulatory roles of SCFAs through modification of cellular processes that include gene expression, differentiation, proliferation and apoptosis. The mechanism of action mostly involves inhibition of histone deacetylase and activation of G protein-coupled receptors (Koh et. al., 2016; Morrison and Preston, 2016). In this study, all major SCFAs and their isoforms were tested for their participation in SOCS1 upregulation. However, only butyrate successfully induced the SOCS1 expression in the Caco-2 cells. Although butyrate is known to exert various physiological effects in the colonic epithelium, such as enhancement of barrier integrity, regulation of cell growth and death, and induction of mucous production (Fatch et. al., 2019; Lee et. al., 2017), the current result shed a new light on the physiological functions of butyrate.

Butyrate is a product of microbial fermentation in the colon. In animal studies, the concentration of butyrate from fermentation of nondigestible polysaccharides, PHGG and GG inclusive, ranges from 3.5 to 7 mmol/L (Hu et. al, 2012; Hung and Suzuki, 2016; Xie et. al., 2019) whereas in human cecum, the concentration is even much higher (26mmol/Kg) (Cumming et. al 1987). Although the absorbed butyrate would be lesser than the cecal concentration, probably 5 mmol/L would be within the range of the physiologically available butyrate to influence SOCS1 expression.

Most animal studies use fiber supplementation of 5-20% w/w dry matter intake. In a review by Morrison and Preston, (2016), they reported that national diet and nutritional survey for the United Kingdom estimated that human daily dry matter intake mean value is 326.3-418.9g/day for both genders aged 19-64 years. In comparison to fiber supplementation in animal experiment, it translates to daily fiber intake of 16.3-83.7g/d. However, the actual measured mean DF intake is 12.8-14.7g/day. In a different study, African rural children, aged 2 to 6 years had a daily intake of 14.2g which showed increased SCFAs in fecal samples (De

Filippo et. al., 2010). Thus, the measured DF intake is close enough to estimated value and 5mmol/L butyrate is likely to be attained at physiological level. Upregulation of SOCS1 by SCFAS, butyrate in particular, means tight control of the cytokine-JAK/STAT pathway thereby regulating intestinal inflammation.

The *in vitro* study using Caco-2 cells, the results showed that not all SCFAs take part in SOCS1 upregulation. Butyrate emerged the possible candidate with it effect measured at a concentration of 5mmol/L. However, more studies are needed to understand how butyrate regulate SOCS1 expression.

CHAPTER 7

OVERALL CONCLUSION

The study investigated the impact of dietary fibers in intestinal SOCS1 regulation. Microbial activity *in vivo* and SCFAs in Caco-2 cells were examined for their involvement in upregulating SOCS1.

The results revealed that GG fiber uses different mechanisms in regulating intestinal SOCS1. In the small intestine the fiber appears to have upregulated SOCS1 in its intact form through TLR-2 and Dectin-1 pathways as reported by previous researchers in our laboratory. In the colon, GG-mediated SOCS1 upregulation was influenced by microbial activity since antibiotic administration reduced SCFAs production and suppressed SOCS1 expression. However, GG hydrolysate could not upregulate SOCS1 in the small intestine, but the colon, suggesting that the specific structure of intact GG may play a role in SOCS1 expression.

For the *in vitro* study, the results suggest that butyrate is the possible candidate responsible for upregulation of SOCS1 in Caco-2 cells at a concentration of 5 mmol/L. Since SOCS1 targets proinflammatory cytokines that lead to inflammatory diseases, dietary interventions that increase intracellular levels of SOCS1 protein may be an alternative approach in the fight against inflammatory diseases. Figure 7.1 is a graphical representation of how intact GG and butyrate may contribute to anti-inflammatory regulation.

Although butyrate showed potential in regulating colonic SOCS1 expression, due to limited time, mechanism of action was not investigated. Further studies are required to establish the mechanism of action used by butyrate in this noble cause. In addition, there is need to investigate the role of SCFAs in other isoforms such as SOCS3 that also function in intestinal epithelial cells. Besides, *in vivo* study used healthy mice. It will be interesting to

investigate if similar trend in SOCS1 upregulation by dietary fiber is maintained in animal models with inflammatory bowel diseases.

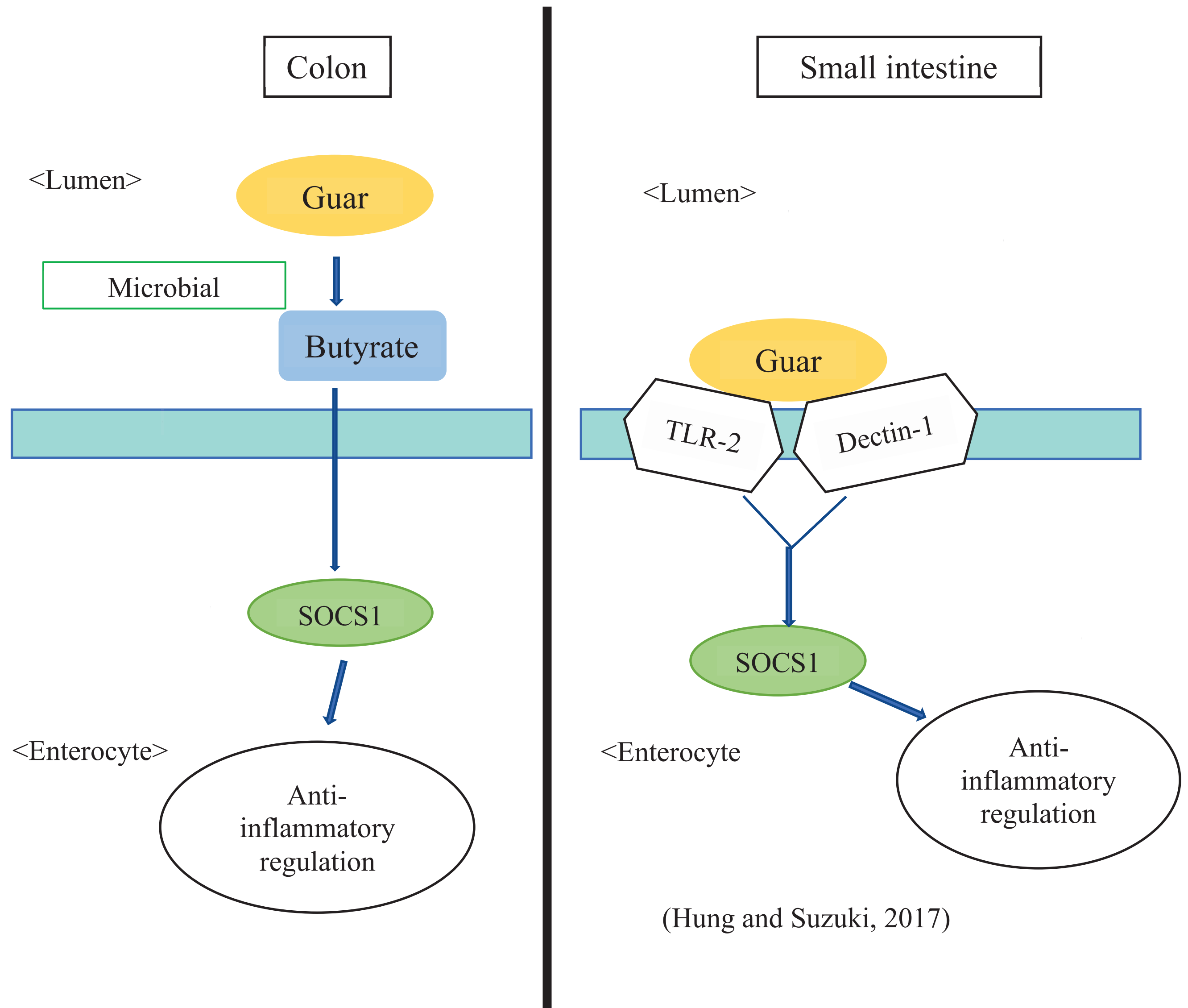


Figure 7.1: Graphical representation of guar gum inducing intestinal SOCS1 expression for anti-inflammatory regulation.

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