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Studies on alleviating intestinal inflammation using probiotic potential of *Saccharomyces cerevisiae* GILA 118 and its cell wall component

프로바이오틱스 후보 *Saccharomyces cerevisiae* GILA 118과 세포벽 성분의 장염 완화에 관한 연구

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Bum Ju Kil

Studies on alleviating intestinal inflammation using probiotic potential of *Saccharomyces cerevisiae* GILA 118 and its cell wall component

지도 교수 윤 철 희

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길 범 주

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2023 년 08 월

위 원 장 김 영 훈 (인)

부위원장 윤 철 희 (인)

위 원 허 철 성 (인)

위 원 강 상 기 (인)

위 원 김 명 후 (인)

Summary

Inflammatory bowel disease (IBD), including crohn's disease (CD) and ulcerative colitis (UC), are idiopathic pathologies characterized by chronic and relapsing inflammation and mucosal tissue damage of the gastrointestinal tract (GIT). Although etiopathogenesis and the exact pathogenesis of IBD are unclear, the main factors of IBD have been considered the dysbiosis of intestinal microbiota, i.e., the disruption of normal composition in the gut microbiota and damage of epithelial cell integrity. When IBD occurs, epithelial migration of neutrophils and can activate pro-inflammatory cytokine such as TNF-α. CD, and UC-induced pro-inflammatory cytokine IL-17 producing $CD4^+$ T cell-related Th17 cells to mediate the immune response. Thus, Th17 and Treg cells in IBD are imbalanced. Long-term drug therapy using sulfa pyridine could be avoided due to side effects. A common alternative treatment is probiotics. Recently, various probiotic products have been developed; however, most probiotic applications have only investigated the use of prokaryotic bacteria. Eukaryotic probiotics have not been as well studied except *Saccharomyces boulardii*.

In the first study, the strain *S. cerevisiae* GILA 118 met the criteria for a probiotic which had alleviating inflammatory effect. Novel yeast strains were isolated from Korean fermented beverages and examined for their

potential probiotic characteristics. Seven isolates showed auto-aggregation tendency, coaggregation with a pathogen, hydrophobicity with n-hexadecane, and 1,1-diphenyl-2-picrylhydrazyl scavenging effect similar to or higher than the control strain *S. boulardii* CNCM I-945. Nitric oxide production by *S. cerevisiae*'s results were significantly lower than control. The selected isolates that showed promise as probiotics were identified as *S. cerevisiae* by internal transcribed spacer sequencing and results suggest that *S. cerevisiae* GILA could be potential probiotic strain. *S. cerevisiae* GILA strains were chosen by *in vivo* screening with 1.5 % dextran sulfate sodium-induced colitis murine model.

Here, *Saccharomyces cerevisiae* GILA strains, particularly GILA118, down-regulate neutrophil, and myeloperoxidase. The expression levels of genes encoding tight junction proteins in the colon were upregulated, cytokine IL-10 was significantly increased, and TNF-α was reduced in the serum.

In the second study, *S. cerevisiae* GILA 118's separate cell wall polysaccharide and protoplast were confirmed the alleviation of inflammation using murine macrophage RAW 264.7 cells, and C57B6/J mouse. *S. cerevisiae* GILA 118 cell wall polysaccharide and protoplast were used by NaOH/HCl separation technique and zymolyase. *S. cerevisiae* GILA 118 cell wall polysaccharide, protoplast verified the mechanism for alleviating intestinal inflammation *in vitro* and *in vivo* model. Nitric Oxide (NO) was reduced, *COX-2* gene expression was reduced by *PPAR-γ*

expression in RAW 264.7 cells, and *S. cerevisiae* GILA 118 cell wall polysaccharide treated with zymolyase (PZ), which IL-10 were significantly increased than DSS mouse model was selected. *S. cerevisiae* GILA 118 PZ induced the proportion of Treg cells and decreased the proportion of Th 17 cells in the spleen. GILA 118 and GILA 118 PZ also managed the gut microbiome to Thus, this study suggests that GILA118 and GILA118 cell wall components are potential alleviative agents in patients with IBD.

Keyword : Intestinal inflammation, Probiotics, Yeast, *Saccharomyces cerevisiae*, Cell wall polysaccharide

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List of Abbreviations

AIEC	Adherent-invasive E. coli
BSA	Bovine serum albumin
CD	Crohn’s disease
CD	Cluster of differentiation
cDNA	Complementary DNA
CBC	Complete blood count
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
CFU	Colony forming unit
COX	Cyclooxygenase
CRP	C-reactive protein
DAI	Disease activity index
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Distilled Water
E-cadherin	Epithelial cadherin
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
Foxp3	Forkhead box protein P3
GIT	Gastrointestinal tract

IBD	Inflammatory bowel disease
IFN	Interferon
IL	Interleukin
iNOS	isoform of Nitric oxide synthase
LAB	Lactic acid bacteria
Lcn	Lipocalin
LPS	Lipopolysaccharides
LTA	Lipoteichoic Acid
MDS	Multi-dimensional scaling
MLCK	Myosin light-chain kinase
mLN	Mesenteric lymph node
MPO	Myeloperoxidase
Muc	Mucin
mRNA	Messenger RNA
NF-κB	Nuclear factor-kappa B
NLR	Neutrophil-lymphocyte ratio
NOS	Nitric oxide synthase
OTU	Operational Taxonomic Unit
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PMA	Phorbol 12-myristate 13-acetate
PLM	Phospholipomannan
PP	Peyer's patches

PPAR	Peroxisome proliferator-activated receptor
PPM	Polyprenylphosphate-based mannose
qPCR	Quantitative PCR
RBC	Red blood cells
RNA	Ribonucleic acid
TGF	Transforming growth factor
Th	Helper T cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tregs	Regulatory T cells
UC	Ulcerative colitis
YPD	Yeast extract–peptone–dextrose
ZO	Zonula occludens

Chapter 1. Review of Literature

1. Probiotics and eukaryotic probiotics

1-1. Probiotics

Probiotics have been defined by various meanings until World Health Organization (WHO) has made a definition of probiotics in 2002[1]. 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 microorganisms having health promoting effects, most studies have focused on examining some bacterial genera, such as species of *Lactobacillus, Enterococcus, Streptococcus* and *Bifidobacterium.* Probiotics were investigated specifically for the lactic acid bacteria (LAB), which are made up of *Lactobacillus* sp.[2,3].

While bacterial probiotics are well known, only limited eukaryotic probiotics are used in human and animal. Nowadays interest in eukaryotic probiotics is on the rise, especially *S. boulardii* were well studied. Both eukaryotic probiotic (yeast) and prokaryotic probiotics (bacteria) is given in Table 1-1[4]. Probiotics have already been shown to have beneficial effects on the host's metabolism and immune system. The presence of beta-glucan and chitin in yeast cell walls, which have immune-modulating properties, contrasts with bacteria's peptidoglycan-based cell wall as one of the primary differences in membrane composition.

Table 1-1. Comparison between bacteria and yeast as probiotic implication.

	Bacteria	**Yeast**	**Probiotic implication**
Presence in human flora	99%	<1%	
Cell size	1 μm	10 μm	Steric hindrance
Cell wall	Peptidoglycan, LPS(Gram-negative), LTA (Gram-positive)	Chitin, mannose (PPM, PLM), glucan	Immune response via TLRs, lectin receptors
pH	6.5–7.5	4.5–6.5	Different sites of action in the GI tract
Effect on host growth and nutrition	Y	Y	Safety in combination with Antibiotherapy
Application as probiotics	Wide range of animals	Limited application	

With regard to pH, most of the yeasts can grow very well in between the pH 4.5–6.5 but nearly all species can survive at low pH

1-2. Eukaryotic Probiotics

Only a small portion of eukaryotic probiotics come from yeasts, molds, or fungi. Yeasts, particularly *Saccharomyces* species, are the most common and widely used eukaryotic probiotics in a variety of hosts[4].

Several clinical studies have been conducted over the years to determine the ability of yeasts to protect against disease. The therapeutic potential of yeasts, particularly their antioxidant, anti-inflammatory, and immunomodulatory properties, has been well documented in animal models and clinical trials. Furthermore, *S. cerevisiae* can reduce *Candida albicans* in the digestive tracts of normal and antibiotic-treated animals[5,6], as well as other pathogens such as *Salmonella typhimurium* and *Shigella flexneri*[7]. *S. cerevisiae* var. *boulardii* has also been reported to decrease the inflammatory reaction and colonization of the mouse intestine by *C. albicans* infection[8].

S. cerevisiae can cause a significant increase in the specific and total activities of brush-border membrane disaccharidases, which can help patients with sucrase–isomaltase deficiency improve their malabsorption. Eukaryotic probiotics are rich sources of biologically active substances that can potentially modulate the immune system of the host. Chitin, mannose, and glucan are the most common yeast cell wall components[4].

When ingested orally as food/feed supplements, eukaryotic probiotics provide various nutritional benefits to hosts. The US Food and Drug Administration (FDA) has approved certain eukaryotic probiotic strains

such as *S. boulardii* as commercially available probiotic yeast species[4]. *S. boulardii* are the only probiotic yeast species that are marketed for human benefits (Table 1-2) despite the FDA having approved certain probiotic strains that can be beneficial to humans. *S. boulardii* has been gradually incorporated into food matrices, involving dairy products (yogurt, cheese whey, ice cream), grains (beer, boza, cornflakes), fruit juices (cashew juice), chocolate, coffee, and tea[9]. This has been in accordance with ongoing developments in eukaryotic probiotic functional foods and inclinations toward health and wellness.

In animals, *Saccharomyces cerevisiae* supplementation frequently leads to increased body weight gain as the feed intake increases. Notably, both whole *S. cerevisiae* and its extract can improve the tenderness and oxidative stability of broiler meat. Certain antioxidant factors found in *S. cerevisiae* are thought to be involved in shifting the oxidative factor of the fatty acid profile in meat[10].

Table 1-2. Commercial Eukaryotic Probiotic Product[11].

Commercial Probiotic Product	**Probiotic Strain**	**Company**
Reflor (Single strain)	*Saccharomyces boulardii lyo*	Biocodex (Turkey)
Inteflor	*Saccharomyces boulardii* + 1 bacterial probiotic strains	Yamamato Research (United States)
Ultimate Flora Probiotic	*Saccharomyces boulardii* + 10 bacterial probiotic strains	RenewLife (United States)
Florstor	*Saccharomyces boulardii lyo*	Biocodex (United States)
Saccharomyces Boulardii	*Saccharomyces boulardii*	Kirkman (United States)
Saccharomyces Boulardii	*Saccharomyces boulardii*	Allergy Research Group (United States)
Saccharomyces Boulardii 10B	*Saccharomyces boulardii*	Pure Therapro Rx (United States)
Nexabiotic	*Saccharomyces boulardii*	DrFlormulas (United States)
Flora	*Saccharomyces boulardii*	Institute Rosell Lafelmanol (Belgium)
Daily Probiotic	*Saccharomyces boulardii*	Florastor (United States)
Saccharomyces Boulardii PLUS MOS	*Saccharomyces boulardii*	Jarrow Formulas (United States)
Perenterol	*Saccharomyces boulardii lyo*	Biocodex (Germany)

Table 1-3. Foods formulated with commercial Eukaryotic Probiotic[9].

Product	Probiotic Strain	Formulation	Main findings
Dairy Yogurt	*S. boulardii* CNCM-I745	Fermented	Inulin reduced syneresis and fat content, while improving survival of *S. boulardii* (>6 Log CFU/g) after 28 days at 4C.
Cheese whey	*S. boulardii* CCT 4308	Fermented	Optimised conditions to obtain simultaneous production of GOS and maintain *S. boulardii* viability: 29.5C and 0.14% w/w lactase.
Ice cream	*S. boulardii* CNCM-I745	Fermented	Inulin improved survival of *S. boulardii* (6.2 Log CFU/g) after 120 days at -18C. Synbiotic ice cream had improved melting properties and stability, with inulin reversing a decline in firmness caused by *S. boulardii*.
Grains Beer	*S. boulardii* (strain unspecified)	Fermented	Sugars, ethanol, hop levels, temperature, and pitching rate affected *S. boulardii* growth, and formation of alcohols and esters. High pressure processing led to 1 Log reduction in cell counts (5.4 Log CFU/mL), while terminating fermentation
Beer	*S. boulardii* CNCM-I745	Fermented	*S. boulardii* maintained >6 Log CFU/mL after 60 days at 0C, even after

			simulated gastrointestinal transit. Acetic acid produced.
Beer	*S. boulardii* 17	Fermented	*S. boulardii* achieved 8.5 Log CFU/mL after fermentation. Acetic acid produced. Beer fermented by *S. boulardii* elicited antidepressant behaviour in mice.
Beer	*S. boulardii* CNCM I-3856	Fermented	*S. boulardii* stimulated *L. paracasei* to produce greater levels of aromatic amino acid catabolites
Boza fortified with chickpea flou	*S. boulardii* CNCM-I745	Fermented	Boza enriched with chickpea flour maintained probiotic cell counts (3.3, 7.9, 7.3 Log CFU/ mL for *S. boulardii, L. acidophilus*, and *B. bifidum* respectively) after storage for 12 days at 4C.
Cornflakes	*S. boulardii* NCYC-3264	Non-fermented	Acacia gum coating protected *S. boulardii* for 90 days at 30C, contact with 80C milk (7.3 Log CFU/mL), and stimulated gastrointestinal transit. Acacia gum reduced water penetration into the cornflakes.
Fruits Cashew juice	*S. boulardii* 17	Non-fermented	*S. boulardii* maintained viabilities >7 Log CFU/mL for 28 days at 7C.

			S. boulardii enhanced protein value and turbidity
Others Chocolate	*S. boulardii* (strain unspecified)	Non-fermented	*S. boulardii* remained stable for 6 months at 4 and 20C, and under simulated gastrointestinal conditions (>8 Log CFU/mL). No significant changes in water activity, pH, total phenolic content, and sensory parameters
Coffee brew	*S. boulardii* CNCM-I745	Fermented	*S. boulardii* maintained viabilities ~7 Log CFU/mL for 6 months at 4 and 25C, while enhancing viability of probiotic lactobacilli. 2-Isopropylmalic acid produced by *S. boulardii.*
Tea	*S. boulardii* CNCM-I745	Fermented	*S. boulardii* maintained >6 Log CFU/mL for 85 days at 25C, while enhancing viability of *L. plantarum* 299v. *S. boulardii* stimulated L. plantarum 299v to produce greater levels of aromatic amino acid catabolites.

1-3. *Saccharomyces cerevisiae*

Two closely related strains of Saccharomyces, *S. boulardii* and *S. cerevisiae*, are used as probiotics or in the preparation of food and wine. The two strains received extensive study and has shown that although they are nearly identical at the molecular level[12]. *S. cerevisiae* is the well-known eukaryote and a valuable yeast for most aspects of basic research on eukaryotic organisms.

Protoplast and cell wall compose *S. cerevisiae*'s structure. *S. cerevisiae*'s cell wall is an elastic structure that provides protection and determines the shape of the cell. Cell wall macromolecules of *S. cerevisiae* are composed of mannoproteins, β1,6-glucan, β1,3-glucan, and chitin. Mannoproteins and β1,6-glucan are highly branched structures, whereas the β1,3-glucan structure is moderately branched. β1,3-glucan molecules are connected by hydrogen bonding between locally aligned chains. Chitin is present inside and β1,6-glucan is present outside of β1,3-glucan molecules[13].

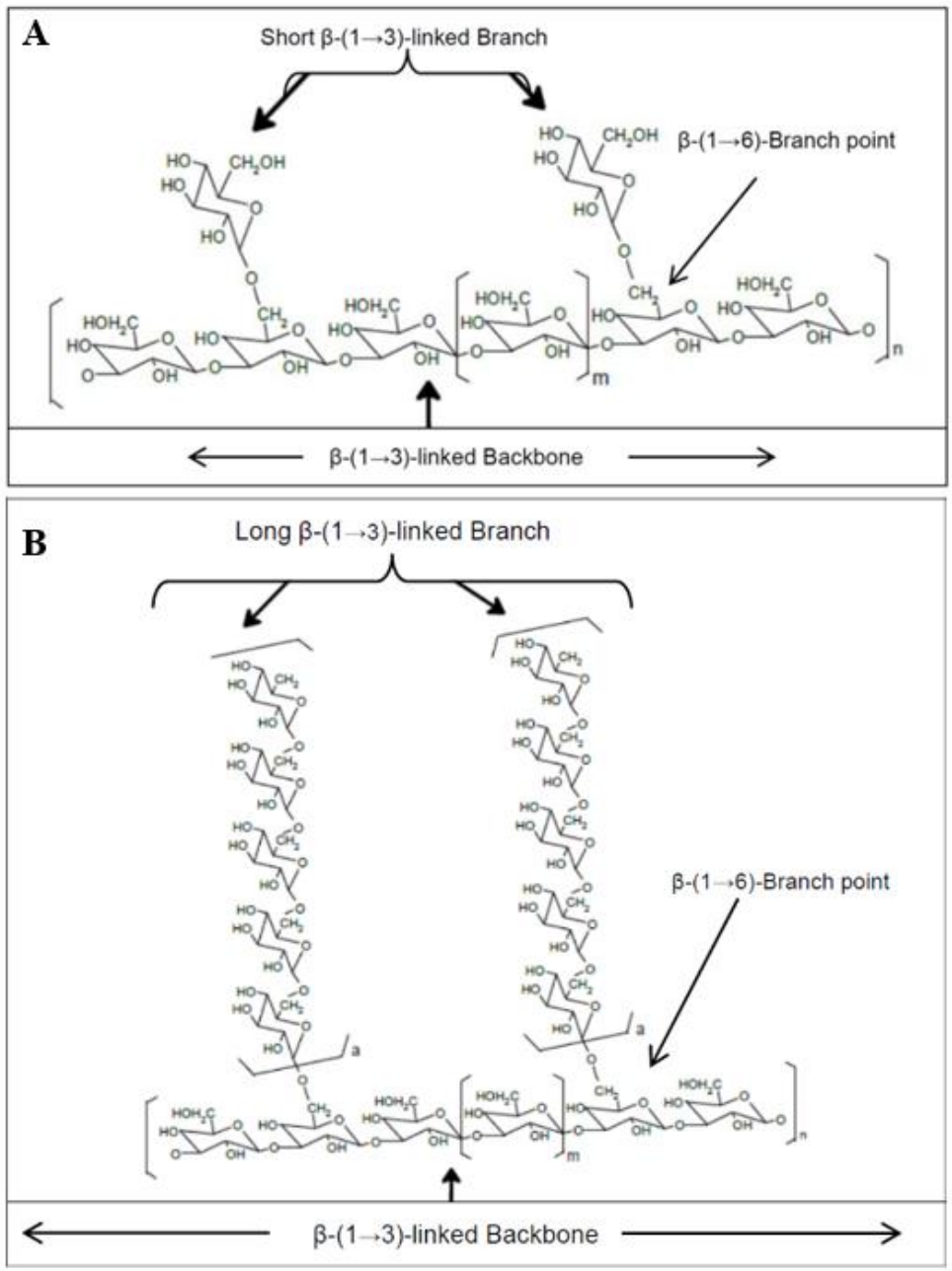


Figure 1-1. The molecular structure of soluble and insoluble yeast β-glucan[14].

The protoplast of *S. cerevisiae* is made up of a nucleus, a mitochondrion, a vacuole, and golgi[15]. In contrast to bacteria, in which the cytoplasmic membrane accommodates all the membrane associated processes, yeasts contain many specialized membranes. The composition of phospholipids, sphingolipids, and sterols in *S. cerevisiae*'s plasma membrane influences the activity of proteins related to the lipid bilayer[16].

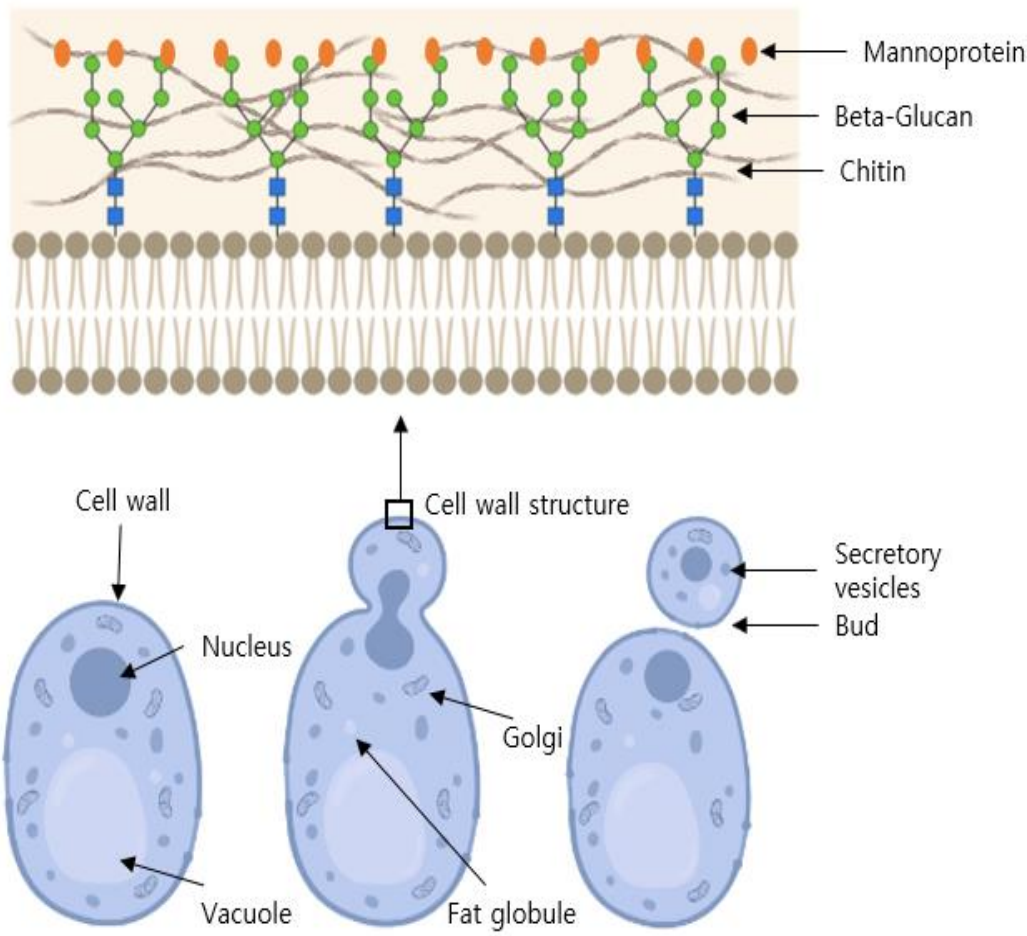


Figure 1-2. *S. cerevisiae* Morphology, Cell wall structure and Life cycle.

2. IBD

2-1. IBD

Inflammatory bowel disease (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[17,18]. They differ in terms of symptoms and sites of inflammation lesions. While CD is characterized by chronic and partial inflammation in the gastrointestinal tract[19], UC is typically characterized by an inflammation condition with ulceration primarily in the colon[20]. Although these two types of IBD share many epidemiologic, therapeutic, and clinical characteristics, the composition and population of gut microbiota differ significantly between them.

IBD is defined as the dysbiosis-associated mucosal immune dysfunction that results from exposure to environmental triggers, such as dietary elements, gastrointestinal infections, drugs, psychological stress, and smoking, in genetically vulnerable individuals. The mucosal immune response becomes dysfunctional under chronic dysbiotic circumstances, which are marked by a rise in aggressive bacterial strains and a decrease in regulatory species[21]. Gut dysbiosis is likely to prolong mucosal inflammation and could result in IBD, along with impaired intestinal barrier function[22].

The main microbiological causes causing persistent low-grade inflammation are pathogen-associated molecular patterns (PAMPs), which are brought on by alterations in intestinal barrier integrity as a result of the disturbed control of intestinal flora. PAMPs are small molecules with conserved patterns that are found in a variety of microorganisms[23]. These include elements that build cell walls (such as bacterial peptidoglycan, lipopolysaccharide [LPS], lipoteichoic acid, and flagellate protein) as well as other elements that are typical of microorganisms (such as viral RNA or DNA)[24].

IBD has been observed for a long time[25], and it is regarded as a growing and significant intestinal disorder in developed countries[26]. Although IBD has been conventionally considered a disorder of Caucasians residing in developed Western countries, as developing countries in the Eastern and Mediterranean worlds have become westernized, the prevalence and incidence rates of IBD have increased in the same trends as in the Western world in 2015[27]. Results Over 4.9 million IBD cases were reported in the world in 2019, with China and the USA reporting the highest rates (911 405 and 762 890, or 66.9 and 245.3 cases per 100,000 persons, respectively)[28].

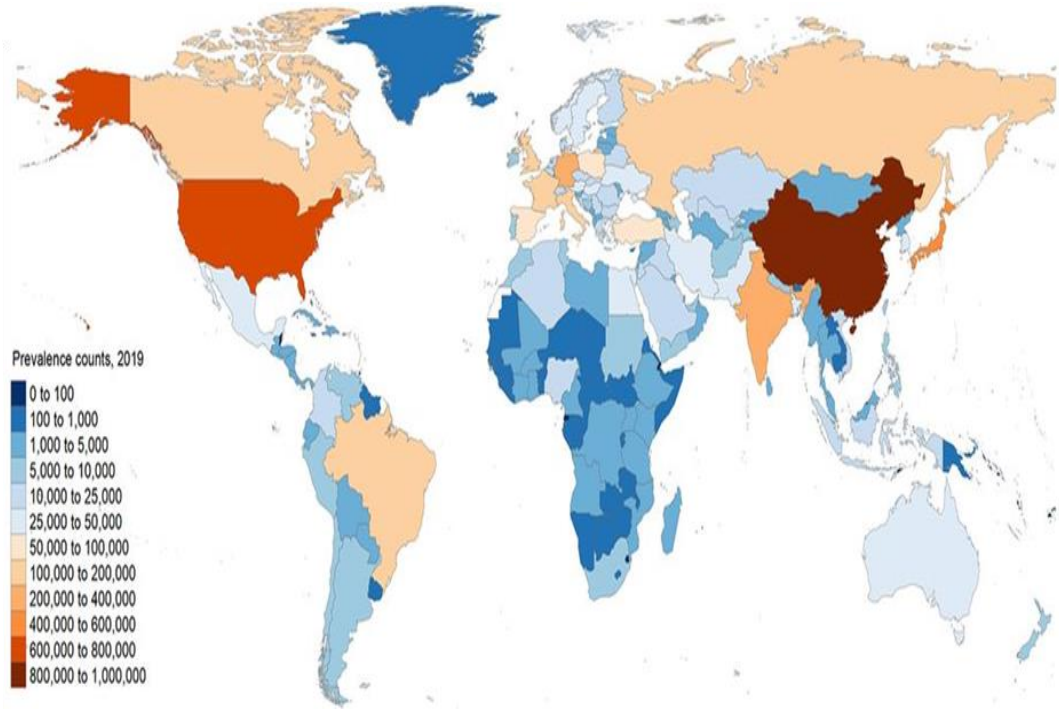


Figure 1-3. The global prevalence of IBD in 2019[28]. The absolute number of IBD prevalent cases in 204 countries and territories.

2-2. Pathogenesis of IBD

2-2-1. Neutrophil and myeloperoxidase infiltration

Neutrophils, also called polymorphonuclear leukocytes, are the most abundant leukocyte population in the blood. Neutrophil transport from circulation to the gastrointestinal tract can be found in inflammation. When intestinal mononuclear phagocytes were depleted, neutrophil infiltration into tissues increased and the severity of injury also increased in a DSS-induced colitis mouse model. Although the role of neutrophils has not been clearly defined, neutrophil infiltration is dependent on the inflammation model. Notably, neutrophils can contribute to disease pathology, where increased recruitment and DSS-like products induce epithelial injury[29].

Calprotectin, lactoferrin, and myeloperoxidase (MPO) are all associated with inflammation and are derived from neutrophils. Calprotectin is found within the cytosol of neutrophils. Studies have successfully shown elevated levels of calprotectin in patients with IBD, and that fecal calprotectin accurately predicts relapses. Lactoferrin is a glycoprotein found in the secondary granules of neutrophils and is resistant to proteolysis in feces. Studies have found it to be both sensitive and accurate in measuring the IBD activity. Calprotectin and lactoferrin are both documented biomarkers for monitoring and diagnosing IBD.

MPO, a lysosomal protein found in neutrophils, could be a viable biomarker for IBD. A researcher measured MPO levels in stool extracts from patients with UC, patients with CD, inflammatory disease control

patients, and healthy individuals. The researcher found decreased levels of MPO in the healthy population and patients with inactive IBD when compared to patients with active IBD[30]. The bowel mucosal MPO activity was higher in patients with active IBD than in patients with inactive IBD.

2-2-2. Inflammatory cytokine and tight junction protein

Inflammatory cytokines have been identified as potential new targets for treating intestinal inflammation in studies using tissue from patients with IBD and animal IBD models. Tumor necrosis factor (TNF) blockade is now commonly used in clinics as a standard therapy for IBD[31]. Cytokines drive intestinal inflammation and diarrhea in IBD while probably regulating extra-intestinal disease manifestations and systemic effects. Furthermore, cytokines seem to play a crucial role in driving the complications of IBD, such as intestinal stenosis, fistula formation, and colitis-associated neoplasia [31]. TNF-α is a major target of human IBD treatment and is highly upregulated in DSS-induced IBD models. When inflammation occurs in the intestine, inflammatory cytokines are upregulated by immune cell infiltration[32]. In patients with IBD, NF-κB signaling is often downregulated, resulting in the production of diverse proinflammatory mediators, such as COX-2, IL-1β, and IL-6[33]. TNF-α can also affect tight junctions and decrease the epithelial barrier function by increasing myosin light-chain kinase (MLCK) phosphorylation. TNF-α-induced MLCK expression may be a critical mechanism for barrier dysfunction in UC and CD[34].

The largest portion of the body's mucosal barriers the intestinal epithelium, is lined with a monolayer of cells containing crypts and villi. By recognizing the stimulation of intestinal commensal bacteria and contributing in the reinforcement of mucosal barrier function, intestinal epithelial cells (IECs) play important roles in separating host and luminal microorganisms. They

continually get replaced by intestinal epithelial stem cells that control critical immune system functions and are found at the base of the crypts. Goblet cells, paneth cells, and enteroendocrine cells—each specialized for digestive or epithelial barrier functions—are among the several cell types found in secretory IECs[35]. Barrier dysfunction is most likely caused by epithelial damage, which is common in IBD. Inflammatory cytokines associated with gut inflammation alter the epithelial barrier function through their effects on junctional complexes[34]. Future studies are required to determine the role of these proteins in modulating tight junctions and the epithelial barrier function in IBDs.

2-2-3. Imbalance of Th17/Treg cell balance

The $CD4^+$ T cells that are actively producing cytokines could develop and recognize the antigens that were transmitted through the epithelium. IBD pathogenesis shows that the imbalance between Th17 and Treg cells, which differentiate from $CD4^+$ T cells, contributes to IBD. Th17 cells, a subset of $CD4^+$ T cells, are primarily proinflammatory. However, Treg cells, another subset of $CD4^+$ T cells, which are characterized by forkhead box protein P3 (Foxp3) expression, play an essential role in maintaining immune tolerance and balance. Th17 and Treg cells are related to differentiation and inhibitory function. They share a common signal pathway mediated by TGF-β. While naïve $CD4^+$ T cells differentiate into Th17 cells in the presence of IL-6 or IL-21 (with TGF-β), they differentiate into Treg cells in the absence of proinflammatory cytokines. This balance is mainly affected by T cell receptor (TCR) signaling, costimulatory signals, cytokines, bile acid metabolites, intestinal microbiomes, short chain fatty acid and other factors. Once the Th17/Treg balance is broken, some autoimmune diseases, including IBD, occur[36].

2-2-4. Gut microbiota imbalance

A large portion of the more than 100 trillion microorganisms that colonize the gastrointestinal and oral tracts are bacteria, which are exceedingly abundant and predominate. In particular, most of them have coexisted in the colon for millions of years. The host has evolved to provide the habitat and nutrition that the microbiota need to survive, and in exchange, the microbiota have a variety of physiological impacts that support the host's health[37]. Microbiota contributes an essential component in the development of lymphoid tissue, particularly Th 17 cells and Treg cells, in the gut immune system.

It is well known that the gastrointestinal microbiome of healthy people maintains gut balance between beneficial and pathogenic bacteria that have the potential to cause infections. Dysbiosis, which is the disturbance of normal gut microbiota composition, can nevertheless be brought on by a number of situations, such as dietary changes, immune system flaws that are innate, and/or inflammatory conditions[38]. Dysbiosis is also linked to aberrant conditions where specific commensal bacteria initiate and become more virulent. These normal conditions can then become pathobionts, which are ongoing colitogenic conditions that cause intestinal inflammation.

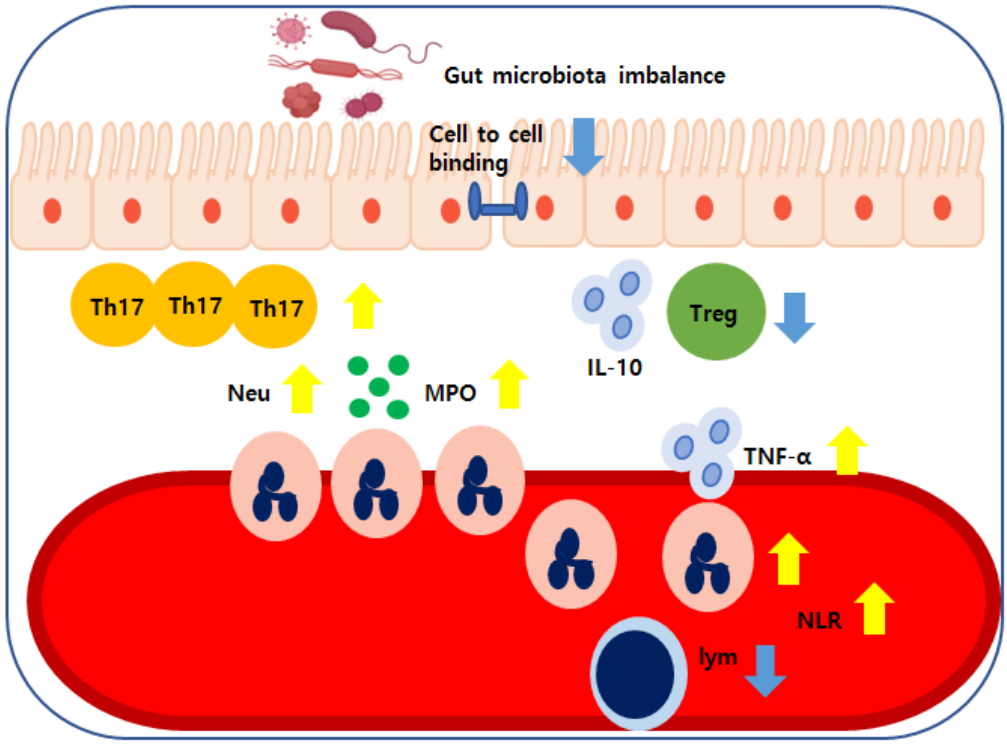


Figure 1-4. IBD pathogenesis includes neutrophil, MPO infiltration, inflammatory cytokine, tight junction protein downregulation, imbalance of the Th17/Treg cell and gut microbiota.

3. *Saccharomyces cerevisiae* as Eukaryotic Probiotics in the Management of IBD

3-1. Medical Treatment for IBD

Among the drugs used to treat IBD are corticosteroids, 5-aminosalicylic acid, immunomodulators, calcineurin inhibitors, antibiotics, and anti-TNF-alpha medicines. However, these medical treatments have some temporary and symptomatic complications due to side effects[39,40]. The effectiveness of these treatments is also limited across a spectrum of IBD patients. The necessity for the development of effective, well-tolerated, and long-term anti-inflammatory medicines for IBD is demonstrated by these deficiencies in our recognition of the underlying causes and anti-IBD medications.

For this reason, there has been great interest in the possibility of treating IBD with probiotics. Probiotics have been studied for IBD treatment since 1997[41].

Table 1-4. Medical Treatment for IBD[37].

Medical Treatment	Clinical indications	Common side effects
5-aminosalicylates	Mild-to-moderate disease	Headaches, nausea, diarrhea, photosensitivity
Corticosteroids	Acute moderate-to-severe disease	Hyperglycemia, hypertension, growth failure, weight gain, osteopenia, mood disturbances
Immunomodulators	Maintenance of remission of moderate-to-severe disease	Bone marrow suppression, hepatitis, pancreatitis, infections, malaise, rashes
Calcineurin inhibitors	Acute severe or fulminant disease	Nephrotoxicity, infections, seizures, hirsutism, hypertension, headaches
Antibiotics	Adjunctive therapy for active disease; pouchitis	Specific to individual antibiotics
Anti-TNF-α agents	Induction and maintenance of remission of moderate-to-severe disease	Hypersensitivity reactions, headaches, nausea, abdominal pain, infections

3-2. Probiotics for IBD treatment

Hegazy and Bedewy[42] discovered that supplementation with *Lactobacillus delbrueckii* and *Lactobacillus fermentum* for 8 weeks in patients with UC receiving sulfasalazine (an anti-inflammatory medicine) reduced several proinflammatory parameters in the colon, including IL-6 levels, TNF-α and NF-kB p65 expression, and leukocyte recruitment, when compared with the placebo and medical treatment groups. In terms of histological parameters, the extent and severity of the lesions were attenuated in the nonprobiotic-treated groups. However, in the groups treated with medicine and probiotics, there was an improvement in UC with the inhibition of lesion extension and the prevention of mucosal aggression, indicating that the concomitant administered interventions had superior therapeutic potential.

Apart from the *Lactobacillus* genus, *Bifidobacterium* has also been extensively studied for UC treatment, particularly its immunomodulatory effects, both in isolated and symbiotic forms. Furrie et al.[43] showed that supplementing patients with UC with a symbiotic containing *Bifidobacterium longum* and inulin–oligofructose for 4 weeks reduced β-defensins, IL-1α, and TNF-α gene expression while improving rectal biopsy, indicating a reduction in the inflammatory response and epithelial tissue regeneration. Another study found that symbiotic administration of *Bifidobacterium breve* and galacto-oligosaccharides to patients with mild to moderate UC for one year significantly decreased inflammatory parameters and improved their clinical status, as evaluated by colonoscopy[44].

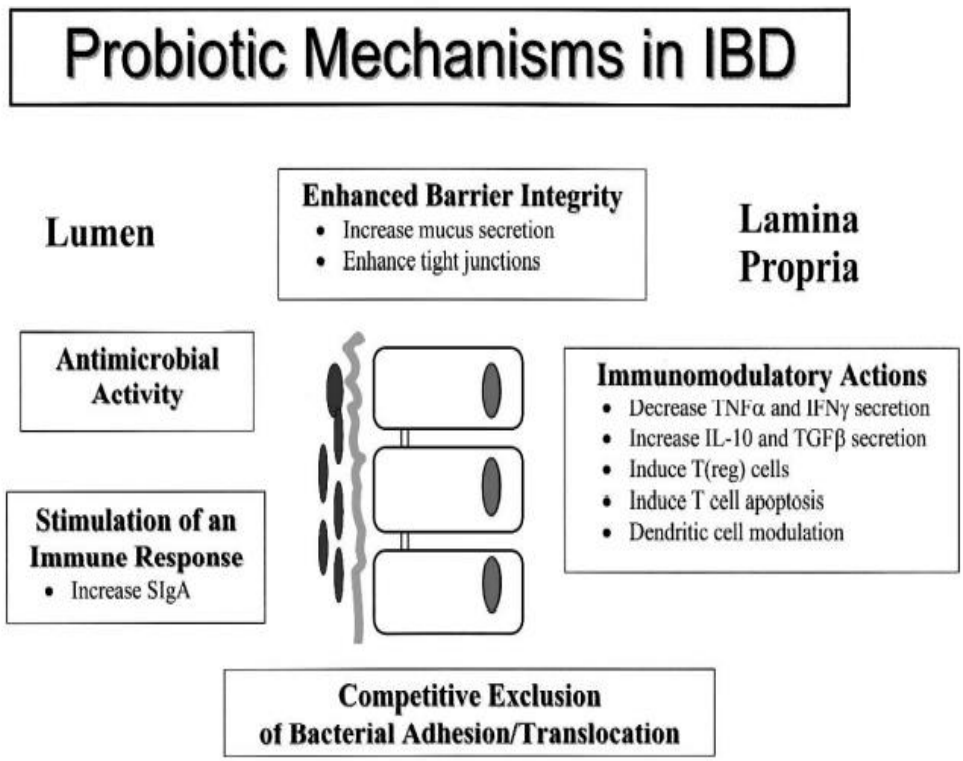


Figure 1-5. Action of probiotics in therapy for IBD[45]. Probiotics have been shown to have the following effects: competitive exclusion, whereby they outcompete microbial pathogens for a limited number of receptors on the surface epithelium; immunomodulation; probiotic-induced antimicrobial activity and suppression of pathogen growth through release of antimicrobial factors; stimulation of T-cell death in the mucosal immune compartment; and probiotic-induced increase of barrier activity through mucin synthesis and secretion.

Probiotics with effects on modifying gut flora have been recommended for treatment or alleviation of chronic intestinal inflammation to be a result of various studies examining the connection between gut microbiota and IBD. Probiotics could modulate the composition of the gut microbiota in addition to inhibiting pathogenic bacteria by interacting with specific pattern-recognition receptors including TLRs located on the surface or inner membrane of immune cells by stimulating immunological responses in the gut[46].

3-3. *Saccharomyces cerevisiae* as eukaryotic probiotics in the management of IBD

In recent mycobiome studies, *Saccharomyces* strains were found in up to 96% of the samples. However, only a few *S. cerevisiae* strains have been shown to benefit the host[47]. Different studies using animal models and clinical trials have been conducted to investigate the potential mechanisms of health benefits for alleviating IBD.

Broad-spectrum antibiotics are conventionally used to treat UC, but due to antibiotic resistance, their efficacy has been reduced to a substantial level. Probiotics, especially *S. boulardii* and its derivatives, act as alternatives for maintaining normal gut microbiota and treating patients with chronic colitis disease[8]. Studies have suggested that the pathogenic strain of *Escherichia coli*, known as the adherent-invasive *E. coli* (AIEC), showed a strong binding affinity with the small intestinal lining in patients with CD. These Gram-negative bacteria can easily invade the intestines of patients. Patients with CD showed strong adherence to AIEC bacteria due to their FimH adhesion potential and overexpressed mannose residues, which are present on the surface of the intestinal glycoprotein CEACAM6 (carcinoembryonic antigen-related cell adhesion molecule). *In vivo* studies have reported that *S. cerevisiae* var. *boulardii* significantly blocked the adherence potential of LF82 to the intestinal brush border. Probiotic yeast also lowered the proinflammatory cytokine level and was confirmed to treat UC

pathogenesis[48].

The difference in the cell wall structure of *S. cerevisiae*, with the β 1,3 glucan structure and without the β 1,3 glucan structure, also had proinflammatory and anti-inflammatory effects[49]. The cell wall polysaccharide of *S. cerevisiae* could be used as a therapeutic application for IBD. Sc1-1 was found to be comparable to *Sb* and to have a beneficial effect on intestinal inflammation. The cell wall components of *S. cerevisiae* are in direct contact with the host, and cell wall fractions from *S. cerevisiae* have been shown to alleviate colonic colitis induced by DSS in mice[50].

This chapter comprises an article, published in **Scientific Reports** with a minor modification as a partial fulfillment of Bum Ju Kil's Ph.D. program

Chapter 2. Probiotic potential of *Saccharomyces cerevisiae* GILA with alleviating intestinal inflammation in a dextran sulfate sodium induced colitis mouse model

1. Introduction

Probiotic research including product development has received increasing attention[51-53]. Probiotics are active microorganisms that improve health and prevent illness[54]. It has been suggested that *Saccharomyces boulardii*, a well-known probiotic, can be effective in IBD by inhibiting the Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κb) signaling pathway[55]. Probiotic research on *S. boulardii* has indicated positive outcomes in treating environment-related guts and anti-inflammatory effects[56,57].

Yeasts are ten times larger than bacteria, and their cell wall comprises chitin, glucan, mannose, phosphopeptidomannan, and phospholipomannan. Glucan, a cell wall component, modulates the immune system to improve immune functions[58], presenting a benefit for probiotic applications. Eukaryotic (yeast) probiotics can tolerate stress, survive, and adhere to the gastrointestinal tract. However, yeast application as a probiotic is limited. Therefore, application as a probiotic need to provide insights into the yeast strain that will benefit the host.

Similar to other microorganisms, the principal function of yeast is fermentation. For instance, *S. cerevisiae* converts glucose to ethanol and carbon dioxide in traditional Korean rice wines such as makgeolli and dongdongju, contributing to their characteristic properties[59]. Yeast also protects rice wine from contamination by other bacteria[59]. Some yeast

strains also have high nutrient value[60], and the ability of yeast to change cereals to fermented and functional foods is closely related to health issue[61]. Because of their safety and technological applications, *S. cerevisiae* species have been extensively studied and used in various sectors, including bakeries and breweries. Several reports have demonstrated and documented the benefits of *S. cerevisiae* strains on human health[62-64]. Functions of *S. cerevisiae*'s including its anti-infective properties[62], antioxidant activities[63], and other probiotic properties, have also been studied. Recently, yeast *S. cerevisiae* UFMG A-905 showed probiotic properties when treated in mice infected by *Salmonella Typhimurium*[65]. *S. cerevisiae* CNCM I-3856 strain has been found to alleviate gastrointestinal discomfort since 2015 in four prospective randomized placebo-controlled experiments[66-69].

The most common fungus discovered in feces samples in investigations have been yeasts from the genera *Saccharomyces*, *Malassezia*, and *Candida*. The question of whether *Saccharomyces* spp. are regarded as natural residents of the gastrointestinal tract becomes pertinent by the evidence that these organisms are linked to certain foods or are considered residents of the skin[70]. Evidently, there is no consensus on the fungi that compose on a "core gut mycobiome" yet. In the human intestines, *Saccharomyces* spp. are not considered of residing naturally[71]. Moreover, using the same samples for real-time qPCR targeting *S. cerevisiae*, Sokol, H *et al*[72] were able to detect a distinct drop in *S. cerevisiae* both in terms of absolute numbers and in terms

of the proportion in IBD, particularly in flare-ups. A previous study revealed disease-specific inter-kingdom network changes in IBD, indicating that, in addition to bacteria, fungi may potentially be involved in the pathogenesis of IBD.

These findings suggest that the probiotic *S. cerevisiae* varieties should be carefully selected for the optimum host health. Therefore, screening of probiotic *S. cerevisiae* is of utmost importance, and our findings highlight the probiotic potential of *S. cerevisiae*.

The objective of the present study demonstrated the probiotic potential of *S. cerevisiae* strains derived from Korean rice wine. The Immunomodulatory activity of *S. cerevisiae* was compared with *S. boulardii* to determine nitric oxide production by RAW 264.7 cells. Furthermore, *in vivo* studies were done to choose the *S. cerevisiae* GILA stain that alleviated intestinal inflammation functionality in a DSS-induced colitis mouse model.

2. Materials and Methods

2-1. Isolation and culture conditions

Eight samples of rice wine were obtained from Gangwon-do and three from Chungcheong-do, both in Korea. Samples were serially diluted 10-fold using 0.85 % NaCl solution. Diluted samples were spread onto yeast extract–peptone–dextrose (YPD) agar to selectively isolate yeast. Ninety-two isolates of yeast were from makgeolli, and eight isolates were from dongdongju. All isolates were confirmed by cell morphology difference (based on color, shape, size) and gram staining[73].

Yeasts were cultured in YPD broth, consisting of 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% dextrose. To screening yeasts that aggregate with a pathogen, *Staphylococcus aureus* ATCC 25922, *Enterococcus faecalis* ATCC 29212, and *Escherichia coli* K88 were cultured in brain heart infusion broth.

S. boulardii CNCM I-745, supplied by Jarrow Formulas (USA), was used as a control for comparison with the isolated yeast strain. The yeast isolates were incubated aerobically at 37℃ for 24 h[74]. All broth and agar materials were obtained from Difco (USA).

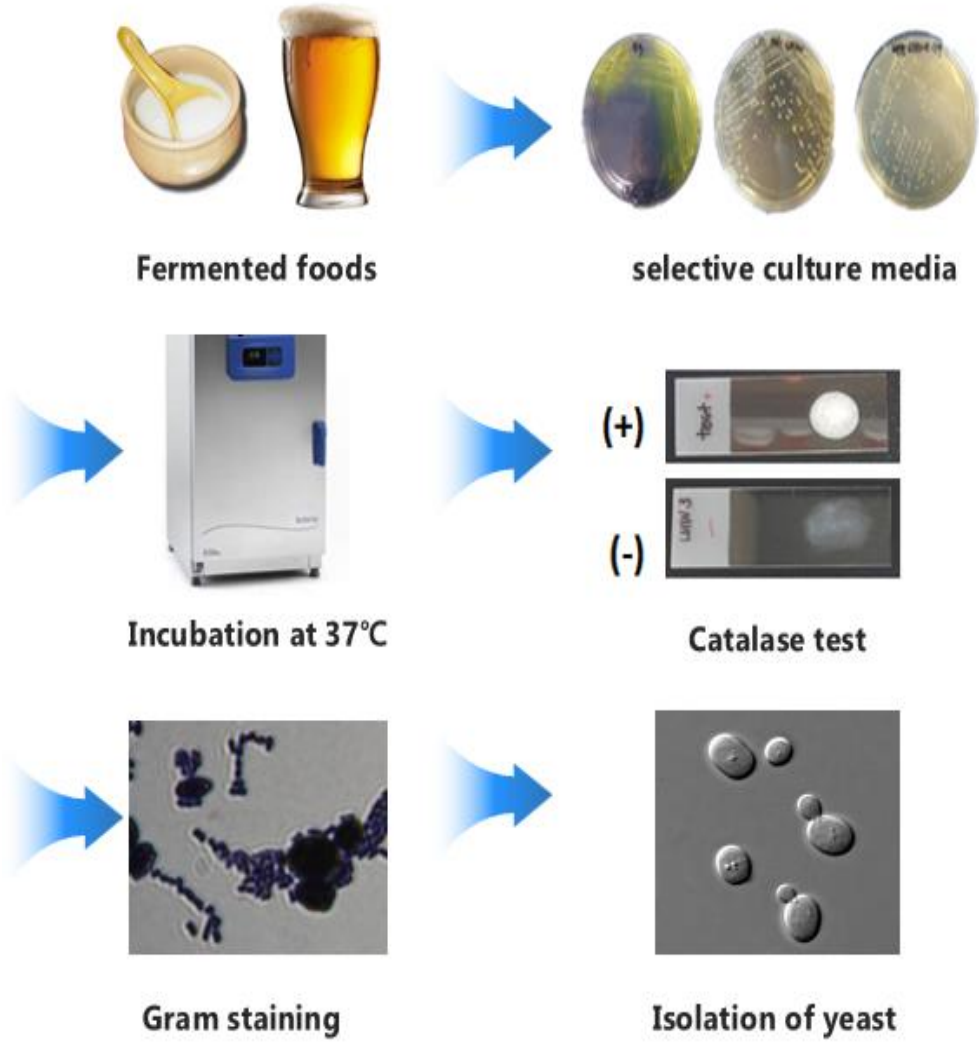


Figure 2-1. Scheme for isolation of yeast.

2-2. Resistance to low pH and bile tolerance

To determine the ability of yeast isolates to survive in GI conditions, the isolates were incubated in YPD broth at 37°C for 24 h. Then, the cultured yeasts were centrifuged at 5,500 x g for 10 min at 4°C. The pellets were incubated for 2 h in YPD broth, and adjusted to pH 2.0 with 1 N HCl. The sample (100 μL), diluted in phosphate-buffered saline (PBS), was spread on YPD agar according to the drop-plating method[75].

After incubation in YPD broth (pH 2.0) at 37°C, the resistance of yeast to bile was estimated similarly. The pellets were incubated for 12 h in YPD broth with 3.0% bovine bile (Oxgall, Difco, USA). The survival rate at pH 2.0 or in 3.0% Oxgall was calculated using the following formula:

Acid and bile tolerance (%) = [yeast after 24 h incubation (log cfu/mL)/yeast after 2 h incubation at pH 2.0 (log cfu/mL) or in 3.0% Oxgall (log cfu/mL)] × 100[76].

2-3. Autoaggregation and coaggregation with pathogen

To estimate the aggregation potential of selected yeast, the following assay was performed. Yeasts were grown for 24 h at 37°C in YPD broth, then harvested by centrifugation at 5,500 x g for 15 min. The pellets were washed twice with PBS, and then resuspended in PBS. Cell suspensions (4 mL) were mixed by vortexing for 10 s, and autoaggregation was determined after 24 h of incubation at 37°C. The upper suspension layer (0.1 mL) was transferred to another tube with 3.9 mL PBS, and the absorbance (A) was measured at 600 nm.

The autoaggregation percentage was calculated as follows:

$[1 - (A_{24} / A_0)] \times 100$[77], where At represents the absorbance at time t = 24 h and A_0 the absorbance at t = 0.

For the coaggregation test, 2 mL each of yeast and pathogen, *Staphylococcus aureus* ATCC 25922, *Enterococcus faecalis* ATCC 29212, and *Escherichia coli* K88 were vortexed together. The level of coaggregation with pathogen was calculated according to the equation of Handley et al.[78] as follows:

coaggregation (%) = $[((Ax + Ay) / 2) - A(x + y) / ((Ax + Ay) / 2)] \times 100$, where x and y represent the yeast and pathogen in the control tube, respectively, and (x + y) is the mixture.

2-4. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging effect

The DPPH scavenging assay was performed to compare the antioxidant abilities of the yeast isolates. The yeast pellets were harvested, washed twice, and resuspended in 1 mL PBS. The resulting suspensions (800 μL) were added to 1 mL DPPH solution (0.2 mM in 80% methanol) and mixed by vortexing, followed by incubation for 30 min in the dark. After the incubation, the solutions were centrifuged at 12,400 x g for 5 min, and 300 μL of each sample was transferred to a 96-well plate to measure the absorbance at 517 nm. The reconstitution of the standard was performed by adding ascorbic acid to 80% (v/v) methanol at a concentration of 1 mg/mL to 400 μL/mL. Five twofold serial dilutions were performed, and 80% methanol served as the zero standard[79].

2-5. Hydrophobicity

The hydrophobicity of the yeast cell surface [H(%)] was estimated using adhesion to n-hexadecane (Sigma-Aldrich, USA) according to the method by Rosenberg et al[80] with a slight modification as follows:

$H(\%) = [(1 - OD_4) / OD_0] \times 100$, where OD is the optical density at 0 and 4 h.

2-6. Hemolytic activity and biogenic amine production

Safety assessments were conducted by measuring the hemolytic activity and biogenic amine production. The hemolytic activity was evaluated using blood agar plates supplemented with 5% (v/v) defibrinated sheep blood (KisanBio, Korea). The appearance of clear zones around the colonies confirmed by β-hemolysis. After the colony of each isolate was streaked on the blood agar, the plates were incubated aerobically at 37°C for 48 h[81].

Biogenic amine production was analyzed according to the method of Bover-Cid and Hozapfel[82]. The isolates were streaked on decarboxylase media and incubated aerobically at 37°C for 4 days. Decarboxylase activity was detected by the color change from yellow to blue.

Table 2-1. Component of decarboxylase medium.

Component	Modified medium (%)
Tryptone	0.5
Yeast extract	0.5
Meat extract	0.5
Sodium chloride	0.25
Glucose	0.05
Tween 80	0.1
$MgSO_4$	0.02
$MnSO_4$	0.005
$FeSO_4$	0.004
Ammonium citrate	0.2
Thiamine	0.001
K_2PO_4	0.2
$CaCO_3$	0.01
Pyridoxal-5-phosphate	0.005
Histidine monohydrochloride	0.25
Tyrosine free base	0.25
Ornithine monohydrochloride	0.25
Lysine monohydrochloride	0.25
Bromocresol purple	0.006
Agar	2
pH	5.3

2-7. ITS region sequencing and Phylogenetic analysis

Single colonies were submitted to SolGent Corporation (South Korea) for ITS sequencing. DNA extraction was performed using a boiling method by Chelex bead. The screened and selected isolates were identified using amplified internal transcribed spacer ITS 1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') sequencing[83]. The polymerase-chain-reaction (PCR) reaction was performed in a BigDye® Terminatorv3.1 cycle sequencing kit. Sequencing was analyzed by ABI 3730XL DNA Analyzer (50 cm capillary). The primary measurement (identity, %) was compared to the yeast strain. Sequences were aligned by the NCBI GenBank database using the BLASTn. Phylogenetic analysis proceeded with MEGA software version 11 with neighbor-joining analysis of the ITS region identified *S. cerevisiae* with their type strains. Bootstrap analysis included 1,000 replicates. In addition, sequences were compared with others in the NCBI GenBank database using the BLASTn technique for identification.

2-8. *In vitro* gastrointestinal tract (GIT) models

Table 2-2. The solution, mixture, pH for *in vitro* gastrointestinal tract (GIT) models.

	Inorganic solution	Organic solution	Add to mixture	pH
Saliva (Mouth)	KCl 89.6 g/L KSCN 20 g/L NaH_2PO_4 88.8 g/L Na_2PO_4 57 g/L NaCl 175.3 g/L NaOH 40 g/L	urea 25 g/L	145 mg α-amylase 15mg uric acid 50 mg mucin	6.5 ± 0.2
Gastric Juice (Stomach)	NaCl 175.3 g/L NaH_2PO_4 88.8 g/L KCl 89.6 g/L $CaCl_2 \cdot 2H_2O$ 22.2 g/L NH_4Cl 30.6 g/L HCl 37% g/g	glucose 65 g/L glucuronic acid 2 g/L urea 25 g/L glucoseamine hydrochloride 33 g/L	1 g BSA 1 g pepsin 3 g mucin	3.2 ± 0.2
Duodenal Juice (Intestine)	NaCl 175.3 g/L $NaHCO_3$ 84.7 g/L KH_2PO_4 8 g/L KCl 89.6 g/L $MgCl_2$ 5 g/L HCl 37% g/g	urea 25 g/L	$CaCl_2 \cdot 2H_2O$ 22.2 g/L 1 g BSA 3 g pancreatin 0.5 g lipase	7.8 ± 0.2

Bile Juice (Intestine)	$NaHCO_3$ 84.7 g/L KCl 89.6 g/L HCl 37% g/g	urea 25 g/L	$CaCl_2 \cdot 2H_2O$ 22.2 g/L 1.8 g BSA 6 g bile	8.0 ± 0.2

The constituents and concentrations of the synthetic juices of the *in vitro* GIT model are shown in Table 2-2. All materials were obtained from Sigma-Aldrich (USA) or Difco (USA). The inorganic and organic solutions are mixed with distilled water. The pH of the juices and incubation time are adjusted to human physiological traits with a minor modification[84,85]. 7 ml of the *S. boulardii* CNCM I-745 and *S. cerevisiae* GILA strains were centrifuged, then resuspended in 1 ml PBS. Saliva was added and incubated for 5 min. After the incubation, gastric juice was mixed and incubated for 2h. 12 ml of duodenum juice with 6 ml of bile juice were mixed and incubated for 2 and 5 h with agitation (60 x g) at 37℃. Yeast isolates were harvested three times and serially diluted and plated onto YPD agar.

2-9. Adhesion assay

The adherence of yeast strains to Caco-2 cell line, obtained from the Korea cell line bank (KCLB, Seoul, Korea), was used between passages 40-60 for all experiments. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; corning, USA) supplemented with 10 % heat inactivated FBS, 2 mM L-glutamine /ml, 100 U penicillin/ml, and 100 µg, streptomycin/ml at 37 ℃ in a 5 % CO_2 atmosphere.

After harvesting yeast, overnight cultures of yeast strains were suspended with preheated fresh DMEM media and adjusted to O.D 600 nm at 2.0 density (approximately 1 X 10^7 cfu/ml). One millimeter of each yeast was inoculated to 12-well cell cultured plates and incubated for 2 h at 37 in a 5 % CO_2 atmosphere. Then, non-adherent yeasts were removed by washing with PBS twice, and the Caco-2 cells and attached yeast were lysed with 1 ml of 0.05 % Trypsin-EDTA (Gibco, USA). The adherent yeast was enumerated by diluting the solution serially (1 : 10) with PBS from the initial and using the drop-plating method on YPD agar[52].

Equation was used to calculate the adhesion rate[86]:

adhesion capacity (%) = (A_0/A_2) ×100, where A_0 represents initial counts and A_2 represents adhesion counts after incubated for 2 h.

2-10. Determination of nitric oxide production

RAW 264.7 cell lines of murine macrophages were obtained from the Korea cell line bank (KCLB, Seoul, Korea). The cells were cultivated in Dulbecco's modified eagles medium (DMEM, Gibco, USA) supplemented with 10 % heat inactivated fetal bovine serum (FBS, Gibco, USA) and 1% antibiotic-antimycotic (Gibco, USA) at 37 ℃ in a 5 % CO_2 atmosphere. RAW 264.7 cells were seeded at 1×10^5 cells ml^{-1} in 24-well plates and stabilized for 2 h. To stabilize overnight cultures of *S. bouladii* CNCM I-745 and *S. cerevisiae* were centrifuged. The cell pellet was washed with PBS twice and adjusted with OD 600nm at 1.0 density. *S. bouladii* CNCM I-745 and *S. cerevisiae* were heated for 15 min at 110 °C to remove activity of *S. bouladii* CNCM I-745 and *S. cerevisiae*, and then the cells were stimulated with 450 μL of lipopolysaccharides (LPS, 1 μg/mL; Sigma-Aldrich, USA) and 50 μL of heat-killed *S. bouladii* CNCM I-745 and *S. cerevisiae* for 48 h.

The incubated cells were centrifuged at 600 x g, 4 ℃ for 10 min, and the cell supernatant was transferred to new tubes. The measurement of nitric oxide (NO) concentration was estimated using the Griess reagent (Promega Inc., USA) as the manufacture's instruction. After mixing the cell supernatant and Griess reagent with the 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, Molecular Devices, USA). The concentration of NO was calculated

by comparing it a standard curve[87].

2-11. *ex vivo* Experimental design

Five-week-old female Balb/c mice were purchased from KOATECH (Pyeongtaek, Korea). After acclimatization for one week, the mice were fed with 50-μg Ovalbumin grade V (Sigma, USA) and 1-mg alum adjuvant (Thermo Scientific, USA) once a week, followed by 14 days, and then sacrificed. The spleen was homogenized with 2 mL RBC lysis buffer and splenocytes were suspended with complete media. *S. bouladii* and *S. cerevisiae* were grown for 24 h at 37°C in YPD broth and then harvested by centrifugation at 5,527 x g for 15 min. The pellet was washed with DPBS by centrifugation. Absorbance was measured when the OD reached 1.0 at 600 nm. The following samples were added to the 96-well plate: 2×10^5 splenocyte, 1-μL Ovalbumin, 2-μL *S. bouladii* or *S. cerevisiae*, and 97-μL complete media. The control did not contain yeast and was co-cultured for 2 days at 37°C and with 5% CO_2. The supernatant was analyzed using Mouse IFN-γ ELISA Kit (BioLegend, San Diego, USA).

2-12. *in vivo* Experimental design

Six-week-old female C57BL/6J mice were purchased from Daehan Bio Link Co., Ltd. (Korea). Mice were randomly distributed into 11 groups. After one week of stabilization, the mice were treated with 1.5% dextran sulfate sodium (DSS, MW 36,000–50,000; MP Biomedicals, USA) in distilled water with *S. boulardii* CNCM I-745 or *S. cerevisiae* strain (10^7 CFU/day, early stationary stage of yeast growth phase) once a day for 14 days, followed by 6 days of recovery (Fig. 2-2). All mice were subsequently euthanized by carbon dioxide (CO_2) asphyxiation.

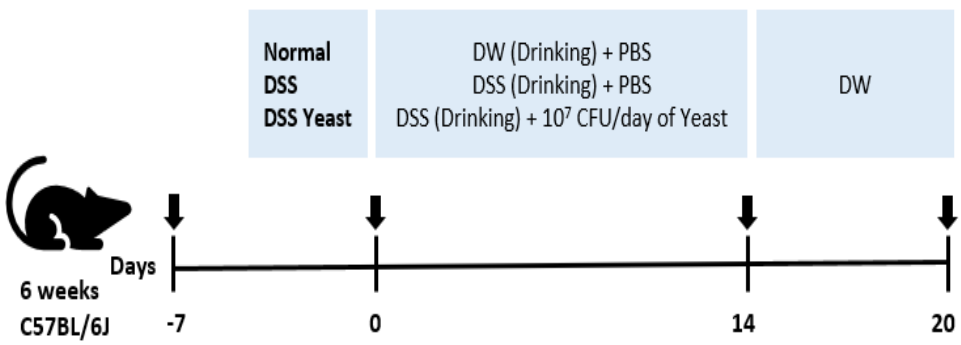


Figure 2-2. Overview of *in vivo* screening. Based on Yeast and DSS treatment, these studies include Normal, DSS and DSS with yeast group.

2-13. Colitis evaluation

Mice were examined daily for weight, stool consistency, and total blood in feces for colitis evaluation. DAI was evaluated using the method of Cooper et al[88] with minor modification. Scores for weight loss, stool consistency, and bleeding (Table 2-3) were monitored after 7 days of DSS treatment. After sacrifice, the mouse, colon length and spleen weight per body weight were compared in each group (n = 8).

Table 2-3. Scoring of the disease activity index (DAI)

Score	Body weight decrease (%)	Stool consistency	Bleeding
0	< 1	Normal	Normal
1	1-5		
2	5-10	Loose stools	
3	10-20		
4	>20	Diarrhea	Gross Bleeding

2-14. RNA isolation and qRT-PCR

Colon tissue in buffer RLT was well homogenized. After disruption, the RNA was isolated by RNeasy Plus Mini Kit (Qiagen, Germany) protocol. cDNA was synthesized by the PrimeScipt RT reagent Kit (Takara Korea Biomedical Inc.,Seoul, Korea) protocol. Gene amplification was done by the iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) protocol. Data were normalized with the housekeeping β-actin expression level. The primers used are listed in Table 2-4.

Table 2-4. Gene primer sequences.

Primer	Sequence	Reference
β-Actin (F)	TCCATCATGAAGTGTGACGT	Yin et al.[89]
β-Actin (R)	GAGCAATGATCTTGATCTTCAT	
Muc-2 (F)	GTGCTGCAATATCACCTCATGT	Floyd et al.[90]
Muc-2 (R)	TGTATGTGATGGAGCCTGAAAC	
ZO-1 (F)	CCACCTCTGTCCAGCTCTTC	Liu et al.[91]
ZO-1 (R)	CACCGGAGTGATGGTTTTCT	
Occludin (F)	CCTCCAATGGCAAAGTGAAT	
Occludin (R)	CTCCCCACCTGTCGTGTAGT	
E-Cadherin (F)	GCAGTTCTGCCAGAGAAACC	Shiohira et al.[92]
E-Cadherin (R)	TGGATCCAAGATGGTGATGA	

2-15. ELISA

Serum was quantified by ELISA kits (R&D Systems, Minneapolis, MN, USA) for inflammatory cytokine. Feces were quantified by ELISA kits (R&D Systems, Minneapolis, MN, USA) for myeloperoxidase (MPO) and Lipocalin-2 (Lcn-2) according to the manufacturer's instructions.

2-16. Fermentative profiling

Fermentative profiles of *S. cerevisiae* GILA were used to investigate selected isolates. The protocol of API kits was performed as manufacturer's instructions. A volume of each suspension was loaded in API 20C AUX kit (Bio-Merieux, France). After incubation, each wall was recorded at 48 and 72 h.

2-17. Cell wall β-glucan

β-Glucan (%, w/w) was measured using the yeast glucan assay kit (Megazyme, Ireland). Before the calculation of β-glucan, the yeast cell wall was autolyzed and hydrolyzed following the procedures of Pengkumsri *et al.*[93]. Briefly, yeast cells were incubated in pH 5.0 water at 50°C for 48 h with shaking at 160 x g, then at 80°C for 15 min in a water bath. After incubation, yeast cells were harvested by centrifugation for 10 min at 4°C at 6,900 x g. The autolyzed yeast cells were mixed with 1.0 M NaOH/HCl and incubated at 80°C with a stirrer for 2 h. Finally, the hydrolyzed cells' β-glucan content (%, w/w) was calculated following the assay kit protocol.

2-18. Statistical analysis

The results are the means ± standard deviations of triplicate analysis. Duncan's test was performed with SPSS (version 18.0), and results were analyzed to ANOVA using the GraphPad Prism software (GraphPad Software, CA, USA).

3. Results

3-1. Isolation of yeasts

Two hundred four isolates of different shapes and sizes were picked to investigate the probiotic characteristics. One hundred isolates were confirmed to be yeast by gram staining and morphology. The isolated strains were used to further the studies.

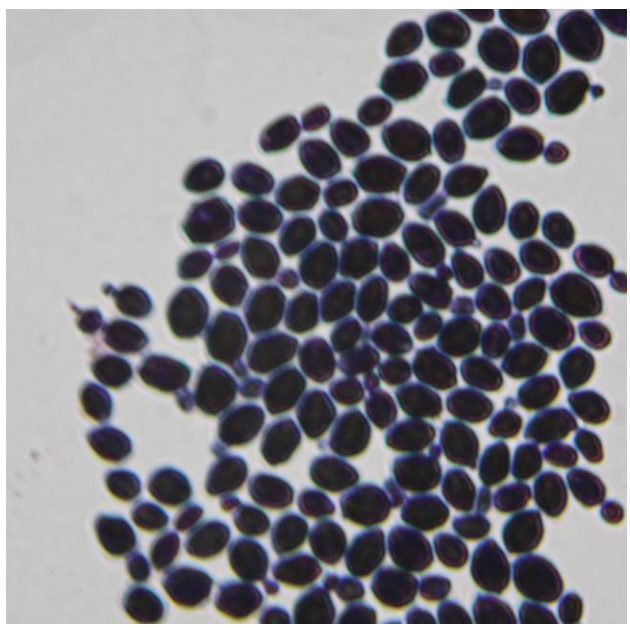


Figure 2-3. Gram positive yeasts are confirmed by a yeast smear's Gram staining.

3-2. Resistance to low pH and bile tolerance

Yeast cell growth was less resistant at pH 2.0 than *S. boulardii* CNCM I-745. According to resistance to acid and bile condition, tested yeast showed a similar survival rate. Yeast was chosen to compare the growth in the bile condition with *S. boulardii* CNCM I-745. *S. boulardii* control strains had 97.85% survivability in 3.0% Oxgall. The isolates with over 90% survivability were selected, where seventy-four isolates showed > 90% survivability (Fig. 2-4).

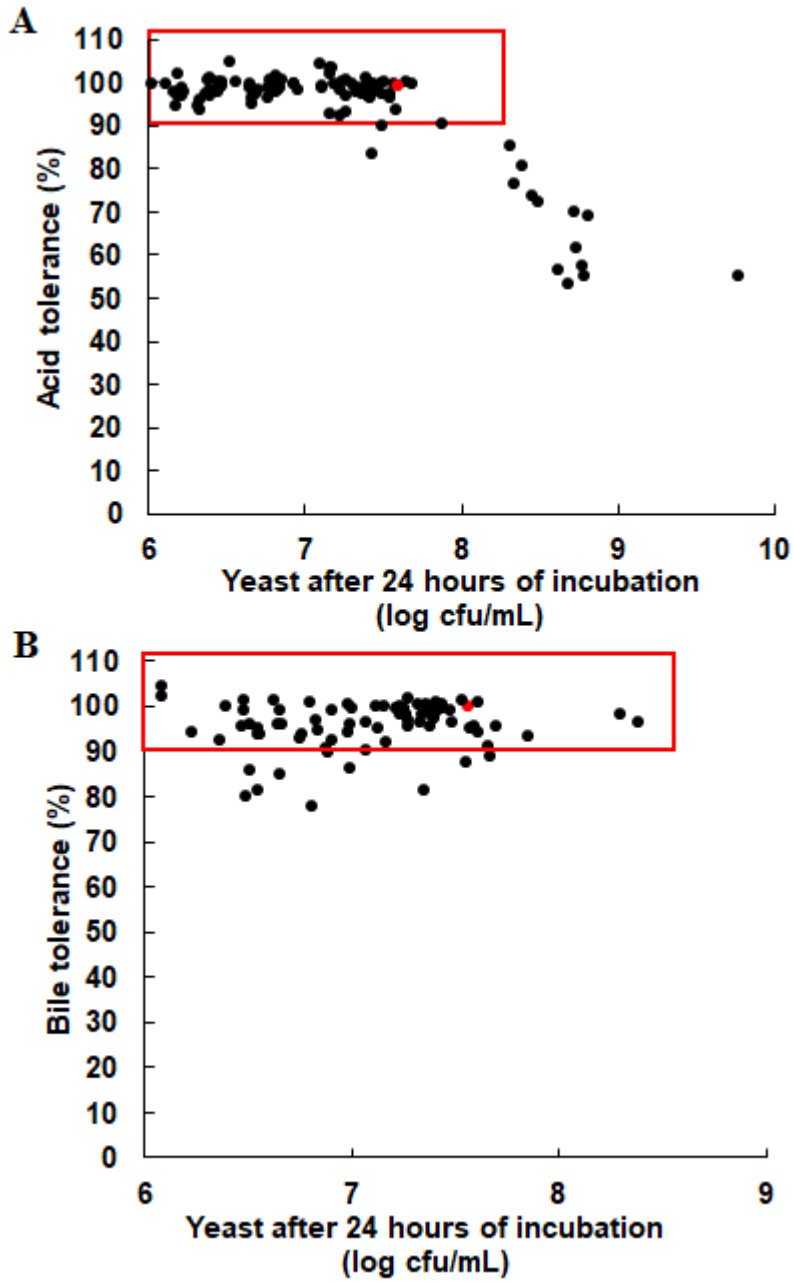


Figure 2-4. Screening of yeast with over 90% acid tolerance (n=82) and bile tolerance (n=74). Control strain (red color) is *S. boulardii* CNCM I-745.

3-3. Auto aggregation and coaggregation with pathogens

Aggregation characteristics of yeast are related to sporulation[94]. Notably, 80% auto aggregation after 24 h of incubation was based on colony formation[95], which affects host colonization after entry. Coaggregation with pathogen was high in *S. boulardii* CNCM I-745. GILA isolates with more significant or similar to coaggregation ability than *S. boulardii* CNCM I-745 were selected (Table 2-5). Sixty-three isolated yeasts showed high auto aggregation properties (Fig. 2-5). Thirty-three isolates with auto aggregation ability could also have coaggregation ability with the pathogen. *S. boulardii* CNCM I-745 had between 60% and 85% coaggregation with the three pathogens, *Staphylococcus aureus* ATCC 25922, *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* K88.

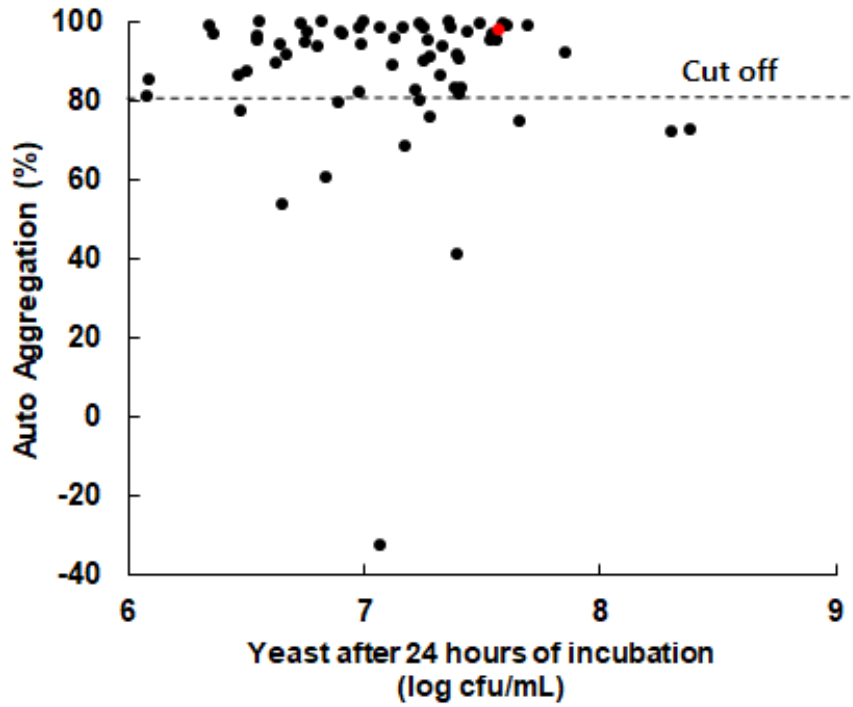


Figure 2-5. Screening of yeast with over 80% auto aggregation (n=63).

Control strain (red color) is *S. boulardii* CNCM I-745.

Table 2-5. Coaggregation of yeast isolates with pathogens (n=33).

Yeast	Coaggregation (%)		
	***S. aureus* ATCC 25922**	***E. faecalis* ATCC 29212**	***E. coli* K88**
S.b CNCM I-745	71.04 ± 28.66	60.31 ± 17.07	68.07 ± 11.78
GILA 59	49.42 ± 23.97	62.39 ± 37.90	55.75 ± 5.30
GILA 100	60.30 ± 29.30	50.37 ± 12.76	44.25 ± 33.50
GILA 102	79.71 ± 2.73	26.63 ± 9.74	67.03 ± 25.09
GILA 106	74.79 ± 8.12	97.65 ± 7.15	57.74 ± 0.57
GILA 109	27.27 ± 12.70	20.62 ± 5. 31	42.50 ± 27.02
GILA 112	86.24 ± 9.08	34.53 ± 19.22	46.21 ± 4.85
GILA 114	49.93 ± 24.25	59.53 ± 20.51	30.82 ± 4.46
GILA 115	73.46 ± 8.64	94.24 ± 1.97	84.43 ± 6.19
GILA 116	55.48 ± 12.18	49.63 ± 6.15	71.43 ± 16.99
GILA 117	75.83 ± 26.43	85.80 ± 15.28	60.81 ± 6.33
GILA 118	73.16 ± 21.88	47.41 ± 9.10	23.23 ± 1.43
GILA 129	60.30 ± 24.12	85.95 ± 39.14	20.28 ± 0.32
GILA 133	55.09 ± 7.07	13.63 ± 3.06	42.76 ± 1.16
GILA 135	75.83 ± 17.55	86.07 ± 4.92	90.56 ± 11.44
GILA 136	74.55 ± 4.87	98.06 ± 6.60	54.62 ± 8.82
GILA 137	22.79 ± 0.97	89.38 ± 7.39	24.79 ± 14.19
GILA 142	60.95 ± 0.29	49.74 ± 21.15	43.80 ± 14.81
GILA 143	51.57 ± 7.47	36.70 ± 20.07	57.07 ± 14.42
GILA 145	47.27 ± 9.51	117.28 ± 28.83	63.53 ± 3.18
GILA 146	52.93 ± 19.87	63.35 ± 11.45	47.29 ± 9.04
GILA 158	101.74 ± 5.28	110.84 ± 19.99	101.05 ± 6.05
GILA 173	52.08 ± 17.05	44.38 ± 23.33	31.16 ± 0.68
GILA 174	53.14 ± 17.05	54.97 ± 5.59	49.66 ± 9.74
GILA 176	53.27 ± 5.44	56.20 ± 1.62	61.64 ± 11.10
GILA 180	54.54 ± 8.19	31.72 ± 16.99	51.40 ± 8.85
GILA 181	73.29 ± 23.99	56.29 ± 7.66	46.64 ± 6.72
GILA 184	41.19 ± 14.95	55.32 ± 10.22	61.35 ± 0.87
GILA 187	49.98 ± 11.88	26.39 ± 9.12	54.16 ± 9.68
GILA 189	73.63 ± 10.89	40.95 ± 9.06	52.54 ± 9.32
GILA 190	47.69 ± 7.50	65.87 ± 6.23	65.05 ± 25.40
GILA 193	87.08 ± 6.88	35.70 ± 13.01	31.52 ± 18.32
GILA 194	54.54 ± 8.19	31.72 ± 16.99	51.40 ± 8.85
GILA 197	50.05 ± 5.36	60.82 ± 9.75	36.14 ± 19.58

3-4. Antioxidant ability by DPPH assay

The DPPH scavenging activity of GILA isolates was evaluated to examine their antioxidant ability. The scavenging activity for *S. boulardii* CNCM I-745 was 91.35 ± 0.13%; results for yeast were less than 90.00%. For this reason, screening of GILA(n=21) had more than 85.00% DPPH scavenging activity (Fig. 2-6).

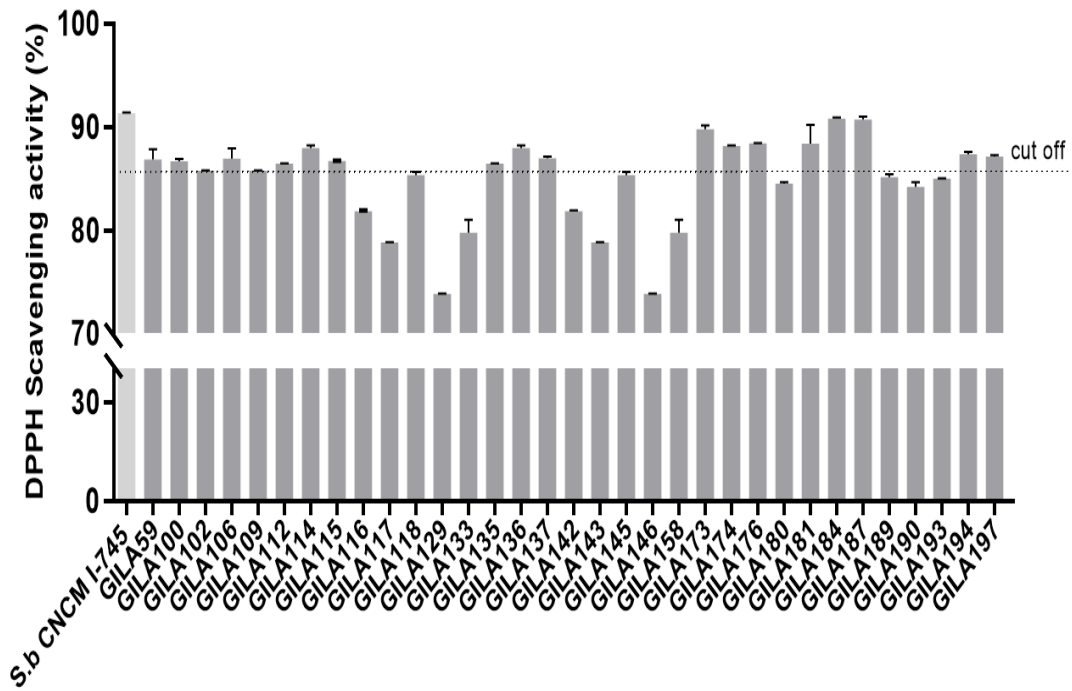


Figure 2-6. Screening of yeast with over 85% DPPH scavenging activity (n=21). Values are mean ± S.D of triplicates for each group.

3-5. Safety assessments

Prior to safety evaluations, twelve isolates with growth characteristics comparable to *S. boulardii* CNCM I-745 were chosen (Fig. 2-7). We measured the hemolytic activity and analyzed biogenic amine production to determine the safety of our isolated yeast for further study. To confirm the safety of yeast, seven isolates were preliminarily screened (n=7).

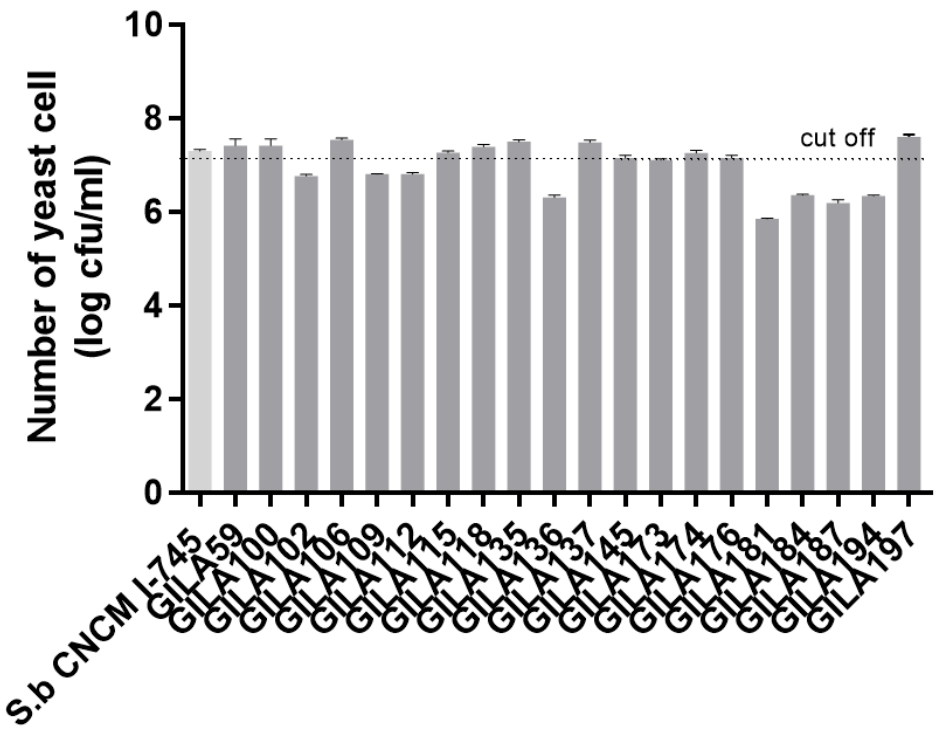


Figure 2-7. Screening of yeast with growth characteristics comparable to *S. boulardii* CNCM I-745 (n=12).

Table 2-6. Safety assessments of isolated yeast (n=7)

Yeast isolates	Hemolytic activity	Biogenic amine
GILA 59	-	-
GILA 100	-	-
GILA 106	-	-
GILA 115	-	-
GILA 118	-	-
GILA 135	-	+
GILA 137	-	-
GILA 145	-	+
GILA 173	-	+
GILA 174	-	+
GILA 176	-	+
GILA 197	-	-

-; negative, +; positive

3-6. Identification and phylogenetic analysis of the ITS region

Internal transcribed spacer (ITS) sequencing identified the selected strains that showed potential as probiotic yeast as *S. cerevisiae.* High homology of ITS sequences was observed closest location of *S. cerevisiae* GILA 137 to *S. cerevisiae* GILA 106 and closer to *S. cerevisiae* GILA 118 in the 15 yeasts (Fig. 2-8). *S. cerevisiae* GILA 100 is closely related to *S. cerevisiae* GILA 106, 118, and 137. Due to the high homology of *S. cerevisiae* GILA, phenotypic differences such as the probiotics potential of *S. cerevisiae* GILA were investigated.

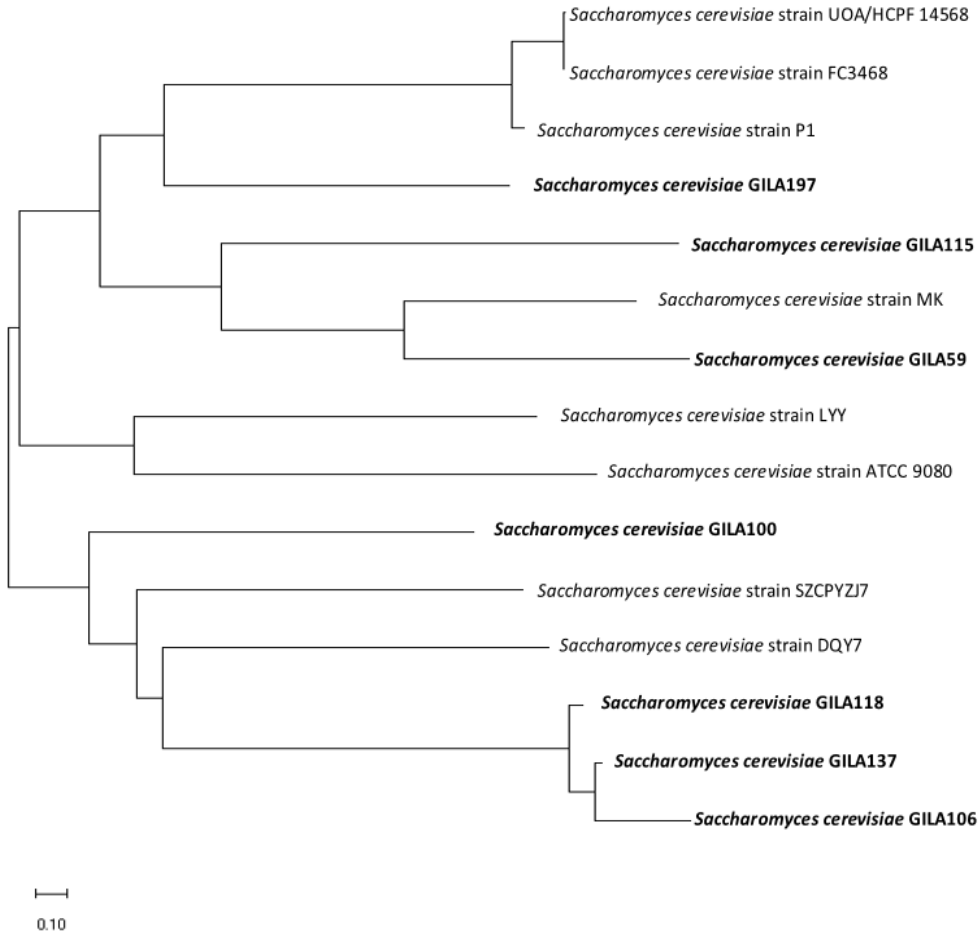


Figure 2-8. The phylogenetic tree of *S. cerevisiae* GILA. Based on ITS region sequencing. Relationships of taxa was inferred using the Neighbor-Joining method. Bar, 0.10 substitutions per nucleotide position

3-7. Survival rate in the GIT model

The survival rates of all *S. cerevisiae* GILA strains were similar when compared with control strain *S. boulardii*, CNCM I-745, which showed over 95% survival in GIT model at mouth, stomach and intestine (Fig. 2-9). *S. cerevisiae* GILA106 showed a significantly lower survival rate ($p < 0.01$ and $p < 0.001$) than other *S. cerevisiae* GILA strain in the stomach and intestine, whereas all strains showed >90.0% survival rate.

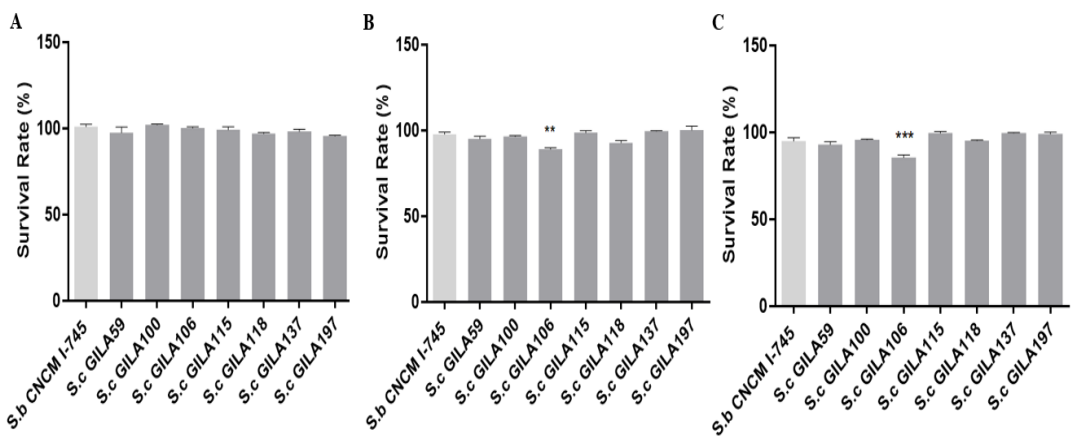


Figure 2-9. The survival rate of selected *S. cerevisiae* GILA strains at (a) mouth, (b) stomach and (c) intestine in the GIT model. Values are mean ± S.D of triplicates for each group. ** p <0.01, *** p <0.001, compared with *S. boulardii* CNCM I-745.

3-8. Adhesion to Caco-2 cell

Adhesion assay to Caco-2 cell show all selected *S. cerevisiae* GILA strains adhesion ability (4% to 15%). When compared with the control yeast strain, *S. boulardii* CNCM I-745 (8%), *S. cerevisiae* GILA strains were not significantly different. (Fig. 2-10).

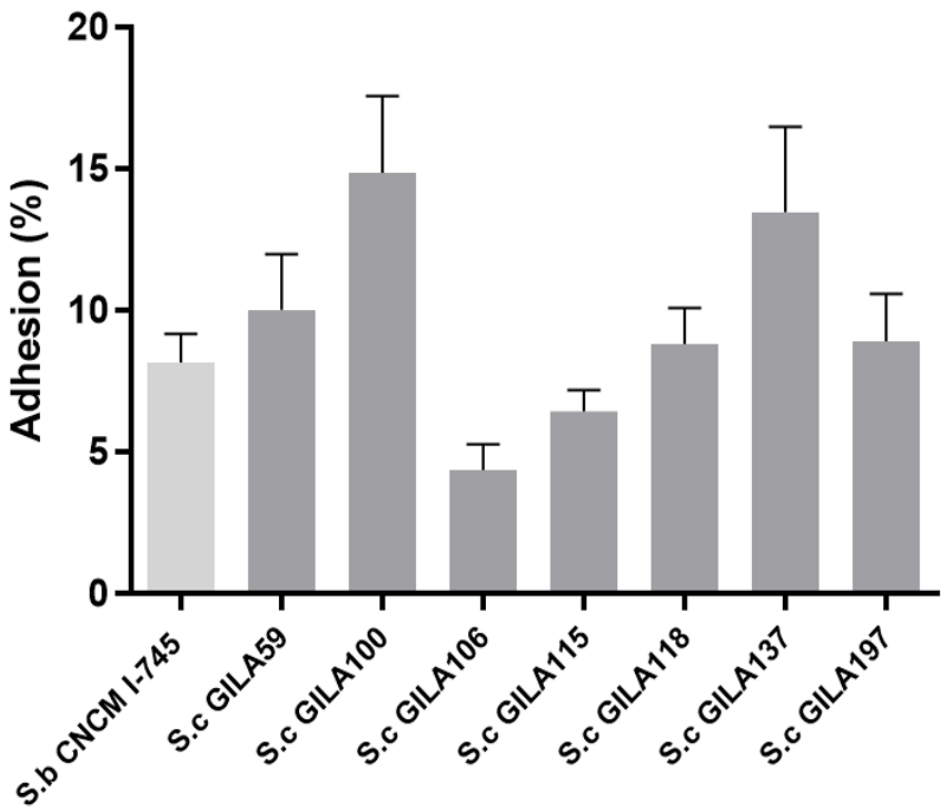


Figure 2-10. Adhesion percentages of *S. cerevisiae* strain on caco-2 cells.

Adhesion percentages are calculated by plate count method.

.3-9. Alleviating the inflammation in RAW 264.7 cells

NO is related to various immunological procedures such as host defense, immunoregulation and signal transduction and are importance mediators triggering gastrointestinal disease[96]. NO is produced from L-arginine by an enzyme of nitric oxide synthase (NOS) and the inducible isoform of NO (iNOS) during inflammation where iNOS is activated by pro-inflammatory cytokines like TNF-α, interleukin-6 IL-6. For examining the effects of selected *S. cerevisiae* GILA, 10 ng/ml of LPS was treated in 264.7 cells for 48 h to induce inflammation and NO production. Selected *S. cerevisiae* GILA significantly ($p < 0.001$) suppressed NO production induced by LPS compared with the positive control treated LPS only. *S. boulardii* CNCM I-745 has shown anti-inflammatory effects compared with the LPS treatment group (Fig. 2-11).

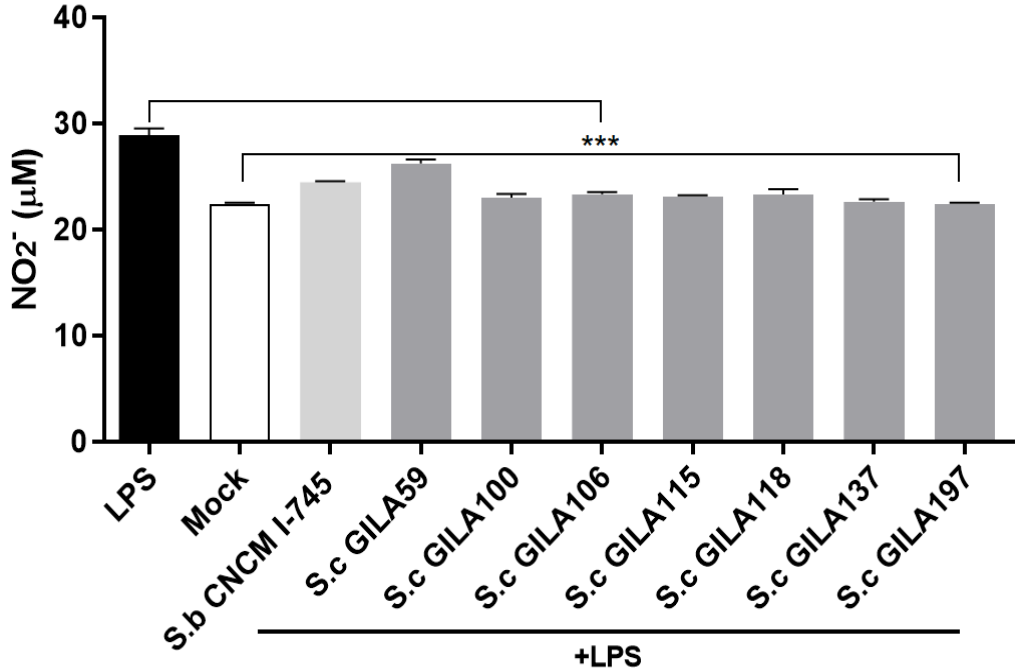
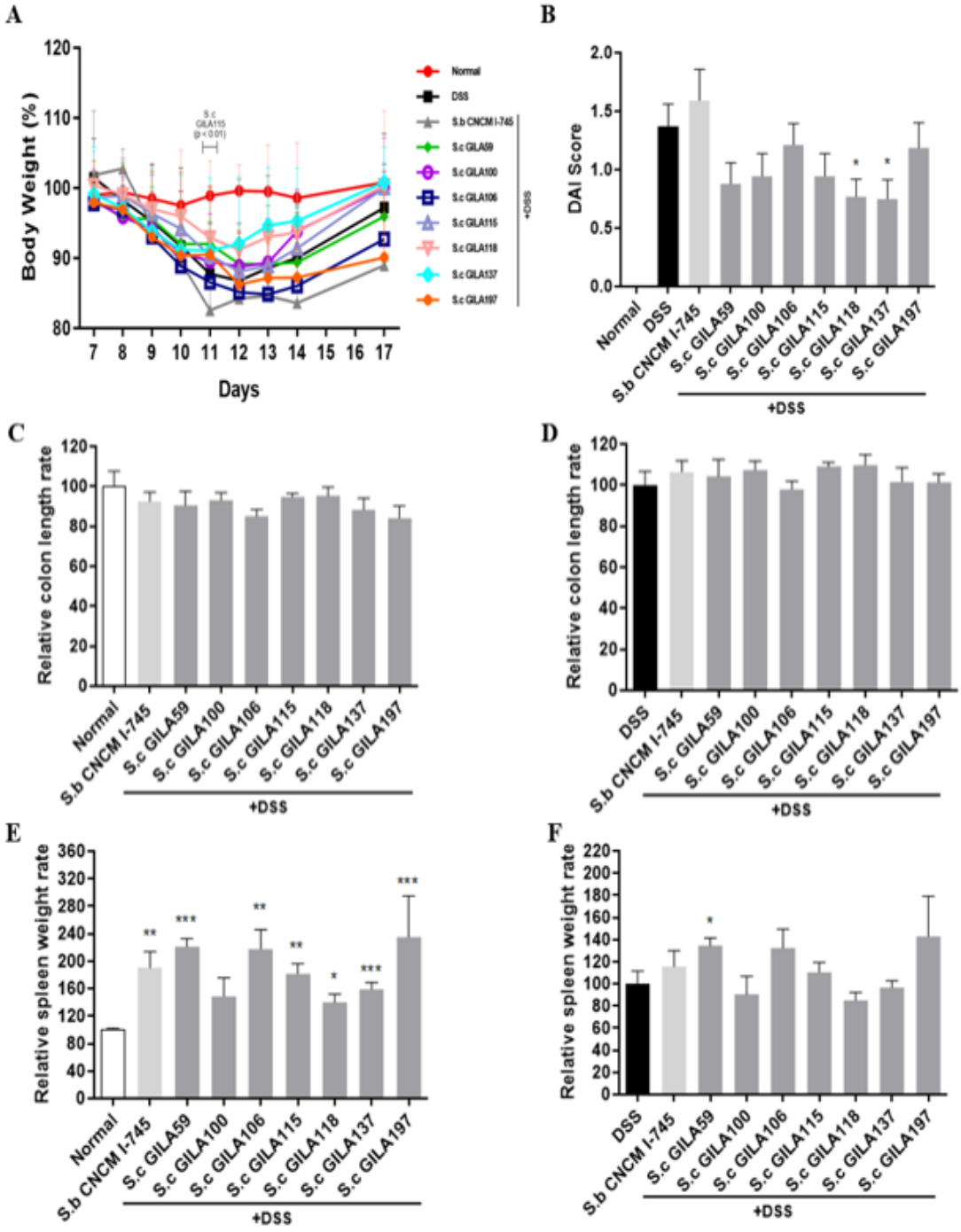


Figure 2-11. Nitric oxide production of heat-killed *S. boulardii* and *S. cerevisiae* strains in LPS (1 μg/ml) induced RAW 264.7 murine macrophage cells. The concentration of Nitric oxide production was determined by calculating standard curve. Values are mean ± S.D of triplicates for each group. ****p* <0.001, compared with treatment of only LPS.

3-10. Alleviating the intestinal inflammation in a dextran sulfate sodium-induced colitis mouse model

During DSS treatment with yeast period, the *S. cerevisiae* GILA115 group showed body weight loss more than the normal group ($p < 0.01$) on day11. In contrast the other *S. cerevisiae* GILA groups were not significantly ($p > 0.05$) different (Fig. 2-12A). Stool consistency and bleeding score were significantly lower in the *S. cerevisiae* GILA 59, 100, 118, and 137 groups than in the DSS group, although *S. cerevisiae* GILA 59 and 100 groups lose more weight (8-10%) than *S. cerevisiae* GILA 118 and 137 groups (4-8%). Consequently, *S. cerevisiae* GILA 118 and 137 groups were significantly ($p < 0.05$) lower than the DSS treatment group when compared with the DAI score (Fig. 2-12B). The relative colon length rate was not significantly different (Fig. 2-12C and D) whereas the relative spleen weight rate was significantly different between the normal and DSS treatment groups (Fig. 2-12E and F). *S. cerevisiae* GILA100 and 118 groups were similar spleen weight rates to the normal group (Fig. 2-12E). Standard scores were calculated using normal and DSS group scores. The total score showed GILA 100, 118, and 137 groups were similar to the normal group (blue line) than the other groups (Fig. 2-12G).


A
Body Weight (%)
Days
Normal
DSS
S.b CNCM I-745
S.c GILA59
S.c GILA100
S.c GILA106
S.c GILA115
S.c GILA118
S.c GILA137
S.c GILA197
+DSS
B
DAI Score
C
Relative colon length rate
D
Relative colon length rate
E
Relative spleen weight rate
F
Relative spleen weight rate

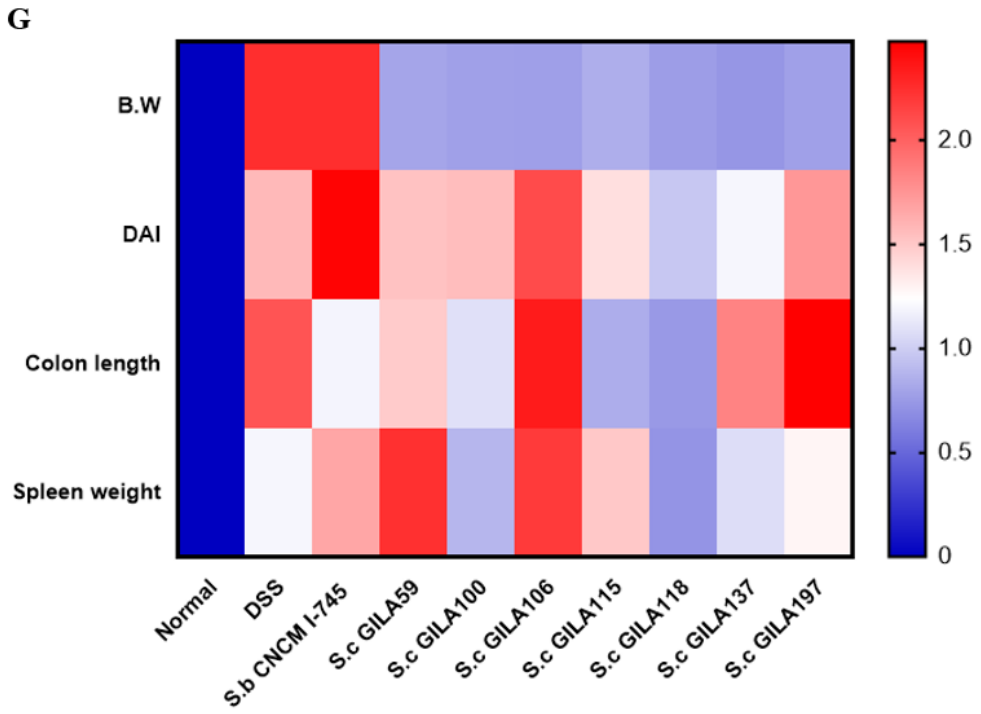


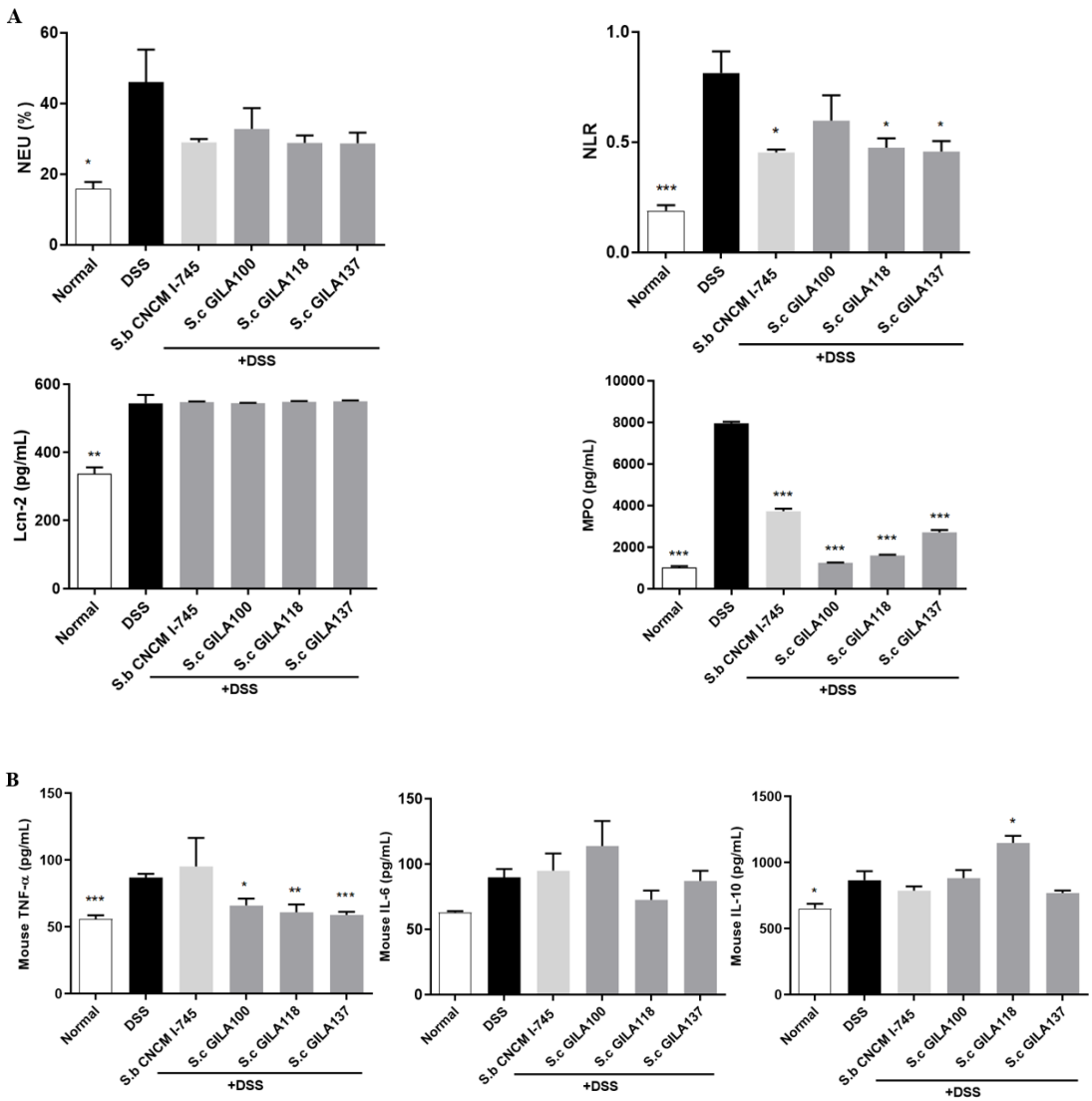
Figure 2-12. *in vivo* Screening of *S. cerevisiae* GILA strain. Pathological and physiological status through the indicators of inflammation. (A) Body weight (%) compared to the normal group. (B) DAI score of disease from C57BL/6J mouse group. Relative colon length rate compared with that of Normal (C) and DSS (D) group. Relative spleen weight rate compared with that of Normal (E) and DSS (F) group. (G) *S. cerevisiae* GILA strain's *in vivo* screening total score. Statical significance is indicated as follows: *p <0.05, **p <0.01 and ***p <0.001.

To investigate the therapeutic properties of *S. cerevisiae* GILA strains *in vivo*, the DSS group showed IBD-colitis symptoms, including increased neutrophil count, neutrophil-lymphocyte ratio (NLR) in blood, myeloperoxidase (MPO) in feces (Fig. 2-13a), and proinflammatory cytokine (TNF-α) in serum (Fig. 2-13b). Stool consistency and bleeding score results were related to NLR from complete blood cell count (Fig. 2-13a)[97,98]. This finding suggested that the DSS-induced increase in neutrophils may affect other biomarkers and cytokines. Neutrophil expression in blood is one of the main features of colitis[29,99,100]. Further analysis was conducted to investigate the amelioration of intestinal inflammation. The gene expression levels of mucin-2 (Muc-2), zonula occludens-1 (ZO-1), occludin and epithelial cadherin (E-cadherin) significantly increased compared with those in the DSS group. (Fig. 2-13c).

S. cerevisiae GILA group had significantly decreased NLR in the blood (p <0.01), MPO in feces (p <0.001), and TNF-α in serum ($p < 0.05$, $p < 0.01$ and $p < 0.001$) (Fig. 2-13a and b). No significant changes were observed for Lcn-2 in feces, IL-6 in serum. Meanwhile, IL-10 in serum significantly increased in the *S. cerevisiae* GILA 118 group compared with that in the other groups.

The *S. cerevisiae* GILA 118 group also showed significantly increased serum IL-10 levels compared with the DSS group (Fig. 2-13b). These results suggested that *S. cerevisiae* GILA 118 effectively inhibits the

biomarker of IBD and the expression of IL-10, thereby ameliorating colitis in mice.



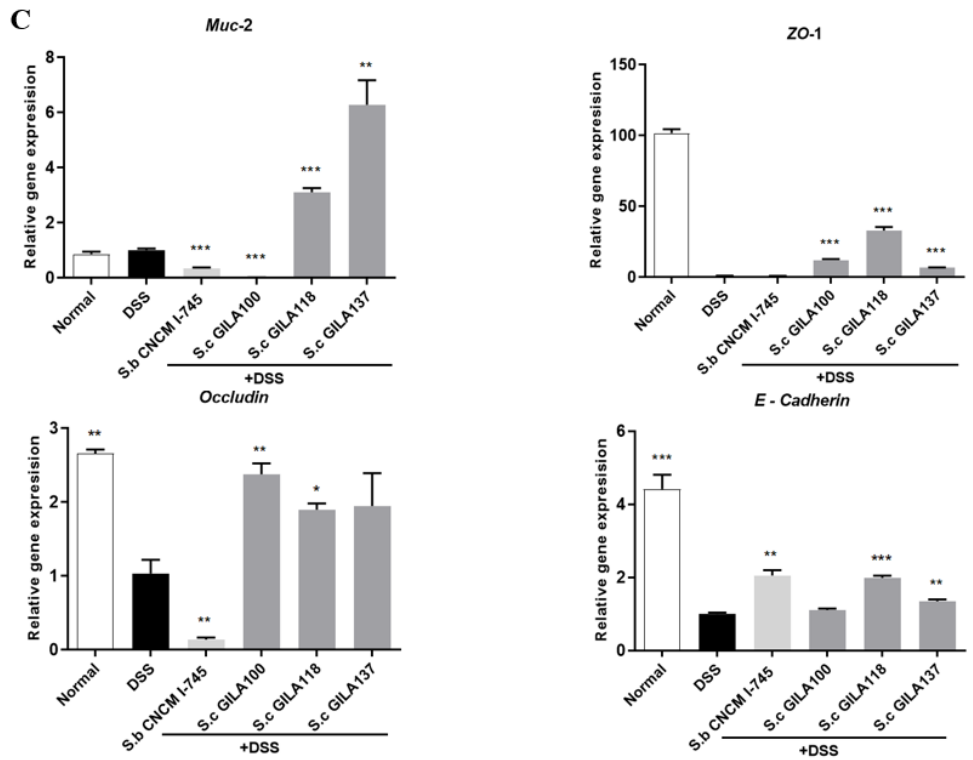


Figure 2-13. Alleviation of Intestinal Inflammation in Mice between Treatments of *S. cerevisiae* GILA.

(A) Neutrophil, Neutrophil–lymphocyte ratio from complete blood cell count and Amount of Lcn-2, MPO in feces. (B) Analysis of pro-inflammatory cytokine TNF-α, IL-6 and anti-inflammatory cytokine IL-10 in serum. (C) Relaive gene expression of Muc-2, ZO-1, Occludin, E-cadherin in colon tissue. Statical significance is indicated as follows: **p* <0.05, ***p* <0.01, ****p* <0.001, compared to treatment of DSS.

3-11. Fermentative profiles

The *S. cerevisiae* GILA strains selected from ameliorating colitis in mice showed the selective fermentative profiles (Fig. 2-14). The strain GILA118 did not ferment Glycerol, α-Methyl-D-Glucosamine and showed Glucose, Galactose, Cellobiose, Maltose, Saccharose and Raffinose activity. Through an exception of glycerol activity, *S. cerevisiae* GILA 118's fermentative profile was similar with the characteristics of *S. boulardii*.

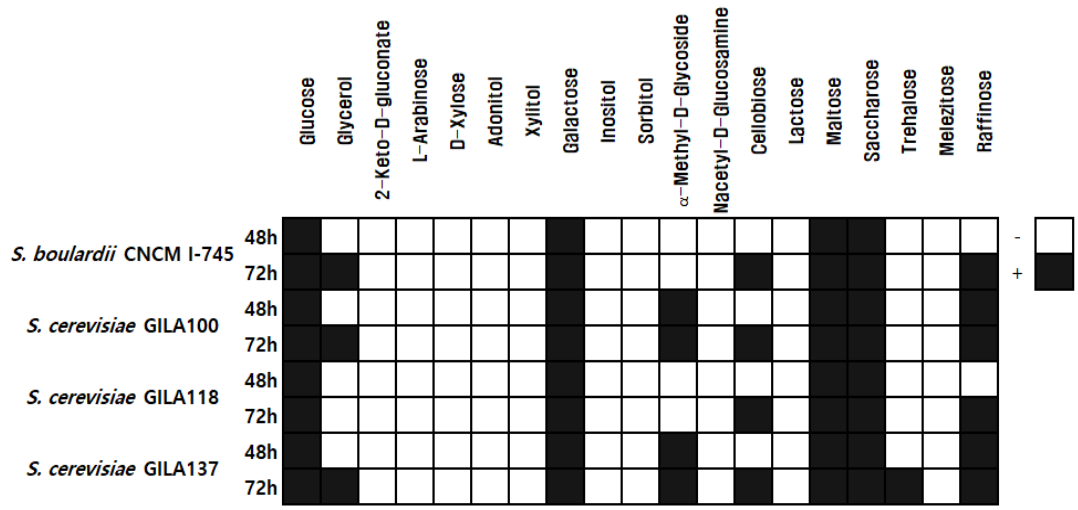


Figure 2-14. Fermentative profiles of *S. cerevisiae* GILA.

3-12. Cell wall β-glucan content

Figure 2-15 shows the *S. cerevisiae* GILA strains β-glucan content. The β-glucan content (w/w) was determined by the total glucan and α-glucan percentages (w/w). *S. boulardii* CNCM I-745 cell walls comprised 41.31% β-glucan *S. cerevisiae* GILA strains had β-glucan contents similar to or higher than *S. boulardii*. Only *S. cerevisiae* GILA 137 showed a lower content of β-glucan than *S. boulardii*.

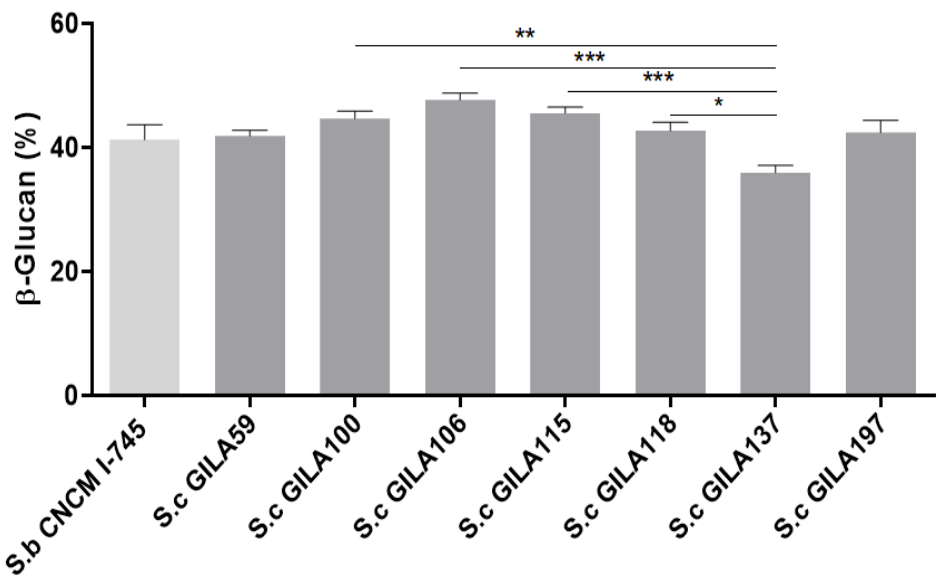
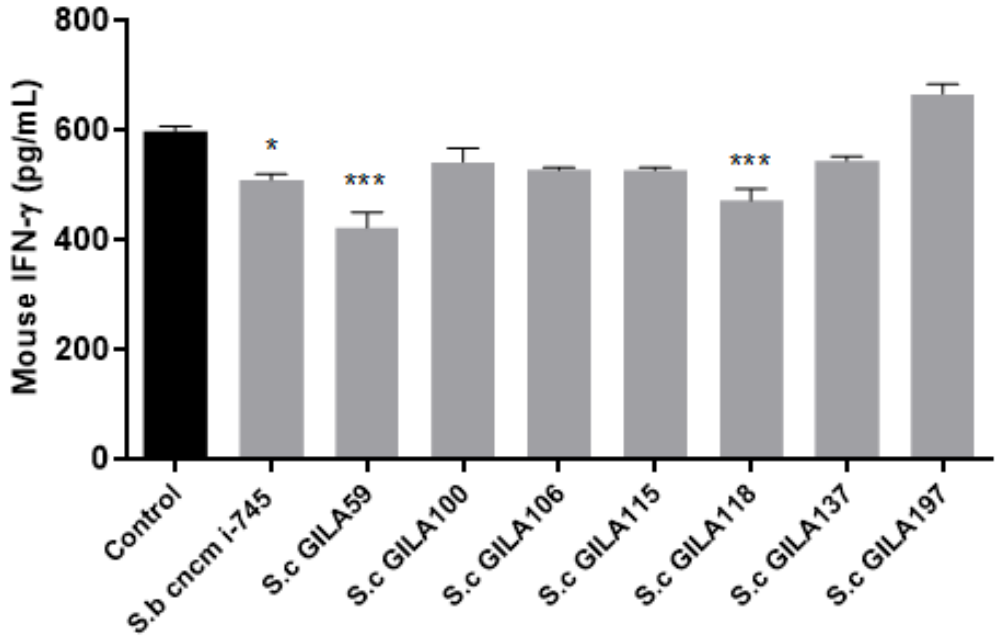


Figure 2-15. Cell wall β-glucan content of *S. cerevisiae* GILA strains. β-glucan content calculated using a yeast glucan assay kit. The data represent standard errors of the means with duplicates (*p <0.05, **p <0.01 and ***p <0.001).

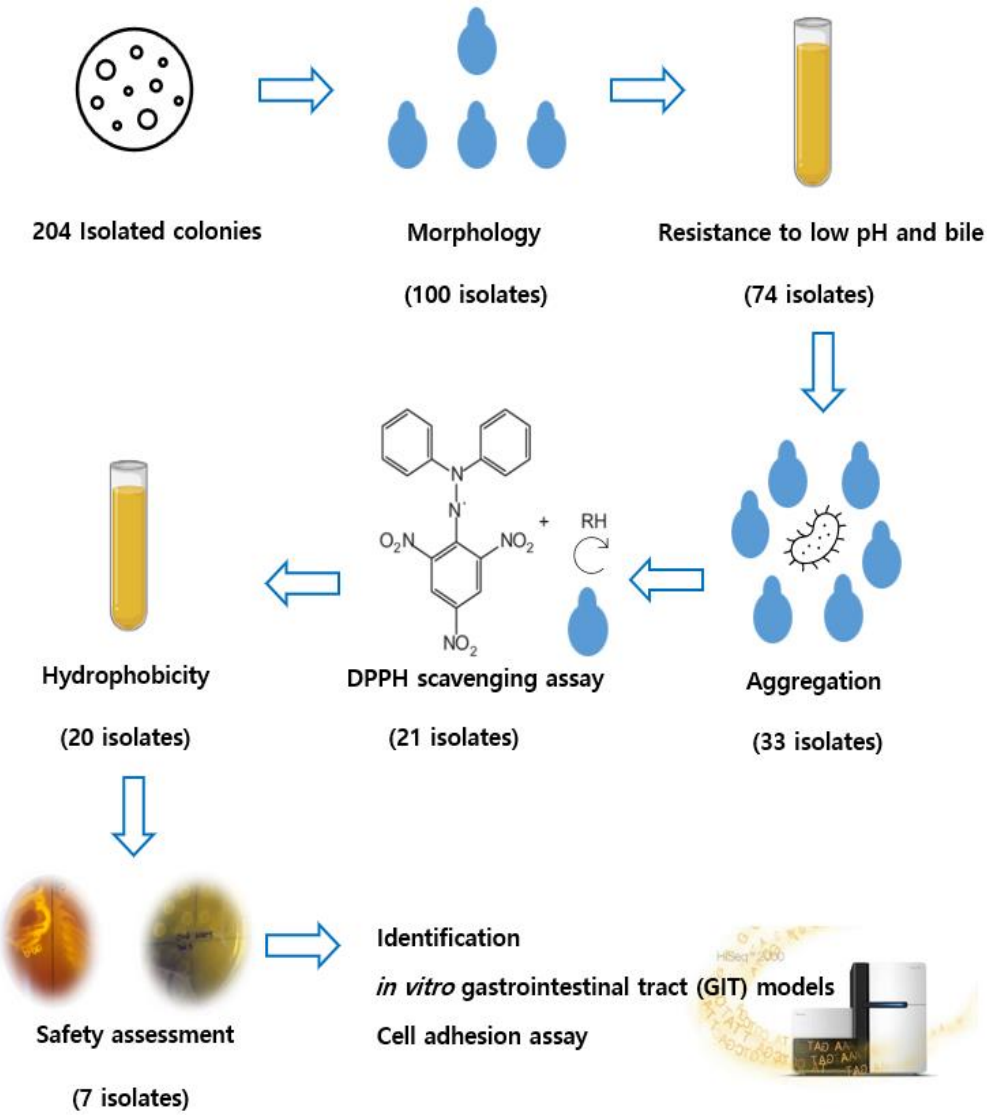
4. Supplementary Figures



Supplementary Figure 2-1. Mouse IFN-γ expression by splenocyte from Balb/c mouse. IFN-γ expression in with *S. boulardii* and *S. cerevisiae* strain. Values are mean ± S.D of triplicates for each group. ***P* <0.01, ****P* <0.001, compared with control (Treatment of only PBS).

Supplementary Table 2-1. Hydrophobicity of yeast isolates

Yeast Strain	Hydrophobicity (%)
S.b CNCM I-745	99.66
GILA 59	99.40
GILA 100	99.46
GILA 102	99.96
GILA 106	99.96
GILA 109	99.90
GILA 112	99.90
GILA 115	99.48
GILA 118	94.41
GILA 135	99.21
GILA 136	98.94
GILA 137	98.14
GILA 145	98.58
GILA 173	99.56
GILA 174	100.12
GILA 176	100.03
GILA 181	99.79
GILA 184	100.05
GILA 187	99.257
GILA 194	98.36
GILA 197	99.60



Supplementary Figure 2-2. The probiotic characteristics of seven *S. cerevisiae* GILA, isolated from Korean fermented beverage.

5. Discussion

In this investigation selected a probiotic candidate *S. cerevisiae* GILA, which had a high survival rate in low pH and bile conditions. These results indicate that yeast which had high resistance to harsh condition also had high survival in GIT simulation model. *S. cerevisiae* GILA has almost over 90.0 % survival rate in GIT model. *S. cerevisiae* has the advantage of better survival than lactic acid bacteria in a GIT environment[101]. Tolerance to GI tract could be considered *in vivo* conditions. Comparing the viable counts in the murine gastrointestinal tract could be shown survival rate *in vivo*[102]. The probiotic function of *S. boulardii* has been attributed to a number of pathways, including regulation of the normal microbiota of the gut, antagonistic activity against pathogens, adherence to mucus, immunological modulation, and trophic effects on the GI tract[103,104]. After receiving antibiotics or undergoing surgery, S. *boulardii* aids in the restoration of the normal gut microbiota in the patients. While the native microbiome is restored, it may also act as a temporary replacement[105]. Survival was an important factor of probiotics, but safety was also studied for selected probiotic potential yeast. None of the selected *S. cerevisiae* GILA strains had hemolytic activity and biogenic amine production. Yeast is also known to have antibiotic resistance.

Neutrophils rule the early phases of inflammation and pave the way for macrophages to heal tissue damage. Numerous cytokines and the expression

of their receptors coordinate these effects, and they may be used to block certain inflammatory processes[106]. Neutrophils can be involved in both tissue damage and repair, and neutrophil-macrophage interaction is essential for the control of the inflammatory, proliferative, and remodeling phases of repair as well as the transition between them[107]. These inflammatory processes were linked to *in vivo* mouse CBC testing and *in vitro* RAW 264.7 cell.

Yeasts aggregate via their cell wall mannose[108]; a prominent aggregation ability indicates ample mannose. GILA isolates showed > 90% hydrophobicity (Table. S2-1). The adhesion ability for Caco-2 cells, such as microbial adhesion to mucosa model[109] was 4%–15%. Aggregation, hydrophobicity, Caco-2 cell adhesion assay, showed that *S. cerevisiae* GILA stain possesses cell adhesion ability to be used in probiotic preparation.

The DPPH scavenging effect measures the antioxidant capacity related to the cell wall β-glucan content. Early research found that the primary active components of yeast cell walls, β-glucan and mannan (also known as mannoprotein), can operate on macrophages to increase the body's non-specific immunity[110,111]. β-Glucan is a β-D-glucose polysaccharide group and a component of the yeast cell wall. Its structure is a long, β-(1,6)-branched, β-(1,3)-glucan[112]. β-Glucan has an excellent antioxidant capacity[113]; however, this could not explain the results for cell wall β-glucan (Fig. 2-15). Yeast itself may then possess an intrinsic antioxidant ability[114].

Therefore, there should be another experiment to quantify cell wall β-glucan. β-Glucan is recognized by the receptors on the host's immune cells[115], enhancing immune function resulting in anticancer and anti-inflammatory effects[115,116]. Furthermore, yeast β-Glucan benefits host health by protecting it against pathogens[117]. The content of β-Glucan was calculated from total glucan by subtracting α-glucan. A large amount of β-glucan in the yeast cell walls was comparable to a previous study[93]. The β-glucan that makes up the yeast cell wall ranges in percentage from 29% to 64%[118,119]. The *S. cerevisiae* GILA strains had more than 36% β-glucan; this result could imply a probiotic potential (Fig. 2-15). *S. cerevisiae* has more than triple the amount of β-glucan than these *Lactobacillus* strains. Moreover, the structure of β-glucan should also be considered in future research. Since β-glucan modulates cytokines in human blood, it should be confirmed whether all structures of β-glucan are beneficial to host health[120]. This result indicated that β-glucan benefits the host's health and immune system. A future probiotic approach could consider yeast's β-glucan characteristics. Our results revealed the quantity of β-glucan in the yeast cell walls. This method could be applied during probiotics-related yeast and β-glucan screening. The result was unexpected from the DPPH scavenging effect, but all experimental strains had more than 36% β-glucan, which is a sufficient level[93].

The cell wall had more than 36% β-glucan, and nitric oxide production was

significantly lower than the control. The β-glucan quantity could be adequate, but results need more evidence of related probiotic functionality. Previous studies have reported the prevention of inflammation by yeast fermentate[121]; there are also reports on β-glucan-mediated induction of proinflammatory cytokines[122]. Given that the quantity of β-glucan did not influence this outcome, another factor, such as the probiotic properties of the *S. cerevisiae* GILA strains, may have been responsible for reducing in proinflammatory cytokines. IFN-γ is key to derived inflammation in GI tract when intestinal barrier impairment[123]. Thus, *S. cerevisiae* GILA strains suppressed the proinflammatory functionality (Fig. S2-1). The physiological impact of yeast probiotics against the host was determined by their ability to relieve oxidative stress measured by fecal MPO level[30]. Activated neutrophils release MPO, a marker of oxidative stress, and destroy epithelial cells[29]. As demonstrated by DPPH scavenging capacity of *Saccharomyces cerevisiae* GILA *in vitro* (Fig. 2-6), *in vivo* results also prove this ability to relieve oxidative stress (Fig. 2-13a). As a result, numerous compounds with antioxidant capabilities are currently being thoroughly researched as a potential treatment for IBD, either in addition to or instead of traditional medication. The scavenging of free radicals, acting as immunomodulators, boosting antioxidant defense capacity, influencing multiple signaling pathways, inhibiting pro-oxidative enzymes and cytokine levels are just a few of the effects that natural and synthetic compounds with antioxidant and anti-inflammatory properties exhibit that may be helpful in the treatment of

UC [124,125]. The *S. cerevisiae* strain was resistant to ETEC infection[62], and *S. cerevisiae* cell wall glucan had an immune-modulatory effect, which could affect colitis reduction[58]. Spleen weight could be due to alleviating intestinal immune response[32]. Moreover, the inflammation biomarkers were similar to those in the CBC test-neutrophil lymphocyte rate results[100] (Fig 2-13a, b). The effects of GILA on the inflammatory markers Lcn-2 and IL-6 were not discovered in the results, nevertheless. The capacity of GILA to reduce oxidative stress was demonstrated in this study, although not all inflammatory markers. There was no significant difference in the relative expression rate of the Muc-2 gene between the normal and DSS groups (Fig. 12c). Recovery through a six-days period is considered maintaining in the DSS group. In this study, an increase in Muc-2 gene expression was regarded as an improved capacity of epithelial protection[126]. As a result, similar to this experiment, colitis was alleviated by increasing Muc-2 expression in intestinal goblet cells[127]. An increase in Muc-2 gene expression is assumed to reduce colitis (Fig. 2-12c). Furthermore, results elucidated that anti-inflammatory cytokine IL-10 in serum was more upregulated by *S. cerevisiae* GILA 118 than other *S. cerevisiae* GILA strains. These findings warrant further experiments, especially *S. cerevisiae* GILA 118 structure study with the DSS-colitis mouse model. For this reason, *S. cerevisiae* could be developed as a useful probiotic in the future. Nevertheless, more evidence as a potential gut microbiota modulator[128] is required for the *S. cerevisiae* GILA strain-related probiotics.

This chapter will be published elsewhere

as a partial fulfillment of Bum Ju Kil's Ph.D. program

Chapter 3. Cell wall component of *Saccharomyces cerevisiae* GILA 118 alleviates intestinal inflammation in a dextran sulfate sodium induced colitis mouse model

1. Introduction

IBD, such as CD and UC, is a chronic relapsing inflammatory disorder of the GIT. The primary IBD symptoms are dysregulated immune response and impairment of the epithelial barrier function[18]. UC damages the barrier, which is localized to the mucous membrane, and the submucosal layer, whereas CD causes transmural inflammation that induces fibrosis, stricture, and fistula[129]. Damages to the surface, which are related to the development of inflammation, are one of the factors that recruit neutrophils and can activate proinflammatory cytokine (TNF-α, IL-6) and the differentiation of inflammatory T cells[130]. CD, and UC-induce Th17 cells to mediate the immune response. Conversely, regulatory T (Treg) secreted anti-inflammatory cytokine IL-10 has immunosuppressive effects. Treg and Th17 cells are therefore different in IBD from a normal state[131,132].

Therapy with drugs related to colitis includes amino salicylates, nicotine, corticosteroids, infliximab, and thiopurines. Long-term drug or overdose therapy use could be a high risk due to side effects[40]. Probiotics are an alternative treatment to IBD. Probiotics refer to living microorganisms that provide beneficial functions to the host when ingested in sufficient amounts. Probiotics exert beneficial effects through different mechanisms, such as anti-inflammatory, and inhibitory effects, reducing pathogen-induced inflammatory responses, improving the epithelial-barrier integrity, and modifying the gut microbiota[133-135]. Research had brought attention to the

potential contribution of cell-wall elements to anti-inflammatory benefits of probiotics[136,137]. *Saccharomyces boulardii*, a well-known probiotic, can be effective in IBD by inhibiting the NF-κB signalling pathway[55]. Among different *Saccharomyces cerevisiae* strains, some also have high nutrient components, of which its positive effect on alleviating inflammation is not widely studied. *S. cerev*isiae strain has potential probiotic quality[138] related to inflammation. *S. cerevisiae* CNCM I-3856 has an antiadhesive property to IBD-related pathogens by a cell wall[48]. One of the yeast cell wall components, mannan has antioxidant activity[139]. *S. cerevisiae* and its cell wall extracts have anti-inflammatory qualities; they have different effects from stain to structure[50]. Mannoproteins and β1,6-glucan are highly branched structures, whereas β1,3-glucan structures are moderately branched. β1,3-Glucan molecules are connected by hydrogen bonding between locally aligned chains. Chitin is found inside, and β1,6-glucan is found outside β1,3-glucan molecules[13]. β-Glucan is the polysaccharide of the *S cerevisiae* cell wall, which modulates immune responses in the host.[140] Polysaccharides have an anti-inflammation effect, and inhibit proinflammatory cytokine, inducible NO synthase, and the NF-κB signaling pathway[141]. β1,3-Glucan obtained from *Agrobacterium* sp. R259 prevents DSS induced IBD by restoring Tregs[116]. *S. cerevisiae* cell surface polysaccharides, which contain mannan/β-1,6-glucan, have potent anti-inflammatory effects on autoimmune diseases[49]. Lentinus edodes β-glucans alleviate colitis in mice by reducing proinflammatory cytokines by

suppressing NF-κB signaling from the activated mitogen-activated protein kinase-peroxisome proliferator-activated receptor-γ (PPAR-γ) pathway[142]. PPAR-γ suppress NF-κB signaling[143], and heme oxygenase (HO-1) controls the neutrophil migration[144]. Using immune cell research, the mechanism through which *S. cerevisiae* stains and cell wall components reduce intestinal inflammation still requires further clarification.

Previous study, the *S. cerevisiae* GILA 118 strain was selected from Korean rice wine based on anti-inflammatory cytokine IL-10 in serum was more upregulated by *S. cerevisiae* GILA 118 than other *S. cerevisiae* GILA strains[145]. This study evaluated which *S. cerevisiae* GILA 118 components alleviate intestinal inflammation more than *S. boulardii* CNCM I-745 in the DSS model. To address the components and intestinal inflammation mechanism, Th17, and Treg cells in the spleen and expression of IBD biomarkers were analyzed in the serum and tissue. Correspondingly, it demonstrates the relationship between the cell wall component of *S. cerevisiae* GILA 118 and the alleviation of intestinal inflammation.

2. Materials and Methods

2-1. The isolation of cell wall component

S. boulardii CNCM I-745 was purchased from Jarrow Formulas. The yeast was used as a control strain for all experiments to compare with a strain of selected yeasts, *S. cerevisiae* GILA 118 in the previous study[145]. The cell walls of *S. cerevisiae* GILA 118 and *S. boulardii* were individually separated from the disrupted of whole cells, cultivated in YPD broth, using the autolysis method[93]. The yeast cells were suspended in DW (pH 5.0, adjusted using 1.0 M HCl), and incubated at 50 °C for 48 h in a shaking incubator with a speed of 120 rpm. The cells were autolyzed by heating at 80 °C for 15 min in a water bath and let the supernatant to cool down to 4 °C. The residual cell walls in the tubes were fractionated from the supernatant by centrifugation at 4,000 rpm, 4 °C for 10 min. The wet pellets of the cell walls were dried at 60°C in a drying oven and stored at 4°C until extraction. The outside, stiff layer of the cell is called the cell wall. It envelops the protoplast and gives the cell a certain form. The cell wall participates in a variety of enzymatic processes. These enzymes take involvement in the breakdown of cell wall elements as well as extracellular components[146].

The autolyzed yeast cells mixed with 1.0 M NaOH solution (Sigma-Aldrich, CA, USA) in the ratio of 1:5 (w/v) were heated at 80 °C by stirring with a magnetic bar for 2 h. Then, the cell walls were precipitated by centrifugation

and suspended in a tube containing a 3-fold volume of DW and thoroughly mixed it. Thorough mixing, the cells were centrifuged to 2 times for washing at 4°C. After washing, the cell pellet was dissolved in 5-fold of 1.0 M HCl (Sigma-Aldrich, USA) incubated at 80 °C in stirrer for 2h. Then the pellet was collected by centrifugation. The obtained cell wall polysaccharide (PS) was washed with water 3 times and dried (Fig. 3-1). The literature data[147] that presented the structural identification and quantitative analysis of each *S. cerevisiae* cell wall fraction served[148] as the foundation for the confirmation of the presence of β-1, 3-glucan, β-1, 6-glucan, and mannoprotein in cell wall polysaccharide (PS). PS was treated with zymolyase 20T (MP biomedical, CA, USA). Zymolyase, an enzyme that hydrolyzes glucose polymers connected by β-1, 3-bonds, is a β-1, 3-glucan laminaripentaohydrolase. Cell wall polysaccharide treated with zymolyase (PZ) has a lower -1, 3-glucan structure than PS due to zymolyase (Fig. 3-1).

To validate the existence of a coating layer on the cell membrane surface, a fourier-transform infrared spectroscopy (FT-IR, VERTEX80v, Bruker, Germany) was used. The comb polymer was identified by its own unique spectrum in the range of 1-10 ppm using a proton nuclear magnetic resonance (1H NMR) using a 600 MHz high-resolution NMR spectrometer (AVANCE 600 FT-NMR, Bruker, Germany).

2-2. The protoplast isolation

Protoplast (PP) isolation from the whole cells followed the method as described previously(Aoyagi, Ishizaka, & Tanaka, 2012). Both the protoplast of *S. cerevisiae* GILA 118 and *S. boulardii* were isolated after the treatment of zymolyase 20T. The zymolyase was diluted in the 1M mannitol containing 100mM 2-mercaproethanol and 0.067M sodium phosphate (pH 7.0), which effectively lyses the cell walls of living yeast cells. To remove the cell wall component, the cells were shaken at 120 rpm for 90 min at 30 °C. After incubation yeast protoplasts (PP) were centrifuged twice with 1.0 M mannitol for washing (Fig. 3-1).

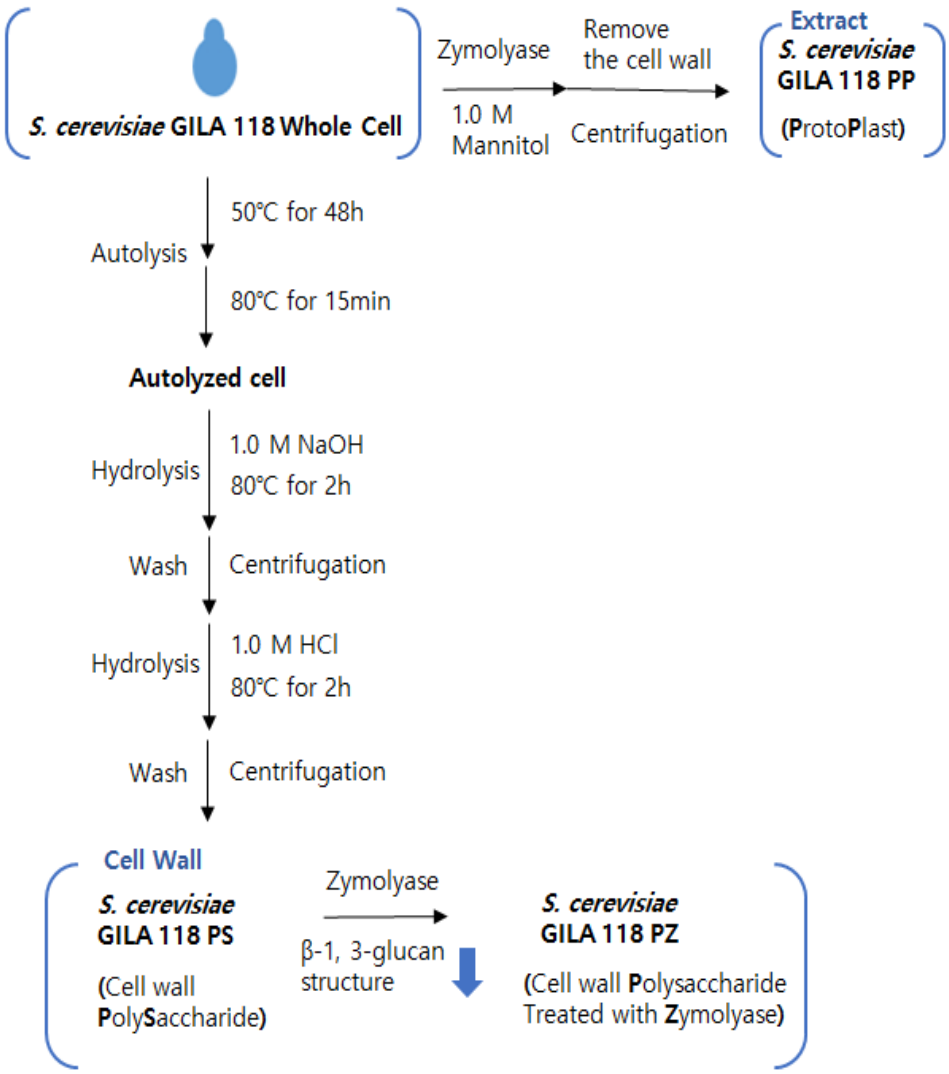


Figure 3-1. Schematic diagram protoplast (PP), cell wall polysaccharide (PS), and cell wall polysaccharide treated with zymolyase (PZ) isolated from *S. cerevisiae* GILA118 whole cell.

2-3. *in vitro* RAW 264.7 cell assay

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 Fetal Bovine Serum (FBS, Gibco, USA), and 1% Antibiotic-Antimycotic (Anti-Anti, Gibco, USA) at 37 ℃ in a 5 % CO2 atmosphere. RAW 264.7 cells were seeded at 1×10^5 cells/ml in 24-well plates and stabilized for 2 h.

S. boulardii CNCM I-745, *S. cerevisiae* GILA118 PS, PZ, and PP 250 μg/mL were treated to 50 μL with 450 μL of lipopolysaccharides (LPS, 1 μg/mL; Sigma-Aldrich, USA).

The cells were then centrifuged at 600 x g for 10 min at 4 °C, and the cell supernatant was transferred in new tubes. According to the manufacturer's instructions, the Griess reagent (Promega Inc., USA) was used to quantify the concentration of nitric oxide (NO). A microplate reader (SpectraMax M4 Microplate/Cuvette Reader, Molecular Devices, USA) was used to measure the absorbance at 540 nm after mixing the cell supernatant and Griess reagent in equal amounts and incubating the combination for 10 min at room temperature.

2-4. *in vivo* Experimental Design for Yeast cell components

Six-week-old female C57BL/6J mice were purchased from Daehan BioLink Co., Ltd., Korea. Mice were randomly distributed into 8 groups (n = 10 per group). After one week of stabilization, the mice were treated with 1.5% dextran sulfate sodium (DSS, MW 36,000–50,000; MP Biomedicals, USA) in distilled water with *S. boulardii* CNCM I-745 and *S. cerevisiae* GILA118 cell wall PS, PP, PZ (5mg/kg)[110,149] once a day for 12 days, followed by 3 days of recovery. The mice from the administered *S. boulardii* and *S. cerevisiae* GILA118 PS, PP, PZ groups were injected orally. For the *in vivo* experiment, this study was carried out in accordance with the guidelines by the Korean Association for Laboratory Animals, and the protocol was approved by the Institutional Animal Care and Use Committee of Seoul National University (Approval No. SNU-210322-3-2).

2-5. qRT-PCR and ELISA

Macrophage RAW 264.7 cells and colon tissue in Buffer RLT were well homogenized. After disrupting the cell RNA isolated by RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) protocol. cDNA was synthesized by the PrimeScipt RT reagent Kit (Takara Korea Biomedical Inc.,Seoul, Korea) protocol. Gene amplification was done by the iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) protocol. Data were normalized with the housekeeping β-actin expression level. The primers used are listed in Table 3-1.

Table 3-1. List of primer sequences

Primer	Sequence	Reference
β-Actin (F)	TCCATCATGAAGTGTGACGT	Yin et al.[89]
β-Actin (R)	GAGCAATGATCTTGATCTTCAT	
Muc-2 (F)	GTGCTGCAATATCACCTCATGT	Floyd et al.[90]
Muc-2 (R)	TGTATGTGATGGAGCCTGAAAC	
ZO-1 (F)	CCACCTCTGTCCAGCTCTTC	Liu et al.[91]
ZO-1 (R)	CACCGGAGTGATGGTTTTCT	
Occludin (F)	CCTCCAATGGCAAAGTGAAT	
Occludin (R)	CTCCCCACCTGTCGTGTAGT	
PPAR-γ (F)	GCCTCCCTGATGAATAAAGATG	Dou et al.[150]
PPAR-γ (R)	AGGCTCCATAAAGTCACCAAAG	
COX-2 (F)	CAGACAACATAAACTGCGCCTT	Kallassy et al.[151]
COX-2 (R)	GATACACCTCTCCACCAATGACC	

Macrophage RAW 264.7 cells supernatants and serum were quantified by ELISA kits (R&D Systems, Minneapolis, MN, USA) for proinflammatory cytokine, according to the manufacturer's instructions. Colon tissues were also quantified by ELISA kits (Abcam, Cambridge, MA, USA) for PPAR-γ and HO-1 according to the manufacturer's instructions.

2-6. Histology

The paraffin-embedded and stained the distal colon tissues with hematoxylin and eosin (H&E) after fixing the samples in 10% buffered formalin phosphate (Sigma-Aldrich, St. Louis, MO, USA).

A previously published criterion[152] was used to calculate the histological score (Table 3-2).

Table 3-2. Scoring scheme for DSS-induced colonic inflammation.

Severity	**Extent**		**Epithelial changes**	**Mucosal architecture**	
Mild	Mucosa	1	Focal erosions		1
Moderate	Mucosa and submucosa	2	Erosions	± Focal ulcerations	2
Marked	Transmural	3		Extended ulcerations ± granulation tissue ± pseudopolyps	3

Sum of scores severity and epithelial changes: 0-6

2-7-1. Isolation of Single cell

Mice were euthanized by CO_2 asphyxiation. Spleen, mesenteric lymph node (mLN) and Peyer's Patches (PPs) were isolated and rinsed with PBS. Then, they were placed on the 70μm cell strainer (BD Biosciences, San Jose, CA, USA) and ground using the back of the syringe to make single cells. Splenocyte were depleted using ACK-lysis buffer and complete media (RPMI-1640 medium with FBS, Abx, HEPES, Sodium pyruvate, 2-Mercaptoethanol and Gentamicin) were added. The cells were stained with the antibodies labeled with fluorescence and examined by using flow cytometry (FACScanto II, BD Biosciences).

2-7-2. Phenotypic and Functional Examination of Immune Cells by Using Flow Cytometry

For the analyzed of regulatory T cells, single cells were stained with anti-mouse CD44-FITC, CD25-PE-cy7, live/dead-APC Cy7, Foxp3-APC, CD8α-PB, amcyan-CD3 (all from BD Biosciences), and CD4-PE (Biolegend, San Diego, MA, USA). After surface staining in a 96 well plate, $CD4^+$ T cells were fixed and stained with anti-mouse Foxp3-APC mAb (BioLegend, San Diego, MA, USA) using Foxp3 Fix/Perm Buffer Set (BioLegend). For *In vivo* examination, spleen, mLN, and PP were isolated from the mice and single cells were prepared.

To examine the subpopulation of $CD4^+$ T cells, splenocytes, and mononuclear cells from mLN and PP were stimulated with 50 ng/ml of phorbol 12-myristate-13-acetate (PMA) and 750 ng/ml of ionomycin (Sigma-Aldrich) in the presence of brefeldin A (Sigma-Aldrich) for 5 h. Then, the cells were stained with appropriate combination of anti-mouse IFN-γ-PE-cy7 (BD), IL-4- APC (tonbobio., San Diego, CA, USA), and IL-17-PerCP-cy mAb (BD Biosciences).

To examine the neutrophil, splenocytes were stained with anti-mouse Ly6G-PE, CD11b-PE-cy7, CD64-PercP Cy5.5 Live/Dead-APC Cy7, F4/80-PB, Amcyan-CD3 (all from BD Biosciences). The cells were washed, and the expression of fluorescence was examined using a FACS-Canto II (BD Biosciences). All flow cytometric data acquired were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

2-8-1. DNA extraction from the fecal sample

The fecal were sampled through dissection after sacrifice. DNA extraction was followed the manufacturer's protocol ZymoBIOMICS^{TM} DNA Miniprep kit (Zymo Research Corp, USA).

2-8-2. Gut microbiota analysis

PCR was used to amplify the template in the DNA sample using 16S V3 and V4 region specific primers with overhang adapters. Amplification was carried out using a 2x KAPA HiFi HotStart Ready Mix (Roche, Switzerland) and the PCR conditions were as follows 94 ℃ for 3 min; followed by 25 cycles at 95 ℃ (30 s), 55 ℃ (30 s), and 72 ℃ (30 s); and a final extension step at 72 ℃ for 3 min. Amplicons were purified from free primers and PCR materials using AMPure XP beads (Beckman Coulter, USA). Then, the amplicons were then attached with the Illumina sequencing adapters to construct DNA libraries using the Nextera XT index kit (Illumina, USA). and the PCR conditions were as follows 95 ℃ for 3 min; followed by 8 cycles at 95 ℃ (30 s), 55 ℃ (30 s), and 72 ℃ (30 s); and a final extension step at 72 ℃ for 5 min. The library was cleaned up using AMPure XP beads before quantification. Finally, the libraries were normalized and pooled, and sequenced by Macrogen (Seoul, Korea). Following sequencing, raw data reads were demultiplexed and given to the appropriate samples using previously assigned barcode pairs that are specific to each sample. A fully modular R-based pipeline (Rhea) was created for the analysis of microbial

profiles for the downstream processing of intermediate files produced by IMNGS.

2-9. Statistical analysis

The results are the means ± standard deviations of triplicate analysis. Results were analyzed to ANOVA using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Differences were considered significant at error probabilities smaller than 0.05.

3. Results

3-1. *S. cerevisiae* GILA 118 cell wall component administration reduced inflammatory biomarkers in murine macrophage RAW 264.7 cells

Murine macrophage cell line, RAW 264.7 cells initiate inflammatory responses when treated with lipopolysaccharides (LPS)[153]. This study evaluated which *S. cerevisiae* GILA 118 component affects murine macrophage cells stimulated by LPS. NO, cyclo-oxygenase-2 (COX-2), and TNF-α expression significantly increased in the LPS group compared to that in the mock group. However, NO expression significantly decreased in the *S. cerevisiae* GILA 118 component group (PS, PP, PZ) compared to that in the LPS group (Fig. 3-2A). Moreover, PPAR-γ gene expression significantly increased compared to that in the LPS group whereas COX-2 gene expression in RAW 264.7 cells significantly decreased compared with that in the LPS group (Fig. 3-2B, C). *S. cerevisiae* GILA 118 component increased PPAR-γ gene expression by decreasing NO and COX-2 (Fig. 3-2A, C). Moreover, TNF-α significantly decreased in GILA 118 cell wall component group (PS, PZ) compared to that in the LPS group (Fig. 3-2D). As a result, the *S. cerevisiae* GILA 118 cell wall component could have anti-inflammatory effects.

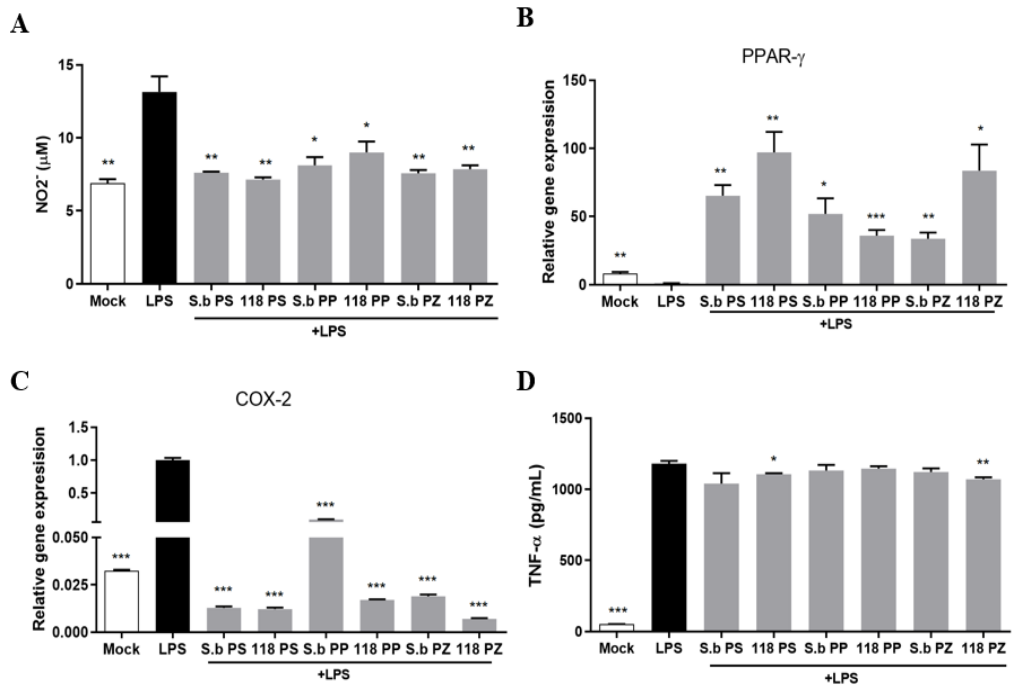


Figure 3-2. The anti-inflammatory effect of *S. cerevisiae* GILA 118 cell wall component on RAW 264.7 cells. (A) NO inhibitory effects. (B) Relative gene expression of PPAR-γ and (C) COX-2. (D) Analysis of proinflammatory cytokine TNF-α. **P* <0.05, ***P* <0.01, and ****P* <0.001, compared with treatment of only LPS.

3-2. *S. cerevisiae* GILA 118 PZ administration alleviated intestinal inflammation in mice compared to PS and PP

To determine which *S. cerevisiae* GILA 118 component alleviates intestinal inflammation, DSS colitis mice were treated with or without the *S. cerevisiae* GILA 118 component. The body weight change of the GILA 118 PS and PZ groups was higher than that of the DSS group (Fig. 3-3A). GILA 118 PZ groups were significantly ($p < 0.05$) lower than the DSS treatment group when compared with the DAI score (Fig. 3-3B). Colon length/body weight ratio (Fig. 3-3C), and spleen/body weight ratio (Fig. 3-3D), were also alleviated in the GILA 118 PZ group compared to those in the DSS group. Gene expression of mucin-2, occludin, and ZO-1 increased in the GILA 118 PS and PZ groups compared to that in the DSS group. In particular, the GILA 118 PZ group showed significantly higher mucin-2 and occludin gene expression than the DSS group (Fig. 3-3E). The results showed GILA 118 PZ groups were significantly different to the DSS group in DAI score (Fig. 3-3B), colon length/body weight ratio (Fig. 3-3C), spleen/body weight ratio (Fig. 3-3D), Gene expression of mucin-2 and occludin (Fig. 3-3E).

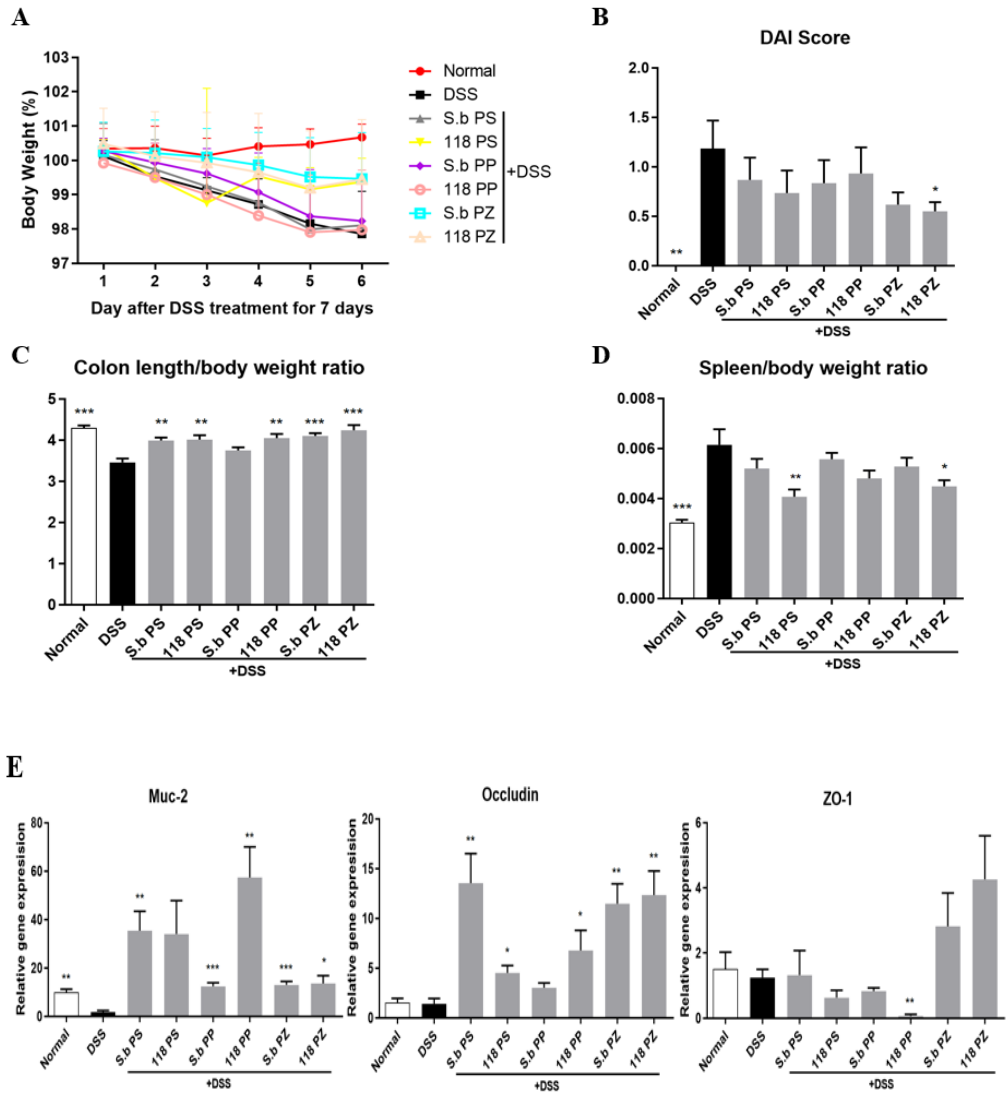
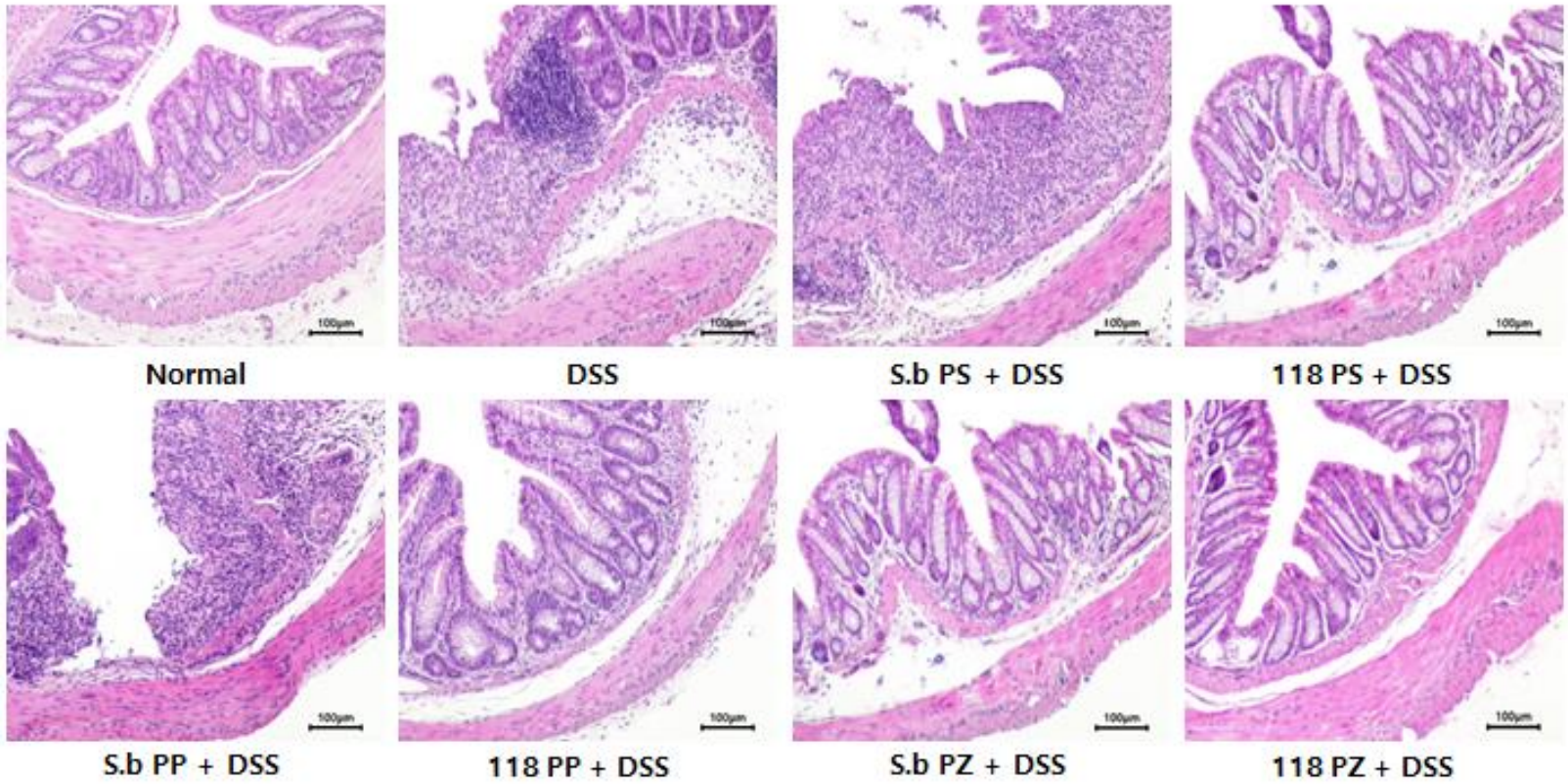


Figure 3-3. *S. cerevisiae* GILA 118 PZ alleviated intestinal inflammation in mice compared to PP and PS. (A) Loss of body weight (%) for 6 days. (B) DAI score of disease from C57BL/6J mice. (C) Colon length and (D) spleen weight with body weight rates were compared to that of the DSS group. (E) Relative gene expression of Muc-2, occluding, ZO-1 in colon tissue. **P* <0.05, ***P* <0.01, and ****P* <0.001, compared with treatment of only DSS.

Consequently, compared to GILA 118 PS and PP, the GILA 118 PZ is more potent at alleviating intestinal inflammation. In the histological analysis, the DSS group showed pathology develops severely with disappearance of crypt structure (Fig. 3-4A). On the other hand, the distal colon in GILA 118 PS and 118 PZ groups showed a relatively intact colonic architecture compared to the DSS group (Fig. 3-4B).

A


Normal
DSS
S.b PS + DSS
118 PS + DSS
S.b PP + DSS
118 PP + DSS
S.b PZ + DSS
118 PZ + DSS

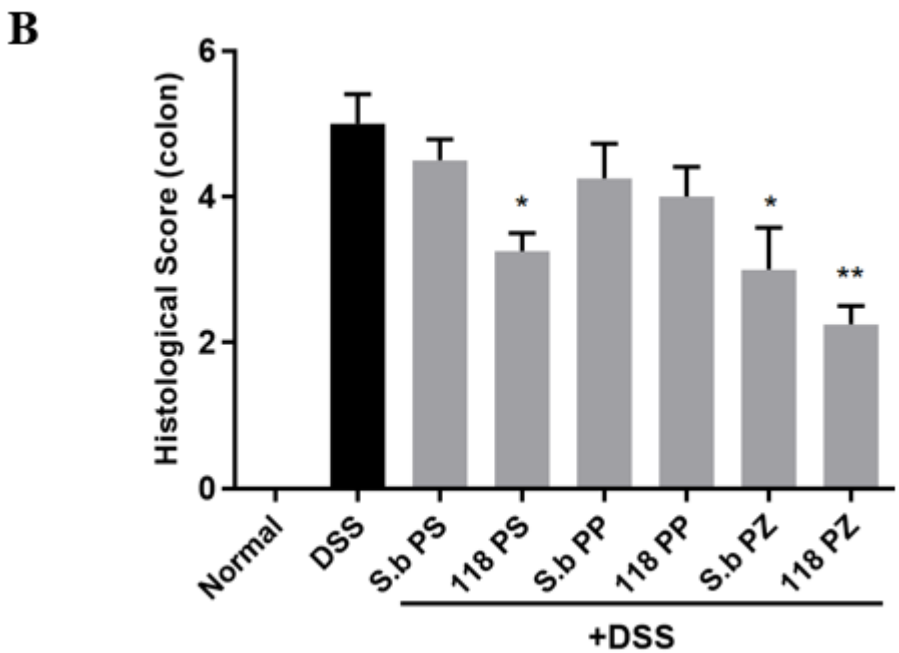
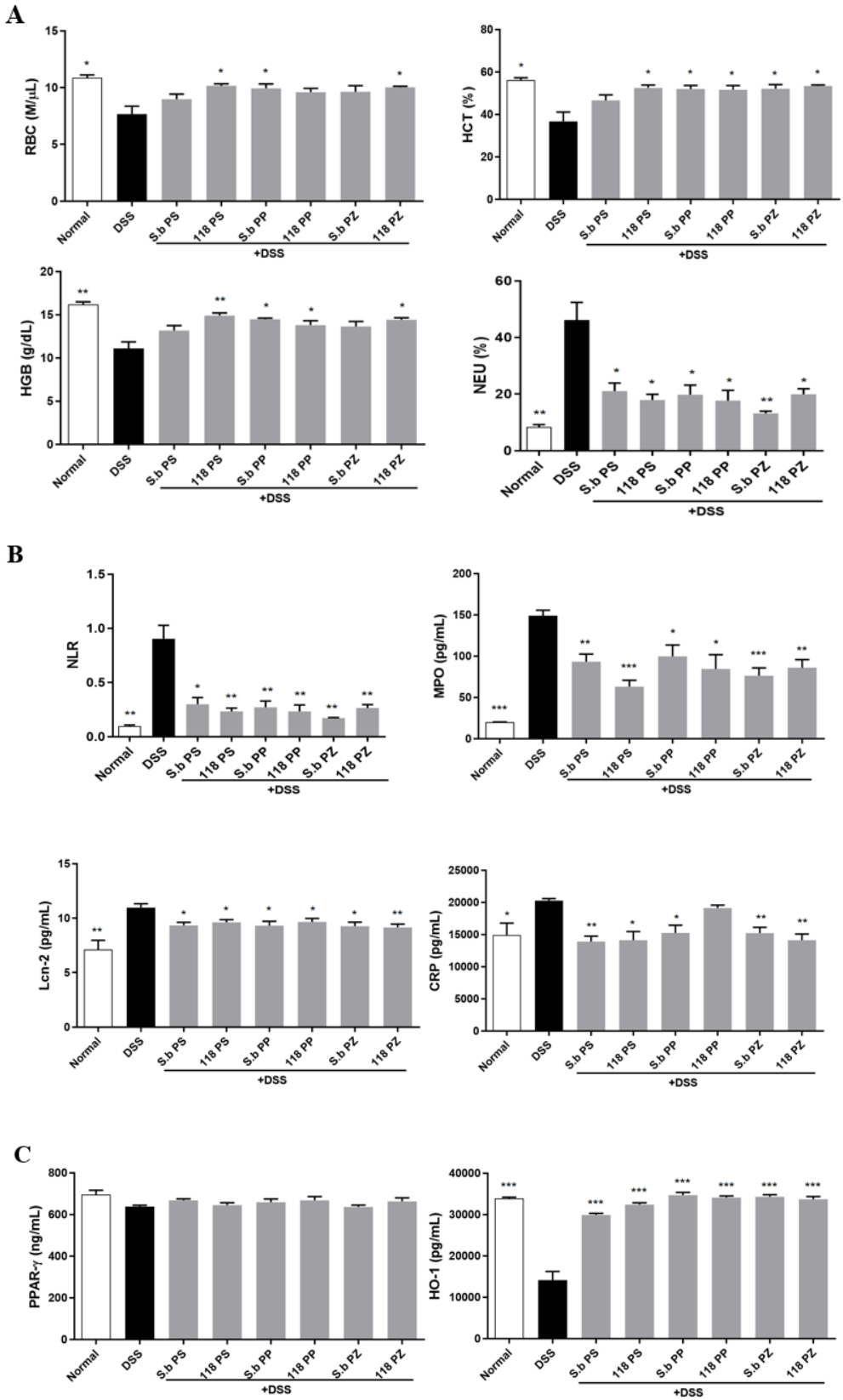


Figure 3-4. *S. cerevisiae* GILA 118 PZ alleviated intestinal inflammation in distal colon in DSS-induced colitis mice (A) Histological architecture. Original magnification, 20X. (Scale bars, 100μm) (B) Histological Score. * P <0.05, and ** P <0.01, compared with treatment of only DSS.

3-3. *S. cerevisiae* GILA 118 PZ administration alleviated intestinal inflammation in mice compared to PS and PP by increasing IL-10

Red blood cell, hematocrit, and hemoglobin levels were significantly low in the DSS group possibly due to intestinal bleeding[154]. Compared to those in the DSS group, red blood cell, hematocrit, and hemoglobin levels were significantly increased in the GILA 118 PS and PZ groups (Fig. 3-5A). Neutrophil and NLR levels were significantly decreased in the GILA 118 PS, PP and PZ groups (Fig. 3-5A, B). Neutrophil-associated molecules namely, MPO, Lcn-2, and C-reactive protein (CRP)[30,155,156] were used as biomarkers to evaluate the severity of intestinal inflammation. MPO and Lcn-2 are secreted more consistently by neutrophils that have been activated by inflammation. As a result, this condition may serve as a quantitative indicator of the severity of colitis. MPO, Lcn-2, and CRP showed a significant decrease in GILA 118 PS and PZ groups compared to those in the DSS groups (Fig. 3-5B). PPAR-γ expression in colon tissue showed no difference, but HO-1 was significantly increased in GILA 118 PS, PP and PZ group (Fig. 3-5C). These results suggested that HO-1 contributes to the alleviation of intestinal inflammation in the colon. Proinflammatory cytokine (TNF-α, IL-6, and IL-17) levels in serum were also significantly lower in the GILA 118 PS and PZ groups than in the DSS-treated groups. IL-10 in serum was significantly increased in the *S. cerevisiae* GILA 118 PZ group than in the other groups (Fig. 3-5D). These results indicated that *S.*

cerevisiae GILA 118 PZ alleviates intestinal inflammation, especially IL-10 cytokine level. Results showed that *S. cerevisiae* GILA 118 cell component alleviated the activated neutrophils and reduced the secretion of inflammation biomarkers MPO, Lcn-2, and CRP.


A
RBC (M/μL)
HCT (%)
HGB (g/dL)
NEU (%)
B
NLR
MPO (pg/mL)
Lcn-2 (pg/mL)
CRP (pg/mL)
C
PPAR-γ (ng/mL)
HO-1 (pg/mL)
Normal
DSS
S.b PS
118 PS
S.b PP
118 PP
S.b PZ
118 PZ
+DSS

D

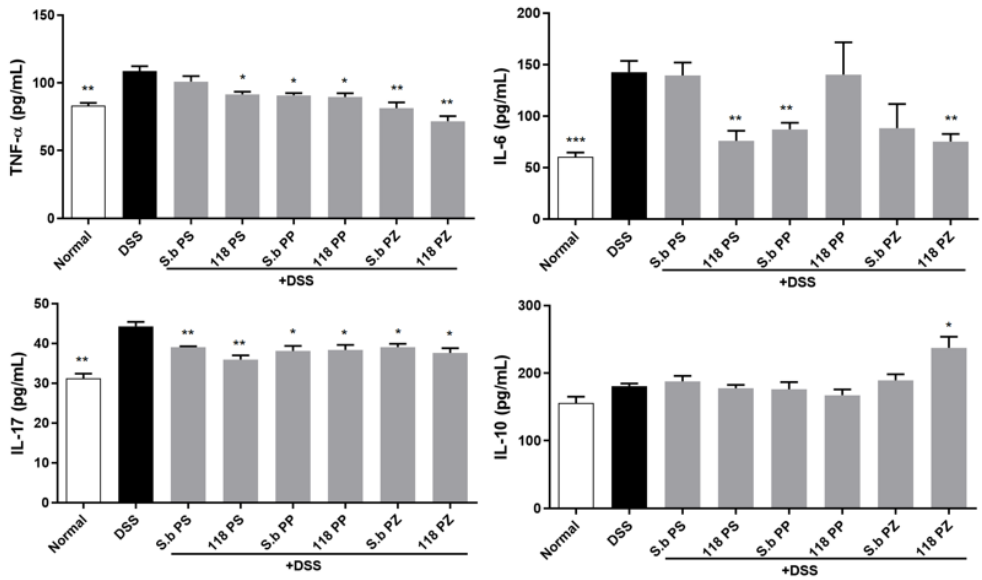


Figure 3-5. *S. cerevisiae* GILA 118 PZ alleviated the intestinal inflammation in mice and increased IL-10 in serum. (A) Complete blood counts (CBC) in whole blood. (B) Analysis of CRP in serum, and Lcn-2, and MPO in fecal. (C) Amount of PPAR-γ and HO-1 in colon tissue. (D) Analysis of proinflammatory cytokines TNF-α, IL-6, and IL-17, and anti-inflammatory cytokine IL-10 in serum. **P* <0.05, ***P* <0.01, and ****P* <0.001, compared with treatment of only DSS.

3-4. *S. cerevisiae* GILA 118 PZ shown more glucose residue than *S. cerevisiae* GILA 118 PS

Fourier transform infrared and Proton nuclear magnetic resonance structure analysis showed similar results for *S. cerevisiae* GILA 118 PS and PZ. No differences in the peaks of C–C and C–H stretching were observed. However, C–O stretching was decreased in *S. cerevisiae* GILA 118 PZ (Fig. 3-6). These results suggested that zymolyase induces a C–O bond cut and cuts down the β-1,3 glucan bond to activate β-1,3 glucanase[157,158]. Based on FTIR data, H NMR shown more glucose residue(1 to 5 ppm) with the proton in *S. cerevisiae* GILA 118 PZ than 118 PS (Fig. 3-6).

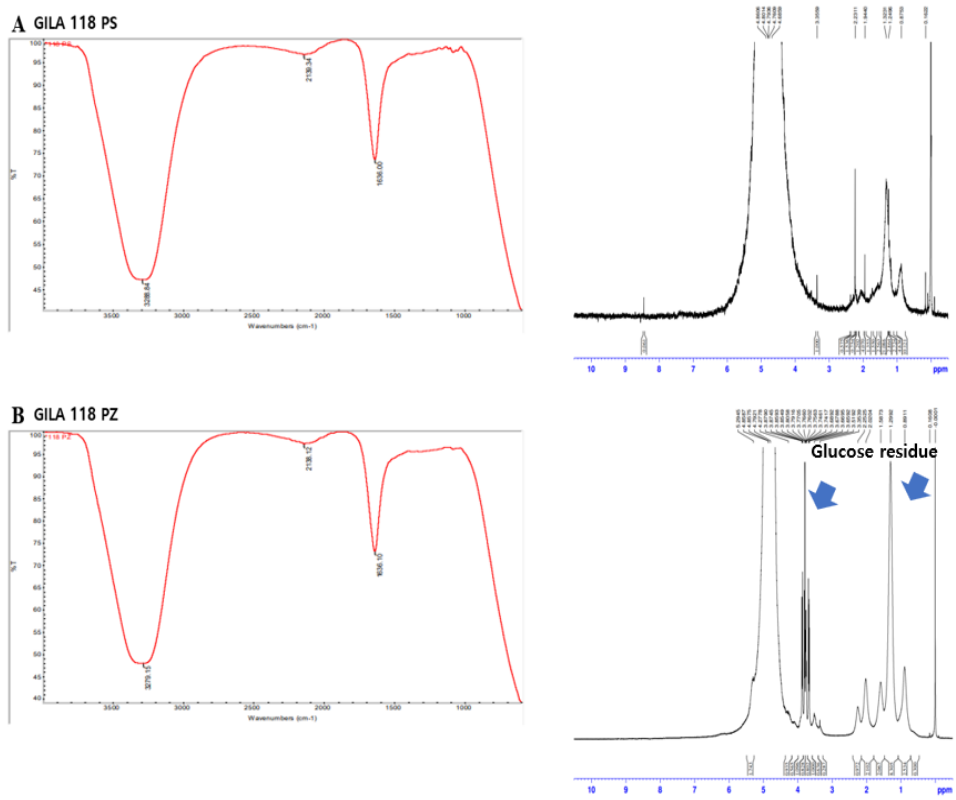
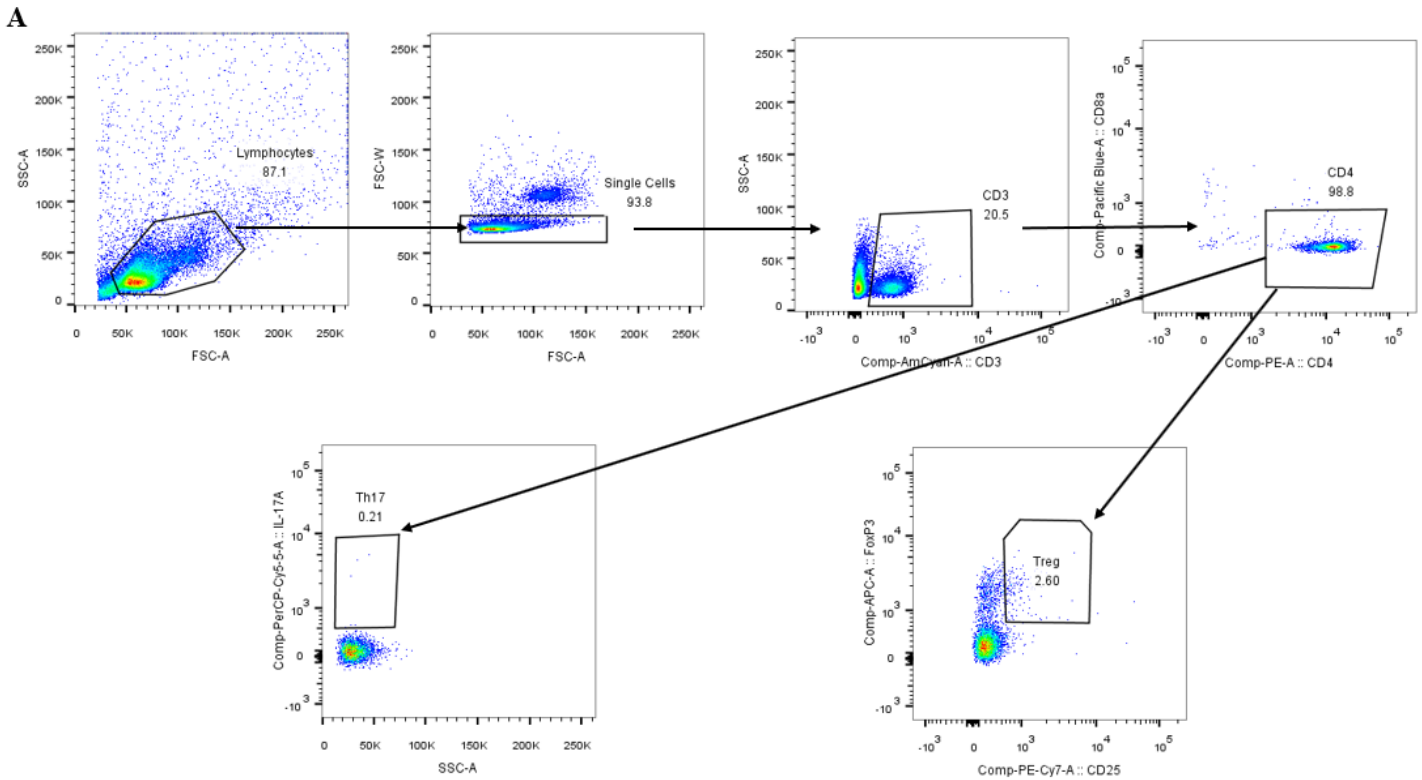


Figure 3-6. The structure of *S. cerevisiae* GILA 118 PS (A) and PZ (B) using FT-IR and H NMR.

3-5. *S. cerevisiae* GILA 118 PZ administration alleviated intestinal inflammation by regulating the Th17/Treg cell balance in spleen

Naive CD4 T cells undergo differentiation into $CD4^+$ T cell subsets, producing a characteristic set of cytokines[159]. $CD4^+$ T cell subpopulations in the spleen were obtained using the gating strategy to determine whether *S. cerevisiae* GILA 118 PZ affects systemic T cell responses (Fig. 3-7A). The Treg cell proportion in $CD4^+$ T cells was significantly increased in *S. cerevisiae* GILA 118 PZ (Fig. 3-7B). The results for the spleen were correlated with those for serum cytokine IL-10. Other subtypes of helper T cells were also examined by testing the intracellular IL-17 expression in $CD4^+$ T cells. The number of Treg cells was higher in the spleen of the DSS mice compared with that in the mice administered with *S. cerevisiae* GILA 118 PZ (Fig. 3-7C). This study examined the population changes of IL-17-producing $CD4^+$ T cells and Treg rate in the spleen of DSS mice administered with *S. cerevisiae* GILA 118 PZ. The IL-17 levels in $CD4^+$ T cells and the Treg population rate in the spleen were rebalanced to similar levels in normal mice (Fig. 3-7D).


A
SSC-A
FSC-A
Lymphocytes
87.1
FSC-W
FSC-A
Single Cells
93.8
SSC-A
Comp-AmCyan-A :: CD3
CD3
20.5
Comp-Pacific Blue-A :: CD8a
Comp-PE-A :: CD4
CD4
98.8
Comp-PerCP-Cy5-5-A :: IL-17A
SSC-A
Th17
0.21
Comp-APC-A :: FoxP3
Comp-PE-Cy7-A :: CD25
Treg
2.60

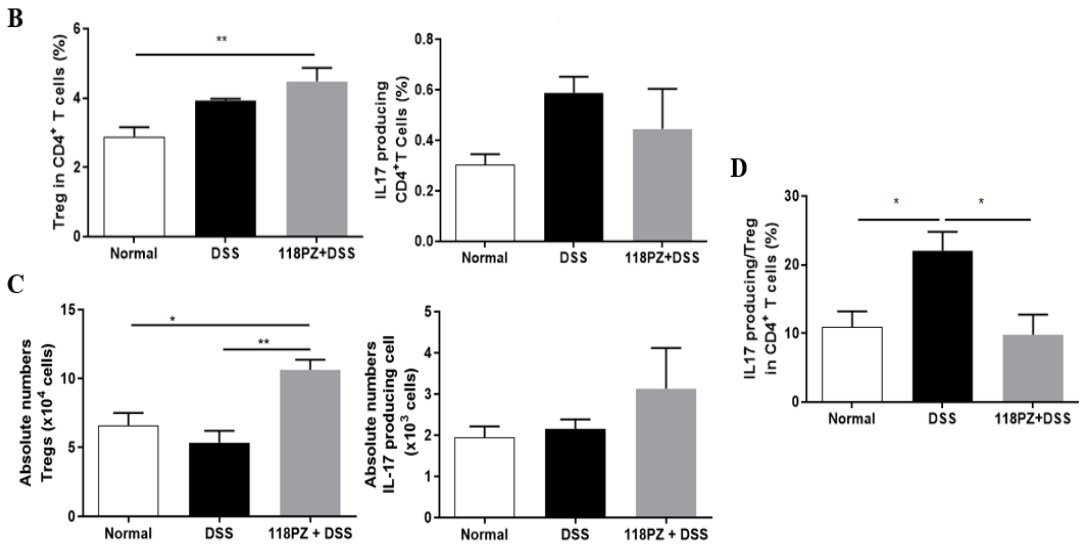


Figure 3-7. *S. cerevisiae* GILA 118 PZ alleviated the intestinal inflammation by regulating Th17/Treg cell balance. (A) Gating strategy for $CD4^+$ T cell subtypes (B) Percentage of Treg and IL-17 producing cells from the spleen in $CD4^+$ T cells. (C) Absolute numbers of Treg and IL-17 producing cells from the spleen. (D) Percentage of IL-17 producing cells/Treg from the spleen in $CD4^+$ T cells. $*P < 0.05$, and $**P < 0.01$

3-6 Comparison of the influence to the gut microbiota on GILA 118 live cells and PZ in mouse feces

DSS colitis-associated bacterial taxa and their relative abundances were proposed for fecal samples from the class to the genus level (Fig. 3-8-1). In particular, a higher relative abundance of the family *Akkermansiaceae* and genus *Akkermansia* were observed in the DSS treated group than other group, however a lower relative abundance of the family *Lactobacillaceae* and genus *Lactobacillus* were observed in the DSS treated group than other group. Yeast treatment groups shown similar taxonomic binning at class, order, family and genus level with normal group.

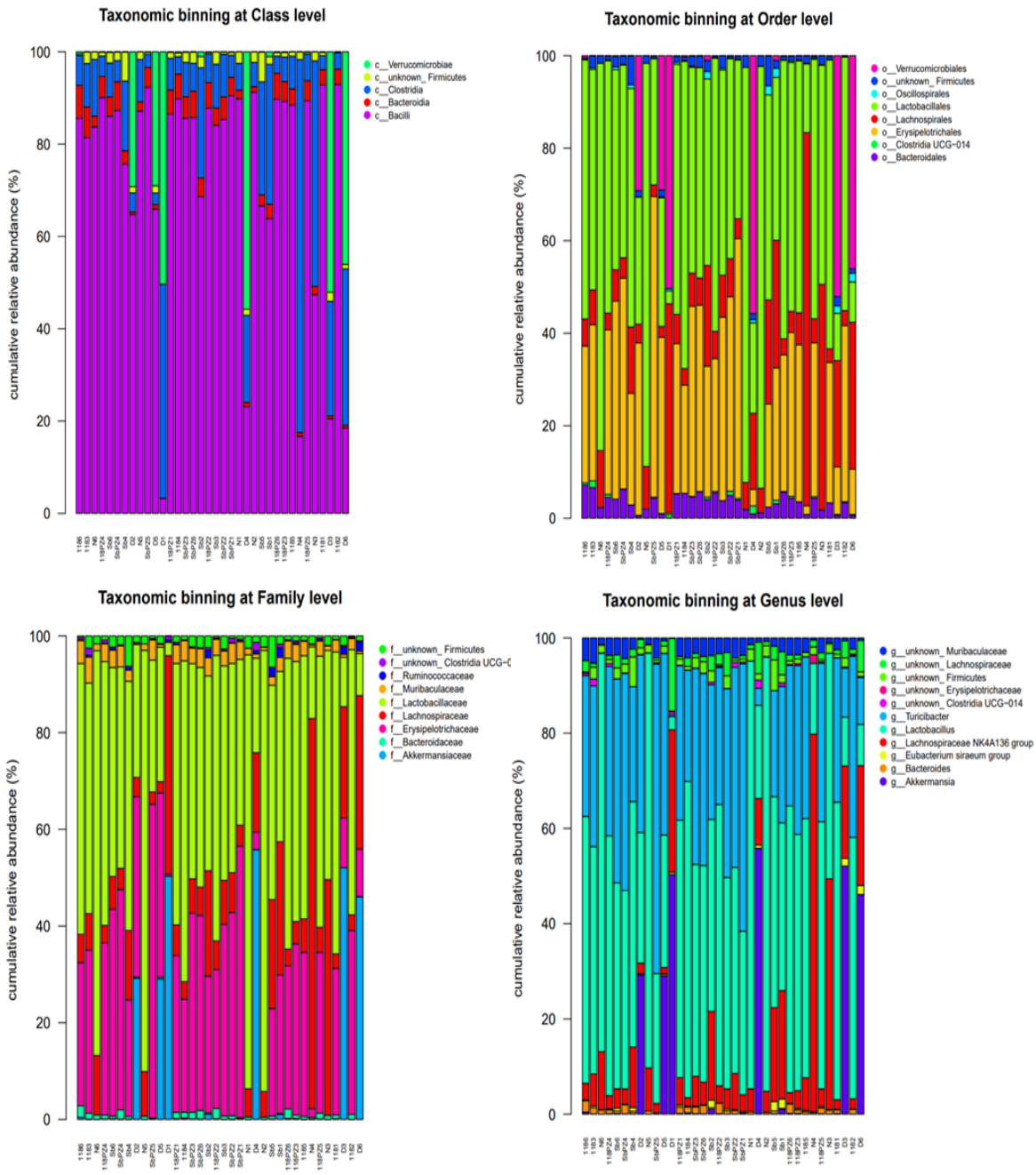


Figure 3-8-1. GILA 118 live cells and PZ-associated bacterial taxa in mouse feces

Akkermansia were significantly increase in the DSS group than the other group, whereas *Bacteroides* were significantly increase in the normal, 118, 118 PZ group than the DSS group. Although *Clostridia*, *Turicibacter* were not shown in the normal group. *Eubacterium siraeum, Firmicutes* were not significantly ($p > 0.05$) different with each group. *Lacchospiraceae* were significantly decrease in the normal, 118 and 118 PZ group than the DSS group. *Lactobacillus* and *Muribaculaceae* were significantly increase in the yeast treatment group than the DSS group, especially *Lactobacillus* were significantly increase in 118 and 118 PZ groups.

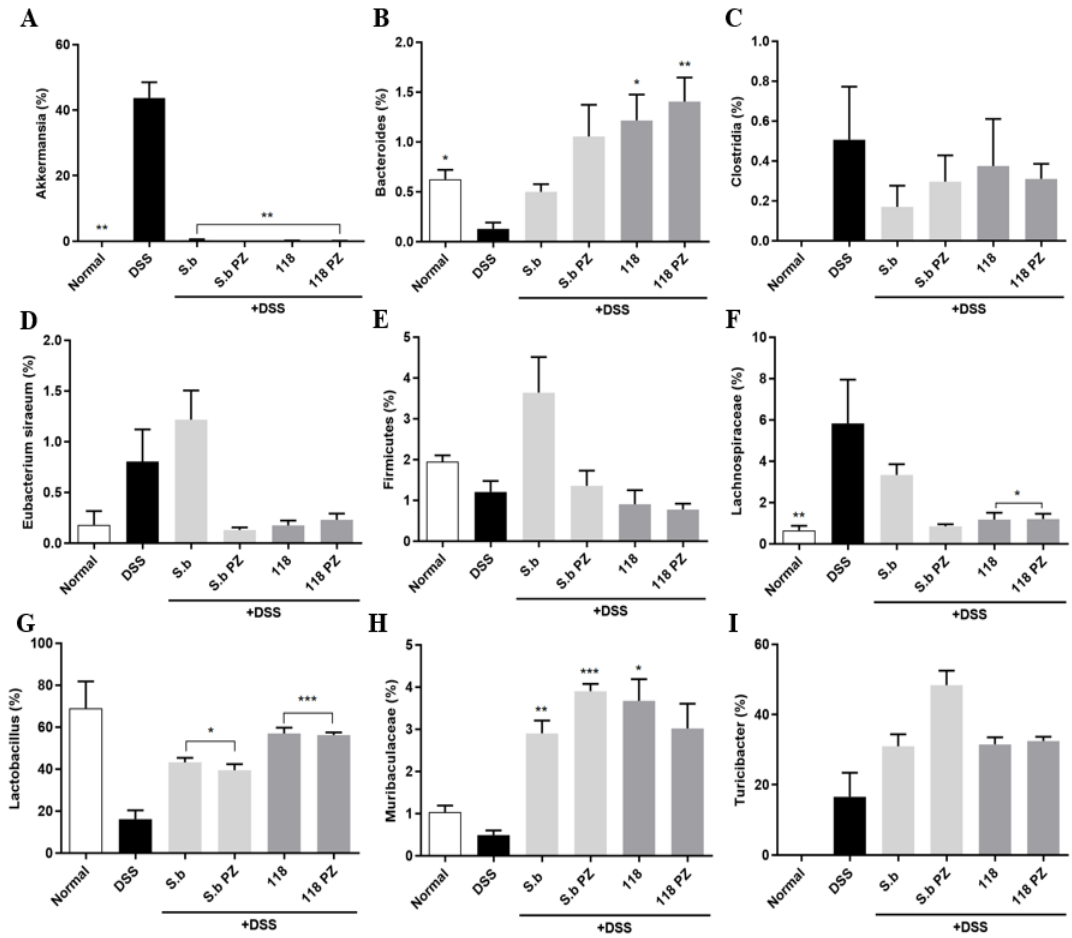


Figure 3-8-2. Relative abundance of specific genus modulated after live cells and PZ administration. (A) *Akkermansia*, (B) *Bacteroides*, (C) *Clostridia*, (D) *Eubacterium siraeum*, (E) *Firmicutes*, (F) *Lacchospiraceae*, (G) *Lactobacillus*, (H) *Muribaculaceae*, (I) *Turicibacter*.

Statistical significance is indicated as follows: **p* <0.05, ***p* <0.01 and ****p* <0.001, compared with treatment of only DSS.

Alpha diversity by 16S amplicon sequencing data within each fecal sample was suggested by eight indices. The richness showed a tendency to increase in DSS group. Meanwhile, 118 PZ group showed a decrease in effective richness than Sb. Evenness and Simpson showed similar results. In the case of Shannon index and Shannon effective analysis, 118 group showed a significantly decrease than S.b group. Yeast administration changes the diversity of gut microbiota.

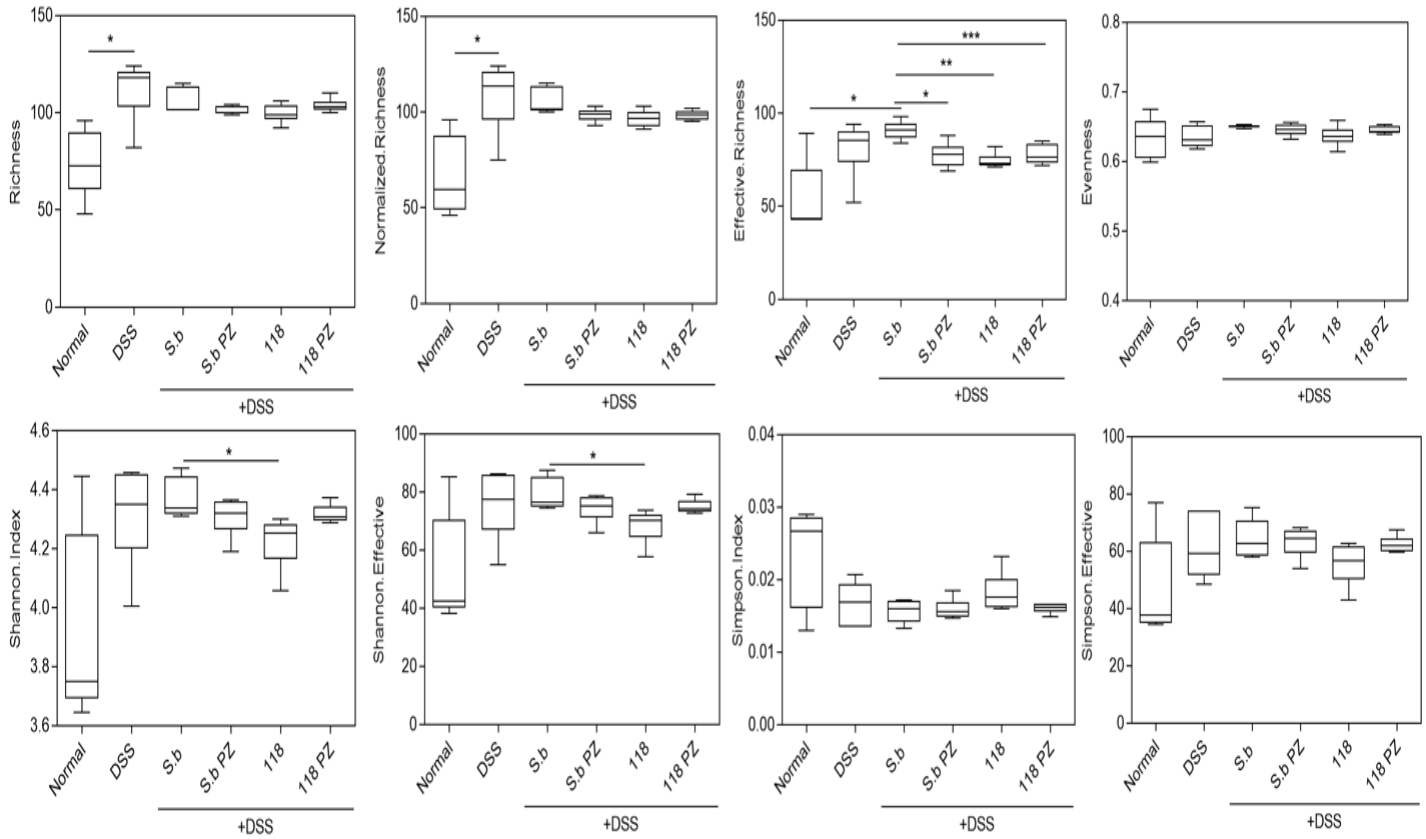


Figure 3-8-3. Alpha diversity after live cells and PZ administration. Statistical significance is indicated as follow: **P* <0.05, ***P* <0.01, and ****P* <0.001

Beta diversity of tested groups show the difference and heterogeneity of OTUs across groups. The PERMANOVA test showed that microbial communities of yeast groups were separated significantly from the normal and the DSS group. Each group also showed clear clustering.

Microbiota modulation effect of *S. cerevisiae* GILA118 and GILA118 PZ were observed in DSS-induced colitis mice model, however group were not separation between GILA118 and GILA118 PZ.

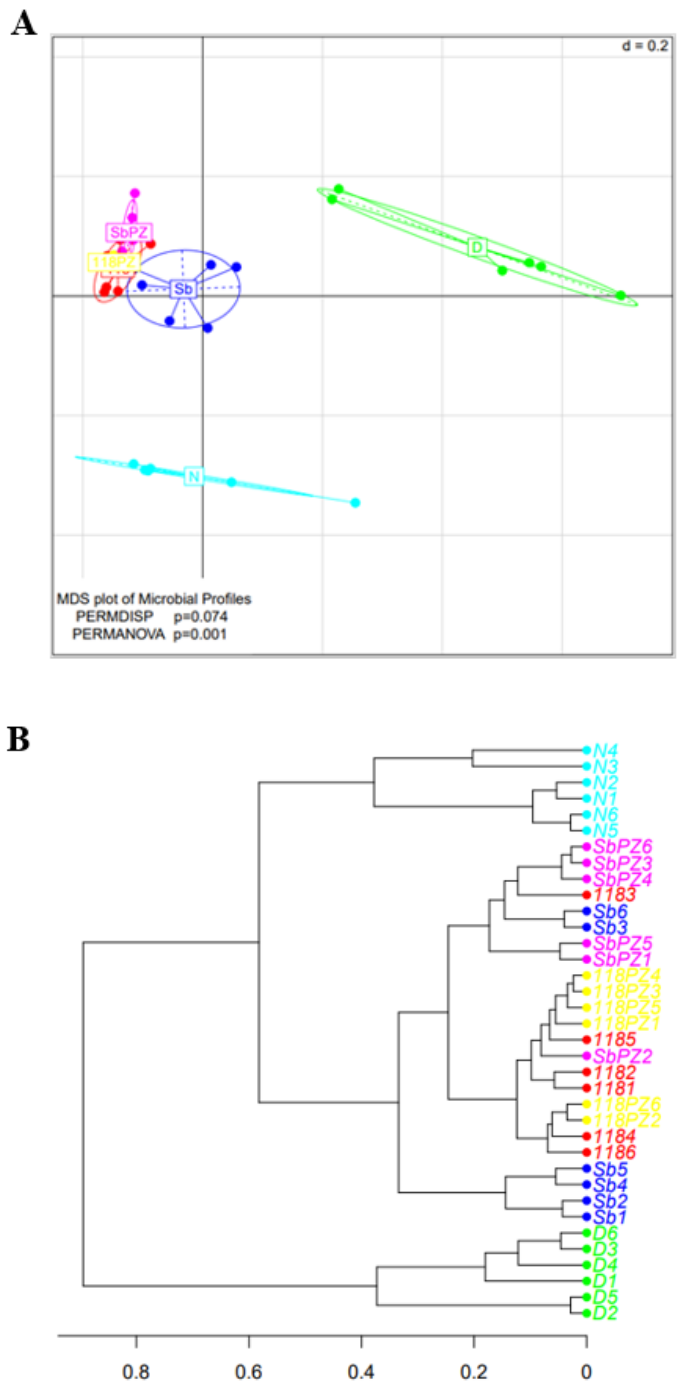
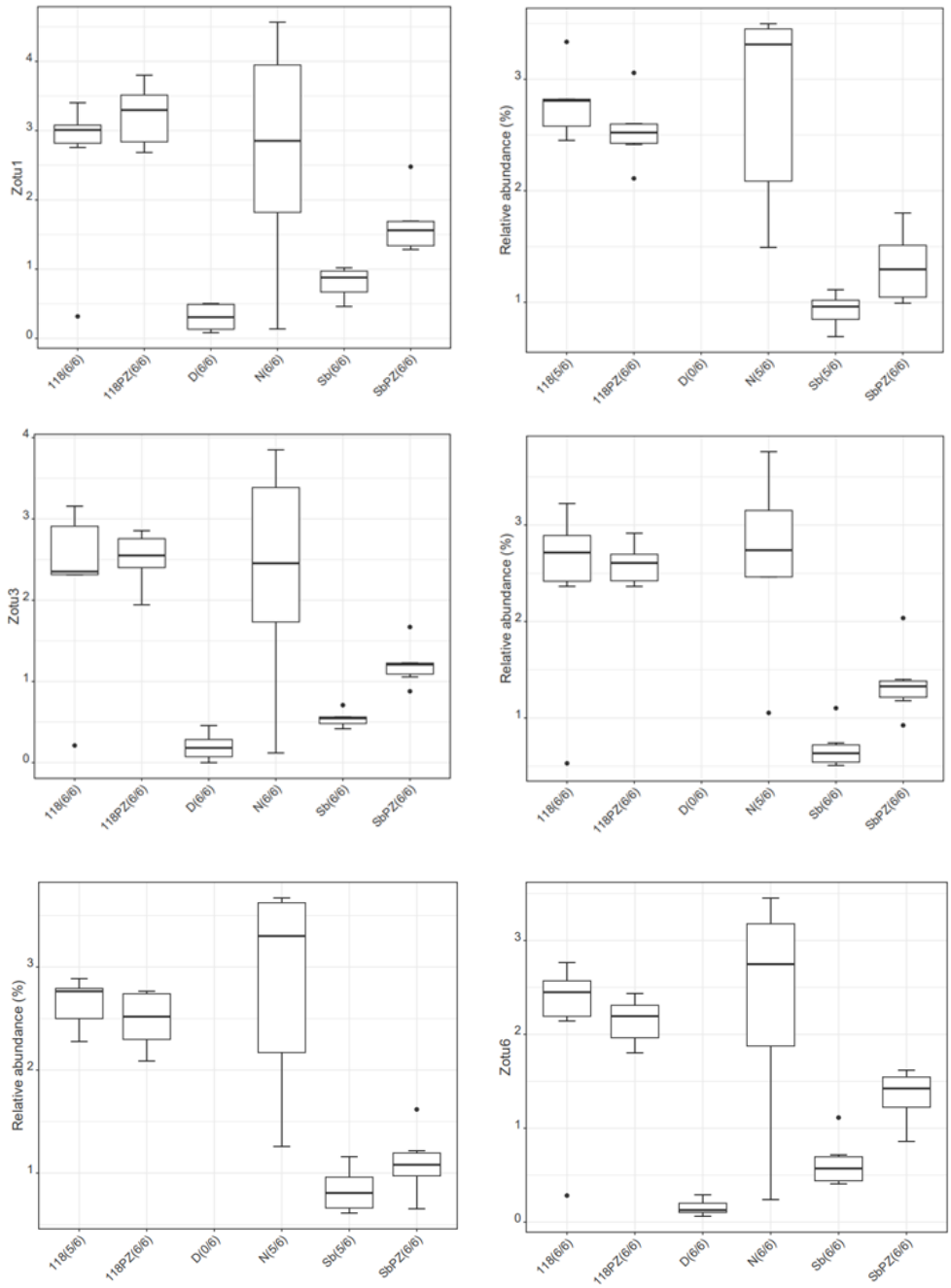


Figure 3-8-4. Beta diversity of tested groups. (A) Multi-dimensional scaling (MDS) and (B) phylogram. Normal (sky blue), DSS (green), Sb (blue), SbPZ (pink), 118 (red), 118PZ (yellow).

Lactobacillus was significantly increase in 118 and 118 PZ groups than the DSS group (Fig. 3-8-2). Figure 3-8-5 show *Lactobacillus johnsonii*, *Lactobacillus animalis*, and *Lactobacillus murinus* showed significantly increase in 118 and 118 PZ groups than the DSS group. These results indicated cell wall components (118 PZ) as well as live cell modulate gut microbiota by increase *Lactobacillus*.

Lactobacillus johnsonii



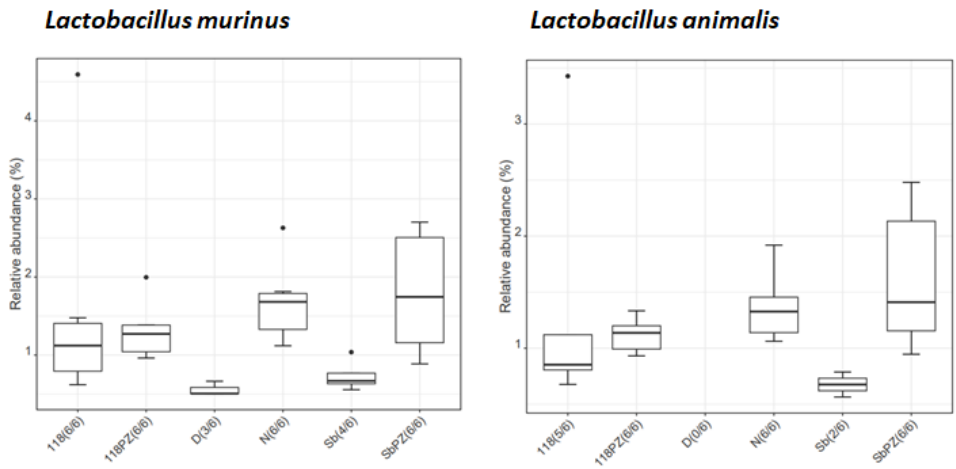
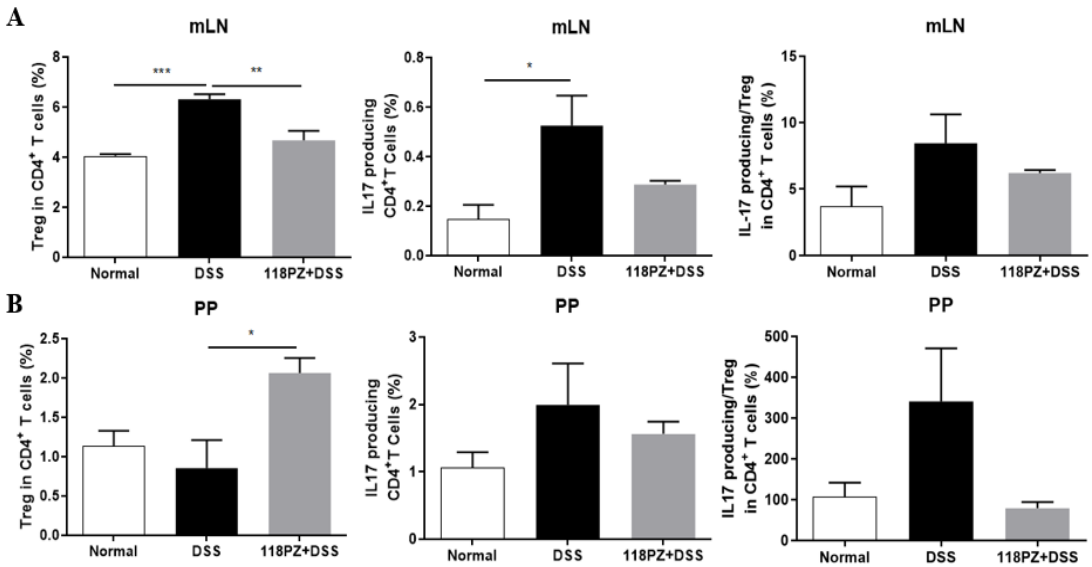
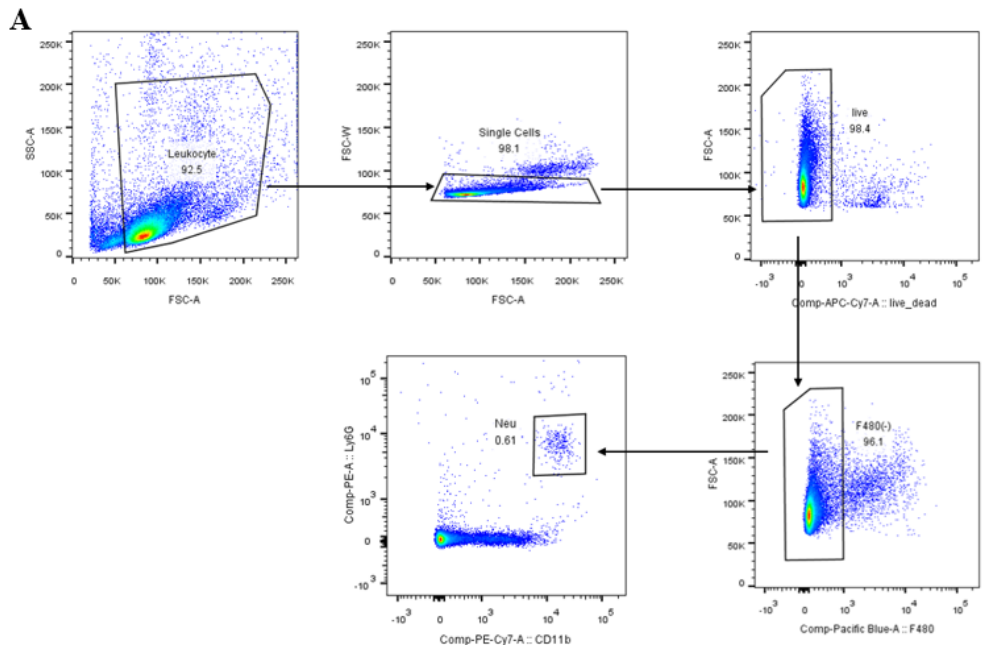


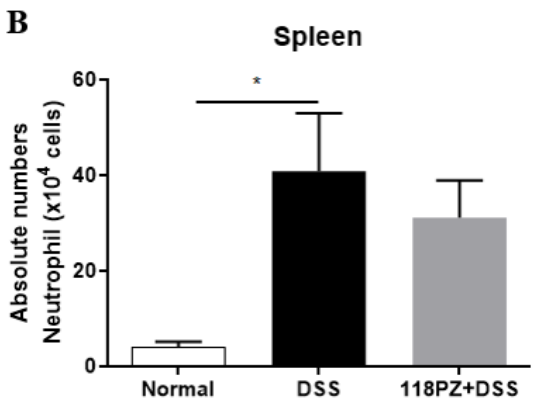
Figure 3-8-5. Relative abundance of specific *Lactobacillus* species modulated after live cells and cell wall components administration.

4. Supplementary Figures



Supplementary Figure 3-1. Percentage of Treg, IL-17 producing cells in CD4$^+$ T cells and IL-17 producing cells/Treg from (A) mLN and (B) PP. **P* <0.05, ***P* <0.01, and ****P* <0.001, compared with treatment of only DSS.





Supplementary Figure 3-2. (A) Gating strategy for neutrophils (B) Percentage of neutrophils from spleen. **P* <0.05 compared with treatment of only DSS.

5. Discussion

The DSS colitis mouse model is easy to establish and most frequently used in IBD-related animal studies. In this study, inflammation was induced using 1.5% DSS in DW[160]. The use of the appropriate DSS brand and concentration, the inclusion of an RNA cleaning method to prevent DSS contamination of the colonic tissue, the selection of adequate reference genes and qRT-PCR chemistry, as well as using the same region of the colon to analyze the different parameters are all issues that require special consideration when designing the protocol in order to obtain accurate results[161]. Despite numerous studies on *S. boulardii*, other yeast strains are not widely used in research related to gut and health. Because they contain a variety of immune-modulating substances (such as glucans) and are unaffected by anti-bacterial substances, yeasts could be regarded as probiotic organisms[162,163]. Previous study elucidated that anti-inflammatory cytokine IL-10 in serum was more upregulated by *S. cerevisiae* GILA 118 than other *S. cerevisiae* GILA strains[145]. These results require for additional research, more especially on the *S. cerevisiae* GILA 118 cell wall component with the DSS colitis model. Moreover, the treatment of live cells should be considered for safety[164]. Therefore, the *S. cerevisiae* GILA component, especially the cell wall, was examined via autolysis, and hydrolysis. However, only a few reference reports are available, and most are concentrated on the β-glucan structure and related Treg cells[49,116]. Chitin

and mannose are also present in the *S. cerevisiae* cell wall. Previous studies on related cell walls focused on β-glucan and mannose[165,118]. This study focused on cell wall component (PS, PZ), protoplast (PP) and anti-inflammatory cytokine IL-10 in serum was more upregulated by *S. cerevisiae* GILA 118 PZ than PS and PP. Cell wall component come in a variety of forms, each with potential impacts. One type of cell wall component treated with zymolyase (PZ) increase anti-inflammatory cytokine IL-10 doesn't necessarily suggest that any other type of cell wall component or protoplast will as well. Zymolyase is an enzyme that hydrolyzes glucose polymers connected by β-1, 3-bonds. Intriguingly, Lee *et al*[49] reported that zymosan's 1,3-glucan was removed by an enzyme, which enhanced Treg cell development *in vitro* and suppressed anti-cancer immunity *in vivo.* Despite these developments, a thorough understanding of how each component of the yeast cell wall affects the immune system is still unknown. The observed anti-inflammatory result appears to be the result of numerous processes involving IL-10 and Treg-mediated suppression in conjunction with its impact on effector T-cell development. Moreover, it has been shown that D-(+)-mannose, the monomeric component of mannan, has the ability to induce Tregs by acting directly on naive T cells in a DC-independent way[166]. Hence, it was possible that the monomeric D-(+)-mannose created after mannan was degraded in PZ may have a role in boosting Treg induction and IL-10 induction. This is highly implausible, given Lee e*t al*[49] show that the Treg-inducing growing property of yeast cell

wall component is a DC-dependent phenomena.

The pathogenesis of inflammation is demonstrated through the nitric oxide (NO) production[167]. Decreased NO production is crucial for regulating the excessive immune response[168]. The *S. cerevisiae* GILA 118 cell component alleviated inflammation by significantly decreasing NO production compared to the LPS group. TNF-α was overexpressed during inflammation[169] and significantly decreased when the *S. cerevisiae* GILA 118 cell wall component PZ was administered. Murine macrophage RAW 264.7 cells exhibited PPAR-γ upregulation when treated with the *S. cerevisiae* GILA 118 cell component. This result coincided with the effects on NO. However, mouse colon tissue did not exhibit PPAR-γ upregulation. HO-1 is related to PPAR-γ in an inflammation environment and is an antioxidant and anti-inflammatory enzyme regulated by PPAR-γ[170,171]. Therefore, the effects of the anti-inflammatory-related component of *S. cerevisiae* GILA cell wall differed from cell to tissue type.

Treatment with the *S. cerevisiae* GILA 118 cell wall component alleviated inflammation to related IBD colitis in RAW 264.7 cells and mice.

The ability of low molecular weight β-glucans derived from *A. pullulans* to reduce LPS-activated nuclear factor-kappa B (NF-kappa B) and mitogen-activated protein kinase pathway (MAPKs) in LPS-stimulated RAW 264.7 cells was examined by No *et al.*[172]. The extracellular polysaccharide's high viscosity is one cause of this ability. Compared to those in the DSS group,

the neutrophil rate was lower in mice in the normal group and those treated with the *S. cerevisiae* GILA 118 cell component. The role of neutrophils during colitis remains unclear; however, infiltration of neutrophils is significantly increased in intestinal inflammation than in normal conditions[99]. Neutrophils are essential to the innate immune system, the first defence against inflammation[29]. After treatment with *S. cerevisiae* GILA 118 PZ, neutrophil levels were reduced in the blood of mice. Further analysis was conducted to compare neutrophil activation under IBD colitis and normal status. Using flow cytometry analysis, the DSS group had significantly increased leukocytes than the normal group, but *S. cerevisiae* GILA 118 PZ administration did not significantly increase the leukocyte count relative to the normal group (Fig. S3-2).

To confirm serum cytokine data (Fig. 3-5), this study obtained spleen $CD4^+$ T cell subsets, especially Treg cells correlated with serum IL-10 cytokine. Treg cells in mLN differed from those in the spleen (Fig. S3-1). DSS directly induced injury to the colon tissue, and colon length decreased compared to that in the normal group[32]. For this reason, Treg was also upregulated in mLN after DSS treatment[173]. Meanwhile, Treg response in the PP decreased in the DSS mice, suggesting that the small intestine and organ immune systems differed from those in mLN (Fig. S3-1). Moreover, Th17 cells increased significantly in the mLN of the DSS group compared to that in the normal group. Treg rate in the spleen, mLN, and PP showed

differences upon treatment with *S. cerevisiae* GILA 118 PZ. However, the *S. cerevisiae* GILA 118 PZ-treated group showed a similar tendency for IL-17 production/Treg in $CD4^+$ T cells (Fig. S3-1). The *S. cerevisiae* GILA 118 PZ group had significantly increased HO-1 in colon tissue (Fig. 3-5C) and IL-10 in serum. HO-1 also regulates Th17/Treg cells[174,175] during inflammation. These results indicated that *S. cerevisiae* GILA 118 PZ increases HO-1 in the colon and IL-10 in serum. For these reasons, HO-1, and Th17/Treg cell balance were related to alleviating intestinal inflammation[175-177].

Relative abundance of *Akkermansia* were significantly increase in the DSS group than the other group[178]. Despite being widely regarded as the new generation of probiotics and having possible anti-inflammatory qualities, *Akkermansia* includes mucolytic enzymes that can cause the intestinal mucus barrier to erode and aggravate colitis[179,180]. *Akkermansia* were upregulated in DSS group[160], whereas *Lactobacillus* were downregulated in DSS group. It was known as *Lactobacillus* alleviated intestinal inflammation[181,182,183]. Relative abundance of specific three *Lactobacillus* strains, especially *Lactobacillus johnsonii* and *Lactobacillus murinus* were upregulated in *S. cerevisiae* GILA 118 and GILA 118 PZ, which were related IL-10 expression results[184,185,186]. The variability in response frequently observed with living probiotic cultures may be caused by varying concentrations of dead cells. However, using cell wall component (118 PZ)

as biological response modifiers has a number of attractive benefits, including very high safety standards and a lengthy shelf life[187]. Specific storage conditions are required for the viability of probiotic microorganisms because probiotic yeast can lose some of its maximum vitality during storage. The ratio of viable to non-viable yeast may vary in probiotic formulations, and the number of dead cells may even be higher than that of living cells.

Chapter 4. Conclusion

Potential probiotic *S. cerevisiae* strains were screened and selected. Their probiotic properties, including survival rate in low pH, bile condition or autoaggregation, co-aggregation with pathogens, survival in the GIT model, adhesion to Caco-2 cells, and the DPPH scavenging effect, were similar to or higher than *S. boulardii* CNCM I-745. *In vivo* Screening saw three *S. cerevisiae* GILA strain saw alleviating inflammation functionality. *S. cerevisiae* GILA 118 administration increased IL-10 in serum and alleviated intestinal inflammation in mice compared with *S. cerevisiae* GILA 100 and GILA137. *S. cerevisiae* GILA 118 PZ, HO-1 regulated the expression of IL-10 in serum. Additionally, proinflammatory cytokine TNF-α and neutrophils were significantly decreased in serums. Therefore, *S. cerevisiae* GILA 118 PZ regulated Th17/Treg cells in spleen similarly to the normal. *S. cerevisia*e GILA 118 and GILA 118 PZ also managed the gut microbiome to upregulate *Lactobacillus johnsonii, Lactobacillus animalis,* and *Lactobacillus murinus*. These results suggested that *S. cerevisiae* GILA 118 and the yeast cell wall *S. cerevisiae* GILA 118 PZ has therapeutic potential to alleviate intestinal inflammation and may act as an immunomodulator for patients with IBD (Fig. 4).

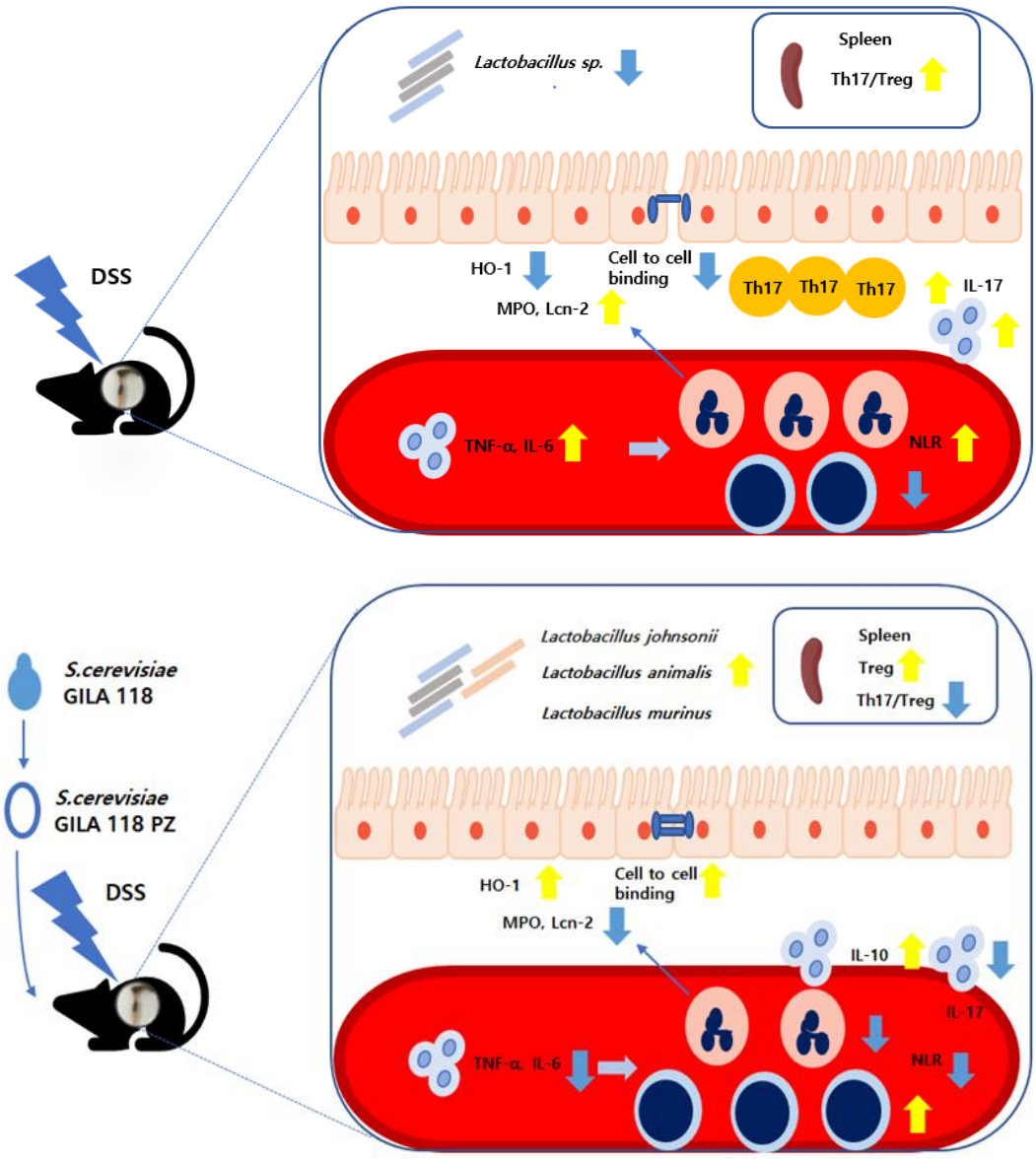


Figure 4. Graphical summary.

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Abstract in Korean

염증성 장 질환 (IBD)은 장관 내 만성 염증에 의해 일어난다고 알려져 있으며 궤양성 대장염과 크론병을 포함한다. IBD의 정확한 발병기전은 밝혀지지 않았으나 장 표피세포의 장벽 손상, 사이토카인 불균형 등이 염증성 장 질환을 일으키는 원인으로 보고되고 있다. 장상피에서 호중구 (neutrophils)가 활성화되면 TNF-α와 같은 염증성 사이토 카인이 방출된다. 크론병과 궤양성 대장염에서는 T helper (Th) 17의 분화가 유도되는데 이로 인해 조절 T 세포 (Treg)와 Th 17의 비율이 불균형해진다. 현재 염증성 장질환의 치료법으로는 일반적으로 항염증제와 항스테로이드, 면역조절제를 투약하는데 장기간 투약시 치료 효과가 감소하고 부작용 문제 또한 제기되었다. 따라서 보다 효율성과 안전성을 강구한 여러 연구에서 프로바이오틱스를 IBD 치료 전략으로 제안되었다. 하지만 대부분의 프로바이오틱스는 락토바실러스 위주의 유용 원핵생물 위주로 연구가 진행되었고 진핵생물인 효모는 *Saccharomyces boulardii* (*S. boulardii*) 외에는 상대적으로 연구가 많이 진행되지 않았다.

이에 본 연구에서는 발효 및 식품 산업에 쓰이는 *Saccharomyces cerevisiae* (*S. cerevisiae*) 효모를 전통주인 막걸리로부터 분리하였고 *S. boulardii*를 비교 균주로 설정하여 분리된 효모와 장내

환경에서의 생존성을 기반으로 안정성, 장세포 부착성과 같은 프로바이오틱 특성에 근거하여 선발하였다. 마우스 대식세포 RAW 264.7에서 Nitric oxide (NO)와 마우스의 splenocyte에서 인터페론감마 (IFN)-γ 수치를 염증 유발 그룹보다 감소시키는 것을 확인하였다. ITS identification을 통해서 선발 효모가 *S. cerevisiae* strain인 것을 확인하였고, 장내 염증을 감소시킬 수 있는 잠재적인 프로바이오틱스로서 *S. cerevisiae* GILA 로 명명하였다. *S. cerevisiae* GILA를 1.5% dextran sulfate sodium (DDS)로 대장염을 유발한 마우스(C57BL/6J)에 처리하여 대장염 완화 효능을 확인하였다. *S. cerevisiae* GILA 중 특히 GILA118은 호중구 (neutrophils)의 활성화를 감소시키고 골수세포형과산화효소 (Myeloperoxidase, MPO)를 감소시켰다. 또한, 대장에서 밀착연접단백질 (tight junction protein, Occludin과 ZO-1)의 mRNA 발현을 유의적으로 증가시켰으며 혈청에서는 IL-10의 유의적인 증가 및 TNF-α의 유의적인 감소가 나타났다. 이로서 *S. cerevisiae* GILA 118을 최종 선발하였다.

최종 선발된 *S. cerevisiae* GILA 118 세포벽 분리물을 제조 후 염증을 완화하는 효능을 마우스 대식세포 RAW 264.7과 일반 마우스 (C57BL/6J)를 통하여 검증하였다. GILA118를 산, 염기 분리법과 자이모레이즈 (Zymolyase)를 이용하여 세포벽 분리물을 제조하였다. 본 분리물은 Lipopolysaccharide (LPS)를 처리한

대식세포 RAW 264.7과 DSS 1.5 % 급이로 유도한 IBD 마우스 모델을 이용하여 염증을 완화하는 기전을 확인하였다. RAW 264.7 cell에서 nitric oxide (NO) 발현 감소 및 *PPAR-gamma* gene expression을 통해 *COX-2* gene expression 발현을 완화시켰으며, 마우스 혈청에서 IL-10이 DSS 유도 IBD 마우스보다 유의적으로 높아지는 세포벽 성분인 GILA118 PZ를 선발하였다. Treg 군집과 IL-17 사이토카인을 생성하는 $CD4^+$ T 세포의 군집의 비율을 비교해본 결과 전신 면역 조직인 비장에서 Treg 군집의 증가와 IL-17 사이토카인을 생성하는 $CD4^+$ T 세포의 비율이 감소하였다. 이후 생균과 세포벽 분리물 (PZ)의 처리군에서 장내 미생물총 조성이 안정화된 것을 확인하였다.

종합하면, 본 연구는 IBD를 완화하는 물질로 효모인 *S. cerevisiae* GILA를 분리하였고 GILA 118을 선발하여 세포벽 성분인 GILA 118 PZ를 제시하였다. 이는 향후 염증성 장질환의 완화와 관련 있는 프로바이오틱 특성을 가진 효모 및 효모의 세포벽에 대한 연구의 가능성을 시사한다.