Master's Thesis of Science in Agriculture

Studies on the Potential Probiotic Strains Isolated from Korean Traditional Fermented Foods for Alleviating the Intestinal Inflammation

한국 전통 발효 식품으로부터 분리한 장내 염증 완화를 위한 프로바이오틱스 후보 균주에 관한 연구

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Abstract

Inflammatory bowel disease (IBD) including ulcerative colitis and crohn's disease is caused by chronic inflammation in the digestive tract. Even though the exact pathogenesis remains unclear, dysbiosis of gut microbiota, damage of epithelial integrity, imbalance of cytokine profile and infection of specific pathogenic bacteria have been considered as the primary factors for triggering IBD. For developing probiotics which could positively modulate gut microbiota and immune response, this study aimed to screen and characterize putative probiotics with various functions to alleviate intestinal inflammation associated with IBD. First, using selective and differential media, 399 lactic acid bacteria (LAB) were isolated from 77 traditional fermented food samples produced in Gangwon province, Korea. Second, 10 strains with putative functional LAB were screened and selected based on the probiotic properties, such as resistance to intestinal conditions, safety assessment and adhesion ability for human epithelial cell line. Then, the selected bacterial strains were evaluated for their antimicrobial activities against pathogens, particularly related to IBD by disk diffusion and agar-well diffusion methods and ATP assay. Almost every strains showed inhibition activities against pathogen indicators except the strain of B4 compared to reference strain, LGG. Anti-inflammatory effects of the selected strains suppressing

excessive intestinal inflammation were assessed by nitric oxide (NO) assay using murine macrophage RAW264.7 cell line. In addition, concentration of IL-8 produced by HT-29 cells and the expression levels of tight junction proteins in caco-2 cells which were both treated by 10 ng/ml of TNF-α were significantly regulated positively. After inducing colitis in mice by administration of 2.5 and 5 % DSS for 3 days, LGG, HS-2 and NG-4 strains were treated orally for 14 days to verify the attenuation effects of intestinal inflammation. The groups of NG-4 treatment showed increased average feed intake, better survival, less DAI scores, shortening of the colon length, modulating gut microbiota positively and upregulating the expression of TJ proteins compared to LGG group. In conclusion, *Pediococcus acidilactici* NG-4 is anticipated to have possibilities and apply as probiotics for IBD patients to alleviate the symptoms of intestinal inflammation followed by clinical tests.

Keyword : Bowel disorders, Inflammatory bowel disease, lactic acid bacteria, probiotics

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Chapter 1. Introduction

Bowel disorders, encompassing irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD), are considered as a general concern as the incidence and prevalence rates of these disorders have increased annually all over the world for decades. IBD, which is mainly comprised of ulcerative colitis (UC) and crohn's disease (CD), is a group of intestinal disorders that chronically cause inflammation through the intestinal tract. Although the exact pathogenesis of IBD is still unclear, the main factors of IBD have been considered as the dysbiosis of intestinal microbiota which means the disruption of normal composition in gut microbiota, damage of epithelial cell integrity and infection of specific pathogens including *Campylobacter coli, Campylobacter jejuni, Escherichia coli* and *Helicobacter pylori* (Hold *et al.*, 2014). The gut microbiota, a complex community of microorganisms residing in the gastrointestinal tracts of mammals including human, have been suggested as a key effector in pathogenesis and prevention of IBD (Guinane and Cotter, 2013).

Probiotics are recognized as live microorganisms that are believed to provide health benefits when consumed in sufficient quantities (Butel, 2014). According to recent studies, probiotics confer various health promoting effects, such as modification of the gut microbiota, improvement of epithelial barrier integrity, and anti-inflammatory and inhibitory effects against the stimulation of

inflammatory response induced by pathogens (Sánchez *et al.*, 2017).

In this study aimed to screen potential probiotic bacteria isolated from Korean traditional fermented foods and investigate functionalities of selected lactic acid bacteria, such as gut microbiota modulation, antimicrobial activity, anti-inflammatory effects and regulation of tight junction proteins expression, leading to attenuation of intestinal inflammation in both *in vitro* and *in vivo* study.

Chapter 2. Review of Literature

2.1. Gut immunity

2.1.1. Mucosal immune system

The mucosal immune system of vertebrates directly contacts with the external environment and constitutes mucosal barrier surfaces. It includes the mucosa-associated lymphoid tissues (MALT) related to mucosal surfaces and is classified with several constituents containing gut-associated lymphoid tissue (GALT), nasopharinx-associated lymphoid tissue (NALT), the salivary and mammary glands, the urogenital organs and bronchus-associated lymphoid tissue (BALT). The mucosal tissues populated with immune cells are crucial to prevent infection from the external surroundings and to provoke more antibodies production than any other parts in the body. Furthermore, they contain the specialized epithelial cell barrier exhibiting surface protection to protect the pathogens from penetrating the internal lining of tissues and administering tolerance against intact antigens such as commensal bacteria (McGhee *et al.*, 1992).

The lymphoid components of GALT are classified with organized mucosa associated lymphoid tissue which contains mucosal follicles acting as inducers of the primary immune response, and the diffuse mucosa associated lymphoid tissue which is composed of scattering leukocytes through the lamina propria and epithelial lines of mucosa

(Baumgart and Dignass, 2002). The organized tissues include Peyer's patches which aggregated lymphoid tissue compounds in the submucosa parts of small intestine and mesenteric lymph nodes (MLNs) connected to both vilus lamina propria and Peyer's patches (Mowat, 2003). The diffuse mucosa-associated lymphoid tissue, the less organized components, encompasses the lamina propria and intestinal epithelium. Various immune cells, such as T cells, B cells, dendritic cells, neutrophils, macrophages and mast cells can be found in the intestinal lamina propria of adult human (Prosper and Boyaka, 2013). The antigens penetrated through the epithelium may be progressed and recognized by the $CD4^+$ T cells proliferating cytokines extensively.

The intestinal epithelium, largest part of the body's mucosal sides, is lined with a monolayer of cells derived from villi and crypts. Intestinal epithelial cells (IECs) have key roles to separate host and luminal microorganisms by recognizing stimulation of intestinal commensal bacteria and facilitating to reinforce mucosal barrier function. They are constantly replaced by intestinal epithelial stem cells located at the base of the crypts and regulate essential immune functions. There are several types of cells in secretory IECs, containing goblet cells, peneth cells, and enteroendocrine cells specialized to digestive or epithelial barrier functions (Peterson and Artis, 2014). Secretory goblet cells and peneth cells settles and reinforces a biochemical and physical barrier by producing mucus and

antimicrobial proteins (AMPs) to inhibit translocation of luminal microbial and food antigens (Kim and Ho, 2010).

2.1.2. Immune homeostasis

IECs have the central capacity of maintaining barrier function against external environment and intestinal homeostasis by performing as a primary sensors to segregate signals of luminal commensal bacteria from that of microbial encounters and control anti-microbial and immune responses.

IECs exhibit pattern-recognition receptors (PRRs) that are receptor molecules to identify pathogen-associated molecular patterns (PAMPs), well-known as flagellin, lipopolysaccharide, bacteria-derived DNA and RNA. PRRs are specialized to induce innate immunity system and act as a crucial sensor for protection against pathogens. Majority of PRRs can be classified with three groups: Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and NOD-like receptors (NLRs). These PRRs induce individual pathways to recognize ligands or inherent signals of pathogenic bacteria related to pathogenesis (Kamada *et al.*, 2013). Specifically,

TLR families are presented on the bacterial surfaces and interior side of endosomes and prompt activation of myeloid differentiation primary response 88 protein, known as downstream adaptor molecules and TIR-domain-containing adaptor protein inducing

interferon-β (TRIF) while stimulated by bacterial components. They have been estimated to induce proliferation of epithelium cells, IgA production, conservation of tight junction integrity and antimicrobial molecules secretion, which are crucial roles to maintain homeostasis in intestine when recognizing signals of TLR. There are various sorts of TLRs in humans, including TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, TLR11(Mahla *et al.*, 2013). TLR2 and TLR 4 are exhibited by IECs at low levels in human colon tissues with normal condition (Abreu, 2010). While TLR3 has shown to be plentifully expressed in small intestine and colon of normal human, TLR5 is mainly represented in the colon(Cario and Podolsky, 2000).

Recently, several studies using germ-free mice have demonstrated that the intestinal epithelium and commensal bacteria stimulate a complex progress that is featured by exhibition of genes enhancing tight junction proteins (Hooper *et al.*, 2001). The apical pole of epithelial cells is connected by tight junction (TJ) complexes to block the transportation of bacteria and PAMPs through the passages between IECs (Bischoff *et al.*, 2014). The major TJ complex includes transmembrane proteins characterized with occludin, junctional adhesion molecules (JAM) and claudins family members depending on the position and permeability regulation. Zonula occludens located in the upper part of epithelium cell interact with occludin and claudins as connected with the actin cytoskeleton and regulate cycle and polarity in cell functions. TJs has a fundamental role to control paracellular

permeability by transferring selectively ions, solutes, small sized molecules between epithelium cells. Assembly and disassembly of TJ complexes exert an energetic process including migration, endocytosis, and recycling (Konig *et al.*, 2016). TJ dynamics can be modulated by several cytokines (Petecchia *et al.*, 2012). For instance, tumor necrosis factor-α (TNF-α) can modulate and promote permeability in TJ proteins through upregulating the expression of claudin-2 which controls selectively cation-channel through phosphatidylinositol-3-kinase signaling (Mankertz *et al.*, 2009).

2.1.3. Gut microbiota

The surfaces of vertebrates including skin and mucosal layer are settled by large amounts of microorganisms such as bacteria, viruses, fungi, parasites which normally represented to the microbiota. There are extremely plenty of microorganism, mainly bacteria, most of which colonize gastrointestinal and oral tract more than 100 trillion microorganisms. Particularly, most of them have resided in the colon for millions of years by establishing a cooperative relationship. The host has evolved to supply microbiota habitation and nutrients as necessary requisite for survival of bacteria and, in turn the microbiota serve many physiological effects for contributing health of host (Hooper and Macpherson, 2010). The leading roles of the microbiota for promotion of health in host contain abilities to fermentation and digestion of carbohydrates, the supply of vitamins, the development of

gut immune responses which modulate the formation of the microbiota and inhibition of pathogen colonization (Renz *et al.*, 2012). In gut immune system, microbiota have an essential role of developing lymphoid tissue including GLATs, T_H 17 cells, T_{reg} cells, IgA-producing B cells, plasma cells and innate lymphoid cells through plenty of *in vivo* studies using germ free mice (Kamada *et al.*, 2013).

It has been understood the importance and relationships between gut microbiota and immune function by investigating that disruption of gut microbiota composition can influence gastrointestinal conditions and cause various disease associated with immune dysfunction, such as inflammatory bowel disease and intestinal inflammation (Frank *et al.*, 2007) and systematic diseases encompassing diabetes (Qin *et al.*, 2012) and obesity (Zhang *et al.*, 2009).

Along with development of metagenomics technology to analysis the full genetic composition of community, the studies of gut microbiota have been extremely activated to determine potential functions as microbes constitution for decades (Ley *et al.*, 2008). Using these newly developed technologies, it has been known that gastrointestinal microbial community of healthy individuals maintains gut homeostasis between beneficial bacteria and harmful bacteria which are potential to bring about infections induced by pathogens. However, the disruption of normal composition in gut microbiota, designated as dysbiosis, can be caused under specific states including changes of diet, intrinsic immune defects and/or inflammation

conditions (Honda and Littman, 2012). Dysbiosis also associated with the abnormal conditions of accumulating and increasing virulence certain kinds of commensal bacteria and can transform these normal conditions into pathobionts which are constant colitogenic conditions triggering intestinal inflammation. Therefore, maintaining gut homeostasis through regulation of these microbiota composition is extremely crucial for exerting health benefits and preventing intestinal inflammation (Kamada *et al.*, 2013).

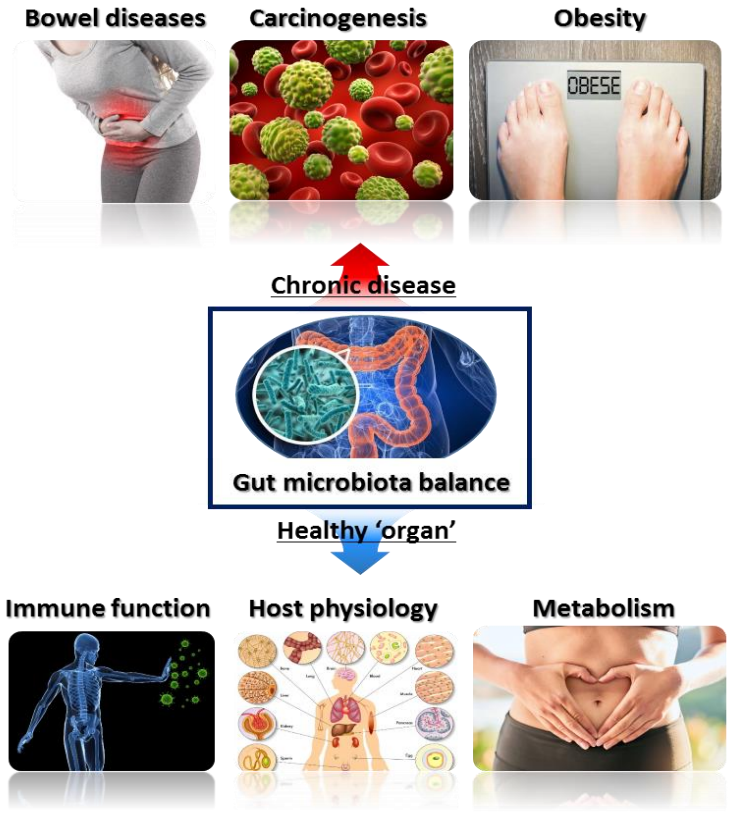

Figure 1. The role of gut microbiota association with health and intestinal disease.

2.2. Bowel disorders

2.2.1. Irritable bowel syndrome (IBS)

Irritable bowel syndrome (IBS) belongs to functional bowel disorders which accompany persistent conditions characterized with physical and mental illness, abdominal discomfort, constipation and diarrhea induced by uncertain bowel motility. It can be diagnosed solely using symptoms-based criteria, referred to as Rome diagnostic criteria due to absence of obvious organic lesion (Häuser *et al.*, 2012). Although IBS is not recognized as severe illness, its incidence has increased about 10 ~15 % between adults suffered from this disorder (Longstreth *et al.*, 2006).

There have been numerous attempts to investigate the pathophysiology of IBS, but the exact cause of it is still unclear. While changed brain-gut relations has been considered as a main mechanisms of pathogenesis in IBS for decades, recently studies have suggested changes of gut microbiota composition and inflammation associated immune activation at the low level as novel mechanisms of IBS (Brint *et al.*, 2011). Dysbiosis of gut microbiota also is speculated to facilitate adhesion ability of pathogens to wall of bowel surfaces in pathogenesis of IBS (Ghoshal *et al.*, 2012). Particularly, the gut microbiota profile of IBS patients has specific features of high proportion of *Fimicutes* genus, such as *Ruminococcus*, *Clostridium* and *Dorea* species and a significant reduction of

Bifidobacterium and *Faecalibacterium* species (Rajilić-Stojanović *et al.*, 2011).

2.2.2. Inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD) has been intermittently observed since long before (Mulder *et al.*, 2014) and considered as increasing and significant bowel disorders in developed countries (Molodecky *et al.*, 2012). The worldwide incidence of IBD has been investigated by an organized review of over 200 countries-based researches. In Western world, IBD is characterized by highest prevalence, influencing over 0.5 % of the ordinary population, whereas it occurs at low level in Mediterranean and Eastern Asia. Although IBD has been conventionally considered as a disorder of Caucasian residing in developed countries of the Western part, as developing countries in Eastern and Mediterranean world has become westernized, prevalence and incidence rates of IBD has increased the same trends of Western world (Kaplan, 2015).

IBD encompassing two main groups of ulcerative colitis (UC) and crohn's disease (CD) is characterized with chronic relapsing conditions of inflammation through small intestine and colon, which accompanies inflammation response and epithelial injury (Szkaradkiewicz *et al.*, 2009). Although both UC and CD are classified with IBD categories, they have distinct features with symptoms and sites of inflammation lesion. While CD is has a feature of chronic and

partial inflammation through the gastrointestinal tracts (Loftus, 2004), UC is typically characterized by occurring inflammation condition with ulceration mainly through the colon (Lepage *et al.*, 2011). Although these two types of IBD share manifold epidemiologic, therapeutic and clinical traits, CD and UC have distinct differences in the composition and population of gut microbiota. Especially in patients with CD, *Faecalibacterium*, *Peptostreptococcaceae, Anaerostipes, Methanobrevibacter* and *Christensenellaceae* genus are deficient in comparison with healthy controls and UC patients (Pascal *et al.*, 2017). It has also been revealed that the proportion of Firmicutes are decreased, wheareas gammaproteobacteria are increased in CD and Enterobacteriaceae has speculated to be associated with the pathogenesis of UC patients (Li *et al.*, 2012). A highlighted feature in microbiome level has shown that F. prausnitsii which is classified with C. leptum group has reduced abundance in both CD and UC (Sokol *et al.*, 2009). In addition to recent study, examining biopsies of intestine and fecal samples from IBD patients and healthy controls demonstrated that alterations abundance of gut microbiota at the phylum level, such as *Leuconostocaceae*, *Ruminococcacea* and *Enterobacteriaceae*, while *Clodstridium* genus increased, but *Roseburia* which acts as a butyrate producer and phascolarctobacterium genus for producing succinate considerably decreased in IBD conditions (Morgan *et al.*, 2012).

2.2.3. IBD and gut immune response

Cytokines have been considered as key factors of pathogenesis of IBD in accordance with recent immunological and genetic studies by controlling inflammation response in gut and the related clinical indication of IBD. IBD patients have distinct characterization of altered cytokine patterns produced by immune cells from the lamina propria under the gut epithelial cells. Although there has been some attempts to treat IBD patients consuming recombinant anti-inflammatory cytokines including IFNβ, IL-10 or specific antibodies for suppressing pro-inflammatory cytokines such as TNF, only antibody specific binding TNF (infliximab) showed alleviating effects for crohn's disease through clinical trials (Van Dullemen *et al.*, 1995). TNF-α is a well-known proinflammatory cytokine produced by macrophages, monocytes, and T lymphocytes activated with immune response (Papadakis and Targan, 2000). Its expression in human macrophages was increased in colonocytes and macrophages in IBD patients (Stucchi *et al.*, 2006) and high concentration of that in serum levels could be used to diagnose IBD as clinical and laboratory criteria of intestinal disease acitivity (Reimund *et al.*, 1996). In addition, some kinds of pro-inflammatory cytokines, expecially TNF, have been observed to modify tight junction activity and to cause apoptosis of IECs (Su *et al.*, 2013).

Maintenance of epithelial integrity by proliferation and expansion of IECs is fundamentally important traits for regulating excessive

inflammation response for enhancement of intestinal barrier function and treatment of the inflamed mucus layer in IBD (Neurath and Travis, 2012). When the intestinal barrier function is impaired by pathogens, allergens or external elements such as diets or antibiotics, intestinal components and microbiota are easily translocated, which is known as the cause of chronic inflammation in IBD patients (Wallace *et al.*, 2014). However, in normal gut homeostasis condition, only some kind of gut bacteria could selectively translocate through tight junctions of the epithelial cell, this translocation can be used as a way of sampling for antigens and immune observation to maintain the host's homeostasis (Slack *et al.*, 2009).

2.3. IBD and probiotics

2.3.1. Probiotics

Probiotics have been defined by various meanings until World Health Organization (WHO) has made a definition of probiotics in 2002 (Azizpour *et al.*, 2009). According to WHO, the term of probiotics represents "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host." which means they can survive in gastrointestinal environment and keep viability during storage and safety properties when ingested for human. Although there are many microorganism having health promoting effects, most studies have focused on examining only some bacterial genera, such as species of *Lactobacillus*, *Enterococcus*, *Streptococcus* and *Bifidobacterium*. However, some kinds of these genera, such as *Enterococcus* and *Bacillus* have increased concerns of their safety traits for human health (Hempel *et al.*, 2011). There are several isolated strains of lactic acid bacteria conferring many health effects in clinical trials, including species of *Lactobacillus plantarum* 299v, *L. rhamnosus* GG, *L. acidophilus* La5 and *B. lactis* Bb-12 (Bengmark, 2013).

Probiotics have been related to many health benefits, fundamentally focused on the health promoting effects in gastrointestinal tract through several regulating mechanisms. Probiotic strains can replace harmful bacteria with harmless bacteria by competitive adhesion on the epithelium cells or inhibition of

pathogenic bacteria by secreting antimicrobial compounds or short chain fatty acids (SCFAs) such as butyrate, lactate and acetate, which reinforce gut barrier function and are used by the colonocytes as the main source of energy. They have been also demonstrated immune-modulating effects by stimulating immune cells and inducing cytokine production or increasing IgA secretion (Steidler, 2001). In addition to these benefits, some probiotic bacteria can improve the mucosal barrier by prevention and repair of mucosal damage led to pathogens, food antigens, or antibiotics (Saxelin *et al.*, 2005).

Apart from immunomodulation and improvement gut barrier function, they conduct various health improvement by reducing cholesterol, alleviating lactose intolerance, preventing diarrhea, inhibiting *helicobacter pylori* and treating allergy (Singh *et al.*, 2011).

2.3.2. Relationships between probiotics and IBD

Potential mechanisms of health benefits for improvement and treatment of IBD symptoms have been explored through extremely many kinds of studies using animal models and clinical trials. Probiotics can be applied for exerting their prevention and treatment effects with three mechanisms of action.

First of all, probiotics can inhibit domination of pathogenic bacteria in intestine by competing with nutrients and adhesion sites or secreting antimicrobial compounds. For instance, high adherence properties of probiotics to intestinal epithelial cells, suppressing the

access and invasion of intestinal pathogens or external pathogens which could induce chronic and acute inflammation such as *Helicobacter*, *Campylobacter*, pathogenic *Escherichia* species (Allen *et al.*, 2010). In addition to inhibition of pathogenic bacteria, probiotics could modulate the composition of the gut microbiota by interacting with specific pattern-recognition receptors including TLRs located on the surface or inner membrane of immune cells by inducing immune responses in the gut (Bouskra *et al.*, 2008).

Second mechanism includes regulation and development effects of some parts of the human GIT, such as mucus layer, epithelial cells, and GALT. The mucus layer, which is subdivided into outer layer with mucin molecules and inner mucin sublayer for restricting translocation of gut bacteria. Probiotics can exert the prevention effects of IBD by strengthening tight junction complexes such as occludin and claudin groups and promoting production of mucins (Sánchez *et al.*, 2017).

Finally, some probiotics have immunomodulating effects by regulating various cytokine production through gut immune cells, especially T regulatory cells associated with the maintenance of gut homeostasis and establishment of tolerance against the residing intestinal bacteria (Wang *et al.*, 2014).

2.3.3. Probiotics products for IBD treatment

On account of numerous studies involving the relationship between gut microbiota and IBD, probiotics with effects of modifying the gut flora have been suggested for treatment or alleviation of chronic intestinal inflammation. There have been various attempts to develop different preparation of probiotics including *Lactobacillus rhamnosus* GG, *L. johnsonii* LA1, *E. coli* Nissle 197, and VSL#3 either in animal models or in clinical trials, which are summarize in Table 1. VSL#3 is a probiotic product developed by Alfasigma Inc. from USA, which contains *S. thermophilus*, *B. breve*, *B. longum*, *B. infantis, L. acidophilus, L. plantarum, L. casei* and *L. bulgaricus* and has been demonstrated its beneficial effects by normalizing gut barrier function, reducing pro-inflammatory cytokines and alleviating histological disease in colitic mouse model (Madsen, 2001).

Table 1. Probiotics in clinical trials and animal models.

Probiotic strain	Effects &mechanism	Reference
Lactobacillus	Mucosal Ig A ↑	(Malin *et al.*, 1996)
rhamnosus GG	Similar endoscopic recurrence	(Prantera *et al.*, 2002)
(LGG)	Intestinal permeability & clinical disease activity ↓,	(Gupta *et al.*, 2000)
VSL #3	75 % still in remission and changed fecal flora	(Venturi *et al.*, 1999)
	TLR-9 mediated anti-inflammatory effect of DNA	(Rachmilewitz *et al.*, 2004)
	Improvement of recurrent colitis by IL-10 dependent induction of regulatory cells	(Madsen, 2001).
Lactobacillus	Improvement of IBD by	(Matsumoto *et al.*, 2005)
casei shirota	downregulation of pro-inflammatory cytokines (IL-6)	
(LcS)		

Chapter 3. Materials and methods

3.1. Isolation and identification of lactic acid bacteria

3.1.1. Collection of traditional fermented foods

The traditional fermented foods for isolating lactic acid bacteria were collected from Ganwon-do, South Korea. 52 samples of Kimchi, 20 samples of Jeotgal, 4 samples of Makgeolli, 1 sample of Chenggukjang were prepared for this study (Table 2). After samples of collected fermented foods were transported within 4 hours, the 10 g of samples were separated in 50 ml conical tubes and stored at 4 ℃ in refrigerator or at −80 ℃ in deep-freezer before proceeding isolation of lactic acid bacteria.

Table 2. Origin of the samples for isolation of lactic acid bacteria (LAB) in this study.

Sample	Gangwon-do, South Korea				
	Pyeongchang	Hoengseong	Gangneung	Other districts	No.
Kimchi	27	·	13	· 12	52
Jeotgal	9	·	11	·	20
Makgeolli	·	2	·	2	4
Cheonggukjang	·	·	1	·	1
Total					77

3.1.2. Isolation of lactic acid bacteria

After each fermented foods sample (1 g) was weighted, the sample was serially diluted 10-fold using 0.85 % NaCl solution (Sigma-Aldrich, USA) and mixed well by hand. An amount of 0.1 ml of 0.85 % NaCl serial dilution were spread on de Man, Rogosa and Sharp (MRS) (Difco, USA) agar for selectively isolating lactobacilli, M17 (Difco, USA) with 10 % lactose (Difco, USA) agar for lactococci isolation. The inoculated MRS plates were incubated at 37 ℃ under anaerobic condition used an anaerobic chamber (Coy Laboratory products, AnnArbor, MI, USA) and M17 plates were incubated at 37 ℃ and 30 ℃ under aerobic condition for 48 h. After incubation, single colonies with different morphologies were collected and inoculated to same broth media. Inoculated MRS broth and M17 broth were incubated for 24 h at 37 ℃ under aerobic condition. Overnight incubated broth of isolates were streaked on plate count agar containing bromocresol purple (BCP) (Eiken chemical co., Ltd, Japan) to confirm single strains at 37 ℃ and 30 ℃ aerobically for 24h. Subsequently, catalase test using 3 % hydrogen peroxide (H_2O_2) and gram staining (Becton, Dickinson and Company, USA) were done for screening LAB. Only catalase negative and gram positive isolates were selected and maintained at −80 ℃ in 20 % glycerol stock for long term storage (Figure 2).

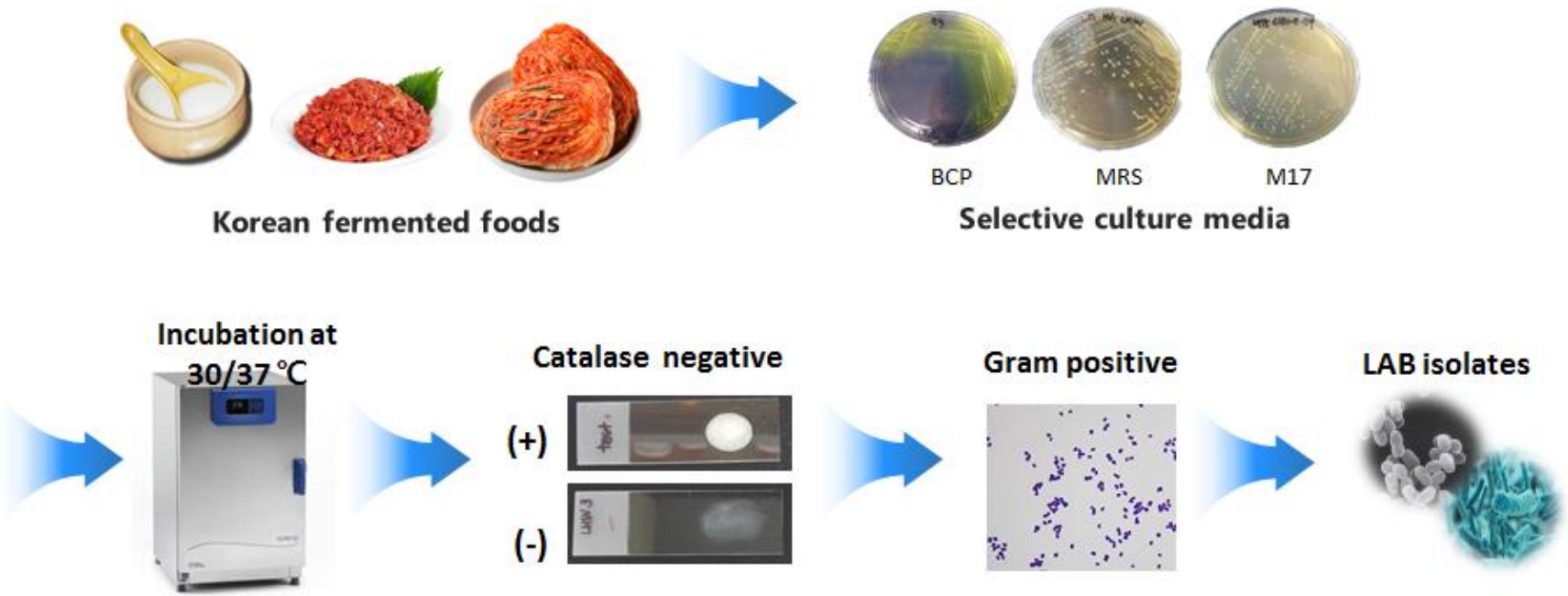

Figure 2. Scheme for isolation of lactic acid bacteria.

3.1.3. Resistance to low pH and bile salts

Resistance to low pH Frozen isolates were activated by subsequently inoculation into MRS broth twice every 24 h for assessment of acid and bile tolerance of LAB. All strains were prepared in MRS broth for 18 h at 37 ℃. To examine survival of LAB strains under acidic condition, 50 ㎕ (1 %) of cultures were inoculated into 5 ml of MRS broth adjusted pH at 3.0 with 1 N HCl. The number of initial inoculum was counted immediately by drop plating on MRS agar. The inoculated samples were incubated at 37 ℃ under aerobic conditions for 2 h. After incubation, 0.1 ml of sample was transferred to 1 X phosphate buffered saline (PBS) solution, and then serially diluted solution was spread on MRS agar by drop-plating methods. The MRS agar plates were cultured for 24 h at 37 ℃. The colonies grown on MRS agar were counted and compared with initial counts (Yeo *et al.*, 2016).

Bile resistance To estimate bile tolerance of LAB, 50 ㎕ (1 %) of overnight cultures (18 h) were inoculated to 5 ml of MRS broth supplemented with 0.3 % bovine bile (Oxgall, Difco) or without bile as a control group simultaneously and incubated at 37 ℃ under aerobic conditions for 12 h. The treatment and control groups, each with a triplicate, were compared by measuring the optical density for detection of cell growth between two groups. The inoculated MRS plates were incubated at 37 ℃ aerobically for 24 h. The survival rate in bile salts were calculated with the following formula: Relative bile

tolerance rate = OD_{600nm} of 0.3 % oxgall MRS culture in treatment group/ OD_{600nm} of MRS culture in treatment (Hassanzadazar *et al.*, 2012).

3.1.4. Safety assessment

Antibiotic Resistance Antibiotic susceptibility for selected antibiotics was tested by measuring minimal inhibitory concentration (MIC) and microbiological cut-off values (mg/L) of antibiotics were assessed based on the European Food Safety Authority guidelines (EFSA, 2012). Ampicillin, streptomycin, tetracycline, erythromycin, kanamycin, gentamycin, chloramphenicol were prepared for two fold dilutions with concentrations between 2 and 512 ㎍/㎖. Overnight (18h) cultures grown in LAB susceptibility medium (LSM) constituted by 90 % IsoSensitest broth (Oxoid, USA) and 10 % MRS broth were adjusted with optical density at 600 nm ($O.D_{600nm}$) of 1 density (SPECTROstar nano, BMG LABTECH, Germany). A volume of 0.1 ml of the prepared antibiotics and 0.1 ml of bacteria cultures were inoculated to a 96-well microplates and diluted serially by two-fold and incubated at 37 ℃ for 24 h. The final concentrations were adjusted from 1 to 256 ㎍/㎖ and the MIC were evaluated as the lowest antibiotic concentration at which no growth of strains was observed (Leite *et al.*, 2015). All strains for antibiotic susceptibility assay were conducted in triplicate.

Hemolytic activity The hemolytic activity was evaluated using blood

agar plates supplemented with 5 % (v/v) defibrinated sheep blood (KisanBio, Korea). The β-hemolysis was indicated by appearance of clear zone around the colonies. After the colony of each strain were streaked on the blood agar, the plates were incubated aerobically at 37 ℃ for 48 h (Ji *et al.*, 2015). *Enterococcus faecalis* ATCC 29212 were tested as positive control.

Biogenic amine production Analysis of biogenic amine production was conducted by method described as Bover-Cid and Hozapfel (1999). The isolates were streak on the decarboxylase media, followed by aerobic incubation at 37 ℃ for 4 days. *Enterococcus faecalis* ATCC 29212 were tested as positive control. Decarboxylase activity was detected with the color change from yellow to blue (Bover-Cid and Holzapfel, 1999).

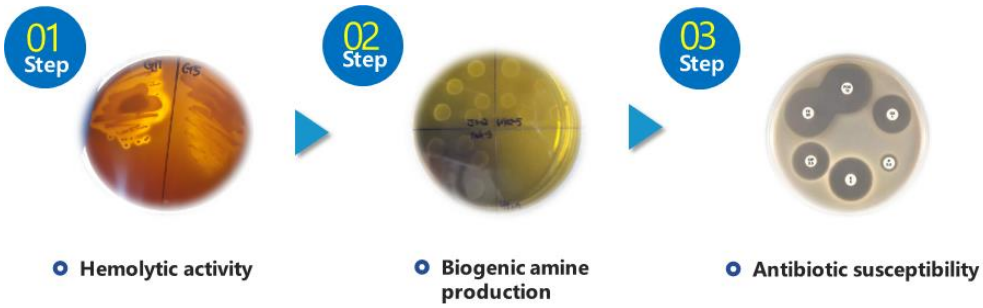

Figure 3. Sammary of the safety assessment methods of lactic acid bacteria.

3.1.5. 16S rRNA sequencing

The screened and selected strains were identified using amplified 16S rRNA gene sequencing. The target 16S rRNA gene of selected

isolates was amplified by using universal primers, 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492 R (5'-TAC GGY TAC CTT GTT ACG ACT T-3'). The PCR was conducted using thermal cycler (iCycler, Bio-Rad, Hercules, CA) at 95 ℃ for 3 min, 20 cycles of denaturation for 30 sec at 95 ℃, annealing for 30 sec at 55 ℃, and extension for 1 min at 72 ℃ and the final elongation for 5 min at 72 ℃. The PCR products was transferred to Macrogen corporation (South Korea) and analyzed by 16s rRNA gene sequencing. The analyzed sequences were compared with those collected in the GenBank database with the BLAST program (http://www.ncbi.nlm.nih.gov).

3.1.6. Enzyme and fermentation test

API 50 CH and API ZYM (Bio-Merieux, France) were used to investigate carbohydrate fermentation and enzymatic profiles of selected isolates, respectively. The protocol of API kits was performed as manufacturer's instructions. The activated cultures of isolates were centrifuged at 12,000 rpm, 4 ℃ for 5 min and washed twice with 0.85 % NaCl. Each of the strains was adjusted with OD_{600nm} at 1 density. A volume of 65 ㎕ of each suspension was loaded in API ZYM kit wells respectively, and after the ZYM test strips were incubated after 4 h of incubation at 37 ℃, changes of color were recorded to determine the enzyme activity (Lee *et al.*, 2012).

3.2. Characterization and validation of LAB for functionality

3.2.1. Bacterial adhesion assay

Cell culture The epithelial cell line of HT-29 derived from human colon adenocarcinoma were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were grown in RPMI 1640 medium (Gibco, USA) containing 10 % heat inactivated fetal bovine serum (FBS; Gibco, USA), L-glutamine (2 mmol l^{-1}), 100 μg streptomycin ml^{-1}, 100 U penicillin ml^{-1}, and 0.25 μg amphotericin B ml^{-1} (Gibco, USA) at 37 ℃ in a 5 % CO_2 atmosphere. To evaluate adhesion ability of LAB, HT29 cells were seeded at 1.0 X 10^6 cells ml^{-1} in 12-well plates and incubated until fully differentiated for 10 days by changing the media every two days. The cells were washed twice with preheated PBS and replaced with fresh non-supplemented RPMI for 2 h prior to the assay.

Adhesion assay of LAB After harvesting the cells of LAB, overnight cultures of LAB strains were suspended with preheated fresh RPMI 1640 media and adjusted to $O.D_{600nm}$ at 1.0 density (aaproximately 1 X 10^8 cfu ml^{-1}). The 1 ml of each bacteria were inoculated to 12-well plates and incubated for 2 h at 37 in a 5 % CO_2 atmosphere. Then, the non-adherent LAB were removed by washing with PBS two times and the HT29 cells and attached bacteria were lysed with 1 ml of 0.05 % Trypsin-EDTA (Gibco, USA). The adherent LAB were enumerated by diluting the solution

serially (1 : 10) with PBS from the initial and using drop-plating method on MRS agar (Xu *et al.*, 2009). Experiments were conducted in duplicate and relative adhesion rates were estimated by comparing with that of *Lactobacillus rhamnosus* GG.

3.2.2. Antimicrobial activity

Disk diffusion assay For screening lactic acid bacteria strains which has potential inhibitory effects against specific major pathogens recognized as the cause of IBD and indicator strains, the paper disc method was carried out on a 1:1 mixture agar of brain heart infusion (BHI) and MRS (Karska-Wysocki *et al.*, 2010). The pathogens and indicators were as follows: indicator strains, such as *Listeria innocua, Listeria monocytogenesis, Staphylococcus aureus, Pseudomonas aeruginosa* and *Enterococcus faecalis* and pathogens known as pathogenesis of IBD including *Helicobacter pylori, Campylobacter coli, Campylobacter jejuni,* and *Escherichia coli*. Overnight cultures of each strain were adjusted into OD_{600nm} at 1 density. 100 ㎕ of the bacteria cultures were inoculated and spread with sterilized cotton swab on the agar media. The paper disks (6mm; oxoid, USA) were placed on the BHI and MRS mixture agar plates. 10 ㎕ of LAB cultures that probably have antibacterial activity were inoculated on the each disk. Inhibitory zone of LAB strains against microaerophilic bacteria strains were checked after 72 hour incubation in mircoaerobic conditions in gas jars using the GasPak

system (CampyGen, Oxoid) at 37 ℃. For assessing antimicrobial activity against indicator strains, the inoculated agar plates were incubated aerobically for 24 h at 37 ℃. The inhibitory effects were determined by measuring the diameters of inhibition zones.

Agar well diffusion assay All pathogens and LAB strains were propagated overnight in BHI broth and MRS broth, respectively. The pathogen strains were centrifuged for 10 min at 12,000 rpm and suspended twice with PBS, and then the bacteria were adjusted to OD_{600nm} of 0.2. A volume of 100 ㎕ of each pathogen culture was combined with 4 mL of LB (Difco, USA) soft agar (0.7 %) and poured onto LB agar plates. After the plates were dried for 30 min, wells was made on plate with sterile pipet. The 100 ㎕ overnight cultures of LAB isolates were inoculated in the wells. The microaerophilic bacteria plates were co-incubated for 72 h at 37 ℃ under microaerophilic conditions and indicator strains were co-incubated aerobically for 24 h at 37 ℃. The inhibition activity was determined by measuring the diameters of inhibition zones (Balouiri *et al.*, 2016).

ATP assay To determine antimicrobial activities of LAB supernatant against specific major pathogens causing IBD, intracellular adenosine triphosphate (ATP) of *Helicobacter pylori, Campylobacter coli, Campylobacter jejuni,* and *Escherichia coli* strains was measured. The pathogenic bacteria were prepared overnight and harvested by centrifugation 10,000 X g, 4 ℃ for 8 min. The harvested cell was washed with PBS and adjusted with

OD_{600nm} values of 1 density. BacTiter-Glo reagent which is an ATP-dependent luciferase-luciferin reagent mixture (Promega Inc., USA) was used in this study for measuring intracellular ATP contents. 50 ㎕ of pathogenic bacteria and each LAB supernatant was added to opaque 96 well plate, respectively, and then 100 ㎕ of the reagents was loaded to each well without light. After incubation for 5 min at 37 ℃ for lysing the bacteria and triggering the enzymatic reaction, the emission of photons was assessed with luminometer (SpectraMax M4 Microplate/Cuvette Reader, Molercular Devices, USA) (Schweinitzer *et al.*, 2008).

3.2.3. Anti-inflammatory effects

Culture of RAW 264.7 cells and HT-29 cells RAW 264.7 cell line of murine macrophages were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were cultivated in Dulbecco's modified Eagles medium (DMEM, Gibco, USA) supplemented with 10 % heat inactivated FBS, L-glutamine (2 mmol l^{-1}), 100 ㎍ streptomycin ml^{-1}, 100 U penicillin ml^{-1}, and 0.25 ㎍ amphotericin B ml^{-1} (Gibco, USA) at 37 ℃ in a 5 % CO_2 atmosphere. HT29 cell line were cultured with complete DMEM and seeded in 24-well plates at a concentration of 1.0 X 10^5 cells ml^{-1} for 7 days at 37 ℃ in a 5 % CO_2 atmosphere until fully confluence for assessing immunomodulation effects of LAB.

Determination of nitric oxide production RAW 264.7 cells were

seeded at 1 X 10^5 cells ml^{-1} in 24-well plates and stabilized for 2 h. For stabilization of the cells, overnight cultures of LAB were centrifuged and the cell pellet were washed with PBS two times and adjusted with OD_{600nm} at 1.0 density. The bacteria were heated for 15 min at 110 ℃ to remove activity of bacteria, and then the cells were stimulated with 900 ㎕ of lipopolysaccharides (LPS, 1 ㎍/ml; sigma-aldrich, USA) and 100 ㎕ of heat-killed LAB for 48 h. The incubated cells were centrifuged at 600 X g, 4 ℃ for 10 min and the cell supernatant were transferred to new tubes. The measurement of Nitric oxide (NO) concentration was estimated by using the Griess reagent (Promega Inc., USA) as the manufacture' s instruction. After mixing the cell supernatant and griess reagent with same volumes, the mixture was incubated for 10 min at room temperature and absorbance at 540 nm was determined by a microplate reader (SpectraMax M4 Microplate/Cuvette Reader, Molercular Devices, USA). The concentration of NO was calculated by comparing to a standard curve (Lee *et al.*, 2008).

Enzyme-linked immunosorbent assays (ELSIA) The HT-29 cells were plated at 1 X 10^5 cells/well and incubated until fully confluence condition by changing media every two days. 24 h before coculture, media was changed with DMEM containing 5 % heat inactivated FBS and no antibiotics solution. The LAB were propagated by subculturing twice and adjusted with OD_{600nm} at 1.0 (aaproximately 1 X 10^8 cfu ml^{-1}) resuspended in fresh DMEM media. After the cells

were incubated with 10 ng ml^{-1} of human tumor necrosis factor-α (TNF- α; PeproTech, INC., USA) and 100$\mu\ell$ of LAB strains for 6 h, the cell supernatants were collected and centrifuged at 13,000 X g at 4 ℃ for 5 min. The cytokines of IL-8 in the supernatants were determined by ELISA as the manufacturer's protocols (Koma Bio-Tech, Seoul, Korea) and measured by the microplate reader (SpectraMax M4 Microplate/Cuvette Reader, Molercular Devices, USA))at 450 nm (Shi *et al.*, 2017).

3.2.4. Evaluation of tight junction integrity

Cell cultures and bacterial administration Caco-2 cell line obtained from Korean Cell Line Bank (KCLB, Seoul, Korea) was originated from a human colonic adenocarcinoma and used between passages 40-60 for all experiments. Cells were maintained in minimal essential medium (MEM; corning Costar Corporation, USA) supplemented with 20 % heat inactivated FBS, 2 mM L-glutamine l^{-1}, 100 U penicillin ml^{-1}, and 100 μg streptomycin ml^{-1}. Lactic acid bacteria cultures were prepared by adjustment of OD_{600nm} at 0.1. TNF- α (10 ng ml^{-}) were treated on caco-2 cell monolayers and the cells were incubated for 24 h. After incubation with TNF- α, the 1 ml of bacterial suspensions with fresh MEM media were inoculated on the cell monolayers and co-cultured for 6 h.

RNA isolation and reverse transcription The caco-2 cells were treated with 0.05 % Trypsin-EDTA (Gibco) and harvested by

centrifugation at 1,000 X g for 3 min. RNA of the cells were treated and extracted with AccuZol™ (Bioneer, USA) according to manufacturer's protocol. For cDNA synthesis, the ReverTra Ace® qPCR RT Master Mix with gDNA Remover kit (TOYOBO, Japan) was used following the manufacturer's instruction. After mixing every reagents of the kit, amplification was conducted using a C1000 Touch thermal cycler (Bio-Rad, USA). It was incubated at 37 ℃ for 15 min, at 50 ℃ for 5 min and at 98 ℃ for 5 min. The synthesized cDNA was stored at 4 ℃ before the experiment.

Quantification of mRNA expression using real-time PCR (qRT-PCR) qRT-PCR was carried out using TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX) kit (Enzynomics, Korea) according to manufacturer's protocol. Oligonucleotide sequences for ZO-1 (TJP1), claudin-1 (CLDN1), occludin (OCLN) and β-actin used to analyze tight junction proteins expression are indicated in Table 3. (Sanchez-Muñoz *et al.*, 2008)

Table 3. Primers of RT-PCR for analyzing tight junction proteins expression.

Gene	Forward primer	Reverse primer	Product size (bp)
CLDN1	GTGGTTGGCATCCTCCTG	AATTCGTACCTGGCATTGACTGG	232
TJP1	CAAGATAGTTTGGCAGCAAGAGATG	ATCAGGGACATTCAATAGCGTAGC	182
OCLN	TCAGGGAATATCCACCTATCACTTCAG	CATCAGCAGCAGCCATGTACTCTT	189
β-actin	AAGGACCTCTACGCCAACAC	CTGCTTGCTGATCCACATCTG	210

3.3. Validation of selected strains in *in vivo* model

3.3.1. Mice and experimental design

8 week-old female C57BL/6 mice were randomly distributed in 6 groups and housed at cages in the animal facilities under the specific pathogen free environment and standard conditions (50-60 % humidity, 12-h dark/12-h light cycles). To induce acute colitis, 2.5 % dextran sulphate sodium (DSS, MW 36,000-50,000; MP Biomedicals, USA) was added into the drinking water for one day, then the concentration of DSS was elevated with 5 % for two days. After constructing IBD model by adding DSS into the drinking water for three days, mice were randomly allocated to three groups for testing the probiotic effects of selected strains. The mice of LGG group (9 mice) were injected with *L. rhamnosus* GG by oral administration every day, whereas the mice of HS-2 (9 mice) and NG-4 groups (9 mice) were gavaged with *Weissella cibaria* HS-2 and *Pediococcus acidilactici* NG-4, respectively every day for two weeks. After 2 weeks of feeding, animals were sacrificed by injecting tribromoethanol overdose. The animal experiment in this study were approved by the Institutional Animal Care and Use Committee of Seoul national university (Approval No. SNU-170912-14-1).

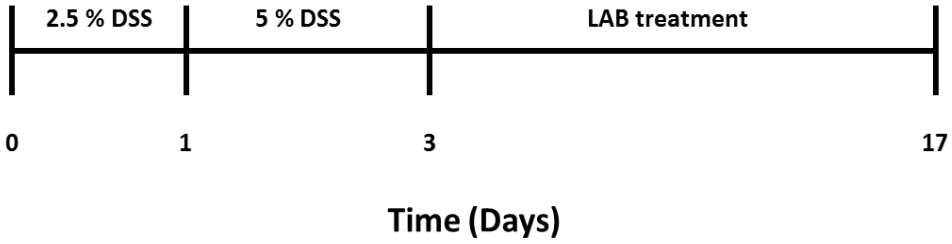

Figure 4. Time schedule for the study of *in vivo* IBD model.

Table 4. Experimental design of the groups and LAB treatment.

Group	Cage	Mice/cage	LAB treatment	Cfu/ml	Treatment periods (Days)
LGG (Control)	3	3	*Lactobacillus rhamnosus* LGG	$(3.0 \pm 1.2) \times 10^8$	14
HS-2	3	3	*Weissella cibaria* HS-2	$(3.7 \pm 1.4) \times 10^8$	14
NG-4	3	3	*Pediococcus acidilactici NG-4*	$(4.6 \pm 1.8) \times 10^8$	14

3.3.2. Probiotic preparation

W. cibaria HS-2 and *P. acidilactici* NG-4 selected from *in vitro* study and *L. rhamnosus* GG (LGG) which is a well-known commercial strain, were cultivated in MRS broth for 18 h at 37 ℃. After bacterial cells were harvested by centrifugation at 14,000 X g for 7 min and washed twice with PBS. The LAB cells were prepared to a final dose of 1.0 X 10^8 CFU/mouse/day.

3.3.3. Sampling of fecal, serum and colons

The fecal samples were collected once a week during the experiment period and After sacrifice on day 17, the samples were collected directly from the large intestine and immediately moved into Eppendorf tubes and stored at −80 ℃. Genomic DNA from the fecal samples was extracted by using the commercial FastDNA Spin Kit for Soil (MP biomedicals, USA) according to the manufacturer' s protocols. The blood was harvested by vein puncture in silca coated tubes (BD, USA) for serum determination. After the blood samples were left for 30 minutes at room temperature for allowing to clot, the serum was collected by centrifugation (4000 X g, 4 ℃, 10 min) and stored at −80 ℃ until analyzed by using ELISA kits. The experimental mice were sacrificed and the colon samples were obtained by dissecting sacrificed mice and the samples were directly treated with AccuZol^{TM} (Bioneer, USA) and stored at −80 ℃ until analyzed. Frozen tissue samples were homogenized by using mini homogenizer and stored at −80 ℃ until extracting RNA from the samples.

3.3.4. Evaluation of colitis

For evaluating colitis during consumption of DSS, animals were checked daily for weight, water/food consumption, stool consistency and existence of total blood in feces. The disease activity index (DAI) was assessed by the following parameters: a) weigh loss (0 point =

none, (1 point = none, 2 point = 1%–2% weight loss, 3 points = 2%–5 % weight loss, 4 points = 5 %–10 % weight loss and 5 points = more than 10% weight loss); b) stool consistency/diarrhea (1 point= normal, 3 points = loose stools, 5 points = watery diarrhea); c) bleeding (1 points = no bleeding, 3 points = slight bleeding, 5 points = gross bleeding) as described by Murthy *et al.* (1993).

3.3.5. Measurement of cytokines

The cytokines of TNF-α, IL-10 and IL-17 and in the collected serum samples were determined by using ELISA kits according to the manufacturer's protocols (Koma Bio-Tech, Seoul, Korea) and measured by the microplate reader (SpectraMax M4 Microplate/Cuvette Reader, Molercular Devices, USA)) at 450 nm (Shi *et al.*, 2017). The levels of detected cytokines were calculated from the standard curves.

3.3.6. RNA Extraction and Real-time PCR

For RNA extraction from the colon tissue samples, total RNA was isolated using AccuZol^{TM} (Bioneer, USA) according to manufacturer's protocol. For cDNA synthesis, the ReverTra Ace® qPCR RT Master Mix with gDNA Remover kit (TOYOBO, Japan) was used following the manufacturer's instruction. After mixing every reagents of the kit, amplification was conducted using a C1000 Touch thermal cycler (Bio-Rad, USA). It was incubated at 37 ℃ for 15 min, at 50 ℃ for 5

min and at 98 ℃ for 5 min. The synthesized cDNA was stored at 4 ℃ before the experiment. The relative expression of tight junction proteins in colon was detected by qRT-PCR which was carried out using TOPreal^{TM} qPCR 2X PreMIX (SYBR Green with low ROX) kit (Enzynomics, Korea) according to manufacturer's protocol. Oligonucleotide sequences for ZO-1 (TJP1), claudin-1 (CLDN1) and GAPDH which was used to analyze tight junction proteins expression are indicated in Table 5.

Table 5. Primers of RT-PCR for analyzing tight junction proteins expression.

Gene	Forward primer	Reverse primer	Product size (bp)
CLDN1	TATGACCCCTTGACCCCCAT	TTGTTTTCCGGGGACAGGAG	127
TJP1	GCAGACTTCTGGAGGTTTCG	CTTGCCAACTTTTCTCTGGCAAC	194
GAPDH	CCAGGATTATGGGCTTGAGA	TCTGACACAAGACGCACTCC	135

Table 6. 16S rRNA gene targeted group specific primers of RT-PCR for analyzing intestinal bacterial flora of mouse feces.

Gene	Forward primer	Reverse primer	Product size (bp)
Weissella cibaria	TTGATTGACGTAGAACCTGAT	TTC GGT GCT AGT TCT TCA ATA	596
Akkermansia muciniphila	CAGCACGTGAAGGTGGGG	CCTTGCGGTTGGCTTCAGAT	327
Enterobacteriaceae	CATTGACGTTACCCGCAGAAGA	CTCTACGAGACTCAAGCTTGC	195
Lactobacillus spp.	TGGATGCCTTGGCACTAGGA	AAATCTCCGGATCAAAGCTTACTTAT	92
Bifidobacteria spp.	CGCGTCYGGTGTGAAAG	CCCCACATCCAGCATCCA	244
Pediococcus spp.	GGACTTGATAACGTACCCGC	GTTCCGTCTTGCATTTGACC	449
Universal primer sets	CCTACGGGAGGCAGCAG	GGACTACHVGGGTWTCTAAT	450

The relative expression of the intestinal bacterial flora were analyzed by qRT-PCR by detecting 16S rRNA gene targeted group specific primers (Table 6).

Chapter 4. Results

4.1 Isolation and identification of lactic acid bacteria

4.1.1. Lactic acid bacteria isolation

77 samples of Korean traditional foods were assembled from mainly the province of Gangwon, South Korea. Total 399 isolates were obtained from the samples by using culture dependent methods with selective media, such as MRS medium and all isolates were confirmed to be LAB by conducting catalase test, gram staining and assessing and physiological traits.

Table 7. The number of isolates obtained from Korean fermented foods.

Sample	No. of samples	No. of isolates
Kimchi	52	· 283
Jeotgal	20	· 68
Makgeolli	4	42
Cheonggukjang	1	· 6
Total	**77**	**399**

4.1.2. Screening and identification of LAB with probiotic properties

For screening every isolate from the samples with fast and simple ways, 399 isolates were sorted out for resistance rate in condition of acid and bile salt. 38 isolated strains were selected with higher relative survival rates when compared to those of *Lactobacillus rhamnosus* GG, which was used as a standard reference culture in this study and commercial lactic acid bacteria.

Antibiotic resistance properties of the selected isolates were determined by MIC of each type of antibiotics which were recommended to assess susceptibility according to microbiological breakpoints of European Food Safety Authority (EFSA). 7 isolates of the selected bacteria were excluded by determining resistance of some antibiotics and cut off by the microbiological breakpoints

4 isolates of 21 LAB which were selected by demonstrating traits antibiotic susceptibility based on criteria of EFSA were shown $\beta-$ hemolytic activity, known as complete hemolysis by breaking down of red blood cells in blood agar and producing clear zone around the cultivated colonies.

4 isolates of the screened bacteria by demonstrating negative hemolysis activity were characterized with expressing properties of biogenic amine production.

Finally, 23 isolates of LAB were examined and selected for identification using 16s rRNA gene BLAST by assessment of

antibiotic susceptibility, hemolytic activity and biogenic amine production property.

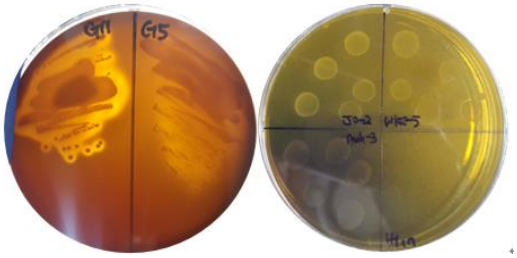

Figure 5. The examples of the result of hemolytic activity and biogenic amine production of test bacteria (Positive reaction of β-hemolytic activity, left; *L. sakei* G7, production of biogenic amin, right; *E. faecium* M0h-3).

Selected 23 strains with evaluation of *in vitro* intestinal survival rate and safety features for human use were identified by analyzing 16s rRNA gene sequence using BLAST database. Only 10 strains were selected for further studies by the criteria on the basis of considering tolerance properties in acid and bile environments and species diversity of identified strains (Table 8).

The identified strains include *Enterococcus*, *Lactobacillus*, *Pediococcus*, *Weissella* species which isolated from Kimchi and Jeotgal which compromised with morphologies of 4 rod and 6 cocci

Table 8. Identification and characterization of 10 selected isolates.

No	16s rRNA sequence analysis (Closest known species)	Strain	Origin	Catalase test	Gram staining	Morphology	Culture condition[a]	
				(+/−)	(+/−)		Anaerobic	Aerobic
1	*Enterococcus faecium*	M7k−1	Kimchi	−	+	Cocci	+	+
2	*Lactobacillus brevis*	B4	Kimchi	−	+	Rod	+	+
3	*Lactobacillus paracasei*	Mr−1	Kimchi	−	+	Rod	+	+
4	*Lactobacillus plantarum*	M0u−3	Kimchi	−	+	Rod	+	+
5	*Lactobacillus sakei*	RS6	Kimchi	−	+	Rod	+	+
6	*Pediococcus acidilactici*	NG−4	Jeotgal	−	+	Cocci	+	+
7	*Pediococcus pentosaceus*	B7	Kimchi	−	+	Cocci	+	+
8	*Pediococcus pentosaceus*	HS−1	Jeotgal	−	+	Cocci	+	+
9	*Weissella cibaria*	HS−2	Jeotgal	−	+	Cocci	+	+
10	*Weissella hellenica*	WKC−4	Kimchi	−	+	Cocci	+	+

[a] Each isolate was incubated both aerobically and anaerobically.

All of 10 selected strains evaluated to have tolerance ability to acidic conditions, pH 3. After incubation for 2 h in MRS medium which pH value is 3, the survival rates of M7k-1, B4, Mr-1 and WKC-4 strains showed significantly higher survivability compared to LGG, reference strains. The LGG as positive control were demonstrated as survival rate of 73.41 %. Though all strains including LGG tested in this study show low relative survival rates in MRS medium with 0.3 % (w/v) oxgall, eight strains of selected LAB except RS6 and WKC-4 showed significantly higher resistance to bile salts than that of LGG (Table 9).

Table 9. *In vitro* intestinal survival rate and minimum inhibitory concentrations (㎍/ml) of antibiotics to selected lactic acid bacteria.

Species	Strains	*In vitro* intestinal survival rate		Antibiotics susceptibility						
		Bile tolerance[a] (%)	Acid tolerance[b] (%)	AMP	GEN	KAN	STR	ERY	TET	CHL
E. faecium	M7k-1	64±4.64**	103.19±12*	2	32	256	8	<2	<2	<2
L. brevis	B4	67±14.25**	100.00±7**	<2	16	64	64	<2	<2	<2
L. paracasei	Mr-1	43±2.32**	122.16±26*	4	16	64	64	<2	<2	2
L. plantarum	M0u-3	36±5.71**	83.33±15	<2	4	32	4	<2	2	8
L. sakei	RS6	23±9.08	72.83±16	4	16	64	64	<2	4	4
P. acidilactici	NG-4	46±2.87**	107.84±29	4	4	64	64	<2	4	4
P. pentosaceus	B7	32±4.31**	73.68±24	4	16	64	64	<2	<2	4
P. pentosaceus	HS-1	39±3.32**	81.82±14	4	16	64	4	<2	4	4
W. cibaria	HS-2	42±4.29**	75.00±10	4	4	64	32	<2	8	2
W. hellenica	WKC-4	46±16.93	100.00±3**	2	16	256	16	<2	8	4
L. rhamnosus	GG	18±0.88	73.41±7							
Suggested breakpoint in accordance to the European Food Safety Authority (EFSA)										
Pediococcus				4	16	64	64	1	8	4
Lactobacillus plantarum				2	16	64	64	1	32	8
Lactobacillus paracasei				4	32	64	64	1	4	4
Lactobacillus facultative heterofermentative				4	16	64	64	1	8	4
Enterococcus faecium				2	32	1024.	128	4	4	16

AMP, GEN, KAN, STR, ERY, TET, and *CHL* refer to ampicillin, gentamycin, kanamycin, streptomycin, erythromycin, tetracycline and chloramphenicol, respectively

[a] Relative bile tolerance (%) in MRS media supplemented with 0.3 % (w/v) oxgall,

[b] Bacterial cell survival rate (%) at pH 3.0

The data represent the means and standard errors of three replicates. Significance is indicated as follows: *P < 0.05; **P < 0.01

4.1.3. Enzymatic and fermentation profiles

The selected 10 LAB strains showed completely dissimilar patterns of fermentation which means that every isolates are identified as distinct strains even though B7 and HS-1 are classified with same species, *Pediococcus pentosaceus.* All strains are characterized with fermentation ability of D-SACcharose (sucrose) (Figure 6).

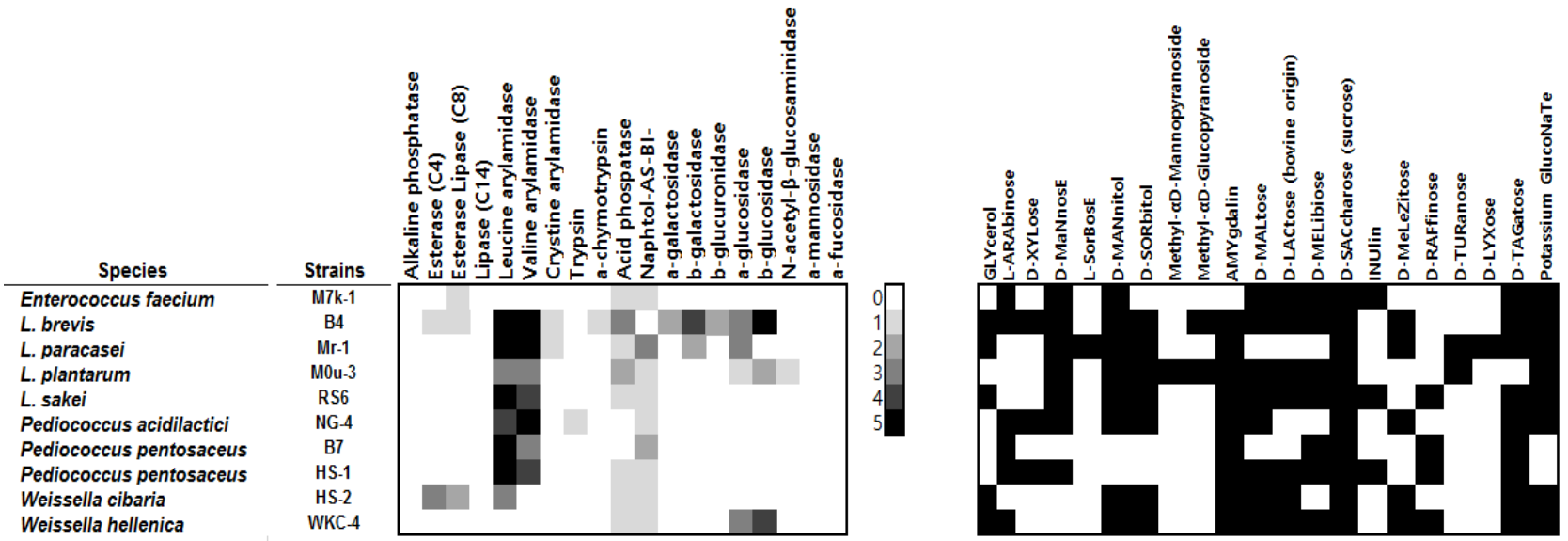

Figure 6. Enzyme patterns and carbohydrate fermentation of selected LAB were assessed by API ZYM kit, and API 50 CHL, respectively. The biochemical properties are shown in a black–white color scale, where black represents positive activity and white represents negative activity.

4.2. Characterization and validation of functionality

4.2.1. Adherence ability to epithelial cells

For evaluation of each adherence ability of 10 selected LAB, all experiments were conducted in 12 well plates with duplicate. The relative adhesion rates of selected bacteria were evaluated by comparing with LGG as a control strain using HT-29 epithelial cell line. *L. brevis* B4 strains showed unequaled adhesion ability higher than almost 7-fold to intestinal epithelial cell compared with LGG. In addition, *E. faecium* strain M7k-), *L. paracasei* Mr-1, *P. acidilactici* NG-4, *P. pentosaceus* B7, *W. cibaria* HS-2 has significantly higher adhesion rates than control strain. Strain of HS-1 showed similar adhesion ability when compared with LGG.

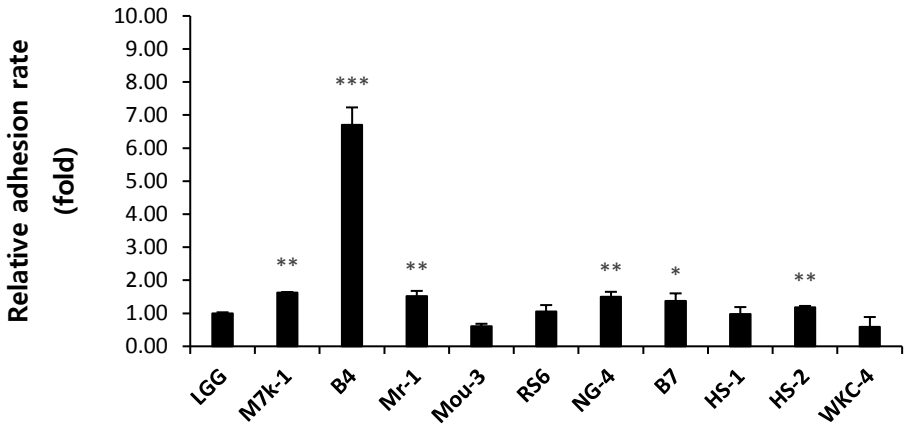

Figure 7. The relative adhesion rate of lactic acid bacteria strains on HT 29 cells. Relative adherence was represented by a fold change compared with that of LGG. The data represent the means and standard errors of duplicates. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

4.2.2. Antimicrobial activity

The objective of high speed screening LAB and confirmation with antimicrobial activity, two kinds of methods were used in this study. Disk diffusion assay were used for screening LAB fast and simply and the degree of antimicrobial activity were assessed by measuring length of clear zone around the well using agar well diffusion method.

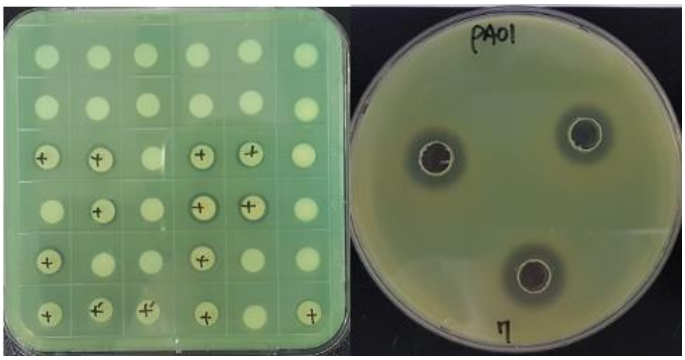

Figure 8. The examples of result for disk diffusion assay (left) and agar well diffusion assay (right).

The antimicrobial activity of the disk diffusion of LAB were assessed against food-born pathogen indicators and major pathogens in pathogenesis of IBD except helicobacter pylori and *campylobacter coli* as these pathogens didn't grow on the agar plates with 1:1 mixture of MRS and BHI medium. Every selected strains showed antagonistic effects against *campylobacter coli*, Staphylococcus aureus, *Enterococcus faecalis,* and *Peudomonas aeruginsa* (Table 10).

Table 10. The assessment of antimicrobial activity by disk diffusion assay.

Species	Name	Existence of the zone of inhibition[a]						
		SA	K88	EF	LM	LI	PAO1	C. coli
E. faecium	M7k-1	+	+	+	+	+	+	+
L. brevis	B4	+	+	+	+	+	+	+
L. paracasei	Mr-1	+	+	+	+	+	+	+
L. plantarum	M0u-3	+	+	+	+	+	+	+
L. sakei	RS6	+	+	+	+	+	+	+
P. acidilactici	NG-4	+	+	+	+	+	+	+
P. pentosaceus	B7	+	+	+	+	+	+	+
P. pentosaceus	HS-1	+	+	+	+	+	+	+
W. cibaria	HS-2	+	+	+	+	+	+	+
W. hellenica	wkc-4	+	+	+	+	+	+	+
L. rhamnosus	LGG	+	+	+	+	+	+	+

C. coli; *Capmpylobacter coli* KTCC 15212, K88; *Escherichia coli* K88 SA; *Staphylococcus aureus* ATCC 25922. EF; *Enterococcus faecalis* ATCC 29212, LI; *Listeria innocua* ATCC 33090, LM; *Listeria monocytogenes* KCTC 13064, PAO1; *Pseudomonas aeruginosa* PAO1

[a] +, > 1cm; w, < 1cm; -, no clear zone

The results of antimicrobial activity for LAB cultures on growth of pathogens were evaluated by the agar well diffusion assay (Table 11). Almost every LAB strains have antimicrobial activities against major pathogen groups in the pathogenesis of IBD and food-born pathogen indicator groups, except the strain of *L. brevis* B4. As it' s shown almost every strains except B4 has antimicrobial activity by forming clear zone around the well, ranging from 1.16 cm to 2.57 cm. *L. paracasei* Mr-1 has the strongest antimicrobial activities among the tested LAB strains when compared with the reference culture, LGG.

Table 11. Diameter of the zone of inhibition against pathogens.

Species	Name	Major pathogens in the pathogenesis of IBD[a] (mm)				Foodborne pathogen indicators[b] (mm)				
		C. coli	HP	CJ	K88	SA	EF	LM	LI	PAO1
E. faecium	M7k-1	1.76±0.09	1.42±0.1	1.63±0.15	1.81±0.15	1.47±0.02	1.6±0.2	1.49±0.06	1.51±0.08	1.43±0.07
L. brevis	B4	1.62±0.12	-	-	1.76±0.11	1.53±0.09	-	1.71±0.13	1.59±0.09	1.76±0.11
L. paracasei	Mr-1	2.5±0.25	1.5±0.07	1.91±0.08	1.92±0.09	1.71±0.05	1.29±0.04	1.63±0.01	1.58±0.05	1.75±0.06
L. plantarum	M0u-3	2.1±0.09	1.3±0.13	1.74±0.18	1.75±0.09	1.46±0.14	1.16±0.03	1.37±0.1	1.64±0.04	1.43±0.1
L. sakei	RS6	1.98±0.07	1.46±0.13	1.62±0.13	1.74±0.17	1.48±0.02	1.21±0.03	1.46±0.12	1.57±0.09	1.29±0.03
P. acidilactici	NG-4	2.05±0.19	1.54±0.05	2.28±0.13	1.9±0.11	1.5±0.04	1.44±0.12	1.43±0.13	1.54±0.05	1.88±0.08
P. pentosaceus	B7	2.01±0.13	1.56±0.06	1.85±0.08	1.67±0.03	1.39±0.1	1.32±0.01	1.5±0.16	1.69±0.12	1.67±0.03
P. pentosaceus	HS-1	1.99±0.08	1.67±0.07	1.82±0.13	1.56±0.17	1.38±0.07	1.3±0.08	1.39±0.05	1.47±0.06	1.56±0.17
W. cibaria	HS-2	1.8±0.15	1.74±0.21	1.69±0.11	1.83±0.13	1.48±0.07	1.2±0.08	1.44±0.12	1.59±0.03	1.39±0.09
W. hellenica	WKC-4	1.58±0.15	1.79±0.17	2.19±0.18	1.69±0.08	1.41±0.09	1.56±0.06	1.33±0.11	1.55±0.07	1.9±0.03
L. rhamnosus	GG	2.57±0.19	1.58±0.1	1.72±0.16	1.72±0.18	1.7±0.04	1.41±0.15	1.5±0.04	1.45±0.05	1.79±0.12

[a] C. coli; *Capmpylobacter coli* KTCC 15212, HP; *Helicobacter pylori* KTCC 12083, CJ; *Campylobacter jejuni* subsp. *Jejuni* ATCC 33560, K88; *Escherichia coli* K88

[b] SA; Staphylococcus aureus ATCC 25922, EF; *Enterococcus faecalis* ATCC 29212, LI; *Listeria innocua* ATCC 33090, LM; *Listeria monocytogenes* KCTC 13064, PAO1; *Pseudomonas aeruginosa* PAO1, *Lactobacillus rhamnosus* GG, a commercial lactic acid bacteria, was used as a standard reference culture,.-; no clear zone

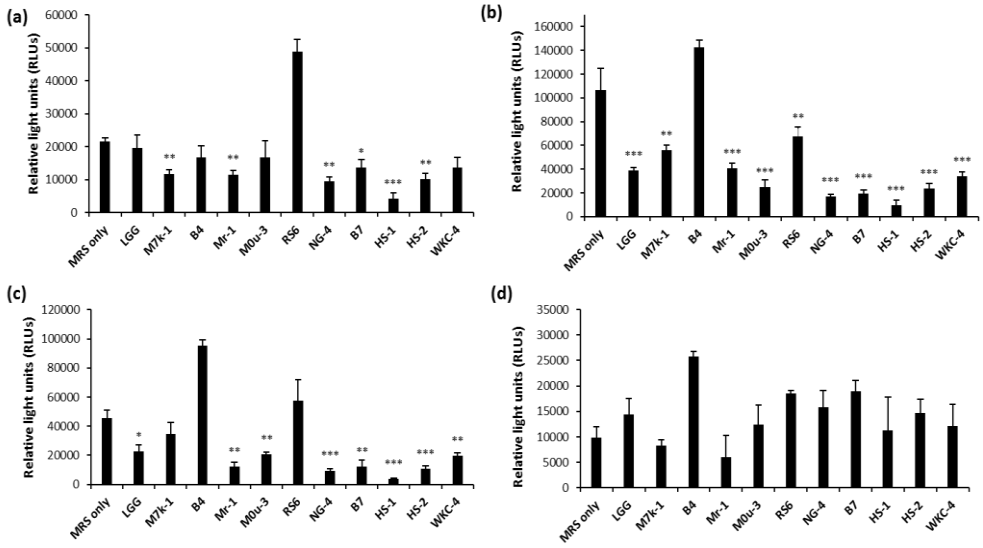

Figure 9. Measurements of intrabacterial ATP levels in the presence of target LAB strains. (A) *Escherichia coli* K88 (B) *Helicobacter pylori* ATCC 12083 (C) *Campylobacter coli* KTCC 15212 (D) *Campylobacter jejuni* subsp. *jejuni* ATCC 33560. The data represent the means and standard errors of duplicates. Significance is indicated as follows; $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

To evaluate the antimicrobial activity against main pathogens known as causing IBD, level of ATP produced from pathogens using bioluminescence assay was measured by the amount of relative light unit (RLU) which can be transformed into the RLU/mole of ATP.

The RLUs of *Escherichia coli* K88 was significantly suppressed by the strains of M7k-1, Mr-1, NG-4, B7 and HS-2 compared to negative control which contains viable pathogen cultures without

inoculating any LAB cultures, whereas LGG, M0u-3, RS6 and WKC-4 cultures didn't express inhibitory effects. The strain of HS-1 was the most powerful inhibition effects against *Escherichia coli* among the tested LAB (Figure 9 (A)). As shown in the figure 9 (B) which was measured for inhibition effects of *Helicobacter pylori*, almost every strains determined to have antimicrobial effects except B4 strain. HS-1 significantly repressed the level of ATP and showed the strongest inhibition effects against *Helicobacter pylori*. The level of RLUs detected from *Campylobacter coli* was reduced by most LAB except M7k-1, B4 and RS6 and HS-1 strain also expressed the lowest level of RLUs, which has the most powerful inhibition effects (Figure 9 (C)). Although the results of other pathogen species by measuring intrabacterial ATP showed significant reduction effects, the results of *Campylobacter jejuni* didn't expressed significant inhibition effects (Figure 9 (D)).

4.2.3. Anti-inflammatory activity

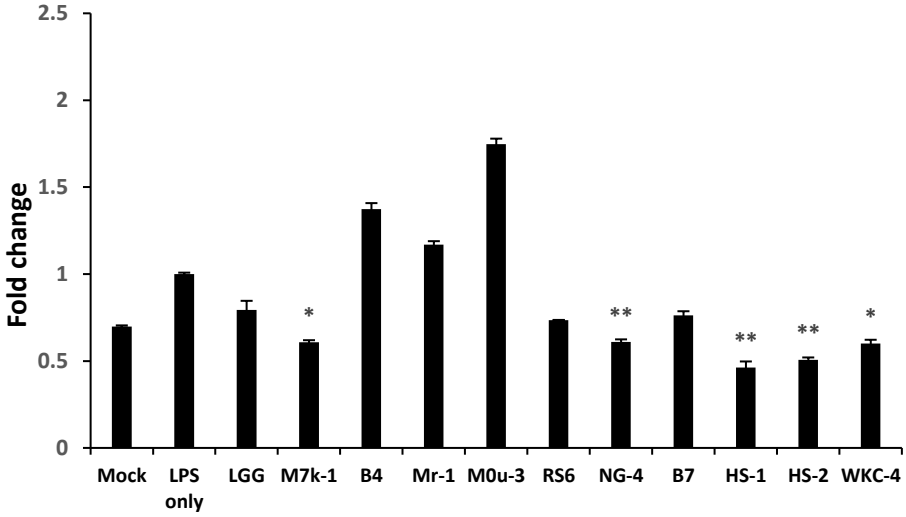

Figure 10. Anti-inflammatory effects of heat-killed lactic acid bacteria in LPS (1 μg/ml) induced RAW 264.7 murine macrophage cells. The concentration of nitrile production was determined by calculating standard curve. Values are mean ± S.D of triplicates for each group. * $P < 0.05$, ** $P < 0.01$, compared with treatment of only LPS.

To examine the effects of selected lactic acid bacteria, 10 ng/ml of LPS was treated to 264.7 cells for 48 h to induce inflammation and NO production. 5 strains of 10 selected bacteria have shown the inhibition effects of NO production induced by LPS. *E. faecium* M7k-1 was evaluated to have immune-regulating effects by significantly suppressing NO production compared to LGG and other strains. *Pediococcus* and *Weissella* species also have shown anti-

inflammatory effects compared with the positive control treated only LPS (Figure 10).

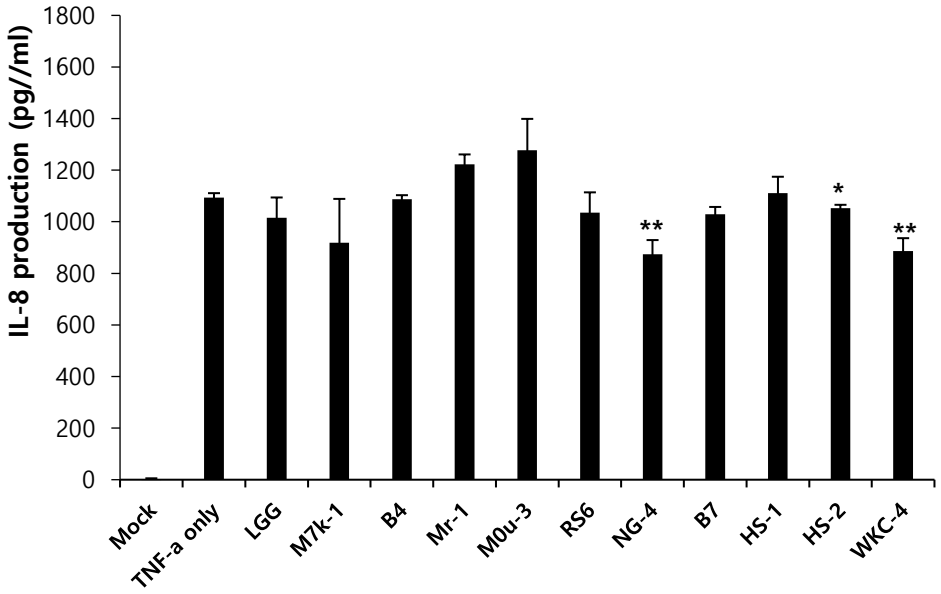

Figure 11. Attenuation effects of TNF-α (10 ng/ml) mediated suppression of IL-8 production for 6 hours by co-incubation with selected lactic acid bacteria in HT-29 cells. Supernatant were harvested for IL-8 ELISA. *p<0.05, **p<0.01 by using t-test analysis.

As shown in Fig. 11 which is the result of measuring IL-8 production by ELISA, there was a significant regulating effects for secretion of IL-8 in TNF-α activated HT-29 cells by the strains of NG-4, HS-2 and WKC-4 compared to the treatment group of only TNF-α (10 ng/ml).

4.2.4 Evaluation of tight junction integrity

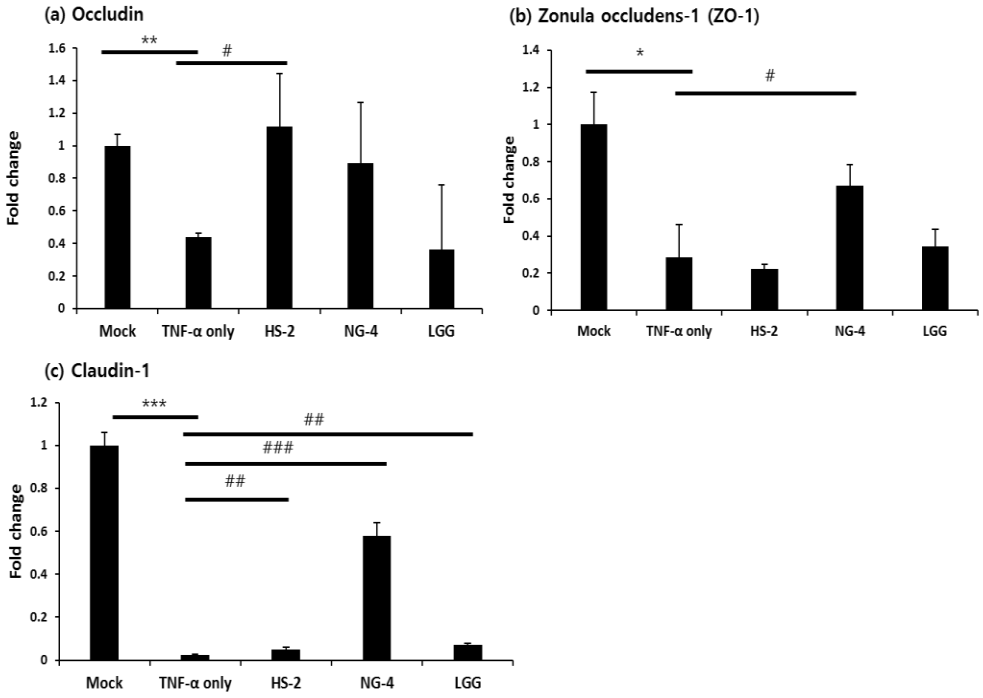

Figure 12. Tight junction-related mRNA gene expressions by treatment of two selected lactic acid bacteria in TNF-α (10 ng/ml)-induced Caco-2 monolayer. (a) Occludin (b) Zonula occludens-1 (c) Claudin-1. The monolayers were stimulated with TNF-α, and then incubated for 24 h. After 24 h, LAB ($O.D_{600nm}$ = 0.1) were added on the cells, and incubated for 6 h. Data are expressed as mean ± SD. of triplicate experiments. $*P<0.05$, $*P<0.01$; Mock versus TNF-α, $\#P<0.05$, $\#\#P<0.01$, $\#\#\#\ P<0.001$; LAB versus TNF-α treatment only.

As shown in Fig. 12, the changes in the expression of tight junction proteins genes were characterized by performing quantitative RT-PCR (qRT-PCR) analysis. TNF-α treatment caused significant decrease in mRNA gene expressions of all kinds of tight junction proteins in caco-2 monolayers. The administration of HS-2 strain significantly increased the occludin and claudin-1 mRNA expression after 24 hours, whereas the treatment of NG-4 strain significantly enhanced mRNA gene expression of zonula occludens-1 and claudin-1.

4.3. Validation of selected strains in *in vivo* model

4.3.1. Evaluation of colitis

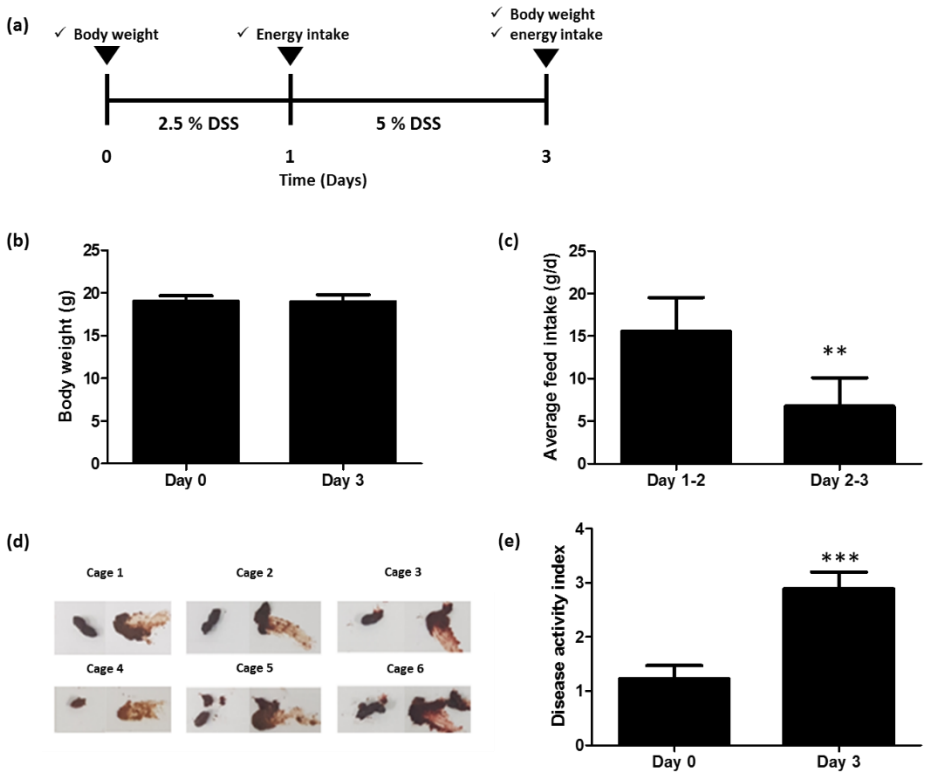

Figure 13. Evaluation of DSS-induced colitis. 5 % DSS were added in the drinking water for 3 days, and then water without DSS in all groups. (a) Time schedule of DSS treatment, (b) body weight and (c) average feed intake during administration of DSS, (d) photographs of fecal samples on day 3 of DSS administration and (e) disease activity index on day 0 and day 3 of DSS treatment. Vertical columns and error bars represent mean ± SD. *$p<0.05$ versus control group by using t-test analysis.

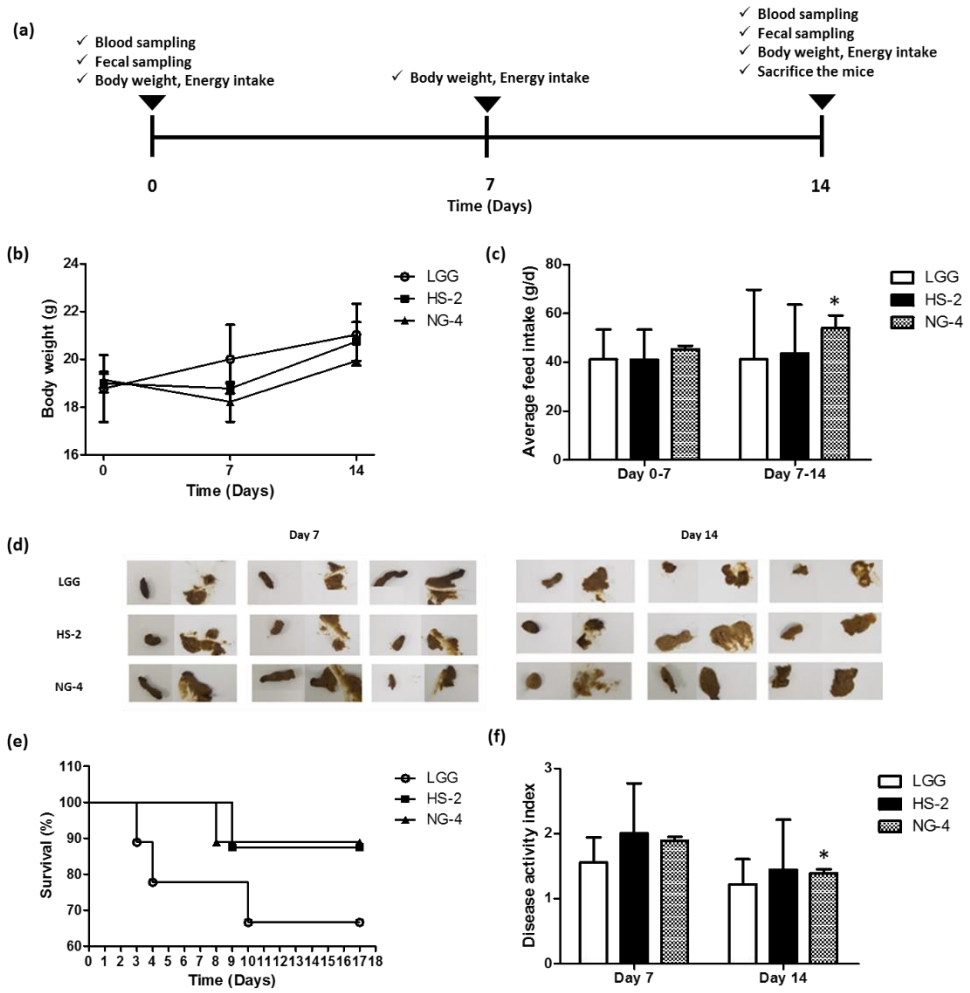

Figure 14. Lactic acid bacteria ameliorates DSS-induced colitis in mice. In parallel, they were administrated daily LGG, HS-2 or NG-4 intragastrically for 14 days. (a) Time schedule of LAB treatment, (b) Body weight, (c) Average feed intake,(d) Disease activity index, (e) Survival rate for 17 days (f) Photographs of fecal samples on day 7 and day 14 during LAB administration. Vertical columns and error bars represent mean ± SD. *$p<0.05$ by using t-test analysis

The body weight of animals didn't show any changes over three days of observation, whereas average feed intake showed significant reduction during DSS administration over same time. Though the body weight changes were not observed during the 3 day period of DSS treatment, the photographs of feces on the last day of DSS treatment and the results of disease activity index represented that animal models of intestinal colitis established well during 3 days of DSS treatment (Figure 13).

Though all groups of LAB treatment didn't show any body weight changes for 14 days, the photographs of feces on 7 day and 14 day of LAB treatment exhibited clearly reduced bleeding and diarrhea. The administration of LAB for 14 days exhibited significant increase of average feed intake and disease activity index in NG-4 group. HS-2 and NG-4 groups showed better survival than LGG group. Disease activity indexes (DAI) in every LAB groups displayed a tendency to reduce the scores of that (Figure 14).

4.3.2. Colon length

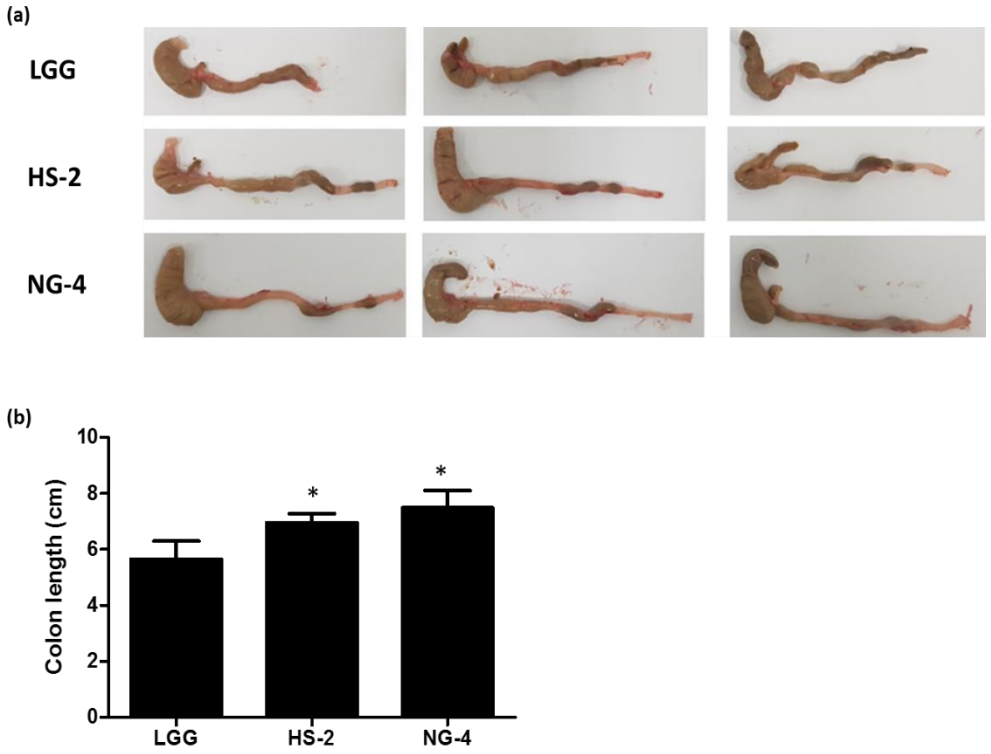

Figure 15. Colons of mice on day 14 of DSS-induced colitis in LGG (n=3), HS-2 (n=3) and NG-4 (n=3) groups. (a) Photographs of dissected colons on day 14 of LGG, HS-2 or NG-4 strain administration after inducing colitis by treatment of DSS. (b) Colon length in mice on day 14. Vertical columns and error bars represent mean ± SD. *$p<0.05$ versus LGG group by using t-test analysis.

Markedly less reduction of the colon length was evident in the both selected lactic acid bacteria, HS-2 and NG-4 group than LGG group (Figure 15).

4.3.3. Analysis on cytokine profile

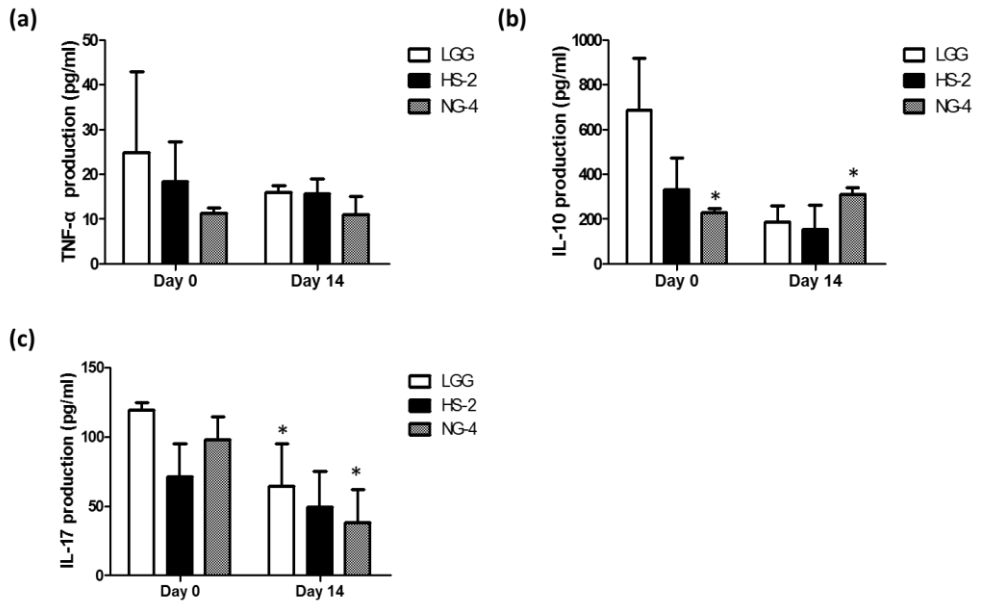

Figure 16. Serum levels of Cytokines in LGG, HS-2 and NG-4 strain group. (a) IL-10 (b) IL-17. Vertical columns and error bars represent mean ± SD. $*p<0.05$ by using t-test analysis.

To investigate the attenuation effects of the selected lactic acid bacteria in DSS models, we analyzed serum cytokine profiling in TNF-α, IL-10 and IL-17. The production of TNF-α which is a typical pro-inflammatory cytokine was not changed significantly in all groups. Levels of IL-10, well-known anti-inflammatory cytokine, was significantly increased in NG-4 group after treating LAB for 14 days. Significant reduction of IL-17 levels was observed in LGG and HS-2 group compared to that of on the day 0 (Figure 16).

4.3.4. Microbiology analysis of mouse feces

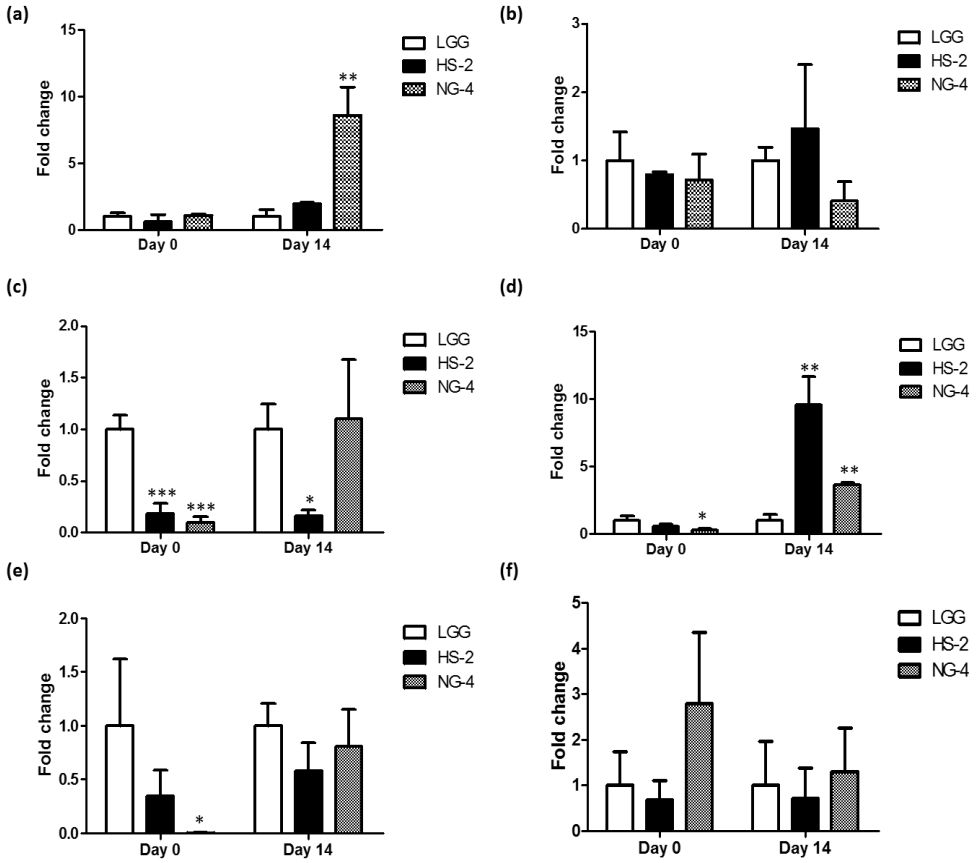

Figure 17. Relative bacterial quantitation in fecal samples at day 0 and at day 14 as determined by qPCR. (a) *Lactobacillus* spp. (b) *Weissella* spp. (c) *Pediococcus* spp. (d) *Bifidobacteria* spp. (e) *Akkermansia muciniphila* (f) *Enterobacteriaceae*. Differences with in groups (day 0 and day 14) were determined by the paired *T* test. . $*p \leq 0.05$, ** ≤ 0.01, *** ≤ 0.001.

DSS-induced colitis was related to total alterations in the fecal microbiota. Levels of Lactobacillus have a tendency to increase in both HS-2 and NG-4 group as well as significantly increased in the animals in the NG-4 group compared to LGG group at day 14. HS-2 group are shown to have higher levels of *Weissella* spp. than LGG group at day 14. NG-4 group are seen to be similar levels of *Pediococcus* spp. at day 14 whereas the levels of that was significantly decreased at day 0 compared to LGG group, which means that the amounts of *Pediococcus* spp. have estimated to increase due to the consumption of NG-4 strain. In both probiotic group, levels of *Bifidobacterium* were increased after 14 days compared to the LGG control group. No significant differences were seen in levels of *Akkermansia muciniphila*. Though the levels of *Enterobacteriaceae* were not significantly varied in both LAB group at day 14, they have a tendency to decrease in NG-4 group at day 14 than at day 0 compared to LGG group (Figure 17).

4.3.5. Evaluation of tight junction integrity

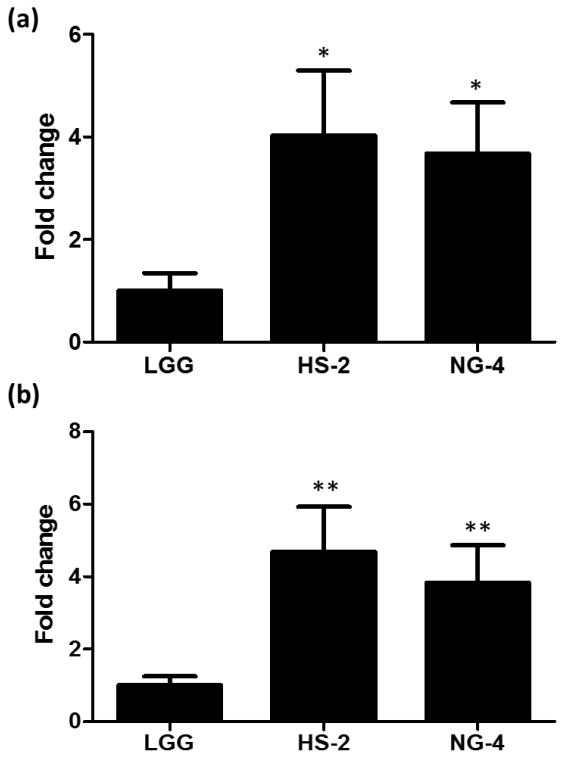

Figure 18. Colonic expressions of tight junction proteins. Quantitative real time PCR for determination of relative mRNA gene expression (a) Claudin-1, (b) Zonula occludens-1 (ZO-1) after 14 days of LAB treatment. $*p<0.05$, $**p<0.01$ compared with LGG group by using t-test analysis.

Quantification of tight junction proteins (claudin-1 and ZO-1) were conducted by real-time PCR. Significant increase of relative TJ proteins mRNA gene expression were seen when comparing the LGG group with each test group (Figure 18).

Chapter 5. Discussion

Several studies have shown that a variety of factors and mechanisms, such as the actions of gut microbiota (Nell *et al.*, 2010), regulatory T cells and cytokines playing a crucial role to modulate inflammation (Renz *et al.*, 2012), and pathogenic infection including *Helicobacter, Campylobacter, Salmonella* species (Nell *et al.*, 2010) are closely related to triggering IBD. As previously studied (Delcenserie *et al.*, 2008), probiotics can healthfully modulate the composition of gut microbiota and the excessive immune response corresponding with the factors to cause IBD. Though many attempts to cure IBD by using probiotics have been recently attained (Damaskos and Kolios, 2008), there is still significant controversy regarding its effectiveness. Moreover, there is still no medication for treatment of IBD up to now according to Prisciandaro *et al.* (2009). Therefore, the objective of this study is to develop putative probiotics for alleviating intestinal inflammation with *in vitro* complicated methods by screening lactic acid bacteria isolated from Korean traditional fermented foods and to verify the attenuation effects of selected probiotics by using dextran sodium sulfate (DSS)-induced colitis models *in vivo* study.

In this study, 399 lactic acid bacteria (LAB), well-known generally

recognized as safe (GRAS) microbes, were isolated from 77 fermented food samples with using selective medium. The first step of the study, isolated colonies of LAB were screened on the basis of acid and bile tolerance, that allow to evaluate survivability in the gastrointestinal tract and are key features to conserve health effects for their host in accordance with Dunne *et al.* (2001). *Lactobacilli*, *Enterococcus* and *Pediococcus* species generally showed resistance to low pH because of their functions to produce short chain fatty acids including lactic acid, acetate and butyrate, which agrees with previous studies (Pinto *et al.* 2006, Zago *et al.* 2011). It was assessed that 38 LAB isolates were characterized with outstanding viability under acidic and bile salts conditions compared to reference strain, *Lactobacillus rhamnosus* GG, which is widely investigated of various probiotic properties (Näse *et al.*, 2001).

Safety assessments of the LAB isolates according to criteria suggested from EFSA were conducted as an essential stage for selection and validation of probiotics according to the study by Gueimonde and Salminen (2006). Owing to bacterial resistance to antibiotics is considered as a worldwide issue for both human and animal use with regard to a report of Yüceer and Özden Tuncer (2015), the safety assessment of LAB were conducted by antibiotic susceptibility, hemolytic and biogenic amine production ability. In this

study, 23 isolates including *Lactobacillus*, *Pediococcus*, *Weissella* and *Enterococcus* spp. were evaluated to be safe even when consumed with high amounts, similar to other results of studies (Riboldi *et al.* 2009, Toomey *et al.* 2010). Finally, 10 strains of LAB were selected for the further study for evaluating an adhesion ability to the epithelial cells, which is an important factor to prevent adhesion of pathogens from infections by competitively colonizing to intestinal mucosa in agree with Lee *et al.* (2008). It has been reported that the human colonic adenocarcinoma cell line HT-29 have been widely used to examine the adhesion rates of possible probiotic bacteria (Ayeni *et al.*, 2011). *Lactobacillus brevis* B4 strain in this study showed exceptional adhesion rates and 6 strains (M7k-1, RS6, NG-4, B7 and HS-2) of 10 selected LAB performed similar adhesion values compared to LGG, signifying that these strains have great possibilities of colonization in *in vivo* intestinal environments.

Selected LAB strains were evaluated with three main functions, including antimicrobial, anti-inflammatory activity and reinforcement ability of tight junction integrity in epithelial cells, to validate alleviating effects of intestinal chronic inflammation, based on the main factors of influencing IBD. By investigating antimicrobial activities using three different methods, most selected strains except *Lactobacillus brevis* B4, displayed inhibition effects against *Escherichia coli*, *Helicobacter pylori*, *Campylobacter coli*,

Campylobacter jejuni that possibly explain the cause of pathogenesis of IBD as well as foodborne pathogens according to reports of Hansen *et al.* (2010). Considering these results, we could anticipate health-promoting effects of the probiotic candidates by preventing against recurrence of diarrhea, food poisoning, enteric colonization and dominance of the pathogens.

To examine the anti-inflammatory effect of LAB in intestinal tract, nitric oxide (NO) and cytokine productions were determined by using microplate reader. NO production was measured by treating heat-killed LAB on the lipopolysaccharide (LPS)-activated RAW264.7 cells. It has been reported that NO is related to various immunological procedures such as host defence, immunoregulation and signal transduction and are importance mediators triggering IBD (Schoedon *et al.* 1995). Dijkstra (2004) reported that NO is produced from L-arginine by enzyme of nitric oxide synthase (NOS) and the inducible isoform of NO (iNOS) during inflammation and iNOS is activated by pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α), interferon-gamma (IFN-γ) and LPS. In lined with the previous studies, suppressing these inducers and NO production is crucial to inhibit the excessive inflammatory response as present in IBD (Cross and Wilson, 2003). For the immunomodulation experiment, the HT-29 cellular model stimulated with TNF-α is widely used to approve anti-inflammatory properties of LAB, similarly as previously studied

(Kechaou *et al.*, 2013). It has been well known that TNF is produced by lamina propria mononuclear cells and the levels of that markedly increased in IBD patients according to Atreya *et al.* (2011). Nielsen *et al.* (1997) reported that IL-8, well known as neutrophil chemotactic factor and basically secreted cytokines during inflammation response, has been also increased in the inflamed intestine of patients with IBD. In this study, both the levels of NO in LPS-activated RAW264.7 cells and IL-8 in TNF-α stimulated HT-29 cells were significantly suppressed by *Weissella cibaria* HS-2 and *Pediococcus acidilactici* NG-4 strains. This result is implicated that both strains have the anti-inflammatory property and modulating effects of excessive immune response with *in vitro* study. Besides, both selected strains demonstrated an ability to upregulating mRNA expression of TJ proteins against TNF-α induced injury. It is well proved that treatment of TNF-α to intestinal epithelial cells encourages reconstruction of several TJ proteins such as claudin-1, occluding and ZO-1 and disrupts epithelial barrier integrity (Cui *et al.*, 2010). There are some reports regarding to therapeutic effects of probiotics on impaired epithelial barrier function in vitro and in vivo study (Gong *et al.* 2016, Hsieh *et al.* 2015).

Because the cause and definite diagnosis of IBD is not clear, there has been necessity of a reliable means to quantitate inflammatory activity for IBD patients and alleviate the symptoms of IBD (Nikolaus

and Schreiber, 2007). As previously characterized (Strober *et al.*, 2002), DSS-induced murine colitis models have many common traits to human IBD which includes cytokine dysfunction and alteration of intestinal microbiota. Therefore, experimental colitic mice induced by DSS have been studied effectively as an IBD model and the influence of LAB on intestinal inflammation.

To verify the attenuation effects of selected two kinds of LAB on DSS-induced colitis, we treated three kinds of LAB including LGG, HS-2 and NG-4 strain daily for 14 days after administration 2.5 % and 5 % DSS for three days to C57BL/6 mice. Through the results of increased average feed intake, better survival, less DAI scores and shortening of the colon length compared to LGG group, these findings shows that administration of NG-4 strain significantly attenuates inflammation in mice colitis models induced by DSS compared to LGG strain.

With regard to a study of Alex *et al.* (2008), acute DSS colitis was marked by higher production of pro-inflammatory cytokine such as IL-6 and TNF-α and regulatory cytokines IL-17 generated by Th-17. Although our cytokine analysis profile were not accordance with these results because there is no control group which was treated only DSS, the levels of IL-17 was significantly decreased whereas IL-10 production was significantly increased in NG-4 groups at day 14 compared to LGG groups without affecting the levels of TNF-α.

This implicates that NG-4 strain have a tendency to regulate inflammatory responses similar to other LABs with previous studies (Park *et al.* 2017, Park *et al.* 2017).

According to Herias *et al.* (2005), a significant increase in the levels of Enterobacteriaceae was observed when treatment of DSS. Our results which have a tendency to reduce more the levels of that in NG-4 group compared to LGG group after 14 days indicate that NG-4 strain could regulate and recover the gut microbiota more effectively than LGG strain. Based on the report by Vlasova *et al.* (2016), *Lactobacillus* and *Bifidobacteria* spp. are recognized as one of the main human intestinal microbiota exerting various health-promoting effects by colonization in the gastrointestinal tracts. The treatment of NG-4 strain also showed higher levels of *Lactobacillus* and *Bifidobacteria* spp. on the day of 14 compared to day of 0, which indicates that this strain has probiotic traits to regulate gut microbiota positively.

In line with *in vitro* results in this study, both HS-2 and NG-4 groups showed strengthened TJ proteins including claudin-1 and occludin compared to LGG groups. According to Zeissig *et al.* (2007), TJ proteins expression in intestine is altered and levels of claudin-1 expression is decreased significantly. Moreover, the expression of ZO-1 have been reported to decline in DSS-induced colitis mice as well as in IBD patients (Poritz *et al.*, 2007). Correspondingly these

results, both selected LAB are anticipated to have restoration effects for impaired TJ proteins.

In conclusion, our results of *in vitro* study indicated that selected HS-2 and NG-4 strains have been expected to modulate the gut microbiota by colonizing well in the host intestine, inhibit colonization of pathogens as well as exert anti-inflammatory effects against intestinal inflammation by regulating NO and IL-8 productions and restoring impaired TJ proteins by TNF-α targeting for IBD patients. This previously characterization of selected LAB were verified by application to DSS-induced colitis mice models. The groups of NG-4 treatment showed increased average feed intake, better survival, less DAI scores, shortening of the colon length, modulating gut microbiota positively and regulating expression of TJ proteins compared to LGG group. These results also provide the further proof of *Pediococcus acidilactici* NG-4 as the putative probiotics effective for alleviating intestinal inflammation by affecting various mechanisms causing IBD. Despite of these encouraging results, additional studies using *in vivo* experiments are requested to deeply elucidate their probiotic mechanisms for the further study followed by clinical attempts for IBD patients.

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Abstract in Korean

궤양성 대장염과 크론병을 포함하는 염증성 장질환 (IBD)은 장관내 만성 염증에 의해 일어난다. 비록 아직까지 염증성 장질환의 명확한 발병기전은 밝혀져 있지 않으나, 장내 세균총의 비정상, 장 표피세포의 장벽 손상, 사이토카인 불균형 그리고 특정 병원균의 감염이 염증성 장질환을 일으키는 주요한 요인으로 간주되고 있다. 이러한 원인과 관련하여, 장내 세균총을 유익하게 조절할 수 있는 프로바이오틱스를 개발하기 위하여 본 실험에서는 염증성 장질환과 관련이 있는 장내 만성 염증을 완화하는 다양한 기능을 가진 프로바이오틱스 후보군을 선발하고 특징을 평가하는 것을 목적으로 한다. 먼저, 선택 및 분별 배지를 이용하여 77개의 전통 발효 식품에서 399개의 유산균을 분리하였다. 두번째, 기능성이 있는 것으로 기대되는 10개의 균주를 장내 생존성, 안정성, 장세포 부착성과 같은 기본적인 프로바이오틱 특성에 근거하여 선별 및 선발하였다. 다음으로, 선발된 유산균 균주가 염증성 장질환과 관련이 있는 특정 병원균에 대해 항균 활성이 있는지를 평가하기 위해 disk diffusion, agar well diffusion과 ATP 분석 방법을 통해 확인하였다. B4 균주를 제외하고 선발된 거의 모든 균주가 병원균 및 식중독균에 대해 기준 균주인 LGG와 비교했을 때 저해능을 보였다. 과도한 염증 반응을 억제하는 선발된 균주의 항염증 효과는 산화질소 분석 방법을 쥐 대식세포 유래의 RAW264.7 세포주에 적용함으로써 평가되었다. 또한 HT-29 세포와 caco-2 세포에 10ng/ml 농도의 TNF-α를

처리함으로써 HT-29 세포에서 생성되는 IL-8 농도와 tight junction 단백질의 발현 수준이 유의적으로 긍정적인 효과를 가지게 조절되었다. 장내 염증을 완화하는 효과를 가지는지 확인하기 위해 2.5 % 와 5 % 농도의 DSS를 3일동안 처리함으로써 쥐에 장염을 유발한 이후, LGG, HS-2, NG-4 세가지 균주를 14일동안 쥐에 경구투여하였다. NG-4 균주를 투여한 그룹이 LGG 그룹에 비해 유의적으로 높은 평균 사료 섭취량과 생존률 및 낮은 DAI 점수를 보였고 장의 길이가 덜 짧아졌으며, 장내 세균총과 TJ 단백질의 발현을 유익하게 조절하는 효과를 보였다. 결론적으로, *Pediococcus acidilactici* NG-4 균주가 임상 실험 이후, 장내 염증 증상을 완화하기 위해 프로바이오틱스로써 염증성 장질환 환자에게 적용될 가능성이 있을 것으로 기대된다.