A DISSERTATION

FOR THE DEGREE OF MASTER OF SCIENCE

Alleviation of colitis in mice administered with vitamin D-loaded resistant starch nanoparticles internalized in *Lactobacillus plantarum*

락토바실러스 플란타룸에 내재화된 비타민 D 담지 난소화성 전분 나노입자 급이에 의한 쥐의 대장염 완화

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Abstract

Inflammatory bowel disease (IBD) is chronic inflammation of the gastrointestinal tract. Although the prevalence of IBD is increasing internationally, there is no ideal cure owing to side effects. Therefore, research on alternate therapy using probiotics, prebiotics, and Vit D has been actively conducted. In this study, vitamin D_3 (Vit D)-loaded acetyl resistant starch (ARS) nanoparticles (NPs), used as nanoprebiotics, were internalized in *Lactobacillus plantarum* (*L. plantarum*). Also, Vit D was released from *L. plantarum* in a controlled manner. Effects of *L. plantarum* internalized with Vit D-loaded ARS NPs in Caco-2 cells and dextran sodium sulfated (DSS)-induced colitis model were examined thoroughly.

The results showed that the treatment of Vit D-loaded ARS NPs-internalized in *L. plantarum* (LPSV) were mitigated the deleterious effects of DSS on monolayer cell permeability by enhancing tight junction proteins and alleviated colitis effects by enhancing the epithelial barrier function in conjunction with inhibition of inflammatory responses in the gut. It was intriguing that there was no significant change in intestinal barrier damage in LPSV treated group by increased occludin genes in colon. In addition, pro-inflammatory cytokines such as tumor necrosis factor-α (*TNF-α*) and interferon-γ (*IFN-γ*) were significantly low in concordance with enhanced proportion of Tregs in peripheral lymph nodes (pLN) and mesenteric lymph nodes (mLN) by the treatment of LPSV. Collectively, LPSV contributes to colitis alleviation, which can be utilized as a potential therapeutic agent against IBD.

Keywords: Inflammatory bowel disease, nanoprebiotics, polymeric nanoparticles, probiotics

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List of Abbreviations

ACSS2	Acyl-CoA synthetase short-chain family member 2
APC	Antigen presenting cell
ARS	Acetyl resistant starch
ARS NPs	Acetyl resistant starch nanoparticles
Calcidiol	25-OH-D_3
Calcitriol	$1,25(OH)_2D_3$
CD	Crohn's disease
CLSM	Confocal laser scanning microscopy
CPT1A	Carnitine palmitoyltransferase 1
DAPI	$4',6$-diamidino-2-phenylindole
DCM	Dichloromethane
DLS	Dynamic light scattering
DMAP	Dimethyl aminopyridine
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DS	Degree of substitution
DSS	Dextran sulfate sodium
ELS	Electron light scattering
FBS	Fetal bovine serum
FITC	Fluorescence isothiocyanate

GPR	G protein-coupled receptor
H&E	Hematoxylin and eosin
HDAC	Histone deacetylase
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFN	Interferon
IL	Interleukin
LP	*Lactobacillus plantarum*
LPS	lipopolysaccharide
LPSA	*L. plantarum* internalized with ARS NPs
LPSV	*L. plantarum* internalized with Vit D-loaded ARS NPs
MEM NEAA	MEM Non-Essential Amino Acids
MHC	Major histocompatibility complex
NF-κB	Nuclear factor-κB
NK	Natural killer
NMR	[1]H-nuclear magnetic resonance
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
PGN	Peptidoglycan
Pro-Vit D$_3$	7-dehydrocholesterol
PUMA	p53-upregulated modulator of apoptosis
PVA	Poly (vinyl alcohol)

qRT-PCR	Quantitative real-time PCR
RS	Resistant starch
SCFA	Short chain fatty acids
SCID	Severe combined immunodeficiency
TBP	TATA-binding protein
TEER	Transepithelial electrical resistance
TEM	Transmission electron microscopy
TLR	Toll-like receptor
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF	Tumor necrosis factor
Tregs	Regulatory T cells
UC	Ulcerative colitis
UV	Ultraviolet
VDR	Vitamin D receptor
Vit D	Vitamin D$_3$
ZO	Zonula occludens

I. Review of Literature

1. Inflammatory bowel disease

1.1 Causes and immune responses in IBD patients

Inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory diseases in the gastrointestinal tract that have become a global disease where the incidence rises in newly industrialized countries as society westernizes in the 21st century [1]. UC is characterized by its occurrence only in the rectum, extending proximally to the colon and causing superficial inflammation of the intestine wall. On the other hand, CD is characterized by transmural inflammation that occurs throughout the intestine, causing fibrosis, stricture and fistula in the bowel. The exact pathogenesis of CD and UC is still unknown, but genetic susceptibility, dysregulated immune responses, environmental factors, and altered gut microbiota are known to contribute the most to the disease onset and progression [2, 3].

In a genetically susceptible host, environmental factors mainly affect the CD's onset and progression. Polymorphism of the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) which is required to bacterial invasion resistance, occurs in Crohn's disease patients, impairing its ability to activate NF-κB in response to lipopolysaccharide (LPS) and peptidoglycan (PGN). Thus, NOD2 polymorphism disrupts epithelial barrier function. It causes luminal contents to enter the lamina propria, activating effector T cells via dendritic cells, which then produce

pro-inflammatory cytokines such as interleukin (IL)-17, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, exacerbating inflammation [4]. Regulatory T cells (Tregs) from patients in IBD are less effective on suppressing effector T cell responses due to deficiencies in Treg numbers and ability to produce IL-10, resulting in the persistence of excessive immune responses [5, 6]. On the other hand, proinflammatory cytokines, IL-12 and IL-23 produced by activated macrophages trigger the activation of natural killer (NK) cells that prolongs the intestinal inflammation (Figure 1A) [4].

Pathogenesis of UC is caused by combinations of unknown multiple factors. The composition and diversity of gut bacteria together with their production of short-chain fatty acids (SCFAs) are reduced. As the expression of mucin 2 is downregulated due to the decrease in the number of goblet cells, the mucous layer's ability to protect against exogenous antigens is also reduced [7, 8]. Due to changes in the expression of tight junction proteins and apoptotic foci, the epithelium is destroyed and external antigens infiltrate lamina propria, causing an immune response. Antigen presenting cells (APCs) are activated and neutrophils are attracted by chemokine expression at the damaged area. Type 1 T helper (T_H1) cells become polarized as a result of the production of TNF-α, IL-12, IL-23, and IL-6 by activated macrophages. IL-36 derived from epithelium limits Tregs and induces fibroblast. NK T cells in the lamina propria are induced by foreign antigens such as glycolipid antigens [9]. IL-13 derived from NK T cell enhances colon epithelial permeability by the production of claudin-2, the pore forming tight junction protein and delays repair of colon epithelial cells by the induction of apoptosis (Figure 1B) [10, 11].

By using various colitis models, understanding of IBD has significantly advanced in recent years. The most commonly used mouse models to study colitis are those caused by dextran sulfate sodium (DSS), 2,4,6-trinitrobenzene sulfonic acid (TNBS), oxazolone, adoptive transfer of naïve $CD4^+$ T cells ($CD4^+CD45RB^{high}$ T cells) into syngeneic immunodeficient severe combined immunodeficiency (SCID) or $Rag1^{-/-}$ recipient mice, and genetically modified model causing spontaneous colitis. The DSS-induced colitis model is probably the most widely used because it has a pathogenesis resembling UC, a simple induction method with consistent outcome. The DSS-induced colitis model uses a method of directly damaging the colonic epithelium by administering sulfated polysaccharide with water. In this model, the expression of tight junction proteins in mice is reduced, which not only weakens the barrier function but also increases permeability. As a result, invasion of foreign antigens into the lamina propria occurs and causes an immune response [12].

(A) Crohn's disease

Epithelial Permeability ↑

Epithelial barrier dysfunction

Gut lumen

Lamina propria

Entering foreign antigens

Macrophage

NK cell

Activated NK cell

APC

T_H17 cell — IL-17, IL-21, IL-22

T_H1 cell — TNF-α, IFN-γ

pro-inflammatory cytokine

NOD2 ↑

(B) Ulcerative colitis

Epithelial Permeability ↑

Reduced mucus layer

Impaired barrier function

TNF-α, IL-12, IL-23, IL-6

Activated APCs

T_H1 cell — TNF-α, IFN-γ, IL-6

pro-inflammatory cytokine

NK T cell — IL-13

Neutrophil recruitment

IL-36

T_reg cell

Fibroblast

Legend:

Innate immune cells

Activated macrophage

Neutrophil

Regulatory T cell

Effector T cell

NK cell

NK T cell

Bacteria

4

Figure 1. Schematic diagram for Crohn's disease and ulcerative colitis pathogenesis

The pathogenesis of UC and CD patients is triggered by a number of unknown factors. (A) NOD2 variants impair the IEC's barrier function in CD patients, and luminal contents invasion accelerates the CD pathogenesis. APCs recognize invaded antigens and activate effector T and NK cells, which cause chronic inflammation by continuously producing pro-inflammatory cytokines. (B) Due to the decrease in mucin and tight junction protein and the increase in apoptotic foci, many antigens can enter the lamina propria. This causes the synthesis of chemokines, which activate APCs. Activated APCs eventually induce neutrophil recruitment and activate effector T cells. The IL-36 induced by the damaged epithelium directly suppresses Treg induction and promotes the expression of fibrogensis genes. NK T cells induced by foreign antigens damage epithelial cells by inducing IL-13.

1.2 Limitation of IBD therapeutics

Due to its importance in human health, preclinical and clinical studies on IBD are actively ongoing, however it is unfortunate that there is no perfect cure for IBD. In the last two decades, patients with IBD generally have received monoclonal antibody therapy targeting TNF-α (i.e., infliximab, adalimumab and certolizumab). Patients at the early stage of an illness, exhibiting significant disease progression, are frequently given anti-TNF drugs that help avoiding the administration of corticosteroids or/and surgery. However, when using anti-TNF treatment, about 30-40% of patients developed antibodies against it, resulted in ineffectiveness as IBD treatment [13, 14]. Other biologic treatment for IBD patients includes IL-6 receptor antibodies that block T-cell signaling, preventing leucocyte adhesion, and targeting cytokines like IL-12/23. These medications have a variety of adverse effects and limitations, particularly when combined with traditional immunosuppressive medicines or corticosteroids [15-17]. Opportunistic infections, cancers, and other problems such as injection/infusion responses and autoimmunity as well as contraindications like cardiac failure and acute infectious disorders can occur in patients receiving biologic treatment for IBD [17]. Due to the side effects of conventional therapy, alternative strategies are being actively investigated. It has been suggested that probiotics, dietary fiber, and fat-soluble vitamins can significantly alleviate the symptoms of IBD due to their immune modulation capabilities [18].

2. Immune modulator treatment for inflammatory bowel diseases

2.1 Vitamin D₃

Vitamin D₃ (Vit D) is fat-soluble vitamin that is produced endogenously by ultraviolet (UV) radiation and found in food ingredients. The biologically active form of Vit D, calcitriol, is produced in the liver and kidneys through a two-step metabolic process from naturally obtained Vit D. In general, 80% of Vit D is absorbed through the skin and 20% through the diet [19]. In the skin epidermis, exposure to ultraviolet B (UV-B, 290-320 nm) converts 7-dehydrocholesterol (pro-Vit D₃) into pre-Vit D. And pre-Vit D is processed into Vit D by heat. Obtained Vit D from the skin and diet binds to Vit D binding protein and is transported to the liver [10]. Vit D is converted by the enzyme CYP2R1 in the liver to the predominant circulating form, 25-OH-D₃ (calcidiol). In kidney, calcidiol is converted to $1,25(OH)_2D_3$ form (calcitriol) by the enzyme CYP27B1 (Figure 2) [20].

Calcitriol, an active form of Vit D, circulates in the blood by its binding protein. Calcitriol can bind to the vitamin D receptor (VDR), found in a variety of organs and cell types, including immune and intestinal cells, involving a variety of physiological functions [19]. When calcitriol binds to the cytosolic VDR, calcitriol-VDR complex form a heterodimerization with the retinoid-X receptor. The heterodimer, then targets to specific DNA location known as Vit D responsive elements, which causes the expression or suppression of specific gene products [21]. Calcitriol can modulate antigen-presenting cells, particularly dendritic cells, to tolerogenic state with the reduction of major histocompatibility complex (MHC)-II, CD80 and CD86, and induction of IL-10 [22]. Tolerogenic DCs, influenced by Vit D, play a role in the

differentiation of naïve T cells into the Tregs [23]. As a result, Vit D modulates to reduce the development of effector T cells, helping to prevent the onset of autoimmune disease.
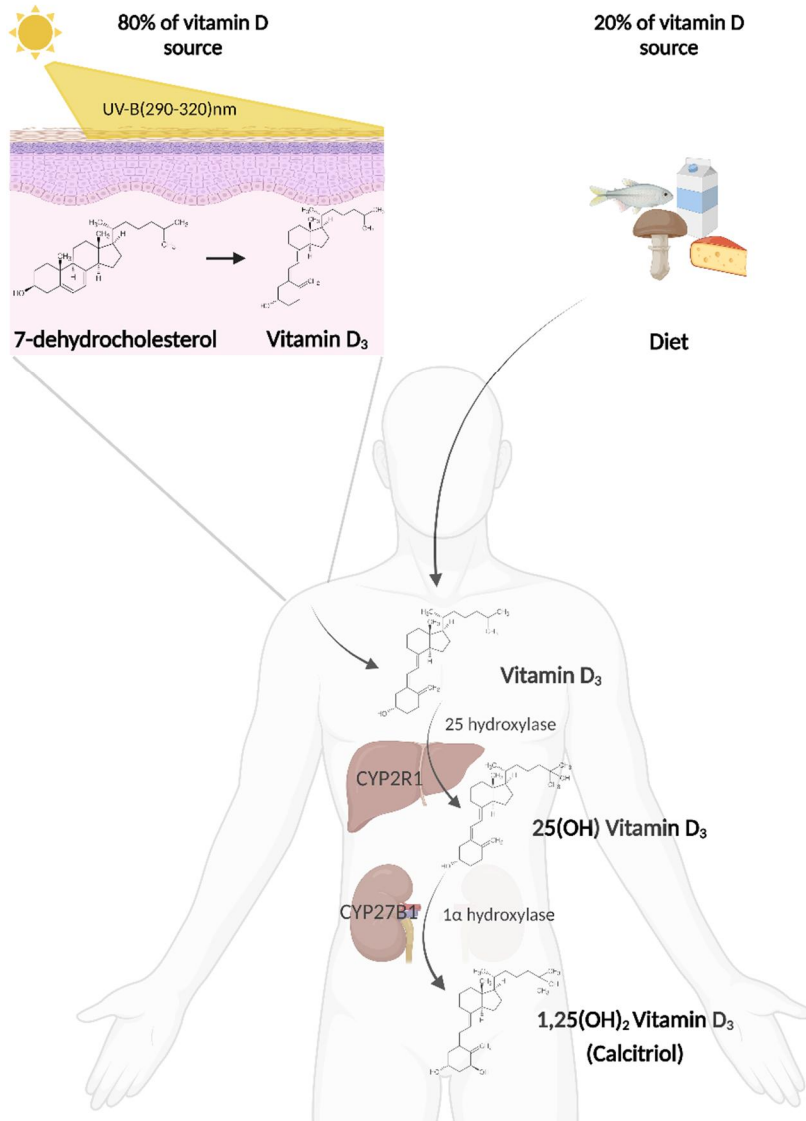

Figure 2. Metabolism and structural formula of Vit D

Vitamin D_3 is obtained from sunlight absorption and food. Obtained vitamin D_3 is converted to 25(OH) vitamin D_3 by CYP2R1 in the liver and converted to the active form of calcitriol by CYP27B1 in the kidney.

VDR expression on colonic epithelial cells is decreased in patients with CD or UC. By inhibiting NF-kB activation, VDR signaling reduces p53-upregulated modulator of apoptosis (PUMA) in intestinal epithelial cells (IECs), resulted in decrease of IEC apoptosis [24]. Through the regulation of tight junction proteins such as occludin, zonula occludens (ZO)-1, ZO-2, claudin-2, and claudin-12, VDR contributes to the preservation of the structural integrity of the epithelial barrier [25]. Pretreatment with calcitriol prevents DSS-induced inflammation from enhancing cell permeability [26]. Consequently, colitis is inhibited by VDR signaling in gut epithelia, which protects the mucosal epithelial barrier. VDR expression is also known to maintain microbial homeostasis by lowering pathogenic bacteria and enhancing probiotics. Many studies reported that deletion of VDR gene results in an imbalance in the gut microbiota consisting of more proteobacteria and less Firmicutes, a situation typically reported in IBD patients [27-29]. Vit D supplementation reduces proteobacteria and increases beneficial organisms, such as members of the Firmicutes phyla, in humans and CYP27B1 knockout mice [30, 31].

Although calcitriol is known to play a role in immune regulation and maintaining the homeostasis of the microbial composition, it has the drawback of having a very short half-life and a retention duration. After the administration of calcitriol, it is eliminated within 5-8 hours and the half-life in plasma is 3-5 hours only [32]. Furthermore, there is a limited bioavailability of Vit D correlated with dose, thus a greater dose should be administered [33].

2.2 Probiotics

Probiotics are living microorganisms that, when administered in adequate amount, confer a health benefit to the host [34]. Probiotics are acid resistant, and thus survive better and colonize in the intestine [35]. With the binding force of pili, probiotics could attach to the intestinal mucosa. Probiotics are known to influence the gut in a variety of ways. Probiotics release a number of metabolites such as SCFAs, vitamins, and anti-microbial peptides [36]. By boosting epithelial cells for their proliferation and tight junction protein expression, probiotics could induce epithelial regeneration and restore the epithelial integrity in colitis [37]. Additionally, probiotics could induce gastrointestinal immune modulation, secretion of defensin by paneth cells, and induction of IgA by plasma cells [38].

The majority of probiotic strains consist of the *Lactobacillus* spp. and *Bifidobacterium* spp. One of the most widely studied and well-known *Lactobacillus* species is *L. plantarum* that is frequently present in a variety of fermented food products. It is a member of the phylum Firmicutes, which are one of the two dominants primarily composed of the human intestine, along with Bacteroidetes [39, 40]. *L. plantarum* modulates T cell differentiation by inducing Treg differentiation. It helps to suppress pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IFN-γ, thereby preventing IEC apoptosis by inhibiting pro-apoptotic signaling pathway [39, 41-43]. The administration of *L. plantarum* also enhances intestinal barrier function by increasing the expression of epithelial tight junction proteins ZO-1 and occludin through Toll-like receptor (TLR) 2 signaling (Figure 3) [44]. The use

of probiotics as alternative therapy in IBD has been examined, however, its mechanism of action appears to be complex and poorly understood.
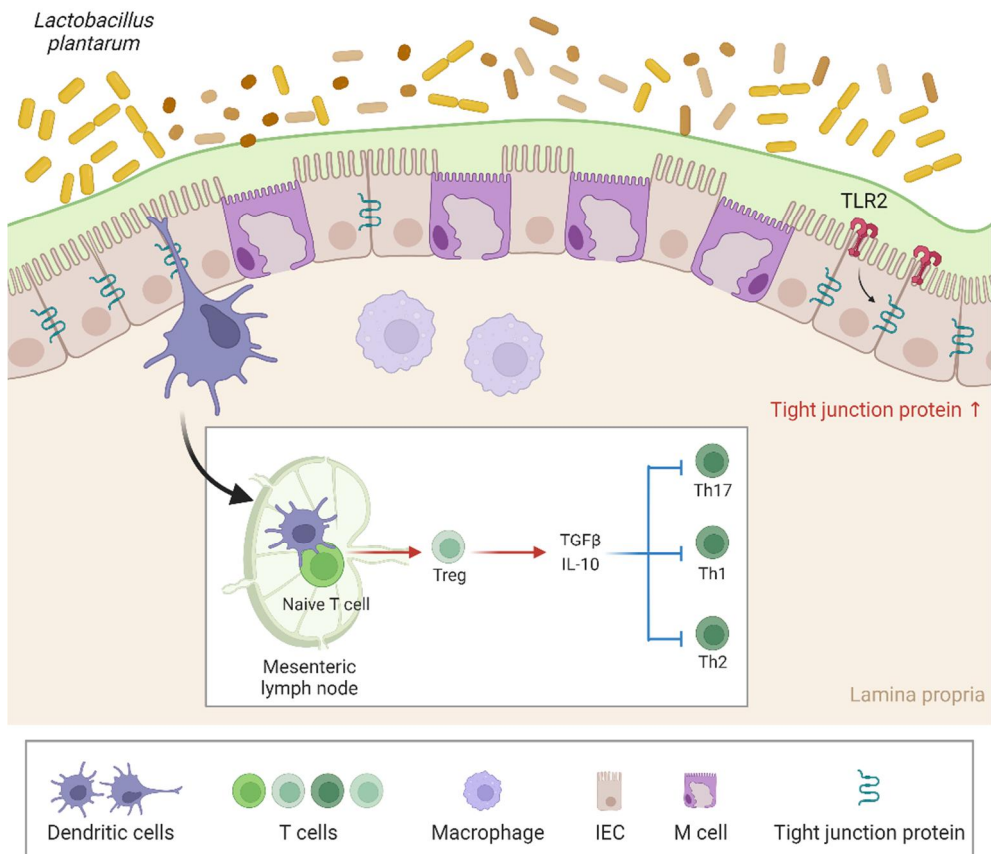

Figure 3. Probiotics modulate intestinal immune response and tight junction protein expression

Probiotics induce the tight junction proteins through TLR2 signaling and modulate intestinal immune response by inducing Tregs via producing IL-10 and TGF-β.

2.3 Prebiotics

Prebiotics is a substrate that is selectively utilized by microorganisms for conferring a health benefit of the host. It is non-viable substrates that serve as food for supplied probiotic strains as well as resident microorganisms that are kept alive by the host [45].
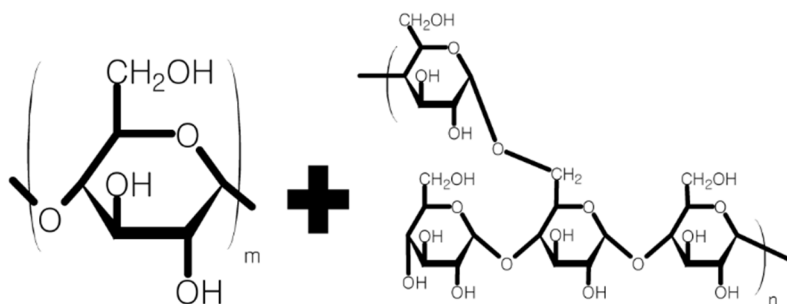
Figure 4. Chemical structure of resistant starch

Resistant starch is composed of 40 wt.-% of amylopectin which has a branched structure with α-1,4 as well as α-1,6 glycosidic linkages and 60 wt.- % of amylose which has a linear structure with α-1,4 glycosidic linkage.

A large number of natural foods such as potato, corn, banana, and seeds contain resistant starch (RS), containing long polymers of glucose subunits. The structure of RS is composed of amylose and amylopectin (Figure 4). The majority of RS consumed is not easily broken down by digestive enzymes and is instead fermented by microbiota in the large intestine, which has an impact on bacterial development and intestinal health status [46, 47]. *Bifidobacteria* and *Ruminococcus*, acting as the first degrader in the colon, decompose the RS into glucose and malto-oligosaccharides. Secondary fermenters, such as the *Bacteroidetes phylum* and

firmicutes phylum, break down intermediate metabolites into propionate and butyrate. Butyrate is the key component to break down RS, and it has a variety of anti-inflammatory effects in the gut. G protein-coupled receptor (Gpr)109a and Gpr43, found on macrophages and dendritic cells, are butyrate receptors. Once butyrates are uptaken by APCs, the anti-inflammatory cytokine IL-10 is produced [48]. On the other hand, butyrate plays a role in the differentiation of naive T cells into Tregs by up-regulating histone acetylation for gene expression as a histone deacetylase (HDAC) inhibitor and accelerating fatty acid oxidation. Butyrate is converted to butyryl-CoA by acyl-CoA synthetase short-chain family member 2 (ACSS2), and butyryl-CoA increases the activity of carnitine palmitoyltransferase 1 (CPT1A) by inhibiting the activity of malonyl-CoA, a representative inhibitor of CPT1A. Finally, when butyryl-CoA enhances CPT1A activity, fatty acid oxidation is promoted, which increases the differentiation of Tregs [49, 50].

3. Improvement of intestinal immune regulation ability through a nano-delivery system

3.1 Enhancement of bioavailability using nano-delivery system

Vit D is a fat-soluble, and thus its use in the body requires an organic solvent to be dissolved. However, most, if not all, organic solvents have toxicity and can cause a variety of serious diseases. The conventional method to deliver therapeutics used to be dissolved in a solvent, but it has a disadvantage of low bioavailability. Such limitation can be overcome by using nano-delivery system. For instance, polymeric nanoparticles, made of synthetic materials or natural materials such as resistant

starch, pullulan, inulin, and dextran, have several advantages over conventional therapeutics. The first, because polymeric nanoparticles are biocompatible and have simple formation parameters, the properties of nanoparticles can be easily controlled depending on how they are synthesized [51]. The second, depending on the synthesis method, they can enhance solubility of hydrophobic drugs and stabilization of loaded drugs. The third, the loading efficacies and release kinetics of hydrophobic materials-loaded nanoparticles can be precisely regulated by modulating their characteristics such as composition, stability, responsivity, and surface charge [51-53]. The fourth, polymeric nanoparticles can overcome extracellular and intracellular barriers which restrict their ability to reach the site of action. For example, once entered into the body, they protect the substances from extracellular enzymatic breakdown and undesired environment until they reach the target cells [54]. The fifth, polymeric nanoparticles can regulate cellular uptake pathway based on the designed functional molecules [55]. Therefore, polymeric nanoparticles can be used as effective delivery vehicles. Many of them are being approved for clinical use and seeing increase in therapeutic applications [51].

3.2 Nanoprebiotics with probiotics

Nanoprebiotics, synthesized from inulin [56], dextran [57], pullulan [58], and resistant starch [59], can be internalized into probiotics by time-, energy-, and transporter-dependent manner. After the internalization, the nanoprebiotics could cause the development of natural antibacterial peptides such as bacteriocin, which can directly kill pathogens and limit bacterial colonization [58]. The improved

antibacterial properties of probiotics internalized with nanoprebiotics have altered microbial composition and reduced the number of pathogens while increasing beneficial bacteria species [57]. Furthermore, intestinal microbes, such as *Firmicutes*, degrade nanoprebiotics to boost the generation of butyrate which is known to be beneficial for gut health of the host, and the proliferation of intestinal probiotics [60].

Synbiotics mean a mixture comprising live microorganisms and substrate(s) that confers a health benefit on the host. Synbiotics are divided into synergistic synbiotics and complementary synbiotics. Synergistic synbiotics are comprised of living bacteria and a selectively used substrate, and designed to work together, with the co-administered microbe using the substrate in a selected manner. On the other hand, complementary synbiotics are a combination of probiotics and prebiotics which can work independently to achieve one or more health benefits [61]. Depending on the circumstance, it is possible to create synbiotics that contain nanoprebiotics in a suitable form, and to construct a therapeutic strategy that is appropriate for the host's health.

Probiotics, prebiotics, and Vit D, the three representative immune modulators used in the alternative treatment for IBD. However, it has not yet been explored whether excessive immunological activity may be controlled by utilizing all three of these candidates at once. By adopting nano-delivery technology, Vit D, a hydrophobic drug, can be loaded into nanoprebiotics and then these nanoprebiotics are internalized into probiotics. Therefore, it is vital to investigate whether Vit D, probiotics, and prebiotics with immunomodulatory properties may be used effectively through nano-delivery technology to treat IBD symptoms.

II. Introduction

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a disease that causes chronic inflammatory state of the digestive tract and has an increasing incidence worldwide. Major symptoms of IBD include weight loss, severe diarrhea, rectal bleeding, fever, and abdominal pain, which are caused mainly by genetic, environmental, and immunological factors [62, 63]. Recently, investigations on the therapy of IBD have been intensively conducted, but unfortunately, there is currently no perfect cure for IBD. Most commercial drugs for IBD focus on slowing the onset or alleviating the symptoms by suppressing inflammatory responses [64]. However, due to the inefficiency of conventional drugs and their various side effects [65], researches on alternative therapies have recently increased [14]. Above all, because of their capacity to modulate the immune system, it has been reported that probiotics, dietary fiber, and Vit D could significantly reduce the symptoms of IBD [14, 18].

Probiotics are living microorganisms that provide the host for health benefits [34]. Probiotics improve the intestinal barrier function, modulate host immune system, and produce antimicrobial peptides [38]. Among the many probiotics, *Lacobacillus plantarum* is one of the most well-studied probiotics in humans and animals and is known to alleviate IBD symptoms by modulating host immune response and tight junction protein expression [41-44]. Although probiotics are the most frequently used alternative therapy, their mechanism of action in IBD is complex and currently poorly understood.

Prebiotics are non-digestible food ingredients that benefit host's health by acting as stimulants for the activity and proliferation of bacterial species found in the large intestine [66]. Among the various types of prebiotics, resistant starch (RS) is composed of two kinds of polysaccharide chains, amylose which possesses a linear structure with α-1,4 glycosidic linkage and amylopectin which possesses a branched structure with α-1,4 as well as α-1,6 glycosidic linkages [67]. RS, resistant to digestion by digestive enzymes, is fermented by microbes in the large intestine. It has beneficial effects primarily on gut health of host through increase of beneficial gut commensal bacteria and the production of SCFA such as butyrate [47, 68].

Synbiotics refer to a combination of live microorganisms and substrate(s) that are specifically used by host microorganisms that eventually causes health benefit of the host [61]. Indeed, the use of synbiotic treatment has suggested as an approach for combining the benefits of probiotics and prebiotics. A synbiotic treatment induces engraftment in mice, has beneficial effects in a colitis model [69], inhibits enteropathogens, and induces butyrate production in mice colonized with dysbiotic human microbiota [70].

Vit D is a fat-soluble that can be obtained from dietary supplements or internally synthesized by exposure to UV light [20]. Additionally, Vit D is known as an immune modulator that maintains immune homeostasis in the intestine and alleviates IBD symptoms by inducing proliferation of Tregs and production of anti-inflammatory cytokines [71, 72]. However, there is a problem with low bioavailability for use as conventional therapeutics due to short half-life and retention time, and low solubility in water [32].

Polymeric nanoparticles have several benefits, including biocompatibility, the capacity to overcome extracellular and intracellular barriers, and controlled cellular uptake, especially enhancing bioavailability and controlling the release of hydrophobic molecules such as Vit D. Recently, researches on nanoprebiotics prepared with polymeric nanoparticles have been actively conducted to increase the antibacterial efficiency of prebiotics. Nanoprebiotics prepared by inulin [56], dextran [57], pullulan [58], or resistant starch [59] through self-assembly mechanism were internalized into probiotics via time-, energy-, and/or transporter-dependent manner. Once internalized, the nanoprebiotics caused each probiotic to produce higher amount of anti-microbial peptides through an induction of mild stress than each probiotic itself, which modified the intestinal microbial composition to prevent harmful bacterial infection [57].

In this study, acetyl resistant starch nanoparticles (ARS NPs) and Vit D-loaded ARS NPs were prepared by a single emulsion method. It was examined whether ARS NPs and Vit D-loaded ARS NPs were internalized into LP as nanoprebiotics and whether Vit D could be released from Vit D-loaded acetyl resistant starch nanoparticles internalized in *L. plantarum* (LPSV) in a controlled manner. This study aimed to evaluate whether LPSV regulates intestinal barrier function and modulates the intestinal immune system in terms of tight junction protein expression and Tregs, alleviating colitis symptoms in mice.

III. Materials and Method

1. Materials

RS (mixture of 40 wt.-% of amylopectin and 60 wt.- % of amylose) was provided by Ingredion (IL, USA). The other chemicals were provided by Sigma-Aldrich (MO, USA). Lactobacilli MRS broth and agar were purchased from BD Difco™ (MD, USA) for bacterial culture.

2. Synthesis of acetyl resistant starch (ARS)

One gram of resistant starch (RS) was dissolved in 10ml of dimethyl sulfoxide (DMSO) at 54°C, and 0.1 mol% dimethyl aminopyridine (DMAP) per RS sugar residues as a catalyst and 10 ml of acetyl anhydride were added to the above solution. The reaction was done at 54°C for 48 h with nitrogen conditions. After the reaction, the above reactant was firstly dialyzed in DMSO to remove unreacted acetic acid and dialyzed in distilled water, and the obtained precipitates were freeze-dried.

3. Preparation of ARS NPs

ARS NPs were prepared by a single emulsion method. Briefly, 100 mg of ARS were dissolved in 5 ml of dichloromethane (DCM) to prepare the organic solution. The ARS solution was added dropwise into 16 ml of 2% (w/v) poly (vinyl alcohol) (PVA) and the mixture was sonicated with the ultrasonic processor (Sonics Vibra Cell™, Newtown, Australia) at 60 amplitude, 4 min, and 10-sec pulser. The emulsion was firstly dropped into 40 ml of 1 % (w/v) PVA solution and then stirred with an

IKA RW20 mechanical stirrer (IKA-Werke, Staufen, Germany) at 600 rpm for 4 h at room temperature to evaporate the DCM. The NPs thus formed were collected with ultracentrifugation at 14,000 rpm for 10 min and rinsed with distilled water three times.

4. Preparation of Vit D-loaded ARS NPs

Vit D-loaded ARS NPs were also prepared by a single emulsion method. The mixture of 4 µg of Vit D dissolved in 1ml of DMSO and 100mg of ARS dissolved in 5 ml of DCM was obtained to prepare the organic solution. The mixture solution was added dropwise into 10 ml of 2% (w/v) PVA and the mixture was sonicated with the ultrasonic processor at 60 amplitude, 4 min, and 10-sec pulser. The emulsion was firstly dropped into 40 ml of 1 % (w/v) PVA solution and then stirred with an IKA RW20 mechanical stirrer at 600 rpm for 4 h at room temperature to evaporate the DCM. The NPs thus formed were collected with ultracentrifugation at 14,000 rpm for 10 min and rinsed with distilled water three times.

5. Characterization of ARS NPs and Vit D-loaded ARS NPs

ARS synthesis and content of acetyl groups in the ARS were confirmed by 600 MHz [1]H-nuclear magnetic resonance (NMR) spectroscopy (AVANCE 600, Bruker, Germany). The morphologies of ARS NPs were observed by transmission electron microscopy (TEM) (Talos L120C, FEI, Czech). The sizes of ARS NPs were measured by a dynamic light scattering (DLS) spectrophotometer (DLS-7000, Otsuka Electronics, Osaka, Japan). The zeta potentials of ARS NPs were measured

by electron light scattering (ELS) spectrophotometer (ELS Z-1000, Otsuka Electronics, Osaka, Japan).

6. Confirmation of the internalization of ARS NPs and Vit D-loaded ARS NPs into _Lactobacillus plantarum_ (LP)

Fluorescence isothiocyanate (FITC)-labeled ARS NPs and Vit D-loaded ARS NPs were prepared ahead. 100 mg of ARS and 100 mg of Vit D were dissolved in 1 ml DMSO with 5 mg of FITC, respectively, and stirred at room temperature for 4 hours. The solutions were dialyzed with distilled water for 24 hours at 4°C and the pellets were harvested by ultracentrifugation and the freeze-dried to be stored at -20°C till use. FITC-labeled ARS NPs and FITC-labeled Vit D-loaded ARS NPs were prepared by the single emulsion method described above. _L. plantarum_ was treated with 0.5% (w/v) FITC-labeled ARS NPs and FITC-labeled Vit D-loaded ARS NPs for 2 hours at 37°C. The samples were washed with PBS and fixed using 10% paraformaldehyde. The internalization of Vit D-loaded ARS NPs and ARS NPs in _L. plantarum_ was observed using confocal laser scanning microscopy (CLSM) (SP8X STED, Leica, Wetzlar, Germany).

7. _In vitro_ Vit D release from Vit D-loaded ARS NPs internalized in LP

Dialysis bag method was used to study the _in vitro_ release profile of Vit D from Vit D-loaded ARS NPs internalized in the _L. plantarum_. The release was measured in PBS. The _L. plantarum_, Vit D-loaded ARS NPs, and Vit D-loaded ARS NPs internalized in _L. plantarum_ were suspended in PBS (pH 7.4) with 0.01% (v/v)

Tween 80 and sealed into dialysis bags (molecular weight cut-off with 12-14 kD, Repligen Corporation, AS, USA). The bags were then immersed into 40 ml of the PBS (pH 7.4) and were incubated in a shaking incubator at 37°C at a rate of 100 rpm. At specified time intervals (h), 1 ml of the sample was withdrawn and replaced with fresh buffer. The amount of released Vit D was quantified spectrophotometrically at 265 nm. Vit D release measured at 265 nm was normalized by that of the LP group [73, 74].

8. Preparation of LP, LP internalized with ARS NPs (LPSA), LP internalized with Vit D-loaded ARS NPs (LPSV)

All groups were prepared as a lyophilized powder form. The LP group was prepared a single dose of 2 x 10^8 CFU *L. plantarum*. The LPSA and LPSV groups were prepared by single dose of ARS NPs (0.5 wt. %) treated LP or Vit D-loaded ARS NPs (0.5 wt. %) treated LP for 2 hours at 37°C.

9. Cell culture

Human intestinal epithelial cell lines, Caco-2, were obtained from the American Type Culture Collection (VA, USA) and cultured in complete high glucose Dulbecco's modified Eagle medium (DMEM) (Gibco, ON, Canada), supplemented with 10% heat-inactivated fetal bovine serum (Gibco), MEM Non-Essential Amino Acids (Gibco), and 1% penicillin/streptomycin (Invitrogen, AS, USA). The cells were grown at 37℃ in a 5% CO_2-humidified incubator. For differentiation, Caco-2 cells were seeded at 1×10^5 cells/mL in 500 μL DMEM medium, as described above,

on 12-mm Transwell® with 0.4 μm pore polycarbonate membrane insert (Corning, NY, USA); the basolateral side was filled with 1 mL DMEM. The medium was changed 3 times a week and the cells were cultured for 21 days to allow full differentiation. The cell monolayers were kept in the serum-free medium overnight before the experiments, to avoid possible interferences with serum proteins [75, 76].

10. Tight junction protein analysis

In order to examine the effect of LP, LPSA, LPSV, ARS NPs, and Vit D-ARS NPs on tight junction proteins, Caco-2 cells were seeded at 5×10^4 cells/well in a 96-well plate for 24 h until reach the confluence. The cells were then treated with 1/10,000 diluted LP, LPSA, LPSV, ARS NPs, or Vit D-loaded ARS NPs for 24h at 37°C and washed with PBS. Then, the cells were fixed and stored for 20 min at room temperature in a fixation buffer (eBiosciences, CA, USA). The cells were washed with permeabilization buffer (eBiosciences) and stained with primary polyclonal antibodies to rabbit anti-ZO-1, -occludin, and -claudin-3 IgG (all from Invitrogen) for 20 min at 4°C. Then, the cells were rinsed with a permeabilization buffer and incubated with Goat anti-rabbit IgG APC (Abcam, CB, UK) [77]. All samples were acquired with a FACS Canto II (BD Biosciences, NJ, USA) and the data were analyzed by using FlowJo software (Tree Star, OR, USA).

11. Cell viability analysis

The attached cells were trypsinized for 5 min and then PBS-washed. And the cells were stained with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Thermo

Scientific, AS, USA) for 20 min at 4℃ in the dark. After surface staining, all samples were washed and measured with a FACS Canto II (BD Biosciences) and the data were analyzed by using FlowJo software (Tree Star).

12. Cell permeability assessment

Cell membrane permeability was assayed by measuring the transepithelial electrical resistance (TEER) and expressed as Ohm (resistance) X cm^2 (surface area of the filter). TEER was monitored every three days and the differentiation of the cells was confirmed by measuring TEER (> 1000 Ω/cm2) with EVOM2 (World Precision Instruments, FL, USA). The differentiated Caco-2 cells were treated with 1/10,000 diluted LP, LPSA, LPSV, ARS NPs, and Vit D-loaded ARS NPs for 24h. Then, the medium containing 1% DSS (wt%) was replaced to induce inflammation. During the experiments, TEERs were monitored every 60 min [75, 76].

13. Animal experimental procedures and measurements

Female, six-week-old C57/BL6 (B6) mice from Raon Bio (Yongin, Korea) were purchased. All the experimental procedures were conducted in accordance with the guidelines provided by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University, Seoul, Korea (Approval No. SNU-211019-2-1). Mice were administered with PBS (200 μl/day), LP (2 x 10^8 CFU/200 μl/day), LPSA (2 x 10^8 CFU/200 μl/day), and LPSV (2 x 10^8 CFU/200 μl/day) using feeding needle for 14 days. To induce acute colitis, mice were provided by 2.5% (w/v) DSS (molecular weight 36-50 kDa, MP Biomedicals, CA, USA) in drinking water for the last 7 days.

Daily clinical assessment of DSS-treated animals included body weight loss measurement, stool consistency, and detection of blood in the stool. Experimental samples were collected on day 7 after the DSS treatment.

14. Histology

For histological analysis, large intestine taken from mice were perfused and fixed with 4 % paraformaldehyde and embedded into paraffin block for hematoxylin and eosin (H&E) staining. Samples were examined under the light microscopy (Leica Microsystems, Wetzlar, Germany). All clinical and histological evaluations were performed in blinded manner.

15. Gut permeability test

FITC-dextran (molecular weight 4 kDa, Sigma-Aldrich) was dissolved in PBS, then administered to mouse with 400 μg/g of body weight by using oral gavage. All mice used in the study were fasted for 4 hours before the administration of the FITC-dextran. Blood samples were collected by eye bleeding into heparinized tubes at 4 hours after the administration. Blood samples were centrifuged, and serum were collected for the measurement of FITC by using a multiple plate reader (Perkin Elmer, AS, USA) at 485 nm for excitation and 528 nm for emission wavelength. Concentration of FITC-dextran in serum was calculated by using a standard curve.

16. Quantitative real-time PCR

Total RNA was extracted from mouse colon tissue using the RNeasy Plus mini kit (QIAGEN, Hilden, Germany). Lithium chloride purification was performed to exclude the possibility of DSS contamination that might decrease the activity of polymerase and reverse transcriptase [78]. For quantitative real-time PCR, cDNA was synthesized from 1 µg total RNA using M-MLV reverse transcriptase. Real-time qRT-PCR was performed using a StepOne Plus real-time PCR system (Applied Biosystems, AS, USA). The PCR reaction was conducted in a 96-well reaction plate using 9 µL SYBR green PCR Master Mix, 1 µL each of forward and reverse primers, 1 µL cDNA template, and 8 µL nuclease-free H_2O. The PCR conditions included 40 thermal cycles of 2 min at 50°C, 10 min at 95°C, 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Relative quantification of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ method. Target gene expression was normalized to *TATA-binding protein (TBP)* mRNA level. Primer sequences used in the experiment are shown in Table 1.

Table 1. Nucleotide sequence of the real-time PCR primers

		Nucleotide Sequence (5' → 3')	Gene ID	Size (nt)
TBP	F	CAAACCCAGAATTGTTCTCCTT	21374	22,802
	R	ATGTGGTCTTCCTGAATCCCT		
Occludin	F	GTCCGTGAGGCCTTTTGA	18260	73,079
	R	GGTGCATAATGATTGGGTTTG		
IFN-γ	F	TGAACGCTACACACTGCATCTTGG	15978	6,303
	R	TGACTCCTTTTCCGCTTCCTGAG		
TNF-α	F	GGCAGGTCTACTTTGGAGTCATTGC	21926	3,433
	R	ACATTCGAGGCTCCAGTGAATTCGG		

17. Examination of Tregs population by using flow cytometry analysis

In order to examine population changes of Tregs, mLN and pLN were isolated from the mice and single cells were prepared. Single cells were stained with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Thermo scientific), anti-mouse CD4-PE, CD25-PE-cy7, CD3-BV605 (all from BD Biosciences), CD8-PerCP-Cy5.5 (TONBO Bioscience, CA, USA). After surface staining, cells were fixed and stained with anti-mouse Foxp3-APC mAb (Biolegend, CA, USA) using Foxp3 Fix/Perm Buffer set (Biolegend). The expression of fluorescence was measured with a FACS Canto II (BD Biosciences) and the data were analyzed by using FlowJo software (Tree Star).

18. Statistical Analysis

The levels of significance for comparison between samples were determined by ANOVA test by GraphPad Prism software (Ver 7, GraphPad). The data in the graphs were expressed as the mean ±SEM. Significant differences using one-way ANOVA followed by Tukey test for multiple comparison were compared at $P < 0.05$.

IV. Results

1. Synthesis and characterization of ARS NPs and Vit D-loaded ARS NPs

The reaction scheme for ARS synthesis is shown in Figure 5A. To prepare the ARS NPs, acetyl groups were introduced into resistant starch. The hydroxyl groups in resistant starch and the carboxylic groups in acetyl acid obtained by acetic anhydride were reacted via esterification. The synthesis of ARS was confirmed by ^{1}H-NMR measurement (Figure 5B). Peaks of 1.9-2.1 ppm were assigned to acetyl protons, and peaks of 4.58-5.50 ppm were assigned to sugar protons, which belong to the C_1 position at α-1,6 and α-1,4 glycosidic bonds, respectively, in the NMR spectrum of ARS. The degree of substitution was confirmed as 7.1 [79].

ARS NPs and Vit D-loaded ARS NPs were prepared by using a single emulsion method. As shown in Figure 5E, the morphologies of both ARS NPs and Vit D-ARS NPs, as observed by TEM were spherical with diameters of around 150 nm. It was noting that due to the retention of the bound water in the ARS NPs to form hydrogen bonds among hydroxyl groups in the ARS NPs in the dry state, ARS NPs and Vit D-loaded ARS NPs appear to aggregate [80]. The particle sizes of ARS NPs and Vit D-loaded ARS NPs measured through DLS were 173.4 nm with polydispersity index of 0.096, and 208.3 nm with polydispersity index of 0.207, respectively, and the particles were well dispersed (Figure 5C). The surface charges of ARS NPs and Vit D-loaded ARS NPs measured through ELS were -17.46 mV and -23.11 mV, respectively due to the unreacted carboxylic acids in the ARS NPs and Vit D-loaded ARS NPs (Figure 5D).
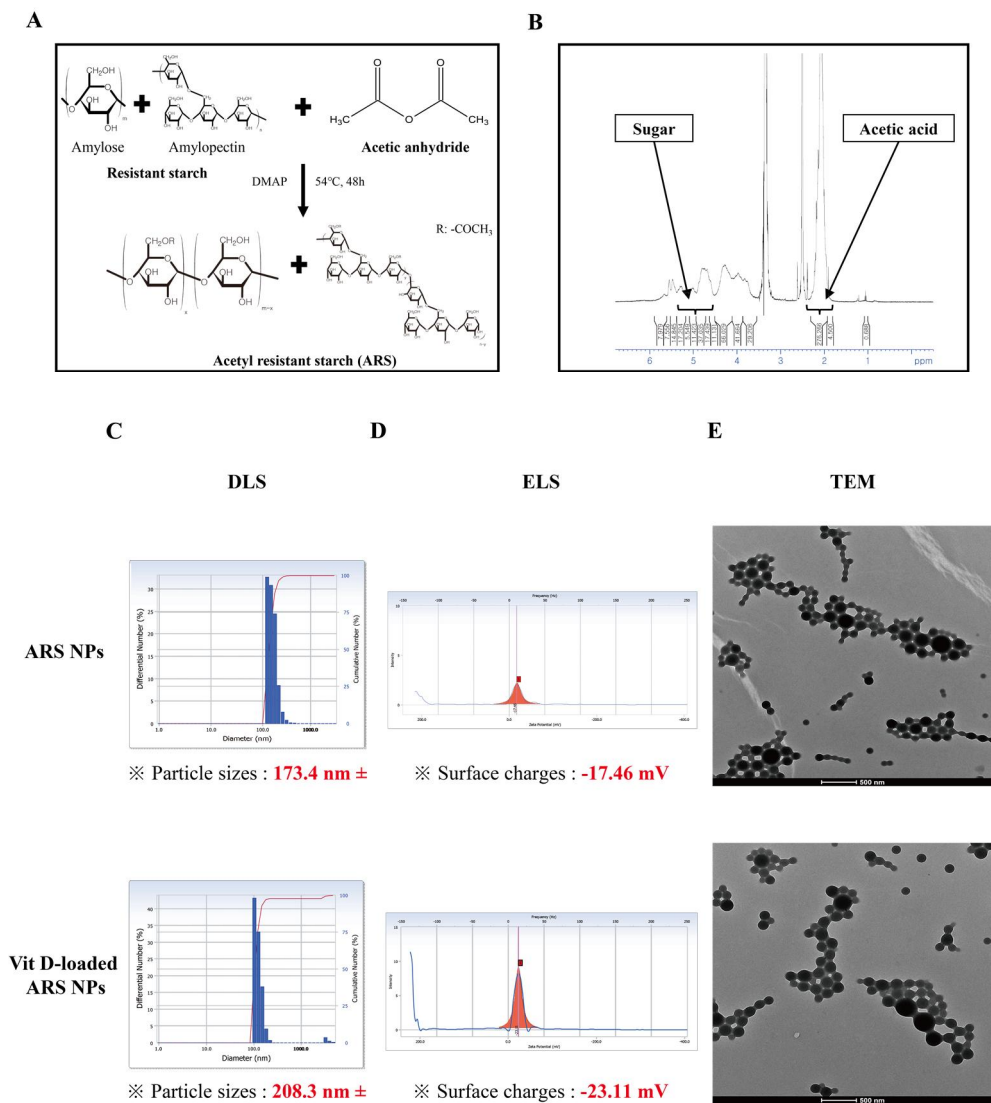
2 9

A

Amylose   Amylopectin   +   Acetic anhydride

Resistant starch

DMAP   54℃, 48h

R: -COCH₃

Acetyl resistant starch (ARS)

B

Sugar   Acetic acid

C   **D**   **E**

DLS   ELS   TEM

ARS NPs

※ Particle sizes : **173.4 nm ±**   ※ Surface charges : **-17.46 mV**

Vit D-loaded ARS NPs

※ Particle sizes : **208.3 nm ±**   ※ Surface charges : **-23.11 mV**

Figure 5. Characteristics of ARS NPs and Vit D-loaded ARS NPs

(A) Chemical reaction scheme for the synthesis of ARS. (B) Confirmation of synthesis of ARS by ^{1}H-NMR spectroscopy. Measurement of (C) particle size observed by DLS and (D) zeta potentials observed by ELS of ARS NPs and Vit D-loaded ARS NPs. (E) The morphologies of ARS NPs and Vit D-loaded ARS NPs was observed by using TEM. Scale bar = 500 nm.

2. Internalization of ARS NPs and Vit D-loaded ARS NPs by LP, and release of Vit D from Vit D-loaded ARS NPs and LPSV

To investigate the internalization, ARS and Vit D were conjugated with fluorescence isothiocyanate (FITC) and nanoparticles were prepared by the single emulsion method. Then, the internalization of FITC-labeled ARS NPs and FITC-labeled Vit D-loaded ARS NPs in LP for 2h was analyzed by confocal laser scanning microscopy (CLSM). As shown in Figure 6A and B, the CLSM showed that FITC-labeled ARS NPs and FITC-labeled Vit D-loaded ARS NPs were found at the same location as in the LP stained with 4′,6-diamidino-2-phenylindole (DAPI), indicating that ARS NPs and Vit D-loaded ARS NPs were internalized into LP.

To further verify Vit D release from the Vit D-loaded ARS NPs and LPSV, the dialysis was performed in a shaking incubator at 37°C for 72h. Released Vit D was measured at 265nm using UV spectra. The amount of released Vit D from other groups was normalized using the LP group. The amount of Vit D released from the LPSV was more released compared to the Vit D-loaded ARS NPs (Figure 6C).
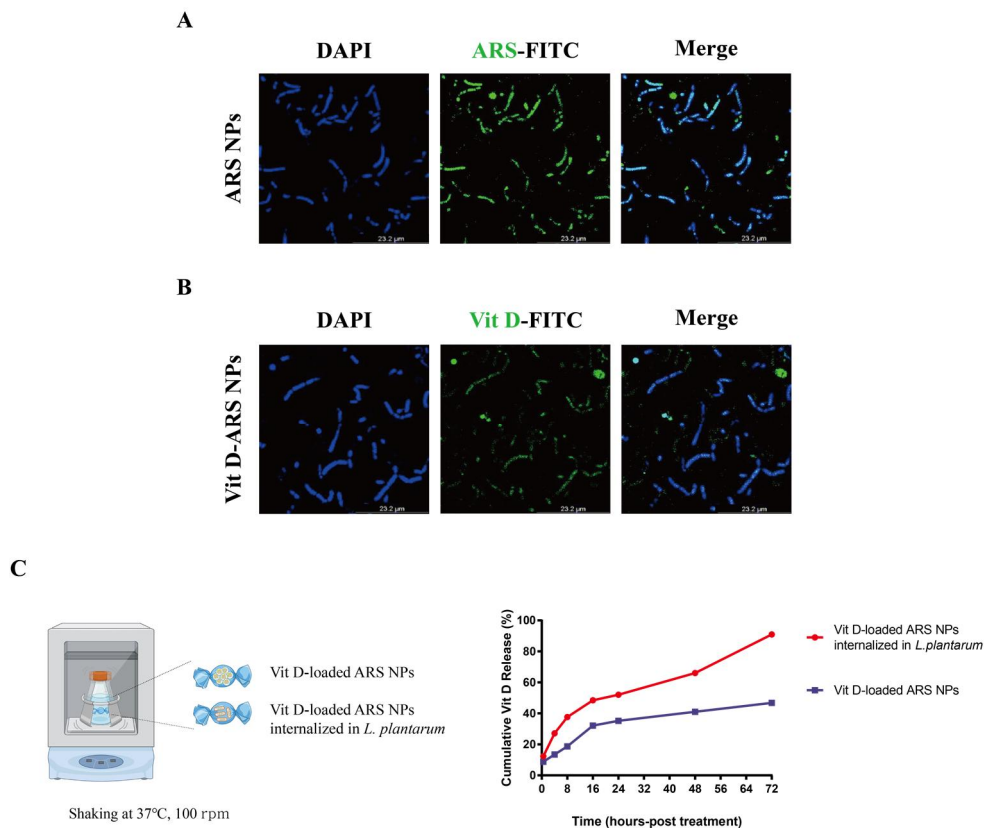
Figure 6. Confirmation of the internalization of ARS NPs and Vit D-loaded ARS NPs by LP and *in vitro* release profile of Vit D

The internalization of (A) ARS NPs and (B) Vit D-loaded ARS NPs was confirmed by CLSM. FITC-labeled ARS NPs or FITC-labeled Vit D-loaded ARS NPs are shown in green, and LP stained with DAPI are shown in blue. Scale bar = 23.2 μm. (C) Experimental scheme for the release of Vit D from Vit D-loaded ARS NPs and Vit D-loaded ARS NPs internalized in LP [left]. Cumulative release profile of Vit D at 37°C [right].

3. LPSV exerted a protective effect against DSS-induced membrane barrier damage

Cell viability was examined in Caco-2 cells treated with LP, ARS NPs internalized in LP (LPSA), Vit D-loaded ARS NPs internalized in LP (LPSV), ARS NPs, or Vit D-loaded ARS NPs. The results showed that treatment of LP, LPSA, LPSV, ARS NPs, or Vit D-loaded ARS NPs minimally, if any, affected survival of Caco-2 cells (Figure 7A). Next, differentiated Caco-2 cells were treated with LP, LPSA, LPSV, ARS NPs, or Vit D-loaded ARS NPs for 24 h, and the expression of tight junction proteins including claudin-3, occludin, and ZO-1 was examined. Claudin-3 expression was significantly increased in LPSA and LPSV treatment groups compared to other groups (Figure 7B). Occludin expression was significantly increased in LPSV treatment group as well as in cells treated with LP, LPSA, or Vit D-loaded ARS NPs (Figure 7C). On the other hand, the expression of ZO-1 was not affected (Figure 7D). These results suggest that LPSV treatment may efficiently improve intestinal barrier function by causing an increase in tight junction proteins like claudin-3 and occludin.

To evaluate protective effect against inflammatory responses, differentiated Caco-2 cells pretreated with LP, LPSA, LPSV, ARS NPs, or Vit D-loaded ARS NPs for 24 h were treated with 1% DSS for 5 h to induce inflammation. As shown in Figure 7E and F, the TEER relative level decreased by about 30% in all groups in which inflammation was induced, except for LPSV being marginally declined, and gradually recovered to about 80% at 5 h. The LPSV-treated group displayed a less severe decrease in TEER value, which was recovered to a level comparable to the

NT group at 5 h. Taken together, the results suggest that LPSV treatment could enhance the expression of tight junction proteins, which in turn has a protective effect against DSS-induced intestinal barrier damage.
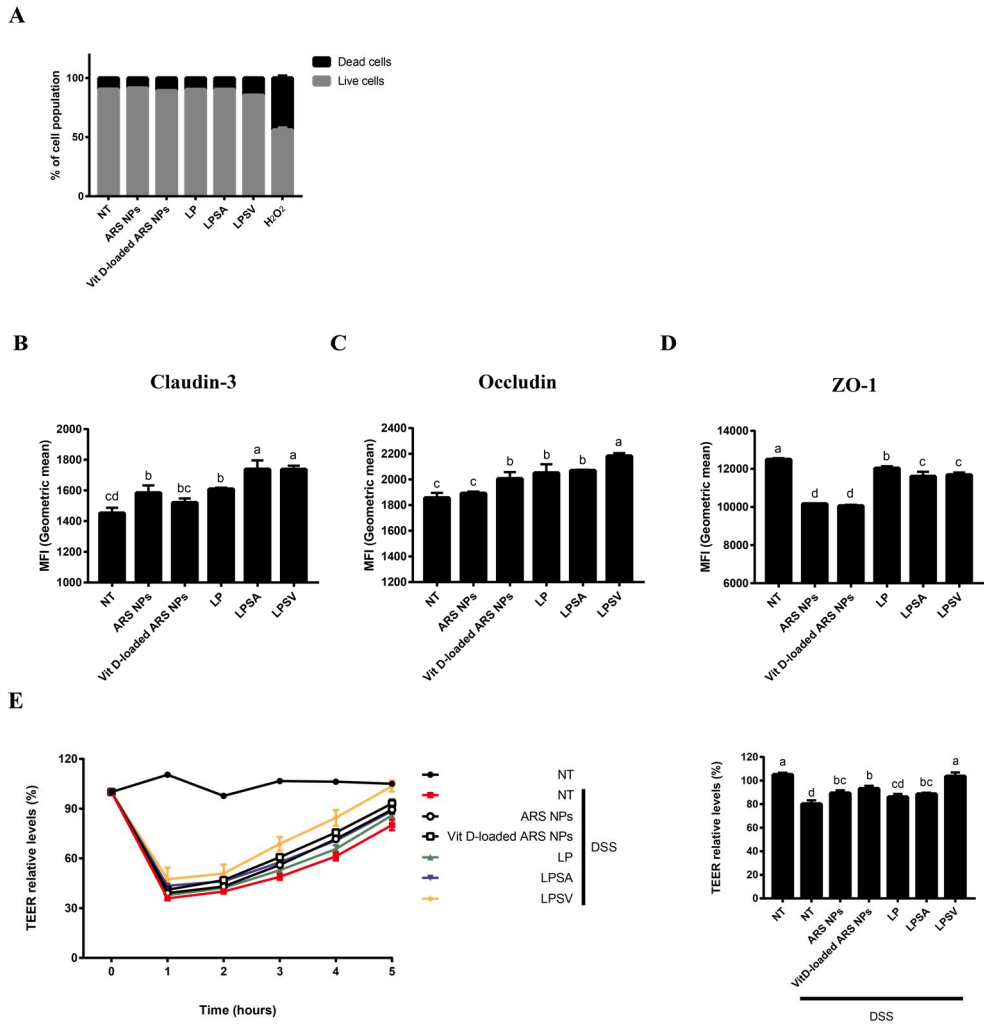
Figure 7. Protective effect of LP, LPSA, LPSV, ARS NPs, and Vit D-loaded ARS NPs against DSS-induced membrane damage in Caco-2 cells

Caco-2 cells were treated with LP, LPSA, LPSV, ARS NPs, and Vit D-loaded ARS NPs for 24h and (A) the percent of live cells was measured by flow cytometry. H_2O_2 treatment was used as a positive control. Tight junction proteins (B) Claudin-3, (C) Occludin, and (D) ZO-1 in Caco-2 cells were examined by intracellular staining using flow cytometry. (E) Differentiated Caco-2 cells were treated with LP, LPSA, LPSV, ARS NPs, or Vit D-loaded ARS NPs followed by DSS-contained media for 5 h. TEER was reported as Ohm $\times$ cm^2 for every 1h [left]. TEER relative levels (%) were compared in 5 h [right]. The results are presented as the mean $\pm$ SEM.

3 5

Significant differences using one-way ANOVA followed by Tukey's multiple comparison test are shown with different letter at P<0.05.

4. Administration of LPSV mitigated the symptom of colitis-induced mice

To determine whether LPSV can alleviate intestinal inflammation, acute colitis was induced in the mice pretreated with PBS, LP, LPSA, or LPSV. As shown in Figure 8A, mice were administered with PBS, LP, LPSA, and LPSV by intragastric route with 24 h interval for 14 days and treated with or without DSS for the last 7 days. As shown in Figure 8B, the DSS+PBS group, which were administered PBS for 14 days and treated with DSS for the last 7 days, have lost weight the most. However, the results showed that among the mice treated with DSS, the weight of LPSV group changed the least. As shown in Figure 8C-E, the length of the intestine was significantly reduced while the weight of the spleen was significantly increased in the DSS+PBS group. Length of the intestine was reduced the least in the LPSV group, and spleen weight was similar to that of the NT group in all groups except the DSS+PBS group being significantly increased. In the colon of DSS+PBS, crypt and villi was most severely damaged when compared to the other groups. However, colon damage in the LPSV group was restored to NT levels (Figure 8F).

Gut permeability has been assessed using FITC-dextran in mouse model [81]. To investigate whether LPSV enhances the gut barrier function, colitis was induced in mice treated with DSS, then gut permeability was examined by FITC-dextran in serum (Figure 8G). The results showed that the DSS-induced control group had the most severe barrier damage, and LP and LPSV group were also comparable in terms of intestinal barrier function as determined by FITC-dextran permeability. On the other hand, the barrier damages in the LPSV group were not as severe as in the NT group (Figure 8H). In order to investigate whether LPSV increases the expression of

tight junction proteins involved in the intestinal barrier function, colon tissue was analyzed by quantitative real-time PCR (qRT-PCR). As shown in Figure 8I, the expression of occludin was the lowest in the DSS+PBS group, but tended to increase in the LPSV group. The results showed that DSS-induced colitis significantly impaired intestinal barrier function, whereas LPSV enhanced the barrier function from damage done by intestinal inflammation. Taken together, these results suggest that LPSV administration effectively alleviate colitis symptoms.

A

		0	7	14	(Days)
C57BL/6					

NT	PBS treatment
PBS	PBS treatment
LP	*L. Plantarum*
LPSA	ARS NPs internalized in *L. plantarum*
LPSV	Vit D-loaded ARS NPs internalized in *L. Plantarum*

DSS

B

NT
PBS
LP
LPSA   DSS
LPSV

a
b
c
c
d

C

NT
PBS
LP   DSS
LPSA
LPSV

D

E

F

DSS

NT   PBS   LP   LPSA   LPSV

50μm

G

NT
PBS
LP   DSS
LPSA
LPSV

Fasting

(hours)

0   4   8

FITC-dextran
oral gavage (400ug/g)

Serum FITC-dextran
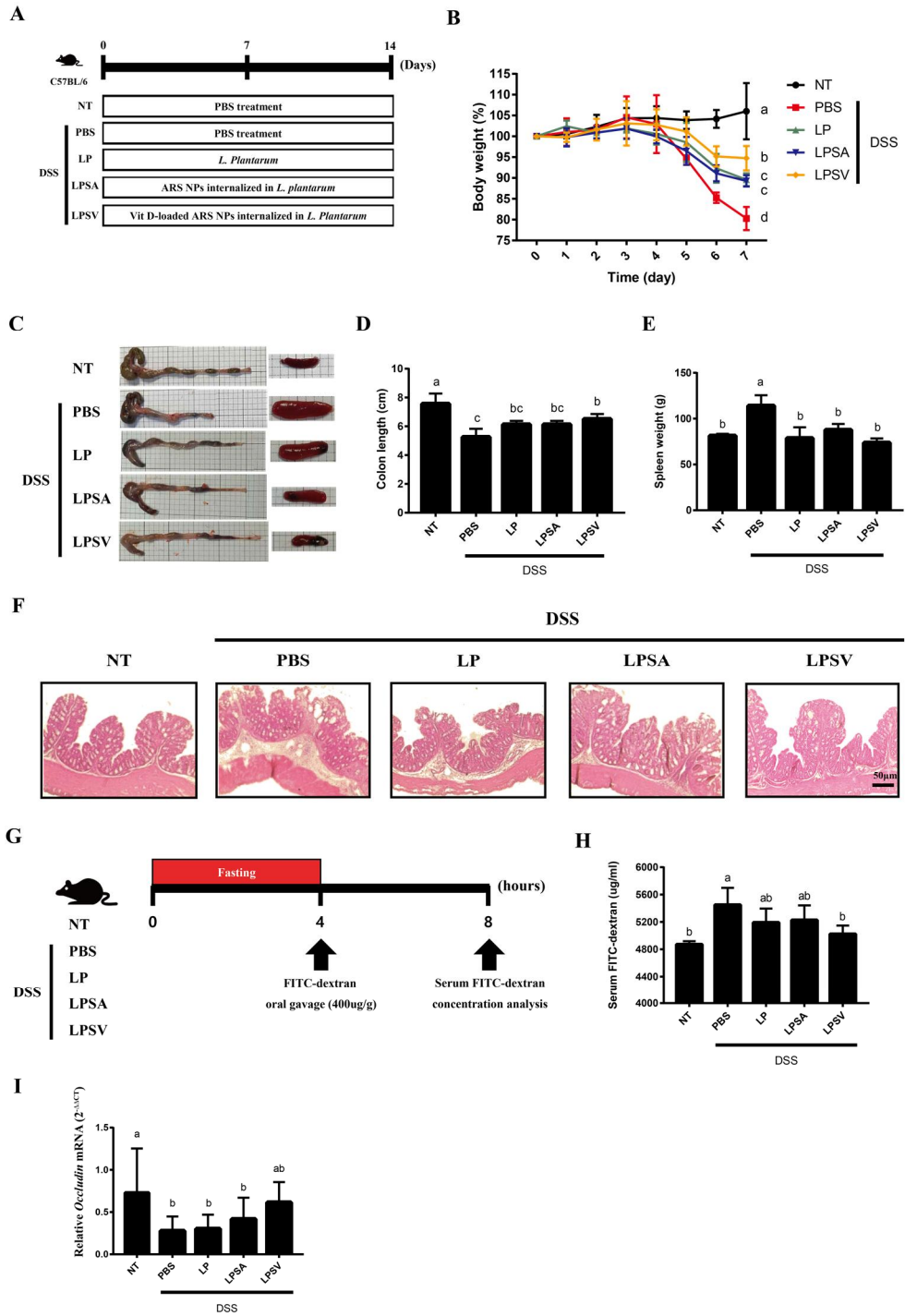concentration analysis

H

I

3 9

Figure 8. Amelioration effects in colitis-induced mice treated with LP, LPSA, or LPSV

(A) Experimental scheme for oral administration of PBS, LP, LPSA, and LPSV for 14 days and DSS for the last 7 days to induce colitis in C57/BL6 mice. The mice were divided into five groups, (1) PBS control (2) DSS+PBS (3) DSS+LP (2 x 10^8 CFU/day) (4) DSS+LPSA (2 x 10^8 CFU/day) (5) DSS+LPSV (2 x 10^8 CFU/day) (B) Body weight changes of mice during the last 7 days of DSS treatment. (C) The spleen and colon were taken and photographed. To determine inflammatory response, (D) spleen weight and (E) colon length was measured. (F) Colonic epithelium was stained by H&E staining. (G) Experimental scheme for gut permeability test. (H) Level of serum FITC-dextran was examined. (I) For cDNA synthesis, RNA was extracted from colons. Occludin, tight junction protein, was examined by using qRT-PCR. The results are presented as the mean ± SEM. Significant differences using one-way ANOVA followed by Tukey's multiple comparison test are shown with different letter at P<0.05.

5. Administration of LPSV enhanced the intestinal immunity in colitis-induced mice

To further investigate whether LPSV administration affects intestinal immunity, TNF-α and IFN-γ, pro-inflammatory cytokines, were quantified using qRT-PCR in the colon tissues of DSS-induced colitis mice. Expression of TNF-α was significantly decreased in the LPSV-treated group, when compared to other groups (Figure 9A). Expression of IFN-γ was significantly increased in the DSS+PBS treatment group while it was at a similar level in the NT, LP, LPSA, and LPSV treatment groups (Figure 9B).

Tregs are essential for maintaining immune homeostasis by suppressing excessive immune responses [82]. Thus, changes of Tregs from pLN and mLN in mice were examined by using flow cytometry. As shown in Figure 9C-F, Treg proliferation in pLN and mLN was significantly high in mice administered LPSV than in other groups. These results suggested that the administration of LPSV maintained immune homeostasis by decreasing pro-inflammatory cytokine expression through increased Treg proliferation.
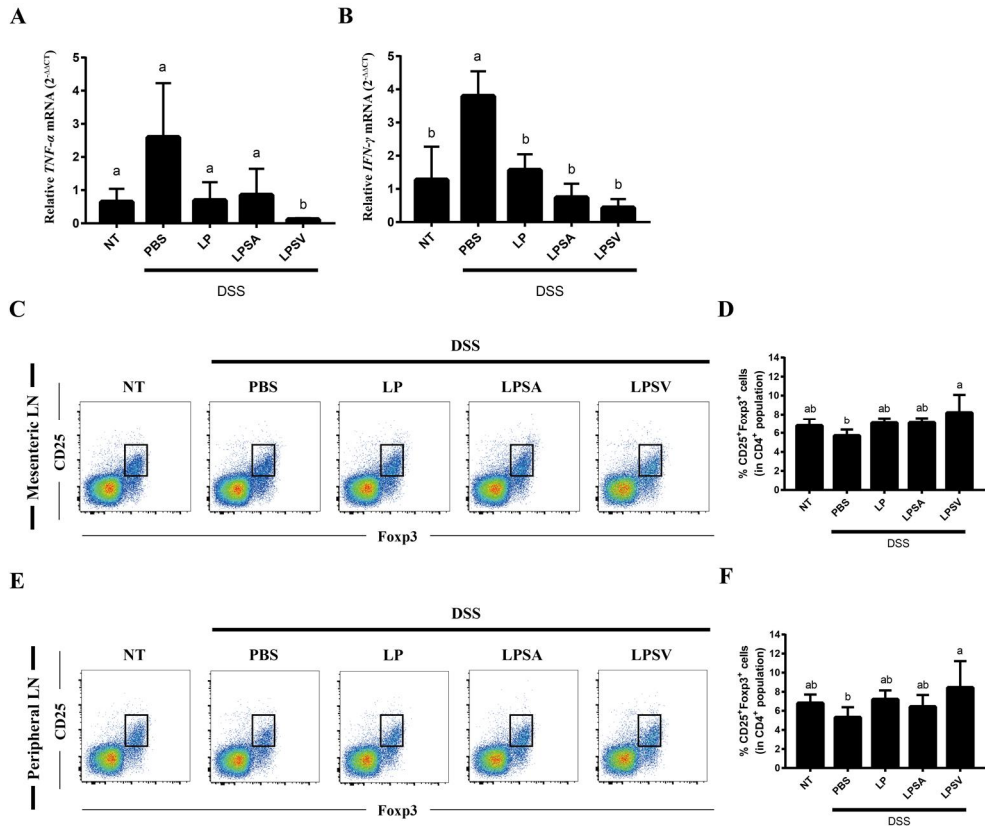
Figure 9. Analysis of cytokines and proliferation of regulatory T cells in colitis-induced mice treated with LP, LPSA, or LPSV

C57/BL6 mice were treated with PBS, LP, LPSA, and LPSV for 14 days and administered with DSS for the last 7 days to induce colitis. Then, colon, pLN, and mLN were collected and RNA was extracted for cDNA synthesis. (A) TNF-α and (B) IFN-γ were examined by using qRT-PCR. The percentages of Tregs (Foxp3$^+$CD25$^+$CD4$^+$ T cells) were analyzed by flow cytometry via intracellular staining of the cells from (C-D) mLN and (E-F) pLN. The results are presented as the mean ± SEM. Significant differences using one-way ANOVA followed by Tukey's multiple comparison test are shown with different letter at P<0.05.

V. Discussion

Due to the inefficiency and side effects of conventional treatment for IBD, research on alternative strategies is being actively conducted. In this study, synbiotics using LP as probiotics and Vit D-loaded ARS NPs as nanoprebiotics were devised for the purpose of alleviating colitis symptoms. Polymeric nanoparticles were prepared using resistance starch as nanoprebiotics. When phthalic resistance starch was used to prepare nanoparticles using the self-assembly method, which has been tried in the previous studies as nanoprebiotics, there were several drawbacks in that the hydrophobic drug loading efficiency and loading content were remarkably low. However, in the present study, when Vit D was loaded on polymeric nanoparticles with a single emulsion, the loading efficiency was almost 100%. Furthermore, low drug loading content by self-assembly method has been overcome (data not shown). To prepare acetyl resistance starch nanoparticles using the single emulsion method, ARS was synthesized by the reaction between the primary hydroxyl groups of the RS and carboxylic acids of acetic anhydride after ring-opening of acetic anhydride via an esterification mechanism. Then, Vit D-loaded ARS NPs were also prepared using a single emulsion method. It was confirmed that nanoparticles were prepared as suitable for nanoprebiotics, similar to the materials used in previous studies [56-59].

Previous studies have reported an observation on the internalization of polymeric nanoparticles into probiotics [56-59]. However, the mechanism for internalization of polymeric nanoparticles into prokaryotes as prebiotics rather than endocytosis into

mammalian cells [83] is not yet fully understood. In the present study, The internalization of ARS NPs and Vit D-loaded ARS NPs into probiotics was checked. It is important to note that three combinations of Vit D, probiotics, and prebiotics effects can simultaneously be used for inducing immunomodulatory effects via nano-delivery system.

Controlled release of drug delivery systems using polymeric nanoparticles have many advantages over conventional therapeutics, such as targeted and sustained delivery and enhanced bioavailability of hydrophobic drugs, which enable them to overcome pharmacological limitations [84]. Resistant starch nanoparticles are efficiently degraded by gut microbes, including firmicutes, and have been shown to increase butyrate production when combined with LP [60]. In this study, release of Vit D from Vit D-loaded ARS NPs internalized in LP was checked in vitro. Interestingly, Vit D was more released from Vit D-loaded ARS NPs internalized in LP than Vit D-loaded ARS NPs. The results suggested that probiotics functioned to accelerate the release of Vit D from Vit D-loaded ARS NPs internalized in LP, but further research is needed to check the exact mechanism why Vit D was more released from Vit D-loaded ARS NPs internalized in LP.

Epithelial tight junctions, responsible for maintaining the intestinal barrier, are constituted of various junctional substances. Among them, claudin and occludin are primary junctional regulators of the paracellular permeability of water, ions, and macromolecules into the body [85]. Damage to these junctions alters the mucosal immune system, for instance causing an inflammation, which can often lead to the onset of intestinal diseases [86]. In this study, LPSV treatment induced increase of

tight junction proteins and enhance protective and restorative effects in DSS-induced membrane barrier damage of intestinal epithelial cells. Based on these results, it is hypothesized whether LPSV can alleviate colitis.

IBD is thought to be caused by a disturbance in the intestinal barrier, which results in a change in the intestinal flora and an abnormal activation of the mucosal immune system [64]. An increase in pro-inflammatory cytokines is the initiation of chronic inflammation by inducing apoptosis of intestinal epithelial cells [87] and weakening the barrier function due to the reduction in tight junction proteins [65, 88]. For instance, IFN-γ could induce the decrease of occludin expression, which in turn increases permeability of epithelial barrier via the paracellular pathway [89]. In the present study, LPSV treatment improved gut barrier function by increasing occludin expression and decreasing pro-inflammatory cytokines, alleviating colitis. Furthermore, the differentiation of T cells into Tregs, which suppresses the differentiation of effector T cells that induce excessive immune responses, is essential for alleviating the symptoms of colitis [82, 90]. In the present study, colitis-induced mice treated with LPSV showed increase of Treg concordance with alleviation of excessive immune responses. When resistant starch nanoparticles were used with *L. plantarum*, bacterial growth and production of butyrate together were increased [60]. Butyrate is known to promote the differentiation of naive T cells into Tregs by increasing histone acetylation as histone deacetylase inhibitor and accelerating fatty acid oxidation [50]. On the other hand, tolerogenic DCs induced by Vit D treatment could cause the differentiation of naive T cells into Tregs [23]. The results suggested that LPSV administration could be used as a therapeutic

strategy to treat colitis through increase of Treg production and enhancement of tight junction protein expression.

It is seemingly necessary to further examine the microbial composition in multiple taxonomic levels in relation to the intestinal immunity. Dysbiosis in IBD patients commonly results in a decline in commensal microbial diversity and an increase in pathogenic bacterial species [91]. The microbiome, which is crucial for regulating effector T cell response and maintaining tolerance to microbial antigens [90], can be controlled by administering synbiotics containing nanoparticles. In the previous studies, synbiotics containing nanoparticles revealed the additive effects of enriching several well-known beneficial bacteria, including *Lactobacillus*, *Bifidobacterium*, and butyrate-producing bacteria, such as *Faecalibacterium* [57, 92]. Therefore, it is likely that LPSV contributes to changes in the intestinal flora, resulting alleviation of colitis, which should perform further investigation.

In summary, the present study showed that Vit D-loaded ARS NPs prepared by single emulsion methods can be internalized into probiotics and Vit D was more released from Vit D-loaded ARS NPs internalized in probiotics than Vit D-loaded ARS NPs. In addition, LPSV alleviated colitis symptoms through the regulation of intestinal barrier function by the expression of tight junction proteins and an increase of Tregs (Figure 10). Taken together, the results suggest that synbiotics with hydrophobic drug loaded-nanoprebiotics may be used as potential therapeutic agents and immune modulators to treat IBD symptoms.
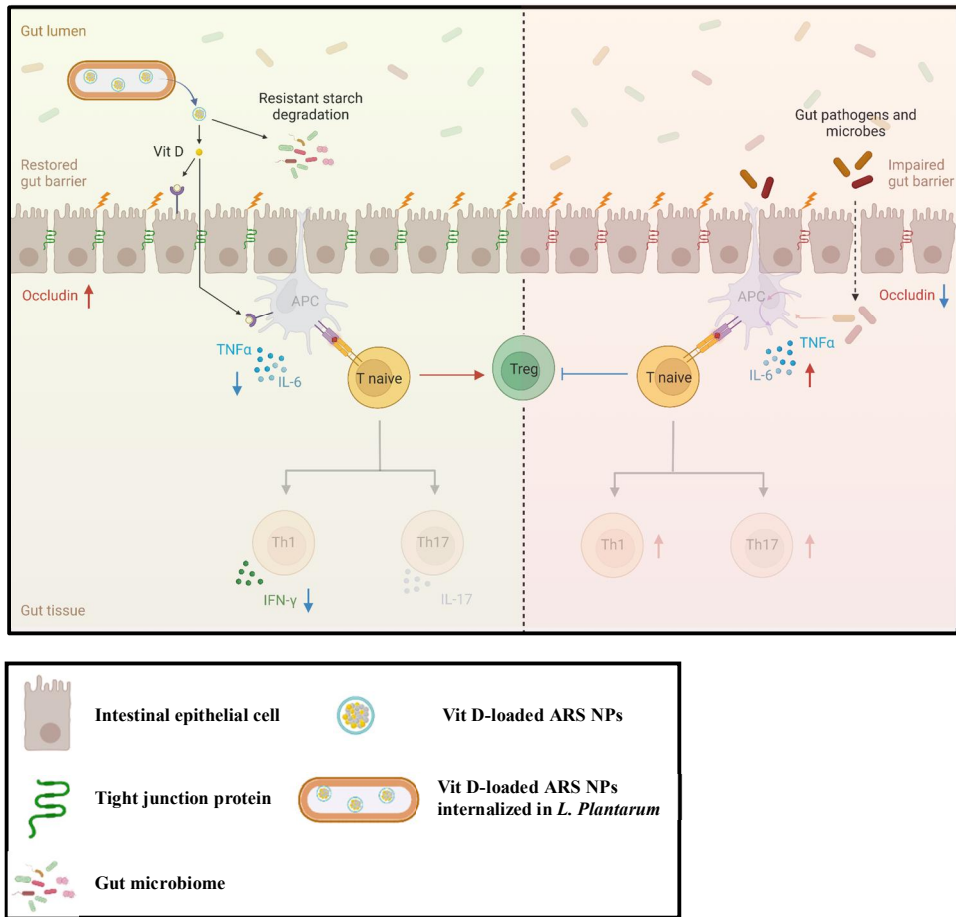
Figure 10. A postulation for immunological mechanism of LPSV in colitis-induced mice

Immune response to colitis in the intestines [right]. Synbiotics with Vit D-loaded acetyl resistant starch nanoparticles internalized in *Lactobacillus plantarum* induced the expression of tight junction proteins and the proliferation of Tregs, thereby alleviating colitis symptoms in mice [left].

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VII. Summary in Korean

본 연구에서는 프로바이오틱스, 나노프리바이오틱스, 면역 조절 물질을 조합한 신바이오틱스 활용을 통해, 장내 불균형한 면역반응으로 인해 염증이 유발된 장내에서 대장염 완화에 기여할 수 있음을 살펴보았다. 신바이오틱스는 살아있는 미생물인 프로바이오틱스와 숙주의 미생물에 의해 이용되어 건강상의 이점을 주는 난소화성 물질인 프리바이오틱스의 조합을 의미한다. 최근 프리바이오틱스의 기능을 향상하기 위한 목적으로 나노 프리바이오틱스의 활용도가 높아지고 있으며, 해당 연구에서는 면역 조절 물질로 알려진 Vit D 를 난소화성 전분 나노입자에 담지하여 나노 프리바이오틱스로 활용하였다.

Vit D 와 난소화성 전분 입자는 싱글 에멀전 방식을 통해 나노입자로 형성되었으며, 해당 나노입자는 락토바실러스 플란타룸에 내재화되어 신바이오틱스로 이용되었다. Vit D 가 담지된 난소화성 전분 나노입자가 내재화된 락토바실러스 플란타룸 (LPSV)에서 Vit D 가 담지된 나노입자보다 Vit D 방출량이 더 증가하였음을 확인할 수 있었다. 또한 인간 대장 선암 세포주 (Caco-2) 와 DSS 로 장염이 유발된 마우스 모델에서 LPSV 의 효능을 검증하였다.

LPSV 는 Caco-2 세포 내에서 밀착 연접 단백질을 증가시켜 단층 세포 투과성에 대한 DSS 의 유해한 영향을 완화했다. 또한 대장염이 유발된 마우스에서 몸무게 변화, 장 길이, 비장 무게, 대장의 조직검사, 장내 투과성 검사를 통한 현상적 비교를 진행하였을 때, 흥미롭게도 LPSV 를 처리한 그룹에서 음성대조군과 비슷한 수준으로 대장염이 완화되었음을 확인할 수 있었다. 또한 장에서 밀착 연접 단백질인 occludin 이 증가하였으며, 종양괴사인자 (Tumor necrosis factor)-α와 인터페론 (Interferon)-γ 와 같은 전염증성 사이토카인이 감소하였다. 이와 더불어 말초 림프절과 장간막 림프절에서 조절 T 세포의 비율이 증가함을 확인할 수 있었다. 결론적으로, Vit D 가 담지된 난소화성 전분 나노입자가 내재화된 락토바실러스 플란타룸은 장벽 강화 및 면역 항상성 유지를 통해 대장염 완화에 기여하며, 이는 염증성 장 질환에 대한 잠재적 치료제로 활용될 가능성을 시사한다.