A Dissertation

for the Degree of Doctor of Philosophy

Research on synbiotic combination of *Lactobacillus* spp. and polysaccharides for enhancing protective function of intestinal barrier

장벽 보호 기능 강화를 위한 락토바실러스 종과 다당류의 신바이오틱스 조합에 대한 연구

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Summary

For digestion the food and absorption the nutrient, mammals have developed a very complex and highly specialized gastrointestinal system maintained by the intestinal barrier. The gastrointestinal epithelium forms the body's largest interface with the external environment, and the intestinal barrier separates our body from the external environment. However, in addition to the function of absorption, the intestinal barrier also faces a large number of external antigens, including food particles, commensal bacteria, virus, and toxins. The intestinal barrier function is required to prevent the entry of multiple external antigens while absorbing nutrients. The first line of this barrier is benefit commensal bacteria like *Lactobacillus*.spp. The second line of this barrier is maintained only by a special layer of epithelial cells that are linked together by tight junction proteins. Many other factors help support this barrier, including mucins, antimicrobial peptides, and immunoglobulin A. The third line of this barrier is made up of immune cells (dendritic cells, macrophage and B cell). Under pathological conditions, the permeability of the intestinal barrier may be compromised, allowing the

toxins, antigens, and bacteria in the lumen to enter the bloodstream, and causes a “leaky gut syndrome”.

Growing evidence shows that gut microbiota is very important in supporting the intestinal barrier and plays a key role in regulating environmental factors that enter the bloodstream. Recent reports indicate that probiotics can enhance the expression of tight junction proteins to reverse tight junction proteins by its own fermentation of short chain fatty acids and also have antimicrobial ability through their antimicrobial molecules (lactic acid and bacteriocin). Given that prebiotics increase the growth and/or activity of probiotics in the gut and have beneficial health effects on the host, many researchers have chosen prebiotics to increase the growth or activity of probiotics. In order to improve the protective function of the intestinal barrier, a novel synbiotic combination was selected to inhibit the pathogenic bacteria and promote the formation of the tight junction.

In the first study, through the test of the API 50 CH kit as a standardized system associating 50 biochemical tests for the study of the carbohydrate metabolism of microorganisms, five kinds of *Lactobacillus.spp* were used as probiotics to select their corresponding prebiotics. The results showed that

twenty-four kinds of carbohydrate can be fermented by LAB. Among them, the carbohydrate which cannot be used as a prebiotic, seven kinds of prebiotics or their building block were selected as candidates. Next, three kinds of synbiotic combinations were selected in terms of the total fermentation of short-chain fatty acids and the additional effect of prebiotics. In order to validate the efficacy of the synbiotics, a leaky gut cell model was constructed by treating LPS (100μg/ml) for twenty-four hours. And then, the SCFA in cell free supernatant of three kinds of synbiotics were quantified. The selected prebiotics showed increased fermentation of acetate by the lactic acid bacteria. However, the pullulan itself increased the fermentation of propionate and butyrate by the *Lactobacillus plantarum*. And the results of synbiotics functional assay *in vitro* showed that the combination of *Lactobacillus plantarum*(LP) and pullulan(P) had a significant positive effect on the expression of tight junction protein mRNA（ZO-1 and claudin-1) compared with *Lactobacillus plantarum* alone, also in TEER value change. It was found that butyrate played the most critical role in the formation of the tight junction so that LP/P was selected as a synbiotic combination.

In the second study, pullulan was used to make three kinds of phthaly-pullulan nanoparticles (PPNs) because previous studies have shown that prebiotic nanoparticles can improve the antimicrobial activity of lactic acid bacteria. The PPNs were developed as prebiotics, and their effect were observed on the cellular and antimicrobial activities of LP were investigated. The antimicrobial activities against pathogens were tested after the treatment with those nanoparticles by LP. All three types of pullulan nanoparticles increased the antimicrobial property of LP. Therefore, a mechanism study was performed to examine how the PPNs could increase the antimicrobial property of LP. The internalization of the PPNs into LP was firstly assessed. The internalization of the PPNs was mostly regulated by galactose transporters in LP, and the process was energy-dependent. After the internalization of the PPNs, a substantial amount of antimicrobial peptide (plantaricin) was produced by LP. Furthermore, the higher amounts of plantaricin could be more effective against both Gram-positive (*L. monocytogenes*) and Gram-negative (*E.coli K99*) pathogens than LP alone or

LP treated with pullulan. The increase in plantaricin expressioin in LP treated with PPNs was accompanied by the enhanced expression of stress

response genes (*danJ* and *dnaK*) and plantaricin biosynthesis genes (*planS*). Overall, the results suggest that the internalization of the PPNs by LP causes mild stress in LP through the defense mechanism which leads to an increase of plantaricin production.

Form the results of study 1 and 2, in study 3 i*n vivo* validation of novel synbiotic combinations including LP, pullulan and PPNs was studied. This combination enhanced antimicrobial ability against pathogenic *E.coli* by enhancing microbial barrier function and they also reduced the FITC-dextran and endotoxin going into serum through enhanced physical barrier. Also, pullulan and PPNs treatment changed the composition of gut microbiota increasing *Lactobacillus*.spp and *Bifidobacterium*.spp *in vivo*.

The results of study suggested that pullulan and PPNs treated in LP enhanced the intestinal barrier function, by the synbiotic combination including LP, PPN, and pullulan can be a therapeutic agent to modulation the intestinal barrier function.

Keywords: Probiotics, Prebiotics, Synbiotics, SCFA, Bacteriocin, Tight junction, Antimicrobial ability, Intestinal barrier function.

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List of Abbreviations

ABC: Antibiotic cocktail

CFU: Colony forming unit

CFS: Cell free supernatant

CLSM: Confocal laser scanning microscopy

DAPI: 4',6-diamidino-2-phenylindole

DLS: Dynamic light scattering

DSS: Dextran sulfate sodium

ELS: Electrophoretic light scattering

FASC: Fluorescence-activated cell sorting

FITC: Fluorescein isothiocyanate

GIT: Gastrointestinal tract

H-NMR: Nuclear magnetic resonance

HSP: Heat shock protein

IBD: Inflammatory bowel disease

IgA: Immunoglobulin

IPEC-J2: Intestinal porcine epithelial cells from jejunum

LAB: Lactic acid bacteria

L. monocytogenes: *Listeria monocytogenes*

LPS: Lipopolysaccharide

LP: *Lactobacillus plantarum*

LRe: *Lactobacillus reuteri*

LPa: *Lactobacillus paraaceri*

LS: *Lactobaciilu salivarius*

LRh: *Lactobacillus rhamnosus*

qRT-PCR: Quantitative real-time polymerase chain reaction

P: pullulan

PPN: phtahlyl pullulan nanoaparticls

RT-PCR: Quantitative real-time polymerase chain reaction

SCFA: Short chain fatty acid

SEM: Scanning electron microscope

TJ: Tight junction

TNF: Tumor necrosis factor

TEER: Trans-epithelial electrical resistance

LP/P: Combination of *Lactobacillus plantarum* and pullulan

LP/PPNs: Combination *of Lactobacillus plantarum* and pullulan nanoparticles

LP/P/PPNs: Combination of *Lactobacillus plantarum*, pullulan and pullulan nanoparticles

Introduction

The gastrointestinal epithelium is the largest exchange surface between the host and the external environment (Brandtzaeg, 2011), it is composed of a monolayer of intestinal epithelia cells, a mucus layer, a microbial layer, and an immunological layer. The intestinal barrier enables the absorption of nutrients in the diet and prevents the passage of pathogens and toxins into systemic circulation. It is a well-known truth that an intact and healthy intestinal barrier is necessary for optimal health (Neunlist et al., 2013). The intestinal barrier defects is characterized by increased intestinal permeability and it is positively correlated with a variety of gastrointestinal dysfunctions and diseases. In humans, an impaired intestinal barrier function is associated with a wide range of diseases, such as inflammatory bowel disease(Turner, 2009), necrotizing enterocolitis (Clayburgh et al., 2004), diabetes mellitus (Vaarala, 2008), and rheumatic diseases (Weber et al., 2003). In pigs, pathogenic enteric bacteria like *E.coli*, mycotoxin, and various stresses especially weaning stress, are potent disruptors of the intestinal barrier function, leading to impaired growth and digestive disorders, diarrhea and

other gastrointestinal disorders (Vellenga et al., 1992;Peace et al., 2011;Hu et al., 2012). In poultry, *Salmonella* infection, toxins and stress induce and increase the intestinal epithelial permeability, resulting in nutrient malabsorption, mortality and potential human foodborne salmonellosis (Quinteiro et al., 2010;Awad et al., 2012;Murugesan et al., 2015). Intestinal epithelial cells are mainly sustained by tight junction proteins. Tight junctions hold adjacent epithelia cells at the apical side of the later membrane and anchor transmembrane proteins (claudin and occludin) to intracellular actin cytoskeleton (Hammer et al., 2015). They have a crucial role in the maintaining the intestinal integrity (Gumbiner, 1996;Ashida et al., 2012). Growing evidence shows that the gut microbiota is important in supporting the intestinal barrier and has a key role in regulating environmental factors that enter the bloodstream. Therefore, intestinal tight junctions and a healthy gut microbiota are considered as therapeutic targets for the modulation of the intestinal barrier function and the prevention of various gastrointestinal diseases.

Recent reports have indicated that probiotics can enhance the expression of tight junction proteins to reverse tight junction proteins by their own fermentation of short chain fatty acids and also have an antimicrobial ability through their antimicrobial molecules (lactic acid and bacteriocin). Given that prebiotics increase the growth and/or activity of probiotics in the gut and have beneficial health effects on the host, many researchers have been chosen to use prebiotics to increase the growth or activity of probiotics. Thus, to improve the protective function of the intestinal barrier, a novel synbiotic combination was selected to inhibit the pathogenic bacteria and to promote the formation of the tight junctions. This research was divided into three studies.

In study 1, IPEC-J2 cells originally from the jejunum of a neonatal piglet were used as the experimental model to investigate the functional of synbiotics on the intestinal integrity, because these cells are highly sensitive to LPS stimulation leading to induction of inflammation and the impairment of the intestinal epithelia integrity. First, synbiotics were selected based on their fermentation ability of SCFA; SCFA are products of microbial fermentation of indigestible carbohydrates in the gut, and recent studies have

confirmed that SCFA, especially butyrate, may have an important role in the maintenance of the intestinal barrier function. Furthermore, synbiotics were used to investigate the effect on the LPS induced intestinal impairment.

In study 2, the aim was to investigate the antimicrobial activities of phthalyl pullulan nanoparticle (PPN)-treated LP. PPNs were synthesized and developed as a new type of prebiotic for LP. In addition, antimicrobial assays were checked whether the internalization of the PPNs by LP led to an enhanced antimicrobial activity by LP against the Gram-negative bacteria *Escherichia coli K99* and the Gram-positive bacteria *Listeria.monocytogenes* (LM) compared to pullulan treated LP or pullulan alone. The mechanism of the antimicrobial activity of the PPN-treated LP by the internalization of the PPNs by LP was further validated

In study 3, to confirm the suppression of the pathogenic induced the intestinal barrier dysfunction and the alteration in the gut microbiota, a novel synbiotic combination was developed and orally administered to mice. The antimicrobial ability of the synbiotics against *E.coli* and the change in specific microbes in the microbiota were investigated. Furthermore, the gut

physical barrier function was checked by serum endotoxin and serum FITC-dextran.

The aim of this research was to find a strategy that enhancing the protective function of the intestinal barrier by synbiotics; as a result, the prebiotic pullulan enhanced butyrate fermentation in the probiotic bacterium *Lactobacillus plantarum*; furthermore, the pullulan-based nanoparticles PPNs, enhanced the antimicrobial ability of *Lactobacillus plantarum.* Moreover, the synbiotic combination LP/P/PPNs prevented the intestinal barrier dysfunction by *E.coli in vivo.*

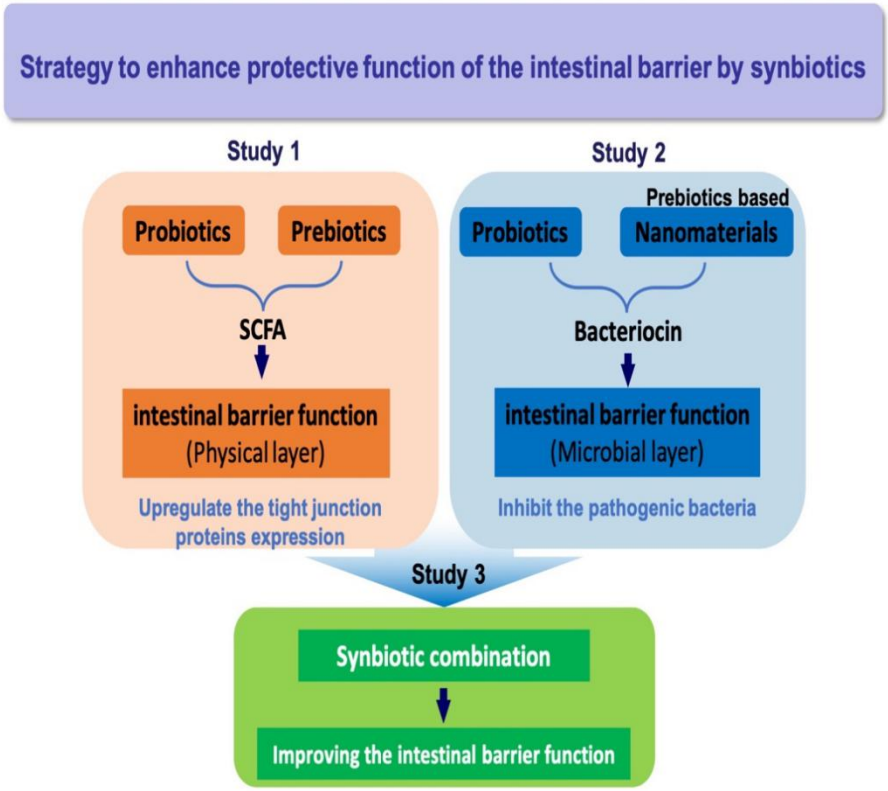

Figure. 1 Aim of the study and research organization.

The research consists of study 1, 2 and 3. Each study is a strategy to enhance protective function of the intestinal barrier by synbiotics.

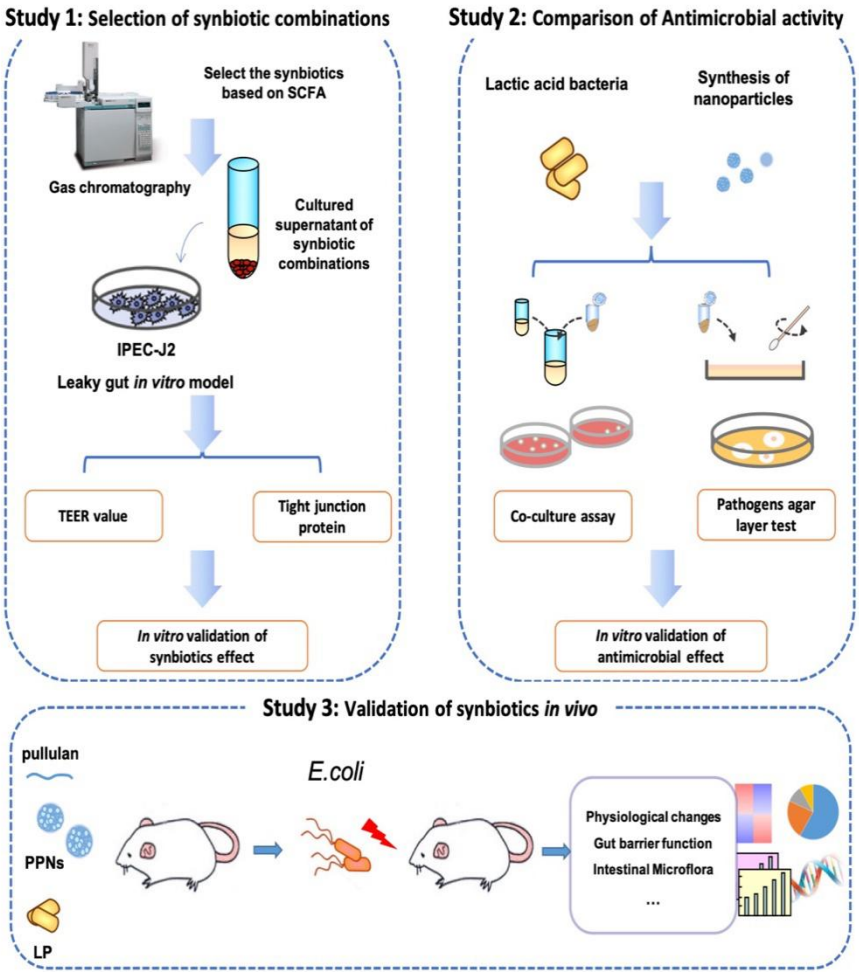

Figure. 2 Experimental flow chart.

In study 1, synbiotics were selected based on their fermentation ability of SCFA and investigate the effect on the LPS induced intestinal impairment in the leaky gut in vitro model. In study 2, PPNs were synthesized and developed as a new type of prebiotic for LP and antimicrobial assays were checked. In study 3, synbiotics functional assay was carried out *in vivo*.

Review of Literature

1. Intestinal barrier function

1) Intestinal barrier composition

A lot of exogenous antigens colonize in the intestinal lumen such as microorganisms, toxins, viruses, and food particles. Without the complete function of the intestinal barrier, these antigens penetrate the tissue beneath the intestinal epithelia disrupting the homeostasis. However, there is an effective intestinal barrier system (Figure. 3) whose, microbial, chemical, physical and immunological components prevent most antigens from penetrating. The following is a brief overview of the major composition of the intestinal barrier.

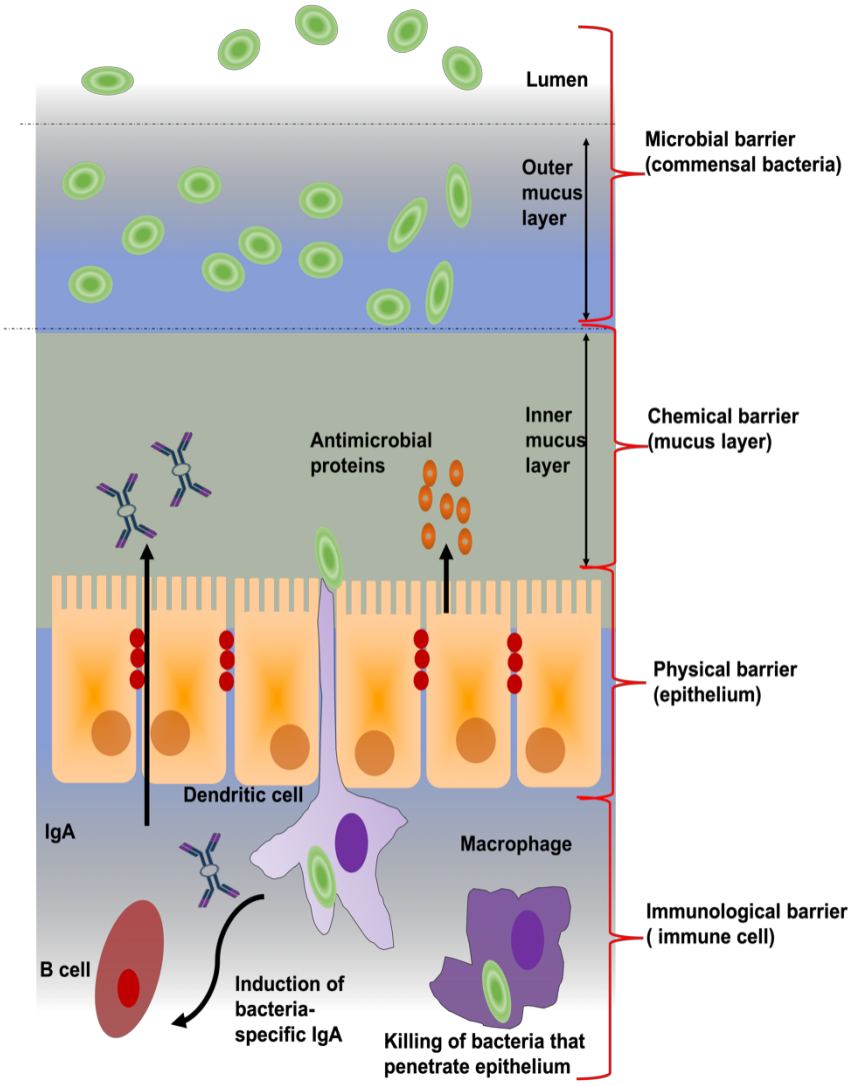

Figure. 3 Intestinal barrier composition.

The intestine is the body's most important immune function-related organ, and intestinal barrier is consisted by four parts: microbial barrier, chemical barrier, physical barrier, immunological barrier.

(1) Microbial barrier

The gut commensal bacterial have been described as one component of the intestinal barrier primarily due to their two major functions (Sekirov et al., 2010). The first one is to promote resistance to the colonization of harmful or pathogenic bacteria species by competing for nutrients, occupying the colonization sites, and releasing antimicrobial substances to kill them (Ming et al., 2015; Baumler and Sperandio, 2016). The second one is that the gut microbiota regulates the digestion of the substances that cannot be used by host and supply energy to epithelia cells, which are a major component of the physical barrier (Ramakrishna, 2013). Short chain fatty acids produced by the microbiota are used by colonocytes in their development (Krajmalnik-Brown et al., 2012).

(2) **Chemical barrier**

On the top of the gut epithelium, there are two mucus layers, the inner and outer layers. They cover the whole intestinal epithelium and provide protection to separate the commensal microorganisms from the epithelium. The major component of the mucus layers is a highly glycosylated gel forming mucin. The mucus contains diverse molecules including IgA and

enzymes and proteins (Singh et al., 2002). Chemical molecules with antimicrobial properties exist in the mucus and in the lumen, which include bile acid and AMPs (Dupont et al., 2014). These diverse chemical molecules form a complicated network to reduce the colonization of bacteria and to decrease the chance of contact between luminal antigens and host cells. The chemical barrier is a good supplement to the physical barrier and an indispensable component of the intestinal barrier function. Because of the large number of microorganisms, multiple AMPs are generated to fight against invaders. These AMPs are divided into several types, including defensins, lectin, lysozyme and intestinal alkaline phosphatase. The detailed antimicrobial mechanisms of AMPs are not discussed in this section. As a major, but not exclusive producer of AMPs, Paneth cells support and regulate the chemical barrier function.

(3) **Physical barrier**

The intestinal epithelium covers a large surface area of the host, and in humans, it covers up to 400 m^2 (Peterson and Artis, 2014). Though it only consists of a singer layer of cells, the intestinal epithelia cells are the main of the intestinal barrier and serve as a physical barrier. There are five types of

functional IECs: enterocytes, goblet cells, Paneth cells, microfold cells, and enteroendocrine cells. Among these cells, enterocytes represent the majority of the IECs, accounting for at least 90% of the crypt cells and villus cells. Enterocytes are responsible for absorbing the nutrients. However, the growing evidence shows that the function of enterocytes is not only limited to absorption. Enterocytes control the abundance of Gram-positive cells by the expression of RegIIIγ, one of the antimicrobial proteins. (Vaishnava et al., 2008;Vaishnava et al., 2011). The IEC layer is continuous, and it is held together by tight junctions (Anderson and Van Itallie, 2009). The paracellular pathway enables the transport of substances across the epithelium through the spaces between the IECs. Mainly proteins control the tight junctions. More than forty TJ proteins have been recognized, including occludin, claudins, and junctional adhesion molecule A (Yamazaki et al., 2008). Under pathological conditions, paracellular permeability increases, resulting in the entry of the luminal antigens into the body.

(4) **Immunological barrier**

The intestinal immune systems compose are the last barrier of the intestinal barrier**.** Below the intestinal epithelium, there are organized lymphoid

follicles, including the Peyer's patches and isolated lymphoid follicles. There is a variety of immune cells, including dendritic cells (DCs), macrophages, T cells and B cells inside the follicles. Dendritic cells in immunoglobulin A(IgA) induction by sampling bacteria at the apical epithelial surface and carrying small numbers of live bacteria to the inductive site (Macpherson and Uhr, 2004). IgA directed against intestinal bacteria is produced by B cells, transcytosed across the epithelial layer and secreted from the apical surface of the epithelial cells. This secreted IgA reduces the numbers of epithelia-adherent bacteria (Suzuki et al., 2004) and limits bacterial translocation across the epithelium (Macpherson et al., 2000). In addition, macrophages can also directly kill invading bacteria.

2) Gut microbiota and intestinal barrier

Microbiota can be recognized by the host through pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). In the gut, the bacteria–host communications are largely dependent on the recognition of microbe-associated molecular patterns by PRRs expressed on immune and non-immune cells. Microbiota, bacterial products, and metabolites affect the

intestinal barrier function and are responsible for the gut immune homeostasis. When there is a leaky gut, commensal bacteria in the gut lumen, together with their products, are able to escape the lumen of the gut, which may induce inflammation and cause systemic tissue damages if translocated into the blood stream.

Many of the germ free (GF) animal studies suggest that the development and function of the intestinal barrier rely on the microbiota. In GF animals, due to the absence of bacterial stimulations, the thickness of the mucus layers is extremely reduced, and the thinner mucus layer would enable bacteria penetration (Johansson et al., 2015;Desai et al., 2016). A balance exists between commensal bacteria and the mucus layers and they maintain the gut homeostasis (Wrzosek et al., 2013). Within the mucus layers, there are AMPs secreted from Paneth cells, which clear pathogens and control the colonization of commensal bacteria.

Intestinal epithelial cells contribute the to the single layer of the intestinal epithelium, and the generation of new IECs from the local stem cells is critical in maintaining the barrier function. As much as 10% of all gene

transcriptions associated with immunity, cell proliferation, and metabolism in IECs are regulated by gut microbiota (Sommer et al., 2015).

3) Leaky gut syndrome

The father of modern medicine, Hippocrates, said “All disease begins in the gut.” More than two millennia after his death, scientific research has now proven he was onto something all those years ago. For over three decades, study after study has been published discussing our growing understand of immunity, gut function and how modern diets and lifestyles negatively contribute to overall health by damaging our digestive system.

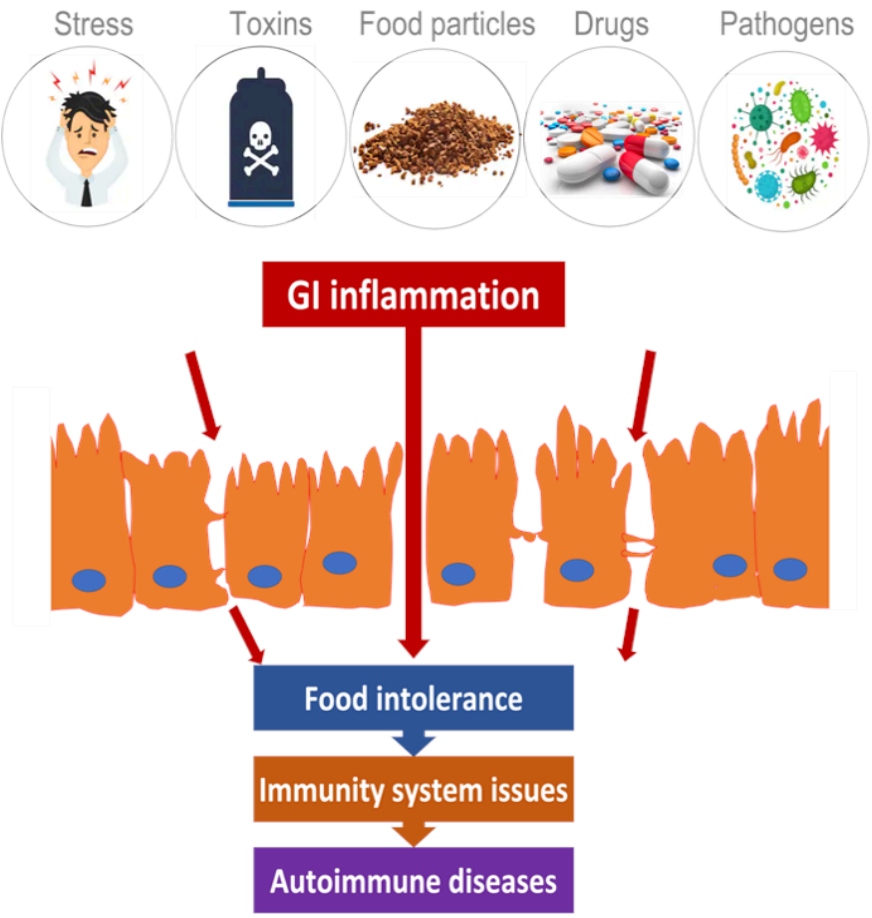

Figure. 4 Leaky intestinal progression.

Stress, toxin overload and microbiota dysbiosis induce intestinal lining inflamed and the barrier break down formed passageways for bacteria, toxins and even food particles to enter the bloodstream and these foreign matter in the blood triggers the body's immune system, resulting in widespread inflammation in the body.

What causes a leaky gut? Some of underlying causes of a leaky gut include the following: **Genetic predisposition**: certain people may be more predisposed to developing a leaky gut because they are sensitive to environmental factors that "trigger" their bodies into initiating an autoimmune response; **Poor diet**: especially a diet that includes allergens and inflammatory foods such as un-sprouted grains, added sugar, GMOs, refined oils, synthetic food additives and conventional dairy products; **Chronic stress**; **Toxin overload**: including drug at a high concentration and alcohol consumption. We come into contact with over 80,000 chemicals and toxins every single year, but the worst offender for causing a leaky gut include antibiotics, pesticides, tap water, aspirin and NASIDS, and **Bacterial imbalance:** also called dysbiosis, which means an imbalance between beneficial and harmful species of bacteria in the gut. A large body of evidence now shows that the gut microbiota is important in supporting the epithelia barrier and preventing autoimmune reactions. At least 10 percent of all gene transcriptions found in intestinal epithelia cells that are related to immunity, cell proliferation and metabolisms are regulated by gut microbiota.

What are the symptoms of leaky gut? Some of the most prominent signs of a leaky gut are listed in the Table 1

Table 1. Diseases related to intestinal permeability.

Intestinal	*Extra-intestinal*
Gastric ulcers	Allergies
Infectious diarrhea	Infection (e.g. respiratory)
Irritable bowel syndrome; Functional GI diseases	Acute inflammation (sepsis, SIRS, MOF)
Inflammatory bowel disease, Celiac disease	Chronic inflammation (e.g.arthritis)
Cancer (esophagus, colorectal)	Obesity-associated metabolic diseases(NASH, diabetes, CVD)

(Fasano and Shea-Donohue, 2005;Odenwald and Turner, 2013;Bischoff et al., 2014)

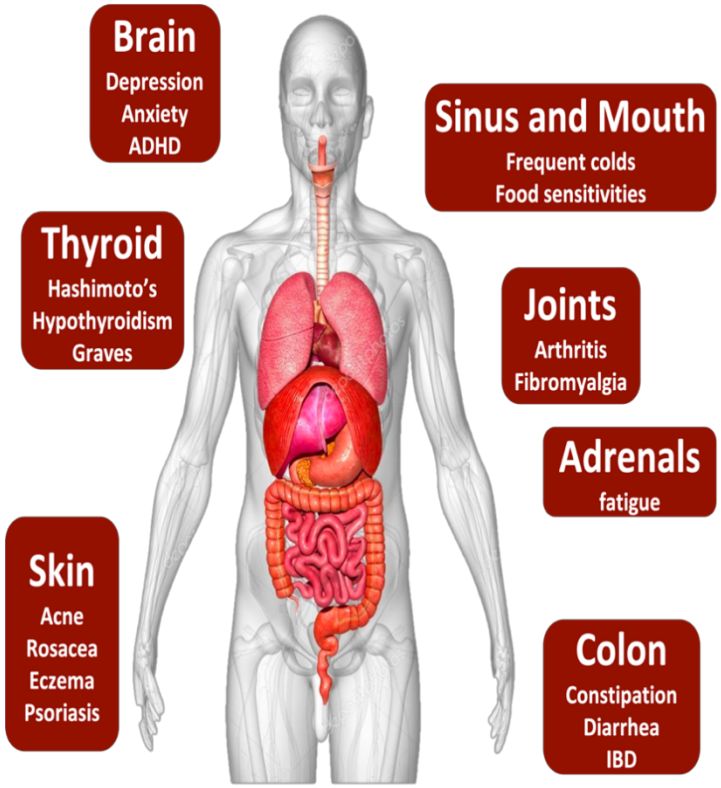

Figure. 5 Leaky intestine effects the whole body.

The leaky gut is the root of modern health problems has yet to be proven by science. However, many studies have connected increased intestinal permeability with multiple chronic diseases

While these diseases are linked to leaky gut syndrome, it has not been proven that there is a causal relationship; in other words, it has not yet been established that a leaky gut causes any of these conditions. Instead, people who have a leaky gut are more likely to have a number of other health problems as well. Thus, while the scientific evidence has not yet proven that intestinal hyper-permeability (leaky gut syndrome) is actually responsible for these conditions, it strongly suggests that a leaky gut and another dysfunction tend to occur simultaneously.

2. Probiotic

1) Definition of probiotic

In the past few decades, there has been increasing interest in the use of probiotics as potential alternative for synthetic antibiotics and anti-inflammatory drugs, not only because of the side effects of antibiotics but also because of improper use of antibiotics which has promoted the development of antibiotic-resistant bacteria. According to the Food and Agriculture Organization of United Nations (FAO) and the World Health Organization (WHO), probiotics are defined as live microorganisms, which when administered in adequate amounts, confer a health benefit on the host (FAO/WHO 2001). Various microorganisms are known as probiotics although most typical probiotics are lactic acid bacteria (LAB), bacilli and yeast.

There are specific strains with probiotic properties in the genus *Lactobacillus*. The genus *Lactobacillus* includes various Gram-positive facultative anaerobic rod-shape bacteria. They are a major part of the LAB group that can convert hexose sugars to lactic acid and forming an acid

Table 2. Microorganisms applied in probiotic products.

***Lactobacillus* species**	***Bifidobacterium***	**Other LAB**	**Non-lactics**
L.Acidophilus	*B.adolescentis*	*Enterococcus faecalis*	*Bacillus* species
L.casei	*B.animalis*	*Pediococcus acidlactici*	*Escherichia coli*
L.crispatus	*B.bifidum*	*Lactococcus lactis*	
L.gallinarum	*B.breve*		
L.gasser	*B.infantis*		
L.johnsonii	*B.lactis*		
L.paracasei	*B.longum*		
L.plantarum			
L.reuteri			

environment which can inhibit the growth of several species of harmful bacteria (Makarova et al., 2006). These probiotics are currently used in fermented foods, drinks, cosmetics and medicines and as animal feed additives.

2) Characteristics of probiotics

Probiotics confer a health benefit to the host through various mechanisms. The effects of probiotics can be classified into their modes of action (Figure.6). These modes of action are also the reasons why probiotics can be used as antibiotics substitutes.

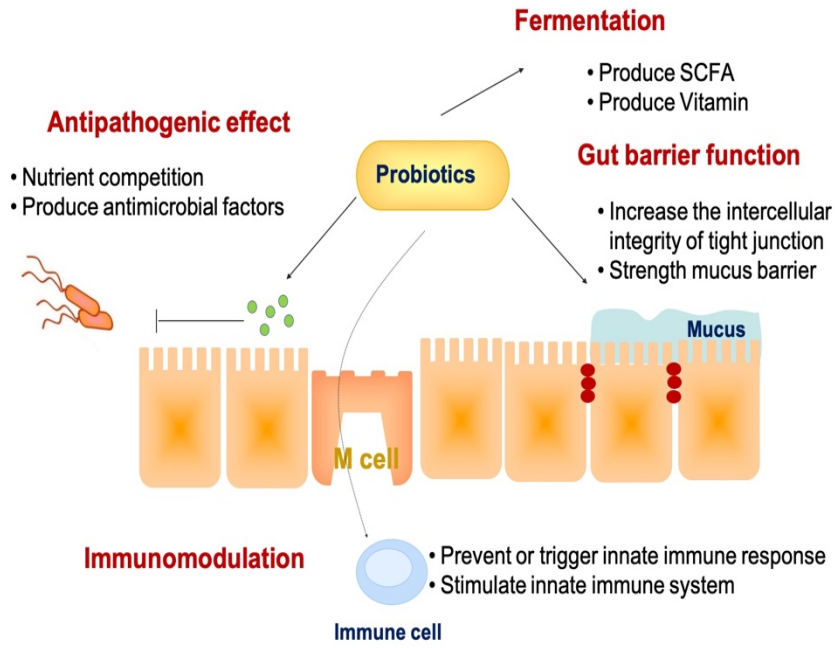

Figure. 6 Biological effects and mechanism of probiotics.

Probiotics affect host in several mode of actions: anti-pathogenic effect, fermentation, gut barrier reinforcement, and immune-modulation

The first mode of action is to promote resistance against the colonization of harmful or pathogenic bacteria species by competing for nutrients, and releasing antimicrobial substances to kill them directly (Prabhurajeshwar and Chandrakanth, 2017). Second one is that probiotics can modulate host defenses including the innate and acquired immune system (Plaza-Diaz et al., 2014; Giorgetti et al., 2015). The third one is that probiotics can increase the

intercellular integrity and strength the mucus layer (Alemka et al., 2010; Oelschlaeger, 2010). The last one is that probiotics can break down the polysaccharides that mammals cannot directly absorb into SCFA (Fernando et al., 2018).

3) *Lactobacillus* as probiotics

Lactobacillus is a genus of the Gram-positive, facultative anaerobic or microaerophilic, rod shaped, non-spore forming bacteria (Makarova, et al. 2006b)) They are a major part of the lactic acid bacteria. In humans, they constitute a significant component of the microbiota at a number of body sites, such as the digestive system, urinary systems, and genital system. In women, *Lactobacillus* species are normally a major part of the vaginal microbiota (Ma et al., 2012). *Lactobacillus* forms biofilms in the vaginal and gut microbiota, enabling them to persist during harsh environmental conditions and to maintain ample populations (Salas-Jara et al., 2016). *Lactobacillus* exhibit a mutualistic relationship with the human body, because it protects the host against potential invasions by pathogens, and in turn, the host

provides a source of nutrients (Martin et al., 2013). *Lactobacillus* is the most common probiotics found in food such as yogurt, and it has diverse applications for maintain human well-being such as treating diarrhea, vaginal infections and skin disorders (Sharafi et al., 2013; Seddik et al., 2017).

(1) *Lactobacillus plantarum*

Because it is abundant and easy to grow, *Lactobacillus plantarum* has been tested for its health effects. It has been identified as a probiotic, which suggests its value for further research and applications. It has significant antioxidant activities and also helps to maintain intestinal permeability (Bested et al., 2013a). It is also to suppresses the growth of gas-producing bacteria in the intestines and may confer health benefits to IBS patients (Stevenson et al., 2014). Lactobacillus plantarum has been found to increase brain derived neurotrophic factor, which means *Lactobacillus plantarum* may have a beneficial role in the treatment of depression (Bested et al., 2013b). The ability of *L.plantarum* to survive in the human gastro-intestinal tract makes it a possible *in vivo* delivery vehicle for therapeutic compounds.

The ability of *Lactobacillus plantarum* to produce antimicrobial substances (bacteriocin, lactic acid, H_2O_2) helps it to survive in the gastrointestinal tract. The antimicrobial substances produced have shown significant effects on Gram-positive and Gram-negative bacteria.

4) Veterinary importance of probiotics

In animal nutrition, microorganisms used as probiotics have been shown to have proven efficacy on the gut microflora. Administration of probiotics strains separately and in combination was significantly improved the feed intake, feed conversion rate, daily weight gain and total body weight in chickens, pigs, sheep, goats, cattle and equines (Samli et al., 2007).

Probiotics have been used as an alternative to antibiotic growth promoters which has led to and improved the growth performance of the animal. Probiotics have been incorporated through diets, with the objective to keep intestinal microbiota balance of the animals, preventing the digestive tract diseases, improve feed digestibility, increase the efficient use of nutrients an improve the performance of the animals.

Supplemental probiotics are now widely used in quality animal production

and accepted as agents that can bring significant health benefits such as follows: enhancement of the immune systems; antimicrobial effects inhibiting pathogens in the intestine and feed; improvement of gut functions by normalizing the microbiota balance, reducing constipation and improving intestinal mobility; improved nutrition through the enhanced breakdown of vitamins, polysaccharides, minerals and amino acids and their absorption, and prevention of infection by harmful bacteria (Rautray, et al. 2011).

3. Prebiotic

1) Definition of prebiotic

Improving the health through modulation of the microbiome is an evolving strategy that is part of a comprehensive, holistic approach to lifestyle wellness. Over 20 years ago, a class of compounds, termed prebiotics, were recognized for their ability to manipulate the host microbiota to the benefit of the host (Gibson and Roberfroid, 1995b).

Today, the prebiotic concept has expanded, because of advances in tools for microbiome research, for example, next generation sequencing, which have improved our knowledge of the composition of the microbiota and enabled the identification of additional substances influencing colonization. The latest definition proposed was “a substrate that is selectively utilized by host microorganisms conferring a health benefit.” (Gibson et al., 2017). Prebiotics must be selectively utilized and have adequate evidence of a health benefit for the target host, and dietary prebiotics must not be degraded by the target host enzymes. However, there are still conflicting interests in terms of the concept and range of prebiotics, and the definition of prebiotics seems to be expanding over time.

Because selective utilization of prebiotics by host microorganisms is key to their physiological effects, the metabolic results of this utilization must, by deduction, be the main factors. Some organic acids, for example, are principal end products of non-digestible carbohydrate or dietary fiber fermentation by host microorganisms. The main SCFAs generated mostly in the colon and caecum as a result of several bacterial metabolic pathways are acetate, propionate, and butyrate. These SCFAs are crucial for intestinal health and their activity can subsequently influence sites distant to the gut, with different SCFAs having various functions. SCFAs can modulated certain aspects of metabolic activity including colonocyte function, gut homeostasis, energy gain, the immune system, blood lipids appetite and renal physiology (Roberfroid et al., 2010; O' Keefe, 2016; Pluznick, 2016).

Table 3. Prebiotics use in animals.

Animal species	Prebiotics used	Outcomes
Dog	Short chain FOS Short chain MOS	Improved insulin response Reduced pathogen infections
Cat	Fructans and galactans	Increased level of organic acid, increased bifidobacterial
Piglets	Soy polysaccharides, FOS, chito-oligosaccharides and MOS	Reduced pathogen load Improved weight gain
Poultry	Inulin, lactulose and GOS	Improved growth, reduced infection, improved egg quality
Farmed fish	FOS, GOS and MOS	Improved survival rate, growth rate, pathogen resistance
Pre-weaned calves	Cello-oligosaccharides, galactosyl-lactose, yeast cell wall extracts and MOS	Reduced pathogen load Improved weight gain
Horses	Short chain FOS	Increased nutrient digestibility, improved insulin sensitivity

FOS, fructo-oligosaccharides; GOS, galactooligosaccharides; MOS, mannanoligosaccharides

Modified from Gibson (Gibson et al., 2017)

2) Pullulan as a prebiotic

Pullulan is a polysaccharide polymer consisting of maltotriose units, and three glucose units in maltotriose are connected by an α-1,4 glycosidic bond, whereas the consecutive maltotriose units are connected to each another by and by an α-1,6 glycosidic bond (Figure. 7). It is secreted by the fungus *Aureobasidium pullulans* (Catley et al., 1986).

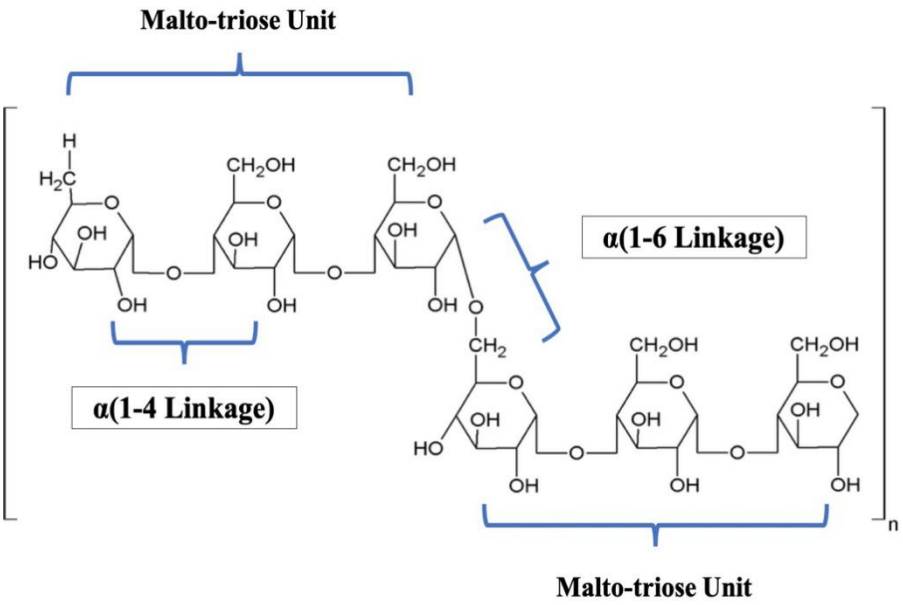

Figure. 7 Chemical structure of pullulan.

Pullulan is an a-1,6 linked polymer of maltotriose subunits.

Pullulan is widely used as a food additive, because of it is an excellent film former. In addition to food additives, pullulan also serves as dietary food such as a starch substitute because of its resistance to human intestinal enzymes, thus, pullulan is considered to be a non-digestible carbohydrate (Cheng et al., 2011). Pullulan serves as a prebiotic later in the composition of the intestinal microbiota and promotes the growth of the beneficial *Bifidobacteria* in the human intestines (SUGAWA-KATAYAMA, Yohko, et al. 1994). Pullulan is poorly metabolized and is mainly only metabolized by *Bifidobacteria* (Ryan et al., 2006), but there is increasing evidence that pullulan may also promote the growth of fecal *Lactobacilli* (Spears et al., 2005).

4. Synbiotics

1) Definition of synbiotics

In 1995, Gibson and Roberfroid introduced the term “synbiotics” to describe a combination of synergistically acting probiotics and prebiotics (Gibson and Roberfroid, 1995b). The word “synbiotics” means synergy, the term should be reserved for those products in which a prebiotic component selectively favours probiotic microbe (Cencic and Chingwaru, 2010), the primary purpose of the synbiotic combination is the improvement of survival of probiotic microbe in the gastrointestinal tract.

Synbiotics have both probiotic and prebiotic properties and were combined in order to overcome some possible difficulties in the survival of probiotics in the GI tract (Rioux et al., 2005). Therefore, an appropriate combination of both components in a single product should ensure a superior effect, compared to the activity of the pro or prebiotic alone (Bengmark, 2005).

2) Synbiotics selection criteria

The first aspect to be taken into account when composing a synbiotic formula should be a selection of an appropriate probiotic and prebiotic,

exerting a positive effect on the host's health when used separately. The determination of specific properties to be possessed by a prebiotic to have a favourable effect on the probiotic seems to be the most appropriate approach. A prebiotic should selectively stimulate the growth of microorganisms, having a beneficial effect on health, with simultaneous absent stimulation of other microorganisms.

3) Synbiotics in use

Previous sections discussed probiotic microbes and prebiotic substances most commonly used. A combination of *Bifidobacterium* or *Lactobacillus* spp, bacteria with FOS in synbiotic products seems to be most popular. Table 4 presents the most commonly used combinations of probiotics and prebiotics (Markowiak and Slizewska, 2017).

Table 4. Most commonly used synbiotic combinations.

Synbiotics
Lactobacillus genus bacteria +inulin
Lactobacillus, streptococcus and *Bifidobacterium* genus bacteria + FOS
Lactobacillus, Bifidobacterium, Enterococcus geneus bacteria + FOS
Lactobacillus and *Bifidobacterium* genus bacteria + oligofructose
Lactobacillus and *Bifidobacterium* genus bacteria + inulin

5. Short chain fatty acid

1) Microbial fermentation products: SCFA

Dietary fibers that can escape the digestion by host enzymes in the upper gut are metabolized by the host gut microbiota in the cecum and colon (Kaiko et al., 2016). The major products from the microbial fermentative activity in the gut are SCFAs, particular acetate, propionate and butyrate. One of the major SCFAs, acetate, can be produced from pyruvate by many gut bacteria either via acetyl-CoA or via the Wood-Ljungdahl pathway (Ragsdale and Pierce, 2008). Another major SCFAs, propionate, is produced form succinate conversion to methylmalonyl-CoA through the succinate pathway. Propionate can also be synthesized from acrylate with lactate as a precursor through the acrylate pathway (Hetzel et al., 2003) and via the propanediol pathway, in which deoxyhexose like fucose and rhamnose are substrates (Scott et al., 2006). The third one, butyrate is formed from the condensation of two molecules of acetyl-CoA and subsequent reduction to butyryl-CoA, which can be converted to butyrate via the so-called classical pathway, by phosphotransbutyrylase and butyrate kinase(Louis et al., 2004). Butyryl-CoA

can also be transformed to butyrate by the butyryl-CoA: acetate CoA-transferase route (Duncan et al., 2002). Some bacteria in the gut can use both acetate and lactate to synthesize butyrate, which prevents the accumulation of lactate and stabilizes the intestinal environment.

2) SCFA as signaling molecules

(1) *HDAC* inhibitors

Acetyl groups are added to histone tails by histone acetyltransferases (HATs) and are removed by histone deacetylases (HDACs). HDAC inhibitor have been widely used for cancer therapy. Their anti-inflammatory or immune-suppressive function has also been reported. Butyrate and propionate are known to act as HDAC inhibitors (Johnstone, 2002). Therefore, SCFAs may act as modulators of cancer and immune homeostasis. Among the SCFAs, butyrate has been investigated most extensively. Present at high levels (mM) in the gut lumen, butyrate is the primary energy source for colonocytes. It also protects against colorectal cancer and inflammation by inhibiting HDACs (Flint et al., 2012) and altering the gene expression of diverse functions include cell proliferation, apoptosis and differentiation.

Table 5. SCFAs production by microbes in the gut.

SCFAs	Producers	Reference
Acetate	Most of the enteric bacteria, e.g., *Akkermansia muciniphila*, *Bacteroides* spp., *Bifidobacterium* spp., *Prevotella* spp., *Ruminococcus* spp.	(Louis, et al. 2014) (Rey, et al. 2010)
Propionate	*Bacteroides* spp., *Phascolarctobacterium succinatutens*, *Dialister* spp., *Veillonella* spp. *Megasphaera elsdenii*, *Coprococcus catus* *Salmonella* spp., *Roseburia inulinivorans*, *Ruminococcus obeum*	(Louis, et al. 2014) (Scott, et al. 2006)
Butyrate	*Anaerostipes* spp. (A, L), *Coprococcus catus* (A), *Eubacterium* transferase route *rectale* (A), *Eubacterium hallii* (A, L), *Faecalibacterium prausnitzii* (A), *Roseburia spp.* (A)	(Louis, et al. 2014) (Duncan, et al. 2002)

A, acetate is the substrate for producing butyrate; L, lactate is the substrate for producing butyrate.

Butyrate consumption by normal colonocytes protects stem cells in the colon from exposure to high butyrate concentrations and alleviates butyrate-dependent HDAC inhibition and impairment of stem cells function (Kaiko et al., 2016). In contrast, butyrate-induced HDAC inhibitions in the small intestinal stem cells promotes the stem cell population (Yin et al., 2014). Butyrate can induce different effects in a cell-and environment-specific context.

In addition, SCFAs-mediated HDAC inhibition is also a potent anti-inflammatory agent. Butyrate suppressed proinflammatory effectors in lamina propria macrophages (Yin et al., 2014) and the differentiation of dendritic cells form bone marrow stem cells (Singh et al., 2010) via HDAC inhibition, making our immune system hypo-responsive to beneficial commensals. SCFAs also regulate the cytokine expression in T cells and generation of regulatory T cells through HDAC inhibition. Interestingly, acetate, which is not regarded as a HDAC inhibitor- was also found to inhibit HDACs in activated T cells (Park et al., 2015).

(2) Ligands for GPCRs

G-protein-coupled receptors are also called free fatty acid receptors (FFARs) and include GPR41, GPR43 and GPR103. GPR41 and GPR43 can be efficiently activated by acetate, propionate and butyrate (Brown et al., 2003). SCFAs could regulate the immune response by activating GPCRs which are expressed on almost all the immune cells, such as epithelia cells, neutrophils and macrophages (Brown et al., 2003).GPR43 on intestinal epithelial cells activates the NLRP3 inflammasome and enhances the production of IL-18, which is critical for maintaining the epithelial integrity and intestinal homeostasis (Macia et al., 2015). The protection of $GPR43^{-/-}$ mice against inflammatory tissue destruction in chronic DSS colitis was due to the diminished intestinal migration of PMNs (Sina et al., 2009). Furthermore, acetate could inhibit LPS-induced TNF-a secretion from both mice and human PBMCs by the GPR43 pathway (Masui et al., 2013). GPR43 on colonic T cells induces the differentiation and enhances the suppressive function of Foxp3 Tregs through epigenetic modifications (Smith et al., 2013). In summary, SCFA-sensing GPCRs have an important role in regulation of immunity and inflammation.

The gut is the primary site where SCFAs mediate their effect on either the intestinal epithelial integrity or mucosal immune responses. Disorders of the gut microbiota leading to decreased SCFAs are associated with colonic diseases, including IBD. A SCFA-sensing GPCR protects against the intestinal inflammation not only by maintain the intestinal epithelial barrier but also by regulating the immune system. Both GPR43 and GPR109a are important for the regulation of the gut immunity. It was found that GPR43$^{-/-}$ and GPR109a$^{-/-}$ mice suffered from more severe DSS-induced colitis (Maslowski et al., 2009; Singh et al., 2014). The intestinal epithelial barrier has a critical role in preventing the intestinal inflammation of IBD. SCFAs affect intestinal epithelial cells that highly expressed GPR43, functioning as regulators of the physical barrier and secretion of mucin, antimicrobial peptides, chemokines and cytokines. SCFAs might activate NALP6 through the GPCR pathway, further promoting gut goblet cells to secret mucus, which is an important barrier to separate the bacteria and epithelial cells (Thorburn et al., 2014; Wlodarska et al., 2014). Several recent studies also have suggested that butyrate could upregulate the tight junction and regulate epithelial permeability (Peng et al., 2009)

Chronic intestinal inflammation such as IBD increases the risk of colorectal carcinogenesis, which is called colitis-associated cancer. The gut microbiota is associated with the process of inflammation and tumorigenesis. Thus, their metabolites, SCFAs, were observed to confer protection against development of colon cancer, partially in a GPCR- dependent manner. The beneficial effects of SCFAs have also been reported in patients with IBD. It was proposed a long time ago to treat patients with IBD by administrating of SCFAs or prebiotics that are known to enhance SCFA production (Scheppach et al., 1996; Breuer et al., 1997). Overall, SCFAs have profound effects on the regulation of gut immunity and integrity.

Microbial interactions with dietary polysaccharides and the resulting SCFAs are important energy and signaling molecules. It is becoming increasingly accepted that butyrate-producing bacteria and butyrate itself may be beneficial for human health. Fermentative bacteria mostly target the colon, whereas the effects of exogenously administered SCFAs may be dependent on the route of administration and thus different from microbially produced metabolites. For example, oral delivery of butyrate may target the small intestine and reach supraphysiological concentrations in the periphery

because it is not consumed by colonocytes. It is necessary to know the spatiotemporal concentration of metabolites and their functional capacity will hopefully lead to general principles for microbial metabolite actions affecting the host physiology.

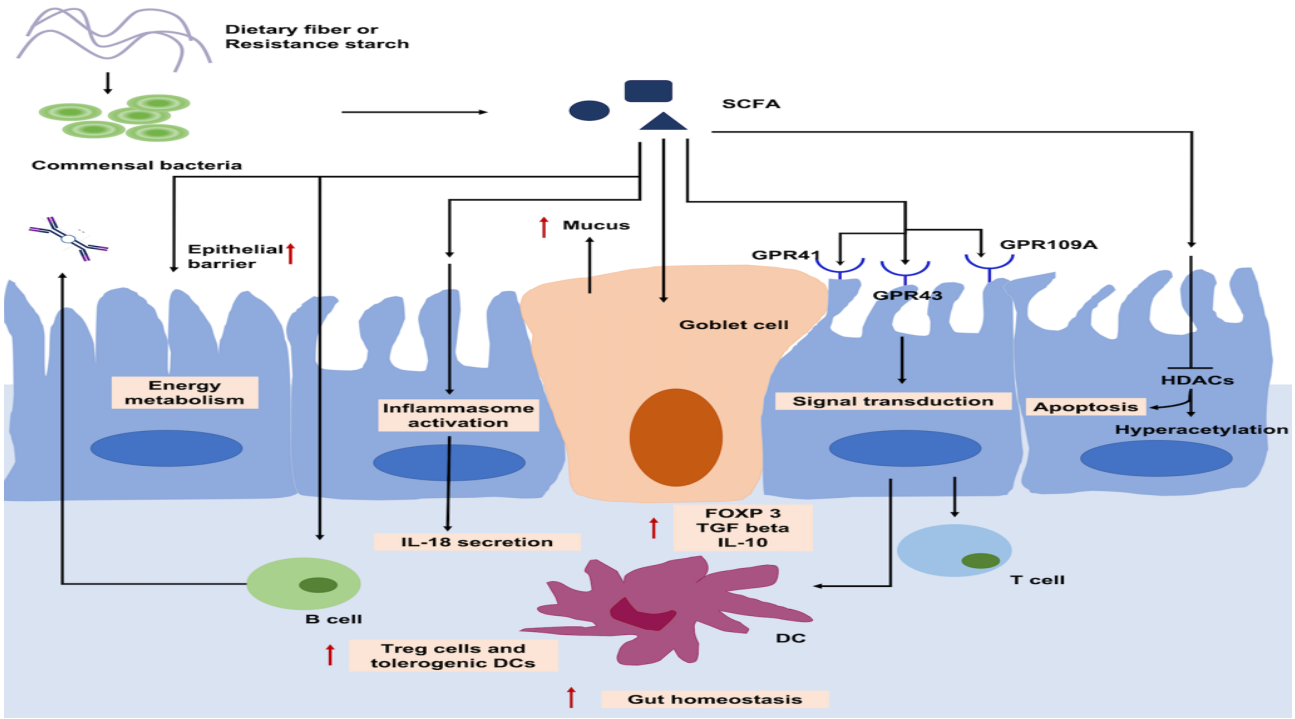

Figure. 8 SCFAs, host physiology and gut immunity.

The intestine is the primary site where SCFA mediate their effect on either intestinal epithelial integrity or mucosal immune response.

Short-chain fatty acids (SCFAs) — such as butyric acid, propionic acid and acetic acid are produced by colonic microbial fermentation of undigested or partially digested dietary fibers and have broad effects on the development and function of the host immune system.

6. Bacteriocin

1) Definition of bacteriocin

Bacteriocins were first identified in 1925 and a are defined as synthesized from ribosome, proteinaceous substances that inhibit the growth of closely related species through various mechanisms (Inglis et al., 2013; Micenkova et al., 2014). Production of these proteins is widespread among bacterial species and it is suggested that virtually all bacterial species synthesise bacteriocins (O'Connor et al., 2015), Such production is made possible by relatively simple biosynthetic machineries that are often associated with elements such as plasmids and conjugative transposons (Yamashita et al., 2011;Phelan et al., 2013)

There have been multiple classifications for bacteriocins. This controversy has led to such divisions as ‘true bacteriocins’ such as colicins, and those

more recently discovered from *Lactobacillus* spp. (Cui et al., 2012) and other lactic acid bacteria (LAB), bacteriocins from LAB have undergone several classifications from being placed into four groups to more recent groupings (Klaenhammer, 1993) .

Table 6. Classification of bacteriocins.

Class	Property	Example
Class I	lanthionine-containing LANTIBIOTICS Small(<5kDa)	Nisin A, Z, lacticin 3147 Salivaricin A mersacidin cytolysin
Class II	Non-lanthionine containing bacteriocins Small (<10kDa) Heat-stable	Entericin A, P Pediocin PA-1,AcH, SJ-1 AcidocinJ1132 Plantaricin EF,JK,S,A
Class III	Large (>30kDa) Heat-labile murein hydrolases BACTERIOLYSINS	Acidophilucin
Class IV	Non-proteinaceous moieties for activity	Reuterin, Reutericyclin
Class V	Circular LAB bacteriocins a head-to-tail peptide bond formation of a prepeptide	Gassericin A

2) Mechanism of antimicrobial ability

Bacteriocins are capable of inhibition of four of these pathways (Figure. 9)

(1) Inhibition of cell wall biosynthesis

Nisin A, produced by *Lactococcus lactis*, one of the most frequently referenced bacteriocins, possesses multiple modes of action. This lantibiotic docks to lipid Ⅱ, a membrane-bound precursor of the cell wall, and inhibits cell well synthesis. In addition, following lipid Ⅱ docking, pore formation by nisin molecules arranged as pore-forming unit can be induced, which can rapidly kill the cells (Modi et al., 2000). Similarly, nukacin ISK-1 produced by *Staphylococcus warneri*, inhibit cell wall synthesis by binding lipid Ⅱ but it has not seen to induce pore formation (Islam et al., 2012).

These aforementioned bacteriocins show great promise in inhibiting the cell well biosynthesis by binding to lipid Ⅱ.

(2) Inhibitory and destructive effects on DNA structure

During the DNA replication, positive supercoil relaxation results in a superhelical tension that facilitates the movement of polymerases down the open frame. This is an ideal and safe target because there are differences in the structure of DNA gyrase between eukaryotic and prokaryotic organisms

(Collin et al., 2013). Bacterial DNA gyrase is targeted through competitive inhibition of the ATPase active site on the GyrB subunit and by binding and preventing decatenation of replication DNA. Quinolones represent a large group of antibiotics that target DNA gyrase.

(3) Inhibition of protein synthesis

Specifically, colicins E3, E4 and E6 and cloacin DF13 show 16S rRNase activity. These bacteriocins cleave the 16S rRNA at the 3' end of the coding sequence, which inhibits translation (Akutsu et al., 1989).

Similar to the RNase colicins are the tRNases (colicin D and E5) which act by accelerating the exhaustion of tRNA in the cytoplasmic pool and limiting protein synthesis (Ogawa et al., 2006).

(4) Disruption of bacterial membrane integrity

Bacteriocins that act on the targeted cells by forming pores in their membranes do not always dock to lipid Ⅱ. Lacticin Q, produced by *L.lactis* QU 5, forms toroidal pores due to lipid flip-flop, which causes proteins leakage and cell death without a specific receptor (Li et al., 2013).

Furthermore, lacticin Q shows selectivity in inhibition for Gram-positive bacteria but not for Gram-negative bacteria owing to physiochemical

differences in the outer membrane (Yoneyama et al., 2011). Carnocyclin A, a 60-amino acid circular bacteriocin form *Carnobacterium maltaromaticum* UAL307, is another lipid Ⅱ independent bacteriocin capable of direct interaction with the lipid bilayer, causing formation of ion-specific pores (Gong et al., 2009).

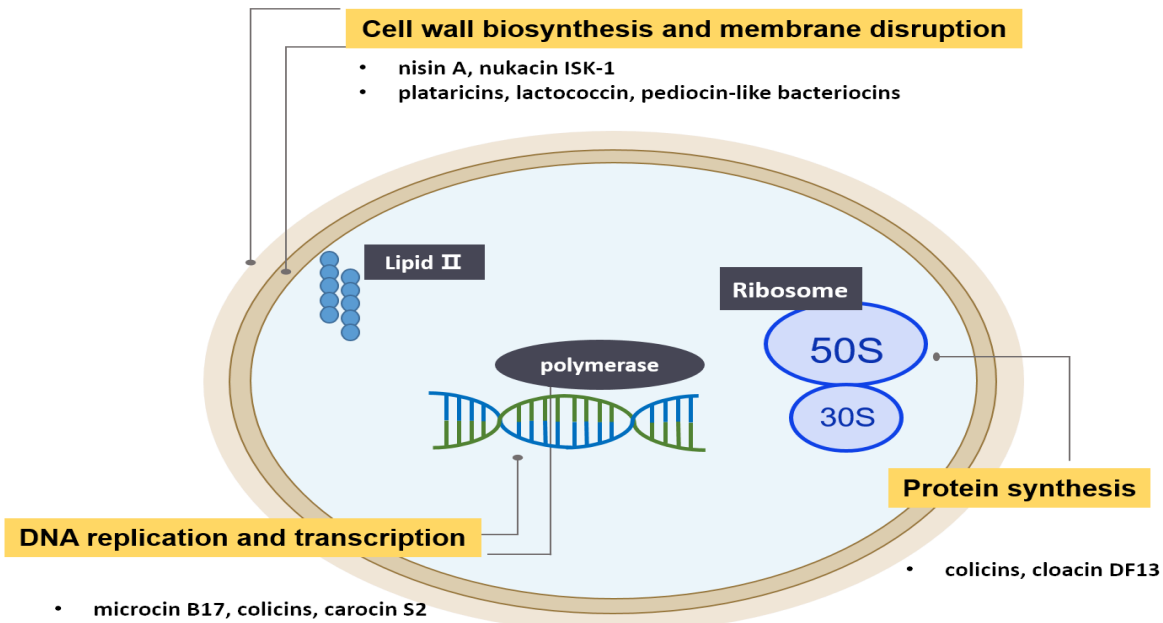

Figure. 9 Bacteriocins and their inhibition target.

Bacteriocin has the antibacterial ability due to they can inhibit the cell wall biosynthesis, disrupt the cell membrane, inhibit the DNA replication and transcription and inhibit the protein synthesis.

Study 1. *In vitro* screening of synbiotics according to the fermentability of SCFAs and validation using a leaky gut cell model.

1. Introduction

Human inflammatory bowel diseases (IBDs), including Crohn's disease and ulcerative colitis, are a group of chronic inflammatory disorders of the intestine characterized by the inflammation and mucosal damage (Quetglas et al., 2015). It is known that the destruction of the intestinal barrier function destruction, dysbiosis of the gut microbiota, and dysfunction of the immune system have critical roles in the pathogenesis of IBDs (Guinane and Cotter, 2013;Loddo and Romano, 2015). In the production animals such as pigs, and chickens, intestinal barrier dysfunction lead LPS or toxin exposure, and it antagonizes appetite, digestion, and skeletal muscle protein synthesis which ultimately leads to the diversion of nutrients and energy away from important production orientated pathways and systems (skeletal muscle, reproductive tract) to support the immune systems. There are some immunosuppressive drugs that have been used for the treatment and maintenance of IBDs.

However, clinical application for these drugs is limited by their adverse effects (Mao and Hu, 2016). For livestock animals, preventive treatment to maintain the health of the intestinal barrier function is more important than clinical treatment for the destruction of the intestinal barrier function. Therefore, there is an urgent need to seek alternative remedies.

Increasing evidence shows that the intake of dietary fibers and probiotics seems to beneficial in improving of the intestinal barrier function, because the their fermentation products, SCFAs, have clinical benefits in the treatment of intestinal colitis (Cabre and Domenech, 2012). Depending on diet and gut microbiota composition, the intestinal SCFA concentration can range from 60 to 150 mmol/L (Hill, 1995), with the butyrate, propionate, and acetate in a nearly constant molar ratio of 15:25:60, respectively (D'Argenio and Mazzacca, 1999). The physiological effects of SCFAs have been well documented by many researchers who have proven that SCFAs, especially butyrate, were conducive in the assembly of intestinal tight junctions (Figure. 10) and enhanced the intestinal barrier function (Mariadason et al., 1997;Peng et al., 2007;Suzuki et al., 2008;Van Deun et al., 2008;Eamin et al., 2013).

The intestinal barrier function of the small intestine is more important than that of the colon or cecum, because the small intestine is well organized for the digestion the of food and the absorption of nutrients, and the surface area of small intestine is also much larger than that of the colon. Thus, *Lactobacillus* species were selected as candidates for probiotics because their major habitat is the proximal part of the GIT. *Lactobacillus spp.* are the most common bacteria in the duodenum, and jejunum (Walter, 2008).

The aims of the study 1 were to screening the synbiotic combinations according to the fermentability of SCFAs and to investigate the effect of the synbiotics on an *in vitro* leaky gut cell model induced by LPS treatment.

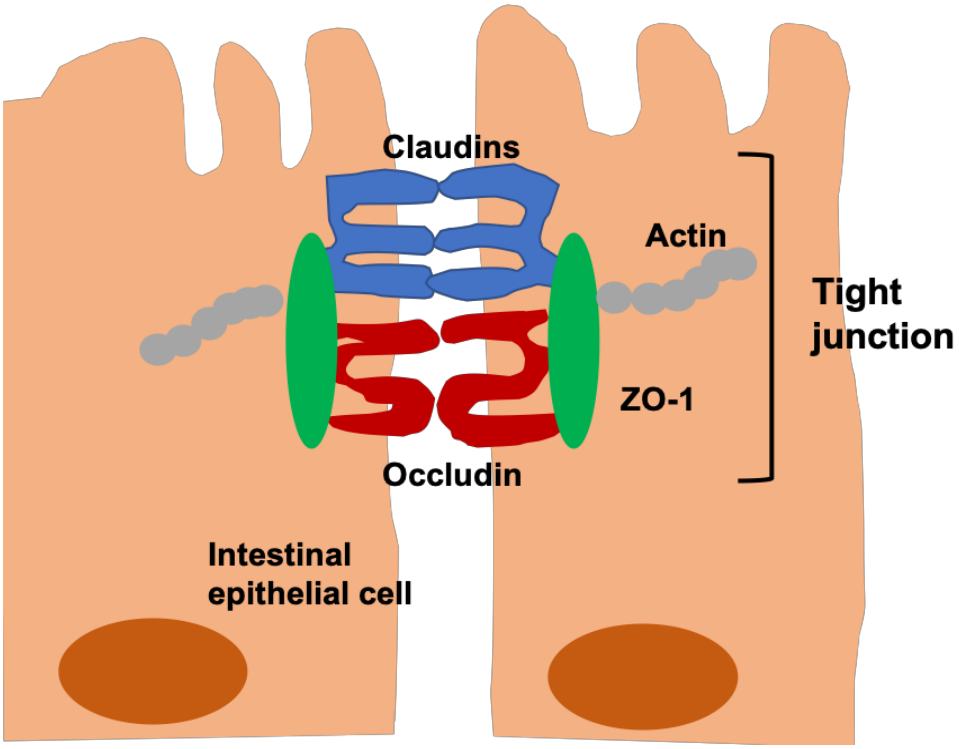

Figure. 10 Illustration of epithelial tight junction.

Tight junctions are cell-cell adhesion structures present in epithelia cells at the limit between the apical plasma membrane that faces the exterior environment.

Table 7. Contents of study 1.

Study	Category	Item
Screening for prebiotics candidates	Screening the prebiotics candidates by API kit	API kit for Lactobacillus spp.
	SCFA fermentation profile of the synbiotic combinations	Detection SCFA fermentation profile by gas chromatography
In vitro synbiotics functional assay	in vitro leaky gut cell model construction	Treatment LPS to IPEC-J2 cell
		Monolayer permeability and TEER value
		mRNA expression of TJ protein
	Effects of SCFA to in vitro leaky gut model(IPEC-J2 cell)	sodium acetate , sodium propionate, sodium butyrate
		mRNA expression of TJ protein
	Test of curing effects	mRNA expression of TJ protein
		Change of TEER value
	Test of preventing effects	mRNA expression of TJ protein
		Change of TEER value
	Test of protecting effects	mRNA expression of TJ protein
		Change of TEER value

2. Materials and Methods

1) Materials

All the materials and chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. De Man, Rogosa and Sharpe agar (MRS) broth were purchased from BD Difco (Sparks, MD, USA) for bacterial cultures.

2) Growth and culture conditions of microorganisms

The three strains, *Lactobacillus plantarum* 177*; Lactobacillus salivarius* KLW001*; Lactobacillus reuteri* KLR3004 were isolated from swine feces and two strains, *Lactobacillus paracaeei* KCTC3510, *Lactobacillus rhamnosus* KCTC5033 were purchased from Korean Collection for Type Cultures (KCTC). Pure cultures were stored at -70°C in MRS broth supplemented with 20% v/v glycerol for further use.

3) Fermentation profiles of *Lactobacillus* species

Carbohydrate fermentation profiles were obtained with API Rapid CH fermentation strips (bioMérieux, Marcy l'Etoile, France) in duplicate at 37°C,

in *Lactobacillus* identification medium (CHL broth, API 50 CHL; bioMérieux) as specified by the manufacturer.

4) Analysis of SCFA production of *Lactobacillus* species

To detect the production of SCFAs by gas chromatography (GC), the cultured supernatants of synbiotic combinations were mixed with an internal standard (propionic acid-2,2-d2) and methanol. GC was performed under the following conditions. The Thermo Scientific Trace 1310 system was used for GC, comprising a Thermo ISQ LT mass selective detector with a TG-5MS (Mass spectroscopy) column (30 x 0.25 mm (5 %-phenyl)–methylpolysiloxane capillary column, film thickness of 0.25 lm). The temperature of the oven was programmed as follows: initial temperature of 50 °C for 5 min, then increases of 4 °C/min up to 250 °C. The carrier gas was helium, and the flow rate was 1.0 ml/min. Samples were injected in a volume of 1 μl, and the ionization energy was 70 eV. SCFAs were identified based on their retention time and by comparison of their mass spectral pattern with the National Institute of Standards and Technology library.

5) IPEC-J2 cell culture conditions and MTT assay

IPEC-J2 cell line (intestinal porcine epithelial cells from jejunum) were used between passage 45 and 60. Cells were cultured with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% insulin/transferrin/selenium (ITS) (100X; Gibco, Germany). Cells were grown in 37°C, 5% CO_2, and 95% relative humidity. They were fed every other day. Trypsinized cells were added to 6-well tissue culture-treated plates (200μl; Costar, Corning) and Trans-well filters (500μl, 6.5 mm × 0.33 cm^2, 0.3um pore size; Costar, Corning). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2Htetrazolium bromide) assay was performed as described before. A stock solution of MTT (1mg/ml distilled water) was prepared immediately prior to use, the culture medium of IPEC-J2 cell was replaced by 1.5ml DMEM/F12 which is added with 0.1ml MTT stock solution. Cells were incubated for 4h at 37°C, after which the medium was removed and the culture washed with phosphate buffered saline (PBS). For the viability assay, the formazan product was dissolved in 1.5 ml dimethylsulfoxide (DMSO) and the absorbance measured at 540 nm with a plate reader (SpectraFluor, Tecan, Männedorf, Switzerland).

Cell survival rate was expressed as the relative percentage of the non-treat control group.

6) Measurement of trans-epithelial electrical resistance (TEER)

IPEC-J2 cells were seeded at 10^5 cells in culture medium in 0.33cm^2 polyethylene terephthalate membrane inserts with 0.4um pores. The medium was changed every other day. The electrical resistance was measured using the epithelial voltohmmeter (World Precision Instruments). During measurement, both apical and basolateral sides of the epithelium were bathed with a buffer solution. Electrical resistance was measured until similar values were recorded for three consecutive measurements. The TEER was measured at 24 h after treatment. Measurements were performed on 3 replicates from 3 independent experiments.

7) Determination of the para-cellular permeability

IPEC-J2 cell monolayers were grown on Trans-well plates and treated as described above. By the end of TEER measurements, 1 g/L fluorescein isothiocyanate-labeled dextran 4 kDa (FITC-D4; Sigma-Aldrich) was added to the apical compartment and incubated for 4h at 37°C. Then, 100μl of medium in basal compartments were collected in 96-well plates, and FITC-D4 was measured spectrophotometrically at an excitation wavelength of 498 nm and an emission wavelength of 540 nm. Mono-layer permeability was quantified of FITC-D4 permeating from the apical to the basal compartment.

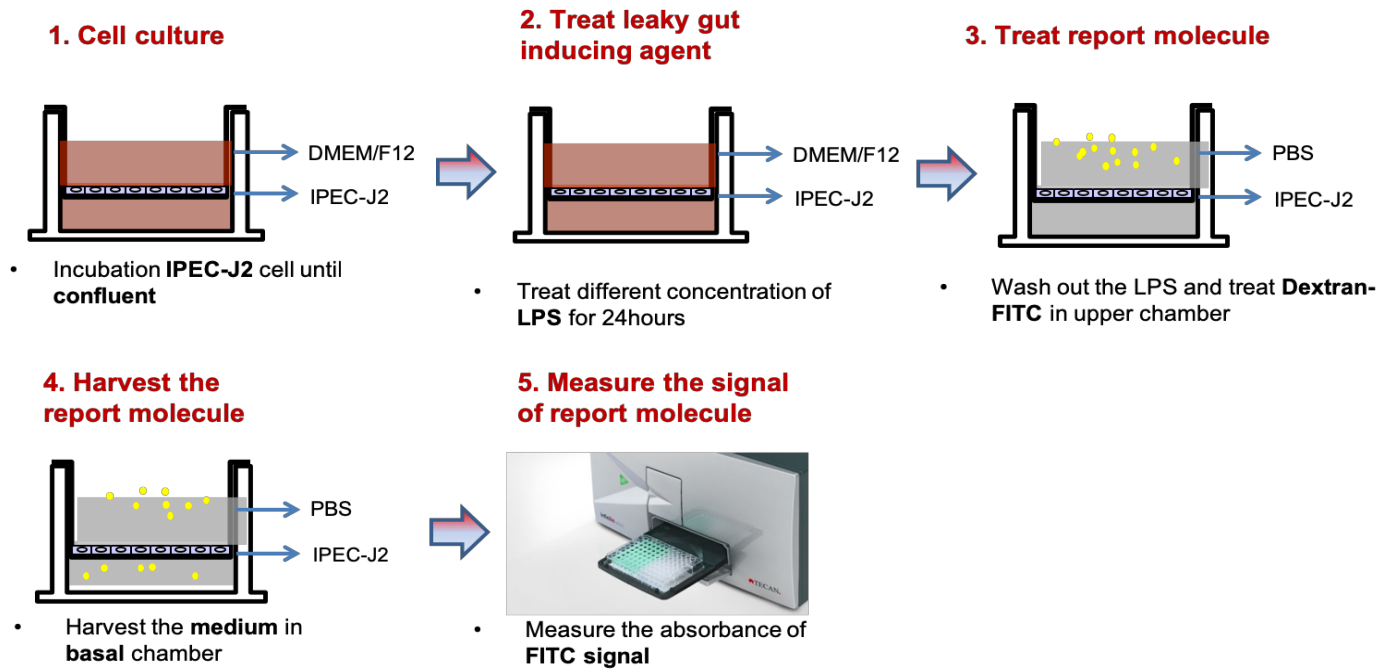

Figure. 11 Procedure of measuring paracellular permeability.

8) mRNA extraction and qRT-PCR

IPEC-J2 cells were grown to confluence, then they were washed three times with PBS and immediately lysed and collected using TRIzol reagent (Invitrogen Life Technologies Ltd., Carlsbad, CA, USA). Total RNA was extraction and quality were checked cDNA was synthesized from 1 μg of RNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover purchased from TOYOBO CO., LTD (Dojima, Osaka, Japan). Quantitative real-time PCR (qRT-PCR) was performed with SYBR qPCR Mix using one-step real-time PCR. The primer sequences are listed in Table 8.

The relative gene expression was calculated using the ΔΔCt method. The target gene expression was normalized to the relative expression of beta-actin.

Table 8. Primers used in this study.

Gene	Primer sequence (5`-3`)	TM (◦C)
Beta-actin	F: TGCGGGACATCAAGGAGAAG	58.8
	R: AGTTGAAGGTGGTCTCGTGG	58.6
Occludin	F: ATCAACAAAGGCAACTCT	50.5
	R: GCAGCAGCCATGTACTCT	56.1
ZO-1	F: GAGTTTGATAGTGGCGTT	51.4
	R: GTGGGAGGATGCTGTTGT	55.9
Claudin-1	F: TTTCCTCAATACAGGAGG	56.1
	R: CCCTCTCCCCACATTCGA	58
TNF-alpha	F: ATTCAGGGATGTGTGGCCTG	59
	R: CCAGATGTCCCAGGTTGCAT	59

9) Statistical analysis

Data are presented as the mean ± SEM of three independent experiments. The statistical significance was analyzed between each group by one-way ANOVA and Tukey's test (*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$).

3. Results

1) Screening the prebiotic candidates by API kit

The API CH strip consists of 50 microtubes used to study fermentation of substrates belonging to the carbohydrate family and its derivatives. The following table 9 and 10 listed the substrates that can be fermented by five kinds of *Lactobacillus* species. Among the substrates that can be fermented, toxic substances, substances that go against the concept of prebiotics, and substances that are not economically viable were excluded. Maltose was confirmed to be fermented by four species in five *Lactobacillus*, therefore, pullulan which composed by alpha1-4 linkage maltotriose like maltose, and can be used as prebiotics was selected as prebiotic candidates. Xylose can be fermented by *Lactobacillus reuteri*. So that xylo-oligosaccharide which considered as prebiotic (Lecerf et al., 2012;Ribeiro et al., 2018), was selected as its prebiotic candidate.

Table 9. Check the fermentation ability of five kinds of *Lactobacillus* spp.

	Glucose	Fructose	Galactose	Mannose	Ribose	Rhamnose	Sorbitol	Glucopyranoside	Mannitol	Acetyl-glucosamine	Amygdalin	Arbutin
L.plantarum	+	+	+	+	+		+			+	+	+
L.reuteri	+		+									
L.salivarius	+	+	+	+			+		+	+		
L.paracasei	+	+	+	+	+				+	+		
L.rhamnosus	+	+	+	+	+	+	+	+		+		+

Symbol '+' in the table means fermented by the corresponding *Lactobacillus.*spp

Rhamnose, Sorbitol, Mannitol were selected as prebiotic candidates.

Table 10. Check the fermentation ability of five kinds of *Lactobacillus* spp. (continued)

	Salicin	Cellobiose	Maltose	Tagatose	Xylose	Lactose	Melibiose	Sucrose	Trehalose	Raffinose	Turanose	Gentiobiose
L.plantarum	+	+	+			+	+	+	+	+		+
L.reuteri			+		+	+	+	+		+		
L.salivarius	+		+			+	+	+		+		
L.paracasei	+	+		+			+		+		+	+
L.rhamnosus	+	+	+	+		+	+		+		+	

Symbol '+' in the table means fermented by the corresponding *Lactobacillus.*spp

Maltose (Pullulan), Xylose (xylo-oligosacchaide), Trehalose, Raffinose were selected as prebiotic candidates.

2) SCFA fermentation profile of synbiotic combinations

SCFA fermentation ability of synbiotic combinations were verified by gas chromatography. *Lactobacillus plantarum* can ferment pullulan to acetate, propionate and butyrate, except pullulan, terhalose can be fermented to acetate, sorbitol and raffinose can be fermented to propionate and butyrate. *Lactobacillus reuteri* can ferment raffinose to acetate, and *Lactobacillus reuteri* itself was found to ferment a large amount of acetate. (Figure. 12). According to figure 13, mannitol and trehalose can't be fermented by *Lactobacillus paracasei* to SCFA. And pullulan can be fermented by *Lactobacillus salivarius* to acetate. Rhamnose was well-known prebiotics to *Lactobacillus rhamnosus*, it was found that rhamnose can be fermented to butyrate, except rhamnose, sorbitol and trehalose were also can be fermented to butyrate by *Lactobacillus rhamnosus* (Figure. 14).

Three kinds of synbiotic (*Lactobacillus plantarum*/pullulan; *Lactobacillus salivarius*/pullulan, *Lactobacillus reuteri*/raffinose) combinations were selected considering the total fermentation of SCFA and the additional effect of prebiotics.

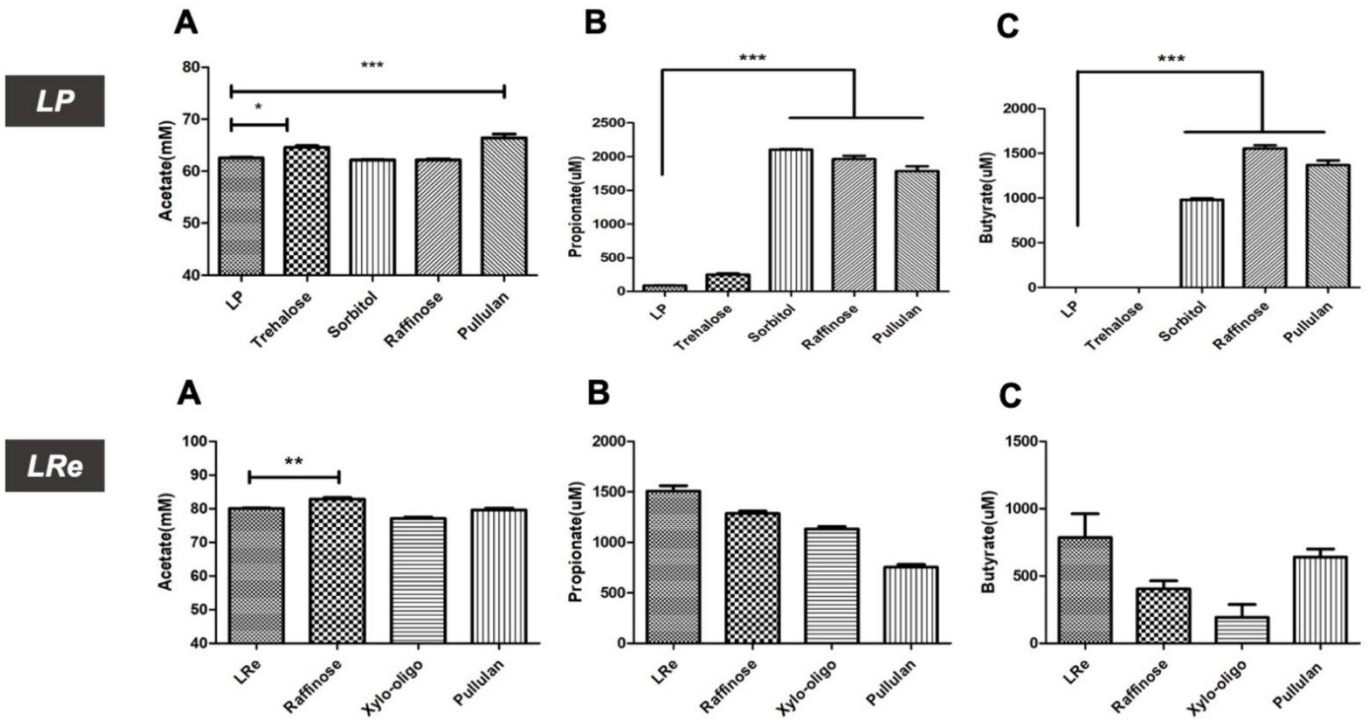

Figure. 12 SCFA fermentation ability of LP and LRe combine with their prebiotic candidates.

Acetate. fermentation profile (A), Propionate fermentation profile (B), Butyrate fermentation profile (C).

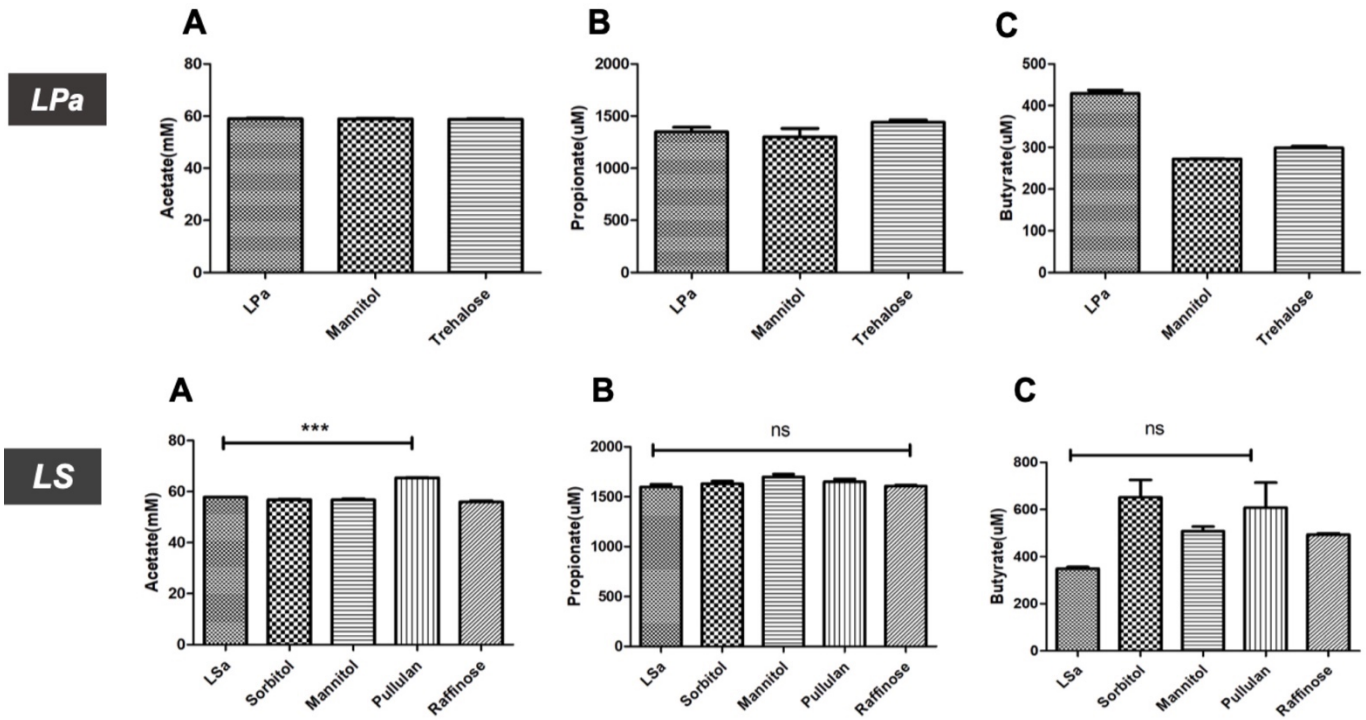

Figure. 13 SCFA fermentation ability of LPa and LS combine with their prebiotic candidates.

Acetate fermentation profile (A), Propionate fermentation profile (B), Butyrate fermentation profile (C).

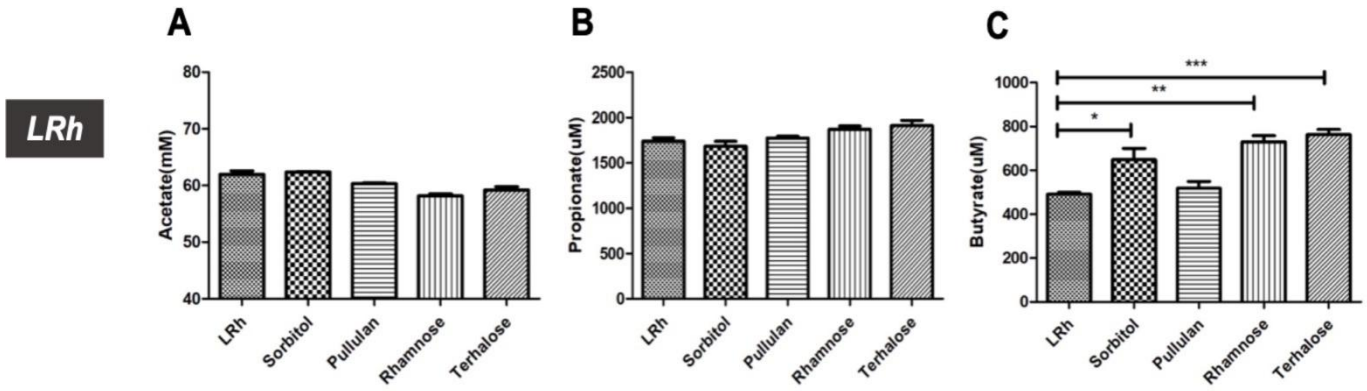

Figure. 14 SCFA fermentation ability of LRh combine with its prebiotic candidates.

Acetate fermentation. profile (A), Propionate fermentation profile (B), Butyrate fermentation profile (C).

3) *In vitro* leaky gut cell model construction

In order to verify the effectiveness of synbiotics on the intestinal barrier function, especially the tight junction of intestinal epithelial cells, a leaky gut cell model was established. IPEC-J2 cell line (intestinal porcine epithelial cells from jejunum) were used, and lipopolysaccharide (LPS) from *E.coli* was used to induce the cell dysfunction which can lead inflammation through the TLR4 mediated pathway. LPS was treated to IPEC-J2 cells from 0.1μg/ml to 200μg/ml after incubation for 24 hours the monolayer permeability; TEER value change and mRNA expression of tight junction protein were verified. When the LPS concentration was up to 100μg/ml and 200μg/ml, the integrity of IPEC-J2 monolayer was effectively destroyed due to the most dextran transmitted monolayer and the TEER value was decreased the most (Figure. 15). The mRNA expression associated the tight junction protein occludin and ZO-1 were down-regulated by LPS treatment. Similarly, the most disruptive levels of mRNA expression were found in the 100μg/ml and 200μg/ml (Figure. 16A, B).

MTT assay was performed to determine the final concentration of LPS treatment, cell viability of IPEC-J2 cell began to decrease at LPS concentration at 200μg/ml (Figure. 15C).

The mRNA expression of TNF-alpha (Figure. 16C), well-known pro-inflammatory cytokine, was also approved to check whether the LPS was working well in the IPEC-J2 cell line.

Overall, LPS can induce the dysfunction of IPEC-J2 cell, and *in vitro* leaky gut cell model can be constructed by treating LPS 100μg/ml for 24 hours.

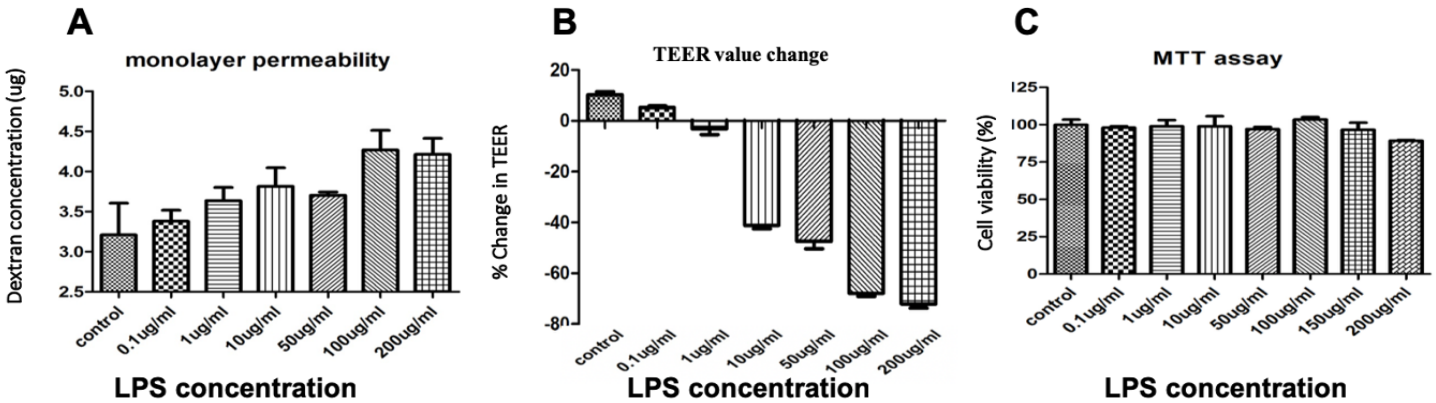

Figure. 15 *In vitro* leaky gut cell model construction.

Monolayer permeability after LPS treatment were determined by dextran concentration (A), TEER value change (B), cell viability check by MTT assay(C).

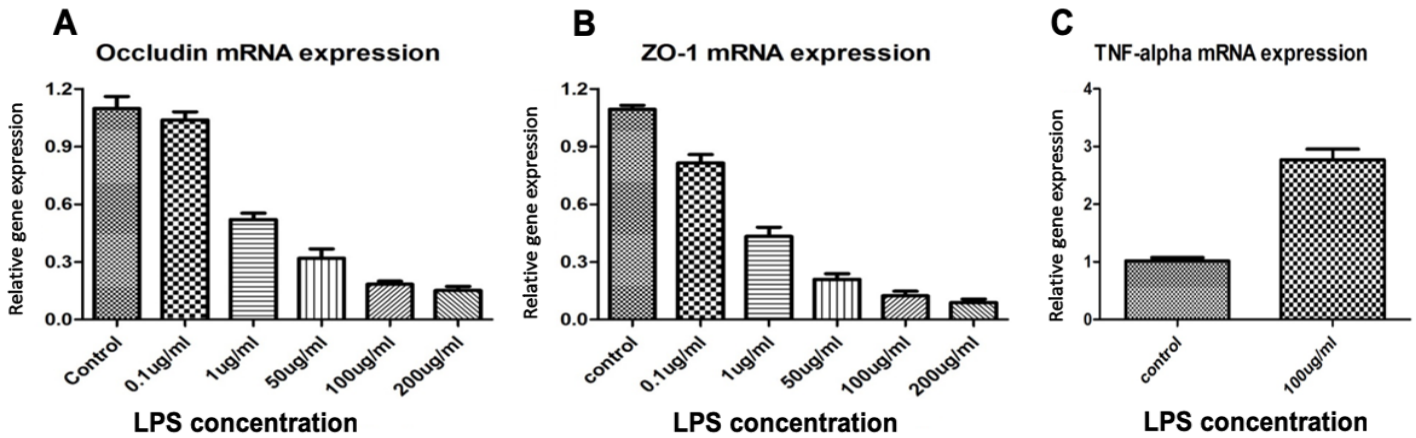

Figure. 16 mRNA expression of IPEC-J2 cell after LPS treatment.

Occludin mRNA expression (A), ZO-1 mRNA expression (B), TNF-alpha mRNA expression(C).

4) Effect of SCFA to *in vitro* leaky gut cell model

To check the effectiveness of SCFA in IPEC-J2 cells, purified sodium acetate(C2), sodium propionate(C3), and sodium butyrate(C4) were treated with low (100μm/ml) concentration and high (1mM/ml) concentration (Figure. 17). It was found that occludin did not response to the low concentration of SCFA, ZO-1 was up-regulated by all kinds of SCFA, and claudin-1 was the most sensitive to butyrate and thus the most frequently up-regulation. The high concentration of SCFA up-regulated all kinds of tight junction associated proteins mRNA expression. Among the SCFA, butyrate was found to be the most effective.

In conclusion, the in vitro IPEC-J2 leaky gut cell model was confirmed to be suitable for verifying the function of synbiotics which mediated SCFA, and butyrate was the most effective SCFA.

5) Quantification of short chain fatty acid in CFS

All prebiotics could increase the fermentation of acetate in probiotics, and *Lactobacillus reuteri* had the most superb competence in the fermentation of acetate. Pullulan improved fermentation of propionate and butyrate in LP. It has been confirmed that the combination of LP and pullulan significantly fermented more butyrate than any probiotics or synbiotic combinations (Figure. 18). Based on the results of the functional verification of SCFA above, it was believed that LP and pullulan combination would have the most effective result i*n vitro* functional assay.

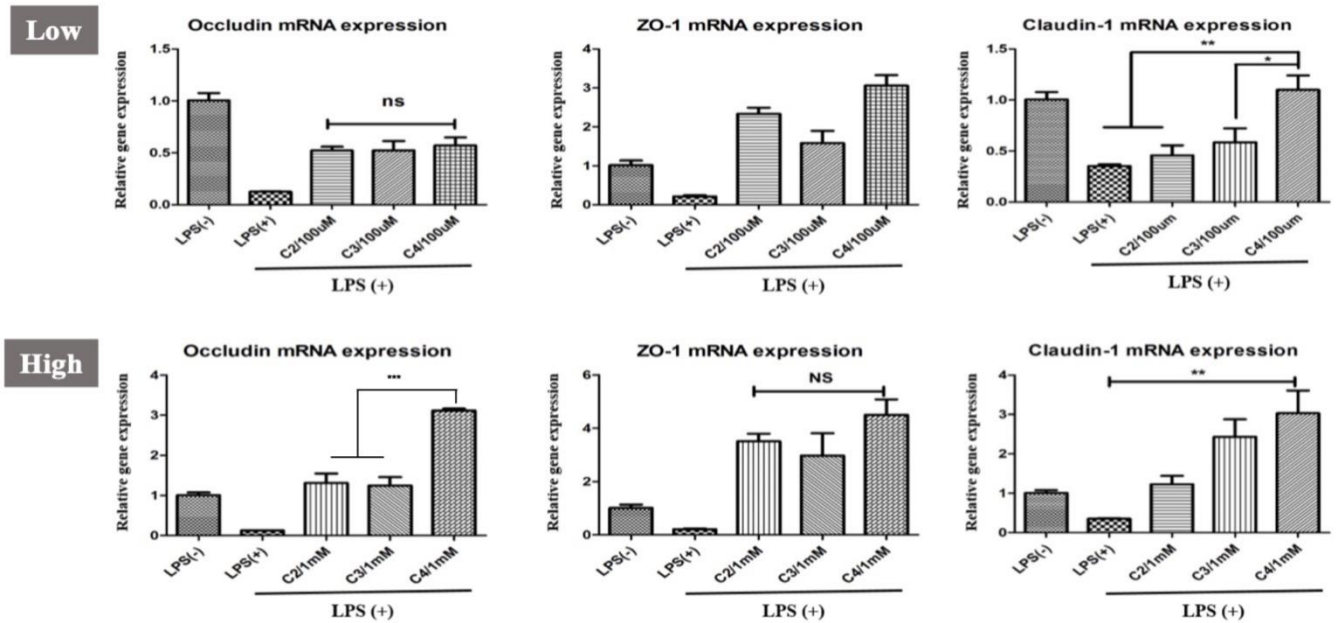

Figure. 17 Effect of SCFA on in vitro leaky gut cell model.

Low concentration (100μM) and high concentration(1mM), C2: sodium acetate; C3: sodium propionate; C4: sodium butyrate.

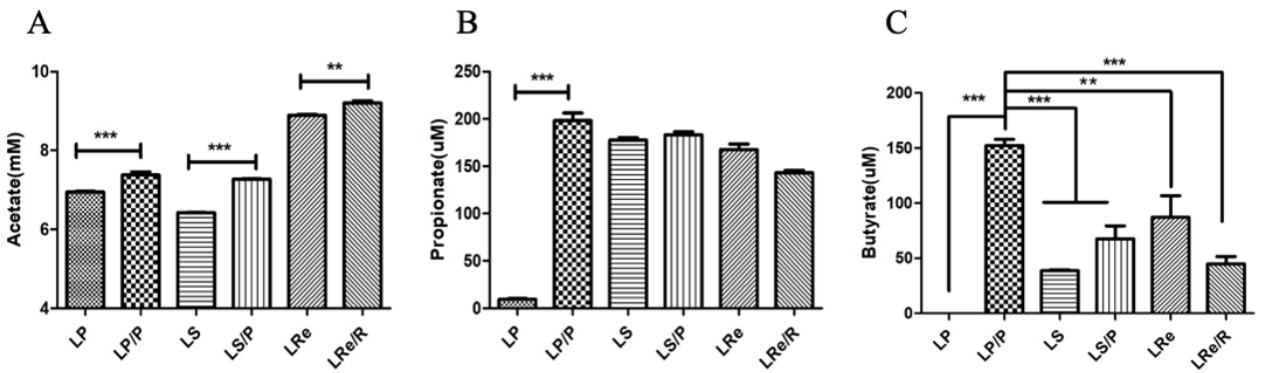

Figure. 18 Quantification of SCFA in CFS.

Acetate (A), propionate (B), butyrate (C).

6) *In vitro* synbiotics functional assay

Three methods (curing test, preventing test, protecting test) were used for functional evaluation of synbiotics *in vitro* (Figure. 19). In curing test, the apical side of the cells was first treated with LPS 100μg/ml for 24h, after which cells were washed with PBS and treated with 10% v/v CFS of synbiotic or probiotics. In the preventing test, the order of the treatment was reversed, and the treatment with LPS 100μg/ml was followed by treatment with 10% v/v CFS of synbiotics or probiotics. In the protecting test, 10% v/v CFS of synbiotics or probiotics and 100ug/ml of LPS were treated to cell and incubated 48h.

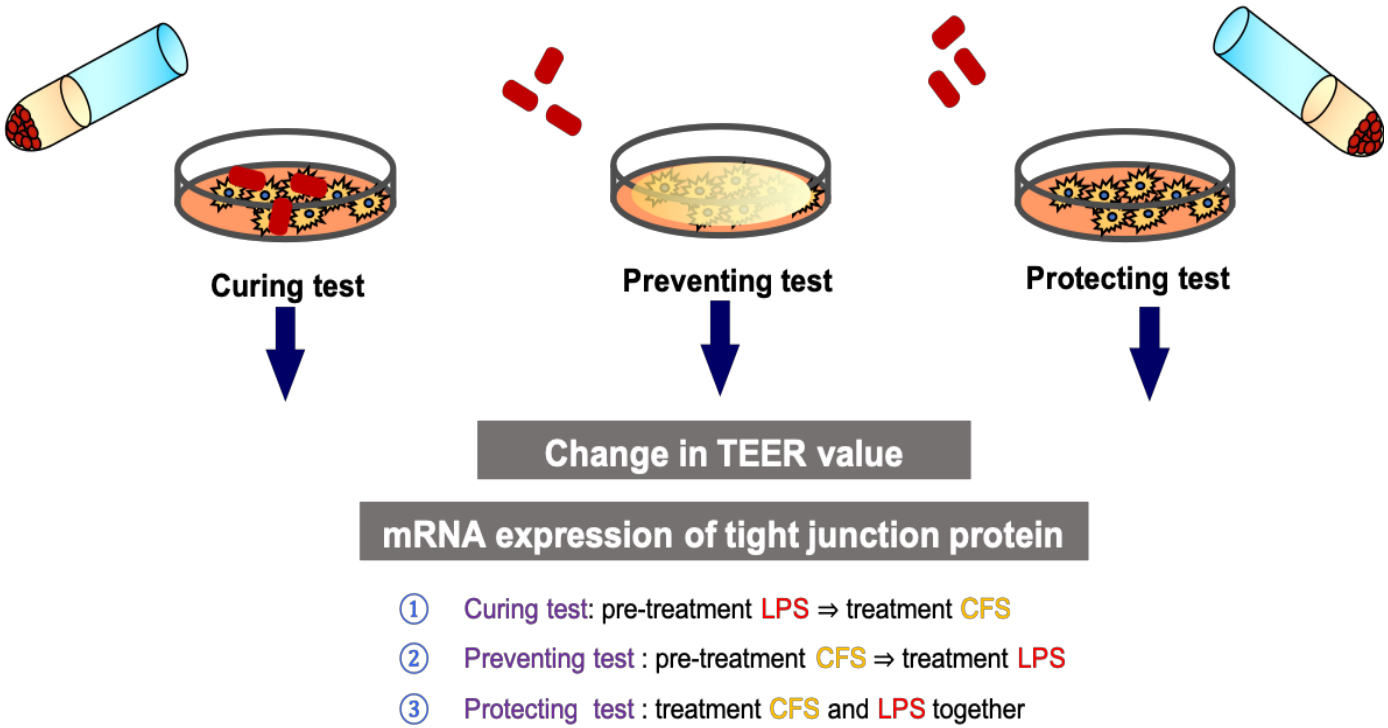

Figure. 19 Procedure of synbiotics in vitro functional assay.

(1) Curing test

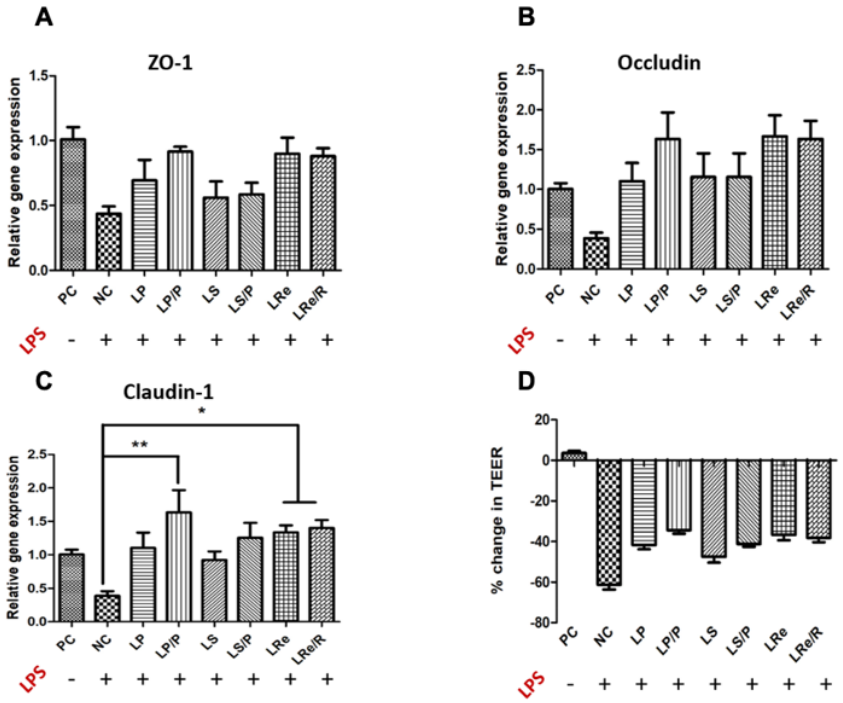

Figure. 20 Test of curing effect.

ZO-1 mRNA expression (A), occludin mRNA expression (B), Claudin-1 mRNA expression (C), TEER value change (D).

There is no additional prebiotics effect was found on the combination of synbiotics as compared to probiotics alone in mRNA expression of tight junction protein and change in TEER value. LP and pullulan significantly up-regulated claudin-1 (Figure. 20C).

(2) Preventing test

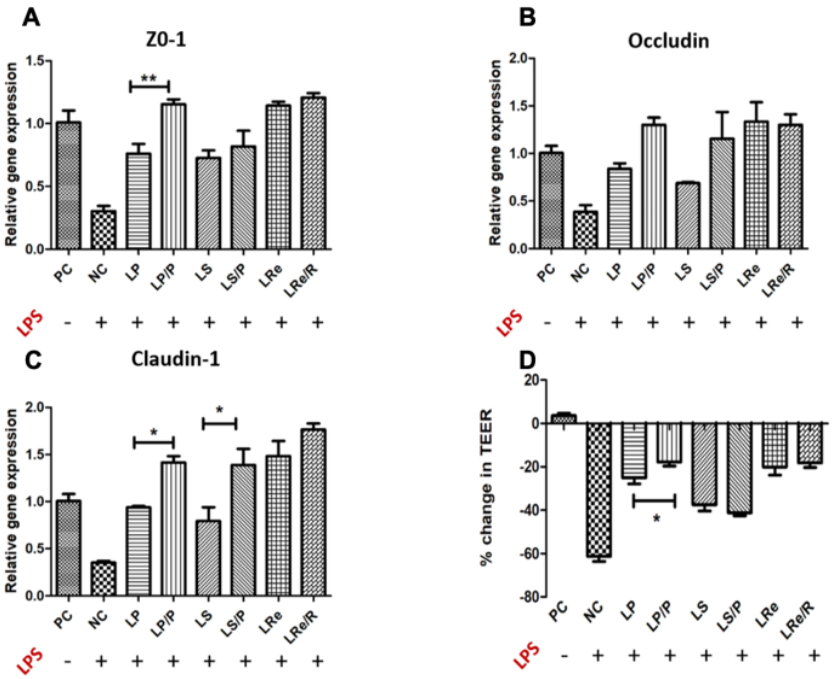

Figure. 21 Test of preventing effect.

ZO-1 mRNA expression (A), occludin mRNA expression (B), Claudin-1 mRNA expression (C), TEER value change (D).

LP with pullulan had a significant positive effect on the up regulating of tight junction mRNA compared with LP alone, it was also confirmed that LP/P prevented the decrease of TEER value more efficiently than LP alone. LS with pullulan up-regulated claudin-1 mRNA expression more efficiently

than LS, but there was no difference between LS and LS/P in the TEER value change (Figure. 21).

(3) Protecting test

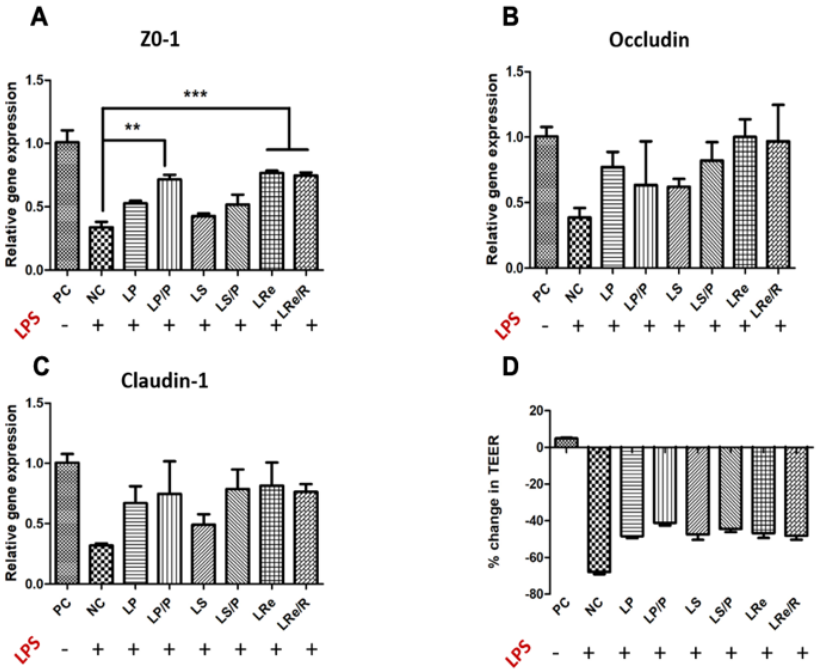

Figure. 22 Test of protecting effect.

ZO-1 mRNA expression (A), occludin mRNA expression (B), Claudin-1 mRNA expression (C), TEER value change (D).

There is no additional prebiotics effect was found on the combination of synbiotics as compared to probiotics alone in mRNA expression of tight

junction protein and change in TEER value. Therefore, LP/P, LR and LR/R could significantly up-regulate ZO-1 mRNA expression (Figure. 22).

4. Discussion

The intestinal epithelial barrier selectively regulates epithelial permeability to luminal substances and antigens. The disruption of the intestinal barrier induced by toxins and pathogens contributes to the development of severe intestinal inflammation and digestive disorders (Arrieta et al., 2006; Oswald, 2006). SCFA, the products of bacterial fermentation, has been found to exert profound influences on intestinal barrier function (Oswald, 2006; von Martels et al., 2017).

In the present study, the synbiotic combination was selected based on SCFA fermentation ability, and demonstrate that Lactobacillus plantarum treated with pullulan fermented most butyrate and enhanced the intestinal barrier integrity against LPS-induced impairment. The IPEC-J2 cell model is a well-established model for studying intestinal barrier function (Brosnahan and Brown, 2012)

Lactobacillus treated with prebiotic promoted intestinal barrier integrity, indicated by the inhibited the decreased in TEER, and inhibited the down-regulation of tight junction proteins mRNA (ZO-1, claudin-1). Claudins are

the main structural and functional components of tight junctions, selectively preventing the passage of luminal substances through paracellular routes (Hammer et al., 2015). The decrease in protein expression of claudins is highly correlated to impaired intestinal barrier function (Pinton et al., 2010). Therefore, the protective effect of LP/P on intestinal barrier against LPS damage may be partly explained by the increase in the expression of claudin proteins.

LPS activated inflammatory pathway through binding and activation TLR-4, its cognate receptor (Schlegel et al., 2012; Guo et al., 2015). Yan and Ajuwon indicated that butyrate could downregulate the expression of TLR-4 (Yan and Ajuwon, 2017). Thus, downregulation of expression of TLR-4 may partly explain the suppression of LPS effect by butyrate. So that LP/P had the most preventing effect in LPS induced intestinal dysfunction.

In conclusion, LP/P fermented most butyrate and it restored LPS-induced impairment of the intestinal barrier by promoting tight junction (especially claudins) expression. Results obtained agree with the observed enhancement of epithelial barrier integrity in CACO-2 cells by butyrate (Valenzano et al., 2015). Butyrate was indicated that regulate of AMPK phosphorylation and

activation (Yan and Ajuwon, 2017) and may result from its role in the regulation of energy homeostasis, agreeing with the recognized the importance of butyrate as an energy source for epithelial cell proliferation and maintenance of intestinal barrier integrity. LP/P could be developed as synbiotic additives which can increase intestinal butyrate level.

Table 11. Summary of study 1.

Contents	Results
Screening for prebiotics candidates	Rhamnose, Sorbitol. Mannitol, Maltose(pullulan) Xylose(xylo-oligosacharide), Trehalose, Raffinose
The synbiotics was selected based on SCFA fermentation profiles	Lactobacillus plantarum/pullulan(LP/P) Lactobacillus salivarius/pullulan(LS/P) Lactobacillus reuteri/raffinose(LR/R)
in vitro leaky gut cell model construction	Construction in vitro model by treating LPS 100ug/ml for 24hrs
In vitro synbiotics functional assay	Curing effect : LP/P had the most curing effect on claudin-1 expression
	Preventing effect : LP /P had the most preventing effect on ZO-1 and claudin-1 expression
	Protecting effect : LR had the most protecting effect on ZO-1 expression

Study 2. Pullulan nanoparticles as prebiotics enhance the antibacterial properties of *Lactobacillus plantarum*

1. Introduction

According to the World Health Organization (WHO), the use of antibiotics as growth promoters for livestock is a major cause of antibiotic resistance. Antibiotic resistance affects not only livestock health but also human health. Therefore, finding alternatives to antibiotics and addressing drug resistance have become important issues for scientists (de la Fuente-Nunez et al., 2012; Allen et al., 2014). Recently, several studies have demonstrated the potential of probiotics are potential candidates as antibiotic alternatives due to their ability to inhibit bacterial colonization on the intestinal barrier or to directly kill pathogens through their secreted bacteriocins (Gillor et al., 2008). Therefore, there have been many attempts to increase the production of bacteriocins, including biological and physical methods. A biological engineering strategy as one of biological methods enhanced production of bacteriocins in probiotics with higher stability and good characteristics

(Papagianni and Anastasiadou, 2009) ; however, the method is very complex, and consumers are increasingly concerned about genetically modified products. The physical methods used to optimize the production of bacteriocins include changing pH, temperature, pressure, oxygen content, and incubation time during probiotic culture. Interestingly, in our previous studies (Cui et al., 2018; Kim et al., 2018), pediocin production in *Pediococcus acidilactici* (PA) was markedly enhanced through intracellular stimulation by internalized inulin nanoparticles used as a synbiotic.

Among the probiotics, *Lactobacillus plantarum* (LP) is a versatile and abundant microorganism found in several environments ranging from food to animal gastrointestinal tracts (de Vries et al., 2006). It is also known that some strains of LP are capable of producing several natural antimicrobial substances, such as bacteriocins and organic acids (lactic acid and acetic acid), thereby inhibiting competitors in the same niche (Todorov et al., 2011; Reis et al., 2012). It was previously reported that LP 177 isolated from pig intestines exhibited strong antibacterial activity against *E. coli K99*, which can cause bacterial diarrhea in pigs (Yun 2007).

Prebiotics used as non-digestible food additives beneficially affect the host by selectively stimulating the growth and/or activity of a limited number of microorganisms in the colon (Gibson and Roberfroid, 1995a). Most prebiotics are inulin-based fructose oligomers or galacto-oligosaccharides. Among potential prebiotic compounds, pullulan has long been applied to food additives (Cheng et al., 2011). Pullulan is an α- 1,6 linked polymer of maltotriose subunits and is secreted by the fungus *Aureobasidium pullulans* (Catley et al., 1986). Due to its high molecular weight and slow hydrolysis by α-amylase and glucoamylase, pullulan is considered to be a non-digestible carbohydrate (Leathers, 2003).

In recent years, many researchers have begun to synthesize and apply drug delivery systems based on pullulan-based self- assembled nanoparticles (Na et al., 2003; Jeong et al., 2006;Zhang et al., 2010). By contrast, our synthetic PPN application is not a drug or gene carrier but a new type of prebiotic.

One of the simplest ways to synthesize polymeric nanoparticles is the self-assembly of hydrophobically modified hydrophilic polymers. Self-assembled polymeric nanoparticles, consisting of a hydrophobic core and a hydrophilic shell, have been used as promising drug carriers because they can be rapidly

internalized by mammalian cells after loading drugs into their hydrophobic cores (Zhang et al., 2008).

In study 2, the aim of the study was investigating the antimicrobial activities of phthalyl pullulan nanoparticle (PPN)-treated LP. PPNs was synthesized and developed as a new type of prebiotic for LP. In addition, antimicrobial assays were checked whether the internalization of PPNs by LP led to enhanced antimicrobial activity by LP against Gram-negative bacteria *Escherichia coli K99* and Gram-positive bacteria *Listeria.monocytogenes* (LM) than LP or pullulan alone. The mechanism of the antimicrobial activity of PPN-treated LP by the internalization of PPNs by LP was further validated.

Table 12. Contents of study 2.

Study	Category	Item
Development and phenotypic analysis of prebiotic nanoparticles	Synthesis of pullulan nanoparticles	pullulan, hydrophobic group (phthalyl anhydride)
	Characterization	H-NMR, SEM, DLS, ELS
	Antimicrobial property	Co-culture assay (viable cell counts)
		Pathogen agar layered test (inhibition zone)
	Tracking internalization of PPNs	Confocal microscope (FITC, FM4-64)
		FACS
	Antimicrobial property of PPNS alone	Co-culture assay (viable cell counts)
Mechanism study of enhanced antimicrobial property	Antimicrobial property of different kinds of nanoparticles	Co-culture assay (viable cell counts)
	Effects of PPNs on probiotics	Viable cell counts and pH
		Proteinase K
		Plantaricin (protein, mRNA)
		Stress response gene (mRNA)

2. Materials and Methods

1) Materials

All the materials and chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Lysogeny *broth* (LB), LB agar, De Man, Rogosa and Sharpe agar (MRS) broth, MacConkey agar and brain heart infusion (BHI) broth were purchased from BD Difco (Sparks, MD, USA) for bacterial cultures.

2) Synthesis of phthalyl pullulan nanoparticles (PPNs)

Phthalyl pullulan nanoparticles were synthesized according to a previously described method(Na and Bae, 2002),with a slight modification. One gram of pullulan was dissolved in 10 ml of dimethyl formamide (DMF), and 0.1 mol-% dimethylaminopyridine per pullulan sugar residue was added to the solution as a catalyst., and then phthalic anhydride was added to the above solution at different molar ratios per pullulan, including 6:1 (phthalic anhydride: pullulan) (named PPN1), 9:1 (phthalic anhydride: pullulan) (named PPN2), and 12:1 (phthalic anhydride: pullulan) (named PPN3), to produce PPNs with different degrees of substitution of phthalic groups. The

reaction was performed at 54°C for 48 h under nitrogen. The produced PPNs were dialyzed first in DMF to remove unreacted phthalic anhydride and then in distilled water at 4°C for 24 h to form self-assembled nanoparticles of phthalyl pullulan. The unreacted pullulan was removed after ultra-centrifugation of prepared PPNs. Finally, the PPNs were freeze-dried and stored at −20° C until use.

3) Characterization of PPNs

The content of the phthalyl groups in PPNs was confirmed by 600 MHz ^{1}H-nuclear magnetic resonance (NMR) spectroscopy (AVANCE 600, Bruker, Germany). The surface topography of PPNs was analyzed using a field-emission scanning electron microscope (FE-SEM) with SUPRA 55VP-SEM (Carl Zeiss, Oberkochen, Germany). The PPNs were mounted onto stubs with adhesive copper tape and coated with platinum under a vacuum using a coating chamber (CT 1500 HF, Oxford Instruments, Oxfordshire, United Kingdom). The sizes of the nanoparticles were measured with a dynamic light scattering (DLS) spectrophotometer (DLS-7000, Otsuka Electronics, Japan). The zeta potential of the nanoparticles was measured

with an electrophoretic light scattering (ELS) spectrophotometer (ELS- 8000, Otsuka Electronics, Japan).

4) Confirmation of internalization of PPNs by LP

First, the fluorescence isothiocyanate (FITC)-labeled PPNs were prepared as follows. Five mg of FITC was mixed with 100 mg PPNs or pullulan dissolved in 2 ml dimethyl sulfoxide (DMSO). After stirring for 4 h in an opaque tube at room temperature, the products were dialyzed against distilled water at 4°C for 24 h. Finally, FITC-labeled PPNs and pullulan were lyophilized and stored at −20°C until use.

To observe the internalization of PPNs and pullulan by probiotics, LP 177 (2.0×10^6 CFU/ml) was inoculated into 1 ml of MRS broth, treated with 0.5% (w/v) FITC-PPNs or FITC- pullulan, and incubated for 2 h at 37°C. The samples were then washed with PBS and analyzed by flow cytometry and confocal laser scanning microscopy (CLSM) (SP8X STED, Leica, Wetzlar, Germany). To confirm the internalization of nanoparticles into the probiotics, LP treated with FITC-PPN3 was observed by Z-section mode in CLSM.

To confirm the temperature-dependent internalization of nanoparticles, three separate cultures of LP were treated with 0.5% (w/v) FITC-PPN3 and incubated at 4, 20, and 37°C for 2 h. The samples were further washed with PBS and analyzed by flow cytometry and CLSM. To confirm further the transporter- dependent internalization of nanoparticles into probiotics, and glucose, galactose, fructose and PPN3 were used as blocking agents. LP (2.0×10^6 CFU/ml) was inoculated into 1 ml of PBS and pre-treated with 10% (w/v) glucose, galactose, fructose or PPN3 for 10 min at 37°C before treatment with 0.5% (w/v) FITC-PPN3. After 2 h of incubation at 37°C, the samples were washed three times with PBS, and the internalization of PPN3 was analyzed by flow cytometry and CLSM.

5) Bacterial cultures

Escherichia coli (E. coli) K99 and *Listeria monocytogenes* (LM) were used as representative Gram-negative and Gram-positive pathogens, respectively. MRS, LB, and BHI broths were used for LP 177, *E. coli* K99, and LM, respectively. All bacteria cultures were incubated at 37°C in a

shaking incubator (250 rpm) for 24 h prior to experimental procedures or stored at −70°C in 15% glycerol for further use.

6) Co-culture Assay and Agar Diffusion Test for Antimicrobial Ability

Antimicrobial activity of LP against *E. coli* and LM was determined using co-culture assays (Ditu et al., 2011) and agar diffusion tests (Driscoll et al., 2012), with some modifications. To compare the antimicrobial activity of LP against *E. coli* by co- culture assay, 2.0×10^6 CFU/ml of *E. coli* was co-cultured with 2.0×10^5 CFU/ml LP treated with or without 0.5% (w/v) PPNs or pullulan in MRS broth for 8 h at 37°C under aerobic conditions in a shaking incubator (250 rpm). The antimicrobial activity was determined by the survival rate of *E. coli*. The co-cultured samples were spread on MacConkey agar and incubated for 24 h at 37°C, and the number of *E. coli* colonies was counted. The antimicrobial activity of LP against LM was also determined by co-culture assay. LP and LM were co-cultured in BHI broth under similar conditions as described above. Finally, the co-cultured samples were spread on Oxford agar, and the number of LM colonies was counted.

The agar diffusion test was used to determine whether the cultured medium of LP treated with or without PPNs and pullulan was able to inhibit the growth of pathogens on an agar plate. First, 100 μl *E. coli* stock (2.0×10^8 CFU/ml) was spread onto LB agar. A paper disk was placed on the *E. coli*-spread plate, then 120 μl 8 h-cultured LP media of LP treated with or without (0.5% w/v) PPNs and pullulan was dropped onto the paper disk. After drying at room temperature, the plate was cultured overnight at 37°C. The zone of inhibition of *E. coli* growth was used as a direct measurement of antimicrobial activity. The same protocols were followed to test the inhibitory effect of LP treated with or without 0.5% (w/v) PPNs or pullulan on LM growth on BHI agar plates.

To confirm plantaricin activity, agar diffusion tests of LP against *E. coli* and LM were performed using the same protocols described above after the culture medium was treated with 1 mg/ml proteinase K and incubated at 37°C for 2 h, and then each culture supernatant was heated at 100°C for 30 min.

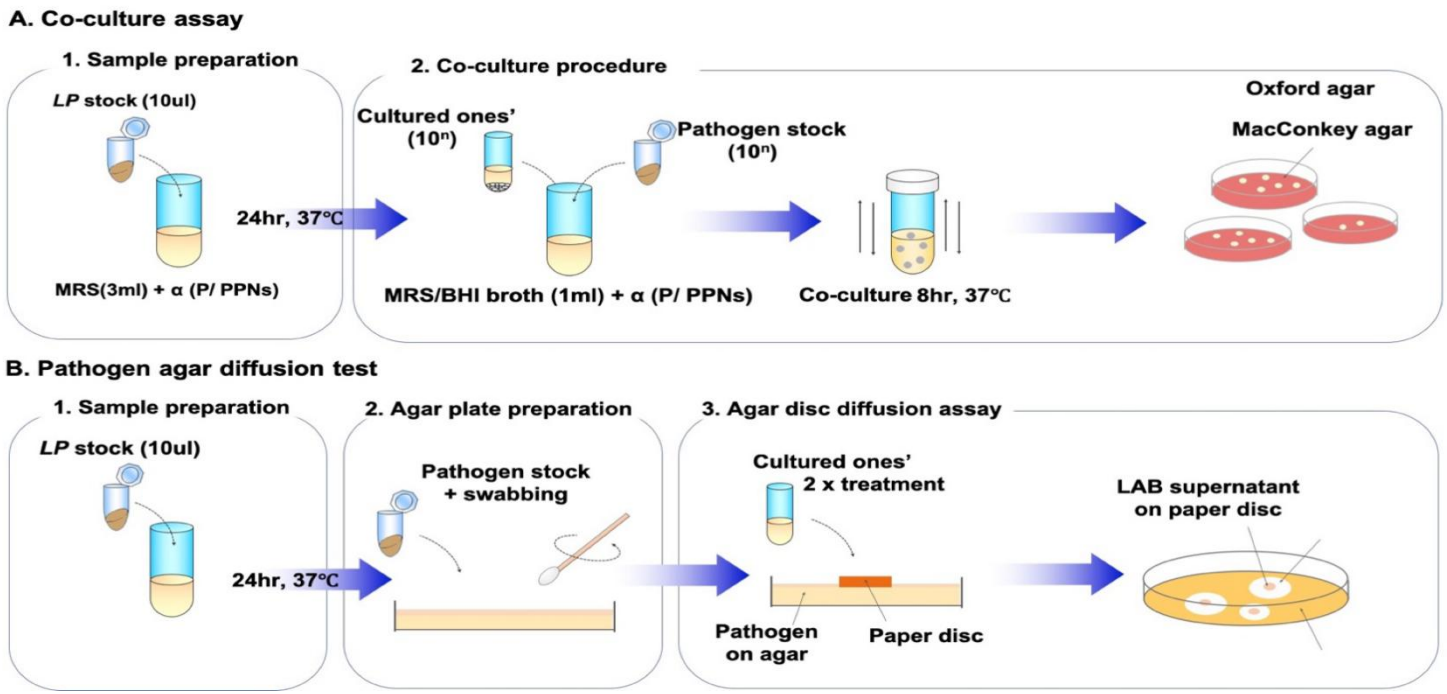

Figure. 23 Procedure of measuring antimicrobial activity.

(A) Quantitative analysis for measuring antimicrobial activity of LP against pathogens. Viable cell counts of pathogen was measured using selective agar. (B) Agar diffusion test was used to determine cultured LP can inhibit the growth of pathogens. The zone of inhibition was measured.

7) Analysis of the Growth Conditions of LP

After treatment of LP with or without PPNs or pullulan as described above, the growth characteristics of the LP were checked by measuring the pH of growth medium and viable cell counts at the indicated time points.

8) Protein Isolation and Identification by SDS–PAGE

Plantaricin was isolated and purified as described in a previous study(Song et al., 2014) with some modifications. Supernatants from the cocultured medium were stirred with ammonium sulfate (80% saturation) for 2 h at room temperature. The precipitated proteins, collected by centrifugation, was dissolved in citrate phosphate buffer (50 mM) and desalted by dialysis (1 kDa cut-off membrane, Spectrum Lab, United States). Proteins were lyophilized and stored at 4°C for further analyses.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was used to observe the isolated plantaricin.

9) Analysis of Stress Response and Plantaricin Genes by Quantitative Real-Time PCR

RNA extraction was performed using the TRIzol® Max™ Bacterial RNA Isolation Kit purchased from Thermo-Fisher Scientific Inc. (Waltham, MA, USA). Total RNA extraction was conducted according to the manufacturer's instructions. LP was treated with or without PPNs or pullulan as described above. After the isolation of RNA, cDNA was synthesized from 1 μg RNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover purchased from TOYOBO CO., LTD (Dojima, Osaka, Japan). Quantitative real-time PCR (qRT-PCR) was performed with SYBR qPCR Mix using one-step real-time PCR. The primers are listed in Table 13. Relative gene expression was calculated using the -2ΔΔCt method. The target gene expression was normalized to the relative expression of 16s rRNA as an internal control in each sample.

Table 13. Primers used in this study.

Gene	Primer sequence (5`-3`)	Size (bp)
planS	F:GCCTTACCAGCGTAATGCCC	450
	R:CTGGTGATGCAATCGTTAGTTT	
dnaK	F:ATTAACGGACATTCCAGCGG	600
	R:TTGGCCTTTTTGTTCTGCCG	
dnaJ	F:GGAACGAATGGTGGCCCTTA	474
	R:CTAGACGCACCCACCACAAA	
16S rRNA	F:GATGCGTAGCCGACCTGAGA	113
	R:TCCATCAGACTTGCGTCCATT	

10) Statistical analysis

Data are presented as the mean ± SEM of three independent experiments. The statistical significance was analyzed between each group by one-way ANOVA and Tukey's test (*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$).

3. Results

1) Synthesis and characterization of PPNs

The reaction scheme of PPN synthesis is shown in figure 24.

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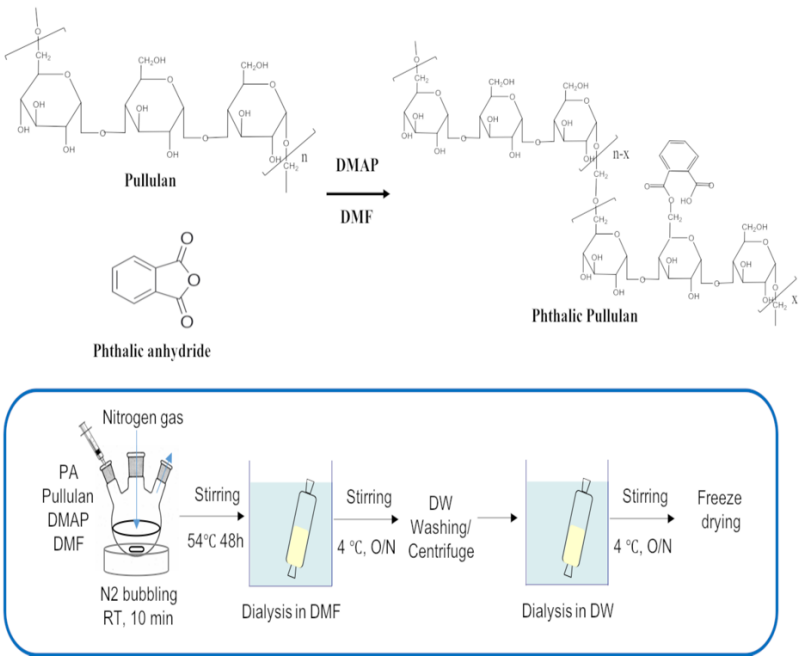


Figure. 24 Chemical reaction scheme for the synthesis of PPNs.

The degree of substitution of phthalic moieties in pullulan was confirmed by ^{1}H-NMR spectroscopy and calculated by determining the ratio of phthalic acid protons (7.4–7.7 ppm) to sugar protons (C_1 position of α-1,6 and α-1,4 glycosidic bonds, 4.68 and 5.00 ppm, respectively) as described by Tao et al.(Tao et al., 2016). According to the degrees of substitution of phthalic acid,

the PPNs were named as follows: PPN1 (DS: 30.90 mol. −%), PPN2 (DS: 50.45 mol. −%) and PPN3 (DS: 68.50 mol. −%). Using SEM, PPN3 was determined to be spherical and sized between 100 and 150 nm. The sizes of nanoparticles measured by DLS were 140.0, 108.9, and 82.1 nm for PPN1, PPN2, and PPN3, respectively indicating that the particles sizes decreased with an increase in the number of conjugated phthalic acid groups in pullulan. Furthermore, the surface charges of the PPNs, measured by ELS, were −38.09, −38.45, and −33.84 mV for PPN1, PPN2, and PPN3, respectively Due to the unreacted carboxyl groups in phthalic acid, the PPNs showed negative zeta potential (Figure. 25).

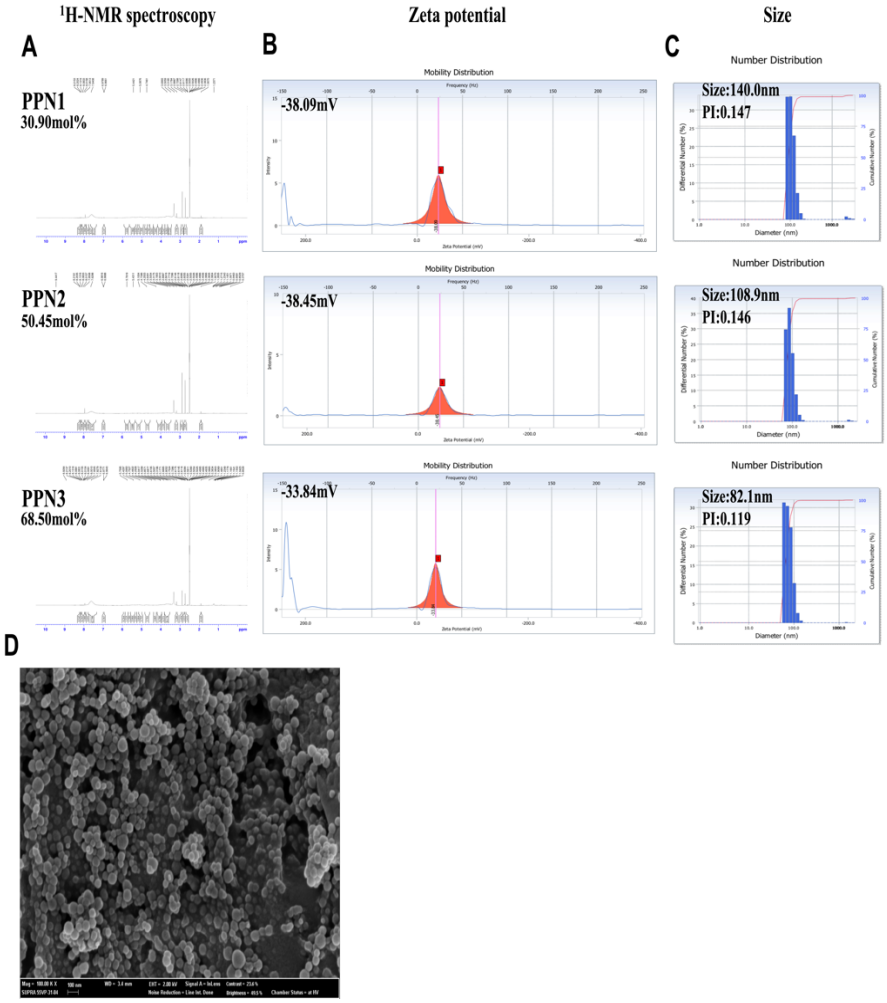


Figure. 25 Characteristics of PPNs.

Calculation of mol.-% phthalic acid in PPNs by ^{1}H-NMR spectroscopy (A). Measurement of the zeta potential of PPNs by ELS (B) and size by DLS (C). Morphologies of PPNs observed by SEM (D). Magnification: 100,00K, scale bar = 100 nm.

2) Internalization of PPNs by LP

To confirm the internalization of PPNs by LP, PPNs were conjugated to fluorescence isothiocyanate (FITC). The internalization of FITC-PPNs by LP was analyzed by CLSM and quantified by fluorescence-activated cell sorting (FACS). It was observed by CLSM that FITC-PPNs and FITC-pullulan were able to enter LP after incubation at 37°C for 2 h. The internalization of PPNs into LP was not much different among the PPNs due to the not much differences of the particle sizes of the PPNs (Figure. 26A), although pullulan alone entered LP through a diffusion mechanism. To further confirm whether the PPNs were located at the cell surface or were internalized by LP, LP was treated with FITC-PPN3 and the location of FITC-PPN3 was identified by Z-section mode of CLSM. As shown in figure 27A, the fluorescence intensity of FITC and DAPI was the highest at the center of LP, indicating the internalization of PPNs by LP. The membrane binding dye (FM4-64) was also used to performed the experiment as a negative control. As shown in figure 28A the fluorescence intensity of FITC was the highest at the center of LP, and confirmed that the FITC fluorescence appeared inside the bacteria (Figure. 27B).

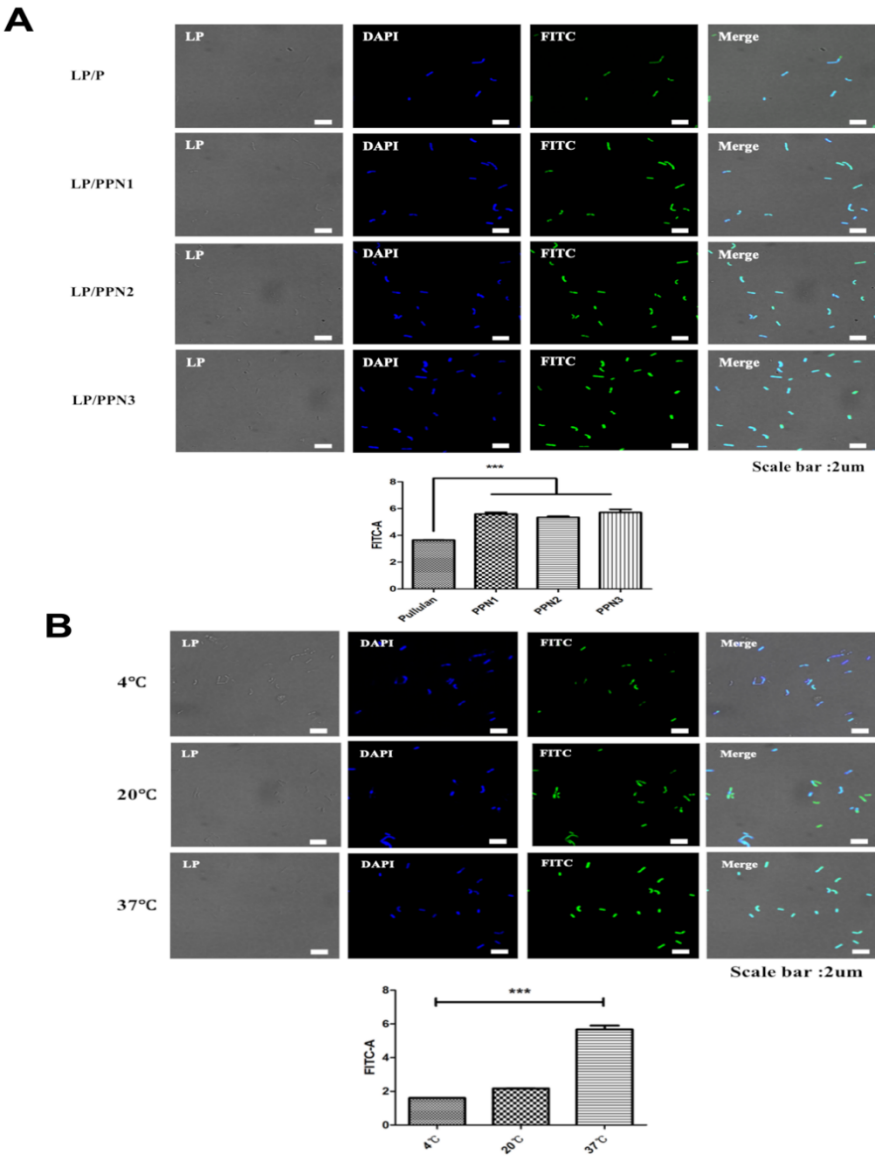


Figure. 26 Analysis of the internalization of PPNs by LP.

The internalization of pullulan and PPNs after 2 h of treatment was quantified by FACS and statistically analyzed (A). Next, LP was treated with 0.5% (w/v) FITC-PPN3 at different temperatures (4, 20, or 37°C) for 2 h (B)

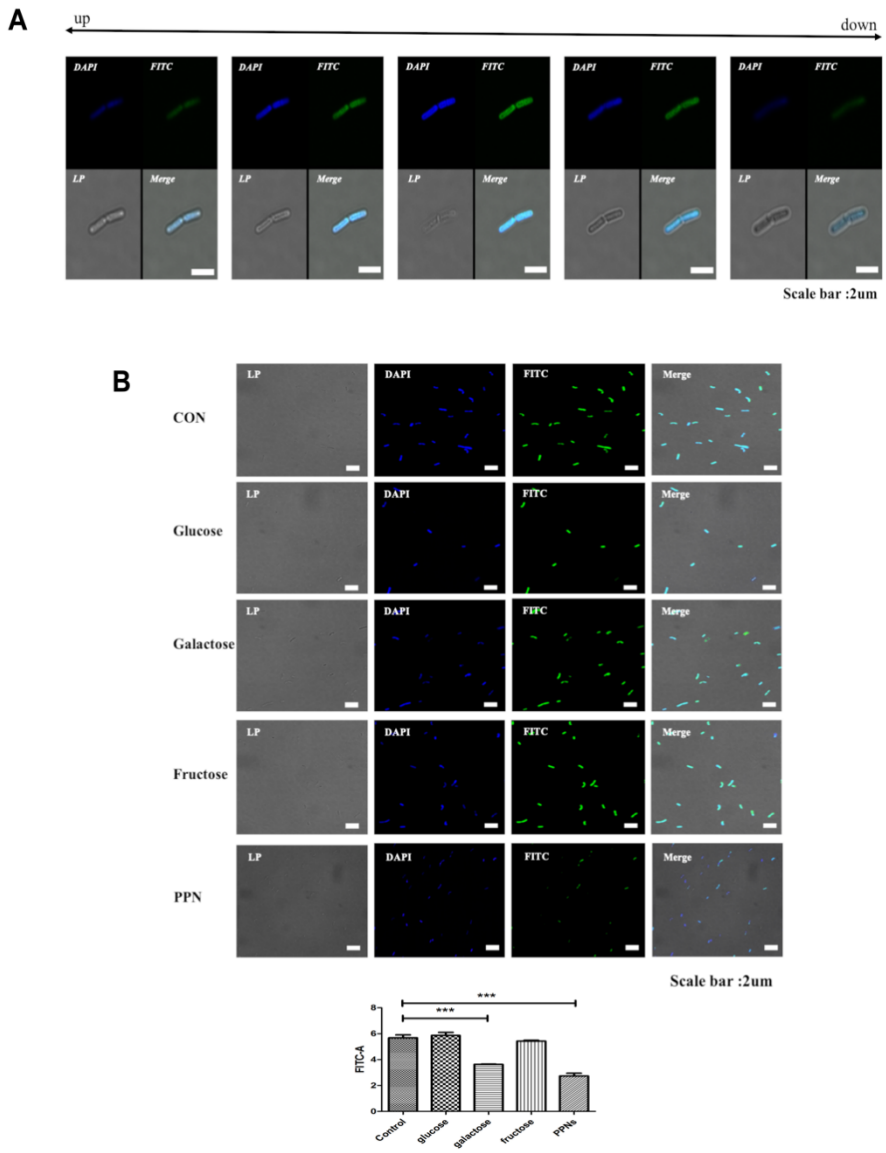


Figure. 27 Analysis of the internalization of PPNs by LP.

Z-section images show the internalization of corresponding PPNs into LP (A). Analysis of the internalization of PPNs by LP depending on transporters (B)

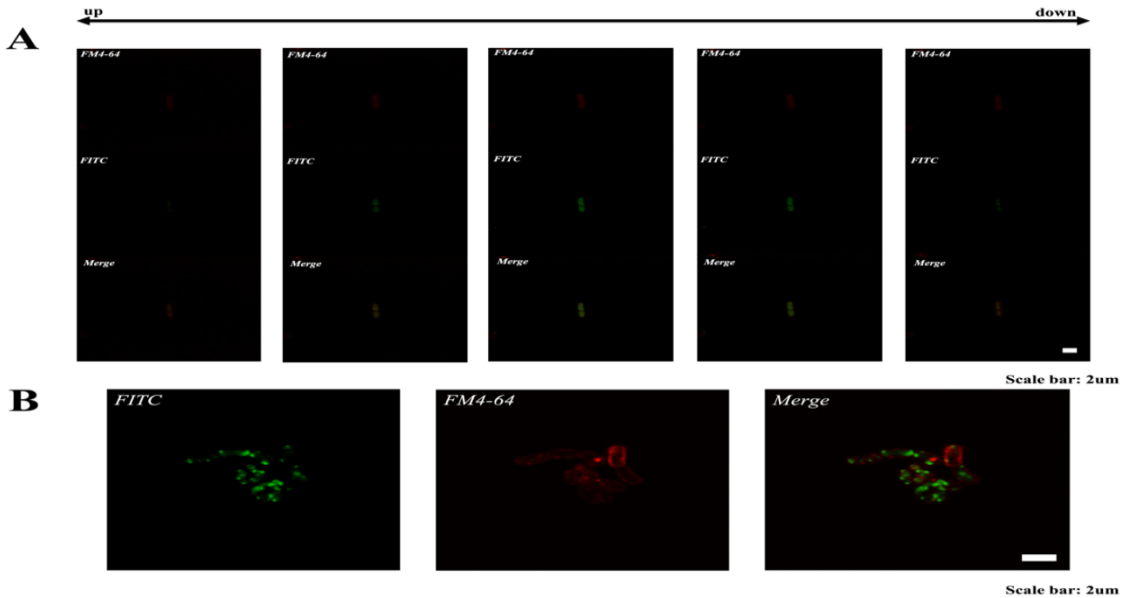


Figure. 28 Analysis of the internalization of PPNs by LP.

FITC-PPNs are shown in green, and membrane was stained red with FM4-64. Z-section showed that fluorescence intensity of FITC was highest at the center of LP (A). Confirmed that the FITC fluorescence appeared inside the bacteria (B)

Further studies were performed to determine the effect of incubation temperature and the role of sugar transporters in the internalization of PPNs by LP. To determine if the internalization of PPN3 by LP was temperature-dependent, LP was treated with FITC-PPN3 at 4°C, room temperature or 37°C for 2 h and subsequently analyzed by CLSM and FACS. The internalization of PPN3 by LP was highest at 37°C (Figure. 26B), suggesting that the internalization of PPN3 was energy-dependent.

Furthermore, to determine whether the internalization of PPN3 was via a sugar transporter, LP was pre-treated with 10% (w/v) glucose, galactose, fructose, and PPN3, and then treated with 0.5% (w/v) FITC-PPN3 for 2 h. The internalization was then observed by CLSM and FACS. The results showed that the internalization of PPN3 was predominantly dependent on the galactose transporter of LP because pre-treatment with galactose blocked approximately 40% of the internalization of PPNs by LP (Figure 27B). And it was found that the internalization of PPN3 was also blocked by pre-treatment of PPNs.

3) Effects of PPNs on antimicrobial activity

To evaluate whether the internalization of PPNs by LP affected its antimicrobial activity, LP was treated with three types of PPNs or pullulan itself. The antimicrobial activity of PPN-treated LP (LP/PPNs) was then tested against *E. coli* and LM and compared with that of untreated or pullulan-treated (LP/P) LP. The antimicrobial activity of the LP/PPNs groups was higher than that of untreated LP or LP/P against both *E. coli* and LM by co-culture assays (Figure. 29). Interestingly, stronger antimicrobial activity was observed when LP was treated with smaller nanoparticles. To determine whether the PPNs alone had antimicrobial activity, *E. coli* and LM were treated with PPNs PPNs alone displayed no antimicrobial activity (Figure. 30), indicating that the antimicrobial activity must be derived from the internalization of the PPNs by LP. In addition, the antimicrobial activity of LP/PPNs against *E. coli* and LM was further evaluated by agar diffusion tests. The inhibition zone was relatively larger when LP was treated with smaller PPNs suggesting that the agar diffusion tests showed similar antimicrobial patterns of LP/PPNs against *E. coli* and LM (Figure. 29).

In order to understand whether the improved antimicrobial ability is limited to PPNs nanoparticles, I tested the antimicrobial ability of LP treated with different kinds of nanoparticles against *E.coli*, and LP was co-cultured with these nanoparticles. The results (Figure. 31A) showed that not all kinds of nanoparticles can improve the anti-bacterial ability of LP and some nanoparticles can directly kill LP. (Figure. 31B).

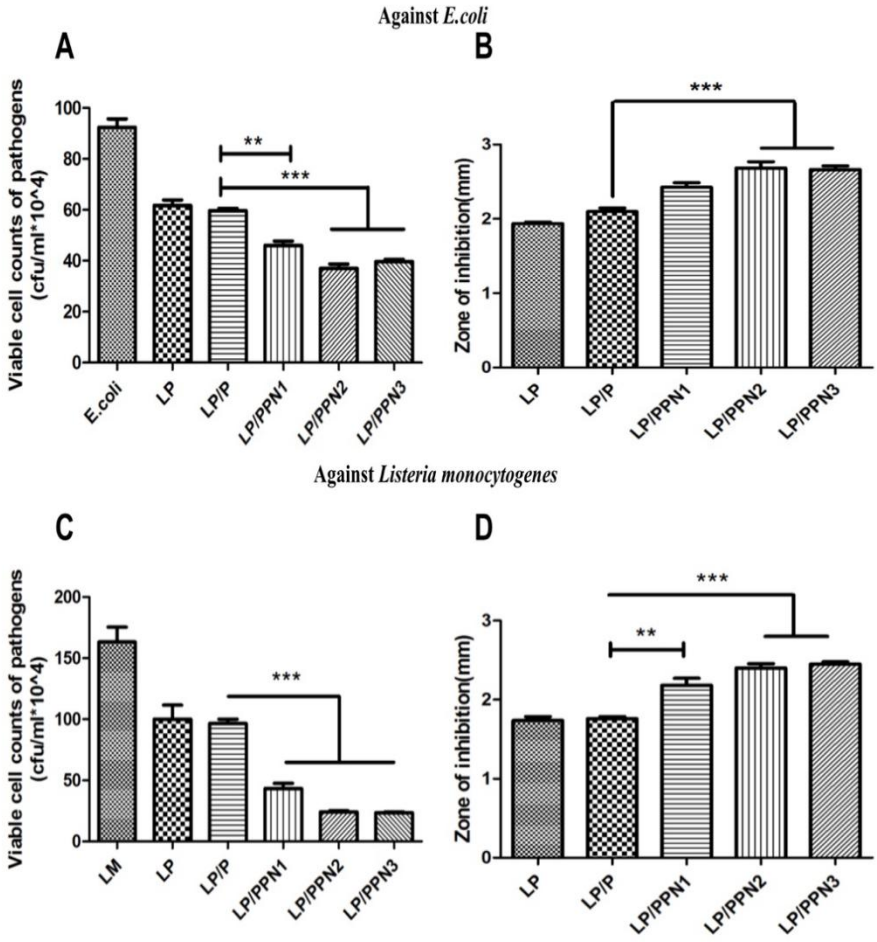


Figure. 29 Antimicrobial property of LP treated with PPNs.

LP treated with PPNs or pullulan were cultured with Gram-negative *E. coli* or Gram-positive LM, and the growth inhibition was calculated by CFU for *E. coli* (A) and LM (C). Similarly, the diameters of the growth inhibition of *E. coli* (B) and LM (D) on LB and BHI agar plates, respectively, were measured.

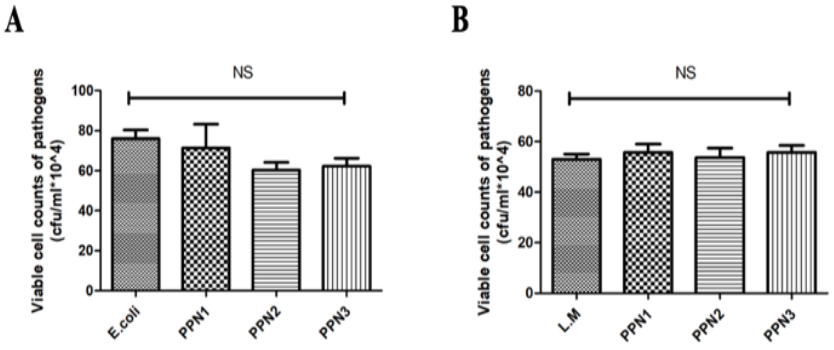


Figure. 30 Antimicrobial property of PPNs against pathogens.

PPNs were cultured with Gram-negative *E. coli* or Gram-positive LM, and the growth inhibition was calculated by CFU for *E. coli* (A) and LM(B)

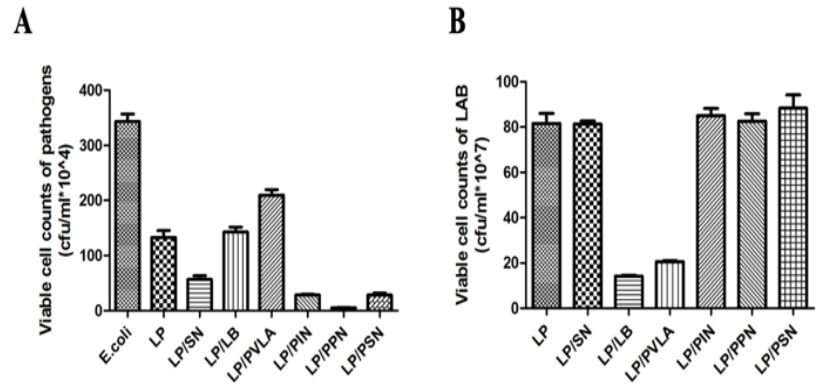


Figure. 31 Antimicrobial property of different kinds of nanoparticles and toxicity analysis.

LP treated with different kinds of nanoparticles were cultured with Gram-negative *E. coli* and the growth inhibition was calculated by CFU for *E. coli* (A), furthermore, LP was co-cultured with these nanoparticles and the viable cell was calculated by CFU (B).

4) Biological effects of PPNs on LP

To test the growth of LP after treatment with PPNs or pullulan, cell colonies were counted at different time points (Figure. 32A). The results showed no differences in LP growth with or without PPNs or pullulan treatment. The pH of the culture media of LP after treatment with PPNs or pullulan was also measured to evaluate lactic acid production. Consistent with the growth curve, the pH curve of the LP with or without PPNs or pullulan also showed no significant changes between the groups (Figure. 32B). Therefore, the internalization of PPNs by LP had no negative effects on the growth of LP, and improved antimicrobial ability was not because of more lactic acid produced from LP.

To test whether LP exerts its antimicrobial activity via plantaricin, a natural peptide, proteinase K was added to the medium of LP during agar diffusion tests. The antimicrobial activity of the proteinase K-treated group was significantly reduced compared with the untreated group (Figure. 32 C, D), suggesting the degradation of plantaricin by proteinase K.

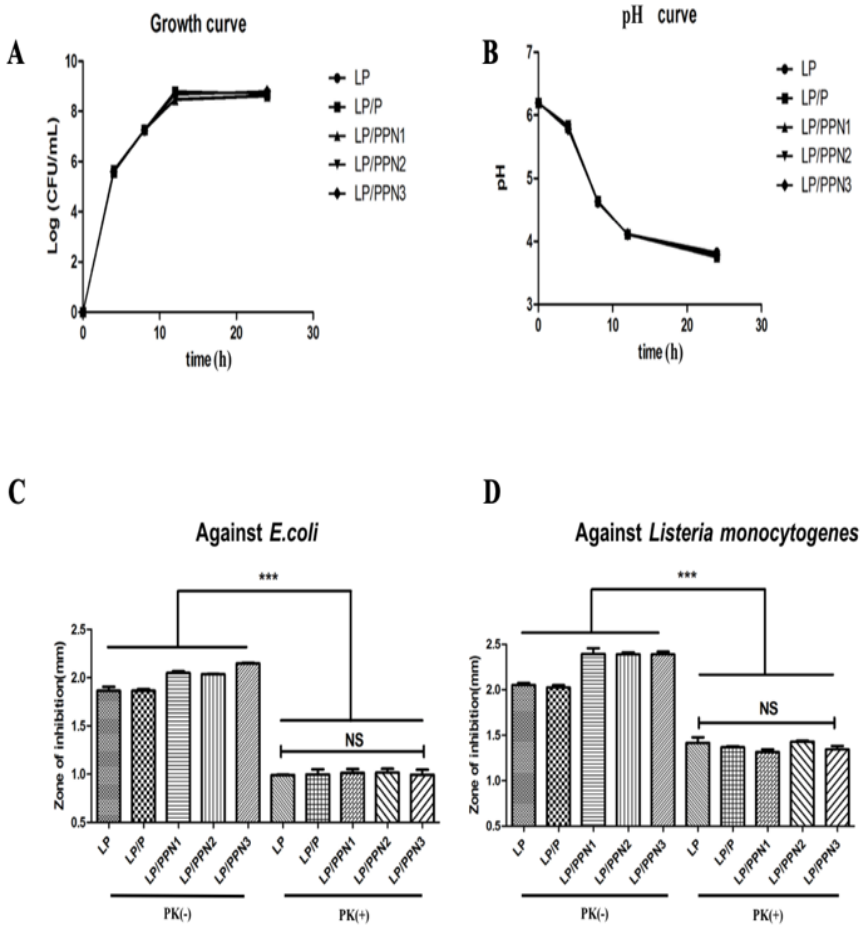


Figure. 32 Analysis the mechanism of enhanced antimicrobial ability.

Measurement of the growth of LP (A) and pH of the culture medium (B) among LP groups with internalized PPNs or pullulan. Antimicrobial efficacy of LP/PPNs against *E. coli* (C) and LM (D) was measured after proteinase K treatment

5) Effects of PPNs on plantaricin production by LP

To evaluate production of plantaricin by PPN-treated LP at the mRNA level, the expression level of plantaricin mRNA in PPN-treated LP was compared with that of untreated LP using qRT-PCR. The plantaricin gene planS was selected, and 16s rRNA was used for normalization. After treatment with PPNs or pullulan for 8 h, the expression level of planS was higher in PPN-treated LP than in untreated or pullulan-treated LP (Figure. 33A). The expression level of planS clearly demonstrated the enhanced antimicrobial activity of PPN-treated LP.

To determine the variations in the production of plantaricin in LP by PPNs, the plantaricin from LP, LP/P, LP/PPNs was isolated and observed by SDS–PAGE. As results, the molecular weight of isolated plantaricin was between from 2.5 to 6.5 kDa because it was already reported by Jimenez-Diaz et al.(Jimenez-Diaz et al., 1993). Also, the SDS–PAGE showed that LP/PPNs increased the production of plantaricin compared with the LP and LP/P groups under the same isolation conditions (Figure. 33B)

6) Expression of Stress response genes by qRT-PCR

In our previous study, it was found that internalized inulin nanoparticles induced a stress response in PA. To verify whether similar behavior occurs in LP, the expression levels of genes related to heat shock proteins, dnaK and dnaJ, were determined. After treatment with PPNs or pullulan for 8 h, the expression levels of dnak and dnaJ were significantly higher in PPN-treated LP than those of untreated LP (Figure. 34). The results suggested that the internalization of PPNs by LP induced a stress response.

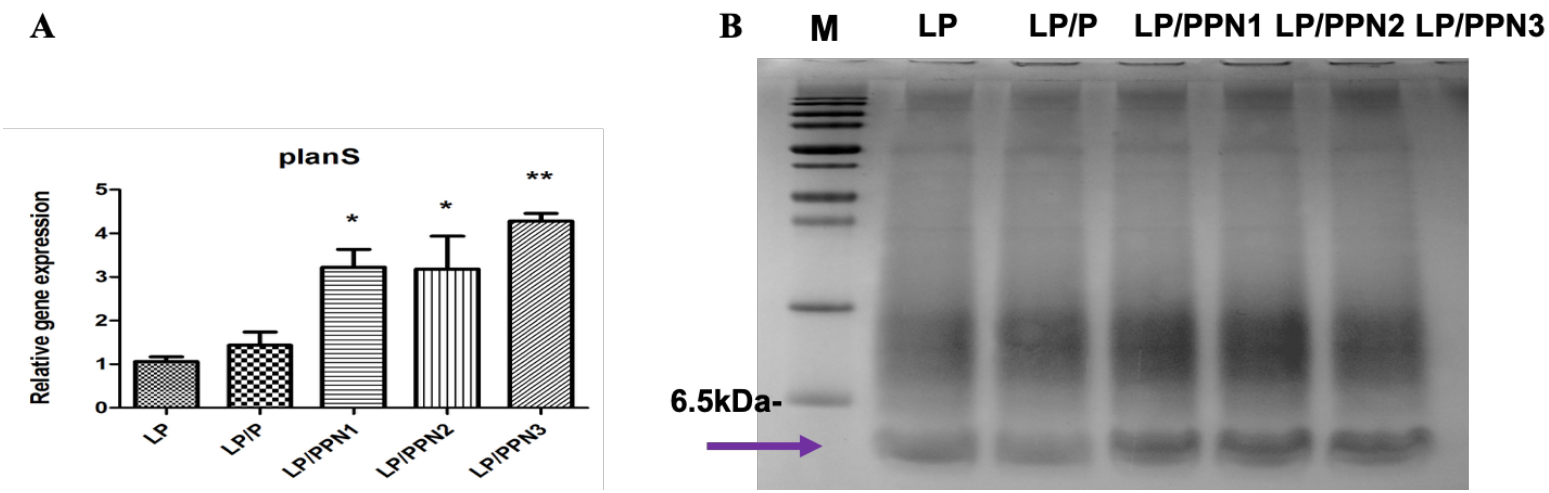


Figure. 33 Analysis the mechanism of enhanced antimicrobial ability.

Relative mRNA expression levels of *planS* compared with 16S rRNA expression levels (A). The isolated plantaricin was determined by SDS–PAGE (B)

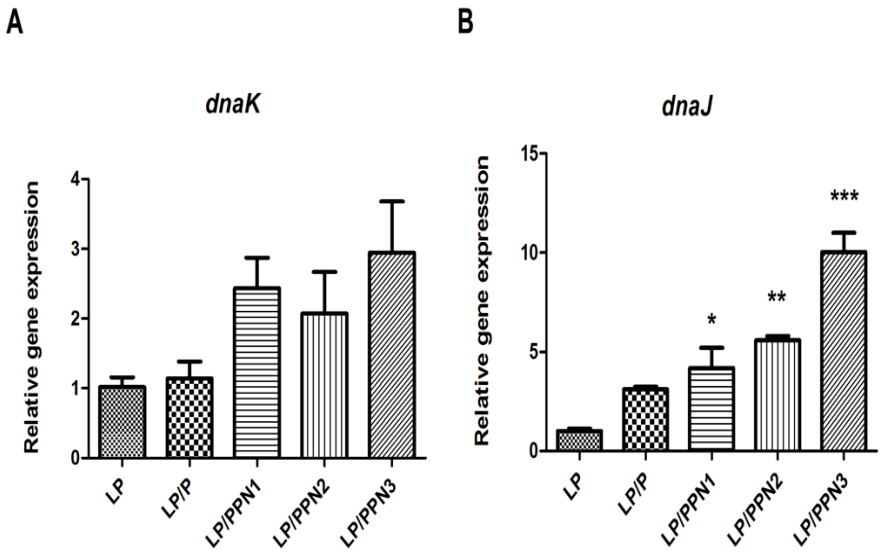


Figure. 34 Analysis of genes related to the stress response in LP treated with PPNs.

The expression levels of *dnaK* (A) and *dnaJ* (B) relative to 16s rRNA was quantified by qRT-PCR.

4. Discussion

Many researchers have been interested in the use of probiotics as a promising alternative for synthetic antibiotics because synthetic antibiotics cannot only elicit negative side effects but, with improper use, can also lead to antibiotic-resistant bacteria (Witte, 1998). Prebiotics are generally defined as non-digestible compounds that stimulate the activity and/or growth of probiotics and other microorganisms in the gastrointestinal (GI) tract and have favorable effects on the health of the host (Gibson, 1999), which are often mediated by short chain fatty acids (SCFAs) derived from the metabolism of prebiotics by the gut microbiota (Gourbeyre et al., 2011).

In study 2, PPNs was developed as a new formulation of prebiotics to increase the antimicrobial activity of probiotics. The PPNs were prepared by self-assembled nanoparticles after conjugation of hydrophobic phthalic anhydride to hydroxyl groups in pullulan through hydrophobic interactions. It is believed that the reaction occurs between the primary hydroxyl groups of the pullulan and the carboxylic acids of phthalic anhydride through esterification. Furthermore, increased conjugation of phthalic groups resulted

in smaller PPN sizes due to the increased hydrophobic interactions among phthalic moieties in the PPNs.

A large number of researchers have been interested in how polymeric nanoparticles are internalized into mammalian cells through endocytosis (Oh and Park, 2014). Because polymeric nanoparticles can deliver therapeutic drugs to the necessary place of action (Zhang et al., 2010), and can be used to overcome cellular barriers when delivering hydrophobic drugs (Blanco et al., 2015) ; however, research on the internalization of polymeric nanoparticles by prokaryotes as a prebiotic, except for metal nanoparticles, is still in an early stage. Thus far, much of the research on prebiotics has focused on their fermentation by the microbiota (Slavin, 2013), polymeric nanoparticles were developed as a prebiotic and to elucidate their internalization by probiotics. Interestingly, it is assumed that PPNs enter through galactose transporters on the cell surface of probiotics because pretreatment with galactose significantly decreased the internalization of PPN3 by LP, whereas glucose and fructose inhibited internalization to a lesser degree.

The other goal of our research was to evaluate the effect of prebiotics on the antimicrobial properties of probiotics. Many studies have described the antimicrobial properties of metal nanoparticles against pathogens due to their abrogation of bacterial growth by ionic interactions with the bacterial membrane (Sanyasi et al., 2016) . However, metal nanoparticles can cause serious side effects in the host (Roy et al., 2003) and can inhibit both pathogens and beneficial microbes (Travan et al., 2009). Hence, the treatment of LP with PPNs enhanced its antimicrobial activity against both Gram-negative *E. coli* and Gram-positive *L. monocytogenes* compared to pullulan or LP alone although the effect of PPNs internalization in probiotics on observed antimicrobial activities was mild and PPNs did not show toxicity to the host. Particularly, LP/PPN3 showed the highest antimicrobial activity. The results indicated that the increased antimicrobial activity was dependent on the size of the PPNs taken up by LP.

The advantages of probiotics as a food and feed additive have been mostly focused on their antimicrobial properties, suggesting that the enhancement of antimicrobial properties is of importance to probiotics researchers.

Plantaricin is a natural peptide produced by LP and was reported to possess strong antimicrobial properties(Nes and Hole, 2000). In this study, treatment of LP with PPNs markedly increased the production of plantaricin, which was confirmed by the mRNA expression of planS. It is hypothesized that the internalization of PPNs directly affected the production of plantaricin. Therefore, we hypothesize that the internalization of PPNs by LP contributes to the enhanced antimicrobial properties of LP via increased expression of the plantaricin.

Notably, probiotics produce bacteriocins as their first defense mechanism (Cleveland et al., 2001;Castro et al., 2015). Several factors, such as culture pH, temperature, and pressure (Kalchayanand et al., 1998) , affect the expression of bacteriocins by the upregulation of genes related to heat shock proteins (HSPs) (Bove et al., 2013) and the stress response. The mRNA expression levels of dnaK and dnaJ in PPN-treated LP were significantly higher than those of untreated LP. The results indicated that the internalization of PPNs by LP induced a mild intracellular stress response to stimulate antimicrobial activities without death of the host. Therefore, the

internalization of PPNs by LP enhanced the expression of the plantaricin gene to activate the host's defense system. Further research is needed to determine the precise mechanism of the internalization of PPNs by LP.

Ultimately, polymeric nanoparticles as prebiotics can exert substantial effects on probiotics, which lead to the increased production of an antimicrobial peptide that is powerful against Gram-positive and Gram-negative pathogens. Therefore, this study shows a new way to produce antimicrobial peptides in probiotics through mild intracellular stimulation by the internalization of PPNs into probiotics, suggesting that PPNs have great promise as an alternative to synthetic antibiotics in veterinary, dairy, and human applications.

* This study had published in frontier in microbiology. *Hong L, Kim W-S, Lee S-M, Kang S-K, Choi Y-J and Cho C-S (2019) Pullulan Nanoparticles as Prebiotics Enhance the Antibacterial Properties of Lactobacillus plantarum Through the Induction of Mild Stress in Probiotics. Front. Microbiol. 10:142.*

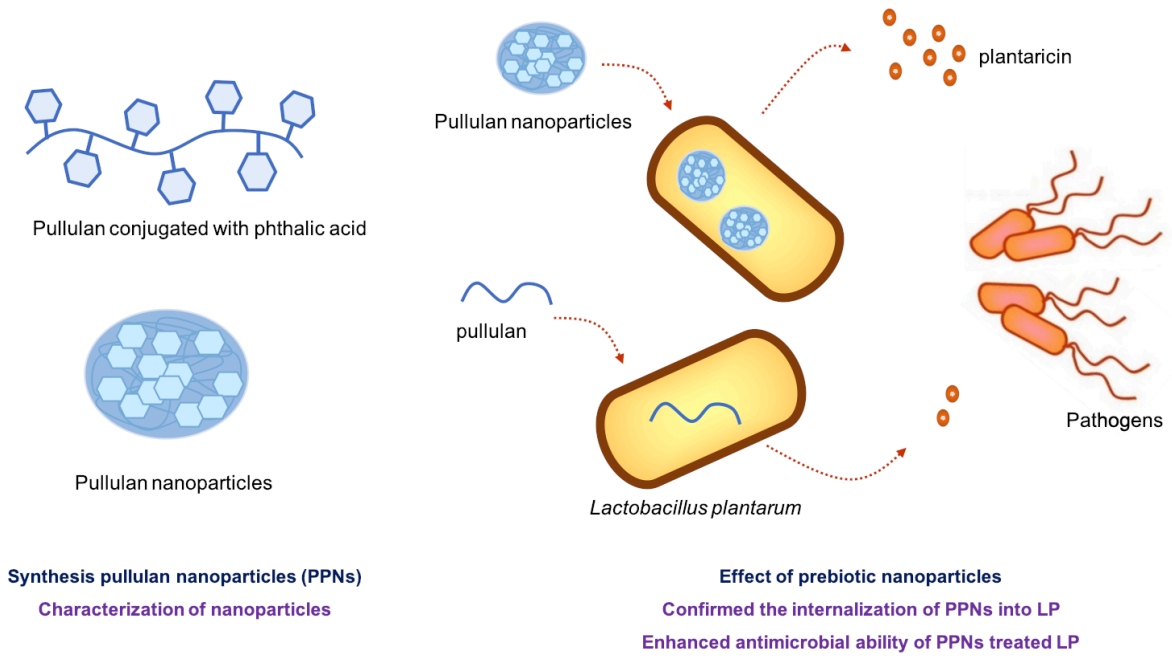


Figure. 35 Graphical summary of study 2.

Table 14. Summary of study 2.

Contents	Results
Synthesis and characterization of PPNs	PPN1(30.9 mol.-%, 140.0nm), PPN2(50.45, 108.9)
	PPN3(68.50, 82.1)
Effect on antimicrobial activity	Gram (-) bacteria (E,coli K99)
	Gram (+) bacteria (L.monocytogenes)
Investigation on internalization of PINs	temperature dependent (Internalized highest in 37℃)
	Transporter dependent (Highest blocking with galactose, PPN)
Mechanism analysis	plantaricin expression (protein, mRNA)
	Stress response proteins (mRNA)

Study 3. Investigation on the effects of synbiotics on the intestinal barrier function in an animal model

1. Introduction

Enteric pathogens are a major cause of infections in the gastrointestinal track worldwide, and they also induced inflammation condition of the gut caused intestinal barrier defects. To control these gastrointestinal infections, many strategies have been focused on using synthetic antibiotics. However, the emergence of antibiotic-resistant bacteria is a serious problem and in animal feeds, the use of antibiotics is prohibited. Therefore, finding efficient alternatives to antibiotics is a global issue (Czaplewski et al., 2016). Because probiotics are generally considered safe and can confer health benefits to the host when adequate amounts are administered, interest has been growing in the use of probiotics as a replacement of antibiotics (Wan et al., 2018). Lactic acid bacteria are commonly used as probiotics for alternatives to antibiotics. Among the lactic acid bacteria, *Lactobacillus* spp. are used widely because these probiotics have antimicrobial properties in many pathogens

(Mountzouris et al., 2007;Cotter et al., 2013). These probiotics produce antimicrobial molecules (e.g., lactic acid and bacteriocins) that enable them to inhibit the colonization of pathogens (Mayakrishnan et al., 2018;Le et al., 2019) and to enhance the intestinal barrier function (Anderson et al., 2010;Wang et al., 2018;Zhou et al., 2018).

Among the many strategies used for prebiotics, increasing the growth or activity of probiotics is one of them. Prebiotics are generally defined as indigestible food ingredients that increase the growth or activity of beneficial microorganisms in the gastrointestinal tract and provide favorable health effects to the host (Gibson et al., 2017). In study 1, pullulan was used as a prebiotic for LP. Because it increases the butyrate and propionate fermentation of LP. These SCFAs are crucial for intestinal health, and their activity can subsequently influence sites distant to the gut. Especially, butyrate is well-known because it can regulate the intestinal barrier function (Han et al., 2015;Yan and Ajuwon, 2017;Knudsen et al., 2018), and propionate also improved DSS-induced intestinal gut dysfunction (Tong et al., 2016). In recent years, many researchers have begun to synthesize and apply drug delivery systems based on pullulan-based self-assembled

nanoparticles (Na et al., 2003;Jeong et al., 2006;Zhang et al., 2010). In contrast, in this study, application of the synthetic PPN was not as a drug or gene carrier but as a new type of prebiotic which can increase the antimicrobial ability of LP (Hong et al., 2019)

In this study, to confirm the suppression of the pathogenic induced the intestinal barrier dysfunction, and the alterations in the gut microbiota, a novel synbiotic combination was developed and orally administered to mice. The antimicrobial ability of the synbiotics against *E.coli* and the change in specific microbes in the microbiota were investigated. Furthermore, the gut physical barrier function was checked by serum endotoxin and serum FITC-dextran.

Table 15. Contents of study 3.

Study	Category	Item
Investigation effect of synbiotics on intestinal barrier function in animal model	Experimental design	Animal (murine)
		Sampling (body weight, colon, serum, cecum, feces)
	Physiological changes	Body weight, Feed intake
		Colon length, Cecum weight
	Microbial barrier function	Viable cell counts of pathogens and LAB
	Physical barrier function	Serum endotoxin, Serum FITC-dextran
	Specific microbial analysis	Viable cell counts, Fecal DNA (qPCR)

2. Materials and Methods

1) Materials

All of the materials and chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. For bacterial cultures, lysogeny broth (LB), LB agar, De Man, Rogosa and Sharpe agar *(*MRS) broth, MacConkey agar broth were purchased from BD Difco (Sparks, MD, USA).

2) Synthesis of phthalyl pullulan nanoparticles (PPNs)

Phthalyl pullulan nanoparticles were synthesized according to the method described in study 2, One gram of pullulan was dissolved in 10 ml of dimethyl formamide (DMF), and 0.1 mol-% dimethylaminopyridine per pullulan sugar residue was added to the solution as a catalyst, and then phthalic anhydride was added to the above solution at 9 molar ratio per pullulan. The reaction was performed at 54°C for 48 h under nitrogen. The produced PPNs were dialyzed first in DMF to remove unreacted phthalic anhydride and then in distilled water at 4°C for 24 h to form self-assembled

nanoparticles of phthalyl pullulan. The unreacted pullulan was removed after ultra-centrifugation of prepared PPNs. Finally, the PPNs were freeze-dried and stored at −20° C until use.

3) Bacterial cultures

All bacterial strains were cultured in their corresponding medium: *Lactobacillus plantarum* (LP) in MRS broth, *E.coli K99* in LB broth. Both bacteria were cultured at 37 °C in a shaking incubator (255 rpm) for 24 h prior to being used in subsequent experiments or being stored at -70 °C in 15 % (v/v) glycerol.

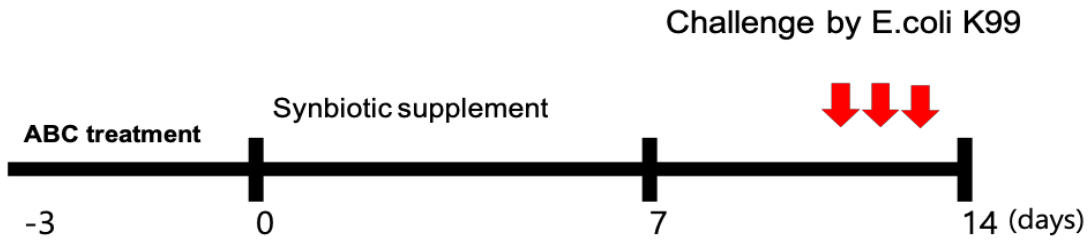
4) Animal experimental procedures and measurements

Studies were performed using four-week-old BALB/C male mice in accordance with international ethical guidelines. The Institutional Animal Care and Use Committee (IACUC) at Seoul National University approved the animal experiments (SNU-180904-2-1). Mice were housed at a controlled temperature (22 ± 2 °C) on a 12 h light/dark cycle. Animals were fed standard mouse chow *ad libitum* and were provided distilled water at all times. After

7 days of acclimation, mice were randomly allocated into six groups (6 BALB/c mice per group). The control group continued to be fed as before. All groups except the control group were administered with an antibiotic cocktail (ampicillin:gentamicin:neomycin:vancomycin=2:2:2:1 total 20mg/mice) three days at the beginning of the experiment to induce the microbiota dysbiosis. The T2 group was administered a single dose of 10^8 CFU *Lactobacillus plantarum* (LP) in saline solution via oral gavage. The T3 and T4 groups were single doses of pullulan (0.5 wt.-%)-treated LP or PPNs (0.5 wt.-%)-treated LP, respectively. The T5 groups were single dose pullulan (0.5 wt.-%) and PPNs (0.5 wt.-%)-treated LP as described above. After 11 days on the test diets, *E.coli K99* (10^9 CFU) was administered with 0.2 ml of 1% $NaHCO_3$ (treated before administrating *E.coli K99* for 30 min) to the mice from T1 to T5 group via oral gavage for three days.

The body weights and food intakes of mice were monitored daily over the entire experimental period. Beginning from the first day of pathogen administration. LP and *E.coli* were counted as viable CFU from the fecal sample on the last day of the experiment. Feces were spread onto both MRS agar and MacConkey agar and incubated for 20 h at 37 °C. At the end of the

experiment, mice were sacrificed by CO_2. Intestinal samples, feces, colons, serum were collected for further analysis.



Group	trial
C	Non treat
T1	antibiotics—E.coli k99
T2	antibiotics—E.coli k99---LP
T3	antibiotics—E.coli k99---LP/P
T4	antibiotics—E.coli k99---LP/PPNs
T5	antibiotics—E.coli k99---LP/P/PPNs

Figure. 36 Experiment schedule and group organization.

5) Detection serum FITC-dextran level

On the day of sacrifice, deprive mice of food for 4 hr, administer FITC-dextran tracer (0.6mg/g body weight) intra-gastrically in 0.1ml PBS, and bleed the mice after 3hr in serum separator tubes to collect hemolysis-free serum. Measure intestinal permeability, which correlates with fluorescence intensity of appropriately diluted serum, using a Hitachi F-4500 fluorescence spectrophotometer.

6) Detection serum endotoxin level

Serum endotoxin levels were detected according to the manufacturer's protocol form 50ul of diluted serum samples using the Pierce™ Chromogenic Endotoxin Quant Kit, Endotoxin levels are determined by measuring the activity of Factor C in the presence of a synthetic peptide substrate that releases p-nitroaniline (pNA) after proteolysis, producing a yellow color that can be measured at an absorbance of 405 nm.

7) DNA extraction and qRT-PCR

DNA was extracted according to the manufacturer's protocol from 50 mg of each fecal sample using the AccuPrep® Stool DNA extraction kit (Bioneer, Daejeon, Republic of Korea), followed by storage at -20 °C until further analysis. For species-specific quantitative PCR, the primers used were designed based on the sequences (Table 16).

Table 16. Primer used in the study.

Target strains/genes	Sequences (5`-3`)	Size(bp)
E.coli	F:CATGCCGCGTGTATGAAGAA R:CGGGTAACGTCAATGAGCAAA	470
***Lactobacillus* spp.**	F:AGCAGTAGGGAATCTTCCA R:CACCGCTACACATGGAG	341
***Bifidobacterium* spp.**	F:GCGTGCTTAACACATGCAAGTC R:CACCCGTTTCCAGGAGCTATT	126
16S rRNA	F:CCTACGGGAGGCAGCAG R:GGACTACGGGTTCTAAT	450

8) Statistical analysis

Data are presented as the mean ± SEM of three independent experiments. The statistical significance was analyzed between each group by one-way ANOVA and Tukey's test (*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$).

3. Results

1) Physiological changes in mice

The Mouse feeding experiment was performed, to evaluate the effect of synbiotic on the intestinal barrier functions in animals. Interestingly, groups that were fed diets supplemented with PPNs had increased body weight at the end of experiment compared with the day 12 (Figure 37A). In particular, the body weights were increased the most for the T5 groups which supplemented wich LP/Pullulan/PPNs combinations, while the body weights were decreased for the T2 group on day 14 compared to day 12. Moreover, the average food intake per animal for the probiotics supplemented group was higher than other groups (Figure 37B).

Change in colon length following the trial were also measured. The T4 group had the longest colon length, all groups supplemented with probiotics or synbiotics had a longer colon length compared which T2 group (Figure. 38A, B), and it seems to have positively correlated with the body weight. Cecum weight was also measured. The group T2 which administrated with *E.coli K99* had the heaviest cecum weight and the other groups supplemented

with probiotics or synbiotics had lower cecum weight, the cecum weight of these groups more close to the control group (Figure. 38C).

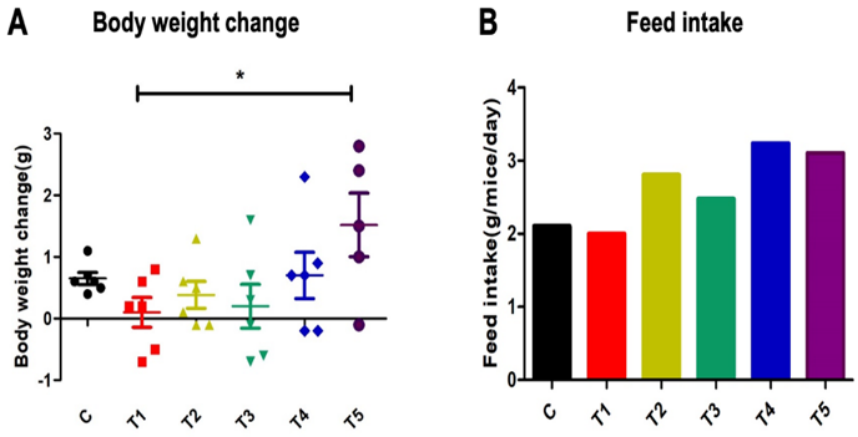


Figure. 37 Body weight change and feed intake in mice.

Body weight change during the *E.coli* K99 challenge (A). Average feed intake per mice per day (B).

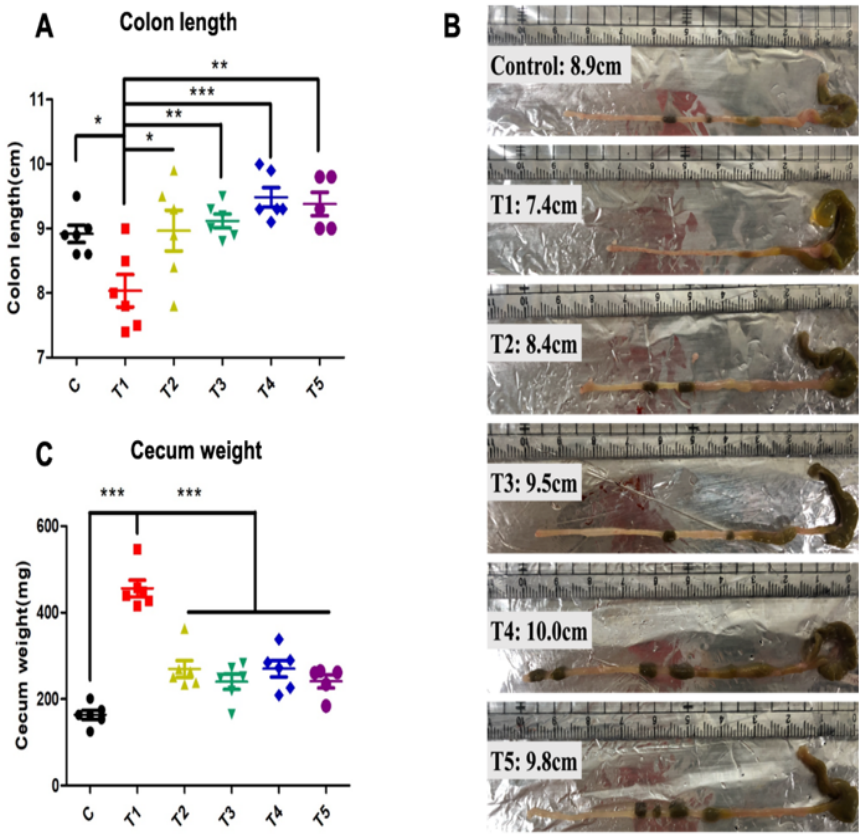


Figure. 38 Conlon length and cecum weight in mice.

Length of colon after the trial (A) (B). Cecum weight after the trial(C).

2) Microbial analysis in a murine model

Viable cell count, real-time PCR (qPCR) was used to identify the quantities of specific microbes from murine fecal samples. To track intestinal colonization, viable cell counts were assessed by plating fecal samples onto MacConkey agar and MRS agar. Viable *E.coli* was higher in T1 group approximately 7 log 10(CFU/mg of feces) compared with other groups treated with probiotics or synbiotics which was approximately 3~4 log 10(CFU/mg of feces). Interestingly, the group T4 and T5 had the lowest *E.coli* compared with T2 and T3. T4 and T5 were feed with PPNs and its induced higher antimicrobial ability against *E.coli* (Figure. 39A).

Overall viable LAB was counted in MRS agar plates, of course, more LAB was found in the groups which were feed with LP. There were approximately 5~6 log 10(CFU/mg of feces), in the control group, there was approximately 3~4 log 10(CFU/mg of feces). Treatment of antibiotics seemed to have definitely destroyed the microbiota in group T1, there was only 2~3 log 10(CFU/mg of feces). Interestingly, a significant number of LAB were found in group T3 (Figure. 40A).

To support the viable cell count results and due to the limited selectiveness of MRS agar and MacConkey agar, qPCR was used. *E.coli* levels were significantly lower for the groups that were orally administered probiotics (Figure. 39B). indicating that probiotics eliminated pathogens from the gut.

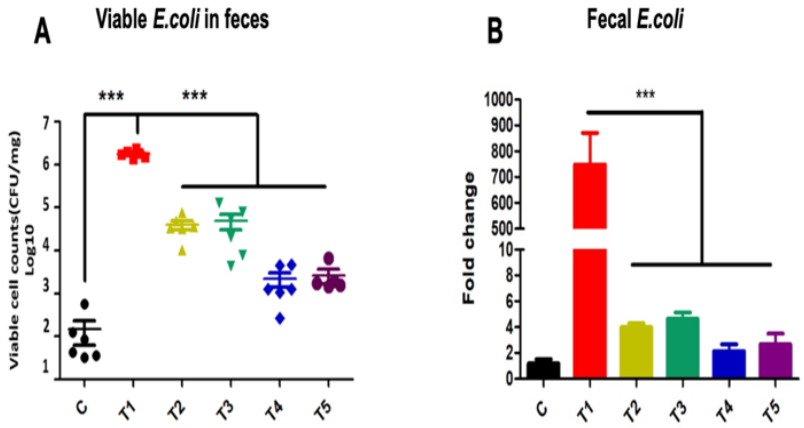


Figure. 39 Number of the *E.coli* cells in the intestinal microflora .

The viable cells count of *E.coli* (A). DNA based *E.coli* in feces(B). To tract the intestinal colonization, viable cell counts were assessed by plating fecal sample onto MacConkey agar. To support the viable cell count results of qPCR was used to confirmed the data.

The amount of *Lactobacillus.*spp was higher for the groups which were orally administered LP, especially in group T3 (Figure. 40B). From fecal samples, the relative abundance of *Bifidobacterium* spp. was also analyzed by qPCR. It was found that *Bifidobacterium* spp. were higher in the T4 and T5 groups than other groups (Figure. 40C).

In the group T4 and T5 had the lowest level of *E.coli*. it was a negative correlation with the number of *Bifidobacterium* spp. These results indicated that feeding mice LP/PPNs improved the antimicrobial activity of LP more than feeding them pullulan itself or LP alone. Therefore, LP/Pullulan increased the viable LAB in the gut microbiota.

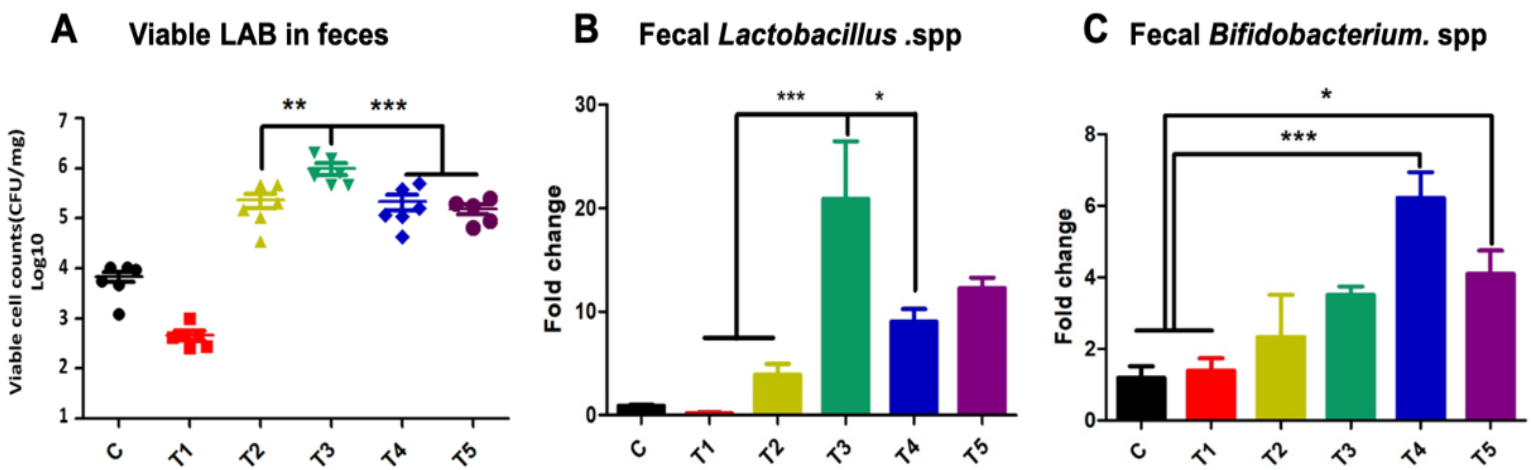


Figure. 40 Number of the beneficial microbe in the intestinal microflora.

The viable cells count of LAB (A). DNA based *Lactobacillus*.spp in feces (B). DNA based *Bifidobacterium.*spp *in feces* (C).

The levels of LAB and *Lactobacillus* spp were analyzed by viable cell counts and fecal DNA, the levels of *Bifidobacterium* spp. were analyzed by fecal DNA.

3) Effect of synbiotics on the intestinal barrier function

The intestinal barrier function plays an important role in maintaining normal bowel function by preventing the harmful substance such as intestinal bacteria, toxins, and LPS of gram-negative bacteria going into other tissues or blood circulation.

Serum LPS level were higher in the group T1 which just orally administered *E.coli K99*, and the serum LPS level was lower in the group T4 and T5 which administered with LP/PPNs and LP/P/PPNs (Figure. 41A).

Serum FITC-dextran in the group T1 was higher than that in control group, and probiotics inhibited the increase of FITC-dextran in serum and Synbiotic combinations significantly inhibited the increase of FITC-dextran in serum (Figure. 41B).

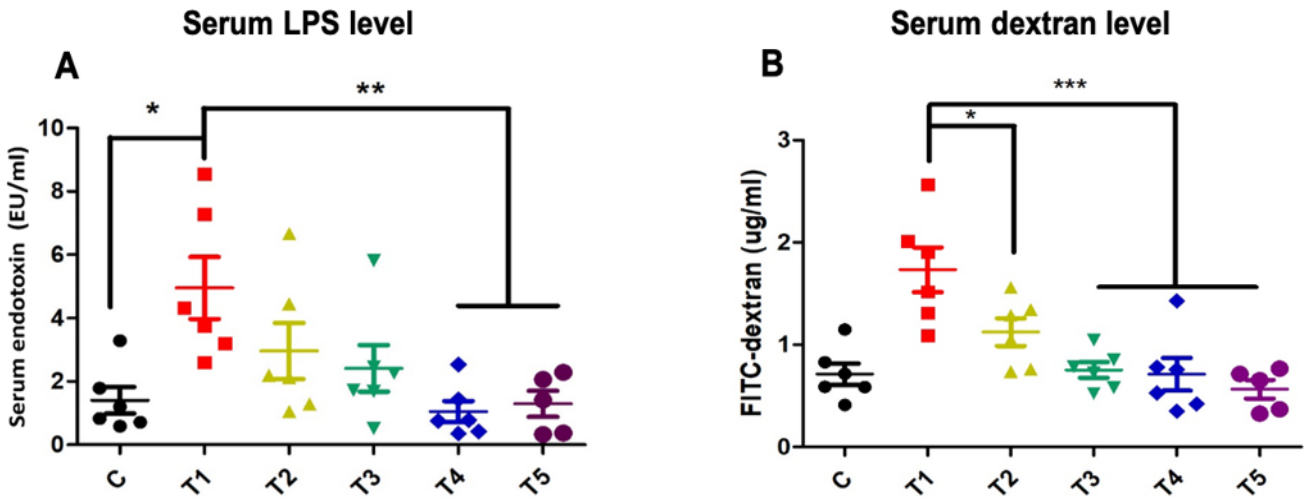


Figure. 41 Effect of synbiotics on the intestinal barrier function.

LPS detected in serum (A). FITC-dextran.detected in serum (B).

4. Discussion

In previous study 1, it was found that *Lactobacillus plantarum* with pullulan inhibited the down-regulation of tight junction proteins such as ZO-1, occludin, and claudin-1. In study 2, pullulan nanoparticles increased the antimicrobial ability of LP.

In the present study, *Lactobacillus plantarum*, pullulan and PPNs were orally administered in adequate amounts and evaluated the health benefit effect especially the intestinal barrier function to the mice.

To reduce the pathogenic infection, LP treated with or without pullulan or PPNs were pre-feed before the administration of pathogen was because the domestic microflora plays an important role in inhibiting pathogenic infection. Overall, results showed that group with oral supplementation of PPNs reduced the infection by *E.coli* K99 in mice. Reduced inflammation agent caused the longer colon length, feeding probiotics with PPNs seem to alleviate the infection of *E.coli* and reduce the inflammation. The T5 group had the highest increased in body weight and increased in feed intake. Moreover, the body weights of the group T2, T3 and T4 groups were also

higher than the T1 groups .it is known that *E.coli* was infected in the intestinal tract, it induced inflammation of the intestine.

To see if the increased antimicrobial property *in vitro* is maintained *in vivo*, antimicrobial properties of the groups which LP treated with PPNs were analyzed by counting the number of the *E.coli*. By counting the viable cells of coliform bacteria in MacConkey agar plates and counting the DNA of *E.coli* in fecal samples, the highest antimicrobial activity was shown in the T4 and T5 which LP treated with PPNs. Viable cell counts and qPCR were also used to analyze the levels of LAB and *Lactobacillus.*spp. Interestingly, in the group T3 *Lactoabillus plantarum*/pullulan shown a significantly higher level of LAB and *Lactobacillus.*spp. The results indicated that pullulan worked as prebiotics increased the LAB level *in vivo*, or pullulan helped the colonization of *Lactobacillus plantarum* in intestine like other prebiotic did (Monaco et al., 2018). Moreover, *Bifidobacterium* spp. were increased in the PPNs treatment groups (T4 and T5 groups). Based on the influence of prebiotics on the growth of *Bifidobacterium* spp. and LAB, this result implied that PPNs and pullulan treatment might also work as a prebiotic in the intestine.

In addition, the cecum, for mice, is the reservoir of intestinal flora and the largest fermentation tank in the gut and studies performed by other researchers have identified increased cecum weight following inulin or FOS treatment (Chassaing B, et al. 2015, Moen, B, et al. 2016, Verghese, M et al. 2002), probably as a result of increased SCFA fermentation and growth of cecum. However, in the present study the T1 group had the highest cecum weight and the administration of pro or synbiotics showed to restore the cecum weight to a level comparable to that of the control group, it was reported that swelling of the cecum was observed in the antibiotic administered mice (Nameda, et al. 2007) and this observation is similar to the germ-free mice (Okada Y, et al. 1994), it suggested that the sterilization by the antibiotics was successful and considered that the diversity of microflora have been improved in groups administered with LP, LP/P, LP/PPNs or all of them.

To verify the intestinal barrier function, in this study, serum endotoxin levels were meseraed and FITC-dextran were detected. Increased serum endotoxin and serum FITC-dextran reflected the dysfunction of the intestinal

barrier function. It was found that serum endotoxin level was lower in T4 and T5 groups which corresponding to the viable *E.coli* in feces. The results indicated that the “microbial barrier” were composite with fewer *E.coli* and “physical barrier” were up-regulated. It was found that probiotics or synbiotic treatment inhibited the increase of serum FITC-dextran level. Moreover, pullulan and PPNs had an additional effect on LP with inhibiting the increase of serum FITC-dextran.

Study 3 reports that *in vivo* validation of novel synbiotic combinations including LP, pullulan and PPNs. This combination enhanced antimicrobial ability against pathogenic *E.coli* to enhanced microbial barrier functions and it also reduced the FITC-dextran and endotoxin going into serum through enhanced physical barrier. Furthermore, pullulan and PPNs treatment can change the composition of gut microbiota increased *Lactobacillus*.spp and *Bifidobacterium*.spp. The results of study 3 suggested that pullulan and PPNs treated with LP can enhance the intestinal barrier function.

For further study, in order to achieve the ultimate goal of the study, novel synbiotic combination (LP/P/PPNs) should be directly compared with

antibiotics through animal experiments, and the effect of antibiotic substitution should be tested.

Table 17. Summary of study 3.

Contents	Results
Physiological change	Body weight: most increased in group T5 Feed intake: higher in groups administered with pro or sybiotics
	Colon length: longer in group administered with PPNs Cecum weight: E.coli increased the cecum weight, and synbiotics inhibited
Microbial barrier function	Viable cell counts of *E.coli*: T4, T5 administered with PPNs inhibited more *E.coli* Viable cell counts of LAB: T3 administered with pullulan increased viable LAB
Physical barrier function	Serum endotoxin and FITC-dextran: Lowest serum endotoxin and dextran were detected in T4 and T5
Specific microbial analysis	Fecal *E.coli*: Synbiotics inhibited *E.coli* most effectively Fecal *Lactobacillus* spp.: T3 administered with pullulan increased *Lactobacillus* spp. Fecal *Bifidobacterium* spp. T4 and T5 group administered with PPNs increased *Bifidobacterium* spp.

Overall Conclusion

The intestinal barrier selectively regulates epithelial permeability to luminal substances and antigens. The disruption of the intestinal barrier induced by toxins, pathogens and stress contributes to the development of severe intestinal inflammation. SCFAs, the products of bacteria fermentation, have been found to exert profound influences on the intestinal barrier function, particular the physical barrier.

In study 1, synbiotic combination was selected based on the fermentability of the SCFAs, and demonstrated that the fermentation of *Lactobacillus plantarum* treated with pullulan produced the most butyrate and enhancing the intestinal barrier integrity against LPS-induced impairment. *Lactobacillus* treated with a prebiotic promoted the intestinal barrier integrity, indicated by the inhibited the decreased in TEER, and inhibited the down-regulation of mRNA for tight junction proteins (ZO-1 and claudin-1). Claudins are the main structural and functional components of tight junctions Therefore, the protective effect of LP/P on the intestinal barrier against LPS damage may be partly explained by the increase in the expression of claudin proteins

In conclusion, LP/P fermentation produced the most butyrate and it restored the LPS-induced impairment of the intestinal barrier by promoting tight junction (especially claudins) expression

In study 2, PPNs were developed as a new formulation of prebiotics to increase the antimicrobial activity of probiotics. The PPNs were prepared by self-assembled nanoparticles after conjugation of tje hydrophobic phthalic anhydride to the hydroxyl groups in the pullulan through hydrophobic interactions. Many studies have described the antimicrobial properties of metal nanoparticles against pathogens due to their abrogation of bacterial growth by ionic interactions with the bacterial membrane However, metal nanoparticles can cause serious side effects in the host and can inhibit both pathogens and beneficial microbes.Hence, the treatment of LP with PPNs enhanced the antimicrobial activity against both Gram-negative *E. coli* and Gram-positive *L. monocytogenes* compared to pullulan or LP alone.

Ultimately, polymeric nanoparticles as prebiotics can exert substantial effects on probiotics, which can lead to the increased production of an antimicrobial peptide which is effective against Gram-positive and Gram-negative pathogens. Therefore, this study shows a new way to produce

antimicrobial peptides in probiotics through mild intracellular stimulation by the internalization of PPNs into probiotics.

In study 3, *Lactobacillus plantarum*, pullulan and PPNs were orally administered in adequate amounts, and then, the health benefit effect, especially the intestinal barrier function, in mice was measured.

Overall, the results show that the group with oral supplementation of PPNs had reduced the infection by *E.coli* K99 in mice. The reduced inflammation agent caused the longer colon length and feeding probiotics with the PPNs seemed to alleviate the infection of *E.coli* and reduce the inflammation. The T5 group had the highest increased in body weight and increased in feed intake. To see if the increased antimicrobial property *in vitro* is maintained in *in vivo*, the antimicrobial properties of the groups treated LP and PPNs were analyzed by counting the number of the *E.coli*. By counting the viable cells of coli form bacteria on MacConkey agar plates and measuring the DNA of *E.coli* in fecal samples, the highest antimicrobial activity was seen in the T4 and T5 which were treated with LP and PPNs. Viable cell counts and qPCR were also used to analyze the levels of LAB and *Lactobacillus.*spp. Interestingly, in the T3 group *Lactobacillus plantarum*/pullulan had a

significantly higher level of LAB and *Lactobacillus spp*. The results indicated that, pullulan worked as a prebiotic increased the LAB level *in vivo*. Moreover, *Bifidobacterium* spp. were increased in the PPNs treated groups (T4 and T5 groups). Based on the influence of the prebiotics on the growth of *Bifidobacterium* spp. and LAB, this result implies that PPNs and pullulan treatment might also work as a prebiotic in the intestine. To verify the intestinal barrier function, in this study, the serum endotoxin levels and FITC-dextran were detected and measured.

It was found that the serum endotoxin level was lower in the T4 and T5 groups which corresponded to the viable *E.coli* in feces. The results indicated that the "microbial barrier" consisted of less *E.coli* and the "physical barrier" was up regulated. It was found that probiotics or synbiotic treatment inhibited the increase in the serum FITC-dextran level. Moreover, pullulan and PPNs had an additional effect on LP by inhibiting the increase of serum FITC-dextran.

The combination of LP, pullulan and PPNs, enhanced the antimicrobial ability against pathogenic *E.coli* which enhanced the microbial barrier functions and it also reduced the FITC-dextran and endotoxin going into the

serum due to the enhanced physical barrier. Furthermore, the pullulan and PPNs treatment can changed the composition of gut microbiota increasing *Lactobacillus* spp. and *Bifidobacterium* spp. *in vivo*. The results of study 3 suggested that the combined treatment of pullulan, PPNs and LP can enhance the intestinal barrier function.

It is not yet economical to use PPNs as the livestock feed additives due to it caused a lot of economic loss when removing the unreacted pullulan and phthalic acid. For further study, in order to develop the pullulan nanoparticles as feed additives, pullulan can be conjugated with butyrate, which is the fermented product of pullulan by LP, and omits the step of remove of unreacted substances, because all substances included in the reaction can be used as “prebiotics”. So that, the synbiotic combination of *Lactobacillus plantarum*, pullulan and pullulan nanoparticles could be a therapeutic agent to enhance the protective function of the intestinal barrier and substitute of antibiotic growth promoters.

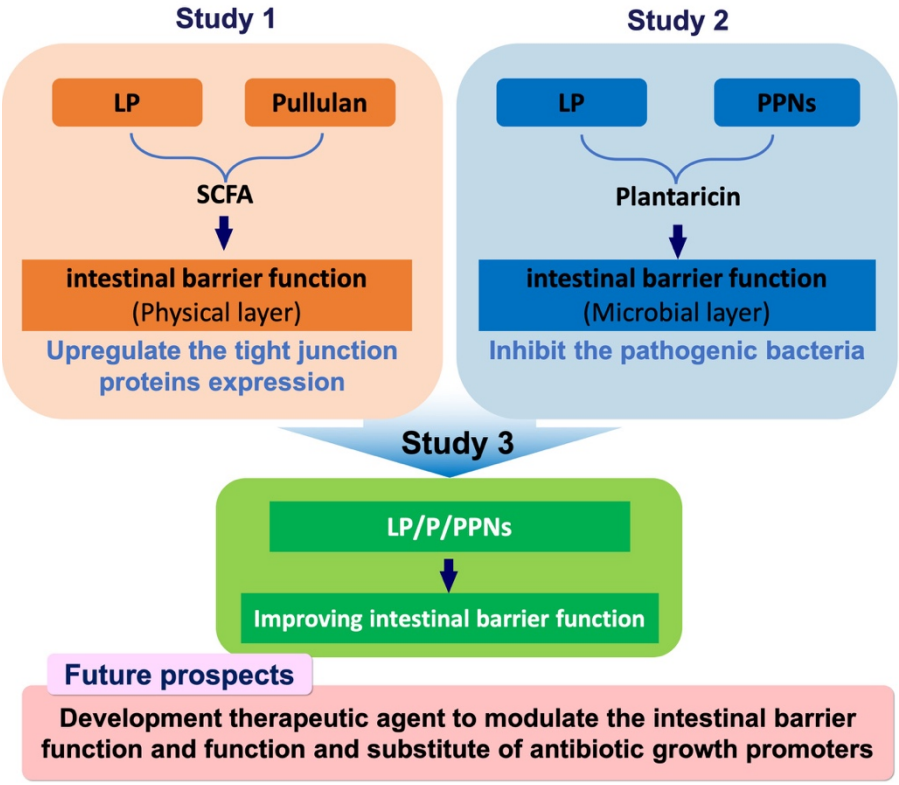


Figure. 42 Overall conclusion.

Synbiotic combination of *Lactobacillus plantarum*, pullulan and pullulan nanoparticles could be a therapeutic agent to enhance the protective function of the intestinal barrier and substitute of antibiotic growth promoters.

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Summary in Korean

음식을 소화시키고 영양분을 흡수하기 위해 포유류는 내장의 장벽에 의해 유지되는 매우 복잡하고 고도로 전문화된 위장 시스템을 개발해왔다. 위장 상피는 신체 중 외부 환경과 가장 큰 접점을 형성하면서 우리 몸을 외부 환경으로부터 분리시키면서 영양분을 흡수한다. 그러나 이 장벽은 흡수의 기능 외에도 음식물 입자, 세균, 바이러스, 독소 등 외부 항원을 많이 마주하고 있고 내장의 장벽 기능은 영양분을 흡수하면서 여러 개의 외부 항원이 유입되는 것을 막기 위해 필수적이다.

위장 상피 장벽의 첫 번째 층은 락토바실러스와 같은 유익한 박테리아들로 이루어진 미생물 층이고, 두 번째 층은 밀착연접 단백질에 의해 연결된 상피세포가 이루고 있는 물리적 층이다. 또한 점액, 항균 펩타이드, 면역 글로불린 A 를 포함한 많은 화학적 요소들로 이루어진 화학적 층도 존재한다. 마지막으로 면역 세포 (수지상세포, 대식세포, B 세포)로 이루어진 면역층 또한 장벽의 구성요소이다. 병리적 상태에서는 장내 장벽의 투과성이 저하되어 장내 독소, 항원, 박테리아가 혈류로 유입되어 '장 누수 증후군'을 일으킬 수 있다.

많은 연구 증거에 따르면 장내 미생물들이 내장의 장벽을 지지하면서 혈류로 들어가는 환경적 요인을 규제하는 데 중요한 역할을 한다는 것을 보여준다. 최근 보고에 따르면 생균제는 짧은 사슬 지방산의 자체 발효로 밀착연접 단백질의 발현을 향상시키고

또한 그것의 항균분자 (유산 과 박테리오신)를 통해 항균 능력을 가질 수 있다고 알려졌다.

프리바이오틱스는 장내 생물의 성장 및/또는 활성을 증가시키고 숙주에게 유익한 건강 영향을 미친다는 점을 감안할 때, 많은 연구자들이 프리바이오틱스를 사용하여 유익균의 성장이나 활성을 증가시키도록 많은 노력을 하였다. 장벽의 보호기능을 향상시키기 위해 병원균을 억제하고 밀착연접 단백질의 발현을 촉진하기 위하여, 본 연구에서는 새로운 probiotic 와 prebiotic 의 조합을 개발하였다.

연구 1 에서는 미생물의 탄수화물 대사 연구를 위한 50 가지 생화학실험 기반 표준화 시스템인 API 50 CH 키트를 이용하여 5 가지 종류의 락토바실러스 생균제와 조합을 이루는 프리바이오틱스를 선정하였다. 그 결과 24 종의 탄수화물이 유산균에 의해 발효될 수 있는 것을 확인했고, 그 중 프리바이오틱스로 사용할 수 없는 탄수화물을 제거했으며, 최종적으로 7 종의 프리바이오틱스와 그 단위체 블록이 후보로 선정되었다. 다음으로, 단쇄지방산의 총 발효량을 기준으로 프리바이오틱스 첨가 시 그 발효량이 증진되는 3 종류의 신바이오틱스 조합을 선정하였다. 세 종류의 신바이오틱스를 키운 상등액에 있는 SCFA 를 정량화하였다. 선발된 프리바이오틱스는 유산균에서의 아세테이트 발효가 증가했음을 보여주었다. 그러나 오직 풀루란만이 락토바실러스 플란타룸의 프로피오네이트와 뷰티레이트의 발효를 증가시키는 것을 확인하였다. 신바이오틱스의 효능은 24 시간 LPS 100μg/ml 를 처리하여 구축한

장 누수 세포 모델을 이용하였다. 세포모델에서 신바이오틱스의 기능을 검증한 결과, 락토바실러스 플란타룸 (LP)과 풀루란 (P)의 조합은 락토바실러스 플란타룸 단독보다 TEER 값 변화와 밀착연접 단백질의 mRNA (zo-1/claudin-1)의 발현에 유의미한 긍정적 영향을 주었다. 뷰티레이트는 밀착연접 단백질 형성에 가장 중요한 역할을 한다는 사실을 발견했고. LP/P 가 가장 많은 뷰티레이트를 발효하기에, 연구 2 의 재료로 선정하였다.

연구 2 에서 풀루란은 세 종류의 PPN (Phthaly-Pullulan Nanoparticle)으로 명명된 나노입자로 개발되었다. 이는 이전의 연구에서 프리바이오틱스 나노입자가 유산균의 항균 능력을 증가할 수 있다는 것이 밝혀졌기 때문이다. 개발된 PPN 역시 LP 유산균에 미치는 영향과 항균 활성에 미치는 영향이 관찰되었다. 병원균에 대한 항균 활성은 LP 에 나노입자를 처리한 후에 테스트되었고, 그 결과 세 가지 종류의 풀루란 나노입자는 모두 LP 의 항균 활성을 증가시켰다. 이에, PPN 이 LP 의 항균 활성을 증가시킬 수 있는 메커니즘에 대한 연구가 수행되었다. 먼저, PPN 의 LP 내로 내재화되는 지 여부를 평가했다. PPN 의 내재화는 LP 의 갈락토스 수용체에 의해 크게 규제되었고, 그 과정은 에너지 의존적이었다. PPN 이 LP 에 유입되면서 상당량의 항균 펩타이드 (플랜타리신)가 생산되었다. 따라서, 더 많은 양의 플랜타리신은 LP 단독이나 풀루란으로 처리된 LP 보다 그람 양성 병원균과 그람 음성 병원균 모두에 대해 더 효과적일 수 있다. PPN 으로 처리된 LP 에서 플랜타리신의 증가는 스트레스 반응 유전자 (dnaJ 와 dnaK)와 플랜타리신 생합성 유전자 (PlanS)의

발현력 향상과 함께 나타났다. 전반적으로 결론을 내리자면 LP 내로 유입된 PPN 의 LP 에 있어 스트레스원으로 작동하여, LP 의 방어 메커니즘을 활성화하므로 플랜타리신의 생성이 증가함을 시사한다.

연구 1 과 2 의 결과를 토대로 연구 3 에서 LP, 풀루란 및 PPN 을 포함한 새로운 신바이오틱스 조합의 생체내 유효성을 확인하였다. 이 조합은 병원성 대장균에 대한 항균 능력을 향상시켜 미생물 장벽 기능을 강화했으며, 강화된 물리적 장벽을 통해 혈청으로 들어가는 FITC-dextran 과 체내 내독소 수준을 감소시켰다. 게다가, 풀루란과 PPN 을 급여 받은 그룹에서 장내 유익균으로 알려진 락토바실러스.spp 와 비피도박테륨.spp 을 증가시킴을 확인하였다.

결론적으로, LP, 풀루란, PPN 으로 구성된 신바이오틱스 조합은 내장의 장벽 기능을 강화시킬 수 있으며, 내장의 장벽 기능을 조절하는 치료제가 될 수 있다는 가능성을 제시했다.

키워드: 프로바이오틱스, 프리바이오틱스, 신바이오틱스, 단쇄지방산, 박테이로신, 밀착연접, 항균능력, 위장벽기능.

학번: 2015-22384

Appendix. Phthalyl starch nanoparticles as prebiotics enhanced nisin production in *Lactococcus lactis* through the induction of mild stress in probiotics

Introduction

Nisin produced by *Lactococcus lactis* (LL) as one of the bacteriocins was firstly approved by the Food Drug Administration as a GRAS additive (Luck and Jager 1995) because it shows wide spectra of antimicrobial activities against Gram-positive bacteria, including pathogenic food bacteria such as *Listeria monocytogenes, Staphylococcus aureus,* and some *Bacillus species,* and also can prevent its spore germination and Gram-negative pathogen species such as *Escherichia coli* and *Salmonella* (Belfiore et al., 2007) although it was used since the 1950s to solve *Clostridium tyrobtricum* in cheese. Recently, it has been widely used in the production of various foods such as cheese, ready-made soups and canned foods (Gharsallaoui et al., 2016). And it has also been shown to have potential uses for the biomedical application and other fields (Takala and Saris 2007) although the enhancement of nisin production and reduction of costs have been concerned

with many researchers (Simsek et al., 2009b;Papagianni and Avramidis, 2012;Zhang et al., 2014;Kordikanlioglu et al., 2015).

Two methods have been mainly approached to increase the production of nisin. One is the batch fermentation system to use whole milk or skimmed milk to grow LL for industrial nisin production. However, it is complex to find out the optimum production conditions including pH, temperature, substrate, aeration, pressure, and incubation period. The other is to construct producer strains which have high yields of nisin production through genetic manipulation and results in improved nisin production according to the nisin producer (Simsek et al., 2009a;Ni et al., 2017). However, there are concerned about regulatory issues and consumer acceptance by GMO approaches although non-GMO ones as alternative methods have been developed to improve the adaptation abilities of native strains (Ozel et al., 2018).

Recently, we reported a new way of producing pediocin in Pediococcus *acidilactici* (PA) and plantaricin in Lactobacillus *plantarum* (LP) by internalized inulin and pullulan nanoparticles as prebiotics (Kim et al., 2018;Hong et al., 2019) into probiotics, respectively, through the induction of mild stress in probiotics, which is a new avenue for the biological

production of antimicrobial peptides. Interestingly, the self-assembled polymeric nanoparticles consisted of the hydrophobic core and hydrophilic shell were rapidly internalized into the probiotics.

In this study, we are aimed to investigate the antimicrobial activities of phthalyl starch nanoparticles (PSNs)-internalized LL. We synthesized and characterized PSNs to develop a new chemical inducer of nisin production in LL. Also, we checked whether the internalization of PSNs into LL enhanced antimicrobial activities against Gram-negative bacteria *Escherichia coli* (*E.coli), Salmonella Gallinarum* and Gram-positive bacteria *Listeria.monocytogenes* (LM) than LL or starch itself by antimicrobial assays. We further validated the nisin production of internalized PSNs into LL and made clear the mechanism of the antimicrobial ability by the internalization of PSNs into LL.

Materials and methods

Materials

Maize starch (Himaize 1043K) used in the study was provided by Ingredion (Seoul, Korea), and other chemicals were provided by Sigma-Aldrich (St. Louis, MO, USA). Lysogeny broth (LB), LB agar, De Man, Rogosa and Sharpe agar (MRS) broth, MacConkey agar and brain heart infusion (BHI) broth were purchased from BD Difco (Sparks, MD, USA) for bacterial cultures.

Synthesis of PSNs

The PSNs were synthesized according to the method described by Namazia (Namazia et al., 2011) with a slight modification. One gram of maize starch was dissolved in 10 ml of dimethyl sulfoxide (DMSO) at 54°C, and 0.1 mol-% dimethylaminopyridine as a catalyst per starch sugar residues was added to the solution, and then, phthalic anhydride was added to the above solution at different molar ratios per starch, such as 1.5:1(phthalic anhydride: starch) (named as PSN1), 3:1(phthalic anhydride: starch) (named as PSN2), and 6:1(phthalic anhydride: starch) (named as PSN3) to produce PSNs with different degrees of substitution of phthalic groups in starch. The

reaction was performed at 54°C for 24 h under nitrogen. The produced PSNs were dialyzed in dimethyl formamide (DMF) to remove unreacted phthalic anhydride and distilled water at 4°C for 24 h to form self-assembled nanoparticles of phthalyl starch. Finally, the PSNs were freeze-dried and stored at -20°C until use.

Characterization of PSNs

The substitution contents of phthalyl group in the phthalyl starch were confirmed by 600 MHz ^{1}H-nuclear magnetic resonance (NMR) spectroscopy (AVANCE 600, Bruker, Germany). The topography of PSNs was confirmed by field-emission scanning electron microscope (FE-SEM) with SUPRA 55VP-SEM (Carl Zeiss, Oberkochen, Germany). PSNs were coated with platinum under a vacuum using a vacuum chamber (CT 1500 HF, Oxford Instruments, Oxfordshire, UK). The sizes of the PSNs were measured by dynamic light scattering (DLS) spectrophotometer (DLS-7000, Otsuka Electronics, Osaka, Japan). The zeta potential of the PSNs was measured with an electrophoretic light scattering (ELS) spectrophotometer (ELS-8000, Otsuka Electronics, Osaka, Japan).

Tracking the internalization of PSNs into probiotics

To confirm the internalization of PSNs into LL. First, the fluorescence isothiocyanate (FITC)-conjugated PSNs were obtained as follows. Five mg of FITC and 100 mg PSNs were mixed and dissolved in 2 ml DMSO. After reaction for 4 h in an opaque tube at room temperature, and then the product was dialyzed against distilled water at 4°C for 24 h. After dialysis, the FITC-labelled PSNs were lyophilized, and unreacted FITC was removed after washing with ethanol several times.

To observe the internalization of PSNs into LL. LL was inoculated into 1 ml of MRS broth, treated with 0.5% (w/v) FITC-PSNs, and incubated for 2h at 37°C with shaking, and then washed with PBS several times and analyzed by confocal laser scanning microscopy (CLSM) (SP8X STED, Leica, Wetzlar, Germany) and flow cytometry. To confirm the internalization of nanoparticles by LL clearly, LL treated with FITC-PSN3 were observed by Z-section mode of the CLSM.

Bacterial cultures

All bacterial strains were cultured in the corresponding medium: *Lactococcus lactis* (LL) in MRS broth, Gram-negative *Salmonella Gallinarum* (SG) and *Escherichia coli (E.coli)* in LB broth, and Gram-positive *Listeria monocytogenes* (LM) in BHI broth. All bacteria were incubated at 37℃ in the shaking incubator (250 rpm) for 24 h before being applied to subsequent experiments or stored at -70°C in 15% (v/v) glycerol.

Co-culture assay and agar diffusion test

Antimicrobial activities of LL against *E.coli* , SG and LM were determined by co-culture assays (Ditu et al., 2011) and agar diffusion tests (Driscoll et al., 2012) with some modifications. To compare the antimicrobial ability of LL against *E.coli* and SG by co-culture assay, 2.0 x 10^6 CFU/ml of *E. coli* and SG were co-cultured with 2.0 x 10^5 CFU/ml LL treated with or without 0.5% (w/v) PSNs or starch in MRS broth for overnight culture under aerobic conditions at 37°C shaking incubator (250rpm). The antimicrobial ability was determined by the survival rate of pathogen strains. The co-cultured samples were spread on MacConkey agar and incubated for 24 h at 37°C, and the colonies of *E.coli* and SG were counted. The antimicrobial ability of LL

against LM was also determined by co-culture assay. LL and LM were co-cultured in BHI broth under the same condition described above, and the co-cultured samples were spread on Oxford agar after incubated for 24 h, the colonies of LM were counted.

The agar diffusion test was used to determine whether the cultured medium of LL with or without PSNs can inhibit the growth of pathogens on the agar plates. First of all, the 100 ul of *E.coli* and SG stock (2.0 x 10^8 CFU/ml) were spread onto LB agar, the paper disk was placed on the pathogen-spread plate, 120ul of 8 h cultured medium of LL or LL treated with or without PSNs and starch were dropped onto the paper disk, and then, the plates were incubated overnight at 37°C . The zone of inhibitions of *E.coli* and SG was used as a direct measurement of antimicrobial ability. The same methods were performed to test the antimicrobial ability of LL treated with or without PSNs or starch to LM after the LM were spread onto BHI agar.

Antimicrobial abilities of PSNs against *E.coli*, SG and LM were determined by co-culture assay described above. After 2.0 x 10^6 CFU/ml of *E.coli* and SG were co-cultured with 0.5 % (w/v) PSN3 in MRS broth for 8h at 37°C under aerobic conditions in a shaking incubator (250rpm), the co-

cultured samples were spread on MacConkey agar, were incubated for 24 h at 37°C, and the number of E.coli and SG colonies were counted. The antimicrobial ability of PSNs against LM was also determined by the co-culture assay. PSN3 and LM were co-cultured in BHI broth, the co-cultured samples were spread on Oxford agar, and then the number of LM colonies was counted.

Growth conditions of LL

After LL were treated with or without PSN3 and starch as described above, the growth characteristics of the LL were checked by measuring the pH of growth medium and viable cell counts at the indicated time points.

Nisin isolation and identification by SDS-PAGE

Nisin was isolated and purified as described in a previous study (Choi et al., 2001) with some modification, LL were treated with or without 0.5% (w/v) PSNs or starch in MRS broth for 24h under aerobic conditions at 37°C, and then LL were removed by centrifugation (8000g for 10 min at 4°C). The supernatant was gently stirred with ammonium sulfate (35% saturation) overnight at 4°C. Subsequently, the mixture was centrifuged at 12 000g for 20 min at 4°C, and then the pellet was dissolved in 20 ml of 50 mmol sodium

acetate buffer. This solution was desalted by dialysis (1kDa cut-off membrane, Spectrum Lab, city, state, USA). Proteins were lyophilized and stored at -20°C for further analyses.Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was used to observe the isolated nisin.

Analysis of the stress response genes by quantitative real-time PCR

RNA extraction was performed using the TRIzol® Max™ Bacterial RNA Isolation Kit purchased by Thermo Fisher Scientific Inc. (Waltham, MA, USA). Bacterial RNA extraction was conducted according to the manufacturer's instructions. LL were treated with or without PSNs or starch as described above. After the extraction and purification of RNA, cDNA was synthesized from 1 μg of RNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover purchased by TOYOBO CO., LTD (Dojima, Osaka, Japan). Quantitative real-time PCR (qRT-PCR) was performed with SYBR qPCR Mix using one-step real-time PCR (primers used in the study are listed in Table 1). The relative gene expression was calculated using the ΔΔCt method. The target gene expression was normalized to the relative expression of 16s rRNA as an internal control in bacteria samples.

Table 1. Primers used in this study.

Gene	Primer sequence	Product size (bp)
groES	F:5`-TGAAGTTGTTGCCGTCGG-3` R:5`-CATTTTTACGGTCGTTCCAGC-3`	118
dnaK	F:5`-CAGCTTGCCGTTCATCACAG-3` R:5`-TTGCTTGTCCAGCTTCACCT-3`	1007
dnaJ	F:5`-CCGCCAAAAGCGACTTTTACT-3` R:5`-TGGTGGCCGTGGACAAATTA-3`	641

Results

Synthesis and characterization of PSNs

The chemical reaction scheme of the synthesis of PSNs was shown in Fig.1. The substitution of phthalic moieties in starch confirmed by ^{1}H-NMR spectroscopy was shown in Figure S1A. The degree of substitution (DS) of phthalic moieties in starch was calculated by the ratio of phthalic acid protons (7.4-7.7ppm) to sugar protons (4.58-5.50ppm) using the equation described by Namazia (Namazia et al., 2011). According to the amount of the hydrophobic phthalic moieties in the PSNs, they were named as followed: PSN1 (DS: 14.3 mol.-%), PSN2 (DS: 17.8 mol.-%) and PSN3 (DS: 30.4 mol.-%). The surface morphologies of PSNs observed by SEM looked as spherical shapes (Fig.2B). The sizes of PSN1, PSN2, and PSN3 measured by DLS were 364.7, 248,4 and 213.4 nm respectively (Fig.2A), indicating that the introduction of more hydrophobic phthalic moieties in the starch made the nanoparticles smaller. Also, the surface charges of three kinds of PSNs measured by ELS were -23.47, -28.83, -29.83 mV for PSN1, PSN2 and PSN3 respectively as shown in Figure S1B, the indication of negative surface

charges due to the non-conjugated carboxylic groups in the phthalic acid of PSNs.

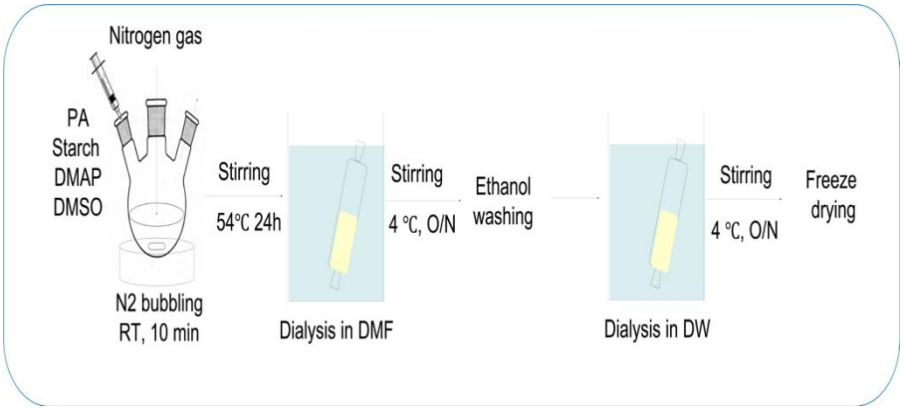
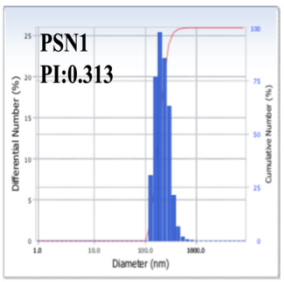
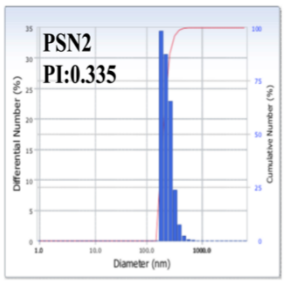


Figure. 1 Reaction scheme for the synthesis of PSNs (PA: Phthalic anhydride, DMAP: dimethylaminopyridine, DMSO: dimethyl sulfoxide. DMF: dimethylformamide).

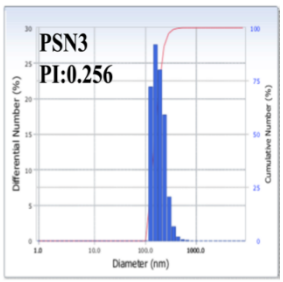
A



Peak	Diameter (nm)	Std. Dev.
1	364.7	173.3
2	0.0	0.0
3	0.0	0.0
4	0.0	0.0
5	0.0	0.0
Average	364.7	173.3



Peak	Diameter (nm)	Std. Dev.
1	248.4	66.1
2	0.0	0.0
3	0.0	0.0
4	0.0	0.0
5	0.0	0.0
Average	248.4	66.1



Peak	Diameter (nm)	Std. Dev.
1	213.4	67.0
2	0.0	0.0
3	0.0	0.0
4	0.0	0.0
5	0.0	0.0
Average	213.4	67.0

B

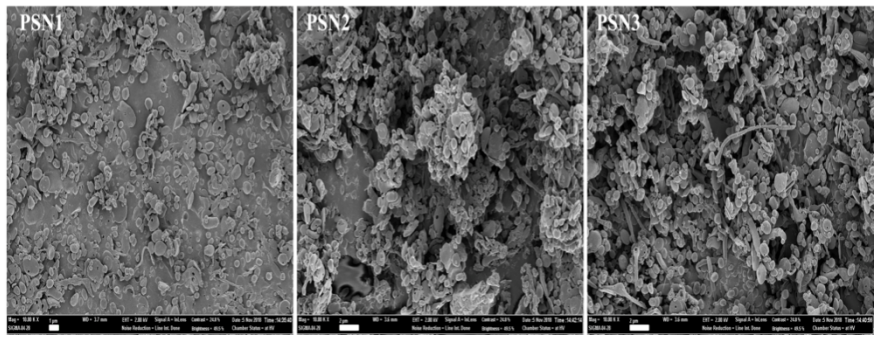


Figure. 2 Characteristics of PSNs. Measurement the size of PSNs by DLS (A). Morphologies of PSNs observed by SEM (B). Magnification:10.00K scale bar=2um. (PSNs: phthalyl starch nanoparticles, DLS: dynamic light scattering, SEM: scanning electron microscope).

Internalization of PSNs into LL

To confirm the internalization of PSNs into LL, fluorescence isothiocyanate (FITC) was conjugated to PSNs. The internalization of FITC-PSNs into LL was analyzed by confocal laser scanning microscopy (CLSM). The CLSM images showed that the green fluorescence of FITC-PSNs and the blue DAPI fluorescence of LL appeared in the same location (Fig.3A). To further confirm whether the FITC-PSNs were located at the surface of LL or inside of LL. LL were treated with FITC-PSN3 and the location of the FITC-PSN3 was observed by the Z-section mode of CLSM. As shown in Figure 3B, the fluorescence intensity of FITC was the highest at the center of the LL, indicating that internalization of PSNs into LL occurred.

Further studies were performed to investigate the internalization phenomena of PSNs into LL. First of all, to determine how long it takes internalization of PSNs into LL. LL were treated with FITC-PSNs for 5 min, 30 min or 1 h at 37°C and the internalization was confirmed by the fluorescence-activated cell sorting (FACS). The results showed that the PSNs were able to internalize into the LL very quickly because most of the PSNs were internalized into the LL within 5 min. Secondly, to determine the effect

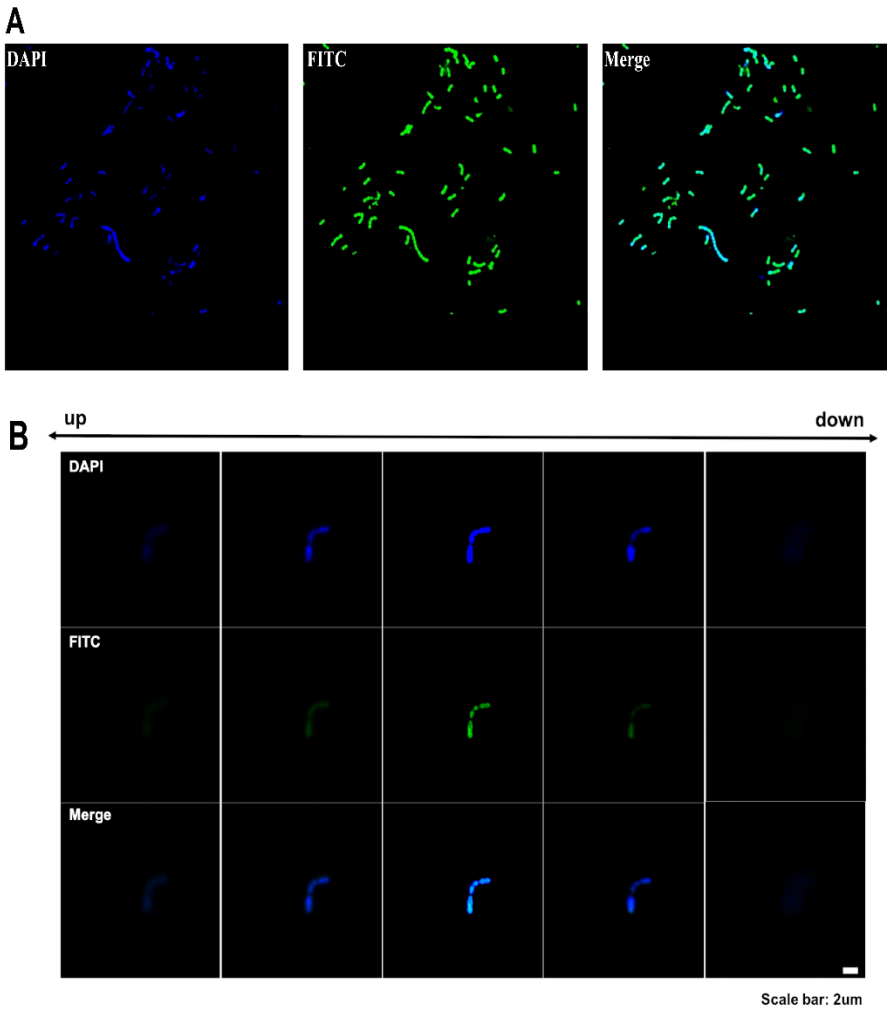


Figure. 3 Analysis of the internalization of PSNs by CLSM. FITC-PSNs and are shown in green, and LL was stained blue with DAPI. The internalization of PSNs after 2 h of treatment was shown in **A**. Z-section images show the internalization of corresponding PSNs into LL**(B)**. (LL: *Lactococcus lactis,* PSN: phthalyl starch nanoparticle, CLSM: confocal laser scanning microscopy, FITC: fluorescein isothiocyanate, DAPI: 4',6-diamidino-2-phenylindole).

of incubation temperature, LL were treated with FITC-PSNs at 4℃, room temperature or 37°C for 2h and subsequently observed by the FACS. The internalization of PSNs into LL was the highest at 37℃, indicating that the internalization of PSNs into LL was energy-dependent. Thirdly, to figure out the internalization of PSNs via which sugar transporter, LL were pre-treated with each 10 % (w/v) glucose, galactose, fructose, and PSN3 for 10 min, and then LL were treated with 0.5% (w/v) FITC-PSN3 for 2 h. FACS confirmed the internalization of PSNs into LL. The results showed that the internalization of PSNs was mostly via glucose transporter of LL because pre-treatment of glucose blocked nearly half of internalization of PSNs into LL. And it was shown that the internalization of PSNs was also blocked by pre-treatment of PSNs themselves (Data showed in Figure.S2).

Effect of PSNs on the antimicrobial ability of LL

To determine whether the internalization of PSNs into LL affect the antimicrobial ability of LL. LL were treated with three types of PSNs or starch itself. The antimicrobial abilities of LL were tested against Gram-negative bacteria *E.coli* (Fig.4A,4B), SG(Fig.4C,4D) and Gram-positive

bacteria LM(Fig.4E,4F). The antimicrobial abilities were compared among PSNs-treated LL(LL/PSN) and untreated LL or starch-treated LL(LL/S). The antimicrobial ability of LL/PSN groups was higher than that of untreated or starch-treated LL against three kinds of pathogens. Also, it was found that more strong antimicrobial ability was observed when LL was treated with smaller nanoparticles. Also, the antimicrobial ability of PSN-treated LL was further evaluated by agar diffusion assay. The inhibition zone was relatively wider when LL was treated with PSN3 as the smallest nanoparticles because the width of the inhibition zone reflected the antimicrobial ability.

To determine whether the PSNs alone have the antimicrobial ability, *E.coli*, SG and LM were treated with PSN3. It was found that the PSN3 alone did not have the antimicrobial ability (Fig.5C), indicating that antimicrobial ability comes from the PSNs-internalized LL.

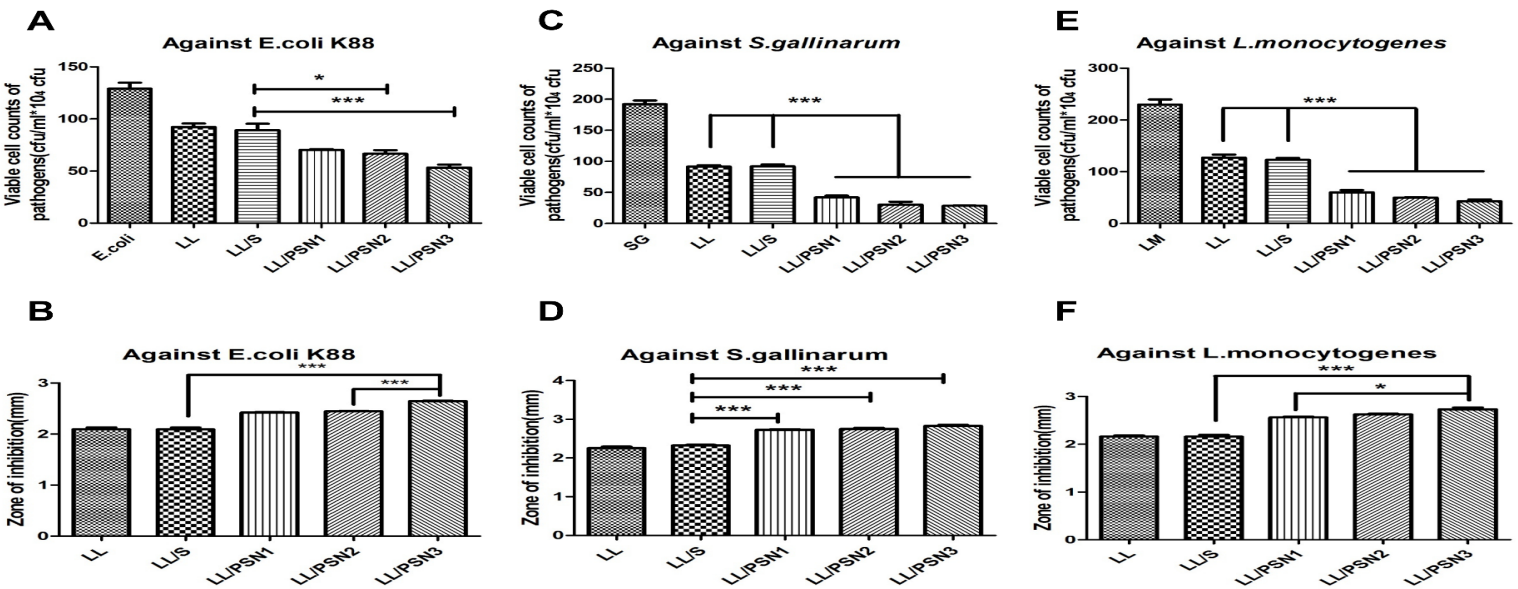


Figure. 4 Antimicrobial ability of LL/PSNs against *E. coli*, SG and LM (A-F). LL treated with PSNs or starch were cultured with Gram-negative *E.coli* and SG or Gram-positive LM, and the growth inhibition was calculated by CFU of E.coli **(A)**, SG**(C)** and LM**(E)**.Similarly, the diameters of growth inhibiton of E.coli**(B)**, SG**(D)** and LM**(F)** on LB and BHI agar plates, Respectively, were measured. Data are presented as the mean ± SEM of three independent experiments. Statistical significance was analysed between LL or LL treated with S and LL treated with PSNs by one-way ANOVA and Tukey's t test (*p<0.05, **p<0.01, ***p<0.001). (LL: *Lactococcus lactis,* PSN: phthalyl starch nanoparticle, S: starch, CFU: colony forming unit)

Effects of PSNs on growth and lactic acid production of LL.

To check the growth of LL when treated with PSNs or starch, cell colonies were counted at different time points after LL treated with PSN3, starch or LL alone. The results showed that not many differences in LL growth with or without PSNs or starch treatment were found (Fig.5A). The pH of culture media of LL after treatment PSN3 or starch was also measured at the same time to determine lactic acid production. The pH curve of LL treatment with or without PSNs or starch showed no significant changes among the groups as same as the growth curve (Fig.5B). Therefore, the internalization of PSNs by LL did not affect the growth and lactic acid production of LL.

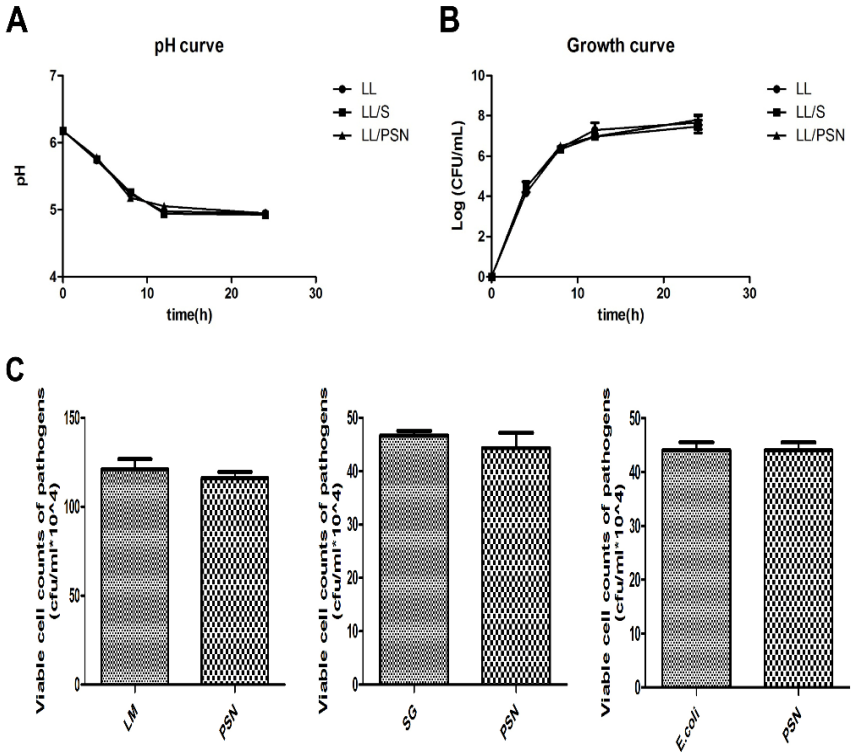


Figure. 5 Analysis the mechanism of enhanced antimicrobial ability. Measurement of the growth of LL **(A)** and pH of the culture medium **(B)** among LL groups with internalized PSNs or starch. Antimicrobial ability of PSNs against *LM, SG and E.coli* was measured**(C)**.

Effect of PSNs on nisin production

To determine the production of nisin in LL by PSNs, the nisin from LL, LL/PSNs and LL/S was isolated and observed by SDS-PAGE. As results, it is thought that the molecular weight of isolated nisin was between from 3.3 to 6.5 kDa, which is similar results reported by Gharsallaoui (Gharsallaoui et al., 2016). We also found that LL/PSNs increased the production of nisin compared with the LL and LL/S groups. Altogether, the production of nisin in LL/PSNs increased with a decrease in the size of internalized PSNs into LL(Fig.6A).

Effect of PSNs on the expression of stress response genes by qRT-PCR

In our previous studies, it was found that internalized nanoparticles into probiotics induced a stress response in lactic acid bacteria (Kim et al., 2018; Hong et al., 2019). To verify whether a similar response occurs in LL. The heat shock protein-associated genes such as dnaK, dnaJ and groES were determined. LL were treated with PSNs or starch for 8h, the expression levels of dnaK, dnaJ and groES were significantly higher in PSNs-treated groups compared with a starch-treated group or LL alone. Also, it showed that the

expression levels of stress-associated genes were much higher when LL were treated with smaller PSNs (Fig.6B). The results indicated that the internalization of nanoparticles into LL induced mild stress response to the LL.

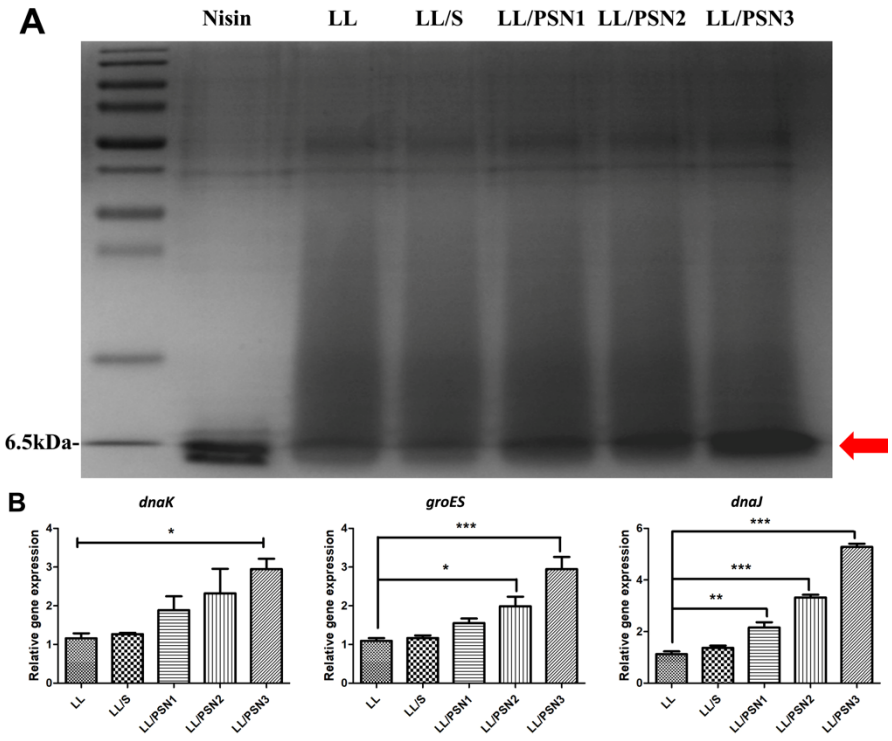


Figure. 6 Analysis the isolated nisin and genes related to the stress response in LL treated with PSNs. The isolated nisin was determined by SDS-PAGE **(A).** The expression levels of *dnaK*, *dnaJ and groES* **(B)** relative to 16s rRNA was quantified by qRT-PCR. Data are presented as the mean ± SEM of three independent experiments. Statistical significance was analysed between LL and other groups by one-way ANOVA and Tukey's t test (*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$). (*dnaK*, *dnaJ and groES*: heat shock proteins**,** qRT-PCR: quantitative real-time polymerase chain reaction).

Discussion

Many scientists have been interested in using the probiotics as alternatives for antibiotics because according to the World Health Organization (WHO) the use of antibiotics as a growth promoter for livestock animals results in the main reason of antibiotics resistance (Witte, 1998). Antibiotics resistance affects not only animal health but also human life. Recently, many scientists have reported that probiotics can inhibit the colonization of pathogens on the the intestinal barrier or directly kill pathogens through their secreted bacteriocin to make the host welfare (Gillor et al., 2008). Therefore, there have been many attempts to increase the production of bacteriocin by probiotics.

In this study, we developed the starch nanoparticles as a novel formulation of prebiotics to increase the production of bacteriocin from LL. The self-assembled PSNs were prepared after conjugation of hydrophobic phthalic anhydride with hydroxyl groups in starch due to the hydrophobic interaction during the dialysis in DW. It is believed that chemical reaction occurs between the primary hydroxyl groups of starch and the carboxylic acids of phthalic acid through esterification. It was found that more phthalic groups

in the PSNs resulted in smaller sizes of PSNs due to the increased hydrophobic interactions.

In recent years, many polymeric nanoparticles have been commercialized for drug delivery systems (Na et al., 2003;Zhang et al., 2010;Blanco et al., 2015;Masood, 2016) because they can deliver therapeutic drugs to necessary place of action and can overcome cellular barriers when delivering hydrophobic drugs. However, researches on the internalization of polymeric nanoparticles into bacteria except for metal nanoparticles, is still in an early stage. The internalization of metal nanoparticles into bacteria generally occurred via electrostatic interactions (Sanyasi et al., 2016). Many studies have described that metal nanoparticles have antimicrobial ability due to their abrogation of bacterial growth by ionic interaction with the bacterial membrane. But metal nanoparticles inhibit both pathogens and beneficial microbes (Travan et al., 2009). In this study, we developed PSNs as a new formulation of prebiotics and found that PSNs were internalized into LL via size of PSNs and energy dependence. Interestingly, PSNs were internalized into LL through glucose transporter of LL because pretreatment of glucose significantly decreased the internalization of PSN3 into LL.

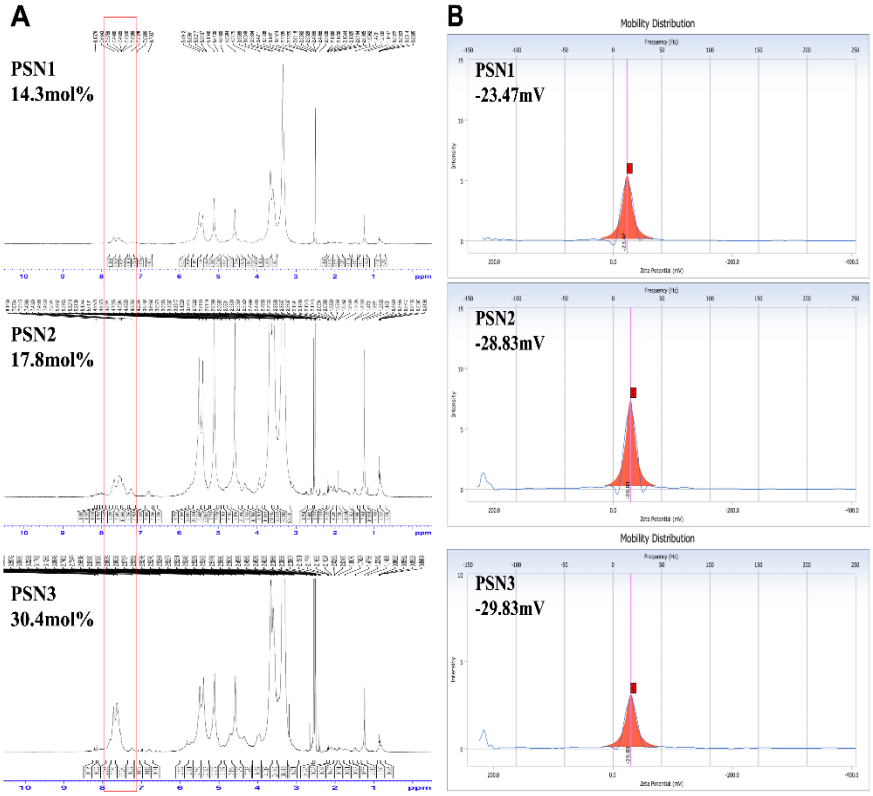
Interestingly, the treatment of LL with PSNs enhanced antimicrobial activities against Gram-negative and Gram-positive pathogens than starch or LL themselves although the used PSNs did not much show toxicity to the LL. Particularly, LL/PSN3 showed the highest antimicrobial activity due to the more internalization of PSNs into LL.

The enhancement of antimicrobial properties by the probiotics is very important to probiotics researchers because the advantages of probiotics as food additives have been mostly focused on their antimicrobial ability. Nisin is a natural bacteriocin produced by *Lactococcus lactis*, which has been approved by the Food and Drug Administration (FDA) as GRAS food additive, due to its broad-spectrum antimicrobial ability. In this study, PSNs treatment on LL markedly increased the production of nisin through confirmation by the SDS-PAGE. Therefore, it is hypothesized that the internalization of PSNs into LL is contributed to the higher antimicrobial properties of LL via the production of nisin.

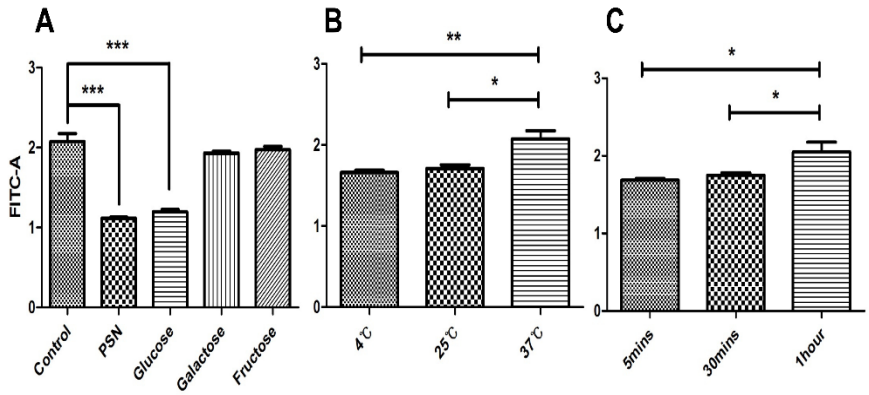
In this study, we found that the expression of heat shock proteins such as dnaK, dnaJ and groES in PSNs-treated group was significantly higher than those of untreated LL. The results indicated that the internalization of PSNs

into LL induced mild stress to stimulate nisin production through their defense mechanism.

Ultimately, it is concluded that the polymeric nanoparticles as the prebiotics can exert interesting effects on probiotics leading to increased production of an antimicrobial peptide that is powerful against Gram-positive and Gram-negative pathogens. Therefore, our study shows a new way to produce antimicrobial peptide in probiotics through mild intracellular stimulation by the internalization of PSNs into probiotics, suggesting that they have great potentials as an alternative for the synthetic antibiotics.



Supplementary Fig 1. Characteristics of PSNs. Calculation of mol.-% phthalic acid in PSNs by ^{1}H-NMR spectroscopy **(A)**. Measurement of the zeta potential of PSNs by ELS **(B)** (H-NMR: nuclear magnetic resonance, ELS: electrophoretic light scattering)



Supplementary Fig. 2 Analysis of the internalization of PSNs by LL. Analysis of the internalization of PSNs by LL depending on transporters **(A).** LL, pre-incubated with 10% (w/v) glucose, fructose, galactose or PSN3 was treated with 0.5% (w/v) FITC-PSN3 for 2 h at 37 °C, and internalization was quantified by FACS. LL was treated with 0.5% (w/v) FITC-PSN3 at different temperatures (4, 20, or 37°C) for 2 h **(B)**, and internalization was quantified by FACS. LL was treated with 0.5% (w/v) FITC-PSN3 for 5 mins, 30mins and 1hour**(C)**, and internalization was quantified by FACS. FACS data are representative of three independent experiments, and the average values are presented as the mean ± SEM of three independent FACS experiments in a bar chart. Statistical significance was analysed between each group by one-way ANOVA and Tukey's t test (*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$). (FACS: fluorescence-activated cell sorting)

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